

APPLICATION OF ACQUITY TQD FOR THE ANALYSIS OF NITROFURAN VETERINARY DRUG RESIDUES IN SHRIMP

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INTRODUCTION

This application note describes the transfer of an existing UPLC® method¹ to the tandem quadrupole mass spectrometer, Waters® ACQUITY® TQD (Figure 1). Standard solutions spiked into blank matrix extract were used to assess chromatography as there will not be changes to the extraction of nitrofurantoin metabolites from edible tissues.

The four drugs furazolidone, furaltadone, nitrofurazone, and nitrofurantoin are veterinary drugs that belong to the family of nitrofuran antibiotics and are banned in meat destined for human consumption². These nitrofurans are readily metabolized in animals to undetectable levels, metabolizing to 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SC) and 1-aminohydantoin (AHD), respectively. The structures of the nitrofuran antibiotics and their free metabolites are shown in figure 2. The metabolites are more persistent in edible tissues and can be used to detect use of the parent drug. Proportions of these metabolites exist as protein adducts and the established method of analysis contains acid hydrolysis and derivatization steps prior to extraction³.

The European Union (EU) has banned the use of these substances, documenting them in Council Regulation 2377/90, annex IV.



Figure 1. ACQUITY TQD featuring the TQ detector.

A Minimum Required Performance Limit (MRPL) of 1 µg/kg has been set to which laboratories should be able to detect and confirm these compounds. Concerns over nitrofurans are related to potential carcinogenicity (causing cancer) and genotoxicity (causing damage to genetic material in cells) of the drugs and their metabolites as well as the ability to cause allergic reactions⁴.

The introduction of the ACQUITY TQD (Figure 1) allows scientists to perform nitrofuran metabolite analysis while harnessing all the benefits that this new instrument brings to the laboratory. The IntelliStart™ technology in this instrument is designed to reduce the burden of complicated operation, time-intensive troubleshooting, and upkeep. Its small footprint will give any laboratory an advantage as this powerful tool removes the need for larger instrumentation.

This note describes an extended nitrofuran method, for four residues and their associated internal standards, which exceeds the requirements for analysis of these regulated compounds.

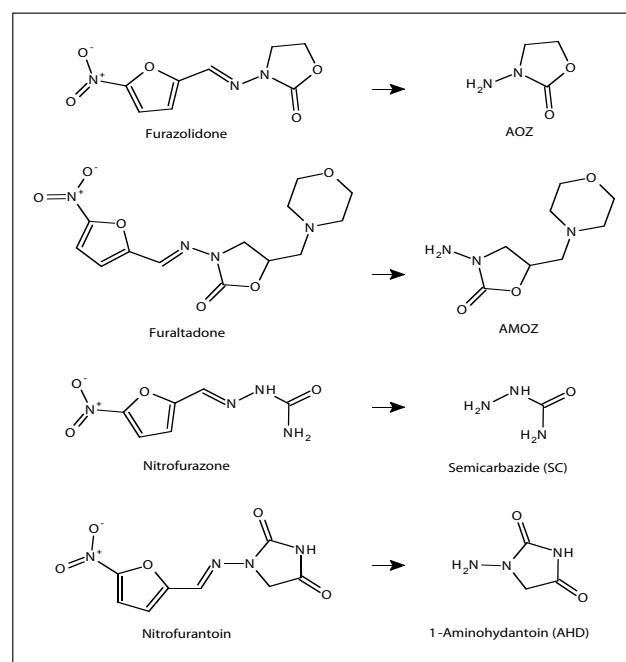


Figure 2. Nitrofuran antibiotics and their free metabolites.

EXPERIMENTAL

Solvent solutions were produced in 1:4 methanol: water (v/v) containing the four derivatized metabolites. These solutions were further diluted in extracted blank matrix to form a calibration curve over the range 0.05 to 5 ng/mL. Deuterated internal standards, 2-NP-AOZ-D₄, 2-NP-AMTZ-D₅, 2-NP-SC-¹³C₁₁N₂, and 2-NP-HD¹³C₃ were also added. The chromatography was assessed for each compound at each level. All concentrations reported in this application note are comparable to the nitrofurantoin antibiotics (pre-extraction) as correction factors, were applied to the volumes of derivatized metabolites. These correction factors also took into account the 2x concentration step during sample extraction. This means that the 0.1 ng/mL solution injected is directly comparable to shrimp containing 0.05 µg/kg.

UPLC CONDITIONS

| | |
|-----------------|---|
| LC system: | Waters ACQUITY UPLC® |
| Column: | ACQUITY UPLC BEH C ₁₈ column 2.1 x 100 mm, 1.7 µm |
| Column temp: | 40 °C |
| Flow rate: | 450 µL/min. |
| Mobile phase A: | 1:4 methanol: water (v/v) + 0.5 mM ammonium acetate |
| Mobile phase B: | 9:1 methanol: water (v/v) + 0.5 mM ammonium acetate |

MS CONDITIONS

| | |
|--------------------|--------------------------------------|
| MS system: | Waters ACQUITY TQ detector |
| Ionization mode: | ESI positive |
| Capillary voltage: | 1 KV |
| Cone voltage: | See table 1 |
| Desolvation gas: | Nitrogen, 1000 L/Hr, 450 °C |
| Cone gas: | Nitrogen, 5 L/Hr |
| Source temp: | 120 °C |
| Acquisition: | Multiple Reaction Monitoring (MRM) |
| Collision gas: | Argon at 3.5 x 10 ⁻³ mBar |

All compounds were optimized and two Multiple Reaction Monitoring (MRM) transitions were obtained for each derivatized metabolite and one for each internal standard. These two MRM transitions were monitored for each derivatized metabolite in accordance with European Union guidelines⁵. The primary transition is used for quantification and the secondary transition is used for confirmation purposes. These MRM transitions are listed in table 1, along with their respective cone voltages and collision energies.

Acquisition and Processing Methods

Waters MassLynx™ software v4.1 was used for data acquisition and the TargetLynx™ application manager was used for data processing.

| Metabolite | MRM Transition | Cone Voltage (V) | Collision energy (eV) |
|--|----------------|------------------|-----------------------|
| 2-NP-AOZ | 236.0>134.0 | 35 | 14 |
| | 236.0>104.0 | | 20 |
| 2-NP-AMTZ | 335.1>291.2 | 31 | 12 |
| | 335.1>100.0 | | 26 |
| 2-NP-SCA | 209.0>166.1 | 25 | 8 |
| | 209.0>192.1 | | 10 |
| 2-NP-AHD | 249.0>133.9 | 32 | 10 |
| | 249.0>104.0 | | 20 |
| 2-NP-AOZ-D ₄ | 240.0>134.0 | 35 | 13 |
| 2-NP-AMTZ-D ₅ | 340.1>296.2 | 30 | 12 |
| 2-NP-SCA- ¹³ C ₁₁ ¹⁵ N ₂ | 212.0>168.0 | 30 | 10 |
| 2-NP-AHD- ¹³ C ₃ | 252.0>134.0 | 30 | 11 |

Table 1. MRM transitions for the derivatized metabolites and their internal standards.

RESULTS AND DISCUSSION

All nitrofurans were separated successfully. Figure 3 shows the total ion chromatogram (TIC) for all derivatized metabolites. Only one time window was required for the eight compounds being analyzed. A dwell time of 0.025 seconds was used giving approximately 14 data points across each peak.

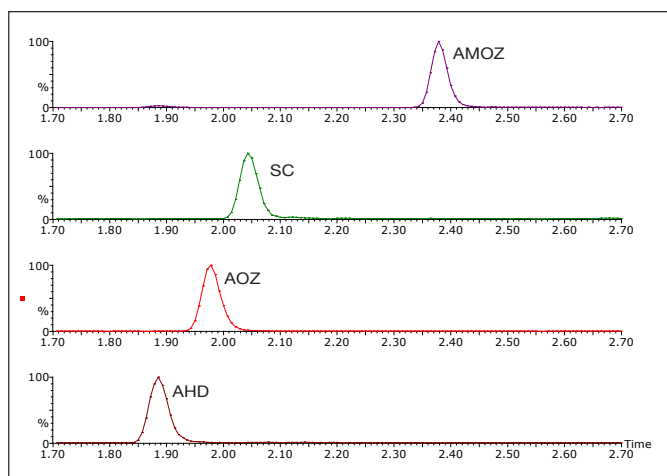


Figure 3. TIC showing the four nitrofuran metabolites in shrimp matrix at 1 ng/mL (equivalent to 2 µg/kg in shrimp).

The limit of detection achieved for all components of the derivatized metabolite mixture was less than 0.05 ng/mL (equivalent to 0.025 µg/kg in shrimp). This is forty times below the EU minimum required performance limit (MPRL) guideline. The 0.05 ng/mL matrix-matched standard for 2-NP-AMAZ is illustrated in Figure 4.

A calibration curve was spiked over the range of 0.05–5 ng/mL which gave a good linear response ($r^2=0.9972$). Good peak shape was achieved for both analyte and internal standard. The lowest quantifiable level achieved by the TQD in this analysis was 0.05 ng/mL. This level gave an acceptable S:N of 5:1 or greater for all four nitrofuran metabolites in shrimp with 50 µL injection volume. This level is twenty times lower than the MPRL set by the EU.

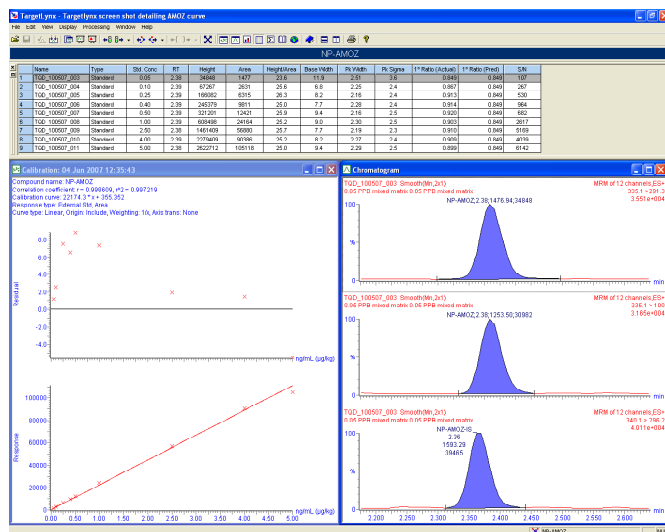


Figure 4. TargetLynx screen shot showing response for 2-NP-AMAZ at 0.05 ng/mL (0.025 µg/kg in shrimp).

A run of 50 samples in matrix at a concentration of 5 ng/mL was injected. This was used to assess robustness of ACQUITY TQD response over a four hour run. Injections of 10 µL were performed as a limited amount of sample was present. Figure 5 shows a plot of injection number vs. response ratio (analyte peak area/internal standard peak area). There is no noticeable change in response for any compound over the course of the run.

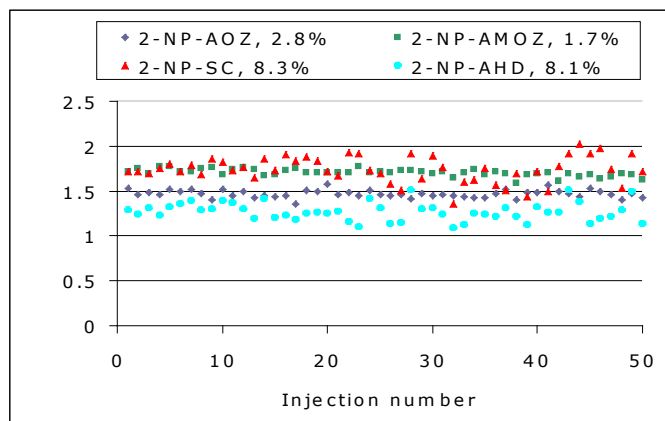


Figure 5. Plot showing peak area over 50 injections in shrimp. Extracts were injected at a concentration of 5 ng/mL. The percent relative standard deviation (% RSD) is shown in the graph header.

Figure 6 illustrates the robustness of the ion ratios over the same 50 injections. This plot shows that there is no significant change in ion ratio for any compound over the course of the run. A stable ion ratio is fundamental when confirming the identity of compounds.

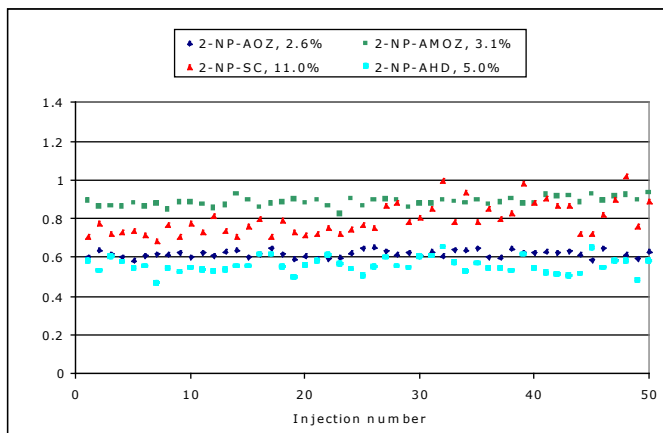


Figure 6. Plot showing peak area over 50 injections in shrimp. Extracts were injected at a concentration of 5 ng/mL. All injections lie within the $\pm 20\%$ boundary required for confirmation with the average difference shown in the header of the graph.

CONCLUSION

The established UPLC method for the determination of nitrofurantoin veterinary drug residues, which are hazardous to human health, has been successfully transferred to ACQUITY TQD.

The ACQUITY TQD provides sensitivity that allows quantifiable detection at twenty times below the EU minimum required performance limit (MPRL) guideline for the four nitrofurantoin metabolites.

Robustness of the ACQUITY TQD was proven with a 50 injection sequence that did not show a major change ($<20\%$) in response

ratio with relative standard deviations being 8.3% or less for all compounds.

Confirmation was achieved using a secondary MRM transition over a 50 injection sequence, where the variance was 11% or less in all cases showing it to be robust.

This multiple nitrofurantoin method obsoletes the use of several single nitrofurantoin methods where repeat analysis is required.

The method allows the determination of multiple contaminants per sample which enables a complete picture to be obtained of exposure to these compounds from the human diet.

The benefits of UPLC for a revenue conscious laboratory are shown with increased speed, while further reducing solvent usage and therefore the costs of solvents and solvent disposal.

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