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Comparison of two different isolation methods of benzimidazoles and their metabolites in the bovine liver by solid-phase extraction and liquid chromatography–diode array detection

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ABSTRACT

A new analytical method that uses high performance liquid chromatography–diode array detector (HPLC–DAD) was developed for the analysis of 14 benzimidazoles residues, including metabolites, in bovine liver. Samples were extracted using two different extraction procedures: with phosphate buffer after enzymatic hydrolysis (method A) or using organic solvent, i.e. acetonitrile (method B). Then, samples were purified on a strong cation exchange (SCX) cartridge and analyzed in HPLC/DAD. The recovery percentages, obtained spiking the matrix (liver) at concentrations of 500 and 100 μg kg^{-1} with a standard mixture of benzimidazoles, were in the range 6–101% and 80–102% for methods A and B, respectively. The repeatability of the methods was assessed in all cases by the % of correlation value (CV) that was lower than 19%. The limits of quantification (LOQs) in the matrix for methods A and B were in the range 40–60 and 20–50 μg kg^{-1}, respectively. The best of the two methods, method B, was used for the analysis of 10 bovine liver samples.

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1. Introduction

Benzimidazoles are anthelmintic agents widely used for prevention and treatment of parasitic infections in agriculture and aquaculture. Some benzimidazoles have also found applications as pre- or post-harvest fungicides for control of a wide range of fungi affecting field crops, stored fruits and vegetables [1]. In particular, they are widely used in veterinary medicine against gastrointestinal nematodes and lungworms that affects cows. Enhancement of milk production of 0.35–0.63 kg day^{-1}, after anthelmintic treatment of naturally infected lactating dairy cows, has been shown [2,3]. But a number of these compounds have been shown to cause teratogenic and embryotoxic effects in some species; their use in farm animals raises the possibility that residues may be found in food for human consumption [4]. In addition, metabolism of the drugs is extensive. Found metabolites depend on the structure of the parent drug, the tissue, and the animal species. One or more metabolites can be found in animal tissues for most drugs [5]. The EU has set Maximum Residues Limits (MRLs) for benzimidazoles and their metabolites in animal products [6]. The MRL values range within 10–1000 μg kg^{-1}, depending on the compound and on the matrix (Table 1).

Several methods exist in the literature for the analysis of one or more benzimidazoles as residues in a variety of food types. Most of the authors used LC with UV detection [7–13], in two cases [11,12] compared with HPLC/ESI/MS, while other researchers used both HPLC/ESI/MS and HPLC/ESI/MS/MS [13,14,15,16].

The extraction methods vary from liquid–liquid extraction (LLE) with conventional organic solvent followed by liquid–liquid partition (LLP) [17–21] or solid-phase extraction (SPE) [22–25]. Several authors used hydrolysis as a pre-treatment step to release residues bound to proteins and drug conjugates or simply convert residues to a common structure. Tocco et al. [26] found that urinary metabolites of thiabendazole (TBZ) mainly occurred as glucuronide and sulphate conjugates of 5-hydroxythiabendazole (5-OH-TBZ), while VandenHeuvel et al. [27] also deconjugated 5-OH-TBZ by enzymatic and acid hydrolysis in a study in bluegill sunfish.

The aim of this work was to set up a new and simple method of screening for the analysis of benzimidazole residues and their main metabolites. Since the method is to be used by territorial agencies assessing food safety, we choose to set up a method based on the diode array detector, a low cost and very common HPLC detector.

For that, we compared two different extraction procedures for the analysis of 14 benzimidazoles in bovine liver sam-

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Table 1
EU MRL values for benzimidazole anthelmintic drugs (based on Council Regulation No. 2377/90 and Commission Regulation Nos. 508/1999, 2385/1999, 2393/1999 and 807/2001).

Benzimidazole	Marker residue	Animal species	MRL ($\mu g\,kg^{-1}$)	Target tissue
Netobimin (NETO), albendazole (ABZ) albendazole sulphoxide (ABZ-SO)	Sum of ABZ-SO, albendazole sulphone ($ABZ\text{-}SO_2$) and amino-albendazole sulphone ($NH_2ABZ\text{-}SO_2$), expressed as albendazole	Bovine, ovine	100	Milk
			100	Muscle
			100	Fat
			1000	Liver
			500	Kidney
Febantel (FEB), fenbendazole (FBZ) and oxfendazole (OFZ)	Sum of extractable residues that may be oxidised to fenbendazole sulphone ($FBZ\text{-}SO_2$)	Bovine, ovine	10	Milk
		Bovine, ovine, porcine, equidae	50	Muscle
			50	Fat[a]
			500	Liver
			50	Kidney
Flubendazole (FLU)	Sum of FLU and amino-flubendazole (NH_2FLU)	Porcine, game birds, chicken, turkey	50	Muscle
			50	Fat[a]
			400	Liver
			300	Kidney
		Chicken	400	Eggs
Thiabendazole (TBZ)	Sum of TBZ and hydroxy-thiabendazole (TBZ-OH)	Bovine	100	Muscle
			100	Fat
			100	Liver
			100	Kidney
			100	Milk
Oxibendazole (OXI)	OXI	Porcine	100	
			500	Muscle
			200	Fat[a]
			100	Liver
				Kidney
Triclabendazole (TCB)[b]	Sum of extractable residues that may be oxidised to keto-triclabendazole	Bovine, ovine	100	Muscle
			100	Liver
			100	Kidney
Mebendazole (MBZ)[b]	Sum of hydroxy-mebendazole (MBZ-OH) and amino-mebendazole (NH_2-MBZ), expressed as mebendazole equivalents	Ovine, caprine, equidae	60	Muscle
			60	Fat[a]
			400	Liver
			60	Kidney

[a] For porcine and/or poultry species, this MRL relates to "skin and fat in natural proportion".
[b] Not for use in animal from which milk is produced for human consumption.

ples. One method is, new and based on enzymatic hydrolysis with protease (method A), to increase free drug or to release drug conjugates, and the second, modified in laboratory from that reported by Rose [9] is based on solvent extraction with acetonitrile (method B). Most of the published methods deal with the determination of only few benzimidazoles. Using the approach of identifying residues most likely to occur in different animal species, a multi-residue method might be developed that would provide more complete surveillance for these compounds. Hence, another objective of this work was to develop an improved quantitative method for the determination of an extended range of benzimidazoles within a multi-residue method. Subsequently, method B was chosen to analyze in bovine liver the 14 above indicated benzimidazoles and their metabolites, i.e. 5-hydroxythiabendazole, albendazole 2-aminosulphone, albendazole sulphoxide, thiabendazole, albendazole sulphone, oxfendazole, 2-ammino flubendazole, oxfendazole sulphone, oxibendazole, mebendazole, flubendazole, albendazole, fenbendazole and triclabendazole. Finally, the selected method was applied to the analysis of 10 real samples of bovine liver.

2. Experimental

2.1. Materials and standards

Albendazole 2-aminosulphone (CAS No. 80983-34-2), albendazole sulphone (CAS No. 75184-71-3), albendazole sulphoxide (CAS No. 54029-12-8), and 2-amino flubendazole (CAS No. 82050-13-3) were purchased from LGC-Promochem (Milan, Italy). Albendazole (CAS No. 54965-21-8), oxibendazole (CAS No. 20559-55-1) and thiabendazole (CAS No. 148-79-8) were obtained from SIGMA (St. Louis, MO, USA). Oxfendazole (CAS No. 53716-50-0) and triclabendazole (CAS No. 68786-66-3) were from Dr. Ehrenstorfer (Augsburg, Germany). 5-Hydroxythiabendazole (CAS No. 948-71-0) and oxfendazole sulphone (CAS No. 54029-20-8) were obtained from the "Bank of Reference Standards" (RIVM, the Netherlands). Fenbendazole (CAS No. 43210-67-9), flubendazole (CAS No. 31430-15-6) and mebendazole (CAS No. 31431-39-7) from Riedel-de-Haën (Seezle, Hannover, Germany).

Individual stock solutions were prepared by dissolving 10 mg of each compound in 10 ml of dimethylformamide and stored in

glass-stopper bottles at 4 °C. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution with methanol of aliquots of the stock solutions.

Hydrochloric acid 37% was supplied by Carlo Erba Reagenti spa (Milano, Italy). Ammonia solution 28% was supplied by Prolabo (Fontenais sous Bois Cedex, France). Acetic acid and HPLC-grade ammonium acetate were supplied by Mallinckrodt Baker (Deventer, Netherlands). Acetone and *n*-hexane solvents for residue analysis were supplied by Fluka-Riedel-deHaën (Milano, Italy). HPLC-grade methanol, HPLC-grade acetonitrile, protease from *Rhizopus* sp. and sodium sulphate >99% were supplied by Sigma–Aldrich (Milano, Italy). HPLC-grade formic acid was supplied by Merck (Darmstadt, Germany). Deionized water (<8 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). Prior to HPLC injection, the samples were filtered through a 0.2-μm PTFE filter from Supelco (Bellefonte, PA, USA). Cartridges SPE Bond Elute SCX Varian (1 g/6 ml) were purchased from Varian (Palo Alto, CA, USA).

2.2. Sample collection

The bovine liver samples were purchased from different butcheries of the Camerino area. Liver samples were homogenized in Ultraturrax (IKA, Staufen, Germany) and stored in different food containers in a refrigerator at −20 °C. They were allowed to stand at room temperature for some time before use. In this work we used spiked blank samples to determine the recovery values, while incurred samples containing analytes were not available.

2.3. Sample preparation

2.3.1. Method A

Method A, applied to the analysis of benzimidazoles in bovine liver, was developed and optimized in our laboratory. Five grams of liver was weighed in a 50 ml centrifuge tube and homogenized with Ultraturrax with 15 ml phosphate buffer pH 3. Then, 1.5 ml of protease solution (800 mg of protease from *Rhizopus* sp. dissolved in 10 ml of HCl 0.002 M) was added and the sample was soaked twice in a vortex mixer for 2 min. Liver–phosphate buffer solution was adjusted at pH value of 3.78 with hydrochloric acid 25% and homogenized again for 10 s using a vortex mixer. Sample was kept overnight at 37 °C for enzymatic hydrolysis. The following morning, sample was cooled down to room temperature, adjusted at pH value of 1.5 with hydrochloric acid 25% and centrifuged at 3000 rpm for 15 min at 4 °C. Supernatant acqueous solution was transferred into another centrifuge tube. The residue was treated twice with 6 ml of hydrochloric acid 0.1N, manually shaken for 1 min, sonicated for 30 min and then centrifuged at 4000 rpm for 15 min at 4 °C. When supernatants were combined, 10 ml hexane were added and the mixture was gently shaken. Afterwards, the solution was centrifuged at 3000 rpm for 15 min at 4 °C and the supernatant hexane was discarded. The defattening step was repeated with 10 ml hexane. After that, the combined extracts were purified on SCX cartridges (6 ml, 1 g). Firstly, the cartridge was conditioned with 5 ml of an acetonitrile/acetic acid mixture (95/5, v/v); the liver–phosphate buffer solution, adjusted at pH 1.2 with hydrochloric acid solution 25%, was tranferred onto the SCX cartridge at a flow rate lower than 0.5 ml min^{-1}. Afterwards, the cartridge was washed with 5 ml acetonitrile and finally the elution was performed with 10 ml of a acetonitrile/ammonia solution 28% mixture (95/5, v/v) at a flow rate lower than 0.5 ml min^{-1}. The eluate was totally evaporated under vacuum (60 mbar) at 40 °C by a Büchi apparatus (Büchi R200, Labortechnik, Flawil, Switzerland), the residue was dissolved in 0.5 ml of methanol and filtered through a 0.2-μm PTFE filter from Supelco (Bellefonte, PA, USA) before LC/DAD analysis. 25 μl were injected into the LC–DAD system. Extraction recoveries were determined by spiking liver–phosphate buffer solution with standard benzimidazole mixture at level of 500 and 100 μg kg^{-1}.

2.3.2. Method B

Method B used for the analysis of benzimidazoles in bovine liver was modified in laboratory that was reported by Rose [9]. Five grams of liver was weighed in a 50 ml centrifuge tube and homogenized in Ultraturrax with 20 ml acetonitrile and 2 g sodium sulphate. Then, the sample was soaked for 1 min in a vortex mixer, sonicated for 30 min and centrifugated for 5 min and 20 °C at 1700 rpm. The supernatant was then filtered. After that, the extracts were purified on SCX cartridges (6 ml, 1 g), which we conditioned with 5 ml of an acetonitrile/acetic acid mixture (95/5, v/v); the liver–acetonitrile solution, acidified with 5 ml acetic acid, is tranferred onto the SCX cartridge at a flow rate lower than 0.5 ml min^{-1}. Afterwards, cartridge was washed with 2.5 ml of acetone, 5 ml of methanol, 5 ml of acetonitrile and elution was performed with 10 ml of an acetonitrile/ammonia solution 28% mixture (95/5, v/v) at a flow rate lower than 0.5 ml min^{-1}. The eluate was totally evaporated under vacuum (60 mbar) at 40 °C by a Büchi rotavapor (Büchi R200, Labortechnik, Flawil, Switzerland), the residue was dissolved in 0.5 ml of methanol and filtered through a 0.2-μm PTFE filter from Supelco (Bellefonte, PA, USA) before LC/DAD analysis. 25 μl were injected into the LC–DAD system.

Extraction recoveries were determined by spiking the liver–acetonitrile solution with standard benzimidazoles mixture at level of 500 and 100 μg kg^{-1}.

2.4. HPLC/DAD analysis

The separation was performed on Zorbax SB-C_{18} Agilent (Santa Clara, CA, USA) 250 mm × 4.6 mm, 3 μm column in the gradient mode with acetonitrile/methanol 9/1, v/v (phase A) and ammonium dihydrogenophosphate buffer at pH 4.78 (phase B). The gradient program was: 0 min 20% A, 0–12 min 30% A, 12–30 min 50% A, 30–35 min 80% A. Finally, phase A was decreased at 20% from 35 to 40 min and held at 20% until end of the run at 45 min. The flow rate during analysis was 1 ml min^{-1}.

LC/DAD studies were performed using a Hewlett Packard (Palo Alto, CA, USA) HP-1090 Series II, made of an autosampler, a binary solvent pump, and a diode-array detector (DAD).

LC/DAD analyses were performed monitoring four different wavelengths: 310 nm for 5-hydroxythiabendazole, mebendazole, and flubendazole, 298 nm for thiabendazole, 2-amino flubendazole, oxfendazole sulphone, oxibendazole, albendazole, fenbendazole, and triclabendazole, 290 nm for albendazole sulphoxide, albendazole sulphone, and oxfendazole, 285 nm for albendazole 2-aminosulphone. The reported chromatograms (Figs. 1–4) of the bovine liver samples and of the standard mixture of benzimidazoles are presented at one wavelength, for sake of clarity of the figures.

3. Results and discussion

3.1. Comparison of two extraction procedures

Fig. 1 reports an HPLC–DAD chromatogram of standard mixture of the analyzed benzimidazoles at a concentration of 1000 μg l^{-1} monitored at 298 nm.

The best of the two optimized extraction procedures is the one that uses acetonitrile as extraction solvent (method B), both for method performances (trueness and precision) and for the simplicity of sample preparation. In fact, in this procedure, the recoveries obtained by spiking the bovine liver at level of 500 μg kg^{-1} were in the range 88–102%, with CVs lower than 10% (Table 2). The recoveries obtained by spiking the bovine liver at level of 100 μg kg^{-1} were

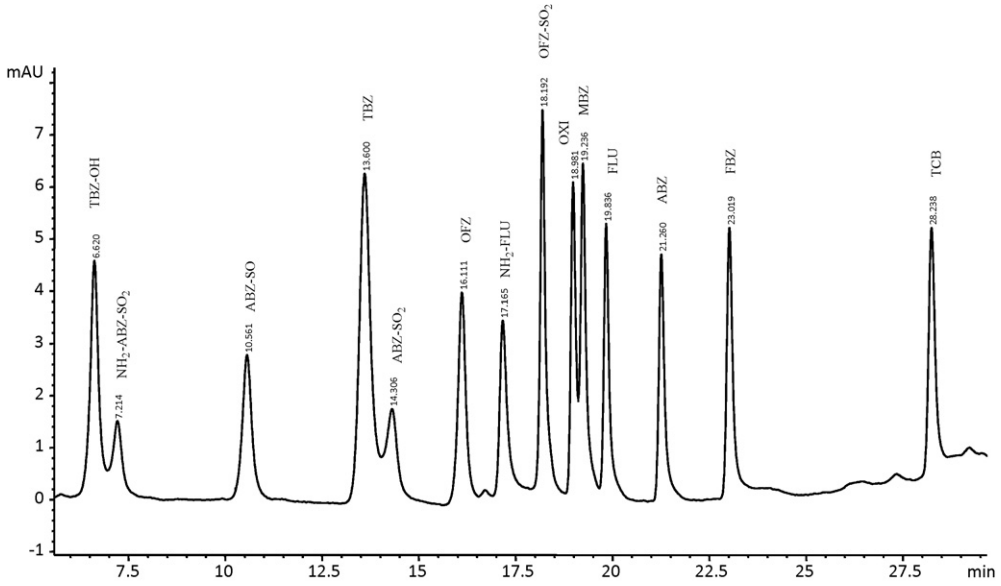

Fig. 1. HPLC–DAD chromatogram of standard mixture of the analyzed benzimidazoles at concentration of 1000 μg kg^{-1} and monitored at 298 nm.

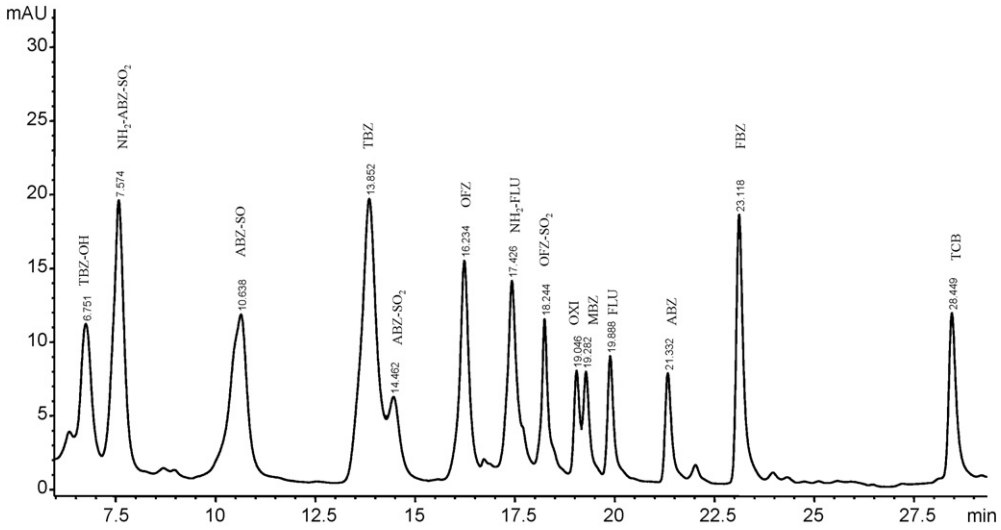

Fig. 2. HPLC–DAD chromatogram of bovine liver sample prepared by method B, fortified by standard mixture of benzimidazoles at concentration of 500 μg kg^{-1} and monitored at 298 nm.

Table 2
Comparison of percent recovery and reproducibility with-in lab of the two methods at two fortification levels.

Compounds		Fortification level 100 μg kg^{-1} (n = 10)				Fortification level 500 μg kg^{-1} (n = 10)			
No.	Benzimidazoles	Method A		Method B		Method A		Method B	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
1	TBZ-OH	97	5	93	6.2	98	3.2	95	8
2	NH_2-ABZ-SO_2	99	1	99	1.7	99	0.5	100	0.4
3	ABZ-SO	101	1	99	2.6	89	8.4	99	0.5
4	TBZ	89	8.5	97	2.2	95	4.4	97	1.8
5	ABZ-SO_2	63	5.5	101	1.1	47	10	98	4.5
6	OFZ	97	2.7	100	0.7	82	16	100	3.7
7	NH_2-FLU	65	17.4	93	9.3	63	19	99	9.3
8	OFZ-SO_2	73	11.5	97	5.9	64	12	92	5.7
9	OXI	66	15.7	100	1.2	65	9	102	6.2
10	MBZ	61	19	98	3.7	53	16	99	2
11	FLU	49	18.7	97	4.7	40	19	97	3
12	ABZ	50	15.8	80	16.7	38	18	88	9.7
13	FBZ	37	17.5	99	2.3	16	18.4	100	2
14	TCB	9	9.1	93	2.3	6	8	95	5.2

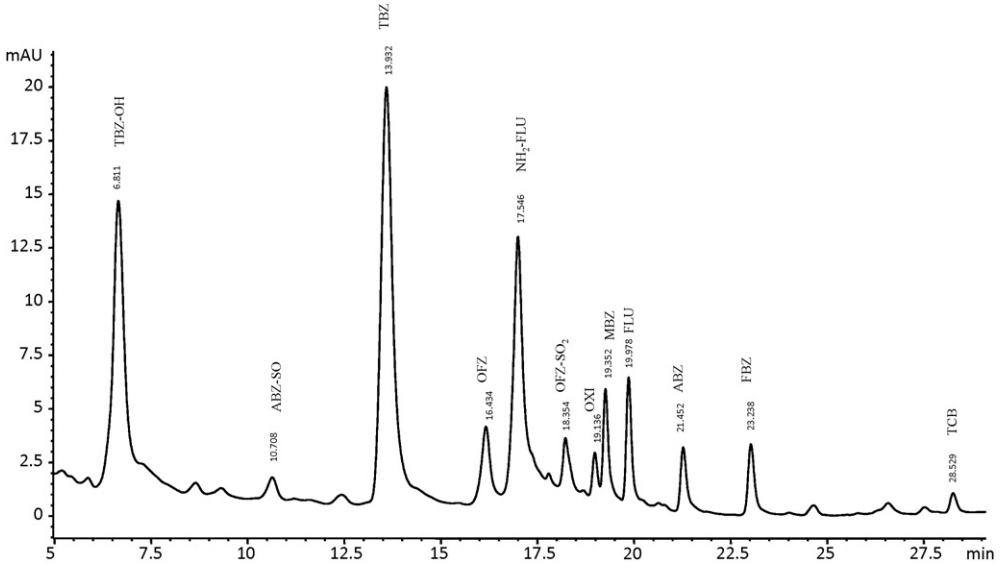

Fig. 3. HPLC–DAD chromatogram of bovine liver sample prepared by method A, fortified by standard mixture of benzimidazoles at concentration of 500 μg kg^{-1} and monitored at 298 nm. Compounds no. 2 (NH_2-ABZ-SO_2) and 5 (ABZ-SO_2) are not visible because they have been monitored at 285 and 290 nm, respectively.

in the range 80–101%, with CVs lower than 10% except for albendazole (Table 2). As an example, in Fig. 2 is a reported HPLC/DAD chromatogram of bovine liver sample prepared by method B, fortified by standard mixture of benzimidazoles at concentration of 500 μg kg^{-1}, and monitored at 298 nm. On the contrary, applying the procedure "A", which includes the enzymatic hydrolysis, we obtained lower recoveries and higher coefficients of variation (CV%) by analyzing the spiked bovine liver samples. In fact, in this procedure, the recoveries obtained by spiking the bovine liver at level of 500 μg kg^{-1} were in the range 6–99%, with coefficients of variation lower or equal to 19 (Table 2). The recoveries obtained by spiking the bovine liver at level of 100 μg kg^{-1} were in the range 9–101%, with coefficients of variation lower or equal to 19 (Table 2). As an example, in Fig. 3 an HPLC/DAD chromatogram of bovine liver sample prepared by method A, fortified by standard mixture of benzimidazoles at concentration of 500 μg kg^{-1} and monitored at 298 nm is reported.

Method "B", that was developed starting from that reported by Rose [9], presents significative improvements. Our method analyzed simultaneously 14 analytes vs. 11 of Rose, showing, at the same fortification level (100 μg kg^{-1}), much higher recoveries values (80–101%) for all analytes with respect to those obtained by Rose et al. (34–96%). In particular, fenbendazole recovery was 99%, vs. 41–49%. Moreover, the obtained CV% values are lower than 10%, except for albendazole (16%), while Rose obtained most of CV% around 20%. Hence, a significative novelty of this work is the evident improvement obtained with respect to the methods present in literature.

In addition, we can say that method A is more time-consuming and laborious than method B. In fact, during enzymatic hydrolysis an exact pH (3.78) and temperature (37 °C) control is required. After hydrolysis, in the following morning it is important to adjust pH value at 1.5 with hydrochloric acid 25% because the solubility of benzimidazoles in water is related to the pH [1]. Adjusting pH of liver–phosphate buffer solution at higher values (we tried with 1.8, 2, 2.6) leads to negative values for percentage recovery. Moreover, after centrifugation it is very important to wash the liver residues two times (one wash is not enough to extract analytes from matrix) with hydrochloric acid 0.1N (better than with HCl 0.01N) and to sonicate for extracting analytes bound to the matrix. The

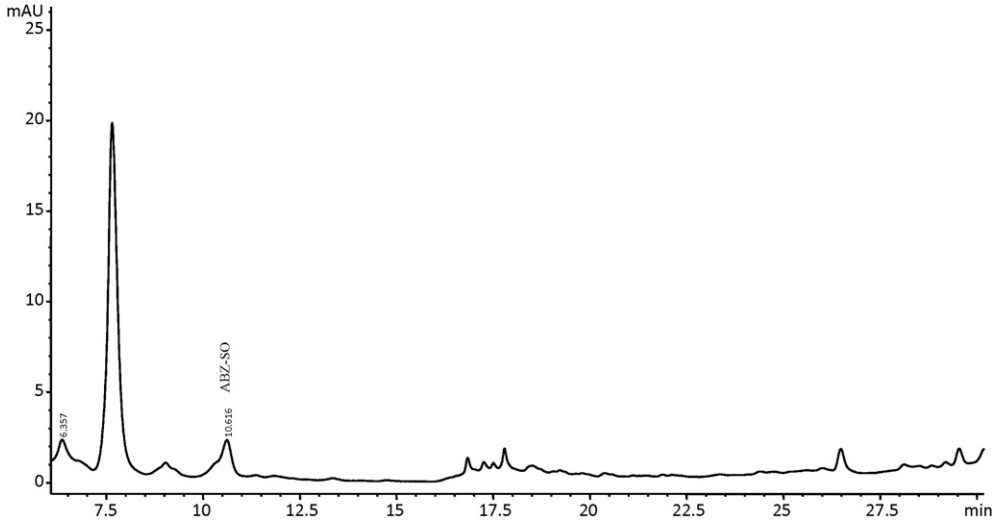

Fig. 4. HPLC–DAD chromatogram of blank sample of a bovine liver monitored at 290 nm with suspect contamination of albendazole sulphoxide.

two defattening steps allow to eliminate fats and to obtain a cleaner sample. Before SPE-SCX purification it is necessary to adjust pH of liver–phosphate buffer solution at the value of 1.2 with hydrochloric acid, in such a way that protonated analytes are able to interact with stationary phase of SPE SCX cartridges. Higher pH values of the liver–phosphate buffer solution (we tried 1.5, 2, 2.5 and 3) brought about decreasing recovery of the 14 analytes.

On the contrary, method B is simple and fast. During sample preparation for the solubility of analytes it is important to perform the 30 min sonication step before centrifugation and the acidification with 5 ml of acetic acid before SPE-SCX purification in such a way that protonated analytes are able to interact with the stationary phase of cartridges [9]. In addition, the chromatograms obtained with method B showed less interferences of matrix and presented peaks having a higher chromatographic resolution. On the basis of the above mentioned facts, we have chosen method B for the analyses of real bovine liver samples.

At the beginning of the work, we planned to analyze 17 benzimidazoles and metabolites, including, besides the 14 reported compounds, triclabendazole sulphoxide, triclabendazole sulphone and 5-hydroxy mebendazole. Unfortunately, recovery studies on these three metabolites furnished very low values (1–7%) with both sample preparation methods (A and B). Moreover, the obtained chromatograms including these three molecules were of scarce quality, with a systematic overlay of peaks referred to these three metabolites with other compounds. For this reason we decided not to include them among the monitored molecules.

Moreover, we optimized different analytical parameters. The gradient program was: 0 min 20% A, 0–12 min 30% A, 12–30 min 50% A, 30–35 min 80% A. Finally, phase A was decreased at 20% from 35 to 40 min and held at 20% until end of the run at 45 min. In our experiments we found that a long time of conditioning is indispensable for reproducibility of retention times of the monitored analytes. In addition, we tried to use various aqueous mobile phases, as water/ammonium dihydrogenphosphate at different pH values (for example pH 2, 3, 4.6 and also higher than 5) but with decreased separation of benzimidazoles. In particular, the peaks corresponding to the following pairs of compounds: 5-hydroxythiabendazole and albendazole 2-aminosulphone, thiabendazole and albendazole sulphone, and mebendazole and flubendazole showed a partial overlap. Danaher et al. [28] proposed a method for quantification of 10 benzimidazoles, showing a good separation between analytes, especially for thiabendazole and albendazole sulphone, by using a ternary gradient elution. Our method, that is able to analyze 14 benzimidazoles by using a binary gradient elution, shows a quite good separation of thiabendazole and albendazole sulphone, as is evident by analyzing the chromatograms at the specific wavelength (298 and 290 nm, respectively) for each compound.

3.2. Analysis of liver samples

The LC/DAD analysis was performed choosing wavelengths (four in total) in the maximal absorbance zone for each molecule: 310 nm for 5-hydroxythiabendazole, mebendazole, and flubendazole, 298 nm for thiabendazole, 2-amino flubendazole, oxfendazole sulphone, oxibendazole, albendazole, fenbendazole, and triclabendazole, 290 nm for albendazole sulphoxide, albendazole sulphone, and oxfendazole, 285 nm for albendazole 2-aminosulphone.

Ten bovine liver samples were analyzed using the selected method B, which showed the highest recovery and the lowest CV percentages obtained, and which was also quite simple in terms of sample preparation. Due to the very complex composition of the matrix liver and due to the not identical composition of the liver, among samples, it could be expected that, in some cases, interferents could be found. In our analysis, the data show that seven samples did not show any suspected peak for the presence of benzimidazoles residues, while three liver samples showed a suspected peak, with retention time similar to that of albendazole sulphoxide (Fig. 4).

Table 3
Limits of detection (LODs) and limits of quantification (LOQs) obtained for the 14 benzimidazoles, expressed in $\mu g\,kg^{-1}$, calculated in the matrix prepared with methods A and B.

Compounds		Method A		Method B	
No.	Benzimidazoles	LOD	LOQ	LOD	LOQ
1	TBZ-OH	10	40	8	30
2	NH_2-ABZ-SO_2	18	60	15	50
3	ABZ-SO	18	60	12	50
4	TBZ	10	40	8	30
5	ABZ-SO_2	12	50	12	50
6	OFZ	12	50	12	50
7	NH_2-FLU	18	60	12	50
8	OFZ-SO_2	12	50	12	50
9	OXI	18	60	12	50
10	MBZ	18	60	12	50
11	FLU	12	50	12	50
12	ABZ	18	60	12	50
13	FBZ	12	50	8	30
14	TCB	10	40	5	20

We tried to exclude the suspected presence of the benzimidazole derivative by comparing the UV–vis spectrum of the sample peak and that of the standard analyte, but a most efficient analytical method that uses LC–MS-MS is required to exclude false positive. Hence, we can conclude that this is a valid method of screening for the analysis of benzimidazole residues in a very complex matrix such as liver.

3.3. Method validation

The methods were validated by determining linearity, recovery at two fortification levels, repeatability and with-in reproducibility, limits of detection (LODs) and limits of quantification (LOQs) (instead of CCs alpha and CCs beta). Calibration curves of the analyzed compounds were constructed injecting 25 μl of standard solutions at five different concentrations, i.e. 100, 500, 1000, 5000 and 10,000 $\mu g\,l^{-1}$ in HPLC/DAD technique. Five replicates for each concentration were performed and the coefficients of variation (CVs) ranged from 1.0 to 5.3% for run-to-run precision, and from 3.5 to 6.5% for day-to day precision. All the calibration curves of the analyzed benzimidazoles showed a correlation coefficient greater than 0.998. The recovery percentages, obtained spiking the matrix (liver) at concentrations of 500 and 100 $\mu g\,kg^{-1}$ with a standard mixture of 14 benzimidazoles, were in the range 6–101% or 80–102%, for methods A and B, respectively. The repeatability of the methods was calculated on fortified samples at 500 and 100 $\mu g\,kg^{-1}$ ($n = 3$), giving CV% that were in a range 0.2–3.6% and 0.1–4.1%, respectively. The reproducibility with-in lab of the methods, reported in Table 2, was calculated on fortified samples at 500 and 100 $\mu g\,kg^{-1}$ ($n = 10$), giving CV% that was in all cases lower than 19%.

In Table 3 are reported the limits of detection and the limits of quantification of the 14 benzimidazoles, expressed in $\mu g\,kg^{-1}$, calculated in the matrix for methods A and B. LOD and LOQ were estimated on the basis of 3:1 and 10:1 S/Ns. For method A, LODs and LOQs of benzimidazoles were in the range 10–18 and 40–60 $\mu g\,kg^{-1}$, respectively. For method B, LODs and LOQs of benzimidazoles were in the range 5–15 and 20–50 $\mu g\,kg^{-1}$, respectively. By comparison of these two different approaches, we can conclude that the matrix can interfere with the signal of selected compounds but in all cases the highest limit (60 $\mu g\,kg^{-1}$) is lower than the MRLs reported in European Regulation No. 2377/90.

4. Conclusions

A comparison of two different isolation methods (A and B) of benzimidazoles and their metabolites in the bovine liver by solid-phase extraction and liquid chromatography–diode array detection has been performed. The most efficient and simpler of the two methods, method B, was chosen to analyze 10 samples of bovine liver, using HPLC/DAD to quantify the benzimidazoles.

From our results, analyzed samples of bovine livers, bought in local shops in Camerino area, are free of contamination by benzimidazoles and/or their main metabolites. Moreover, we set up an analytical screening method (method B), which, being DAD based, could be applied by the territorial agencies that are in charge of analysis of residues in food samples.

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