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Review

Review of methodology for the determination of benzimidazole residues in biological matrices

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Abstract

Benzimidazoles are anthelmintic agents widely used in the treatment of parasitic infections in a range of species and as fungicidal agents in the control of spoilage of crops during storage and transport. In this paper, the more important benzimidazoles are introduced and their pharmacological effects and physiochemical properties discussed. The metabolism of these drugs is described relating to the occurrence and persistence of residues in biological matrices, providing information for selection of suitable matrices and target residues for testing. Methods for determination of benzimidazoles are reviewed for a range of biological matrices. The importance of selecting suitable extraction and clean-up procedures is discussed, along with the difficulties encountered in adapting single residue methods to multi-residue methods. The importance of suitable detection systems for determination of benzimidazoles, namely, screening, HPLC, GC and confirmatory methods is described in detail. The future for benzimidazole residue analysis is discussed, focusing on selection of appropriate residues for screening methods and protocols for confirmation of benzimidazole residues.
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Keywords: Benzimidazole residues; Animal tissues; SPE; HPLC; LC–MS/MS

Contents

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1. Introduction

Benzimidazoles are veterinary drugs 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 fruit and vegetables. Thiabendazole (TBZ) was the first benzimidazole to be marketed over 40 years ago [1]. It has been used widely for control of gastrointestinal nematodes, lungworms and as a fungicidal agent. After its introduction, a number of alternative benzimidazoles offering similar activity came on the market, such as parbendazole (PAR) [2] cambendazole (CAM) [3] mebendazole (MBZ) [4] and oxibendazole (OXI) [5].

Benzimidazoles possessing sulphide and sulphoxide functional groups were subsequently introduced, offering a wider spectrum of activity and improved efficacy. Albendazole (ABZ) [6], fenbendazole (FBZ) [7] and oxfendazole (OFZ) [8] were the first such benzimidazoles to be successfully used in the treatment of all growth stages of gastrointestinal nematodes. They may be

used also in the treatment of lungworms, tapeworms and adult stages of liver fluke. The benzimidazole, triclabendazole (TCB) was later introduced as an anthelmintic agent for treatment of all stages of liver fluke, but it is ineffective against nematodes [9]. Luxabendazole (LUX) is another benzimidazole-sulphide used in the treatment of food-producing animals but is not licensed for use in the EU [10]. The low solubility of benzimidazole sulphides and sulphoxides leads to their low absorption from the gut, resulting in low bioavailability [11,12]. Netobimin (NETO) [13] and febantel (FEB) [14], which are the pro-drugs of ABZ and FBZ, respectively, have greater water solubility resulting in improved absorption and increased bioavailability. Similar pro-benzimidazoles have found widespread use as fungicidal agents, including benomyl (BEN) and thiophanate-methyl (TM), which are precursors of carbendazim (MBC).

In this paper, a comprehensive review of methodologies for the determination of benzimidazole residues in biological matrices is presented. Firstly, an introduction is given on benzimidazole residues in relation to their mode of action, activity and toxicity. The metabolism and pharmacokinetics of these substances is then presented and discussed in relation to MRLs, and the selection of appropiate residues to monitor the presence of residues in food. Methodology for determination of benzimidazole residues in food is subsequently discussed in terms of sample handling, analysis, residues included and sensitivity. The paper concludes with comments on method validation, a general summary and notes on future perspectives.

2. Mode of action, biological activity and toxicity

2.1. Mode of action

A number of different modes of action have been proposed for benzimidazole drugs. ABZ has been shown to block glucose uptake in the larval and adult stages of susceptible parasites, by depleting their glycogen reserves and thereby decreasing ATP formation [15]. Blocking of glucose uptake was shown to be a mechanism of action for MBZ and TBZ [16]. A number of researchers have shown that benzimidazoles inhibit the enzyme fumaric acid reductase in the parasite, thus blocking the formation of succinic acid [16–18]. Consequently, this disturbs the normal operation of the dicarboxylic acid cycle, which replaces the Krebs cycle in anaerobic parasites [19,20]. The dicarboxylic acid cycle starts by fixing CO_2 on pyruvate, or preferably on phosphoenolpyruvate, to form oxaloacetate, and proceeds via malate and fumarate to form succinate as an end-product. Therefore, this cycle runs in anaerobic parasites in an opposite direction to the Krebs cycle, and its final electron receiver is fumarate. It is also coupled with a special electron transport system particular to anaerobic parasites for production of ATP. By blocking the cycle at the fumarate level, TBZ deprives the parasites of a significant source of energy, thus ultimately causing their paralysis and death [21,22].

Inhibition of microtubule formation has been identified as the primary mode of action of benzimidazole drugs by a number of researchers [15,23,24]. Many of the processes proposed as targets for benzimidazoles are dependent on the integrity of the microtubule matrix. Microtubules are associated with the following cellullar functions—formation of the mitotic spindle in cell division, maintenance of cell shape, cell motility, cellular secretion, nutrient absorption and intracellular transport. In view of the crucial roles microtubules play in many cellular processes, their drug-induced destruction eventually leads to the death of the organism. The dynamics of microtubule formation has been described in a number of papers, which should be consulted for more detail [25–28]. Inhibition of microtubule formation has been investigated in detail with the classical antimitotic agent colchicine showing that this compound binds to tubulin prior to its polymerisation [29]. Inhibition of microtubule assembly appears to be achieved through addition of colchicine-bearing tubulin to the end of a growing microtubule and loss of the ability of these subunits to accept other tubulin molecules for further microtubule growth. Data obtained from fluorescence spectroscopy and labelling experiments have indicated that the colchicine binding site is on the β-tubulin monomer, obviously close to the α,β subunit interface [30,31]. Other spectroscopic analyses suggest that inhibition of tubulin polymerisation is due to a drug-induced local unfolding of a small region within the β-tubulin monomer [29]. A similar mechanism may be responsible for inhibition of tubulin polymerisation by benzimidazoles, as the binding site of these compounds appears also to be located on the β-tubulin monomer [32–34].

The reason for selective effect of benzimidazoles towards parasites and high safety in host species is not well understood. Friedman and Platzer [35] demonstrated that the binding of FBZ and MBZ was 250- to 400-fold greater in parasite tubulin than mammalian tubulin. This provided the first evidence that the species selectivity of the benzimidazoles may derive from differences in their affinities for host and parasite tubulin. Kohler and Bachmann [36] showed in subsequent work that selectivity was more likely achieved by differential drug pharmacokinetics in the host and parasite. Russell et al. [37] found that the rate of dissociation of MBZ from parasite tubulin was much lower than the rate for dissociation of MBZ from mammalian tubulin. Therefore, it would appear that the selective toxicity of benzimidazoles may be due to differences in the strength of binding in parasite and host tubulin.

2.2. Activity

A summary of the activity of different benzimidazole drugs in the major food-producing animals is presented in this section. More detailed reports of drug activity are described elsewhere [11,38]. At the recommended dosage rates in cattle, TBZ (66 mg/kg bw) and OXI (10 mg/kg bw) are effective only against adult and developing larval stages of the common gastrointestinal (G.I.) roundworms. FEB shows additional activity against lungworms. ABZ-SO_2, FBZ and OFZ have broader spectra of activity which include adult larvae. ABZ-SO and OFZ are also effective against tapeworms. The spectra of activity are extended further to include adult fluke for NETO and ABZ although the dosages have to be increased to 20 and 10 mg/kg, respectively, for efficacy against this parasitic disease. TCB is ineffective

against nematodes and cestodes but has excellent activity against all stages of the fluke (a trematode).

2.2.1. Sheep

OXI is effective against adult and developing larval stages of the common G.I. roundworms. TBZ and MBZ have additional activity against lungworms, although TBZ has to be given at a higher dose rate for treatment of G.I. roundworms and lungworm. At a higher dose rate, TBZ is also effective against developing G.I. roundworms. MBZ is also active against tapeworms. ABZ-SO, FEB, FBZ and OFZ are effective against all stages of G.I. roundworms, they are also effective against lungworms and tapeworms. NETO and ABZ have similar activity to this group but are also effective against adult stages of fluke if given at an increased dose rate. TCB is effective against all stages of fluke.

2.2.2. Goats

MBZ and FBZ are effective against all the major G.I. roundworms, lungworms and tapeworms in goats.

2.2.3. Horses

All benzimidazoles and probenzimidazoles licensed for use in horses are effective against adult G.I. roundworms, although TBZ needs to be given at an increased dose rate for efficacy against *P. equorum*. TBZ, OXI and FBZ are effective against G.I. roundworms if given at an increased dose. FBZ and OFZ are effective against migrating G.I. roundworm larva, although FBZ must be given at a much higher dose rate.

2.2.4. Pigs

OXI, flubendazole (FLU) and FEB are effective against G.I. roundworms in pigs. FLU, FEB and FBZ are also effective against lungworm. FBZ should be given at a higher dose rate for lungworms if treatment is being carried out on a single occasion.

2.2.5. Poultry

CAM is effective against larval and adult stages of G.I. roundworms in pigeons at a dose rate of 75 mg/kg given on two consecutive days. In pheasants, partridges and waterfowl, MBZ is effective against gapeworm and G.I. roundworms. FLU offers the broadest spectrum of activity of benzimidazoles in poultry and game birds, showing activity against G.I. roundworms, gapeworms and tapeworms.

2.3. Toxicity

McKellar and Scott [11] have reviewed the toxicity of benzimidazole veterinary drugs in detail. Excerpt from this review are summarised in this section. Benzimidazoles are regarded as safe up to 20 to 30 times the recommended dose. Acute toxicity is difficult to induce with these drugs and LD_{50} values are almost impossible to define for drugs such as TBZ and FBZ. Reports of acute toxicity of benzimidazoles in animals are very limited. The main toxic effect of the benzimidazole compounds involves their teratogenic effect, which was first reported for PAR [39]. Congenital malformations resulting from administration of benzimidazole anthelmintics during gestation in ewes have been observed with CAM [40], OFZ [41] and ABZ [42]. However, FEB [43], FBZ [44], MBZ [45] and OXI [46,47] do not appear to exert a teratogenic effect in sheep when administered in early gestation. Polyploidy was observed in cultured hamster ovary cells incubated with OXI and, as a result, OXI has been restricted to use in weaning piglets or at batch formation [48]. In breeding poultry, treatment with FLU in feed caused transient diarrhoea but there was no effect on egg production, fertility and hatchability [49]. In general, if these drugs are to be used in early pregnancy, it must be for good reason and at the lowest recommended doses. Seiler observed anaemia in dogs after prolonged treatment with TBZ [50]. In calves, treatment with cambendazole caused pulmonary oedema and necrotic lymphadenopathy, which was fatal in some cases [51].

Of particular note is the fact that some benzimidazole metabolites are more toxic than the parent drug; for example, hydroxymebendazole (MBZ-OH) has been found to be more embryotoxic than MBZ, in rat [52]. In rat, OFZ is teratogenic at about one-half the highest reported no-effect level for FBZ [52].

3. Important physical and chemical properties of benzimidazoles

The benzimidazoles are the largest chemical family used to treat endoparasitic disease in domestic animals. This group includes thiabendazole analogues and benzimidazole carbamates; substitution of various side chain and radicals on the parent benzimidazole nucleus produces the individual members. Newer benzimidazole carbamates are characterised by novel substituents on the benzimidazole nucleus and replacement of the thiazole ring by methylcarbamate. Such modifications have given rise to a new generation of benzimidazoles with much slower rates of elimination, higher potencies and broader activity spectra.

Much information about the physical and chemical properties of benzimidazoles can be found in reference books, from chemical suppliers and by simple calculations. However, information relating to important properties such as octanol–water partition coefficients and pK_a values are not readily available. Some researchers have referenced pK_a values in published papers for determination of benzimidazole residues in biological matrices, but information regarding determination of pK_a values (either by experimentation or computer software) is generally not quoted [12,53–56]. These properties, pK_a values in particular, provide important information regarding solubility and ion exchange properties of residues. It is known that benzimidazoles possess an imidazole ring containing both acidic and basic nitrogen atoms. Under suitable conditions, the molecule may be protonated (pK_a ~5–6) or deprotonated (pK_a ~12), as shown in Fig. 1 [57].

The pK_a values of a limited number of benzimidazoles have been quoted in the literature but mostly only one pK_a value, is listed when two or more usually exist. A summary of the pK_a values and octanol–water partition coefficients (K_{ow}) are listed in Table 1 (N = acidic or basic nitrogen groups and

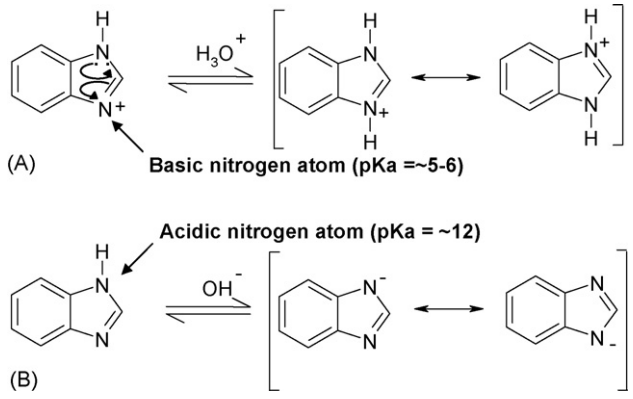


Fig. 1. Ionisation of the benzimidazole molecule under acidic (A) and basic (B) conditions.

OH = hydroxyl groups) [57]. Experimental (from literature) and calculated (using computer software) values are quoted for each benzimidazole listed, where available. Each benzimidazole in Table 1 has pK_a values listed relating to acidic and basic nitrogen atoms on the imidazole ring. Other benzimidazoles, namely CAM, TBZ, TCB, FLU reduced metabolite (FLU-RMET) and hydroxy mebendazole (MBZ-OH), are more complex molecules possessing more than two ionisable groups. FLU-RMET and MBZ-OH possess acidic hydroxyl groups, with pK_a values of 13.1 and 13.8, respectively. This has important consequences for extraction and clean-up of 5-hydroxy thiabendazole (5-OH-TBZ) residues from biological matrices. A common approach is to carry out aqueous extraction of residues under acidic or, more commonly, alkaline conditions, before adjusting the pH and partitioning into ethyl acetate. Unfortunately, because 5-OH-TBZ is ionised over the complete pH range, it is readily soluble in most acidic or basic solutions and, therefore, difficult to extract into ethyl acetate. In the case of CAM, TCB and TCB metabolites, two or more pK_a values are indicated for each substance. Again, for these molecules it can be seen that the pH of the extraction solvent needs careful manipulation. Triclabendazole (TCB), triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO_2) and CAM are neutral in the ranges of pH 7.3–10.9, 5.0–9.0, 3.8–8.0 and 7.4–11.1, respectively. Therefore, the only pH range that would give non-ionised forms of these molecules is 7.4–8.0. The problem gets more complicated when the other

Table 1
Listing of important physical and chemical properties of benzimidazoles

Benzimidazole	CAS number	Octanol–water partition coefficient		pK_a values[a]			pH range at which substance is in neutral state
		Experimental	Calculated[b]	Experimental pK_a	Calculated		
					Basic groups	Acidic groups	
ABZ	54965-21-8		2.2–2.92		N: 5.54	N: 13.11	7.7–11.2
ABZ-SO	54029-12-8		0.82–0.94	7.8	N: 5.69	N: 13.25	7.6–11.2
ABZ-SO_2			0.9–1.01		N: 3.50	N: 11.20	5.5–9.2
ABZ-NH_2-SO_2	80983-34-2		0.69–0.75		N: 5.98	N: 13.30	7.9–11.3
FBZ	43210-67-9	1.95	3.07–4.01		N: 5.12	N: 12.72	7.1–9.8
OFZ	53716-50-0		1.88–2.13		N: 4.13	N: 11.79	6.1–9.8
FBZ-SO_2			2.13–3.30		N: 3.41	N: 11.12	5.4–9.2
FLU	31430-15-6		1.98–2.41		N: 4.10	N: 11.76	6.1–9.8
FLU-HMET			1.72		N: 6.58	N: 13.87	6.5–11.9
FLU-RMET			1.94		N: 5.57	N: 13.14 OH: 13.68	7.6–11.2
MBZ	31431-39-7		2.44–2.52	3.5	N: 4.13	N: 11.79	6.1–9.8
MBZ-OH			2.22–2.61	9.8	N: 5.60	N: 13.16 OH: 13.77	7.6–11.2
MBZ-NH_2			1.84–2.27	5.5	N: 6.61	N: 13.89	8.6–11.9
OXI	20559-55-1		1.86–2.63		N: 6.26	N: 13.78	8.2–11.8
TBZ	148-79-8	5.3–6.2	1.58–1.76	2.5,4.7	N: 5.22	N: 12.83	7.2–10.9
5-OH-TBZ			1.29–1.37	4.5	N: 7.65	Phenol: 5.46 N: 15.10	Always in ionised state
TCB	68786-66-3		4.90–6.66	10.5, 2.5	N: 5.31 N: 1.82	N: 12.91	7.3–11.0
TCB-SO			3.39–3.66		N: 3.00 N: 1.82	N: 10.95	5.0–9.0
TCB-SO_2			3.58–5.14		N: 1.86 N: 1.82	N: 9.99	3.8–8.0
CAM	26097-80-3		1.81–1.91		N: 5.44 N: −4.62	N: 13.04 N: 15.97	7.4–13.1
MBC	10605-21-7		1.29–1.69	9.52	N: 5.52	N: 13.09	7.5–11.1
Benzimidazole		1.5	0.8–0.95	4.36, 16.4	N: 5.30	N: 12.90	7.3–10.9
PAR	14255-87-9		1.86–2.63		N: 5.99	N: 13.53	7.9–11.6

N, ionisable nitrogen atom. OH, ionisable hydroxyl group.

[a] pK_a values calculated using Pallas Software Version 3.0 (CompuDrug International Inc., Grandview Drive, South San Francisco, CA 94080, USA).

[b] Octanol–water partition coefficient calculated using ChemDraw Ultra Software Version 6.0.1 (CambridgeSoft, 100 Cambridge Park Drive, MA 02140, USA).

benzimidazoles are included; predicted pK_a values indicate that at a pH value of 8.0 most molecules are in their non-ionised state. However, a few exceptions exist, such as amino mebendazole (MBZ-NH_2) and OXI which exist in a non-ionised form at pH values of >8.6 and 8.3, respectively.

The octanol–water partition coefficient, K_{ow}, although not generally quoted in the scientific literature, is also a very important property. It can provide an indication of the solubility of these residues in different solvents and give an indication of the elution conditions necessary for liquid chromatography. Most of these molecules have similar K_{ow} values, which are generally in the range 0.8–3.3. However, FBZ and the TCBs residues appear to be much more lipophilic in nature than the other benzimidazoles; these properties appear to be in good agreement with the polarity of the molecules, as indicated by chromatographic elution order in reversed phase LC systems.

4. Factors affecting the occurence of benzimidazoles in tissues of animal origin

4.1. Route of administration

Benzimidazoles are typically administered as an oral or intraruminal dose or in feed. The bioavailability of benzimidazoles depends greatly upon the route of adminstration and the drug in question. After administration of intraruminal doses of FBZ and ABZ to sheep, improved bioavailability of the drug was observed compared to oral doses [58]. Earlier work proposed that a portion of the oral dose can on occasion bypass by the rumen and rapidly enter the abomasum by closure of the oesophageal groove, reducing bioavailability [59]. In the case of NETO, the drug rapidly cleared from the plasma 12 h post-treatment, when administered parentally in sheep and cattle [60–62]. It was found that over 94% of the dose was excreted in urine as parent drug, with ABZ metabolites making up the remainder of residues [60]. This metabolism profile was reversed after intra-ruminal administration at the same dose [60]. In contrast to the other pro-benzimidazole drugs, FEB and NETO are not transformed by liver microsomal enzymes. NETO is converted in the gastro-intestinal tract, leading to greater bioavailability of the active form of the drug after intraruminal administration [63]. Group administration entails the inclusion of drugs in feed supplements and drinking water. Results show that there is great variation between farms in rate of consumption of feed blocks and between sheep on the same farm in their plasma levels of the drug [64,65].

4.2. Physiochemical properties of the drug

Drug particles must dissolve in the enteric fluids to facilitate absorption of the benzimidazole molecule through the GI mucosa. The dissolution rate influences the rate and extent of its absorption (systemic bioavailability), its maximal plasma concentration and its subsequent distribution and disposition kinetics. Drugs such as TBZ and CAM, the most hydrosoluble moeities among the benzimidazole anthelmintics, are extensively dissolved in aqueous ruminal fluid and rapidly absorbed reaching maximal plasma concentrations as early as 4 h post administration to cattle [11]. Newer benzimidazole compounds show limited GI absorption due to their poor solubility in water, resulting in extended residence times for active metabolites of the substituted benzimidazole compound compared with those of TBZ or CAM [66,67]. Prichard et al. [67] showed that maximal plasma concentrations of TBZ in cattle occurs 4 h post treatment, compared with 24 h for FBZ, and that FBZ persists much longer than does TBZ in plasma and the GI tract. Consistently, TBZ and its metabolites are recovered in urine much more rapidly than are FBZ and its metabolites. This difference in pharmacokinetics in many ways accounts for the greater anthelmintic potency of FBZ compared with TBZ. Similar slow absorption of MBZ has been observed and residues can be found for 15 and 30 days post-treatment [68]. Different distribution patterns among benzimidazole sulphides (FBZ, ABZ), sulphoxides (OFZ, ABZ-SO) and sulphone (FBZ-SO_2, ABZ-SO_2) metabolites, based on their differential lipophilicities, may be expected. The distribution rate depends on molecular weight, lipid solubility, and plasma protein binding of each drug metabolite. The majority of benzimidazole compounds show a binding of less than 50% to plasma protein, relatively high volume of distribution, and a relatively fast elimination rate [11]. In contrast, TCB is strongly bound to plasma proteins, especially albumin which reduces its distribution in the body and increases its elimination half-life [69,70].

4.3. Animal species

On administration of either NETO prodrug or ABZ, ABZ-SO and ABZ-SO_2 are the major metabolites recovered in plasma of both sheep and cattle. However, pronounced differences in the plasma disposition and in the bioavailability of these metabolites between sheep and cattle have been shown [60–62,71,72]. Studies have shown the ratio of plasma area under curve (AUC) for ABZ-SO_2/ABZ-SO obtained in cattle to be higher in comparison to sheep, after treatment with NETO [60–62]. In addition, the elimination half-life and mean residence of the ABZ-SO metabolite were significantly longer in sheep compared to cattle [73]. The bioavailability of OFZ and its metabolites after oral administration was significantly lower in goats than in sheep [74,75]. However, the intravenous administration of OFZ in both species resulted in similar AUC values. In addition, FBZ was more rapidly cleared from plasma in goats than in cattle, and the detection of the sulphone metabolite [FBZ-SO_2] in plasma was delayed in cattle compared to goats [76,77].

4.4. Animal husbandry

Animal husbandry factors can have major effects on the disposition of benzimidazole drugs. Prichard et al. [78] found that the passage of digesta could influence the kinetics of orally administered benzimidazole drugs via binding of benzimidazoles to dietary fibre, modifiying bioavailability. Hennessy et al. [79] demonstrated that an increased rate of digesta passage through increased feed intake reduced bioavailability of OFZ and its metabolites in sheep. Lanusse et al. [73] proposed that

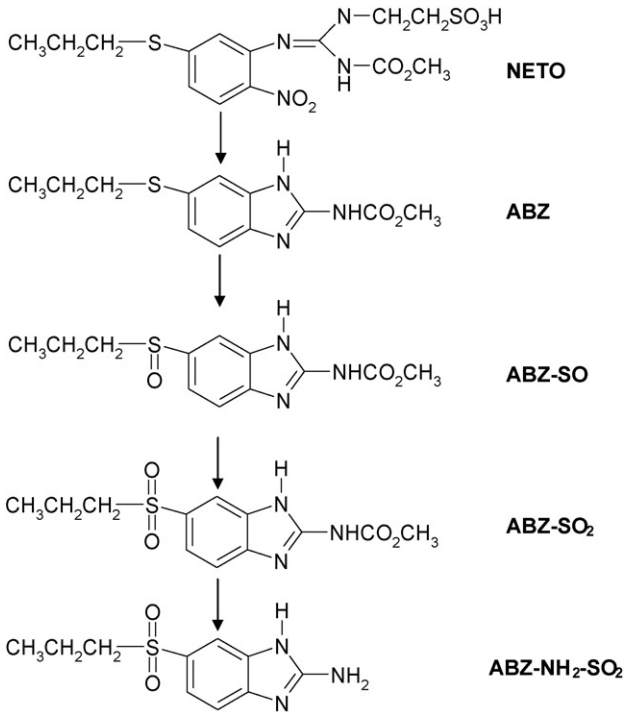


Fig. 2. Major metabolites of netobimin and albendazole related drugs in tissue and milk.

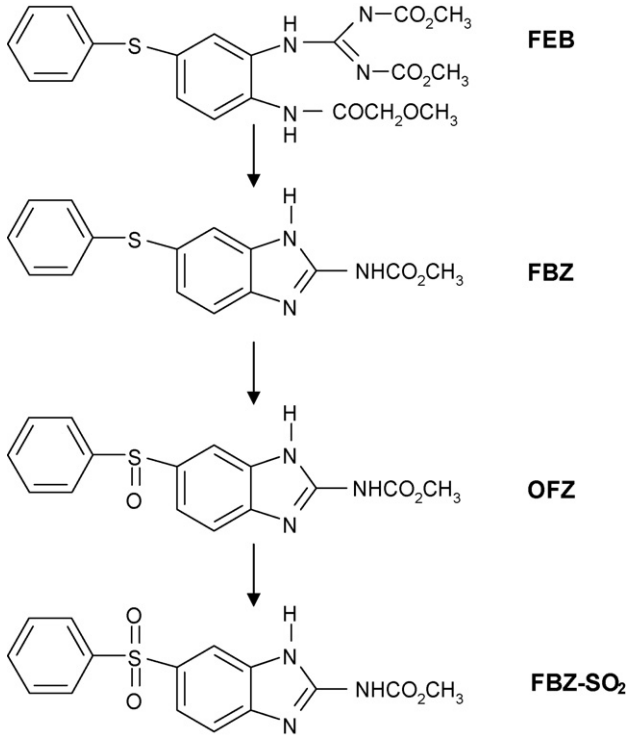


Fig. 3. Major metabolites of febantel and fenbendazole related drugs in tissue and milk.

bacterial population in microflora can play an important role on biotransformation of benzimidazole drugs in the gasrointestinal tract. In particular, bacteria-mediated reduction of benzimidazole sulphoxides (OFZ and ABZ-SO) back to the parent drug (FBZ and ABZ), extending residence time of the active drug in the animal's body. Therefore, changes in microflora populations and variations in ruminal pH, based on dietary modifications, may affect the reductive activity of the GI fluids. This could lead to differences in pharmacokinetic behaviour and clinical efficacy between animals fed on grain-based diets and those reared exclusively on pasture.

5. Metabolism and distribution of benzimidazole residues in tissues

5.1. Metabolism of benzimidazoles

It has been found in animal studies that metabolism of these drugs is extensive (Figs. 2–7), with one or more major metabolites found in animal tissue or milk for each drug [11,52,80–82]. The metabolites found depend on the structure of the parent drug, the tissue and animal species. The drugs ABZ, FBZ, and TCB possess a sulphide linkage, which is susceptible to oxidation. As a result, two metabolites of the parent sulphide are possible, a sulphoxide (SO) and a sulphone (SO_2). MBZ and FLU possess a carbonyl group that may be reduced to form a hydroxy group. ABZ, FBZ, MBZ and FLU possess carbamate groups that may be hydrolysed to form an amino-benzimidazole. TBZ may be oxidised, resulting in the formation of 5-OH-TBZ. A 4-hydroxy metabolite of TCB and a keto metabolite have also been observed in rat, goat and sheep [18]. For other drugs, such as CAM, LUX, OXI and PAR, little information has been reported. The structures of these drugs, along with some benzimidazole fungicides, are shown in Figs. 8 and 9. In the case of the pro-drugs FEB and NETO, they are converted into their benzimidazole form after dosing. FEB is rapidly converted into FBZ and does not persist as a major residue. NETO is rapidly converted into ABZ with only low levels of NETO residues occurring in tissue.

5.2. Albendazole, albendazole sulphoxide and netobimin

In cattle treated with single oral doses of radiolabelled ABZ, it was shown that residues mainly occur in the liver followed by the kidney with lower levels of residues detected in other tissues [83]. In calves, 90% of residues were extractable at 1 day after treatment with an oral dose of radiolabelled ABZ [83]. However, 4–10 days post-treatment only 20–30% of residues were extractable. The parent drug and its marker metabolites (sulphoxide, sulphone and amino-sulphone) accounted for 27

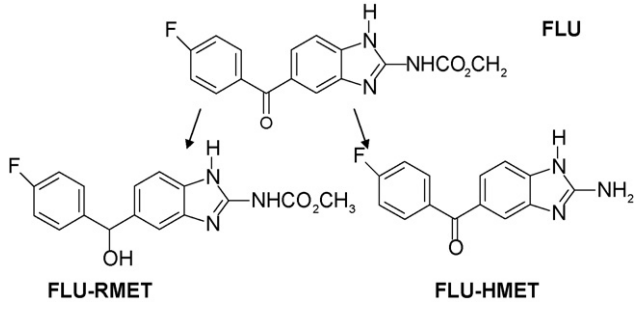


Fig. 4. Major metabolites of flubendazole in tissue and eggs.

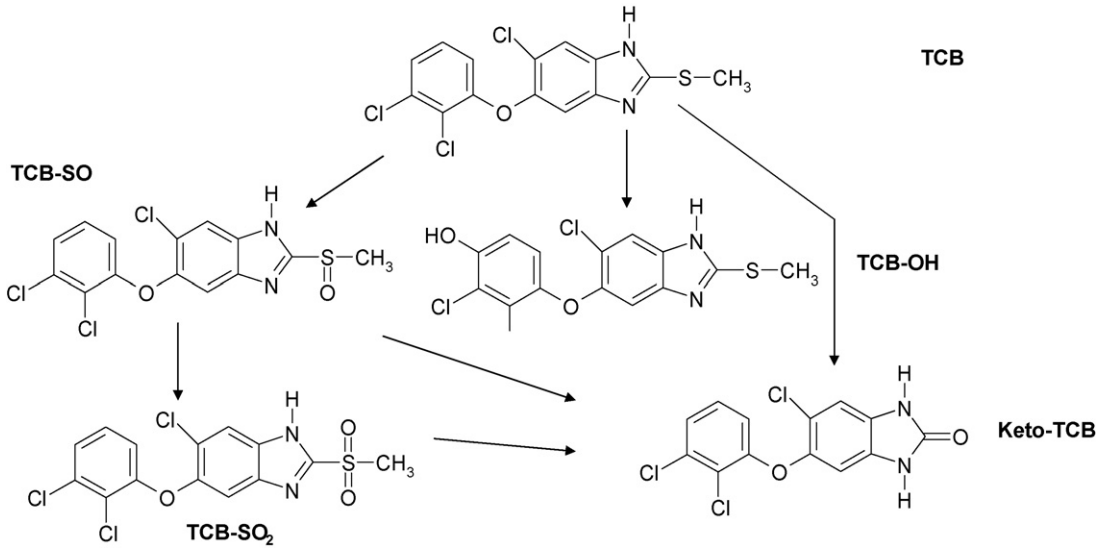


Fig. 5. Major metabolites of triclabendazole in rats, goats and sheep.

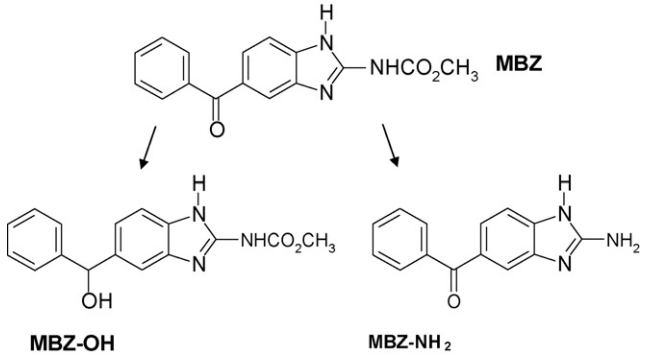


Fig. 6. Major metabolites of mebendazole in tissue.

and 52% of total extractable residues at 1 day post-treatment, respectively. At 4 days post-treatment, ABZ was not detected in tissues and marker metabolites accounted for 40–50% of the total extractable residues. In sheep treated with a single oral dose of ABZ, 100% of residues were extractable at 1 day post-treatment. The distribution of residues in tissues was similar to that in cattle. However, only 37 and 13% of residues were extractable at 4 and 8 days post-treatment, respectively. In sheep treated with an intra-ruminal dose of ABZ for 7 and 14 days, it was shown that the marker residues accounted for 80–100, 52–58 and 47–74% of total extractable residues in muscle, liver and kidney at zero days post-treatment, respectively [83]. In sheep treated with an oral dose of ABZ-SO, it was shown that ABZ-SO_2 was the major metabolite detected at 1 day post-treatment in liver and kidney [84]. A similar residue depletion profile was observed in poultry and pheasant [84]. In cattle and sheep treated with oral doses of NETO, ABZ was the major residue detected at 18 h post-treatment [85]. ABZ-SO and ABZ-NH_2-SO_2 were the major residues found at 3 days post-treatment [85]. Depletion studies of ABZ in trout, tilapia and salmon up to 120 h after treatment showed that ABZ-SO and ABZ-NH_2-SO_2 were the major residues found in trout and tilapia muscle and skin tissue. In salmon ABZ and ABZ-SO were the major residues found [86].

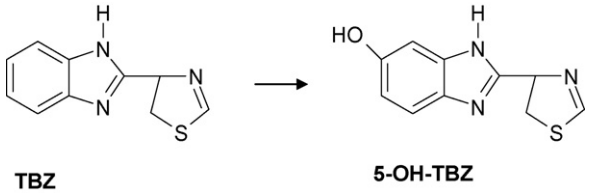


Fig. 7. Major metabolites of thiabendazole in tissue and milk.

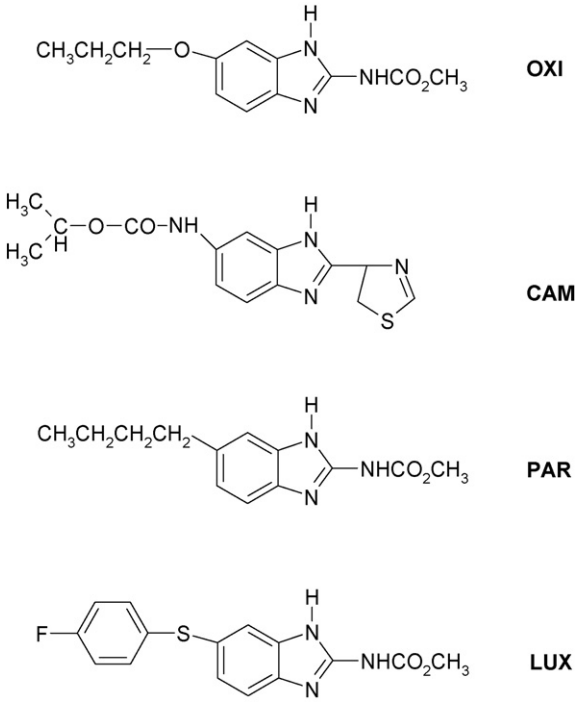


Fig. 8. Structures of oxibendazole, cambendazole, parabendazole and luxbendazole.

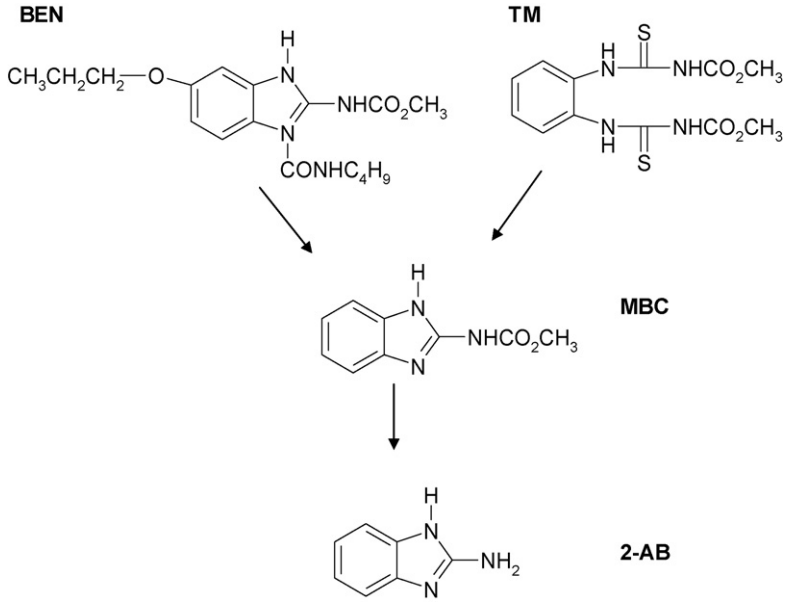


Fig. 9. Structures of some benzimidazole fungicides and their breakdown products.

5.3. Febantel, fenbendazole and oxfendazole

Studies on animals treated with FEB, FBZ and OFZ have shown that FBZ, OFZ and FBZ-SO_2 were the major residues [25–29]. Similarly to ABZ, FBZ and related drug residues are mainly found in the liver and kidney, with lower levels found in the muscle and fat tissues. In cattle treated with an oral dose of FEB, 90% of residues were readily extractable 18 h post treatment [87]. At this time-point, FBZ, OFZ, FBZ-SO_2 and FEB accounted for 30–41, 4–19, 14–15 and 3–6% of total extractable residues. At 10 days post-treatment, <25% of residues were readily extractable from liver. The major residue found at 10 days post-treatment was the FBZ amino-sulphone (FBZ-SO_2-NH_2), which accounted for 12–35% of total residues. In sheep treated with an oral dose of FEB, residues were shown to occur mainly in liver, with much lower levels of residues being found in other tissues [88]. Separate studies in cattle and sheep treated with oral doses of FBZ reported approximately 20 and 50 times higher residue levels, respectively in liver than in the next highest residue containing tissue [89]. Blanchflower et al. [90] found that OFZ was the major residue found in the liver of sheep after administration of an oral dose of FBZ (5 mg/kg bw); FBZ was also detected but at lower levels. In pigs treated with an oral dose of FEB (5 mg/kg bw), residue levels in liver were 10 times higher in liver than in other edible tissues [88]. Capece et al. [91] investigated the distribution of oxidised FBZ residues in tissues after treatment of pigs with FBZ in medicated feed (5 mg/kg bw per day for 5 days). The study showed that highest residue levels were detected in the liver with lower levels found in kidney, followed by fat and muscle [91]. Sorensen and Hansen [92] investigated the distribution of FBZ and metabolites in the muscle and skin of trout after administration in medicated feed (1 g/kg feed for 1 week). The study showed that FBZ and OFZ residues were slightly higher in skin compared to muscle tissue, with lower levels of FBZ-SO_2 found.

5.4. Flubendazole

In pigs treated with FLU in medicated feed (1.2 mg/kg bw per day for 5 days), 71, 80, 90 and 89% of residues were shown to be readily extracted from liver, kidney, muscle and fat tissues, respectively [93]. At 5–30 days post-treatment, approximately 50% of residues were readily extractable from liver and kidney. At 6 h post-treatment, the hydrolysed metabolite of FLU (FLU-HMET) was the major residue found and accounted for 47, 94, 94 and 31% of total extracted residues in liver, kidney, muscle and fat tissues, respectively. At 10 days post-treatment, FLU-HMET accounted for 18 and 23% of residues in liver and kidney. In laying hens treated with FLU (2.7 mg/kg bw per day for 7 days), it was shown that 76–79% of residues were extractable at 24 h post-treatment from muscle, skin and fat tissues [93]. At this time-point, 49 and 61% of residues were extractable from kidney and liver tissues, respectively. At later time-points, 30–35% of residues were extractable from liver and kidney. Residues were distributed mainly in the liver and kidney with much lower levels being found in muscle, skin and fat. It was reported that the parent drug accounted for <3% of total extracted residues in liver and kidney tissues at 24 h post-treatment. The majority of residues comprised of FLU-HMET and FLU-RMET (the reduced metabolite of FLU). De Ruyck et al. [94] investigated the fate of FLU residues in turkey after administration of FLU in medicated feed (1.2 mg/kg bw for 7 days). They found that FLU-HMET was the major residue present in turkey breast muscle with lower levels of the parent drug being present. De Ruyck et al. investigated the fate of FLU residues in the tissues of guinea fowl in a separate study, finding that FLU-RMET was the major residue present in liver and muscle tissues [95]. FLU was also detected with lower levels of FLU-HMET present. It was shown that 80% of FLU are readily extractable from the eggs of laying hens treated with this drug. FLU was the major residue found in eggs, accounting for 40% of total residues at 1 and 9 days post-

treatment [93]. A study carried out by Kan et al. [96] supported the findings of this study.

5.5. Mebendazole

In sheep treated with an oral dose of mebendazole (15 mg/kg bw), 74, 95, 92 and 99% of residues were extractable from liver, muscle, kidney and fat tissues at 1 day post-treatment, respectively [97]. At 7 and 14 days post-treatment, 87 and 74–78% of residues were extractable from liver and kidney, respectively. The parent drug, accounted for 8, 3, 20 and 30% of total residues in liver, muscle, kidney and fat, at 1 day post-treatment. At the same time-point, MBZ-OH accounted for 47, 90, 14 and 67% of total residues in liver, muscle, kidney and fat, respectively. At 3 days post-treatment, MBZ and MBZ-OH accounted for 2 and 3.5% of total residues. De Ruyck et al. [98] found a similar distribution pattern of residues in the tissues of sheep treated with an oral dose of MBZ (20 mg/kg bw). The fate of MBZ residues in goat was found to be similar to that of sheep [97]. In horse, it was shown that the major residue found in edible tissues is MBZ-NH_2, with much lower levels of MBZ and MBZ-OH detected [97]. Residues were found at highest levels in the liver and kidney, with lower levels found in muscle and fat. Iosifidou et al. [99] investigated the fate of MBZ in farmed eel, finding that residues mainly occurred in the liver and kidney with lower levels found in skin and muscle. MBZ-NH_2 was the major residue found with lower levels of MBZ and MBZ-OH present.

5.6. Thiabendazole

In calves treated with an oral dose of TBZ, it was found that residues mainly occurred in the liver and kidney [100]. At 1 day post-treatment, residues were found to occur mainly in the kidney with lower levels found in liver. At later withdrawal periods (>2 days), residues were mainly found in the liver [100]. Chukwudebe et al. [101] investigated the fate of TBZ residues in goats treated with oral doses (120 mg per animal daily for 7 days). Residues were shown to occur mainly in the liver and kidney. The major residue found in tissue at 1 day post-treatment was TBZ, with lower levels of 5-OH-TBZ present. The same study showed that at 1 day post-treatment, 5-OH-TBZ was the major residue detected in the tissues of laying hen, with lower levels of TBZ found [101]. Residues occurred mainly in the kidney with lower levels found in the liver. Wilson et al. [102] investigated the fate of TBZ in cattle, sheep and pig liver. In sheep treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 7 and 10 days post-treatment. TBZ was the only residue found and in one animal at 10 days post-treatment. In pigs, treated with an oral dose (50 mg/kg bw), residues were not detected in liver at 7 and 10 days post-treatment. In cattle treated with an oral dose (50 mg/kg bw), TBZ was the major residue found with lower levels of 5-OH-TBZ. Coulet et al. [103] carried out an in vitro study of the metabolism of TBZ in the cultured hepatocytes of rats, rabbits, sheep, calves and pigs, finding 5-OH-TBZ to be the major metabolite in calf and pig hepatocytes. Several metabolites were found in sheep, consisting mainly of 5-OH-TBZ (40%) and three unknown metabolites representing 26, 25 and 15% of residues found.

5.7. Triclabendazole

In cattle and sheep treated with oral doses of TCB it was shown at early withdrawal periods (2 days) that residues occurred at higher levels in the liver and kidney, with lower levels found in muscle and fat [104]. At longer withdrawal periods (>28 days), it was found that similar levels of residues could be found in muscle, liver and kidney. The contribution of individual TCB metabolites to the marker residues in animal tissues has not been reported. Most studies report the depletion of total marker residues and not individual components.

5.8. Oxibendazole

There is very little information available on the metabolism of OXI, but studies indicate that the drug is significantly metabolised in the liver and kidney [47,105]. Two metabolites 5-OH-OXI and 6-OH-OXI have been identified in urine and tissues, but only accounted for 15–20% of the total residues (the remainder are unidentified) [105].

5.9. Cambendazole

Early studies on the drug found that it was rapidly metabolised producing at least 13 urinary metabolites [106,107]. The identification of marker residues in animal tissues is not well described because the drug is not marketed for use in food producing animals.

5.10. Benomyl

Gardiner et al. [108] investigated the elimination of benomyl (BEN) residues in animals, finding 5-OH benzimidazole carbamate to be the major metabolite, with lower levels of the 4-OH metabolite present.

5.11. Summary

In cattle and sheep treated with ABZ, ABZ-SO or NETO, it has been shown that ABZ-SO or ABZ-SO_2 are the most likely residues to be found in the case of an MRL violation. ABZ-NH_2-SO_2 is perhaps the most persistent residue in tissue but generally occurs at levels below the MRL. There is very little information available on the presence of individual FBZ residues in animal tissues, although Blanchflower et al. [90] found that OFZ was the major residue present in the tissues of sheep treated with FBZ. In cattle treated with FEB, FBZ was the major residue found at early withdrawal periods, with lower levels of OFZ and FBZ-SO_2 found.

FLU parent drug is the major residue found in the eggs of laying hens, while FLU-HMET is the major metabolite present in pig and turkey tissues. A recent study has shown that FLU-RMET is the major residue found in the tissue of guinea fowl, indicating that it should be listed as a marker residue. MBZ-OH

and MBZ-NH_2 are the major MBZ residues found in sheep and horse liver, respectively. In cattle, goats and sheep treated with TBZ, the parent drug was shown to be the major residue found in liver after treatment. The 5-OH-TBZ metabolite occurs only at low levels in these species, but was shown to be the major metabolite in poultry at early withdrawal periods. There is very little information available on the occurrence of individual TCB residues in tissues. However, pharmacokinetic data indicates that TCB-SO and TCB-SO_2 would be the most likely residues to be found. Very little data has been published on the metabolism and distribution of CAM and OXI residues in tissues.

6. Depletion of benzimidazole residues in tissues

6.1. Netobimin, albendazole and albendazole sulphoxide

In cattle treated with an oral dose of ABZ-SO (12 mg/kg bw), ABZ-SO marker residues were below the MRLs in liver and kidney at 2 days post-treatment [84]. At 1 day post-treatment, ABZ-SO_2 was detected in liver and kidney at levels of 2953 and 1355 μg/kg, respectively. ABZ-SO was detected at 1 day post-treatment in liver and kidney at levels of 294 and 233 μg/kg. In cattle treated with an oral dose of netobimin (20 mg/kg bw), marker residues were below the MRL at 3 days post-treatment [85]. At 10 h post-treatment, ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, were found at levels of 7354, 771, 5289 and 0 μg/kg in liver, respectively. These parent/metabolite residues were at levels of 3285, 467, 1879 and 0 μg/kg in kidney, 792, 1909, 1740 and 0 μg/kg in muscle; and 556, 195, 474 and 0 μg/kg in fat, respectively. ABZ residues were detectable at 1 day post-treatment with NETO but were not detected at 3 days. At 3 days post-treatment and thereafter, the only residue present was ABZ-NH_2-SO_2, which could be found at levels of 243, 213 and 81 μg/kg at 3, 7 and 14 days post-treatment. In sheep treated with an oral dose of NETO (20 mg/kg bw), marker residues were below the MRL at 3 days post-treatment [85]. At 18 h post-treatment, ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, were found at levels of 18,000; 3100; 4200 and 78 μg/kg in liver, respectively. At 18 h post-treatment, these residues were at levels of 7400, 2400, 110 and 110 μg/kg in kidney, 2200, 3400, 1100 and 0 μg/kg in muscle, and 303, 983, 555 and 0 μg/kg in fat, respectively. At 3 days post-treatment, ABZ-SO and ABZ-NH_2-SO_2 were found at levels of 150 and 770 μg/kg in liver tissues, respectively. At 3 days post-treatment, ABZ-NH_2-SO_2 was found in kidney at a level of 226 μg/kg. At 10 days post-treatment, ABZ-NH_2-SO_2 was the major residue found in liver and kidney at levels of 150 and 31 μg/kg, respectively.

In pheasant treated with ABZ-SO in medicated feed (17 mg/kg bw per day for 3 days), residues were found to be below the MRL at 1 day post-treatment [84]. At this time-point, combined ABZ-SO and ABZ-SO_2 residues were found at levels of 168, 479 and 366 μg/kg in muscle, liver and kidney tissues, respectively. Combined ABZ-SO and ABZ-SO_2 residues were more persistent in liver than other tissues and were found at levels of 90 and 45 μg/kg at 3 and 7 days post-treatment, respectively. ABZ-NH_2-SO_2 was only found in liver tissue and at levels of 24, 168 and 99 μg/kg at 1, 3 and 7 days post-treatment, respectively. Shaikh et al. investigated the depletion of ABZ and its metabolites in salmon, tilapia, trout after treatment with ABZ (oral dose 10 mg/kg bw) [86]. In salmon, ABZ and ABZ-SO were found in muscle tissue at levels of 22 and 39 μg/kg, respectively at 24 h post-treatment. ABZ-SO_2 and ABZ-NH_2-SO_2 were also present but at lower levels, typically less than 5 and 10 μg/kg, respectively. ABZ was found in tilapia at a level of 678 μg/kg at 8 h post-treatment but was not present at later time-points. ABZ-SO was found at levels of 39 and 31 μg/kg at 8 and 12 h post-treatment, respectively. ABZ-SO was generally at 7 μg/kg or less at 24 h. ABZ-SO_2 was at or below 7 μg/kg at all time-points. ABZ-NH_2-SO_2 was the more persistent residue, present at levels of 18, 100 and 53 μg/kg at 8, 72, and 120 h post-treatment, respectively. ABZ parent drug was not found in trout muscle. The major residues found were ABZ-SO (18–47 μg/kg) followed by ABZ-SO_2 (5–14 μg/kg) and ABZ-NH_2-SO_2 (3–10 μg/kg).

6.2. Febantel, fenbendazole and oxfendazole

In cattle treated with FBZ (oral dose 7.5 mg/kg bw), animals were sacrificed at 7 and 21 days post-treatment. Marker residues (FBZ, OFZ and FBZ-SO_2) were below the MRL in all tissues at 7 days post-treatment at levels of 5, 7, 8 and 194 μg/kg in fat, kidney, muscle and liver, respectively [89]. In sheep treated with FBZ (oral dose 10 mg/kg bw), animals were sacrificed at 5 and 9 days post-treatment [89]. At 9 days post-treatment, marker residues were below the MRL in all tissues, with the exception of liver. At this time, combined marker residues were present at levels of <5, <6, 6 and 745 μg/kg in kidney, fat, muscle and liver, respectively. At 5 days post-treatment, marker residues were found to be at levels of 79 and 3659 μg/kg in kidney and liver. In sheep treated with an oral dose of OFZ (5 mg/kg bw), animals were sacrificed at 10 and 24 days post-treatment [109]. Marker residues were below the MRLs at 10 days post-treatment. Highest residue levels (at 10 days post-treatment) were found in the liver at a level of 476 μg/kg. In sheep treated with an oral dose of FEB (5 mg/kg bw), animals were sacrificed at 3 and 7 days post-treatment [88]. Marker residues were above the MRL at 7 days post-treatment. At this time-point, marker residues were found at a level of 942 μg/kg in liver (nearly twice the MRL). At 3 days, marker residues were found at levels of 40, 4617, 199 and 133 μg/kg in muscle, liver, kidney and fat, respectively.

In pigs treated with an oral dose of FEB (5 mg/kg bw), animals were sacrificed at 12, 20 and 34 days post-treatment [88]. Marker residues were below the MRL at 34 days post-treatment. At 24 days post-treatment, marker residues were found at levels of 402, 6, 12 and 100 μg/kg in liver, muscle, kidney and fat, respectively. The marker residues were above the MRL in fat, which is 50 μg/kg. Blanchflower et al. [90] investigated the depletion of FBZ and OFZ in sheep treated with an oral dose of FBZ (5 mg/kg bw). Animals were sacrificed at 1, 2, 4, 7, 10 and 15 days post-treatment. It was shown in this study that marker residues were typically below the MRL of 500 μg/kg at 14 days post-treatment in liver. At 2 days post-treatment, OFZ and FBZ were both found at levels of approximately 3000 μg/kg. OFZ was shown to be a much more persistent residue in liver tissue than FBZ. Capece et al. [91] monitored the depletion of oxi-

dised FBZ residues in pigs after treatment with an oral dose of FBZ (5 mg/kg bw per day for 5 days). Marker residues were below the MRLs in all tissues at 2 days post-treatment. At 1 day post-treatment, marker residues were found in liver, kidney, muscle and fat, at levels of 5552, 917, 623 and 1423 μg/kg, respectively. Sorensen and Hansen [92] investigated the depletion of FBZ, OFZ and FBZ-SO_2 in trout after treatment with an oral dose of FBZ in medicated feed (1 g/kg feed for 1 week). At 1 day post-treatment, FBZ, OFZ and FBZ-SO_2 were found at levels of 56–389, <2–252 and <4–53 μg/kg, respectively. At 6 weeks post-treatment, only low concentrations of residues were reported.

6.3. Flubendazole

Pigs treated with FLU in medicated feed (1.2 mg/kg bw per day for 7 days), animals were sacrificed at 6 h and 10 days post-treatment [93]. Residue depletion data showed that FLU marker residues (FLU and FLU-HMET) were below the MRL at 10 days post-treatment. At 6 h post-treatment, FLU-HMET was found at levels of 1817, 2517, 246 and 66 μg/kg in liver, kidney, muscle and fat, respectively. FLU was found at levels of 39, 51, 30 and 62 μg/kg in liver, kidney, muscle and skin-fat, respectively. In turkeys treated with FLU in medicated feed (30 mg/kg feed per day for 7 days), birds were sacrificed at 6 h, 1 day, 3 days, 7 days and 9 days post-treatment [93]. Marker residues were found to be below the MRL at 1 day post-treatment. At 6 h post-treatment, FLU was found at levels of 64, 67, 18 and 60 μg/kg in liver, kidney, muscle and skin-fat, respectively. At 6 h post-treatment, FLU-HMET was found at levels of 29, 11, 0 and 0 μg/kg in liver, kidney, muscle and skin-fat, respectively. At 6 h post-treatment, FLU-RMET was found at levels of 200, 80, 42 and 32 μg/kg in liver, kidney, muscle and skin-fat, respectively. FLU and FLU-HMET are the marker residues for FLU in all species. De Ruyck et al. [94] monitored the depletion of FLU marker residues in turkeys (1.2 mg/kg bw per day for 7 days). At 1 day post-treatment, combined FLU and FLU-HMET residues were at levels of 102 and 120 μg/kg in thigh muscle, respectively. In liver, combined FLU and FLU-HMET residues were found at a level of 2334 μg/kg. At 2 days post-treatment, marker residues were below the MRL in all tissues.

In laying hens treated with FLU in medicated feed (3.6 mg/kg bw per day for 7 days) eggs were collected at 7 and 11 days post-treatment [93]. FLU levels in egg ranged between 118 and 230 μg/kg at 7 days post-treatment to 13 μg/kg at 11 days post-treatment. Kan et al. [96] investigated the depletion of FLU residues in eggs collected from laying hens treated with rations of medicated feed containing different concentrations of FLU (2.6, 9.4 and 27 mg/kg feed) for 21 days. The study showed that FLU residues in egg reached a maximum level of 150 μg/kg in whole egg during the treatment period after consuming the ration containing the highest levels of FLU. In pheasant treated with FLU in medicated feed (3.6 mg/kg bw per day for 7 days), birds were sacrificed at 6 h, 1 and 10 days post-treatment [93]. This group monitored the depletion of FLU only and not metabolites. At 6 h post-treatment, FLU was found at levels of 35, 58, 18.5 and 76 μg/kg in liver, kidney, muscle, and skin-fat, respectively. FLU was more persistent in skin-fat and could be found at levels of 29 and 12 μg/kg at 1 and 7 days post-treatment, respectively. De Ruyck et al. [95] investigated the depletion of FLU and it's metabolites in guinea fowl after administration in medicated feed (56 mg/kg feed for 7 days). The highest concentration in thigh muscle during the treatment period for FLU-RMET (Day 3) and FLU plus FLU-HMET (Day 4) was 312 and 114 μg/kg, respectively. At 1 day post-treatment, FLU-RMET and combined FLU plus FLU-HMET residues were detected in thigh muscle at levels of 81 and 50 μg/kg, respectively. In liver, highest levels of FLU, FLU-HMET and FLU-RMET during treatment were 108, 23 and 1043 μg/kg, respectively. At 1 day post-treatment, FLU, FLU-HMET and FLU-RMET were 17, 11 and 283 μg/kg in liver, respectively.

6.4. Mebendazole

In horse treated with an oral dose of MBZ (8.8 mg/kg bw), animals were sacrificed at 1 and 28 days post-treatment [97]. At 1 day post-treatment, MBZ-NH_2 was the major residue, which was found at concentrations of 5047, 5851, 497 and 156 μg/kg in liver, kidney, muscle and fat, respectively. At the same time-point, MBZ was found at levels of 728, 29, 16 and 57 μg/kg in liver, kidney, muscle and fat, respectively. MBZ-OH was found at levels of 293, 85, 84 and 60 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, MBZ-NH_2 was the only marker residue detected in liver and kidney, at levels of 182 and 23 μg/kg, respectively. In sheep treated with an oral dose of MBZ (20 mg/kg bw), animals were sacrificed at 1, 7, 14, 21 and 28 days post-treatment [97]. At 28 days post-treatment, marker residues (MBZ, MBZ-OH and MBZ-NH_2) were below the MRL in all tissues. At 1 day post-treatment, MBZ was at 1016, 1460, 21 and 343 μg/kg; and MBZ-OH was at 7582, 1531, 1783 and 758 μg/kg; and MBZ-NH_2 was at 18, 147, 0 and 0 μg/kg in liver, kidney, muscle and fat, respectively. At 7 days post-treatment, residues were only detectable in liver and kidney. MBZ, MBZ-OH and MBZ-NH_2 were determined in liver at levels of 56, 272 and 27 μg/kg, respectively. In kidney, MBZ, MBZ-OH and MBZ-NH_2 were determined at 27, 38 and 44 μg/kg, respectively. De Ruyck et al. [98] carried out a similar study in sheep at the same dosage. However, in this study residues were shown to be below the MRL in all tissues at 7 days post-treatment. In goats treated with an oral dose of MBZ (20 mg/kg bw), animals were sacrificed at 1 and 28 days post-treatment [97]. At 28 days post-treatment, marker residues were below the MRL in all tissues. In liver, MBZ-OH and MBZ-NH_2 were found at levels of 308 and 19 μg/kg, respectively. At 1 day post-treatment, highest residues levels were found in the liver, where MBZ, MBZ-OH and MBZ-NH_2 were present at levels of 1020, 7502 and 151 μg/kg, respectively.

6.5. Thiabendazole

A number of studies reporting the depletion of combined marker residues (TBZ and 5-OH-TBZ) have been described in different species after treatment with TBZ [100]. In sheep treated with an oral dose of TBZ (44 mg/kg bw), marker residues were

found in liver at levels of 4680, 326, 825 and <50 μg/kg at 1, 2, 3 and 4 days post-treatment, respectively [100]. In calves treated with an oral dose of TBZ (75 mg/kg bw), combined marker residues at 1, 2 and 6 days post-treatment were 472, 215 and 63 μg/kg in liver; 638, 97 and <50 μg/kg in kidney; 75, 50 and <28 μg/kg in muscle and 89, <50 and <50 μg/kg in fat [100]. Wilson et al. [102] reported the depletion data for individual TBZ marker residues in a study in sheep and cattle. In sheep treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 7 and 10 days post-treatment. TBZ was found in one animal at a level of 72 μg/kg at 10 days post-treatment. In cattle treated with an oral dose of TBZ (50 mg/kg bw), animals were sacrificed at 1 and 2 days post-treatment. Marker residues were below the MRL in the liver of cattle at 2 days post-treatment. TBZ was the major residue found at this withdrawal period at a level of 71 μg/kg. At 1 day post-treatment, TBZ and 5-OH-TBZ were found in one animal at levels of 677 and 259 μg/kg, respectively.

6.6. Triclabendazole

In cattle treated with an oral dose of TCB (12 mg/kg bw), animals were sacrificied at 2 and 42 days post-treatment [104]. At 2 days post-treatment, combined marker residues (TCB, TCB-SO) and TCB-SO_2) were found at levels of 5870, 4300, 1420 and 2470 μg/kg in liver, kidney, muscle and fat, respectively. At 42 days post-treatment, combined marker residues were found at levels of 80, 75, 100 and <60 μg/kg in liver, kidney, muscle and fat, respectively. In a second study, cattle treated with an oral dose of TCB (12 mg/kg bw) showed, at 2 days post-treatment, combined marker residues at levels of 3250, 3050, 875 and 1800 μg/kg in liver, kidney, muscle and fat, respectively [104]. At 28 days post-treatment, combined marker residues were found at levels of 52, 47, 23 and <3 μg/kg in liver, kidney, muscle and fat, respectively. In a study on cattle treated with an oral dose of a combination product (12 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the cattle were slaughtered at 1, 21 and 28 days post-treatment [104]. At 1 day post-treatment, marker residues were found at levels of 7050, 5800, 1400 and 5800 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at levels of 144, 102, 142 and <40 μg/kg in liver, liver, kidney, muscle and fat, respectively. In a similar study on sheep treated with an oral dose of a combination product (12 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the animals were sacrificed at 2 and 28 days post-treatment [104]. At 2 days post-treatment, TCB marker residues were found at levels of 3500, 3100, 1420 and 1350 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at levels of 127, 119, 95 and <29 μg/kg in liver, kidney, muscle and fat, respectively. In a study on sheep treated with an oral dose of a combination product (10 mg/kg bw (TCB) and 7.5 mg/kg bw (levamisole)), the animals were slaughtered at 1, 21 and 28 days post-treatment [104]. At 1 day post-treatment, TCB marker residues were found at levels of 7400, 6800, 2100 and 10,100 μg/kg in liver, kidney, muscle and fat, respectively. At 28 days post-treatment, TCB marker residues were found at levels of 238, 198, 321 and 23 μg/kg in liver, kidney, muscle and fat, respectively.

6.7. Summary

The marker residues of ABZ, ABZ-SO and netobimin are rapidly cleared from the edible tissues of cattle and sheep; and are typically below the MRL at 4 days post-treatment. Results of studies verify the suitability of the withdrawal periods that are specified in cattle (14 days) and sheep (3–10 days) [110]. FLU marker residues were shown to be below the MRLs in turkey and pigs at 1 and 10 days post treatment, respectively. The withdrawal period specificed for FLU marker residues in turkey and pig is 7 days. Two separate studies have been carried out in eggs, showing that FLU residues do not occur at levels above the MRL during or after treatment. As a result, a zero withdrawal period has been specified for FLU in eggs. Marker residues for FBZ and related drugs (OFZ and FEB) were shown to be below the MRL in the edible tissues of cattle, sheep and pig at 10, 7 and 2 days post-treatment, respectively. The withdrawal periods for FBZ related drugs in cattle, sheep and pigs range between 14–35, 7–21 and 3–35 days, respectively. MBZ and TCB have been identified as the most persistent benzimidazole drug in animal tissues. In horse and sheep, MBZ marker residues were below the MRL at 28 days post-treatment. TCB residues were shown to be below the MRL in cattle at 28 to 42 days (two separate studies) and above the MRL in sheep at 28 days post-treatment. As a result, withdrawal periods for MBZ and TCB are typically >14 and 28 days, respectively. TBZ is rapidly cleared from the edible tissues of cattle and sheep, to below the listed MRLs, at 6 and 4 days post-treatment, respectively. No withdrawal period has been specified for TBZ, which may be an indication that this drug has been withdrawn from the market. The depletion of others benzimidazole drugs, namely OXI and CAM, are not well described for the edible tissues of food-producing animals. The withdrawal period for OXI is 7 days in pig, indicating that it is one of the least persistent benzimidazole residues. CAM is not approved for treatment of food-producing species and detailed residue depletion studies have not been conducted.

7. Depletion of benzimidazole residues in milk

7.1. Netobimin, albendazole and albendazole sulphoxide

In dairy cows treated with NETO (oral dose 20 mg/kg bw), ABZ-SO residues were found at levels of 7374, 2278 and 210 μg/kg at 7.5, 22 and 31.5 h post-treatment, respectively. ABZ-SO_2 residues were found at levels of 2336, 3410 and 81 μg/kg at 7.5, 22 and 31.5 h post-treatment, respectively. Residues of NETO, ABZ and ABZ-NH_2-SO_2 were not detectable at any timepoint [85]. Fletouris et al. [111] investigated the depletion of ABZ, ABZ-SO and ABZ-SO_2 in the milk of dairy cows treated with ABZ (oral dose 15 mg/kg bw). At 12 h post-treatment, ABZ-SO and ABZ-SO_2 were found at levels of 163 and 931 μg/kg, respectively. At 24 h post-treatment, ABZ-SO and ABZ-SO_2 residues were found at levels of 800 and 853 μg/kg, respectively. ABZ-SO and ABZ-SO_2 residues were

below the MRL at 36 h post-treatment. Cinquina et al. [112] investigated the depletion of ABZ residues in the milk of sheep and goats treated with ABZ (oral dose 3.75 mg/kg bw). At 24 h post-treatment, ABZ-SO residues were found approximately at 1100 and 1700 μg/kg in goat and sheep milk, respectively. At 24 h post-treatment, ABZ-SO_2 residues was found at an approximate level of 500 μg/kg in goat and sheep milk. At 48 h post-treatment, ABZ-SO and ABZ-SO_2 were the major residues found in sheep milk. At 48 h post-treatment, ABZ-NH_2-SO_2 was the major residue found in goat milk, with lower levels of ABZ-SO_2 present. At 72 h post-treatment, residues were typically at less than 100 μg/kg in goat and sheep milk. De Liguoro et al. [113] investigated the depletion of ABZ marker residues in milk from sheep treated with ABZ (oral dose 12.5 mg/kg). ABZ residues were not detected in milk at any time-point. At 12 h post-treatment, ABZ-SO and ABZ-SO_2 were found at levels of 3896 and 902 μg/kg, respectively. At 48 h post-treatment, ABZ-SO and ABZ-SO_2 were found at levels of 62 and 106 μg/kg, respectively. ABZ-NH_2-SO_2 was only found at 36 h post-treatment and at a level of 89 μg/kg. Chu et al. [114] investigated the depletion of ABZ marker residues in cows milk after treatment with ABZ (oral dose 10 mg/kg bw). At 24 h post-treatment, combined marker residues were found at a level of 576 μg/kg. At 48 h post-treatment, combined marker residues were found at a level of 67 μg/kg.

Moreno et al. [115] compared the milk residue profiles of ABZ marker residues in dairy cows treated with ABZ oral (oral dose 5 mg/kg bw) and ABZ-SO aqueous injectable (3 mg/kg bw) formulations. At 12 h post-treatment, ABZ-SO and ABZ-SO_2 residues were found at levels of 280 and 860 μg/kg after oral administration, respectively. After administration by subcutaneous injection ABZ-SO and ABZ-SO_2 were at levels of 180 and 80 μg/kg, respectively. In both studies, residues were not detectable at 36 h post-treatment.

7.2. Febantel, fenbendazole and oxfendazole

Fletouris et al. [111] investigated the depletion of FBZ marker residues in the milk of a dairy cow treated with FBZ (oral dose 10 mg/kg bw). At 12 h post-treatment, FBZ, OFZ and FBZ-SO_2 residues were found at levels of 85, 196 and 48 μg/kg, respectively. Marker residues were shown to be below the MRL at 84 h post-treatment. In dairy cows treated with FEB (undescribed dosage), marker residues depleted from 268 to <5 μg/kg at 24–130 h post-treatment, respectively [89]. Residues were below the MRL at 106 h post-treatment. In sheep treated with FEB (undescribed dosage), marker residues depleted from 357 to <5 μg/kg at 10–130 h post-treatment, respectively [88]. Residues were below the MRL at 120 h post-treatment. Tai et al. [116] investigated the depletion of FBZ and OFZ residues in the milk of dairy cows treated with FBZ (oral dose 10 mg/kg bw). At 28 h post-treatment, FBZ and OFZ residues were found at levels of 105 and 441 μg/kg, respectively. OFZ residues were found to be more persistent in milk than FBZ residues and OFZ could be found at a level of 29 μg/kg at 76 h post-treatment. Moreno et al. [115] compared the milk residue profiles of OFZ marker residues in dairy cows treated with oral (5 mg/kg bw) and aqueous injectable (3 mg/kg bw) formulations. At 12 h post-treatment, OFZ, FBZ and FBZ-SO_2 residues were found at levels of 390, 90 and 70 μg/kg after oral administration, respectively. Residues were not detectable after 72 h post-treatment. After administration by subcutaneous injection OFZ and FBZ-SO_2 were the only residues detected. OFZ and FBZ-SO_2, reached maximum milk levels at 12 h (30 μg/kg) and 36 h (42 μg/kg), respectively. In both studies, residues were not detectable after 72 h post-treatment.

7.3. Thiabendazole

In cows treated with TBZ (oral dose 66 mg/kg bw), marker residues (TBZ and 5-OH-TBZ) were found at a level of 5175 μg/kg at 12 h post-treatment [100]. Residues were found to be present at a level of 45 μg/kg at 84 h post-treatment (below the MRL of 100 μg/kg). Tocco et al. [66] investigated the depletion of TBZ residues in the milk of dairy cows and goats treated with TBZ (oral dose 66 and 150 mg/kg bw, respectively). The sulfate conjugate of 5-OH-TBZ was found to be the major and most persistent residue found in milk. At 6 and 72 h post-treatment, 5-OH-TBZ was found in goat milk at levels of 39,000 and and 800 μg/kg, respectively. In cow's milk, TBZ was only found to be present at a level of 60 μg/kg at 12 h post-treatment. At 12 and 48 h post-treatment, 5-OH-TBZ was found at a level of 1860 and 80 μg/kg, respectively. Tai et al. [116] found that the sum of TBZ and 5-OH-TBZ residues were below the MRL of 100 mg/kg in cows milk at 48 h post-treatment (oral dose of 100 μg/kg bw).

7.4. Triclabendazole

Kinabo and Bogan [117] showed that TCB-SO and TCB-SO_2 could be detected up to 3 and 6 days in the milk of goats treated with an oral dose of TCB (12 mg/kg bw). TCB parent drug was not detectable at any timepoint. Highest residue levels of TCB-SO and TCB-SO_2 were found at 1 (160 μg/kg) and 3 (640 μg/kg) days post-treatment, respectively. Counotte et al. [118] investigated the depletion of TCB residues in the milk of cows treated with an oral dose of TCB (12 mg/kg bw). TCB-SO_2 was the major residue present with lower levels of TCB-SO determined. At 50 h post-treatment, TCB-SO_2 and TCB-SO were found at levels of 1415 and 100 μg/kg. Residues of TCB-SO and TCB-SO_2 could be detected up to 45 and 240 h post-treatment. The study showed that 1.5% of the administered dose was excreted in milk. Takeba et al. [119] carried out a similar study in cows treated with an oral dose of TCB (12 mg/kg bw). This group only reported residue data for the first 48 h post-treatment. At 48 h post-treatment, TCB, TCB-SO and TCB-SO_2 were found at levels of 6–35, 17–114 and 51–595 μg/kg, respectively.

7.5. Summary

In dairy cows, sheep and goats treated with ABZ, levels of marker residues in milk were typically below the MRL at 72 h post-treatment [83,111–113]. At early withdrawal periods, ABZ-SO was found to be the major residue, ABZ-SO_2 was

found to be the pre-dominant residue at later timepoints. The withdrawal periods for ABZ and related drugs in species producing milk for human consumption range between 36 and 72 h. In dairy cows treated with FBZ, OFZ and FEB, marker residues were shown to be below the MRL at 84, 72 and 120 h post-treatment, respectively [89,115,116]. The withdrawal periods for FBZ and related drugs in milk range between 2 and 7 days. In dairy cows treated with TBZ, marker residues were typically below the MRL at 84 h post-treatment. 5-OH-TBZ was found to be the major residue in cow and goat milk [66,100,116]. TCB is not licensed for use in lactating species. Kinabo and Bogan [117] found that TCB residues could be detected in goat milk for up to 6 days post-treatment. Counotte et al. [118] found that TCB residues could be detected in cow milk up to 10 days post-treatment. TCB-SO_2 was shown to be the most persistent residue in the milk of cows and goats.

8. Monitoring for benzimidazole residues in food

It has been shown that some twenty or so different benzimidazole residues could occur in animal tissue. However, it would be difficult to develop and maintain a robust residue method that would cover all of these residues. Such a method would probably require the application of expensive LC–MS/MS technology. An alternative approach would be to select the most likely residues of a particular benzimidazole drug that would give rise to a MRL violation or that would be most persistent in tissue (to identify unapproved use). It is important to be aware of the depletion pattern of benzimidazole residues in tissues before developing a residue method. Non-compliant benzimidazole residues may occur in tissues because products are administered to the wrong species, are administered at more than the recommended dose, withdrawal periods are not observed or because animals consume contaminated feed.

For most benzimidazoles, the marker residue is defined as the sum of the parent drug or/and its' major or most persistent metabolites. After administration of ABZ related drugs, the residues found depend on the drug used (ABZ, ABZ-SO or NETO), route of administration, target tissue or time that has elapsed since treatment. At early withdrawal periods, the most likely marker residues to be found are ABZ-SO and ABZ-SO_2, while at longer withdrawal periods the most prevalent residue is ABZ-NH_2-SO_2. As a result, the marker residue is defined as the sum of ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, expressed

Table 2
MRL listings for benzimidazole anthelmintic drugs

Drug	Marker residue	Animal species	MRL (μg/kg)	Target tissue
NETO, ABZ and ABZ-SO	Sum of ABZ-SO, ABZ-SO_2, and ABZ-NH_2-SO_2, expressed as ABZ	Bovine, ovine	100	Milk
			100	Muscle
			100	Fat
			1000	Liver
			500	Kidney
FEB, FBZ and OFZ	Sum of extractable residues which may be oxidised to FBZ-SO_2	Bovine, ovine	10	Milk
			50	Muscle
		Bovine, ovine, porcine and equidae	50	Fat
			500	Liver
			50	Kidney
FLU	Sum of FLU and FLU-HMET	Porcine, poultry and game birds	50	Muscle
			50	Skin and fat
			400	Liver
			300	Kidney
		Chicken	400	Eggs
TBZ	Sum of TBZ and 5-OH-TBZ	Bovine	100	Muscle
			100	Fat
			100	Liver
			100	Kidney
			100	Milk
OXI	OXI	Porcine	100	Muscle
			500	Skin and fat
			200	Liver
			100	Kidney
TCB	Sum of extractable residues that may be oxidised to ketotriclabendazole	Bovine and ovine	100	Muscle
			100	Liver
			100	Kidney
MBZ	Sum of MBZ, MBZ-OH and MBZ-NH_2 expressed as MBZ	Ovine, caprine and equidae	60	Muscle
			60	Fat
			400	Liver
			60	Kidney

as ABZ. The MRLs for ABZ related drugs have been set at 100 μg/kg for milk, muscle and fat, 500 μg/kg for kidney, and 1000 μg/kg for liver in bovine and ovine species (Table 2) [120]. Similarly, for FEB, FBZ and OFZ the marker residue is defined as the sum of FBZ, OFZ and FBZ SO_2, expressed as FBZ-SO_2. Data from residue depletion studies indicate that OFZ and FBZ-SO_2 are the most suitable residues to test for in tissues. The MRLs range between 10 μg/kg for milk and 500 μg/kg for liver, in the listed species.

The marker residue for FLU is defined as FLU parent drug in eggs and as the sum of FLU and FLU-HMET in other edible tissues of avian and porcine species. The MRLs range between 50 μg/kg for muscle and 400 μg/kg for eggs and liver. The marker residue for MBZ is defined at the sum of MBZ, MBZ-OH and MBZ-NH_2, expressed as MBZ. Residue depletion data indicate that MBZ-NH_2 or MBZ-OH are the most persistent residues and most likely contribute to an MRL violation. The MRLs in ovine, caprine and equine species are set at 60 μg/kg for muscle, fat and kidney and 400 μg/kg for liver. The marker residue for TBZ is defined as the sum of TBZ and 5-OH-TBZ. In edible tissues, the parent drug is the most likely residue to be found. While the sulfate conjugate of 5-OH-TBZ is the predominant metabolite in milk. The MRL for bovine tissues and milk is 100 μg/kg. The marker residue for TCB is defined as the sum of extractable residues (TCB, TCB-SO and TCB-SO_2) that may be derivatised to form ketotriclabendazole. Residue depletion studies in tissue have been published reporting the depletion of the sum of extractable residues derivatised to form ketotriclabendazole, but do not indicate the contribution of individual residues. Studies indicate that because TCB is not licensed for use in lactating species and it should be best monitored as its' most persistent residue, TCB-SO_2. The MRL for TCB is 100 μg/kg for muscle, liver and kidney. The marker residue for OXI is the parent drug and MRLs are set at 100 μg/kg for muscle and kidney, 200 μg/kg for liver, and 500 μg/kg for skin and fat. Other benzimidazoles such as CAM, LUX, PAR and BEN have no EU MRL listings because they are not licensed as veterinary drugs in food producing animals. It is recommended that target certain target residues (as indicated in Table 3) should be selected in the event that all marker residues of benzimidazole are not included in a method. In the event that a positive residue is detected, a method capable of detecting the complete range of marker residues of a drug should be applied.

9. Sample extraction and clean-up procedures

9.1. Sample pre-treatment

9.1.1. Incorporation of hydrolysis steps

Hydrolysis may be used as a pre-treatment step to release protein bound residues, drug conjugates or simply convert residues to a common structure. However, in the area of benzimidazole analysis most hydrolysis steps have been used to release conjugates. Tocco et al. [121] found that urinary metabolites of TBZ mainly occurred as glucuronide and sulfate conjugates of 5-OH-TBZ. Conjugates were released using enzymatic (β-glucoranidase or glucosulase) or acid hydrolysis (refluxing in 6N HCl (1 h)). Tocco et al. [66] later used similar procedures to deconjugate 5-OH-TBZ residues present in the milk of cows and goats treated with TBZ. Chukwudebe et al. [101] completed a more detailed study into the metabolism of TBZ in laying hens and lactating species, using enzymatic and acid hydrolysis, and found that conjugates were mainly present as sulfates. Vanden-Heuvel et al. [122] also deconjugated 5-OH-TBZ by enzymatic and acid hydrolysis in a study in bluegill sunfish. Kriedel et al. [123] deconjugated the glucuronide of MBZ-OH after digestion with β-glucuronidase. Gyurik et al. [124] used glucosulase to enzymatically hydrolyse ABZ metabolites in urine before detection by TLC. Short et al. [76] incubated urine samples from animals treated with FBZ with β-glucuronidase and sulfatase before extraction and determination by mass spectrometry, showing that the majority of residues present in urine were conjugated.

As a result of these studies, hydrolysis steps have been included in some residue methods. Markus and Sherma [125] subjected liver sample extracts to acid hydrolysis to allow conversion of ABZ metabolite residues to a common ABZ-NH_2-SO_2 residue. Arenas and Johnson [126] used enzymatic hydrolysis to deconjugate 5-OH-TBZ sulfate, in a method for determination of TBZ residues in milk. Coulet et al. [103] used basic hydrolysis to release protein bound residues of TBZ from tissue.

9.2. Extraction and clean-up of benzimidazole residues

Liquid extraction is a common approach used to extract benzimidazoles, usually with aqueous extraction at high pH with partitioning into an immiscible organic solvent [53,96,102].

Table 3
Identification of target analytes for monitoring residues of benzimidazoles in liver and milk

Drug	Target analytes	
	Liver	Milk
NETO, ABZ or ABZ-SO	ABZ-SO_2, and ABZ-NH_2-SO_2	ABZ-SO_2, and ABZ-NH_2-SO_2
FEB, FBZ or OFZ	FBZ, OFZ and FBZ-SO_2	OFZ and FBZ-SO_2
FLU	FLU and FLU-HMET	Not defined
TBZ	TBZ	5-OH-TBZ
OXI	OXI	Not defined
TCB	TCB-SO and TCB-SO_2	TCB-SO_2
MBZ	MBZ-OH, MBZ-NH_2	Not defined

However, polarity and pK_a can differ greatly between parent benzimidazoles and metabolites, not only making it difficult to develop a multi-residue method for different benzimidazole drugs, but also for individual drugs and their metabolites. This has been observed by many researchers developing methodology for determination of FBZ and its two metabolites OFZ and FBZ-SO_2 [127–131]. FBZ has differing physical properties to its two metabolites, making it difficult to extract and determine by LC [128,129,132,133]. Similar difficulties have been encountered with MBZ and its two metabolites MBZ-OH and MBZ-NH_2 [53]. MBZ and MBZ-NH_2 are basic in nature with pK_a values of 3.5 and 5.5, respectively. Therefore, only at pH levels >7.5 are both metabolites non-ionised. MBZ-OH possesses an acidic OH group with a pK_a of 9.8 which is non-ionised below pH 7.8. This indicates that only in the pH range 7.5–7.8, are all three residues non-ionised. Alternatively, extraction may be carried out using a more polar organic solvent without pH manipulation. Some researchers have extracted these drug residues using a single solvent system, such as acetonitrile [119,134], or binary water/organic solvent mixtures which would be around neutral pH [131,135–137]. Extraction with acidified extractants has only been used by a small number of researchers for methods requiring hydrolysis or digestion steps [101,125,136,137] or methods including only a limited number of residues [138].

9.2.1. Plasma, serum or other biological fluids

Benzimidazole residues may be extracted from biological fluids using water immiscible organic solvents with or without pH adjustment and analysed without further purification. Bogan and Marriner [139] adjusted the pH of plasma to pH 7.4 before extracting FBZ, OFZ and ABZ residues with diethyl ether. Bogan's procedure has been applied for isolation of benzimidazole residues from plasma and blood [140–142]. Other groups have developed alternative procedures, while substituting solvents such as chloroform [143,144] and ethyl acetate [145–147] for diethyl ether. Hoaksey et al. [148] extracted ABZ and ABZ-SO from plasma samples with dichloromethane after protein precipitation with acetonitrile. Alvinerie and Galtier [149] extracted TCB, TCB-SO and TCB-SO_2 directly from plasma without pH adjustment using a simple ethyl acetate extraction procedure. Galtier et al. [150] later applied this method for determination of TBZ and 5-OH-TBZ in plasma.

Allan et al. [151] simply diluted plasma samples in acid prior to application onto a C_{18} SPE cartridge. The SPE cartridge was washed with water (20 ml), methanol + water (40 + 50, 0.5 ml) and methanol (0.4 ml) prior to elution with 1.6 ml of methanol. Recovery of mebendazole and its metabolites was typically greater than 80%. The procedure was subsequently applied by Galtier et al. [150] and Behm et al. [152] to determine mebendazole in plasma. Hennessy et al. [70] subsequently modified this procedure and applied it to investigate the pharmacokinetics of TCB. Hennessy et al. also found the procedure to be suitable for determination of TCB in bile (after pH adjustment) but samples required liquid–liquid extraction (LLE) clean-up into ethyl acetate before SPE. This procedure was later applied by other researchers to determine ABZ related residues in plasma [60,153].

The use of automated purification systems has been shown to enhance reproducibility, improve throughput of samples and reduce manual handling. Rouan et al. [154] described an automated C_{18} clean-up procedure for isolation of TCB residues on an ASPEC system. They demonstrated, with a limited number of samples, that recovery values greater than 90% could be obtained under optimal conditions. Chiap et al. [155] developed an automated procedure based on dialysis clean-up for isolation of albendazole and its metabolites from plasma with on-line determination by HPLC through the use of switching valve technology. Recovery of ABZ residues from plasma was typically greater than 65%. Negro et al. [56] isolated TCB-SO and TCB-SO_2 from serum and urine samples using a combination of protein precipitation and ultra-filtration through a 30,000 molecular mass cut-off filter. This approach was shown to greatly reduce sample preparation time in comparison to conventional off-line clean-up procedures. Recovery of TCB marker residues from serum and urine were typically greater than 82%.

9.2.2. Tissue

Benzimidazole residues are typically extracted from animal tissues using organic solvent and purified by LLP and/or SPE. Some researchers have opted to carry out comprehensive purification of residues through LLP and SPE, usually with determination by HPLC-UV. Other groups have moved towards the development of simple clean-up procedures based on LLP only, with determination by more selective fluorescence and LC–MS/MS systems. These simpler clean-up procedures (although less selective) may be more suitable to allow for isolation of a broad range of benzimidazoles, which are difficult to recover using more comprehensive clean-up procedures. Brandon et al. [135] extracted TBZ residues from liver tissues using DMSO-H_2O (10:90, v/v) and diluted extracts in PBS Tween prior to determination by ELISA. Brandon et al. [156] developed a similar type of extraction procedure for determination of some 11 benzimidazole residues in liver tissues. To allow recovery of a range of benzimidazole residues including the poorly water soluble FBZ, samples had to be extracted in DMF-H_2O (10:90, v/v).

Originally procedures for tissue samples were based on multiple LLP and/or SPE clean-ups. Marti et al. [134] extracted eight benzimidazole residues from muscle, liver and kidney tissues using acetonitrile. Extracts were subsequently purified using a series of LLP steps which included washing with hexane, saturated NaCl and dichloromethane. Extracts were concentrated and further purified using a combination of C_{18} and Florisil SPE prior to analysis by HPLC-UV or confirmation by GC–MS. Recovery of benzimidazole residues was typically >65% for all residues with the exception of OFZ, which gave a lower recovery of 45 and 39% for liver and kidney, respectively. Farrington et al. [157] extracted five benzimidazole residues from liver tissue using ethyl acetate and purification on acid alumina and C_{18} SPE before determination by HPLC-UV. Recovery of benzimidazoles ranged between 73 and 90%. Blanchflower et al. [90] extracted FBZ and OFZ from liver and muscle tissues using methanol–water (20:7, v/v). Extracts were washed with light petroleum, diluted with phosphate buffer and extracted with

diethyl ether–ethyl acetate (60:40, v/v). Recovery of FBZ and OFZ was typically greater than 80 and 70%, respectively. de Buyanski et al. [158] developed a simple procedure for extraction of TBZ and levamisole from muscle tissue at alkaline pH using ethyl acetate with subsequent partitioning into 0.5 M HCl. The ethyl acetate layer was discarded before alkalisation of the aqueous phase and extraction with chloroform. Levan and Barnes [159] developed a labour intensive procedure for extraction of five benzimidazole residues from liver tissue using large volumes of organic solvent. Samples were purified using LLP steps prior to purification on silica SPE cartridges.

Wilson et al. [102] developed a simpler procedure based on ethyl acetate extraction for isolation of eight benzimidazole residues from muscle and liver tissues. Ethyl acetate was chosen as the extraction solvent because of its good solvating power for weakly basic drugs and its ability to form emulsion-free interfaces with muscle and liver tissues. Samples were subsequently purified using LLP (acidified ethanol versus hexane wash) and C_2 SPE clean-up prior to determination by HPLC-UV or GC–MS. This group concluded that C_2 was the most effective sorbent material for removing polar matrix interference while giving good recovery of benzimidazole residues in comparison to other SPE sorbent materials evaluated. Wilson's method is the most widely used method for isolation of benzimidazole residues from animal tissues. In some cases, researchers have reported modifications of this method, such as size of sample used, extraction pH and SPE cartridge clean-up.

A number of researchers have applied the Wilson method, modified to exclude the SPE clean-up. Domany and Kovacsics [160] found that the method could be extended to 10 benzimidazole residues in liver and muscle tissues, while excluding SPE clean-up. Shaikh et al. [86] found that ABZ marker residues could be determined in salmon, tilapia and trout without the need for SPE clean-up through the application of a more selective HPLC fluorescence detection system. They found that ABZ recovery increased after adding dimethyl sulphoxide (0.5 ml) to the extraction solvent (ethyl acetate, 5 ml), while extraction efficiency for ABZ marker residues did not vary greatly over the pH range from 9 to 11. It was also found that addition of sodium metabisulfite to the extraction medium inhibited the oxidation of ABZ-SO to ABZ-SO_2, resulting in increased recovery for ABZ-SO. Recovery of ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 was typically greater than 82, 78, 75 and 63%, respectively.

Hajee and Haagsma [53] extracted MBZ, MBZ-OH and MBZ-NH_2 from eel muscle with ethyl acetate after adjusting the pH to 7.5 with phosphate buffer. They concluded that only in the narrow pH range between 7.5 and 7.8 were all MBZ marker residues in a non-ionised state thereby facilitating their extraction using ethyl acetate. Ethyl acetate extracts were modified with hexane prior to application onto a preconditioned aminopropyl SPE cartridge. The authors evaluated a range of normal phase and reverse phase SPE systems but found aminopropyl to be most suitable. After loading of sample extract, the aminopropyl cartridge was washed with iso-octane and MBZ marker residues were eluted with methanol. Mean recovery of MBZ, MBZ-NH_2 and MBZ-OH was typically greater than 80, 60 and 85%, respectively. Cannavan et al. [161] developed a simple procedure for isolation of TBZ and 5-OH-TBZ from liver, kidney and muscle tissues based on an ethyl acetate extraction at pH 7.0, and SPE clean-up on cyanopropyl (CN) SPE cartridges. Deuterated TBZ was used as an internal standard to improve reproducibility. Recovery of TBZ and 5-OH-TBZ ranged between 96–103 and 70–85%, respectively. It was concluded that reproducibility of 5-OH-TBZ recovery could be improved through the introduction of a deuterated internal standard for 5-OH-TBZ.

Acetonitrile was found to be an effective solvent for extraction of benzimidazoles from animal tissues by some researchers. Sorensen and Hansen [92] extracted the marker residues of FBZ from the muscle and skin tissues of trout using acetonitrile. Extracts were washed with hexane prior to purification on C_{18} and CN SPE cartridges. Under acidic conditions benzimidazole residues may become ionised making them amenable for more selective ion exchange SPE clean-up. Rose extracted nine metabolites of OFZ from liver using acetonitrile prior to purification on strong cation exchange (SCX) SPE cartridges [133]. Acetonitrile extracts were modified using acetic acid and applied onto the SCX cartridge, which was subsequently washed with acetone, methanol and acetonitrile. The cartridge was eluted with acetonitrile containing 5% ammonia (5 ml). Recovery of benzimidazoles varied between 28 and 117%. Su et al. [162] extracted six benzimidazole residues from muscle and liver tissues using acetonitrile after adjusting the pH to 10. Dibutyl hydroxytoluene (BHT) was added to prevent oxidation of 5-OH-TBZ. Extracts were further purified on C_{18} SPE prior to determination by HPLC. Recovery ranged between 71 and 105%.

Polymeric sorbents have been successfully applied to isolate benzimidazole residues from animal tissue in some multi-residue methods. Roudaut and Garnier [163] extracted 10 benzimidazole residues from liver using the Wilson procedure, while substituting Oasis HLB for C_2 SPE cartridges. Sample extracts were loaded onto HLB cartridges in methanol–0.1 M ammonium acetate (50:50, v/v, 1.0 ml) and eluted with methanol (1 ml) before determination by HPLC-UV. They compared a number of other polymeric SPE cartridges including Isolute 101 and Nexus but found that Oasis HLB gave the cleanest sample extracts and most satisfactory recovery. Balizs loaded muscle extracts onto styrol-divinyl-benzene SPE cartridges in methanol–0.1 M ammonium acetate (50:50, v/v, 1.0 ml) and eluted them with methanol–ethyl acetate (1:4, v/v, 3 ml) before analysis by LC–MS/MS [164]. Balizs applied the method to isolate 15 benzimidazole residues from muscle tissues. Recovery of benzimidazole residues was typically in the range of 36–117%. A low recovery of 8% was obtained for FEB.

Capece et al. [91] extracted FBZ marker residues from muscle, fat and kidney samples using ethyl acetate after adjusting the pH to 8.0. Extracts were analysed by HPLC without further purification. De Ruyck et al. [94] extracted FLU, FLU-HMET and FLU-RMET from egg and muscle tissues with ethyl acetate at alkaline pH. Extracts were subsequently evaporated to dryness and purified by LLP (methanol versus hexane) before analysis by LC–MS/MS. Recovery of FLU marker residues was typically greater than 70% (eggs) and 87% (muscle). De Ruyck et al. [98] subsequently applied this method to the determina-

tion of MBZ marker residues in sheep liver. Mean recovery of MBZ marker residues was typically greater than 85%. Edder et al. [165] extracted 11 benzimidazoles and levamisole (LEV) from liver and muscle tissues at alkaline pH while using MBC as an internal standard. Extracts were further purified by LLP (methanol versus hexane), before determination by LC–MS/MS. Recovery of residues was typically greater than 70%.

Some novel procedures have been developed for isolation of benzimidazole residues from animal tissues. Fletouris et al. [166] isolated ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues from muscle, liver, kidney and fat tissues using a LLP procedure based on ion-pair extraction with octane sulphonate under acidic conditions. Extracts were further purified by partitioning between phosphate buffer pH 8.5 and ethyl acetate. However, this group concluded that the ion pair extraction was critical for purification of tissue extracts. Recovery of ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 was greater than 81, 88 and 76%, respectively. Techniques such as matrix solid phase dispersion (MSPD) [77,129,131,167–171] and supercritical fluid extraction [172] have been applied for isolation of benzimidazoles residues from liver. Long et al. [168] developed an MSPD method for isolation of five benzimidazole residues from liver tissue. Liver samples (0.5 g) were blended with C_{18} material and packed into a SPE column between two frits. The column was washed with hexane and eluted with acetonitrile prior to determination by HPLC-UV. Recovery of the benzimidazole residues ranged between 55 and 93%. Danaher et al. extracted 13 benzimidazoles from fortified liver samples using unmodified supercritical CO_2 (60 l, 690 bar and 80 °C) [172]. Benzimidazole residues were trapped off-line on a neutral alumina SPE cartridge and further purified on a SCX cartridge prior to determination by HPLC-UV. Mean recovery for 10 benzimidazoles ranged between 51 and 113%. TBZ, CAM and TCB-SO_2 residues were also extracted but results were not quantitative.

Some groups have implemented automated SPE clean-up procedures. Stubbings et al. [173] developed an automated on-line clean-up system for isolation of benzimidazole residues from liver and tissue samples. Benzimidazole sample extracts were purified on SCX cartridges prior to on-line clean-up on cation exchange trace enrichment cartridges. Dowling et al. [174] modified the method developed by Wilson and extended it to isolate 12 benzimidazole residues from liver tissue. Purification of samples was carried out on C_{18} SPE cartridges using an automated SPE system (ASPEC^{TM}). They found that this automated system allowed unattended clean-up of samples and enhanced reproducibility of assay results. Mean recovery of residues was typically in the range of 60–100%. Lower recovery of 25% was observed for ABZ-NH_2-SO_2. HPLC-UV chromatograms of negative control and fortified liver samples are presented in Fig. 10.

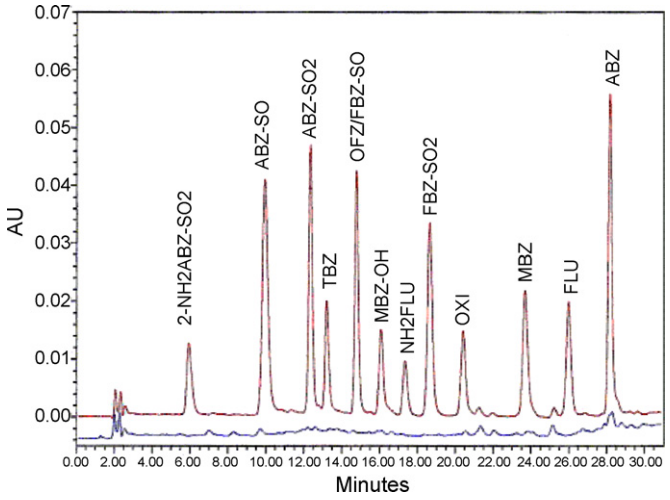


Fig. 10. Chromatogram of blank liver sample and liver sample fortified at MRLs (Reprinted from reference [174], with permission from Elsevier, Copyright 2005).

9.2.3. Milk and milk products

Milk samples are generally easier to manipulate than corresponding solid tissue samples. In addition, milk contains less pigments and matrix interence components that typically occur in tissue. Important purification issues that need to be overcome with milk samples include removal of proteins and fat through introduction of precipitation and defatting steps. A number of groups have simply adjusted the pH of samples prior to extraction with an immiscible solvent such as ethyl acetate, chloroform or dichloromethane. The required pH adjustment may depend on the type or number of drug residues included in a particular method. Tocco et al. [66] found that TBZ and 5-OH-TBZ could be successfully extracted from milk samples at pH 6.0 with ethyl acetate. Fletouris et al. [175] showed that ABZ, ABZ-SO and ABZ-SO_2 were best extracted at pH 9.8 with ethyl acetate, giving recovery values of 81, 78 and 100%, respectively. Fletouris et al. [111] later extracted 10 benzimidazole residues from milk samples at pH 10 with ethyl acetate. They evaluated extraction efficiency over the range pH 3–11.5, finding pH 10 gave best recovery for the majority of benzimidazole residues selected. Mean recovery for benzimidazole residues was typically in the range of 79–100%. A lower recovery in the range of 56–66% was obtained for FBZ. Fletouris et al. [128] developed an alternative acetonitrile based extraction procedure that gave recovery near to 100% for FBZ over the pH range 2–9, indicating pH adjustment of milk samples was unnecessary with acetonitrile. They later used a similar extraction procedure for determination of ABZ residues in cheese [176], and FBZ metabolites in milk [128]. It was found that solvent extraction with ethyl acetate extraction gave dirtier extracts for cheese samples than acetonitrile, while dichloromethane gave low recovery. Samples (0.5 g) were extracted with acetonitrile (3 ml), defatted with hexane, evaporated to dryness and further purified by LLP (ethyl acetate versus phosphate buffer pH 8.5). Recovery of ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 were typically greater than 83, 80 and 74%, respectively. De Ruyck et al. [177] later scaled this method up (larger volmes of solvent and sample) and applied it for extraction of four benzimidazole residues from milk. Mean recovery of FBZ, TBZ, ABZ and OXI was typically between 68 and 77%. De Ruyck et al. [178] subsequently developed a procedure to isolate eight benzimidazoles and levamisole from milk samples at alkaline pH using ethyl acetate with determination by LC–MS/MS. Recov-

ery of benzimidazole residues was typically in the range of 79–110%.

The amount of purification required to successfully isolate benzimidazole residues from milk samples may depend on the number of residues included and the detection system being used. Brandon et al. [179] simply diluted milk samples in phosphate buffer saline prior to direct determination by ELISA. They found that milk samples had to be diluted 10-fold to eliminate matrix effects and ensure good agreement between standards prepared in buffer and fortified or incurred samples. Fletouris et al. [111] extracted 10 benzimidazoles from milk samples (1 ml) at pH 10 with ethyl acetate (6 ml). Samples extracts were subsequently evaporated to dryness and reconstituted in mobile phase before determination by HPLC. Other researchers have developed more labour intensive sample preparation procedures using multiple LLE/LLP/SPE steps that require larger quantities of organic solvent, glassware and cumbersome rotary evaporation steps [116,180]. Tai et al. [116] extracted four benzimidazole residues from milk at alkaline pH using large volumes of ethyl acetate (150 ml). Sample extracts were purified by LLP (phosphoric acid versus hexane wash) and back extracted into ethyl acetate after alkalisation. Sample extracts were further purified on silica SPE prior to determination of residues by HPLC-UV. Recovery of FBZ, OFZ, TBZ and 5-OH-TBZ was greater than 78, 86, 81 and 25%, respectively. They proposed that the poor recovery of 5-OH-TBZ might be due to degradation during the long extraction procedure. Constantinou et al. [180] developed a similarly labour intensive procedure for isolation of OFZ, FLU, ABZ and FBZ from milk based on LLE, with further purification on C_{18} SPE. Recovery of TBZ, OFZ, FLU, ABZ and FBZ were greater than 68, 63, 57, 24 and 29%, respectively.

Other researchers have developed simpler sample preparation procedures based on solvent extraction and SPE. Moreno et al. [115] deproteinised milk samples (0.5 ml) with acetonitrile (0.5 ml) and applied the supernatant layer onto a preconditioned C_{18} SPE cartridge, which was washed with water (0.5 ml) and eluted with methanol (2 ml). The method was suitable for determination of ABZ, FBZ and their metabolites. Mean recovery ranged between 77 and 97%. Sorensen and Petersen [181] mixed milk samples (5 ml) with phosphate buffer pH 11 (5 ml) and incubated the mixture in a water bath (45 °C, 20–30 min) prior to application onto a C_{18} SPE cartridge. The cartridge was washed with water (5 ml) and eluted with acetonitrile (6 ml). Samples were evaporated to dryness and further purified by LLP (phosphate buffer pH 9 versus dichloromethane extraction) before determination by HPLC-UV. Mean recovery of ABZ-SO, ABZ-SO_2, OFZ, OXI, ABZ, MBZ, FBZ and FEB was in the range 81–96%. Sorensen and Petersen [182] developed an alternative procedure for determination of TBZ and LEV in milk. Defatted milk samples (10 ml) were mixed with phosphate buffer pH 6.5 (10 ml) and incubated in a water bath (45 °C, 20–30 min.) prior to passing through a C_{18} SPE cartridge. The cartridge was washed with phosphate buffer (5 ml) and eluted with methanol (2 ml). Sample extracts were evaporated to dryness and further purified by LLP (phophate buffer pH 10 versus dichloromethane extraction) before determination by HPLC-UV. Mean recovery of TBZ and LEV was 81–89%, respectively.

A number of novel methods, including use of MSPD, have been developed to allow determination of benzimidazole residues in milk and milk products. Long et al. [129] mixed milk samples (0.5 ml) with C_{18} material (2 g) and packed the mixture between two filter paper discs in a 10 ml syringe barrel and compressed to a volume of 4.5 ml. The column was washed with hexane (8 ml), air-dried and eluted with dichloromethane–ethyl acetate (1:2, v/v, 8 ml). Recovery of the benzimidazole residues was typically in the range 81–108%. A slightly lower recovery of 69% was obtained for FBZ. De Liguoro et al. [113] extracted ABZ residues from milk, whey and whey solubles using acetonitrile, prior to defatting with hexane, LLP clean-up and C_{18} SPE. Recovery of ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 were typically greater than 78, 82, 79 and 81%, respectively. De Liguoro et al. [113] developed an MSPD procedure for determination of ABZ residues in cheese. Cheese samples (1 g) were mixed with C_{18} material and packed between two filter paper discs in a 10 ml syringe barrel and compressed to a volume of 7 ml. The column was washed with hexane (10 ml), air-dried and eluted with methanol–acetic acid (97:3, v/v). The eluate was defatted with hexane and further purified by C_{18} SPE prior to determination by HPLC. Recovery of ABZ metabolites were similar to those achieved by the procedure developed for milk (77–86%).

Residue scientists face similar problems when trying to determine benzimidazole residues in milk as in animal tissue. Development of methods that will cover the complete number of marker residues or most persistent or predominant metabolites is most difficult. As a result, a number of important benzimidazole residues are not included in multiresidue methods. TCB and it's metabolites have been included in few milk residue methods. De Ruyck et al. [178] developed a method that included TCB parent drug but not the more important TCB-SO_2 metabolite, which is the most persistent residue of TCB in milk. Some researchers have developed methods to isolate TCB and its metabolites from milk but methods do not include other benzimidazole drugs. Takeba et al. [119] extracted TCB, TCB-SO and TCB-SO_2 from milk using acetonitrile with subsequent defatting with hexane. The acetonitrile fraction was modified with carbonate buffer and extracted with dichloromethane prior to further purification on C_{18} SPE and determination by HPLC-UV. Mean recovery was in the range of 89–95%. Kinabo and Bogan [117] extracted TCB and it's metabolites from milk (4 ml) with acetone (4 ml). Acetone extracts were simply diluted with water and applied onto C_{18} SPE cartridges, which were washed with water (5 ml) and eluted with methanol (3 ml). Purified extracts were subsequently determined by HPLC fluoresence. Su et al. [162] demonstrated that their earlier method for the determination of benzimidazole residues in animal tissues was also applicable to milk samples with minor modification.

Furthermore, few multi-residue methods for determination of benzimidazole residues in milk include a deconjugation step. Inclusion of such a step is particularly important when testing for residues of TBZ in milk, which mainly occur as the sulfate conjugate of 5-OH-TBZ. Arenas and Johnson [126] subjected milk samples (5 ml) to acid hydrolysis in the presence of concentrated HCl (85–90 °C, 4 h) to free the sulphate conjugate of 5-OH-TBZ.

Hydrolysed extracts were neutralised with NaOH and adjusted to pH 8.0 before extraction with ethyl acetate. Ethyl acetate extracts were subsequently purified on PRS cation exchange cartridges prior to determination by HPLC fluorescence. Recovery of TBZ, 5-OH-TBZ and the sulphate conjugate of 5-OH-TBZ were typically greater than 87, 98 and 96%, respectively. Arenas and Johnson proposed enzymatic hydrolysis as an alternative to acid hydrolysis but required an overnight incubation. One factor to be considered when including an acid hydrolysis step is that benzimidazole carbamate residues (ABZs, FBZs, MBZs and FLUs) may be hydrolysed to amino benzimidazoles. Chu et al. [114] included an acid hydrolysis in a method for determination of ABZ metabolites in milk samples. Milk samples were subjected to acid hydrolysis (1 h, 100 °C), followed by purification on SCX SPE cartridges. In this procedure, acid hydrolysis was used to convert ABZ metabolites to a common residue (ABZ-NH_2-SO_2). The procedure should be suitable for determination of ABZ-SO_2, ABZ-NH_2-SO_2 and their conjugates as ABZ-NH_2-SO_2. However, the procedure was not demonstrated to be suitable to allow determination of ABZ, netobimin or ABZ-SO as ABZ-NH_2-SO_2. Chu et al. demonstrated that the method was suitable for determination of more than 40% of ABZ residues in cow's milk 36–120 h post-treatment. Recovery by the method was typically in the range of 91–105%.

9.2.4. Plant matrices

There are fewer benzimidazole compounds used in the treatment of crops compared to animal health. TBZ and thiophanate-methyl (TM) are used in crop protection and animal health, while BEN is used in crop protection only. The purpose of this section is to give an overview of methods for determination of BEN, TBZ, TM and their breakdown products in crops. Those interested in reading in more detail should consult the reviews by Gorbach [183] and Singh and Chiba [184]. It will be found throughout this section that the techniques used to isolate benzimidazole fungicides from crops are typical of those applied in a pesticide laboratory. In general, larger weights of sample and volumes of solvent are used in procedures for isolation of benzimidazoles from crops. Samples are typically purified by LLE/LLP steps, which require relatively large volume glassware and concentration of samples one at a time using rotary evaporation. In recent years, researchers in this area of testing have moved towards scaling down methods to reduce solvent usage and increase sample throughput.

The control of sample pH is critical to ensure satisfactory recovery of benzimidazole residues from crops. Benzimidazole residues have been extracted from certain crops with [185–187] or without [188–190] adjustment of sample pH. However, when extracting acidic samples such as citrus fruits, sample pH should be controlled to ensure a robust method [188–190]. A principle applied in most LLE/LLP procedures for isolation of benzimidazole residues is that they are highly soluble in dilute acid and less soluble in aqueous solutions at higher pH values (>7.0). This allows the washing or extraction of water immiscible organic extracts with basic or acidic aqueous solutions, respectively. In a typical ethyl acetate extraction, extracts may be washed with water or alkaline buffers (to remove polar interference) and extracted with 0.1N HCl (while non-polar matrix components remain in the ethyl acetate). The acidified extracts are subsequently adjusted to alkaline pH with NaOH and re-extracted with ethyl acetate, which is evaporated to dryness or reduced to a small volume prior to analysis. Alternative extractions have been developed by other researchers using solvents such as ethanol–dichloromethane [191] or hexane–ethyl acetate [192]. Corti et al. [193] extracted TBZ from samples using a saturated sodium chloride-dichloromethane system. Other researchers have extracted samples with more polar solvents such as methanol [191] or water [194], prior to dilution of sample extracts and determination by ELISA.

Extraction and clean-up procedures that include BEN, TM and their marker residue (MBC) are more complex than those used to isolate TBZ. The approach adopted in most methods is to convert the parent fungicides to MBC or 2-aminobenzimidazole (2-AB) through acid or basic hydrolysis prior to analysis. Acid hydrolysis converts BEN to MBC, while basic hydrolysis converts MBC to amino-benzimidazole. Pease and Gardiner [195] extracted BEN residues from fruit and vegetable matrices using ethyl acetate. Samples were subjected to acid and basic hydrolysis prior to LLP and determination by fluorescence spectrophotometry. Recovery of BEN from fortified fruit, vegetable, animal tissue and soil was typically greater than 76%. Pease and Holt [196] later improved this clean-up procedure through the introduction of alkaline wash steps to remove matrix components that interfered with fluorescent quenching, allowing the more effective determination of BEN in oranges.

Kirkland et al. [136] extracted BEN from soil and crops by refluxing in the presence of methanol and 1N HCl. Hydrolysed extracts were purified by a series of LLP steps prior to HPLC determination. The method allowed the determination of BEN and MBC (both as MBC), and 2-aminobenzimidazole. Recovery of the respective residues was 92, 88 and 71%, respectively. Physalo [197] later modified this clean-up procedure and used it to isolate MBC and 2-AB from crops, prior to determination by GC. Recovery ranged between 70 and 90%. Tjan and Jansen [186] extracted BEN, MBC and TBZ from crops with ethyl acetate–0.1N HCl. After adjustment of the acidified fraction to pH 7.5, residues were extracted with ethyl acetate, concentrated, derivatised and determined by GC. Recovery of BEN, MBC and TBZ were typically greater than 91, 93 and 95%, respectively. Farrow et al. [198] extracted BEN, MBC, TM, TBZ and some non-benzimidazole fungicides from citrus fruit by refluxing in 2N HCl. Sample extracts were washed with chloroform and adjusted to a high pH prior to extraction with chloroform. Recovery of BEN, MBC, TM and TBZ were typically greater than 70, 66, 32 and 84%, respectively.

Gilvydis and Walters [199] extracted five benzimidazole residues from crops using acidified methanol and partitioning into dichloromethane after dilution in NaCl solution, prior to HPLC determination. This fraction contained TM. The acidified fraction (containing the other benzimidazoles) was adjusted to pH 7.7–8.0 and extracted with dichloromethane. Recovery of BEN, MBC, TBZ and TM were typically greater than 78, 87, 81 and 63%, respectively. Corti et al. [200] extracted BEN, MBC and TBZ from fruit and vegetable samples using satu-

rated NaCl solution–chloroform. The chloroform extract was re-extracted with 0.1N HCl and incubated at 50 °C (30 min.). Acidified extracts were alkalised and extracted with chloroform prior to determination by HPLTC or HPLC. Recovery of BEN, MBC and TBZ was typically greater than 91, 93 and 94%, respectively.

The application of sorbent based clean-up for isolation of benzimidazole residues has become more popular in recent years. However, there was some application of sorbent clean-up in early methods, using either column chromatography or preparative TLC. Aharonson and Ben-Aziz [201] extracted BEN, MBC and TBZ from crops using acetone, with subsequent purification on magnesium oxide-celite-alumina columns prior to determination by TLC or fluorescence spectrophotometry. Recovery of BEN, MBC and TBZ were typically greater than 68, 60 and 70%, respectively. White and Kilgore [202] extracted BEN from different fruits with benzene and partitioned residues into 0.1N HCl, before converting the BEN to MBC at room temperature. Sample extracts were purified by LLP and preparative TLC prior to determination by UV spectrophotometry. Recovery of BEN was >87%. More recently, groups have developed procedures on commercial SPE materials. Oishi et al. [203] adjusted fruit juice samples to pH 8–9 before application onto an Extrelut^{TM} cartridge (supported LLE), which they subsequently eluted with hexane–ethyl acetate (3:1, v/v). This group found that the highest recovery was achieved at pH values >4. Recovery of TBZ ranged between 90 and 96%.

The development of chemically more sophisticated cation exchange and polymeric sorbent materials has resulted in improved procedures for isolation of benzimidazole fungicide residues from crops. Di Muccio et al. [204] extracted MBC and TBZ from crops using an aqueous-acetone mixture. A portion of the aqueous-acetone extract (20 ml) was dispersed over the diatomaceous earth material (Extrelut-20) packed into a SPE cartridge. Acetone was removed by passing nitrogen through the cartridge from bottom to top. Residues were subsequently eluted with 0.1 M phosphoric acid and isolated on a phenyl-SCX cartridge attached to the end of the Extrelut^{TM} cartridge, while leaving material poorly soluble in dilute phosphoric acid retained on the Extrelut^{TM} cartridge. The Extrelut^{TM} cartridge was disconnected and the SCX cartridge was washed with water, methanol and eluted with acetonitrile–methanol–ammonium formate buffer pH 6.8 (50:25:25, v/v/v). Arenas and Johnson [205] extracted TBZ from banana with ethyl acetate and applied extracts onto PRS-SCX cartridges. TBZ was eluted with 0.1 M KH_2PO_4 in acetonitrile and purified extracts were determined by HPLC with fluorescence detection. Recovery of TBZ ranged between 87 and 101%. Arenas et al. [206] later modified this procedure through the inclusion of sample pH control and applied it to determine TBZ in citrus fruits. Young et al. [207] developed two procedures for the isolation of TBZ and MBC residues from fruit juice samples using Oasis® mixed mode cation exchange (MCX) SPE cartridges. They found that isolation of TBZ and MBC residues from orange juice could be effectively achieved using MCX operating sequentially in SCX and reversed phase retention modes. Orange juice samples were loaded at pH 2 and catridges were washed with 0.1N HCl, methanol and high aqueous basic solutions, prior to elution with NH_4OH in methanol. In contrast, MCX operating sequentially in reversed phase and SCX modes provided a more effective clean-up for apple and grape juice samples. It was proposed that the apple and grape juice samples contained polyphenolic (acidic) interferences which were strongly retained after loading under acidic conditions. Young et al. [208] later applied this clean-up in an LC–MS assay, showing that the matrix effect from purified extracts had little or no ion suppression effects. Recovery of MBC and TBZ in both methods was typically greater than 74 and 90%, respectively. Zrostlíková et al. [209] isolated 17 crop protection agents including TBZ and MBC from fruit samples using Oasis® MCX cartridges. Capitan-Valvey et al. [210] developed a novel sorbent extraction procedure for the determination of TBZ in fruit. Samples were extracted using aqueous buffer before dilution of the extract in water and mixing with Sephadex G-15 gel beads. The Sephadex gel beads were isolated by filtration and packed into a glass cuvette, before determination using a luminescence spectrometer.

There have been some applications of automated extraction and clean-up procedures for isolation of benzimidazole residues from crops in recent years. Hiemstra et al. [54] developed an automated on-line clean-up procedure to isolate MBC and TBZ residues from crops. Samples were extracted using acetone and partitioned into dichloromethane–petroleum ether. Sample extracts were evaporated to dryness and reconstituted in methanol (2 ml) and an aliquot (1 ml) was applied onto a Diol SPE cartridge using an ASPEC^{TM} system. The cartridge was washed with methanol (1 ml) and eluted with methanol–0.1 M H_3PO_4 (2 ml) into a vial containing 1 M NaOH (100 μl). Recovery of MBC and TBZ ranged between 97 and 108%. Supercritical fluid extraction (SFE) has been used by some researchers to isolate benzimidazole residues from crops. Aharonson et al. [211] mixed samples (2 g) with hydromatrix (HMX) prior to extracting BEN, MBC, TM and TBZ residues using supercritical carbon dioxide modified with 3% methanol. Optimum extraction was achieved using 90 g of SF-CO_2 (400 atm, 60 °C). Residues were trapped off-line on a C_{18} trap maintained at 25 °C and desorbed with methanol (10 ml). It was found that recovery of residues was affected by the water content in samples but this variable could be controlled using a HMX to sample ratio of 1:1. At higher ratios of HMX recovery of residues was reduced and at ratios greater than 3:1 residues were not recovered. Mock and Scherbaum [212] extracted TBZ and other non-benzimidazole fungicides from fruit using unmodified SF-CO_2 (350 bar, 50 °C), with off-line trapping on a C_{18} trap maintained at 20 °C. Recovery of TBZ ranged between 63 and 70%. Anastassiades and Schwack [213] extracted MBC and TM from crops using unmodified SF-CO_2 (329 atm, 55 °C). Extracts were trapped off-line on a C_{18} trap maintained at 10 °C before desorption with acetonitrile (1.3 ml, 50 °C). This group investigated the influence of pH on the extraction of residues from fortified fruit juice samples, finding that sample pH had to be adjusted to pH 7 to ensure quantitative extraction of MBC. Mean recovery of MBC and TM ranged between 85 and 93%. Kakimoto et al. [214] extracted TBZ and other fungicides from fruit and vegetable samples and processed products using accelerated

solvent extraction (ASE). Samples were mixed with diatomaceous earth prior to extraction with acetonitrile or methanol (100 °C, 2000 psi) over a 20 min period. Mean recovery of TBZ ranged between 90 and 110%. Pawlowski and Poole [215] extracted MBC and TBZ from crops using subcritical water (40 ml, 75 °C, 50 bar). The pH of aqueous extracts was adjusted to pH 8–9 with 1 M NaOH before partitioning into ethyl acetate. Mean recovery of MBC and TBZ ranged between 80 and 101%.

Some methods have been described for isolation of benzimidazole veterinary residues from medicated feed. Shah et al. [216] extracted OFZ from medicated feeds using methanol–acetic acid (90:10, v/v) at 45 °C. Plant pigments and other feed excipients were removed using zinc acetate treatment and LLP prior to determination by HPLC-UV. Mean recovery of OFZ from fortified feed samples were at 98%. Botsoglou et al. [217] extracted ABZ residues from medicated feed using acetonitrile and washing with hexane. Recovery of ABZ was typically greater than 98%. This group later developed a method for the isolation of FBZ from medicated feed using acetonitrile before washing with iso-octane and determination by HPLC-UV [218]. Mean recovery of FBZ from fortified feed samples ranged between 95 and 100%.

9.3. Summary on sample preparation

The extraction and purification of benzimidazole residues from biological tissues offers a difficult challenge. However, some of these molecules possess common functional groups, which in some cases allows the selective isolation of a range of residues through extraction at a particular pH or through ion exchange SPE clean-up. However, to extract a wide range of residues a more generic acetonitrile based extraction procedure might be required, with hexane defatting and washing with saturated sodium chloride to remove polar components. Extracts may invariably require further purification by SPE prior to determination of residues.

At present, the majority of methods used for isolation of benzimidazole residues from animal tissues and milk may be described as screening methods. This is because the majority do not generally include the complete range of marker residues

Table 4
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from animal tissues

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
ABZ, CAM, MBC, MBZ, OFZ, FBZ, 5-OH-TBZ, TBZ	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane C_2 SPE	81–100	[102]
ABZ, MBZ, OFZ, FBZ, OXI, FLU, TBZ, TCB	M, L, K	Acetonitrile–H_2O	LLP C_{18}, Florisil SPEs	65–87 39–80 (OFZ)	[134]
CAM, TBZ, 5-OH-TBZ, 5-NH_2-TBZ	L	DMSO–H_2O			[135]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2 *(MBC, MBZ, FBZ-OH, FLU, 5-OH-TBZ, TBZ)	L	DMF–H_2O			[156]
ABZ, CAM, MBZ, OFZ, TBZ	L	Ethyl acetate	Alumina C_{18} SPE	67–90	[157]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CAM, FBZ-SO_2, MBZ, OXI, 5-OH-TBZ, TBZ	Mk	Acetonitrile	LLP	62–108	[160]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, OXI, 5-OH-TBZ, TBZ	M, L, K	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane Oasis HLB SPE	44–87	[163]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FEB, FBZ, OFZ, FBZ-SO_2, MBZ, OXI, FLU, CAM, TBZ, 5-OH-TBZ, TCB	M, L	Ethyl acetate at alkaline pH	Acidified ethanol vs. hexane SDB SPE	36–117 8 (FEB)	[164]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, MBZ, OXI, 5-OH-TBZ, TBZ, TCB, LEV	M, L, fish	Ethyl acetate at alkaline pH	Methanol vs. hexane	75–109	[165]
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, MBZ, TBZ	M	MSPD	MSPD	63–86	[167]
ABZ, FBZ, OFZ, MBZ, TBZ	L	MSPD	MSPD	55–93	[168]
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, TBZ	L	MSPD	MSPD	61–92	[170]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, MBZ, MBZ-OH, FLU, OXI, TBZ		SF-CO_2	SCX	56–109	[172]
ABZ, ABZ-SO, ABZ-SO_2, CAM, FBZ, OFZ, FBZ-SO_2, MBZ, LEV	L, M, Mk, Fish	Acetonitrile	SCX SPE On-line TEC	57–89	[173]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, FLU, FLU-NH_2, MBZ, MBZ-OH, OXI, TBZ	L	Ethyl acetate at alkaline pH	C_{18} on ASPEC™	27–120	[174]
ABZ, ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2, MBZ, OXI, FLU	L	Dilution juice			[230]

[a] Liver, fat, kidney, muscle and milk are abbreviated as L, F, K, M and Mk.

Table 5
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from milk

Residues	Extraction	Clean-up	Recovery (%)	Ref.
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, FBZ-OH, MBZ, OXI	Ethyl acetate at pH 10		79–100	[111]
ABZ-NH_2-SO_2	Acid hydrolysis (H_3PO_4) Acetonitrile (pH 5–7)	SCX SPE	>91	[114]
ABZ-SO, ABZ-SO_2, FBZ, OFZ, FBZ-SO_2	Acetonitrile	C_{18}	77–97	[115]
FBZ, OFZ, TBZ, 5-OH-TBZ	Ethyl acetate at alkaline pH	LLP Silica SPE	>80 56 (5-OH-TBZ)	[116]
ABZ, FBZ, OFZ, FBZ-SO_2, FBZ-OH, MBZ, TBZ	MSPD	MSPD	70–107	[129]
ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, 5-OH-TBZ, TBZ, MBZ	Acetonitrile at pH 10 in presence of BHT	C_{18}	71–105%	[162]
ABZ, ABZ-SO, ABZ-SO_2	Ethyl acetate at alkaline pH	LLP	78–100	[175]
ABZ, FBZ, TBZ, OXI, LEV	Acetonitrile-ethyl acetate	LLP	68–85	[177]
ABZ, FEB, FBZ, OFZ, OXI, TBZ, TCB, LEV	Ethyl acetate at alkaline pH		79–110	[178]
FBZ, OFZ	None	Diluted 1:10	95–122	[179]
ABZ, ABZ-SO, ABZ-SO_2, FEB, FBZ, OFZ, MBZ, OXI	Dilute buffer pH 11.0	C_{18} SPE	81–96	[181]
TBZ, LEV	Defat Dilute buffer pH 6.5	C_{18} SPE	81–89	[182]

for each substance. Some researchers have developed methods for a wide range of substances using LC–MS/MS but these methods do not include the complete range of marker residues [164,165,178]. These multi-residue screening methods are summarised in Tables 4 and 5. In some cases, it can be seen from the methods that they are suitable for determination of the marker residues of certain substances such as ABZ, FBZ, OXI and TBZ. However, the majority of these methods fall short when it comes

Table 6
Summary of methods suitable for the isolation of marker residues of individual benzimidazole drugs from food of animal origin

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
MBZ, MBZ-OH, MBZ-NH_2	Eel	Ethyl acetate at pH 7.8	NH_2 SPE	>83, >83, >59	[53]
ABZ, ABZ-SO, ABZ-SO_2 ABZ-NH_2-SO_2	Fish	Ethyl acetate-DMSO at pH 11		82, 78, 75 and 63	[86]
FBZ, OFZ	L	Methanol–H_2O	LLP	80, 70	[90]
FBZ, OFZ, FBZ-SO_2	M, F, K	Ethyl acetate at pH 8.0			[91]
FBZ, OFZ, FBZ-SO_2	Fish, M, Sk	Acetonitrile and hexane wash	C_{18} and CN SPE		[92]
FLU, FLU-HMET, FLU-RMET	E, M	Ethyl acetate at pH 10	Methanol vs. hexane	>70 (E) and >87 (M)	[94]
MBZ, MBZ-NH_2, MBZ-OH	L	Ethyl acetate at pH 10	Methanol vs. hexane	>85	[98]
ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2	Mk	Acetonitrile	LLP	74–90	[112]
ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2	Dairy products	Acetonitrile	LLP	78–86	[113]
TCB, TCB-SO, TCB-SO_2	Mk	Acetone	C_{18}	76–92	[117]
TCB, TCB-SO, TCB-SO_2	Mk	Acetonitrile and hexane wash	C_{18}	89–95	[119]
ABZ marker residues	L	Acid hydrolysis Ethyl acetate at pH 8–9.5	C_{18} SPE	>75	[125]
TBZ, 5-OH-TBZ, 5-HSO_4-TBZ	Mk	Acid hydrolysis (HCl) Ethyl acetate at pH 8.0	PRS SPE	87–115	[126]
Nine metabolites of OFZ	L	Acetonitrile	SCX SPE	28–118	[133]
TBZ, 5-OH-TBZ	M, L, K	Ethyl acetate at pH 7.0	CN SPE	>96, >70	[161]
ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2	M, L, F, K	Acetonitrile	Ion-pair extraction LLP	>81, >88, >76	[166]
ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2	Mk	Acetonitrile	LLP	73–85	[175]

[a] Egg, fat, kidney, liver, muscle, milk and skin, are abbreviated as E, F, K, L, M, Mk and Sk.

Table 7
Summary of extraction and clean-up procedures used in selected multi-residue methods for isolating benzimidazole residues from crops

Residues	Matrix[a]	Extraction	Clean-up	Recovery (%)	Ref.
MBC, TBZ	F, V	Acetone	LLE Diol SPE on ASPEC	97–108	[54]
BEN, MBC, 2-AB	F, V	Ethyl acetate	LLP	71–92	[136]
TBZ, MBC	F, V	LLE (ethyl acetate vs. 0.1N HCl)	LLP	91–97	[186]
BEN, TBZ	F, V	Reflux with ethyl acetate at alkaline pH		73–109	[227]
BEN, MBC, 2-AB	F, V	Benzene at alkaline pH	LLP	70–90	[197]
BEN, MBC, TBZ	F, V	Reflux in 2N HCl Chloroform at pH 9.5			[198]
BEN, MBC, TBZ	F, V	Methanol	LLP	64–105	[199]
BEN, MBC, TBZ	F	LLE (chloroform vs. aqueous NaCl)	LLP	>90	[200]
BEN, MBC, TBZ	F, V	Acetone	LLP, normal phase column chromatography	48–86	[201]
BEN, MBC, TBZ	F, V	Acetone	Extrelut and SCX SPE	>80	[204]
MBC, TBZ	Fruit juice	Dilution	Oasis® MCX	74, 97	[207,208]
MBC, TBZ	F	Acetonitrile	Oasis® MCX	>88	[209]
BEN, MBC, TBZ, T-M	F, V	SF-CO_2	C_{18} Trap	78–102	[211]
BEN, MBC, T-M	F, V	SF-CO_2	C_{18} Trap	85–93	[213]
TBZ, MBC	F, V	H_2O (100 °C, 50 bar)	LLP	80–101	[215]

[a] Fruit and vegetables are abbreviated as F and V, respectively.

to the inclusion of the marker residues of FLU, MBZ and TCB. It may be proposed that these methods are suitable for residue monitoring purposes. However, in some cases, if the most persistent and/or predominantly occurring residues such as MBZ-OH in sheep, MBZ-NH_2 in horse, and the sulphate conjugate of TBZ and TCB-SO_2 in milk are not included in methods then this is not strictly true. It may be concluded that for more effective determination of benzimidazole residues in food of animal origin, more residues need to be included in methods. Alternatively, laboratories should apply methods that are suitable for the determination of individual marker residues such as those outlined in Table 6, in combination with multi-residue methods.

The methodology developed for the isolation of benzimidazole fungicides from crops is generally fit for purpose. In general, there are only a few benzimidazole fungicides used in crop protection, requiring methods for the isolation of four residues BEN, MBC, TBZ and TM. A summary of these methods are outlined in Table 7. It may be concluded that methods that have been developed for the determination of MBC and TBZ are suitable for the determination of benzimidazole fungicides in crops. Some have looked at the researcher's applicability of these extraction and purification procedures also for recovery of the pre-benzimidazoles (BEN and TM).

10. Methods for measurement of benzimidazole residues

10.1. Bioassays

Bioassays have been used to detect benzimidazole residues in food but are more routinely used to evaluate the potency of anthelmintic substances. A common approach adopted in most bioassays is to first separate residues on a TLC plate and subsequently spray the plate sequentially with nutrient agar and a solution containing an indicator organism. The presence of benzimidazole residues is indicated by a zone of inhibited growth on the TLC plate. The size of the zone of inhibition is related to the concentration of the benzimidazole residue. Erwin et al. [219] developed a bioassay method for determination of BEN. Later Petersen and Edginton [220] developed an assay to detect BEN and MBC in crops. BEN and MBC residues were extracted and separated on a silica gel TLC plate, which was subsequently sprayed with a mixture of agar and *Penicillium* spores prior to incubation (ambient, 20 h). The authors concluded that the bioassay was 10 times more sensitive than detection by UV spectrometry. The procedure offered a number of advantages over an alternative bioassay procedure developed by Erwin et al. [219]. These advantages included the possibility to allow separation of different metabolites and improved sensitivity. Dew et al. [221] developed a multiwell screening assay for TBZ, ABZ, ABZ-SO, ABZ-SO_2 and MBZ using larvae of *Ascaris suum*. The effect of different drugs and concentrations was evaluated on the different larval development stages (L2–L4). L2 is defined as larvae hatched from eggs, L3 the first moult and L4 the second moult. All of the benzimidazole residues selected were shown to inhibit development of the L2–L3 stage at a concentration of 10 ng/ml. The L3–L4 stage was also inhibited by each of the benzimidazole residues. However, inhibition of this larval stage was strongly influenced by the concentration and benzimidazole type. ABZ, MBZ, TBZ, ABZ-SO and ABZ-SO_2 inhibited larval development at concentrations of 10, 10, 100, 100 and 1000 ng/ml, respectively. The bioassay was proposed as a low cost screening system for detection of benzimidazole residues

in food. However, it can be concluded that the variable sensitivity of test organisms to benzimidazole metabolites or marker residues is a key issue that needs to be evaluated more closely.

10.2. Direct spectrometric detection

In early work, a number of groups developed spectrometric methods for determination of benzimidazole residues in crops. Pease and Gardiner [195] determined BEN, after conversion to 2-aminobenzimidazole (2-AB), by fluorescence spectrophotometry (λex 285 nm and λem 335 nm) or by colorimetric detection at 445 nm after derivatisation with bromine. The limit of detection was less than 100 μg/kg. The three benzimidazole fungicides, 2-AB, TBZ and 2-(2-furyl)-benzimidazole all fluoresce naturally; however, only 2-AB reacts with bromine to produce coloured derivatives. As a result, colorimetric detection after bromination is a means of discriminating between 2-AB and other benzimidazole fungicide residues. Aharonson and Ben-Aziz [201] similarly determined BEN and TBZ residues in crops by fluorescence spectroscopy after selective clean-up by column chromatography. Baeyens et al. [222] determined MBZ and FLU in pharmaceutical preparations by fluorescence (λex 300 nm and λem 400 nm) after reaction with 0.3% hydrogen peroxide in alkaline solution. This group indicated that the assay could be applied also to tetramisole, dexamisole and levamisole. In summary, spectrometric methods may be suitable for quantitation of high levels of benzimidazole residues in food. However, when quantitation of benzimidazoles in the low μg/kg range and greater selectivity is required, chromatographic separation of residues prior to spectrometric detection is generally required.

10.3. Thin layer chromatography

TLC is a suitable technique to detect benzimidazole residues in food, while providing semi-quantitative analysis. The technique has been applied to allow cost-effective detection of benzimidazole residues in crops and animal tissues. Norman et al. [185] determined TBZ in citrus fruits by TLC with fluorescence detection (λex 302 nm and λem 360 nm). White and Kilgore [202] determined MBC in crops at a concentration lower than 50 μg/kg on polyamide plastic TLC plates coated with a fluorescent indicator. Plates were developed using a solvent of chloroform–ethyl acetate–acetic acid (190:10:4, v/v/v) and visualised under UV light at 254 nm. Corti et al. investigated the use of high performance TLC (HPTLC) for determination of TBZ and other residues in potatoes [193]. The residues were separated on C_8 plates using a mobile phase of methanol–water (10:3, v/v) and quantitation was completed using a scanning densitometer at 248 nm. The sensitivity of the method was compared with HPLC, but HPLC was found to be much more sensitive. The same researchers later developed a method for determination of BEN, MBC and TBZ in fruit [200]. Residues were separated on NH_2 HPTLC plates (derivatised with polyamine) using a solvent of chloroform–cyclohexane–methanol (6:1:0.1, v/v/v) for MBC and TBZ. Residues were detected using a scanning densitometer at 285 nm. More recently, Abjean [223] developed a TLC screening method for detection of ABZ-NH_2-SO_2 in liver. ABZ-NH_2-SO_2 was separated using a mobile phase of acetonitrile–ammonium hydroxide (10:0.6, v/v) on a silica TLC plate coated with a fluorescent indicator. The limit of detection of the method was less than 500 μg/kg.

10.4. Immunochemical methods

Immunoassays can provide simple, sensitive and selective detection of benzimidazole residues in biological matrices. In some cases, samples such as plasma, serum and milk may be analysed directly or diluted prior to analysis. In early work, radioimmunoassays were applied to detect benzimidazole residues in animal tissue and crops. Nerenberg et al. [224] developed a radioimmunoassay for determination of OFZ in plasma. Polyclonal antibodies were raised in rabbits after immunisation with OFZ coupled to a polylysine carrier. In subsequent work, the assay was applied to OFZ residues in sheep fat tissue [137]. Newsome and Shields [225] developed a radioimmunoassay using a polyclonal antibody for determination of BEN in crops, as it's major residue MBC. Polyclonal antibodies were raised in rabbits after immunisation with the hapten 2-amino benzimidazole coupled to a human serum albumin (HSA) carrier.

In recent years, enzyme linked immunoassays (ELISAs) have found widespread application and have largely taken over from radioimmunoassays. There have been a number of significant applications of ELISA technology in the area of benzimidazole analysis. Brandon et al. [135] developed a competitive ELISA for determination of TBZ in liver, using a monoclonal antibody raised in mouse. Haptens of TBZ and 5-OH-TBZ were prepared and coupled to the carrier bovine serum albumin (BSA) for use as immunogens. It was found that antibodies raised after immunisation with the conjugate prepared from TBZ were more specific for TBZ and 5-OH-TBZ, while antibodies raised from the conjugates prepared with 5-OH-TBZ were found to be less specific and also showed good cross-reactivity to CAM and TBZ-NH_2. Cross-reactivity to benzimidazoles not possessing a thiazolyl ring was also evaluated, but none of these residues (namely ABZ, MBZ and FBZ) showed significant cross-reactivity. Competitive ELISAs were developed based on the horseradish peroxidase (HRP) conjugate and were applied to liver samples extracted using water. They later applied this ELISA for determination of TBZ residues in fruit, vegetables and fruit juices [226]. It was found that simple dilution of fruit juice samples in buffer was sufficient prior to determination by ELISA. Brandon et al. [156] later produced a mouse monoclonal antibody that showed good cross-reactivity to 11 benzimidazole carbamate residues including ABZ, FBZ, OXI, MBZ, FLU, MBC and some metabolites. The antibody did not show cross reactivity to thiazolylbenzimidazoles (TBZ, CAM and 5-OH-TBZ). Antibodies were raised in mice using conjugates prepared after coupling the succinamide hapten of ABZ to BSA carrier protein. They developed a competitive ELISA based on HRP for determination of benzimidazole residues in bovine liver samples after aqueous extraction and later developed a method for determination of FBZ residues in milk using this ELISA [179]. Milk samples were simply diluted in PBS-Tween-BSA before determination by ELISA.

Bushway et al. [191] evaluated a commercially available enzyme immunoassay tube kit for determination of TBZ residues in processed/unprocessed fruit and vegetables. The polyclonal antibody used in the assay was raised against BEN-MBC, but the antibody demonstrated sufficient cross-reactivity towards TBZ to allow its sensitive detection in food. Newsome and Collins [227] developed ELISAs for determination of BEN and TBZ in crops, using polyclonal antibodies raised in rabbits. Immunogens were prepared by coupling the carrier human serum albumin (HSA) to the succinamide haptens of TBZ and MBC (the breakdown product of BEN), and were used in rabbits for raising antisera. Abad et al. [228] produced monoclonal antibodies raised against a TBZ-ovalbumin conjugate. They indicated that this was a very novel conjugate because it was prepared from a hapten functionalised at the nitrogen atom of the 1-position of the TBZ structure. They developed an indirect ELISA based on HRP for determination of TBZ residues in fruit juices. Moran et al. [229] produced mouse monoclonal antibodies raised against the novel immunogen 5-benzimidazole–carboxylic acid conjugated to the lipopeptide $Pam_3Cyst\text{-}T_H$. The antibodies were produced for development of ELISAs for determination of protein-bound residues of TBZ in tissue, although no applications were described.

Crooks [230] later produced a polyclonal antibody that showed good cross-reactivity to eight benzimidazole carbamate residues. Antibodies were raised in sheep using conjugates prepared after coupling the succinamide hapten of ABZ to HSA carrier protein. This group developed a competitive ELISA based on HRP for determination of benzimidazole residues in the tissue juice produced from bovine liver. Johnsson et al. [231] later applied the antibody produced by Crooks in a Biacore® biosensor assay for detection of benzimidazole residues in bovine serum. They evaluated the effect of different sample processing conditions on the biosensor assay response, finding that benzimidazole standard curves prepared in serum, without protein precipitation did not agree with standards in buffered solutions. The assay was shown to give good cross-reactivity (>74%) to five benzimidazoles. The results also indicated an improved cross-reactivity to FLU (75%) compared to the original ELISA developed by Crooks (23%). The LOD and LOQ of the method were determined using 20 blank serum samples to be 2.6 and 4.8 ng/ml, respectively.

10.5. Liquid chromatography based separations

Benzimidazole drugs and their metabolites may differ greatly in terms of physical and chemical properties. As a result, development of a chromatographic method for a range of benzimidazole residues is a challenging process. A number of problems need to be overcome including resolution, peak shape, peak sharpness and reasonable run time. The majority of LC methods for determination of benzimidazoles have been developed using reversed phase columns (C_8 or C_{18}) and ion suppression based mobile phase systems. In early work, some methods were developed using alternative column types such as silica or cation exchange. Karlaganis et al. [144] determined MBZ in plasma using a silica column with a mobile phase of acetonitrile–water saturated chloroform–formic acid (75:92.5:0.25, v/v/v). Alvinerie and Galtier [149] developed a normal phase separation of TCB, TCB-SO, TCB-SO_2 and OFZ on a silica column achieving good separation in a 15 min run. Kirkland et al. [136] used a cation exchange column for determination of BEN residues in plants and soil. Arenas and Johnson [126] determined TBZ and 5-OH-TBZ in milk using a strong cation exchange column. Hiemstra et al. [54] used a polymeric column for analysis of TBZ and MBC residues in crops giving better peak shape, separation and longer column lifetime than silica-based columns.

In early work, Bogan and Marriner [139] developed an isocratic separation for eight benzimidazole residues on a C_{18} column in 14 min, but the substances were poorly resolved. Bull and Shume [130] later separated metabolites of FBZ and TCB in a 40 min run time. Long et al. [131] and Wilson et al. [102] developed multi-residue LC methods for determination of 7 and 8 benzimidazoles in less than 13 and 35 min, respectively. Other researchers have developed gradient separations achieving improved separation or inclusion of more residues. Allan et al. [151] compared isocratic and gradient systems for determination of five benzimidazoles in plasma. Sorensen and Petersen [181] developed a gradient separation of eight benzimidazoles in a 40 min run with additional time needed for equilibration. Rose [133] separated OFZ and nine metabolites in less than 35 min using a binary gradient on a C_8 column. Similarly, Stubbings et al. developed a gradient separation for levamisole and eight benzimidazoles on a C_8 column [173]. More recently, Roudaut and Garnier [163] separated 10 benzimidazoles using a binary gradient on a base deactivated C_{18} column, all residues with the exception of OXI and OFZ were separated in less than 16 min.

Rouan et al. separated TCB, TCB-SO, TCB-SO_2 and an internal standard on a short C_{18} column (33 mm × 4.6 mm) in less than 10 min [154]. Porter and Johnston [232] evaluated narrow bore columns (2.1 mm) for determination of TBZ, MBZ, ABZ and FBZ residues. This group found that the peak heights for analytes increased by a factor of five using narrow bore columns compared to a column of standard diameter (4.6 mm). This group concluded that use of narrow bore columns resulted in a reduction in solvent usage, analysis time and pump wear. Kan et al. [96] and Hajee and Haagsma [53] developed isocratic separations on columns of intermediate diameter (3 mm) for FLU and MBZ metabolites, respectively. Likewise, Domany and Kovacsics [160] developed an isocratic separation of 10 benzimidazoles on a narrow bore (2.1 mm) C_{18} column. Shaikh et al. found that ABZ and its three major metabolites could not be eluted in less than 20 min without the use of gradient elution [86]. This group chose to determine ABZ and its three metabolites using two separate isocratic systems. Danaher et al. [172] separated 14 benzimidazole residues on an Xterra® C_{18} column using gradient elution in a run time of 60 min.

Negro et al. [56] evaluated the use of ion-pair chromatography for determination of TCB-SO and TCB-SO_2 in plasma and urine. They investigated the effect of pH, organic modifier content and the chain length of the sodium alkylsulphonate ion-pair reagent on chromatography. The optimum mobile phase consisted of 0.05 M phosphate buffer pH 7.0–acetonitrile (55:45, v/v) con-

taining 0.001 M sodium decanesulphonate. De Ruyck et al. [177] developed an ion pair gradient separation for FBZ, TBZ, ABZ, OXI and LEV. Satisfactory separation conditions were achieved with sodium 1-octanesulphonate as the ion-pairing agent and adjustment of the mobile phase buffer solution to pH 3.5 with ortho-phosphoric acid. Botsoglou et al. [132] investigated the effect of ion-pair reagents and pH on the retention time and peak height of FBZ and OFZ, and showed that decreasing the pH to 2.2 shortened the retention time of FBZ markedly and OFZ slightly. Addition of octane-sulphonate anion increased retention time and tetrabutyl ammonium cation decreased retention time. Over the range of pH 3.7 to 6.5 no change in retention time was observed with or without the addition of ion-pair reagents. It was found that the peak shape of FBZ was poor over a range of pH values in the absence of ion-pair reagents, but addition of ion-pair reagents greatly enhanced peak shape at pH 2.2, even at longer retention times. The same group developed a range of separations for FBZ residues using ion-pair chromatography. A multi-residue method was later developed for separation of 10 benzimidazoles in milk in less than 30 min, using an isocratic mobile phase. Macri et al. [233] developed an ion-pair separation of eight benzimidazoles using a mobile phase containing 0.01 M pentane-sulphonate and 0.5% triethylamine at pH 3.5.

In the area of LC–MS, most groups carry out separations using shorter columns (less than 150 mm in length). These columns require shorter equilibration times during gradient chromatography because of the lower column volume, which increases LC–MS throughput. Blanchflower et al. [90] developed an isocratic separation of FBZ and OFZ, finding it gave more reproducible MS results compared to gradient separation. Cannavan et al. [161] developed a gradient method for separation of TBZ and 5-OH-TBZ, using a mobile phase consisting of acetonitrile and 0.1 M ammonium acetate. Balizs [164] developed a method for determination of 15 benzimidazole residues by LC–MS/MS with a gradient separation carried out on a narrow bore column (2.1 mm). The mobile phase flow rate ranged between 0.04 and 0.2 ml/min depending on the ionisation interface used. The benzimidazoles were eluted from the column in less than 6 min but were not resolved. It was claimed that, because individual benzimidazoles have different mass-to-charge values, chromatographic separation was unnecessary. De Ruyck et al. [94] developed an LC–MS/MS method for determination of benzimidazole residues using a column of similar dimensions for determination of FLU, FLU-HMET and FLU-RMET in eggs and muscle tissue. Later, they developed a method for determination of levamisole and seven benzimidazole residues in milk [178].

10.5.1. Liquid chromatography detection systems

The determination of benzimidazoles in milk, tissue and crops is routinely carried out using UV, fluorescence and mass spectrometric (MS) detection systems coupled to LC. Benzimidazoles possess a strong UV chromophore and may be determined using LC–UV. However, it has been found that certain benzimidazoles (namely ABZ, CAM, FLU, TCB, TBZ, and their metabolites) possess naturally fluorescing chromophores, making them suitable for detection by LC fluorescence. Fluorescence is more sensitive and selective, but does not have the same range of applicability as UV detection. As a result, UV is the most widely applied system to detect benzimidazole residues in biological extracts. MS detection is becoming more widely used for determination of benzimidazole residues in biological matrices, offering the capability for combined quantitative-confirmatory analysis.

10.5.1.1. Detection by UV. Alton et al. [146] determined MBZ residues in plasma by UV detection at 313 nm. Karlaganis et al. [144] determined MBZ in plasma, finding less interference at a wavelength of 307 nm compared to 247 nm. Bogan and Marriner [139] developed a method for determination of eight benzimidazoles in body fluids, using UV detection at 292 nm. Mellergaard et al. [138] determined MBZ residues in eel muscle at 254 nm. Hajee and Haagsma [53] determined MBZ, MBZ-NH_2 and MBZ-OH in eel muscle at 289 nm. Kan et al. [96] developed a method for analysis of FLU and its metabolites in eggs using UV detection at 250 nm. Chiap et al. [155] determined ABZ, ABZ-SO and ABZ-SO_2 in plasma at 295 nm.

Long et al. [131] determined seven benzimidazole residues in tissue using UV photodiode array (PDA) detection. They also investigated the use of PDA as a confirmatory tool, but found differences between spectra at lower and higher concentration levels. The PDA detector was not suitable for confirmatory analysis at lower concentrations (5 ng on-column). They later developed methodology for determination of these residues in milk and animal tissues. De Buyanski et al. [158] determined TBZ and levamisole in tissue using PDA detection at 300 and 240 nm, respectively. Danaher et al. [172] determined 14 benzimidazole residues in liver tissue using UV detection at 298 nm.

Tai et al. [116] adopted a dual wavelength approach for determination of FBZ, OFZ, TBZ and 5-OH-TBZ residues in milk. FBZ and OFZ residues were monitored at 298 nm, while TBZ and 5-OH-TBZ were monitored at 318 nm. Similarly, Sorensen and Petersen [182] determined TBZ and levamisole residues in milk using a PDA detector at wavelengths of 300 and 220 nm, respectively. This group also developed a method for determination of eight other benzimidazoles in milk (ABZ, ABZ-SO, ABZ-SO_2, OFZ, OXI, MBZ, FBZ and FEB) [181].

A range of derivatisation methods can be used for determination of benzimidazoles. Most of these have been used for GC applications and only two papers on derivatives for LC have been published. Capece et al. [91] converted FBZ and OFZ residues to FBZ-SO_2 and measured the sum of total residues as this peak. Residues were derivatised by reaction with percetic acid and purified by liquid–liquid partitioning prior to determination by LC–UV. Tafuri et al. [234] confirmed the presence of TBZ residues, by derivatising with *p*-nitrobenzyl bromide in the presence of potassium carbonate (110 °C, 3 h) with detection by LC–UV. The derivatisation procedure was used to provide quantitative and confirmatory analysis of TBZ residues in suspect samples.

10.5.1.2. Detection by fluorescence. A number of researchers have developed methods for determination of benzimidazole residues using HPLC coupled to fluorescence detection. Wilson

et al. [102] found that sample extracts needed further purification on C_2 SPE cartridges to remove matrix components that interfered with UV detection of ABZ-NH_2-SO_2 and 5-OH-TBZ. However, the influence of this matrix interference could be greatly reduced using fluorescence detection. It was proposed because less clean-up is required with fluorescence that recovery and reproducibility of assays should improve. Le Boulaire et al. [169] determined TBZ and MBZ in tissue, finding fluorescence to be 20 times more sensitive than UV detection for TBZ. Kinabo and Bogan [117] developed a method for determination of TCB and its metabolites in milk using fluorescence detection (λex 300 and λem 676 nm). The limit of detection of the method was 20, 40 and 40 μg/l for TCB-SO_2, TCB-SO and TCB, respectively. Arenas and Johnson determined TBZ and 5-OH-TBZ in milk using fluorescence detection with λex 305 and 318 nm and λem 380 and 515 nm, respectively [126]. Two separate chromatographic runs were required to achieve more sensitive detection, but it was concluded that these residues could be determined at their optimum wavelengths in a single run using a dual grating monochromator. The LOD and LOQ were 5 and 50 μg/l, respectively. Markus and Sherma [125] used fluorescence detection for determination of ABZ-NH_2-SO_2 in bovine liver (λex 300 nm and λem 320 nm). Shaikh et al. [86] determined ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 and three metabolites in fish muscle using fluorescence detection with λex 290 nm and λem 330 nm. LOQs for the method were 20, 1.5, 0.5 and 5 μg/kg for ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2, respectively. Aharonson et al. [211] determined benzimidazole fungicides in fruit and vegetable matrices using UV and fluorescence detection. TBZ, MBC and TM were determined by UV at 285 nm while TBZ and MBC were determined also by fluorescence (λex 305 nm, λem 345 nm and λex 282 nm, λem 307 nm, respectively). It was found that detection of TBZ residues could be enhanced using fluorescence instead of UV detection, but detectability of MBC was similar using both detectors. However, matrix interference was found to be less significant with fluorescence detection.

10.5.1.3. Detection by UV and fluorescence in series. The use of fluorescence detection can offer added sensitivity and selectivity to methods, potentially reducing the need for extensive sample clean-up. These added advantages may allow the development of multiresidue methods that would allow the determination of all major benzimidazole residues, a task that has not yet been completed by any researcher. However, since some benzimidazoles do not fluoresce naturally (namely FBZ, OFZ, FBZ-SO_2, MBZ, FLU, MBZ-NH_2 and FLU-NH_2), UV is still required as it applicable to all benzimidazole residues.

Some researchers have developed methods for the determination of benzimidazole residues using UV and fluorescence detection in series, these methods are described in this section. Farrington et al. [157] determined benzimidazole residues (ABZ-SO_2, OFZ, CAM and MBZ) using UV and fluorescence detectors in series. ABZ-SO_2, TBZ and CAM were monitored using fluorescence detection (λex 312 nm, λem 355 nm), while OFZ and MBZ were monitored using UV detection at 290 nm. Su et al. [162] developed a method for determination of benzimidazole residues using UV and fluorescence detection. A generic wavelength of 290 nm was found to be suitable for detection of ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2 and TBZ. This group found that more selective detection of 5-OH-TBZ and MBZ could be achieved using UV at 320 nm. Fluorescence detection (λex 290 nm, λem 320 nm) was found to be suitable for the determination of ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2 and TBZ. Fluorescence detection was found to be unsuitable for the detection of 5-OH-TBZ and MBZ because of weak signal and lack of fluorescent characteristics, respectively. Constantinou et al. [180] determined five benzimidazole residues in milk using a similar approach. TBZ and ABZ residues were monitored using fluorescence (λex 312 nm, λem 355 nm) while other residues were detected by UV at 290 nm. Hiemstra et al. [54] determined MBC residues in crops using UV (280 nm) and fluorescence (λex 235 nm, λem 280 nm) detectors in series. Residues were confirmed by comparing the relative UV and fluorescence responses for positive samples with standards.

10.5.1.4. Detection by electrochemical potential. There are few reported applications of electrochemical detection for benzimidazole residues. Oosterhuis et al. [143] determined MBZ and MBZ-OH residues in biological fluids by electrochemical detection. The electrochemical detector consisted of a carbon paste electrode and a saturated calomel electrode; MBZ residues were determined at 950 mV versus the reference electrode. FLU, which possesses similar oxidative properties to MBZ, was used as internal standard. Leenheers et al. [235] similarly developed a method for determination of a metabolite of MBC in urine by HPLC with electrochemical detection.

10.5.1.5. Detection by mass-spectrometry. It will be seen from the applications reviewed in this section that LC–MS is the technique of choice for sensitive and selective detection of a wide range of benzimidazole residues in complex biological matrices. In contrast to traditional LC–UV methods, LC–MS can be used to allow stand-alone determination and confirmation of benzimidazole residues. LC-fluorescence could be applied as an alternative technique to allow determination and confirmation of selected benzimidazole residues. However, not all benzimidazole residues fluoresce naturally. In this section, the suitability of LC–MS methods for determination and confirmation of benzimidazole residues will be reviewed.

Some early methods were reported for determination of benzimidazole residues in animal tissues using single quadrupole MS instruments equipped with thermospray ionisation interfaces, while operating in selected ion monitoring (SIM) mode. The disadvantage with such systems is that insufficient fragmentation of molecules is achieved and resulting mass spectra do contain a suitable number of diagnostic ions for confirmatory purposes. Blanchflower et al. [90] used LC–MS with a thermospray interface for determination of FBZ and OFZ residues in tissue. FBZ and OFZ were determined in SIM mode as their $[M+H]^+$ ions. Cannavan et al. [161] used LC–MS with a thermospray interface for determination of TBZ and 5-OH-TBZ residues in bovine tissue, while using a deuterated TBZ internal standard to improve reproducibility. TBZ and 5-OH-TBZ were

monitored in SIM mode as their $[M+H]^+$ ions. Cannavan et al. confirmed the presence of residues on a single quadrupole instrument equipped with an atmospheric pressure chemical ionisation (APCI) interface. They demonstrated that suitable fragmentation could be induced with an APCI interface to produce four diagnostic ions, which were suitable for confirmation of residues. Other researchers have developed methods for determination or confirmation of benzimidazole residues using single quadrupole instruments equipped with electrospray ionisation (ESI) interfaces. Takeba et al. briefly described a method for confirmation of TCB marker residues, which were monitored as their $[M-H]^-$ ions [119]. The LOD of the method ranged between 4 and 6 μg/kg in bovine milk. The protocol used for confirmation of residues was poorly described and it was not clear if residues could be confirmed at these low levels. Young et al. [208] determined TBZ and MBC residues in fruit juices using a single quadrupole LC–MS instrument equipped with an ESI interface in positive ion mode. TBZ and MBC were monitored as their $[M+H]^+$ ions. The LOQ of the method was 2 μg/kg in fruit juice. TBZ and MBC residues were fragmented through in-source collision induced dissociation at different cone voltages to produce five and six diagnostic ions for confirmation of MBC and TBZ, respectively.

In the last decade, the application of tandem mass spectrometry (MS/MS) has resulted in improved determination and confirmation of benzimidazole residues in biological matrices. Most of these methods have been reported on triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode or using the MS/MS capabilities of ion trap instruments. Balizs [164] developed a method for determination of 15 benzimidazoles using a triple quadrupole instrument equipped with ionspray and turbospray interfaces while operating in positive ion mode. $[M+H]^+$ ions were fragmented through CID and monitored as a single daughter transition through MRM. The LOQ ranged between 5 and 30 μg/kg in meat. De Ruyck et al. [94] developed an LC–MS/MS–ESI method for determination of FLU and metabolites in muscle, liver and eggs. Residues were monitored as their $[M+H]^+$ ions and confirmed using MRM of two daughter ion transitions. Quantitation was carried using the most abundant daughter ion while using chlorobenzimidazole as an internal standard and matrix matched calibration. The LOQ was approximately 1 μg/kg in egg and meat tissues. They also reported an LC–MS/MS–ESI for determination of MBZ, MBZ-OH and MBZ-NH_2 in sheep muscle, liver, kidney, liver and back fat [98]. The limit of detection of the method was less than 1 μg/kg. De Ruyck et al. [178] later developed a multi-residue method based on the LC-MS/MS-ESI for determination of eight anthelmintic drugs (LEV, TBZ, OFZ, FBZ, OXI, ABZ FEB and TCB) in milk (Fig. 11). Residues were monitored as their $[M+H]^+$ ions and confirmed using MRM. The decision limit (CCα) and the detection capability (CCβ) were calculated by analysing samples fortified at 1 μg/kg (for non-permitted substances) and at the MRL (for permitted substances). Edder et al. [165] determined levamisole and 11 benzimidazole residues in meat and liver by LC–MS/MS using positive ESI and MRM mode. They similarly confirmed the presence of residues based on two daughter transitions. The LOD and LOQ of the method were 5 and 10 μg/kg (except for ABZ, which were 10 and 20 μg/kg), respectively.

A limited number of LC–MS methods have been developed for determination of benzimidazole residues on ion trap instruments. Zrostlíková et al. [209] reported a method for determination of TBZ, MBC and other crop protection agents in fruit using an LC–MS system equipped with an ESI interface in positive ion mode and an ion trap detector. Benzimidazole parent $[M+H]^+$ ions were fragmented using collision induced dissociation through the MS/MS capability of the ion trap and monitored as a single product ion transition. The LOQ of the method for MBC and TBZ was 2 and 4.8 μg/kg, respectively.

10.6. Gas chromatography

GC determination of benzimidazoles is difficult because of the basic nature and low volatility of these substances. However, TBZ and TCB are sufficiently volatile to allow their determination by GC without derivatisation. The main applications of GC methods have been in determining benzimidazole fungicides in crops. Some resreachers have found GC coupled to mass spectrometery useful for confirmation of the presence of benzimidazole residues. However, GC–MS procedures usually require derivatisation of residues to induce volatility and allow the generation of suitable MS spectra for confirmatory analysis and, as a result, have been largely been replaced by LC–MS/MS. Quantitative GC methods have been developed by some researchers to determine the presence of benzimidazole fungicides in crops. Lafuente et al. [192] determined TBZ residues in fruit by direct GC using nitrogen–phosphorous detection (NPD) and electron capture detection (ECD). Separation was carried out on a wide-bore capillary column (10 m × 0.53 mm) with a silicone-bonded phase. NPD was found to be more sensitive than ECD. The LOD of the method was 10 μg/kg. Oishi et al. [203] similarly determined TBZ (without derivatisation) by GC–NPD. The separation was carried out on a fused silica capillary column (25 m × 0.2 mm), giving a LOD of 10 μg/kg. However, most GC methods for determination of benzimidazoles include derivatisation steps, where more polar amino or hydroxyl groups are reacted with suitable alkylating/acylating reagents. An overview of these derivatisation procedures is outlined in Fig. 12 and Table 8. Some researchers hydrolysed the carbamate functional group to form an amino functionality prior to reaction with the alkylating/acylating reagent. Alternatively, residues may be derivatised directly without hydrolysis. A wide range of alkylating and acylating reagents have been used to derivatise benzimidazoles, these are summarised in Table 8. These allow the introduction of functional groups ranging from simple methyl esters to more complex pentafluorobenzyl groups.

Jacob et al. [236] confirmed the photolysis products of TBZ residues by GC–MS after derivatisation with bis(trimethylsilyl)acetamide. They later applied the same method to confirm the structures of urinary metabolites of CAM [106,237]. VandenHeuvel et al. [238] developed an on-column methylation procedure for confirmation of CAM residues by GC–MS–EI (electron impact mass spectrometry) in SIM mode. Residues were dissolved in 0.125 M triethylammonium hydrox-

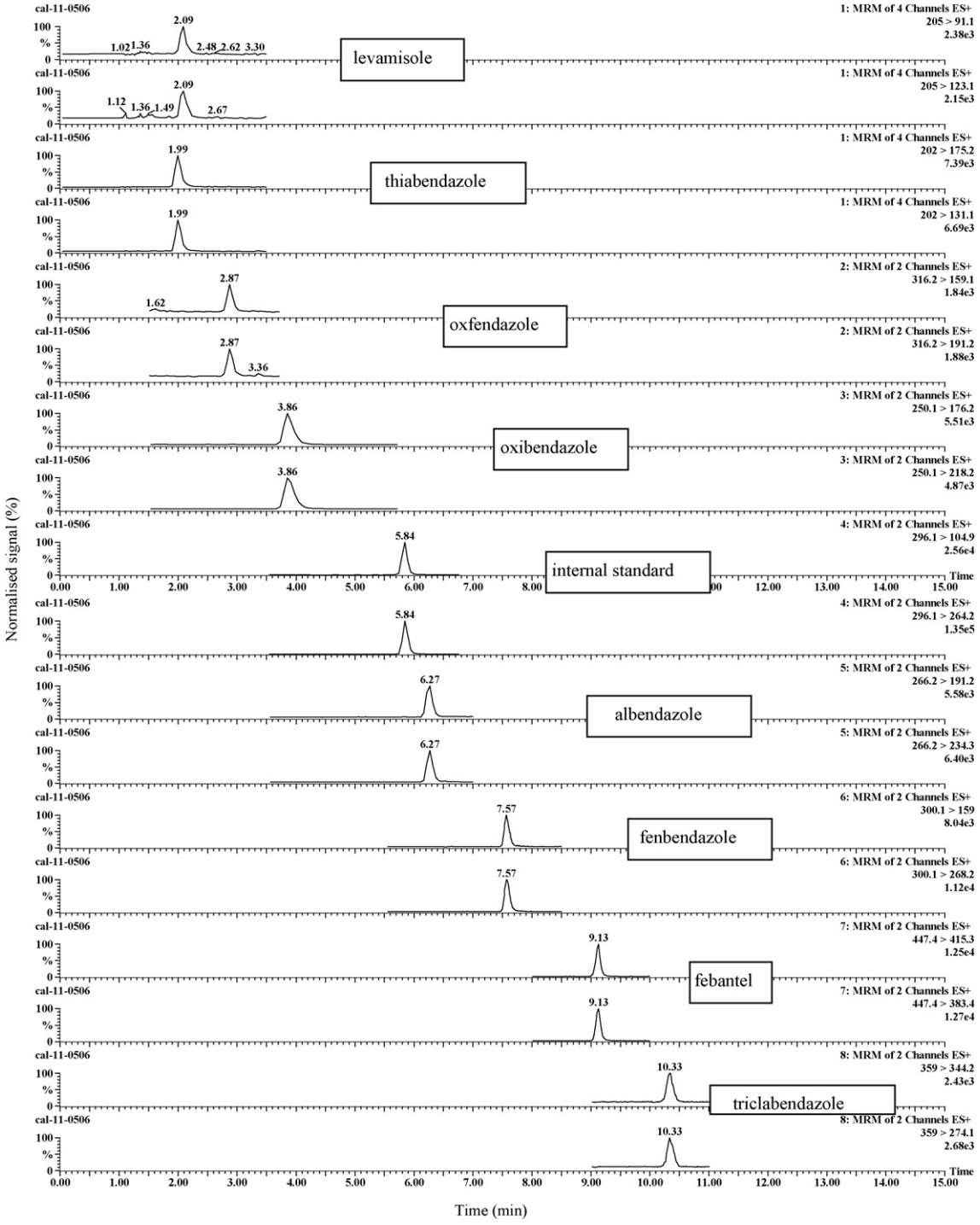
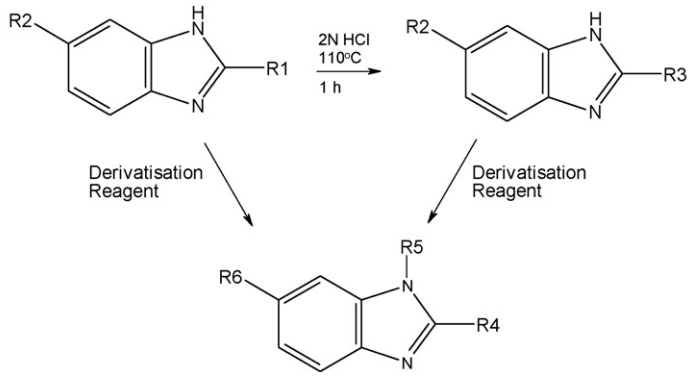


Fig. 11. Chromatogram of a blank milk sample spiked with a mixture of the anthelmintics at 1 μg/l and with the internal standard mebendazole at 10 μg/l (reprinted from reference [178], with permission from Elsevier, Copyright (2002)).

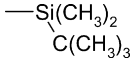
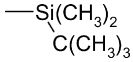
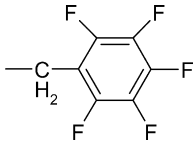
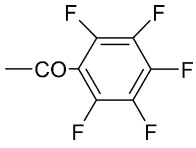
ide in methanol just prior to injecting into the GC–MS system. They later applied this method for TBZ and 5-OH-TBZ [239]. They used a column with a polysulfone stationary phase because its background bleed and adsorption characteristics were more desirable than others tested. Tanaka and Fujimoto [188] determined the methyl derivative of TBZ by GC with flame ionisation detection (FID). Derivatives were prepared by reacting with dimethylformamide and dimethyl acetal in acetonitrile (120 °C, 40 min). The procedure was used to determine TBZ residues in fruit with a limit of detection of 100 μg/kg. Nose et al. [190] acylated TBZ by reaction with pentafluorobenzyl-bromide PFB-Br (120 °C, 30 min) before determination by GC–ECD and confirmation by GC–MS–EI in full scan mode. Physalo derivatised BEN residues with acetic anhydride (100 °C, 30 min) before determination by capillary GC-NPD [197]. The LOD was 10 μg/kg. GC–ECD was also evaluated for determining residues but was 10 times less sensitive. Bardalaye and Wheeler later modified this procedure and applied it to the determination of



Residue	R1	R2	R3	R4	R5	R6
MBC	-NHCOOOCH$_3$	-H	-NH$_2$	-NH-R5	-R5	-H
TBZ		-H			-R5	-H
5-OH-TBZ		-OH			-R5	-O-R5
ABZ-NH$_2$-SO$_2$	-NHCOOOCH$_3$	-SO$_2$(CH$_2$)$_2$CH$_3$	-NH$_2$	-NH-R5	-R5	-SO$_2$(CH$_2$)$_2$CH$_3$
FBZ	-NHCOOOCH$_3$		-NH$_2$	-NH-R5	-R5	
MBZ	-NHCOOOCH$_3$		-NH$_2$	-NH-R5	-R5	
OFZ	-NHCOOOCH$_3$		-NH$_2$	-NH-R5	-R5	

Fig. 12. Overview of derivatisation procedures used in the preparation of benzimidazole derivatives for gas chromatography. The R1 functional group remains unchanged if residues are not hydrolysed prior to alkylation/acylation. The structures for the R5 functional group are listed in Table 8 (reprinted from reference [102] with permission from AOAC International, Copyright (1991)).

Table 8
Summary of derivatisation reagents, reaction conditions and functional group modifications

Derivatisation reagent	Residues	Conditions	R5	Ref.
MTBSTFA	MBC, TBZ, 5-OH-TBZ, ABZ-NH$_2$-SO$_2$, FBZ, MBZ, OFZ	110 °C, 2 h		[102,213,241]
BSA in pyridine	CAM, TBZ	60 °C, 30 min		[106,236,237]
PFB-Br + K_2CO_3	TBZ, TCB, ABZ, FBZ, OFZ, MBZ, FLU, OXI, MBC	100 °C, 3 h or 60 °C, 3 h		[134,186,240]
PFB-CO-Cl + Na_2CO_3 (TBZ)	TBZ	120 °C, 30 min or 100 °C, 45 min		[134,187,190]
PFB-CO-Cl + Na_2CO_3 (other benzimidazoles)			No reaction	[134]
CH3-I	TBZ, TCB, ABZ, FBZ, OFZ, MBZ, FLU, OXI	60 °C, 30 min	—CH_3	[134]
Trimethylphenylammonium hydroxide in methanol	CAM, TBZ, 5-OH-TBZ	On-column methylation	—CH_3	[102,238,239]
DMA-DMF	TBZ	120 °C, 40 min	—CH_3	[188]

TBZ in fruits [187]. Tjan and Janssen [186] determined TBZ and MBC in fruit using GC–ECD after derivatisation with PFB-Br. The procedure was also applicable to BEN, which was converted to MBC during the extraction and clean-up procedure. The LOQ of the method was 50 μg/kg. Tjan and Janssen also developed a confirmatory procedure using GC–MS–EI. Cline et al. [240] later modified this procedure and applied it to determine MBC in fruit treated with BEN. The LOD of the method was 10 μg/kg.

Marti et al. [134] developed GC methods to confirm the presence of eight benzimidazole residues in animal tissue, while evaluating a range of derivatisation procedures used for substances with amino functional groups. They found that acylation with PFB-Br and methylation with methyl iodide gave satisfactory results. Derivatised extracts were determined by GC–NPD and GC–ECD; GC–NPD chromatograms were found to contain less matrix interference than GC–ECD chromatograms. However, derivatives were found to decompose during injection and chromatography because of the high temperature used, making quantitative determination difficult. The main advantages of these derivatives could be seen with GC–MS–EI and GC–MS–positive ion chemical ionisation (PICI) determination where particularly useful structural information was provided for confirmatory analysis. Electron impact spectra of the methyl derivatives produced fewer ions than the pentafluorobenzyl derivatives. Positive ion chemical ionisation provided a similar number of ions suitable for confirmatory analysis, while negative ion chemical ionisation produced only one or two ions for most residues. Wilson et al. [102] used capillary GC–MS–EI in SIM mode for confirmation of benzimidazole residues in tissue. Residues were derivatised in a two-step procedure, firstly involving hydrolysis with 2N HCl (110 °C, 1 h) to convert the carbamate group to an amino functionality. Hydrolysed residues were reacted with *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), converting the primary and secondary amines to a silyl amine. In the case of 5-OH-TBZ, the phenol functional group was converted to a silyl ether. The method was not applicable to CAM, which was alternatively derivatised using flash alkylation in the GC injection port (260 °C) after injection in triethylammonium hydroxide in methanol. Markus and Sherma [241] developed a method for confirmation of ABZ-NH_2-SO_2 in tissue based on this derivatisation procedure, with determination by GC–MS–EI. Balizs and Erbach [242] determined TBZ and ABZ residues in tissue using capillary GC with high-resolution mass spectrometry (HRMS), after derivatisation with PFB-Br. Anastassiades and Schwack later applied a similar GC–MS procedure for confirmation of benzimidazole fungicides in crops after SFE [213].

10.7. Summary on detection systems

At present most methods for the determination of benzimidazole residues in biological matrices using HPLC with UV and/or fluoresence detection. HPLC-UV is a more universal detection system but fluoresence is generally more selective and in some cases more sensitive. In the last 10 years, LC–MS(–MS) has found more widespread application in benzimidazole residue analysis offering more sensitive detection and increased confidence in reporting results. In the future, with an increasing demand to include additional residues in methods there should be an increase in the number of LC–MS/MS methods. Such methods can offer the ability to include additional residues in methods and offer improved limits of detection. In particular, when testing for low levels of residues, such as those that may occur in milk due to unapproved used of licensed veterinary drugs, LC–MS/MS is the technique of choice. Alternatively, HPLC coupled to UV and fluorescence detection in series may offer a low cost alternative to LC–MS/MS, and may be particularly effective for independently confirming the presence of MRL substances in food.

It is important to emphasise the need for rapid methods to detect the presence of residues in food prior to its entry into the marketplace. There have been considerable developments in the area of immunochemical screening methods. No antibody has been developed yet that shows cross-reactivity to the complete range of benzimidazole residues. Future work in this area should concentrate on exploring the cross-reactivity of antibodies to important residues and improvement of cross-reactivity through genetic engineering of antibodies. The application immunobiosensor assays has been explored in a limited study in serum. However, future work should investigate the application of this technique to allow more sensitive detection of residues and reduce non-specific binding effects.

11. Conclusions

There has been intensive research in the last decade into developing methods for the determination of benzimidazole residues in food. At present, methodology for the determination of benzimidazoles in crops would appear to be fit for purpose. However, methodology for determination of benzimidazole residues in foods of animal origin needs improvement. This is not a reflection on the quality of the research that has been published but of the difficult challenge faced, owing to the extensive metabolism of these molecules.

The authors of this paper have carefully considered these problems and have come up with the following recommendations:

- There is a need to develop multi-residue methodology that will allow the determination of the complete range of benzimidazole veterinary drug residues in foods of animal origin.
- Sample preparation techniques need to be developed to allow simultaneous extraction of polar residues such as TBZ and non-polar residues such as TCB.
- These methods may require the application of more selective detection such as LC–MS/MS or a combination of HPLC-UV-fluoresence or through the application of a combination of more specific analytical methods.
- An alternative approach might be to include a rational scientific selection of benzimidazole residues according to their occurrence/persistence in the tissues of treated animals. However, in the event that a drug residue is found the presence of other marker residues of the drug should be checked. The disadvantage of such an approach is that it would necessitate the

validation of alternative residue methods for confirming the marker residues of each drug.

- The need for rational selection of residues particularly applies when testing for benzimidazole residues in milk. In the case of TBZ and TCB the most prevalent and persistent residues in milk are the sulfate conjugate of 5-OH-TBZ and TCB-SO_2, respectively. This would indicate that hydrolysis steps should be included to allow the effective determination of TBZ residues in milk. To monitor for the unapproved use of TCB in dairy animals, TCB-SO_2 in milk would be the most appropriate residue to select.
- One problem encountered when selecting residues for a method is that the most prevalent/persistent residue is not always clearly identifiable. This is the case with benzimidazole veterinary drugs, where the residue is often expressed as the sum of marker residues. The marker residues of ABZ may be expressed as the hydrolysed/oxidised residues and FBZ as the sum of oxidised residues. In the case of TCB, residues are expressed as the sum of residues, which may be derivatised to form keto-TCB. It is not clear whether keto-TCB is a metabolite or an artifact of derivatisation. In the interest of food safety, it would be more appropriate if residues were expressed as the individual metabolites to allow rational selection of marker residue(s) for analysis.
- Another problem faced, is that current validation criteria for testing of foods of animal origin, as outlined in Commission Decision 2002/657/EC, are not appropriate for all approved benzimidazoles because MRLs are often expressed as the sum of marker residues. This indicates that determination of CCα and CCβ is not always appropriate. This is a particular problem for MBZ and FLU because no method is available that allows the determination of total marker residues.

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