Short communication

Determination of benzimidazole residues in bovine milk by ultra-high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method based on ultra-high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UHPLC–MS/MS) for the simultaneous determination of benzimidazole residues in bovine milk has been optimized and validated. Rapid chromatographic separation of 13 analytes in 8 min was obtained by means of UHPLC. The samples were subject to Oasis MCX solid-phase extraction cartridges for extraction and clean-up. Matrix-matched calibration curves were performed to compensate for the matrix effect and loss in sample preparation. Mean recoveries ranged from 80% to 101% and inter-day precision was lower than 14%. Limit of detection and limit of quantification of the method ranged from 0.01 to $0.5\,\mu g\,L^{-1}$ and from 0.1 to $1.0\,\mu g\,L^{-1}$, respectively.

1. Introduction

Benzimidazole anthelmintic drugs are commonly used for prevention and treatment of parasitic infections in the veterinary practices. Incorrect use of these drugs can possibly leave residues in edible tissues or food products, which may have a potential risk to consumers [1,2]. The European Union has set maximum residue limits (MRLs) for benzimidazoles in animal food products [3]. In China, similar MRLs have also been established in muscle, liver, kidney, and milk [4]. Therefore, reliable analytical methods are required to monitor these drug residues in foods such as milk and to ensure the safety of food supply.

Different methods, such as thin layer chromatography [5] and direct spectrometric detection [6,7], have been developed for analysis of benzimidazoles, but they are not suitable for residual analysis for sensitivity reason. For screening purposes, enzyme-linked immunosorbent assay (ELISA) [8,9] and surface plasmon resonance-biosensor [10] have been used. However, the confirmation of suspect positive samples must be conducted by mass spectrometry coupled to the adequate chromatographic separation. Several methods using liquid chromatography–mass spectrometry (LC–MS) have been published [11–13]. The combination of liquid chromatography and tandem mass spectrometry (LC–MS/MS) tended to be a sensitive method that was capable of detecting various benzimidazoles at trace levels [14–18].

Kinsella et al. [19] described a LC–MS/MS method for the determination of benzimidazoles, macrocyclic lactones, and flukicides in bovine milk and liver using QuEChERS (quick, easy, cheap, effective, rugged and safe) method for sample preparation. The QuEChERS approach involved liquid extraction with acetonitrile and clean-up by dispersive solid phase extraction. Later, the group applied ultra-high performance liquid chromatography coupled to tandem MS (UHPLC–MS/MS) to the detection of anthelmintic drug residues with rapid polarity switching [20]. In recent years, large-scale multi-residue screening methods have been developed for the analysis of different classes of veterinary drugs including benzimidazoles. Based on UHPLC and time-of flight mass spectrometry (TOF MS), these methods are very powerful to detect more than 100 veterinary drugs [21,22]. However, the limitations of TOF MS, compared to triple-quadrupole MS, are the lower sensitivity, worse quantitative accuracy, and higher price.

The aim of this study was to develop and validate a rapid and sensitive method for the determination of benzimidazole residues in bovine milk by UHPLC–MS/MS. Milk samples were cleaned up by one step solid phase extraction (SPE) using mixed mode cartridge without liquid extraction. UHPLC conditions were optimized to obtain fast analytical time of 8 min for each injection as well as excellent peak shape and separation. The sensitivity of the method was demonstrated by validation of fortified samples at $0.1\,\mu g\,L^{-1}$.

2. Experimental

2.1. Materials and reagents

HPLC grade methanol, acetonitrile, and formic acid were purchased from Dima Technology Inc. (Muskegon, MI, USA).

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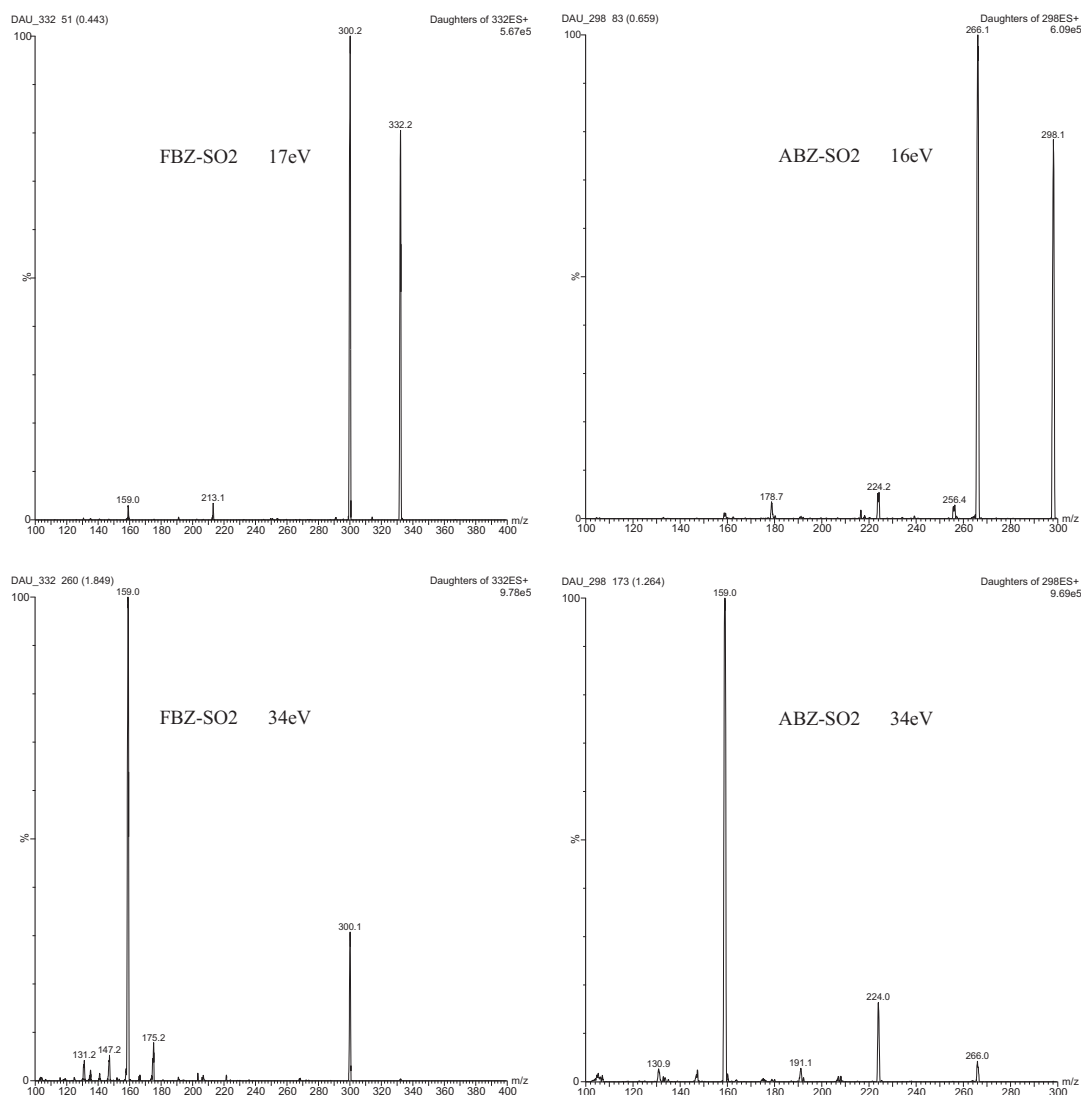[1] These authors contributed equally to this work.

Fig. 1. MS/MS spectra of benzimidazoles acquired at different collision energies.

Hydrochloric acid and ammonia were obtained from Alfa-Aesar (Ward Hill, MA, USA). Water was purified using a Milli-Q Synthesis system from Millipore (Bedford, MA, USA). Oasis MCX (60 mg) extraction cartridges were supplied by Waters (Milford, MA, USA). Syringe filter was purchased from Pall Corporation (Ann Arbor, MI, USA). Albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ), thiabendazole (TBZ), oxfendazole (OFZ), 5-hydroxythiabendazole (5-OH–TBZ), albendazole sulfoxide (ABZ–SO), albendazole sulfone (ABZ–SO$_2$), fenbendazole sulfone (FBZ–SO$_2$), flubendazole (FLUB), febantel (FBT), oxibendazole (OBZ), 2-amino-albendazole sulfone (ABZ–NH$_2$–SO$_2$) were kindly provided by Institute of Veterinary Drug Control of Henan Province (Zhengzhou, Henan, China). Individual stock solutions (1 mg mL^{-1}) were prepared by dissolving 10 mg of compound in 10 mL of methanol. Mixed working standard solutions were prepared by diluting stock solution with methanol. These solutions were stored in dark glass bottles at −20 °C and were stable for at least 6 months.

2.2. Sample preparation

The milk was centrifuged (9000 rpm, 10 min, 4 °C) and then the upper fat layer was removed. Two mL of milk sample was transferred into a centrifuge tube and 2 mL of 0.1 M hydrochloric acid was added. After vortex mixing, the mixture was loaded onto an Oasis MCX cartridge previously conditioned with 3 mL of methanol and 3 mL of 0.1 M hydrochloric acid. The cartridge was washed with 3 mL of 0.1 M hydrochloric acid, followed by 3 mL of methanol. The compounds of interest were eluted with 4 mL of 10% ammonia in acetonitrile. The eluate was evaporated to dryness at 40 °C, under nitrogen flow. The residues were dissolved in 400 μL of 0.1% formic acid in water–0.1% formic acid in acetonitrile (90:10, v/v). The resulting solution was filtered through a 0.2 μm filter into a LC vial.

2.3. Instrumental conditions

LC analyses were performed on a Waters Acquity ultra-performance liquid chromatography system with column oven temperature maintained at 30 °C, using an Acquity BEH C$_{18}$ column (50 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The mobile phase was constituted by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The flow rate was 0.3 mL min^{-1} with a linear gradient at the following conditions: 0–0.2 min, 90% A; 0.2–4 min, 90–60% A; 4–5 min, 60–5% A; 5–6 min, 5% A; 6–6.1 min, 5–90% A; 6.1–8 min, 90% A. The injection volume was 10 μL.

The UHPLC system was coupled to a Micromass Quattro Premier XE triple quadrupole mass spectrometer (Waters, Manchester, UK) fitted with an electrospray ionization (ESI) source and controlled
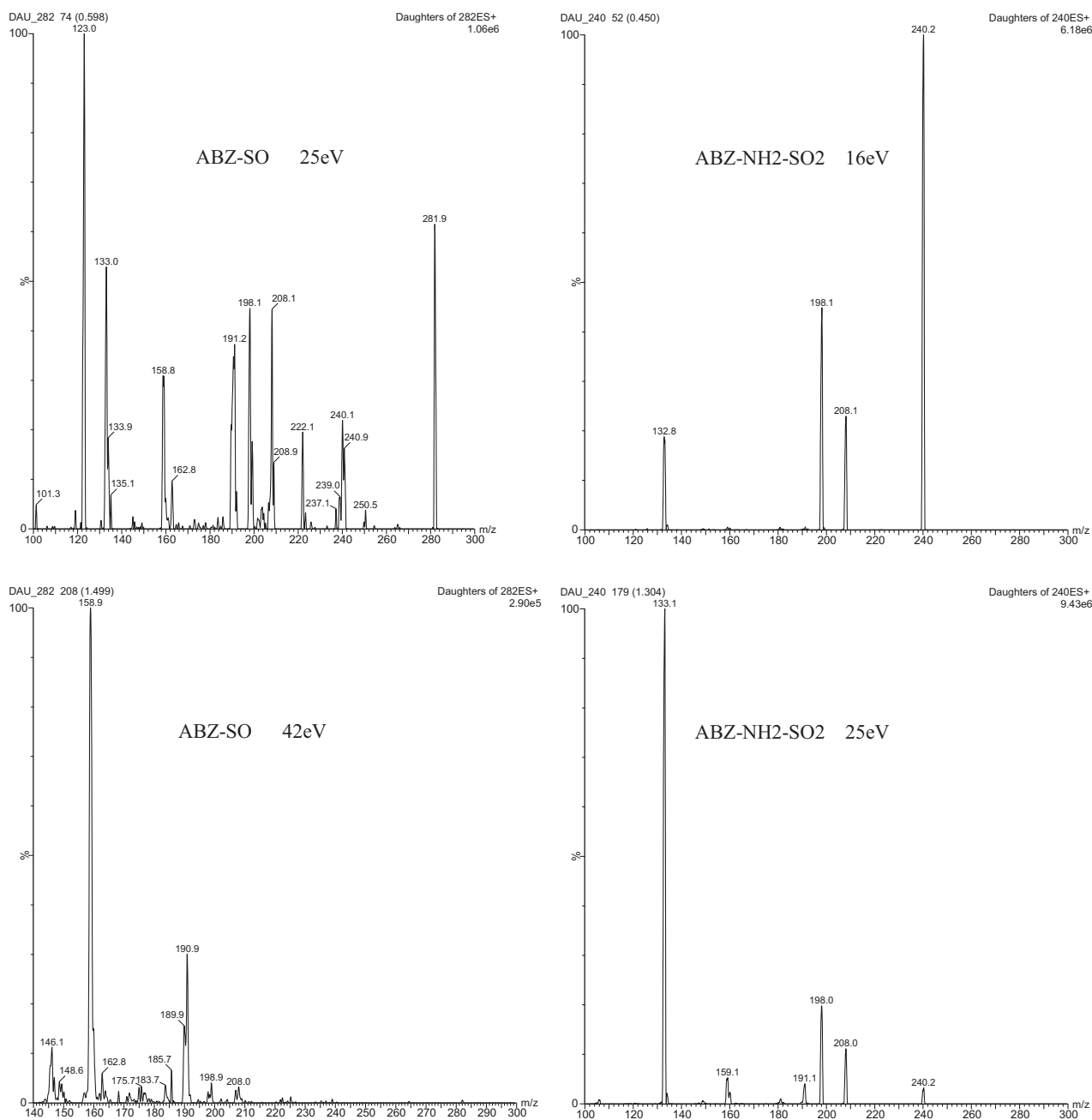
Fig. 1. (*Continued*).

by MassLynx software (version 4.1). Typical source conditions for maximum intensity of precursor ions were as follows: capillary voltage, 2.8 kV; source temperature, 100 °C desolvation temperature, 350 °C; cone gas (N$_2$) flow rate, 30 L h^{-1}; desolvation gas (N$_2$) flow rate, 600 L h^{-1}. For all compounds, the MS instrument was operated in the ESI positive mode and the data was acquired in multiple reaction monitoring (MRM) mode. The argon pressure in the collision cell was 3.7×10^{-3} mbar. Optimized MS/MS transitions as well as specific cone voltages and collision energies are summarized in Table 1.

2.4. Method validation

To evaluate the performance of the proposed method, the method was validated in terms of specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). Twenty blank samples obtained from local supermarkets were prepared and analyzed to verify the absence of interfering substances around the retention time of analytes. The linearity of the method was evaluated by linear regression analysis of matrix-matched calibration curves. The recovery experiments were performed to investigate the method accuracy and precision. Six replicates of spiked samples at three levels each were prepared on four different days. The precision, expressed as relative standard deviation (RSD), was determined by the intra-day and inter-day assays. The LOD was defined as the concentration with a signal-to-noise ratio (S/N) of 3. The LOQ, based on the recovery and precision data, was defined as the lowest validated fortified level meeting the requirements of a recovery within the range of 80–120% and a RSD ≤ 20%.
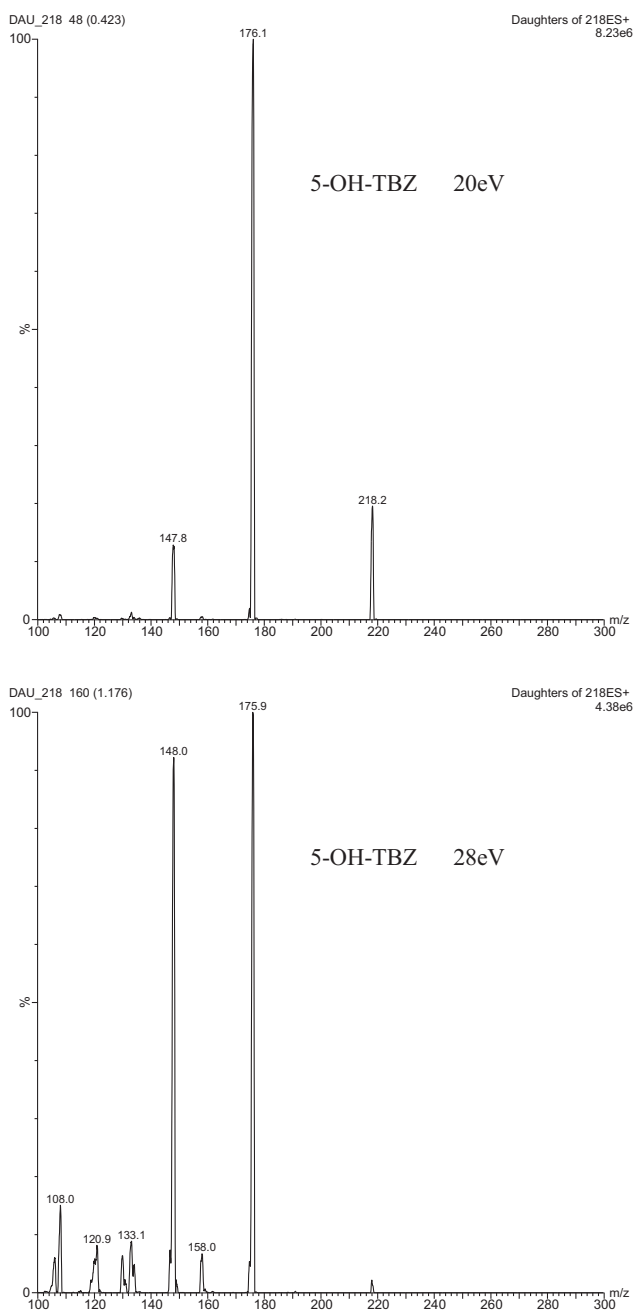
Fig. 1. (*Continued*).

3. Results and discussion

3.1. Sample preparation

To detect trace levels of the target compounds, it is necessary to remove proteins and fat from milk samples. Benzimidazoles were usually extracted from milk at high pH using ethyl acetate [11,12,17]. In this study, milk samples were centrifuged and then acidified and directly loaded onto the mixed-mode solid-phase extraction cartridges. Preliminary experiments were performed to investigate the effect of centrifugation step on recovery. Samples fortified before and after centrifugation were analyzed and no different results of recovery and precision were observed. And this centrifugation step was necessary to prevent the clogging of SPE cartridge. Although the polarity and pK_a of benzimidazoles and their metabolites differ greatly, Oasis MCX cartridge provides sufficient retention for all the analytes. It was found that the use of

acetonitrile at the elution step, instead of methanol, could obtain better recovery. The proposed SPE procedure was rapid and effective for sample cleanup and enrichment.

3.2. UHPLC–MS/MS analysis

Selection and tuning of MRM transitions were performed by direct infusion of standard solution of each analyte. Different collision energies were tested in order to find the most abundant product ion. The fragmentation pathways of nine benzimidazoles have been studied in a previous publication [17], while several metabolites were not covered in that study. The loss of $-OCH_3$ is found to be common and leads to m/z 300 and 266 for FBZ–SO$_2$ and ABZ–SO$_2$, respectively. For FBZ–SO$_2$, a subsequent elimination of $-SO_2C_6H_5$ from the ion at m/z 300 gives the fragment at m/z 159. A similar fragmentation pathway, the elimination of $-SO_2C_3H_7$, is
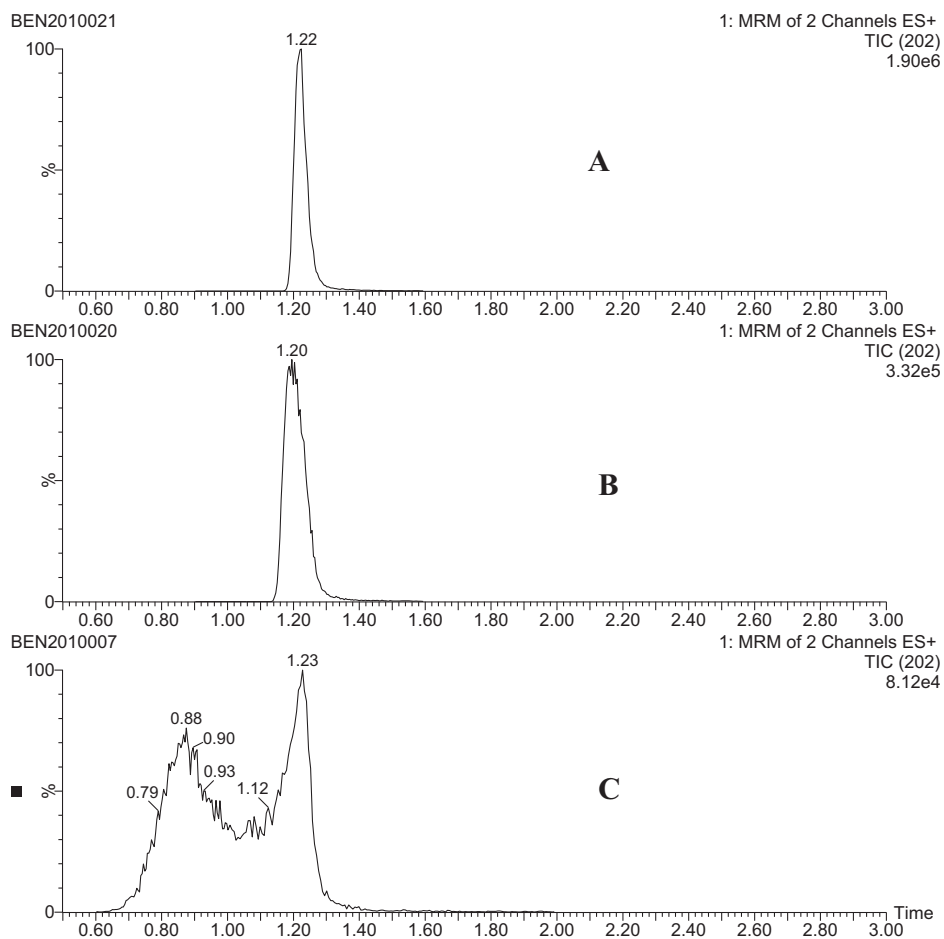
Fig. 2. Chromatograms of TBZ dissolved in different final solutions. (A) acetonitrile:H$_2$O, 10:90; (B) acetonitrile:H$_2$O, 15:85; (C) acetonitrile:H$_2$O, 50:50.

Table 1
MS/MS transitions and optimal conditions used for MS/MS analysis.

Compound	Precursor ion (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)
TBZ	202	175[a]	50	24
	202	131	50	30
ABZ–NH$_2$–SO$_2$	240	133[a]	50	25
	240	198	50	16
ABZ–SO	281.5	208[a]	55	25
	281.5	159	55	42
ABZ–SO$_2$	298	159[a]	40	34
	298	266	40	16
OFZ	316	159[a]	50	33
	316	284	50	17
OBZ	250	218[a]	40	20
	250	176	40	24
5-OH–TBZ	218	176[a]	55	20
	218	148	55	28
FBZ–SO$_2$	332	159[a]	45	34
	332	300	45	17
MBZ	296	105[a]	50	32
	296	264	50	20
ABZ	266	234[a]	40	17
	266	191	40	31
FLUB	314	282[a]	45	20
	314	123	45	32
FBZ	300	159[a]	40	29
	300	268	40	15
FBT	447	383[a]	40	17
	447	280	40	31

[a] Transitions for quantification.

observed for ABZ–SO$_2$ and results in the fragment at m/z 159. For ABZ–SO, the ion at m/z 208 results from the loss of –NHCO$_2$CH$_3$, whereas m/z 159 is obtained from the successive loss of –SOC$_3$H$_7$. For ABZ–NH$_2$–SO$_2$, the fragments of interest (m/z 198 and m/z 133) originate from the loss of C$_3$H$_7$ followed by that of the SO$_2$ group. For 5-OH–TBZ, the ion at m/z 176 results from the loss of –OH and the ring opening followed by the loss of –CN, and the ion at m/z 148 is obtained from consecutive loss of –CS. Product ion spectra of these compounds are shown in Fig. 1.

Chromatographic conditions were optimized to obtain minimal run time and best peak shape. During the method development, it was found that the component of final dissolving solution influenced the peak shape of TBZ and ABZ–NH$_2$–SO$_2$ significantly. As shown in Fig. 2, the standard dissolved in acetonitrile–H$_2$O (10:90, v/v) containing 0.1% formic acid produces good peak shape and sensitivity. After optimization of the gradient profile, the analysis time for each injection was 8 min, which was faster than the existing multi-residue method for benzimidazoles.

3.3. Matrix effects

Matrix effects are commonly encountered in LC electrospray mass spectrometry analysis of target compounds in complex sample matrices. Large amounts of endogenous compounds may potentially co-elute with target analytes and significantly affect the efficiency of the ionization process. In this study, the signal intensity of standard solution was compared with that of the matrix-matched standard solution at different concentrations. The approximate ratio of matrix-matched standard versus standard in
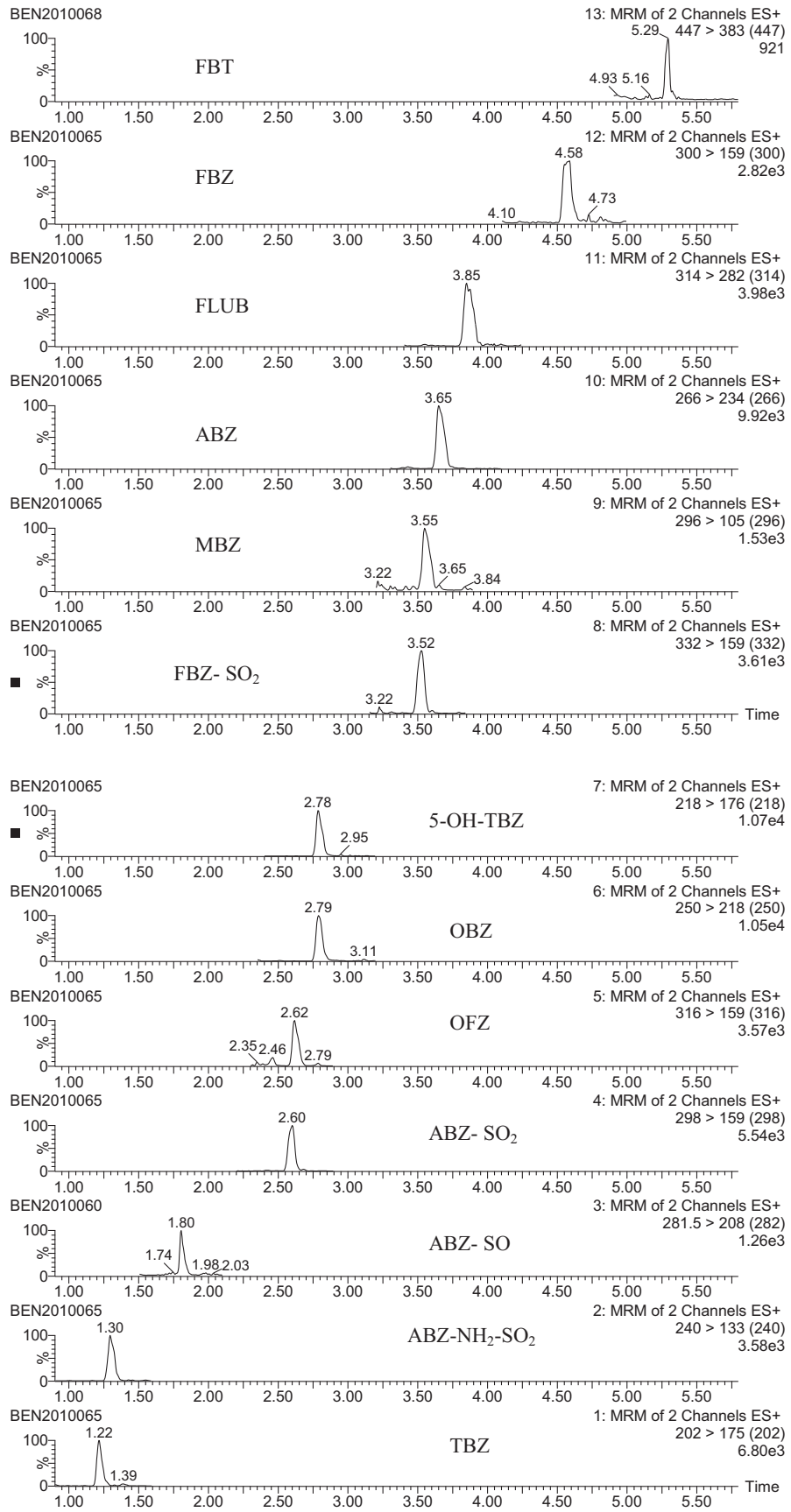
Fig. 3. MRM chromatograms of milk sample fortified at LOQ level.

Table 2
Recovery and precision of the method.

Compound	Fortified level (μg/L)	Mean recovery (%)	Intra-day RSD (%)	Inter-day RSD (%)
TBZ	0.1	95.0	5.5	7.2
	50	94.2	3.4	5.0
	100	93.3	6.3	8.3
ABZ–NH$_2$–SO$_2$	0.1	93.1	11.2	14.1
	50	84.8	5.0	8.5
	100	92.2	2.4	6.1
ABZ–SO	1	101.3	2.6	5.7
	50	93.5	4.9	5.9
	100	94.1	5.0	7.2
ABZ–SO$_2$	0.1	95.0	6.6	8.1
	50	89.7	1.4	4.0
	100	95.2	2.1	4.5
OFZ	0.1	82.0	7.0	8.6
	5	85.4	2.9	5.4
	10	95.5	2.1	5.7
OBZ	0.1	90.7	4.7	7.8
	5	94.8	3.8	6.0
	10	94.2	1.7	5.2
5-OH–TBZ	0.1	87.0	1.6	4.9
	50	89.9	7.5	10.5
	100	93.0	1.1	7.3
FBZ–SO$_2$	0.1	81.4	11.3	13.1
	5	83.3	7.2	8.6
	10	93.9	10.3	12.7
MBZ	0.1	83.0	12.6	14.0
	5	89.2	7.0	10.6
	10	92.0	10.4	11.8
ABZ	0.1	85.1	7.8	8.8
	50	84.5	10.0	12.6
	100	90.1	4.8	8.7
FLUB	0.1	81.5	2.6	5.0
	5	86.7	4.9	5.6
	10	86.6	3.8	5.6
FBZ	0.1	81.2	7.8	9.3
	5	82.6	5.2	8.4
	10	81.4	2.8	6.2
FBT	0.2	81.7	9.1	12.8
	5	80.5	10.6	12.4
	10	82.2	9.8	10.7

Table 3
LOD and LOQ of the method.

Compound	LOD (μg/L)	LOQ (μg/L)
TBZ	0.03	0.1
ABZ–NH$_2$–SO$_2$	0.05	0.1
ABZ–SO	0.5	1.0
ABZ–SO$_2$	0.05	0.1
OFZ	0.05	0.1
OBZ	0.01	0.1
5-OH–TBZ	0.01	0.1
FBZ–SO$_2$	0.05	0.1
MBZ	0.05	0.1
ABZ	0.02	0.1
FLUB	0.03	0.1
FBZ	0.05	0.1
FBT	0.1	0.2

of 34 milk samples purchased from local supermarkets (Beijing, China) were analyzed with the method during the spring 2010. No detectable benzimidazole residue was found in the samples.

4. Conclusion

A relatively fast, simple and selective method has been developed for the simultaneous determination of benzimidazoles in milk. The application of UHPLC allows a short chromatographic run time of 8 min, which is significantly lower than those of between 25 and 40 min applied in the previous multi-residue HPLC–MS/MS methods. Satisfactory recoveries with associated relative standard deviations were obtained at the level of 0.1 μg L^{-1} for most of the analytes. The method can be advantageously applied in routine analysis of benzimidazole residues in bovine milk samples.

Acknowledgements

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solvent for each analyte was as follows: ABZ–NH$_2$–SO$_2$, ABZ–SO, OBZ, 5-OH–TBZ, FBZ–SO$_2$, ABZ, FBZ, 101–109%; ABZ–SO$_2$, OFZ, MBZ, FLUB, 113–120%; TBZ, 86%; FBT, 31%. Ion suppression and enhancement were both observed for different compounds of interest. Therefore, matrix-matched calibration curves were used for quantification to compensate for the matrix effects.

3.4. Method validation

Specificity was found to be satisfactory, with no chromatographic interference being observed at the retention time of the target compound. All matrix-matched standard calibration curves with linear function resulted in the correlation of coefficient values (r^2) consistently above 0.991. Table 2 reports the average recoveries and relative RSDs of fortified samples. Benzimidazoles were fortified in milk samples at levels of 0.1, 5, 10 μg L^{-1} or 0.1, 50, 100 μg L^{-1} in six replicates, considering their MRL at 10 or 100 μg L^{-1}, on four different days for inter-day repeatability study, in addition to the same day for intra-day repeatability study. The lowest fortification level of ABZ–SO and FBT was 1 and 0.2 μg L^{-1}, respectively, because their sensitivity was not as high as other analytes. Mean recoveries ranged between 80.5 and 101.3% with inter-day RSD values of ≤14.1%, demonstrating the good accuracy and precision of the method. The method LOD and LOQ values are summarized in Table 3. Typical MRM chromatograms of fortified samples are shown in Fig. 3.

The described analytical method has been applied to real samples to assess the occurrence of the anthelmintic drugs. A total