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Development of a rapid method for the determination and confirmation of nitroimidazoles in six matrices by fast liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed to identify and to quantify nitroimidazoles, metronidazole (MNZ), ronidazole (RNZ) and dimetridazole (DMZ) and their corresponding hydroxy metabolites, MNZ-OH and 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMNNI) in plasma, milk, muscle, egg, honey and feed samples. The same sample clean-up procedure including a novel solid-phase extraction (SPE) on polymeric Strata-SDB cartridges was used for each matrix. The analytes were separated on Kinetex XB C-18 core–shell type HPLC column using isocratic elution mode with a mobile phase containing 0.1% formic acid in water/methanol (88/12, v/v, pH 2.6) at a flow rate of 0.7 ml/min. The main advantage of the developed method is that the analysis time of only 3 min, which is about three to ten times shorter than in other reported HPLC methods. The developed method was validated using a matrix-comprehensive in-house validation strategy. The matrix effect of LC–MS/MS analysis was also investigated. Results are presented from the successful application of the developed method to an incurred pork meat certified reference material and to incur porcine plasmas in a proficiency test in year 2011.

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1. Introduction

Metronidazole (MNZ), ronidazole (RNZ) and dimetridazole (DMZ) have antibiotic activities and are used for therapeutic and prophylactic treatments in diseases in animals and farmed fish [1,2]. These nitroimidazoles possess mutagenic and carcinogenic properties [3] and their use in veterinary drugs are prohibited for food producing products in the European Union. According to Annex IV to No. 2377/90, these must not be present in tissues of food-producing animals or in products intended for human consumption [3]. Nitroimidazoles belong to A6 group and therefore, the Federal Office of Consumer Protection and Food Safety (BVL, European Reference Laboratory for Residues, Berlin, Germany) set a limit of 3 μg/kg for nitroimidazoles in the matrices considered in the present paper. CCβ for screening methods or CCα for confirmatory methods should be lower than this value [4].

The analysis of banned substances requires a highly selective and accurate determination. A number of methods have been developed for analyzing nitroimidazoles and their metabolites in different matrices by using chromatographic techniques [1,3,11–19]. Gas chromatography (GC) requires a time-consuming derivatization step to improve the sensitivity of analysis [3], hence it is not preferred. The HPLC-UV method is not allowed by the EU (2002/657/EC) to confirm A group substances. LC–MS/MS is thus the preferred technique to analyze low levels of banned residues in complex matrices [5–10]. Recently, several LC–MS/MS methods have been reported for analyzing nitroimidazoles in plasma, muscle and egg samples, which were applicable for a maximum of four matrices and the analysis times were in the range of 8–30 min [1,3,11–19]. The present paper demonstrates for the first time analysis of nitroimidazoles in six matrices (pig plasma, bovine milk, chicken muscle, chicken egg, honey and maize-based feed) in much shorter time than reported in literature.

In recent years, we have made efforts to analyze pharmaceuticals at low levels in biological and water samples [6–10]. Application of the Phenomenex Kinetex columns made a great stride in achieving enhanced separation for fast analysis of pharmaceuticals [9,22–25,32]. These columns are packed with the

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Table 1
Structures and log *P* values of analyzed nitroimidazoles.

Compound	Structure	Log *P*	CCα (μg/kg)	CCβ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
MNZ		−0.46	0.10	0.13	0.10	0.33
MNZ-OH		−1.76	0.10	0.13	0.10	0.33
RNZ		−0.83	0.05	0.08	0.05	0.17
HMNNI		−0.94	0.10	0.15	0.10	0.33
DMZ		0.37	0.10	0.12	0.10	0.33

CCα, decision limit; CCβ, detection capability; LOD, limit of detection; LOQ, limit of quantification.

superficially porous particles, which are made of solid silica core and homogenous porous shell sorbent. These core–shell packings have shown excellent efficiency for separation [22–25,32]. In the present study, a Kinetex XB C-18 core shell type column was used for separation of nitroimidazoles. The analyzed compounds were metronidazole (MNZ), hydroxymetronidazole (MNZ-OH), ronidazole (RNZ), 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMNNI) and dimetridazole (DMZ) (Table 1). RNZ and DMZ have the same hydroxy metabolite, HMNNI. The LC–MS/MS method was optimized for determining nitroimidazoles and their corresponding hydroxy metabolites in six matrices. The simultaneous validation of matrices using a matrix-comprehensive strategy and Interval software was conducted [9,20,21]. Significantly, the demonstrated method was successfully applied to analyze real samples (pork meat certified reference material and porcine plasma proficiency test samples).

2. Experimental

2.1. Reagents, equipments and instruments

HPLC grade acetonitrile, methanol and ethyl acetate were obtained from Promochem (Wesel, Germany). Suprapur formic acid (98–100%) was purchased from Merck (Budapest, Hungary). Ammonia solution (0.25%) was obtained from Scharlau (Barcelona, Spain). Analytical standards, including the internal standard DMZ-d_3 were purchased from Sigma–Aldrich (Budapest, Hungary). Stock solutions (0.1 mg/ml) were prepared by dissolving 5.0 mg standard in 50 ml of methanol. These solutions were stored at −20 °C for a maximum of one month. A working standard solution, which contained all nitroimidazoles in 0.25 μg/ml concentration, was prepared daily by diluting 25 μl of the stock solution to 10 ml with distilled water. The blank and fortified samples, which were used for method development and validation, originated from Hungarian residue control monitoring program from March 2011 to September 2011 and were stored at −20 °C until analysis.

Phenomenex Strata-SDB (3 ml, 200 mg and 100 μm) SPE cartridges and Phenomenex Kinetex XB C-18 (100 mm × 3 mm, 2.6 μm) HPLC column were purchased from Gen-lab Ltd. (Budapest, Hungary). Validation software was Interval 3 (Berlin, Germany). LC–MS/MS separation was carried out with an Agilent 6410A Triple Quad LC/MS, which had an Agilent 1200 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat) and a directly connected Agilent 6410A tandem mass spectrometric detector, equipped with an Agilent multimode ion source (G1978B) (Agilent Technologies, Palo

Alto, CA, USA). Data acquisition, qualitative and quantitative evaluation were performed using the Agilent Mass Hunter B.01.04 Software.

2.2. Preparation of samples

2.2.1. Plasma, milk, muscle, egg and feed

Samples (5.0 g) were weighed into 50 ml polypropylene (PP) centrifuge tubes and extracted with 15 ml acetonitrile by vortex-mixing for 30 s, followed by shaking on a Janke & Kunkel IKA KS125 shaker (Staufen, Germany) at 700 min^{-1} for 60 min at ambient temperature. Samples were then centrifuged at 10,000 rpm speed with a Sigma 3–18 K centrifuge (Osterode am Harz, Germany) at 25 °C for 10 min. The supernatans were transferred into glass tubes and evaporated to 50–100 μl (not to dryness) in a TurboVap LV (Hopkinton, MA, USA) under a gentle nitrogen stream at 50 °C. Then samples were cooled down to ambient temperature. Distilled water (6 ml) was added to the samples and then re-dissolved by vortex-mixing for 20 s. Samples were cleaned-up and concentrated on SPE cartridges.

2.2.2. Honey

Samples (5.0 g) were weighed into 50 ml centrifuge tubes and dissolved in 15 ml 0.1% formic acid in water solution (v/v, pH 2.6) by hand-shaking for 30 s. Samples were vortex-mixed for 10 s, followed by shaking at 700 min^{-1} for 60 min to hydrolyze the occurring N-glycoside bounds between primary amino group of RNZ and sugars. Then the pH of hydrolyzed samples was adjusted to 7.0 ± 0.2 with 10 mM ammonia solution. Samples were then centrifuged at 10,000 rpm at 25 °C for 10 min and purified with SPE cartridges.

2.3. Solid-phase extraction clean-up

All samples were subjected to Strata-SDB (3 ml and 200 mg) SPE clean-up. Cartridges were previously conditioned three times with 3 ml methanol followed by three times with 3 ml water. Samples were passed through the cartridges drop wise (~0.3 ml/min). SPE columns were then washed three times with 3 ml water. Columns were dried under vacuum for 1 min and samples were eluted twice with 2.5 ml ethyl acetate into receiving glass tubes that already had 4 μg/kg DMZ-d_3 internal standard (ISTD). The eluted samples were evaporated under a gentle nitrogen stream to dryness at 45 °C. The samples were re-dissolved in 1.0 ml 0.1% formic acid in water solution by vortex-mixing for 20 s and filtered through on 0.45 μm Phenex nylon filters (Gen-lab Ltd., Budapest, Hungary) into HPLC vials.

2.4. LC–MS/MS analysis

Nitroimidazoles were separated on Kinetex XB C-18 (100 mm × 3 mm, 2.6 μm) column using an isocratic elution. The mobile phase was a mixture of 0.1% formic acid in water–methanol (88/12, v/v, pH 2.6). The flow rate was 0.7 ml/min and the analysis time was 3.0 min. The injection volume was 10 μl and the thermostat of the analytical column was set at 35 °C.

The compounds were detected in the MRM (multiple reaction monitoring) mode in the MS/MS analyzer by applying two ion transitions (quantitative and qualitative) for a molecule. 75 ms dwell time and 200 V delta electron multiplier voltage were used for all ion traces. The multimode ion source (MMI) was set in the positive ESI mode (Table 2). The MMI settings were: gas temperature: 350 °C and gas flow: 5 l/min; vaporizer: 150 °C; nebulizer pressure: 413.7 kPa (60 psi); capillary voltage: 2500 V; charging voltage: 2000 V. These settings were optimal for multimode ion source [26]. Nitrogen was the collision and drying gas. The collision gas pressure was 1.07 Pa.

2.5. Quantification

Six points matrix-matched curves were prepared for quantification. Calibration samples were made by spiking prepared blank samples, which were already cleaned-up using the same procedure, with different volumes of working standard solution. Calibration samples were fortified at the end of sample preparation procedure to calculate the absolute recovery of determination [5]. Calibration levels were 0, 1.5, 3.0, 4.5, 6.0 and 9.0 μg/kg for each compound. Standard curve in neat solution (matrix-free curve) at the same concentration levels was also prepared and analyzed to calculate the absolute matrix effect of analysis. Matrix effect (ME%) was calculated as [(slope of matrix − matched curve)/(slope of matrix − free curve) − 1] × 100 in the concentration range from 0 to 9 μg/kg [5,27]. Results were evaluated by internal standard (ISTD) method using a linear regression between relative areas and relative concentrations. DMZ-d_3 as the ISTD was used at 4 μg/kg concentration for each analyte to calibrate the ionization source response and to facilitate the quantitative determination [31].

3. Results and discussion

3.1. Optimization of LC–MS/MS separation

Initially, the ion transitions (precursor ion ≫ product ion) were optimized using individually diluted 1 μg/ml methanolic standards. Flow injection analysis (FIA) was carried out by connecting the injector outlet of the HPLC system directly to the nebulizer of the mass spectrometer. Methanol–0.1% formic acid in water (70/30, v/v) mobile phase was used at flow rate of 0.3 ml/min. Mass spectra of nitroimidazoles were recorded in both ESI and APCI mode using positive ionization. Firstly, MS2 scan mode was used to find the precursor ions. $[M+H]^+$ precursor ions were found for each compound in both ion modes, however, ESI mode showed higher responses. We therefore optimized the ion traces in positive ESI mode.

Selected precursor ion was optimized employing different fragmentor voltage between 60 and 150 V. After selecting the best fragmentor voltage for the precursor ion, the collision energies (CEs) of the ion traces were optimized between 0 and 30 V using the product ion scan mode. After choosing the two product ions with their optimal CEs, the detector was set to the MRM mode. The more intensive ion transition was used for quantification and the other one for qualification. The MRM ion transitions were further enhanced using different vaporizer temperatures between 150 and 250 °C. There were no differences between detector responses, however, vaporization temperature of 150 °C resulted in lower noise level for all ion transitions and was used for subsequent experiment. It should be pointed out that Agilent also recommends the usage of low vaporizer temperature in ESI mode [26].

For HPLC separation we used the Kinetex XB C-18 column, which contains protective butyl side chains and is developed especially for basic compounds [9]. The application of Kinetex XB column enabled improved sensitivity [9] and therefore, isocratic elution could be used for separation. Using methanol as organic modifier in mobile phase and 35 °C column temperature resulted in minimal overlapping between the peaks (Fig. 1), so the disturbance of ionization between investigated analytes could be minimized. Since, the first and last eluted compounds, MNZ-OH (1.4 min) and DMZ (2.3 min), eluted within one min (Fig. 1), DMZ-d_3 can be used as internal standard for all studied nitroimidazoles resulting in good recovery and reproducibility. In the case of honey matrix, an unacceptable high background was observed in DMZ-d_3 145.3 ≫ 99.3 *m*/*z* ion transition. Therefore the second most intense ion trace was applied for DMZ-d_3 (145.3 ≫ 56.2 *m*/*z*) in honey because it resulted in clear baseline and good quantification (Table 2).

Table 2
MS/MS detector parameters and calculated ion ratios (IR). Weaker ion transition of DMZ-d_3 (145.3 ≫ 56.2 m/z) was used only for honey matrix.

Time (min)	ΔEMV	Compound	Precursor ion (m/z)	Product ions (m/z)	Dwell time (ms)	Fragmentor (V)	CE (V)	IR in standard solution (average ± SD) (%)	Permitted tolerance according to EU 2002/657/EC (%)	IR in samples (range) (%)
0–3.0	200 V	MNZ	$[M+H]^+$ 172.3	128.3 82.3	75	90	10 25	51.0 ± 0.6	40.8–61.2	45.3–52.5
		MNZ–OH	$[M+H]^+$ 188.3	144.2 123.3	75 75	90	10 10	35.1 ± 0.9	26.3–43.9	30.2–36.3
		RNZ	$[M+H]^+$ 201.3	140.3 55.3	75 75	90	10 25	41.3 ± 0.5	31.0–51.6	38.1–45.5
		HMNNI	$[M+H]^+$ 158.3	140.3 55.3	75 75	90	10 15	52.0 ± 0.9	41.6–62.4	47.0–55.3
		DMZ	$[M+H]^+$ 142.3	96.3 54.3	75 75	90	10 30	36.0 ± 1.8	27.0–45.0	29.5–39.9
		DMZ–d_3	$[M+H]^+$ 145.3	99.3 (56.2)	75 (75)	90 (90)	15(30)	ISTD		

Scan type, MRM; ion mode, ESI+.

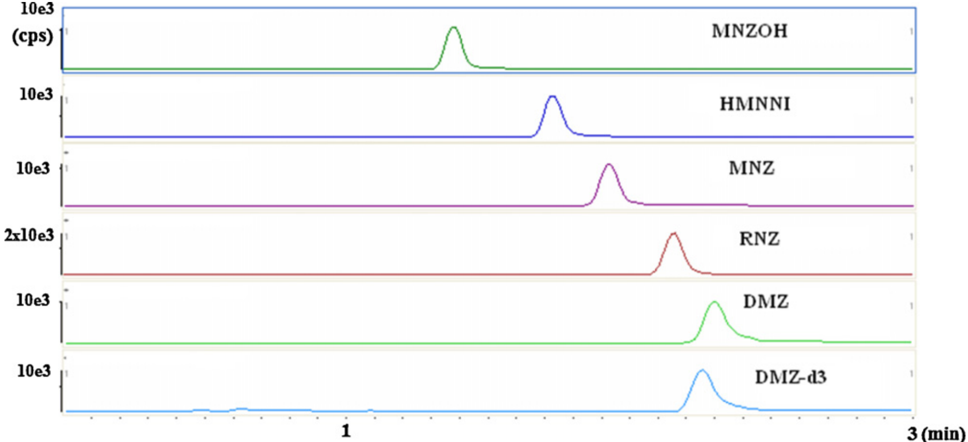

Fig. 1. Quantification MRM chromatograms of a spiked (3 μg/kg for all nitroimidazoles) plasma sample.

3.2. Optimization of SPE clean-up

Some previous methods applied only sample extraction and no further SPE clean-up in sample preparation for nitroimidazole determination [11,15,17,18]. Because of weak basic (pK_a 1.32–2.81) and polar characteristic of nitroimidazoles, some study groups include silica based SPE, strong cation exchange (SCX) clean-up, or polymeric mixed-mode cation exchange (MCX) and HLB (hydrophilic–lipophilic balance) SPE clean-up for nitroimidazole sample preparation [1,3,12,13,16]. Mohamed et al. used special molecularly imprinted SPE for cleaning egg-based foods that resulted clear samples prior to LC–MS/MS separation [19]. The polymeric Strata SDB SPE cartridges were tested at pH 7 for the extraction of six matrices. Strata SDB can adsorb nitroimidazoles well at this pH and consequently, other pH adjustment was not investigated. The other advantages of Strata-SDB cartridges are the large pore size (100 μm) that protects the SPE column from clogging.

The matrix effects were calculated for each compound and for each matrix by comparing the slope of matrix-matched curve to matrix-free curve (calibration in neat solution) [5,9,27]. Generally, ion enhancement was observed for each matrix (Table 3). In the case of muscle, a relatively high ion enhancement was seen for MNZ-OH and DMZ, +37% and +48%, respectively. Other ME% in the different matrices were between −22 and +26% for nitroimidazoles (Table 3) that are acceptable when complex matrices are analyzed [5–9,27]. Ion effects during the validation procedure and in real sample analysis were compensated using matrix-matched curve for calibration.

3.3. Validation

Method was validated in accordance with EU 2002/657/EC Decision and LC–GC International using a matrix-comprehensive in-house validation [9,20,21,28,29]. Two factors were set. Matrix was defined as first (leading) factor with six levels (plasma, milk, muscle, egg, honey and feed). The other factor was the operator (different person). The fortification levels were 3 μg/kg (MRPL), 4.5 μg/kg (1.5 MRPL) and 6 μg/kg (2 MRPL) for each compound that met the EU standards [28]. Each matrix was analyzed on different days. Each day eight parallel samples were analyzed at each concentration level by two different operators. During the validation, 144 samples (24 samples/matrix) were analyzed.

Selectivity was proven by analyzing blank samples that were spiked with other polar and basic antibiotics (amoxicillin, sulfadiazine, sulfadimethoxine, streptomycin) before the sample preparation. There were no any interfering matrix peaks in the time window where nitroimidazoles eluted, consequently, the selectivity met the EU standards. All compounds were detected with two

Table 3
Matrix effect (ME%) in the investigated matrices.

	Plasma (%)	Milk (%)	Muscle (%)	Egg (%)	Honey (%)	feed (%)
MNZ	+10	+19	+22	+23	+9	−5
MNZ-OH	+13	−10	+37	+13	−6	−12
RNZ	+26	+15	+5	+12	+6	−22
HMNNI	+12	+10	+23	+20	+3	−1
DMZ	−8	−22	+48	+11	+24	+26

Table 4
Validation results in different matrices: linearity between 0 and 9 μg/kg for each matrix, recovery at 3, 4.5 and 6 μg/kg levels and repeatability, reproducibility.

Matrix		MNZ	MNZOH	RNZ	HMNNI	DMZ
Plasma	Slope	1.2299	0.2080	0.3433	0.4320	0.5662
	Intercept	−0.1194	−0.0286	−0.0567	−0.0586	−0.2107
	r^2	0.9973	0.9952	0.9933	0.9963	0.9736
	Recovery%	88.0–97.2	68.9–91.1	99.7–105.1	86.8–92.8	90.7–96.4
	Repeatability (RSD%)	1.4–2.5	5.6–8.4	1.4–2.7	1.6–3.0	7.8–14.6
	Reproducibility (RSD%)	2.1–10.6	6.0–23.6	2.5–6.2	2.1–6.2	10.9–15.5
Milk	Slope	1.4156	0.1947	0.4919	0.4784	0.4504
	Intercept	−0.1398	−0.021	−0.0613	−0.0643	0.0832
	r^2	0.9978	0.9969	0.9969	0.9950	0.9613
	Recovery%	93.2–99.2	93.3–100.6	93.1–102.4	92.0–101.6	108.6–111.9
	Repeatability (RSD%)	3.5–7.5	3.4–7.2	3.7–8.2	4.9–10.9	10.2–18.8
	Reproducibility (RSD%)	6.3–8.6	3.6–7.7	4.0–8.7	5.2−–11.5	10.7–19.8
Muscle	Slope	1.1774	0.2320	0.3819	0.4141	0.6630
	Intercept	−0.1632	−0.0537	−0.0430	−0.0665	−0.2364
	r^2	0.9957	0.9856	0.9963	0.9947	0.9672
	Recovery%	76.6–78.6	59.6–65.0	82.6–83.1	70.2–71.6	62.0–68.7
	Repeatability (RSD%)	2.5–5.1	8.4–15.2	3.2–6.4	3.6–7.1	10.4–22.9
	Reproducibility (RSD%)	4.2–5.5	8.9–16.0	4.8–6.9	4.1–7.6	11.0–24.2
Egg	Slope	1.2207	0.2011	0.3966	0.4093	0.5167
	Intercept	−0.2430	−0.0504	−0.0728	−0.0999	−0.1167
	r^2	0.9869	0.9837	0.9879	0.9813	0.9840
	Recovery%	77.0–82.7	56.8–61.6	78.3–85.0	74.5–80.3	78.6–100.6
	Repeatability (RSD%)	4.1–7.6	16.2–23.8	1.7–3.1	6.9–12.9	4.5–7.0
	Reproducibility (RSD%)	6.2–8.0	18.9–20.7	3.5–5.3	9.7–13.6	5.1–7.4
Honey	Slope	6.6046	1.2020	2.4663	2.2779	3.1845
	Intercept	−1.2847	−0.2688	−0.1230	−0.2668	−0.6446
	r^2	0.9842	0.9607	0.9745	0.9879	0.9720
	Recovery%	61.1–69.7	<35	55.6–60.9	64.3–68.8	64.4–86.8
	Repeatability (RSD%)	15.3–26.4	Not calculated	15.5–24.0	14.5–24.2	8.6–23.3
	Reproducibility (RSD%)	16.1–27.2	Not calculated	16.3–25.8	15.3–24.9	16.9–24.5
Feed	Slope	0.8856	0.1706	0.3326	0.3031	0.53262
	Intercept	0.0893	0.0113	0.0429	0.0234	0.0568
	r^2	0.9860	0.9889	0.9829	0.9906	0.9649
	Recovery%	60.6–63.2	<35	59.4–62.6	59.2–63.4	73.4–83.6
	Repeatability (RSD%)	4.3–10.2	Not calculated	5.7–12.7	4.3–10.2	10.2–16.3
	Reproducibility (RSD%)	9.6–14.3	Not calculated	11.0–13.5	9.6–14.3	18.6–24.5

ion transitions, which means four identification points and fulfil the EU guidelines [28]. The identification was based on the ion ratio (IR) that is the intensity ratio of qualify and quantify ion transitions (Table 2).

Linearity was investigated for each compound at six concentration levels between 0 and 9.0 μg/kg for each matrix using the matrix-matched curve. The slopes and intercepts of calibrations are presented in Table 4. Recovery, repeatability and within laboratory reproducibility were calculated for each matrix and summarized in Table 4. The absolute recovery for plasma and milk samples ranged from 68.9 to 105.1% and from 92.0 to 111.9%, respectively. In the case of muscle and egg samples, lower absolute recoveries were observed (59.6–83.1 and 56.8–100.6%, respectively). For the honey samples, absolute recoveries were found between 55.6 and 86.8%, while in the feed samples ranged from 59.2 to 83.6% (Table 4). These results satisfy the validation strategy of LC–GC International [29]. Repeatability and reproducibility are expressed as relative standard deviation (RSD%) and were calculated by Interval 3 as 100 × (standard deviation)^2/fortification level concentration [9,20,21]. Repeatability was under 20% for plasma, milk and feed samples, but for other matrices lower repeatability was observed (up to 26.4%). According to the EU standards, the repeatability and reproducibility shall be as low as possible under 100 μg/kg level [28]. The reproducibility of milk sample was found to be less than 20%. In other matrices reproducibility was sometimes higher than 20% (up to 27.2%) (Table 4), but it did not exceed 30%; consequently, these values are acceptable based on the EU standards [29].

Although the analyses of MNZ-OH in plasma, milk, muscle and egg were successful, but low absolute recoveries (<35%) were observed for MNZ-OH in honey and feed samples (Table 4). Acetonitrile is a common extraction solvent for nitroimidazoles [13,15,18], so it could not cause the low recovery in the feed. The spiked nitroimidazoles in dissolved and hydrolyzed honey did not degrade, consequently the ion suppression or low SPE capacity could give rise to the low recoveries. The matrix effects for MNZ-OH were −6 and −12% ion suppressions for honey and feed, respectively (Table 3). These results are not high, therefore, ion suppression would not result in the low recovery. The low SPE capacity could be the reason for the unacceptable low recovery in these two matrices. MNZ-OH therefore was validated only for four matrices (plasma, milk, muscle and egg). However, in honey and feed samples, MNZ-OH can only be screened with this method.

Decision limit (CCα) was calculated for all nitroimidazoles along with the limit of detection (LOD) as three times the signal to noise ratio (SNR) and were between 0.05 and 0.1 μg/kg (Table 1). These results were confirmed by analyzing blank samples that were spiked to individual CCα values before the sample preparation. CCα was accepted when the SNR of ion transitions were higher than three and ion ratios were in the acceptable range. Detection capability (CCβ) was calculated as CCα plus 1.64 times the standard deviation (SD) of the within-laboratory reproducibility at the decision limit [28]. Limit of quantification (LOQ) was determined as 10 times the signal to noise ratio (Table 1).

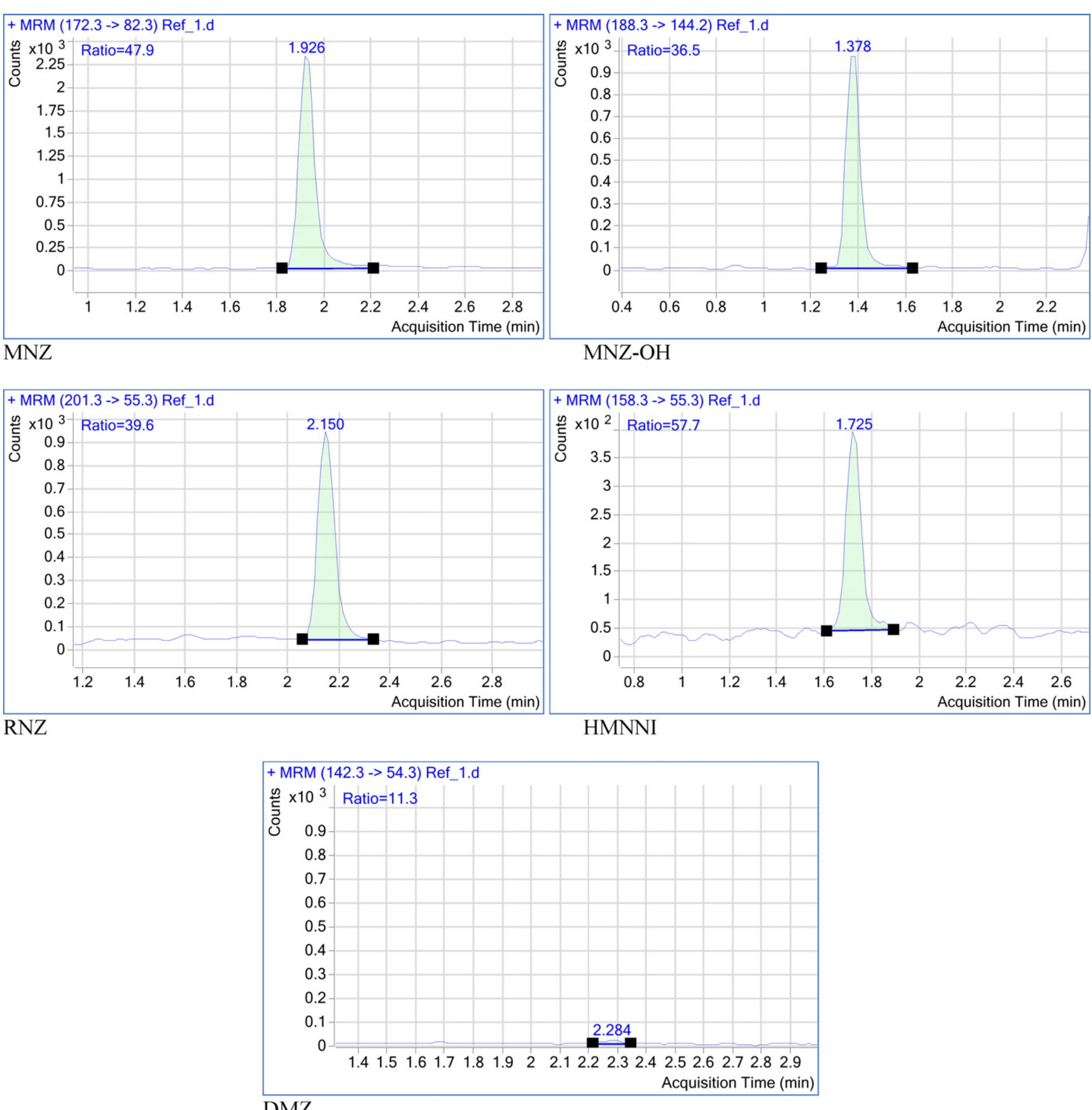

Fig. 2. MRM chromatograms of a reference incurred pork muscle containing 1.92 μg/kg MNZ, 6.13 μg/kg MNZ-OH, 1.93 μg/kg RNZ, 0.74 μg/kg HMNNI and <0.1 μg/kg DMZ. Chromatograms show the qualification ion transitions and the ion ratios.

3.4. Comparison of the developed method to the existing ones

In the present study, we used methanol as an organic modifier in the mobile phase and applied isocratic separation for nitroimidazoles, while previous studies preferred applying acetonitrile as the organic modifier and gradient elution to obtain sharp peaks (Supplementary Table). Mottier et al. [1] and Mohamed et al. [19] used the same separation that included an HPLC determination using the fully porous SymmetryShield C-18 column with analysis time of 18 min. These methods could be applied to egg and meat samples. Xia et al. analyzed liver [12] and meat [16] samples using fully porous C-8 HPLC column (Sunfire) for nitroimidazole separation. These separations resulted in 18 and 25 min run times for liver and meat samples, respectively (Supplementary Table). Cronly et al. investigated plasma and egg samples for nitroimidazoles in two different studies [15,18] using the same separation. Gradient elution and a fully porous Luna C-18 HPLC column were applied in these studies that resulted in 20 min analysis time. Fully porous Gemini C-18 column was tested in two studies for nitroimidazoles [13,17]. Zeleney et al. [17] applied this column only for meat samples, however, Mitrowska et al. [13] could used this column for separating nitroimidazoles in meat, plasma and egg samples. Both

Table 5
Detected compounds and concentrations in certificate reference materials.

Compounds	Concentrations (μg/kg)		Acceptable range (μg/kg)
	Sample 1	Sample 2	
MNZ	1.92	1.88	1.78–2.08
MNZ-OH	6.13	6.27	5.3–6.9
RNZ	1.93	2.0	1.84–2.34
HMNNI	0.74	0.74	0.60–0.78
IPZOH	Not analyzed	Not analyzed	1.55–1.79
DMZ	<0.1	<0.1	<0.25

studies report long analysis times (29 and 30 min, respectively). Mitrowska et al. were the first to determine nitroimidazoles from three matrices using the LC–MS/MS technique.

Hurtaud-Pessel et al. [11] used isocratic elution with ternary mobile phase (methanol–acetonitrile–0.2% formic acid), but only for meat samples. This ternary separation on a fully porous column (Symmetry C-18) resulted in 8 min analysis time (Supplementary Table). The application of Kinetex XB core–shell type column in this study enabled an analysis time of 3 min that is approximately three times faster than in an earlier study [11]. This determination can be applied to six matrices for the first time. The effectiveness of Kinetex C-18 column in antibiotic separations had already been published [23,32]. The Kinetex column used in this study (XB C-18) is the latest generation of Kinetex family and has not been used for nitroimidazoles determination previously. Moreover the better sensitivity of core–shell type column [33] allowed us to obtain similar analytical limits in isocratic separation to those studies that applied gradient elution (Supplementary Table).

Since previous studies calculated relative recoveries (calibration samples were spiked at the beginning of clean-up procedure) and we calculated absolute recoveries (calibration samples were spiked at the end of clean-up procedure), hence comparison of the recoveries is not possible. In general, the relative recovery is higher than absolute recovery. It should be pointed out that we may have got higher recoveries for MNZ-OH in honey and feed samples, if we had calculated relative recovery. Significantly, the analysis of incurred samples (porcine plasma and meat) provides evidence for the goodness of the present method. The application of more internal standards could result in better reproducibility than in the previous methods. Other studies applied multiple ISTDs to compensate the losses during the extractions which makes this approach more expensive. Only Hurtaud-Pessel et al. [11] used one ISTD (RNZ-d_3) adding at the beginning of sample preparation. In the described method ISTD (DMZ-d_3) was added at the end of clean-up procedure, only to calibrate the ion source response. Since background matrices with similar properties could enter the ion source in the time window where nitroimidazoles eluted (Fig. 1), DMZ-d_3 can be used for each compound to compensate the matrix effect.

4. Analysis of real samples

4.1. Certified reference material

A lyophilized incurred pork muscle reference material was obtained from European Reference Materials (ERM, Geel, Belgium) [30]. Lyophilized sample was reconstituted in distilled water. Duplicate samples were analyzed in parallel for nitroimidazoles with the described LC–MS/MS method. Quantitative results were corrected, based on recovery. According to the certificate of the material, the sample contains 1.93 ± 0.15 μg/kg MNZ, 6.2 ± 0.9 μg/kg MNZ-OH, 2.09 ± 0.25 μg/kg RNZ, 0.69 ± 0.09 μg/kg HMNNI, 1.67 ± 0.12 μg/kg IPZOH (hydroxyipronidazole) and <0.25 μg/kg DMZ (Table 5). Our identification was satisfactory. MNZ, MNZ-OH, RNZ and HMNNI were detected (Fig. 2). IPZOH

Table 6
Detected compounds and mean concentrations in proficiency test samples based on preliminary report. "*n*" value means the number of independent analysis and RSD% is the reproducibility of analysis.

Sample	MNZ		MNZ-OH		RNZ		HMNNI	
	Our results (μg/kg)	Median of all labs (μg/kg)	Our results (μg/kg)	Median of all labs (μg/kg)	Our results (μg/kg)	Median of all labs (μg/kg)	Our results (μg/kg)	Median of all labs (μg/kg)
1	**1.46** (*n* = 3) RSD = 9.2%	**1.67** (*n* = 129) RSD = 14.1%	**4.69** (*n* = 3) RSD = 4.9%	**5.20** (*n* = 134) RSD = 24.6%	**2.18** (*n* = 3) RSD = 7.1%	**2.53** (*n* = 133) RSD = 15.6%	**1.81** (*n* = 3) RSD = 3.2%	**2.01** (*n* = 127) RSD = 20.0%
2	**0.64** (*n* = 3) RSD = 0.9%	**0.68** (*n* = 125) RSD = 16.9%	**1.97** (*n* = 3) RSD = 3.2%	**2.16** (*n* = 129) RSD = 22.2%	**0.80** (*n* = 3) RSD = 3.1%	**1.05** (*n* = 124) RSD = 18.5%	**0.73** (*n* = 3) RSD = 8.6%	**0.84** (*n* = 93) RSD = 27.4%
3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

was not analyzed and DMZ was not detected. All detected concentrations were in the acceptable range, therefore, the quantitative analysis was also successful (Table 5). The real sample analysis showed that high ion enhancement of MNZ-OH, which was observed in muscle matrix during the method development (Table 3), could be well compensated with matrix-matched curve.

4.2. Proficiency test

A performance test called "Proficiency Test on Nitroimidazoles in Porcine Plasma NIIM_05/2011" was organized by BVL (Berlin, Germany) in the summer of 2011. Reconstituted incurred plasma samples have been analyzed in three different batches. Quantitative and exact qualitative information was not provided to participants at the beginning of the proficiency test. At least two independent analyses have been made for each sample. In samples 1 and 2 MNZ, MNZ-OH, RNZ and HMNNI were detected. The detected compounds were the same in two different samples, but the concentrations were different. Sample 1 contained higher concentration of each analyte: 1.46 ± 0.13 μg/kg MNZ, 4.69 ± 0.23 μg/kg MNZOH, 2.18 ± 0.15 μg/kg RNZ and 1.81 ± 0.06 μg/kg HMNNI, while sample 2 contained lower values: 0.64 ± 0.006 μg/kg MNZ, 1.97 ± 0.063 μg/kg MNZOH, 0.80 ± 0.025 μg/kg RNZ and 0.73 ± 0.063 μg/kg HMNNI. Sample 3 was identified as the blank sample (Table 6). DMZ was not detected in the samples. A preliminary report was sent to the participants in September 2011, which showed that the mean concentrations in sample 1 were: 1.67 μg/kg MNZ, 5.20 μg/kg MNZOH, 2.53 μg/kg RNZ and 2.01 μg/kg HMNNI. The concentrations in sample 2 were: 0.68 μg/kg MNZ, 2.16 μg/kg MNZOH, 1.05 μg/kg RNZ and 0.84 μg/kg HMNNI. The samples also contained IPZOH, but it was not analyzed with the developed method in the present study. Our identification and quantification using this method were satisfactory in the test.

5. Conclusions

A novel LC–MS/MS method was successfully developed to determine nitroimidazoles (MNZ, MNZ-OH, RNZ, HMNNI and DMZ) in six matrices. All samples were cleaned-up on polymeric Strata-SDB cartridges and subsequently separated on Kinetex XB HPLC column. The investigated analytes could be separated with minimal overlapping, therefore, they should not interfere with each others' ionization in the MMI. The fast separation enabled the application of only one ISTD (DMZ-d_3) for all compounds that could well compensate the ionization source response from sample to sample and the analysis time was only 3.0 min. Additionally, the method was validated based on a matrix comprehensive method and EU 2002/657/EC Decision. All parameters met the EU guideline. Moreover, the method showed excellent performance in analyzing incurred pork muscle and plasma samples. The confirmatory validation of MNZ-OH in honey and feed was not achievable; therefore, further investigation is required to obtain good absolute recovery for it. In the future the method will be tested for ipronidazole and hydroxyipronidazole to gain the number of compounds in this optimized separation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2012.02.013.

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