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Development of a liquid chromatography–tandem mass spectrometry with pressurized liquid extraction method for the determination of benzimidazole residues in edible tissues

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ABSTRACT

A confirmatory and quantitative method of liquid chromatography–tandem mass spectrometry (LC–MS/MS) combined with a pressure liquid extraction (PLE) was developed for the determination of 11 benzimidazole and 10 metabolites of albendazole, fenbendazole and mebendazole in the muscles and livers of swine, cattle, sheep and chicken. For sample preparation, we used an automated technique of PLE method. The optimum extraction conditions were obtained using an 11 ml Accelerated Solvent Extraction (ASE) cells, acetonitrile/hexane as the extraction solvent. HPLC analysis was performed on a C18 column with gradient elution using acetonitrile and 5 mmol l^{-1} formic ammonium as mobile phase. The analytes were detected in the positive ion multiple reaction monitoring (MRM) mode by the LC–ESI–MS/MS analysis. The recoveries of benzimidazole (BZDs) spiked at the levels of 0.5 μg kg^{-1} ranged from 70.1% to 92.7%; the between-day relative standard deviations were no more than 10%. The limits of quantification were 0.02–0.5 μg kg^{-1}. The optimized method was successfully applied to monitor real samples containing BZDs, demonstrating the method to be simple, fast, robust and suitable for identification and quantification of BZDs residues in animal products.

1. Introduction

Benzimidazoles (BZDs) are anthelmintic agents widely used for the treatment of parasitic infections in food-producing animals. Widespread use of BZDs increases the possibility of residues appearing in food-producing animals, and several toxic effects have been associated with chronic exposure to benzimidazole compounds, such as teratogenicity, embryotoxicity, and anemia [1–3]. For the food safety risks, the use of BZDs in food-producing animals is controlled in China and the European Union. China and the European Union established the recommended maximum residue limits (MRL) for BZDs ranged from 50 to 400 μg kg^{-1} and 60 to 5000 μg kg^{-1}, depending on the compound and matrix. Consequently, there is an urgent need to develop comprehensive control measures to monitor residues of BZDs in animal products.

BZDs (Fig. 1) including thiabendazole analogs and benzimidazole carbamates have a bicyclic ring system in their structures in which benzene is fused to the 4 and 5 positions of the heterocycle (imidazole) [4]. The marker residues of most BZDs are defined as the sum of the parent or/and its persistent metabolites [5,6]. For the effective control of the residues of BZDs in foods, methods capable of detecting the complete range of marker residues should be applied. As to the determination of BZDs residues, liquid chromatography methods based on ultraviolet detection [7–10] and fluorometric detection [11] were frequently used. However, these detectors have low sensitivity and specificity. The combination of HPLC and MS for the determination of a wide range of BZDs and their metabolites has become a popular technique for sensitive and selective detection in complex biological matrices [12–20]. Kinsella et al. established the method for 18 BZDs in bovine liver [20]. However there are no published reports for simultaneously measuring 21 BZDs in diverse tissue by HPLC–MS/MS until now.

Sample preparation is the crucial technique in the simultaneous determination of multiple analytes in complex matrices. Although liquid–liquid extraction (LLE) and solid-phase extraction (SPE) can be used to extract BZDs in edible tissues [7,10,21], they are time-consuming and require a large amount of solvents some of which are toxic. QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) has been used to analyze BZDs in milk and liver [20,22]. The approach used very little organic solvent, but did not realize automated sample-handling. Pressurized liquid extraction (PLE) is a sample preparation technique that combines elevated tempera-

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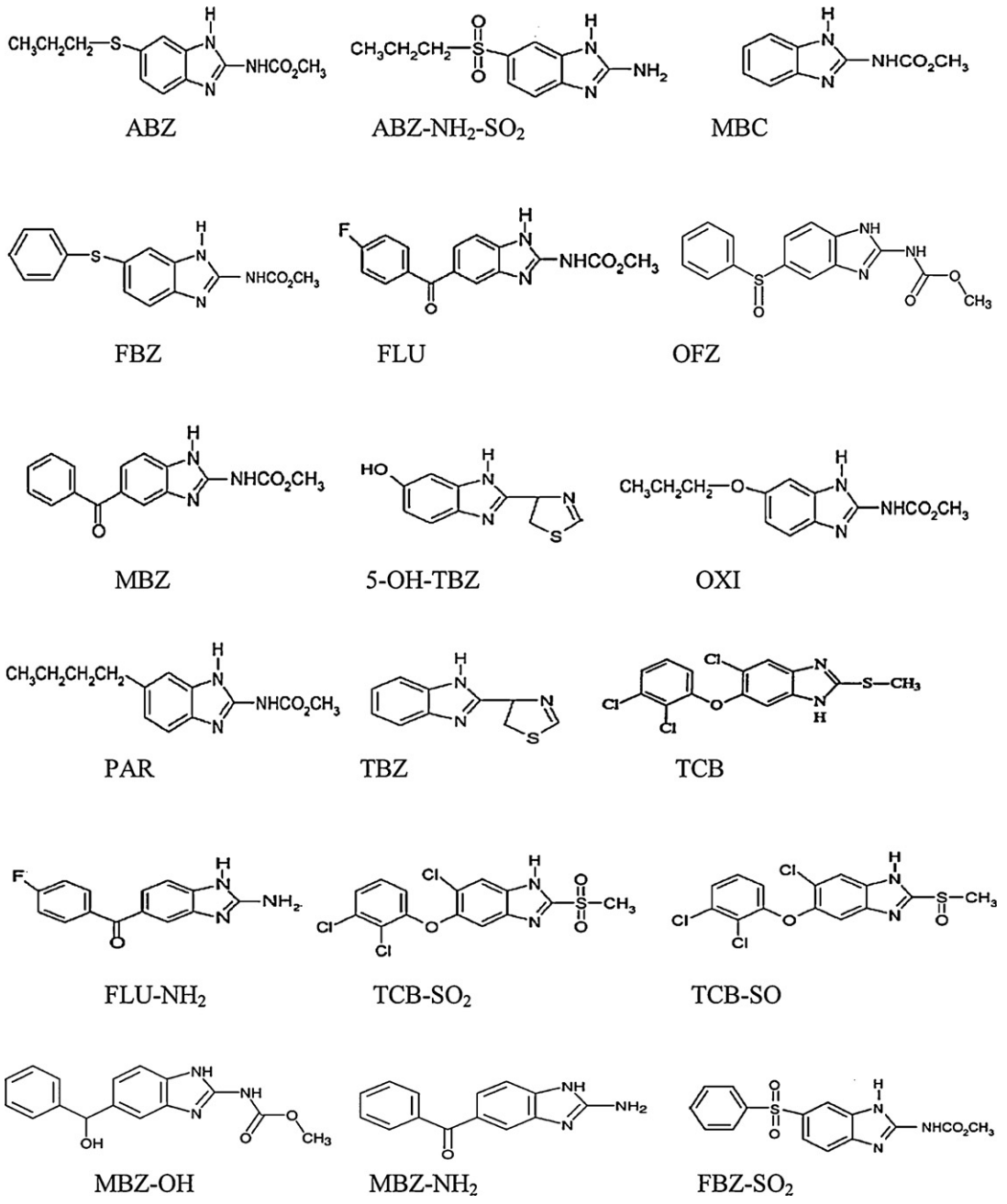

Fig. 1. Chemical structures of benzimidazoles.

ture and pressure with liquid solvent to achieve fast and efficient extraction of the analytes from the solid matrix. PLE is a robust and time-saving alternative that would enable automated sample-handling as well as to avoid health risks caused by both the analytes and the solvent. However, only several PLE methods have been applied in recent year for the analysis different analytes in food samples [23–28]. Furthermore, there was no method to apply PLE to analyze BZDs residues in animal origins food.

The purpose of this work was to develop a simple and sensitive LC–MS/MS method with a PLE extraction for the simultaneously confirmatory detection of the residues of 21 BZDs in edible tissues. Different variables affecting PLE efficiency including extraction solvent, extraction temperature, extraction pressure and static cycles were optimized. Extracts were filtered and directly analyzed by HPLC–MS/MS without any other clean-up procedures. Thus, the method reported here is a simpler, faster, more sensitive and economic tool to monitor the residues of BZDs in edible tissues.

2. Experimental

2.1. Drugs and reagents

Analytical standards of albendazole (ABZ), thiabendazole (TBZ), fenbendazole sulphoxides (oxfendazole, OFZ), mebendazole (MBZ), and carbendazim (MBC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cambendazole (CAM), 5-hydroxy thiabendazole (5-OH-TBZ), oxibendazole (OXI), flubendazole (FLU), fenbendazole (FBZ), and triclabendazole (TCB) were obtained from Dr. Ehrenstorfer (Gmbh, Augsburg, Germany). Albendazole sulphoxides (ABZ-SO), albendazole sulphone (ABZ-SO_2),

albendazole-2-amino-sulphone (ABZ-NH_2-SO_2), amino mebendazole (MBZ-NH_2), hydroxy mebendazole (MBZ-OH), fenbendazole sulphone (FBZ-SO_2), Amino-flubendazole (FLU-NH_2), triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO_2), and FBZ-D_3 were purchased from Witega (Belin, Germany). Parbendazole (PAR) was purchased from USP (St. Rockville, MD, USA).

Water was purified with a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). Acetonitrile and methanol were HPLC-grade and obtained from Fisher (Bar-Bel, France). Other solvents of analytical reagent grade including ethyl acetate, hexane, formic acid, ammonium formate and diatomaceous earth, were supplied by Shanghai Guoyao Company (Shanghai, China).

2.2. Standard solutions

Individual stock standard solutions (100 μg ml^{-1}) of all analytes were made by dissolving each pure standard in methanol. A 1.0 μg ml^{-1} mixed standard fortification solution was prepared by combining 1.0 ml of each stock standard and dilute to 100 ml with methanol. Tuning solution of each analyte (100 μg l^{-1}) was prepared by diluting individual stock solution with acetonitrile–water (20:80, v/v). A 1.0 μg ml^{-1} labeled internal standard solution (IS: FBZ-D_3) was prepared with acetonitrile–water (20:80, v/v), respectively. Stock and mixed standard solution were prepared every 3 and 1 months and were stored in amber vials at or below −20 °C.

2.3. Sample preparation

Samples as muscles and livers of swine, cattle, sheep and chicken were collected from local markets. After being homogenized in a high-speed food blender, the samples were stored below −20 °C in a freezer.

The extraction of BZDs from tissues was performed by a PLE. An ASE 200 system (Dionex, Sunnyvale, CA, USA), equipped with a 24-sample carousel, 11-ml stainless steel cells, and 40-ml collection vials was used for the PLE. Two cellulose filters (Dionex) were placed at each end of the PLE cell.

Two grams of sample spiked with 10 μl of IS solution and 3 g diatomaceous earth were mixed and grounded into powder using a pestle. The mixtures were weighed in 11-ml stainless steel cells capped with two glass–fiber filters, and the cells were capped and placed on an extractor. Extraction was carried out at the temperature of 80 °C using acetonitrile/hexane (80/20, v/v) as extraction solvent. After the injection of the solvent into the cell, a pressurized static extraction phase lasting 5 min was carried out at 1500 psi, followed by a flow of fresh acetonitrile/hexane (80/20, v/v). The extract was then centrifuged at 8470 × *g* for 10 min, and hexane was discarded. The acetonitrile was evaporated to dryness in a water bath at 40 °C. The dry residues were dissolved in 2 ml of acetonitrile/water (20/80, v/v) solution. The resulting solutions were vortexed for 1 min and then centrifuged at 10,200 × *g* for 10 min. The acetonitrile/water solution was transferred to another sample vial for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

The liquid chromatography system (Shimadzu Corp., Kyoto, Japan) was equipped with a solvent delivery pump (LC-20AD), an auto-sampler (SIL-20AC), and a column oven (CTO-20AC). The chromatographic separation was accomplished on a Thermo Hypersil Gold C18 column (3.5 μm, 150 mm × 2.1 mm i.d.) maintained at 40 °C. The mobile phase of component A was a buffer solution consisting of 5 mmol l^{-1} ammonium formate and that of component B was acetonitrile. The mobile phase gradient profile was as follows (*t* in min): $t_{0'}$, A = 85%, B = 15%; $t_{15'}$, A = 20%, B = 80%; $t_{21'}$, A = 85%, B = 15%. The mobile phase was delivered to the LC column at a flow rate of 200 μl min^{-1} and the injected volume was 10 μl.

MS/MS analysis is performed on an API 5000 triple quadrupole mass spectrometer instrument (Applied Biosystems, Foster City, CA) operated in the positive (ESI^+) electrospary ionization mode. Applied Biosystems Sciex Analyst software version 1.5 was employed for data acquisition and processing. Analysis was performed with nitrogen used as: curtain gas with a pressure of 6 psi, ion source gas 1 with a pressure of 50 psi, and ion source gas 2 with a pressure of 60 psi, respectively. The source temperature and ionspray voltage (IS) were set at 600 °C and 5500 V, respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode that selects one precursor ion and two product ions for each target compound. The values of entrance potential and collision cell exit potential were 10.0 V and 12.0 V. The mass spectrometer was operated at unit mass resolution for both Q1 and Q3 in the MRM mode using a dwell time of 20 ms for all analytes. The precursor ion and two product ions for each target compound and its labeled internal standard are listed in Table 1. The choice of fragmentation products for each substance and the optimization of energy collisions and other instrument parameters were done in a flow injection analysis mode with standard solution at concentrations of 100 μg l^{-1}.

2.5. Method validation

For the study of specificity, which is the ability to differentiate between target analytes and interference, 20 blank samples for each matrix originating from different animals were analyzed. The analytes were identified by matching retention times of peaks with the values of the corresponding standard analyzed under the same experimental conditions. For confirmation purposes, specific fragmentation pattern of individual analyte (Table 1) was used for distinguishing the analyte from the matrix interferences thus allowing for greater evidence in compound identification.

The limits of detection (LOD) and limits of quantification (LOQ) values of each analyte were considered as the concentrations giving a signal-to-noise ratio of 3 and 10, respectively.

The linearity of the standard mixtures was obtained by analyzing standards in acetonitrile–water (20:80, v/v) at concentration levels from 0.5 to 50 μg l^{-1} for benzimidazoles. Matrix-match calibration curves were prepared daily by spiking blank control samples of tissue with mixtures of benzimidazoles at concentration levels from 0.5 to 50 μg l^{-1}. Curves were constructed by linear regression of the ratios of chromatographic peak area of the standards and suitable IS (5 μg l^{-1}) versus nominal concentrations. The correlation coefficients and the slope were calculated. Such calibration curves were prepared with each series of samples.

Accuracy and precision of the analytical method were calculated by the determination of six replicates of blank samples fortified at three different spiked levels (0.5, 50 and 100 μg kg^{-1}) over a period of 3 days. Recovery was performed by comparing the analytical results of extracted BZDs from fortified whole samples with matrix-match calibration curves. Intra-day precision was conducted on the same day. Inter-day precision was determined by repeating the study on 3 consecutive days.

3. Results and discussion

3.1. LC–MS/MS optimization

3.1.1. Selection of mobile phase

The choice of the mobile phase should be based on the consideration of the ionization efficiency and retention time before the analytes enter the MS/MS system in order to obtain good res-

Table 1
Optimum precursor and product ions with the respective collision energy (eV) for MS/MS.

Compound	Precursor ions (*m/z*)	Product ions 1[a]			Product ions 2			t_R (min)
		m/z	CE (eV)	DP (V)	*m/z*	CE (eV)	DP (V)	
MBC	192.1	160.2	43	100	132.2	26	95	3.9
TBZ	202.0	175.2	47	130	131.2	38	125	4.4
5-OH-TBZ	218.0	191.2	48	15	146.8	38	140	2.9
MBZ-NH_2	238.1	105.1	52	145	77.3	37	145	7.2
ABZ-NH_2-SO_2	240.0	133.0	40	100	198.0	27	107	3.9
PAR	248.0	216.2	28	120	145.1	55	142	11.0
OXI	250.1	218.2	28	117	176.2	41	120	8.6
FLU-NH_2	256.1	123.3	39	156	95.2	58	148	7.7
ABZ	266.4	234.1	28	121	191.3	45	127	10.9
ABZ-SO	282.2	208.3	50	125	160.0	35	127	6.2
MBZ	296.1	264.2	32	132	105.1	47	112	10.0
MBZ-OH	298.3	266.3	31	135	160.0	49	135	7.4
ABZ-SO_2	298.4	159.0	52	190	224.0	36	135	7.8
FBZ	300.3	268.2	30	145	159.0	50	148	12.5
CAM	303.2	217.1	40	125	261.1	28	130	7.8
FLU	314.3	282.3	32	150	122.9	52	145	10.7
OFZ	316.3	159.1	50	145	283.9	30	150	8.0
FBZ-SO_2	332.2	300.2	31	142	159.1	52	140	9.4
TCB	359.2	274.1	52	168	344.0	35	160	15.7
TCB-SO	377.1	362.3	31	135	152.1	60	128	14.0
TCB-SO_2	393.2	241.9	52	168	258.0	47	168	14.9
FBZ-D3	303.3	268.5	32	176	159.5	50	186	12.5

[a] Quantification ions.

olution and high sensitivity. Acetonitrile and methanol are usually used in the mobile phase for reversed-phase (RP) HPLC separation of various compounds. Therefore, the two solvents were tested in this study. When acetonitrile was used as an organic solvent in the mobile phase, the sensitivity of the analytes was better. According to the structure of benzimidazoles, the pH of the mobile phase may be a critical factor in achieving the chromatographic separation of the BZDs studied. Several mobile phase additives such as formic acid and ammonium formate were comprehensively investigated. The maximum sensitivities of the most of analytes exclude MBZ-NH_2 and FLU-NH_2 were achieved when using 5 mM ammonium formate buffer and acetonitrile as mobile phase. The gradient was optimized to provide the maximum separation possible in a minimum time period.

3.1.2. Optimization of the MS/MS conditions

The stock solutions (100 μg ml^{-1}) of 21 BZDs standards were prepared with acetonitrile–water (20:80, v/v). As for the selection of parent ions, the ionization mode (ESI+/ESI−) should be decided first according to chemical ionization characteristics of BZDs. The parent ion *m/z* of each BZDs was subsequently confirmed by infusion with a syringe pump based on the optimization of MS/MS parameters and solvent medium. The result showed that BZDs could form [M+H]+ parent ions with high abundance under the ESI+ mode. The final selection of parent ions was summarized in Table 1.

Based on the confirmation of parent ions, more than two daughter ions should be selected when using low resolution LC–MS analysis in accordance with relevant EU legislation. Therefore, the optimization of daughter ions and their collision energy was performed under the daughter scan mode soon. Table 1 shows MS/MS transitions for quantification and confirmation as well as declustering potential (DP) and collision energy (CE) values optimized for each of the selected compounds. For instance, it can be observed that typical product ions correspond with the loss of CH_3OH $[M+H-32]^+$ for the benzimidazole carbamates including MBC, PAR, OXI, ABZ, MBZ, MBZ-OH, FBZ, FLU, OFZ, and FBZ-SO_2. The transition of $[M+H-HCN]^+$ was used for the quantification of thiabendazole analogs, such as CAM, TBZ, and 5-OH TBZ. The ion at *m/z* 344 for TCB corresponds to the loss of CH_3 from the ring structure.

3.1.3. Selection of sample solvent medium before injection

The composition of sample solvent medium before injection directly affects the separation behavior of BZDs in the HPLC column and their sensitivity during LC–MS/MS detection. To select the optimal solvent medium, different ratios of water, formic acid, ammonium formate solution, and acetonitrile were compared in this study. The relative peak height of each analyte was increased when choosing acetonitrile–water (20:80, v/v) compared to other solvent mediums.

3.2. PLE optimization

In the optimization of the PLE procedure, all important parameters affecting extraction efficiency were evaluated: extraction solvent, temperature, pressure and number of the extraction cycles.

The use of solvents at elevated temperatures increases the capacity of solvents to solubilize analytes and increases the diffusion rate. In addition, increased temperatures also decrease the viscosity of liquid solvents (better penetration of matrix particles) and can disrupt the strong solute–matrix interactions caused by van der Waals forces, hydrogen bonding and dipole attractions of the solute molecules and active sites of the matrix [29]. The choice of the extraction solvent is probably one of the most critical parameters in the PLE procedure. Previous studies indicated that acetonitrile and ethyl acetate [7,10,21] were generally the preferred solvents for BZDs extraction. The extraction efficiency of the three solvents of methanol, acetonitrile, and ethyl acetate was evaluated. The PLE conditions used were as follows: 60 °C as extraction temperature, 1500 psi, 5 min static time, flushing volume of acetonitrile 60% of cell size, and one cycle. With acetonitrile as extraction solvent, statistically higher recoveries for compounds studied were obtained than using methanol and ethyl acetate (Fig. 2). After the extraction of BZDs the fat that remains in the sample extract must be removed. In order to obtain an optimum solvent ratio for defatting, experiments were carried out with the ratio of acetonitrile and hexane continuously varied from 50% acetonitrile and 50% hexane to 90% acetonitrile and 10% hexane. The results showed that the best extraction and cleanup for BZDs was obtained when acetonitrile/hexane is at a ratio of 80:20 (v/v).

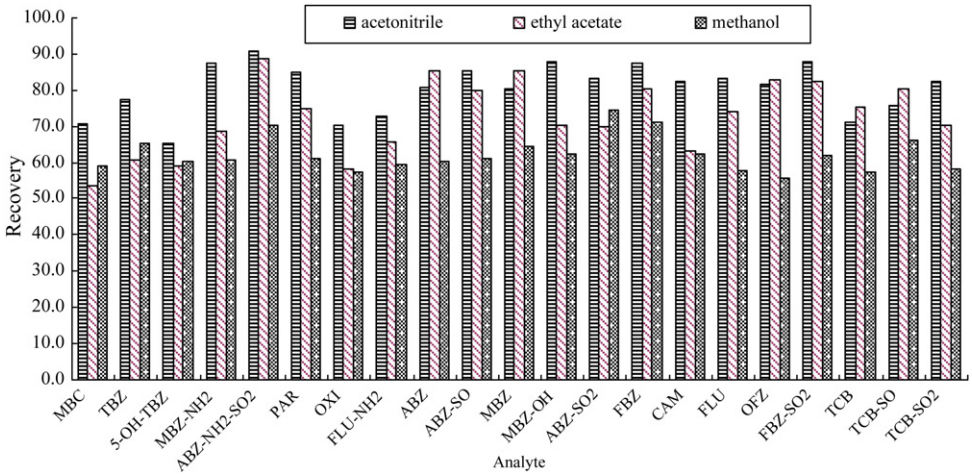

Fig. 2. Effect of extraction solvent on the PLE extraction of BZDs in muscle of swine. Temperature: 60 °C; pressure: 1500 psi; 1 cycle of 5 min.

Since temperature strongly affects the extraction efficiency, a series of experiment at different temperatures (40–100 °C) was performed to determine the best extraction temperature. The effect of extraction temperature on the extraction efficiency of PLE is presented in Fig. 3. The results indicated that the recovery of the analytes stay constant up to 60 °C, and then decrease at higher temperatures. The decrease in the observed extraction efficiency was possibly due to the degradation of these compounds at temperatures above 60 °C. Thus, 60 °C was chosen as the extraction temperature.

Static cycles introduce fresh solvent during the extraction process and have been proved to be useful for difficult to penetrate matrices. The number of the extraction cycles was tested to assure a rapid extraction as well as high recovery. The number of extraction cycles was varied between one and three. In general, an increment of the number of extraction cycles allows the exposure of the matrix to fresh solvent and favors the solvent/sample equilibrium, improving partition into the liquid phase and increasing the analyte recoveries. Fig. 4 shows that the extraction cycles did not significantly affect recoveries of BZDs. One cycle was selected in order to minimize the time of analysis.

Three different pressures, 1500, 1800, 2000 psi, were tested. The results showed that the recovery was not affected by pressure. The extraction pressure was set as 1500 psi for further experiments.

To evaluate if extraction time could influence extraction efficiency, different extraction times (5, 7, 10, 12 min) were used. Increasing the static extraction time from 5 to 10 min did not affect the extraction of target compounds. Consequently, a 5 min static time was selected in order to minimize the time of analysis.

The flush percentage refers to the amount of solvent flushed through the cell following the static heating step, expressed as a percentage of the cell volume. Increasing the flush volume allowed more solvent to pass through the sample, but it also increased the final volume of the extract. The flush volume (20, 40, 60, 80%) did not significantly affect the extraction efficiencies of the analytes. So the flush volume was set at their default values (60%).

Based on the test results, we have selected the optimized PLE extraction as follows: using acetonitrile/hexane (80/20, v/v) to extract BZDs by one static cycle of 5 min at 60 °C. The PLE entire procedure was completely automated and required about 30 min for a single sample.

3.3. Comparison of PLE and shaking extraction

The extraction efficiency of PLE for BZDs was compared with those obtained by shaking extractions. The relative yields of the BZDs extracted from edible tissues samples were compared (Table 2). PLE extraction was done within about 30 min, while shak-

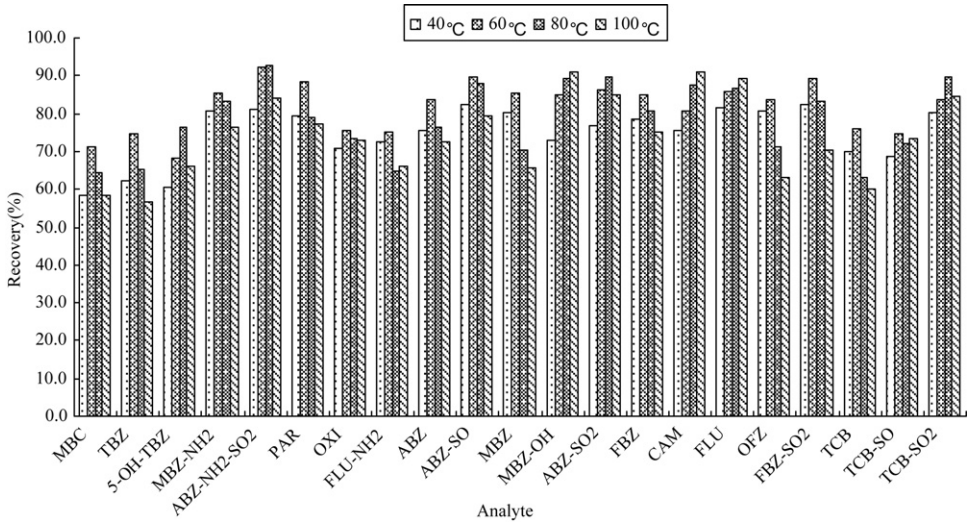

Fig. 3. Effect of temperatures on the PLE extraction of BZDs in the muscle of swine. Solvent: acetonitrile; pressure: 1500 psi; 1 cycle of 5 min.

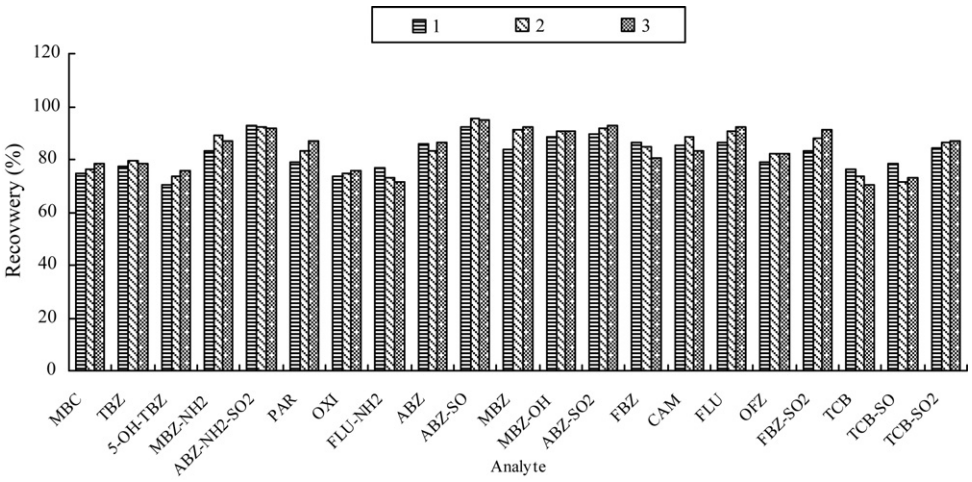

Fig. 4. Effect of the number of cycles on the PLE extraction of BZDs in the muscle of swine. Solvent: acetonitrile; temperature: 60 °C; pressure: 1500 psi.

Table 2
Relative amounts of BZDs in edible tissues by ASE and shaking extraction methods.

Parameters	ASE	Shaking extraction
Extraction solvent	Acetonitrile/hexane	Acetonitrile
Extraction time	30 min	2 h
Acetonitrile consumption	12 ml	20 ml
Defatting method	ASE	LLE
Defatting solvent	Hexane	Hexane
Hexane consumption	3 ml	5 ml
Automatic/manual	Automatic	Manual
Inter-day RSD	Low	High
Capital investment	High	Low

ing extraction required about 2 h. The extraction efficiency of PLE was slightly higher than those of shaking extraction, and PLE has the advantage of shorter extraction time than the other, which is the main advantage of PLE over shaking extraction. PLE was conducted automatically, using less solvent with higher sensitivity. In addition, the defatting step was simultaneous to the extraction of target drugs in PLE, whereas, in the shaking the step was posterior to the extraction of target drugs. The shaking method used acetonitrile to extract target drugs, after that, liquid–liquid partition was used to eliminate the fat. The clean-up efficiency of PLE was better than that of shaking. It was obvious that the interference peaks in PLE extracted sample chromatogram were fewer than those in shaking extracted sample chromatogram.

3.4. Matrix effect

When using electrospray ionization, the presence of matrix components that may affect the ionization of the target analytes may be a significant problem, by either reducing or increasing analytes response. For assessment of matrix effect, signal suppression/enhancement was estimated by comparing standard solution and matrix-matched solution calibration curves. The results revealed a significant ion suppression for MBC, PAR, and a slight ion suppression for 5-OH-TB, FLU-HMET, TCB-SO2, a significant ion enhancement for MBZ-NH2, TCB, TCB-SO, and a slight ion enhancement for TBZ, ABZ-SO, ABZ-SO2, FBZ, OFZ, whereas no matrix effects were observed for ABZ-NH_2-SO_2, CAM, MBZ, FLU, OXI, ABZ, MBZ-OH and FBZ-SO_2. For this reason matrix matched calibration was selected for quantification in real samples.

Table 3
The validation results of BZDs (50 μg kg^{-1}) spiked in the muscles of swine, bovine, ovine and chicken.

Analyte	Average recovery (%)				Inter-RSD (%)			
	Porcine muscle	Bovine muscle	Sheep muscle	Chicken muscle	Porcine muscle	Bovine muscle	Sheep muscle	Chicken muscle
MBC	98.6	93.0	90.2	91.4	7.2	6.3	7.8	6.5
TBZ	90.2	93.1	96.4	89.0	7.5	6.8	8.1	6.1
5-OH-TBZ	87.2	96.9	97.3	88.4	6.3	6.9	7.4	7.8
MBZ-NH_2	103.3	94.6	102.2	96.4	6.5	8.1	6.7	7.2
ABZ-NH_2-SO_2	111.4	112.8	109.3	117.0	7.8	6.4	6.1	7.0
PAR	92.3	88.0	92.3	95.9	7.8	6.9	7.4	7.5
OXI	89.7	82.9	87.9	92.7	8.3	7.0	6.6	7.2
FLU-NH_2	103.1	90.2	96.4	93.4	7.4	8.6	8.0	8.7
ABZ	104.9	103.3	101.9	106.8	6.5	7.2	6.8	7.7
ABZ-SO	105.4	103.1	109.5	112.8	7.1	7.3	6.6	6.0
MBZ	96.6	95.8	100.3	102.0	7.4	9.2	6.6	6.8
MBZ-OH	97.1	99.4	102.4	93.7	8.5	7.3	7.2	8.1
ABZ-SO_2	109.3	104.2	106.5	103.2	8.0	8.8	7.5	6.7
FBZ	102.9	105.1	100.3	105.5	7.9	7.5	6.9	8.2
CAM	112.1	109.5	112.5	111.2	6.4	7.8	7.2	6.8
FLU	103.3	104.2	95.7	108.2	6.6	7.5	6.8	6.9
OFZ	102.4	91.4	96.9	92.1	8.9	9.2	7.6	7.7
FBZ-SO_2	104.1	99.1	112.4	102.8	8.2	6.4	6.6	7.1
TCB	80.2	87.9	81.9	81.2	7.2	8.4	7.9	8.3
TCB-SO	88.5	90.0	79.9	82.0	7.4	8.5	8.2	8.5
TCB-SO_2	80.8	81.5	89.7	79.9	8.6	9.0	9.3	8.2

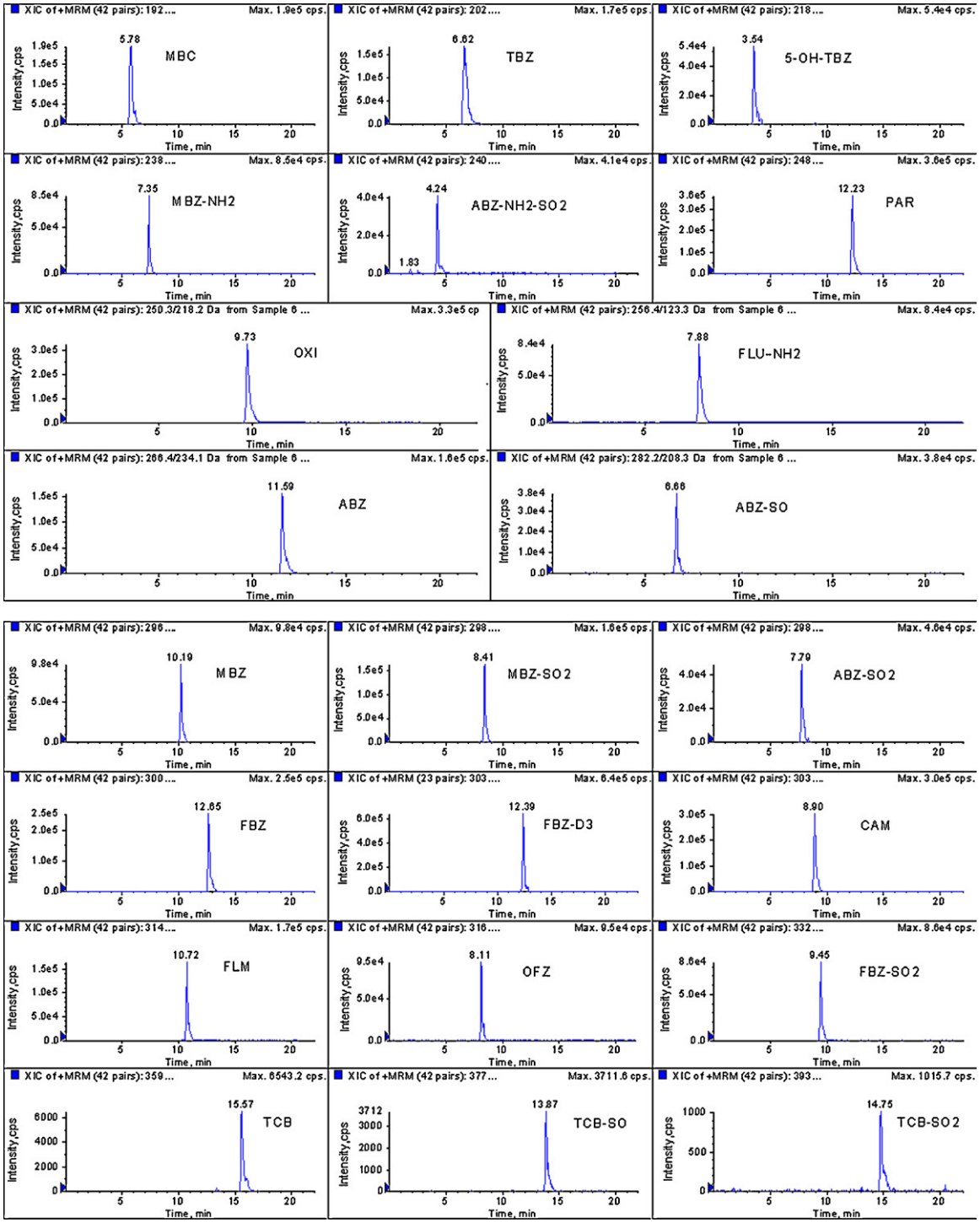

Fig. 5. Selected reaction monitoring LC–MS/MS chromatograms of BZDs spiked in the muscle of swine (0.5 μg kg^{-1}).

3.5. Validation of the analytical method

The selectivity of the method was demonstrated through the analysis of 20 blank samples of each matrix originating from different animal tissues, which were found to be free of interference from endogenous materials. The specificity of the assay was demonstrated by checking interfering peaks at the retention time of target analytes. The results showed that there were no interference peaks co-eluting with target analytes. The developed LC–MS/MS method is capable of separating all analytes under the given gradient condition within 15 min. Representative chromatograms of fortified porcine muscle are shown in Fig. 5.

Measurements of linearity were carried out with standard mixtures and the matrix-matched mixed standard solutions at a series of concentrations 0.5–50 μg l^{-1}. The calibration curves were constructed using peak areas from six concentration levels versus the concentration of analytes. The linearity of the standard mixtures was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients being greater than 0.997 for 63 calibration lines (21 analytes × 3 validation days).

Table 4
The validation results of BZDs (50 μg kg^{-1}) spiked in the livers of swine, bovine, ovine and chicken.

Analyte	Average recovery(%)				Inter-RSD(%)			
	Porcine liver	Bovine liver	Sheep liver	Chicken liver	Porcine liver	Bovine liver	Sheep liver	Chicken liver
MBC	87.0	89.5	94.1	83.3	6.7	7.2	8.1	7.6
TBZ	91.1	96.8	87.1	92.2	5.9	6.3	8.2	6.4
5-OH-TBZ	92.1	95.3	88.4	94.0	8.5	6.2	7.2	8.1
MBZ-NH_2	104.6	100.6	96.9	92.5	8.8	7.4	7.8	6.6
ABZ-NH_2-SO_2	107.4	110.6	103.1	109.8	8.0	8.2	8.3	6.7
PAR	98.6	99.7	89.4	95.5	8.1	8.9	6.5	7.2
OXI	89.8	89.1	93.2	96.2	8.4	8.5	7.6	7.2
FLU-NH_2	102.3	98.1	100.6	95.5	8.5	8.6	7.1	5.6
ABZ	106.9	102.9	110.7	111.4	6.3	8.3	7.5	6.3
ABZ-SO	108.0	112.0	111.7	109.1	7.6	6.5	6.2	7.1
MBZ	92.1	94.0	90.0	94.4	7.1	8.2	6.4	7.1
MBZ-OH	96.6	104.2	97.7	93.4	7.9	8.2	7.5	6.4
ABZ-SO_2	106.1	110.0	106.6	109.7	8.8	6.6	8.4	7.4
FBZ	98.3	102.8	104.9	97.6	6.2	7.0	7.9	6.5
CAM	90.4	93.7	93.1	90.9	7.3	5.8	7.2	8.1
FLU	103.8	107.2	108.8	108.0	8.2	9.1	7.3	7.3
OFZ	89.7	90.3	96.0	97.2	6.4	7.0	8.3	6.8
FBZ-SO_2	99.9	95.0	97.7	107.5	6.8	7.5	8.0	7.1
TCB	86.8	90.9	88.5	92.5	7.6	6.3	8.4	7.7
TCB-SO	85.6	84.5	84.0	88.5	7.1	8.8	7.9	8.2
TCB-SO_2	83.0	79.6	87.2	89.5	8.8	7.8	8.5	7.9

Table 5
The ion ratios of BZDs in the muscle of swine.

Compound	Product ions	Average of ion ratios (%) of standard solution	Maximum permitted tolerances (%)	Average of ion ratios (%) of spiked porcine muscle
MBC	132.2/160.2	78.6	62.8–94.3 (1 ± 20%)	70.3
TBZ	131.2/175.2	83.5	66.8–100.2 (1 ± 20%)	73.2
5-OH-TBZ	146.8/191.2	45.4	34.1–56.7(1 ± 25%)	40.6
MBZ-NH_2	77.3/105.1	64.4	51.5–71.3 (1 ± 20%)	50.2
ABZ-NH_2-SO_2	133.0/198.0	38.1	28.6–47.6 (1 ± 25%)	30.5
PAR	216.2/145.1	21.6	16.2–27.0 (1 ± 25%)	18.5
OXI	176.2/218.2	91.0	728.8–109.2 (1 ± 20%)	82.2
FLU-NH_2	95.2/123.3	44.5	33.4–555.6(1 ± 20%)	52.4
ABZ	191.3/234.1	54.7	43.8–65.6 (1 ± 20%)	53.6
ABZ-SO	160.0/208.3	13.5	9.5–17.6 (1 ± 30%)	11.4
MBZ	105.1/264.2	72.5	58.0–87.0 (1 ± 20%)	63.4
MBZ-OH	160.0/266.3	75.8	60.6–91.0 (1 ± 20%)	62.1
ABZ-SO_2	224.0/159.0	57.6	46.1–69.1 (1 ± 20%)	66.0
FBZ	268.2/159.0	81.0	64.8–97.2 (1 ± 20%)	68.6
CAM	217.1/261.1	13.3	9.3–17.3 (1 ± 30%)	7.6
FLU	282.3/122.9	82.2	68.8–98.6 (1 ± 20%)	92.3
OFZ	159.1/283.9	69.0	55.2–82.8 (1 ± 20%)	80.2
FBZ-SO_2	300.2/159.1	42.6	32.0–52.3 (1 ± 25%)	43.8
TCB	274.1/344.0	26.7	20.0–33.4 (1 ± 25%)	21.4
TCB-SO	362.3/152.1	23.3	17.4–29.1 (1 ± 25%)	18.3
TCB-SO_2	258.0/241.9	66.8	83.4–80.2 (1 ± 20%)	62.6

Matrix-matched calibration curves were prepared daily by spiking blank control samples of tissue with mixtures BZDs. For each compound eight matrix-match calibrations of different tissue sample standard curves were calculated. The correlation (r) with each calibration curves was higher than 0.99. However, to avoid matrix effects, matrix-match calibrations curves were chosen as reference curves throughout this study.

Recovery experiments were performed by comparing the analytical results of the extracted veterinary drug from fortified whole tissue samples with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. The results of sample are summarized in Tables 3 and 4. The overall recoveries of edible tissues ranged from 70.1% to 92.7% and inter-day precisions were less than 10%. The results demonstrated that the precision and the accuracy of the present method were acceptable for routine monitoring purpose.

The LOQ of the examined BZDs extracted from edible tissues ranged from 0.02 to 0.5 μg kg^{-1}, values much lower than the MRLs established by the European Union for BZDs in tissues. The LOD of BZDs ranged from 0.01 to 0.2 μg kg^{-1} in edible tissues. For the confirmation of the presence of BZDs residues, their identity could be determined according to European Union Commission Decision 657/2002 (European Commission 2002) with a minimum total score of three identification points. Since one precursor ion and two product ions were monitored, this requirement is fulfilled. Each analyte ion ratio was effectively measured on each chromatogram, corresponding to the less intense signal against the most intense one. During the validation the ion ratios measured on the spiked samples were compared with those obtained from the calibration curve standards (Table 5). The calculated results from this work are in compliance with European Union Commission Decision 657/2002.

3.6. Application of the method

This method has been applied for daily analysis of 40 real samples collected from local markets including 20 porcine muscles and 20 porcine livers. All samples were processed according to the

method described. No detectable residues of the target analytes were found in any of the 40 samples. Only ABZ-SO_2 and ABZ-NH_2-SO_2 have been detected in some porcine livers, but values much lower than the MRLs established by the European Union for ABZ in tissues. In terms of overall data, this method has proved to be suitable for identifying real samples of BZDs in tissues.

4. Conclusions

The major goal of this research was to investigate for the first time the suitability of PLE for the extraction of 21 BZDs from edible tissues samples. This method allows the extraction of the analytes without purification, and it has the main advantage of reducing the analyte loss during sample handling, time required for the analytical procedure, and costs for material and manpower. The identification and quantification of multiple BZD residues in edible tissues were successfully achieved using LC–MS/MS. The method has satisfactory validation characteristics with respect to specificity, accuracy, precision, and sensitivity. Therefore, we conclude that this LC–MS/MS method is suitable for routine determination of BZD residues in food products.

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