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Multi-residue monitoring for the simultaneous determination of five nitrofurans (furazolidone, furaltadone, nitrofurazone, nitrofurantoine, nifursol) in poultry muscle tissue through the detection of their five major metabolites (AOZ, AMOZ, SEM, AHD, DNSAH) by liquid chromatography coupled to electrospray tandem mass spectrometry—In-house validation in line with Commission Decision 657/2002/EC

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Abstract

Following the ban of four nitrofurans in the mid-90s (furazolidone, furaltadone, nitrofurantoine, nitrofurazone), the nifursol, a veterinary drug from the nitrofuran class of antibacterials which has been used prophylactically as feed additive for treating turkeys against histomoniasis (blackhead disease) was also declared in Annex IV of the European Union Directive no. 90/2377/EC in 2002 according to the Regulation no. 1756/2002/EC. As for the four other nitrofurans, nifursol disappears from tissues within a few days after treatment of food-producing animals. But toxic metabolites are still present for longer periods (several weeks or even months). The major metabolite that can readily be monitored in the tissues following nifursol abuse is the 3,5-dinitro-salicylic acid hydrazine (DNSAH). This article displays some improvements and the revalidation of the analytical method by liquid chromatography coupled to electrospray tandem mass spectrometry (LC-esiMS/MS) already in use in our laboratory for monitoring nitrofuran metabolites but also including the nifursol metabolite at the confirmatory minimum required performance level (MRPL) of 1 $\mu g\,kg^{-1}$. The validation is applied both to artificially and to naturally incurred turkey muscle.

Keywords: Nitrofuran metabolites; Furazolidone; Furaltadone; Nitrofurantoine; Nitrofurazone; Nifursol; Turkey muscle; LC–MS/MS

1. Introduction

The nitrofurans are antimicrobial drugs that have been widely used as veterinary therapeutics or feed additives for treating bacterial diseases in cattle, swine and poultry production. Due to the toxicological hazard for human consumers (carcinogenicity and mutagenicity) provoked by these drugs [1], their ban in food animal production was effectively declared in the European Union in the mid-90s for furaltadone, nitrofurantoine, nitrofurazone and further for furazolidone. The four compounds were put into the Annex IV of the European Union Directive no. 90/2377/EC in 1993 and 1995 [2–4].

In accordance with European Directive 96/23/EC [5] and Decision 657/2002/EC [6], a definitive minimum required performance limit (MRPL) was finally set for these drugs (markers = metabolites of nitrofurans) at 1 $\mu g\,kg^{-1}$ in March 2003 [7]. Analytical methods essentially based on liquid chromatography coupled to electrospray tandem mass spectrometry (LC-esiMS/MS or LC-esiTandemMS) instrumentation as presented in the article of Leitner et al. [8] were developed to

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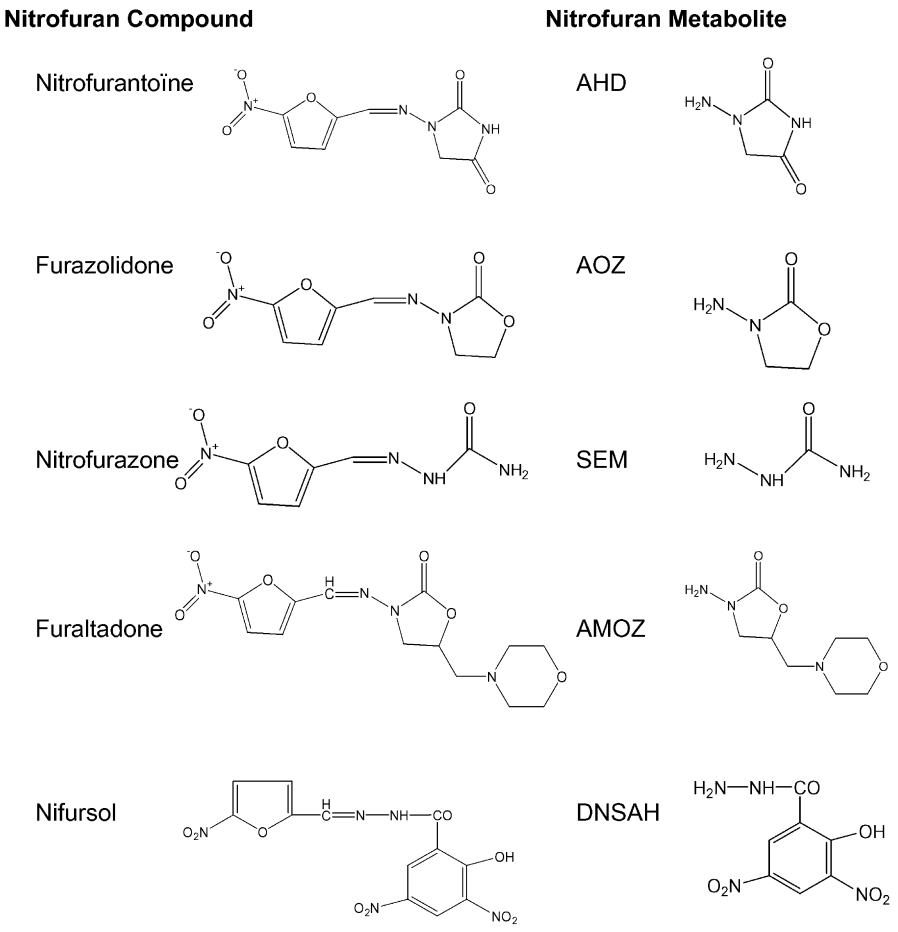

Fig. 1. Metabolites considered for the analysis of the residues of the five nitrofurans in food from animal origin.

monitor these drugs in food from animal origin. The nitrofuran antibacterials are rapidly biochemically transformed in still toxic metabolites which have the property to be highly bound to proteins and thus stable for longer periods (several weeks or even months) in the food producing animals [9–12]. These metabolites are 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) for furaltadone, 1-aminohydantoïne (AHD) for nitrofurantoine, semicarbazide (SEM) for nitrofurazone and 3-amino-2-oxazolidinone (AOZ) for furazolidone (Fig. 1). For their analysis either by HPLC-UV [13] or by LC–MS/MS [14], they absolutely need to be derivatized to finally attach a mass portion for enhancing their detection (Fig. 2). The method was transferred at the Laboratory for Research and Study on Veterinary Drug and Disinfectant of the French Food Safety Agency (AFSSA-LERMVD) in 2002, qualified and further validated on a LC-esiMS/MS instrument [15,16] according to the methods developed in the framework of the Foodbrand European project [17].

Following the ban of the four nitrofurans in the mid-90s, a 5th member of the family, the nifursol (3,5-dinitro-*N*′-(5-nitrofurfurylidene) salicylhydrazide), still in use for prophylactic treatment as a feed additive for treating turkeys against histomoniasis (black-head disease) was finally also withdrawn from the authorized list of additives in feedingstuffs [18] following the 2001–2002 meetings of the Standing Committee for Animal Nutrition of European Commission-Directorate General for Health and Consumer Protection (EC-DGSANCO SCAN) [19] and the Commission Regulation no. 1756/2002/EC [20]. As for the other nitrofurans, nifursol disappears rapidly from tissues within a few days after treatment of the food-producing animals. But toxic metabolites are still present for longer periods [21–25] and the major metabolite that can readily be monitored in the tissues following nifursol abuse is the DNSAH, 3,5-dinitro-salicylic acid hydrazine (Fig. 1). It can be detected with the same principle as for the previously banned nitrofurans (Fig. 2). The present article demonstrates the feasability of a multi-nitrofuran residue monitoring taking into account the metabolites (AMOZ, AOZ, AHD, SEM, DNSAH) of the five banned compounds within a unique method [26,27]. The demonstration displays some improvements of our previous 2002-method particularly at the sample preparation and extraction steps. The plan for revalidation of our LC-esiTandemMS analytical method is applied both to artificially and to naturally incurred turkey muscle [28]. The scope of the method also extends to other species and animal food products such as chicken, quails, swines, shrimps, whole egg and honey for which testing assays were satisfactorily undertaken.

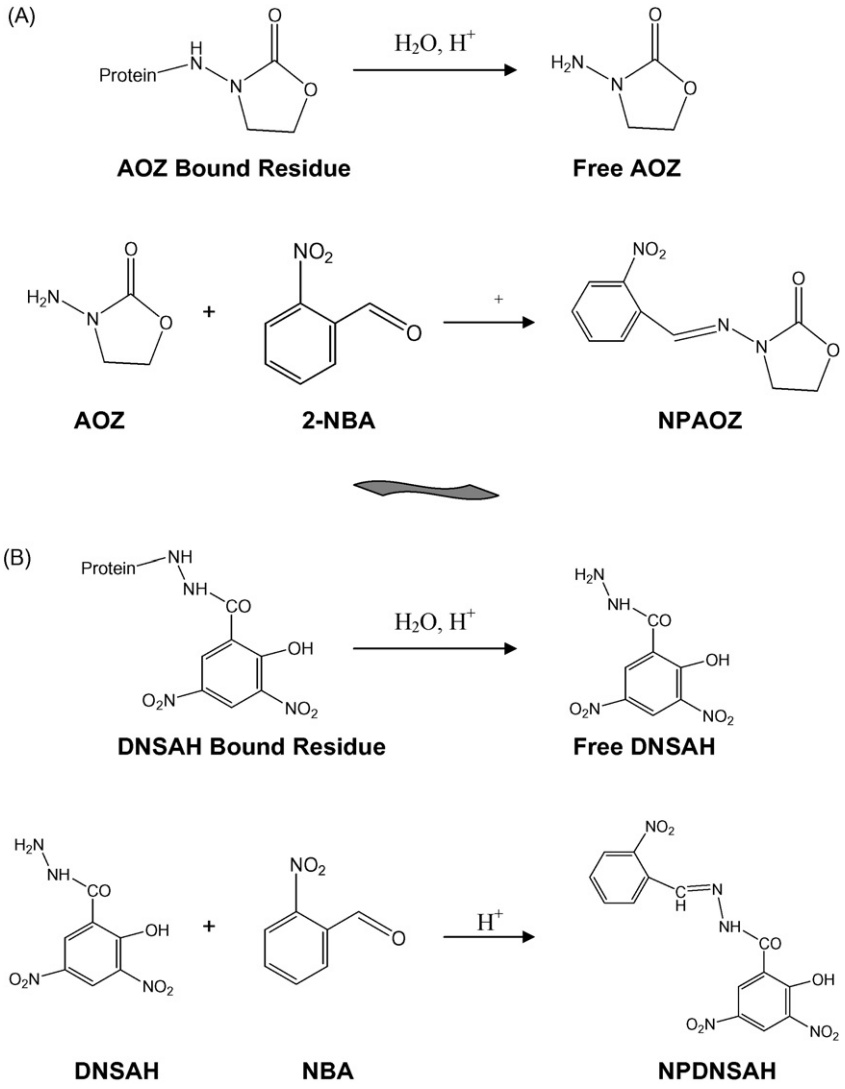

Fig. 2. Derivatizing reactions leading to the nitrophenyl derivatives of AOZ (A) and DNSAH (B).

2. Experimental

2.1. Reagents and standards

All reagents and solvents were of analytical grade or better. Methanol was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). 2-nitrobenzaldehyde and ammonium formiate were obtained from Fluka-Sigma–Aldrich Chimie (St Quentin-Fallavier, France). Sodium hydroxide (NaOH) and anhydrous dipotassium hydrogenophosphate were from Prolabo (Fontenay-sous-bois, France). Ethyl acetate and 12 N hydrochloric acid (HCl) were from Merck (Darmstad, Germany). Ultra-pure water was demineralised using an Alpha-Q Millipore purification system (Molsheim, France). 3,5-dinitrosalicylic acid hydrazine (DNSAH) was obtained from Mikromol (Luckenwalde, Germany), 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ), semicarbazide (SEM·HCl) and nifuroxazide, a compound further hydrolysed and derivatised as salicylic acid hydrazine (SAH), were purchased from Sigma–Aldrich Chimie (St Quentin-Fallavier, France), d_4-3-amino-2-oxazolidinone (D_4-AOZ) was obtained from CSS (Belfast, Northern Ireland), 1-aminohydantoin (AHD·HCl), ^{13}C isotope of 1-aminohydantoin ($^{13}C_3$-AHD), d_5-3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ), ^{13}C and ^{15}N isotope of semicarbazide ($^{13}C^{15}N_2$-SEM·HCl) were purchased from Witega (Berlin, Germany). Individual stock standard solutions of the four nitrofuran metabolites (AOZ, AMOZ, AHD and SEM) were prepared at 1.0 mg mL^{-1} by dissolving with methanol the appropriate amount of analytical standards in 25 mL volumetric flasks. The stock standard solution of DNSAH was specifically prepared at 0.04 mg mL^{-1} by dissolving with methanol the appropriate amount of analytical standards in 250 mL volumetric flasks. Individual stock standard solutions for the five internal standards, either isotopic ($^{13}C^{15}N_2$·SEM, $^{13}C_3$-AHD), deuterated (D_5-AMOZ, D_4-AOZ) or analogue (nifuroxazide to be transformed in SAH) were also prepared at 0.1 mg mL^{-1} by dissolving with methanol the appropriate amount of analytical standards in 100 mL volumetric flasks. These stock standard solutions were all stored in a cool dark room and were considered

stable for more than 6 months. Intermediate working standard solutions were prepared in order to finally give the following two working standard solutions used for the fortification of the tissue samples: a solution at 10 ng mL^{-1} of DNSAH, AOZ, AMOZ, AHD and SEM in methanol (called 5WSTD) and a solution of the five internal standards (named 5IS) containing nifuroxazide at 20 ng mL^{-1}, D_5-AMOZ at 100 ng mL^{-1}, D_4-AOZ at 100 ng mL^{-1}, $^{13}C^{15}N_2$·SEM at 100 ng mL^{-1} and $^{13}C_3$-AHD at 1000 ng mL^{-1}.

2.2. Apparatus

An Agilent Technologies (Waldbronn, Germany) liquid chromatograph (HP1100 series) fully automated and run in gradient mode was coupled to a Micromass (Altrincham, UK) tandem mass spectrometer (Quattro LCZ). Chromatography was performed on a Symmetry® C8 50 mm × 2.1 mm column with 3.5 μm particle size connected to a guard column Symmetry® C18 10 mm × 2.1 mm with 3.5 μm particle size (Waters, Milford, Massachussetts, USA). The chromatographic separation was achieved by a gradient elution using a flow rate of 0.2 mL min^{-1} and segmented in four parts over a total period of 16 min of analysis. The first segment was a linear gradient of 20% methanol mixed with 80% 1 mM ammonium formiate at $T = 0$ min to 95% methanol mixed with 5% 1 mM ammonium formiate at $T = 7$ min. The second segment was a 1 min gradient stop ($T = 7$ min to $T = 8$ min) at 95% methanol mixed with 5% 1 mM ammonium formiate. The third segment was a linear gradient of 95% methanol mixed with 5% 1 mM ammonium formiate at $T = 8$ min decreased to 20% methanol mixed with 80% 1 mM ammonium formiate at $T = 10$ min. The fourth segment was a 6 min gradient stop ($T = 10$ min to $T = 16$ min) at 20% methanol mixed with 80% 1 mM ammonium formiate.

The interface between LC equipment and tandemMS apparatus was operated in a positive electrospray ion mode. The parameters of the source were optimised for the five compounds and their five internal standard counterparts with a capillary voltage of 3.2 kV, a source temperature of 150 °C, a desolvation temperature of 350 °C, a nitrogen desolvation gas flow of 770 L h^{-1} and a nitrogen nebulizing gas flow of 80 L h^{-1}. The molecular ions were selected as precursor ions and fragmentation was operated in the collision induced dissociation cell using argon as the collision gas at a pressure of 2.2×10^{-3} mbar. Data acquisition is performed in MRM mode using the parameters listed in Table 1.

2.3. Sample preparation, extraction, derivatization, clean-up and analysis (for total residues)

The turkey muscle tissue sample is defrozen, thoroughly grinded and homogenized and a portion of 1.0 ± 0.02 g is then weighed and distributed in a 50 mL disposable polypropylene screw-capped tube. Appropriate amount (50 μL) of the working internal-standard solution (5IS) is added to every 1 g tissue portion. For calibration purpose, appropriate amounts of the working solution made of the five analytes (5WSTD) are added to the six 50 mL-tubes containing each a 1 g portion of blank muscle tissue matrix in order to get a matrix-match calibration at 0.0; 0.5; 1.0; 1.5; 2.0 and 5.0 μg kg^{-1} for the five analytes. Fifteen minutes later, 4 mL ultra-pure water are added to the sample followed by 0.5 mL of 1 M HCl and 150 μL of 50 mM methanolic 2-nitrobenzaldehyde (prepared from 189 mg of 2-nitrobenzaldehyde dissolved with 25 mL of methanol in a glass-ambered volumetric flask). The 50 mL-tube is then capped, vortex-mix for 10 s and finally incubated either in a +37 °C agitated water bath for 16 h (one night) or in a +55 °C agitated water bath for 4 h and protected from light. The next step is the neutralisation of the solution by adding 5 mL of 0.1 M di-potassium hydrogenophosphate followed by 300 μL of 1 M NaOH. Swirling the tube for few seconds, the pH is con-

Table 1
Diagnostic ions for the five nitrofuran metabolites and their internal standard counterparts

Nitrophenyl derivatized analytes	Parent ion > daughter ion	Dwell (s)	Cone voltage (V)	CID (eV)
NP-AHD	249 > 134.1	0.25	25	12
	249 > 178.1	0.25	25	15
$^{13}C_3$-NP-AHD	252.1 > 179.3	0.15	25	15
NP-AOZ	236 > 134.1	0.25	25	12
	236 > 104	0.25	25	22
D_4-NP-AOZ	240 > 134	0.15	25	12
NP-SEM	209 > 192.1	0.25	20	12
	209 > 166.1	0.25	20	12
$^{13}C^{15}N_2$-NP-SEM	212 > 168	0.15	20	10
NP-AMOZ	335 > 291.1	0.25	25	12
	335 > 262.1	0.25	25	17
D_5-NP-AMOZ	340 > 296.1	0.15	25	12
NP-DNSAH	376 > 166	0.35	25	17
	376 > 211	0.35	25	15
NP-SAH	285.8 > 121.1	0.15	32	18

trolled at 7.0 ± 0.5 with pH strips adding few drops of NaOH if necessary. The subsequent step is a liquid–liquid extraction with 5 mL ethyl acetate added to the neutralized solution and mixed for 20 min on a rotary homogenizer at about 100 rd min^{-1}. The ethyl acetate portion is then extracted by centrifugation at 3000 × *g* for 10 min at 4 ± 1 °C and the supernatant transferred into a clean 10 mL disposable polypropylene tube. A second liquid–liquid extraction with a 3 mL volume of ethyl acetate is applied to the neutralized solution and mixed for 20 min on a rotary homogenizer at about 100 rd min^{-1}. Centrifugation at 3000 × *g* for 10 min at 4 ± 1 °C and transfer of the new supernatant into the same 10 mL disposable polypropylene tube are then carried out in order to recover a 8 mL ethyl acetate volume containing the nitrophenyl derivatized residues of the nitrofuran metabolites. Evaporation at 45 °C under gentle nitrogen stream down to nearly dryness is implemented leading to an oily extract to be redissolved into a 400 μL solution of methanol/1 mM ammonium formiate (60/40; v/v) thanks to a 1 min water bath ultra-sonication. The recovered extract is transferred to a clean eppendorf tube and ultra-centrifuged at 19200 × g during 20 min at 4 ± 1 °C. Then the supernatant is filtered onto a 0.45 μm PVDF filter and the 300 μL filtrate is transferred to a microvial capped and adapted to the LC autosampler.

2.4. A specific sample preparation for tissue-bound residues (also called washing procedure)

The preceeding sample preparation is applied when a total nitrofuran metabolite residue monitoring is requested. But when a specific 'confirmatory' information on the tissue bound residues of nitrofuran metabolites is needed then a specific, time-consuming (2 h more), sample preparation (hereafter described) must be applied prior to the extraction-derivatization and analytical process.

After defrozing and thoroughly grinding of the turkey muscle tissue sample, the homogenized 1.0 ± 0.02 g portion distributed in a 50 mL disposable polypropylene screw-capped tube is submitted to a series of 4 solvent washing. The first wash is carried out adding 6 mL of a methanol/water solution (50/50; v/v) to the sample followed by a 15 min 100 rd/min rotary homogenisation and then by a 10 min 3500 × *g* centrifugation at 4 ± 1 °C. The supernatant is discarded and the sample is processed to the next washing step. The second wash is carried out adding 6 mL of a methanol/water solution (75/25; v/v) to the sample followed by the same rotary homogenisation and centrifugation as for the first wash. The supernatant is once more discarded and the sample is processed to the next washing step. The third wash consists to add 6 mL of pure methanol followed by the same rotary homogenisation and centrifugation as for the first and second washes. Once more, the supernatant is discarded and the sample is processed to the last washing step. The fourth and last wash consists to add 2 mL of ultra-pure water followed by a 20 s vortex-mix and the same centrifugation as for the previous washes. The supernatant is discarded and the sample is then ready for the analytical preparation as already described here above for the total residues. This washing procedure is expected to remove large amount (near 100%) of any free nitrofuran metabolite that might still be present into the tissue sample. High percentage of nitrofuran-free metabolite in a sample is now widely considered not to be sufficiently specific of a veterinary drug treatment abuse with banned nitrofuran compounds, especially in the case of nitrofurazone abuse (i.e. monitoring of the semicarbazide metabolite) [29–37].

2.4.1. Validation scheme and statistical analysis

The validation is performed with taking into account the criteria and the recommendations of the European Commission Decision 2002/657/EC [6] implementing the Council Directive 96/23/EC [5] and concerning the performance of analytical methods and the interpretation of results. The fully validated matrix is poultry muscle tissue (turkey, duck, guinea fowl) and with satisfactory qualification, the method has been extended to different other species and animal food products: chicken, quails, swines, shrimps, whole egg and honey. The method is validated on a multi-residue scale with simultaneous analysis of DNSAH, AOZ, AMOZ, SEM, AHD (Fig. 1) as their derivatized nitrophenyl counterparts: NPDNSAH, NPAOZ, NPAMOZ, NPSEM, NPAHD (Fig. 2). The calibration of the five analytes is carried out taking into account an internal standard correction as follows: d_4-AOZ for the AOZ, d_5-AMOZ for the AMOZ, $^{13}C_3$-AHD for the AHD, $^{13}C^{15}N_2$-SEM for the SEM and nifuroxazide (further hydrolyzed in salicylic acid hydrazine SAH) for the DNSAH.

For each analyte, the performance of the method is assessed through its qualitative parameters: analyte specificity, molecular identification in term of retention time (RT), of signal-to-noise ratio and of transition ion ratios, and also through its quantitative parameters: linearity, accuracy in term of trueness and of precision expressed as the intra- and inter-day/series repeatabilities, and analytical limits (limit of decision CCα and capacity of detection CCβ). The validation is planned with a set of eight series of analyses dispatched in four batches of different muscle tissues (two turkey batches, one guinea fowl batch and one duck batch) analysed against two different parameters (temperature/time conditions of hydrolysis and solvent washing/non washing conditions prior to analysis) (Table 2). For each of the eight series, the experimental plan (Table 3) comprises of 2 sets of 14 samples: 6 calibrating samples (further named standard of

Table 2
Planification of the eight series of analyses

Turkey batch 24	Washing + hydrolysis 37 °C	No washing + hydrolysis 37 °C	No washing + hydrolysis 55 °C
Turkey batch 27	Washing + hydrolysis 37 °C	No washing + hydrolysis 37 °C	No washing + hydrolysis 55 °C
Guinea fowl batch 25	Washing + hydrolysis 37 °C	Not tested	Not tested
Duck batch 42	Washing + hydrolysis 37 °C	Not tested	Not tested

Hydrolysis at +37 °C is carried out during 16 h (1 night); Hydrolysis at +55 °C is carried out during 4 h.

Table 3
Experimental plan for validation

Samples	Level	Concentration ($\mu g\,kg^{-1}$)	Parameters estimated
Calibrating samples (SC or SE)	1	0.0 (Fortified matrix)	- Specificity
	2	0.5 (Fortified matrix)	- Identification criteria (2 transitions, RT, ion ratio, S/N)
	3	1.0 (Fortified matrix)	- Regression parameters for each batch of analyses: linearity
	4	1.5 (Fortified matrix)	- Analytical limits: CCα and CCβ for each batch of analyses
	5	2.0 (Fortified matrix)	
	6	5.0 (Fortified matrix)	
Validating samples (SV)	1	0.3 (Fortified matrix)	- Specificity
	1	0.3 (Fortified matrix)	- Identification criteria (2 transitions, RT, ion ratio, S/N)
	2	0.7 (Fortified matrix)	- Back-calculation of estimated concentrations
	2	0.7 (Fortified matrix)	- Linearity of the validating standards
	3	1.2 (Fortified matrix)	- Accuracy in term of trueness
	3	1.2 (Fortified matrix)	- Precision in term of repatability intra-series (r)
	4	3.8 (Naturally incurred matrix)	- Precision in term of repatability inter-series (R)
	4	3.8 (Naturally incurred matrix)	

calibration SE) and 8 validating samples (further called standard of validation SV). These two different sets of samples allow to simulate their analysis on a routine basis, i.e. the SE samples simulating the routine calibration and the SV samples (possibly analysed in blind) simulating the real-life control samples.

2.4.1.1. Specificity. This parameter is assessed for each of the analytes directly onto the chromatograms obtained from standard solutions, blank tissue matrices, and fortified tissue matrices; and when possible also from naturally incurred tissue matrices. It consists of both detecting any extra-peaks in the retention time window of the analyte for the two multiple reaction monitoring (MRM) transitions of interest onto the blank matrix chromatograms and also checking the matching of the retention time observed for the spiked analytes compared to the aqueous standard analytes with a tolerance of ±2.5% according to CD 657/2002/EC (point 2.3.3.1).

2.4.1.2. Identification. The criteria for identification are those of liquid chromatography coupled to tandem mass spectrometry. Identification of the analyte in the matrix is based on four criteria: the stability of the chromatographic retention time better than 2.5% when compared to the retention time in the standard solution, the presence of the two relevant transitions from the analyte molecular peak (Table 1), a signal-to-noise ratio of the ionic transitions greater than 3, and the stability of the ion ratio between the two transitions for each analyte in accordance with the tolerances recommended by the CD 657/2002/EC (point 2.3.3.2) (Table 4). The ion ratio of each analyte is effectively measured on each of the chromatograms. It always corresponds to the less intense signal (low transition) against the most intense one (high transition). The mean ion ratio is calculated taking into account the calibrating standards from 0.5 to 5.0 $\mu g\,kg^{-1}$. The relative deviation (%) of the ion ratios measured on the SVs, spiked or naturally incurred, compared to the mean ion ratio of the SEs is then calculated and compared to the acceptable tolerances.

Table 4
Tolerances for ion ratios according to Commission Decision no. 657/2002/EC

Ion ratios (less intense signal/most intense signal)	Tolerances in LC–MS/MS (%)
≥50%	±20
≥20% et <50%	±25
≥10% et <20%	±30
<10%	±50

2.4.1.3. Statistical analysis of the linearity. The adjustment of the linearity of the estimated concentrations for the validating standards, SVs, at 0.3, 0.7 and 1.2 $\mu g\,kg^{-1}$ and also at 3.8 $\mu g\,kg^{-1}$ for DNSAH specifically, back-calculated from the matrix-match calibrating standards, SEs, at 0.5, 1.0, 1.5, 2.0 and 5.0 $\mu g\,kg^{-1}$, are investigated using the following statistical tools: a linear regression (by analysis of the mean least squares), the calculation of the coefficient of determination (R^2), the analysis of variance (ANOVA) of the linear regression and the testing of the slope and of the intercept (Student's t-test).

2.4.1.4. Accuracy in term of trueness and recovery. The percentage of trueness of the estimated concentration of the SVs back-calculated from the matrix-match SEs and expressed as the bias to the real spiked concentration was estimated for each analyte and at each level of concentration of the SVs at 0.3, 0.7 and 1.2 $\mu g\,kg^{-1}$ and also at 3.8 $\mu g\,kg^{-1}$ for DNSAH specifically. The estimated concentrations of the SVs were calculated from the most intense signal (Transition no. 1) after correction from the appropriate internal standard. The estimation of the recovery for the five analytes was not considered because the calibration is effectively including this parameter through the matrix-match calibrating standards (SEs) corrected by the appropriate internal standards.

2.4.1.5. Precision. The precision in terms of repeatability and intra-laboratory reproducibility was evaluated by calculating the relative standard deviation (R.S.D.) of the results obtained from the SVs and for each analyte and at each level of concentration. The repeatability and reproducibility were particularly examined to evaluate the within- and between-day/series variations. One batch (series) is analysed each day.

2.4.2. Analytical limits

2.4.2.1. Limit of decision CCα, and capacity of detection CCβ. The two analytical limits recommended in the European decision no. 657/2002/EC [6], **CCα**, the critical concentration at risk alpha also called the limit of decision (parag. 3.1.2.5 of CD 657/2002/EC), and **CCβ**, the critical concentration at risk beta also called the capacity of detection of the method (parag. 3.1.2.6 of CD 657/2002/EC), were both calculated taking into account the two possible routes of calculation proposed in the European Decision, i.e. either as it is stated in the ISO Standard 11843 [38] or with the calculation of the signal-to-noise ratio for 20 blank materials per matrix (samples from different origin).

The Decision limit (CCα) means: "... the limit at and above which it can be concluded with an error probability of α (1% for non-authorized substances) that a sample is non compliant ..." (EC 657/2002 Annex 1, 1.11). The principle of the calculation is:

$$\mathrm{CC}\alpha = \mathrm{Cy_o} + 2.33 \times \mathrm{Sy_o}$$

with $\mathrm{Cy_o}$ the concentration back-calculated at the intercept of the regression line, and $\mathrm{Sy_o}$ the standard deviation obtained from the regression line for the intercept.

In the case of the ISO Standard 11843, the calculation derived from the analysis of variance of the regression leads to the following equation [39]:

$$\mathrm{CC}\alpha = t_{1-\alpha}(IJ-2)\frac{\hat{\sigma}}{\hat{b}}\sqrt{\frac{1}{K}+\frac{1}{IJ}+\frac{X^2}{\sum(x_{ij}-X)^2}}$$

where $\boldsymbol{t_{(\alpha,IJ-2)}}$ is the Student's t at risk of α for $\boldsymbol{IJ-2}$ degrees of freedom,

$\boldsymbol{\sigma}$ is an estimation of the residual standard deviation of the regression function;
$\boldsymbol{b}$ is the slope of the regression function;
$\boldsymbol{I}$ the number of levels of calibration;
$\boldsymbol{J}$ the number of replications of the calibrants;
$\boldsymbol{K}$ the number of replication of the control sample(s) analysed on a routine basis;
$\boldsymbol{X^2}$ the grand mean square of the concentrations;
$\boldsymbol{\Sigma(x_{ij}-X)^2}$ the sum of the squares of the residual variations of the concentrations.

In the case of the estimation based on the 20 blank materials, the calculation leads to the following equation:

$$\mathrm{CC}\alpha = 3 \times \frac{S}{N_{20\ \mathrm{blank\ samples}}}$$

with S/N the signal-to-noise ratio obtained as the mean of 20 representative blank samples.

The capacity of detection (CCβ) means: "... the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (5% for non-authorized substances) ...". In the case of substances for which no permitted limit has been established (i.e. MRPL substances), this means that detection capability is the lowest concentration at which the method is able to detect truly contaminated samples with a statistical certainty of $1-\beta$ (95%) (EC 657/2002 Annex 1, 1.12). For non-authorized substances, the detection capability is calculated above the CCα and is also depending on the precision of the method. CCβ can be calculated as follows:

$$\mathrm{CC}\beta = \mathrm{CC}\alpha + 1.64 \times \mathrm{S.D._{CC\alpha}}$$

with $\mathrm{S.D._{CC\alpha}}$ the standard deviation at the CCα level of concentration which can also be estimated by calculating the within-lab reproducibility of the method at this level of concentration.

In the case of the ISO Standard 11843, the calculation derived from the analysis of regression leads to the following equation [39]:

$$\mathrm{CC}\beta = \delta\frac{\hat{\sigma}}{\hat{b}}\sqrt{\frac{1}{K}+\frac{1}{IJ}+\frac{X^2}{\sum(x_{ij}-X)^2}}$$

where $\boldsymbol{\delta_{(IJ-2;\alpha;\beta)}}$ is a statistical function that can be approximated by using $\boldsymbol{2.t_{1-\alpha}(IJ-2)}$.

$\boldsymbol{t_{(\alpha,IJ-2)}}$ is the Student's t at risk of α for $\boldsymbol{IJ-2}$ degrees of freedom;
$\boldsymbol{\sigma}$ is an estimation of the residual standard deviation of the regression function;
$\boldsymbol{b}$ is the slope of the regression function;
$\boldsymbol{I}$ the number of levels of calibration;
$\boldsymbol{J}$ the number of replications of the calibrants;
$\boldsymbol{K}$ the number of replication of the control sample(s) analysed on a routine basis;
$\boldsymbol{X^2}$ the grand mean square of the concentrations;
$\boldsymbol{\Sigma(x_{ij}-X)^2}$ the sum of the squares of the residual variations of the concentrations.

In the case of the estimation based on the 20 blank materials, the calculation leads to the following equation:

$$\mathrm{CC}\beta = \mathrm{CC}\alpha + 1.64 \times \mathrm{S.D._{20\ CC\alpha\ samples}}$$

with CCα expressed as $3 \times \mathrm{S/N_{20\ blank\ samples}}$, and $\mathrm{S.D._{20\ CC\alpha\ samples}}$ expressed as the standard deviation obtained from the 20 representative blank samples spiked at CCα level.

All the limits discussed in this paper (CCα, CCβ) are calculated for each of the eight series and are taking into account the less intense and/or the most variable signal for which the most critical analytical limit shall always be met. The limits for CCα and for CCβ accepted for the validation are the medians of the eight results. Moreover, the limits obtained through each of the two routes of calculation (either by the regression line and ISO Standard 11843 or by the signal-to-noise ratio and 20 blank materials) are further subjected to comparison. The calculation took into account only four different blank materials and analysed them twice in three different conditions (Table 2).

3. Results and discussion

3.1. Applicability

The method was successfully assessed by analyzing the five nitrofuran metabolites after their spiking in poultry muscle tissues (turkey, guinea fowl, duck) during the validation but also after their spiking in chicken, quail and swine muscle tissues, in deshelled shrimp, in whole egg (yolk and white in natural proportion) and in natural honey during several routine analyses performed further on after the validation. The method was also successfully implemented during the validation for analyzing DNSAH metabolite in a set of frozen samples of turkey muscle tissue obtained from a natural contamination during an animal study on turkeys fed with nifursol additive about 1 year ago in Germany [22].

The eight different series of analysis ($n = 112$ samples) show the possibility to modify the hydrolysis-derivatization step in terms of temperature and time moving from 16 h at +37 °C (six series—$n = 84$ samples) to 4 h at +55 °C (two series—$n = 28$ samples) without any significant change in the resulting data. The scope of the method can easily be extended from turkey muscle tissue to other poultry species and also to other matrices such as honey, whole egg and swine tissue. The method can either be implemented on a tissue-bound nitrofuran metabolite residue analysis scheme with solvent washing steps for confirmation of a nitrofuran veterinary treatment (Fig. 3A and B) or it can also be simply applied for a total (free + protein-bound) nitrofuran metabolite residue analysis scheme with no solvent washing steps prior to hydrolysis-derivatisation (Fig. 4A and B). The method is then more dedicated to screening for large quantities of samples in a more reduced frametime.

3.2. Stability

The stability of the metabolites in matrix sample was not studied specifically during this validation period but as an example the samples with nifursol natural contamination have been successfully analysed for their DNSAH content in the range of concentrations expected from the first analyses carried out more than 1 year ago in Germany (2–6 μg kg^{-1}) [22]. The other metabolites (AOZ, AMOZ, SEM, AHD) have also been examined for their stability in different biological matrices even though not extensively during several experiments or material

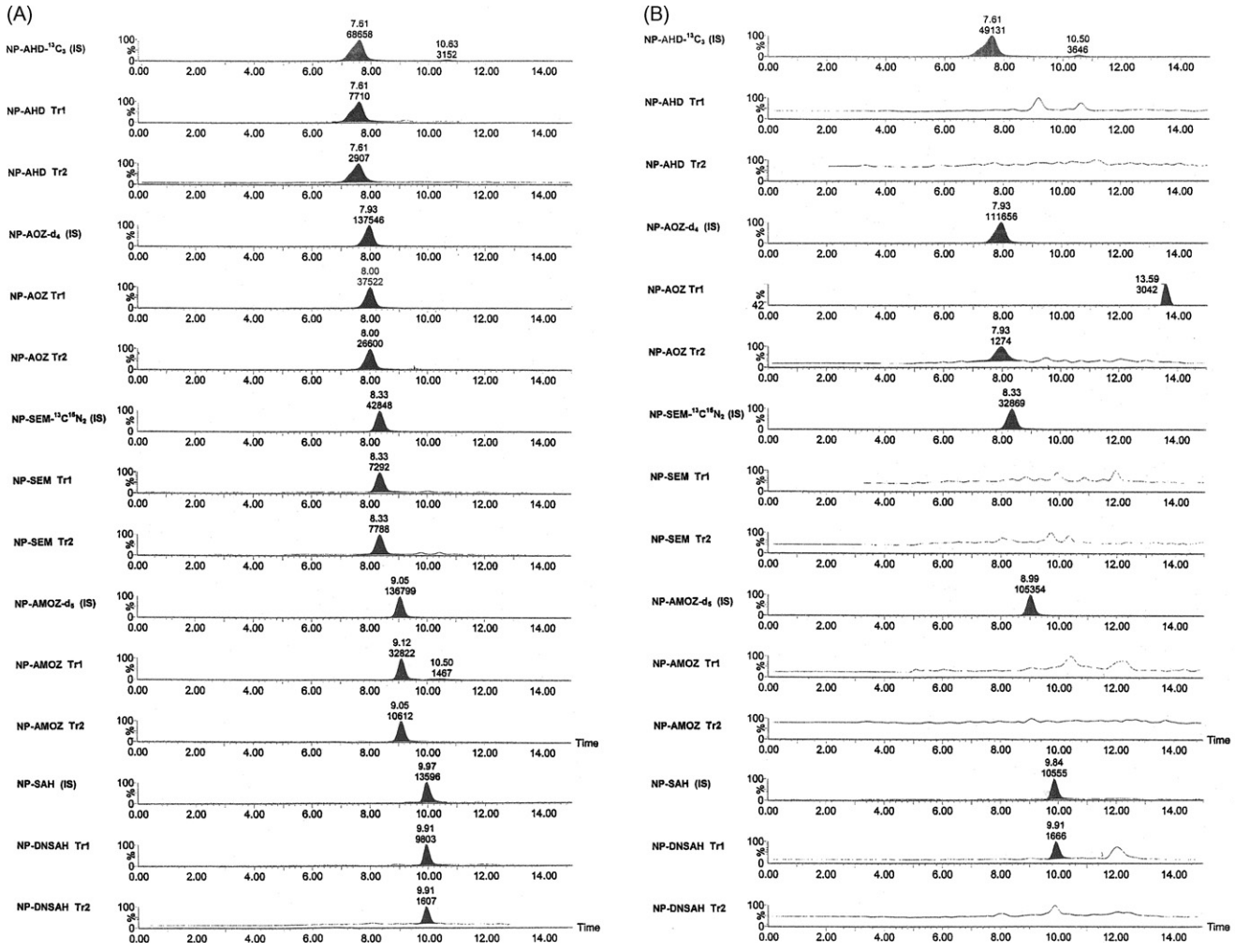

Fig. 3. (A) Chromatogram of a spiked turkey muscle tissue sample spiked at 1 μg kg^{-1} (with washing process applied). (B) Chromatogram of a blank turkey muscle tissue sample (with washing process applied).

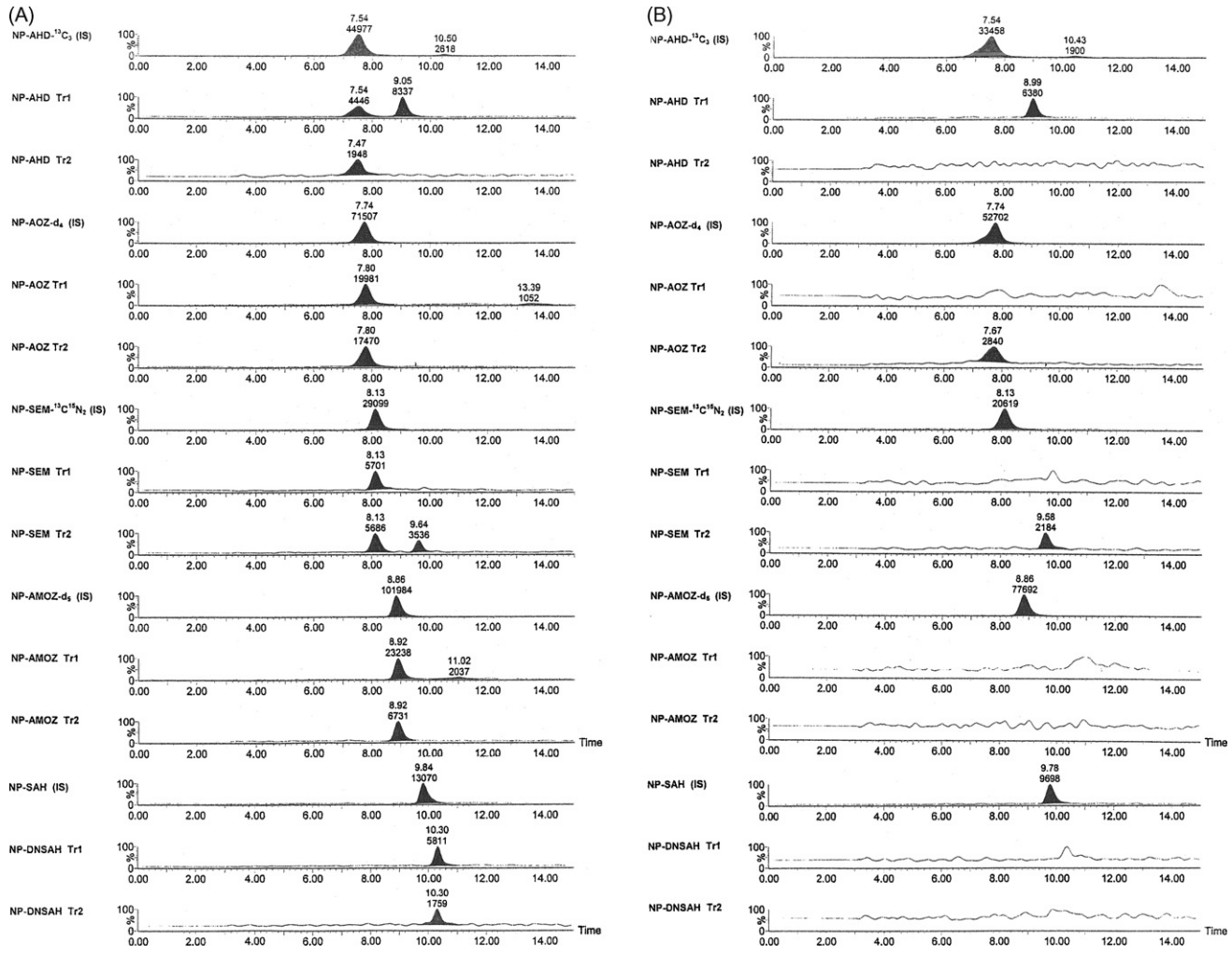

Fig. 4. (A) Chromatogram of a spiked turkey muscle tissue sample spiked at 1 μg kg^{-1} (with no washing process). (B) Chromatogram of a blank turkey muscle tissue sample (with no washing process).

preparation for proficiency testing [40–42]. The recognized persistence of these protein-bound metabolites in tissues for several weeks or even month after cessation of the treatment strongly speaks for a good stability observed with the samples stored in frozen conditions. "Even for the nifursol metabolite which is more quickly depleted than the furazolidone one [21–22], it is also observed a good stability of the residues when the samples are stored in frozen conditions."

3.3. Specificity and selectivity

Every analyte is structurally identified with two ion transitions each (Table 1). None of the analytes shares the same molecular mass or even the same transition. No interference from the matrix was observed that might disturb the signals except for one transition of the AOZ metabolite for which an interfering signal may appear in some matrices in a close vicinity of the retention time of the second transition (236 > 104). Another matrix interference was also considered for one of the transitions for DNSAH (376 > 166) specifically in the case of the tissue-bound residue analysis after the solvent washing steps (see Fig. 3B). All the chromatograms obtained throughout the validation study show a very good stability of the retention times for all the analytes both for the spiked samples and for the naturally contaminated samples with relative deviations always better than ±2.5% (Table 5). Consequently, the method is considered very specific for each of the five nitrofuran metabolites.

Table 5
Relative deviations of the chromatographic retention times for the five analytes and their internal standard counterparts

Analyte as their nitrophenyl derivatives	Mean Retention time (min)	Relative deviation
AHD	7.8	From −1.0% to +0.9%
$^{13}C_3$-AHD (IS)	7.8	–
AOZ	8.1	From −1.2% to +0.9%
D_4-AOZ (IS)	8.1	–
SEM	8.5	From −1.2% to +1.0%
$^{13}C^{15}N_2$-SEM (IS)	8.5	–
AMOZ	9.1	From −1.0% to +0.9%
D_5-AMOZ (IS)	9.1	–
DNSAH	10.0	From −0.9% to +0.6%
SAH (IS)	9.9	–

3.4. Identification

The mean ion ratios measured for the five analytes on the calibrating standards (SEs) were 22.3% for DNSAH, 28.4% for AHD, 89.1% for AOZ, 88.2% for SEM and 31.4% for AMOZ, respectively. The relative deviations of the ion ratios observed during the validation on the SVs ($n = 48$ for AHD, SEM, AMOZ and AOZ and $n = 64$ for DNSAH) were very satisfactory with regard to DNSAH, AHD, SEM and AMOZ. Only for AOZ were observed 2/48 analyses with unacceptable ion ratios at the smallest tested concentration of 0.3 μg kg^{-1} (Table 6).

3.5. Linearity

The 40 calibrating regression lines were all satisfactory with a coefficient of determination (R^2) always higher than 0.9970 for all the eight series over the five analytes. The tests for the linearity of the estimated concentrations of the SVs are satisfactory except for AMOZ for which the slope is considered different from 1 and the intercept different from 0. Consequently, the calibration for AMOZ might be performed with preferably taking into account a blank matrix calibrant. Moreover, care must be taken to never apply a single-level calibration for the estimation of the AMOZ concentration (Table 7).

3.6. Trueness

The results displayed in Table 6 show a satisfactory match of the estimated concentrations of the SVs for all the levels of concentration tested and for all the analytes monitored with this method. The worse bias of −4.9% is observed at 0.3 μg kg^{-1} for DNSAH but is still very acceptable with regard to the criteria of CD 657/2002/EC (point 2.3.2.1).

3.7. Precision

The results displayed in Table 6 show a satisfactory performance of the method in term of repeatability intra-day/intra-series and also in term of repeatability inter-day (8 days) and inter-series (8 series). This intra-laboratory precision is here accepted by the authors as the intermediate reproducibility of the method and successfully compared both to the criteria of CD 657/2002/EC (point 2.3.2.2) and to the published data on precision of analytical methods at ppb and sub-ppb levels from M. Thompson [43]. Only the samples containing DNSAH at 0.3 μg kg^{-1} level of concentration were not sufficiently precise on a inter-day/inter-series basis with an unacceptable intermediate reproducibility of 42.3%.

3.8. Limit of decision CCα, and capacity of detection CCβ

According to the two routes proposed in CD657/2002/EC for the calculation of the critical limits CCα and CCβ, the results obtained during this validation are summarized in Table 8. It is demonstrated that both CCα and CCβ critical limits are always in line with the minimum required performance limit set for the nitrofurans at MRPL = 1.0 μg kg^{-1}. Nevertheless, the nifursol

Table 6
Overview of the validation parameters in term of trueness, precision and relative ion ratios

Analyte	SV[a] (μg kg^{-1})	No. of samples	No. of series × replicates	Estimated concentration ± S.D. (μg kg^{-1})	Trueness (%)	Minimum trueness 657/2002/EC (%)	r intra-series (%)	R inter-series (%)	Precision 657/2002/EC r (%)	Precision 657/2002/EC R (%)	Ion ratio mean (Tr_2/Tr_1)	Relative ion ratios Minimum (%)	Relative ion ratios Maximum (%)	Tolerance ion ratios 657/2002/EC (%)
DNSAH	0.3	16	8 × 2	0.25 ± 0.10	−4.9	−50 to +20	11.1	42.3	15	22	22.5	−8.7	+11.9	±25
	0.7	16	8 × 2	0.70 ± 0.04	−0.1		5.7	5.7	15	22	22.0	−5.9	+11.7	
	1.2	16	8 × 2	1.21 ± 0.05	+0.6	−30 to +10	3.6	4.3	15	22	22.6	−3.4	+6.5	
	3.8[b]	16	8 × 2	3.81 ± 0.78	+0.7		3.5	21.2	15	22	22.2	−7.7	+5.3	
AHD	0.3	16	8 × 2	0.28 ± 0.03	−1.8	−50 to +20	9.8	12.4	15	22	23.6	−16.6	+15.1	±25
	0.7	16	8 × 2	0.71 ± 0.04	+1.0		3.9	6.5	15	22	27.0	−10.8	+9.0	
	1.2	16	8 × 2	1.20 ± 0.09	−0.1	−30 to +10	5.4	7.5	15	22	34.6	−4.3	+19.1	
AOZ	0.3	16	8 × 2	0.30 ± 0.04	+0.3	−50 to +20	3.8	14.4	15	22	99.2	−9.6	+40.9	±20
	0.7	16	8 × 2	0.71 ± 0.02	+1.3		2.3	3.0	15	22	85.5	−17.1	+6.7	
	1.2	16	8 × 2	1.21 ± 0.03	+1.1	−30 to +10	1.7	2.5	15	22	82.4	−19.2	+5.2	
SEM	0.3	16	8 × 2	0.29 ± 0.04	−0.7	−50 to +20	5.6	15.2	15	22	88.5	−19.1	+15.2	±20
	0.7	16	8 × 2	0.71 ± 0.03	+1.1		3.2	4.2	15	22	86.8	−12.1	+9.9	
	1.2	16	8 × 2	1.20 ± 0.04	0.0	−30 to +10	2.3	3.8	15	22	89.3	−7.1	+17.2	
AMOZ	0.3	16	8 × 2	0.27 ± 0.03	−3.4	−50 to +20	5.1	11.7	15	22	31.8	−3.6	+4.9	±25
	0.7	16	8 × 2	0.70 ± 0.02	+0.2		2.7	3.5	15	22	30.7	−4.2	+2.7	
	1.2	16	8 × 2	1.21 ± 0.04	+1.3	−30 to +10	1.7	3.2	15	22	31.5	−2.9	+2.7	

[a] Standards of validation.
[b] Nifursol naturally incurred turkey tissue samples.

Table 7
Overview of the validation parameters in term of linearity of the calibration

Analyte	No. of analyses	No. of series	Mean Slope S.D.	Slope test (calc. Student's *t*)	Mean intercept S.D.	Intercept test (calc. Student's *t*)	
DNSAH	64	8	1.1512	0.2795	−0.1210	1.615	Student's *t* factor
			0.0407	Accepted	0.0749	Accepted	(62,0.95) = 1.999
AHD	48	8	1.0171	0.7176	−0.016	0.802	Student's *t* factor
			0.0238	Accepted	0.0195	Accepted	(48,0.95) = 2.013
AOZ	48	8	1.0086	0.6800	0.0027	0.260	Student's *t* factor
			0.0126	Accepted	0.0103	Accepted	(48,0.95) = 2.013
SEM	48	8	1.0065	0.4132	−0.003	0.272	Student's *t* factor
			0.0156	Accepted	0.0128	Accepted	(48,0.95) = 2.013
AMOZ	48	8	1.0515	4.1208	−0.044	4.312	Student's *t* factor
			0.0125	Denied	0.0103	Denied	(48,0.95) = 2.013

Table 8
Analytical critical limits obtained from the validation

According to CD657/2002/EC		DNSAH ($\mu g\,kg^{-1}$)	AOZ ($\mu g\,kg^{-1}$)	AMOZ ($\mu g\,kg^{-1}$)	AHD ($\mu g\,kg^{-1}$)	SEM ($\mu g\,kg^{-1}$)
According to ISO11843 and calibrating samples	CCα (n = 8 series × 5 calibrants) with Tr1	0.19	0.08	0.13	0.20	0.20
	CCβ (n = 8 series × 5 calibrants) with Tr1	0.24	0.10	0.17	0.25	0.25
	CCα (n = 8 series × 5 calibrants) with Tr2	0.54	0.21	0.25	0.40	0.24
	CCβ (n = 8series × 5 calibrants) with Tr2	0.66	0.26	0.31	0.50	0.29
According to signal-to-noise ratio of 20 blanks[a]	CCα (n = 20 blank samples) with Tr1	0.47	0.14	0.01	0.01	0.02
	CCα (n = 20 blank samples) with Tr2	0.65	0.68	0.02	0.10	0.03

[a] Only CCα is calculated according to the signal-to-noise ratio of 20 blanks.

metabolite (DNSAH) still remains the most critical analyte to monitor with a CCα edging at 0.5–0.6 $\mu g\,kg^{-1}$. The comparison of the two routes for calculating the critical limits satisfactorily leads either to a close agreement between the values for DNSAH or to a more realistic higher critical value obtained when using the regression function (ISO Standard11843) for AOZ, AMOZ, SEM and AHD. These observed discrepancies can be explained by the less stringent calculation applied with the signal-to-noise ratio route. Actually, this calculation is essentially based on the mean deviation of the different noises of the 20 materials. With our method in all the different analysed samples, the noise is found to be very low for AMOZ, SEM and AHD signals (for transition no. 1 as for transition no. 2) and for AOZ (for transition no. 1). Conversely, the noise is found higher for AOZ signal (transition no. 2) because of the co-extractive interfering signal sometimes observed on the transition no. 2 (236 > 104) as it has been specified previously in this paper. As a consequence of this observation, it can be assumed that the CCα and CCβ calculation derived from the mean signal-to-noise ratio of 20 blanks is realistic in evaluating the analytical limits only from a range of signals all displaying a significant range of noises.

4. Conclusion

The monitoring of five nitrofuran metabolites in muscle tissue within the same method on a multi-residue frame is demonstrated to be acceptable and is validated according to the CD 657/2002/EC. The different series of analysis show the possibility to adjust the time frame of the method to the capacity of the laboratory. The hydrolysis-derivatization step can be changed from 16 h at +37 °C to 4 h at +55 °C with no significant change in the resulting data. The scope of the method can easily be extended from turkey muscle tissue to other poultry species and also to other matrices such as honey, whole egg and swine tissue. The method can also be implemented on a tissue-bound residue scheme, with solvent washing steps for confirmation of a nitrofuran veterinary treatment. Or it can be applied on a total nitrofuran residue analysis scheme, with no solvent washing steps prior to hydrolysis-derivatisation and then more dedicated to screening for many samples in a more reduced frametime.

The assessment of performance of the method demonstrated that the more variable signal and thus less precise analysis is clearly operated for the DNSAH, the nifursol metabolite. This can certainly be attributed to less intense ionic signals with regard to the other nitrofuran metabolites and also because of the lack of an isotopic internal standard which is substituted in this study by an analogue of the DNSAH: the SAH, obtained from another nitrofuran compound, the nifuroxazide. Yet, all the ionic signals (two transitions) are sufficiently expressed to give a satisfactory analysis for all the five nitrofuran metabolites and down to 0.5 $\mu g\,kg^{-1}$ as it was calculated through the analytical critical limits reported in Table 8.

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