Universität für Bodenkultur Wien

BEHAVIOUR OF VETERINARY DRUGS IN SOIL, PLANT AND WATER SYSTEM

Transport investigation of antiparasitic drugs in soil-plant-water

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Abstract

The use of anthelmintics in food producing animals could affect the ecological system due to the application of manure containing drugs to the land. Translocation of antiparasitic drugs from animal excrement through soil and water to crops and forages and their recycling to food (crops and grazing animals) is also a potential concern for the food chain. To investigate the behaviour of some drugs in soil, plant and water, a lysimeter experiment was designed. Slurry containing three representative anthelmintics, levamisole (LEV), fenbendazole (FBZ), and eprinomectin (EPR), was applied to lysimeters containing sandy or loamy soil and samples of alfalfa and water were collected at different time intervals, and soil samples at different intervals and depths. The samples were analysed for the target analytes and the sulphoxide (FBZ-SO) and sulphone (FBZ-SO_2) metabolites of fenbendazole using a previously validated LC-MS/MS method. Results showed that in the upper horizon (0-10 cm) of soils the target analytes and metabolites of FBZ were found at high concentrations even at the final sampling time (day 290). In the lower horizon (10-30 cm), all target compounds were detected on day 10 and both FBZ-SO and FBZ-SO_2 were found even at day 290 only in loamy soil. The concentration of FBZ-SO was higher at soil depth 10-20 cm and the FBZ-SO_2 at 20-30 cm. In seepage water LEV (0.8 µg l^{-1}) was the only compound detected (day-36). In plants the maximum concentration of EPR (40.2 µg kg^{-1}) was found in sandy soil at day 32, LEV (37.0 µg kg^{-1}) at day 32 in loamy soil and FBZ-SO metabolite (19 µg kg^{-1}) at day 63 in loamy soil, at approximately 4 times the concentration of FBZ or FBZ-SO_2. A radiotracer experiment using ^{14}C-LEV showed that LEV and its transformation products were transported into the lower horizon.

Keywords: Antiparasitic drugs; lysimeter; soil; plants; water

Zusammenfassung

Die Verwendung von Entwurmungsmitteln bei Nutztieren kann die Umwelt beeinträchtigen aufgrund der Ausbringung von organischen Düngern die die Medikamente enthalten. Boden und Wasser, Getreide und Grünfutter sind potenziell kontaminationsgefährdet. Um das Verhalten einiger dieser Medikamente im Boden, Pflanze und Wasser zu untersuchen, wurde ein Lysimeter-Experiment durchgeführt. Gülle mit drei repräsentativen Entwurmungsmitteln, Levamisol (LEV), Fenbendazol (FBZ) und Eprinomectin (EPR), wurde auf Lysimeter mit einem sandigen und einem lehmigen Boden ausgebracht. Proben von Luzerne und Sickerwasser wurden zu verschiedenen Terminen gesammelt. Ebenso wurden nach unterschiedlichen Zeitintervallen Bodenproben in verschiedenen Bodentiefen gesammelt. Die Proben wurden auf die Ziel-Analyten und die Metaboliten Fenbendazol Sulfoxid (FBZ-SO) und Sulfon (FBZ-SO_2) analysiert mittels einer zuvor validierten LC-MS/MS-Methode. Die Ergebnisse zeigten, dass im oberen Horizont (0-10 cm) der Böden die Substanzen und die Metaboliten von FBZ in hohen Konzentrationen auch zum letzten Zeitpunkt der Probenahme (Tag 290) vorlagen. In dem tieferen Horizont (10-30 cm) wurden alle Verbindungen nur am 10. Tag nach der Ausbringung nachgewiesen. FBZ-SO und FBZ-SO_2 wurden nur im lehmigen Boden auch noch am 290. Tag nachgewiesen. Die Konzentration von FBZ-SO war in der Bodentiefe von 10-20 cm am höchsten, jene von FBZ-SO_2 in 20-30 cm. Im Sickerwasser konnte nur LEV (0,8 µg l^{-1}) als einzige Verbindung nachgewiesen werden (am 36. Tag). In Pflanzen wurden die höchsten Konzentrationen von EPR (40,2 µg kg^{-1}) auf sandigem Boden am Tag 32 gefunden, für LEV (37,0 µg kg^{-1}) am 32. Tag auf lehmigem Boden und für den FBZ-SO Metaboliten (19 µg kg^{-1}) am 63. Tag am lehmigen Boden, was ca. der 4-fachen Konzentration von FBZ oder FBZ-SO_2 entspricht. Ein ^{14}C-LEV Radiotracer-Experiment zeigte, dass LEV und seine Umwandlungsprodukte in die unteren Bodenhorizonte transportiert wurden.

Stichwörter: Antiparasitenmittel; Lysimeter; Boden; Pflanzen; Wasser

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1 Introduction

Anthelmintics are economically important pharmaceuticals used to treat parasitic infections of humans and domestic pets, and also for the treatment of livestock, poultry animals and even crops, thereby having an effect on food production (HOLDEN-DYE and WALKER 2007). The use of anthelmintics, as well as other veterinary pharmaceuticals, is crucial in modern production practices. Since the administered drugs are usually eliminated from the body in the animals' faeces, they can be found in manure (DU and LIU 2011; KIM et al. 2011). The use of animal manures fertilizer, along with run-off from intensive or semi-intensive farming systems in which such drugs are used, and excreta from grazing animals treated with the drugs, may result in increasing amounts of these drugs in the environment (GERBER et al. 2005). When found in the environment as pollutants, they can be distributed in soil, water, sediment, biota and plants, causing possible threats to aquatic and terrestrial ecosystems (HORVAT et al. 2012). The increasing use of anthelmintics with farm animals to improve animal production could, therefore, affect not only the ecological system (KAY et al. 2005; VAN HORN et al. 1994) but also raises the possibility that residues may be found in food produced for human consumption (ROSE 1999).

Soil provides habitats for numerous organisms and microorganisms and the quality and quantity of trace elements and organic chemicals found in soil resulting through natural processes or anthropogenic activities can be influenced in various ways. It has been demonstrated that water sources used for irrigation can contain a wide range of organic contaminants (CHEN et al. 2005; ESTEVEZ et al. 2012; PEREZ-CARRERA et al. 2010). When water percolates into the soil, it causes both mechanical and chemical translocation of materials, which may lead to leaching (JECFA 1999). Both soil and water can be significantly affected by the use of veterinary drugs in agriculture production and their inadvertent application to the land.

In pasture fields, alfalfa (*Medicago sativa L.*), also called lucerne, is known as the —ing of forages" and is one of the more widely grown grazed grasses throughout the world as forage for cattle (YUEGAO and CASH 2009). It is a versatile crop that can be used as pasture, hay, silage, green chop and as a cash crop. Due to its high quality, digestibility, good roughage value and yield potential, alfalfa is used

successfully in many types of livestock feeding programs. Successful production of this crop requires attention to a variety of details including proper fertilization, which can be achieved by applying manure and slurry to reduce cost of agricultural inputs (LACEFIELD et al. ; SHEWMAKER et al. 2011). However, some chemicals, such as veterinary drugs, contained in these fertilizers may be transported to the plants that are not only grazed or fed to animals in other forms of feedstuffs, but may also be consumed by humans as fresh or as processed supplementary products (INRA et al. 2013). Thus, the translocation of antiparasitic drugs from animal excrement through soil and water to crops and forages and their recycling to food-producing animals is a potential concern for the food chain (BOXALL et al. 2004b). The content of organic contaminants and residues found in these plants may be associated with the transport behaviour of the chemicals in the environment. This may also lead to recycling of drug substances contained in food, triggering concerns over food or feed safety and production practices (WU et al. 2010; ZURER 2010).

Unlike pesticides, which have been extensively studied in the past, the impact of veterinary drugs on the environment and the extent to which they are transported in the ecological system has not been adequately considered. The impact and magnitude of the effects depend upon factors such as the drugs' physico-chemical properties, the amount used and method of administration, treatment type and dose, metabolism within the animal, animal husbandry practices, manure storage and handling practices, degradation rates in manure, the types of soil to which the manure is applied and, the types of plants and prevailing climatic conditions during vegetation period (BOXALL et al. 2004a; BOXALL et al. 2006; BOXALL et al. 2004c).

The antiparasitic drugs that are used for different purposes are classified into several classes although they are sold under different brand names (SCHOENIAN 2008). Levamisole, fenbendazole (including its metabolites fenbendazole sulphoxide and fenbendazole sulphone) and eprinomectin are amongst the most widely used and important veterinary drugs, representing the imidazothiazole, benzimidazole, and the macrocyclic lactone classes of anthelmintics, respectively. They are the most important chemical groups used to control parasitic infections in domestic animals (ALVAREZ et al. 2007; MOTTIER et al. 2006). Each group class has a different specific mode of action.

Imidazothiazoles

Discovered in the 1960´s, imidazothiazoles are compounds with broad-spectrum anthelmintic efficacy against roundworms (nematodes) vastly used on livestock and pets (JUNQUERA 2013a). The imidazothiazoles cause the neuromuscular paralysis of gastrointestinal nematodes and lungworms in cattle and sheep (MARTIN 1997).

Belonging to a class of synthetic imidazothiazole derivatives, levamisole (LEV) is a levo enantiomer of tetramisole, which is a nicotinic receptor antagonist causing spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine receptors on body wall muscle (ACEVES et al. 1970). It depolarises the ganglions and nervous cells of the worms, which made them paralyzed and die or are expelled. LEV acts as a broad spectrum anthelmintic and has been used as a good dewormer (lungworms and other roundworms) for livestock for the prevention and treatment against nematodes and various species of lungworms. It has the narrowest margin of safety amongst all anthelmintics.

Levamisole salts are soluble in water making them easily absorbed to blood and distributed to other parts of the body after administration through oral drench or in-feed, topical (pour-on) and subcutaneous or intramuscular injection. The absorption and excretion of levamisole is rapid and not affected by the route of administration or ruminal bypass because it is highly soluble. It is mainly metabolized in the liver and usually excreted through urine (83 and 84 %) and faeces (11 and 9 %) when given orally and subcutaneously respectively (PAULSON and FEIL 1996). In soil, levamisole has a half-life of five to seventy five days depending on sunlight, soil type and climatic conditions (NOVARTIS 2009).

Benzimidazoles

The benzimidazoles represent the only class of truly broad-spectrum antiparasitic drugs which interrupt parasite-energy metabolism by binding to tubulin, thereby disrupting microtubular cell structure and preventing nutrient uptake and other actions. Benzimidazole anthelmintics work through selective binding to nematode β-tubulin, and consequent inhibition of microtubule formation (MARTIN 1997), which inhibits glucose uptake, blocking the energy management mechanism of the worms that are paralyzed and die or are expelled. The lack of water solubility and poor drug absorption of the benzimidazoles are problems that limit the use of most

benzimidazole carbamates against intestinal parasites. However their use became widespread due to their extent of spectrum and efficacy against immature stages as well as the safety of host animals.

Benzimidazoles are extensively metabolized in animals and humans. The parent compound is generally short lived and metabolised predominantly in plasma, tissues and excreta. The primary metabolites usually results from normal oxidative and hydrolytic processes and are more soluble than the parent compound. Metabolites have been isolated from both urine and faeces with the latter being mostly attributed to limited absorption, although biliary excretion can contribute to faecal levels. The metabolic profile of individual benzimidazole follows similar pattern across species but metabolite percentage do vary substantially (DUBEY and SANYA 2010).

A representative compound, fenbendazole (FBZ), is effective for the control of sensitive gastrointestinal roundworms and lungworms in many mammalian species such as cattle, sheep, goats, horses and pigs. It is a highly hydrophobic compound, which has a polar carbamate side chain that is extremely insoluble (BOGAN and MARRINER 1983; SHORT et al. 1987). This compound was found to be acutely toxic to a fresh water invertebrate, Daphnia magna (EC50s:16.5 µg l^{-1}) (OH et al. 2006). Both in vivo and in vitro studies of FBZ metabolism have been performed in multiple species (GOZALO et al. 2006). In vivo, FBZ mainly exists in its oxidised form, as the sulphoxide (FBZ-SO) or sulphone (FBZ-SO_2) (EMEA 2004). FBZ-SO, also known as oxfendazole, is an anthelmintic by itself and is the most toxic form of the compound (EMA and CVMP 2013). The oxidised metabolites are the main active products found systematically after FBZ administration to sheep and cattle (LANUSSE et al. 1995; SHORT et al. 1987). FBZ-SO and FBZ-SO_2 metabolites have been detected as trace metabolites in urine or faeces of cattle, sheep, chickens, goats, and rabbits after dosing with FBZ (SHORT et al. 1988a; SHORT et al. 1988b). It is primarily eliminated through a function of oxidative metabolism and is excreted through the liver. As much as 44% to 50% of the dose is found unchanged in the faeces, whereas a small concentration (0.1%) is excreted through the kidneys in the urine (SHORT et al. 1988b).

The interspecies metabolism and distribution of fenbendazole has been extensively studied. Results of a detailed in vivo study in goats showed that the metabolism of fenbendazole in ruminants is qualitatively similar, and that the primary metabolites include the sulfoxide, sulfone, and parahydroxy metabolites, plus a very small

amount of decarboxylation metabolism to the amine. The rates of formation of the parahydroxy, sulphoxide, and sulphone metabolites differed substantially between species (CRAIGMILL and CORTRIGHT 2002). A disposition study in cattle showed that all known metabolites were identified in urine and feces except for fenbendazole amine and that the major excretory product was FBZ-OH (SHORT et al. 1987). Sulphate and glucuronide conjugates of the hydroxyl metabolite were found in urine, together corresponding to < 15% of the dose. In a study using hepatic functions from livers of cattle, sheep, goats, chickens, ducks, turkeys, rats, rabbits and catfish, all species were found to produce the sulphoxide and sulphone metabolites, and *p*-hyfroxyfenbendazole except in sheep (SHORT et al. 1988b).

Figure 1 shows the metabolism process of fenbendazole and fenbendazole sulphoxide. The primary metabolism and toxicity of the benzimidazoles is generally controlled by the substituent at position 5- of the benzimidazole ring (GOTTSCHALL et al. 1990). The principal metabolic route is by oxidation of the sulphide to the sulphoxide (oxfendazole) and sulphone. The first sulphoxidation step is catalysed by a microsomal flavin-containing monooxygenase (albendazole monooxygenase) to produce fenbendazole sulphoxide. This step is reversible, with both compounds existing in equilibrium. The second sulphoxidation is mediated by cytochrome P450, and is irreversible. Formed by cytochrome P450 mediated *p*-hydroxylation of the aromatic ring, *p*-hydroxyfenbendazole, has also been reported as a metabolite of fenbendazole and fenbendazole sulphoxide.

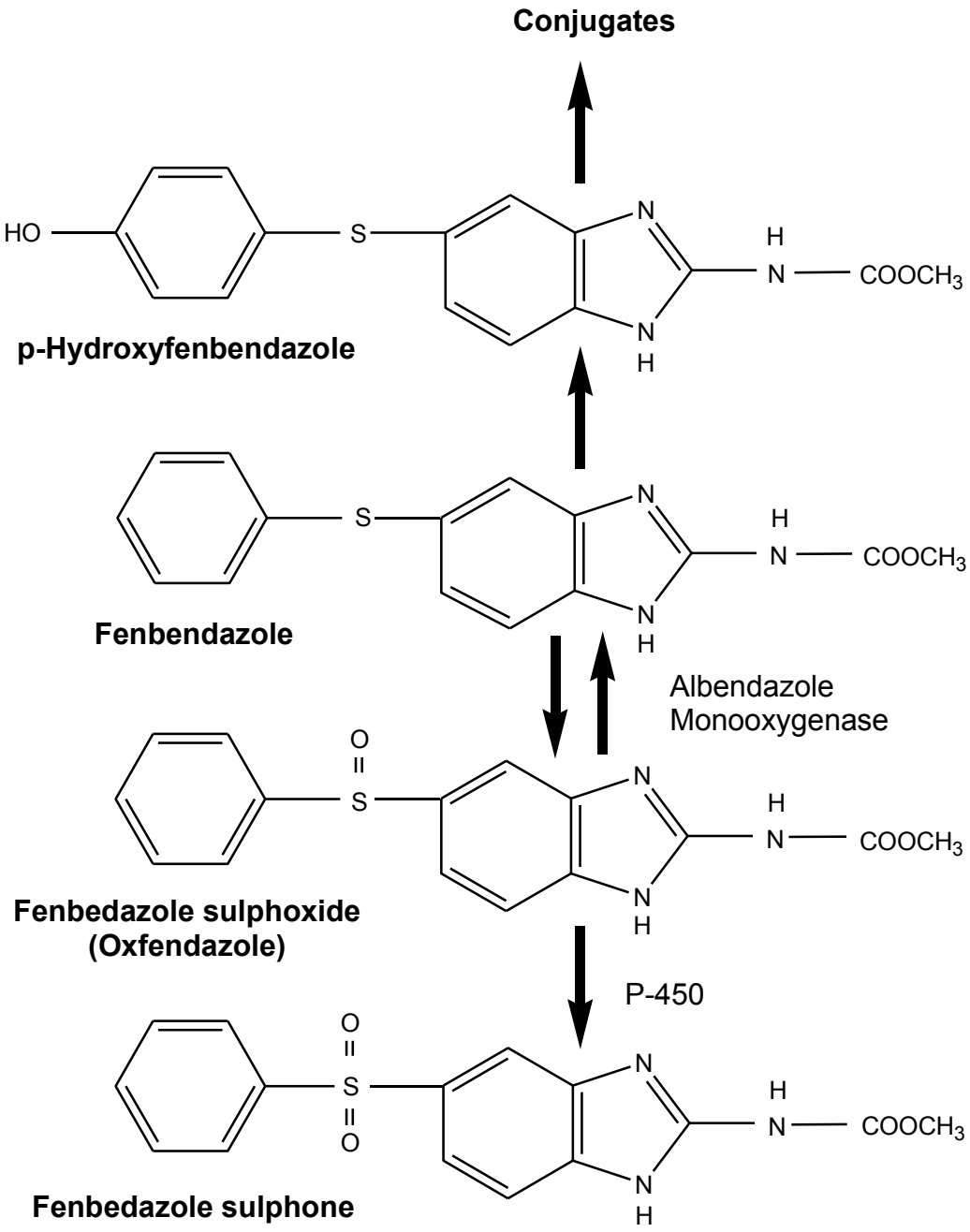

Figure 1: The metabolic pathways of fenbendazole and oxfendazole

Macrocyclic lactones

The macrocyclic lactones (avermectins and milbemycins) are products or chemical derivatives of soil microorganisms belonging to the genus *Streptomyces*. Macrocyclic lactones (MLs) are GABA (gamma-aminobutyric acid) agonists which block the transmission of neuronal signals nerve and muscle cells of invertebrates

(JUNQUERA 2013b). This particular group of anthelmintic interfere with parasite nerve transmission by indirectly opening chloride channels in the post synaptic membrane. The MLs have a potent, broad antiparasitic spectrum at low dose levels. Regardless of the route of administration, MLs are extensively distributed throughout the body and concentrate particularly in adipose tissue. The commercially available MLs are primarily excreted in the faeces, and a broad range of insecticidal activities have been observed against dung-inhabiting insect species.

Eprinomectin (EPR) belongs to a family within this group collectively referred to as avermectins. It consists of a mixture of two homologous components, B1a (not less than 90%) and B1b (not more than 10%), which differ by a single methylene group at the 25-carbon position (JIANG et al. 2005). EPR is a polar ML, with a lower association with lipids, resulting in a shorter drug half-life compared with other topically applied MLs in cattle (ALVINERIE et al. 1999). It is used as both endo- and ectoparasiticides and is usually marketed as a pour-on formulation for use in beef and dairy cattle (SHORT et al. 1988a). It is approved for topical use in all cattle including lactating cows without any milk withdrawal periods (BAOLIANG et al. 2006; LANUSSE et al. 2009). Compared with oral and intravenous treatment, topical veterinary medicines like EPR have greater potential to be released to the environment (BOXALL et al. 2004a).

EPR is excreted in the bile and faeces and approximately 86% of the applied dose is excreted in faeces as unchanged drug (MERCK & CO. 1996b). As a highly hydrophobic compound, based on its octanol/water coefficient (Log Kow = 5.4), EPR is expected to be strongly bound in the soil. Under aerobic conditions, the half-life of EPR in soil is about 64 days (MERCK & CO. 1996a).

The pathway by which organic pollutants enter the vegetation is a function of chemical and physical properties of each pollutant, such as hydrophobicity, water solubility, and vapour pressure, as well as the conditions of the receiving environment like temperature, organic content of the soil, and plant species. Uptake of organic chemicals into plants can occur from air or soil, depending on the properties of the compounds (HELLSTRÖM 2004). Organic chemicals bound to soil particles may be deposited on the vegetation as a result of wind re-suspension or rain splash (SMITH and JONES 2000). But in general, organic chemicals are taken up by plants through the soil pore water.

Currently, there is limited information available to concerned regulatory bodies regarding the fate of anthelmintic substances in environmental compartments such as soil, plant and water (KELLY et al. 2003). Published environmental fate assessment data on anthelmintic drugs is not as extensive as some other pharmaceutically active substances such as antibiotics. Most literature on fate of anthelmintics dealt only with the metabolic pathways in living organisms. However, the reactions and the transformation products they may produce in the environment could be identical to the metabolites found in the organisms (HORVAT et al. 2012). The high octanol-water partition coefficient values for most of the anthelmintics indicates that these compounds are strongly adsorbed to sediment/soils and therefore not mobile. But it is rather difficult to assess movement and bioavailability of such compounds through the soil profile (LITSKAS et al. 2010b). Moreover, unlike other organic substances, such as pesticides and industrial chemicals, the sorption behaviour of many pharmaceuticals cannot simply be derived from the substance's hydrophobicity or the organic carbon content of the solid material (TOLLS 2001). It is therefore the aim of this study to generate additional information concerning the behaviour of the three major groups of anthelmintics in the three environmental compartments: soil, plant and water. However, analytical methodologies must be developed to elaborate and characterise the risks associated with the exposure of these organic contaminants in the environment.

This work presents two major phases of the study: firstly, the development and validation of a liquid chromatography tandem mass spectrometric (LC-MS/MS) methods for the determination of anthelmintic compounds described above in soil (loamy and sandy), water and plants with associated extraction/concentration/clean-up regimes for each matrix and secondly, the investigation into the transport of these antiparasitic drugs in soil, plant and water system.

2 Objective and definition of the research topic

The general objective of this study is to evaluate the behaviour of anthelmintic drugs and the possible ecological impact to the environment.

2.1 Objective of the research topic

The specific objectives are the following:

- To develop, adapt and validate analytical methods on the multi-residue (+multi class) analysis of selected veterinary pharmaceutical compounds in soil, water and plant matrices
- To investigate the fate of the tested substances in two types of soil (loamy and sandy) at different horizon level and seepage water.
- To examine uptake of these drugs from soil into plants and concentration of anthelmintic drug residues that may impact food producing animals.

2.2 Definition of the research topic

- Several methods for anti-parasitic compounds are available for animal tissues since these compounds are administered to animals to kill or expel worms from the body to promote growth and improve animal food production. Monitoring of these drugs in animal tissues used for food consumption is important to ensure that any amounts detected are within the permitted tolerance limits. However to date, very limited and mostly single residue methods on anti-parasitic drugs are available for soil, water and plants. Multi-class anthelmintic methods are necessary to study the residual concentrations of exposed veterinary pharmaceuticals (applied as fertilizers or normal droppings in grazing lands) that are transported into the different environmental compartments.
- Most agricultural lands in Austria are made up of silt-loam and loamy sand soils. It is therefore interesting to investigate how these veterinary drugs behave in these types of agricultural soils used for crop production at different depth level and the possibility of contaminating ground water.
- Parasite resistance has emerged as a continuing problem in the livestock industry because many parasites are becoming more resistant to all classes of anthelmintics. The increasing use of anthelmintics over time could also lead to accumulation of the chemical concentration and the consequent uptake of the drugs by plants. Recycling

of the drugs in food by grazing livestock or in feeds through hays or forage may not only affect or induce the already existing problem on anthelmintic resistance but can also add up to the drug residue levels present in the animal tissues, a human health concern.

3 Data basis, Materials and Methods (published sections: 3.1 - 3.2[1] and 4.1 - 4.2[2])

The data for this research study was based on an experiment conducted in an open field cyclindrical lysimeter blocks where veterinary drugs and radiolabelled compound were applied to check for the transfer of targeted compounds into the different ecosystems. For this reason, the first part of the study was focused on developing a multi-class method for the determination of anthelmintics in soil, water and plants specifically for each matrix group using liquid chromatography-tandem mass spectrometry. After validating each of the developed method, it was applied into the analysis of soil, water and plant (green chops) samples collected following the designed sampling regimes. The said analytical methods and lysimeter experiments are therefore described in details in the succeeding sections. The method for soil and water was combined in one published manuscript (ISLAM et al. 2013) whereas the method for plants was published as a single paper (ISLAM et al. 2012).

3.1 Determination of anthelmintics in soil and water

3.1.1 Standards and chemicals

All solvents were of high-performance liquid chromatography (HPLC) grade and other chemicals were of analytical reagent grade, unless otherwise stated. Methanol, acetonitrile, formic acid (99.8%, w/v), hydrochloric acid (32%, w/v) and sodium chloride were all purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced in the laboratory with a Milli-Q gradient system produced by Millipore (Molsheim, France). LEV (98.5%), FBZ-SO (99%), FBZ-SO_2 (99%), FBZ (99%), and EPR (97%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).The deuterated internal standards, tetramisole-d5 (LEV-d5), FBZ-SO-d3, FBZ-SO_2-d3, and FBZ-d3 were obtained from Witega (Berlin, Germany), all with 99% P.

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[1] ISLAM, M. D.; HABERHAUER, G.; KIST, A.; RATHOR, M. N.; GERZABEK, M.; CANNAVAN, A. (2013): Multi-class determination of anthelmintics in soil and water by LC-MS/MS. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 30:1128-1137.

[2] ISLAM, M. D.; HABERHAUER, G.; GERZABEK, M.; CANNAVAN, A. (2012): Liquid chromatography-tandem mass spectrometry method for the determination of anthelmintics in alfalfa plants. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 29:1679-1688.

Ammonium formate (99%), magnesium sulphate (98%), sodium citrate dihydrate (99.5%), sodium citrate sesquihydrate (99%), and dimethylsuphoxide (99.9%) were purchased from Sigma Aldrich (Steinheim, Germany). Primary secondary amine (PSA) was purchased from Varian (Yarntorn, Oxford, UK). Solid phase extraction (SPE, 500 mg, 6 ml) cartridges, Strata-X and Oasis-HLB were purchased from Phenomenex (Aschaffenburg, Germany) and Waters (Dublin, Ireland) respectively.

3.1.2 Sample sources and preparation

Soil samples were collected using GPSmap76CSx referencing from Garmin (Romsey, UK) to source the same soil as was used in a lysimeter study to which the methods developed were applied in a second step (manuscript in preparation, to be reported elsewhere). Loamy soil was obtained at Fuchsenbigl/T (Marchfeld), in the northeastern part of Austria whereas sandy soil was taken at Marchegg, in lower Austria. A layer of soil approximately 15–20 cm deep was excavated, mixed, filtered through a 2mm sieve and well mixed in a rotary blender. Sample portions were transferred into plastic containers, taken to the laboratory and air-dried in porcelain dishes at room temperature. The air-dried soil portions were then mixed and stored in a closed container under controlled conditions (22 + 2°C). Tap water was used for method development and validation. Volumes of water were collected directly from the faucet into amber bottles (1 l) and acidified with concentrated hydrochloric acid (32%) to pH~3 prior to analysis.

3.1.3 Preparation of solutions

Stock solutions (1 mg ml^{-1}) of FBZ, FBZ-SO, FBZ-SO_2 and their deuterated internal standards were prepared individually in dimethylsulphoxide (DMSO). Stock solutions (1 mg ml^{-1}) of LEV and EPR were prepared in methanol and acetonitrile respectively. All solutions were stored at -20°C. Stock standards (250 µl LEV, FBZ, FBZ-SO, FBZ-SO_2; 500 µl EPR) were diluted in methanol to a final volume of 25 ml to prepare a mixed intermediate standard solution containing the five target analytes (10 µg ml^{-1} LEV, FBZ, FBZ-SO, FBZ-SO_2, 20 µg ml^{-1} EPR). The intermediate mixed standard was stable for at least 1 month at 4°C. A mixed internal standard solution containing the four internal standards was prepared similarly. Working standard solutions (1.0 µg ml^{-1} LEV, FBZ, FBZ SO, FBZ-SO_2, 2.0 µg ml^{-1} EPR) were then prepared by diluting the intermediate mixed standard solution (1 ml LEV, FBZ, FBZ-SO, FBZ-SO_2,

2 ml EPR) to volume (10 ml) with methanol and stored at 3–8°C. Calibration solutions (equivalent to 5, 10, 20, 40, and 160 µg kg^{-1} FBZ, FBZ-SO, FBZ-SO_2 and10, 20, 40, 80 and 160 µg kg^{-1} EPR) were prepared by pipetting aliquots of working standard solution (10, 20, 40, 80 and 160 µl) and internal standard mixture (80 µl, 1.0 µg ml^{-1}) into individual tubes and each tube was made up to a final volume (500 µl) with blank matrix extract.

3.1.4 Extraction and sample processing

3.1.4.1 Soil

The analytical method employed dispersive solid phase extraction. Portions (10 g) of the air-dried soil sample were weighed into polypropylene centrifuge tubes (Sarstedt, Wr. Neudorf, Austria). For analyte recovery experiments samples were spiked by the quantitative addition of standards in methanol. Each sample was vortexed for a few seconds and allowed to stand at room temperature for at least 20 min. An acetonitrile/methanol mixture (1:1, v/v, 15 ml) was added and the tube was capped and shaken vigorously by hand for 30 seconds. A mixture of anhydrous magnesium sulphate (4 g), sodium chloride (1 g), sodium citrate dihydrate (1 g), and sodium citrate sesquihydrate (0.5 g) was then added and the tube was shaken vigorously for another 30 seconds followed by vortexing for 1 min. The mixture was centrifuged (3620g, 4°C, 10min) and an aliquot (6 ml) of supernatant was transferred into a 15 ml plastic centrifuge tube and cleaned-up by shaking for 1 minute with primary secondary amine (PSA, 150mg) and magnesium sulphate (900 mg). The mixture was centrifuged and an aliquot (3.0 ml) of each supernatant was transferred into a 15 ml conical glass test tube and evaporated almost to dryness using a Turbo-Vap (Zymark, Hopkinton, MA, USA) at 45°C under a stream of nitrogen. A mixture of internal standards was added to the evaporated sample just prior to analysis to compensate for matrix and instrument variations. The residue was then reconstituted in 500 µl mobile phase (A/B, 20:80, v/v, see 2.5 for mobile phase composition) with vigorous vortex mixing. The dissolved extract was transferred into an auto-sampler vial (Waters, Manchester, UK) for LC-MS/MS analysis.

3.1.4.2 Water

A simple extraction method using solid phase extraction cartridges was used to adsorb and concentrate the analytes. For recovery experiments, the pre-acidified sample was spiked with a known amount of analytes and mixed thoroughly using a magnetic stirrer. The sample was loaded onto a SPE cartridge (Oasis HLB 500 mg, 6 ml) previously conditioned with methanol, followed by methanol/water (1:1, v/v) and finally with acidified water (pH~3). Using a vacuum manifold, the sample was drawn under negative pressure from the 1 l bottle through a 100 ml plastic reservoir, tightly fitted on top of the SPE cartridge, at a flow rate of approximately 6 ml per minute. Sample flow was controlled through a customized electronic timer connected to the pump, set with an interval delay of 1 minute per 2min continuous run (this precaution was taken to avoid overflow of the reservoir tube connected to the SPE cartridges). The SPE cartridge was dried with a stream of nitrogen for about 10 minutes to avoid the presence of water during evaporation step. Adsorbed analytes were then eluted with 5 mL methanol/acetonitrile (1:1, v/v) at a controlled flow (1 ml min^{-1}) and the eluate was collected in 15 ml glass centrifuge tubes. The eluate was evaporated in a Turbo-Vap under a stream of nitrogen gas at 50°C almost to dryness. A mixture of internal standards was added and the residue was dissolved in 1 ml of mobile phase (see 2.4.1) with vigorous vortex mixing. For the highest spiking level, the sample was diluted first to bring into a concentration within the range of the calibration standards prior to addition of internal standards. The dissolved extract was transferred into an auto-sampler vial for LC-MS/MS analysis.

3.1.5 Liquid chromatography-MS/MS analysis

Chromatography was performed using a HPLC Alliance 2695 attached to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Manchester, UK). The separation module was equipped with automated sample injector and cooling system, quaternary pump, degassing system and a column heater. The compounds were separated using an Atlantis T-3, 2.1 x 100 mm x 3 µm analytical column (Waters, Dublin, Ireland) attached to a C8, 4 x 2 mm guard cartridge (Phenomenex, Aschaffenburg, Germany). The column oven was maintained at 45°C and the auto-sampler compartment at 15°C. Gradient elution was employed with mobile phase (A) comprising water/acetonitrile (90:10, v/v) and (B),

methanol/acetonitrile (50:50, v/v), both containing 10 mM ammonium formate. The mobile phase was pumped at a flow rate of 350 µl min^{-1}. The gradient was initially 30% B ramping to 100% from 0.5–2.1 minutes, remaining at 100% B from 2.1–5.5 min, returning to 30% B between 5.5 and 5.6 min and equilibrating at this composition from 5.6–10 min. The volume of sample injected was 10 µl. The mobile phases were filtered by vacuum through a 0.45 mm HVLP filter (Millipore) before use and degassed by sonication in an ultrasonic bath (USC-500, Belgium).

Mass tuning of all compounds was obtained by setting the capillary at 3kV, extractor at 2 V, RF lens at 0.4 V, source temperature at 120°C, desolvation temperature at 400°C, desolvation gas at 600 l $hr.^{-1}$, cone gas at 50 l $hr.^{-1}$ and interscan delay of 0.1 s. To optimize the MS conditions for each analyte, a standard solution (10 µg ml^{-1}) prepared in mobile phase was infused at a rate of 20 µl min^{-1}. Positive electrospray ionization mode (ESI+) was used for all compounds. Selected reaction monitoring (SRM) was used to monitor LEV, FBZ-SO, FBZ-SO_2, FBZ and EPR. Eprinomectin concentrations were calculated as the EPR B1a component. For each analyte, two transitions were monitored. The identification of individual analytes was based on the matching of chromatographic retention times and ion ratios in accordance with Commission Decision 2002/657/EC.

The analyte concentration was determined by taking the ratio of the quantification ion abundance of the analyte to that of the internal standard and calculated against a matrix matched calibration standard/internal standard curve for soil, or solvent calibration standard/internal standard curve for water. The stable isotope internal standards (LEV-d5, FBZSO-d3, FBZ-SO_2-d3, and FBZ-d3) were used to compensate for run-to-run variation in instrument response. Since no internal standard was used for eprinomectin, quantification was performed using an external matrix-matched standard calibration. Matrix-matched calibrators were prepared by addition of standard solution to negative matrix extracts. Both calibration curves covered a concentration range from 50% of the lowest to twice highest spiking concentration, linearly fitted to a polynomial curve using a 1/x weighting. MassLynx® software (version 4.1) was used to control all instrument conditions and for processing of the mass chromatographic data.

3.1.6 Method optimization

3.1.6.1 Soil

During the preliminary studies different solvent ratios of methanol, acetonitrile, acetone, dichloromethane and water were tried out to optimize the extraction of analytes from both types of soils. The combination of methanol/acetonitrile (1:1, v/v) was found to be the most effective amongst all solvents tested therefore this was the extraction solvent chosen for the analytical method (Appendix Table 1). Sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate were used to buffer the extraction solution, which helps prevent the breakdown of pH sensitive/basic analytes such as levamisole (ANAGNOSTOU et al. 1996; HANSEN et al. 2012; RIVIERE and PAPICH 2009). Magnesium sulphate was used to help in partitioning of polar analytes in the extraction phase and as a dessicant in the clean-up step, and sodium chloride was used to reduce polar co-extractables. Primary secondary amine (PSA) was used to reduce levels of organic acids that could be present in soil matrix (LEHOTAY 2012). Typical SRM chromatograms for a negative control soil matrix, a negative soil sample spiked with the target compounds at 10 µg kg^{-1} and a matrix-matched calibration standard equivalent to the lowest spiking level (10 µg kg^{-1}) are shown in Figure 2. While there was no obvious interference found in the blank matrix, two peaks (RTs: 3.8, 5.2) were present in the time window chosen for monitoring fenbendazole at m/z 159. The presence of the first peak was attributed to a fragmentation product of deuterated $FBZSO_2$. The second peak, at the retention time of fenbendazole, was a product ion of the deuterated FBZ internal standard, but was below the LOD for fenbendazole and was considered negligible.

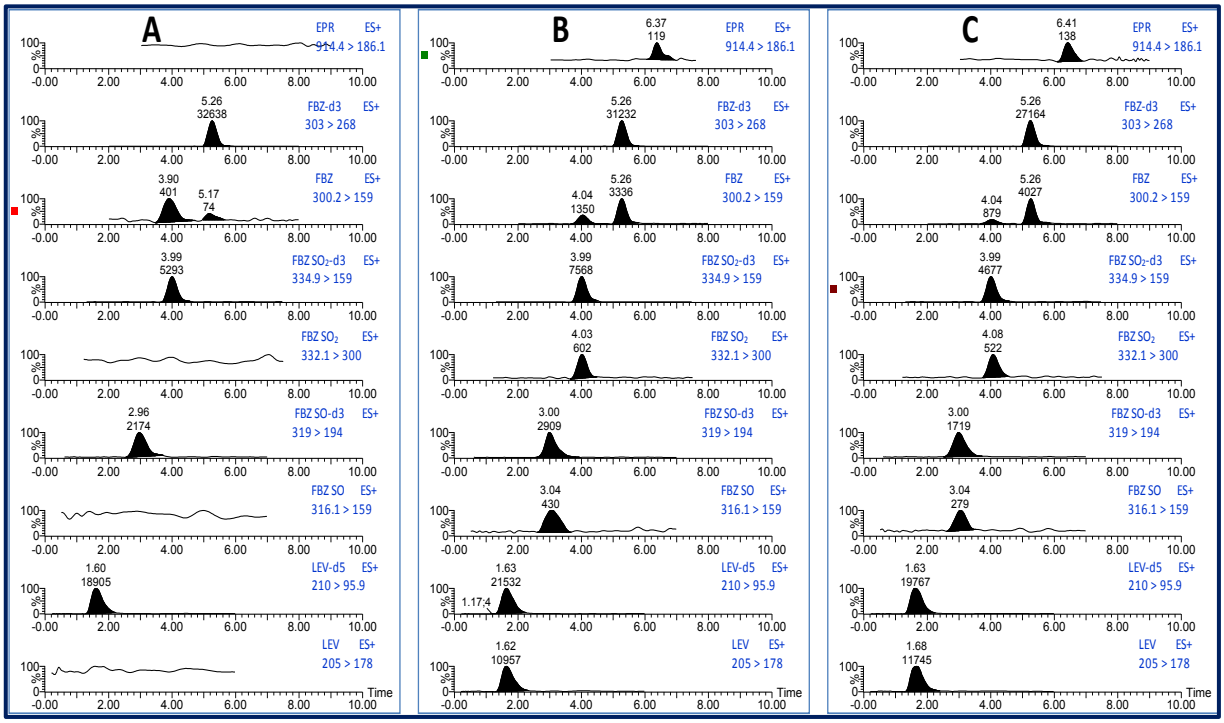


Figure 2: Extracted ion chromatograms from SRM transitions of 5 anthelmintics and deuterated internal standards of: (A) a control soil matrix, (B) negative soil fortified with anthelmintics mixture at 20 µg kg^{-1}, and (C) matrix standard equivalent to 20 µg kg^{-1}.

3.1.6.2 Water

Figure 3 presents the typical SRM chromatograms of anthelmintic compounds in water. The LOD and LOQ for all analytes were 0.1 µg l^{-1} and 0.25 µg l^{-1}, respectively. The LOQ for all compounds for which both methods were validated was the lowest point of the calibration curves.

Two types of cartridges were used to compare the efficiency of extraction and enrichment of five anthelmintic compounds covering the mid-polar to non-polar analytes. Strata-X and HLB-Oasis cartridges were selected at the initial stage of method development because both are known for their durable capacity for hydrophilic and hydrophobic compounds (DAVIS et al. 2006).

Prior to loading of water sample (100 ml) into SPE cartridges, pre-treatment of tap water with acid (HCl) to pH~3.0 was necessary to adjust the ionization state of the analytes before extraction. Most pharmaceutical compounds are known to have acidic and/or basic properties and their ionization rate has to be controlled by solution pH (see Figure 4 for the structures and physico-chemical properties of the target anthelmintics). Without acid treatment, eprinomectin was not recovered at all from Strata-X while an appreciable amount was still obtained from HLB. Lowering the sample pH from 3 to 2.5 led to reduced recovery values of most analytes and caused an early shift of retention time especially with levamisole, the first compound eluted from the analytical column. Two elution solvents were also compared; methanol (100%) and methanol/acetonitrile (1:1, v/v) with both types of adsorbents.

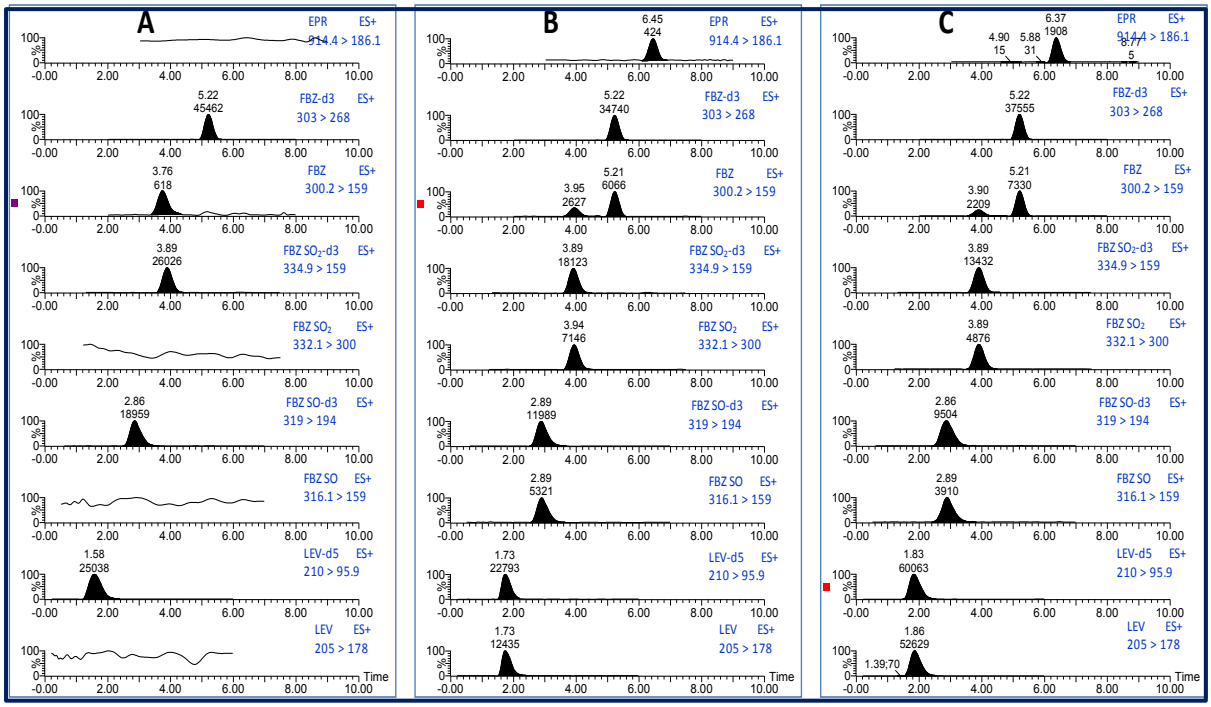
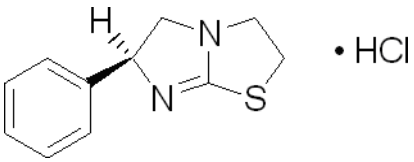
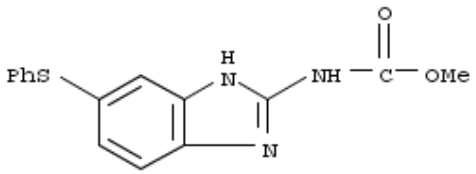
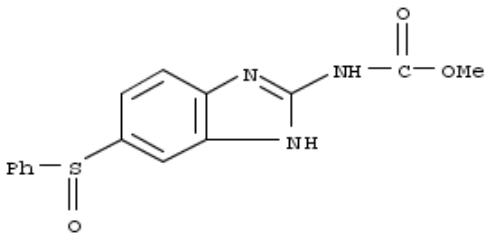
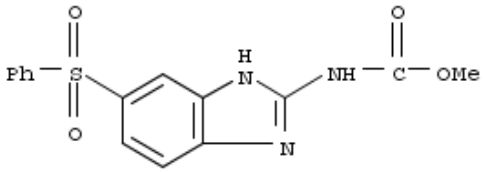
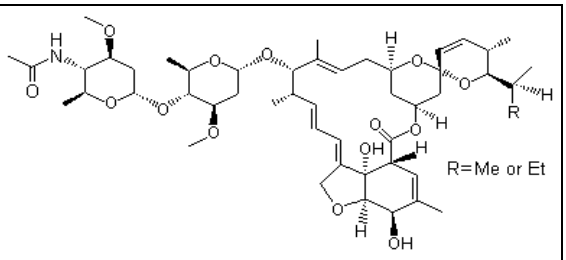


Figure 3: Extracted ion chromatograms from SRM transitions of 5 anthelmintics and deuterated internal standards of: (A) a control water matrix, (B) negative control water fortified with anthelmintics mixture at 0.5 µg l^{-1}, and (C) solvent standard equivalent to 0.5 µg l^{-1}.

Results showed that the latter was the best eluent for all studied compounds. Mean recoveries were in the range 67.2–122.5% and 87.8–125.3% using Strata-X and Oasis-HLB, respectively, therefore Oasis-HLB was the SPE cartridge of choice for this method (Appendix Table 2). Verifying these results, the method was run in

parallel using small and large volumes of water sample and performed in duplicate for each of the following: a) 1 l sample using normal load rate, b) 100 ml sample loaded slowly to finish at the same time as 1 l sample and c) 100 ml sample by normal load rate. Both (a) and (b) aimed to determine whether there was breakthrough during the loading process while (c) was used to check if there was degradation of analytes in water during the process and whether there was a difference in performance with varying loading rates of the same sample volume. From this test, we have verified that there was no degradation of analytes taking place and that there was no difference in using variable loading rates with a small volume of sample. However, obvious breakthrough was observed in EPR with 64% loss while LEV may be assumed to have experienced the same phenomenon with about 6% loss (Appendix Table 3).

1. Levamisole $C_{11}H_{13}C_lN_2S$ MW: 204.29 Log P: 1.84±0.57 Pka_2: 10.00±0.4	
2. Fenbendazole $C_{15}H_{13}N_3O_2S$ MW:299.35 Log P: 2.3±0.49 Pka_1: 10.80±0.10 Pka_2: 5.25±0.10	
3. Fenbendazolesulphoxide $C_{15}H_{13}N_3O_3S$ MW:315.35 Log P: 2.003±0.855 Pka_1: 10.27±0.10 Pka_2: 4.47±0.10	
4. Fenbendazolesulphone $C_{15}H_{13}N_3O_4S$ MW: 331.35 Log P: 1.632±0.855 Pka_1: 10.14±0.1 Pka_2: 4.04±0.10	

5.	Eprinomectin $C_{50}H_{75}NO_{14}$;$C_{49}H_{73}NO_{14}$ MW:914.14; 900.12 Log Kow: 5.4±0.3 No Pka between 3 and 10 http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5080442	

Pka_1 – at most acidic T@25°C

Pka_2 – at most basic T@25°C

Figure 4: Molecular structures and physico-chemical properties of LEV, FBZ, FBZ-SO, FBZ-SO_2 and EPR

3.2 Determination of anthelmintics in alfalfa plants

3.2.1 Standards and chemicals

LEV, FBZ-SO, FBZ-SO_2, FBZ, EPR standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Tetramisole-D5 (LEV-d5), FBZ-SO-d3, FBZ-SO_2-d3, and FBZ-d3 were obtained from Witega (Berlin, Germany). All solvents were of high-performance liquid chromatography (HPLC) grade and other chemicals were of analytical reagent grade, unless otherwise stated. Methanol, acetonitrile, formic acid (99.8%, w/v), hydrochloric acid (32%, w/v) and sodium chloride were all purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced in the laboratory with a Milli-Q gradient system produced by Millipore (Molsheim, France). Ammonium formate, magnesium sulphate, sodium citrate dihydrate, sodium citrate sesquihydrate, and dimethylsuphoxide were purchased from Sigma Aldrich (Steinheim, Germany). Solid phase extraction (SPE) cartridges (Strata-X, 500 mg, 6 ml) were purchased from Phenomenex (Aschaffenburg, Germany). Disposable syringe filters (PTFE, 13 mm x 0.45 µm) were obtained from Whatman (New Jersey, USA). Omnifix luer lock syringes (3 ml) were purchased from B Braun (Bad Arolsen, Germany).

3.2.2 Sample sources and preparation

Plant samples (green leaves and stalks) of about one half the height of the plants were cut and collected within the perimeter of a lysimeter field at the research facility of the Austrian Institute of Technology (AIT). These were sheared into small pieces and frozen (-80°C ± 2°C) to facilitate mass blending. The frozen chops were

thoroughly homogenized using a Stephan universal chopper UM-12 (Hameln, Germany). Several analytical portions were prepared and sub-sampled into food plastic jackets and stored in a freezer (-20°C ± 2°C).

3.2.3 Sample Preparation of solutions

Individual stock solutions (1 mg ml^{-1}) of FBZ, FBZ-SO, FBZ-SO_2 and their deuterated internal standards were prepared in dimethylsulphoxide (DMSO). LEV (and its deuterated form) and EPR were prepared in methanol (MeOH) and acetonitrile (MeCN) respectively. All solutions were stored at -20°C. Stock standards were diluted in methanol to prepare a mixed intermediate standard solution containing the five target analytes (10 µg ml^{-1} LEV, FBZ, FBZ-SO, FBZ-SO_2, 20 µg ml^{-1} EPR). The intermediate mixed standard was stable for at least 1 month at 4°C. A mixed internal standard solution containing the four internal standards was prepared similarly. Working standard solutions (1.0 µg ml^{-1}, 2.0 µg ml^{-1} EPR) were then prepared by diluting the intermediate mixed standard solution with methanol and stored at 3–8°C. Calibration solutions (equivalent to 5, 10, 20, 40, and 160 µg kg^{-1} FBZ, FBZ-SO, FBZ-SO_2 and 10, 20, 40, 80 and 160 µg kg^{-1} EPR) were prepared by pipetting aliquots of working standard solution (10, 20, 40, 80 and 160 µl) into individual vials and adding internal standard mixture (80 µl, 1.0 µg ml^{-1}) to each vial. Each solution was made up to a final volume of 500 µl with blank matrix extract.

3.2.4 Extraction and sample processing

The analytical method employed a solid phase extraction clean-up and concentration step using Strata-X (500 mg, 6 ml). Portions (10 g) of the homogenized plant sample were weighed into polypropylene centrifuge tubes (Sarstedt, Wr.Neudorf, Austria). For analyte recovery experiments samples were spiked by the quantitative addition of standards in methanol. Each sample was vortexed for a few seconds and allowed to stand at room temperature for at least 20 minutes. A methanol/acetonitrile mixture (7:3, v/v, 15 ml) was added and the tube was capped and shaken vigorously for 30 seconds. A mixture of salts comprising anhydrous magnesium sulphate (4 g), sodium chloride (1 g), sodium citrate tribasic dihydrate (1 g), and sodium citrate dibasic sesquihydrate (0.5 g) was then added and the tube was shaken vigorously for 1 minute followed by strong vortexing for another 2 minutes.

The mixture was centrifuged and an aliquot (3 ml) of supernatant was transferred into a 15 ml conical glass test tube and evaporated to approximately 1 ml using a Turbo-Vap (Zymark, Hopkinton, MA, USA) operated at 50°C under a stream of nitrogen. The diminished sample volume was loaded onto a SPE cartridge (Strata-X, 500 mg, 6 ml) previously conditioned sequentially with methanol, acetonitrile and water (5 ml each). The cartridge was washed with diluted methanol (5%, 2.5 ml) to remove polar interferences and the eluate discarded. The SPE cartridge was dried with a stream of nitrogen for about 10 minutes. Adsorbed analytes were eluted with methanol/acetonitrile mixture (1:1, v/v, 5 ml) followed by 4 ml acetonitrile at a controlled flow (1 ml min^{-1}). The combined methanol/acetonitrile and acetonitrile eluates were collected in a 15 ml glass centrifuge tube and evaporated in a Turbo-Vap under a stream of nitrogen gas at 50°C almost to dryness. Internal standard mixture (80 µl, 1.0 µg ml^{-1}) was added to the evaporated sample and the residue was reconstituted in 500 µl of mobile phase (A/B, 20:80, v/v, see 1.5 for mobile phase composition) with vigorous vortex mixing. The dissolved extract was filtered using a luer lock syringe fitted with a PTFE filter (0.45 µm) and was transferred into an auto-sampler vial (Waters, Manchester, UK) for LC-MSMS analysis.

3.2.5 Liquid chromatography-MSMS analysis

The same instrumentation method as for soils was used to analyse plant extracts except for the MS part using desolvation gas at 400 l hr^{-1}.

The analyte concentration was determined by taking the ratio of the quantification ion abundance of the analyte to that of the internal standard and calculated against a matrix-matched calibration standard/internal standard curve. The stable isotope internal standards (LEV-d5, FBZ-SO-d3, FBZ-SO_2-d3, and FBZ-d3) were used to compensate for run-to-run variation in instrument response and to improve the precision of the results. Since no internal standard was used for eprinomectin, quantification was performed using an external matrix-matched standard calibration. Matrix-matched calibrators were prepared by addition of standard solution to negative matrix extracts. All instrument conditions and the processing of mass chromatographic data were controlled using MassLynx® software (version 4.1).

3.2.6 MSMS optimization/analysis

The optimized conditions of selected SRM transitions for the precursor and complementary product ions of the five anthelmintic compounds studied in plants are the same as for soils. The precursor ions for all compounds analyzed are $[M+H]^+$. Two product ions were selected to confirm the molecular ion by comparison of their ion ratios. This is in accordance with relevant guidelines when a low resolution liquid chromatography-mass spectrometry analysis is used (EUROPEAN COMMISSION 2009). A wide retention time window was used during method development to investigate possible cross talk and interferences. This was not further optimized for the final method, since the method performance was fit for purpose. Further optimization of the acquisition windows for each ion may improve the method performance slightly. The precursor and transition ions monitored for each compound are listed in Table 1.

Table 1: Optimized MS/MS parameters of the five target anthelmintic compounds and their deuterated analogues using the positive ESI mode

Compound	Dwell time (s)	RT window (min)	SRM transition (*m/z*)					
			Precursor ion	Cone voltage(V)	Quantifier ion	Collision energy(eV)	Qualifier ion	Collision energy(eV)
LEV	0.05	0.2 – 6.0	205.0	37	178	21	90.7	36
LEV-d3	0.05	0.2 – 6.0	210.0	37	95.9	32	183.1	19
FBZ-SO	0.05	0.5 – 7.0	316.1	33	191	21	159	35
FBZ-SO-d3	0.05	0.5 – 7.0	319.0	34	194	20	159	32
FBZ-SO_2	0.05	1.2 – 7.5	332.1	35	300	21	159	38
FBZ-SO_2-d3	0.05	1.2 – 7.5	334.9	35	159	35	300	21
FBZ	0.05	2.0 – 8.0	300.2	35	159	20	268	35
FBZ-d3	0.05	2.0 – 8.0	316.1	34	268	20	159	20
EPR	0.20	3.0 – 9.0	914.4	25	186.1	22	298.2	23

The mass spectra of the molecular ions for all analytes are presented in Figure 5. Individual injection of standards prepared both in methanol and buffered mobile

phase were examined over the mass range m/z 50 – 1000. The transition m/z 159 was the common product ion observed from the fragmentation of fenbendazole and its metabolites (FBZ-/SO/SO_2). The ion at m/z 159 from fenbendazole may be due to the splitting of the -OCH_3 from the protonated molecule. For fenbendazole, there was a small degree of cross talk at m/z 159 at the retention time of the compound, attributed to the fragmentation of deuterated fenbendazole. However, using the selected conditions it did not significantly affect the FBZ signal intensity (about 1%), so m/z 159 was used as the quantifier ion for FBZ.

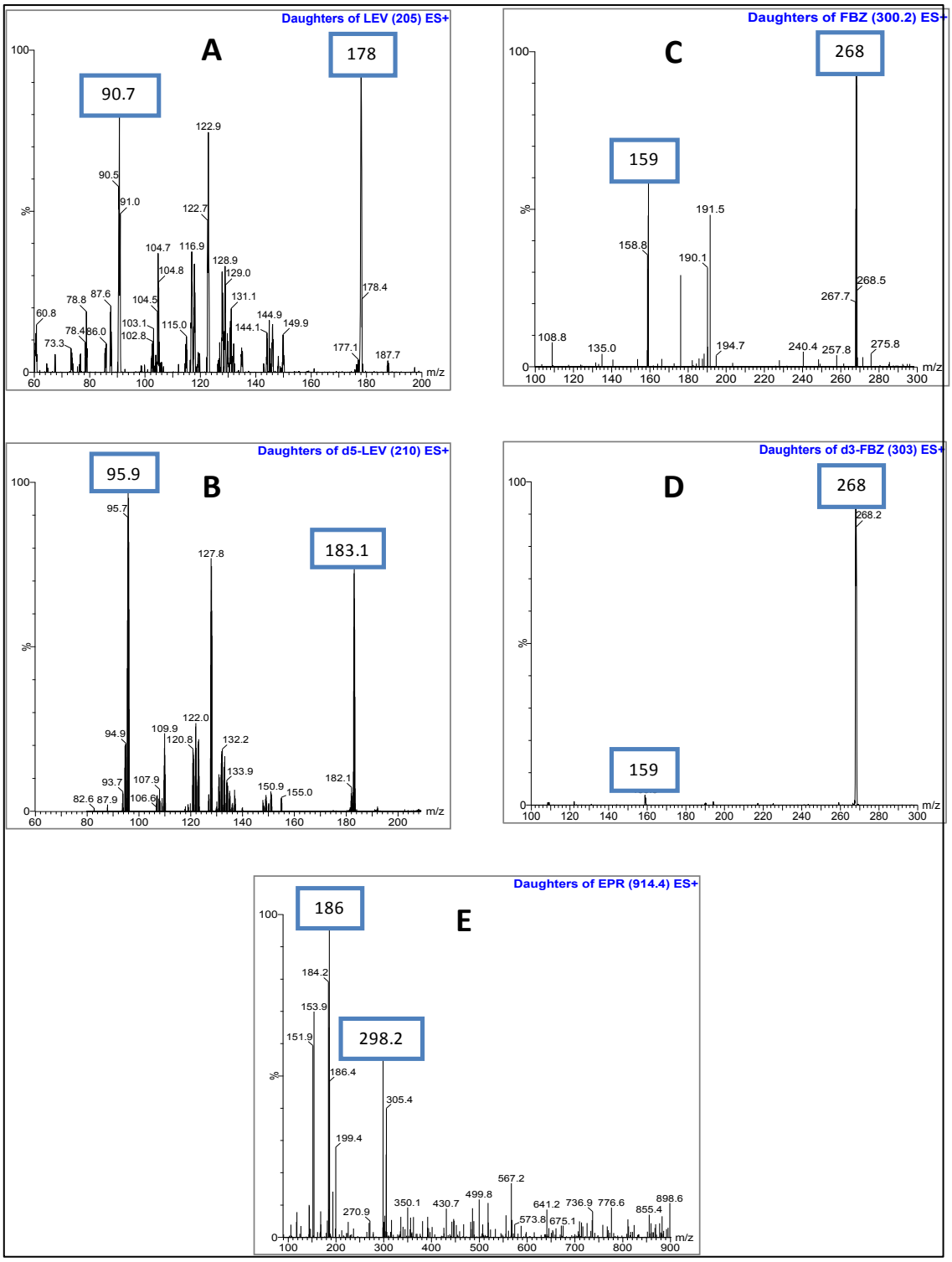


Figure 5: MS/MS spectra of fragmented transitions of (A) LEV (*m/z* 205), (B) LEV-d5 (*m/z* 210), (C) FBZ (*m/z* 300.2), (D) FBZ-d3 (*m/z* 303), and (E) EPR (*m/z* 914.4)

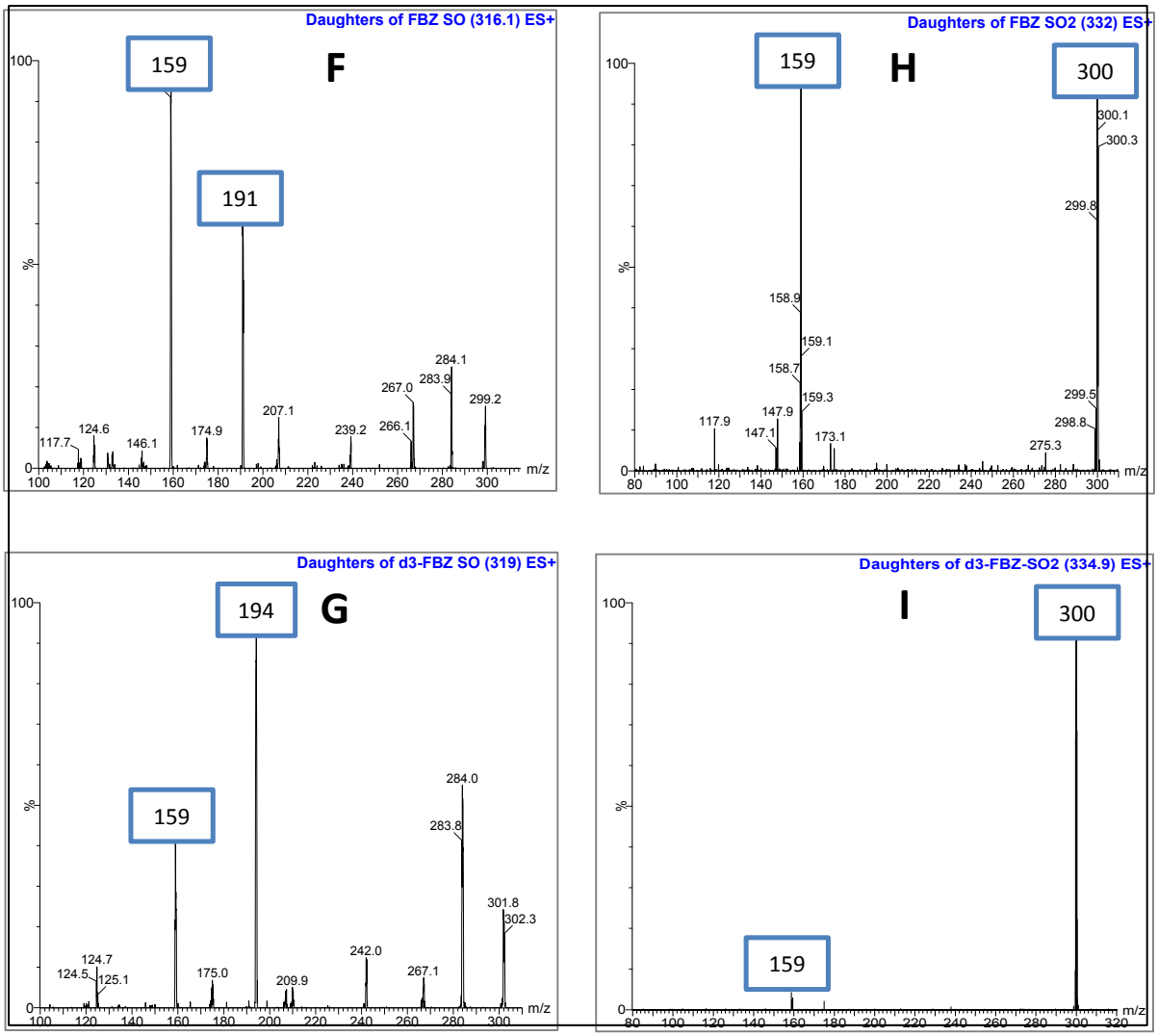


Figure 5 continued:

MS/MS spectra of fragmented transitions of (F) FBZ-SO (*m/z* 316.1), (G) FBZ-SO-d3 (*m/z* 319), (H) FBZ-SO_2 (*m/z* 332), (I) FBZ-SO_2 -d3 (*m/z* 334.9)

Product fragments from $FBZSO_2$-d3 likewise produced a cross-talk effect for fenbendazole sulphone at m/z 159 to about 20.6%. The same effect was revealed in previous study (BALIZS and HEWITT 2003) and was addressed by choosing an alternative transition ion at m/z 300, which was also the quantification ion used in this experiment. The fragment m/z 159 was likely due to the consecutive losses of sulfinyl (C_6H_5-/SO/SO_2) and the -OCH_3 group from $[M + H]^+$ ion of FBZ-/SO/SO_2 (DE RUYCK et al. 2002). For levamisole, a prominent fragment at m/z 178 was obtained. This could be attributed to the breakdown of $[M + H - HCN]^+$ ion in the 3,4 position of the thiazole ring (CHAPPELL et al. 1992). For eprinomectin, a protonated molecule at m/z 914.4 was obtained, as reported in other studies that have isolated the molecular

ion $[M + H]^+$ at m/z 914 – 914.5 (BALLARD et al. 1997; KINSELLA et al. 2009; RUDIK et al. 2002). Using collision-induced fragmentation over the mass range m/z 100 – 900, the most abundant fragment obtained is the product ion at m/z 186, which may have resulted from $C_9H_{15}O_3N$ fragment cleavage from the monosaccharide moieties. Fragmentation patterns for macrocyclic lactones have been suggested elsewhere (ALI et al. 2000; BALLARD et al. 1997; SHERIDAN and DESJARDINS 2006).

The LOQ was established at 10 µg kg^{-1} for LEV, FBZ-SO, FBZ-SO_2 and FBZ and at 20 µg kg^{-1} for EPR. Chromatographic peaks are presented in Figure 6 showing the typical selected reaction monitoring (SRM) ion chromatograms for the blank plant matrix, a negative plant sample fortified with the anthelmintics mixture at 20 µg kg^{-1}, and a matrix-matched calibration standard equivalent to 20 µg kg^{-1}.

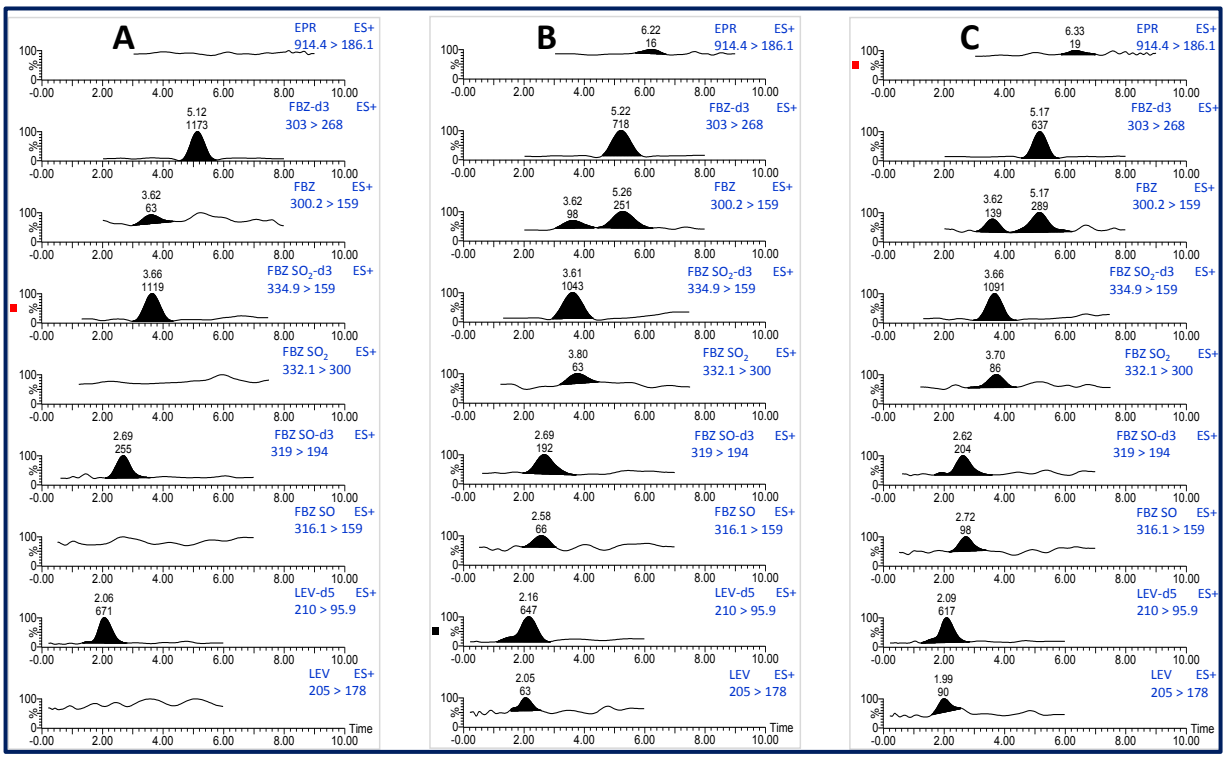


Figure 6: Extracted ion chromatograms from SRM transitions of 5 anthelmintics and deuterated internal standards of: (A) a control plant matrix, (B) negative plant fortified with anthelmintics mixture at 20 µg kg^{-1}, and (C) matrix-matched standard equivalent to 20 µg kg^{-1}

3.2.7 Method optimization

Different solvents and mixtures of methanol (MeOH), acetonitrile (MeCN), water, acetone, hexane, and dichloromethane were investigated to optimize the extraction of the target analytes from plant material. Various solvent combinations of the last four solvents listed did not effectively extract the target substances, as indicated by low recoveries from the fortified plant extract. Single solvent extractions were also tried out but were no better.

Mixtures of MeOH/MeCN (7:3, v/v) and MeOH/MeCN (1:1, v/v) gave good recoveries. The former mixture was selected as the preferred extraction solvent for this analytical method because it offered the advantage of minimizing the extraction of strong green pigments from plant matrix, which could potentially cause problems during the SPE clean-up procedure and analysis. The green pigments caused the rapid contamination of the sample cone of the mass spectrometer resulting in a significant decrease in the sensitivity of analyte detection.

Sodium citrate dibasic sesquihydrate and sodium ctirate tribasic dihydrate were used to buffer the extraction solution, which helps prevent the breakdown of pH sensitive/basic analytes such as levamisole. Magnesium sulphate was used to help in partitioning of polar analytes in the extraction phase and as a dessicant in the clean-up step, and sodium chloride was used to reduce polar co-extractables. Primary secondary amine (PSA) was used to reduce levels of organic acids that could be present in plant matrix (LEHOTAY 2012).

Using SPE to remove matrix interferences and concentrate the sample, the recovery of analytes was investigated by eluting the adsorbed anthelmintic compounds from the SPE cartridge with MeOH/MeCN (5 ml, 1:1, v/v) and MeCN (2 ml). Results indicated that fenbendazole was retained significantly when the first elution solvent was passed through the stationary phase, as was shown by its low recovery (24.6%). Using MeCN as a second eluent, fenbendazole was recovered to a greater extent (66.6%) while small amounts of all other analytes were still detected (Table 2). It was therefore decided to use both solvents in sequence to increase the elution efficiency for the targeted compounds. For the final method, the volume of MeCN was increased to 4 ml to ensure the optimal elution of fenbendazole.

Table 2: Recoveries of five anthelmintic compounds after a SPE clean-up using eluting solvent 1 (MeOH/MeCN, 1:1, v/v, 5 ml) and solvent 2 (MeCN, 2 ml) at method development stage

Analytes	Eluting solvent 1	Eluting solvent 2
LEV (205 > 178)	73.1%	4.6%
FBZ (300.2 > 159)	24.6%	66.6%
EPR (914.4 > 186.1)	89.2%	15.4%
FBZ-SO (316.1 > 191)	73.1%	17.5%
FBZ-SO_2 (332.1 > 300)	84.7%	18.5%

3.2.8 Matrix effect

Significant ion suppression in the LC-MS/MS was observed for the majority of analytes in extracts of homogenized green chops. Levamisole showed the largest decrease in m/z abundance followed by eprinomectin. However, the two oxidised metabolites of fenbendazole demonstrated some ionisation enhancement. The suppression effect on levamisole could be explained by the presence of low retention time interferences from the plant matrix, which eluted largely in the area of the solvent front where LEV also eluted.

Alfalfa has a high level of proteins and is a vitamin rich, and could therefore be a difficult matrix for analysis (HURLEY 2011). Relatively simple clean-up techniques may not be sufficient to remove such complex interferences, especially for LC-MS/MS analysis. Ion suppression is a common effect observed in LC-MS detection using electrospray ionization mode. This phenomenon is a manifestation of matrix effects, generally a result of endogenous material present in biological samples, as explained in a study related to ion suppression (JESSOME and VOLMER 2006; MALLET et al. 2004).

Since the problem of matrix suppression (LEV, EPR, FBZ) and enhancement (FBZ-SO, FBZ-SO_2) could not be eliminated in this analytical method, the effects were compensated by using stable isotope-labelled internal standards added at the final evaporation stage, just before instrumental analysis, and matrix-matched standard calibration. The linearity of response was acceptable for all analytes, with correlation coefficients typically ≥ 0.98 using internal standard corrected matrix-matched standard curves at concentrations ranging from 5.0 to 300 µg kg^{-1}.

The limited amounts of stable-isotope labelled compounds available to us at the time of this study were used to compensate for matrix effects and instrumental variation. Addition of the internal standards before the extraction and clean-up stages would also improve the trueness and precision of the method.

It was considered that the matrix effects for the species of alfalfa used in the study would most probably be similar to those of other alfalfa species. However, if different alfalfa species were to be analysed within the same analytical batch, the authors recommend that an aliquot of each species should be analysed as a spiked sample and the recovery obtained checked for consistency with the species used for the matrix-matched calibrators. The use of deuterated internal standards should minimize or negate any minor variations that may be caused by differences between alfalfa matrices.

3.3 Transport investigation of antiparasitic drugs in soil-plant-water

3.3.1 Materials and experimental design

Five lysimeters (1.13 m^2) in the Austrian Institute of Technology (AIT, Seibersdorf, Austria) were used in this research. Lysimeter experiments were conducted using two types of soil, loamy and sandy, with two replicate lysimeters for each and one lysimeter containing sandy soil as a control. The soil profiles are presented in Table 3. The soils were classified as loam/silt-loam (WRB: Calcic Chernozem) and loamy sand (WRB: Eutric Arenosol) according to their texture and properties (ISRIC 2013; JUMA 2002). Circular metal grids (1 m^2 x 14.1 cm internal grids) were commercially fabricated by the AIT workshop (see Figure 7). A stainless steel soil borer (30 cm x 10 mm i.d.), which could take a 20-30 g soil sample, was fabricated by the machine shop at the IAEA Seibersdorf laboratories. The radio-isotope marker, ^{14}C-labelled levamisole (~ 2.0 mCi), used as a tracer in this study was synthesized by International Isotopes Clearing House (Leawood, Kansas). Panacur (fenbendazole, 100 mg ml^{-1}) was obtained from Intervet (Igoville, France), Levamisol (levamisole, 100 mg ml^{-1}) from Intervet (Burgdorf, Germany) and Eprinex (eprinomectin, 5 mg ml^{-1}) was purchased from Merial (Lyon, France). The physico-chemical properties and molecular structure of these target compounds are shown in Figure 4.

Figure 7: Lysimeters containing loamy and sandy soils fitted with circular metal internal grids for application and sample collection guide.

Table 3: Characteristic profile of sandy and loamy soils contained within the lysimeters

Depth	Particle size (Wt. %)			OM	pH	$CaCO_3$
(cm)	Sand	Silt	Clay	(Wt. %)	$CaCl_2$	(Wt. %)
Fuchsenbigl (silt loam)						
0 – 20	22	55	23	2.7	7.9	18
20 – 40	21	54	25	2.3	7.9	27
40 – 60	20	60	20	0.4	7.9	35
Marchegg (loamy sand)						
0 – 20	78	12	10	0.5	6.5	-
20 – 30	78	10	12	0.5	6.2	-
30 – 45	75	16	9	0.3	6.3	-

3.3.2 Preparation and application

All weeds, leaves, and stones were removed from the top soil of the lysimeters and the soil surfaces were raked to obtain a homogenous flat surface. The metal grids were then fitted onto each lysimeter. Target concentrations of radiotracer (~0.412 mCi; 6000 dpm g^{-1}) and non-labelled substances (1500 µg kg^{-1}) for each lysimeter were mixed with cow slurry (equivalent to 120 kg N per hectare) and measured

amounts of the compounds in slurry were transferred into bottles (100 ml). The content of one bottle was carefully applied over the surface of each individual 14.1 x 14.1 cm grid sector using a multi-channel pipette (see Figure 8). The bottles were rinsed with water and applied to the same grid sector. After application of the mixture, a plastic sheet (~35 cm in height) was placed around the perimeter of each lysimeter to avoid possible transfer and consequent contamination of the adjacent land by wind drift. Deep rooted alfalfa plants, previously growing in the lysimeters, started to grow a few days after application of the slurry.

Figure 8: Positioning and application of veterinary pharmaceuticals mixed in cow slurry

Figure 9: Fencing the lysimeter with plastic sheets after application of veterinary drugs and ^{14}C-labelled compound.

3.3.3 Sampling regime and sample preparation

Immediately after application, soil samples were randomly taken from different grid sectors using a soil borer at depth 0–10 cm (Figure 9). The sampled point was marked and recorded after each withdrawal to avoid repetition of sampling at the same place. Sampling was repeated 10, 30, 80 and 300 days after application (DAA). Samples were withdrawn each time from six different grid locations within the lysimeter. Samples taken randomly from two non-adjacent grid locations were pooled, giving a total of three replicate samples per horizon at each sampling time. Horizon levels were assigned at soil depths 0-10 cm (horizon 1), 10-20 cm (horizon 2) and 20-30 cm (horizon 3). Sampling at the second horizon was initiated on 10 DAA and at the third horizon on 30 DAA.

The soil samples were air-dried in the laboratory in a fume cupboard. Sample preparation was performed by removing leaves, roots and other unwanted objects prior to grinding by mortar and pestle. The equipment was washed and dried between samples to avoid carry over. Each of the replicates of soil was then further sub-sampled into duplicate portions (10 g) and transferred to polypropylene tubes pending analysis.

For alfalfa plants, samples were collected by cutting the tops of the plants, at half of their height, at early, middle and late vegetation stages. Stems and leaves were chopped and immediately frozen at –80°C. The next day, the frozen plant samples were blended using a Stephan universal chopper UM-12 (Hameln, Germany) to obtain a powdery, homogenized sample. Three replicate analytical portions (10 g) were weighed and set aside for extraction and the rest of homogenized samples were distributed into several polypropylene tubes and stored in a freezer.

Water samples were taken when one litre of seepage water in the lysimeter had accumulated from natural precipitation. The water was filtered through a Buchner funnel into an amber bottle (1 l) and adjusted to pH~3.0 using hydrochloric acid (Merck, Darmstadt, Germany).

3.3.4 Extraction, sample preparation and analysis

All methods used for chemical analysis of the unlabelled compounds were based on our own published procedures (ISLAM et al. 2012; ISLAM et al. 2013). These methods were described in details in the previous sections (3.2.4 – 3.2.5 and 3.1.4 –

3.1.5). Typical recoveries for the analytes in soil were between 75 and 88%, in plants between 76 and 83% and in water between 62 and 142%. Because of changes in instrument performance and sensitivity subsequent to the method validation reported, the limits of detection (LOD) and quantitation (LOQ) were recalculated for the sample analyses on the basis of signal to noise ratios of 3 and 6, respectively, and the limits were adopted on the basis of the least sensitive analyte. For the analysis of soil and plant material, the limit of detection (LOD) and limit of quantitation (LOQ) were 2 and 4 µg kg^{-1}, respectively. In water, the LOD and LOQ for all analytes were 0.05 µg l^{-1} and 0.10 µg l^{-1}, respectively.

3.3.5 Incubation experiment

A quantity (400 g) of sandy and loamy air-dried soil was weighed in duplicate into a plastic container with a tightly fitting lid. The soil was treated with ^{14}C-LEV at a targeted concentration of 7500 dpm g^{-1} and a mixture of the three targeted veterinary drugs (LEV, FBZ, and EPR) at 200 µg kg^{-1} soil. An appropriate volume of water based on the previously determined withholding capacity was added to sandy (84 ml) and loamy (118 ml) soils. One control sample for sandy soil was also prepared where only water was added to the soil. The soil mixture was thoroughly shaken to ensure a homogenous distribution of the solutes into the soil. A small portion of soil (≈ 40–50 g) was withdrawn and air-dried in a fume cupboard for day-0 analysis. About half of the remaining portion of the treated sandy and loamy soils, including the control sample was transferred into another container. One container was kept at 4°C and the other at 20°C in an environmental chamber under controlled humidity (~ 60 %). The soils were sampled weekly for 6 weeks. The processed soil samples (in triplicates) were analysed by LC-MS/MS for unlabelled compounds and liquid scintillation counter (LS 6000 TA, USA) for the measurement of ^{14}C-LEV radioactivity.

3.3.6 Adsorption-desorption experiment

1. From the preliminary study, a soil/solution ratio of 1:5 was used to determine the equilibrium time of the solute between the sorptive material and the liquid phase. In all experiments, a portion of soil (10 g) was weighed into a Nalgene flask (100 ml) and a volume of $CaCl_2$ solution (0.01 M, 50 ml) prepared in deionized water was added. The equilibrium time determination was performed in duplicate for sandy and loamy soils. A radioactivity count of ≈ 267000 dpm ml-1 $CaCl_2$ solution was used for

^{14}C-LEV while a concentration of 3 µg ml-1 soil solution was used individually for FBZ and EPR. After spiking replicate portions of each soil type, a volume (50 ml) of $CaCl_2$ solution was added. The soil-solution mixture was shaken intermittently for 72 hours, during which samples were taken after 15 m, 30 m, 1, 2, 3, 4, 5, 6, 7, 26, 28, 30, 32, 46, 48, 50, 54, and 72 hrs. At each sampling time the flask was centrifuged (10 min) and an aliquot was transferred directly to HPLC vials for the analysis of unlabelled compounds by LC-MSMS. For the analysis of ^{14}C-LEV, an aliquot (50 µl) was mixed with liquid scintillation cocktail (12 ml) and the radioactivity measured using a liquid scintillation counter. The radioactivity count (dpm) or equivalent concentration in soils for ^{14}C-LEV, FBZ and, EPR was plotted against time. The time at which the concentration curve for each compound started to plateau was taken as the equilibrium time for determining the sorption isotherms, as described below.

To determine the sorption isotherms, 5 duplicate portions of each soil type were mixed with a $CaCl_2$ solution containing each target analyte at 5 different concentrations. For ^{14}C-LEV, activity counts of 1336 (C1), 13361 (C2), 52634 (C3), 107293 (C4), and 133610 (C5) dpm ml^{-1} in $CaCl_2$ solutions were used. For the unlabelled compounds the targeted concentrations in $CaCl_2$ solution were 0.05, 0.5, 1.0, 2.0 and, 5.0 µg ml^{-1}. One blank sample (soil + $CaCl_2$) to check for soil interferences, and one control sample (target analyte + $CaCl_2$) to check for adherence of the substance to the container, were analysed simultaneously. This experiment was performed individually for LEV, FBZ and EPR. The flask was shaken (210 rpm) using a horizontal shaker (Certomat® S II, Germany) at the pre-determined equilibrium time (described above) for each compound. The flask was centrifuged (3620 g, 10 min, 20ºC). Aliquots of the aqueous phase were transferred to HPLC vials and analysed by LC-MS/MS. For ^{14}C-LEV, aliquots (500 µl for C1/C2 and 100 µl for C3-C5) were transferred to LSC vials and mixed with liquid scintillation cocktail (12 ml) for radioactivity measurement by liquid scintillation counter. The sorption isotherm was plotted based on the calculated concentration of substance adsorbed on soil (µg/g) against the concentration of the substance in aqueous phase (µg/ml) at adsorption equilibrium.

From the preliminary study, a soil/solution ratio of 1:5 was used to determine the equilibrium time of the solute between the sorptive material and the liquid phase. In all experiments, a portion of soil (10 g) was weighed using a Nalgene flask (100 ml) and a volume of $CaCl_2$ solution (0.01 M, 50 ml) prepared in deionized water was

added. To determine the sorption isotherms, 5 duplicate portions of the previously weighed soil were added with same $CaCl_2$ solution containing test substance of 5 different but increasing concentrations. Simultaneously analysed were one blank run (soil + $CaCl_2$) to check for soil interferences and one control sample (test substance + $CaCl_2$) to check adsorption of the substance on the container wall. This experiment was performed individually for compounds (LEV, FBZ, EPR) used in this study. The flask was shaken (210 rpm) using a horizontal shaker (Certomat® S II, Germany) at a pre-determined equilibrium time for each compound. The same flask was centrifuged (3620 g, 10 min, 20ºC) using a bench-top centrifuge machine (Sigma®, UK). An aliquot of the aqueous phase was transferred to HPLC vials and directly analysed using a LC-MS/MS. For ^{14}C-LEV, an aliquot was transferred to LSC vials and mixed with liquid scintillation cocktail for radioactivity measurement using the same liquid scintillation counter mentioned above. The sorption isotherm was plotted based on the calculated concentration of substance adsorbed on soil (µg g^{-1}) against the concentration of the substance in aqueous phase (µg ml^{-1}) at adsorption equilibrium.

3.3.7 Radiotracer analysis

The analysis of the radioactive compound in the soil and plant samples was done simultaneously with the extraction of non-labelled compounds. Aliquots of soil (3 x 1 ml) and plant (2 x 0.5 ml) centrifugates were transferred into plastic vials and shaken with 12 ml liquid scintillation cocktail (Ultima Gold, Canberra). For water samples, aliquots (3 x 1 ml) were directly pipetted from the seepage water and analysed after shaking with liquid scintillation cocktail (12 ml). The amount of radioactivity present in the samples was measured using a Beckmann liquid scintillation counter (LS 6000 TA, USA). Table 4 shows the results for mean recoveries and precision of the extraction method used in soil and plants.

Table 4: Method recovery and precision of ^{14}C-LEV in loamy soil and, ^{14}C-LEV in alfalfa plants at 3 spiking levels in three different occasions (*n = 6*) using Liquid Scintillation Counter

Spiking levels	100 dpm g^{-1}		200 dpm g^{-1}		400 dpm g^{-1}		Overall spiking levels	
^{14}C-LEV Loamy soil	Mean	RSD	Mean	RSD	Mean	RSD	Overall Rec.	Overall RSD
Day-1	81.6%	2.2%	80.5%	1.0%	87.0%	1.5%	83.0%	1.6%
Day-2	70.8%	3.3%	69.2%	1.8%	71.6%	1.2%	70.5%	2.1%
Day-3	79.4%	3.2%	81.9%	2.4%	85.8%	2.5%	82.4%	2.7%
^{14}C-LEV Alfalfa plants	1000 dpm ml^{-1}		2000 dpm ml^{-1}		4000 dpm ml^{-1}		Overall spiking levels	
Day-1	65.4%	2.3%	64.3%	3.6%	62.4%	5.8%	64.0%	3.9%
Day-2	79.4%	3.5%	71.5%	3.4%	70.9%	1.6%	73.9%	2.8%
Day-3	109.6%	10.8%	80.6%	1.2%	73.0%	1.9%	87.7%	4.6%

3.3.8 Internal quality control

Reagent blank and negative controls of the sample matrix were analysed along with a series of environmental samples subjected for residues testing. The performance of the respective methods used to analyse a set of samples was controlled through the addition of mixture of standards to blank soil, plant or water samples at a concentration expected to be present in the sample. Measured sample concentrations were corrected for the mean recovery obtained from validated methods mentioned above to provide for the approximate real values of the samples. The same quality control step was adapted for the measurements of radioactivity in the samples.

3.3.9 Data Analysis

The IBM SPSS Statistics software package was applied for data analysis. ANOVA and Student's t-tests were applied to compare the results found for each compound at different sampling periods, soil depths and replicate lysimeters.

4 Results and discussion

The pathway by which organic pollutants enter vegetation is a function of their chemical and physical properties, such as hydrophobicity, water solubility, and vapour pressure, as well as the conditions of the environment, including temperature, organic content of the soil, and plant species. Uptake of organic chemicals into plants can occur from air or soil, depending on the properties of the compounds (HELLSTRÖM 2004). Organic chemicals bound to soil particles may be deposited on the vegetation as a result of wind re-suspension or rain splash (SMITH and JONES 2000). However, in general, organic chemicals are taken up by plants through the soil pore water.

4.1 Determination of anthelmintics in soil and water

The optimized parameters for the five anthelmintic substances and the selected SRM transitions for the precursor and complementary productions are the same as for plants (Table 1). The precursor ions for all compounds analyzed were $[M+H]^+$. Analysis of all compounds using electrospray ionization in positive mode provided high sensitivity detection. Ion suppression was observed in some analytes in the soil matrix, which was overcome by using stable isotope-labelled internal standards. For the present study, the internal standard was added immediately prior to instrumental analysis to compensate for matrix and instrument variations. If readily available, addition of the internal standard at the start of the sample extraction process would also allow correction for variable losses during extraction, and would improve method performance.

The linearity of response for soil was acceptable for all analytes with correlation coefficients typically ≥ 0.98 using matrix matched standard curves at concentrations ranging from 5.0 to 400 µg kg^{-1} (Appendix Figure 2). Good linearity was also achieved for water samples by using solvent standard curves giving a correlation coefficient of ≥ 0.99 over a concentration range of 0.1 to 0.8 µg l^{-1}.The selectivity of the methods was demonstrated by analyzing blank soil and water samples to investigate the potential occurrence of analogous compounds both for solvent and matrix-matched calibration standards in the area of anthelmintics' retention times. The limits of detection (LOD) and quantification (LOQ) were determined by fortifying six analyte-free samples with decreasing amounts of the target compounds and

measuring the response at signal-to-noise ratio (S/N) of ≥ 3 and ≥ 6 respectively in accordance with recent guidelines (NATA 2012). In soil the LOD and LOQ for all analytes except for eprinomectin was established at 5 and 10 µg kg^{-1}, respectively; eprinomectin had a LOD and LOQ of 10 and 20 µg kg^{-1}, respectively (Figure 2). In water the LOD and LOQ for all analytes were 0.1 µg l^{-1} and 0.25 µg l^{-1}, respectively. Typical chromatograms are presented in Figure 3. The LOQ for all compounds for which both methods were validated was the lowest point of the calibration curves.

4.1.1 Soil method

All anthelmintic compounds eluted on a reversed phase column within 6.5 min and all peaks for selected quantification transitions were chromatographically separated. Referring to Commission Decision 2002/657/EC (EUROPEAN COMMISSION 2002), which provides method validation guidelines for veterinary drug residues in animal-derived foods, but which was considered a good basis for the validation of this method for drug residues in environmental samples, the trueness and precision were measured by the analysis of six negative soil samples fortified with each of the anthelmintics mixture at three concentrations for LEV, FBZ-SO, FBZ-SO_2, FBZ (10, 20 and 40 µg kg^{-1}) and for EPR (20, 40 and 80 µg kg^{-1}) on three separate occasions using same equipment and same operator at short intervals of time. Results are presented in Table 5. Trueness, defined as the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value (BIPM 2008), is expressed here as the mean recovery, and precision (repeatability and reproducibility) is expressed as relative standard deviation (% RSD). The repeatability of the analytical method was measured by the analysis of six negative soil samples fortified with each of the anthelmintics at three concentrations and analyzed on three consecutive days using the same method and the same equipment. The within laboratory reproducibility was determined in same way but with different batches of materials used and over large intervals of time.

Good recovery (and RSDr) values were obtained for all anthemintics though levamisole was relatively lower in sandy, 79.9% (11.7), and loamy soils, 76.5% (6.9), as compared to other compounds. Mean recoveries (and RSDr) for EPR were in the range 82–86% (7–10) in loamy soil and comparing favourably to the recovery obtained by another study (KROGH et al. 2008), which were 63–80% (9–15).These were also comparable to the values obtained by Litskas, et al. at 89% (8) using

HPLC with fluorescence detection (LITSKAS et al. 2010a). FBZ, FBZ-SO and FBZ-SO_2 recoveries were in the range 80–99% in both types of soil.

The overall mean recovery resulting from repeatability and reproducibility experiments in loamy soils was between 76.1 to 89% with repeatabilities ranging 5.3–16.8% and reproducibilities in the range 3.9–14.7% (Table 5). In sandy soils mean recovery was between 79.9 to 96.9% with repeatabilities ranging 7.6–13.2%. Statistically, there was no significant difference (P = 0.07) observed between the performances of the method in two types of soil for most of the studied compounds and, for this reason reproducibility experiments were not set up separately for sandy soils. In general, however, the extraction efficiency of all analytes was slightly better in sandy than in loamy soils, especially in the case of fenbendazole. This could be attributed to the fact that loamy soil contained more organic matter and clay materials, responsible for the greater binding capacity (sorption capacity) of organic compounds to its soil particles (BLACKWELL et al. 2004; THIELE-BRUHN et al. 2004).

Table 5: Trueness (Rec. %), repeatability (RSD_r %) and reproducibility (RSD_R %) of the validated method in sandy and loamy soils performed at 3 fortification levels on three different days (*n = 18*)

Spiking levels	10.0 µg kg^{-1}		20.0 µg kg^{-1}		40.0 µg kg^{-1}	
Repeatability						
Analytes	Rec.	RSD_r	Rec.	RSD_r	Rec.	RSD_r
	Sandy soil					
LEV	80.1	10.3	77.9	11.8	81.7	13.2
FBZ-SO	91.1	8.3	91.3	10.7	88.4	7.6
FBZ-SO_2	94.1	10.3	91.3	8.9	93.8	10.6
FBZ	98.9	9.6	94.1	9.9	97.6	13.2
EPR[a]	85.5	9.9	90.4	8.4	84.5	11.5
	Loamysoil					
LEV	77.9	8.2	74.8	6.5	77.0	5.3
FBZ-SO	87.7	16.8	85.3	11.4	82.9	14.8
FBZ-SO_2	91.1	7.5	89.9	6.1	89.8	6.1
FBZ	81.6	11.8	79.9	6.8	84.1	11.2
EPR[a]	83.4	7.1	82.2	8.9	85.7	10.1

Reproducibility						
Analytes	Loamysoil					
LEV	74.5	8.7	76.6	8.2	75.7	3.9
FBZ-SO	85.8	6.7	80.7	10.1	84.8	9.6
FBZ-SO_2	94.5	12.0	85.5	11.0	83.5	9.3
FBZ	88.2	12.1	89.4	14.7	82.6	6.3
EPR[a]	85.0	7.3	85.0	11.0	84.2	11.4

[a]fortification level: 20, 40, 80 µg kg^{-1}

4.1.2 Water method

Validation experiments were carried out by fortifying known concentrations of LEV, FBZ-SO, FBZ-SO_2, FBZ (0.25, 0.5, and 1.0 µg l^{-1}) and EPR (0.5, 1.0, and 2.0 µg l^{-1}) into one liter of acidified tap water (pH~3.0) and mixing well prior to sample concentration. Repeatability, RSDr, was checked based on six replicates of each fortification level and reproducibility, RSDR, was calculated based on pooled relative standard deviation of 18 replicates (3 spiking levels) analyzed on three separate days. Validation results are presented in Table 6. Mean recovery (and RSD) of the method was between 35.4% (28) to 125.1% (16) with repeatability standard deviation (RSDr) values ranging from 5.5–22.8% and reproducibility standard deviation (RSDR), which ranged from 6.1 to 28%. EPR recovery (RSD) ranged between 27.8 to 39.8% (19–23) but was comparable with that of Krogh et al. (2008), which were 38 to 67% (9–26) whereas LEV recovery were in the range 62.5 to 66.2% with RSD between 5.5 and 6.1. The confirmation on breakthrough as the main cause of loss of our analytes, allowed us to consider correction of results obtained from lysimeter seepage water samples based on these recovery experiments.

Table 6: Method validation results of five anthelmintics in 1 l water sample analyzed at 3 spiking levels on three different days using liquid chromatography tandem mass spectrometry (*n = 18*)

Compound	Trueness (% Rec.)			Precision (% RSD)		
	0.25 µg l^{-1}	0.5 µg l^{-1}	1.0 µg l^{-1}	0.25 µg l^{-1}	0.5 µg l^{-1}	1.0 µg l^{-1}
LEV	66.2	63.8	62.5	5.5	5.6	6.1
FBZ-SO	112.2	117.4	130.7	10	8.1	7.3
FBZ-SO_2	104.0	130.1	141.2	14.4	8.1	8.6
FBZ	107.0	98.9	116.6	8.2	10.6	11.9
EPR[b]	27.8	38.6	39.8	22.8	24.0	18.9

[b]spiking levels: 0.5, 1, 2 µg l^{-1}

4.2 Determination of anthelmintics in alfalfa plants

4.2.1 Method validation

The selectivity of the method was demonstrated by analyzing blank plant samples to determine the analytes in the presence of possible co-eluting substances. The limits of detection (LOD) and quantification (LOQ) were determined by fortifying six analyte-free samples with decreasing amounts of the target compounds and measuring the response at signal-to-noise ratio (S/N) of ≥ 3 and ≥ 6 respectively. The LOD for all analytes except for eprinomectin was established at a level of 5 µg kg^{-1}; eprinomectin had a LOD of 10 µg kg^{-1}.

The trueness and precision of the method were measured by the analysis of six negative plant samples fortified with each of the anthelmintics mixture at three concentrations for LEV, FBZ-SO, FBZ-SO_2, FBZ (10, 20 and 40 µg kg^{-1}) and for EPR (20, 40 and 80 µg kg^{-1}) on three separate occasions using same equipment and same operator at specified intervals of time (Table 7). Trueness, defined as the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value (BIPM 2008), is expressed here as the mean recovery and precision (repeatability and reproducibility) is expressed as relative standard deviation (% RSD). The repeatability of the analytical method was measured by the analysis of six negative plant samples fortified with each of the anthelmintics at three concentrations and analyzed on three consecutive days using the same method and the same equipment. The within laboratory reproducibility was determined in same way but with different batches of materials used and over large intervals of time. The overall recovery for each compound was calculated as the average recovery from the repeatability and reproducibility experiments of 18 replicate samples over three different concentration levels that were analyzed on three different occasions.

Table 7: Trueness (Rec. %) and repeatability-/reproducibility (RSD %) of the finalized, validated method in green plants at 3 fortification levels on three different days (*n = 18*)

Spiking levels	Quantification ion	10.0 µg kg^{-1}		20.0 µg kg^{-1}		40.0 µg kg^{-1}	
Analytes	*m/z*	Rec.	RSD	Rec.	RSD	Rec.	RSD
		repeatability					
LEV	205>178	73.0	2.5	72.6	2.2	71.8	4.9
FBZ	300.2>159	89.3	11.2	85.5	8.1	79.3	12.5
EPR[a]	914.4>186.1	78.9	7.8	76.5	6.1	79.9	6.0
FBZ-SO	316.1>191	79.2	7.8	71.8	19.1	77.4	9.1
FBZ-SO_2	332.1>300	81.0	7.2	79.2	12.8	75.7	10.4
		reproducibility					
LEV	205 > 178	77.8	6.1	74.2	3.8	76.0	6.5
FBZ	300.2 > 159	81.4	7.4	77.0	5.6	76.1	4.9
EPR[a]	914.4 > 186.1	82.7	8.7	80.9	7.2	81.8	5.1
FBZ-SO	316.1 > 191	79.4	5.4	80.0	5.2	79.0	6.6
FBZ-SO_2	332.1 > 300	82.3	4.8	83.0	5.8	81.3	5.6

[a]fortification level: 20, 40, 80 µg kg^{-1}

The overall recovery values ranged from 74.8 to 81.4% for the five target compounds, with repeatability between 2.2–19.1% and reproducibility between 3.8–8.7% were obtained. The fenbendazole recovery was slightly lower in the validation experiments compared with that obtained in the method development stage; however, the difference was not significant. Levamisole showed the lowest recovery amongst all analytes investigated.

The validated method has sufficient sensitivity to discriminate the signal response of the quantification ion for each analyte from co-extracted materials and to confirm the identity of the substance by measurement of the product ion ratios at the LOQs mentioned above. Although no maximum residue limits have been established for anthelmintics in alfalfa green forage in food and feed, the method fulfilled the required performance criteria set by Codex Alimentarius (EUROPEAN COMMISSION 2009) for the concentration levels measured (10–100 µg kg^{-1}). The precision (repeatability and reproducibility) we obtained in this study is far below the range recommended by Codex (20–32%) and the recovery is within the guideline range (70–120%).

The method was not only applied in our laboratory for research into the transfer of anthelmintics in a soil/water/plant system (ISLAM et al. 2012), but could also be applied to support food and feed safety agencies for regulatory control purposes.

4.3 Mobility and transport of antiparasitic drugs in soil, plant and water

4.3.1 Soil

Figure 10 illustrates the concentrations measured in horizon 1 for the three targeted veterinary pharmaceuticals in each soil type at each sampling time. At horizons 2 and 3, concentrations were lower and variable, and are not presented graphically. Table 8 presents the concentrations of extractable drug and metabolites at each horizon over the duration of the experiment. Values in Table 8 that are < LOQ but > LOD are reported in italics and are intended to be taken as semi-quantitative, indicative only of the general trend and not as accurate values. For each of the compounds, there was a highly significant difference (P=0.001) between the mean concentrations found at each of the sampling times in both sandy and loamy soils.

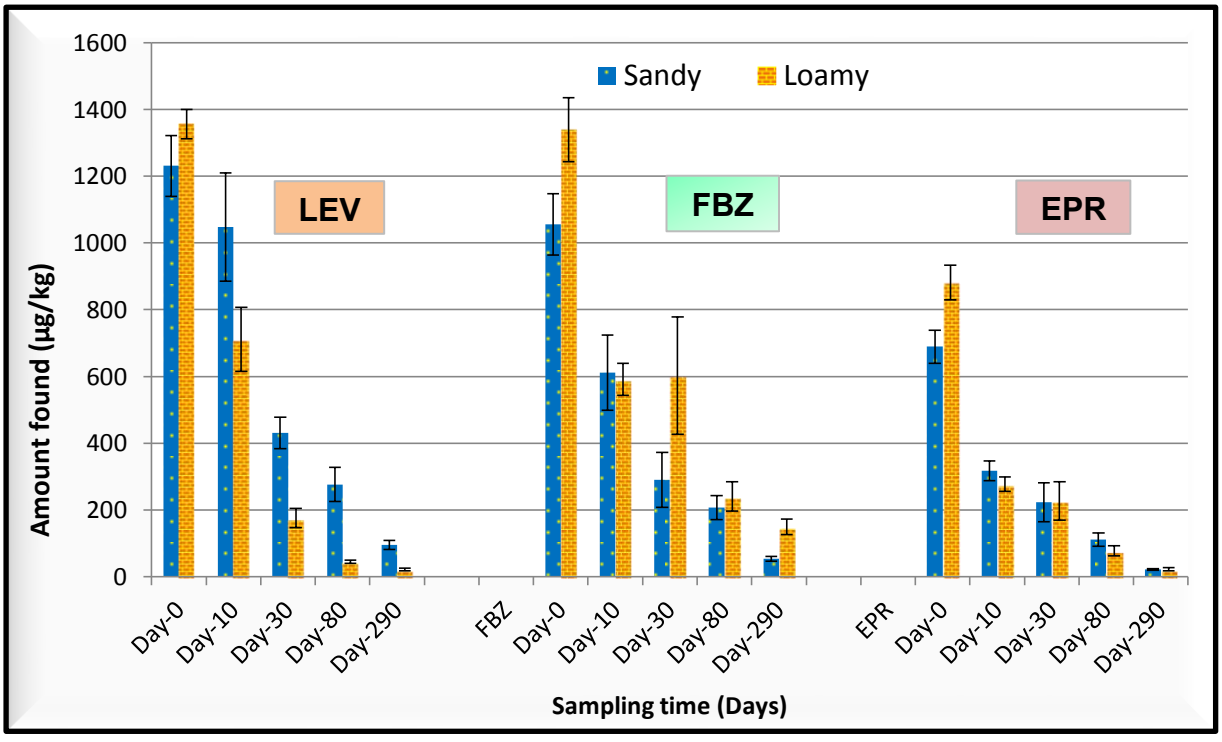


Figure 10: Concentrations of extractable levamisole, fenbendazole and eprinomectin at different sampling periods at horizon 1 (0 – 10 cm) in sandy and loamy soil. Error bars represent SD, n=12.

Table 8: Concentration (µg kg^{-1}) of extractable anthelmintic drugs in sandy and loamy soils at different collection periods and soil depths. Figures in italics are < LOQ and are indicative only that some analyte was detected and the general trend; the values are not intended to be taken as accurate.

Cpds.	LEV - Sandy			LEV - Loamy			EPR - Sandy			EPR - Loamy		
Sampling Times	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3
D-0	1215	-	-	1241	-	-	689	-	-	881	-	-
D-10	1048	32.1	-	711	22.4	-	318	14	-	278	22	-
D-30	430	5.0	4.6	175	4.7	3.6	223	*	*	227	*	*
D-80	276	2.3	1.9	44	3.0	*	111	*	*	77	*	*
D-290	94	2.5	*	21	2.9	1.4	21	*	*	21	*	*

Sampling Times	FBZ - Sandy			FBZ - Loamy			FBZ-SO -Sandy			FBZ-SO -Loamy			$FBZ-SO_2$-Sandy			$FBZ-SO_2$-Loamy		
	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H2	H3
D-0	1056	-	-	1340	-	-	285	-	-	212	-	-	5.6	-	-	5.2	-	-
D-10	611	14	-	699	33	-	423	17	-	263	19	-	23	*2.5*	-	12	5.0	-
D-30	290	*3.2*	*3.0*	322	9.0	14.7	314	5.3	*2.3*	279	13	5.6	51	*2.8*	*2.2*	57	*1.7*	4.9
D-80	206	*2.3*	*0.6*	240	5.0	6.7	226	9.7	5.4	142	11	8.0	68	*2.6*	*1.6*	69	*	15.4
D-290	88	4.0	*0.8*	149	18	7.1	156	6.3	5.0	113	31	13	93	*3.1*	*1.2*	67	11	35.2

H1: 0 – 10 cm (*) :<< LOD (-) : Soil sample not collected according to experiment design
H2: 10 – 20 cm
H3: 20 – 30 cm

4.3.1.1 Levamisole

After application of the slurry mixture (day-0), the mean concentrations of LEV found in horizon 1 were 1357 µg kg^{-1} (loamy soil) and 1231 µg kg^{-1} (sandy soil). For subsequent sampling events the amount of extractable LEV was higher in sandy than in loamy soil at horizon 1, decreasing with time until the final sampling at day-290, when the levels found were 94 µg kg^{-1} and 21 µg kg^{-1} in sandy and loamy soils, respectively.

Sampling at the second horizon commenced 10 days after sowing (DAS). At this time, the LEV concentrations found were 32 µg kg^{-1} and 22 µg kg^{-1} in sandy and loamy soils, respectively. By 30 DAS, although LEV was still detectable in both sandy and loamy soils, the concentrations were 5 µg kg^{-1} or less. The LEV concentration was below the LOD of the method at horizon 3 throughout the course of the experiment.

The higher concentrations of extractable LEV in sandy soil than in loamy soil at the upper horizon is probably attributable to its hydrophilic (log P=1.840±0.57) character (SARI et al. 2004). The relatively polar species would have a high binding affinity for the hydrophilic materials in the organic matter, clay and silt particles of the loamy soil. The amount of these particles is about 2-5 times higher in loamy than sandy soils, as shown in Table 1. An adsorption-desorption experiment conducted as part of this study using ^{14}C-LEV further demonstrated that this compound was adsorbed more in loamy than in sandy soil (Figure 11) .

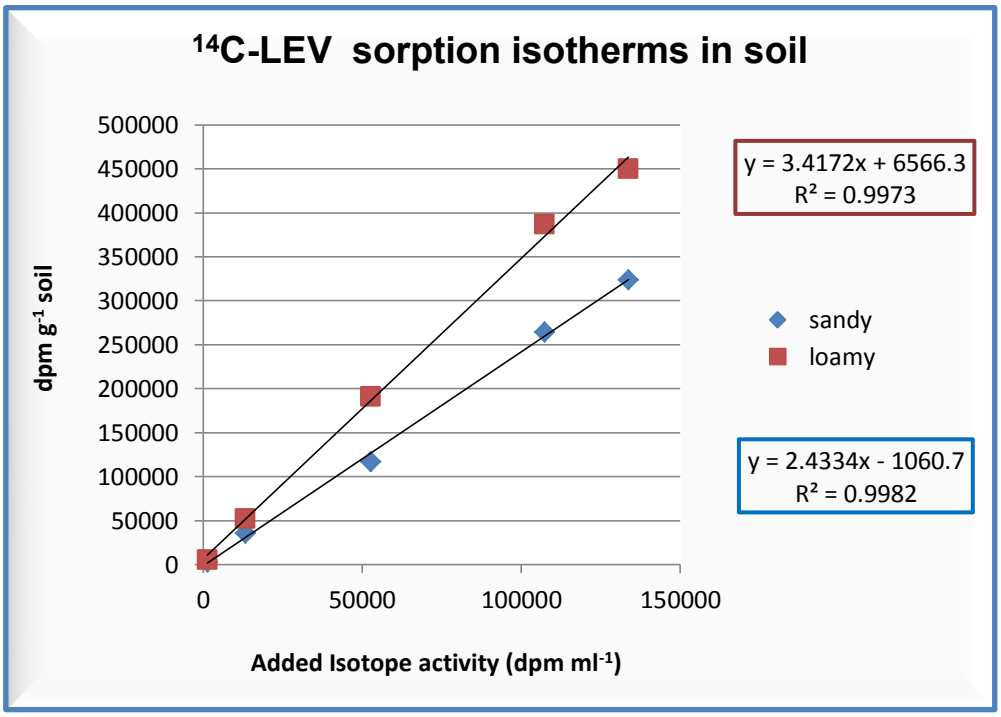


Figure 11: Adsorption-desorption study on ^{14}C-LEV in sandy and loamy soils

4.3.1.2 Radioactive tracer (^{14}C-levamisole)

Results of radioactivity measurements showed similar trends to the non-labelled LEV (Figure 12). The use of a radioisotope tracer in this study offered a comparative advantage over the use of non-labelled substances in that detection was still possible even at the last sampling period in the lowest horizon (20-30 cm). The radioactivity measured could have been attributable to transformation products of levamisole, which would not have been detected by the mass spectrometric method for the unlabelled compound.

The radiotracer experiment also provided useful information on unavoidable losses that occurred a few days after the application due to heavy precipitation.

Measurements made on swipes taken from the lysimeter ring covers revealed that splashed radiolabelled compound was present on the ring covers at higher levels for the lysimeters containing sandy soils than for those containing loamy soil. This splash effect would have resulted in a reduced amount of both hot and non-labelled compound in the lysimeter soils. The hot and cold LEV concentrations are plotted in Figure 4 at the different sampling stages and horizons. There was no significant difference (P=0.05) between the means of the concentrations of radioactive LEV in loamy and sandy soil at any sampling time. At horizon 1 (0-10 cm) a consistent decrease in radioactivity was observed from day 0 to day 290. At horizon 2 (10-20 cm) there was a marked decrease from day-10 to day-30, and the concentration varied little thereafter, with an apparent slight increase at day 290. At horizon 3 measurable radioactivity was found at days 30, 80 and 290, again with a slight apparent increase at day 290.

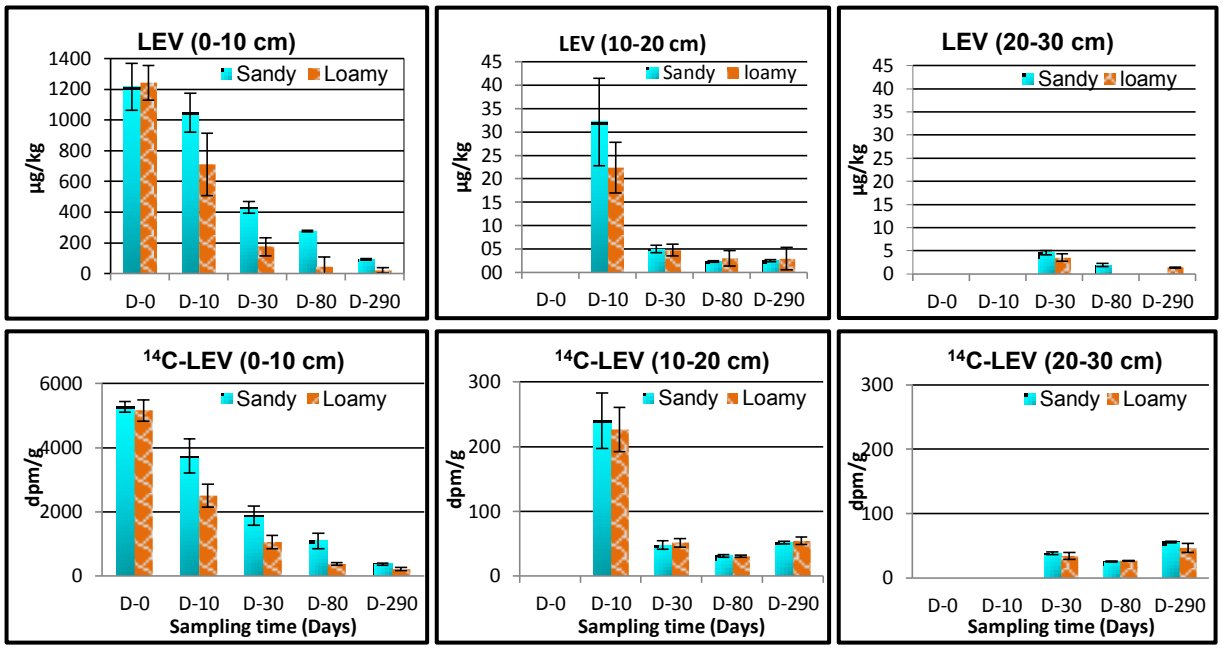


Figure 12: Comparison of the hot (^{14}C-LEV) and cold (non-labelled LEV) compound detected in soils taken at various sampling periods in soil horizon 1, 2 and 3. Error bars represent SD, n=12.

4.3.1.3 Fenbendazole and its metabolites

In contrast to the results for LEV in soil, at horizon 1 the concentration of FBZ was lower in sandy than in loamy soil at nearly all sampling times (Table 8, Figure 10).

This may be partly due to its less hydrophilic character (log P = 2.3±0.49), but also by the greater degradation of FBZ in sandy soils into its product metabolites, FBZ-SO and FBZ-SO_2.

Figure 13 shows the concentration of extractable FBZ metabolites in both sandy and loamy soil at the different sampling periods and horizons. On day 0 at horizon 1, the parent FBZ had already degraded to produce a significant amount of FBZ-SO; approximately 27% of the amount of parent compound in sandy soil and 16% in loamy soil. A detectable amount of the sulphone metabolite was also present. In both types of soil, the concentration of the primary metabolite, FBZ-SO, increased until the second to third sampling (day10-30) then started to decrease, whereas FBZ-SO_2 concentrations increased over the course of the experiment, probably due to the continued conversion of FBZ-SO to FBZ-SO_2. At all sampling times at the first horizon, the concentrations of sulphoxide and sulphone metabolites were higher in sandy than in loamy soils. These results were in agreement with the observation that the concentration of the parent compound was lower at day-0 in sandy soil than in loamy soil. Although this may have been partially due to loss of the applied solutions due to splashing during the heavy rainfall a few days after application, which was greater for sandy than loamy soil, it also suggests that more of the FBZ was metabolised to the sulphoxide and sulphone species in sandy soil. This was further confirmed by the results of the incubation experiments in an environmental chamber under temperature and humidity controlled conditions (Figure 6). The results showed that the sulphoxide product had higher concentrations in sandy than in loamy soil.

Previously published data from absorption, distribution, metabolism, and excretion (ADME) studies of FBZ in various animal species showed that FBZ is metabolised by the oxidation of the sulphur molecule, hydroxylation of the phenyl ring and degradation of the carbamate to the amine (FAO/WHO 1991). It is metabolised in mammals into a series of benzimidazoles, including FBZ-SO. The same process may also take place in organism-rich soils. FBZ, FBZSO and $FBZSO_2$ share a common metabolism (EMA and CVMP 2013; GOKBULUT et al. 2007). The transformation of FBZ into its product metabolites may be due to microorganism activity in the wet soil environment. In the study presented, breakdown of the compound could have commenced in the wet soil prior to sample preparation and extraction.

At horizon 2, FBZ could be reliably quantified only on day 10 in sandy soil (14 µg kg^{-1}) decreasing thereafter to below the LOQ. In loamy soil, the concentrations

detected were significantly higher and decreased from day 10 (33 µg kg^{-1}) to 5 µg kg^{-1} at day 80, with an apparent increase at day 290 to18 µg kg^{-1}. The higher amount observed generally for FBZ and its metabolites in loamy soil at the middle horizon (10–20 cm) could have resulted from the continuous accumulation of solutes in loamy soil through transport of substances into the lower horizon. This is specifically obvious for the three-fold increase in the amount of FBZ on the last day of sampling. As observed from the soil profile, the amount of organic matter and silt in loamy soils, which probably impacts the adsorption of applied compounds, decreases in the lower part of the horizon. The extent of compound adsorption to soil particulates may therefore have been reduced.

The concentrations of extractable FBZ and both metabolites at horizon 2 was higher in loamy than in sandy soil (Figure 14). The accumulated concentration of FBZ may have triggered the increase in the bioavailability of the compound to soil microorganisms, causing enhanced transformation into product metabolites in soils. On day 10 the concentration of extractable FBZ-SO was 17 µg kg^{-1}, in sandy soil, which was 17% higher than the parent compound (14 µg kg^{-1}) , whereas the FBZ-SO concentration in loamy soil was 19 µg kg^{-1}, 44% lower than the parent compound (33 µg kg^{-1}). The extractable FBZ-SO_2 at horizon 2 remained below the limit of quantification from day 10 to day 80 in sandy soil and was measurable only in loamy soil at days 10 (5 µg kg^{-1}) and 290 (11 µg kg^{-1}). At horizon 2 the metabolite FBZ-SO was found in greater concentrations in both types of soil than the parent FBZ or the FBZ-SO_2, whereas in horizon 3 the concentrations of FBZ-SO_2 increased in loamy soil.

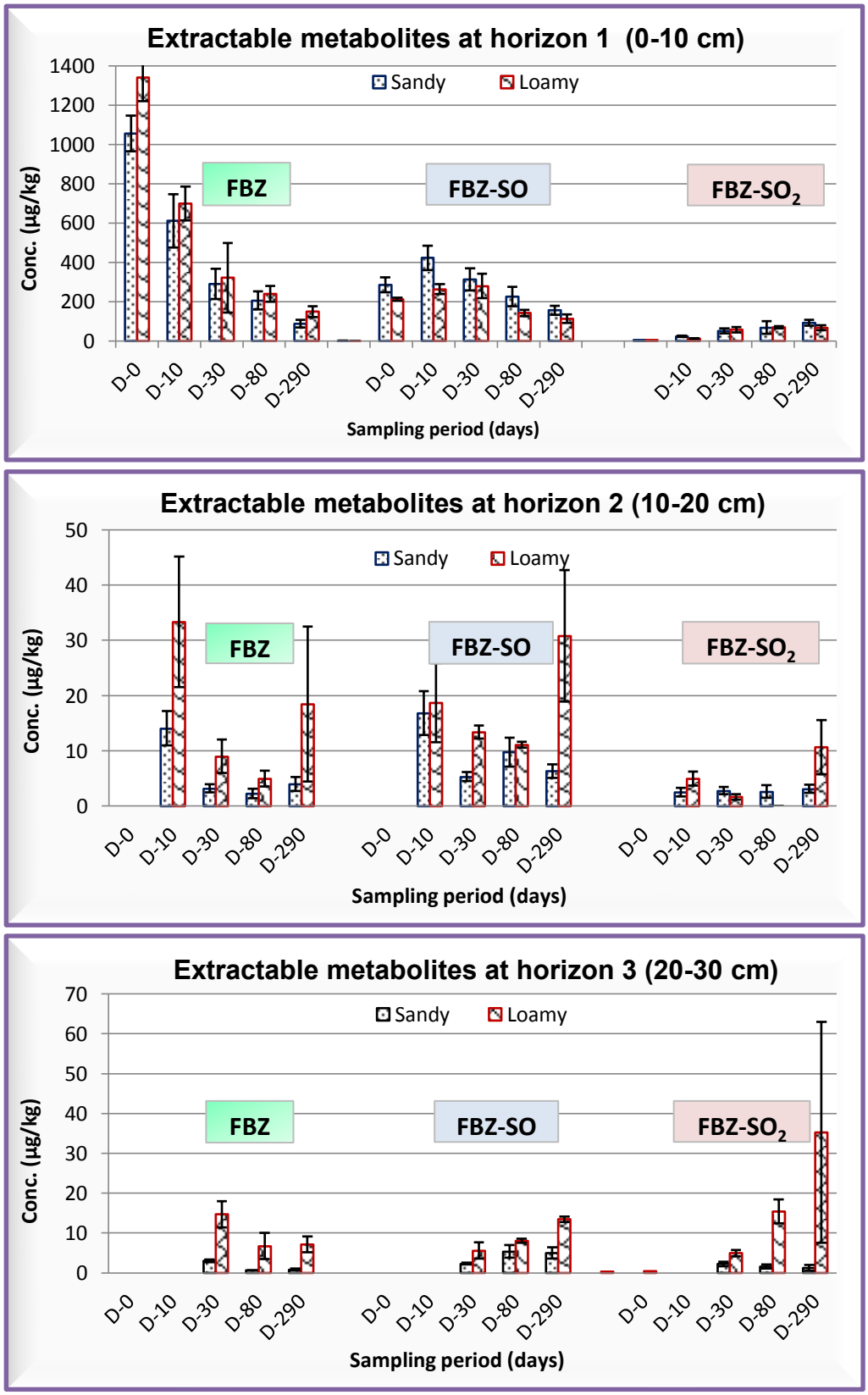


Figure 13: The concentrations of extractable transformation products (FBZ-SO and FBZ-SO_2) of FBZ at various sampling periods and different soil horizons. Error bars represent SD, n=12.

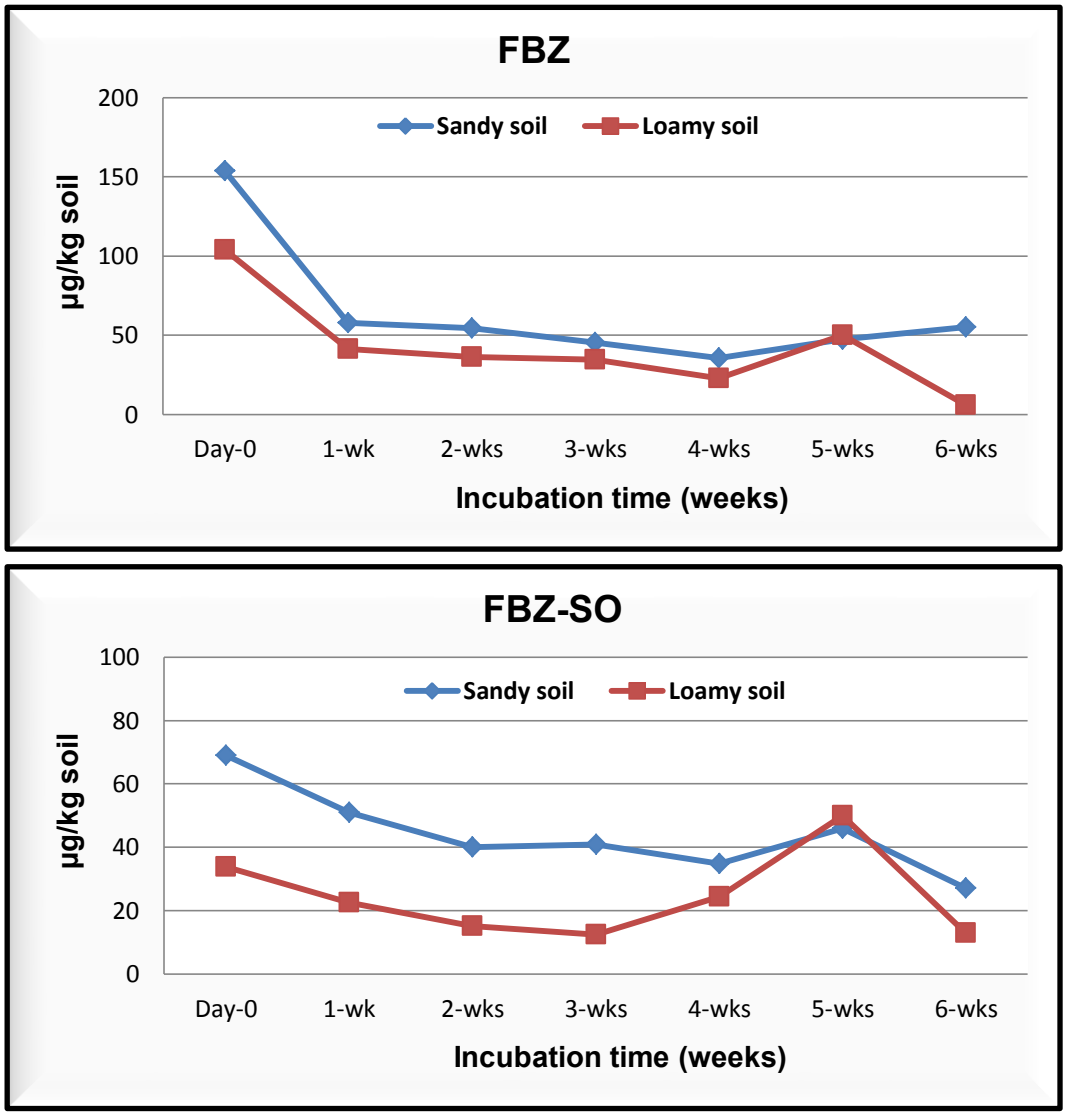


Figure 14: Incubation study of FBZ and the sulphoxide (FBZ-SO) metabolite in sandy and loamy soils in environmental chamber at controlled condition (20ºC, RH ≈ 60%). FBZ-SO_2 was detectable but < LOQ at all sampling times.

4.3.1.4 Eprinomectin

The mean concentrations of EPR found at horizon 1 on day 0 were 689 µg kg^{-1} (loamy soil) and 881 µg kg^{-1} (sandy soil). The concentrations decreased steadily to 21 µg kg^{-1} in both soil types by day 290. There was no significant difference between the concentrations found in sandy and loamy soil at any sampling time.

The concentration of EPR in both soil types was lower than both FBZ and LEV, especially at the 1st and 2nd sampling times. On day 10 the concentrations of extractable EPR had decreased by 50-70% from the concentrations measured at day 0. This sharp decrease may be due to various factors. EPR is likely to bind strongly to soil particles due to its hydrophobic character (log P=5.4), reducing the extractable amount of the drug. The short half-life of EPR in water (0.29/1.1 days in summer/winter) and in soil (64 days) under aerobic condition at 22°C (MERCK & CO. 1996a), especially considering exposure to sunlight under open field conditions during and after application, may have contributed through degradation of the drug, as may metabolism in the soil. Though the sorption study indicated that EPR was adsorbed to a slightly greater degree in sandy soils (Figure 15), the concentrations of extractable EPR in sandy and loamy soils were not significantly different at any of the sampling times. Litskas and others (2010) found that EPR binds to vermiculite and to riparian (sandy) soils with low organic matter content. In addition, they suggested that the observed correlation between concentration specific adsorption and cation exchange capacity could indicate cation exchange between inorganic soil components and avermectins. The sorption characteristics of EPR to both types of soil may also be influenced by the compound's relatively weak lipohilicity. Although macrocyclic lactones are generally lipophilic, EPR is relatively weakly lipophilic and has the lowest mean physiological residence time amongst the drugs in the macrocyclic lactone group; for this reason, it is the preferred macrocyclic lactone for treating dairy cattle (CILLIERS 2012). A previous study on the leaching behaviour (OPPEL et al. 2004) of veterinary pharmaceuticals also suggested specific binding of ivermectin, a related macrocyclic lactone, to soil due to its capability to form adducts with cations such as sodium or ammonium (ALI et al. 2000). Eprinomectin is likely to behave in a similar manner, forming adducts or complexes with immobile inorganic soil matter. The sorption behaviour of this group of compounds is, therefore, influenced by various physico-chemical properties.

At horizon 2, EPR was detected only at the first sampling (day 10) at concentrations of 14 μg kg^{-1} and 22 μg kg^{-1} in sandy and loamy soils, respectively. At all subsequent sampling times, and in horizon 3, no EPR was detected.

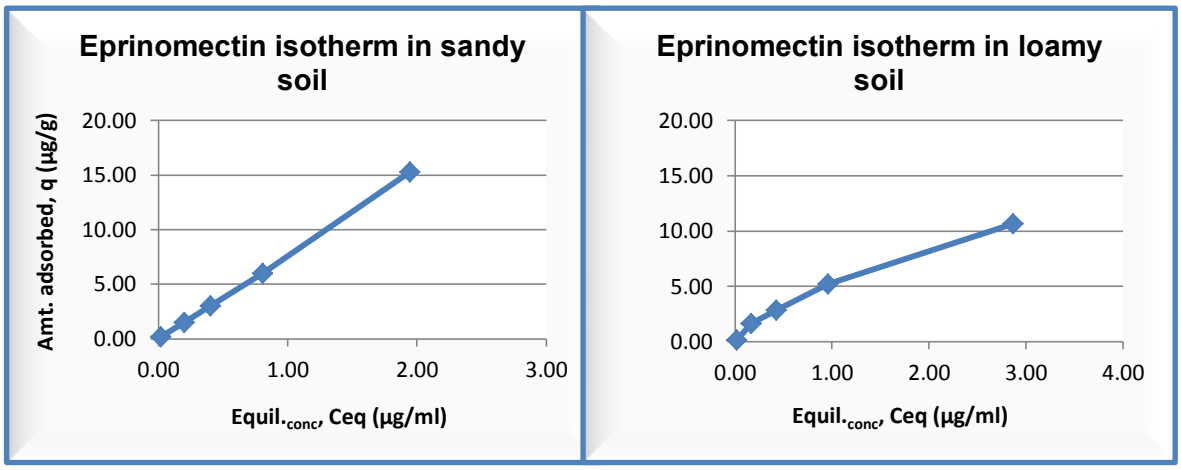


Figure 15: Adsorption-desorption study on EPR in sandy and loamy soils

4.3.2 Plant

4.3.2.1 Levamisole

The uptake of veterinary pharmaceuticals in alfalfa plants (Table 9) was observed to be significantly higher for LEV (log P = 1.8) and EPR (log P = 5.4) than for FBZ (log P = 3.8) for most of the sampling times. Studies on the translocation of pesticides into plant shoots indicate that uptake is related to the octanol-water partition coefficient (log P), by a Gaussian curve distribution (BRIGGS et al. 1983; BURKEN and SCHNOOR 1998). Boxall et al (2006) stated that maximum translocation is observed at a log P of ~1.8. More polar compounds are taken up less well by shoots, and uptake of highly lipophilic compounds (log P > 4.5) is low. Amongst the compounds studied, only LEV showed a significant difference (P=0.05) in uptake between plants grown in loamy and sandy soil at early vegetation stage (1st cutting: day 32). The concentration of extractable LEV from the 1st cutting was about 35% higher in plants grown in loamy (37 µg kg^{-1}) than in sandy (24 µg kg^{-1}) soils. These results may relate to the lower LEV concentrations detected in soil in the 1st horizon indicating that this could have been partly attributable to translocation of the drug into the plants. In a previous study (BOXALL et al. 2006) it was demonstrated that levamisole was one of 3 compounds taken up into lettuce leaves when a mixture of 10 veterinary medicines was applied to a loamy sand soil, the same type of soil used in this experiment. A negative correlation was found between LEV concentration and the harvesting time of plants in both loamy and sandy soils, possibly partly due to dilution of the

compound as the plant mass increased. No significant correlation was found for the other compounds.

Table 9: Uptake of LEV (cold), FBZ/metabolite products, and EPR (µg kg-1) in alfalfa plants grown in 2 types of soil at different vegetation stages. Figures in italics are < LOQ and are indicative only that some analyte was detected and the general trend; the values are not intended to be taken as accurate.

Harvest period	LEV		FBZ		FBZ-SO		FBZ-SO_2		EPR	
	Sandy	Loamy	Sandy	Loamy	Sandy	Sandy	Loamy	Loamy	Sandy	Loamy
Day-32	23.9	37.0							40.2	32.9
Day-63	12.8	17.3	4.2	4.9	14.5	19.4	4.9	5.0	27.7	35.0
Day-314	6.1	10.3	4.7	7.0			3.5	3.7		

4.3.2.2 Radioactive tracer (^{14}C-levamisole)

Typical recoveries of the ^{14}C-labelled levamisole using the extraction procedure described above were 78.6 % (RSD = 2.1 %) for soil (loamy) and 75.2 % (RSD = 3.8 %) for plants. Since no significant difference was found between the recovery of non-labelled compound in loamy and sandy soils in the validated analytical procedure (ISLAM et al. 2013), the extractability of the labelled compound in sandy soil was assumed to be the same as for loamy soil.

The mean differences in measurable radioactivity between plants grown in loamy and sandy soils,–across all vegetation stages, were not significant (P=0.05). Like the results obtained for the cold compound, the concentration of the radioactive levamisole (or its transformation products) decreased with the growth of the plant. Figure 16 shows the uptake of the target compounds, including the radiolabeled levamisole, in plants grown in loamy and sandy soils. The total ^{14}C-LEV uptake into the whole plants grown on sandy soil and loamy soil lysimeters, however, could not be taken into account for any of the harvesting periods in this study since the whole biomass was not removed from the soil base. Thus, mass balances could not be estimated in general for the overall components of this research study.

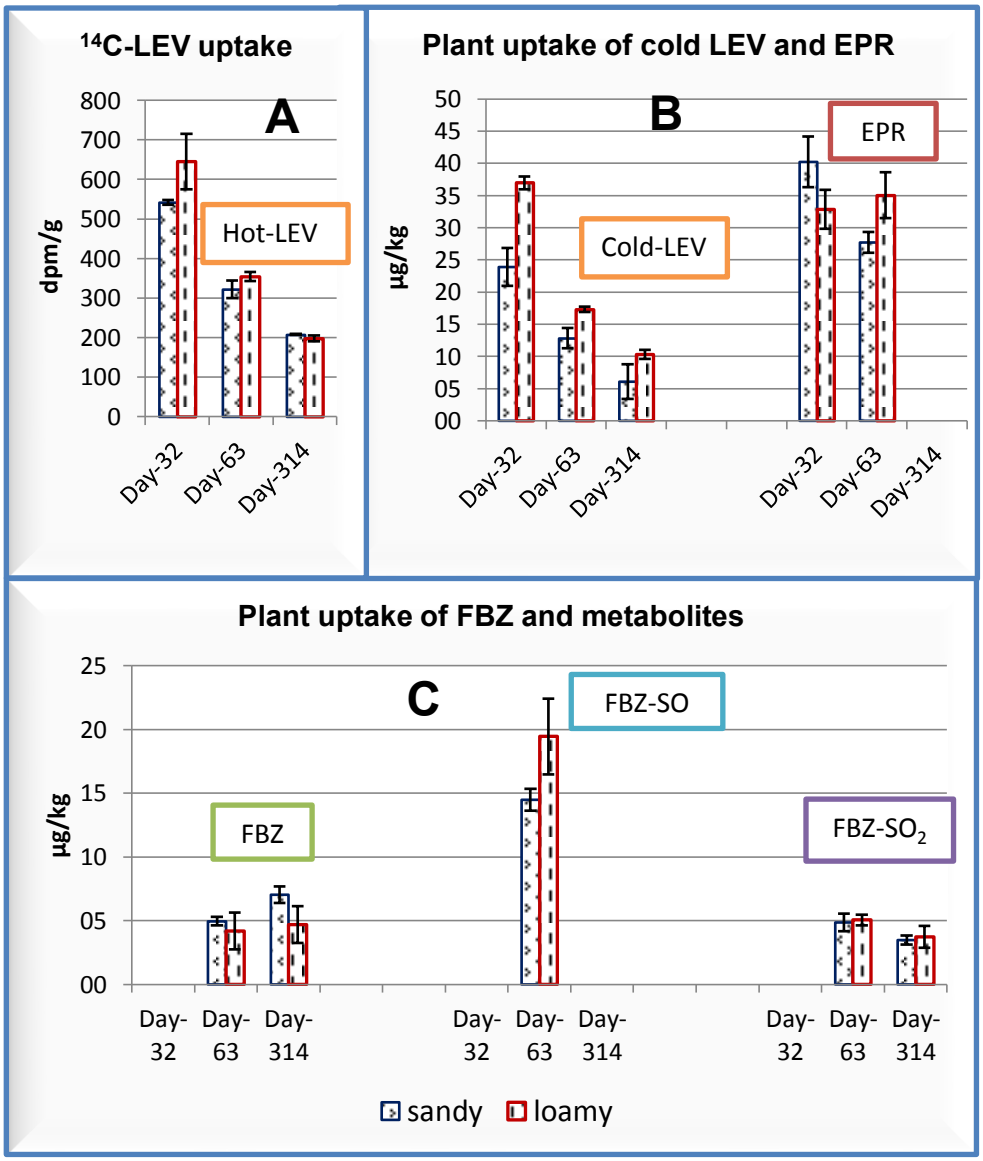


Figure 16: Concentration of anthelmintic compounds (and ^{14}C-LEV) in alfalfa plants on loamy and sandy soils. Error bars represent SD, n=12.

4.3.2.3 Fenbendazole and its metabolites

No FBZ was detected at the early vegetation period (day 32), but for the last two cuttings (days 62 and 310) the concentration of FBZ was close to the LOQ of the method (estimated at between 4 and 7 µg kg^{-1}). The concentration of extractable FBZ was found to be lower in plants grown in sandy soil than in loamy soil at all sampling times.

Like the parent compound, neither of the metabolites, FBZ-SO or FBZ-SO$_2$ were detected in the first cuttings (day 32). But for plant samples analysed from the

second cuttings (day 62), FBZ-SO was observed to be higher in plants grown in loamy (19 µg kg^{-1}) than in sandy (14.5 µg kg^{-1}) soil. As found in the soil matrix, the sulphoxide was the major metabolite observed in plants. The concentration of FBZ-SO in loamy-grown plants was about 4-5 times more than the concentration of either the parent FBZ or the sulphone metabolite in samples from the 2^{nd} cutting (each estimated at approximately 4-5 µg kg^{-1}). In alfalfa samples of the third cutting (day-311), the sulphoxide was no longer detectable. However, the sulphone metabolites were detected at lower concentrations than at the 2nd cutting.

4.3.2.4 Eprinomectin

Eprinomectin (log P ~ 6.2) was detected at early and mid-vegetation stages of plants grown in sandy (28–40 µg kg^{-1}) and loamy (33–35 µg kg^{-1}) soils, but was not detected at the last cutting period (Figure 8). Given a significantly higher octanol-water partition coefficient than LEV (log P~1.8), it was interesting to find that the concentration of eprinomectin was in most cases higher than for levamisole in plants. One possible explanation for this is that the uptake of EPR may have occurred more through the aerial pathway rather than from root to shoot translocation. Plant parts above the ground may become contaminated with organic chemicals via pathways involving direct contact between soil particles and plant surfaces. Being retained on plant surfaces, they may subsequently be taken up into the plant cuticle where the chemical transfer occurs via diffusion when it is adsorbed to lipophilic tissues (RIEDERER and SCHONHERR 1984). The fact that no EPR was detected in the last cuttings (day 314) may be attributable to the short half-life of EPR (64 days) in soil, as well as and other contributing factors such as soil adsorption and degradation of compound into other transformation products

4.3.3 Water

Water samples could be collected only from the sandy soil lysimeters; no leachate was produced in the lysimeters containing loamy soil. Amongst the target compounds, only LEV could be detected above the LOQ. Table 10 presents the concentration and estimated total amount of the veterinary drugs transported through ground water based on the total volume of seepage water collected from sandy soil lysimeters.

The mean concentration of LEV for the two lysimeters increased over the first 3 collections (days 13, 20, 36) of seepage water. However, for the 4th collection (day 304) the concentration decreased by > 80% for both lysimeters. This may be attributable to the half-life (5-75 days) of LEV in soil (MERIAL 2008); at this last sampling time, almost no LEV remained to be carried down the horizon by preferential water flow. Between the two sandy lysimeters a significant difference in results was observed except on day 36 where LEV was detected at the highest concentration (0.7-0.8 µg l^{-1}). The significant differences can probably be explained by the differences in their monolith structure as evidenced by the varying volumes of seepage water measured.

Radioactive measurements showed a moderate but significant negative correlation from the second (day 20) until the last (day 306) collection between the isotope tracer radioactivity and collection period indicating that the amount of radioactive substance decreased with time. The fraction of tracer detected in seepage water was quantified based on the radioactivity measured on day 0 at soil depth 10 cm. The trends in results (Table 11) obtained are comparable with those from the cold veterinary drug compounds. The FBZ metabolites could not be quantified in water as they were far below the LOD.

Table 10: Concentrations and total amount of veterinary drugs transported to ground water through sandy soil lysimeters.

Compounds Collection time	Preci-pitation (mm)*	Seepage H_2O (l)	Conc. (µg/l)	Tot. amount (µg)	Seepage H_2O (l)	Conc. (µg/l)	Tot. amount (µg)
		Sandy lysimeter 1			Sandy lysimeter 2		
LEVAMISOLE							
Day-13	76.8	9	*0.09*	0.84	18	*0.05*	0.84
Day-20	75.6	46	0.19	8.60	48	*0.05*	2.24
Day-36	9.5	9	0.80	7.19	10	0.70	7.04
Day-304	13.0	5	0.25	1.26	14	*0.08*	1.07
FENBENDAZOLE							
Day-13		9			18		
Day-20		46	*0.01*	0.46	48	*0.01*	0.48
Day-36		9			10		
Day-304		5	*0.01*	*0.03*	14	*0.01*	0.11
EPRINOMECTIN							
Day-13		9			18		
Day-20		46	*0.06*	2.60	48	*0.03*	1.36
Day-36		9		0.03	10	*0.01*	0.08
Day-304		5			14		

*Rain amount is same for all compounds per collection

Table 11: Fraction of isotope tracer (^{14}C-LEV) detected in seepage water

Col. time	Sandy 1	Sandy 2	Average
D-13	0.27%	0.25%	0.26%
D-20	1.22%	0.83%	1.03%
D-36	0.16%	0.11%	0.14%
D-308	0.08%	0.10%	0.09%

5 Summary

Anthelmintics are one of the main groups of veterinary drugs that are vital in the production of livestock. Since the administered drugs are usually eliminated from the body in the animals' faeces, they can be found in the manure. The use of anthelmintics in food producing animals could, therefore, affect the ecological system due to application of the animals' excreta to the land. Translocation of antiparasitic drugs from animal excrement through soil and water to crops and forages and their recycling to food (crops and grazing animals) is also a potential concern for the food chain. In this research work, analytical methodologies were developed to elaborate and characterise the risks associated with the exposure of these organic contaminants in the environment specifically looking into the mobility and transport of antiparasitic drugs in soil, water and plants.

A method was developed and validated for the determination of three different classes of anthelmintic compounds in soil and water. Analysis of soil samples involved a simple solvent extraction using acetonitrile/methanol mixture (1:1, v/v) and a dispersive clean-up technique using primary secondary amine (PSA) and magnesium sulfate ($MgSO_4$). Analysis of acidified water samples was performed by extraction/concentration on an Oasis-HLB cartridge. A methanol/acetonitrile mixture (1:1, v/v) was used to elute the adsorbed analytes from the dried stationary phase of the SPE cartridge. Deuterated levamisole, fenbendazole, fenbendazole sulphoxide and fenbendazole sulphone were used as internal standards. Both methods employed gradient chromatography to permit the simultaneous analysis of all analytes. The extracts were analyzed using a liquid chromatography tandem mass spectrometry (LC-MS/MS). The method for soil was very fast, precise and effective, allowing an analyst to extract and inject approximately 30 samples a day. The good recoveries obtained for representative compounds show the potential of this method in determining a range of anthelmintics of different physico-chemical properties in sandy and loamy soils. The relatively low recoveries of eprinomectin and levamisole in water were, to some extent, compensated by the fact that individual extraction was not necessary for these two compounds. The method could be improved through the addition of stable isotope labelled internal standards prior to sample loading into the SPE cartridge.

For alfalfa (*Medicago sativa* L.), anthelmintics in plant leaves and stems (green chops) were extracted with methanol/acetonitrile (7:3, v/v) followed by a concentration and clean-up step using solid-phase extraction (Strata-X, 500 mg, 6ml cartridge). Elution of adsorbed analytes was done using a methanol/acetonitrile mixture (1:1, v/v) followed by 100% acetonitrile. The extracted analytes were separated by gradient reversed-phase chromatography and the transition ions monitored by a Waters triple quadrupole mass spectrometer operated in positive electrospray ionisation mode with selected reaction monitoring. The validated method demonstrated the potential of determining multi-class anthelmintics in different forage crops used for animal food production. In the guidance document for Directive 91/414, it is stated that beef and dairy cattle can be fed on 100% grass, silage or hay. Therefore, monitoring drug residues in feed is of great relevance (EURL 2010). The validated method complied with Codex performance criteria for residues of veterinary drugs in foods. The validation data presented demonstrate the feasibility of the method for use in research into the occurrence of veterinary drugs in the food chain or for routine regulatory or control purposes. It is also envisaged that the method for alfalfa plants could be extended to other forage crops and relevant drug groups, with the appropriate validation steps.

All three validated methods (soil, water and plants) were successfully applied alongside direct ^{14}C measurements of labelled compound in lysimeter studies to elaborate and characterise the behaviour of anthelmintics in a soil-water-plant system.

A designed lysimeter experiment made use of 5 cyclindrical blocks (1.13 m^2) containing two types of soil (silt loam and loamy sand), with 2 replicate lysimeters for each type of soil and one lysimeter containing sandy soil, used as a control. Veterinary drugs and radiolabelled compound were applied through a slurry mixture as fertilizer to check for the transfer/distribution of the exposed compounds in the soil surface into the different ecosystems. Sampling regime for soil was designed from day 0 application to day 290 at 3 different soil depths. The use of small internal grids (14.1 cm) was useful for the quality control of sampling points and making sure that withdrawal of samples will not be repeated at the same site. The entire experiment lasted for about a year from the time of sowing (early summer). Using the above

methods, samples taken from three compartments were analysed thus, generating numerous information pertaining the distribution of the target drugs in soil.

The general findings was that most of the compounds and including the product metabolites were located at the upper horizon of the soils (height = 10 cm). In the lower horizon, metabolites could still be detected in a much higher concentration than the parent compound. The concentration of sulphoxide (FBZ-SO) metabolite was abundant in horizon 2 (height = 20 cm) whereas the sulphone (FBZ-SO_2) metabolite was greater in horizon 3 (height = 20 cm). The concentration of these metabolites were higher in loamy than in sandy soils at all sampling times.

Sampling of alfalfa grasses was planned by cutting during early, mid and late vegetation stages of the plants. A long-standing alfalfa plant in these lysimeters, which started to grow about 2 weeks after slurry application, was the species used to determine if any uptake could be observed from any of the compounds exposed in the soil. The imidazothiazole LEV and macrocyclic lactone EPR were both found during the early and mid-vegetation of the forage crop. Also found in mid-vegetation of the crop was FBZ-SO metabolite, which was significantly higher in concentration than FBZ and FBZ-SO_2 but not significantly different than LEV concentration. The concentration for most of these compounds were higher in loamy- than in sandy-grown plants.

The water collected, which represented the seepage water and which was only found from sandy lysimeter, was used to determine if there is leaching of target compounds into the lower horizon of the soil. Minute amounts were detected for all target compounds (LEV, FBZ and EPR) especially on days 20 and 36 after application but higher concentrations of levamisole were consistently detected at all sampling periods. LEV was the only target compound having the lowest log P.

6 Conclusions

Based on the experimental design of this lysimeter study and using a range of anthelmintic drugs with varying physico-chemical properties, the results clearly showed that soil sorption was the prevailing mechanism taking place in the terrestrial system. The degree of adsorption varied amongst the drugs and between types of soil. Uptake of the drugs by alfalfa was demonstrated. The main metabolite of fenbendazole was detected in alfalfa, the sulphoxide, at about 3-4 times (15-20 µg kg^{-1}) the concentration of the parent compound, which could be alarming if the metabolite was more active and toxic than its original form, as in this case. However, the sulfoxide form is not stable since it is further oxidised to its sulphone molecule, which is the inactive form. Understanding the uptake of the targeted anthelmintic drugs is challenging specifically for eprinomectin, which has a higher log P than levamisole and fenbendazole, but was taken up more than the other two compounds. The presence of these drugs in alfalfa grasses, which are commonly grazed in pasture fields, could pose a possible threat to livestock and other food producing animals. It is a well-known fact that anthelmintic resistance is prevalent not only in sheep but also in livestock industry (COLES et al. 2006; EDMONDS et al. 2010; SUTHERLAND and LEATHWICK 2011). Therefore findings of this study could raise more concern for the farm growers whose main target is to avoid losses in animal food production. Reduced efficacy of anthelmintic drug could suggest increased dosage of drugs thereby instigating more concentration of these drugs in the environment.

The results of the study indicate that the possibility of the drugs contaminating the ground water to any significant degree is remote. However, there is a risk of ground water contamination in the case of accidental spillage or very poor farm management practices. The risk may be greater for polar substances or compounds with lower log P, as indicated by the fact that levamisole was detected in seepage water at all sampling times.

The results of this study suggest that further research is needed to investigate more veterinary pharmaceuticals used in food-animal production to provide data for risk assessment regarding the behaviour of these drugs in the environment. Such

assessment is needed to ensure food safety and the protection of human and animal health, as well as the ecological system.

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10 Appendix

Appendix Table 1: Extraction recovery (%) of ^{14}C-LEV using LSC and the cold compounds using LC-MS/MS based on various organic solvents and different solvent mixtures

Organic solvents	^{14}C-LEV	LEV	FBZ-SO	FBZ-SO_2	FBZ	EPR
Acetone (100%)	24.1	-	-	-	-	-
ACN (100%)	39.7	7.7	138.7	64.4	107.5	79.1
MeOH (100%)	62.7	44.8	59.2	104.6	136.7	66.9
MeOH/Acetone (1:1, v/v)		92.8	91.7	97.0	102.4	61.1
MeOH/DCM (7:3, v/v)	67.8	-	-	-	-	-
MeOH/H2O (9:1, v/v)	70.9	-	-	-	-	-
MeOH/ACN (7:3, v/v)	72.7	79.4%	73.1%	84.7%	66.6%	89.2%
MeOH/ACN (1:1, v/v)	78.9	78.8%	99.7%	92.5%	98.3%	69.9%

Appendix Table 2: Extraction efficiency of anthelmintics from a 100 ml pre-treated water sample using two different SPE sorbents

SPE type	Strata-X	Oasis-HLB	Strata-X	Oasis-HLB
Eluting solvents	100 % Methanol		Methanol/Acetonitrile (50:50, v/v)	
LEV	33.5	69.4	67.2	97.0
FBZ-SO	77.3	95.0	96.3	116.7
FBZ-SO_2	91.3	88.2	117.5	117.1
FBZ	68.2	63.9	84.0	87.8
EPR	47.1	17.4	122.5	125.3

Appendix Table 3: Comparison of analyte recoveries (%) for the method optimization of water extracted through SPE cartridge using different sample volumes and loading rates

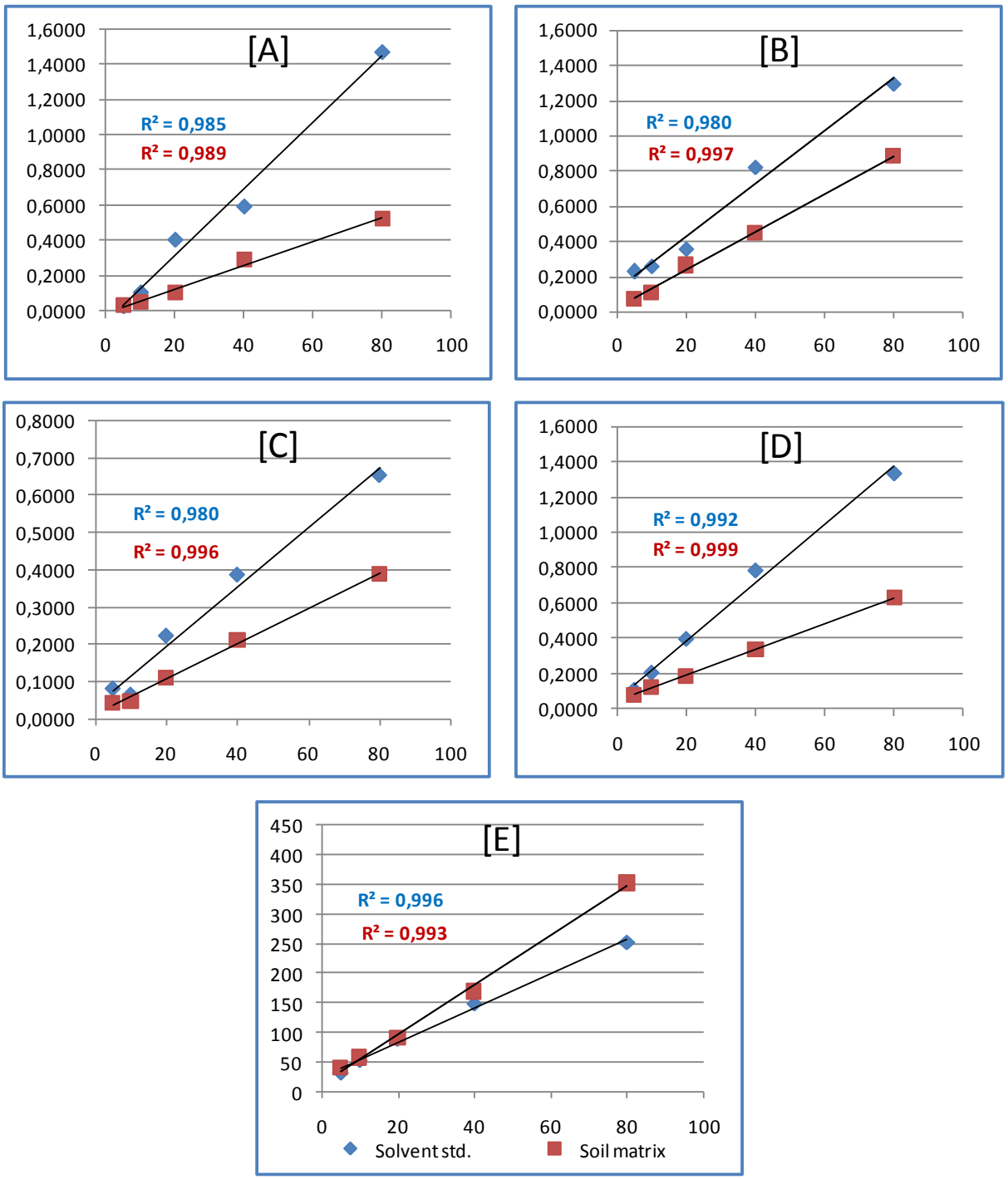
Sample ID	LEV	FBZ-SO	$FBZSO_2$	FBZ	EPR
Wat-R1[a] (Vol: 1000 ml)	60.1	84.9	92.9	74.7	17.9
Wat-R2[a] (Vol: 1000 ml)	60.2	87.3	99.6	81.2	13.6
Wat-R3[b] (Vol: 100 ml)	58.3	77.4	87.7	44.3	45.3
Wat-R4[b] (Vol: 100 ml)	56.2	76.7	83.2	45.9	42.6
Wat-R5[c] (Vol: 100 ml)	57.3	88.1	88.3	52.8	44.1
Wat-R6[c] (Vol: 100 ml)	55.2	73.5	82.1	44.7	43.8

a - extracted by pump loading

b - extracted by manual loading and finish at the same time as (a)

c - extracted by manual loading and finish in about 3 minutes

Appendix Figure 1: Comparison of solvent and matrix-matched standard calibration curves of [A] LEV, [B] FBZ-SO, [C] FBZ-SO_2, [D] FBZ, and [E] EPR for the quantification ion (m/*z*) abundance ratio against concentration of analytes in soil equivalent to 5.0 – 80 µg kg^{-1}



11 CV

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