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Multiresidue method for the determination of nitroimidazoles and their hydroxy-metabolites in poultry muscle, plasma and egg by isotope dilution liquid chromatography–mass spectrometry

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ABSTRACT

A multiresidue analytical procedure for the determination of four nitroimidazoles (metronidazole, dimetridazole, ronidazole, ipronidazole) and their hydroxy-metabolites in poultry muscle, plasma and egg is presented. The procedure is based on ion-exchange solid phase extraction with acetonitrile as an extractant followed by liquid chromatography–mass spectrometry. The separation of analytes was performed on a C18 column using a mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in water with gradient elution. The electrospray ionization was used to obtain the protonated molecules $[M+H]^+$ and two product ions were monitored for each compound. For the quantification stable isotope-labelled analogues of the analytes were used as internal standards. The whole procedure was evaluated according to EU Commission Decision 2002/657/EC requirements. Specificity, decision limit (CCα), detection capacity (CCβ), recovery and precision were determined during validation process. The overall recoveries ranged between 93 and 103% with a good coefficient of variation, less than 14.0% under within-laboratory reproducibility conditions. CCα and CCβ were 0.05–0.44 and 0.08–0.90 μg kg^{-1} depending on analyte and matrix.

1. Introduction

Nitroimidazoles are an important group of antibacterial compounds from a historical point of view. In the past they were used for the treatment and prevention of histomoniasis in poultry and haemorrhagic in pigs. The main nitroimidazole compounds: metronidazol (MNZ), dimetridazole (DMZ), ronidazole (RNZ) and ipronidazole (IPZ) show mutagenic and carcinogenic properties [1–3]. For this reason, the European Union has prohibited their use as veterinary drugs for food-producing species and all of them are currently in Annexe IV to Council Regulation (EEC) No. 2377/90 [4–7], meaning that any residues of parent nitroimidazole compounds or their metabolites in tissues of food-producing animals or in products intended for human consumption has to be considered as a violation of the EU regulation. However the technical recommendation for the analytical methods has been given by the Community Reference Laboratory (CRL) in Berlin for residues of beta-agonists, anticoccidials including nitroimidazoles, anthelmintics and non-steroidal anti-inflammatory drugs and the so-called "recommended concentration" for the most important nitroimidazoles and their hydroxy-metabolites is 3 μg kg^{-1} [8].

Nitroimidazoles are characterized by their rapid metabolism within a few hours after their administration. For DMZ and RNZ, the major metabolite formed by hydroxylation is 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI). In the same way, MNZ gives the hydroxymetronidazole (MNZOH) and IPZ is metabolized into hydroxyipronidazole (IPZOH). The metabolites formed may have a similar mutagenic potential as the parent compounds. Depletion studies have demonstrated rapid elimination of nitroimidazoles and their metabolites from the animal body. Additionally, nitroimidazoles are inhomogenously distributed in muscle and liver tissue and they are sensitive to rapid degradation in unfrozen incurred tissues. Plasma was found as good matrix to check for the presence of nitroimidazoles, due to good stability of the compounds during storage and a larger concentration than other tissues [9,10].

Several analytical procedures have been developed for the determination of nitromidazoles and their metabolites in animal tissues [10,12,13,21], eggs [14,15] and plasma [9,16]. Liquid chromatography coupled with ultra-violet detection [19–21] and gas chromatography with nitrogen-phosphorus detection [18] have been developed for determination of two or four analytes in one or two matrices. After the use of nitroimidazoles as veterinary drugs in food-producing animals was banned, the analytical procedures utilized gas chromatography–mass spectrometry [9,11,12] and liquid chromatography–mass spectrometry [13,17,21] have been devel-

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Table 1
MS/MS parameters used for nitroimidazole compounds confirmation.

Analyte	Ion transition (*m*/*z*)	Declustering potential (V)	Collision energy (eV)	Cell exit potential (V)	Peak area ratio ± tolerance (%)
MNZ	172 → 128[a]	45	22	13	0.44 ± 25
	172 → 82	45	36	15	
MNZ-d3	175 → 131	45	22	13	
MNZOH	188 → 126[a]	40	26	12	0.91 ± 20
	188 → 123	40	20	12	
MNZOH-d2	190 → 125	40	20	12	
DMZ	142 → 96[a]	45	24	18	0.31 ± 25
	142 → 81	45	38	15	
DMZ-d3	145 → 99	45	24	18	
RNZ	201 → 140[a]	40	17	14	0.17 ± 30
	201 → 55	40	35	10	
RNZ-d3	204 → 143	40	17	14	
HMNNI	158 → 140[a]	40	18	14	0.21 ± 25
	158 → 55	40	30	10	
HMNNI-d3	161 → 143	40	18	14	
IPZ	170 → 124[a]	55	26	12	0.95 ± 20
	170 → 109	55	36	10	
IPZ-d3	173 → 127	55	26	12	
IPZOH	186 → 168[a]	45	20	17	0.44 ± 25
	186 → 122	45	30	12	
IPZOH-d3	189 → 171	45	20	17	

[a] Ion transition used for quantification.

oped. Most of these methods are focused on the determination of parent nitroimidazole compounds and their metabolites in one or two matrices. Up to now only one analytical method has been described for the determination of nitromidazoles in so various matrices such as muscle, plasma and retina after using the same sample preparation protocol [9].

In sample preparation procedures mainly ethyl acetate or acetonitrile as organic extraction solvent have been used followed by solid phase extraction (SPE) clean-up [12,19–21] or without further clean-up [6,13,14,17]. Moreover an extensive and complicated sample preparation procedure has been developed using protease for the matrix decomposition [16].

The present work reports a simple and fast analytical procedure for the determination of MNZ, DMZ, RNZ, IPZ and their metabolites (MNZOH, HMMNI, IPZOH,) in poultry muscle, plasma and egg by LC–MS/MS utilizing isotope dilution. Moreover the method is suitable for the determination of nitroimidazole compounds from different kinds of matrices after using the same sample preparation protocol and has been validated according to the quality criteria of Commission Decision 2002/657/EC [22] showing that such sample processing can provide the proper results.

2. Experimental

2.1. Reagents

Acetonitrile, acetone, methanol, formic acid, acetic acid (99.5%) and ammonium hydroxide (25%) (HPLC grade) were provided by J.T. Baker (Deventer, The Netherlands). Anhydrous sodium sulphate was obtained from Sigma–Aldrich (Steinheim, Germany). Water was purified trough a Mili-Qplus system from Millipore (Bedford, MA, USA). Analytical standards of MNZ (purity 99.9%), DMZ (purity >98%), RNZ (purity 99%) were from Riedel-de Haën (Seelze, Germany) whereas IPZ (purity >99%), MNZOH (purity >99.5%), HMMNI (purity >99%), IPZOH (purity >99.5%), DMZ-d3, RNZ-d3, IPZ-d3, MNZOH-d2, HMMNI-d3, IPZOH-d3 were obtained from Witega Labs. (Berlin, Germany). MNZ-d3 was purchased from Chimete Srl (Rivalta Scrivia, Italy). All deuterated nitroimidazoles were of chemical and isotopic purity >99.9%.

Individual stock solutions of reference compounds at 1000 μg ml^{-1} were prepared in acetonitrile (stable for at least 6 months at −20 °C). Stock solutions of nitroimidazoles were combined and diluted in acetonitrile to prepare intermediate standard solution of 10 μg ml^{-1} (stable for at least 6 months at −20 °C). For sample fortification working standard solutions of nitroimidazoles at 1 and 0.1 μg ml^{-1} were prepared in 0.1% formic acid in water (stable for at least 3 months at 4 °C).

Stock solutions of deuterated nitroimidazoles were combined and diluted in acetonitrile to prepare intermediate internal standard solution of 10 μg ml^{-1} (stable for at least 6 months at −20 °C). For sample fortification purpose, working internal standard solution at 1 and 0.1 μg ml^{-1} was prepared in 0.1% formic acid in water (stable for at least 3 months at 4 °C).

Working standard solutions of nitroimidazoles with working internal standard solution of deuterated nitroimidazoles were further diluted to produce LC–MS/MS calibration standards at concentrations of 0, 25, 50, 75, 100, 250 ng ml^{-1}. The concentration of all internal standards was 50 ng ml^{-1}.

Table 2
Robustness test (minor changes): selected factors and the levels of variation.

Selected factor	Abbreviation	High level	Low level
Incubation time of fortifying (min)	A, a	20	10
Acetonitrile (ml)	B, b	60	40
Extraction time (min)	C, c	2.0	0.5
Sodium sulphate (g)	D, d	20	10
SPE cartridge elution (ml)	E, e	6	4
Ammonium hydroxide (%)	F, f	5.5	4.5
Eluate evaporation	G, g	To dryness	To dryness + 5 min

Table 3
The mean amount of co-extracted endogenous compounds from matrix ($n = 6$).

Matrix	Extractant	Amount of co-extracted compounds (%)
Muscle	Ethyl acetate	4.29
	Dichloromethane	6.32
	Acetonitrile	1.08
Plasma	Ethyl acetate	0.97
	Dichloromethane	1.09
	Acetonitrile	0.56
Egg	Ethyl acetate	9.04
	Dichloromethane	9.88
	Acetonitrile	1.20

2.2. Sample preparation

Samples were taken from chicken never treated with any nitroimidazole compounds. Muscle and egg were homogenised whereas the whole plasma was centrifuged and stored at −20 °C.

A portion of each sample (5 g) was weighed into a 100 ml polypropylene centrifugal tube. Then, 100 μl of a 0.1 μg ml^{-1} internal standard solution was added to each sample to yield 2.0 μg kg^{-1}. After 15 min equilibration time, 50 ml of acetonitrile was added into the tube. The sample was homogenised at high speed for 1 min, and 5 g of anhydrous sodium sulphate was added. The tube was sealed and shaken vigorously for 1 min, then centrifuged at 2200 × *g* for 10 min at 4 °C.

The upper layer was filtered through anhydrous sodium sulphate (15 g in Whatman No. 4 filter paper) directly into SCX solid phase extraction cartridge fitted with a reservoir and preconditioned with the mixture of acetonitrile and acetic acid (95:5, v/v). Then, 5 ml of acetic acid was added to the reservoir and the content was well mixed. The solution obtained was allowed to run through the SPE cartridge at a rate of approximately 50 drops per min. The cartridge was washed sequentially with 2.5 ml of acetone, 5 ml of methanol and 5 ml of acetonitrile. The residual acetonitrile was removed under vacuum and the nitroimidazoles were eluted into a glass test tube with 5 ml of the mixture of acetonitrile and ammonium hydroxide (95:5, v/v). The content of the test tube was evaporated to dryness under a stream of nitrogen at 40 °C and the residue was dissolved with 200 μl of the mobile phase.

2.3. Liquid chromatography–mass spectrometry equipment and conditions

The LC–MS/MS system consisted of an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, a degasser, an autosampler, a column heater, a switching valve (Valco Instruments Co., Inc., USA) and an API 4000 triple quadrupole mass analyser with a TurboIonSpray source (Applied Biosystems/MDS SCIEX, Toronto, Canada). All the experiments were carried out in the positive ion electrospray mode. The Analyst 1.4.2 software controlled the LC–MS/MS system and processed the data. The TurboIonSpray source was operated at 400 °C with the capillary voltage set at 5500 V. Nitrogen was used as nebuliser gas (1.77 l min^{-1}), curtain gas (1.70 l min^{-1}) and collision gas (0.76 l min^{-1}). The mass spectrometer settings were optimised both with direct infusion of each standard solutions (0.1 μg ml^{-1}) from a syringe pump at the rate of 10 μl min^{-1} and with a LC-injection. The fragmentation reactions used for monitoring were selected on the basis of their significance in the product ion spectra. The analytes were quantified using selected reaction monitoring (SRM) mode with a dwell time 100 ms used for each transition to obtain between 15 and 25 data points per chromatographic peak (Table 1). Both Q1 and Q3 quadrupoles were maintained at unit resolution. The chromatographic separation was performed on a Gemini octadecyl analytical column (150 mm × 2.0 mm, 5 μm) with an octadecyl guard cartridge (4 mm × 2 mm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in water). The linear gradient was 10% A at 0 to 1 min, 90% A from 8 to 11 min and then 10% A from 18 to 30 min. The flow rate was 200 μl min^{-1} at ambient temperature and the injection volume was 30 μl.

2.4. Method validation

The whole procedure was validated in accordance with Commission Decision 657/2002/EC [22]. The validation study was performed in terms of specificity, recovery, repeatability, within-laboratory reproducibility, decision limit, detection capability, stability and ruggedness.

Method quantification was based on peak area and was performed using internal standard solvent-based calibration curve with 1/*x* weighting obtained from analysing LC–MS/MS calibration standards.

To evaluate possible interferences encountered in the method, the specificity of the method was verified by analysing 10 different blank samples of each tested matrices.

For the matrix-matched calibration curves, blank samples fortified with working standard solutions of analytes at six concentration levels (0, 1, 2, 3, 4, and 10 μg kg^{-1}) were analysed on three different days.

Table 4
Performance data of the LC–MS/MS method for the analysis of nitroimidazole compounds in poultry muscle, plasma and egg samples at concentration levels: 1, 2, and 3 μg kg^{-1} (mean values, $n = 54$).

	MNZ	MNZOH	DMZ	RNZ	HMNNI	IPZ	IPZOH
Muscle							
Recovery (%)	98.4	97.7	98.9	99.3	94.4	100.8	104.1
Repeatability, (CV,%)	5.3	6.8	6.6	8.5	4.5	8.0	6.6
Reproducibility, (CV,%)	10.1	10.4	8.7	9.4	4.9	13.2	13.5
Uncertainty (%)	10.5	12.0	9.0	8.0	6.5	16.0	16.5
Plasma							
Recovery (%)	103.4	97.9	97.9	96.1	102.4	96.8	94.1
Repeatability, (CV,%)	3.7	5.9	8.5	3.1	2.5	9.0	7.5
Reproducibility, (CV,%)	11.0	13.0	11.0	6.9	3.5	13.6	14.0
Uncertainty (%)	11.5	15.0	10.0	7.5	6.0	16.5	16.5
Egg							
Recovery (%)	93.4	97.5	99.5	96.1	102.0	99.0	97.8
Repeatability, (CV,%)	7.7	9.9	6.5	2.1	3.5	9.4	6.5
Reproducibility, (CV,%)	10.0	13.3	7.0	6.8	5.5	12.6	13.2
Uncertainty (%)	13.0	17.0	6.5	7.0	7.5	15.0	16.0

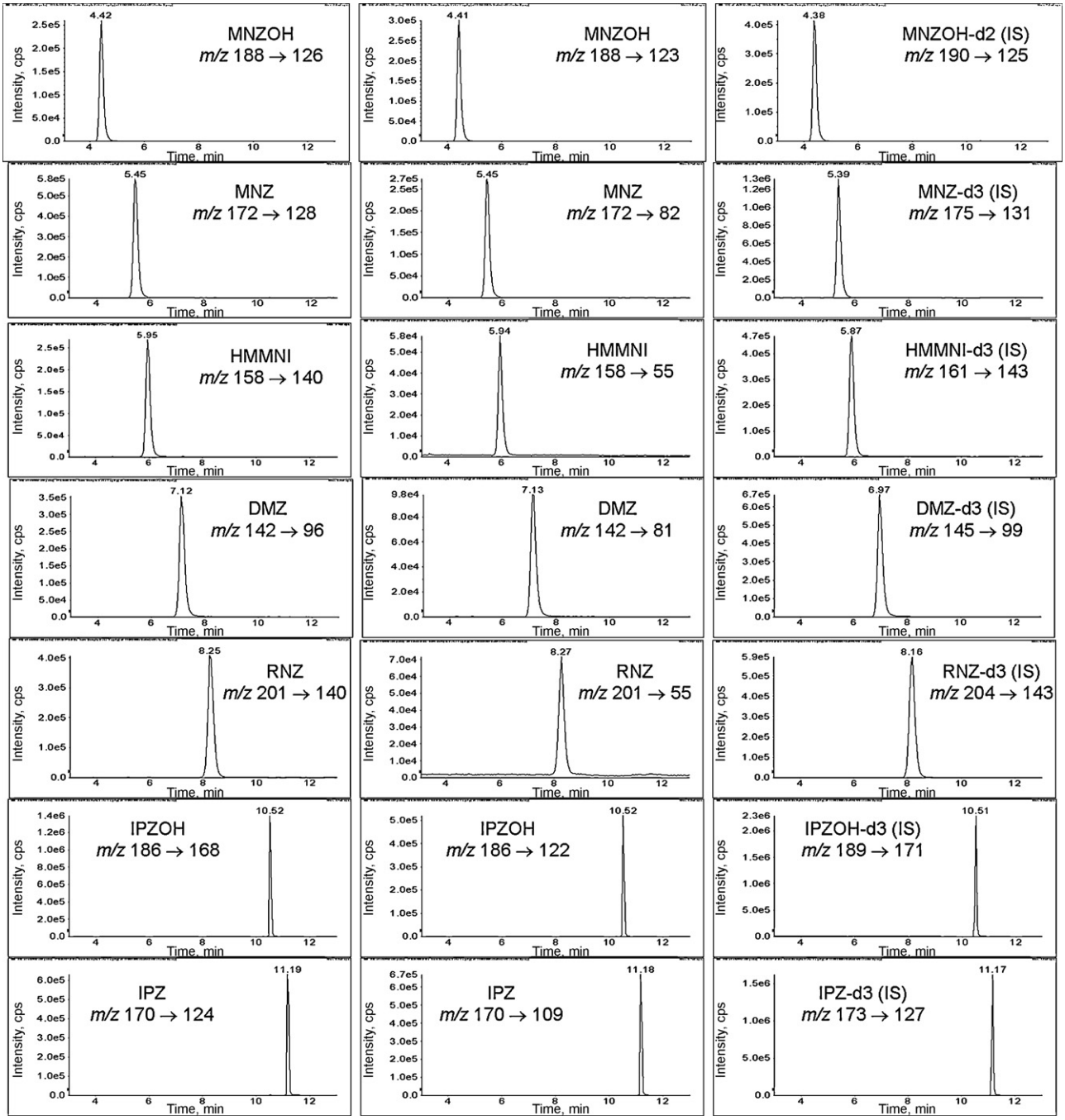

Fig. 1. Chromatograms of analytes and internal standards in poultry muscle fortified at the 1 μg kg^{-1} concentration level.

The repeatability was determined by fortifying six blank samples at each of three concentration levels 1, 2 and 3 μg kg^{-1} with nitroimidazole compounds. The samples were analysed on the same day with the same instrument and the same operators and the coefficients of variation (CV, %) of the fortified samples were calculated.

The within-laboratory reproducibility was determined by fortifying another two sets of blank samples at the same concentration levels of analysed compounds as for the repeatability and analysing on two different days with the same instrument and different operators and the overall coefficients of variation of the fortified samples were calculated.

The percentage recovery was evaluated in the same experiment as repeatability by comparing the mean measured concentration with the fortified concentration of the samples.

The decision limit (CCα) and the detection capability (CCβ) were determined by the matrix-matched calibration curve procedure according to the ISO 11843 [23]. CCα was calculated with a statistical certainty of 1-α ($\alpha = 0.01$) whereas CCβ was calculated with a statistical certainty of 1-β ($\beta = 0.05$).

The stability of nitroimidazoles in matrix was investigated in fortified poultry muscle samples (3 μg kg^{-1}). The material was analysed twice on the day of preparation and after 1, 2, 6 and 12 weeks of storing at −20 °C.

Method ruggedness was estimated for both minor and major changes according to the Youden robustness test by adopting the experimental design described in Commission Decision 657/2002/EC [22]. Eight experiments for each tested matrices (fortified at 3 μg kg^{-1}) were carried out in order to estimate the effect on method robustness of minor changes in seven vari-

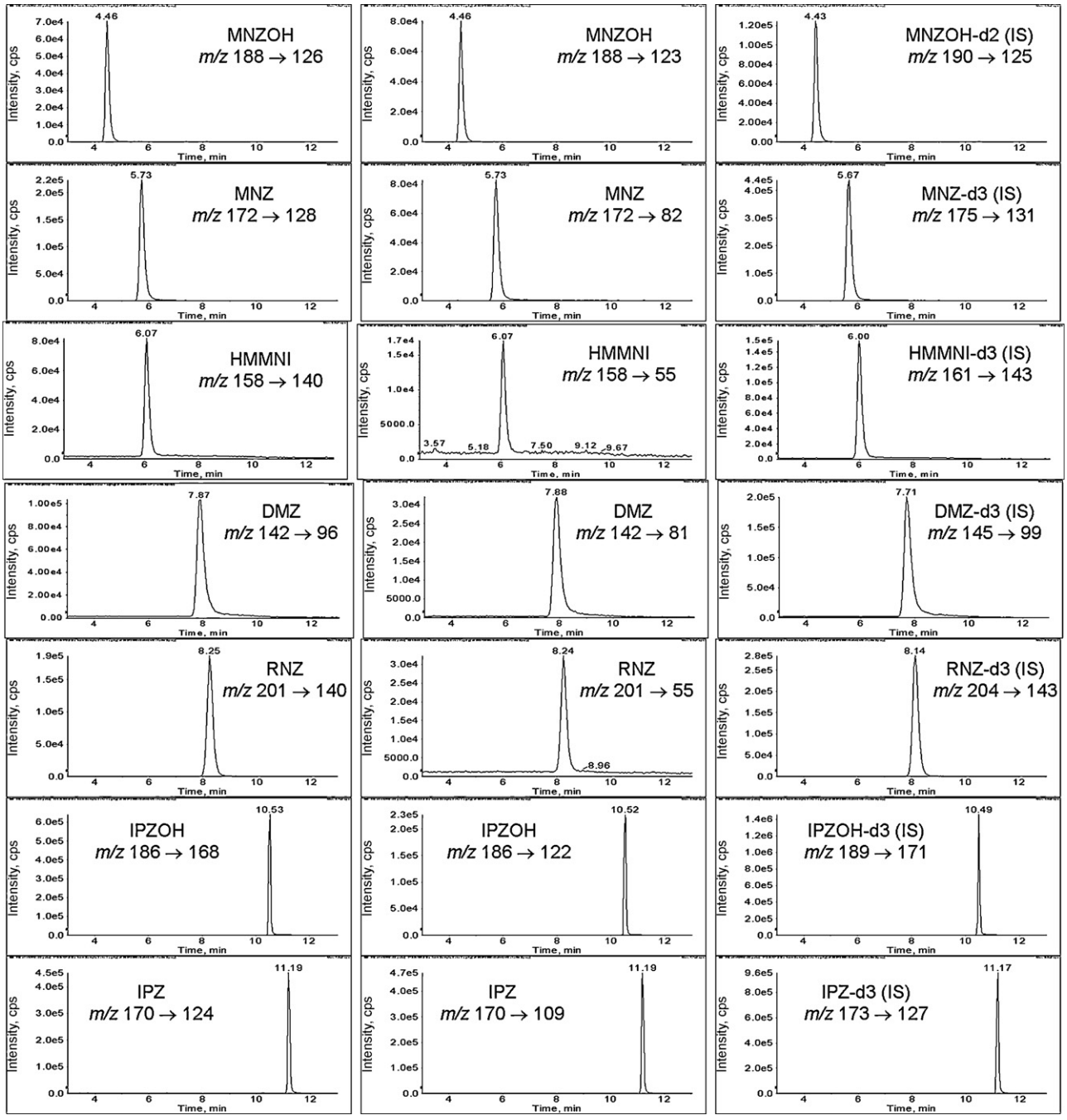

Fig. 2. Chromatograms of analytes and internal standards in poultry plasma fortified at the 1 μg kg^{-1} concentration level.

ables (Table 2) chosen from the sample preparation step. The major changes introduced included different species such as swine, bovine and fish samples. The standard deviation of the differences between recoveries at the two levels of each factor was calculated and compared to the standard deviation of the method carried out under within-laboratory reproducibility conditions.

3. Results and discussion

3.1. Sample preparation

Traditional sample preparation strategies for nitroimidazoles in animal tissues involve isolation with organic solvent (e.g. acetonitrile, dichloromethane, toluene or ethyl acetate) usually followed by SPE with polar, non-polar or ion-exchange sorbent materials [11–21].

In our preliminary studies, the extraction steps were performed with different organic solvents (ethyl acetate, dichloromethane, acetonitrile), and co-extraction of endogenous compounds from poultry muscle, plasma end egg samples was evaluated. In this experiment, the samples were treated with different solvents and centrifuged. After that, organic layers were separated, evaporated to dryness in preweighed test tubes, and the amount of co-extracted matrix was determined by differences in mass. As it was found, the most amounts of co-extracted compounds were isolated after sample treatment with dichloromethane as well as with ethyl acetate. In difference to non-polar solvent, the smallest ones were isolated after application of acetonitrile (Table 3), so we decided to use acetonitrile as the extraction solvent.

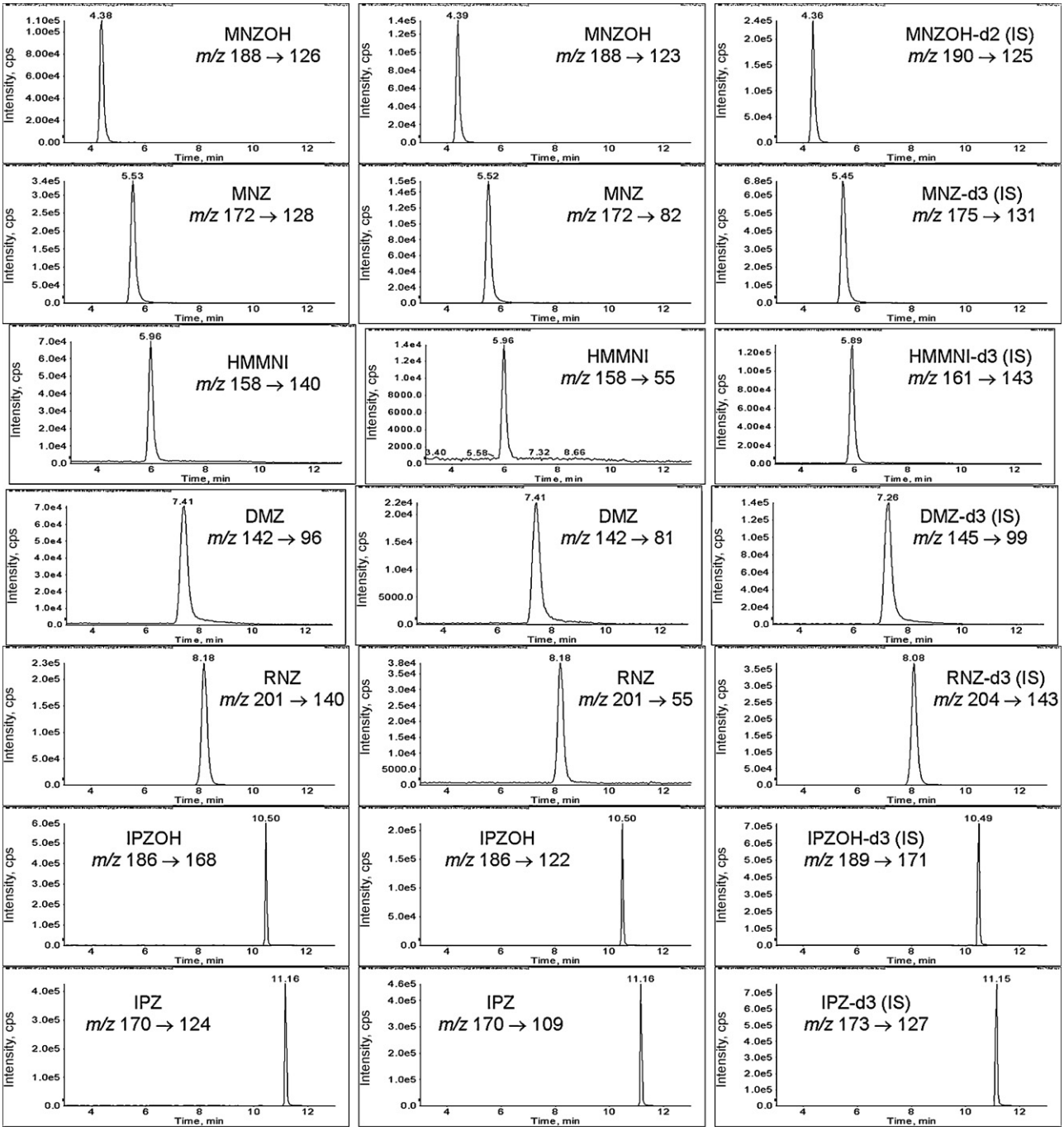

Fig. 3. Chromatograms of analytes and internal standards in poultry egg fortified at the 1 μg kg^{-1} concentration level.

Additionally, the use of acetonitrile allows for quantitatively isolating the analytes from fortified samples with simultaneous deproteinization of biological matrix, and coupling with the strong in-exchange SPE sorbent allows for omitting the critical evaporation of extraction solutions.

In this procedure, the protonation of the analytes was involved by addition of acetic acid as it was described previously [18,21]. The protonated analytes were retained on the cartridge by ionic interaction with the benzenesulphonic group bonded to the silica packing material. These ionic interactions are much stronger than the non-polar interactions taking place in reversed phase SPE, allowing for the use of an extensive wash sequence and giving cleaner extracts. The elution is achieved by addition of ammoniacal methanol, which deprotonates the analytes and breaks the ionic interaction. The final evaporation step at lower temperature (40 °C) improved absolute recoveries of analytes and was identified as critical for good recovery.

3.2. Liquid chromatography–mass spectrometry

The ion transitions monitored by LC–MS/MS and peak area ratios with their maximum permitted tolerances based on Commission Decision 2002/657/EC [22] obtained for nitroimidazole compounds confirmation are presented in Table 1 whereas typical SRM chromatograms of analytes and internal standards in poultry muscle, plasma and egg samples are presented in Figs. 1–3, respectively.

When analysing nitroimidazoles in muscle samples by LC–MS/MS with an ESI source, a negative matrix effect of ion

Table 5
CCα and CCβ obtained for nitroimidazole compounds determined in poultry muscle, plasma and egg samples by LC–MS/MS.

	MNZ	MNZOH	DMZ	RNZ	HMNNI	IPZ	IPZOH
Muscle							
CCα (μg kg^{-1})	0.20	0.22	0.14	0.16	0.17	0.37	0.42
CCβ (μg kg^{-1})	0.34	0.38	0.24	0.28	0.29	0.63	0.72
Plasma							
CCα (μg kg^{-1})	0.21	0.27	0.10	0.24	0.09	0.41	0.53
CCβ (μg kg^{-1})	0.36	0.46	0.17	0.41	0.16	0.70	0.90
Egg							
CCα (μg kg^{-1})	0.14	0.10	0.18	0.05	0.06	0.44	0.39
CCβ (μg kg^{-1})	0.23	0.16	0.30	0.08	0.11	0.76	0.66

suppression is a common problem. In this study, the matrix suppression was tested by comparing the intensity of signal from standard solution with those coming from analysis of blank matrix extracts fortified with standard solutions. The most evidence of ion suppression was found for IPZ and IPZOH in the extract prepared from muscle samples. It was interesting that the extract prepared from poultry muscle, plasma and egg had the same adverse effect. Slight ion suppression was determined for MNZ, MNZOH and RNZ in extract from egg. No matrix effect could be detected for DMZ and HMMNI in any tested matrices. The results described above can be mainly explained by the process of ionization of the sample at the entrance of the mass spectrometer; this process is largely analyte/matrix dependent.

However, satisfactory recoveries were obtained by quantification using solvent-based calibration curves due to the use of stable isotope-labelled analogues of the analytes used as internal standards. The application of isotope dilution mass spectrometry offers the advantage of compensation for the signal loss resulting from matrix effect and the analyte loss during sample preparation. There is no significant difference between the slopes and intercepts of solvent-based and matrix-matched calibration curves. The solvent-based calibration curves were linear with *P*-value >0.10 for the lack-of-fit *F*-test over the range 0–250 ng l^{-1} and matrix-matched calibration curves were linear over the range 0–10 μg kg^{-1}. The correlation coefficients of both calibration curves were between 0.995 and 0.998.

3.3. Method validation

The method performance was investigated with respect to various parameters such as specificity, recovery, precision, decision limit, detection capability, stability and ruggedness. The specificity was evaluated by the analysis of 10 different blank samples of poultry muscle, plasma and egg. No interfering peaks from endogenous compounds were found in the retention time of the target analytes for analysed samples.

The recovery, and the precision as repeatability and within-laboratory reproducibility at three concentration levels in poultry muscle, plasma and egg samples were summarized in Table 4. The results showed good recovery ranging from 73.2 to 110.6% with a good CV, less than 14.0% under within-laboratory reproducibility conditions at all fortification levels and in all tested matrices.

The decision limit means that the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Likewise the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β. The "recommended concentration" for nitroimidazoles proposed by the CRL Berlin is 3 μg kg^{-1} [8]. From the laboratory point of view it means that CCβ for screening methods or CCα for confirmatory methods should be lower than this recommended value. The calculated critical concentrations of CCα for nitroimidazoles were between 0.05 and 0.53 μg kg^{-1} while CCβ were between 0.08 and 0.90 μg kg^{-1}. All of those values are in accordance with the CRL's recommendation (Table 5).

Seven different variables (minor changes) chosen from the sample preparation step were taken into account in the evaluation of method ruggedness (Table 2). It was demonstrated that all selected factors did not significantly affect the analytical performance. Youden approach was also used to verify the method performances when applied to swine, bovine and fish samples (major changes). Consequently the method proved to be fairly robust and able to withstand minor fluctuations in the operating variables that may occur in its routine application and the different animal species did not have significantly effect on the analytical results.

The stability of nitroimidazole compounds in fortified muscle samples stored at −20 °C was observed for at least 3 months. After that period the degradation of the analytes was less than 10%.

3.4. Measurement uncertainty

In this work, the bottom-up approach together with validation data and the use of formal uncertainty propagation principles was applied for the evaluation of measurement uncertainty [24]. The steps involved were specification of the measurand, identification of uncertainty sources and quantification of uncertainty components and calculation of combined uncertainty. The expanded uncertainty for each matrix was calculated with applying a coverage factor of 2, which gives a level of confidence of approximately 95% (Table 4).

3.5. Method evaluation

The method developed in this study has been used to monitor for the presence of nitroimidazole residues in egg, muscle and plasma of poultry, bovine, porcine and fish as part of the National Monitor-

Table 6
Results of the proficiency tests.

Proficiency test	Analyte	Assigned value (μg kg^{-1})	*Z*-score
FAPAS 0296 (2007)	DMZ	2.64	0.6
	HMNNI	14.7	−0.5
	RNZ	13.9	−0.1
FAPAS 02120 (2008)	MNZ	3.50	0.6
	MNZOH	2.67	0.5
CRL Berlin (NIIM_09/07) Sample P06365	DMZ	0.587	−0.515
	HMNNI	3.226	−0.785
	IPZOH	2.792	−1.192
	IPZ[a]	0.078	
	MNZOH	1.615	−0.204
	MNZ	1.831	−1.141
	RNZ	1.799	0.199

[a] Value <CCα (0.44 μg kg^{-1}).

ing Plan in Poland since 2007 and no non-compliant samples have been found for the past years for this method.

The method performance has been successfully evaluated through the participation in Food Analysis Performance Assessment Scheme (FAPAS), showing our data with *Z*-scores of 0.6, −0.5, −0.1 and for DNZ HMNNI and RNZ, respectively (test 0296, 2007) and 0.6 for MNZ and 0.5 for MNZOH (test 02120, 2008). In the interlaboratory comparison organised by the CRL Berlin the test material consisted of five samples of egg containing incurred residues of nitroimidazoles and one blank sample. *Z*-Scores achieved for MNZ, DMZ, RNZ, IPZ, MNZOH, HMNNI and IPZOH in this comparison were in the range from −1192 to 0831 (NIIM_09/07). Moreover in all the interlaboratory studies neither false positives nor false negatives were found. These results prove good accuracy and reproducibility of the developed method (Table 6).

4. Conclusions

In this study a relatively fast LC–MS/MS method for detection and confirmation nitroimidazoles and their hydroxy-metabolites in poultry muscle, plasma and egg has been developed. The application of isotope dilution mass spectrometry allowed an accurate and precise quantification of nitroimidazole compounds in analysed samples. The validation results obtained indicate the accordance with requirements of Commission Decision 2002/657/EC. The CCα and CCβ for all the nitroimidazoles and their hydroxy-metabolites are below the recommended concentration of 3 $\mu g\,kg^{-1}$. The applicability of the method for the use on different species (poultry, bovine, porcine and fish) was demonstrated by the satisfactory results obtained from ruggedness tests and ongoing data gathered as part of National Residue Monitoring Plan in Poland for all the different species mentioned above.

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