

DETERMINATION OF NITROFURAN METABOLITE RESIDUES IN EGGS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed for the determination of metabolites of furazolidone, furaltadone, nitrofurantoin, and nitrofurazone in eggs. The procedure requires an acid-catalysed release of protein-bound metabolites, followed by their *in situ* conversion into the 2-nitrobenzaldehyde (NBA) derivatives. The sample cleanup was performed on a solid phase extraction (SPE) cartridge. LC-MS/MS analysis was performed by positive electrospray ionisation (ESI) applying multiple reaction monitoring (MRM) of two transition reactions for each compound. The validation of the method was conducted following the European Union criteria for the analysis of veterinary drug residues in foods. The decision limits (CC α) were 0.16-0.24 $\mu\text{g}/\text{kg}$, and the detection capabilities (CC β) 0.22-0.36 $\mu\text{g}/\text{kg}$.

Key words: eggs, nitrofuran, metabolites, liquid chromatography, mass spectrometry.

The use of the four main nitrofurans (furazolidone, furaltadone, nitrofurazone, and nitrofurantoin) has been banned within the European Union (Annex IV of Regulation 2377/90/EEC) due to their toxicological influence on the health of consumers of food from animal origin (5). Previously, these antibacterial agents were added to feeds to stimulate growth or/and to prevent and treat several bacterial and protozoan infections, such as fowl cholera, coccidiosis, and blackhead.

After administration to poultry, the nitrofurans are quickly metabolised to more stable products, as follows: nitrofurantoin to 1-aminohydantoin (AHD); furazolidone to 3-amino-2-oxazolidone (AOZ); furaltadone to 5-methylamorpholino-3-amino-2-oxazolidone (AMOZ); and nitrofurazone to semicarbazide (SEM). The metabolised products are bound to protein and stay in animal body or animal products (*e.g.* eggs) for a few days (elimination half-life time - between 4 and 9 d) (2, 10-15).

Because the protein bound nitrofuran metabolites may persist in food of animal origin for a considered time, they can be used as the markers of residues for the detection of illegal use of nitrofuran in food producing animals. Several analytical procedures have been described for the detection of nitrofuran metabolites as target analytes in biological samples. All of them utilise hydrolysis under acidic conditions, derivatisation with 2-nitrobenzaldehyde (NBA), and extraction with ethyl acetate. Following extraction and/or further cleanup, the residue content is analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (8-14, 16). The EU Commission Decision (2003/181/EC) has set up a minimum required performance limit (MRPL) of 1 $\mu\text{g}/\text{kg}$ for nitrofuran metabolites in poultry meat and aquaculture products. However, up until today, an MRPL for nitrofuran metabolites in eggs has not been laid down.

In this paper, the procedure described for the determination of SEM (6) was modified for the simultaneous determination of four target analytes (AOZ, AMOZ, AHD, and SEM) in whole eggs. The nitrofuran metabolites were analysed by LC-MS/MS after electron spray ionisation (ESI) in the positive mode using multiple reaction monitoring (MRM). The procedure was validated in accordance with Commission Decision 2002/657/EC employing isotope dilution (4).

Material and Methods

Reagents and chemicals. Ammonium formate, acetic acid (99.5%), hexane (95%), ethyl acetate, and methanol (HPLC grade) were from J.T. Baker (The Netherlands). Ultrapure water was filtered through a Milli-Q system Millipore (USA). Anhydrous dipotassium hydrogen orthophosphate, 35% hydrochloric acid, and sodium hydroxide were supplied from POCH (Poland). 2-nitrobenzaldehyde (2-NBA) was from Sigma Aldrich Chemical Company (Germany).

Standard solutions. The metabolites AOZ, AMOZ, AHD, and SEM, and the internal standards AOZ-d4, AMOZ-d5, and SEM (C13, N15, N15) 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.

Extraction. An amount of 2.0 ± 0.05 g of fresh egg portion of each samples were transferred to centrifuge tubes. The samples were submitted to hydrolysis and derivatisation processes, by adding internal standard mixture (AMOZ-d5, AOZ-d4, and 1,2-[15N2, 13C] SEM), 10 ml of the 0.2 mol/L hydrochloric acid and 240 μl of 2-NBA (10 mg/mL in methanol). The samples were capped the centrifuge tubes securely and vortexed for 30 s, then were placed in water bath at $40 \pm 3^{\circ}\text{C}$ and incubated overnight. After the incubation, the samples were removed from the water bath and they were allowed to cool to room temperature. The pH-value of about 7.0, and was adjusted by the addition of 10 mL of 0.2 mol/L di-potassium hydrogen orthophosphate solution and 800 μL of 2 mol/L sodium hydroxide solution. The samples were centrifuged at 4500 g for 15 min and transferred to the SDB-L cartridges.

Cleanup. SPE cartridges were placed on vacuum manifold and the cartridges were conditioned (3 ml of ethyl, 3 ml of methanol, and 5 ml of water, sequentially), a small portion (0.5 ml) of water should remain on the cartridges until the sample extracts were applied. The samples extracts were passed through the SDB-L cartridges, followed by washing with 5 ml of water and 5 ml of hexane, and elution with ethyl acetate. The eluates were vortexed for 10 s and then evaporated to dryness on a heating block at 40°C under a gentle stream of nitrogen. The residues were dissolved in 200 μl of 60% methanol solution in water and filtered through 0.45 μm filters, before injecting onto the LC column.

LC-MS/MS. The LC-MS/MS system consisted of an Agilent Series 1100 HPLC system (Agilent Technologies, Germany) connected to a PE Sciex API 3000 triple quadruple mass spectrometer (PE Sciex, Canada) in electrospray positive ionisation mode. The chromatography was performed in 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 composed by two solutions: A (ammonium formate – 5 mmol/L) and B (methanol) in a gradient that started with 80% of A and 20% of B; from 0 to 5 min the concentration of B was raised to 95% and remained for 9 min. Finally, after 9-10 min, the B concentration was decreased to 20%. The column was operated at 40°C and a flow rate of 0.2 ml/min. The ions were monitored by a multiple reaction monitoring (MRM, Table 1). The source block temperature was set to 450°C and the electrospray capillary voltage to 4.5 kV.

Validation scheme. The evaluation of the suitability of the whole procedure for the determination

of nitrofuran metabolite residues in the eggs was carried out in accordance with the Commission Decision 2002/657/EC (5). Quantification was performed using internal standards and was based on the peak area. Recoveries and precisions (within- and between-day) were calculated from the analysis of four blank eggs fortified with each derivatised nitrofuran metabolite at four fortification levels (0.5, 1.0, 1.5, and 2.0 $\mu\text{g}/\text{kg}$ - and performed by the same operator on three separate occasions. Within the laboratory, precision was obtained by following the same protocol but analyses were performed by two different operators. Repeatability at the 95% confidence level was counted from the within-day precision using an expansion factor of 2.77. Similarly, the intermediate reproducibility was calculated from the within-laboratory precision using the same expansion factor.

Results

Preliminarily, standard solutions were used to optimise cone voltages and collision energies for MS-MS transitions of each nitrofuran analyte. Finally, blank and spiked sample extracts were injected and their corresponding sensitivities and interferences in each transition channel were compared in order to choose the most suitable transitions for the analysis of nitrofurans in eggs. Nitrofuran metabolites were considered as positively identified in egg samples when: (a) the ratio of the retention time of the analyte to that of the corresponding IS corresponded to that of the calibration solution within a $\pm 2.5\%$ tolerance for liquid chromatography, and (b) the peak area ratios of the various transition reactions were within the tolerances set by the EU criteria (4). The results are shown in Table 1 as ratios between the MRM channels that were used for analyte confirmation.

Repeatability and reproducibility, studies and overall mean recovery rates, are reported in Table 2. The decision limits and detection capability, expressed as $\text{CC}\alpha$ and $\text{CC}\beta$, are summarised in Table 3.

Samples for the generation of calibration curves were prepared by adding aliquots of blank egg homogenate to the residue of evaporated standard solutions of AOZ, AMOZ, AHD, and SEM. Linearity was tested in the range of 0.5-100 ng/g. The limits of the detection were determined in experiments in the range of 0.5-2 ng/g in egg.

Typical chromatograms obtained for the extract of blank egg (Fig. 1), and the extract of egg spiked with AOZ, AMOZ, AHD, and SEM at the level of 1 $\mu\text{g}/\text{kg}$ are shown in Fig. 2.

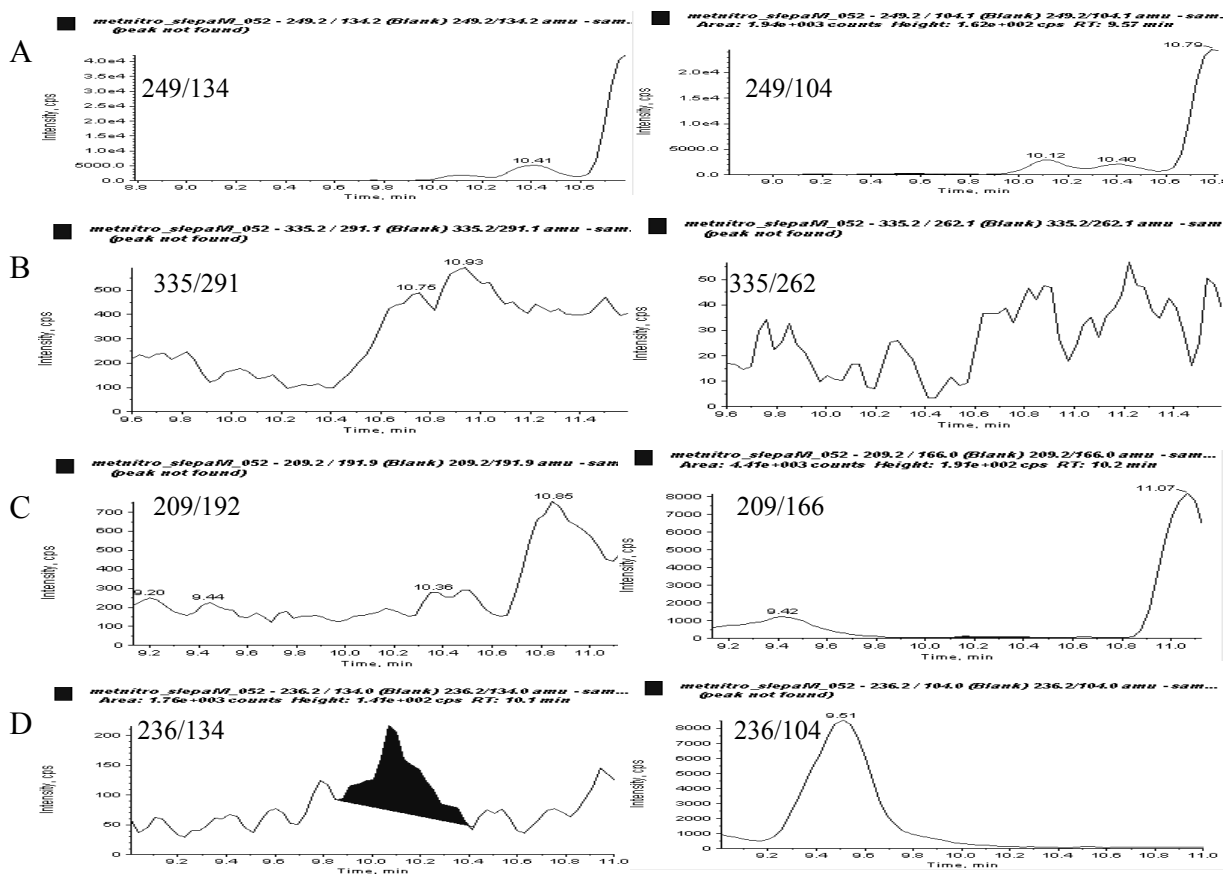


Fig. 1. LC-MS/MS chromatograms for eggs free from nitrofurantoin metabolites. A) AHD, B) AMAZ, C) SEM, D) AOZ.

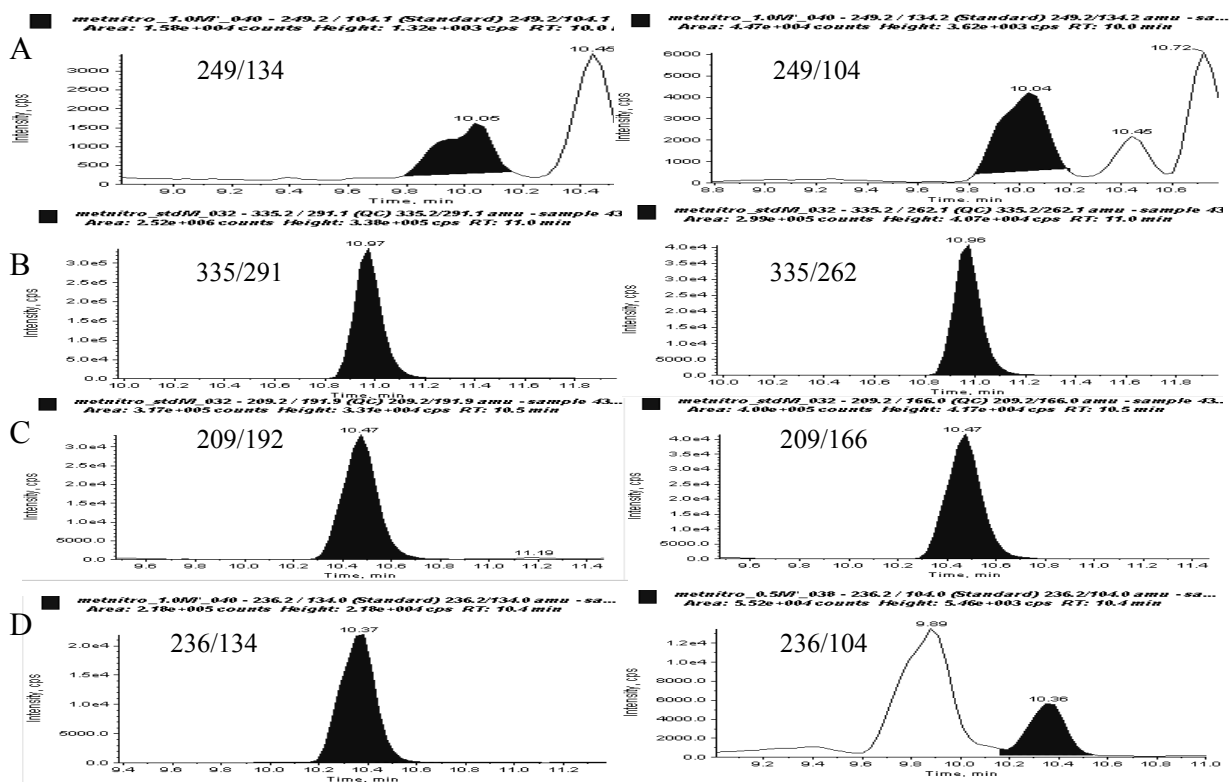


Fig. 2. LC-MS/MS chromatograms for eggs spiked with nitrofurantoin metabolites at 1.0 µg/kg. A) AHD, B) AMAZ, C) SEM, D) AOZ.

Table 1
Transition reactions monitored by LC-ESI-MS/MS

Analytes	Retention time (min)	Q1	Q3	Ion ratio (%)	Internal standard	Internal standard Q1/Q3
AOZ	10.8	335.2	291.1	9.3	AMOZ-d ₅	340.3/296.2
			262.1			340.3/25.2
AMOZ	10.2	209.2	191.9	60.4	SEM-C ₁₃ , 2 N ₁₅	212.0/195.0
			166.0			212.0/168.0
SEM	9.8	249.2	134.2	46.7	AOZ-d ₄	240.0/134.0
			104.1			240.0/104.0
AHD	10.4	236.2	134.0	36.0	AOZ-d ₄	240.0/134.0
			104.0			240.0/104.0

Table 2
Recovery, repeatability, and reproducibility - validation data for egg matrix

Analytes	Recovery (%)	Repeatability				Reproducibility			
		0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
AOZ	102.8	7.4	10.4	14.2	8.2	7.6	9.0	13.3	7.1
AMOZ	110.8	3.9	4.8	7.8	7.5	4.3	4.6	7.6	7.8
SEM	102.0	4.7	3.0	7.2	7.4	4.1	4.0	7.0	7.7
AHD	101.0	8.9	4.4	10.6	4.3	11.6	3.9	10.4	3.8

^afortification level in $\mu\text{g kg}^{-1}$

Table 3
CC α and CC β validation data for egg matrix

Analytes	CC α , $\mu\text{g/kg}$	CC β , $\mu\text{g/kg}$
AOZ	0.16	0.22
AMOZ	0.25	0.36
SEM	0.17	0.24
AHD	0.24	0.33

Discussion

Only single analytical procedures have been evaluated for the estimation of the contents of nitrofurans metabolites in eggs. Two of them (1, 10) were signed for the determination of the all four target metabolites as analytes and they utilise liquid-liquid partitioning for cleanup of the obtained extracts. The third is developed for the determination of SEM after application of solid phase extraction (SPE) with polymeric sorbent (6). The use of SPE technique was previously described by Leitner *et al.*, (9), and they indicated that sample preparation protocol including cleanup with polymeric sorbent is simple and robust.

In this study, a polystyrene-divinylbenzene copolymer (SDB-L) was used as sorbent material, because it enables a strong and quite selective retention of the nitro-aromatic derivatives (π - π -interaction) while most of the matrix compounds are more weakly retained (6). We observed that the procedure of solvent pre-washing by water and especially hexane reduced the amount of matrix co-extractives and permitted adequate

clean up of sample extracts by SPE. Quantitative desorption of the analytes from the SPE column was achieved with 3 ml of ethyl acetate as eluent.

The chromatographic system applied in our method exhibited enough resolution to separate the analyte peaks from those resulting from matrix interference, and separation was performed with gradient elution on a Luna C18 analytical column, and the mobile phase for LC analyses consisted of ammonium formate and methanol (80:20, v/v).

All nitrofurans metabolites have low molecular masses, from 75 m/z (SEM) to 201 m/z (AMOZ). In liquid chromatography coupled to mass spectrometry analysis of low-molecular-mass substances, promotes a relatively poor sensitivity due to high abundant mass spectrometric background noise in this mass range and non-specific fragmentation behaviour (*e.g.* loss of ammonia, water or carbon dioxide). The derivatisation of the free amino groups with 2-nitrobenzaldehyde normally increases the sensitivity of the MS analysis significantly, which has been described by various authors (1, 2, 6, 14, 15). This simple approach has the additional advantage of simultaneous hydrolysis of the

protein-bound metabolites and rapid derivatisation with 2-nitrobenzaldehyde since the chemical attack of the nucleophile, R-NH₂, on the 2-nitrobenzaldehyde carbonyl group is catalysed by acid.

The best MS sensitivity for the four analytes was obtained using the positive ESI mode. APCI interface was not used due to its poor sensitivity in this ionisation technique. The thermal degradation of the analytes might be a reason for this observation. Generally, ionisation in positive ion mode gave better results compared to the negative ion mode. To reach the highest sensitivity, optimal MS-MS parameters proved to be quite different for the four analytes. Substance identification and quantification was performed by a multiple reaction monitoring selecting one parent ion and two product ions for each analyte, which is in accordance with the EU guidelines for unambiguous positive identification and quantification of analytes (4).

In conclusion, the method for the determination of residual nitrofuran metabolites in eggs was successfully developed. The selection of an adequate clean-up procedure using SPE polymeric cartridges made the method suitable for routine analysis of nitrofuran metabolites in eggs. The applied LC-MS/MS conditions allow for satisfactory separation and analysis of target agents with accordance to considered conditions.

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