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Molecularly imprinted solid phase extraction method for simultaneous determination of seven nitroimidazoles from honey by HPLC-MS/MS

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ABSTRACT

In this work, a selective sample cleanup procedure that combined molecular imprinting technique with solid phase extraction was developed for the simultaneous extraction of the seven nitroimidazoles (NMZs) from honey samples. The molecular imprinting polymers for NMZs were prepared through bulk polymerization method using 2-methyl-5-nitroimidazole as template molecule, methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the cross linking agent. The obtained molecular imprinting polymers showed high affinity to template molecule and was used as selective sorbent for simultaneously selective extraction of the seven NMZs from honey matrix. An off-line molecularly imprinted solid phase extraction (MISPE) method followed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) for simultaneous determination of the seven NMZs from honey samples was also established. The proposed method was validated at 1.0, 2.0 and 10.0 μg/kg, obtaining recoveries in the range of 79.7–110%, with repeatability and interday precision values (expressed as relative standard deviation) ≤11.4% and ≤15.2%, respectively. Limits of quantification for different NMZs were 1.0 μg/kg, which were always below the minimum required performance limits established by the European Community Reference Laboratories (Commission Decision 2002/657/EC). It was demonstrated that this proposed MISPE-HPLC-MS-MS method could be applied to direct determination of NMZs from honey samples.

1. Introduction

With the development of modern agriculture and animal husbandry, several kinds of veterinary medicines have been used to prevent infection or for growth promotion. However, the improper use of permitted chemotherapeutics and/or illegal use of prohibited chemotherapeutics may lead to the presence of drug residues in foods of animal origin, posing potential hazards to human. Nitroimidazoles (NMZs) are a series of imidazole derivatives that contain a nitro group in different positions. Different NMZs have been widely used to combat anaerobic bacterial and parasitic infections [1]. In recent years, the use of NMZs in beekeeping is being practiced in many countries. For example, NMZs are widely and effectively used to prevent and control *Nosema apis*, which is one of the most serious diseases of honeybee [2]. However, NMZs are believed to be mutagenic and carcinogenic to humans [3–5]. So, the use of NMZs is strictly banned in different countries, including the USA [6], the European Union [7] and China [8]. The recommended minimum required performance limit for the determination of NMZs in different matrices is 3 μg/kg (or 3 μg/L) [9]. Such ultra-low level always required sophisticated sample preparation and/or analysis techniques. It is worth noting that NMZs accumulates easily in animal organs and can enter the human body through the food chain. So, there is also a need for rapid multi-residue analytical methods that have capabilities to determine a wide range of NMZs in foods of animal origin.

Presently, there are several analytical methods that have been proposed for the determination of NMZs in a quite variety of matrices such as plasma, milk, muscle, egg, honey, feed samples and fish [10,11]. At present, the most widely used methods for the analysis of different NMZs are based on chromatographic techniques such as HPLC [12], LC-MS [10,11,13,14], GC [15] and GC-MS [16]. In addition to these methods, electrochemical method [17,18], surface-enhanced Raman scattering (SERS) [19], enzyme-linked immunosorbent assay (ELISA) [20], spectrophotometric method [21] and capillary

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electrophoresis [22,23] also have been used for the determination of NMZs.

Honey is a complex biological matrix with large variety in composition due to honey coming from a great variety of plants and/or origins. In view of the above mentioned, pretreatment in order to concentrate and purify NMZs should be executed. SPE is a common method that is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the NMZs [11,24]. However, SPE still has several limitations. One of the main disadvantages of the classical SPE sorbents (C_{18}, Florisil, Alumina etc.) is low selectivity. Molecularly imprinted polymers (MIPs) are synthetic polymers having a predetermined selectivity for a given analyte or group of structurally related compounds. So combining MIPs with SPE technique is possible to combine the advantages of both molecular recognition and traditional separation methods. And molecularly imprinted solid phase extraction (MISPE) presents the high specificity, selectivity and sensitivity of the molecular recognition mechanism and the high resolving power of separation methods [25,26]. Though MISPE has been developed for extraction of different NMZs from egg, muscle and serum [27,28], these above-mentioned methods cannot be easily adopted to honey due to the very high viscosity of honey, thus there are few report of this MISPE for honey.

In this work, MIPs for NMZs was prepared through bulk polymerization method using 2-methyl-5-nitroimidazole as template molecule, methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the cross linking agent. The obtained MIPs showed high affinity to template molecule and were successfully applied as special SPE sorbents for selective extraction of different NMZs from honey samples. The method was validated and applied to determine NMZs from honey samples with satisfactory results. To the best of our knowledge, this work represents the first attempt using a universal MISPE column for simultaneous selective determination of seven different NMZs from honey samples by HPLC-ESI-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

Nitroimidazoles standards: Metronidazole [MNZ, 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol], Dimetridazole (DMZ, 1,2-Dimethyl-5-nitroimidazole), Ipronidazole (IPZ, 2-Isopropyl-1-methyl-5-nitro-1H-imidazole), Ronidazole [RNZ, (1-Methyl-5-nitro-1H-imidazol-2-yl)methyl carbamate], Ornidazole [ONZ, 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl)propan-2-ol], Tinidazole [TNZ, 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole], Carnidazole {CNZ, O-Methyl [2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl]carbamothioate} and 2-methyl-5-nitroimidazole (Tempalte) were purchased from Sigma Aldrich or Dr. Ehrenstorfer GmbH. Isotope-labeled internal standards (ISs) of nitroimidazole parent drugs: MNZ-$13C_2$,$15N_2$, DMZ-D_3, RNZ-D_3, IPZ-D_3 at chemical and isotopical purities > 99% were obtained from Witega. Methacrylic acid (MAA), Ethylene glycol dimethacrylate (EGDMA) were purchased from Alfa Aesar and distilled to remove the polymerization inhibitor before use. Azobisisobutyronitrile (AIBN) was the product of Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China) and was recrystallized prior to use. Acetonitrile, methanol, N,N-dimethylformamide (DMF) and formic acid were all of HPLC grade and procured from Merck. All water used was obtained from a Millipore Milli-Q purification system. NMZs standard stock solutions were respectively prepared in methanol at a concentration of 1000 mg/L and placed in the dark. Isotope-labeled internal standards of nitroimidazole parent drug solutions were respectively prepared in methanol at a concentration of 100 mg/L and placed in the dark. More dilute standard solutions were prepared by appropriate successive dilutions.

2.2. Instrument and conditions

All separations were carried out on an Agilent 1100 series HPLC system containing a G1322A Vacuum Degasser, a G1312A Binary Pump, a G1313A ALS Autosampler, a G1316A Column Compartment and a G1315B diode array detector. Separation of NMZs was carried out on a CAPCELL PAK® C_{18} MGIII column (150 mm×2.0 mm-i.d., particle size 5 μm). For HPLC separation, the mobile phase consisted of a binary mixture of solvent A (acetonitrile) and B (0.1% formic acid in water) with a gradient elution program: 0–0.5 min, 3% A; 0.5–4 min, 3–90% A; 4–5.5 min, 90–3% A; 5.5–12 min, 3% A. The mobile phase flow rate was 0.3 mL/min, elution was monitored at 278 nm on the diode array detector, the column was maintained at 40 °C and the injection volume was 5 μL.

An API 4000Q triple-quadrupole mass spectrometry (Applied Biosystems/MDS Sciex, USA) with a turbo ion spray (TIS) interface operating in positive ionization mode was used for the multiple reaction monitoring (MRM) analyses. Nitrogen was used as the collision gas. The detection of NMZs was carried out in positive ionization mode (ESI+). The mass spectrometer conditions were optimized for NMZs detection as follows: The temperature of the ion source heater was 550 °C. The nebulizer gas pressure was 0.31 MPa, the auxiliary gas pressure was 0.345 MPa, while the curtain gas pressure was 0.138 MPa. The ion spray voltage was 5.5 kV. The MRM settings for NMZs and internal standards are shown in Table 1.

2.3. Preparation of MIPs for NMZs

Generally, MIPs were prepared using a bulk polymerization method by dissolving 1 mmol of template (2-methyl-5-nitroimidazole, in this case), 4 mmol of functional monomer MAA and 20 mmol of cross-linker EGDMA in 10 mL of DMF in a 20 mL borosilicate glass bottle. This mixture was stirred over night at room temperature for the formation of a complex of template and monomers. After added 0.25 mmol of initiator AIBN, the solution was saturated with dry nitrogen for 10 min, then the bottle was equipped with a rubber cap. At last, the bottle was placed in a thermostated oil bath and polymerized at 60 °C for 24 h. After polymerization, the polymer was ground with a mortar and pestle, and sieved between 200 mesh and 400 mesh screens to give particles with size dimensions between 37 and 74 μm. After that, the particles were repeatedly suspended in acetone to remove the small particles. Then the product was extracted with ethanol containing 20% acetic acid using a Soxhlet apparatus for 48 h to make sure that the template could not be detected in the filtrate. At last, the product was washed with ethanol for three times and dried under vacuum at room temperature. The non-imprinted polymer (NIP) particles were prepared and washed using the same recipe but without the addition of the template.

Table 1
The MRM settings for different NMZs and ISs.

NMZs	t_R (min)	AIT (*m/z*)	DP (V)	CE (V)	CCEP (V)
MNZ-13C2,15N2	5.79	176.1/132.0	60	20	12
MNZ	5.83	172.0/128.1	65	33	16
DMZ-D3	6.08	145.0/99.1	80	25	22
RNZ-D3	6.10	204.0/143.1	50	18	15
DMZ	6.12	142.0/96.1	75	21	14
RNZ	6.14	201.0/140.1	50	16	12
TNZ	6.45	248.1/121.1	70	24	12
ONZ	6.61	220.2/128.2	63	23	11
CNZ	6.89	245.2/118.0	40	15	15
IPZ	7.00	170.2/109.3	60	35	12
IPZ-D3	7.00	173.2/127.1	66	25	11

Note: AIT=Acquired ion transition, DP=Declustering potential, CE=Collision energy, CCEP=Collision Cell exit potential