Review

The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food – Challenges and applications

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A B S T R A C T

In the field of food contaminant analysis, the most significant development of recent years has been the integration of ultra-high pressure liquid chromatography (UHPLC), coupled to tandem quadrupole mass spectrometry (MS/MS), into analytical applications. In this review, we describe the emergence of UHPLC through technological advances. The implications of this new chromatographic technology for MS detection are discussed, as well as some of the remaining challenges in exploiting it for chemical residue applications. Finally, a comprehensive overview of published applications of UHPLC–MS in food contaminant analysis is presented, with a particular focus on veterinary drug residues.

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Contents

1. Introduction ............................................................................................................................ 84
2. The introduction of UHPLC and its impact on residue analysis .............................................. 85
3. Coupling UHPLC with MS/MS detection ................................................................................. 85
   3.1. Acquisition rate and method sectoring .............................................................................. 86
   3.2. UHPLC–MS/MS versus HPLC–MS/MS ......................................................................... 86
4. Challenges inherent in UHPLC–MS/MS techniques ............................................................... 87
   4.1. Frictional heating effects .................................................................................................. 87
   4.2. Band broadening effects .................................................................................................. 87
   4.3. Narrow analyte peaks ...................................................................................................... 88
   4.4. Matrix effects .................................................................................................................. 88
   4.5. Column blockage ............................................................................................................ 89
5. Recent applications of UHPLC–MS technology in the analysis of agrochemical residues and mycotoxins ................................................................. 90
   5.1. UHPLC–MS/MS methods for prohibited substances ....................................................... 90
   5.2. UHPLC–MS/MS methods for substances with an established permitted limit ................. 91
   5.3. Multi-class multi-residue analysis .................................................................................... 92
6. Conclusion............................................................................................................................... 93
Acknowledgements .................................................................................................................... 93
References ..................................................................................................................................... 93

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1. Introduction

Within the past decade, the introduction of ultra-high pressure liquid chromatography (UHPLC) and rapid-scan and sensitive mass spectrometry (MS) instruments has resulted in a seismic shift away from traditional chromatographic techniques in the field of food contaminant analysis, towards multi-class, multi-residue methods with short injection cycle times and minimal sample preparation. The competing pressures of improving sample throughput, reducing analysis costs and complying with regulatory requirements have led to the continued adoption of UHPLC–MS/MS as the technique of choice in the analysis of veterinary drug residues and related substances. In the present review, a comprehensive overview of published UHPLC–MS/MS food contaminant applications is presented, with a particular focus on veterinary drug residues. However, this is still a relatively new development, challenges associated with the technology are still emerging, and some of these will also be detailed and discussed.

Analytical strategies applied in this field have evolved, not only to respond to both statutory requirements for the scope of analytes detected and the needed method detection limits to meet enforcement action levels, such as those stipulated by the European Commission in 2002/657/EC [1], but also in response to the adoption of MS as the most frequently used detection method. The improved selectivity offered by MS/MS methods means that the analysis can still be conducted with a lesser degree of chromatographic resolution between target analytes, or between matrix components and analytes. LC–MS/MS also offers greater versatility than that of GC–ECD (gas chromatography–electron capture detection) or GC–MS methods, where the analyte must be volatile, or else put through a derivatisation process to permit volatilisation of the analyte. With the specificity of detection greatly improved and sample preparation time reduced, innovation in the field of chromatographic separation became a research focus. Since 2003, more commercial UHPLC systems continue to become available (Table 1).

While the development of ever-more efficient monolithic silica HPLC columns has been an area of extensive chromatographic research activity, the development and refinement of UHPLC, employing columns packed with sub-2 µm particles, has had the largest impact on analytical separation science. The Van Deemter equation for describing band-broadening mechanisms relates separation efficiency of a method to the linear velocity of the mobile phase, which changes according to the size of the stationary phase carrier particles. Thus, lower particle size permits an increase in separation efficiency, and consequently, conventional HPLC separation time can be reduced, or resolution between analytes increased. This has beneficial ramifications for the field of chemical residue analysis, where frequently, analytical methods test for analytes that are similar in chemical nature.

For example, the epimeric compounds dexamethasone and betamethasone (Fig. 1) are licensed for therapeutic use against inflammation in bovine animals in the European Union (although use as growth promoters is banned). Chromatographic resolution of these compounds can be difficult to achieve, due to their structural similarity. Separation of these compounds has been performed using a special column containing Hypercarb™ porous graphite [2]. Applying UHPLC conditions to this separation, Decueuninck et al. [3] attained resolution of the dexamethasone/betamethasone pair using an Acquity™ BEH C18 column (1.7 µm, 2.1 mm × 100 mm), in a method validated according to Commission Decision 2002/657/EC [1]. The same column chemistry was employed by Touber et al. in their earlier work [4] on developing a multi-residue method for corticosteroids, including dexamethasone and betamethasone. Chromatographic resolution of dexamethasone and betamethasone is only one example of where the power of UHPLC has been successfully applied to solve challenges in multi-residue analyses. Elsewhere, it has been shown that UHPLC–MS/MS can be used to separate and identify as many as 107 pesticide compounds in strawberries, using a run time of just 6.5 min [5]. Further examples of successful application of UHPLC–MS/MS in food contaminant applications will be discussed in the review.

The number of published methods exploiting UHPLC–MS/MS continues to grow each year as the technology matures (see Fig. 2). However, there remain outstanding challenges to be addressed. The reduced sample preparation approach which has become standard in analytical strategies (e.g. QuEChERS) poses questions as to what extent the matrix effect influences the outcome of analyses, and whether generic clean-up methods are necessarily compatible with MS detection. The application of UHPLC-type separation can also lead to chromatographic peaks which have a width of 1–3 s. The analyst must then examine whether the data acquisition rate is appropriate for compliance with quality control requirements, especially in applications where analytes co-elute or elute within a time segment where data is collected for several analytes. The application of high pressure to separations that is the core of UHPLC technology can also result in the creation of “frictional heating” effects, with possible ramifications for chromatographic methods. This review will examine these aspects of UHPLC–MS/MS application, followed by a detailed examination of how UHPLC–MS/MS has been exploited in various food contaminant analyses, through an overview of work published in the field, as well as work of general relevance which is of importance to method development using this new technology. However, we first describe the emergence and development of UHPLC, after two decades of the

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>UHPLC system</th>
<th>Maximum pressure (bar)</th>
<th>Maximum flow rate (mL min⁻¹)ᵃ</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent</td>
<td>Infinity 1290</td>
<td>1200</td>
<td>2</td>
<td>Pump delay volume of &lt;10 µL, depending on solvent mixing unit</td>
</tr>
<tr>
<td>Hitachi</td>
<td>LaChromUltra</td>
<td>600</td>
<td>2.5</td>
<td>System delay volume as low as 266 µL</td>
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<tr>
<td>Jasco</td>
<td>X-LC</td>
<td>1000</td>
<td>1.35</td>
<td>Minimum injection interval of 30 s</td>
</tr>
<tr>
<td>Shimadzu</td>
<td>Nexera</td>
<td>1300</td>
<td>3</td>
<td>Minimum injection interval of 14 s</td>
</tr>
<tr>
<td>Thermo Scientific</td>
<td>Accela</td>
<td>1250</td>
<td>2</td>
<td>Pump delay volume of 70 µL</td>
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<tr>
<td>Waters</td>
<td>Acquity H-Class</td>
<td>1000</td>
<td>1</td>
<td>System delay volume &gt;400 µL; injection interval of 30 s</td>
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<tr>
<td>Waters</td>
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<td>1</td>
<td>System delay volume of &lt;100 µL; injection interval of 15 s</td>
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<td>System delay volume of &lt;1 µL</td>
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<td>Dionex</td>
<td>UltiMate</td>
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<td>Injection cycle time of 30 s</td>
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<tr>
<td>PerkinElmer</td>
<td>Flexar</td>
<td>1240</td>
<td>5</td>
<td>Injection cycle time of 8 s</td>
</tr>
<tr>
<td>SSI LabAlliance</td>
<td>UHP</td>
<td>1720</td>
<td>5</td>
<td>Flow rates of up to 12 mL min⁻¹ possible</td>
</tr>
<tr>
<td>Eksigent</td>
<td>UltraLC</td>
<td>1240</td>
<td>5</td>
<td>Mixer volume 60 µL Injection cycle time of &lt;60 s</td>
</tr>
<tr>
<td>Knauer</td>
<td>PLATINblue</td>
<td>1000</td>
<td>2</td>
<td>System delay volume of 110 µL; 15s injection cycle time</td>
</tr>
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ᵃ Maximum flow rate achievable at maximum pressure.
predominance of HPLC in fields such as veterinary drug residue analysis, environmental analysis and pesticide residue testing.

2. The introduction of UHPLC and its impact on residue analysis

The utility of very high pressures (in combination with small particles) in improving chromatographic efficiency, as well as reducing analysis time, was first explored by Jorgenson and colleagues in the 1990s [6,7]. Soon after this work, the first commercial UHPLC systems came on to the market in 2004, along with Agilent RRHT columns (1.8 μm particle size) and Waters Acquity BEH columns (1.7 μm). The successful uptake of this technology was attributed to the benefits associated with the sub-2 μm column technology. In order to utilise sub-2 μm columns, new pumping technology had to be developed, which could pump mobile phase at pressures up to 1000 bar and above. This represented a major improvement in pumping technology, as until then LC systems were rated up to approximately 400 bar and separations were typically run on columns containing 5 μm particles at 170 bar to prolong pump seal lifetimes. As indicated in Table 1, several manufacturers have entered the UHPLC market, producing systems which operate at even greater pressures and thus providing even greater chromatographic power.

Many (UHP)LC–MS/MS applications to date have been in the area of food safety because of the requirement to analyse a wide range of residues down to low ng/g levels. HPLC coupled with UV or fluorescence detection continues to be used in some laboratories, but most groups are progressing to (UHP)LC–MS/MS platforms. Although it is difficult to measure the number of laboratories that are using UHPLC–MS/MS compared to LC–MS/MS without a comprehensive survey of end-users, the FAPAS proficiency test (PT) reports issued by the Food and Environmental Research Agency in the United Kingdom offer an indication of UHPLC–MS/MS implementation among food safety laboratories. In the 2012 anticoccidial PT study 02183, it was shown that 48 laboratories participated. Only two of the 38 laboratories that completed the questionnaire reported that they used UHPLC [8]. Similarly, in the 2012 Nitrofurans PT study 02179, two out of 35 laboratories reported that they used UHPLC–MS/MS [9]. More interestingly, a PT study was carried out on avertin in corned beef (FAPAS Round 02175), where 17 laboratories used HPLC-fluorescence and 13 reported the use of LC–MS [10]. This can be ascribed to the sensitivity offered by HPLC–Fluorescence towards avermectin compounds, while analysis by MS poses challenges due to their propensity to form sodium adducts. UHPLC–MS/MS was reported to be used in two laboratories in the avertin study. Cumulatively, these results offer some insight on the adoption of UHPLC in residue laboratories, although reported usage of “LC” as the separation technique is ambiguous, and may be either HPLC or UHPLC. In the future, it is expected that more laboratories will move to UHPLC–MS/MS because the technique can be applied to multiple analytes in shorter run times compared to traditional HPLC-UV or fluorescence systems. In addition, because of the increased sensitivity of UHPLC–MS/MS, less complicated sample preparation procedures can be used. We anticipate additional developments in the area of online SPE combined with UHPLC–MS methods to further improve method reproducibility and speed of analysis. Some examples of such applications have already been published [11,12]. The peak broadening inherent in such methods may be addressed by using advanced chromatographic techniques such as the solvent plug injection technique described by Gode et al. [13]. Moreover, the application of UHPLC–MS/MS can reduce solvent waste by a factor of 10 and increase the sample throughput by a factor of 5 [14]. Typically, the cost per analyte can be reduced by a factor of 15 and can be further reduced by including additional analytes. In the future, it is expected that UHPLC will be implemented in more residue control laboratories in order to reduce cost and hazardous waste. The many advantages offered by UHPLC are thus expected to result in widespread use of the technology in all areas of chemical residue analysis.

3. Coupling UHPLC with MS/MS detection

UHPLC coupled with MS/MS is becoming an increasingly used instrumental set-up for researchers due to the advantages such as increased sensitivity, selectivity, resolution and throughput compared with conventional HPLC instruments. The sub-2 μm particles used in UHPLC columns offer increased efficiency and peak capacity. Peaks can be as narrow as 1–3 s wide and coupling with

![Fig. 1. Structures of epimeric synthetic glucocorticosteroids dexamethasone and betamethasone.](image1)

![Fig. 2. Growth in the number of publications on UHPLC, and UHPLC combined with mass spectrometry, 2003 – 2011. Data was sourced from Thomson Reuters Web of Science® (keywords: “UHPLC” or “UPLC”; “MS” or “mass spec”).](image2)
modern, fast scanning mass spectrometers allows for the detection of hundreds of analytes in a short retention window, even in complicated biological matrices. Modern MS/MS analysers are capable of full scan acquisition rates of up to 10,000 m/z per s, dwell times of 1 ms and polarity switching in 30 ms or less (Table 2). Although the focus of this review is the combination of UHPLC technology with tandem mass spectrometers, it is important to note that high resolution mass spectrometry (HRMS) continues to evolve as a powerful alternative to MS/MS detection. A careful study by Kaufmann and colleagues, comparing LC–MS/MS and LC-HRMS for the analysis of seven veterinary drugs in a variety of matrices, showed that once HRMS resolution attains 50,000 full width at half maximum (FWHM), selectivity for the two techniques is comparable [15]. MS/MS has been widely adopted in the food analysis and pharmaceutical industries, due to advantages such as its robust tolerance of different matrices [16], but HRMS retains distinct advantages such as the ability to retrospectively analyse historical data for non-preselected compounds [17]. Kaufmann et al. [15] also noted that at a high resolution, HRMS could identify a matrix component which behaved similarly to a banned nitroimidazole compound, which in MS/MS analysis produced a false positive. HRMS detection may eventually be the dominant detection method in pesticide testing in fruits and vegetables, mainly due to its potential ability to identify unknown chemicals [18].

3.1. Acquisition rate and method sectoring

The acquisition rate in SIM (selected ion monitoring) and SRM (selected reaction monitoring) modes is determined by the switching time and the dwell time per SIM or SRM channel. Dwell time is the amount of time spent collecting a data point at a set transition or peak before switching to the next value in the SIM or SRM method; this dwell time is set by the analyst. If necessary, dwell time can be increased to collect fewer data points. Conversely, if a paucity of data points is collected, dwell time may be decreased to improve peak definition. Dwell time represents a compromise between signal-to-noise (S/N) ratio and the number of data points acquired across a chromatographic peak. Generally, decreased acquisition time results in increased sensitivity, as less time is required to acquire adequate mass spectral data. Background noise is decreased as a result of the increased peak height of the analyte in UHPLC, and its increased chromatographic resolving power may offer a reduction in ion suppression, also leading to an increase in the S/N ratio compared with HPLC.

Time-sectoring is another important factor when setting up UHPLC–MS/MS methods. If the S/N ratio is poor for an analyte, it will require an increase in dwell time to increase the S/N ratio. However, increasing dwell time may result in the detection of too few data points across the peak, due to insufficient acquisition or cycle time. If this is the case, the number of transitions within a SIM or SRM should be reduced to increase the cycle time and obtain more points across the chromatographic peak. Alternatively, if the analytes are sufficiently separated, the SIM or SRM channel can be time-sectored to increase the cycle time for that analyte and acquire a sufficient number of points across that chromatographic peak. Time-sectoring also places less pressure on the instrument and allows more time for the raw data to be processed [19].

As well as sectoring the method and setting adequate dwell times, the inter-channel and inter-scan delays are important. Interscan delay must be set to leave enough time for the instrument electronics to switch from one transition to the next within an SIM or SRM and inter-channel delay to leave enough time between successive SIM or SRM channels. If the method contains analytes that need different ionisation polarities, extra time is required for the electronics to switch between one channel and the next, as well as change polarities. Typically with newer MS/MS instruments, inter-scan and inter-channel delays are set to 5 ms when switching between successive channels and 20 ms is required for inter-channel delay when switching from positive ionisation to negative ionisation mode between successive channels.

3.2. UHPLC–MS/MS versus HPLC–MS/MS

UHPLC–MS/MS yields many advantages over HPLC and HPLC–MS/MS. It is more environmentally friendly, as it reduces the volume of chemicals used and waste produced in the laboratory. It also offers increased sensitivity, resolution, S/N ratio and shorter injection cycle times. UHPLC–MS/MS is capable of detecting analytes at a lower limit of quantification (LOQ) due to the increased resolution of the system. Yu et al. demonstrated the advantages of UHPLC–MS/MS approach over HPLC–MS/MS in a quantitative analytical method for five drug compounds in rat plasma. A shorter run time and narrower peaks were achieved with UHPLC, which lowered the LOQ and increased the sensitivity, as well as considerably improving accuracy and precision compared with conventional HPLC [20]. The authors proposed that this was due to a narrow band of analyte entering the mass spectrometer, resulting in increased S/N. The throughput of samples is dramatically increased for quantitative analysis due to the shorter separation time of the UHPLC coupled with the faster acquisition capability of MS/MS. Recent examples of high throughput methods include that of Whelan et al., who developed a multi-residue method for the determination of 38 anthelmintic drug residues in milk (as well as 20 internal standards) using UHPLC–MS/MS with rapid polarity switching; all analytes were eluted within 8 min [21]. Ventura et al. detected 34 forbidden drugs and metabolites in a total run time of 5 min by UHPLC–MS/MS [22]. A comparison of UHPLC–MS/MS and HPLC–MS/MS methods for the determination of pesticide residues in baby foods demonstrated that the UHPLC approach was 2.5 times faster than the corresponding HPLC method [23]. Aside from the clear benefit of shorter run times, it has also been noted that use of UHPLC in place of HPLC can also improve method performance via reducing matrix effects, such as in the study of pharmaceutical drugs in surface water described by Van de Steene and Lambert [24]. Here, the reduction in observed matrix effect permitted the authors to replace standard addition with a simpler internal standardisation approach for analyte quantification. Other examples of methods that have been improved by use of UHPLC have been well described in a recent review by Guillarme et al. [25].

A major consideration in selecting UHPLC in place of conventional HPLC is that the higher throughput obtained from using
UHPLC coupled to MS/MS increases the requirement to perform regular preventative maintenance on the instrument to ensure continued optimal performance. This is especially important when working with more complex biological samples: if the orifice of the MS/MS is not routinely cleaned, accumulated residue on the front end could potentially enter the quadrupoles, which would lead to reduction in sensitivity or the drop-off of less-intense analytes. A full system clean would then be required by engineers to regain sensitivity; this may not be covered under service contracts and will also result in unnecessary down-time of the instrument due to negligence. If the instrument is analysing a large number of samples daily, the sample cone of the MS/MS should be cleaned daily at the end of the sequence, although it should be noted that UHPLC methods typically involve lower injection volumes than HPLC methods. However, there are some disadvantages to using UHPLC–MS/MS over HPLC–MS/MS. For one, a significant disadvantage is the high cost of the sub-2 μm columns. Also, there is a much smaller selection of UHPLC column chemistries available – although the range is steadily increasing each year, this could prevent a straight transfer from HPLC to UHPLC–MS/MS for some methods. Furthermore, the narrow plumbing and small volumes inherent in UHPLC systems have practical implications. For example, samples must be filtered through 0.2 μm filters prior to use, as solid material may easily precipitate out of solution at column frits, especially when a steep gradient is employed. Mobile phases may also require filtration, to exclude particulate matter, although care must be taken to avoid introducing additional contamination from filtration glassware, filters, etc. Although the instrumentation can be capital-intensive initially, increased market competition is reducing prices, while gains in productivity can offset high instrument costs [26]. However, the benefits far outweigh the disadvantages, e.g. the columns may cost more than traditional HPLC columns but the increased throughput obtained counteracts the increased column cost.

4. Challenges inherent in UHPLC–MS/MS techniques

4.1. Frictional heating effects

As observed by Churchwell et al. [27], UHPLC offers improvements in both the duration of a separation and in sensitivity – although this is compound dependent and not necessarily straightforward. One of the issues which may present difficulty in transferring a method from HPLC to UHPLC is frictional heating [28]. This is a physical phenomenon whereby the interaction between the mobile phase and the stationary phase creates heat. This occurs with liquid chromatography in general, but is far more pronounced with UHPLC. Potential, this phenomenon could mean molecules travelling in a band through the column experience greater heat at the centre of the column than at the walls, with the net result being band broadening (Fig. 3).

The effect of the heat can also have an impact on method performance – de Villiers and co-workers determined that when a radial temperature gradient is prevalent within a column, the efficiency may be seriously affected, particularly with (relatively) larger columns (internal diameter (ID) = 2.1 mm). However, in their work, it was observed that use of a still-air column heater meant that a longitudinal temperature gradient was prevalent along the length of the column, with no considerable impact on separation efficiency. For routine use, the implication is that pre-heating of mobile phase and use of a column oven are essential in controlling heat flow. Work by Novakova et al. [29], using a selection of pharmacologically relevant molecules, investigated the importance of the frictional heating effect for method selectivity. Using a column of ID = 2.1 mm, a pressure drop of 1000 bar over the column resulted in an observed temperature increase of 16 °C. Changes in retention time were not considered significant however, with changes of 5–10% observed for most of the compounds analysed in the range 30–60 °C. The effect was more pronounced for acidic compounds, with changes of 25% for ascorbic acid and 41% gallic acid. The authors commented that the effects of frictional heating may be mitigated through several means, including use of narrow-bore columns; use of several shorter columns in place of one long column; a decrease in column oven temperature in UHPLC when transferred from HPLC; or modification to the method gradient or mobile phase pH to compensate for changes in resolution between HPLC and UHPLC. In a study comparing the efficiency of sub-2 μm porous and sub-3 μm shell particles in reverse phase separations, McCalley noted that when using 100% acetonitrile with sub-2 μm columns, the detrimental effects of frictional heating are enhanced [30]. However, aqueous content in the mobile phase was found to moderate this effect, due to greater thermal conductivity. For larger (3–5 μm) particles, the effects of frictional heating were also significant [31]. The authors observed that the impact of heating effects were proportional to the capacity factor of a compound and the column inner diameter, but inversely proportional to column particle size and the set column oven temperature.

4.2. Band broadening effects

The use of UHPLC–MS/MS technology has afforded, among other advantages, a particular benefit when it comes to sample injection volumes. The greater sensitivity achievable with MS/MS combined with the chromatographic power of UHPLC technology means that the analyst may use smaller injection volumes than was previously required, to attain acceptable detection limits. The small diameter of the columns typically employed in UHPLC means that a small injection volume is required, to avoid possible column overload. Smaller quantities of matrix are thus loaded on to the column than with standard HPLC, meaning matrix suppression effects are minimised. However, to ensure good chromatographic performance, the system must contain the absolute minimum
amount of dead volume to minimise extra-column broadening effects. As system volume is low, extra-column volume can represent a large percentage of overall system volume. Consequently, extra-column volume becomes a significant source of band broadening. As noted by McCalley [32], the influence of dead volume has an inversely proportional relationship to column internal diameter. Dead volume effects have been observed to be more detrimental to performance in narrow bore (2.1 mm) columns employed with a UHPLC system than with a standard bore (4.6 mm) column operated on a typical HPLC system [30].

As well as tubing diameter, connectors for tubing must be carefully selected so as to avoid contributing extra-column volume. The narrow peaks found in UHPLC analyses mean that band broadening can have a large effect on resolution and overall performance. Gritti and Guiochon [33] have observed that for some modern high-pressure LC systems, the largest contributing factor to band broadening was the inner diameter of the needle seat capillary tube (along with detector cell volume). One possible path to reducing extra-column band broadening due to sample injection is described in detail by Sanchez et al. [34]. Their use of a “performance optimising injection sequence” (POISE) allowed the reduction or elimination of the effect of sample injection on the chromatographic performance. This method involves the injection of a defined amount of a weak solvent (relative to the mobile phase) along with the sample. This has the effect of minimising the extra-column dispersion taking place ahead of the analytical column, while having the advantage of not requiring any physical modification to the chromatographic system. Analyte bands are compressed at the top of the analytical column, in proportion to their retention factor. This is referred to as isocratic focussing. It was found that band compression could be achieved in both isocratic and gradient elution modes.

4.3. Narrow analyte peaks

The very short dwell times possible with modern MS instruments (as low as 1 ms, as indicated in Table 2) mean that many analytes can be rapidly detected, e.g. 55 anabolic and androgenic steroids in a 5-min injection cycle [35]. Software-based optimisation of dwell times, cycle times and multiple reaction monitoring windows (dynamic multiple reaction monitoring) has permitted the analysis of up to 11 pesticides which elute within a one-min time window [36]. However, as well as possible peak overlap, chromatographic systems may also produce peaks of very narrow width, particularly in UHPLC [37]. Improvements in data-handling capabilities permit the collection of detector data from compounds where the UHPLC peak profile is only about 2 s across. A multi-residue qualitative MS/MS method for the identification of drugs of abuse in urine by Maquille et al. [38] clearly indicates the challenge this can pose. The application of a small-particle (1.7 μm) column and high pressures caused all compounds to elute within approximately 2 min. However, as all compounds exhibited narrow peaks (a few seconds), the authors employed rapid data collection parameters – selected reaction monitoring dwell-time was set at 5 ms, while inter-channel delay was also set to 5 ms. Time-segmenting of the compounds according to their retention time was also essential to achieving adequate numbers of points across peaks. Although not defined in the relevant legislation, a general reference for peak definition is that a minimum of 5–6 data points should be achieved per peak, to ensure reliable integration [39]. Vidal et al. [40] exploited an alternative means of data collection in their work on determination of veterinary drug residues in milk, which permits rapid screening of samples. A MS/MS screening method was designed based on the selection of a neutral loss or product ion which is selective for a family of compounds. If a positive sample is detected, the sample can then be retested using a confirmatory (SRM) method. The run time for this particular screening method was 3 min, and 21 veterinary drug residues were included in its scope.

Kovalczuk et al. have presented a comprehensive study of the chromatographic output of a UHPLC–MS/MS multi-residue analysis [41]. This represents one of the few examples of multi-residue methods where the authors have assessed chromatographic parameters such as peak capacity, peak symmetry and height of theoretical plates. Direct comparison of HPLC and UHPLC analysis (with MS/MS detection) of a multi-pesticide mixture highlighted some of the key chromatographic considerations when selecting a UHPLC approach over a HPLC approach. Peak compression on the UHPLC system meant that S/N at low concentrations improved, with a consequent reduction in the method LOQ versus the HPLC method. For the compound tebuconazole (the matrix analysed was apple), S/N improved by a factor of 10 going from HPLC to UHPLC, while for several compounds, the LOQ was as much as four times lower using UHPLC. Peak capacity was comparable in both techniques under optimised conditions, although it is noted that UHPLC offers shorter analysis time (and reduced solvent consumption). Height of theoretical plates was greater in UHPLC, but with significant variability.

4.4. Matrix effects

Matrix effects are a common phenomenon when using MS for chromatographic detection, as well as for screening methods. These effects can result from a number of factors, generally the presence or co-elution of interfering compounds, resulting in enhancement or suppression of the analyte of interest. Interfering analytes or compounds can be extracted from the original sample matrix or from extraction equipment such as plastic tubing. The presence of interfering analytes along with the analyte of interest in the ionisation source increases the competition for ionisation and typically reduces ion efficiency of the analyte. The effect of ion suppression in residue analysis applications has been investigated in detail by Antignac et al. [42]. Using a method for the analysis of the β-agonist isoproterenol in bovine meat samples, it was illustrated how a polar analyte that eluted early in a typical reverse-phase separation may potentially co-elute with matrix interferences which come with the solvent front. The signal for this compound was shown to reduce by as much as 95% from the anticipated response due to matrix interference, indicating how severe the effects may be. The choice of ionisation technique can be a major factor in eventual matrix effects in an analysis – it has been observed that atmospheric pressure chemical ionisation (APCI) is, in some cases, less prone to matrix effects than electrospray ionisation (ESI) [43], although ESI is more widely applicable. This has been attributed to the differences in ionisation techniques, in that using ESI, the charged analyte is formed in the liquid phase, while with APCI, the analyte is transferred into the gas phase in the neutral form before ionisation occurs [44]. Several possible mechanisms that lead to matrix effects in ESI are described by Trufelli et al. [44]. High analyte concentration (>10−5 M) can lead to a loss of sensitivity, possibly due to saturation of the electrospray ionisation droplet surface with analyte. Other possible causes include the formation of analyte-inclusion particles from non-volatile additives and analytes of interest, with resultant hindrance of ionisation. It has also been postulated that the presence of non-volatile interferents affects the viscosity and surface tension of ESI droplets, which thus affects ionisation efficiency and the eventual signal recorded [45]. Hydrophilic analytes may also be significantly suppressed in the presence of surfactants in the sample matrix. The presence of lipophilic components in the sample matrix is another factor which can have a large impact on method response, as observed in a method for the analysis of veterinary drug residues in meat-based baby food [46]. Another
known issue in the LC–MS/MS analysis of samples of biological origin is the effect of phospholipids on ionisation efficiency [47]; these compounds can cause significant matrix ionisation effects in positive electrospray ionisation mode. Phospholipids represent a main constituent of cell membranes. These compounds can present very broad elution profiles, requiring the analyst to either monitor these compounds chromatographically to ensure they do not coelute with analytes [48], or employ advanced sample preparation techniques to remove the interfering compounds [49].

Highly selective sample preparation techniques are important for removing compounds from the matrix that produce matrix effects – although more elaborate sample preparation procedures do have a bearing on sample throughput, and are more costly. Chambers et al. carried out a systematic and comprehensive strategy for reducing matrix effects in LC–MS/MS as applied in bioanalytical assays. A paired t-test illustrated a statistically significant improvement in matrix effects using UHPLC when compared with traditional HPLC [50]. The evolution of UHPLC–MS/MS, which has permitted the analysis of multiple drug residues from multiple classes, as well as the development of fast generic clean-up and extraction techniques, means that highly selective sample preparation techniques are no longer critical. A consequence of employing these generic methods is that matrix effects can have a large bearing on method performance. This is particularly the case when using solvent standard calibration curves, and where possible, matrix matched calibration curves offer a more accurate means of quantification. Alternatively, stable isotopically labelled standards can be employed to negate matrix effects to improve both accuracy and precision. This is demonstrated in work by Varga et al. [51], on the determination of various mycotoxins in maize, where the use of $^{13}$C-labelled mycotoxin analogues permitted the development of the first multi-target method capable of determining all mycotoxins regulated in the EU for maize.

The likelihood of matrix effects is high with the analysis of samples of biological origin, such as food of animal origin in veterinary drug residue analysis. These effects may be somewhat mitigated when using UHPLC – as mentioned in Section 4.2, UHPLC methods require smaller injection volume than comparable HPLC methods, to avoid overloading the column. A major advantage of this reduced injection size is that matrix effects are reduced, as smaller quantities of matrix are injected onto the source. This, in turn, leads to less competition for ionisation in the ESI droplets, increased resolution between the analytes of interest, and a much cleaner mass spectrum compared to that of HPLC–MS/MS. The smaller quantity of matrix passing through the system means less accumulation of non-volatile components and other contaminants on the sample cone ahead of the quadrupoles, as well as on the quadrupoles themselves. Such accumulations can lead to charging effects with a resultant loss of sensitivity, so this is a further benefit of UHPLC interfaced with MS/MS. Given the extreme impact on analyte signal which is possible with matrix effects, quantification/evaluation of matrix effects in a given analysis can provide the analyst with essential detail in ensuring consistent method performance. This may be assessed either through spiking of samples with analyte following extraction, or through infusion of analyte post-column into the mass spectrometer detector system, and examining the effect on signal when a negative control sample is injected (see Fig. 4). The possibility of matrix interference with analyte signal is one reason why validation procedures (such as those indicated in 2002/657/EC [1]) require that a method be validated for each compound in a multi-analyte method, rather than selecting one representative compound.

4.5. Column blockage

Due to the narrow plumbing employed in UHPLC, a high level of chromatographic hygiene is required to avoid problems with blockages. In addition, sample matrix and analytes can contribute to column blockage. In an application for detection of steroid esters, columns were susceptible to blocking when solvent standards were injected onto the column in a gradient that started with a high aqueous mobile phase, going to high organic content [52]. It was proposed that the waxy ester substances crashed out of solution, when mixed with the mobile phase during the injection cycle. The solution to the problem was simple; by elevating column temperature to 60 °C, and including isopropyl alcohol in mobile phase and needle washes, the problem was circumvented. In the authors’ laboratory, a number of methods have been developed using high temperature [14,21,53,54]. However, increasing temperature may not be suitable for all substances, e.g. tetracycline antibiotics have to be separated at 40 °C or less, as they can form epimers [55].

Fig. 4. Experiment for observation of suppression effects. The signal corresponds to the introduction into the mass spectrometer of a mobile phase or blank sample extract injection (from the LC) and standard solution (via direct infusion). When just mobile phase is introduced from the LC, signal remains constant (a and b). However, upon injection of blank sample extract, although the TIC increases (c), the specific transition monitored for the analyte (d) is suppressed due to matrix effects. Adapted from [20] with permission from Elsevier.
5. Recent applications of UHPLC–MS technology in the analysis of agrochemical residues and mycotoxins

As noted in Section 1, there has been significant uptake of UHPLC–MS/MS technology in areas of analytical research such as pesticide testing, veterinary drug residue analysis and environmental contaminant detection, and Fig. 2 clearly indicates how widely adopted the technique has become. In this section, an overview of some of these published applications is given. Methods have been published for many classes of compound; these may be broadly grouped into substances with an established permitted limit [e.g. maximum residue limits (MRLs)], and substances which are prohibited. In veterinary drug residue testing, Council Directive 96/23/EC [56] lists prohibited compounds as Group A substances, covering stilbenes, antihelminthic agents, steroids, resorcinol acid lactones, β-agonists and banned veterinary drugs. Other substances with a permitted limit are found in Group B substances (anthelmintics, anticoagulants, nitroimidazoles, carbamates, pyrethroids, sedatives, non-steroidal anti-inflammatory drugs, sulphonamides, quinolones and others). Examples of UHPLC–MS/MS methods for different veterinary drug classes are summarised in Table 3. For pesticides, there are 1289 active substances that are controlled under EU regulation 1107/2009 [57]. A total of 511 substances have been given MRLs under EU regulation 396/2005 [58, 59], while for pesticides without an established MRL, a default limit of 0.01 mg kg⁻¹ is set [60]. The sheer diversity of pesticides in use underlines the need for a robust, versatile analytical approach, as offered by UHPLC–MS techniques. Examples of such methods are described in the following sections.

5.1. UHPLC–MS/MS methods for prohibited substances

Analysis for banned compounds represents an area of continued research focus, as these are typically chemical entities which pose a direct risk of harm to human health. UHPLC–MS/MS presents a very attractive option for the analysis of complex biological samples for residual quantities of zero-tolerance substances. This can be attributed to the inherent resolving power, permitting resolution of chemically similar compounds, and sensitivity. This is crucial in areas such as environmental analysis, where the ability to specifically detect small polar organic molecules within complex sample matrices poses a particular challenge [61]. In the area of veterinary drug analysis, UHPLC–MS/MS analytical power has been applied to most banned substances including thyreostats [62], as well as stilbenes and resorcinol acid lactones [63]. The bulk of published work, however, has focussed on two main sub-categories of Group A substances, namely the steroids and β-agonists.

Steroid hormones are banned from use in food-producing animals, and fall into three distinct groups: estrogens, gestagens and androgens (EGAs) [64]. Exploitation of these compounds for the purposes of growth promotion in food-producing animals is strictly prohibited within the European Union by Directive 96/22/EC [65]. Application of UHPLC–MS/MS to determine the presence of these compounds in bovine hair has been described by Duffy et al. [52], who found that the method performed comparably to the more conventionally employed GC–MS/MS methods. In their method for the detection of boldenone (an androgen) and metabolites in cattle, Van Poucke et al. [66] used an UHPLC–MS/MS, with a short column (50 mm) containing particles of 1.7 μm diameter. Gradient optimisation permitted the polar analyte β-hydroxy-β-boldenone to be separated from the solvent front. The authors noted that the limiting factor in speeding up their method beyond the 5-min injection cycle achieved was the number of data points being acquired for peaks. Covering a broader range of compounds, Malone and colleagues analysed for 13 synthetic growth promoters in bovine muscle, applying a 1.8 μm particle-size column
(2.1 mm × 50 mm) and achieving separation of all compounds in 14 min [67]. Wang et al. described a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) extraction procedure to extract nine estrogens from milk powder. Two UHPLC C₁₈ columns were evaluated, the first was an ethylene bridged hybrid (BEH) column, and the second was a HSS T3 column. Results from this study showed that the BEH-C₁₈ column was not suitable as it was not capable of separating all nine estrogens [68], but the HSS T3 column was successfully employed, using an appropriate gradient. You et al. extracted 10 anabolic steroids (AASs) from equine plasma using methyl tert-butyl ether (MTBE) prior to separation on a C₁₈ reversed-phase column [69]. This extraction procedure was again employed by Guan et al., who developed a method to isolate 55 anabolic and androgenic steroids in equine plasma. Separation was performed using a Hypersil Gold™ C₁₈ column. Amongst the 55 AASs were epimer pairs (testosterone and epitestosterone, boldenone and epiboldenone, and anadrolone and epiandrolone) which were chromatographically baseline separated. This high-throughput method had a cycle time of 5 min per sample [35]. Finally, Wong et al. [70] described a UHPLC–MS/MS method for the analysis of 140 compounds including anabolic steroids in equine urine, with an injection cycle time of 13 min. The authors noted how a combination of fast chromatography and rapid polarity switching in MS detection has allowed them to condense what was formerly four individual test methods into one comprehensive method.

Also falling into the category of banned (Group A) substances, β-agonists are synthetic derivatives of catecholamines, used at lower quantities as tocolytics and bronchodilators in veterinary medicine [71]. However, for the last 20 years there has been frequent reports of their misuse as growth-promoting agents in food producing animals [72]. These compounds may be divided into two groups: the substituted anilines, including clenbuterol, and the substituted phenols, which include salbutamol [64]. Yang et al. developed a method capable of detecting 13 β-agonists in milk. Ion suppression was overcome by adding perchloric acid during the sample extraction step to precipitate protein out of the samples [72]. Separation was carried out using an Acquity™ BEH C₁₈ column. Shao et al. isolated 16 β-agonists from porcine tissue using enzymatic hydrolysis. Maximum sensitivity in this method was achieved by optimising the solvent type and pH of the mobile phase. Methanol-water containing 0.1% formic acid provided good separation and resolution when compared to a comparable acetonitrile–water mobile phase [73]. A similar method was used to determine 19 β-blockers and 11 sedatives in animal tissue, with an injection cycle time of 14 min [74]. Nielen et al. developed a method for 22 β-agonists, which was sufficiently versatile to be applied to the analysis of bovine and porcine urine, feed and hair using a mixed-mode solid-phase extraction procedure. The method was performed by conventional LC–MS/MS and UHPLC–MS/MS. Due to the diverse structure of β-agonists, developing a single analytical method which encompasses multiple compounds is challenging. This issue was overcome by performing two injections of each sample using the same LC gradient whilst acquiring a different set of ion transitions. Run time was reduced from 20.5 min to less than 15 min using UHPLC–MS/MS. Chromatographic resolution, peak shape and signal-to-noise ratio improved with the introduction of a high-resolution column [75].

5.2. UHPLC–MS/MS methods for substances with an established permitted limit

The presence of a MRL, ‘tolerance’ or recommended limit (RL) for a compound of interest has somewhat different implications – methods have a well-defined target concentration for analysis, rather than the criterion of ‘as low as possible’ as with banned substances. An example of a class of veterinary drug which possess MRLs is the quinolone group. These are potent antibacterials used in the therapeutic treatment of infections in both human and veterinary medicine [76]. MRLs to monitor quinolone residues in edible animal tissue are controlled by European Union Directive 2377/90/EC [77]. Zhang et al. extracted 22 fluoroquinolones from milk using an EDTA-McIlvaine buffer solution and purified using Bond Elut™ Plexa SPE cartridges. Zhang et al. examined the separation capabilities of four different reversed-phase chromatographic columns which varied in particle size and bonded phase chemistry. The highest sensitivity and acceptable peak shape was observed with a Waters Acquity™ Shield RP18 Column (2.1 mm × 100 mm, 1.7 μm). Recoveries of 61.9–94.6% were observed for 19 of the 22 fluoroquinolones included in the study [76]. Lombardo-Agui et al. described the determination of eight quinolones in bee products. The separation capabilities of three different C₁₈ columns were compared, where the particle size of each varied from 1.7 μm to 1.9 μm. An intermediate particle size of 1.8 μm delivered slightly better separation of two of the quinolones [78].

Sulphonamides represent a class of drugs used to prevent and treat bacterial infections. They are amongst the most widely used veterinary drugs because of their broad-spectrum antimicrobial activity, low cost, and their effectiveness as growth promoters in livestock [79]. The MRL for individual sulphonamides in food of animal origin has been set at 100 μg kg⁻¹ by the European Union [77]. Careful selection of gradient conditions by She et al. permitted the separation of 24 sulphonamides in bovine milk in 10 min [80]. Although C₉ and phenyl stationary phases were investigated, an Acquity™ BEH C₁₈ column proved most suitable. Zhao et al. employed an immunoaffinity chromatography (IAC) technique to purify muscle and milk extracts containing 12 sulphonamides [79]. McDonald et al. developed a rapid method to isolate 19 sulphonamides from muscle matrix using 0.1 M EDTA/acetonitrile mix, with a total injection cycle time of 15 min. Two sets of sulphonamides had similar molecular weights and parent–daughter transitions (tetracycline/doxycycline and sulfamethoxypyridazine/sulfamonomethoxine). The authors distinguished between these compounds using retention time [81]. Tamosiunas et al. compared the performance capabilities of LC–MS/MS to UHPLC–MS/MS. A simple acetonitrile extraction procedure was carried out to extract ten sulphonamides from egg and honey matrix, followed by SPE clean-up with Strata-X™ cartridges. Similar recoveries (80–110%) were obtained by HPLC and UHPLC–MS/MS determination for all analytes. However, narrower peaks, and consequently better chromatographic resolution was observed with UHPLC–MS/MS [82]. Cai et al. developed a simple method for isolating 24 sulphonamides in muscle followed by analysis via UHPLC–MS/MS. Three columns (C₁₈ 5 μm, C₁₈ 1.7 μm, and C₈ 1.7 μm) were tested for signal intensity and separation efficiency. The columns containing smaller particle size displayed improved sensitivity and separation efficiency, when compared to the C₁₈ 5 μm column [83].

A large number of anthelmintic drugs are licensed for the treatment of gastrointestinal nematodes and trematodes in food producing animals [84]. They include nematicides, fluocicides and endectocides [14,21]. The EU, through Council Regulation 2010/37, established MRLs for a limited number of anthelmintics in food of animal origin [85,21]. Zhang et al. described a procedure for isolating albendazole (ABZ) and three of its metabolites in fish muscle using UHPLC–MS/MS equipped with an electrospray ionisation (ESI) source [86]. The separation capabilities of three columns (BEH C₈, BEH C₁₈, and CSH C₁₈) were compared. Identical retention times (BEH C₈), poor separation and double-humped peaks (BEH C₁₈) were observed for two of the three columns. Following optimisation of the gradient profile, the CSH C₁₈ column was selected and used for the study. A study of extraction behaviour was necessary due to the variation in lipophilicity and
pK₄ among ABZ and its metabolites. Optimum peak shape and sensitivity were obtained through optimisation of the reconstitution solution. Subsequent to a series of optimisation experiments where mean recoveries were monitored, ethyl acetate was selected as an extraction solvent. Whelan et al. extracted 38 anthelmintic drugs from milk using a modified QuECHERS-type extraction method. Factorial design experiments were carried to determine the optimum mobile phase composition. Results from these experiments indicated that ionisation of analytes improved when the mobile phase contained less additive. However, it was necessary to add ammonium formate to the mobile phase at a low concentration to improve the sensitivity of several of the anthelmintic drugs [21]. Whelan et al. further optimised this method by including an enzymatic hydrolysis step to investigate the prevalence of nitroxylin and its conjugate forms in lactating dairy cows [87]. Xia et al. described the rapid chromatographic separation of 13 benzimidazoles using UHPLC–MS/MS [88]. The analysis time for each injection was 8 min, which was substantially lower than an earlier HPLC–MS/MS method [89]. UHPLC–MS/MS methods for other members of group B2 have also been published, including anticoccidials [90] and non-steroidal anti-inflammatory drugs (NSAIDs) [91]. Hu et al. have recently described a UHPLC–MS/MS method for the determination of thirty NSAIDs in swine muscle, in a run time of 16 min [92].

Other possible contaminants and substances of environmental origin are covered within group B3 in Council Directive 96/23/EC [56], such as mycotoxins. As is commonly the case with multi-residue methods, the chemical diversity found within this group of compounds represents a challenge for analysis. However, combined with an effective clean-up technique, UHPLC–MS/MS has provided a means of rapidly and sensitively determining these contaminants. Van Pamel et al. developed a UHPLC–MS/MS method for 26 different mycotoxins in maize silage [93]. The polarity-switching capability of the mass spectrometer system used (a Waters Xevo™ TQ mass spectrometer) permitted detection of positively and negatively ionised analytes within the same run, avoiding the need for two separate injections of each sample and giving a total run time of 9 min.

5.3. Multi-class multi-residue analysis

Finally, the expansion of methods to cover ever greater numbers of compounds means that certain methods cannot be simply categorised as being specific towards banned or MRL compounds. We here examine recent methods which are capable of simultaneously determining several classes of analyte. The advantages of such an approach are high sample throughput, simplicity and reduced cost. The bulk of methods reported use tandem mass spectrometry (MS/MS), as multiple transitions can be monitored for compounds simultaneously, providing unambiguous identification. Perhaps one of the most accomplished methods published to date is the generic extraction and analysis procedure published by Moi et al. [94]. With careful examination of extraction parameters, as well as matrix effects, existing multi-analyte methods were combined to give a method capable of analysing for 172 pesticides, mycotoxins and plant toxins. Separation and detection of the analytes was performed via UHPLC–MS/MS. Herrmann et al. also demonstrated how a generic sample extraction procedure combined with UHPLC–MS/MS analysis could be used to test for a range of 108 compounds (out of 127 targeted compounds) related to emergency situations [95]. One of the methods displaying the widest scope of analytes to date is the UHPLC–MS/MS method for analysis of contaminants in milk published by Zhan et al. [96]. The method covered 255 veterinary drug residues, pesticide residues, mycotoxins and other potential chemical contaminants, using a generic extraction technique based on a two-step precipitation, followed by sample drying and then reconstitution in injection solvent.

For screening of large numbers of compounds, time-of-flight (ToF) mass spectrometry has also been successfully applied. Kaufmann et al. developed a method for isolating 100 veterinary drug residues from muscle, liver and kidney prior to UHPLC–ToF MS analysis. Samples were extracted using liquid–liquid–solid extraction (bi-polarity extraction), a technique which is known for its ability to recover polar, and medium polar compounds. Kaufmann encountered difficulties when attempting to isolate polar analytes. Evaluation of the method indicated that these polar analytes were adsorbing on proteins which precipitated during the extraction stage. This issue was overcome by rinsing the sample and glassware with DMSO and complexing buffer. Poor recovery of at high spiking concentration was attributed to detector saturation and a mass shift, a phenomenon not observed at lower concentrations [97]. Recently, Kaufmann et al. modified and improved this procedure to isolate over 100 residues in muscle, kidney, liver, fish and honey using a single stage Orbitrap MS. Co-eluting proteins or peptides resulted in extensive signal suppression effects, which is an issue that had not been typically encountered when using ToF [18]. In the area of environmental contaminant analysis, UHPLC–ToF MS has been successfully applied to the screening of organic pollutants in water [98].

Also selecting ToF MS for detection, Ortelli et al. reported a method for extracting 150 residues in milk which involved protein precipitation using acetonitrile and ultrafiltration. The group encountered difficulties in the isolation of avermectin residues, where recoveries (≤ 10%) did not fulfill the criteria of validation. This result was attributed to losses during ultrafiltration. A post-column infusion system was used to evaluate matrix effects on response. Signal response was sample-dependent for two of the compounds (febantel and ivermectin), while a significant signal enhancement (1300%) was observed for enrofloxacin [99]. Peters et al. developed a simple extraction procedure to isolate 100 veterinary drug residues in egg, fish and muscle by extracting with acetonitrile and purifying using Strata-X™ SPE cartridges. Separation was performed using a Waters Acquity™ UPLC BEH C₁₈ column, and detection was via ToF MS. The authors constructed the method using retention times, specific accurate mass and isotope pattern ratios calculated by SigmaFit™ [100]. Rapid quantification and accurate mass measurement for 166 pesticides in fruits and vegetables was reported by Wang et al. [60]. Following sample extraction via a QuEChERS approach, samples were tested using a full-scan MS detection strategy for quantification, with confirmation via a UHPLC–Q-Orbitrap MS/MS data-dependent scan. This represents an efficient means of confirmatory analysis of a large number of pesticides, with the additional benefit of circumventing the need for lengthy compound-dependent parameter optimisation as is necessary with MRM approaches. QuECHERS in particular has proven to be an excellent means of sample preparation for pesticides, providing clean sample extracts for analysis by UHPLC–MS/MS, such as in the recent work described by Arienza et al. on the testing of pesticides in fresh cut vegetables [101].

Using the more commonly employed LC–MS/MS method, Rezende et al. described a simple extraction procedure for 12 β-lactams and tetracyclines (in bovine muscle) which traditionally rely on time-consuming, expensive SPE approaches. Tetracyclines can be difficult to analyse for, due to the ability of tetracycline to epimerise to 4-epitetracycline at pH 4–8. The method involved extraction with water/acetonitrile followed by purification with dSPE (dispersive solid phase extraction) C₁₈ and finally defatting with hexane. Rezende studied the matrix effect using an F-test to examine the homoscedasticity of data and a t-test to compare average results obtained in matrix samples and solvent. Results from these tests suggested there was a matrix effect. To overcome this
issue, samples were quantified using matrix-matched calibration curves [102]. Lehotay et al. have recently reported a streamlined method designed to detect residues of as many as 60 priority veterinary drugs in bovine kidney, using UHPLC–MS/MS [103], with an injection cycle time of under 10 min. The authors determined that their method was suitable for screening of 54 of the 62 drugs studied, qualitative identification of 50 compounds, and quantification of 30. For bovine muscle [104], a multi-class, multi-residue UHPLC–MS/MS method was designed for the purpose of monitoring over 100 veterinary drug residues. The importance of the choice of the dispersive solid-phase extraction sorbent used was investigated, as was the influence of post-column infusion of ammonium formate on ionisation enhancement for anthelmintic compounds. The final method presented was capable of screening for 113 compounds, identification of 98 compounds, and quantification of 87.

When MRLs have not been established for compounds, researchers typically attempt to develop analytical methods capable of detecting these compounds at extremely low levels. Aguilera-Luiz et al. encountered this problem when attempting to develop a multiclass method for 39 veterinary drugs in meat-based baby food and powdered milk-based infant formulae [46]. Separation was carried out on a C18 column (100 mm x 2.1 mm, 1.7 μm particle size) using a previously developed chromatographic procedure [94]. Chromatographic separation was optimised by evaluating two solvents, acetonitrile and methanol. Retention time decreased with acetonitrile; however sensitivity was significantly better when methanol was used in the mobile phase. The authors also compared two additives, formic acid and acetic acid. Ionisation efficiency improved with the addition of formic acid at a concentration of 0.05%.

Matrix effects associated with milk can pose a significant challenge in creating multi-residue methods. Tang et al. described the difficulties encountered when attempting to extract veterinary drugs from milk. A relatively high level of acetonitrile was used to isolate 23 veterinary drugs from milk. Matrix effects were quantified in milk, and matrix-matched calibration curves were employed for accurate quantitation [105]. Vidal et al. developed two screening methods to identify 21 veterinary drug residues in milk. The first method used parent ion and neutral loss scan whereas the second method only used one transition per compound. Both methods were separately validated but it was ultimately decided that the second method was more suitable as it was capable of screening at lower concentration levels [40]. Wang et al. compared the separation of ten antibiotic residues in milk using three kinds of columns (C18, C8 and phenyl). The investigation indicated that satisfactory separation and resolution could only be achieved using a C18 column. Samples were extracted using Mcllvaine buffer: methanol (8:2), and purified with HLB SPE cartridges [106].

The QuEChERS technique has proved suitable when it comes to cleaning up milk samples prior to UHPLC–MS/MS analysis. Aguilera-Luiz et al. developed a method for the analysis of 18 veterinary drugs in milk. Initially, extraction was carried out using acidified acetonitrile followed by clean-up using QuEChERS DSPE. Separation was performed using an Acquity™ UHPLC BEH C18 column. Poor recoveries were attributed to the propensity macrolides have to form chelation complexes with cations present in solution. To overcome this problem, EDTA was added to the extractant solution prior to QuEChERS clean-up [107]. Vidal et al. utilised this method to isolate 17 antibiotic residues in honey. To avoid contamination of the column from lipids and waxes present in honey, the organic solvent content was quickly raised during the gradient profile [108]. Frenich et al. compared several extraction procedures (solvent extraction, SPE, MSPD, and a modified QuEChERS procedure) to analyse 25 veterinary drugs in eggs. A uniform dwell time of 0.015 s was used for most of the compounds except for levamisole, marbofloxacin and sulfadimidine which had a dwell time of 0.025 s, whereas fenbendazole, emamectin and ivermectin were monitored at 0.100 s. Results suggested that solvent extraction was the most reliable procedure for the simultaneous extraction of several classes of veterinary drugs (tetracyclines, macrolides, quinolones, sulphonamides and anthelmintics), where recoveries ranged from 60% to 119% [109].

As previously mentioned, the tetracycline compounds pose particular challenges for multi-compound methods, as very specific analysis conditions are required. Shao et al. described the difficulties encountered when developing an extraction procedure for 21 veterinary drugs from two classes (tetracyclines and quinolones). Quinolones are typically extracted solvents acidified at a pH of less than 3. However under these acidic conditions, tetracyclines will reversibly form 4-eptetrazyclines and anhydro-tetracyclines. An investigation was carried out, where three different extraction solvents (methanol, acetonitrile and EDTA-Mcllvaine buffer) were acidified at pH 4 prior to sample clean-up with HLB SPE. Results indicated that acceptable repeatability was achieved with the use of EDTA-Mcllvaine (pH 4) [110].

### 6. Conclusion

The emergence and rapid development of UHPLC–MS/MS technology which we have documented in this review has had a large impact on the area of veterinary drug residue analysis and related compounds. Injection cycle times have shortened, sensitivity has improved, chromatographic resolution of compounds has increased and the number of compounds included in multi-residue methods continues to climb. The combination of these advantages (along with peripheral advantages such as lower solvent consumption) has led to the increasing adoption of UHPLC–MS/MS as the default approach for confirmatory analysis of multiple compounds. In this review, we have examined the technical aspects of coupling UHPLC instrumentation to mass spectrometric detection systems, as well as highlighting some of the inherent challenges that come with the technology, such as fractional heating effects, narrow chromatographic peaks and the impact of sample matrix effects. Despite these challenges, the arenas of veterinary drug residue analysis, pesticide analysis and mycotoxin testing have shown prolific uptake of the technology, and we have detailed some of the most accomplished methods which employ UHPLC–MS/MS as the separation and detection system. Comprehensive methods for some of the more important contaminant groups in residue analysis have been developed for UHPLC–MS/MS, including anthelmintics, β-agonists, steroids, quinolones and others. Expected future developments include evolution of methods to include larger numbers of compounds and classes of compound; the use of ever higher temperatures and pressures to create more effective separation methods; and further reduction in sample preparation via online SPE and other techniques, to increase the speed of analysis beyond current standards.

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