Application Note: 442

LC-MS/MS Analysis of Malachite Green, Leucomalachite Green, Ciprofloxacin, and Tetracycline in Food Samples using a TurboFlow Method

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Key Words

- Aria™ TLX System
- Food Residue Analysis
- TSQ Quantum Access™
- TurboFlow™ Technology
- Veterinary Drugs

Introduction

Accurate monitoring of chemical residue levels in food and agriculture products is essential to assure the safety of the food supply and manage global health risks. The analysis of chemical residues requires techniques sensitive enough to detect and quantify contaminants at or below the maximum residue limit (MRL) of the compound in a given sample matrix. In addition, because of increased food safety regulations and the growing numbers of samples to be analyzed, it is critical that the analytical techniques provide high sample throughput.

With the continuing rapid growth of the aquaculture industry, there is increasing concern about the use of unapproved drugs and unsafe chemicals in aquafarming operations. Malachite green (MG), a triphenylmethane dye, is an effective and inexpensive fungicide used in aquaculture, particularly in Asian countries (Figure 1).

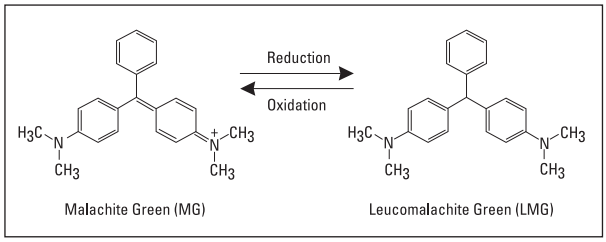

Figure 1: Structures of malachite green and leucomalachite green

During metabolism, malachite green reduces to leucomalachite green (LMG), which has been shown to accumulate in fatty fish tissues and can be found long after MG may no longer be detected.[1] Both MG and LMG have demonstrated putative carcinogenic activity, and thus MG has been banned for use as an aquaculture veterinary drug in many countries including the United States and Canada, as well as the European Union (EU). For substances that are banned from use in food producing animals, EU legislation defines minimum required performance limits. For malachite green, an analytical test method must be able to determine the sum of MG and LMG residues in fish muscle at the minimum required performance limit of 2 µg/kg (ppb).[2]

Ciprofloxacin is a broad-spectrum antibiotic belonging to the fluoroquinolone group (Figure 2). Fluoroquinolones have been shown to be very effective in combating various diseases in animal husbandry and aquaculture and are used extensively worldwide. However, because of concerns that fluoroquinolone residues in food products may lead to the development of antibacterial resistance to these drugs in humans, the FDA has prohibited extra-label use of fluoroquinolones in food animals.[3] According to the EU legislation on veterinary drug residues, the maximum residue limits for the sum of enrofloxacin and its metabolite ciprofloxacin are 100 µg/kg (ppb) in muscle for all food producing species and 200 µg/kg (ppb) in pork liver.[4]

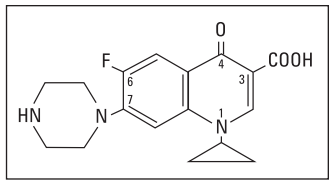

Figure 2: Structure of Ciprofloxacin

Tetracycline is a polyketide antibiotic that is highly effective against a number of gram-positive and gram-negative bacteria (Figure 3). As with other veterinary antibiotics, when tetracycline is used in food animals, it has the potential to generate drug residues in the animals and animal products which can lead to increases in microbial resistance. The MRLs for tetracycline are 100 µg/kg (ppb) in muscle and 300 µg/kg (ppb) in liver for all food producing species.[5]

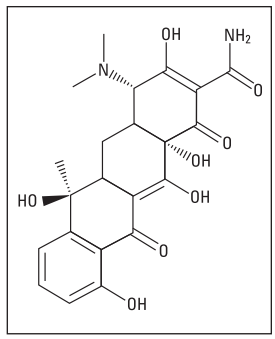

Figure 3: Structure of Tetracycline

Here we show a solution that combines the Thermo Scientific Aria TLX system utilizing TurboFlow technology with a Thermo Scientific TSQ Quantum Access mass spectrometer. Compared to traditional offline extraction methods, this solution provides fast and reliable sample analysis of chemical residues in food by online sample extraction followed by LC-MS/MS. The Aria TLX system uses TurboFlow technology to retain small molecules and filter out proteins and larger materials by diffusion, size exclusion, and column chemistry. This enables users to directly inject samples into the LC-MS system for analysis, greatly simplifying sample preparation and increasing throughput.

Goal

To demonstrate a reduction in overall analytical time compared to traditional methods, such as liquid-liquid or solid phase extraction, while also minimizing ion suppression and matrix interference in food samples.

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Experimental Conditions

Sample Preparation

Samples were prepared for analysis using a simple extraction procedure with acetonitrile. Individual samples, weighing 3 to 4 g, of shrimp (with head attached), tilapia, and pig liver were homogenized with approximately 30 mL of acetonitrile and then left at room temperature for 10 minutes. The liquid phase was then aspirated from the sample into a 0.22 µm pore centrifuge tube and spun at 10,000 rpm for approximately 10 minutes. The supernatant was aspirated into a 50 mL scintillation vial. This portion of the sample preparation took 25 minutes.

A stock mix solution of malachite green, leucomalachite green, ciprofloxacin, and tetracycline was prepared at a concentration of 1 mg/L. All four analytes were mixed in one vial at 0.1 mg/mL (1000 µg/mL) in methanol. Calibration solutions in the concentration range 10 µg/kg to 5 ng/kg were prepared by serial dilution of the stock solution into the three sample matrices. The total sample preparation time was approximately 30 to 40 minutes.

TurboFlow Method Conditions

The samples were processed on an Aria TLX-1 System (Thermo Fisher Scientific, Franklin, MA). The Multiple Column Module (MCM), which allows 6 loading columns and 6 analytical columns to be tested at once, was used to facilitate method development. First, the loading column that gave the best recovery of each analyte was selected. Because all of the analytes were in one stock solution, the run time was minimized. Then the analytical column that gave the best performance was selected. The final TurboFlow method conditions were as follows:

Loading Column:	TurboFlow XL C18 column (Thermo Fisher Scientific, Franklin, MA)
Analytical Column:	50 x 3 mm, 5 µm Hypersil GOLD™ column (Thermo Fisher Scientific, Bellefonte, PA)
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile
Mobile Phase D:	20% acetone/40% methanol/40% acetonitrile
Autosampler Injection Size:	10 µL
Sample Extraction Solution:	50:50 (A/B)

The Aria TLX TurboFlow method is shown in Figure 4. The analytical run was completed in less than 6 minutes.

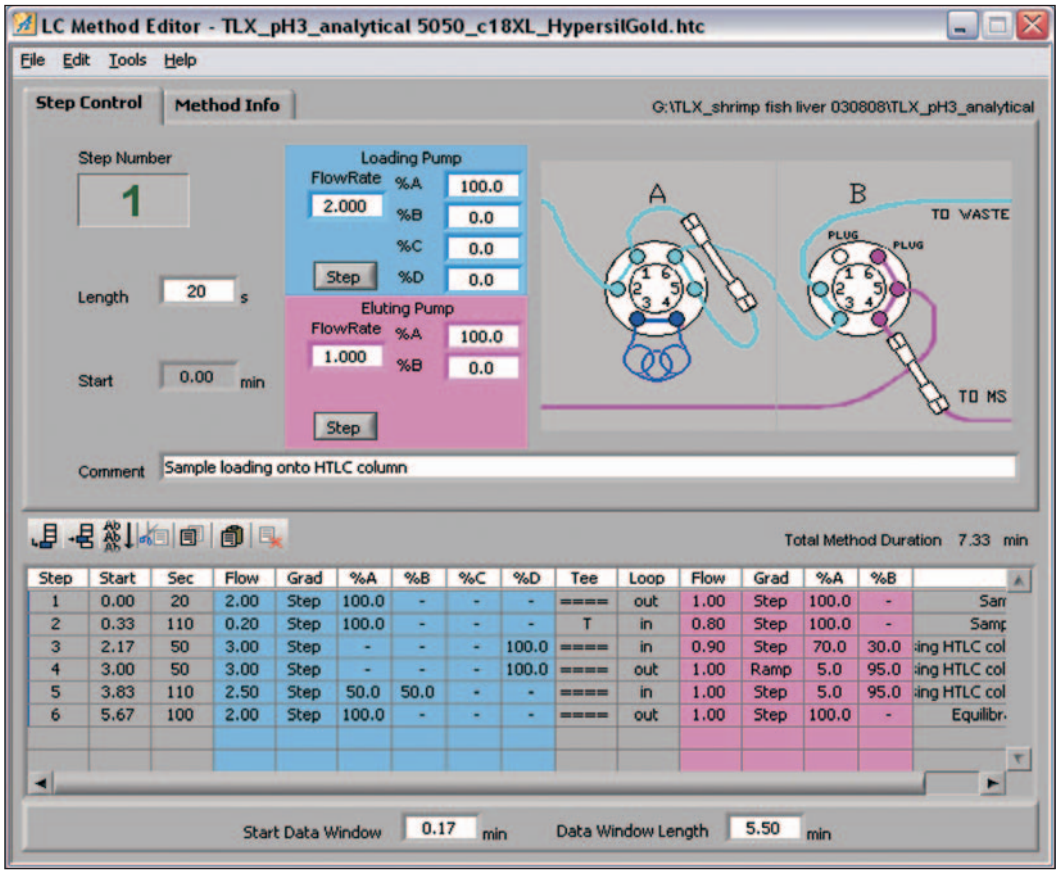

Figure 4: Aria TLX method

MS Conditions

MS analysis was carried out on a TSQ Quantum Access triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ion Source Polarity:	Positive ion mode
Spray Voltage:	3500 V
Vaporizer Temperature:	472 °C
Sheath Gas Pressure (N_2):	40 arb
Auxiliary Gas Pressure (N_2):	50 arb
Ion Transfer Tube Temperature:	270 °C
Skimmer Offset:	5 V
Collision Gas (Ar):	1.5 mTorr
Q1/Q3 Peak Resolution:	0.7 u (unit mass resolution)
Scan Mode:	Selected Reaction Monitoring

The MS method is shown in Figure 5.

Results and Discussion

As the international food trade continues to grow, so does the need for careful monitoring of the food supply to ensure that levels of drug residues and other chemical contaminants are below established standards. In this study, ciprofloxacin, tetracycline, and malachite green/leucomalachite green were examined in several food matrices.

LC-MS/MS assays of chemical residues in food matrices typically require extensive sample preparation prior to analysis, which can be time consuming and expensive. The Aria TLX system with TurboFlow technology eliminates the need for lengthy sample preparation steps. In this study, each sample was centrifuged once to clean out any floating particles and then immediately injected onto the column. Most of the time-consuming steps in the sample preparation process were removed, which increased sample throughput and helped to minimize errors and variability.

The Aria TLX system software allows both HPLC and TurboFlow methods to be run on a single system, injection to injection. To evaluate the differences in performance between a standard HPLC

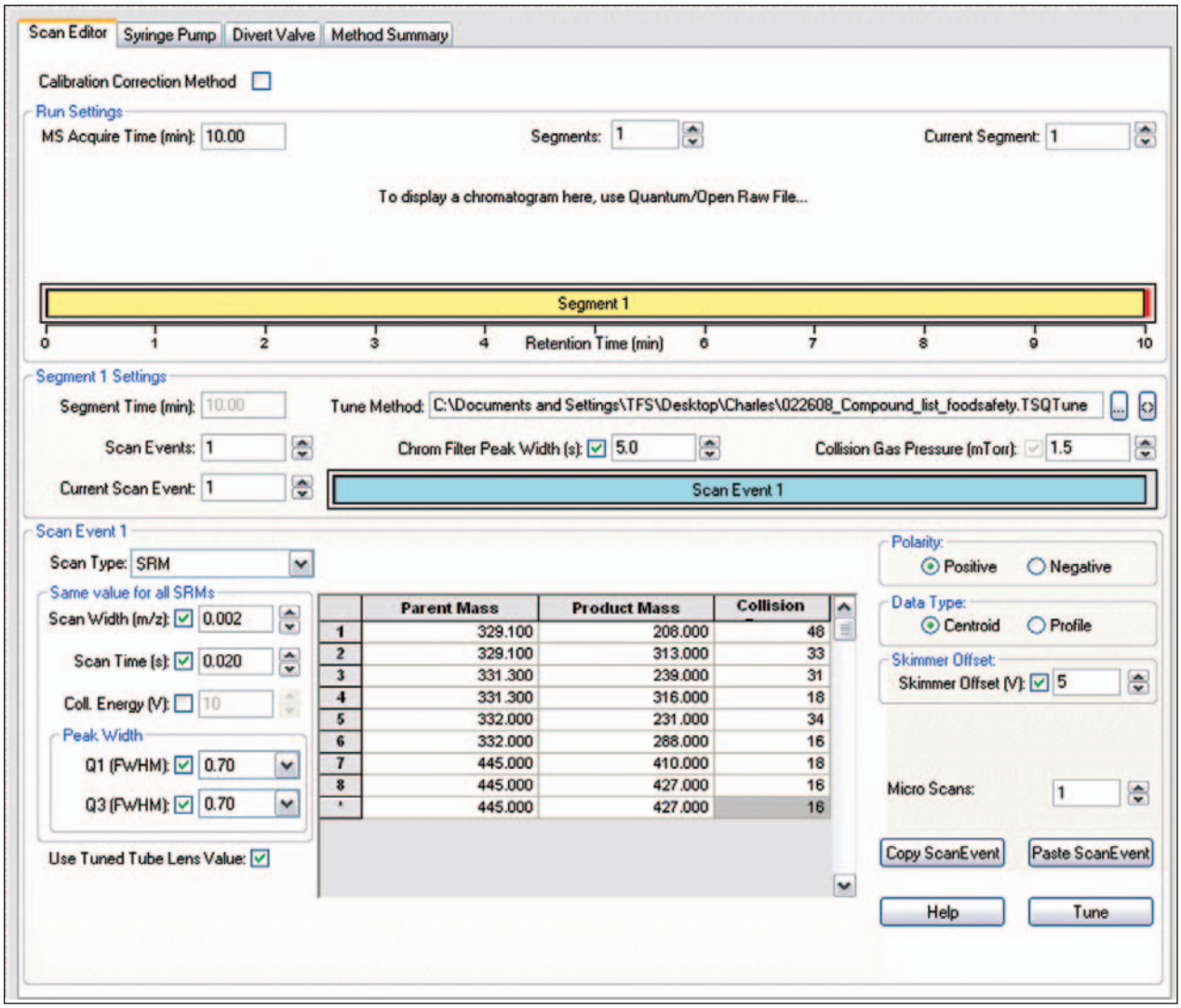

Figure 5: MS method showing the SRM transitions that were monitored

method and a TurboFlow method, each sample was analyzed by both methods. In the standard HPLC method, only the analytical HPLC column was used. In the TurboFlow method, the TurboFlow column and the analytical column were used. Figure 6 compares representative standard HPLC and TurboFlow method chromatograms of 500 ng/kg (parts per trillion) tetracycline in the fish matrix. The TurboFlow method chromatogram shows that interferences present in the standard HPLC chromatogram have been removed. The turbulent flow properties successfully remove matrix interferences that cause ion suppression.

Similar results were observed in the analysis of drugs in other food matrices. Figure 7 compares representative standard HPLC and TurboFlow method chromatograms

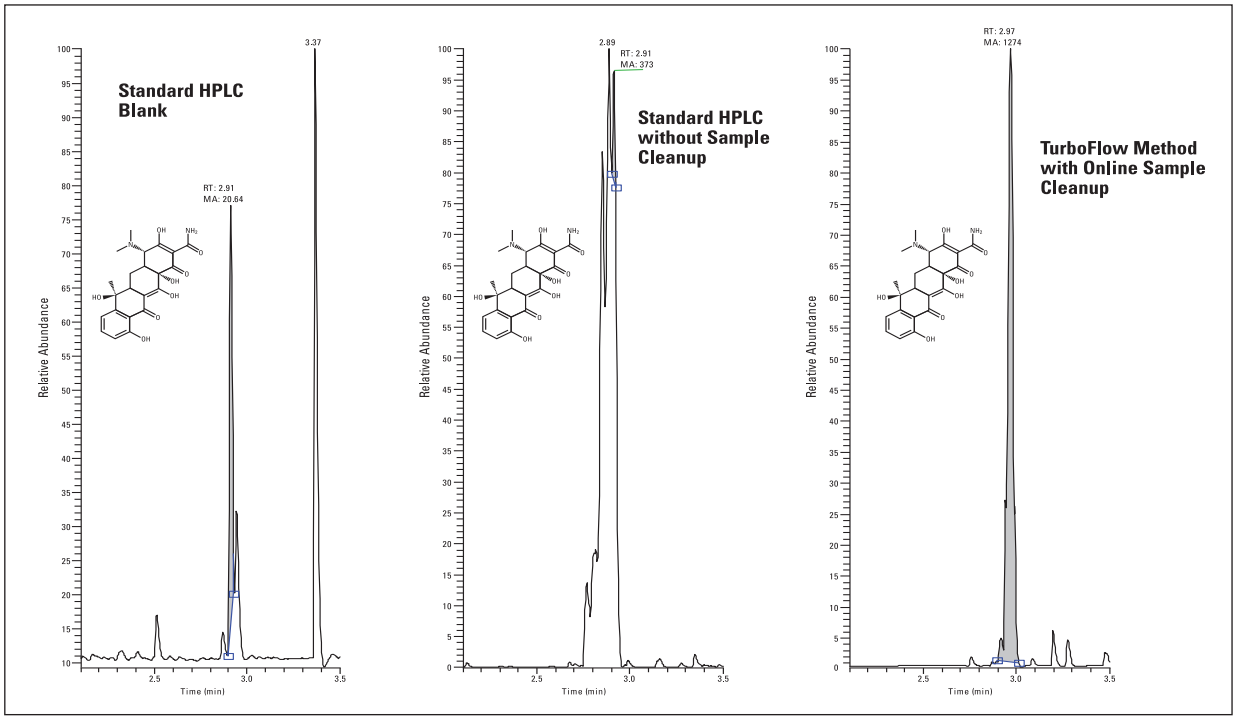

Figure 6: Chromatogram comparison of tetracycline at 500 ng/kg in fish (tilapia) matrix in standard HPLC and TurboFlow method

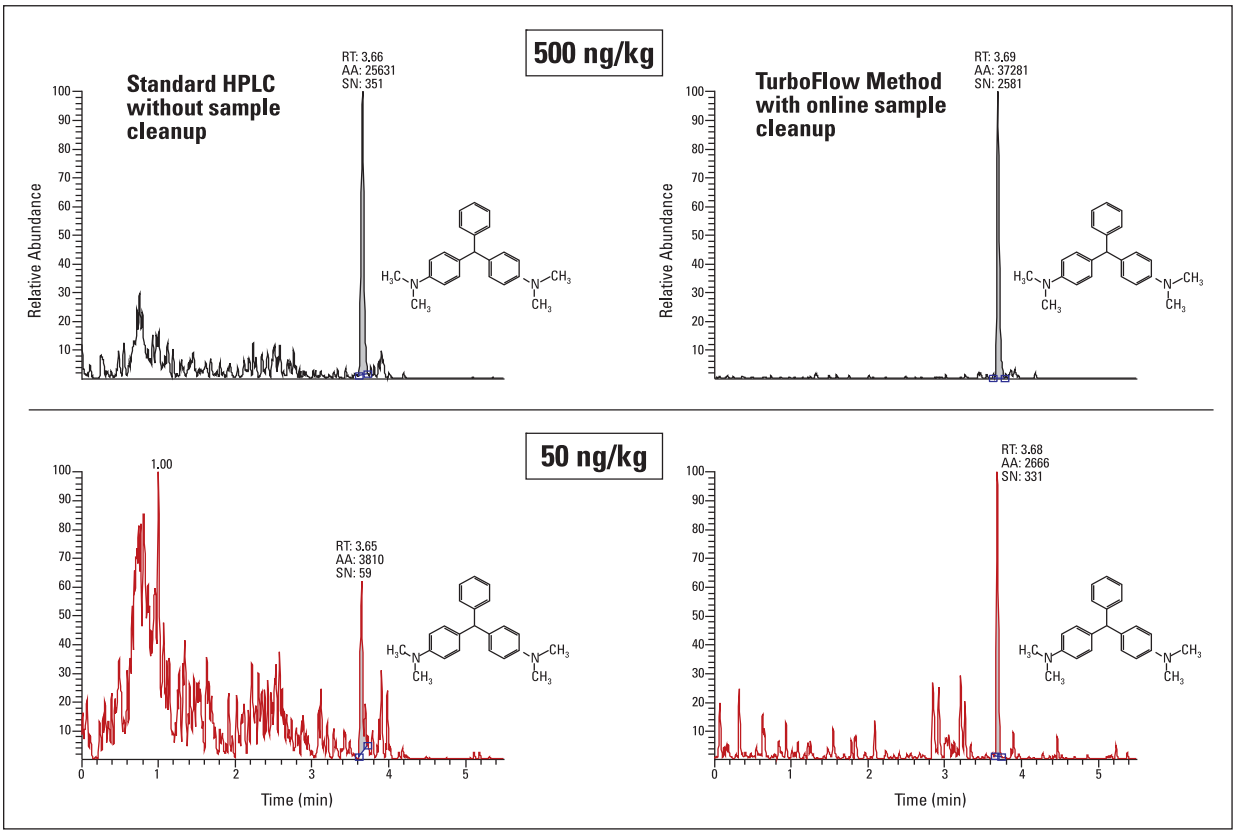

Figure 7: Chromatogram comparison of LMG at 500 ng/kg and 50 ng/kg in shrimp matrix in standard HPLC and TurboFlow method

for leucomalachite green at both 500 ng/kg (ppt) and 50 ng/kg (ppt) in the shrimp matrix. The TurboFlow method chromatograms show improved signal-to-noise ratios and significant reduction in ion suppression. The most dramatic results are shown in the analysis of tetracycline at 500 ng/kg (ppt) in pig liver (Figure 8).

Peak areas were used for quantitation and the resultant linearity of responses is plotted in Figure 9 for both the standard HPLC and the TurboFlow methods for ciprofloxacin in pig liver. Excellent R^2 values were observed for the TurboFlow method. A data summary showing the improvement in the TLX system results over those of the standard HPLC results is shown in Table 1. The R^2 values for the TLX system results were all greater than 0.99 for the linear regression equations (1/x weighted) in the concentration ranges tested.

Table 1 shows the results of the assay for ciprofloxacin, MG, LMG, and tetracycline in fish, shrimp, and pork liver extracts. The limits of quantitation (LOQs) achieved for all four analytes using online extraction followed by LC-MS/MS were significantly better compared to that achieved by standard HPLC. This indicates the removal of endogenous tissue by the Aria TLX system, thus reducing ion suppression effects and increasing detection limits.

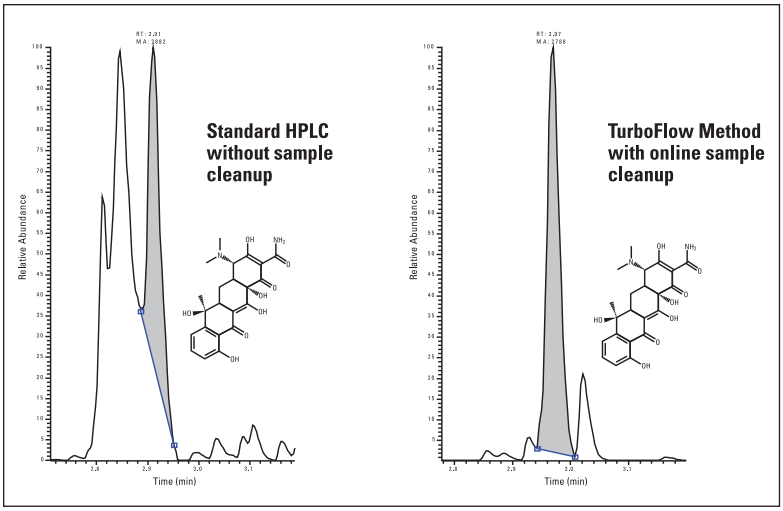

Figure 8: Chromatogram comparison of tetracycline at 500 ng/kg in pig liver matrix in standard HPLC and TurboFlow method

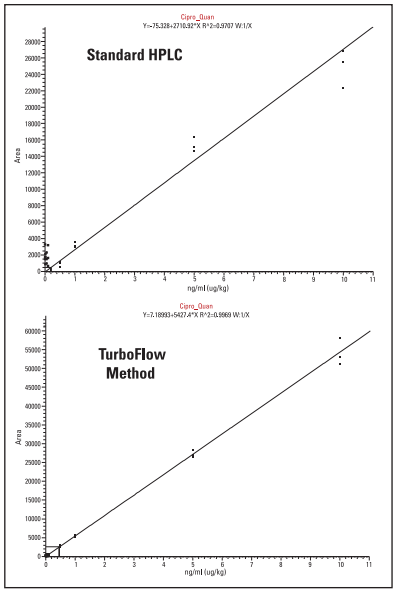

Figure 9: Ciprofloxacin calibration 1/x on standard HPLC vs. TurboFlow method in pig liver matrix

	Standard HPLC					TurboFlow Method				
	LOD (µg/kg)	%RSD n = 3	LOQ (µg/kg)	%RSD n = 3	R^2	LOD (µg/kg)	%RSD n = 3	LOQ (µg/kg)	%RSD n = 3	R^2
Fish										
Ciprofloxacin	0.5	32.0	1.0	8.0	0.9875	0.1	15.9	0.5	7.1	0.9968
MG	0.1	23.0	0.5	1.4	0.9988	0.1	6.8	0.1	8.2	0.9984
LMG	0.5	7.4	0.5	7.4	0.9990	0.1	12.0	0.1	12.0	0.9983
Shrimp										
Ciprofloxacin	5.0	10.5	5.0	10.5	0.9580	0.5	16.0	1.0	2.0	0.9906
MG	0.1	21.4	0.5	7.2	0.9991	0.05	7.9	0.05	7.9	0.9990
LMG	0.1	20.5	0.5	11.0	0.9975	0.05	13.5	0.1	11.2	0.9988
Pig Liver										
Ciprofloxacin	0.5	38.8	1.0	10.4	0.9707	0.1	29.0	0.5	8.6	0.9969
Tetracycline	0.5	14.8	1.0	10.5	0.9932	0.1	11.5	0.5	11.3	0.9953

Table 1: Data summary showing the improvement in the TurboFlow method results over those of the standard HPLC results. Note that results are only shown for a compound in the matrix in which it would be found; for example, MG and LMG would be found in fish but not in pig liver.

Conclusion

A rapid, sensitive and reliable method for the quantitation of veterinary drugs in food matrices was developed using the Aria TLX-1 system with the TSQ Quantum Access mass spectrometer. Minimal sample preparation was required because the TurboFlow method allows direct injection of samples into the system. The overall processing time for analysis was significantly shortened compared to methods using offline sample preparation. In addition, the Aria TLX system reduced ion suppression and matrix effects compared to standard HPLC runs.

References

1. Plakas, S. M.; Doerge, D. R.; Turnipseed, S. B. Disposition and Metabolism of Malachite Green and Other Therapeutic Dyes in Fish. In *Xenobiotics in Fish*; Smith, D. J., Gingerich, W. H., Beconi-Barker, M. G., Eds.; Plenum Press: New York City, 1999; p. 149-166.
2. European Commission, Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, as amended by Decision 2003/181/EC(4), (*Official Journal of the European Communities* L 221, 17.08.2002, p. 8-36).
3. U.S. Food and Drug Administration. Federal Register: May 22, 1997 (Volume 62, Number 99).
4. European Parliament and Council, Regulation (EC) 1181/2002 of 1 July 2002, amending Annex I of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin food (*Official Journal of the European Communities* L 172, 2.07.2002).
5. European Parliament and Council, Regulation (EC) 508/1999 of 4 March 1999, amending Annex I of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin food (*Official Journal of the European Communities* L 60, 9.3.1999).

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