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Determination of four nitroimidazole residues in poultry meat by liquid chromatography–mass spectrometry

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Abstract

A multi-residue liquid chromatography–mass spectrometry (LC–MS) assay method is described for the determination of four nitroimidazoles in poultry muscle. The extraction procedure is based on liquid–liquid extraction with ethyl acetate followed by an evaporation step. A deuterated internal standard is used. The LC separation was made on a C_{18} bonded silica column with an aqueous formic acid (0.2%) solution–methanol–acetonitrile (81:13:6) mobile phase. Following electrospray ionization, the protonated molecular ion $[M+H]^+$ is obtained for each compound. Monitoring several ions for each nitroimidazole provides the specificity required for confirmatory assay. Validation of the method was performed to estimate linearity, intra-day and inter-day repeatability, accuracy and detection limit. The present method is capable of identifying nitroimidazole residues in muscle at levels below 5 μg/kg.

Keywords: Food analysis; Meat; Nitroimidazoles; Imidazoles

1. Introduction

Nitroimidazoles are a class of veterinary drugs used for the treatment and prevention of certain bacterial and protozoal diseases in poultry (histomoniasis in turkeys, trichomoniasis in pigeons etc.) and for swine dysentery [1].

Mutagenic, carcinogenic and toxic properties of these compounds have been reported [2]. This potentiality led the European Union Commission to include them in Annex IV to Council Regulation (EC) 2377/90 [3]. This means that their use in food-producing species is prohibited within the EU. This decision was made for ronidazole (RNZ) in 1993 [4], for dimetridazole (DMZ) in 1995 [5] and for metronidazole (MNZ) in 1998 [6]. However, DMZ may still be used as feed additives [7].

The 5-nitroimidazoles are known to be rapidly metabolised. The main metabolites result from oxidation of the side-chain in the C2 position of the imidazole ring. For DMZ, the major metabolite is formed by hydroxylation of the 2-methyl group to give the hydroxydimetridazole DMZOH [8]. In the same way, MNZ gives the hydroxylated metabolite MNZOH. MNZ gives another metabolite by oxidation of the N-2hydroxyethyl group to give the acetylmetronidazole. The metabolites formed may have a similar mutagenic potential as the parent compound [9].

Several published methods are able to detect the parent compound nitroimidazoles with or without their metabolites.

In our laboratory, a sensitive screening TLC method was available for the detection of RNZ, DMZ and DMZOH in pig and poultry muscle with detection limits between 2 and 5 μg/kg [10].

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Another screening method using immunoassay was described for the detection of dimetridazole and other nitroimidazole residues in turkey at ppb level [11]. Several liquid chromatography (LC) methods applied to determination of nitroimidazoles in animal tissues have also been reported. Most of them concerned DMZ and DMZOH. These LC methods used electrochemical [12] or UV detection [13,14] and allowed determination at levels of less than 2 ppb in muscle. Some methods used mass spectrometry detection either with GC [15] or LC separation [16–18]. For banned substances, unambiguous identification of the suspect residues found in the sample is necessary. Due to its high specificity and sensitivity, mass spectrometry is well suited for confirmation of such analytes.

As in our laboratory we had only a screening method, a confirmatory method was required. The purpose of this work was to develop a multi-residue confirmatory method for nitroimidazole residues in poultry muscle. The work presented here uses LC–MS with electrospray ionization for the determination of DMZ, RNZ, MNZ and DMZOH following a liquid–liquid extraction. This method was validated and used to confirm nitroimidazole residue in a plan survey.

2. Experimental

2.1. Chemicals and solvents

Methanol, acetonitrile, carbon tetrachloride, hexane, ethyl acetate, all analytical grade, and formic acid (98–100%, for analysis) were obtained from Merck (Darmstadt, Germany). Dipotassium hydrogenphosphate was obtained from Prolabo (Paris, France). Water was purified with a Milli-Q apparatus (Millipore, Molsheim, France). Nitrogen C gas for the mass spectrometer was purchased from Air liquide (St. Quentin en Yvelines, France).

For the extraction procedure, a 0.5 *M* phosphate buffer (pH 8.8) was prepared by dissolving 17.4 g of K_2HPO_4 in water and diluting to 100 ml.

2.2. Standards

Dimetridazole and metronidazole were obtained from Sigma (Saint-Quentin-Fallavier, France) and ronidazole and hydroxydimetridazole from Merck Sharp and Dohme (Bruxelles, Belgium). [$^2H_3$]Ronidazole (RNZ-d_3; 2-carbamoyloxymethyl-1-methyl(d_3)-5-nitroimidazole) was obtained from RIVM (Bilthoven, The Netherlands).

Individual stock standard solutions (1 g/l) of DMZ, MNZ, RNZ, and DMZOH were prepared by dissolving each pure reference compound in methanol. These solutions were stored at −20°C in the dark and warmed up to room temperature before use. An intermediate composite standard solution (10 mg/l) of DMZ, MNZ, RNZ, and DMZOH was prepared in methanol and was stored at +4°C in the dark. The working standard solution (0.1 mg/l) was prepared daily in an amber-coloured vial by diluting the intermediate composite standard solution in water.

A stock solution of internal standard RNZ-d_3 at 10 mg/l was prepared in methanol. Another working standard solution containing RNZ-d_3 at 0.2 mg/l and DMZ, MNZ, RNZ, and DMZOH at 0.1 mg/l was prepared in water.

2.3. Tissue sample

For the validation, a range of spiked samples was prepared as follows: to 4 g of minced muscle weighed in 100×18 mm PPCO centrifuge tubes, 120, 200, 300, 400, 600 μl of 0.1 mg/l working standard solution of DMZ, MNZ, RNZ, and DMZOH and respectively 480, 400, 300, 200 and 0 μl of water were added to obtain muscle samples spiked at 3, 5, 7.5, 10 and 15 μg/kg. Blank sample was prepared by adding 600 μl of water to 4 g of minced muscle.

2.4. Sample extraction

Nitroimidazoles are very light sensitive, therefore it is essential to protect the solutions and the extracts from light (dark room, amber-coloured vials, amber foil).

To the sample (4 g), 10 μl of internal standard RNZ-d_3 (10 mg/l) and if necessary tissue sample solutions (as above) were added. The sample was mixed and allowed to stand for 10 min in the dark before extraction. Then, 1.6 ml of phosphate buffer

(0.5 *M*, pH 8.8) were added to the sample and the sample was vortex-mixed for 20 s. Then 8 ml of ethyl acetate were added and vortex-mixed vigorously for 20 s at maximum speed. The tube was shaken for 10 min at 100 rev./min with a rotating stirrer. Following centrifugation (10 min, 8000×*g*), 6.8 ml of the organic phase (upper layer) were pipetted and transferred into a 25-ml round-bottomed flask. The organic phase was evaporated just to dryness with a vacuum evaporator (water bath at 35°C). Then the remaining oily residue was mixed with 400 μl of a solution of hexane–CCl_4 (1:1) and with 400 μl of formic acid (0.2% in water). The mixture was transferred into an Eppendorf tube and centrifuged (2 min, 17 300×*g*). The aqueous phase (upper layer) was pipetted and transferred into microvials for analysis.

2.5. HPLC–MS equipment and conditions

The HPLC system was a Hewlett-Packard pump (Type 1050) equipped by a Rheodyne injector with a 100-μl sample loop. Separation was performed on a Waters Symmetry C_{18} column (150×3.9 mm I.D., particle size 5 μm, 100 Å) protected by a guard column Symmetry C_{18}. The mobile phase consisted of acetonitrile–methanol–formic acid 0.2% in water (6:13:81) and the flow-rate was 0.6 ml/min. No split was necessary to introduce the LC eluent in the mass spectrometer.

The HPLC was coupled to a Finnigan SSQ 7000 mass spectrometer with an electrospray interface. The capillary temperature was 220°C and the quadrupole temperature was 70°C. The spray voltage was set at 5000 V. Nitrogen sheath gas pressure was fixed at 80 p.s.i. and no auxiliary gas was used (1 p.s.i.= 6894.76 Pa). The offset voltage was set at −0.2 V and the collision-induced dissociation (CID) voltage was 10 V. The system was operated with positive ion detection. The data system was the standard digital DEC 3000 station.

Different parameters of the mass spectrometer like the tube lens voltage and the capillary voltage were optimized by loop injection of the nitroimidazoles. The LC effluent was connected to the interface via a divert valve from 3.5 min to 7 min after the injection of the extract into the LC system to avoid the contamination of the system. The instrument was operated in selected ion monitoring (SIM) mode with a dwell time of 62.5 ms per ion.

2.6. Validation of the method

Validation of the method was carried out as follows: a range of muscle samples spiked at 3, 5, 7.5, 10 and 15 μg/kg and four control samples spiked at 5 μg/kg were analysed per day for 5 days. Each day, least-squares linear regression analysis was used to calculate the equation $y = mx + b$ (where y was the ratio of the peak area of the $[MH]^+$ ion of the nitroimidazole to that of the internal standard and x was the sample concentration) for the range 3–15 μg/kg. The levels in the control samples fortified at 5 μg/kg were back-calculated using the calibration curve of the day.

The data obtained from these assays allowed the assessment of linearity, intra-day and inter-day repeatability at one level of 5 μg/kg, accuracy at 5 μg/kg, detection and determination limits.

3. Results and discussion

The structures of DMZ, DMZOH, RNZ, MNZ and RNZ-d_3 are given in Fig. 1 and their full scan spectra in positive ion mode in Fig. 2. For all these compounds, the full scan spectra exhibited an intense pseudo-molecular ion $[M+H]^+$ and some fragment or adduct ions which were less abundant. The acetonitrile adduct $[M+CH_3CN+H]^+$ is obtained for each compound. For confirmatory analysis, it was necessary to monitor several ions for each analyte [19]. Table 1 shows the four chosen ions to be monitored for each compound with their respective retention time.

The nitroimidazole residues are usually extracted from meat with an organic solvent like dichloromethane, toluene, acetonitrile and further clean-up is made by solid-phase extraction (SPE) with C_{18}, silica or strong cation-exchange (SCX) cartridges. The selectivity and specificity of mass spectrometry detection made possible the reduction of the extraction steps. Here, samples were prepared by liquid–liquid extraction without SPE clean-up step. We observed that DMZOH and RNZ partitioned well into the organic phase under basic conditions. There-

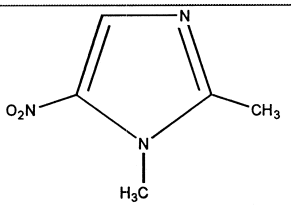
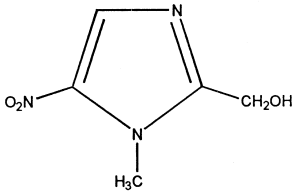
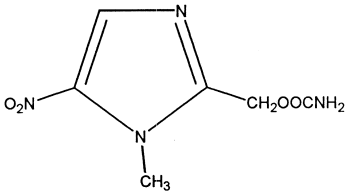
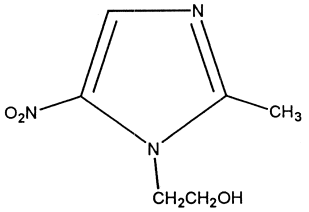
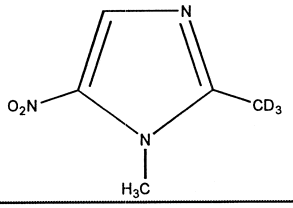
Compound	Structure	Molecular mass	Formula
Dimetridazole		141	$C_5H_7N_3O_2$
Hydroxydimetridazole		157	$C_5H_7N_3O_3$
Ronidazole		200	$C_6H_8N_4O_4$
Metronidazole		171	$C_6H_9N_3O_3$
Deuterated ronidazole		203	$C_6H_5D_3N_4O_4$

Fig. 1. Molecular structures and molecular masses of DMZ, DMZOH, RNZ, MNZ and RNZ-d_3.

fore, the extraction of DMZOH and RNZ into ethyl acetate was improved by the addition of dipotassium hydrogenphosphate. Ethyl acetate was chosen as extraction solvent because it can be evaporated rapidly under vacuum at 35°C. This evaporation step is critical. Excessive heat and drying reduced recovery. Therefore immediately after the evaporation was completed, the oily residue was dissolved in hexane–CCl_4 and formic acid. These extracts give relatively clean SIM chromatograms shown in Fig. 3.

Initially, an RP18 encapped column was used for the liquid chromatographic separation of the four nitroimidazoles. This gave good separation and resolution except for DMZ, for which the peak was broad. To overcome this problem of peak tailing for DMZ, we selected a Waters Symmetry C_{18} column, which exhibited good peak shapes.

The method was validated for poultry muscle samples. A deuterated internal standard was used and quantification was based on the peak areas of the pseudo-molecular ions $[MH]^+$.

The linearity of the mass spectrometer was

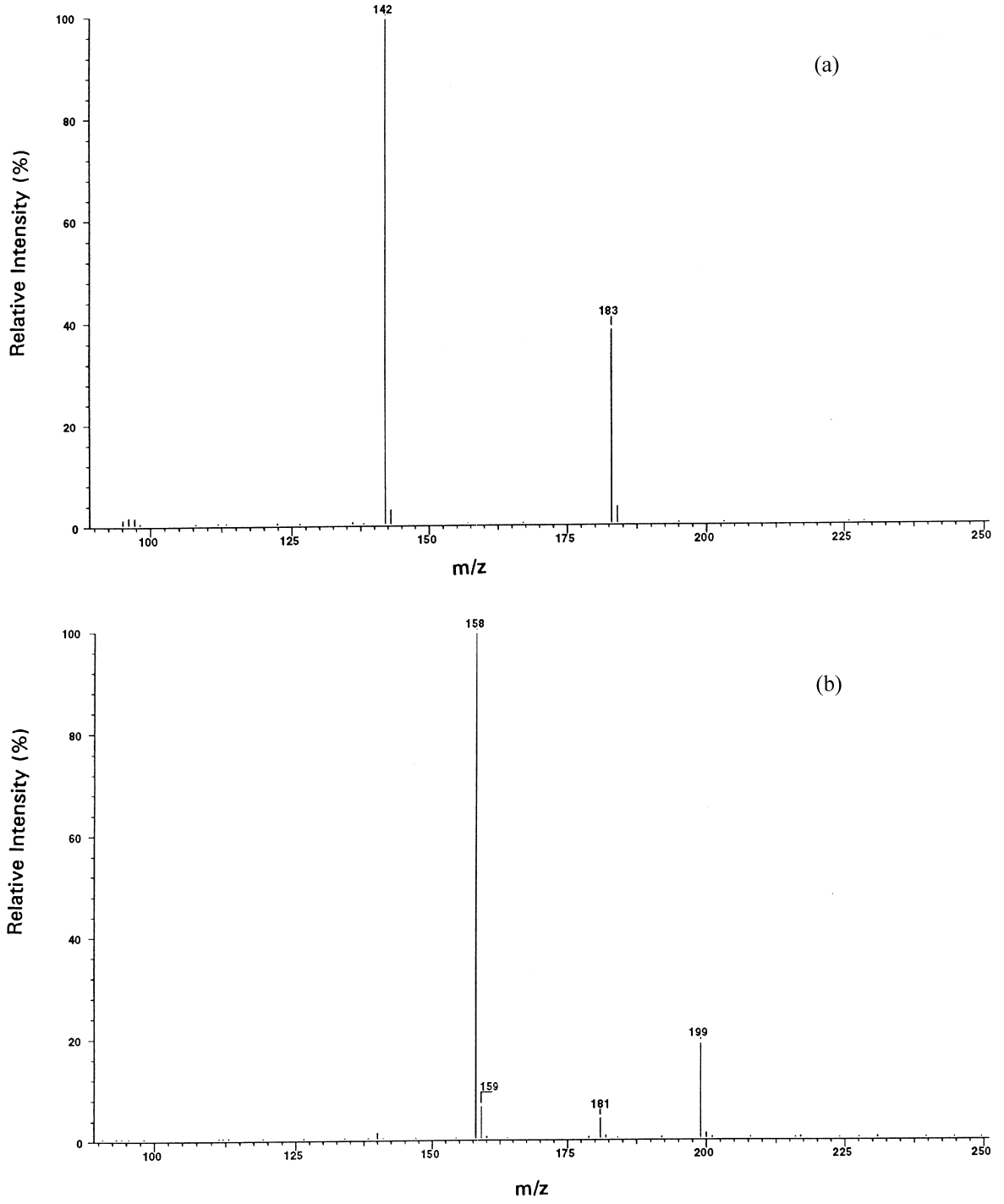
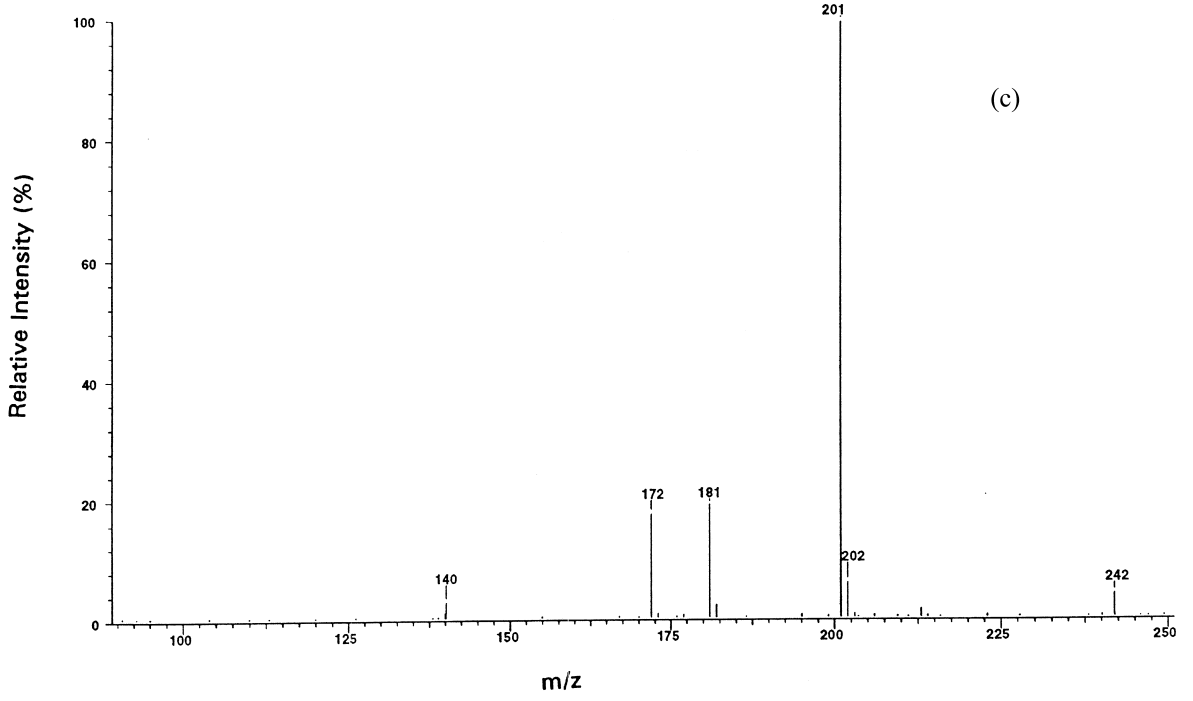


Fig. 2. Full scan spectra of (a) DMZ, (b) DMZOH, (c) RNZ, and (d) MNZ.



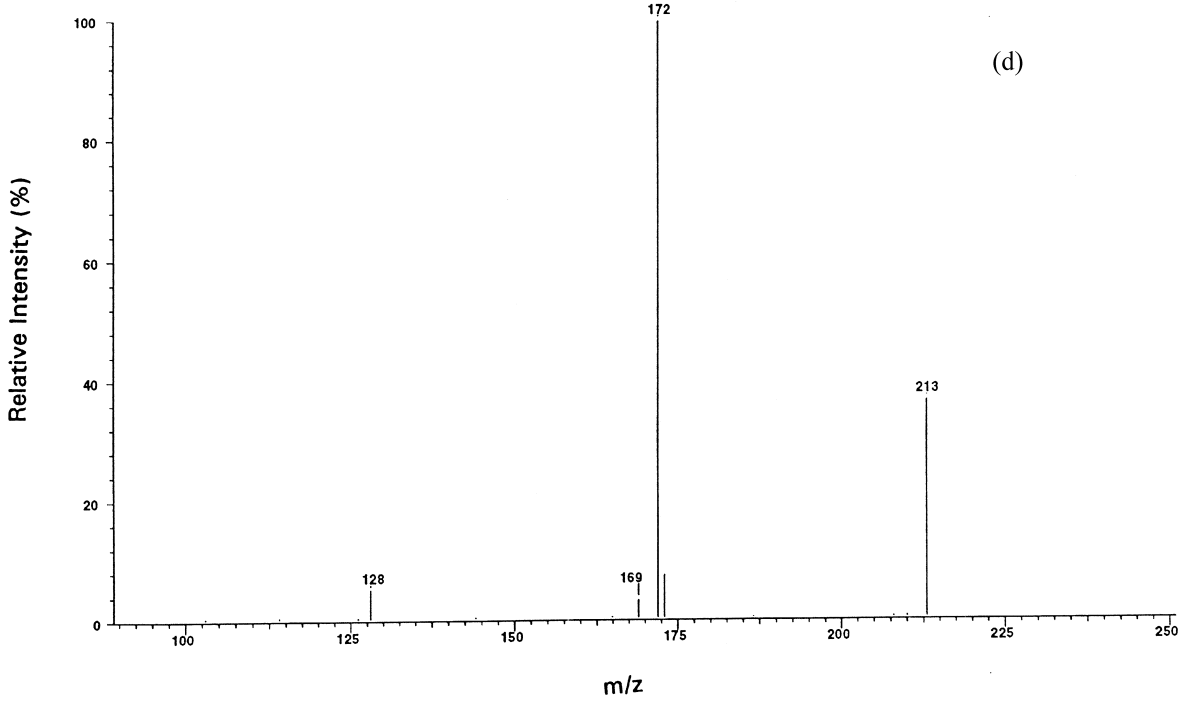


Fig. 2. (*continued*).

Table 1
Ions monitored for each compound with their respective retention time

Compound	Ions monitored in SIM		Retention time (min)
	m/z	Postulated ions	
DMZ	142	$[M+H]^+$	5.7
	143	$[M+H]^+$ isotopic	
	183	$[M+H+CH_3CN]^+$	
	184	$[M+H+CH_3CN]^+$ isotopic	
DMZOH	158	$[M+H]^+$	5.2
	159	$[M+H]^+$ isotopic	
	181	$[M+H+CH_3CN-H_2O]^+$	
	199	$[M+H+CH_3CN]^+$	
	140	$[M+H-H_2O]^+$	
MNZ	172	$[M+H]^+$	6.2
	173	$[M+H]^+$ isotopic	
	213	$[M+H+CH_3CN]^+$	
	128	$[M+H-C_2H_4O]^+$	
RNZ	201	$[M+H]^+$	4.8
	202	$[M+H]^+$ isotopic	
	181	$[M+H+CH_3CN-CONH-H_2O]^+$	
	172		
	140	$[M+H-CONH-H_2O]^+$	
RNZ-d_3	204	$[M+H]^+$	6.2

checked by analysing a set of standard solutions at five levels of concentration from 0.025 to 0.04 mg/l. Calibration curves were constructed by plotting peak area ratio of each nitroimidazole to that of the internal standard versus the corresponding concentrations. The regression curves obtained were represented by the equation $y = mx + b$ for each nitroimidazole. The correlation coefficients r were >0.997, demonstrating good linearity of the detector. To assess the linearity of the analytical method, blank muscle was spiked with solutions of nitroimidazoles to give muscle samples at 3, 5, 7.5, 10 and 15 μg/kg. One series of spiked muscle samples was analysed on 5 separate days. The regression curves obtained gave a correlation coefficient $0.95 < r_{moy} < 0.98$.

Intra-day and inter-day repeatability were assessed by analysing four spiked muscle samples at 5 μg/kg on 5 days. The levels in the samples were calculated using a calibration curve constructed from a range of spiked samples in the range 3 to 15 μg/kg. The results are presented in Table 2. The intra-day repeatability of the method expressed by the RSD was $<11.9\%$ and the inter-day RSD was $<16.1\%$ for all nitroimidazoles. Mean recoveries calculated at the concentration of 5 μg/kg were 73% for DMZOH, 75% for MNZ, 89% for RNZ and 97% for DMZ with RSDs of 19%, 19%, 26% and 17%, respectively.

The limits of detection were calculated on the SIM chromatogram of the most abundant ion. The limits of detection, corresponding to a signal-to-noise ratio 3:1, were ~1 μg/kg for DMZ, 3 μg/kg for MNZ and 4 μg/kg for RNZ and DMZOH. The determination limit which corresponds to the presence of several ions to identify the suspected nitroimidazole was estimated at 5 μg/kg for each nitroimidazole. These limits are not strictly constant because they are instrument-condition-dependent.

The accuracy of the method was calculated on muscle sample at 5 μg/kg and is presented in Table 3. Inter-day accuracy ranged from 89.9% to 98.6% with RSD$<15.4\%$.

For regulatory confirmation analysis, the presence of nitroimidazoles in a sample would be based on the following criteria: (1) correct retention time, (2) presence of several ions specific to the nitroimidazole, (3) relative abundance of each ion in the sample should be the same as in the standard. If quantification were necessary, the concentration

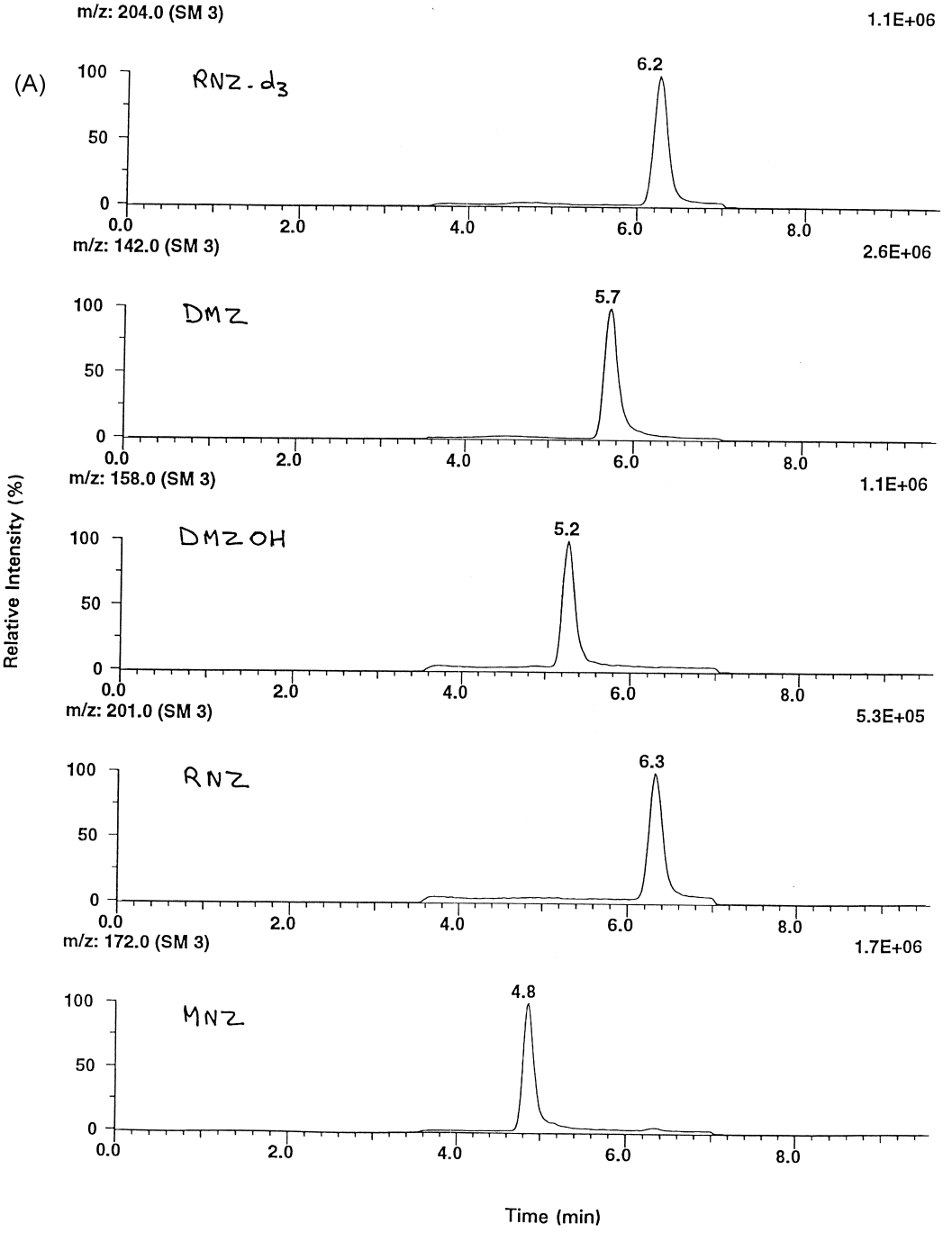


Fig. 3. SIM chromatograms of RNZ-d_3 at m/z 204, DMZ at m/z 142, DMZOH at m/z 158, RNZ at m/z 201 and MNZ at m/z 172. (A) Standard solution containing RNZ-d_3 at 0.2 mg/l and DMZ, MNZ, RNZ, and DMZOH at 0.1 mg/l in water. (B) Extract of muscle fortified at 5 μg/kg.

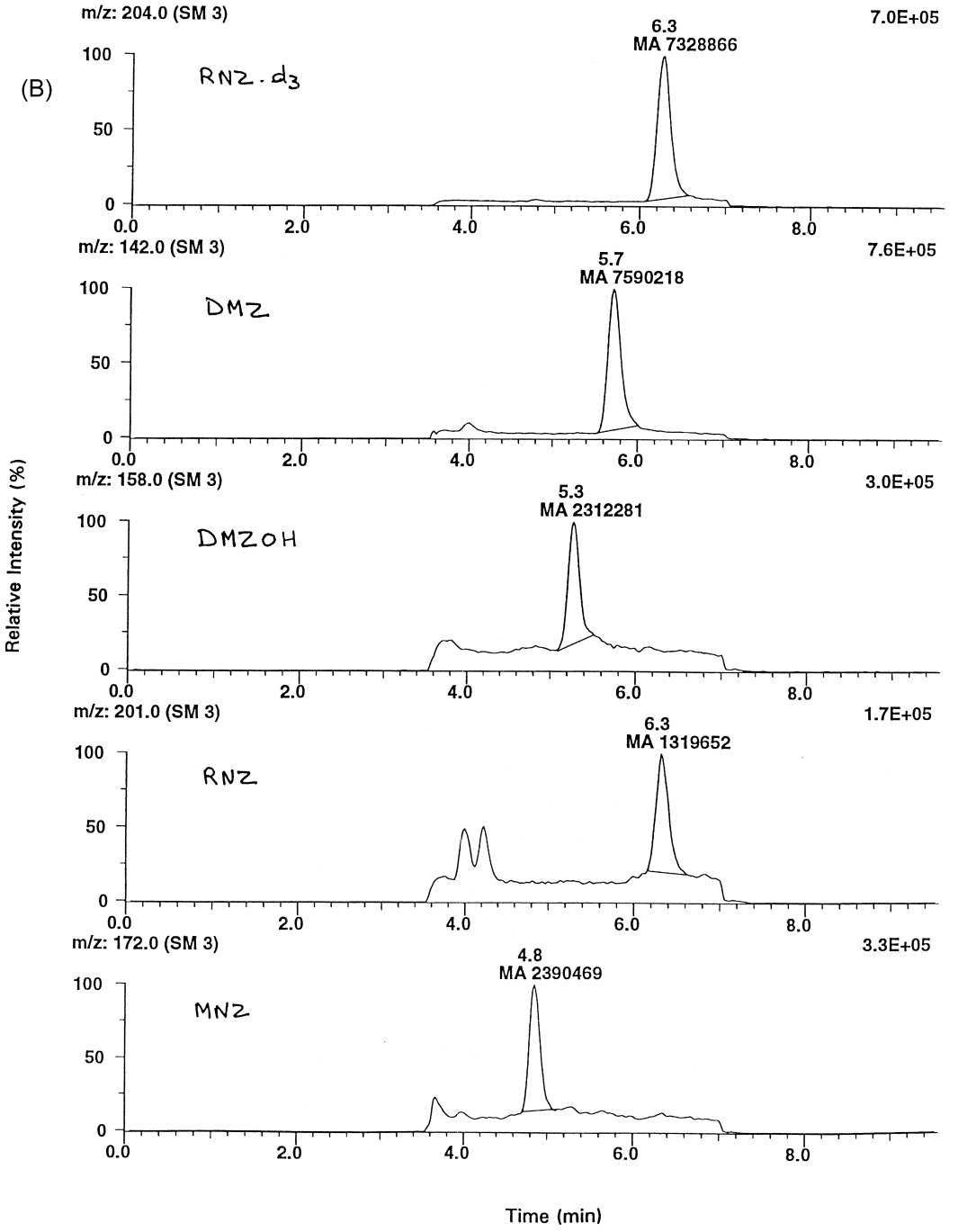


Fig. 3. (*continued*).

Table 2
Values of RSD for intra-day repeatability (RSD_r) and for inter-day repeatability (RSD_R) for muscle fortified at 5 μg/kg (four repetitions on 5 days)

Compound	RSD_r (%)	RSD_R (%)
DMZ	10.6	12.6
DMZOH	11.9	14.7
RNZ	10.9	16.1
MNZ	10.4	15.9

would be calculated using three sample concentrations to construct a calibration curve.

This multi-residue method has been applied in our laboratory to confirm and measure nitroimidazole residues in authentic poultry samples. Suspected samples found by the TLC screening method in the National Surveillance Plan were analysed and confirmation of the presence of DMZ and DMZOH in some cases or of RNZ in other cases was made.

4. Conclusion

This LC–MS method using electrospray ionization allows the simultaneous determination of four nitroimidazole residues in poultry muscle. The sample preparation is simple and rapid and no extensive sample clean-up is necessary. This method is suitable for routine analysis of a large number of samples. Because of its specificity and sensitivity, mass spectrometry detection is well adapted for confirmation of banned substances which need a high degree of certainty in the identity.

Table 3
Accuracy values for muscle fortified at 5 μg/kg

	DMZ	DMZOH	RNZ	MNZ
Day 1 ($n=4$)				
Mean (μg/kg)	5.19	5.10	5.18	5.14
SD	0.52	0.47	0.63	0.53
RSD (%)	10.01	9.18	12.18	10.32
Day 2 ($n=4$)				
Mean (μg/kg)	5.30	4.83	4.68	5.48
SD	0.45	0.40	0.39	0.47
RSD (%)	8.57	8.29	8.36	8.56
Day 3 ($n=4$)				
Mean (μg/kg)	5.19	4.38	4.74	3.97
SD	0.46	0.42	0.62	0.33
RSD (%)	8.91	9.51	13.14	8.19
Day 4 ($n=4$)				
Mean (μg/kg)	4.60	3.90	3.68	5.00
SD	0.79	0.84	0.46	0.49
RSD (%)	17.18	21.52	12.47	9.78
Day 5 ($n=4$)				
Mean (μg/kg)	4.36	4.25	5.18	4.30
SD	0.27	0.43	0.42	0.63
RSD (%)	6.12	9.99	8.04	14.68
Overall ($n=20$)				
Mean (μg/kg)	4.93	4.49	4.69	4.78
SD	0.61	0.65	0.72	0.72
RSD (%)	12.30	14.4	15.4	15.1
Accuracy (%)	98.5	89.8	93.8	95.6

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