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Review

Review of methodology for the determination of macrocyclic lactone residues in biological matrices

Martin Danaher[a,*], Laurence C. Howells[b], Steven R.H. Crooks[c], Vesna Cerkvenik-Flajs[d], Michael O'Keeffe[a]

[a] *Teagasc, Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland*
[b] *Department of Risk Research, Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK*
[c] *Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stormont, Belfast BT4 3SD, UK*
[d] *Institute of Food Hygiene and Bromatology, Veterinary Faculty of the University of Ljubljana, Gerbičeva 60, SI-1000, Ljubljana, Slovenia*

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Abstract

The macrocyclic lactones (MLs) are probably the anti-parasitic agents most widely used in the treatment of food producing animals, poultry, aquaculture and crops. Ivermectin was the first macrocyclic lactone product to be licensed for use about 20 years ago. A number of alternative products such abamectin, doramectin, emamectin, eprinomectin, moxidectin, milbemycin and selamectin, have been marketed since. Because of the increase in the number of ML drugs, there has been a steady increase in the number of published analytical methods for determination of their residues. In this paper, the structure and properties of the different ML drugs available on the market are described. The occurrence and persistence of ML residues in food is discussed in relation to marker residues and current maximum residue limits (MRLs) as defined in the European Union (EU). Methodologies for determination of ML residues in biological matrices are described in terms of extraction and clean-up methods used for different matrices. Detection systems for determination of ML residues are discussed with a particular emphasis placed on new developments in screening technologies and liquid chromatography with fluorescence or mass spectrometry.
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Keywords: Avermectins; Milbemycins; Extraction; SPE; Derivatisation; HPLC Fluorescence; LC–MS/MS

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* Corresponding author. Tel.: +353 1 8059919; fax: +353 1 8059550.
E-mail address: martin.danaher@teagasc.ie (M. Danaher).

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1. Introduction

The avermectins and milbemycins belong to a family of compounds called the macrocyclic lactones (MLs) and are natural fermentation products of soil dwelling streptomycete micro-organisms [1]. MLs have found widespread application in human and animal health and crop protection. MLs were initially investigated for treatment of parasitic infections in animals but their potential for use in human medicine was quickly identified [2]. The most important application of MLs in human health is the use of ivermectin against the human parasitic infection, *Onchocerca volvulus* (river blindness) [3]. It has been donated free of charge by the Merck Corporation for treatment of river blindness to those who need it and for as long as it is needed [2]. Other applications in human health include the treatment of scabies [4], head lice [5] and parasitic infections in AIDS patients [6].

The discovery of ivermectin and its subsequent commercialisation led to considerable chemical and microbiological efforts to explore the general structure. As a result more drugs having specialist applications have been developed. Six avermectins and two milbemycins are currently licensed as veterinary drugs and insecticides. Abamectin [7] and ivermectin [8] are the best known, because of their long and widespread usage. A num-

ber of other compounds have been marketed more recently, doramectin [9], emamectin benzoate [10], eprinomectin [11], moxidectin [12], milbemycin oxime [13] and selamectin [14]. Eprinomectin [15] was the first avermectin drug licensed for treatment of parasitic infections in lactating cows and emamectin [16] was licensed for treatment of sea-lice on farmed salmon. Milbemycin oxime and selamectin have found application in the treatment of *Dirofilaria immitis* (heartworm) in dogs and cats [13,14,17].

The aim of this paper is to give a comprehensive overview of methodologies for determination of ML residues in biological matrices, with a focus on food safety. Background information is presented on the mode of action, activity and toxicity of ML veterinary drugs. Current listings of MRLs for ML veterinary drugs are presented and discussed in relation to different animal species and tissues. The influence of the pharmacokinetics of ML veterinary drugs on the presence of residues in food of animal origin is described, along with factors that can affect pharmacokinetics. Methodology for determination of ML residues is presented, covering extraction and clean-up procedures and detection methods. The need for continued development of improved multi-residue methods for determination of ML residues is highlighted, together with the requirement to validate methods according to validation criteria established in the EU.

2. Mode of action, biological activity and toxicity

2.1. Mode of action of macrocyclic lactones

Avermectins and milbemycins are believed to have a common mode of action against parasites. Initially it was thought that milbemycins had a different mode of action from the avermectins, suggesting that milbemycins could be used against parasites that had built up a resistance to avermectins [1,18].

It has been proposed that the mode of action of MLs is based on their interaction with the receptor channels for inhibitory neurotransmitters. An overview of the operation of neuromuscular transmission is well described by Bloomquist [19]. In vertebrates, gamma-amino butyric acid (GABA) and glycine are known to block electrical activity in nerve and muscle cells by increasing the conductance of chloride ions. In invertebrates, GABA and glutamate block electrical activity via a similar mechanism. During this process, the inhibitory neurotransmitter (GABA, glycine or glutamate) is released from the presynaptic nerve terminal and it binds to a postsynaptic receptor protein containing an intrinsic chloride channel. When the inhibitory neurotransmitter binds to the receptor, the channel is opened and chloride ions flow into the postsynaptic neuron. This chloride permeability can significantly hyperpolarize (make more negative) the membrane potential and have a dampening effect on nerve impulse firing. This process may be reversed by the addition of picrotoxin [19].

MLs also bind to these receptors but with high affinity in an irreversible process. Binding of MLs to GABA [20], glutamate [21] and glycine receptors [22] has been observed. Interaction of MLs with GABA receptors was observed only at very high doses, indicating that the mechanism of action is not solely explained by interaction with these receptors. MLs have been found to bind to glycine receptors, which are found only in the musculature of vertebrates but not in invertebrates [22]. In contrast, glutamate receptors are present in the musculature of invertebrates but not in vertebrates. Schaeffer and Haines showed that ivermectin binds with high affinity to glutamate receptors at therapeutic doses [20]. Electrical studies have shown that this results in an irreversible increase in membrane conductance leading to paralysis of the somatic musculature and particularly of the pharyngeal pump. As a result, it has been proposed that the mode of action of MLs is related to their interaction with glutamate receptors. It is difficult to say if this is the only mechanism of action of these drugs but it is the most important one identified.

2.2. Activity

Ivermectin was the first anti-parasitic agent that showed broad-spectrum activity against both nematodes and arthropods [8]. This unique broad spectrum activity against both endo- and ecto-parasites has resulted in the MLs being classified as endectocides. MLs are not effective against trematodes and cestodes. To compensate for this, some manufacturers have combined them with other drugs to broaden their spectrum of activity. In one such product, ivermectin has been combined with clorsulon to add liver fluke efficacy in treatment of cattle, goats, pigs and horses [17]. Since the introduction of ivermectin, a number of alternative ML drugs have been developed [1]. Each drug has its own fingerprint spectrum of activity against parasites, showing activity against certain parasites at low dose (5 μg/kg), while for other parasites a much higher dose is required (100 μg/kg) [1]. Essentially, all MLs show broad spectrum activity at a dose of 200 μg/kg. Most products used for treatment of food producing animals are formulated as broad-spectrum products at doses of ≥200 μg/kg. However, a number of narrow spectrum products have been formulated at low doses because of risk of toxicity to the host, as is the case in certain genetic lines of collie dogs. In these narrow spectrum applications, products have been formulated at low doses of 3–6 μg/kg to offer specific activity against heartworm in dogs, with a reasonable margin of safety [13,14,17].

2.3. Toxicity

MLs are toxic and their safe use in animals depends on the therapeutic dose against the parasite being much lower than their toxic effect level for treated animals [17]. MLs are relatively safe for cattle, pigs, sheep and other approved species. Toxic effects have been observed in certain genetic lines of collies [23] and Murray Grey cattle [24] treated with ivermectin and abamectin, respectively. In both of these studies, very high levels of the drugs were detected in the brain tissues of animals after death. It was proposed that the elevated levels of the drugs in the brain tissue could be related to a deficiency of P-glycoprotein in these species [25]. P-glycoprotein acts as a transmembrane protein, transporting certain drugs in and out cells. This reduces tissue distribution and bioavailability of the drug, and enhances the elimination of the drug. It also limits the entry of drugs into potentially

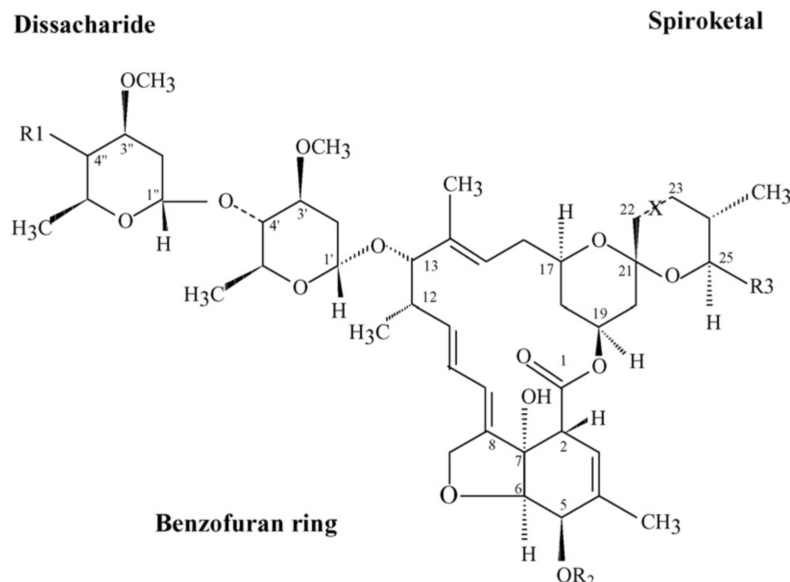

Fig. 1. Chemical structures of avermectins. (Adapted and modified from reference [26], with permission from Springer–Verlag, Copyright (1989).)

Avermectin	R1	R2	R3	C_{22}–x–C_{23}
A_{1a}	OH	CH_3	$CHCH_3CH_2CH_3$	—CH=CH—
A_{1b}	OH	CH_3	$CHCH_3CH_3$	—CH=CH—
Avermectin B_{1a}	OH	H	$CHCH_3CH_2CH_3$	—CH=CH—
Avermectin B_{1b}	OH	H	$CHCH_3CH_3$	—CH=CH—
A_{2a}	OH	CH_3	$CHCH_3CH_2CH_3$	—CH_2—CHOH—
A_{2b}	OH	CH_3	$CHCH_3CH_3$	—CH_2—CHOH—
B_{2a}	OH	H	$CHCH_3CH_2CH_3$	—CH_2—CHOH—
B_{2b}	OH	H	$CHCH_3CH_3$	—CH_2—CHOH—
Doramectin	OH	H	C_6H_{11}	—CH=CH—
Emamectin Benzoate B_{1a}	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	—CH=CH—
Eprinomectin B_{1a}	$NHCOCH_3$	H	$CHCH_3CH_2CH_3$	—CH=CH—
Ivermectin B_{1a}	OH	H	$CHCH_3CH_2CH_3$	—CH_2—CH—

sensitive areas such as the central nervous system (CNS). Animals showing decreased P-glycoprotein activity show greater bioavailability of drug after oral administration and accumulate greater levels of drugs in their CNS tissue. This potential toxicity has raised concern over the use of macrocyclic lactones in human medicine. However, evidence from the use of ivermectin in worldwide onchocerciasis treatment programmes suggests that hypersensitive individuals, if they exist, must be quite rare [2].

3. Properties of macrocylic lactones

MLs are large complex ringed structures; avermectins have a 16-membered macrocyclic ring, containing a spiroketal group, a benzofuran ring and a disaccharide functionality (Fig. 1) or, in the case of selamectin, have only a monosaccharide group (Fig. 2A) [17,26]. Milbemycins are structurally similar to avermectins but lack the disaccharide group (Fig. 2B–E). A number of different avermectins have been identified in the fermentation products of *Streptomyces avermitilis* [26]. The following nomenclature has been assigned to these compounds A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a} and B_{2b} [26]. Three of these compounds A_{2a}, B_{1a} and B_{2a}, are major fermentation products. However, B_{1a} showed much greater activity than the other homologues. After purification of the fermentation broth, only the B_1 isomers remained in large quantities. The B_{1a} and B_{1b} homologues have almost identical activities but B_{1a} is produced in much greater amounts than B_{1b}. As a result, avermectin B_1 (generically known as abamectin) is the most important naturally produced avermectin. Ivermectin is it's semi-synthetic derivative, produced by saturation of a double bond between C22 and C23. With this selective hydrogenation ivermectin keeps a "chair" conformation, typical of the B_2 avermectin family. Ivermectin keeps excellent antiparasitic activity (typical for B_1 avermectins) and lower toxicity (typical for B_2 avermectins) [1]. The specification for most avermectins is normally defined as greater than 80% B_{1a} and less than 20% B_{1b} [26].

4. Factors affecting the occurrence of macrocyclic lactones in animal tissues

4.1. Route and mode of administration

It has been shown that the route of administration for ML drugs greatly affects pharmacokinetic parameters such as max-

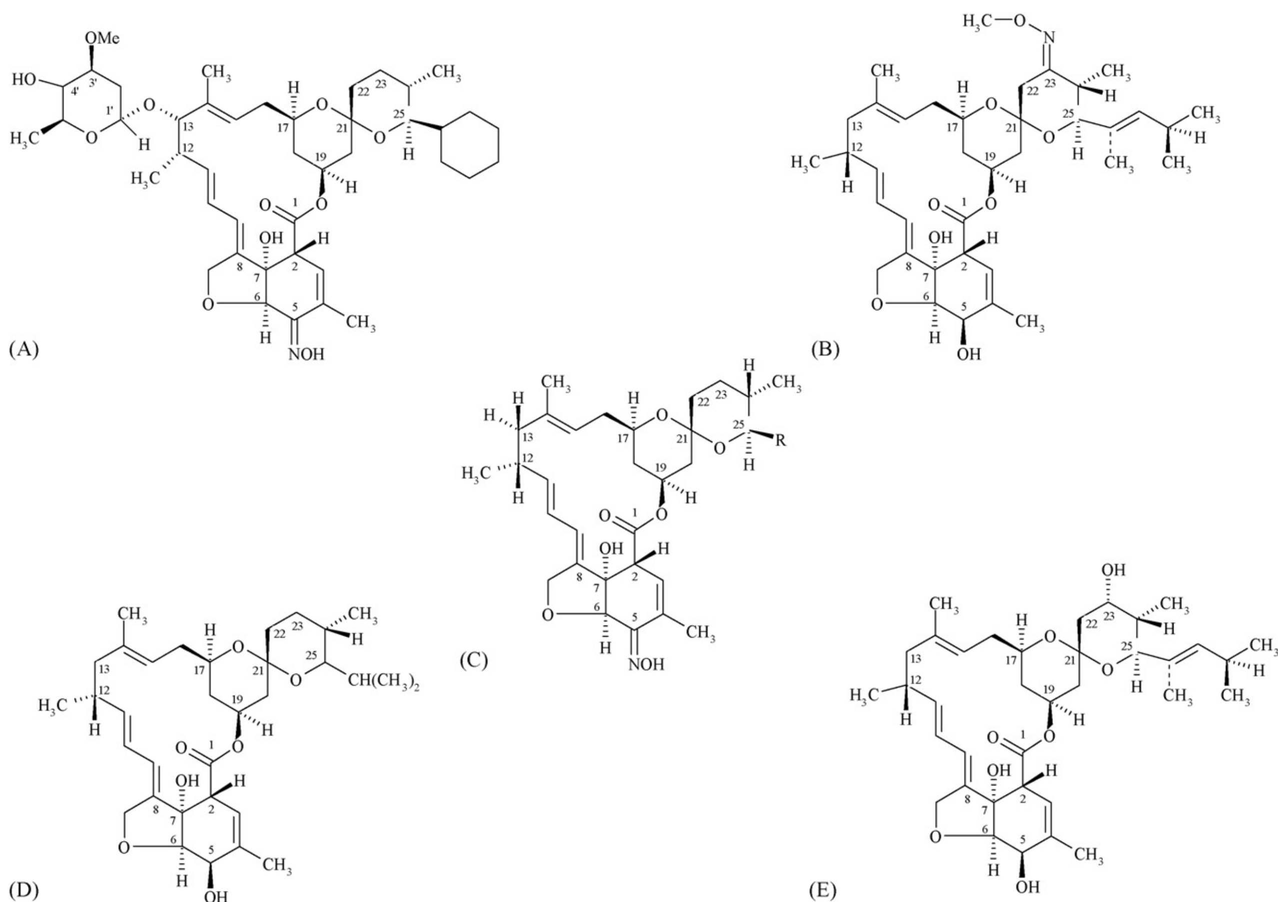

Fig. 2. Structures of (A) selamectin, (B) moxidectin, (C) milbemycin oxime A3 (R = CH_3) and A4 (R = C_2H_5), (D) milbemycin D and (E) nemadectin.

imum concentration (C_{max}), time of C_{max} (T_{max}), and drug half-life. The half-life of ivermectin in cattle after administration via oral, subcutaneous and topical routes is 2.7, 5.5 and 5.3 days, respectively [27,28]. In dog and cat, similarly high C_{max} values and short T_{max} values were observed after administration of oral doses of selamectin [29]. However, lower C_{max} values and longer T_{max} times were observed in dog in comparison to cat, after topical administration of selamectin. It has been proposed that the difference in selamectin pharmacokinetics in cat may be due to self-grooming. The practice of feed withdrawal before oral treatment has been shown to increase the absorption of ivermectin, leading to increased anthelmintic efficacy [30].

4.2. Drug formulation

The liquid in which the drug is formulated affects the distribution of ivermectin after subcutaneous injection. Lo et al. showed that ivermectin was absorbed three-times more slowly when administered in propylene glycol-glycerol formal compared to an aqueous-based formulation [31]. Hayes et al. found that when moxidectin was administered in an aqueous injectable-based formulation the rate of absorption into the bloodstream increased dramatically compared with oil-based formulations [32].

4.3. Physiochemical properties of the drug

Lower C_{max} and longer T_{max} values have been observed for moxidectin in comparison to ivermectin [32]. It has been concluded that this is because moxidectin is 100-times more lipophilic than ivermectin and accumulates in adipose tissue. This results in a lower partitioning between fat and plasma resulting in a slower elimination of residues from the body. In contrast, eprinomectin is a polar ML, with a lower association with lipids, resulting in a shorter drug half-life and higher C_{max} values compared with other topically applied MLs in cattle [33].

4.4. Animal species

Chiu et al. demonstrated that the half-life for ivermectin was shorter in pigs compared to cattle [34]. It was proposed that the rate of metabolism in pigs [34,35], and possibly in goat [36], is faster than in cattle [37,38]. Similarly, short drug half-lives have been observed for doramectin in goat [39] and pig [40] compared to cattle [41], sheep [42].

4.5. Body condition

It has been shown by a number of researchers that body condition (fat versus lean tissue) has an influence on drug phar-

macokinetics. In the case of ivermectin, longer half-lives have been observed in fatty species (pig and sheep) compared to cattle [43]. Craven et al. found for pigs (lean versus fatty animals) that shorter elimination half-lives were observed for lean animals [44]. Similar profiles have been observed for moxidectin in fatty and lean species. A lower area under the curve (AUC) has been observed for moxidectin in lean goats compared to fatty goats, whereas AUC values were similar for eprinomectin in these animals [43].

5. Metabolism and distribution of macrocyclic lactone residues

5.1. Ivermectin

Campbell et al. showed that ivermectin is not extensively metabolised in mammals, with 90% of the dose being excreted in the faeces and tissue residues occurring mainly in the form of the parent drug [45]. Chiu et al. demonstrated that ivermectin residues were present at higher concentrations in the liver and fat tissues of cattle, with lower levels being detected in muscle tissue [46]. The parent drug accounts for at least 50% of total residues in tissues from cattle, sheep, pigs and rats up to 14, 5, 7 and 3 days after treatment, respectively. Hoy et al. investigated the fate of ivermectin in salmon and found that the parent drug accounted for 42% of residues 15 days after treatment [47]. Unidentified polar metabolites were also observed in the tissues of salmon.

The major metabolites of ivermectin in the liver of cattle, sheep and rat have been identified as 24-hydroxymethyl-H_2B_{1a} and the monosaccharide of 24-hydroxymethyl-H_2B_{1a}. In fat tissue, more non-polar products were found. Chiu et al. proposed that these non-polar products were due to fatty acid esterification of the 24-hydroxymethyl metabolites [48,49]. The half-life of ivermectin in the liver and fat tissues of cattle was 118 and 182 h, respectively. In pigs, the metabolism pattern in the fat tissue is different to that observed in cattle, sheep and rats. The major metabolites have been identified as 3″-*O*-desmethyl metabolites, rather than 24-hydroxymethyl identified in cattle, sheep and rats. In contrast to the 24-hydroxymethyl metabolites, the 3″-*O*-desmethyl is less available for esterification. As a result, non-polar ester metabolites are not formed, reducing the deposition of ivermectin in fat. Accordingly, the half-life of ivermectin in fat and liver is of similar duration (120 h) in pig.

5.2. Abamectin

In a study in sheep, results indicated that unmetabolised abamectin accounted for majority of residues in tissues [50]. Unmetabolised abamectin was found to be the major residue found in liver and kidney, along with lower levels of an unidentified polar metabolite. Maynard et al. investigated the fate of abamectin in lactating goats administered with abamectin daily for 10 days [51]. It was shown that 85–99% of the residues in edible tissue, milk and faeces were in the form of unmetabolised abamectin, 24-hydroxymethyl and 3″-*O*-desmethyl B_{1a} metabolites. 24-hydroxymethyl and 3″-*O*-desmethyl B_{1a} metabolites accounted for 3–10 and 1–5% of total residues, respectively.

5.3. Doramectin

In cattle, doramectin was shown to account for 70% of residues in liver 3 days after treatment [52]. In studies carried out in rats and dogs, doramectin accounted for 18 and 28% of residues in liver 2 days after treatment [52]. The major metabolite identified in cattle liver was 3″-*O*-desmethyl-doramectin (8–19% of total residues) with 24-hydroxymethyl-doramectin and 24-hydroxymethyl-3″-*O*-desmethyl-doramectin being identified as minor metabolites. A 2-epimer metabolite of doramectin was identified in cattle fat.

In pigs, unmetabolised doramectin was shown to account for 45–59% of the total residues, 7–21 days after treatment [53]. 3″-*O*-desmethyl-doramectin was identified as the major metabolite in edible tissue. Residues were found at highest levels in fat followed by liver, kidney and muscle. In sheep, the parent drug accounted for 67–92% of total residues in fat, liver, kidney and muscle 14 days after treatment [53]. The major metabolite identified in liver was 3″-*O*-desmethyl-doramectin. Low levels of the 2-epimer of doramectin were detected in fat.

5.4. Moxidectin

Stout et al. proposed that monohydroxy metabolites were the principal metabolites of moxidectin found in tissues and that dihydroxy and *O*-desmethyl-dihydroxy metabolites were found at lower levels [54]. Zulalian et al. investigated the distribution of moxidectin residues in cattle, identifying a number of monohydroxy, dihydroxy and *O*-desmethyl-dihydroxy metabolites [55]. However, the parent drug was found to be the major residue present in fat, liver, kidney and muscle, accounting for 90, 36, 77 and 50% of total residues in these tissues, respectively. Highest residue levels were detected in fat tissue. Afzal et al. found that moxidectin accounted for 91, 51, 52 and 92% of total residues in the fat, liver, kidney and muscle tissues of sheep, respectively [56]. Subsequently, Afzal et al. found that moxidectin accounted for 85–89, 60, 80 and 48% of total residues in horse fat, liver, kidney and muscle, respectively [57].

5.5. Eprinomectin

In cattle, unmetabolised eprinomectin accounted for 80, 100, 78 and 75% of total residues in liver, fat, kidney and muscle tissue, respectively, 21 days post-treatment [15]. Seven minor metabolites of eprinomectin were identified in edible tissue (most in the range 1–2% of total residues) with one metabolite accounting for 3.9% of total residues.

5.6. Emamectin

In salmon, unmetabolised emamectin was the major residue found in muscle and skin tissue, accounting for 98 and 83% of total residues at 12 h and 90 days, respectively [16]. The major metabolite identified in tissue was *N*-desmethylated emamectin,

which accounted for 6 and 15% of residues at 7 and 90 days, respectively. A second metabolite, 4″-deoxy-4″-epi-(*N*-formyl-*N*-methyl)amino-avermectin B_1, was found to be present at 1% of total residues, but only at early times post-treatment.

5.7. Summary

The metabolism of ML drugs in animal tissues is well described. It has been shown that the parent drugs are the major residues found in tissues and, as a result, parent drugs are the most suitable marker residues. ML residues are found to occur in liver and fat tissues at higher levels than in kidney and muscle tissues.

6. Depletion of macrocyclic lactone residues in tissues

6.1. Ivermectin

Chiu et al. investigated the depletion of ivermectin in the tissues of cattle, pigs and sheep [58]. In cattle administered with a subcutaneous (sc) injection of 0.3 μg/kg bodyweight (bw) the level of marker residue, ivermectin H_2B_{1a}, was below the MRLs in liver and fat at 14 days post-treatment, respectively. The MRL for ivermectin is 30, 100 and 100 μg/kg in the kidney, liver and fat tissue in all mammalian food-producing species, respectively. At 7 days post-treatment, ivermectin H_2B_{1a} was determined at levels of 348 and 134 μg/kg in liver and fat, respectively. In sheep treated with an intraruminal dose (300 μg/kg bw), ivermectin H_2B_{1a} was below the MRL in liver and fat tissues at 3 days post-treatment. Highest residue levels were determined in these tissues at 1 day post-treatment, 114 and 174 μg/kg in liver and fat, respectively. In pigs (sc 400 μg/kg bw), ivermectin residues were below the MRL in liver and fat tissues at 1 and 7 days post-treatment, respectively. Highest residue levels were detected at 1 day post-treatment, 72 and 165 μg/kg in liver and fat, respectively.

Two studies have been carried out on the depletion of ivermectin residues in deer and reindeer after administering topical (1 mg/kg bw) and sc (0.2 mg/kg bw) doses, respectively [59]. In deer and reindeer, ivermectin residues were below the MRL at 28 and 17 days post-treatment, respectively. In red deer, highest residue levels were found in fat, followed by liver, kidney and muscle tissues. At 7 days withdrawal, ivermectin H_2B_{1a} levels in the fat, liver, muscle and kidney tissues of red deer were 294, 180, 78 and 78 μg/kg, respectively. In reindeer, at 10 days withdrawal, ivermectin H_2B_{1a} residues in the back fat, liver, muscle and kidney were 362, 71, 40 and 54 μg/kg, respectively.

Roth et al. investigated the depletion of ivermectin residues in salmon tissue [60]. Ivermectin was administered to salmon in medicated feed at a rate of 50 μg/kg bw per day once a week for 9 weeks. Degree days (°D) were used to account for variability in water temperature during experiments (3 days at a water temperature of 10 °C would equal 30°D). In this study it was defined as the cumulative daily water temperature for each sample day. Ivermectin H_2B_{1a} was determined at levels of 117 and 83 μg/kg in muscle and skin tissue, respectively, at 10°D post-treatment. Ivermectin H_2B_{1a} was determined in salmon muscle (7.2 μg/kg) and skin tissues (17.2 μg/kg) at 250 and 750°D post-treatment.

6.2. Abamectin

In sheep (oral dose 286 μg/kg bw), avermectin B_{1a} was below the MRL in all tissues at 10 days withdrawal with the exception of liver, which contained residues at levels of <10–31 μg/kg [50]. The MRL for abamectin is 20, 25, 20 and 50 μg/kg in the muscle, liver, kidney and fat of sheep, respectively. Highest residue levels were determined at 3 days post-treatment at levels of 37, 226, 74 and 307 μg/kg in muscle, liver, kidney and fat tissues, respectively.

6.3. Doramectin

In cattle (intramuscular injection 200 μg/kg bw), doramectin was below the MRL in tissues at 42 days post-treatment [61]. The MRLs for doramectin in cattle are 10, 100, 30 and 150 μg/kg in muscle, liver, kidney and fat tissues, respectively. Highest residue levels were detected in muscle, liver, kidney and fat tissues at 7 days post-treatment, 33, 319, 96 and 493 μg/kg, respectively. In pigs (intramuscular injection 200 μg/kg bw), doramectin was below the MRL in tissues at 21 days post-treatment [53]. The MRLs for doramectin in pig, sheep and deer are 20, 50, 30 and 100 μg/kg in muscle, liver, kidney and fat tissues, respectively. Highest residue levels were reported in muscle, liver, kidney and fat tissues at 7 days post-treatment, 7, 66, 23 and 242 μg/kg, respectively. In crossbred pigs (intramuscular injection 375 μg/kg bw), doramectin was below the MRL in tissues at 35 days post-treatment. Highest residue levels were reported in the muscle, liver, kidney and fat tissues at 7 days post-treatment, 40, 160, 80 and 470 μg/kg, respectively. In sheep (sc injection 300 μg/kg bw), doramectin was below the MRL at 14 days post-treatment. Highest residue levels were reported in muscle, liver, kidney and fat tissues at 14 days post-treatment, 14, 48, 18 and 63 μg/kg, respectively. In deer (sc injection 200 μg/kg bw), doramectin was below the MRL in tissues at 21 days post-treatment [53]. Highest residue levels were detected in muscle, liver, kidney and fat tissues at 10 days post-treatment, 13, 66, 31 and 66 μg/kg, respectively.

6.4. Moxidectin

In cattle (sc injection 200 μg/kg bw), highest residues levels (total radioactive residues) of 898, 636 and 275 μg/kg were detected in abdominal fat at 7, 14 and 28 days post-treatment [54]. Much lower residue levels were detected in liver (8–9 times lower) and muscle (40–70 times lower) at these time-points. Results were reported for the marker residue (moxidectin parent drug) at 28 days post-treatment only, at which time levels were below the MRL in all tissues. The MRLs for moxidectin cattle, sheep and horse are 50, 100, 50 and 500 μg/kg in muscle, liver, kidney and fat tissues, respectively. Moxidectin levels in abdominal fat, back fat, liver, muscle and kidney at this time-point were 250, 159, 11, 2 and 10 μg/kg, respectively.

In sheep (sc injection 200 μg/kg bw), residues were below the MRL in all tissues 21 days post-treatment [62]. At 21 days post-treatment residues were determined in fat, liver and muscle tissues at levels of 212, 23 and 8 μg/kg, respectively. In a separate study in sheep (sc injection 200 μg/kg bw), moxidectin was below the MRL in tissues at 10 days post-treatment [63]. At 10 days post-treatment, moxidectin was detected in fat, muscle, liver and kidney tissues at levels 222, 41, 21 and 13 μg/kg, respectively. In sheep administered with two doses of moxidectin (sc 200 and 200 μg/kg bw 10 days apart), residues were found to be below the MRL in tissues at 10 days post-treatment [63]. Moxidectin was detected at this withdrawal period in fat, muscle, liver and kidney tissues at levels 324, 29, 29 and 13 μg/kg, respectively. In horses (oral dose 400 μg/kg bw), moxidectin was below the MRL in tissues at 28 days post-treatment [64]. At this time-point, moxidectin was detected in fat at levels of 221 μg/kg. Residues were all below the limit of quantitation in other tissues and no studies were carried out at earlier withdrawal periods.

6.5. Eprinomectin

In cattle (pour-on, 500 μg/kg bw, radiometric study), residues were found to be below the MRL in tissues at 7 days post-treatment [15]. At this time-point, eprinomectin B_{1a} was detected in muscle, liver, kidney and fat tissues at levels of 6, 807, 161 and 30 μg/kg, respectively. The MRLs for eprinomectin in cattle are 50, 1500, 300 and 250 μg/kg in muscle, liver, kidney and fat tissues, respectively. At 21 days post-treatment, eprinomectin B_{1a} residues had depleted to levels of 3, 369, 54 and 14 μg/kg, respectively. In a non-radiometric study on cattle (pour-on 500 μg/kg bw), eprinomectin B_{1a} was detected at 10 days post-treatment in muscle, liver, kidney and fat tissues at levels of 8, 748, 74 and 26 μg/kg, respectively [15]. At 17 days post-treatment, eprinomectin B_{1a} residues had depleted to levels of <2, 237, 40 and 8 μg/kg, respectively. At 44 days post-treatment only trace residues were detectable in liver tissue.

In non-ruminating calves (pour-on, 500 μg/kg bw), residues were found to be below the MRL in tissues at 7 days post-treatment [65]. At this time-point, eprinomectin was detected in muscle, liver, kidney and fat tissues at levels 48, 1220, 237 and 287 μg/kg, respectively. At 14 days post-treatment, eprinomectin residues had depleted to 22, 803, 120 and 103 μg/kg, respectively.

6.6. Emamectin

In salmon (50 μg/kg bw per day for 7 days), emamectin residue levels were reported as separate results in muscle and skin but not the target tissue, which is described as muscle and skin in natural proportions [16]. At 12 h and 30 days post-treatment, emamectin was detected in muscle at 67 and 20 μg/kg and skin at 124 and 39 μg/kg, respectively.

6.7. Summary

The persistence of ML residues in animal tissues depends on the drug, route of administration and animal species. MLs may be administered to cattle in pour-on, sc injection, intramuscular injection or bolus formulation. It has been shown in the studies described that ML residues are more persistent when administered by sc injection compared to pour-on. As a result, longer withdrawal periods in the range of 34–45 days are required to ensure that ML residues are below the MRLs after sc injection. Shorter withdrawal periods are typically required for pour-on compared to other treatment routes. Ivermectin, moxidectin, and eprinomectin pour-on formulations have withdrawal periods of 28, 14 and 17 days, respectively. However, doramectin is much more persistent than other ML pour-on formulations and a withdrawal period of 35 days is specified. In sheep, similar withdrawal periods (to cattle) are required after administration of MLs by sc injection and pour-on. In pigs, MLs may be administered in feed, by sc injection or by intramuscular injection. After sc injection of ivermectin, a shorter withdrawal period of 28 days (compared to cattle) is required. However, after intramuscular injection of doramectin to pigs a withdrawal period of 49 days is required compared to cattle.

7. Depletion of macrocyclic lactone residues in milk

7.1. Ivermectin

Ivermectin is not licensed for use in lactating species in the EU. However, a number of studies have been carried out into the depletion of ivermectin residues after administration to lactating cows [37,66], goats [67], sheep [68] and buffalo [69]. Alvinerie et al. found that residue levels of ivermectin (sc 200 μg/kg bw), reached a maximum concentration of 7.26 μg/kg in goat milk at 2.8 days post-treatment and residues were detectable at >0.05 μg/kg for 25 days post-treatment [67]. Toutain et al. found that residue levels of ivermectin (sc 200 μg/kg bw), reached a maximum concentration of 41 μg/kg in cows milk at 1.8 days post-treatment and residues were detectable for 29 days after treatment [37]. They estimated that 5% of the ivermectin dose was secreted in the milk. Cerkvenik et al. found that residue levels of ivermectin (sc 200 μg/kg bw) reached a maximum concentration of 23 μg/kg in sheep milk at 1.3 days post-treatment and residues were detectable 23 for days after treatment [68]. Anastassio et al. found that residue levels of ivermectin (sc 200 μg/kg bw), reached a maximum concentration of 29 μg/kg in buffalo milk at 2.5 days post-treatment and residues were detectable at 20 days post-treatment at a level of 0.6 μg/kg [69]. Cerkvenik-Flajs et al. investigated the fate of ivermectin residues in ewes' milk and dairy products (raw bulk milk, yoghurt from raw and pasteurised milk, cheese and whey) from ewes undergoing a residue depletion trial [70]. Highest levels of ivermectin residues were determined at 2 days withdrawal in raw milk (22 μg/kg), yoghurt (23 μg/kg), cheese (96 μg/kg) and at 1 day withdrawal in albumin cheese (31 μg/kg). This work demonstrates the high stability of ivermectin during lactic acid fermentation and thermal treatment processes.

7.2. Abamectin

Cerkvenik-Flajs et al. monitored the depletion of abamectin residues in sheep milk after administration of the drug (sc

200 μg/kg bw), finding that residue levels fell below the limit of detection of the method (0.04 μg/kg) at 23 days post-treatment [71]. Abamectin is not licensed for application to lactating species in the EU. The highest concentration of abamectin detected in milk from an individual sheep was 37.5 μg/kg. The C_{max} and T_{max} values for abamectin in sheep's milk, as calculated from data collected in this study, were 26.8 μg/kg and 2 days, respectively.

7.3. Doramectin

Imperiale et al. found that residue levels of doramectin (sc 200 μg/kg bw), reached a maximum concentration of 79.8 μg/kg in sheep milk at 3 days post-treatment and residues were detectable (>1.0 μg/kg) for 30 days after treatment [72]. Doramectin is not licensed for application to lactating species in the EU. It was estimated that 2.4% of the total dose of doramectin was excreted in milk. Cerkvenik-Flajs et al. investigated the pharmacokinetics of doramectin residues in ewes' milk (sc 200 μg/kg bw), finding that residue levels fell below the limit of detection of the method at 37 days post-treatment [73]. The maximum concentration of doramectin found in ewes' milk was 31 μg/kg. The C_{max} and T_{max} values for doramectin in ewes' milk, as calculated from data collected in this study, were 31 μg/kg and 3 days, respectively. The authors concluded that the half-life of doramectin was 1.6-times longer than that for abamectin and ivermectin in milk.

7.4. Moxidectin

Three studies have been carried out on the depletion of moxidectin residues in dairy cows after treatment with a pour-on dose of 500 μg/kg [74]. The highest level of moxidectin detected in milk in the three studies was 34 μg/kg at 5.5 days post-treatment (or the 11th milking), which was less than the MRL of 40 μg/kg. Residues were found to be less than 10 μg/kg at 10–13 days post-treatment.

7.5. Eprinomectin

In dairy cows (pour-on, 500–547 μg/kg bw), highest levels of eprinomectin were found at the first 5–6 milkings post-treatment at levels of 5 μg/kg and residues were below 0.5 μg/kg at the 13th milking [15]. The MRL for eprinomectin in bovine milk is 20 μg/kg. Alvinerie et al. found similar milk depletion characteristics for eprinomectin in dairy cows with C_{max} values ranging from 3 to 8 μg/kg after 1.5–2.5 days [33]. They estimated that only 0.1% of the eprinomectin dose was eliminated in the milk of dairy cows compared with as much as 5% of the dose for ivermectin and moxidectin. It was proposed that lower levels of eprinomectin occur in milk because eprinomectin has a lower milk–blood distribution constant than ivermectin and moxidectin (0.1 versus 1.0).

7.6. Summary

After treatment of cows, buffalo, goats and sheep with ivermectin, residues were found at levels of 41, 29, 7 and 23 μg/kg in milk at early withdrawal periods. Ivermectin residues were detectable in cows, buffalo, goats and sheep milks for 29, 20, 25 and 23 days post-treatment. In sheep milk, abamectin residues were detectable for 23 days post-treatment. Doramectin residues were shown to be more persistent in sheep milk being determined at levels of 80 μg/kg at early withdrawal periods and being detectable at 30–37 days post-treatment. In dairy cows, it was shown that moxidectin could be detected at a level of 34 μg/kg at early withdrawal periods and was detectable for 10–13 days post-treatment.

8. Monitoring for macrocyclic lactone residues in food

Chiu et al. demonstrated, using radiolabelled ivermectin, that bound residues are not an issue and that residues containing the radiolabelled fraction of the molecule are readily extracted from incurred tissue using organic solvent [46,75]. MLs are not extensively metabolised and the marker residue is defined as the parent drug [46,54–57,62,75–77]. In the case of certain avermectins, the marker residue is more specifically defined as the B_{1a} homologue. The liver and fat tissues are normally chosen as the target tissues for monitoring because residues are more persistent in these tissues and they are the only tissues that have MRLs defined for all animal species [78]. The MRLs defined for MLs in fat and liver are higher than kidney and muscle tissues, indicating that fat and liver are more suitable tissues for residue analysis (Table 1). In crop protection, the parent drug, generally, is the major residue that occurs after application of MLs to crops. On exposure to light, it has been shown that these molecules can isomerise to form an 8,9-*Z*-isomer [79–82]. On derivatisation, abamectin and it's 8,9-*Z*-isomer were converted into the same fluorescent derivative [83].

In recent years, there have been concerns over the presence of ivermectin residues in milk and farmed salmon. Residues of this drug should not be detected in either of these matrices, as it is not licensed for use in the treatment of lactating species or farmed fish. Previously, ivermectin was used in the treatment of parasitic infections in farmed salmon on the basis that no other suitable product was available. However, because of fears of bioaccumulation of ivermectin residues in marine sediment [84] and possible risk to sediment dwelling organisms [85], this practice has been stopped. An alternative product, emamectin, has been approved for treatment of farmed salmon in recent years. The MRL for emamectin has been defined as 100 μg/kg in salmon muscle and skin in natural proportions.

There have been a number of publications on the depletion of ML residues in milk. However, eprinomectin is still the only ML product licensed for treatment of lactating species in the EU. The pharmacokinetic properties of eprinomectin are such that only a small fraction of the dose (0.1%) is excreted in milk after pour-on application to dairy cows and a zero withdrawal period has been set for milk. The MRL for eprinomectin in milk from dairy cows is 20 μg/kg. An MRL of 40 μg/kg has been set for moxidectin in bovine milk but no withdrawal period has been set. This may be because the withdrawal period for currently reg-

Table 1
MRL listings for macrocyclic lactone anthelmintic drugs

Drug	Marker residue	Animal species	MRL (μg/kg)	Target tissue
Abamectin	Avermectin B_{1a}	Bovine	10	Fat
			20	Liver
		Ovine	20	Muscle
			50	Fat
			25	Liver
			20	Kidney
Doramectin	Doramectin	Bovine	10	Muscle
			150	Fat
			100	Liver
			30	Kidney
		Porcine, ovine and deer, including reindeer	20	Muscle
			100	Fat
			50	Liver
			30	Kidney
Emamectin	Emamectin B_{1a}	Salmonidae	100	Muscle and skin in natural proportions
Eprinomectin	Eprinomectin B_{1a}	Bovine	50	Muscle
			250	Fat
			1500	Liver
			300	Kidney
			20	Milk
Ivermectin	Ivermectin H_2B_{1a}	All mammalian food-producing species	100	Fat
			100	Liver
			30	Kidney
Moxidectin	Moxidectin	Bovine, ovine	40	Milk
		Bovine, ovine	50	Muscle
		Equidae	500	Fat
			100	Liver
			50	Kidney

Data taken from reference no. [78].

istered moxidectin products would be overly long to support it's practical application in lactating species. Alternatively, it may be that sufficient data has not been provided for defining withdrawal period. It is expected that a ML product containing moxidectin will be developed and marketed for use on dairy animals in the near future. No other MLs are approved for treatment of dairy animals. There is concern that unapproved ML products may be used in dairy animals, especially when there are claims that use of the approved (and more expensive) drug eprinomectin can increase milk yield [86,87].

Concerns over the presence of ML residues in milk are supported by the fact that as much as 5% of the drug dosage may be excreted in milk after treatment with certain unapproved ML products [33,37,72]. In addition, a number of ML products are used for dry cow therapy in the winter months when cows are brought in from pasture. It is quite common to treat in-calf cows for parasitic infections during this period with an ML product because they will not be producing milk for a period of 8–10 weeks. If cows calve early, an adequate withdrawal period may not be met and residues may occur in milk. In some cases, cows have been known to calve as much as 30 days early. New sensitive methodologies are available that can detect ML residues in milk up to 37 days post-treatment, increasing the potential to detect ML abuse in lactating species [73].

9. Extraction of ML residues from biological matrices

A number of methods have been developed for determination of individual ML residues in milk [88–91], salmon [60,92–94] animal tissue [95–107], crops [108–116] and animal feed [117–119]. More recently, multi-residue methods, capable of determining two or more residues, have been reported [120–134]. Samples are typically extracted using organic solvent [83,88,92] and cleaned up by liquid–liquid partitioning [95], solid phase extraction [96,97,100] or immunoaffinity chromatography [116,128]. Alternatively, samples may be extracted using matrix solid phase dispersion (MSPD) [93,104] or supercritical fluids [135]. In some methods involving derivatisation prior to chromatography, sample extracts may require further post-derivatisation clean-up [95,136]. A number of methods involve simple solvent extraction and purification by a single SPE step prior to determination [76,92,96,120,126,129]. However, different combinations of liquid–liquid partitioning or SPE have been used also to purify sample extracts [88,95,96,98,137].

9.1. Blood and plasma

Early methods for determination of MLs in plasma usually involved extensive clean-up based on different combina-

tions of liquid–liquid extraction (LLE), liquid–liquid partitioning (LLP) and sorbent chromatography. Tolan et al. extracted abamectin and ivermectin from plasma using ethanol/water with partitioning into ethyl acetate [136]. Extracts were further purified before and after derivatisation using florisil and silica chromatography, respectively. Schnitzerling et al. extracted ivermectin from blood with ethanol followed by partitioning into acetone [137]. Protein was precipitated at 4 °C before addition of ethanol/water and partitioning into hexane. The hexane was extracted using acetonitrile, dried and reconstituted in tetrahydrofuran, before determination by LC-UV.

A number of methods have been published more recently based on a SPE clean-up using bonded silica phases. Chiou et al. extracted ivermectin from plasma and milk with methanol, before purifying extracts on C_2 SPE cartridges [138]. Extracts were derivatised and further purified on a diol SPE cartridge prior to determination. Nowakowski et al. later modified this method and applied it to the determination of doramectin in plasma samples [41]. Oehler et al. found C_{18} SPE to be a more effective purification sorbent for isolation of ivermectin from serum than C_2 SPE [139]. Dickinson et al. extracted ivermectin from blood serum and muscle tissue using acetonitrile, before purification on C_{18} SPE [97]. De Montigny et al. extracted ivermectin from plasma using acetonitrile/water and purified the extracts on C_{18} SPE [140]. Alvinerie et al. used a variation of this method for determination of ivermectin in goat plasma and milk [67]. Recently, Chen et al. developed a clean-up method using a polymeric SPE cartridge (Oasis HLB®) for determination of moxidectin in human plasma [141]. Moxidectin was extracted from plasma samples using acetonitrile/water, applied onto the SPE cartridge, washed with water and methanol/water and eluted with isopropyl alcohol.

The main application for plasma testing is in residue depletion studies. Because of the very large numbers of samples involved in such studies, researchers working in this area tend towards use of automated and miniaturised clean-ups. Lanusse et al. [38] automated the earlier method by Alvinerie et al. [66] and applied it to the determination of moxidectin, doramectin and ivermectin residues in cattle plasma, and subsequently to eprinomectin in plasma [142]. In separate work, Antonian et al. also developed automated clean-up procedures for eprinomectin in plasma [143]. Harrison et al. developed an automated 96-well plate extraction and clean-up procedure for determination of doramectin in plasma [144]. Plasma samples were extracted with acetonitrile/water, before transfer to a 96-well C_{18} SPE block and loaded under vacuum. The sorbent bed was washed with water, dried under vacuum and eluted with methanol into the wells of a 96-well plate. Extracts were evaporated to dryness and derivatised. The plates were sealed and inserted into the autosampler of a LC system for determination. Mitsui et al. found that a simple dilution of serum samples in phosphate buffer-BSA was sufficient for determination by immunoassay [145].

9.2. Tissue

Tissue samples need to be finely chopped or homogenised prior to extraction. In the case of homogenised samples, solvent may be added and extraction may occur with or without a further homogenisation step. Finely chopped samples are normally homogenised using a probe blender in the presence of solvent. Methodology has been developed for extraction and clean-up of ML residues from muscle, liver, kidney, and fat. Most reported tissue methods for determination of ML residues have been developed for liver and fat tissue, because these are the only tissues to have MRL listings for all mammalian species. Liver tissue is regarded as the matrix of choice for determination of residues but is particularly difficult to work with due to the complex matrix interference that may be present. Methods for determination of MLs in salmon muscle have been developed; in this species the muscle tissue is very oily and lipophilic in nature and contains higher levels of ML residues generally compared to animal muscle.

Methods for determination of ML residues in tissue are more involved than for most other matrices and generally require extensive clean-up. Tway et al. extracted ivermectin from muscle, liver, fat and kidney tissues with acetone/water followed by partitioning into isooctane [95]. Extracts had to be further purified using additional precipitation and partitioning steps prior to determination. Prabhu et al. [98] and Reising et al. [99] later applied this method to studies on ivermectin residues in tissues. Khunachak et al. extracted moxidectin from liver, muscle, fat and kidney using acetonitrile, before clean-up using hexane partitioning prior to determination [102]. Stout et al. later used the same procedure for determination of moxidectin in fat [54].

MSPD methods have been applied for isolation of residues from tissue [93,104]. In this procedure, samples are finely chopped before blending with C_{18} material prior to solvent extraction. Iosifidou et al. developed an MSPD method for determination of ivermectin in salmon muscle [93]. Salmon muscle was blended with C_{18} material and packed into a column between two filter paper frits. The column was washed with hexane and eluted with dichloromethane/ethyl acetate. Alvinerie et al. later modified this method and applied it to determine moxidectin residues in bovine liver [104].

Extract purification on SPE cartridges is currently one of the most widely used clean-up strategies. Norlander et al. developed one of the most widely used methods for determination of ML residues in biological matrices [96]. Ivermectin was extracted from pig tissues using acetonitrile and the extracts were purified on C_8 SPE. The method was subsequently applied by two groups to determine ivermectin residues in salmon tissue [60,92]. Dusi et al. modified the clean-up procedure developed by Norlander et al., finding that substituting C_{18} for C_8 provided cleaner extracts [126]. Salisbury et al. extracted ivermectin residues from muscle and liver using acetonitrile and passed extracts through deactivated alumina cartridges [100]. Extracts were further purified by C_{18} SPE, prior to determination. Similar procedures were developed by other researchers for determination of ivermectin in liver and fat [101,103,105]. Abjean et al. purified liver extracts on C_{18} SPE, derivatised the extracts and further purified the

derivatised extracts on the same C_{18} cartridges, which had been reconditioned [103]. The objective of this work was to separate ivermectin derivatives from the derivatising reagents, which were found to affect the thin layer chromatography determination step.

In recent years, ML drugs possessing more specific functional groups have been developed, such as emamectin benzoate and eprinomectin. The functionality of these compounds has made it possible to apply ion exchange SPE to their determination. Payne et al. extracted eprinomectin from bovine tissue using acetone/dichloromethane before purification on an aminopropyl SPE cartridge prior to determination [76]. This procedure was also used by Ballard et al. to prepare samples for confirmation by LC–MS/MS [77]. Kim-Kang et al. extracted emamectin residues using ethyl acetate, before purification on a cation exchange cartridge [146].

Li et al. used immunoaffinity chromatography (IAC) for purification of ivermectin and abamectin residues from plasma [147,148], tissue [116,128,148] and fruit [147]. In initial work samples were purified by C_{18} SPE before application onto the immunoaffinity columns [147]. The C_{18} SPE step was later found to be unnecessary and determination by LC-UV following IAC was possible without significant interference [148]. In further work, a method was developed for isolation of ivermectin from liver, using an alternative antibody coupling procedure for preparation of the immunoaffinity columns that aided removal of matrix interference [116]. In addition, a larger volume of wash solvent was required to produce clean chromatograms. Crooks et al. extracted ivermectin from liver samples using acetonitrile [106]. Extracts were washed with hexane and purified on aminopropyl SPE cartridges, prior to determination by ELISA.

9.3. Milk and milk products

Surprisingly, few methods have been developed for determination of ML residues in milk. Alvinerie et al. [66] developed a method for determination of ivermectin in milk, using the liquid–liquid partitioning procedure developed by Tway et al. [95] and determination by LC–MS. Kijak et al. mixed milk samples with ammonium hydroxide and ethanol before extracting ivermectin with ethyl acetate/isooctane [88]. The ethyl acetate/isooctane extract was evaporated to dryness before reconstitution in hexane and partitioning of ivermectin residues into acetonitrile. Cerkvenik et al. [149] developed a method for determination of ivermectin in a range of dairy products, based on the procedure developed by Norlander et al. [96]. Heller et al. extracted ivermectin residues from milk (and liver) using acetonitrile, before purification on basic alumina [150]. Crooks et al. extracted ivermectin from milk samples using acetonitrile [89]. Extracts were washed with hexane and purified on aminopropyl SPE cartridges, prior to determination by ELISA. Eprinomectin was extracted by Dusi et al. using acetonitrile and cleaned up on C_{18} SPE, prior to determination by LC [151]. Pollmeier et al. extracted eprinomectin from milk using acetonitrile and analysed extracts without further purification [90].

9.4. Crops, fruit and vegetables

Some of the more interesting developments in the area of extraction and clean-up for MLs have been in the area of plant matrices. Some of the problems that have to be overcome include the removal of pigments and control of sample moisture. Vuik et al. extracted abamectin from cucumber and lettuce using ethyl acetate, with further purification on a Sep-Pak^{TM} silica cartridge [108]. Prabhu et al. extracted emamectin benzoate and its 8,9-isomer from celery and lettuce using methanol [109]. Extracts were purified by a combination of C_8 SPE, liquid–liquid partitioning and cation exchange SPE. Valenzuela et al. applied MSPD to determination of abamectin in citrus fruits [114,115]. Samples were blended with C_{18} material and packed into a glass column. The column was eluted with dichloromethane; extracts were evaporated to dryness and reconstituted in acetonitrile before determination by LC–MS.

Prabhu et al. extracted abamectin and its 8,9-isomer from tomatoes using acetonitrile/water before purification by liquid–liquid partitioning and aminopropyl SPE [110]. Chamkasem et al. later modified this method and applied it to the determination of abamectin in oranges, pears, spinach and celery [111]. They found that incorporation of a salting out step removed much of the ionic species and hydrophilic pigments. With spinach and celery, it was found that most of the pigment stayed in the acetonitrile layer. The aminopropyl clean-up was shown to remove most of the pigments, but a small portion of pigment was found to elute with abamectin. This procedure was later used by Cobin et al. for determination of abamectin and its 8,9-*Z*-isomer in apples [83], hops [112] and wine [113]. This group found that the extraction procedure had to be modified depending on the moisture content of samples. With hops, water had to be added to samples, which were extracted using methanol and partitioned into hexane. Acetonitrile had to be added to wine samples before partitioning into hexane, drying over sodium sulphate and clean-up on an aminopropyl SPE cartridge.

9.5. Animal feed

MLs may be administered through feed in both agriculture and aquaculture. As a result, a number of methods have been developed for quantitative determination of these drugs in medicated feeds. Fox et al. extracted ivermectin from medicated feed using methanol, followed by clean-up on a deactivated alumina column and determination by LC-UV [117]. They later developed a more sensitive method including additional C_{18} and silica SPE steps [118]. Farer et al. extracted emamectin benzoate from feed by mixing overnight with methanol/water [119]. They compared four different SPE methods for these compounds (strong cation exchange, silica, C_8 and C_{18}); C_{18} was found to give the best retention and recovery.

9.6. Multi-residue extraction and clean-up procedures

The methods already discussed are single residue methods, which satisfy the requirements for specific residue testing

applications. However, multi-residue methods are preferred for surveillance of residues in foods.

9.6.1. Tissues

Roudaut extracted moxidectin, abamectin, doramectin and ivermectin from liver tissue before purification on C_{18} SPE. This extraction and clean-up procedure has been applied more recently to isolate ML residues from trout and salmon samples [152]. The method uses a combination of a small sample size (1 g) and selective SPE elution with a low volume of acetonitrile–water from a 1 ml C_{18} cartridge. After elution, extracts are evaporated to dryness, derivatised and determined by LC fluorescence. Ishii et al. extracted moxidectin, abamectin, doramectin and ivermectin from liver and fat using a simple extraction and LLP procedure [121]. Samples were mixed with sodium sulphate and extracted with acetonitrile, before partitioning with hexane to remove non-polar interference prior to determination by LC fluorescence. Danaher et al. developed a robust alumina SPE clean-up procedure for determination of moxidectin, abamectin, doramectin and ivermectin residues in liver samples [153].

Rupp et al. extracted ivermectin and doramectin from salmon tissue using acetonitrile before purification of extracts using C_8 and silica SPE [94]. They found that an additional silica clean-up step was needed to remove tissue pigments remaining after the C_8 clean-up. Van De Riet et al. extracted emamectin and ivermectin residues from salmon tissue using acetonitrile prior to clean-up on C_{18} [129]. Turnipseed et al. developed multi-residue methodology for determination of eprinomectin, moxidectin, doramectin and ivermectin in liver and salmon muscle prior to determination by LC–MS [123]. They adopted the sample preparation procedures developed by Rupp et al. [94] and Salisbury et al. [100] for isolation of residues from salmon and liver tissue, respectively. Howells et al. [127] applied the procedure developed by Norlander et al. [96] to isolate eprinomectin, moxidectin, abamectin, doramectin and ivermectin residues from liver. They developed an on-line clean-up procedure on a Prospekt^{TM} solid phase extraction system to improve sample throughput for LC–MS. In this procedure, sample extracts in acetonitrile/water/triethylamine (30 + 69.9 + 0.1, v/v/v) were injected onto HySphere C_8 cartridges and eluted directly onto the LC–MS. The programme allowed the sequential extraction of samples in parallel with LC–MS analysis.

Ali et al. extracted eprinomectin, moxidectin, abamectin, doramectin and ivermectin from liver using acetonitrile, before purification by deactivated alumina and C_{18} SPE steps [125]. They also used an alternative clean-up procedure to purify samples for LC–MS confirmation [154]. Liver extract was loaded onto a C_8 cartridge, and washed with water/acetonitrile/triethylamine and hexane. The C_8 cartridge was subsequently eluted with dichloromethane onto an attached basic alumina cartridge. The C_8 cartridge was discarded and the alumina column was eluted sequentially with acetone and methanol. Danaher et al. [155] later applied the alumina/C_{18} clean-up procedure developed by Ali et al. [125] to purification of liver sample extracts, while using a newly developed derivatisation procedure for fluorescent derivatives. Danaher et al. subsequently developed a multi-residue supercritical fluid extraction (SFE) procedure for extraction and isolation of eprinomectin, moxidectin, abamectin, doramectin and ivermectin residues from animal liver [156]. Liver samples were mixed with hydromatrix and packed into a vessel containing 2 g basic alumina as a trap. The samples were extracted at 100 °C using unmodified supercritical carbon dioxide (SF-CO_2) at a pressure of 300 bar and flow rate of 5.0 l/min. Residues were adsorbed in-line on the basic alumina trap, which was later eluted with 4 ml of methanol–ethyl acetate (70 + 30, v/v). They evaluated

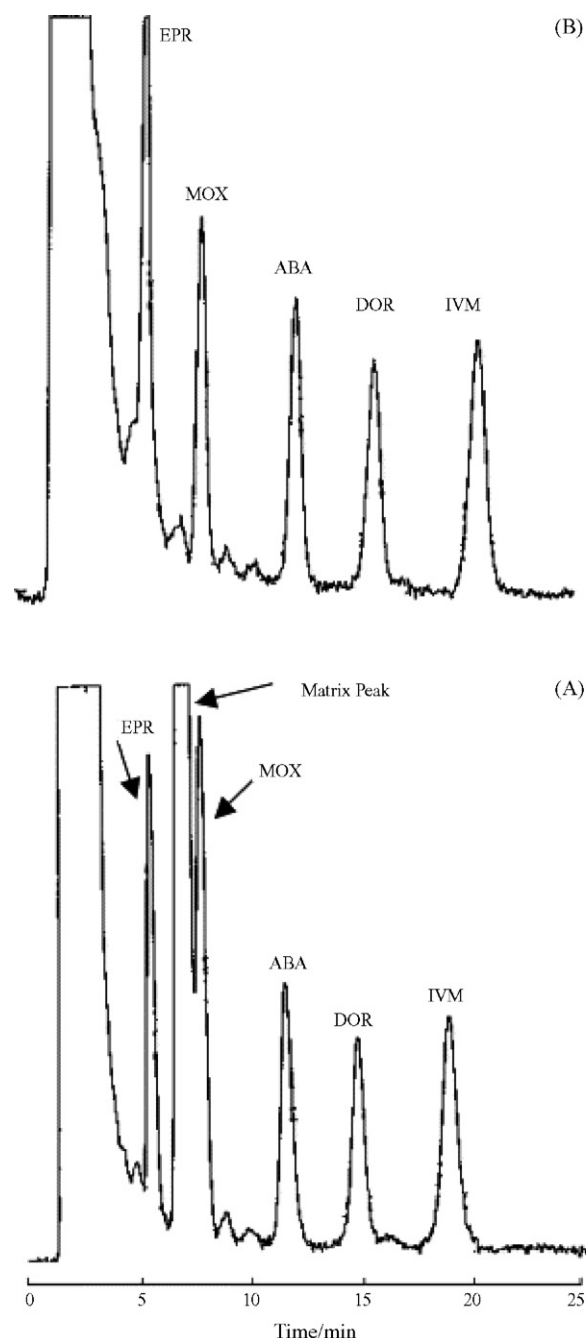

Fig. 3. Chromatograms of porcine liver extracts fortified with 20 μg/kg of eprinomectin (EPR), moxidectin (MOX), abamectin (ABA), doramectin (DOR) and ivermectin (IVM), (A) off-line trap and (B) in-line trap. (Reprinted from reference [156], Copyright (2001), with permission from Elsevier.)

off-line trapping of residues also but found that that in-line trapping reduced the presence of a matrix interference peak eluting close to moxidectin (Fig. 3). The method gave improved clean-up and extracts did not require further clean-up prior to determination.

Coles et al. extracted emamectin, doramectin and ivermectin residues from liver and salmon muscle using acetonitrile and purified the extracts on deactivated alumina and C_{18} SPE clean-up prior to determination by LC–MS/MS [157]. Daeseleire et al. extracted eprinomectin, moxidectin, abamectin, doramectin and ivermectin residues from meat samples using acetonitrile and purified the extracts on C_{18} SPE cartridges prior to determination by LC–MS/MS [158]. They also digested meat samples in the presence of proteinase in a buffered solution for 2 h at 60 °C prior to extraction with diethyl ether and determination by LC–MS/MS. Selamectin was used as internal standard to increase the accuracy and precision of the method.

Li et al. developed a multi-residue procedure for determination of abamectin and ivermectin in pig liver based on immunoaffinity chromatography, with determination by LC–MS [116]. He et al. recently developed a multi-residue method for isolation of abamectin, ivermectin, doramectin and eprinomectin from bovine liver based on immunoaffinity clean-up [159]. Recovery of residues ranged between 79 and 116%. Knold et al. extracted ivermectin and doramectin from porcine liver using acetonitrile with abamectin as an internal standard [133]. They centrifuged the samples at $21{,}000 \times g$ and took an aliquot of the supernatant for determination. A late eluting peak was observed in the chromatogram but no interference was observed in the region of interest. Rudik et al. recently developed a simple procedure for determination of eprinomectin, moxidectin, doramectin, selamectin and ivermectin in serum and liver [132]. Samples were extracted using acetonitrile and sodium chloride was added to allow separation of the acetonitrile and aqueous layers. The aqueous layer was discarded and the acetonitrile layer was filtered before residue determination by LC–MS.

Nagata et al. developed a multi-residue method for extraction and clean-up of six ML drugs (eprinomectin, moxidectin, milbemycin A3 and A4, abamectin, doramectin and ivermectin) in bovine liver and muscle tissues [160]. Pre-homogenised tissue samples were re-homogenised in the presence of anhydrous sodium sulphate and acetonitrile. Sample extracts were subsequently defatted with hexane and purified by aminopropyl SPE prior to determination.

9.6.2. Milk and milk products

Roybal et al. isolated eprinomectin, moxidectin, doramectin and ivermectin residues from milk using a combination of C_{18} and carbon column SPE clean-up [122]. Turnipseed et al. [123,161] developed multi-residue methodology for determination of ML residues in milk using the extraction and clean-up procedure developed by Roybal et al. [122] with detection by LC–MS. They adopted the sample preparation procedures developed by Roybal et al. [122] for isolation of residues from milk. Daeseleire et al. [158] extracted six ML residues from milk samples using acetonitrile and purified the extracts on C_{18} SPE cartridges prior to determination by LC–MS/MS.

Schenck et al. developed a method for determination of moxidectin, abamectin, doramectin and ivermectin in milk, after extraction and purification by liquid–liquid partitioning [124]. Dusi et al. extracted eprinomectin, moxidectin, abamectin, doramectin and ivermectin from milk using acetonitrile before purification on a C_{18} SPE cartridge [126]. Purified extracts were derivatised by the procedure developed by Tway et al. and further purified on silica prior to determination [95]. Capurro et al. developed a multi-residue extraction procedure for determination of six MLs in milk and milk products (cheese, butter and skimmed milk powder) [162]. Milk samples were extracted and purified using the clean-up procedure developed by Nordlander et al. [96]. For samples with a high fat content, modification of the method was necessary. Modifications included addition of water to samples, incubation at 80 °C and blending of samples.

9.6.3. Fruit and vegetable matrices

Yoshii et al. developed LC–MS and LC-fluorescence methods for determination of nine MLs in tea and vegetable samples [130,131]. Samples were extracted using acetone and purified using a stacked SPE system consisting of C_{18} and aminopropyl cartridges. Radish samples required an additional propylsulphonic acid (PRS) SPE clean-up step to eliminate a component that inhibited the formation of emamectin derivatives. The stacked cartridges were used for clean-up because they reduced the number of evaporation steps required and analyte degradation.

9.6.4. Summary

A summary of the extraction and clean-up procedures used in some of the more important multi-residue methods is shown in Table 2. The sample preparation applied depends on the sample matrix and the number of residues to be included. For residue monitoring and surveillance applications, methods that are applicable to the largest number of residues are most desirable. Therefore, the most suitable methods are those that include at least five residues. Such methods have been developed by Nagata et al. [160], Howells et al. [127] and Danaher et al. [155] for five to six ML residues in muscle or liver tissues, by Dusi et al. [126], Daeseleire et al. [158], and Capurro et al. [162] for six ML residues in milk and milk products, by Roudaut and Garnier [152] for five ML residues in fish tissue, and by Yoshii et al. for five MLs and metabolites in plant samples [130,131].

An acetonitrile extraction followed by C_8 SPE clean-up is most suitable for isolation of ML residues from milk. However, in the case of liver samples, a combination of deactivated alumina and C_{18} SPE is required for purification of the sample extracts. Difficulties may be observed with some species; for example, equine liver is a difficult matrix because of the presence of late eluting peaks in chromatograms. These problems may be overcome by more selective elution from C_{18} SPE cartridges. Alternatively, the development of more selective clean-up procedures using on-line trace enrichment systems may be effective. Such on-line sample purification systems may prove to be most effective when coupled to LC–MS/MS. Improved clean-up could potentially reduce ion-suppression effects in the

Table 2
Summary of extraction and clean-up procedures used in selected multi-residue methods for analysis of ML residues in food matrices

Residues[a]	Matrix[b]	Extraction	Clean-up	Recovery (%)	Ref.
	Animal tissue				
Mx, A, D, I	L	Acetonitrile	C_{18}	>77	[120]
Mx, A, D, I	L, F	Acetonitrile, Na_2SO_4	LLP	>80	[121]
Mx, A, D, I	L	LLE	Alumina	>90	[153]
Ep, Mx, D, I	M	Acetonitrile	C_{18} + Carbon	Non-quantitative	[123]
Ep, Mx, A, D, I	L	Acetonitrile	C_8	>65	[127]
Ep, Mx, A, D, I	L	Acetonitrile	Alum. + C_{18}	>70	[125]
Ep, Mx, A, D, I	L	Acetonitrile	Alum. + C_{18}	>73	[155]
Mx, A, D, I	L	Acetonitrile	Alum. + C_{18}	55–104	[157]
Ep, Mx, A, D, I	L	Acetonitrile	C_8 + Alumina	Non-quantitative	[154]
A, I	L	Methanol	IAC	>85	[116]
I, D	L	Acetonitrile, 21000 g	None	>70	[133]
Ep, Mx, D, I, S	Ser, L	Acetonitrile, NaCl		61–91	[132]
Ep, Mx, A, D, I	L	SF-CO_2	In-line alumina trap	>76	[156]
Epr, A, D, I	L	Methanol	Immunoaffinity	>79	[159]
Epr, Mx, Mi, A, D, I	L, M	Acetonitrile, Na_2SO_4	LLP, NH_2	60–80	[160]
Epr, Mx, A, D, I, S	M	Protease digestion	LLE (diethyl ether)	>74	[158]
	Milk				
Mx, A, D, I	Mk	LLE	LLP	>80	[124]
Ep, Mx, A, D, I, S	Mk	Acetonitrile	C_{18}	>70	[126]
Ep, Mx, A, D, I	Mk	Acetonitrile	C_{18}	>70	[158]
Ep, Mx, Em, A, D, I	Mk, dairy products	Acetonitrile	C_8	>75	[162]
Ep, Mx, Em, A, D, I	Mk	Acetonitrile	C_8	50–106	[122]
Ep, Mx, D, I	Mk	Acetonitrile	C_{18} + Carbon	Non-quantitatively	[123]
Ep, Mx, D, I	Mk	Acetonitrile	C_{18} + Carbon	>60 (typical)	[158]
Ep, Mx, A, D, I	Mk	Acetonitrile	C_8	Not available	[172]
	Fish				
I, D	Salm.	Acetonitrile	C_8 and silica	>75	[94]
Em, I	Salm.	Acetonitrile	C_{18}	>85	[129]
Mx, Em, A, D, I	Salm. trout	Acetonitrile	C_{18}	>79	[152]
	Plant-based matrices				
Em (X4), Mi, A, D, I	Tea + Veg. Radish	Acetone	C_{18} + NH_2 C_{18} + NH_2 + PRS	>80	[130,131]

[a] Eprinomectin, moxidectin, emamectin, milbemectin, abamectin, doramectin, selamectin and ivermectin are identified using the abbreviations Epr, Em, Mx, Mi, A, D, S and I, respectively.
[b] Liver, fat, muscle, serum, milk, salmon and vegetables are abbreviated as L, F, M, Ser, Mk, Salm. and Veg.

MS leading to a more stable signal and improved signal to noise.

10. Methods for measurement of macrocyclic lactone residues

Residues in sample extracts are typically determined quantitatively directly by liquid chromatography (LC) with UV [97,101,110] or mass spectrometric (MS) [54,66,77,116, 127,130] detection. Alternatively, extracts may be derivatised to produce a fluorescent molecule before determination by LC with fluorescence detection [94,95]. The detection method largely depends on the sensitivity required in the assay. For example, for medicated feed samples where drugs are present in the mg/kg region, UV detection is most suitable [117–119]. However, when determination of residues is required in the low μg/kg region, fluorescence and MS detection are more suitable because of their greater sensitivity and selectivity. A number of screening assays have been developed for detection of ML residues using thin layer chromatography- (TLC) and immunochemical-based methods.

10.1. Thin layer chromatography

Malanikova et al. used TLC for determination of avermectins produced using different strains of *Streptomyces avermitilis* [163]. Sample extracts were applied onto silica plates coated with a fluorescent indicator. Plates were developed for 30–40 min, with best elution conditions being hexane/acetone/methanol or hexane/isopropyl alcohol/methanol. Quantitative determination was carried out using a TLC scanner at 254 nm. Avermectin B_{1a} and B_{1b} were eluted as a single peak as were the B_2, A_1, A_2 homologues. The method could determine avermectin residue levels at less than 4 μg on plate. Hoy et al. used silica TLC plates coated with a fluorescent indicator and a development solvent of chloroform/ethyl acetate/methanol/dichloromethane for ivermectin determination, with visualisation under UV light at 254 nm [47].

Taylor et al. [164] used TLC for determination of ivermectin in cattle serum. In this procedure, sample extracts were derivatised using the method developed by De Montigny et al. [140] followed by reaction with ammonia in methanol to produce a more stable derivative of ivermectin. Plates were developed

using an elution solvent of hexane/acetone/decane/methanol and visualised under UV light. It was found that the intensity of the ivermectin diminished on exposure to UV light, but incorporation of decane into the elution solvent slowed down this degradation. The method was also evaluated using high performance silica TLC plates and quantified using a TLC scanner. The sensitivity of the method was comparable on TLC plates with or without the fluorescent indicator. The limit of detection was between 1 and 2 ng/ml in cattle serum, equivalent to 100 pg ivermectin on the plate. The method was later used to evaluate the degradation of ivermectin in cattle dung over time [165].

Abjean et al. found that ivermectin derivatives had to be purified by C18 SPE before application onto a silica gel TLC plate [103]. Plates were developed using chloroform/ethyl acetate, air-dried and dipped in a tank containing paraffin/hexane. The plate was visualised under UV light at 366 nm. It was found that derivatives degraded on exposure to light and determination had to be carried out promptly to avoid this degradation.

10.2. Immunochemical methods

Schimdt et al., during ivermectin monoclonal antibody production in mice, evaluated a number of different ivermectin conjugate/carrier combinations as immunogens [166]. Protein conjugates using both BSA and conalbumin were prepared at the 4″- and 5-hydroxyl positions. Conjugates produced as an ivermectin hemisuccinate derivative formed through the oxygen on carbon 5 were virtually non-immunogenic. However, the conjugates produced by reaction at the 4″-hydroxyl position were immunogenic. Precise cross-reactivities for the monoclonal antibodies produced were not determined although it was suggested that the unique structure of the avermectins would limit cross reactivity to these compound and their metabolites. Mitsui et al. developed a competitive ELISA for determination of ivermectin in biological fluids, using polyclonal antibodies raised in rabbits against an ivermectin-BSA conjugate [145]. In this case, conjugation took place at the carbon 5 position but using an oxime rather than a hemisuccinate derivative. They found the antibody raised to be highly specific with cross-reactivity limited to the ivermectin oxime derivative. The method was applied to residue detection in serum. The limit of detection of the method was 0.1 μg/kg. The ELISA was a competitive sandwich assay with competition between an ivermectin–biotin conjugate and free ivermectin for antibody binding, with avidin peroxidase conjugate as a tracer. In addition, they evaluated an ivermectin-poly-L-lysine conjugate, but found the assay to be 5–10 fold less sensitive in this format. It was postulated that the better sensitivity observed in the original format was because of the lower molecular weight of biotin, which allowed the ivermectin–biotin conjugate to compete more successfully with ivermectin for antibody binding, or due to amplification of the enzyme reaction because of high-affinity binding between biotin and avidin.

Crooks et al. developed a competitive ELISA for determination of ivermectin in bovine liver, using a polyclonal antiserum raised in rabbits [106]. It was found that by using a 5-*O*-succinoyl-ivermectin derivative, as first described by Schmidt et al., and coupling it to an alternative carrier protein, apo-transferrin, an immunogenic conjugate was formed [166]. The antibodies produced showed cross-reactivity with doramectin, the only avermectin assessed, but none with moxidectin. The limit of detection of the method was 1.6 μg/kg. Li et al. raised polyclonal antibodies in rabbits after immunisation with 4″-*O*-succinoyl abamectin BSA [167]. This group developed an indirect ELISA for determination of abamectin residues using this polyclonal antibody. The antibody also showed cross-reactivity to ivermectin.

Crooks et al. later developed a dissociation enhanced lanthanide fluorescence immunoassay (DELFIA®) method for determination of ivermectin residues in milk [89]. Monoclonal antibodies were raised in Balb C mice after immunisation with a 5-*O*-succinoyl-ivermectin-transferrin conjugate. Significant antibody cross-reactivity was reported for eprinomectin (92%), abamectin (82%) and doramectin (16%). The limit of detection of this method for ivermectin was 4.6 μg/kg. In a study carried out on milk samples taken from ivermectin treated animals, no ivermectin residues were detected by LC 14 days after treatment. However, the DELFIA® assay showed that residues could be detected for up to 35 days after treatment. They suggested that one possible explanation for these results could be detection of drug metabolites by the immunoassay, which were not detectable by the LC method.

Dubois et al. developed the first ELISA for determination of moxidectin in bovine tissues and milk using a polyclonal antibody raised in rabbits against a moxidectin-BSA conjugate [168]. The method was validated and shown to be capable of detecting moxidectin residues to 2, 19 and 1 μg/kg in milk, fat and muscle, respectively. This ELISA has been developed into a kit and is commercially available.

Samsanova et al. developed an immunobiosensor-based method (Biacore AB) for determination of ivermectin in bovine liver, based on 96-well plate technology and detection using a surface plasmon resonance optical biosensor [107]. In this procedure specific antibody is combined with sample extract prior to injection of an aliquot of this mixture over the surface of a sensor chip which is comprised of a glass slide coated with a thin layer of gold on one side. The gold is coated with a covalently bound hydrophilic dextran matrix which when activated is suitable for immobilisation of biomolecules—in this case an ivermectin derivative. As the mixture is injected across the chip surface the binding of free antibodies to the immobilised ligand on the sensor chip surface is monitored. Detection is based on the principle of surface plasmon resonance (SPR), which allows minute changes in refractive index to be monitored. The limit of detection of the method was calculated to be 20 μg/kg. The cross-reactivity of the antibody, which was the one previously developed by Crooks et al., in the immunosensor assay was determined as abamectin (151%), eprinomectin (78%), emamectin benzoate (15%), doramectin (10%) and moxidectin (<0.1%), relative to ivermectin [89]. The immunobiosensor method was claimed to require a quicker and simpler extraction procedure than previously published methods and was suitable for analysis of 20 liver samples within a single working day. Samsanova et al. later applied this immunosensor technology for detection of ivermectin in milk samples.

Using the same monoclonal antibody as in their previous study, a method suitable for detection of ivermectin (LOD 16.2 μg/kg) in 20 milk samples per day was reported [91].

There have been a number of developments in the area of ELISA methods but no antibody has been produced that shows cross-reactivity to both the avermectins and the milbemycins. The first antibody that shows cross-reactivity to moxidectin has been recently produced [168]. It is likely in the future that a moxidectin antibody will be produced and genetically engineered to extend activity to the avermectins. Alternatively, acid hydrolysis of avermectin molecules in the presence of sulphuric acid to produce an aglycone structure could extend the activity of the existing moxidectin antibody to avermectins. However, a limitation associated with immunochemical methods at present is the requirement for SPE clean-up in the procedure, thereby increasing the analysis time for these methods. It can be concluded that for immunochemical methods for detection of ML residues to be more widely applied improvements need to be made in the area of sample preparation. Possibilities include ultra-filtration or immunoaffinity extraction-based sample preparation procedures.

10.3. Liquid chromatography

10.3.1. Liquid chromatography separations

It has been found that four of these drugs (moxidectin, abamectin, doramectin and ivermectin) can be easily analysed on C_{18} columns using 90% or more organic modifier in the mobile phase. The organic content in the mobile phase may be constituted of methanol, acetonitrile or a mixture of the two. Schenck and Lagman used a mobile phase, consisting of acetonitrile/tetrahydrofuran/water for separation of four MLs, giving better chromatography and a greater than two-fold increase in peak heights [124]. C_8 packing has been used by De Montigny et al. for determination of ivermectin in plasma, using a similar mobile phase composition [140]. The percentage of water used in the mobile phase depends on the number of drugs and the detection system. With fluorescence detection, these drugs are derivatised to form more non-polar molecules. As a result, the organic content of the mobile phase may be increased to elute these compounds in a comparable time to non-derivatised drugs. Separations are normally carried out on standard LC columns, 150–250 mm in length, with diameters ranging from 3.0 to 4.6 mm and particle sizes are 3.0–5.0 μm.

Addition of buffer to the mobile phase is generally not required for most MLs and good chromatography can be achieved using an organic solvent/water mobile phase mixture. However, Alvinerie et al. used a mobile phase of acetic acid/methanol/acetonitrile for determination of ivermectin in goat plasma [67]. They had previously developed an LC–MS method for determination of ivermectin, finding that addition of acetic acid was necessary to provide adequate ionisation in the MS [66]. Heller et al. found that addition of ammonium acetate buffer to the mobile phase was necessary for MS, with separation on a narrow bore column (150 mm × 2 mm) [150]. Chiou et al. developed a fast gradient method for determination of ivermectin in milk and plasma, using a short column (50 mm × 4.6 mm) that required only a short equilibration time [138]. Stout et al. used a short column for determination of moxidectin with a gradient mobile phase containing ammonium acetate for LC–MS [54]. Cobin et al. used triethylamine to enhance chromatographic separation [83,112,113]. Valenzuela et al. found that methanol had to be included in the mobile phase to meet MS ionisation requirements [114,115].

Payne et al. found that the best chromatography for eprinomectin was achieved on a C_8 column after addition of triethylamine and phosphoric acid to the mobile phase [76]. Others found that this compound could be analysed on a C_{18} column using a similar mobile phase with higher organic solvent content [90,143]. Sutra et al. found that suitable chromatography could be achieved for using an ion-pairing reagent with separation on a base deactivated C_{18} column [142]. Two research groups found it necessary to include phosphoric acid in the mobile phase to achieve suitable chromatography for emamectin [119,146]. Some researchers have found with UV detection that matrix interference is a problem. This may be overcome using appropriate column selection or careful manipulation of the mobile phase [101,108]. Matrix interference can generally be overcome using suitable clean-up methods, derivatisation for fluorescence detection, or MS detection.

Separation may be obtained for six of these drugs using an isocratic mobile phase [155]. However, gradient elution has been used by a number of researchers [54,70,119,126,131, 138,143,169]. Gradient elution may be used to separate the residues or to remove late eluting interferences from the column that may interfere with subsequent determination [131,132, 138,143,169]. In the case of LC–MS, gradients are quite frequently used because late eluting interferences can interfere with MS ionisation processes. As a result, the LC column flow is often directed away from the MS when the analytes are not eluting off the column [169]. In the case of fluorescence detection, isocratic mobile phases are generally used. Knold et al. developed a LC method for determination of ivermectin, doramectin and abamectin in liver, using a flow gradient to remove late eluting interferences from the column [133]. Farer et al. separated emamectin from interfering peaks present in medicated feed by using gradient elution, with detection by UV [119]. Yoshii et al. recently developed a method for separation of nine ML residues in plant-based matrices by using both fluorescence and MS detection [130,133]. The composition of the mobile phase indicates that a gradient is necessary for chromatographic separation of these nine residues. The gradient conditions were at the following compositions of acetonitrile + water—(80 + 20, v/v) at 0 min, (90 + 10, v/v) at 5 min, (93 + 7, v/v) at 20 min and (100 + 0, v/v) at 22 min. From experience in our laboratory, elution of ML residues from HPLC columns in less than 40 min is difficult to achieve with less than 90% organic solvent in the mobile phase.

Roudaut et al. separated five ML residues in less than 20 min using a gradient based on acetonitrile–water [152]. The authors observed an interfering peak in the tissue extract chromatograms, eluting close to moxidectin. Danaher et al. observed the same interfering peak in chromatograms of liver sample

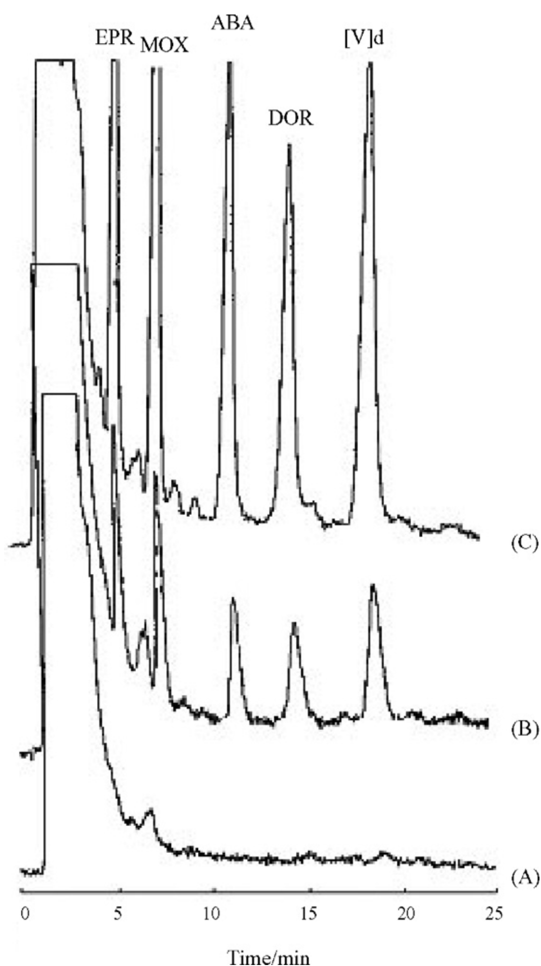

Fig. 4. Chromatograms of bovine liver samples fortified with 0 μg kg^{-1} (A), 4 μg/kg (B) and 20 μg/kg (C) of eprinomectin (EPR), moxidectin (MOX), abamectin (ABA), doramectin (DOR) and ivermectin (IVM). (Figure reproduced from reference [155], by permission of The Royal Society of Chemistry, Copyright (2001).)

extracts but managed to separate the peak from residues using an isocratic separation (Fig. 4) [156]. In general, for a more robust separation and better peak shape of ML residues, a gradient separation is recommended.

10.3.2. UV detection of underivatised residues

UV detection is known to be very robust and can be readily applied to MLs because of their strong UV chromophore. However, when analysing for low levels of these drugs in plasma, tissue and milk, UV detection may not provide the specificity required. Schnitzerling et al. determined ivermectin in cattle blood and plasma; detection at 5 μg/kg could be achieved in blood at a wavelength of 254 nm [137]. Oehler et al. determined ivermectin in bovine serum to 2 μg/kg using a wavelength of 245 nm [139]. Reuvers et al. developed a method for ivermectin in liver using UV detection at 254 nm, but found that the method sometimes yielded false positive results for ivermectin [101]. However, by using an alternative LC column, discrimination could be achieved between ivermectin and this matrix interference peak. The limit of detection of the method was 5–10 μg/kg.

10.3.3. Fluorescence detection of derivatised residues

Methods have been developed using pre-column derivatisation with fluorescent detection providing greater sensitivity and selectivity than UV detection. As a result, fluorescence is often preferred to UV for detection of these residues at low μg/kg concentrations. Derivatisation involves reacting the MLs with non-fluorescent reagents to produce fluorescent derivatives. A number of derivatisation procedures have been developed over the past 20 years based on this principle (Fig. 5). The early trend was to use more reactive reagents to shorten the reaction time, reduce the derivatisation temperature and eliminate the need for post derivatisation clean-up [95,136]. More recently, a number of groups have studied the production of more stable fluorescent derivatives [41,94,111,125]. Stability problems have been observed with many derivatives and, in particular, the fluorescent derivative of eprinomectin is quite unstable and degrades rapidly over a short period [76]. Stability problems have also been observed for derivatives of abamectin [111], doramectin [41] and ivermectin [94]. This factor prevents long-term storage of derivatised samples or standards, which require determination within 24 h of derivatisation.

Tolan et al. developed the first derivatisation procedure for determination of abamectin and ivermectin in plasma [136]. In this derivatisation procedure, residues were reacted with acetic anhydride in pyridine, resulting in the loss of two water molecules and acetylation of the hydroxy group at the 4″ position. Dehydration of the molecule results in the production of an aromatic ring, which is in conjugation with a diene functional group, resulting in fluorescence. The reaction took 22–24 h to go to completion and a temperature of 105–110 °C gave best results. The resulting fluorescent derivatives (both standards and samples) required purification on silica prior to determination. The excitation wavelength used was 364 nm and the emission wavelength was 480 nm. The limit of detection of the method was 0.5 μg/kg.

Tway et al. modified this derivatisation procedure to produce the same fluorescent derivative in 60 min using a derivatisation temperature of 90 °C [95]. The dramatic decrease in the reaction time was achieved by reacting with acetic anhydride in dimethylformamide in the presence of 1-methylimidazole, a more powerful nucleophilic catalyst. Derivatives were purified on silica SPE cartridges prior to determination. The method has been used by a number of groups for determination of ML residues in animal liver [96], salmon muscle [60], milk [126] and cheese [170].

De Montigny et al. further modified this method, by introducing a better leaving group in the form of trifluoroacetyl, replacing acetic anhydride with trifluoroacetic anhydride and shortening the reaction time to less than 30 s [140]. Detection limits were at about 20 pg on column. It is important to note that the fluorescent derivatives produced for abamectin, doramectin and ivermectin are not the same derivatives as produced in the Tway method. This is because a trifluoroacetyl ester is formed at the 4″ position rather than the acetyl ester produced by the Tway method. However, the derivatives produced for eprinomectin and moxidectin are the same using both derivatisation procedures. This reaction is almost instantaneous; it takes place under ambient

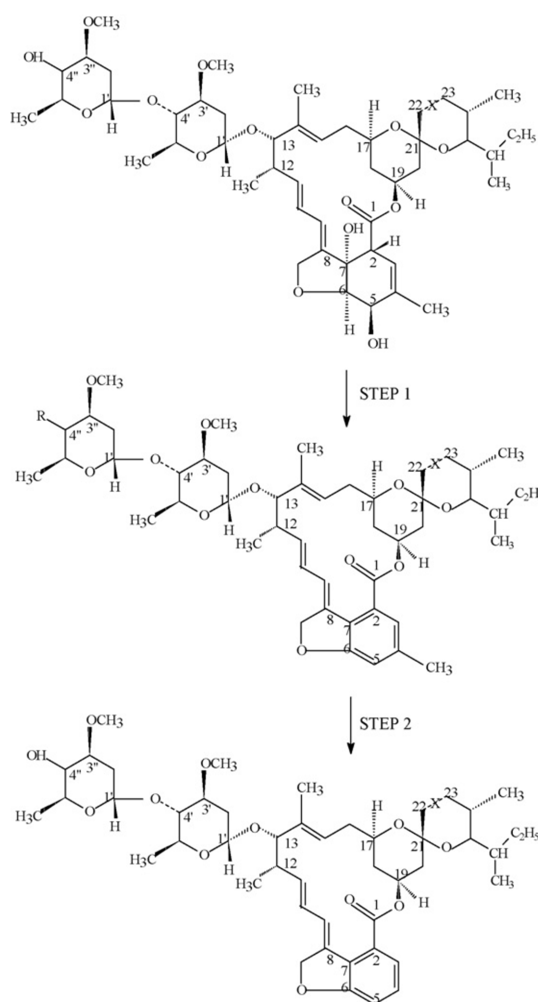

Fig. 5. Outline of derivatisation reactions for the preparation of fluorescent derivatives of ivermectin and abamectin.

Route	Acetylation reagent	Catalyst	Time	Temperature (°C)	R
Reaction step 1					
A	Acetic anhydride	Pyridine	24 h	100	CH_3CO
B	Acetic anhydride	Methylimidazole	1 h	95	CH_3CO
C	Trifluoroacetic anhydride	Methylimidazole	30 s	Ambient	CF_3CO
C	Trifluoroacetic anhydride	Triethylamine	30 s	Ambient	CF_3CO

Route	Stabilisation reagent	Time	Temperature (°C)
Reaction step 2			
A	2.0 M NH_3 in methanol	1 h	30
B	Methanol + water + triethylamine	10 min	Ambient
C	Ammonium acetate in methanol	15 min	50–55

conditions and requires no post-derivatisation purification. Furthermore, because of the rapid nature of this derivatisation procedure, fewer secondary derivatisation products are produced. As a result, it has become the derivatisation procedure of choice for determination of ML residues in plasma, animal tissue, salmon muscle, milk and crops.

Although the fluorescent derivatives of abamectin, doramectin and ivermectin are known to be unstable, it is possible to analyse these compounds in an overnight run without observing any degradation. However, instability problems may be overcome by using on-line derivatisation with programmable autosamplers with built in aliquoting and mixing capabilities, which provide derivatisation of each sample, or standard, individually immediately prior to LC. Payne et al. developed an automated procedure for derivatisation of eprinomectin because of the poor stability of the trifluoroacetyl derivative of this residue [76]. The fluorescent derivative of eprinomectin produced by this method is unstable, degrading by 50% in 2 h. In this procedure, sample extracts were reconstituted in methylimidazole/acetonitrile, transferred to LC vials and stored in the autosampler of a LC system. Trifluoroacetic anhydride was added and the vial contents were mixed, 2–7 min before injection, using the mixing capability of the autosampler. De Montigny's group developed methods for determination of eprinomectin in milk and plasma based on this automated derivatisation system [90,143]. Limits of detection for methods in milk and plasma were 80 and 20 pg on column, respectively.

The derivatisation procedure developed by De Montigny et al. [140] has been modified by other researchers with the aim of producing more stable fluorescent derivatives. The trifluoroacetyl derivatives of abamectin, doramectin, ivermectin and selamectin are susceptible to hydrolysis on storage, forming more stable alcohol derivatives. This may be due to the unstable nature of this derivative and reagents present in the derivatisation mixture. Chamekasem et al. derivatised abamectin by incubating with a mixture of methylimidazole/trifluoroacetic anhydride/dimethylformamide for 1 h at 30 °C [111]. The trifluoroacetyl derivatives were further reacted with 2.0 M ammonia in methanol to form more stable alcohol derivatives. Similar modifications have been made in procedures for determination of doramectin and selamectin residues. Nowakowski et al. substituted triethylamine for methylimidazole, while using trifluoroacetic anhydride as the acetylating reagent [41]. This procedure was later used by Walker and Fenner for determination of doramectin and selamectin residues [171]. Cobin et al. found that a more stable alcohol derivative of abamectin could be produced by mixing derivatised extracts with mobile phase (methanol/water/triethylamine) for 10 min at room temperature [112]. It was found that abamectin and its 8,9-*Z*-isomer were both converted to the same derivative using this procedure. Similarly Rupp et al. showed that more stable fluorescent derivatives of ivermectin and doramectin could be produced by reacting with methanolic ammonium acetate [94].

Roybal et al. developed a method for derivatisation of eprinomectin and other MLs, using a post-column photochemical reactor [122]. This instrumentation may not be available in most laboratories so modifications have been made to the De Mon-

tigny method [140] to produce a more stable derivative of eprinomectin. Ali et al. found that the derivatisation of eprinomectin was influenced by temperature and demonstrated that heating at 65 °C for 90 min produced a stable derivative [125]. The method was also applicable to moxidectin, abamectin, doramectin and ivermectin which were unaffected by the additional step. Dusi et al. [126] applied the derivatisation procedure developed by Tway et al. [95] to the determination of eprinomectin and the other four MLs analysed by Ali et al. [125] in milk. Nagata et al. [160] similarly applied the derivatisation developed by Tway et al. [95], for determination of six ML residues in tissue. This group found that extracts could be simply diluted in acetonitrile, eliminating the need for subsequent clean-up of derivatised extracts. The method produced a stable derivative of eprinomectin but both samples and standards had to be purified on silica cartridges after derivatisation. Danaher et al. [155] described modifications made to the derivatisation procedure developed by De Montigny et al. [140] that allowed the production of a stable derivative of eprinomectin. The procedure uses a combination of elevated temperature and acid to produce the stable derivative of eprinomectin. The derivatisation procedure was optimised using response surface methodology, which showed that derivatising eprinomectin in the presence of 50 μl of acetic acid, at 65 °C for 30 min gave suitable results. The derivatisation procedure has been applied in a multi-residue method for determination of eprinomectin, moxidectin, abamectin, doramectin, and ivermectin residues in liver samples [155,156]. Berendsen et al. developed a method for determination of the same five residues in milk [172]. Triethylamine and trifluoroacetic acid were included in this derivatisation procedure to accelerate the formation of the initial derivatives and subsequently convert them to more stable derivatives. This group demonstrated the stability of the derivatives over an 80 h period.

Downing outlined a novel derivatisation procedure for verification of ivermectin residues in liver [173]. Liver sample extracts were derivatised using the derivatisation procedure developed by Tway et al. [95] and subjected to further acid hydrolysis to produce more non-polar derivatives. It was shown that the fluorescent derivatives could be further reacted with sulphuric acid in propanol or methanol to produce monosaccharide or aglycone derivatives, respectively (Fig. 6). Chiu and Liu outlined a similar type of hydrolysis scheme for identification of non-polar metabolites of ivermectin in fat tissue [58]. In this work, they found that fatty acid drug conjugates could be selectively deconjugated using enzymatic hydrolysis with cholesterol esterase without removal of the saccharide functional groups. Alternatively, hydrolysis under harsher conditions with *p*-toluene sulfonic acid resulted in the cleavage of fatty acid esters and both saccharide groups (Fig. 7).

10.3.4. Mass spectrometry

A number of different MS configurations have been coupled with LC and used for determination of ML residues in biological matrices. Some groups have developed LC–MS for purely confirmatory purposes, with LC fluorescence being used for quantitative determination [54,77,102,123,150,154,169]. More recently, LC–MS methods have been developed that provide both quantitative and confirmatory information on ML residues [114,127,131,132,157,158,161]. A number of different MS ionisation sources have been applied to these residues, including particle beam [150], thermospray (TS) [102], electrospray (ES) [77,114,115,128,130,131,157,158,169], atmospheric pressure chemical ionisation (APCI) [123,127,128,132,154,169] and atmospheric pressure photoionisation (APPI) [161]. It has been found that TS and ES work best in positive ion mode. However, APCI can operate in negative [123,127,128] or positive [132,154,169] ion modes with modification of mobile phase constituents and MS conditions to give satisfactory performance. Turnipseed et al. found that APPI was most sensitive when operating in negative ion mode [161]. However, APCI in positive ion mode gave the best overall response with the corona discharge needle switched off.

The different mass analysers that have been used for determination of ML residues include quadrupole, triple quadrupole, time-of-flight and ion trap. Valenzuela et al. determined abamectin in fruit using a single quadrupole LC–MS system equipped with an electrospray ionisation source operating in positive ion mode [114,115]. Abamectin was monitored as the sodium adduct $[M+Na]^+$ ion. The limit of quantitation of the method was 2.5 μg/kg. Yoshii et al. developed a method for confirmation of nine ML residues in crops using a single quadrupole LC–MS system equipped with an electrospray ionisation source [130,131]. Residues were monitored as their $[M+Na]^+$ ions, with limits of detection of 2–50 pg on column (≤1 μg/kg).

Turnipseed et al. developed a multi-residue method for confirmation of ML residues in animal tissue and milk, using a single quadrupole instrument equipped with an APCI source operating in negative mode with selective ion monitoring to increase the detectability [123]. ML residues could be confirmed at between 20 and 40 μg/kg using this method. It was found that the response for moxidectin was not as intense as that for eprinomectin, doramectin and ivermectin. They concluded that MS/MS would be needed if greater sensitivity was to be achieved.

Ali et al. confirmed eprinomectin, moxidectin, abamectin, doramectin and ivermectin, using a single quadrupole LC–MS system equipped with an APCI source operating in positive ion mode, down to 25 μg/kg in tissue [154]. Rudik et al. developed a multi-residue method for determination of eprinomectin, moxidectin, doramectin, selamectin and ivermectin in serum and liver, using a single quadrupole LC–MS system equipped with an APCI interface operating in positive ion mode [132]. This group found that confirmation of doramectin and ivermectin was more difficult than for other drugs due to limited fragmentation on the single quadrupole instrument. Detection limits of 10, 10, 10, 50 and 100 μg/kg for moxidectin, doramectin, ivermectin, selamectin and eprinomectin, respectively, were obtained.

Ballard et al. developed a method for detection and confirmation of eprinomectin in bovine liver by LC–MS/MS [77]. The LC–MS/MS system was equipped with an electrospray ionisation source and operated in positive ion mode. This group selected the parent $[M+H]^+$ ion of eprinomectin at *m/z* 914 in the first quadrupole. The ion at *m/z* 914 was fragmented in second quadrupole and the ions at *m/z* 896, 468 and 330 monitored in the third quadrupole. Residues could be confirmed at levels greater

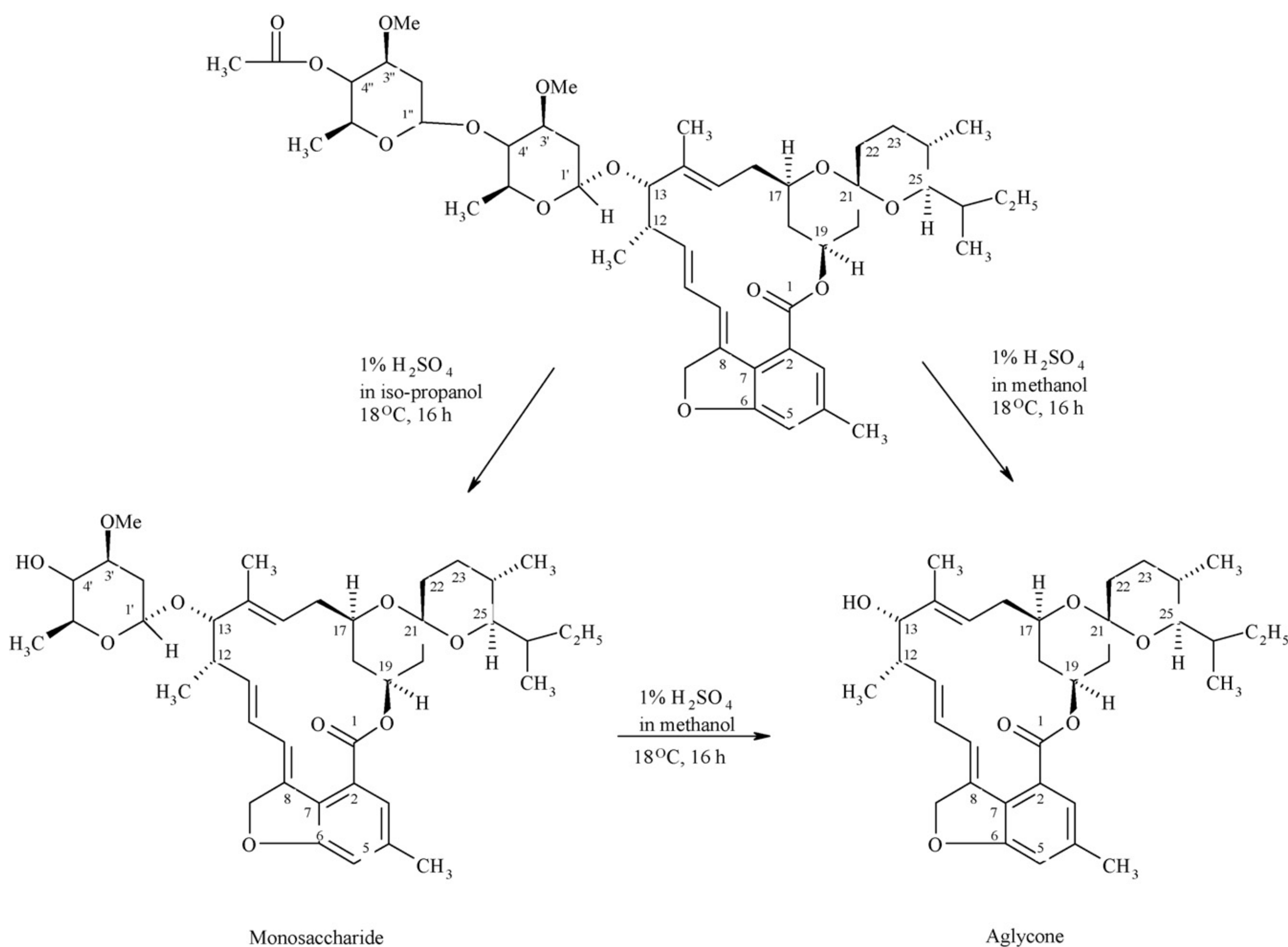

Fig. 6. Derivatisation procedures for the preparation of monosaccharides and aglycone fluorescent derivatives of ivermectin.

than 2400 μg/kg. At concentrations less than 1000 μg/kg, problems were encountered when trying to confirm residues because of poor matching between relative ion intensities for standards and samples. The MRL for eprinomectin in bovine liver is 1500 μg/kg. It was found that certain relative ion intensities (undescribed) were outside the ±10% tolerance limits. It was thought overloading the column with matrix was the cause of the failure in some cases to meet the relative ion intensity criteria. They highlighted that good performance from the system could be achieved by limiting the size of the injection volume to 10 μl. In addition, daily regeneration of the column by reversing the flow and passing sequential 10 ml volumes of methanol, acetonitrile, tetrahydrofuran, acetonitrile and methanol through the column, ensured good reproducibility.

Stout et al. evaluated different LC–MS configurations for determination of moxidectin residues in cattle fat [169]. Using LC–MS with an ESI source operating in positive ion mode, it was found that the $[M+H]^+$ ion at *m/z* 640 was only 20% of the most abundant ion. The main ions produced were an $[M+Na]^+$ ion at *m/z* 662, an $[M+H-H_2O]^+$ ion at *m/z* 622 and $[M+H]^+$ at *m/z* 640. The $[M+Na]^+$ parent ion could not be fragmented on a triple quadrupole instrument, but could be fragmented using the MS/MS capability of an ion trap. It was found that the $[M+H]^+$ ion was the main ion produced after incorporating ammonium acetate in the mobile phase, allowing confirmatory determination with ES. Collision induced dissociation (CID) was also evaluated using a single quadrupole instrument. However, the ratio of the $[M+Na]^+$ ion to the fragment ions was markedly higher in samples compared to the ratio for standards, due to the presence of traces of sodium in the sample extracts. Using APCI, this $[M+Na]^+$ ion did not occur but loss of a water molecule was observed; the extent of this neutral loss could be reduced by incorporating ammonium acetate into the mobile phase and reducing the temperatures of the heated capillary and vaporiser.

Daeseleire et al. developed a multi-residue method for determination of five ML residues in milk, using selamectin as an internal standard on a triple quadrupole instrument equipped with an ESI source operating in positive ion mode [158]. Residues were monitored as their $[M+Na]^+$ ions. The method was validated to allow determination of eprinomectin and moxidectin at the MRLs of 20 and 40 μg/kg, respectively and for detection and confirmation of other ML residues, namely abamectin, doramectin and ivermectin, to 4 μg/kg. The method was validated to allow determination of eprinomectin, moxidectin, abamectin, doramectin and ivermectin residues at the MRLs 50, 50, 20, 10 and 20 μg/kg, respectively, in bovine muscle tissues. Coles et al. determined doramectin, emamectin and ivermectin residues in liver and fish muscle using a LC–MS/MS

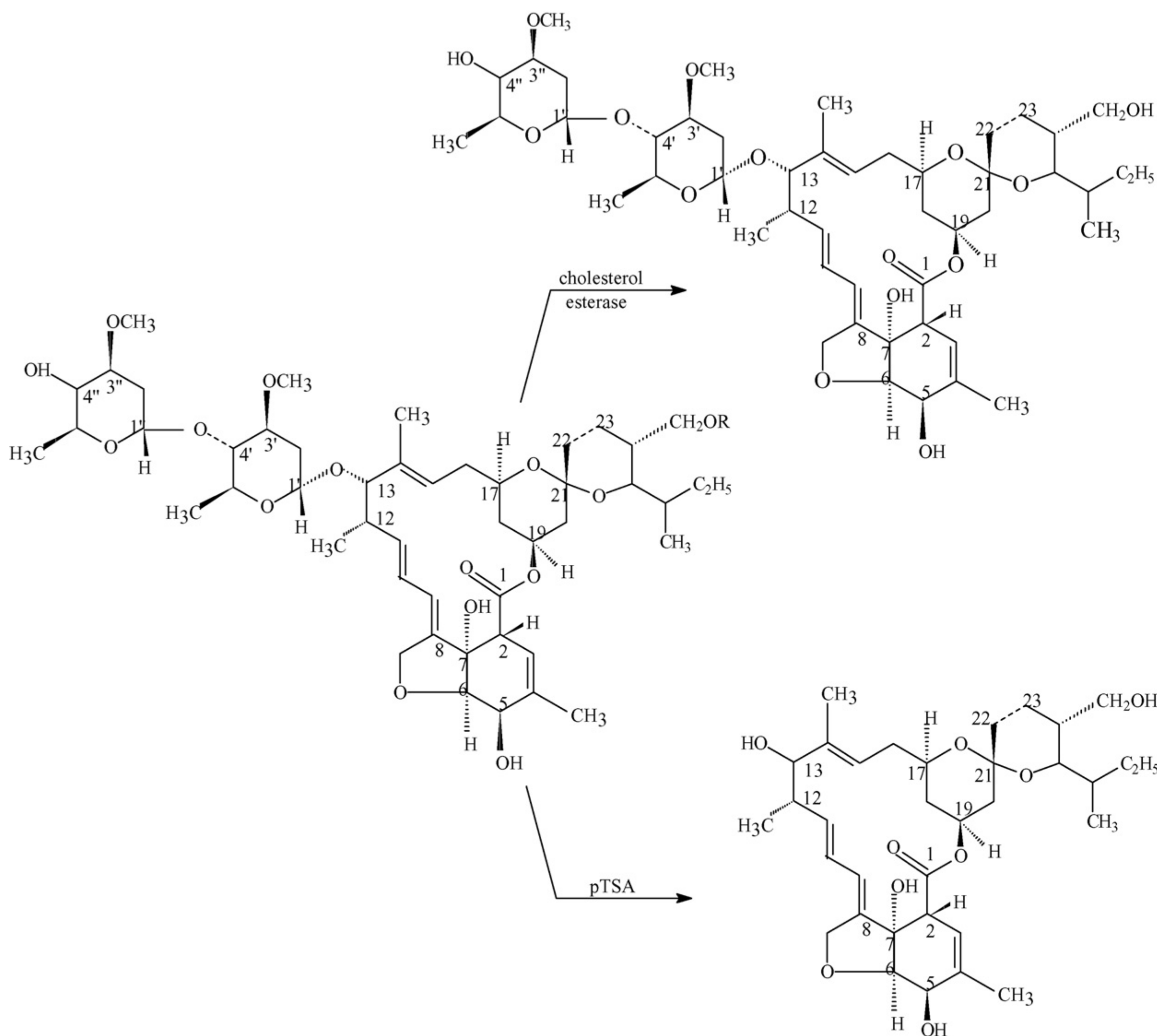

Fig. 7. Scheme of hydrolysis reactions of ivermectin H_2B_{1a} in fat tissue with p-toluenesulfonic acid (pTSA) or cholesterol esterase. (Reprinted from reference [58], with permission from Springer–Verlag, Copyright (1989).)

system equipped with an ESI source operating in positive ion mode [157]. Residues were monitored as their $[M+H]^+$ ions. The method was validated for determination of emamectin and ivermectin in salmon tissue at 100 and 0.2 μg/kg, respectively. Emamectin is approved for use in salmon and has an MRL of 100 μg/kg. However, ivermectin is not approved in aquaculture and lower limits of detection are required to monitor for abuse of this drug in aquaculture products. The method allows for determination of abamectin, doramectin and ivermectin in liver tissue to levels of 7.5 μg/kg based on the lowest standard in the calibration curve. The MRLs for these drugs in the liver vary with species, the lowest being for ivermectin in bovine liver at 15 μg/kg.

Howells et al. developed a more sensitive method for quantitation and confirmation of these residues in bovine liver, with limits of detection between 2.2 and 4.0 μg/kg (40–80 pg on column), allowing accurate determination at the MRLs [127]. This was achieved using the MS/MS capability of an ion trap mass spectrometer with an APCI interface in negative ion mode (Fig. 8). The parent $[M-H]^-$ ion was fragmented using collision induced dissociation, with the most abundant daughter ion being used for confirmation while the daughter ion that could be determined with greatest precision was used for quantitation.

Wu et al. developed an LC–MS method for determination of abamectin and ivermectin in pig liver using a time of flight instrument mass analyser equipped with an APCI source operating in negative ion mode, with selective ion monitoring of the $[M-H]^-$ ion [128]. With APCI, no ions were observed in positive ion mode and negative ion mode was found to be the most suitable technique. Using an ESI source operating in positive ion mode, it was found that the most abundant ions for standards were $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$. However with samples it was found that the $[M+Na]^+$ and $[M+K]^+$ ions were most abundant. Such adducts are undesirable in LC–MS and can lead to poor reproducibility in ionisation efficiencies between samples. This can result in suppression or ion enhancement phenomena that require the incorporation of internal standards to increase reproducibility in the MS. ESI was also investigated in negative ion mode but sensitivity was poor.

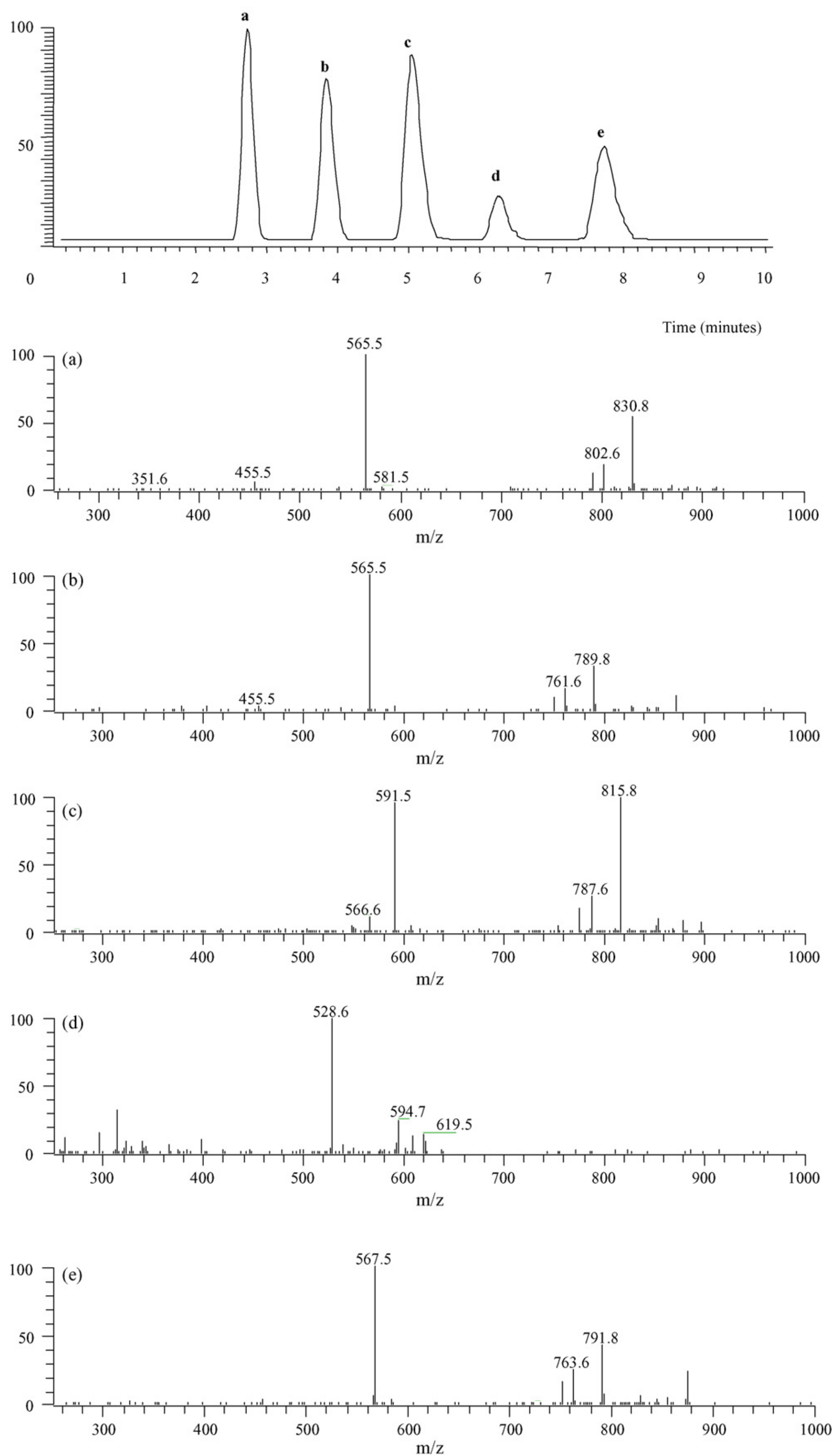

Fig. 8. LC–MS/MS total negative ion current chromatogram of avermectin mixed standard (10 ng ml^{-1}) using an LCQ ion trap with APCI interface (Thermoelectron Corp.) and full scan MS^2 spectra of avermectin parent ion fragmentations. (a) Eprinomectin, (b) abamectin, (c) doramectin, (d) moxidectin and (e) ivermectin. (Reprinted from reference [127], by permission of The Royal Society of Chemistry, Copyright (2001).)

In recent years, a new ionisation source, APPI, has been proposed for LC–MS. This source induces ionisation of compounds by irradiating them with UV light. The technique offers the potential to analyse residues that are poorly ionised by traditional ionisation procedures. It offers higher sensitivity for non-polar compounds, weaker fragmentation of compounds and reduced chemical noise. Turnipseed et al. evaluated APPI and compared results to APCI for determination of ML residues in milk while using an ion trap LC–MS system [161]. It was shown in negative ion mode that photoionisation of the residues using UV light and an acetonitrile-based mobile phase increased the MS response. However, the best response for these compounds was obtained in positive ionisation mode without any discharge current applied to the corona needle and with the UV light source switched off. The use of methanol in the mobile phase with acetone as a post-column dopant also enhanced the signal. This group also evaluated ESI in positive ionisation mode with sodium acetate in the mobile phase. However, it was found that APCI in positive ionisation mode was 2–3 times more sensitive. A sensitive method was developed for determination of the $[M+Na]^+$ molecular ions using an APCI source and the MS/MS capabilities of an ion trap instrument. The limit of quantitation of the method for eprinomectin, moxidectin, doramectin and ivermectin was 1, 5, 5 and 1 μg/kg, respectively. It was concluded that the performance of the method could be further improved using a triple quadrupole instrument to monitor a fragment ion in SIM mode, while using an internal standard and/or a matrix curve.

Single quadrupole instruments may be used in selected ion monitoring (SIM) mode to provide both quantitative and confirmatory identification of residues. According to current EU criteria, a minimum three diagnostic ions have to be selected for MLs to give three identification points (one point for each ion) required for Group B substances [174]. In addition, a minimum of one ion ratio has to be calculated, relative to the most intense ion in a suspect test sample. This ion ratio should correspond to those of standards at an equivalent concentration. Most of the methods developed on single quadrupole instruments typically monitor for four or more ions.

Triple quadrupole instruments are becoming more widely used for determination of ML residues [77,157,158,169], offering increased sensitivity over single quadrupole instruments through reduction in the noise that results from the increased specificity afforded by the extra fragmentation and selection stages. The second quadrupole acts as a collision cell to fragment the parent ions from the first quadrupole through the process of collision induced dissociation and these fragments are monitored in the third quadrupole. Two parent ions can be selected in the first quadrupole, with fragmentation to produce suitable transitions that are monitored in the third quadrupole. According to the criteria for identification proposed by the European Commission, such an approach would typically give five identification points (two for parent ions and 1.5 for each transfer) [174]. A number of researchers have developed LC–MS procedures for determination of ML residues using ion trap instruments [123,127,161,169]. Ion trap instruments have been shown to give good sensitivity [123,127,161], offer full scan spectra without huge loss in sensitivity and allow fragmentation of certain ions (namely sodium adducts) that are difficult to fragment using the MS/MS capability of a triple quadrupole [169]. The disadvantage with ion traps is that there is a limited mass range and that isobaric interference can seriously reduce sensitivity with consequential loss of repeatability. Overall, triple quadrupole mass spectrometers when linked to HPLC systems are the mass detectors of choice for quantitation of MLs, being less susceptible to matrix interference and having a larger dynamic range than the alternatives. Furthermore, the reduction in their price over recent years is bringing them within the price range of most analytical laboratories.

10.3.5. Summary for detection

The most suitable methods at present for determination of ML residues would appear to be LC fluorescence or LC–MS/MS. These methods are summarised in Table 3. LC fluorescence would be the method of choice for determination of ML residues because of its low cost and good sensitivity. A problem that has been encountered by a number of researchers developing LC fluorescence methods has been the instability of fluorescent derivatives. In particular, the susceptibility of the ester derivatives of avermectins to hydrolysis and poor stability of the fluorescent derivative of eprinomectin has been noted. This may be overcome by using on-line derivatisation or by use of alternative derivatisation procedures that produce more stable fluorescent derivatives.

LC–MS/MS is becoming widely used for determination of ML residues. Suitable determination of ML residues (in terms of sensitivity and selectivity) has been achieved on ion trap instruments using APCI operating in negative ion mode while monitoring for $[M-H]^-$ molecular ions or in positive ion mode while monitoring for $[M+Na]^+$ molecular ions. Alternatively, methods for determination of ML residues using LC–MS/MS may use an ESI source while monitoring for $[M+H]^+$ or $[M+Na]^+$ molecular ions using a triple quadrupole analyser. Sample matrix effects may cause difficulties when trying to meet relative intensity tolerances for confirmation. Such problems may be reduced using spiked or extracted matrix calibration curves. However, the most efficient means of allowing for these variations is by the use of deuterated internal standards. Alternatively, an internal standard that displays similar properties to the analytes may be used.

11. Practical aspects of residue testing

11.1. Development of methods

Several factors need to be considered when developing methodology for determination of ML residues. These factors include the number of residues to be included in the method (parent drug or metabolite), target matrix, sample preparation, method of determination or confirmation, required sensitivity and method validation. Because of the number of ML veterinary drugs available on the market, it has become apparent that where possible, multi-residue methods should be applied in laboratories. Six MLs (eprinomectin, moxidectin, emamectin, abamectin, doramectin and ivermectin) are approved for treatment of food producing species in the EU. Methods have been

Table 3
Summary of selected multi-residue detection systems for the determination of ML residues

Residues	Derivatisation reagent	Reaction (temp., time) (°C, min)	LOQ (μg/kg)	Detection system	Ref.
	Animal tissue				
Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	7.5	LC-Fl	[120]
Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	≤5	LC-Fl	[121]
Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	2	LC-Fl	[153]
Ep, Mx, D, I	Non-derivatised		Confirmatory	LC–MS	[123]
Ep, Mx, A, D, I	Non-derivatised		≤4	LC–MS	[127]
Ep, Mx, A, D, I	TFAA/MIM/ACN	65, 90	25	LC-Fl	[125]
Ep, Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	2	LC-Fl	[155]
	Acetic acid	65, 30			
Ep, Mx, A, D, I	Non-derivatised		1–50	LC–MS	[157]
Mx, A, D, I	Non-derivatised		Confirmatory	LC–MS	[154]
A, I	Non-derivatised		5	LC-UV<5	[116]
I, D	TFAA/MIM	Ambient, 3		LC-Fl	[133]
Ep, Mx, D, I, S	Non-derivatised		10–100	LC–MS	[132]
Ep, Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	2	LC-Fl	[156]
	Acetic acid	65, 30			
Epr, Mx, Mi, A, D, I	TFAA/MIM/ACN	Ambient, 3	5	LC-Fl	[160]
Epr, Mx, A, D, I, S	Non-derivatised		Not available	LC–MS	[158]
	Dairy products				
Mx, A, D, I	TFAA/MIM/ACN	Ambient, 3	1	LC-Fl	[124]
Ep, Mx, A, D, I	AA/MIM/DMF	100, 60	5	LC-Fl	[126]
Ep, Mx, A, D, I, S	Non-derivatised		4–40	LC–MS	[158]
Ep, Mx, Em, A, D, I	TFAA/MIM	Ambient, 3	1	LC-Fl	[162]
	Acetic acid	65, 30			
Ep, Mx, Em, A, D, I	Photochemical		10	LC-Fl	[122]
Ep, Mx, Em, A, D, I	Non-derivatised		Confirmatory	LC–MS	[123]
Ep, Mx, Em, A, D, I	Non-derivatised		1–5	LC–MS	[158]
Ep, Mx, A, D, I	TFAA/MIM/TEA	Ambient, 3	<1	LC-Fl	[172]
	Trifluoroacetic acid	70, 30			
	Fish				
I, D	TFAA/MIM/ACN	Ambient, 3	1	LC-Fl	[94]
	Ammon. Ac. MeOH	50–55, 15			
Em, I	TFAA/MIM/ACN	Ambient, 3	1.5	LC-Fl	[129]
Mx, Em, A, D, I	TFAA/MIM/ACN	Ambient, 3	≤1	LC-Fl	[152]
	Plant-based matrices				
Em (X4), Mi, A, D, I	Non-derivatised		≤1	LC–MS	[130]
Em (X4), Mi, A, D, I	TFAA/MIM/ACN	Ambient, 3	≤1	LC-Fl	[131]

developed for determination of ML residues in tissue on the basis that the drugs are used in a particular species (for example, emamectin and ivermectin in salmon, and doramectin and ivermectin in pig) [129,133]. Because unapproved used of MLs has been identified in the past, particularly the use of ivermectin in lactating species [175] and in farmed salmon [176,177], methods are required that are capable of determining residues occurring due to unapproved use of drugs or accidental contamination.

ML residues are more concentrated and persistent in fat and liver tissues than in muscle. Therefore, fat and liver are more suitable matrices to screen for ML residues than muscle, particularly when testing for unapproved use of MLs. This review has shown that there are a number of suitable methods for determination of ML residues in milk, tissue and plant-based matrices. At present, no universal sample preparation procedure is available that covers all matrices. It is recommended to develop methods suitable for individual matrices. For example, a method for milk may require only a single SPE step whereas two SPE steps may be required for liver tissue.

Several different detection systems have been applied for determination of ML residues that offer sensitivity suitable for residue testing. The most effective system for determination of ML residues at present is LC fluorescence. LC–MS/MS is also suitable but is more expensive. A number of developments have been made in recent years in the area of immunochemical methods. Antibodies have been developed that show cross-reactivity to the individual avermectins and milbemycin classes. However, no antibody has yet been produced that shows cross-reactivity to both classes.

11.2. *Validation of methods according to Commission Decision 2002/657/EC*

Procedures for validation of methods are documented in Commission Decision 2002/657/EC [174]. Prior to the development of the 2002/657/EC criteria, a number of different approaches were adopted for validation of methods. Typically, validation included intra- and inter-assay validation and calcu-

lation of limits of detection and quantitation. Since the implementation of the 2002/657/EC criteria, a systematic approach to evaluate the performance of residue methods is in place. A number of methods have been validated for determination of MLs in tissue and milk using this approach, as it is now a requirement for residue methods in the EU. Important validation concepts that have been included in the 2002/657/EC criteria are the decision limit (CCα) and the detection limit (CCβ).

Coles et al. used the calibration curve method to calculate CCα and CCβ for ivermectin and emamectin residues in liver salmon [157]. For ivermectin (an unapproved substance in salmon), blank salmon muscle samples were fortified at and above the lowest calibrated level in equidistant steps. CCα was determined by plotting the measured signal against the added concentration and calculating the corresponding concentration at the y-intercept plus 2.33-times the standard deviation of the within-laboratory reproducibility of the intercept. CCβ was calculated as CCα plus 1.64-times the standard deviation of the within-laboratory reproducibility. For emamectin (an MRL substance in salmon), blank samples were fortified around the MRL in equidistant steps. CCα was calculated by plotting the measured signal against the added concentration and CCα was determined as the concentration at the MRL plus 1.64-times the standard deviation of the within-laboratory reproducibility. CCβ was calculated as the measured content at CCα plus 1.64-times the standard deviation of the within-laboratory reproducibility. Using this approach, CCα and CCβ were determined to be 0.2 and 0.3 μg/kg for ivermectin in salmon. In the case of the licensed veterinary drug, emamectin benzoate, CCα and CCβ were calculated to be 108 and 112 μg/kg.

Daeselaire et al. adopted an alternative approach to validate a method for determination of six MLs in bovine muscle [158]. In this study, 20 blank muscle samples were fortified at the MRL and CCα was calculated as the level determined plus 1.64-times the within laboratory reproducibility. CCα values (MRL values in brackets) were calculated to be 55 (50), 64 (50), 21 (20), 12 (10) and 23 (20) μg/kg for eprinomectin, moxidectin, abamectin, doramectin and ivermectin, respectively. CCα values were determined for unapproved substances in milk (abamectin, doramectin and ivermectin) by fortifying 20 blank milk samples at a concentration that would give a signal to noise ratio of at least three (4 μg/kg). For CCβ values, blank muscle and milk samples were fortified at the CCα level and calculated as the level determined plus 1.64-times the within laboratory reproducibility.

CCα or CCβ can be determined using the calibration curve procedure or by spiking blank samples. In the case of the calibration curve approach, the linearity of the calibration curve should be verified by regression analysis. It is recommended using either validation approach that CCα and CCβ values be verified by fortification of blank samples. Spiking of 20 blank samples is probably the most common procedure for determination of CCα and CCβ. A common pitfall with this procedure is to select "clean" blank samples for validation studies. Ideally, blank materials should be representative of the population, and picked from animals of different breed, condition (fat and lean), age, feeding regime, etc.

12. Conclusions

A comprehensive review is presented on methodology for determination of ML residues in biological matrices. Recommendations are made on multi-residue methods that are considered to be most suitable for surveillance of ML residues in food. Acetonitrile (liver and milk) and acetone (plant material) have been shown to be suitable for extraction of ML residues. The clean-up step, subsequently required for purification of sample extracts, is dependent on the sample matrix. At present, many methods use isocratic chromatographic systems for separation of ML residues. However, due to the increasing number of ML residues being included in analytical methods, separation of ML residues in a reasonably short chromatographic run time is becoming more difficult. In future, it may be expected that more methods will use gradient systems to separate residues and to wash non-polar matrix interference from HPLC columns. This approach could lead to a reduction in the extent of sample clean-up required prior to analysis. LC fluorescence and LC–MS are presently the techniques of choice for determination of ML residues. LC fluorescence has advantages over mass spectrometry in terms of cost but mass spectrometry is more sensitive and specific. At present, the widespread application of immunochemical methods is restricted by the limited cross-reactivity of antibodies, particularly between avermectins and milbemycins.

There have been a number of developments in methodology for determination of ML residues in recent years, particularly in multi-residue applications. In the future, it is expected that developments will continue in the areas of sample preparation and detection. In particular, research should focus on the development of automated or on-line clean-up procedures that allow unattended purification of sample extracts. Alternatively, 96-well plate technology that has found application in plasma analysis may find application in testing for ML residues in milk and tissues. In the area of detection systems, the production of an antibody showing cross-reactivity to avermectins and milbemycins would appear to be a priority. Such an antibody may be applied in a biosensor assay to give equivalent sensitivity to chromatographic detection systems. Automated pre-column derivatisation has not found widespread application in multi-residue methods. It is expected that researchers may apply this technique in future to overcome stability problems that have been encountered with the off-line derivatisation procedures. Many of the latest multi-residue methods developed for determination of MLs use LC–MS/MS for detection of residues. It is expected that researchers will develop LC–MS/MS methods offering improved reproducibility and reliability. This might be achieved through the introduction of suitable internal standards (such as nemadectin or selamectin) and/or improvements in sample preparation procedures.

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