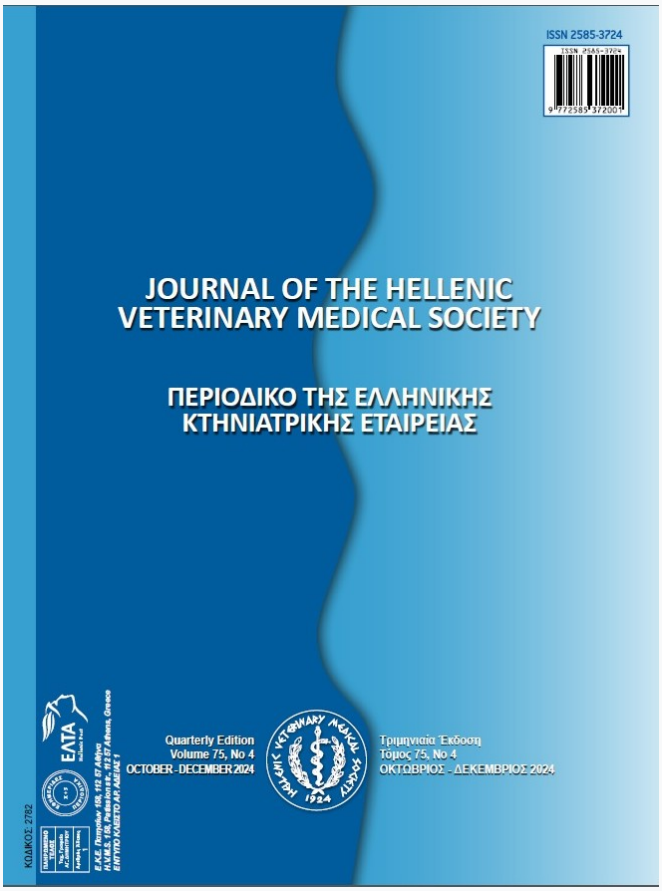
Journal of the Hellenic Veterinary Medical Society

Vol 75, No 4 (2024)



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doi: 10.12681/jhvms.36689

To cite this article:

Ozdemir, O., & Ince, S. (2025). Determination of nitrofuran residues in milk by LC/MS/MS and assessment of human health risks in Ankara region, Turkey. *Journal of the Hellenic Veterinary Medical Society*, *75*(4), 8275–8280. https://doi.org/10.12681/jhvms.36689

J HELLENIC VET MED SOC 2024, 75 (4): 8275-8280
ΠΕΚΕ 2024, 75 (4): 8275-8280

Research article
Ερευνητικό άρθρο

Determination of nitrofuran residues in milk by LC/MS/MS and assessment of human health risks in Ankara region, Turkey

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ABSTRACT: In this study, we investigated the occurrence of nitrofuran (NF) metabolites, namely 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ), and 1-aminohydantoin (AHD), in raw cow's milk and assessed the potential health risks to humans in the Ankara region, Turkey. The analytical methodology employed liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to evaluate the milk samples. The results demonstrated that the methodological parameters, including linearity and recovery, along with other validation metrics, met the necessary criteria for the analysis. Raw milk samples collected from farms within Ankara province were subjected to LC-MS/MS analysis. Upon completion of the sample analyses, NF metabolites were not detected in any of the raw milk samples. Therefore, we conclude that the NF metabolites can be accurately quantified in milk using LC/MS/MS, and the milk available for consumption in this region does not present a health risk concerning NFs to consumers.

***Keywords*:** LC/MS/MS; milk; nitrofuran; residue

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Date of initial submission: 29-1-2024
Date of acceptance: 17-6-2024

INTRODUCTION

Nitrofurans (NFs) are synthetic, broad-spectrum antibacterial, and antiparasitic agents that have been in use for over six decades. These drugs exhibit efficacy against a wide range of microorganisms, including numerous gram-positive and gram-negative bacteria (such as *Streptococci, Staphylococci, Escherichia coli, Salmonella,* and *Proteus*), as well as against spore-forming anaerobes. Additionally, NFs are effective in treating infections caused by parasites responsible for diseases such as trichomoniasis, leishmaniasis, and trypanosomiasis, including strains that are resistant to antibiotics and sulfonamides (Zuma et al., 2019; Melekhin et al., 2022). Historically, NFs have been widely utilized in veterinary medicine for the prevention and treatment of bacterial infections in farm animals, aquatic species, and bees. Furthermore, these compounds have been employed as growth stimulants in animal husbandry. NFs, upon accumulation in foods of animal origin, are metabolized into hazardous compounds that may adversely affect human health due to their carcinogenic and mutagenic potential (McCalla, 1983). Consequently, the use of NFs in intensive animal farming and aquaculture has been prohibited within the European Union (EU) and the United States (US), reflecting concerns over their health impacts (Rodziewicz, 2008; Pearson et al., 2016; Tripathi et al., 2023). Although NFs are rapidly metabolized and excreted via bile and urine, their metabolites can bind to animal tissues, remaining detectable at low concentrations for weeks following administration (Conklin, 1978). Among the NFs commonly utilized in veterinary medicine for livestock treatment are furaltadone, furazolidone, nitrofurazone, and nitrofurantoin, which are transformed into side-chain metabolites: 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ), semicarbazide (SEM), 3-amino-2-oxazolidinone (AOZ), and 1-aminohydantoin (AHD) (Ramos et al., 2017). The European Union has established minimum required performance levels (MRPLs) for the detection of AMOZ, AOZ, SEM, and AHD in animal products at a threshold of 1 µg/kg (EUC, 2003).

Numerous studies have focused on the development of innovative chromatographic techniques for the identification of side-chain metabolites of NFs, rather than the parent compounds themselves (Chu and Lopez, 2007). These chromatographic procedures often entail elaborate sample preparation steps, including extended acid hydrolysis followed by the conversion of the resultant chains into nitrophenyl derivatives for subsequent chromatographic identification (Hoogenboom and Polman, 1993; Mottier et al., 2005). Within the scope of chromatographic analyses targeting NFs, various methodologies have been proposed for the analysis of animal tissues (McCracken et al., 1995; Leitner et al., 2001; Conneely et al., 2003), honey (Khong et al., 2004), shrimp (Chu and Lopez, 2005), milk (Chu and Lopez, 2007), and eggs (McCracken et al., 2001).

Despite regulatory prohibitions, the utilization of NFs persists globally, not only in developing nations but also in developed ones. This widespread use can be attributed to their potent antibacterial properties, ease of access, and affordability. Consequently, developing sensitive and reliable chromatographic techniques for the detection of NF metabolites in foodstuffs has emerged as a critical concern for public health. In this investigation, we explore the presence of NF metabolites in raw milk intended for consumption, utilizing chromatographic methods. Furthermore, we assess the potential health risks associated with the residual presence of these metabolites for consumers.

MATERIALS AND METHODS

Chemicals

5-Methylmorpholino-3-amino-2-oxazolidinone (AMOZ), semicarbazide (SEM), 3-amino-2-oxazolidinone (AOZ), and 1-aminohydantoin (AHD) were sourced from the Pendik Veterinary Control Institute (İstanbul, Turkey). Methanol, ethyl acetate, potassium phosphate (K2PO4), n-hexane, 2-nitrobenzaldehyde, hydrochloric acid (HCl), and the other chemicals were of analytical reagent grade purchased from commercial sources. Each compound was dissolved in methanol, and a mixed solution containing 0.1 mg/L of each compound was prepared. An internal standard solution containing four internal standards ((C13)3-AHD, AMOZ-d5, AOZ-d4, and C13N15-N15-SEM)) was similarly prepared in methanol, achieving a concentration of 0.1 mg/L.

Preparation of samples for analysis

To ascertain the validation parameters, residue-free milk samples were subjected to centrifugation at +4°C and 3500 g for duration of 15 minutes. The supernatant was subsequently removed. A volume of 2 ml from these samples was allocated into centrifuge tubes. To these samples, additions were made sequentially: standard mix solution in volumes of 50 µL, 100 µL, 150 µL, and 200 µL; 100 µL of

an internal standard mix solution; and 5 mL of 0.1 M HCl. The mixture was then homogenized using a vortex for 2 minutes, followed by the incorporation of 300 µL of 50 mM 2-nitrobenzaldehyde, and vortexed again for 2 minutes for thorough mixing.

Subsequent to this vortexing step, the tubes were incubated at 37°C for a period of 16 hours. Post-incubation, the samples were cooled and neutralized by the addition of 1 mL of 1 M K2PO4, and vortexed for 2 minutes to ensure uniformity. Following neutralization, 5 mL of ethyl acetate and 3 mL of n-hexane were added, vortex mixed for 15 minutes, and then centrifuged at 4000 g for 15 minutes. The resultant organic phase was transferred into a 15 mL glass tube and subjected to evaporation under a nitrogen stream.

The residual content was reconstituted in 2 mL of n-hexane, vortex mixed for 2 minutes, after which 0.75 mL of a methanol (5%)/water (95%) solution was added and vortex mixed for an additional 2 minutes. From the resultant lower phase, a 0.5 mL sample was collected and passed through a 0.2 µm syringe filter into a vial for further analysis.

Between August and October 2023, a total of 60 raw cow milk samples were systematically collected from five distinct districts in Ankara, Turkey, namely Haymana, Ayaş, Akyurt, Bala, and Gölbaşı (Figure 1). For each sampling, a minimum of 15 ml of milk was aseptically collected directly from the cows' udders into sterile containers. The samples were immediately placed in ice packs and transported to the laboratory in an insulated ice box, where they were subsequently stored at -20°C until the analysis for NFs could be conducted. The extraction process for the milk samples, with the exception of those subjected to standard addition, adhered to the methodologies previously established for standard analyses.

Calibration

The linearity of the calibration curve was assessed by calculating the peak area following five applications of the sample at two distinct concentrations. The detection limit (LOD) is defined as the lowest concentration that can be confidently identified, measured, and reported with 99% confidence. The relative standard deviation (RSD) for 10 replicate measurements was calculated using the formula LOD = RSD × concentration × 3, assuming the blank response to be zero. From these calculations, the optimum LOD level was established. Furthermore, the quantification limit (LOQ) was determined using the equation LOQ = 10 × RSD × concentration, representing the lowest level at which quantification is feasible.

Instrumental conditions

Chromatographic analyses were conducted utilizing an LC/MS/MS system (Thermo Electron TSQ Quantum Access Max, San Jose, CA, USA), with data management and analysis facilitated by Xcalibur software (version 2.2 SP1). Chromatographic separation was achieved on a C-18 column (4 µm Synergy Hydro-RP 80 Å, 150 × 2 mm, Phenomenex®, Torrance, California, USA). The mobile phase comprised deionized water and methanol (with 0.1% acetic acid) in an 80/20 ratio. The gradient conditions were set as

Figure 1. Location map of the raw milk samples collected in the study area.

follows: 100% for 0-2 minutes, 10% for 2-9 minutes, and back to 100% for 9-15 minutes, with a flow rate of 0.25 mL/min and an injection volume of 50 µL. Sample analyses were conducted in the positive ionization mode (ESI-MS/MS). The precursor ions and MS/MS parameters were optimized in Selected Reaction Monitoring (SRM) mode, including Vaporizer and Capillary Temperature, Spray Voltage, Sheath Gas, Aux Gas, and Ion Sweep Gas, which were set at 300°C, 3000 V, 35 psi, 15 psi, and 0.5 psi, respectively. Quantitative analyses were performed under the specified conditions, detailed in Table 1.

RESULTS

When evaluating the correlation coefficients derived from the analyses, the calibration standards exhibited excellent linearity ($r^2 > 0.99$) across the examined range in ESI mode, as shown in Table 2. The retention times (RT) for AOZ, AMOZ, AHD, and SEM were identified as 6.05, 5.34, 5.95, and 6.07 minutes, respectively, illustrated in Figure 2. The decision limit (CCα), detection limit (LOD), and quantification limit (LOQ) for NFs in milk, as determined for the analyzed standards, ranged from 0.33 to 0.51 µg/kg, 0.30 to 0.44 µg/L, and 0.34 to 0.58 µg/L, respectively, detailed in Table 2. Recovery rates of NFs, assessed through five repeated measurements at two distinct concentrations, were also observed to be high, as documented in Table 3. Samples presenting NF levels beneath the detection limit were classified as negative. Consequently, no concentrations exceeding the LOD were detected in the cow milk samples collected from farms within the Ankara region, leading to the conclusion that NFs were not present in these samples.

Table 1. LC-MS/MS conditions of NF metabolites

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision Energy	Width	Tube Lens	Dwell Time
AOZ	236	134	13	0.05	64	0.1
		104	22			
AMOZ	335	291	12	0.06	70	0.1
		261	17			
AHD	249	134	12	0.07	71	0.1
		104	22			
SEM	209	166	11	0.08	98	0.1
		192	13			

Table 2. Linearity, CCα, CCβ, LOD, and LOQ values of NF metabolites

Analyte	Linearity (r^2)	CCα (µg/kg)	CCβ (µg/kg)	LOD (µg/L)	LOQ (µg/L)
AOZ	0.998	0.33	0.35	0.30	0.34
AMOZ	0.998	0.22	0.23	0.20	0.22
AHD	0.999	0.34	0.37	0.32	0.40
SEM	0.995	0.47	0.51	0.44	0.58

Table 3. Recovery values of NF metabolites

Analyte	Amount (µg/kg)	Mean estimated value (µg/kg)	Recovery (%)	CV (%)
AOZ	0.3	0.29	97.0	4.02
	0.6	0.59	99.6	2.51
AMOZ	0.2	0.19	94.9	3.00
	0.4	0.39	96.6	5.06
AHD	0.3	0.29	97.3	4.45
	0.6	0.59	97.8	2.62
SEM	0.4	0.39	99.6	5.35
	0.8	0.78	98.3	2.88

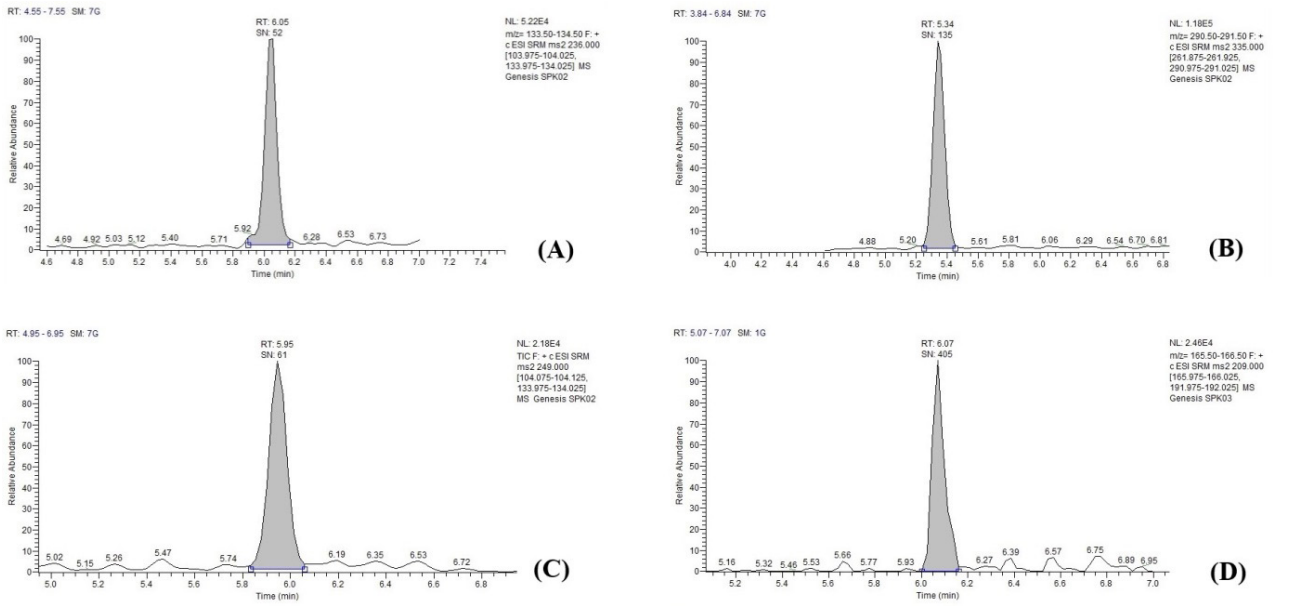

Figure 2. Chromatography of 3 amino2 oxazolidinone (AOZ; A), 5 methylemorpholino 3 amino 2 oxazolidinone (AMOZ; B), 1 aminohydantoin (AHD; C) and semicarbazide (SEM; D).

DISCUSSION

Despite their therapeutic benefits, NFs have been identified as having mutagenic and carcinogenic properties. Consequently, the Food and Drug Administration (FDA) prohibited all off-label applications of NFs in animal feed products in 2002 (Federal Register, 2002). Additionally, NFs were included in the list of substances banned in Turkey, as stipulated by the Turkish Food Codex Regulation, which was published in the Official Gazette No. 30000 on March 7, 2017. Despite these prohibitions, the detection of NF residues in food and food products in various countries has necessitated the continued inclusion of NFs in national residue monitoring programs (Khong et al., 2004).

In recent studies, researchers have focused on analytical methods for the rapid and precise detection of NF metabolites. Alkan et al. (2016) isolated NF metabolites from milk, fish, meat, honey, and poultry through acidic hydrolysis, followed by derivatization with nitrobenzaldehyde and liquid extraction using ethyl acetate. They utilized LC/ESI-MS/MS for the analysis of NF metabolites and reported the capability to detect low levels of these metabolites across all samples with a 99% recovery rate, enabling rapid and quantitative confirmation. Kaufmann et al. (2015) developed a methodology to determine residues of NF and chloramphenicol in various matrices including muscle, milk, kidney, honey, fish, liver, and eggs. Their approach involved ultra-high-performance liquid chromatography combined with high-resolution mass spectrometry (UHPLC-HRMS), based on hydrolysis of covalently bound metabolites and subsequent derivatization with 2-nitrobenzaldehyde. Similarly, in our study, NF metabolites were obtained through acidic hydrolysis, derivatized with nitrobenzaldehyde, and effectively analyzed using LC-MS/MS. The study demonstrated that the linearity, recovery, and other validation parameters of the analyzed milk samples met the required levels for analysis. In parallel, Chu and Lopez (2007) established a method for analyzing furaltadone, furazolidone, nitrofurantoin, and nitrofurazone residues in cow milk, which also involved acid hydrolysis and derivatization with 2-nitrobenzaldehyde, with the analyses conducted via LC-MS. They reported method accuracies ranging from 83% to 104%, and coefficients of variation for all four analytes were under 13%.

In addition to method development studies, there have been relatively few screening studies for NFs both domestically and internationally. One such study conducted in Iran utilized the ELISA method to screen 81 milk and egg samples for NF residues. The findings of this research indicated that while no residues were detected in the egg samples, two milk samples were found to contain NF residues at levels of 216.6 and 208.1 ng/kg (Raziabad et al., 2022). Conversely, in our study, the examination of milk samples from the Ankara province using the LC/MS/MS technique a method more sensitive than ELISA revealed no presence of NF metabolites.

CONCLUSION

Consequently, this study suggests that this methodology applied is very suitable for the identification of NFs, as well as the milk available for consumption in the Ankara region is safe and does not pose a health risk to consumers. However, despite their status as banned substances, it has remained imperative to conduct regular monitoring of NFs in animal milk and/or feed to mitigate the risk of NF contamination. Furthermore, dairy producers and relevant regulatory bodies must be cognizant of the detrimental health impacts associated with NFs. To support these objectives, the adoption and further development of highly sensitive analytical methods for the detection of NFs are recommended.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

ÖÖ and SI conceived and planned the experiments. ÖÖ carried out experiments. ÖÖ and SI contributed to the interpretation of the results. ÖÖ and SI took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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