Sorbent selection for solid-phase clean-up of nitrofuran metabolites from meat

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Abstract

Introduction. In this study there are presented the results of selecting a sorbent for clean-up of an extract of 4 nitrofuran metabolites extracted from the meat, preparing a solid-phase column with this sorbent, and confirming the advantages of this column using validation of method.

Materials and methods. The extract was clean-up using a solid-phase column, prepared by us with the sorbent Silica gel 60; identification and quantitative determination of nitrofuran metabolites: 3-amino-2-oxazolidinone (AOZ), 3-amino 5-methylmorpholino-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM), and their nitrophenyl derivatives was carried out using a high-performance liquid chromatography with diode array detector.

Results and discussion. Based on the physicochemical properties of the analyzed substances, sorbents and literature data, two sorbents were selected – silica gel 60 and a polymer sorbent – polypropylene, which is most often used in ready-made columns for nitrofuran purification. Columns were made from the selected sorbents and their suitability was determined. The advantage of the manufactured columns using silica gel was experimentally proven. 96-98% extraction of nitrofuran metabolites was achieved with good reproducibility (RSD $\leq$ 2.0%), since when using a polymer sorbent, the extraction does not exceed 94%.

When using silica gel 60, the limit of quantification for metabolites – AOZ, AMOZ, AHD and SEM is within 0.1–0.12 μg/kg, for their nitrophenyl derivatives (NP) within – 0.11–0.14 μg/kg. The decision limit is within 0.01–0.07 μg/kg. When using a polymer sorbent: the limit of quantification for metabolites – AOZ, AMOZ, AHD and SEM is within 0.21–0.32 μg/kg; for their NPs within 0.25–0.43 μg /kg. The decision limit is within 0.13–0.28 μg/kg.

Conclusions. Silica gel 60 and columns prepared with this sorbent were identified as preferable for the purification and concentration of nitrofuran metabolites isolated from meat. The advantages of the proposed column over disposable, expensive, commercially available columns were demonstrated through method validation.

Abbreviations:

SPE – solid-phase extraction;
HPLC – high-performance liquid chromatography;
DAD – diode array detector;
HPLC/DAD – high-performance liquid chromatography with diode array detector;
HPLC-MS/MS – liquid chromatography-tandem mass spectrometry;
UPLC-MS/MS – ultra-high-performance liquid chromatography-tandem mass spectrometry;
AOZ – 3-amino-2-oxazolidinone;
AMOZ – 3-amino 5-methylmorpholino-2-oxazolidinone;
AHD – 1-aminohydantoin;
SEM – semicarbazide;
NP-derivatives – nitrophenyl derivatives;
NPAMOZ – 5- (morpholinomethyl) – 3 – (2 – nitrobenzylidenamino)-2-oxazolidinone;
NPAOZ – 3-(2-nitrobenzylidenamino)-2-oxazolidinone, NPSEM – 2-nitro-benzaldehyde-Semicarbazone;
NPAHD – 1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione;
MRL – Maximum Residual Limit;
MRPL – Minimum Required Performance Limit;
LC-MS/MS – Liquid chromatography- tandem mass spectrometry;
2-NBA – 2-nitrobenzaldehyde;
RSD – Relative standard deviation;
LOQ – Limit of quantification;
LOD – Limit of detection.

Introduction

Nitrofurans are antibacterial drugs, which can be considered one of the most used drugs for the treatment of microbial infections in farm animals (Torre et al., 2015; Wang et al., 2017). However, the most commonly used nitrofurans, such as furazolidone, furaltadone, nitrofurantoin, and nitrofurazone, have been banned worldwide for farmed animals due to their carcinogenic and teratogenic risks (Commission implementing regulation (EU) 2021/808; García et al., 2017; Yuan et al., 2020; Zuma et al., 2019). However, these drugs continue to be used in some countries because of their low cost, effectiveness against infections, and availability (Antunes et al., 2006; Gotsiridze et al., 2022, 2023; Yu et al., 2019; Zhang et al., 2016; Zhao et al., 2016). Therefore, it is necessary to control their illegal use in food products by establishing effective and reliable methods. As of today, the analytical strategy for the quantification of nitrofurans is based on the determination of four metabolites: AOZ, AMOZ, AHD, and SEM (Alkan et al., 2016; Craken et al., 2001; Tsai al., 2010).

Many analytical methods have been developed to determine nitrofuran metabolites, such as LC-MS (Gong X. et al., 2022), HPLC-MS/MS (Park et al., 2017), UPLC-MS/MS (Gong J. et al., 2020; Noelia et al., 2013), enzyme-linked immunosorbent assay (Cháfer et al., 2010), HPLC/DAD (Kangkang et al., 2020). However, not all of these methods are suitable due to the very low detection limit required for the analyte, with an MRPL of 0.5 µg/kg (Luo et al., 2019); therefore, the HPLC-MS/MS method is mainly used due to its high sensitivity and accuracy (Shi et al., 2016). The use of this analytical method is limited

in economically developing countries due to the expensive equipment and high maintenance costs of this equipment (Ong et al., 2013).

The HPLC/DAD method is convenient and relatively less expensive compared to other methods, but it is difficult to achieve the required detection limit using a diode detector (Luo et al., 2019). For the determination of nitrofuran metabolites in food, sample preparation is of particular importance. Most often, for clean-up of samples solid-phase purification is used through various expensive cartridges (Barbosa et al., 2007; Conneely et al., 2002; Kaufman et al., 2015; Tripathi et al., 2023), where polymers are used as sorbents, followed by determination by HPLC-MS/MS. Currently, commercially available ready-made cartridges used for sample cleaning are disposable and expensive. Moreover, for commercial purposes, sorbents are often designed for a wide range of substance groups and are primarily polymer-based, which limits the ability to account for the specific chemical nature of individual target compounds.

Based on the above, the aim of this study was to select an appropriate sorbent and prepare a column for solid-phase sample purification in accordance with MRPL (Minimum Required Performance Limits) requirements. The goal was to achieve specificity and selectivity toward nitrofuran metabolites, thereby enhancing the sensitivity of the HPLC method and enabling the use of a diode array detector instead of a more costly mass spectrometric detector.

Materials and methods

Materials

Reagents and chemicals

Acetonitrile (HPLC grade), potassium dihydrogen orthophosphate, 35% hydrochloric acid, sodium hydroxide, 2-nitrobenzaldehyde (2-NBA), dimethyl sulfoxide anhydrous, ≥99.9%, ethyl acetate, methanol (HPLC grade), were from Sigma Aldrich Chemical Company (Germany) and Silica gel 60 from Roth (Germany). Ultrapure water was filtered through a Milli-Q system Millipore (USA). The metabolites AOZ, AMOZ, AHD, SEM, NP-AOZ, NP-AMOZ, NP- AHD and NP- SEM were supplied by Sigma (Aldrich Chemical Company, Germany). Stock solutions may be stored, refrigerated below -14°C for at least 12 months, and standard solutions should be stored refrigerated from 2°C to 8°C. Intermediate standards should be replaced at least monthly and working solutions at least weekly.

Standard solutions

Individual standard stock solutions of 1 mg/mL were prepared in acetonitrile. Working solutions of 10 ng/mL were diluted by water. All standard stock solutions were stored -20 ºC, and the working solutions were stored in refrigerator.

The concentration and content of mix standard solution were used to spiked samples with AMOZ, AOZ, AHD, NP-AOZ, NP-AMOZ, NP- AHD, NP- SEM and SEM at an 8.0, 9.0, 10.0, 11.0 and 12.0 ng/mL respectively.

Sample preparation end extraction

2.0±0.05 g of homogenized meat was weighed into a 50 mL polypropylene centrifuge tube. A mix of standard spiking solutions 50.0 µL, 5 mL of 0.1 M hydrochloric acid solution and 50 µl of a solution of 2-nitrobenzaldehyde in dimethyl sulfoxide (DMSO) (8 mg±0.6 mg in 5 mL of DMSO) were added. Thoroughly mixed for 1 minute and incubated for 12 hours at 37° C to hydrolyze the protein-bound metabolites and convert the metabolites to their nitrophenyl derivatives. After the sample solution was cooled to room temperature, 500 µL of potassium dihydrogen orthophosphate solution, 300 µL of sodium hydroxide solution were added to adjust the pH to 7.0±0.5, and 5 mL of acetonitrile were added. Thoroughly stirred for 1 minute, centrifuged at 4000 rpm for 10 minutes (Bongers et al., 2021; Śniegocki et al., 2018; Zuma al., 2019) and the acetonitrile layer was transferred to a pre-activated silica gel column, prepared by us, at a flow rate of 5 ml/min. After sample loading, the column was washed with 10 mL of ethyl acetate and eluted with 10 mL of acetonitrile. The column was then evacuated, the eluates were combined and evaporated under a moderate nitrogen flow.The residue was dissolved in 1 ml acetonitrile, 1 ml of the mobile phase was added, mixed for 1 minute, filtered through a Syringe Filter PTFE for HPLC 0.22um and chromatographed.

Selection of sorbent and column preparation for solid phase purification

The choice of sorbent was made based on the physicochemical properties of the analyzed substances, sorbents, literary and experimental data. The columns with the selected sorbent silica gel 60 were prepared by the dry method. For activation, the prepared column was washed with 5 mL of methanol, dried under vacuum, then washed with 10 mL of acetonitrile, and again dried under vacuum.

Quantitative determination of metabolites

Quantitative of AOZ, AMOZ, AHD, SEM, NP-AOZ, NP-AMOZ, NP-AHD and NP-SEM were determined by high-performance liquid chromatography with diode array detector. The LC/DAD system consisted of an Agilent Series 1260 HPLC system (Agilent Technologies, Germany) with DAD detector.

The chromatography was performed on a C18 column 3 µm x 2 mm 150 mm (Phenomenex, Torrance, CA, USA), connected to a C18 precolumn 3 µm x 2 mm x 4 mm (Phenomenex, USA). The mobile phase was Acetonitrile: 0.01 M sodium acetate buffer, pH 6. 0 – 250:750, λ 376 nm, flow rate of 1 mL/min, Injection volume was 50 µL. The column was thermostated at 30^0C. All determinations were carried out under standard conditions: Air temperature – (20±5) ^{0}C, atmospheric pressure – 84.0 – 106.7 kPa (630 – 800 mm Hg), air humidity no more than – 80%, mains voltage 198 – 242 V, frequency AC – 50±1 Hz. Acceptance criteria: Reference points for action (RPA) 0.5 µg/kg for each of the metabolites (Commission implementing regulation (EU) 2023/411).

Validation

The HPLC/DMD method for the determination of nitrofuran metabolites in meat extract purified using our column was validated in accordance with the European Union criteria for the analysis of veterinary drug residues in food (Commission implementing regulation (EU) 2021/808; Commission implementing regulation (EU) 2024/2052).

The validation was carried out according to the following parameters: Specificity/selectivity. The limit of detection (LOD) and the limit of quantification (LOQ), Recovery, Linearity, Trueness and Accuracy, Repeatability and Reproducibility also determined Decision limit (CC_α) and detection capability (CC_β), Stability and Matrix effects,%.

Specificity/selectivity

The specificity of the method was tested by analyzing 20 different blank meat samples. These samples were randomly selected from previously analyzed nitrofuran-free samples (eight pork, seven beef, and five poultry) and 20 matrix samples spiked with nitrofuran metabolite standards (concentration 0.5 μg/kg each). Acceptance criteria: No interference.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD: smallest measured content, from which it is possible to deduce the presence of the analyte with reasonable statistical certainty.

LOQ: Analyte content that can be determined with a certain level of precision.

The calculation of LOD was performed according to equation (1) under the condition of a pseudo-blank sample and equal probabilities (α=ß=0.05) for false positive and false negative decisions.

$$x_{LOD} = 3.3 \times \frac{S_{y,b}}{b},$$

where: X_{LOD} is limit of detection;

$S_{y,b}$ is standard deviation of the blank (pseudo-blank) signals;

b is a slope of the calibration curve;

3.3 is the multiplication factor.

The calculation of LOQ was performed according to equation:

$$X_{LOQ} = 10 \times \sigma / S,$$

where: σ is SD of the obtained results;

S is a slope of the calibration curve.

Acceptance criteria: LOD is three times the standard deviation of the mean of blank determinations (n > 20) and S/N (Signal/Noice) > 2 or 3; LOQ is numerically equal to 10 times standard deviation of the mean of blank determinations (n > 20) and S/N > 10 (ISO 11843-2).

Recovery

The recoveries and RSDs were determined from 6 replicates at four concentration levels spiking blank samples over three days.

The absolute recovery was calculated as:

Rec (analyte) = (area matrix-fortified standard) / (area matrix-matched standard)×100.

Acceptance criteria: 70-120%, % RSD of recovery concentration must be < 2;

Decision limit (CC_α) and detection capability (CC_β)

The CC_α and CC_β were calculated with the application of the following formulas:

$$CC\alpha = t_{(\alpha,IJ-2)} \frac{\sigma}{b} \sqrt{\frac{1}{K} + \frac{1}{IJ} + \frac{\bar{x}^2}{\sum_{i=1}^{I}(x_i - \bar{x})^2}}$$

$$CC\beta = \delta \frac{\sigma}{b} \sqrt{\frac{1}{K} + \frac{1}{IJ} + \frac{\bar{x}^2}{\sum_{i=1}^{I}(x_i - \bar{x})^2}}$$

where: σ is an estimation of the residual standard deviation of the regression function;

b is an estimation of the slope of the calibration curve obtained through regression calculation;

$\overline{X}$ is the mean of the x_{ij} values;

$t_{(\alpha,I\,J-2)}$ – is the Student's t at risk of α and for degrees of freedom (dof) equal to IJ-2 with I the number of calibrating levels of concentration and J the number of replicates per level of concentration;

K is the number of replicates for the real state;

$\delta_{(I\,J-2;\alpha;\beta)}$ is a statistical function that can be fairly approximated by using $2t_{(\alpha,I\,J-2)}$ (ISO 11843-2; Verdon et al., 2007).

Acceptance criteria: For prohibited substances (Nitrofuran metabolites), for which an Reference points for action is established under Regulation (Commission regulation (EU) 2023/411) CCα shall be lower than or equal to the reference point for action (MRL of nitrofurans -0.5 µg/kg).

Linearity

Calibration curves were constructed on extracts from AOZ, AMOZ, AHD, SEM, NP-AOZ, NP-AMOZ, NP-AHD and NP-SEM spiked matrices with the following analyte concentrations: 0.4, 0.45, 0.5, 0.55 and 0.6 µg/kg for each metabolites.

Acceptance criteria: Range – concentration where data can be reliable detected (MRL – 0.5 µg/kg) 80 – 120%; Linearity -Correlation coefficient – NLT 0.999.

Trueness and accuracy

Trueness and accuracy were validated in the studied matrices using blank samples fortified at three concentration levels: 0.25, 0.5, and 0.75 µg/kg; which correspond to 0.5, 1, and 1.5 times the RPA (0.5 µg/kg) value, respectively. At each concentration level 20 replicate analyses were performed.

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

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

where $C_{analyte}$ is the found analyte concentration using standard calibration with matrix-fortified standards;

C_{spiked} is the known spiked concentration.

The obtained accuracy values (%) were expressed as the average value±standard deviation ($n \geq 20$).

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

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

Acceptance criteria: −50% to +20% for levels ≤1 µg/kg, and -30% to +20% for levels >1 µg/kg to 10 µg/kg.

Repeatability

A set of identical blank matrix samples were spiked with the analyte to obtain concentrations equivalent to 0.5, 1.0 and 1.5 times the RPA i.e. for furan metabolites (RPA is 0.5 μg/kg) 0.25, 0.5, and 0.75 μg/kg. At each level, the analysis was performed at least six times. The concentration found in each sample, the mean concentration, the standard deviation and the coefficient of variation (%) of the spiked samples were calculated. These steps were repeated twice. Overall, mean concentrations, standard deviations (by averaging the squares of the standard deviation of individual cases and taking the square root of it) and the coefficients of variation for the spiked samples were calculated. Acceptance criteria: $CV_R \leq 30\%$ for concentration levels <10 μg/kg

Reproducibility

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 operator applying the following equations:

$$S_R^2 = S_L^2 + S_r^2,$$

where S_R^2 is the variance of reproducibility;

S_L^2 is the variance of the different analytical series;

S_r^2 is the variance of repeatability.

From these values, reproducibility (CV_R) was calculated:

$$CV_R\,(\%) = S_R/C_M \times 100,$$

where S_R is the standard deviation for reproducibility;

C_M is the average found concentration considering all analytical series.

Acceptans critetia: CVr values should be $\leq 2/3\ CV_R$ (i.e., $\leq 20\%$).

Stability

The stability of the stored analyte in solution and analyte(s) in matrix was tested under four different conditions: storage in light for 8 hours per day at 25°C; in the dark at 25°C; in the dark at 4°C; and in the dark at -20°C (Tsai C, 2010). The storage time was extended to 30 days until degradation phenomena became visible during quantification. The maximum storage time and optimal storage conditions were recorded.

The calculation of the concentration of the analyte(s) in each aliquot was carried out by using the solution of the analyte freshly prepared at the time of analysis as 100%.

$$\text{Analyte Remaining}\,(\%) = Ci \times 100/C_{fresh},$$

where Ci is a concentration at time point;

C_{fresh} is a concentration of fresh solution.

Acceptance criteria: The mean value of five replicate solutions, which were stored, shall not differ by more than 15% from the mean value of five freshly prepared replicate solutions. The mean value of the five freshly prepared solutions shall be used as the basis for calculating the percentage difference.

Matrix effects, %

The calculation of the matrix effect, %, was carried out at 20 different blanks lots (matrix/species).

The blank matrix was fortified after extraction with the analyte and was analysed together with a pure solution of the analyte.

The matrix effect, % was calculated as:

Matrix effect, % = (peak area of solution standard – peak area of extract of fortified matrix) / peak area of solution standard x 100.

Acceptance criteria: RSD < 20%.

Statistical analysis

The software package SPSS Statistics 2015 was used for the statistical analysis.

Results and discussion

Selection of sorbent and conditions of solid-phase purification of nitrofuran metabolite extract from meat

Based on the physicochemical properties of the analyzed substances, sorbents, literature, and experimental data, silica gel 60 with a particle size of 0.2–0.5 mm was selected for purifying nitrofuran metabolite extracts. Solid-phase extraction (SPE) columns for nitrofuran metabolite purification were prepared using this sorbent, with cartridge dimensions of 20 × 400 mm. Ethyl acetate was chosen as the washing solvent, and acetonitrile as the elution solvent.

Validation

Specificity/Selectivity. According to the results of the analysis for the investigated substances, the specific wavelength is 376, there were no significant peaks with an S/N ratio (signal/noise) of 3 and chromatographic interference during the retention times of the target metabolites of nitrofurans;

The coefficient of variation of the specificity of the obtained results during the working day is within 0.02–0.18, during the working week – within 0.02–0.29. That is satisfactory as required by Commission implementing regulation (EU) 2021/808/EC.

Limit of Detection (LOD) and (Limit of Quantitation). When using silica gel 60, the limit of detection for metabolites is – 0.08, 0.05, 0.01 and 0.03 μg/kg, for their NP- derivatives 0.10, 0.09, 0.11 and 0.10 μg /kg respectively; the limit of quantification for metabolites – is 0.10, 0.09, 0.11 and 0.10 μg /kg, for their NP- derivatives is – 0.13, 0.11, 0.14 and 0.12 μg /kg respectively.

When using a polymer sorbent: the limit of detection for metabolites is – 0.20, 0.17, 0.21 and 0.14 μg/kg, for their NP- derivatives 0.34, 0.18, 0.13 and 0.15 μg/kg respectively; the limit of quantification for metabolites – is 0.32, 0.23, 0.29 and 0.21 μg/kg, for their NP- derivatives is – 0.43, 0.29, 0.25 and 0.36 μg /kg respectively. The mean value of LOD and LOQ (Table 1 and 2).

Recovery. The recovery was observed within 96.27 – 98.79% and%RSD < 2 when used sorbent silica gel, 89.56 – 91.24% and%RSD < 2 when used polymer sorbent.

Despite the fact that the data both when using silica gel 60 and when using a polymer sorbent meet the acceptability criteria of Commission implementing regulation (EU) 2021/808, the advantage of using silica gel 60 is obvious (Table 1 and 2).

Table 1

Mean value of recovery, LOD, LOQ, CCα and CCβ of nitrofuran metabolites using silica gel 60

Analytes	Calibration range (μg/kg)	Mean value of recovery (%)*	LOD (μg/kg)	LOQ (μg/kg)	CCα (μg/kg)	CCβ (μg/kg)
AOZ	0.1–1.0	96.55±0.97	0.08	0.12	0.04	0.10
AMOZ	0.1–1.0	97.49±1.04	0.05	0.11	0.01	0.09
AHD	0.1–1.0	97.28±1.36	0.01	0.12	0.05	0.07
SEM	0.1–1.0	98.79±1.29	0.03	0.10	0.03	0.09
NP-AOZ	0.1–1.0	96.27±0.37	0.10	0.13	0.06	0.12
NP-AOZ	0.1–1.0	98.81±0.41	0.09	0.11	0.04	0.08
NP-AHD	0.1–1.0	97.54±1.38	0.11	0.14	0.07	0.14
NP-SEM	0.1–1.0	97.20±1.23	0.10	0.12	0.05	0.08

*μg/kg

Table 2

Mean value of recovery, LOD, LOQ, CCα and CCβ of nitrofuran metabolites using polymer sorbent

Analytes	Calibration range (μg/kg)	Mean value of recovery (%)*	LOD (μg/kg)	LOQ (μg/kg)	CCα (μg/kg)	CCβ (μg/kg)
AOZ	0.1–1.0	90.30±0.63	0.20	0.32	0.28	0.54
AMOZ	0.1–1.0	90.61±3.65	0.17	0.23	0.17	0.36
AHD	0.1–1.0	90.63±2.05	0.21	0.29	0.23	0.43
SEM	0.1–1.0	90.36±2.65	0.14	0.21	0.19	0.27
NP-AOZ	0.1–1.0	90.96±2.17	0.34	0.43	0.26	0.39
NP-AOZ	0.1–1.0	91.24±2.24	0.18	0.29	0.16	0.30
NP-AHD	0.1–1.0	89.56±2.44	0.13	0.25	0.22	0.47
NP-SEM	0.1–1.0	90.24±2.85	0.15	0.36	0.13	0.31

*Standard 1 μg/kg

Decision limit (CC_α) and detection capability (CC_β). In all cases, CCα is below the MRL (0.5 μg/kg). Despite the fact that the data both when using silica gel 60 and when using a polymer sorbent meet the acceptability criteria of Commission implementing regulation (EU) 2021/808, the advantage of using silica gel 60 is obvious (Table 1 and 2).

Linearity. The plots are linear over the reported range,% RSD of peak area of 6 standard samples – 0.98%,% RSD of peak area of 6 standard samples relative to bracketing peak area – 1.08% and acceptable as the correlation coefficient r^2 is above 0.999.

Trueness and Accuracy

The data for determining the Trueness values ranged and Accuracy values ranged when using silica gel 60 and polymer sorbent are presented in Table 3 and 4. As can be seen in Table 3 and 4, the obtained accuracy values: when using silica gel 60, ranged from 95.99 to 98.64% for 0.4 μg/kg; from 95.81 to 99.09% for 0.5 μg/kg and from 95.49 to 100.08% for 0.6 μg /kg. Trueness values ranged from −2 to -5% for 0.4 μg/kg; from −1 to -4 for 0.5 μg/kg and from 0 to -5 for 0.6 μg/kg, meeting in all cases the acceptability criteria of Commission implementing regulation (EU) 2021/808.

Table 3

Mean value of Accuracy and Trueness of nitrofuran metabolites using silica gel 60

Analytes	Accuracy (%)*			Trueness (bias, %)**		
	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg
AOZ	97.24±0.35	96.92±4.44	95.49±2.18	-3	-4	-5
AMOZ	96.62±0.16	97.24±2.31	98.61±6.52	-4	-3	-2
AHD	98.37±0.49	95.81±5.56	97.66±5.14	-2	-5	-3
SEM	97.53±0.52	98.75±6.12	100.08±3.39	-3	-2	0
NP-AOZ	96.01±1.08	96.68±8.29	96.12±4.52	-4	-4	-4
NP-AOZ	98.64±0.67	98.52±1.45	99.28±7.74	-2	-2	-1
NP-AHD	96.82±1.23	99.09±3.57	96.71±9.17	-4	-1	-4
NP-SEM	95.99±0.24	97.22±2.63	98.39±8.81	-5	-3	-2

*Accuracy values (%) obtained in the analysis of fortified samples, expressed as the average value±standard deviation (n ≥ 20);
**Trueness values (%) expressed as bias (100 – Accuracy%).

Table 4

Mean value of Accuracy and Trueness of nitrofuran metabolites using polymer sorbent

Analytes	Accuracy (%)*			Trueness (bias, %)**		
	0.4 µg /kg	0.5 µg /kg	0.6 µg /kg	0.4 µg /kg	0.5 µg /kg	0.6 µg /kg
AOZ	90.62±1.28	89.65±2.41	90.64±1.90	-10	-11	-10
AMOZ	93.44±0.99	91.42±3.85	86.98±1.49	-7	-9	-14
AHD	92.37±1.39	90.86±4.16	88.67±2.72	-8	-10	-10
SEM	90.09±1.42	88.11±2.92	92.88±3.27	-10	-12	-12
NP-AOZ	89.74±0.89	93.23±4.53	89.90±4.14	-11	-7	-7
NP-AOZ	91.25±0.97	89.19±3.17	93.27±3.56	-9	-11	-11
NP-AHD	90.88±1.11	87.04±1.08	90.76±2.68	-10	-13	-13
NP-SEM	87.63±1.48	90.33±1.11	92.77±3.02	-13	-10	-10

*Accuracy values (%) obtained in the analysis of fortified samples, expressed as the average value±standard deviation (n ≥ 20);
**Trueness values (%) expressed as bias (100 – Accuracy%).

When using polymer sorbent, ranged from 87.63 to 93.44% for 0.4 µg /kg; from 87.04 to 91.42% for 0.5 µg/kg and from 86.98 to 92.88% for 0.6 µg/kg. Trueness values ranged from −7 to -13% for 0.4 µg/kg; from −7 to -11 for 0.5 µg/kg and from -7 to -14 for 0.6 µg/kg, meeting in all cases the acceptability criteria of Commission implementing regulation (EU) 2021/808.

Despite the fact that the data both when using silica gel 60 and when using a polymer sorbent meet the acceptability criteria of Commission implementing regulation (EU) 2021/808, the advantage of using silica gel 60 is obvious.

Repeatability and reproducibility

The data for determining the repeatability values ranged and reproducibility values ranged when using silica gel 60 and polymer sorbent are presented in Table 5 and 6.

As shown in the tables, the obtained accuracy values met the acceptability criteria established by Commission Implementing Regulation (EU) 2021/808 in all cases. When using silica gel 60, repeatability ranged from 1.14% to 8.57%, and reproducibility ranged from 1.98% to 8.99%. In comparison, the use of a polymer sorbent resulted in repeatability values ranging from 7.77% to 19.25%, and reproducibility values from 12.55% to 22.11%. Despite the fact that the data both when using silica gel 60 and when using a polymer sorbent meet the acceptability criteria of Commission implementing regulation (EU) 2021/808, the advantage of using silica gel 60 is obvious.

Table 5

Mean value of Accuracy and Trueness of nitrofuran metabolites using silica gel 60

Analytes	Repeatability (CV_r, %)*			Reproducibility (CV_R, %)**		
	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg
AOZ	8.57	2.92	2.16	8.99	3.27	4.29
AMOZ	6.33	7.26	4.65	7.22	7.64	5.23
AHD	5.08	4.83	1.98	6.18	4.93	3.61
SEM	7.64	3.75	5.78	8.27	4.29	6.43
NP-AOZ	2.67	2.94	3.48	3.13	3.65	5.55
NP-AOZ	3.43	1.45	6.23	4.21	1.98	8.63
NP-AHD	1.14	5.86	5.88	2.38	6.81	7.28
NP-SEM	1.19	7.35	2.99	3.45	7.97	5.33

*Repeatability values (CV_r,%) obtained in the replicate analysis of fortified samples ($n \geq 20$).
**Reproducibility values (CV_R,%) obtained in the replicate analysis of fortified samples in different analytical series (4 series, n = 5).

Table 6

Mean value of Accuracy and Trueness of nitrofuran metabolites using polymer sorbent

Analytes	Repeatability (CV_r, %)*			Reproducibility (CV_R, %)**		
	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg	0.4 µg/kg	0.5 µg/kg	0.6 µg/kg
AOZ	12.26	17.11	14.56	15.54	21.42	16.31
AMOZ	11.58	9.24	12.87	13.22	16.27	15.72
AHD	15.57	14.45	9.93	18.43	18.32	10.57
SEM	18.71	12.28	11.69	21.61	15.19	13.59
NP-AOZ	9.26	9.99	7.77	15.18	13.76	15.43
NP-AOZ	14.73	13.17	10.44	17.24	16.25	12.55
NP-AHD	16.54	11.32	16.08	19.76	14.39	19.23
NP-SEM	17.08	16.67	19.25	22.11	19.42	20.45

*Repeatability values (CV_r, %) obtained in the replicate analysis of fortified samples ($n \geq 20$).
**Reproducibility values (CV_R, %) obtained in the replicate analysis of fortified samples in different analytical series (4 series, n = 5).

Stability

The mean value of five stored duplicate solutions was within 2.66 – 7.89%, i.e. did not differ by more than 15% from the mean value of five freshly prepared duplicate solutions. The mean value of five freshly prepared solutions was used as the basis for calculating the percentage difference.

Matrix effects, %

The calculated ion inhibition of the matrix effect was in the range of 5.98 – 8.47%, which is acceptable for the validated method (N ≤ 20%).

For the solid-phase extraction of nitrofurans, ready-made columns with polymer-based sorbents – typically polypropylene cartridges containing a polystyrene-divinylbenzene copolymer – are most commonly used (Barbosa et al., 2007; Conneely et al., 2002; Kaufman et al., 2015; Leitner et al., 2001; Śniegocki et al., 2018; Tripathi et al., 2023). According to these authors, such sorbents enable strong and selective retention of nitroaromatic derivatives through π–π interactions.

Oasis MAX cartridges contain mixed- mode polymeric sorbent with reversed phase and anion exchange function. During sample loading, NBA may be retained by the ion exchange groups, while the NP derivative of nitrofurans may be retained by the reversed-phase sorbent. Ammonia (2%) can be used to remove the matrix co-extractive from the cartridge while enhancing the bonding of the NBA to the sorbent by the ion exchange mechanism. NP gets held by hydrophobic interaction with reversed phase functionality of the sorbent, which can be eluted with methanol.

In case of dual MAX/HLB SPE, while SPE on Oasis™ MAX cartridges gave good recovery of NP derivative a further clean-up step was needed to remove excess NBA. Although the MAX cartridge had the capacity to remove up to 93% NBA a significant amount of NBA still remained, as typically, NBA is added in1000-fold excess to that of NPAOZ to ensure complete derivatization of any AOZ present (Tripathi, 2023).

When choosing the right SPE sorbent, there are four main considerations to consider the type of sorbent, the sorption mechanism appropriate for the sample and analyte to be tested, the type of matrix and the method of extraction (GL Science, 2025).

As of today, the most widely used nitrofurans in the treatment of cattle are furaltadone, furazolidone, nitrofurazone and nitrofurantoin, all of which are broad-spectrum antibiotics containing a 5-nitrofuran ring structure and contain an azomethine bond ~C=N~. The presence of this bond and the nitro group causes the similarity of their physicochemical properties (Betsy, 2006).

The parent nitrofurans are rapidly metabolized to protein-bound metabolites, making the use of the parent drugs as marker residues ineffective; however, tissue-bound metabolites are stable in the body for several weeks after treatment and thus represent a better choice as marker residues (Gaastra et al., 2024; Ramos et al., 2017; Rixt et al., 2018). Based on this, the analytical strategy for the quantitative assessment of nitrofurans is based on the determination of 4 stable and persistent metabolites that can be released from proteins (Alkan et al., 2016). These stable metabolites are 3-amino-2-oxazolidone AOZ, 3-aminomorpholinomethyl-2-oxazolidinone AMOZ, 1-aminohydantoin AHD and semicarbazide SEM (Craken et al., 2001) (Figure 1).

Nitrofuran metabolites in a living organism meet in protein-bound forms and are released in an acidic environment, forming free compounds with a low molecular weight, are well soluble in polar solvents, and can be easily ionized, which complicates their analysis (Kaufmann et al., 2015; Xiaoing et al., 2022).

The relative molecular weights of AOP, AMOZ, AGD and CEM are 102.1, 201.2, 115.1 and 75.1, respectively, with no characteristic ion spread, making qualitative and quantitative analysis difficult. Derivatization increases the side chain molecular weight and, accordingly, the relative molecular weights of the nitrofuran metabolites to 248.2, 334.3, 235.2 and 208.2, respectively. Due to the covalent bonding of the metabolites to the tissue, solvent extraction of the sample will not recover the residues. Instead, an acid hydrolysis step is required to cleave the covalently bound marker residues from the tissue prior to analysis (Craken et al., 2001).

The metabolites are simultaneously treated with acid hydrolysis and derivatization reagents to increase the relative molecular weights of the target compounds, which, under appropriate conditions, allows for quantitative determination (Leitner et al., 2001). Derivatization of nitrofuran metabolites is performed using 2-nitrobenzaldehyde to yield the NP- derivatives shown in Figure 1, after which the sample is incubated at 37◦C for 16 hours. The process using furazolidone as an example is shown in Figure 2 (Verdon et al., 2007).

Nitrofuran metabolites are Hydrogen Bond Acceptor Count and generally polar. This is because they contain functional groups such as amines, hydroxyls, and other polar groups that make them soluble in polar solvents. For example, main metabolites, SEM, AOZ and AMOZ are highly polar (NCBI, 2025).

Nitrofuran metabolites have a different structure than the parent compound, and these structural change significantly are changing affect polarity. The polarity of nitrofuran metabolites is a crucial factor in their analysis. Liquid chromatography (LC), in particular reversed-phase LC, is often used for their separation and detection. However, precisely because of the high polarity of these metabolites, derivatization with 2-nitrobenzaldehyde (2-NBA) is used to improve their retention and separation in reversed-phase columns (Arystanova, 2022; Vass et al., 2008).

Nitrofuran metabolites are generally stable in nature, meaning that they can persist in the environment and food products. Their polarity also affects how they interact with various matrices and how they are extracted and analysed (Moragues et al., 2024). The main goal in selecting the sorbent was to minimize the loss of nitrofuran metabolites directly related to the sample preparation procedure. To effectively isolate nitrofuran metabolites using solid-phase extraction (SPE), an appropriate sorbent must be selected based on the chemical properties of the analytes and the characteristics of the sample matrix.

Generally, sorbents like C18 or polymer material are used for non-polar solutions, while silica gel, diol, aminopropyl, or cyanopropyl are preferred for polar solutions. The specific sorbent should be tailored to the target analyte, considering its solvent solubility and the sample matrix's nature.

Nitrofuran metabolites have different solubility in different solvents and therefore it is necessary to select a sorbent that will ensure effective binding of analytes while minimizing the retention of interfering compounds (NCBI, 2025). Since nitrofuran metabolites are polar, a sorbent with polar characteristics (e.g. silica gel, diol) is recommended. Silica gel is a polar sorbent because its surface is covered with polar silanol (Si-OH) groups. These silanols act as hydrogen bond donors and acceptors, allowing silica gel to interact strongly with polar compounds. If the solvent polarity is weak, the adsorbent will exhibit a strong adsorption capacity for the solute (Shimadzu, 2025).

The polar nature of the Si-O bonds in the bulk structure of silica gel makes the entire material polar. Because of this polarity, silica gel can interact with other polar substances. While the bulk structure of silica gel is polar, what makes silica gel exceptionally versatile is its surface properties. Silica gel has a vast surface area due to its porous nature, and this surface can be modified in various ways to tailor its polarity for specific applications (Shimadzu, 2025).

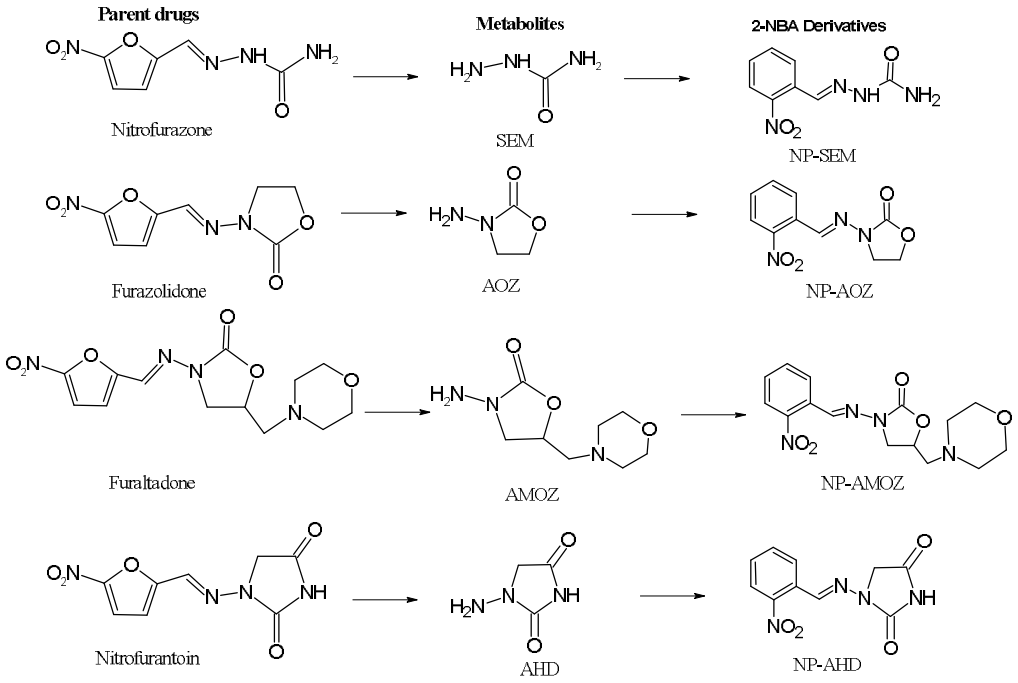

Figure 1. 2-NBA derivative products

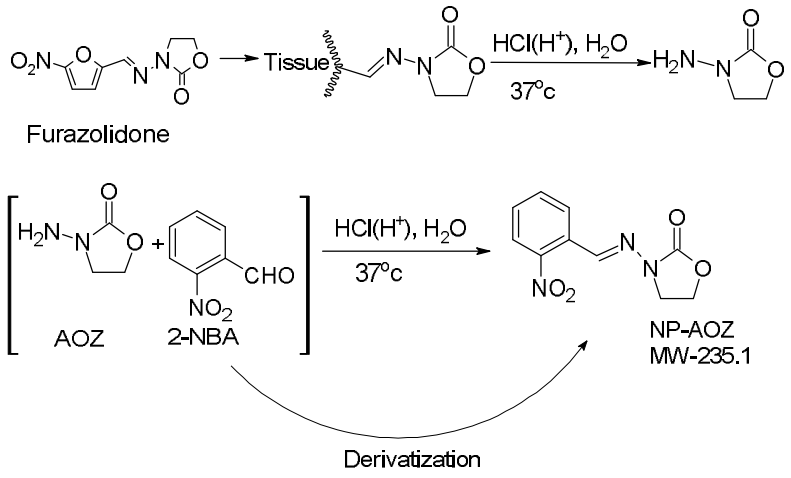

Figure 2. Derivatization of Furazolidone

In desiccation, silica gel's polar nature, combined with its high surface area, makes it highly effective at selectively adsorbing polar substances. The polar nature of silica gel allows it to interact strongly with polar compounds, causing them to be retained on the column's

surface. Meanwhile, non-polar substances are less attracted to the silica gel and tend to pass through the column more quickly. This differential interaction helps separate complex mixtures into their individual components, a fundamental process in many analytical and preparative chemistry procedures.

Silica gel 60 is a widely used silicon dioxide (SiO_2) based sorbent with high adsorption capacity and stability. It is characterized by a small pore size (60 angstroms), which allows it to adsorb molecules of various sizes. Silica gel 60 also has a high specific surface area (about 500 m^2 g^{-1}) and pore volume (0.75 mL g^{-1}), which makes it effective for various applications. The characteristics of the adsorbent ensure high resolution and flow coefficient, maximum reproducibility of analytical results, as well as scalability of the method without the need for adjustments.

Based on the above, the choice of Silica gel 60 as a sorbent, rather than polymers, is justified (Besbes et al., 2009). The size of the molecules of the substance purified by the SFE method should be smaller than the pore size of the sorbent. The mass of the target component retained by the sorbent from the solution subjected to SFE, including retained impurities, is close in chemical nature to this component (approximately 5% of the sorbent mass). Thus, a 100 mg cartridge can retain approximately 5 mg of dissolved substances, which made it possible to calculate the required sorbent mass. The free volume of the sorbent is the sum of the volume of space between the particles and the pore volume of the sorbent. The free volume of the sorbent determines how much solvent is needed for SPE. To create optimal conditions for dilution, purification and elution, it is required that the volume of solvent exceeds the free volume of the sorbent by 4-8 times. Otherwise, the risk of incomplete solvation and low analyte recovery increases. Silica-based SPE products usually have a free volume to sorbent mass ratio of approximately 150 µL per 100 mg. Polymer sorbents require a larger volume of solvent. It is recommended to use 250 µL per 100 mg of sorbent.

Silica gel is a polar sorbent that can be used to extract nitrofuran metabolites from polar solutions. Selecting appropriate wash and eluent solvents for solid phase extraction (SPE) of nitrofuran metabolites requires consideration of both the analyte and sample matrix properties. Wash solvents are used to remove interfering compounds while eluents selectively extract the target nitrofuran metabolites.

Polar solvents have high permittivity, which allows for efficient separation of ionic charges and dissolution of polar molecules. Polar solvent molecules have partial charges, which makes them capable of interacting with polar solutes (Thermofisher, 2025).

The purpose of wash solvents is to remove co-extracted compounds that are not of interest while preserving the target analytes on the SPE cartridge. Connelly et al. (2002) used 2% acetic acid in 50% methanol, which served to disrupt the interaction of acidic analytes such as NBA, and used 2% ammonia in the wash solution for the dual effect of firstly cleaning the sample and secondly improving the binding of NBA to the sorbent for more selective elution of NPAOZ. Hexane along with water removes excess water trapped in the polymer phase of the cartridge before eluting the compounds (Khong et al., 2004).

Wash solvents should be less polar than the eluent to remove interferences without eluting the target analytes. Less polar solvents are usually non-polar. They include alkanes (such as pentane, hexane, and heptane) and aromatic compounds (such as benzene, toluene, and xylene). Other such solvents include diethyl ether, ethyl acetate, methylene chloride, chloroform, and others. Among these solvents, ethyl acetate is a moderately polar substance. The polar carbonyl group and polar oxygen atom create a dipole moment in the molecule, making it polar. At the same time, the ethyl group, which is non-polar, partially compensates for the polarity of other groups, so ethyl acetate is a moderately polar compound (Thermofisher, 2025)

Weak organic solvents, such as lower concentrations of methanol or acetonitrile in water, may also selectively remove certain types of interferences. The volume of wash solvents should be sufficient to remove the interferences, typically 2–5 times the void volume of the SPE cartridge.

The purpose of the eluent is to selectively and efficiently elute nitrofuran metabolites from the SPE cartridge into the final extract. To effectively wash nitrofuran metabolites from silica gel in solid-phase extraction (SPE), a solvent with a good balance of polarity and ability to dissolve the target compounds is required. Solvents with moderate to high polarity, such as methanol or acetonitrile, are usually more effective in dissolving them.

The solvent chosen should be compatible with subsequent analytical methods (e.g., LC) to ensure accurate and reliable quantification. It is necessary to elute with a smaller volume of solvent to obtain a more concentrated extract (Thermofisher, 2025).

To sum it up, we can say that, methanol can be a versatile solvent for washing polar substances, especially when used in combination with other polar solvents or water, acetonitrile is another good choice for washing polar substances due to its ability to dissolve polar compounds and its relatively low boiling point.

The polarity of the sample matrix can influence the choice of solvents. If the sample contains particles, filtration or centrifugation may be required prior to SPE to avoid clogging of the cartridge. The meat matrix exhibits both polar and non-polar characteristics due to the presence of both hydrophilic and hydrophobic components. This is because meat proteins, like other proteins, contain amino acid side chains that can be polar or non-polar. In addition, the fats and lipids in meat also contribute to the non-polar aspects of the matrix (García et al., 2023).

Meat contains water, which is a polar solvent. In addition, some of the amino acid side chains in meat proteins are polar, which contributes to the overall polarity of the matrix. Meat also contains fats and lipids, which are non-polar and hydrophobic. These contribute to the overall non-polar nature of the meat matrix. Meat proteins, like many other proteins, are amphipathic, meaning they have both polar and non-polar regions. This allows them to interact with both water and fat. Meat is therefore not simply polar or non-polar, but rather a complex matrix with hydrophilic and hydrophobic properties (Decker et al., 2022).

By carefully considering these factors, suitable solvents were selected for efficient and accurate washing and elution of nitrofuran metabolites from the solid-phase column. In particular, acetonitrile was used for washing and methanol was used for elution.

In conclusion, it should be repeated that the selected sorbent and solid-phase purification mode are optimal, which has been confirmed experimentally, in particular, 89–96% extraction of nitrofuran metabolites with good reproducibility (RSD $\leq$ 2.0%) was achieved.

The SPE purification column we propose is specifically designed for nitrofuran metabolites, offering high selectivity and specificity. It enhances the quantitative detection limit of the HPLC method, enabling the use of a diode array detector instead of more expensive cartridges and mass spectrometers. This approach achieves the determination of nitrofuran metabolite levels below the established MRL of 0.5 μg/kg, while maintaining relatively low financial costs.

The proposed conditions for determining nitrofuran metabolites are relatively accessible, enabling small farmers and producers in developing countries and regions to implement the method, thereby ensuring the safe use of meat and meat products.

Conclusions

The following conclusions can be drawn from the conducted studies:

(1) a sorbent – silica gel 60, particle size 0.2 – 0.5 mm, – was selected for purification of the extract of nitrofuran metabolites and confirming the advantages of using this column using validation;

(2) columns for solid-phase purification of nitrofuran metabolites were prepared from the selected sorbent, cartridge size – (20 x 400 mm);

(3) selected solvents for washing nitrofuran metabolites from the solid-phase column – ethyl acetate and for elution – acetonitrile;

(4) the advantage of the columns manufactured using silica gel compared to the polymer sorbent – polypropylene was experimentally proven. 96-98% extraction of nitrofuran metabolites was achieved with good reproducibility (RSD ≤ 2.0%), since when using a polymer sorbent, the extraction does not exceed 94%;

(5) the advantage of the proposed column over disposable, expensive, ready-made columns for purification and concentration of four nitrofuran metabolites isolated from meat is its significantly lower cost combined with high specificity and selectivity. This enhances the sensitivity of the HPLC method, enabling the use of a diode array detector instead of a costly mass spectrometer to detect nitrofuran metabolites below the EU's Minimum Required Performance Limit of 0.5 μg/kg in the complex meat matrix. As a result, the method becomes financially acceptable for small farmers, developing countries and regions, which in turn will ensure the safe use of meat and meat products in these regions.

Evaluation of results

The method has been validated in accordance with the requirements of European Commission Decision 2021/808/EC. The effectiveness of the proposed column and the use of a diode detector for HPLC determination of nitrofuran metabolites was confirmed by the results of professional testing by the Globaltest testing laboratory accredited according to ISO 17025 by the Accreditation Agency of Georgia.

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