# Quantitation Method for Nitrofuran Metabolites in Milk using SCIEX Triple Quad™ 3500 System

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## Overview

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of Nitrofuran metabolites in milk was developed. The method presented adequate linearity with correlation coefficients above r ≥0.99 for both analytes in the dynamic range of 0.50–20.0 µg/kg, with average accuracies for matrix based recovery were in the range 85%–120%. The results qualified the method for the quantification and confirmation of the analytes in milk at concentrations lower to the established Minimum Required Performance Limit (1.0µg/kg).

## Introduction

Nitrofurans are synthetic chemotherapeutic agents which have a broad spectrum of bacteriostatic activity. Nitrofurans mainly inhibit the enzymes involved in the carbohydrate metabolism. These bacteriostatic compounds are prohibited in livestock production by EU which is listed in Annexe IV of EC Council Regulation 2377/90. No MRLs have been established for Nitrofurans hence it is necessary to have sensitive confirmatory analytical methods for the detection of nitrofuran residues in food commodities. Further the detection of Nitrofurans has been shown to be difficult as they are quickly metabolized. Therefore the analysis of the protein bound, solvent extractable metabolites of Nitrofurans have been reported as the ideal choice of analysis. Analytically, residues are checked only for marker metabolites of the 4 nitrofuran chemicals, in particular: 3-amino-2-oxazolidinone (AOZ) for furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) for furaltadone,1-aminohydantoin (AHD) for nitrofurantoin and Semicarbazide (SEM) for Nitrofurazone (Figure 2).

**Figure 1. SCIEX Triple Quad™ 3500**

In general the study of nitrofuran metabolites in food samples requires incubation period for derivatization with nitrobenzaldehyde for 16hr at 37ºC in dark. The quantitative and confirmatory determination of nitrofuran metabolites was performed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) in positive ion mode, according to European Decision 2002/657/EC. The MRPL for nitrofuran metabolites (individual) is 1.0µg/kg as per RMP/EU/2016-17.

The present application note describes a method which is sensitive and selective enough to meet the global guidelines analyze the nitrofuran metabolites in milk using SCIEX Triple Quad™ 3500 LC-MS/MS System.

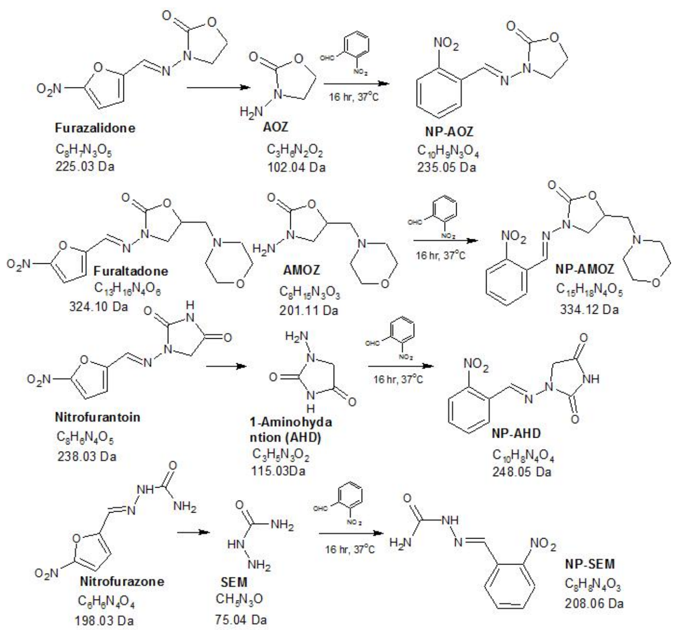


**Figure 2. Structures of Nitrofuran, Nitrofuran metabolites and Nitrophenyl derivatives**.

## Materials and Methods

### *Chemicals*

Nitrofuran metabolites Standards were purchased from Clearsynth and 2-Nitrobenzaldehyde was purchased from Sigma Aldrich ≥99% Purity. All other chemicals used were of LC-MS grade.

### *Sample Preparation*

Milk sample (3 ml) was mixed with 1ml of HCl (0.1M) and 50mM of 2-Nitrobenzaldehyde (0.3ml), vortexed and incubated on ultrasonic bath for 16hr added 0.6ml of 1M K2HPO4 solution and 10 ml of ethyl acetate, vortexed, followed by centrifugation at 4000 rpm, the supernatant was evaporated to dryness reconstituted with 1ml of Methanol: water (5:95) and 10µl is used for LC-MS/MS analysis.

## Experimental Conditions

### *LC Conditions*

LC separation was performed on a Shimadzu instrument using Zorbax Eclipse Plus C18(150 X 4.6)mm 5.0µ and a fast gradient of 1mM Ammonium acetate(Mobile Phase A) and Methanol(Mobile Phase B) at a flow rate of 0.4ml/min (Table 1).

| Time (min) | Mobile phase A% | Mobile phase B% |
|---|---|---|
| 0.01 | 95 | 5 |
| 0.50 | 45 | 55 |
| 3.50 | 45 | 55 |
| 4.00 | 95 | 5 |
| 12.00 | Controller | Stop |

**Table 1: Mobile Phase Gradient**

### *MS/MS Conditions*

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The TurboV™ source was used with an Electrospray Ionization (ESI) probe in positive ionization mode at 5500 ion spray voltage. Two selective MRM transitions were monitored and ion ratio was calculated automatically by software for compound identification (Table 2). Analyst® 1.6.2 Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.2

| Compound | Precursor ion | Product ion Quantifier | Product ion Qualifier |
|---|---|---|---|
| AOZ | 236.0 | 104.0 | 78.0 |
| AMOZ | 335.0 | 291.1 | 128.2 |
| SEM | 209.0 | 166.0 | 192.0 |
| AHD | 249.1 | 134.0 | 104.0 |

**Table 2: MRM transitions**

## Results and Discussions

### *Sensitivity, Reproducibility, Linearity and Accuracy*

The developed method showed signal-to-noise ratio > 23 for all the analytes with sample extracted at a level of 1.0 µg/kg (Spiked) which meets the regulatory criterion (Figure 3)

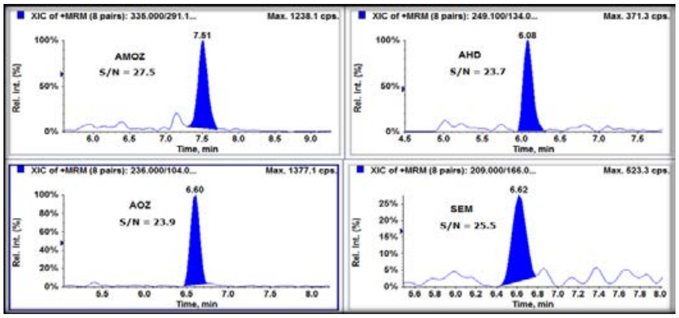


**Figure 3: Chromatogram (1.0 µg/kg) showing signal to noise**

Matrix based Calibration curve was plotted, found linear in the range of 0.50 µg/kg (ppb) to 20.0µg/kg (ppb) and correlation regression co-efficient r > 0.98 for both quantifier and qualifier ions by applying weighing factor of 1/X2 (Table 4).

Repeatability at three levels (1/2 MRPL, MRPL, 1.5MRPL) were evaluated for 6 injections and %relative standard deviation (%CV) was observed to be less than 10 (Table 3). Accuracies observed were in the range from 85% to 120%.

| Analyte | Repeatability | | | Recovery (n=6) | | |
|---|---|---|---|---|---|---|
| | ½ MRPL (0.5ppb) | MRPL (1.0ppb) | 1.5MRPL (1.5ppb) | ½ MRPL (0.5ppb) | MRPL (1.0ppb) | 1.5MRPL (1.5ppb) |
| **AOZ** | 3.41 | 5.04 | 1.72 | 98.83 | 105.00 | 107.89 |
| **AMOZ** | 5.98 | 2.89 | 3.49 | 94.37 | 92.82 | 101.67 |
| **SEM** | 8.70 | 8.84 | 3.30 | 92.37 | 102.75 | 108.33 |
| **AHD** | 7.12 | 5.86 | 8.00 | 92.57 | 86.93 | 96.56 |

**Table 3: Repeatability (%CV) and recovery statistics and in Milk**

| Analyte | Calibration Range (ppb) | Linearity (r) | CCα | CCβ |
|---|---|---|---|---|
| AOZ | 0.5 -20 | 0.9994 | 0.54 | 0.57 |
| AMOZ | 0.5 -20 | 0.9994 | 0.57 | 0.61 |
| SEM | 0.5 -20 | 0.9964 | 0.59 | 0.65 |
| AHD | 0.5 -20 | 0.9992 | 0.58 | 0.63 |

**Table 4: Summary of CCα, CCβ and linearity in milk Sample**

Decision limit (CCα) and detection capability (CCβ) were calculated for AOZ, AMOZ, SEM and AHD derivatives of Nitrofuran in milk samples. The calculation was based on using linear regression model analyzing spiked milk samples at below MRPL level (Van Loco et al, 2007).

The calculated value of CCα and CCβ are given in Table 4. The decision limit (CCα) and detection capability (CCβ) of all the compounds were well below the MRPL.

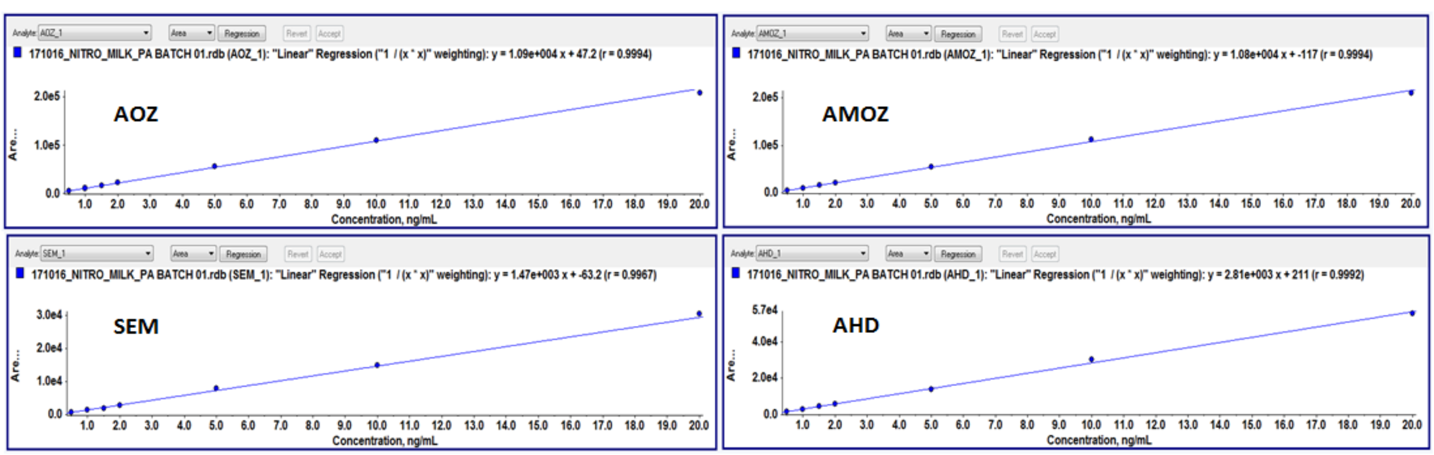


**Figure 4: Matrix based calibration curve AOZ, AMOZ, SEM and AHD**

## Conclusion

The method and data acquired here gives sensitive and accurate solution for the quantitation and confirmation of Nitrofuran metabolites in Milk samples by LC-MS/MS. The SCIEX™ 3500 system provides good sensitivity and selectivity for this analysis, allowing maximum output for the analysis of a bigger batch of samples in a short time period. Automatic ion ratio calculation in MultiQuant™ software can be used for confirmation of compound. The method showed acceptable accuracies (85%-120%), linearity with r >0.99 for both quantifier and qualifier, repeatability (%CV) observed was less than 10. The method allows high throughput, selective, rapid and sensitive LC-MS/MS identification and quantitation of banned Nitrofuran metabolites meeting EU MRPL of 1.0 µg/kg.

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