JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of Four 5-Nitroimidazoles and Their Corresponding Hydroxylated Metabolites in Egg, Processed Egg, and Chicken Meat by Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry

PASCAL MOTTIER,* ISABELLE HURÉ, ERIC GREMAUD, AND PHILIPPE A. GUY

Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

An isotope dilution liquid chromatography−electrospray ionization−tandem mass spectrometry method is presented for the simultaneous analysis of several 5-nitroimidazole-based veterinary drugs, which are dimetridazole (DMZ), ronidazole (RNZ), metronidazole (MNZ), ipronidazole (IPZ), and their hydroxylated metabolites (DMZOH, MNZOH, and IPZOH), in egg (fresh egg, whole egg powder, and egg yolk powder) and chicken meat. Data acquisition was achieved by applying multiple reaction monitoring, and quantitation was performed by means of five deuterated internal standards (ISs), namely, DMZ-d_3, RNZ-d_3, IPZ-d_3, DMZOH-d_3, and IPZOH-d_3, whereas MNZ and MNZOH were quantitated using DMZOH-d_3. At the lowest fortification levels (i.e., 0.5 μg/kg for fresh egg and chicken meat and 1.0 μg/kg for other egg-based matrices) and for compounds having their own corresponding deuterated analogue used as an IS, acceptable performance data were obtained (corrected recoveries, 88−111%; decision limits, 0.07−0.36 μg/kg; detection capabilities, 0.11−0.60 μg/kg; and within-lab precision, ≤15%). The method failed to give acceptable quantitative results for MNZ and MNZOH due to the unavailability of the corresponding deuterated ISs. Nevertheless, a reliable identification of these two analytes at levels ≤1 μg/kg was still feasible.

KEYWORDS: Veterinary drug; 5-nitroimidazole; dimetridazole; metronidazole; ipronidazole; ronidazole; DMZOH; MNZOH; IPZOH; egg; meat; fish; liquid chromatography tandem mass spectrometry; LC-MS/MS

INTRODUCTION

Dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ) are 5-nitroimidazole-based drugs with antibiotic and anticoccidial activities. They are primarily used for the prophylactic and therapeutic treatments of diseases such as histomoniasis and coccidiosis in poultry, genital trichomoniasis in cattle, and hemorrhagic enteritis in pig and for the treatment of parasitic infections in farmed fish. These drugs are suspected to be human carcinogens and mutagens (*1*). DMZ, RNZ, and MNZ are thus included in the European Union (EU) list of pharmacologically active substances for which no maximum residue limit can be fixed (*2*) and are, consequently, banned in food-producing animals. IPZ has never been authorized as a veterinary drug and is also considered a banned substance. The 5-nitroimidazoles are rapidly metabolized. Thus, the main metabolite of DMZ, IPZ, and MNZ results from oxidation of the side chain in the C-2 position of the imidazole ring (**Figure 1**). RNZ, on the other hand, has a different degradation pathway and shows only a minor metabolite containing the imidazole ring, identical to that of DMZ. The metabolites retaining the nitroimidazole ring are carcinogenic and mutagenic in some animal species (*1*). This fact emphasizes the need to consider not only the parent drugs but also their metabolites during residue controls. Furthermore, treatments of turkeys with DMZ and IPZ have shown that their respective metabolites are the relevant target analytes in muscle samples, whereas for animals treated with RNZ and MNZ, the parent drug itself is the most relevant analyte (*3*). A similar fact was previously observed in eggs, after a single dose of DMZ, IPZ, and RNZ to laying hens (*4*). In trout treated with MNZ, the fraction of the metabolite to the drug itself was found to be low (<2%), leaving the parent drug as the analyte of interest (*5*). Depending on the method sensitivity, the simultaneous detection of a parent drug and that of its metabolite may provide additional proof of misuse when positive samples are depicted. Depletion studies have demonstrated a rapid elimination of DMZ and its metabolite, DMZOH, in muscle and liver tissues from turkey, with no detectable residues (<1 μg/kg) being present after 5 days following the withdrawal period. Additionally, an inhomogeneous analyte distribution in muscle and liver samples and a rapid degradation of the drugs in unfrozen incurred tissues have been highlighted. Plasma and retina were found better matrices to check for the presence of 5-nitroimidazole compounds, due to both a better stability of the drugs during storage and a larger concentration than in other tissues (*3*). However,

* To whom correspondence should be addressed. Tel: (+41/21)785 8231. Fax: (+41/21)785 8553. E-mail: pascal.mottier@rdls.nestle.com.

10.1021/jf052907s CCC: $33.50
Published on Web 02/17/2006

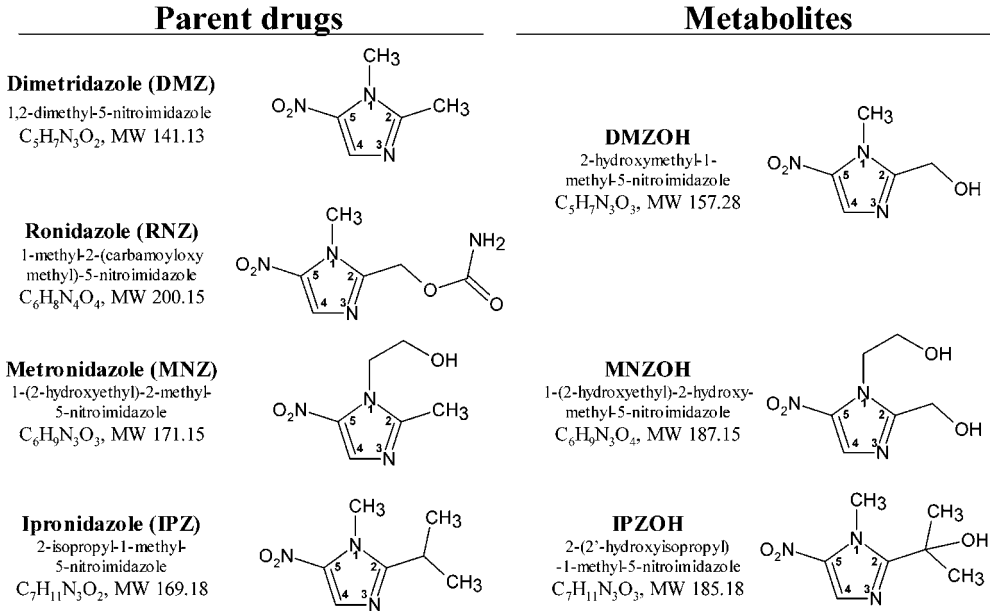

Figure 1. Chemical structures of 5-nitroimidazole parent drugs along with their metabolites. DMZ and RNZ have the same metabolite (DMZOH).

such matrices are rarely available for control laboratories linked to the food industry. In eggs from laying hens fed a diet containing DMZ, IPZ, and RNZ, detectable residues (>10 μg/kg level) were found for more than 5 days after the end of the medication, showing identical concentrations of drugs in both white and yolk compartments (*4*). The persistence of DMZ, RNZ, and DMZOH in cooked food has been discussed elsewhere (*6*).

Several methods have been proposed for the analysis of one or several 5-nitroimidazoles in food and other biological matrices (*7*), but few have described the simultaneous determination of the four parent compounds along with one or more metabolites using confirmatory MS techniques. Thus, gas chromatography coupled to MS has been used for the control of turkey and swine muscles following trimethylsilylation of four 5-nitroimidazoles and three metabolites (*8*). Analysis of 5-nitroimidazoles (four parent drugs and one metabolite) in water from different sources was conducted using a single-stage liquid chromatography−mass spectrometry (LC-MS) instrument (*9*). This technique was employed to elucidate their fragmentation pathways using in-source fragmentation experiments (*10*), whereas LC-tandem mass spectrometry (MS/MS) was proposed for the monitoring of poultry and pig tissues (four parent drugs and one metabolite) (*11*).

The present work describes a method for the determination of four 5-nitroimidazoles and three metabolites employing isotope dilution LC-MS/MS. The matrices considered were fresh eggs but also other forms of egg such as whole egg powder, egg yolk powder, or liquid egg yolk, as these processed raw materials are frequently used by the food industry in a broad range of commodities. The procedure was also adapted for the analysis of meat and fish. Validation was conducted according to the EU Commission Decision 2002/657/EC guidelines (*12*). Finally, the applicability of this analytical procedure was demonstrated in a minisurvey of eggs, meat, and fish samples of different origins.

MATERIALS AND METHODS

Safety. 5-Nitroimidazoles are possible mutagens. Avoid inhalation and use only in a chemical fume hood.

Chemicals and Reagents. DMZ and MNZ were obtained from Riedel-de-Haën (Fluka, Buchs, Switzerland). RNZ was from Sigma (Fluka, Buchs, Switzerland). IPZ, 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZOH), 1-(2-hydroxyethyl)-2-hydroxy-methyl-5-nitroimidazole (MNZOH), and 2-(2′-hydroxyisopropyl)-1-methyl-5-nitroimidazole (IPZOH) were supplied by Witega (Berlin, Germany). Deuterated internal standards (ISs) containing a CD_3 in position N-1 (DMZ-d_3, RNZ-d_3, IPZ-d_3, DMZOH-d_3, and IPZOH-d_3) at chemical and isotopical purities >99% were also from Witega. Acetonitrile, methanol, hexane, ethyl acetate, concentrated formic acid (98%), dipotassium hydrogen phosphate (K_2HPO_4), and sodium chloride were purchased from VWR (Darmstadt, Germany) and were of analytical reagent grade. Oasis HLB (60 mg/3 cm^3) solid phase extraction (SPE) cartridges were supplied by Waters Corp. (Milford, MA). Deionized and distilled water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA).

Standard Solutions. Unlabeled 5-nitroimidazoles (DMZ, MNZ, RNZ, IPZ, DMZOH, MNZOH, and IPZOH) stock standard solutions were prepared individually by dissolving each pure compound in methanol at a concentration of 500 μg/mL. Composite standard solutions of the seven compounds were then obtained by successive dilutions (10 μg/mL in methanol and 500 and 25 ng/mL in water, respectively). These solutions were aliquoted and stored at −20 °C in darkness and allowed to warm at room temperature before use. IS solutions were prepared and stored similarly (composite solutions at 10 μg/mL in methanol and at 1 μg/mL and 125 ng/mL in water, respectively).

Food Samples. Fresh egg samples were purchased from local supermarkets whereas processed eggs were obtained from different suppliers in Europe, the United States, Vietnam, and India. Meat and fish were from local supermarkets and were first minced using a kitchen homogenizer (MultiMoulinette, Moulinex, France) and then stored at −20 °C until use. For method validation, chicken meat from animals grown under strictly controlled conditions ("bio" label) was used.

Sample Preparation. *Fresh Egg*. A well-homogenized test portion (5.0 g) was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France) and fortified with an aliquot of 100 μL containing the deuterated ISs (125 ng/mL stock standard solution, 2.5 μg/kg). The sample was mixed and allowed to stand for 10 min in darkness. Acetonitrile (10 mL) was added, and the slurry was vigorously hand-shaken for 2 min and then placed in an ultrasonic bath (Branson 2510, Branson Ultrasonics Co., Danburry, CT) for 5 min. The tube was centrifuged at 4000*g* at room temperature for 15 min (centrifuge Multifuge 3s, Heraeus, Geneva, Switzerland), and the resulting supernatant was collected in a second 50 mL Falcon tube containing sodium chloride (ca. 2 g). The solution was first vortexed and then centrifuged at 4000*g* for 5 min. The upper organic phase was collected in a 15 mL Falcon tube and further evaporated almost to dryness under a stream of nitrogen at 45 °C. Care was exercised to

avoid excessive drying time. The yellowish dry residue obtained was reconstituted in distilled water (2 mL), and this solution was loaded onto an Oasis HLB cartridge, conditioned sequentially with methanol (1 mL) and water (2 mL). The cartridge was further rinsed with water−methanol 95:5 (v/v) (3 mL) and then dried by sucking air through it for a few seconds (Visiprep vacuum manifold, Supelco, Buchs, Switzerland). The analytes were finally eluted with methanol (2 mL), which was evaporated under a stream of nitrogen at 45 °C up to a ca. 5−10 μL remaining volume. After reconstitution with water (500 μL), the extract was filtered through a 0.45 μm nylon filter (Nalgene, Rochester, NY) directly into a high-performance liquid chromatography (HPLC) vial.

Other egg-containing matrices were analyzed as described above but with the following modifications: The test portion size was 2.5 g, distilled water (10 mL) was added after the IS fortification (5 μg/kg), and the mixture was hand-shaken to obtain a homogeneous slurry.

Meat and Fish. The well-homogenized test portion (5 g) was weighed into a 50 mL Falcon tube, fortified with 100 μL of an aliquot containing the deuterated ISs (125 ng/mL stock standard solution, 2.5 μg/kg), and diluted in a solution of 0.5 M K_2HPO_4 (10 mL). The mixture was thoroughly mixed until a homogeneous slurry was obtained, and this was left in darkness for 10 min. Ethyl acetate (10 mL) was added to the slurry before it was thoroughly hand-shaken for 2 min and centrifuged at 4000*g* for 15 min. The upper organic phase was collected, and the liquid−liquid extraction was repeated with a second 5 mL portion of ethyl acetate. The combined organic phases were then evaporated to dryness under a stream of nitrogen at 45 °C. The oily residue obtained was reconstituted in hexane (500 μL) and transferred into a 2 mL Eppendorf tube to which 0.2% formic acid (500 μL) was then added. The mixture was first vortexed for 20 s and then centrifuged at 8400*g* for 15 min (Centrifuge Eppendorf 5415C, Hamburg, Germany). The hexane phase was pipetted to waste, and the aqueous phase (recentrifuged if still cloudy) was filtered through a 0.45 μm nylon filter directly into an HPLC vial.

LC-Electrospray Ionization (ESI)-MS/MS. Analyses were performed on a Perkin-Elmer 200 Micro Pump series system (Perkin-Elmer, Uberlingen, Germany) coupled to an API 3000 triple stage quadrupole mass spectrometer equipped with a TurboIonSpray ionization source (Applied Biosystems, Foster City, CA). The HPLC column was a SymmetryShield C_{18} reversed phase (15 cm × 2.1 mm, 3.5 μm) connected to a SymmetryShield RP_{18} guard column (1 cm × 2.1 mm, 3.5 μm) (Waters). The mobile phase was constituted by solvent A, water containing 0.1% formic acid (v/v) (pH 3), and solvent B, acetonitrile containing 0.1% formic acid (v/v). A linear gradient program was realized with 0−1 min of 5% B, 1−8 min of 100% B, then hold at 100% B for 4 min before coming back to 5% B in 1 min (the HPLC column was reconditioned at 5% B for an additional 5 min of time). The flow rate was 0.3 mL/min, and 30 μL of the extract was injected onto the column. The HPLC flow was directed into the MS detector between 2 and 7 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX). MS tuning was performed in positive ESI by infusing solutions of analytes (5 μg/mL in methanol) at a flow rate of 10 μL/min mixed with an HPLC flow made of solvents A and B (50/50, v/v; 0.3 mL/min) using a T-connector. Nitrogen was used both as TurboIonSpray and curtain gases at flow rates of 7.5 L/min and 10 mL/min, respectively. The nebulizer and collision gases (both nitrogen) were used under a pressure of 776 Torr (15 psi) and 5 mTorr, respectively. The block source temperature was maintained at 350 °C, and the electrospray capillary voltage was set at 1.2 kV. The declustering potential (similar to cone voltage for Waters MS instruments) was set at 20 V for all analytes (optimal value). Other parameters whose values were common to all analytes were the entrance potential (10 V), the focusing potential (100 V), and the collision exit potential (15 V). The quantitative analysis was performed using MS/MS in multiple reaction monitoring (MRM) mode alternating two transition reactions for each 5-nitroimidazole and their corresponding ISs with a dwell time of 10 ms (**Table 1**). Data processing was done using Analyst software 1.4.1.

Calibration. 5-Nitroimidazoles were quantitated with six concentration calibrants (external calibration curves in water) ranging from 0 to 0.75 ng (0.75 ng of each deuterated IS) injected on-column. The analyte/IS area ratio (= *y*) was plotted against the analyte/IS concentration ratio (= *x*). DMZOH-d_3 was employed as the IS for DMZOH, MNZ, and MNZOH. Standards were injected before and after each series of samples, and both sets of data were compiled to establish the calibration curves. The linearity of MS responses was checked by calculating the relative standard deviation (RSD) of the average of response factors (RFs; RF = *y*/*x*), which should be below 15% (*13*). Calibration curves were also constructed in various matrices to check whether any potential matrix effects could be identified.

Table 1. Transition Reactions Monitored by LC-ESI-MS/MS (Collision Energies in eV Are Reported within Brackets) and Peak Area Ratios along with Their Limit of Acceptance (*12*)[a]

	transition reaction (*m*/*z*) used for		
analyte	quantitation	analyte confirmation	peak area ratio ± limit (%)
DMZ	142 → 96 (25)	142 → 81 (38)	0.35 ± 25
DMZ-d_3	145 → 99 (25)	145 → 83 (38)	0.14 ± 30
DMZOH	158 → 140 (18)	158 → 55 (30)	0.17 ± 30
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.27 ± 25
IPZ	170 → 124 (25)	170 → 109 (36)	0.96 ± 20
IPZ-d_3	173 → 127 (25)	173 → 112 (36)	0.71 ± 20
IPZOH	186 → 168 (20)	186 → 122 (29)	0.56 ± 20
IPZOH-d_3	189 → 171 (20)	189 → 125 (29)	0.42 ± 25
MNZ	172 → 128 (23)	172 → 82 (40)	0.58 ± 20
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.27 ± 25
MNZOH	188 → 126 (26)	188 → 123 (20)	0.92 ± 20
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.27 ± 25
RNZ	201 → 140 (18)	201 → 55 (35)	0.13 ± 30
RNZ-d_3	204 → 143 (18)	204 → 58 (35)	0.19 ± 30

[a] DMZOH-d_3 is used as an IS for DMZOH, MNZ, and MNZOH.

Confirmation Criteria. The compounds were considered as positively identified in food when criteria were met as follows: (i) the ratio of the chromatographic retention time of the analyte to that of the corresponding IS, i.e., the relative retention time of the analyte, corresponded to that of the averaged retention time of the calibration solutions within a ±2.5% tolerance; (ii) the presence of a signal at each of the two diagnostic transition reactions for the analyte and at each of the two transition reactions for the corresponding IS [the use of two transition reactions for each compound counts for four identification points as defined and required by the EU Commission Decision 2002/657/EC (*12*) and is a warranty for the selectivity of the method]; and (iii) the peak area ratio from the different transition reactions recorded for each analyte was within the tolerances fixed by the EU criteria (*12*) as shown in **Table 1**.

Method Validation. Recoveries and precisions (within- and between-day) were calculated from the analysis of six aliquots from a blank matrix, fortified with each analyte at three fortification levels (1.0, 2.0, and 3.0 μg/kg for powdered eggs and at 0.5, 1.0, and 1.5 μg/kg for fresh egg and chicken meat) and performed by the same operator on three separate occasions in a 1 week period (thus a total of 18 experiments for each concentration level). Within-laboratory precision was obtained similarly, but analyses (i.e., six blank aliquots spiked at each of the three fortification levels) were performed each by two different operators on one occasion in a 2 week period (thus a total of 12 experiments for each concentration level). The repeatability limit at the 95% confidence level was deduced from the within-day precision using an expansion factor of 2.77 (*14*). Similarly, the intermediate reproducibility limit at the 95% confidence level was calculated from the within-laboratory precision using the same expansion factor (*14*).

Measurement of Uncertainties. The estimation of measurement uncertainty was based on the results of in-house testing of spiked samples following the bottom-up approach (*15*−*17*). This methodology involves the specification of the measure, the identification and quantification of the uncertainty sources, and finally the calculation of the combined uncertainty according to the rules of errors propagation. Previous published studies have shown that the contributions of some

uncertainty sources (mass of sample, sample dilution, etc.) were so low that they could easily be neglected (*18*, *19*). The analytical parameters taken into account in this study were repeatability, recovery, and calibration data (linear regression), and their contributions were shown to represent 99% of the final uncertainty (*20*). This final value was calculated using an expansion coefficient of 2, which represents a confidence interval of 95%. The following formula was thus applied for each fortification level (*20*):

$$U(\%) = 2 \times \frac{u(x)}{x} = 2 \times \sqrt{\mathrm{RSD}(r)^2 + \left[\frac{u(\mathrm{Rec})}{\mathrm{Rec}}\right]^2 + \left[\frac{u(c)}{c}\right]^2}$$

where $u(x)/x$ = relative standard uncertainty of the analyte x; RSD(r) = RSD of repeatability; [u(Rec)/Rec] = relative uncertainty of recovery; and [$u(c)/c$] = relative uncertainty due to calibration (model/linearity).

The standard deviation of the uncertainty of recovery was calculated at the repeatability level and was defined as

$$\mathrm{SD}(\mathrm{Rec}) = \bar{\mathrm{R}}\mathrm{ec} \times \sqrt{(1/n) \times [\mathrm{SD}(\bar{C}_{\mathrm{observed}})/\bar{C}_{\mathrm{observed}}]^2}$$

where $\bar{\mathrm{R}}$ec is the mean of recovery experiments for each fortification level, n is the number of replicates, and $\bar{C}_{\mathrm{observed}}$ is the calculated concentration. However, this formula cannot apply as such if the recovery is statistically different from 100%. To check for this deviation, the significance t-test is used and is calculated using the following equation

$$t = |1 - \bar{\mathrm{R}}\mathrm{ec}|/\mathrm{SD}(\mathrm{Rec})$$

where t should be not bigger than the critical value for a two-tailed test at the 95% confidence level and for n replicates. When this happens, an extended standard deviation SD(Rec)* should be used and is defined as

$$\mathrm{SD}(\mathrm{Rec})^* = \sqrt{[(1 - \mathrm{Rec})/k]^2 + [\mathrm{SD}(\mathrm{Rec})]^2}$$

where k is 2 for a coverage factor of 95%.

The relative standard uncertainty due to the calibration model (least-squares method) was calculated as the averaged uncertainty of all calibration levels for each analyte. At each calibration level, the uncertainty was estimated as follows:

$$u(C_{\mathrm{level}})/C_{\mathrm{level}} = [\mathrm{SD}(\sqrt{(Y_{\mathrm{obs}} - Y_{\mathrm{cal}})^2})]/\mathrm{slope} \times C_{\mathrm{IS}}/C_{\mathrm{level}} \times 100$$

where C_{level} is the nominal concentration of the considered level, C_{IS} is the nominal concentration of the IS, and $\mathrm{SD}(\sqrt{(Y_{\mathrm{obs}}-Y_{\mathrm{cal}})^2})$ is the standard deviation of residuals from duplicate injection of the considered standard solution. For each analyte, the results from seven calibration curves were used for this uncertainty evaluation.

RESULTS AND DISCUSSION

Method Development. Initially, the extraction method considered for the analysis of egg-containing matrices was that described by Sams et al. (*21*), which employs a strong cation-exchange SPE cartridge to purify acidified acetonitrile extracts of fresh eggs. However, applied to the analysis of egg powders (after reconstitution with water in a proportion similar to that contained in fresh egg), this method failed to give satisfactory results, as most of 5-nitroimidazole compounds showed no retention on this SPE cartridge. No further improvement was obtained either by using cation-exchange SPE cartridges from different suppliers or by altering the test portion size or the volumes of solvents used. Finally, the method applied consisted of a water addition to the powdered samples, a removal of proteins by means of acetonitrile, followed by a sodium chloride addition, which permitted the elimination of water coextracted with the organic solvent. A final cleanup on an Oasis HLB SPE cartridge was found mandatory to reduce the background noise before the LC-ESI-MS/MS analysis and thus allowed more than 150 sample injections to be performed without excessive pollution of the mass spectrometer or loss of LC column performance. Wang et al. (*22*) have demonstrated a similar extraction procedure for the analysis of macrolides in fresh egg, but the acetonitrile extract was subsequently washed with hexane to remove the lipidic content before SPE cleanup. This defatting step applied to the analysis of our analytes was shown to reduce the overall recovery of the less polar 5-nitroimidazole compounds, e.g., IPZ and IPZOH, as also evidenced by other authors (*8*). Our previous experiences when analyzing powdered eggs have demonstrated a better extractability of contaminants (veterinary drugs and pesticides) when the matrix was first water- or buffer-slurried before analyte(s) extraction. Additionally, this step permits the fortified ISs to be placed in a chemical environment similar to that of the endogenous drugs, which is a mandatory condition when using the isotopic dilution method for quantitation. The extraction procedure described provides apparent recoveries for our targeted analytes of 19−24, 33−48, and 27−38% for whole egg powder, fresh eggs, and egg yolk powder, respectively. These apparent recoveries were calculated from the ratio of the peak area of the IS in the sample to the mean peak area of the IS of the corresponding standard calibration and thus included the loss of analyte during the extraction/purification steps as well as ion suppression effects observed during the analysis by LC-ESI-MS/MS. Attempts to improve these yields by repeated extractions with acetonitrile or by first solubilizing egg powders in a phosphate buffer (*23*) before liquid−liquid extraction showed no significant improvements. However, as such, this extraction procedure still allowed the 5-nitroimidazoles under survey to be unambiguously detected at the ≤ 1 μg/kg levels.

The extraction of the seven 5-nitroimidazoles from meat and fish samples was adapted from that proposed by Hurtaud-Pessel et al. (*23*) for the analysis of DMZ, MNZ, RNZ, and DMZOH in poultry meat using single quadrupole LC-MS. Thus, the use of ethyl acetate as the extracting solvent applied to our study provided apparent recoveries ranging from 52 to 66%. Comparatively, when acetonitrile was involved with these matrices, lower extraction yields (ca. −20%) were obtained, showing thus ethyl acetate as a better extraction solvent for 5-nitroimidazole drugs than acetonitrile. However, when applied to the analysis of eggs, the use of ethyl acetate leads to the formation of stable emulsions, preventing the extraction of these analytes (*4*). Both extraction procedures allowed ca. 20 samples to be extracted within 6 h.

LC-ESI-MS/MS Acquisition. Both ESI and atmospheric pressure chemical ionization sources were tested, showing nearly identical responses for all analytes in the positive mode. The ESI source was considered for convenience since it is frequently used for other types of analysis. Protonated molecules $(M + H)^+$ were thus obtained for the different 5-nitroimidazoles and their metabolites. Collision-induced dissociation (CID) mass spectra were then recorded for each analyte and their corresponding IS at various collision energies before selecting the two major characteristic transition reactions for our confirmatory method. The fragmentation pathway of the main fragments depicted for each molecule is tentatively explained hereafter (**Figure 2**). For DMZ, the fragment at m/z 96 represents the elimination of NO_2 (46 Da) from the protonated molecule (m/z 142) and is thus a radical cation (nitrogen rule). The ion at m/z 81 ($C_4H_5N_2$) originates from the imidazole ring opening followed by the loss of CH_3NO_2 (61 Da). A similar fragmenta-

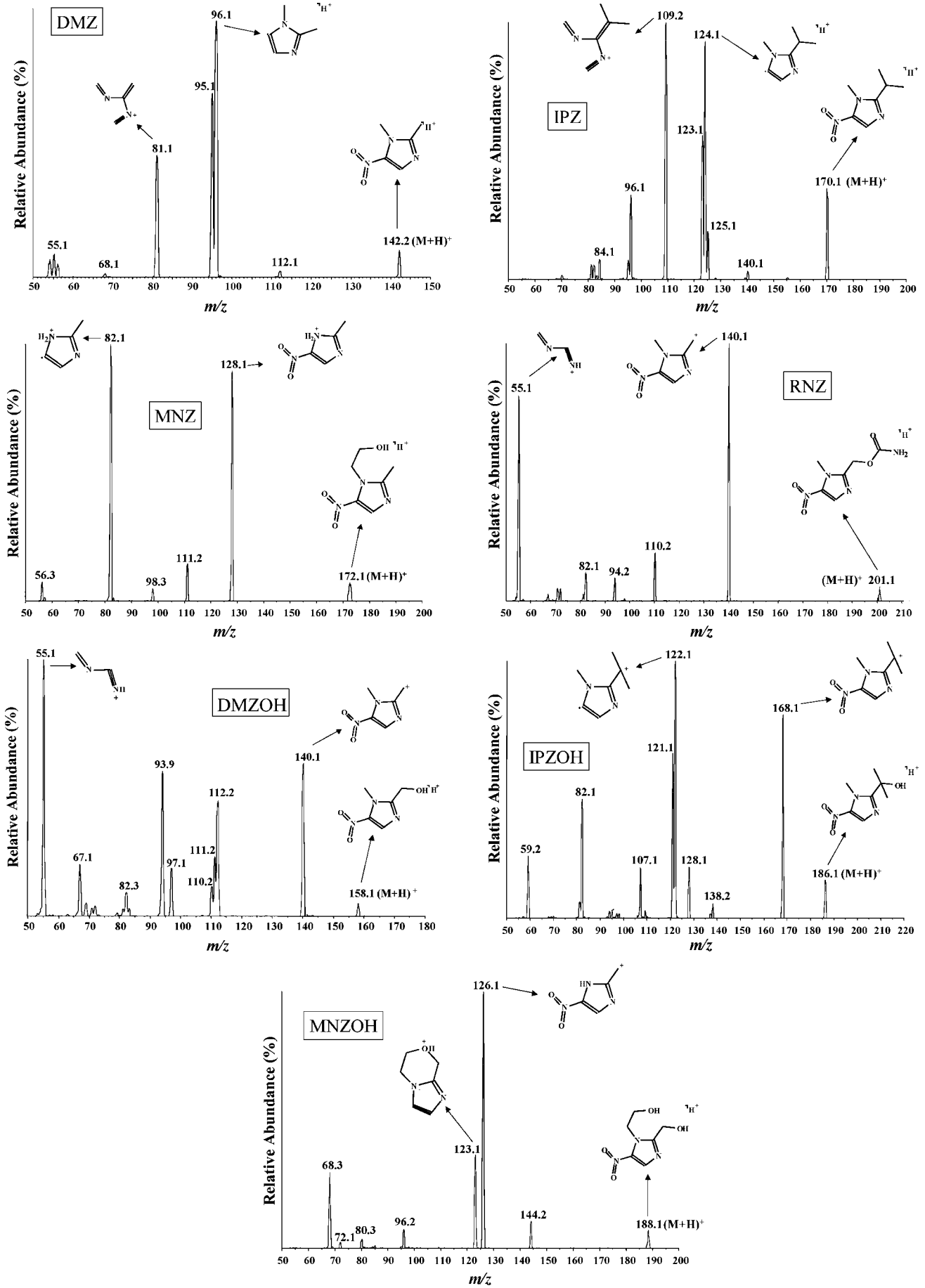

Figure 2. CID spectra of the seven 5-nitroimidazoles acquired with a collision energy set at 30 eV (collision gas pressure of 5 mTorr). The chemical structures of the two fragment ions selected for the confirmatory method are inserted for each compound.

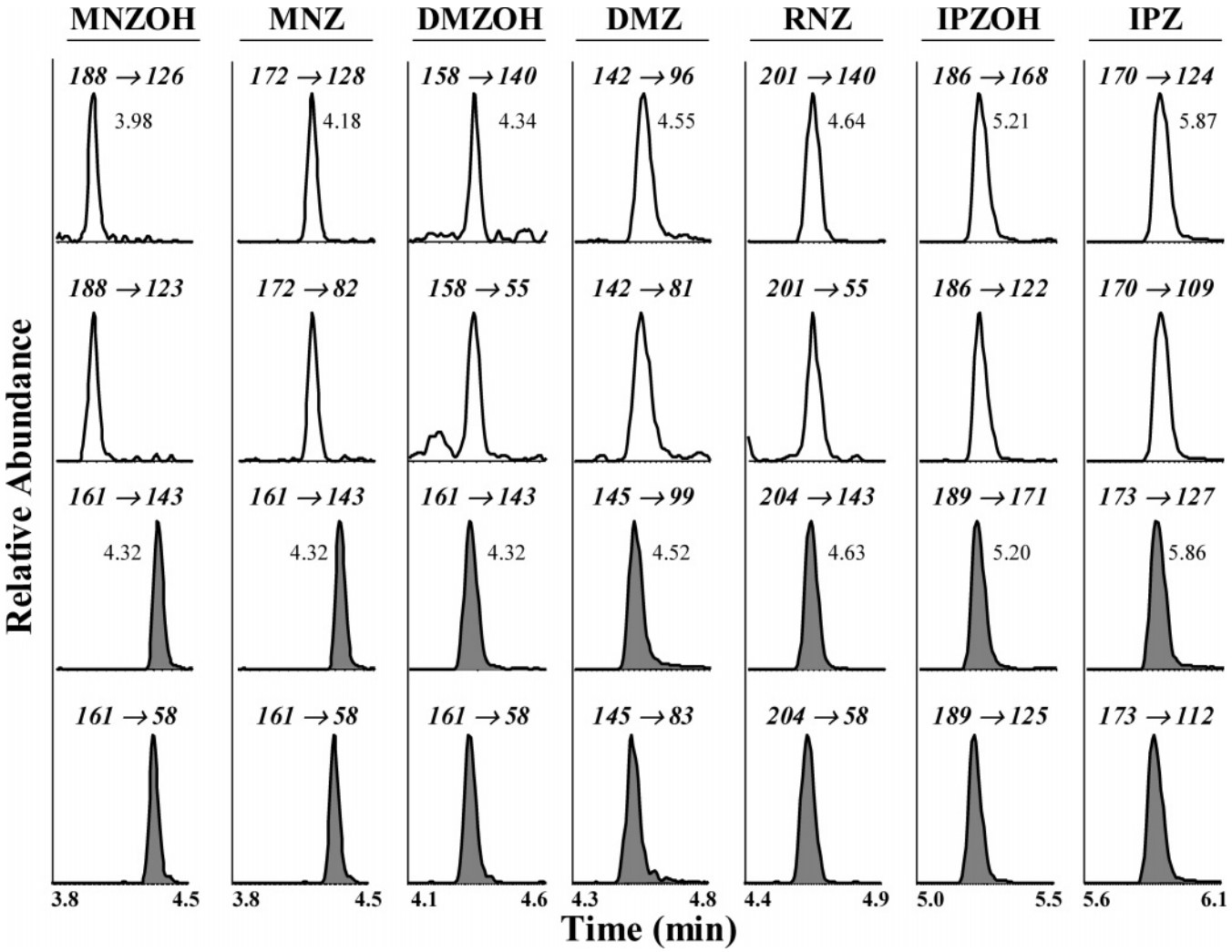

Figure 3. MRM chromatograms of a spiked fresh egg extract (0.5 μg/kg of each analyte and 2.5 μg/kg of each IS). IS peaks are darkened. DMZOH-d_3 was used as the IS for DMZOH, MNZ, and MNZOH.

tion pathway is observed for IPZ (*m*/*z* 124 and *m*/*z* 109, respectively). For MNZ (*m*/*z* 172), the loss of $H_2C{=}CHOH$ (44 Da) leads to *m*/*z* 128. A subsequent elimination of NO_2 from this fragment then gives a radical cation at *m*/*z* 82. For RNZ (*m*/*z* 201), the ion at *m*/*z* 140 results from the loss of the carbamate group ($HO{-}CO{-}NH_2$, 61 Da), whereas *m*/*z* 55 is obtained from the ring opening followed by the loss of $C_4H_6O_4N_2$ (146 Da). For DMZOH (*m*/*z* 158), the elimination of a water molecule accounts for the ion at *m*/*z* 140, whereas *m*/*z* 55 (loss of 103 Da) originates from the ring opening followed by the successive losses of $NO_2{-}C{=}CH$ and methanol. For IPZOH (*m*/*z* 186), the fragments of interest (*m*/*z* 168 and *m*/*z* 122) result from the loss of H_2O followed by that of the nitro group to give the radical cation. For MNZOH (*m*/*z* 188), the concomitant elimination of H_2O and $H_2C{=}CHOH$ (62 Da) affords the fragment ion at *m*/*z* 126. The losses of NO_2 and water followed by a cyclization of the residual fragment give *m*/*z* 123. The relevance of these fragments was confirmed by the CID spectra analysis of the deuterated ISs. Thus, for DMZ-d_3, a fragment at *m*/*z* 83 ($C_4D_2H_3N_2$) was evidenced and the discrepancy between this ion and the expected *m*/*z* 84 was explained by the loss of one deuterium atom.

Through chromatographic separation as well as MS detection, the signals of the seven analytes were well-separated in 6 min (**Figure 3**) with a total run time of 18 min (including the reequilibration time) enabling >30 extracts to be injected in an overnight sequence. No interference at the expected retention times was noticed in blank matrices. Matrix effects were evaluated by building matrix-matched calibration curves (obtained by spiking the analytes and their corresponding ISs at the beginning of the sample workup) and comparing their slopes with those of water-based curves. Deviations from the slopes of water-based curves were observed for all analytes in the range of −18 to +7, +1 to +11, 0 to +10, and −2 to −7% for whole egg powder, fresh egg, egg yolk powder, and chicken meat, respectively. However, considering these small differences and their weak impact on the final results, only water-based calibration curves were used for convenience during this validation. The stability of the 5-nitroimidazoles in final food extracts was assessed by reinjecting a series of processed samples left at room temperature onto the autosampler for a 36 h period. No significant difference (peak area comparison) was observed between the two injected batches.

Method Performance Characteristics. The overall performance data of this LC-ESI-MS/MS procedure at the lowest fortification levels are summarized in **Table 2**. According to the EU Commission Decision 2002/657/EC (*12*), recoveries for addition with known amounts of the analytes to a blank matrix should fall within −50 to +20% of the spiked concentration for values ≤1 μg/kg and within −30 to +10% for values between 1 and 10 μg/kg. In egg-based matrices, this requirement is satisfied for all analytes having their own corresponding

Table 2. Performance Data of the LC-ESI-MS/MS Method for the Analysis of Seven 5-Nitroimidazoles in Different Matrices at the Lowest Fortification Levels Considered (Concentrations in Brackets)

	DMZ	RNZ	MNZ	IPZ	DMZOH	MNZOH	IPZOH
			fresh egg (0.5 μg/kg)				
overall recovery ± SD (%)	94 ± 11	98 ± 6	79 ± 44	90 ± 6	97 ± 9	31 ± 24	88 ± 7
within-day precision (%)	10	6	>20	7	8	>20	8
between-day precision (%)	15	6	>20	8	10	>20	9
r (μg/kg)[a]	0.13	0.08	0.56	0.08	0.11	0.30	0.09
within-lab precision (%)	14	6	>20	7	7	19	5
iR (μg/kg/%)[b]	0.19/38	0.09/18	0.77	0.09/20	0.10/20	0.15	0.06/13
U (μg/kg/%)[c]	0.11/22	0.09/18		0.09/18	0.08/16		0.10/20
			whole egg powder (1.0 μg/kg)				
overall recovery ± SD (%)	103 ± 8	98 ± 9	131 ± 43	89 ± 15	111 ± 9	81 ± 41	95 ± 6
within-day precision (%)	8	7	>20	7	8	>20	4
between-day precision (%)	8	9	>20	20	8	>20	6
r (μg/kg)[a]	0.23	0.20	0.76	0.18	0.25	0.83	0.11
within-lab precision (%)	8	6	13	11	6	>20	7
iR (μg/kg/%)[b]	0.23/23	0.15/16	0.36	0.27/30	0.18/18	0.66	0.18/20
U (μg/kg/%)[c]	0.17/17	0.21/21		0.21/21	0.22/22		0.12/12
			egg yolk powder (1.0 μg/kg)				
overall recovery ± SD (%)	95 ± 6	96 ± 5	136 ± 20	97 ± 6	95 ± 8	56 ± 12	94 ± 5
within-day precision (%)	6	5	14	6	7	>20	4
between-day precision (%)	6	6	14	7	8	19	4
r (μg/kg)[a]	0.16	0.14	0.54	0.15	0.18	0.32	0.11
within-lab precision (%)	4	5	14	5	8	15	5
iR (μg/kg/%)[b]	0.11/12	0.14/14	0.47	0.12/13	0.21/21	0.23	0.12/13
U (μg/kg/%)[c]	0.14/14	0.18/18		0.13/13	0.16/16		0.13/13
			chicken meat (0.5 μg/kg)				
overall recovery ± SD (%)	99 ± 5	97 ± 5	91 ± 7	95 ± 5	97 ± 7	52 ± 5	101 ± 7
within-day precision (%)	5	5	8	6	7	8	5
between-day precision (%)	6	5	9	6	8	9	7
r (μg/kg)[a]	0.07	0.06	0.10	0.08	0.09	0.06	0.07
within-lab precision (%)	7	7	7	4	8	9	7
iR (μg/kg/%)[b]	0.09/20	0.09/20	0.09/19	0.06/12	0.11/23	0.07	0.10/21
U (μg/kg/%)[c]	0.05/10	0.09/18	0.12/24	0.07/14	0.08/16		0.06/12

[a] Repeatability limit at 95% confidence level. [b] Intermediate reproducibility limit at 95% confidence level. [c] Expanded uncertainty at 95% confidence level.

deuterated analogue used as IS (e.g., DMZ, RNZ, IPZ, DMZOH, and IPZOH). Thus, at the lowest fortification levels, recovery values of these analytes, corrected by the relevant IS, ranged between 88 and 98% for fresh egg, 89 and 111% for whole egg powder, and 94 and 97% for egg yolk powder. Furthermore, both within- and between-day precisions were <15% and considered as satisfactory at these low concentrations. In chicken meat, correct recoveries were found for the same compounds but also for MNZ and values ranged between 91 and 101% (at a spiking level of 0.5 μg/kg), along with within- and between-day precisions ≤10%. The closeness between repeatability and intermediate reproducibility values shows that the method is not operator-dependent.

Accreditation bodies have to look for procedures to estimate the uncertainty, since it is a requirement from ISO 17025 (*24*). However, the whole procedure of calculating this value following the bottom-up approach (*16*) is often a time-consuming task added to the regular laboratory workload. It has been proposed that the RSD of intermediate reproducibility (RSD_{iR}), when >3%, could be used as an estimation of the uncertainty (*U*) (*20*). In our study (**Table 2**), this statement is almost verified for all analytes/matrices considered with differences between RSD_{iR} and *U* ranging from −8% up to +16% (mean ± SD, 2 ± 6%, $n = 21$). One drawback of this time-saving approach is that it will not provide information about the contributions of the different method parameters to the combined uncertainty.

The requirement for MNZ and its metabolite MNZOH to be quantitated against their corresponding deuterated IS for accurate measurements was apparent in egg-based samples but also for MNZOH in chicken meat. Indeed, these two 5-nitroimidazoles were quantitated using DMZOH-d_3 as the IS, which was selected due to its chromatographic closeness with MNZ ($\Delta R_T = 0.14$ min) and MNZOH ($\Delta R_T = 0.33$ min). However, as demonstrated, a complete compensation of both losses during sample workup and matrix effects was not systematically obtained using this IS. Attempts to use one of the other available deuterated ISs (e.g., DMZ-d_3, RNZ-d_3, IPZ-d_3, or IPZOH-d_3) failed to improve these recovery values. Nevertheless, the method still allows a correct identification of MNZ and MNZOH in egg-based matrices and MNZOH in chicken meat at concentrations corresponding to the lowest levels of fortification. However, a reliable quantitation of these matrix/analyte combinations would involve the time-consuming approach of matrix-matched calibration curves (without any of the ISs used in this study), once identified by the described procedures.

The determination of decision limits (CCα) and detection capabilities (CCβ) was calculated following the calibration curve procedure as described in the EU guidelines (*12*), and their values are summarized in **Table 3**. It is worth noting that these limits should not be considered as steady values but rather as variables reflecting a spot check of the method instrument's performances. This fact was recently demonstrated by Verdon et al. (*25*) during routine controls of banned nitrofuran veterinary drugs. In egg-based matrices and for DMZ, RNZ, IPZ, DMZOH, and IPZOH, CCα and CCβ values ranged from 0.09 to 0.36 and from 0.14 to 0.60 μg/kg, respectively. CCα values for MNZ and MNZOH were arbitrarily set at the lowest levels of fortifications, e.g., 0.5 μg/kg for fresh egg and 1.0 μg/kg for the other egg-containing matrices, and no CCβ value was attributed. In chicken meat and for DMZ, RNZ, IPZ, DMZOH, IPZOH, and MNZ, CCα and CCβ values ranged from 0.07 to 0.10 and from 0.11 to 0.18 μg/kg, respectively. For MNZOH

Table 3. Decision Limits (CCα) and Detection Capabilities (CCβ) for the 5-Nitroimidazoles under Survey (Values in μg/kg)

	DMZ	RNZ	MNZ	IPZ	DMZOH	MNZOH	IPZOH
egg yolk powder							
CCα	0.13	0.14	1.0	0.14	0.20	1.0	0.12
CCβ	0.22	0.25		0.23	0.35		0.20
whole egg powder							
CCα	0.21	0.22	1.0	0.36	0.32	1.0	0.15
CCβ	0.37	0.36		0.60	0.54		0.26
fresh egg							
CCα	0.18	0.09	0.5	0.13	0.10	0.5	0.13
CCβ	0.28	0.14		0.20	0.17		0.18
chicken meat							
CCα	0.07	0.07	0.10	0.07	0.08	0.5	0.09
CCβ	0.12	0.12	0.18	0.11	0.14		0.15

in this latter matrix, the CCα value was set at 0.5 μg/kg and no CCβ value was given.

Validation of the method in fresh egg was further performed through participation in a FAPAS ring test (24 laboratories involved), showing our data with *z*-scores of -0.1 and -0.5 for DMZ and DMZOH, respectively (*26*). The applicability of the developed procedures in routine quality controls was demonstrated by the analysis of 25 egg-based (whole egg powder, egg yolk powder, liquid egg yolk, and crystallized egg albumin), meat (chicken, pork, beef, and horse), and farmed fish (trout and salmon) samples. None of the samples tested showed contamination with 5-nitroimidazoles.

Conclusions. A LC-MS/MS method has been developed for the analysis of trace levels of four 5-nitroimidazole-based veterinary drugs and three of their metabolites in egg-based raw materials and in meat and fish samples. MS/MS using MRM transitions on analyte-specific fragment ions enables selective and confirmatory detection of the seven analytes considered at levels ≤ 1 μg/kg. A precise and sensitive quantitation (≤ 0.6 μg/kg) was obtained for five out of seven compounds, i.e., those having their own isotopically labeled analogue used as ISs. On the other hand, MNZ and its metabolite MNZOH, once identified by the described method, should be quantitated by means of the matrix-matched calibration curve quantitation procedure, without the use of any of the deuterated ISs considered in this study.

Supporting Information Available: MRM chromatograms of spiked extracts of egg yolk powder, whole egg powder, egg albumin powder, liquid egg yolk, meat (chicken, horse, beef, and pork), and fish (salmon and trout). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Raether, W.; Hänel, H. Nitroheterocyclic drugs with broad spectrum activity. *Parasitol. Res.* **2003**, *90*, 19−39.

(2) Commission Regulation No. 508/1999 of 4 March 1999 amending Annexes I to IV to Council Regulation No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Commun.* **1999**, *L060*, 16−52.

(3) Polzer, J.; Stachel, C.; Gowik, P. Treatment of turkeys with nitroimidazoles. Impact of the selection of target analytes and matrices on an effective residue control. *Anal. Chim. Acta* **2004**, *521*, 189−200.

(4) Aerts, R. M.; Egberink, I. M.; Kan, C. A.; Keukens, H. J.; Beek, W. M. J. Liquid chromatographic multicomponent method for determination of residues of ipronidazole, ronidazole and dimetridazole and some relevant metabolites in eggs, plasma and feces and its use in depletion studies in laying hens. *J. AOAC Int.* **1991**, *74*, 46−55.

(5) Sorensen, L. K.; Hansen, H. Determination of metronidazole and hydroxymetronidazole in trout by a high performance liquid chromatographic method. *Food Addit. Contam.* **2000**, *17*, 197−203.

(6) Rose, M. D.; Bygrave, J.; Sharman, M. Effect of cooking on veterinary drug residues in food. Part 9. Nitroimidazoles. *Analyst* **1999**, *124*, 289−294.

(7) Mortier, L.; Daeseleire, E.; Delahaut, P. Simultaneous detection of five cocciodiostats in eggs by liquid chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2003**, *483*, 27−37.

(8) Polzer, J.; Gowik, P. Validation of a method for the detection and confirmation of nitroimidazoles and corresponding hydroxy metabolites in turkey and swine muscle by means of gas chromatography-negative ion chemical ionization mass spectrometry. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *761*, 47−60.

(9) Capitan-Vallvey, L. F.; Ariza, A.; Checa, R.; Navas, N. Determination of five nitroimidazoles in water by liquid chromatography−mass spectrometry. *J. Chromatogr. A* **2002**, *978*, 243−248.

(10) Radeck, W. Determination of nitroimidazoles using APCI+ mass spectrometry. In *Proceedings of the Euroresidue IV Conference, Veldhoven, The Netherlands*; Van Ginkel, L. A., Ruiter, A., Eds.; 2000; pp 878−884.

(11) Govaert, Y.; Degroodt, J. M.; Srebrnik, S. Nitroimidazole drug residues in poultry and pig tissues: Adaptation and validation of a methodology designed for the routine analysis. In *Proceedings of the Euroresidue IV Conference, Veldhoven, The Netherlands*; Van Ginkel, L. A., Ruiter, A., Eds.; 2000; pp 470−475.

(12) Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *L221*, 8−36; http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/l_221/l_22120020817en00080036.pdf.

(13) Rodriguez, M.; Orescan, D. B. Confirmation and quantitation of selected sulfonylurea, imidazolinone, and sulfonamide herbicides in surface water using electrospray LC/MS. *Anal. Chem.* **1998**, *70*, 2710−2717.

(14) *Accuracy (Trueness and Precision) of Results and Test Methods. Part 6: Practical Use of Accuracy Values*; ISO 5725-6; International Standards Organization: Geneva, Switzerland, 1994.

(15) *Guide to the Expression of Uncertainty*, 1st ed.; International Standards Organization: Geneva, Switzerland, 1993.

(16) Ellison, S. L. R., Roesslein, M., Williams, A., Eds. Quantifying uncertainty in analytical measurement. *Eurachem/CITAC Guide*, 2nd ed.; 2000. http://www.eurachem.ul.pt/guides/QUAM2000-l.pdf.

(17) Barwick, V. J.; Ellison, S. L. R. Protocol for uncertainty evaluation from validation data. *VAM Project 3.2.1. Development and Harmonisation of Measurement Uncertainty Principles*; LGC/VAM: 2000. http://www.caeal.ca/assessor_training/at01_VAM_uncertainty.pdf.

(18) Barwick, V. J.; Ellison, S. L. R. Estimating measurement uncertainty using a cause and effect and reconciliation approach. Part 2: Mesurement uncertainty estimates compared with collaborative trial expectation. *Anal. Commun.* **1998**, *35*, 377−383.

(19) Hund, E.; Massart, D. L.; Smeyers-Verbeke, J. Comparison of different approaches to estimate the uncertainty of a liquid chromatography assay. *Anal. Chim. Acta* **2003**, *480*, 39−52.

(20) Campos Gimenez, E.; Populaire, S. Use of validation data for fast and simple estimation of measurement uncertainty in liquid chromatography methods. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28*, 3005−3013.

(21) Sams, M. J.; Strutt, P. R.; Barnes, K. A.; Damant, A. P.; Rose, M. D. Determination of dimetridazole, ronidazole and their common metabolite in poultry muscle and egg by high performance liquid chromatography with UV detection and confirmatory analysis by atmospheric pressure chemical ionisation mass spectrometry. *Analyst* **1998**, *123*, 2545−2549.

(22) Wang, J.; Leung, D.; Butterworth, F. Determination of five macrolide antibiotic residues in eggs using liquid chromatography/electrospray ionisation tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 1857−1865.

(23) Hurtaud-Pessel, D.; Delépine, B.; Laurentie, M. Determination of four nitroimidazole residues in poultry meat by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **2000**, *882*, 89−98.

(24) *General Requirements for the Competence of Testing and Calibration Laboratories*; ISO 17025; International Standards Organization: Geneva, Switzerland, 2000.

(25) Verdon, E.; Hurtaud-Pessel, D.; Sanders, P. Evaluation of the limit of performance of an analytical method through a statistical calculation of its critical concentrations according to the European Decision 657/2002EC. Poster presented at the 3rd AOAC-Eurachem Symposium, Brussels, Belgium, 2005; http://crl.fougeres.afssa.fr/publicdoc/POSTER-AFSSA-EVerdon-2005.pdf.

(26) Food Analysis Performance Assessment Scheme (FAPAS) Veterinary Drug Residues Report No. 0267, 2005.

Received for review November 22, 2005. Revised manuscript received January 24, 2006. Accepted January 24, 2006.

JF052907S