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Short communication

Determination of nitrofuran metabolites in milk by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

An LC–ESI–MS–MS method for the analysis of metabolites of four nitrofurans (furazolidone, furaltadone, nitrofurazone and nitrofurantoin) in raw milk has been developed. The samples were achieved by hydrolysis of the protein-bound drug metabolites, derivatization with 2-nitrobenzaldehyd (2-NBA) and clean-up extraction liquid–liquid with ethyl acetate. LC separation was achieved by using a Phenomenex Luna C-18 column. The mass spectrometer operated in multiple reaction monitoring mode (MRM) with positive electro-spray interface (ESI). The method validation was done according to the criteria laid down in Commission Decision No. 2002/657 EC. The validation includes the determination of linearity, repeatability, within-laboratory reproducibility, accuracy, decision limit (CCα) and detection capability (CCβ). The calibration curves were linear, with typical (R^2) values higher than 0.991. The coefficient of variation (CV, %) was lower than 9.3% and the accuracy (RE, %) ranged from −9.0% to 7.0%. CV within-laboratory reproducibility was lower than 13%. The limits of decision (CCα) and detection capability (CCβ) were 0.12–0.29 μg/kg and 0.15–0.37 μg/kg, thus below the minimum required performance limit (MRPL) set at 1 μg/kg by the UE. This validated method was successfully applied for the determination of nitrofuran metabolites in a large number of milk samples.

Keywords: Milk; Nitrofuran metabolites; Residue; LC–ESI–MS–MS

1. Introduction

Furazolidone, furaltadone, nitrofurazone and nitrofurantoin are veterinary drugs and have been widely used in the treatment of gastrointestinal infections in cattle, pigs and poultry. Due to the carcinogenic and mutagenic features, the nitrofurans were placed in Annex IV to Council Regulation 2377/90/EEC [1] which prohibits the use of certain chemicals in food producing animals in the EU.

The nitrofurans are known to be unstable and rapidly metabolised, with *in vivo* half-lives of less than a few hours. Their metabolites are highly stable. Thus the analysis of nitrofurans is based on the determination of the metabolites. In the case of the furazolidone, furaltadone, nitrofurazone and nitrofurantoin, they were analysed by detecting 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM), respectively [2].

For nitrofuran metabolites the maximum residue limit (MRL) could not be set in food-producing animals. The European Commission Decision 2003/181/EC [2] established the minimum required performance level (MRPL) at 1 μg/kg for each nitrofuran metabolite in poultry meat and aquaculture products [3]. Only tandem LC–MS–MS could meet these criteria. Single LC–MS is not sufficiently sensitive to reach the MRPL. No LC–MS method for detection of nitorofuran metabolites has been published so far.

Several LC–MS–MS methods have been developed for determination of nitrofuran metabolites in different matrices: tissues [4–6], eggs [5,7] and honey [8,9]. Only one method could be found for the milk matrix [10]. This method for determination of nitrofuran metabolites in milk involves an acid hydrolysis and derivatization, liquid–liquid extraction, clean-up by solid phase extraction (SPE) and analysis by LC–MS–MS with atmospheric pressure chemical ionization (APCI) in the positive mode.

The aims of this study were to introduce simplified milk sample preparation for use with LC–MS–MS detection. In our

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paper we described the method for the determination of nitrofuran metabolites in milk which does not require an additional chemistry step, such as SPE. The acid hydrolysis and derivatization were adapted from the procedure published by Leitner et al. [2]. The samples were analysed by LC–MS–MS after electrospray ionization (ESI) in the positive mode using multiple reaction monitoring (MRM). LC–ESI–MS–MS was equipped with a two position cut-off valve. Two internal standard AOZ-d4 and AMOZ-d5 were used.

2. Experiment

2.1. Reagents

The metabolites 3-amino-2-oxazolidinone, 3-amino-5-morpholinomethyl-2-oxazolidinon, 1-aminohydantoin were obtained from Witega and semicarbazide hydrochloride (SEM) from Sigma–Aldrich. The internal standards AOZ-d4, AMOZ-d5 and 2-nitrobenzaldehyde (2-NBA) were obtained from Sigma–Aldrich. The 2-NBA derivatives of nitrofuran metabolites NPAOZ, NPAHD, NPAMOZ and NPSEM were supplied by Sigma–Aldrich. The purity of these compounds was greater than 99%.

Methanol LC–MS grade, methanol LC grade, ethyl acetate LC grade and hydrochloric acid were supplied by Baker. Trisodium phosphate dodecahydrate p.a., sodium hydroxide p.a. and ammonium acetate p.a. were obtained from Baker. The water used was purified with a Milli-Q water purification system from Millipore. The filters for the filtration of extract were from Millipore (Millex GV, 0.45 μm).

2.2. Standard solution

Standard stock solutions of 1.0 mg/ml were prepared by dissolving 50 mg of AMOZ, AOZ, AHD, SEM in 50 ml of methanol and this solution was diluted 100 times in methanol obtaining an intermediate standard solution of 10 μg/ml. Working standard solutions of 10 ng/ml were made by diluting stock solution with methanol. Internal standards of AMOZ-d5 and AOZ-d4 were prepared by dissolving 10 mg in methanol, which was adequately diluted until a working solution of 10 ng/ml was obtained. The standard stock solution was stored at −20 °C in the dark and warmed up to room temperature before use. The working standard solution was stored at +4 °C in the dark.

2.3. Samples

Blank control samples of raw milk were collected in compliance with the procedures provided by the national program for residue control in Poland. The milk samples were stored in the dark at −20 °C for analysis.

2.4. Sample preparation

The milk was centrifuged (3500 rpm, 10 min, 4 °C) and then the upper fat layer was removed. The amount of 1 g of raw milk was weighed into a 25 ml centrifuge tube with screw caps, 100 μl of internal standard mixture was added (10 ng/ml of AOZ-d4 and AMOZ-d5). Nine milliliters of 0.1 M hydrochloric acid and 100 μl of 100 mM 2-NBA were added to the samples and followed by vortex mixing. The caps were capped tightly and incubated overnight (16 h) using a thermostat set at 37 ± 1 °C. After incubation time, the samples were cooled down to room temperature and neutralized by the addition of 1 ml of 0.3 M trisodium phosphate and adjusted to pH 7 ± 0.5 with 2 M sodium hydroxide. The samples were extracted twice with ethyl acetate (2 ml × 5 ml) and centrifuged (3500 rpm, 10 min) before removing the ethyl acetate supernatant. The ethyl acetate was evaporated to dryness under a stream of nitrogen using a heating block at 45 °C. The dry residues in milk were redissolved in 0.5 ml mobile phase and filtered through a 0.45 μm filter, before injecting onto the LC–MS–MS.

2.5. LC–ESI–MS–MS

LC analyses were performed on a Luna C18(2) column (150 mm × 2 mm i.d., 3 μm) (Phenomenex, Torrance, USA) using an Agilent 1100 series liquid chromatograph, equipped with a binary pump and an autosampler. The column was thermostated at 40 °C. Flow rate was set at 200 μl/min and the injection volume was 30 μl. The mobile phase was 0.5 mM acetate ammonium containing 20% methanol (A) and methanol

Table 1
MRM transitions reactions monitored by LC–ESI–MS–MS

Compound	MRM transition (*m/z*)	Collision energy (eV)	Peak area ratio ± limit (%)
NPAHD	249 → 134	15	
	249 → 178	28	30 ± 25
NPAOZ	236 → 134	19	
	236 → 192	19	10 ± 50
NPSEM	209 → 166	15	
	209 → 192	25	32 ± 25
NPAMOZ	335 → 291	18	
	335 → 262	24	33 ± 25
NPAOZ-d4 (IS)	240 → 134	19	
NPAMOZ-d5 (IS)	340 → 296	24	

(B). The linear gradient was: 0–0.1 min 95% A, 0.1–9.3 min 0% A, 9.3–15 min 0% A, 15–15.3 min 95% A, 15.3–25 min 95% A.

MS analyses were carried out on an API 3000 triple stage quadruple mass spectrometer (Applied Biosystems, Foster City, CA, Canada) equipped with a turbo–ionspray interface and two position microelectric valve. The entire LC flow was directed into the MS detector between 8 and 15 min using a cut-off valve (Valco instrument Co. Inc., Huston, TX, USA). The source block temperature was set at 450 °C and the electrospray capillary voltage was 5500 V. Nitrogen was used as curtain and collision gas. MS detection was performed in positive mode using MRM. The declustering, focusing potential and collision energy were optimized for MRM transitions for each compound. The dwell time for each transition reaction was set to 150 ms. Resolution was set to Q1 at unit and Q3 to low. The MRM transitions and their collision energies (CE) are shown in Table 1.

2.6. Calibration curves

The calibration curves at five concentration levels were prepared by adding a standard solution mixture at the following concentrations: 0.0 (blank sample) 0.5, 1.0, 1.5 and 2.0 μg/kg to the milk. A fixed amount of internal standard (AOZ-d4 and AMOZ-d5) was added to all the samples. The AOZ-d4 was used for AHD, AOZ, and SEM and AMOZ-d5 for AMOZ. The calibration curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. Peak-area ratios of analyte to internal standard were calculated using Analyst 1.4 Software.

3. Results and discussion

According to Decision 2002/657/EC [10] for banned substances a minimum of four identification points were required. The four identification points were obtained using LC–MS–MS with one precursor and two product ions. The presented research method detected two product ions for each nitrofuran metabolite. The peak area ratios of the various transition reactions were calculated from the spiked samples. The ratios vary from 28% to 33% for 249/178 (AHD), 8–11% for 236/192 (AOZ), 28–36% for 209/192 (SEM) and 30–35% (AMOZ). The mean ratios are presented in Table 1 together with their tolerances given in Decision 2002/657/EC. The signal-to-noise ratio was checked by the analysis of 20 different blank and spiked samples milk at 1 μg/kg. The signal-to-noise ratio for each diagnostic ion was >3:1. Fig. 1 shows MRM chromatograms of blank and spiked milk samples. The spiked samples contained 1.0 μg/kg of each analyte.

The method was based on the validation according to the criteria of Decision 2002/657/EC [11]. According to these criteria,

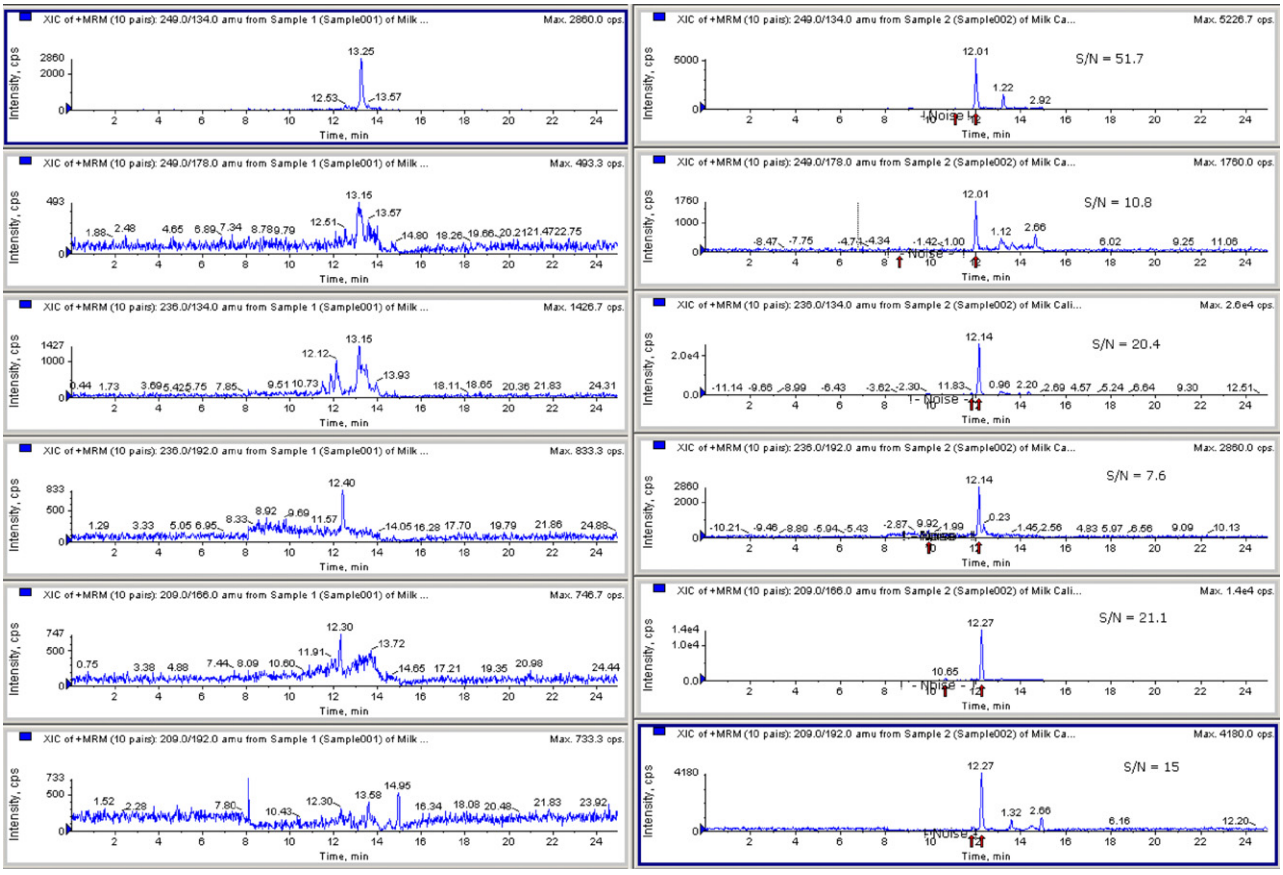

Fig. 1. MRM chromatograms of blank and spiked milk samples at 1.0 μg/kg.

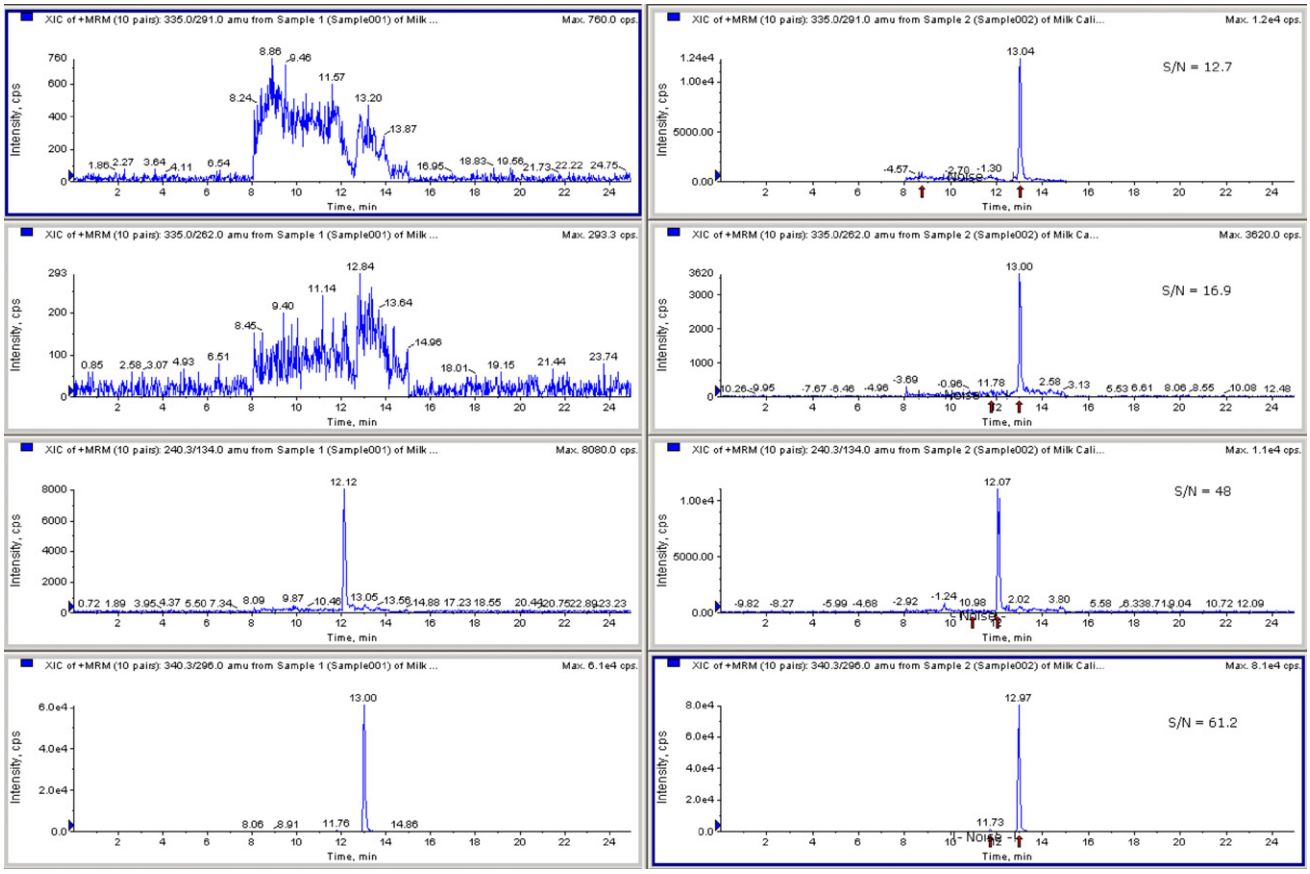

Fig. 1. (*Continued*)

the validation includes the determination of linearity, repeatability, within-laboratory reproducibility, accuracy, decision limit (CCα) and detection capability (CCβ).

Response linearity was evaluated by calibration curves. The calibration curves for AHD, AOZ, SEM and AMOZ were prepared by using spiked in 0.0–2.0 μg/kg milk and were repeated for three different days. For each compound, three calibration curves were made. No significant differences were found between the curves. Linearity was good for all the analytes throughout all the tested concentrations. The corresponding correlation coefficients (R^2) for the curves prepared were higher than 0.991.

According to Decision 2002/657/EC repeatability and accuracy of the method were calculated from the analysis of blank spiked milk samples at three levels 1, 1.5, and 2 times the MRPL (which corresponded to 1, 1.5 and 2.0 μg/kg). Different milk samples were analysed by the same instrument and the same operator. Twelve replicates were obtained for each concentration. The repeatability and within-laboratory reproducibility were determined by calculating the coefficient of variation (CV, %). The accuracy (relative error RE, %) was calculated by the agreement between the measured and the nominal concentrations of the spiked samples. Results of repeatability and accuracy are presented in Table 2.

As shown in Table 2, the CV was below 10% and accuracy ranged from −9.0% to 7.0% for all analytes.

The within-laboratory reproducibility was calculated in different spiked milk samples at concentration of 1 μg/kg (MRPL level). They were analysed for three different days, with the same instrument and different operators. The within-laboratory reproducibility was found satisfactory for the four compounds. CV (%) were 12.2, 5.7, 8.9 and 2.2 for AHD, AOZ, SEM and AMOZ, respectively. According to Decision 2002/657/EC, the CV from method does not cover the value calculated by the Hor-

Table 2
Repeatability and accuracy in spiked milk samples

Compound	Parameter	Level of spiking (μg/kg)		
		1.0	1.5	2.0
AHD	Average (μg/kg)	0.95	1.62	1.82
	CV (%)	7.8	7.4	9.3
	Accuracy (RE, %)	−5.0	−8.0	−9.0
AOZ	Average (μg/kg)	0.99	1.52	1.89
	CV (%)	3.7	5.9	7.7
	Accuracy (RE, %)	−1.0	1.3	−5.5
SEM	Average (μg/kg)	1.07	1.41	1.86
	CV (%)	6.8	7.4	7.0
	Accuracy (RE, %)	7.0	−6	−7.0
AMOZ	Average (μg/kg)	0.98	1.42	1.85
	CV (%)	1.85	4.7	4.2
	Accuracy (RE, %)	−2.0	−5.3	−7.5

Table 3
CCα and CCβ obtained in milk

Compound	CCα (μg/kg)	CCβ (μg/kg)
AHD	0.29	0.37
AOZ	0.12	0.15
SEM	0.26	0.34
AMOZ	0.20	0.25

witz equation. The equation is: $CV = 2^{(1-0.5\log C)}$, where C is the mass fraction expressed as the power of 10. However, for the mass fraction below 1 μg/kg, this equation gives unacceptable high CV values. CV should be as low as possible in the cases.

The values CCα and CCβ were determined by the matrix calibration curve procedure according to ISO 11843 [12]. The CCα and CCβ were calculated using two calibration curves (at four levels 0.0, 1.0, 1.5 and 2.0 μg/kg) from three different experiments on different milk matrix and different days. Curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. CCα and CCβ were calculated for m/z 249 → 134, 236 → 134, 209 → 166 and 335 → 292 ion transition for AHD, AOZ, SEM and AMOZ, respectively. The mean value CCα and CCβ are presented in Table 3.

4. Conclusion

This LC–ESI–MS–MS method allows the simultaneous determination four nitrofuran metabolites residues in milk. The method avoids the use of clean-up by SPE and should be performed quickly. The obtained validation results indicate the accordance of the method with Decision 2002/657/EC. The repeatability and within-laboratory reproducibility (precision) of the method are less than 13% for all analytes. The CCα and CCβ are below the MRPL of 1 μg/kg. This method has been used for routine analysis of nitrofuran metabolites in milk samples.

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