Application Note

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Veterinary Drug Analysis in Seafood Tissue

Direct Analysis in Real Time (DART) and High Performance Liquid Chromatography (HPLC) Coupled with the Agilent 6400 Series Triple Quadrupole Mass Spectrometer (QQQ-MS)

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Introduction

Aquaculture is a growing global industry; the probability of >93,000 tons of global production is estimated beyond 2030[1]. As production increases, the increased use of veterinary aquaculture drugs has become a worldwide concern. Despite the controls and regulations of the use of veterinary aquaculture drugs and nonprescribed antibiotics, in some countries the concerns over antimicrobial resistance and toxicity are increasing. U.S. food imports continue to grow, increasing the need for a rapid and sensitive screening technique for the detection of unapproved veterinary aquaculture drugs in seafood. Several manufacturers have developed rapid screening protocols using enzyme-linked immunosorbent assays (ELISAs) to determine veterinary drug residues. The optimal, sensitive instrument method is high-pressure liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS or triple quadrupole/MS), as this instrument platform significantly reduces background signal, and allows accurate, quantitative measurement of veterinary drugs at very low levels in sample matrices[2].

However, Direct Analysis in Real Time Mass Spectrometry (DART-MS), Ionsense, Inc., facilitates a real-time laboratory analysis of a wide range of veterinary drug classes found in tissue products, while eliminating standard protocols and procedures required of typical HPLC-MS. Usually, time-consuming ELISA kits are used to target individual veterinary drugs-of-interest. Analysis using DART-MS affords an instantaneous, real-time, simultaneous detection of many veterinary drug classes.

Ion source technology, such as DART-MS, was developed for rapid, noncontact analysis of materials and sample matrices at ambient, pressure/ground potential, which is the minimum quantity of energy expended for molecular ionization to a charged state. Volatile samples are introduced under a vacuum source where they are ionized by reactions with metastable atoms. The technology, created in 2005, is used as a first screen for human-used illegal drugs in both state and federal laboratories, and has been validated with HPLC-MS technology as a confirmatory method. Some examples of successful applications of the technology include the detection of pharmaceutics, metabolites, peptides and oligosaccharides, synthetic organics, organometallics, drugs of abuse, explosives, and toxic industrial chemicals. The DART-MS, currently used for various surfaces, such as concrete, asphalt, human skin, currency, airline boarding passes, business cards, fruits, vegetables, spices, beverages, bodily fluids, horticultural leaves, cocktail glasses, and clothing, provides instantaneous response in real time, which is critical for high-throughput and enhanced analytical capability[3].

Triple quadrupole (QQQ) MS technology has been used successfully. This includes the Agilent 6495 Triple Quadrupole LC/MS System, which can detect very low levels of veterinary drugs, and is built on the performance of Agilent iFunnel Technology. The 6495 System extends analytical sensitivity, dynamic range, robustness, precision, and accuracy to previously unreachable levels[4]. This Application Note describes the novel use of the Agilent proprietary iFunnel QQQ technology, in combination with the real-time analytical capability of the DART ion source, to analyze shrimp and fish seafood matrices with a standard mix of veterinary drugs spiked into the tissues.

To comprehensively examine both DART and HPLC-ESI (Electron Spray Ionization) capabilities using the Agilent 6400 Series Triple Quadrupole/MS, a series of veterinary drug analyses were completed in parallel using the Agilent 1290 HPLC-6495 System along with the DART-6495 QQQ/MS (Figure 1).

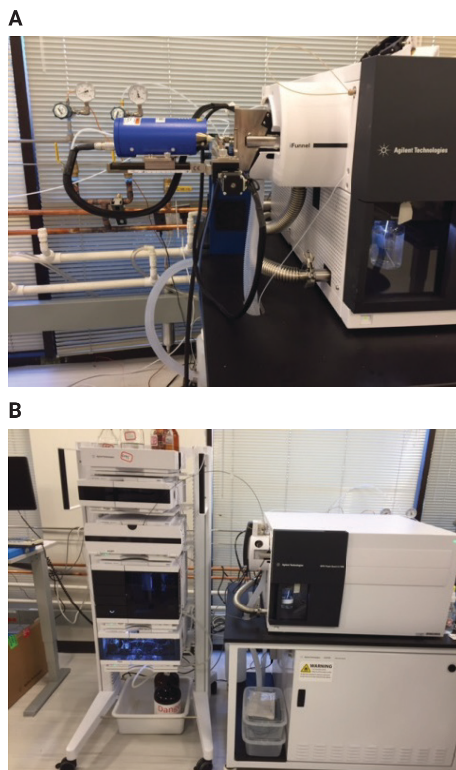

Figure 1. A) Ionsense, Inc., SVP-DART coupled to an Agilent 6495 Triple Quadrupole MS.
B) Agilent 1290 HPLC coupled to an Agilent 6495 Triple Quadrupole MS.

Experimental

Separations of analytes were carried out using both an Agilent 6495 iFunnel (triple quadrupole MS, model G6495A) and an Ionsense, Inc., SVP-DART with Vapur Interface. A combined standard of nine veterinary drugs (Figure 2, Sigma-Aldrich) was analyzed to determine a baseline for quantitation, detection, linear dynamic range, and analytical sensitivity of the DART-QQQ platform to these components.

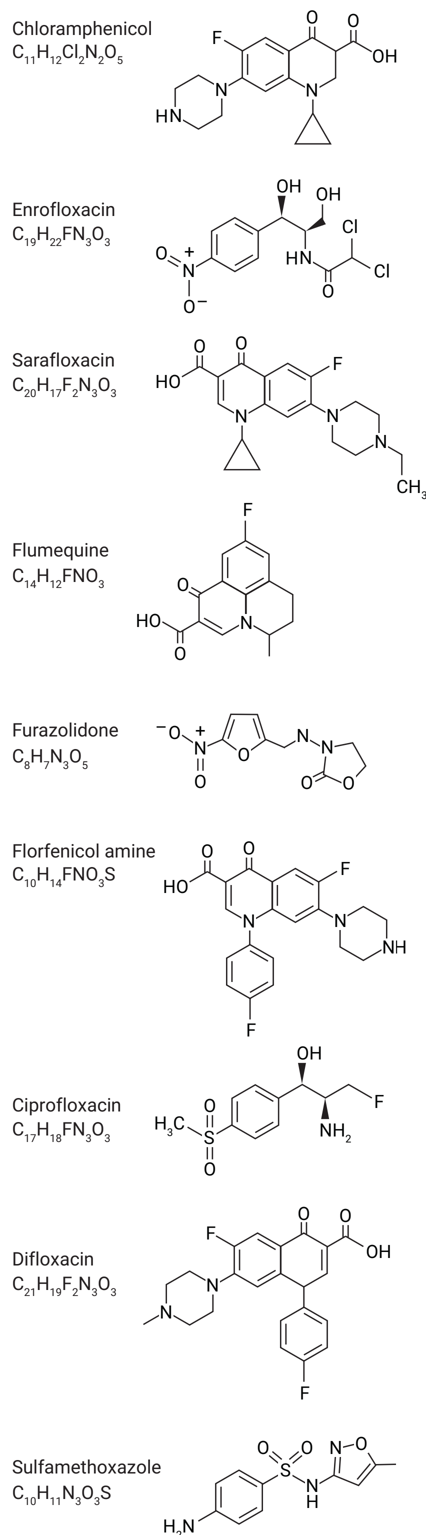

Figure 2. Veterinary aquaculture drugs analyzed using DART-MS and HPLC-MS.

Agilent Bond Elut Enhanced Matrix Removal–Lipid (EMR—Lipid) was used for fish and shrimp tissue extraction. The EMR—Lipid dSPE kit includes a dispersive sorbent, premeasured in a 15-mL centrifuge tube (EMR—Lipid, p/n 5982-1010), as well as a prepacked $MgSO_4$ polish powder (p/n 5982-0102). Approximately 15 g each of *l. setiferus* (white shrimp) and 5 g of *Oreochromis spp*. (tilapia) were transferred to ceramic mortars and thoroughly macerated to lyse the cell walls of the tissue. Aliquots of 2 g each of the macerated tissue were transferred to 50-mL polypropylene centrifuge tubes. A combined spiking solution with the nine drugs listed in Figure 2 was prepared in deionized water after separate spike compound preparations in water (with the exception of chloramphenicol, prepared in EtOH, Fisher Chemical, LC/MS grade) were made. The combined solution spike concentrations were: 500 parts per billion (ppb), 250 ppb, 100 ppb, 50 ppb, and 10 ppb.

After tissue spiking, the samples were allowed to sit for 15 minutes. Subsequently, the spiked tissue was vortexed for 30 seconds, then 10 mL of 5 % formic acid in ACN (Fisher Chemical LC/MS Grade) was added to the centrifuge tube. The tube was vortexed for 1 minute and centrifuged at 4,000 rpm for 10 minutes. Following centrifugation, the dispersive EMR—Lipid sorbent was activated by adding 5 mL of 5 mM ammonium acetate to a prefilled 15-mL dispersive solid phase extraction polypropylene tube (p/n 5982-1010) and vortexing for 1 minute. Alternatively, 18Ω purified water was used in later experiments, and yielded more promising results. Protocols substituting purified water *in lieu* of ammonium acetate in the dispersive step will be published in a later manuscript.

Immediately after vortexing the dispersive sorbent and the ammonium acetate, 5 mL of supernatant from the extracted sample tube was added to the tube with dispersive sorbent and ammonium acetate, vortexed for 1 minute, and centrifuged at 4,000 rpm for 10 minutes. Next, the entire extract was decanted from the EMR—Lipid centrifuged tube into a 50-mL centrifuge tube, and the content of one $MgSO_4$ polish pouch was added. This tube was first shaken, then vortexed for 1 minute and centrifuged for 10 minutes at 4,000 rpm. The upper layer of the centrifuged tube with $MgSO_4$ powder was then transferred into a clean 15-mL polypropylene tube and evaporated with N_2 gas in a water bath at 65 ±5 °C until 10 µL remained in the tube.

The 15-mL tube was reconstituted with 100 µL of 1:1 5 mM ammonium formate/MeOH (Fisher Chemical LC/MS Grade). The contents of the reconstituted tube was vortexed for 30 seconds. A 5 µL amount of solution per screen spot was applied to spot DART QuickStrip Cards. To complete the HPLC analysis, 50 µL of reconstituted solution was transferred to a 2-mL microcentrifuge tube and centrifuged for 30 seconds. Subsequently, 30 µL of the centrifuged solution was transferred to an Agilent HPLC vial with a polymer insert (p/n 5180-1270). Figure 3 illustrates a flowchart diagram for Agilent Bond Elut EMR—Lipid dSPE. Figure 4 graphically demonstrates the beneficial cleanup effect of the Bond Elut Enhanced Matrix Removal Kit.

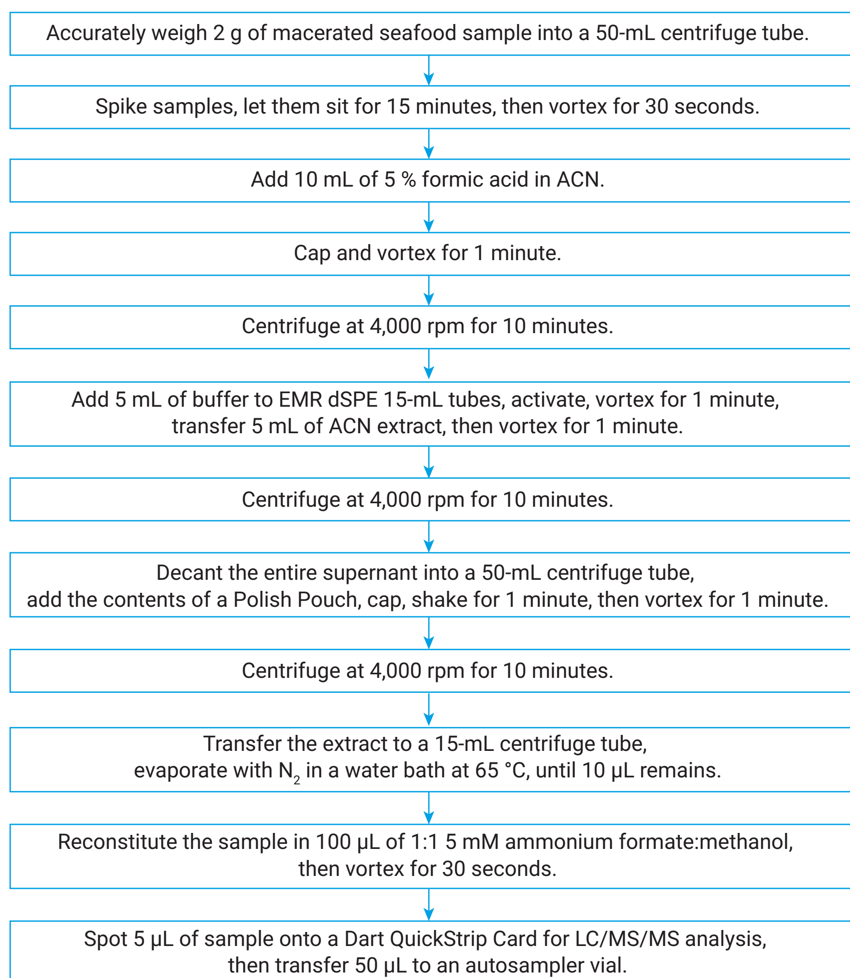

Figure 3. Sample preparation procedure using Agilent Bond Elut EMR—Lipid dSPE for the analysis of veterinary drugs in seafood.

Instrument Platform Analysis Using DART – Agilent 6495 Triple Quadrupole/MS

Figure 5 depicts parameters used for the DART ion source. Positive or negative polarities were set according to analytes of interest. A QuickStrip sampling configuration was used with a run temperature of 400 °C, a heater wait time of 30 seconds, a sample speed of 0.5 mm/s, and a contact closure delay of 2 seconds. Following iterative experimentation, a discovery was made that a second analytical pass of the same DART QuickStrip yielded optimal peak heights and resolution (sulfamethoxazole, 50 ppb, Figure 6). Figure 7 shows the optimized program settings for the 6495 QQQ/MS software. Figures 8, 9, and 10 list the MRM transitions used for the veterinary aquaculture drugs analyzed. Figure 5 illustrates the program settings used for the Ionsense, Inc. DART ion source.

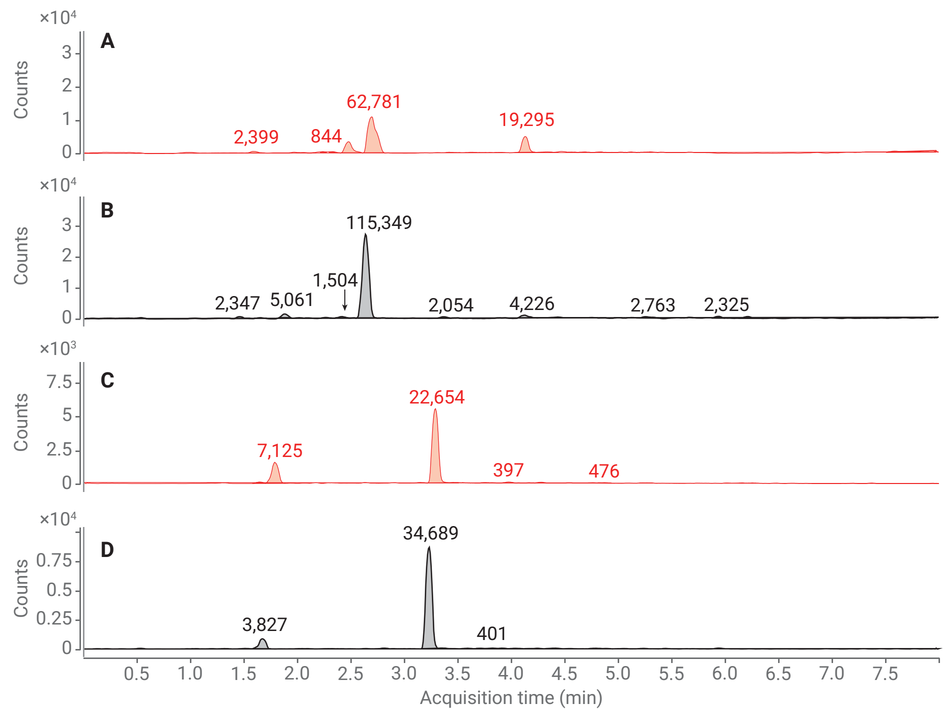

Figure 4. Furazolidone and chloramphenicol before and after EMR—Lipid cleanup. Note that the extra peaks in the chromatogram before cleanup are greatly reduced after cleanup.

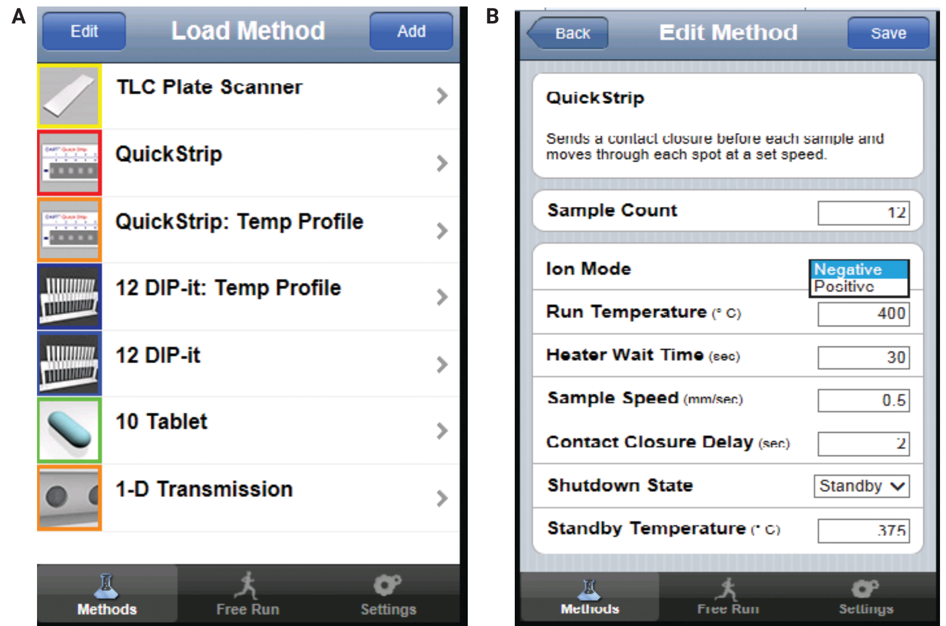

Figure 5. Software program settings for the DART ion source.

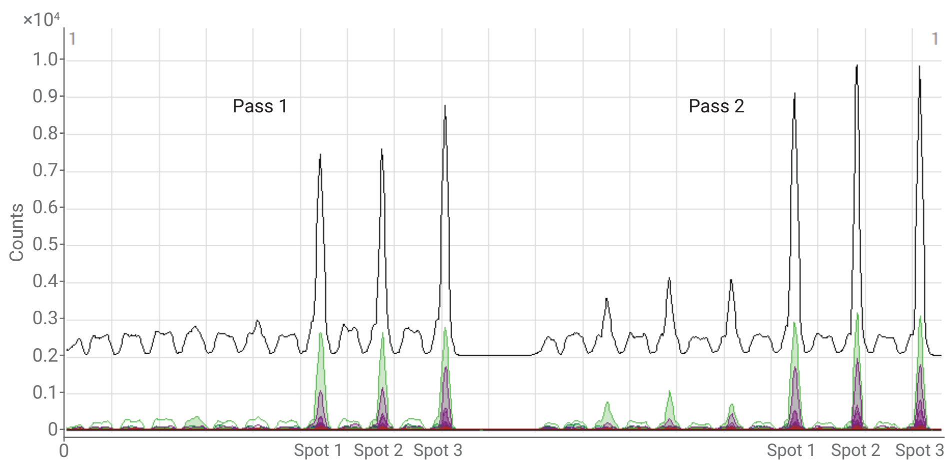

Figure 6. 50 ppb Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$), first and second DART QuickStrip passes.

Acquisition | **Source** | Chromatogram | Instrument | Diagnostics

Source parameters

	Setting	Unit	Actual	Unit
Gas Temp:	200	°C	200	°C
Gas Flow:	11	l/min	11.0	l/min
Nebulizer:	0	psi	1.0	psi

	Positive		Negative		Actual	
Capillary:	1000	V	1000	V	1575	nA

Chamber Current: 0.24 µA

iFunnel parameters

	Positive		Negative	
High Pressure RF	150	V	90	V
Low Pressure RF	60	V	60	V

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Figure 7. Agilent 6495 Triple Quadrupole/MS optimized program method settings.

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Difloxacin	☐	400.1	Unit	382.1	Unit	5	380	20	2	Positive
	Difloxacin	☐	400.1	Unit	356.2	Unit	5	380	16	2	Positive
	Difloxacin	☐	400.1	Unit	334.1	Unit	5	380	32	2	Positive
	Difloxacin	☐	400.1	Unit	306.1	Unit	5	380	36	2	Positive
	Difloxacin	☐	400.1	Unit	299.1	Unit	5	380	32	2	Positive
	Sarafloxacin	☐	386.1	Unit	368.1	Unit	5	380	20	2	Positive
	Sarafloxacin	☐	386.1	Unit	348.1	Unit	5	380	40	2	Positive
	Sarafloxacin	☐	386.1	Unit	342.1	Unit	5	380	20	2	Positive
	Sarafloxacin	☐	386.1	Unit	320.1	Unit	5	380	40	2	Positive
	Sarafloxacin	☐	386.1	Unit	299.1	Unit	5	380	40	2	Positive
	Enrofloxacin	☐	360.2	Unit	342.2	Unit	5	380	20	2	Positive
	Enrofloxacin	☐	360.2	Unit	316.2	Unit	5	380	16	2	Positive
	Enrofloxacin	☐	360.2	Unit	286.1	Unit	5	380	36	2	Positive
	Enrofloxacin	☐	360.2	Unit	245.1	Unit	5	380	32	2	Positive
	Enrofloxacin	☐	360.2	Unit	203.1	Unit	5	380	44	2	Positive
	Ciprofloxacin	☐	332.1	Unit	314.1	Unit	5	380	20	2	Positive
	Ciprofloxacin	☐	332.1	Unit	288.2	Unit	5	380	20	2	Positive
	Ciprofloxacin	☐	332.1	Unit	245.1	Unit	5	380	20	2	Positive

Figure 8. MRM transitions for Difloxacin ($C_{21}H_{19}F_2N_3O_3$), Sarafloxacin ($C_{20}H_{17}F_2N_3O_3$), Enrofloxacin ($C_{19}H_{22}FN_3O_3$), and Ciprofloxacin ($C_{17}H_{18}FN_3O_3$).

Acquisition | Source | Chromatogram | Instrument

Scan segments

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Chloramphenicol	☐	321	Unit	46.1	Unit	5	380	60	4	Negative
	Flumequine	☐	262.1	Unit	244.1	Unit	5	380	12	4	Positive
	Flumequine	☐	262.1	Unit	202	Unit	5	380	32	4	Positive
	Flumequine	☐	262.1	Unit	126	Unit	5	380	52	4	Positive
	Flumequine	☐	262.1	Unit	99.1	Unit	5	380	70	4	Positive
	Flumequine	☐	262.1	Unit	75.1	Unit	5	380	70	4	Positive
	Sulfamethoxazole	☐	254.1	Unit	156	Unit	5	380	12	4	Positive
	Sulfamethoxazole	☐	254.1	Unit	108.1	Unit	5	380	24	4	Positive
	Sulfamethoxazole	☐	254.1	Unit	92.1	Unit	5	380	24	4	Positive
	Sulfamethoxazole	☐	254.1	Unit	80.1	Unit	5	380	56	4	Positive
	Sulfamethoxazole	☐	254.1	Unit	65.1	Unit	5	380	48	4	Positive
	florfenicol amine	☐	248.1	Unit	230	Unit	10	380	12	4	Positive
	florfenicol amine	☐	248.1	Unit	130.1	Unit	10	380	24	4	Positive
	Furazolidone	☐	226	Unit	95	Unit	5	380	20	3	Positive
	Furazolidone	☐	226	Unit	83	Unit	5	380	20	3	Positive
	Furazolidone	☐	226	Unit	67	Unit	5	380	40	3	Positive

Figure 9. MRM transitions for Ciprofloxacin ($C_{17}H_{18}FN_3O_3$), Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$), Flumequine ($C_{14}H_{12}FNO_3$), Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$), and Florfenicol amine ($C_{10}H_{14}FNO_3S$).

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Ciprofloxacin	☐	332.1	Unit	245.1	Unit	5	380	20	2	Positive
	Ciprofloxacin	☐	332.1	Unit	231.1	Unit	5	380	40	2	Positive
	Chloramphenicol	☐	321	Unit	257	Unit	5	380	0	2	Negative
	Chloramphenicol	☐	321	Unit	152.1	Unit	5	380	4	2	Negative
	Chloramphenicol	☐	321	Unit	121	Unit	5	380	36	2	Negative
	Chloramphenicol	☐	321	Unit	46.1	Unit	5	380	60	2	Negative
	Flumequine	☐	262.1	Unit	244.1	Unit	5	380	12	2	Positive
	Flumequine	☐	262.1	Unit	202	Unit	5	380	32	2	Positive
	Flumequine	☐	262.1	Unit	126	Unit	5	380	52	2	Positive
	Flumequine	☐	262.1	Unit	99.1	Unit	5	380	70	2	Positive
	Flumequine	☐	262.1	Unit	75.1	Unit	5	380	70	2	Positive
	Sulfamethoxazole	☐	254.1	Unit	156	Unit	5	380	12	2	Positive
	Sulfamethoxazole	☐	254.1	Unit	108.1	Unit	5	380	24	2	Positive
	Sulfamethoxazole	☐	254.1	Unit	92.1	Unit	5	380	24	2	Positive
	Sulfamethoxazole	☐	254.1	Unit	80.1	Unit	5	380	56	2	Positive
	Sulfamethoxazole	☐	254.1	Unit	65.1	Unit	5	380	48	2	Positive
	florfenicol amine	☐	248.1	Unit	230	Unit	10	380	12	2	Positive
	florfenicol amine	☐	248.1	Unit	130.1	Unit	10	380	24	2	Positive

Figure 10. MRM transitions for Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$), Flumequine ($C_{14}H_{12}FNO_3$), Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$), and Furazolidone ($C_8H_7N_3O_5$).

Instrument Platform Analysis Using the Agilent 1290 Infinity HPLC and Agilent 6495 Triple Quadrupole/MS

Table 1 lists the Agilent 1290 Infinity HPLC column used, and method parameters. The 6495 QQQ/MS optimized program method settings used for HPLC analysis were identical to those used for the DART-QQQ/MS analysis.

Results and Discussion

To correct for sample matrix interference effects, combined matrix-matched standards (tissue samples spiked with veterinary drug standards) of both shrimp and fish tissues were analyzed to determine the veterinary drug limits of detection in the tissues examined. With the second analytical DART pass (Figure 6), all matrix-matched drug standards were detectable at 50 ppb, and several matrix-matched drugs were detectable as low as 1 ppb. Figures 11 and 12 illustrate DART-QQQ/MS data results for ciprofloxacin and flumequine using matrix-matched standards at concentrations of 1 ppb.

To correct for sample matrix interference effects, combined matrix-matched standards were analyzed to determine veterinary drug limits of detection in tissues examined using HPLC-QQQ/MS protocols. Analytes were easily detected in matrix-matched standards at 10 parts per trillion (ppt), as shown in the enrofloxacin example (Figure 13).

Table 1. Column and method parameters.

Parameter	Value
Column	Agilent Pursuit 3 PFP, 100 × 3 mm, 3 µm (p/n A3051100X030)
Mobile phase	Acetonitrile/water in 0.1 % formic acid
Injection volume	2 µL
HPLC separation program	Time (min) %Water %ACN 0.10 95.00 5.00 1.00 80.00 20.00 5.00 40.00 60.00 5.10 10.00 90.00 6.00 10.00 90.00 6.10 100.00 0.00

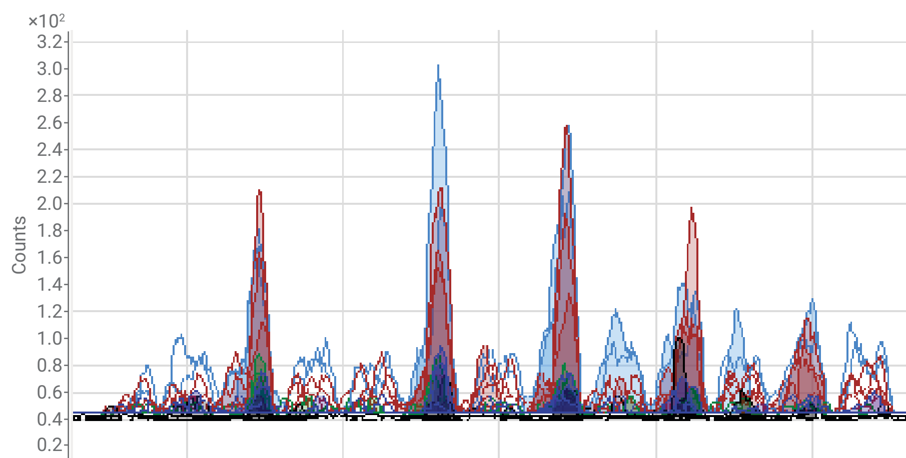

Figure 11. Ciprofloxacin ($C_{17}H_{18}FN_3O_3$), matrix-matched combined standard, 1 ppb.

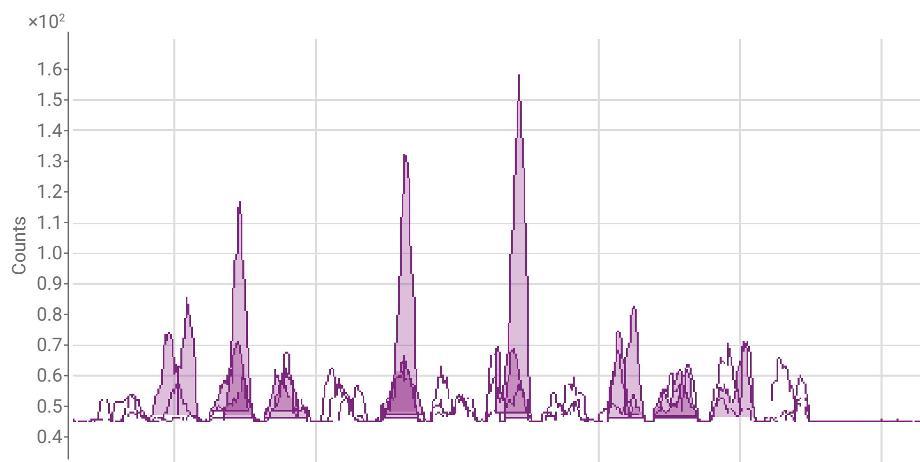

Figure 12. Flumequine ($C_{14}H_{12}FNO_3$), matrix-matched combined standard, 1 ppb.

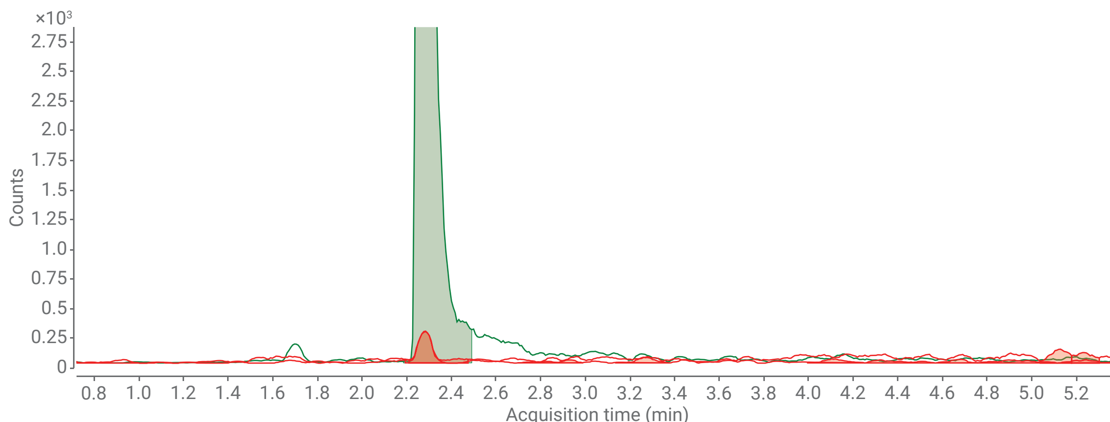

Figure 13. Enrofloxacin ($C_{19}H_{22}FN_3O_3$), 10 ppb standard (green peak) and 10 ppt matrix-matched standard (red peak).

Conclusions

The simplicity, speed, and extremely low reagent use of the DART-QQQ/MS analytical platform to examine harmful levels of veterinary drugs in seafood makes it an ideal initial assessment screen, particularly in tandem with a confirmatory HPLC-QQQ/MS method. Both DART-QQQ/MS and HPLC-QQQ/MS methods were developed for the detection of veterinary drugs using the Agilent 6400 Series Triple Quadrupole/MS; one analysis using DART coupled to an Agilent 6495 Triple Quadrupole/MS as an ion source for a fast, broad analytical drug screen, and another low-level quantitative technique using Agilent 1290 HPLC sample separation with an ESI source and a 6495 Series Triple Quadrupole/MS. Further experimentation should enhance the analyses of aquaculture drugs using the two methods demonstrated in this Application Note.

Anticipated future protocol testing includes comparing water versus ammonium acetate as a buffer in the dispersive extraction step of the analysis and further optimization of the DART ion source sample method and program profile to enhance screening results.

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