

## ORIGINAL ARTICLES

### Analysis of nitrofurans metabolic residues in tissues by liquid chromatography–tandem mass spectrometry

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#### ABSTRACT

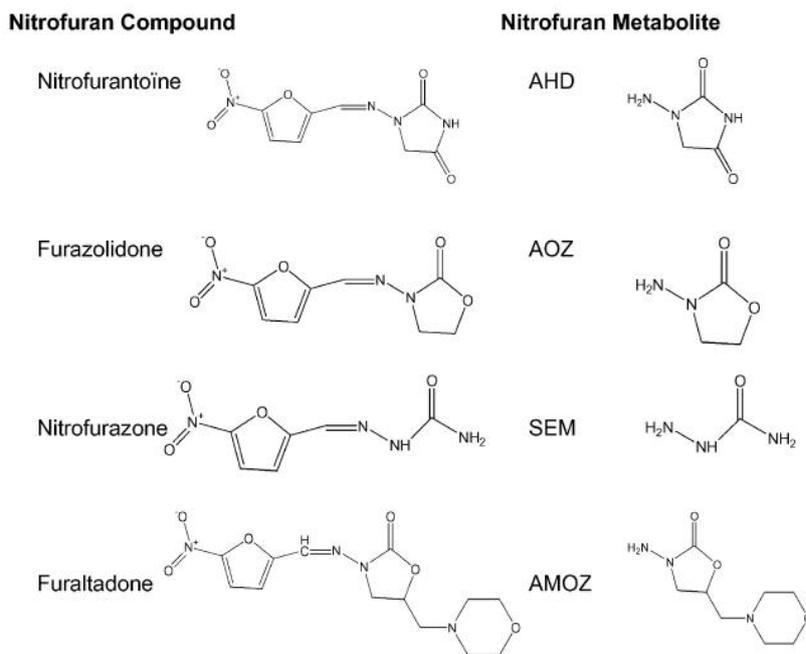
This method is considered as applicable good method validated in QCAP laboratory for determination of four Nitrofurans veterinary drugs (Furazolidone, Furaltadone, Nitrofurazone, Nitrofurantoin) in animal origin foods through the detection of their four major metabolites; 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) for Furaltadone, 1-aminohydantoin (AHD) for Nitrofurantoin, Semicarbazide (SEM) for nitrofurazone and 3-amino-2-oxazolidinone (AOZ) for Furazolidone. The method performance was tested using recovery tests of 4 compounds on chicken samples. The average recoveries at different concentration levels varied between 70 -120%. The reproducibility expressed as relative standard deviation was less than 20 %. The method showed to be linear at least up to 10 µg/kg level. The limit of quantitation was 0.5µg/kg. The measurement uncertainty expressed as expanded uncertainty and in terms of relative standard deviation (at 95% confidence level) is within the range of  $\pm 40$  %. The method is based on the acid-catalysed hydrolysis of protein-bound metabolites by hydrolysis with hydrochloric acid, followed by their conversion into NBA imine-type derivatives using Nitrobenzaldehyde (NBA). After hydrolysis and derivatization, the solution is neutralized using Sodium Hydroxide and Nitrophenyl (NP)-derivatives are extracted by liquid-liquid partition with Ethyl Acetate. Ethyl Acetate is evaporated to dryness and the residue is re-dissolved in buffer solution and injected into LC system. Quantitation and confirmation of each compound was done by reversed phase HPLC coupled to tandem mass spectrometry (LC-MS/MS) with Electro Spray ionization (ESI) in positive mode, the mass spectrometer was operated in multiple reactions monitoring (MRM) mode and two different MRM are used for confirmation.

**Key words:** Analysis, nitrofurans, residues, tissues, liquid, Chromatography, spectrometry

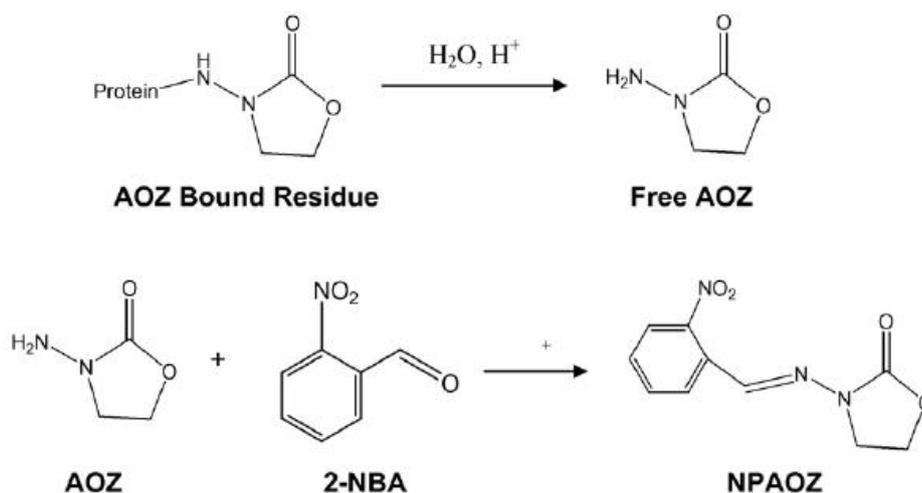
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#### Introduction

The use of Nitrofurans such as Furazolidone, Furaltadone, Nitrofurazone and Nitrofurantoin has been banned in the European Union since 1993 (1995 for Furaltadone) ( McEvoy, 2002). These compounds are antibacterial drugs and are often added to feeds to stimulate growth and to prevent and control several bacterial and protozoan infections, such as fowl cholera and coccidiosis black-heads. Because of their potentially harmful effects on human health the European Union prohibited its use in food-producing animals (Commission Regulation, 1995). 3-amino-2-oxazolidone (AOZ; metabolite of Furazolidone), 5-methylamorpholino-3-amino-2-oxazolidone (AMOZ; metabolite of Furaltadone) and Semicarbazide (SEM; metabolite of nitrofurazone). Many laboratories have been focused on development of analytical methods for identification of these Nitrofurans metabolites (Leitner *et al.*, 2001 and Connely *et al.*, 2003). The most recent and accurate analysis methods described in the literature are based on determination of the 2-nitrobenzaldehyde imine-type of the metabolites using liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS). All four compounds are simultaneously analyzed by these methods (Leitner *et al.*, 2001 ; Connely *et al.*, 2003 and Pereira *et al.*, 2004)). The minimum required performance limit (MRPL), specified by the European Commission 2003/181/EC, for nitrofurans metabolites in poultry meat is 1µgkg<sup>-1</sup> (Commission Decision, 2003), although an MRPL for nitrofurans metabolites in eggs has not yet been established.



**Fig. 1:** Nitrofurans veterinary drugs and their metabolites



**Fig. 2:** Acid-catalyzed of protein-bound metabolites and NBA derivatization

*Experimental:*

*Reagents and materials:*

Methanol and ethyl acetate (HPLC grade) and hydrochloric acid were obtained from Merck (LOBA CHEMIE,  $\geq 99\%$ ); AOZ, AMOZ, AHD hydrochloride, 3-amino-2-oxazolidone-d4 (AOZ-d4), 5-methylamorfolino-3-amino-2-oxazolidone-d5 (AMOZ-d5) and the nitrophenyl derivatives of AOZ, AMOZ, AHD and SEM were obtained with 99% purity from (LOBA CHEMIE  $\geq 99\%$ ). A stock standard solution was prepared by 100  $\mu\text{g/ml}$  reference standard solutions of each compound in 100 ml volumetric flask and dissolve in methanol and working standards was prepared 1  $\mu\text{g/ml}$  reference standard solution for the four compounds in one mixture by diluting appropriate volume of the stock solution with Methanol. Calibration mixture solutions was prepared by 1 ml of the mixture working solution and derivatized with 2-nitrobenzaldehyde. The produced NP-derivatives will be used to prepare the calibration mixtures at concentration levels 0.25, 0.50, 1.0, 2.0, 5.0

and 10ng/ml by making appropriate dilutions with dilution solvent (methanol-buffer). Calibration mixture solutions must be prepared daily with each set of samples.

#### *HPLC analysis:*

HPLC analyses were performed on a C18 reversed-phase column (100mm × 2.1mm, 4<sub>μ</sub>m from Jones Chromatography) and were operated at room temperature. The LC Mobile phase was composed of (5 mM Ammonium format solution in Methanol/Buffer (1:9): dilute 50 ml of stock buffer with 450 ml Methanol/buffer (1:9), the P<sup>H</sup> should be 2.78 ± 0.1, adjust if necessary, stable for one week in refrigerator. The total run time was set for 5 min. The temperature of the auto sampler was maintained at 4 °C and the injection volume was 40 <sub>μ</sub>l.

#### *Sample Preparation:*

Samples were immediately frozen to reduce degradation of the antibiotics during transport and they were kept at -20 °C not more than 2 days till sample treatment and analysis. Before analysis, samples were partially thawed at room temperature and muscle tissue was taken for analysis. Preparation of sampling was taken place according to Codex Alimentarius Vol 2; (1993), the edible part was separated and homogenized well.

#### *Electro spray ionization (ESI) MS–MS:*

MS detection was done on a Sciex API 4000 triple stage quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA). Nitrogen was used for the gas nebulizer. The ions were monitored by Multiple Reaction Monitoring (MRM). The source block temperature was set at 450°C and the electro -spray capillary voltage to 5.5 kV.

#### *Sample preparation:*

1.0g ± 0.01 g sample tissue was weighed in 50 ml plastic tube. In case of spiking, fortify with 100 <sub>μ</sub>l of 10 ng/ml to get an expected concentration level of 1.0<sub>μ</sub>g/kg (at the MRPL), 4mL de-ionized water was added to the sample followed by 0.5mL of 1M HCl and 150 <sub>μ</sub>l of 50 mM methanolic 2-nitrobenzaldehyde. The tube is then capped, vortex-mix for 10 s and finally incubated in 55°C agitated water bath for 4 h and protected from light. The solution was neutralized by adding 5mL of 0.1M di-potassium hydrogen phosphate followed by 300<sub>μ</sub>l of 1M NaoH. The tube was swirled for few seconds; the pH was adjusted at 7.0±0.5 with pH strips after adding few drops of NaoH. 5mL ethyl acetate was added to the neutralized solution and mix for 20 min. The ethyl acetate portion is then extracted by centrifugation at 2500 rpm for 10 min at 4±1 °C and the supernatant transferred into a clean 15mL disposable polypropylene tube. The neutralized solution was re-extracted with 3mL of ethyl acetate and mix for 20 min. Centrifugation at 2500 rpm for 10 min at 4±1 °C and was transferred of the new supernatant into the same 15mL disposable polypropylene tube then carried out in order to recover a 8mL ethyl acetate volume containing the Nitrophenyl derivatized residues of the Nitrofurans metabolites. Then evaporated at 45 °C under gentle nitrogen stream down to nearly dryness and re-dissolved into a 1.0 ml dilution solvent (Methanol-buffer 1:1, P<sup>H</sup> = 4) and 1 min water bath ultra-sonication then centrifugation at 2500 rpm for 10 min at 4±1 °C. The sample was detected using disposable acrodisc 0.45 <sub>μ</sub>m and injected 25 <sub>μ</sub>l of the sample into LC-MS/MS system.

#### *Determination of tissue-bound residues:*

For determination of tissue-bound residues, the 1.0g ± 0.01 g sample tissue in 50 ml plastic tube was subjected to the following 4 steps washing procedure; The first wash was carried out by adding 6ml of a methanol/water solution (50/50; v/v) to the sample followed by shaking for 15 min and then by a 10 min 2500 rpm centrifugation at 4±1 °C. The supernatant was discarded and the sample was processed to the next washing step. The second wash was carried out by adding 6ml of a methanol/water solution (75/25; v/v) to the sample then followed by the same shaking and centrifugation as for the first wash. The supernatant is once more was discarded and the sample was processed to the next washing step. The third wash was carried out by adding 6ml of pure methanol followed by the same shaking and centrifugation as for the first and second washes. Once more, the supernatant was discarded and the sample was processed to the last washing step. The fourth and last wash was carried out by adding 2ml of de-ionized water followed by a 20 s vortex-mix and the same centrifugation as for the previous washes. The supernatant was discarded and the sample was ready for the analytical preparation as previously described.

*Regression curve:*

An external regression curve was prepared using blank salt samples fortified with the four Nitrofurans metabolites to obtain the final concentrations 0.25, 0.5, 1, 2, 5 and 10 ng g<sup>-1</sup>. All samples for regression curve were prepared in duplicate and injected in singular [Pereira *et al.*, (2004)].

*Recovery experiments:*

Six blank samples of chicken were fortified with a mixture of the Nitrofurans metabolites (SEM, AHD, AOZ, and AMOZ) to 0.5 MRPL, IMRPL, 2 MRPL and 10 MRPL final concentrations. These samples were extracted according to the method described in Section 2.5. The average recoveries and relative standard deviation on each level were calculated. The following table shows the recovery test results. Recovery results are based on matrix-matched standard calculations.

**Table 1:** Recoveries and CV% of the analyzed drugs in chicken samples.

No.	Chicken		0.5 µg/kg		1.0 µg/kg (MRPL)		2 µg/kg		10 µg/kg	
	Drug	n	Mean Rec.%	CV%	Mean Rec.%	CV%	Mean Rec.%	CV%	Mean Rec.%	CV%
1	NP-AHD	6	105%	6%	99%	12%	94%	7%	85%	9%
2	NP-AMOZ	6	109%	6%	102%	9%	102%	12%	78%	5%
3	NP-AOZ	6	93%	19%	95%	15%	84%	21%	80%	7%
4	NP-SEM	6	92%	8%	102%	15%	99%	13%	91%	6%

**Results And Discussion***Optimization of the method:*

All Nitrofurans metabolites have low molecular masses, between 75 g mbl(SEM) and 201 g mol<sup>-1</sup> (AMOZ). In liquid chromatography coupled to mass spectrometry analysis of low-molecular-mass substances promotes a relative poor sensitivity due to low ionization efficiency and non-specific fragmentation behavior (e.g. loss of ammonia, water or carbon dioxide). The derivatization of the free amino groups normally with 2-nitrobenzaldehyde normally increases the sensitivity of the MS analysis significantly and this approach is normally used in routine laboratory analysis (Leitner *et al.*, 2001; Connely *et al.*, 2003; Commission Decision, 2002 and Pereira *et al.*, 2004). This simple approach has the additional advantage of simultaneous hydrolysis of the protein-bond metabolites and rapid derivatization with 2-nitrobenzaldehyde since the chemical attack of the nucleophile, R-NH<sub>2</sub>, on the 2-nitrobenzaldehyde carbonyl group is catalyzed by acid.

*Validation results:**Linearity:*

External calibration curves obtained for the Nitrofurans metabolites ( $n = 5$ ) in the 0.25–10 µg g<sup>-1</sup> range were linear. The calculations are based on matrix-matched standard calculations method using one concentration level. Calculations are based on drawing the calibration between the two levels that bracketing the concentration of the sample under test. Method linearity was tested by performing recovery tests at different concentration levels on chicken samples. The method showed to be linear from the LOQ up to 10 µg/kg. Recovery results for the different four levels are shown in table (1).

*Precision and accuracy in spiked samples:*

Accuracy expresses the closeness of a result to a true value. Accuracy is expressed in terms of two components: "Trueness" and "Precision"

*Trueness:*

The trueness of a method is an expression of how close the mean of a set of results (produced by the method) to the true value. To check trueness of the method, spiked samples are used at different levels on chicken samples. The data for bias are described in the following table.

**Table 2:** Method bias

Chicken			0.5 ug/kg		1.0 ug/kg		2.0 ug/kg		10 ug/kg	
No.	Compound	n	Mean Rec.%	Bias						
1	NP-AHD	6	105%	5%	99%	-1%	94%	-6%	85%	-15%
2	NP-AMAZ	6	109%	9%	102%	2%	102%	2%	78%	-22%
3	NP-AOZ	6	93%	-7%	95%	-5%	84%	-16%	80%	-20%
4	NP-SEM	6	92%	-8%	102%	2%	99%	-1%	91%	-9%

Also, the method trueness was tested using the FAPAS proficiency testing sample (round 02138) used as CRM. The following table shows accepted z-score for the total and bound veterinary metabolite residues, the sample was pig kidney and contained AOZ metabolite.

**Table 3:** Results of Certified Reference Material CRM

Compound	Assigned value(mg/kg)	Found(mg/kg)	St.dev	z-score	remarks
AOZ(bound)	0.71	0.412	0.155	-1.9	Satisfactory
AOZ(total)	2.26	1.228	0.496	-2.1	Satisfactory

**Precision:**

Precision is a measure of how close results are to one another. The two most common precision measures are (repeatability) and (reproducibility)

**Repeatability:**

Qualitatively is the closeness of agreement between successive results obtained with the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory and short intervals of time). Repeatability experiments were done by fortification on chicken and honey samples at different levels. CV% results for the different three levels are shown in tables (1). The CV% for repeatability should be lower than 20%.

**Reproducibility:**

Reproducibility is the precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment. In this study intra-laboratory reproducibility will only be considered, spiking chicken samples are analyzed by different analysts on several days. Reproducibility was estimated from validation data by pooling the variances of the different levels. The pooled CV% for reproducibility should be lower than 25%.

**Table 4:** The pooled CV% of the reproducibility experiments.

No.	Compound	Pooled CV%
1	NP-AHD	9%
2	NP-AMAZ	9%
3	NP-AOZ	16%
4	NP-SEM	11%

\*\*RSD<sub>pooled</sub>: Pooled standard deviation and can be calculated from following equation;

$$RSD_{pooled} = \sqrt{\frac{(RSD_1^2(n_1 - 1) + (RSD_2^2(n_2 - 1) + \dots))}{(n_1 - 1) + (n_2 - 1) + \dots}}$$

**Reporting limit, decision limit (CC $\alpha$ ) and the detection capability (CC $\beta$ ):**

The limit of reporting level (LRL) for the method, established as the lowest fortified level for which recovery and precision were acceptable, was 0.5 mg/kg for each analysed drug. The method decision limit (CC $\alpha$ ) and the detection capability (CC $\beta$ ) were evaluated in accordance with the EU guidelines 2002/657/EC.

**Decision limit (CC $\alpha$ ):**

Decision limit is the limit above which it can be decided with a statistical certainty of 95% that the identified analyte concentration is truly above the MRL. ( $\alpha$  error = 5% in the case of MRL compounds)

$$CC_{\alpha} = \text{MRL} + 1.64 \sqrt{\text{S.D.}_{\text{std}}^2 + \text{S.D.}_{\text{R}}^2}$$

MRL: is the maximum residue value ( $\mu\text{g}/\text{kg}$ );

$\text{S.D.}_{\text{std}}$ : the allowed variation in concentration of the standard solution, is typically set to 5%

$\text{SD}_{\text{R}}$ : is the within-house reproducibility for the analysis of different samples.

The equation is derived from Commission Decision 2002/657/EC. In order to harmonize  $CC_{\alpha}$  with the measurement uncertainty, the two major sources of variation, i.e. the maximum allowed variation in standard concentration and the reproducibility from the validation, were both included in the calculation of the within-laboratory reproducibility. The allowed variation in concentration of the standard solution is typically set to 5%.

#### The Detection Capability ( $CC_{\beta}$ ):

is the concentration of analyte, at which the method is able to detect MRL concentrations with a statistical certainty of 95%. ( $\beta$  error = 5% in the case of MRL compounds)

$CC_{\beta}$  was calculated from  $CC_{\alpha}$  according to Commission Decision 2002/657/EC:

$CC_{\beta} = CC_{\alpha} + 1.64 \text{SD}$  (within-laboratory reproducibility)

**Table 5:** The values of  $CC_{\alpha}$  and  $CC_{\beta}$

No.	Pesticide	MRPL ( $\mu\text{g}/\text{kg}$ )	SDR	SDstd	$CC_{\alpha}$ ( $\mu\text{g}/\text{kg}$ )	$CC_{\beta}$ ( $\mu\text{g}/\text{kg}$ )
1	NP-AHD	1	9%	5%	1.2	1.3
2	NP-AMOZ	1	10%	5%	1.2	1.3
3	NP-AOZ	1	18%	5%	1.3	1.6
4	NP-SEM	1	13%	5%	1.2	1.4

#### Measurement Uncertainty:

#### Relative Standard Uncertainty:

#### Precision:

The random effects were estimated as the relative standard deviation of repeated spike samples at different concentration levels. Relative standard uncertainty due to precision experiments ( $U_{\text{prec}}$ ), expressed as relative standard deviation was found to be less than 16 % (the highest RSD%).

#### Bias:

The bias of the analytical procedure was investigated during the in-house validation study using spiked samples. The lowest mean recovery (88%) with standard deviation  $s = 14\%$  and  $n = 24$ .

The standard uncertainty was calculated as the standard deviation of the mean using;

$$U_{(\text{Rec})} = \frac{s}{\sqrt{n}}$$

A significance test was applied to test if the recovery is significantly different from 100%. For 23 degrees of freedom  $t_{\text{tab}} = 2.07$  and  $t_{\text{calc}} = 4.2$ .

The relative standard uncertainty ( $U_{\text{rec}} = 3.2\%$ )

In this case (since  $t_{\text{calc}}$  is greater than  $t_{\text{tab}}$ ) the recovery is statistically significantly different from 100%, but in the normal application of the method no correction is applied. The uncertainty must be increased to take account of the fact that the recovery has not been corrected for.

#### Other sources:

All balances and the important volumetric measuring devices are under regular control. Precision and recovery studies take into account the influence of the calibration of the different volumetric measuring devices because during the investigation various volumetric flasks and pipettes have been used.

The uncertainty due to reference standard preparation was estimated by accounting for reference standard purity tolerance, balance, volumetric flask and pipettes. The uncertainty component due to reference standard preparation was found to be 0.7 %.

**Combined Uncertainty (UC):**

Combined uncertainty, is the positive square root of the sum of the squares of different uncertainty components, was found to be less than 16.3 %.

The following equation is used for combined uncertainty calculations

$$U_C = \sqrt{(U_p)^2 + (U_{Rec})^2 + U_{Ref}}$$

**Expanded Uncertainty:**

Expanded uncertainty is obtained by multiplying the combined uncertainty, by a coverage factor k, for confidence level of 95% k is 2.

The expanded uncertainty (at 95 % confidence level) was found to be less than 33 %.

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