Nota applicativa

Xevo TQ-S cronos for the Analysis of Banned Veterinary Drug Residues: Determination of Nitrofuran Metabolites and Chloramphenicol in Chicken Muscle at Regulatory Limits

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Abstract

The use of certain antibacterial compounds in livestock and aquaculture production for human consumption is prohibited due to concerns about their toxicity in the major global geographies, according to regulations established by the EU, Food and Drug Administration, CODEX Alimentarius, and Joint FAO/WHO Expert Committee on Food Additives (JECFA). Here we describe the method and report the performance characteristics of the ACQUITY UPLC I-Class PLUS System coupled to the Xevo TQ-S cronos for the quantitation of banned veterinary residues (chloramphenicol and four nitrofuran metabolites) in food of animal origin. The calibration characteristics and repeatability were all shown to be suitable for determination of residues at the respective EU Reference Point for Action in chicken muscle. The results of the performance evaluation show the Xevo TQ-S cronos to be robust for routine operation, with minimal operator intervention or maintenance during extended periods of analysis even for the challenging small, relatively polar analytes in complex extract.

Benefits

- Reliable, routine quantitative analysis of banned drug residues in foods of animal origin combined with Oasis solid phase extraction sample preparation products for compliance with stringent EU Regulations
- Demonstrating robust performance in complex matrix extract maximizing instrument uptime with minimal requirements for operator intervention over the run times typically required during compliance monitoring analysis

Introduction

Historically, there has been global concern about the safety of foods of animal origin contaminated with antibacterial residues. Within the European Union, for specific prohibited, or unauthorised pharmacologically active substances. Reference Points for Action (RPAs) in food have been established under Regulation (EU) 2019/1871.[1] RPAs are set at the lowest level which can analytically be achieved by the official control laboratories [Regulation (EU) 2017/625].[2] By consequence, the RPAs also define the minimum method performance requirements (MMPRs) for these substances in food. Food of animal origin found to contain these residues at concentrations equivalent to, or above the RPA, are considered not to comply with the legislation and shall not enter the food chain.

In 2019, RPAs of 0.15, 0.5, and 0.5 µg/kg were established for chloramphenicol, malachite green, nitrofurans, and their metabolites, respectively[1] within the EU. The use of these antibacterial compounds in livestock and aquaculture production is also banned in major global geographies by regulations established by the Food and Drug Administration, CODEX Alimentarius, and Joint FAO/WHO Expert Committee on Food Additives (JECFA).[3-5]

Nitrofurans are metabolised rapidly *in vivo* but stable tissue-bound metabolites are formed. Fragments of these metabolites may be released by mild acid hydrolysis and monitored as marker residues.[6] For example, 3-amino-2-oxazolidinone (AOZ) is monitored as a marker residue for the drug furazolidone. 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) is the marker residue for furaltadone, and 1-aminohydantoin (AHD) for nitrofurantoin and semicarbazide (SCA) for nitrofurazone. To complete the analysis and present extracts that are amenable to reverse phase chromatography, the resulting metabolites are normally derivatized with 2-nitrobenzaldehyde yielding the 'NB' derivatives.

The Xevo TQ-S cronos instrument was developed as a reliable system for robust, routine quantitative analysis in complex matrices. The Xevo TQ-S cronos incorporates a similar sample cone design that has been previously utilized in the extremely popular ACQUITY QDa. In addition to the reverse cone design, established Waters patented technology also contributes towards the robust performance of the Xevo TQ-S cronos including orthogonal geometry ion source, StepWave ion guide, and the T-Wave enabled collision cell. Previously published application notes have reported benefits for other quantitative residue analysis applications including pesticides, acrylamide, and the trimethylphenyl dyes.[7-9]

This study investigates the performance of the Xevo TQ-S cronos Tandem Quadrupole Mass Spectrometer coupled with the ACQUITY UPLC I-Class PLUS System for the determination of the banned veterinary drug residues of chloramphenicol (CAP) and the nitrofuran metabolites (NF).

Experimental

For this study, in-house methodology for sample extraction and SPE based clean-up was utilized. The UPLC gradient, ESI source, and MS/MS conditions were optimised for the analytes prior to the evaluation study. The performance of the Xevo TQ-S cronos was assessed according to the EU RPAs for CAP and the NF metabolites using pre-derivatized standards (as appropriate).

Preparation of Chicken Extracts

The general workflow scheme for the preparation of chicken muscle extracts is shown in Figure 1. Nitrofuran metabolites require a derivatization step to make them amenable for reverse mode chromatographic separation. For purpose of this study, pre-derivatized NF standards (purchased from Merck and LGC) were used to assess the performance of Xevo TQ-S cronos when spiked in extract of chicken muscle.

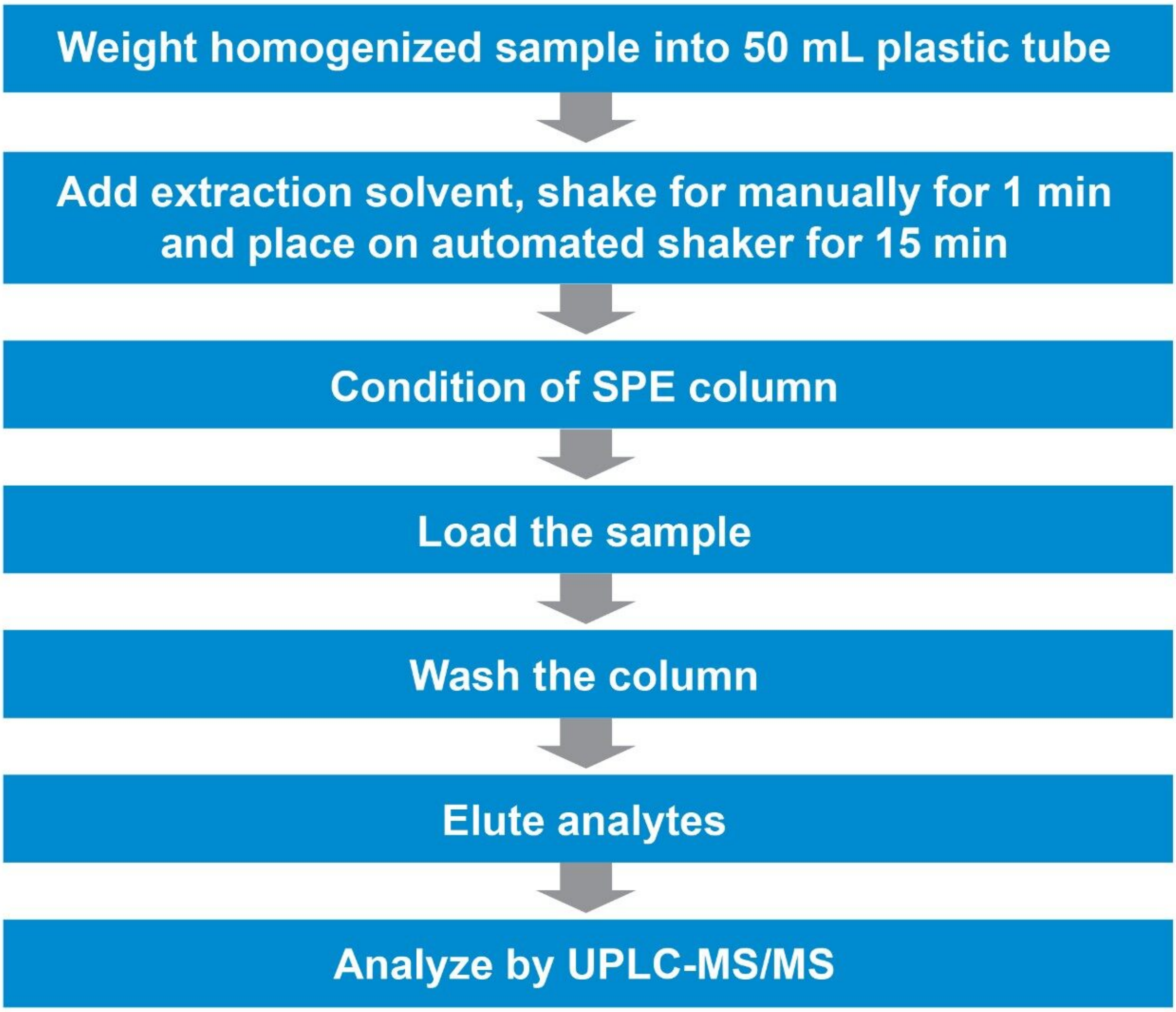


Figure 1. Workflow for sample preparation for determination of banned vet drugs in chicken by SPE in trap and elute mode.

Two different SPE sorbent chemistries were used to prepare chicken extract for spiking with CAP and NFs: Oasis HLB (P/N WAT094226 <https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat094226-oasis-hlb-3-cc-vac-cartridge-60-mg-sorbent-per-cartridge-30--m-1.html>) for CAP and Oasis MCX (P/N 186000254 <https://www.waters.com/nextgen/us/en/shop/sample-preparation--

filtration/186000254-oasis-mcx-3-cc-vac-cartridge-60-mg-sorbent-per-cartridge-30--m-1.html>) for NF. Both were used in trap and elute mode to allow concentration of the analytes and maximize the removal of co-extracted molecules.

For CAP, 5.0 g of blank sample was put into 50 mL plastic centrifuge tube. 6 mL of eTrac was added, then the mixture was shaken for 1 min manually and 5 min on automated shaker. The mixture was centrifuged, and 5 ml of the solvent was evaporated to dryness and reconstituted in 5 mL of 10% methanol in water and used for SPE procedure. The SPE cartridge was conditioned with 2 mL methanol and 2 mL of water. 3 mL of sample was loaded on the conditioned cartridge and washed with 2 mL of 5% acetic acid. 3 mL of methanol/NH_3 90/10 *v*/*v* was used as elution solvent, evaporated, and reconstituted in 1 mL of was evaporated to dryness, The extract was reconstituted in 1 mL of water/methanol 80/20 *v*/*v*, centrifuged at 15,000 rpm, filtered, and placed in autosampler vials for analysis by UPLC-MS/MS.

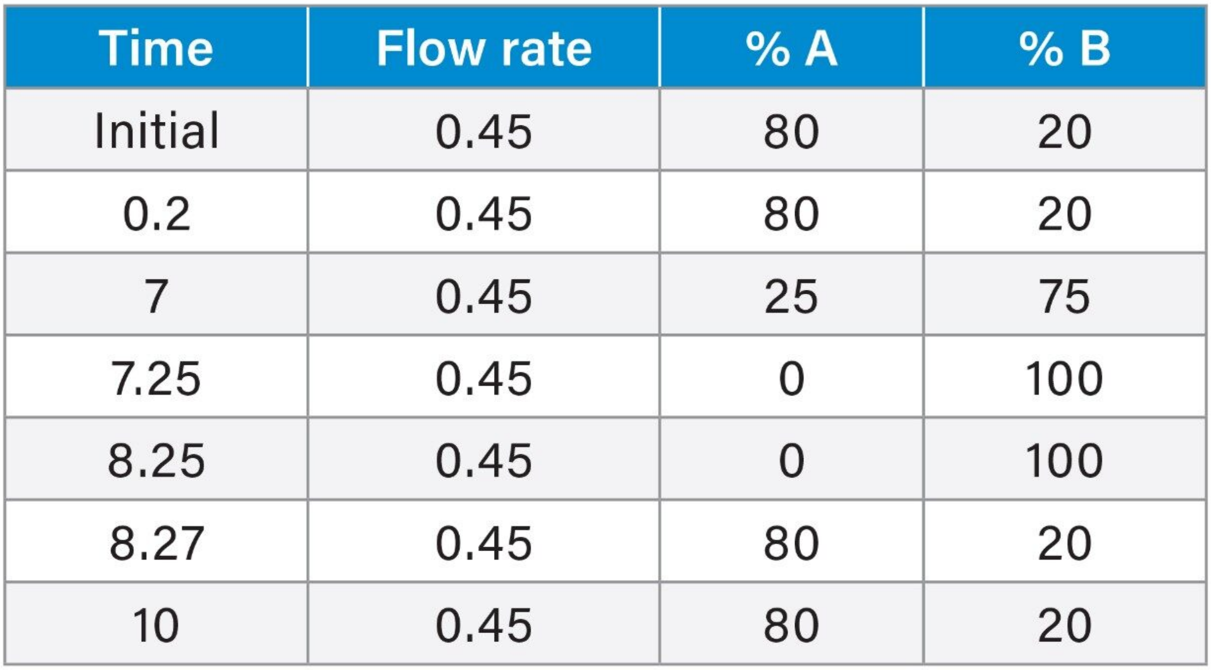
To prepare blank chicken extract for spiking with NFs, 2 g of sample was put into 50 mL plastic centrifuge tube and mixed with 10 mL of methanol. The sample was shaken for 1 min manually and 5 min on automated shaker, centrifuged and used for SPE procedure. The SPE cartridge was conditioned with 3 mL of ethyl acetate, 3 mL of methanol, and 3 mL of water. 3 mL of extract was loaded on the conditioned cartridge and washed with 2 mL of water and 2 mL of 30% methanol in water. 3 mL of ethyl acetate was used as elution solvent and evaporated, reconstituted in 1 mL of water/methanol 60/40 *v*/*v*, centrifuged at 15,000 rpm, filtered, and placed in autosampler vials for analysis by UPLC-MS/MS.

UPLC-MS/MS Conditions

UPLC system:	ACQUITY UPLC I-Class PLUS with FTN Sample Manager
Column:	ACQUITY UPLC BEH C_{18} Column (1.7 µm, 2.1 × 100 mm, P/N: 186002352)
Mobile phase A:	0.5 mM Ammonium formate
Mobile phase B:	Methanol
Flow rate:	0.45 mL/min
Column temp.:	45 °C

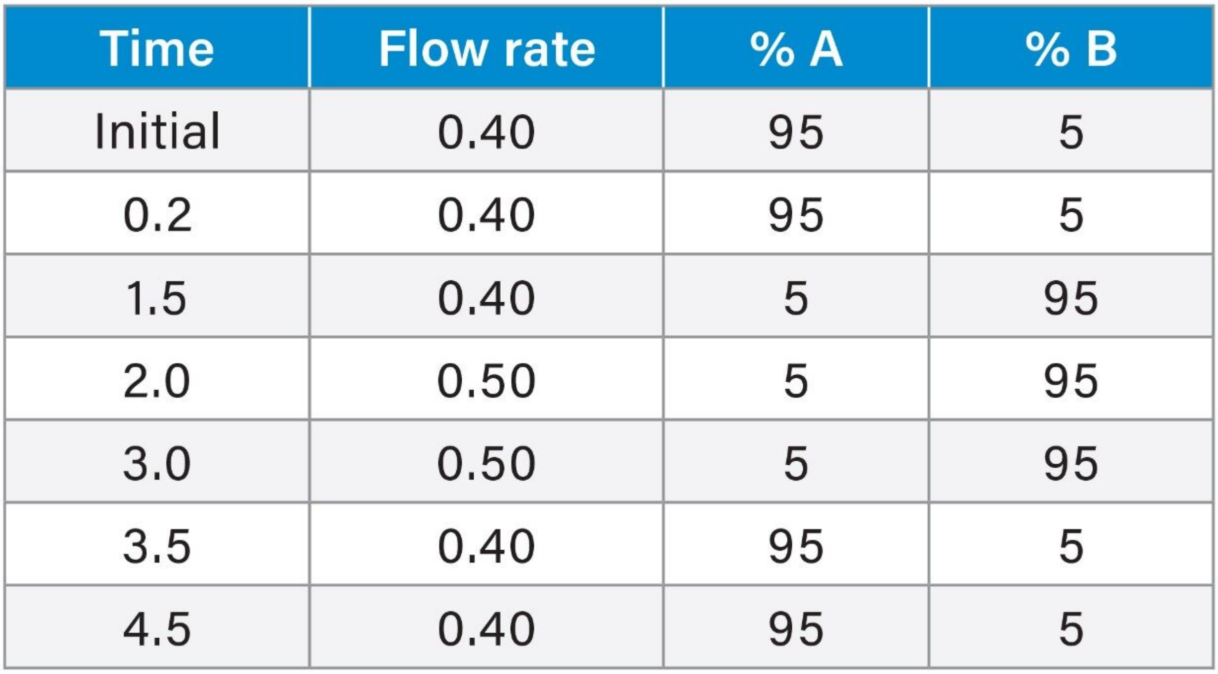
Sample temp.: 10 °C

Gradient for nitrofuran metabolites



Time	Flow rate	% A	% B
Initial	0.45	80	20
0.2	0.45	80	20
7	0.45	25	75
7.25	0.45	0	100
8.25	0.45	0	100
8.27	0.45	80	20
10	0.45	80	20

Gradient for chloramphenicol



Time	Flow rate	% A	% B
Initial	0.40	95	5
0.2	0.40	95	5
1.5	0.40	5	95
2.0	0.50	5	95
3.0	0.50	5	95
3.5	0.40	95	5
4.5	0.40	95	5

MS Conditions

MS instrument:	Xevo TQ-S cronos
Ionization:	Electrospray
Desolvation temperature:	600 °C
Desolvation gas flow:	1000 L/Hr
Source temperature:	150 °C
Cone gas flow:	0 L/Hr

The NFs were analyzed in positive ESI mode, whereas CAP was analyzed in negative ESI mode in a separate run. Capillary voltage optimized at -0.3kV for CAP and +0.5kV for NFs.

The optimized cone voltages and collision energies for the 4 nitrofuran metabolites and chloramphenicol, along with associated internal standards are summarized in Table 1. Calculation of the MS cycle of the final multiple reaction monitoring (MRM) method in positive mode was automatically optimized in MassLynx Software using peak width = 4.5 s and required points per peak = 12 processing of results was done in TargetLynx using the Apex Track function for consistent and time-saving integration in routine analysis. The Apex Track algorithm enables the data processing software to automatically locate a peak by thresholding the second derivative of the chromatogram.

Compound	Q (Q)	CV	CE
2NP-AHD	249.1>134.0	40	10
	(249.1>104.0)		22
2NP-AHD^{13}C$_3$	252.1>134	40	10
2NP-SCA	209.1>166.1	30	8
	(209.1>192.1)		8
2-NP-SCA-^{13}CN$_2$	212.1>168.1	25	8
2NP-AOZ	236.1>134.0	40	10
	(236.1>104.0)		20
2NP-AOZ-D4	240.1>134	40	8
2NP-AMOZ	335.1>291.1	30	8
	(335.1>128.0)		20
2NP-AMOZ-D5	340.1>296.0	30	8

Compound	Q (Q)	CV	CE
CAP	321>152	40	14
	(321>257)		8
CAP-D5	326>157	45	15

Table 1. Optimized MRM conditions for detection of nitrofuran metabolites and chloramphenicol in chicken muscle.

Results and Discussion

Spiked chicken extracts with nitrofuran metabolites and chloramphenicol were analyzed by UPLC-MS/MS and the results were assessed for linearity, matrix effects, ion ratios and retention time stability, precision and sensitivity. The BEH C_{18} Column provided excellent retention and peak shape and could be used with the same mobile phase for both analyses. All peaks eluted between 1.7 and 4 minutes with total run times of 10 minutes.

Linearity and Matrix Effects

For plotting the internally standardised calibration curves, weighting factor 1/x was applied. The linearity was assessed across the whole concentration range (typically 3 orders of magnitude) and R^2 factor was found to be greater than 0.99 and residuals expressing deviation of calculated value from the true value below 20% for nitrofuran metabolites for concentrations ranging between 0.05 and 10 µg/kg and for SCA between 0.25 and 10 µg/kg. For chloramphenicol in chicken, R^2 factor >0.99 using correction by internal standard was found to be in the range of 0.02 and 10 µg/kg with residuals below 15 %. Typical examples of multiple averaged calibration curves for all analytes are in Figure 2.

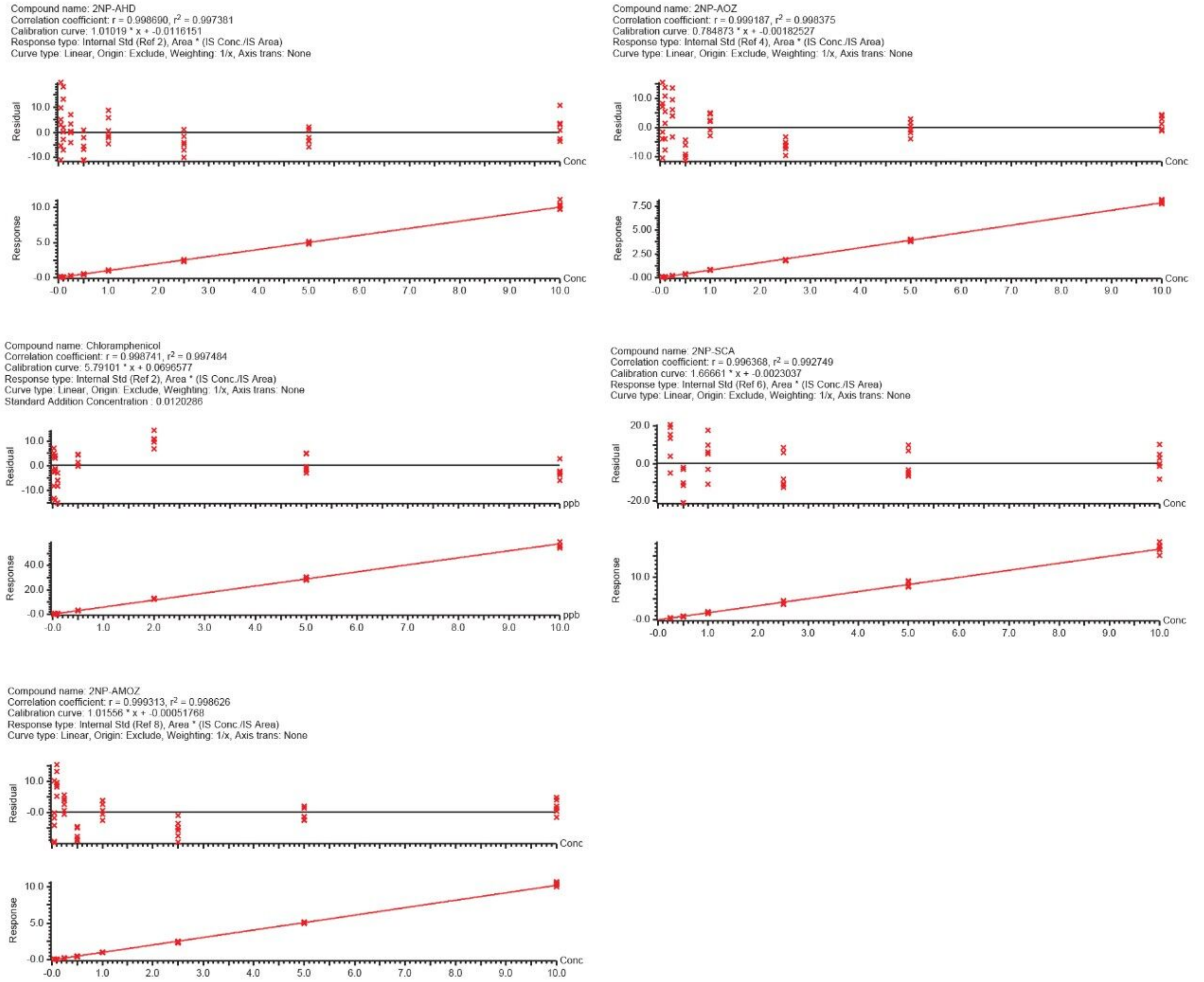
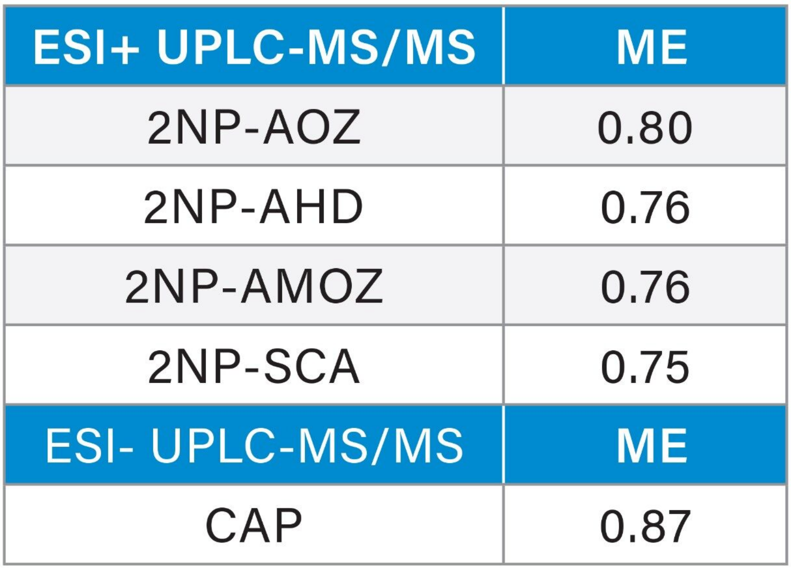


Figure 2. Calibration curves for analytes prepared in extract of chicken.

Matrix effects (ME) were assessed by calculating of ratio of the slope of calibration curve prepared in matrix and its equivalent in solvent. Matrix effects were evaluated for both methods and are summarized in Table 2. To compensate for ME in routine quantitative LC-MS/MS it is possible to apply matrix matched calibration curve. Although often it is necessary to use isotopically labelled analogues as internal standards in order to achieve as precise quantitation of analytes in unknown samples as possible.



ESI+ UPLC-MS/MS	ME
2NP-AOZ	0.80
2NP-AHD	0.76
2NP-AMOZ	0.76
2NP-SCA	0.75
ESI- UPLC-MS/MS	**ME**
CAP	0.87

Table 2. Matrix effects measured for analytes prepared in extract of chicken.

Ion Ratios and Retention Time Stability

Confirmatory multi-residue analysis of banned drugs in complex food matrices involves the comparison of several physico-chemical parameters described in regulatory guidelines Commission Decision 2002/657/EC. [10] Ion ratios calculated from two MRM channels within the spiked samples at the two concentration levels were well within the tolerance of those determined from the matrix matched calibration series for NFs analysed in ESI+ and chloramphenicol in ESI-. Retention time of the spiked samples were within the 0.2 min tolerance of retention time obtained from the matrix matched calibration series.

Precision of The Measurement and Sensitivity

To investigate the inter-day repeatability of the measurements, multiple injections (n=36) were acquired over three typical batches for matrix-matched standards at two concentrations in chicken at 0.5 and 5.0 µg/kg for nitrofurans and 0.15 and 5.0 µg/kg for chloramphenicol. The total run time for CAP was 16 hours and for NFs 36 hours (3 subsequent analytical batches). During this time, no user intervention such as source clean or maintenance was performed.

The specificity was good as blank samples were prepared and analyzed with no significant interferences were found in the region of expected elution of the target analytes. Calculated %RSD for peak areas of NFs and CAP are shown in Table 3 and typical chromatograms for all nitrofuran metabolites and chloramphenicol is in

Figure 3, which shows this method to be suitable for checking regulatory compliance for these analytes in chicken.

ESI+ UPLC-MS/MS	%RSD 0.5 µg/kg (n inj. = 36)	%RSD 5 µg/kg (n inj. = 36)
2NP-AOZ	3.1	3.8
2NP-AHD	7.1	7.1
2NP-AMOZ	3.1	2.5
2NP-SCA	7.1	6.8
ESI- UPLC-MS/MS	**%RSD 0.15 µg/kg (n inj. = 36)**	**%RSD 5 µg/kg (n inj. = 36)**
CAP	2.8	3.0

Table 3. %RSD for NFs and CAP in chicken obtained during the analysis by ACQUITY UPLC I-Class PLUS coupled to Xevo TQ-S cronos.

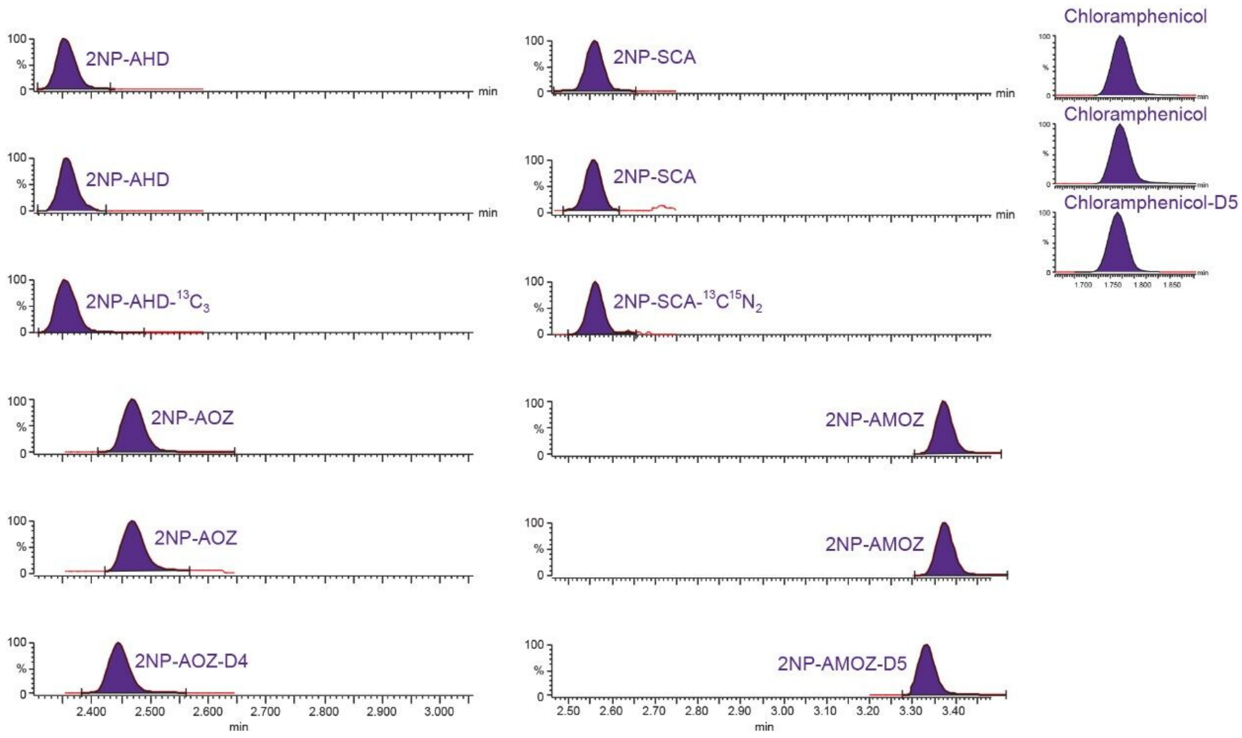


Figure 3. Example of chromatogram obtained for NFs (0.5 µg/kg) and CAP (0.15 µg/kg) in chicken.

Figure 4 shows an example of a QC chart plotted in TrendPlot (TargetLynx) including “warning zones” determined by two and three times multiplied standard deviation. Injections approaching/entering the "warning zone" (>2SD) can be a signal for investigating the system and possible maintenance including cleanliness of the source.

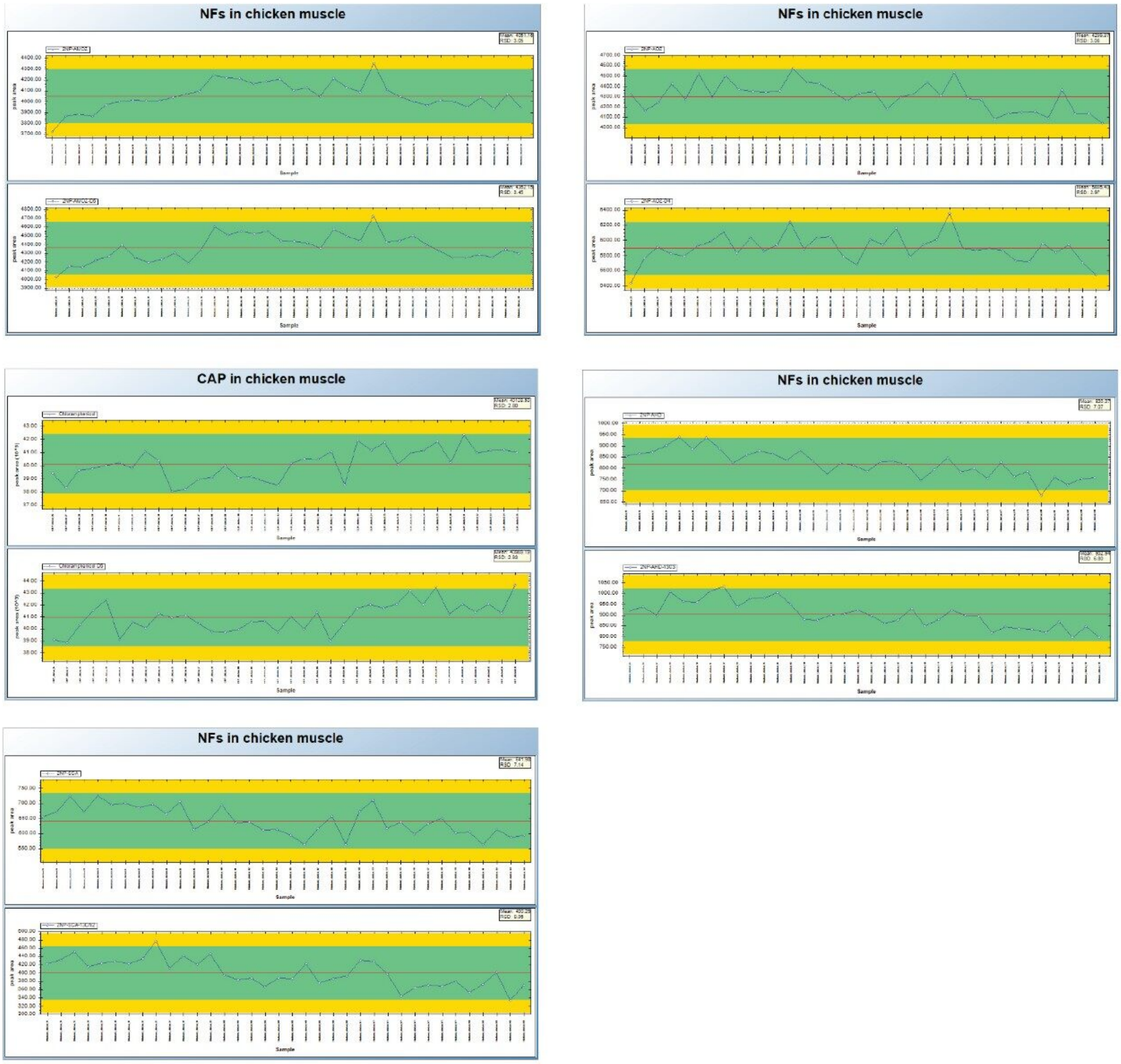


Figure 4. TrendPlot chart for NFs (0.5 µg/kg) and CAP (0.15 µg/kg) in chicken. 36 injections over 3 analytical batches.

Conclusion

This application note shows the performance of an ACQUITY UPLC I-Class System coupled to the Xevo TQ-S cronos is suitable for the quantitative analysis of banned veterinary drug residues in extracts from food of animal origin at the concentration levels required for EU Regulatory compliance.

Calibration characteristics (linearity, sensitivity), ion ratio criteria, and retention time stability and inter-day repeatability were all shown to be within the parameters defined by the 2002/657 guidelines for method validation.

This superior performance (without the requirement for operator maintenance) can be attributed to the technology features of the Xevo TQ-S cronos, including the reverse cone design (shielding the inner cone from contamination), orthogonal geometry ion source, StepWave ion guide, and the T-Wave enabled collision cell.

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