A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Residue Analysis of Cyazofamid, and Its Metabolism by Soil Fungus *Cunninghamella elegans* and Human Liver Microsomes

토양 곰팡이 *Cunninghamella elegans* 및 인체 간 마이크로좀에 의한 살균제 Cyazofamid 의 대사 및 잔류분석법 연구

By

Hyeri Lee

Department of Agricultural Biotechnology

The Graduate School of Seoul National University

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By
Hyeri Lee

Department of Agricultural Biotechnology
The Graduate School of Seoul National University
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APPROVED AS A QUALIFIED THESIS OF HYERI LEE
FOR THE DEGREE OF DOCTOR OF PHOLOSOPHY
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Hyeri Lee
Department of Agricultural Biotechnology
The Graduate School
Seoul National University

ABSTRACT

Cyazofamid, a sulfonamide fungicide, has been used for the protection of several vegetables and fruits from various diseases. In the present study, the analytical method of cyazofamid and its metabolite by HPLC and LC-MS/MS was established. And metabolism of cyazofamid by soil fungus *Cunninghamella elegans* and by *in vitro* human mimicking system was investigated to understand bioenvironmental fate of cyazofamid. Apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean were chosen as the representative crop samples for analytical study. LOQ and MLOQ for cyazofamid were 2 ng and 0.02 mg/kg, respectively, on HPLC. Recoveries of cyazofamid at three spiking levels were reasonable (75.3 - 98.5%). A rapid and effective LC–MS/MS analytical method of cyazofamid and its metabolite, CCIM (4-chloro-5-*p*-tolylimidazole-2-carbonitrile), was established for soil and water samples in addition to the crop samples using QuEChERS sample treatment. On LC-MS/MS, MLOQ of cyazofamid and CCIM were 2 ng/g and 5 ng/g for crop/soil samples, respectively, while 0.02 ng/mL and 0.05 ng/mL were achieved, respectively, for water samples. The recoveries of target analytes in crop/environmental samples were 80.2% - 105.1% for cyazofamid and 75.1% - 99.1% for CCIM. CCIM was observed

in the metabolism of cyazofamid by artificial gastric juice and intestinal juice. CCHS (4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)N,N-dimethyl-imidazole-1-sulfonamide) was identified unambiguously as a noble metabolite in the metabolism of cyazofamid by soil fungus *Cunninghamella elegans*. It could be degraded to 4-chloro-5-(4-hydroxymethylphenyl)imidazole-2-carbonitrile (CHCN) and further oxidized to 4-(4-chloro-2-cyanoimidazole-5-yl)benzoic acid (CCBA). *In vitro* metabolism of cyazofamid by HLMs, CCHS was also produced as the only metabolite. Among 10 types of recombinant CYPs (rCYPs), the formation of CCHS was observed only in CYP2B6, 2C9, and 2C19, contributing 27.4%, 66.1% and 6.5% in metabolism. In molecular docking study of crystal structure of cyazofamid with rCYPs 2B6, 2C9, 2C19 and 3A4, the metabolic reactivity order of three rCYPs was well correlated with the distance between the heme iron of rCYPs and hydroxylated carbon of cyazofamid. In phase II metabolism of cyazofamid no conjugate metabolite was observed in reaction with UDP-glucuronosyl transferases (UGTs) however, CCIM was observed in reaction with glutathione S-transferases (GSTs).

KEYWORD : *Cunninghamella elegans*, Cyazofamid, HPLC, Human liver microsomes, LC-MS/MS, Metabolism, QuEChERS

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

1. Pesticides residues analysis

Pests are organisms that are competitive to mankind or his interests in some aspect. The world's main source of food is plants, but they are susceptible to 80,000 - 100,000 kinds of diseases, 1,800 weed species, 10,000 insect species, and 1,000 nematode species as pests. Pesticides are any substance or mixture of substances intended for preventing, destroying, repelling or migrating any pest (Ware 2004) and are applied to protect food crops from the pests at various stages of cultivation and during post-harvest storage, and therefore pesticides are essential in agricultural production. However, with their use, the risk of residues remaining on the food is of major concern in food safety issues. Legislations were enacted throughout the world to regulate pesticides in food products (Ahmed 2001), and maximum residue limits (MRLs) in foodstuffs have been set by government agencies to guarantee consumer safety and to regulate international trade.

Pesticide residue analysis of food and environmental samples has been performed in numerous government and private laboratories throughout the world for approximately 40 years (Anastassiades et al. 2003).

1.1 General and conventional pesticide residue analysis

A variety of analytical methods have been developed and applied routinely for the control of pesticide residue in food. Analysis of pesticide residues is extremely difficult, because sample matrix is complex, pesticides consist of many types of compounds, and its residues exist at ppm level or lower. Therefore, analytical methodologies employed must be capable of

residue measurement at very low levels and must also provide unambiguous evidence to confirm both the identity and the magnitude of any residues detected (Taylor et al. 2002).

The first multiresidue method for pesticide extraction was developed by Paul A. Mills in 1963 (Mills et al. 1963). In the Mill's method, organochlorine pesticides were the focus for analysis in non-fatty foods. In the 1970s, new methods were developed to extend the analytes, such as organochlorine, organophosphorus, and orgnonitrogen pesticides in a single procedure (Luke et al. 1975). Since the 1980s, environmental and health concerns related to the use of chlorinated solvents have led to the development of many new methods in which such solvents were avoided. Procedures in which operations and processes are introduced that do not require the use of large amounts of chlorinated solvents; these are replaced by others (Specht et al. 1995). During the 1990s, increased urgency to further reduce solvent usage and manual labor in analytical laboratories led to the commercial introduction of several alternative extraction approaches, including supercritical fluid extraction (SFE) (Lehotay 1997), matrix solid-phase dispersion (MSPD) (Barker 2000), microwave-assisted extraction (MAE) (Pylypiw et al. 1997), solid-phase microextraction (SPME) (Wardencki et al. 2004), and pressurized liquid extraction (PLE), also known commercially as accelerated solvent extraction (ASE) (Lehotay and Lee 1997).

Pesticides residue analytical method may contain several discrete steps, as is true also for analytical methods for metal, drugs, and other agents of concern when they are present at relatively low levels.

The steps are as follows.

1) Matrix modification : Various matrix pretreatment methods are employed for foods containing pesticides residues to ensure correct samples mass to take depending on the heterogeneity of matrix. Representative portions of the solid sample (e.g. whole fruits or vegetables) are weighed; chopped, homogenized in a mortar, blender or stirrer; or ultra-sonicated with a solvent (or a sorbent) to disintegrate the matrix (Ahmed 2001).

2) Extraction : Extraction of the analyte from the matrix is needed with a minimum extraction of extraneous materials that might interfere in the analysis (Fong 1999). The necessity of using water-miscible solvents (acetone, methanol and acetonitrile) to extract pesticide residues for high moisture products has been established. Supercritical fluid extraction (SFE) (Lehotay 1997), matrix solid-phase dispersion (MSPD) (Barker 2000), microwave-assisted extraction (MAE) (Pylypiw et al. 1997), solid-phase microextraction (SPME) (Wardencki et al. 2004), and pressurized liquid extraction (PLE), also known commercially as accelerated solvent extraction (ASE) (Lehotay and Lee 1997) are alternative extraction methods.

3) Liquid-liquid partitioning : This step is to reduce the amount of polar impurities that partition into the organic phase. Therefore partitioning used immiscible solvents such as water and dichloromethane, *n*-hexane, ethyl acetate. Liquid-liquid partitioning can be improved by the addition of water-soluble salts such as sodium chloride. Adjust pH can increase efficiency of partitioning. If the analyte is acidic, adjustment to pH ~3 with acid will protonate the analyte and reduce its water solubility (Fong 1999).

4) Solvent evaporation : Essentially residue analytical methods require removal of solvent at some point in order to increase the concentration of analytes in extracted solution. Several different evaporation techniques are

available, each with advantage and disadvantages. The best techniques in particular situation depend on the physical and chemical characteristics of the analyte and the solvent that must be evaporated (Kim 2008).

5) Clean-up : The analyte is concentrated and purified through clean-up procedures such as column chromatography and solid phase extraction (SPE) cartridge. Clean-up system can remove the coextractives which were not removed by liquid-liquid partitioning step, such as lipids and pigments (Fong 1999).

6) Derivatization : Conversion of the chemical of interest into a derivative, in order to enhance extractability, clean-up, or subsequent resolution and determination steps (Fong 1999). This is an optional step, required for some chemicals and some methods, but not all.

7) Resolution : The analyte is resolved from remaining coextractives, so that it may be subsequently measured without significant interference. This is usually done by some form of refined chromatography, for examples, GC or HPLC (Kim 2008).

8) Detection : Obtaining a response (usually and electronic signal) that is proportional to the amount of analyte present. By selective detection, the analyte will produce a signal several times higher than those originating from the background (Kim 2008).

9) Determination : Calculating an amount of analyte present by reference to a standard, either external or internal (Fong 1999).

1.2 QuEChERS method

Traditional and conventional procedures mentioned above are time-consuming, labor-intensive, complicated and expensive; moreover, they

produce considerable quantities of wastes, and frequently, a sufficiently low limit of detection is unobtainable (Beyer and Biziuk 2008).

Recently, a rapid and inexpensive approach to the analysis of pesticide in fruits and vegetables was reported (Anastassiades et al. 2003; Schenck and Hobbs 2004) as 'QuEChERS' (quick, easy, cheap, effective, rugged, and safe)method, which extract pesticides with acetonitrile from fruit and vegetables and then separates organic phase from water by salting before purified by dispersive SPE (d-SPE) such as primary secondary amine (PSA), C18, graphitised carbon black (GCB) and so on (Anastassiades et al. 2003; Lehotay et al. 2005; Lehotay et al. 2010; Wiilkowska and Biziuk 2011). QuEChERS method was then modified into the acetate-buffering version (Lehotay et al. 2005) and the citrate-buffering versions (Anastassiades et al. 2007). This method applied successfully in not only crops but also environmental samples (Kvicalova et al. 2012; Lee et al. 2014a; Liu et al. 2014) for pesticide residue analysis.

In QuEChERS method, LC-MS/MS or GC-MS/MS has been used for the analytical instrument because these instruments are much more sensitive and selective than conventional HPLC or GC by using scheduled selective reaction monitoring (SRM) mode which combines two selective ion monitoring (SIM) modes *via* collision induced dissociation (CID).

1.3 Method validation

Analytical method must be validated to secure the reliability of analytical results. The following method validation parameters are extracted from the published papers as the minimum analytical method validation requirements.

1) Accuracy : It is determined (average of a replicated set of trials) by use

of certified reference materials, use of reference method of known uncertainty, or use of recovery from spiked samples. Reference material and spiked samples should be carried through the entire procedure (from matrix modification to determination). The method of fortification of spiked samples should be described (Fong 1999).

2) Recovery : It can be determined by the amount of recovered analyte after spiking over an appropriate range of concentrations (Fong 1999).

Percent of recovery = (analyte recovered / analyte added) × 100

3) Calibration curve and linearity : It is defined as the signal responses to a number of concentrations of the analyte standards. Linearity is assessing the correlation of signal responses with a range of concentrations (Hernando et al. 2007). A minimum linear correlation is 0.99 (Kanrar et al. 2010). Responses at various concentrations in pure solvents and in matrix should be studied (Fong 1999).

4) Limit of detection (LOD) : There are several ways to define the LOD. Two examples are illustrated as follows a) the mean value of the matrix blank readings plus 3 standard deviations of the mean, expressed in analyte concentration. b) The amount, expressed in ppm or ppb, equivalent to 3 times the background signal contributed by the matrix blank (Fong 1999; Miller 2005).

5) Limit of quantitation (LOQ) : There are several ways to define the LOQ. The examples are illustrated as follows a) The substrate blank plus 10 deviations b) The amount, expressed in ppm or ppb, equivalent to 10 times the background signal contributed by the matrix blank (Fong 1999; Miller 2005).

6) Precision : The precision of a method is assessed as the tightness of

replicate fortifications measured by the relative standard deviation or coefficient of variation (CV). The precision of the method was evaluated by the determination of the intra- and inter-day variabilities. The precision calculated as RSD did not exceed the 15% for each concentration level tested (Hernando et al. 2007).

7) Sensitivity and method limit of quantitation (MLOQ) : It is defined as the ability of the method to detect the analyte at the concentration of interest (Fong 1999). MLOQ is not an instrumental LOQ, but instead is a practical LOQ for the total analytical method. It is calculated using LOQ, injection volume, final extract volume, and sample weight in analytical method (Lee et al. 2008).

8) Specificity : It is defined as the ability of the method to actually determine the analyte, not interfering with the other compounds. Chromatograms of reagent blanks and sample matrix blanks must be free of interfering peaks at the retention time(s) of interest (Fong 1999).

9) Scope : Scope refers to the number of different sample matrix to which the method can be successfully applied. To extend the scope of the method, additional method validation work must be performed on the sample matrix of interest (Fong 1999).

2. Degradation of pesticides in gastrointestinal tracts

Digestion is a series of mechanical and chemical catabolism of food into smaller components that are more easily taken up into a blood stream.

Mastication or chewing is the first step in the food digestion process that reduces food particle size and mixes food particulates with saliva to create a bolus. Saliva is produced by three pairs of salivary glands that drain into the oral cavity. It has a number of organic constituents that serve to initiate digestion (particularly of starch, mediated by amylase) and which also protect the oral cavity from bacteria (such as immunoglobulin A and lysozyme) (Barrett and Ganong 2010).

Food is stored in the stomach; mixed with acid, mucus, and pepsin; and released at a controlled, steady rate into the duodenum. Contents of normal gastric juice are cations (Na^+, K^+, Mg^{2+}, H^+ (pH approximately 1.0), anions (Cl^-, HPO_4^{2-}, SO_4^{2-}), pepsins, lipase, mucus, and intrinsic factor (Barrett and Ganong 2010).

The pancreatic juice contains enzymes that are of major importance in digestion. Its secretion is controlled in part by a reflex mechanism and in part by the gastrointestinal hormones secretin and cholecystokinin. The pancreatic juice is alkaline and has a high HCO_3^- content. Bile and intestinal juices are also neutral or alkaline, and these three secretions neutralize the gastric acid, raising the pH of the duodenal contents to 6.0 - 7.0. By the time the chyme reaches the jejunum, its pH is nearly neutral, but the intestinal contents are rarely alkaline (Barrett and Ganong 2010). Compositions of normal

human pancreatic juice are cations (Na^+, K^+, Ca^{2+}, Mg^{2+} (pH approximately 8.0), anions (HCO_3^-, Cl^-, SO_4^{2-}, HPO_4^{2-}, digestive enzymes (95% of protein in juice), and other proteins (Barrett and Ganong 2010).

An additional secretion important for gastrointestinal function, bile, arises from the liver. The bile acids contained therein are important in the digestion and absorption of fats. Bile is made up of the bile acids, bile pigments, and other substances dissolved in an alkaline electrolyte solution that resembles pancreatic juice.

In vivo approaches for investigating food disintegration in the gastrointestinal tract are conducted by a feeding study, and acquiring the digesta samples using naso-gastric and naso-jejunal tube (Kong and Singh 2008).

In vivo feeding methods, using animals or humans, usually provide the most accurate results, but they are time consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures (Boisen and Eggum 1991). In principle, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients. Compared with *in vivo* study, *in vitro* techniques can save labor and time, reduce cost, and improve accuracy and reproducibility (Kong and Singh 2008). The ideal *in vitro* digestion method would provide accurate results in a short time (Coles et al. 2005) and could thus serve as a tool for rapid screening foods or delivery systems with different compositions and structures. Another advantage for *in vitro* test is that there is no ethical constraint that often limits human experimentation. A number

of *in vitro* gastrointestinal tract models are currently available for nutrition, toxicology, pharmacology, and safety assessments (Kong and Singh 2008).

For all *in vitro* digestion models surveyed in the review research (Hur et al. 2011), the digestion temperature was 37℃ despite the variations in the enzymes employed. The length of the incubation times of samples in the various simulated digestive fluids should mimic the reported digestion times in humans. The digestion time for each step (mouth, stomach, and small intestine) is an important factor to establish when designing an appropriate *in vitro* digestion model (Mcclements et al. 2009). A short transit time of a food within the small intestine may limit the absorption of bioactive lipophilic compounds, thereby reducing their bioavailability (Dahan and Hoffman 2007). A digestion time (the stomach, small intestine and large intestine each) of 2 hr was usually used in many *in vitro* digestion models (Adenugba et al. 2008; Laurent et al. 2007; Lee et al. 2014c; Versantvoort et al. 2005).

3. Metabolism of pesticides by phase I and II reactions

It should be emphasized that, although pesticides and their use have many positive attributes, in terms of their interactions with living organisms, pesticides are xenobiotics and are processed (metabolized) in the same way as other xenobiotics such as clinical drugs and industrial chemicals (Hodgson 2010a).

The metabolism of pesticides involves three phase process; in first phase process (phase I) the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent, in second phase process (phase II) involves conjugation of a pesticide to a sugar or amino acid, which increases the water solubility and reduces toxicity compared with the parent pesticide, and in third phase process helps in conversion of second phase metabolites into secondary conjugates, which are also non-toxic (Van Eerd et al. 2003; Verma et al. 2014).

Williams (1959) first suggested that the metabolism of xenobiotics generally occurs in two phases (Table 1). Phase I reactions involve hydrolysis, reduction, and oxidation. These reactions expose or introduce a functional group (–OH, $–NH_2$, –SH or –COOH), and usually result in only a small increase in hydrophilicity. Phase II biotransformation reactions include glucuronidation, sulfonation (sulfation), acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis), and conjugation with amino acids (such as glycine, taurine, and glutamic acid). The cofactors for these reactions react with functional groups that are either presented on the xenobiotic or are introduced/exposed during phase I biotransformation. Most phase II metabolic reactions result in a large increase in xenobiotic

hydrophilicity, hence they greatly promote the excretion of foreign chemicals (Parkinson 2001).

In vitro metabolism studies are generally carried out using isolated metabolic enzymes and the aim of such *in vitro* characterization is to produce relevant and useful information on metabolism and interactions to anticipate, and even to predict, what happens *in vivo* in human. An overview of different *in vitro* studies for the characterization of metabolism and metabolic interactions of xenobiotics are collected in Table 2.

During the recent years, a large number of papers have been published on the metabolism of pesticides by human CYPs and HLMs involved in (Table 3).

Table 1. General pathways of xenobiotic metabolism and their major subcellular location (Parkinson 2001)

Reaction		Enzyme	Location
Phase I	Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
		Peptidase	Blood, lysosomes
		Epoxide hydrolase	Microsomes, cytosol
	Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
		Carbonyl reduction	Cytosol, blood, microsomes
		Disulfide reduction	Cytosol
		Sulfoxide reduction	Cytosol
		Quinone reduction	Cytosol, microsomes
		Reductive dehalogenation	Microsomes
	Oxidation	Alcohol dehydrogenase	Cytosol
		Aldehyde dehydrogenase	Mitochondria, cytosol
		Aldehyde oxidase	Cytosol
		Xanthine oxidase	Cytosol
		Monoamine oxidase	Mitochondria
		Diamine oxidase	Cytosol
		Prostaglandin H synthase	Microsomes
		Flavin-monooxygenases	Microsomes
		Cytochrome P450	Microsomes
Phase II	Glucuronide conjugation		Microsomes
	Sulfate conjugation		Cytosol
	Glutathione conjugation		Cytosol, microsomes
	Amino acid conjugation		Mitochondria, microsomes
	Acylation		Mitochondria, cytosol
	Methylation		Cytosol, microsomes, blood

Table 2. *In vitro* studies for the characterization of the metabolism and metabolic interactions of xenobiotics (Abass 2010; Pelkonen and Raunio 2005)

In vitro test	Preparations	Parameters	Extrapolations
Metabolic stability	Microsomes Homogenates Cells Slices	Disappearance of the parent molecule or appearance of (main) metabolites	Intrinsic clearance Interindividual variability
Metabolite identification	Microsomes Homogenates Cells Slices	Tentative identification of metabolites (LC-TOF-MS and LC-MS/MS)	Metabolic routes Qualitative (semi-quantitative, if possible) metabolic chart
Identification of metabolizing enzymes	Microsomes with inhibitors or inhibitory antibodies Recombinant individual enzymes	Assignment and relative ability of enzymes to metabolize a compound	Prediction of effects of various genetic, environmental and pathological factors Interindividual variability
Enzyme inhibition	Microsomes Recombinant enzymes	Inhibition of model activities by a substance	Potential drug–drug interactions
Enzyme induction	Cells Slices Permanent cell lines (if available) Constructs	Induction of model activities (or mRNA) Receptor binding (e.g., PXR or CAR)	Induction potential of a substance

CAR : Constitutive androstane receptor, PXR : Pregnane X receptor

Table 3. Metabolism studies of pesticides by human CYPs and HLMs

Pesticide	Chemical class	Usage	Reference
2,4-D	Phenoxy	Herbicide	(Ohkawa et al. 1998)
Acetochlor	Chloroacetanilide	Herbicide	(Kale et al. 2008) (Coleman et al. 2000)
Alachlor	Chloroacetanilide	Herbicide	(Coleman et al. 1999) (Kale et al. 2008)
Ametryne	Triazine	Herbicide	(Cresteil et al. 1979)
Atrazine	Triazine	Herbicide	(Cresteil et al. 1979) (Joo et al. 2010)
Azinphos-methyl	Organophosphate	Insecticide	(Buratti et al. 2003)
Benfuracarb	Carbamate	Insecticide	(Abass et al. 2014a) (Abass et al. 2014b)
Bifenthrin	Pyrethroid	Insecticide	(Scollon et al. 2009)
Bioresmethrin	Pyrethroid	Insecticide	(Scollon et al. 2009)
Butachlor	Chloroacetanilide	Herbicide	(Coleman et al. 2000)
Carbaryl	Carbamate	Insecticide	(Tang et al. 2002)
Carbosulfan	Carbamate	Insecticide	(Abass et al. 2009)
Chlorfenvinphos	Organophosphate	Insecticide	(Hutson and Logan 1986)
Chlorpyrifos	Organophosphate	Insecticide	(Tang et al. 2001) (Buratti et al. 2003) (Sams et al. 2004) (Mutch and Williams 2006)
Chlorpyrifos	Organophosphate	Insecticide	(Choi et al. 2006) (Foxenberg et al. 2007) (Croom et al. 2010) (Smith et al. 2011)
β-Cyfluthrin	Pyrethroid	Insecticide	(Scollon et al. 2009)
λ-Cyhalothrin	Pyrethroid	Insecticide	(Scollon et al. 2009)
Cypermethrin	Pyrethroid	Insecticide	(Scollon et al. 2009)
Deltamethrin	Pyrethroid	Insecticide	(Godin et al. 2006) (Godin et al. 2007)
Diazinon	Organophosphate	Insecticide	(Kappers et al. 2001) (Buratti et al. 2003) (Sams et al. 2004) (Mutch and Williams 2006) (Ellison et al. 2012)

Pesticide	Chemical class	Usage	Reference
Dimethoate	Organophosphate	Insecticide	(Buratti and Testai 2007)
Disulfoton	Organophosphate	Insecticide	(Usmani et al. 2004)
Diuron	Phenylurea	Herbicide	(Abass et al. 2007)
Endosulfan	Cyclodiene	Insecticide	(Lee et al. 2006)
Esfenvalerate	Pyrethroid	Insecticide	(Godin et al. 2006) (Godin et al. 2007)
Fenthion	Organophosphate	Insecticide	(Furnes and Schlenk 2005) (Leoni et al. 2008)
Fipronil	Phenylpyrazole	Insecticide	(Tang et al. 2004) (Joo et al. 2007)
Flucetosulfuron	Sulfonylurea	Herbicide	(Lee et al. 2014b)
Furametpyr	Anilide	Fungicide	(Nagahori et al. 2000)
Imidacloprid	Neonitotinoid	Insecticide	(Schulz-Jander et al. 2002)
Isocarbofos	Organophosphate	Insecticide	(Zhuang et al. 2014)
Malathion	Organophosphate	Insecticide	(Buratti et al. 2005)
Methiocarb	Carbamate	Insecticide	(Usmani et al. 2004)
Methoxychlor	Organochlorine	Insecticide	(Stresser and Kupfer 1998) (Hu and Kupfer 2002)
Metolachlor	Chloroacetanilide	Herbicide	(Coleman et al. 2000)
Molinate	Thiocarbamate	Herbicide	(Jewell and Miller 1999)
Myclobutanil	Triazole	Fungicide	(Barton et al. 2006)
Parathion	Organophosphate	Insecticide	(Butler and Murray 1997) (Buratti et al. 2003) (Sams et al. 2004) (Mutch and Williams 2006) (Foxenberg et al. 2007)
Parathion-methyl	Organophosphate	Insecticide	(Ellison et al. 2012)
Permethrin	Pyrethroid	Insecticide	(Scollon et al. 2009) (Lavado et al. 2014)
Phorate	Organophosphate	Insecticide	(Hodgson 2003) (Usmani et al. 2004)
Sulprofos	Organophosphate	Insecticide	(Usmani et al. 2004)

Pesticide	Chemical class	Usage	Reference
Terbuthylazine	Triazine	Herbicide	(Cresteil et al. 1979)
Terbutryne	Triazine	Herbicide	(Cresteil et al. 1979)
Thiamethoxam	Neonicotinoid	Insecticide	(Swenson and Casida 2013)
Triadimefon	Triazole	Fungicide	(Barton et al. 2006)

3.1 Phase I metabolic reaction

Pesticide absorption occurs through the skin as well as the respiratory and gastrointestinal tracts, with eventual disposition to the liver from all routes of exposure, the liver being the primary site of pesticide biotransformation for the purpose of facilitating clearance through excretion of water-soluble products detoxification (Hodgson 2010c).

The majority of oxidation reactions which xenobiotics undergo are catalysed by one enzyme system, the cytochrome P450 (CYP) mono-oxygenase system (Guengerich 2008). However, there are a number of other oxidative enzyme systems whose importance in the biotransformation of xenobiotics is increasingly being recognized (Timbrell and Marrs 2009). P450 enzymes are categorized into families and subfamilies by their sequence similarities. The human genomes comprise 57 CYP genes and about the same numbers of pseudogenes, which are grouped according to their sequence similarity into 18 families and 44 subfamilies (Abass et al. 2012).

The crystal structures of human P450s are available for human P450s 1A2 (Sansen et al. 2007), 2A6 (Yano et al. 2005), 2C8 (Schoch et al. 2004), 2C9 (Wester et al. 2004; Williams et al. 2003), 2D6 (Rowland et al. 2006), and 3A4 (Ekroos and Sjogren 2006; Williams et al. 2004b; Yano et al. 2004), which are the major P450s involved in drug metabolism (~75%, Figure 1) (Guengerich 2008). Such crystal structures could be used for the prediction of protein-ligand docking study.

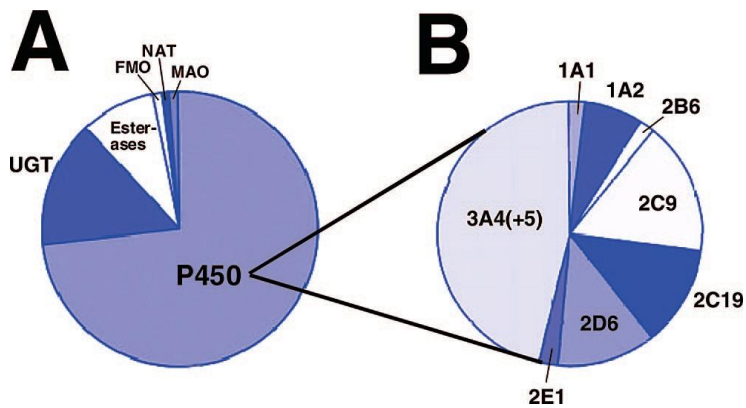


Figure 1. Contributions of enzymes to the metabolism of marketed drugs. P450 : Cytochrome P450, UGT : UDP-glucuronosyl transferases, FMO : Flavin-containing monooxygenase, NAT : N-acetyltransferase, and MAO : Monoamine oxidase (Guengerich 2008; Wienkers and Heath 2005; Williams et al. 2004a).

Many different pesticide monooxygenation reactions are attributed to CYPs, including epoxidation (e.g., aldrin), N-dealkylation (e.g., alachlor, atrazine), O-dealkylation (e.g., chlorfenvinphos), S-oxidation (e.g., phorate), and oxidative desulfuration (e.g., parathion) (Hodgson 2010b).

Like CYP, the flavin-containing monooxygenase (FMO) is a microsomal enzyme, a monooxygenase requiring NADPH and oxygen, and exists as multiple isoforms in various tissues. The FMO is found in highest levels in the liver, but is also found in significant levels in the lung and kidney (Hodgson 2010c). Recent studies have identified five forms of FMO (FMO1 - FMO5), which are differentially expressed with respect to species and tissue (Lawton et al. 1994; Philpot et al. 1995).

The importance of the FMO in pesticide metabolism was established when it was discovered that the FMO oxidizes a variety of thioether

containing pesticides (Chambers and Levi 2013; Hajjar and Hodgson 1980; Hajjar and Hodgson 1982).

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 and Hodgson 1985; Smyser et al. 1985) as well as the oxidation of pesticides from a number of different chemical classes (Tynes and Hodgson 1985).

Other phase I reactions, except CYPs and FMOs, were epoxide hydrolases (e.g., carbaryl) (Dorough and Casida 1964), prostaglandin synthetase (e.g., parathion) (Hodgson 2010b), hydrolases and amidases (e.g., dimthoate, malathion) (Hodgson et al. 2007), and etc..

3.2 Phase II metabolic reaction

Most phase II biotransformation reactions result in a large increase in xenobiotic hydrophilicity, and a formation of the conjugation metabolite. The xenobiotics takes place with glucuronic acid to form glucoronides, 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. In fact, the actual conjugations often occur with derivatives of the conjugating molecule, for example, with glutathione, uridine diphosphate glucuronic acid, or phosphoadenine phosphosulfate (Hodgson 2010b).

With the exception of glutathione conjugation, most conjugation reactions involving pesticides are secondary, involving, as substrates, the products of phase I reaction. They include glucoside, glucuronide formation, sulfate

formation, and conjugation with amino acids.

Glucuronide conjugates represent one of the major types of naturally occurring phase II metabolites of xenobiotics and endobiotics (Ritter 2000). A wide variety of chemicals are metabolized by UDP-glucuronosyl transferases (UGTs) (Ritter 2000), the reaction can occur at hydroxyl, carboxylic acid, amino acid, and even carbon centers (Miners and Mackenzie 1991). UGTs are a superfamily of membrane-bound enzymes whose active site is localized inside the endoplasmic reticulum (Mackenzie et al. 1997). The xenobiotic metabolizing UGTs exist in two subfamilies, designated 1A and 2B (Jedlitschky et al. 1999).

In UGTs assay, the pore forming peptide alamethicin is used *in vitro* metabolism test, because UGTs are localized inside the endoplasmic reticulum. In three assays of UGT catalytic activity estradiol-3-glucuronidation, acetaminophen-O-glucuronidation, and morphine-3-glucuronidation by human UGT1A1, 1A6, and 2B7 isoforms, respectively, treatment of micorosomes with alamethicin (50 mg/mg protein) resulted in conjugation rates 2 - 3 times the rate observed with untreated microsomes (Fisher et al. 2000). And addition of physiological concentrations of Mg^{2+} to the alamethicin-treated microsomes yielded rates that were 4 - 7 times the rates with untreated microsomes (Fisher et al. 2000).

The glutathione S-transferases (GSTs) are an abundant family of dimeric proteins that have the capacity to conjugate glutathione (GST) with a variety of compounds containing electrophilic centers. The major hepatic cytosolic GSTs in mammalian liver can be divided into three classes, alpha (α), mu (μ), and pi (π), based on sequence similarity and catalytic activity (Mannervik et al. 1985).

4. Metabolism of pesticides by microorganisms

Smith and Rosazza (1974) first explored the possibility of using microorganisms as models of mammalian drug metabolism having recognized the potential similarity between microbial and mammalian cytochromes P450 (CYP) (Murphy 2015).

There are clearly a number of practical advantages in the use of microbial systems as models for drug metabolism 1) simple culture media are easily prepared at low cost, and screening for the ability of a large number of strains to metabolize the drug is a simple repetitive process, requiring only a periodical sampling of incubation media, 2) concentrations used (generally ranging from 0.2 to 0.5 g/L) are much higher than those employed in other cell or tissue models; as metabolic capabilities of microorganisms can be high, the amount of metabolites formed is currently in the 20 - 200 mg/L scale, allowing easier detection, isolation and structural identification. The only constraint is the maintenance of stock cultures of microorganisms, which is clearly simpler and cheaper than the maintenance of cell or tissue cultures or laboratory animals (Azerad 1999).

The studies of microbial degradation pesticides have two objectives. First, they are aimed at gaining a basic understanding of how biodegradation activities arise, evolve, and are transferred among members of the soil microflora. This information provides the basis upon which the environmental fate of a crude array of compounds can be predicted. The second objective is to devise bioremediation methods for removing or detoxifying the dangerously high concentrations of pesticide residues (Kumar et al. 1996).

In these metabolic processes fungi and bacteria are involved producing

intracellular or extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, and so on (Van Eerd et al. 2003; Verma et al. 2014). Microbial pesticide conjugation reactions include xylosylation, alkylation, acylation, and nitrosation and can occur intra- or extracellularly (Van Eerd et al. 2003). Especially, bacterial GST-mediated herbicide metabolism is important because herbicide metabolites with thiol, thioester, and sulfoxide moieties have been identified in soil (Feng 1991; Field and Thurman 1996).

The representative microbial model of mammalian drug metabolism is *Cunninghamella* species. They have the ability to metabolize a wide variety of xenobiotics in regio- and stereo-selective manners that are similar to those in mammalian enzyme systems (Davis 1988). It is a filamentous fungus found in soil and plant material, particularly at Mediterranean and subtropical zones. It has also been recovered from animal material, cheese and Brazil nuts (Asha and Vidyavathi 2009; Larone 1993; St-Germain et al. 2011)

The genus *Cunninghamella* currently contains 15 species (Cunninghamella 2015; Zheng and Chen 2001). There are *C. antarctica*, *C. bainieri*, *C. bertholletiae*, *C. binariae*, *C. blakesleeana*, *C. clavata*, *C. echinulata*, *C. elegans*, *C. homothallica*, *C. intermedia*, *C. multiverticillata*, *C. phaeospora*, *C. polymorpha*, *C. septata*, and *C. vesiculosa*.

Among them *C. bertholletiae*, *C. elegans* and *C. echinulata* are the most common species. *C. bertholletiae* is the only known human and animal pathogen. *C. elegans* and *C. bertholletiae* show similar reproductive structures making their identification difficult (Asha and Vidyavathi 2009; Weitzman 1984).

Species of the genus, in particular *C. elegans*, *C. blakesleeana* and *C. echinulata*, have been employed extensively in the transformation of drugs and xenobiotics via phase I (oxidative) and phase II (conjugative) routes (Asha and Vidyavathi 2009; Murphy 2015).

Unlike fungal CYPs, which are typically membrane bound proteins, bacterial CYPs are soluble and thus easier to purify and characterize. *Streptomyces griseus* enables to transform a range of drug and xenobiotics in an analogous fashion to mammals (Taylor et al. 1999). Other species of Streptomyces have also been examined for their abilities to transform drugs *in vivo* and demonstrate similar capabilities for phase I oxidation as *S. griseus* and *Cunninghamella* sp. (Bright et al. 2011). *Bacillus megaterium* ATCC14581 produces CYP BM3 (or 102A1), which in the wild type strain hydroxylates fatty acids (Narhi and Fulco 1986). The wild type enzyme also transforms some drugs that are substrates for CYP3A4, CYP2E1 and CYP1A2 (Di Nardo et al. 2007).

The pesticides metabolism studies of microorganisms such as fungi and bacteria are enumerated in Table 4.

Table 4. Metabolism studies of pesticide in microorganisms

Pesticide	Chemical class	Usage	Microorganism	Reference
2,4,-D	Phenoxyacetate	Herbicide	*Alcaligenes eutrophus* *Flavobacterium* *Arthrobacter* *Pseudomonas cepacia* *Dichomitus squalens*	(Pemberton and Fisher 1977) (Chaudhry and Huang 1988) (Sandmann and Loos 1988) (Bhat et al. 1994) (Reddy et al. 1997)
2,4,5-T	Phenoxyacetate	Herbicide	*Dichomitus squalens*	(Reddy et al. 1997)
Acephate	Organophosphate	Insecticide	*Pseudomonas* sp.	(Rosenberg and Alexander 1979)
Acibenzolar-S-methyl		Fungicide	*B. pumilus* SE34	(Myresiotis et al. 2012)
Alachlor	Chloroacetanilide	Herbicide	*Enterobacteriaceae* and *Pseudomonaceae* *Cunninghamella elegans*	(Zablotowicz et al. 1995) (Pothuluri et al. 1993)
Aldicarb	Carbamate	Insecticide	*Pseudomonas sp.*	(Read 1987)
Aldrin	Cyclodiene	Insecticide	*Pseudomonas fluorescens*	(Bandala et al. 2006)
Atrazine	Triazine	Herbicide	*Norcardia* *Pseudomonas*	(Cook 1987) (Mandelbaum et al. 1995) (Behki et al. 1993) (Behki and Khan 1986)
Carbaryl	Carbamate	Insecticide	*Pseudomonas aeruginosa* *Bacillus sp.* *Micrococcus sp.*	(Chapalmadugu and Chaudhry 1993) (Rajagopal et al. 1986)

Pesticide	Chemical class	Usage	Microorganism	Reference
Carbofuran	Carbamate	Insecticide	*Achromobacter*	(Karns et al. 1986)
			Pseudomonas	(Chaudhry and Ali 1988)
			Flavobacterium	(Head et al. 1992)
			Streptomyces sp.	(Venkateswarlu and Sethunathan 1984)
			Azospirillum lipoferum	
Chlorpyriphos	Organophosphate	Insecticide	*Klebsiella* sp.	(Ghanem et al. 2007)
			Serratia and *Trichosporon* sp.	(Xu et al. 2007)
			Synechocystis sp. strain PUPCCC 64	(Singh et al. 2011)
			Phanerochaete chrysosporium	(Bumpus et al. 1993)
			Acremonium sp. strain GFRC-1	(Kulshrestha and Kumari 2011)
			Bacillus licheniformis ZHU-1	(Zhu et al. 2010a)
			Cladosporium cladosporioides Hu-01	(Gao et al. 2012)
			Bacillus pumilus C2A1	(Anwar et al. 2009)
			Azospirillum lipoferum and *Paenibacillus polymyxa*	(Romeh and Hendawi 2014)
Cyanophos	Organophosphate	Insecticide	*Azospirillum lipoferum* and *Paenibacillus polymyxa*	(Romeh and Hendawi 2014)
Cyprodinil	Anilinopyrimidine	Fungicide	*Cunninghamella elegans* ATCC36112	(Schocken et al. 1997)
			Absidia pseudocylindraspora ATCC 24169	
			Streptomyces rimosus ATCC 10970	
DDT	Organochlorine	Insecticide	*Trichoderma viride*	(Matsumura and Boush 1968)
			Ralstonia eutropha strain A5	(Hay and Focht 2000)
			Eubacterium limosum	(Yim et al. 2008)
			Azoarcus	(Ortiz et al. 2013)

Pesticide	Chemical class	Usage	Microorganism	Reference
DEET		Insect repellent	*Cunninghamella elegans* ATCC 9245	(Seo et al. 2005)
Diazinon	Organophosphate	Insecticide	*Flavobacterium* ATCC 27551 *Pseudomonas* sp. *Serratia marcescens* DI101	(Sethunathan and Yoshida 1973) (Rosenberg and Alexander 1979) (Abo-Amer 2011)
Dieldrin	Cyclodiene	Insecticide	*Bacillus* sp. *Pseudomonas* sp. *Pseudomonas fluorescens*	(Matsumura and Boush 1967) (Matsumura et al. 1968) (Bandala et al. 2006)
Dimethoat	Organophosphate	Insecticide	*Pseudomonas* sp.	(Rosenberg and Alexander 1979)
Endosulfan	Organochlorine	Insecticide	*Klebsiella* sp. M3 *Alcaligenes faecalis* JBW4	(Singh and Singh 2014) (Kong et al. 2013)
EPTC	Carbamate	Herbicide	*Arthrobacter* *Rhodococcus* *Rhodococcus*	(Tam et al. 1987) (Behki et al. 1993) (Behki and Khan 1994)
Ethaboxam	Amide	Fungicides	*Cunninghamella elegans*	(Park et al. 2003)
Fenitrothion	Organophosphate	Insecticide	*Burkholderia* sp. strain NF100	(Hayatsu et al. 2000)
Fenoxaprop-ethyl	Phenoxypropionate	Herbicide	*Pseudomonas fluorescens*	(Hoagland and Zablotowicz 1998)
Fenthion	Organophosphate	Insecticide	*Bacillus*	(Hoagland and Zablotowicz 1998)
Fensulfothion	Organophosphate	Insecticide	*Pseudomonas* sp.	(Rosenberg and Alexander 1979)
Fipronil	Pyrazole	Insecticide	*Bacillus thuringiensis*	(Mandal et al. 2013)
Fonofos	Organophosphorous	Insecticides	*Phanerochaete chrysosporium*	(Bumpus et al. 1993)
Furalaxyl	Acylamino acid	Fungicide	*Brevibacillus brevis*	(Sulimma et al. 2013)

Pesticide	Chemical class	Usage	Microorganism	Reference
Glyphosate	Organophosphorus	Herbicide	*Flavobacterium* sp. *Pseudomonas* sp. PG2982 *Alcaligenes* isolate *Bacillus megaterium* strain 2BLW *Arthrobacter* sp.GLP-1	(Balthazor and Hallas 1986) (Moore et al. 1983) (Talbot et al. 1984) (Quinn et al. 1989) (Pipke et al. 1987)
Heptachlor	Cyclodinene	Insecticide	*Pseudomonas fluorescens*	(Bandala et al. 2006)
Heptachlor epoxide	Cyclodinene	Insecticide	*Pseudomonas fluorescens*	(Bandala et al. 2006)
Isoproturon	Phenylurea	Herbicide	*Cunninghamella elegans*	(Hangler et al. 2007)
Malathion	Organophosphate	Insecticide	*Trichoderma viride* *Pseudomonas* sp. *Arthrobacter* sp. *Aspergilus oryzae* *Lysinibacillus* sp. strain KB1 *Brevibacillus* sp. strain KB2 and *Bacillus cereus* strain PU *Pseudomonas* sp. and *Pseudomonas putida*	(Matsumura and Boush 1966) (Walker and Stojanovic 1974) (Lewis et al. 1975) (Singh et al. 2012b) (Singh et al. 2012a) (Goda et al. 2010)
Mepanipyrim	Anilinopyrimidine	Fungicides	*Cunninghamella elegans* ATCC36112	(Zhu et al. 2010b)
Metalaxyl	Acylamino acid	Fungicide	*Brevibacillus brevis*	(Sulimma et al. 2013)
Methomyl	Carbamate	Insecticide	*Stenotrophomonas maltophilia* M1	(Mohamed 2009)
Methoxychlor	Organochlorine	Insecticide	*Cunninghamella elegans* ATCC36112	(Keum et al. 2009)
Metobromuron	Phenylurea	Herbicide	*Talaromyces wortmanii*	(Tweedy et al. 1970)

Pesticide	Chemical class	Usage	Microorganism	Reference
Metolachlor	Anilide	Fungicide	*Cunninghamella elegans* ATCC36112	(Pothuluri et al. 1997)
Monocrotophos	Organophosphates		*Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423	(Bhadbhade et al. 2002)
Parathion	Organophosphates	Insecticide	*Flavobacterium* ATCC 27551 *Pseudomonas diminuta* *Pseudomonas stutzeri*	(Sethunathan and Yoshida 1973) (Serdar et al. 1982) (Daughton and Hsieh 1977)
Propanil	Acylanilides	Herbicide	*Fusarium oxysporum* Schlecht	(Blake and Kaufman 1975)
Terbufos	Organophosphorous	Insecticides	*Phanerochaete chrysosporium*	(Bumpus et al. 1993)
Vinclozolin	Dicarboximide	Fungicide	*Cunninghamella elegans* Mixed bacteria	(Pothuluri et al. 2000) (Mercadier et al. 1998)

5. Human liver microsomes (HLMs)

In vitro human metabolism studies of pesticides are very important and useful for prediction and management of the hazard and risk arising from introduction of pesticides in that understanding metabolism of pesticides.

Subcellular fractions, which include microsomes, other cellular organelles, and liver homogenates, continue to be the most widely used *in vitro* system for drug metabolism (Ekins et al. 2000). The advantages of using these systems include their ease of preparation, reproducible nature, capacity for long-term storage, and ample characterization of optimal incubation conditions (Sean et al. 1999). Their disadvantages are well understood, in that some enzymes may be more labile and require addition of cofactors for optimal activity to replace those lost due to decreased cell integrity (Ekins et al. 2000). Microsomes remain the most widely used subcellular fraction in use as an *in vitro* system for metabolism studies. It remains to be seen whether other subcellular fractions will achieve a similar level of importance in metabolism studies.

Human liver microsomes can be prepared easily from frozen liver tissue, and enzymatic activities are stable during prolonged storage (Yamazaki et al. 1997). Microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by standard differential ultracentrifugation (Pelkonen et al. 1974). Liver homogenates centrifuged at 9000 ×g results in a supernatant (S9 fraction) rich in drug-metabolizing enzymes and a pellet containing predominately nuclei, lysosomes, peroxisomes and mitochondria. Centrifugation the S9 fraction at 100,000 ×g results in a supernatant (cytosol) and pellet (microsomes). Liver preparations, other than from fresh human liver, can also be used (e.g., liver slices, liver cell lines, and primary

hepatocytes) for the preparation of microsomes (Li 2008; Olsen et al. 1997).

Recombinant P450 isoforms are microsomes derived from organisms transfected with genes for individual human P450 isoforms (e.g., bacteria, yeast, mammalian cells (Barnes et al. 1991; Donato et al. 2004; Friedberg et al. 1999)) and therefore contain only one specific human isoform. The major human P450 isoforms involved in drug metabolism are available commercially as rCYP. The main CYP proteins expressed in human liver are CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, and their expression and activities dictate the pharmacological effect of drugs (Guengerich 2003). This experimental system is widely used to evaluate the drug-metabolizing activities of individual P450 isoforms (Rodrigues 1999).

6. Cyazofamid

Cyazofamid (4-chloro-2-cyano-N,N-dimethyl-5-*p*-tolylimidazole-1-sulfonamide, Figure 2) is a sulfonamide fungicide (Tomlin and British Crop Protection Council. 2009), has been used for the protection of several vegetables and fruits from various diseases, such as tomato late blight (*Phytophthora infestans*), downy mildews (*Pseudoperonospora cubensis* of cucumber) and seedling blight of rice, barley, maize, sweet potato (*Pythium spinosum*), by inhibiting the Qi site (the ubiquinone reducing site) of cytochrome bc1 in complex III (ubiquinol-cytochrome c reductase) of the mitochondrial respiratory chain (Mitani et al. 2001; Mitani et al. 2002; Mitani et al. 2003; Tomlin and British Crop Protection Council. 2009).

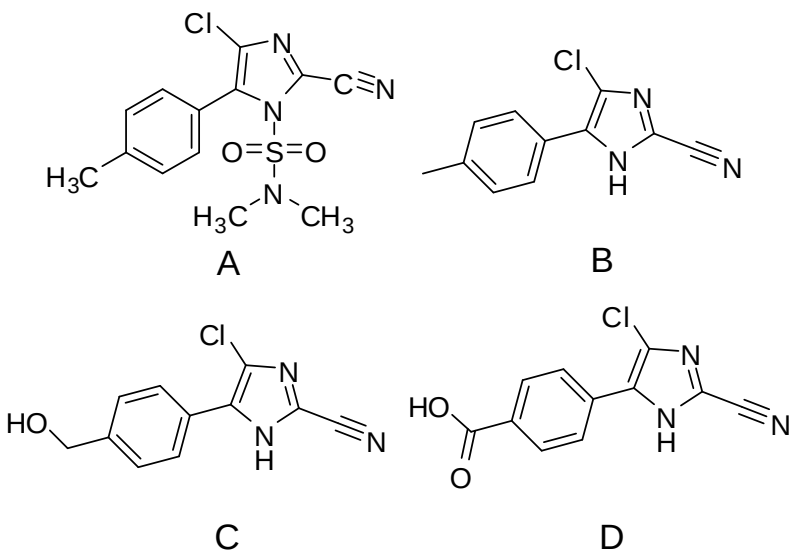


Figure 2. Structures of cyazofamid (A), CCIM (B), CHCN (C), and CCBA (D).

Cyazofamid has minimal to moderate toxicity in acute oral, dermal and inhalation test, LD_{50} for rats are >5,000 mg/kg, >2,000 mg/kg and >5.5

mg/kg, respectively (EPA 2004). In the absorption, distribution, metabolism, and excretion (ADME) of cyazofmaid, 24 hours after administration, 90% or more of the dose was excreted in the urine and feces, and 168 hours after administration less than 0.5% TAR remained in the tissues (Terada et al. 2004). Major metabolites were 4-(4-chloro-2-cyanoimidazole-5-yl)benzoic acid (CCBA), 4-chloro-5-*p*-tolylimidazole-2-carbonitrile (CCIM), 4-chloro-5-(4-hydroxymethylphenyl)imidazole-2-carbonitrile (CHCN) and their conjugates (Terada et al. 2004).

In ecological effect, indicated by low Log P_{ow} (3.2 at 25°C). Pharmacokinetics and metabolism studies in rats showed rapid absorption and elimination. Both the urine and feces were major routes of excretion, and excreted material was mainly 4-(4-chloro-2-cyanoimidazol-5-yl)benzoic acid (Ware 1978). Cyazofamid was degraded rapidly in aerobic soil (DT_{50} in soil : 3 - 5 days) and covalently bound to organic matter after degradation into the major degradates such as 4-chloro-5-*p*-tolylimidazole-2-carbonitrile and 4-chloro-5-*p*-tolulimidazole-2-carboxylic acid (Terada et al. 2004).

The first registration of cyazofamid was received in March 2001 in the United Kingdom. This was followed by registration in Korea and Japan in April 2001, and it is sold as Miricut® in Kyung Nong Corporation in Korea (Mitani et al. 2001; Ware 1994). Maximum residue limits (MRLs) of cyazofamid in several vegetables and fruits is established (Table 5).

Only few reports were available for the analysis of cyazofamid residues in the limited environmental or crop/food samples including grapes (Gonzalez-Alvarez et al. 2012a; Gonzalez-Rodriguez et al. 2009; Gonzalez-Rodriguez et al. 2011), ginseng (Choi et al. 2007), potato plant (Doshi et al. 2011), soil and water (Doshi et al. 2011; Singh and Tandon 2015; Tandon

and Singh 2012; Tandon and Singh 2015). Such methods utilized HPLC–UVD (Choi et al. 2007; Singh and Tandon 2015; Tandon and Singh 2012; Tandon and Singh 2015) or GC–ITMS (Gonzalez-Alvarez et al. 2012a; Gonzalez-Rodriguez et al. 2009; Gonzalez-Rodriguez et al. 2011) after conventional sample treatment, and only cyazofamid was analyzed without metabolites. Some scientists analyzed cyazofamid as one of multiresidue from fruits and vegetables by LC-MS/MS or GC-MS/MS (Pihlstrom et al. 2007; Pizzutti et al. 2009; Tseng et al. 2009).

Table 5. MRLs of cyazofamid in various crops (Ministry of Food and Drug Safety 2015)

Crops	MRLs	Crops	MRLs	Crops	MRLs
Mandarin	0.5	Kimchi cabbage	0.7	Sesame	0.1
Potato	0.1	Peach	1.0	Oriental melon	0.5
Pepper	2.0	Ginger	0.5	Tomato	0.5
Leaf beet	10	Watermelon	1.0	Spring onion	1.0
Others	0.05	Spinach	3.0	Grape (wild grape)	2.0
Jujube (dry)	3.0	Onion	1.0	Bell pepper	2.0
Melon	0.5	Winter grown cabbage	2.0	Crown daisy	15
Pear	0.2	Cucumber	0.5	Dried ginseng	0.3
Ginseng concentrate	1.0	Red ginseng concentrate	1.0	Fresh ginseng	0.3
Red ginseng	0.3				

7. The purposes of the present study

The purpose of the present study was to establish the analytical method of cyazofamid or cyazofamid metabolite, CCIM by HPLC and LC-MS/MS as well as to investigate metabolites formation by metabolism of cyazofamid by soil fungus *Cunninghamella elegans* and *in vitro* human mimicking system including gastrointestinal juice, microsomes and S9 fractions.

The analytical method of cyazofamid residue in crop/food samples using HPLC was established, which is easily accessible for many different crop and food samples. And a rapid and effective analytical method of cyazofamid and its metabolite, CCIM, using LC–MS/MS after QuEChERS sample treatment was established. The metabolism of cyazofamid by soil fungus *Cunninghamella elegans* ATCC36112 was conducted to investigate the metabolites formation and metabolic pattern of cyazofamid. Metabolite identification was carried out by HPLC, LC-MS/MS, and NMR. The metabolism study of cyazofamid by *in vitro* human mimicking system was conducted to identify the metabolite, metabolic pattern, the metabolic kinetics and CYP isoforms responsible for metabolism of cyazofamid.

CHAPTER I

Residue Analysis of Cyazofamid

I. Establishment of Analytical Method for Cyazofamid Residue in Apple, Mandarin, Korean Cabbage, Green Pepper, Potato and Soybean

1. Introduction

Pests are organisms that are competitive to mankind or his interests in some aspect. The world's main source of food is plants, but they are susceptible to 80,000 - 100,000 kinds of diseases, 1,800 weed species, 10,000 insect species, and 1,000 nematode species as pests. Pesticides are any substance or mixture of substances intended for preventing, destroying, repelling or migrating any pest (Ware 2004) and are applied to protect food crops from the pests at various stages of cultivation and during post-harvest storage, and is essential in agricultural production. However, with their use, the risk of residues remaining on the food is of major concern in food safety issues. Legislations were enacted throughout the world to regulate pesticides in food products (Ahmed 2001), and maximum residue limits (MRLs) in foodstuffs have been set by government agencies to guarantee consumer safety and to regulate international trade. For this reason, a variety of analytical methods have been developed and applied routinely for the control of pesticide residue in food. Analysis of pesticide residues is extremely difficult, because sample matrix is complex, pesticides consist of many types of compounds, and its residues exist at ppm level or lower. Therefore, analytical methodologies employed must be capable of residue measurement at very low levels and must also provide unambiguous evidence to confirm both the identity and the magnitude of any residues

detected (Taylor et al. 2002).

Cyazofamid [4-chloro-2-cyano-N,N-dimethyl-5-*p*-tolylimidazole-1-sulfonamide] is a sulfonamide fungicide (Tomlin and British Crop Protection Council. 2009) and registered 2001 in Korea for protection of ginger, potato, tomato, pepper, mandarin, ginseng, grape, Korean cabbage, melon, and green onion from various diseases (KCPA 2010).

It has very low mammalian toxicity (LD_{50} for rats : >5000 mg/kg) and ecological effect, indicated by low Log P_{ow} (3.2 at 25 ℃). Pharmacokinetics and metabolism studies in rats showed rapid absorption and elimination. Both the urine and feces were major routes of excretion, and excreted material was mainly 4-(4-chloro-2-cyanoimidazol-5-yl)benzoic acid (EPA 2004). Cyazofamid degraded rapidly in aerobic soil (DT_{50} in soil : 3–5 days) and was covalently bound to organic matter after degradation into the major degradates such as 4-chloro-5-*p*-tolylimidazole-2-carbonitrile and 4-chloro-5-*p*-tolulimidazole-2-carboxylic acid (Terada et al. 2004).

Only few reports were available for the analysis of cyazofamid residues in crop or food including grapes and ginseng (Choi et al. 2007; Gonzalez-Alvarez et al. 2012a; Gonzalez-Alvarez et al. 2012b; Gonzalez-Rodriguez et al. 2011; 農藥殘留分析法研究班 2006). However, such methods are not suitable for standard analytical method, because the subject crops are limited, not validated in full manner, time-consuming, and labor-intensive due to many steps involved in the clean-up procedure (農藥殘留分析法研究班 2006). In Korea, the analytical method of cyazofamid residues in crop/food is listed in Food Code (KCPA 2010) only as a part of multiresidue method for screening, therefore it cannot be used as a precise and reliable standard analytical method due to lack of method

validation data.

The purpose of the present study is the establishment of a standard analytical method of cyazofamid residues in crop/food using HPLC, which is easily accessible and approved by the goverment for many different crop/food samples through full method validation and improvement for more efficient and simpler clean-up procedures than other existing methods using HPLC. Representative crops were selected among five crop groups such as cereal, fruits, vegetables, beans and oil crops, and potatoes.

2. Materials and methods

2.1 Subject pesticides and crops

Standard material of cyazofamid (98.4%) was purchased from Fluka™ (Buchs, Switzerland). Apple, mandarin, Korean cabbage, green pepper, potato, and soybean of "residue-free grade" were purchased from a local market. They were chopped, macerated, and kept in a freezer at a temperature below -20℃ in polyethylene bags.

2.2 Chemicals, reagents, and standard solutions

Acetonitile, acetone, *n*-hexane, and ethyl acetate were HPLC grade (Burdick and Jackson®, Ulsan, Korea). Sodium sulfate (GR grade) and sodium chloride (GR grade) were from Junsei Chemical Co. Ltd. (Tokyo, Japan). Florisil® (60 - 100 mesh) was purchased from Fluka™ (Saint Louis, MO) and activated by drying at 130℃ for over 5 hr. Filter papers (GF/A) were from Whatman International Ltd. (Maidstone, England). Fat-removing solvent (FR solvent) was prepared by saturating of *n*-hexane in acetonitrile. A stock solution of cyazofamid was prepared in acetonitrile at 1000 mg/L,

and the working solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile.

2.3 Measurement of instrumental sensitivity, reproducibility, and calibration curve linearity

Cyazofamid standard solutions (1, 0.1, and 0.05 mg/L) were analyzed with HPLC and the signal/noise ratio (S/N) of cyazofamid peak on chromatograms was calculated for LOD (S/N of 3) and LOQ (S/N of 10). For the assessment of reproducibility, a standard solution (0.05 mg/L) was analyzed with HPLC in seven replicates, and variations of retention time (t_r), peak area, and symmetry were examined. The standard solutions at 0.05, 0.1, 0.5, 1, 10, and 20 mg/L were analyzed with HPLC, and calibration curve linearity (R^2) was measured.

2.4 Establishment of the HPLC condition for separation of cyazofamid in crop samples

HPLC analysis was performed using an Agilent HPLC 1100 series system (Santa Clara, CA) with Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm i.d., 5 μm particles), and column temperature was maintained at 35℃. Mobile phase was acetonitrile-water and the flow rate was 1 mL/min. For the analysis of apple, Korean cabbage, potato, and soybean, 65 : 35 (acetonitrile : water) was used as mobile phase, 62 : 38 for mandarin, and 63 : 37 for green pepper samples. The injection volume was 20 μL, and the detection wavelength of cyazofamid in crop samples was 280 nm.

For the selection of optimum detection wavelength of cyazofamid, aliquot (20 μL) of a standard solution (1 mg/kg) was analyzed to obtain a full UV

spectrum with diode-array detector (DAD; 180 - 400 nm) under isocratic elution (acetonitrile : water = 70 : 30).

2.5 Establishment of sample preparation procedure for cyazofamid

For establishment of optimum clean-up system, a glass column (35 cm × 1.5 cm i.d.) was filled with activated Florisil® (10 g) and added with anhydrous sodium sulfate (3 g). The column was pre-conditioned with *n*-hexane (100 mL) before loading the cyazofamid standard solution (5 mL, 1 mg/L). The column was eluted with 50 mL of 10, 20, 30, 40, and 50% ethyl acetate/*n*-hexane mixture sequencially. Each eluate was evaporated under 40℃ to dryness; the residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

For the optimization of the liquid-liquid partitioning system, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to water (25 mL) and left standing about 30 min in a separatory funnel before water (50 mL) and saturated sodium chloride solution (50 mL) were added. The mixture was extracted with each portion of three solvents (dichloromethane, *n*-hexane and ethyl acetate; 100 and 50 mL for each solvent). Organic phases were dried over anhydrous sodium sulfate and evaporated under 40℃ to concentration. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

To establish a fat removal system for soybean, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to 50 mL of *n*-hexane saturated with acetonitrile, and extracted with FR solvent (50, 30 mL). Acetonitrile layer was evaporated under 40℃. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

To select the proper sample extraction solvent, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to green pepper sample (25 g) and left standing fot about 30 min in flask. The flask was shaken at 180 rpm (1 hr) for extraction after three solvents (acetone, acetonitrile, and methanol; 100 mL each) were added. The extract was filtrated, evaporated, partitioned, purified, and concentrated as mentioned in recovery procedure. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

2.6 Recovery test of cyazofamid in crop samples

Samples (25 g) of apple, mandarin, Korean cabbage, green pepper, soybean, and potato were macerated and fortified with cyazofamid standard solution at 0.02, 0.2, and 2 mg/kg levels before the samples were extracted with shaking in the reciprocal shaker (SA-2s, Taitec, Japan) at 180 rpm for 1 hr with acetone (100 mL). The mixture was filtered under reduced pressure through a Whatman™ GF/A filter paper, and the filter cake was rinsed with acetone (30 mL). The filtrate was concentrated under vacuum at 40℃ (R-114, Büchi, Switzerland). The concentrate was dissolved in dichloromethane (100 mL), water (50 mL), and saturated sodium chloride solution (50 mL) for partitioning by shaking. Partitioning was repeated once more with 50 mL of dichloromethane and the combined dichloromethane layer was dried over anhydrous sodium sulfate, concentrated, and dissolved in *n*-hexane (5 mL). After loading the extract on the Florisil® column, which was conditioned with *n*-hexane (100 mL), the column was washed with 100 mL of ethyl acetate/*n*-hexane (10 : 90, v/v) and eluted with 100 mL of ethyl acetate/*n*-hexane (30 : 70, v/v). The eluate was concentrated, dissolved with acetonitrile (5 mL) and analyzed with HPLC.

2.7 Retention factor of cyazofamid of chromatogram

Retention factor (capacity factor, *k*) was calculated from equation using retention time (t_r) and adjusted retention time (t_r').

$k = t_r'/t_m$

t_r = retention time (min)

t_m = retention time of a non-retained compound (min)

$t_r' = t_r - t_m$ = adjusted retention time (min)

2.8 Number of theoretical plate (*N*) and height equivalent to a theoretical plate (*H*)

N was calculated using t_r and peak width. *N* and column length was used for calculation of H (Rood 2007).

$N = 5.545\ (t_r/W_h)^2$

W_h = peak width at half height

H (mm) = column length (mm)/*N*

3. Results and discussion

3.1. Optimum detection wavelength of cyazofamid

Optimum detection wavelength of cyazofamid in HPLC was investigated for sensitive detection. When a full UV spectrum of cyazofamid was recorded using DAD, λ_{max} was observed at 280 nm, therefore 280 nm was used as a detection wavelength in the present study (Figure 3). Other reports also engaged 280 nm (農藥殘留分析法研究班 2006) and 290 nm (Suciu et al. 2011) as a detection wavelength.

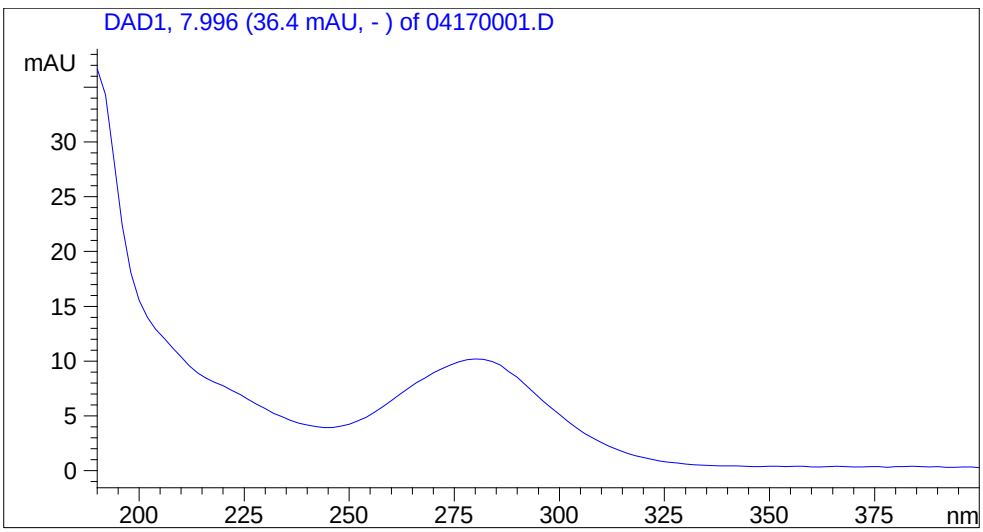


Figure 3. UV spectrum of cyazofamid.

3.2 Clean-up method with Florisil® column chromatography

In pesticide residue analysis, adsorption chromatography is generally used for clean-up of the interfering coextractives (e.g. lipids and pigments), which were not removed by liquid-liquid partitioning. Florisil® , silica gel, and alumina were used traditionally as column chromatography sorbents. On the other hand, Choi et al. (2007) used NH_2 cartridge. In the present

study, Florisil® , the most popular material for clean-up in pesticide analysis, was chosen for absorption column chromatography. Various combinations of ethyl acetate/*n*-hexane mixture were used in sequence after loading of cyazofamid on Florisil® column. As a result, a mixture of 30 : 70 (ethyl acetate/*n*-hexane, v/v) gave a good recovery, indicating a mixture of such combination of 100 mL could be used as an elution solvent, whereas a mixture of 10 : 90 (ethyl acetate/*n*-hexane, v/v) be used for washing of the column to remove early eluting impurities without losing cyazofamid during clean-up procedure (Table 6).

3.3 Liquid-liquid partitioning of cyazofamid

After successful establishment of the clean-up procedure, liquid-liquid partitioning system was examined. Through this procedure, the polar interfering coextractives (e.g. carbohydrates) could be removed from sample extract (Fong 1999). Sodium chloride was added in partitioning system, because as more ‘salt’ dissolves in the aqueous phase, more of the pesticide is partitioned into the organic phase (Fong 1999). In the present study, three organic solvents such as dichloromethane, ethyl acetate, and *n*-hexane, were used with water (Table 7). Cyazofamid was well partitioned with dichloromethane, giving a total recovery of 105.4%. Therefore, dichloromethane (100, 50 mL) was selected as the organic solvent for liquid-liquid partitioning system.

For the fat removal system of soybean samples, the recovery rate of cyzofamid extracted with FR solvent (50, 30 mL) were 100.8% and 3.5%, respectively.

3.4 Selection of extraction solvent

Three common solvents such as acetone, acetonitrile, and methanol were used to extract cyazofamid from crop samples. All three solvents gave reasonable recoveries (94.2 - 97.8%); however, acetone (94.9%) was chosen as the extraction solvent.

Table 6. Recovery rate by sequential elution of ethyl acetate/*n*-hexane

Ethyl acetate/*n*-hexane		Recovery (%)
v/v	Volume (mL)	Florisil®
10 : 90	50	-
20 : 80	50	-
30 : 70	50	91.7
40 : 60	50	4.3
50 : 50	50	-
Total		96.0

Table 7. Efficiency of liquid-liquid partitioning with three different solvents

Solvents	Recovery (%)		
	100 mL	50 mL	Total
n-Hexane	98.8	3.1	101.8
Ethyl acetate	92.2	5.6	97.8
Dichloromethane	100.3	5.1	105.4

3.5 Method validation

Method validation is a set of procedures to evaluate the performance characteristics such as recovery, reproducibility, linearity and range of calibration, limits of detection, and quantitation of a method for specific analyte and sample types (Guidelines on food laboratory practice in residue analysis 2010).

Instrumental LOD and LOQ express the sensitivity of analytical instruments (Fong 1999; Miller 2005). Based on the analysis of several concentrations, 0.4 and 2 ng was determined as LOD and LOQ, respectively, which are satisfactory for sensitive analysis of cyazofamid residue. Choi et al. (2007) reported that LOQ was 1.6 ng using HPLC.

For reproducibility study, LOQ level of cyazofamid solution (2 ng) was analyzed seven times (Table 8). Good reproducibility was observed with small coefficient of variation (<4%) for retention time (t_r), peak area, and peak symmetry, providing a good stability of instrument for reliable analysis. Good peak shape was also observed within values of 0.9 - 1.1 (Rood 2007).

Excellent linearity was achieved between 0.05 and 20 mg/kg of cyazofamid standard solutions, with coefficient of determination (R^2) of 1.000. The regression equations were $y = 50.7810x - 0.3448$ for apple, Kimchi cabbage, potato, and soybean, $y = 51.8090x + 0.2325$ for mandarin, and $y = 51.1591x - 0.9011$ for green pepper (Figure 4).

MLOQ (Method limit of quantitation) is not an instrumental LOQ, but instead is a practical LOQ for the total analytical method. It is calculated using LOQ, injection volume, final extract volume, and sample weight in analytical method (Lee et al. 2008).

$$\text{MLOQ (mg/kg)} = (2\ \text{ng} \times 5\ \text{mL})/(20\ \mu\text{L} \times 25\ \text{g}) = 0.02\ \text{mg/kg}$$

MLOQ value for cyazofamid calculated using the above equation was 0.02 mg/kg. This value satisfied the criteria of KFDA (Korea Food and Drug Administration) which are below 0.05 mg/kg or half of MRL (Lee 2011). Compared to other works with grapes (MLOQ = 0.72 mg/kg) (Gonzalez-Alvarez et al. 2012a), our result is relatively more sensitive.

Table 8. LOD, LOQ and reproducibility of analysis of cyazofamid

LOD	LOQ	Reproducibility		
		Factors	Average	C.V (%)
0.4 ng	2 ng	t_r (min)	8.78	0.08
		Area	5.03	1.81
		Peak symmetry	1.08	3.20

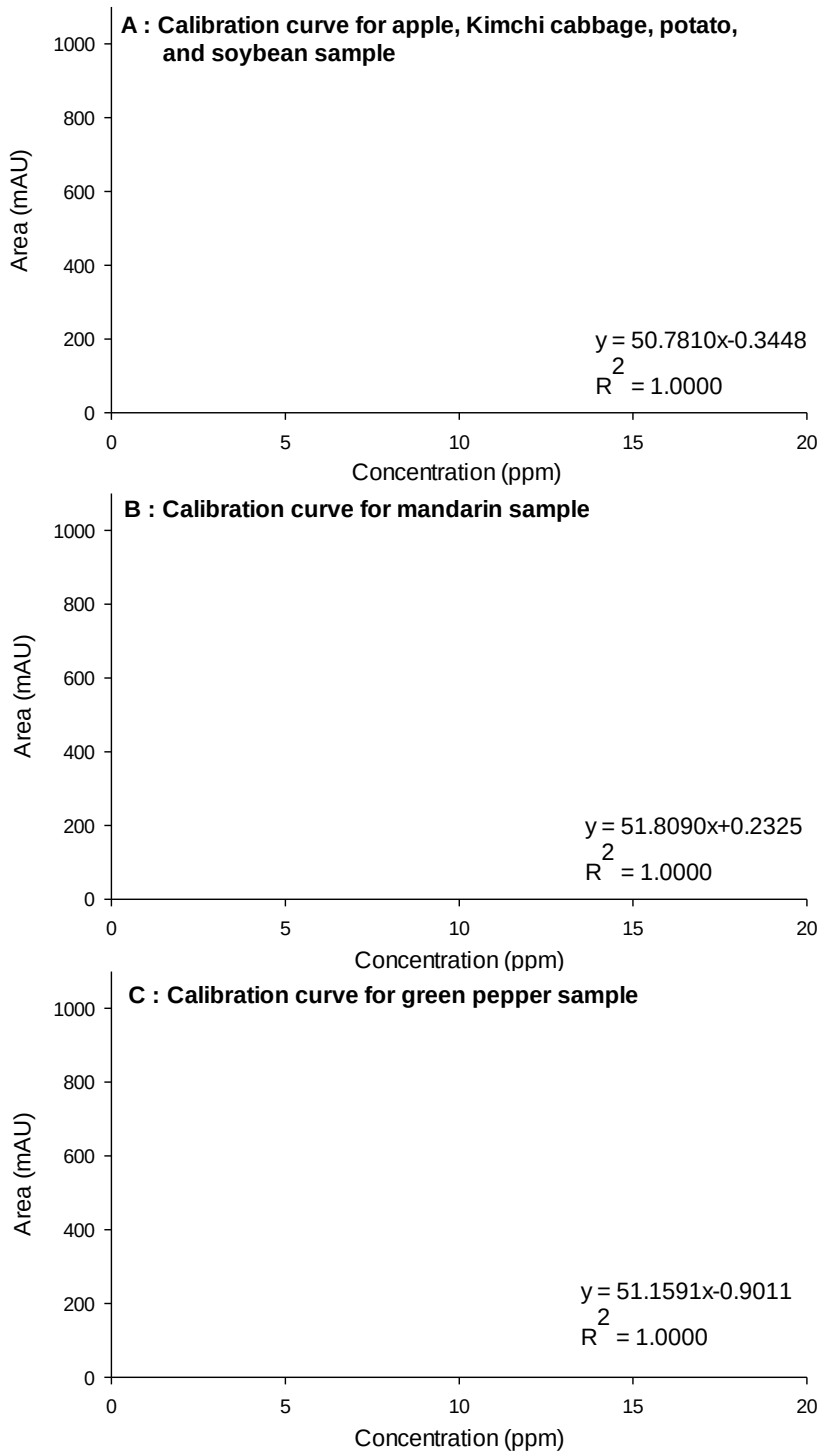


Figure 4. Calibration curves of cyazofamid. A : For apple, Kimchi cabbage, potato, and soybean sample, B : For mandarin samples, C : For green pepper samples.

3.1.6 Recoveries of cyazofamid from crop samples

Recovery test can provide accuracy and precision of sample preparation method by recovered rate (accuracy, %) and C.V (precision, %) (Fong 1999). Untreated samples were spiked with MLOQ (0.02 mg/kg), 10MLOQ (0.2 mg/kg) and MRL (2 mg/kg) levels of cyazofamid standard solutions, and the analysis was performed using the established method of extraction, partitioning and clean-up to give reasonable recoveries (75.3 - 98.5%) and low C.V (0.5 - 9.2%) (Table 9, Figure 5 - 10).

Other studies also reported reasonable recoveries; Gonzalez-Rodriguez et al. (2011) reported that recoveries were 85 - 103% in grapes and wines by HPLC-DAD and GC-ITMS, and Choi et al. (2007) reported that recovery was 80.6% in ginseng by HPLC-UVD.

Table 9. Recovery and MLOQ for cyazofamid in crops

Crops	Recovery (%)/C.V (%)			MLOQ (mg/kg)
	0.02 mg/kg	0.2 mg/kg	2 mg/kg	
Apple	79.1/5.2	95.2/0.8	91.7/0.5	0.02
Mandarin	75.3/6.0	78.5/4.5	86.1/0.5	
Kimchi cabbage	92.0/2.6	89.9/1.3	88.0/2.7	
Green pepper	90.1/1.3	86.1/3.7	88.6/3.4	
Potato	87.0/3.4	83.0/4.7	78.9/9.2	
Soybean	98.5/4.4	89.8/2.7	82.6/5.9	

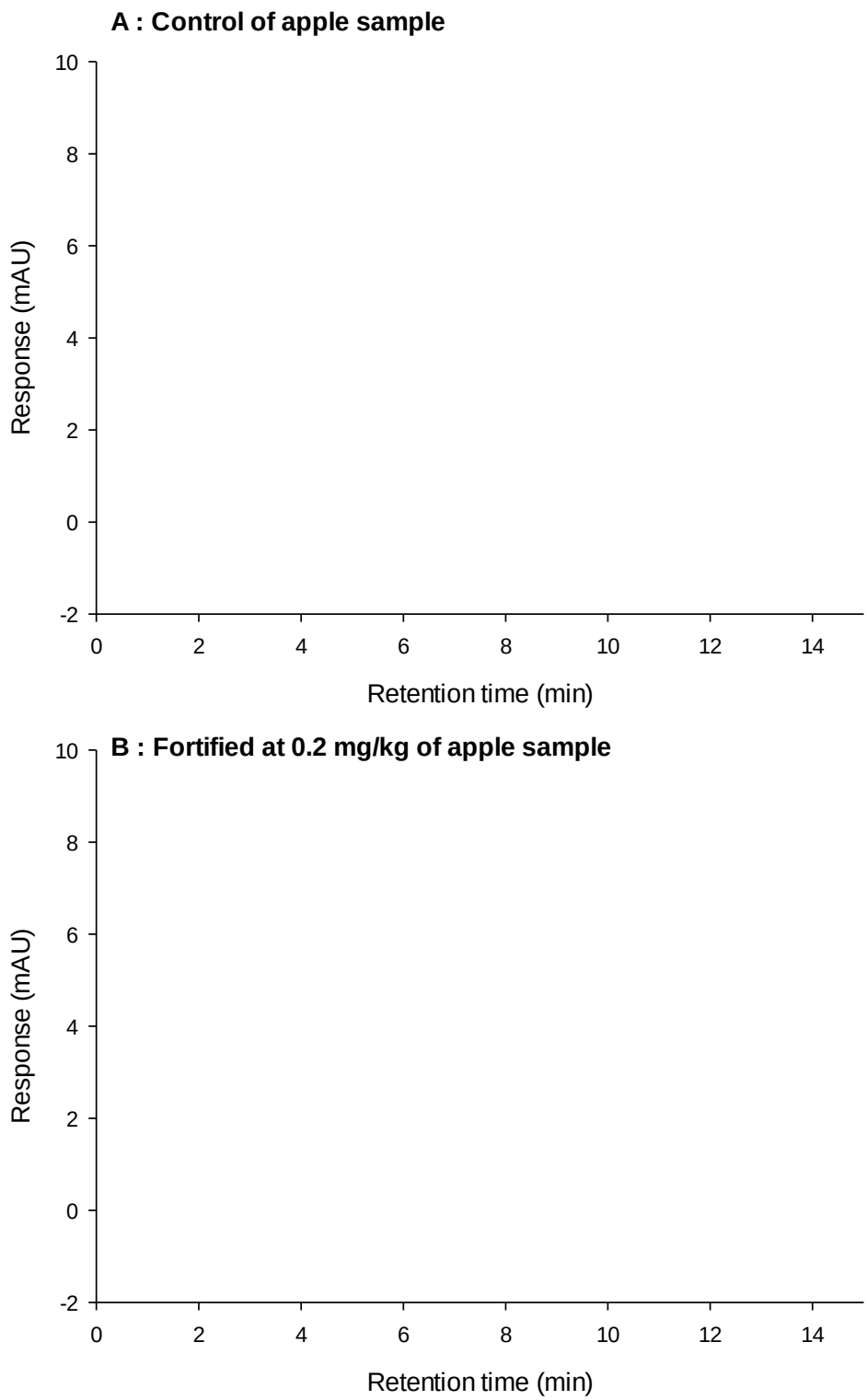


Figure 5. Chromatograms of control (A) and recovery (B) cyazofamid in apple extracts (fortified at 0.2 mg/kg).

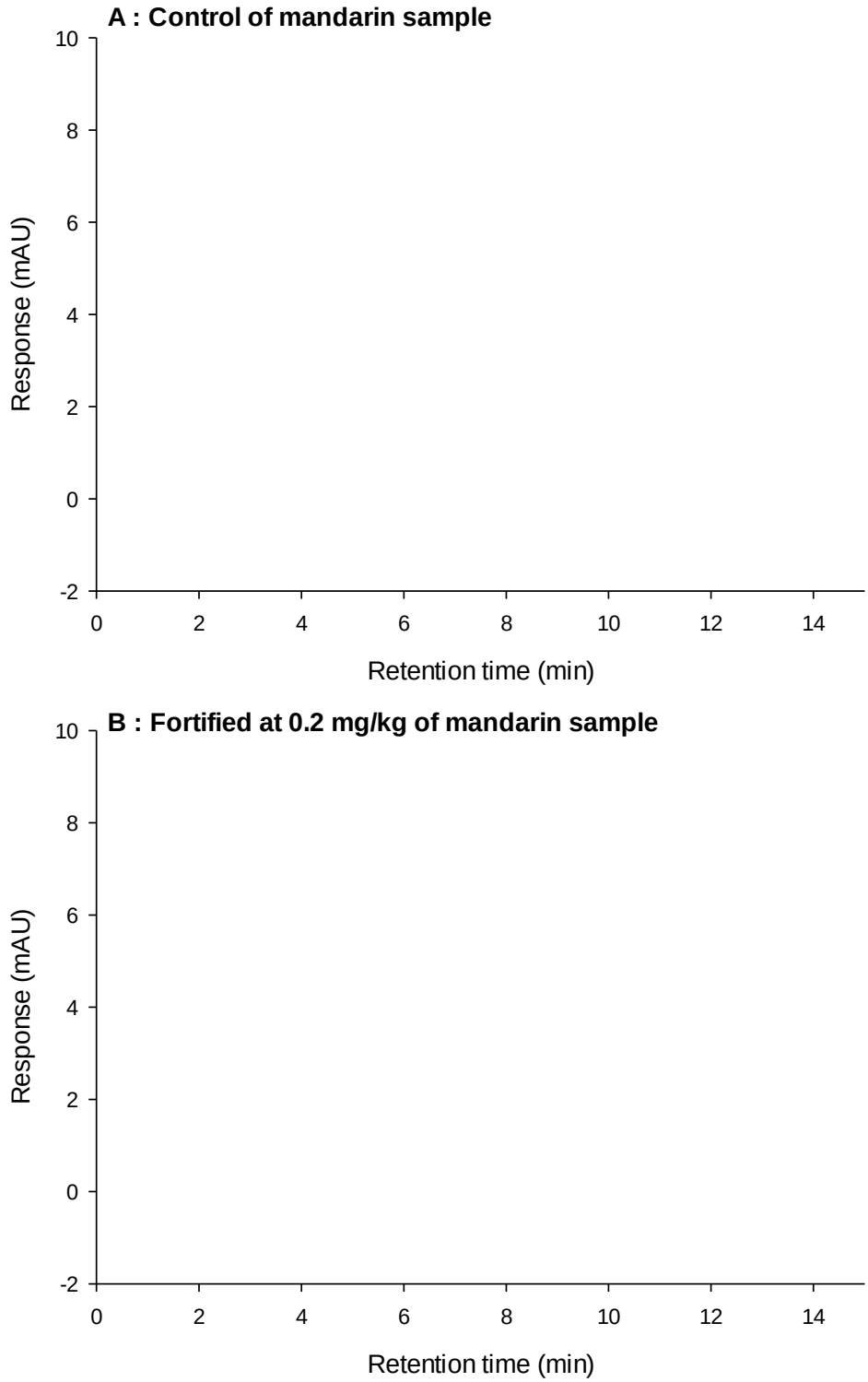


Figure 6. Chromatograms of control (A) and recovery (B) cyazofamid in mandarin extracts (fortified at 0.2 mg/kg).

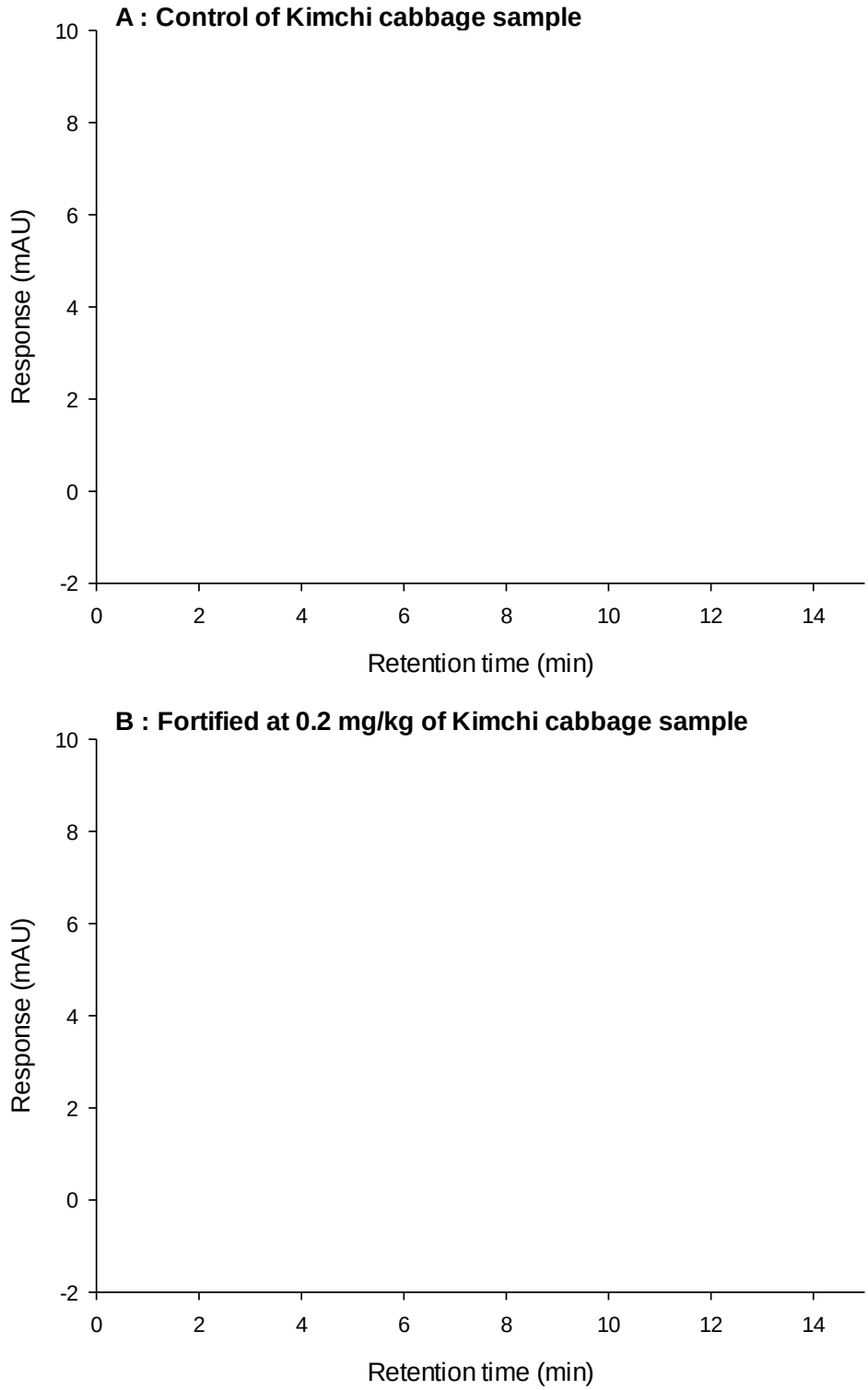


Figure 7. Chromatograms of control (A) and recovery (B) cyazofamid in Kimchi cabbage extracts (fortified at 0.2 mg/kg).

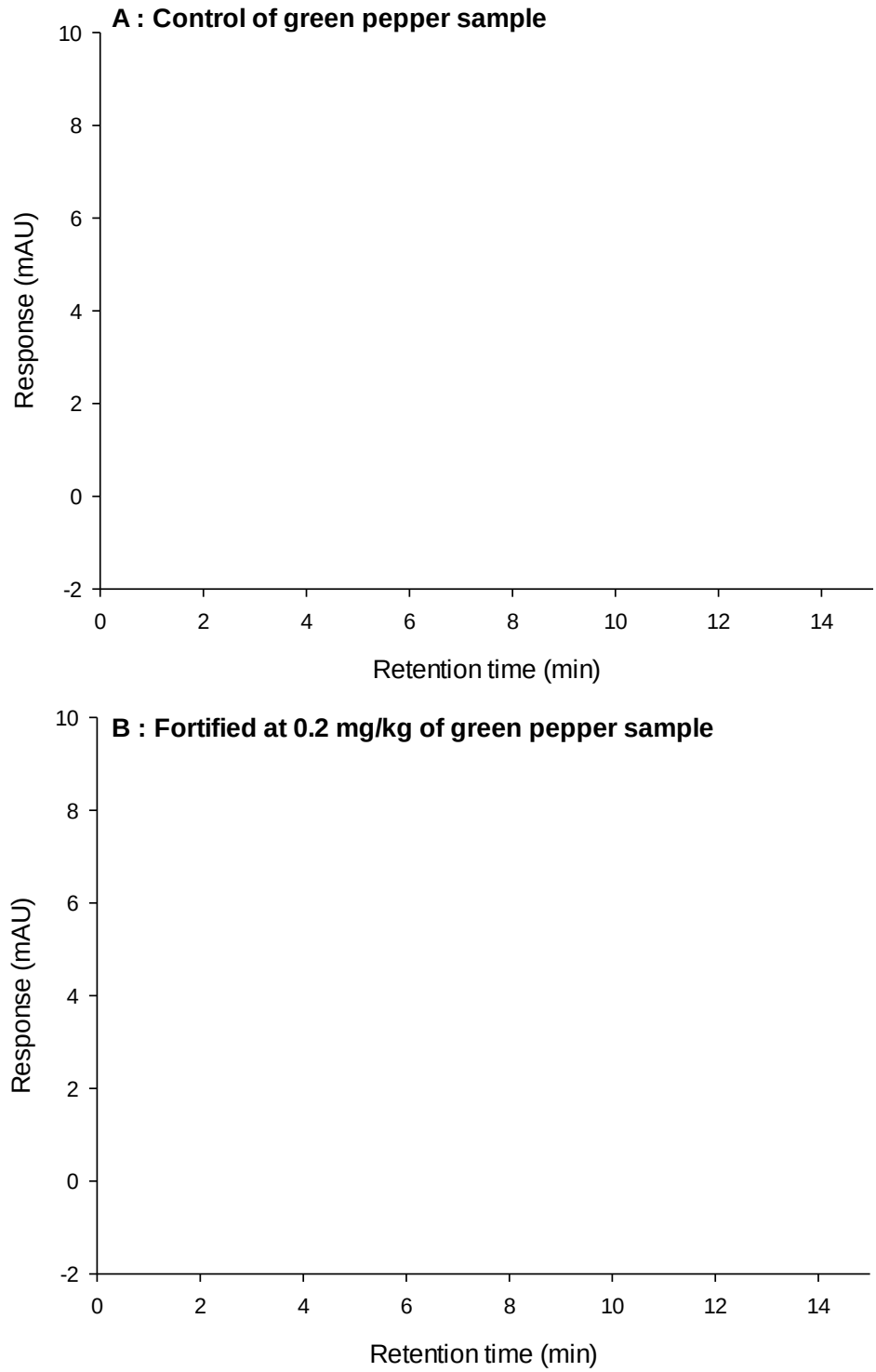


Figure 8. Chromatograms of control (A) and recovery (B) cyazofamid in green pepper extracts (fortified at 0.2 mg/kg).

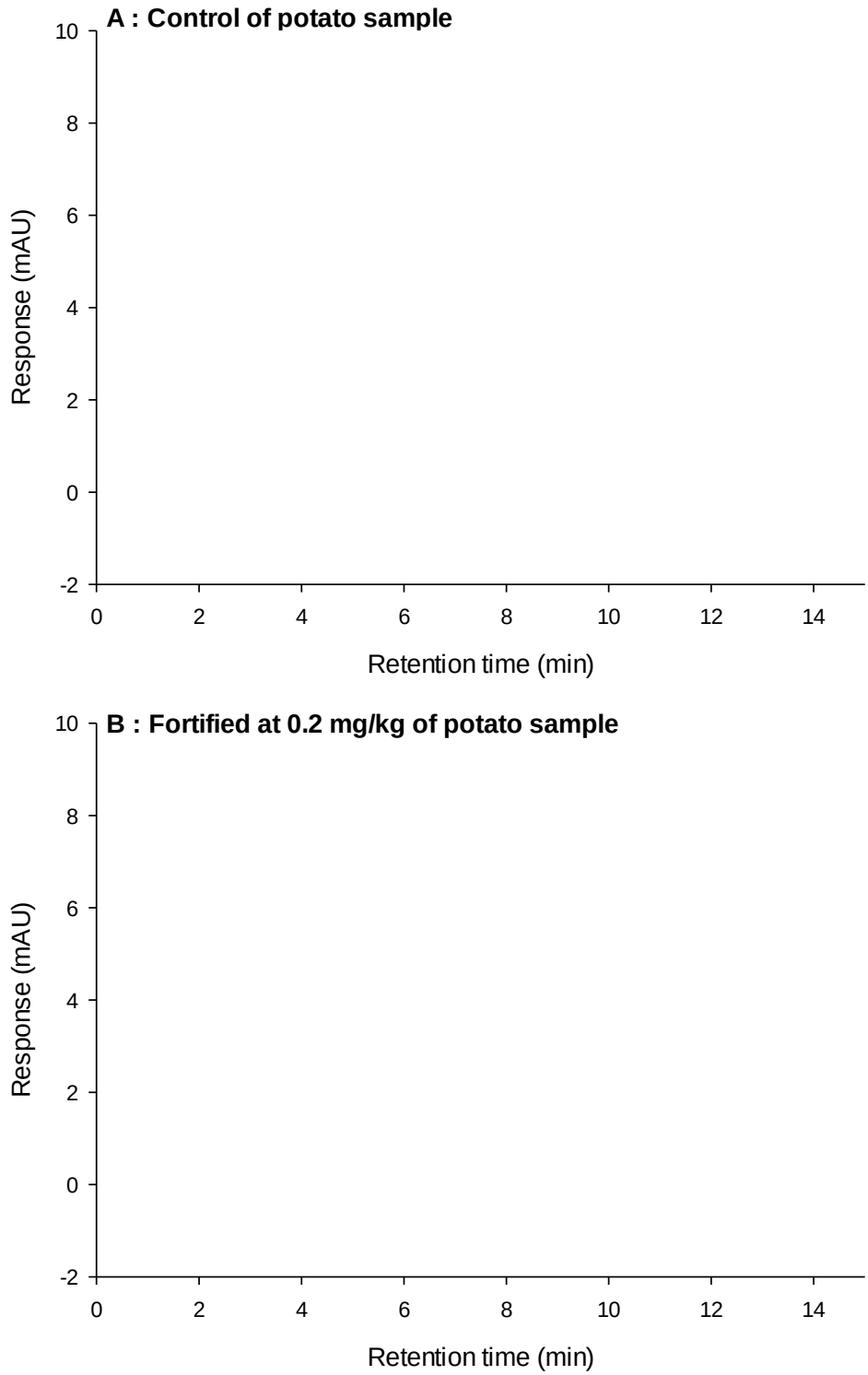


Figure 9. Chromatograms of control (A) and recovery (B) cyazofamid in potato extracts (fortified at 0.2 mg/kg).

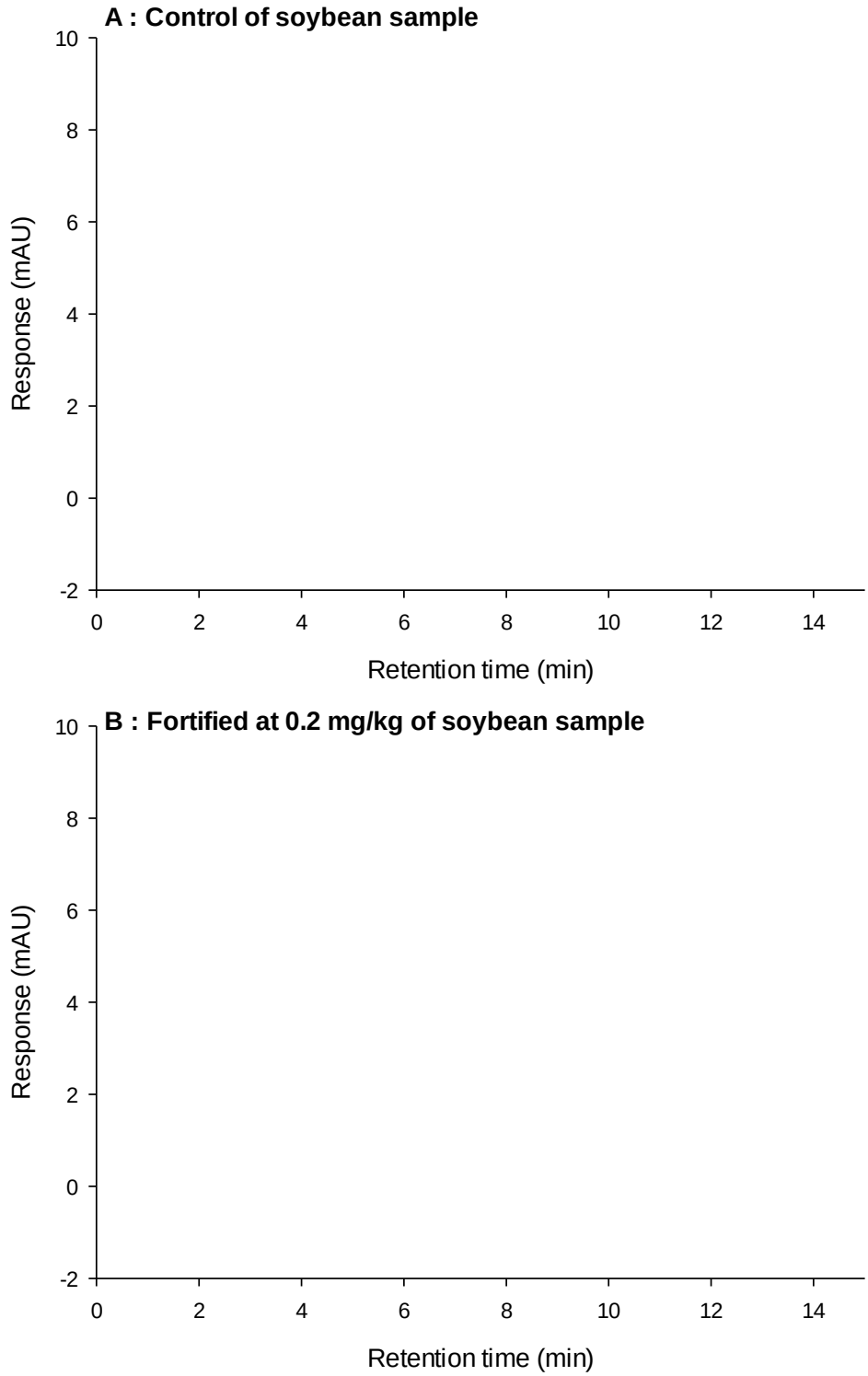


Figure 10. Chromatograms of control (A) and recovery (B) cyazofamid in soybean extracts (fortified at 0.2 mg/kg).

3.7 Retention factor (*k*) and chromatographic efficiency in resolution of cyazofamid on HPLC

Retention factor (*k*) measures the extent to which a solute (cyazofamid) is retained and is commonly called the partition ratio or capacity factor (McNair and Miller 1998; Miller 2005; Rood 2007). It is proportional to the time a compound spends in the stationary phase (t_r') relative to the time it spends in the mobile phase (t_m). *k* value was 4.165 − 4.880 for cyazofamid (Table 10), indicating enough retention for good separation.

The most common measure of the efficiency of a chromatographic system is plate number (*N*) and a related parameter, which expresses the efficiency of a column as the plate height (*H*) (McNair and Miller 1998; Miller 2005; Rood 2007). The greater the number of total theoretical plate a unit length (mm) of column, the shorter each theoretical plate. Therefore, high efficiency columns have large *N* and small *H* values. For cyazofamid, *N* was 10110 − 22413 and *H* was 0.0112 − 0.0247 mm in each crop sample (Table 10), suggesting high column efficiency.

Table 10. Retention times (t_r), retention factor (*k*), number of plates (*N*), and height of theoretical plate (*H*) of cyazofamid (each analytical condition)

Crops	Apple, Kimchi cabbage, potato and soybean	Mandarin	Green pepper
t_r (min)	8.78	10.82	9.96
t_m (min)	1.7	1.84	1.86
t_r'	7.08	8.98	8.10
k	4.165	4.880	4.355
N	22413	10110	10600
H (mm)	0.0112	0.0247	0.0236

II. Analysis of Cyazofamid and Its Metabolite in the Environmental and Crop Samples Using LC–MS/MS

1. Introduction

Cyazofamid is a sulfonamide fungicide (Tomlin and British Crop Protection Council. 2009) which has been used for protection of several vegetables and fruits from various diseases (KCPA 2010).

It has very low mammalian toxicity (LD_{50} for rats : >5,000 mg/kg) and ecological effect [LC_{50} (96 h) for carps : >0.14 mg/L] (Tomlin and British Crop Protection Council. 2009). In aerobic soil, cyazofamid degraded rapidly (DT_{50} in soil : 3 - 5 days) into the major degradates such as CCIM, 4-chloro-5-*p*-tolylimidazole-2-carboxamide (CCIM–AM), and 4-chloro-5-*p*-tolylimidazole-2-carboxylic acid (CTCA) which were covalently bound to organic matter (Terada et al. 2004). When cyazofamid was treated on field for crop residue trial, CCIM was found as a major metabolite (Terada et al. 2004).

Only few reports were available for the analysis of cyazofamid residues in the limited environmental or crop/food samples including grapes, ginseng, soil and water (Choi et al. 2007; Gonzalez-Alvarez et al. 2012a; Gonzalez-Rodriguez et al. 2009; Gonzalez-Rodriguez et al. 2011; Lee et al. 2012; Tandon and Singh 2012). Such methods utilized HPLC–UVD (Choi et al. 2007; Lee et al. 2012; Tandon and Singh 2012) or GC-ITMS (Gonzalez-Alvarez et al. 2012a; Gonzalez-Rodriguez et al. 2009; Gonzalez-Rodriguez et al. 2011) after conventional sample treatment, and only cyazofamid was analyzed without metabolites.

The objective of this study is to establish a rapid and effective analytical method of cyazofamid and its metabolite, CCIM, using LC–MS/MS after QuEChERS sample treatment. Upland soil and deionized water were used for environmental samples, while apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean were selected for the representative crops.

2. Materials and methods

2.1 Chemicals and reagents

Cyazofamid (98.4 %) was purchased from Fluka™ (Buchs, Switzerland) and its metabolite, CCIM was kindly donated from the manufacturer. All of solvents (HPLC grade) were obtained from Burdick and Jackson® (Korea) and other reagents were purchased from Sigma Aldrich (USA). Water was distilled and purified with a LaboStar™ TWF UV7 Ultra-pure water system (Siemens Water Technologies LLC, USA). Upland soil sample was collected from a local province. Apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean of 'Residue free grade' were purchased from a local market. The QuEChERS materials were obtained from commercial suppliers. For extraction of crops, 'Ultra QuECh extract kit' (Ultra Scientific, USA), which is containing 4 g of $MgSO_4$, 1 g of NaCl, 1 g of $Na_3Citrate \cdot 2H_2O$ and 0.5 g of $Na_2HCitrate \cdot 1.5H_2O$ was used. For the dispersive SPE (d-SPE) cleanup of crop extracts from apple, mandarin Kimchi cabbage, green pepper, and potato samples, 'Ultra QuECh dSPE-General' (2 mL centrifuge tubes containing 150 mg of $MgSO_4$ and 25 mg of PSA, Ultra Scientific, USA) was used. For soybean samples, 'Agilent Dispersive SPE 2 mL Fatty Samples' (2 mL centrifuge tubes containing 150

mg of $MgSO_4$, 25 mg of PSA and 25 mg of C18, Agilent Technologies, USA) was chosen.

2.2 Analytical instruments and conditions

LC–MS/MS analysis was performed on LCMS-8030 (Shimadzu, Japan) coupled to Nexera UHPLC (Shimadzu, Japan) with electrospray (ESI, positive mode). The analytical column was a Kinetex C18 (100 × 2.1 mm i.d., 2.6 μm, Phenomenex®, USA) and the column oven temperature was 40℃. The injection volume was 4 μL and the mobile phases were eluted at a 0.2 mL/min. Mobile phases were 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). For gradient elution, the initial combination was 40 : 60 (A : B, v/v) and the B solution was increased to 100 % in duration of 2 min, holding for 1 min. To establish the multiple reaction monitoring (MRM) condition on LCMS-8030, precursor ions, product ions, Q1 and Q3 pre bias voltage, and collision voltage were optimized through the flow injection of cyazofamid and CCIM standard solutions (1 μg/mL).

2.3 Stock and working solutions

Standard stock solutions of cyazofamid and CCIM were prepared at the concentrations of 1,000 and 100 mg/L with acetonitrile, respectively. Those two stock solutions were combined to make standard mixture solution, and then it was diluted with acetonitrile to prepare the standard working solutions of various concentrations.

2.4 Matrix effect

The calibration curves were constructed in solvent (acetonitrile), or in matrix extract for matrix matched calibration at 5, 10, 20, 50, 100, and 200 ng/mL levels. Matrix effect (ME, %) was calculated by the equation of ME (%) = $[(S_m/S_s)-1] \times 100$ (Lozano et al. 2012). S_s is the slope of solvent calibration curve, while S_m is the slope of matrix matched calibration curve.

2.5 Recovery test of cyazofamid and its metabolite in crop and environmental samples

A 10 g of homogenized crop sample was placed in a 50 mL conical tube. And the sample was fortified with the standard mixture solution at 10 and 100 μg/kg levels before the crop was extracted with acetonitrile (10 mL) by shaking with the reciprocal shaker (SA-2s, Taitec Corporation, Japan) at 250 rpm for 10 min. Then, 'Ultra QuECh extract kits' were added in the conical tubes, shaken for 2 min, and centrifuged for 5 min at 3,500 rpm (Combi 408, Hanil Science Industrial Co., Ltd., Korea). In the case of soybean sample, it was soaked in water (10 mL) for 20 min before extraction. An aliquot (1 mL) of extract was transferred in 2 mL d-SPE tube, before centrifuged for 5 min at 15,000 rpm (Micro 17TR, Hanil Science Industrial Co., Ltd., Korea). The aliquots (400 μL) of crop extracts from d-SPE cleanup were also mixed with 50 μL acetonitrile with 1% formic acid and 50 μL of acetonitrile for matrix matching before analyzed with LC–MS/MS. For crop matrix matched calibration, an aliquot (400 μL) of control crop extract was mixed with 50 μL acetonitrile which contains 1% formic acid and 50 μL of standard working solution. A dried and sieved (2 mm) soil (10 g, sandy loam, pH 6.5) was placed into a 50 mL conical tube, and

was fortified with the standard mixture solution at 10 and 100 μg/kg levels. And then it was extracted with 10 mL acetonitrile containing 1% acetic acid by shaking at 250 rpm for 5 min with the reciprocal shaker. Four grams of $MgSO_4$ and 1 g of NaCl were added into extraction mixture, and the mixture was shaken with the reciprocal shaker for 5 min, before centrifuged at 3,500 rpm for 5 min. The aliquots (800 μL) were mixed with 200 μL of acetonitrile, and analyzed with LC–MS/MS. For soil matrix matched calibration, an aliquot (800 μL) of control soil extract was mixed with 200 μL of standard working solution. The water sample (500 mL) was placed into a 1,000 mL separatory funnel and fortified with the standard mixture solution at 0.1 and 1 μg/L levels. Then the sample was added with 20 g of NaCl before extracting with 100 and 50 mL of dichloromethane successively, by hand shaking. Two dichloromethane extracts were combined, dried over anhydrous Na_2SO_4, and concentrated with rotary evaporator (Rotavapor R-114, Büchi, Switzerland) under reduced pressure. The residue was dissolved with 5 mL of acetonitrile and an aliquat (400 μL) was mixed with 50 μL of acetonitrile which contains 1% formic acid and 50 μL of acetonitrile before analyzed with LC–MS/MS. For water matrix matched calibration, an aliquot (400 μL) of control water sample extract was by mixed with 50 μL of acetonitrile which contains 1% formic acid and 50 μL of standard working solution.

2.6 Real sample test

For the true sample applications, grapes were purchased from local market and 10 g of homogenized grape sample were processed and analyzed according to the crop method.

For the field sample applications, orchard soil was collected and the dried and sieved (2 mm) soil (10 g, sandy loam, pH 6.5) was processed and analyzed according to the soil method.

3. Results and discussion

3.1 Instrumental conditions by LC-MS/MS

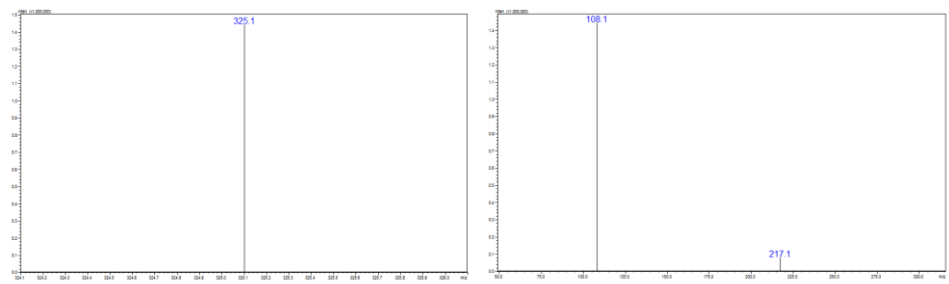
Generally, LC–MS/MS offers very sensitive, selective and rapid analysis compared to the conventional HPLC and GC. When MRM mode was used in this study few ppb levels for soil and crop samples, and sub-ppb levels for water samples were observed as MLOQs.

On LC–MS/MS, the protonated molecular ion $[M + H]^+$ at *m/z* 325.0 for cyazofamid, and *m/z* 218.0 for CCIM were obtained in full scan spectra at 4,500 v of needle voltage at the positive ESI mode. And product ions of cyazofamid and CCIM were selected in product scan during the MRM optimization of Q1 pre bias, Q3 pre bias and collision voltages (Table 11 and Figure 11). The other conditions such as DL temperature, nebulizing gas flow, heat block temperature were set at the recommended values of the instrument. Good and clear separation was observed on MRM for cyazofamid and CCIM in a variety of samples, showing better sensitivity for cyazofamid than CCIM (Figure 11). Method limit of quantitations (MLOQs) were 2 ng/g (cyazofamid) and 5 ng/g (CCIM) for soil/crop samples, while 0.02 ng/mL (cyazofamid) and 0.05 ng/mL (CCIM) for water samples.

Table 11. MRM conditions for cyazofamid and CCIM on LC–MS/MS

Name	Transition (m/z)	Dwell time (sec)	Q1 pre bias (v)	CE (v)	Q3 pre bias (v)
Cyazofamid	325.0 > 108.1	100	-12	-14	-18
CCIM	218.0 > 139.1	100	-10	-23	-24

A : Full spectrum and product scan spectrum of cyazofamid



B : Full spectrum and product scan spectrum of CCIM

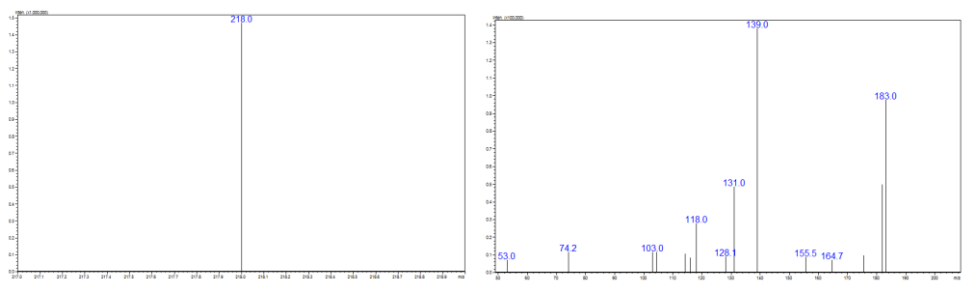


Figure 11. LC-MS spectra of cyazofamid (A) and CCIM (B).

3.2 Matrix effect

Linearity of matrix matched standard solutions (5 - 200 ng/mL) of cyazofamid and CCIM was good, showing higher than 0.999 of coefficients of linear determination (Table 12, Figure 12, and 13). In water samples ME (%) was generally enhanced, while those of upland soil, mandarin, green pepper, and soybean samples were suppressed for the both of cyazofamid and CCIM. On the other hand in apple, Kimchi cabbage, and potato samples, enhancement for cyazofamid, and suppression for CCIM were observed.

Table 12. Regression equation and matrix effect (ME, %) for cyazofamid and CCIM

Crops	Cyazofamid		CCIM	
	Regression equation	ME (%)	Regression equation	ME (%)
Solvent	y = 2,598.3x + 810.6	-	y = 560.8x + 143.4	-
Apple	y = 2,662.9x + 90.2	2.5	y = 548.0x - 201.5	-2.3
Mandarin	y = 2,294.1x + 2,554.1	-11.7	y = 412.8x + 211.1	-26.4
Kimchi cabbage	y = 2,714.2x - 577.1	4.5	y = 512.6x - 146.1	-8.6
Green pepper	y = 2,485.7x + 2,219.8	-4.3	y = 446.2x + 293.6	-20.4
Potato	y = 2,849.1x + 873.9	9.7	y = 548.4x - 225.5	-2.2
Soybean	y = 2,337.4x + 1,669.0	-10.0	y = 503.4x + 179.4	-10.2
Soil	y = 2,446.7x + 406.0	-5.8	y = 531.4x - 177.9	-5.2
Water	y = 2,976.1x - 2,093.3	14.5	y = 565.4x - 533.3	0.8

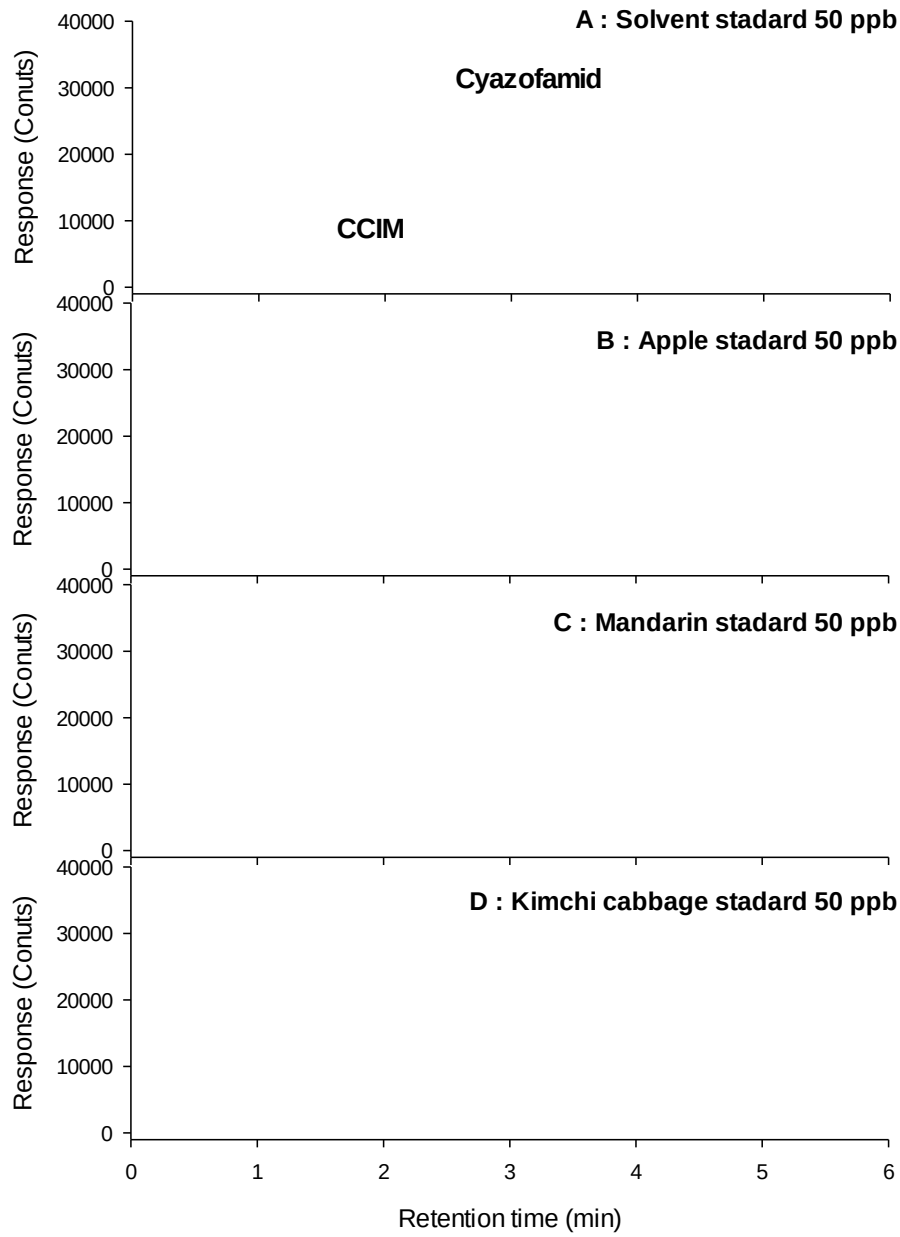


Figure 12. MRM chromatograms of matrix matched calibration (50 ppb) for cyazofamid and CCIM in solvent (acetonitrile, A), apple (B), mandarin (C), and Kimchi cabbage (D) extract.

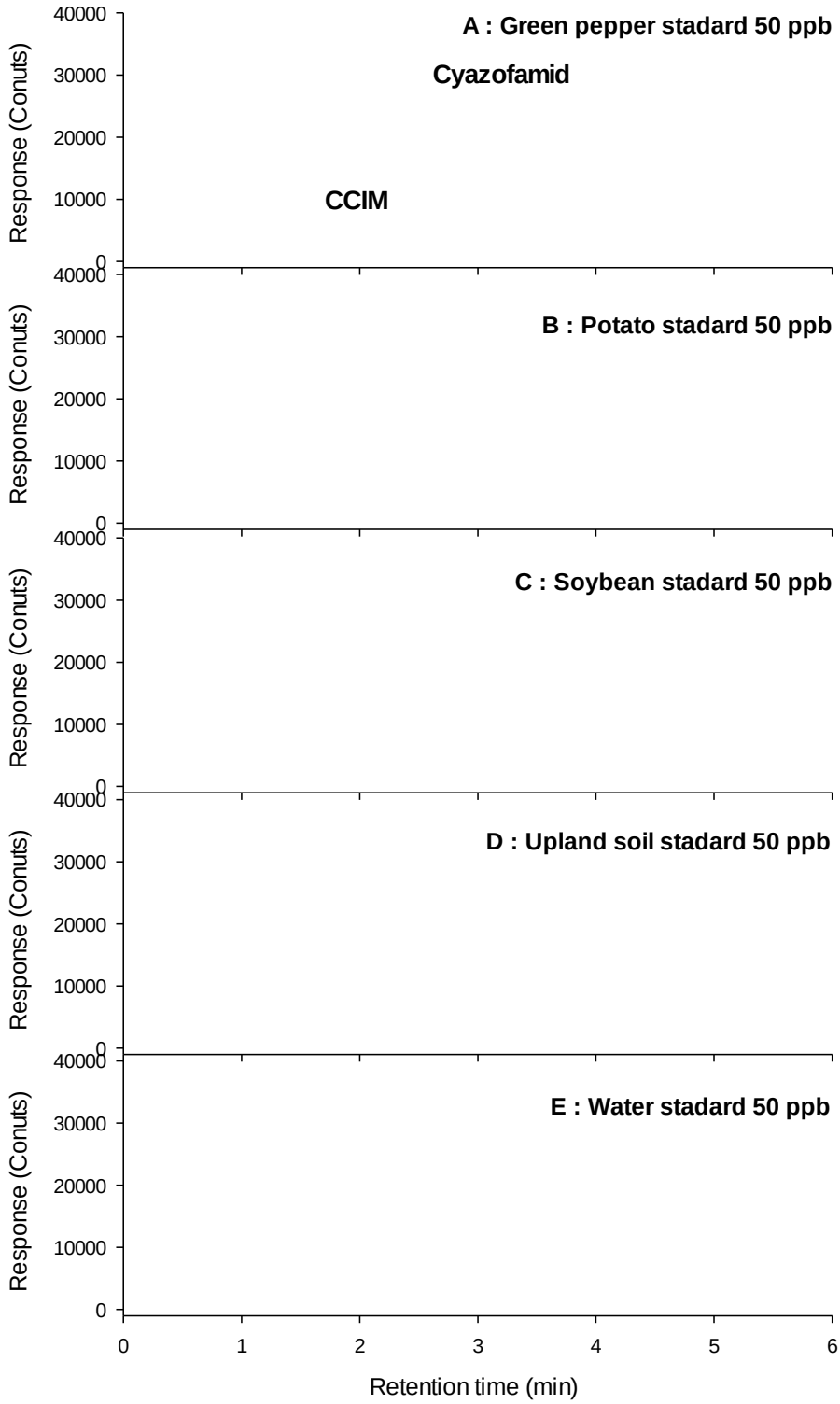


Figure 13. (Continued) A : Green pepper, B : potato, C : soybean, D : upland soil, and E : water extract.

3.3 Recoveries of cyazofamid from crop and environmental samples

Most of the previous analytical studies dealt with only cyazofamid (Choi et al. 2007; Gonzalez-Alvarez et al. 2012a; Gonzalez-Rodriguez et al. 2009; Gonzalez-Rodriguez et al. 2011; Lee et al. 2012; Tandon and Singh 2012) however, in this study, metabolite CCIM was also analyzed together with cyazofamid because it has been known as the major and common metabolite in soil and crop (EPA 2004; Terada et al. 2004) and in the processed food (e.g. wine) (European Food Safety Authority 2013).

The recoveries of crop samples were from 80.2% to 105.1% for cyazofamid (C.V; 0.5% - 16.4%) and from 75.1% to 99.1% for CCIM (C.V; 0.8% - 15.1%). For soil recovery tests, good recoveries from 91.1% to 96.5% (C.V; 1.0% - 9.5%) were obtained, while the recoveries were from 91.3% to 99.0% (C.V; 1.7% - 4.7%) for water samples (Table 13 and Figure 14 - 21).

For the rapid and efficient sample treatment, QuEChERS method was used because the conventional extraction, liquid–liquid partitioning, column chromatography purification and concentration procedures are tedious and time/labor consuming. The popular QuEChERS method was originally introduced in 2003 by Dr. Steven J. Lehotay's team of USDA ARS (Anastassiades et al. 2003) for the multiresidue analysis of pesticides in food and cops, and was modified into the acetate-buffering version (Lehotay et al. 2005) and the citrate-buffering versions (Anastassiades et al. 2007). This method applied successfully in not only crops but also environmental samples (Kvicalova et al. 2012; Liu et al. 2014) for pesticide residue analysis.

In this study for crop samples, citrate-buffering QuEChERS version was

used successfully, giving a reasonable recoveries (75.1% – 105.1%) and precisions for two target analytes. The other studies (Choi et al. 2007; Lee et al. 2012) with the conventional sample treatment and HPLC analysis of crops reported that the recoveries were 75.3% - 98.5 for several crops and 80.2% - 80.6% for ginseng. As expected, the analytical time, cost, reagents and labor were saved greatly compared to conventional procedures, still keeping reasonable recovery rates.

In soil recovery tests, the modified QuEChERS method (Caldas et al. 2011) which utilizes $MgSO_4$ and NaCl in extraction process was used without d-SPE procedure since the extract was less complicated than crop extracts. By omitting d-SPE step, the analytical time and cost were reduced again, maintaining of good recoveries (91.1% - 96.5%). In case of field soil analysis, the established soil method was applied nicely and no cyazofamid and CCIM was observed. Tandon and Singh (2012) also obtained the reasonable recoveries (86.4%) of cyazofamid from soil samples after the conventional extraction and SPE cartridge purification.

However for water samples, the traditional liquid–liquid partitioning of large volumes of sample (500 mL) and concentration step were carried out in order to reach the sub-ppb level of MLOQ because the analytes concentration in field water samples could be significantly low by dilution. Recoveries from 91.3% to 99.0% were obtained with MLOQs of 0.02 - 0.05 ng/mL in this study, while 95.3% of recovery of cyazofamid was reported by Tandon and Singh (2012), which used the partitioning with chloroform of water samples. However, MLOQ of this study was significantly lower than that of their work (Tandon and Singh 2012).

Table 13. Recoveries of the cyazofamid and CCIM residues in crop, soil, and water samples

Crops	Cyazofamid		CCIM	
	Recovery (%)/C.V. (%)		Recovery (%)/C.V. (%)	
	10 μg/kg	100 μg/kg	10 μg/kg	100 μg/kg
Apple	105.1/2.6	103.6/1.1	86.0/5.8	88.1/3.8
Mandarin	88.1/3.6	101.8/3.4	90.3/4.1	98.2/2.4
Kimchi cabbage	101.0/1.4	95.7/16.4	92.3/0.8	91.0/15.1
Green pepper	80.2/1.2	90.5/1.8	75.1/4.1	85.5/2.1
Potato	96.4/1.9	99.0/0.5	82.4/10.7	91.1/2.6
Soybean	98.1/15.7	96.9/2.6	99.1/8.9	89.9/2.5
Soil	95.3/2.1	96.5/1.0	91.1/9.5	91.6/3.7
Water*	99.0/2.3	91.3/2.2	92.1/4.7	94.9/1.7

* : The water samples were fortified at 0.1 and 1.0 μg/L instead fortified at 10 and 100 μg/kg, respectively

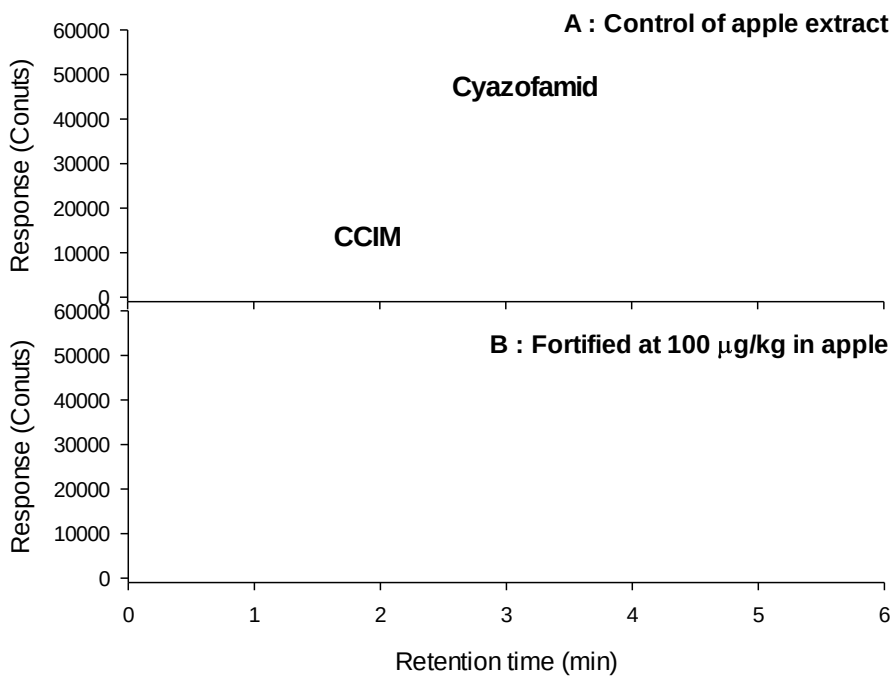


Figure 14. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in apple extract (Fortified at 100 μg/kg).

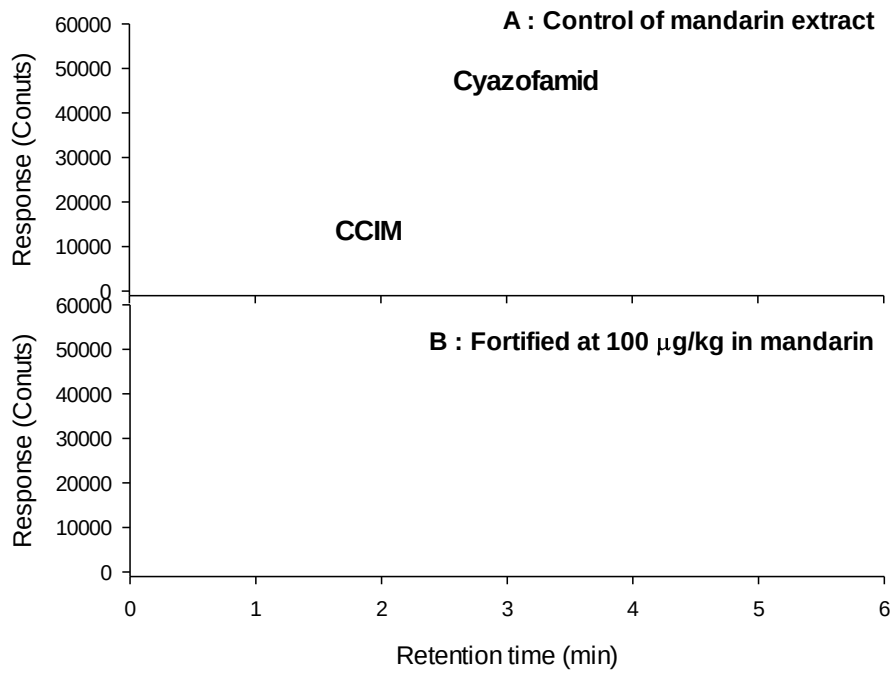


Figure 15. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in mandarin extract (Fortified at 100 μg/kg).

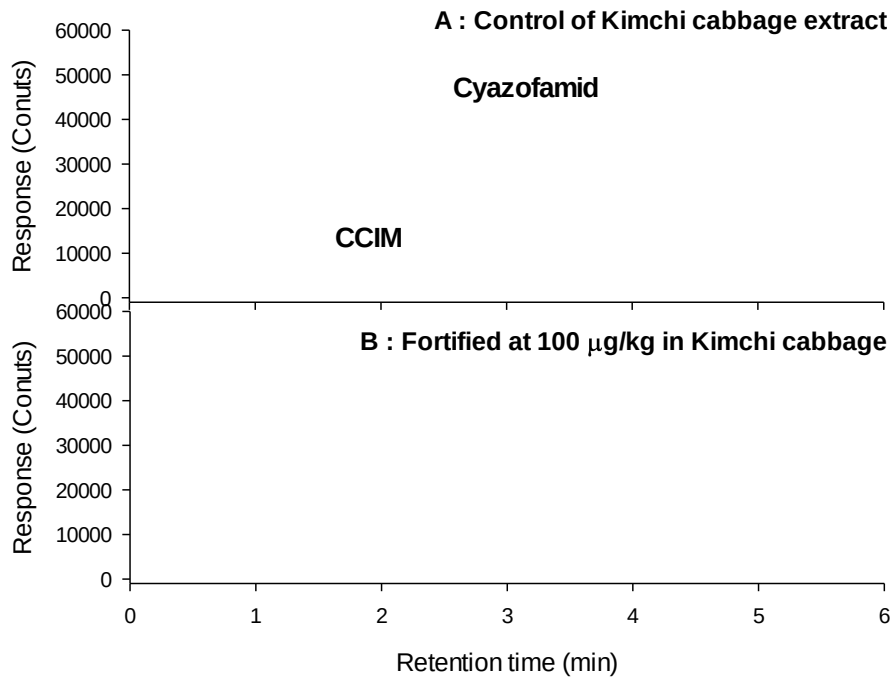


Figure 16. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in Kimchi cabbage extract (Fortified at 100 μg/kg).

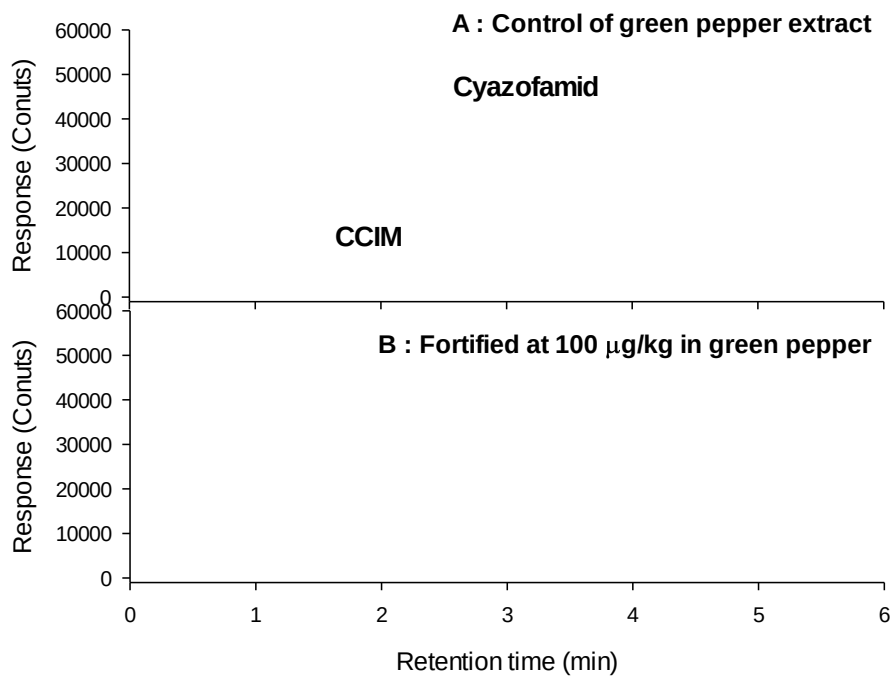


Figure 17. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in green pepper extract (Fortified at 100 μg/kg).

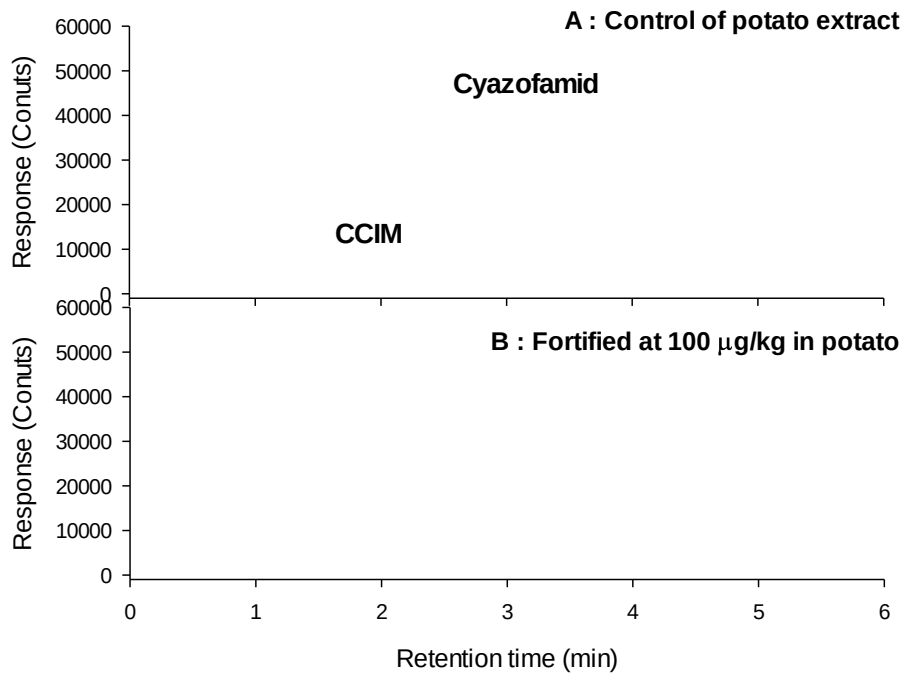


Figure 18. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in potato extract (Fortified at 100 μg/kg).

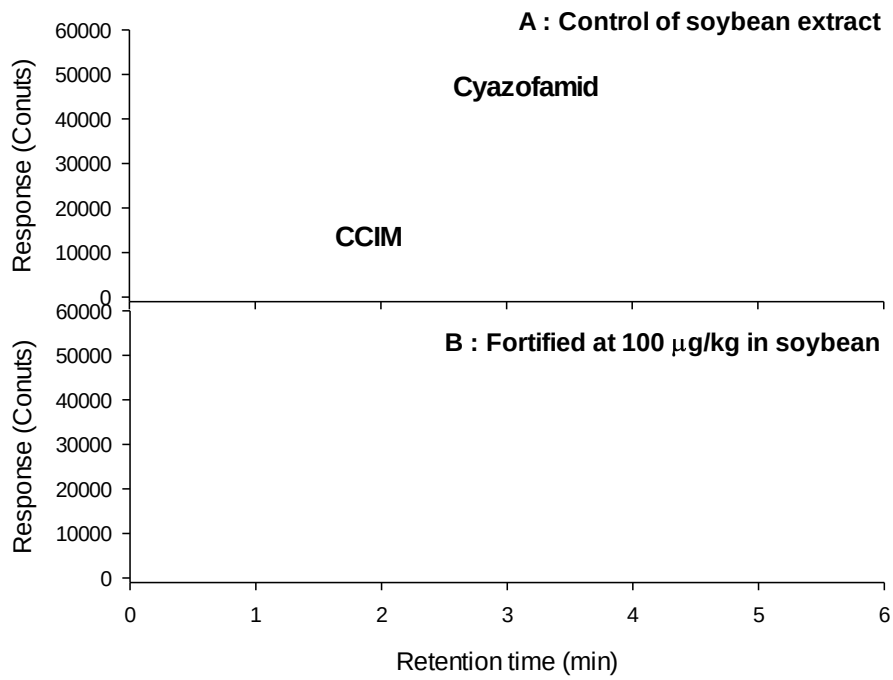


Figure 19. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in soybean extract (Fortified at 100 μg/kg).

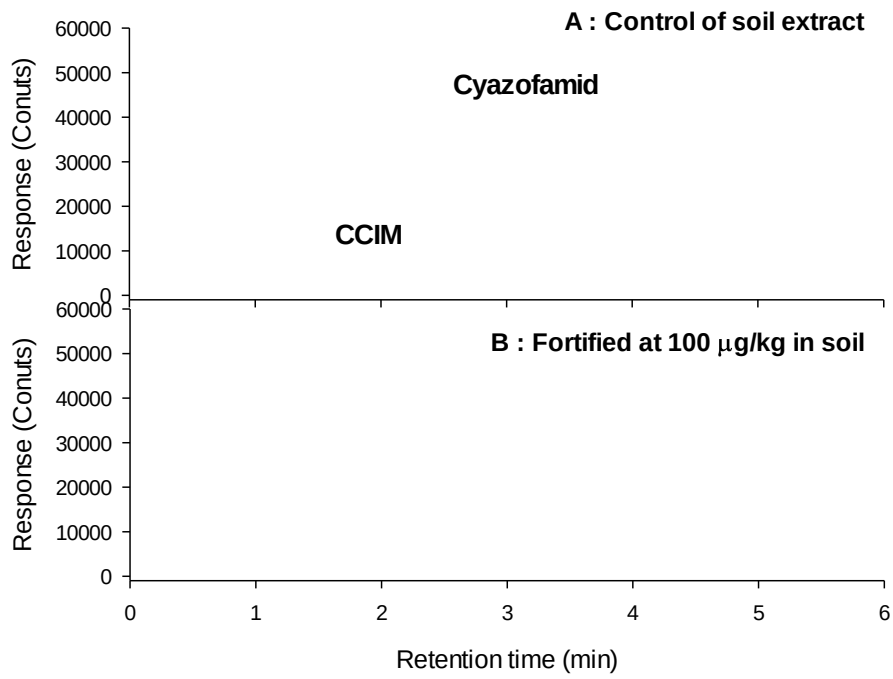


Figure 20. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in upland soil extract (Fortified at 100 μg/kg).

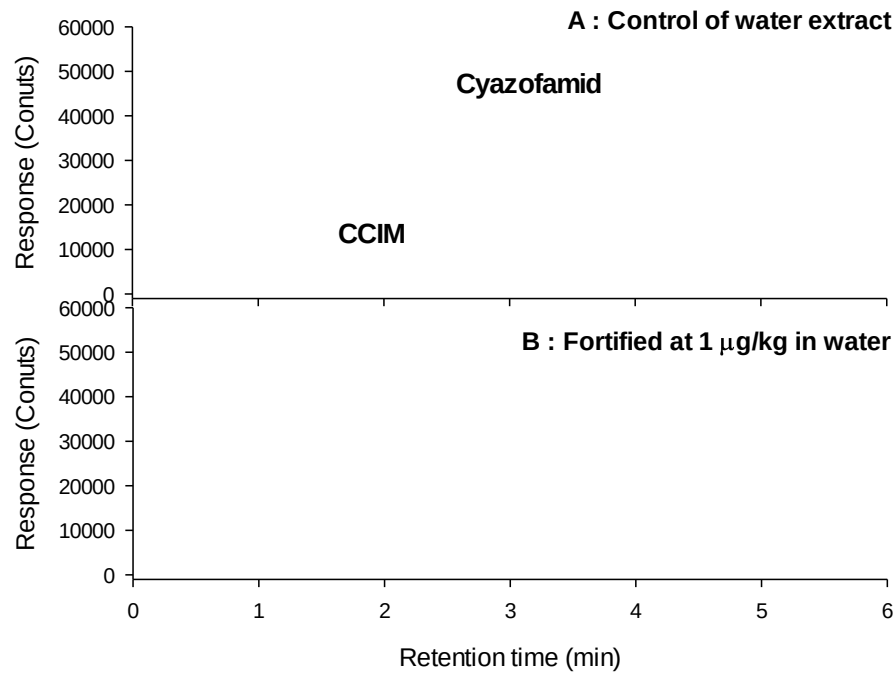


Figure 21. MRM chromatograms of control (A) and recovery (B) for cyazofamid and CCIM in water extract (Fortified at 1 μg/kg).

3.4 Application of established method for real samples

In order to apply the established method, the cyazofamid residue from grape and soil samples were analyzed. When grape sample from market was analyzed, crop method was applied successfully and no cyazofamid and CCIM was observed.

As a conclusion, a rapid and effective LC–MS/MS method for the analysis of cyazofamid and its metabolite CCIM in environmental samples (soil and water) and crops (apple, mandarin, Kimchi cabbage, green pepper, potato and soybean) was established in this study. By employing QuEChERS process for the crop and soil sample treatment, and MRM on positive mode ESI for MS/MS analysis, the method was very sensitive, quick and selective. Matrix matched calibration was used for the proper quantitation and the recoveries of analytes from matrix samples satisfied the criteria of CODEX guideline (Guidelines on food laboratory practice in residue analysis 2010). Applicability and robustness of established method was proved through true samples (grapes and soil) analysis.

CHAPTER II

Metabolism of Cyazofamid

by Soil Fungus *Cunninghamella elegans*

I. Introduction

Many pesticides undergo metabolism in mammals, microorganisms, soil and plants, and metabolism study is very important for the understanding of pesticide toxicity and safety. *In vivo* ADME studies of cyazofamid, the major route of excretion for the low dose group (0.5 mg/kg) was urine, and the major route for the high dose group (1,000 mg/kg) was feces with 4.4 - 11.6 hours of half-life in whole blood concentration. The major metabolites in urine were 4-(4-chloro-2-cyanoimidazole-5-yl)benzoic acid (CCBA), 4-chloro-5-[β-(methylsulfinyl)-*p*-tolyl]imidazole-2-carbonitrile (CH_3SO-CCIM), 4-chloro-5-[β-(methylsulfonyl)-*p*-tolyl]imidazole-2-carbonitrile (CH_3SO_2-CCIM), and those in bile were CCBA (Terada et al. 2004). It was reported that in aerobic soil, cyazofamid degraded rapidly (DT_{50} in soil : 3 - 5 days) into the major degradates, such as 4-chloro-5-*p*-tolylimidazole-2-carbonitrile (CCIM), 4-chloro-5-*p*-tolylimidazole-2-carboxamide (CCIM–AM), and 4-chloro-5-*p*-tolylimidazole-2-carboxylic acid (CTCA), which were covalently bound to organic matter (Terada et al. 2004).

The soil fungus *Cunninghamella* species have the ability to metabolize a wide variety of xenobiotics through phase I and phase II metabolic reactions, similar to those in mammalian metabolism systems (Asha and Vidyavathi 2009; Keum et al. 2009; Zhang et al. 1996a; Zhang et al. 1996b). Among these species, *C. bertholletiae*, *C. elegans* and *C. echinulata* are the common species (Asha and Vidyavathi 2009), and *C. elegans* is the most useful fungus for mimicking mammalian metabolism of xenobiotics, including pesticides

(Hangler et al. 2007; Keum et al. 2009; Park et al. 2003; Pothuluri et al. 1997; Pothuluri et al. 1993; Pothuluri et al. 2000; Zhu et al. 2010b)

The present study was conducted to elucidate the metabolites and metabolic pattern for metabolism of cyazofamid with *C.elegans*.

II. Materials and Methods

1. Chemicals and reagents

Cyazofamid (98.4%) was purchased from Fluka™ (St. Louis, MO, USA) and CCIM was kindly donated from manufacture. All solvents (HPLC grade) were obtained from Burdick and Jackson® (Ulsan, Korea).

Sodium chloride was purchased from Samchun pure chemical co., Ltd. (Pyeongtaek city, South Korea). Dichloromethane-d_2 ($CDCl_3$), methanol-d_4 (MeOD), and benzene-d_6 was purchased from Merck (Germany). *Cunninghamella elegans* ATCC36112 was provided by National Center for Toxicological Research in U.S. FDA (AR, USA). Potato dextrose agar (PDA) and broth (PDB) were purchased from BD Korea Ltd. (Seoul, South Korea). Fungal cultures were typically maintained on PDA, while the corresponding liquid culture was performed on PDB at 27℃, 200 rpm. To stabilize the metabolic reaction system, the PDB seed culture was incubated for 2 days.

2. Analytical instruments and conditions

2.1. HPLC

The metabolism reaction samples of cyazofamid from the incubation of and *C. elegans* were measured using an Agilent HPLC 1100 series (Santa Clara, CA, USA) equipped with a Kinetex C18 column (2.1 mm i.d. × 100 mm, 2.6 μm; Phenomenex®, Torrance, CA, USA) at 40℃. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient conditions used were as follows : 30% B at 0

min to 2 min, 95% B at 13 min to 18 min, and 30% B at 20 to 25 min. The injection volume was 2 μL, and UV detector wavelength was 280 nm.

2.2 LC-MS/MS

Varian 500-MS IT-MASS spectrometer (Walnut Creek, CA, USA) equipped with Agilent 1100 HPLC was used with Luna C18 column (2.0 mm i.d. × 150 mm, 3.0 μm; Phenomenex®) at 40℃. The mobile phases and the gradient conditions were identical with the HPLC condition. The sample was analyzed by ESI positive mode (needle voltage : 4000 V) from m/z 190 to m/z 350. Drying gas temperature, drying gas pressure, and nebulizer gas pressure were 350℃, 40, and 30 psi, respectively. Turbo Data Dependent Scanning (TurboDDS^{TM}, Varian) was used to obtain MS^3 spectra of ion *m/z* 341. The proposed structures for the fragment ions of the ion m/z 341 were determined by using Mass Frontier^{TM} software (version 6.0, HighChem Ltd., Bratislava, Slovakia).

2.3 Preparative HPLC

On HPLC, one metabolite peak (retention at 10.19 min) was fractionated with fraction collector FC 205 (Gilson, Middleton, WI, USA) after multiple injection of 25 μL. The separation column was CAPCELL PAK C18 UG120 column (4.6 mm i.d. × 250 mm, 5 μm; Shiseido, Tokyo, Japan). The mobile phase, gradient condition and detector wavelength were identical with the analytical HPLC condition.

2.4 ^{1}H, ^{13}C, DEPT, and 2D ^{1}H-^{13}C HSQC-NMR

^{1}H, ^{13}C, DEPT (distortionless enhancement by polarization transfer), and 2D ^{1}H-^{13}C HSQC (heteronuclear single-quantum correlation) NMR spectra were recorded on a 400 MHz NMR spectrometer (Jeol JNM-LA400, JEOL Ltd., Tokyo, Japan) in $CDCl_3$ (99.8%, Merck) at 292 K. Residual $CHCl_3$ in $CDCl_3$ was used as reference ($\delta = 7.27$).

3. Metabolism of cyazofamid by *Cunninghamella elegans*

Culture medium with mycelia (10 mL) was added in fresh PDB (250 mL), supplemented with cyazofamid (1 mg in 250 μL acetonitrile), and cultured at 27℃, 200 rpm for 10 days.

Each culture sample (10 mL) was extracted with ethyl acetate (20 mL × 2) at 0, 1, 2, 3, 5, 7, and 10 days after treatment and the extracts were combined and dried under pressure at 40℃, before dissolved with 1 mL of acetonitrile. An aliquot (2 μL) of the sample was analyzed with HPLC. Control incubations were conducted with the sterilized culture medium or in the absence of cyazofamid.

III. Results and Discussion

1. Degradation of cyazofamid and formation of metabolites

Microorganisms, such as *Cunninghamella*, can be used as a reliable alternative to *in vitro* models for drug metabolism studies (Asha and Vidyavathi 2009; Rydevik et al. 2013). The HPLC analysis of the culture extract indicated that cyazofamid was rapidly degraded to three metabolites 1, 2, and 3 (CM1, CM2, and CM3) while no appreciable degradation was observed in an absence of cyazofamid or a sterilized control experiment (Figure 22). Approximately 50% of cyazofamid degraded in 1 day, and after 7 days of incubation the entire cyazofamid disappeared. CM1 was observed from 1 day until 5 days after treatment. CM2 was detected from 1 day until 10 days after treatment, while CM3 was detected from 3 days until 10 days after treatment (Figure 23). When cyazofamid disappeared (7 days of incubation) the ratio of CM1, CM2, and CM3 was 21.5%, 71.3%, and 5.6%, respectively. These results of three different metabolites production suggest that *C. elegans* is a powerful tool for efficient biotransformation system as previously indicated in studies with methoxychlor (Keum et al. 2009), cyprodinil (Schocken et al. 1997), and vinclozolin (Pothuluri et al. 2000). Additionally, *C. elegans* metabolism experiments have obtained some major mammalian metabolites from various drugs and other metabolites with high yield at low cost, for example, amoxapine (Moody et al. 2000), mirtazapine (Moody et al. 2002), flurbiprofen (Amadio et al. 2010), and gemfibrozil (Kang et al. 2009).

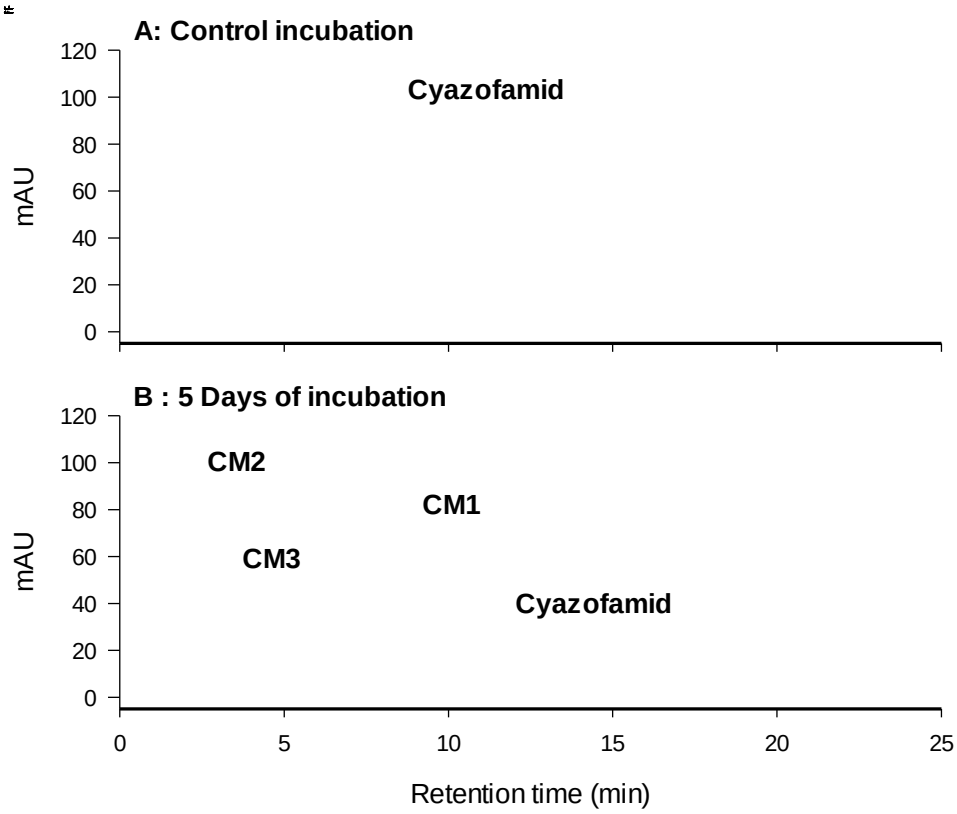


Figure 22. Formation of metabolite CM1, CM2, and CM3 from cyazofamid when it was incubated with *C. elegans* for 5 days at 27℃. A : Control incubation, B : 5 days of incubation.

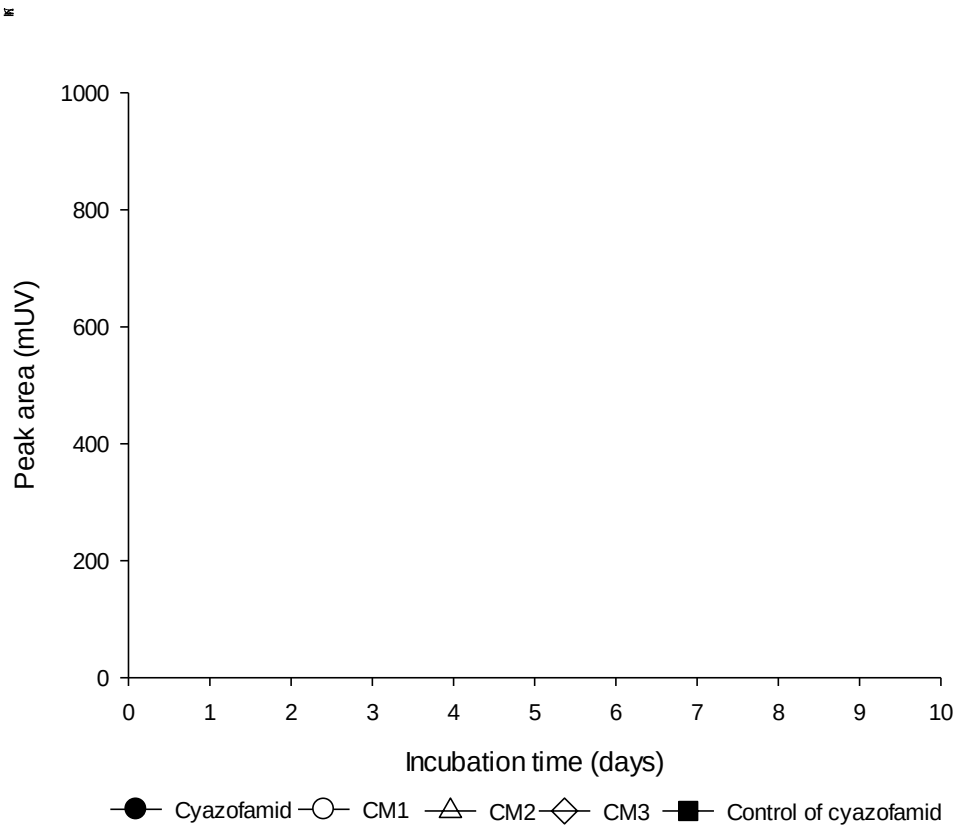


Figure 23. Degradation of cyazofamid and formation of metabolites (CM1, CM2, and CM3) from cyazofamid when cyazofamid (4 μg/L) were incubated with *C. elegans* for 10 days at 27℃.

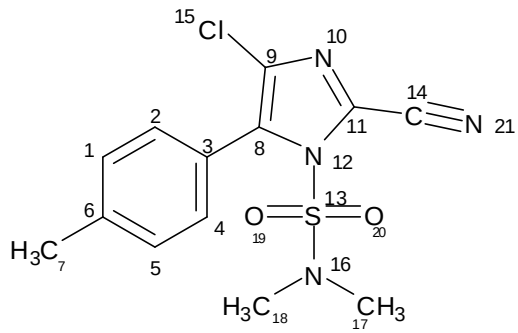
2. Confirmation of cyazofamid structure with NMR

To confirm the structure of cyazofamid, ^{1}H, ^{13}C, DEPT, and 2D ^{1}H-^{13}C HSQC were conducted on NMR (Appendix 1 – 4).

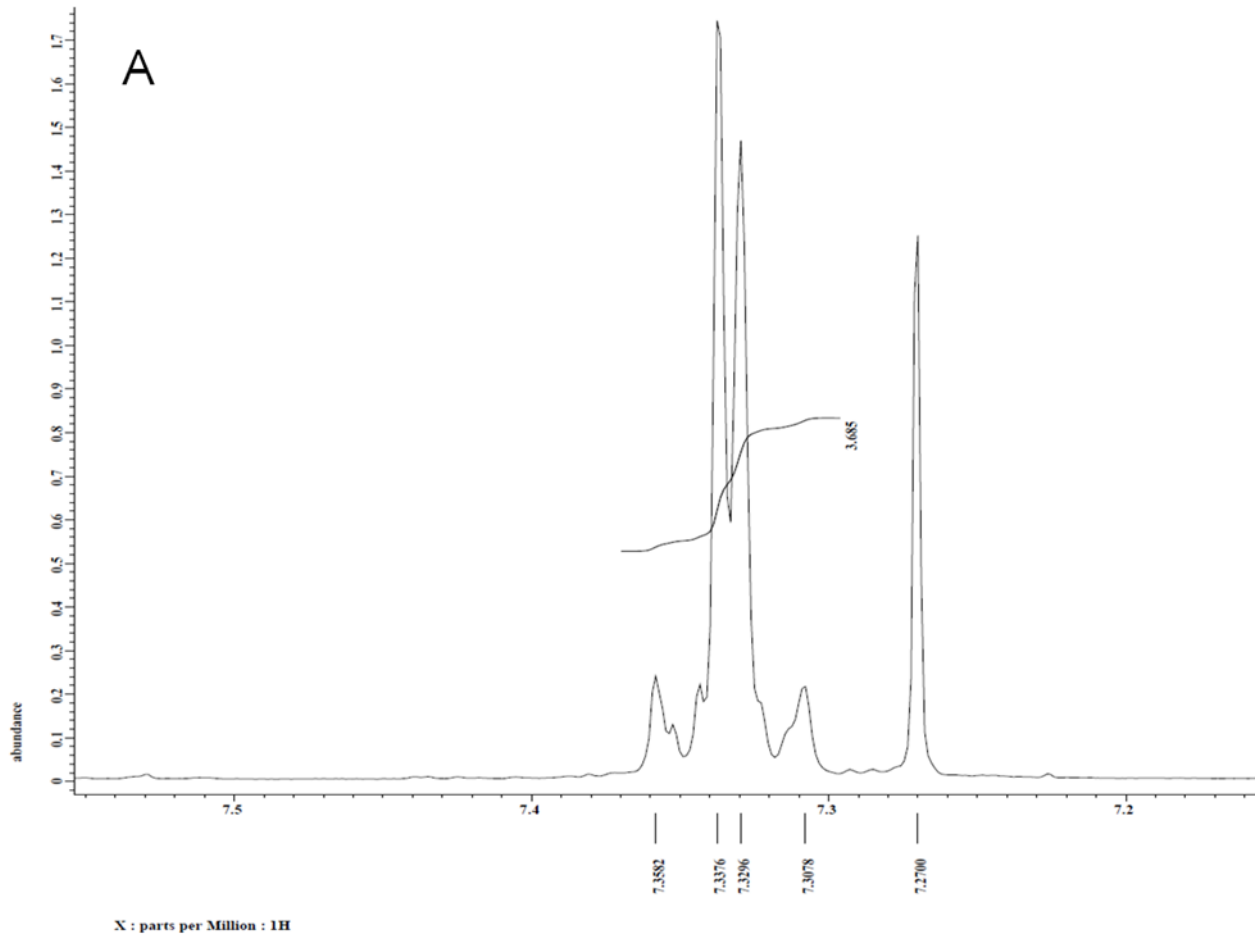
By combining ^{1}H, ^{13}C and DEPT results, protons and carbons in cyazofamid are identified as in Table 14. And 2D ^{1}H-^{13}C HSQC results also helped the identification of them.

In ^{1}H NMR 'benzene trick' was tried to get better resolution of multiplets such as benzene ring protons in cyazofamid (Figure 24) because it was reported that addition of a small amount (up to 20%) of a benzene-d_6 or pyridine-d_5 to the $CDCl_3$ solution of a sample, overlapping multiplets may be separated from one another sufficiently (Pavia et al. 2001). Therefore, the results of benzene-d6 was added to the cyazofamid sample about 10% to separate multiplets from benzene ring protons of cyazofamid (Figure 24).

Table 14. ^{1}H and ^{13}C NMR data for cyazofamid



	^{1}H NMR	
	δ ($CDCl_3$)	*J* (Hz)
1, 5	7.32 (2H, d)	8.72
2, 4	7.35 (2H, d)	8.24
7	2.44 (3H, s)	-
17, 18	2.67 (6H, s)	-
	^{13}C NMR	
	δ ($CDCl_3$)	*J* (Hz)
1, 5	131.18	-
2, 4	129.28	-
3	131.09	-
6	132.21	-
7	21.53	-
8	119.84	-
9	122.16	-
11	141.08	-
14	109.91	-
17, 18	37.96	-


A
abundance
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
3.685
7.5
7.4
7.3
7.2
7.3582
7.3376
7.3296
7.3078
7.2700
X : parts per Million : 1H

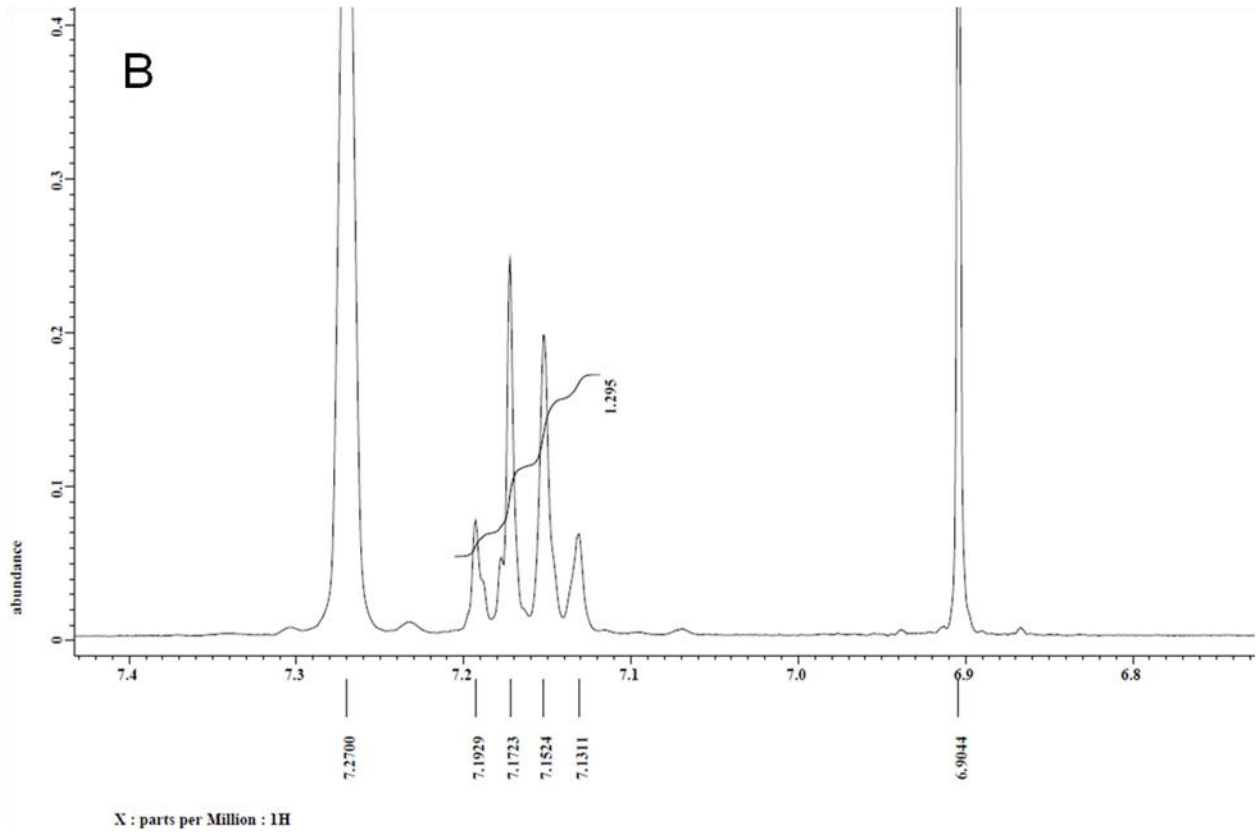


Figure 24. ^{1}H NMR spectra for cyazofamid. A : In $CDCl_3$, B : In $CDCl_3$ and benzene-d_6.

3. Metabolites identification

To identify the metabolites from cyazofamid, the incubation mixtures of *C. elegans* were analyzed by LC-MS/MS (Figure 25). As predicted, CM1 in metabolic mixture of *C. elegans* gave LC-MS spectrum, giving the $[M+H]^+$ at *m/z* 341 with $[M+H+2]^+$ at *m/z* 343 (intensity of 341 : 343 = 3 : 1). These results suggest that CM1 contains a chlorine atom in the molecule because cyazofamid also shows the $[M+H]^+$ at *m/z* 325 and the $[M+H+2]^+$ at *m/z* 327 (intensity of 325 : 327 = 3 : 1). An increase of molecular weight of 16 indicates that insertion of one oxygen atom into the cyazofamid molecule to produce CM1 by oxidation of N-methyl group (Abass et al. 2007; Suzuki and Casida 1981) or a tolyl group (Slade and Casida 1970). In Data Dependent Scanning mode on the ion trap MS, the critical MS^2 fragment ion *m/z* 296 from $[M+H]^+$ *m/z* 341, and a MS^3 fragment ion *m/z* 232 from the MS^2 fragment ion *m/z* 296 (Figure 26 and 27) indicated the oxidation of tolyl group ($CH_3 \rightarrow CH_2OH$) to produce CM1. If CM1 is N-hydroxy derivative, there must be a MS^2 fragment ion *m/z* 280 from $[M+H]^+$ *m/z* 341, instead of the MS^2 fragment ion *m/z* 296. This result was supported by the work on the microsomal metabolism study with diuron which reported the oxidation of N-methyl group to yield N-hydroxy derivative [$-N(CH_3)CH_2OH$] was not observed due to its instability (Suzuki and Casida 1981).

To confirm the structure of CM1, its peak on HPLC was isolated by fractionation to be investigated by NMR. ^1H-^{13}C HSQC (Figure 28) revealed that three protons and ^{13}C of C-7 in cyazofamid provided a ^1H singlet at 2.44 ppm and a ^{13}C singlet at 21.53 ppm, respectively. However in CM1, ^1H and ^{13}C peaks shifted to ^1H singlet at 4.77 ppm and a ^{13}C singlet at

64.54 ppm, respectively, suggesting an electro-negative oxygen must be attached at C-7 as predicted by LC-MS/MS data. These results from LC-MS/MS and ^{1}H-^{13}C HSQC clearly indicated CH_3 of cyazofamid oxidized to CH_2OH, yielding CM1. Slade and Casida (1970) also observed the oxidation of tolyl group of landrin in the mice liver microsome metabolism study. Recently, the rapid oxidation of ring methyl group in the gemfibrozil to hydroxymethyl group by *C. elegans* was reported (Kang et al. 2009). The identified metabolite in this study CM1, 4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)N,N-dimethyl-1H-imidazole-1-sulfonamide (CCHS) has not been reported in any other studies to date.

The two metabolites CM2 and CM3 from *C. elegans* incubation gave $[M+H]^+$ at *m/z* 234 and 248, respectively (Figure 25). Judging from molecular weight, CM2 must be 4-chloro-5-(4-(hydroxymethyl)phenyl)-imidazole-2-carbonitrile (CHCN), and CM3 must be CCBA [4-(4-chloro-2-cyano-imidazol-5-yl)benzoic acid] which must be produced from CHCN by further oxidation. Those metabolites CHCN and CCBA were observed and identified as the parent compounds and conjugated form from *in vivo* (rats) metabolism studies of cyazofamid and CCIM (Terada et al. 2004). These results also indicate the new metabolite CCHS must be formed *in vivo* (rats) even though it was not observed in blood and urine, probably due to the rapid degradation to CHCN and CCBA. *C. elegans* metabolic system provided the critical evidence to elucidate the metabolites of the first stage of oxidation reaction, CCHS, and two degradation metabolites.

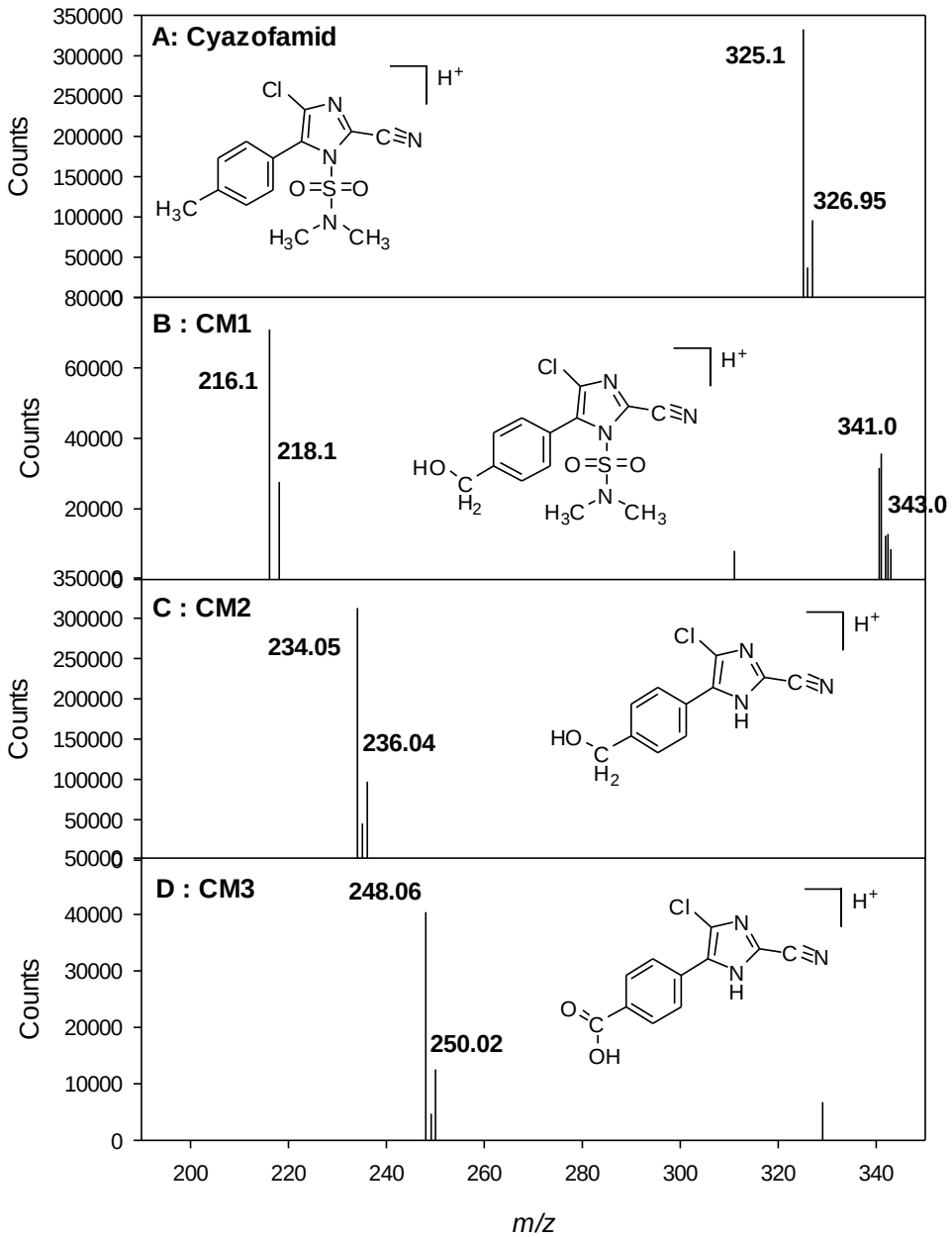


Figure 25. LC-MS/MS spectra and structures of cyazofamid (A) and metabolite CM1 (B), CM2 (C), and CM3 (D).

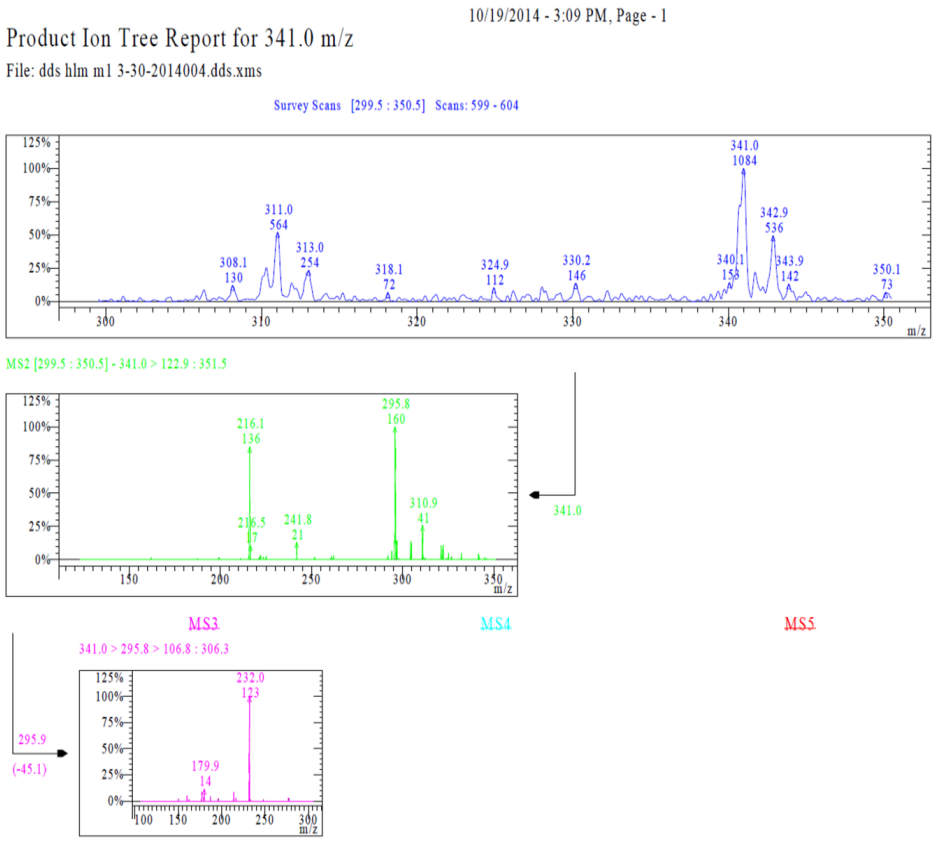


Figure 26. Ion tree report for MS^3 fragmentation of CM1 by Turbo data dependent scanning (TurboDDS™).

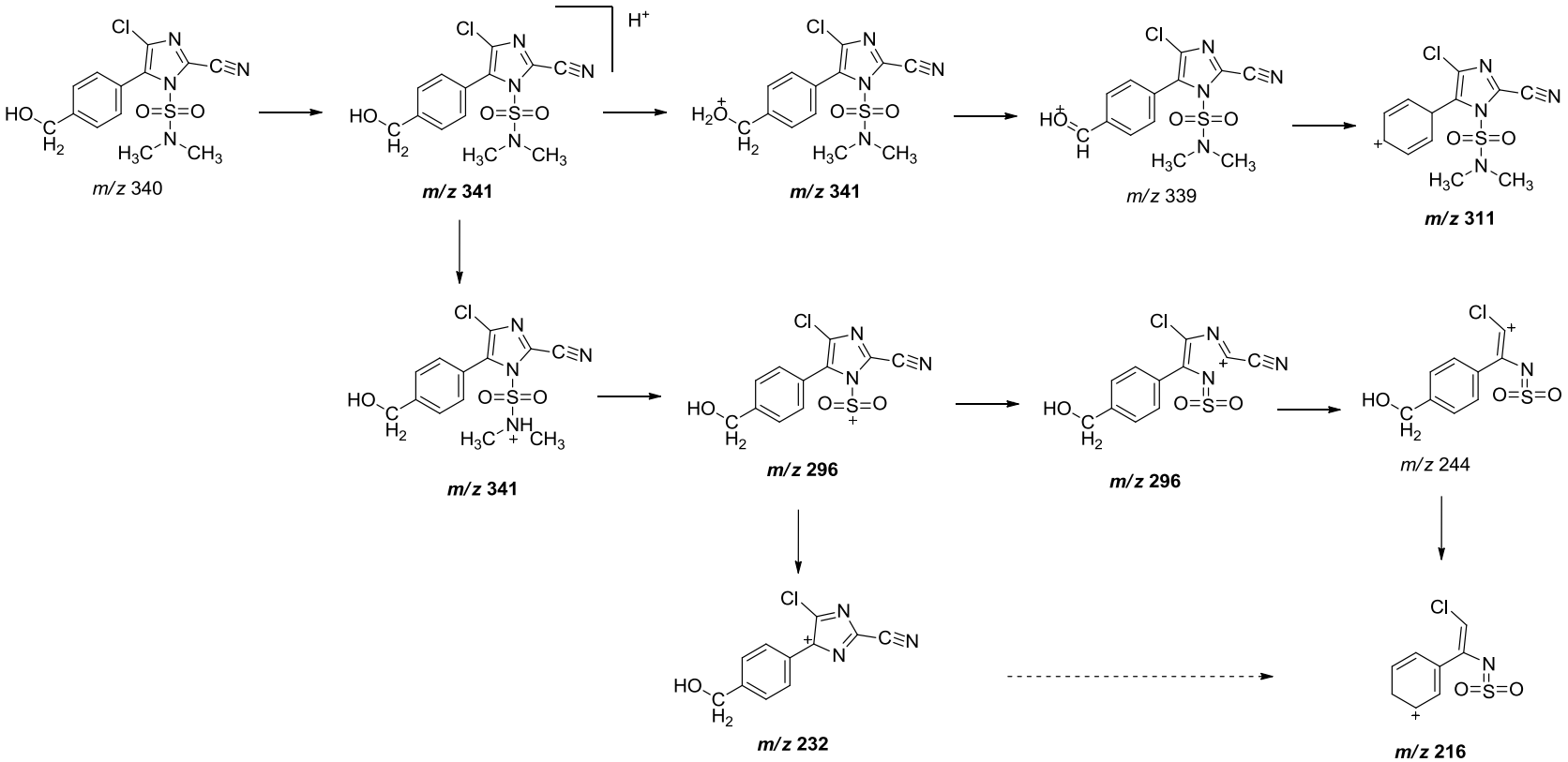


Figure 27. MS^3 fragmentation scheme of CM1 (*m/z* 341).

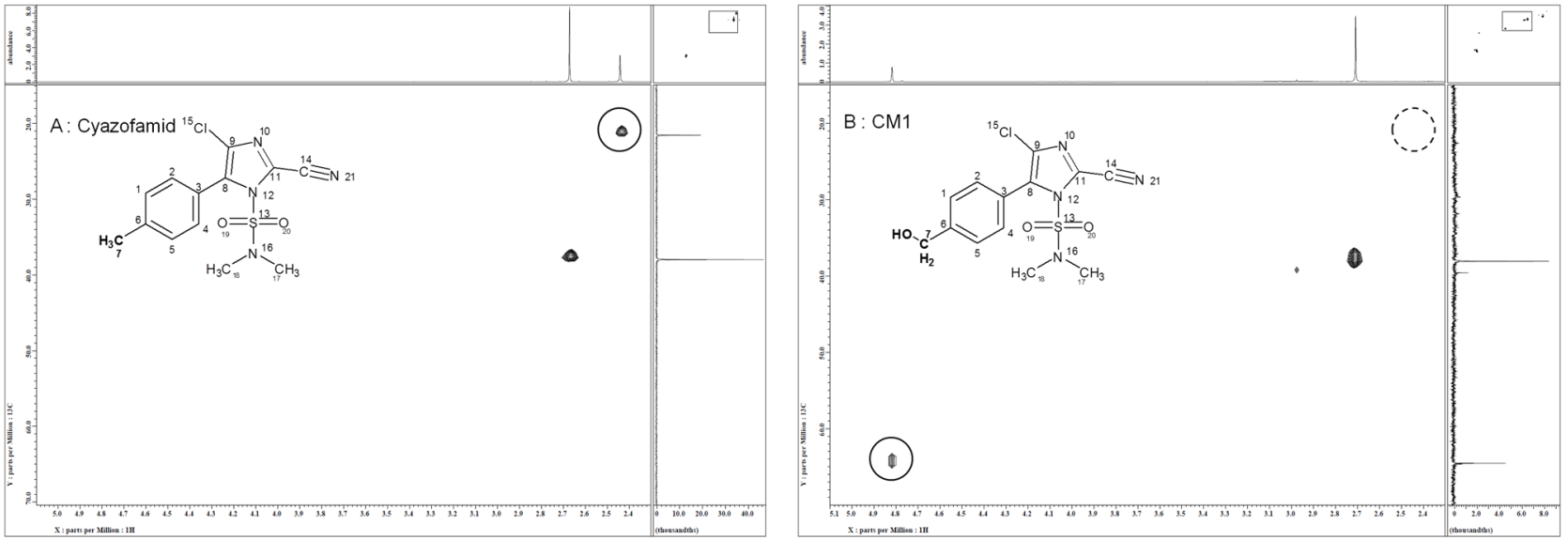


Figure 28. 2D ^{1}H-^{13}C HSQC spectra of cyazofamid (A) and CM1 (B).

IV. Conclusion

In conclusion for the possible metabolic pathway (Figure 29), cyazofamid was first oxidized to CCHS in a *C. elegans* metabolic system, and it could be degraded to CHCN and further oxidized to CCBA, judging from their structural relationship and the formation pattern of those three metabolites, and from the report of Slade and Casida (1970) which observed further oxidation of landrin-alcohol to landrin-acid.

CCIM from *in vivo* (rats) metabolism studies of cyazofamid (Terada et al. 2004) was not observed in this study probably because it is a simple hydrolysis product of cyazofamid in urine and bile. The *C. elegans* metabolic reaction provided the critical evidence to elucidate the metabolites of the first stage of oxidation reaction, CCHS, and two degradation metabolites. The metabolic system of *C. elegans* will be a powerful tool for predicting and identifying phase I metabolites that could be formed in mammalian system.

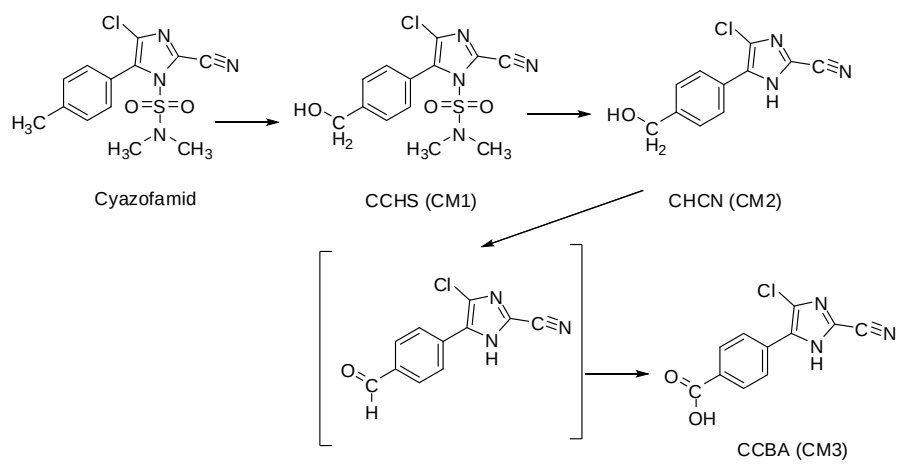


Figure 29. Proposed metabolic pathway of cyazofamid by *C. elegans*.

CHAPTER III

Metabolism of Cyazofamid

by *in vitro* Human Mimicking System

I. Introduction

Chewing is the first step in the food digestion process that reduces food particle size and mixes food particulates with saliva (Barrett et al. 2010), then food is mixed with gastric juice in stomach before contacting with intestinal juice in intestine.

Therefore, pesticide residues in food may react with gastrointestinal juices such as saliva, gastric juice, and intestinal juice to be transformed to metabolites depending on their chemical/biological stability because of pH of juices or enzymes in juices.

In vitro artificial gastrointestinal juices system provide a useful alternative to animal and human models in metabolism study by saving labor and time, reducing cost, and improving accuracy and reproducibility (Kong and Singh 2008) since *in vivo* feeding methods, using animals or humans, are time consuming and costly (Boisen and Eggum 1991).

There are a large number of both phase I and phase II xenobiotic metabolizing enzyme, and most exist in the same organism and/or the same tissue as several polymorphic forms (Hodgson 2012).

During the past decade, because of the availability of human liver cells, cell fractions and recombinant human xenobiotic metabolizing enzymes, there has been an increasing number of studies of the human metabolism of pesticides (Hodgson 2012).

The most of the biotransformation studies of pesticides are those resulting from metabolism catalyzed by the CYP dependent monooxygenases (Buratti

et al. 2005; Hodgson 2010c). The extensive knowledge base for this large group of enzymes is established and the substantial number of known CYP catalyzed metabolic reactions of pesticide (Hodgson 2003; Hodgson 2010c; Hodgson 2012). Metabolism by FMO isoforms is also frequently studied (Hodgson 2012). Glucuronidation is an important phase II reaction for the carbamate pesticides such as banol, carbaryl, carbofuran (Mehendale and Dorough 1972), some organophosphate compounds (Dorough 1979) and other chemicals. Glutathione conjugation played major role in the metabolism of organophosphates (Motoyama 1980) and the conjugated products of glutathione adducts may be further metabolized to mercapturic acids, the N-acetyl cysteine derivative of the original xenobiotic substrate. The mu (μ) class of GSTs is responsible for conjugating benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) as well as a wide variety of pesticides such as the organophosphorus pesticides (Abel et al. 2004a; Choi et al. 2006), the halogenated pesticides, the S-triazine herbicides (Abel et al. 2004b; Cho and Kong 2007; Hodgson 2012), and thiocarbamate herbicides (Campbell et al. 2008).

In this study, the metabolisms of cyazofamid by human mimicking system including artificial gastrointestinal juice, HLMs, cDNA-expressed human recombinant P450s (rCYPs), and S9 fractions were conducted to identify the metabolites, metabolic pattern and CYP isoforms responsible for the formation of metabolites. The molecular docking study was attempted to confirm the metabolic reactivity difference between cyazofamid and rCYPs.

II. Materials and Methods

1. Chemicals and reagents

Cyazofamid (98.4%) were purchased from Fluka™ (St. Louis, MO, USA) and CCIM was kindly donated from manufacture.

α-Amylase, bile, mucin, pancreatin, pepsin, trypsin, urea, and uric acid for the preparation of artificial gastrointestinal juices were products of Sigma-aldrich.

Calcium chloride ($CaCl_2$), sodium thiocyanate (NaSCN), sodium sulfate (Na_2SO_4), sodium hydrogen carbonate ($NaHCO_3$), magnesium chloride ($MgCl_2$), and potassium phosphate monobasic (KH_2PO_4) for the preparation of buffer solution or artificial gastrointestinal juices were products of Sigma-aldrich. And sodium chloride (NaCl) and hydrochloric acid (HCl) were purchased from Samchum pure chemical co., Ltd. and potassium chloride (KCl) was obtained from Kanto chemical co., Inc. (Tokyo, Japan).

Pooled HLMs (Table 15), S9 fraction and 10 different cDNA-expressed human recombinant P450s, CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Supersomes) were purchased from BD Gentest (Woburn, MA, USA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide adenine dinucleotide phosphate reduced (NADPH), alamethicin (from *Trichoderma viride*), uridine-5-diphosphoglucuronic acid trisodium salt (UDPGA), glutathione reduced/oxidized form (GSH/GSSG), potassium phosphate monobasic/dibasic, and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All solvents (HPLC grade) were obtained from Burdick and Jackson® (Ulsan, Korea)

Table 15. Characteristics of BD Ultra Pool HLM 150*

Enzyme Measured	Assay	Enzyme Activity [in pmol/(mg×min)]
Total P450	Omura and sato	350 pmol/mg
OR**	Cytochrome c Reductase	270
Cyt. b_5	Spectrophotometric	520 pmol/mg
CYP1A2	Phenacetin O-deethylase	690
CYP2A6	Coumarin 7-hydroxylase	830
CYP2B5	(S)-Mephenytoin N-demethylase	40
CYP2C8	Paclitaxel 6α-hydroxylase	170
CYP2C9	Diclofenac-4'-hydroxylase	3100
CYP2C19	(S)-Mephenytoin N-hydroxylase	57
CYP2D6	Bufuralol 1'-hydroxylase (The amount of activity inhibited by 1 μM quinidine)	71
CYP2E1	Chlorozoxazone 6-hydroxylase	2400
CYP3A4	Testosterone 6β-hydroxylase	4600
CYP4A11	Lauric acid 12-hydroxylase	1700
FMO	Methyl p-Tolyl Sulfide Oxidase	1100
UGT1A1	Estradiol 3-Glucuronidation	940
UGT1A4	Trifluoperazine Glucuronidation	580
UGT1A6	Serotonin Glucuronidation	10000
UGT1A9	Propofol Glucuronidation	3700
UGT2B7	AZT Glucuronidation	760

* : Provided from BD Biosciences

** : OR, Oxido-reductase

2. Analytical instruments and conditions

2.1. HPLC

The digestion samples of cyazofamid by gastrointestinal juice were analyzed using an Agilent HPLC 1100 series (Santa Clara, CA, USA) equipped with a Kinetex C18 column (2.1 mm i.d. × 100 mm, 2.6 μm; Phenomenex® , Torrance, CA, USA) at 40℃. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient conditions used were as follows : 0% B at 0 min to 1 min, 95% B at 10 min to 17 min, and 0% B at 20 to 25 min. The injection volume was 5 μL, and UV detector wavelength was 280 nm.

The metabolism reaction samples of cyazofamid from the incubation of HLMs were measured using an Agilent HPLC 1100 series equipped with a Kinetex C18 column (2.1 mm i.d. × 100 mm, 2.6 μm; Phenomenex®, Torrance, CA, USA). The analytical conditions include the column oven temperature, the mobile phase conditions, the detector wavelength were identical with the conditions for the metabolism study of *C. elegans*. The metabolites were quantitated using the calibration curve of cyazofamid because their authentic standards were not available (Kim et al. 2011).

The metabolism reaction samples of cyazofamid from the incubation of S9 fraction were measured using an Agilent HPLC 1100 series. The analytical conditions were same with the condition of artificial gastrointestinal metabolism study.

2.2 LC-MS/MS

Varian 500-MS IT-MASS spectrometer (Walnut Creek, CA, USA) equipped with Agilent 1100 HPLC was used with Luna C18 column (2.0

mm i.d. × 150 mm, 3.0 μm; Phenomenex®) at 40℃. To confirmation of metabolites by HLM metabolism study, the LC-MS conditions were identical with the conditions for the metabolism study of *C. elegans.*

3. Metabolism of cyazofamid by artificial gastrointestinal juice

3.1 Preparation of artificial gastrointestinal juices

Artificial saliva, gastric juice, and intestinal juice were prepared as the artificial gastrointestinal juice. Each composition of artificial gastrointestinal solutions was weighed and solubilized to 100 mL volume (Table 16). The artificial saliva and intestinal juices were adjusted to pH 7.4 with 1N $NaHCO_3$, and artificial gastric juice was adjusted to 2.0 with 1 N HCl. The pH adjusted gastrointestinal solutions were finally made up to total volume 100 mL (100%). The mixture of saliva + gastric juice (1:4, v/v) was prepared by mixing artificial saliva and gastric juice with a ratio of 1:4 (v/v).

Phosphate buffer at 50 mM (pH 7.4) was used as neutral control. As an acidic control, pH 2 buffer solution was prepared by adjusting the pH of 50 mM phosphate buffer to 2.0 with 1 N HCl. All solutions were stored at 4℃, and used within 24 hr.

Table 16. Composition of gastrointestinal model juices in 100 mL (Adenugba et al. 2008)

Salivary juice	mg	Gastric juice	mg	Intestinal juice	mg
NaCl	50	NaCl	290	KCl	30
NaSCN	15	KCl	700	$CaCl_2$	50
Na_2SO_4	55	KH_2PO_4	270	$MgCl_2$	20
NaHCO3	15	Pepsin	100	$NaHCO_3$	100
KCl	45	Mucin	300	Trypsin	30
KH_2PO_4	60			Pancreatin	900
$CaCl_2$	15			Bile	900
Mucin	75			Urea	30
α-amylase	25				
Urea	10				
Uric acid	1				

3.2 *In vitro* metabolism of cyazofamid by gastrointestinal juices

The concentration of cyazofamid in each gastrointestinal solution (neutral control, acidic control, saliva, saliva + gastric juice (1:4, v/v), gastric juice, intestinal juice) was 10 μM and final volume of each gastrointestinal juice was adjusted to 500 μL. The metabolic reaction was carried out in a shaking water bath with 65 cycle/min at 37℃. The times for each metabolic reaction were 5 min for saliva, 2 hr for acidic control, saliva + gastric juice (1:4, v/v) and gastric juice, and 6 hr for neutral control and intestinal juice. After metabolic reaction for each designed time, the reaction samples were terminated by addition of 500 μL acetonitrile. After immediately vortexing for 30 sec, the samples were centrifuged at 13,000 rpm for 7 min. The metabolic reaction was carried out in triplicate.

After identification of metabolites, quantitative analysis was performed with an external standard calibration method. Standard solutions of cyazofamid and CCIM were prepared as 1 mM stock solutions in acetonitrile. The stock solution was diluted at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μM with acetonitrile. Concentrations of cyazofamid and CCIM were obtained based on the peak area from each calibration standard. The calibration standards were fitted with high linearity (R^2 > 0.99909; cyazofamid, 0.99891; CCIM).

4. Metabolism of cyazofamid by HLMs and S9 fractions

4.1 Metabolism of cyazofamid in HLMs (Phase I reaction)

To determine the metabolite formation from cyazofamid, the incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), 10 mM

magnesium chloride, pooled HLMs (0.5 mg/mL), NADPH-generating system (1 mM $NADP^+$, 5 mM glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, and 1 mM NADPH), and 100 μM cyazofamid were prepared in a total incubation volume of 500 μL. The reaction mixtures were incubated at 37℃ for 0, 30, 60, and 120 min in a shaking water bath before terminating the reaction by the addition of 500 μL of acetonitrile on ice. The reaction mixture was centrifuged at 13,000 rpm for 7 min at 4℃, and the 2 μL of supernatant was subsequently analyzed with HPLC. Control incubations were conducted in the absence of an NADPH-generating system or with the denatured HLMs at 80℃. HLMs were heated for 30 min at 45℃ before the incubation to confirm whether FMOs are involved in the metabolite formation or not.

4.2 Metabolite identification

The acetonitrile supernatant (200 μL) of the pooled HLMs reaction mixture was dried with the gentle nitrogen stream, and the residue was dissolved with a 50 μL of acetonitrile to be analyzed with LC-MS/MS scan mode.

4.3 Optimization of metabolic conditions and kinetic studies

The metabolic reactions were performed with various concentrations of HLMs (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) for 0, 5, 10, 15, 20, and 30 min in the same manner as the above method to determine the optimal reaction conditions.

To analyze enzyme kinetics such as V_{max}, K_m, and CL_{int}, a range of cyazofamid concentrations (5, 10, 20, 50, 100, and 200 μM) were used in

metabolic reactions under the optimized conditions (HLMs concentration of 0.2 mg/mL and an incubation time of 10 min).

4.4 Metabolism of cyazofamid by cDNA-expressed CYP450 isoforms and kinetic studies

The metabolic reactions were performed with ten types of cDNA-expressed CYP450 isoforms (10 pmol) and 10 μM of cyazofamid for 10 min to identify CYP isoforms responsible for metabolite formation.

Ten pmol of each CYP2B6, 2C9, and 2C19, which produced metabolite, were incubated with various concentrations of cyazofamid (1, 5, 10, 20, 50, and 100 μM) for 10 min to obtain the values of V_{max}, K_m, and CL_{int}.

4.5 Metabolism of cyazofamid in S9 fractions (Phase II reaction)

To investigate the metabolism of cyazofamid by UGT, the incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), 10 mM magnesium chloride, S9 fractions (0.5 mg/mL), 25 μg/mL alamethicin, NADPH-generating system (1 mM $NADP^+$, 5 mM glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, and 1 mM NADPH), 5 mM UDPGA, and 100 μM cyazofamid were prepared in a total incubation volume of 500 μL.

For the metabolism study of cyazofamid by GST, the incubation mixture containing 50 mM potassium phosphate buffer (pH 7.4), 10 mM magnesium chloride, S9 fraction (0.5 mg/mL), 5 mM GSH, and 10 μM cyazofamid were prepared in a total incubation volume of 500 μL. The reaction mixtures were incubated at 37℃ for 0, 30, 60, and 120 min in a shaking water bath before terminating the reaction by the addition of 500 μL of acetonitrile on ice. The

reaction mixture was centrifuged at 13,000 rpm for 7 min at 4℃, and the 5 μL of supernatant was subsequently analyzed with HPLC. Control incubations were conducted in the absence of an NADPH-generating system/UDPGA and GSH or S9 fraction.

5. Determination of crystal structure for cyazofamid

Small clear crystals were obtained by slow evaporation in a mixture of acetone and hexane. The structure of cyazofamid was determined by single crystal X-ray diffraction methods by professor Hoseop Yun group of Ajou University. Data collection was performed with Mo $K\alpha_1$ on an RIGAKU R-ASXIS RAPID diffractometer. Although we were not able to obtain good quality single crystals, structural studies conducted on cyazofamid gave enough information : a = 6.8160(10) Å , b = 13.396(2) Å , c = 16.462(2) Å, α = 90°, β = 92.993(3) °, γ = 90° and 1501.0(4) $Å^3$. Intensity data were collected with the ω-2θ scan techniques. The intensities of two standard reflections, showed no significant deviations during the data collection. A summary of the crystallographic data, data collection and structure refinement for the cyazofamid is given in Table 17.

The centrosymmetric space group P 21/n was assumed and the satisfactory refinement confirmed the choice of this space group. The initial position for all non hydrogen atoms were obtained by using direct methods of the SHELXS-86 program. The structure was refined by full matrix least squares technique with the use of the SHELXL-93 program. Anisotropic thermal motion for non-hydrogen atoms and extinction parameters were included. The final cycle of refinement performed on Fo^2 with all 5349

unique reflections afforded residuals wR_2 = 0.3307 and the conventional R index based on the reflections having Fo > 2σ(Fo^2) is 0.1050.

Table 17. Crystal data and structure refinement for cyazofamid

Empirical formula	$C_{13}H_{13}ClN_4O_2S$
Formula weight	324.78
Temperature (K)	293(2)
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	P 21/n
Unit cell dimensions (a, b, c (Å), α, β, γ (°))	a = 6.8160(10), b = 13.396(2), c = 16.462(2) α = 90, β = 92.993(3), γ = 90
Volume (Å^3)	1501.0(4)
Z	4
Calculated density (Mg/m^3)	1.437
Absorption coefficient (mm^{-1})	0.403
F(000)	672
θ range for data collection (°)	3.284 to 22.500
Limiting indices	$-7 \le h \le 6$, $-12 \le k \le 14$, $-17 \le l \le 17$
Reflections collected / unique	5349 / 1952 [R(int) = 0.0452]
Completeness to θ = 25.242 (%)	72.0
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1952 / 0 / 190
Goodness-of-fit on F^2	0.995
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.1050, wR_2 = 0.3144
R indices (all data)	R_1 = 0.1256, wR_2 = 0.3307
Extinction coefficient	n/a
Largest diff. peak and hole (e Å^{-3})	1.167 and -0.442

6. Molecular docking study

Molecular docking experiments of cyazofamid with CYP were performed on an Intel Core 2 Quad Q6600 (2.4 GHz) Linux PC with the Sybyl 7.3 software (Tripos, USA) by professor Yoongho Lim group of Konkuk University. The three dimensional (3D) structures of CYP 2B6, 2C9, 2C19, and 3A4 were adapted from the Protein Data Bank (3IBD, 1OG5, 4GQS, and 2J0D, respectively). The binding pocket was determined from 3IBD.pdb, 1OG5.pdb, 4GQS.pdb, and 2J0D.pdb using the LigPlot program (Wallace et al. 1995) and all 3D images were constructed using PyMOL program (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.).

III. Results and Discussion

1. Metabolism of cyazofamid by artificial gastrointestinal juice

1.1 Preparation of artificial gastrointestinal juices

Prior to study on *in vitro* metabolism of cyazofamid by gastrointestinal juices, attempt was made to prepare similar artificial gastrointestinal juices to those in the human body. Because the human saliva, with pH ranging from 5.3 to 7.8, is 99.5% water and 0.5% other components such as enzymes, mucins, nitrogenous products, and electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates, the artificial saliva that we prepared had pH 7.4 and contained all of the above components (Adenugba et al. 2008; de Almeida Pdel et al. 2008)

The gastric juice was prepared to pH 2 by using HCl, KCl, and NaCl with pepsin and mucin because the gastric juices is strongly acidic (pH 1 - 3.5) for the denaturation of proteins from food intake and reaction of the enzyme pepsin (Adenugba et al. 2008; Barrett et al. 2010; Talwar and Srivastava 2002; Ulleberg et al. 2011). Intestinal juice was prepared with pancreatin, trypsin, bile, and electrolytes. Pancreatin and trypsin are secreted by the pancreas, whereas bile is released by the liver through the bile duct. Other components in intestinal juice are secretions by the intestinal glands in the small intestine. Therefore, the intestinal juice was prepared by mixing pancreatin, trypsin, bile, urea, and electrolytes, and the pH was adjusted to 7.4 (Adenugba et al. 2008; Rao et al. 1989). The compositions of each gastrointestinal juice were modified according to the purpose of the study. In this study, the composition of gastrointestinal juice from the studies of

Adenugba et al. (2008) and Lee et al. (2014c) was adopted, because several major enzymes and salts of this composition were similar to the gastrointestinal juice content in the human body.

1.2 *In vitro* metabolic reaction of cyazofamid by artificial gastrointestinal juice

To determine the cyazofamid metabolism in artificial saliva, saliva + gastric juice (1:4, v/v), gastric juice, and intestinal juice, the gastrointestinal solutions with 10 μM of cyazofamid was incubated for a specific time. Metabolic reaction times to satisfy the proper digesting time in the body were determined to 5 min for saliva, 2 hr for acidic control, saliva + gastric juice (1:4, v/v), and gastric juice, and 6 hr for neutral control and intestinal juice, by considering the digesting time required by the gastrointestinal organs such as the mouth, stomach, and small intestine. The gastrointestinal metabolite (GM) peaks in the HPLC chromatogram of the incubated reaction with 10 μM cyazofamid were identified (Figure 30). The cyazofamid did not show any metabolite in the control, which was a pH 7.4 buffer solution, whereas one unknown GM was observed under acidic conditions (pH 2). In artificial saliva (5 min) cyazofamid was stable. The reaction with artificial saliva + gastric juice (1:4, v/v) and artificial gastric juice for 2 hr each produced identical metabolite GM (T_r : 12.70 min), with no interfering peaks. GM was identified as CCIM, the degradate of cyazofamid, based on its retention time and LC-MS spectrum (Figure 31).

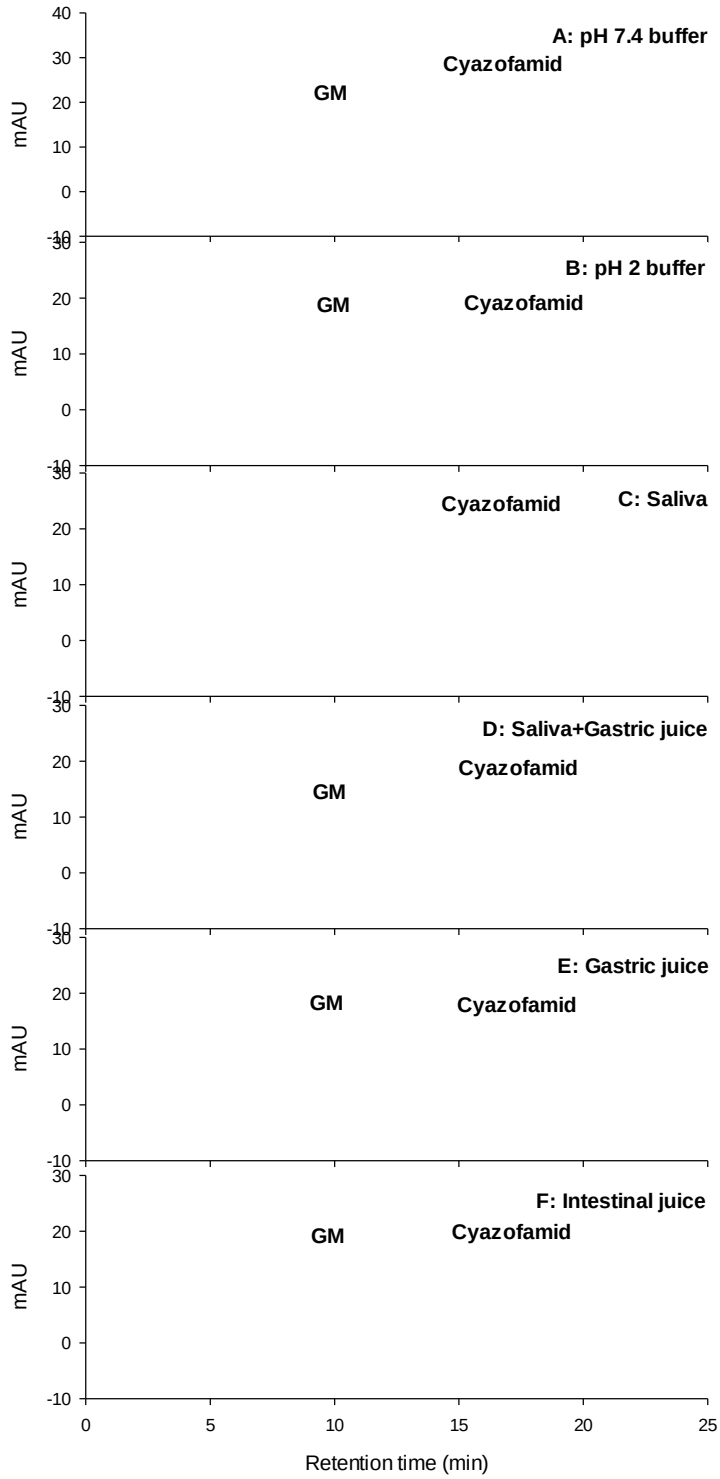


Figure 30. HPLC chromatogram of cyazofamid with gastrointestinal juice, buffer solution pH 7.4 (A), buffer solution pH 2 (B), saliva (C), saliva + gastric juice (1:4, v/v) (D), gastric juice (E), and intestinal juice (F).

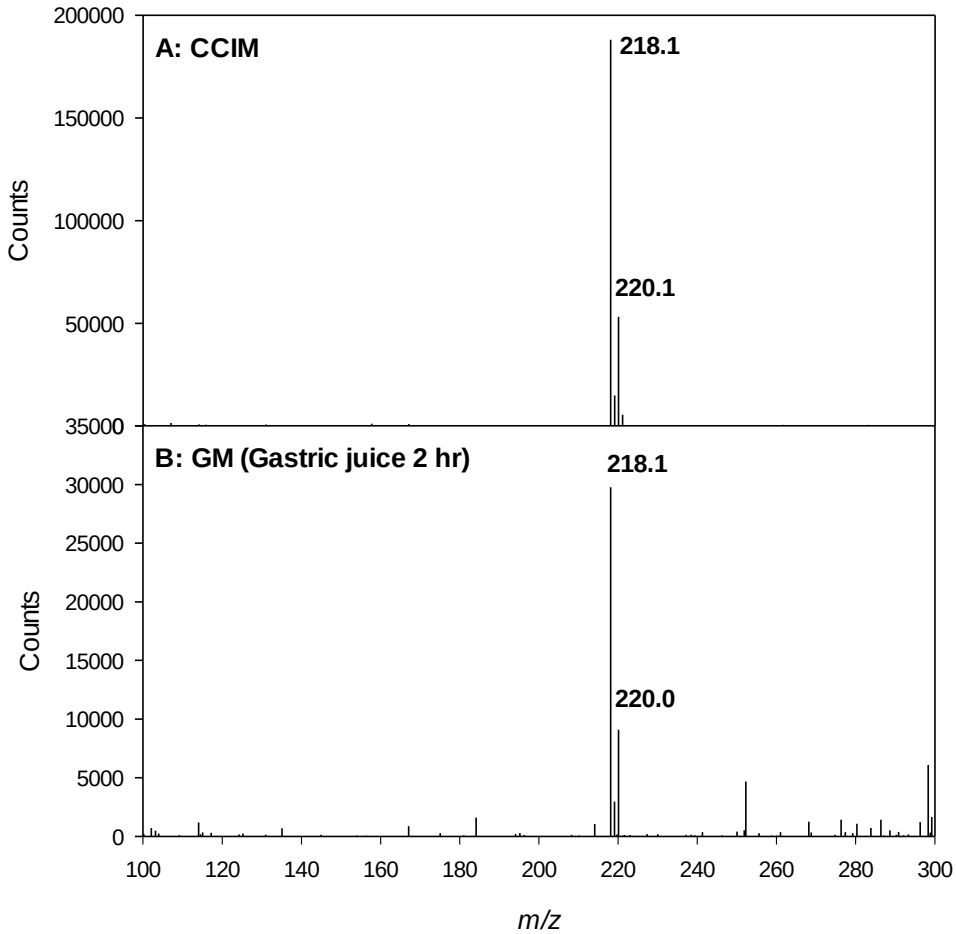


Figure 31. LC-MS spectra of CCIM (A) and GM (B), the degradate of cyazofamid from the gastrointestinal incubation

1.3 Metabolic pattern of cyazofamid by artificial gastrointestinal juices

Because it is impossible to perform *in vivo* metabolism of contaminants in humans (Versantvoort et al. 2005), the current *in vitro* metabolism of cyazofamid using artificial gastrointestinal solutions was designed to provide an ideal estimation of the *in vivo* situation in humans.

To date, some researchers have suggested pesticide biomonitoring approach by using the animal/human saliva, or artificial gastrointestinal juices. But, only few reports were available for the metabolism of pesticides by gastrointestinal juices (Esteban and Castano 2009; Lee et al. 2014c; Lu et al. 2003; Nigg et al. 1993; Timchalk et al. 2007).

The degradation patterns of cyazofamid in each artificial gastrointestinal juice are shown in Table 18 and Figure 32. Cyazofamid was observed to be stable in saliva. In the saliva + gastric juice, gastric juice and intestinal juices, approximately 8%, 17%, and 15% of cyazofamid was degraded, respectively. However, cyazofamid was considered not to be degraded by the artificial gastrointestinal juices, but to be degraded by simple aquatic environmental condition, because neutral and acidic control experiments also showed similar degradation pattern with the artificial juices.

Our study showed that cyazofmaid could be degraded to CCIM in the gastrointestinal organs, especially in the stomach (about 17% degradation) and intestines (about 15% degradation), before reaching other metabolic organs such as the liver or cytosol through the blood stream. Considering the degradation of cyazofamid by artificial gastrointestinal juices in the *in vitro* metabolism study, the availability of cyazofamid from the gastrointestinal tract into the blood stream is considered to be limited to some extent.

Table 18. Metabolism of cyazofamid (10 μM) by artificial gastrointestinal juices

Gastrointestinal juices	Optimal time	Concentration (μM)	
		CCIM	Cyazofamid
Buffer (pH 7.4)	6 hr	0.40 ± 0.05	3.69 ± 0.40
Buffer (pH 2)	2 hr	0.58 ± 0.17	3.35 ± 0.88
Saliva	5 min	-	4.27 ± 0.12
Saliva + gastric juice (1:4)	2 hr	0.24 ± 0.04	4.26 ± 0.09
Gastric juice	2 hr	0.62 ± 0.02	3.67 ± 0.16
Intestinal juice	6 hr	0.84 ± 0.03	3.56 ± 0.08

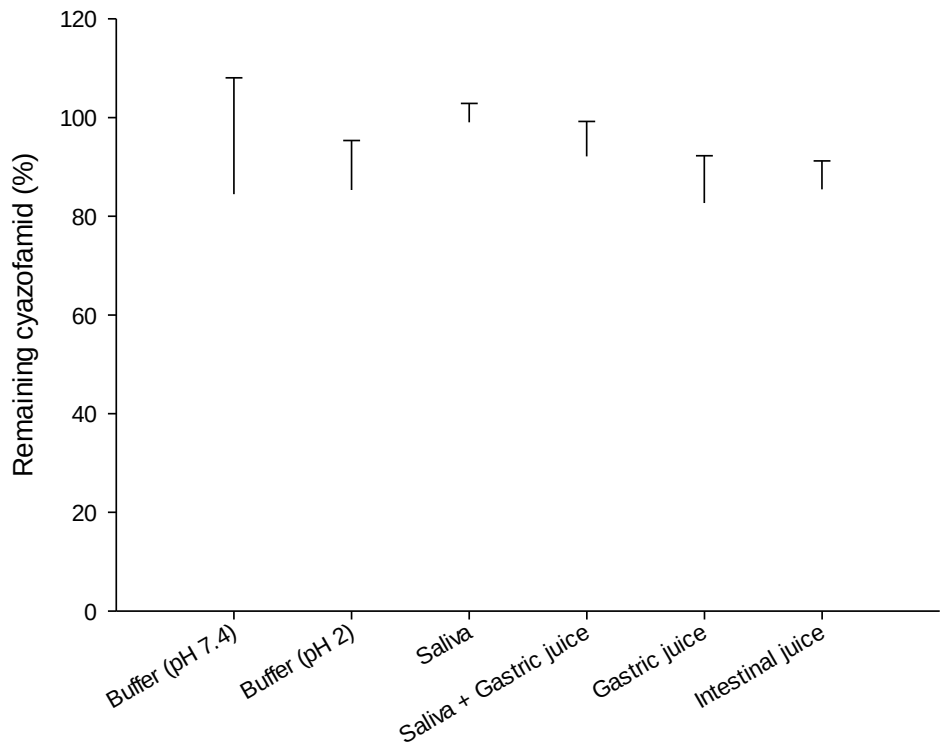


Figure 32. Remaining cyazofamid after metagolic reaction with artificial gastrointestinal juices.

2. *In vitro* Metabolism of cyazofamid by HLMs and S9 fraction

2.1 Phase I metabolism of cyazofamid by HLMs

2.1.1 Formation of the cyazofamid metabolite by HLMs

HLMs incubation of cyazofamid in the presence of NADPH resulted in the formation of one metabolite 1 (M1, Figure 33). No metabolites were observed in control reactions with denatured HLMs or with the absence of the NADPH generating system, suggesting that M1 was formed from cyazofamid by the HLMs metabolic reaction. To confirm whether FMOs mediate the formation of M1 from cyazofamid in HLMs, HLMs was heated to deactivate the heat-labile FMOs. Heated HLMs with NADPH generating system also produced M1, demonstrating that M1 was formed by the activity of CYPs.

The metabolite, M1 from HLMs incubation gave $[M+H]^+$ at *m/z* 341 (Figure 34). Judging from molecular weight and mass fragment pattern, M1 must be 4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)N,N-dimethyl-1H-imidazole-1-sulfonamide (CCHS) which is same metabolite with the CM1 of *C. elegans* metabolism study. Consequentially, the formation of M1 or CM1 from cyazofamid is resulted in the metabolic reaction by CYPs, resulting in oxidation.

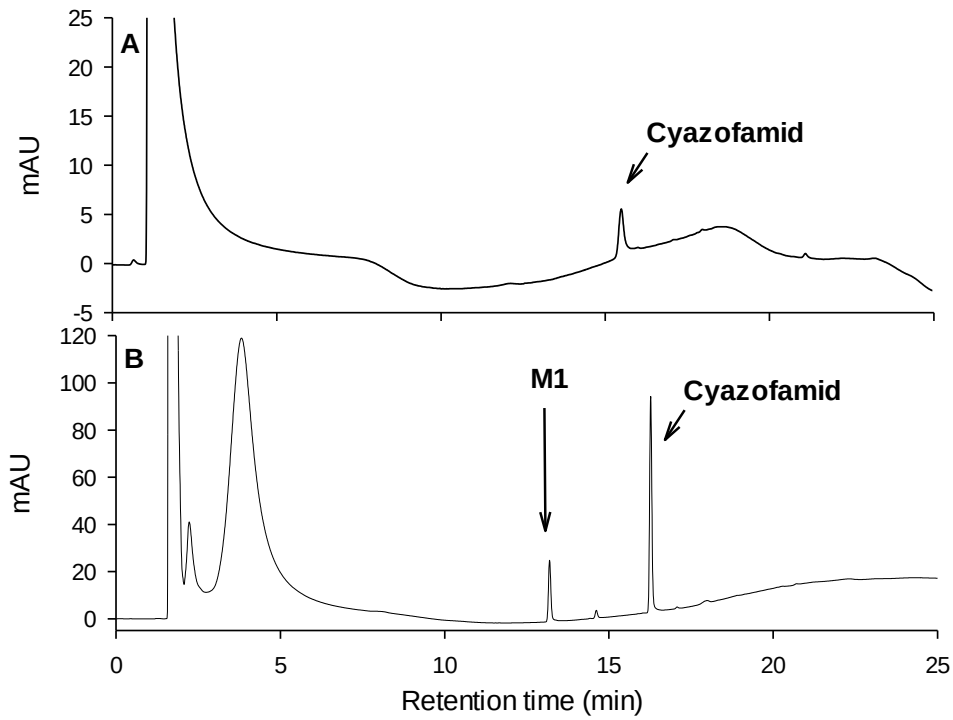


Figure 33. Formation of metabolite M1 from cyazofamid when it was incubated with human liver microsomes and NADPH-generating system for 10 min at 37℃. A : Control incubation, B : 10 min of incubation (The sample was concentrated at 5 times).

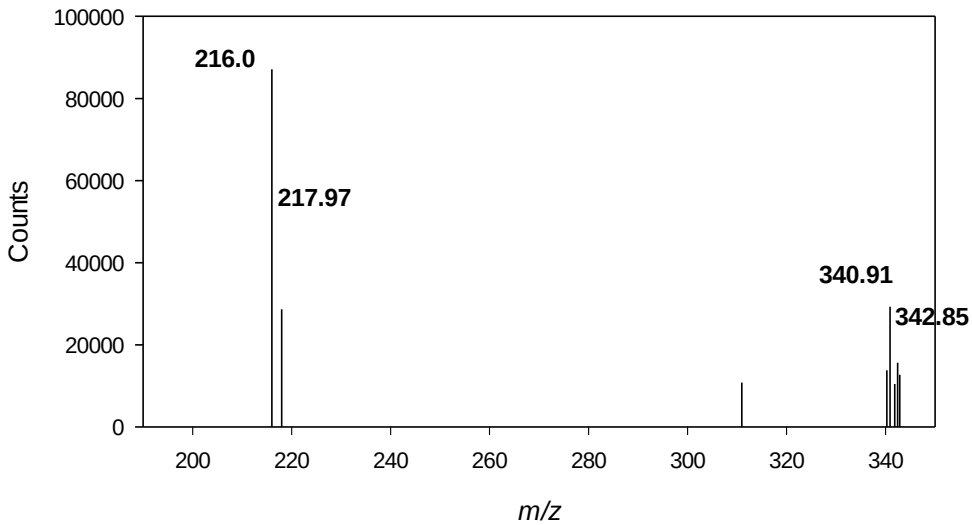


Figure 34. LC-MS spectrum of M1 from cyazofamid by metabolism of HLM with NADPH-generation system.

2.1.2 Optimization of metabolic conditions and kinetic studies

A range of HLMs protein concentrations and incubation time were used in metabolic reactions to obtain 0.2 mg/mL of protein and 10 min of incubation time as the optimal metabolic conditions of cyazofamid in HLMs. Under these optimized metabolic conditions, the formation pattern of M1 from cyazofamid by HLMs was best fitted to a Michaelis-Menten equation [$V = V_{max} \times [S] / (K_m + [S])$] to yield 1.963 pmol/min/mg proteins of V_{max} and 3.627 μM of K_m values. In endosulfan sulfate formation from α-endosulfan and β-endosulfan by HLMs, V_{max} were 1.48 ± 0.07, 4.40 ± 0.18 pmol/min/pmol P450, respectively while K_m were 7.34 ± 1.29 μM, 6.37 ± 0.88 μM, respectively (Lee et al. 2006). The CL_{int} (mL/min/ mg proteins) is a pure measure of enzyme activity towards a compound and acts as a proportionality constant to describe the relationship between rate of metabolism of a drug and its concentration at the enzyme site. It is not influenced by other physiological determinants of liver clearance such as hepatic blood flow or drug binding within the blood matrix (Houston 1994; Rane et al. 1977; Wilkinson 1987). In this study, the CL_{int} value of M1 was 0.541 μL/min/mg proteins for cyazofamid, while those of the other studies were 13.9-789 μL/min/mg proteins for benfuracarb (Abass et al. 2014b), 48.02-51.20 μL/min/mg proteins for *erythro*, *threo*-flucetosulfuron (Lee et al. 2014b), and 0.20-0.69 μL/min/ mg proteins for α, β-endosulfan (Lee et al. 2006).

2.1.3 Metabolism of cyazofamid in cDNA-expressed CYP450 isoforms and kinetic studies

When M1 formation from cyazofamid was investigated using recombinant CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, the formation of M1 was observed only in CYP2B6, 2C9, and 2C19 (Figure 35). In 63 pesticide metabolism studies using human recombinant cytochrome P450 isoforms, CYP 2C19 was involved in metabolism by 15%, CYP2B6 by 12%, CYP2C9 by 10%, and 24% by CYP3A4, which is the most abundant isoform (Abass et al. 2014b). In this study CYP3A4 was not involved in metabolism of cyazofamid to form M1.

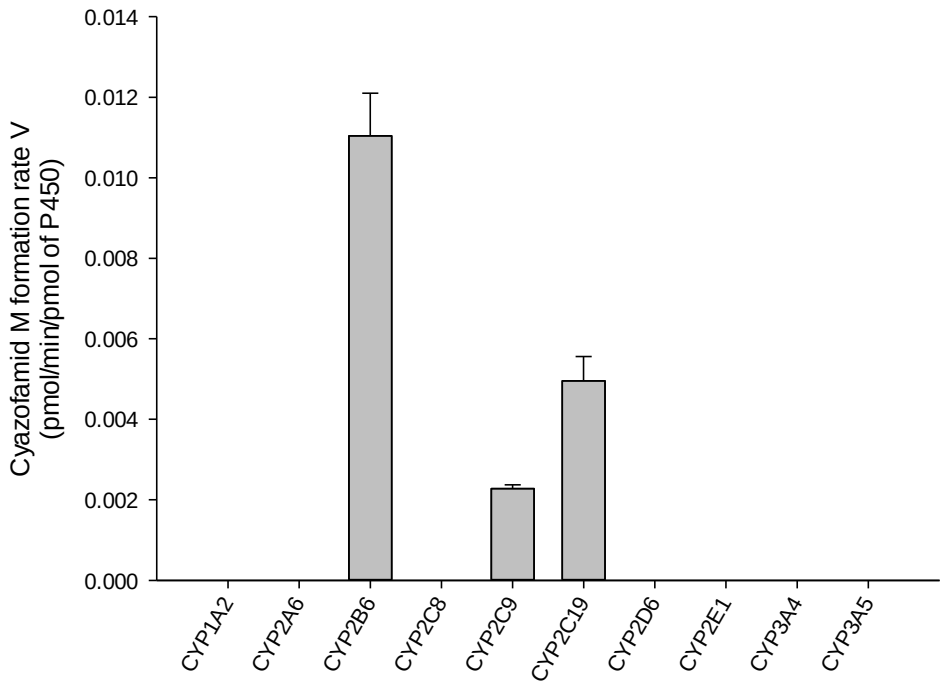


Figure 35. The formation of M1 from cyazofamid by cDNA-expressed P450 isoforms when those were incubated with 20 μM of cyazofamid at 37℃ for 10 min. Data shown are averages of triplicate experiments.

When the enzyme kinetic parameters for the formation of M1 were examined with CYP2B6, 2C9, and 2C19 (Figure 36, Table 19), the metabolism of cyazofamid by these P450 isoforms was best described by the Michaelis-Menten equation. The V_{max} values were 0.011, 0.002, and 0.005 pmol/min/mg proteins for CYP2B6, 2C9, and 2C19, respectively while 1.447, 0.507, and 0. 987 μM of K_m values were calculated for CYP2B6, 2C9, and 2C19, respectively. As a result, the total CL_{int} value of three P450 isoforms was 0.018 μL/min/mg proteins. The most efficient CYP isoform for cyazofamid was CYP2B6, while CYP2C9 and 2C19 showed relatively lower efficiency. The relative importance of CYP2B6 in drug and pesticide metabolism has only recently become apparent. Recent studies have demonstrated that CYP2B6 is an important isoform in human metabolism of pesticides including alachlor, metolachlor, acetochlor, and butachlor (Coleman et al. 2000). Meanwhile, CYP2B6 is now known to vary in protein expression depending on gender and ethic difference (Lamba et al. 2003). It is likely that many of the described metabolic differences are due to polymorphic variants of CYP2B6 (Hodgson and Rose 2007)

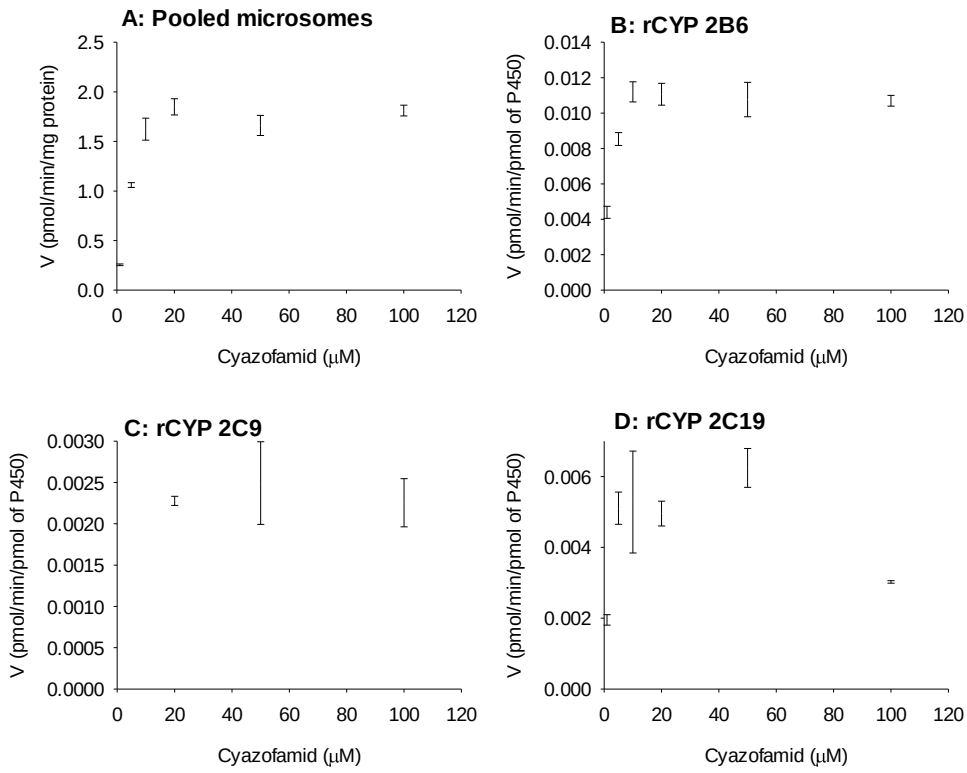


Figure 36. Kinetics for the formation rate of M1 from cyazofamid in pooled human liver microsomes (A) and cDNA-expressed P450 isoforms (B : CYP2B6, C : CYP2C9, and D : CYP2C19). An increasing concentration of cyazofamid (0 - 100 μM) was incubated with HLMs (0.2 mg/mL of pooled microsoms or 10 pmol of P450) and an NADPH-generating system for 10 min at 37℃. Data shown are averages and standard deviations of triplicate experiment.

Table 19. Mean enzyme kinetic parameters of the formation of metabolite M1 from cyazofamid in HLMs

	Pooled microsomes	CYP2B6	CYP2C9	CYP2C19
V_{max} (pmol/min/mg proteins)	1.963	0.011	0.002	0.005
K_m (μM)	3.627	1.447	0.507	0.987
CL_{int} [V_{max}/K_m (μL/min/mg proteins)]	0.541	0.008	0.005	0.005
Total CL_{int}	-		0.018	

To measure the contributions of each cytochrome to cyazofamid metabolite formation, the reaction rates measured with individual CYP isoforms were normalized with respect to the nominal specific content of the corresponding P450 in native human liver microsomes (Rodrigues 1999). Among the several reports on the contents of CYP isoform in liver (Zanger and Schwab 2013), we adapted LC-MS/MS quantification data reported by Kawakami et al. (2011). According to their work, the percent of CYP2C9 in pooled HLMs is 19.5%, while those of CYP2B6 and CYP2C19 are 1.67 and 0.886%, respectively. After normalization (Figure 37), the contribution values show that CYP2C9 was the main isoform responsible for M1 formation (66.1%), and CYP2B6 contributed by 27.4%, while CYP2C19 was the least contributor (6.5%).

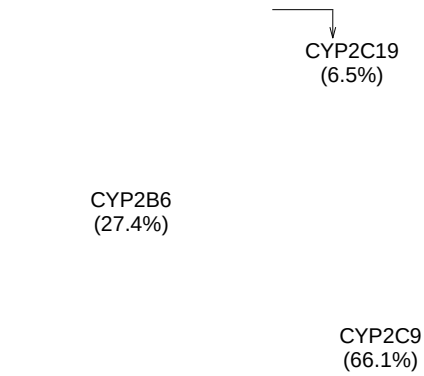


Figure 37. The relative contributions of P450 isoforms (CYP2B6, 2C9, and 2C19) to the M1 formation in 20 μM of cyazofamid.

2.2 Phase II metabolism of cyazofamid by S9 fractions

Phase II reaction, consisting primarily of conjugation reactions, involves the combination of the products of phase I reactions with several endogenous molecules to form water soluble products being able to be excreted. Phase II reactions important for pesticide metabolism include those important for the products of other xenobiotic oxidations, especially those catalyzed by the N-acetyl-, sulfo-, uridine diphosphate UDP-glucuronyl-, methyl- and amino acid taurine, glycine transferases (Carrera and Periquet 1990; Hodgson 2010c).

2.2.1 Phase II metabolism of cyazofamid by UGT

Glucuronidation is one of the most important reactions for the elimination of xenobiotics from the body, although not as yet well studied in the case of pesticides or the phase I metabolites of pesticides (Hodgson 2012).

In this study, cyazofamid was incubated with NADPH-generating system, UDPGA, and S9 fractions. After incubation for 120 min, one metabolite was observed in NADPH or NAPDH/UDPGA dependent metabolic system (Figure 38).

The pattern of metabolite formation in the metabolism reaction by NADPH and NADPH/UDPGA was similar (Figure 39). After the incubation of the NADPH metabolism mixture at 37℃, cyazofamid transformed to the metabolite, CCHS by approximately 85%. However, no glucuronidation metabolite was detected in the incubation of NADPH reaction mixture containing metabolite CCHS with UDPGA (Figure 38), suggesting no glucuronic acid conjugation occurred or its concentration was too low to detect.

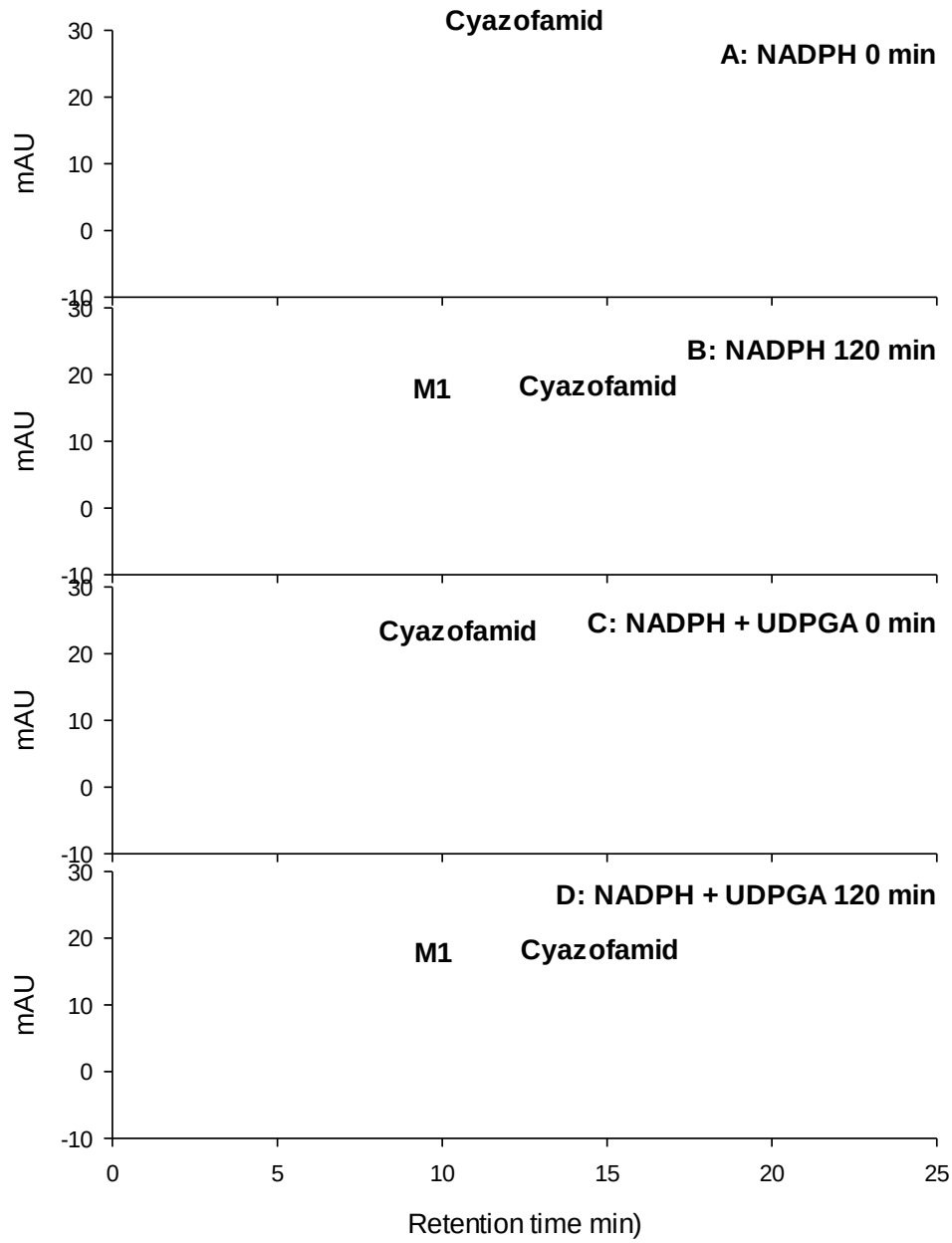


Figure 38. Formation of metabolite M1 from cyazofamid when it was incubated with S9 fractions and NADPH-generating system or NADPH/UDPGA for 120 min at 37℃. A : Control incubation with NADPH, B : 120 min of incubation with NADPH. C : Control incubation with NAPDH/UDPGA, D : 120min incubation with NADPH/UDPGA.

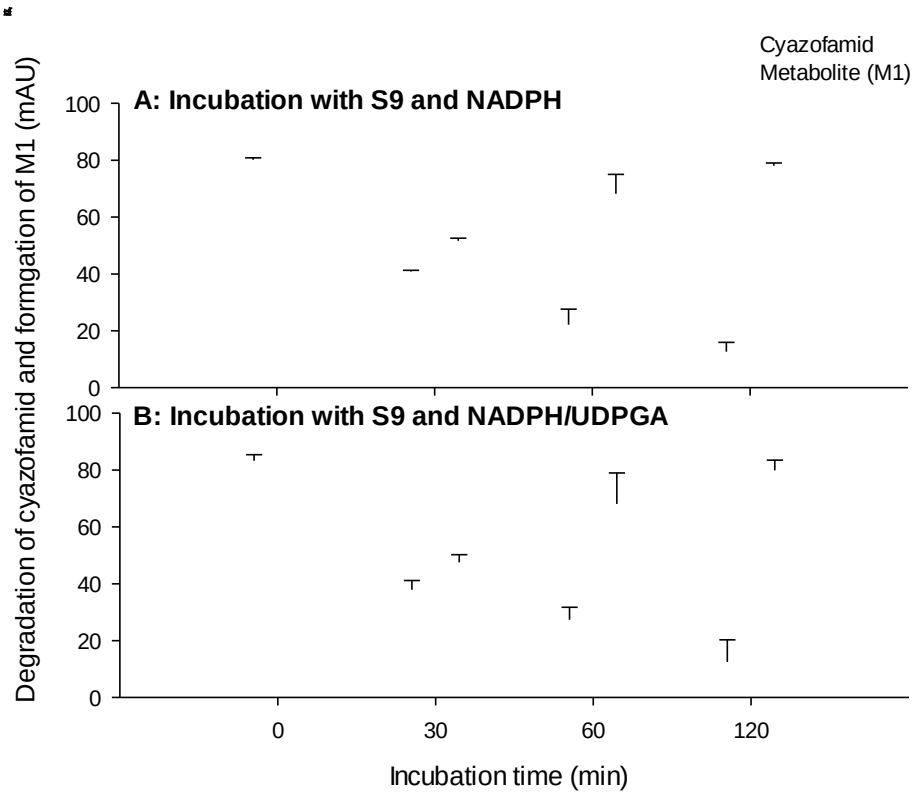


Figure 39. Degradation of cyazofamid and formation of metabolite M1 from cyazofamid by the metabolism with S9 fractions and NADPH or NADPH/UDPGA depending on incubation times (0, 30, 60, and 120 min) at 37℃. A : The incubation mixture with S9 and NADPH, B : The incubation mixture with S9 and NAPDH/UDPGA.

2.2.2 Phase II metabolism of cyazofamid by GST

Conjugation with glutathione (GSH), mediated by one of the glutathione S-transferase (GST), is important for organophosphorus compounds, DDT, HCH, and organothiocyanates (Abel et al. 2004a; Motoyama 1980). Members of the mu (μ) class GSTs are responsible for conjugating a wide variety of pesticides such as, the halogenated hydrocarbon insecticides (Hodgson 2010b).

Cyazofamid was presumed to be metabolized by glutathione conjugation, because it has one chlorine element. *In vitro* metabolism of cyazofamid was conducted to confirm the formation of GSH conjugate. After 120 min of incubation reaction with S9 fraction and GSH, approximately 70% of cyazofamid degraded and one metabolite M2 was formed (Figure 40 and 41) while no change of cyazofamid was observed in the control metabolism mixture consisting of cyazofamid, GSH, and denatured protein (heating at 80℃) for 120 min. Metabolite M2 was analyzed with LC-MS to be identified as CCIM instead of GSH conjugate.

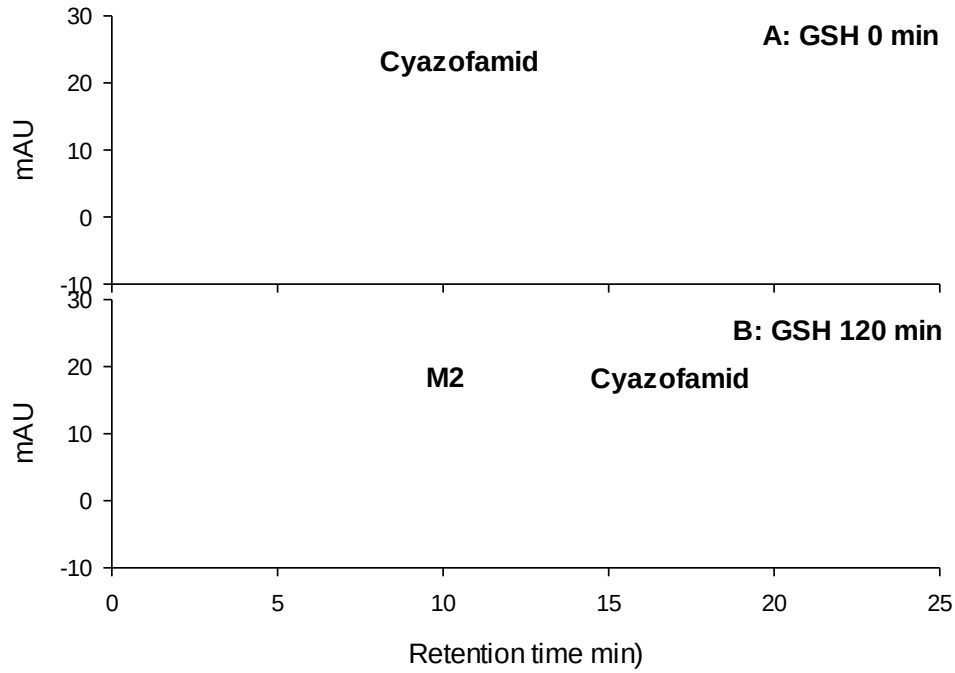


Figure 40. Formation of metabolite M2 from cyazofamid when it was incubated with S9 fractions and GSH for 0 min (A) and 120 min (B) at 37℃.

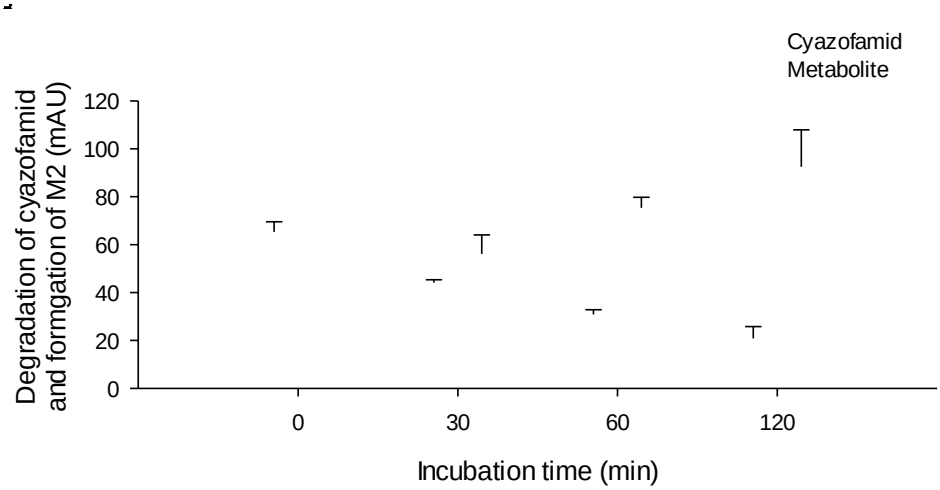


Figure 41. Degradation of cyazofamid and formation of metabolite M2 from cyazofamid by the metabolism with S9 fractions and GSH depending on incubation times (0, 30, 60, and 120 min) at 37℃.

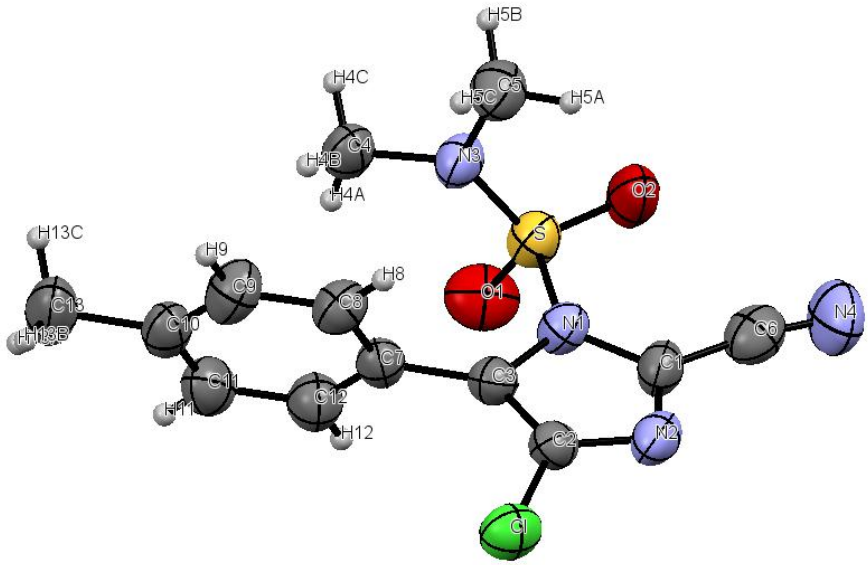
3. Determination of crystal structure for cyazofamid

An ORTEP diagram and the packing mode for cyazofamid are shown in Figure 42. Although good quality single crystal was not obtained, enough information to understand cyazofamid structure was available. However, further synthetic and structural studies will be necessary, though.

The imidazole group and benzene group were nearly perpendicular to each other.

The mean C-C single bond length is 1.466 Å, in good agreement with the expected range for a C-C single bond. Double bond length of carbon-carbon and bond length of benzene ring carbons are about 1.381 and 1.38 5Å, respectively. Bond angles of sp^2 hybrid of benzene ring are around 120°. The values of selected bond lengths, angles and torsion angles are given in Table 20. Figure 42 (B) shows the packing mode of molecules in the unit cell. Four molecules of cyazofamid were packed with a center of symmetry. The arrangement of the molecular units suggests that the packing could be stabilized by a network of intermolecular hydrogen bonding between the sulfate group and the methyl group of tolyl group, in addition between the cyano group and the methyl groups of sulfonamide group.

A



B

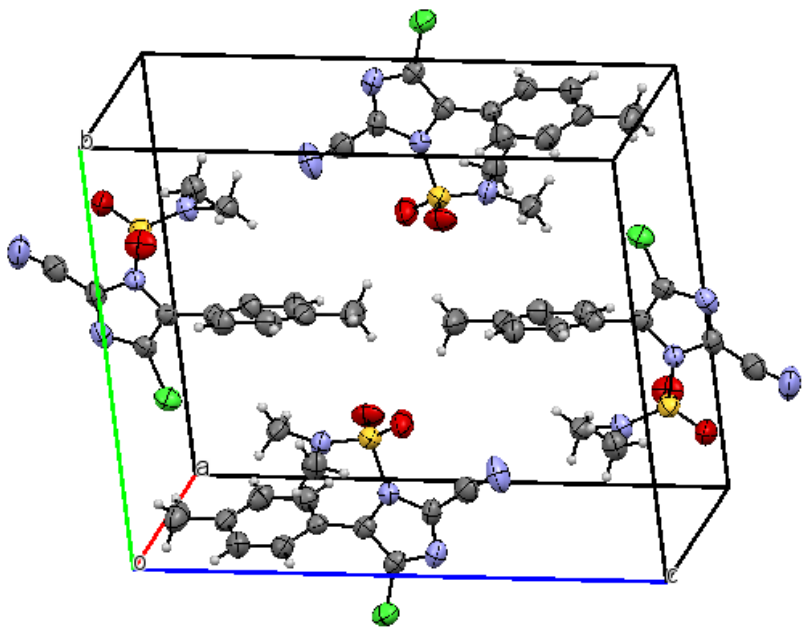


Figure 42. ORTEP diagram and numbering scheme (A) and packing diagram (B) for cyazofamid.

Table 20. Bond lengths (Å), angles (°) and torsion angles (°) for cyazofamid

Bond length (Å)		Angle (°)		Torsion angle (°)	
C1-N2	1.309(14)	N2-C1-N1	112.2(9)	N1-C1-C6-N4	-155(10)
C1-N1	1.393(12)	N2-C1-C6	122.9(10)	N2-C1-C6-N4	28(11)
C1-C6	1.416(17)	N1-C1-C6	124.9(11)	C6-C1-N1-C3	-178(1)
C2-C3	1.357(14)	C3-C2-N2	114.0(9)	C6-C1-N1-S	7(2)
C2-N2	1.360(13)	C3-C2-Cl	125.6(8)	N2-C1-N1-C3	-2(1)
C2-Cl	1.726(11)	N2-C2-Cl	120.4(8)	N2-C1-N1-S	-176.5(7)
C3-N1	1.397(13)	C2-C3-N1	103.7(8)	C6-C1-N2-C2	178(1)
C3-C7	1.471(13)	C2-C3-C7	130.0(9)	N1-C1-N2-C2	1(1)
C4-N3	1.480(13)	N1-C3-C7	126.2(9)	N2-C2-C3-C7	-177(1)
C5-N3	1.482(13)	N4-C6-C1	172.3(13)	N2-C2-C3-N1	-0(1)
C6-N4	1.156(15)	C8-C7-C12	119.5(9)	Cl-C2-C3-C7	4(2)
C7-C8	1.379(14)	C8-C7-C3	119.6(9)	Cl-C2-C3-N1	-179.4(7)
C7-C12	1.388(15)	C12-C7-C3	120.9(9)	C3-C2-N2-C1	-1(1)
C8-C9	1.392(15)	C7-C8-C9	119.8(10)	Cl-C2-N2-C1	178.4(8)
C9-C10	1.377(16)	C10-C9-C8	121.7(11)	C2-C3-C7-C8	74(1)
C10-C11	1.374(16)	C9-C10-C11	117.9(10)	C2-C3-C7-C12	-106(1)
C10-C13	1.512(15)	C9-C10-C13	121.3(11)	N1-C3-C7-C8	-102(1)
C11-C12	1.397(15)	C11-C10-C13	120.8(11)	N1-C3-C7-C12	79(1)
N1-S	1.741(8)	C10-C11-C12	121.7(11)	C2-C3-N1-C1	1(1)
O1-S	1.417(8)	C7-C12-C11	119.3(11)	C2-C3-N1-S	176.0(7)
N3-S	1.544(8)	C1-N1-C3	106.1(8)	C7-C3-N1-C1	177.9(9)
S-O2	1.417(8)	C1-N1-S	126.8(7)	C7-C3-N1-S	-7(1)
		C3-N1-S	126.9(7)	H4A-C4-N3-C5	180

Bond length (Å)	Angle (°)		Torsion angle (°)	
	C1-N2-C2	104.0(8)	H4A-C4-N3-S	-1
	C5-N3-C4	116.1(8)	H4B-C4-N3-C5	-60
	C5-N3-S	119.6(7)	H4B-C4-N3-S	118.7
	C4-N3-S	124.3(7)	H4C-C4-N3-C5	60
	O2-S-O1	120.3(5)	H4C-C4-N3-S	-121.3
	O2-S-N3	112.3(5)	H5A-C5-N3-C4	-179.9
	O1-S-N3	110.4(5)	H5A-C5-N3-S	1
	O2-S-N1	101.6(5)	H5B-C5-N3-C4	-60
	O1-S-N1	105.0(5)	H5B-C5-N3-S	121
	N3-S-N1	105.5(4)	H5C-C5-N3-C4	60
			H5C-C5-N3-S	-119
			C3-C7-C8-H8	1
			C3-C7-C8-C9	-179(1)
			C12-C7-C8-H8	-179
			C12-C7-C8-C9	1(2)
			C3-C7-C12-C11	178(1)
			C3-C7-C12-H12	-2
			C8-C7-C12-C11	-1(2)
			C8-C7-C12-H12	179
			C7-C8-C9-H9	179
			C7-C8-C9-C10	-1(2)
			H8-C8-C9-H9	-1
			H8-C8-C9-C10	179
			C8-C9-C10-C11	2(2)
			C8-C9-C10-C13	-179(1)
			H9-C9-C10-C11	-178
			H9-C9-C10-C13	1

Bond length (Å)	Angle (°)	Torsion angle (°)	
		C9-C10-C11-H11	178
		C9-C10-C11-C12	-2(2)
		C13-C10-C11-H11	-2
		C13-C10-C11-C12	178(1)
		C9-C10-C13-H13A	-180
		C9-C10-C13-H13B	-60
		C9-C10-C13-H13C	60
		C11-C10-C13-H13A	-0
		C11-C10-C13-H13B	119
		C11-C10-C13-H13C	-120
		C10-C11-C12-C7	2(2)
		C10-C11-C12-H12	-178
		H11-C11-C12-C7	-178
		H11-C11-C12-H12	2
		C1-N1-S-O1	107.4(9)
		C1-N1-S-N3	-136.0(9)
		C1-N1-S-O2	-18.6(9)
		C3-N1-S-O1	-66.2(9)
		C3-N1-S-N3	50.4(9)
		C3-N1-S-O2	167.8(8)
		C4-N3-S-N1	-110.4(8)
		C4-N3-S-O1	2(1)
		C4-N3-S-O2	139.7(8)
		C5-N3-S-N1	68.3(9)
		C5-N3-S-O1	-178.9(8)
		C5-N3-S-O2	-41.6(9)

4. Molecular docking of cyazofamid with rCYPs

The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure (Morris and Lim-Wilby 2008)

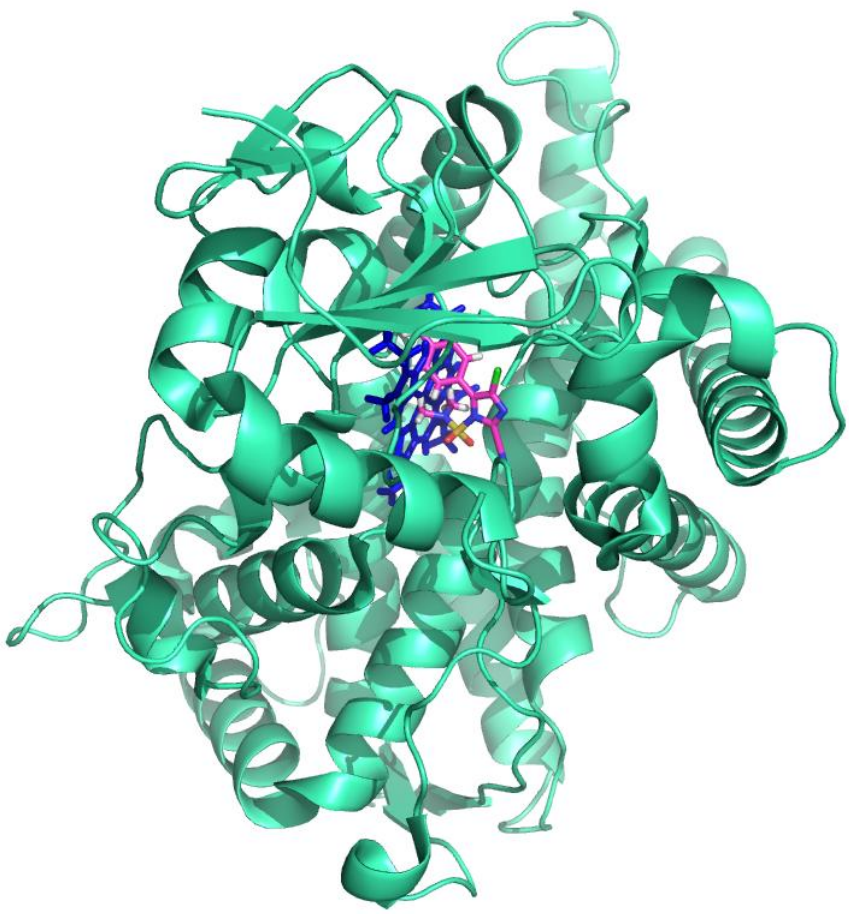
Because the CYP2B6, 2C9, and 2C19 resulted in metabolite formation with different rate, and CYP3A4 did not give any metabolites even though it is the most abundant CYP isoform in human liver, the crystal structure of cyazofamid were docked with rCYPs, 2B6, 2C9, 2C19, and 3A4 by Sybyl 7.3 software to elucidate the reactivity differences between them.

The distances between the carbon atom of methyl group (C13 in Figure 43 - 46), which was oxidized to form metabolite CCHS by responsible rCYPs, and iron center of a heme in CYPs were 4.949, 14.209, 14.266, and 15.397 Å for CYP 2B6, 2C19, 2C9, and 3A4, respectively. These results were correlated well with the result of metabolism reaction with rCYPs (the metabolic reaction ratio : CYP2B6 > 2C19 > 2C9, Figure 35).

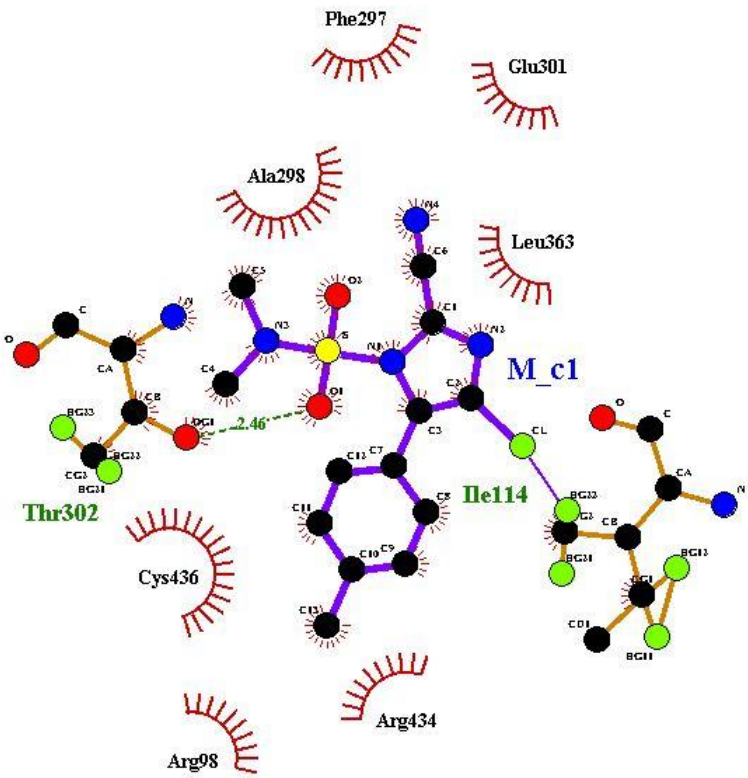
The binding pocket was determined from 3IBD, 1OG5, 4GQS, and 2J0D.pdb using the LigPlot program (Wallace et al. 1995). In CYP2B6, the binding pocket of 3IBD.pdb was found to include Arg98, Ile114, Phe297, Ala298, Glu301, Thr302, Leu363, Arg434, and Cys436, where Thr302, and Ile114 residues formed hydrogen bonds with cyazofamid and the others participated in the hydrophobic interactions (Figure 43). The residues of 1OG5.pdb neighboring the binding site in CYP2C9 were Ile99, Phe100, Ala103, Leu208, Gln214, Asn217, Ser365, Leu366, Pro367, Asn474, Phe476, and Ala477, where Gln214 and Asn217 residues formed hydrogen bonds with cyazofamid and the others participate in the hydrophobic interactions (Figure 44). In case of CYP2C19, the residue of 4GQS.pdb

neighboring the binding site were found to be Leu102, Val208, Ile205, Leu233, Gly296, Ala297, Glu300, Thr301, and Phe476, where all of the residues showed hydrophobic interactions with cyazofamid (Figure 45). However, in CYP3A4 (the binding pocket of 2J0D.pdb, Figure 46), those residues include only Leu211, Arg212, Phe213, and Phe220, where Leu211 and Phe220 interacted with cyazofamid by hydrophobic binding and Arg212 and Phe213 interacted with cazofamid by hydrogen bonds. These results also demonstrated the reason why CYP3A4 could not produce metabolite.

A



B


Phe297
Glu301
Ala298
Leu363
M_c1
2.46
Thr302
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Arg434
Arg98

C

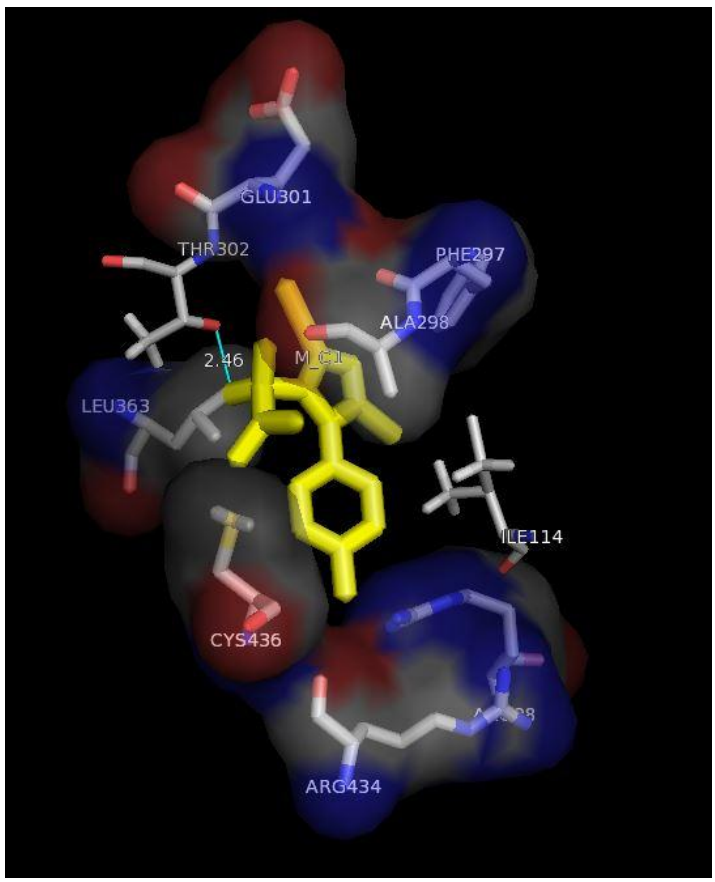
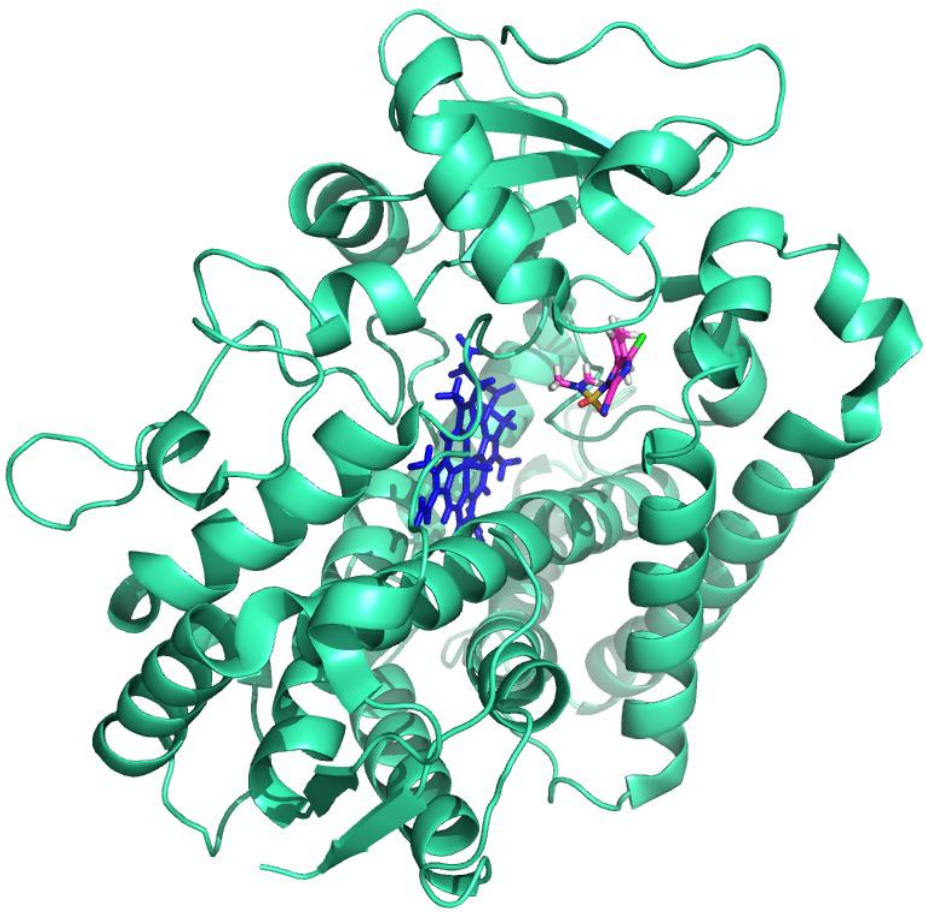
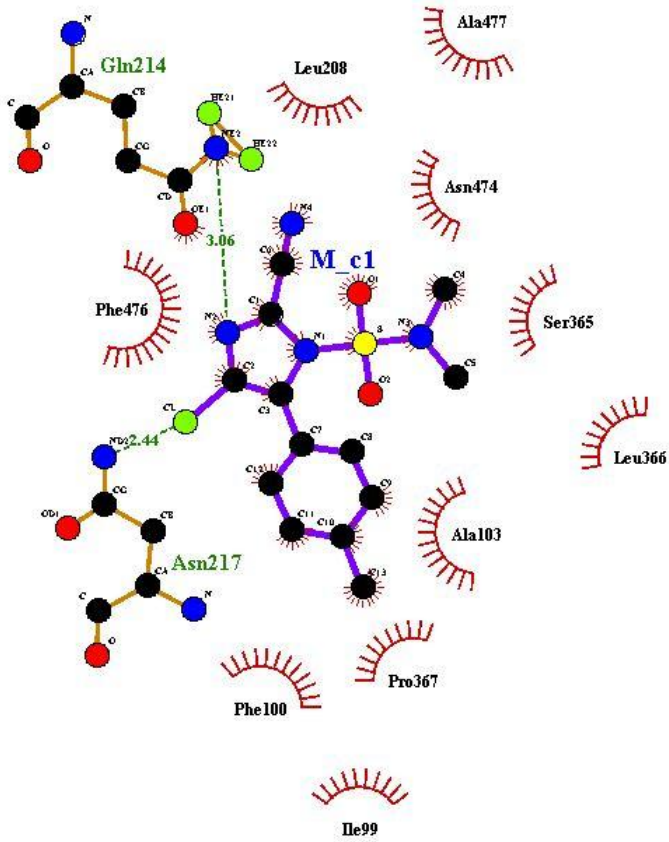


Figure 43. Interaction of cyazofamid and rCYP2B6. A : Protein structure with the heme group (a blue ball-and-stick) and cyazofamid (a pink carbon ball-and-stick), B : LigPlot analysis, C : Binging site of cyazofamid and rCYP2B6 by PyMOL.

A



B


Ala477
Gln214
Leu208
Asn474
3.06
M_c1
Phe476
Ser365
Leu366
2.44
Ala103
Asn217
Pro367
Phe100
Ile99

C

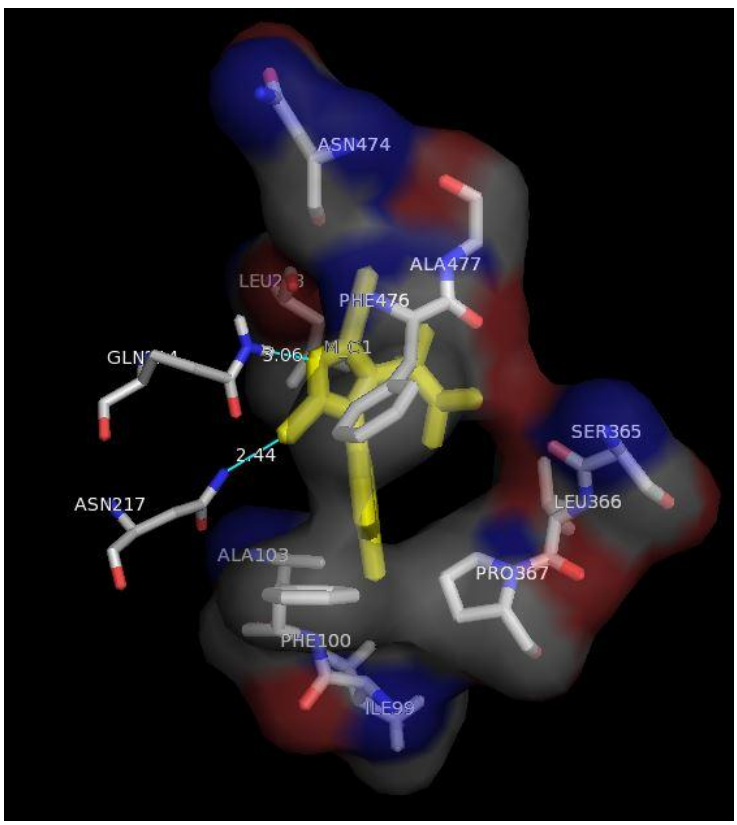
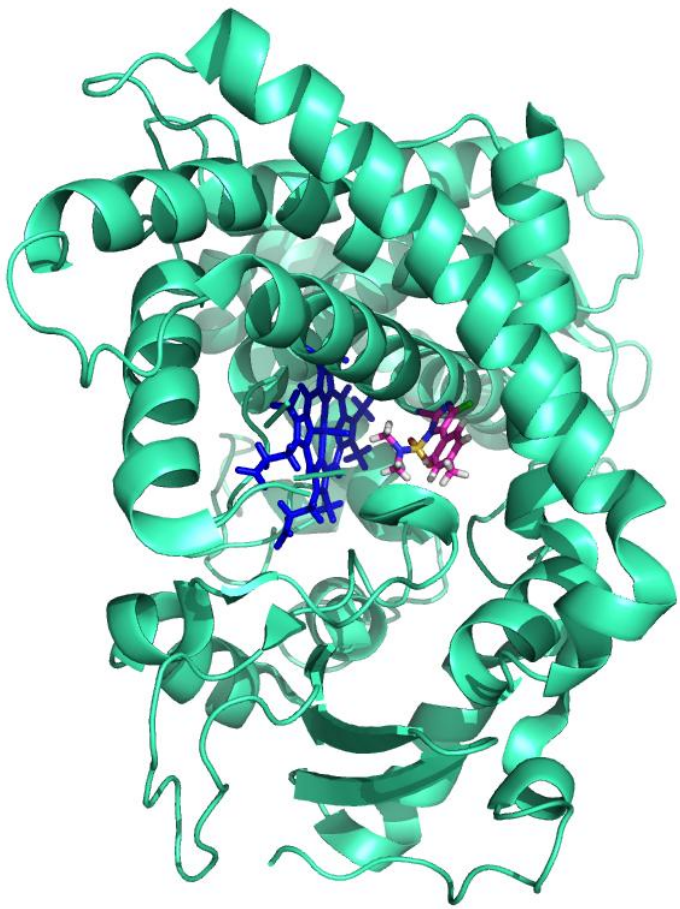
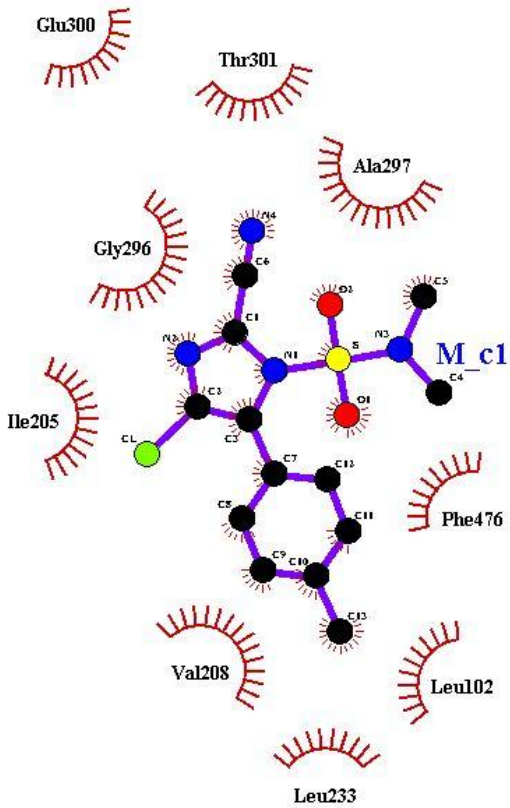


Figure 44. Interaction of cyazofamid and rCYP2C9. A : Protein structure with the heme group (a blue ball-and-stick) and cyazofamid (a pink carbon ball-and-stick), B : LigPlot analysis, C : Binging site of cyazofamid and rCYP2C9 by PyMOL.

A



B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Gly296
Ile205
Val208
Leu102
Leu233
Phe476
M_c1

C

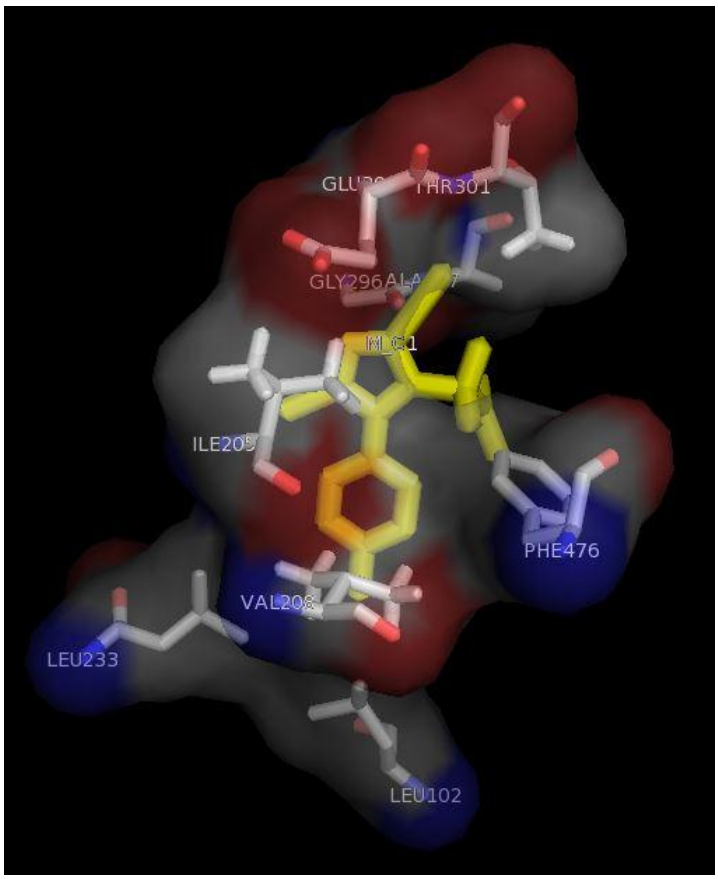
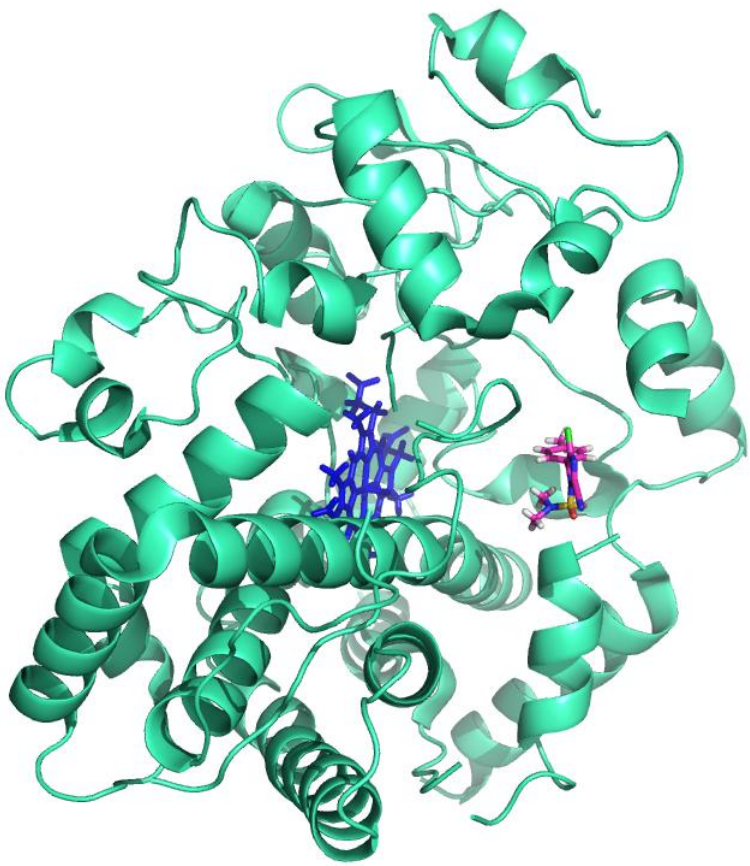
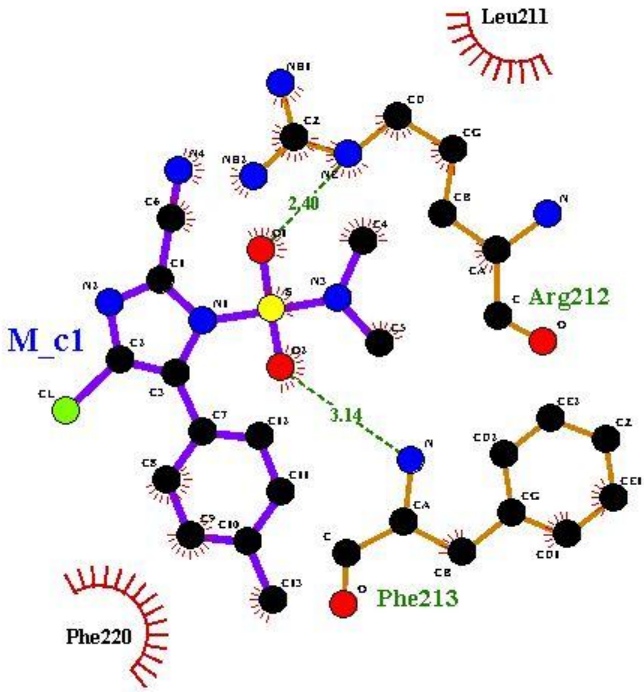


Figure 45. Interaction of cyazofamid and rCYP2C19. A : Protein structure with the heme group (a blue ball-and-stick) and cyazofamid (a pink carbon ball-and-stick), B : LigPlot analysis, C : Binging site of cyazofamid and rCYP2C19 by PyMOL.

A



B



C

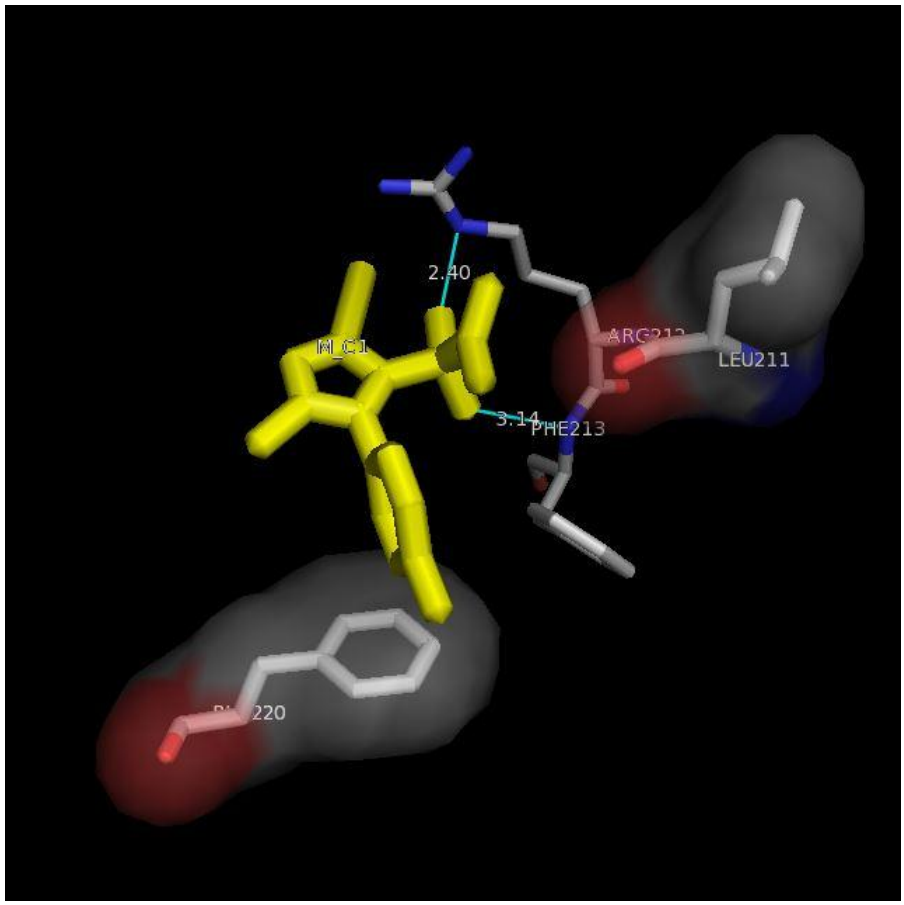


Figure 46. Interaction of cyazofamid and rCYP3A4. A : Protein structure with the heme group (a blue ball-and-stick) and cyazofamid (a pink carbon ball-and-stick), B : LigPlot analysis, C : Binging site of cyazofamid and rCYP3A4 by PyMOL.

IV. Conclusion

In vitro metabolism of cyazofamid was investigated by artificial gastrointestinal juices, human microsomes, tissue homogenate (S9 fractions). The structure of cyazofamid was determined by single crystal X-ray diffraction methods, molecular docking study was carried out for elucidation the reactivity differences between cyazofamid and rCYPs.

In vitro metabolism of cyazofamid by gastrointestinal juices produced GM, which is identified as CCIM using liquid chromatography-tandem mass spectrometry. Cyazofamid was observed to be stable in saliva. In the saliva + gastric juice, gastric juice and intestinal juices, approximately 8%, 17%, and 15% of cyazofamid was degraded, respectively. However, cyazofamid was considered not to be degraded by the artificial gastrointestinal juices, but to be degraded by simple aquatic environmental condition, because neutral and acidic control experiments also showed similar degradation pattern with the artificial juices.

In vitro metabolism of cyazofamid by HLMs produced M1, which was identified as CCHS. After optimization of incubation time, protein concentration, and cyazofamid concentration, kinetics parameters of cyazofamid were obtained from Michaelis-Menten plot. The estimated *Vmax* and *Km* values were calculated 1.963 pmol/min/mg proteins and 3.627 μM, respectively. When M1 formation from cyazofamid was investigated using recombinant CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, the formation of M1 was observed only in CYP2B6, 2C9, and 2C19. The V_{max} values were 0.011, 0.002, and 0.005 pmol/min/mg

proteins for CYP2B6, 2C9, and 2C19, respectively while 1.447, 0.507, and 0. 987 μM of K_m values were calculated for CYP2B6, 2C9, and 2C19, respectively. The structure of cyazofamid was determined using X-ray crystallography, predicted interactions with rCYPs (2B6, 2C9, 2C19 and 3A4) by molecular docking study. In the same manner *in vitro* metabolism of cyazofamid using HLMs, molecular docking study was related with CYP2B6, 2C9, and 2C19, not CYP3A4. To investigate conjugated metabolite, *in vitro* metabolism of cyazofamid by S9 fractions was carried out; however no metabolite was observed by the metabolism study of UGTs while CCIM was observed by that of GSTs.

Therefore, cyazofamid was oxidized to CCHS in the HLMs and *C. elegans* metabolic system, and it could be degraded to CHCN and further oxidized to CCBA.

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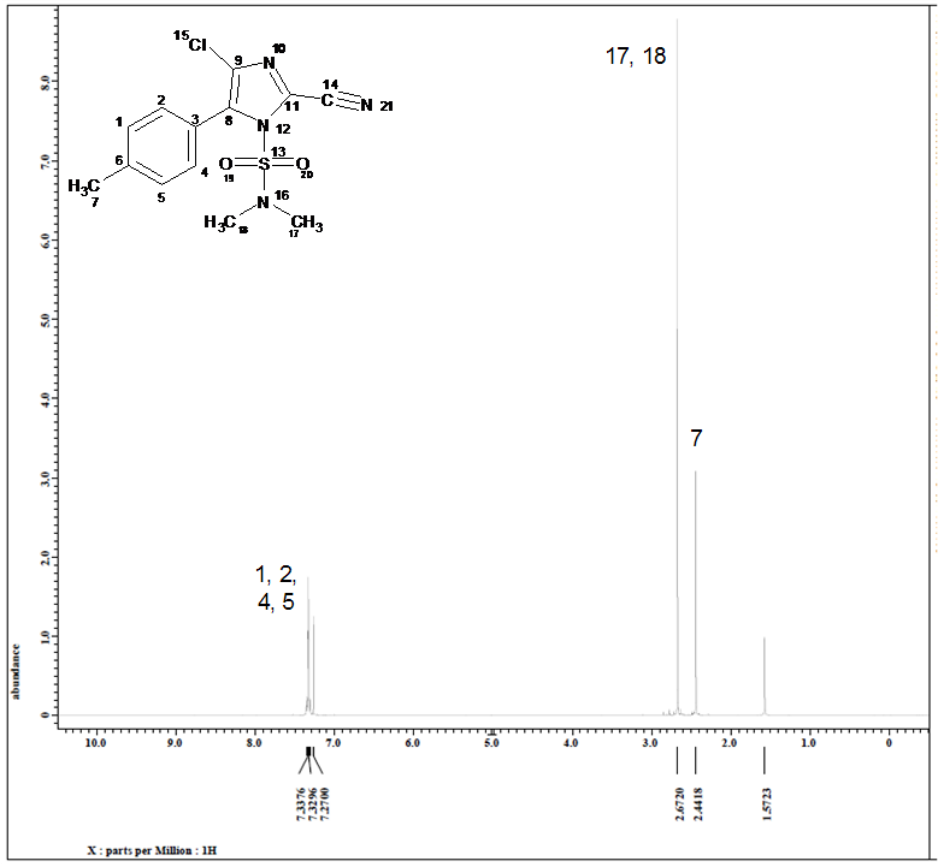
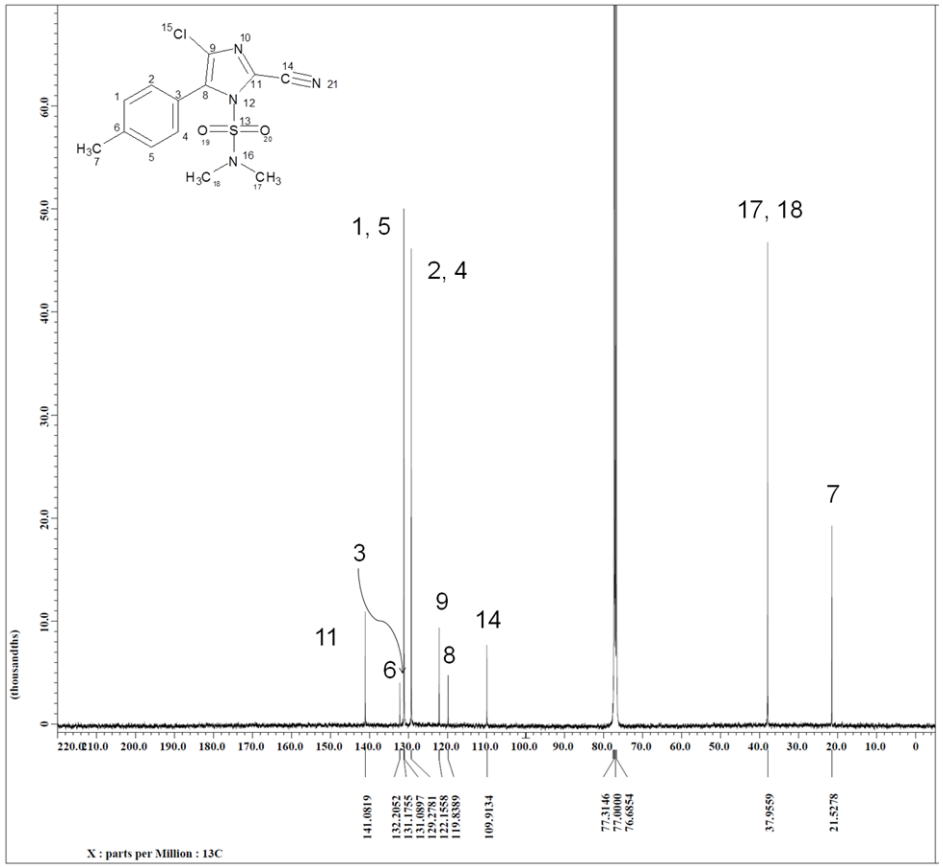
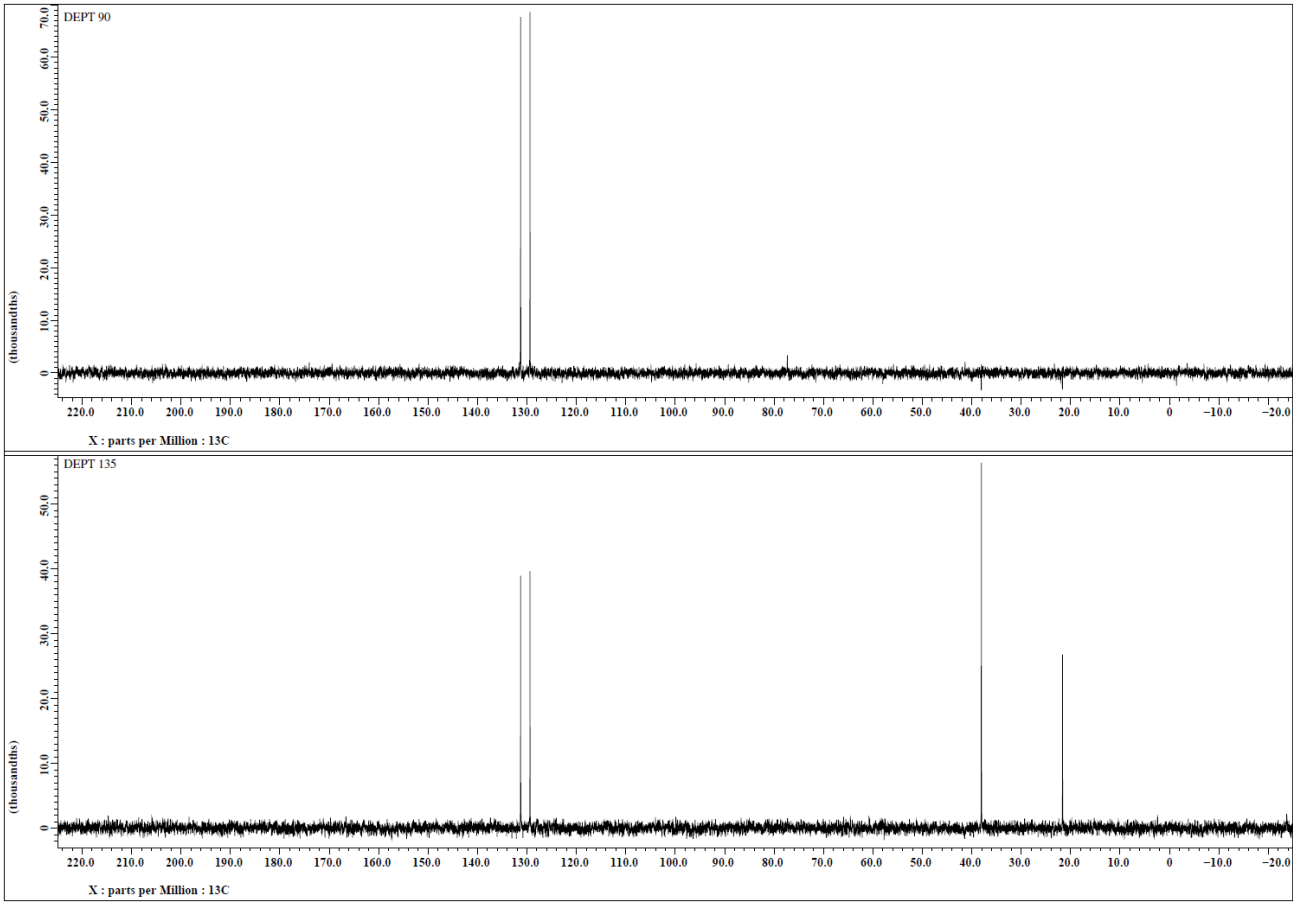
APPENDICES



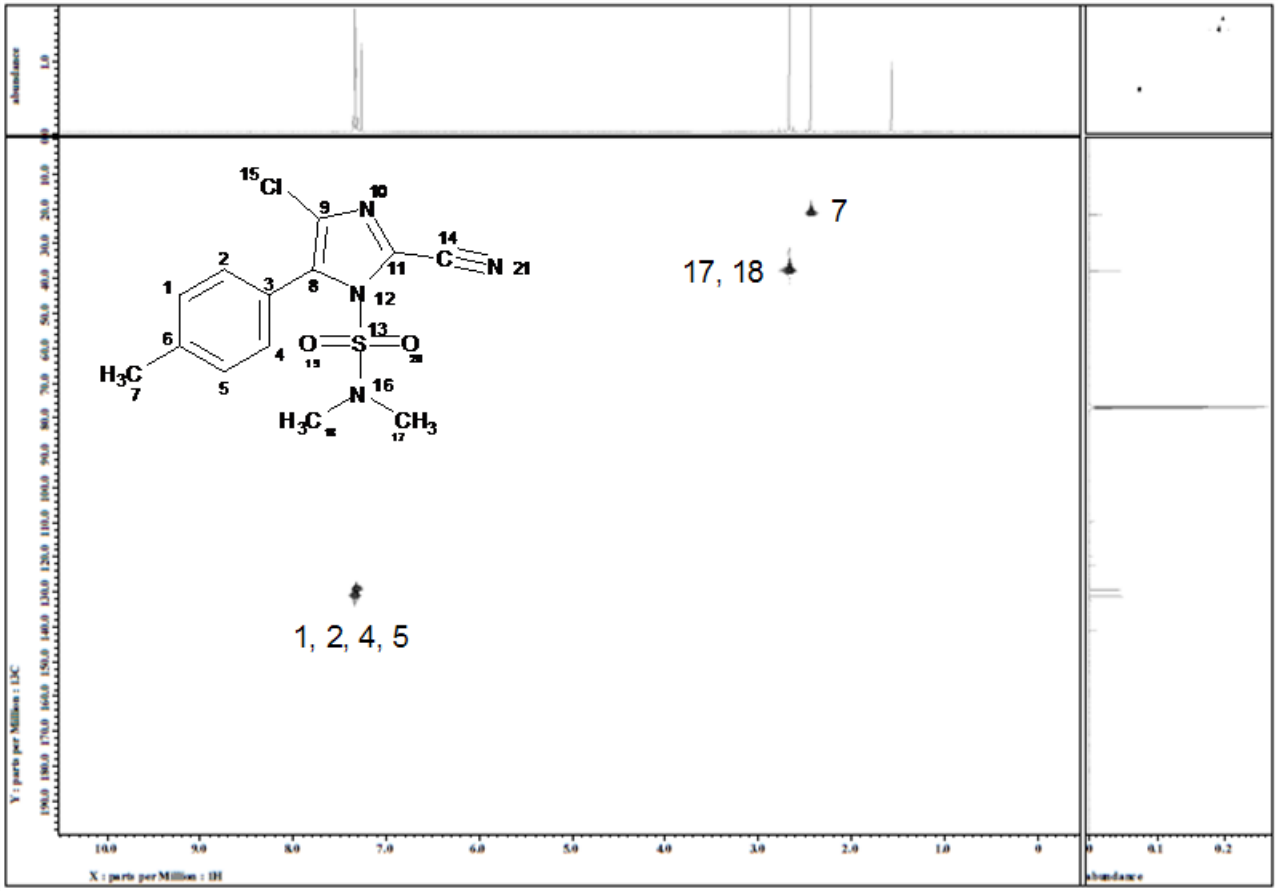
Appendix 1. ^{1}H NMR spectrum for cyazofamid.



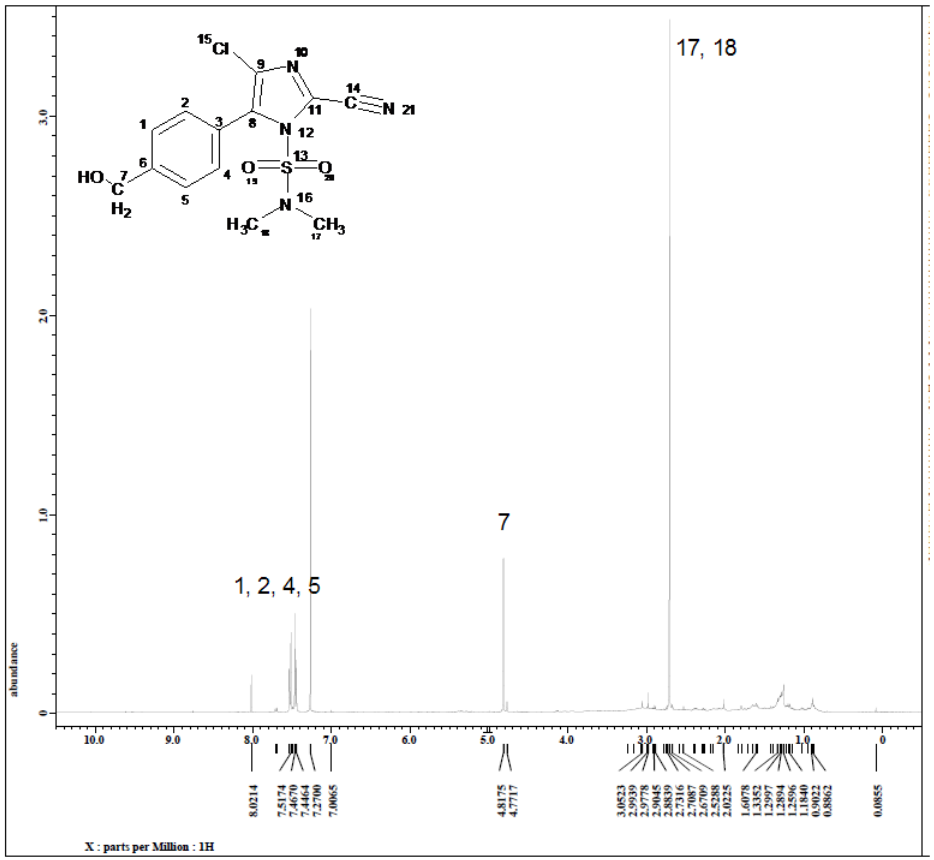
Appendix 2. ^{13}C NMR spectrum for cyazofamid.



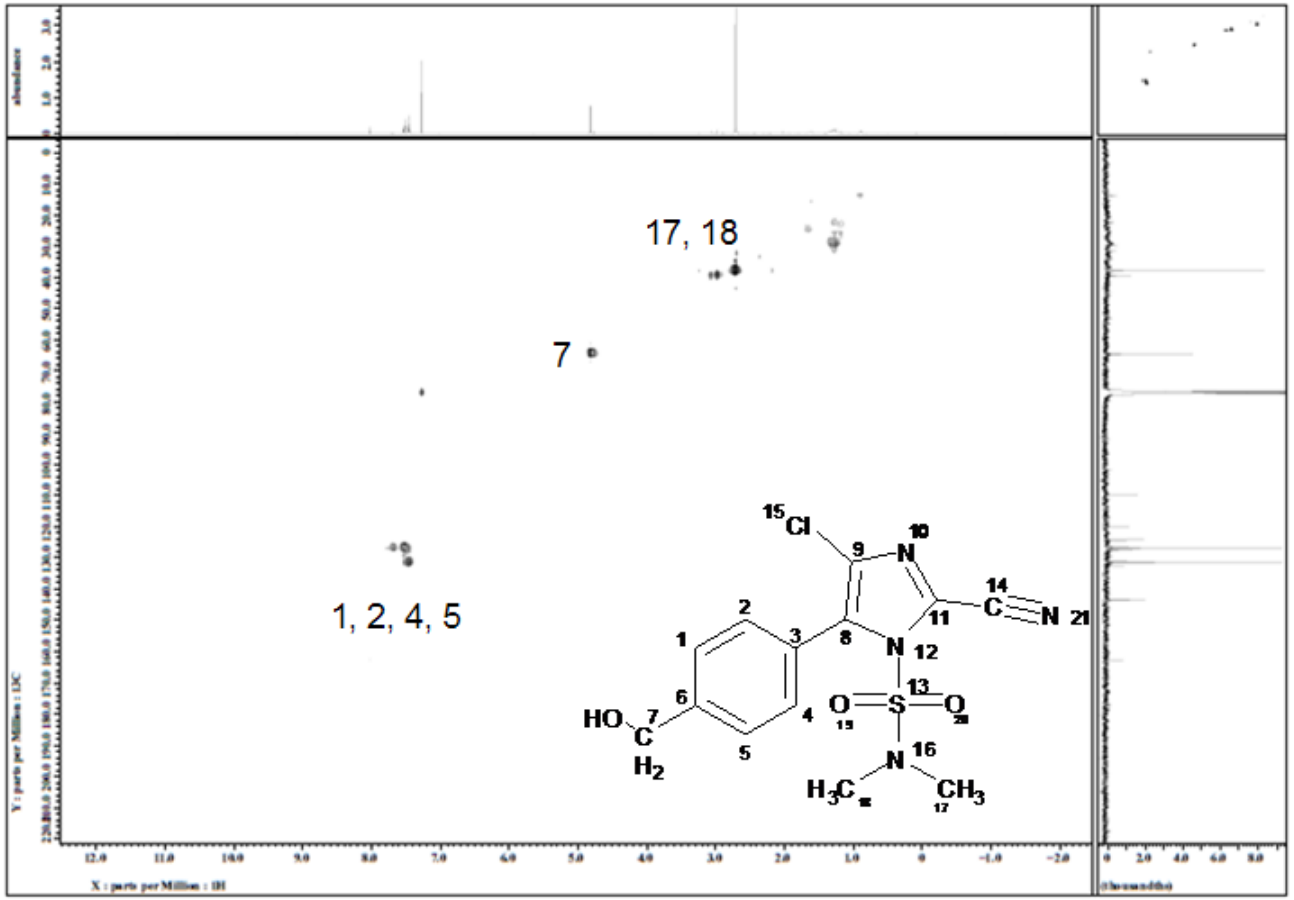
Appendix 3. DEPT NMR spectrum for cyazofamid.



Appendix 4. 2D ^{1}H-^{13}C HSQC spectrum for cyazofamid.



Appendix 5. ^{1}H NMR spectrum for CM1 from *C. elegans* incubation.



Appendix 6. 2D ^{1}H-^{13}C HSQC spectrum for CM1 from *C. elegans* incubation

ABSTRACT IN KOREAN

토양 곰팡이 *Cunninghamella elegans* 및 인체 간 마이크로좀에 의한 살균제 Cyazofamid의 대사 및 잔류분석법 연구

서울대학교 대학원
농생명공학부
이 혜 리

국문초록

Cyazofamid는 다양한 채소 및 과일의 병을 방제하는 설폼아미드계의 살균제이다. 본연구에서는 cyazofamid와 그 대사체를 HPLC와 lc-MS/MS를 이용하여 분석법을 확립하고, 토양 곰팡이 *Cunninghamella elegans*와 *in vitro* 인체 모사 시스템의 대사연구를 통하여 cyazofamid의 생물환경 중 변화에 대하여 연구하였다. 대표작물로 사과, 감귤, 배추, 고추, 감자, 대두를 선정하였다. Cyazofamid의 정량한계(LOQ)와 분석법의 정량한계(MLOQ)는 HPLC 분석법에서 각각 2 ng 및 0.02 mg/kg이었으며, 회수율은 세 농도에서 75 - 98.5%이었다. 또한 cyazofamid와 그 대사체, CCIM(4-chloro-5-*p*-tolylimidazole-2-carbonitrile)을 QuEChERS법으로 전처리하고, 작물 외에 토양 및 수질시료를 포함하여 빠르고 효율적인 LC-MS/MS 분석법을 확립하였다. LC-MS/MS 분석법에서의 cyazofamid의 CCIM의 분석법의 정량한계는 작물 및 토양에서 2 ng/g 및 5 ng/g이었고, 수질에서는 각각 0.02 ng/mL, 0.05 ng/mL이었다. 이때의

작물과 환경시료 중 cyazofamid와 CCIM의 회수율은 각각 80.2% – 105.1%와 75.1% – 99.1%이었다. Cyazofamid는 인공위액 및 장액에 의하여 CCIM으로 대사되었다. 또한 토양 곰팡이 *C. elegans*에 의하여 대사되어 새로운 대사체인 CCHS(4-chloro-2-cyano-5-(4-(hydroxymethyl)-phenyl)N,N-dimethyl-1H-imidazole-1-sulfonamide)를 확인하였으며, 이는 점차 대사되어 CHCN(4-chloro-5-(4-hydroxymethylphenyl)imidazole-2-carbonitrile)과 CCBA(4-(4-chloro-2-cyanoimidazole-5-yl)-benzoic acid)로 분해되었다. 생체 외 인체 간 마이크로좀 대사연구를 통하여 CCHS가 cyazofamid의 유일한 대사체임을 확인하였고, 10가지의 재조합 CYPs(rCYP)을 이용하여 CCHS의 생성을 살펴본 결과, 27.4%, 66.1%, 6.5%의 기여도로 CYP2B6, 2C9, 2C19에 의한 대사반응이 진행됨을 밝혔다. Cyazofamid의 결정구조와 CYP2B6, 2C9, 2C19, 3A4의 molecular docking 실험결과, rCYP의 heme과 산화반응이 진행되는 탄소간의 거리가 대사반응 순서와 상관성이 있음을 확인하였다. 2차대사 연구결과, UGT(UDP-glucuronosyl transferases)에 의한 콘쥬게이션 대사체는 확인되지 않았으나, GST(glutathione S-transferases)에 의한 CCIM을 대사체로 확인하였다.

Keywords : *Cunninghamella elegans*, Cyazofamid, HPLC, 인체 간 마이크로좀, LC-MS/MS, 대사, QuEChERS

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