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High-resolution mass spectrometry for bioanalytical applications: Is this the new gold standard?

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Abstract

Liquid chromatography coupled to quadrupole-based tandem mass spectrometry (QqQ) is termed the "gold standard" for bioanalytical applications because of its unpreceded selectivity, sensitivity, and the ruggedness of the technology. More recently, however, high-resolution mass spectrometry (HRMS) has become increasingly popular for bioanalytical applications. Nonetheless, this technique is still viewed, either as a screening technology or as a research tool. Although HRMS is actively discussed during scientific conferences, it is yet to be widely utilised in routine laboratory settings and there remains a reluctance to use HRMS for quantitative measurements in regulated environments. This paper does not aim to comprehensively describe the potential of the latest HRMS technology, but rather, it focuses on what results can be obtained and outlines the author's experiences over a period of many years of the routine application of various forms of HRMS instrumentation. Fifteen years ago, some nine different QqQ methods were used in the author's laboratory to analyse a variety of different veterinary drug resides. Today, many more analytes are quantified by seven HRMS methods and just three QqQ methods remain in use for the analysis of a small set of compounds yet to be upgraded to HRMS analysis. This continual upgrading and migration of analytical methods were accompanied by regularly participating in laboratory proficiency tests (PTs). The PT reports (covering a range of analytes and analytical methods) were used to compare the accuracy of HRMS- versus QqQ-based measurements. In the second part of this paper, the particular strengths and limitations of HRMS for both method development and routine measurements are critically discussed. This also includes some anecdotal experiences encountered when replacing QqQ assays with HRMS methods.

KEYWORDS

high-resolution mass spectrometry, proficiency tests, tandem mass spectrometry, technology comparison

1 | INTRODUCTION

Bioanalysis can be challenging because low concentrations of specific analytes have to be identified and/or quantified within samples which typically contain thousands of other similar compounds present at concentrations that are orders of magnitude higher. Historically, liquid chromatography (LC) coupled to ultraviolet (UV) detection was used for this work, but the limited selectivity and sensitivity of combined LC-UV soon lead to the development of some innovative strategies to improve performance. These included

an array of selective chemical derivatisation approaches for different analytes and to more specific detection methods (e.g., fluorescence and electrochemical detection). In the ongoing quest for improved selectivity and sensitivity, the successful direct coupling of LC to mass spectrometry (MS) resulted in a sea change in testing. The first generation of LC-MS-based applications used either a single quadrupole analyser or, more commonly, the more powerful triple quadrupole assembly (QqQ). LC-MS/MS, delivered by way of the LC-QqQ, offered significant advantages over alternative analytical options and has become the golden standard for sensitive, precise, and accurate biochemical analysis. Quickly, commercial tandem quadrupole instruments (QqQ) became widely perceived as the ultimate analytical tool, and the phrase "golden standard"[1] was hastily adopted by instrument vendors and users alike to describe LC-QqQ instruments.

LC-MS/MS has been applied to a broad range of analytical challenges, but the limited mass resolving power of the quadrupole analyser, even when incorporated in the QqQ configuration, constrains its utility for some applications. While the LC-QqQ has comfortably occupied its place as the "go-to" or golden standard instrument for several decades now, the field of MS has not stood still. There have been ongoing developments in instrument design, most notably, the commercialisation of alternative analysers/analyser configurations that offer improved selectivity through enhanced mass resolving power. These alternative analysers/analyser configurations have now reached a high level of maturity and are increasingly adopted.

The value of enhanced resolving power has been highlighted in proteomics,[2–4] metabolomics,[5,6] residue analysis,[7] and drug discovery,[8–11] by numerous groups, but its routine adoption in highly regulated environments (e.g., good laboratory practice) such as drug stability testing and clinical chemistry settings has been slow. This is likely because of the resistance of scientists to leave trusted technologies behind,[9] the absence of guidelines written by authorities/administrative bodies,[9,10] and the difficulties in introducing changes in highly regulated environments.[10] Nevertheless, high-resolution mass spectrometry (HRMS) has been increasingly accepted in fields where an ever-increasing number of new analytes has had to be covered, for example, environmental chemistry,[12–14] forensic chemistry,[15–17] food analysis,[18] pesticide residue analysis,[19–21] and veterinary drug residue analysis.[21–26] HRMS has also been used for "multi-multi" residue methods, where, besides veterinary drugs, pesticides, contaminants, plant toxins, and alkaloids have been determined.[27]

The adoption of HRMS in nonresidue food analysis is relatively recent. HRMS has been used to replace cumbersome derivatisation techniques (e.g., for biogenic amines)[28] or to quantify a large number of anionic food additives[29] which previously had to be analysed by several individual methods. Synthetic food dyes[30] as well as synthetic antioxidants and preservatives[31] have been analysed by HRMS as well. In fact, there is no lack of peer-reviewed papers that show the opportunities provided by HRMS versus QqQ.[5,6,8,9,15,18,20,27,32,33] Further, there are also many reports claiming both techniques produce identical quantitative results,[9,23,34–41] and some investigators have called for a shift from QqQ to HRMS technolgy.[9,42] Nonetheless, there is still a large discrepancy between claims regarding the professed potential benefits of the technique and the real penetration of HRMS technology into measurement laboratories. For this reason, the first part of this paper focuses on a comparison of validation data obtained by HRMS versus QqQ methods. These methods were used for analysing laboratory proficiency test (PT) samples, and the dataset presented here consists of some 300 PT results (HRMS versus QqQ) from the author's laboratory, obtained while participating in various veterinary drug residue PTs over a period of 15 years. During this timeframe, the QqQ methods were gradually replaced by HRMS (time-of-flight [TOF] and Orbitrap)–based methods. Consequently, the data presented here reflect how well a routine laboratory can introduce HRMS as its new workhorse. The HRMS methods were all developed in-house and were either based on existing QqQ methods, or in most cases, they were developed entirely from scratch. Therefore, the wealth of acquired experience during this transition process offers a deeper insight into the inherent strengths and limitations of HRMS versus QqQ. Some highlights experienced during this method-development process are given in the second part of this paper. Finally, the paper critically discusses the differences and technological features of these two MS technologies in detail. This discussion certainly reflects the positive attitude of the author towards HRMS as a technology, but at the same time, all known limitations and shortcomings (e.g., sensitivity, dynamic range, and coalescence) are critically discussed in detail. It is assumed that the reader is familiar with the basic principle of HRMS technology, otherwise a review of the following Orbitrap[43–45] or TOF[46,47] reviews may serve as useful background.

In summary, in this review, I highlight the utility of several alternatives to the QqQ analyser. Specifically, I objectively access the benefits arising from increased resolving power. This enhanced resolving power can be achieved in several ways, depending on the analyser/analysers assembled, and a range of different modes of operation are possible. Specifically, I review the power of three mature, commercially available instrument options: that is, the Q-TOF, Orbitrap, and Q-Orbitrap configurations—all of which offer "high-resolution" capabilities. I illustrate the power of these configurations as alternatives to the QqQ analyser by way of practical examples derived from my own workplace. While some of these configurations incorporate a quadrupole, it is used as a wide-pass filter to eliminate very light and very heavy ions, or it is used to permit the passage of only a particular mass range of interest.[4] In none of these alternative options is the quadrupole used as the primary analyser.

2 | MATERIALS AND METHODS

2.1 | Method validation

Several different methods were validated in parallel. Parallel validation refers to a procedure whereby all final injection-ready extracts are

split and afterwards, injected into two LC-MS systems utilising identical analytical columns and elution parameters, yet the two LC instruments are connected to two different MS systems. All validation protocols were based on the European Commission Decision CD 2002/657/EC. The pesticide validation[48] compared a QqQ instrument (TQD from Waters Milford, USA) with a single-stage Orbitrap (Exactive HCD, Thermo-Scientific, Bremen, Germany), the nitrofuran chloramphenicol method[35] compared a QqQ (TSQ Quantum Access MAX, Thermo-Scientific) with a Q-Orbitrap (Q-Exactive Plus, Thermo-Scientific), the anthelmintic method[38] compared a QqQ (TSQ Quantum Access MAX, Thermo-Scientific) with a single-stage Orbitrap (Exactive HCD; Thermo-Scientific), and the steroid method[49] compared a Q-Orbitrap (Q-Exactive Plus, Thermo-Scientific) with a Q-TOF (X-500R, Sciex, Concord, Canada). The detailed technical information relating each of these analyses (i.e., extraction, clean-up, chromatography, and detection parameters) is noted elsewhere.[35,38,48,49]

2.2 | Proficiency tests

All the veterinary drug PTs that the author's laboratory participated in within the last 15 years were used for the investigation. Very few individual test results had to be excluded, but this was the case when deviating results could clearly be linked to a methodological problem (e.g., degradation of the labile analyte in the standard, precipitation of a poorly soluble analyte in the stock solution stored in the freezer, and a low derivatisation reaction yield due to the insufficient acidification of a high pH sample). The majority of the PT samples were provided by the Food Analysis Performance Assessment Scheme (FAPAS), and others were organised by various European reference laboratories (BVL Berlin, ANSES Fougères and RIKILT Wageningen). The group of analytes quantified included nitroimidazoles, aminoglycosides, tetracyclines, illegal dyes, chloramphenicol, penicillins, macrolides, quinolones, sulfonamides, avermectines, anthelminthics, nitrofurans, and nonsteroidal anti-inflammatory drugs (NSAIDs). The analytes were present at food-safety-relevant concentrations: that is, depending on the compound, from 0.1 μg/kg up to 1,000 μg/kg, and they were present in a variety of matrices: that is, pure standard solution, muscle, liver, kidney, milk, honey, fish, shrimps, eggs, and urine. A variety of analytical methods were used to analyse the different analyte and matrix combinations. All of these methods have been developed and fully validated in the author's laboratory, and some of these methods have been published in peer-reviewed papers.[26,35,38,49–57] During the 15 years under investigation, the existing methods were modified and additional analytes were included. Primarily, these modifications focused on improving the recovery, sensitivity, and clean-up stages. What is most relevant is that 15 years ago, all analyses were undertaken on two QqQ and two LC-UV/fluorescence instruments, but currently, a single-stage Orbitrap, a Q-Orbitrap, and two Q-TOFs together with a single remaining QqQ instrument are used. Consequently, the workload (an average of 1,500 samples per year) and, with this, the related PTs, have gradually shifted from QqQ instruments towards HRMS systems.

3 | RESULTS AND DISCUSSION

3.1 | Precision and accuracy of results

3.1.1 | Precision (method-validation studies)

The validation data produced by four multiresidue methods (i.e., nitrofurans,[35] anthelmintics,[38] pesticides,[48] and steroids[49]) were compared. Each method utilises a different extraction and clean-up procedure. Validation was based on the European Commission Decision 2002/657/EC. The procedure is based on repeated multiple fortification levels into a variety of different food matrices. The final extracts that were produced were split into two high-pressure liquid chromatography (HPLC) vials and then analysed by two different LC-MS instruments. Because an identical type of chromatography was used, the differences between the observed results are entirely due to the detection system.

Table 1 shows the comparative performance of the four methods. The data are provided for the average and median of several method-performance values. In the case of the pesticide studies, this refers to the average and respective median performance of more than 200 analysed compounds.

Pesticides: At the lowest spiking level (A = 1 $\mu g kg^{-1}$), "higher recoveries" and better precision were observed for QqQ-based measurements. This was no longer the case for the higher B and C levels. This can be explained by the poorer limit of detection produced by HRMS full-scan acquisition as compared with QqQ multiple-reaction monitoring (MRM). The underreporting of peak areas at low ion abundances can frequently be observed when utilizing Orbitrap-based instrumentation. (This will be discussed in more detail in the sensitivity section.) The nitrofuran and chloramphenicol method requires a complex derivatisation and clean-up step. It is clearly visible that compounds quantified by incorporating an isotopically labelled internal standard produce noticeably superior results, and this is independent of the detection technology used. The HRMS method (Q-Orbitrap) was based on a combination of scan and targeted experiments: that is, precursor isolation by the quadrupole, followed by fragmentation and detection of HRMS-resolved product ions. In this case, the targeted experiments produce a much higher selectivity (i.e., HRMS detection of product ions) than that available from QqQ instruments operated in the MRM mode. This is probably the main reason why Q-HRMS data based on isotopically labelled internal standards show a better low-fortification level precision than data generated on a QqQ. The anthelmintic method included some compounds (avermectins) that produce a weak $[M + H]^+$ but an intensive $[M + Na]^+$ signal. (The formation of the sodiated species likely occurs in the interface.) These difficult-to-fragment sodium adducts limit the value of QqQ-based MRM detection, but the HRMS method relied on the direct detection of the intact $[M + Na]^+$ precursor ions. Detection of the relatively high mass sodiated analyte produced better selectivity and sensitivity results than those generated on the QqQ instrument operated in the MRM mode.

TABLE 1 Validation data from four different multiresidue methods

	Method	Matrix	Calibration	Detection	r^2 average	r^2 median	Recovery (%) average			Precision (RSD, %) average		
Concentration	Pesticide	Rocket	External				0 μgkg^{-1}	10 μgkg^{-1}	100 μgkg^{-}	0 μgkg^{-1}	10 μgkg^{-1}	100 μgkg^{-1}
	Pesticide	Rocket		QqQ MRM	0.9939	0.9980	80.5	99.5	105.0	19.8	9.0	6.0
	Pesticide	Rocket	External	Orbitrap scan	0.9938	0.9977	53.2	80.3	102.7	28.4	9.1	5.8
Concentration	Nitrofurans	Liver					0.25 μgkg^{-1}	1.25 μgkg^{-1}	5 μgkg^{-1}	0.25 μgkg^{-1}	1.25 μgkg^{-1}	5 μgkg^{-1}
	Nitrofurans	Liver	Internal	QqQ MRM	0.9973	0.9978	101.7	106.7	99.2	10.6	3.9	3.8
	Nitrofurans	Liver	Internal	Q-Orbitrap scan/targeted	0.9986	0.9993	110.9	108.2	99.8	4.2	2.9	2.1
	Nitrofurans	Liver	External	QqQ MRM	0.9456	0.9814	147.7	123.8	167.4	31.1	20.3	16.4
	Nitrofurans	Liver	External	Q-Orbitrap scan/targeted	0.9781	0.9785	90.7	95.8	116.7	31.2	30.5	9.4
Concentration	Anthelmintics	Milk					1 μgkg^{-1}	10 μgkg^{-1}	200 μgkg^{-1}	1 μgkg^{-1}	10 μgkg^{-1}	200 μgkg^{-1}
	Anthelmintics	Milk	External	QqQ MRM	0.9962	0.9993	75.5	88.5	84.8	19.3	8.0	3.7
	Anthelmintics	Milk	External	Q-Orbitrap scan	0.9958	0.9971	82.1	86.7	79.7	5.3	4.1	5.2
Concentration	Steroids	Liver					0.25 μgkg^{-1}	1 μgkg^{-1}	100 μgkg^{-1}	0.25 μgkg^{-1}	1 μgkg^{-1}	100 μgkg^{-1}
	Steroids	Liver	External	Q-Oribtrap scan	0.9754	0.9973	59.8	65.9	71.6	20.4	7.0	4.6
	Steroids	Liver	External	Q-Oribtrap target	0.9932	0.9957	40.9	54.1	74.4	45.9	19.7	5.6
	Steroids	Liver	External	Q-TOF scan	0.9850	0.9881	67.5	75.5	86.1	41.2	16.7	10.6
	Steroids	Liver	External	Q-TOF target	0.9984	0.9898	70.9	73.7	84.7	23.4	16.6	9.3

Note. All final extracts were split and analysed by two different detection technologies (see "detection" column). The given values reflect the average and respective median of all the analysed analytes. In the case of the pesticide method, this represents a set of some 200 compounds.

Abbreviations: MRM, multiple-reaction monitoring; QqQ, triple quadrupole assembly; RSD, relative standard deviation; TOF, time-of-flight.

The steroid method is based on Q-TOF and Q-Orbitrap measurements. Some steroids produce a multitude of nonspecific low-abundance fragments. These compounds are therefore detected with higher sensitivity in the scan mode. Other compounds, however, have to be detected in the targeted mode to achieve the required selectivity in a particular matrix. There was a higher recovery but somewhat poorer TOF precision when quantifying steroids. However, this can partially be explained by the calibration curves that were used. The Orbitrap software permits the definition of quadratic calibration curves with a forced zero intercept, whereas the TOF software can handle such curves but a zero intercept is not enforceable. Generally speaking—with the increasing penetration of software into MS systems—a strict hardware comparison is becoming increasingly difficult.

In conclusion, precision and accuracy are affected by a variety of factors (e.g., the source geometry, ion optics, the availability of isotopically-labelled standards, the extent of signal suppression, the detection selectivity, the complexity of the matrix, the fragmentation properties of the precursor ions, and the stability of the electrospray). The contribution of different mass analyser configurations (e.g., QqQ versus Orbitrap or TOF-based HRMS) does not have a significant effect on the overall accuracy and precision.

3.1.2 | Accuracy (laboratory PTs)

Laboratory PTs are the most important indicator that a laboratory or an analytical technology is truly capable of reproducibly generating the correct analytical results. It not only addresses the question of precision, but more importantly, the question of accuracy. Good method-validation results may partially be the result of careful organisation of the validation process, but such data also reflect past and not necessarily present capabilities (e.g., a new instrument, skilled personnel, and sufficient timeframe). On the other hand, PT samples frequently arrive during a time when the laboratory manager is engaged attending to numerous pressing problems and is likely facing a backlog of unanalysed samples. Consequently, PT reports reflect the capability of a laboratory or the analytical technology more so than a detailed method-validation report does.

Food-analysis PTs are organised and processed in the following manner: A professional scheme provider sets up programmes that interested laboratories can register for. Particular matrix-analyte combinations are prepared (e.g., a set of pesticides in oranges). These samples contain an undisclosed number of analyte(s). Participating laboratories are anxious not to report false-positive or false-negative findings (i.e., a sample may contain a dozen analytes out of a list of several hundred potential pesticides). Last but not least, the quantitative results reported to the organiser should not deviate outside of a predefined acceptance range. Most PT organisers consider a *z*-score in the range −2 to 2 as acceptable: that is, $Z = (x\text{-}x_{pt})/\sigma_{pt}$, where x is the analyte concentration reported by the participating laboratory and x_{pt} is the assigned value measured by the scheme provider. Here, σ_{pt} is the standard deviation determined by the scheme provider. Alternatively, some scheme providers derive σ_{pt} from outlier-cleaned participants data.

Theoretically, only one out of 20 participating laboratories should produce a result outside this *z*-score range. However, the reports released by the scheme providers almost always show that there are a significantly larger number of "failing" laboratories. This is due to the fact that for most PTs, laboratories are free to choose the analytical method they employ, even though any systematic bias among the organiser and participating laboratory's method will inflate the *z*-score. In other words, participants know that the use of an analytical method which significantly differ from that used by the scheme provider could lead to stronger deviating *z*-scores. They are aware that they get the best scores when they analyse their sample in a very similar way to that adopted by the scheme provider. The PT results provide important diagnostic information within regulated laboratory quality control systems. Depending on the *z*-score deviations obtained, the involved analytical method may no longer be applied until the reason for the deviation has been found and corrective measures have been taken. Consequently, there is little motivation to obtain better analyte extraction efficacy or to employ an innovative analytical technology. It is more prudent to wait until the scheme provider and the majority of the participating laboratories have adopted a new analytical method than it is to be the first laboratory to introduce it.

In our laboratory, we began replacing QqQ-based methods by HRMS equivalents more than 10 years ago. Our decision was based on the knowledge that the QqQ methods as used by the scheme providers, as well as our utilized HRMS methods, show little bias from the true analytical concentrations. Last but not least, our validation data indicated that the precision of QqQ- and HRMS-based measurements was comparable.[30,33] Figure 1 lists all the *z*-scores (i.e., for various analytes in various matrices) obtained over 15 years. What is clearly evident is the gradual replacement of QqQ methods with HRMS equivalents (the top and bottom of Figure 1). On visual inspection, the results are heavily biased by some "outliers"; hence, the moving averages are given in Figure 2. The averages and medians of the *z*-scores and the absolute *z*-scores are given in Table 2.

The comparison of the absolute *z*-scores shows that more accurate measurements were obtained with HRMS than with QqQ instruments. The same improvement is observed for the untreated *z*-scores. Based on these data, the selectivity of the HRMS approach was fit for the proposed applications. Insufficient selectivity would lead to overlapping/integrated chromatographic peaks comprised of the analyte plus some coeluting matrix compounds. This is clearly not the case. Twenty-two percent of all QqQ measurements produced poor results (*z*-score > ±2). This is higher than the theoretical value of 5%, but as stated above, such differences are expected when participants use different analytical methods. The 22% value is still slightly better (i.e., lower) than the average of all the other participating laboratories (Table 2). In addition, the HRMS measurements produced better results (7.9%) than the QqQ measurements did.

It would be wrong to claim that this improvement is directly linked to the HRMS technology alone. All of the methods used were developed in our laboratory, and many of the HRMS methods were

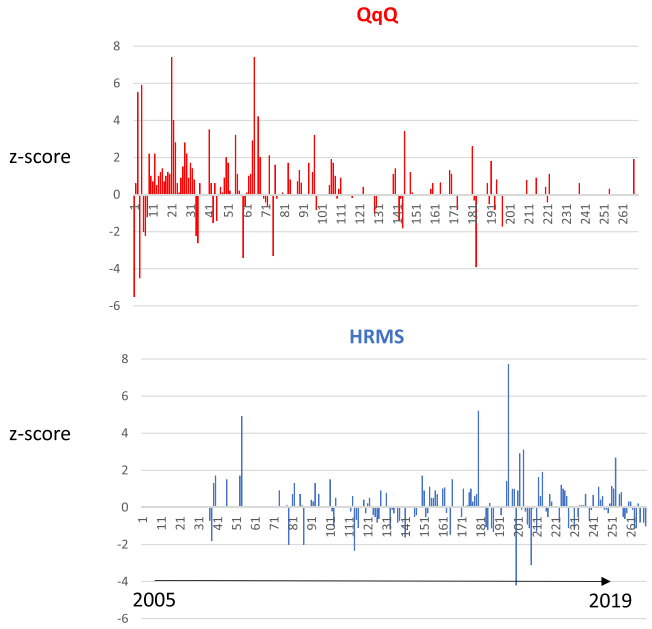

FIGURE 1 *z*-scores (261 measurements) of some 100 proficiency tests obtained in the author's laboratory. The graph compares triple quadrupole assembly (QqQ)– and high-resolution mass spectrometry (HRMS)–derived results (time series) over a period of more than 10 years (*x* axis).

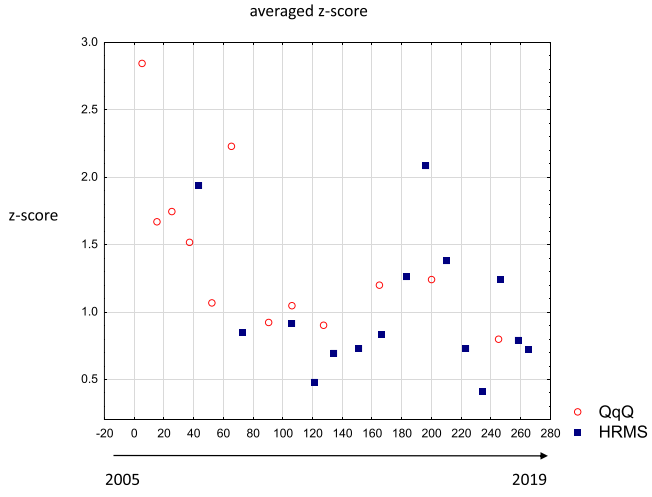

FIGURE 2 A different view of the data shown in Figure 1. The moving smoothed averages of absolute *z*-scores are shown over a period of more than 10 years (time series).

based on older QqQ methods. The evolution of the methods we utilised not only involved the use of a new detection technology (i.e., MS), but in most cases, it also involved modifications to the extraction and clean-up steps. Many of the methods could be simplified, recoveries could be improved, and signal suppression could be reduced. In addition, by the time we made these changes, the laboratory had developed considerable skill and expertise in performing the specific methods.

Figure 2 gives the moving average of absolute *z*-scores across the years. The trend towards lower (i.e., better) *z*-scores is most evident for the QqQ-based results. This is likely also the case for the HRMS-based results, but the presence of some outliers (e.g., a *z*-score of 2.1 as shown in Figure 2) obscures this trend. These outliers could be linked to specific occurrences. For example, a new method was developed for analyte groups which, up to that point in time, had not been analysed in our laboratory. Poor results were most likely due to our limited knowledge regarding the chemical and physical properties of these new compounds. In addition, some HRMS-based results included compounds that had not been formally validated at the time; nevertheless, because of their HRMS-based detectability, they were quantified and reported. Based on these data, results derived by HRMS-based methods and QqQ methods showed similar accuracy and precision. In this instance, poor precision is not linked to the detector technology, but rather is associated with variance introduced during the extraction and clean-up steps and most likely to the expertise and skill of the analyst performing the analytical work. This opinion is shared by several other working groups.[9,17,23,32,36,37]

3.2 | Technological capabilities and limitations of HRMS

3.2.1 | Sensitivity

Limits of detection achievable on a QqQ when operating in the MRM mode are typically better than those generated on a Q-TOF or Q-Orbitrap operated in the full-scan mode. This is, however, a complex issue requiring a more detailed investigation. The previously mentioned pesticide-validation study[48] provides some insights. Sensitivity comparisons reflect the performance of two particular instruments at a given time in the past, but such comparisons can still answer some underlying issues. As can be seen in Figure 3, there are significantly more analytes detected at lower concentrations by QqQ in the MRM mode than by HRMS utilising a full-scan signal. Yet, there are some compounds which can be detected by 10 (or even more) times higher signal to noise when using either of the two technologies. An analyte may just produce one or two fragments. The corresponding high yield of product ions leads to high MRM sensitivity. On the other hand, some compounds produce many fragments, and therefore the total ion current is distributed across these many low intensity species. Figure 4 illustrates this behaviour. The second group of compounds—producing a large number of low-abundance fragments—give superior HRMS sensitivity since the compounds are preferably detected as unfragmented precursor ions. An example of such compounds are the avermectins—they produce low-abundance $[M + H]^+$ ions, but a high abundance of stabile $[M + Na]^+$ precursor ions.[38]

Typically, nontargeted acquisitions (e.g., a full scan by QqQ, Q-Orbitrap, or Q-TOF) are less sensitive than targeted acquisitions (e.g., MRM by QqQ or PRM by Q-Orbitrap). This is and will likely

TABLE 2 PT data from the author's laboratory obtained over a period of more than 10 years

	z-score		Abs (*z*-score)		Questionable *z*-scores [%]
	Average	Median	Average	Median	
QqQ	0.71	0.7	1.49	1.05	22.2
HRMS	0.27	0.2	0.99	0.7	7.9
Other participants	-	-	-	-	25.7

Note. Statistical data (average and median) reflect a total of 261 *z*-scores (*x* axis). More than 100 PTs covered different families of veterinary drugs in different food matrices.

Abbreviations: HRMS, high-resolution mass spectrometry; PT, proficiency test; QqQ, triple quadrupole assembly.

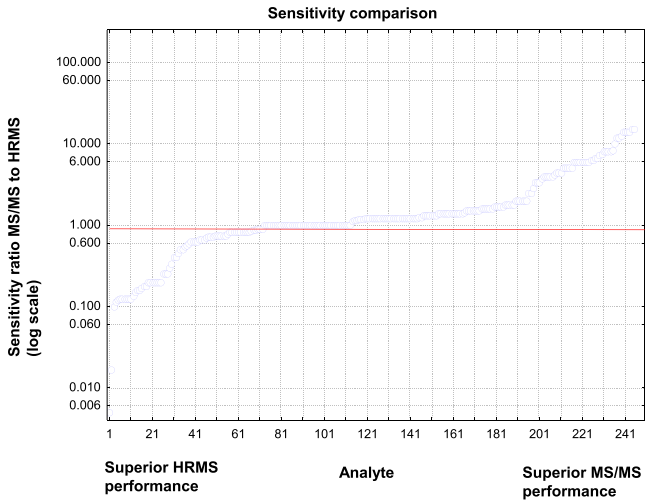

FIGURE 3 The relative detection sensitivity of 241 pesticide analytes in a matrix (high-resolution mass spectrometry [HRMS] versus triple quadrupole assembly [QqQ]). Note the logarithmic relative intensity scale (*y* axis).

remain a limitation. Using the quadrupole of a Q-TOF or Q-Orbitrap to isolate a narrower mass range frequently improves HRMS sensitivity. This is regardless of whether the quadrupole-isolated precursor ion is fragmented, the resulting product ions are detected by the HRMS, or if no fragmentation energy is applied and the intact precursor ion is monitored by HRMS.

Orbitrap and TOF sensitivity are affected in a different way. The Orbitrap's sensitivity is directly proportional to the number of ions collected within the C-trap. Because there is an upper limit on the number of ions that can be successfully trapped within the C-trap, it is the number of analyte ions injected into the analyser that determines the detection sensitivity. Using the quadrupole to isolate a narrower mass range permits longer C-trap filling times and therefore enables the collection of more analyte ions. This is different for TOF instruments. On a Q-TOF operating in full-scan mode, sensitivity is enhanced as the scanned mass range is reduced. Using the quadrupole to prevent the entrance of high *m/z* ions into the TOF flight tube permits an increase in the pusher frequency: that is, a new push can be initiated as soon as the highest *m/z* ion has reached the detector plate. Therefore, Q-TOF scan modes based on the isolation of a restricted mass range can significantly improve the sensitivity of HRMS detection: that is, the ion statistics are improved by summing more individual TOF pushes to produce the final spectrum. In contrast, utilizing a Q-TOF or Q-Orbitrap quadrupole in the same way, where the first quadrupole in a QqQ instrument selects a single m/z value, leaves all other ions unsampled. Consequently, full-scan information is no longer available. Multiresidue methods (i.e, utilizing a TOF and Orbitrap) frequently contain some particular analytes that require extra sensitivity or selectivity, and therefore such methods frequently consist of a full scan (i.e., suitable for most analytes), followed by one or several targeted acquisitions (i.e., designed for the problematic analytes). In the case of many problematic compounds, retention-time-controlled acquisition settings—as are commonly used for MRMs in QqQ instrumentation—can be used to accommodate a large number of targeted acquisitions within a chromatographic run.[49] However, this can finally lead to the situation where the HRMS instrument virtually becomes a QqQ instrument.

One important limitation of the current HRMS technology is the poorer limit of detection of a targeted compound present in a "dirty" matrix versus present in a pure standard solution. In other words, a given (low) concentration of an analyte can produce a sufficiently intensive signal when present in a standard solution, but no signal is observable when present at the same concentration in an injection-ready sample extract. This outcome is not only to be explained by interface related signal suppression, but is frequently linked to the dynamic range of the analyser and is therefore comprehensively discussed within the "linear dynamic range" section of this paper.

Detection sensitivity is not only limited by the detection technology but also by the interface and the ion optics. The enormous improvements in QqQ sensitivity over the last two decades would not have been possible without the development of interfaces with improved ionisation, desolvation, and ion transfer. In the past, many MS companies have outfitted their high-end QqQ instruments with their latest interface technologies, but frequently, their HRMS instruments are still sold with earlier generation interfaces.

The sensitivity gap between QqQ and HRMS has probably narrowed over the last decade. This is indicated by the observation that over the last few years, published sensitivity-critical applications are now more frequently attempted by Q-HRMS—whether with or without utilizing the quadrupole as isolation device. It may be that this process will not only continue, but that HRMS may ultimately take the lead.[58] The remarkable increase in QqQ sensitivity over the last two decades has not been accompanied by related selectivity

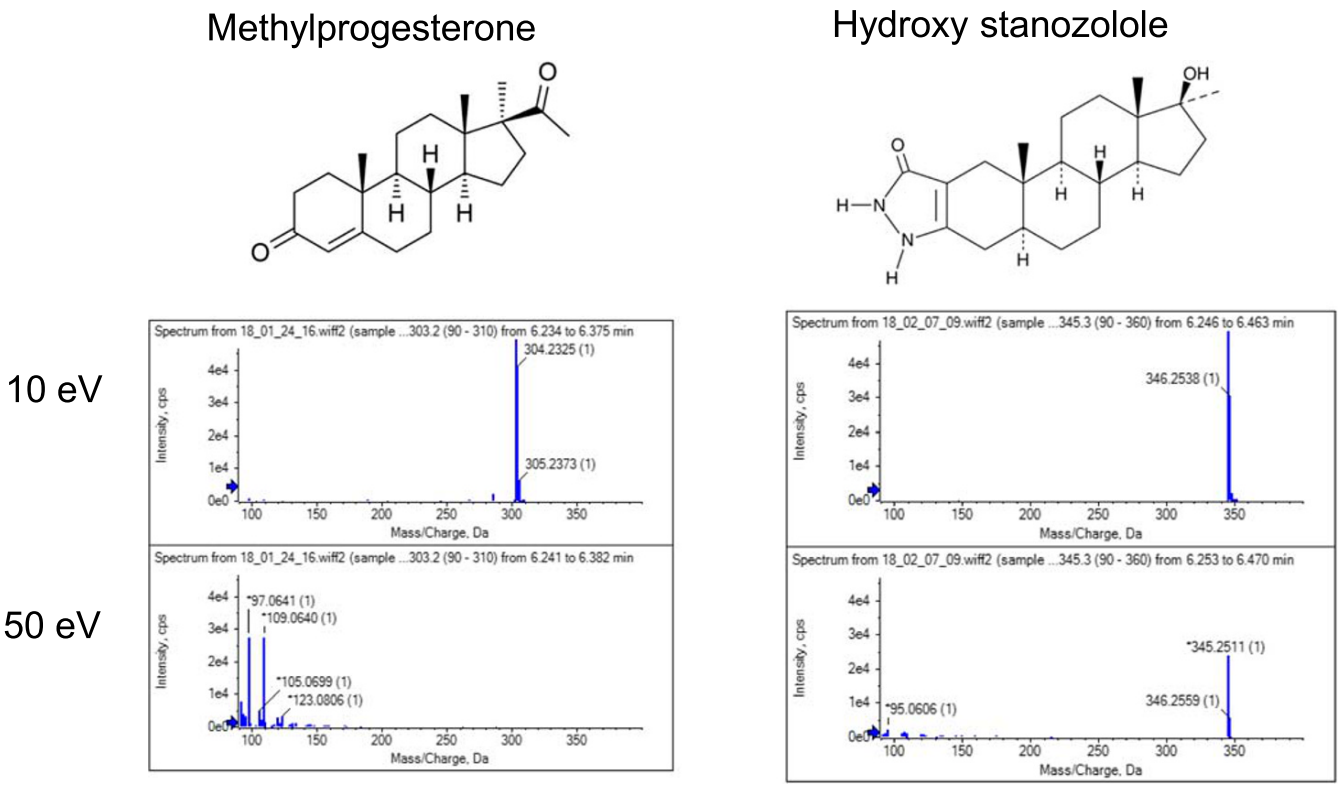

FIGURE 4 Examples of two similar compounds showing entirely different fragmentation properties. The compound on the left (methylprogesterone) produces two distinctive intensive product ions. This translates into high multiple-reaction monitoring (MRM)–based sensitivity.

improvements. Some quadrupoles (e.g., those utilising hyperbolic rods) permit the isolation of subunit-mass isolation. This can improve the selectivity of detection for these analytes showing a significant mass defect (e.g., containing halogens), but this comes at the price of ion transmission losses. Historically, chromatograms based on MRMs consisted of a completely flat baseline from which a single analyte peak emerged. Looking at more recently acquired high-sensitivity residue chromatograms acquired by QqQ-based MRM reveals a different picture. These chromatograms increasingly resemble those of a UV detector used for trace analysis. Consequently, reliable detection and confirmation requires the monitoring of two or more transitions. In regulated residue analysis, analysts have to prove the presence of a compound by showing two transitions, as well as showing that the MRM ion-abundance ratio does not deviate by more than 20% or 30% from the ion ratio observed when comparing results with those obtained from a comparable matrix sample fortified with the analyte of interest. The higher the QqQ-based MRM sensitivity, the more likely it is that coeluting matrix compounds will produce a detector signal—that is, coisolated by the quadrupole and producing a product ion with the same unit mass as that of the targeted analyte)—thereby affecting the MRM ion ratio of one or both MRMs. This can lead to situations where a truly present analyte can no longer be confirmed. It has been estimated[58] that at a true analyte concentration of 10 μgL^{-1}, it is very unlikely (p = 0.001) that a coeluting compound will be present capable of shifting the MRM ratio by more than 20% and therefore leading to a false-negative finding. At 0.05 μgL^{-1}, the likelihood increases to p = 0.1 (10%), and therefore additional sensitivity will no longer arise from having access to instruments producing an improved signal-to-noise (S/N) ratio, but by instruments with increased selectivity. As the mass-resolving power of HRMS instrumentation is constantly improving, HRMS is more likely to evolve in this direction. This development is likely the reason why the introduction of an additional degree of hyphenation (e.g., ion mobility) to QqQ technology has gained momentum.

Detector noise is not directly related to the detection sensitivity, but frequently, the limit of detection is quoted as the analyte concentration where the S/N approaches 3. Many HRMS instruments (e.g., Orbitraps in general) do not report true white noise; rather, the signal seems to emerge directly out of a noise-free baseline.[6,7] In many cases, noise may be detected by the instrument but may not be reported to the analyst. There are different reasons for this. The Fourier transformation algorithm is utilised for processing the Orbitrap extracts sinusoidal frequencies out of a very complex signal. A transient length may contain thousands of periodic oscillations. Theoretically, failing to detect a single maximum or minimum may prevent the Fourier transformation from extracting this frequency (ion) from the complex detector signal, and therefore the reported ion abundance will be zero. In reality, the reporting of Orbitrap and TOF signals is governed by several superimposed filters. Spurious signals or detection artefacts (e.g., side lobes) are eliminated, and signals are

smoothed and otherwise manipulated. This is done in order to reduce the flow and storage of data. Measuring and storing all the noise would challenge currently available high-end computers, including their short- and long-term storage capabilities. There is a relevant difference between Orbitrap and TOF mass peaks. A TOF peak may be skewed or contain spikes, but this is generally not the case for Orbitrap, where Gaussian peaks are reported. Reducing the concentration of a low-level compound detected with an Orbitrap does not lead to a chromatogram where a peak disappears within a noisy baseline. Rather, a clearly visible Gaussian-shaped peak disappears as a whole. In other words, a repeated injection of the same sample containing a low-level analyte may produce several chromatograms where the peak is clearly visible with a perfect Gaussian shape. Yet, in other injections, no analyte signal at all will be detectable. Frequently, this can also lead to chromatographic peaks which consist of only one to three data points around the chromatographic peak apex. The absence of the remaining (below detection threshold) data points leads to the underreporting of chromatographic peak area and can affect quantification at low ion abundances. As a consequence, the conventional measurement of sensitivity based on the S/N can become impossible. Statistical measurements that are based on the increase in the relative standard deviation of average peak areas[59] when approaching the limit of detection were therefore suggested.

3.2.2 | Selectivity

HRMS selectivity is given by the mass-resolving power and mass accuracy of the instrument in combination with the user-defined mass-extraction window. There are no clear-cut rules to guide the user in the process of selecting these parameters.[32,60–64] It is very tempting to use increasingly narrower mass-extraction windows in order to improve selectivity.[18] Yet, mass-extraction windows approaching, or exceeding the physical resolving power or the mass accuracy of the analyser, can lead to false-negative findings. It is also important to treat centroided and continuum data in a different way.[67] Centroids are intended to decrease the size of the datafile. The mass peak is not stored as a two-dimensional object (continuum) but just as a stick with an *m/z* value and an ion abundance. Applying a mass-extraction window on continuum data cuts out the total ion abundance (integrates) of that mass window and reports a summed ion abundance for that *m/z* value (range). A mass shift of the measured peak (regardless of the underlying reason) may lead to the situation where the applied mass-extraction window only extracts the front or the tail of the targeted analyte mass peak. This is different for a centroided peak. The whole ion abundance of the mass peak is represented by a single stick (no summation is anymore required). A slight mass shift in that centroid does not affect the reported chromatographic peak area as long as the applied mass-extraction window still overlaps with the *m/z* of the centroid. In such cases, it is always the full ion abundance of the centroid that is displayed. However, there is a digital (complete) loss of ion abundance if the mass-extraction window no longer overlaps with the *m/z* of the centroid. This is graphically shown in Figure 5. Poor mass calibration may lead to such a shift of an analyte ion out of the mass-extraction window. Additionally, isobaric interferences or coalescence may be other reasons for such an undesirable situation (a false-negative finding). Isobaric interferences (the analyte coelutes with a matrix ion with a very similar *m/z* value) likely inflates the measured peak area of an analyte measured in the continuous mode. However, depending on the centroiding algorithm, the two partially resolved mass peaks may be correctly recognised by their measured accurate mass or merged into a single centroid with the "accurate" mass representing the area-weighted average of both analyte mass peaks. Hence, working with centroided data requires the use of conservative (wider) mass-extraction windows to prevent false-negative findings.

HMRS selectivity has been well investigated.[33,61,63,65,66] A comparison with the QqQ gold standard has been attempted by many authors. A scientific comparison of QqQ versus HRMS selectivity may be perceived to resemble the comparison of apples and oranges. Probably the first systematic comparison, "the crossover point," was based on a comparison between the average analyte peak area versus the average matrix peak area.[65] This was based on the measurement of a bovine liver extract fortified with a known concentration of various veterinary drugs. The average analyte peak areas were obtained by averaging the measured area/amount of a number of analyte peaks belonging to different analyte compound groups. Potentially interfering matrix peak areas were obtained by extracting 100 so-called dummy signals from a truly blank bovine liver extract. For HRMS, this consisted of randomly generated accurate masses within the analyte mass range of interest. For QqQ, this consisted of MRMs made up of randomly generated precursor and corresponding product ion masses. Some "chemical intelligence" was used to produce realistic transitions. This was due to the large number of tested transitions and accurate

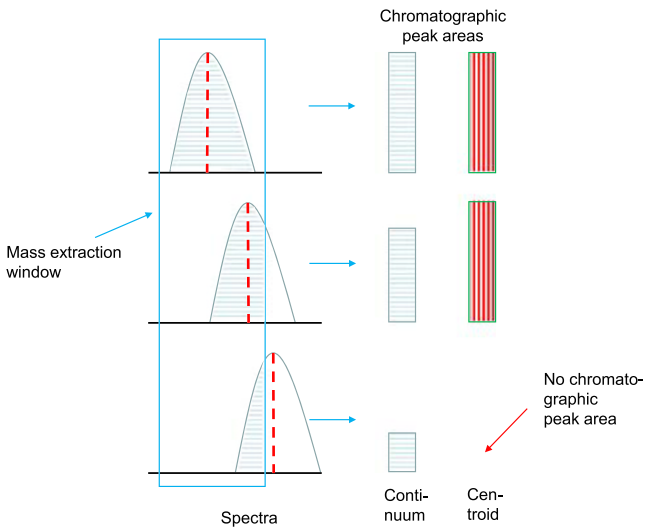

FIGURE 5 The difference between centroid- and continuum-based high-resolution mass spectrometry (HRMS) detection. A centroid shifting out of a user-defined mass-extraction window leads to the absence (false-negative) of a chromatographic peak (bottom).

masses where some of these dummy traces was found to be populated by matrix-related signals. These chromatographic matrix peaks were integrated, and their summed peak areas were compared with the analyte-related peak areas. A normalisation permitted the comparison of analyte versus matrix abundance for QqQ- and HRMS-based acquisitions. In other words, this approach tested how likely a MRM, or a narrow HRMS mass window, extracts by chance a matrix peak signal and how intense this "false detect" is in relation to the analyte peaks abundances. It was concluded that a mass-resolving power of 50,000 full width at half maximum (FWHM) in combination with corresponding mass-window widths produces a selectivity which equals that of a QqQ-based MRM. This finding was later repeated and confirmed by other investigations.[66–68] A recent global interlaboratory study[33] investigated HRMS residue-screening results. The interesting aspect of this study is the fact that the participants used different types of newer and older HRMS instrumentation to analyse a set of complex samples. The aspect of the orthogonality of detection (QqQ versus HRMS) has also been investigated for isobaric pesticides in a pepper matrix.[64]

The system of a two-unit mass-based transition, as used for QqQ-based MRM measurements, was once considered to prevent any false-positive findings being reported.[69] However, later, the false-positive detection of the pesticide sebuthyalazin in tarragon[70] was reported, although the confirmation procedure was strictly followed (retention time, and two MRMs including their ratios, corresponded closely with the pure reference standard). The responsible interfering compound was identified by the use of a now outdated single-stage LC-TOF instrument providing only a mass resolving power of 12,000 FWHM. A. This compound (an endogenic insecticide) is present in tarragon at high concentrations and was able to produce two MRMs mimicking the exogenous sebuthyalzine. Interestingly, both compounds show no structural similarity at all.[70] False negatives were also reported when applying the MRM ratio to the analysis of ronidazole residues in muscle tissue[68] and benzophenone in food stuffs.[71] It was concluded that the likelihood of false-positive findings increases for low-mass ions. These examples certainly represent rare cases where the use of two QqQ-based MRMs can still lead to false-positive or false-negative results, yet the availability of higher sensitivity instrumentation will increase the likelihood of such undesirable findings in the future. Hence, additional QqQ sensitivity should be paired with an increase in selectivity. This could be done in some cases with a third or fourth fragment. When using Q-HRMS instrumentation, the use of a unit-resolution-selected precursor and two HRMS-resolved product ions is certainly a step in the right direction.?

Figure 6 shows a trace analysis of banned nitrofuran drugs in animal-based food. A full-scan-based HRMS (Orbitrap) acquisition at 70,000 FWHM produced insufficient selectivity at the lowest fortification level (0.25 µg/kg). Yet, using product-reaction monitoring (PRM) where the quadrupole of the Q-HRMS instrument selectively isolates the precursor ion produced sufficient selectivity to improve the sensitivity in the matrix for two compounds. However, one analyte remained below the ion-abundance level required for detection

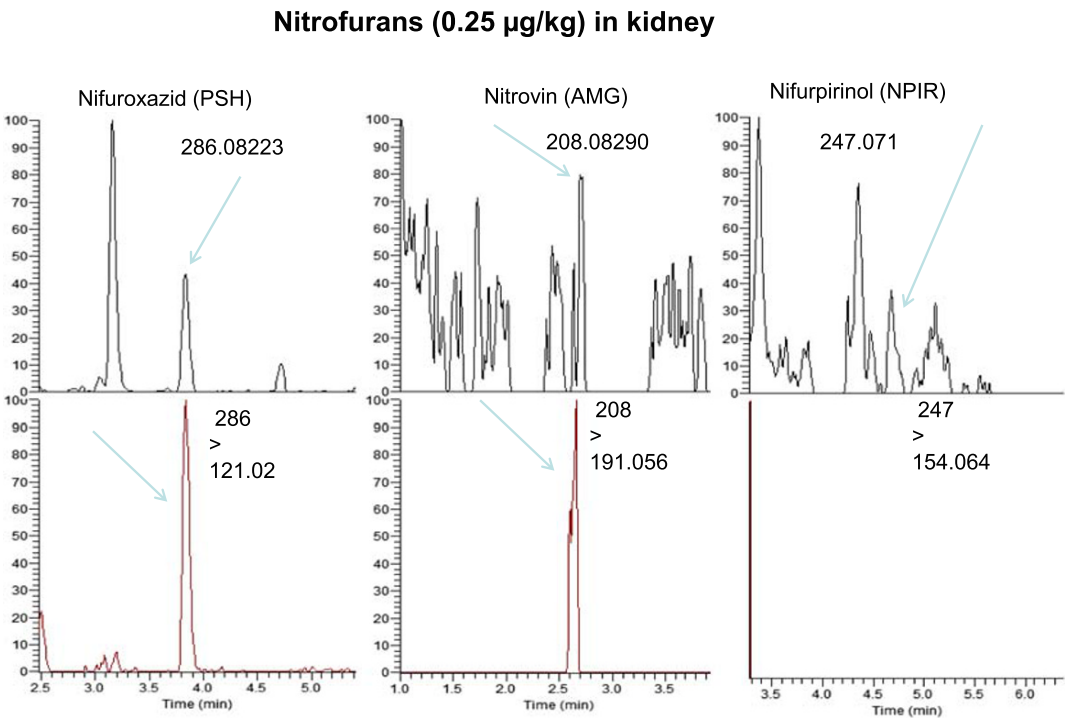

FIGURE 6 Sensitivity and selectivity differences for three selected trace-level analytes (selected nitrofurans) when using a full scan (top) and PRM (bottom).

(note the absence of any baseline). As discussed below, the combination of a full-scan acquisition and targeted acquisition is possible. Unfortunately, the speed of the analyser limits the number of targeted experiments used for the detection of critical (requiring extra selectivity and sensitivity) analytes.

3.2.3 | Speed

The speed of QqQ instruments has been dramatically improved over the last decade. This refers to the number of MRMs that can be monitored within a given unit of time. On the other hand, the scan-based speed as required for conventional, precursor, or product ion scans is significantly lagging behind the MRM-acquisition speed. The situation is different for HRMS instrumentation. Here, a "scan" requires (at least for the Orbitrap technology) no more time than for the measurement of a single *m/z* value. A TOF is extremely fast, yet it is still not advisable to migrate a QqQ-based multiresidue method directly into a TOF method, where each analyte uses another targeted acquisition. Commercial TOF instruments are marketed as capable of recording a 100-Hz acquisition rate; however, a TOF obtains its sensitivity and mass accuracy by summing up many consecutive TOF pushes into a single spectrum. Hence, the ion statistic improves with the number of pushes available for summation. In other words, accurate masses and good sensitivity require sufficiently long acquisition times even though short acquisition times do not negatively affect the mass-resolving power. This is different for the Orbitrap. Two ions with a nearly equal *m/z* require many oscillations along the central spindle of the Orbitrap analyser until they are physically separated. In other words, the Orbitrap's mass-resolving power is proportional to the transient time. Acquiring many precursor-isolated PRM scans prolongs the cycle time. This reduces the number of datapoints collected across a chromatographic peak. Nevertheless, the user can define a shorter transient time that restores the cycle time at the price of the mass-resolving power. Unlike TOF detectors, Orbitrap mass accuracy and sensitivity are hardly affected when selecting lower mass-resolving settings (shorter transients). A lot of technological effort has been put into improving Orbitrap duty cycles (e.g., the size of the analyser cell, faster stabilisation of ions collected in the analyser, and an improved Fourier transformation algorithm), yet Orbitrap instrumentation is still slower than TOF-based instruments. This becomes relevant when developing methods with many targeted experiments.

Another aspect related to speed is the time required to switch from positive ionisation to negative ionisation. QqQ instruments are the clear leaders in this regard. It is possible to use the latest generation of Orbitraps in a continuous polarity switching mode; but this leaves little time for targeted experiments outside of undertaking a full scan for each polarity. The situation is even worse for TOF instruments. A polarity change requires the inversion of thousands of volts within the voluminous flight tube. The stabilisation of the applied voltages and the subsequent mass calibration puts severe limitations on polarity switching. This situation has improved with the latest TOF instruments. For example, the Agilent 6546 specifications claim the instrument is able to do a complete cycle of positive and negative spectral acquisitions within 1.5 s. This is still slower than the latest Orbitraps (e.g., Exploris 480) where this can be done in 0.7 s.

3.2.4 | Linear dynamic range

There is no generally accepted definition of a linear dynamic range, but the term refers to the width of the ion-abundance range showing acceptable correlation with the analyte concentration. The linear dynamic range is more complex for HRMS than it is for QqQ. There is a need to acknowledge two different types of dynamic ranges when talking about HRMS spectra. There is the within-spectra dynamic range (i.e., the intra-dynamic range) and the between-spectra dynamic range (i.e., the inter-dynamic range). The inter-dynamic range is generally wider than the intra-dynamic range.[72] A weak point of HRMS is the fact that unbiased mass accuracy and correct ion-abundance measurements are only possible if the ion count for all (Orbitrap) or for any specific (TOF) ion species remains below a certain number. Exceeding this number can lead to several undesirable effects (see below) such as mass shifts. Consequently, most HRMS instruments use an ion-abundance measurement device to determine the abundance of incoming ions and to regulate their flow into the analyser in such a way that no detrimental overfilling results. This feature is well known for the Orbitrap and is termed "automatic gain control" (AGC). An Orbitrap will sample a shorter time segment of the continuously incoming ion beam, while a TOF may use a defocusing device that dilutes the ion beam sampled by the orifice of the ion optics. Such an attenuation or restricted sampling of the ion beam not only prevents the overfilling of the analyser but reduces the signal from all the ions (analytes and matrix ions). Consequently, the reported analyte ion abundance would be reduced by the applied attenuation factor, and this makes quantification impossible. However, the truly measured ion abundance of every mass peak within a spectrum is automatically multiplied by the applied attenuation factor. This approach maintains the quantitative nature of the data and at the same time prevents the overfilling of the analyser. A very intensive matrix peak may require the attenuation of the incoming ion beam by a factor of 20 or more. In the case of an analyte coeluting with a abundant matrix compound, the abundance of both ions will be equally attenuated. Nonetheless, a low-abundance analyte ion coeluting with an intensive matrix ion species may be so attenuated that the resulting analyte ion abundance falls below the physical detection threshold for the analyte of interest. As a result, the matrix ion species abundance is correctly restored by multiplying with the attenuation factor. Yet, the more important abundance of the now undetectable analyte ion cannot be restored. In other words, the detection sensitivity of an analyte in pure solvent may be significantly better than in a complex matrix extract. This is schematically visualised in Figure 7. The practical effect on analyte sensitivity is illustrated in Figure 8. A matrix extract and a standard

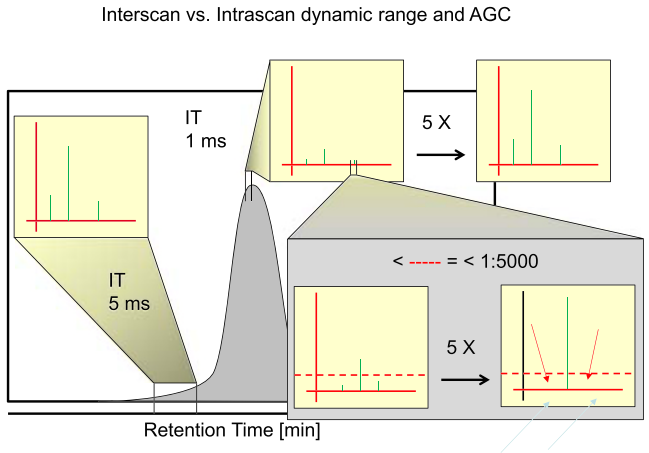

FIGURE 7 The behaviour of "automatic gain control" methodologies. A spectrum taken at the foot of a chromatographic peak needs no attenuation due to the low ion abundance (left). A spectrum taken at the apex of a chromatographic peak may require attenuation (top) and subsequent multiplication with the applied attenuation factor (top right). Notice the loss of some low-abundance signals in the spectrum (bottom right).

solution were injected. Both samples contained an identically low concentration of a sulfonamide (sulfadiazine). What was extracted from the two full-scan acquisitions were the monoisotopic, the first isotopic, and the second isotopic signals. The monoisotopic signal is clearly detectable in both chromatograms, but the first and second isotopic signals are completely absent when extracting from the fortified matrix sample (right side). Hence, the analysis of a low analyte concentration in a complex matrix may create problems when using full-scan HRMS detection. There are several strategies to mitigate against or solve such problems. First, the quadrupole should be used as a wide-pass filter so as to prevent the passage of ions below and above the mass range of interest. A good chromatographic procedure where the matrix compounds and the analytes analytes are resolved and are spread over a wide retention-time range should be attempted for every analysis. Finally, using the quadrupole to isolate a single particularly negatively affected analyte may make sense. Figure 9 shows the (double-logarithmic) calibration curve of an analyte when using the scan and the selected ion monitoring (SIM) mode. The blue circles show the chromatographic peak area of the analyte when injecting a standard. The red filled squares show the same calibration curve with the postcolumn infusion of a single high-abundance compound (simulating the impact of a matrix). The continuous presence of this high-abundance ion reduces the sensitivity when relying on a full-scan acquisition (left side of Figure 9). On the other hand, nearly equal calibration curves are obtained when measuring with SIM (right side of Figure 9) or any other mode where the quadrupole is used to isolate only a section of the mass range

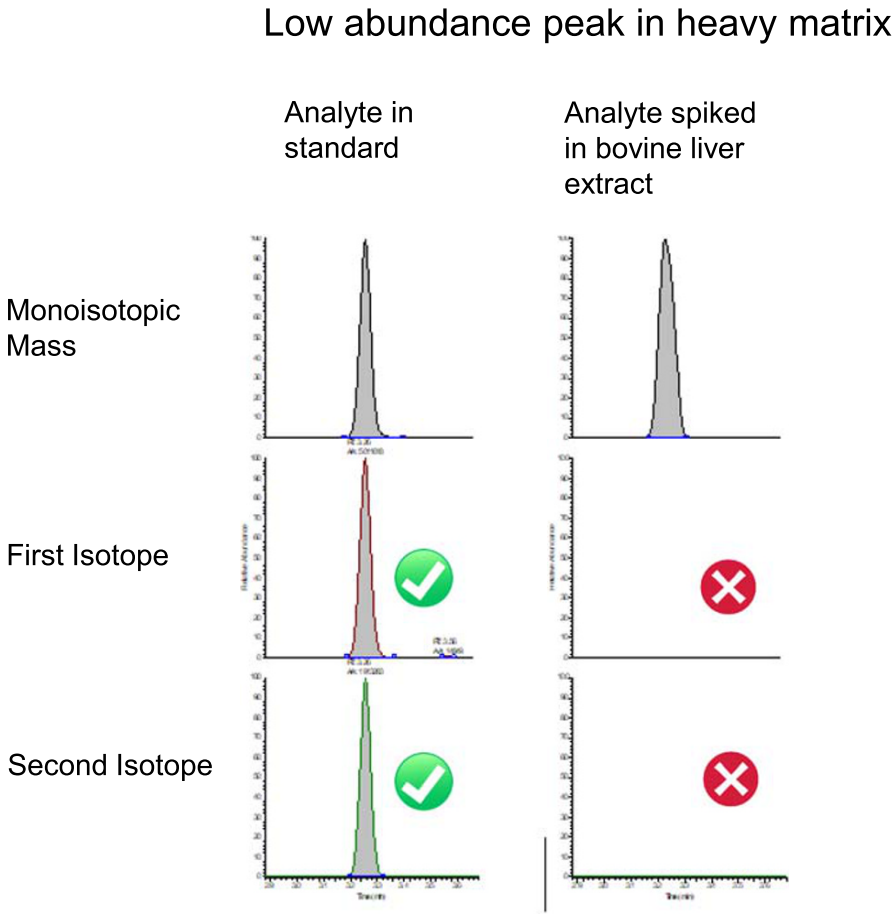

FIGURE 8 A sulfonamide present at an equally low concentration in a standard (left) and matrix extract (right) was injected and detected by an Orbitrap full scan. The monoisotopic signal (top) as well as the normalised signals for the first (middle) and second isotope (bottom) are given. Note the absence of both isotopic signals when analysing the analyte present in the matrix.

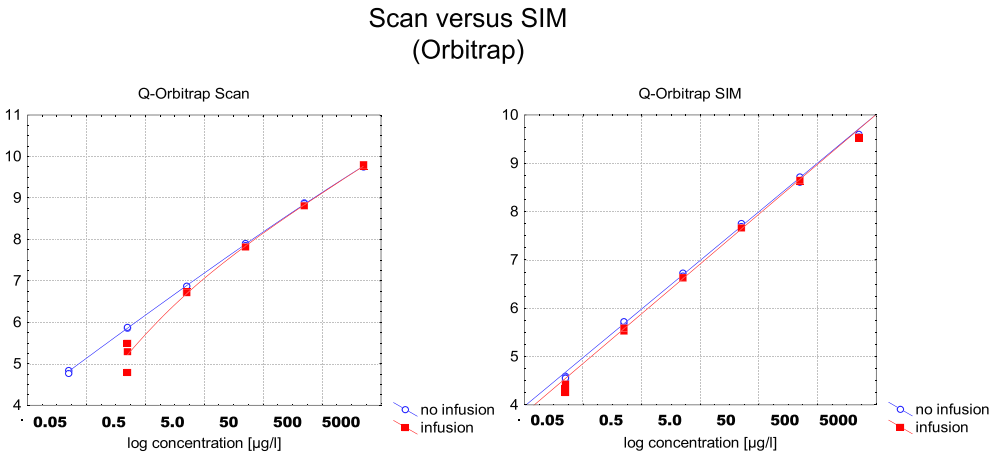

FIGURE 9 An Orbitrap full-scan-based linear dynamic range of an analyte (albendazole aminosulfone) with and without the presence of a very intensive base peak. Note the loss of the low-abundance analyte signal in the presence of an intensive base peak.

(e.g., sequential windowed acquisition of all theoretical fragment ion mass spectra [SWATH] or operating the quadrupole as a wide mass pass filter intended to prevent the collection of the mobile phase or column bleed-related ions).

3.2.5 | Signal suppression

Signal suppression is a phenomenon occurring during the ionisation process. The presence of a matrix can reduce (or in some situations, increase) the efficiency of the analyte ionisation and desolvation process. This is strictly a source/interface issue and is unrelated to the mode of detection. A phenomenon termed "postcolumn interface signal suppression"[73] occurs in single-stage Orbitrap instruments and is distinct from the loss of low-abundance analyte sensitivity associated with that related to the AGC. (See the discussion above.) Post-interface signal suppression was observed when the analyte eluted together with a high abundance of multiple-charged ions (e.g., proteins), and it was hypothesised that the presence of multiple-charged ions creates an environment within the C-trap where the trapping efficacy of single-charged, low *m/z* ions is dramatically reduced. This was a technical issue restricted to an early generation of single-stage Orbitrap instruments and is not observed in later released instruments.[7]

3.2.6 | Detector saturation and coalescence

As discussed above, most HRMS instruments use an AGC-like device to extend the interscan dynamic range of the instrument and to prevent undesirable saturation effects. Detector saturation differs for TOF and Orbitrap instruments. Older TOF instruments used a so-called time-to-digital detector (TDC). This device can precisely detect the arrival time of an ion, but the detector needs a certain "recovery" time to be able to detect the next ion hitting the detector plate. Out of this digital signal, an analogue spectrum is generated by summing hundreds of individual TOF pushes. This detector experiences two major problems. First, two or more concurrently incoming ions produce the same signal intensity as that caused by a single ion. This results in nonlinear calibration curves (i.e., saturation at high ion abundances). Second, if two or three identical ions hit the detector plate with a slight time spread, the detector will only trigger a signal for the first incoming ion and neither the signal intensity nor the time of the following ions will be recorded. Because there is no statistical averaging of the flight time of these nearly simultaneously arriving ions, a slightly biased flight time will be recorded. This leads to lower than true *m/z* values being reported. The consequences are shown in Figure 10, where a high concentration of analyte is injected and

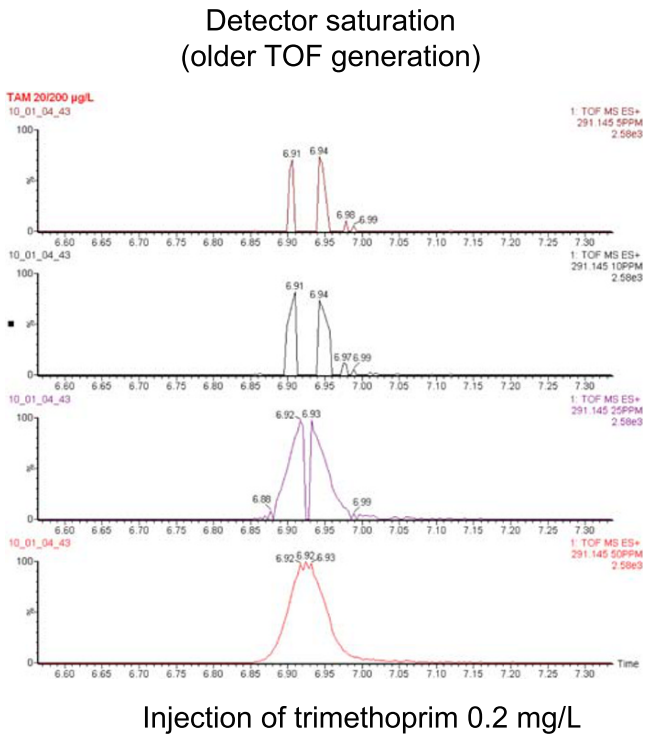

FIGURE 10 The effect of narrowing down the mass-extraction window on the chromatographic analyte peak when using an old time-to-digital-based time-of-flight (TOF) detector.

chromatographed. The chromatographic peak (bottom) looks perfect when applying a wide mass-extraction window, but a gap appears when narrowing the mass-extraction window (see upper traces). It is not that the analyte signal disappears, but that the mass shift (the TDC bias) causes the biased mass trace to disappear from the user-selected mass-extraction window. The introduction of analogue-to-digital detectors and hybrid detectors has significantly improved these problems.

TOF saturation only affects the ions exceeding a certain ion abundance. This is different for the Orbitrap where the C-trap defines an upper limit for the number of concurrently collected ions. This limit is given by the ion storage capacity of the C-trap. As mentioned, the use of the AGC should prevent such issues. Nonetheless, mass accuracy and the trapping efficiency of low *m/z* ions would be affected when exceeding such a limit. In addition, there is an Orbitrap analyser-related abundance limitation for adjacent *m/z* ions. This issue is not linked to the C-trap's ion capacity but to the analyser itself. The Orbitrap instrument's control software automatically proposes lower C-trap capacities when moving from a full scan towards SIM or PRM. This measure is imposed to prevent the possible "crowding" of ions within adjacent orbits within the analyser. By permitting a sufficient number of oscillations around the central Orbitrap spindle, ions with nearly identical *m/z* values can be separated and detected within the analyser, but exceeding certain ion abundances of these near isobaric ions can lead to detrimental effects. The high charge density between narrowly spaced orbits causes the ions to affect each other's flight trajectory. The repulsion of equally charged ions leads to a phenomenon called coalescence. Initially, it may be considered counter-intuitive that electrostatic repulsion leads to the merging of two or more isobaric ions into a very narrow single peak.[74,75] It has to be noted that coalescence is only detectable above a certain ion abundance. However, this is seldom observed in routine everyday work. Yet, coalescence may be a problem when using the quadrupole to isolate an analyte in order to determine the elemental composition by analysing the isotopic fine structure.

3.2.7 | Ruggedness and applicability

Beyond scientific limitations and capabilities, there are other factors that determine the acceptance and fate of a particular technology. Such factors have to be known because they may affect daily routine work more than technical specifications will.

Mass-axis calibration is an aspect of particular importance. Mass-axis calibration is also required for QqQ instruments, although it is of minor importance because QqQ mass-axis calibrations stay stable for a long time. On the other hand, the high mass-resolving power of HRMS is only an asset when accompanied by high mass accuracy. Commercial HRMS instruments are provided with a more or less user-friendly mass-axis calibration routine. A previously calibrated Orbitrap mass axis stays stable for an extended period of weeks. Temperature changes or normal net voltage fluctuations barely affect the mass accuracy. This is different for TOF analysers. Temperature-dependent flight-tube expansions and contractions used to be a problem for older generation instruments. This problem has been successfully resolved by a number of engineering solutions. However, short- and long-term mass stability is still affected by many other factors. Hence, depending on the particular instrumentation, partial or even full mass-axis calibration is required at periodic intervals. Most commercial TOF instruments use a feature called lock mass spray. This was historically done by continuously infusing a mass calibrant. Such a technique had a number of disadvantages. An insufficient ion abundance of the calibrant could lead to the loss of the lock mass signal (e.g., during the elution of an intensive matrix peak). On the other hand, continuously infusing a mass calibrant pollutes every acquired spectrum not only with the mass peak of the calibrant, but most likely with other coinfused contaminants. Hence, most vendors maintain a system whereby a number of sample spectra are taken, and this acquisition sequence is interrupted at regular periods to enable a rapid switching on of the lock spray solution. The mass-axis stability of the latest commercial TOF instruments is now that high that such a lock spray infusion can be done before or after a chromatographic run. In other words, the situation has greatly improved. However, TOF mass-axis calibration is still an issue whereas Orbitrap users have fewer concerns with mass-axis stability.

The Orbitrap analyser contains no mechanical features nor any other parts that are affected by wear and tear. This is somewhat different for TOF. The detector plate is sensitive to humidity and oxygen. This is not an issue while under operation, but after venting, most detector cells require a constant stream of nitrogen to prevent degradation. In addition, detector plates age during normal operation. This can be compensated for by increasing the applied voltage. Nonetheless, after reaching a certain upper voltage limit, the detector plate has to be replaced. The associated costs are far from negligible.

The initial investment required for an HRMS instrument was often considered to be a major reason for continuing with QqQ technology. The price difference between these two technologies has certainly narrowed so that high-end QqQ instruments currently cost more than mid-range Q-HRMS instrumentation does. During the last few years, retail prices for some instrument configurations have changed markedly. This has probably less to do with the development and production costs of HRMS instrumentation and more to do with the pricing politics of vendors that sell both types of instrument. The monopoly situation with the Orbitrap instrument initially led to significantly higher Orbitrap than TOF retail prices. High speed, higher mass-resolving TOF instruments can compete with Orbitraps for some application and has likely created some pressure to lower Orbitrap prices.

The regulatory framework has frequently been cited as an aspect favouring time-trusted QqQ technology. The way in which compounds have to be detected and, most importantly, confirmed is governed by a number of regulatory documents. It is obvious that

such infrequently updated documents cover well-established technologies more extensively than they do newer or emerging technologies. In addition, such documents may lack supporting interpretative guidelines, thus leading to a situation whereby local regulatory authorities in one country will consider a certain analytical procedure as compliant, while in another country, the responsible authority will interpret the very same method in a different way. Such fears constitute a reason for the reluctance to adopt HRMS in regulated environments.

Discussions regarding HRMS versus QqQ frequently mention a higher level of user knowledge required to successfully implement HRMS as a detection and quantification technique. We consider this questionable. Our laboratory participates in an apprentice training programme where young people with minor or no previous experience in MS receive training. Based on this, setting up a QqQ-based MRM method is considered more intellectually challenging than developing a simple HRMS method. Transitions, especially elucidating the best product ions, are clearly less straightforward than calculating the accurate mass and, depending on the ionisation mode, including or excluding the mass of a hydrogen. Many analysts who have their initial contact with MS by utilizing HRMS consider QqQ-based MRMs as a difficult concept.

The currently still thinly populated user base of HRMS analytes is certainly a limitation. In addition, HRMS is further split into TOF and Orbitrap instruments. Hence, it is much easier to find somebody working with the same analytical problem by using QqQ than someone employing TOF or an Orbitrap. Not being able to use developed methods is one issue; troubleshooting technical aspects may constitute another issue. Many HRMS vendors are aware of this issue and have increased the staff in their application laboratories. However, this may remain an issue for contract laboratories that expect paid-for customer samples to be run directly after having the newly bought instrument installed. Finally, perceived and experienced difficulties with a new technology are frequently linked to limited training and familiarisation.

The data size and the time to process the data (extraction of mass traces, analyte concentration calibration, and reporting) are important issues. The data-processing speed and the ease of use of the data-processing software (especially for the multiresidue method) have been significantly improved over the last few years. This certainly reflects the growing HRMS user base that has permitted the development of improved software versions. There is, however, still a long list of software-related wishes, but this has increasingly less to do with essential tools and more to do with nice-to-have features. A problem remains with the large datafile size. The increasing mass-resolving power and the higher speed of HRMS instrumentation demand large data storage devices. Finally, old raw data should be properly archived to remain available for retrospective review. This aspect still represents a bottleneck and will become even more pressing when hyphenating HRMS with techniques such as ion mobility. As mentioned, the storage of centroids instead of continuum data and the application of a threshold below which no data is recorded are crude measures imposed to keep the datafile sizes within manageable dimensions.

3.3 | Unique benefits provided by HRMS

3.3.1 | Information available from sensitive full-scan data

The availability of sensitive, highly mass resolved full-scan data is the main advantage of HRMS- versus QqQ-based data. Quadrupole scans are faster than highly mass-resolving Orbitrap scans, but they are clearly slower than TOF scans. However, quadrupole scans (including those obtained by using hyperbolic rod-based QqQ instruments) are incapable of providing the sensitivity and selectivity in combination with the wide scan range available when using HRMS instrumentation. Although QqQ instruments are capable of using a variety of acquisition modes (scan, precursor scan, product ion scan, neutral loss scan), it is the MRM which is by far the most frequently used QqQ acquisition mode. An HRMS instrument (Orbitrap or TOF) operated in the scan mode with a mass-resolving power of greater than 50,000 FWHM provides a selectivity that rivals or exceeds[65] a QqQ MRM. In many cases, the sensitivity may be somewhat inferior to that of a QqQ-based MRM, yet the freedom to run an acquisition without having to define any compound-specific settings is a great asset. This "inject first, think later" mantra is absolutely essential for nontargeted analysis. Nonetheless, even targeted analysis benefits from this concept.

As discussed above, some compounds may require an additional degree of sensitivity and selectivity which can be obtained by targeted acquisitions (PRM) where the quadrupole is used to isolate a narrow mass range (e.g., a particular precursor ion). Ions may be fragmented and detected by the HRMS analyser. Using such modes basically converts the instrument into a highly resolving QqQ instrument and will require setting up a compound-specific isolation window and fragmentation energy. In the case of several PRMs, retention-time windows will be required. Even in such cases, it is important to maintain a full-scan signal for the whole chromatographic run time. At a glance, a number of critical issues can be spotted when having access to a full-scan trace. The presence of an injection signal shows that a sample and not an empty vial has indeed been injected. The rising baseline due to column bleed at the end of the chromatogram indicates that the gradient has worked properly and again that a sample and not an empty vial has been injected. The appearance of a large, wide, and shifting peak within a chromatogram points to a matrix carry-over from a previous injection. An unusually large peak appearing at a given retention time or a significantly elevated peak is most likely due to contamination being present in the mobile phase or the sample. By observing such issues, the underlying problem can be addressed quickly. This information was available when analysts were still working with LC-UV technology. Many chromatographers bemoaned that loss when they started utilising QqQ detector technology. Currently, LC-HRMS is providing analysts with this previously lost diagnostic information.

There are chromatographic separations where an analyte tends to split into different chromatographic peaks. Figure 11 shows two chromatograms for acepromazine. The compound elutes at 3.8 min, but a

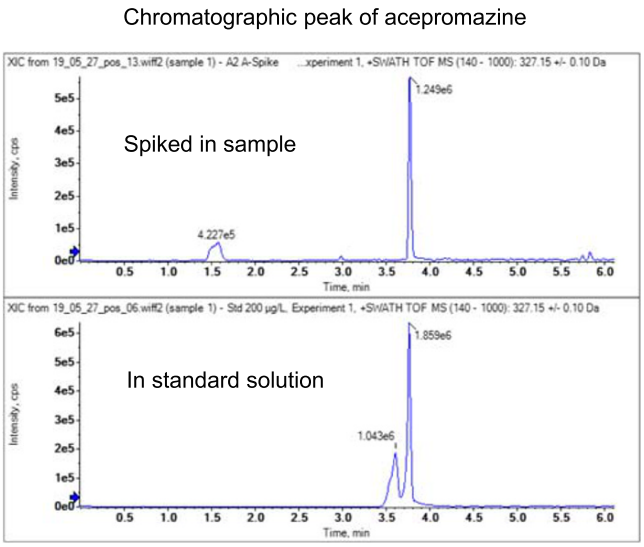

FIGURE 11 An example of an amphoteric analyte producing more than one chromatographic peak. Using retention-time windows may not spot this behaviour.

variable amount of the analyte appears within the column void volume. This can frequently be observed for dissociable compounds that are dissolved in a solvent that is slightly stronger than the initial mobile phase strength is. Such conditions should be avoided, but multiresidue methods which cover compounds with a wide polarity range may require the use of a relatively strong solvent in order to recover polar analytes after an evaporation and solvent-exchange step. Behaviour like the splitting of chromatographic peaks would be much less likely to be noticed when relying on a retention-time-based acquisition window as is typically used for QqQ-based MRM acquisitions.

It may also be relevant to be able to perform a retrospective analysis[76] of existing data. Discovering a new contaminant or residue in a particular sample may lead to questions regarding that analyte also perhaps being present in other, already analysed samples. Furthermore, it may be of interest to find out how long the compound has been in the environment for or how long it has been present in a particular food commodity for.

3.3.2 | Detection of compounds in the absence of reference compounds and generic fragments

An answered question often creates a set of new questions. Finding a particular pesticide may lead to questions about known degradation products also being present. After finding a particular active substance, literature research may reveal that the found compound is frequently used together with some other compounds. Or, when finding a veterinary drug, confirmation of its presence may be made through detecting metabolites which are reported in the literature. Finding a

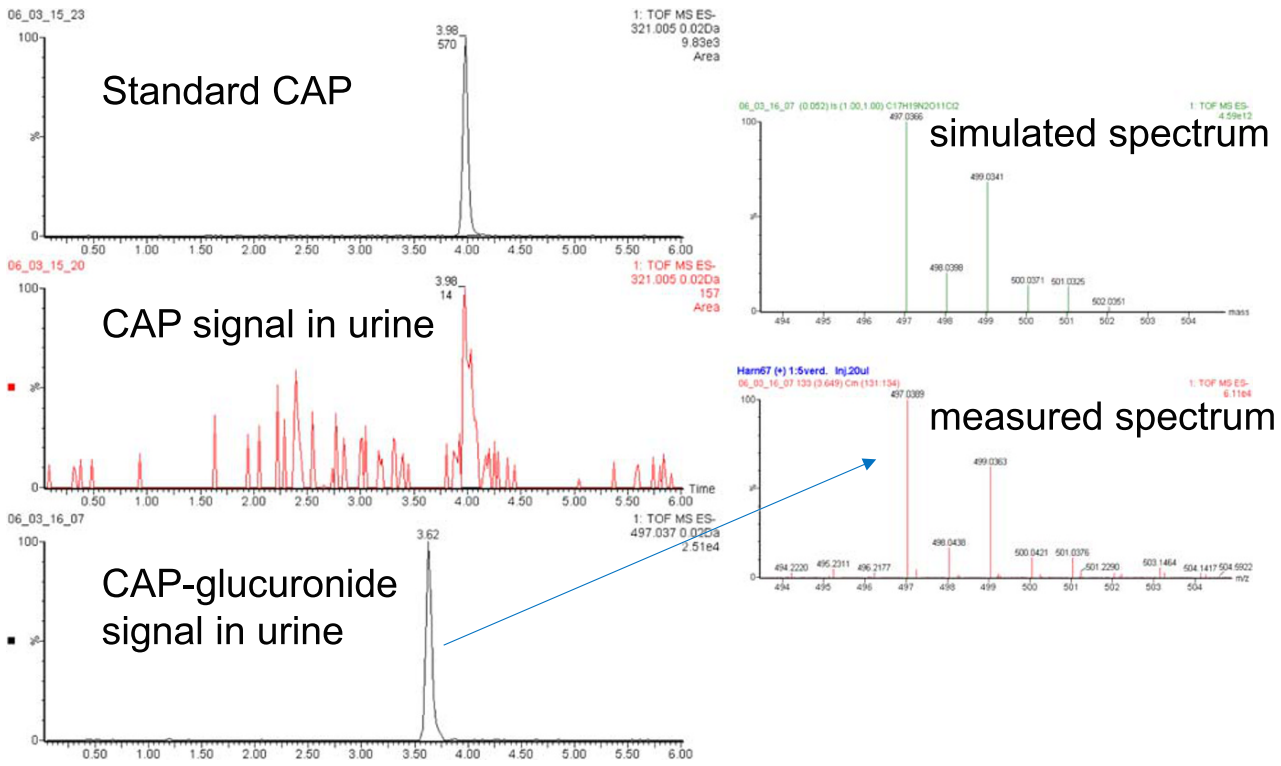

FIGURE 12 Low signal-intensity-based detection of chloramphenicol in urine (middle left). Extracting a narrow mass window around the calculated mass of the chloramphenicol metabolite produces a distinct signal and a spectrum that corresponds well with the simulated spectrum (right).

parent drug together with a metabolite is not only confirmation of the presence of the active drug; detecting a metabolite is the proof that the found active drug is not the product of sampling contamination but that the drug has truly passed into the body of a living animal. Since most metabolites are not commercially available substances, they are generally not present in the analysis laboratory. Therefore, false-positive findings due to carry-over or contamination within the laboratory can clearly be ruled out. Hence, detecting a suspected compound where no physical reference substance is available is an important capability of HRMS. QqQ instruments are much less suitable for this task. This is due to their poor full-scan sensitivity and the lack of selectivity (the unit-mass resolving power of quadrupoles).

Two examples showing the power of extracted mass traces from full-scan acquisitions are given herein. Chloramphenicol (CAP) is an antibiotic that is used for animal treatment and has been banned due to toxicological reasons. Hence, such a banned compound has to be detected at low concentrations in complex matrices. Bovine urine is one of the sample matrices to be analysed. CAP given to animals is metabolised and excreted in the urine in the glucuronidated form. The parent drug for which commercial reference standards are available is present in urine at a much lower concentration than the metabolite is. Hence, due to sensitivity reasons, the proof of the illegal use of CAP may be based on detecting the metabolite rather than the parent drug. Unfortunately, like many other drug metabolites, reference standards are either difficult to obtain or are not commercially available. This limits the utility of QqQ for this application. Even if transitions (MRM) are available from a literature search, the conditions (the collision energy and, frequently, the obtained fragments) can often not be transferred between two different QqQ instruments (e.g., different vendors). An example is shown where an ELISA screening test produced a suspicious CAP response in the particular sample. The sample was confirmed by LC-HRMS where again a very weak signal at the retention time for CAP was detected (Figure 12). This still questionable finding could be further confirmed by extracting a narrow mass

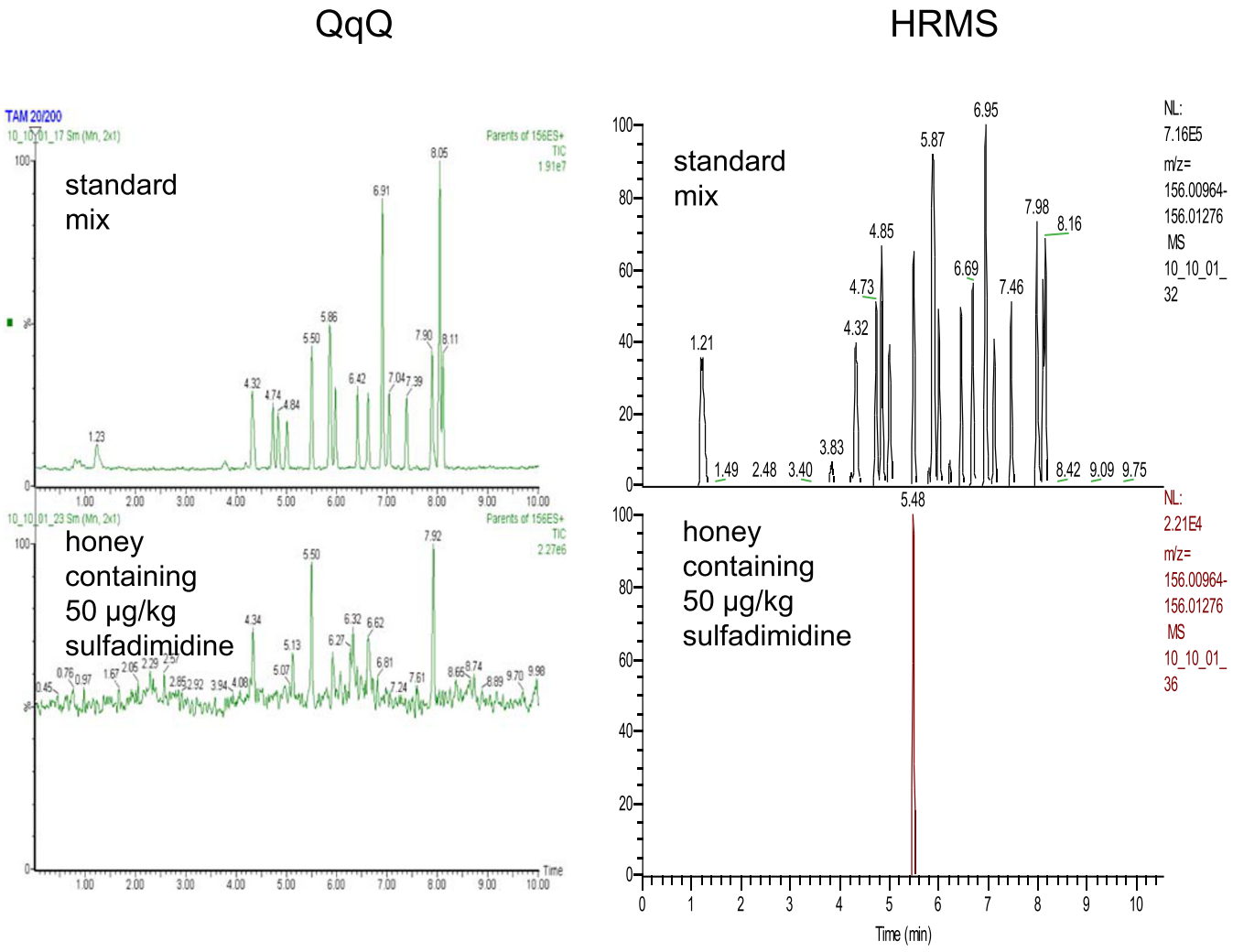

FIGURE 13 Triple high-resolution mass spectrometry (HRMS)– (right) based generic detection of sulfonamides by all-ion fragmentation. The chromatograms show the generic sulfonamides m/z = 156 when present in the standard (top) and in the matrix (bottom) extract. The honey sample contains only a single sulfonamide that is much more clearly visible when using HRMS than when using QqQ.

trace representing the calculated exact mass of CAP glucuronide. A single chromatographic peak emerged. The low energy peak apex spectrum (Figure 12) was compared with the theoretical (based on the elemental composition) spectrum of CAP. In this case, confirmation was possible, although neither a physical reference standard nor a spectral library entry was available.

QqQ-based precursor ion scans were developed to detect all compounds that share a particular chemical moiety (substructure), but QqQ-based precursor ion scans are slow, insensitive, and insufficiently selective. The top row of Figure 13 shows the chromatograms obtained when injecting a standard solution containing a number of different sulfonamides. The left side of Figure 13 shows the QqQ precursor ion scan, and the right side of Figure 13 shows the HRMS trace. The HRMS instrument did not use the quadrupole (rf mode only). A so-called all-ion fragmentation was applied to all incoming ions, and the accurate mass of the generic sulfonamide fragment (m/z = 156.011) was extracted. HRMS permits the definition of narrow mass-extraction windows and therefore produces better selectivity and sensitivity and shows a lower baseline level than QqQ does (this without using the quadrupole as an ion selection device). This becomes even clearer when injecting a blank honey sample spiked with a single sulfonamide at a trace-level concentration (the bottom of Figure 13). The QqQ precursor scan-based trace shows a high baseline and a number of false detections. A significantly better performance is observed when using the HRMS instrument. Hence, HRMS is clearly the better screening tool when compared with QqQ-based precursor ion scans. In addition, even finding a precursor ion based on a precursor ion scan would only reveal the nominal mass of a suspected sulfonamide, which is insufficient for structural elucidation. On the other hand, the HRMS instrument could be used to elucidate the precursor ion (e.g., by SWATH-based approaches). This would finally permit the determination of the elemental composition based on accurate masses and associated relative isotopic abundances.

3.3.3 | Determination of the elemental composition

An important capability of HRMS instrumentation is that it can indicate the elemental compositions of analytes present at trace levels. It is not impossible to determine accurate masses from unit-mass-resolved quadrupole-based measurements, but at unit-mass resolution, coeluting isobaric component can lead to incorrect accurate mass assignments. This will be the case for all residue applications in complex matrices. Hence, the elucidation of elemental compositions requires the use of HRMS instrumentation. For small molecules, accurate mass values in combination with isotopic ratios can produce a single possible elemental composition. However, above m/z > 300, the number of possible combinations of C, H, N,

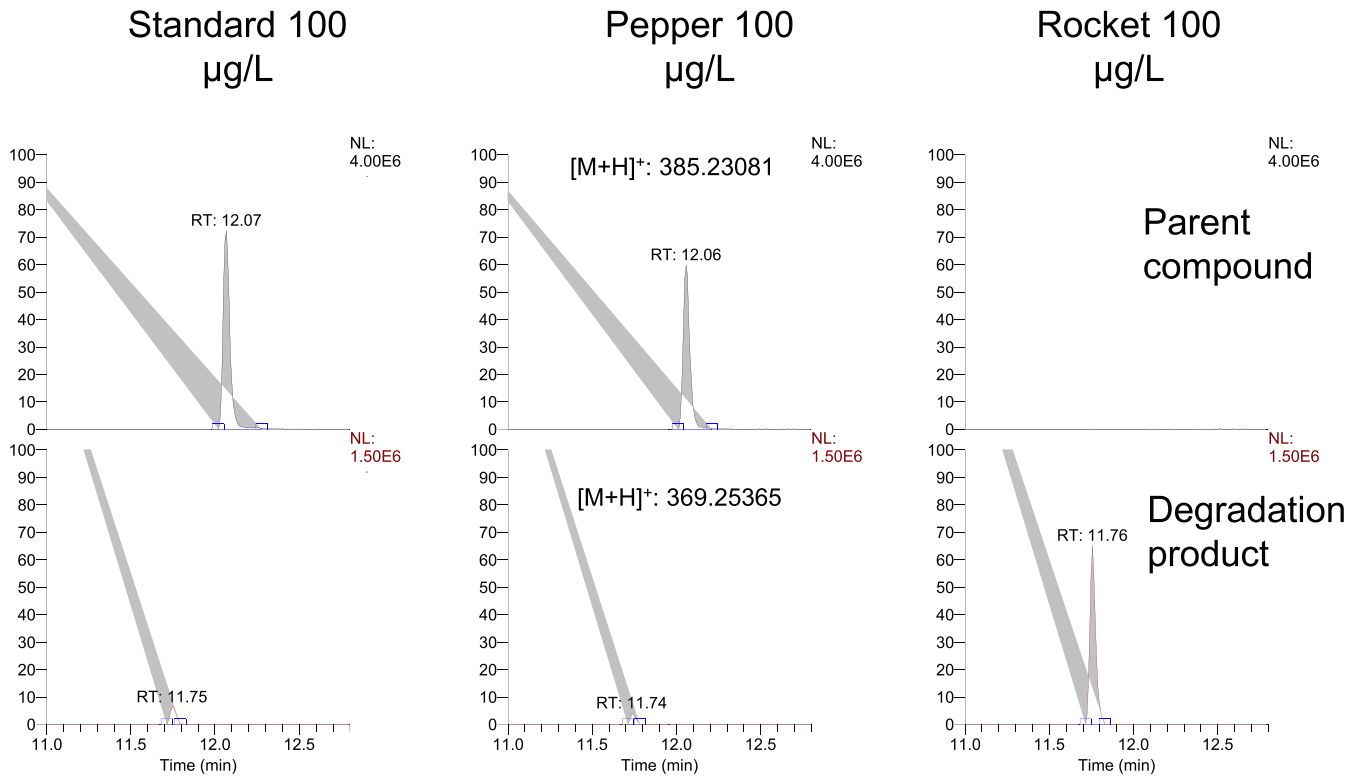

FIGURE 14 The signal for the diafenthiuron pesticide in a standard (left), pepper (middle), and rocket (right) matrix. The top traces show the parent compound, and the bottom traces the degradation compound. Note the absence of the parent drug when fortified into the rocket matrix and the emergence of the degradation product for which no reference compound was available.

O, and so forth, becomes so large that the elucidation of a single elemental composition requires physically unattainable mass accuracies.[77] Nonetheless, the use of the isotopic fine structure[78] where the appearance of nitrogen and sulphur masses, as well as their relative ion abundances, permits the extension of the upper mass range. It is important to appreciate, however, that even the elucidation of the correct elemental composition does not define a unique structure: that is, there may be more than 100 listed database compounds sharing the same elemental composition. Under these circumstances, the investigation of product ions may be helpful. Nevertheless, it is commonly recognised that MS alone is not always capable of determining the chemical structure of completely unknown compounds. This still remains the domain of (low-sensitivity) nuclear resonance spectrometry in combination with MS.

3.3.4 | HRMS-based method development and troubleshooting

It is common that during analytical method development, a set of initially unexplainable observations or results come up. MRM-based QqQ instrumentation only provides the method developer with information regarding the analyte. The data may show that some analytes may produce unexpected low recoveries or poor reproducibility in some matrices. How can this be explained or even rectified? Having access to HRMS data provides the method developer with an additional set of diagnostic tools. Two examples where the availability of full-scan HRMS acquisitions was essential for elucidating and finally solving underlying problems are given below.

Diafenthiuron in rocket (arugula or *Eruca vesicaria*): The dataset[48] has been discussed above. Diafenthiuron was detectable with QqQ and HRMS in lettuce and pepper, yet it remained undetectable for both detection technologies when it was spiked into a rocket matrix. Hence, the existing QqQ method constantly reported diafenthiuron in rocket as "undetectable." A quick web-based investigation showed that diafenthiuron can be degraded into two degradation products.[48] These two compounds are not commercially available, therefore the QqQ instrument could not be easily tuned for their detection. On the other hand, calculating their exact masses permitted the extraction of narrow HRMS mass traces which revealed the presence of one of the postulated degradation products (see Figure 14). Certainly, the absence of a reference substance does not permit the quantification of this compound,[79] yet a possible treatment of rocket with diafenthiuron became detectable.

Clean-up improvement of a steroid method: A novel steroid multiresidue method[49] was about to be validated, but the

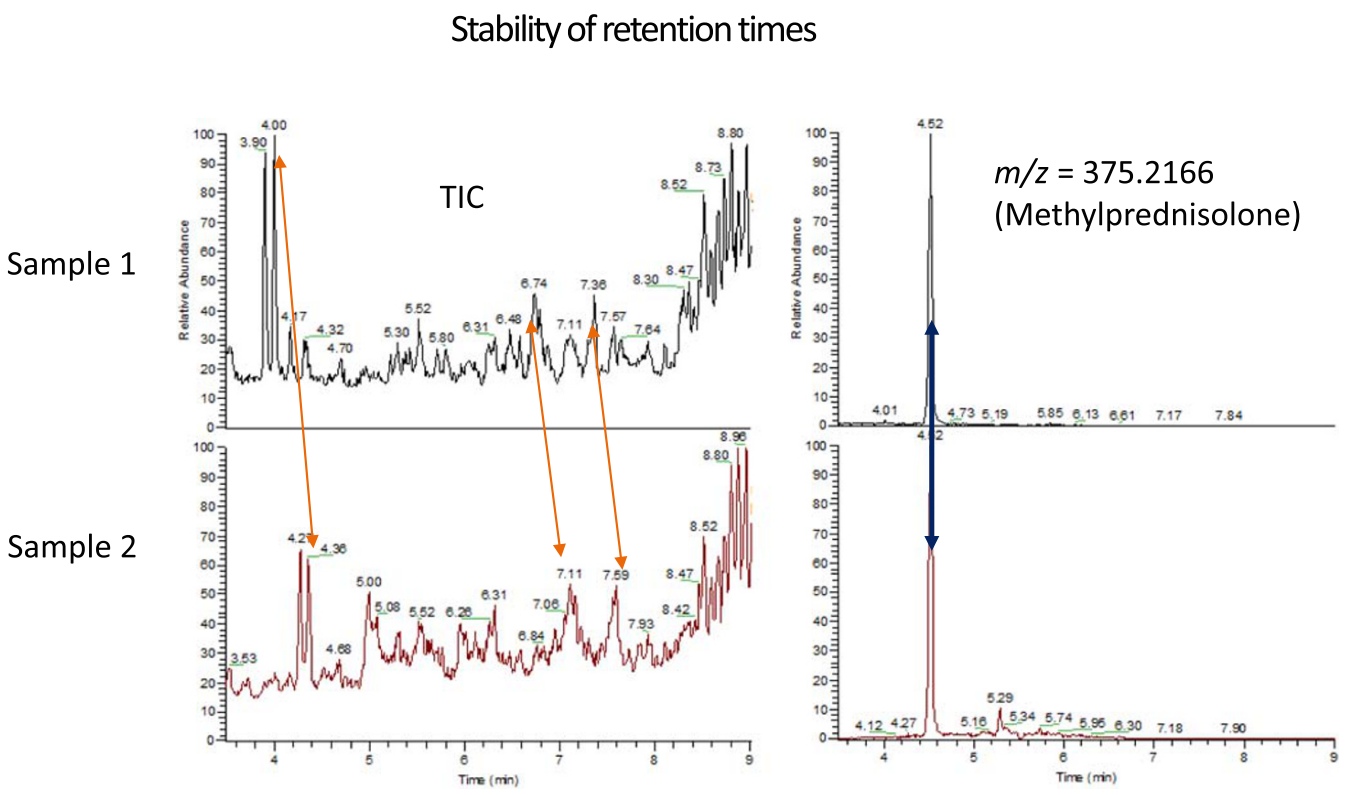

FIGURE 15 Injection of two steroid-spiked bovine urine sample extracts. The differences between the two samples caused the shift of retention times for some matrix peaks (left), but the retention time of the nondissociatable analyte (methylprednisolone) is not affected (right). This behaviour can lead to irreproducible signal-suppression effects.

prevalidation data showed poor precision. There were poorly reproducible signal-suppression effects (between, but also within, a given type of matrix). A closer investigation of the full-scan signal showed that the retention time of many intensive matrix compounds differed from sample to sample. On the other hand, the retention times of the various analytes were very stable (see Figure 15). This ruled out the pump as the source but pointed to the chromatography. The dominating matrix peaks were quickly identified as partially unsaturated fatty acids by accurate mass as partially unsaturated fatty acids. The method under development had to detect analytes which require positive as well as negative ionisation for their sensitive detection. Enabling both detection modes with a single mobile phase prevents the use of acidic or basic additives. However, a small amount of ammonium fluoride present in the mobile phase can significantly improve the detectability of sensitivity-critical oestrogens. Yet, the low modifier concentration does not anymore sufficiently buffer the mobile phase. It was therefore concluded that the eluting fatty acids modify the mobile phase pH value along the length of the separation column in a dynamic way. This pH change does not change the retention time of the undissociable steroids. Yet, the pH change induced by the dissociable fatty acids affects their own retention time. Therefore, a fatty acid matrix peak may coelute in one sample with an analyte peak, while no such coelution is observed in another sample. This leads to irreproducible signal-suppression effects. This knowledge led to the concept of a modified sample clean-up. Instead of performing the liquid/liquid extraction (aqueous/ethyl acetate) at pH 5.0, a higher value of 9.0 was selected. At that pH value, significantly fewer fatty acids moved into the analyte-containing organic phase. This resulted not only in a less intensive total ion current, but it significantly improved the reproducibility and precision of the analyte peak areas.

4 | CONCLUSIONS

Over the last decade, MS technology has significantly progressed. Lower concentrations of analytes in more complex matrices can be analysed with more reliable and user-friendly instruments. QqQ sensitivity and the number of MRMs acquired within a set period of time have been greatly improved. This trend is likely to continue. Yet, due to the virtually unchanged unit-mass resolution selectivity, QqQ technology will soon reach a point where matrix interference and not the S/N will be limiting. This bottleneck does not yet exist for HRMS because the S/N has increased in parallel with the mass-resolving power. Triple quadrupole instruments were originally not developed because it was thought that using two quadrupoles in sequence represented a very elegant way of obtaining more selectivity. It was rather the realisation that physics prevents a quadrupole from filtering a mass peak with a width clearly below 1 Da. The inherent ion-transmission loss and the difficult fragmentation properties of some compounds had to be accepted in the absence of an alternative. It is remarkable that such a concept, which was born out of a need, has become the widely accepted gold standard. It is even more remarkable that such a concept has not only been promoted to analyse a few analytes but has been expanded to monitor greater than 500 analytes within a single chromatographic run. The analysis of greater than 500 pesticides may still be considered as a targeted approach, but it is approaching a nontargeted concept and should therefore be preferably attempted by such a technology. Technical history is full of examples where a proven technology was constantly improved, but nevertheless, was finally squeezed into a niche existence by an emerging technology. This was the case for the replacement of horsepower by engines, typewriters by computers, vinyl discs by CDs, and possibly the more recently initiated change from cars running on gas versus being powered by an electric engine. These changes never go smoothly because it is more than just a financial or technological question. People become emotionally attached to a technology and there is a reluctance to adopt change. There may also be a fear that well-established structures will be challenged not only by a new technology but by a new generation of analysts who are faster in embracing and understanding this new world. Frequently, the increasingly dense regulatory framework prevents the better technology from being readily accepted. Or worse, the regulatory framework serves to prevent technological changes.

Thus, there is still a need to show that HRMS can produce equally reliable, accurate, and precise results as QqQ can.

It was my aim to provide solid data and illustrative anecdotal evidence that HRMS offers a performance that can excel that of the so-called gold standard. This is not only partially due to the resolving power and related selectivity enhancement. Even more important is the fact that HRMS provides several tools that enable researchers to develop robust methods and help analysts to identify and resolve potential problems in a much faster and more efficient manner. This paper addresses known positive and negative aspects of historical and present HRMS technology. My hope is that it enables newcomers, not only to decide to solve their daily analytical problems with HRMS, but also to become faster and more productive after having received the instrumentation.

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