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Use of Molecularly Imprinted Solid-Phase Extraction Sorbent for the Determination of Four 5-Nitroimidazoles and Three of Their Metabolites from Egg-Based Samples before Tandem LC−ESIMS/MS Analysis

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A nitroimidazole, molecularly imprinted polymer (MIP) was tested to extract four 5-nitroimidazoles (i.e., dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ)) and three of their metabolites (i.e., DMZOH, IPZOH, and MNZOH) from egg powder samples. Various MIP templates were produced, and their selectivity was assessed on nitroimidazole standard solutions using liquid chromatography coupled with ultraviolet detection. The optimal cleanup was then used for the extraction of nitroimidazole in egg powder samples, and their quantification was achieved by isotope dilution LC−ESIMS/MS. The sample preparation entails a solubilization of the samples with water and acetonitrile followed by a MISPE cleanup step before LC−ESIMS/MS analysis. Data acquisition was achieved using selected reaction monitoring, and quantification was done with five deuterated analogues (i.e., DMZ-d_3, RNZ-d_3, IPZ-d_3, DMZOH-d_3, and IPZOH-d_3). DMZOH-d_3 was used to quantify MNZ and MNZOH since they do not have their corresponding internal standards. The method was validated according to the European Union criteria by spiking experiments at concentration levels of 1, 2, and 3 μg/kg. At these three levels and for compounds having their own internal standards, acceptable performance data were obtained, with internal standard corrected recoveries ranging from 91 to 111%, and decision limits (CCα) and detection capabilities (CCβ) were below 0.34 and 0.39 μg/kg, respectively.

KEYWORDS: Molecularly imprinted polymer; solid-phase extraction; nitroimidazole; liquid chromatography tandem mass spectrometry; egg

INTRODUCTION

Synthesis of molecularly imprinted polymers (MIP) has been made in an attempt to mimic the high selectivity afforded by immunoextraction tools. MIPs are made by synthesizing highly cross-linked polymers in the presence of a "fingerprint" or template. This template molecule needs to contain similar chemical functionality to the targeted analyte(s). After removal of the template, the provided polymer can be used as a selective binding medium for the targeted analyte(s) and other structurally related compounds. The mechanisms by which these MIPs are believed to specifically bind the targeted ligands are attributed to the formation of functional groups in a specific arrangement within the polymer sitting within shape-selective cavities created by the template. In addition, retention of analytes on the sorbent relative to other unrelated analytes can be attributed to the presence of other physicochemical properties, such as hydrogen-bonding and hydrophobic interactions (*1*). Therefore, in a MIP sorbent a key recognition element is based on shape selectivity. MIPs offers additional advantages over immunoadsorbents, such as the ability of being rapidly and easily synthesized and showing a broad stability over a wide range of pH, solvents, and temperature. On the other hand, a synthetic challenge lies in the efficient removal of the template after the polymer synthesis. This removal is important since otherwise the potential risk of

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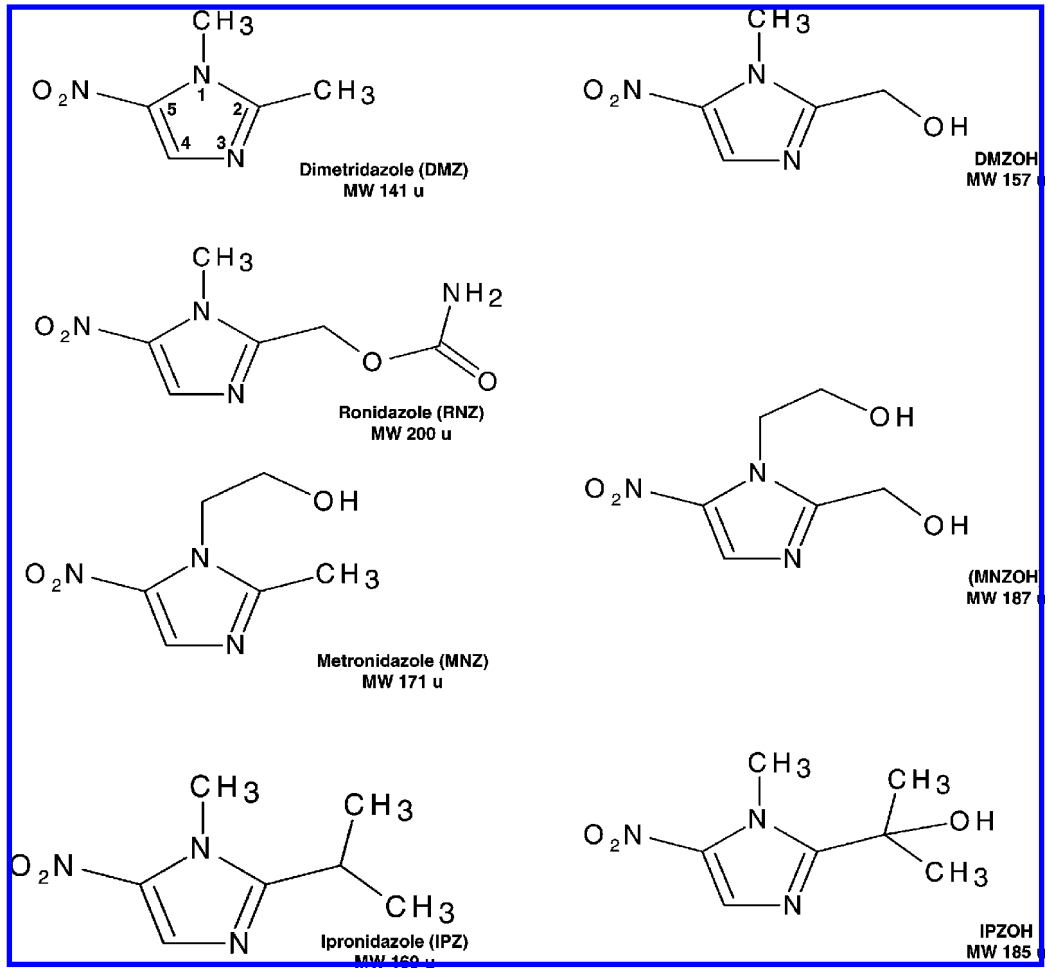

Figure 1. Chemical structures of 5-nitroimidazole parent drugs along with their metabolites.

leaking during the analysis would make trace determination difficult. To avoid this "carryover", structural analogues of the analyte can be used as the template for the sorbent cavity (*2, 3*). Because of the nature of their selectivity once developed, MIPs can be used for a number of applications such as chromatography (*4*), immunoassays and sensors (*5, 6*), or as SPE sorbents (*7*). Molecularly imprinted solid-phase extraction (MISPE) has been used as a selective sorbent method in numerous applications (*8, 9*), which also include food applications (*10, 11*). However, the use of MIPs in egg analysis has been reported only once for the selective extraction of cholesterol from different food matrices (*12*). The use of MIP sorbents in egg analysis is expected to result in selective binding of the targeted analytes while removing egg-interfering compounds such as proteins (i.e., albumin) and fat, thus providing a cleaner extract.

Dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ) are 5-nitroimidazole-based drugs possessing antibiotic and anticoccidial properties. They have been widely used for therapeutic treatment in poultry, cattle, and farmed fish. The 5-nitroimidazoles DMZ, IPZ, and MNZ are rapidly metabolized into DMZOH, IPZOH, and MNZOH, resulting from the side-chain oxidation at the C_2 position of the imidazole ring (**Figure 1**). In addition, RNZ has a different metabolic pathway and shows only a minor metabolite containing the imidazole ring, similar to that of DMZ metabolite (i.e., DMZOH). These drugs and their metabolites are suspected to be human carcinogens and mutagens (*13*). Consequently, DMZ, MNZ, and RNZ are classified in the European Union (EU) list of pharmacologically active substances for which no maximum residue limit can be fixed (*14*), meaning that their use is forbidden in food-producing animals. IPZ has never been authorized as a veterinary drug and therefore is also considered as a forbidden compound. The presence of DMZ, IPZ, and RNZ (>10 μg/kg) in eggs has been demonstrated in laying hens fed with a diet containing these drugs more than five days after the end of the medication, which showed identical concentrations in both egg white and egg yolk parts (*15*).

The detection of nitroimidazoles has been reported in several biological and food matrices by gas chromatography coupled to tandem mass spectrometry (*16*), liquid chromatography

Table 1. Transition Reactions Monitored by LC−ESIMS/MS (Collision Energies Expressed in Electronvolts Are Reported within Parentheses) along with Their Area Ratios (± Allowed Tolerance Limit in Absolute and Percentage Values) (*19*)

	transition reaction (*m*/*z*) used for		
analyte	quantitation	confirmation	area ratio ± limit (%)
DMZ	142 → 96 (25)	142 → 81 (38)	0.33 ± 0.09 (25%)
DMZ-d_3	145 → 99 (25)	145 → 83 (38)	0.15 ± 0.05 (30%)
DMZOH	158 → 140 (18)	158 → 55 (30)	0.14 ± 0.04 (30%)
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.15 ± 0.05 (30%)
IPZ	170 → 124 (25)	170 → 109 (36)	0.89 ± 0.18 (20%)
IPZ-d_3	173 → 127 (25)	173 → 112 (36)	0.88 ± 0.17 (20%)
IPZOH	186 → 168 (20)	186 → 122 (29)	0.37 ± 0.09 (25%)
IPZOH-d_3	189 → 171 (20)	189 → 125 (29)	0.39 ± 0.10 (25%)
MNZ	172 → 128 (23)	172 → 82 (40)	0.54 ± 0.11 (20%)
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.15 ± 0.05 (30%)
MNZOH	188 → 126 (26)	188 → 123 (20)	0.75 ± 0.15 (20%)
DMZOH-d_3	161 → 143 (18)	161 → 58 (30)	0.15 ± 0.05 (30%)
RNZ	201 → 140 (18)	201 → 55 (35)	0.12 ± 0.04 (30%)
RNZ-d_3	204 → 143 (18)	204 → 58 (35)	0.13 ± 0.04 (30%)

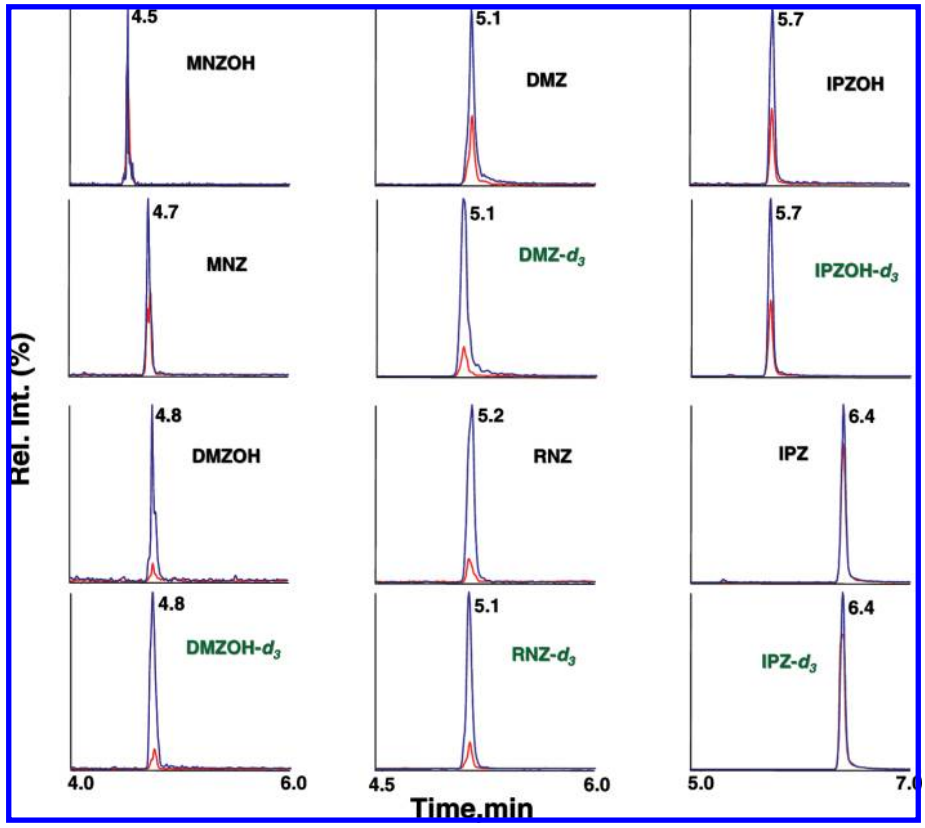

Figure 2. SRM chromatograms of a standard solution containing all analytes at 2.5 pg/μL (i.e., 75 pg injected on column) while their internal standard is at 25 pg/μL (i.e., 750 pg injected on column).

coupled to ultraviolet detection (LC−UV) (*17*), and liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) (*18*). However, few of these methods include a multiresidue approach in which the parent drugs are included along with their metabolites.

This work describes the development of a MISPE protocol for the selective extraction of four different 5-nitroimidazoles and three of their metabolites in egg powder matrices based on a MIP material newly synthesized by MIP Technologies. Quantification of the analytes was carried out by isotope dilution and detection by LC−electrospray (ESI)MS/MS. Particular attention to cleanup of the extracted sample was taken in development of the MISPE procedure, and validation of the method was performed according to EU criteria (*19*).

MATERIALS AND METHODS

Chemicals and Reagents. DMZ and MNZ were purchased from Riedel de Haën (Fluka). RNZ was purchased from Sigma (Fluka). IPZ, 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZOH), 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (MNZOH), and 2-(2′-hydroxyisopropyl)-1-methyl-5-nitroimidazole (IPZOH) were supplied by Witega. Deuterated internal standards (IS) containing the CD_3 group at 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 (ACN), hexane, toluene, methanol, concentrated formic acid, acetic acid (AcOH, 98%), and sodium chloride were purchased from VWR and were all of analytical grade. MIP4SPE (lot number: MG02-0548, 25 mg, 10 mL) targeted for nitroimidazoles were provided by MIP Technologies (quality report and certificate of analysis has been included as Supporting Information for this work). Deionized and distilled water were obtained from a Milli-Q water purification instrument (Millipore).

Standard Solutions. 5-Nitroimidazoles (DMZ, MNZ, RNZ, IPZ, DMZOH, IPZOH, and MNZOH) stock standard solutions were prepared by individually diluting each pure compound in methanol at a concentration of 500 μg/mL. Standard solutions of the seven compounds were then obtained by successive dilutions (5 μg/mL and 125 ng/mL in methanol).

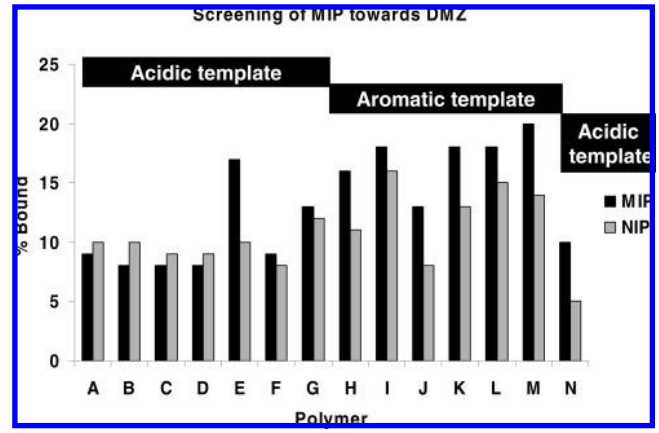

Figure 3. Bound percentage toward DMZ for the different MIPs and NIPs synthesized.

Preparation of the MIP Nitroimidazoles. The developmental MIP for nitroimidazole was prepared by MIP Technologies, using a template analogue different from the analyte studied (proprietary information). The synthesis of the polymer was performed using methacrylic acid as the monomer and divinylbenzene as cross-linker reagent in chloroform. The nonimprinted polymer (NIP) was synthesized in the same manner but without using the template.

Sample Preparation. Processed eggs powder was obtained from European suppliers. The extraction procedure was an adaptation from Mottier et al. (*18*). A well-homogenized portion (2.5 g) was weighed in a 50-mL Falcon polypropylene tube (Becton Dickinson) and fortified with 40 μL of a solution of IS at 125 ng/mL (2 μg/kg). A subsequent addition of 10 mL of water was done and followed by vigorous manual shaking. Acetonitrile (10 mL) was added, and the slurry was hand-shaken for 2 min. The tube was then centrifuged at 4000*g* for 15 min

Table 2. Combinatorial Selection of the Molecularly Imprinted Polymer for DMZ Binding; Independent Triplicate Experiments Were Realized for Each Polymer

polymer batch	mean IF[a]	STDEV	batch-to-batch variation(CV, $n = 3$) (%)
A	0.89	0.079	8.9
B	0.84	0.007	0.9
C	0.90	0.065	7.2
D	0.89	0.031	3.5
E	1.57	0.354	22.6
F	1.39	0.773	55.5
G	1.03	0.067	6.5
H	1.40	0.111	7.9
I	1.15	0.049	4.3
J	2.03	0.139	6.8
K	1.33	0.295	22.2
L	1.25	0.072	5.7
M	1.49	0.026	1.7
N	2.05	0.832	40.6

[a] IF, imprinting factor.

(Centrifuge Multifuge 3s, Heraeus), and the supernatant was transferred to a 50-mL Falcon polypropylene tube containing NaCl (ca. 2 g). The solution was then vortexed and centrifuged at 4000*g* for 5 min. The upper organic phase was transferred to a 15-mL Falcon tube and evaporated to dryness at 50 °C under a light stream of nitrogen. The residue was solubilized in 2 mL of distilled water using a vortex and an ultrasonic bath for a few seconds, and this solution was loaded onto the MISPE cartridge.

MISPE Procedure. The cartridge was previously conditioned with 1 mL of toluene, 1 mL of acetonitrile, and 1 mL of water. The 2-mL extract was loaded and then washed with 1 mL of water and 2 × 1 mL of hexane. The elution step was carried out with 1 × 2 mL of ACN/H_2O (60/40, v/v) containing 0.5% of AcOH. The eluate was then evaporated to dryness under a light stream of nitrogen at 50 °C and resuspended in 500 μL of distilled water. After being vortexed, the final extract was placed in an ultrasonic bath (Branson 2510, Branson Ultrasonics Co.) for 5 min and filtered through a 0.45-μm nylon filter (Nalgene) directly into an HPLC vial.

LC−ESIMS/MS. Analyses of nitroimidazoles were carried out on a Perkin-Elmer Micro Pump Series 200 HPLC system. The HPLC column was a SymmetryShield RP18 (2.1 × 150 mm, 3.5 μm; Waters) equipped with a SymmetryShield RP18 guard column (2.1 × 10 mm, 3.5 μm). The mobile phases were constituted by solvent A: distilled water containing 0.1% formic acid and solvent B: acetonitrile containing 0.1% formic acid. The linear gradient program was: 0−1 min 5% B; 1−8 min 100% B; 8−12 min 100% B; 12−13 min 5% B and 13−18 min 5% B, running at a constant flow rate of 0.3 mL/min. An identical injection volume of 30 μL was used for the various egg extracts. Analytes were detected on an API 3000 triple stage quadrupole mass spectrometer (Applied Biosystems) in positive electrospray ionization using selected reaction monitoring (SRM) acquisition mode. Nitrogen gas was used as TurboIonSpray and curtain gases at a flow rate of 8 L/min and 10 mL/min, respectively. The nitrogen gas pressure of the nebulizer and collision gases were respectively set at 0.77 and 5 mTorr. The block source temperature was maintained at 350 °C with a capillary voltage of 1.2 kV. Optimization of the MS and MS/MS parameters was performed in positive electrospray ionization mode by infusing individual solutions of the analyte (5 μg/mL) at a flow rate of 10 μL/min mixed with a HPLC flow made of solvents A and B (50/50, v/v, 0.3 mL/min) using a T connector. Collision energies of selected precursor ions for each SRM experiments of the different 5-nitroimidazoles and their deuterated analogues are given in **Table 1**. Quantitative analysis was carried out using the most intense SRM signal (SRM1), while the second signal (SRM2) was used for analyte confirmation based on appropriate area ratio calculated from those of standards. Data processing was performed using Analyst 1.4.2 software.

Calibration. 5-Nitroimidazoles were quantified from five concentration calibrants (external calibration curves in water) ranging from 0 to 0.75 ng injected on column. The analyte/IS area ratio (i.e., y) was plotted against the analyte/IS concentration ratio (i.e., x). DMZOH-d_3 was employed as IS for DMZOH, MNZ, and MNZOH. Standards were injected before and after each series of samples, and both sets of data were collected to build the calibration curves. The linearity of the MS response was checked by calculating the relative standard deviation (RSD) of the average of response factors (RFs; RF = y/x), which should be below 15% (*20*).

Confirmation Criteria. 5-Nitroimidazoles were considered as positively identified in egg samples when the following criteria were met: (i) the ratio of the chromatographic retention time of the analyte to that of labeled IS (i.e., the relative retention time of the analyte corresponded to that of the averaged retention time of the calibration solution within ±2.5% tolerance), (ii) the presence of a signal at each of the two SRMs for the analyte and its IS (the use of two SRMs per compound counts for four identification points as defined and required by the EU Commission Decision 2002/657/EC (*19*) to give an added certainty for confirming the presence of the monitored analytes), and (iii) the peak area ratio from the different transition reactions recorded for each analyte was within the tolerances fixed by the EU criteria.

Method Validation. Precision and accuracy (within- and between-day) were calculated from the analysis of six aliquots from a blank matrix, spiked with each analyte at three concentration levels (1.0, 2.0, and 3.0 μg/kg and 2.0 μg/kg of IS) and performed by the same operator on three separate occasions in a week period. Within-laboratory precision was obtained similarly, but analyses were performed by two different operators on one occasion in a two-week period. The within-day precision was calculated from the square root of the averaged variances obtained at the three concentration levels (on three independent occasions). The repeatability limit at the 95% confidence level was deduced from the within-day precision using an expansion factor of 2.77 (*21*). Similarly, the within-laboratory precision was calculated for each concentration level from the square root of the averaged variances calculated from the three different operators, and the intermediate reproducibility limit was calculated, at 95% confidence level, from the within-laboratory precision using the same expansion factor of 2.77.

Decision Limit (CCα) and Detection Capability (CC*β*). These limits were calculated following the calibration curve approach, as described in the 2002/657/EU document (*19*). CCα was calculated from the within-laboratory reproducibility data of full egg powder sample spiked at 1.0, 2.0, and 3.0 μg/kg. For banned compounds such as nitroimidazoles, the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. CCβ was calculated from the corresponding concentration at the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility.

RESULTS AND DISCUSSION

Evaluation of the MISPE Cartridges. LC−ESIMS/MS. The analytes were analyzed in both positive and negative ionization modes, but the former one was used for the rest of the work because of higher signal response. Protonated $(M + H)^+$ molecules were obtained for the studied nitroimidazoles and their deuterated analogues. Collision-induced dissociation spectra were then recorded for each analyte at different collision energies before we selected the two major characteristic fragment ions needed for the confirmatory method (**Table 1**). The fragmentation pathways of the protonated nitroimidazoles have been previously described (*18*) and will not be elaborated further. It is worth mentioning that the fragmentation pattern for DMZ and its deuterated analogue differs as a result of different fragment ions monitored for the confirmation SRM (i.e., m/z 81 for DMZ and m/z 83 for DMZ-d_3). This discrepancy explains the distinct area ratio obtained with DMZ and its deuterated analogue (*18*).

The chromatographic performances enabled the separation of the seven compounds in 7 min (**Figure 2**), within a total run time of 18 min (including the column re-equilibration time).

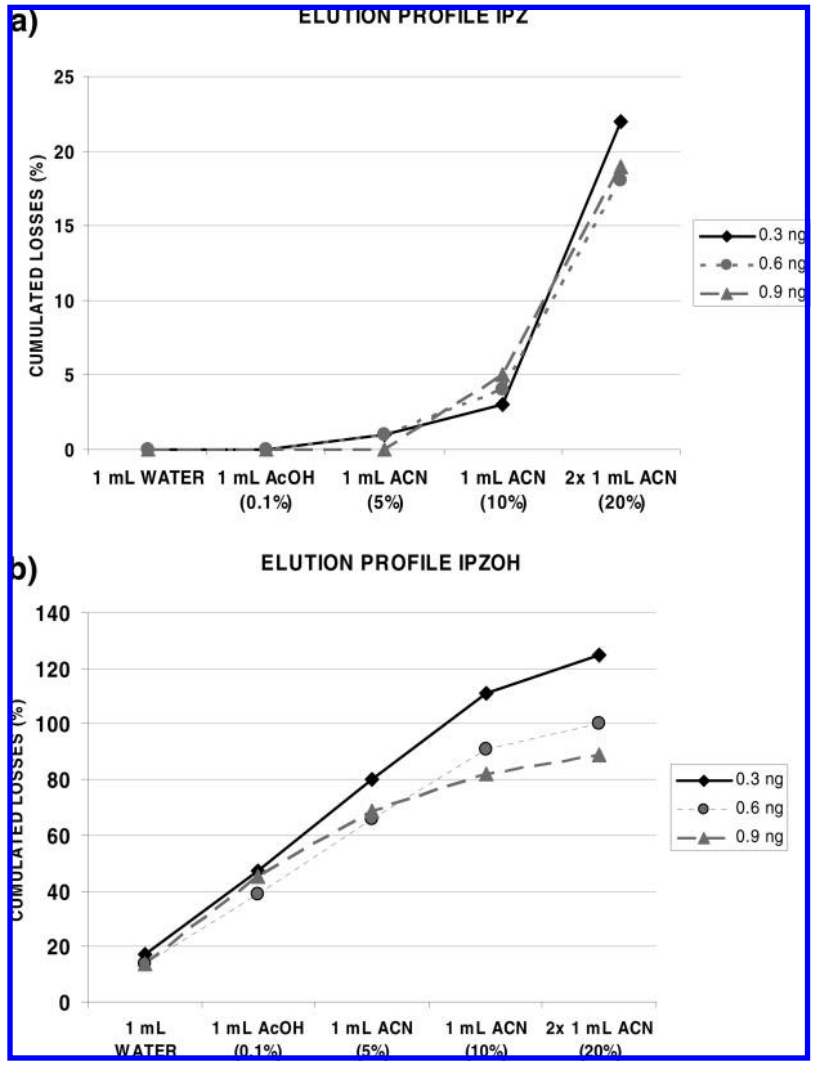

Figure 4. Elution profile obtained for (**a**) IPZ and (**b**) IPZOH, obtained at three concentration levels corresponding to 0.3, 0.6, and 0.9 ng injected on the column.

Blank matrices were extracted to check whether chemical interferences occurred at the expected analyte retention times, as well as to confirm that the egg matrix chosen was free of nitroimidazoles.

Preparation of the MIP Nitroimidazoles. The choice of the template was determined by synthesizing several MIPs using different templates, monomers, and cross-linker reagents. A solution of DMZ (1.5 mM) in acetonitrile was then added to vials containing 60 mg of MIP or NIP. The solution was incubated overnight and the supernatant analyzed by LC−UV (λ = 278 nm). The polymer presenting the highest bound percentage of DMZ on the MIP and the lowest bound percentage on the NIP was then selected as the final sorbent for the MISPE. For confidential reasons, the synthesis conditions are not reported in this article and each polymer is coded from A to N. Three template molecules that had slightly different chemical properties, but functionalities closely related to the targeted nitroimidazole compounds, were selected for the synthesis of polymers. Indeed, polymers A to G were synthesized using the same template that contained an acidic function linked to an imidazole core and where various combinations of monomers, solvents, and cross-linkers were evaluated. Similarly, the template used to synthesize polymers H to M used a different imidazole core, and finally template N possessed aromatic and acidic functionalities. **Figure 3** depicts the average percentage of DMZ bound onto the MIP and the NIP cartridges for the different polymers synthesized. The interactions between DMZ and the polymers synthesized from acidic templates seemed not to be selective (except polymer E) as DMZ was also retained on the NIP. This combinatorial approach to select the best polymer involves the determination of an "imprinting factor" (*22*). The imprinting factor (IF) is a numerical expression of the imprinting effect (the difference in binding between the MIP and corresponding NIP) and is calculated based on the weight of the polymer and the original concentration of the template in the rebinding solution. For these series of polymers, a similar principle is applied to determine the IF with respect to DMZ as opposed to the polymer template. The calculated IF for each polymer is shown in **Table 2**. Values of IF $\leq$ 1.0 revealed no selective imprinted interactions between the MIP and DMZ. The best imprinting effects are observed for polymer J (IF = 2.03) and polymer N (IF = 2.05). In addition, each polymer was tested in triplicate (independent polymer synthesis). The repeatability data are also reported in **Table 2**. Polymer J has a much better batch-to-batch repeatability when compared to that of polymer N (CV of 6.8% against 40.6%, respectively). Therefore, polymer J was selected for further work as it gave the best combination of a high IF and low batch-to-batch variations (CV). After the polymer was selected, the washing and elution conditions on the MIP cartridge were optimized to enable good retention behavior for all the analytes and to improve the analyte recovery.

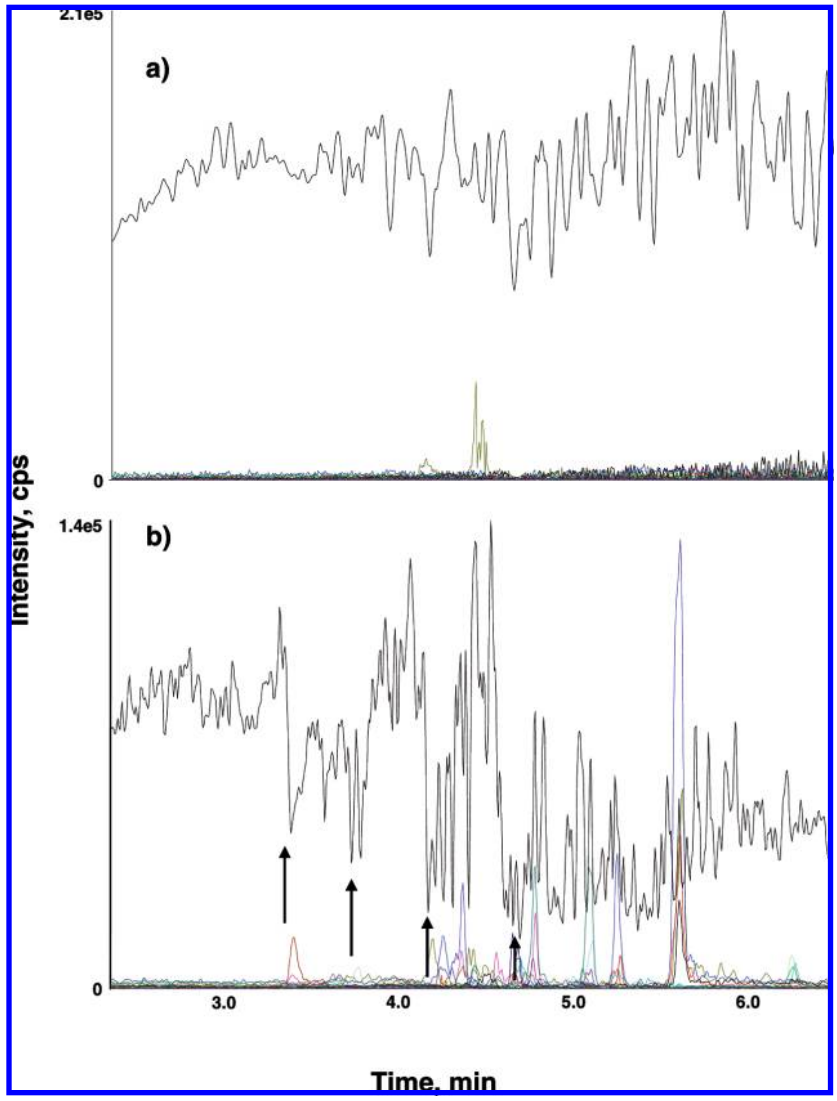

Figure 5. LC−ESIMS/MS analysis of a (**a**) blank water sample and a (**b**) full egg powder sample spiked at a concentration level of 2 μg/kg (IS = 5 μg/kg). Sulfathiazole (5 μg/mL) was continuously infused post column at a flow rate of 10 μL/min, and *m*/*z* 256 → 156 was monitored along with the analytes and IS signals.

Table 3. Performance Data of the LC−ESIMS/MS Method for the Analysis of Seven 5-Nitroimidazoles in Full Egg Powder at the Three Fortification Levels

		DMZ	RNZ	MNZ	IPZ	DMZOH	MNZOH	IPZOH
1 μg/kg	overall recovery ± SD (%)	111 ± 12	95 ± 16	82 ± 19	106 ± 14	102 ± 15	52 ± 16	107 ± 12
	within-day precision (%)	9	16	>20	13	15	>20	11
	between-day precision (%)	11	16	>20	16	15	>20	11
	r (μg/kg)[a]	0.29	0.43	0.47	0.38	0.44	0.37	0.32
	within-laboratory precision (%)	11	13	>20	13	18	>20	12
	iR (μg/kg)[b]	0.34	0.42	0.56	0.44	0.46	0.44	0.39
2 μg/kg	overall recovery ± SD (%)	105 ± 8	99 ± 12	72 ± 19	102 ± 11	103 ± 10	42 ± 13	99 ± 10
	within-day precision (%)	7	12	>20	10	8	>20	10
	between-day precision (%)	9	11	>20	11	10	>20	11
	r (μg/kg)[a]	0.41	0.66	1.03	0.56	0.47	0.67	0.52
	within-laboratory precision (%)	13	15	>20	12	18	>20	10
	iR (μg/kg)[b]	0.58	0.78	0.93	0.72	1.01	0.63	0.55
3 μg/kg	overall recovery ± SD (%)	100 ± 6	111 ± 15	86 ± 15	100 ± 11	96 ± 11	39 ± 21	91 ± 10
	within-day precision (%)	6	11	18	9	11	>20	10
	between-day precision (%)	6	14	18	12	11	>20	11
	r (μg/kg)[a]	0.51	1.02	1.30	0.78	0.90	1.40	0.79
	within-laboratory precision (%)	9	11	>20	11	20	>20	11
	iR (μg/kg)[b]	0.68	0.81	1.77	0.93	1.38	0.55	0.88

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

Effect of Solvents on Nitroimidazoles Binding to Imprinted Polymers. To determine the effect of the solvents used on the nitroimidazole MIP, the cartridge was conditioned as described previously before 500 μL of a mixture of standard solution analytes was loaded (0.01, 0.02, and 0.03 ng/μL; i.e., 0.3, 0.6, and 0.9 ng injected on column). The washing was

Table 4. Decision Limits (CCα) and Detection Capabilities (CCα) for the 5-Nitroimidazoles Calculated in Full Egg Powder (Values Expressed in Micrograms per Kilogram)

	DMZ	RNZ	MNZ	IPZ	DMZOH	MNZOH	IPZOH
CCα	0.14	0.23	0.34	0.22	0.21	0.73	0.21
CCβ	0.23	0.39	0.57	0.37	0.36	1.00[a]	0.36

[a] CCα for MNZOH was arbitrarily set at the lowest fortification level to ensure good confidence on the quantitative results.

performed successively with 2 mL of hexane (to remove the lipids present in the food matrix), 1 mL of H_2O, 1 mL of H_2O containing 0.5% of AcOH, 1 mL of H_2O containing 5% of ACN, 1 mL of H_2O containing 10% of ACN, and 1 mL of H_2O containing 20% of ACN. All fractions issuing from the different washing and eluting steps were collected, evaporated, resuspended, and analyzed by LC−ESIMS/MS. The analytes recovered in each fraction were compared as a percentage from the area ratio of the similar amount directly analyzed into the LC−ESIMS/MS. From this set of data, losses were calculated by plotting the area of the analytes recovered in each fraction versus the area of the similar amount directly analyzed into the LC−ESIMS/MS. The cumulated losses represent the sum of losses occurring subsequently at each step and then the total cumulated losses correspond to the recovery of the analytes on the MIPSPE cartridge.

During the loading and the subsequent hexane wash steps, no loss was recorded for all the studied analytes. However, the addition of 1 mL of water was already enough to partially elute all targeted compounds except for IPZ. The washes with acidified water and water containing acetonitrile at different percentage levels enabled the total elution of all the analytes except for IPZ. **Figure 4** depicts the elution profile of IPZ and IPZOH at three concentration levels, the latter representing the general trend for all the targeted analytes. Clearly, IPZ has a different behavior on the polymer and all washes and elutions performed were not efficient enough to completely release this analyte from the MISPE cartridge as only 20% was recovered compared to standard directly injected at the same concentration (**Figure 4a**). This difference in behavior may be explained by the higher hydrophobicity of IPZ compared to the other compounds (**Figure 1**). On the other hand, the rest of the analytes presented a behavior similar to that of IPZOH (**Figure 4b**) as they were all released from the MIP cartridge by the action of the successive solvents addition. To improve the method, other trials were performed to increase the recovery of IPZ. It was observed that, for IPZOH, RNZ, MNZOH, and MNZ, 1 mL of water was already enough to partially elute the analytes. The eluting properties of water were, however, not of concern here, as the analytes were diluted in water during the loading step and no losses were registered. Thus, the presence of water should not interfere with the retention of the analytes. Consequently, the performance of the hexane wash added just after the loading step might induce some losses recorded during the subsequent aqueous wash. Similar losses also occurred with the wash using 1 mL of acidified water. Here, the use of acid clearly helped to elute the analytes. A final procedure was established incorporating the following variations: (i) permute the order of the hexane/water washes, (ii) remove the washing step with acidified water, (iii) increase the percentage of acetonitrile in the elution solution to 60%, and (iv) add 0.1% of acetic acid to the elution solvent system to increase the elution of IPZ.

The final percentage of ACN was evaluated by testing several amounts from 0 to 90% (with a 10% step). These experiments showed that above 60% ACN no significant improvement was obtained. Using our optimized conditions, we calculated the recovery of standard nitroimidazole solutions by plotting the area obtained for the standard solution loaded onto the MISPE cartridge against the one obtained for the same amount of standard directly analyzed by LC−ESIMS/MS. Overall, the recoveries obtained ranged between 55% for IPZ to 77% for RNZ. Under these conditions, IPZ was correctly released; therefore, this procedure was kept for the extraction of nitroimidazoles in egg powders. The final procedure is described in Materials and Methods.

These sets of experiments enabled us to draw several conclusions regarding the mechanistic interactions between the MIP cavities and the analytes. The use of hexane may activate the specific sites of the MIP, because in the first experiment elution of the analytes with water occurred just after the hexane wash. It is reported in the literature and widely observed that the selective interactions of certain MIP sorbents are stronger for some analytes in nonpolar media (*1*); such MIPs may not exhibit as high selectivity in water. To overcome this, it is possible in such MIPs to retain the target analytes by nonspecific interactions (aqueous wash) and then to trap the analyte using a solvent that promotes selective binding (hexane wash) (*23*). This would explain why a partial elution was observed when the aqueous wash was performed immediately after the hexane wash.

Furthermore, the use of acid seems to also influence the analyte retention behavior. The effect of the elution phase pH (i.e., ACN/H_2O, 60/40, v/v) was checked by eluting the analytes using an elution media with or without 0.5% AcOH acid. The recoveries obtained with the acidified elution system were higher than those obtained without acidification (data not shown). Here, the use of acid helped to disrupt the interaction between the analytes and the specific sites of the MIP cartridges. This allowed us to speculate that, in addition to hydrophobic interactions, secondary hydrogen bonding occurs, with both controlling the retention of the nitroimidazoles on the MISPE cartridges.

Linearity and Loading Capacity of the MIP. To check the saturation level of the MISPE cartridge and also the linearity, six standard solutions of nitroimidazoles were prepared at different concentration ranging from 2.5 to 50 ng and loaded onto the cartridge. The samples were then processed as described above. All analytes presented a good linearity within the concentration range evaluated. The coefficient of determination R^2 was calculated as 0.9693, 0.9943, 0.9524, 0.9719, 0.9937, 0.9900, and 0.9910 for, respectively, DMZ, DMZOH, IPZ, IPZOH, MNZ, MNZOH, and RNZ. In addition, no saturation effect was observed at the highest amount loaded. Similar experiments were carried out by spiking egg powder samples to check if the matrix environment had an influence on the retention of the analytes onto the MISPE cartridge. Indeed, egg samples are very complex matrices containing, among other things, proteins (essentially albumin) and lipids. These macromolecules might then influence the interaction of the analyte with the specific sites of the MISPE. Thus, six full egg powder samples were spiked at different concentration levels ranging from 1.25 to 20 ng loaded onto the MISPE (i.e., 0.5−8 μg/kg for 2.5 g of initial sample weight). Here again, a good linearity was observed for all analytes with R^2 values of 0.9994, 0.9982, 0.9836, 0.9512, 0.9873, 0.9873, and 0.9478 for, respectively, DMZ, DMZOH, IPZ, IPZOH, MNZ, MNZOH, and RNZ. In addition, these results highlight the close behavior of the

analytes and their deuterated analogues on the MISPE cartridges. Indeed, plotting the area ratio of the analyte against the corresponding IS (= y) versus the concentration ratio of the analyte against the corresponding IS (= x) resulted in slope values in standards solution and matrix close to 1 for the analytes having their own deuterated analogue (i.e., 1.01/0.91, 0.41/0.63, 1.96/1.72, 1.16/1.03, 1.04/0.97, 1.05/0.89, and 1.03/0.81) for, respectively, RNZ, MNZOH (IS: DMZOH-d_3), MNZ (IS: DMZOH-d_3), IPZOH, IPZ, DMZOH, and DMZ, respectively, for matrix/standards. Consequently, a calibration curve built with standard solutions can be used to quantify nitroimidazoles in full egg powder.

Post Column Infusion. To evaluate the selectivity of the MIP cartridge, we investigated the possible matrix effects generated with the MISPE sample preparation procedure. This evaluation was done by infusing a molecule not present in the study (5 μg/mL of sulfathiazole at a flow rate of 10 μL/min) post column, while injecting egg powder extract samples (*24*). The traces of sulfathiazole transition reaction (*m/z* 256 → 156) continuously infused post column revealed punctual ion suppression effects at the following retention times: 3.4, 3.8, 4.2, and 4.6 min compared to a blank sample (water) (**Figure 5a**). In addition, the MS/MS response of sulfathiazole is continuously affected from 4.8 to 6.4 min, revealing a general matrix effect within this period (**Figure 5b**). This set of experiments shows that, despite the selectivity afforded by the MISPE cartridge, ion suppression phenomena still occurred. However, the sensitivity reached with the MISPE method is still acceptable for these banned compounds.

Method Performance Characteristics. The overall performance data of the LC−ESIMS/MS procedure is summarized in **Table 3** for the three concentration levels. As MNZ and MNZOH did not possess their own deuterated analogue, the corrected recoveries was calculated with DMZOH-d_3 as internal standard and ranged between 39 and 86% with within- and between-day precision up to 20%. For the other analytes, the recovery corrected by the relevant IS ranged between 70 and 110%. This is in agreement with the requirements stated by the European Union (*19*); values ranged between 95 and 111% at 1 μg/kg, 99 and 105% at 2 μg/kg, and 91 and 111% at 3 μg/kg. In addition, both within- and between-day precisions were below 16%. The results obtained for the intermediate reproducibility revealed values close to the between-day precision, showing that the method is not operator-dependent.

The choice of the appropriate IS for MNZ and MNZOH was guided by two criteria: (i) close chromatographic behavior of the analyte and the IS and (ii) close MS/MS signal intensity. Regarding these criteria, the areas obtained for MNZ and MNZOH for standard calibration solution were plotted individually against the areas of all IS standards to evaluate the linearity obtained. From these sets of experiments, MNZOH shows a good linearity when its area is plotted against the area of DMZOH-d_3. MNZ shows a good linearity when plotted against either DMZ-d_3 or DMZOH-d_3. Regarding the chromatographic behavior, both MNZ (RT 4.7 min) and MNZOH (RT 4.5 min) elute closer than DMZOH-d_3 (RT 4.8 min), meaning that they are more likely sensitive to the same matrix effect, whereas DMZ elutes later (5.1 min). Finally, DMZOH-d_3 was chosen as IS for both MNZ and MNZOH. However, there is a requirement for MNZ and MNZOH to be quantified either using calibration curves built into the matrix or using the corresponding internal standard to improve their quantitative measurements. In fact, these two compounds were quantified using DMZOH-d_3, even though this was not enough to completely compensate the losses occurring during the sample extraction. Similar phenomena were previously observed by Mottier et al. using a classical SPE sample preparation before LC−MS/MS analysis (*18*).

Decision Limits (CCα) and Detection Capabilities (CCβ). CCα and CCβ were calculated following the calibration curves as described in the EU guidelines (*19*), and their values are summarized in **Table 4**. In full egg powder, CCα and CCβ values ranged from 0.14 to 0.73 μg/kg (MNZOH) and from 0.23 to 0.57 μg/kg (MNZ), respectively. It is worth mentioning that, for MNZOH, CCβ was arbitrarily set at the lowest level of spiking (i.e., 1 μg/kg).

Conclusions. In this study, a MISPE extraction method for four 5-nitroimidazoles and their three metabolites in egg powder samples was developed. This is the first report of the use of MIP as a solid-phase extraction sorbent for nitroimidazoles in egg-based samples. The MISPE procedure was developed to exploit the different interactions occurring between the analytes and the specific sites in the MIP material. The 5-nitroimidazoles and their deuterated analogues showed similar and good behavior on the MISPE cartridges. The linearity of the response along with their potential saturation effects, both assessed in solvent and matrix, showed satisfactory results within the concentration studied.

The method was validated according to EU criteria, and the results showed that the method is accurate, reproducible, and sensitive enough. The lack of real internal standard for MNZ and MNZOH highlights the need for a matrix-matched calibration curve to provide better quantification results. For the other analytes, the internal standard corrected recoveries were calculated above 91% at the three concentration levels, and the CCα and CCβ values ranged, respectively, below 0.23 and 0.39 μg/kg.

Supporting Information Available: Quality assurance report for nitroimidazole MIP. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Hennion, M. C. Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr., A* **1999**, *856*, 3–54.

(2) Andersson, L. I. Molecular imprinting: Developments and applications in the analytical chemistry field. *J. Chromatogr., B* **2000**, *745*, 3–13.

(3) Andersson, L. I. Molecular imprinting for drug bioanalysis. A review on the application of imprinted polymers to solid-phase extraction and binding assay. *J. Chromatogr., B* **2000**, *739*, 163–173.

(4) Mayes, A. G.; Mosbach, K. Molecularly imprinted polymers: Useful materials for analytical chemistry. *Trends Anal. Chem.* **1997**, *16*, 321–332.

(5) Haupt, K.; Dzgoev, A.; Mosbach, K. Assay system for the herbicide 2,4-dichlorophenoxyacetic acid using a molecularly imprinted polymer as an artificial recognition element. *Anal. Chem.* **1998**, *70*, 628–631.

(6) Andersson, L. I. Application of molecular imprinting to the development of aqueous buffer and organic solvent based radioligand binding assays for (*S*)-propranolol. *Anal. Chem.* **1996**, *68*, 111–117.

(7) Ariffin, M. M.; Miller, E. I.; Cormack, P.; Anderson, R. A. Molecularly imprinted solid-phase extraction of diazepam and its metabolites from hair samples. *Anal. Chem.* **2007**, *79*, 256–262.

(8) Zander, A.; Findlay, P.; Renner, T.; Sellergren, B.; Swletlow, A. Analysis of nicotine and its oxidation products in nicotine chewing gum by a molecularly imprinted solid-phase extraction. *Anal. Chem.* **1998**, *70*, 3304–3314.

(9) Mosbach, K.; Yu, Y.; Andersch, J.; Ye, L. Generation of new enzyme inhibitors using imprinted binding sites: The anti-idiotypic approach, a step toward the next generation of molecular imprinting. *J. Am. Chem. Soc.* **2001**, *123*, 12420–12421.

(10) Muldoon, M. T.; Stanker, L. H. Molecularly imprinted solid phase extraction of atrazine from beef liver extracts. *Anal. Chem.* **1997**, *69*, 803–808.

(11) Schirmer, C.; Meisel, H. Synthesis of a molecularly imprinted polymer for the selective solid-phase extraction of chloramphenicol from honey. *J. Chromatogr., A* **2006**, *1132*, 325–328.

(12) Shi, Y.; Zhang, J. H.; Shi, D.; Jiang, M.; Zhu, Y. X.; Mei, S. R.; Zhou, Y. K.; Dai, K.; Lu, B. Selective solid-phase extraction of cholesterol using molecularly imprinted polymers and its application in different biological samples. *J. Pharm. Biomed. Anal.* **2006**, *42*, 549–555.

(13) Raether, W.; Hanel, H. Nitroheterocyclic drugs with broad spectrum activity. *Parasitol. Res.* **2003**, *90* (Suppl. 1), S19–S39.

(14) Council Regulation 2377/90/EC laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Communities* **1990**, 1–8.

(15) Aerts, R. M.; Egberink, I. M.; Kan, C. A.; Keukens, H. J.; Beek, W. M. 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. Assoc. Off. Anal. Chem.* **1991**, *74*, 46–55.

(16) 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* **2001**, *761*, 47–60.

(17) 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 eggs by high performance liquid chromatography with UV detection and confirmatory analysis by atmospheric pressure chemical ionisation mass spectrometry. *Analyst* **1998**, *123*, 2545–2549.

(18) Mottier, P.; Hure, I.; Gremaud, E.; Guy, P. A. 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. *J. Agric. Food Chem.* **2006**, *54*, 2018–2026.

(19) Council Regulation 2002/657/EC Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Communities 2002, *L221*, 8−36.

(20) 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.

(21) *Accuracy (trueness and precision) of measurement methods and results. Part 6: Use in practice of accuracy values*; ISO 5725−6; International Organization for Standardization: Geneva, Switzerland, 1994.

(22) Lanza, F.; Sellergren, B. Method for synthesis and screening of large groups of molecularly imprinted polymers. *Anal. Chem.* **1999**, *71*, 2092–2096.

(23) Ridgway, K.; Lalljie, S. P.; Smith, R. M. Sample preparation techniques for the determination of trace residues and contaminants in foods. *J. Chromatogr., A* **2007**, *1153*, 36–53.

(24) Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1175–1185.

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