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Quantitative Determination of Six Nitrofuran Parent Drugs in Shrimp Muscle Using Ultra-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry According to European Commission Implementing Regulation (EU) 2021/808

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Abstract

A method validated in accordance with Commission Implementing Regulation (EU) 2021/808 was developed for the detection of six nitrofuran parent compounds, including furazolidone, furaltadone, nitrofurazone, nitrofurantoin, nifursol, and nitrovin in shrimp muscle to meet the food safety requirements of importing countries, particularly those of the European Union and the Korean Ministry of Food and Drug Safety (MFDS). Samples were extracted using acetonitrile, and the extracts were dried under a nitrogen stream. The dried extract was reconstituted with 1 mL of n-hexane and 1 mL of methanol/0.1% formic acid in water (90:10, v/v) and then defatted with n-hexane. Analysis was performed using UPLC-MS/MS. This method achieved limits of quantification below 0.5 μg kg^{-1} for all nitrofuran parent compounds. The decision limit (CCα) ranged from 0.30 to 0.34 μg kg^{-1} for all six compounds, and within-laboratory reproducibility was below 12%. Linear calibration curves for fortified shrimp muscle ranged from 0.25 to 5 μg kg^{-1}, with R^2 values between 0.995 and 0.998 for all six compounds.

Keywords: Nitrofurans, decision limit, UPLC-MS/MS, nitrovin, method validation, reference point of action

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Introduction

Nitrofuran parent (NFP) drugs are a family of chemical compounds that have broad-spectrum antimicrobial activities and can be used in both humans and animals, including fish. Nitrofuran antimicrobial drugs are used in veterinary practice, rapidly degrading in treated animals and accumulating in the tissues, where they remain for a long time. As a result, residues have been found in many foods. NFPs are listed as prohibited substances for use in food-producing animals in the European Union (EU) member states, where a maximum residue limit (MRL) cannot be established, according to Regulation (EU) 37/2010. In addition, the use of these substances is also prohibited in Japan, the USA, Korea, China, and other countries. The stability of nitrofuran residues bound to tissues has been studied widely, and it was demonstrated that with the most common food processing techniques, the residues were not significantly degraded.

NFP compounds are rapidly metabolized after ingestion, forming corresponding tissue-bound metabolites. The short *in vivo* half-life of the parent drugs (7 to 63 minutes) leads to the rapid depletion of nitrofurans in blood and tissue (Nouws & Laurensen, 1990). Although the metabolism of nitrofurans is not well-documented, a suggested mechanism involves the cleavage of the nitrofuran ring, leaving the specific tail group covalently bound to tissue (Leitner, Zöllner, & Lindner, 2001). Studies have shown that furaltadone, nitrofurantoin, and nitrofurazone are rapidly converted into their metabolites, with furazolidone being an exception.

According to Jia, Zhang, Qu, Wang, and Xu (2022), although the parent drugs are rapidly metabolized *in vivo*, tissue-bound metabolites cannot undergo further metabolism, leading to significant amounts of these metabolites remaining in the body. As per Cooper, McCracken, Buurman, and Kennedy (2008), nitrofuran parent compounds were also detected in the eyes of broiler chickens, with furaltadone being identified in individual eyes after a 21-day withdrawal following a diet containing 6 mg/kg of furaltadone.

The addition of nitrofurans to animal samples must therefore be strictly controlled and screened, as toxicological assays have demonstrated their potential mutagenic, carcinogenic, and teratogenic effects. Overseas studies showed that nitrofurans had been used in pigs, poultry, shrimps and fish. The presence of nitrofuran residues in food can lead to allergic reactions and contribute to the development of antimicrobial resistance (AMR). AMR occurs when microorganisms such as bacteria, fungi and viruses develop tolerance to antimicrobial substances, especially nitrofurans. According to the World Health Organization (WHO), AMR represents a serious threat to global health, development, and food security (WHO, 2023). As per Melekhin, Tolmacheva, Apyari, and Dmitrienko (2022), nitrofuran parent compounds (NFPs) are transformed into carcinogenic and mutagenic metabolites, which can accumulate in foods of animal origin and adversely affect human health. Studies have revealed that nitrofurans may cause cancer in both animals and humans (Zhou et al., 2012). Nitrofurans have been prohibited for use in food-producing animals in many countries, including the members of the European Union, the United States, Canada, Japan, Korea, and China. However, because of the low price and significant efficacy, NFP drugs are still illegally used as veterinary drugs in some developing countries. Some hatcheries are using nitrofuran self-destructively for the treatment of brood stocks as well as prawn larvae. This later sustains as residue in the seeds and travels to the culture pond. Global Nitrofuran ''crisis'' came to light in 2002–03 when nitrofuran metabolites were reported in shrimp and aquaculture products from Thailand, Vietnam, and Brazil. According to Antunes, Machado, and Peixe (2006), the use of nitrofuran drugs in food animal production has been banned in the EU since 1993 and 1995 (Council Regulation (EEC) No. 2901/93, 1993; Commission Regulation (EC) No. 1442/95, 1995) (The European Commission, 1993, 1995).

The European Union (EU) has banned many additives used in samples for animal growth promotion and established a zero tolerance for these compounds and their metabolites in Annex IV of Regulation 2377/90/EEC (1995). The European Commission Decision (EU) 2019/1871 established a Reference Point of Action (RPA) of 0.5 μg kg^{-1} for unauthorized pharmacologically active substances, such as nitrofurans found in food of animal origin (The European Commission, 2019). A similar target testing level (TTL) is set by the USFDA Compliance Program Guidance Manual, Program No. 7304.018.

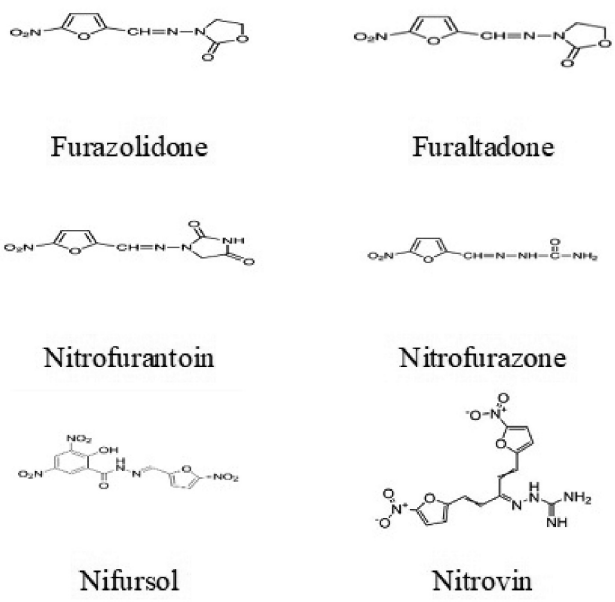

Fig. 1. Molecular structures of nitrofuran parent compounds (furazolidone, furaltadone, nitrofurantoin, nitrofurazone, nifursol and nitrovin)

Many analytical methods have been developed to detect nitrofuran metabolites (NFM) in various matrices, such as shrimp, meat, milk, and honey. However, few methods specifically target the detection of four nitrofuran parent compounds—furazolidone, furaltadone, nitrofurazone, and nitrofurantoin—using LC-MS/MS. This new method addresses this gap by including six NFP compounds, adding nifursol and nitrovin to align with EU and MFDS (Korea) guidelines, and employs Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). These six nitrofurans are commonly used as additives in livestock and aquatic products (Fig.1). The method is designed to meet the exceptionally low detection requirements for nitrofurans, with detection limits

as low as 0.5 μg kg^{-1} (RPA/TTL). Given these strict detection needs, developing a cost-effective, simple, and sensitive analytical method for NFM detection in shrimp is essential. UPLC-MS/MS is widely used for its high sensitivity and accuracy, making it a reliable tool for detecting nitrofuran parent drugs and addressing food safety concerns.

Materials and Methods

Acetonitrile, Methanol, and Water (LC-MS grade) were supplied by Merck (India). Acetic acid, and n-Hexane (LC grade) and Dimethyl sulfoxide (LiChrosolv) were supplied by Merck (India). The reference standards, Furazolidone, Nitrofurantoin, Nifursol and Nitrovin hydrochloride were purchased from HPC (Germany), Furaltadone was purchased from Sigma Aldrich (USA), and Nitrofurazone was purchased from Dr. Erhenstorfer (Germany). Stock standard solutions of the above six NFP compounds (1000 mg/L) were prepared by dissolving them in methanol and stored in a refrigerator at -18 °C. The working solutions were prepared through appropriate dilutions of the stock solutions. Working standard solutions were stable at least for 2 months when stored in the dark at 4 °C, and stock solutions were stored in the dark at -18 °C and were stable at least for 6 months.

The equipments used for sample preparation are Refrigerated centrifuge (Eppendorf, 5810 R, Germany), a weighing balance (Shimadzu, AUW220D, Uni Bloc, Japan), and a nitrogen evaporator (Turbo Vap LV, Caliper Life Sciences, USA). The water purification system (Evaqua Water Technologies, Inexus Biotech, India) was used to get demineralized water. Homogenizer (Preeti, India) was used for homogenizing the shrimp meat. The liquid chromatographic analysis was performed using an Acquity ultra-high-performance liquid chromatography (Waters, Singapore) comprising a Binary solvent manager (Waters, I-class), an autosampler (Waters, Acquity UPLC sample Manager FTN), and a column oven. The chromatographic separation was carried out using an XBridge BEH C18, 2.5 ìm, 2.1×100 mm (Waters) with a mobile phase consisting of 0.1% acid in water (phase A) and Methanol (phase B) at a flow rate of 0.4 mL min^{-1}. The gradient elution program was as follows: 0 min, 98% A; 0.5 min, 98% A; 3.0 min, 2% A; 4.5 min, 2% A; 4.6 min, 98% A; 7.0 min, 98% A. The total chromatographic run time was 7 min. The injection volume was 10 μL, and the column temperature was 40 °C.

Mass spectrometry analysis was performed using an Xevo TQ-S Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) interface; the capillary voltage was 3.0 kV in positive and negative mode. The ion source block temperature was set at 150 °C, dissolvation temperature was set at 550 °C and dissolvation and cone gas flow were set at 1000 L/hr and 50 L/hr, respectively. The nitrogen was used as the collision gas at a pressure of 5m Torr. Each target compound was detected by using two multiple reaction monitoring (MRM) channels, of which one was used for quantification and the other for confirmation. Instrument settings, data acquisition and processing were controlled by the software Masslynx (version 4.1, Waters). The complete Mass Spectrometry (MS) parameters, including cone voltage, MRM transitions and collision energies are shown in Table 1.

Shrimp samples were collected from the local markets of Chennai, India. The shells were removed, and the edible parts were used for sample homogenization. The sample was homogenized in a laboratory homogenizer and stored at -18 °C until analysis. Antibiotic-free samples were used as blanks for validation experiments. The samples were kept for one hour after spiking to equilibrate before extraction.

1 ± 0.1 g of the shrimp sample was taken into 50 mL polypropylene centrifuge tubes. Further, 8 mL of acetonitrile was added to the sample. Further, the mixture was mixed vigorously for 30 seconds. The mixture was centrifuged at 10,000 rpm for 5 min. The acetonitrile layer (supernatant) was transferred into a 15 mL glass test tube, and the extraction procedure was repeated.

The combined supernatant was evaporated to dryness at 45 °C with a nitrogen stream using Turbo vap. The dried extract was reconstituted with 1 mL of n-hexane and 1 mL of 1:9 Methanol/0.1% formic acid in water and vortexed for one minute, and then the mixture was transferred to a 2 mL polypropylene centrifuge tube (Tarson, India) and centrifuged at 8,000 rpm for 5 min. The bottom layer was transferred and filtered through a 0.20 μm polytetrafluoroethylene (PTFE) filter (Agilent, USA). The collected filtrate was injected into the UPLC-MS/MS system under the optimized analytical conditions.

Method validation was performed according to European Commission Implementing Regulation

(CIR) (EU) 2021/808 (The European Commission, 2021) in order to establish the performance characteristics of the method, ensuring the adequate identification, confirmation and quantification of target compounds. The performance characteristics established were specificity, linearity, absolute recovery, matrix effect, trueness, retention time, relative intensities, identification points, within-laboratory repeatability, within-laboratory reproducibility, ruggedness and decision limit (CCα).

For specificity as per CIR (EU) 2021/808, a minimum of twenty samples is to be evaluated. In this study, a total of 21 representative blank shrimp muscle samples were analyzed and checked for any interferences (signals, peaks, ion traces) in the region of interest, where the target analyte is expected to elute.

The detection and quantitation limits were determined according to the signal-to-noise ratio. The limit of detection was set as a signal-to-noise ratio of 3:1, while the limit of quantification was set as a signal-to-noise ratio of 10:1 and from the standard deviation of the intercepts limit of detection (LOD = 3σ/S) and limit of quantitation (LOQ = 10σ/S) were calculated (where, σ –Standard deviation of the intercepts and S - Slope off the regression).

As per EU regulation, at least five preferably equidistant levels (including zero level) need to be used for the construction of the calibration curve, and the curve will be a linear (y = mx + c) with a 1/x fit. The mathematical formula of the curve and the goodness-of-fit of the data (coefficient of determination R^2) are required to be described.

Absolute recovery (RE) indicates the influence of the sample components on the entire determination method (Caban, Migowska, Stepnowski, Kwiatkowski, & Kumirska, 2012). The absolute recovery was calculated in six representative sample matrices. An aliquot of blank matrix was fortified with the analyte before extraction, and a second aliquot of blank matrix was fortified after sample preparation at a relevant concentration level, and the concentration of the analyte was determined.

The recovery was calculated as:

Eq.1. Absolute Recovery (RE) = (area matrix-fortified standard)/ (area matrix-matched standard) × 100

In liquid chromatography–tandem mass spectrometry (LC–MS/MS), matrix effects from biological samples can interfere with the ionization of target analytes, leading to either enhancement or suppression of ion intensity (Williams, Olomukoro, Emmons, Godage, & Gionfriddo, 2023). Compounds with high mass and polarity are more likely to trigger matrix effects (Wong et al., 2022). To evaluate ME, in this study, 21 fortified different lots of shrimp samples were analysed and bracketed with neat solution standards of the equivalent concentration of the fortified samples. The matrix effect was estimated by comparing the slope of the external calibration curve with that of the standard addition calibration curve covering the concentrations from 0.25 to 5 μg kg^{-1} for each NFP drug.

The recovery was calculated as:

Eq. 2. Matrix effect (ME) % = (Slope of the matrix matched curve/ Slope of the external standard curve) x 100

The acceptance criterion as per CIR (EU) 2021/808 specifies that the coefficient of variation should be less than 20% for the Matrix Factor.

Trueness of an analytical method measures the method's correctness with respect to the analyte of interest (González-Mariño et al., 2009). It reflects how closely the mean of a set of measurements aligns with the expected reference value and is influenced by systematic errors. Trueness is a key factor in evaluating the accuracy of a UPLC-MS/MS method for food analysis (Pizzutti et al., 2016). In this study, since no certified reference material (CRM) is available, trueness was assessed by recovering fortified samples of six nitrofuran standards at each concentration level of the analytes in blank shrimp muscle. At each level, the analysis was performed with seven replicates and the recovery was calculated using the following formula:

Eq. 3. Trueness % = (mean recovery-corrected concentration detected) × 100/fortification level

The acceptable ranges should fall between –50% to +20% as per point No. 1.2.2.1 of CIR (EU) 2021/808.

For liquid chromatography analysis, as per CIR (EU) 2021/808, the minimum acceptable retention time for the analyte(s) under examination is twice the retention times corresponding to the void volume of the column. The RT requirements for chromato-

graphic separation of the analyte in the extraction analysis correspond to those of the calibration standard, a matrix-fortified standard with a tolerance of ± 0.1 minute (Arrizabalaga-Larrañaga, van Doorn, & Sterk, 2024).

The relative intensities of the diagnostic ions are expressed as a percentage of the intensity of the most abundant ion or transition (Ferrer & Thurman, 2013). In this study, the ion ratios were determined by integrating the signals of the extracted ion mass traces. The relative intensities of the ion ratio of the analyte were confirmed and correspond to matrix fortified standards solutions at comparable concentrations, measured under the same conditions, within ± 40% relative deviation as per CIR (EU) 2021/808.

The identification points system must be applied to select appropriate acquisition modes and evaluation criteria. All mass spectrometric analyses should be coupled with a separation technique that provides sufficient separation power and selectivity for the specific application (Criscuolo, Zeller, Cook, Angelidou, & Fedorova, 2019). According to the European Union's CIR (EU) 2021/808, at least four identification points are required to confirm the identity of substances in a matrix with an established Maximum Residue Limit (MRL). For each analyte in a confirmatory method for prohibited substances, a minimum of five identification points is necessary.

Within-laboratory repeatability studies were carried out to contribute to the evaluation of the method's trueness and precision. Repeatability studies were performed at three concentration levels—0.5, 1.0 and 1.5 times the RPA for the six nitrofuran prohibited substances considering the applicable RPA. All repeatability analyses were carried out by the same analyst. In each WL_r run, a total of 21 samples were analyzed, comprising seven samples at each level, spiked at 0.25, 0.5, and 0.75 μg kg^{-1}. The fortified samples were evaluated against an extracted matrix-based (procedural) calibration curve prepared from the same control sample used for the 21 samples in that run, covering all concentration levels. The mean concentration and recovery of the fortified samples were calculated for each run.

A within-laboratory reproducibility (WL_{WR}) study was carried out to support the evaluation of the method's trueness and precision. The WL_{WR} was determined at three different levels by carrying out 21 shrimp samples at each level. These analyses were carried out by different analysts on three different days. The WL_{WR} studies were determined by carrying out at three different levels for shrimp muscle samples at 0.5, 1.0 and 1.5 times the RPA for the six nitrofuran prohibited substances considering the applicable RPA. In each WL_{WR} run, seven samples per concentration level were spiked at 0.25, 0.5, and 0.75 μg kg^{-1}. Across three days, a total of 21 fortified samples at each level were analyzed. The fortified samples were evaluated against an extracted matrixfortified calibration curve prepared from the same control shrimp muscle sample. The coefficient of variation (CV) for the repeated analysis of a sample, under within-laboratory reproducibility conditions, shall not exceed the level calculated by the Horwitz Equation.

The equation is: $CV = 2^{(1 - 0{,}5 \log C)}$

Where C is the mass fraction expressed as a power (exponent) of 10 (e.g., 1 mg/g = 10^{-3}).

The maximum allowable coefficient of variation (CV%) shall not exceed 30% for unauthorized banned substances with limits of ≤ 10 μg kg^{-1}.

The decision limit (CCα) is defined as the threshold at or above which it can be concluded, with an error probability of α, that a sample is non-compliant. The value 1 - α represents the statistical confidence (in percentage) that the permitted limit has been exceeded (de Oliveira, Vieira, Orlando, & Faria, 2017). CCα for all substances is calculated using Method 1 for unauthorised or prohibited pharmacologically active substances as described in CIR (EU) 2021/808. Method 1 uses the calibration procedure for marker residues according to ISO 11843, procedure. CCα is calculated by plotting the signal against the added concentration corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory of the intercept equals the decision limit (α = 1%) are calculated using the statistical calculation..

The decision limit is calculated as:

CCα = yintercept + 2.33 x SD reprod

where yintercept - The concentration at the y-intercept of the plotted calibration curve (using recalculated concentrations from blank material fortified at RPA

2.33 - The statistical factor (k-value) corresponding to a one-sided 99% confidence level (α = 0.01)

SD reprod - The standard deviation of the within-laboratory reproducibility at the intercept.

For NFP in shrimp muscle, the results align with Section 2.6.1(c) of EU 2021/808, and the calculated CCα values must be lower than or equal to the RPA. These calculated CCα values are presented in Table 3.

In LC-MS/MS, ruggedness refers to the ability of a method to remain consistent when small, deliberate variations are made to method parameters (Li, Zhang, Bai, & Lu, 2024). This characteristic is crucial, as LC-MS/MS involves numerous system parameters that can be challenging to control, and certain key performance features are sensitive to slight alterations. It is assessed by intentionally adjusting method parameters to observe the method's stability. For example, the column temperature was adjusted from 40 °C to 37 °C, and the mobile phase composition was changed from 0.1% acetic acid in water to 0.15% acetic acid in water and methanol. Additionally, the room temperature was increased from 22 °C to 28 °C to ensure that these variations did not affect the analytical method. Each modification was assessed using the standard addition method at a concentration of 0.5 $\mu g\ kg^{-1}$, and recovery was calculated accordingly.

A spreadsheet (Excel 2013 version) was used to calculate CCα, repeatability, and within-lab reproducibility, expressed as a percentage of the coefficient of variation (CV).

Results and Discussion

In this study, we also examined the sample preparation step, which is crucial for obtaining reliable and significant results. Sample preparation plays a vital role, especially during LC-MS/MS method development for analyzing biological samples. The goal of sample preparation is to ensure that the analytical method remains robust and uniform. Shrimps from aquaculture ponds contain various organic and inorganic impurities. These co-existing substances can block the HPLC column, and during sample preparation, they may be co-extracted with the target compounds, interfering with the ionization of analytes in mass spectrometry (You et al., 2009). Biological matrices are complex and often contain proteins, lipids, salts, acids, bases, and other organic and inorganic impurities with properties similar to those of the target analytes, which can affect the analytes' detection (Abdel-Rehim et al., 2020). Therefore, sample preparation is an essential step before instrumental analysis. However, it remains the most time-consuming and labour-intensive step in biochemical analysis.

Bourdat-Deschamps, Leang, Bernet, Daudin, and Nélieu (2014) and Mahdavi and Talebpour (2023) reviewed several biological sample preparation techniques, including solid-phase extraction (SPE), liquid–liquid extraction (LLE), protein precipitation (PP), and solid-phase microextraction (SPME). Different sample weights (0.1, 1, and 5 g), various extraction solvents (methanol, water, and acetonitrile), and different concentrations of formic acid were evaluated to determine the optimal extraction efficiency. Previous studies have reported lower recoveries when using acetonitrile; however, in the present study, repeated extraction with acetonitrile acidified with formic acid in water consistently yielded high extraction recoveries, minimized lipid co-extraction, and effectively denatured proteins, in agreement with earlier findings of Pati, Nie, Arnold, & Cummings (2016) and Saxena et al. (2018). The method was optimized using a smaller sample size (1.0 ± 0.1 g) and involved defatting with n-hexane after reconstitution in methanol/0.1% formic acid (90:10, v/v), which reduced matrix effects and minimized ion suppression and enhancement during mass spectrometric analysis. This method achieved a lower limit of quantification at 0.25 $\mu g\ kg^{-1}$ for all six nitrofuran parent compounds and provided satisfactory recoveries for all six NFP compounds.

For each target analyte, the MRM conditions of the mass spectrometer were optimized to provide the best performance for all six NFP drugs quantification. To achieve high sensitivity, each analyte prepared as a 100 $\mu g\ L^{-1}$ standard solution was individually injected directly into the mass spectrometer by the direct infusion method, and the mass spectrum was identified in full scan mode. In the Q1 mass spectrum, all analytes showed a high signal intensity in the form of $[M + H]^+$ (for compounds viz; furaltadone, furazolidone and nitrovin) and $[M - H]^-$ (for compounds viz; nitrofurantoin, nitrofurazone and nifursol) and the corresponding ion was selected as the precursor ion. To acquire the MRM conditions, the m/z value of the precursor ion was input into the tuning

programs of the mass spectrometer and the product ion was identified according to the precursor ion. Consequently, the detection sensitivity of the product ions was augmented by altering the declustering potential energy (DP) in terms of Cone voltage, entrance potential energy (EP), collision energy (CE), and collision cell exit potential energy (CXP); three optimal product ions were selected. Among the three product ions, the ion with the highest signal strength was selected as the quantitative ion, and the remaining two product ions were chosen as qualitative ions (Table 1).

In terms of chromatographic optimization, several gradient profiles were checked to improve peak resolution and minimize the run time. To obtain good resolution of the analyte, different combinations of solvents were tested, using gradient mode: a mixture of methanol-water containing 0.1% of formic acid; methanol-water; acetonitrile-water containing 0.1% formic acid and the same conditions with acetonitrile-water. The use of methanol–water, acetonitrile–water containing 0.1% formic acid, and acetonitrile–water resulted in poor peak resolution for nifursol and nitrofurazone. In contrast, a mobile phase consisting of methanol–water containing 0.1% formic acid provided improved peak shape, higher peak intensity, and better resolution. The use of methanol as a mobile phase component for analysis of antibiotics has been reported previously by Sniegocki, Giergiel, Sell, & Posyniak (2018), as has the use of acetonitrile (Vincent, Chedin, Yasar & von Holst, 2008). The LC column with smaller particles (2.5 μm) also improved peak separation, peak sharpness and reduced run time. The use of UPLC gave the possibility of having short run time, reducing mobile phase solvent consumption, higher resolution and sensitivity, which is in agreement with earlier studies (Geis-Asteggiante et al., 2012). The gradient program described in this study enabled the determination of all six NFP compounds in 7 min with a 1 min delay in MS for equilibrium.

The ionization efficiency of analytes in the ESI source may be influenced by matrix effects. Therefore, evaluating matrix effects is crucial. Slope ratio percentage (Table 2) was calculated for each pair of curves to the target compounds using Eq. (1) and (2). To evaluate ME, in this study, 21 fortified different lots of shrimp samples were analysed and bracketed with neat solution standards of the equivalent concentration of the fortified samples. Matrix effects were evaluated by determining the percentage difference between the mean peak area counts of the solvent standards and those of the fortified samples, as well as by calculating the coefficient of variation for the fortified samples. As observed from equations, ME% varied from 92 to 97% for all six compounds. The absolute recovery for regulatory compliance, expressed as RE%, was slightly low, ranging from 77 to 91%; however, the recovery values were within the acceptable range of

Table 1. MS/MS transitions and optimal operational conditions used for nitrofuran parent analysis

Compound(s)	Molecular formulae	ESI	Retention Time, RT (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Collision energy (eV)	Cone Voltage (eV)
Furaltadone	$C_{13}H_{16}N_4O_6$	ESI^{+ve}	1.76	325.08	99.98 (Quantifier)	0.023	21	40
					251.96 (Qualifier)	0.023	11	40
Furazolidone	$C_8H_7N_3O_5$	ESI^{+ve}	2.05	225.89	94.88 (Quantifier)	0.023	15	50
					67.00 ((Qualifier)	0.023	20	50
Nitrofurantoin	$C_8H_6N_4O_5$	ESI^{-ve}	1.95	236.83	151.91 (Quantifier)	0.023	10	20
					123.93 (Qualifier)	0.023	16	20
Nitrofurazone	$C_6H_6N_4O_4$	ESI^{-ve}	1.88	197.43	149.97 (Quantifier)	0.023	08	20
					123.83 (Qualifier)	0.023	10	20
Nifursol	$C_{12}H_7N_5O_9$	ESI^{-ve}	2.43	363.86	182.20 (Quantifier)	0.023	20	44
					209.93 (Qualifier)	0.023	14	44
Nitrovin	$C_{14}H_{12}N_6O_6$	ESI^{-ve}	2.48	361.05	57.94 (Quantifier)	0.023	28	30
					222.06 (Qualifier)	0.023	18	30

70–120% for all six banned substances. Despite the low injection volume (10 μL), significant matrix effects were observed for most analytes. These results are consistent with observations reported in other studies (Lopes, Reyest, Romero-Gonzalez, Vidal, & Frenich, 2012; Fedorova, Nebesky, Randak, & Grabic, 2014; Hoff et al., 2015). The magnitude of the matrix effect depends on the sample matrix, the sample preparation procedure used for clean-up, the chromatographic separation (column, mobile phase), and the ionization interface. Matrix effects can dramatically impact the performance of analysis, leading to false negative or false positive results (Zhou, Yang, & Wang, 2017). Since, multiple residues were analyzed in a single run, finding an internal standard (IS) for each analyte was difficult and, in some cases, not possible. Consequently, understanding the matrix effect is crucial when simultaneously analyzing multi-residue antibiotics. Therefore, the matrix effect was compensated for by using a recovery calibration curve. Evidently, a matrix fortified calibration curve was used for accurate quantification of target compounds in real samples, as ion suppression effects were spontaneously corrected. These results confirm the observation of other studies by Koike et al. (2018).

The method was validated according to the Commission Implementing Regulation (EU) 2021/808. The analysis of 21 blank shrimp muscle samples for each of the matrices did not reveal any interference found at the expected RT of all the analytes. The specificity analysis concluded that there were no interfering peaks and cross-interference in the area of the analyte of interest. The LOD and LOQ values were determined according to the signal-to-noise ratio and were found to be in the range of 0.1 μg kg^{-1} and 0.25 μg kg^{-1} for each of the six NFP compounds, respectively. The method was also able to detect the antibiotics spiked in the samples without obtaining any false negatives (Fig. 2). An extracted matrix fortified calibration curve (Procedural calibration) with a minimum of six levels (including the origin) was injected in the calibration ranges of 0, 0.25, 0.5, 1.0, 1.5, 2.0, and 5.0 μg kg^{-1}. The mathematical formula of the curve and the goodness-of-fit of the data (coefficient of determination R^2) to the curve were described. The linearity (R^2) for all concentration levels (0.25 - 5.0 μg kg^{-1}) was found between the ranges of 0.995–0.998 for all six NFP drugs.

The accuracy of the measurements was evaluated by recovering six nitrofuran standards, each fortified at different concentration levels of the analytes in blank shrimp muscle. The analysis was conducted in seven replicates per concentration level, with recovery calculated using Eq. (1). Trueness, evaluated as recovery across all three concentration levels, ranged from 89.2% to 96.5% for furaltadone, 87.4% to 98.8% for furazolidone, 85.5% to 95.2% for nitrofurantoin, 87.6% to 97.4% for nitrofurazone, 87.5% to 98.2% for nifursol, and 89.3% to 95.6% for nitrovin. All recovery values were within the acceptable range of 50–120% for the six banned substances. The coefficient of variation (CV) for repeated analyses under within-laboratory reproducibility conditions, calculated using the Horwitz equation, did not exceed 12%, in agreement with previous reports by Luo et al. (2012) and Shendy, Al-Ghobashy, Alla, and Lotfy (2016).

The retention time requirements in chromatographic separation ensure that the analytes in the sample are correctly identified and quantified. For all six

Table 2. Slope, regression coefficient (R^2), matrix effect (ME %) and Absolute recovery (RE %) estimated for antibiotics in shrimp muscle

Compound (s)	Solvent standard calibration (S)		Matrix fortified (Procedural)		Matrix matched calibration (MM)		Matrix Effect (ME) %	Absolute Recovery (RE) %
	Slope	R^2	Slope	R^2	Slope	R^2		
Furaltadone	278009	0.997	229090	0.999	256302	0.998	92.19	82.40
Furazolidone	31046	0.998	26996	0.998	29633	0.996	95.45	86.95
Nitrofurantoin	8026	0.995	7143	0.996	7623	0.996	94.98	89.00
Nitrofurazone	3102	0.996	2400	0.997	2856	0.995	92.07	77.38
Nifursol	6125	0.998	5505	0.992	5960	0.997	97.31	89.89
Nitrovin	76392	0.996	69949	0.998	72963	0.998	95.51	91.57

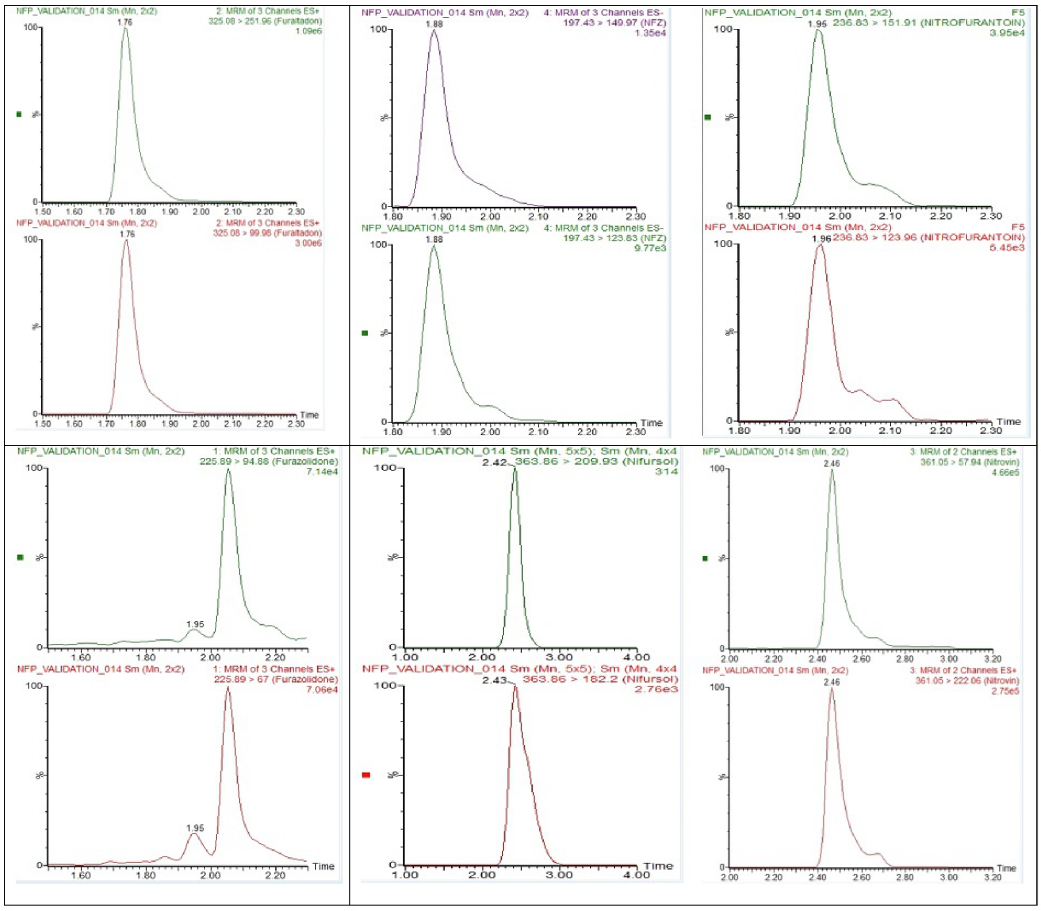

Fig 2. MRM chromatograms of six nitrofuran parent drugs showing precursor-to-product ion (m/z) transitions for identification in shrimp samples spiked at 0.5 μg kg^{-1}

nitrofuran parent drugs, the retention times closely matched those of the calibration standard, which is typically a matrix-fortified standard. The acceptable tolerance is within ±0.1 minute of the calibration standard's retention time, ensuring accurate analyte identification and reducing the risk of false positives or negatives. The retention time results showed that all six matrix-fortified standards were within the acceptable tolerance limits and complied with Commission Implementing Regulation (EU) 2021/808, point 1.2.3, consistent with previous findings reported by Lozano, Hernando, Uclés, Hakme, and Fernández-Alba (2019).

In chromatographic and mass spectrometric analysis, the relative intensities of diagnostic ions are crucial for confirming the identity of an analyte (Thevis & Schänzer, 2005). The relative intensity ratios are often used in conjunction with retention times to provide a robust identification of the analyte. In this method for all nitrofuran parent drugs, the relative intensities of the ion ratio of the analyte were confirmed and correspond to matrix fortified standards solutions at comparable concentrations, measured under the same conditions and falling within ± 40% relative deviation and met the commission implementing regulation point 1.2.4.1 of (EU) 2021/808. The results of relative intensities provide a standardized comparison across various analyses, ensuring that the identification of the analyte is based on consistent and reproducible criteria. This also confirms the analyte's presence in the sample and that the detected ions are not artefacts or noise, aligning with previous studies by Chamberlain, Rubio, and Garrett (2019).

As per CIR point 1.2.4.2 of (EU) 2021/808, confirmatory methods for detecting prohibited substances require a minimum of five identification points for each analyte. This ensures the validity and reliability

of the results, confirming the presence of a substance with a high degree of accuracy in regulatory analyses. In this method, five identification points were applied to minimize the risk of false positives and to ensure that the detected substance is indeed the target analyte from the NFP prohibited substances in shrimp complex matrices.

Table 3. Validation level, limit of quantitation (LOQ), and decision limit (CCα) for six nitrofuran parent compounds in shrimp muscle

Compound (s)	Validation level (μg kg^{-1})	LOQ (μg kg^{-1})	CCα (μg kg^{-1})
Furaltadone	0.5	0.25	0.32
Furazolidone	0.5	0.25	0.34
Nitrofurantoin	0.5	0.25	0.32
Nitrofurazone	0.5	0.25	0.33
Nifursol	0.5	0.25	0.34
Nitrovin	0.5	0.25	0.32

Precision was evaluated through within-laboratory repeatability and reproducibility analysis. Within-laboratory repeatability (WL_r) was assessed by fortifying 21 blank shrimp muscle samples with nitrofuran (NFP) drugs. The WL_r analysis was conducted at three different levels: 0.5, 1.0 and 1.5 times the RPA for the six nitrofuran prohibited substances considering the applicable RPA. The samples were analyzed on the same day, using the same instrument and operators, and the overall coefficient of variation (CV) percentage of the fortified samples was calculated.

Within-laboratory reproducibility (WL_{WR}) was evaluated by fortifying 7 blank shrimp muscle samples with six NFP drugs at three different levels: 0.5, 1.0, and 1.5 times of RPA. This process was repeated over three separate days using the same instrument but with different operators. The reproducibility was assessed by calculating the CV% for all 21 fortified samples across each level. The CV% results were satisfactory for all analytes, with values below 20%, consistent with previous findings (Moragues, Miralles, Igualada, & Coscollà, 2024; Saluti et al., 2024). The WL_r and WL_{WR} results also complied with the recommendations outlined in Commission Implementing Regulation (EU) 2021/808.

In accordance with Method 1 (Calibration Curve Procedure) of Commission Implementing Regulation (EU) 2021/808, the decision limit (CCα) for unauthorized substances was calculated using the intercept of the calibration curve plus 2.33 times the standard deviation of the within-laboratory reproducibility. This corresponds to a 1% false-compliant probability (α=0.01). By utilizing this calibration-based approach, the calculated CCα values incorporate measurement uncertainty, ensuring the statistical confidence required for confirming non-compliance.

In this study, the method was validated at the Reference Point for Action (RPA) level of 0.50 μg kg^{-1} for NFP compounds. The resulting CCα values ranged from 0.30 to 0.34 μg kg^{-1}, which are well below the RPA, thereby demonstrating high sensitivity and full compliance with CIR (EU) 2021/808 criteria. These CCα values were further verified by analyzing 21 independent blank matrix samples fortified at the calculated decision limit, with all samples showing detectable residues.

The specificity of this method was evaluated and successfully applied to determine NFP drugs in shrimp from various aquaculture species, including prawns, fish, and crab meat. No differences were observed in the method's performance across these fishery species. The applicability of the method was further tested using real samples, where it proved effective in accurately detecting and quantifying NFP drugs in shrimp. This process involved assessing the method's accuracy, reliability, and suitability for complex shrimp matrices, thereby supporting food safety monitoring and regulatory compliance.

Few methods have been published for the determination of nitrofuran parent compounds in fish matrices using LC–MS/MS. However, these methods focus on only four nitrofuran compounds: furaltadone, furazolidone, nitrofurantoin, and nitrofurazone. To address this gap, a relatively fast, precise, and low-cost method has been developed for the determination of six nitrofuran parent drug residues, including nifursol and nitrovin, in shrimp. This method supports the analysis of a large number of samples (25–35) within a working day. The purpose and effectiveness of the method are demonstrated by verifying various performance characteristics as per CIR (EU) 2021/808. The method complies with EU analytical requirements in terms of specificity, linearity, absolute recovery, matrix effect, trueness, retention time, relative

intensities, identification points, within-laboratory repeatability (WL_r), within-laboratory reproducibility (WL_{WR}), ruggedness and decision limit (CCα). It meets all the required performance criteria for quantifying and confirming nitrofuran drug residues in food of animal origin and is suitable for official control and ensures compliance with international safety standards for nitrofuran residues, offering high sensitivity, precision, and accuracy in detecting these banned substances in shrimp.

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