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Evaluation of the selectivity of molecularly imprinted polymer cartridges for nitroimidazoles. Application to the simultaneous extraction of nitroimidazoles and benzimidazoles from samples of animal origin

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ABSTRACT

In this work the selectivity of commercial molecularly imprinted polymer (MIP) cartridges for nitroimidazoles has been evaluated. For this, a sample treatment based on molecularly imprinted solid phase extraction (MISPE) has been developed and applied to the simultaneous extraction of nitroimidazoles and benzimidazoles. These cartridges were compared with other sorbents commonly used for the solid phase extraction (SPE) of veterinary drugs such as C18, hydrophilic/lipophilic balanced (HLB), and strong cation exchange (SCX). Analysis of the extracts was carried out by HPLC-MS/MS. The use of MIP cartridges provided good results for all nitroimidazoles and for most of the benzimidazoles studied (except for the most apolar analytes), both in terms of recovery and precision and in the cleaning of the extracts. Thus, although MIPs have been designed to specifically bind to a target molecule, i.e., nitroimidazoles, they are able to extract other analytes, such as benzimidazoles, with high efficiency. The developed method was applied to the determination of four nitroimidazoles and ten benzimidazoles in samples of animal origin. All nitroimidazoles and eight of the benzimidazoles were successfully determined. Validation of the method afforded values in the range of 91–111% for the recovery studies. The analysis of intra-day and inter-day precision showed relative standard deviation values lower than 9.5 % and 12 % respectively. These results indicate that the proposed method is suitable for the determination of nitroimidazoles and benzimidazoles in samples of animal origin.

1. Introduction

Veterinary drugs are widely used to treat and prevent diseases and infections in animals. The inappropriate use of these substances both in human and veterinary medicine can lead to the appearance of drug-resistant parasites and bacterial resistance. This is considered a major problem for public health on a worldwide scale by the World Health Organisation. Large quantities of these compounds can be excreted through urine and faeces. For this reason, the study of these drugs in biological samples of animal origin is necessary.

Nitroimidazoles are wide-spectrum antibiotics used mainly for the treatment and prevention of anaerobic protozoan and bacterial infections. These drugs are used in both human and veterinary medicine. However, the use of these compounds in animals intended for human consumption is forbidden because of their carcinogenic, mutagenic and genotoxic properties [1]. Nitroimidazoles are rapidly metabolized and their metabolites are both carcinogenic and mutagenic [2]. 5-nitroimidazoles are imidazole heterocycles that contain a NO_2 group in fifth position on this ring (Fig. 1A). The most representative compound, metronidazole, has been included in the World Health Organization (WHO) Model List of Essential Medicines [3].

Benzimidazoles (Fig. 1B) are broad spectrum anthelmintic veterinary drugs used for the prevention and treatment of parasitic infections in veterinary medicine. The inadequate use of anthelmintics can produce adverse effects on human health such as congenic malformations, teratogenicity, diarrhoea, anaemia, pulmonary oedemas among others [4]. To assure human food safety, the European Union has set maximum residue limits (MRLs) for benzimidazoles and their metabolites in foodstuffs of animal origin [5]. Therefore, specific, and sensitive analytical methods are necessary to guarantee both compliance with MRLs and the absence of banned drugs.

Methodologies for the determination of nitroimidazoles [2] and benzimidazoles [4] have been reviewed in detail. Many methods have been published about the analysis of each of these classes of compounds.

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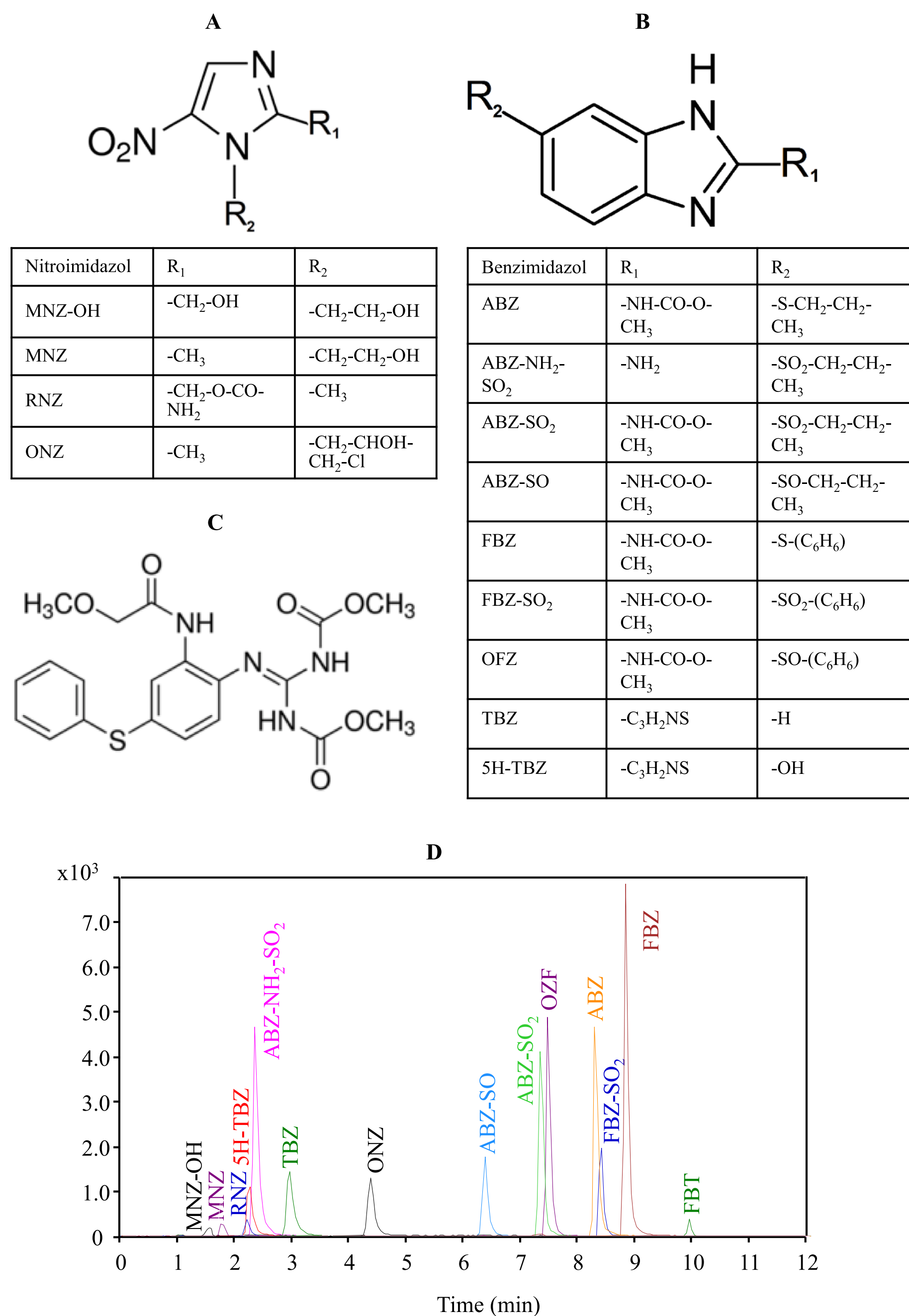
Nitroimidazoles have been mainly determined by liquid chromatography with tandem mass spectrometry [6-17], although UV detection has also been used [18-20]. LC-MS/MS methods have been developed for the analysis of nitroimidazoles and metabolites in foods, tissues, and plasma. Benzimidazoles have been widely determined in food samples using liquid chromatography coupled with ultraviolet [21], diode array [22], or fluorescence [23,24] detection; although most methods include mass spectrometry [25-27] or tandem mass spectrometry [28-33]. Regarding the simultaneous determination of nitroimidazoles and benzimidazoles, many multiresidue methods have been developed for the determination of different classes of veterinary drugs using liquid chromatography–tandem mass spectrometry. Most of these have been applied to food matrices like milk [34-36], infant formula [37], eggs [38], royal jelly [39], and other food matrices [40-44], tissues [45-47], and environmental samples [48,49].

In biological and food sample analysis, the most critical step in the development of an analytical method is sample preparation, due to the complexity of these matrices. Biological and food samples may contain organic compounds with properties similar to the analytes, with concentrations often being higher than those of the analytes. Therefore, it is important to have sample preparation procedures that remove these interferences, as well as being able to isolate and preconcentrate target analytes. One of the most used methods in the case of veterinary drug extraction is solid phase extraction (SPE) [2], which usually involves the use of hydrophilic/lipophilic balanced (HLB) [49] or strong cation exchange (SCX) cartridges [35,36,46]. Recently, molecularly imprinted polymers (MIPs) have been developed as selective sorbents in SPE. MIPs are synthetic materials with artificially generated recognition sites able



Nitroimidazol	R_1	R_2
MNZ-OH	$-CH_2-OH$	$-CH_2-CH_2-OH$
MNZ	$-CH_3$	$-CH_2-CH_2-OH$
RNZ	$-CH_2-O-CO-NH_2$	$-CH_3$
ONZ	$-CH_3$	$-CH_2-CHOH-CH_2-Cl$

Benzimidazol	R_1	R_2
ABZ	$-NH-CO-O-CH_3$	$-S-CH_2-CH_2-CH_3$
$ABZ-NH_2-SO_2$	$-NH_2$	$-SO_2-CH_2-CH_2-CH_3$
$ABZ-SO_2$	$-NH-CO-O-CH_3$	$-SO_2-CH_2-CH_2-CH_3$
ABZ-SO	$-NH-CO-O-CH_3$	$-SO-CH_2-CH_2-CH_3$
FBZ	$-NH-CO-O-CH_3$	$-S-(C_6H_6)$
$FBZ-SO_2$	$-NH-CO-O-CH_3$	$-SO_2-(C_6H_6)$
OFZ	$-NH-CO-O-CH_3$	$-SO-(C_6H_6)$
TBZ	$-C_3H_2NS$	-H
5H-TBZ	$-C_3H_2NS$	-OH

Fig. 1. Structure of analyzed compounds: A) nitroimidazoles, B) benzimidazoles, C) febantel (precursor of benzimidazoles); D) extracted ion chromatograms of a 10 μg L^{-1} standard solution of nitroimidazoles and benzimidazoles. Analytes: hydroxymetronidazole (MNZ-OH), metronidazole (MNZ), ronidazole (RNZ), 5-hydroxy-thiabendazole (5H-TBZ), albendazole-2-aminosulfone (ABZ-NH2-SO_2), thiabendazole (TBZ), ornidazole (ONZ), albendazole-sulfoxide (ABZ-SO), albendazole-sulfone (ABZ-SO_2), oxfendazole (OFZ), albendazole (ABZ), fenbendazole-sulfone (FBZ-SO_2), fenbendazole (FBZ), and febantel (FBT).

to specifically bind to a target molecule in preference to other closely related compounds. Combining MIP sorbents together with SPE has led to a technique known as molecularly imprinted solid phase extraction (MISPE). This technique has the advantages of high specificity, selectivity and sensitivity of the molecular recognition mechanism, and the high resolving power of separation methods [15].

The main aim of this work was to evaluate the selectivity of commercial MIP cartridges for nitroimidazoles. For this purpose, a method based on molecularly imprinted solid phase extraction (MISPE) was developed using these cartridges. To check the selectivity of the MIP cartridges, the developed method was applied to the determination of nitroimidazoles in the presence of other compounds also used in veterinary medicine, such as benzimidazoles. It should be noted that both types of compounds present an imidazole group in their structure.

In addition, the extraction efficiency achieved with commercial MIP cartridges was compared with that obtained with other non-selective sorbents commonly used for solid-phase extraction (SPE) of veterinary drugs. Thus, MIP cartridges for nitroimidazoles were compared with SPE materials such as C18, hydrophilic/lipophilic balanced sorbents (HLB) and MCX, a mixed-mode polymeric sorbent with reversed-phase strong cation exchange retention feature. The extraction efficiency of the different sorbents for the extraction of nitroimidazoles and benzimidazoles was evaluated by HPLC-MS/MS.

The results obtained showed that, despite being a selective material designed to extract nitroimidazoles, MIPs were also capable of retaining other structurally similar compounds, such as benzimidazoles, with similar efficiency to other non-selective sorbents. The MIP cartridges provided similar results to the other materials, both in terms of recovery and precision, as well as cleaning of the extracts. Furthermore, compared to traditional SPE sorbents, MIPs, being more selective towards specific analytes, may be able to better remove other interferences from the matrix. In view of these results, the applicability of commercial MIP cartridges for nitroimidazoles was evaluated. For this, a procedure was optimized to carry out the simultaneous extraction of nitroimidazoles and benzimidazoles using MISPE. The developed method was validated and applied to the determination of four nitroimidazoles and ten benzimidazoles in samples of animal origin. To the best of our knowledge, this work reports for the first time the use of MIP sorbents for nitroimidazoles for the simultaneous extraction of other types of compounds. In addition, the proposed method uses commercial MIP cartridges, which avoids the complex sorbent preparation step that is carried out in most cases when MISPE is used.

2. Material and methods

2.1. Chemicals and materials

Albendazole (ABZ) CAS RN [54965–21-8], albendazole-2-aminosulfone (ABZ-NH2-SO2) CAS RN [80983–34-2], albendazole-sulfone (ABZ-SO2) CAS RN [75184–71-3], and albendazole-sulfoxide (ABZ-SO) CAS NR [54029–12-8] were supplied by Dr. Ehrenstorfer (Augsburg, Germany). Febantel (FBT) CAS NR [58306–30-2], fenbendazole (FBZ) CAS NR [53571–02-1], fenbendazole-sulfone (FBZ-SO2) CAS NR [54029–20-8], oxfendazole (OFZ) CAS NR [53716–50-0], thiabendazole (TBZ) CAS NR [148–79-8], 5-hydroxy-thiabendazole (5H-TBZ) CAS NR [948–71-0], metronidazole (MNZ) CAS NR [443–48-1], hydroxy-metronidazole (MNZ-OH) CAS NR [4812–40-2], ornidazole (ONZ) CAS NR [16773–42-5], and ronidazole (RNZ) CAS NR [7681–76-7] were purchased from Sigma-Aldrich (Steinheim, Germany). Individual stock solutions of analytical standards were prepared in dimethylsulfoxide (Panreac, Barcelona, Spain) at 100–500 mg L-1.

Acetonitrile (MeCN), methanol (MeOH), dimethylsufoxide (DMSO) and acetic acid were of HPLC grade. Ultra-high quality (UHQ) water was obtained with a Wasserlab UHQ (Noain, Spain) water purification system. All other chemicals were of analytical reagent grade. MeCN and MeOH were supplied by Merck (Darmstadt, Germany). DMSO, formic acid and ammonium hydroxide were purchased from Sigma-Aldrich. Toluene was obtained from Acros Organics (Geel, Belgium). Acetic acid, ammonium acetate and n-hexane were supplied by Scharlau (Barcelona, Spain).

The following extraction cartridges were used: Sep-Pak® tC18 (3 mL, 200 mg), Oasis® HLB (3 mL, 60 mg), and Oasis® MCX (3 mL, 60 mg) were obtained from Waters (Barcelona, Spain), and SupelMIP® SPE-nitroimidazoles (3 mL, 50 mg) were from Sigma-Aldrich.

2.2. Instrumentation

Preliminary studies were performed on a HP 1100 Series liquid chromatograph from Agilent (Waldbronn, Germany) equipped with a diode-array detector (DAD). Quantitative analyses were carried out with a HPLC-MS/MS system which consisted of a HP 1200 Series system (Agilent) and a 6410 Triple Quad mass spectrometer (Agilent) equipped with an electrospray ionization source (ESI). HPLC analysis were performed on a Kinetex® 2.6 μm EVO C18 Polar 100 Å (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA) at 40 °C. The mobile phase consisted of MeCN (solvent A) and 0.1% formic acid in water (solvent B). Linear gradient was used at the following conditions: 0–1.5 min, 10% A; 1.5–2.5 min, 10–30% A; 2.5–4 min, 30% A; 4–5 min, 30–65% A; 5–8 min, 65% A; 8–10 min, 65–80% A; 10–12 min, 80–10% A. Mobile phase flow rate was 0.3 mL min^{-1}. The injection volume was 20 μL. DAD was set at 292, 300 and 320 nm, corresponding to the wavelength of maximum sensitivity of the analytes. ESI conditions for maximum intensity of precursor ions were as follows: capillary voltage, +4000 V; drying gas flow rate, 12 L min^{-1}; ion source temperature, 350 °C; and nebulizing pressure, 35 psi. ESI-MS spectra were acquired in positive-ion multiple reaction monitoring (MRM) mode.

2.3. Sample preparation

Urine samples were collected from male and female dogs. The samples were collected by a veterinarian, among animals owned by the veterinarian and patients. Whole and semi-skimmed cow's milk were purchased at a local supermarket. Both types of samples were stored at 4 °C. One millilitre aliquots of each sample type were spiked with nitroimidazoles and benzimidazoles at the desired concentration (5 to 500 μg L^{-1} for linearity assessment, 10 to 100 μg L^{-1} for other validation studies), diluted with 1 mL of UHQ water, and allowed to stand for 15 min.

2.4. SPE conditions

The extraction conditions were different depending on the type of cartridge tested.

2.4.1. C18 and HLB cartridges

The cartridges were conditioned using 2 mL of methanol and 2 mL of UHQ water. A volume of 2 mL of sample was passed through the cartridge at a flow rate of 1 mL min^{-1}. Then, the cartridges were washed with 2 mL of water. After application of a strong vacuum for 5 min, elution of the analytes was performed using acetonitrile:water (95:5, v/v) containing 0.5 % of acetic acid at a flow rate of 1 mL min^{-1}.

2.4.2. MCX cartridges

Before applying the samples, the cartridges were conditioned with 2 mL of methanol and 2 mL of 5 % aqueous acetic acid. Then, 2 mL of sample were loaded into the cartridge at a flow rate of 1 mL min^{-1}. The cartridges were washed by passing 2 mL of methanol, followed by the application of a vacuum for 5 min. 2 mL of acetonitrile:water (95:5, v/v) containing 2 % ammonium hydroxide were used to elute the analytes, at a flow rate of 1 mL min^{-1}.

2.4.3. MIP cartridges

In this case, the protocol recommended by the supplier was followed, with some modifications. The cartridges were conditioned by the sequential passage of 1 mL of toluene, 1 mL of acetonitrile, and 1 mL of 10 mM ammonium acetate buffer (pH 6). Similar to the other types of cartridges, 2 mL of each sample were passed through at a flow rate of 1 mL min^{-1} in the loading step. In all cases, the sorbent was not allowed to dry during the conditioning and loading steps. The sorbent was washed with 1 mL of water followed by the application of a strong vacuum for 5 min to remove residual moisture and ensure a dry cartridge. Then, a second washing step was carried out twice using 1 mL of hexane. Between these two washes, a gentle vacuum was applied for 10 s. Before the elution step, a strong vacuum was applied to the cartridge for 5 min. Finally, the analytes were eluted at a flow rate of 1 mL min^{-1} with 2 mL of acetonitrile:water (95:5, v/v) containing 0.5 % acetic acid.

In all cases, the elution solvent was evaporated under N_2 at 50 °C and reconstituted in 500 μL of UHQ water containing 0.1 % formic acid. The extracts were filtered through 0.22 μm PVDF filters prior to injection into the LC system.

In the studies of evaluation of the selectivity of the sorbents (3.2.1) and optimization of the SPE procedure for MIP cartridges (3.2.2), recovery studies were carried out using standard solutions. The recovery values were calculated as the ratio between the signals obtained using the standard solution extracts, after SPE, and those corresponding to the direct injection of standard solutions at the same concentrations.

For the optimization of the procedure for MISPE using samples of animal origin (3.2.3), recovery values were calculated as the ratio between the signals corresponding to blank urine samples spiked with the analytes before MISPE and those corresponding to the direct injection of standard solutions at the same concentrations.

2.5. Validation

The proposed SPE-HPLC-MS/MS method was validated in terms of linearity, detection and quantification limits, matrix effect, process efficiency, precision, and applicability.

Prior to the validation studies, all natural samples were evaluated for the possible presence of the compounds under study. None of the analytes studied were detected in any of the samples above the detection limit, so some of them were used as blank samples in subsequent studies.

Linearity was evaluated by analysis of blank urine samples spiked with standard solutions of the nitroimidazoles and benzimidazoles at six levels of concentration, ranging from 5 to 500 μg L^{-1}. These values were chosen to cover the range of MRLs established for these analytes. Two samples per level were subjected to the MISPE treatment and analyzed in triplicate by HPLC-MS/MS under the described conditions. Calibration curves were constructed by plotting the peak area versus the analyte concentration, and the equation curves and coefficients of determination were obtained by linear regression.

Detection and quantification limits of the method were calculated as the minimum analyte concentration yielding a signal-to-noise ratio (S/N) of 3 and 10, respectively.

The matrix effect was evaluated by comparing the analytical response of blank urine samples spiked with the analytes, after MISPE, at three levels of concentration (10, 50 and 100 μg L^{-1}) with those of standard solutions of the analytes at the same concentrations. This method for estimating the matrix effect has been proposed by Matuszewski et al. [50] to evaluate the matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.

To evaluate the efficiency of the extraction process, a recovery study was conducted. For this, the analytical signals obtained from blank urine samples spiked with the analytes before MISPE (subjected to the full proposed method) were compared with those obtained from blank urine samples spiked after MISPE, and before injection into the LC system. Urine samples were spiked (before and after MISPE) at three levels of concentration (10, 50 and 100 μg L^{-1}) and analyzed in triplicate.

Precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) for the complete analytical method. Intra-day precision was evaluated by analysing, in triplicate and on the same day, six blank urine samples spiked at three concentration levels. Inter-day precision was calculated in triplicate at three concentration levels on four different days.

The applicability of the proposed method was evaluated by means of a recovery study performed on dog urine and cow's milk samples. Urine samples from four different dogs (two males and two females) and four samples of cow's milk (two whole and two semi-skimmed) were used. None of the samples analyzed showed concentrations of the analytes studied above the limit of detection. Three replicates of each urine and milk samples were spiked at three concentration levels and treated using the MISPE procedure as described in section 2.4.3. The extracts were analyzed by LC-MS/MS in triplicate. Recoveries were evaluated by comparing these signals with those obtained using blank samples spiked after being treated using the MISPE method and prior to analysis.

3. Results and discussion

3.1. Optimization of HPLC-MS/MS conditions

In order to optimize the chromatographic separation, three analytical columns were tested: Waters Cortecs C18+ (50x2.1 mm, 2.7 μm), Phenomenex Kinetex EVO C18 (100x2.1 mm, 2.6 μm) and Phenomenex Kinetex Polar C18 (100x2.1 mm, 2.6 μm). Although the three columns gave quite similar results, the Kinetex Polar C18 column was chosen because it provided the best retention and peak shape for the most polar analytes (MNZ-OH, MNZ, RNZ and 5H-TBZ).

Mobile phase composition was also studied. Different additives (formic acid, acetic acid and ammonium formate) were tested in mixtures of MeCN and water. Finally, a mobile phase consisted of 0.1 % formic acid in water and acetonitrile resulted in the most satisfactory separation and peak shape (Fig. 1C). The elution gradient was also optimized, obtaining an adequate separation of the target analytes under the conditions described in section 2.2.

Optimization of MS/MS parameters was carried out by direct infusion of the standard solutions of the analytes. The optimized parameters are shown in Table 1.

3.2. Optimization of sample treatment

3.2.1. Evaluation of the selectivity of MIP sorbents

In order to evaluate the selectivity of molecularly imprinted polymers as sorbents towards the target analytes, a sample treatment based on molecularly imprinted solid phase extraction (MISPE) was developed

Table 1
MS/MS parameters for nitroimidazoles and benzimidazoles.

Analyte	t_R (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)
MNZ-OH	1.66	188.07	123.0	81	9
MNZ	1.90	172.07	128.1	81	13
RNZ	2.27	201.06	140.0	70	10
5H-TBZ	2.31	218.04	147.1	121	37
ABZ-NH_2-SO_2	2.59	240.08	133.1	108	28
TBZ	3.08	202.05	131.1	121	37
ONZ	4.69	220.05	128.1	81	13
ABZ-SO	6.48	282.09	159.1	92	32
ABZ-SO_2	7.43	298.09	159.1	124	32
OFZ	7.57	316.08	159.0	124	36
ABZ	8.34	266.10	234.1	124	16
FBZ-SO_2	8.37	331.90	158.9	120	36
FBZ	8.85	300.08	268.1	124	16
FBT	10.0	447.14	383.0	108	12

using commercial MIP cartridges for nitroimidazoles. The extraction of nitroimidazoles was carried out in the presence of other compounds also used in veterinary medicine, such as benzimidazoles. Using the protocol indicated by the supplier, standard solutions containing the 4 nitroimidazoles and the 10 benzimidazoles under study were passed through the MIP cartridges. Chromatographic analysis of the extracts confirmed that the MIP sorbents were capable of retaining both types of compounds.

The extraction efficiency of nitroimidazoles and benzimidazoles using these selective sorbents was compared with that of other materials commonly used in SPE. As discussed above, in the determination of benzimidazoles and nitroimidazoles from biological samples, the extraction and clean-up of the extracts using SPE often involves reversed phase C18, hydrophilic/lipophilic balanced (HLB) or strong cation-exchange (SCX) sorbents. Thus, in this work, four types of cartridges were examined and compared: SupelMIP® SPE-Nitroimidazole, Sep-Pak® tC18, Oasis® HLB, and Oasis ® MCX, a mixed-mode polymeric sorbent with reversed-phase and strong cation exchange retention feature.

The extraction efficiency of the four types of SPE cartridges cited was tested using aqueous solutions of the analytes at different pH values (1, 3, 5, and 7). After taking into account the pKa values of the analytes, the results obtained coincided with those that had been predicted. For the MCX cartridges, the highest recoveries were obtained at pH 1, since all the analytes are in cationic form at this pH. For the rest of the sorbents, similar results were obtained at pH values above 3, a pH at which the analytes are in neutral form. The recoveries obtained at the most suitable pH in each case are shown in Fig. 2. The results showed that there were no great differences among MIP and the rest of the SPE sorbents used. The best performance was provided by MIP and HLB sorbents, both in terms of recovery and precision. Contrary to what might be expected, molecularly imprinted polymers cartridges are capable of extracting benzimidazoles in the same way as the other cartridges tested, which do not have restrictive properties. This may be due to the fact that benzimidazoles, like nitroimidazoles, have an imidazole group in their structure (Fig. 1), which would have served as a template for cavities formation. After removing the template from the crosslinked polymeric networks, MIPs binding sites complementary to the template in size, shape and position of chemical functionality are obtained. In this way, MIPs designed to extract nitroimidazoles are also capable of retaining other types of structurally similar compounds. On the other hand, compared to traditional SPE sorbents, MIPs, being more selective towards specific analytes, may be able to better remove other matrix interferences. Recovery values higher than 80% were obtained for all nitroimidazoles and six out of the 10 benzimidazoles tested (except for the less polar benzimidazoles: ABZ, FBZSO2, FBZ and FBT) using MIP sorbents.

3.2.2. Optimization of the SPE procedure for MIP cartridges

Considering that the protocol recommended by the supplier was designed for the extraction of nitroimidazoles, and that in this work benzimidazoles and nitroimidazoles were extracted simultaneously, all steps were evaluated so as to obtain the highest extraction efficiencies for both analytes. To do so, aqueous solutions of the 14 analytes were extracted using MIP cartridges based on the protocol recommended by the supplier, but with modifications to the conditioning, loading, washing, and elution conditions.

Firstly, the influence of the addition of toluene in the conditioning step was assessed. This study was aimed to evaluate if this solvent could be in fact eliminated due to its toxicity. The results obtained showed that the use of toluene was favourable for nitroimidazoles, obtaining signals between 2 and 5 times higher when toluene was used in the conditioning step. In the case of the benzimidazoles, significantly similar results (p-values > 0.05 in t-student comparison studies) were obtained in both cases (with and without addition of toluene). For this reason, the sample conditioning step was maintained according to the protocol established by the supplier: 1 mL of toluene, 1 mL of acetonitrile, and 1 mL of 10 mM ammonium acetate buffer (pH 6) were passed through the cartridge in this step.

Then, the sample loading step was optimized by introducing different sample volumes (1, 2, 3, 4, and 5 mL) of standard solution of the analytes at 100 $\mu g\ L^{-1}$ into the MIP cartridges. The highest recoveries were obtained when 2 mL of sample was introduced into the cartridge, as indicated in the protocol.

In the washing step, the addition of 1 mL heptane:toluene 3:1 (v/v) was considered to be unnecessary, because neither the extraction efficiency nor the cleaning of the extracts were significantly improved (p-values < 0.05 in t-student comparison studies). This step was subsequently eliminated due to the toxicity of these solvents. Thus, the cleaning step was carried out using 1 mL of ultra-pure water followed by twice 1 mL of hexane.

To optimize the elution step, methanol and acetonitrile were tested as solvents, both pure and mixed in different proportions with water. In all cases, cleaner extracts were obtained when acetonitrile was used.

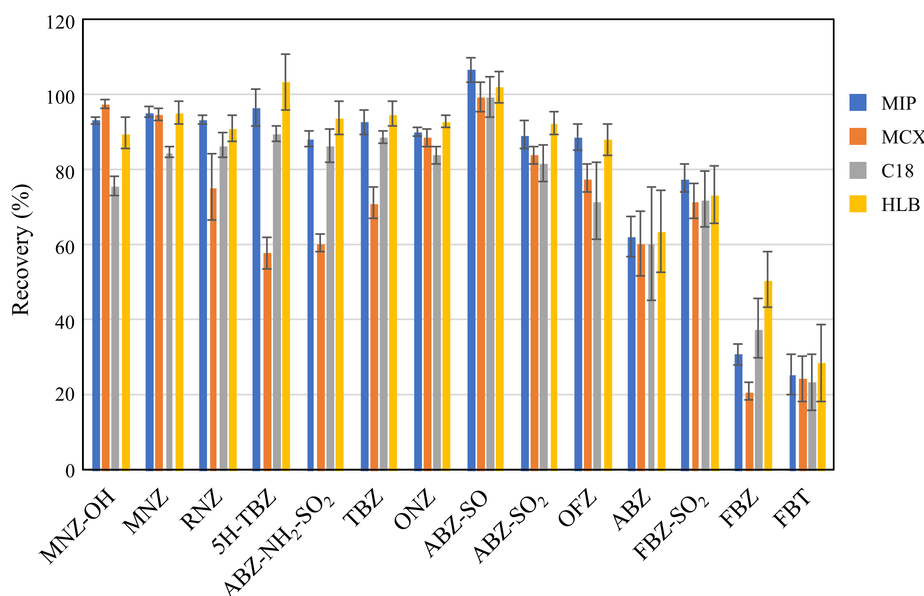


Fig. 2. Extraction efficiency of the cartridges assayed for nitroimidazoles and benzimidazoles. Analyte abbreviations as in Fig. 1.

According to the protocol, sample elution must be carried out in two steps using acetonitrile:water (60:40, v/v) containing 0.5 % (v/v) of acetic acid. Due to the fact that most of the benzimidazoles have lower polarity than the nitroimidazoles, the composition of the eluent was studied using different acetonitrile:water mixtures. In all cases, 0.5% acetic acid was used as recommended by the supplier. For all analytes, the best results were obtained with acenotitrile:water (95:5, v/v) containing 0.5% acetic acid (Fig. 3). Consequently, this mixture was chosen as the eluent.

The method used for carrying out the elution step was also evaluated using two different conditions: 1) different volumes of the eluent mixture (1, 2, and 3 mL of acenotitrile:water (95:5, v/v) containing 0.5% acetic acid) were used in one single step; and 2) by varying the number of times the elution step was carried out (1, 2 or 3 times) using 1 mL of the eluent mixture, drying the cartridge between successive elutions. The highest extraction efficiency was provided when using a single step with 2 mL of the abovementioned eluent.

3.2.3. Optimizing the procedure for MISPE using samples of animal origin

Once the use of the MIP cartridges was verified as being suitable for extracting nitroimidazoles and benzimidazoles, their application was tested using samples of animal origin. This study was carried out using dog urine samples, as urine is more complex than cow's milk.

Firstly, the extraction efficiency of these cartridges was compared with the other types of sorbents previously tested for the standard solutions (C18, HLB and MCX). This study was carried out using the SPE method, described in section 2.4., on blank urine samples enriched with the 14 analytes, including nitroimidazoles and benzimidazoles. The best extraction efficiencies were obtained with MIP and HLB sorbents, which was the same result obtained when using the standard solutions. Moreover, the cleaning of the extracts is also an important step that should be taken into consideration. Thus, the chromatograms obtained for blank urine samples passed through the four types of cartridges were compared. As can be seen in Fig. 4, cleaner extracts were obtained with MCX and MIP sorbents. In view of the above, it can be concluded that MIP cartridges provide similar results than the rest of the sorbents, both in terms of recovery and precision, as well as cleaning of the extracts. In order to evaluate the applicability of commercial MIP cartridges for nitroimidazoles, a MISPE-based procedure was optimized for the simultaneous extraction of nitroimidazoles and benzimidazoles from samples of animal origin. Although this type of sorbent was designed for specifically extracting nitroimidazoles, it can also selectively retain similar compounds such as benzimidazoles, where other types of compounds present in urine samples are excluded.

Other experimental parameters affecting the extraction of analytes by MISPE, such as pH, sample loading volume, elution solvent volume and composition, and the elution flow rate, were also evaluated using spiked blank urine samples. To optimize the pH of the loading sample, blank urine samples, diluted in a 1:1 (v/v) ratio with buffer solutions of pH 1, 3, 5 and 7, respectively, were prepared. These sample solutions were passed through MIP cartridges under the conditions described in section 2.4. As can be seen in Fig. 5, the extraction efficiency increased by increasing the pH of the loading solution. Since similar results were obtained when the blank urine samples were diluted 1:1 (v/v) with pH 7 buffer solution or water, we decided to use water for dilution.

Loading step was also evaluated, both in terms of sample volume and sample dilution. Higher extraction yields were obtained when the sample was diluted in a 1:1 (v/v) ratio with water and when the sample volume passed through the cartridge was 2 mL. The most suitable solvent for elution of benzimidazoles and nitroimidazoles was 2 mL of acetonitrile:water (95:5, v/v) containing 0.5% acetic acid, as for standard solutions.

It is known that the eluent flow rate has a great influence on analyte recovery, so this parameter was also evaluated. For this, the elution step was carried out at flow rates 0.2, 0.5, and 1.0 mL min^{-1} and by applying a weak vacuum. Fig. 6 shows the results obtained, where it is observed that the recovery of the analytes increases as the elution speed increases. Because the application of vacuum was difficult to fully control, the analytes were eluted at a rate of 1.0 mL min^{-1}.

3.3. Method validation

Matrix-matched calibration curves showed good linearity (R^2 > 0.992) over the calibration range selected for the target compounds (Table 2). The LOD values ranged from 0.028 to 1.6 μg L^{-1} (Table 2). The LOQ values varied from 0.092 to 5.3 μg L^{-1}. These values are below the lowest maximum residue limits allowed for benzimidazoles (10 μg L^{-1}). Considering that there is a difference between LOQ values and the lower limit of the linear range, linearity in this interval was studied, finding variations in the slopes of the calibration curves ranging from 4% for ABZ-SO to 10% for FBZ, the analyte with the lowest LOQ.

The matrix effect was calculated as a percentage. The results showed

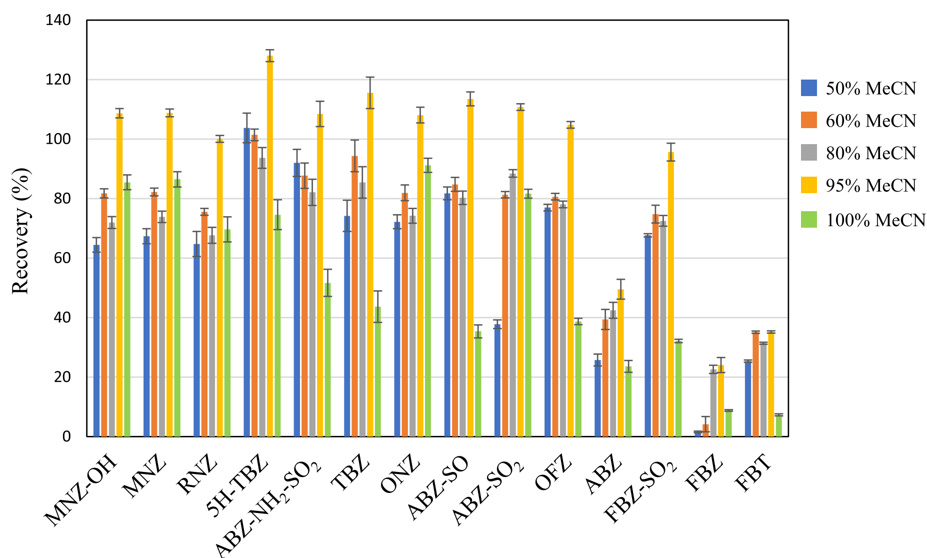


Fig. 3. Influence of the percentage of acetonitrile in the eluent using different acetonitrile:water mixtures all containing 0.5% acetic acid. Analyte abbreviations as in Fig. 1.

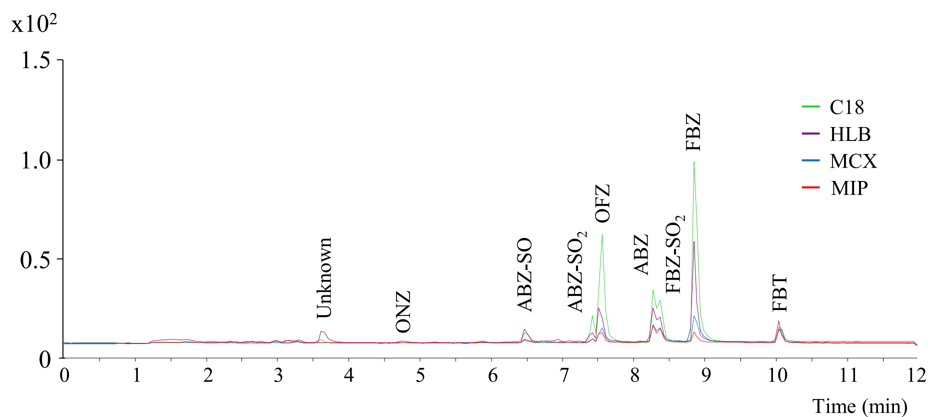


Fig. 4. Chromatograms obtained from urine samples passed through the four types of cartridges. Analyte abbreviations as in Fig. 1.

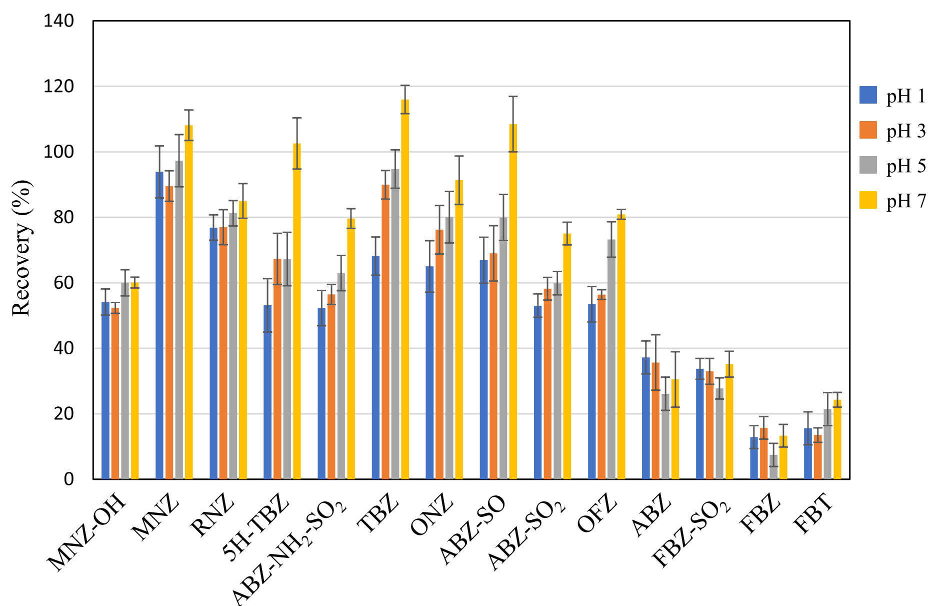


Fig. 5. Influence of the pH of the loading sample on MISPE extraction of urine samples.

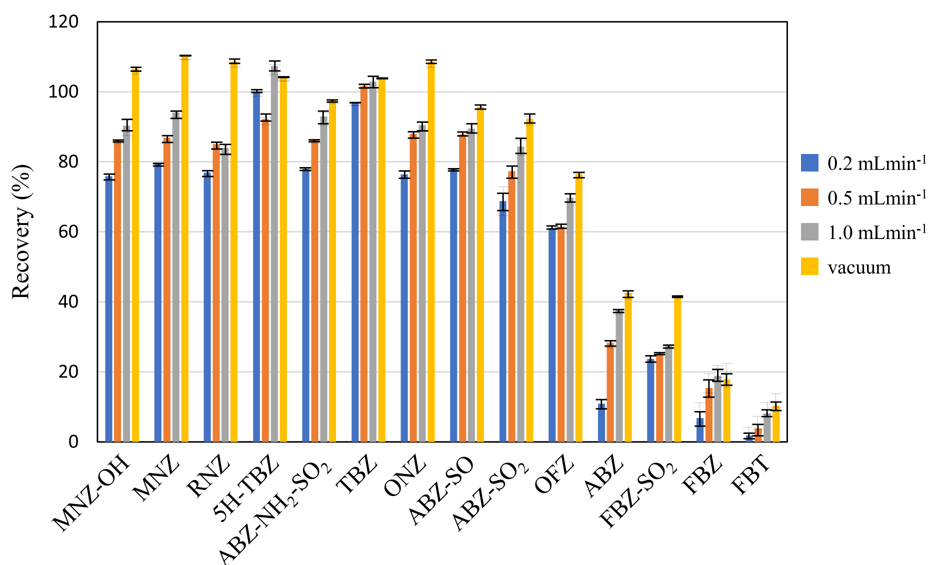


Fig. 6. Influence of elution flow rate on the recovery of nitroimidazoles and benzimidazoles from urine samples. Analyte abbreviations as in Fig. 1.

Table 2
Analytical characteristics of the proposed method (linear range 5 – 500 μg L^{-1}).

Analyte	Slope	Intercept	R^2	LOD(μg L^{-1})	LOQ(μg L^{-1})
MNZ-OH	67.5 ± 0.4	75 ± 37	0.9995	0.54	1.82
MNZ	297 ± 2	−241 ± 166	0.9994	1.60	5.32
RNZ	172 ± 1	−298 ± 75	0.9997	0.59	1.98
5H-TBZ	410 ± 2	−532 ± 145	0.9998	0.68	2.25
ABZ-NH_2-SO_2	1272 ± 3	435 ± 305	0.9999	0.16	0.53
TBZ	865 ± 6	1881 ± 575	0.9992	0.20	0.67
ONZ	1025 ± 4	−1582 ± 334	0.9998	0.21	0.70
ABZ-SO	554 ± 5	1841 ± 467	0.9984	0.04	0.12
ABZ-SO_2	1788 ± 34	3205 ± 7180	0.9921	0.05	0.17
OFZ	1496 ± 18	10866 ± 1669	0.9973	0.09	0.30
ABZ	597 ± 3	5369 ± 1324	0.9995	0.05	0.15
FBZ-SO_2	871 ± 14	−20150 ± 5876	0.9942	0.50	1.68
FBZ	1063 ± 12	26746 ± 4760	0.9981	0.17	0.57
FBT	4345 ± 24	−4002 ± 9992	0.9996	0.03	0.09

in Table 3 represent the average for the three concentration levels assayed. Values lower than 64 % were obtained in all cases in the estimation of the matrix effect. This study shows that there is a great ionic suppression in the determination of these analytes. Due to this large matrix effect, matrix-matched calibration curves should be used when analysing nitroimidazoles and benzimidazoles in samples of animal origin according to the proposed method.

The efficiency of the extraction process was evaluated as the ratio between the analytical signals of blank urine samples spiked with the analytes, at three concentration levels (10, 50 and 100 μgL^{-1}) before MISPE, and those of blank urine samples enriched at the same concentration levels after MISPE, as a percentage. Table 3 shows the average value obtained in this study for each analyte. Recovery values ranging between 91 and 111 % were obtained for all analytes, except for ABZ and FBZ.

The repeatability and intermediate precision results obtained for the three concentration levels tested (10, 50 and 100 μgL^{-1}) are expressed as the relative standard deviation (RSD) of analytical signals in Table 3. Values lower than 13 % were obtained for repeatability and lower than 24 % for intermediate precision. The highest values were obtained for the analytes that are not well retained by the MIPs cartridges (ABZ and FBZ). Values lower than 9.5 % and 12% were obtained for repeatability and intermediate precision, respectively, when considering only those analytes that can be successfully determined by the proposed method.

The applicability of the developed method was evaluated by the analysis of real samples of animal origin. Urine samples collected from four different dogs (two males and two females) and four cow's milk samples (two whole and two semi-skimmed) were subjected to the applicability study. None of the analytes was detected in the analyzed samples. Recovery values were calculated as the ratio between the signal obtained in the analysis of each sample enriched at three concentration levels (10, 50 and 100 μgL^{-1}) and treated using the MISPE procedure with those corresponding to blank samples spiked at the same concentration levels after the MISPE treatment and before the LC-MS/MS analysis. The data listed in Tables 4 and 5 showed that the recoveries, except for ABZ and FBZ, are in the ranges of 70.2–124 % and 75.5–118 %, for dog urine and cow's milk samples, respectively. The satisfying analytical performance indicated that the developed method can be applied to the determination of the four nitroimidazoles (MNZ-OH, MNZ, RNZ, and ONZ) and eight of the ten of the benzimidazoles studied (5H-TBZ, ABZ-NH_2-SO_2, TBZ, ABZ-SO, ABZ-SO_2, OFZ, FBZ-SO_2, and FBT) in dog urine and cow's milk samples.

4. Conclusions

In this work, four types of sorbents for SPE have been compared for the simultaneous extraction of nitroimidazoles and benzimidazoles: commercial MIP cartridges for nitroimidazoles, C18, HLB and MCX. Although MIP cartridges have a predetermined selectivity for a certain type of analyte, the MIP sorbents assayed were able to efficiently extract

Table 4
Analysis of dog urine samples spiked at three concentration levels. Recovery values (%).

Analyte	Recoveries for male dog (%)			Recoveries for female dog (%)		
	10 μgL^{-1}	50 μgL^{-1}	100 μgL^{-1}	10 μgL^{-1}	50 μgL^{-1}	100 μgL^{-1}
MNZ-OH	101	87.3	108	89.0	90.3	116
MNZ	74.6	101	111	104	98.8	115
RNZ	105	104	124	107	98	115
5H-TBZ	96.2	91.3	105	70.4	83.8	107
ABZ-NH_2-SO_2	102	96.2	104	78.5	85.9	102
TBZ	99.8	106	97.8	74.9	91.4	99.0
ONZ	89.3	91.9	101	98.5	90.8	106
ABZ-SO	78.7	80.4	83.8	80.7	79.1	96.9
ABZ-SO_2	80.8	88.2	87.9	91.4	89.2	91.6
OFZ	79.8	87.8	86.2	83.6	81.1	95.2
ABZ	43.0	32.2	41.9	44.6	43.7	46.9
FBZ-SO_2	72.9	70.2	76.6	96.7	94.5	91.5
FBZ	59.0	37.3	33.9	58.8	41.2	39.2
FBT	83.4	76.3	97.6	101	95.4	101

Table 3
Matrix effect, efficiency of the extraction process and precision studies for urine samples.

Analyte	Matrix effect (%)	Efficiency of extraction process (%)	Repeatability (RSD, %)			Intermediate precision (RSD, %)		
			10 μgL^{-1}	50 μgL^{-1}	100 μgL^{-1}	10 μgL^{-1}	50 μgL^{-1}	100 μgL^{-1}
MNZ-OH	5.1	98.7	9.1	7.4	3.3	6.8	7.2	4.2
MNZ	19.8	102.4	4.2	5.9	2.4	8.7	9.2	6.1
RNZ	25.4	111.3	9.0	3.1	4.8	11.8	3.1	4.2
5H-TBZ	37.2	97.7	5.4	1.7	1.9	5.9	7.6	8.2
ABZ-NH_2-SO_2	53.1	94.6	5.3	2.0	2.5	7.5	5.0	4.6
TBZ	48.7	95.2	7.4	2.8	3.1	9.0	10.0	9.1
ONZ	63.6	94.1	9.0	4.5	3.5	5.3	4.9	8.2
ABZ-SO	58.7	98.0	6.3	1.9	2.2	5.5	2.0	3.2
ABZ-SO_2	48.8	91.1	6.9	2.0	2.9	3.8	3.6	1.5
OFZ	50.6	95.6	6.5	2.4	4.2	5.4	4.4	3.8
ABZ	11.5	49.0	12.7	4.8	10.6	23.9	16.7	17.4
FBZ-SO_2	24.1	94.4	5.0	6.6	5.0	7.0	10.8	10.5
FBZ	8.4	47.1	8.7	7.9	5.5	14.3	18.9	7.9
FBT	24.4	103.5	9.5	9.0	8.2	3.5	10.9	8.4

Table 5
Analysis of cow's milk samples spiked at three concentration levels. Recovery values (%).

Analyte	Recoveries for semi-skimmed milk (%)			Recoveries for whole milk (%)		
	10 $\mu g L^{-1}$	50 $\mu g L^{-1}$	100 $\mu g L^{-1}$	10 $\mu g L^{-1}$	50 $\mu g L^{-1}$	100 $\mu g L^{-1}$
MNZ-OH	88.8	94.5	96.9	84.2	89.9	104
MNZ	89.3	101	97.6	77.8	91.5	93.7
RNZ	90.6	93.2	80.6	90.5	89.6	98.6
5H-TBZ	89.3	84.9	111	87.6	84.4	98.8
ABZ-NH_2-SO_2	84.4	80.3	118	82.5	86.5	115
TBZ	75.5	78.2	108	79.4	82.2	103
ONZ	88.3	94.8	110	83.3	89.1	111
ABZ-SO	87.6	89.5	91.2	87.9	88.7	105
ABZ-SO_2	92.4	92.0	113	95.0	94.0	88.9
OFZ	82.7	84.2	102	88.3	86.2	98.2
ABZ	48.1	47.0	55.2	51.0	49.3	54.1
FBZ-SO_2	80.9	107	93.7	92.7	113	99.7
FBZ	52.2	43.3	42.1	46.5	35.0	42.0
FBT	88.1	91.8	93.9	98.0	108	105

both nitroimidazoles and most of the benzimidazoles tested. To the best of our knowledge, this work reports for the first time the use of MIP sorbents for nitroimidazoles for the simultaneous extraction of other types of compounds. The results obtained in this work showed that the proposed method is suitable for the determination of nitroimidazoles and benzimidazoles in samples of animal origin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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