16 July 2018 Volume 14 Issue 7

The Column

the interactive e-publication for the global separation science industry

Cover Story

Features

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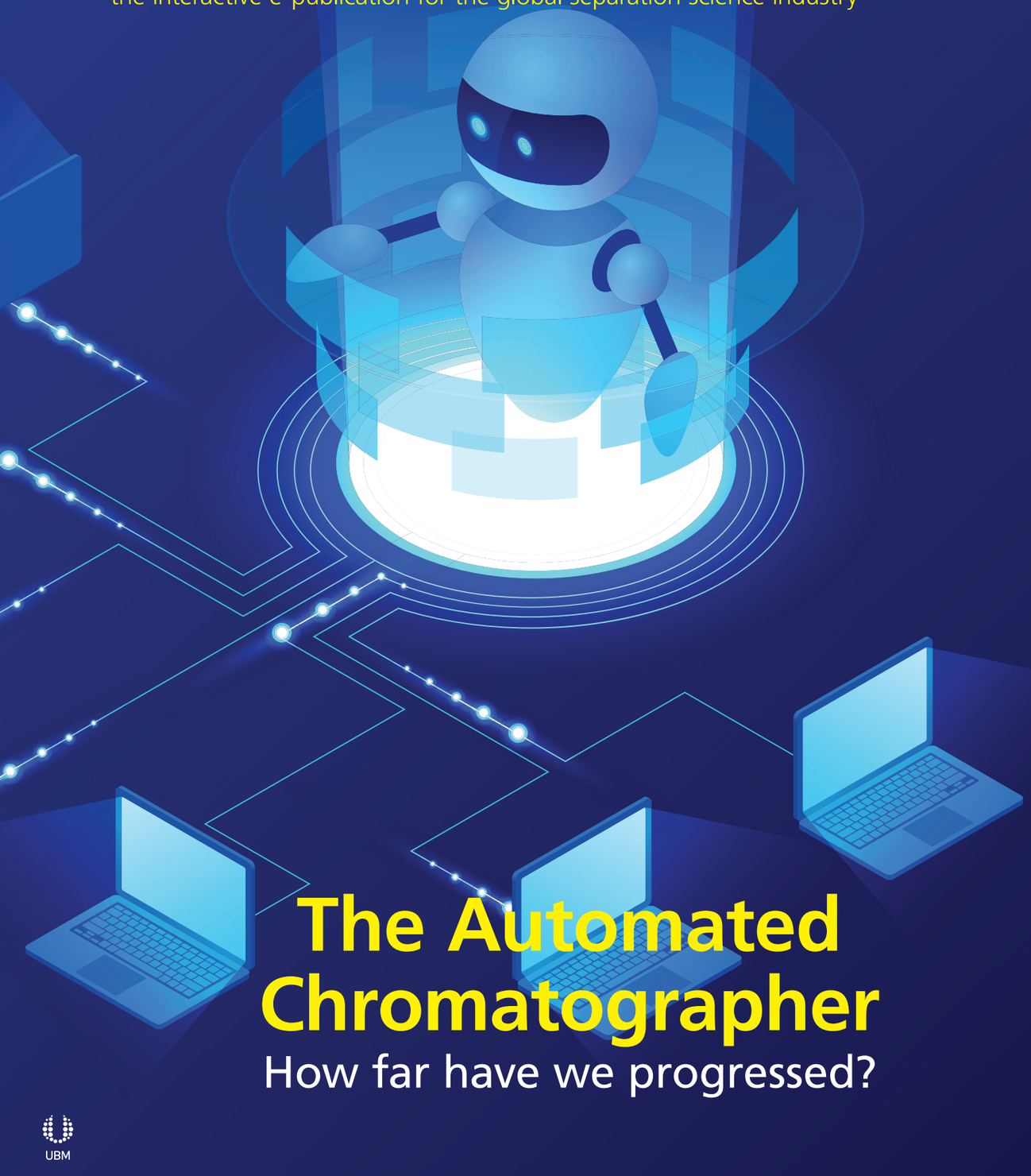

The Automated Chromatographer

How far have we progressed?

UBM

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The *LCGC* Blog: Quantitative Analysis Using Hybrid Mass Analyzers: Mass Accuracy Versus Tandem Mass Spectrometry

Kevin A. Schug, Department of Chemistry and Biochemistry, The University of Texas (UT) at Arlington, Arlington, USA

What type of mass spectrometry (MS) instrumentation provides the best specificity during trace quantitative analysis from complex mixtures?

It's a debate that has been going on for some time. I would have to speculate it is an argument that was initially propagated by a purveyor of one technology provider over other. The overarching issue is what type of mass spectrometry (MS) instrumentation provides the best specificity during trace quantitative analysis from complex mixtures. Electrospray ionization (ESI), being the most common ionization source for liquid-phase sampling into MS, is a very

efficient means for producing gas phase ions; as such, complex mixtures will yield a plethora of signals, and those signals for the analytes of interest must be reliably differentiated from those from potential interferences. Obviously, MS provides strong qualitative information in the form of mass-to-charge ratios (*m/z*) to aid compound identification, but in a complex mixture there can be a large number of species with very similar or identical masses.

If you want to differentiate target analytes from interferences, is it better to have an instrument that provides high resolution and high mass accuracy, or one that offers tandem mass spectrometry (MS/MS)? Let's start where the mainstream debate started—comparing the high-resolution mass spectrometry (HRMS) capabilities of an orbital trap mass analyzer versus the MS/MS capabilities of a triple-quadrupole (QQQ) mass analyzer. Which provides better specificity? Let's also put sensitivity aside, because nowadays modern ESI-MS instruments can all generally detect very low abundance species.

QQQ-MS has long been referred to as the gold standard for quantitative analysis. When operated in selected reaction monitoring (SRM) (or multiple reaction monitoring [MRM]) mode, a QQQ-MS system provides specificity through first isolating a mass of interest and then generating a unique diagnostic high-abundance fragment ion from that analyte of interest by collision-induced dissociation (CID). By focusing on a specific fragment of an ion, virtually all isobaric interferences in a sample will be excluded from detection, because only the analyte of interest should yield that fragment after it is dissociated. A wide linear range is inherent to quadrupole instruments, relative to other mass analyzers, and many modern QQQ-MS instruments have very high scan speeds. This wide linear range and high scan speed enable the instruments to monitor many—even hundreds or thousands—of specific precursor to product ion MRM transitions in a single run. QQQ-MS instruments are also relatively affordable, and they are widely distributed through routine analytical laboratories, primarily for the purpose of carrying out quantitative analysis. The biggest drawback is that QQQ-MS systems are low, unit-resolution instruments, so only integer *m/z* values can generally be reliably assigned to detected ions.

The orbital trap, released commercially 13 years ago, operates on a different

principle. Ions are made to orbit a spindle electrode in the high-vacuum region of the instrument. As they orbit this spindle, they are separated from other ions having different *m/z* values with extremely high resolution. The frequency of ion movement is measured, and then a Fourier transform is applied to convert this information into mass spectra. As a result, ions that reach the detector can be assigned very accurate *m/z* values, down to four or five decimal places. With sub-parts-per-million error in mass accuracy, the number of ways that you can put together multiple carbon, oxygen, nitrogen, and other atoms to generate a specific molecular formula decreases dramatically. Thus, high specificity is achieved by ensuring that molecular formula of the ion detected well matches that of the target analyte ion. Yet, high resolution comes at a cost. Orbital mass analyzers are generally much more expensive than QQQ-MS systems, and they are also relatively slower. Trap instruments are also characterized as having reduced linear operating ranges compared to quadrupole instruments. Even so, most versions of commercial orbital trap instruments also incorporate the capability for tandem mass analysis, which broadens their application base considerably. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) systems can be characterized similarly. However, without some capability to fragment ions, no amount of resolution can resolve isobaric species.

A quick search will show that some significant work has been performed previously to directly compare MS/MS with HRMS for quantitative performance. In many cases, comparable performance can be claimed (1), but performance is also dependent on the nature of the analyte and sample in question—I think one could always find applications where one or the other approach wins out. One study determined that a single *m/z* measured by HRMS at >50,000 resolution is more selective than performing SRM on a QQQ-MS system (2). With such a report, one might consider this debate case closed in favour of HRMS, but cost is still an important driver in most people's purchasing decisions. As a result, the market for QQQ-MS instruments is still quite a bit larger than for HRMS systems.

The truth is, ideally you would like to have both capabilities. Nowadays, with continued and growing interest in the vast array of "omics" topics, researchers would like to combine both targeted and untargeted data collection capabilities.

A targeted method is one for which you know what you are looking, whereas an untargeted method is designed to collect signals for anything that might be in a sample. In that case, it is reasonable to argue that a quadrupole time-of-flight (QTOF) mass spectrometer provides the best compromise between the need for HRMS and MS/MS.

In a QTOF system, a fast scanning quadrupole can be interfaced, through a CID cell, with a TOF mass analyzer. TOF measures a flight time to determine *m/z* values, and this can be done with quite good resolution and mass accuracy. Flight-time measurements are a bit less accurate than ion frequency measurements, but the former are much simpler and less expensive to perform than the latter. QTOF systems maintain a wide linear range, are fast, and provide for MS and MS/MS measurements with standard resolutions in the 30,000–50,000 range. Although QTOF systems can be quite versatile and powerful, a common issue with TOF systems is the stability of their calibration. Often small changes in temperature over time can cause significant drift in mass accuracy. Either the temperature of the laboratory needs to be carefully maintained, or the TOF system needs to be calibrated often. In many cases, calibration mixtures for tuning TOF flight times can be quite sticky, making them difficult to clean from the system after use. A stable QTOF, which does not have to be calibrated often, is certainly a desirable item.

One thing is for sure—there are a lot of choices out there in the mass spectrometer marketplace. It is extremely important to assess the planned primary use of the system to make the correct choice. You can never have too much resolution, but it comes at a cost (both analysis time and money). I would also be remiss if I didn't mention the critical role of the sample preparation and chromatographic systems that often come before the mass spectrometer. If sample collection, preparation, and separation are done poorly, no mass spectrometer will provide good quality data, no matter how much money you spend.

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News

Agilent Announces ProZyme Acquisition

Agilent Technologies (Santa Clara, California, USA) has announced that it has entered into a definitive agreement to acquire the privately held ProZyme, Inc. (Hayward, California, USA), a provider of glycan analysis reagents, kits, and standards. The acquisition will expand Agilent's portfolio of biopharmaceutical consumables in the growing glycan market.

Founded in 1990, ProZyme is a developer and manufacturer of glycan reagents, kits, and standards, which are required for efficient sample preparation in the analysis of free glycans. Glycobiology, which is the study of the structure, function, and biology of carbohydrates, also called glycans, is a fast-growing and important field of study in life sciences. Glycans play diverse roles in biotherapeutics, novel drug development, the study of bacterial physiology, and proteomics research.

"Glycan analysis is essential to the discovery, development, and quality control testing of the ever-growing pipeline of biotherapeutic drug products," said Padraig McDonnell, Vice President and General Manager of Agilent's Chemistries and Supplies Division. "This acquisition provides greater scale to our biopharma consumables business and enhances our value proposition by enabling complete glycan liquid chromatography and mass spectrometry workflow solutions."

For more information, please visit **www.agilent.com** and **www.prozyme.com**

Generic GC–FID for Volatile Amine Quantitation in Pharma

Researchers from Merck and Agilent Technologies have developed a simple and fast generic gas chromatography–flame ionization detection (GC–FID) method for the quantitation of volatile amines in pharmaceutical drugs and synthetic intermediates (1).

Among the most frequently used compounds in pharmaceutical chemistry, volatile amines offer chemists the ability to control the pH of reaction mixtures and improve product yield because of their basic properties and low boiling point. However, the selection of the "optimal" amine for a particular production also becomes a bottleneck in synthetic route development process. Many hours are devoted to the development of new analytical methods for the quantitation of residual amine content prior to each analysis session—hours and resources that could be used elsewhere if an alternative option existed.

This issue has been well documented with a wide spectrum of extraction procedures already existing for each separation technique. However, most require detailed sample preparation and specific instrumentation, thereby not addressing the time issue they were intended to solve. Furthermore, many of the procedures are specific and are focused on a narrow group of amines, lacking the potential to be used universally. The added speed with which chemists can generate accurate and quality data with generic or more universal chromatographic methods has led to their popularity in recent years and hence the need for such procedures in the production of pharmaceuticals.

The method developed by researchers analyzes over 25 volatile amines and other basic polar species in a single 16-min chromatographic run using conventional and readily available GC–FID instrumentation and using either He or H_2 as a carrier gas.

The validation experiments showed excellent sensitivity, precision, linear correlation, and accuracy for all the amines. Researchers also believe the method can be used as an effective starting point for solving challenging separations and for the analysis of volatile polar species beyond the list of amines described in the study.—L.B.

Reference

1. G.C.Graffius *et al., J. Chromatogr. A* **1518**, 70–77 (2017).

Photo Credit: Planar/Shutterstock.com

Hamilton Announces European Relocation

Hamilton Storage GmbH has announced the total relocation of its operations to a high-technology industrial park in Domat/Ems, Switzerland. The company is the first business to open within the industrial park and will be followed by other Hamilton affiliate entities.

"This is a great milestone in the history of Hamilton Storage that opens the door to many exciting opportunities," said Martin Frey, Vice President, Hamilton Storage. "The Domat/Ems location gives us leverage to maintain strong growth and facilitate extraordinary customer support in Europe and beyond," continued Frey.

The newly built 15,000 m^2 building is commuter friendly and includes an automated parts warehouse, customized production area for large compound storage systems, as well as sustainable features such as solar panels and a geothermal heating and cooling system.

For more information, please visit **www.hamiltoncompany.com/samplestorage**

The Chromatographic Society: "Grass Roots 3" Educational Event

The Chromatographic Society has announced the "Grass Roots 3" Educational Event is to be held at the Waterhead Hotel, Ambleside, Cumbria, UK, Friday 5–Monday 8 October 2018.

The event looks to build on the two previous events with the first being organized in October 2016 as part of the society's Diamond Anniversary celebrations. Held in Grasmere in the Lake District, the course focused on teaching the fundamentals of liquid chromatography (LC) to graduate students and novice chromatographers from the industry. The event proved to be a success with over 20 attendees taking part spurring a repeat of the course, which was held in Church Stretton in October 2017.

This new event will build upon the fundamentals taught on the previous Grass Roots courses, focusing on reversed-phase method development for small molecules.

"The subject matter will be of particular relevance for attendees working with pharmaceutical compounds, but the concepts and approaches will be equally relevant to those working in the agrochemical, food, environmental, and other industries," said Paul Ferguson, AstraZeneca.

The course will be delivered by a number of very experienced chromatographers with industrial experience including: Mel Euerby (Shimadzu), Tony Taylor (Crawford Scientific), Roman Szucs (Pfizer), and Paul Ferguson (AstraZeneca).

The lecture programme includes approximately 20 hours of lectures alongside a walking programme and evening events. The programme will be "fixed" content, however, there is significant scope to include additional topics as requested by attendees as well as informal discussion during social elements.

"We would also be happy to discuss any specific chromatographic queries or issues that attendees bring from their workplaces," said Ferguson. "Attendees who wish to bring chromatography posters to the event are also welcome to do so and these can be discussed at appropriate times linked to the programme."

The course will cover many aspects of method development such as sample preparation, gradient chromatography, modifying selectivity, detection options, quantification, method robustness, validation, and interactive practical exercises using *in silico* retention modelling software. The course fee includes:

- Three night's accommodation (Friday, Saturday, and Sunday evenings)
- All meals and course refreshments (breakfast, lunch, and dinner)
- Course notes
- Certificate of attendance
- Three month's ChromSoc membership

Full course details, bursary information, and registration may be found on our website: **http://chromsoc.com/event/grass-roots-2018/**

For sponsorship and other queries, please contact **paul.ferguson@chromsoc.com**

Photo Credit: The Chromatographic Society

Peaks of the Month

- **The *LCGC* Blog: Understanding Stationary Phases for HILIC Separations**—HILIC is not straightforward and there may be a number of mechanisms in play which need to be considered. **Read Here>>**

- **New Gas Chromatography Products for 2017–2018**—John Hinshaw presents his annual review of new developments in the field of gas chromatography seen at Pittcon and other venues in the past 12 months. **Read Here>>**

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- **Monitoring of Oxidation in Biopharmaceuticals with Top-to-Bottom High Performance Liquid Chromatography–Mass Spectrometry Methodologies: A Critical Check**—This article details the potential of HPLC and MS with regard to revealing methionine oxidation, a chemical modification that may be induced during downstream processing and storage of biopharmaceuticals. The benefits and limitations of bottom-up, middle-down, and top-down HPLC–MS analysis will be demonstrated for the detection of oxidation variants in a therapeutic monoclonal antibody (mAb). **Read Here>>**

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- **A Fast, Robust, and Reliable Method for Sensitively Screening Drugs of Abuse in Human Urine for Forensic Toxicology**—Analyzing drugs of abuse (DoA) in human bodily fluids is crucial for clinical research and forensic toxicology. In these routine analyses, a large number of samples must be investigated, with a potentially high laboratory cost for each sample. As such, a reliable and affordable method is required for analysis. In this article, a fast, robust, and reliable method is presented for routine, high-throughput drug screening of urine samples. **Read Here >>**

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- **Investigating the Flavour Profiles of E-Cigarettes**—Researchers from Gdansk University of Technology, Poland, have investigated the flavour profiles of e-cigarette refill solutions using gas chromatography–tandem mass spectrometry (GC–MS/MS). **Read Here >>**

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News In Brief

Research conducted at Texas Tech University has investigated whether alloimmunization with boar sperm membranes or lipid rafts affects the fertility of female pigs, and identified the major membrane and lipid raft alloantigens. Sperm antigens have previously shown potential as targets for fertility control, offering an inexpensive, long-acting, nonhormonal control of reproduction in many species. Researchers used anion-exchange chromatography, electrophoresis, immunoblotting, and mass spectrometry to investigate and concluded that alloimmunizing female swine with sperm membranes or lipid rafts evokes the formation of antibodies to a relatively small number of dominant alloantigens with possible functions in fertilization and potential utility as targets for immunocontraceptions.
DOI.10.1371/journal.pone.0190891

Phenomenex (Torrance, California, USA) has announced the opening of a new office in Singapore to serve customers directly. Active in Singapore through distributors since 1993, this new direct presence will allow Phenomenex customers access to comprehensive training and support services, live technical chat, improved product availability, and faster shipping times. "With Singapore's position as a leading pharmaceutical and biotech hub within Southeast Asia, there is great demand for the latest innovations in separation science technology. We are excited to be expanding our global operations into the Asia Pacific region," said Shane Lyons, President of Phenomenex. For more information, please visit **www.phenomenex.com**

Photo Credit: Golden Sikorka/Shutterstock.com

Do Chromatographers Need More Automation?

Incognito asks if chromatographers are behind the curve when it comes to automation.

I've seen a real drive in recent years towards letting the scientists concentrate on science while the more menial tasks in the analytical laboratory are taken care of by staff employed by outsourcing or facilities management companies. This move is generally driven by two factors: the need to reduce costs and the requirement to be more productive.

In an ideal world, we would request an eluent and chromatography system setup this afternoon and come into the laboratory tomorrow to find the system ready to go—already qualified by test injections of a generic system suitability standard. By having the outsourced staff start work earlier than the "scientists" this is very much a reality for staff within some of the larger research and development companies that I've visited. But is this really "ideal"?

Most folks wouldn't need a kaizen burst or a spaghetti diagram derived by their local six sigma expert to tell them where the bottlenecks are in their processes, and the areas in which outsourcing may make a positive impact should be fairly obvious.

However, I can't shake the notion that as an industry we are lagging behind in the degree of automation that is used to achieve cost reduction and improved throughput. By no means do I want to see the outsourcing company staff become unemployed, but by the same token I really do see a great opportunity to further automate many of our operations and to address the areas in which the real bottlenecks lie.

Most of you reading this article will be able to identify the efficiency pinch points in your laboratory, which I believe will include:

- Sample and eluent preparation
- Data processing
- Data reporting
- Instrument preparation
- Instrument (hardware) failures
- Chemistry failures
- Non-availability of equipment or reagents
- Method development and validation

With the level of sophistication that is possible in automation (think of the complexity of some manufacturing plants for example), the power of modern data management systems and the interconnectedness of the Internet of Things (IoT), surely we must be able to overcome some of these challenges.

I wish I had some insightful answers for you, but frankly I have only questions and suggestions and will need to leave it to the specialists to build the solutions. But I can't be the only laboratory dweller who yearns for answers to the bottlenecks outlined above?

Let's take a brief look at some of the issues that I believe we should have tackled by now, in the hope that someone somewhere can let us all know that there is hope, or maybe even that the problem has been solved—we just haven't found out about it yet.

Eluent Preparation

I'd really like to know why I can't dial up an eluent recipe and have it delivered in whatever volume required and degassed. I appreciate that there several discrete steps such as weighing of additives, volumetric accuracy, and pH adjustment but all of these are capable of automation—aren't they? Whilst some laboratories will have a very diverse set of reagents, solvents, and additives making it very tricky to store them all in reservoirs or silos ready for use, a large number use a fairly standard set of reagents, which would not require such a large number of containers. I also believe that automated eluent preparation would reduce the number of errors in manual eluent preparation. I'm also aware that prep-stations of this type have been attempted in the past, but I don't see a ready source of this equipment and wonder what were the issues with the implementation or commercialization?

Sample Preparation

I admit that in some industries I do see great advances in automated sample preparation and there are some vendor companies that really excel at automating these processes. But if I take the pharmaceutical industry as an example, there are very few systems that I know of which can take tablets, sterile injectable solutions, or powders at one end and come out of the other with a sample ready for injection into the chromatography system, either in a bulk format or with a just-in-time approach. Why is this? What are the barriers to implementation? I really can't believe it's a technology hurdle because these automation companies can do pretty much anything with their robots including

weighing, accurate volumetric operations, centrifuging, solvent extraction, and solid-phase extraction (SPE). I must admit I haven't come across a solution for grinding tablets, but I'm sure they will let me know if this is possible. So why so little automation of sample preparation in the pharmaceutical industry?

Instrument Preparation

Why do I need to "set up" the instrument? Why haven't we come up with a solution for automated (robotic?) column changing (high performance liquid chromatography [HPLC] or gas chromatography [GC]) that can load an acquisition method and flush the instrument with the eluent that has been automatically prepared for me, then inject the generic system suitability standard to check that the system as a whole is performing to the required standards?

I know that various parts of what I have described are possible, but why have we not yet combined these into a viable solution?

In terms of telemetry, surely it would also be possible to test the various parts of the system for leaks and go through a self-check routine to not only check the detector function (as is possible in most instruments today), but also for susceptibility for leaks as the eluent viscosity changes and pressure increases, or the likelihood of a column to fail during an analysis based on the back pressure history of that column.

Automated Troubleshooting

If an instrument fails, I would like it to do some more advanced diagnostics to tell me exactly why it failed. Where is the leak and why did it occur? Why is the sensitivity of the detector not as it should be? If there is pressure ripple, where is it coming from? Why won't the flame ionization detector (FID) flame light or why has it gone out? Why is the GC–mass spectrometry (MS) system not seeing any peaks?

If we were to reach advanced levels of automation, it should be possible to simply swap the faulty component from an HPLC system (pump, degasser, detector) and get on with the job rather than having to come into the laboratory the next day only to be disappointed that the "batch has failed" overnight because of an instrument error. My pet hate is that the autosampler has failed to recognize or pick up a vial by the way—surely this should be a trivial fix?

In a similar fashion, with the IoT so much in focus these days, why is it not possible to discover an issue with my system via my smartphone and then, in conjunction with the fully automated (multiplexed if you will) system, simply divert the eluent flow via another pump or detector within the matrix so that the system continues to collect data and not leave me fretting about all the remedial work I'm going to have to get through the next day?

Furthermore, when my problems are associated with "chemistry" issues, why haven't we employed machine learning to better interpret and solve these problems? In troubleshooting classes we teach folks how to recognize "symptoms" related to baseline appearance, peak shape, changes in selectivity, and drifting retention times and then relate them back to the issues with the chemistry of the separation. There will be very little which has never been seen before, and so why can we not harness the power of "big data" to associate "pictures with causes" and at the very least give suggestions for the causes or perhaps even one day to fix the issue on the fly. Making up a new batch of eluent or changing the column would fix many of the issues that I see. We are constantly sending data back to Microsoft so that they can improve our experience on the Windows operating system—is this really so far removed from what we are trying to achieve here in terms of harnessing the power of big data?

Again, I realize that some equipment manufacturers have begun to implement some ideas around remote diagnostics and telemetry, but what exists is still a long

way short of the ideal described above and there isn't anything that I'm aware of which can take a chromatogram (or trends over a number of chromatograms) and tell us what might be wrong with our separation.

Data Management

I realize that data processing systems are capable of a very high level of automation. Integration algorithms are very advanced and the level of sophistication in terms of "custom calculations" that can be performed is high. Why then do I still see people using spreadsheets for calculation of results or collation of data into useable tables? Perhaps this says more about our adoption of the technology rather than its availability.

However, if one of my quality control (QC) results is out of specification or my system suitability contains a problem or the calibration function does not meet specification limits, it's usually off to the spreadsheet and some head scratching over whether there is enough evidence for me to scientifically and statistically justify that, actually, I have fit for purpose data. Can we not build algorithms that can interrogate the data within some basic statistical framework to do this job for us? Do I have an outlier or a batch failure?

Most of us will have a protocol for method validation and anyone doing method development work will be quite aware of what we consider to be satisfactory in terms of the number and types of data that we need to properly validate a method to the regulatory framework in which we operate. So why is it that I seem to have so many conversations about what data is required and the experiments needed to generate the data, in order to properly validate a method to, say, ICH Q2 standards? Why can I not simply load an autosampler with samples and standards and press the "ICH Q2" button on my CDS and come back some time later to see the data collected and collated? I know there will be challenges with the production of data for intermediate precision and robustness, but surely we should be able to automate a design of experiments (DOE) program that automatically varies the parameters according to a generated factorial design in order to carry out an analysis of variance (ANOVA) and show me the quality by design (QbD) type map of the method design and control spaces? Could an "instrument matrix" be used alongside automated eluent preparation equipment and column changers to produce satisfactory data on intermediate precision? One for debate I feel.

I know that some pieces of this puzzle have already been solved and that software and equipment are available to partially solve these

challenges, but I still don't see the "validate to ICH Q2" button in any data systems.

Method Development

I believe we are "almost" there in terms of analysts being able to put a sample or standard onto the autosampler and return to a fully developed method. But when I say "almost" there I mean exactly that. Some laboratories are pretty close to full automation, as are some software and equipment combinations, but I don't think we are "completely" there yet. The methods created, in my humble opinion, are sometimes more complex than they need to be and a fully viable method is not "always" reached. Again, if you know differently I'm sure readers would love to hear about it.

Further, whilst I refer to HPLC applications above, I don't see any fully formed solutions for LC–MS (automated optimization) or for GC or GC–MS method development.

Why are we not as fully automated as we need to be? Perhaps the answer lies within the question—do we actually need this level of automation?

Is it truly easier or more cost-effective for outsourced (and therefore less expensive?) workers to perform some of our more menial tasks in the hours we are not present? If that is the case, why are these folks less expensive? Are they less qualified or less well trained? Is this a situation that we can live with from a quality perspective? Are these people not who we used to call *technicians* and if so, why do we not have technicians anymore?

Whilst automation companies are fantastic at engineering and coding, do they truly understand our scientific requirements or applications enough to deliver a fully formed solution that truly meets our requirements? Is the investment required in getting someone "on the inside" trained and developed to properly integrate the automation solution the real barrier to adoption?

I apologize for the number of unanswered questions in this article, however, I've been struggling with these automation concepts for a long time and wanted to get all of my thoughts down on paper in the hope that perhaps I can spark a debate on the "big picture" solutions and bring some of the existing solutions together to solve the bigger issues—if indeed they are issues!

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Two-Dimensional Liquid Chromatography Does More Than Increase Peak Capacity

Andreas Borowiak and Jens Hühner, Agilent Technologies, Inc., Waldbronn, Germany

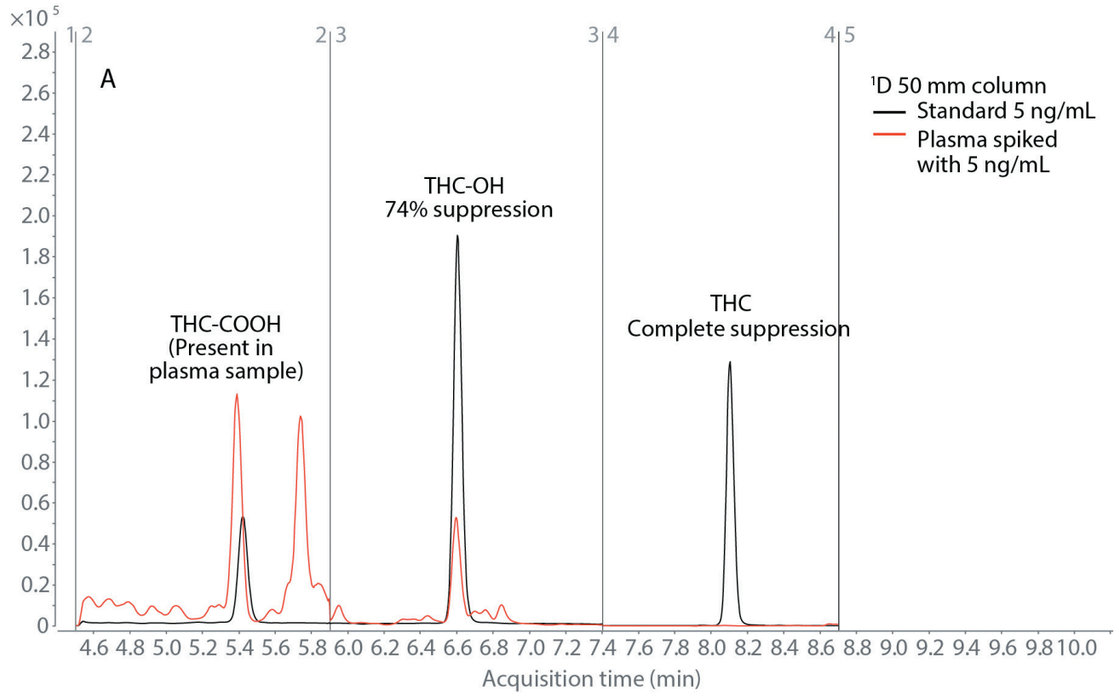
Two-dimensional liquid chromatography (2D-LC) is drawn out of the chromatographic toolbox if resolution for compounds of interest is insufficient. Recently, several studies have started to highlight 2D-LC as a tool of choice to streamline analytical workflows to increase automation making them less time-consuming. This article highlights two proven cases where 2D-LC does more than simply increase peak capacity.

Two-dimensional liquid chromatography (2D-LC) can be seen as the next logical step in liquid chromatography. To date, 2D-LC is predominantly considered when addressing unsolved chromatographic challenges; 2D-LC is considered the technique of choice when chromatographic resolution is insufficient, for example, when sample complexity is high and the number of analytes exceed peak capacity. In some cases (especially with comprehensive 2D-LC) this may result in a complex set of data requiring advanced results evaluation capabilities.

On the other hand, it is less well known that 2D-LC can also be used to streamline existing challenging analytical procedures, turning complex, manual interaction processes into automated, less time-consuming workflows. Decreased analysis time and substitution of manual sample preparation workflows, for example, when relying on manual sample desalting or fractionation-reinjection steps, have increasingly become areas of interest in the chromatographic community. One of the two

Photo Credit: Olarn Meesang/Shutterstock.com

Figure 1: Comparison of a 1D analysis of a standard solution and a plasma spiked solution containing THC, THC-COOH, and THC-OH demonstrating the 74% suppression for THC-OH and the complete suppression for THC.



chromatographic dimensions can be used to perform a sample preparation step that can be performed on-line, automatically and without time spent on sample transfer. This article highlights two case studies where significant reduction in analysis time was achieved by either substituting multiple one-dimensional (1D) separations or manual sample preparation by 2D-LC.

Case Study 1: Online Desalting Using 2D-LC for the MS Determination of Glucagon Analysis in Accordance with *USP* 39

Desalting often requires repetitive protocols, which are monotonous and time-consuming. Such protocols are error-prone and lack good reproducibility, which is critical, especially in profiling or quantitation workflows. Moreover,

they rely on disposable supplies and consumables, which are neither economical nor ecological when compared to on-line sample preparation workflows. A method was developed for the analysis of organic impurities in glucagon applying mass spectrometry (MS) detection (1). The first-dimension separation was in accordance with *USP* 39 but lacked MS compatibility, and was then followed by on-line desalting to enable MS detection in the second dimension of the 2D-LC analysis. The 1D assay and the analysis of the complex glucagon profile rendered it almost impossible to find an MS-compatible eluent.

For an acceptable separation, the *USP* 39 LC–UV method used potassium phosphate in the mobile phase. This mobile phase was not compatible with MS detection but did provide excellent separation of glucagon and the four deamidated variants of glucagon. Deamidation is the loss of ammonia from asparagine or glutamine, respectively, and resulted in a +1 Da mass shift. Glucagon contains one asparagine and three glutamine side chains and deamidation is a common protein reaction during thermal stress. This glucagon analysis provided sufficient separation between the different analytes of interest, but only with ultraviolet (UV) detection. For more information on the analytes, MS detection and 2D-LC was required. An automated multiple heart-cut (MHC) approach was used, whereby all analytes were transferred into the second dimension. Automated desalting (diverting of the salt fraction off-line) and MS detection was then performed after the second dimension. Diverting the salt prior to the MS detection required a complete separation of the salt plug from the analyte of interest. This was possible by using a 2.1 × 12.5 mm column in the second dimension. Completed in accordance with *USP* 39, the retention time relative standard deviation (RSD) (n = 5) was 0.27% and peak area RSD (n = 5) was 0.11% compared to the suitability requirement of ≤2.0%.

In general, MHC 2D-LC can be used as an effective desalting solution to allow hyphenation of chromatographic methods applying MS-incompatible mobile phase to MS detection (2,3).

Case Study 2: 2D-LC–MS to Reduce Ion Suppression in the Determination of Cannabinoids in Blood Plasma

The analysis of cannabinoids in biological fluids, such as blood plasma or urine, is an important workflow for forensic

toxicology laboratories. Detection of Δ-9 tetrahydrocannabinol (THC) and the corresponding metabolites in plasma are indicators of drug abuse. The main metabolites are carboxy-THC (THC-COOH) and hydroxy-THC (THC-OH). Both can be detected in biological matrices over a long period, therefore indicating a history of drug usage. THC itself is quickly metabolized and can be found only for a short time after THC consumption (4,5).

Analysis of these three substances in crude matrices, such as blood plasma, requires extensive sample preparation techniques such as protein precipitation, liquid–liquid extraction, solid-phase extraction (SPE), or a combination of the above. The primary reason for not applying direct LC–MS analysis for these types of samples is the large amount of matrix entering the ionization source. This impacts the ionization process, which can result in ion suppression. Moreover, the sample matrix can also lead to overall sensitivity reduction of the MS instrument by contaminating the ion source and the MS lens entrance over time. Therefore, matrix reduction or elimination is mandatory. On-line approaches to speed-up sample analysis time are therefore an attractive option. In another developed method, it was shown that MHC on the analytes of interest and analysis of the cut(s) in the second dimension with orthogonal separation principle significantly reduced the ion suppression effects for THC and the metabolites. In contrast, utilizing only a one-dimensional method resulted in an ion suppression for THC-OH of 74% and a complete suppression for the THC signal. This effect is illustrated in Figure 1. Without matrix removal, quantification was not possible. The only alternative approach to quantify THC in matrix following this workflow would be via the collection of fractions and re-injection on a second column–mobile phase combination with different selectivity. The complete THC peak was sampled in small fractions, and each of these fractions were analyzed automatically on a second-dimension column. In this case, a phenyl-hexyl stationary phase with formic acid and methanol as the mobile phase were selected. No ion suppression effects were observed using this 2D-LC approach. Good precision, linearity, and excellent recovery values were found for all three analytes (1).

Conclusions

In summary, 2D-LC will not only increase the peak capacity for complex samples, it can also reduce the overall analysis time and substitute manual steps by using the second dimension for desalting or removal of matrix. Moreover, the use of 2D-LC for desalting or sample clean-up workflows is gaining more attention in the chromatographic community. As Petersson *et al.* state, they were able to optimize an existing protocol for the characterization of biopharmaceuticals in salt-based separation methods and shorten their workflow from days to hours by avoiding fraction collection and solvent exchange steps (6).

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Solvents: An Overlooked Ally for Liquid Chromatography–Mass Spectrometry

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Liquid chromatography–mass spectrometry (LC–MS) platforms are continually developing to offer improved sensitivity to meet the analytical demands of today's laboratories. However, choosing an inappropriate solvent can significantly undermine the quality of results, even when using the most advanced technology; a high-purity mobile phase with excellent batch-to-batch consistency is essential for reliable and reproducible results. This article discusses the importance of selecting the correct grade of solvent for LC–MS analyses and some of the challenges arising from an insufficiently pure mobile phase.

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Analytical laboratories currently face ever-growing demands for increased sample throughput and faster turnaround times. At the same time, advances in technology enable analytes to be detected at lower concentrations than ever before, presenting a challenge to analysts striving to maintain the accuracy and reproducibility of their results. Liquid chromatography–mass spectrometry (LC–MS) has become the technique of choice for many laboratories working in a broad range of scientific fields, including pharmaceutical, food and beverage, forensics and toxicology, environmental monitoring, and R&D. Its popularity is largely because of its low limits of detection for both qualitative and quantitative analyses. As instrument manufacturers continue to improve the sensitivity and mass accuracy of their LC–MS systems, analyte detection limits are set to decrease even further, highlighting the need to ensure that any potential interferences are reduced as much as possible.

The Impact of Solvent Choice on Potential Interferences

Choosing an ultrapure solvent is critical to reliable and reproducible instrument operation and high-quality results. However, the choice of available solvents can be overwhelming, sometimes creating the temptation to cut corners. For example, while it is obvious that a laboratory-grade solvent is unsuitable for highly sensitive analytical techniques, people still wonder if choosing a high performance liquid chromatography (HPLC)-, rather than LC–MS-, grade solvent makes any difference. In fact, the consequences can

be considerable, because LC–MS-grade solvents will have undergone additional purification steps to remove trace metals and other impurities that could potentially interfere with the analysis.

Purification Methods

Purification methods will vary between manufacturers, and some will be more effective than others. In many cases, solvents are batch selected, with suppliers testing each batch for suitability on delivery before repackaging it into smaller containers for laboratory use. However, while the solvent may be optically pure and appear clean to the naked eye, it is likely that a considerable non-volatile residue will have accrued during shipping. This can be problematic, potentially causing blockages in HPLC lines, pumps, and columns. In contrast, extensive purification by chemical treatment and glass distillation significantly reduces impurities and enhances batch-to-batch consistency, reducing unnecessary instrument downtime and the need to troubleshoot ghost peaks.

The Ideal Solvent

Ideally, the solvent should have been functionally tested to ensure that it is suitable for use as a mobile phase. Typically, functional testing is done using the reserpine test. A reserpine standard of a specified concentration is run in both electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) modes to evaluate the presence of any impurities and establish that the signal intensity to background noise ratio meets the requirements for successful LC–MS analyses.

Particulates can block inlet and in-line filters and LC columns—reducing column life span and causing costly unscheduled downtime—so it is important to select LC–MS solvents that have been prefiltered with a 0.2 µm (or smaller) filter to eliminate these impurities. This is especially important for users of ultrahigh-performance liquid chromatography (UHPLC)–MS systems, which use narrower bore columns and smaller particle sizes.

Solvent Choice and Background Noise

LC–MS systems may be equipped with diode-array detection, and a stable, flat baseline with minimal drift is essential. The solvent should be free from impurities showing UV–vis absorbance, such as organic acids. Similarly, solvents offering the lowest possible mass background are crucial for trace MS analyses, maximizing the signal intensity at low analyte concentrations. Contaminants—such as plasticizers or phthalates—can be introduced during the manufacturing process, for example, from production area surfaces and equipment, and manufacturers must be vigilant and implement measures to ensure these are tightly controlled.

The Impact of Trace Metals on MS Spectra

The formation of metal adduct ions is a particular problem for ESI analyses. Typically, this occurs with alkali metal ions, such as sodium and potassium, although adduct ions may also arise from other species, from salts such as ammonium or chloride, or even solvent molecules. Adduct ions in APCI must be volatile and so, while ammonium, chloride, or water adducts can arise, metal ion adducts do not.

The formation of adduct ions has a twofold effect. First, they lead to complex MS spectra and a reduction in the signal intensity to background noise ratio, resulting in less sensitivity. Second, adduct ion formation is inconsistent from run to run, likely leading to a decrease in the accuracy and reproducibility of the analytical data generated. To reduce this effect, it is vital to choose a solvent that has undergone stringent testing for metal ions, as well as other ionic species that could cause ion suppression.

Best Practices

Correct storage and handling is essential to maintaining the quality of a solvent once it is in use, because it is easy to forget that contaminants can be absorbed from the environment. Analysts should also take care not to inadvertently introduce contamination when handling and preparing mobile phases, remembering that, for example, plasticizers can leach from plastic gloves. In addition, using pipettes to add reagents such as trifluoracetic, acetic, or formic acids to the mobile phase can be a potential source of contamination. Ideally, different sets of pipettes should be used to prepare the mobile phase and the stock solutions for analysis.

LC–MS mobile phases should be stored on the instrument for the minimum amount of time, and solvent reservoirs should always be capped. The choice of storage container should also be considered carefully. For example, plastic bottles leach plasticizers into aqueous solvents over time, while glass containers release ionic contaminants and organics, although at a lower level than their plastic counterparts. Generally, this makes glass reservoirs—preferably borosilicate glass—the material of choice.

Aqueous mobile phases and water are prone to microbial growth during storage, which can cause blockages and result in spurious peaks. Regular replacement of the mobile phase, accompanied by flushing the LC system to ensure the removal of any residual solvent from the previous batch, will help to reduce the likelihood of microbial contamination. Equally importantly, analysts should resist the temptation to "top up" solvent reservoirs, rather than replacing them with a fresh bottle of mobile phase. And, of course, storage reservoirs should be thoroughly cleaned before reuse.

Conclusions

State-of-the-art LC–MS instruments demand mobile phases prepared from ultrapure solvents to achieve the best possible sensitivity, accuracy, and reproducibility. While this begins with careful choice of manufacturer, laboratories need to follow best practice to avoid contamination during the preparation and use of mobile phases. By using LC–MS-grade solvents and ensuring that mobile phases are correctly handled and stored in the laboratory, analysts can be certain of getting the most out of their LC–MS systems.

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Robustness Modelling in Ultrahigh-Pressure Liquid Chromatography Methods

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Many workers in pharmaceutical laboratories are unable to change any aspect of their methods, although they often encounter severe problems and create many out-of-specification (OoS) results. They are particularly afraid to investigate these problems from a chromatographic perspective in case they generate new unforeseen problems. In the literature, however, there are numerous examples showing that it is worthwhile trying to understand the reasons for "unexplainable" behaviour in ultrahigh-pressure liquid chromatography (UHPLC) using modelling. By using modelling, problems can be recognized and often eliminated with legal operations according to the allowed tolerance limits mentioned in pharmacopoeia descriptions. The following article aims to show that "visual chromatographic modelling" can be a useful aid.

A. Schmidt *et al.* (1) discussed the drug pramipexol, which was showing repeated out-of-specification (OoS) results. Sometimes it was a turnover in peak elution order of two neighbouring peaks, sometimes they coeluted. The reason for this observation was that the set point (SP) was close to the edge of failure (EoF) at an extreme part of the design space (DS). This unstable condition could be found with the help of modelling software and corrected by reducing the concentration

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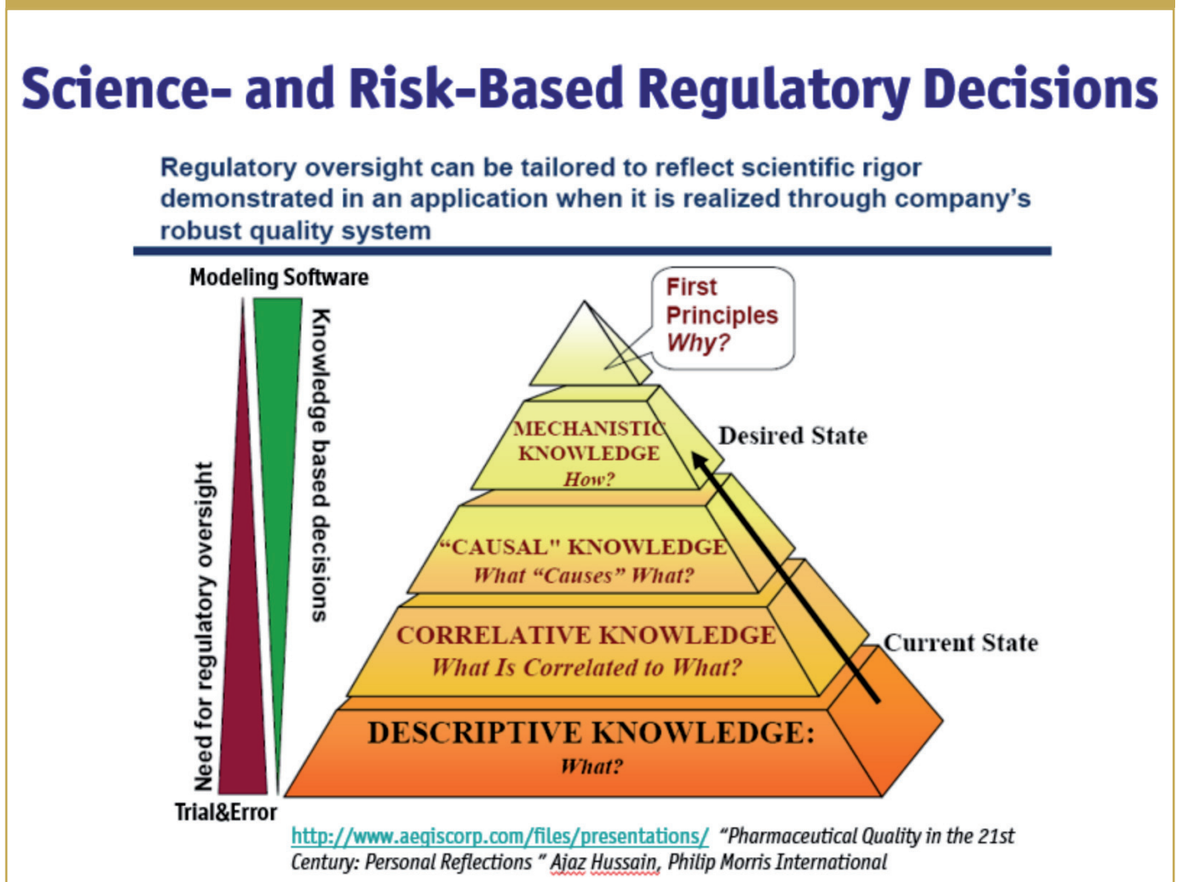
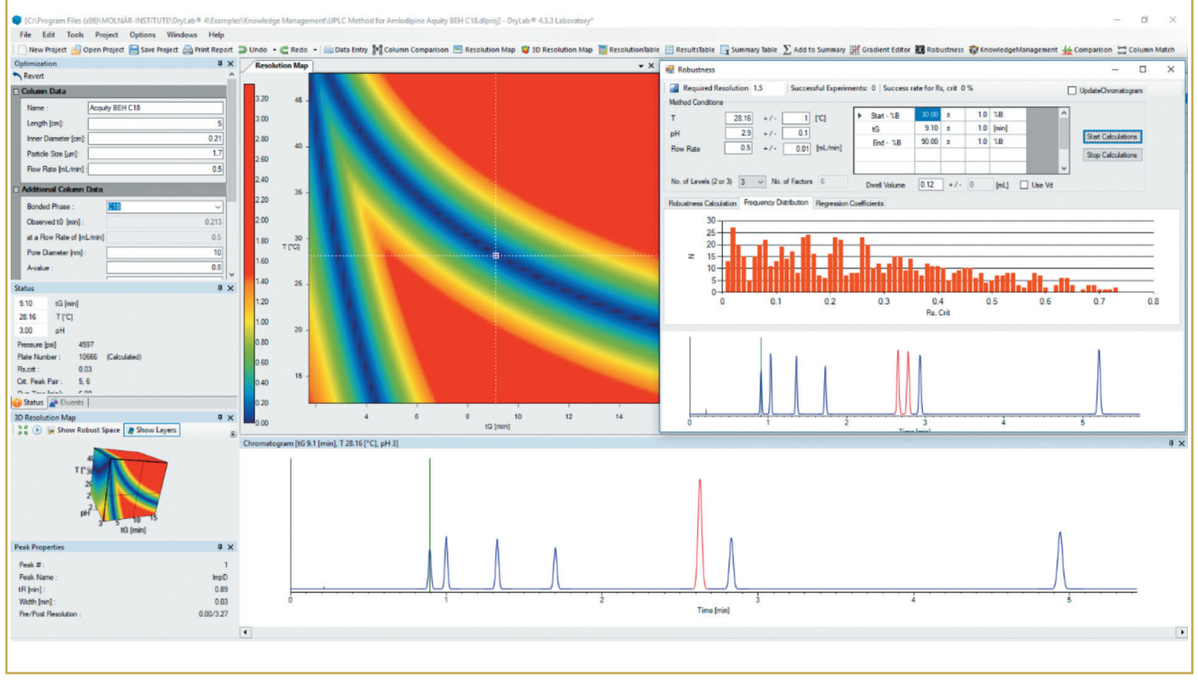
Figure 1: The more we know (the higher we are in the pyramid), the less regulatory oversight is to be expected. In other words, the regulatory agency becomes active if they sense that the applicant doesn't really know what they are doing. As UHPLC is sometimes complicated, understanding can be gained by modelling, where generating a chromatogram takes typically less than one second. Adapted with permission from reference 5.



of the ion pair reagent to prevent the coelution and peak turnover happening again.

Another common problem is the fear of changing a method, even when it is obvious that the method is out of date, takes too

Figure 2: Robustness calculations at the wrong location. The set point is at a blue coelution line, consequently the robustness is insufficient. The success rate in routine QC = 0, as the ATP (the critical resolution) is between 0 and 0.75, way below the requested range of 1.5 or higher (2). The process capability is insufficient.

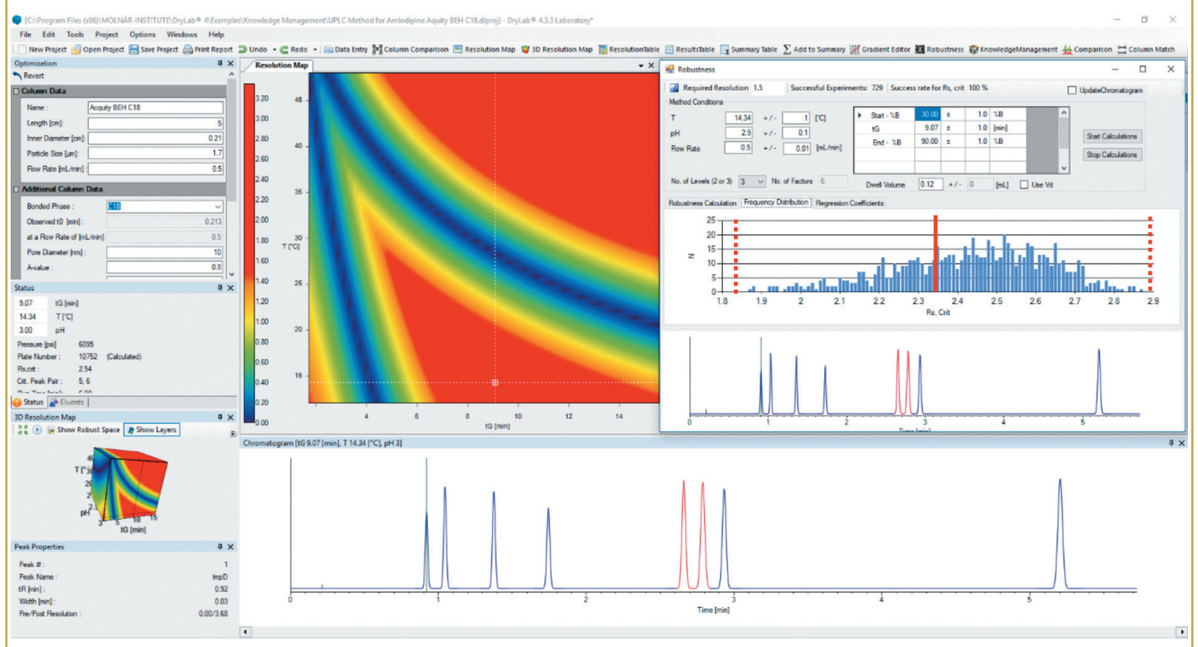


long, and exhibits often unexplainable OoS findings, resulting in weeks of lost time and a lot of confusion (1,2). With modelling these problems can often be resolved in a fast and efficient way (3).

What Can We Change in a Validated Method?

What can be legally changed in methods can be found in the *United States Pharmacopeia (USP)* and in the *European Pharmacopoeia (Ph. Eur.).* Chapter 2.2.46 of *Ph. Eur.* "Chromatographic Separation Methods" contains the following description: "These allowed adjustments may be necessary because the stationary phases are described in a general way, and there are a variety of phases available commercially that meet these general descriptions, which can result in

Figure 3: After changing the temperature to a lower value, there is an improvement in success rate to 100% (2) in the range of $R_{s,crit}$ 2.34 +/- 0.5, corresponding to a process capability of Cpk > 1.0.



chromatographic behaviour differences" (4). Chapter 2.2.46 is similar to the *USP* Chapter 621, concerning the adjustments allowed.

Many workers are concerned that quality by design (QbD) compatible modelling in the analytics means that criticism of QbD in the production would also follow, but ICH Q8R2 states explicitly that more knowledge based on solid science causes less regulatory oversight (Figure 1).

UHPLC Method Adjustments

Heiko Behr wrote in his blog: "The last liquid chromatography allowed adjustments revision in 2010, stated that adjustments for gradient methods are more critical than isocratic methods. These changes can lead to shifts in peaks and to a different step of the gradient. This then leads to the incorrect assignment of peaks, peak masking, or an elution shift that occurs beyond the prescribed elution time. As a result, the allowed adjustments

were class-divided for isocratic and gradient methods, with minor allowed adjustments for the latter. Effective from August 2014 (*USP37-NF32*, *1st supplement*) the *USP* split the allowed adjustments into isocratic and gradient sections. In addition, the *USP* introduced a substantial change in the column related to allowable adjustments for isocratic methods to improve user flexibility" (4).

The primary focus is keeping the column plate number, and thus resolution, fairly constant. Similarly, to the last revision of the *USP* 621 chapter, the *L/dp* ratio was introduced for maintaining nearly constant efficiency and, therefore, resolution. But this change is not only valid for isocratic elution (like in the *USP*), it is also customized to gradient methods. This explanation is very much valid in gas chromatography (GC). In reversed phase chromatography, the selectivity is governed by interactive forces in the aqueous eluent (6).

Behr is showing the differences between the allowed adjustments for isocratic and gradient liquid chromatography methods for the new *Ph. Eur. Draft*, the current *Ph. Eur. Supplement 9*, and the current *USP 40-NF35* in useful tables (4). The "allowable changes" are sometimes vague and would lead in several cases to OoS results. The only way to make changes should be done by modelling based on a few basic experiments around the SP, which would discover how peak movements occur and from there one can return in modelling to the original validated system suitability test (SST), which is validated. If the adjustment is not possible because the deviation is too large, then a revalidation has to be performed. This is currently not a problem because the 50–100-mm-long columns of small particle sizes (d_p: 1.7–1.8 µm) have analysis times typically below 10–15 min, so the validation would not require more than a few days. It is also important that the model is included in the new master file, allowing later adjustments because alterations of the SP inside of the DS are not considered to be a "change", but an adjustment to meet system suitability parameters.

UHPLC Method Robustness

The communication with regulatory bodies includes relatively high handling charges for "post approval changes" and are connected with a great deal of bureaucracy. Therefore, pharmaceutical and food companies are working to improve the robustness of their methods, so they can safely be used in routine work. Nevertheless, methods developed by trial and error are always subject to many OoS events, causing a breakdown in production and interrupting

the provision of new, promising drugs to patients.

Regulatory agencies are therefore required to prove the robustness of new UHPLC methods. Earlier it was sufficient to submit methods with decent statistics, today the regulatory agencies want to know how the method was developed and why the pH, gradient shape, temperature, or the flow rate were selected and localized specifically at a given SP in the submissions.

In 2006 the regulatory expectations changed to more scientific fundamentals. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) mentions the regulatory expectations in the ICH Q8, R2 towards more scientifically reliable drug master files. QbD should now be introduced in the analytical work.

What is QbD?

QbD is the end of trial and error. The UHPLC method development process with QbD principles should be properly documented and the method should be based on solid science. The work should be carefully planned, instead of based on the "intuitions" of "experienced" users in the laboratory. This includes registering all starting factors, such as the batch number of the stationary phase, the serial number of the column, the instrument configuration, type of pumps, injection devices, and detectors, all in one data set, including parameter tolerances. These are included in the installation qualification (IQ) and should be regularly revisited. Another problem is that laboratory workers often forget some details and therefore have to repeat the experiments several times. The best way to get a precise description of the method conditions is to collect them from the instrument in a method report.

All this was leading to an automated generation according to different design of experiments (DoEs), which was started by modelling tools, where the UHPLC instrument was preprogrammed to perform a number of runs in a sequence, followed by an evaluation of the results. The question is, however, how to get as close as possible to the separation optimum? Even in the case of a sufficient separation, variabilities of the parameter could interact with each other and the analytical target profile (ATP), often the critical resolution, would fall below the validated level of 1.5 (baseline separation).

Currently, a submitted method has to be validated, which means the applicant has to approve that the method is well suited for its intended use. However, many times in the validation process, which consists of a large number of runs for statistical purposes, one obtains OoS results.

One realizes, often too late, that the method was not robust at all and the method has to be redeveloped. Therefore, we should now include the robustness tests before the validation step. Modelling software can test all the eventual problems in a much shorter time than one would be able to do in the laboratory.

What is More Important, Knowledge of Statistics or Knowledge of UHPLC?

The experienced user says that if the chromatographic method is robust, one would never have any problems with the statistical evaluation of the method in the production process.

How is it Possible to Make a New Submission According to the Above Principles?

In the ICH Q8, R2 there is a request to use solid science in the submissions. Therefore, a scientific model of the chromatographic history of the method should be included in the submission in a "Knowledge Management Document", including the experiments for the model, which would allow later potential changes in the SP. As far as the SP remains inside of the DS, no revalidation will be necessary. This will allow production costs to be reduced and make method handling more flexible. The cooperation with regulatory agencies will become more relaxed and harmonious.

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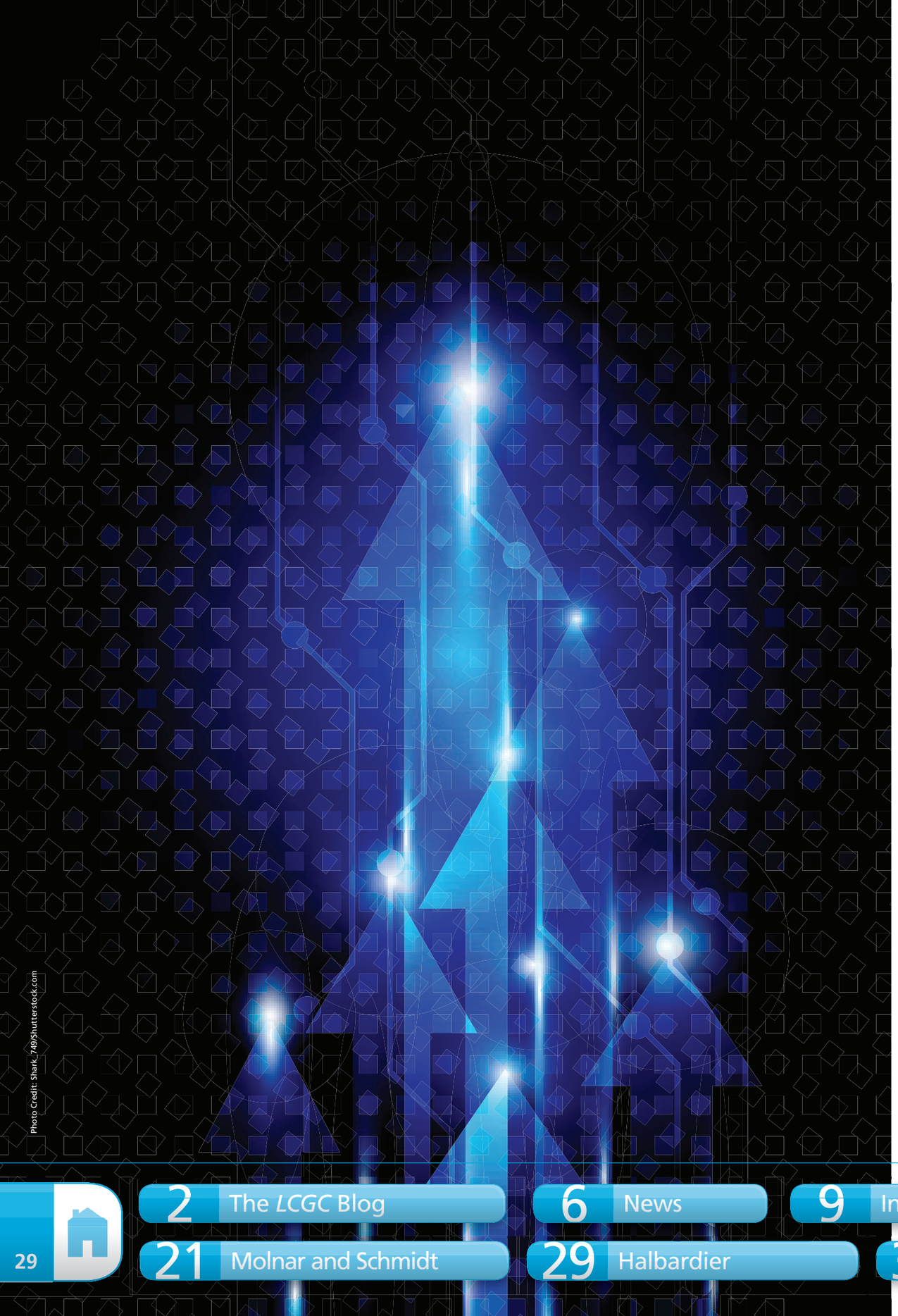

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Application of Novel Balance Systems: Next Step Towards Laboratory Automatization?

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The key differences between a classical balance and currently available automated systems from the good weighing practice (GWP) perspective and within the scope of ISO9001:2015 quality standard are discussed. The systems under review are: (i) manual analytical balances; (ii) semi-automatic systems; (iii) fully automatic systems; and (iv) integrated systems that use on-line coupling of the analytical balance with the instrument used for analytical measurements. The parameters defined in GWP guidelines, such as accuracy, uncertainty, minimum weight, and risks (including out-of-specification results), will be evaluated for all of these systems. Additionally, data integrity, costs, and time demands associated with respective approaches will be discussed. Finally, the weighing process efficiency will be reviewed in three diverse example applications including gravimetric methods for pharmaceutical analysis, semi- or fully automatic balance in preparation of reference standard solutions in pesticide residue analysis, and the use of a fully integrated system in a high-throughput good manufacturing practices (GMP) release laboratory.

Despite substantial advances in mechanization and automatization of repetitive, labour-intensive, or hazardous tasks in the laboratory environment, the work of the analytical chemist remained largely manual for most of the 20th century. Indeed, the process of analytical laboratory automatization, particularly in sample

preparation and instrumental analysis, began in the 1980s (1). The use of automated analytical balance systems remains a tool that is sporadically implemented in various fields of analytical chemistry. When an analytical result depends on the weigh precision, a question arises: Why don't analytical laboratories invest in versatile automatization systems to improve analytical results to avoid errors incurred during manual weighing?

The Automated System in Analytical Chemistry

In the 15th century, the first development of a "robot-like" system was reported (2,3). Then, with the Industrial Revolution, system automatization and mechanization developments led to humans being replaced for repetitive, labour-intensive, or hazardous tasks. However, the analytical chemistry laboratory remained manual for a large part of the 20th century. Despite automatization being recognized as reducing errors and improving accuracy and reproducibility, only tasks such as pipetting, centrifugation, mixing, and autosampling were automated early. Currently, a variety of automated systems (autosampler, micropipette) find their place in different analytical laboratories (1), but the automatic analytical balance remains a tool that is used sporadically.

An automated instrument can be either "off-line" or "on-line". For the standalone or "off-line" instrumentation, human manipulation is still required for moving the samples from the preparation system, for example, dilution, transfer, solid-phase extraction (SPE), to the analytical measurement system. In the integrated instrumentation or "on-line", the samples carry on automatically, without human intervention, from the preparation system to the analytical measurement system (1).

The benefits of automatization in the analytical laboratory are not only a reduction in manual labour and the risks involved in hazardous tasks, but also an improvement in data integrity, downscaling, improvement in accuracy, speeding up of analysis processes, a reduction in expenditure on costly chemicals, and a reduction in sample contamination and human error (1). Specifically, balance automatization and gravimetric methods reduce error risk from using a volumetric flask (calibration, filling to the line, contamination, cost, and mixing), the labels, and the calculated final concentration (4,5).

Weight Accuracy and Good Weighing Practice

As a reliable analytical result depends on the weight precision, authorities and

Figure 1: Pfizer example of manual and automatic balance comparison. Adapted with permission from reference 7.

	Manual Prep	Automated Prep	Difference
Amount of substance	20 mg solid +50 mL diluent	5 mg solid +12.5 g diluent	Save 75% substance
Time	50 mins (total) 35 mins (FTE)	30 mins (total) 10 mins (FTE)	Save 70% labour time
Precision	%RSD = 1.67	%RSD = 0.49	Improve precision x 3

Reproducibility and precision:
Comparison between manual and automated gravimetric sample preparation.

	Manual Prep	Automated Prep	Difference
Sample size	100 mL diluent	10 g diluent	Save 90% substance
Time	60 mins (total)	45 mins (total)	Save 25% time
Correlation coefficient	0.99473	0.99998	Improved to near perfect
Unknowns (% intent)	97 – 100 %	100 %	Improved to near perfect

Linearity:
Comparison between manual and automated gravimetric sample preparation.

accreditation bodies encourage quality control to be set up. Generally, duplicate preparation on a calibration system and solution comparisons are required. For example, ISO9001:2015 enforces a process approach for risk management and quality. In parallel, good weighing practice (GWP) guidance in the form of a science-based global standard for efficient life cycle management of weighing systems was introduced in 2013 (6). This guide includes the routine testing of equipment that will not be discussed in this article. However, the measurement uncertainty and minimum weight concept will be detailed below.

True and Precise

The International Organization for Standardization (ISO) defined accuracy as true and precise. The trueness refers to the closeness of agreement between the arithmetic mean of many test results and the true or accepted reference value. The precision refers to the closeness of agreement between test results. This accuracy is the main factor of measurement uncertainty, meaning that a normal distribution around the measure weight can be observed. This distribution widens with the small masses measurement. The minimum weight is defined by the minimum weight that a system can measure within an acceptable uncertainty. In addition, the manufacturer adds a safety factor to this minimum weight to take into consideration changing environmental conditions that affect the performance of the instrument, such as vibrations, drafts, wear and tear, or temperature changes. As mentioned, the system drifts over time and needs to be checked and recalibrated in a defined time scale (6).

Accuracy, uncertainty, minimum weight, and risks (including out-of-specification [OOS]) will be evaluated throughout the different automated systems.

The Different Weighing Systems

This article will focus on analytical balances, micro balances, and ultra-micro balances, with readability from 0.1 mg for the analytical balance to 0.1 µg for the ultra-micro balance. Semi-automatic, automatic, or integrated systems are upgrades from the manual system. Therefore, the instrument description will be limited to the option to improve weight accuracy and the automatization option. But first, the definition and advantage of gravimetric methods versus volumetric will be explained.

Gravimetric Method Versus Volumetric Method: The definition of a gravimetric method is weighing not only the solid but also the solvent to enable a specific concentration to be prepared accurately and precisely, whilst a volumetric method uses an accurate volume container. Generally, a volumetric method is performed in a volumetric flask, however, to reduce contamination risk, an exact volume can be dispensed by a positive displacement pipette. Indeed, a volumetric flask is a potential source of contamination, interferents, or noise (from electrolytes for a more selective detector) (7).

Why are gravimetric methods more accurate than volumetric? Volumetric flasks are not recalibrated in-house, and there are also error risks associated when using a volumetric flask. These are numerous: (i) the weighing boat: weighing by difference (transfer of the contents, weigh the remaining), or transfer all the contents with a liquid; (ii) the volumetric flask: the working temperature and the meniscus reading. On the other hand, gravimetric methods reduce contamination risks as well as error

Table 1: Mettler Toledo and Anatune data summary (6,7,8)

	Manual Weight	Automated Weight	On-Line System
Targeted Value	0.6 mg/mL	0.6 mg/g	5 g
n	10	9	32
Average	12.906	10.176	4.941 g
Deviation	0.076	0.018	0.012
RSD (%)	0.57	0.21	0.224

risks, giving only a known uncertainty on both the compound and the solvent weight (7).

The Manual Balance: Apart from the accuracy of the different systems (understanding the accuracy improvement from analytical to micro or ultra-micro balances), different options can be added to improve manual weighing, for example, isolation of environmental variation, the plate, and the anti-static charge.

Analytical balances are sensitive to environmental factors including air flow, bench vibration, or movement. To decrease this effect, the balance can be isolated on a specific table; a graphite plate can also isolate the balance from the bench environment. The weighing plate can be isolated by suspension to decrease static elements such as powder between the plate, and the measurement system or wall around the plate (a double wall for micro balance, or a wider one for ultra-micro balance) can decrease the air flow effect.

Plate suspension has already been discussed, however, a grid plate (with a centre mark for a large plate) or smaller plate can be used. The first one can be used only for a suspended plate on analytical balance whilst the size reduction is used for more accurate micro- and ultra-micro balances.

Generally, modern balances are electronic and sensitive to electrostatic charge. These charges can come from the weighing product itself, the weighing container, or the manipulator. There are different options to reduce it, for example, using an anti-static gate, either off or on the system.

The Semi-Automatic System: The system is qualified as semi-automatic because the final container of the preparation has to be loaded manually between each weighing. There are three different systems: one for liquid dispensing, one for solid dispensing,

Table 2: Mettler Toledo data from automated gravimetric sample preparation data (7)

# Solution	Dose API (mg)	Solvent Added (g)	Solution Concentration (mg/g)	Area Correlated to 0.6 mg/g
1	10.105	16.7481	0.60299	2584.00634
2	10.320	17.1048	0.60298	2582.52818
3	10.140	16.8063	0.60298	2584.69296
4	10.125	16.7815	0.60298	2582.72885
5	10.250	16.9885	0.60299	2584.24611
6	10.200	16.9058	0.60298	2583.2152
7	10.130	16.7895	0.60299	2589.51769
8	10.040	16.6408	0.60297	2596.79455
9	10.275	17.0297	0.60299	2591.82747
Mean	**10.176**	**16.866**	**0.603**	**2586.617**
RSD (%)	**0.894**	**0.893**	**0.001**	**0.19**

and a combination of liquid and solid dispensers. One of the typical applications for the combined system is standard preparation.

The combined system includes a manually adjustable dispenser at head height (the height is manually commutable) to weigh directly into the destination container, a suspension plate with a reduced size compared to a manual balance, optional anti-static gate, and double wall for micro-automatic balance. The accuracy is improved by reducing the manual operation and thus reducing electrostatic interferences. The accuracy is improved by a factor of three compared to manual weighing. Another advantage is that the final concentration is recorded directly into the electronic laboratory notebook, which is easily transferable to a laboratory information management system (LIMS) and printable on a label.

The Fully Automatic System: This system has the same technical parts as the semi-automatic but with an autosampler. Sample weighing or small-scale production are two possible applications for this system.

On-Line System: A manual balance is used within a robotic system and thus without human manual handling. It offers the same accuracy as a semi-automatic balance. A typical application example is difficult weighing samples (that is, sticky samples).

Balance Comparison: A comparison between the accuracy of manual balances and automatic balances and a comparison between volumetric methods on manual balances (traditional across the sector and laboratories) and gravimetric methods on automatic balances are summarized in Table 1 (7,8,9).

A precision of 0.57% is observed for manual balances whilst a precision of around 0.2% is observed for an automatic system, including the integrated system (5). The trueness (recovery) is three times closer to the true value for automatic systems than manual. Better accuracy of the automatic system led to a smaller minimum weight. If for a manual system the minimum height is X, for the same system under automatic conditions the minimum weight will be around 0.67 X. A smaller minimum weight allows the method to be scaled down and made more cost-effective in term of solvent, waste, and costly reference compounds (7,8,9).

Applications

Pharmaceutical Sector: Expensive reference materials, active pharmaceutical ingredients (APIs), or compendium standards in parallel with the huge amounts of volumetric flasks used, unknown peak identification (contamination from volumetric flasks), and a request for accurate (precise and accurate) data make automatic balances a useful tool for an analytical chemistry laboratory performing pharmaceutical release, method development, or small-scale research and development.

The example presented here focuses on data released in parallel with method uncertainty (as defined in ORA-LAB. 5.4.6) (10).

First, FDA<1251> (11) defines the use of automatic balances, meaning that automatic can be used in line with regulations. Data integrity is a regulation hot topic, whilst an automatic balance integrated easily into all the systems can be configured to communicate directly to a LIMS system. In addition, printing accurate labels can guarantee a better traceability. Therefore, the use of an automatic balance is recommended by FDA<1251> (11).

A further reason why FDA<1251> recommends automatic balances is related to method uncertainty (11). Indeed, one of the tightest release criterion on a drug is ± 2% of the certified value. With a precision of approximately 0.5%, a liquid chromatography–ultraviolet (LC–

UV) analytical measurement is often performed. However, the weighing methods will have an impact on the overall uncertainty. Table 2 presents a compound analyzed by LC–UV (injector precision of 0.21%) with a gravimetric preparation on an automatic system. A 0.2% precision is achieved with this system without any volume or weight outside the method procedure criteria (7). Compared to the 0.6% relative standard deviation (RSD) on the weight, a tolerance of 0.4% on the volume of a volumetric flask, and the 0.5% of the analytical measurement system—cumulated up to 1.5% for a manual volumetric method—the automatic system provided a tolerance of approximately ten times smaller than the criterion limit whilst the manual system uncertainty represented 75% of the tolerance limit. Using an automatic balance improved the closeness between the value measure and the true value ensuring patient safety. This is also illustrated in the example from Pfizer (Figure 1) (7).

The other important aspect is the scale-down process and the lower minimum weight to support expensive or rare API research and development.

Standard Preparation for Pesticides Analysis: Multiresidue analysis methods like pesticide residue methods or QuEChERS (quick, easy, cheap, effective, rugged, and safe) can include more than 500 different reference materials to weigh. On a manual instrument, all of these compounds have to be weighed in duplicate (generally a laboratory goes for a commercial solution as a second duplicate), the concentration calculated, and then transferred to a labelled vial. The process is time-consuming and can occur every year for a high-throughput laboratory.

The concentration of working solutions is in the ηg/mL scale, whilst stock solution preparations are in the mg/mL scale or commercial solutions in the µg/mL scale. What are the advantages of using an automatic balance? First, an automatic system is fully compliant under ISO 17025 (normal for pesticides analysis). Second, it allows better data integrity in terms of concentration and solution labels, but also accelerates the process with the possibility to store the reference materials inside the weighing head of the freezer. Third, it allows mixes to be prepared at lower concentrations. Finally, a weighing scale allows more accurate solutions to be prepared. For example, a 25-mg reference material can be weighed three times accurately (minimum weight of 7 mg) in a unique stock solution of 1 mg/mL, 21 mL stock solutions can be prepared, and 2.1 L of solutions at 10 µg/mL (commercial mix solution currently available). A higher volume and a longer use of the reference standard is therefore observed compared to manual weighing.

With a full automatic system that includes an autosampler for 30 vials, multiple stocks or mix reference solutions can be completed and dispatched easily. These solutions have their weight and concentration directly recorded and associated to each vial filled in the automatic system.

High-Throughput GMP Laboratory: This example focuses on a high throughput GMP release. Indeed, clinical laboratories and other sectors can find benefits to an integrated system (7).

In addition to high accuracy, the integrated system can reduce sample handling by the operator and can run in parallel to the analytical measurement system, reducing degradation overtime and overall sample preparation time. This last benefit gives the analyst more time to interpret the data, which can have an improved traceability over the sample preparation.

Conclusion

Versatile weighing automatic systems exist and their applications look promising in terms of accuracy, time savings, cost, and data integrity. Instrument cost remains acceptable compared to the potential source of revenue and the day-to-day cost reduction (solvent used and waste reduction included). However,

a full evaluation of the investment cost and revenue must be calculated on a case-by-case basis.

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Direct Liquid Chromatography Tandem Mass Spectrometry Analysis of Glyphosate, AMPA, Glufosinate, and MPPA in Water Without Derivatization

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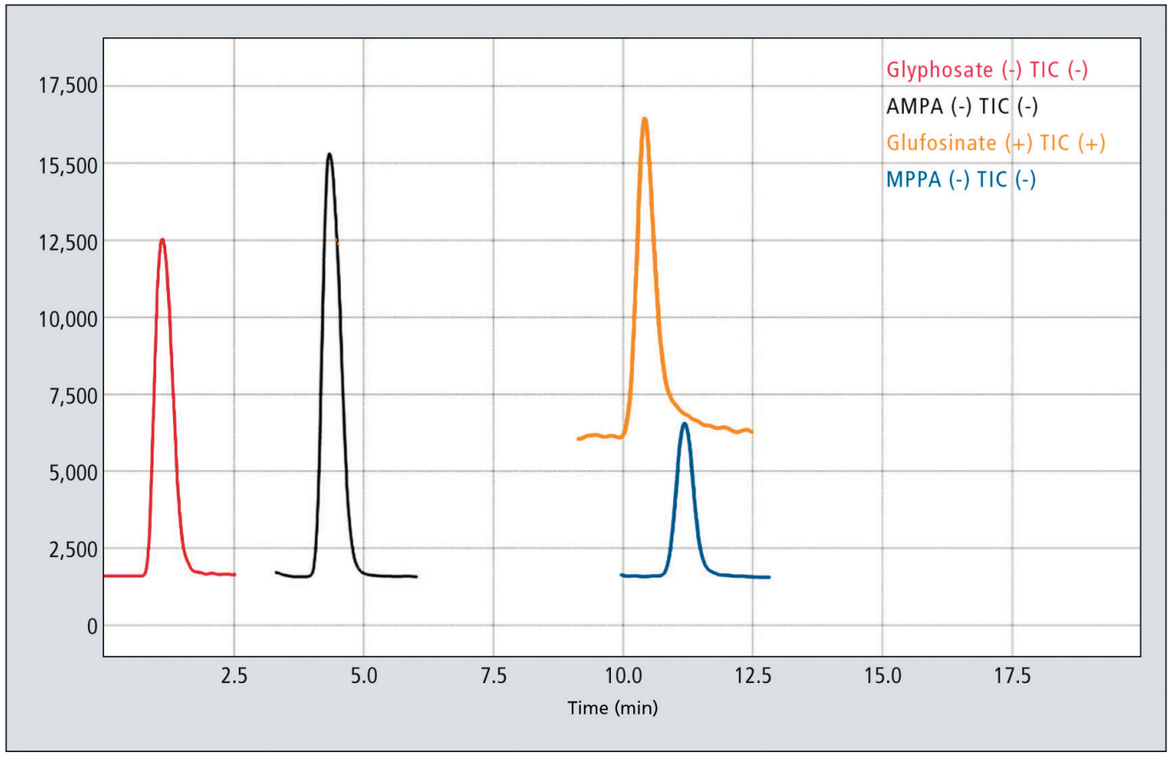
This article describes a direct analysis of glyphosate, aminomethylphosphonic acid (AMPA), glufosinate, and 3-methylphosphinicopropionic acid (MPPA) in water by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) without derivatization. The chromatographic separation was performed using a hydrophilic interaction liquid chromatography (HILIC) column and typical LC–MS mobile phases. Method performance was evaluated, showing excellent results. The low limits of quantification (LLOQs) obtained meet the requirements of EU guidelines and could also be used to get an agreement in France where regulations require lower LLOQs (NOR: DEVL1703763V).

Glyphosate and glufosinate are two broad-spectrum systemic herbicides, aminomethylphosphonic acid (AMPA) and 3-methylphosphinicopropionic acid (MPPA), their two major metabolites, respectively. Glufosinate, also known

Figure 1: Typical MRM chromatogram.



as phosphinothricin, is a naturally occurring broad-spectrum systemic herbicide produced by several species of *Streptomyces* soil bacteria. Glyphosate is the most frequently used herbicide globally and in the EU.

Glyphosate and glufosinate are in much discussion in Europe, particularly regarding their use in farming. Glufosinate, registered for use as an herbicide in Europe, has been withdrawn from the French market since 2017 by ANSES (French Agency for Food, Environmental and Occupational Health & Safety) because of its classification as a possible reprotoxic chemical (R1b).

As a result of the wide use of these herbicides and increasingly restrictive regulations, very sensitive methods for their determination are required.

Table 1: Calibrators accuracies (%)

Concentration (ppb or ng/mL)	Accuracy (%)			
	Glyphosate	AMPA	Glufusinate	MPPA
0.02	-	-	102.7	-
0.03	106.4	100.1	99.6	-
0.05	94.7	100.4	97.2	97.9
0.1	89.4	101.3	94.2	103.9
0.2	95.6	99.6	97.4	102.2
0.5	107.5	84.8	99.3	97.0
1	108.2	106.2	105.7	98.6
2	95.2	104.8	100.6	97.6
5	103.6	104.6	105.1	103.9
10	98.7	97.6	97.6	98.9

In March 2015, Japan's Ministry of Health, Labour and Welfare, Health Service Bureau, Water Supply Division issued a notification (No. 0325 Item 3–6) specifying the use of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) for water quality control inspections of glufosinate, but without specifying the method.

Meanwhile in Europe, guidelines were established. For example, the low limits of quantification (LLOQ) needed for glyphosate and AMPA in water was set at 0.1 ppb (ng/mL), and even lower (0.03 ppb) in France for conformity to get an agreement.

Reaching such low LLOQs is very challenging because of the ionic character, low volatility, low mass, and high polarity of these substances. Derivatization quickly became a standard for their determination (1), but the derivatization process is exhaustive and time-consuming. This article describes a direct analysis of these pesticides in water.

Methods

Quantitative analysis of glyphosate, AMPA, glufosinate, and MPPA was performed with commercially available mineral water. Sample pretreatment consisted of a 0.2 µm filtration directly into LCMS filter vials (Thomson Instrument Company). Chromatographic separation was performed using a 2.0 × 150 mm, 5-µm HILIC column (Shodex) heated at 40 °C and typical LC–MS mobile phases (water, acetonitrile, formic acid) in a 20-min gradient run. Samples were monitored using a high sensitivity UHPLC–MS/MS system (Nexera X2 and LCMS-8060, Shimadzu Corporation). MRM transitions were optimized using LabSolutions software (Shimadzu Corporation). For glyphosate analyzed in negative mode, the first transition (168>63) followed by (168>79) were used. For AMPA, also analyzed in negative mode, MRM (110>79) and (110>63) were selected. Glufosinate was ran in positive mode with the transitions (182>56) and (182>136). Finally, MPPA was analyzed in negative mode with MRM (151>63) and (151>78).

Regarding the tandem mass spectrometry detector, the following parameters were used. The LC–MS system ran with a heated electrospray ionization (ESI) at 350 °C. The nebulizing gas was set at 3 L/min, the heating gas at 15 L/min, and the drying gas at 5 L/min. With a pause time of 3 msec and a polarity switching time of 5 msec, a minimum of 30 points for each peak was reached. See reference 2 for more details on that method.

The four analytes were quantified simultaneously in less than 12.5 min. Figure 1 shows a typical MRM chromatogram of the analytes.

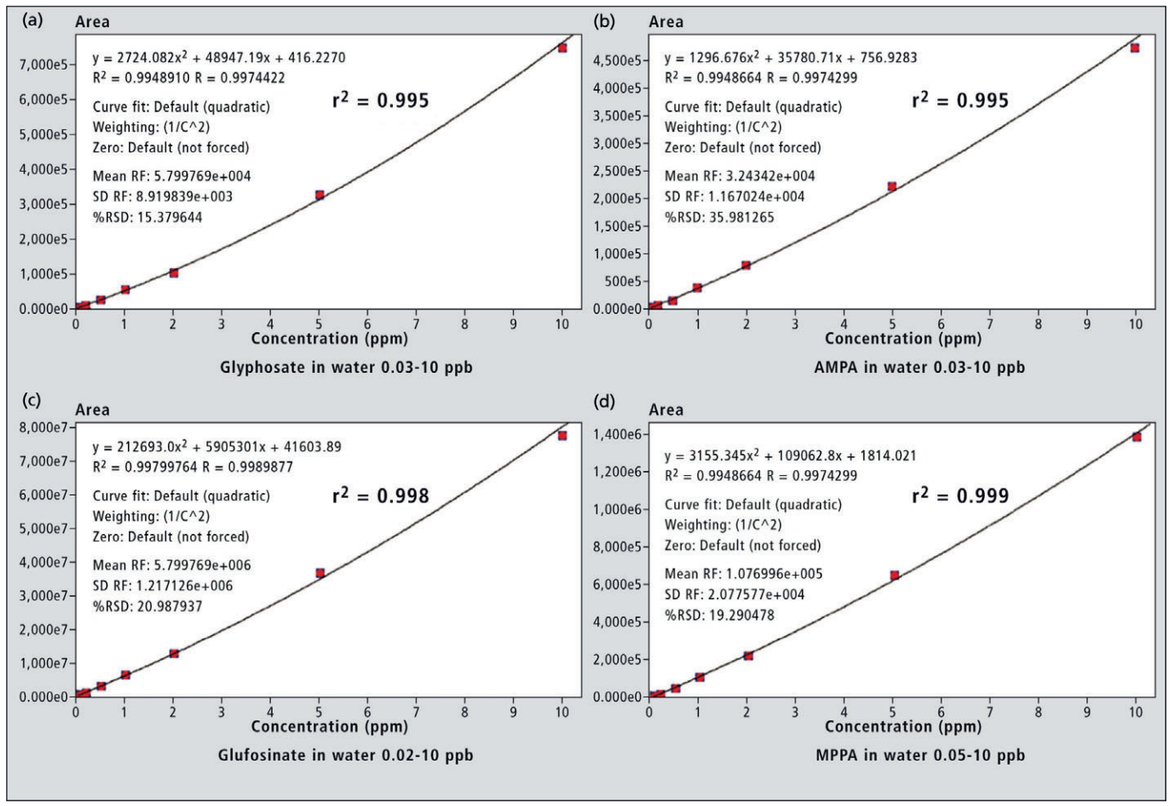
Table 2: QC samples accuracies and RSD

Compound	Accuracy (min–max) %	RSD intra-day (n = 5)%	RSD inter-day (n = 3) %
Glyphosate	106.6 (96.8–115.0)	4.7	0.7
AMPA	106.8 (98.0–112.7)	5.3	1.9
Glufosinate	95.7 (90.0–100.8)	1.4	5.4
MPPA	100.4 (92.5–108.9)	6.6	1.0

Results

The developed method enabled the quantification of glyphosate, glufosinate, and their metabolites AMPA and MPPA in mineral water without derivatization. The calibration range was 0.03–10 ppb for glyphosate (Figure 2[a]) and AMPA (Figure 2[b]), 0.02–10 ppb for glufosinate (Figure 2[c]), and 0.05–10 ppb for MPPA

Figure 2: Calibration curves.



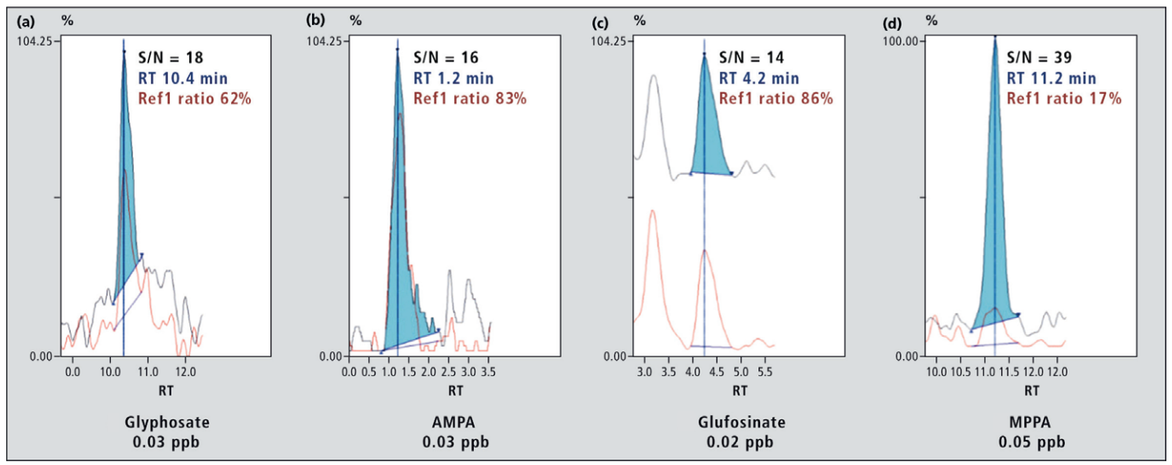
(Figure 2[d]). For all analytes, the r^2 values of the calibration models were above 0.995, with signal-to-noise ratios (S/N) above 10 for all LLOQ levels. Accuracies are detailed in Table 1.

LLOQs in water were 0.03 ppb (ng/mL) for glyphosate (Figure 3[a]) and AMPA (Figure 3[b]), 0.02 ppb for glufosinate (Figure 3[c]) and 0.05 ppb for MPPA (Figure 3[d]). Signal-to-noise ratio (S/N) was above 10 for all LLOQ levels (ASTM, 4 blocks of 0.25 min).

Analytical performance of the method was monitored using calibrators in LCMS water and QCs in mineral water at several levels.

Accuracies of calibrators (Table 1) and QC samples (Table 2) were between 85%

Figure 3: MRM chromatograms at LLOQs.



and 115% for all analytes, and relative standard deviation (RSD) values (n = 5 intra-day, over 3 days for inter-day) at 0.1 ppb in mineral water were below 10% (Table 2). To conclude, this method enables the quantification of the pesticides of interest without derivatization. The LLOQs obtained meet the requirements of the EU and French guidelines.

Conclusion

Using ultrafast tandem mass spectrometry technology and hydrophilic interaction liquid chromatography (HILIC) separation conditions, this method enables the 0.03 ppb LLOQs required in France—which has currently the most stringent regulations—to be reached. This method allows routine laboratory testing of water quality to fulfill not only the EU guidelines, but also worldwide regulations regarding drinking water. In addition, by eliminating the derivatization step, this method reduces maintenance on the LC–MS system and make the analysis faster and simpler.

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