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From The Editor

By Samara E. Kuehne

Pesticide residues that remain in or on vegetables, fruits, herbs, honey, oil, seeds, and food of animal origin can pose major threats to human health and to the environment. Improperly used veterinary drugs and antibiotics can accumulate in food derived from animals, which can also adversely affect consumers. Additionally, mycotoxins, produced primarily by the *Aspergillus*, *Penicillium*, and *Fusarium* fungi, have the potential to contaminate a variety of common foods, such as grains, nuts, cocoa, and milk, and present an ongoing challenge to food safety all along the food chain.

Limiting exposure to these potentially life-threatening contaminants in food and animal feed is critically important. However, there are hundreds of compounds that should be actively monitored. To tackle this challenge, high performance triple quadrupole liquid chromatography/mass spectrometry (LC/MS) is a powerful analytical tool for food contaminant detection of this nature.

In this special collection, we bring together articles from Agilent Technologies and Wiley publications that detail how LC/MS is a gold standard analytical tool in food safety and how the technology can be used to detect a large number of undesirable chemical residues with a high degree of confidence.

You'll read about how LC/MS can be used to detect and quantify mycotoxins and pesticide residues and also screen for veterinary drugs and antibiotics. We've also included articles on how the technology can be used specifically in analyzing contaminants in wines and coffee.

We think this series of important articles will serve as a useful resource in your workflow, and serve as a tool in mitigating contamination to protect your consumers.

Kuehne is the professional editor of *Food Quality & Safety*. Reach her at skuehne@wiley.com.

Application Note

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Comprehensive LC/MS/MS Workflow of Pesticide Residues in Food Using the Agilent 6470 Triple Quadrupole LC/MS System

Pesticides residue workflow in high water content, high oil content, and high starch content samples

Authors

Aimei Zou, Sashank Pillai, Peter Kornas, Melanie Schober, Limian Zhao, Marco Zanotti, and Chee Sian Gan

Agilent Technologies, Inc.

Abstract

A comprehensive LC/MS/MS workflow was developed for the quantitation of 510 pesticide residues with the intention to accelerate and simplify routine laboratory food testing. Compound transitions and optimized parameters were developed based on the Agilent Pesticide Dynamic MRM Database, which has over 750 pesticides including curated parameters for fast and easy transfer into the analytical method. The workflow includes sample preparation, chromatographic separation, mass spectrometry (MS) detection, data analysis, and interpretation. The workflow applicability was demonstrated using an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS on three food matrices with different content types: tomato (high water content), wheat (high starch content), and olive oil (high oil content). For sample preparation of the tomato and wheat samples, an Agilent QuEChERS kit was used with dSPE cleanup. Extraction was performed with the QuEChERS kit followed by Agilent Captiva EMR—Lipid cleanup for preparing olive oil samples.

Workflow performance was evaluated and verified according to SANTE/12682/2019 based on limit of detection (LOD) and limit of quantitation (LOQ), calibration curve linearity, and recovery and precision using matrix-matched calibration standards from 1 to 100 µg/L. Over 95% of analytes demonstrated linearity with R^2 ≥0.99, with calibration curves plotted from LOQ to 50 or 100 µg/L. Method precision was assessed using recovery repeatability (RSD_r) and intralaboratory reproducibility (RSD_{iR}). It was assessed at three levels of fortified quality control (QC) samples at 1, 5, and 10 µg/kg in three matrices. RSD_r and RSD_{iR} at 10 µg/kg for 90% of compounds were within the limit of 20%. The method performance across tomato, wheat, and olive oil matrices demonstrated the method applicability for quantitative analysis of multiresidue pesticides in high water, high oil, and high starch contents with potential implication for use on other food matrices.

Introduction

Pesticides used to protect crops from disease or harmful organisms during production, storage, and transportation have potential toxicity. Pesticide residues remaining in or on commodities such as vegetables, fruits, herbs, honey, oil seeds, cereals, and food of animal origin can cause adverse health effects and environmental concerns as well. Organizations including the World Health Organization (WHO), the Food and Agricultural Organization (FAO), the U.S. Environmental Protection Agency (EPA), and the European Union (EU) have developed and published policy statements to guide agricultural organizations on the proper use of pesticides. For example, according to EU regulation, a maximum residue level (MRL) is the highest level of a pesticide residue legally tolerated in or on food or animal feed when pesticides are applied.[1] The amount of pesticide residues allowed in food must be as low as possible to ensure food safety for consumers. Ten µg/kg (10 ppb) is the MRL for most pesticides except for explicitly prohibited compounds.

This points to the demand and need for highly sensitive analysis methods of multiresidue pesticides in food matrices.

High performance liquid chromatography coupled to triple quadrupole mass spectrometry (LC/TQ) is a widely accepted modern technique that works with a broad range of pesticides for quantitative analysis. This is because of its high sensitivity, selectivity, and accuracy that ensure high quality data for meeting MRL requirements in complex food matrices. A comprehensive LC/MS/MS workflow has been developed for an accurate and reliable analysis of more than 500 pesticide residues in various plant origin food matrices. This workflow, including sample preparation, chromatographic separation, and MS detection targets quantitation and results interpretation, helps streamline routine pesticide analysis, and therefore accelerates lab throughput and productivity.

The LC/TQ method and a method protocol with details on sample preparation, acquisition, and data analysis steps are available from Agilent.[2]

Experimental

Chemicals and reagents

Agilent LC/MS-grade acetonitrile (ACN), methanol (MeOH), and water were used for the study. LC/MS-grade formic acid and ammonium formate were purchased from Sigma-Aldrich. All other solvents used were HPLC-grade from Sigma-Aldrich.

Standards and solutions

The ready-to-use and custom premixed pesticide standards were acquired from the vendors listed in Table 1.[3]

An intermediate standard mix comprised of 510 targets at a concentration of 1,000 µg/L was prepared in ACN from stock standard solutions and used for the rest of experiment. Working standard solutions at 50 µg/L and 500 µg/L were diluted from the intermediate standard solution and used for the preparation of prespiked QCs.

Solvent calibration standards were prepared in ACN for the purpose of matrix effect assessment.[1] Serial dilutions were done from 1000 µg/L

Table 1. Pesticide standards.

Vendor	Part Number	Part Description	Analyte Concentration	Matrix	No. of Vials	Total No. of Analytes
Agilent Ultra (Rhode Island, USA)	5190-0551	LC/MS pesticide comprehensive test mix	100 µg/mL	Acetonitrile	8	254
	CUS-00000635	Custom pesticide test mix #1	100 µg/mL	Acetonitrile	1	27
	CUS-00000636	Custom pesticide test mix #2	100 µg/mL	Acetonitrile	1	26
	CUS-00000637	Custom pesticide test mix #3	100 µg/mL	Acetonitrile	1	27
	CUS-00000638	Custom pesticide test mix #4	100 µg/mL	Acetonitrile	1	28
	CUS-00000639	Custom pesticide test mix #5	100 µg/mL	Acetonitrile	1	25
	CUS-00000640	Custom pesticide test mix #6	100 µg/mL	Acetonitrile	1	26
	CUS-00000641	Custom pesticide test mix #7	100 µg/mL	Acetonitrile	1	28
	CUS-00000642	Custom pesticide test mix #8	100 µg/mL	Acetonitrile	1	29
	CUS-00000643	Custom pesticide test mix #9	100 µg/mL	Acetonitrile	1	30
Accustandard (Connecticut, USA)	ACCU S-85870-R1-10X	Custom pesticide test mix #10	100 µg/mL	Acetonitrile	1	26

intermediate standard to prepare seven calibration concentration levels of 1, 2, 5, 10, 25, 50 and 100 µg/L into Eppendorf tubes. Calibration standard solutions must be prepared freshly and stored in the refrigerator at 4 °C if not used immediately.

Sample preparation

Pesticide-free and organically labeled fresh tomato, wheat powder, and olive oil were obtained from local grocery stores. The tomato was homogenized using a domestic blender and stored in the refrigerator at 4 °C if it was unable to be analyzed immediately.

The following products and equipment were used for sample preparation:

- Agilent QuEChERS EN extraction kits (part number 5982-5650CH)
- Agilent universal QuEChERS dispersive SPE kits (part number 5982-0028)
- Agilent Captiva EMR—Lipid 6 mL cartridges (part number 5190-1004)
- Agilent positive pressure manifold PPM-48 processor (part number 5191-4101)
- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centrifuge (Eppendorf, Centrifuge 5804R and 5430R)
- Vortexer and multitube vortexer (VWR, Plainfield, NJ, USA)

Ten ±0.1 g of homogenized fresh tomato, 2 ±0.1 g of dry wheat powder, and 5 ±0.1 g of olive oil were weighed into a 50 mL tube, respectively. Prespiked QC samples were fortified by spiking an appropriate amount of pesticide working standard solution to make low QC at 1.0 µg/kg (LQC), mid QC at 5.0 µg/kg (MQC), and high QC at 10.0 µg/kg (HQC) solutions. After spiking standard into the matrix, the samples were capped tightly, vortexed, and equilibrated for 15 to 20 minutes. It was recommended to add water to the dry wheat powder before extraction to improve the extraction efficiency of low moisture commodities. QuEChERS extraction followed by universal dSPE cleanup was applied for tomato and wheat sample preparation, while Captiva EMR—Lipid cleanup was used for olive oil sample preparation with assistance from the Agilent positive pressure manifold PPM-48 processor for eluting. The preparation procedure is illustrated in Figure 1.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards (postspiked standards) were used and prepared for the assessment of workflow performance in this study. Matrix blank was prepared using unfortified blank samples of tomato, wheat, and olive oil. Preparation of matrix-matched calibration levels was identical to solvent standards preparation by replacing ACN solvent with matrix blank accordingly. The matrix-matched standards were used to evaluate the matrix effect by comparing responses in the corresponding solvent standards.[1]

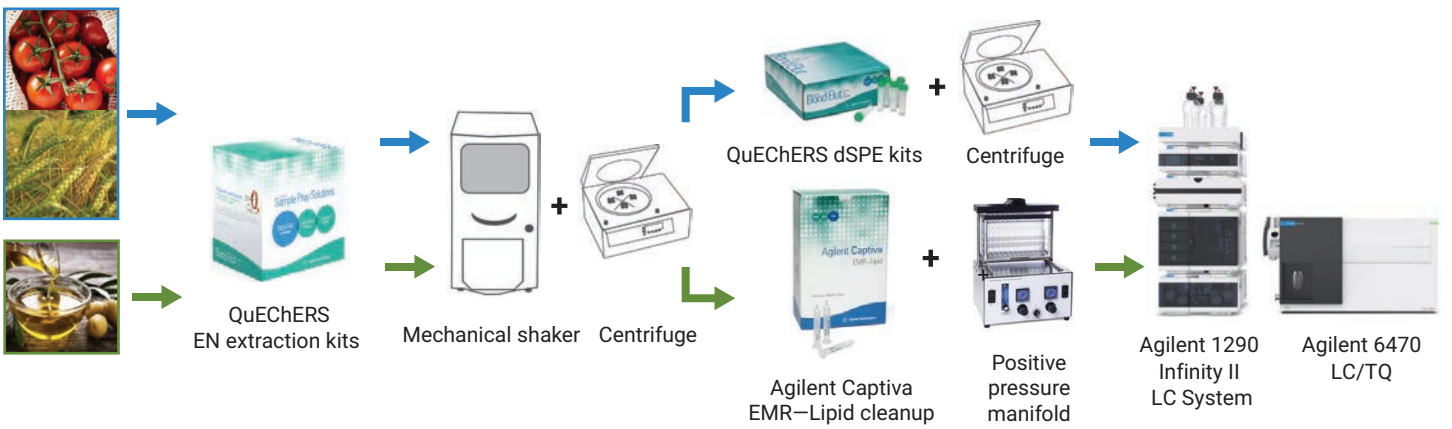

Figure 1. Sample preparation procedure for tomato, wheat, and olive oil samples.

Instrumentation

Chromatographic separation was performed using an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm column (part number 959759-902) installed on an Agilent 1290 Infinity II LC system.

The individual modules of the 1290 Infinity II LC system included:

- Agilent 1290 Infinity II high-speed pump (G4220A)
- Agilent 1290 Infinity II autosampler (G4226A)
- Agilent 1290 Infinity II thermostatted column compartment (G1316C)

The LC system conditions are listed in Table 2.

An Agilent 6470 LC/TQ mass spectrometer with an Agilent Jet Stream (AJS) electrospray ion source was operated in dynamic MRM (dMRM) mode. The LC/TQ autotune was performed in unit and wide modes. All data acquisition and processing were performed using the Agilent MassHunter software (version 8.0 or higher). The 6470 LC/TQ parameters are shown in Table 3.

Results and discussion

Development of LC/TQ method

A major part of this work was the development of dynamic MRM transitions for 510 pesticide compounds. For each compound, MRM transitions, as well as fragmentor voltages, collision energies, and ionization polarity were optimized using Agilent MassHunter optimizer software by flow injection. The four most abundant product ions per compound were selected automatically. More than 1,000 MRM transitions from 510 pesticides were stored in the dMRM method. Depending on the fragmentation behavior of the individual compound, two or three target-specific MRM transitions were selected per pesticide (except for EPTC and procymidone where only one transition was stable enough to be monitored). This was done to satisfy regulatory requirements for identification and confirmation by LC/MS/MS.[1] The two most abundant fragments were defined as primary transitions that were acquired over the retention time window and subsequently used as the quantifier and qualifier ion.

The chromatographic method was optimized using the ZORBAX RRHD Eclipse Plus C18 column, which resulted in good separation and distribution of 510 pesticide residues within a 20-minute HPLC gradient. The 0.4 mL/min flow rate offered effective desolvation of target ions using the AJS ion source. A dMRM method with a cycle time of 500 ms was used. Typical chromatographic peak widths observed were between 8 to 12 seconds. Figure 2A shows a representative MRM chromatogram for all 510 pesticide targets postspiked at 10 µg/L in olive oil matrix extract. The dMRM statistics diagram with the concurrent MRMs plot and min/max dwell time is captured in

Table 2. 1290 Infinity II LC conditions.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)
Column Temperature	40 °C
Injection Volume	2 µL
Autosampler Temperature	10 °C
Mobile Phase A	5 mM ammonium formate in water with 0.1 % formic acid
Mobile Phase B	5 mM ammonium formate in MeOH with 0.1 % formic acid
Mobile Phase Flow Rate	0.4 mL/min
Gradient Program	Time/min %A %B 0 95 5 3 70 30 17 0 100 20 0 100
Postrun	3 minutes
Needle Wash	Standard wash: flush port (12 s)

Table 3. Agilent 6470 LC/TQ parameters.

Parameter	Value
Software Version	Agilent MassHunter version B.08
Ionization Mode	Simultaneous positive/negative ESI with Agilent Jet Stream (AJS)
Scan Type	Dynamic MRM
Cycle Time	500 ms (Total MRMs = 1,023 Min/Max Dwell = 0.90 ms/248.28 ms)
Stop Time	20 minutes
MS1/MS2 Resolution	Unit/Wide
Gas Temperature	200 °C
Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,500 (+)/3,000 (–) V
Nozzle Voltage	0 V

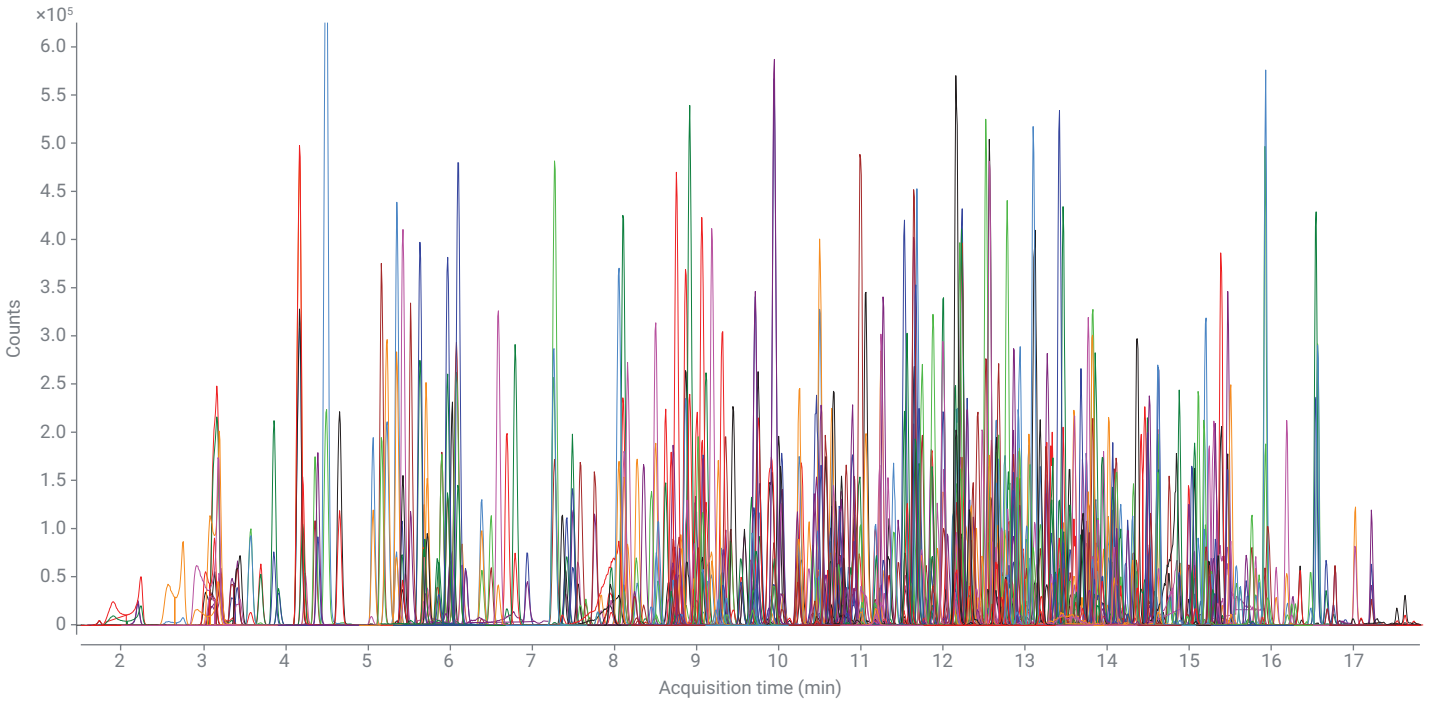

Figure 2A. Representative MRM chromatogram of 510 pesticides postspiked at 10 µg/L in olive oil matrix extract. The symmetric sharp peaks demonstrate the efficient chromatographic separation of targets within the retention time window.

Figure 2B. This shows that the dMRM method accurately quantifies more than 500 individual analytes in a relatively short LC run.

The full list of 510 compounds in the dMRM method, together with retention time, collision energy, fragmentor voltage, and MRM transitions is available in the method. Some compounds including acephate, brodifacoum, difenoconazole, etaconazole, halfenprox, iprovalicarb, omethoate, orbencarb, propamocarb, pymetrozine, resmethrin, thiobencarb, thiofanox sulfone, and triadimenol showed split peaks in all three matrices. Other compounds including butachlor, cycloprothrin, dimethachlor, imazamox, methamidophos, oxadixyl, pretilachlor, and tridemorph, showed peak tailing or broadening in all three matrices.

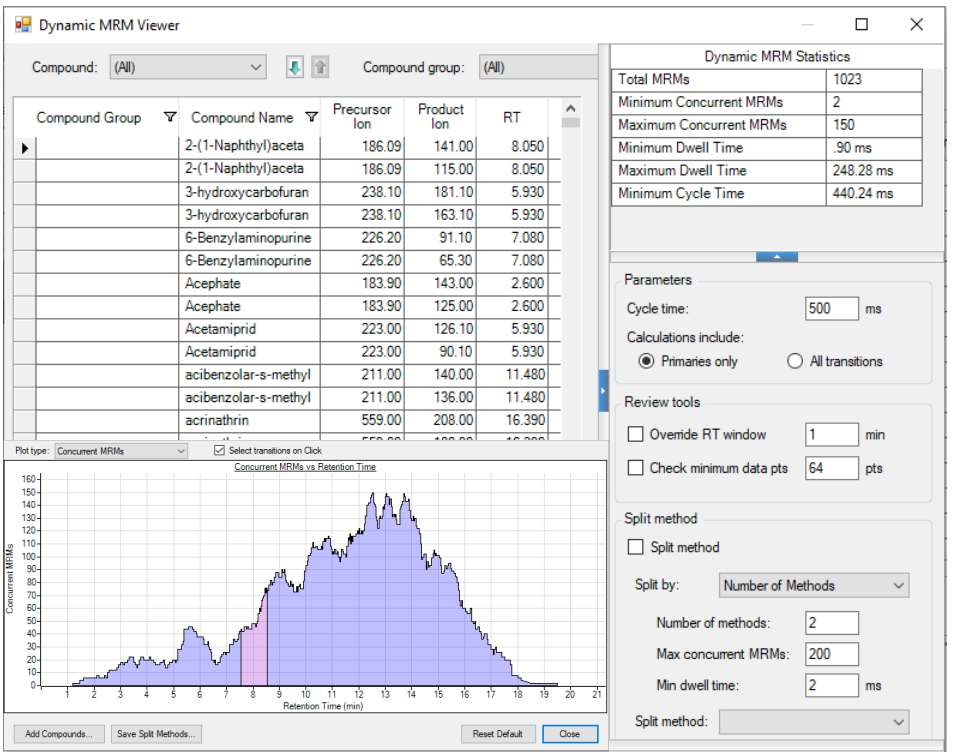

Figure 2B. 510-Compound acquisition method shown in Dynamic MRM Viewer software illustrating efficient management of more than 1,000 MRMs.

Matrix effect assessment

Matrix effects (ME) caused by sample matrix are frequent and behave in terms of suppression or enhancement of the MS detection system response.[1] ME was assessed by the ratio of target response in matrix-matched standards to that in corresponding solvent standards. Typically, there is no strict requirement on acceptance ME criteria, because ME can be corrected by the matrix-matched calibration curve. However, ME is an important parameter for method sensitivity and reliability assessment, and less than 20% signal suppression or enhancement is usually considered as insignificant ME.[1] In this study, ME was investigated using seven levels of matrix-matched calibration standards in comparison to the corresponding same levels of solvent standards. ME at calibration level 4 (10 µg/L), which is the MRL for all 510 pesticides in this study, was considered in the final compilation.

70% to 90% of 510 targets in tomato showed insignificant ME at 10 µg/L. For analytes with relatively significant ME in the tomato matrix, most of them showed matrix enhancement. For the dry wheat powder, insignificant ME was observed for 90% to 95% of total 510 targets at 10 µg/L. As for olive oil, insignificant ME was obtained for 70% to 85% of all 510 pesticides at 10 µg/L. Due to the complexity of oil matrix, more targets were negatively impacted by ion suppression. Based on the result of ME at 10 µg/L in tomato, wheat, and olive oil, matrix-matched calibration standards were finally used to compensate ME in this study.

As an example, the calibration curve of 2-(1-naphthyl)acetamide in solvent calibration standards and matrix-matched standards is plotted in Figure 3. This demonstrates good agreement across solvent standards and tomato, wheat, and olive oil matrices.

Verification of workflow performance

The workflow performance criteria was verified based on linearity, method sensitivity, recovery, and precision. Considering the dilution factor of 1:5 and 1:2 introduced for wheat and olive oil during sample preparation, the final result was corrected accordingly, based on dilution factors. Two batches of analyses were carried out for each matrix. The batch run for each sample matrix included solvent blank, matrix-matched calibration standards, matrix blank, postspiked QCs, and prespiked QCs. At least six technical replicates were prepared for prespiked QCs per level.[1] Each were injected into MS at least once.

1. Linearity: A calibration curve for the majority of targets was generated using matrix-matched standards from the defined LOQ to 100 µg/L, while the range from LOQ to 50 µg/L was applied to some of the compounds due to saturation at 100 µg/L. To determine the best linearity response function, various regression models were evaluated, and the best calibration model was with Type: Linear, Origin: Ignore, Weight: $1/x^2$, while a few compounds showed better linear regression with Weight: 1/x. More than 95% targets met the calibration curve linearity requirement of $R^2 \geq 0.99$.

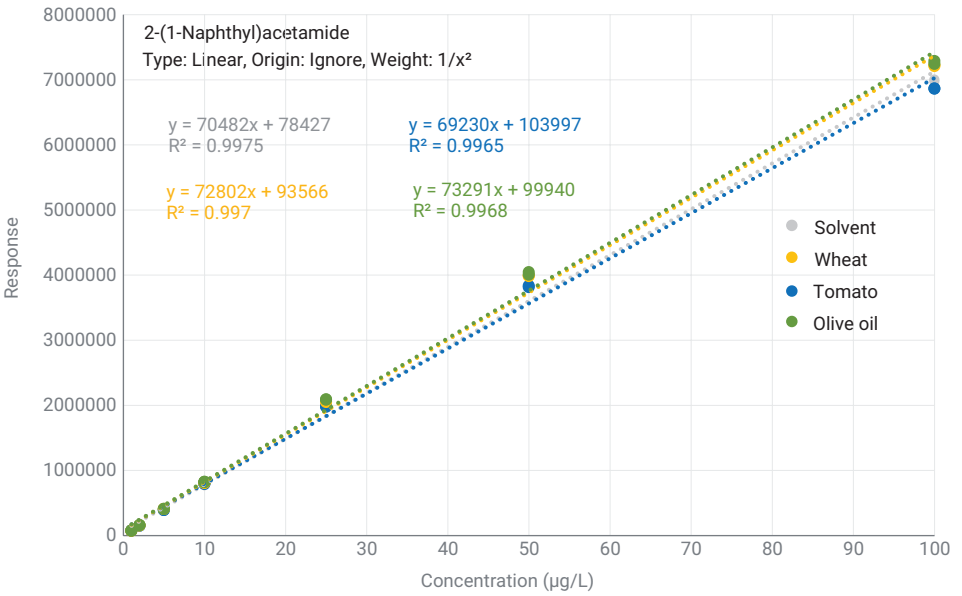

Figure 3. Overlay of calibration curve in solvent standards, tomato, wheat, and olive oil matrices.

2. Limit of quantification (LOQ) and instrument limit of detection (LOD):
A sensitive workflow for pesticide residue analysis is beneficial for users to perform routine operations following various regulatory guidelines. Workflow LOQ and instrument LOD were used to evaluate the method sensitivity. Instrument LOD was established based on matrix-matched calibration standards for signal-to-noise ratio (S/N) of three and up, while workflow LOQ was obtained from the prespiked samples going through the entire workflow procedure for S/N of 10 and up. The S/N was defined using the peak height and auto-RMS algorithm embedded in Agilent MassHunter Quantitative Analysis software. For defining LOQ, additional assessments including target selectivity in sample matrix and precision of analyte response and analytes recovery, were also considered. This is because LOQ is more important for quantitative methods. According to the guidance across the European Union (EU), the lowest spiking level within calibration range meeting the identification and method performance criteria was claimed as LOQ in this study.[1] Precision was obtained from six replicates of prespiked QCs, and %RSD was less than or equal to 20%. Figures 4A and 4B show an MRM chromatogram overlay of 2-(1-naphthyl)acetamide and acetamiprid for six technical replicates at pre-spiked QC 1 µg/kg and 5 µg/kg, respectively. This indicates high sensitivity and good precision at LOQ level across three matrices.

3. Method precision and recovery:
Method precision was estimated using recovery repeatability (RSD_r) and intralaboratory reproducibility (RSD_{iR}) based on the variation of recovery values from technical replicates of pre-spiked QC at 10 µg/kg in two batches across three matrices. RSD_r was determined by calculating percent relative standard deviation (%RSD) of recovery using six technical preparations of HQC within a batch. RSD_{iR} was measured as %RSD of recovery from a total of 12 technical preparations of HQC across two batches.

Typically, the acceptable RSD_r limit at 10 ppb is 20%. The RSD_r values of more than 91% of all targets in three different matrices were within 20%, demonstrating consistent behavior with each technical preparation. These results confirmed the high repeatability of analyte recovery using Agilent Universal QuEChERS dSPE and Agilent Captiva EMR—Lipid sample preparation.

Intralaboratory reproducibility for three matrices was assessed in two batches with the consideration of potential variables for the sample preparation and analysis, including different lots of sample matrix and consumables for extraction, different analytical columns and different days. RSD_{iR} was obtained for all matrices from total 12 technical preparations conducted in two batches. Among 510 targets, results of more than 90% of targets were within 20% RSD_{iR}. These results confirm the precision of workflow performance across different experimental conditions.

Variation of retention time (RT) for all targets in different batches across three matrices was also monitored to evaluate the chromatographic method precision. RT tolerance of all targets in three different matrices was within ±0.1 minutes. The precision results of RT confirm the reliability of the elution profile and MS detection.

Recovery was used in this experiment to evaluate the capability of a quantitative analytical workflow for more than 500 pesticides.[1] Three levels of prespiked QCs were used to evaluate analytes recovery across three different matrices, including 1, 5, and 10 µg/kg. Recovery was calculated based on analytes responses ratio between prespiked QCs and corresponding matrix-matched calibration levels. Mean recovery at each spiking level was obtained for six technical replicates. Given to the MRL for the majority of pesticides, the recovery results of 10 µg/kg spiking level were used to report workflow recovery performance. According to SANTE/12682/2019, mean recoveries can be accepted within the range of 40 to 120% if they are consistent (RSD_r ≤20%). Based on these criteria, the mean recovery results for 92%, 82%, and 86% of targets in tomato, wheat, and olive oil at 10 µg/kg met acceptance criteria, respectively.

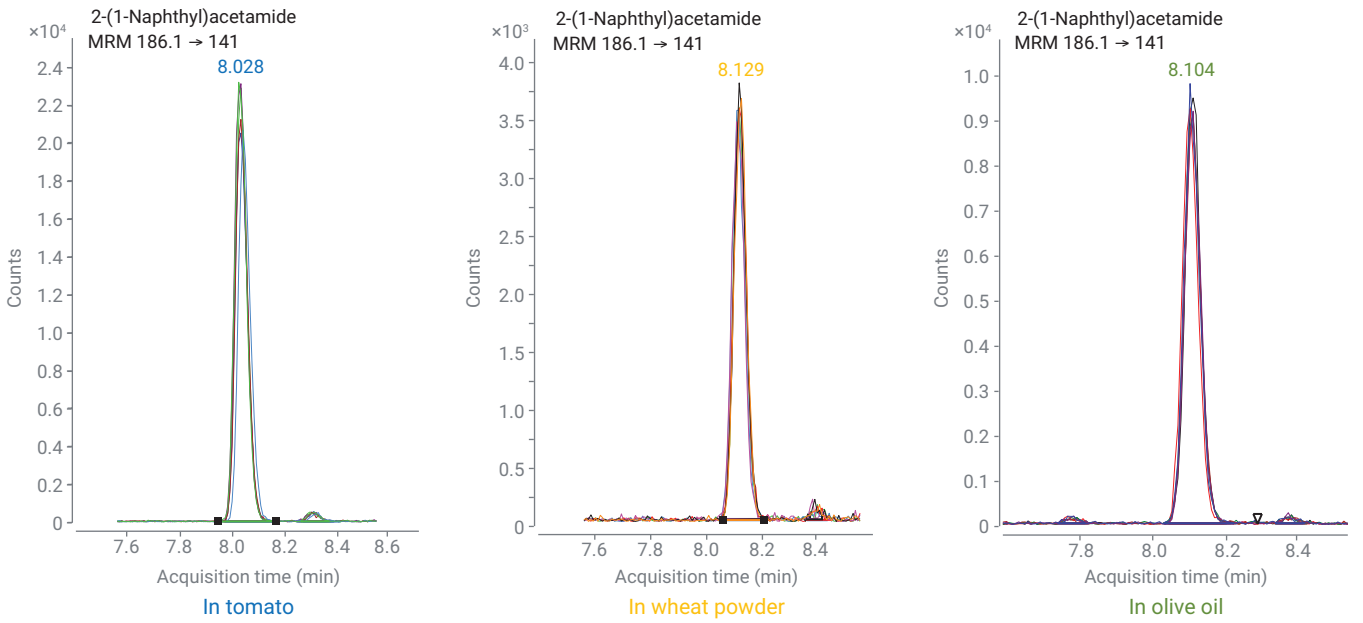

Figure 4A. MRM chromatograms overlay of 2-(1-naphthyl)acetamide for six techincal replicates at 1 µg/kg (prespiked QC) in three matrices.

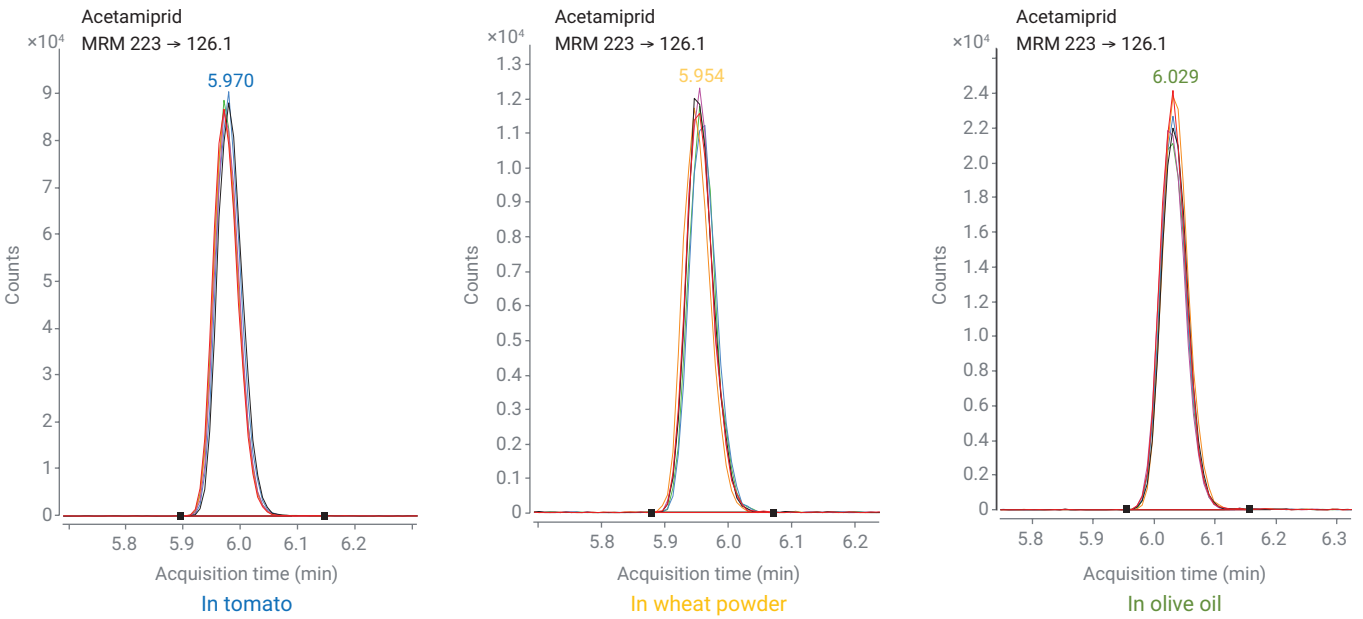

Figure 4B. MRM chromatograms overlay of acetamiprid for six technical replicates at 5 µg/kg (prespiked QC) in three matrices.

4. Robustness assessment

Robustness is the ability of a system and a method to produce a reliable response and result when a long run is required in the laboratory. In this study, robustness was evaluated by two days' (48 hours) continuous injection of olive oil extract spiked with pesticides at 50 µg/L. Nine compounds were selected to represent different classes of pesticides from fungicide, insectidie, herbicide, acaricide, and nematicide. The retention time window of these nine compounds covers from 12.5 to 15.0 minutes, the busiest window where the number of concurrent MRM is 150 (the maximum concurrent MRM). The large concurrent MRM transitions resulted in decreased dwell time for each compound within this window. Thefore, these nine compounds with shorter dwell times were selected to evaluate the performance of the dynamic MRM method in a long run. The analyte responses of nine representative compounds over >100 injections are displayed in Figure 5. Over two days' continuous running, the analyte responses were observed in good consistency with RSD <3.5%. This demonstrates that the use of dMRM mode can produce consistent responses with very short dwell time, which supports the reliable method robustness for the large number of sample injections.

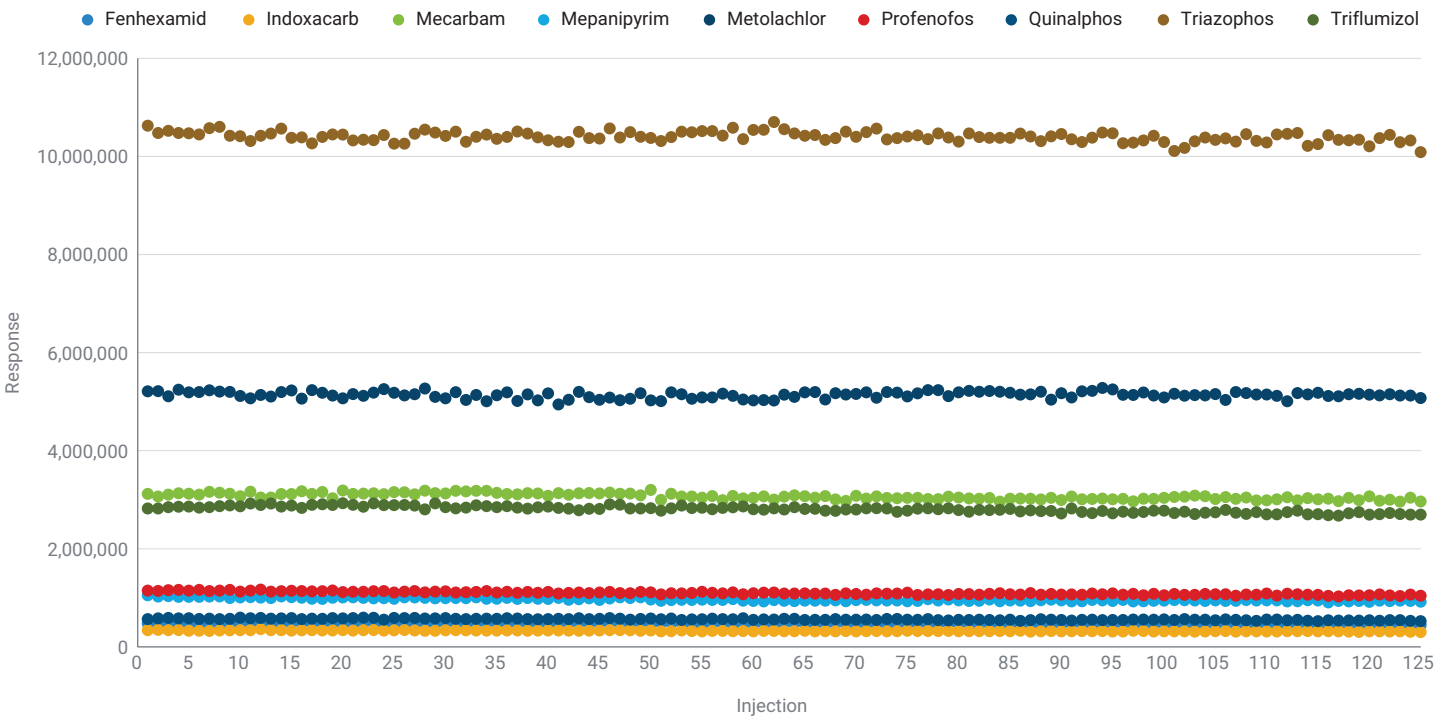

Figure 5. Response of representative compounds for 48 hours of continuous injections in olive oil extract spiked at 50 µg/L.

Conclusion

This study describes a highly sensitive and reproducible workflow for the fast and reliable quantitation of 510 pesticide residues in tomato, wheat, and olive oil matrices. The dMRM method was created and developed based on Agilent Pesticide Database including over 750 pesticides that can be saved to any name for customization by re-optimization of compounds in the database or addition/deletion of those present. The simplified sample preparation protocols included extraction with the Agilent QuECheRS kit followed with Agilent Bond Elut universal dSPE cleanup to prepare tomato and wheat powder samples. QuECheRS extraction followed with Agilent Captiva EMR—Lipid cleanup was used to prepare olive oil samples, providing highly efficient, selective, and reproducible pesticides extraction and complex food matrix cleanup.

The Agilent 1290 Infinity II LC coupled to the Agilent 6470 Triple Quadrupole LC/MS was used for over 500 pesticide residues analysis, which is easily and readily scalable to Agilent 6495 for achieving additional sensitivity if desired. The 20-minute LC gradient method using an Agilent ZORBAX RRHD Eclipse Plus C18 column offered good chromatographic separation and even RT distribution of all targets. LC/TQ data acquisition was in the dMRM mode with fast polarity switching for the most efficient use of instrument cycle time.

The workflow performance was verified in three different matrices based on matrix-matched calibration curve linearity, instrument LOD and workflow LOQ, recovery, and precision. The results in alignment across two batches demonstrate the applicability of the quantitative analytical workflow for more than 500 pesticide residues in high water, high oil, and high starch content with possibility to extend to various other food matrices.

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February / March 2021

Application Note

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An End-To-End Workflow for Quantitative Screening of Multiclass, Multiresidue Veterinary Drugs in Meat Using the Agilent 6470 Triple Quadrupole LC/MS

Authors

Siji Joseph, Aimei Zou, Chee Sian Gan, Limian Zhao, and Patrick Batoon
Agilent Technologies, Inc.

Abstract

A comprehensive LC/MS/MS workflow was developed for targeted screening or quantitation of 210 veterinary drug residues in animal muscle prepared for human consumption, with the intention to accelerate and simplify routine laboratory testing. The workflow ranged from sample preparation through chromatographic separation, MS detection, data processing and analysis, and report generation. The workflow performance was evaluated using three muscle matrices—chicken, pork, and beef—and was assessed on two different Agilent triple quadrupole LC/MS models (an Agilent 6470 and a 6495C triple quadrupole LC/MS). A simple sample preparation protocol using Agilent Captiva EMR—Lipid cartridges provided efficient extraction and matrix cleanup. A single chromatographic method using Agilent InfinityLab Poroshell 120 EC-C18 columns with a 13-minute method delivered acceptable separation and retention time distribution across the elution window for reliable triple quadrupole detection and data analysis.

Workflow performance was evaluated based on evaluation of limit of detection (LOD), limit of quantitation (LOQ), calibration curve linearity, accuracy, precision, and recovery, using matrix-matched spike samples for a range from 0.1 to 100 µg/L. Calibration curves were plotted from LOQ to 100 µg/L, where all analytes demonstrated linearity R^2 >0.99. Instrument method accuracy values were within 73 to 113%. Target analytes response and retention time %RSD values were ≤19% and ≤0.28% respectively. Analyte recovery and reproducibility at three levels of fortified quality control (QC) samples—1, 10, and 25 µg/kg in meat—were used to validate the method applicability for confident routine screening of veterinary drugs. The recovery repeatability (intrabatch technical replicates) and recovery reproducibility (interbatch technical replicates) were calculated using QC samples, and the results were within acceptable limits of 20 and 32%, respectively.[1] The workflow method performance results across the chicken, beef, and pork muscle matrices showed excellent overlap, and confirm the method applicability for routine multiresidue screening in various animal origin matrices.

Introduction

Veterinary (vet) drugs are commonly used to improve the growth and health outcomes of farm animals. Improper use of vet drugs in animal farming can result in the accumulation of these drugs in animal-derived foods, causing adverse effects to consumers. Global regulations define limits for vet drugs in food of animal origin to protect public health. As a gold standard for chemical quantitation, triple quadrupole LC/MS (LC/MS/MS) is a widely accepted technique for this analysis. However, laboratories traditionally use chemistry-specific extraction procedures and run individual LC/MS analyses based on compound class. This can be inefficient for productive lab operations and result in diminished throughput and high operating costs. To streamline day-to-day operation, a comprehensive workflow has been developed for the accurate and reliable analysis of >200 multiclass veterinary drugs in various animal-origin food matrices using LC/MS/MS. The end-to-end workflow includes sample extraction and matrix cleanup, chromatographic separation, MS detection, target quantitation, and reporting templates. Table 1 lists the veterinary drug classes covered using this workflow.

Table 1. Classification of 210 vet drugs based on functional use/ chemical class, and the number of target compounds in each class.

No.	Functional Use/Chemical Class	Number of Targets
1	Anesthetic	1
2	Anthelmintic	16
3	Anthelmintic/Avermectins	3
4	Anthelmintic/Benzimidazoles	14
5	Anthelmintic/Nitroimidazoles	5
6	Anti-herbivore	1
7	Anti-inflammatory	2
8	Antibiotic	7
9	Antibiotic/Aminoglycosides	5
10	Antibiotic/Amphenicols	3
11	Antibiotic/Beta-Lactam	16
12	Antibiotic/Macrolides	10
13	Antibiotic/Quinolones	10
14	Antibiotic/Sulfonamides	27
15	Antibiotic/Tetracycline	6
16	Antiemetic	1
17	Antimicrobial	6
18	Antimicrobial /Furans	1
19	Coccidiostats	14
20	Dopamine receptor	1
21	Fungicides and dyes	3
22	Growth promoters/Anabolic steroids	3
23	Growth promoters/Beta-agonists	4
24	Growth promoters/Corticosteroids	4
25	Hormones	9
26	Insecticide	15
27	NSAIDs	14
28	Quinoxalines	1
29	Tranquilizer	8

Experimental

Standards and reagents

Veterinary drug standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), Toronto Research Chemicals (Ontario, Canada), and Alta Scientific (Tianjin, China). Agilent LC/MS-grade acetonitrile (ACN), methanol (MeOH), and water were used for the study. All other solvents used were HPLC-grade from Sigma-Aldrich. LC/MS additives for mobile phases were also purchased from Sigma-Aldrich. Stock solutions of individual veterinary standards were prepared from powdered or liquid veterinary drug standards at 1,000 or 2,000 µg/mL using an appropriate dissolving solvent (methanol, dimethyl sulfoxide, acetonitrile, or water individually or in combination). A few stock standard solutions were purchased as ready-made solutions with a concentration of 100 µg/mL from the above-listed suppliers.

A comprehensive standard mix (1 µg/mL of each target analyte in 50/50 acetonitrile/water) was prepared from individual stock solutions and used for this experiment.

Sample preparation

Chicken, beef, and pork muscle matrices were used to assess the method performance. Fresh chicken (antibiotic-free), beef, and pork were obtained from local grocery stores. Samples were homogenized using a domestic blender. A 2±0.1 g portion of blended meat was weighed in a 50 mL conical polypropylene tube. Homogenized meat samples were stored at −20 °C, if not analyzed immediately.

Sample preparation was based on solvent extraction followed by Agilent Captiva EMR—Lipid (p/n 5190-1003) SPE cleanup. Sample elution was aided using the Agilent positive pressure manifold system (PPM-48, p/n 5191-4101).

Pre-extraction (matrix-spiked) QC samples were fortified by spiking appropriate veterinary standard solution into the homogenized muscle matrices at three levels: 1 µg/kg for low QC (LQC), 10 µg/kg for mid QC (MQC), and 25 µg/kg for high QC (HQC) in meat. Pre-extraction LQC and MQC samples were used to evaluate method recovery and reproducibility. After spiking standards into the matrix, samples were vortexed for 30 seconds and equilibrated for 15 to 20 minutes. This allowed the spiked standards to infiltrate the sample matrix and equilibrate before sample extraction.

The sample preparation procedure is summarized in Figure 1. The detailed procedure is included in the workflow guide included with the Comprehensive Veterinary Drug dMRM Solution (G5368AA).

Postextraction calibration standards

Matrix blank was prepared using unfortified meat samples. Matrix-matched calibration standards were prepared by spiking appropriate standards into the matrix blank. The targeted concentrations of calibration levels in muscle matrix were 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/kg. Considering the 1:10 dilution factor introduced during sample preparation, the actual matrix-matched calibration standard levels were 0.01, 0.025, 0.05, 0.10, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/L in matrix blank extract.

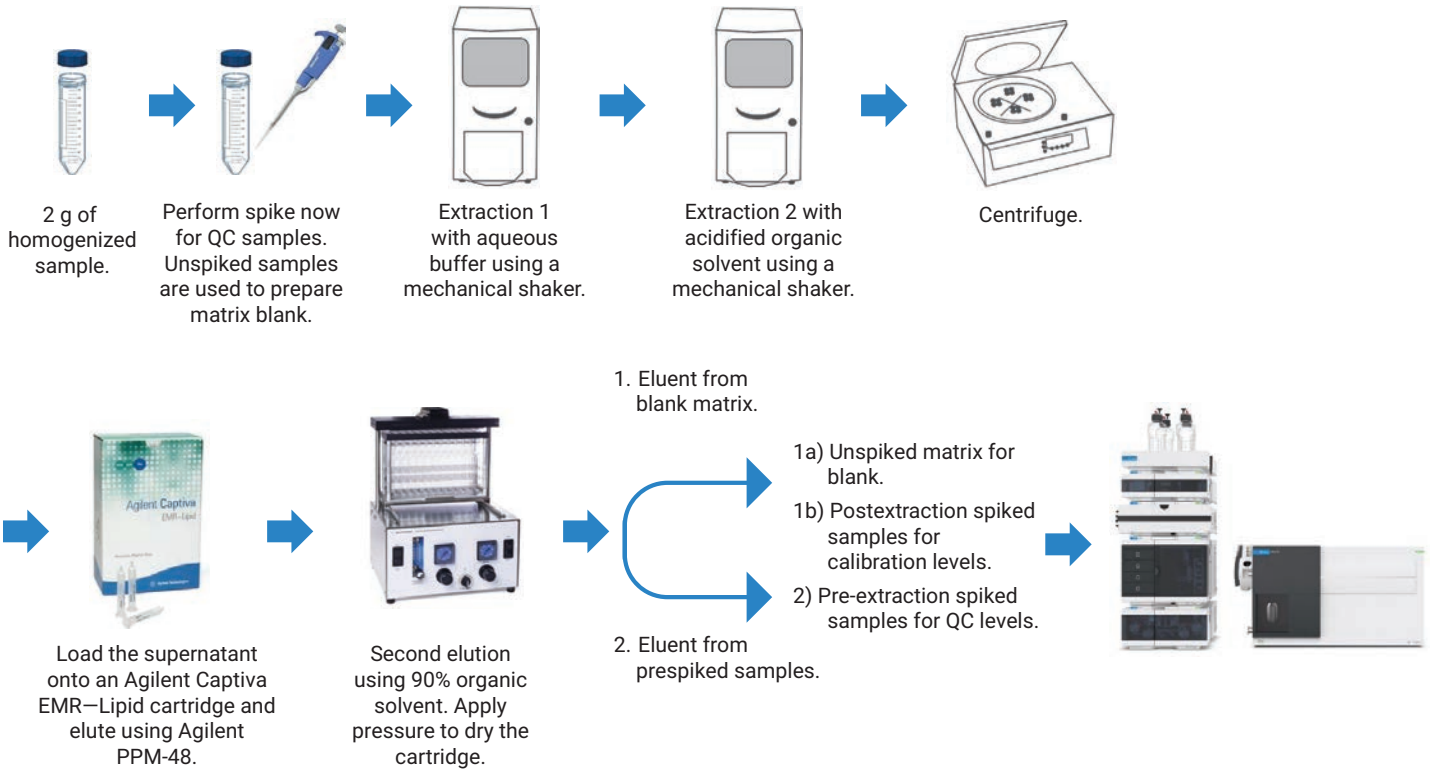

Figure 1. Flowchart of sample extraction and Agilent Captiva EMR—Lipid cleanup protocol. (The size of images is not to any scale.)

Neat solutions at 0.1, 1.0, and 2.5 µg/L in 50/50 acetonitrile/water were used to evaluate matrix effects by comparing the responses in the corresponding matrix-matched calibration standards.

Instrumentation

Chromatographic separation was performed using an Agilent InfinityLab Poroshell 120 EC-C18 column (p/n 695575-302) installed on an Agilent 1290 Infinity II LC. The individual modules of the 1290 Infinity II LC were:

- Agilent 1290 Infinity II high-speed pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)

The LC system was equipped with a 20 µL injection loop and multiwash capability. Mobile phase A was water with 4.5 mM ammonium formate, 0.5 mM ammonium fluoride, and 0.1% formic acid; and mobile phase B was 50/50 ACN/MeOH with 4.5 mM ammonium formate, 0.5 mM ammonium fluoride, and 0.1% formic acid.

A 6470 LC/TQ with an Agilent Jet Stream (AJS) ion source was operated in dynamic MRM (dMRM) mode. The LC/TQ autotune was performed in unit mode with *report m/z <100* mode enabled. Data acquisition and processing were performed using Agilent MassHunter software (version 10.0). Please refer to the workflow guide included with the Comprehensive Veterinary Drug dMRM Solution, for more information on non-Agilent laboratory equipment and supplies used in this study. The methods for the 6470 LC/TQ and 6495C LC/TQ are included in the Comprehensive Veterinary Drug dMRM Solution, allowing users to copy and use the acquisition method directly.

Application of the workflow for the screening of veterinary drugs

Reporting limits are implemented from different regulatory organizations to control the veterinary drug residues in animal-origin food matrices. Depending on the regulatory organization and sample matrix, the acceptable residue limit of veterinary drugs may vary. The 210 targeted veterinary drugs were selected based on a combinatory study of the vet drug monitoring lists recommended by US FDA-CFR,[2] US FSIS,[3] EU,[4] and AOAC.[5] A Venn diagram of target distribution across various organizations is given in Figure 2. Of the total 210 target analytes, 168 of them have maximum residue limits (MRLs) established in three muscle matrices regulated by AOAC, EU, and US regulation/guidelines. The remaining 42 targets with no MRL established are specified under *monitoring* category in muscle matrix per the requirement of these regulations/guidelines. The workflow applicability for a specific regulation/guideline-based routine screening is demonstrated by evaluating the analytical characteristics of the appropriate fortified QC samples.

Results and discussion

Simple workflow method for the screening of multiclass veterinary drugs

A sensitive and robust workflow for vet drug analysis is beneficial for users to perform routine screening following various regulatory guidelines. The applicability of the newly developed workflow for guideline-based routine analysis is demonstrated by carrying out a screening of chicken muscle matrix for the AOAC recommended target list. Out of 168 targets, 86 targets are specifically required for chicken screening, with results summarized in Table 2 (found at the end of this document). The sensitivity of the workflow method was established using postextraction spiked calibration levels, and applicability for routine screening was demonstrated using recovery analysis at three pre-extraction QC levels: 1 (LQC), 10 (MQC), and 25 µg/kg (HQC). Based on the MRL value of a target, one of the QC levels was chosen to demonstrate the screening aspects. The MRL for most targets (85 out of 86) listed in the AOAC guidelines for chicken matrix is ≥10 µg/kg, and recovery analysis using MQC (10 µg/kg) is appropriate to screen all these targets.

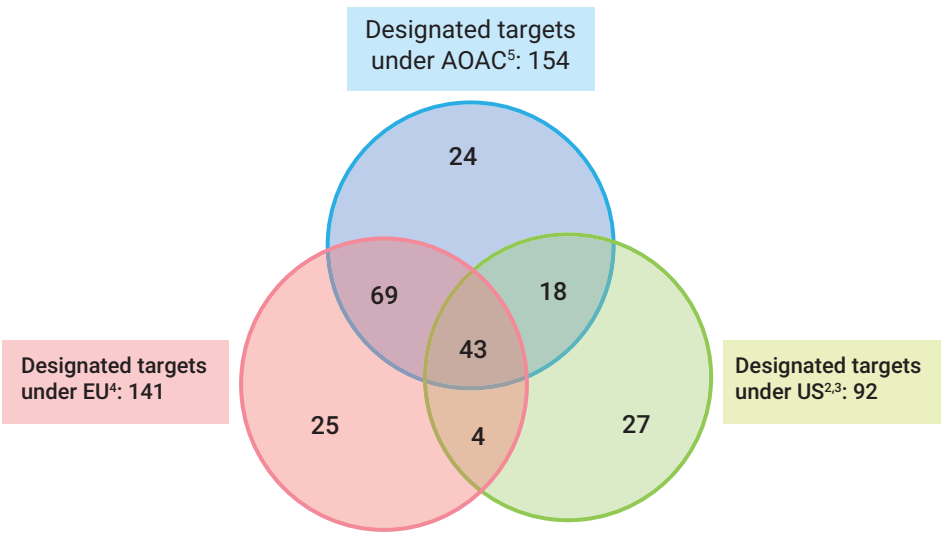

Figure 2. Venn diagram of 210 targets distribution across various regulations.

For the target prednisone, the MRL in chicken matrix is ~1 µg/kg, and for this target, LQC (1 µg/kg) was used to estimate target performance metrics such as recovery, repeatability, and reproducibility. Similarly, for cefalexin, the MRL is 200 µg/kg, and HQC was used to assess the target performance. In summary, the proposed workflow method can successfully be used to screen all 86 targets in chicken matrix as per AOAC guidelines. The results on intraday repeatability and interday reproducibility of recovery values confirmed the consistent and reproducible results for confident day-to-day screening analysis.

LC/TQ method development and performance evaluation

Compound-specific parameters including precursor ion, most abundant product ions, and collision energies were optimized using the MassHunter MRM Optimizer. Two or three target-specific MRM transitions were selected for each compound to satisfy regulatory requirements for identification and confirmation by LC/MS/MS. The method included in the Comprehensive Veterinary Drug dMRM Solution is comprised of MRM transitions for each compound and all relevant MS parameters.

Chromatographic separation using the InfinityLab Poroshell EC-C18 column resulted in good separation and retention time distribution of 210 veterinary drugs with a 13-minute gradient. The 0.5 mL/min flow rate offered easy desolvation of target ions on the AJS source. The addition of ammonium fluoride in the mobile phase helped to improve the sensitivity of negative ionization and reduced the formation of adducts. A dMRM method with a cycle time of 750 ms was used, with dwell times between 7 to 370 ms.

Typical chromatographic peak widths observed were between 8 to 12 seconds. Figure 3 shows a representative MRM chromatogram for all veterinary drug targets postspiked at 2.5 µg/L concentration in chicken matrix.

Early-eluted polar compounds such as piperazine, amprolium, and nicotine have acceptable peak shapes. However, a few of the mectins, such as emamectin and moxidectin, eluted towards the end of the chromatographic run. Targets such as 2,4,6-triamino-pyrimidine-5-carbonitrile, amoxicillin, baquiloprim, cefapirin, cotinine, deacetylcefapirin, dicloxacillin, dicyclanil, diminazene, ractopamine, salbutamol (albuterol), sulfaguanidine, tilmicosin, and zilpaterol showed split peaks. This issue can be overcome by using a higher aqueous solvent percentage in the final, ready-to-inject sample.

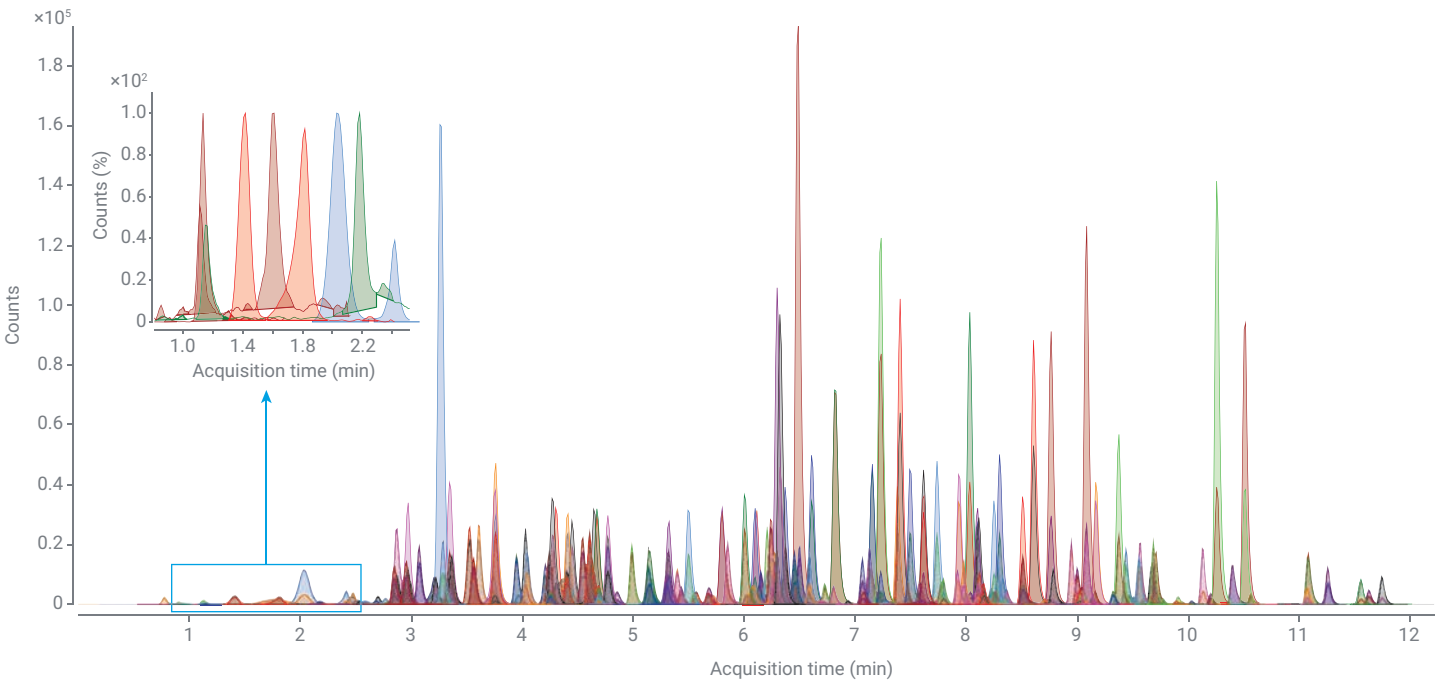

Figure 3. MRM chromatogram of 210 veterinary drug targets postspiked at 2.5 µg/L in blank chicken matrix. Considering the dilution factor was 1:10, this 2.5 µg/L postspike is equivalent to 25 µg/kg spike in chicken. The symmetric sharp peaks demonstrate the efficient chromatographic separation of targets within the retention time window. The inset plot is the zoomed-in view of normalized peaks corresponding to six early-eluting targets.

The workflow performance was assessed based on method sensitivity, linearity, accuracy, precision, analyte recovery, repeatability, and reproducibility. Workflow performance was evaluated using five batch analyses in three different muscle matrices (3× batches for chicken matrix, 1× batch for beef, and 1× batch for pork, respectively). Two different model instruments, the 6470 LC/TQ and 6495C LC/TQ, were used to verify the workflow method performance. The results were cross-verified with a second set of instruments from both models. The batch run for each sample matrix included solvent blank, matrix blank, matrix-matched calibration standards, and pre-extraction QC samples. Matrix-matched calibration standards were run in triplicate and matrix-spiked QC samples were run in duplicate. Neat QC samples were also run to assess the matrix effect.

LOD, LOQ, and calibration curve linearity

LOD and LOQ were established using the various lower levels of postextraction calibration levels. For each compound, the minimum signal-to-noise ratio (S/N) defined for LOD was >3, and >10 for LOQ, using the peak height and the auto-RMS algorithm embedded in Agilent MassHunter Quantitative Analysis software. For claiming LOQ, additional measures such as target selectivity for sample matrix and analyte response reproducibility were also considered. The LOD and LOQ calculation based only on S/N may be impacted if there is matrix contribution due to the endogenous presence of targets in the matrix. When there was a contribution from the matrix to the target analyte, LOD was defined as the three-fold peak area of matrix contribution, and LOQ was defined as the five-fold area of matrix contribution. Analyte response reproducibility calculated from three replicate injections was another important consideration for LOQ, and %RSD was less than the typical acceptance criteria of 25%. Considering the regulatory MRLs requirement for most vet drugs, the lowest postspiking level in matrix extract was 0.01 µg/L, corresponding to 0.1 µg/kg in meat. However, the intensity of many target MRM signals showed the potential to reach lower LODs and LOQs.

A calibration curve for each target was generated using postextraction samples from the defined LOQ to the highest spiked level. For example, for a target with LOD at 0.1 µg/kg, the calibration curve was constructed from 0.25 to 100 µg/kg; for a target with LOD at 1 µg/kg, the calibration curve range was 2.5 to 100 µg/kg; for a target with LOD at 10 µg/kg, the calibration curve range was 25 to 100 µg/kg. To determine the best linearity response function, various regression models were evaluated, and the best calibration model was with Type: *Linear*, Origin: *Ignore*, Weight: *1/x*. All targets met the calibration curve linearity requirement of R^2 >0.99. Table 2 shows the LOD, LOQ, and calibration curve data of all targets in the chicken matrix.

Instrument method accuracy and precision

The average accuracy value for each postextraction (matrix-matched) calibration level was calculated from triplicate injections. Observed accuracy values for all targets across the calibration range were well within the range of 70 to 120%.

Precision was determined by calculating percent relative standard deviation (%RSD) of the target response and retention time (RT) using triplicate injections for the postextraction calibration levels. Good RTs and response precision values for all targets in all matrices were observed. Response %RSD for all targets in the chicken matrix was <20%, and RT %RSD of all targets was within 0.5%. The precision results confirm the reproducibility of the elution profile and MS detection. For targets having LOQs at 25 µg/kg, the RT %RSD and area %RSD were calculated at 25 µg/L.

Target recovery/extraction efficiency

In this experiment, the impact of sample preparation on target recovery was assessed using three levels of pre-extraction QC samples (LQC, MQC, and HQC). Percent recovery was calculated using "target response" in pre-extraction QCs and "measured response" using postextraction spiked calibration curve equations. Figure 4 shows a MRM chromatogram overlay for the three targets trimethoprim, oxibendazole, and febantel for postextraction sample (black trace) and pre-extraction sample (blue trace) at a concentration corresponding to 1 µg/L in chicken. The response counts comparison between postextraction calibration level and pre-extraction QC samples indicates good recovery (106 ±1%) of these targets. For LQC and MQC, the average recoveries were calculated from duplicate injections of three technical preparations, while for HQC, the average recoveries were calculated from duplicate injections on one technical preparation. Recovery values of over 97% of the targets met the acceptable range of 60 to 120%. Recovery values for targets such as amproilum, cefapirin, erythromycin, malachite green, narasin, and nicotine were within a range of 30 to 60%. However, the results were reproducible over three different batches of study. The results for all target recoveries are listed in Table 2.

Workflow intrabatch repeatability

In this study, the variation of target recovery results between technical preparations of QC levels within a batch was estimated. Recovery repeatability was measured as %RSD of recovery values calculated using intraday technical preparations of QC levels using the chicken matrix. Sample preparation conditions were kept as constant as possible. Captiva EMR—Lipid extraction was performed in triplicate each technical preparation of LQC (1 µg/kg) and MQC (10 µg/kg) levels. Each technical preparation was injected into the mass spectrometer in duplicates. The %RSD was calculated for each QC level and expressed as repeatability. Typically, the acceptable recovery repeatability limit at 10 ppb is 21% and at 1 ppb the limit is 30%.[1] The recovery repeatability %RSD values of all targets were within the acceptable limits, and the results are included in Table 2. The recovery value of a few targets was less than 60%; however, the recovery repeatability for these targets was within 10% RSD, demonstrating consistent behavior with each technical preparation. These results confirm the repeatability of analyte recovery using Captiva EMR—Lipid sample preparation.

Workflow interbatch reproducibility

In this study, precision of recovery results obtained among three different chicken matrix batches across different laboratory conditions was assessed. The potential variables for the sample preparation and analysis were kept as different as possible, including different lots of sample matrix, different analysts, different instruments, different days, and different laboratory environments. Target recovery reproducibility was measured for all three pre-extraction spiked levels: LQC (1 µg/kg), MQC (10 µg/kg), and HQC (25 µg/kg). Each technical preparation was injected in duplicate, and %RSD of calculated concentrations resulting under different laboratory conditions was reported as reproducibility.

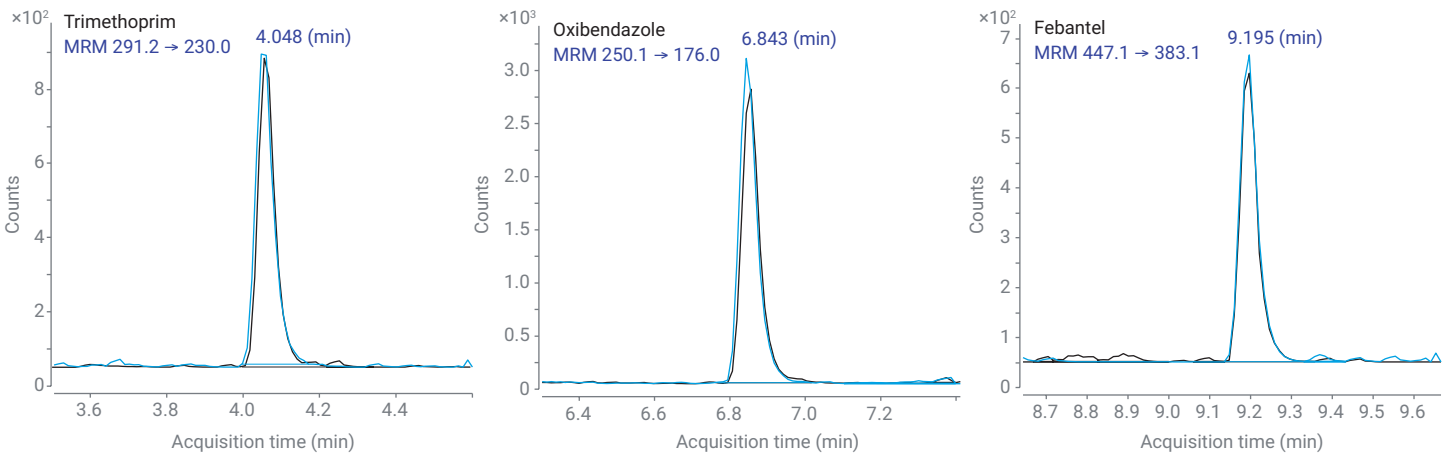

Figure 4. MRM chromatograms overlay of three selected veterinary drug targets corresponding to 1 µg/kg in chicken across the method retention window. The black trace is the MRM for 1 µg/L postspike calibration level, the blue trace is for 1 µg/kg prespike.

The observed results are summarized in Table 2. All 210 targets met the recovery reproducibility limit[1] of <32% RSD and among that, results of >91% of targets were within 15% RSD. The recovery reproducibility results confirm the precision of Captiva EMR—Lipid sample preparation across different laboratory conditions.

Matrix effect assessment

Matrix effect (ME) was assessed by the ratio of target response in postspiked samples to that in corresponding neat standards. Typically, there is no strict requirement on acceptable ME criteria, because the matrix effect can be corrected by the matrix-matched calibration curve. However, the matrix effect is an important parameter for method sensitivity and reliability assessment. In this study, ME was investigated using the postspiked calibration levels at 2.5 µg/L level in comparison to the corresponding neat standards. Within the total of 210, >93% of targets did not show any significant matrix suppression; for these targets, ME was >75%. Approximately 3% of targets resulted in ME within 50 to 70%, indicating low ion suppression; 1% of targets showed ME within 25 to <50%, indicating relatively medium level ion suppression; and 3% of targets exhibited significant ion suppression with MEs <25%. Targets such as cyromazine, dicyclanil, sulfacetamide, sulfaguanidine, sulfisomidine, and tolfenamic acid were affected by low ion suppression. Targets such as erythromycin and fluralaner were affected with relatively medium level ion suppression, and 2,4,6-triamino-pyrimidine-5-carbonitrile, amprolium, cotinine, deacetylcefapirin, metronidazole, metronidazole-OH, and nicotine showed significant ion suppression.

Method performance comparison across three muscle matrices

The performance results from chicken, beef, and pork muscles were in good agreement. As an example, the recovery results for targets in chicken, beef, and pork muscle at 10 µg/kg are shown in Figure 5. The recoveries of >97% of targets in chicken were within the acceptable range of 60 to 120%, while the recoveries of >94% of targets in beef and pork meet the criteria. The results verified the workflow applicability for various meat matrices. Dipyrone hydrate and cefuroxime showed matrix interference in beef and pork matrices, and quantitation results were negatively impacted. Acepromazine, chlorpromazine, and propionyl promazine showed poor recoveries in beef and pork matrix, but still with acceptable 7% RSD reproducibility.

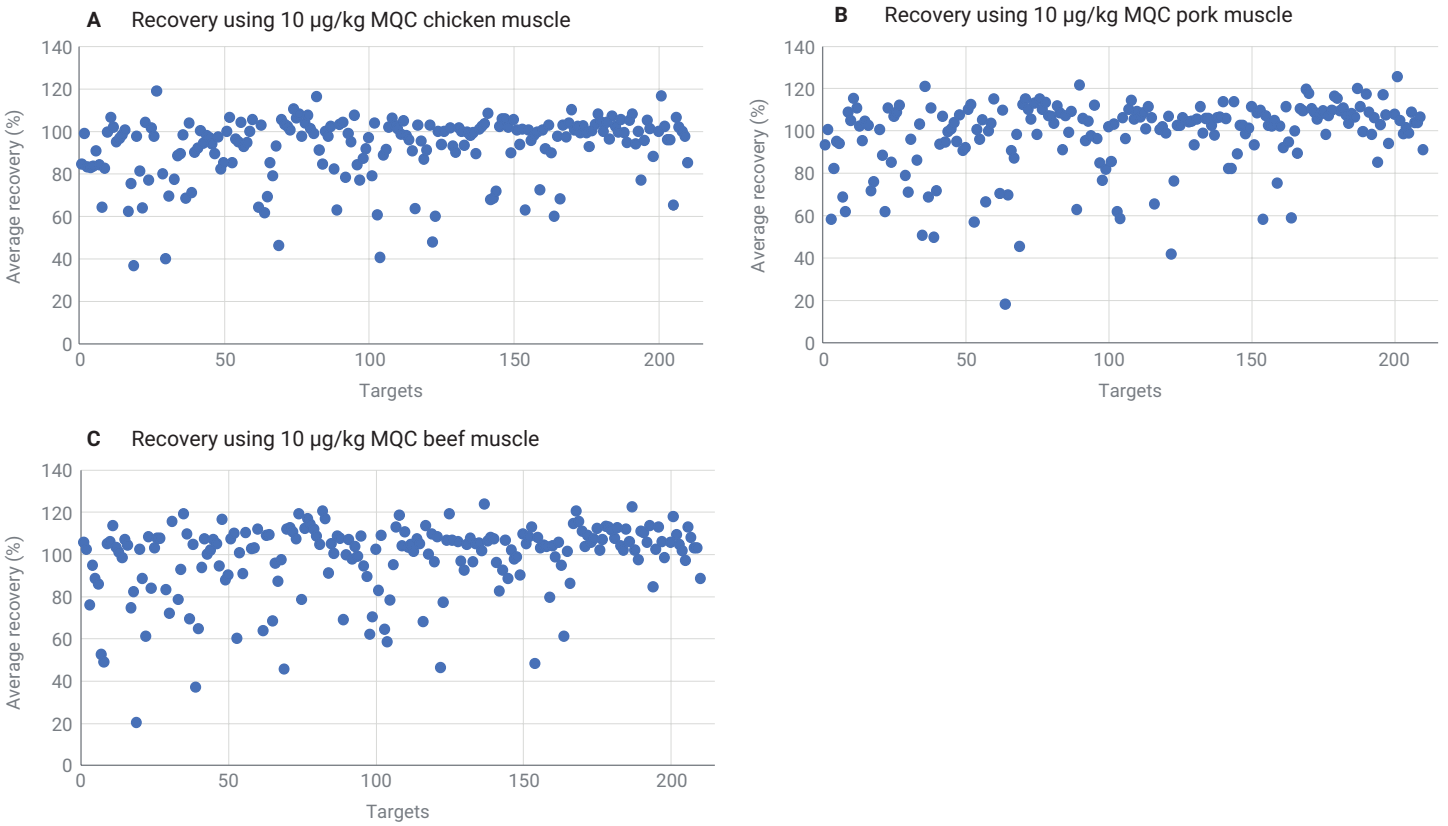

Figure 5. Target recovery from chicken (A), pork (B), and beef (C) muscle matrices using 10 µg/kg prespiked MQC samples.

Conclusion

This study describes a highly sensitive and reproducible workflow for fast and reliable screening and quantitation of 210 multiclass veterinary drugs in meat using a 6470 LC/TQ. The workflow uses a solid/liquid extraction with Captiva EMR—Lipid sample cleanup followed by analysis using a 1290 Infinity II LC coupled to a 6470 LC/TQ. The applicability of the workflow solution for routine veterinary drug screening analysis was demonstrated by performing screening of AOAC-listed targets in chicken matrix.

A simple sample preparation protocol based on solvent extraction and Captiva EMR—Lipid cleanup provides highly efficient, selective, and reproducible matrix/lipid removal without impacting the target analyte recoveries. The 13-minute LC method using an InfinityLab Poroshell EC-C18 column offered good chromatographic separation and even RT distribution of all targets. LC/TQ data acquisition was in dMRM mode with fast polarity switching for the most efficient use of instrument cycle time. The method's sensitivity helped to achieve sub-5 ng/mL LODs for most analytes.

The workflow performance was verified using two different triple quadrupole models (6470 LC/TQ and 6495C LC/TQ). The method performance evaluation based on calibration curve linearity, accuracy, precision, and recovery results from both models was in alignment with the additional benefit of improved sensitivity on the 6495C LC/TQ. The method was cross-verified using a second set of instruments on both models. The workflow applicability in other meat matrices was demonstrated in beef and pork.

Table 2. Target screening results based on AOAC guidelines in a chicken matrix.

No.	Compound Name	RT (min)	Functional Use/ Chemical Classes	CAS Number	AOAC MRL (µg/kg)	LOD (µg/L)	Linear calibration curve Range with R^2 > 0.99 (µg/L)	MQC Recovery (%) (*LQC, #HQC)	MQC Recovery Repeatability (%) (*LQC)	MQC Recovery Reproducibility (%) (*LQC, #HQC)
1	2, 4, 6-triamino-pyrimidine-5-carbonitrile	1.58	Insecticide	465531-97-9	N/A	5	10 to 100	85	8%	5%
2	2,4-DMA [Amitraz Metabolite]	4.34	Insecticide	33089-74-6	N/A	0.1	0.25 to 100	99	1%	1%
3	2-Quinoxalinecarboxylic acid [QCA]	4.13	Quinoxalines	879-65-2	N/A	5	10 to 100	83	6%	13%
4	4-epi-oxytetracycline	4.26	Antibiotic/ Tetracycline	14206-58-7	200	0.5	1 to 100	83	5%	14%
5	4-epi-tetracycline	4.17	Antibiotic/ Tetracycline	79-85-6	200	0.25	0.5 to 100	83	1%	15%
6	5-Hydroxy thiabendazole	3.52	Anthelmintic/ Benzimidazoles	948-71-0	N/A	0.25	0.5 to 100	91	1%	3%
7	5-Hydroxyflunixin	8.29	NSAIDs	75369-61-8	N/A	0.1	0.25 to 100	84	2%	10%
8	Acepromazine	7.34	Tranquilizer	61-00-7	N/A	0.1	0.25 to 100	64	7%	11%
9	Acetyl isovaleryl tylosin [Tylvalosin]	8.71	Antibiotic/ Macrolides	63409-12-1	40	1	2.5 to 100	82	1%	10%
10	Albendazole	8.01	Anthelmintic/ Benzimidazoles	54965-21-8	N/A	0.1	0.25 to 100	100	1%	2%
11	Albendazole sulfone	6.14	Anthelmintic/ Benzimidazoles	75184-71-3	N/A	0.25	0.5 to 100	107	2%	6%
12	Albendazole sulfoxide	5.54	Anthelmintic/ Benzimidazoles	54029-12-8	N/A	1	2.5 to 100	102	2%	3%
13	Albendazole-2-aminosulfone	3.71	Anthelmintic/ Benzimidazoles	80983-34-2	N/A	0.5	1 to 100	95	2%	4%
14	Alpha Zearalanol	8.25	Hormones	26538-44-3	N/A	2.5	5 to 100	97	12%	6%
15	Altrenogest	8.96	Hormones	850-52-2	N/A	0.25	0.5 to 100	98	2%	2%
16	Aminoflubendazole	6.08	Anthelmintic/ Benzimidazoles	82050-13-3	50	0.1	0.25 to 100	101	0%	1%
17	Amoxicillin	2.78	Antibiotic/ Beta-Lactam	26787-78-0	10	2.5	5 to 100	62	9%	23%
18	Ampicillin	3.94	Antibiotic/ Beta-Lactam	69-53-4	10	2.5	5 to 100	75	2%	16%
19	Amprolium	1.19	Antimicrobial	13082-85-4	500	1	2.5 to 100	36	7%	14%

No.	Compound Name	RT (min)	Functional Use/ Chemical Classes	CAS Number	AOAC MRL (µg/kg)	LOD (µg/L)	Linear calibration curve Range with $R^2 > 0.99$ (µg/L)	MQC Recovery (%) (*LQC, #HQC)	MQC Recovery Repeatability (%) (*LQC)	MQC Recovery Reproducibility (%) (*LQC, #HQC)
20	Azaperone	5.76	Tranquilizer	1649-18-9	N/A	0.1	0.25 to 100	98	2%	1%
21	Azithromycin	6.16	Antibiotic/ Macrolides	83905-01-5	N/A	0.25	0.5 to 100	81	2%	3%
22	Baquiloprim	2.63	Antimicrobial	102280-35-3	N/A	0.5	1 to 100	64	2%	5%
23	Betamethasone	7.77	Growth promoters/ Corticosteroids	378-44-9	N/A	0.5	1 to 100	104	3%	3%
24	Cabergoline	4.58	Dopamine receptor	81409-90-7	N/A	0.25	0.5 to 100	77	3%	4%
25	Carazolol	6.06	Tranquilizer	57775-29-8	N/A	0.1	0.25 to 100	102	1%	1%
26	Carbadox	4.41	Antimicrobial	6804-07-5	N/A	0.5	1 to 100	98	3%	4%
27	Carprofen	9.00	NSAIDs	53716-49-7	N/A	10	25 to 100	119	0%	4%
28	Cefalexin	3.91	Antibiotic/ Beta-Lactam	15686-71-2	200	10	25 to 100	74 (#)	_	29% (#)
29	Cefalonium	3.91	Antibiotic/ Beta-Lactam	5575-21-3	N/A	5	10 to 100	80	20%	15%
30	Cefapirin	3.19	Antibiotic/ Beta-Lactam	21593-23-7	N/A	0.5	1 to 100	40	6%	32%
31	Cefazolin	4.31	Antibiotic/ Beta-Lactam	25953-19-9	N/A	5	10 to 100	70	16%	6%
32	Cefoperazone	5.14	Antibiotic/ Beta-Lactam	62893-19-0	N/A	10	25 to 100	88 (#)	_	10% (#)
33	Cefquinome	3.69	Antibiotic/ Beta-Lactam	84957-30-2	N/A	1	2.5 to 100	77	9%	6%
34	Ceftiofur	6.27	Antibiotic/ Beta-Lactam	80370-57-6	N/A	1	2.5 to 100	89	5%	11%
35	Cefuroxime	4.40	Antibiotic/ Beta-Lactam	55268-75-2	N/A	5	10 to 100	89	17%	11%
36	Chloramphenicol	6.24	Antibiotic/ Amphenicols	56-75-7	N/A	2.5	5 to 100	98	4%	5%
37	Chlorhexidine	7.08	Antimicrobial	55-56-1	N/A	0.25	0.5 to 100	69	4%	1%
38	Chlormadinone	9.45	Hormones	1961-77-9	N/A	1	2.5 to 100	104	2%	1%
39	Chlorpromazine	8.06	Tranquilizer	50-53-3	N/A	0.1	0.25 to 100	71	12%	13%
40	Chlortetracycline	5.94	Antibiotic/ Tetracycline	57-62-5	200	1	2.5 to 100	90	2%	9%
41	Ciprofloxacin	4.43	Antibiotic/ Quinolones	85721-33-1	N/A	0.25	0.5 to 100	92	2%	2%
42	Clenbuterol	5.28	Growth promoters/ Beta-Agonists	37148-27-9	N/A	0.1	0.25 to 100	100	2%	4%
43	Clindamycin	6.45	Antibiotic/ Macrolides	18323-44-9	N/A	5	10 to 100	94	1%	3%
44	Clopidol	3.56	Coccidiostats	2971-90-6	5000	0.5	1 to 100	98	3%	1%
45	Closantel	10.54	Anthelmintic	57808-65-8	N/A	1	2.5 to 100	97	3%	2%
46	Colchicine	6.72	NSAIDs	64-86-8	N/A	0.5	1 to 100	94	3%	3%
47	Cotinine	2.35	Insecticide	486-56-6	N/A	0.5	1 to 100	89	2%	2%
48	Coumaphos	9.58	Anthelmintic	56-72-4	N/A	1	2.5 to 100	97	3%	10%
49	Cyromazine	2.47	Anthelmintic	66215-27-8	100	1	2.5 to 100	82	3%	3%
50	Danofloxacin	4.63	Antibiotic/ Quinolones	112398-08-0	200	0.1	0.25 to 100	85	1%	2%
51	Dapson	4.67	Antibiotic/ Sulfonamides	80-08-0	N/A	0.1	0.25 to 100	100	3%	3%
52	Dapson N-Acetyl	5.40	Antibiotic/ Sulfonamides	565-20-8	N/A	0.5	1 to 100	107	2%	1%
53	Deacetylcefapirin	2.30	Antibiotic/ Beta-Lactam	104557-24-6	N/A	5	10 to 100	85	8%	2%

No.	Compound Name	RT (min)	Functional Use/ Chemical Classes	CAS Number	AOAC MRL (µg/kg)	LOD (µg/L)	Linear calibration curve Range with $R^2 > 0.99$ (µg/L)	MQC Recovery (%) (*LQC, #HQC)	MQC Recovery Repeatability (%) (*LQC)	MQC Recovery Reproducibility (%) (*LQC, #HQC)
54	Diaveridine	3.73	Antimicrobial	5355-16-8	50	0.1	0.25 to 100	97	2%	1%
55	Diazinon	9.64	Insecticide	333-41-5	N/A	0.25	0.5 to 100	95	2%	8%
56	Diclofenac	9.14	NSAIDs	15307-86-5	N/A	0.5	1 to 100	104	5%	7%
57	Dicloxacillin	8.11	Antibiotic/ Beta-Lactam	3116-76-5	300	5	10 to 100	93	2%	23%
58	Dicyclanil	2.93	Insecticide	112636-83-6	N/A	0.5	1 to 100	95	2%	2%
59	Difloxacin	5.29	Antibiotic/ Quinolones	98106-17-3	300	0.25	0.5 to 100	100	1%	1%
60	Diflubenzuron	9.11	Insecticide	35367-38-5	N/A	2.5	5 to 100	105	7%	3%
61	Dimetridazole	3.66	Coccidiostats	551-92-8	N/A	10	25 to 100	87 (#)	_	7% (#)
62	Diminazene	2.96	Coccidiostats	536-71-0	N/A	2.5	5 to 100	64	9%	8%
63	Dinitolmide [Zoalene]	5.56	Coccidiostats	148-01-6	3000	2.5	5 to 100	103	1%	5%
64	Dipyrone hydrate- metabolite [4-Methylaminoantipyrine]	3.34	NSAIDs	519-98-2	N/A	0.1	0.25 to 100	62	2%	3%
65	Doxycycline	6.26	Antibiotic/ Tetracycline	564-25-0	100	0.5	1 to 100	69	3%	17%
66	Emamectin B1a benzoate	10.09	Anthelmintic/ Avermectins	121124-29-6	N/A	0.25	0.5 to 100	79	2%	4%
67	Emamectin B1b benzoate	9.90	Anthelmintic/ Avermectins	121424-52-0	N/A	2.5	5 to 100	85	7%	5%
68	Enrofloxacin	4.74	Antibiotic/ Quinolones	93106-60-6	100	0.25	0.5 to 100	93	2%	2%
69	Erythromycin	7.40	Antibiotic/ Macrolides	114-07-8	100	0.5	1 to 100	46	7%	3%
70	Ethopabate	6.60	Coccidiostats	59-06-3	500	0.1	0.25 to 100	106	2%	3%
71	Famphur	8.18	Insecticide	52-85-7	N/A	1	2.5 to 100	103	4%	6%
72	Febantel	9.15	Anthelmintic/ Benzimidazoles	58306-30-2	N/A	0.25	0.5 to 100	102	6%	2%
73	Fenbendazole	8.59	Anthelmintic/ Benzimidazoles	43210-67-9	N/A	0.1	0.25 to 100	100	1%	3%
74	Fenbendazole Sulfoxide [Oxfendazole]	6.44	Anthelmintic/ Benzimidazoles	53716-50-0	N/A	0.25	0.5 to 100	110	1%	1%
75	Firocoxib	7.96	NSAIDs	189954-96-9	N/A	2.5	5 to 100	106	5%	6%
76	Florfenicol	5.55	Antibiotic/ Amphenicols	73231-34-2	100	0.5	1 to 100	108	5%	4%
77	Fluazuron	10.17	Insecticide	86811-58-7	N/A	0.5	1 to 100	98	2%	4%
78	Flubendazole	7.72	Anthelmintic/ Benzimidazoles	31430-15-6	50	0.1	0.25 to 100	104	1%	5%
79	Flugestone acetate	8.35	Hormones	2529-45-5	N/A	1	2.5 to 100	108	3%	2%
80	Flumequine	7.39	Antibiotic/ Quinolones	42835-25-6	400	0.1	0.25 to 100	101	2%	1%
81	Flunixin	8.75	NSAIDs	38677-85-9	N/A	0.1	0.25 to 100	99	2%	1%
82	Fluralaner	9.89	Insecticide	864731-61-3	N/A	2.5	5 to 100	116	4%	9%
83	Furazolidone	4.68	Antimicrobial/Furans	67-45-8	N/A	2.5	5 to 100	91	4%	16%
84	Gamithromycin	6.44	Antibiotic/ Aminoglycosides	145435-72-9	N/A	0.25	0.5 to 100	85	11%	1%
85	Gonadotropin	7.57	Hormones	33515-09-2	N/A	0.5	1 to 100	100	4%	4%
86	Halofuginone	6.44	Coccidiostats	55837-20-2	10	0.5	1 to 100	98	1%	3%
87	Haloperidol	7.11	Tranquilizer	52-86-8	N/A	0.1	0.25 to 100	102	1%	1%
88	Haloxon	8.58	Anthelmintic	321-55-1	N/A	2.5	5 to 100	82	8%	10%
89	Imidocarb	3.20	Coccidiostats	27885-92-3	N/A	0.5	1 to 100	63	3%	7%

No.	Compound Name	RT (min)	Functional Use/ Chemical Classes	CAS Number	AOAC MRL (µg/kg)	LOD (µg/L)	Linear calibration curve Range with $R^2 > 0.99$ (µg/L)	MQC Recovery (%) (*LQC, #HQC)	MQC Recovery Repeatability (%) (*LQC)	MQC Recovery Reproducibility (%) (*LQC, #HQC)
90	Ipronidazole	6.04	Anthelmintic/ Nitroimidazoles	14885-29-1	N/A	5	10 to 100	103	13%	11%
91	Ipronidazole-OH	4.85	Anthelmintic/ Nitroimidazoles	35175-14-5	N/A	1	2.5 to 100	104	3%	1%
92	Isometamidium	5.98	Anthelmintic	20438-03-3	N/A	2.5	5 to 100	78	3%	10%
93	Josamycin	8.22	Antibiotic/ Macrolides	16846-24-5	40	0.5	1 to 100	99	3%	2%
94	Ketamine	4.74	Anesthetic	6740-88-1	N/A	0.1	0.25 to 100	95	2%	1%
95	Ketoprofen	8.20	NSAIDs	22071-15-4	N/A	0.5	1 to 100	107	1%	4%
96	Kitasamycin A5 [Leucomycin A5]	7.70	Antibiotic/ Aminoglycosides	18361-45-0	200	1	2.5 to 100	84	1%	4%
97	Lasalocid A	10.99	Coccidiostats	25999-31-9	20	0.25	0.5 to 100	77	2%	4%
98	Leuco Crystal violet	10.36	Fungicides and dyes	603-48-5	N/A	0.5	1 to 100	87	3%	1%
99	Leucomalachite green	10.48	Fungicides and dyes	129-73-7	N/A	0.1	0.25 to 100	92	0%	4%
100	Levamisole	3.58	Anthelmintic	14769-73-4	10	0.25	0.5 to 100	97	2%	2%
101	Lincomycin	3.74	Antibiotic/ Aminoglycosides	154-21-2	100	0.1	0.25 to 100	79	1%	2%
102	Lufenuron	10.11	Insecticide	103055-07-8	N/A	10	25 to 100	104	5%	0%
103	Maduramicin Ammonium	11.59	Coccidiostats	79356-08-4	100	1	2.5 to 100	61	1%	4%
104	Malachite green	8.21	Fungicides and Dyes	10309-95-2	N/A	0.1	0.25 to 100	40	2%	10%
105	Malathion	8.92	Insecticide	121-75-5	N/A	0.25	0.5 to 100	89	2%	4%
106	Marbofloxacin	4.00	Antibiotic/ Quinolones	115550-35-1	N/A	0.25	0.5 to 100	91	4%	2%
107	Mebendazole	7.49	Anthelmintic/ Benzimidazoles	31431-39-7	N/A	0.1	0.25 to 100	102	1%	6%
108	Mefenamic acid	9.68	Anti-inflammatory	61-68-7	N/A	0.25	0.5 to 100	106	1%	6%
109	Megestrol acetate	9.43	Hormones	595-33-5	N/A	0.25	0.5 to 100	103	5%	1%
110	Melengestrol acetate	9.55	Hormones	2919-66-6	N/A	0.25	0.5 to 100	101	4%	3%
111	Meloxicam	8.10	NSAIDs	71125-38-7	N/A	0.1	0.25 to 100	99	1%	5%
112	Methylprednisolone	7.78	Growth promoters/ Corticosteroids	83-43-2	N/A	0.5	1 to 100	105	3%	3%
113	Metoserpate	6.55	Tranquilizer	1178-28-5	20	0.25	0.5 to 100	98	3%	3%
114	Metronidazole	3.22	Anthelmintic/ Nitroimidazoles	443-48-1	N/A	0.5	1 to 100	96	5%	4%
115	Metronidazole-OH	2.77	Anthelmintic/ Nitroimidazoles	4812-40-2	N/A	2.5	5 to 100	91	8%	5%
116	Monensin	11.22	Coccidiostats	17090-79-8	10	0.5	1 to 100	63	1%	2%
117	Monepantel	9.45	Anthelmintic	851976-50-6	N/A	1	2.5 to 100	103	1%	23%
118	Morantel tartrate	5.27	Anthelmintic	20574-50-9	N/A	0.5	1 to 100	95	2%	2%
119	Moxidectin	11.04	Anthelmintic/ Avermectins	113507-06-5	N/A	5	10 to 100	87	14%	23%
120	Nafcillin	8.02	Antibiotic/ Beta-Lactam	147-52-4	N/A	0.5	1 to 100	91	2%	5%
121	Nalidixic acid	7.21	Antibiotic	389-08-2	N/A	0.1	0.25 to 100	103	3%	1%
122	Narasin	11.71	Coccidiostats	55134-13-9	15	0.5	1 to 100	48	2%	7%
123	Neo-Spiramycin	5.71	Antibiotic/ Macrolides	70253-62-2	200	0.5	1 to 100	60	5%	4%
124	Nequinate	9.35	Anthelmintic	13997-19-8	100	0.1	0.25 to 100	100	4%	1%
125	Netobimin	7.06	Anthelmintic	88255-01-0	100	2.5	5 to 100	94	8%	16%
126	Nicarbazine	8.76	Coccidiostats	587-90-6	200	0.5	1 to 100	100	2%	2%

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127	Nicotine	1.44	Anti-herbivore	54-11-5	N/A	10	25 to 100	54 (#)	_	20% (#)
128	Niflumic Acid	9.07	Anti-inflammatory	4394-00-7	N/A	0.25	0.5 to 100	102	3%	1%
129	Nitroxynil	6.67	Anthelmintic	1689-89-0	N/A	2.5	5 to 100	93	5%	3%
130	Norfloxacin	4.28	Antibiotic/ Quinolones	70458-96-7	N/A	0.25	0.5 to 100	90	3%	1%
131	Norgestomet	9.31	Hormones	472-54-8	N/A	1	2.5 to 100	102	3%	3%
132	Novobiocin	9.75	Antibiotic	303-81-1	1000	1	2.5 to 100	100	2%	5%
133	Olaquindox	3.00	Growth promoters/ Anabolic steroids	23696-28-8	N/A	0.5	1 to 100	93	2%	2%
134	Oleandomycin	7.03	Antibiotic/ Aminoglycosides	3922-90-5	150	0.25	0.5 to 100	100	1%	2%
135	Orbifloxacin	4.97	Antibiotic/ Quinolones	113617-63-3	20	0.25	0.5 to 100	98	2%	1%
136	Ormetoprim	4.39	Antibiotic	6981-18-6	100	0.25	0.5 to 100	99	5%	1%
137	Oxacillin	7.51	Antibiotic/ Beta-Lactam	66-79-5	300	5	10 to 100	89	11%	11%
138	Oxibendazole	6.79	Anthelmintic/ Benzimidazoles	20559-55-1	N/A	0.1	0.25 to 100	101	1%	1%
139	Oxolinic acid	6.29	Antibiotic/ Quinolones	14698-29-4	100	0.25	0.5 to 100	102	2%	1%
140	Oxyclozanide	9.49	Anthelmintic	2277-92-1	N/A	2.5	5 to 100	103	4%	2%
141	Oxyphenbutazone	8.09	NSAIDs	129-20-4	N/A	0.5	1 to 100	108	2%	2%
142	Oxytetracycline	4.46	Antibiotic/ Tetracycline	79-57-2	200	1	2.5 to 100	68	3%	19%
143	Penicillin G	6.92	Antibiotic/ Beta-Lactam	61-33-6	N/A	1	2.5 to 100	68	2%	20%
144	Penicillin V [Phenoxymethylpenicillin]	7.33	Antibiotic/ Beta-Lactam	87-08-1	25	2.5	5 to 100	72	2%	25%
145	Phenylbutazone	9.01	NSAIDs	50-33-9	N/A	1	2.5 to 100	102	3%	1%
146	Phosalone	9.69	Insecticide	2310-17-0	N/A	1	2.5 to 100	106	5%	3%
147	Phoxim	9.63	Insecticide	14816-18-3	25	2.5	5 to 100	106	8%	2%
148	Piperonyl butoxide Ammonia	10.24	Insecticide	51-03-6	500	0.1	0.25 to 100	102	3%	7%
149	Pirlimycin	5.70	Antibiotic/ Aminoglycosides	79548-73-5	N/A	2.5	5 to 100	90	5%	10%
150	Praziquantel	8.49	Anthelmintic	55268-74-1	N/A	0.1	0.25 to 100	106	3%	2%
151	Prednisolone	7.22	Growth promoters/ Corticosteroids	50-24-8	N/A	0.5	1 to 100	101	0%	6%
152	Prednisone	7.06	Growth promoters/ Corticosteroids	53-03-2	0.7	0.5	1 to 100	102 (*)	24% (*)	24% (*)
153	Progesterone	9.53	Hormones	57-83-0	N/A	0.5	1 to 100	101	3%	1%
154	Propionylpromazin	7.90	Antiemetic	3568-24-9	N/A	0.1	0.25 to 100	63	5%	11%
155	Propyphenazone	7.61	NSAIDs	479-92-5	N/A	0.1	0.25 to 100	101	0%	2%
156	Pyrantel	4.15	Anthelmintic	15686-83-6	N/A	0.5	1 to 100	96	2%	2%
157	Pyrimethamine	6.20	Antimicrobial	58-14-0	50	0.1	0.25 to 100	98	3%	1%
158	Ractopamine	4.55	Growth promoters/ Beta-agonists	97825-25-7	N/A	0.25	0.5 to 100	100	2%	2%
159	Rafoxanide	11.03	Anthelmintic	22662-39-1	N/A	0.5	1 to 100	72	5%	4%
160	Rifaximin	9.00	Antibiotic	80621-81-4	N/A	1	2.5 to 100	101	4%	4%
161	Robenidine	8.48	Coccidiostats	25875-51-8	100	0.5	1 to 100	92	2%	2%
162	Ronidazole	3.34	Anthelmintic/ Nitroimidazoles	7681-76-7	500	0.25	0.5 to 100	103	2%	2%

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163	Salbutamol [Albuterol]	2.93	Growth promoters/ Beta-agonists	18559-94-9	N/A	0.1	0.25 to 100	90	2%	2%
164	Salinomycin	11.52	Coccidiostats	53003-10-4	100	0.5	1 to 100	60	1%	2%
165	Sarafloxacin	5.29	Antibiotic/ Quinolones	98105-99-8	10	0.25	0.5 to 100	98	2%	2%
166	Spiramycin I	6.03	Antibiotic/ Macrolides	24916-50-5	200	0.5	1 to 100	68	5%	4%
167	Sulfabenzamide	5.99	Antibiotic/ Sulfonamides	127-71-9	100	0.1	0.25 to 100	103	3%	4%
168	Sulfacetamide	3.06	Antibiotic/ Sulfonamides	144-80-9	100	0.25	0.5 to 100	97	3%	2%
169	Sulfachloropyridazine	5.16	Antibiotic/ Sulfonamides	80-32-0	100	0.25	0.5 to 100	104	2%	9%
170	Sulfaclozine	6.21	Antibiotic/ Sulfonamides	102-65-8	100	0.5	1 to 100	110	3%	6%
171	Sulfadiazine [Silvadene]	3.36	Antibiotic/ Sulfonamides	68-35-9	100	0.25	0.5 to 100	101	1%	4%
172	Sulfadimethoxine	6.39	Antibiotic/ Sulfonamides	122-11-2	100	0.1	0.25 to 100	102	1%	3%
173	Sulfadimidine [Sulfamethazine]	4.54	Antibiotic/ Sulfonamides	57-68-1	100	0.25	0.5 to 100	99	1%	4%
174	Sulfadoxine	5.49	Antibiotic/ Sulfonamides	2447-57-6	100	0.1	0.25 to 100	102	2%	1%
175	Sulfaethoxypyridazine	5.84	Antibiotic/ Sulfonamides	963-14-4	100	0.1	0.25 to 100	99	3%	6%
176	Sulfaguanidine	1.82	Antibiotic/ Sulfonamides	57-67-0	100	0.5	1 to 100	93	1%	2%
177	Sulfamerazine	3.94	Antibiotic/ Sulfonamides	127-79-7	100	0.25	0.5 to 100	100	2%	3%
178	Sulfameter [sulfamethoxydiazine]	4.40	Antibiotic/ Sulfonamides	651-06-9	100	0.25	0.5 to 100	103	1%	4%
179	Sulfamethizole	4.43	Antibiotic/ Sulfonamides	144-82-1	100	0.25	0.5 to 100	108	3%	5%
180	Sulfamethoxazole	5.39	Antibiotic/ Sulfonamides	723-46-6	100	0.25	0.5 to 100	105	3%	5%
181	Sulfamethoxypyridazine	4.60	Antibiotic/ Sulfonamides	80-35-3	100	0.25	0.5 to 100	100	3%	4%
182	Sulfamonomethoxine	5.14	Antibiotic/ Sulfonamides	1220-83-3	100	0.25	0.5 to 100	104	3%	7%
183	Sulfamoxole	4.24	Antibiotic/ Sulfonamides	729-99-7	100	0.25	0.5 to 100	96	2%	6%
184	Sulfanitran	7.25	Antibiotic/ Sulfonamides	122-16-7	100	5	10 to 100	107	6%	7%
185	Sulfaphenazole	6.26	Antibiotic/ Sulfonamides	526-08-9	100	0.25	0.5 to 100	102	3%	3%
186	Sulfapyridine	3.75	Antibiotic/ Sulfonamides	144-83-2	100	0.25	0.5 to 100	100	3%	3%
187	Sulfaquinoxaline	6.44	Antibiotic/ Sulfonamides	59-40-5	100	0.1	0.25 to 100	105	3%	7%
188	Sulfathiazole	3.55	Antibiotic/ Sulfonamides	72-14-0	100	0.25	0.5 to 100	99	2%	4%
189	Sulfisomidine	3.27	Antibiotic/ Sulfonamides	515-64-0	100	0.25	0.5 to 100	95	2%	2%
190	Sulfisoxazole	5.67	Antibiotic/ Sulfonamides	127-69-5	100	0.5	1 to 100	105	2%	5%

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191	Sulindac	7.97	Antibiotic/ Sulfonamides	38194-50-2	100	0.25	0.5 to 100	108	1%	2%
192	Teflubenzuron	10.01	Insecticide	83121-18-0	N/A	5	10 to 100	94	4%	5%
193	Testosterone	8.49	Growth promoters/ Anabolic steroids	58-22-0	N/A	0.25	0.5 to 100	100	3%	2%
194	Tetracycline	4.67	Antibiotic/ Tetracycline	60-54-8	200	0.5	1 to 100	77	1%	15%
195	Thiabendazole	4.26	Anthelmintic/ Benzimidazoles	148-79-8	N/A	0.1	0.25 to 100	96	4%	3%
196	Thiamphenicol	4.25	Antibiotic/ Amphenicols	15318-45-3	50	0.5	1 to 100	105	2%	6%
197	Tiamulin	7.56	Antibiotic	55297-95-5	100	0.1	0.25 to 100	101	1%	2%
198	Tilmicosin	6.76	Antibiotic/ Macrolides	108050-54-0	75	1	2.5 to 100	88	3%	6%
199	Tolfenamic acid	9.86	NSAIDs	13710-19-5	N/A	10	25 to 100	120 (#)	_	7% (#)
200	Trenbolone	7.91	Growth promoters/ Anabolic steroids	10161-33-8	N/A	0.5	1 to 100	100	4%	4%
201	Trichlorfon [DEP]	5.20	Tranquilizer	52-68-6	N/A	1	2.5 to 100	117	0%	16%
202	Triclabendazole	9.67	Anthelmintic/ Benzimidazoles	68786-66-3	N/A	0.25	0.5 to 100	102	2%	1%
203	Trimethoprim	4.02	Antibiotic	738-70-5	50	0.25	0.5 to 100	96	2%	1%
204	Tripelennamine	6.28	Anthelmintic	91-81-6	N/A	0.1	0.25 to 100	96	3%	1%
205	Tylosin	7.56	Antibiotic/ Macrolides	1401-69-0	100	1	2.5 to 100	65	5%	10%
206	Valnemulin	8.30	Antibiotic	101312-92-9	N/A	0.5	1 to 100	106	5%	3%
207	Vedaprofen	9.00	NSAIDs	71109-09-6	N/A	0.5	1 to 100	102	2%	1%
208	Virginiamycin M1	8.15	Antibiotic/ Macrolides	21411-53-0	100	0.5	1 to 100	100	2%	2%
209	Xylazine	5.11	Tranquilizer	7361-61-7	N/A	0.25	0.5 to 100	98	3%	2%
210	Zilpaterol	2.93	Growth promoters/ Beta-agonists	119520-05-7	N/A	0.25	0.5 to 100	85	2%	4%

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Powerful LC/MS/MS Approaches for Detection and Quantitation of Mycotoxins

Control of these potentially life-threatening fungal toxins in food and animal feed is vitally important

BY **THOMAS GLAUNER, PHD**

Mycotoxins are produced primarily by *Aspergillus*, *Penicillium*, and *Fusarium* fungi growing on a variety of agricultural commodities worldwide. They pose a major threat to human and animal health, as they have been implicated as causes of cancer and mutagenicity, as well as estrogenic, gastrointestinal, urogenital, vascular, kidney, and nervous disorders. Some may also impair resistance to infectious disease by compromising the immune system. Their impact on human health, animal productivity, and international trade results in significant economic losses.

The mycotoxins that pose the biggest threat to food safety include the aflatoxins, ochratoxin A, and toxins produced by *Fusarium* molds, including fumonisins, trichothecenes, and zearalenone. Aflatoxins (B_1, B_2, G_1, G_2, and M_1), are the most toxic, including damage to DNA that can cause cancer in animals. In fact, AFB_1 and mixtures of AFB_1, AFG_1, and AFM_1 are proven human carcinogens, and AFM_1 and AFB_2 are designated as probable human carcinogens by the International Agency for Research on Cancer (IARC). They contaminate many crops grown in hot and humid regions of the world, including peanuts, corn, cottonseed, and pistachios.

Ochratoxin A is produced by several *Penicillium* and *Aspergillus* fungal strains, and it occurs in a large variety of foods. It is classified by the IARC as a probable human carcinogen and is also implicated in kidney damage, birth defects, and immune deficiency.

Fumonisins are the result of fungal infection of maize, tomatoes, asparagus, and garlic, but maize-containing foods are the major food safety concern for fumonisin contamination. There are at least 15 related fumonisin compounds, and fumonisin B_1 can cause necrotic lesions in the cerebrum in horses, and pulmonary edema in swine. The fumonisins are weak carcinogens in rodents and probable human carcinogens that have been associated with esophageal cancer in South Africa and China. The level of fumonisin contamination in corn was relatively high in the U.S. between 1988 and 1991, but has been low in recent years.

Only a few of the nearly 200 trichothecenes occur at concentrations high enough to pose significant threats to human health. The most prevalent of these is deoxynivalenol (DON), also known as vomitoxin. DON occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and it is immunotoxic in animal models. It is not a known carcinogen and its major symptom in animals is reduced feed intake. Large amounts of grain with vomitoxin would have to be consumed to pose a health risk to humans. Type A trichothecenes like T-2 toxin or HT-2 toxin are more toxic to mammals than type B trichothecenes such as DON, but fortunately often occur in lower concentrations. Oats are the most prone cereals for contamination by trichothecenes, followed by barley and maize.

Zearalenone is an estrogenic compound found almost entirely in grains that has received recent focus due to concerns that environmental estrogens can disrupt sex steroid hormone functions. In fact, occasional outbreaks of zearalenone mycotoxicosis in livestock have caused infertility. Zearalenone has also been reported to have genotoxic activity.

Regulating Levels in Food and Feed

Limiting mycotoxin exposure to humans and agricultural animals is paramount, and more than 100 countries regulate levels of mycotoxins in foods and feed because of their public health significance and commercial impact. The U.S. FDA has established advisory levels for DON and fumonisins and action levels for aflatoxin, but regulatory limits have not been established in the U.S. for mycotoxins. China, Brazil, and Mexico have the most comprehensive legislation on aflatoxin. China and Russia have established limits for ochratoxins in cereals and other products. Several countries, including India and Japan, have maximum limits for DON. However, in the international markets, no maximum limits for fuminisins exist in several countries, including Russia, Canada, and many Latin American countries. Several countries do have maximum limits for zearalenone.

The European Union (EU) has comprehensive regulations that are referenced by several other countries for establishment of their own limits. Commission Regulation (EC) No. 1881/2006 and its amendments set out specific rules in relation to mycotoxins and other contaminants. It includes specific maximum levels for 11 mycotoxins, including aflatoxins, ochratoxin A, type A and B trichothecenes, fumonisins, and zearalenone. This regulation applies to all food business operators involved, for example, in the import, production, processing, storage, distribution, and sale of food.

Efficient Testing

Most traditional methods for the determination of mycotoxins in food or feed have been single-analyte methods, and few of them used liquid chromatography

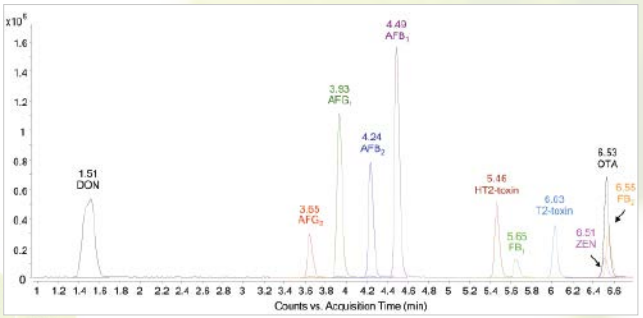

Figure 1: Chromatogram of a calibration sample containing all 11 EU regulated mycotoxins illustrating the separation efficiency of the UHPLC method run on an Agilent 1290 Infinity LC system and an Agilent 6490 Triple Quadrupole LC/MS.

coupled to tandem mass spectrometry (LC/MS/MS) until a few years ago. However, tandem mass spectrometry is a powerful tool capable of accurately detecting and quantitating the levels of mycotoxins that are dictated by the regulations. Several LC/MS/MS methods have been developed that enable high throughput analysis of food products for accurate and reproducible quantitation of very low levels of several mycotoxins at once. A few are presented here.

Accurate quantitation in complex food matrices can be hampered by suppression or enhancement of the analyte signal due to matrix effects during the mass spectrometry ionization process. Differences in the degree of matrix effects cannot only be expected between different commodities but, to a lesser extent, also between individual samples of one matrix type.

There are different strategies to compensate for matrix effects such as the dilution of the sample, matrix-matched calibrations, standard addition, or the use of internal standards. For busy routine testing laboratories, the use of internal standards which behave exactly like the target compounds but are still distinctive, is most attractive. In the past, internal standards have often been analogs of a single compound or group of compounds. However, this has limited value when the intention is to compensate for matrix effects, since such effects are retention time dependent and target compounds rarely elute concurrent with such analogs.

Stable isotopically-labeled compounds are ideally suited as internal standards since they share the same physicochemical properties (meaning they elute together with the target compound) but are still distinguishable by MS due to their different molecular mass. In addition, they are not present in naturally contaminated samples. Since the naturally abundant isotopic distribution of the analyte is diluted by the addition of stable isotopically labeled compounds, this procedure is often referred to as stable isotope dilution assay (SIDA).

A SIDA LC/MS/MS assay has been developed for the analysis of the 11 mycotoxins regulated by the EU in maize. To assure accurate quantitation, a uniformly (^{13}C)-labeled homolog for each target analyte was used as the internal standard (Figure 1). A two-step extraction without further cleanup was combined with ultra high performance liquid chromatography (UHPLC) separation and highly sensitive MS/MS detection using Dynamic Multiple Reaction Monitoring (dMRM). This method was successfully validated for maize based on method performance parameters

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including linearity of response, the limit of quantitation (LOQ) based on the signal-to-noise (S/N) ratio, and repeatability. The accuracy and reliability of the method were proven by analyzing several test materials with well-characterized concentrations. The key benefits of this method are the simple and complete extraction, the improved accuracy for a wide variety of matrices enabled by efficient compensation of all matrix effects, and high sensitivity.

Providing feed to cows that is contaminated with mycotoxins can result in the contamination of products processed from their milk, including infant formula. The EU regulation for the presence of mycotoxins in formula is quite stringent, limiting the maximum concentrations of aflatoxin M_1, aflatoxin B_1 and ochratoxin A, for example, to 0.025, 0.1, and 0.5 microgram/kilogram, respectively. Most current methods for this analysis involve labor intensive and time consuming sample purification and concentration steps required to achieve these detection levels using liquid chromatography with fluorescence detection or LC/MS.

A UHPLC/MS/MS assay for the EU regulated mycotoxins in baby formula has been developed that uses a simple extraction without a concentration step to attain the sub-part per billion detection limits required by the regulation. This method utilizes triggered MRM acquisition (tMRM) for ultimate confidence in the identification of the mycotoxins. Pre-selected MRM transitions trigger the collection of additional MS/MS transitions, each with optimized collision energy and maximized dwell time to enable the highest sensitivity. The collected ions are formulated into a spectrum, which is compared to a triggered MRM library spectrum for confirmation. This method enables the detection of the regulated mycotoxins in infant formula at levels below the maximum allowable limits, as is demonstrated by the results for aflatoxin M_1, which is typically associated with mycotoxin contamination of milk (Figure 2). In addition to the ideal sensitivity and precision of the method, its key benefit is the high confidence in the result due to the availability of high quality spectra down to very low concentration levels, which is only possible with triggered MRM.

Expanding Detection Capabilities

A method for the analysis of mycotoxins in nuts exploits the power of UHPLC and tandem mass spectrometry by enabling the detection and semi-quantitation of 191 mycotoxins and other fungal metabolites, in just two chromatographic runs per sample. UHPLC allows better separation of the analytes from the matrix, when compared to other LC/MS/MS methods, and the overall repeatability is superior to other published methods. This method features fast and easy sample preparation that includes only a single extraction step before injection of the diluted raw extract into the UHPLC/MS/MS. The multiplex analysis capability of the method enables a throughput of 25 samples per day.

This method has been utilized to survey 53 different nut samples for the presence of the 191 fungal compounds (Figure 3). The importance of using multi-mycotoxin methods was demonstrated by the detection of 40 different analytes in the nut samples. The key benefit of this method is the ability to detect mycotoxins in unlikely matrices. By applying comprehensive screening methods, the availability of occurrence data is greatly improved. In addition, this method is a good repository of MRM transitions for method extension of, for example, one of the two methods mentioned previously.

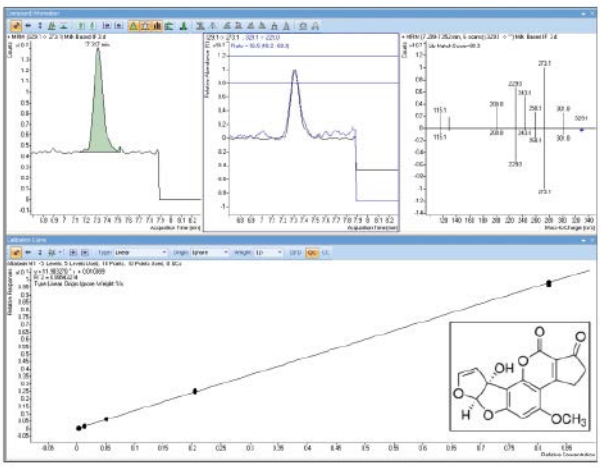

Figure 2: Extracted quantifier ion peak, qualifier to quantifier ion ratios, triggered spectra library matching (upper panel) and calibration curve and structure for aflatoxin M1 (lower panel), using the UHPLC/MS/MS method for infant formula. An Agilent 1290 Infinity LC system and Agilent 6490 Triple Quadrupole LC/MS with triggered MRM was used.

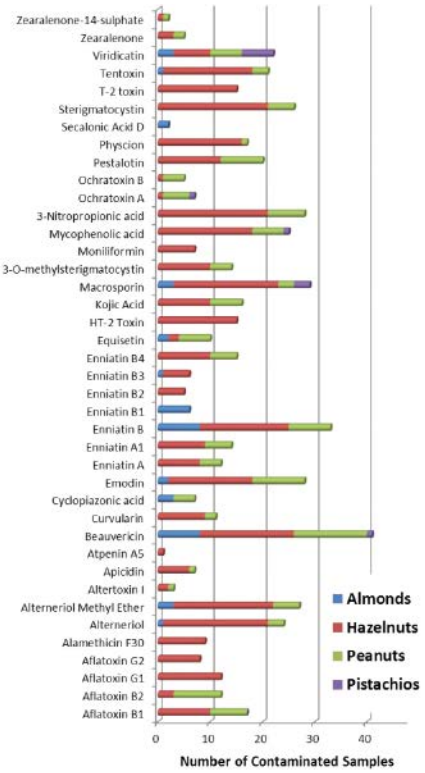

Figure 3: Forty analytes could be identified in different kinds of nut samples. The chart shows the number of each kind of nut sample that contained the given fungal compound. An Agilent 1290 Infinity LC system and Agilent 6460 Triple Quadrupole LC/MS was used.

Although aflatoxins are the only mycotoxins regulated in nuts in the EU, these results suggested that other toxins may also be relevant. Major mycotoxins found in more than 50 percent of the samples were beauvericin, enniatin B, macrosporin, 3-nitropropionic acid, emodin, and alternariol methyl ether. These results also confirmed for the first time the presence of HT-2 and T-2 toxins in hazelnuts. Analysis of such a large number of fungal toxins might be useful in the future since possible toxic effects on humans are still not fully evaluated and additive or synergistic effects of such toxins are largely unknown. ■

Dr. Glauner is a senior LC/MS applications scientist for Agilent Technologies, Inc.,Waldbronn, Germany. Reach him at thomas_glauner@agilent.com.

In The Lab

Antibiotics in Vegetables

Using QuEChERS and liquid chromatography mass spectrometry to detect antibiotics | BY XIAOWEI LIU

A study analyzing 60 samples of vegetables obtained from local markets in China, including cabbage, cucumber, cauliflower, leek, and other commonly consumed vegetables, found that a 33 percent of the samples contained detectable levels of antibiotics (*Food Analytical Methods* 2018;11:2857–2864). The vegetables are likely to have absorbed the antibiotics from soil contaminated by antibiotics.

Antibiotics are still routinely added to animal feed to prevent or treat microbial infections, as well as promote animal growth in livestock production. Most (50 to 90 percent) antibiotics and their primary metabolites are rapidly excreted and ultimately end up in sewage and manure. Some of this is then spread on agricultural fields as fertilizer for growing crops. Vegetables elsewhere, including corn, potatoes, and lettuce, have also been found to contain antibiotic residues. Worryingly, there are currently no regulations to check and monitor for antibiotics in food products. Moreover, antibiotics have been detected in groundwater leading to concerns over their entry into food chain. Antibiotic residue levels should be monitored in fertilizer, the soil, and vegetables for risk assessment and control (*Environ Pollut*. 2006;143:565–571, *Scientific American*. January 2006).

Analysis

Despite efforts to curtail the use of antibiotics in the era of antibiotic-resistant microorganisms, antibiotics are still widely used to treat human and animal diseases. Antibiotic resistance poses a global threat to public health; antibiotic resistance is responsible for 25,000 annual deaths in the European Union and 23,000 annual deaths in the U.S. There are numerous causes of antibiotic resistance, including over-prescribing, patients not taking antibiotics as prescribed, poor infection control in hospitals, poor hygiene and sanitation practices, lack of rapid laboratory tests, and unnecessary antibiotic use in agriculture.

The analysis to detect the antibiotics in the vegetables used a novel highly sensitive method devised to detect 49 target antibiotics, which fall into different classes, including sulfonamides, quinolones, macrolides, beta-lactams, and tetracyclines. Of these 49 antibiotics, five were most commonly detected across 20 samples: oxytetracycline, doxycycline, sulfamethoxazole, enrofloxacin, and chlortetracycline.

The highest concentration was of oxytetracycline in cabbage, found to be 126 µg/kg and roughly 1% of the usual daily dose (1000 mg) for an adult. While this does not sound like much, it could become substantial if exposure is chronic. Oxytetracycline is a broad-spectrum antibiotic and is associated with gastrointestinal and skin-sensitivity side effects. It is contraindicated in pregnancy because it can cross the placenta and may have toxic effects on fetal tissues (*Natl Health Stat Report*. 2018;122:1–16). Although lower compared with the oxytetracycline, doxycycline, sulfamethoxazole, enrofloxacin, and chlortetracycline were also detected, at concentra-

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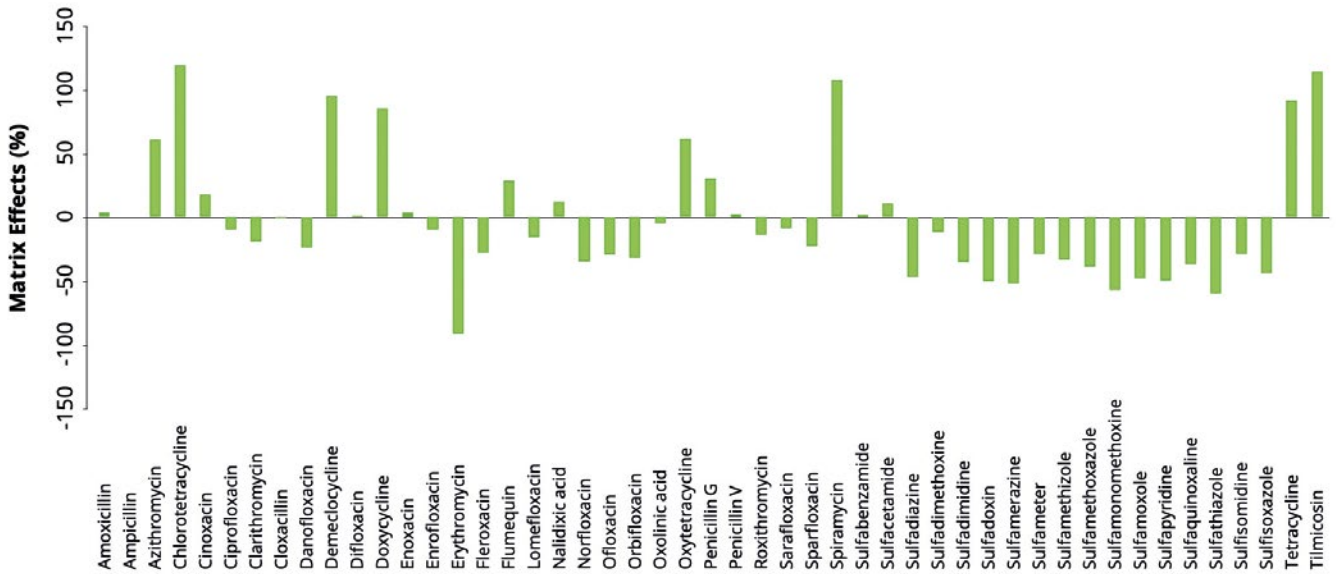

Figure 1: Matrix effects of selected antibiotics in cabbage

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tions ranging between 2.0 and 12.8 µg/kg in the vegetables (*Food Analytical Methods* 2018;11:2857–2864).

Method

The method used to detect and identify this wide range of antibiotics in vegetable samples is a relatively new one, involving the quick, easy, cheap, effective, rugged, and safe (so-called QuEChERS) procedure to prepare the sample for liquid chromatography and mass spectroscopic analysis using SCIEX ExionLC and QTRAP 4500 systems (*Food Analytical Methods* 2018;11:2857–2864). The QuEChERS technique is a simple, rapid, and cost-efficient method of extracting and preparing the sample for liquid chromatography tandem mass spectrometry (LC-MS/MS) (*Annals Chem.* 2012;84(13):5677–5684). It requires less time and solvent than other methods to detect antibiotics, including solid-phase extraction (SPE) after ultrasonic, vortex, or vibration extraction. For the LC-MS/MS analysis of multiple antibiotic residues in different vegetable samples, the extraction timing and buffer system, dispersive solid-phase extraction (d-SPE) clean-up, and other parameters, such as those controlling for matrix effects, were also optimized (see Figure 1).

Along with the improved extraction procedure, the research team also optimized the LC-MS/MS technique. It is common practice to use LC to separate out the analytes in the sample, and then transfer them into a triple quadrupole-based mass spectrometer (triple-quad) to further separate and scan the discrete analytes using a multiple reaction monitoring (MRM). However, using the triple-quad approach to detect and identify multiclass antibiotics can result in type I errors (false positives) due to interferences that have MRM transition signatures that coincide with those of the antibiotics. Type II errors (false negatives) may also occur, should the antibiotic analyte be present at a very low concentration, thus producing a weak response in the second transition (*Food Analytical Methods* 2018;11:2857–2864; *Annals Chem.* 2012;84(13):5677–5684). Therefore, the team used a quadrupole linear ion trap mass spectrometer, which combines the rapid, multiple scanning functionality of a triple-quad with the sensitivity of a linear

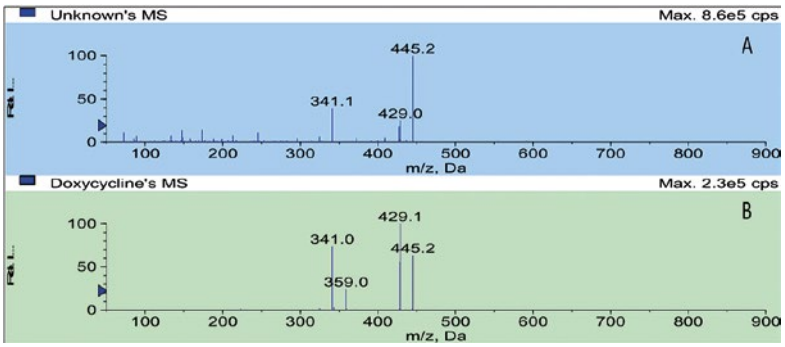

Figure 2: Doxycycline identified in sample through search against MS/MS library. A) EPI spectrum of doxycycline in vegetable; B) EPI spectrum of doxycycline standard in library.

ion trap mass spectrometer (*Food Analytical Methods* 2018;11:2857–2864; *Anal Chem.* 2007;79(24):9372–9384). With such an advanced hybrid system, the SCIEX QTRAP 4500, coupled with the SCIEX ExionLC ultra-high performance LC system, the team were able to develop and validate their method to simply and reliably detect and identify multiple antibiotic residues from different classes (*Food Analytical Methods* 2018;11:2857–2864).

The method was validated by analyzing 17 sulfonamides, 16 quinolones, 6 macrolides, 5 beta-lactams, and 5 tetracyclines, with 7 isotope-labelled internal standards for all the antibiotic classes tested. The QuEChERS-based LC-MS/MS method was confirmed to be highly accurate and precise with recoveries of 70–100 percent and reproducibility of less than 20 percent for relative standard deviation (RSD) for most of the sulfonamide, macrolide, beta-lactam, and tetracycline antibiotics. Although they are still considered acceptable at higher than the SANTE/11813/2017 guideline standard of 30 percent, the recoveries of the quinolones were lower than those of the other antibiotic classes in different vegetables. However, this was not unexpected as similar findings have been reported with both SPE and QuEChERS methods (*Food Analytical Methods* 2018;11:2857–2864). The reproducibility and thus, precision was especially good for the analyses of the macrolide and beta-lactam antibiotic residues, with RSDs that were lower than the other antibiotic classes, particularly at low concentrations of 5 µg/kg. The limit of quantification (LOQ) was 2 µg/kg for most (~74 percent) of the antibiotics tested, and 5 µg/kg for the remaining (~26 percent) residues. The method is accurate for a wide range of concentrations, with the linearity range being 1–200 µg/L. The coefficient of determination (r2) was the requisite value higher than 0.995 for each residue; which guarantees the accurate quantification of each of the 49 antibiotics through the application of this method (*Food Analytical Methods* 2018;11:2857–2864).

To confirm the accuracy of the qualitative results, the MS/MS spectra of the putative antibiotic residues in the positive samples were compared with the spectra of known target analytes housed in a reference library. This helped disqualify type I errors and confirm true positives. This final step, was facilitated by the simultaneous acquisition of the MRM scan data alongside the full scan MS/MS spectra in enhanced product ion (EPI) mode using information-dependent acquisition (IDA), which was uniquely possible with the use of the SCIEX QTRAP instruments. This final confirmatory step helps validate the utility and reliability of this method (*Food Analytical Methods* 2018;11:2857–2864).

Fulfilling a Need

According to research, antibiotic resistance may cause 10 million deaths annually by 2050 (*PLOS Medicine.* 2016;13(11):e1002184). The startling figures show that greater efforts need to be made to eliminate the injudicious application of antibiotics. Moreover, further research and understanding of the presence of antibiotics in the environment is required since antibiotics can leach from the soil into aquifers or groundwater due to run-off. All organisms—human, animal, or vegetable—are therefore susceptible to being exposed unnecessarily and unknowingly to antibiotics. As such, they can unwittingly contribute to the development of antibiotic-

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resistant bacteria and other microbes (*Scientific American*. January 2006).

Not only is there a need for better standards and regulation, there is also a need for tools such as the method described here to allow scientists, regulators, farmers, retailers and even consumers to identify antibiotics in their food. A united effort needs to be made to protect our environment as well as human and animal health, while maintaining food safety. This could include the exploration of other ways to combat bacterial infection, using innovative new technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) and the development of precision medicines (*Nature Medicine*. 2019;25:730–733). The development of our methodology, using QuEChERS and LC-MS/MS, is just one tool in the arsenal in the fight against antibiotic resistance. ■

Professor Liu is executive deputy director of the Agro-environmental Quality Supervision and Testing Center at the Agro-Environmental Protection Institute (AEPI), Ministry of Agriculture and Rural Affairs, in China.

Monitoring of 49 Pesticides and 17 Mycotoxins in Wine by QuEChERS and UHPLC–MS/MS Analysis

Jia He, Bo Zhang, Huan Zhang, Lan-Lan Hao, Teng-Zhen Ma, Jing Wang, and Shun-Yu Han

Abstract: An effective method for the determination of 49 pesticide residues and 17 mycotoxins in wine by a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) method and ultrahigh-performance liquid chromatography–tandem mass spectrometry was developed. The target compounds were extracted with 1% (v/v) formic acid–acetonitrile, and no cleanup steps were required. The extracts were separated on a C18 chromatographic column (2.1 mm × 50 mm, 1.7 μm) with acetonitrile and water with 0.2% formic acid solution and ammonium acetate (10 mM) as the mobile phases under gradient elution at a flow rate of 0.2 mL/min. The determination was conducted using electrospray ionization in positive ion mode with multiple reaction monitoring. The analytes were quantified by comparison with matrix-matched standard solutions. The good linearities were obtained in the range of 0.05 to 500.0 μg/kg, and the correlation coefficients were all greater than 0.9935. The average recoveries of the 66 target compounds ranged from 69% to 119%, and the RSDs were in the range of 1% to 10%. The limits of detection were in the range of 0.05 to 20.0 μg/kg. The method was proved to be rapid, selective, sensitive, and stable, and it has been applied to analysis of 64 wine samples.

Keywords: mycotoxins, pesticide residues, ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), wine

Introduction

Globally, wine is one of the most popular alcoholic beverages. However, with the continuous increases in people's standards of living, more consumers are focusing on health and safety. Two important classes of toxic organic compounds that could be present in wine are pesticides and mycotoxins.

Applying pesticides such as fungicides and insecticides to improve grape yields in vineyards is common practice (Carpinteiro, Ramil, Rodríguez, & Cela, 2010). As a highly processed agricultural product, the pesticides may permeate the plant tissues during fruit growth, especially before harvesting, and may ultimately be present in the processed products, such as grape juice and wine (Wang & Telepchak, 2013). When the residual amount reaches a certain level, the contaminant not only inhibits fermentation but also affects the sensory quality of the wine (A, 2016; Wang et al., 2016). In addition, such contaminants can pose certain risks to consumers' health (Li et al., 2012). To date, nearly 200 pesticide residues have been detected in grapes and wine (Han & Li, 2016).

Moreover, agricultural products used to prepare wine can also be contaminated by various fungi during growth, storage, and processing, which in turn leads to contamination by various mycotoxins. These compounds are toxic secondary metabolites produced by filamentous fungi under suitable ambient temperature and humidity conditions. At present, more than 300 kinds of mycotoxins have been reported (Chen, 2017). Most mycotoxins show high biological toxicity, can inhibit the immune system, and have adverse health effects such as carcinogenicity and teratogenicity as well as reproductive and developmental toxicities (Jestoi, 2008). Although mycotoxins are extremely dangerous, the European Union (EU) has only established limits for ochratoxin A in wine and grape juice (2 μg/kg). Similar limits have been set by the International Organization of Vine and Wine (OIV, 2008).

Despite few countries having specific regulations for hazardous substance in wine, many pesticides have been reported based on gas chromatography–mass spectrometry (GC–MS; Chen, Wu, Wu, Jin, Xie, Feng, & Ouyang, 2016; Jeancarlo & Jailson, 2015; Maja, Gorana, Dragana, & Dubravka, 2016; Wang, Yan, He, & Niu, 2016) and liquid chromatography–mass spectrometry (LC–MS) analyses (Christodoulou, Kanari, Hadjiloizou, & Constantinou, 2015; Pérez-Mayán et al., 2019; Rodríguez-Cabo, Casado, Rodríguez, Ramil, & Cela, 2016). GC–MS(/MS) with a quadrupole filter is the most common method (Pérez-Ortega, Gilbert-López, García-Reyes, Ramos-Martos, & Molina-Díaz, 2012). Ultrahigh-performance liquid chromatography (UHPLC) (Nistor et al., 2017), LC–tandem mass spectrometry (LC–MS/MS; Han, Liu, Wang, Lv, & Wang, 2013; Pizzutti et al., 2014), and direct analysis in real-time MS/MS (DRAT-MS/MS; Gong et al., 2017) have been used for the detection of mycotoxins.

Generally, GC–MS methods require a long detection time and are mostly used to detect volatile substances, such as pyrethroid pesticides (Wang et al., 2016), and such methods are not suitable for determining mycotoxins. Because of its sensitivity and wide detection range, LC–MS is the best detection method for determining multiple residues in complex matrices (Liu, 2016). Clearly, a variety of detection methods are available, but identifying a new method for the simultaneous detection of pesticide residues and mycotoxins in wine is necessary to improve the efficiency of detection, and limited research on the subject is available.

At present, there are at least three multiresidue methods for the simultaneous determination of pesticides and mycotoxins in plant matrices have been reported (Amate, Unterluggauer, Fischer, Fernández-Alba, & Masselter, 2010; Mol et al., 2008; Romero-González, Frenich, Martínez Vidal, Prestes, & Grio, 2011), and only one of these has been validated in wine (Romero-González et al., 2011). Pérez-Orteg et al. (2012) used LC–electrospray

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ionization (ESI)–time-of-flight-MS combined with solid-phase extraction to determine 60 representative pesticides and nine mycotoxins in wine; however, this method requires the use of expensive extraction columns, and the sample preparation method is cumbersome.

The aim of this work was to develop and validate a UHPLC–MS/MS method for the simultaneous identification and determination of pesticide residues and mycotoxins in wine. Multiple reaction monitoring (MRM) with multireaction detection was used. Several representative multiclass pesticides and relevant mycotoxins (50 and 17, respectively) were included in the study. Different extraction solvents and cleanup methods were optimized using a QuEChERS treatment procedure. The proposed method was simple, fast, accurate, and environmentally friendly, and it was used to analyze 64 wine samples produced in the Hexi Corridor region and in various regions in France.

Experimental

Reagents and chemicals

High-purity (>98%) analytical standards of pesticides and mycotoxins were purchased from MANHAGE (Beijing, China), Sigma-Aldrich (Steinheim, Germany) and J & K Scientific Ltd. (Beijing, China). An individual stock solution of each compound (100 mg/L) was prepared in acetonitrile or methanol and stored at −20 °C in the dark. A multicompound working standard solution (5 mg/L of each compound) was prepared by appropriately diluting the stock solutions with methanol, and the solution was stored at 4 °C in silanized screw-capped vials with solid PTFE-lined caps. According to the instrumental response values of each compound, the 67 target compounds were divided into three groups of different concentrations to prepare mixed standard solutions. Details of the groups are shown in Table 1.

HPLC-grade acetonitrile, methanol, and formic acid were obtained from Merck KGaA (Germany). Acetic acid (HPLC grade) was purchased from Dengfeng Chemical Co., Ltd. (China). Ammonium acetate, anhydrous magnesium sulfate ($MgSO_4$), and powdered sodium chloride (NaCl, > 99.0% purity) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Ultra-pure HPLC-grade water was acquired from Watsons (China).

Instrumentation and conditions

Chromatographic analyses were performed using an Agilent 1290 series UHPLC system (Agilent Technologies, Germany) equipped with a binary solvent delivery system, a degasser, an autosampler, and a column heater. UHPLC separations were performed using an Agilent C18 chromatographic column (2.1 mm × 50 mm) a with particle size of 1.7 µm. MS/MS detection was performed using an Agilent 6460 Triple Quadrupole (QqQ) mass spectrometer (Agilent Technologies) equipped with Agilent Jet Stream ion funnel technology and an ESI interface operating in positive ion mode.

A Genie 2 vortex mixer (Scientific Industries, USA) and a TE124S-balance (Sartorius, Germany) were used. Centrifugation was performed in a Sigma 3K30 centrifuge (Sigma).

Chromatographic and mass spectrometric conditions

A gradient program consisting of water with 0.2% formic acid and ammonium acetate (10 mM; eluent A) and acetonitrile (eluent B) was used. The gradient program was as follows: 0.0 min, 5% B; 2.0 min, 5% B; 5.0 min, 50% B; 15.0 min, 90% B; 18.0 min, 90% B; and 25.0 min, 5% B. The column was re-equilibrated for 6 min before the next injection. The flow rate was 0.2 mL/min, the injection volume was 5 µL, and column temperature was 30 °C.

MS/MS analyses of the pesticides and mycotoxins were performed on a 6460 QqQ mass spectrometer with Agilent Jet Stream Technology under MRM conditions in ESI+ mode. The following settings were used: nebulizer, 45 psi; drying gas temperature, 300 °C; drying gas flow rate, 10 L/min; and capillary voltage, 4,000 V. Agilent's Mass Hunter Quantitative Analysis Software (version B.07.00) was used for instrument control, data acquisition, and data processing. Moreover, the optimization in details for the fragmentation and collision energies is shown in Table 1.

QuEChERS extraction procedure

The method was based on the report by Zhang et al. (2018). Homogenized sample (5.0 g) and 5.0 g of distilled water were mixed in a 50 mL polypropylene centrifuge tube, and 10 mL of acetonitrile (containing 1% formic acid) was added. The mixture was vigorously shaken (4,367 × *g*) for 1 min at room temperature, then 1.0 g of NaCl and 4.0 g of $MgSO_4$ were sequentially added. The sample was shaken for an additional 1 min and centrifuged for 5 min (18,924 × *g*) at 10 °C, and then 3 mL of the upper organic phase was transferred into a 10-mL polypropylene tube containing 450 mg of $MgSO_4$. This mixture was shaken for a further 30 s and centrifuged for 5 min at 7,279 × *g* (10 °C). Afterward, approximately 0.5 mL of the extract was taken for analysis. Prior to UHPLC–MS/MS measurements, the extract was passed through a 0.22-µm filter (Anpel, Shanghai, China) and diluted with 0.5 mL of methanol. The sample was diluted four times in the final extracts.

A Cabernet Sauvignon red wine was selected as a blank sample. After testing, the sample contained fewer target substances to be detected. The blank sample was used for standard addition recovery test, and matrix correction solution was prepared.

Method validation

The standard working fluid was prepared by using blank wine matrix solution as solvent. Seven different concentration gradients of standard solution were selected according to Table 2. With the mass concentration X (µg/kg) of target substance as abscissa and the peak area Y of quantitative ion as ordinate, seven levels and three repeated standard curves were established in the concentration range of 0.05 to 500.00 µg/kg. The regression equation of quasi curve is used to obtain the correlation coefficient and quantify it by external standard method. Sixty-seven mixed standard solutions of 1 mg/kg were diluted and detected by instrumental method. The limit of detection (LOD) was the lowest when the signal-to-noise ratio (S/N) was equal to 3.

The accuracy and precision of the method were evaluated by standard addition recovery test. The mixed standard solution was added to the blank wine matrix at three different concentration levels (see Table 2). The results were compared with the blank samples and the recovery of 67 target compounds was calculated. The average repetition of each added concentration was three times. Operations ($n = 6$) are performed to calculate relative standard deviation (RSD).

Results and Discussion

Optimization of the QuEChERS procedure

Selection of the extraction solvent. In the established methods for analyzing multipesticide residues and mycotoxins,

Table 1–Retention times and mass spectrometric parameters for 50 pesticides and 17 mycotoxins ("GROUP" means different concentrations to prepare mixed standard solutions, see Table 2).

Number	Compound	Category	Adduct ion	Transition (*m/z*)	DP (V)	CEs (eV)	Group
1	Abamectin	P	$[M + H]^+$	890.5/305.1*, 890.5/567.4	155	8*, 0	GROUP3
2	Acetamiprid	P	$[M + H]^+$	223.1/126.0*, 223.1/90.0	80	27*, 45	GROUP1
3	Aflatoxin B1	M	$[M + H]^+$	313.0/285.2*, 313.0/241.1	130	20*, 20	GROUP1
4	Aflatoxin B2	M	$[M + H]^+$	315.1/287.1*, 315.1/269.1	130	30*, 30	GROUP1
5	Aflatoxin G1	M	$[M + H]^+$	329.1/243.1*, 329.1/311.1	130	25*, 20	GROUP1
6	Aflatoxin G2	M	$[M + H]^+$	331.1/245.1*, 331.1/217.1	130	30*, 30	GROUP1
7	Aflatoxin M1	M	$[M + H]^+$	328.9/237.0*, 328.9/228.9	165	25*, 50	GROUP2
8	Azoxystrobin	P	$[M + H]^+$	404.1/372.1*, 404.1/344.1	120	10*, 20	GROUP1
9	Benalaxyl	P	$[M + H]^+$	326.2/148.1*, 326.2/91.1	90	27*, 48	GROUP1
10	Bifenazate	P	$[M + H]^+$	301.2/198.1*, 301.2/170.1	25	14*, 28	GROUP1
11	Boscalid	P	$[M + H]^+$	343.2/307.2*, 343.2/140.1	53	29*, 31	GROUP2
12	Buprofezin	P	$[M + H]^+$	306.2/106.1*, 306.2/116.1	18	41*, 21	GROUP1
13	Carbaryl	P	$[M + H]^+$	202.1/144.9*, 202.1/127.0	60	5*, 30	GROUP1
14	Carbendazim	P	$[M + H]^+$	192.1/160.0*, 192.1/131.9	102	15*, 30	GROUP1
15	Carbofuran	P	$[M + H]^+$	222.1/123.1*, 222.1/165.1	80	20*, 20	GROUP1
16	Chlorophos	P	$[M + H]^+$	256.9/109.0*, 256.9/221.0	107	18*, 6	GROUP2
17	Citrinin	M	$[M + H]^+$	250.9/233.0*, 250.9/205.0	80	15*, 25	GROUP1
18	Clofentezine	P	$[M + H]^+$	303.1/137.9*, 303.1/101.9	85	15*, 40	GROUP2
19	Demeton	P	$[M + H]^+$	259.1/89.0*,259.1/61.0	28	25*, 23	GROUP1
20	Deoxynivalenol	M	$[M + H]^+$	297.2/281.0*, 297.2/249.1	120	5*, 30	GROUP1
21	Diacetoxyscirpenol	M	$[M + NH_4]^+$	384.0/307.0*, 384.0/247.0	65	8*, 10	GROUP2
22	Diafenthiuron	P	$[M + H]^+$	385.0/329.2*, 385.0/278.2	140	15*, 35	GROUP2
23	Diethofencarb	P	$[M + H]^+$	268.1/152.1*, 268.1/180.2	41	24*, 14	GROUP1
24	Dimethoate	P	$[M + H]^+$	230.0/124.9*, 230.0/79.0	72	21*, 37	GROUP2
25	Dimethomorph	P	$[M + H]^+$	388.1/301.1*, 388.1/165.1	145	20*, 32	GROUP2
26	Diquat dibromide	P	$[M + H]^+$	217.2/55.1*, 217.2/82.8	105	40*, 30	GROUP1
27	Emamectin benzoate	P	$[M + H]^+$	886.5/158.0*, 886.5/82.1	190	40*, 60	GROUP1
28	Fenpropidin	P	$[M + H]^+$	274.0/147.2*, 274.0/117.1	140	25*, 65	GROUP1
29	Fenthion	P	$[M + H]^+$	279.0/169.1*, 279.0/105.0	125	15*, 25	GROUP3
30	Flusilazole	P	$[M + H]^+$	316.0/165.0*, 316.0/246.9	130	30*, 15	GROUP1
31	Fumonisin B1	M	$[M + H]^+$	722.5/352.3*, 722.5/334.1	142	35*, 45	GROUP3
32	Haloxyfop-methyl	P	$[M + H]^+$	376.1/91.0*, 376.1/316.0	115	35*, 20	GROUP1
33	Hexythiazox	P	$[M + H]^+$	353.1/168.1*, 353.1/228.1	50	35*, 21	GROUP1
34	HT-2 toxin	M	$[M + Na]^+$	447.2/345.1*, 447.2/285.1	135	15*, 18	GROUP3
35	Imazalil	P	$[M + H]^+$	297.0/159.0*, 297.0/255.0	45	36*, 25	GROUP2
36	Imidacloprid	P	$[M + H]^+$	256.0/208.9*, 256.0/175.0	80	12*, 12	GROUP2
37	Indoxacarb	P	$[M + H]^+$	528.0/249.0*, 528.0/218.0	45	24*, 32	GROUP2
38	Isazophos	P	$[M + H]^+$	314.1/120.0*, 314.1/162.1	47	41*, 21	GROUP1
39	Isoprothiolane	P	$[M + H]^+$	291.1/188.9*, 291.1/145.0	65	18*, 40	GROUP1
40	Malathion	P	$[M + H]^+$	330.9/98.9*, 330.9/126.9	65	22*, 10	GROUP1
41	Metalaxyl	P	$[M + H]^+$	280.1/220.1*, 280.1/192.1	85	10*, 15	GROUP1
42	Methomyl	P	$[M + H]^+$	163.1/88.0*, 163.1/106.0	50	0*, 4	GROUP2
43	Myclobutanil	P	$[M + H]^+$	289.1/70.2*, 289.1/125.0	126	16*, 38	GROUP1
44	Mycophenolic acid	M	$[M + NH_4]^+$	337.1/196.0*, 337.1/182.0	125	20*, 15	GROUP2
45	Neosolaniol	M	$[M + NH_4]^+$	400.0/185.0*, 400.0/305.0	95	15*, 10	GROUP1
46	Novaluron	P	$[M + H]^+$	493.0/158.1*, 493.0/141.1	90	15*, 55	GROUP3
47	Ochratoxin A	M	$[M + H]^+$	404.0/238.9*, 404.0/357.9	127	20*, 10	GROUP2
48	Ochratoxin B	M	$[M + H]^+$	369.9/205.0*,369.9/324.0	115	20*, 10	GROUP2
49	Omethoate	P	$[M + H]^+$	214.1/ 124.9*, 214.1/109.0	70	30*, 20	GROUP2
50	Oxamyl	P	$[M + NH_4]^+$	237.1/72.0*, 237.1/90.0	60	12*, 0	GROUP1
51	Paclobutrazol	P	$[M + H]^+$	294.1/70.1*, 294.1/57.2	115	16*, 20	GROUP2
52	Phosalone	P	$[M + H]^+$	367.9/181.9*, 367.9/110.9	76	12*, 40	GROUP2
53	Pirimicarb	P	$[M + H]^+$	239.2/72.0*, 239.2/182.1	100	25*, 10	GROUP1
54	Propiconazole	P	$[M + H]^+$	342.0/158.9*, 342.0/69.1	135	32*, 15	GROUP1
55	Propineb	P	$[M + H]^+$	316.1/165.1*, 316.1/247.1	31	40*, 23	GROUP1
56	Pyridaben	P	$[M + H]^+$	365.1/147.1*, 365.1/309.2	107	22*, 6	GROUP1
57	Pyrimethanil	P	$[M + H]^+$	200.2/82.0*, 200.2/107.0	125	25*, 25	GROUP1
58	Sterigmatocystin	M	$[M + H]^+$	325.0/280.9*, 325.0/252.9	135	35*, 55	GROUP1
59	Sulfotep	P	$[M + H]^+$	323.0/97.0*, 323.0/114.9	132	38*, 30	GROUP1
60	T-2 toxin	M	$[M + NH_4]^+$	484.1/215.0*, 484.1/305.0	115	18*, 10	GROUP2
61	Tebuconazole	P	$[M + H]^+$	308.2/125.0*, 308.2/151.0	125	40*, 23	GROUP2
62	Tebufenozide	P	$[M + H]^+$	353.3/133.1*, 353.3/297.2	28	28*, 11	GROUP1
63	Thiamethoxam	P	$[M + H]^+$	292.03/211.1*, 292.03/181.1	85	8*, 20	GROUP1
64	Thiophanate-methyl	P	$[M + H]^+$	343.1/151.1*, 343.1/93.1	100	16*, 60	GROUP1
65	Triadimefon	P	$[M + H]^+$	294.2/69.1*, 294.2/197.1	100	20*, 12	GROUP2
66	Tricyclazole	P	$[M + H]^+$	190.1/135.9*, 190.1/163.0	75	30*, 20	GROUP1
67	Zearalenone	M	$[M + H]^+$	319.0/187.1*, 319.0/184.9	70	25*, 40	GROUP3

*Quantification transition.
DP, declustering potential; CE, collision energy; P, pesticides; M, mycotoxins.

Table 2–Concentrations of calibration solutions for linearity study and selected levels for recovery study.

	Concentrations of calibration solutions (µg/kg)									
Compounds group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Spiking levels (µg/kg)**		
Group 1	0.05	0.1	0.2	0.5	1	2	5	1	3	5
Group 2	0.5	1	2	5	10	20	50	10	30	50
Group 3	5	10	20	50	100	200	500	100	300	500

dimethoate, carbendazim, fenthion, haloxyfop-methyl (Wang & Cheung, 2016), aflatoxin, and ochratoxin (Jestoi, 2008) were extracted with acetonitrile. Due to the mutual solubility of methanol and water, extraction with methanol and acidic aqueous solutions precludes the use of salting out during the sample preparation, which is not conducive to subsequent extraction, cleanup, and other steps; thus, methanol extraction is not used.

The extraction of target compounds from wine samples may be more effective at low pH (Pizzutti et al., 2014). This test compared five extraction solvents with different acidities ([1] acetonitrile, [2] 0.5% [v/v] formic acid in acetonitrile, [3] 1% [v/v] formic acid in acetonitrile, [4] 2% [v/v] formic acid in acetonitrile, and [5] 3% [v/v] formic acid in acetonitrile) for 67 target compounds to determine the effect of the extractant. The sample preparation was carried out at addition levels of 1, 10, and 100 µg/kg (Table 2) according to the method described in QuEChERS extraction procedure section. The recovery and matrix effect distributions of 49 of the pesticide residues and the 17 mycotoxins are shown in Figure 1. Abamectin is not very sensitive compared to other pesticides using ESI(+)–MS/MS, probably due to the unique structure of abamectin, which causes it to be retained and not elute cleanly, resulting in peak splitting and very low signal intensity due to strong ion suppression. The problems with abamectin relate to its adduct formation with NH_4^+ and Na^+, plus its $[M+H]^+$ precursors. It needs to be forced to one precursor, and higher source temperature and ion spray voltage are needed to yield better results for it. Also, it co-elutes with fatty acids, which cause more matrix effects. When pure acetonitrile was used as the extraction solvent, the recovery (76%) of the compound met the requirements, and 58% of the compound was masked by a weak matrix effect; abamectin could be extracted with solutions of 0.5%, 2%, or 3% (v/v) formic acid in acetonitrile. The standard of this compound showed recovery between 69% and 82% and weak matrix effects between 60% and 73%. Abamectin was extracted with 1% (v/v) formic acid in acetonitrile from the 66 wine samples. Of the tested pesticides and mycotoxins, 63 showed recoveries ranging from 70% to 120%, and 48 showed matrix effects ranging from –20% to 20%, which generally meet the requirements. Therefore, we selected acetonitrile with 1% (v/v) formic acid as the extractant.

Selection of the cleanup procedure. Removal of the matrix interferences is the key to determining pesticide residues and mycotoxins in wine matrices, and optimizing the conditions of the dispersed solid-phase extraction substrate to achieve a lower matrix effect is highly desirable. C18 adsorbs nonpolar components, which can disrupt strong hydrophobic interferences, such as fats and organic acids in the matrix (Lan, Lin, Liu, Wang, & Cao, 2018). PSA (Primary Secondary Amine) can adsorb and remove polar interferences, which can effectively eliminate oils, including sugars, fatty acids, organic acids, and anthocyanin-based pigments (Chen, 2017). Mixtures of C18 and PSA can effectively remove matrix interferences with strong to weak polarities (Wang, Liu, Sun, Du, & Xu, 2018). GCB (Carbon SPE Bulk Sorbent) has a high adsorption capacity for organic compounds containing benzene rings, allowing it to effectively separate compounds and remove most pigments (chlorophyll and carotenoids) and sterols (Chu, Meng, Kang, Tang, & Yang, 2016). However, the disadvantage of GCB is that some target analytes are also lost due to irreversible adsorption (Pizzutti et al., 2014).

In this experiment, the cleanup effects of the three kinds of dispersed adsorbents and their combinations ([1] C18, [2] GCB, [3] PSA, [4] C18+GCB, [5] C18+PSA, and [6] GCB+PSA) were investigated by using the recovery and the matrix effects as indicators; the extraction with no dispersed adsorbent was used as the control. Sample preparation was carried out at addition levels of 1, 10, and 100 µg/kg (Table 2) according to the method described in "QuEChERS extraction procedure" section. The above three dispersed adsorbents and their combinations were used for cleanup, and the obtained recoveries and matrix effect distributions are shown in Figure 2.

The best effects were achieved by using PSA alone. Of the tested combinations, the 74% recovery obtained from the spiked sample met the requirements. When using C18 and GCB, the recovery, which is required to be between 70% and 120% of target, was 73% and 62%, respectively. When a combination of all three dispersed adsorbents was used, the matrix effect is more obvious. When using a combination of C18+GCB, some compounds (such as carbaryl and tebufenozide) are almost completely lost. The recovery of AFM1 and AFG1 was only 30% and 43%, respectively; thus, the recoveries obtained for these compounds do not meet the requirements either. When C18+PSA and GCB+PSA were used, only 55% and 58%, respectively, of the obtained target recovery meet the requirements, and FB1 was almost completely lost. When the dSPE cleanup step was omitted, the recoveries of the majority (79%) of the pesticides and mycotoxins remained within the acceptable range. Therefore, for the economical reason and to maximize operational simplicity, no cleanup step was used in further experiments. Similar results were reported by Pizzutti et al. (2014) and Chen (2017).

Optimization of the chromatographic conditions

Because in electrospray MS the ionization is carried out in solution, the composition and additives in the mobile phase not only affect the chromatographic retention time and peak shape of the analyte but also affect the ionization of the analyte, in turn impacting the sensitivity of the instrument for the target analyte.

In this experiment, acetonitrile and methanol were used as the organic components of the mobile phase, and the mixed standard solution of pesticides and mycotoxins was analyzed. When methanol was used as the organic phase, the resolution of most of the targets was poor, and the peak shapes were poor (short and split); however, no such phenomenon was observed when acetonitrile was selected as the organic phase.

MS requires that the mobile phase be volatile. To achieve optimal chromatographic separation and peak shape, an acid (formic acid) is commonly added to the mobile phase. In addition, using a small amount of formic acid as an additive in the mobile phase

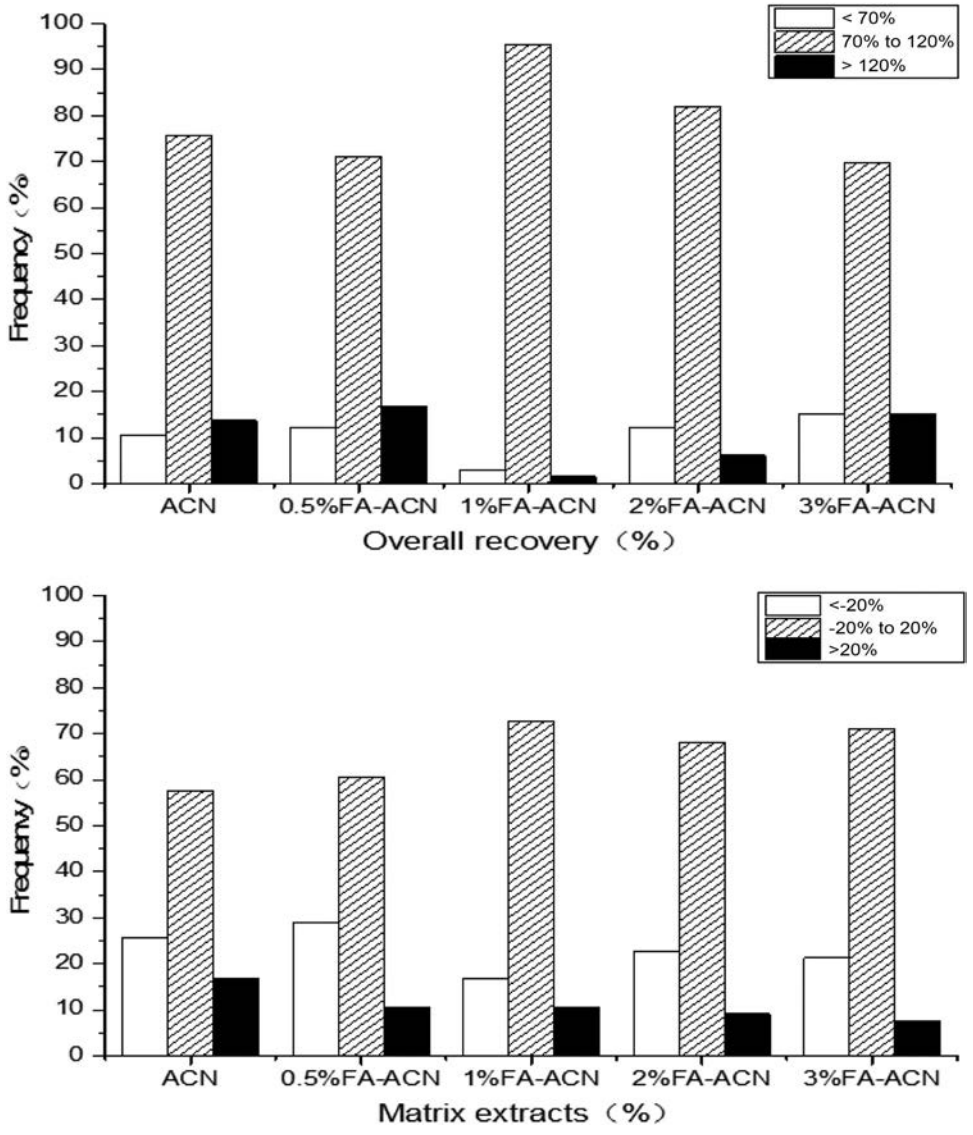

Figure 1–Recoveries and matrix effects of analytes cleaned-up using different extractants.

can reduce the residual silanol activity of the column (Wang et al., 2016). Good peak shapes were observed for the basic compounds, and the analysis was carried out in positive ionization mode. The ionic strength and the pH of the mobile phase can affect both ionization efficiency and chromatographic separation (Carpinteiro et al., 2010). Therefore, we investigated the addition of 0.1% and 0.2% formic acid to the aqueous solution. The higher concentration of formic acid provided better peak shapes (sharp and symmetrical) for most analytes, but some problems remained. For example, pesticides such as carbendazim and omethoate showed premature peaks, and neosolaniol showed substantial tailing. Various salts (ammonium acetate) are commonly used additives in LC–MS to enhance the signal and change the peak shape. After adjusting the flow, all the analytes were better resolved. Therefore, acetonitrile and water with 0.2% formic acid and ammonium acetate (10 mM) were finally selected as the mobile phase.

Optimization of the MS/MS conditions

The optimization of the MS conditions mainly includes the selection of the precursor ion, product ions, fragment ion, and collision energy. First, the standard solution (100 μg/kg) of each pesticide and mycotoxin was directly analyzed by single-needle autoinjection without a column. Positive and negative ESI modes were tested; however, all the compounds were only ionized in a significant extent in positive mode, which is consistent with their basicities. The capillary voltage was fixed at the maximum intensity of the precursor ion $[M + H]^+$, and the molecular ion peak of the target was obtained by first-order MS scanning. The precursor ion of the analyte and the fragment ions were optimized. Then, the molecular ions enter the secondary MS instrument and undergo cracking, rearrangement, or other cleavage reactions to produce fragments ions with different m/z values, and the fragment ions were obtained by scanning the product ions to optimize the collision energy and determine the transitions. The collision energy for the most intense product ions was also optimized. Once the main MS/MS transitions were identified for each compound, their fragmentation patterns were investigated. Finally, the MS parameters were optimized under MRM mode. The results of the optimization tests are shown in Table 1, and the total ion chromatogram is shown in Figure 3.

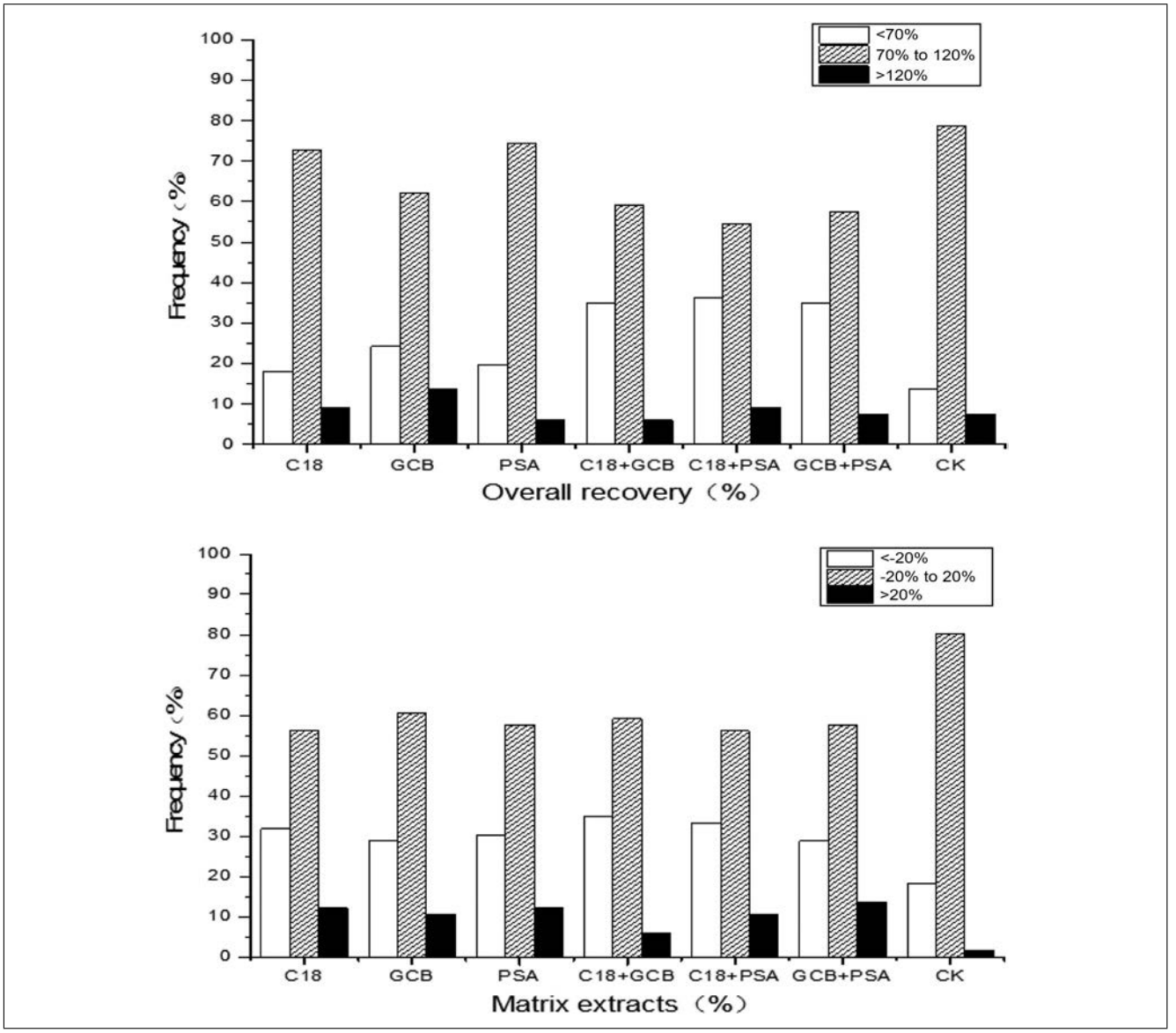

Figure 2–Recovery and matrix effect distributions of 49 of the pesticide residues and the 17 mycotoxins in wine with extraction sorbents with different acidities.

Evaluation of the matrix effects

Matrix effects refer to the effects of the components in the matrix that cannot be detected themselves but influence the analyte response (Pizzutti et al., 2014). In the wine samples, compounds extracted with the pesticide residues and mycotoxins, including fatty acids, esters, alcohols, and sugars, can attenuate or increase the detection responses of the analytes. These effects are difficult or even impossible to eliminate during ionization (Banerjee et al., 2007). The presence of matrix components can have a substantial impact on the ionization of target compounds when ESI is used. This may be due to competition between the analyte and other components available with the same charge, resulting in signal suppression, or components in the matrix affecting the release of ions from the electrospray droplets (Pérez-Orteg et al., 2012). Therefore, to reduce the effects of ion suppression on the quantitative determination, preparing a series of matrix-matched standard working solutions with the extract of a blank wine sample and preparing a standard curve for quantification are of great importance.

Method validation

The standard working solution was prepared by using blank wine substrate solution as the solvent. As shown in Table 2, standard solutions with seven different concentration gradients were selected and determined according to the conditions established in "Chromatographic and mass spectrometric conditions" section. The correlation coefficient (R^2) of the calibration curve of each pesticide and mycotoxin was ≥0.9935. The results are presented in detail in Table 3; refer to Table 2 for spiking level concentrations for each pesticide or mycotoxin.

The recoveries and RSDs were calculated by evaluating blank samples ($n = 6$) spiked at three different concentrations (as shown in Table 2). From Table 3, all 49 pesticides and 17 mycotoxins

Table 3–Recoveries, relative standard deviations (RSDs), and limits of detection (LODs) of 49 pesticides and 17 mycotoxins ($n = 6$).

			Spiking level 1		Spiking level 2		Spiking level 3	
Compounds	R^2	LOD (µg/L)	Rev%	RSD%	Rev%	RSD%	Rev%	RSD%
Acetamiprid	0.9991	0.2	86	2	78	5	82	6
Aflatoxin B1	0.9995	0.1	71	2	71	1	77	1
Aflatoxin B2	0.9998	0.25	89	6	112	4	110	3
Aflatoxin G1	0.9989	0.1	114	4	82	6	84	4
Aflatoxin G2	0.9995	0.25	99	17	111	3	107	10
Aflatoxin M1	0.9973	0.5	69	5	67	3	70	5
Azoxystrobin	0.9992	0.05	99	6	98	11	95	4
Benalaxyl	0.9990	0.05	83	8	111	8	101	3
Bifenazate	0.9986	0.2	95	13	97	14	97	3
Boscalid	0.9994	0.5	101	10	103	8	97	5
Buprofezin	0.9995	0.2	88	6	103	6	102	5
Carbaryl	0.9988	0.1	73	1	86	9	98	4
Carbendazim	0.9884	0.2	100	3	109	1	96	6
Carbofuran	0.9991	0.2	106	10	97	4	101	3
Chlorophos	0.9995	0.5	92	9	109	5	85	6
Citrinin	0.9992	0.2	110	6	105	5	93	5
Clofentezine	0.9994	0.5	86	7	89	4	99	6
Demeton	0.9983	0.2	106	9	115	3	86	1
Deoxynivalenol	0.9989	0.05	96	3	108	9	95	1
Diacetoxyscirpenol	0.9996	0.5	96	2	108	5	96	7
Diafenthiuron	0.9971	0.5	82	9	93	6	94	5
Diethofencarb	0.9994	0.2	89	7	82	1	89	2
Dimethoate	0.9994	0.5	103	3	97	3	100	3
Dimethomorph	0.9995	0.5	128	4	114	7	114	4
Diquat dibromide	0.9995	0.05	97	15	91	2	98	2
Emamectin benzoate	0.9991	0.1	114	9	106	12	100	8
Fenpropidin	0.9991	0.1	104	9	99	2	98	2
Fenthion	0.9991	10	72	3	71	2	90	3
Flusilazole	0.9987	0.05	101	5	107	7	108	2
Fumonisin B1	0.9935	10	109	8	121	7	122	5
Haloxyfop-methyl	0.9994	0.05	121	11	115	3	116	2
Hexythiazox	0.9993	0.2	86	9	90	2	116	2
HT-2 toxin	0.9996	5	100	1	88	10	107	6
Imazalil	0.9994	0.5	103	4	108	3	106	4
Imidacloprid	0.9995	0.5	89	1	106	7	94	5
Indoxacarb	0.9993	2	83	7	93	5	92	2
Isazophos	0.9991	0.1	87	6	89	7	86	14
Isoprothiolane	0.9991	0.05	102	11	101	7	99	6
Malathion	0.9992	0.2	104	11	99	6	102	6
Metalaxyl	0.9994	0.05	93	17	111	5	101	2
Methomyl	0.9971	0.5	111	1	112	3	107	3
Myclobutanil	0.9997	0.2	108	5	114	4	117	3
Mycophenolic acid	0.9983	2	100	5	102	9	115	4
Neosolaniol	0.9947	0.2	112	7	105	5	109	4
Novaluron	0.9994	10	105	10	108	6	110	6
Ochratoxin A	0.9996	1	101	14	111	5	110	9
Ochratoxin B	0.9997	0.5	117	1	110	5	113	2
Omethoate	0.9996	0.5	92	2	83	13	106	6
Oxamyl	0.9981	0.1	76	10	113	3	79	6
Paclobutrazol	0.9973	0.5	119	1	118	1	114	1
Phosalone	0.9995	0.5	84	4	106	7	94	10
Pirimicarb	0.9992	0.05	90	12	88	3	87	6
Propiconazole	0.9990	0.2	94	14	104	11	104	5
Propineb	0.9991	0.1	110	4	111	15	112	2
Pyridaben	0.9995	0.1	111	5	98	1	92	6
Pyrimethanil	0.9991	0.2	107	3	106	3	77	6
Sterigmatocystin	0.9987	0.2	109	18	111	1	104	10
Sulfotep	0.9991	0.2	110	9	115	4	103	2
T-2 toxin	0.9993	0.5	87	9	97	10	90	10
Tebuconazole	0.9995	1	117	2	119	1	111	4
Tebufenozide	0.9971	0.05	115	2	111	6	111	4
Thiamethoxam	0.9951	0.05	104	3	110	5	102	3
Thiophanate-methyl	0.9991	0.2	113	3	97	8	110	6
Triadimefon	0.9998	0.5	88	12	89	3	84	1
Tricyclazole	0.9992	0.05	98	13	83	1	81	5
Zearalenone	0.9995	20	73	5	76	3	72	0

Rev%, recovery (%).

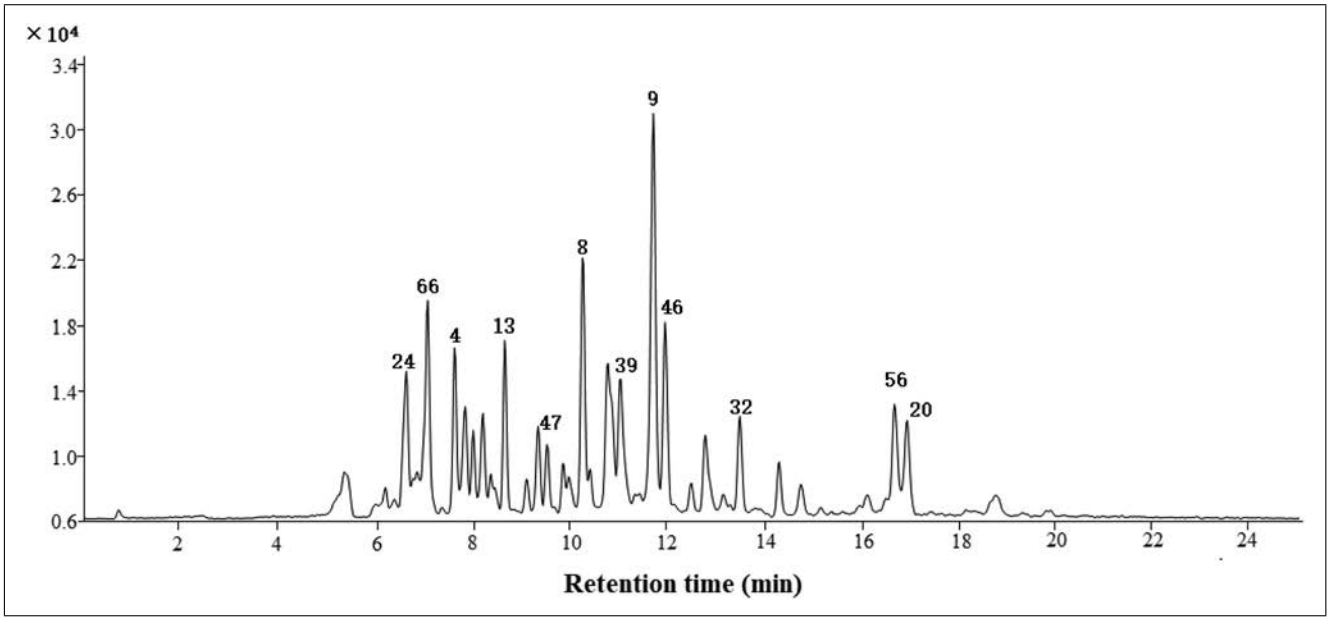

Figure 3–Total ion chromatogram of a mixed standard solution of the 50 pesticides and 17 mycotoxins in MRM mode.

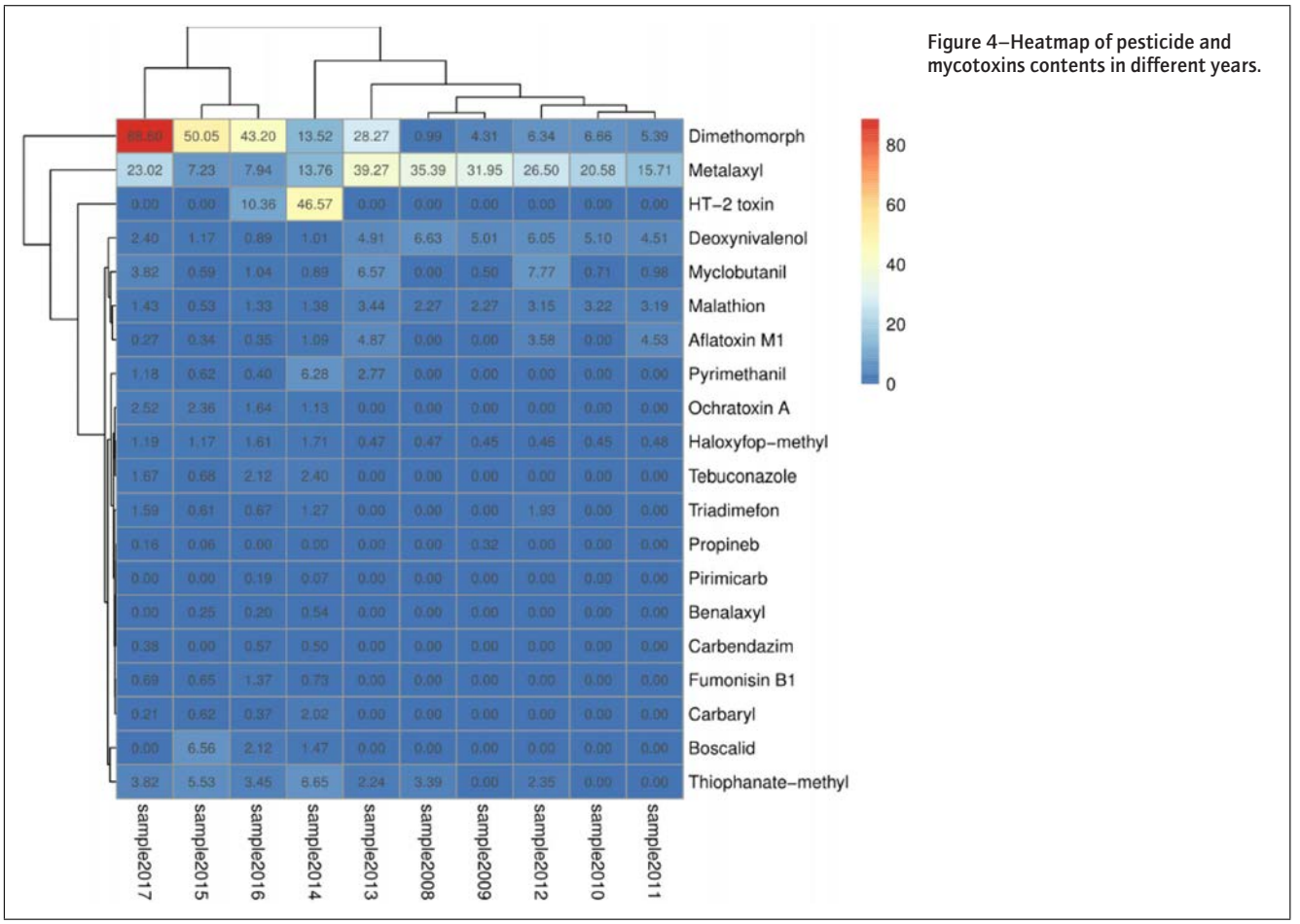

Figure 4–Heatmap of pesticide and mycotoxins contents in different years.

were analyzed in a single, 25-min chromatographic run, and a seven-point calibration curve was prepared for each compound. Matrix-matched standards were used within the range of 0.05 to 500 µg/L. The performance of the method was evaluated by evaluating the accuracy and the precision relative to the SANTE /11813/2017. The 65 compounds gave mean recoveries at the three spiking levels within the range of 70% to 120% with a precision ≤20%.

Analysis of real samples

The 64 wines, which were collected from the Gansu Hexi Corridor production region and foreign wineries from 2008 to 2017, were pretreated according to the method described in "QuEChERS extraction procedure" section, and analyzed according to the instrumental conditions described in "Chromatographic and mass spectrometric conditions" section. The wine samples contained six mycotoxins, namely, fumonisin B1, HT-2 toxin, ochratoxin A, diacetoxyscirpenol, aflatoxin M1, and deoxynivalenol, and 24 pesticide residues, such as dimethomorph, haloxyfop-methyl, thiophanate-methyl, malathion, metalaxyl, and pyrimethanil. Ochratoxin A and deoxynivalenol was found in 42.19% and 53.13%, respectively, of the wine samples. Dimethomorph, haloxyfop-methyl, and metalaxyl was found in 87.5%, 93.75%, and 87.5%, respectively, of the wine samples. The detection frequencies of the other mycotoxins and pesticides were relatively low; omethoate was not observed in any of the samples, which is consistent with the NYB 274–2014. Ochratoxin A in wine samples ranged from 0.76 to 6.40 μg/kg, and 11 (17%) of the samples had levels that exceeded the limit set by EU and OIV; compared with what was reported by Chen, Mo, Xu, Ni, and Chen (2012), contamination by this kind of mycotoxin has become less common. In addition, the contents of metalaxyl ranged from 0.33 to 86.8 μg/kg, meaning that all samples meet the limit set by NYB 274–2014 (no more than 5.0 mg/kg).

Heatmaps have been widely used in statistical analysis in recent years; they simply aggregate a large amount of data, and visually display the results using gradual color changes. The density and frequency of the data can be seen in Figure 4. After clustering the 64 wine samples by year and considering the 20 target compounds with the highest detection frequencies, the results showed that the contents of dimethomorph and metalaxyl were the highest. When comparing wine samples from the same year, the number of types and the contents of pesticide residues and mycotoxins detected in the wine samples from 2015 to 2017 were the highest, whereas relatively fewer compounds were detected in wine samples from 2008 to 2010. These results indicated that younger wines had higher contents of residues and contaminants than old wine. We know that the concentration of pesticide residues is significantly reduced during the solid–liquid separation process during winemaking, especially in the crushing and clarification after fermentation steps, and yeast reduces pesticide residues through metabolic degradation and adsorption (Guo, Zhu, Tian, Zhen, & Tan, 2015). Therefore, some pesticide residues and mycotoxins in wine may undergo significant degradation during storage. This chemical change is affected by many factors, and further research is needed.

Conclusion

Compared with the method (Zhang et al., 2018) for detecting mycotoxins in grape and wine, QuEChERS and UHPLC–MS/MS were used in both methods, and the experimental process and detection effect were comparable. However, in this work, we could simultaneously detect multiple pesticide residues on the basis of detecting mycotoxins, covering a comprehensive range, and the test was simpler and more efficient. Compared with the method of Pérez-Orteg et al. (2012), this experiment did not use solid-phase extraction, avoided the shortcomings of expensive extraction column, and complicated the sample preparation. It was not only simple and safe but also could detect 49 pesticide residues and 17 mycotoxins simultaneously. The detection process only took 25 min, which saved cost and time to a great extent.

In this work, using MRM under gradient elution and ESI (+) detection mode, a reliable UHPLC–MS/MS method for the qualitative and quantitative analyses of 49 pesticides and 17 mycotoxins was developed, which allowed the identification and quantification of these compounds in various types of wine. Sample preparation with QuEChERS makes this method easy, simple, and fast and avoids the use of expensive and cumbersome solid-phase extraction columns while requires less reagents and no additional cleanup steps, making it more economical and environmentally friendly. The method is appropriate for the routine testing of pesticide residues and mycotoxins in actual wine samples and provides a reference for the detection of other pesticides and mycotoxins.

Acknowledgments

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Author Contributions

H.J. and Z.B. conceived and designed the study; H.S.Y. directed the study; H.J. and H.L.L. conducted the study; Z.H. directed the instrument and equipment; M.T.Z. collected the wine samples; H.J. analyzed test data and drafted the manuscript; W.J. critically evaluated the manuscript; all authors approved the final version.

Conflict of Interest

The authors disclose no conflict of interest.

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Research Article

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Determinations of dinotefuran and metabolite levels before and after household coffee processing in coffee beans using solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry

Zhaojie Chen,[a†] Shiming Song,[a†] Lianying Mao,[b] Jie Wei,[a] Yuanfu Li,[a] Huihua Tan[a] and Xuesheng Li[a*]

Abstract

BACKGROUND: Coffee is one of the most popular beverages in the world. However, as daily consumables, coffee beans may contain pesticide residues that are capable of causing adverse health effects. Thus, we investigated residue dynamics in coffee beans using supervised field trials under Good Agricultural Practice conditions and determined the effects of household coffee processing on the coffee-bean pesticide residues dinotefuran and its metabolites 1-methyl-3-(tetrahydro-3-furylmethyl) urea (UF) and 1-methyl-3-(tetrahydro-3-furylmethyl) guanidine (DN).

RESULTS: The recovery rate of dinotefuran and its metabolites UF and DN was in the range 73.5%–106.3%, with a relative SD < 10%. The limits of detection and limits of quantification for dinotefuran, UF and DN were all 0.003 and 0.01 mg kg^{-1}, respectively. Dissipation experiments were conducted over 2015 and 2016 and showed a mean half-life of 40.8 days. Coffee processing procedures were performed as described for traditional household coffee processing in Ethiopia. Dinotefuran contents were reduced by 44.4%–86.7% with washing of coffee beans and the roasting process reduced these contents by 62.2%–100%. DN residues were not detected in roasted coffee beans before day 21 or in brewed coffee before day 35 and UF residues were not detected in brewed coffee before day 35. Kruskal–Wallis analyses indicated large variations in the stability of pesticide residues between processing methods ($P \leq 0.05$). Reductions of pesticide concentrations with washing were also significantly lower than those following roasting ($P = 0.0001$) and brewing processes ($P = 0.002$). Moreover, processing factors were less than one for all processing stages, indicating reductions of pesticides contents for all processing stages.

CONCLUSION: The cumulative effects of the three processing methods are of paramount importance with respect to an evaluation of the risks associated with the ingestion of pesticide residues, particularly those in coffee beans.

Keywords: dinotefuran; metabolites; residues; coffee beans; household coffee processing

INTRODUCTION

Neonicotinoid insecticides that target insect nicotinic acetylcholine receptors[1,2] are by far the most successful insecticides, as indicated by their wide pest spectrum, favourable safety profiles and various applications.[3,4] Neonicotinoids include five-membered ring derivatives such as imidacloprid and thiacloprid, six-membered ring compounds (thiamethoxam), and noncyclic structures such as nitenpyram, acetamiprid, clothianidin and dinotefuran.[5] Dinotefuran (Fig. 1) (RS)-1-methyl-2-nitro-3-(tetrahydro-3-furylmethyl) guanidine is a new furanicotinyl insecticide that represents the third generation of neonicotinoid insecticides.[6] This compound was commercialised by Mitsui Chemicals Agro (Tokyo, Japan) in 2002 and is increasingly used in more than 20 countries, including China, the European Union and the USA,[7,8] where it is commercially available and is widely used for crop protection and the control of various harmful pest species. In 2013, dinotefuran was registered in China for application to several agricultural products, including rice,

* *Correspondence to: X Li, Institute of Pesticide & Environmental Toxicology, Guangxi Key Laboratory Cultivation Base of Agro-Environment and Agro-Product Safety, Guangxi University, Nanning 530005, China. E-mail: lxsngx@163.com*

† *These authors contributed equally to this work.*

a *Institute of Pesticide & Environmental Toxicology, Guangxi Key Laboratory Cultivation Base of Agro-Environment and Agro-Product Safety, Guangxi University, Nanning, China*

b *State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bioresources, Agricultural College, Guangxi University, Nanning, China*

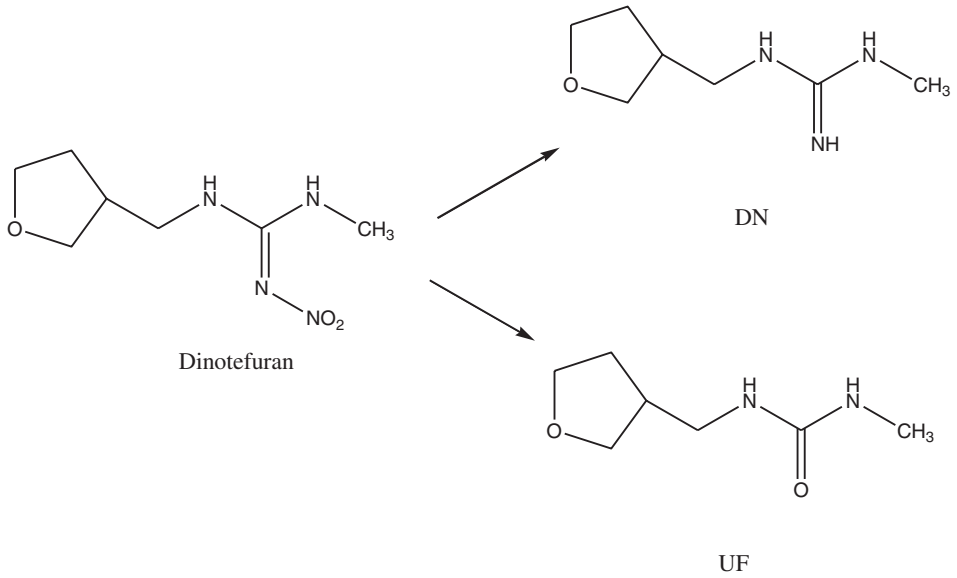

Figure 1. Chemical structure of dinotefuran, DN and UF.

tomato, wheat and cucumber[9] and maximum residue limits for dinotefuran in different agricultural products were established in the range 0.05–25 mg·kg^{-1}.[10] Dinotefuran has been attracting interest as a promising insecticide[11] and has high insecticidal activity at very low application rates.[12] Unfortunately, dinotefuran is highly toxic to bees, with a contact acute half lethal dose at 48 h of 0.023 µg/bee. This compound is also highly susceptible to bioconcentration[13] and acceptable daily intakes and acute reference doses were estimated at 0–0.2 mg kg^{-1} body weight (bw) and 1 mg kg^{-1} bw, respectively.[14] According to a Joint Food and Agriculture Organization of the United Nations and World Health Organization Meeting (JMPR) on Pesticide Residues report,[14] 1-methyl-3-(tetrahydro-3-furylmethyl) guanidine (DN) and 1-methyl-3-(tetrahydro-3-furylmethyl) urea (UF) (Fig. 1) are chiral metabolites that are produced after dinotefuran is applied to plants. A previous study[15] investigated the metabolism of dinotefuran in plants and mammals and, in agreement with other studies,[11,16] showed that 1-methyl-2-nitroguanidine, UF and DN are the major metabolites of dinotefuran in plants. To our knowledge, the toxicities of these metabolites and the parent compound are approximately equal,[14] although the metabolites are reportedly more mobile and persistent than their parent compound,[17] potentially facilitating transfer from plants to water. These metabolites may also be toxic in various foods and beverages that are processed by boiling in water before consumption, such as tea and coffee.

Coffee is one of the most popular beverages and is consumed in high quantities globally. Coffee is also the second most important commodity after oil as a source of foreign exchange for most producing countries.[18,19] As with all crops, coffee plants are susceptible to many pests and plant diseases, and pesticides are commonly applied to avoid these during cultivation of coffee beans. Consequently, residues of active ingredients and metabolites may remain interminal products under certain conditions. Few studies report the presence of residues of dinotefuran and its metabolites in raw and processed (washing, roasting and brewing) coffee beans. By contrast, antioxidant effects and other beneficial biological properties have elicited multiple assessments of quality control and safety of coffee beans. Thus, analyses of residues of dinotefuran and its metabolites in coffee beans and products are urgently required.

During coffee processing, raw beans are washed, roasted and ground into fine powder before brewed coffee is placed into cups and served. Thus, further studies are required to characterise the effects of these processes on pesticide residues. Mekonen *et al.*[18] indicated that concentrations of pesticide residues are reduced by 14.6% to 57.7% by washing of coffee beans, and by up to 99.8% by roasting.

Currently, most analytical methods have been optimised for parent compounds,[11,16,20,21] such as dinotefuran, whereas residue analyses of dinotefuran and its metabolites in coffee beans have not been established or reported. Coffee beans comprise a complex matrix of polyphenols, caffeine and pigments that can be easily extracted simultaneously. Consequently, analysis of exogenous compounds can be hampered by matrix constituents.

In the present study, we developed a sensitive and specific method for determining dinotefuran and its metabolites in coffee beans using Florisil solid phase extraction (SPE) cartridges coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using this method, we determined dinotefuran transformation into its metabolites and identified the predominant metabolic processes and differences in plants. We also investigated the dissipation of dinotefuran in coffee beans in supervised field trials under Good Agricultural Practice conditions in Yunnan province, which is a coffee producing area in China. Finally, we report the effects of household coffee processing (washing, roasting and brewing) on residues of dinotefuran and its metabolites in coffee beans.

Materials and methods

Chemicals and reagents

Standard 99.5% dinotefuran, 99.8% DN and 98.5% UF materials were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile was of high-performance liquid chromatography (HPLC) grade (Merck, Germany). Nitenpyram and dinotefuran were purchased as 60% wettable powders. Analytical-grade acetonitrile, sodium chloride, magnesium sulphate anhydrous, sodium acetate and acetic acid were obtained from Chengdu Kelong

Chemical Reagent Company (Chengdu, China). Analytical-grade ammonium acetate was purchased from Merck Chemicals (Shanghai) Co., Ltd (Shanghai, China). Ultrapure water (0.22 µm) was prepared using a Milli-Q treatment system (Millipore, Billerica, MA, USA). Florisil, C18, Silica, NH_2 and HLB solid-phase extraction columns were provided by Anpel (Shanghai, China).

Field experiments and sampling

Field trials, including studies of dissipation and terminal residues, were designed in accordance with pesticide labels. These supervised field trials were conducted in the Yunnan province of China during 2015 and 2016. The experimental area included three replicated plots and a control plot that was not treated with pesticide. Plots areas contained three trees each and were separated by buffer areas. In dissipation experiments, dinotefuran was distributed at 150 g a.i./ha (1.5 times the highest recommended dosage) in a single spray. Coffee-bean samples of approximately 2 kg were collected randomly from five points in each of the plots at time intervals of 2 h, and 1, 3, 5, 7, 14, 21, 28, 35, 42 and 56 days after application of pesticide. Terminal residue analyses were performed after spraying three times with doses of 100 (highest recommended dosage) and 150 g a.i./ha at intervals of 7 days. Samples of approximately 2 kg were collected from each plot at 7, 14 and 21 days after application. Samples of coffee cherries were cleaned with water and, after separating from peels, samples (0.5 kg) of coffee beans were homogenised. All samples were stored at −20 °C in a freezer and were analysed within 1 month.

Sample preparation and purification

Homogenised coffee-bean samples of 2 g were weighed in 50-mL Teflon centrifuge tubes and 20-mL aliquots of 2% acetic acid in acetonitrile were added. Tubes were then shaken vigorously for 30 min and 2 g of anhydrous magnesium sulphate and 1 g of sodium acetate were added and shaken vigorously for 2 min. Tubes were then centrifuged for 5 min at 2490 × *g*. After centrifugation, 20-mL aliquots of clarified supernatants were transferred to round-bottomed flasks and were evaporated to dryness at < 45 °C under vacuum. Extracts were then dissolved in 2-mL aliquots of acetonitrile and were loaded twice into previously-conditioned 500-mg Florisil SPE cartridges with 5 × 2 mL of acetonitrile. Dinotefuran and its metabolites were then eluted with 5 × 3-mL aliquots of acetonitrile. Elutes were separately evaporated to dryness at < 45 °C under a vacuum and were dissolved in 2-mL aliquots of methanol. Finally, 1.5-mL aliquots of clarified supernatant were filtered through a 0.22-µm filter membranes, and 1-mL extracts were placed into LC vials for chromatographic analyses.

LC-MS/MS analytical conditions

Liquid chromatography was conducted using an Agilent 1290 Series Rapid Resolution LC System (Agilent Technologies Inc., Santa Clara, CA, USA). Analytes were separated on a XBridge C_{18} column (150 mm × 0.21 mm, 3.5 µm; Waters, Milford, MA, USA) in an oven at 30 °C. The binary solvent system comprised acetonitrile (A) and 5-mmol ammonium acetate in water (B) and was applied as a linear gradient starting at 17% A (0–3 min), followed by 40% A (3–6 min), ramping down to 1% A (6–7 min) and then 17% A (7–8 min) at a flow rate of 0.3 mL min^{-1} and an injection volume of 3 µL.

Elutes from the LC system were introduced into an Agilent G6460C Triple Quadrupole LC-MS/MS (QQQ) system in positive electrospray ionisation mode using multiple reaction monitoring with two mass transitions. Among the two mass transitions, the product ion with the highest intensity and another of low intensity were used as quantitative and qualitative ions, respectively. Standard solutions of dinotefuran and its metabolites were directly infused into the QQQ system to optimise MS instrument parameters. Nitrogen was used as the nebuliser and drying gas at 15 psi and 350 °C, and the gas flow rate was 13 mL min^{-1}. The capillary was set to 4 kV. Both MS1 and MS2 quadrupoles were maintained at a single unit resolution. All other experimental conditions are shown in Table 1.

Household processing

Washing of coffee beans. Harvested coffee beans were washed thoroughly for 5 min under tap water (25–30 °C) and all procedures for extraction, clean-up and analysis of the pesticide and its metabolites were applied to unprocessed coffee beans.

Roasted coffee beans. Roasting processes were performed as in a traditional Ethiopian household. Briefly, harvested coffee beans were washed and then roasted on a stove at a temperature of 230–240 °C for an average time of 12–14 min until the characteristic aroma and flavour of coffee beans became apparent. Procedures for extraction and clean-up of raw coffee beans were then applied.

Brewed coffee beans. Some heat-resistant pesticides may be detected in brewed coffee, even after roasting of coffee beans. Thus, to determine the effects of brewing on dinotefuran and its metabolites UF and DN, we ground roasted coffee beans to a fine powder using a coffee grinder and then brewed coffee. The fine coffee powder was added to a coffee pot containing 100 mL of boiled water and was brewed for 10–12 min to emulate the tradition Ethiopian coffee brewing process. Subsequently, the infusion in the coffee pot was cooled on the ground until the coffee sludge settled. The upper liquid layer was then removed carefully and placed in a 50-mL centrifuge tube for extraction, clean-up and analysis of the brewed coffee solution. The coffee sludge was also analysed as described for raw coffee beans above.

Table 1. Multiple reaction monitoring transitions and other LC-MS/MS parameters

Pesticide	Retention time (min)	Precursor ion	Confirmation transition[a]	Quantification transition[a]	Fragmentor (V)
Dinotefuran	1.80	203	114 (5)	129 (10)	110
UF	1.25	159	85 (15)	67 (20)	80
DN	3.60	158	57 (20)	102 (15)	100

[a] Collision energy (eV).

Determination of processing factors (PFs)

The effects of household processing on pesticide contents often correlate with the physicochemical properties of the pesticides, warranting adequate calculation of PF for all transformation steps. PFs were calculated as the ratio between pesticide concentrations in processed and unprocessed commodities (mg kg^{-1}). According to Mekonen *et al.*,[18] PFs of < 1 indicate that pesticide contents are decreased by processing (PF < 1, reduction), whereas PFs > 1 indicate no reduction in weight or volume (PF > 1, concentration). Using PFs, we calculated percentage reductions in pesticide contents for each processing step: % reduction = (1 − PF) × 100.

Analyses of wash water and coffee sludge after coffee brewing

Before roasting, coffee beans were washed with water to remove surface residues. After roasting, grinding and brewing, the coffee grind sludge remaining on the bottom of the coffee pot may contain pesticide residues that were not removed during household processing. Because this coffee sludge is traditionally disposed of into the immediate environment, any remaining pesticide residues may contaminate land that is used to produce food. Thus, we determined the presence of pesticide residues in coffee sludge and in coffee-bean wash water using the extraction, clean-up and analysis procedures described for raw coffee beans.

Statistical analysis

Because matrix effects were observed in coffee-bean samples, we employed a matrix-matched calibration standard to quantify residues in coffee-bean samples using LC-MS/MS analyses.

Dissipation and half-life ($t_{1/2}$) values were calculated using the first-order rate equation $C_t = C_0 e^{-kt}$, where C_t represents the concentration of pesticide residue at time t, C_0 represents the initial concentration after application and k is the per-day degradation rate constant. Half-life ($t_{1/2}$) values were calculated from k values for each experiment as: $t_{1/2} = \ln 2/k$.

All treatments were performed in triplicate, and values are presented as the mean ± SD. Percent reductions in pesticide and metabolite contents following washing, roasting, and brewing were identified using Kruskal–Wallis test. $P < 0.05$ was considered statistically significant. All calculations were performed using Excel 2007 (Microsoft Corp., Redmond, WA, USA) or SPSS, version 19.0 (IBM Corp., Armonk, NY, USA).

Results and Discussion

Development of LC-MS/MS analytical methods

To achieve sensitive, selective and validated LC-MS/MS analyses of dinotefuran, UF and DN residues in coffee beans, the chromatographic conditions were optimised by performing analyses with Agilent ZORBAX Eclipse Plus C_{18} (2.1 × 50 mm, 1.8 µm), Waters XBridge C_{18} (150 × 2.1 mm, 3.5 µm), Agilent Poroshell 120 SB-AQ (100 × 2.1 mm, 2.7 µm) and Agilent ZORBAX SB-C18 (100 × 2.1 mm, 3.5 µm) columns using mobile phases comprising acetonitrile and water, acetonitrile and 0.1% formic acid in water, and acetonitrile and 5 mmol L^{-1} ammonium acetate in water. Optimal separation and peak resolutions were achieved using gradient elution with acetonitrile and 5 mmol L^{-1} ammonium acetate in water through a Waters XBridge C_{18} column (150 × 2.1 mm, 3.5 µm) at a flow rate of 0.3 mL min^{-1}.

Optimisation of extraction and clean-up procedure

In a previous study of dinotefuran and its metabolites,[22] the low sensitivity and shorter wavelength of HPLC-ultraviolet spectrophotometric analyses, and the quick, easy, cheap, effective, rugged and safe sample preparation method resulted in a failure of dispersive clean-up to eliminate interference from complex matrix components.[22] Hence, to achieve higher sensitivity and selectivity and reduce interference from the matrix effect, we performed LC-MS/MS analyses with an SPE cartridge. In another study,[23] the addition of 2%–4% acetic acid to acetonitrile improved the extraction efficiencies of dinotefuran and its metabolites, although DN recovery was not improved under these conditions compared to that following matrix-matched calibration. Thus, to improve DN recovery, we compared extraction efficacies between ultrasound and oscillation methods and extraction times of 15, 30 and 60 min. Recovery of dinotefuran and its metabolites UF and DN was satisfactory (70–110%) following oscillation with an extraction time of approximately 30 min and LC-MS/MS analyses was performed using 2% acetic acid in acetonitrile.

In the present study, we compared the purification effect with that achieved using C18, silica, Florisil, NH_2 and HLB SPE cartridges, and found that elution of Florisil SPE cartridges three times with respect to the use of acetonitrile resulted in a higher purity and recovery of dinotefuran, UF and DN than that following two, three and four elution steps with methanol and acetonitrile using the other SPE cartridges with various elution times.

Matrix effects

Matrix effects influence analytical signals through the actions of co-extracted compounds, although they can vary between matrix types and can be ameliorated by a higher efficiency sample preparation.[24,25] Using the calculations and classifications of matrix effects described by Antignac *et al.*,[25] we calculated matrix effects for dinotefuran, UF and DN in coffee beans. As shown in Table 2, dinotefuran, UF and DN had matrix suppression effects of low recovery compared to the respective solvent calibrations, which showed low to high matrix effects in coffee-bean samples. Therefore, to compensate for matrix effects, matrix-matched calibration curves were established and an acceptable recovery of

Table 2. The results of calculation and classification of matrix effect for the dinotefuran, UF and DN in coffee bean

		Pesticide		
Parameter	Matrix	Dinotefuran	UF	DN
Range	Coffee bean	0.01–5 mg kg^{-1}	0.01–0.5 mg kg^{-1}	0.01–0.5 mg kg^{-1}
Regression equation		$y = 70\,968x - 379.6$	$y = 75\,133x + 225.9$	$y = 9969x + 28.6$
Correlation coefficient		$r^2 = 1$	$r^2 = 1$	$r^2 = 0.999$
Matrix effect (%)		14.56%	48.35%	79.56%
Level		Low	High	High

Table 3. Results of regression coefficient, percentage recovery (LOQ, LOQ × 10 and LOQ × 50) and percentage relative SD of dinotefuran and its metabolites in coffee bean (raw and processed) and water

Pesticide	r^2	Recovery (%)	RSD (%)
Dinotefuran	1.000	77.6–102.0	2.0–4.0
UF	0.998	94.8–97.5	1.4–1.8
DN	0.997	73.5–106.3	1.8–4.2

%RSD, relative SD.

dinotefuran, UF and DN was achieved. Matrix effects were calculated from calibration curve slopes in solvent and in matrix and the horizontal classification of matrix effect described as Economou *et al.*[26] using the equation:

$$\text{Matrix effect } (\%) = \left(S_{\text{matrix}}/S_{\text{solvent}} - 1\right) \times 100$$

Quality control

Calibration curves were prepared for all analytes in both the solvent and matrix to determine the linearity and recovery variations. Recovery experiment were conducted in five replicates at three different fortification levels [limit of quantification (LOQ) (LOQ), 10 × LOQ and 50 × LOQ]. Mean recovery rates for dinotefuran and its metabolites in coffee bean (before and after processing) and water were 73.5–106.3% and all had relative SDs below 10% and regression coefficients (r^2) > 0.997. These data indicate a sufficient accuracy and precision for analysis of pesticides (Table 3), as stipulated in the European Document no. SANCO/12495/2011.[27]

Limits of detection and LOQ for dinotefuran, UF and DN were all 0.003 and 0.01 mg kg^{-1}, yielding signal-to-noise ratios of 3 and 10 relative to blank sample,[28] respectively, for coffee beans.

Digestion dynamics

The methods developed in the present study were successfully applied to samples collected at the indicated times from study fields in Yunnan province that were treated with the dinotefuran formulations over 2 years. Analyses of dinotefuran, UF and DN residues in these coffee-bean samples (Table 4) showed initial deposits of dinotefuran ranging from 2.59 to 2.86 mg kg^{-1} and a mean half-life of 40.8 day over 2015 and 2016. In field tests that were conducted in 2015 and 2016, concentrations of dinotefuran decreased from day 1, whereas those of UF and DN increased from days 1 and 7, respectively. Total residue concentrations decreased over days 1 to 35 during 2015 and 2016. These data indicate that both metabolites form over time, and that DN in particular may form from the degradation and transformation of dinotefuran and UF. In support of this hypothesis, DN concentrations were higher than those of UF at day 28, and total concentrations began to increase again after day 28.

Effect of household processing of coffee beans on concentrations of pesticide residues

Because coffee is one of the most popular drinks globally, analyses of pesticide residues are required in coffee beans before and after processing. In the present study, we individually investigated the effects of washing, roasting and brewing on the stability of dinotefuran and its metabolites. Concentrations of dinotefuran, UF and DN in raw and processed coffee beans (Fig. 2) were decreased by 44.4–86.7% after washing, with maximum reductions in dinotefuran after washing for 1 day. Previous studies[29,18] similarly show that the washing of crop surfaces with tap water decreases the presence of residues by up to 50%. After application to agricultural crops, pesticides are predominantly confined to crop surfaces and much smaller amounts infiltrate into plants.[30] Hence, washing of coffee silverskins effectively reduces pesticide contamination.

Dinotefuran has greater water solubility than UF and DN (dinotefuran, log $k_{ow} = -0.64$; UF, log $k_{ow} > 0.34$; DN, log $k_{ow} < -0.27$) and dinotefuran concentrations were reduced more effectively than those of UF and DN following washing with tap water. However, removal of pesticides by washing with water is not always associated with water solubility, as previously described by Cengiz *et al.*[31]

We show that roasting of coffee beans decreases the concentrations of dinotefuran, UF and DN residues by 62.2–100% and that maximal reductions were observed at 1–14 day. In these experiments, DN levels were undetectable, potentially indicating the instability of this compound under the heated conditions of roasting. Similarly, previous studies of pesticides in coffee beans showed significant decreases following roasting,[18,31] and loss of pesticides under these conditions reportedly depends largely on physicochemical properties that lead to evaporation or thermal degradation.[32]

Table 4. Field-incurred residues (mg kg^{-1}) of dinotefuran, UF and DN (mean ± SD, $n = 3$) in coffee beans

	Dinotefuran		UF		DN		Total amount[b]	
PHI[a] (d)	2015	2016	2015	2016	2015	2016	2015	2016
1	2.86 ± 0.23	2.59 ± 0.21	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	1.88	1.73
3	2.37 ± 0.11	2.07 ± 0.13	0.07 ± 0.01	0.09 ± 0.03	0.03 ± 0.01	0.06 ± 0.02	1.58	1.42
5	1.66 ± 0.14	1.66 ± 0.11	0.07 ± 0.02	0.09 ± 0.02	0.04 ± 0.01	0.06 ± 0.01	1.13	1.16
7	1.60 ± 0.24	1.22 ± 0.15	0.08 ± 0.01	0.09 ± 0.02	0.04 ± 0.01	0.07 ± 0.03	1.10	0.88
14	1.60 ± 0.22	1.11 ± 0.22	0.10 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	1.14	0.86
21	1.35 ± 0.11	1.11 ± 0.18	0.13 ± 0.05	0.13 ± 0.12	0.06 ± 0.02	0.09 ± 0.01	0.99	0.85
28	1.26 ± 0.11	1.07 ± 0.16	0.12 ± 0.06	0.10 ± 0.03	0.14 ± 0.05	0.21 ± 0.05	0.97	0.88
35	1.13 ± 0.14	1.02 ± 0.25	0.19 ± 0.03	0.19 ± 0.05	0.25 ± 0.06	0.30 ± 0.06	1.00	0.97
42	1.10 ± 0.23	0.94 ± 0.08	0.17 ± 0.08	0.19 ± 0.04	0.20 ± 0.03	0.28 ± 0.07	0.94	0.90
56	1.00 ± 0.21	0.76 ± 0.19	0.30 ± 0.04	0.27 ± 0.09	0.46 ± 0.09	0.44 ± 0.02	1.13	0.94

[a] PHI, preharvest interval.
[b] Total amount = residues of the parent compound + residues of the metabolite × (parent molecular weight/metabolite molecular weight).

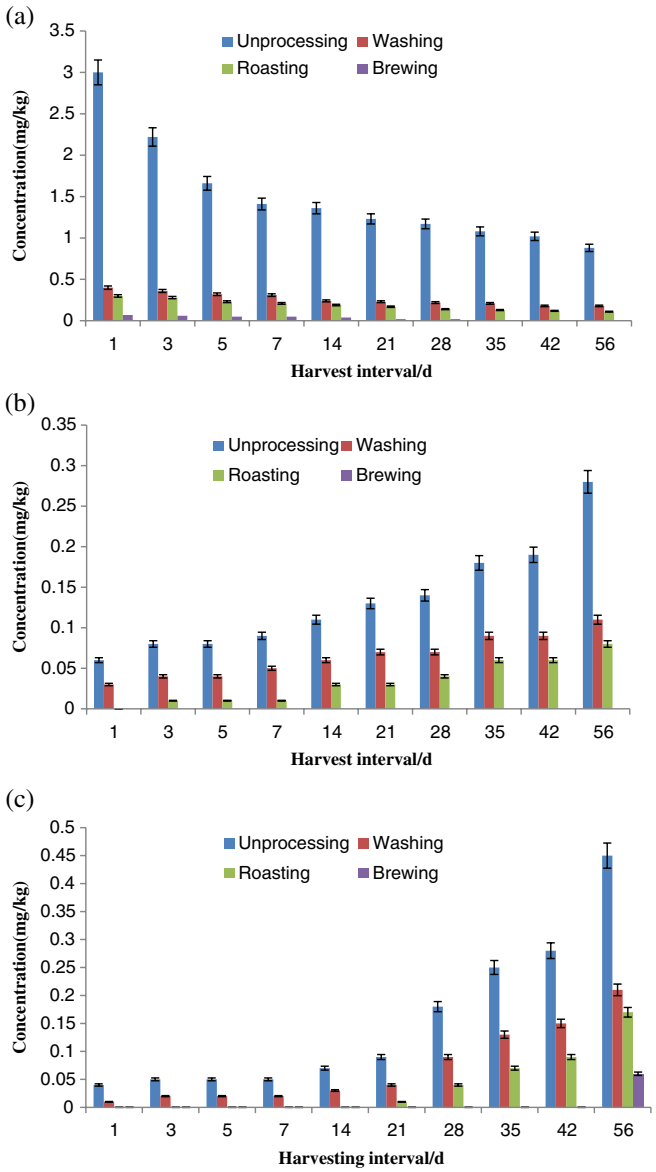

Figure 2. Mean residues of dinotefuran (a), UF (b) and DN (c) at different coffee processing steps. Error bars indicate the SD.

At 28 days after brewing, residues of dinotefuran and UF were not detected in sludge, and residues of dinotefuran, UF and DN were clearly decreased by 92.9–100% in the upper liquid layers. In addition, after 56 days, UF was the only detectable (0.02 mg kg^{-1}) metabolite, suggesting that brewing has less influence on UF than on dinotefuran and DN. The effects of roasting and brewing on residues of dinotefuran, UF and DN were significantly influenced by thermal instability in a previous study.[33] In summary, the three household coffee-bean processing methods cumulatively reduce dinotefuran, UF and DN contents and may prevent the deleterious health effects of coffee-borne pesticide exposures.

Determination of PF

PFs for dinotefuran, UF and DN were < 1 for each processing step, indicating that all traditional coffee processing methods remove these residues from the final consumable. Some similar studies[18,34] show PFs < 1 for pesticides in food processing steps. In the present study, PFs of dinotefuran, UF and DN were lowest for the brewing process and UF was not detected in coffee at days 1–56 after brewing, suggesting that the brewing process is more effective at removing dinotefuran, UF and DN residues from coffee beans than washing and roasting processes. However, PFs were not calculated for pesticide residues at concentrations below 0.01 mg kg^{-1} (Table 5).

Table 5. Processing factor for the effect of washing, roasting and brewing processes

Harvesting interval (days)	Pesticide	PF washing	PF roasting	PF brewing
1–56	dinotefuran	0.13–0.22	0.10–0.15	ND–0.04
	UF	0.39–0.56	ND–0.33	ND
	DN	0.25–0.54	ND–0.38	ND–0.13

ND, not detected.
Calculated by the formula below:
PF = concentration of pesticides in processed coffee beans (mg kg^{-1})/concentration of pesticides in unprocessed coffee beans (mg kg^{-1}).

A Kruskal–Wallis tests showed large differences in the stability of pesticide residues in coffee beans between the processing methods ($P \leq 0.05$). Specifically, reductions in pesticide concentrations as a result of washing were significantly lower than those following roasting ($P = 0.0001$) and brewing ($P = 0.002$). The effects of washing, however, may also depend on factors, such as location, age of residue, water solubility and temperature, as suggested by Thanki *et al.*.[35] These factors likely contributed to the present variable effects of washing.

Pesticide residues in wash water and coffee sludge

We determined pesticide and metabolite levels in wash water and coffee sludge after brewing and showed that dinotefuran, UF and DN were present in wash water at 1–56 days after crop treatments (Fig. 3). Hence, dinotefuran, UF and DN on coffee-bean surfaces are likely removed with the coffee-bean silverskin. Portions of dinotefuran, UF and DN were also removed from coffee beans during roasting. By contrast, dinotefuran and DN concentrations remained detectable in coffee sludge after brewing coffee from samples taken at 1–28 days and at 56 days after pesticide treatments, respectively. These data suggest that percentage reductions of dinotefuran and DN are lower than those of UF (Fig. 3). Further interpretation of these data suggest that coffee sludge should be avoided when pouring brewed coffee into cups for human consumption. Thus, we agree that it is important to wait for coffee sludge to completely precipitate to the bottom of the coffee pot, as suggested by Mekonen *et al.*.[18]

Conclusions

The acceptable daily intakes and acute reference doses were estimated at 0.2 mg kg^{-1} bw and 1 mg kg^{-1} bw, respectively[14] and, in accordance with the calculation method of international acute dietary intake adopted by JMPR, the international estimated daily intake of dinotefuran is 1.0814 mg, which is only 8.6% of the daily allowable intake.[36] Recommended maximum exposures to pesticide residues do not usually pose unacceptable risks to the general population when raw coffee is collected and processed at 28–35 days (the most appropriate harvest days) after pesticide treatment, for which dinotefuran was in the safe range, and

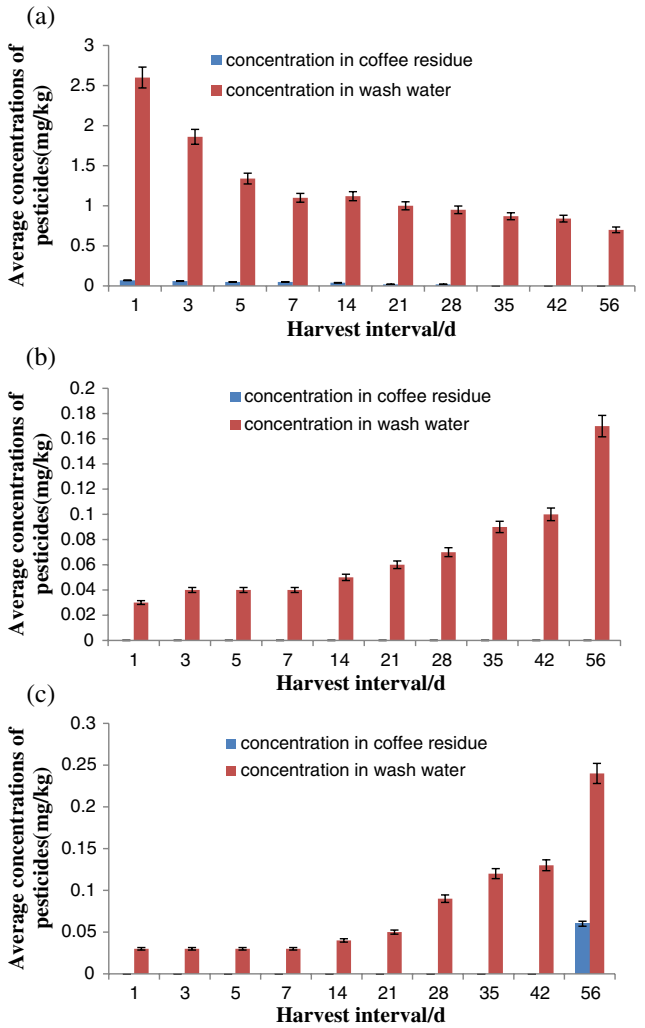

Figure 3. Mean concentrations of dinotefuran (a), UF (b) and DN (c) detected in coffee sludge (after brewing) and wash water. Error bars indicate the SD.

metabolites were not detected at the same time. However, disposal of coffee-bean wash water or coffee sludge into the open environment may cause contamination. Moreover, to ensure safe consumption, when pouring coffee, care must be taken to serve only the upper layer and to avoid the sludge at the bottom of the pot.

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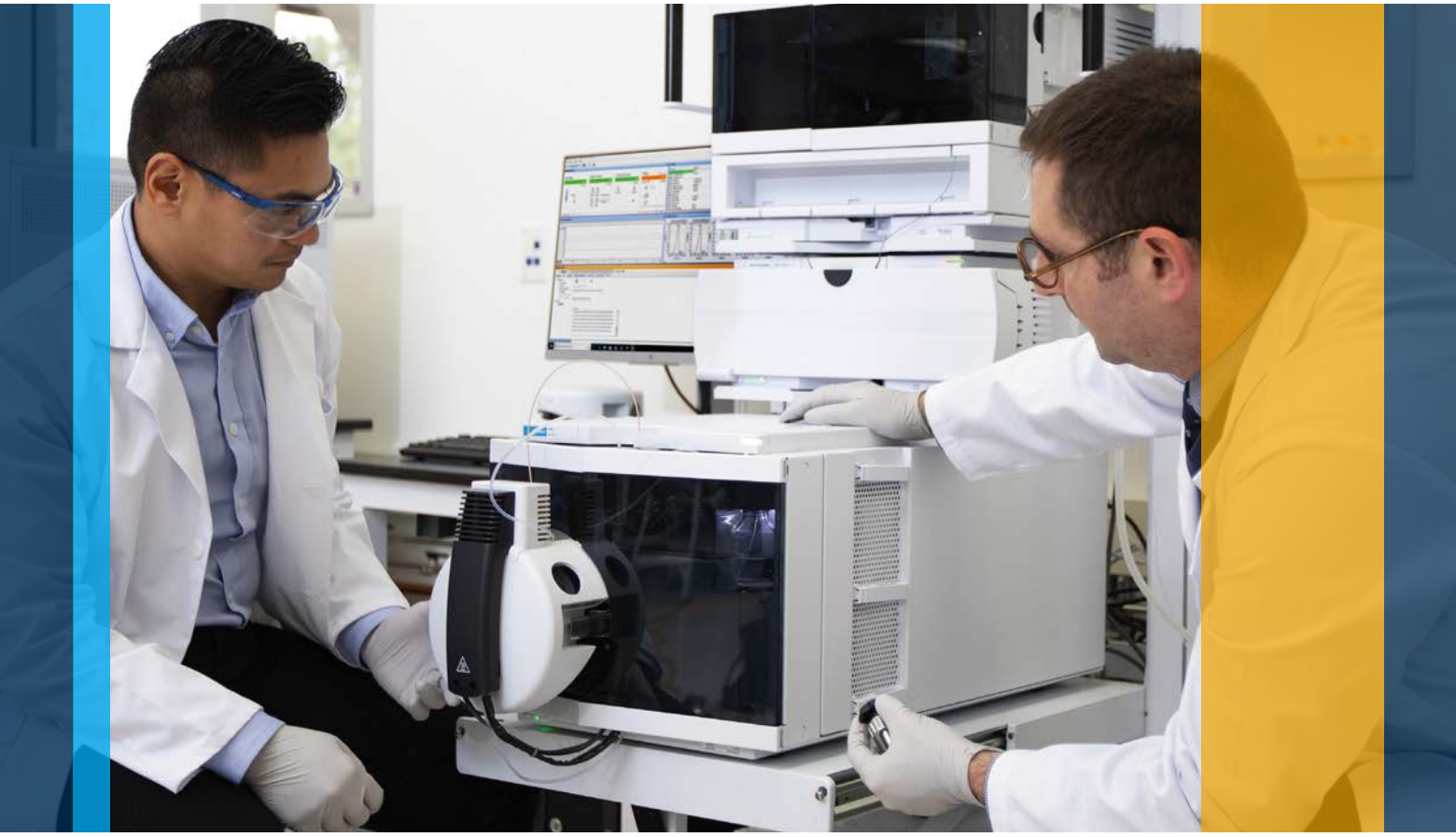

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