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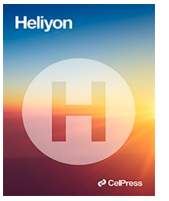

Research article

Determination of nitrofuran metabolites and nifurpirinol in animal tissues and eggs by ultra-high performance liquid chromatography-tandem mass spectrometry validated according to Regulation (EU) 2021/808

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ABSTRACT

In this work, an analytical method for the determination of eight non-allowed nitrofurans, including nifurpirinol and the metabolites of furazolidone, furaltadone, nitrofurantoin, nitrofurazone, nifursol, nitrovin, and nifuroxazide in animal tissues, including muscle (poultry, bovine, ovine, porcine, rabbit, and fish), kidney (bovine, ovine, porcine), and eggs, has been developed and validated according to Regulation (EU) 2021/808. The method was based on derivatization with 2-nitrobenzaldehyde in acid medium, followed by vortex-assisted liquid-liquid extraction and solid phase extraction for sample purification prior to ultra-high performance liquid chromatography-tandem mass spectrometry. Under selected conditions, the method was validated showing satisfactory relative matrix effects (CV $\leq$ 20 %), linearity ($R^2 \geq 0.98$), trueness ($\leq$20 %, expressed as bias), accuracy (83–120 %), repeatability (1.7–19.9 %), reproducibility (1.9–25.7 %), specificity (blank signal $\leq$30 % at the LCL), and ruggedness. The decision limit for confirmation (CCα) for the target analytes ranged from 0.27 to 0.35 μg kg^{-1}, all below the current reference point for action (RPA) of 0.5 μg kg^{-1} for the studied compounds. This validated method is currently accredited according to UNE-EN ISO/IEC 17025 by the Spanish National Accreditation Body (ENAC) to be implemented for official control analyses in the Public Health Laboratory of Valencia (Spain).

1. Introduction

Nitrofurans (NFs) are veterinary drugs exhibiting broad-spectrum antibacterial activity, but these compounds and their metabolites are suspected to produce carcinogenic and mutagenic effects [1,2]. For that reason, NFs are listed as "prohibited substances" from use in food-producing animals in the EU, from which a maximum residue limit (MRL) cannot be established according to Regulation (EU) 37/2010 [3].

Furazolidone, furaltadone, nitrofurantoin, nitrofurazone, and nifursol are the five main veterinary drugs belonging to the NFs group. For these compounds and their metabolites, a new reference point for action (RPA) of 0.5 μg kg^{-1} was established according to

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current Regulation (EU) 2019/1871 [4], lowered from the previous RPA of 1.0 μg kg^{-1}. Furthermore, nitrovin, nifuroxazide, and nifurpirinol are also important NFs, prohibited for use in food-producing animals in the EU and many countries worldwide, whose monitoring in animal products for human consumption should be carried out [5,6].

Parent NFs are rapidly metabolized to their corresponding metabolites, which remain attached for long time in animal tissues due to strong protein binding [7]. These strong covalent unions should be broken by a hydrolysis process to release the free metabolites prior to their determination. In the analytical literature, several works regarding the analysis of these compounds can be found, that were validated according to previous guidelines and regulations [7,8]. In this sense, it is necessary to obtain reliable analytical methods validated according to current Regulation (EU) 2021/808 [9] and updated to comply with the new RPA (0.5 μg kg^{-1}).

Up to date, only few papers have been published on this matter addressing the current requirements of EU regulations. In the work conducted by Regan et al. [10], an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was validated to determine eight NFs (metabolites of nifursol, furazolidone, furaltadone, nitrofurazone, nifursol, nitrofuroxazide, nifuraldezone, and nitrovin) in meat using a microwave reaction to assist the hydrolysis and derivatization of the studied metabolites followed by QuEChERS extraction. Furthermore, Krishnan et al. [5] validated an UHPLC-MS/MS method for the determination of five NFs (metabolites of furazolidone, furaltadone, nitrofurazone, nitrofurantoin, and nifursol) in shrimp and fish samples applying vortex-assisted liquid-liquid extraction (VA-LLE) and solid phase extraction (SPE), being these works the only found antecedents.

The aim of this work was to determine nifurpirinol (NPIR) and the metabolites of furazolidone (3-amino-oxazolidinone, AOZ), furaltadone (3-amino-5-morpholinomethyl-2-oxazolidinone, AMOZ), nitrofurantoin (1-aminohydantoin, AHD), nitrofurazone (semicarbazide, SEM), nifursol (3,5-dinitrosalicylic acid hydrazide, DNSH), nitrovin (aminoguanidine, AMG), and nifuroxazide (4-hydroxybenhydrazide, PSH) in animal tissues (muscle and kidney) of different species (bovine, ovine, porcine, poultry, rabbit, fish) and eggs by VA-LLE followed by SPE purification and UHPLC-MS/MS analysis. The developed method has been validated according to current Regulation (EU) 2021/808 applying a realistic method (Method 3) for CCα calculation [9], being useful to be implemented for official control analyses in public health laboratories.

2. Materials and methods

2.1. Reagents

Analytical standards of AMOZ, AOZ, SEM hydrochloride, AHD hydrochloride, AMG hydrochloride, PSH, NPIR, all from Merck (Darmstadt, Germany), and DNSH, from LGC Standards (Bury, UK), were used.

Analytical standards of 3-amino-oxazolidinone-D_4 (AOZ-D_4), from Merck (Darmstadt, Germany), and 3,5-dinitrosalicylic acid hydrazide-$^{13}C_6$ (DNSH-$^{13}C_6$), from WITEGA (Berlin, Germany), were used as internal standards (IS).

2-Nitrobenzaldehyde (*o*-NBA), also from Merck (Darmstadt, Germany), was used as derivatization reagent.

Ultra-pure water (UW) was obtained using a Purelab Flex water purification system from Elga LabWater (Lane End, UK); MS-grade methanol (MeOH) and acetonitrile (MeCN), HPLC-grade ethyl acetate and dimethyl sulfoxide (DMSO), all from VWR International (Radnor, PA, USA), were used as solvents. Analytical-grade sodium hydroxide (NaOH), sodium chloride (NaCl), trisodium phosphate dodecahydrate, ammonium acetate, and hydrochloric acid (HCl) 37 %, all from Panreac Química (Barcelona, Spain), were also used.

2.2. Samples

For method development and validation, a total of 34 samples of animal tissues and eggs, including kidney and muscle from different species of animals for human consumption were analyzed: 3 poultry muscles, 2 bovine muscles, 2 ovine muscles, 2 porcine muscles, 1 rabbit muscle, 5 fish muscles, 3 porcine kidneys, 4 bovine kidneys, 1 ovine kidney, and 10 hen eggs samples (each egg sample consisting on a pooled mix of 12 eggs from the same producer, according to the Annex II of Regulation (EU) 2021/808 [9]). Additionally, the method was applied in the official control analysis of 95 samples of animal tissues and eggs, including 83 samples of muscle and kidney from different species (bovine, ovine, porcine, equine, poultry, rabbit), 15 aquaculture fish samples (eel, sea bream, sea bass, meagre), 2 frozen raw shrimp samples, and 10 hen egg samples. All samples should be frozen (≤-10 °C) and stored for a maximum period up to 6 months until their analysis.

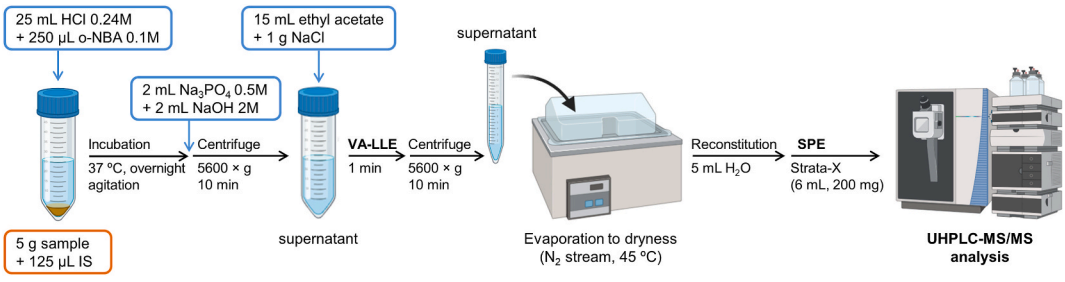

Fig. 1. Scheme of the proposed analytical methodology.

2.3. Proposed method

A scheme of the proposed method is shown in Fig. 1. Briefly, an aliquot of a previously homogenized sample is subjected to a hydrolysis/derivatization step with *o*-NBA in acid conditions at 37 °C and agitation overnight. After incubation, the extract is neutralized, centrifuged and VA-LLE with ethyl acetate is performed. Finally, the extract is evaporated to dryness, reconstituted and purified by SPE prior to UHPLC-MS/MS analysis. The complete procedure is detailed in the following subsections.

2.3.1. Preparation of standard solutions

Individual stock standard solutions of the target analytes (AMOZ, AOZ, SEM, AHD, AMG, PSH and NPIR) and IS (AOZ-D_4 and DNSH-$^{13}C_6$) at 200 μg mL^{-1} were prepared in MeOH (except for DNSH and DNSH-$^{13}C_6$ which were prepared in DMSO). These solutions were stored in darkness at ≤ -10 °C up to 24 months. From these solutions, a multicomponent standard solution containing the target analytes at 1 μg mL^{-1} was prepared in MeOH and stored refrigerated. From this solution, a working standard solution containing the target analytes at 100 ng mL^{-1} was daily prepared using UW as solvent. Additionally, from the individual stock solutions of the IS, a multicomponent solution of AOZ-D_4 and DNSH-$^{13}C_6$ at 40 μg mL^{-1} was prepared in MeOH. Finally, a working solution containing IS at 20 ng mL^{-1} in MeOH was also prepared.

2.3.2. Preparation of samples

Prior to analytical preparation, animal tissues (muscle and kidney) were thoroughly homogenized after the removal of visible parts of fat. In fish samples, head and bones were removed, and eggshells were also removed in egg samples before homogenization.

For sample preparation, 5 g of the homogenized sample were weighed in a 50 mL polypropylene (PP) conical-bottom tube (Falcon type tube) and 125 μL of IS solution at 20 ng mL^{-1} were added (0.5 μg kg^{-1} of IS in sample). For acid hydrolysis and derivatization, 25 mL of HCl 0.24 M in UW and 250 μL of *o*-NBA 0.1 M in MeOH were added. The mixture was incubated overnight (16 h) at 37 °C under constant agitation. After that, 2 mL of trisodium phosphate 0.5 M and 2 mL of NaOH 2 M, both in UW, were added for pH neutralization. Then, the mixture was centrifuged at 5600×*g* for 10 min. The supernatant was collected into a clean 50 mL PP tube and the pH was adjusted to approximately 7.0–7.5 using NaOH 2 M or HCl 1 M, if necessary. Afterwards, a VA-LLE was conducted by adding 15 mL of ethyl acetate and 1 g of NaCl. The mixture was vigorously mixed using a vortex mixer for 1 min and it was centrifuged at 5600×*g* for 10 min. After that, the extract (supernatant phase) was transferred to a 15 mL PP tube, evaporated to dryness in a water bath at 45 °C under a gently N_2 stream, and reconstituted in 5 mL of UW. Later, the extract was purified by reversed phase SPE using Strata-X (6 mL, 200 mg) cartridges from Phenomenex (Torrance, CA, USA). For that, SPE cartridges were conditioned in the vacuum manifold with 3 mL of MeOH and 3 mL of UW. The sample was loaded and washed with 3 mL of UW and 3 mL of MeOH 30 %, *v/v*. Then, it was dried by air for 10 min using vacuum, and the analytes were eluted twice with 3 mL of MeOH. The extract was evaporated to dryness in a water bath at 45 °C under N_2 stream and reconstituted with 250 μL of a mixture of ammonium acetate 5 mM at pH 9 and MeCN (90:10 *v/v*). Finally, the extract was transferred to a 1.5 mL PP microtube (Eppendorf type tube), centrifuged at 14170×*g* for 10 min, and the supernatant was collected into a 250 μL PP injection vial for UHPLC-MS/MS analysis.

2.3.3. Preparation of matrix-fortified standards

In order to construct matrix-fortified standards for calibration, five aliquots of 5 g of blank (analyte-free) samples of the different studied matrices were spiked with the target analytes at 0, 0.25, 0.50, 1.50, and 3.0 μg kg^{-1} (0, 12.5, 25, 75, and 150 μL of the multicomponent 100 ng mL^{-1} standard solution, respectively) and with 0.5 μg kg^{-1} of IS (125 μL of IS solution at 20 ng mL^{-1}). Consequently, the lowest calibrated level (LCL) of 0.25 μg kg^{-1} corresponds to half the current RPA of 0.5 μg kg^{-1}, according to Regulation (EU) 2019/1871 [4].

After that, the matrix-fortified standards were subjected to the same derivatization and extraction procedure described above (see Section 2.3.2).

2.3.4. UHPLC-MS/MS analysis

Twenty microliters of the sample extracts or matrix-fortified standards were injected into the UHPLC-MS/MS system, which consisted of a Dionex Ultimate 3000 LC system coupled to a TSQ Quantiva™ triple quadrupole mass spectrometer, from Thermo Fisher Scientific (Whaltham, MA, USA). The separation was achieved using a Kinetex 2.6 μm EVO18 100A (100 mm × 2.1 mm i.d.) column from Phenomenex (Torrance, CA, USA) at 40 °C with a flow rate of 0.35 mL min^{-1}. The mobile phase consisted of a mixture of

Table 1
Gradient elution program for UHPLC-MS/MS analysis.

Time (min)	Solvent A (%)	Solvent B (%)
0.0	95	5
2.0	95	5
4.0	90	10
5.0	85	15
10.0	80	20
15.0	10	90
15.1	95	5
20.0	95	5

ammonium acetate 5 mM pH 9 (solvent A) and MeCN (solvent B), following the gradient elution program shown in Table 1.

Electrospray ionization (ESI) ion source operated in positive and negative modes with the following parameters: ion spray voltage, +3.6 kV (positive mode), −2.5 kV (negative mode); sheath gas flow, 50 arb.; auxiliary gas flow, 12 arb.; sweep gas flow, 1 arb.; ion transfer tube temperature, 300 °C; and vaporizer temperature, 350 °C.

Acquisition was conducted in multiple reaction monitoring (MRM) mode, using the precursor ions, product ions, and collision energies (CE) shown in Table 2.

2.3.5. Quantification and confirmation

Quantification was achieved applying internal standard calibration in the working range (from 0.25 to 3.0 μg kg^{-1}) using matrix-fortified standards (see Section 2.3.3). The calibration curves were constructed by simple linear regression (5-point calibration curve), being the analytical signal (i.e., peak area ratio between the quantification ion of the target analyte and the IS) the dependent variable, and the spiked analyte concentration (μg kg^{-1}) the independent variable. For that, DNSH-$^{13}C_6$ was used as IS for DNSH, PSH, and NPIR; whereas AOZ-D_4 was used for the others (AMOZ, AHD, SEM, AOZ, and AMG).

For the identification of the target analytes in samples, a retention time match between the quantification ion peak in the sample and in the matrix-fortified standards (±1 % tolerance) was required and at least 1 precursor and 2 product ions should be detected (5 identification points), according to Regulation (EU) 2021/808 [9]. In this work, 3 product ions were used for the identification of the target analytes (except for AOZ and SEM), as can be seen in Table 2, achieving 6.5 identification points [9]. Moreover, the relative intensity between product ions in a suspect sample and in a matrix-fortified standard with a similar concentration level should also match (±40 % tolerance). In all cases, the quantification of a target analyte in a sample should be ≥CCα for that substance (see Section 3.2.6) to be considered as non-compliant.

As an example, the ion chromatograms obtained in the analysis of a blank sample, and blank samples spiked with the analytes at 0.25 and 3.0 μg kg^{-1} are shown in Fig. 2.

3. Results and DISCUSSION

3.1. Preliminary studies

During method development, several preliminary tests regarding experimental variables (sample preparation and analysis) were performed, which are discussed below.

In order to obtain homogeneous and representative analytical samples of the animal tissues (muscle and kidney) and eggs taken by official control agents, the sample amount for subsequent analysis was set at 5 g to ensure homogeneity and reproducibility, especially for suspect or non-compliant samples, as well as to achieve the required sensitivity.

Table 2
Selected ions (*m/z*) and collision energies (CE) for signal acquisition in MRM, polarity mode, and retention times (RT) of the studied compounds.

Compound	Mode	Precursor ion (*m/z*)[a]	CE (eV)	Product ion (*m/z*)	RT (min)
Analytes					
AHD	+	249	18	134[b]	4.5
			20	178	
			18	104	
AMG	+	208	11	191[b]	9.7
			21	119	
			22	91	
AMOZ	+	335	12	291[b]	12.1
			18	262	
			25	128	
AOZ	+	236	15	134[b]	9.1
			23	104	
DNSH	–	374	28	183[b]	13.2
			24	226	
			18	327	
NPIR	+	247	20	154[b]	13.3
			42	115	
			21	158	
PSH	+	286	33	121[b]	11.4
			22	93	
			12	192	
SEM	+	209	10	166[b]	8.3
			15	192	
Internal standards					
AOZ-D_4	+	240	15	134	9.1
DNSH-$^{13}C_6$	–	380	27	189	13.2

[a] Precursor ions correspond to the nitrophenyl derivatives of the target compounds (see Section 2.3).
[b] Product ion used for quantification.

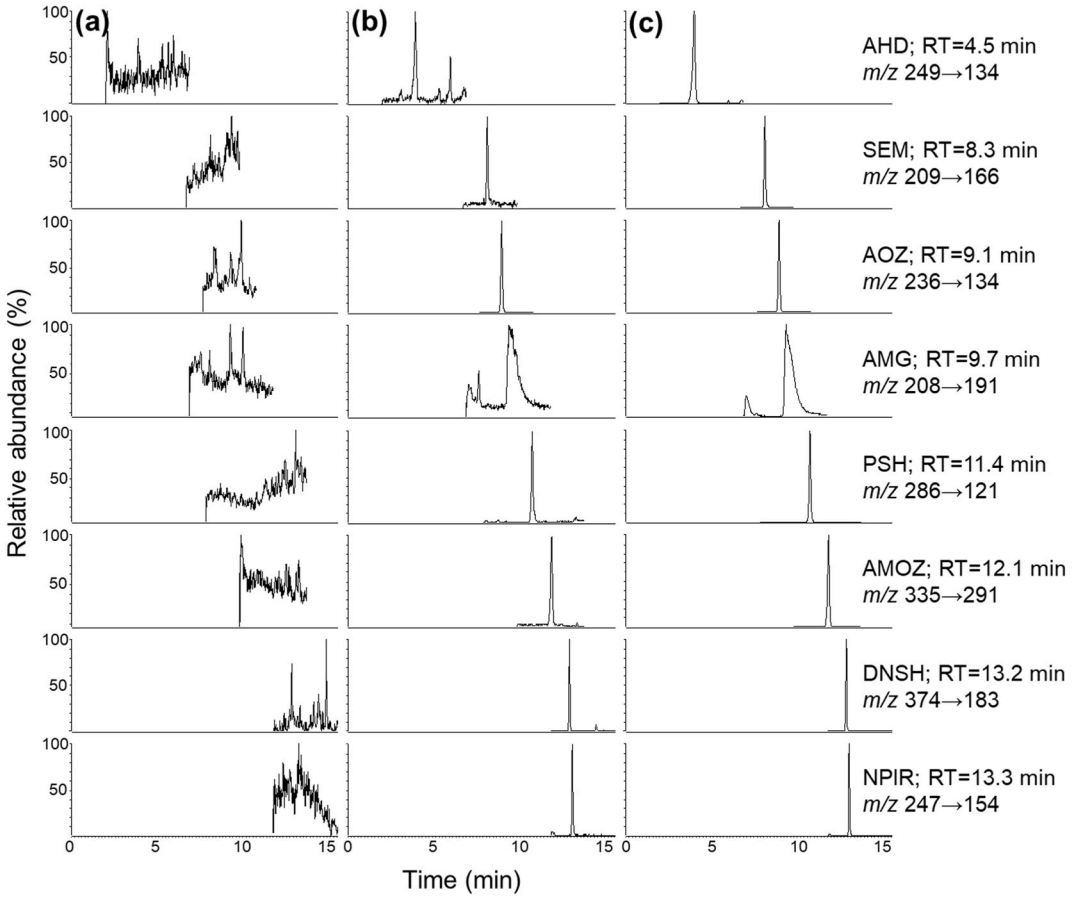

Fig. 2. MRM ion chromatograms obtained in the analysis of: (a) blank sample; (b) blank sample spiked at 0.25 μg kg^{-1}; and (c) blank sample spiked at 3.0 μg kg^{-1}.

To achieve the chemical hydrolysis and derivatization of the target metabolites, acid conditions are needed for breaking the protein-binding interactions with the sample matrix. For that, HCl 0.24 M and *o*-NBA (in MeOH) as derivatization reagent were used to increase their molecular mass (by 133 Da) and modify their polarity, obtaining nitrophenyl derivatives which are more suitable for extraction and reversed phase SPE for the purification of sample extracts. On the other hand, NPIR does not contain the N-terminal amine group (–NH_2) required for derivatization (it is not a metabolite), so it is not affected by the reaction medium and can be determined simultaneously.

After hydrolysis and derivatization, it is necessary to neutralize the acid medium of the obtained solution before extraction and purification. In first place, the purification of supernatant aliquots (5–15 mL) of the sample solution directly by SPE was tested in order to reduce the consumption of reagents and to avoid large amounts of sample matrix in the final extracts. For that, different stationary phases and solvents for conditioning, washing, and elution were considered. In the first place, C_8 SPE cartridges (6 mL, 500 mg) were tested using ethyl acetate for conditioning and elution, and MeOH 30 %, *v/v* for washing. However, low recoveries (<50 %) for the target analytes were observed, so these cartridges were discarded. Then, Strata-X cartridges (6 mL, 200 mg), from Phenomenex; and Oasis Prime cartridges (6 mL, 200 mg), from Waters (Milford, MA, USA), were considered using the same solvents. In both cases, no significant interferences were observed in the chromatograms of blank samples, but the analytical signal of AHD was insufficient for its determination at the LCL (0.25 μg kg^{-1}). To increase the analytical response of AHD, MeOH for conditioning, MeOH 30 %, *v/v* for washing, and MeOH acidified with 1 % formic acid for elution were tested, obtaining satisfactory results. However, the analytical response of DNSH decreased using these solvents, and its determination at the LCL was not possible. After that, the purification of the whole supernatant sample solution (25 mL) by SPE was tested, obtaining lower analytical signals for both AHD and DNSH, probably due to larger amounts of matrix interferences. To reach the required sensitivity, an additional extraction step prior to SPE was proposed.

In this sense, neutralization after incubation was achieved for all sample matrices by adding 2 mL of trisodium phosphate 0.5 M and 2 mL of NaOH 2 M (reaching pH 6.5–7.5), which was finally adjusted to pH 7.0–7.5 using few droplets of NaOH 2 M or HCl 1 M, when necessary. After that, a VA-LLE with 15 mL of ethyl acetate was conducted as described (see Section 2.3.2) using the whole supernatant solution (25 mL) after neutralization, since it enables the extraction of the target nitrofuran metabolites and NPIR while avoiding significant matrix interferences. To further reduce interferences, SPE was conducted after the evaporation of the ethyl acetate extract

and reconstitution in UW. For that, the same SPE cartridges with different conditioning, washing, and elution solvents were tested; obtaining satisfactory results for all the target analytes at the LCL applying the proposed conditions (see Section 2.3.2).

With regard to UHPLC-MS/MS analysis, mobile phase composition using ammonium acetate at 2.5, 5, and 10 mM adjusted at pH 9 was tested to improve ionization in both positive and negative polarity modes. The highest analytical response for all the target analytes was obtained at 5 mM. When 10 mM was used, AHD response was 50 % lower, whereas the SEM response was 30 % lower at 2.5 mM. Thus, 5 mM ammonium acetate at pH 9 was selected as aqueous mobile phase for further analysis. Furthermore, column composition was also studied. Therefore, Kinetex C18 (100 mm × 2.1 mm i.d., 2.6 μm) and Kinetex EVO18 (100 mm × 2.1 mm i.d., 2.6 μm), both from Phenomenex, were tested using the same gradient elution program (see Table 1). With the first column, AHD and SEM showed poor retention and co-eluted with matrix components. Specifically, an interfering compound at *m/z* 134 co-eluting with AHD was observed. With the second column, both AHD and SEM showed adequate retention, being separated from matrix components, including the interference at *m/z* 134. For that reason, Kinetex EVO18 column was selected for further analysis.

3.2. Method validation

The analytical performance of the proposed method was validated according to the performance criteria of Regulation (EU) 2021/808 [9], including the study of relative matrix effect, selectivity, linearity in the working range, trueness, accuracy, repeatability, reproducibility, decision limit for confirmation (CCα), and ruggedness. The obtained analytical features are shown in Table 3 and discussed in the following subsections.

3.2.1. Relative matrix effect

Relative matrix effect was studied for 20 different samples of muscle (bovine, ovine, porcine poultry, and fish), kidney (bovine, ovine, and porcine), and 10 egg samples. For that, blank samples of the selected matrices were spiked with the derivatives at the RPA level (0.5 μg kg^{-1} of the analytes) after sample processing and extraction (matrix-matched standards) and their analytical response was compared with those obtained for a pure (matrix-free) standard solution of derivatized analytes at the same concentration level applying IS correction.

The relative matrix effect or matrix factor (MF) values were calculated applying the following equations:

$$\mathrm{MF\ (standard)} = \mathrm{A_{analyte,mms}} \ / \ \mathrm{A_{analyte,std}} \quad (1)$$

being $A_{analyte,mms}$, the peak area of the analyte in the matrix-matched standard; and $A_{analyte,std}$, the peak area of the analyte in the standard solution.

$$\mathrm{MF\ (IS)} = \mathrm{A_{IS,mms}} \ / \ \mathrm{A_{IS,std}} \quad (2)$$

being $A_{IS,mms}$, the peak area of the IS in the matrix-matched standard; and $A_{IS,std}$, the peak area of the IS in the standard solution.

$$\mathrm{MF\ (normalized\ for\ IS)} = \mathrm{MF\ (standard)} \ / \ \mathrm{MF\ (IS)} \quad (3)$$

According to Regulation (EU) 2021/808 [9], the coefficient of variation (CV) for the MF (normalized for IS) should be ≤ 20 %. The

Table 3
Analytical features of the proposed method.

Analyte	CCα (μg kg^{-1})[a]	Accuracy (%)[b]			Trueness (bias, %)[c]			Repeatability (CV_r, %)[d]			Reproducibility (CV_R, %)[e]		
		0.25 μg kg^{-1}	0.50 μg kg^{-1}	3.00 μg kg^{-1}	0.25 μg kg^{-1}	0.50 μg kg^{-1}	3.0 μg kg^{-1}	0.25 μg kg^{-1}	0.50 μg kg^{-1}	3.00 μg kg^{-1}	0.25 μg kg^{-1}	0.50 μg kg^{-1}	3.0 μg kg^{-1}
AHD	0.29	95 ± 7	100 ± 8	107 ± 10	−5	0	+7	6.5	6.9	6.4	7.9	7.9	10.5
AMG	0.34	92 ± 14	90 ± 21	120 ± 23	−8	−10	+20	12.9	15.2	17.3	15.1	24.8	19.4
AMOZ	0.28	108 ± 5	105 ± 12	108 ± 16	+8	+5	+8	4.4	6.4	7.2	5.2	12.8	16.7
AOZ	0.27	97 ± 3	100 ± 4	101 ± 2	−3	0	+1	1.8	2.1	1.7	2.9	4.4	1.9
DNSH	0.29	96 ± 6	97 ± 6	100 ± 3	−4	−3	0	3.6	3.9	2.1	7.3	6.3	2.9
NPIR	0.35	83 ± 18	119 ± 21	109 ± 23	−17	+19	+9	19.9	15.9	14.0	21.6	18.1	23.4
PSH	0.34	104 ± 14	107 ± 15	97 ± 22	+4	+7	−3	9.8	12.2	8.0	14.8	14.4	25.7
SEM	0.34	99 ± 15	109 ± 13	108 ± 5	−1	+9	+8	11.7	8.0	4.5	16.4	12.5	4.7

[a] Decision limit for confirmation (CCα) values (see Section 3.2.6).
[b] Accuracy values (%) obtained in the analysis of fortified samples, expressed as the average value ± standard deviation (n ≥ 20).
[c] Trueness values (%) expressed as bias (100 - Accuracy (%)).
[d] Repeatability values (CV_r, %) obtained in the replicate analysis of fortified samples (n ≥ 20).
[e] Reproducibility values (CV_R, %) obtained in the replicate analysis of fortified samples in different analytical series (4 series, n = 5).

obtained CV values for the target analytes ranged from 4 % (AOZ) to 20 % (SEM, AMG, NPIR), showing that matrix effects were corrected using the selected IS (see Section 2.3.5).

3.2.2. Specificity

In order to demonstrate the power of discrimination of the proposed method between the target analytes and potential interferences, specificity was studied analyzing 20 blank samples of muscle, kidney and eggs of different animal species. In all the cases, analytical signals ≥30 % of the LCL were not observed at the retention times where the target analytes are expected to elute. These results showed that the quantification and identification of analytes was not affected by interferences and the specificity of the method for all studied species and matrices (see Section 2.2) was demonstrated.

3.2.3. Linearity

Linearity was studied in the calibration curves obtained with matrix-fortified standards in the working range (5-point calibration curve including zero level), from 0.25 to 3.0 μg kg^{-1} (see Section 2.3.3). In all the cases, the coefficient of determination (R^2) values were ≥0.98 and the individual residuals (difference between the predicted analytical response based on the calibration curve and the acquired response) were in the ±20 % tolerance range, showing adequate linearity.

3.2.4. Trueness and accuracy

Trueness and accuracy were validated in the studied matrices using blank samples fortified at three concentration levels: 0.25, 0.50, and 3.0 μg kg^{-1}; which correspond to 0.5, 1, and 3 times the RPA value, respectively. At each concentration level, at least 20 replicate analyses were performed.

Accuracy for each target analyte was determined using the following equation:

$$\text{Accuracy}\ (\%) = C_{\text{analyte}} \ / \ C_{\text{spiked}} \times 100 \tag{4}$$

being $C_{analyte}$, the found analyte concentration using internal standard calibration with matrix-fortified standards; and C_{spiked}, the known spiked concentration. The obtained accuracy values (%) were expressed as the average value ± standard deviation (n ≥ 20).

From these values, trueness (%) was calculated as bias according to the following equation:

$$\text{Trueness(bias, \%)} = 100\text{-Accuracy(\%)} \tag{5}$$

As can be seen in Table 3, the obtained accuracy values ranged from 83 to 108 % for 0.25 μg kg^{-1}; from 90 to 119 % for 0.50 μg kg^{-1}; and from 97 to 120 % for 3.0 μg kg^{-1}. Trueness values ranged from −17 to +8 % for 0.25 μg kg^{-1}; from −10 to +19 for 0.50 μg kg^{-1}; and from −3 to +20 for 3.0 μg kg^{-1}, meeting in all cases the acceptability criteria of Regulation (EU) 2021/808 (−50 % to +20 % for levels ≤1 μg kg^{-1}, and -30% to +20 % for levels >1 μg kg^{-1} to 10 μg kg^{-1}) [9].

3.2.5. Repeatability and reproducibility

Repeatability values, expressed as CV (CV_r, %), were obtained in replicate analyses (n ≥ 20) of blank samples fortified with the target analytes at the three studied concentration levels (0.25, 0.50, and 3.0 μg kg^{-1}). Standard deviations and CV were calculated for all data at the same concentration level, according to the following expression:

$$CV_r\ (\%) = S_r \ / \ C_m \times 100 \tag{6}$$

being S_r, the standard deviation for repeatability; and C_m, the average found concentration.

Moreover, reproducibility values, expressed as CV (CV_R, %), were obtained in repeated series of analyses (4 series of 5 replicates) of blank samples fortified at the studied concentration levels conducted by different operators, applying the following equations:

$$S_R^2 = S_L^2 + S_r^2 \tag{7}$$

being S_R^2, the variance of reproducibility; S_L^2, the variance of the different analytical series; and S_r^2, the variance of repeatability.

From these values, reproducibility (CV_R) was calculated:

$$CV_R\ (\%) = S_R \ / \ C_M \times 100 \tag{8}$$

being S_R, the standard deviation for reproducibility; and C_M, the average found concentration considering all analytical series.

As can be seen in Table 3, repeatability values (CV_r) ranged from 1.7 to 19.9 %, and reproducibility values (CV_R) ranged from 1.9 to 25.7 %, meeting in all cases the acceptability criteria of Regulation (EU) 2021/808 ($CV_R \leq 30$ % for concentration levels <10 μg kg^{-1}) [9]. For repeatability conditions, CV_r values should be ≤ 2/3 CV_R (i.e., ≤20 %), which were also achieved.

3.2.6. Decision limit for confirmation (CCα)

The CCα value is by definition the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant (i.e., that it contains the analyte above this level) and it shall be established considering both identification and quantification requirements.

As stated before, NFs and nitrofuran metabolites are unauthorized substances according to Regulation (EU) 37/2010 [3], and Regulation (EU) 2019/1871 [4] establishes a RPA of 0.5 μg kg^{-1} for each of them. For that, analytical methods are required to identify

and quantify these substances below their reference values.

According to Regulation (EU) 2021/808 [9], CCα should be calculated as follows in the case of unauthorized substances (Method 3):

$$CC\alpha = LCL + \text{k-factor} \times S_{LCL} \quad (9)$$

being the *k-factor*, 2.33; and S_{LCL} the standard deviation in reproducibility conditions at the LCL.

Since a LCL of 0.25 μg kg^{-1} was validated in this study, the obtained CCα values ranged from 0.27 to 0.35 μg kg^{-1}, all below the RPA level.

3.2.7. Ruggedness

The ruggedness of the proposed method vas evaluated to test minor experimental variations, which could occur during routine method application. For that, three series of duplicate blank samples fortified at the RPA level (0.5 μg kg^{-1}) were prepared and analyzed applying slight modifications on the extraction and purification procedure, according to the following conditions: In the first series, the incubation temperature was increased to 40 °C (from 37 °C); in the second series, the volume of UW used to wash the SPE columns was reduced to 2 mL (from 3 mL); and in the third series, the drying time of SPE columns before elution was increased to 15 min (from 10 min). The obtained results for all target analytes in the ruggedness tests were similar to those obtained in the method validation at the same concentration level (0.5 μg kg^{-1}), showing that minor changes in the sample preparation procedure did not affect the obtained results.

3.3. Method applicability to incurred samples

The applicability of the developed method was tested in two FAPAS inter-laboratory test comparisons, during 2022 and 2023, comprising incurred samples (one kidney sample and one shrimp sample) containing known amounts of AHD, SEM, AOZ, and AMOZ. In these tests, z-scores were assigned by the organizers based on measured concentrations. As can be seen in Table 4, the obtained results and z-scores were satisfactory for all analytes and samples. An adequate method performance was observed, with z-scores ranging from −1.1 to +1.2 (absolute z-score values ≤ 2), demonstrating the method applicability.

3.4. Analysis of field samples

The proposed method was applied in the official control analysis of 95 samples of animal tissues and eggs, including 83 samples of muscle and kidney from different species (bovine, ovine, porcine, equine, poultry, rabbit), 15 aquaculture fish samples (eel, sea bream, sea bass, meagre), 2 frozen raw shrimp samples, and 10 hen egg samples, according to the national official control plan derived from Regulation (EU) 2022/1646 [11]. In all of them, the target analytes were not detected, except in a frozen shrimp sample where SEM was detected and identified at levels below the LCL (0.25 μg kg^{-1}), which may be a consequence of the natural occurrence of this nitrofuran metabolite in fishery products [12], as also described in the annex of Regulation (EU) 2019/1871 [4].

3.5. Comparison with other published methods

The developed analytical method was compared with other similar methods published recently in the analytical literature for the analysis of nitrofuran metabolites in animal tissue samples [5,10], validated according to Regulation (EU) 2021/808 [9]. This comparison is shown in Table 5. As can be seen, the method developed in this study showed better or similar analytical performance with a wider applicability range (regarding target analytes, matrices or species) and a higher number of identification points for substance confirmation, constituting a useful and reliable approach. It should be noted that the obtained values for CCα are highly dependent on the method used for its calculation (European Commission, 2021). Current Regulation (EU) 2021/808 [9] establishes three different calculation methods, although only Methods 1 or 3 should be used for methods validated after the entry into force of that regulation. Method 1 is based on the calibration curve procedure according to ISO 11843–1:1997 [13], which consists on determining the concentration at the y-intercept of a calibration curve using fortified blank materials plus 2.33 times the standard deviation at the y-intercept. This approach tends to provide optimistic CCα values, which should be further confirmed experimentally; whereas Method

Table 4
Concentrations (μg kg^{-1}) and z-scores obtained in an interlaboratory test comparison with incurred samples.

Sample	Analyte	Assigned concentration (μg kg^{-1})	Found concentration (μg kg^{-1})	z-score
Kidney	AHD	<0.20	<0.25	N.A.
	AMOZ	2.18	1.65	−1.1
	AOZ	<0.20	<0.25	N.A.
	SEM	2.55	2.09	−0.8
Shrimp	AHD	1.66	1.61	−0.1
	AMOZ	<0.20	<0.25	N.A.
	AOZ	1.76	1.83	+0.2
	SEM	3.30	4.15	+1.2

N.A.: Not applicable.

Table 5
Comparison with other published methods.

Analytes	Studied matrices	Extraction technique	Analytical technique (IP)[a]	Accuracy (%)	Reproducibility (CV_R, %)	CCα (μg kg^{-1})[b]	Reference
AHD, AMOZ, AOZ, DNSH, SEM	Muscle (fish, shrimp)	VA-LLE	UHPLC-MS/MS (5 IP)	82.8–118.1	≤16.9	0.32–0.36 (Method 3)	[5]
AHD, AMG, AMOZ, AOZ, DNSH, OAH, PSH, SEM	Muscle (bovine, ovine, porcine, poultry)	QuEChERS	UHPLC-MS/MS (5 IP)	98–105	0.9–10.7	0.013–0.20 (Method 1)	[10]
AHD, AMG, AMOZ, AOZ, DNSH, NPIR, PSH, SEM	Muscle (bovine, ovine, porcine, poultry, rabbit, fish), kidney (porcine, bovine, ovine), eggs	VA-LLE and SPE	UHPLC-MS/MS (6.5 IP, except AOZ and SEM)	83–120	1.9–25.7	0.27–0.35 (Method 3)	This study

OAH: Oxamic acid hydrazide.
[a] Analytical technique and identification points (IP) presented in parentheses, according to identification criteria of Regulation (EU) 2021/808 [9].
[b] Decision limit for confirmation (CCα) values calculated according to Regulation (EU) 2021/808 [9]. The calculation method is indicated in parentheses.

3 (used in this study) is based on the standard measurement uncertainty at the LCL (as seen in Section 3.2.6) providing more realistic results.

4. Conclusions

The method reported in this study has been validated following the requirements of Regulation (EU) 2021/808 regarding acceptance criteria for several analytical performance parameters, including linearity, specificity, accuracy, trueness, repeatability, reproducibility, and the obtaining of CCα values for the eight studied nitrofuran metabolites and nifurpirinol. After satisfactory validation, the method has been applied for the official control analysis of animal tissues and egg samples according to the national official control plan, and it has been applied in the analysis of incurred samples from inter-laboratory test comparisons, showing adequate analytical performance. This validated method is currently accredited according to UNE-EN ISO/IEC 17025 by the Spanish National Accreditation Body (ENAC) to be implemented for official control analyses in the Public Health Laboratory of Valencia (Spain).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Francisco Moragues: Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pablo Miralles:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Carmen Igualada:** Validation, Supervision, Resources, Project administration, Investigation, Conceptualization. **Clara Coscollà:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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