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Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography-ionspray tandem mass spectrometry

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Abstract

Within the EU, the use of nitrofurans is prohibited in food production animals. For this reason detection of these compounds in feedingstuffs, at whatever limit, constitutes an offence under EU legislation. This detection generally involves the use of analytical methods with limits of quantification lowers than 1 mg kg^{-1}. These procedures are unsuitable for the detection and confirmation of trace amounts of nitrofurans in feedingstuffs due to contamination. It is well known that very low concentrations of these compounds can be the source of residues of nitrofuran metabolites in meat and other edible products obtained from animals consuming the contaminated feed. The present multi-compound method was capable of measuring very low concentrations of nitrofurantoin (NFT), nitrofurazone (NFZ), furazolidone (FZD) and furaltadone (FTD) in animal feed using nifuroxazide (NXZ) as internal standard. Following ethyl acetate extraction at mild alkaline conditions and purification on NH_2 column, the nitrofurans are determined using liquid chromatography with photodiode-array detection (LC-DAD). It was observed a CCα ranged from 50 to 100 μg kg^{-1}. The liquid chromatography-tandem mass spectrometric (LC-MS/MS) procedure was used to confirm the identity of the suspected presence of any of the nitrofuran compounds.
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Keywords: Nitrofurans; Feeds; Liquid-chromatography; Photo-diode array detection; Tandem mass spectrometry

1. Introduction

Nitrofurans are Schiff's base derivatives of nitrofuraldehyde known to have a broad-spectrum of antimicrobial activity. They were widely used as feed additives in food-producing animals like poultry, swine, cultured fish and shrimps, for treatment and prevention of various gastrointestinal infections caused by bacteria or protozoa and as growth promoters [1]. A great advantage of these compounds in comparison with other antimicrobial agents, is the slowly development and only to a limited extent of the in vivo bacterial resistance. The most common nitrofurans are furazolidone (FZD), nitrofurantoin (NFT), nitrofurazone (NFZ) and furaltadone (FTD) (Fig. 1). These compounds are rapidly metabolized in vivo, leading to a significant decrease of their parent compounds levels in plasma. The elimination half-lives of nitrofurans are very short, and some hours after administration it is almost impossible to detect any residues of parent compounds in edible tissues. A concomitant accumulation of their protein-bound residues is observed, and their detection is possible over large periods of time [2–4]. Based on the evidence of carcinogenic and genotoxic effects of these bound metabolites [5], European Union (EU) has forbidden, for more than a decade, the use of nitrofuran drugs in food producing animals [6].

The detection of nitrofuran metabolite residues in large amount of samples from poultry and aquaculture products imported to Europe from some Southeast Asian and South American countries, and also within the EU in pork and poultry meat, provoked the so called nitrofuran crisis during 2002–2003 [7]. This circumstance had demonstrated the necessity of an effective control of the illegal use of these compounds. The EU Commission Decision of 13 March, 2003 [8] was established the minimum required performance limit (MRPL) of 1 μg kg^{-1} for

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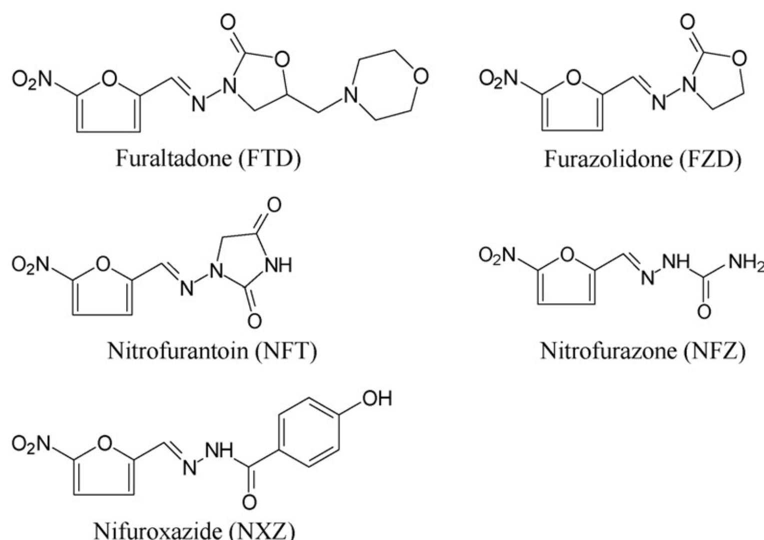

Fig. 1. Chemical structure of furaltadone, furazolidone, nitrofutantoin, nitrofurazone and nifuroxazide (IS).

each nitrofuran metabolite based on the great efficiency of liquid chromatography tandem mass spectrometry (LC-MS/MS) methodologies for detecting bound nitrofuran metabolites in edible tissues.

Nitrofuran compounds were usually administered to animals by means of medicated feeds or at drinking water. Concentrations in feeds ranging from 8 to 400 mg kg^{-1} were considered appropriate depending of the intended use. However, McCracken et al. [9] have confirmed the possibility for the detection of very low concentrations of nitrofuran metabolites in animal tissues exposed to a diet of contaminated feeds with furazolidone and furaltadone at levels as low as 30 $\mu g\ kg^{-1}$.

This means that low concentrations of these compounds can be the source of nitrofuran residue metabolites in meat and other edible products obtained from animals consuming the contaminated feed. So, animal feedingstuffs must be analyzed with analytical procedures capable of measuring very low concentrations of nitrofurans to assure its "fit-for-purpose". Nevertheless, the majority procedures of liquid chromatography with ultraviolet detection (LC-UV) available for nitrofuran feed control, shown limits of quantification around 1 mg kg^{-1}, were usually applied for a single nitrofuran compound and were not suitable for nitrofuran multicomponent screening and confirmation procedures [10–12]. The aim of this paper was to develop a methodology for screening and confirmation the presence of furazolidone (FZD), nitrofurantoin (NFT), furaltadone (FTD) and nitrofurazone (NFZ) at low concentrations in animal feed, in order to satisfy the above referred needs on feed control.

2. Experimental

2.1. Reagents and materials

All chemicals and solvents used were of analytical reagent grade except solvents used in mobile phase that were HPLC grade. Deionized water was obtained from a Mill-Q System (Millipore, Bedford, USA). Ethyl acetate, glacial acetic acid, ammonium hydroxide solution containing NH3 >30%, acetone, methanol, acetonitrile, *N*,*N*–dimethylformamide and ammonium acetate were supplied by VWR (Darmstadt, Germany). HPLC solvents were filtered through a 0.45 μm nylon membrane (Whatman, Maidstone, USA) and Sep-Pack NH2 Cartridges (6 mL, 1 g) were purchase from Waters (Milford, USA). PVDF Mini-uniprep^{TM} vial were purchased from Whatman.

A Retsch ZM 200 miller (Haan, Germany), Mettler Toledo PC2000 and AE100 balances (Greifensee, Switzerland), a Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), a rotary vacuum evaporator (Büchi, Flawil, Switzerland) and a speed-Vac concentrator (Thermo electron corporation, Milford, USA) were used to prepare samples, to perform extraction and clean-up procedures.

Liquid chromatography with diode array detection (LC-DAD) determinations were performed in an HP/Agilent 1100 Series HPLC system with a diode array detector (HP/Agilent Techonologies, Waldbronn, Germany), using a Lichrospher 60, RP-select B, 5 μm, 250 mm × 4 mm analytical column with a Lichrospher 60, RP-select B, 5 μm, 4 mm × 4 mm pre-guard column (Merck, Darmstadt, Germany). Data acquisition was controlled by a ChemStation for LC 3D® software, rev. A.10.01 (Agilent Techonologies, Waldbronn, Germany).LC-MS/MS was performed in an Agilent 1100 Series HPLC system (Agilent Techonologies, Waldbronn, Germany) coupled to a Triple Quadrupole System Sciex API 3000 (Applied Biosystem, Foster City, USA) tandem mass detector, with a Zorbax Eclipse XDB–C18, 5 μm, 150 mm × 2.1 mm column, with a guard column Zorbax Eclipse XDB, C8, 5 μm, 12.5 mm × 2.1 mm (Agilent Technology, Palo Alto, USA). Data acquisition was controlled by a Sciex Analyst® software, Version 1.4.1 (Applied Biosystems, Foster City, CA, USA).

2.2. Standard solutions

Furazolidone, furaltadone, nitrofurantoin, nitrofurazone and nifuroxazide, which were used as internal standard

(IS), were all purchased from Sigma–Aldrich (St. Louis, USA).

Stock standard solutions of the five compounds (100 μg mL^{-1}) were prepared by dissolving each one in *N*,*N*–dimethylformamide:methanol (10:90). Working mixed standard solutions of the compounds were prepared by diluting the stock standard solutions in mobile phase (14 mM ammonium acetate pH 4.6:acetonitrile (70:30, v:v)). Work standard solutions were stable at least for 2 months when stored in the dark at 4 °C. Stock solutions were stored in the dark at −20 °C and were stable at least for 6 months.

2.3. Samples

Poultry, porcine and bovine feed samples were commercial feeds available on the Portuguese market.

2.4. Extraction

An amount of 5.0 g thoroughly minced feed was weighed into a 250 mL polypropylene copolymer centrifuge flask and spiked with IS at a concentration of 500 μg kg^{-1}. Then, 20 mL of ammonium acetate 79 mM solution (pH 4.6) were added and the pH was adjusted to 8 with ammonia hydroxide solution ≥30%. The mixture was allowed to rest for 15 min. Ethyl acetate (30 mL) was added before stirring for 20 min in a rotary shaker and centrifuged for 10 min at 3000 rpm. The organic layer was collected and evaporated to dryness in a rotary vacuum evaporator at 35 °C and 240 mbar. The resulting extract is reconstituted in 2 mL of a mixture of acetone and methanol 80:20 (v:v).

2.5. Clean-up

A Sep-Pack NH2 cartridge was conditioned with 5 mL of a mixture of acetone:methanol (80:20, v:v). The reconstituted extract was put onto the cartridge and, then, the nitrofurans were eluted with 5 mL of the previous mixture. The eluate was evaporated to dryness in the speedVac concentrator and the residue was reconstituted with 500 μL of a mixture of ammonium acetate solution 14 mM (pH 4.6):acetonitrile (70:30, v:v).

The resulting solution was filtered through a 0.45 μm PVDF Mini-uniprep™ vial before chromatography.

2.6. LC-DAD analysis

50 μL of the filtrate was injected into the LC-DAD system. A binary gradient mobile phase composed by a mixture of 14 mM ammonium acetate (pH 4.6) (A) and acetonitrile (B) was used at a flow rate of 1.2 mL min^{-1}. The gradient starts with 70% (A) for 1 min, linearly decreased to 50% (A) in 15 min, brought back to 70% (A) in 0.1 min and maintaining these conditions for 3.9 min with a total run time of 20 min. Detection was made at 375 nm for all the compounds, because all the analyzed nitrofurans show a maximum absorbance near this wavelength.

Table 1
Precursor/product ions monitored in the LC-MS/MS

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Positive mode		
Furaltadone (FTD)	325.00	251.92, <u>280.99</u>
Furazolidone (FZD)	226.20	<u>121.99</u>, 139.16
Nifuroxazide (NXZ) (IS)	276.28	<u>120.98</u>
Negative mode		
Nitrofurantoin (NFT)	236.97	123.60, <u>151.97</u>
Nitrofurazone (NFZ)	196.94	<u>123.78</u>, 149.65
Nifuroxazide (NXZ) (IS)	274.04	<u>153.86</u>

The most intense ion is underlined.

2.7. LC-MS/MS analysis

For LC-MS/MS analysis, injection volume was 50 μL and column temperature was set at 30 °C. The binary gradient phase that was used for HPLC-DAD, was also used for LC-MS/MS but at a flow rate of 0.4 mL min^{-1}. The gradient starts with 90% (A), linearly decreased to 10% (A) in 9 min, brought back to 90% (A) in 0.2 min and maintaining these conditions for 3.8 min, with a total run time of 13 min. The mass spectrometer was operated in electrospray positive mode for FTD, FZD and NXZ (IS) and in electrospray negative mode for NFT, NFZ and NXZ (IS) using multiple reaction monitoring mode (MRM) for data acquisition (Table 1). Nitrogen was used for curtain gas, collision gas and nebulizer gas at flow rates of 7, 4 and 9 L min^{-1}, respectively in both ionization modes. The ion source block temperature was set to 350 °C, and the electrospray capillary voltage to 5.0 kV. Declustering potential, focusing potential, collision energy and cell exit potential MS parameters were shown in Table 2. Dwell times of 600 and 200 ms were chosen, respectively, for nitrofurans and for internal standard evaluation.

2.8. Confirmation

Identification of nitrofurans in samples were performed accordingly Commission Decision 2002/657/EC criteria, namely the ± 2.5% tolerance for relative retention time of the analyte to that of the corresponding internal standard and observation of the tolerances set by EU criteria of peak area ratios from the controlled transitions reactions [13].

3. Results and discussion

Characteristic LC-DAD chromatograms of a standard mixture of the four nitrofuran compounds and internal standard (A), of a blank feed sample (B) and of a spiked feed sample (C) are shown in Fig. 2.

In order to evaluate the best conditions of the mobile phase to achieve the separation of the different nitrofurans, a mixture of acetonitrile and a dilution of primary solution of 250 mM ammonium acetate (pH 4.8) (70:30, v:v) was tested, with an isocratic system. In these conditions a broadening peak of the internal standard (NXZ) was observed.

It was also observed that the ammonium acetate concentration in the mobile phase interfered in the LC-MS/MS analysis,

Table 2
LC-MS/MS parameters for declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP)

Compound	DP	FP	CE	CXP
Positive parameters				
Furaltadone (FTD)	56.0	280.0	29.0, 23.0	8.0, 8.0
Furazolidone (FZD)	61.0	310.0	21.0, 17.0	18.0, 22.0
Nifuroxazide (NXZ) (IS)	61.0	280.0	25.0	8.0
Negative parameters				
Nitrofurantoin (NFT)	−26.0	−150.0	−20.0, −12.0	−7.0, −9.0
Nitrofurazone (NFZ)	−26.0	−150.0	−12.0, −10.0	−9.0, −11.0
Nifuroxazide (NXZ) (IS)	−36.0	−150.0	−18.0	−9.0

by deposition of salt in the curtain plate, so a decrease in the molarity of the ammonium acetate primary solution to 14 mM was preferred, as well as the use of a gradient separation. The pH control of this solution proved to be fundamental in order to attain a proper separation of the analytes. The pH range from 5.0 to 4.5 was tested, and it was concluded that pH 4.6 attained the best separation with sharp and symmetrical peaks (Fig. 3).

For the liquid–liquid extraction of the feed at different pH values with ethyl acetate, pH 8 was the most effective, both in terms of analytes recovery and at attaining cleaner extracts.

In the clean-up step, the use of NH2 columns and elution of the analytes with a solution of methanol and sodium phosphate buffer at 0.1 M pH 8.0 (80:20, v:v), enabled the best recovery levels and the removal of most interfering agents. However, this step turned out to be critical due to the highly variable nature of results, as it seems, from the sample composition. Therefore, a mixture of acetone and methanol as elution solvent was preferred, even though it provided worse recoveries and less clean extracts, but with higher levels of precision.

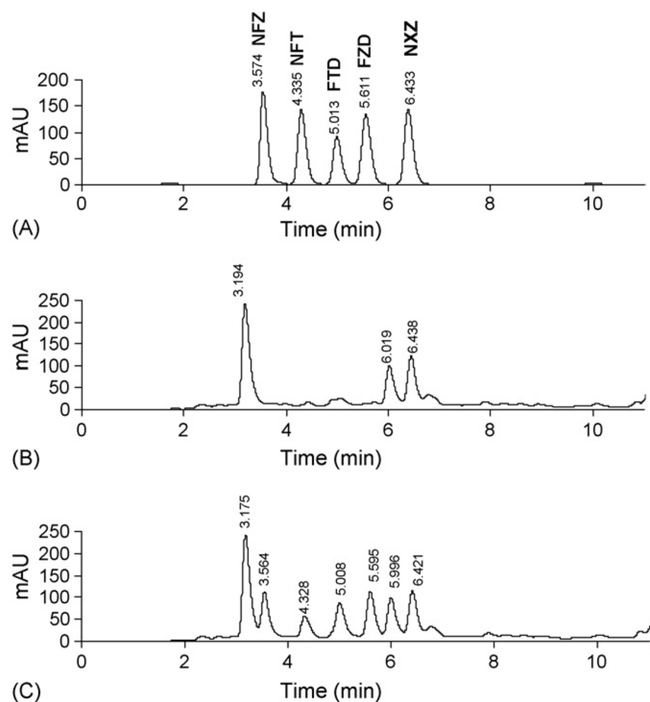

Fig. 2. LC-DAD chromatograms of (A) a mixture of nitrofuran standards at 0.5 μg mL^{-1}; (B) blank feed; (C) blank feed fortification at 500 μg kg^{-1} with the four nitrofurans.

The use of nifuroxazide as IS beside its quantitative and identification purpose, proved to be highly convenient due to the chromatographic signal attained by LC-DAD, at the monitored wavelength, and by LC-MS/MS. After attempting to optimize the conditions for NFT and NFZ by positive electrospray ionization, and as results were far from the expected, optimizing the conditions in negative electrospray ionization turned out to be the solution. It provides good results in both negative and positive ionization modes, respectively for NFT and NFZ and for FZD and FTD as it was shown in Fig. 4.

For validation, decision limit (CCα), detection capability (CCβ), specificity, repeatability, reproducibility and accuracy were determined according to Commission Decision 2002/657/EC [13].

The specificity of the method was checked by analyzing 20 different blank feed samples. These samples were randomly chosen from previously analyzed nitrofuran free samples (six from porcine, six from bovine and eight from poultry). On the expected retention times for the analytes no interfering peaks could be detected by LC-DAD and LC-MS/MS in all the analyzed samples. From these samples it was also evaluated the noise amplitude related to the internal standard signal amplitude.

A sufficient portion of each sample used to evaluate specificity was well homogenized to constituted a representative

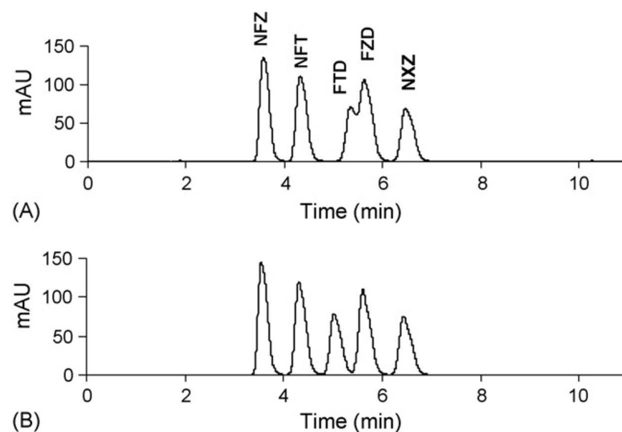

Fig. 3. LC-DAD chromatograms of a mixture of nitrofurans standards at 0.5 μg mL^{-1} with a primary acetate buffer solution at pH 4.9 (A) and with a primary acetate buffer solution at pH 4.6 (B).

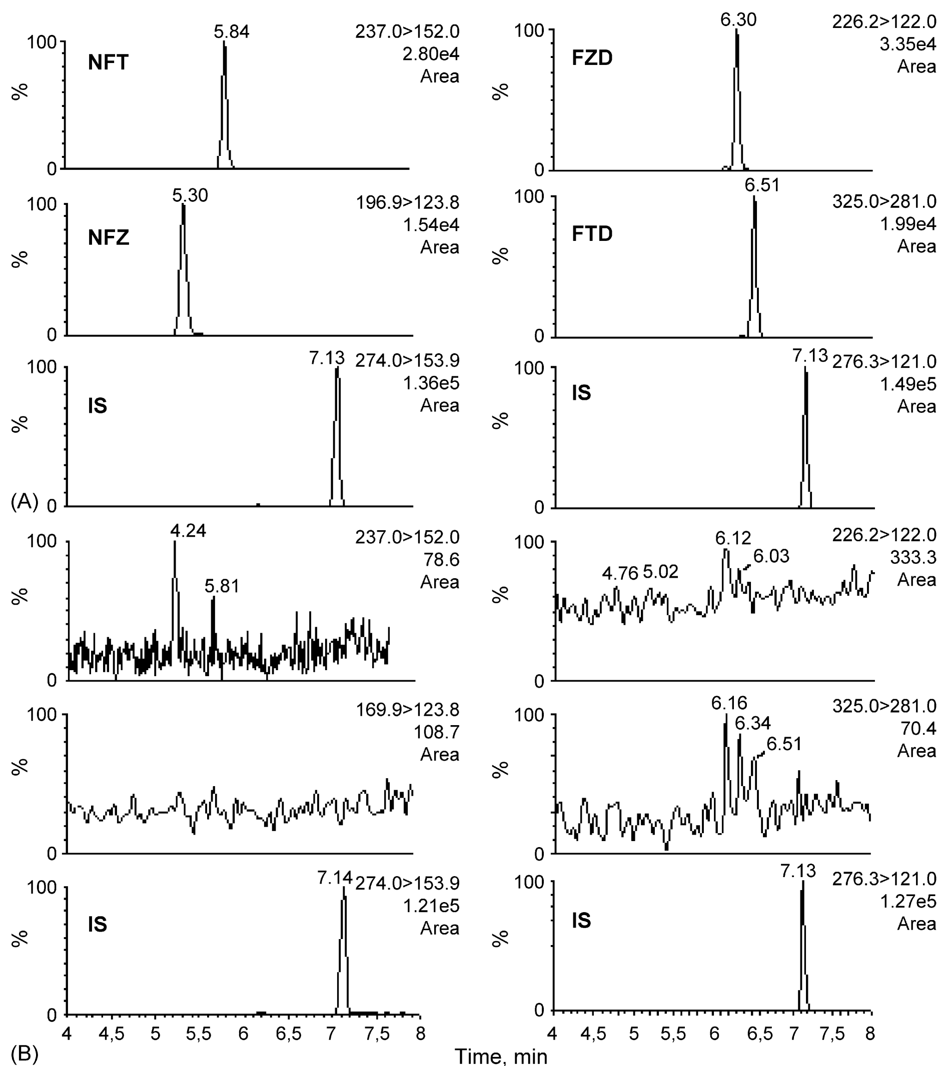

Fig. 4. (A) LC-MS/MS chromatogram of a blank feed fortified at 50 μg kg^{-1} with the four nitrofurans and (B) LC-MS/MS chromatogram of the blank feed.

blank sample, which was then divided into sub-samples to be used for calibration curves. The samples were prepared and analyzed on each day for 3 days. For each calibration curve, samples were fortified at different concentration levels for the four analytes. Number of spiked nitrofuran samples used in calibration curves are shown on Table 3. A standard calibration curve was also prepared at the same range of the fortified curve to be analyzed on each day [14].

Decision limit (CCα) was calculated from results obtained from the 20 blank feed samples and from the calibration curves experiments from the 3 days, and applying the following equation (1)

$$\mathrm{CC}\alpha = \frac{2.33\,\sigma_N}{\varepsilon} \tag{1}$$

where σ_N is the standard deviation of the noise amplitude at the retention time of each analyte and ε the mean slope of calibration curve [15].

For CCβ calculation, the matrix-blank procedure described into Commission Decision 2002/657/EC [13] was used. For this,

Table 3
Number of spiked nitrofuran samples used in calibration curves (spiked levels are expressed in μg kg^{-1})

Spiked levels	LC-DAD						LC-MS/MS					
	0	100	150	200	300	500	0	10	20	30	50	100
Furaltadone	1	6	6	6	1	1	1	6	6	6	1	1
Furazolidone	1	6	6	6	1	1	1	6	6	6	1	1
Nitrofurantoin	1	1	6	6	6	1	1	1	6	6	6	1
Nitrofurazone	1	1	6	6	6	1	1	1	6	6	6	1

Table 4
Validation parameters for LC-MS/MS and LC-DAD

Compound	CCα (μg kg^{-1})		CCβ (μg kg^{-1})		Accuracy at CCβ (%)		Repeatability at CCβ (CV%)		Reproducibility at CCβ (CV%)	
	MS	DAD	MS	DAD	MS	DAD	MS	DAD	MS	DAD
Furaltadone	7	47	20	150	78.1	75.8	9.3	12.3	13.1	19.7
Furazolidone	10	51	30	150	68.1	63.1	8.0	9.1	11.8	15.1
Nitrofurantoin	21	98	50	300	62.1	60.1	7.5	11.4	14.9	18.2
Nitrofurazone	15	76	50	200	67.8	68.8	10.4	10.5	17.6	22.6

Table 5
Calibration curve parameters in fortified feed samples at ranges of 10–100 μg kg^{-1} and 50–500 μg kg^{-1}, respectively for LC-MS/MS and LC-DAD

Compound	R^2		Intercept		Slope	
	MS	DAD	MS	DAD	MS	DAD
Furaltadone	0.992	0.989	2.063	3.976	1.324	3.369
Furazolidone	0.996	0.992	3.143	4.097	1.922	2.922
Nitrofurantoin	0.989	0.985	2.012	5.972	1.840	3.638
Nitrofurazone	0.990	0.981	4.865	4.779	2.701	3.954

20 different blank feed samples where fortified at a level that were estimated during the method development to equal the CCβ for the four analytes. In 95% of the samples was possible to identify the analyte. This CCβ calculation procedure was used for LC-DAD and LC-MS/MS methods. Values obtain for CCα and CCβ are shown in Table 4.

The linearity was evaluated by analyzing the 3 days calibration curves of fortified samples. Peak area ratios between the analytes of the interest and IS were plotted against the concentration ratios. The 3 days resulting calibration curve for each analyte show a coefficient of determination (R^2) better than 0.98. All calibration curve parameters are summarized in Table 5. Repeatability and within-laboratory reproducibility were evaluated at CCβ concentration. For repeatability, the coefficient of variation (CV) of the mean concentration for the repeated analysis within the days ($n = 6 \times 3$) were calculated. For reproducibility, samples were analyzed by different operators on the 3 different days, and expressed as CV of the mean concentration between the days. Considering the application of the Horwitz equation and the criteria defined into Commission Decision 2002/657/EC [13], a CV of 23% was taken as a guide for analyte concentrations ranging from10 to 100 μg kg^{-1}, and all the calculated coefficient of variation were lower than the permitted CV. The accuracy for both methods was evaluated at CCβ concentration by comparing data from the fortified curve and the standard calibration curve (calibration curve without matrix). The values obtained it validation data were summarized in Tables 4 and 5.

4. Conclusions

The LC-UV photodiode array detection (LC-DAD) methodology that was described in this paper was appropriate for simultaneous detection of FZD, NFT, FTD and NFZ in feed samples at levels down to 50–100 μg kg^{-1} depending on the compound. Nonetheless, to have a positive identification by comparing the UV-spectra of the standards with those of the detected analytes, higher concentrations should be present in the sample (300–500 μg kg^{-1}) depending on the compound. When LC-MS/MS methodology described in this paper was used, detection capability is somewhat improved to levels ranging to 20–50 μg kg^{-1} depending on the compound. These levels were appropriate for detection of low-level contamination feed samples. Nifuroxazide (NXZ) was used as internal standard (IS) for both LC-DAD and LC-MS/MS. In routine analysis, the LC-DAD method is used to screen samples for the possible presence of the four nitrofurans analyzed and the LC-MS/MS method is used to confirm any positive finding.

Finally, and based on the obtained values for precision, CCα and CCβ in both LC-DAD and LC-MS/MS methodologies, it has been demonstrated their applicability to measure the presence of the four referred nitrofurans. Therefore, applying both described methodologies in routine analysis was simple and quick, taking into account that the same sample extract could be evaluated by LC-DAD and LC-MS/MS, for screening and confirmation for the presence of one or more of the analyzed nitrofurans.

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