

Article

Development and Validation of a Confirmatory LC-MS/MS Method Using QuEChERS for Determination of Nitrofurans Metabolites in Eggs According to EU Regulation 2021/808

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Abstract

Nitrofurans are banned veterinary medicinal products due to their carcinogenic and mutagenic properties; however, their protein-bound metabolites (AOZ, AMOZ, AHD, SEM, and DNSAH) may persist in food-producing animals, particularly in eggs. Reliable confirmatory methods are therefore essential for residue monitoring under the stringent requirements of Commission Implementing Regulation (EU) 2021/808. This study reports the development and validation of a sensitive and selective LC–MS/MS method combining acid hydrolysis, 2-nitrobenzaldehyde derivatization, and QuEChERS extraction for the determination of nitrofurans metabolites in eggs. Chromatographic separation was carried out using a phenyl-hexyl column, and detection using a tandem mass spectrometer, supported by isotope-labeled internal standards, ensured robust identification and quantification. Linearity was satisfactory over the investigated concentration range ($R^2 > 0.99$), with recoveries between 82 and 109%. The method's precision was acceptable, with repeatability RSD values below 10% and within-laboratory reproducibility RSD values below 22%. Matrix effects were effectively controlled, remaining within $\pm 20\%$ following internal standard normalization. Decision limits ($CC\alpha$) ranged from 0.29 to 0.37 $\mu\text{g}/\text{kg}$, well below the EU reference point for action of 0.5 $\mu\text{g}/\text{kg}$. The method's performance was further confirmed through participation in an accredited proficiency test scheme. Overall, the validated method provides a reliable analytical tool for routine official control laboratories, enabling the sensitive confirmatory detection of banned nitrofurans residues in eggs and supporting food safety and regulatory compliance.

Keywords: validation; nitrofurans; metabolites; LC-MS/MS; eggs; 2-nitrobenzaldehyde derivatization; food safety



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1. Introduction

Nitrofurans (NF) drugs are broad-spectrum antibiotics that were historically used in food-producing animals for the treatment and prevention of bacterial and protozoal infections. Their use in the European Union was prohibited during the 1990s following

evidence of their carcinogenic, mutagenic, and genotoxic potential [1,2]. As a result of these toxicological concerns, nitrofurans are classified as prohibited substances for which no maximum residue limits can be established. However, under Commission Regulation (EU) 2019/1871, a reference point for action (RPA) of 0.5 µg/kg has been established for individual nitrofurans and their marker metabolites in order to harmonise trade within the EU and with third countries [3]. Consequently, food of animal origin containing nitrofurans residues at concentrations below the RPA of 0.5 µg/kg may be placed on the market.

Following administration to food-producing species, nitrofurans drugs are rapidly metabolised *in vivo* and undergo extensive biotransformation, forming reactive intermediates that bind covalently to tissue proteins [4,5]. As a result, parent nitrofurans compounds are usually not detectable in edible tissues shortly after treatment, whereas protein-bound residues persist for extended periods and serve as reliable markers of prior exposure. These bound residues, along with their parent drugs, are now used as marker residues for nitrofurans residue detection as their nitrophenyl ester derivatives [6].

In laying hens, numerous studies have investigated the metabolism, transfer, and persistence of individual nitrofurans drugs in eggs following dietary treatment. These include studies on furazolidone [7–9], nitrofurazone [10], furaltadone [11], and nifursol [11]. These studies have shown that residues of nitrofurans drugs differ in eggs compared to animal tissue, as intact parent nitrofurans compounds can accumulate in eggs and remain detectable for several days following withdrawal of medicated feed. In parallel, protein-bound side chain metabolites shown in Table 1, including 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM), and 5-dinitro-salicylic acid hydrazide (DNSAH), persist for longer periods, and the proportion of bound residue will increase at later treatment or withdrawal periods.

The analysis of nitrofurans residues in eggs typically involves direct acid hydrolysis and derivatization of homogenised samples without a prior washing step. Consequently, both free and protein-bound residues are converted to their corresponding marker metabolites and quantified together. Thus, total nitrofurans residues are analysed by default. Depletion studies conducted using recommended or near-therapeutic treatment regimens consistently report concentrations of total nitrofurans residues in eggs that exceed the current European Union reference point for action (RPA) of 0.5 µg/kg by several orders of magnitude during treatment and for extended periods following withdrawal [9–12]. Residue concentrations reported in the literature for nitrofurans concentrations up to several hundred µg/kg during medication, with levels often remaining above the RPA for several weeks post-treatment, depending on the drug administered.

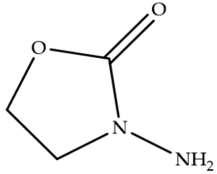
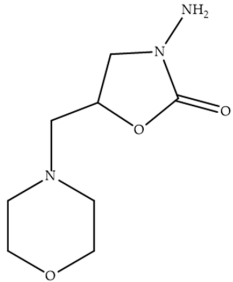
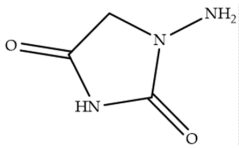
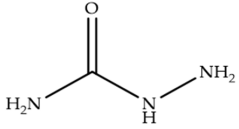
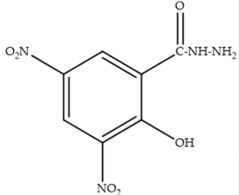
From a regulatory perspective, egg samples containing nitrofurans residues at concentrations below the RPA are considered compliant and may be placed on the market. However, detection of nitrofurans residues, even below the RPA, triggers mandatory follow-up on-farm investigations to determine whether illegal administration has occurred. Where illegal use is suspected or confirmed, the food production establishment may be placed under official restriction, and in severe or unresolved cases, this can ultimately lead to culling of flocks.

Early analytical methods for the determination of total nitrofurans residues in eggs relied on direct acid hydrolysis and derivatization of homogenised samples, followed by liquid–liquid extraction (LLE) using ethyl acetate. This approach enabled the simultaneous determination of free and protein-bound residues and was described in several key papers, including those by McCracken et al. [9,12,13], Cooper et al. [10], Barbosa et al. [11], and Radovnikovic et al. [14]. While these methods provided sufficient sensitivity for early regulatory and research applications, they were often associated with pronounced matrix effects due to the high lipid and protein content of eggs, which could adversely affect

analytical robustness and sensitivity. To address these problems, Śniegocki et al. (2018) employed solid phase extraction as a clean-up step for derivatised egg extracts [15]. The use of solid phase extraction significantly reduced matrix related interferences and improved analytical sensitivity compared with conventional LLE using ethyl acetate.

As an alternative, the QuEChERS approach has emerged as a widely adopted alternative for sample preparation in residue analysis in the mid-2000s [16]. In egg analysis, QuEChERS is based on a single acetonitrile extraction combined with salting out partitioning using magnesium sulfate and sodium chloride [17]. This strategy requires substantially lower solvent volumes than traditional extraction procedures and uses acetonitrile-based extraction, which extracts analytes while co-extracting only low amounts of lipophilic interferences such as fats and phospholipids. As a result, cleaner extracts are obtained, leading to reduced matrix-related ion suppression and improved signal-to-noise ratios in subsequent LC-MS/MS analysis. In addition, QuEChERS offers shorter processing times, reduced solvent consumption, lower operational costs, and improved reproducibility, making it well-suited for routine implementation in official control laboratories.

Table 1. IUPAC name, molecular formula, and structure of nitrofuran metabolites.

	Name	IUPAC Name	Molecular Formula	Parent Drug	Structure
1 AOZ	3-Amino-2-oxazolidinone	3-amino-1,3-oxazolidin-2-one	C ₃ H ₆ N ₂ O ₂	Furazolidone	
2 AMOZ	3-Amino-5-morpholinomethyl-2-oxazolidinone	3-amino-5-(morpholin-4-ylmethyl)-1,3-oxazolidin-2-one	C ₈ H ₁₅ N ₃ O ₃	Furaltadone	
3 AHD	1-Amino hydantoin	1-aminoimidazolidine-2,4-dione	C ₃ H ₅ N ₃ O ₂	Nitrofurantoin	
4 SEM	Semicarbazide	Aminourea	CH ₅ N ₃ O ₃	Nitrofurazone	
5 DNSAH	3,5-Dinitro-salicylik acid hydrazid	2-hydroxy-3,5-dinitrobenzohydrazide	C ₇ H ₆ N ₄ O ₆	Nifursol	

In this paper, we present a new method for the analysis of five nitrofuran residues in eggs using LC-MS/MS. Egg samples are subjected to acid hydrolysis and derivatization at 60 °C for 2 h, representing a substantially shorter derivatization step compared with established protocols, which typically require up to 16 h at 37 °C. Following derivatization,

nitrofuran residues are extracted using a QuEChERS-based procedure, streamlining sample preparation while reducing solvent consumption. The method provides enhanced recovery of nitrofuran residues together with improved analytical sensitivity.

2. Results and Discussion

2.1. Chromatographic Performance and Mass Spectrometric Detection

The LC-MS/MS method provided clear separation of all five NF metabolites and their corresponding isotopically labeled internal standards. The use of a phenyl-hexyl stationary phase resulted in symmetrical peak shapes and efficient resolution between analytes and matrix interferences. Optimization of the gradient, before validation, ensured baseline separation, which is particularly important for structurally similar metabolites such as AOZ and AMOZ. Electrospray ionization in positive mode allowed sensitive detection of AOZ, AMOZ, SEM, and AHD, while DNSAH was more efficiently ionized in negative mode. The high sensitivity is largely attributable to the derivatization step with 2-nitrobenzaldehyde, which forms nitrophenyl derivatives that exhibit enhanced ionization efficiency in mass spectrometry [18]. This step significantly increases signal intensity and improves the reliability of quantification, particularly for SEM and AOZ, which are typically more challenging to detect at trace levels. The use of multiple reaction monitoring (MRM) transitions provided both quantification and confirmation (Figure 1).

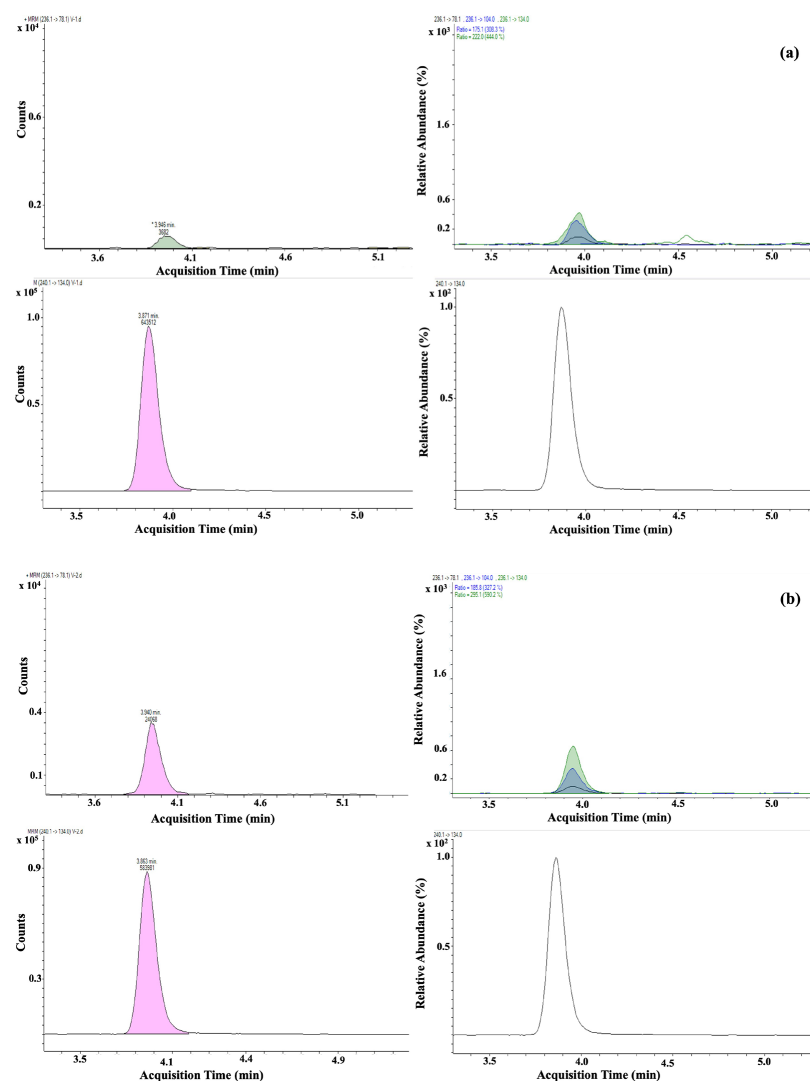


Figure 1. Cont.

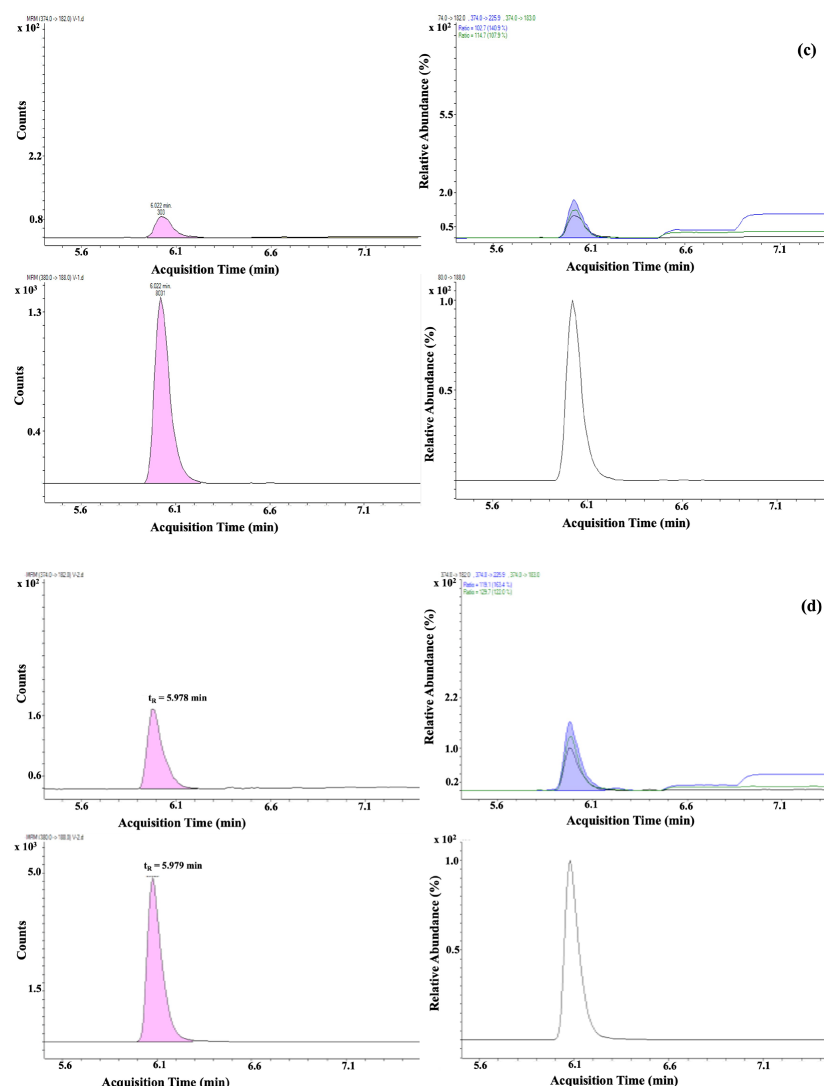


Figure 1. (a) Analyte peak of AOZ (positive mode) in negative control [black—quantifier transition; blue and green—qualifier transitions]. (b) Analyte peak for AOZ (positive mode) spiked at 0.2 $\mu\text{g/kg}$. (c) Analyte peak of DNSAH (negative mode) in negative control [black—quantifier transition; blue and green—qualifier transitions]. (d) Analyte peak for DNSAH (negative mode) spiked at 0.2 $\mu\text{g/kg}$. **Notes:** Quantifier transition: used to determine analyte concentration. Qualifier transitions: used to confirm analyte identity. X-axis: retention time (min), Y-axis: relative response. In the upper side of the picture and in the lower side of the picture, the chromatogram of the internal standard is shown.

2.2. Selectivity and Specificity

Selectivity refers to the ability of the method to clearly distinguish the target analytes from other compounds that may be present in the sample in the retention time windows of the target analytes. To assess this parameter, 40 blank egg samples from different sources were analysed. None of the samples showed interfering peaks at the retention times of the target compounds. This indicates that the method is highly selective and specific for identifying NFs quantitatively from possible interferences, even in egg, which is a complex matrix. The same samples were employed in matrix effects studies (Section 2.3) and recovery and precision studies (Section 2.4).

2.3. Matrix Effect

Matrix effects were evaluated using 36 different blank egg samples spiked with NP2 post-extraction at the RPA level (0.5 $\mu\text{g/kg}$). Matrix effects were calculated as the ratio of analyte peak areas obtained in the matrix to those obtained in the solvent.

The IS-normalized matrix effects are expressed as mean percentage signal variation $\pm$ standard deviation, together with the coefficient of variation (CV). The results demonstrated moderate signal enhancement for all analytes, with mean matrix effects of +10.14% for AHD, +17.31% for AOZ, +14.44% for AMOZ, +10.54% for DNSAH, and +11.62% for SEM. The CV values obtained across the 36 samples confirmed acceptable repeatability of the matrix effect.

All observed matrix effects were within the $\pm 20\%$ acceptance criterion defined in Regulation (EU) 2021/808 [19], indicating that matrix-induced signal variation was effectively controlled using isotopically labeled internal standards and the applied sample clean-up procedure (QuEChERS combined with acid hydrolysis).

2.4. Recovery and Precision

As there are no certified reference materials available, it is acceptable that trueness of measurements is assessed in other ways, such as recovery experiments using blank matrix samples fortified at three concentration levels at concentrations of 0.5, 1.0, and 1.5 RPA. For each sample analysed, the trueness values (Table 2) were calculated based on Equation (1).

$$\text{Trueness (\%)} = \left[\frac{(\text{mean recovery} - \text{corrected concentration detected})}{\text{fortification level}} \times 100 \right]. \quad (1)$$

Table 2. Validation performance parameters: trueness, precision, and decision limits ($CC\alpha$) for NF metabolites in eggs.

Parameters	¹ WLR Trueness (%)			² RSDr (%)			³ RSDR (%)			CC α ($\mu\text{g/kg}$)
Analyte	L1	L2	L3	L1	L2	L3	L1	L2	L3	
AHD	100	103	87	4.7	4.9	5.0	10.8	10.5	9.0	0.31
AOZ	93	97	82	3.5	5.3	5.6	13.2	8.8	9.7	0.32
AMOZ	98	98	86	9.6	7.7	16.1	21.6	12.5	17.3	0.37
DNSAH	107	109	86	4.5	6.8	8.2	6.9	6.7	9.0	0.29
SEM	100	103	87	7.0	6.5	7.5	12.5	16.2	10.1	0.32

¹WLR stands for within-laboratory reproducibility trueness; L1, L2, and L3 stand for different spiking levels, 0.5, 1.0, and 1.5 RPA. ²RSDr stands for within-laboratory repeatability. ³RSDR stands for within-laboratory reproducibility.

Recovery experiments demonstrated that the method could extract and quantify NF metabolites with high accuracy. Mean recoveries across all concentration levels were in the range 82% and 109% (Table 2), which falls well within the range accepted for quantitative confirmatory methods. Acceptable recovery values were based on the criteria outlined in Regulation 2021/808 and complementary guidelines described in SANTE 11312/2021 v2, with typical acceptable ranges of 70–120%. Wider limits (50–120%) were considered acceptable at lower concentration levels or in complex matrices [19,20].

Data was analysed according to ISO 5725 [21] using one-way ANOVA as outlined in Section 3.9. Repeatability precision was estimated for each validation concentration from pooled intra-day replicate measurements ($n = 8$ per concentration replicates per batch $\times$ 3 different times). Reproducibility precision was estimated by one-way ANOVA from between-run and within-run variance. This data is summarized in Table 2.

RSDr values were $<10\%$ (except AMOZ, which was 16% but is still less than 20%), making it an acceptable value of precision under repeatability conditions (Table 2). For inter-day reproducibility, the precision value did not exceed 22%, which is far below the 30% acceptable value of precision (expressed as RSDR) for concentrations $<10 \mu\text{g/kg}$.

These findings indicate that the method is accurate and reproducible under routine laboratory conditions. The relatively low variability is particularly noteworthy considering the complexity of the egg matrix. Egg yolk contains high levels of lipids, while albumen is rich in proteins, both of which can complicate sample preparation and analysis [22,23]. The QuEChERS-based clean-up, combined with derivatization to nitrophenyl derivatives, proved effective in minimizing these matrix effects, resulting in stable recoveries even at the lowest tested concentrations.

2.5. Sensitivity and Decision Limits

The values of decision limits are calculated by combining data from precision in terms of repeatability and reproducibility, which provides the possibility to satisfy the criteria of a confirmatory method, such as ion ratios and satisfactory results for signal-to-noise ratio. The data are obtained from analysing the sample in days separated into more than one week, two different technicians, two different analytical brands of phenylhexyl column (Halo and Kinetex), and two different derivatization conditions (37 °C overnight or 2 h at 60 °C).

The calculated decision limits (CC α) ranged between 0.29 and 0.37 $\mu\text{g}/\text{kg}$ for all analytes (Table 2). These values are below the reference point for action (RPA) of 0.5 $\mu\text{g}/\text{kg}$ set by the European Union. This demonstrates that the method is sufficiently sensitive for enforcement of the EU's zero-tolerance policy on NF residues and is achievable under this method for egg samples.

2.6. Linearity

Linearity for calibration curves (Figure 2a–e) was greater than 0.99, with some exceptional cases for AMOZ, which could be greater than 0.98, which is higher than the values of 0.95 suggested by the EURL methodology. Importantly, the use of matrix-matched calibration compensates for potential ion suppression or enhancement caused by egg components, thereby improving the reliability of quantification in real-world samples [10,12,14].

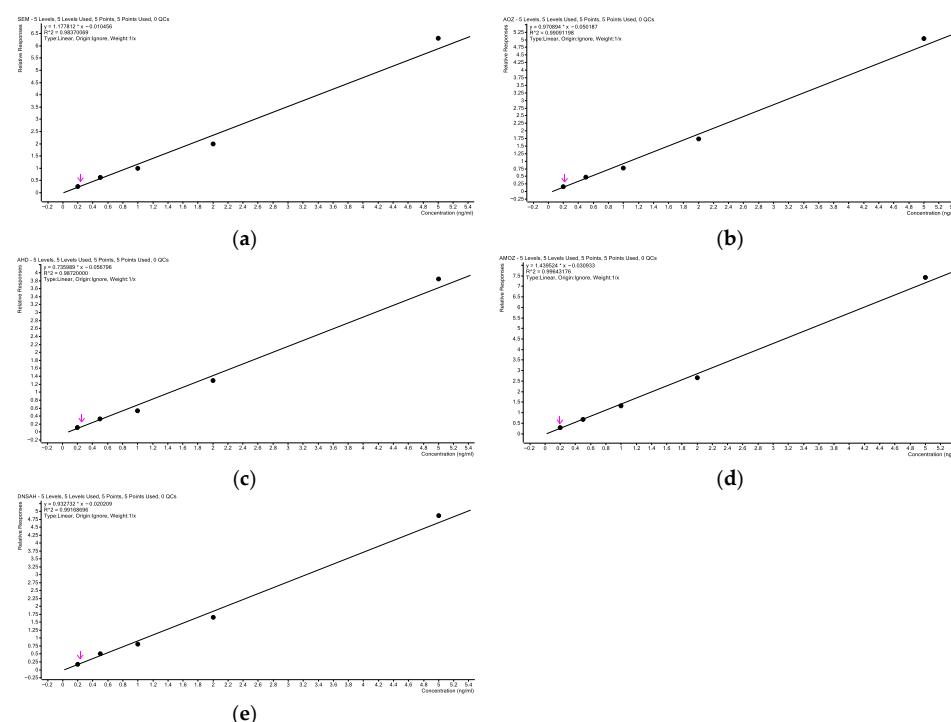


Figure 2. Matrix-matched calibration curves for (a) SEM, (b) AOX, (c) AHD, (d) AMOZ, and (e) DNSAH in blank egg matrix fortified at 0.2–5.0 $\mu\text{g}/\text{kg}$ at the beginning of the analytical procedure and analysed

by LC–MS/MS using isotope-labelled internal standards. The plots illustrate the linear relationship between the instrument’s relative response and the corresponding analyte concentrations over the investigated calibration ranges, demonstrating the suitability of the methods for quantitative analysis.

2.7. Identification

For confirmatory methods, identification of analytes must meet strict requirements. The minimum number of identification points required for prohibited substances in 2021/808 EC was met, as the chromatographic separation (1 point), monitoring one precursor (1 point), and two product ions (1.5 identification points each) achieved a total of 5 identification points. In this study, all their nitrophenyl derivatives of NFs fulfilled these criteria. The use of isotope-labeled internal standards (AOZ-d₄, AMOZ-d₅, AHD-¹³C₃, SEM-¹³C¹⁵N₂, DNSAH-¹³C₆) provided robust quantitation by compensating for matrix effects and signal fluctuations. The relative retention times of analytes compared with their internal standards deviated by no more than 1% during the entire validation process and routine analysis. Diagnostic ion ratios were also stable, with variations remaining within $\pm 40\%$ of the values obtained for calibration standards. Furthermore, the signal-to-noise S/N ratio exceeded the acceptance limit of 3 for all monitored ions. These results demonstrate that the method provides reliable confirmatory identification of NF residues in egg matrices.

2.8. Application of the Method to Spike and Non-Spiked Samples

The results obtained from this study highlight the suitability and robustness of this confirmatory method, given that it was applied to a wide range of different samples. The method was fit to analyse all NF-bound residues, and no additional interferences were observed. This method was verified through participation in a commercial PT scheme organized by Test VERITAS (Table 3) with a sample ID E5106 and laboratory code T046.

Table 3. Results of the proficiency test material.

Analytes	AHD	AMOZ	AOZ
Assigned values (µg/kg)	1.05	1.39	0.82
Measured concentration (µg/kg)	1.01	1.20	0.88
Z-score	−0.17	−0.62	0.34
Measured concentration with Halo column (µg/kg)	1.08	1.43	0.90

Values of Z-score, calculated by the PT Provider, were within the range from ± 1 for three of the analytes that were present in the spiked sample provided by Progeto Trieste, showing that this method is fit for purpose and the method performance is satisfactory. With this PT sample, the reported results were analysed using the Kinetex column. The PT sample was also analysed using the Halo column, which has comparable results to Table 3.

Based on the validated method described in this publication, 16 different samples from different sources (Figure 3), like farms, industry, or markets in different areas of Albania, were analysed. These chromatographic and spectrometric conditions ensured reproducible retention times and stable ion ratios across multiple runs. Almost half of the samples detected some residues of SEM, but these were less than CC α . One sample contained SEM at a concentration of 0.24 µg/kg.

Two of the samples collected were found to contain 3-amino-2-oxazolidinone (AOZ), but again, concentrations were below the CC α and RPA levels, yet clearly identifiable and quantifiable. Details of the measured concentrations and sampling locations are provided in Table 4. To better interpret these findings, additional investigations will be carried out to evaluate possible sources of contamination. SEM residues may arise from sources other than nitrofurazone use, but AOZ is a more specific marker. Nevertheless, in both cases, it

is recommended that more extensive sampling of eggs should be carried out to monitor the situation.

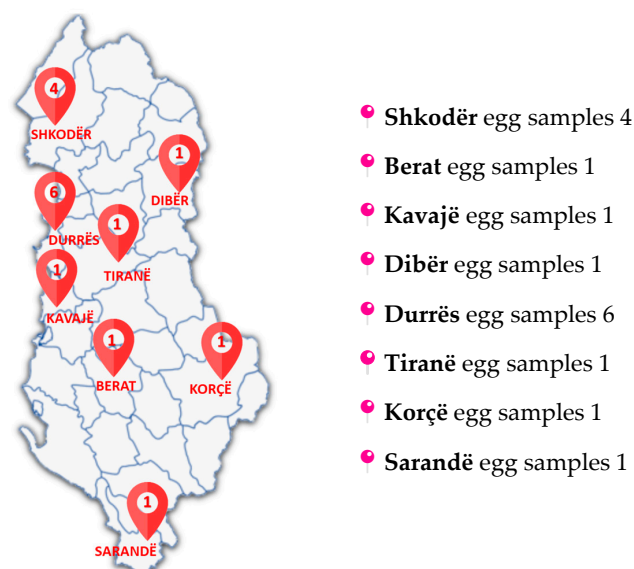


Figure 3. Geo-mapping of the collected eggs throughout Albania.

Table 4. Results of the egg sample with the presence of SEM and AOZ.

	<i>Origin</i>	<i>SEM</i> ($\mu\text{g/kg}$)	<i>AOZ</i> ($\mu\text{g/kg}$)	<i>RPA</i> ($\mu\text{g/kg}$)
1	Shkodër 2	0.240		0.5
2	Durrës 2		0.227	0.5
3	Shkodër 3		0.271	0.5

For those samples in Figures 4 and 5, some details regarding the quantifier transition of SEM and the quantifier and qualifier transition of AOZ compared to the RPA level are shown, which demonstrates the low presence of this nitrofurantoin in the egg. As the values are well below CC α values but above the lowest calibration level, all identification criteria are met.

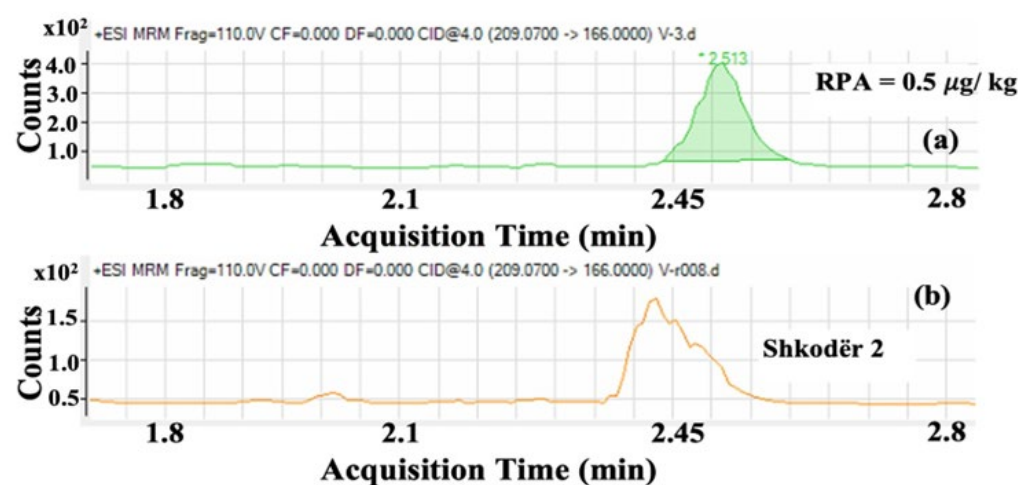


Figure 4. Chromatographic presentation of samples with the presence of the metabolite SEM. (a) Quantifier transition spike at 0.5 $\mu\text{g/kg}$. (b) Quantifier transition found in the region of Shkodër 2.

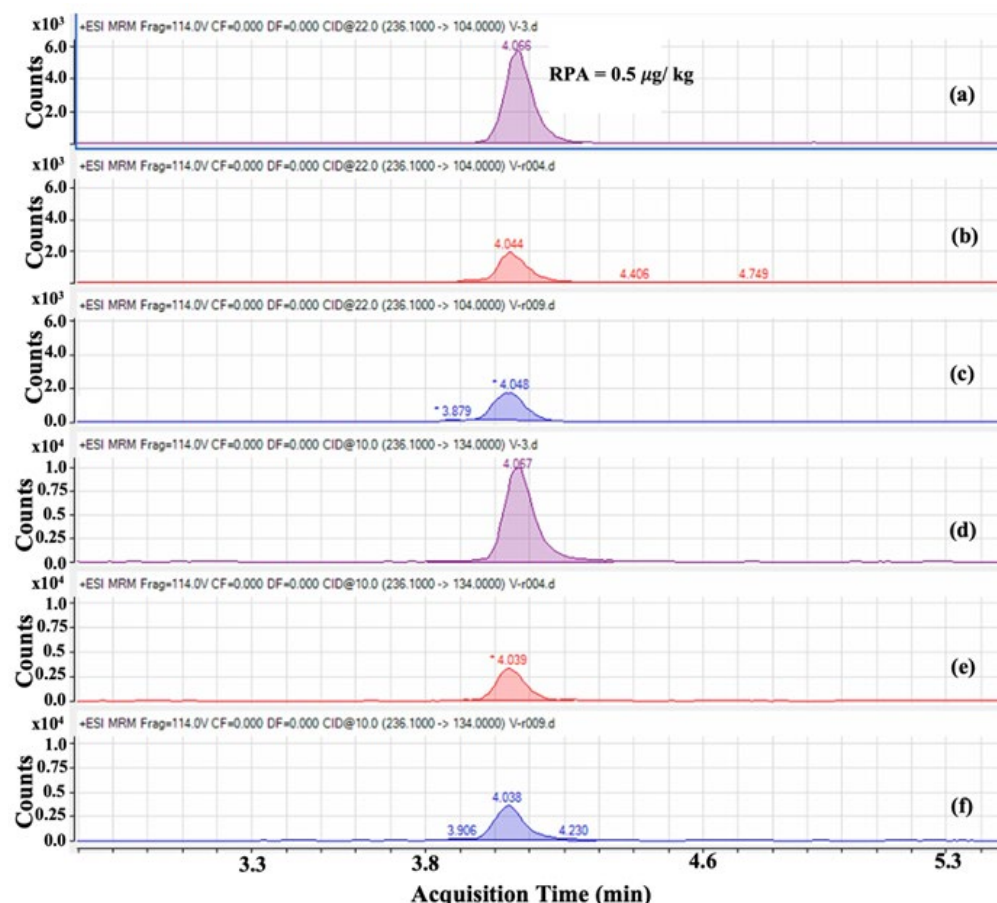


Figure 5. Chromatographic presentation of samples with the presence of the metabolite AOZ in different regions of Albania. Quantifier (d) and qualifier (a) transition spikes at 0.5 µg/kg. Quantifier (e) and qualifier (b) transition found in the region of Durrës 2. Quantifier (f) and qualifier (c) transition found in the region of Shkodër 3.

2.9. Stability Data

Stability testing for nitrofur residues in egg samples is complex because residues can occur as parent drug, protein-bound metabolite, and other forms of the drug that can convert to NP-derivatives. McCracken et al. evaluated the stability of the parent drug furazolidone and its AOZ metabolite in egg homogenate from treated hens after storage at -20°C for 55 days [9]. The egg samples used in their investigation were incurred with furazolidone and AOZ with measured concentrations of approximately 500 and 400 µg/kg, respectively. The study showed that the furazolidone parent drug was unstable and degraded by 44% during storage. In contrast, residues measured as AOZ were found to be stable during storage. Stastny et al. carried out an in-depth stability study on SEM residues in egg samples collected from two groups of birds treated with 30 and 400 mg/kg nitrofurazone in feed for 10 days [24]. Stability was assessed in homogenised egg samples containing SEM in the range 0.47 to 17.67 µg/kg stored at 4°C and -20°C . SEM was found to be stable in eggs for at least 1 year in both storage conditions. There are very few studies published on the fate of other nitrofur drugs in eggs, and reported work has not investigated the stability of drugs in incurred eggs [11].

In this research, the stability of five nitrofur metabolites was evaluated in egg homogenate fortified at 1.0 µg/kg, stored at -20°C , with measurements from day 0 to month 6. The one-way ANOVA showed a significant effect of storage time on SEM concentration ($F(6,28) = 5.00$, $p = 0.001$). Subsequently, Tukey pairwise analysis (95% CI) showed that the mean concentration of SEM on day 0 was measured at 0.89 µg/kg and did not degrade signif-

icantly by week 4, with a level quantified of 0.92 µg/kg ($p > 0.05$). There was a small drop in SEM concentration between weeks 8 and 12, with a mean level of 0.81 µg/kg measured on both days. The mean concentration of SEM was 0.87 and 0.86 µg/kg on week 16 and 24, respectively. Levels dropped in week 16 but recovered in week 24 with higher-than-normal precision (>15%) observed on both weeks, which might indicate a problem with the analyses on both days (Figure S1A). This was despite an isotopically labelled internal standard being used in the method. However, for all other analytes, precision was satisfactory.

The stability of AOZ was assessed using homogenized eggs fortified at 1.0 µg/kg and an incurred egg sample with reported day 0 levels of 0.89 µg/kg and 1.30 µg/kg, respectively. One-way ANOVA appeared to show a significant effect of storage time for fortified egg samples with $F(6,28) = 10.18$ ($p < 0.001$). Tukey analysis (CI 95%) showed no significant difference in AOZ levels between day 0 and week 12. AOZ concentrations were significantly different in weeks 16 and 24, with measured levels of 0.84 µg/kg and 0.92 µg/kg, respectively ($p < 0.05$). This recovery in concentration between weeks 16 and 24 suggests that analytical variability, rather than degradation, may contribute to the observed differences among weeks. For incurred eggs, ANOVA results were highly significant ($F(6,28) = 76.65$, $p < 0.001$). Tukey pairwise comparisons (CI 95%) showed no significant differences between day 0 and weeks 4, 12 and 16. Significant differences were observed between day 0 and weeks 2, 8 and 24, indicating an increase in AOZ concentrations (95% CI). The mean concentration ranged between 1.28 µg/kg (day 0) and 1.37 µg/kg (week 16), which indicates a 7.0% flux in concentration, which is small and falls within laboratory reproducibility precision results reported in Table 2. This agrees with research by McCracken et al. (2001), who investigated the stability of AOZ in eggs but at much higher concentrations, showing that AOZ was stable for at least 55 days [9]. The significantly higher results for AOZ reported in the incurred eggs at week 24 compared to other results might point to issues with the handling of the egg sample. It can be seen from the interval plots shown in the Supplementary Materials that in week 24, AOZ levels were slightly elevated in fortified eggs, but the effect was pronounced in the incurred egg (Figure S1B,C). Thus, care needs to be taken when tempering samples prior to weighing. Alternatively, freeze-drying of samples might produce more reliable analytical results, but adds a layer of complexity to sample and data processing for an already time-consuming assay.

ANOVA showed a statistically significant effect of storage time on the AMOZ levels ($F(6,28) = 7.82$, $p < 0.001$), indicating that the mean response differs for at least some weeks. Tukey's pairwise (95% CI) analysis revealed grouping of day 0 results with weeks 2, 8, 12 and 24, showing that results were not statistically significant from each other. However, Tukey comparisons (95% CI) showed the concentrations measured on weeks 4 and 16 were significantly lower than those on day 0. Average AMOZ results showed a large spread between days, with a minimum value of 0.69 µg/kg (week 16) and a maximum of 1.11 µg/kg (week 4), which indicates a difference of 0.42 µg/kg. AMOZ interval plot shows a spike in concentration in week 4 but a downward trend in concentration values at later storage times (Figure S1D).

Similarly, one-way ANOVA indicated significance between measured AHD during the different weeks of storage ($F(6,28) = 7.82$, $p < 0.001$). Pairwise Tukey comparisons (95% CI) revealed grouping of results with only measurements on weeks 4 and 16 found to be significantly different than day 0. Mean results ranged from 0.94 µg/kg on day 0 to 0.78 µg/kg on week 16 (range of 0.16 µg/kg). However, results appeared to recover on week 24 with a mean measured value of 0.93 µg/kg reported, which was not significantly different than day 0 ($p > 0.05$). The confidence interval plots showed dips in concentration, but results increased at the next time point, which indicates inter-day variation in measurements (Figure S1E).

A highly significant effect was seen in the ANOVA results for DNSAH ($F(6,28) = 36.04$, $p < 0.001$). The overall spread in results ranged from 0.87 µg/kg (day 0) to 1.08 µg/kg

(day 24), with an overall difference of 0.21 µg/kg. Tukey comparative analysis (95% CI) revealed no significant differences between days 0 and weeks 4, 8, 12 and 16, while concentrations were significantly different on weeks 2 and 24. The confidence interval plots showed an increase in concentrations on day 2 but overall low spread in results, except for week 24, which was significantly different from all other weeks (95% CI) (Figure S1F).

The results from this stability study support published results reported for a limited number of nitrofurans in incurred eggs, which indicates that residues of this drug group are very stable in eggs [9,11]. In practical terms, samples should be analysed in as short a turnaround time as possible to ensure that non-compliant samples can be removed from the market and timely follow-up investigations can be carried out on farms. Turnaround times for analysis should be ≤20 working days (or a calendar month) but ideally ≤10 working days. The results from this stability study appear to confirm that nitrofurans metabolites are stable in egg homogenate samples stored at −20 °C, which is a widely used temperature used to preserve samples in laboratories. In this study, stability was assessed using samples fortified with metabolites rather than incurred material.

3. Materials and Methods

3.1. Chemicals and Reagents

All solvents used were of LC–MS/MS grade, and all other reagents were of analytical grade. Distilled water (18.2 MΩ·cm) was prepared using a Milli-Q system (Adrona Crystal). Hydrochloric acid was obtained from VWR International (Radnor, PA, USA). 2-Nitrobenzaldehyde (2-NBA, purity 99.93% was purchased from Apollo Scientifics (Manchester, UK). Acetonitrile 99.95% and trisodium phosphate dodecahydrate were supplied by Titol Chimica (Rome, Italy). Sodium hydroxide (97%) and ammonium formate 99% were from Carlo Erba (Milan, Italy), and HPLC-grade methanol was purchased from Romil (Cambridge, UK). All chemicals were used without further purification and handled according to standard laboratory practices.

3.2. Standards

Analytical standards of the NF metabolites were obtained from commercial suppliers and were purchased from different producers. Semicarbazide (SEM), 3-Amino-2-oxazolidinone (AOZ) and 1-aminohydantoin (AHD) were purchased from CPA Chem (Bogomilovo, Bulgaria), 3-Amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) from HPC (Cunnersdorf, Deutschland) and 3,5-dinitrosalicylic acid hydrazide (DNSAH) from Witega (Berlin, Germany) as well all nitrophenyl derivative marker residues 3-(2-nitro phenyl)methylene-amino-2-oxazolidinone (NPAOZ), 5-Methylmorpholino-3-((2-nitrophenyl)methylene)-3-amino-2-oxazolidinone (NPAMOZ), 1-((2-nitrophenyl) methylene)-amino-2-hydantoin (NPAHD), (2-nitrophenyl) methylene-semicarbazide (NPSEM), 3,5-(2-nitrophenyl)-dinitrosalicylic acid hydrazide (NPDNSAH) and internal standards 3-amino-5-morpholinomethyl-1,3-oxazolidinon-2-one-d₅ (AMOZ-d₅), 1-aminohydantoin-¹³C₃ (AHD-¹³C₃), and 3,5-dinitrosalicylic acid hydrazide ¹³C₆ (DNSAH-¹³C₆) except 3-amino-2-oxazolidinone-d₄ (AOZ-d₄), semicarbazide-¹³C ¹⁵N₂ (SEM-¹³C ¹⁵N₂), which were purchased from HPC (Cunnersdorf, Deutschland).

3.3. Standard Preparation

An individual stock solution of NF metabolites was prepared at 50 µg/mL, based on the batch used as indicated in the certificate of analysis. Individual stock solutions of nitrophenyl (NP) derivatives and corresponding internal standards (ISs) were also prepared at 50 µg/mL. The first mixture of each standard (MM1) was prepared at 1 µg/mL by transferring 200 µL of each stock solution into a 10 mL volumetric flask. A secondary inter-

nal standard solution mixture (IS1) was prepared by diluting primary stocks to 1 µg/mL in methanol.

On the day of analysis, two working mixtures were freshly prepared: MM2 at 5 µg/L (50 µL of the first mixture in a 10 mL flask) and MM3 at 0.5 µg/L (100 µL of the 5 µg/L mixture in a 1 mL flask). A working internal standard solution (IS2) was prepared daily by diluting IS1 to 50 µg/L. All solutions were prepared in methanol.

For NP derivatives, the first mixture (NP1) was prepared by diluting stocks to 1 µg/mL based on free metabolite equivalents. From this, a 10 µg/L solution (NP2) was prepared for spiking recovery controls before the end of evaporation. A system suitability solution for LC-MS/MS analysis was prepared by diluting NP2 solution to 1 µg/L in mobile phase A (5 mM ammonium acetate in water:methanol (90:10, *v/v*). Volumes were adjusted according to the required quantities. The concentrated stock solutions MM1, NP1, and IS1 were stable for at least two years [25]. Particular emphasis is placed on carefully following the protocol for preparation of standard solutions because SEM in working standards (MM2 and MM3) is unstable and must be prepared daily.

3.4. Calibration and Quality Controls

Commercial egg samples showing no detectable quantifier or qualifier peaks in LC-MS/MS traces for the nitrofurans analytes were used as negative controls to prepare matrix-matched calibrants, method quality control samples and for validation studies. A matrix-matched calibration curve was prepared by fortifying egg samples prior to derivatization with MM2 and MM3 standard solutions to give a calibration curve in the range 0.2 to 5 µg/kg. An 80 µL volume of IS2 internal standard solution was also added to each tube (calibrants, controls and test samples) prior to hydrolysis and derivatization. This calibration approach corrects for analyte recovery and matrix effects encountered during LC-MS/MS analysis; consequently, results can be reported directly from the instrument software without applying separate recovery corrections, reducing the risk of transcription errors during subsequent data processing. Quality control was monitored by analysing negative controls and recovery controls spiked with NP2 (0.25, 0.5, 1 and 2 µg/kg) near the end of the nitrogen evaporation step.

3.5. Sample Preparation

Samples were collected based on point 1, Annex II of Regulation EU2021/808, where it is emphasized that the sample size must be at least 12 eggs or more. After removing the shell, the egg samples were mixed and stored at −20 °C or analysed immediately. Egg samples (2.0 ± 0.05 g) were homogenized and transferred into centrifuge tubes. Extracted matrix-matched standards were fortified at this stage, and the internal standard (IS2) was added to every tube (calibrants, controls and test samples). Protein-bound residues were released by adding 9 mL of 0.1 M HCl and 200 µL of 100 mM 2-NBA. The tubes were vortexed for 30 s, sealed, and incubated either overnight at 37 ± 2 °C or for 2 h at 60 ± 2 °C in a water bath. After derivatization, the samples were cooled to room temperature and neutralized by adding 1 mL of tris buffer and 0.57 mL of 1 M NaOH. The pH was further adjusted to pH 6.5–7.5 with 1 M NaOH or 1 M HCl if necessary. Extraction was carried out with acetonitrile, followed by a QuEChERS clean-up using 1 g NaCl and 4 g MgSO₄. After centrifugation at 4500 rpm for 15 min, 5 mL of supernatant was collected, evaporated to dryness in a nitrogen evaporator at 40 °C and reconstituted in 500 µL of mobile phase A. Recovery control samples were spiked with NP2 standards when 1–2 mL of solvent extract remained in the tube. A 20 µL aliquot was injected into the LC-MS/MS system. The schematic workflow of the analytical procedure for detecting protein-bound nitrofurans metabolites in eggs is given in Figure 6.

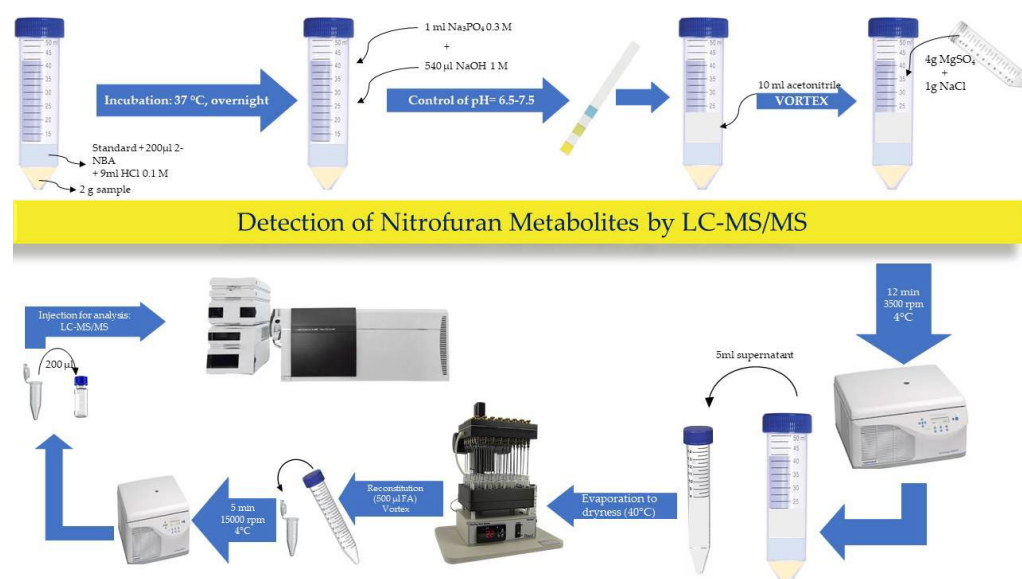


Figure 6. Workflow of sample preparation and LC-MS/MS analysis of nitrofuran metabolites.

3.6. Instrument

A microbalance (Denver Instrument, Bohemia, NY, USA) was used for weighing standards. Other equipment included a water bath (Athena Technology, Herndon, VA, USA), centrifuges (Hettich Rotina 380R and Micro 200R, Beverly, MA, USA), and a Reacti-Therm evaporator (Boston, MA, USA). LC-MS/MS analyses were conducted using an Agilent 1260 HPLC system (San Francisco, CA, USA) coupled to a triple quadrupole 6460 detector.

3.7. LC-MS/MS Conditions

Chromatographic separation was achieved on a phenyl-hexyl column (Kinetex 2.6 μm , 100 $\text{\AA}$, 50 $\times$ 2.1 mm and Halo 2.7 μm , 90 $\text{\AA}$, 50 $\times$ 2.1 mm) maintained at 40 $^{\circ}\text{C}$. The mobile phases consisted of a mobile phase A, which contained 5 mM ammonium formate in water:methanol (9:1, *v/v*) and a mobile phase B containing 5 mM ammonium formate in methanol: water (9:1, *v/v*). The gradient was programmed as follows (*t* in min, %A): 0.0 (90%), 1.5 (90%), 2.5 (70%), 3.0 (60%), 4.0 (50%), 6.5 (50%), 6.51 (0%), 9.5 (0%), 9.51 (90%), 14.5 (90%). The flow rate was 0.6 mL/min, with an injection volume of 20 μL . Detection was performed by electrospray ionization (in positive mode (AOZ, AMOZ, AHD, SEM derivatives) and negative mode (DNSAH derivative). Multiple reaction monitoring (MRM) transitions were optimized for each analyte using MassHunter Optimizer software (Agilent Technologies B.08.00). The source parameters were nebulizer 45 psi, sheath gas heater 400 $^{\circ}\text{C}$ (12 L/min), gas temperature 260 $^{\circ}\text{C}$ (8 L/min), and capillary values in positive and negative were maintained at 3500 V. Precursor and product ions, collision energies, and dwell times optimized for each analyte are shown in Table 5. These parameters were carefully fine-tuned to ensure sensitive detection, accurate quantification, and reliable confirmation of the target compounds in complex egg matrices.

Table 5. Optimized tandem mass spectrometry conditions for nitrophenyl (NP) derivatives for nitrofuran metabolites and their isotopically labelled internal standards.

Analyte	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Fragmentor (V)	CE (V) *	Polarity
NP-AHD	249.1	133.9/103.9	140	8/24	positive
NPA-AHD $^{13}\text{C}_3$	252.1	133.9	140	8	positive
NP-AMAZ	335.1	291.1/262.1	76	6/14	positive
NP-AMAZ- D_5	340.1	296.1	80	8	positive
NP-AOZ	236.1	134/104	114	10/22	positive

Table 5. *Cont.*

Analyte	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Fragmentor (V)	CE (V) *	Polarity
NP-AOZ-D ₄	240.1	134	140	8	positive
NP-DNSAH	374.0	225.9/183	140	24/28	negative
NP-DNSAH ¹³ C ₆	380.0	188	120	20	negative
NP-SEM	209.1	192/133.9	110	8/8	positive
NP-SEM ¹³ C ¹⁵ N ₂	212.1	167.9	110	4	positive

* CE = Collision Energy.

Quantification was carried out using the primary transition, while a secondary transition was employed for confirmation according to Commission Implementing Regulation (EU) 2021/808 criteria [19]. Quantitative analysis was done with the first transition listed in the MRM parameter table. The second transition was used as a qualifier ion for confirmation as per the confirmation criteria.

3.8. Method Validation

The method validation was carried out following the requirements of Commission Implementing Regulation (EU) 2021/808, which specifies the criteria for analytical methods used in official control of veterinary drug residues and is evaluated to be fully in line with Table 5 of this regulation, “Classification of analytical methods by the performance characteristics that have to be determined” [19]. Since NFs are classified as prohibited substances, the method was validated as a quantitative confirmatory method. Accordingly, all mandatory performance parameters were evaluated, including identification, selectivity/specificity, linearity, trueness, precision (repeatability and reproducibility), matrix effects, and decision limit (CC α). The method was validated using matrix-matched samples to ensure applicability to routine analysis under official control conditions. Selectivity and specificity were assessed by analysing 40 blank egg samples from different sources in terms of identifying any interferences. For matrix effect evaluation, 36 blank samples were spiked post-extraction at the RPA level and compared with solvent-based standards and normalized using isotopically labelled internal standards.

Within-laboratory reproducibility (WLR) and within repeatability were determined by analyzing fortified samples over three separate weeks, using different analysts, different incubation times (37 °C overnight or 2 h at 60 °C), and two different chromatographic column producers using the same method and instrumentation. For each level, more than 8 different blank egg samples per batch were spiked at 0.5, 1.0 and 1.5 times the RPA. The mean concentration, standard deviation, and coefficient of variation (%) of the fortified samples were calculated to assess repeatability and reproducibility. In total, 24 different egg samples were used for validation, and an additional three different samples were used for the matrix curves. This data was used to evaluate trueness and precision (both repeatability and reproducibility). Confirmatory identification capability was verified by monitoring one precursor ion and two product ions per analyte, thereby achieving the required number of identification points for prohibited substances. Relative retention time, ion ratio and signal-to-noise ratios were evaluated for each batch of analysis.

In terms of considering a result as compliant or non-compliant, the decision limit (CC α) under conditions complying with the requirements for identification or identification plus quantification as defined under ‘Performance criteria and other requirements for analytical methods’ as laid down in Chapter 1 of the Regulation (EU) 2021/808. The decision limit (CC α) is determined with Method 3 and calculated with Equation (2):

$$CC\alpha = LCL + k \text{ (one-sided, 99\%)} \times \text{(combined) standard measurement uncertainty}_{LCL} \quad (2)$$

where

- * LCL—lowest calibrated level means the lowest concentration on which the measuring system has been calibrated, in our case, 0.2 µg/kg.
- * k-factor—the t-distribution might be reasonably applied, or, if the Gaussian distribution (one-sided, $n = \infty$) is taken as a basis, a k-factor of 2.33 shall be used.
- * (Combined) standard measurement uncertainty at LCL is suitable to be defined by using the within-laboratory reproducibility and the trueness.

Linearity was assessed through inspection of calibration curves prepared using fortifying blank egg samples with NF metabolites across the concentration range of 0.2, 0.5, 1.0, 2.0, and 5.0 µg/kg, spiked at the beginning of the procedure for 12 analytical validation runs. Method applicability and robustness were demonstrated through the analysis of fortified samples, successful participation in a commercial proficiency testing scheme, and the application to real egg samples collected from different regions of Albania.

Method validation in accordance with European Commission Regulation 2021/808 requires assessment of analyte stability in solution and matrix. The first part was determined by Regan et al. (2021), but for the stability of NFs in eggs, no data has been reported so far [26]. Stability testing experiments may seem to be straightforward to conduct, but differences in day-to-day measurements are challenging, and so this type of study is rarely published in the literature [27]. Due to a lack of incurred samples for all analytes, the laboratory determined the stability of one nitrofurans metabolite in an incurred sample and the stability data for all analytes in a fortified sample.

3.9. Data Analysis

Method validation data was analysed using one-way ANOVA using Microsoft Excel. Repeatability was assessed by analysing multiple replicate samples under identical operating conditions (same analyst, instrumentation, reagents, and analytical run). The repeatability standard deviation was calculated from the within-series variance.

Within-laboratory reproducibility (WLR) was evaluated by repeating the analyses over different analytical series performed on separate weeks. Variance components were estimated using a one-way random-effects analysis of variance (ANOVA), in which the analytical series was treated as a random factor. The between-series variance was calculated from the difference between the mean square between series and the mean square within series. The within-laboratory reproducibility variance was obtained by combining the within-series and between-series variance components. Statistical evaluation was carried out in accordance with ISO 5725 and EU Regulation (EU) 2021/808 [19,21].

Storage stability data was analysed using Minitab® 22.2.2 statistical software package. Data was initially analysed using one-way ANOVA, followed by Tukey pairwise comparison with tabular grouping of data to visualize differences and Interval Plots (Figure S1) were graphed to using pooled standard deviations to show significance.

4. Conclusions

In this research, it was demonstrated that faster analysis of nitrofurans residues in eggs could be carried out using higher derivatization reaction temperatures and by using a rapid sample preparation procedure. The method used a phenylhexyl chromatographic column stationary phase that has been previously shown to offer selectivity improvements for nitrofurans residue detection [27]. The method was satisfactorily validated in accordance with Commission Implementing Regulation (EU) 2021/808 while including different analytical conditions, which offer flexibility using different analytical columns and derivatization conditions. Method validation was carried out in accordance with Commission Implementing Regulation (EU) 2021/808. Quantitative data analysis was performed using

matrix-matched calibration curves with isotopically labelled internal standards. Validation experiments were performed under varying conditions.

The method was evaluated in a proficiency test, showing that it could accurately quantify nitrofurans residues in a blind inter-laboratory study. The method was applied to a section of commercially produced table egg samples, which showed that while all samples were compliant, low-level peaks were present in samples below the validated CC α level and RPA. This highlights that further investigations should be carried out using larger sample sets in the different regions of Albania and at different sampling times.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules31040700/s1>, Figure S1: Interval plots for (A) SEM in fortified egg, (B) AOZ in fortified egg, (C) AOZ in incurred egg, (D) AMOZ in fortified egg, (E) AHD in fortified egg, and (F) DNSAH in fortified egg.

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Data Availability Statement: Data will be made available on request.

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Conflicts of Interest: Author Ina Pasho was employed by the company Novartis Pharma Albania & Kosovo & North Macedonia. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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