# Multiclass Accurate Mass Screening of Veterinary Drugs in Animal Tissue – Value of Enhanced Sensitivity and Resolution for Reliable Identification

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## Introduction

Veterinary drugs are pharmacologically active compounds which are used to treat and prevent diseases of animals in livestock. The use of pharmaceuticals can result in non-desirable veterinary drug residues in the food products. European Commission Regulation 37/2010 and its amendments set maximum residue levels (MRLs) for veterinary drugs in the target tissue of various food producing animals. Veterinary drug-food product combinations not mentioned in table 1 of the regulation are not authorized. In addition table 2 of the regulation lists pharmacologically active substances which are completely forbidden for food producing animals. Commission Decision 2003/181/EC sets minimum required performance limits (MRPLs) for the analytical methods used for non authorized or forbidden compounds to 1 µg/kg or even below. These requirements make the multiclass determination of veterinary drugs to a very challenging application especially since there is not yet a generic extraction method available for the wide variety of compounds currently used. For multi-residue pesticide analysis modern LC-(Q)TOF instruments have been successfully used for identification and quantitation. In addition the full scan analysis allows the retrospective data analysis for emerging contaminants.

The goal of this work was the development of a sensitive UHPLC-(Q)TOF method for the identification and quantitation of veterinary drug residues in muscle tissue. The extraction procedure published by Stubbings et al. (2005) as well as the UHPLC separation have been optimized to reduce matrix interferences in the electrospray ionization and (Q)TOF detection. Different generations of (Q)TOF instruments have been used to identify resolving power and sensitivity requirements for this application.

## Experimental

### Sample extraction

Homogenized muscle tissue has been extracted with acetonitrile followed by a de-fatting step with n-hexane. The extracts were further purified by solid phase extraction (SPE) with strong cation exchange materials. After elution of the analytes and the reconstitution of the dried extracts in mobile phase. For validation of the method performance and compound identification extracts have been fortified with 45 veterinary drugs belonging to the classes of benzimidazoles, beta-blockers, fluoroquinolones, nitro-imidazoles, sulfonamides, and tranquilizers. Type A compounds (banned substances and compounds with MRLs < 50 µg/kg) have been spiked between 1 and 20 ng/ml, type B compounds (MRLs > 50 µg/kg) have been spiked between 10 and 200 ng/ml.

## Experimental

### UHPLC-MS/MS parameters

An Agilent 1290 Infinity UHPLC system consisting of a G4220A binary pump, G4226A high performance sampler, and G1316C thermostated column compartment has been coupled to an Agilent G6530A, G6540A, and G6550A QTOF system with electrospray ionization (ESI) via Dual Spray Agilent Jet Stream Technology. Separation has been done on an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column (2.1 x 100 mm, 1.8 µm) @ 20°C with (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile as the mobile phase at a flow rate of 0.4 mL/min.

Gradient program: 0.5 min isocratic at 5% B, linear gradient to 35% B in 6.5 min, linear gradient to 70% B in 5 min, linear gradient to 100% B in 0.5 min, 2.5 min isocratic at 100% B, linear gradient to 5% B in 0.1 min. Total run-time 20 min.

Accurate mass spectra have been acquired in positive mode over the m/z range 50–1000 at an acquisition rate of 3 spectra/sec. To maintain the desired mass accuracy, internal calibration was performed during acquisition via continuous infusion of purine (m/z 121.0509) and HP-921 (m/z 922.0098).

ISTDs: azaperone-$d_4$, carazolol-$d_7$, chloropromazine-$d_6$, dimetridazole-$d_3$, norfloxacin-$d_5$, oxfendazole-$d_3$, sulfamethazine-$d_4$

## Results and Discussion

### Sample preparation and UHPLC separation

Modification of the extraction and clean-up procedure reduced the matrix load of the extract significantly while the majority of the target compounds showed similar or even improved extraction yield.

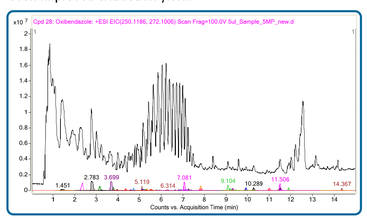


**Figure 1: UHPLC-(Q)TOF-MS chromatogram of a pig muscle extract spiked with type A (20 ng/ml) and type B (200 ng/ml) compounds.**

## Results and Discussion

Figure 1 shows the comparison of the TIC and the target compound chromatograms for a spiked pig muscle extract. The majority of the sample matrix consists of polar compounds. By optimizing the UHPLC method to increase the retention of the early eluting target compounds, matrix effects could be significantly reduced resulting in lower LODs and higher identification rates.

### Impact of resolving power on compound identification

Table 1 shows the identification of the type A compounds in pig muscle extract at different spiking levels for the G6530 and G6540 QTOF. Results have been extracted by using the Find-by-Formula data mining algorithm combined with an accurate mass database for veterinary drugs.

**Table 1: Identification results for type A compounds spiked in pig muscle extract at concentrations ranging from 1 ng/ml to 20 ng/ml.**

| | | G6530A (4GHz) | | | | | G6540A (4GHz) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Type | Compound | 1MP | 2MP | 3MP | 4MP | 5MP | 1MP | 2MP | 3MP | 4MP | 5MP |
| A | Dimetridazole | | | | | | | | | | |
| A | Hydroxy-Dimetridazole | | | | | | | | | | |
| A | Ipronidazole | | | | | | | | | | |
| A | Hydroxy-Ipronidazole | | | | | | | | | | |
| A | Metronidazole | | | | | | | | | | |
| A | Hydroxy-Metronidazole | | | | | | | | | | |
| A | Ronidazole | | | | | | | | | | |
| A | Fenbendazole | | | | | | | | | | |
| A | Flubendazole | | | | | | | | | | |
| A | Levamisole | | | | | | | | | | |
| A | Oxfendazole | | | | | | | | | | |
| A | Oxfendazole-sulfone | | | | | | | | | | |
| A | Acetopromazine | | | | | | | | | | |
| A | Chlorpromazine | | | | | | | | | | |
| A | Promazine | | | | | | | | | | |
| A | Propionylpromazine | | | | | | | | | | |
| A | Triflupromazine | | | | | | | | | | |
| A | Carazolol | | | | | | | | | | |

automatically found; manually found; not found

The G6530 QTOF at m/z 200 typically gave resolution of 12,000 FWHM (full width at half maximum), whereas the G6540 instrument for the same mass gave resolution of 17,000 FWHM. This enhanced resolution allowed for the mass spectral separation of the veterinary drug residues from the sample matrix resulting in significantly higher identification rates.

Figure 2 shows chromatogram, spectrum and identification result for promazine in pig muscle matrix spiked at 5 ng/ml (MP2). The increased resolution of the G6540 QTOF allowed for the separation from a co-eluting matrix compound resulting in an unequivocal identification with a high target score. Mass accuracies typically have been better than 1 ppm. The acquired TOF spectrum was in good agreement with the predicted isotope distribution, indicated by the red bars in the spectrum display.

## Results and Discussion

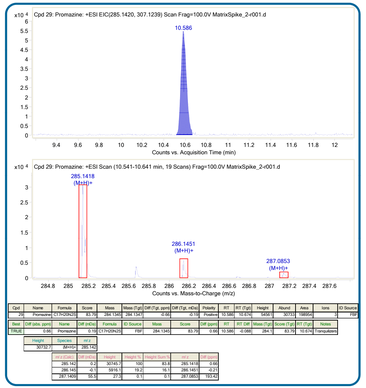


**Figure 2: Chromatogram, spectrum and identification result for promazine in pig muscle extract (sample 2MP, spiked at 5 ng/ml).**

### Impact of enhanced sensitivity on compound identification

By using a dual ion funnel assembly in combination with a hexabore conductive capillary the G6550A QTOF offers by a factor 5 to 10 higher sensitivity compared to the G6540 while maintaining the same mass accuracy and a similar resolving power. Figure 3 shows the two-stage ion funnel of the G6550 QTOF instrument.

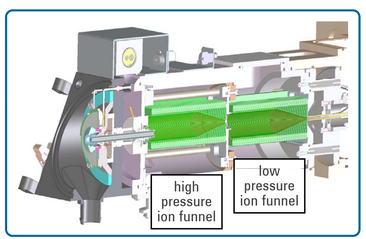


**Figure 3: The dual ion funnel of the G6550 QTOF MS.**

## Results and Discussion

Figure 4 shows the comparison of chromatograms for dimetridazole for sample 2MP for the G6540A and G6550A QTOF. For the ion funnel instrument the peak areas has been increased by a factor of 6 and S/N ratio (peak-to-peak) has been increased by a factor of 4.3. Due to the higher signal the automatic detection rate by the Find-by-Formula algorithm has been further increased and e.g. dimetridazole has been automatically detected in all concentration levels.

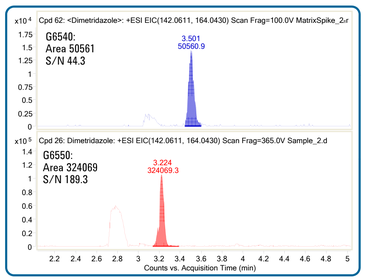


**Figure 4: Comparison of chromatograms of dimetridazole in a spiked pig muscle extract 5 ng/ml) for the two QTOF instruments.**

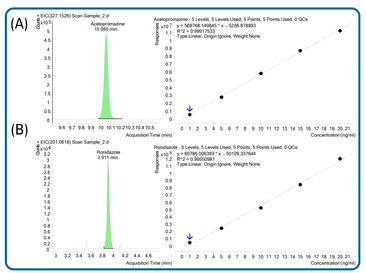


**Figure 5: Chromatograms of a spiked pig muscle extract (5 ng/mL) and calibration curves for acetopromazine (A) and ronidazole (B).**

## Results and Discussion

### Quantitation of veterinary drugs by UHPLC-(Q)TOF

Figure 5 shows chromatograms of a pig muscle extract spiked to a concentration of 5 µg/kg and external calibration curves for acetopromazine and ronidazole acquired with the G6550 QTOF in positive TOF mode. For all type A compounds linear calibration curves have been obtained. For some of the type B compounds the highest calibration standard have been exceeding the linear range of 3 to 4 orders of magnitude.
Several deuterated internal standards have been added after the extraction to compensate for matrix effects in the electrospray ionization. This allows for the accurate quantitation of veterinary drugs even in complex matrices.

## Conclusions

A sensitive and reliable method for the screening and quantitation of various classes of veterinary drugs in meat extract by UHPLC-(Q)TOF has been established. Optimization of the sample preparation and of the UHPLC separation resulted in lower matrix interferences. The impact of higher instrument sensitivity as well as of increased resolving power on the discovery rate of veterinary drugs spiked in muscle tissue has been compared. It has been shown that a resolution of >15,000 FWHM reduced matrix interferences with target compounds and reference mass ions, thus giving better mass accuracies. By using new dual spray Agilent Jet Stream, sensitivity as well as reference mass introduction has been improved significantly resulting in high quality data even in high background matrices. In combination with the dual ion funnel interface the reliable identification of banned and not authorized compounds for which a MRPL of 1 µg/kg has been set, could be achieved.
Data evaluation has been done using the "Find-by-Formula" data mining algorithm in combination with an accurate mass database. By including retention times as well as additional fragments into the database false positive detection rate could be reduced to a minimum. Preliminary validation experiments demonstrated typical analyte real recoveries from 40 to 80 %. The presentation shows that UHPLC/QTOF is a suitable technique for routine screening of a wide range of veterinary drugs in muscle tissue.

References:

1) EU Regulation (EC) 37/2010
2) EU Commission Desicion 2003/181/EC
3) Stubbings et al. *Analytica Chimica Acta*, 547 (2005) 262-268