A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Aerobic and Anaerobic Soil Metabolism of a Herbicide Methiozolin and Its Pharmacokinetics and Metabolism in Rats

제초제 Methiozolin 의 호기성 및 혐기성 토양대사와 흰쥐에서의 약동학 및 대사 연구

By

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Department of Agricultural Biotechnology
The Graduate School of Seoul National University
February 2015

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UNDER THE DIRECTION OF ADVISER JEONG HAN KIM
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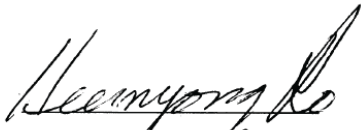
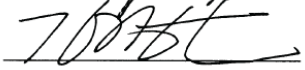
Chairman	Dr. Hee-Myong Ro	
Vice Chairman	Dr. Jeong Han Kim	
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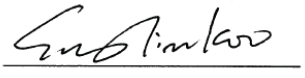
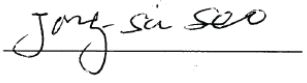
Member	Dr. Suk-Jin Koo	
Member	Dr. Jong-Su Seo	

Aerobic and Anaerobic Soil Metabolism of a Herbicide Methiozolin and Its Pharmacokinetics and Metabolism in Rats

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ABSTRACT

Methiozolin is a new turf herbicide controlling annual bluegrass in various cool- and warm-season turfgrasses. This study was conducted to investigate the fate of methiozolin in soil under aerobic and anaerobic conditions using two radiolabeled tracers, [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin. The mass balance of applied radioactivity ranged from 92.0 to 104.5% in aerobic sterile, 97.5 to 101.2% in aerobic nonsterile, and 93.2 to 102.5% in anaerobic soils. In the aerobic nonsterile soil, [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin degraded with 17.9 and 15.9% remaining, respectively, at 120 days after treatment (DAT). [^{14}C]Carbon dioxide and the nonextractable radioactivity increased up to 41.5 and 35.7% for [benzyl-^{14}C]methiozolin at 120 DAT, respectively, while were 36.1 and 39.8% for [isoxazole-^{14}C]methiozolin, respectively. The nonextractable residue was associated mainly with humin and fulvic acid fractions under aerobic condition. No significant volatile products or metabolites were detected in the soil under aerobic condition. The half-life of [^{14}C]methiozolin was

approximately estimated as 49 days in the nonsterile soil under aerobic condition; however, it could not be estimated in sterile soil under aerobic condition and in the soil under anaerobic conditions, because [^{14}C]methiozolin degradation was limited. Particularly, one significant metabolite was produced in anaerobic soil (7.9% of the applied) and it was identified as 4-(2,6-difluorobenzyloxy)-3-hydroxy-3-methyl-1-(3-methylthiophen-2-yl)butan-1-one. Based on these results, methiozolin is considered to undergo fast degradation by aerobic microbes, but not by abiotic chemical reaction or anaerobic microbes in soil.

The present study reports that elucidate absorption, tissue distribution, excretion, and metabolism of methiozolin in rats following oral administration. The pharmacokinetic parameters in the blood were observed as follows: T_{max} = 6 h, C_{max} = 168.7 μg equiv/mL, $T_{1/2}$ = 49.4 h, AUC_{120} = 9921.5 μg equiv·h/mL, and clearance = 39.2 mL/h/kg. Those parameters and the depletion curve for ^{14}C in the plasma were very similar to those in the blood. The total amounts excreted in urine and feces were 24.3 and 68.9%, respectively, during 120 h after administration; however, no excretion was found in exhaled. The ^{14}C radioactivity excreted through bile was 40.1% of the administered. Excreted radioactivity peaked between 24 and 48 h, showing 51.0% of the total excretion within 48 h. The orally administrated ^{14}C distributed across various tissues within 12 h after administration, showing 14.0% of the dosed, and was eliminated from all the tissues without accumulation. Numerous minor metabolites (<4% of the dosed) in urine and fecal extracts were detected within 72 h and two of those were identified. The identified metabolites in fecal extracts were AMet-1, 6-(5-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)-

4-methylthiophen-2-yloxy)-tetrahydro-3,4,5-trihydroxy-2H-pyran-2-carboxylic acid, and AMet-2, (2-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)thiophen-3-yl)methanol. Conclusively, methiozolin was shown to readily absorb to the gastrointestinal tract, distributed throughout the tissues within 12 h, metabolized extensively, and eliminated through urine and feces mostly within 48 h, without tissue accumulation.

KEYWORD: methiozolin; herbicide; soil metabolism; aerobic; anaerobic; pharmacokinetics; absorption; distribution; excretion; metabolite

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LITERATURE REVIEW

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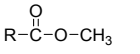
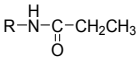
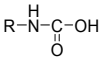
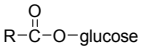
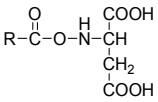
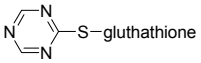
1.1. Metabolism reactions in soil

Understanding environmental fate of agrochemicals is very important for developing chemicals and for defining their application method and optimal use rate. Although the environmental fate of many agrochemicals is affected by chemical and physical processes, such as sorption/desorption and sequestration, knowledge of biological transformation pathways is a prerequisite to understand what metabolites may be present in the environment. Certain agrochemical metabolites have greater plant or mammalian toxicity and the possibility of long-term exposure and chronic effect compared to a parent compound, therefore, these metabolites necessitate a need for their identification and study. It is also important to understand the major pathway of agrochemical degradation in aquatic and terrestrial environment, and to determine the major group of microorganism responsible for such biotransformations.

Agrochemical biotransformations may occur via metabolism or co-metabolism. Metabolism of a given agrochemical in microorganisms is a multi-step process generally. Metabolism of agrochemicals may involve a three-phase process (Hatzios, 1991; Shimabukuro, 1985; Van Eerd et al., 2003) (Table 1). In phase I metabolism, the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to produce a more water-soluble and a less toxic product than the parent.

Table 1. Three phases of pesticide metabolism, with pesticide examples and nonspecific chemical reactions (Van Ered et al., 2003).

Phase	Reaction	Example pesticide	Non-specific example of the chemical scheme		
I	***Oxidation***		Pesticide + O_2 → Pesticide-O + H_2O		
	Aryl/alkyl hydroxylation	Chlortoluron		→	
	O-dealkylation	Ethametsulfuron	R-OCH_3	→	R-OH
	N-dealkylation	Ethametsulfuron	R-$NHCH_3$	→	R-NH_2
	Oxidative deamination	Metribuzin	R-NH-NH_2	→	R-NH_2
	Sulfoxidation	Prometryne	R-S-CH_3	→	
	Nitrogen oxidation	Credazine		→	
	Reduction			→	
	Nitroreduction	Trifluralin	R-NO_2	→	R-NH_2
	Hydrolysis		Pesticide + H_2O	→	Pest-OH + H-icide
	Ester	Diclofop-methyl		→	
	Amide	Propanil		→	
	Nitrile	Cyanazine	R−C≡N	→	R-C-O-NH_2
II	***Conjugation***		Pesticide + molecule to conjugation → Pesticide-conjugate		
	O-glucose	Metribuzin	R-OH	→	R-O-glucose
	N-glucose	Flumetsulam	R-NH_2	→	R-N-glucose
	Glucose ester	2,4-D		→	
	Amino acid	2,4,-D		→	
	Glutathione	Atrazine		→	
III	***Secondary conjugation***		Pesticide + molecule to conjugate → Pesticide-conjugate-conjugate		
	Glucose	Picloram-glucose	R-NH-glucose	→	R-NH-glucose-glucose
	Malonate	Metribuzin-glucose	R-NH-glucose	→	R-NH-glucose-malonate

The phase II involves conjugation of an agrochemical or its metabolite to a sugar, amino acid, or glutathione, which increase the water solubility and reduces toxicity compared with the parent agrochemical. In general, phase III metabolites have little or no phytotoxicity and may be stored of phase II metabolites into secondary conjugates, which are also nontoxic (Hatzios, 1991).

Metabolism pathway diversity depends on the chemical structure of the agrochemicals, the organism, environmental conditions, and metabolic factors regulating expression of these biochemical pathways. Co-metabolism can be defined as the biotransformation of an organic compound that is not used as an energy source, or as a constitutive element of the organism (Alexander, 1994). Initial co-metabolic transformations may render agrochemicals less toxic to target and non-target organisms, and also may enhance agrochemical vulnerability to other biological, chemical, or physical degradative transformations. Enzymes involved in initial co-metabolic transformation include hydrolytic enzymes (esterases, amidases, nitrilases, etc.), transferases (glutathione *S*-transferase, glucosyl transferase, etc.), oxidase (cytochrome P-450s, peroxidases, etc.), and reductases (nitroreductases, reductive dehalogenases, etc.). For complete degradation of an agrochemical, several metabolic and/or co-metabolic processes are usually required (Hatzios, 1991; Shimabukuro, 1985).

Bacteria and fungi can completely metabolize an agrochemical to CO_2, H_2O, inorganic nitrogen and other inorganic components via a process called mineralization (Alexander, 1994). Mineralization is a multi-step process wherein initial catabolism occurs via hydrolytic or reductive reaction, and oxidation of aromatic ring compounds is also usually required.

Ring cleavage is an essential metabolic factor in the mineralization of most aromatic agrochemicals. Aerobic ring cleavage by bacteria requires dihydroxylation, most often catalyzed by dioxygenases (Mason and Cammuck, 1992). There are two general classes of dioxygenases, one catalyzed *ortho* (intra-diol), the other *meta* (extradiol) fission of catechol, thereby forming *cis*-muconic acid or 2-hydroxymuconic acid, respectively. Hydroxylation of aromatic rings may also be catalyzed by cytochrome P-450 monooxygenases and mixed function monooxygenases, in which only one of the oxygen atoms is incorporated into the substrate molecule, and the other is incorporated into water (Mason, 1988).

Many microorganisms are capable of aerobic metabolism under anoxic conditions by using alternate terminal electron acceptors instead of oxygen. Anaerobic microorganisms may use NO_3^-, NO_2^-, SO_4^{2-}, CO_2 and other acceptors (Ehrilich, 1995). Under aerobic conditions, the organic substrates are transformed to CO_2, while under anaerobic respiration the product is either CO_2 or CH_4. Certain microorganisms possess anaerobic or facultative anaerobic metabolism, thus enabling agrochemical biotransformation under anoxic conditions.

Continued use of some soil-applied agrochemicals has resulted in reduced efficacy due to metabolic adaption of microbial populations, and subsequent enhanced biodegradation potential in the exposed soil. A microbial population may also be cross-conditioned for accelerated degradation by exposure to related compounds (Felsot and Shelton, 1993). These relatively rapid microbial adaptations severely limit the effective use of such agrochemicals in these situations.

1.1.1. Oxidative transformations

Oxygenation is the most frequent first step in the biotransformation of agrochemcials and other organic xenobiotics. Many of these reactions are mediated by oxidative enzymes: cytochrome P450s, peroxidases, and polyphenol oxidases. Cytochrome P450s are hemethiolate proteins that have been characterized in animals, plants, bacteria, and filamentous fungi. Cytochrome P450s often catalyze monooxygenase reactions, usually resulting in hydroxylation, according to the following reaction: $RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$. However, there are many other P450-mediated reactions including dehydration, dimerization, deamination, dehydrogenation, heteroatom dealkylation, epoxidation, reduction, and C-C or C=N cleavage. Agrochemicals can influence cytochrome P450 systems by acting as effectors, thereby modifying agrochemical metabolism, or by modulating overall metabolism of an organism. These effects can increase or decrease physiological activities.

Microorganisms produce a wide range of oxidative enzymes, peroxidase, polyphenoloxydase, laccase, and tyrosinase, other than P450s that catalyze the polymerization of various anilines and phenols (Dec et al., 2001). White rot fungi (*Phanerochaete chrysosporium*) offer high potential for xenobiotic transformation because they possess free radical-based lignin degrading systems (lignin peroxidase and manganese-dependent peroxidase) that can degrade a wide range of pollutants such as polychlorinated biphenyls and nitroaromatic explosives (Dec et al., 2001).

In microorganisms, numerous enzymes from many different pathways are capable of oxidizing nitroaromatic compouns. In bacteria, monooxygenases, flavin monooxygenases, and dioxygenases are generally involved in the

initial oxidation of nitroaromatic agrochemicals. For example, 2,4-dinitrophenol can be metabolized by these three enzymes (Cassidy et al., 1999). Depending on the compound, nitrite can be released before, or after ring cleavage.

Hydrolytic enzymes catalyze the cleavage of certain chemical bonds of a substrate by addition of a component of water (H or OH) to each of the products. Many agrochemicals containing amide, carbamate bonds, or esters are subject to enzymatic hydrolysis. Hydrolysis by esterase and amidases from microorganisms can detoxify or activate the mechanism that can govern selectivity or resistance, and initiate or determine the rate of agrochemical biodegradation in environment. Some fungi and bacteria also excrete hydrolytic enzymes that act extracellularly on substrates, and thus agrochemical detoxification and degradation may occur without microbial uptake of the compound.

Esters including carbonyl, phosphoryl, and thionyl linkage are susceptible to hydrolysis by esterase, and to some extent by lipase and proteases. For example, hydrolysis of fenoxaprop-P-ethyl and diclofop-methyl were rapid in soils and this hydrolysis was more rapid in moist and nonsterile soils compared to that in dry or sterile soil (Smith and Aubin, 1990; Gyanor, 1992). Soil pH also affects the degradation pathway of fenoxaprop-P-ethyl. Under acidic conditions, the rate of deesterification was significantly lower than under neutral soil condition, however, the benzoxazoly-oxy-phenoxy ether linkage of fenoxaprop-P-ethyl was prone to non-enzymatic cleavage under acidic conditions (Zablotowicz et al., 2000).

Amide and substituted amide bonds are present in acylanilides, phenylurea, and carbamate agrochemicals. Propanil is the most widely

studied agrochemical with regard to amide hydrolysis, and aryl acylamidases have been characterized in diverse species of algae, bacteria, and fungi. 3,4-Dichloroaniline (DCA) is the major metabolite of propanil in soil (Bartha and Pramer, 1967; Bartha, 1968). Propanil was metabolized to DCA by many microorganisms, but p-chlorophenyl methyl carbamate substantially inhibited the hydrolysis (Kaufman et al., 1971), and carbaryl also inhibited aryl acylamidase activity for propanil hydrolysis in several bacterial strains (Hoagland and Zablotowicz, 1995; Hirase and Matsunaka, 1989). The substituted amide bonds of alachlor and metolachlor were hydrolyzed by certain fungus (Tiedje and hagdorn, 1975), and the benzyl C-N bond cleavage of propachlor was demonstrated (Villareal et al., 1991). However, little is known about its mechanism. The fungicide iprodione undergoes several potential amide hydrolysis reactions, and DCA was also obtained as a major metabolite in soils (Walker, 1987).

Carbamates have three major classes, methyl carbamates, phenylcarbamates, and thiocarbamates. Microbial hydrolysis is the major pathway for the initial breakdown of carbofuran, but little is known about the fate of the metabolites formed by this mechanism (Mateen et al., 1994). Mineralization of the carbonyl group of carbofuran occurs more extensively compared to mineralization of the ring structure (Parkin et al., 1991). The toxicity of aldicarb is greatly reduced when it is hydrolyzed to the oxime and nitrile derivatives (Goldman et al., 1990), but oxidation of aldicarb to the sulfone or sulfoxide yields compounds with similar or greater toxicity. Thiocarbamates have been developed as herbicides and fungicides. Repeated application of these compounds to soil led to the development of microbial population with accelerated thiocarbamate degradation capability.

Soils adapted to EPTC degradation also rapidly degraded a related thiocarbamate, vernolate. However, soils adapted to butylate did not rapidly degrade EPTC and vernolate (Wilson, 1984).

Hydrolysis of the degradation of organophosphate insecticides occurs via nucleophilic addition of water across the acid anhydride bond; thus the enzymes named parathion-hydrolases and phosphotriesterases are actually organophosphorus acid anhydrases (Lewis et al., 1988).

Nitrile groups are essential moieties in the herbicides, bromoxynil, cyanazine, and dichlobenil, and the fungicide chlorothalonil. In bacteria, the cyano group of bromoxynil can also be hydroxylated to the respective carboxylate by several species. For example, *Klebsiella pneumoniae* utilizes bromoxynil as a nitrogen source, rather than a carbon source, with 3,5-dibromo-4-hydroxybenzoate accumulating as an end-product (McBride, 1986).

1.1.2. Conjugation reactions

Metabolic systems protect organisms from toxic substances. Conjugation reactions, found in fungi, bacteria and plants, not only detoxify metabolic wastes, but also form structural molecules. Conjugation has been defined by Dorough (1976) as a metabolic process whereby endogenous and exogenous chemicals are converted to polar components facilitating their removal from sites of continuing metabolism. Generally, as enzymes involved in conjugation are not substrate-specific, they detoxify both exogenous and endogenous compounds. There are many differences among the types of conjugates found in plants and soil microbes. In plants, sugar and amino acid conjugates are formed, whereas, in nutrient-limited soil microbes,

sugars and amino acids are rarely available for conjugation. Microbes use different endogenous acyl conjugates from methanogenesis. Conjugates may be immobilized becoming biologically unavailable, or made more recalcitrant and lipophilic and accumulated in the food chain. Some conjugates of agrochemicals derived by microbes have different chemical properties than their counterparts in plants. Conjugation reaction occurs in fungi and bacteria as one of their many detoxification mechanisms. Microbial conjugation occurs outside when enzymes and substrates from the organism are available.

Conjugation is a metabolic process using existing enzymatic machinery to detoxify xenobiotics, but new enzymes are not synthesized. This type of metabolism is called co-metabolism. Natural substrates are converted to provide energy and a carbon source for primary metabolism, and simultaneously the xenobiotics are transformed into other compounds. The latter transformations do not provide nutrients or energy to the living organisms, however, the organisms conserve energy and resources by not building new pathways for the detoxification of different xenobiotics in which they are exposed. Some of the reactions such as glycosylation and amino acid conjugations are dominant in plants where glucose and amino acids are abundant, whereas, fungi and bacteria are heterotrophs and compete for nutrients in the soil environment where glucose and amino acids are metabolized as soon as they are available. Some microbes prevent toxic compound from entering the cells, for example, extracellular enzymes of fungi conjugate xylose to phenols. Some conjugates are further mineralized to their inorganic components by existing metabolic pathways and thus detoxified.

Although fungi are less abundant than bacterial in soil, fungi have developed metabolic pathways different from bacteria to acquire nutrients and metabolize xenobiotics to prevent exposure to toxic compounds. Most bacteria can completely degrade certain xenobiotics to inorganic compounds to produce energy; however, fungi introduce minor chemical changes to the original compound without subsequent metabolism. This type of fungal metabolism is termed biotransformation, a protective action preventing the accumulation of toxic substances in the organisms while conserving metabolic energy (Cerniglia, 1992).

The basidiomycete *Dichomitus squalens* produces extracelluar enzymes, peroxidase and lactase which depolymerize lignin into monomers for both carbon and nitrogen assimilation (Périé, 1996). The phenolic monomers of lignin are conjugated to a xylose molecule and then the xylosylated phenols become more water-soluble and less toxic to the fungal cells (Reddy et al., 1997). Chlorinated phenoxyacetic acid herbicides are transformed by *D. Squalens*. 2,4-D and 2,4,5-T are degraded to the carbon side chain and chlorinated phenol by cleavage of the ether linkage. The carbon side chain of the herbicide mineralized to CO_2, while the chlorinated phenol is xylosylated (Figure 1).

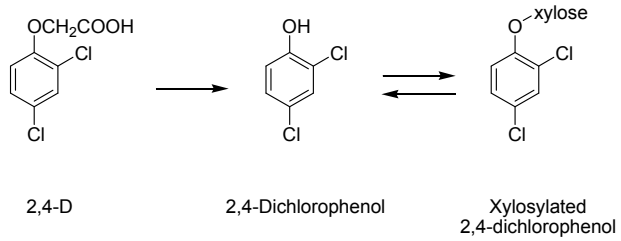
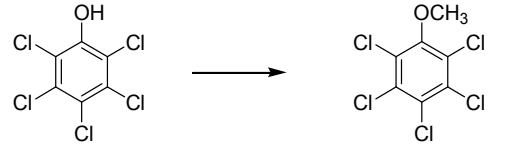


Figure 1. Xylosation of 2,4-D by *Dichomitus squalens*.

Methylation is another fungal conjugation. Pentachlorophenol was methylated to pentachloroanisole in fungal cultures of *Trichoderma virgatum* (Figure 2). Pentachloanisole is more resistant to further chemical and biological degradation but is less toxic towards various organisms than the parent (Reddy and Gold, 2000).



Pentachlorophenol Pentachloroanisole

Figure 2. *O*-Methylation of pentachlorophenol by fungal cultures of *Trichoderma Virgatum*.

Oxadiazon is transformated by *Fusarium solani*. The oxadiazoline heterocycle ring of this herbicide is first cleaved and the intermediate was methylated or decarboxylated before being removed from the aromatic moiety (Figure 3). Phenolic, carboxylic acid and dealkylated derivatives of oxadiazon were identified in soil, but there was no evidence of aromatic ring cleavage or the presence of dechlorinated products (Ambrosi, 1977). It was shown that degradation of the herbicide was a co-metabolic process (Chakraborty et al., 1995).

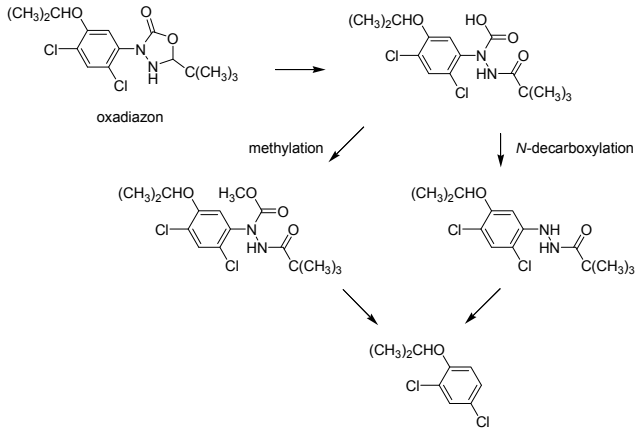


Figure 3. Metabolism of oxadiazon by *Fusarium solani*.

Agrochemicals can be acylated by fungi in a process called acylation. The acylation is observed with anilines and phenols in the soil (Kaufman and Kearney, 1976). Metobromuron is hydrolyzed quickly at the urea bridge to produce 4-bromoaniline in the soil, which is further acetylated to 4-bromoacetanilide by pure fungal cultures of *Talaromyces wortmanni* and *Fusarium oxysporum* (Figure 4). Chlorinated anilines are also conjugated to amino acids during their mineralization by *P. chrysosporium* (Sandermann et al, 1998).

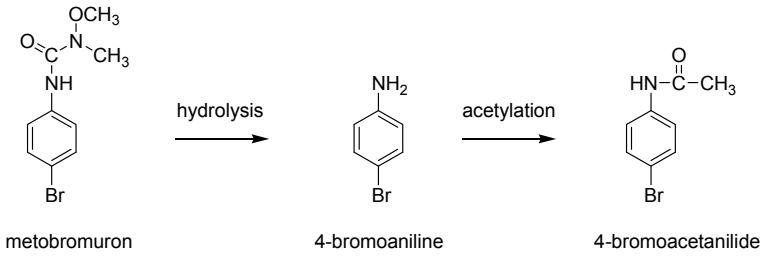


Figure 4. Biotransformation of metobromuron by microorganism.

In the various bacterial metabolisms, *O*-methylation is an environmentally important conjugation reaction of agrochemicals and this reaction increases their lipophilicity and, therefore, increases the potential for bioaccumulation of transformed products. Chloroanisoles are produced by soil bacteria incubated with chlorophenols or chlorophenoxyacetic acids, similar to fungal conjugation reactions. Several species of gram-negative bacteria belonging to the genera *Acinetobacter* and *Pseudomonas*, and gram-positive bacteria belonging to the genera *Rhodococcus* and *Mycobacterium* have been shown to *O*-methylate chlorinated phenols and phenol derivative (Häggblom, 1990). These organisms are widely distributed in nature and, therefore, play an important role of both the degradation and transformation of chlorophenols in the environment. The enzyme responsible for the methyl transfer has not been characterized. However, a wide range for halogenated phenols and phenol derivatives can be *O*-methylated indicating that the enzyme has a wide substrate range (Häggblom et al., 1988).

Nitrosation is another conjugation reaction observed in bacteria in which a nitrile is conjugated to a secondary amine to form introsamine. Most triazines in photosystem II inhibitors contain one or two secondary amine groups. In the soil, nitrate serves as electron acceptor during nitrification and is reduced to nitrite by nitrifying bacteria at alkaline pH in soil. The nitrites formed can conjugate to secondary amine groups of the herbicide and produce the nitrosoamine.

Glutathione (GSH) was first found in cyanobacteria and purple bacteria. This finding led to the hypothesis that GSH was produced to protect the cells from oxygen stress. Glutathione-S-transferases (GSTs), which are also

found in plants and animals, are one of the most extensively studied glutathione-dependent enzymes in microorganisms. Bacterial GSTs have been shown to detoxify various xenobiotics through conjugation with GSH (Feng, 1991). The herbicides of two different families, chloroacetamides and diphenyl ethers, are conjugated with GSH, and the herbicide-GSH conjugates are subsequently metabolized to cysteine conjugates, which are transformed to the corresponding thiols, pyruvates, and ammonia by cysteine β-lyase (Vuilleumier, 1997). In addition, soil bacteria are able to oxidize thiols to sulfonates. Sulfonated metabolites of alachlor and metolachlor have been detected and identified in groundwater and soil (Stamper, 1998).

1.1.3. Formation of bound residues

The term of free residue means that the residual chemicals can be readily extracted from soil without altering their chemical structures, whereas bound residue (nonextractable) is resistant to such extraction. However, the distinction between these two fractions is not always clear, because while they are in soil, even the free or extractable residues are not entirely free from any form of binding. On the other hands, at any time after the chemical enters the soil, the extractable fraction may be sorbed to the soil solid phases and, therefore, show reduced bioavailability and degradation (Alexander, 1994). In other word, free residues may exhibit, to some extent, the properties that traditionally are attributed to bound xenobiotics. It is, therefore, important to bear in mind that extractability of a compound will be defined by the nature of the extractant and the experimental conditions under which an extraction is carried out. Many types of extraction

procedures, such as supercritical fluid extraction (Capriel et at., 1986; Robertson and Lester, 1994; Koskinen et al., 1995), high temperature distillation techniques (Khan and Hamilton, 1980; Khan, 1982b; Worobey and Webster, 1982; You and Bartha, 1982), microwave extraction (Nicollier and Donzel, 1944) and silylisation prior to extraction (Drozd, 1975; Haider et al., 1992, 1993; Dec et al., 1997) can perform differently than conventional Soxhlet procedures.

The definition of bound residues proposed by Roberts (1984), adopted by the International Union of Pure and Applied Chemistry (IUPAC) and generally accepted in the literature is “chemical species originating from agrochemicals, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues”.

The environmental significance of a bound residue, however, depends not on its non-extractability under laboratory test conditions, but on its bioavailability (Khan, 1982a; Calderbank, 1989). Khan (1982a) made a distinction between the bioavailable and non-available fractions of bound residues. The bioavailable bound residue is the fraction of a compound in soils which can be taken up by plants and/or soil-inhabiting animals, while the non-available fraction cannot.

Adsorption is probably the most important mode of interaction between soil and agrochemicals and controls the concentration of the agrochemicals in the soil liquid phase. Adsorption processes may vary from complete reversibility to total irreversibility. The extent of adsorption depends on the properties of soil and the compound, which include size, shape, configuration, molecular structure, chemical functions, solubility, polarity,

polarizability and charge distribution of interacting species, and the acid-base nature of the agrochemical molecule (Bailey and White, 1970; Senesi, 1992; Pignatello and Xing, 1996). Adsorption may be purely physical, as with van der Waals forces, or chemical in nature, as with electrostatic interactions. Chemical reactions between unaltered agrochemicals or their metabolites often lead to the formation of stable chemical linkages, resulting in an increase in the persistence of the residue in soil, while causing it to lose its chemical identity (Berry and Boyd, 1985; Calderbank, 1989; Bollag, 1992; Dec and Bollag, 1997).

Compounds and their metabolites adsorbed by ionic bonding, or cation exchange, exist either in the cationic form in solution or can be protonated and become cationic. Ionic bonding involves ionized, or easily ionizable, carboxylic and phenolic hydroxyl groups of humic substances. Paraquat and diquat bind to soil humic substances by ion exchange via their cationic group. They form highly stable and unreactive bonds with the carboxyl groups of the humic substances. However, possible binding sites on the humic complexes are not utilized due to steric hindrance effects (Senesi, 1993).

The effect of pH on binding has been reported for less basic agrochemicals such as the triazine herbicides, amitrole (Senesi et al., 1986), and dimefox (Grice et al., 1973), which become cationic depending on their basicity and the pH of the system, and also governs the degree of ionization of acidic groups of the humic substances. The study of s-triazine showed that maximum adsorption of basic compounds occurs at pH values close to their pKa value.

Humic substances, with numerous oxygen- and hydroxyl-containing functional groups form hydrogen bonds with complimentary groups on agrochemical molecules. Agrochemical molecules compete with water for these binding sites. Hydrogen bonding is suggested to play a vital role in the adsorption of several non-ionic polar agrochemicals, including substituted ureas and phenylcarbamates (Senesi and Testini, 1980). Acidic and anionic agrochemicals, such as the phenoxyacetic acids (2,4-D and 2,4,5-T) and esters, asulam and dicamba, can interact with soil organic matter by H-bonding at pH values below their pKa in non-ionized forms through their -COOH, -COOR and identical groups (Senesi et al., 1984).

Van der Waals forces consist of weak short-range dipolar or induced-dipolar attractions that exist, in addition to stronger binding force, in all adsorbent-adsorbate interactions. Interactions between non-ionic and non-polar agrochemicals on suitable humic acid molecules are of particular relevance. Since these forces are additive, their contribution increases with the size of the interacting molecules and with its capacity to adapt to the adsorbate surface (Senesi, 1992). Since van der Waals forces are known to decay rapidly with distance, their contribution to adsorption would be greatest for those ions which are in closest contact with the surface, or enable close contact to be maintained with the adjacent adsorbed ions. Although there is paucity of experimental evidence, the involvement of these binding forces has been observed for a large number of compounds, including bipyridilium cations (Burns et al., 1973), cabaryl and parathion (Lenheer and Aldrichs, 1971), benzonitrile and DDT (Pierce et al., 1971), and has been shown to be the major adsorption mechanism for picloram and 2,4-D (Khan, 1973; Kozak, 1983).

Adsorption by ligand exchange involves the replacement of relatively weak ligands, e.g., H_2O partially holding polyvalent cations associated with soil organic matter by suitable adsorbent molecules such as s-triazines and anionic agrochemicals (Nearpass, 1976; Senesi, 1992).

Humic substances contain within their structure both electron-deficient moieties, such as quinines, and electron-rich centers, such as diphenols. Charge transfer complexes are formed via electron donor-acceptor mechanisms, with agrochemicals possessing, alternatively, electron donor or electron acceptor properties. The charge transfer interaction between humics and the agrochemicals can result in an increase in the free radical concentration relative to the unreacted humic acid, due to single-electron donor-acceptor mechanisms.

Hydrophobic retention needs not be an active adsorption mechanism, but can also be regarded as a partitioning between a solvent and a non-specific surface. The partitioning theory, which treats soil organic matter as a water-immiscible liquid phase, has been uses to explain the hydrophobic interaction between agrochemicals and soils (Karickhoff, 1981). This means that humic substances both in the solid- and dissolved-phase are treated as a non-aqueous solvent into which the organic agrochemical can partition from water (Chiou et al., 1986). However, soil organic matter is a solid phase with pH-dependant functional groups and a matrix of internal and external hydrophobic surfaces. Hydrophobic adsorption by soil organic matter and humic substances is suggested as an important mechanism for DDT and other organochlorine insecticides (Lenheer and Aldrichs, 1971), oxadiazinon, butralin, methazole (Carringer et al., 1975), metolachlor (Kozak, 1983), picloram and dicamba (Khan, 1973) and it is considered a possible

interaction mechanism for the s-triazine herbicides and polyureas (Khan and Mazurkevich, 1974).

The formation of covalent bonds between agrochemicals and/or their metabolites and soil humic substances, is often mediated by chemical, photochemical or enzymatic catalysts leading to stable, mostly irreversible incorporation into the soil. The agrochemicals which are most likely to bind covalently to soil humic matter have functionalities similar to the components of humus (Senesi, 1992). Thus, agrochemicals that structurally resemble phenolic compounds can covalently bind to humus. Oxidative coupling is one of the mechanisms that link humus together during humification. This is the process by which phenols, anilines and other compounds are linked together after oxidation by an enzyme or chemical agent. This results in the formation of C-C and C-O bonds between phenolic species and C-N and N-N between aromatic amines (Sjoblad and Bollag, 1977). Oxidative coupling reactions are mediated by a number of biotic and abiotic catalysts, including plant and microbial enzymes, inorganic chemicals, clay and soil extracts (Bollag and Myers, 1992; Dec and Bollag, 1997). Compound classes that can bind covalently to soil humic material without the intervention of microbial activity include acylanilides, phenylcarbamates, phenylureas, dinitroaniline herbicides, nitroaniline fungicides and organophosphate insecticides, such as parathion and methylparathion. They bind by two possible mechanisms involving carbonyl, quinone and carboxyl groups of humic substances leading to hydrolysable and non-hydrolysable bound forms (Parris, 1980).

Non-polar and hydrophobic compounds can undergo sequestration during prolonged residence or ageing in soils. Sequestration is closely related to

sorption phenomena. Pignatello and Xing (1996) in their review of sorption mechanisms referred to sequestration as slow sorption. Sequestered chemicals can be extracted from soil with organic solvents, although the extraction procedures require protracted extraction times compared with other adsorption mechanisms (Dec and Bollag, 1997). Another difference between the two processes is their kinetics. Adsorption is known to occur within a few minutes following addition of the chemical to soil, whereas sequestration may take much longer to become important. Sorption and sequestration can be viewed as one process, which is initially fast and then shifts to a slower sustained rate (Huang et al., 1996; Pignatello and Xing, 1996). This notion does not imply that the slow phase is dependent on the initial fast sorption and will not occur unless the latter is completed. Although sequestration is a form of sorption, there is no chemical or physical barrier that will prevent the two occurring simultaneously and having independent time courses (Dec and Bollag, 1997). Adsorption processes, which are involved predominantly in the initial phase of overall sorption, are generally ascribed to H-bonding, van der Waals forces, electrostatic attraction, and coordination reactions, i.e. to mechanisms that are expected to occur instantaneously upon contact of agrochemical molecules with the active surfaces of soil matrix. Diffusion phenomena, on the other hand, combined with sorption in remote microsites within the soil matrix, appear to be the domain of ageing and sequestration (Alexander, 1995).

1.2. Metabolism reactions of agrochemicals in animals

Generally, the metabolism of xenobiotics including agrochemicals occurs in two phases. Phase I involves predominantly oxidation, reduction, and hydrolysis and serves to introduce a polar group into the molecule. Phase II, consisting primarily of conjugation reactions, involves the combination of the products of phase I reactions with one of several endogenous molecules to form water-soluble, and hence excretable, products.

Chemical reactions reported to occur in the metabolism of agrochemicals are summarized in Table 2 (Hodgson, 2012). It should be noted that biotransformation reactions of agrochemicals may be either activations or detoxifications. The metabolism of most agrochemicals involves a combination of several chemical reactions and products may breakdown in some instances. For example, formaldehyde formed in demethylation reactions may be incorporated into the one-carbon metabolic pool.

A number of phase I and II agrochemicals metabolizing enzymes exist mostly in the same organism and/or the same tissues in several polymorphic forms (Hodgson, 2010). The most important locus for the enzymes is liver, and there are a number of both phase I and II agrochemical metabolizing enzymes in vertebrates. In the past, microsomal cytochrome P450-dependent oxidations and reductions of agrochemicals are mostly emphasized, but other metabolizing enzymes have been found in mitochondria and in the cytosol of hepatocytes and other cells. Recently, the roles of other phase I enzymes, such as flavin-dependant monooxygenases (FMO), hydrolases, and epoxide hydrolases, in cooxidation during prostaglandin synthesis have been studied. Phase II conjugation reactions have also been emphasized as they were applied in agrochemical metabolism.

Table 2. Chemical reactions in agrochemical metabolism (Hodgson, 2012).

Oxidation

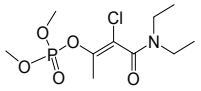
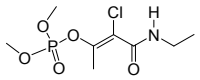
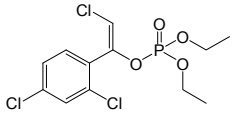
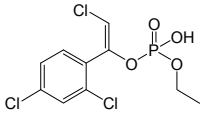
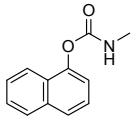
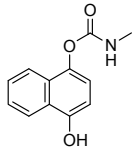
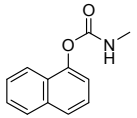
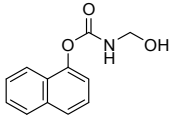
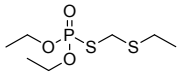
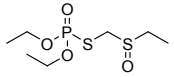
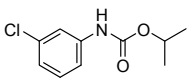
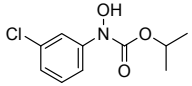
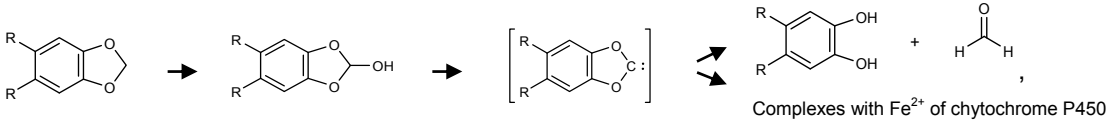
N-Dealkylation			+	CH_3CHO
	Phosphamidon	Desethylphosphamidon		Acetaldehyde
O-Dealkylation			+	CH_3CHO
	Chlorfenvinphos	Desmethyl chlorfenvinphos		Acetaldehyde
Ring hydroxylation (usually via arene oxide formation				
	Carbaryl	4-Hydroxyl-1-naphthyl *N*-methyl carbamate		
Side chain hydroxylation				
	Carbaryl	1-Naphthyl N-hydroxymethyl carbamate		
Sulfoxidation				
	Phorate	Phorate sulfoxide		
N-Oxidation				
	Chlorpropham	Hydroxy chlorpropham		

Table 2. Chemical reaction in agrochemical metabolism (continued).

N-Oxide formation

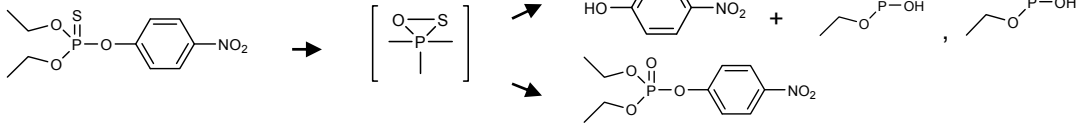
Nicotine Nicotine-1′-*N*-oxide

Methylenedioxy ring cleavage



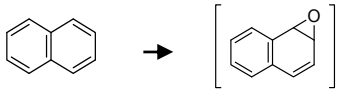
Complexes with Fe^{2+} of chytochrome P450

Desulfuration or dearylation



Epoxidation

Arene oxide formation



Naphthalene Naphthalene 1,2-epoxide

Stable epoxide formation

Heptachlor Heptachlor epoxide

Reduction

Reduction of nitro group

Parathion Aminoparathion

Table 2. Chemical reaction in agrochemical metabolism (continued).

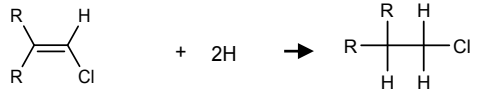
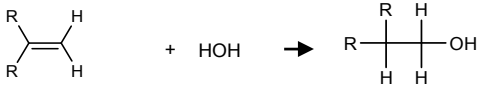
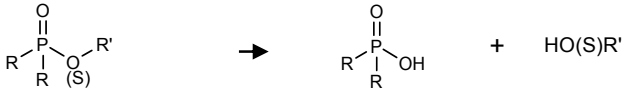
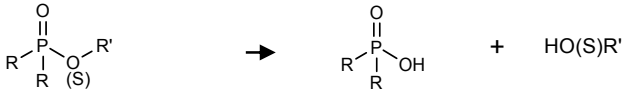
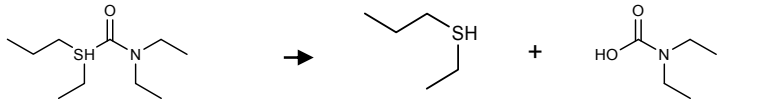
Dechlorination		DDT → DDD
Reduction of a double bond		DDMU + 2H → DDMS
Hydration of a double bond		DDNU + HOH → DDOH
Hydrolysis		
Phosphate ester hydrolysis		Most organic phosphorus esters → Acid + Alcohol
Amide cleavage		Most organic phosphorus esters → Acid + Alcohol
Thioester cleavage		Pebulate → Propyl mercaptide + Diethylcarbamic acid

Table 2. Chemical reaction in agrochemical metabolism (continued).

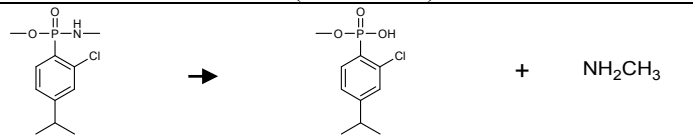
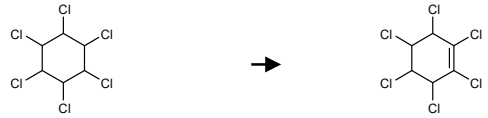
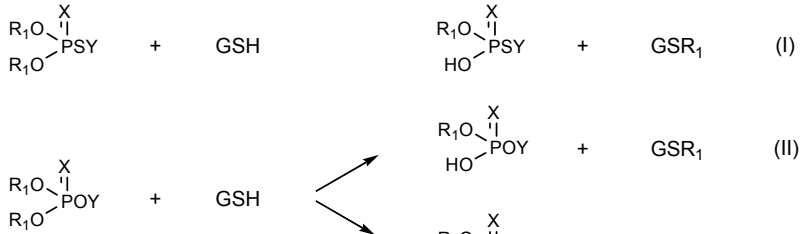
Deamination	 Rulene → Deaminomethyl Ruelne + NH_2CH_3 (Methylamine)
Desaturation	 1,2,3,4,5,6-Hexachlorocyclohexane → 1,2,3,4,5,6-Hexachlorocyclohex-1-ene
With glutathione	RX + $HSCH_2CHC(O)NHCH_2COOH$ (with $NHC(O)CH_2CH_2CH(NH_2)COOH$) ↓ glutathione *S*-transferase $RSCH_2CHC(O)NHCH_2COOH$ ($NHC(O)CH_2CH_2CH(NH_2)COOH$) ↓ γ-glutamyltranspeptidase $RSCH_2CHC(O)NHCH_2COOH$ (NH_2) + glutamate ↓ cysteinyl glycinase + glycine $RSCH_2CHCOOH$ (NH_2) ↓ *N*-acetyl transferase $RSCH_2CHCOOH$ ($NHC(O)CH_3$) Mercapturic acid

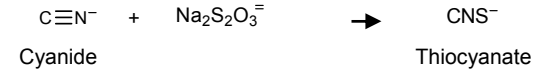
Table 2. Chemical reaction in agrochemical metabolism (continued).

With glutathione

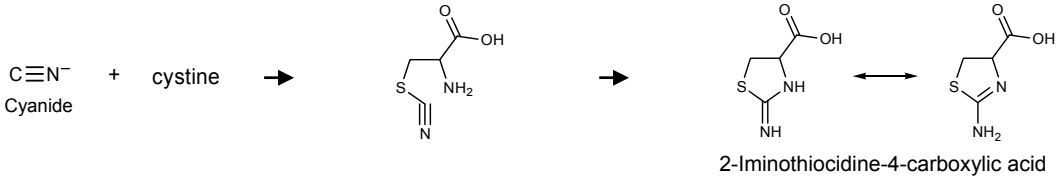


R_1 = alkyl; R_2 = aryl; X=S or O; Y = leaving group

With thiosulfate



With cystine



With acetate

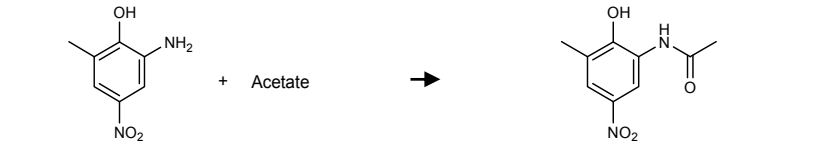
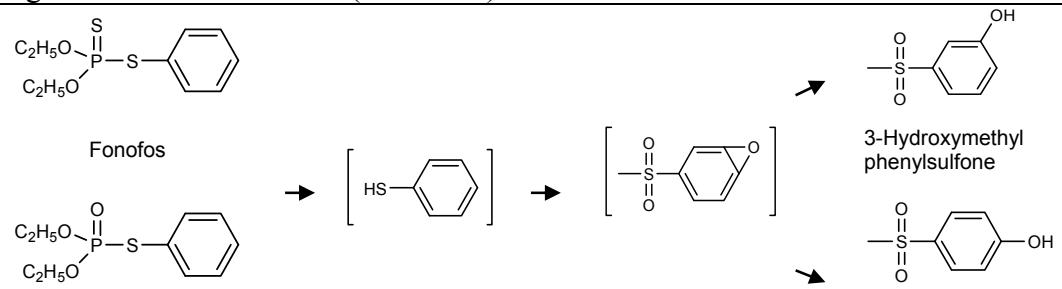


Table 2. Chemical reaction in agrochemical metabolism (continued).

Methylation	

The majority of studies focusing on agrochemical metabolism and the regulation of agrochemical metabolizing enzymes have been conducted in experimental animals, primarily rodents. However, there has been an increase in information about human enzymes, especially the CYP isoform. Much of this information has been gained through the use of specific substrates, antibodies, human hepatocytes, human cell fractions, and recombinant human enzymes. Studies with human CYPs have become more common and have demonstrated that agrochemical metabolism and the regulation and expression of agrochemical metabolizing enzymes may be quite different in human and experimental animals. Such differences make the extrapolation of metabolism studies from experimental animals to humans difficult. A direct research using a human system would only reveal the accurate fate of an agrochemical in human.

1.2.1. Metabolizing enzymes

A series of metabolism reaction of agrochemicals may be required in concert with several enzymes, but the initial reaction usually involves microsomal phase I enzyme catalyzing an oxidation reaction. Reduction reactions are relatively uncommon, although they may also occur. These enzymes include many isoforms of CYP, active in the CYP-dependent monooxygenase systems, as well as FMO isoforms. Many monooxygenation reactions of different agrochemicals are mediated by CYPs including *N*-dealkylation (e.g., alachlor, atrazine), *O*-dealkylation (e.g., chlorfenvinphos), *S*-oxidation (e.g., phorate), epoxidation (e.g., aldrin), and oxidative desulfuration (e.g., parathion) (Hodgson, 1982; Kulkarni and

Hodgson, 1980, 1984). One of the significant features of many microsomal CYPs is their inducibility by agrochemicals; thus, stimulation of metabolism of a chemical by prior administration of the same class chemistry is often regarded as presumptive evidence of its metabolism by microsomal enzymes. For example, phorate metabolism increased in mice pretreated with Phenobarbital, suggesting that CYP isoforms may be important in the metabolism of similar agrochemical substrates (Kinsler et al., 1990).

The microsomal FMO was known for many years as an amine oxidase, but was subsequently shown to be also a sulfur oxidase and a phosphorus oxidase. The FMO is also a microsomal enzyme like CYPs, a monooxygenase requiring NADPH and oxygen, and exists as multiple isoforms in various tissues. However, FMO, unlike CYP catalyzing only oxygenation reactions, has more specific substrate requirements, and is not known to be subjected to induction or inhibition by xenobiotics, apart from competitive inhibition by alternate substrates (Kulkarni and Hodgson, 1984; Ziegler, 1980). In agrochemical metabolism, the importance of FMO was established when it was discovered that the FMO oxidized a variety of thioether-containing agrochemicals (Cherrington et al., 1998a,b; Levi and Hodgson, 1992; Smyser et al., 1985; Tynes and Hodgson, 1985b). It has since been shown that the FMO is capable of oxidative desulfuration (oxon formation) of certain phosphonate insecticides, such as fonofos, through a mechanism distinct from that of oxon formation by CYPs (Smyser et al., 1985) as well as the oxidation of agrochemicals from a number of different chemical classes (Tynes and Hodgson, 1985a,b).

Certain alkene and arene having epoxide rings are hydrated by epoxide hydrolases to form the corresponding *trans*-dihydrodiol. The enzymes are a family known to exist both in the endoplasmic reticulum and in the cytosol. These enzymes are known to attack xenobiotics of many classes, including some agrochemical substrates, although these reactions are subsequent to the initial formation of epoxides, for example, 3,4- and 5,6-epoxides of tridiphane (Magdalou and Hammock, 1987).

Prostaglandins are synthesized in mammals via a reaction sequence starting with arachidonic acid as substrate. Xenobiotics can be co-oxidized to yield products similar to those formed by various isoforms of CYP during the second, or peroxidase, step of prostaglandin synthetase action (Eling et al., 1983; Hodgson et al., 2008; Marnett and Eling, 1983). These reactions may be important in extrahepatic tissues low in CYP and high in prostaglandin synthetase, such as the seminal vesicle and the inner portion of the medulla of the kidney. Many agrochemicals (e.g., aminocarb, parathion) have been shown to act as substrates.

Aldehyde oxidase is a molybdenum-containing oxidoreductase that is similar to xanthine oxidase, also a molybdoenzyme. Although it is clear that aldehyde oxidase plays a role in the metabolism of neonicotinoid insecticides, the extent involved in agrochemical metabolism is uncertain (Shi et al., 2009). The enzyme is cytosolic and appears to be expressed in most tissues to a relatively low extent with the exception of the liver. The enzyme oxidizes aldehydes to their corresponding carboxylic acids, preferring aromatic aldehydes and being relatively inactive toward aliphatic aldehydes such as acetaldehyde. In the presence of a reducing substrate,

aldehyde oxidase can catalyze reduction reaction such as azo-reduction and nitro-reduction.

Hydrolase and amidase are known to be important in the phase I metabolism of xenobiotics. For example, dimethoate is detoxified by amidase activity and the selectivity of malathion is due to the presence in mammals of carboxylesterases not widely distributed in insects. It appears that, in most cases, amidase and esterase activities are different activities of the same enzymes with one or the other activity predominating (Satoh, 1987).

Conjugation may be simple, as in case of phenol, but often they are more complicated processes in which the final product is derived by several steps. Despite this possible complexity, it is useful to think the conjugation of agrochemicals as occurring with glucuronic acid to form glucuronides, *N*-acetylcysteine to form mercapturic acids, glycine to form hippuric and related acids, sulfate to form ethereal sulfates, thiosulfate ions to form thiocyanate, and glutamine to form conjugates of the same name.

Glutathione conjugation is important in the metabolism of organophosphates (Motoyama and Dauterman, 1980) and the conjugated products of glutathione adducts may be further metabolized to mercapturic acids, the *N*-acetylcysteine derivative of the original xenobiotic substrate. While less important in the metabolism of agrochemicals than glucuronides, ethereal sulfates may be formed from carbosulfan and other carbamates (Dorough, 1968). Insect and plants are unusual in forming glucosides rather than glucuronides. With the exception of glutathione conjugation, most conjugation reactions involving agrochemicals are secondary, involving the

products of phase I reactions. They include glucoside formation, glucuronic acid formation, sulfate formation, and conjugation with amino acids.

Glucuronidation is one of the most important reactions for the elimination of xenobiotics including agrochemicals from the body, however, the studies with respect to agrochemicals have not been widely investigated. Glucuronidation involves the reaction of uridine 5′-diphosphoglucuronic acid with one of a number of possible functional group, such as R-OH, R-NH_2, R-COOH, and others (Hodgson and Rose, 2010). Glucuronides are important in the metabolism of carbamates such as banol, carbaryl, and carbofuran (Mehendale and Dorough, 1972) as well as some organophosphate compounds and other chemicals (Hutson, 1981).

Sulfation and sulfate conjugate hydrolysis, catalyzed by various members of the sulfotransferases (SULT) and sulfatase enzyme superfamilies, may play a role in the metabolism and disposition of many xenobiotics including agrochemicals. Reactions of the SULT with various xenobiotics generally produce water-soluble sulfate ester, which are then eliminated. SULTs catalyze the sulfation reaction while the sulfatases catalyze the hydrolysis of the sulfate esters formed by the action of the SULTS. Although not important in agrochemical metabolism, ethereal sulfates may be formed during the oxidative metabolism of organophosphorus agrochemicals (Abel et al., 2004; Choi et al., 2006; Motoyama and Dauterman, 1980) and halogenated herbicides such as the chloroacetanilides and chloro-S-Triazins (Abel et al., 2004; Cho and Kong, 2007) as well as molinate.

Methyltransferase reactions include N-, O-, and S-methylation, and the substrates for these enzymes may be either xenobiotics or endogenous

metabolites. For almost all methylation reactions the methyl donor is *S*-adenosylmethionine formed from methionine and ATP. Cysteine conjugate β-lyase uses cysteine conjugates as substrates, releasing the thiol derivative of the original xenobiotic, ammonia, and pyruvate, the thiol derivative then undergoing S-methylation to give rise to the methylthio derivative.

Acylation reactions are of two types. The first involves transfer of an acetyl group by acetyl-CoA, and the second involves activation of the xenobiotic and subsequent reaction with an amino acid. Deacetylation may also occur. These phase II reactions and enzymes have not been widely studied with respect to agrochemicals. Phosphorylation is not a common reaction in xenobiotic metabolism and, to date, has been described in insects. The enzyme from the cockroach requires magnesium and utilizes ATP. It is known to phosphorylate 1-naphthol, a metabolite of carbaryl, and p-nitrophenol, a metabolite of parathion and methyl parathion.

1.2.2. Biotransformation in extrahepatic tissues

The liver is generally more important than other organs in the biotransformation of xenobiotics, including agrochemicals. However, other organs and tissues may be active to some degree. For example, it was shown early that DDT is degraded by rat diaphragm, kidney, and brain in vitro (Judah, 1949).

The lung is a primary site of exposure to airborne as well as blood-borne environmental pollutants, such as agrochemicals, and for this reason it is a target organ for many chemically induced toxicities (Bond, 1983; Dahl and Lewis, 1993; Ding and Kamienski, 2003). The lung has a full complement

of metabolism enzymes so that it can activate and deactivate agrochemicals. For example, parathion is metabolized to paraoxon and diethylphosphorothioic acid by rabbit lung at about 20% or the rate in liver (Neal, 1972). Several studies reported the importance of pulmonary CYP and FMO enzymes in agrochemical oxidation (Feng et al., 1990; Li et al., 1992). In the lung, FMO showed to play a more important role than CYP in the oxidation of certain agrochemicals (Kinsler et al., 1988; Tynes and Hodgson, 1985a,b). Other studies have shown the existence of an FMO form now known as FMO2 in the lung that is not present in the liver (Lawton et al., 1990; Tynes and Hodgson, 1983; Tynes et al., 1985; Venkatesh et al., 1992; Williams et al., 1984, 1985).

The nasal mucosa is the first tissues of contact for inhaled xenobiotics and agrochemicals. Few agrochemicals are known to cause toxic endpoints in the nasal tissues. However, alachlor was demonstrated to cause rare nasal carcinomas in rats. The putative metabolic product through to be responsible for its carcinogenicity was identified as diethylbenzoquinoneimine, which is produced only after extensive metabolism of alachlor, involving CYPs as well as an aryl amidase.

The skin is often the portal of entry for agrochemicals because it is the largest organ in the human body, is continuous over the surface area of the body, and is in direct contact with the environment. The skin is known to contain many xenobiotic-metabolizing enzymes (XMEs) found in liver. The metabolic capacity of skin for agrochemicals was shown early when slices of rabbit skin were shown to hydrolyze paraoxon. This metabolism may be an important defense mechanism because paraoxon and related compounds

is slowly absorbed (Fredriksson et al., 1961). The skin has been shown to have the capacity to metabolize a variety of agrochemicals, for example, carbaryl and parathion were metabolized by using the isolated perfused porcine skin flap (Chang et al., 1994).

The kidney may also be a site of toxicity from agrochemicals, because of the high blood flow, ability to concentrate chemicals, and the presence of renal XMEs. These toxic effects can be directly attributable to the presence and localization of specific forms of enzymes responsible for activation (Hu et al., 1993; Speerschneider and Dekant, 1987). Several studies have underlined the importance of renal oxidative enzymes, particularly FMO, in the agrochemical metabolism (Kinsler et al., 1988; Tynes and Hodgson, 1983). The renal FMO enzymes played a greater role in mircrosomal systems in the oxidation of several agrochemicals than renal CYP, suggesting an important role for FMO in the extrahepatic metabolism of toxicants. The sulfoxidation of fenthion and methiocarb was demonstrated by kidney FMO (Furnes and Schlenk, 2005).

For xenobiotic metabolism in the gastrointestinal tract, some carbaryl is hydrolyzed and the resulting naphtol is conjugated with glucuronic acid by the intestine (Pekas and Paulson, 1970), and fenthion was sulfoxidated by FMO in the intestine (Furnes and Schlenk, 2005).

2. Soil metabolism study for agrochemicals

2.1. Experimental systems for soil metabolism study

Transformation pathways of agrochemicals in soil can be predicted from

studies with soil incubated under controlled laboratory conditions because field data are less easy to obtain than laboratory data and mass balance studies are difficult to establish under natural field conditions. The laboratory soil metabolism systems include soil perfusion apparatus (Audus, 1946; Lees and Quastel, 1946; Temple, 1951; Sperber and Sykes, 1964; Kimura and Yamaguch, 1978; Fung and Uren, 1997), biometer flask (Bull and Ivie, 1982; Chrzanowsk and Leith, 1982; Betts et al., 1976; Wolt et al., 1996; Wagner et al., 1996; Bartha and Pramer, 1965; Laskowski et al., 1983), flow-through system (Guth, 1981; Kenneth and Joel, 1987; Lee et al., 1989; Schneiders et al., 1993), and intergrated system (Best and Weber, 1974; Lichtenstein et al., 1974; Harvey and Reiser, 1973).

The soil perfusion systems are useful for microbial studies, but are far from natural soil conditions. Biometer flask systems have been commonly used to investigate metabolism of agrochemicals in soil. This system mainly is consisted of the treated soil and CO_2 trapping solutions. The major limitation of the system is the trend to anaerobicity during the study. The integrated systems have been developed for agrochemical transformation studies to introduce the complexity to the environment. Howerver, the systems are unable to simulate accurately environmental situations.

The flow-through system was found to be superior to the other systems. The system has many advantages: controlling gas flow, using various gases, trapping CO_2 and volatile products separately, and handling numerous samples at one time. These advanges make the system to be the most popoular system to the agrochemical metabolism studies despite the complexity and the great cost.

2.2. Soil metabolism study

Soil metabolism studies of agrochemicals in laboratory are very important for predicting the degradation behavior of the parent agrochemical and determining the nature and extent of metabolites, as well as for assessing the potential environmental hazards (OECD guidelines, No. 307, 2002). In soil metabolism studies, non-labeled or labeled test substance can be used to measure the rate of transformation. However, labeled substance is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-Labeled compound is recommended but the use of other isotopes including ^{13}C, ^{15}N, ^{3}H, and ^{32}P may also be informative. As using ^{14}C-labeled compound, the various mineralization rates of the test substace can be measured by trapping evolved $^{14}CO_2$ and a mass balance, including the formation of soil bound residues, can be established.

The position of the radiolabeled is critical for the purpose of the study. The label should be positioned in the most stable moiety of the molecule, for instance, the ring of aromatic or cyclic compounds. If certain molecule having two rings, the label is preferred to be positioned in both or each ring separately. Recoveries should range from 90% to 110% for labeled chemicals, and the limit of detection of the analytical method for the test substance and for the transformation products should be at least 0.01 mg/kg soil or 1% of applied dose whichever is lower. The purity of the test substance should be at least 95% (OECD guidelines, No. 307, 2002).

All test soils should be characterized for texture, pH, cation exchange capacity, organic carbon, bulk density, water retention characteristic, and microbial biomass. Detailed information on the history of the field site from

where the test soil is collected should be available. Details should include exact location, vegetation cover, treatments with chemicals, organic and inorganic fertilizers, and additions of biological materials or other contamination. If soils have been treated with the test substance or its structural analogue within the previous four years, these should not be used for transformation studies. Studies with soils freshly collected from the field are strongly preferred, but if the collected and processed soil has to be stored prior to the start storage conditions must be maintained at 4 ± 2°C for a maximum of three months. Before the start test, the soil should be preincubated to allow germination and removal of seeds, and to re-establish equilibrium of microbial metabolism following the change from sampling or storage conditions to incubation conditions. A preincubation period between 2 and 28 days approximating the temperature and moisture conditions of the actual test is generally adequate. Recent soil metabolism studies and DT_{50} values of agrochemicals are summarized in Table 3.

Table 3. Summary of recent soil metabolism studies for agrochemicals.

Agrochemical	Condition	DT_{50}	Year	Reference
Atrazine	Anaerobic	38 d	2001	Seybold et al.
Bifenox	Aerobic	Stable in soil	2000	Kwak et al.
Cabaryl	Aerobic Anaerobic	1.8-4.9 d 125-746 d	2004	Bondarenko and Gan
Chlorpyrifos	Aerobic Anaerobic	20.3-23.7 d 57.6-223 d	2004	Bondarenko and Gan
Diazinon	Aerobic Anaerobic	14.4-21.1 d 23.7-31.7 d	2004	Bondarenko and Gan
Dicamba	Aerobic	7.6-46.1 d	2007	Kah et al.
Diclosulam	Aerobic	28 d	2000	Yoder et al.
Ethaboxam	Aerobic	8-23 d	2003	Kim et al.
Fenpropimorph	Aerobic	7.9-62 d	2007	Kah et al.
Fluazifop-P	Aerobic	6.0-16.6 d	2007	Kah et al.
Flumioxazin	Aerobic	12.9-17.9 d	2003	Ferrell and Vencill
Flupyrsulfuron-methyl	Aerobic	7.5-21.7 d	2007	Kah et al.
Fluroxypyr	Aerobic	6.6-21.3 d	2007	Kah et al.
IPBC	Aerobic	2.13 h	2000	Juergensen et al.
LGC-42153	Aerobic Flooded	9.0 d 3.0 d	2003	Kim et al.
Malathion	Aerobic Anaerobic	0.8-1.4 d 1.6-2.3 d	2004	Bondarenko and Gan
Methiozolin	Aerobic	49 d	2013	Hwang et al.
Metolachlor	Anaerobic	62 d	2000	Seybold et al.
Metribuzin	Aerobic	9.3-49.4 d	2007	Kah et al.
Metsulfuron-methyl	Aerobic	23.8-175.9 d	2007	Kah et al.
Niclosamide	Flooded Anaerobic	4.91-5.39 d 0.65-2.79 d	2004	Graebing et al.
Paichongding	Aerobic	0.33-20.81 d	2013	Fu et al.
Penoxsulam	Flooded	2-13 d	2006	Jabusch and Tjeerdema
Primicarb	Aerobic	6.6-57.2 d	2007	Kah et al.
Pyribenzoxim	Flooded	1.3 d	2007	Chang et al.
Spinosad	Flooded	23-32 d	2002	Cleveland et al.
Terbutryn	Aerobic	7.1-56.8 d	2007	Kah et al.
Tetrabromobisphenol A	Flooded	20.8 d	2014	Sun et al.
Triadmefon	Aerobic Flooded	14.8-23.1 d 6.1-28.9 d	2005	Singh
Trifloxystrobin	Aerobic	13.4-16.2 d	2006	Banerjee et al.

3. Pharmacokinetics and metabolism study in animals

The study of the absorption, distribution, metabolism, and excretion (ADME) of chemicals is to describe the time course of the chemical in the body. These ADME studies are designed to determine the rate and extent of absorption of the compound, to examine the pattern of tissue distribution of the compound and/or its metabolites, to isolate, identify and quantify the metaboltes, and to determine the rate and route of excretion from the animal.

Basic pharmacokinetic parameters will provide information on the potential for accumulation of the test substance in blood, tissue and/or organs and the potential for induction of biotransformation as a result of exposure to the test substance. These data can be used to assess the adequacy and relevance of the extrapolation of animal toxicity data to human risk assessment. Metabolism data can also be used to assist in determining whether animal toxicity studies have adequately addressed any toxicity concerns arising from exposure to plant metabolites, and in the setting of tolerance, if any, for those metabolites in raw agricultural commodities.

3.1. Absorption

Absorption is translocation of an agrochemical from an external source of exposure to the bloodstream. Typically, agrochemicals are exposed to human by oral, dermal and inhalation routes. Once absorbed into the blood, the chemical is distributed through the body and delivered to tissues, where it may leave the blood and enter the cells of the tissues or it may remain in the blood and simply pass through the tissue. For the routes other than

intravenous administration, a chemical may be absorbed when it crosses the epithelial layers in the skin, small intestine, or alveoli in the lung, and enters the bloodstream from an external site of exposure.

From the site of absorption, agrochemicals are removed by continuous blood flow, maintaining a concentration gradient and enhancing continued absorption. Many lipophilic agrochemicals are thought to enter the systemic circulation at the dermis/epidermis interface in the skin and do not necessarily traverse full thickness of the dermis. For a rapidly absorbed chemical, an equilibrium may be established between the blood and the site of absorption, and the rate of entry in to the blood is limited by the blood flow rate rather than by diffusion across the membrane. In this case, an increase in blood flow increase the rate of absorption, absorption is said to be perfusion- (or blood-flow) limited. However, when absorption is not sensitive to blood flow, it is said to be diffusion-rate limited (Baynes and Riviere, 2012).

The skin is a complex tissue with a large surface area of which primary functions are to protect the body from physical or chemical damages, to thermoregulate, and to simultaneously prevent water loss from the body. Dermal absorption of any chemicals requires movement from the environment across this barrier, which is a biochemical milieu of complex lipids and proteins. Dermal absorption is dependent on the physicochemical properties of the agrochemical, the formulation, and the environmental conditions. Further, dermal absorption assessment is complicated by species, age, and sex differences and differences between anatomical sites within a

species. More importantly, dermal absorption in rodent skin is not always equivalent to that in human skin.

Occupational exposure to agrochemicals is more likely to occur by dermal contact. Dermal absorption is the possible route of entry in 65-85% of all cases of occupational exposure to agrochemicals (Galli and Marinovich, 1987). Spray and dusting can result in disposition of 20-1700 times the amount deposited in the respiratory tract (Feldmann and Maibach, 1974). These anecdotal case reports are often the only available human data with which to perform dermal absorption assessment. Despite such limited data, it is possible to estimate dermal absorption by extrapolation from dermal exposure data. Algebraic equations that take into account exposure time and the chemical nature of the compound (lipophilicity and molecular weight) have been presented for estimating dermal absorption (Cleek and Bunge, 1993; Potts and Guy, 1992).

People are potentially exposed to agrochemicals orally from agrochemical residues in foods such as meat, milk, fruits, and vegetables. The rate and extent of absorption after oral exposure depend on the ability of the chemical to cross the plasma membranes of the gastrointestinal tract (GIT). The rate and extent of absorption of weak organic acids and bases vary with location in the GIS; weak acids are nonionized and are absorbed in the stomach (pH 1-3), whereas weak bases are nonionized and are absorbed in the intestine (pH 6-8). Chemicals absorbed into the bloodstream from the GIS enter the portal circulation and are delivered to the liver, where they may be metabolized before reaching the systemic circulation. This first-pass metabolism decreases the systemic availability of the parent compound.

To be absorbed in the respiration tract, a chemical must be in the form of a gas, vapor, or particulate. Generally, the respiration system can be compartmentalized into the nasopharyngeal, tracheobronchial, and pulmonary regions (Kennedy and Valentine, 1994). Inhaled gases and vapors diffuse across cell membranes in the direction of the concentration gradient until an equilibrium is established. The ration of the gas or vapor equilibrium concentration in blood and air is termed the blood/air partition coefficient. Highly water soluble and reactive gases and vapors tend to be absorbed in the mucus layer of the upper respiratory tract, whereas more lipophilic and nonreactive gases and vapors are absorbed from the deeper regions of the respiratory tract.

3.2. Distribution

Once entered in the bloodstream, the chemical is available for distribution throughout and elimination from the body. Distribution of an agrochemical to and accumulation in the target organ may result in toxicity. Accumulation at nontarget sites, however results in storage of the chemical away from the site of action and ultimately protection from toxicity. The physicochemical characteristics of the agrochemical and the physiology of the organism are important factors in the distribution of absorbed of agrochemicals.

Agrochemicals in the body move throughout the water compartment of the body. The ability of chemicals to move between the various water compartments is limited by the physicochemical properties of the chemical. Total body water consists of plasma water, interstitial water, and intracellular water. Especially plasma water forms 53-58% of blood volume,

playing an essential role in the distribution of absorbed chemicals. For a chemical to move from blood (plasma water) into tissues, it must cross the endothelial cell layer lining the capillaries to enter the interstitial water and then cross the plasma membrane to enter the intracellular water. The capillary wall is permeable to small molecules, but not readily permeable to high molecular weight molecules such as plasma proteins. Only free chemicals that are small enough to pass through the capillaries are available to move from plasma water to interstitial water.

Factors influencing the rate and extent of distribution of a chemical to a particular tissue include the blood flow to the tissue, the mass of the tissue, the ability of the chemical to cross membranes, and the affinity of the chemical for the tissue relative to blood. The rate of distribution of a chemical from blood to tissues can be perfusion- or diffusion-rate limited. For lipophilic chemicals that rapidly cross membranes, the rate of delivery to tissues is limited by blood flow (perfusion-rate limited), whereas for polar and ionized chemicals that do not readily cross the plasma membrane, the rate is limited by diffusion (diffusion-rate limited).

Initial distribution is influenced primarily by blood flow to tissue, whereas final distribution is influenced primarily by the affinity of the chemicals for various tissues relative to blood (i.e. the tissue partition coefficient). In the early phase of distribution, tissues receiving a high blood flow (e.g. liver, kidney, and brain) may achieve high concentrations of the chemical even though the tissue partition coefficient for that chemical is low. Likewise, tissues that are slowly perfused may achieve a low concentration of the chemical in the early phase of distribution even though the tissue

partition coefficient for that chemical is very high. Later in the distribution phase, the chemical redistributed to tissues based on tissue partitioning coefficients, and the chemicals is more concentrated in tissues with relatively high partition coefficients. Agrochemicals and other xenobiotics do not have the same tissue partition coefficient for all tissues.

The tight junctions of the capillary endothelial cells and the surrounding glial cell processes are the main structural features that contribute to the low permeability of the blood-brain barrier. Chemicals that circulate in the blood must pass through the capillary endothelial cell membrane and the glial cell membrane to enter the interstitial fluid of brain. The low protein content of the brain's interstitial fluid limits lipophilic chemicals that are tightly bound to plasma proteins. Chemicals access to the brain, then, is limited to those species that are free, lipophilic, nonionized, and transported by specialized carrier system, whereas ionized and highly plasma protein bound chemicals are excluded by the blood-brain barrier. Another barrier to brain access is the presence of an adenosine 5′-triphosphate-dependent multidrug-resistance protein, which transports intracellular chemicals back into the extracellular space.

Chemicals may accumulate in body compartments because of protein binding, active transport processes, or high solubility in a particular tissue. These sites of accumulation can be considered storage depots. Because a chemical in any tissue compartment is in equilibrium with its free concentration in blood, storage is dynamic. Plasma protein binding plays a very important role in chemical-induced toxicity. If the bound chemical is very toxic, its displacement results in a higher free concentration in plasma,

which results in greater availability for distribution to its site of toxic action. Adipose tissue is a storage depot for a number of highly lipophilic chemicals. Storage in adipose tissue may be considered a protective mechanism, in that the agrochemical is stored in a nontarget tissue, thereby lowering its concentration at the site of toxic action. For example, chlordane (Ambrose et al., 1953), hexachlorobenzene isomers (Davidow and Frawley, 1951), aldrin (Ludwig et al., 1964), and dieldrin (Robinson et al., 1969) are lipophilic and accumulated in fat. Sequestration in tissues (e.g., kidney and liver) may be due to interaction of chemicals with tissue macromolecules such as proteins and nucleic acids, which influences the affinity of a tissue for a given chemical (tissue partition coefficient). Bone tissue is a potential storage depot for heavy metals.

3.3. Metabolism

Xenobiotics including agrochemicals undergo extensive metabolic transformation in living organisms through various metabolic reactions, phase I and II reactions as described above. Although many metabolic products are generally less toxic than the parent compound, more toxic metabolites also may be produced (Chambers et al., 1995; Hodgson and Levi, 1994). For instance, the moiety of P=S in parathion and malathion was metabolized to oxons by oxidative desulfuration. Not only does this reaction produce the oxons, cholinesterase inhibitors responsible for the neurotoxicity of these compounds, but it also release sulfur, a potent inhibitor of cytochrome P450 (Hodgson and Levi, 2001). Therefore, the phase I reactions may be either detoxications or activations. In phase II

reactions, the altered compounds by phase I or parent compounds conbine with and endogenous substrate to produce water soluble conjugation products that are readily excreted. Although conjugation reactions have been considered as detoxication reactions generally, a number of examples of conjugation products that were the ultimate toxicants have been reported (Dauterman, 1994). Threrefore, conjugations can no longer be considered strictly detoxication reactions.

Gut bacteria also are important in metabolism of xenobiotics because of their ability to metabolize chemicals. This may be important when a chemical is administered orally and the bacteria of the gastrointestinal tract convert the compound into metabolites that may be toxic or that may have different absorption characteristics. Scheline (1973) suggested that the role played in xenobiotic biotransformation by intestinal microflora in mammals is quantitatively equivalent to that of the liver. Qualitatively, digestive microorganisms mainly catalyze reductive and hydrolytic reactions, whereas oxidation and conjugation occur principally in liver (Hillenweck et al., 1997). For example, intestinal microflora would appear to play a key role in the biotransformation of chlorothalonil (Hillenweck et al., 1997), genistein (Coldham and Sauer, 2000) or propachlor (Bakke et al., 1980) in the rat. Alternatively the gut bacteria may be important in metabolizing compounds secreted in the bile and eliminated into the gastrointestinal tract. A specific metabolic activity of gut flora is the deconjugation of glucuronide, sulfate, and cystein conjugates (Scheline, 1973). This may result in cleavage of conjugates and reabsorption of the original compound. Metabolism studies with propachlor in bile duct cannulated rats and germfree rats have shown

that biliary secretion of the mercapturate of propachlor followed by metabolisms by the intestinal microflora β-lyase was necessary for the formation of the six methylsulfonyl-containing metabolites that were ultimately excreted in the urine (Bakke et al., 1980; Bakke et al., 1981).

Sex-related differences have been reported for the activity of some phenol sulfotransferases in rat liver (Shiraga et al., 1995). Large amount of phenol sulfotransferase 1 were found for both sexes while large amount of phenol sulfotransferase 2 were restricted to males. For instance, female rats metabolized a fungicide cyprodinil to monosulfate conjugate, while males formed both monosulfate and disulfate conjugate (Müller et al., 1999). The sex dimorphism in the conjugation reaction indicates the involvement of a sex-specific sulfotransferase that catalyzed the transfer of the second sulfate group. Tashiro et al. (1993) also reported the sulfate conjugation was more predominat in the females than males in the metabolism study of pyrachlofos using rats.

3.4. Excretion

Agrochemicals, considered toxicants in general, are taken up by the body in most cases because of their lipophilicity. In general, they are metabolized by phase I and II XMEs to conjugation products that are more polar and hence more hydrophilic than the parent compound, and then excreted primarily by either the renal or the hepatic route. The renal system eliminates molecules of molecular mass smaller than 400-500 Daltons, whereas the liver handles larger molecules (Baynes and Riviere, 2012).

The kidneys are primarily organs of excretion, and elimination by the kidney accounts for most byproducts of normal body metabolism, and are also primary organs for the excretion of polar xenobiotics and polar metabolites of lipophilic xenobiotics. Passive filtration of the blood plasma in the glomerulus, under the influence of the blood pressure generated by the heart, is the initial step in urine formation. All molecules small enough to pass through the glomerular pores (70-100 Å) appear in the ultrafiltration; however, any molecule larger than these pores or bound to molecules larger than these pores will not appear in the ultrafiltrate (Baynes and Riviere, 2012). Tubular reabsorption is the second major step in urine formation. Most of the reabsorption of solutes necessary for normal body function, such as amino acid, glucose, and salts, takes place in the proximal part of the tubule. This reabsorption may be active, as in the case of glucose, amino acids, and peptides, whereas chloride and other ions are passively reabsorbed. Reabsorption of xenobiotics is usually passive and controlled by the same principles that regulate their passage across any membrane. That is, lipophilic compounds cross cell membranes more rapidly than polar compounds; hence, lipophilic toxicants will tend to be passively reabsorbed more than polar toxicants and elimination of polar toxicants and their polar metabolites will be facilitated.

Tubular secretion is also another important mechanism for excretion of solutes by the kidney. Secretion across the wall of the tubule is generally active, using two systems, one for the secretion of organic acids including conjugates, and the other for the secretion of organic bases. Unionized weak acids and based pass across the membrane into the lumen of the tubule and,

depending on the pH of the urine, one or the other may become inonized and unable to diffuse back across the lumen wall. Tubular secretion of organic anions has been known to be of importance in the excretion of certain agrochemicals (Pritchard and James, 1982).

Excretion by the liver, through the biliary system, has been known for a considerable time. Bile is secreted by the liver cells into the bile canaliculi, and then flows into the terminal branches of the bile duct, the hepatic duct, and the gallbladder. The contents of the gallbladder are discharged into the gut under the influence of hormones whose release is triggered by food ingestion. Secretion of xenobiotics or their metabolites into the bile is largely a function of molecular mass and may occur by passive diffusion or by active transport.

Enterohepatic circulation is an important aspect of biliary excretion. Nonpolar xenobiotics are normally oxidized and then conjugated. If the molecular mass of the conjugate is appropriate for biliary excretion, it enters the guts, where hydrolysis by intestinal microflora or gut conditions may occur. The compound, then being again in a less polar form, can be reabsorbed by the intestine and returned to the liver through portal circulation, and the process is repeated. Enterohepatic circulation thus increases the biological half-life and possibly adverse effects of toxicants, particularly to the liver.

Volatile toxicants such as ethanol or fumigants may be eliminated via the lungs, as may volatile metabolites, including acetone and carbon dioxide. Respiratory excretion is not known to be an important route for excretion of agrochemicals or their metabolites.

4. Methiozolin, a new herbicide

Methiozolin is a new turf herbicide developed by Moghu Research Center, and was first commercialized at 2010 in Korea. This molecule is in the isoxazoline chemical family. Rheinheimer et al. (1991) first reported the isoxazoline derivatives of 5-benzyloxymethyl-5-methyl-3-aryl-1,2-isoxazolines. These molecules are known to have the herbicidal activity and selectivity to rice with an unknown mechanism of action. Additionally a large number of the isoxazoline analogues were reported in which various substituents contain alky, alkoxy, allyl or benzene derivatives at the 3 position of isoxazoline ring and methyl or substituted benzyloxymethyl group at 5 position of isoxazoline ring (Nakatami et al., 2003; Yonega et al., 1997). Ryu et al. (2002) reported the analogues substituted with thiophene ring at 3 position of isoxazoline ring, and one of those analogues [5-(2,6-difluorobenzyl)oxymethyl-5-methyl-3-(3-methylthiophen-2-yl)-1,2-isoxazoline] exhibits an excellent herbicidal activity on barnyardgrass at pre- and postemergence stages with high safety to transplanted rice in greenhouse. Hwang et al. (2005) reported that the molecule completely controlled *Monochoria vaginalis*, *Rotala indica*, *Cyperus difformis*, and *Ludwigia prostrata* at a rate of 62.5-125 g ai/ha at 5 days after sowing, and barnyardgrass up to 4-leaf stage without phytotoxicity to transplanted rice. However this molecule as well as any other isoxazoline chemicals was not finally commercialized as a rice herbicide. In a later study, Koo and Hwang (2008) found that the molecule controls annual bluegrass (*Poa annua*) and large crabgrass (*Digitaria sanguinalis*) effectively with high safety to

various cool- and warm-season turfgrasses including creeping bentrgrass, Kentucky bluegrass, perennial ryegrass, zoysiagrass, and bermudagrass, thereafter coined the molecule ‘methiozolin’.

Methiozolin controlled only grass weeds selectively including barnyardgrass (*Echinochloa crus-galli*), yellow foxtail (*Setaria viridis*), fall panicum (*Panicum dichotomiflorum*) and large crabgrass at rates of 125-500 g ai/ha at preemergence application with safety to various crops including corn, rice, wheat, soybean and cotton in greenhouse conditions. For foliar application, methiozolin was safe to soybean, wheat and cotton, and high growth inhibition against grassy weeds as well as broadleaved weeds including velvetleaf (*Abutilon theophrasti*) and Indian jointvetch (*Aeschynomene indica*) at 500 g ai/ha (Koo and Hwang, 2008). In addition, the herbicide controlled annual bluegrass at various growth stages having GR_{50} values of 23, 52, 104, and 218 g ai/ha at preemergence, two-, four- and eight-leaf stage, respectively, and was safe on established zoysiagrass (*Zoysia japonica* Steud.), creeping bentgrass (*Agrostis palustris* Huds.), Kentucky bluegrass (*Poa pratensis* L.), and perennial ryegrass (*Lolium perenne* L.) in the greenhouse (Koo et al., 2014). Applied at early flowering stage, methiozolin suppressed over 80% of annual bluegrass seed head at 2000 g ai/ha in the greenhouse.

Methiozolin has been extensively studied on annual bluegrass control in creeping bentgrass putting greens. Methiozolin, applied at March 2009, reduced annual bluegrass cover by 77-80% at 2 months after treatment at 500 and 750 g ai/ha in creeping bentgrass putting greens; however, any injury on perennial ryegrass, Kentucky bluegrass, or tall fescue was not

observed (McNulty et al., 2010). Methiozolin also controlled annual bluegrass and roughstalk bluegrass >80% and 90%, respectively, at 3000 g ai/ha (McNulty and Askew, 2011). When applied twice at 2000 g ai/ha, methiozolin controlled 31 different annual bluegrass biotype including 28 perennial biotypes in a pot test, and there was no biotype showing tolerance to methiozolin (Kyung and Kaminski, 2012). McCullough et al. (2013) reported that efficacy of methiozolin could be influenced by application timing and temperature in turf. In growth chamber experiments, creeping bentgrass injury from methiozolin at 10°C was 2 and 4 times higher than at 20°C and 30°C, respectively, while annual bluegrass injury was similar across temperature. In the field experiments, sequential methiozolin applications totaling 3360 g ai/ha provide >90% annual bluegrass control at 8 weeks after initial treatment when treatments were initiated in February/March or May. Injury from methiozolin was only observed from February/March applications. As the results of [^{14}C]methiozolin translocation experiments, annual bluegrass and creeping bentgrass showed about 2 times more translocation to shoots from root-applied [^{14}C]methiozolin at 30/25°C (day/night) than 15/10 at 48 hours after treatment. In addition, Koo ea al. (2014) reported that methiozolin at 500 and 1000 g ai/ha proved 80-100% control annual bluegrass when applied in the fall with acceptable and temporary injury to creeping bentgrass, but about 60% control when applied in the spring with no bentgrass injury in the creeping bentgrass putting greens. This result was supportive that herbicidal activity of methiozolin could be affected by temperature and application timing.

When methiozolin was applied only to the foliage, almost no herbicidal activity appeared at 2000 g ai/ha, but when applied only to the soil, efficacy was equivalent to the foliar and soil application (over-top spray). When [^{14}C]methiozolin administered to the leaf, methiozolin was rapidly absorbed, reaching a maximum of approximately 60% of the applied 4 days after treatment, however, translocation to the upper leaf, lower leaves, and root were 1.8, 0.4 and 0.5% of the applied, respectively. By comparison, about 20% of [^{14}C]methiozolin applied translocated to the shoots 48 hours after administration when the herbicide administered hydrophonically to roots. This pattern suggests methiozolin is readily absorbed the root followed by acropetal translocation to the shoot. Therefore, efficacy of methiozolin was clearly dependant on herbicide reaching the soil (Koo et al., 2014).

The mechanism of action of methiozolin and the isoxazolines is not thoroughly understood and appears to be novel. Lee et al. (2007) reported that methiozolin inhibited biosynthesis of both cellulose and hemicelluloses fraction greatly. However, the herbicidal symptom of methiozolin indicated that its mode of action was different from that of inhibitors of cellulose synthesis, microtubule disrupter, or inhibitors of very-long-chain fatty acids (Koo et al., 2008). In a recent study, Grossman et al. (2011) suggested methiozolin might inhibit tyrosine aminotransferase (TAT), an enzyme in the biosynthesis of plastoquinone, in duckweed (*Lemna paucicostata* L.). This suggestion was based on hydroxyphenylpyruvate, the product of TAT, to duckweed, which nullified growth inhibition of methiozolin. They also showed that methiozolin inhibited a recombinant TAT of *A. thaliana*, but with a very high IC_{50} value (about 200 μM). In addition, they suggested that

other TAT isoenzymes in Arabidopsis or from other plants could be more sensitive and the primary *in vivo* target of methiozolin. Up to now, the mode of action of methiozolin might be directly or indirectly associated with cell wall biosynthesis and potentially plastoquinone biosynthesis in susceptible plants, but the primary site of herbicidal action is still unclear.

Toxicological and ecotoxicological properties of methiozolin indicate that methiozolin has very low toxicity to mammals and ecological animals (Table 3). However, little information is available on how methiozolin is absorbed or metabolized in mammals or ecological animals to interpret the low toxicity.

Table 4. Toxicological and ecotoxicological properties of methiozolin active ingredient.

Tests	Test system	Result
Acute oral	Rat	LD_{50} > 2,500 mg/kg b.w.
Acute dermal	Rat	LD_{50} > 2,500 mg/kg b.w.
Skin irritation	Rabbit	Non-irritant
Skin sensitization	Guinea pig	Non-sensitizer
Eye irritation	Rabbit	Mild irritant
Subchronic (13 weeks)	Rat	NOAEL 5,000 ppm
Reverse mutation assay		Negative
Chromosome aberration test		Negative
Micronucleus test	Mouse	Negative
Teratogenecity	Rat	NOAEL (F0) 1,000 mg/kg/d
		NOAEL (F1) 1,000 mg/kg/d
	Rabbit	NOAEL (F0) 1,000 mg/kg/d
		NOAEL (F1) 1,000 mg/kg/d
Fish acute	Medaka	LC_{50} (96 h) = 2.24 ppm
	Rainbow trout	LC_{50} (96 h) = 1.53 ppm
Invertebrate	Daphnia magna	LC_{50} (48 h) = 2.04 ppm
Algal growth inhibition	S. capricornuum	EC_{50} (72 h) = 2.88 ppm
Bee acute/contact	Apis mellifera	LD_{50} (48 h) = 100 μg/bee
Earthworm acute	Eisenia foetida	LC_{50} > 1,000 mg/kg soil
Avian oral	Japanese quail	NOAEL 2,000 mg/kg b.w.
Avian dietary	Japanese quail	NOAEL 5,000 ppm
Silkworm	Bombyx mori	LD_{50} > 1,000 ppm

5. The purposes of the present study

The purpose of the present study was to investigate soil metabolism of methiozolin in laboratory and its *in vivo* pharmacokinetics and metabolism in rats.

The soil metabolism study was conducted to investigate the biotic and abiotic metabolism behavior under aerobic and anaerobic conditions. The investigation included the mass balance, degradation pattern, formation pattern of metabolites, and identification of metabolites.

The pharmacokinetics and metabolism study of methiozolin in rats following oral administration was carried out *in vivo* with male Sprague-Dawley rats. The investigation included total mass balance, absorption, distribution to tissues, excretion kinetics, bile duct secretion, and metabolite identification.

The present studies are the first reports on soil metabolism and animal pharmacokinetics and metabolism of methiozolin. The results will provide basic information and comprehensive understandings of methiozolin to estimate the human, animal, and ecological and environmental risk.

PART I

Soil Metabolism of [^{14}C]Methiozolin under Aerobic and Anaerobic Conditions

I. Introduction

Methiozolin [5-(2,6-difluorobenzyl)oxymethyl-5-methyl-3-(3-methyl-thiophen-2-yl)-1,2-isoxazoline] is a novel turf herbicide developed by Moghu Research Center and registered in 2010 in Korea. This molecule was first invented as a rice herbicide candidate (Ryu et al., 2002), which controlled barnyardgrass (*Echinochloa* sp.) and several annual broadleaved and sedge weeds from 125 g/ha in a paddy condition while showing an excellent safety to transplanted rice up to 1.0 kg/ha (Hwang et al., 2005). Koo and Hwang (Koo and Hwang, 2008) reported that the molecule effectively controlled annual bluegrass (*Poa annua*) and large crabgrass (*Digitaria sanguinalis*) at pre- and post-emergence stages and was highly safe to various cool and warm season turfgrasses including creeping bentgrass, Kentucky bluegrass, perennial ryegrass, zoysiagrass, and bermudagrass. This herbicide greatly inhibited biosynthesis of both cellulose and hemicellulose fractions in corn roots (Lee et al., 2007), however, the morphological symptoms did not resemble those of known cell wall synthesis inhibitors such as dichlobenil (Koo et al., 2008). Up to now, mechanism of action of the molecule is not thoroughly understood and appears to have a new action mechanism.

Koo et al. (2010) reported that methiozolin had low mammalian and ecotoxicity. However, limited information on the environmental fate and metabolism is available until now. In this paper, we report soil metabolism of methiozolin under an aerobic and anaerobic conditions, describing the material balance, the degradation pattern, and formation of metabolites.

II. Materials and Methods

1. Chemicals

The radiolabeled test compounds, [benzyl-^{14}C]methiozolin and [isoxazole-^{14}C]methiozolin (Figure 5), were synthesized at Korea Radiochemicals Center (Suwon, Korea). The purity of the both radiochemicals was >99%, and specific activities were 4.55 and 6.59 MBq/mg for [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin, respectively. They were used without further purification. HPLC-grade acetone, acetonitrile, dichloromethane, and water were purchased from Duksan Co. (Ansan, Korea). All the other reagents and common chemicals were analytical grades and commercially available.

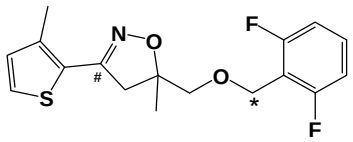


Figure 5. Chemical structure of methiozolin and ^{14}C-labeled position. [Benzyl-^{14}C]methiozolin (∗) and [isoxazole-^{14}C]methiozolin (#).

2. Test soil

A sandy clay loam soil was sampled from top 10 cm of a drained paddy field (582-4, Hakha-dong, Yuseong-gu, Daejeon, Korea). The soil was sieved to remove plant debris and rocky particles, and then air-dried at room temperature for 48 h for soil texture characterization (Table 4), and then stored at 4°C to maintain microbial activity prior to use (Rho, 1988; Nelson

and Sommers, 1996, Gee and Bauder, 1986). Soil and water sterilization was made by autoclaving for 20 min at 120°C (Rueppel et al., 1997).

Table 5. Characterization of the test soil.

Parameter	Soil
Particle size distribution (%)	
sand	53.75
silt	26.25
clay	20.00
texture class (USDA)	sandy clay loam
pH (1:5) in water	5.93
Water holding capacity (%)	38.1
Organic carbon (%)	1.82
Organic matter (%)	3.14
Cation exchange capacity (cmol+/kg)	10.6

3. Radioassay

Radioactivity of all liquid samples was measured by a liquid scintillation counter (LSC; Tri-Carb 2900TR, PerkinElmer, USA). Radioactivity in a gross amount of less than twice the background was considered to be below the limit of determination accuracy. Ultima Gold AB scintillation cocktail (4 mL) was used for the acidic aqueous samples and Insta-Gel Plus (4 mL) was used for the aquatic, organic samples, and CO_2 trapping agent. The nonextractable soil residue (0.2 g) was combusted by a sample oxidizer (Model 307, PerkinElmer, USA) after mixing with Combustaid (0.2 mL). The [^{14}C]carbon dioxide produced was absorbed in Carbo-Sorb E (5 mL) and mixed with PermaFluor E^+ (10 mL) scintillator for LSC counting. All scintillation cocktails and Combustaid used in this study were purchased

from PerkinElmer (USA). The efficiency of the oxidizer was determined using aliquots of the Spec-Chec-^{14}C check source for the automatic sample oxidizer, and was greater than 95%. Measurements of radioactivity were corrected by the oxidizer efficiency.

4. Chromatography

Identification of methiozolin and metabolites, and determination of radioactivity were performed using a PerkinElmer series 200 HPLC system (PerkinElmer, USA) equipped with a UV/vis detector and flow scintillation analyzer (Radiomatic 610TR, PerkinElmer, USA) by cochromatography with an authentic compound. The detection wavelength was 254 nm. A reverse phase C_{18} column (Cosmosil, 5 μm, 250 × 4.6 mm i.d., Nacalai tesque, Japan) was used. The elution solvents were acetonitrile and water (0.1% trifluoroacetic acid). A linear gradient (from 30% acetonitrile to 90% for 27 min) using a flow rate of 1.0 mL/min was adopted to separate the peaks of methiozolin and potential metabolites produced.

5. Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for LSC were calculated from the following expression by using Currie's equation (Currie. 1968). The LOD for radio-HPLC samples was calculated from the lowest peak of sample detected (dpm).

6. Extraction efficiency

To the air-dried soil samples (60 g), 14 mL of distilled water was added to

set the soil moisture at 60% field moisture capacity and to yield an aerobic condition. Alternatively, 44 mL of distilled water was added to flood the soil sample to yield an anaerobic condition. Either [benzyl-^{14}C] or [isoxazole-^{14}C]methiozolin stock solution was prepared at 200 μg/mL in acetone, then 150 μL of the stock solution containing 30 μg of [^{14}C]methiozolin was added to the soil sample. This methiozolin concentration in soil is equivalent to the field use rate (0.5 mg/kg soil). Following [^{14}C]methiozolin addition, the soil was thoroughly mixed. After 30 min, samples of the treated soil were extracted three times sequentially with acetone/water (7:3, v/v, 100 mL), acetone/water (1:1, v/v, 100 mL), and acetone/water/36% HCl (35:35:2, v/v/v, 100 mL). Triplicate aliquots (0.5 mL) of the soil extracts were analyzed by LSC to measure the radioactivity.

7. Soil incubation

For the aerobic condition, the nonsterile soil (60 g, air-dry weight) was weighed into a 100 mL incubation flask and added with 14 mL of distilled water. The sterilized soil was prepared by autoclave and was used for the sterile soil metabolism. For the anaerobic condition, 60 g of the nonsterile soil in the incubation flask was flooded with 44 mL of N_2 gas-purged and sterilized distilled water. The depth of water was maintained at approximately 2 cm (Chang, 2007). All the procedures were performed in a clean bench using a sterilized water to maintain sterility. All soil samples were kept in a flow-through metabolism chamber and preincubated at 25 ± 1°C for 2 weeks in the dark prior to [^{14}C]methiozolin treatment. Air or N_2 gas for the aerobic or anaerobic condition, respectively, was passed through

the system at a flow rate of 20 mL/min through a 0.5 N sodium hydroxide solution to remove carbon dioxide followed by distilled water to humidify. After preincubation, an aliquot of the [benzyl-^{14}C] or [isoxazole-^{14}C]methiozolin solutions was applied to the soil at a rate of 0.5 mg/kg as describe above. The soil samples treated were then again incubated in a flow-through metabolism system at 25 ± 1°C in the dark. The effluent gas from the chamber was passed through two XAD-2 resins (Supelco, USA) and two sodium hydroxide solution traps (0.5 N, 40 mL) in sequence to trap volatile compounds and [^{14}C]carbon dioxide, respectively (Figure 6). The treated soil was sampled at 0, 7, 14, 30, 60, 90, and 120 days after treatment (DAT) for the nonsterile soil under the aerobic and anaerobic conditions, and at 0, 7, 14, and 30 DAT for the sterile soil under the aerobic condition. Readjustment of the soil moisture content and collection of [^{14}C]carbon dioxide and volatile products were carried out every two weeks. Volatile products were extracted with 5 mL of methanol from resins by sonicating for 5 min at ambient temperature. Triplicate aliquots (1 to 4 mL) of the methanol extracts and sodium hydroxide solution from the traps were analyzed by LSC. Redox potential, pH, and dissolved oxygen (DO) were measured at all sampling dates in the anaerobic soil. The redox potential and pH were measured using a pH/mV/ORP meter (model 720A, Orion) equipped with a model 97-98 ORP probe and a model 91-57 pH electrode. The DO was measured using an oxygen meter (Multi 3401, WTW) equipped with a model cell ox 325 oxygen sensor.

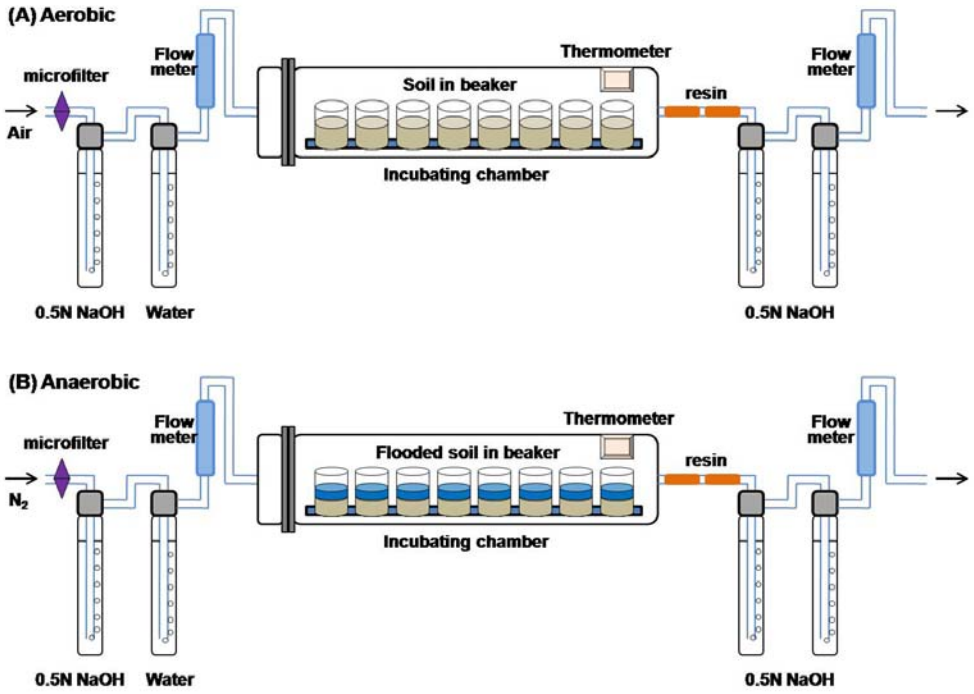


Figure 6. Diagrams and the picture of a flow-through soil metabolism system for the aerobic (A) and anaerobic conditions (B).

8. Extraction and analysis of soil

At each soil sampling date, three flasks per treatment were taken, and soil samples were extracted with acetone/water (7:3, v/v, 100 mL) by sonication (15 min) and shaking (30 min, 250 rpm). The extract was centrifuged at 5000 rpm for 15 min, and then the supernatant was taken and radioactivity was analyzed as described above. After acetone was removed under reduced pressure at 40°C, 20 mL of the saturated sodium chloride solution and 20 mL of the 2 N hydrochloric acid solution were added to the aqueous solution, which was then extracted twice with 100 mL of ethyl acetate. The ethyl acetate solution was concentrated in vacuo at 40°C and dissolved in 2 mL of acetonitrile. An aliquot of the solution was analyzed by radio-HPLC to determine the concentration of the parent and its metabolites.

After extraction with acetone/water (7:3, v/v), the remaining soil was then sequentially extracted with 100 mL of acetone/water (1:1, v/v) and 100 mL of acetone/water/36% HCl (35:35:2, v/v/v). In each procedure, the same extraction was repeated three times, and analysis was done by LSC. All the postextracted soil samples were air-dried, and then homogenized and weighed. Triplicate portions (0.2 g) were combusted with the oxidizer before LSC counting.

9. Distribution of nonextractable radioactivity in soil

The nonextractable radioactivity residue by the extraction solvent system was fractionated with a strong base and acid into three fractions of humin, humic acid, and fulvic acid (Kim et al., 1998; Schnitzer, 1978). Ten grams (dry weight equivalent) of the nonextractable soil residue were extracted

with 30 mL of 0.5 N sodium hydroxide solution by shaking for 24 h at room temperature. The extract was centrifuged at 5000 rpm for 15 min and the supernatant (fulvic and humic acid fraction) was collected. This procedure was repeated until the radioactivity of supernatant reached the background level, and all the supernatants were combined. Triplicate subsamples (0.2 g) of the precipitate (humin fraction) were combusted to determine the radioactivity content. The combined supernatant was added with concentrated hydrochloric acid to adjust the pH to 1.0 and maintained at ambient temperature for 24 h. The mixture was then centrifuged at 5000 rpm for 15 min, and the supernatant (fulvic acid fraction) was collected. The resulting precipitate (humic acid fraction) was washed with 10 mL of 1 N hydrochloric acid solution and centrifuged. The supernatants were combined with the fulvic acid fraction. The volume of the combined solution was measured and 1 mL of the solution was analyzed by LSC. The humic acid precipitate was redissolved in 20 mL of 0.5 N sodium hydroxide solution, the volume of the mixture was measured and then triplicate aliquots (1 mL) were analyzed by LSC.

10. Microorganism population assay

For the assay of microorganism population in the nonsterile soil under the aerobic and anaerobic conditions, triplicate soils (1 g) were taken from the soil sample at each sampling date, and then the soil was suspended in 9 mL of sterilized distilled water. An aliquot (100 μL) of the suspension was diluted 10^4 - 10^5-fold. Triplicates of aliquot (100 μL) were taken from the serial dilutions and placed on agar plates. The agar used in plate for

microorganism population counting was prepared by dissolving 8 g of nutrient broth agar and 15 g of bacto agar in 1 L of distilled water. The agar plates treated with the diluted soil extract were incubated for 3 days at 30°C (Han, 1988). The total number of colonies was recorded using dilution plate method. The dry weight of the soil samples was measured to calculate the number of microorganism per gram of soil dry-based.

11. Isolation of metabolite

Isolation and identification of metabolite from methiozolin was conducted in soil under the anaerobic condition but not in soil under the aerobic condition because major metabolite (10% of the applied) was not detected. Two gram of methiozolin was treated into the 1.5 kg soil under the anaerobic condition to produce metabolites as the same incubation method above. The soil was extracted using acetone/water solution (7:3, v/v) at 150 DAT, the extract was extracted with ethyl acetate twice, the solvent was evaporated in vacuo until complete dryness and dissolved by acetonitrile, and the solution was analyzed by radio-HPLC to determine the metabolite. The extracted metabolite was isolated using precoated silica gel 60 F_{254} chromatomatoplate (20 x 20 cm, 0.5 mm layer thickness, Merck, Germany). The solvent system was n-hexane/ethyl acetate (6:1, v/v) and development was conducted twice. Finally one purified metabolite was identified by nuclear magnetic resonance (NMR) spectrometer (Bruker, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C with acetone-D_6 (99.8%, Merck, Germany) as a solvent at room temperature. EI-MS spectrum was recorded on JMS-700 MS (JEOL, Japan).

12. Calculation of half-life

A pseudo-first-order kinetics model was used to calculate the half-life (Wolt et al. 1996; Han, 1988; Wang et al., 2009; Dictor et al., 2008). The rates of degradation of [^{14}C]methiozolin were calculated by non-linear regression analysis using SigmaPlot 10.0 software (SPSS Science, USA)

III. Results and Discussion

1. Test conditions and microorganism population

Anaerobic condition in water and soil were achieved within 14 days after flooding according to the water and soil redox potential profile (Figure 7, A). [^{14}C]Methiozolin was treated on day 14 after the microcosm soil became anaerobic. The redox potential throughout the study after the treatment ranged -54 to 13 and -86 to -50 mV in water and soil, respectively, and the ranges belong to the highly reduced and intense anaerobic condition (Wolfe et al., 1990). The dissolved oxygen ranged 0.01 to 0.52 mg/L (Figure 7, B) and pH was 7.9 ± 0.5 (Figure 7, C) over the course of the experiment.

The microorganism populations were 2.4 x 10^7, 3.0 x 10^7, and 1.5 x 10^6 cfu/g in the soil under the aerobic condition, repectively, and 1.0 x 10^5, 1.4 x 10^5, and 8.0 x 10^4 cfu/g, respectively, in the soil under the anaerobic condition at 0, 30, and 120 DAT. These results indicated that the soil microorganisms remained viable throughout the studies.

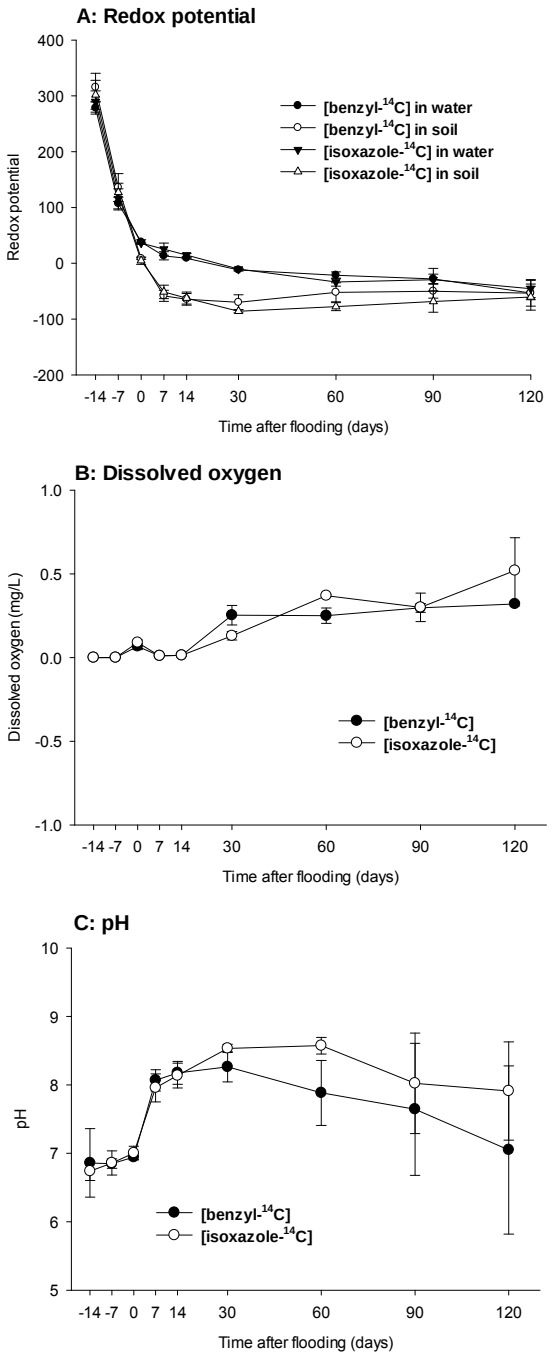


Figure 7. Redox potential (A), dissolved oxygen (B), and pH (C) in soil under the anaerobic condition. Days in negative scale are for preincubation period before treatment. Each data point represents the mean ± SD (n = 3).

2. LOD and LOQ

LOD and LOQ for LSC were 15.1 and 63.0 dpm, respectively, and these values were 0.0002 and 0.0005% of the applied radioactivity (AR), respectively. These results were calculated from untreated control samples of the test soil. The aliquot of the control sample extract was partitioned with ethylacetate, concentrated to dryness in vacuo, and dissolved with 2 ml of acetonitrile to calculate LOD and LOQ of radio-HPLC. The injection volume was 100 μL. LOD of the extract was 1066 dpm, and LOQ were 0.35 and 0.21 % AR for the aerobic and anaerobic soils.

3. Extraction efficiency

[^{14}C]Methiozolin was recovered from the test soil with a high yield (88.7-98.4%) using the acetone/water (7:3, v/v) extraction system under both the aerobic and anaerobic conditions (Table 5). A small amount (<4.2%) was detected in the acetone/water (1:1, v/v) extract. Although most of radioactivity was recovered in the acetone/water fraction, acetone/water/36% HCl (35:35:2, v/v/v) solution was used to extract metabolites, assuming they could be potentially more polar or acidic than the parent compound (Kim et al., 2003).

Table 6. Extractability of [^{14}C]methiozolin with different solvent systems.

Soil condition	Compound	Applied radioactivity (%)			
		Acetone:water (7:3)	Acetone:water (1:1)	Acetone:water:36% HCl (35:35:2)	Total
Aerobic	[Benzyl-^{14}C]	98.4 ± 0.32	4.2 ± 0.15	0.4 ± 0.02	103.0 ± 0.21
	[Isoxazole-^{14}C]	95.7 ± 0.13	4.2 ± 0.09	0.8 ± 0.04	100.8 ± 0.16
Anaerobic	[Benzyl-^{14}C]	88.7 ± 2.00	1.5 ± 0.05	2.2 ± 0.67	92.5 ± 1.35
	[Isoxazole-^{14}C]	91.6 ± 2.69	1.1 ± 0.27	2.4 ± 0.09	95.1 ± 3.04

4. Mass balance

4.1. Aerobic condition

Mass balance was calculated by determining the radioactivity recovered from the solvent extracts, [^{14}C]carbon dioxide, volatile compounds, and solvent nonextractable residues (Wang et al., 2007). The average mass balance throughout the study was 91.7-104.5% of applied radioactivity of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin under both the nonsterile and sterile soil conditions. In the nonsterile soil, the radioactivity of the solvent extract decreased with time and accounted for 21.8 and 18.4% of the applied radioactivity of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin at 120 DAT, respectively (Figure 8). [^{14}C]Carbon dioxide radioactivity levels from the soil steadily increased with time and accounted for 41.5% and 36.1% of the applied radioactivity of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin at 120 DAT, respectively. The large production of [^{14}C]carbon dioxide indicates that rapid and complete mineralization of methiozolin occurs by the aerobic soil microbes (Krueger et al., 1991; Li et al., 1999; Sukul et al., 2010; Anderson, 1984; Knauber et al., 2000). Volatile products from the soil were not detected throughout the study.

The nonextractable radioactivity levels in the nonsterile soil steadily increased as the soil aged, and accounted for 35.7% of [benzyl-^{14}C] and 39.8% of [isoxazole-^{14}C]methiozolin treated at 120 DAT, suggesting the binding of methiozolin or its degradation products to soil had occurred. Many pesticides are partially degraded and the metabolites are involved in the formation of nonextractable residues.

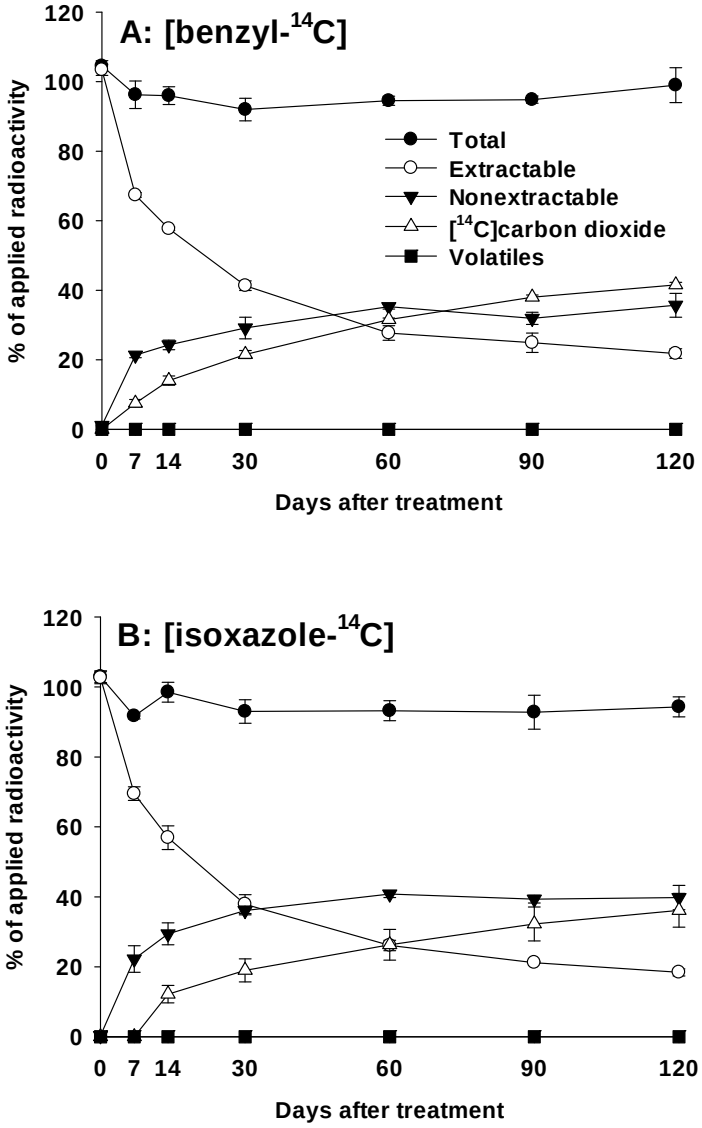


Figure 8. Distribution of radioactivity in the nonsterile soil treated with [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) under the aerobic condition. Each data point represents the mean ± SD (n = 3).

Based on these results, nonextractable formation occurred rapidly especially within the first 7 days, but whether methiozolin or its metabolites were involved in nonextractable residue formation, and which of those was more rapidly formed were unclear (Barriuso et al., 2008). However, the similar rate of formation of the bound residue in soils treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin suggests that the benzyl and isoxazole moiety might have similar binding affinity to the soil matrix, or the binding entity might have both the benzyl and isoxazole moieties.

In the sterile soil treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin, the average mass balances during incubation for 30 days were 97.5-99.1% and 99.1-101.2% of the applied radioactivity, respectively (Figure 9). Solvent-extractable radioactivity from the soil remained consistent (95.5-101.0%) throughout the study. A small amount (<2.5%) was detected in nonextractable radioactivity and [^{14}C]carbon dioxide radioactivity, and no volatiles were detected. These results suggest that aerobic microbial activity contributes to the formation of bound residues and mineralization of methiozolin.

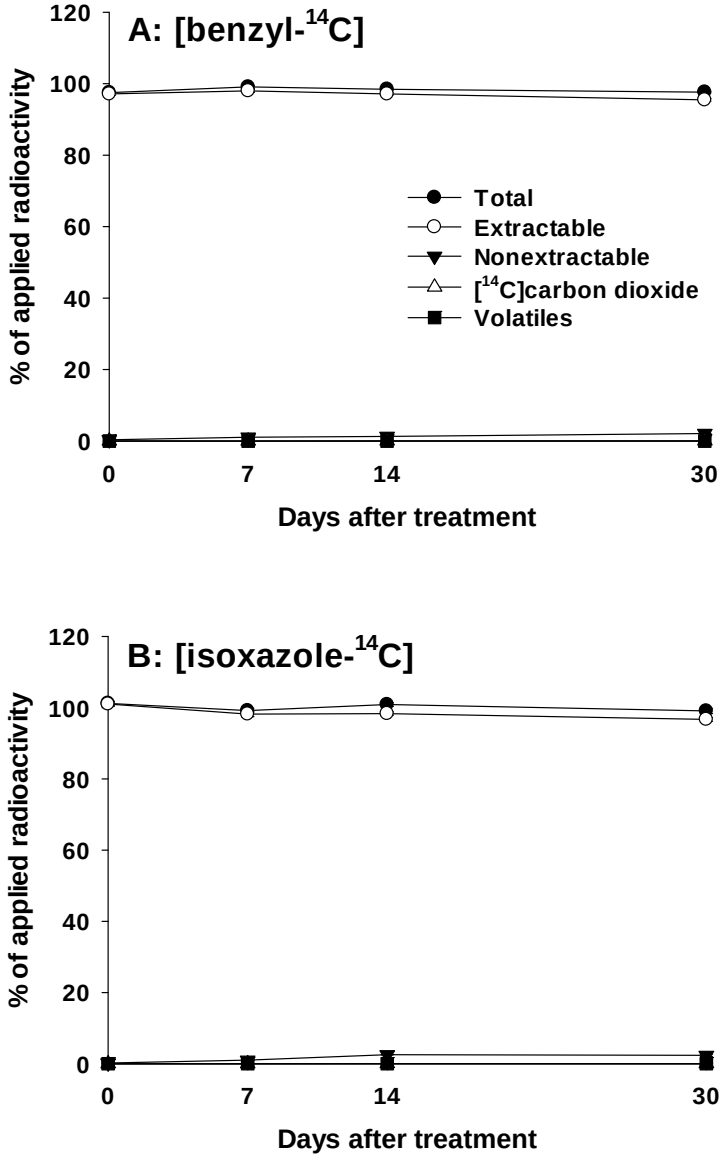


Figure 9. Distribution of radioactivity in the sterile soil treated with [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) under the aerobic condition. Each data point represents the mean ± SD (n = 3).

4.2. Anaerobic condition

In the nonsterile soil under the anaerobic condition, the average mass balances during the study were 93.2-102.5% of the applied radioactivity for both [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin (Figure 10). Solvent-extractable radioactivity levels decreased slightly with time and accounted for 80.8 and 81.1% of the applied radioactivity of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin at 120 DAT, respectively. The nonextractable radioactivity levels were 6.7-8.0% and 6.0-10.5% and [^{14}C]carbon dioxide radioactivity were 0.4-5.2% and 0.02-3.5% of the applied radioactivity of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin, respectively. No volatile products were detected. The results might be due to minimal activity of the soil microbes under an anaerobic condition (Kim et al., 1998).

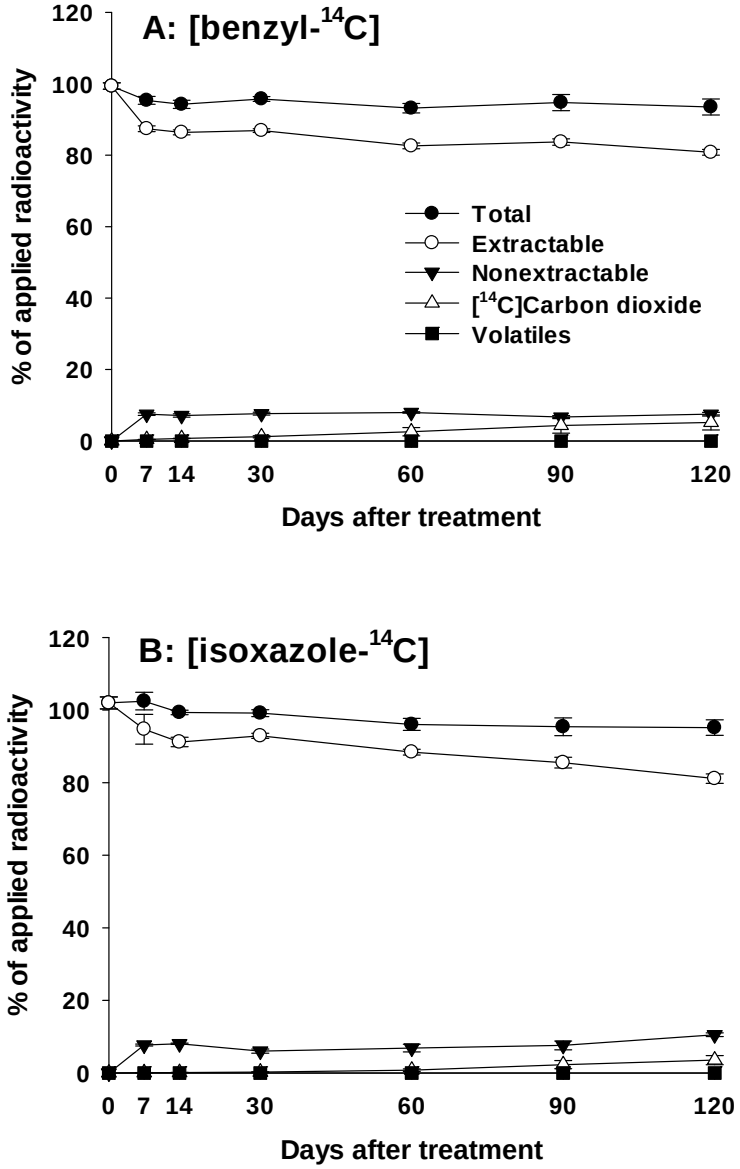


Figure 10. Distribution of radioactivity in the nonsterile soil treated with [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) under the anaerobic condition. Each data point represents the mean ± SD (n = 3).

5. Distribution of nonextractable radioactivity

Humic substance has function to bind with many organic compounds including agrochemicals (Parsons, 1998). To find out the distribution of radioactivity of the applied [^{14}C]methiozolin in the nonextractable soil residues, further fractionation into fulvic acid, humic acid and humin was performed. Of the radioactivity remaining in the nonsterile soil under the aerobic condition treated with [benzyl-^{14}C]methiozolin at 120 DAT, 12.4, 9.7, and 13.6% of the applied radioactivity were found in fulvic acid, humic acid, and humin fractions, respectively (Figure 11). For [isoxazole-^{14}C]methiozolin, 14.0, 9.2, and 16.6% of the applied were founded in fulvic acid, humic acid, and humin fractions, respectively. These results indicated that the bound residue was associated more with humin and fulvic acid fractions (Wang et al., 2007). The fractionation of the bound residues under the anaerobic condition was not conducted because only a small amount of radioactivity was detected.

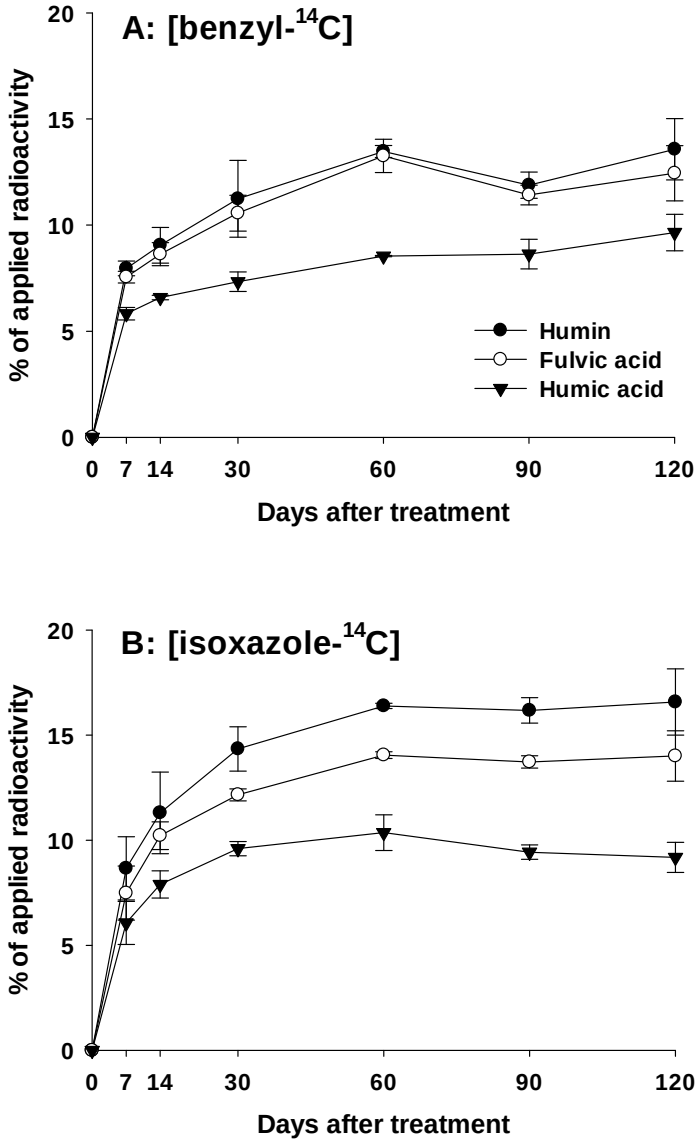


Figure 11. Distribution of nonextractable radioactivity in the nonsterile soil treated with [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) under the aerobic condition. Each data point represents the mean ± SD (n = 3).

6. Degradation of [^{14}C]methiozolin and formation of metabolites

Degradation of [^{14}C]methiozolin and formation of metabolites in the nonsterile and sterile soil under the aerobic, and anaerobic conditions were as shown in Figure 12-14, respectively. In the nonsterile soil under the aerobic condition, both [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin degraded rapidly until 30 DAT, and at that time, the amount of [^{14}C]methiozolin was 37.5 and 34.7% of the applied, respectively (Figure 12). After 30 DAT, [^{14}C]methiozolin degraded relatively slower than the first 30 days, and 17.9 and 15.9% of the applied were detected in the nonsterile soil treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin, respectively, at 120 DAT. The calculated half-lives in the soil were 51.7 and 46.8 days, respectively, for [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin. The similar degradation rate between [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin indicates that both the ^{14}C-labeled moieties have similar susceptibility to degradation by aerobic soil microbes. In the sterile soil under aerobic condition, no degradation of [^{14}C]methiozolin was observed untile 30 DAT (Figure 13). In the nonsterile soil under the anaerobic condition, <25% of applied [^{14}C]methiozolin was degraded until 120 DAT; therefore half-life could not be calculated (Figure 14). The results suggest that methiozolin degradation largely depends on the aerobic microbes, but not on abiotic degradation and anaerobic microbes in soil.

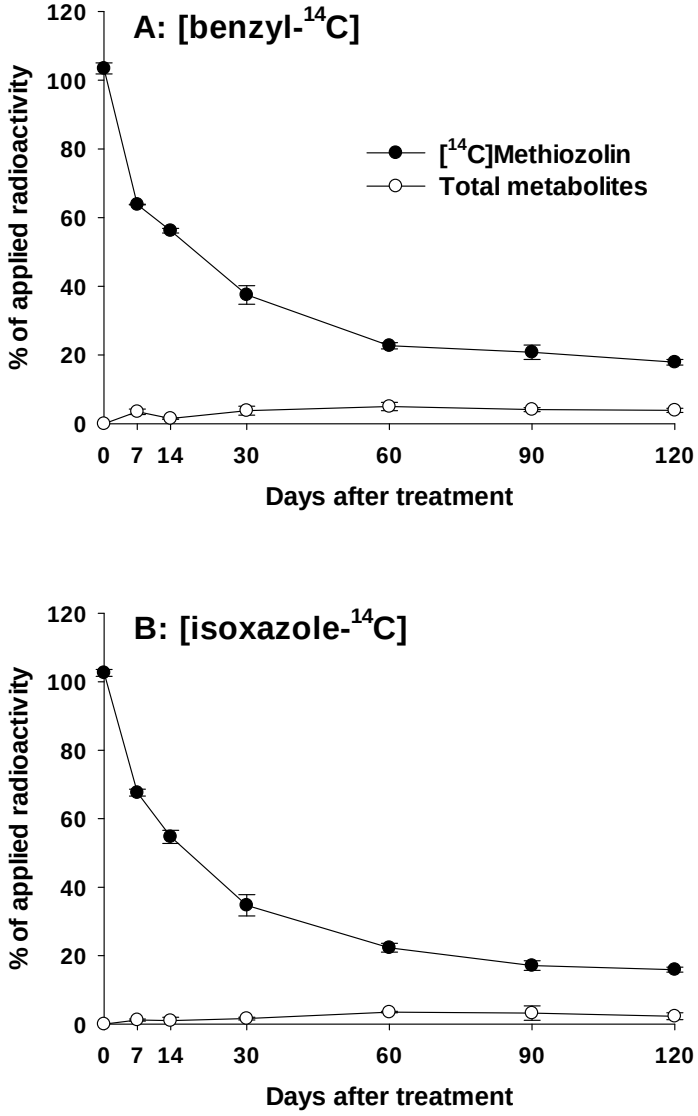


Figure 12. Degradation of [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) and formation of its metabolites in the nonsterile soil under the aerobic condition. Each data point represents the mean ± SD (n = 3).

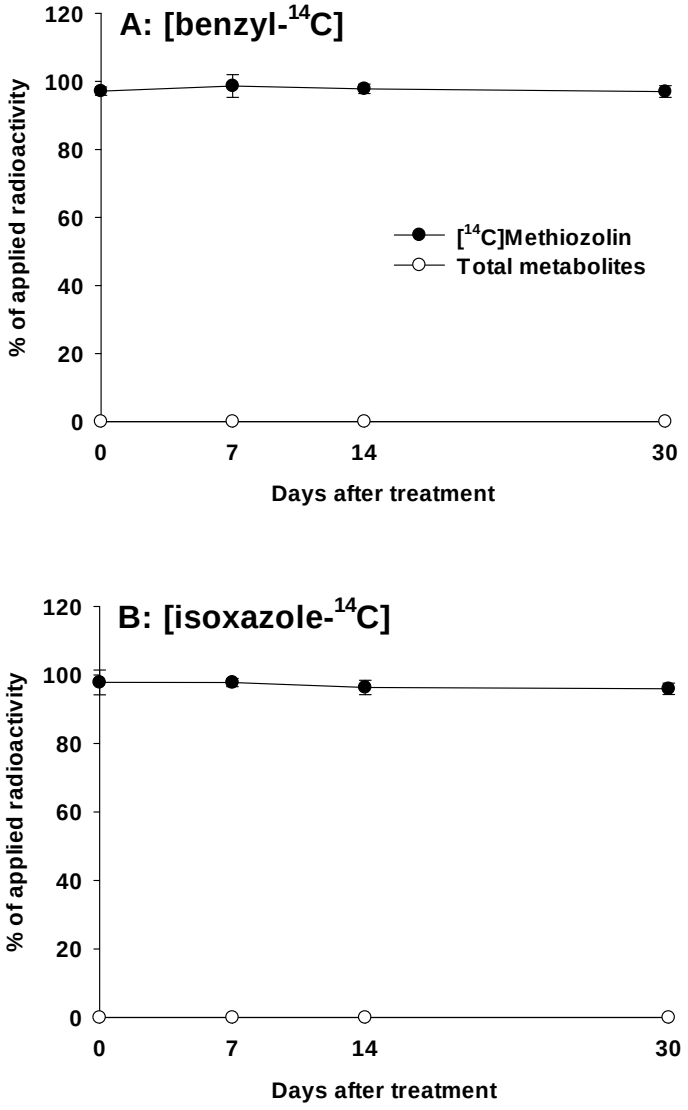


Figure 13. Degradation of [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) and formation of its metabolites in the sterile soil under the aerobic condition. Each data point represents the mean ± SD (n = 3).

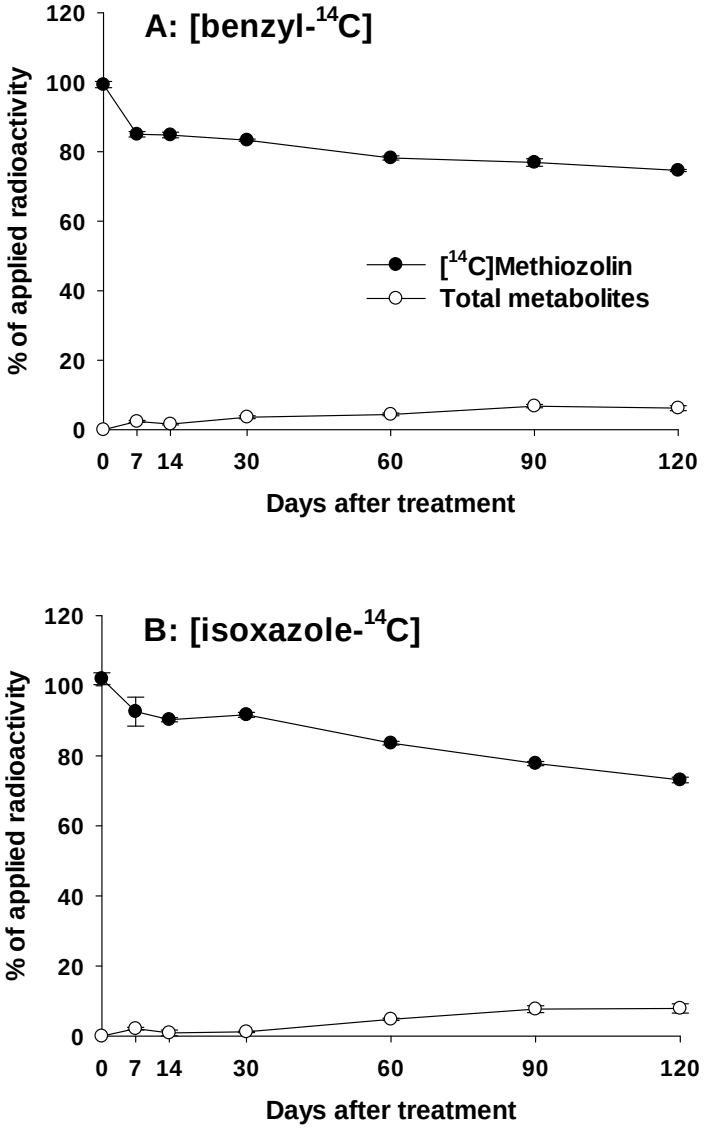


Figure 14. Degradation of [benzyl-^{14}C] (A) and [isoxazole-^{14}C]methiozolin (B) and formation of its metabolites in the nonsterile soil under the anaerobic condition. Each data point represents the mean ± SD (n = 3).

In present study, in both the soil conditions, although the amount of degradation products increased slightly with time, no major metabolite (>10% of the applied) was detected. In the nonsterile soil under the aerobic condition, eight minor metabolites were found in the soil treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin throughout the study (Figure 15). However, the amount of each metabolite was 0.5-2.2% of the applied. The combined percentages for all the metabolites at each sampling date were 1.0-5.0% of the applied radioactivity, and the amount was maximized at 60 DAT in both the soils. In the sterile soil under the aerobic condition, no metabolite was detected untile 30 DAT (Figure 16). In the soil under the anaerobic condition, three minor metabolites were detected and the amount of total metabolites at each sampling date was 0.9-7.9% of the applied radioactivity (Figure 17). Although not considered as a major metabolite, one of the metabolites produced to 7.9% of the applied was isolated to identify and then designated as SMet-1. Consequently, in this study, no major metabolite was found in all the soil conditions, suggesting that metabolites produced from methiozolin may rapidly bind to humic substances, and/or further degraded rapidly into carbon dioxide.

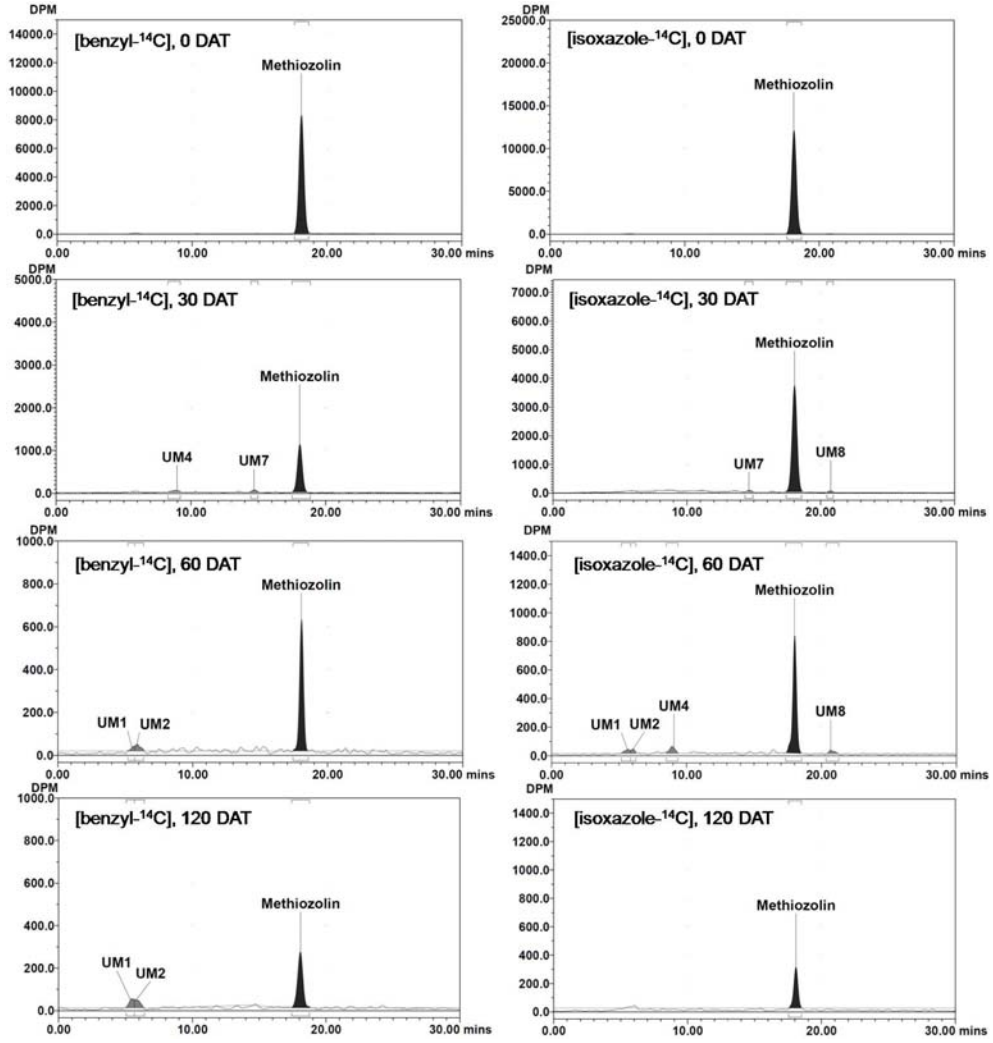


Figure 15. Representative radio-HPLC chromatograms of solvent extracts from the nonsterile soil under the aerobic condition after application of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin. UM: unidentified metabolite.

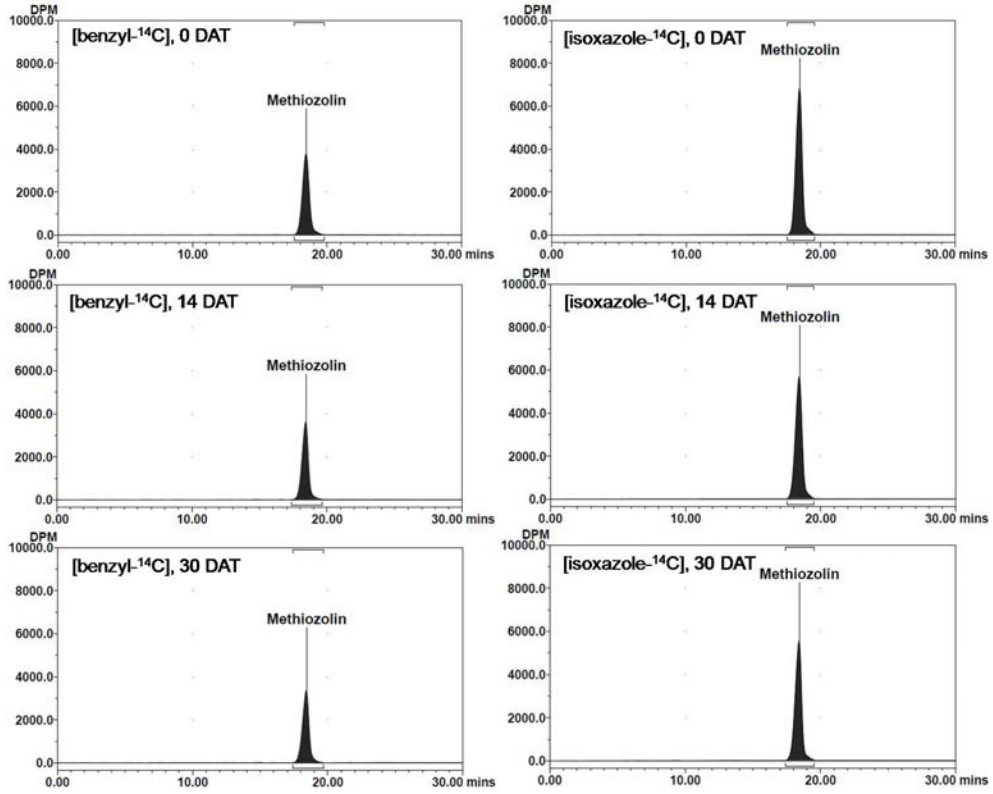


Figure 16. Representative radio-HPLC chromatograms of solvent extracts from the sterile soil under the aerobic condition after application of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin.

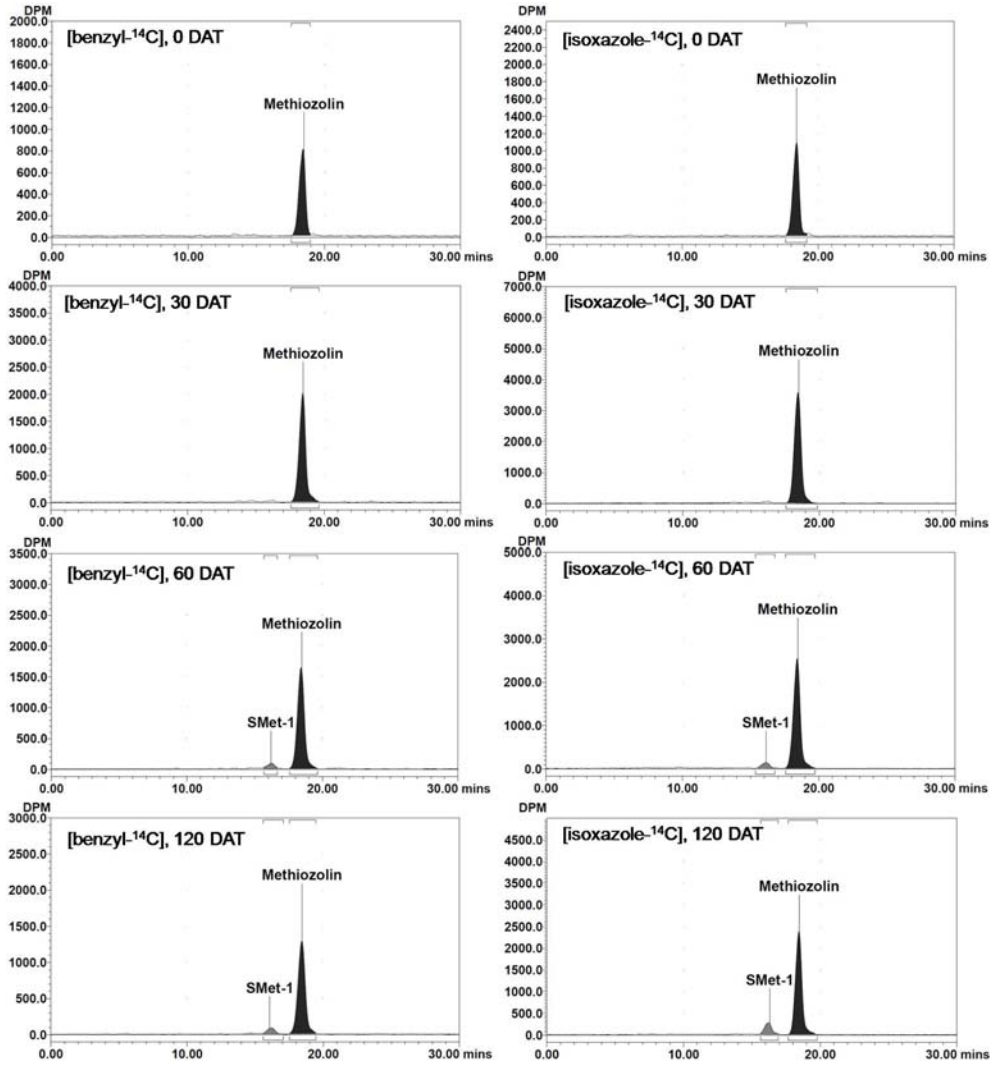


Figure 17. Representative radio-HPLC chromatograms of solvent extracts from the soil under the anaerobic condition after application of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin.

7. Identification of isolated metabolite

The chemical structure of the identified metabolite (SMet-1) is shown at Figure 18, and its ^{1}H-NMR and ^{13}C-NMR data, spectrum, and mass spectrum are shown at Table 6, Figure 19-21, respectively. In the ^{1}H-NMR spectrum (Figure 19), three proton signals at δ 6.98 (2H) and 7.41 (1H) indicated the presence of a difluorobenzene ring. Four proton signals at δ 3.50 (2H) and 4.61 (2H) were assigned to the ether bond. Three proton signals at δ 1.25 were assigned to the methyl moiety of the isoxazole ring. Two proton signals at δ 6.85 (1H) and 7.55 (1H) were indicated the presence of the thiophene ring. However, two proton signals at δ 3.02 (1H) and 3.45 (1H) were shifted to δ 3.14 and 2.98, respectively, and a signal at δ 4.21 (OH) was observed. The results indicated that the N-O bond of isoxazole ring was cleaved and then N was substituted with O finally. In the ^{13}C-NMR spectrum (Figure 20), the signals were assigned as follows: δ 136.5 (C2), 130.7 (C3), 145.1 (C4), 16.1 (C5), 160.8 (C6), 193.6 (C7), 47.8 (C10), 71.6 (C11), 24.7 (C12), 77.0 (C13), 60.0 (C15), 113.9 (C16), 162.8 (C17), 111.3 (C18), 132.7 (C19), 111.3 (C20), and 162.8 (C21). Two carbon signals of the C6 and C7 was changed and compared to those of methiozolin. EI-MS (Figure 21) gave a molecular ion peak at m/z 340 (M^{+}) and their fragment peaks (m/z 322, 183, 140, and 127). From the results, the chemical structure of SMet-1 was identified as 4-(2,6-difluorobenzyloxy)-3-hydroxy-3-methyl-1-(3-methylthiophen-2-yl)butan-1-one. On the basis of these results, the metabolic pathway of methiozolin in the soil under aerobic and anaerobic conditions was proposed as shown in Figure 22.

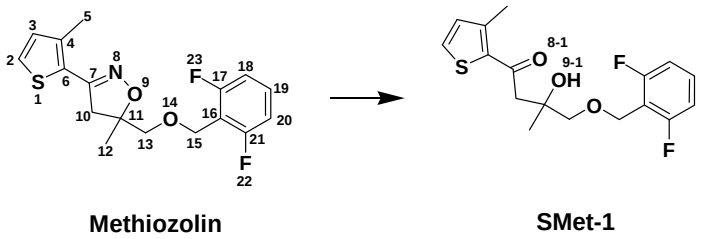


Figure 18. Chemical structures of methiozolin and the identified metabolite (SMet-1) from the soil under anaerobic condition.

Table 7. ^{1}H and ^{13}C-NMR data for methiozolin and SMet-1.

^{1}H-NMR Data

	Methiozolin		SMet-1	
Proton no.	δ ($CDCl_3$)	J (Hz)	δ (Acetone-D_6)	J (Hz)
2	7.28 (m)		7.55 (d)	3.20
3	6.91 (m)		6.85 (d)	5.00
5	2.46 (3H, s)		2.49 (3H, s)	
9	*b*		*b*	
9-1[a]	*b*		4.21 (s)	
10	3.02 (d)	16.45	3.14 (d)	15.80
	3.45 (d)	16.50	2.98 (d)	15.80
12	1.47 (3H, s)		1.25 (3H, s)	
13	3.55 (2H, dd)	9.85, 9.90	3.50 (2H, dd)	9.00, 9.00
15	4.72 (2H, s)		4.61 (2H, s)	
18[c]	6.91 (m)		6.98 (2H, m)	
19	7.28 (m)		7.41 (m)	
20[c]	6.91 (m)		6.98 (m)	

^{13}C-NMR Data

	Methiozolin		SMet-1	
Carbon no.	δ ($CDCl_3$)	J (Hz)	δ (Acetone-D_6)	J (Hz)
2	126.5		136.5	
3	125.8		130.7	
4	138.7		145.1	
5	16.4		16.1	
6	125.8		160.8	
7	152.5		193.6	
10	45.5		47.8	
11	74.3		71.6	
12	23.3		24.7	
13	85.9		77.0	
15	60.9		60.0	
16	113.3		113.9	
17[d]	160.9		162.8	
18	111.4		111.3	
19	131.9		132.7	
20	111.4		111.3	
21[d]	163.3		162.8	

[a] OH, [b] no proton, [c] H-F coupling, [d] no carbon

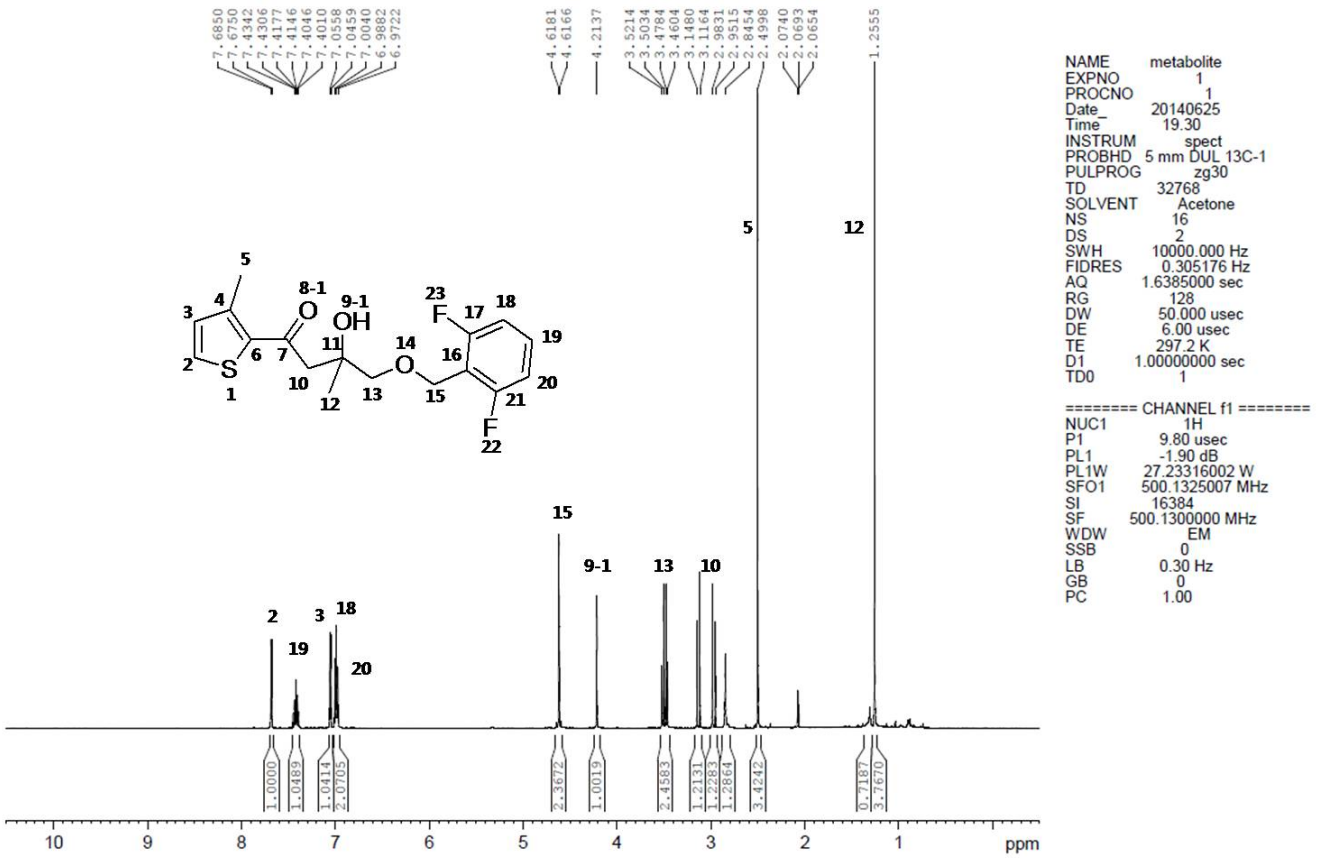


Figure 19. ^{1}H-NMR spectrums for SMet-1 isolated (A) from the soil under anaerobic condition and synthesized (B).

(B) Synthesized SMet-1

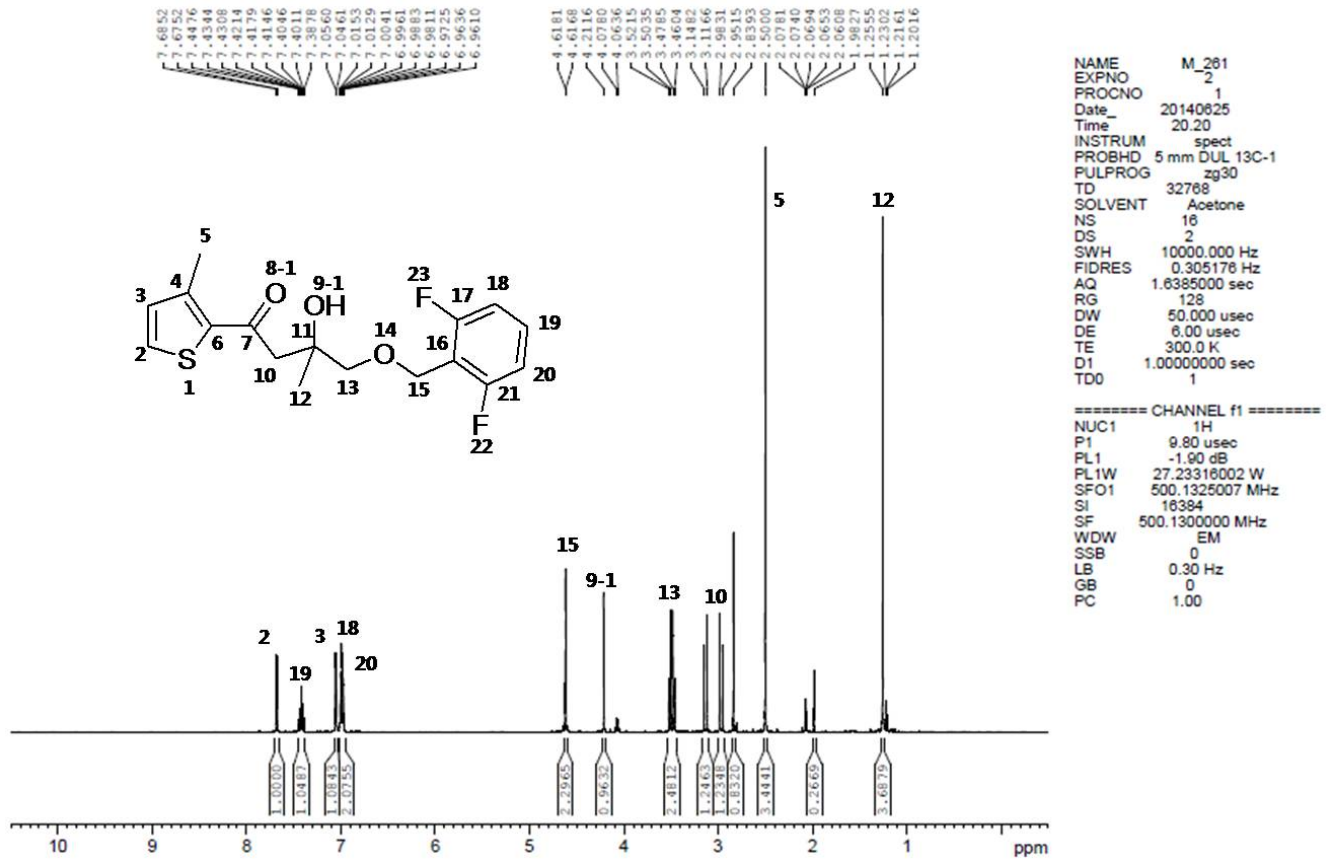


Figure 19. ^{1}H-NMR spectrums for SMet-1 isolated (A) from the soil under anaerobic condition and synthesized (B) (continued).

(A) Isolated SMet-1

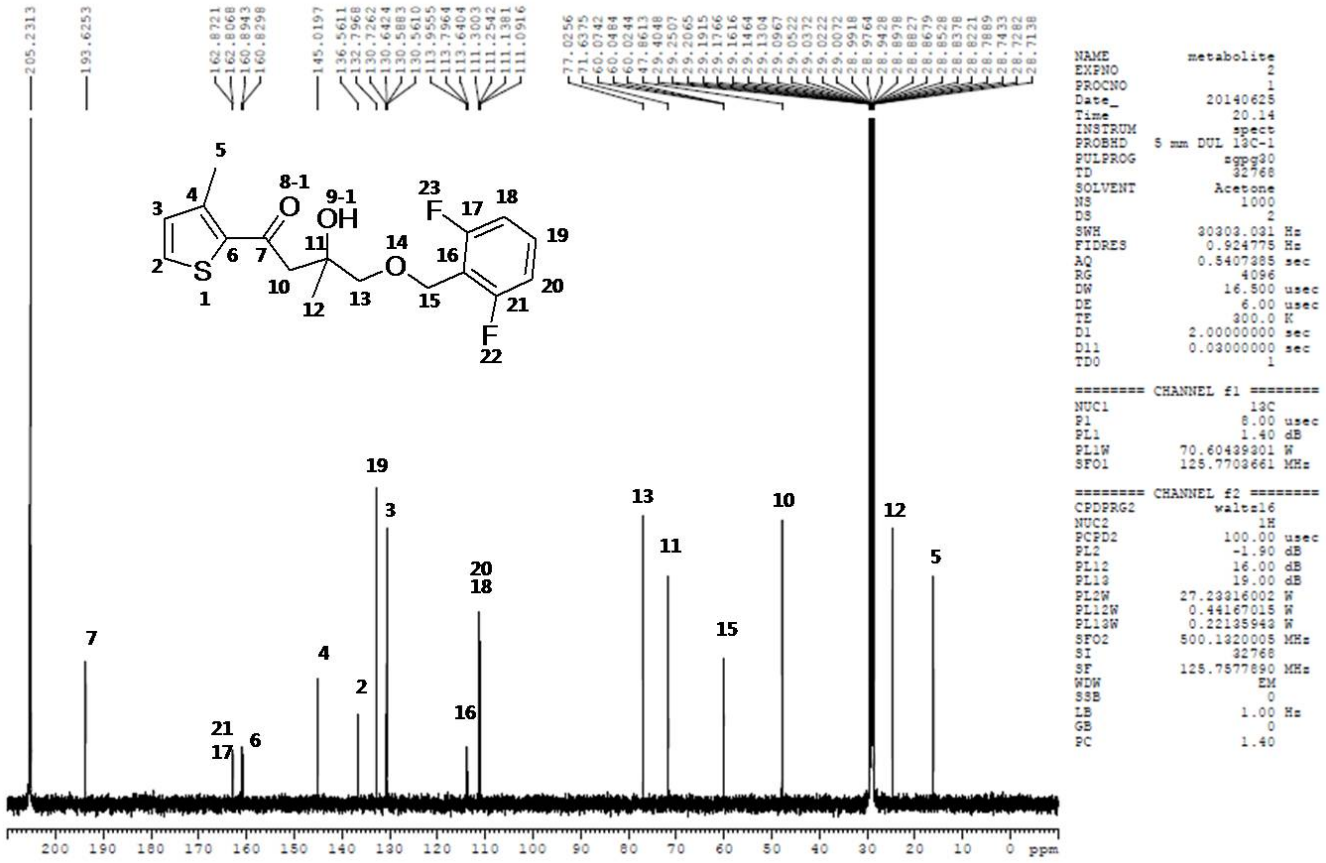


Figure 20. ^{13}C-NMR spectrums for SMet-1 isolated (A) from the soil under anaerobic condition and synthesized (B).

(B) Synthesized SMet-1

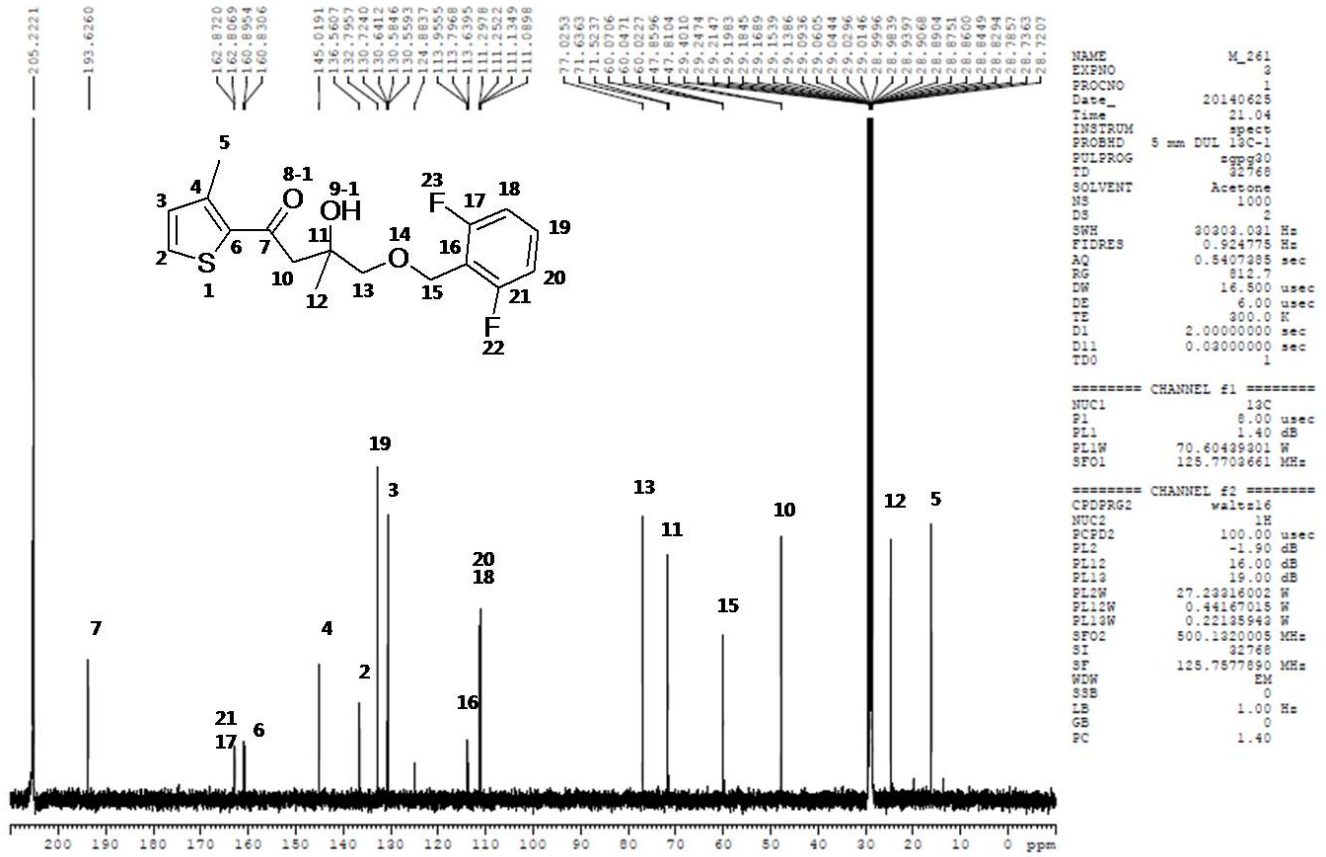


Figure 20. ^{13}C-NMR spectrums for isolated (A) from the soil under anaerobic condition and synthesized SMet-1 (B) (continued).

(A) Isolated SMet-1

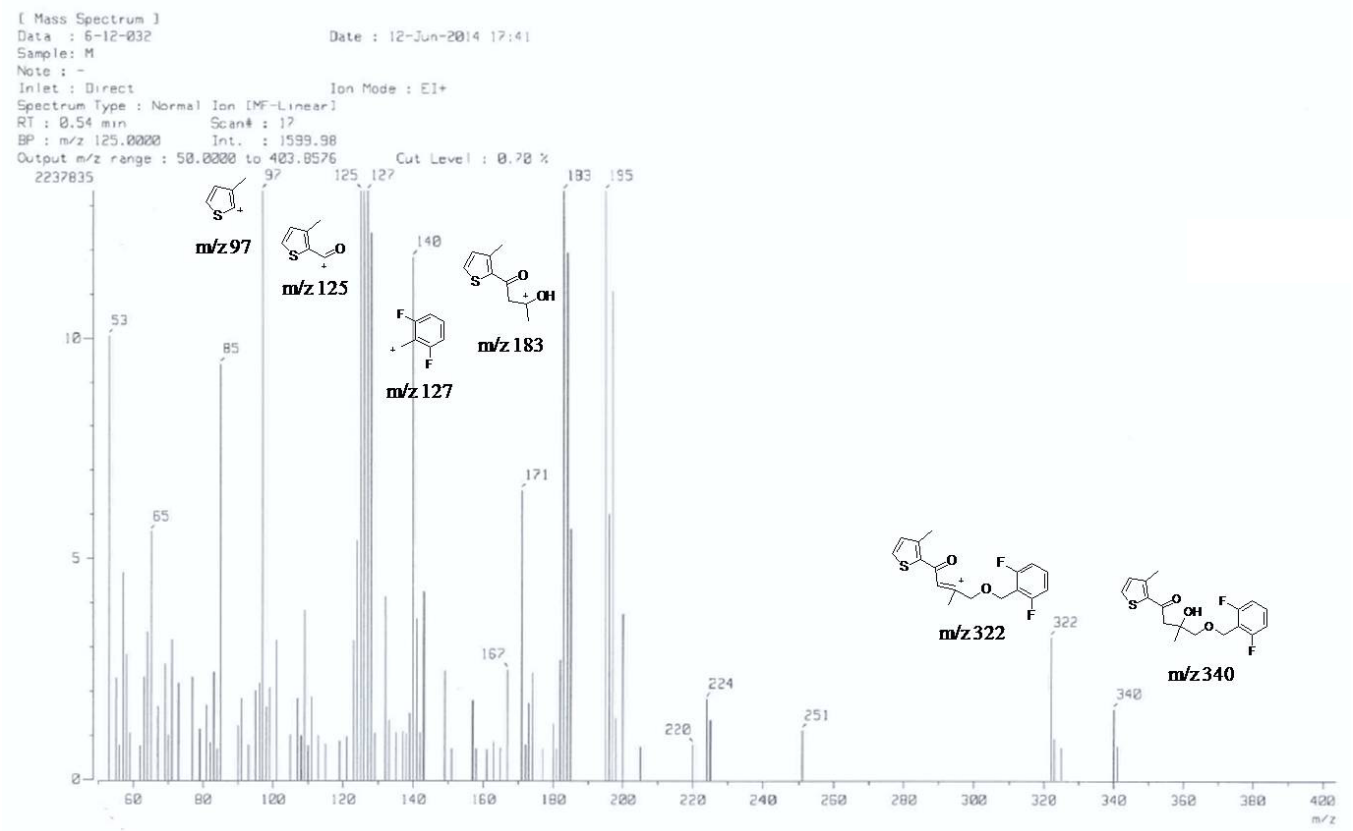


Figure 21. EI-MS spectrums for isolated (A) from the soil under anaerobic condition and synthesized SMet-1 (B).

(B) Synthesized SMet-1

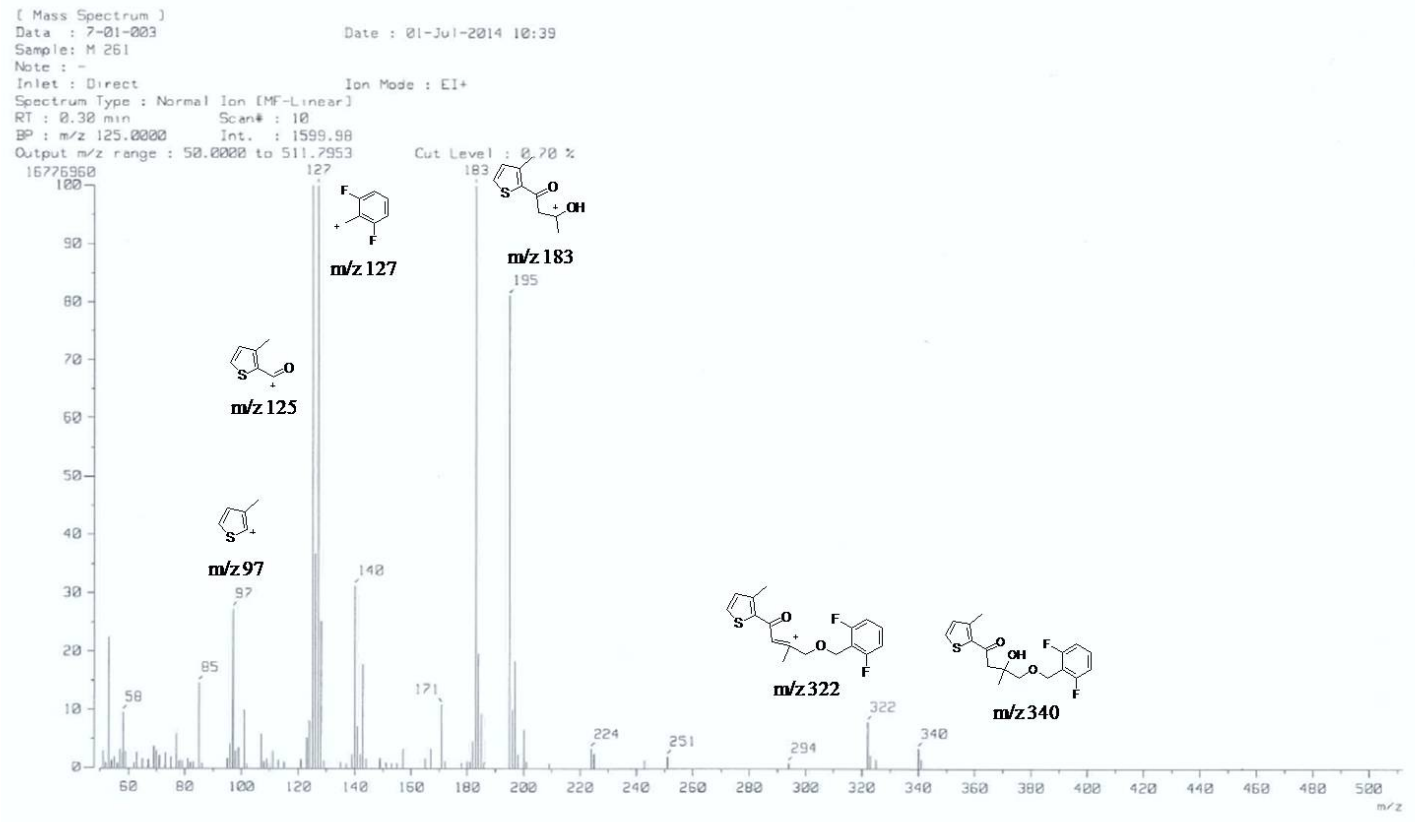
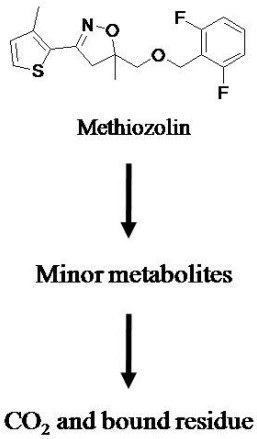


Figure 21. EI-MS spectrums for isolated (A) from the soil under anaerobic condition and synthesized SMet-1 (B) (continued).

(A) Aerobic soil



(B) Anaerobic soil

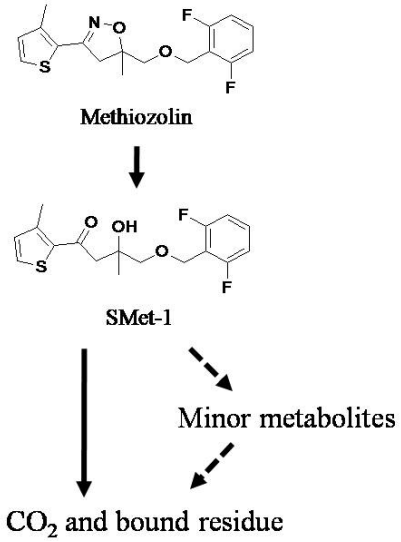


Figure 22. Proposed metabolic pathways of methiozolin in soil under aerobic (A) and anaerobic conditions (B).

IV. Conclusion

Soil metabolism of methiozolin was investigated in nonsterile and sterile soil under an aerobic condition, and nonsterile soil under an anaerobic condition using ^{14}C-labeled compound. Mass balances were 91.7-104.5% under the aerobic and 93.2-102.5% under the anaerobic conditions, justifying the reliability of results obtained in this study. When methiozolin was treated at a concentration of 0.5 kg/ha on a sandy clay loam soil under the aerobic condition at 25 ± 1°C, the half-life of methiozolin was calculated to be approximately 49 day. However, little degradation of methiozolin occurred in the sterile soil under the aerobic, or in the nonsterile soil under the anaerobic condition. The results suggest that metabolism of methiozolin in soil largely depends on the aerobic soil microbes but not on anaerobic microbes or abiotic degradation. In the nonsterile aerobic condition, methiozolin did not produce a major metabolite, but ended up with CO_2 and nonextractable residue. These results suggest that metabolites produced from methiozolin may rapidly bind to humic substance, and/or further degraded rapidly into carbon dioxide in the soil under aerobic condition. In the soil under anaerobic condition, one significant metabolite was very slowly produced, and identified to be 4-(2,6-difluorobenzyloxy)-3-hydroxy-3-methyl-1-(3-methylthiophen-2-yl)butan-1-one. However, little degradation and mineralization of methiozolin and its metabolite occurred.

PART II

Pharmacokinetics and Metabolism of [^{14}C]Methiozolin in Rats Following Oral Administration

I. Introduction

Methiozolin [5-(2,6-difluorobenzyl)oxymethyl-5-methyl-3-(3-methyl-thiophen-2-yl)-1,2-isoxazoline] was developed by Moghu Research Center and registered as a new turf herbicide in 2010 in Korea. This molecule was first invented as a rice herbicide candidate (Ryu, 2002) but not commercialized. Hwang *et al*. (2005) reported that the molecule controls barnyardgrass (*Echinochloa* spp.), sedge weeds and several other annual broadleaved weeds in paddy conditions. In later study, Koo and Hwang found that the herbicide had a potent pre- and post-emergence efficacy on annual bluegrass (*Poa annua*) and large crabgrass (*Digitaria sanguinalis*), with high safety to various warm and cool season turfgrasses including creeping bentgrass, Kentucky bluegrass, perennial ryegrass, zoysiagrass, and bermudagrass (Koo and Hwang, 2008). Lee *et al*. (2007) reported that methiozolin inhibited biosynthesis of both cellulose and hemicellulose fractions greatly. However, the herbicidal symptom of methiozolin indicated that its mode of action was different from that of inhibitors of cellulose synthesis, microtubule disrupter, or inhibitors of very-long-chain fatty acid (Koo et al., 2008). In recent study, Grossman *et al*. suggested that methiozolin might inhibit tyrosine aminotransferase involved in the plastoquinone biosynthesis in duckweed (*Lemna paucicostata* L.) (Grossman et al., 2011). Up to now, the mode of action of the herbicide might be directly or indirectly associated with cell wall biosynthesis and

potentially plastoquinone biosynthesis in susceptible plants, but the primary site of herbicidal action is still unclear.

In various toxicological studies, methiozolin was shown to be practically non-toxic, having acute oral LD_{50} of >2,000 mg/kg body weight, NOAEL of 5,000 mg/kg body weight in 90 days repeated dietary administration, and NOAEL of 1,000 mg/kg body weight in prenatal development toxicity in rats (Koo et al., 2010). However, it is unclear that low toxicity could be due to inherent safety of the molecule, no absorption in gastrointestinal tracts, or metabolic detoxification. In this paper, basic information on the pharmacokinetic parameters, tissue distribution, excretion, and metabolism of methiozolin in rats following oral administration of ^{14}C labeled compound are reported.

II. Materials and Methods

1. Test animals

Male Sprague-Dawley (SD) rats, 8 to 9 weeks old, were purchased from Koatech (Pyeongtaek, Korea). Animals were acclimatized for at least 1 week prior to administration. One day before administration, the animals were kept individually in a closed glass metabolism cage. Sterilized tap water and a laboratory rodent diet (Chunhajeil Feed Company, Daejeon, Korea) were given *ad libitum* to the animals throughout the experiment except for the one night before test substance administration.

2. Test condition and metabolism apparatus

The rats were individually housed in the all-glass metabolism cages, which were designed to collect volatile products, carbon dioxide, urine, and feces separately. Carbon dioxide was collected with two traps of 1N NaOH solution while volatile compounds were collected in two traps containing ethylene glycol. The air was pulled through the closed system continuously by a vacuum pump at a flow rate of 400 mL/min. The urine and feces receivers were cooled in an ice vessel (Figure 23). The whole system was kept in a controlled room at 23 ± 2°C and 60 ± 10% relative humidity. The room was controlled under a 12 h day-night cycle (light from 8 am to 8 pm).

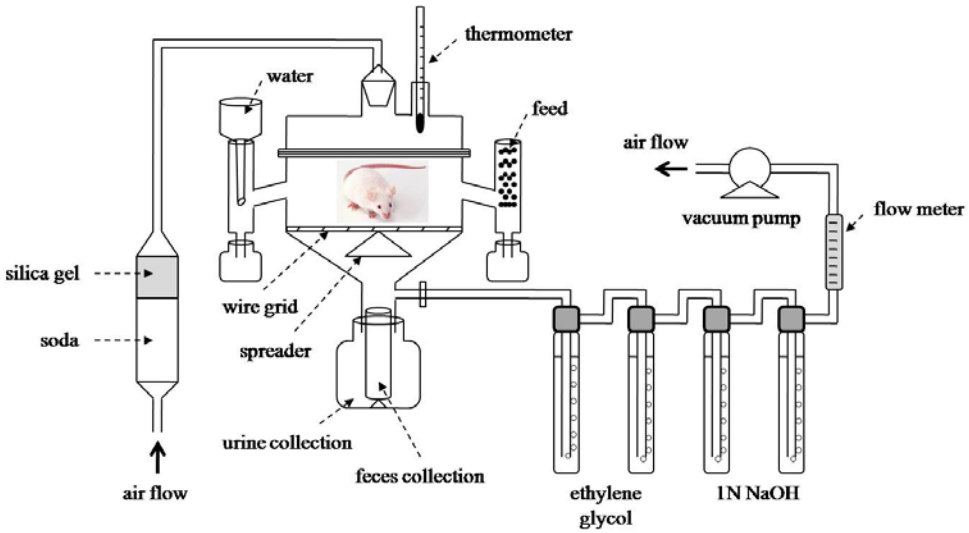


Figure 23. Schematic diagram of the rat metabolism cage.

3. Test materials

The ^{14}C-labeled methiozolin (specific activity: 6.59 MBq/mg, purity: 99.7%) was synthesized at Korea Radiochemicals Center (Suwon, Korea) and an unlabeled methiozolin (purity: 99.8%) was synthesized at Moghu Research Center Ltd (Daejeon, Korea) (Nam et al., 2012). Chemical structure of methiozolin and ^{14}C-labeled position are shown in Figure 24. Corn oil was purchased from Sigma Chemical Co. (St. Louis, USA). The liquid scintillation cocktails (Ultima-Flo M, Insta-Gel Plus, Hionic-Fluor, and Permafluor E^{+}), carbon dioxide absorbent (Carbo-Sorb E), and tissue solubilizer (Soluene-350®) were purchased from PerkinElmer (Waltham, USA). All the other solvents and reagents were commercial products of an analytical grade.

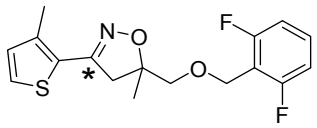


Figure 24. Chemical structure of methiozolin and ^{14}C-labeled position for metabolism study in rats.

4. Measurement of radioactivity

Radioactivity was measured by a liquid scintillation counter (LSC; Tri-Carb 2900TR, PerkinElmer, USA). Radioactivity in gross amounts of less than twice the background was considered to be below the limit of determination accuracy. Aliquots (0.1 to 2 mL) of liquid samples were mixed with 7 to 12 mL of Insta-Gel Plus or Hionic-Fluor prior to LSC

analysis. Portions of the blood, plasma, and fecal residues were combusted using an automatic sample oxidizer (Model 307, PerkinElmer, USA). The combustion products were absorbed into Carbo-Sorb E absorbent (5 mL) and mixed with Permafluor E^+ scintillator (10 mL). The efficiency of the oxidizer was determined using aliquots of Spec-Chec-^{14}C and was greater than 95%. Measurements of radioactivity were corrected for oxidizer efficiency.

5. Administration of test substance

The test article was prepared in a ratio of 125 mg of unlabeled methiozolin and 0.1 mg of [^{14}C]methiozolin (0.66 MBq) per 1 mL of corn oil. It was suspended in corn oil, heated to 60°C to obtain a completely dissolved solution, and was cooled in room temperature before administration. Methiozolin in the cooled corn oil after heating was not chemically changed and not precipitated at room temperature during a day (data not shown). The rats were orally administrated with 1mL of the test solution, which corresponded to a dose of 500 mg/kg body weight of a rat. This dose was a maximum dose level that did not show an acute clinical symptom and diarrhea in a preliminary study conducted in the same experimental condition. The average body weight of 18 rats tested in the following studies was 248.7 ± 6.4 g.

6. Radioactivity in blood and plasma

Blood samples (0.20 to 0.25 mL) were taken from the tail vein at 3, 6, 12,

24, 48, 72, 96, and 120 h after dosing to three rats. Immediately after sampling, the plasma was separated by centrifugation at 10,000 rpm for 5 min. Aliquots of the blood or plasma (0.1 mL) were added to a combustion paper cup, and air-dried. The dried cup was added with 0.1 mL of Combustaid (PerkinElmer, USA), and combusted using the automatic sample oxidizer, then radioactivity was measured by LSC.

7. Urinary and fecal excretion

Each of the three rats received single oral administration of 1 mL of the test solution. Immediately after administration, each rat was housed in a metabolism cage. Urine was collected at 6, 12, 24, 48, 72, 96, and 120 h after initiation. The total volume of each sample was measured. Aliquots (0.5 to 1 mL) of each sample were mixed with 7 mL of Insta-Gel Plus for radioactivity measurement with LSC. Feces were collected at 24, 48, 72, 96, and 120 h after initiation. The feces sample was extracted 2 times, using a homogenizer (Bio Homogenizer, ESGE, Switzerland), with methanol/water (50:50, v/v) solution of at least 2-fold the volume of the sample and then ultrasonicated for 15 min at ambient temperature. The homogenate was centrifuged at 5,000 rpm for 15 min, and duplicate aliquots of 1 mL of each supernatant were mixed with 7 mL of Insta-Gel Plus for measurement of radioactivity. The debris of the extracted feces was dried at 40°C and then homogenized. Triplicate portions (0.2 g) of the feces powder were combusted using the automatic sample oxidizer, and then radioactivity was measured by LSC. Expired air was passed through two traps containing 250

mL of 1N NaOH solution for trapping [^{14}C]carbon dioxide and two traps of 250 mL of ethylene glycol for trapping volatile compounds, and collected at 12, 24, 48, and 72 h after administration. No radioactivity was detected in both trap solutions until 72 h, and thus was not collected after then. Following collection of the feces and urine, each metabolism cage was washed with acetone/water (50:50, v/v) solutions at 120 h after administration, and then duplicate aliquots (4 mL) of the washing solutions were mixed with 12 mL of Insta-Gel Plus for radioactivity measurement.

For quantification and identification of metabolites, an aliquot (100 to 200 μL) of the pooled urine and fecal extracts collected at each sampling time was analyzed using a radio-HPLC (200 Series, PerkinElmer, USA) equipped with a UV/vis detector and a flow scintillation analyzer (Radiomatic 610TR, PerkinElmer, USA). The detection wavelength was 254 nm, and a reverse phase C_{18} column (Cosmosil, 250 × 4.6 mm i.d., 5 μm, Nacalai tesque, Japan) was used. The elution solvents were methanol and water (0.1% formic acid). A linear gradient (from 30% methanol to 90% for 50 min) using a flow rate of 1.0 mL/min was adopted to separate the peaks of methiozolin and potential metabolites produced. The flow rate of scintillation cocktail (Ultima-Flo M) was 3 mL/min.

8. Bile excretion

Three rats were individually bile-duct cannulated, and administered with 1 mL of the test article solution. Each rat was housed in a metabolism cage. Bile was collected from each animal into a separately ice-cooled receiver at

6, 12, 24, 48, 72, 96, and 120 h after initiation. The total volume of each sample was measured. Aliquots (0.5 to 1 mL) of each sample were mixed with 7 mL of Insta-Gel Plus for radioactivity measurement and 100 to 200 μL was analyzed by the radio-HPLC.

9. Tissue distribution

Each of nine rats was orally administered with 1 mL of the test solution, and then anesthetized by diethyl ether inhalation at 12, 48 and 120 h after administration. At each time, the following tissues were obtained from three rats for radioactivity determination: adrenal glands, bladder, bone marrow, brain, epididymis, gastrointestinal tract (GIT), heart, kidneys, liver, lungs, pancreas, pituitary gland, spleen, testes, and thyroid. The separated tissue samples were weighed, chopped and solubilized in 2 to 10 mL of Soluene-350 at 60°C. After complete solubilization, the samples were cooled in room temperature and 2 to 10 mL of methanol was added, and then its aliquots (0.5 to 2 mL) were mixed with 7 to 12 mL of Hionic-Fluor was added for radioactivity measurement.

10. Isolation of metabolites from feces

In present study, isolation and identification of the metabolites was conducted in feces, but not in urine because there were no major metabolites potentially subjected to isolation in the radio-HPLC chromatogram. The feces collected at 24, 48, and 72 h was separately extracted as described above, and the fecal extracts were combined, then concentrated in vacuo.

The concentrated extracts was injected to the HPLC equipped with a column, 250 × 10 mm i.d., 15 μm, Cosmosil 5C_{18}-MS-II (Nacalai tesque, Japan) and eluted by methanol and water (0.1% formic acid) in a linear gradient (from 30% methanol to 90% for 40 min). The detection wavelength was 254 nm and the flow rate was 4.0 mL/min. The eluted solution corresponding to a metabolite peak was collected and concentrated in vacuo. To obtain a single metabolite from the separated eluents, further purification was conducted by thin layer chromatography (TLC) using precoated silica gel 60 F_{254} chromatoplates (20 × 20 cm, 0.5 mm layer thickness, Merck, Germany). The solvent systems were dichloromethane/methanol/acetic acid (6:1:0.6, v/v/v) and dichloromethane/methanol/acetic acid (9:1:0.9, v/v/v, developed triplicate). Each metabolite separated by TLC was extracted with tetrahydrofuran, and the solvent was evaporated in vacuo until complete dryness. Finally, two purified metabolites were identified by NMR spectroscopic and MS spectrometric analysis.

11. Spectroscopic and spectrometric analysis

The chemical structures of purified metabolites were identified by nuclear magnetic resonance (NMR) spectrometer (Bruker, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C with $CDCl_3$ (99.8%, Merck, Germany) and CD_3OD (99.9%, Cambridge Isotope Laboratories, USA) as a solvent, respectively, at room temperature. ESI negative spectra were recorded on 15Tesla Fourier Transform Ion Cyclotron Resonance Mass

Spectrometer (15T FT-ICR MS, Bruker Daltonics, Germany) for AMet-1 and EI-MS spectra on JMS-700 MS (JEOL, Japan) for AMet-2, respectively.

12. Data analysis

Mean and standard deviation calculations were performed using Microsoft Office Excel 2007 (Microsoft Corp., USA) and the total ^{14}C blood concentration-time data was analyzed using the WinNonlin Pro software, Version 3.3 (Pharsight Corp., USA).

III. Results and Discussion

1. Pharmacokinetic parameters

The ^{14}C concentration in the blood and plasma during 120 h following single oral administration of the test article at 500 mg/kg body weight is shown in Figure 25. The estimated pharmacokinetic parameters such as the time showing a maximum concentration (T_{max}, h), maximum concentration (C_{max}, μg equiv of methiozolin/mL), half-life ($T_{1/2}$, h), AUC_{120} (area under the concentration-time curve from 0 to 120 h), and clearance (Cl, mL/h/kg) were calculated (Table 7). T_{max} and $T_{1/2}$ were 6 h and 49.4 h for the blood respectively, indicating that administered ^{14}C was rapidly absorbed from the gastrointestinal tract and eliminated from the body with time. The estimated pharmacokinetic parameters between for blood and plasma were similar. In addition, the depletion curve for ^{14}C concentration in the plasma was very similar to that in the blood. This result suggests that methiozolin or its metabolites were not accumulated in blood cells.

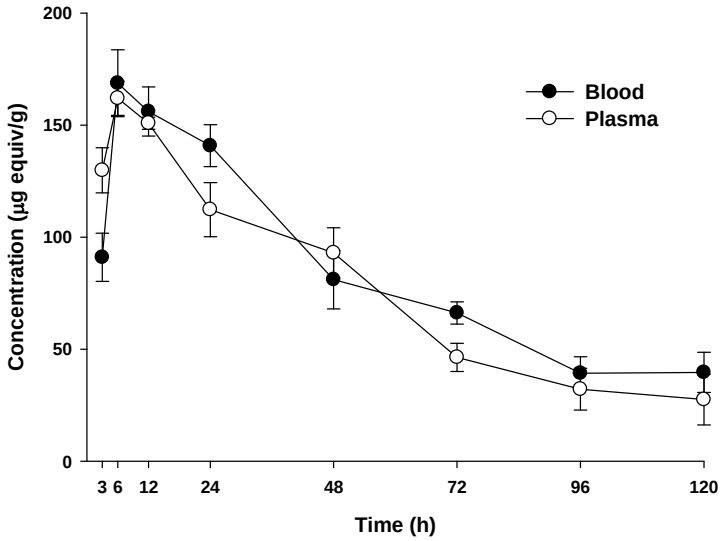


Figure 25. Concentration of ^{14}C in the blood and plasma of rats following single oral administration of methiozolin at 500 mg/kg body weight. Each data point represents means ± SD.

Table 8. Estimated main pharmacokinetic parameters of ^{14}C in blood and plasma after oral administration of [^{14}C]methiozolin at a dose of 500 mg/kg body weight.

	Parameters[a]				
	C_{max} (μg/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{120} (μg·h/mL)	Cl (mL/h/kg)
Blood	168.7	6	49.4	9921.5	39.2
Plasma	162.0	6	41.7	8943.2	47.2

[a] C_{max}, maximum concentration; T_{max}, time of C_{max}; $T_{1/2}$, elimination half-life; AUC_{120}, area under the concentration-time curve; Cl, clearance.

2. Recovery of ^{14}C from various test samples

Recovery test was conducted to provide the reliability of the results obtained from extraction and combustion procedures. The feces and each tissue samples (5 replications/sample) were spiked with [^{14}C]methiozolin (ca. 725,000 dpm), extracted and/or combusted, and LSC counted as the same method described above. The average recovery was 94.18-100.68%, suggesting that the assay was sufficiently precise and accurate to be used for practical analysis (Table 8).

3. ^{14}C Distribution in tissues

Radioactivity distributed rapidly to all the tissues within 12 h after administration, and declined with time thereafter (Table 9, 10). The only exception was kidney. Concentration of ^{14}C in kidney (108.93 μg equiv/g) was the highest at 48 h after dosing (Table 9). At 12 h after administration, the ^{14}C concentration in blood was 156.08 μg equiv/mL, and the concentrations in the most tissues were similar to or lower than the blood concentration. Certain tissues showed higher concentrations than the blood; these include GIT (1413.30 μg equiv/g), lungs (374.70 μg equiv/g), pituitary gland (307.61 μg equiv/g), and adrenal glands (283.50 μg equiv/g). The lowest concentration was measured in testes (5.78 μg equiv/g). Relatively high proportions of radioactivity were measured in GIT (11.684%), liver (1.469%), lungs (0.525%), and kidneys (0.096%) at 12 h.

Table 9. Recovery of ^{14}C in various test samples.

Sample	Average (%)	S.D. [#]
Feces	97.29	3.03
Blood	94.57	3.27
Adrenal glands	94.89	2.46
Bladder	98.42	0.90
Bone marrow	100.68	3.66
Brain	96.54	1.97
Epididymis	97.71	2.88
GIT[*]	98.78	6.44
Heart	95.17	3.37
Kidneys	94.73	1.87
Liver	95.63	3.10
Lungs	95.30	2.97
Pancreas	97.76	1.63
Pituitary gland	98.84	0.49
Spleen	94.18	4.64
Thyroid	100.19	2.41
Testes	99.88	4.02

[*]GIT: gastrointestinal tract

[#] S.D.: standard deviation

At 120 h after administration, the concentrations of ^{14}C in most tissues were several times lower than those at 12 h, suggesting methiozolin or its metabolites were easily eliminated (Table 10). The lower radioactivity in the brain (0.71 μg equiv/g) at 120 h indicated that methiozolin or its metabolites has low permeability on the blood-brain barrier (Hughes, 1996). In this present study, the radioactivity was distributed across the various tissues, indicating that methiozolin or its metabolites were permeated to all the tissues. As the results of the tissue to blood ratio (tissue concentration/blood concentration), the ratio was much higher especially in GIT, lungs, pituitary gland, and adrenal gland at 12 h; however, the values in all tissues declined to 0.02 to 0.55 at 120 h after administration (Table 11). These results suggest that the measured ^{14}C might have low lipophilicity and eliminated easily without accumulation in the tissues (Magnuson et al, 2007).

Table 10. Concentration of ^{14}C in each tissue following oral administration of [^{14}C]methiozolin at a dose of 500 mg/kg body weight to rats.

Tissue	μg equiv of methiozolin/g wet tissue (mg/kg)					
	12 h		48 h		120 h	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Blood	156.08	25.14	81.02	18.02	39.64	8.96
Adrenal glands	283.50	45.32	33.90	2.23	3.48	1.76
Bladder	177.39	37.91	88.15	10.15	7.00	2.05
Bone marrow	101.83	18.78	4.69	1.91	1.27	0.30
Brain	7.01	1.50	2.45	0.69	0.71	0.14
Epididymis	16.29	0.53	15.83	1.17	1.79	0.41
GIT[a]	1413.30	201.55	218.77	32.34	11.46	3.93
Heart	44.06	6.73	9.03	2.58	3.08	0.70
Kidneys	55.58	15.04	108.93	0.91	12.08	3.90
Liver	180.21	39.81	165.10	31.96	21.99	6.11
Lungs	374.70	40.92	32.34	0.13	5.95	1.63
Pancreas	57.23	10.73	24.07	5.05	4.23	1.14
Pituitary gland	307.61	25.28	15.23	3.27	4.17	1.55
Spleen	10.81	3.16	11.23	1.68	5.39	1.07
Testes	5.78	1.88	5.60	1.13	1.29	0.34
Thyroid	12.50	2.10	9.47	1.80	1.89	0.30

[a]Gastrointestinal tract (non-including contents)

Table 11. Proportion of ^{14}C in each tissue following oral administration of [^{14}C]methiozolin at a dose of 500 mg/kg body weight to rats.

Tissue	Proportion (% to administration dose)					
	12 h		48 h		120 h	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Adrenal glands	0.013	0.001	0.002	<0.001	<0.001	<0.001
Bladder	0.021	0.007	0.008	0.002	<0.001	<0.001
Bone marrow	0.009	0.001	<0.001	<0.001	<0.001	<0.001
Brain	0.012	0.003	0.004	0.004	0.001	<0.001
Epididymis	0.010	<0.001	0.008	0.005	0.001	<0.001
GIT[a]	11.684	1.291	1.915	0.054	0.096	0.028
Heart	0.037	0.015	0.008	0.005	0.003	0.001
Kidneys	0.096	0.039	0.202	0.010	0.020	0.012
Liver	1.469	0.383	1.638	0.833	0.168	0.038
Lungs	0.525	0.381	0.048	<0.001	0.009	0.002
Pancreas	0.058	0.041	0.050	0.053	0.009	0.003
Pituitary gland	0.003	<0.001	<0.001	<0.001	<0.001	<0.001
Spleen	0.006	0.002	0.008	0.004	0.003	0.001
Testes	0.018	0.005	0.019	0.009	0.004	0.001
Thyroid	0.006	0.003	0.004	0.002	0.001	<0.001

[a]Gastrointestinal tract (non-including contents)

Table 12. Tissue to blood ratio of ^{14}C following oral administration of [^{14}C]methiozolin at a dose of 500 mg/kg body weight to rats.

Tissue	Tissue to blood ratio					
	12 h		48 h		120 h	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Adrenal glands	1.82	0.29	0.42	0.03	0.09	0.04
Bladder	1.14	0.24	1.09	0.13	0.18	0.05
Bone marrow	0.65	0.12	0.06	0.02	0.03	0.01
Brain	0.04	0.01	0.03	0.01	0.02	<0.01
Epididymis	0.10	<0.01	0.20	0.01	0.05	0.01
GIT[a]	9.05	1.29	2.70	0.40	0.29	0.10
Heart	0.28	0.04	0.11	0.03	0.08	0.02
Kidneys	0.36	0.10	1.34	0.01	0.30	0.10
Liver	1.15	0.26	2.04	0.39	0.55	0.15
Lungs	2.40	0.26	0.40	0.00	0.15	0.04
Pancreas	0.37	0.07	0.30	0.06	0.11	0.03
Pituitary gland	1.97	0.16	0.19	0.04	0.11	0.04
Spleen	0.12	0.03	0.22	0.08	0.07	0.01
Testes	0.07	0.02	0.14	0.02	0.14	0.03
Thyroid	0.04	0.01	0.07	0.01	0.03	0.01

[a]Gastrointestinal tract (non-including contents)

4. Excretion into feces and urine

The overall recovery of total radioactivity from urine and feces was 93.2% of the administrated radioactivity (Table 12). Within the first 48 h, 77.6% of the administered radioactivity was excreted into urine (19.5%) and feces (58.1%), and these amounts accounted more than 83% of the total recovered radioactivity from urine, feces, and cage wash during the entire period. After 120 h, 24.3 and 68.9% of the administered radioactivity were excreted through urine and feces, respectively. When the vapor pressure of the chemical is higher than 1 x 10^{-5} mmHg (20°C), trapping of volatile products is recommended (Martin, 1986), and ethylene glycol (Liu et al., 2011), cold ethanol (Mathews et al., 1991), dry ice-acetone (Marco et al., 1985), Tenax GC and silica gel (Tanaka and Watanabe, 1982) have been used to trap the volatiles in general. Although vapor pressure of methiozolin is 1.9 x 10^{-13} mmHg at 25°C (Koo et al., 2010), traps of ethylene glycol for volatile compounds were used because some volatile products may be generated as metabolites during the study. However, no volatile product was detected during the major absorption and excretion period (0-72 h) after dosing, suggesting that volatile metabolites might not be produced from the rats administered with methiozolin.

Table 13. Mean daily and cumulative percent of ^{14}C in urine and feces after oral administration of methiozolin at a dose of 500 mg/kg body weight to rats[a]

Time (h)	Urine		Feces		Total	
	Daily	Cumulative	Daily	Cumulative	Daily	Cumulative
			% of administrated radioactivity			
0-6	3.54 ± 1.96	3.54 ± 1.96	NA	NA	3.54 ± 1.96	3.54 ± 1.96
6-12	2.51 ± 1.38	6.05 ± 0.77	NA	NA	2.51 ± 1.38	6.05 ± 0.77
12-24	5.02 ± 1.44	11.07 ± 2.00	26.95 ± 1.50	26.95 ± 1.50	31.96 ± 1.48	38.01 ± 2.25
24-48	8.46 ± 2.41	19.52 ± 4.31	31.12 ± 4.70	58.07 ± 3.28	39.58 ± 5.94	77.59 ± 5.45
48-72	2.79 ± 0.85	22.32 ± 4.07	8.18 ± 4.48	66.24 ± 2.42	10.97 ± 5.15	88.56 ± 1.99
72-96	0.85 ± 0.21	23.17 ± 3.88	1.72 ± 0.18	67.96 ± 2.48	2.57 ± 0.31	91.13 ± 1.69
96-120	0.46 ± 0.20	23.63 ± 3.69	0.92 ± 1.03	68.88 ± 1.50	1.38 ± 0.85	92.51 ± 2.42
Cage wash	0.70 ± 0.44	24.33 ± 4.01	NA	NA	0.70 ± 0.44	93.21 ± 2.66
Expired air					ND	ND
Total		24.33 ± 4.01		68.88 ± 1.50		93.21 ± 2.66

[a] Values were expressed as means ± SD; NA, not applicable; ND, not detected

The results suggest that methiozolin is rapidly absorbed and excreted in the first 48 h followed by slow residual excretion. Cumulated recovery was greater than 93% of the administered, justifying the experiment was conducted in a valid condition and the result would represent the true excretion kinetics. The radioactivity recovered in the urine and feces suggested methiozolin is excreted through the fecal and urinary system, but not through the respiratory system. Excreted radioactivity was almost three-fold greater in the feces than in urine, suggesting fecal excretion might be the more important route of elimination of methiozolin in rats. In previous excretion studies with several agrochemicals, compounds such as pyribenzoxim (Liu et al., 2011) and pyridalyl (Nagahori et al., 2009) having relatively low water solubility were excreted through feces mostly, whereas higher water-soluble compounds such as clothianidin (Yokota et al., 2003) and furametpyr (Nagahori et al., 2000) were excreted more through urine. Water solubility of pyribenzoxim, pyridalyl, clothianidin, and furametpyr were 3.0 mg/L, 0.15 μg/L, 340 mg/L, and 225 mg/L (The pesticide manual, 2003), respectively. Methiozolin has relatively low water solubility (1.6 mg/L) (Korea Institute of Toxicology, 2011), and the result of major excretion through the feces is comparable to the previous studies with pyribenzoxim and pyridalyl.

5. Biliary excretion

The cumulative bile excretion during the 120 h was 40.1% of the administered dose (Figure 26). The majority of the bile excretion (94.3% of

cumulative bile excretion for 120 h, or 37.8% of the administered) was measured within the first 48 h after dosing. The excretion rate in feces and urine from the bile-cannulated rats was not determined in this study; however, the absorption rate (64.4%) and the direct fecal excretion rate (28.8%) could be estimated by adding the urinary and the biliary excretion and by deducting the biliary excretion from the fecal excretion, respectively. Biliary excretion is well known to be a major pathway for the elimination of amphipathic, hydrophobic, and high molecular weight xenobiotics (Vore, 1994), and can be linked to a high clearance (Arimori et al., 2003). In previous study, molecular weight was a dominant factor influencing biliary excretion (Wright and Line, 1980; Proost et al., 1997; Han et al., 2001) and foreign compounds having high molecular weight (>325 ± 50) were excreted in bile (Hakk et al., 2000; Mathews et al., 1996). Based on this study, extensive biliary excretion of methiozoin suggests that the produced metabolites might have a high molecular weight.

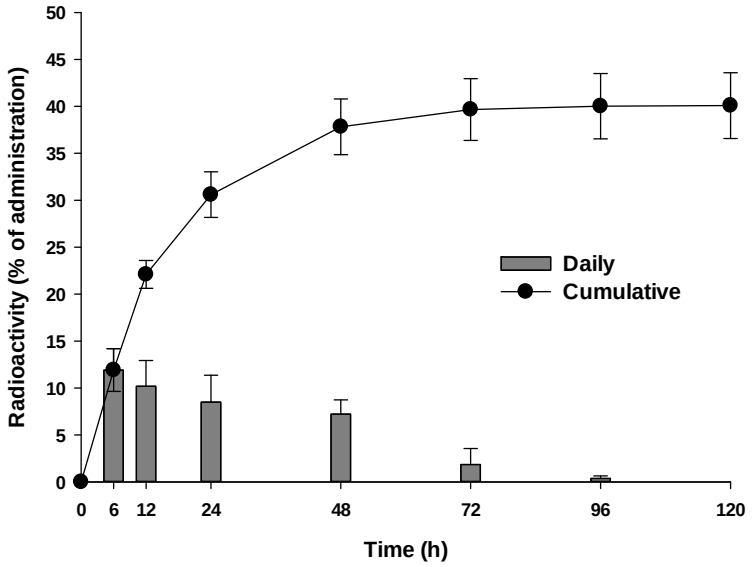


Figure 26. Daily and cumulative amounts of radioactivity excreted from bile of rats after oral administration of methiozolin at 500 mg/kg body weight. Each data point represents means ± SD.

6. Metabolites in urine, feces and bile

Typical radio-HPLC chromatograms of [^{14}C]methiozolin, feces extract, urine, and bile are shown in Figure 27. The amount of metabolite was calculated using the radioactivity according to the metabolite peak integrated by the radio-HPLC and the value was corrected by multiplying the dilution rate (total sample volume/injection volume to the radio-HPLC).

Methiozolin was detected only in the feces extract and was calculated at 2.2% of the administered during 72 h. In the feces extract and urine, numerous minor metabolites were present but the amount of each metabolite was measured below 4% of the dosed. In bile, at least 3 metabolites were found at proportions of 3.9 to 10.9% of the administered during 72 h. These results implied that the absorbed methiozolin was rapidly biotransformated into numerous minor metabolites and excreted through urine and feces in rats.

In this study, two metabolites from the fecal extracts were identified by NMR and MS spectrometry, and were designated as AMet-1 and AMet-2, respectively. AMet-1 and AMet-2 were detected during 72 h after administration and accounted for 3.08 and 1.79% of the dosed, respectively. The highest amount of AMet-1 and AMet-2 was measured at 1.63% and 0.92% of the administered during 0-24 h, respectively. These results indicated that both the metabolites were produced and excreted rapidly within the first 24 h and the production and excretion lasted until 72 h after administration.

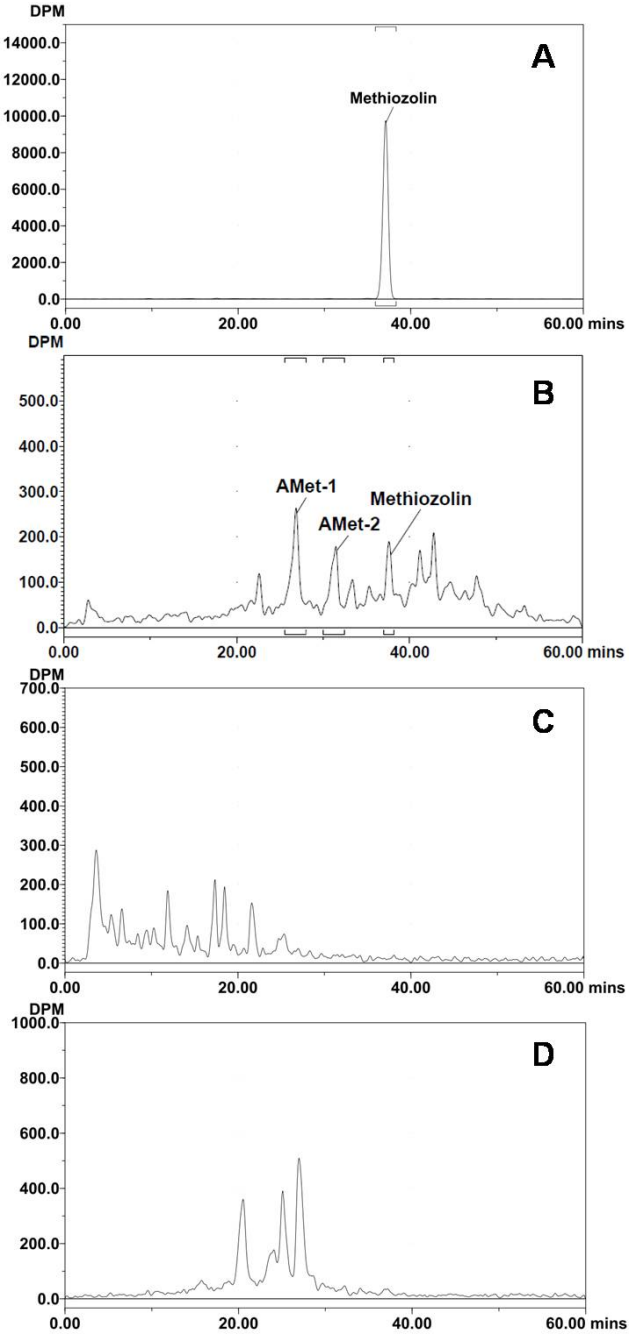


Figure 27. Representative radio-HPLC chromatograms for [^{14}C]methiozolin stock solution (A), fecal extracts (B), urine (C), and bile (D) sampled within 24 h after oral administration to rats.

7. Identification of isolated metabolites

Chemical structures of methiozolin and the identified metabolites, AMet-1 and AMet-2, are shown in Figure 28. ^{1}H-NMR and ^{13}C-NMR data for the metabolites are summarized in Table 13, and NMR and MS spectrums were in Figure 29-34, respectively.

7.1. Identification of AMet-1

In ^{1}H-NMR (Figure 29), three proton signals at δ 6.97 (2H) and 7.38 (1H) and two proton signals at δ 3.03 and 3.35 were indicated the presence of the difluorobenzene ring and isoxazole ring, respectively. Four proton signals at δ 3.52 (2H) and 4.69 (2H) were assigned to the ether bond. Three proton signals at δ 1.38 were assigned to the methyl moiety of isoxazole ring. Three proton signals at δ 2.27 (3H) were assigned to the methyl moiety of thiophene ring. One proton signal at δ 6.91 of C2 in thiophene ring was disappeared compared to methiozolin and five proton signals (δ 4.83, 3.80, 3.35, 3.46, and 3.55) for glucuronic acid were observed. The signals in ^{13}C-NMR for AMet-1 (Figure 30) were assigned as follows: δ 132.1 (C2), 132.2 (C3), 138.7 (C4), 16.8 (C5), 116.0 (C6), 162.9 (C7), 46.2 (C10), 74.5 (C11), 23.2 (C12), 87.5 (C13), 75.6 (C15), 164.0 (C17), 112.5 (C18), 132.1 (C19), 112.5 (C20), and 154.6 (C21). Six carbons signals for glucuronic acid were observed as follows: δ 105.9 (C2-1), 77.6 (C2-3), 176.6 (C2-5), 73.3 (C2-8), 75.6 (C2-9), and 61.8 (C2-11). The ESI negative spectrum showed a molecular ion peak at *m/z* 528.12 ($[M - H]^-$) (Figure 31). From the MSMS analysis, an increase of *m/z* 352.08 produced by glucuronyl moiety cleavage

from AMet-1 was observed (data not shown). On the basis of these results, AMet-1 was identified as a glucuronic acid conjugate, 6-(5-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)-4-methylthiophen-2-yloxy)-tetrahydro-3,4,5-trihydroxy-2H-pyran-2-carboxylic acid.

7.2. Identification of AMet-2

In ^{1}H-NMR (Figure 32), three proton signals at δ 6.89 (2H) and 7.30 (1H) and two proton signals at δ 2.98 and 3.52 (2H, J = 16.64, 16.62 Hz) indicated the presence of difluorobenzene ring and isoxazole ring, respectively. Four proton signals at δ 3.56 (2H) and 4.70 (2H) were assigned to the ether bond. Three proton signals at δ 1.45 were assigned to the methyl moiety of isoxazole ring. Two proton signals at δ 7.30 (1H) and 7.08 (1H) was assigned to thiophene ring. However, two proton signals at δ 2.46 (3H) of methiozolin were shifted to δ 4.70 (2H) and signal at δ 4.16 (OH) was observed. The results indicated that methyl moiety (C5) of thiophene ring was hydroxylated. In the ^{13}C-NMR spectrum (Figure 33), the signals were assigned as follows: δ 128.6 (C2), 126.4 (C3), 143.1 (C4), 59.1 (C5), 128.6 (C6), 152.2 (C7), 44.7 (C10), 73.9 (C11), 23.0 (C12), 86.7 (C13), 60.3 (C15), 113.3 (C16), 161.2 (C17), 111.2 (C18), 130.4 (C19), 111.2 (C20), and 162.6 (C21). One carbon signal of C5 position was changed compared to that of methiozolin. EI-MS gave a molecular ion peat at *m/z* 353 (M^{+}) and their fragment peaks (*m/z* 196, 127 and 112) (Figure 34). From the results, the chemical structure of AMet-2 was identified as (2-(5-((2,6-

difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)thiophen-3-yl)methanol. On the basis of the identification of two metabolites, the metabolic pathway was proposed to hydroxylation of methyl moiety and glucuronic acid conjugation in thiophene ring of methiozolin (Figure 35).

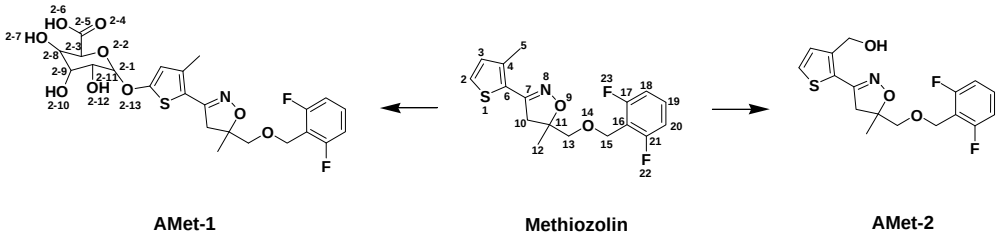


Figure 28. Chemical structures of methiozolin and identified metabolites from the feces in rats following oral administration of methiozolin at 500 mg/kg body weight.

Table 14. ^{1}H and ^{13}C-NMR data for methiozolin, AMet-1 and AMet-2.

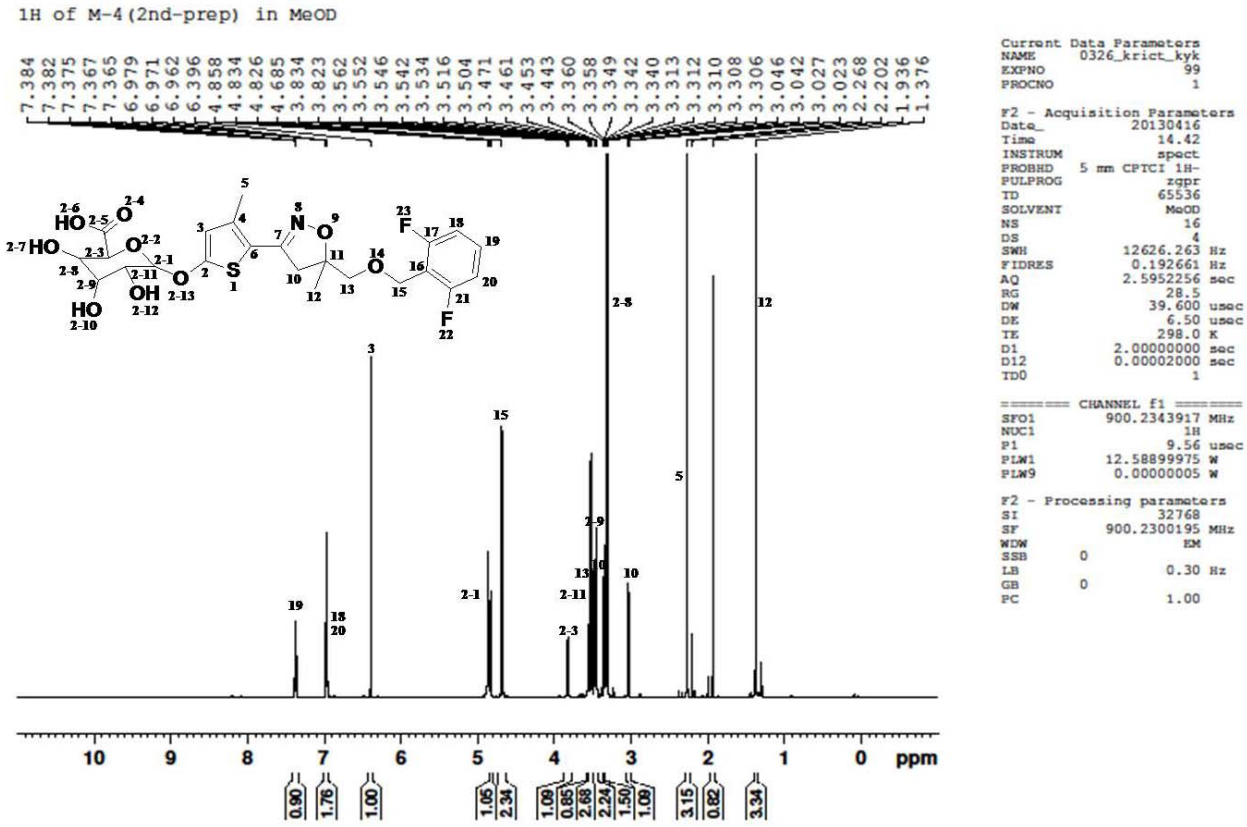
^{1}H NMR Data

	Methiozolin		AMet-1		AMet-2	
Proton no.	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ ($CDCl_3$)	J (Hz)
2	7.28 (m)		*b*		7.30 (m)	
2-1	*b*		4.83 (d)	7.2	*b*	
2-3	*b*		3.80 (d)	9.9	*b*	
2-8	*b*		3.35 (s)		*b*	
2-9	*b*		3.46 (m)		*b*	
2-11	*b*		3.55 (m)		*b*	
3	6.91 (m)		6.40 (s)		7.08 (m)	
5	2.46 (3H, s)		2.27 (3H, s)		4.70 (2H, s)	
5*a*	*b*		*b*		4.16 (br)	
10	3.02 (d) 3.45 (d)	16.45 16.50	3.03 (dd) 3.35 (m)	3.6	2.98 (d) 3.52 (d)	16.64 16.62
12	1.47 (3H, s)		1.38 (s)		1.45 (3H, s)	
13	3.55 (2H, dd)	9.85, 9.90	3.52 (2H, m)		3.56 (2H, m)	
15	4.72 (2H, s)		4.69 (2H, 2H)		4.70 (2H, s)	
18*c*	6.91 (m)		6.97 (2H, m)		6.89 (m)	
19	7.28 (m)		7.38 (m)		7.30 (m)	
20*c*	6.91 (m)		6.97 (2H, m)		6.89 (m)	

^{13}C NMR Data

	Methiozolin		AMet-1		AMet-2	
Carbon no.	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ ($CDCl_3$)	J (Hz)
2	126.5		132.2		128.6	
2-1	*d*		105.9		*d*	
2-3	*d*		77.6		*d*	
2-5	*d*		176.6		*d*	
2-8	*d*		73.3		*d*	
2-9	*d*		75.6		*d*	
2-11	*d*		61.8		*d*	
3	125.8		132.1		126.4	
4	138.7		138.7		143.1	
5	16.4		16.8		59.1	
6	125.8		116.0		128.6	
7	152.5		162.9		152.2	
10	45.5		46.2		44.7	
11	74.3		74.5		73.9	
12	23.3		23.2		23.0	
13	85.9		87.5		86.7	
15	60.9		75.6		60.3	
16	113.3		112.5		113.3	
17e	160.9		164.0		161.2	
18	111.4		112.5		111.2	
19	131.9		132.1		130.4	
20	111.4		112.5		111.2	
21e	163.3		154.6		162.6	

[a] OH, [b] no proton, [c] H-F coupling, [d] no carbon, [e] C-F coupling

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PROCNO                 1

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PULPROG            zgpr
TD                65536
SOLVENT            MeOD
NS                    16
DS                     4
SWH            12626.263 Hz
FIDRES         0.192661 Hz
AQ             2.5952256 sec
RG                  28.5
DW                39.600 usec
DE                 6.50 usec
TE                298.0 K
D1             2.00000000 sec
D12            0.00002000 sec
TD0                    1

======== CHANNEL f1 ========
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NUC1                   1H
P1                   9.56 usec
PLW1          12.58899975 W
PLW9           0.00000005 W

F2 - Processing parameters
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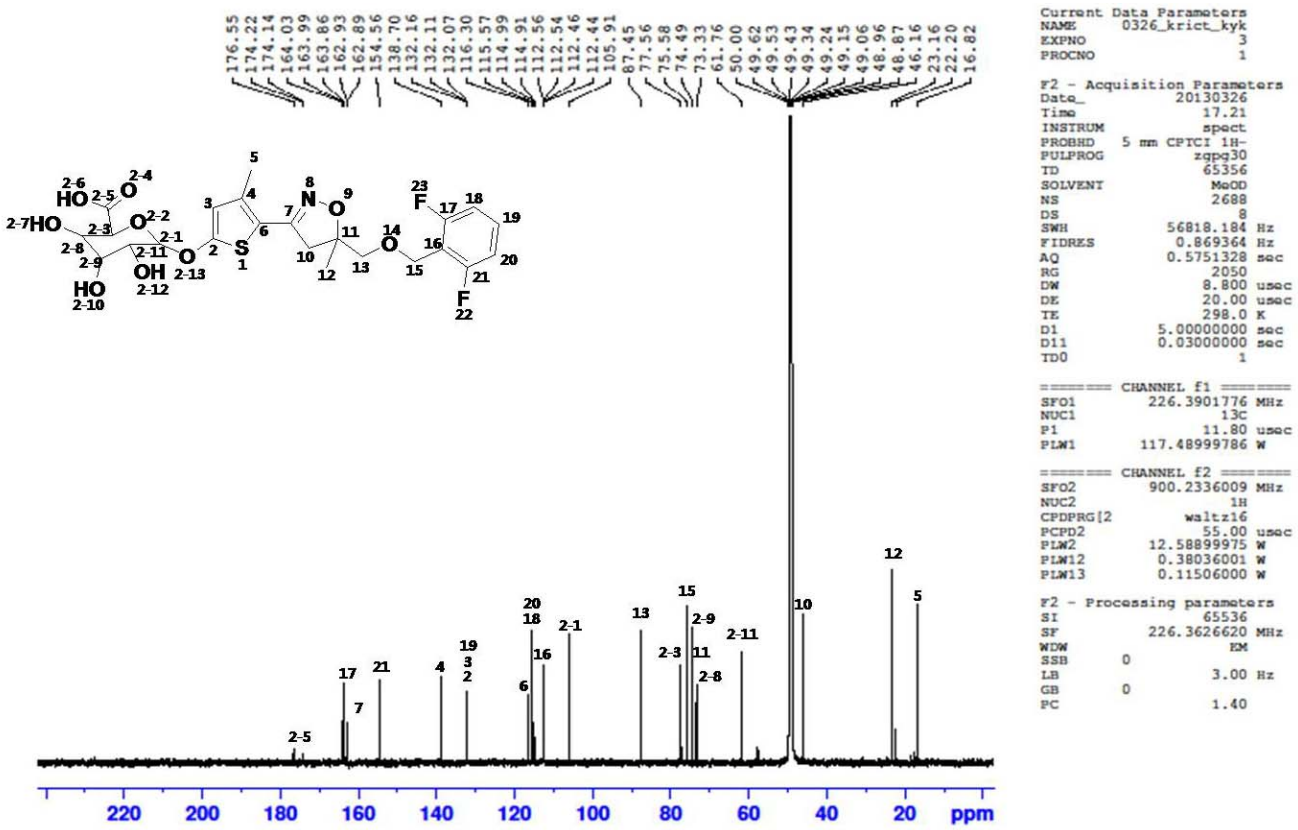
Figure 29. ^{1}H-NMR spectrum for AMet-1 isolated from the feces.

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PROCNO                 1

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SOLVENT           MeOD
NS                2688
DS                   8
SWH           56818.184 Hz
FIDRES        0.869364 Hz
AQ            0.5751328 sec
RG                2050
DW                8.800 usec
DE               20.00 usec
TE               298.0 K
D1           5.00000000 sec
D11          0.03000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1        226.3901776 MHz
NUC1                13C
P1                11.80 usec
PLW1       117.48999786 W

======== CHANNEL f2 ========
SFO2        900.2336009 MHz
NUC2                 1H
CPDPRG[2         waltz16
PCPD2             55.00 usec
PLW2        12.58899975 W
PLW12        0.38036001 W
PLW13        0.11506000 W

F2 - Processing parameters
SI                 65536
SF          226.3626620 MHz
WDW                   EM
SSB      0
LB                 3.00 Hz
GB       0
PC                 1.40
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Figure 30. ^{13}C-NMR spectrum for AMet-1 isolated from the feces.

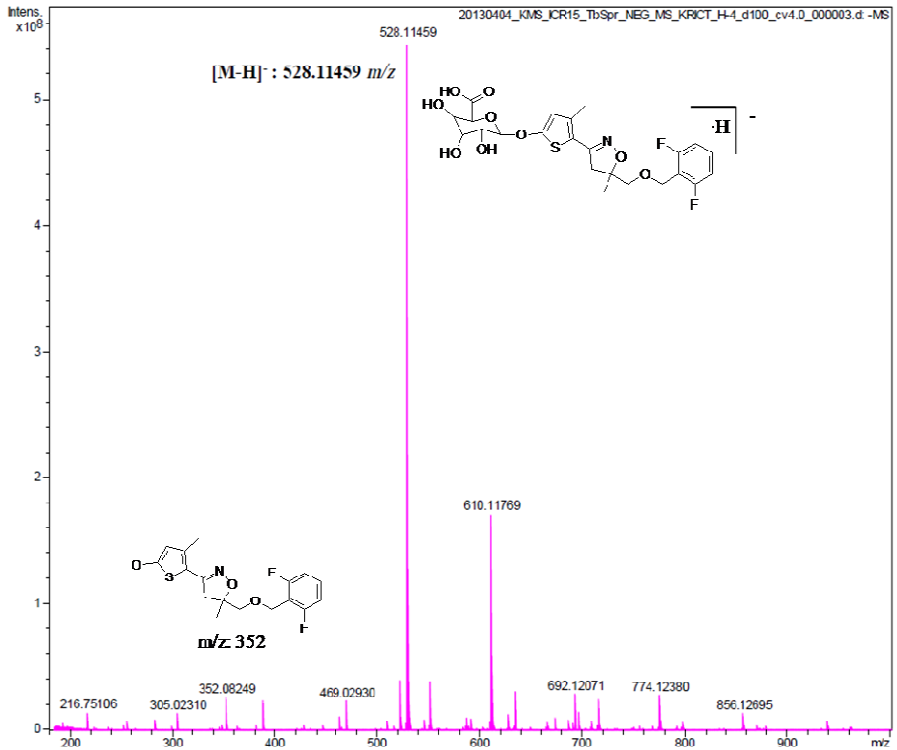

Figure 31. ESI negative MSMS spectrum for AMet-1 isolated from the feces.

(A) Isolated AMet-2

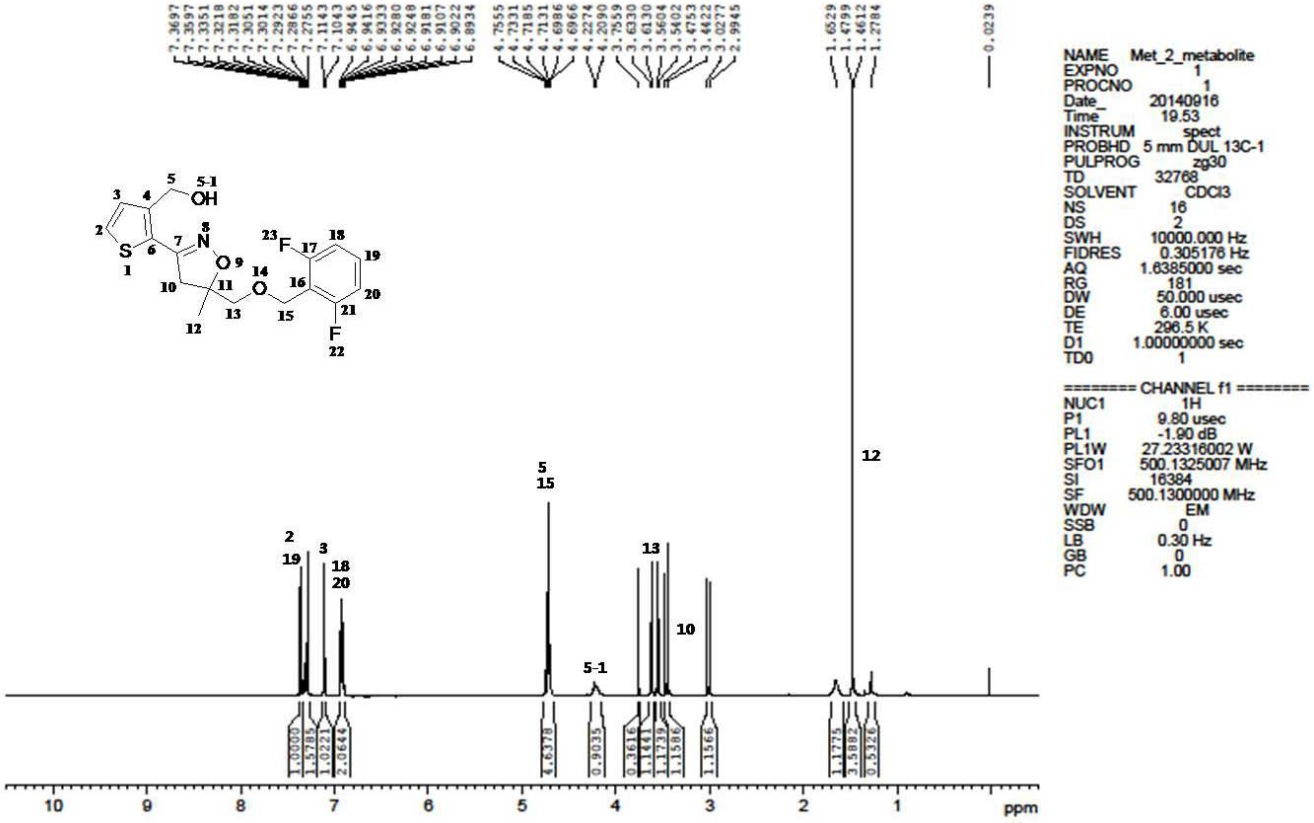

Figure 32. ^{1}H-NMR spectrums for AMet-2 isolated (A) from the feces and synthesized (B).

(B) Synthesized AMet-2

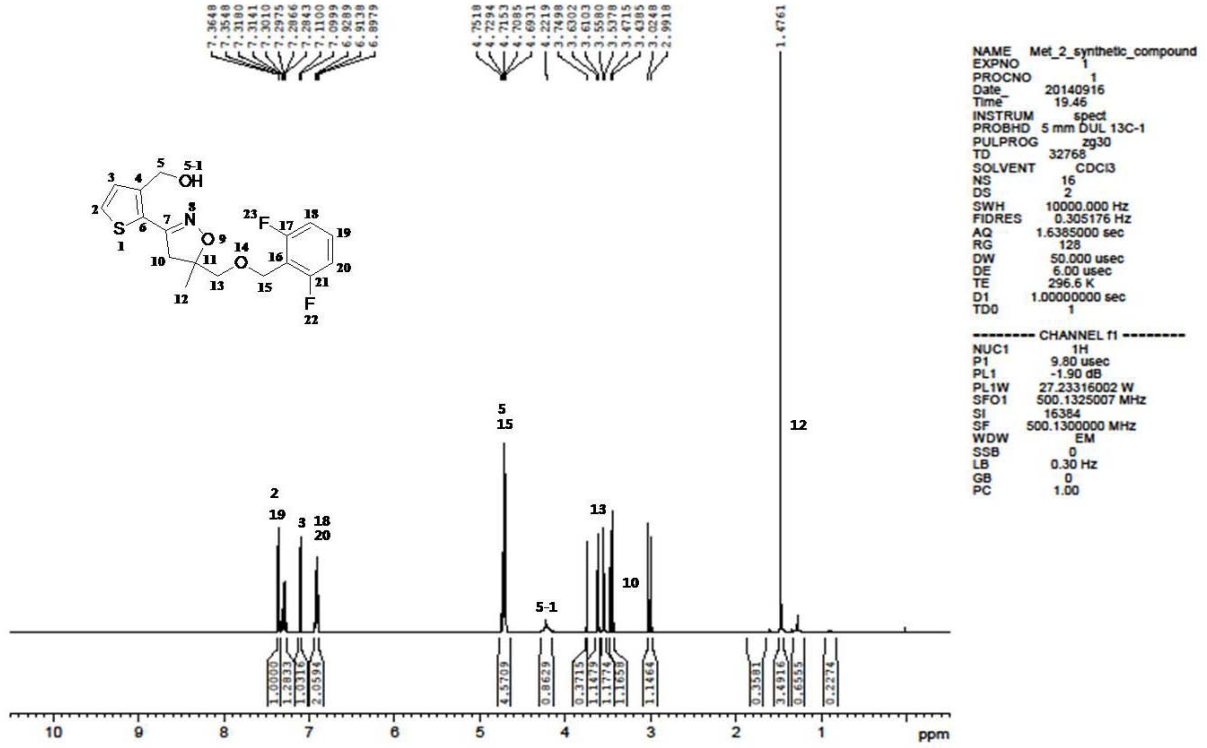

Figure 32. ^{1}H-NMR spectrums for AMet-2 isolated (A) from the feces and synthesized (B) (continued).

(A) Isolated AMet-2

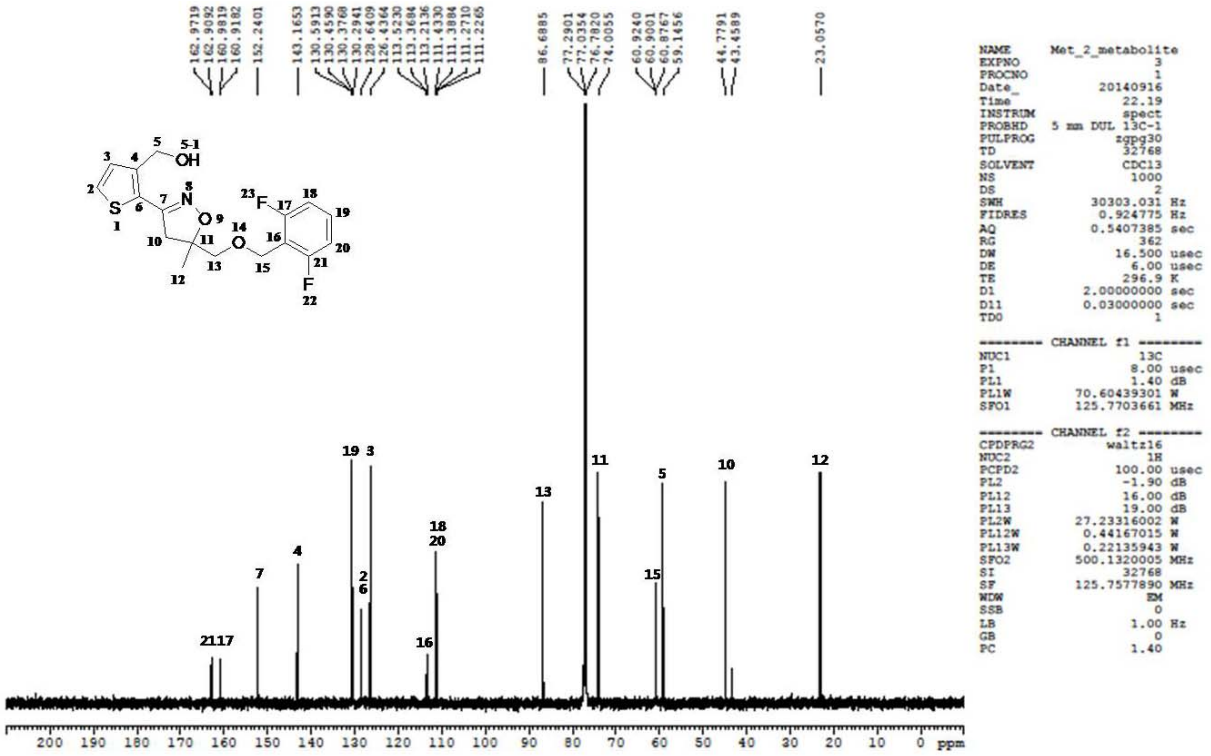

Figure 33. ^{13}C-NMR spectrums for AMet-2 isolated (A) from the feces and synthesized (B).

(B) Synthesized AMet-2

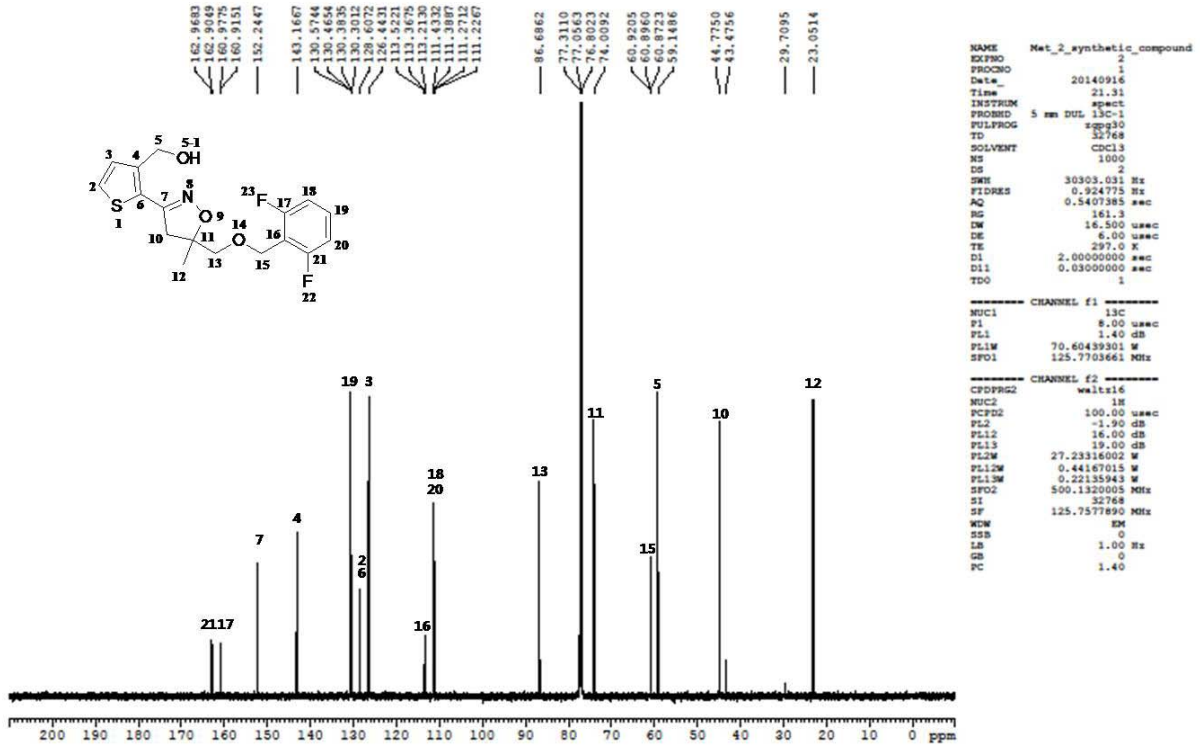

Figure 33. ^{13}C-NMR spectrums for AMet-2 isolated (A) from the feces and synthesized (B) (continued).

(A) Isolated AMet-2

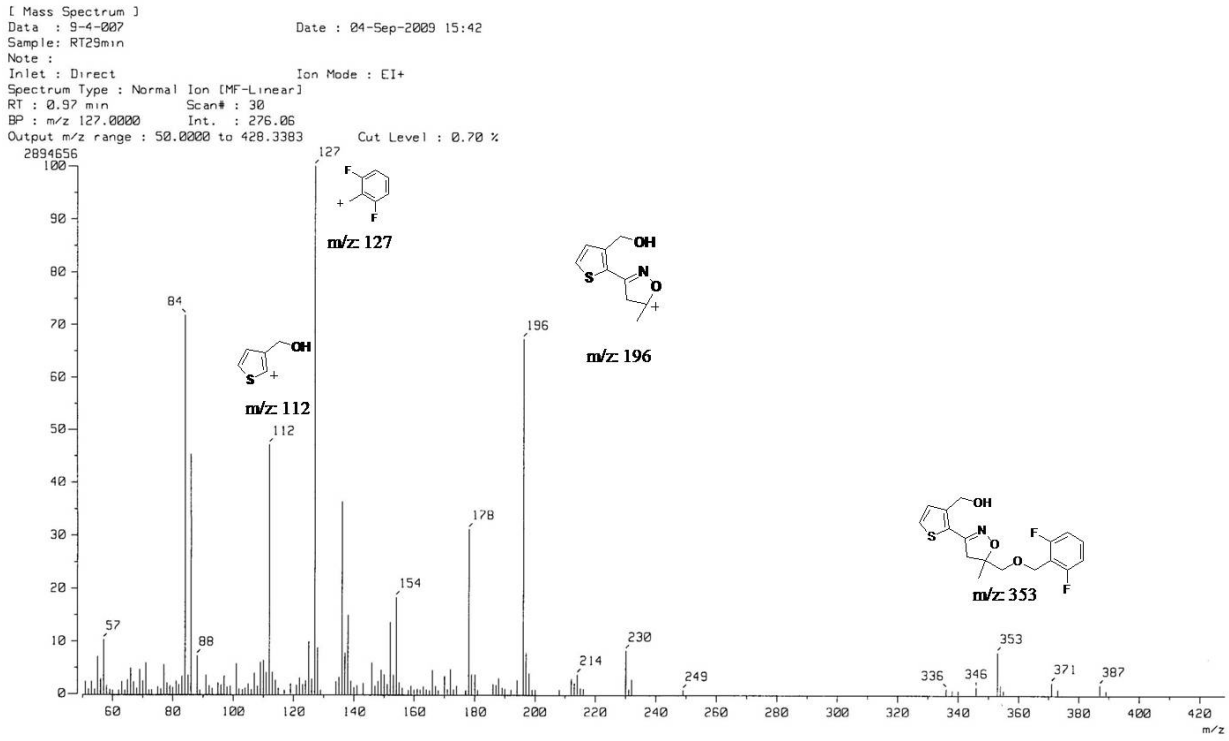

Figure 34. EI-MS spectrums for AMet-2 isolated (A) from the feces (A) and synthesized (B).

(B) Synthesized AMet-2

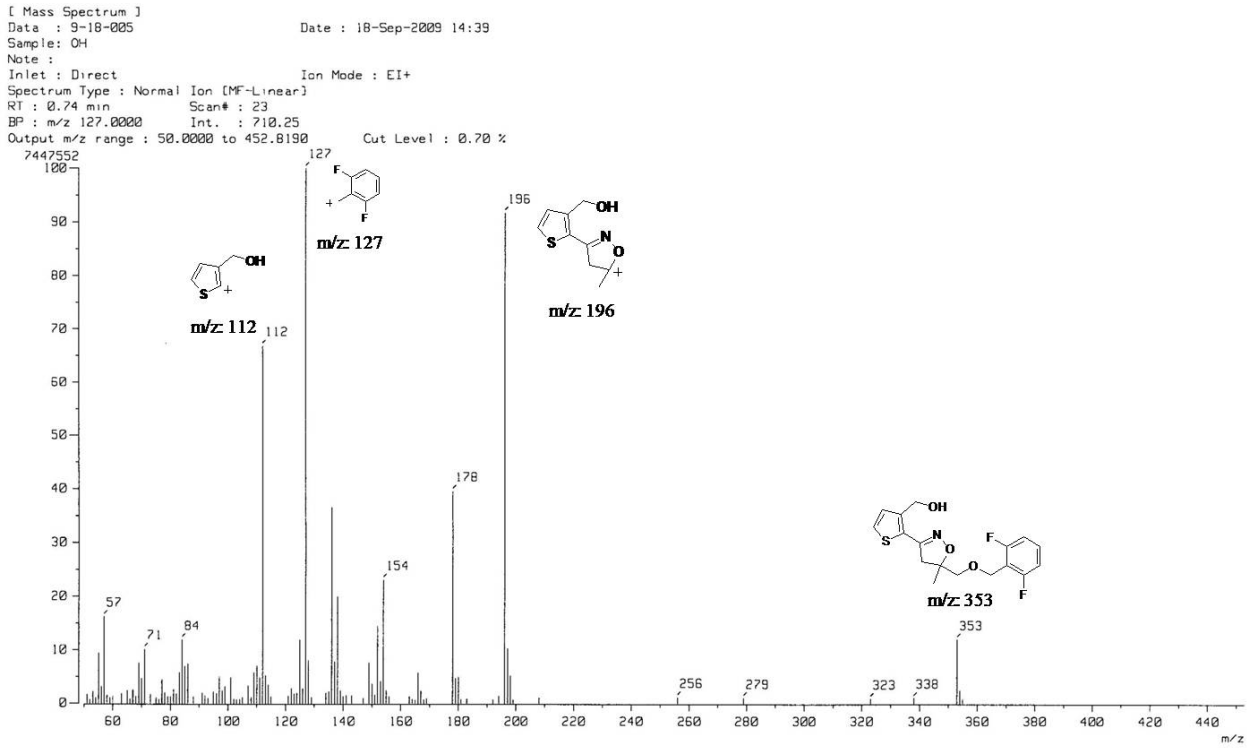

Figure 34. EI-MS spectrums for AMet-2 isolated (A) from the feces (A) and synthesized (B) (continued).

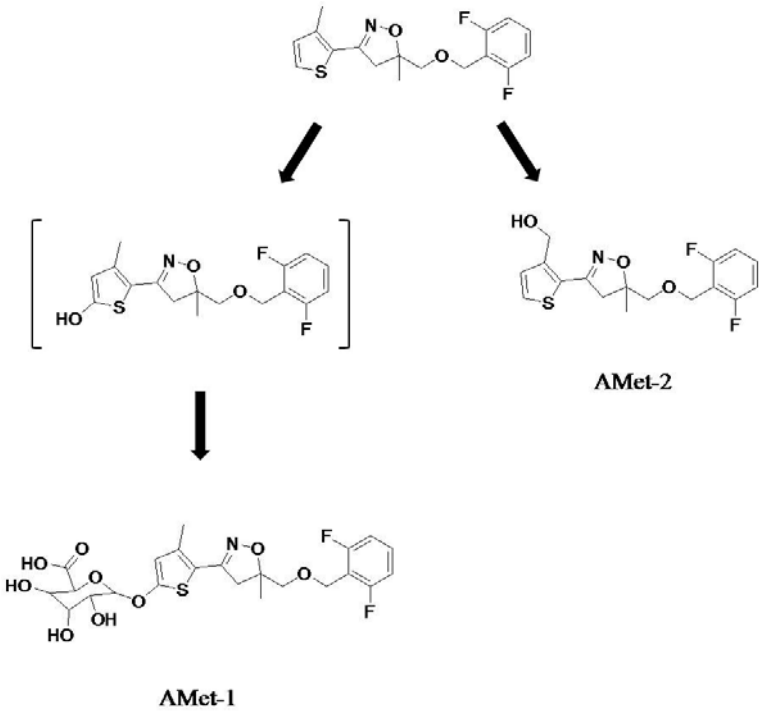

Figure 35. Proposed metabolic pathways of methiozolin in rats following oral administration.

IV. Conclusion

The absorption, tissue distribution, metabolism, and excretion studies of a new herbicide methiozolin in a rat following oral administration were investigated using ^{14}C-labeled compound. The estimated pharmacokinetic parameters and depletion curve for blood and plasma were very similar. Methiozolin was shown to be rapidly absorbed within 12 h, distributed throughout the tissues without a target tissue accumulation, and eliminated through urine and feces mostly within 48 h. The total excretion through urine and feces was 24.3 and 68.9%, respectively, during 120 h after administration, but there was no excretion through the expired air. The absorption was calculated to be 64.4% of the administered. Numerous minor metabolites (<4% of the dosed) in urine and feces extract were detected within 72 h and two of those were identified. The identified metabolites were AMet-1, 6-(5-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)-4-methylthiophen-2-yloxy)-tetrahydro-3,4,5-trihydroxy-2H-pyran-2-carboxylic acid], and AMet-2, (2-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)thiophen-3-yl)methanol. The biotransformation reactions are proposed to hydroxylation of methyl moiety and glucuronic acid conjugation in thiophene ring. In summary, methiozolin was shown to be readily absorbed in the gastrointestinal tract, distributed throughout the tissues within 12 h, metabolized extensively and eliminated through urine and feces mostly within 48h, without tissue accumulation and expired air excretion.

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APPENDICES

Appendix 1. Mass balance of ^{14}C radioactivity in the sterile soil under aerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

[Benzyl-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	97.1 ± 0.4	0.4 ± 0.0	ns	ns	97.5 ± 0.3
7	98.0 ± 0.5	1.1 ± 0.0	nd	nd	99.1 ± 0.4
14	97.1 ± 0.3	1.2 ± 0.0	nd	nd	98.4 ± 0.2
30	95.5 ± 0.4	2.1 ± 0.1	nd	nd	97.6 ± 0.2

[Isoxazole-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	101.0 ± 0.6	0.2 ± 0.1	ns	ns	101.2 ± 0.6
7	98.2 ± 0.7	1.0 ± 0.1	nd	nd	99.2 ± 0.7
14	98.3 ± 0.5	2.5 ± 0.1	nd	nd	100.9 ± 0.5
30	96.7 ± 0.5	2.4 ± 0.3	nd	nd	99.1 ± 0.8

nd, not detected; ns, no sample

Appendix 2. Degradation of [^{14}C]methiozolin and formation of its metabolites in the sterile soil under the aerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

DAT	[Benzyl-^{14}C]		[Isoxazole-^{14}C]	
	Methiozolin	Metabolites (0) [a]	Methiozolin	Metabolites (0)
0	97.1 ± 1.2	0.0 ± 0.0	97.8 ± 3.6	0.0 ± 0.0
7	98.6 ± 3.4	0.0 ± 0.0	97.8 ± 1.2	0.0 ± 0.0
14	97.8 ± 1.4	0.0 ± 0.0	96.4 ± 2.1	0.0 ± 0.0
30	97.0 ± 1.7	0.0 ± 0.0	96.0 ± 1.7	0.0 ± 0.0

[a] total number of detected metabolites

Appendix 3. Mass balance of radioactivity in the nonsterile soil under aerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

[Benzyl-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	103.4 ± 1.5	1.1 ± 0.2	ns	ns	104.5 ± 1.5
7	67.4 ± 0.7	21.3 ± 0.8	nd	7.5 ± 1.1	96.2 ± 4.0
14	57.7 ± 0.5	24.3 ± 1.4	nd	14.0 ± 1.3	96.0 ± 2.5
30	41.3 ± 1.4	29.1 ± 3.1	nd	21.5 ± 1.2	92.0 ± 3.3
60	27.7 ± 2.1	35.3 ± 0.7	nd	31.5 ± 0.5	94.5 ± 1.3
90	24.9 ± 2.8	32.0 ± 1.8	nd	38.0 ± 0.6	94.8 ± 1.0
120	21.8 ± 1.4	35.7 ± 3.4	nd	41.5 ± 0.7	99.0 ± 5.0

[Isoxazole-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	102.6 ± 1.7	0.3 ± 0.0	ns	ns	103.0 ± 1.7
7	69.5 ± 1.9	22.2 ± 3.8	nd	0.0 ± 0.0	91.7 ± 0.8
14	57.0 ± 3.4	29.4 ± 3.1	nd	12.2 ± 2.5	98.5 ± 2.9
30	37.9 ± 2.7	36.1 ± 1.3	nd	19.0 ± 3.3	93.0 ± 3.4
60	26.1 ± 1.5	40.8 ± 1.0	nd	26.3 ± 4.4	93.2 ± 2.9
90	21.2 ± 0.2	39.3 ± 1.1	nd	32.2 ± 4.9	92.8 ± 4.8
120	18.4 ± 1.0	39.8 ± 3.5	nd	36.1 ± 4.8	94.3 ± 2.9

nd, not detected; ns, no sample

Appendix 4. Degradation of [^{14}C]methiozolin and formation of its metabolites in the nonsterile soil under the aerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

DAT	[Benzyl-^{14}C]		[Isoxazole-^{14}C]	
	Methiozolin	Metabolites (7)[a]	Methiozolin	Metabolites (7)
0	103.4 ± 1.6	0.0 ± 0.0	102.6 ± 1.0	0.0 ± 0.0
7	63.8 ± 0.1	3.5 ± 0.8	67.6 ± 1.0	1.2 ± 0.3
14	56.2 ± 0.7	1.5 ± 0.2	54.7 ± 1.9	1.0 ± 1.0
30	37.5 ± 2.7	3.8 ± 1.3	34.7 ± 3.1	1.6 ± 0.4
60	22.7 ± 0.9	5.0 ± 1.2	22.3 ± 1.3	3.5 ± 0.2
90	20.8 ± 2.1	4.1 ± 0.6	17.1 ± 1.4	2.3 ± 1.0
120	17.9 ± 0.8	3.9 ± 0.6	15.9 ± 0.7	2.2 ± 0.4

[a] total number of detected metabolites

Appendix 5. Characterization of nonextractable radioactivity in the nonsterile soil under the aerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

[Benzyl-^{14}C]Methiozolin

DAT	% Humin	% Fulvic acid	% Humic acid	Total
7	8.0 ± 0.3	7.5 ± 0.3	5.8 ± 0.3	21.3 ± 0.8
14	9.1 ± 0.8	8.6 ± 0.5	6.6 ± 0.1	24.3 ± 1.4
30	11.2 ± 1.8	10.6 ± 0.8	7.3 ± 0.5	29.1 ± 3.1
60	13.5 ± 0.3	13.3 ± 0.8	8.5 ± 0.0	35.3 ± 0.7
90	11.9 ± 0.6	11.4 ± 0.5	8.6 ± 0.7	32.0 ± 1.8
120	13.6 ± 1.4	12.4 ± 1.3	9.7 ± 0.9	35.7 ± 3.4

[Isoxazole-^{14}C]Methiozolin

DAT	% Humin	% Fulvic acid	% Humic acid	Total
7	8.7 ± 1.5	7.5 ± 1.3	6.1 ± 1.0	22.2 ± 3.8
14	11.3 ± 1.9	10.2 ± 0.7	7.9 ± 0.6	29.4 ± 3.1
30	14.3 ± 1.1	12.2 ± 0.3	9.6 ± 0.3	36.1 ± 1.3
60	16.4 ± 0.1	14.1 ± 0.2	10.4 ± 0.8	40.8 ± 1.0
90	16.2 ± 0.6	13.7 ± 0.3	9.4 ± 0.3	39.3 ± 1.1
120	16.6 ± 1.2	14.0 ± 1.2	9.2 ± 0.7	39.8 ± 3.5

Appendix 6. Mass balance of radioactivity in the nonsterile soil under the anaerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

[Benzyl-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	99.3 ± 0.9	0.0 ± 0.0	ns	ns	99.3 ± 0.9
7	87.4 ± 0.8	7.5 ± 0.4	nd	0.4 ± 0.0	95.3 ± 1.1
14	86.4 ± 0.7	7.1 ± 0.5	nd	0.7 ± 0.0	94.2 ± 1.1
30	86.9 ± 0.5	7.6 ± 0.4	nd	1.2 ± 0.3	95.7 ± 0.7
60	82.6 ± 0.8	8.0 ± 0.2	nd	2.6 ± 1.2	93.2 ± 1.3
90	83.7 ± 0.9	6.7 ± 0.4	nd	4.3 ± 2.2	94.7 ± 2.2
120	80.8 ± 0.8	7.5 ± 0.5	nd	5.2 ± 2.1	93.5 ± 2.2

[Isoxazole-^{14}C]Methiozolin

DAT	Extractable	Non-extractable	Volatile		Total radioactivity
			Organic	CO_2	
0	102.0 ± 1.7	0.0 ± 0.0	ns	ns	102.0 ± 1.7
7	94.7 ± 4.1	7.7 ± 0.3	nd	nd	102.5 ± 2.4
14	91.2 ± 1.3	8.0 ± 0.2	nd	0.1 ± 0.0	99.3 ± 0.6
30	92.9 ± 0.7	6.0 ± 0.6	nd	0.2 ± 0.1	99.1 ± 1.0
60	88.4 ± 0.8	6.9 ± 1.1	nd	0.8 ± 0.3	96.0 ± 1.7
90	85.5 ± 1.5	7.6 ± 1.2	nd	2.3 ± 1.1	95.4 ± 2.5
120	81.1 ± 1.3	10.5 ± 0.5	nd	3.5 ± 1.2	95.2 ± 2.1

nd, not detected; ns, no sample

Appendix 7. Degradation of [^{14}C]methiozolin and formation of its metabolites in the nonsterile soil under the anaerobic condition treated with [^{14}C]methiozolin at 500 g ai/ha incubated at 25°C. Results are expressed as % of applied radioactivity (% AR).

DAT	[Benzyl-^{14}C]		[Isoxazole-^{14}C]	
	Methiozolin	Metabolites (1)[a]	Methiozolin	Metabolites (1)
0	99.3 ± 0.9	0.0 ± 0.0	102.0 ± 1.7	0.0 ± 0.0
7	85.0 ± 0.8	2.4 ± 0.3	92.6 ± 4.1	2.1 ± 0.4
14	84.8 ± 0.8	1.6 ± 0.2	90.3 ± 0.6	0.9 ± 0.8
30	83.3 ± 0.3	3.6 ± 0.4	91.7 ± 0.7	1.2 ± 0.2
60	78.2 ± 0.6	4.4 ± 0.4	83.6 ± 0.5	4.8 ± 0.3
90	76.9 ± 1.1	6.8 ± 0.5	77.8 ± 0.6	7.7 ± 1.0
120	74.6 ± 0.3	6.2 ± 0.7	73.1 ± 0.8	7.9 ± 1.3

[a] total number of detected metabolites

Appendix 8. Concentration of ^{14}C radioactivity in the blood and plasma of rats following oral administration of [^{14}C]methiozolin at 500 mg/kg body weight.

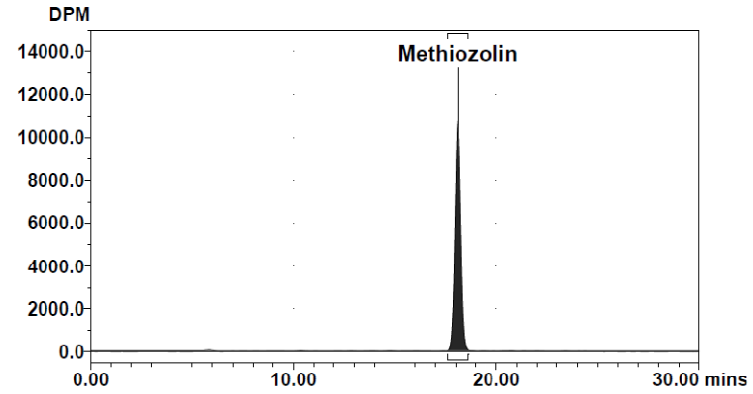
Time (h)	Radioactivity	
	Blood	Plasma
	(μg methiozolin equivalent/g)	
0-3	91.0 ± 10.8	129.8 ± 10.1
3-6	168.7 ± 14.9	162.0 ± 7.6
6-12	156.1 ± 11.0	150.9 ± 2.7
12-24	140.8 ± 9.3	112.3 ± 12.1
24-48	81.0 ± 13.0	93.0 ± 11.2
48-72	66.2 ± 5.0	46.3 ± 6.3
72-96	39.2 ± 7.4	32.1 ± 9.3
96-120	39.6 ± 9.0	27.5 ± 11.3

Appendix 9. Daily and cumulative amounts of ^{14}C radioactivity excreted from bile of rats after oral administration of [^{14}C]methiozolin at 500 mg/kg body weight.

Time (h)	Radioactivity in bile	
	Daily	Cumulative
	(% of administrated radioactivity)	
0-6	11.9 ± 2.3	11.9 ± 2.3
6-12	10.2 ± 2.8	22.1 ± 1.5
12-24	8.5 ± 2.9	30.6 ± 2.4
24-48	7.2 ± 1.5	37.8 ± 2.3
48-72	1.8 ± 1.7	39.7 ± 3.3
72-96	0.4 ± 0.3	40.0 ± 3.5
96-120	0.1 ± 0.1	40.1 ± 3.5

Appendix 10. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

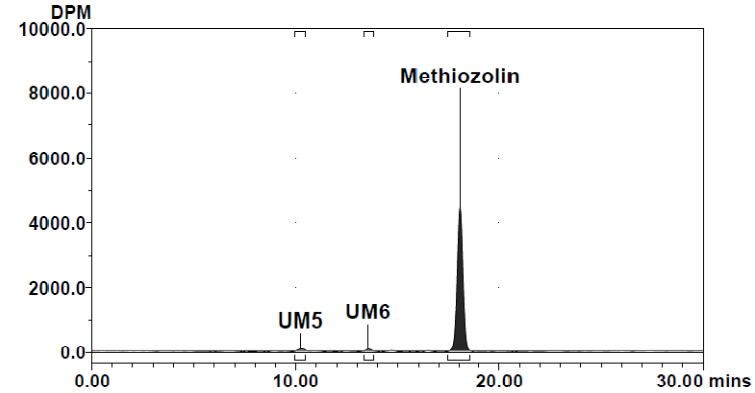


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	5.43	5.47	5.43			
Methiozolin	17.60	18.63	18.10	106528.8	100.00	98.75
Bkg 2	18.63	19.00	18.83			
1 Peak				106528.8	100.00	98.75

Total Area: 107078.5 DPM
Average Background: 47.6 DPM
Unallocated Area: 1349.7 Counts

[7 DAT]



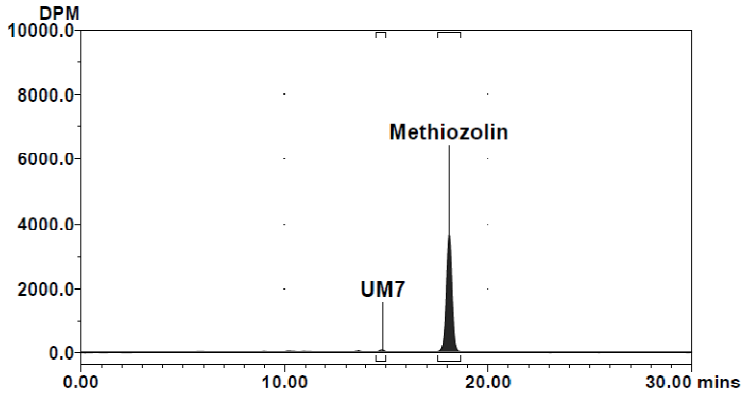
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
UM5	9.97	10.50	10.23	773.3	1.74	1.70
Bkg 1	10.63	11.00	10.83			
UM6	13.33	13.80	13.53	586.7	1.32	1.29
Methiozolin	17.43	18.53	18.07	42986.7	96.93	94.66
3 Peaks				44346.7	100.00	97.65

Total Area: 45413.3 DPM
Average Background: 53.3 DPM
Unallocated Area: 1066.7 Counts

Appendix 10. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

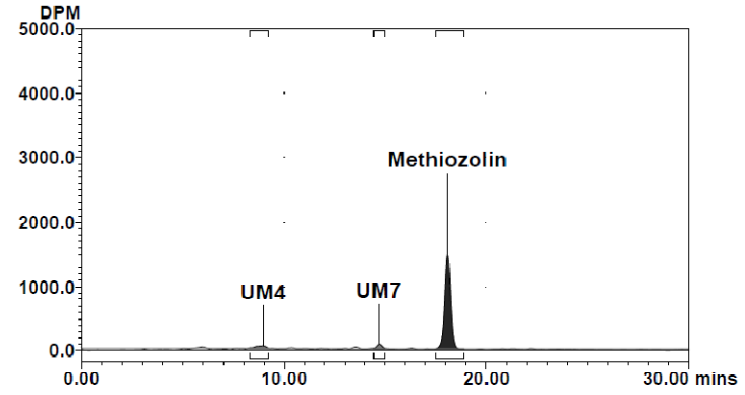


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
UM7	14.47	15.00	14.83	635.2	1.76	1.56
Methiozolin	17.53	18.63	18.07	35538.2	98.24	87.56
Bkg 1	20.60	20.97	20.73			
2 Peaks				36173.3	100.00	89.13

Total Area: 40586.7 DPM
Avcragc Background: 31.5 DPM
Unallocated Area:4413.3 Counts

[30 DAT]



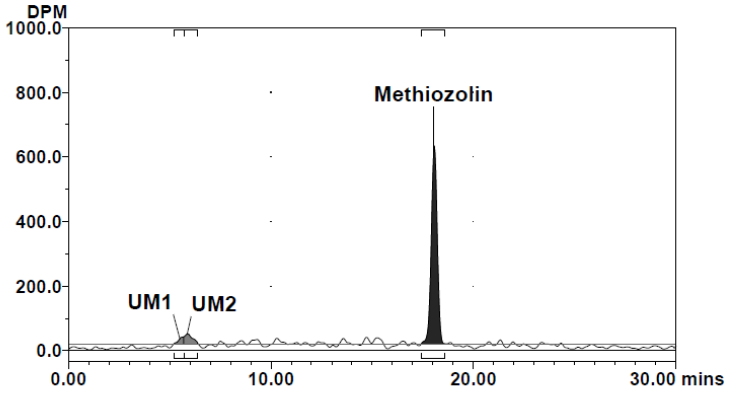
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	7.60	7.97	7.93			
UM4	8.30	9.20	8.97	781.7	4.92	4.16
UM7	14.43	14.97	14.67	652.4	4.11	3.47
Bkg 2	14.97	15.33	15.10			
Methiozolin	17.50	18.87	18.07	14442.4	90.97	76.84
3 Peaks				15876.5	100.00	84.47

Total Area: 18794.9 DPM
Average Background: 27.8 DPM
Unallocated Area:2918.4 Counts

Appendix 10. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[60 DAT]

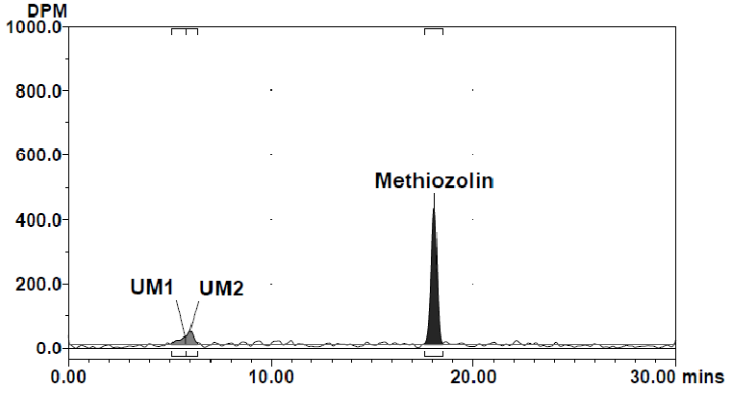


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
UM1	5.20	5.70	5.50	240.0	3.39	2.10
UM2	5.70	6.37	5.87	486.7	6.87	4.25
Methiozolin	17.43	18.57	18.03	6353.3	89.74	55.54
Bkg 1	28.80	28.87	28.83			
3 Peaks				7080.0	100.00	61.89

Total Area: 11440.0 DPM
Average Background: 20.0 DPM
Unallocated Area:4360.0 Counts

[90 DAT]



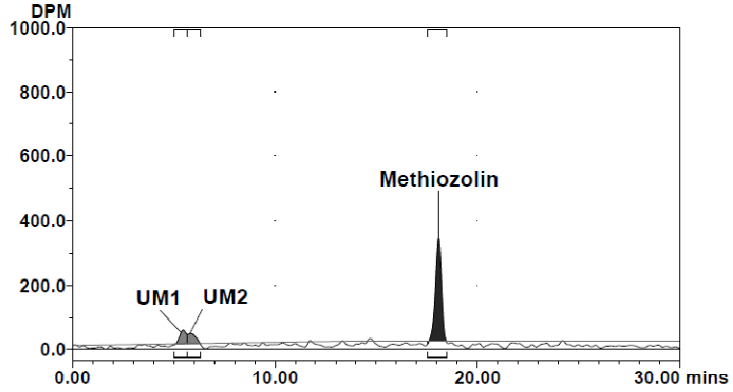
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
UM1	5.10	5.80	5.77	360.0	7.28	4.12
UM2	5.80	6.37	6.00	426.7	8.63	4.89
Methiozolin	17.57	18.47	18.07	4160.0	84.10	47.63
Bkg 1	29.60	29.63	29.60			
3 Peaks				4946.7	100.00	56.64

Total Area: 8733.3 DPM
Average Background: 13.3 DPM
Unallocated Area:3786.7 Counts

Appendix 10. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[120 DAT]



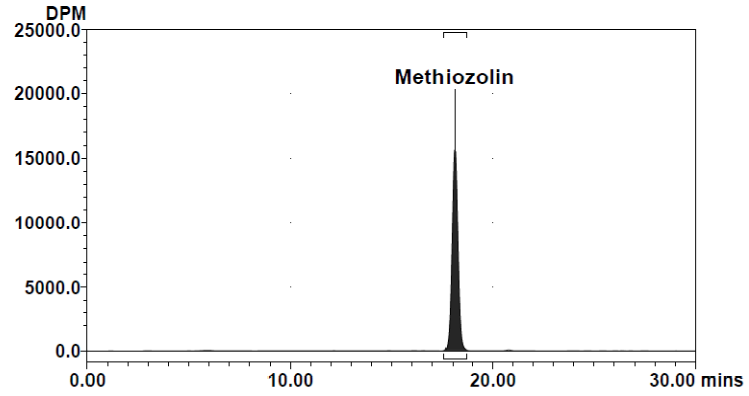
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.43	0.47	0.43			
UM1	5.03	5.67	5.37	510.8	11.72	8.40
UM2	5.67	6.33	5.80	514.3	11.80	8.46
Bkg 2	14.20	14.23	14.20			
Methiozolin	17.53	18.47	18.10	3333.3	76.48	54.83
3 Peaks				4358.5	100.00	71.69

Total Area: 6079.9 DPM
Average Background: 23.4 DPM
Unallocated Area: 1721.4 Counts

Appendix 11. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

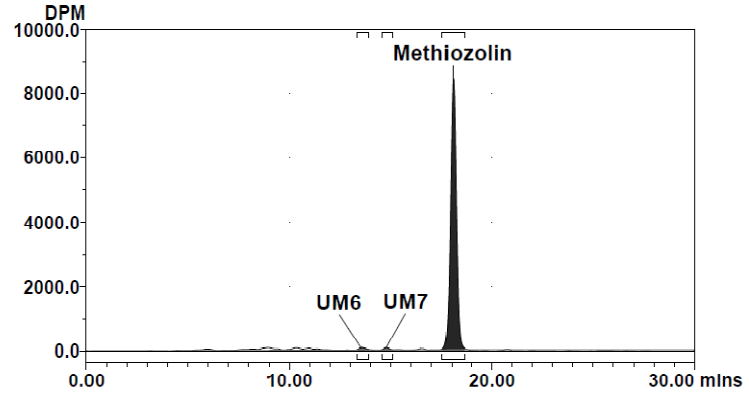


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.13			
Methiozolin	17.60	18.67	18.10	154873.7	100.00	97.08
Bkg 2	18.67	19.03	18.67			
1 Peak				154873.7	100.00	97.08

Total Area: 159530.3 DPM
Average Background: 23.5 DPM
Unallocated Area: 4656.6 Counts

[7 DAT]



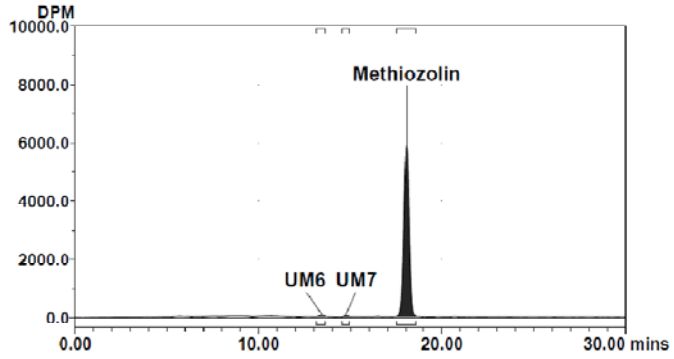
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.23			
UM6	13.33	13.93	13.53	807.4	0.97	0.89
UM7	14.60	15.13	14.80	637.6	0.77	0.70
Methiozolin	17.57	18.63	18.07	81859.0	98.27	89.82
Bkg 2	18.63	19.00	18.63			
3 Peaks				83304.0	100.00	91.41

Total Area: 91132.0 DPM
Average Background: 33.7 DPM
Unallocated Area: 7828.0 Counts

Appendix 11. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

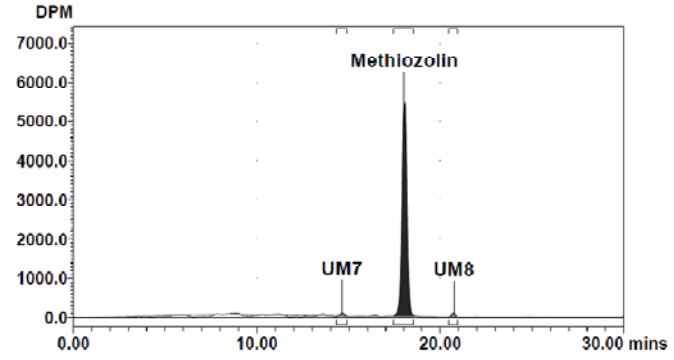


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.20			
Bkg 2	8.33	8.70	8.47			
UM6	13.17	13.63	13.47	702.1	1.17	1.12
Bkg 3	13.63	14.00	13.77			
UM7	14.53	14.93	14.73	567.7	0.95	0.90
Bkg 4	14.93	15.30	15.20			
Methiozolin	17.50	18.53	18.07	58512.3	97.88	93.18
Bkg 5	18.68	18.90	18.67			
3 Peaks				59782.1	100.00	95.20

Total Area: 62797.8 DPM
Average Background: 54.2 DPM
Unallocated Area:3015.8 Counts

[30 DAT]



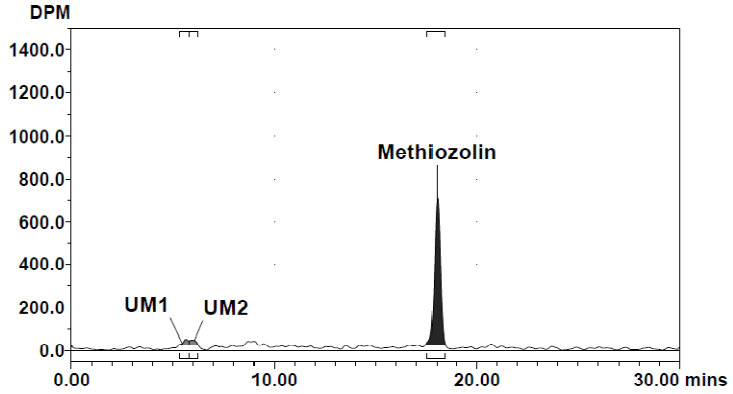
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.00			
Bkg 2	7.70	8.07	7.77			
Bkg 3	10.23	10.60	10.33			
Bkg 4	13.80	14.17	14.03			
UM7	14.33	14.93	14.63	754.3	1.50	1.37
Bkg 5	14.93	15.30	15.20			
Methiozolin	17.43	18.57	18.03	48793.5	97.21	88.69
Bkg 6	18.57	18.93	18.63			
UM8	20.47	20.93	20.73	646.1	1.29	1.17
Bkg 7	20.93	21.30	21.23			
3 Peaks				50193.8	100.00	91.24

Total Area: 55015.9 DPM
Average Background: 43.0 DPM
Unallocated Area:4822.1 Counts

Appendix 11. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[60 DAT]

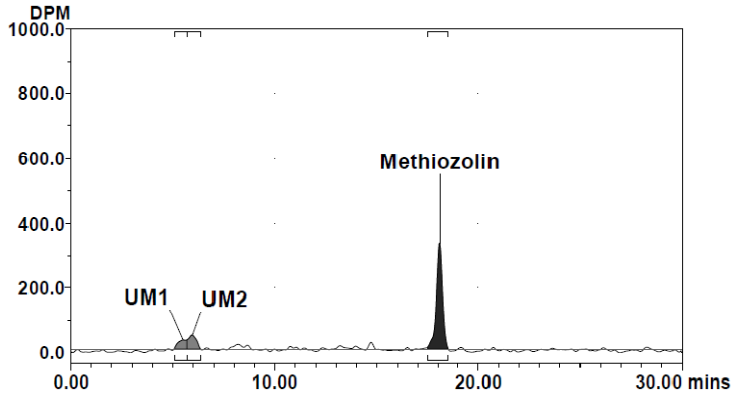


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
UM1	5.33	5.80	5.47	306.7	4.21	3.39
UM2	5.80	6.23	6.07	333.3	4.58	3.69
Bkg 1	10.87	10.90	10.87			
Methiozolin	17.53	18.40	18.03	6640.0	91.21	73.45
3 Peaks				7280.0	100.00	80.53

Total Area: 9040.0 DPM
Average Background: 26.7 DPM
Unallocated Area:1760.0 Counts

[90 DAT]



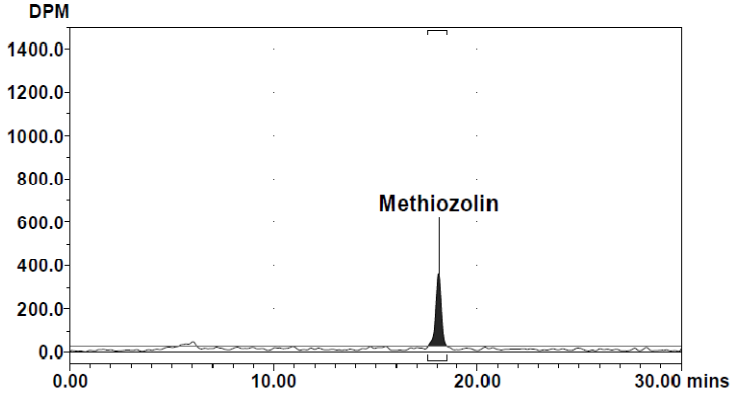
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.33	0.37	0.33			
UM1	5.10	5.70	5.50	440.0	9.79	5.76
UM2	5.70	6.37	5.97	600.0	13.35	7.85
Methiozolin	17.50	18.50	18.10	3453.3	76.85	45.20
3 Peaks				4493.3	100.00	58.81

Total Area: 7640.0 DPM
Average Background: 13.3 DPM
Unallocated Area:3146.7 Counts

Appendix 11. Representative radio-HPLC chromatograms for the extract of the nonsterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[120 DAT]



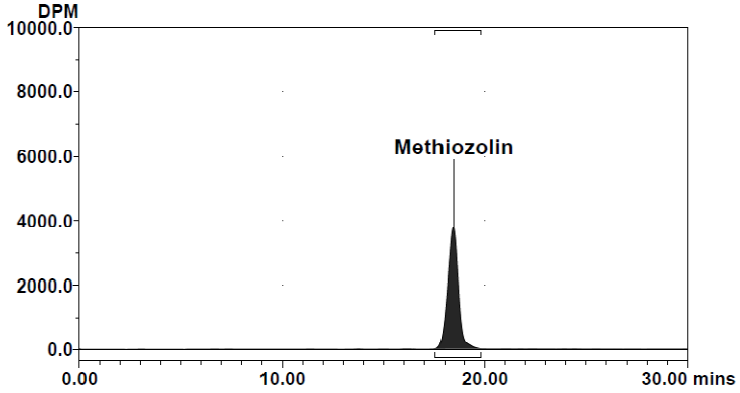
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Rkg 1	1.53	1.57	1.53			
Methiozolin	17.53	18.50	18.10	3280.0	100.00	70.89
1 Peak				3280.0	100.00	70.89

Total Area: 4626.7 DPM
Average Background: 26.7 DPM
Unallocated Area: 1346.7 Counts

Appendix 12. Representative radio-HPLC chromatograms for the extract of the sterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

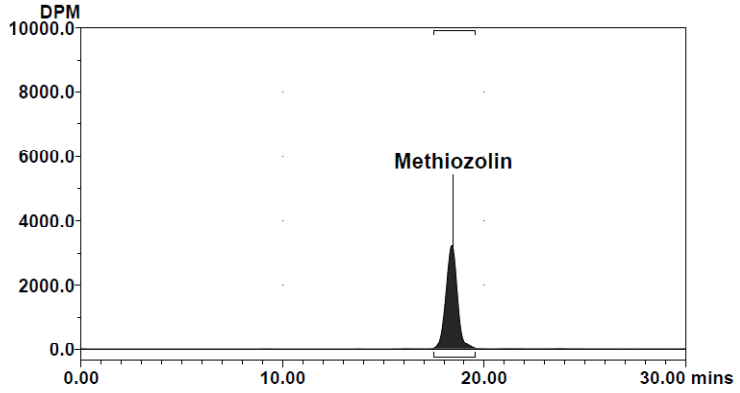


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.07			
Methiozolin	17.57	19.77	18.47	68190.2	100.00	95.62
Bkg 2	19.77	20.13	19.93			
1 Peak				68190.2	100.00	95.62

Total Area: 71310.1 DPM
Average Background: 16.5 DPM
Unallocated Area:3119.9 Counts

[7 DAT]



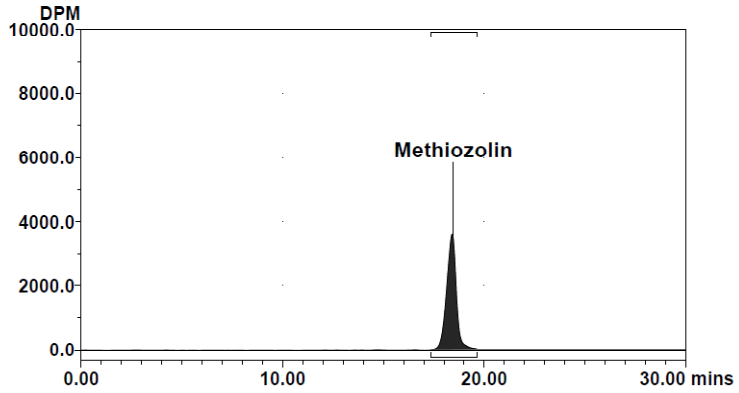
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.13			
Methiozolin	17.50	19.53	18.47	62794.6	100.00	96.39
Bkg 2	19.53	19.90	19.70			
Bkg 3	21.57	21.93	21.67			
1 Peak				62794.6	100.00	96.39

Total Area: 65146.2 DPM
Average Background: 20.9 DPM
Unallocated Area:2351.6 Counts

Appendix 12. Representative radio-HPLC chromatograms for the extract of the sterile soil under aerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

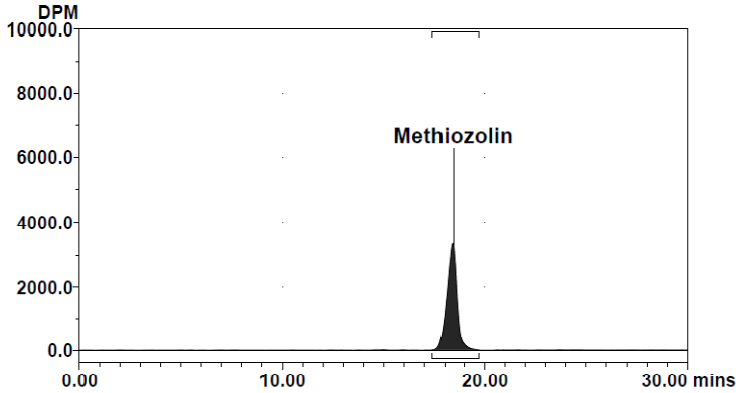


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.20			
Bkg 2	16.70	17.07	16.70			
Methiozolin	17.37	19.63	18.47	58978.1	100.00	95.59
Bkg 3	19.63	20.00	19.80			
1 Peak				58978.1	100.00	95.59

Total Area: 61700.0 DPM
Average Background: 18.2 DPM
Unallocated Area:2721.8 Counts

[30 DAT]



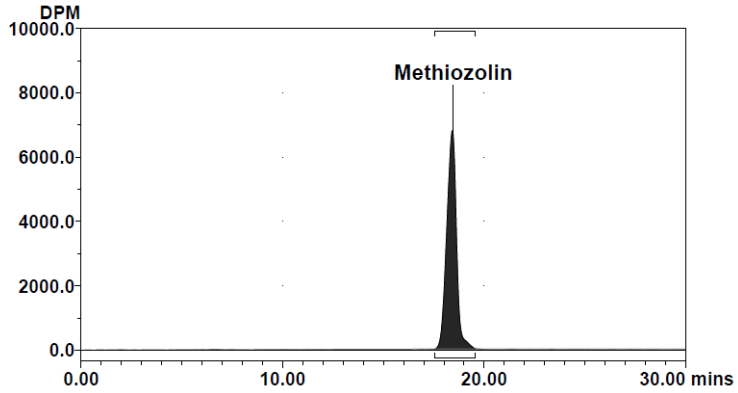
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.17			
Bkg 2	12.23	12.60	12.37			
Methiozolin	17.40	19.70	18.43	57235.1	100.00	94.31
Bkg 3	19.70	20.07	19.87			
Bkg 4	23.63	24.00	23.63			
1 Peak				57235.1	100.00	94.31

Total Area: 60687.5 DPM
Average Background: 15.9 DPM
Unallocated Area:3452.4 Counts

Appendix 13. Representative radio-HPLC chromatograms for the extract of the sterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

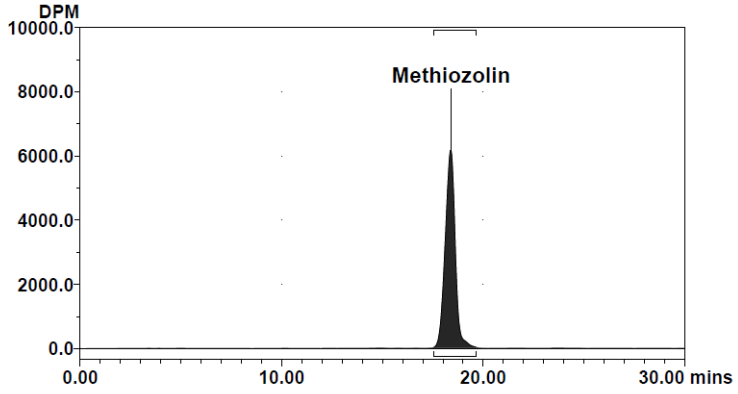


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.23			
Methiozolin	17.53	19.53	18.47	113247.1	100.00	98.25
Bkg 2	19.53	19.90	19.70			
1 Peak				113247.1	100.00	98.25

Total Area: 115260.6 DPM
Average Background: 27.4 DPM
Unallocated Area:2013.4 Counts

[7 DAT]



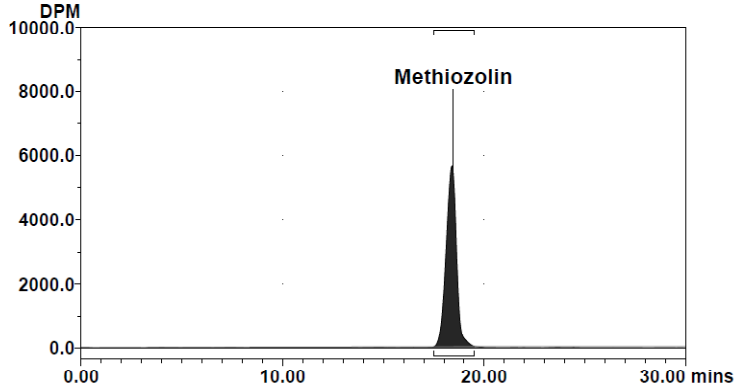
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.17			
Methiozolin	17.53	19.67	18.40	106638.6	100.00	96.69
Bkg 2	19.67	20.03	19.73			
1 Peak				106638.6	100.00	96.69

Total Area: 110294.0 DPM
Average Background: 12.1 DPM
Unallocated Area:3655.5 Counts

Appendix 13. Representative radio-HPLC chromatograms for the extract of the sterile soil under aerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

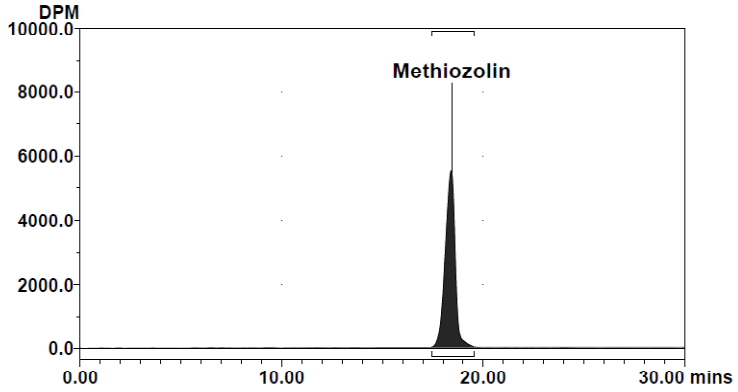


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.13			
Methiozolin	17.50	19.50	18.47	99742.4	100.00	99.08
Bkg 2	19.50	19.87	19.53			
1 Peak				99742.4	100.00	99.08

Total Area: 100669.8 DPM
Average Background: 33.0 DPM
Unallocated Area: 927.3 Counts

[30 DAT]



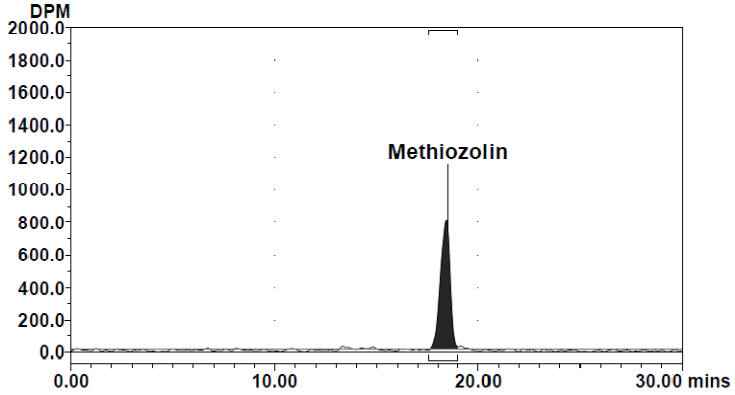
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.17			
Methiozolin	17.43	19.53	18.43	93282.9	100.00	97.32
Bkg 2	19.53	19.90	19.60			
1 Peak				93282.9	100.00	97.32

Total Area: 95852.7 DPM
Average Background: 23.3 DPM
Unallocated Area: 2569.7 Counts

Appendix 14. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

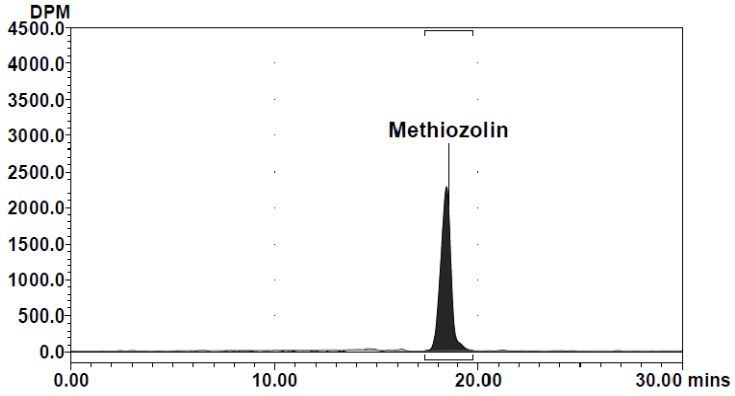


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	16.13	16.50	16.37			
Methiozolin	17.57	18.97	18.50	13291.2	100.00	76.88
1 Peak				13291.2	100.00	76.88

Total Area: 17288.1 DPM
Average Background: 17.2 DPM
Unallocated Area:3996.9 Counts

[7 DAT]



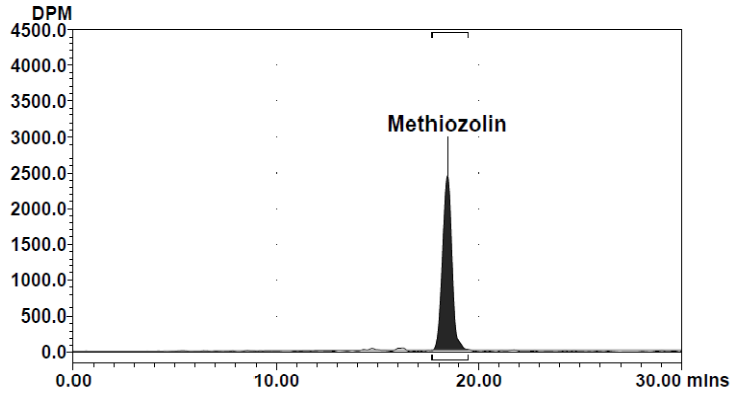
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.10	0.47	0.20			
Bkg 2	13.77	14.13	14.03			
Bkg 3	16.43	16.80	16.53			
Methiozolin	17.33	19.73	18.53	40118.3	100.00	91.69
Bkg 4	20.47	20.83	20.57			
1 Peak				40118.3	100.00	91.69

Total Area: 43752.7 DPM
Average Background: 19.6 DPM
Unallocated Area:3634.4 Counts

Appendix 14. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

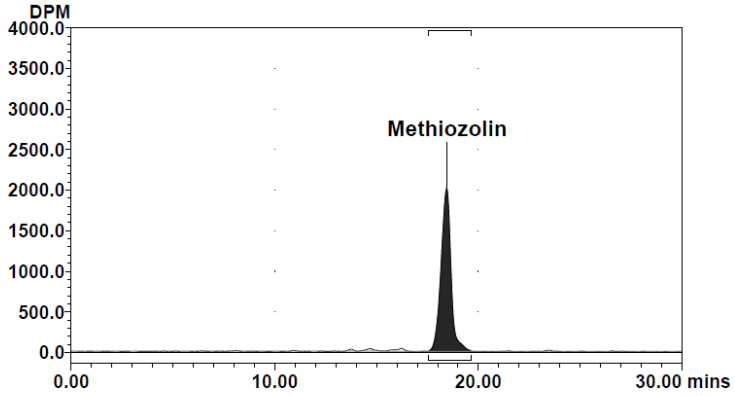


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	3.50	3.87	3.60			
Bkg 2	14.97	15.33	15.03			
Methiozolin	17.70	19.43	18.43	38754.8	100.00	92.39
1 Peak				38754.8	100.00	92.39

Total Area: 41944.9 DPM
Average Background: 24.4 DPM
Unallocated Area:3190.0 Counts

[30 DAT]



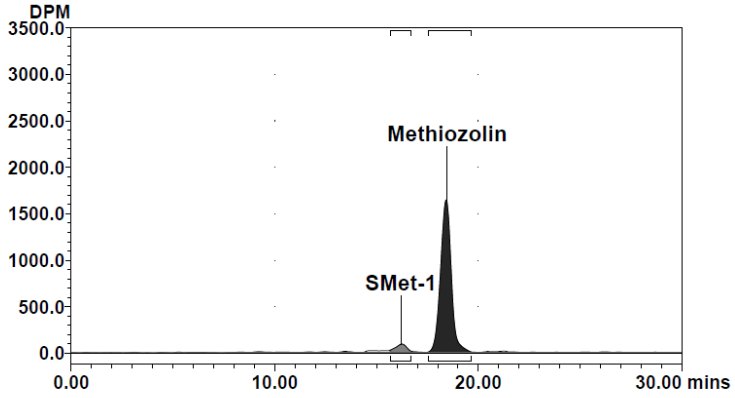
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	8.67	9.03	8.83			
Methiozolin	17.57	19.63	18.47	33982.3	100.00	87.88
Bkg 2	19.63	20.00	19.63			
1 Peak				33982.3	100.00	87.88

Total Area: 38669.4 DPM
Average Background: 16.4 DPM
Unallocated Area:4687.1 Counts

Appendix 14. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[60 DAT]

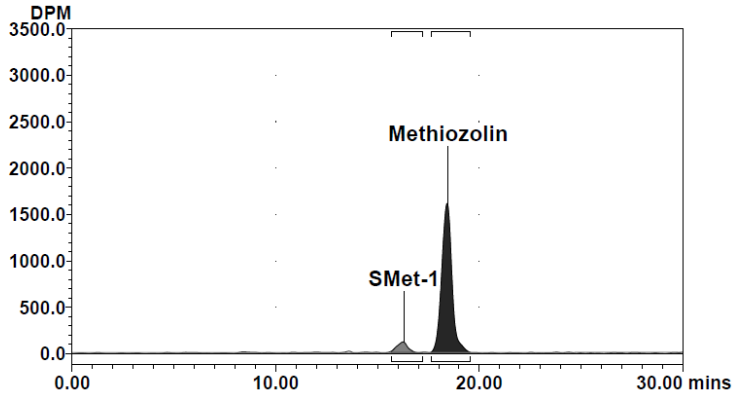


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.17			
SMet-1	15.67	16.67	16.20	1737.5	5.49	4.70
Methiozolin	17.57	19.63	18.47	29900.6	94.51	80.81
Bkg 2	19.63	20.00	19.87			
2 Peaks				31638.1	100.00	85.51

Total Area: 37000.4 DPM
Average Background: 10.4 DPM
Unallocated Area:5362.3 Counts

[90 DAT]



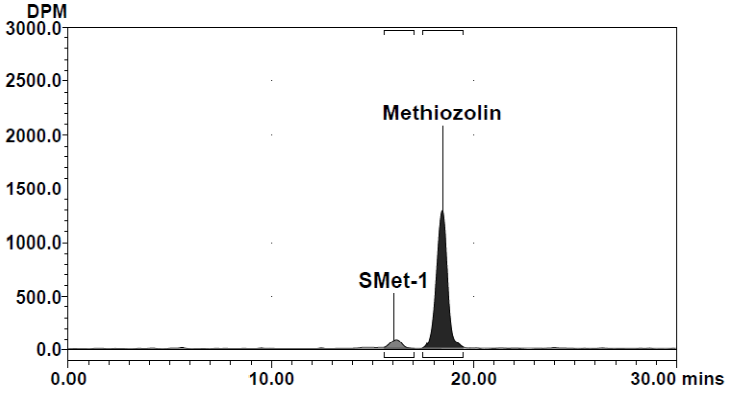
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	13.00	13.37	13.30			
SMet-1	15.67	17.20	16.30	2219.3	7.33	6.58
Methiozolin	17.63	19.53	18.47	28040.8	92.67	83.10
Bkg 2	27.43	27.80	27.53			
2 Peaks				30260.1	100.00	89.67

Total Area: 33744.7 DPM
Average Background: 13.1 DPM
Unallocated Area:3484.5 Counts

Appendix 14. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [benzyl-^{14}C]methiozolin at 500 g ai/ha (continued).

[120 DAT]



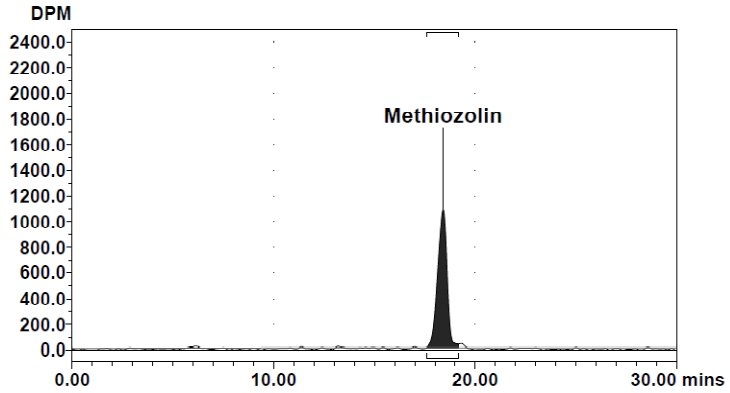
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.20			
Bkg 2	11.53	11.90	11.57			
SMet-1	15.60	17.07	16.07	1766.8	6.78	5.76
Methiozolin	17.50	19.47	18.43	24310.6	93.22	79.28
Bkg 3	19.57	19.93	19.63			
Bkg 4	24.13	24.50	24.27			
2 Peaks				26077.3	100.00	85.05

Total Area: 30662.5 DPM
Average Background: 10.3 DPM
Unallocated Area: 4585.1 Counts

Appendix 15. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha.

[0 DAT]

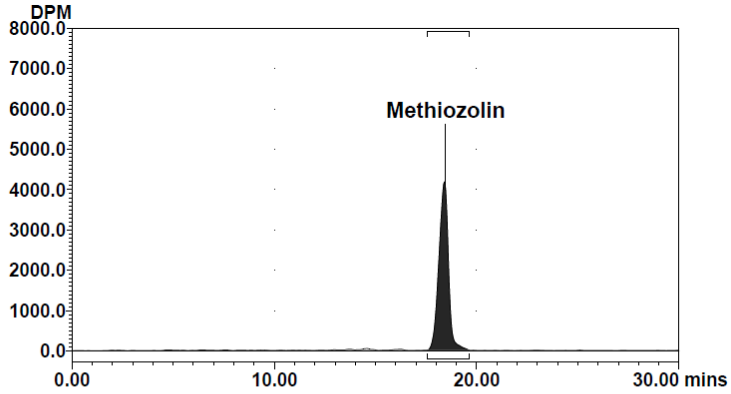


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	1.37	1.73	1.40			
Bkg 2	13.67	14.03	13.83			
Methiozolin	17.60	19.17	18.40	17343.0	100.00	82.79
Bkg 3	22.13	22.50	22.33			
1 Peak				17343.0	100.00	82.79

Total Area: 20947.7 DPM
Average Background: 16.1 DPM
Unallocated Area:3604.6 Counts

[7 DAT]



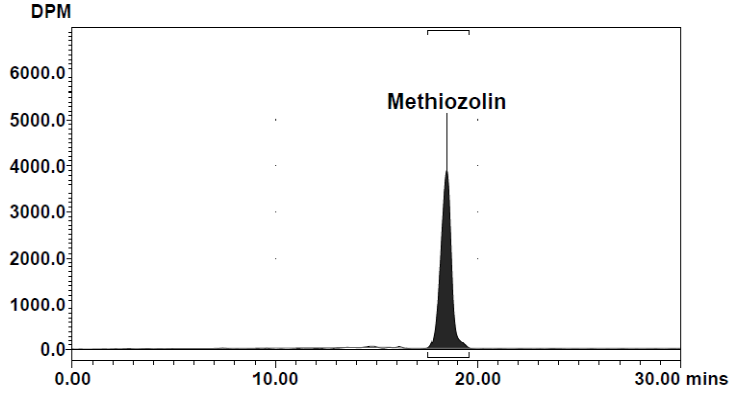
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.23			
Bkg 2	15.10	15.47	15.33			
Methiozolin	17.57	19.63	18.47	68107.1	100.00	90.59
1 Peak				68107.1	100.00	90.59

Total Area: 75181.6 DPM
Average Background: 13.5 DPM
Unallocated Area:7074.5 Counts

Appendix 15. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[14 DAT]

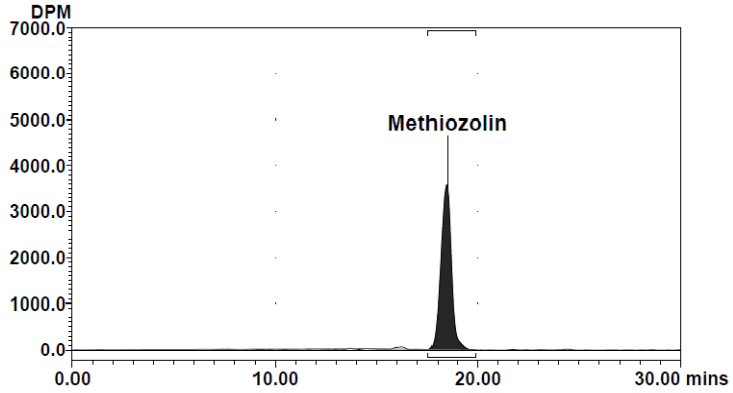


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.17			
Bkg 2	15.50	15.87	15.57			
Bkg 3	16.33	16.70	16.50			
Methiozolin	17.53	19.57	18.47	69202.4	100.00	95.23
1 Peak				69202.4	100.00	95.23

Total Area: 72667.0 DPM
Average Background: 26.2 DPM
Unallocated Area:3464.6 Counts

[30 DAT]



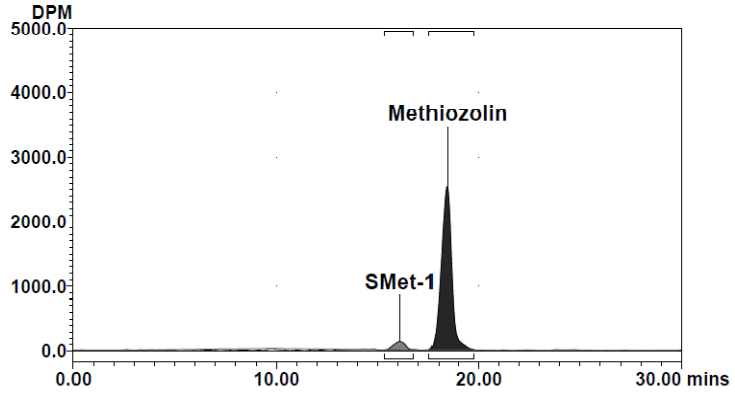
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	0.00	0.37	0.13			
Bkg 2	14.37	14.73	14.70			
Methiozolin	17.57	19.87	18.50	63545.9	100.00	92.64
Bkg 3	19.87	20.23	20.20			
1 Peak				63545.9	100.00	92.64

Total Area: 68591.7 DPM
Average Background: 19.7 DPM
Unallocated Area:5045.7 Counts

Appendix 15. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[60 DAT]

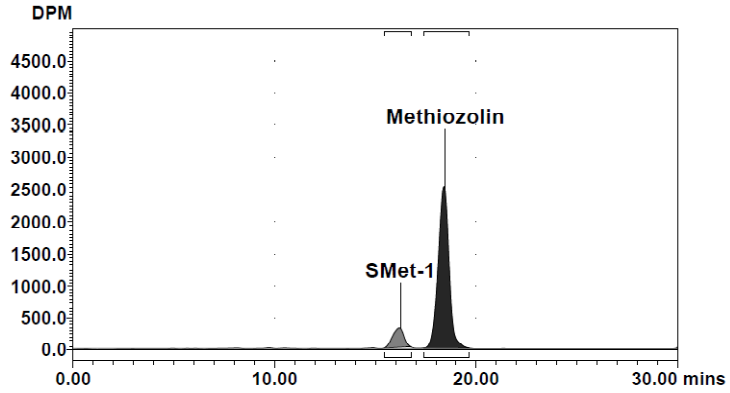


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	2.67	3.03	2.70			
Bkg 2	9.60	9.97	9.70			
SMet-1	15.37	16.77	16.13	2664.0	5.55	5.19
Bkg 3	17.07	17.43	17.30			
Methiozolin	17.57	19.73	18.47	45312.0	94.45	88.22
2 Peaks				47976.0	100.00	93.40

Total Area: 51363.7 DPM
Average Background: 19.5 DPM
Unallocated Area:3387.7 Counts

[90 AT]



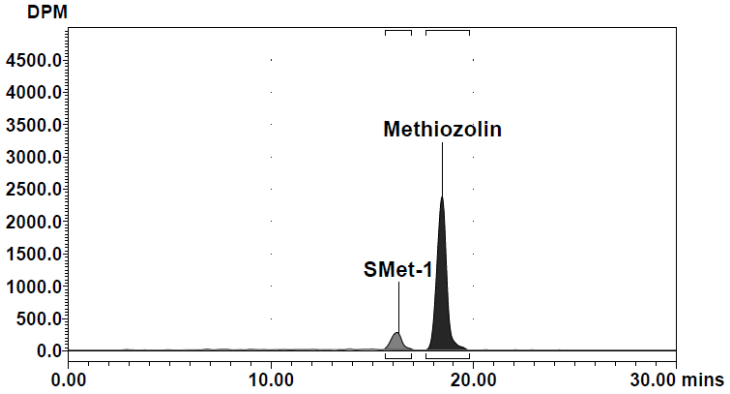
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
SMet-1	15.43	16.77	16.23	5497.0	10.34	9.56
Bkg 1	16.90	17.27	17.07			
Methiozolin	17.40	19.67	18.47	47678.7	89.66	82.91
Bkg 2	19.67	20.03	19.83			
2 Peaks				53175.7	100.00	92.47

Total Area: 57506.9 DPM
Average Background: 18.2 DPM
Unallocated Area:4331.2 Counts

Appendix 15. Representative radio-HPLC chromatograms for the extract of the soil under anaerobic condition treated with [isoxazole-^{14}C]methiozolin at 500 g ai/ha (continued).

[120 DAT]



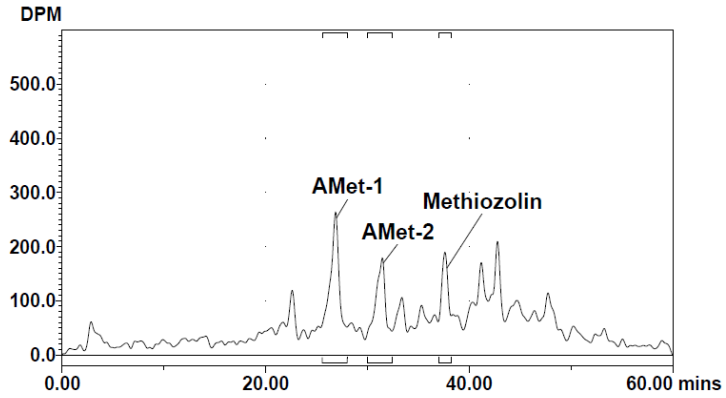
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
Bkg 1	2.20	2.57	2.30			
Bkg 2	14.93	15.30	15.17			
SMet-1	15.63	16.93	16.30	5023.2	11.20	10.21
Bkg 3	17.00	17.37	17.17			
Methiozolin	17.67	19.77	18.47	39808.2	88.80	80.92
2 Peaks				44831.3	100.00	91.13

Total Area: 49192.9 DPM
Average Background: 16.3 DPM
Unallocated Area: 4361.5 Counts

Appendix 16. Representative radio-HPLC chromatograms of the solvent extract from the feces following oral administration of methiozolin at 500 mg/kg body weight in rat.

[0-24 h]

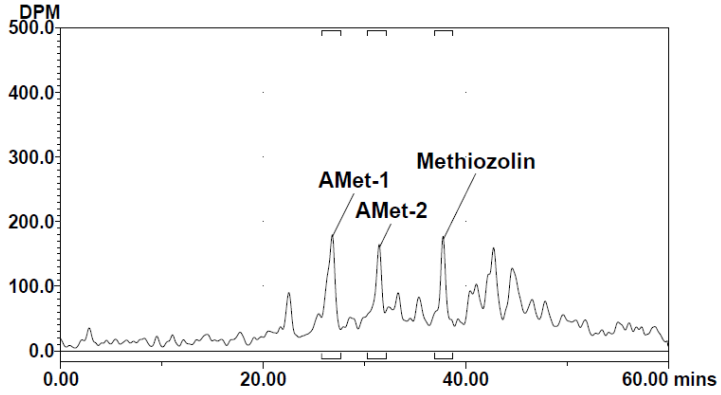


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
AMet-1	25.55	28.00	26.95	4753.3	45.57	7.67
AMet-2	30.00	32.45	31.55	3505.6	33.61	5.66
Methiozolin	37.00	38.20	37.80	2172.0	20.82	3.51
3 Peaks				10430.9	100.00	16.83

Total Area: 61962.0 DPM
Average Background: 0.0 DPM
Unallocated Area:51531.1 Counts

[24-48 h]



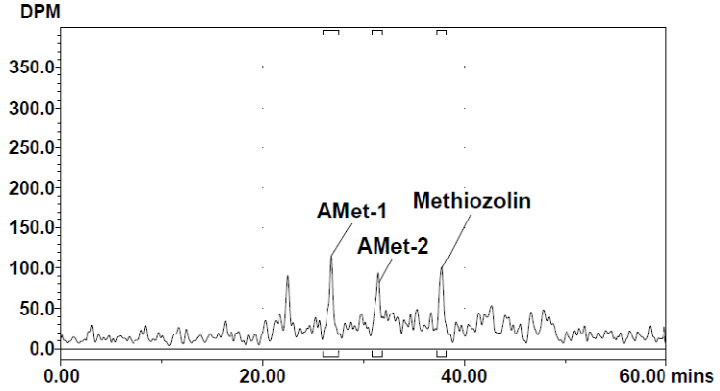
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
AMet-1	25.75	27.65	26.85	2963.1	43.64	5.69
AMet-2	30.30	32.10	31.50	1527.2	22.49	2.93
Methiozolin	36.95	38.65	37.80	2299.1	33.86	4.42
3 Peaks				6789.3	100.00	13.04

Total Area: 52072.0 DPM
Average Background: 0.0 DPM
Unallocated Area:45282.7 Counts

Appendix 16. Representative radio-HPLC chromatograms of the solvent extract from the feces following oral administration of methiozolin at 500 mg/kg body weight in rat (continued).

[48-72 h]

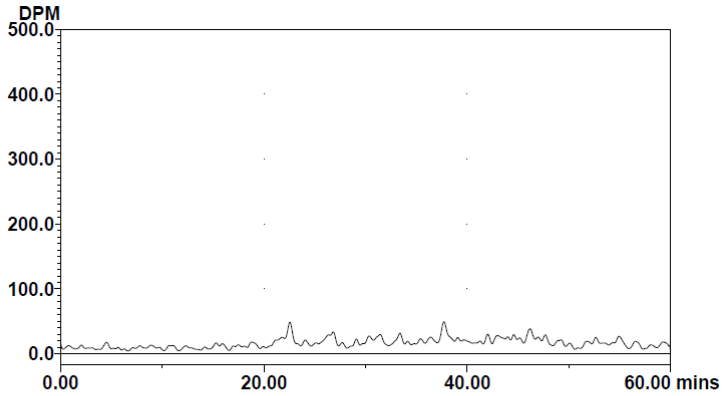


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
AMet-1	25.95	27.55	26.80	1533.3	44.84	5.45
AMet-2	30.80	31.90	31.55	889.3	26.01	3.16
Methiozolin	37.30	38.20	37.80	996.7	29.15	3.54
3 Peaks				3419.3	100.00	12.15

Total Area: 28136.7 DPM
Average Background: 0.0 DPM
Unallocated Area: 24717.3 Counts

[72-96 h]



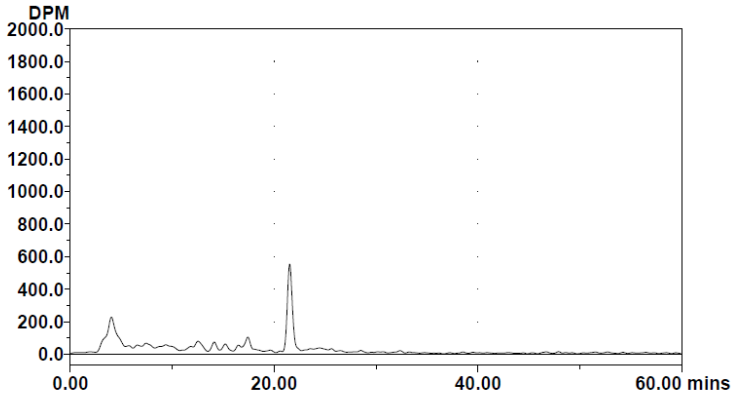
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 19493.3 DPM
Average Background: 0.0 DPM
Unallocated Area: 19493.3 Counts

Appendix 17. Representative radio-HPLC chromatograms of the urine following oral administration of methiozolin at 500 mg/kg body weight in rat.

[0-6 h]

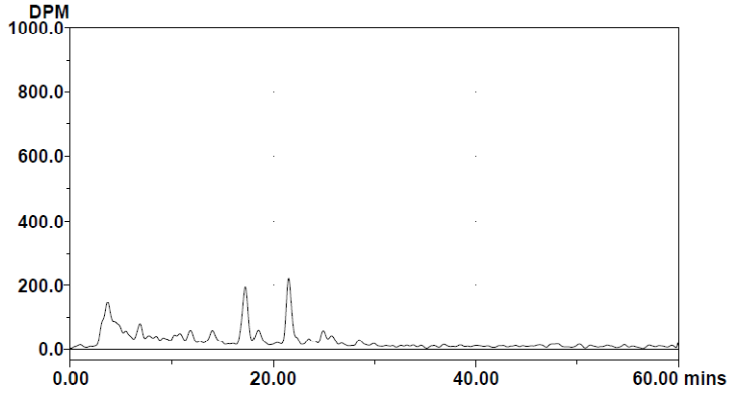


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 35306.7 DPM
Average Background: 0.0 DPM
Unallocated Area:35306.7 Counts

[6-12 h]



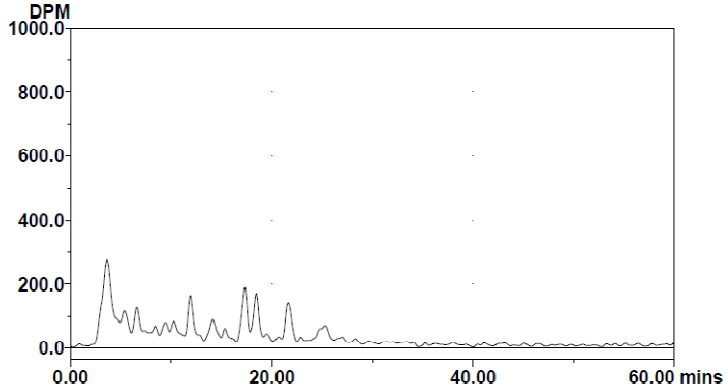
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 30840.0 DPM
Average Background: 0.0 DPM
Unallocated Area:30840.0 Counts

Appendix 17. Representative radio-HPLC chromatograms of the urine following oral administration of methiozolin at 500 mg/kg body weight in rat (continued).

[12-24 h]

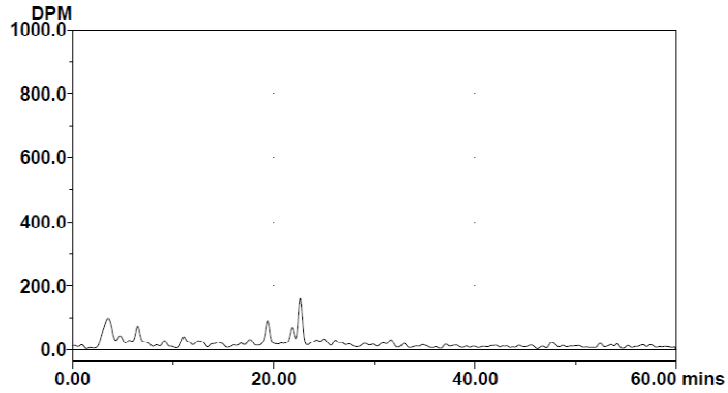


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 42200.0 DPM
Average Background: 0.0 DPM
Unallocated Area:42200.0 Counts

[24-48 h]



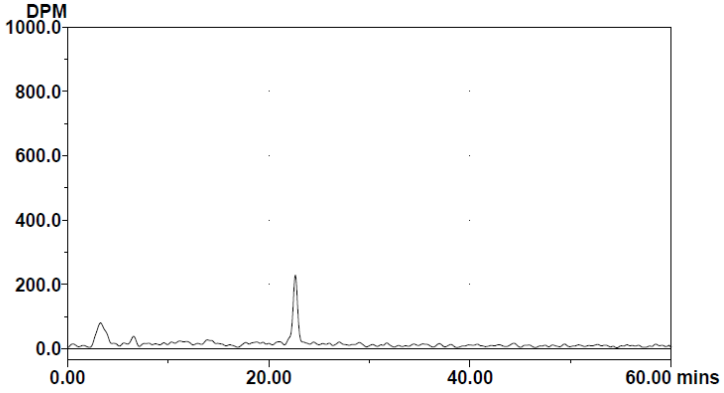
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 23653.3 DPM
Average Background: 0.0 DPM
Unallocated Area:23653.3 Counts

Appendix 17. Representative radio-HPLC chromatograms of the urine following oral administration of methiozolin at 500 mg/kg body weight in rat (continued).

[48-72 h]

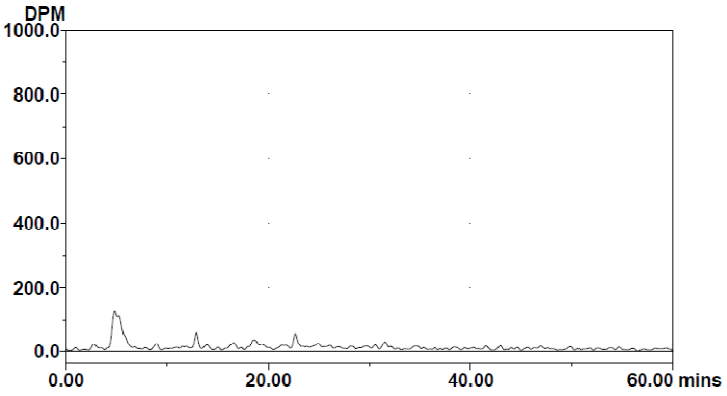


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 19093.3 DPM
Average Background: 0.0 DPM
Unallocated Area: 19093.3 Counts

[72-96 h]



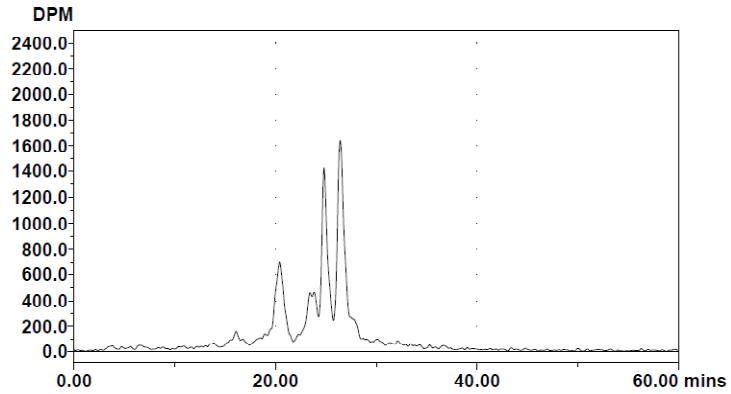
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 17653.3 DPM
Average Background: 0.0 DPM
Unallocated Area: 17653.3 Counts

Appendix 18. Representative chromatograms of the bile following oral administration of methiozolin at 500 mg/kg body weight in rat.

[0-6 h]

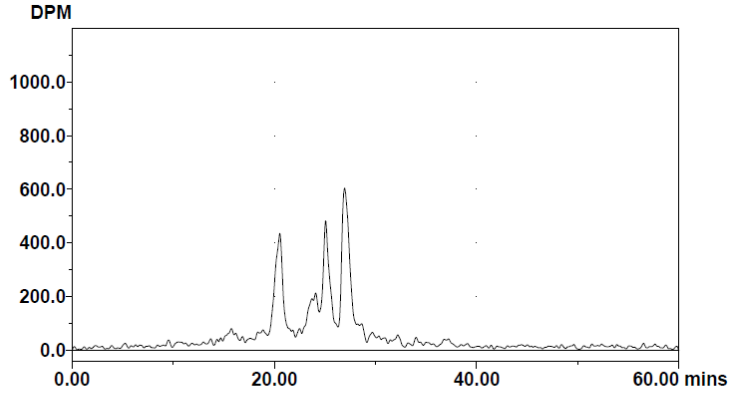


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 117085.3 DPM
Average Background: 0.0 DPM
Unallocated Area: 117085.3 Counts

[6-12 h]



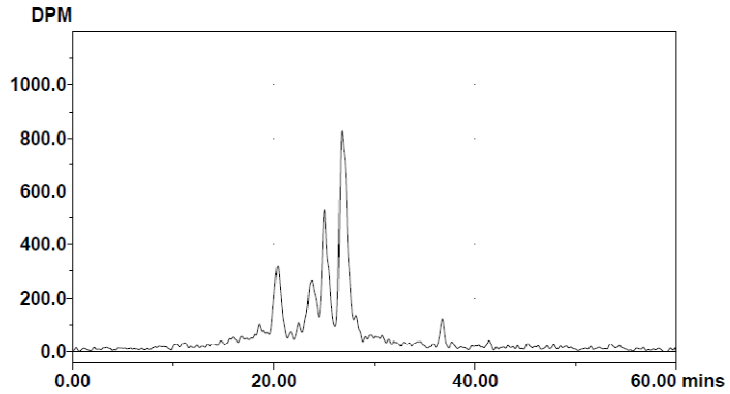
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 60490.0 DPM
Average Background: 0.0 DPM
Unallocated Area: 60490.0 Counts

Appendix 18. Representative chromatograms of the bile following oral administration of methiozolin at 500 mg/kg body weight in rat (continued).

[12-24 h]

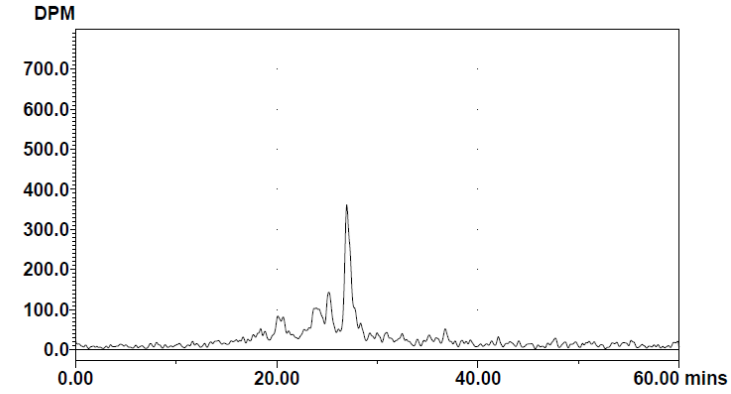


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 64998.0 DPM
Average Background: 0.0 DPM
Unallocated Area:64998.0 Counts

[24-48 h]



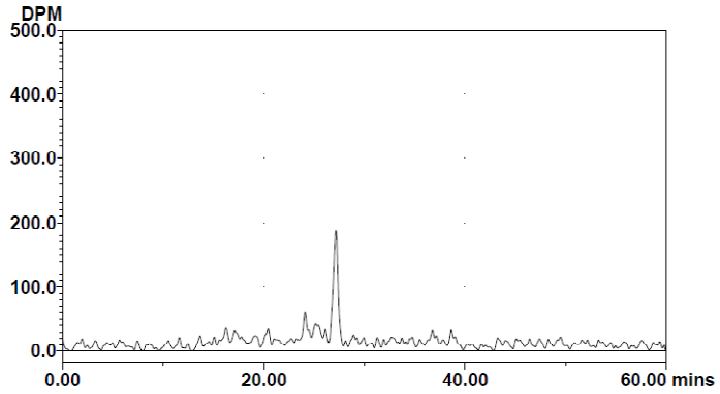
Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 31847.3 DPM
Average Background: 0.0 DPM
Unallocated Area:31847.3 Counts

Appendix 18. Representative chromatograms of the bile following oral administration of methiozolin at 500 mg/kg body weight in rat (continued).

[48-72 h]

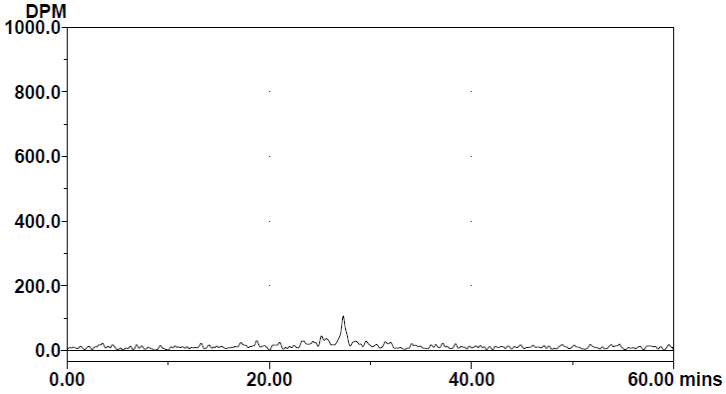


Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 17710.0 DPM
Average Background: 0.0 DPM
Unallocated Area: 17710.0 Counts

[72-96 h]



Regions: ^{14}C Detector: FSA

Name	Start (mins)	End (mins)	Retention (mins)	Area (DPM)	%ROI (%)	%Total (%)
No ROIs allocated.						
0 Peaks				0.0	100.00	0.00

Total Area: 15854.7 DPM
Average Background: 0.0 DPM
Unallocated Area: 15854.7 Counts

제초제 **Methiozolin**의 호기성 및 혐기성 토양대사와 흰쥐에서의 약동학 및 대사 연구

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국문초록

Methiozolin은 다양한 한지형과 난지형 잔디밭의 문제잡초인 새포아풀(annual bluegrass, *Poa annua*), 바랭이(crabgrass, *Digitaria sanguinalis*), 왕바랭이(goosegrass, *Eleusine indica*) 등의 화본과잡초를 선택적으로 방제하는 신규 잔디제초제이다. 본 연구에서 방사선 동위원소가 표지된 [^{14}C]methiozolin ([benzyl-^{14}C]과 [isoxazole-^{14}C]methiozolin)을 이용하여 호기성 및 혐기성 토양대사를 수행하여 반감기를 산출하고, 또한 주요 대사체(처리량 대비 10% 이상)를 동정하여 methiozolin의 대사경로를 제안하고자 하였다. 호기조건의 비살균토양 및 살균토양 그리고 혐기조건 비살균토양의 mass balance는 각각 92.0 ~ 104.5, 97.5 ~ 101.2, 93.2 ~ 102.5%이었다. 호기조건의 비살균토양에 처리된 [benzyl-^{14}C] 또는 [isoxazole-^{14}C]methiozolin은 처리 120일 후 처리량 대비 각각 17.9와 15.9%만이 토양에서 검출되었고, 최종 분해산물인 $^{14}CO_2$는 각각 41.5와 36.1%가 검출되었으며, 비추출성 잔류물은 각각 35.7과 39.8%에 달하였다. 반면, 시험기간 동안 휘발성 물질과 주요대사체는 생성되지 않았다. 위의 조건에서 [^{14}C]methiozolin의 반감기는 약 49일로 산출되었다. 그러나 호기조건의 살균토양에서는 [^{14}C]methiozolin의 분해가 거의 일어나지 않았다.

혐기조건의 비살균토양에서는 각각 [benzyl-^{14}C]과 [isoxazole-^{14}C]methiozolin 처리 120일 경과 후 처리량 대비 각각 74.6과 73.1%, $^{14}CO_2$는 0.4 ~ 5.2%과 0.02 ~ 3.5%, 비추출성 잔류물은 6.7 ~ 8.0과 6.0 ~ 10.5%가 검출되어 호기조건에 비하여 분해율, 최종분해산물 생성율, 그리고 비추출성 잔류물 생성율이 모두 크게 낮았다. 그러나 각각 [benzyl-^{14}C]과 [isoxazole-^{14}C]methiozolin이 처리된 토양에서 동일한 대사체가 각각 6.2와 7.9% 수준 생성되었는데 이는 isoxazole ring의 가수분해에 의한 것으로, 구조동정 결과 4-(2,6-difluorobenzyloxy)-3-hydroxy-3-methyl-1-(3-methyl-thiophen-2-yl)butan-1-one이었다. 이와 같은 결과로 볼 때, methiozolin은 호기조건 토양에서는 미생물에 의해 빠른 분해양상을 보이는 반면, 혐기 미생물에 의한 분해는 매우 느리며 비생물학적 분해는 거의 발생하지 않는 것으로 사료된다.

본 연구에서는 또한 [^{14}C]methiozolin을 흰쥐에 경구투여한 후 흡수, 분포, 배설양상을 조사하고, 체내에서 형성된 각종 대사체를 확인하여 대사경로를 제안하고자 하였다. 혈액에서의 약동학 상수를 산출한 결과 AUC_{120}, T_{max}, C_{max} 및 반감기가 각각 9921.5 μg equiv·h/mL, 6시간, 168.7 μg equiv/mL, 및 48.4시간이었다. 혈장의 약동학 상수는 혈액과 거의 유사하였다. 투여 후 120시간 동안 오줌과 변으로 배설되는 양은 투여량 대비 각각 24.3과 68.9%로 변으로 배설되는 양이 2.8배 높았다. 담즙관을 통한 배출은 40.1%이었으며, 호흡을 통한 이산화탄소 및 휘발성 물질은 검출되지 않았다. 오줌과 변으로의 배설량은 투여 후 24 ~ 48시간에 가장 높았으며 투여량의 약 50.1%에 달했다. 투여된 ^{14}C는 12시간 이내에 다양한 장기에 높은 수준(투여량 대비 14.0%)이 분포하였으나 120시간 후에는 0.3% 수준으로 감소하여 장기 내 methiozolin의 축적 가능성은 매우 낮은 것으로 추정된다.

오줌과 변으로 배설되는 [^{14}C]methiozolin의 대사체가 다수 검출되었으나 모두 투여량 대비 4% 이하의 낮은 수준으로 주요 대사체는 생성되지 않았다. 그러나 이 중 변으로 배설되는 대사체 2개를 분리 및 구조동정하여 AMet-1과 AMet-2로 명명하였다. AMet-1은 methiozolin의 thiophene ring에 glucuronic acid 가 conjugation된 구조로써 화학명은 6-[5-(5-((2,6-difluorobenzyloxy)-methyl)-4,5-dihydro-5-methylisoxazol-3-yl)-4-methylthiophen-2-yloxy]-tetrahydro-3,4,5-trihydroxy-2H-pyran-2-carboxylic acid 이다. AMet-2는 thiophene ring의 methyl기에 수산화기가 도입된 구조로써 화학명은 [2-(5-((2,6-difluorobenzyloxy)methyl)-4,5-dihydro-5-methylisoxazol-3-yl)thiophen-3-yl]methanol 이다. 결론적으로 methiozolin은 흰쥐에 경구투여되었을 때 소화기관으로 빠르게 흡수되어 12시간 이내에 다양한 장기에 분포하지만, 어떠한 장기에도 축적없이 주로 48시간 이내에 빠르게 오줌과 변으로 배설된다는 것을 확인하였다.

주요어: methiozolin, 제초제, 토양대사, 대사체, 호기성, 혐기성, 약동학, 흡수, 분포, 대사, 배설

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