Assessing Near-Field Naturally Occurring Isothiocyanates Emissions after Mustard Green Manure Cover Crop Incorporation

By

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The members of the Committee appointed to examine the thesis of Donna M. Trott find it satisfactory and recommend that it is accepted.

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Assessing Near-Field Naturally Occurring Isothiocyanates Emissions after Mustard Green Manure Cover Crop Incorporation

Abstract

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An assessment of near-field naturally occurring isothiocyanate air emissions was conducted in Benton County, Washington, in the fall of 2008. The purpose of the study was to assess and quantify selected natural isothiocyanate (NITC) compounds in emissions from green manure soil amendments and to develop a method to detect and quantify multiple NITCs in ambient air for human inhalation risk assessment. An analytical method was developed specific to three selected NITC compounds commonly known to be associated with the mustard green manure cover crop: allyl-, benzyl-, and 2-phenethyl isothiocyanate. Air samples were taken in and around a crop circle sown with high biomass-producing brassicas typically used in the Columbia Basin of Washington state before, during and after cover crop incorporation. A total of eight discrete intervals of approximately four hours each were evaluated for assessing potential human inhalation exposure. Emissions of the selected NITCs were quantified or detected from samples taken before and during cover crop incorporation and when field operations ceased. Allyl isothiocyanate and benzyl isothiocyanate concentrations were

observed during the period of active cover crop incorporation, while phenethyl isothiocyanates were detected below the analytical method's Limit of Quantification (LOQ). The selected NITCs were not detected during the pre-incorporation period or on post green manure incorporation days 1 through 3. On the fourth day, allyl isothiocyanate was again observed at levels above LOQ. This observation occurred on a day with light precipitation and suggests increased surface moisture may have enhanced formation and emission of NITCs from previously unhydrolyzed glucosinolates residing on the incorporated land surface. We found the time weighted average (TWA) amounts of allyl- were greater than the benzyl- and phenethyl isothiocyanates by an order of magnitude. The maximum observed TWA air concentration for the allyl-, benzyl-, and phenethyl isothiocynanates were observed during soil incorporation and were respectively 47, 1.0, and 0.1 ppb. Values for acute or chronic inhalation exposure for these NITCs are not available in public databases.

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Table of Contents

TABLES

Figures

Acronyms and Abbreviations

Allyl Isothiocyanate	AITC
Analytical Summary Report	ASR
Benzyl Isothiocyanate	BITC
Environmental Protection Agency	EPA
Food and Environmental Quality Laboratory	FEQL
Glucosinolate	GSL
Isothiocyanate	ITC
Kilopascals	kPa
Lethal Dose	LD
Limit of Detection	LOD
Limit of Quantitation	LOQ
Methyl Isothiocyanate	MITC
Natural Isothiocyanate	NITC
Nitrogen Phosphate Detector	NPD
No Observable Adverse Effect Level	NOAEL
Oil of Mustard Seed	OMS
Oxazolidinethione	OZT
Parts per billion	ppb
Phenethyl Isothiocyanate	PEITC

Temporary Emergency Exposure Limits	TEELs
Thermal Specific Detection	TSD
Time Weighted Average	TWA
Washington State University	WSU

Preface

A crop grown to be tilled back into the ground is called a green "manure" since it provides nutrients and organic matter to improve soil tillage. Mustard and other plants from the Brassicaceae family are currently being grown as cover crops for the biocidal compounds produced as a de novo response to predation or tissue disruption. Naturally occurring isothiocyanates (NITCs) from Brassicaceae tissues can provide biofumigation effects in the soil and aid an integrated pest management program.

This study was designed to provide quantitative analysis of three selected naturally occurring isothiocyanates (NITCs) that are typically found in mustard green manure, and to develop a rugged and reliable method for analyzing multiple NITCs in single samples using Gas Chromatography. The purpose of this study was to estimate near-field NITC emissions after mustard green manure cover crop incorporation and to provide preliminary data for understanding if human risks may occur from inhalation exposure to naturally occurring aliphatic and aromatic isothiocyanates (ITCs). The study anticipates the need for data that has not been compiled previously for use in determining possible non-target and off-site human inhalation of NITCs from large-scale use of green manure technology.

The data for this study was collected in and around an irrigated crop circle under mustard cultivation. The pest management strategies and crop rotation used on this farm are typical of the region. In the fall of 2008, we sampled the air in and around the field before, during, and four days following cultivation of two species of intermixed mustard, *Brassica juncea* and *Sinapis alba*. We developed an analytical method specifically to target multiple

NITC compounds, selecting 3 NITCs commonly known to be formed after green manure soil incorporation: allyl-, benzyl-, and 2-phenethyl isothiocyanate. The reader will find the compiled data from field sampling and method development in the Appendix to this document.

Chapter 1 addresses the literature concerning topics relevant to the use of green manures: the glucosinolate-myrosinase system and isothiocyanate production particular to the Brassicaceae family; the biocidal activity of isothiocyanates; a review of biofumigation, glucosinolate degradation emissions, and soil chemistry; and possible hazards to bystanders as currently understood. My concluding remarks summarize the applicability of the literature review to the stated purpose of this thesis.

Chapter 2 is being developed for submission to the Bulletin of Environmental Contamination and Toxicology, written in the format specified for the journal. This chapter contains a description of the study, its method and results. A discussion of the results, conclusions and future work is presented in Chapter 3.

Appendix A is an Analytical Summary Report (ASR) which will be posted on the Washington State University's pesticide air monitoring website later in 2009 (http://www.doh.wa.gov/ehp/Pest/drift.htm). This summary is a detailed description of the 2008 field emissions monitoring study and analytical methods from initiation to completion for all aspects of field/analytical work. This appended ASR was submitted to the Washington State Department of Health for their consideration of possible inhalation risks from exposure to the three examined isothiocyanates from this field study.

Chapter 1

Introduction

In Washington state, an increasingly common practice is to use mustard, radish and other crops from the Brassicaceae family as green manure in a rotation with crops such as wheat and potatoes (McGuire, 2003). A crop grown to be tilled back into the ground is called a green "manure" since it provides nutrients and organic matter to improve soil tillage. The benefit of using Brassicaceae is that this family can emit volatile sulfur-based allelotoxins, notably isothiocyanates (ITCs), as a localized, *de novo* response to the disruption of plant tissue by predation or by cultivation (Bones and Rossiter, 1996; Rosa et al., 1997; Table 1). The disrupted mustard tissue produce "biofumigation" activated compounds that can control a range of soil-borne fungal and bacterial plant pathogens.

Isothiocyanates are one of several aglucan sulfur chemicals resulting from the hydrolysis of β-thioglucosides called glucosinolates (GSLs), secondary metabolites kept in reserve within vacuoles of specialized cells as a localized pathogen response system. When cell membranes are disrupted, sequestered glucosinolates can encounter the glucosidase enzyme myrosinase and produce biocidal compounds designed to deter further attack (Brown and Morra, 2005; Figure 1).

Isothiocyanates as a group are primary mucous membrane irritants. Toxicity and volatility increase inversely with the length of the aliphatic side chain (Mithin, 2001, Sarwar et al, 1998). The GSL cognate form of the simplest ITC, methyl isothiocyanate (MITC), has not been found in Brassicaceae but is a distinctive component of the closely related Capparaceae (Fahey et al., 2001); both families are members of the order Capparales. MITC

Table 1: Members of the Brassicaceae family of plants cultivated and/or used for scientific study: Botanical and Common Names

Botanical Name	**Common Names**
Brassica juncea	Oriental Mustard, Brown Mustard
Sinapis alba *Brassica hirta*	White Mustard, Yellow Mustard
Brassica napus	Rapeseed (Canola)
Brassica nigra	Black Mustard
Brassica rapa *Brassica campestris*	Turnip, Turnip Rape, Chinese Cabbage
Brassica oleracea	Cabbage, Broccoli, Cauliflower, Kale, Brussel Sprouts
Raphanus sativa	Radish
Armoracea rusticana	Horseradish
Arabidopsis thaliana	Mouse Ear Cress
Camelina sativa	False Flax, Gold of Pleasure
Lepidium sativum	Garden Cress
Eruca vesicaria	Rocket Salad
Diplotaxis erucoides	White Wallrocket
Crambe abyssinica	Crambe

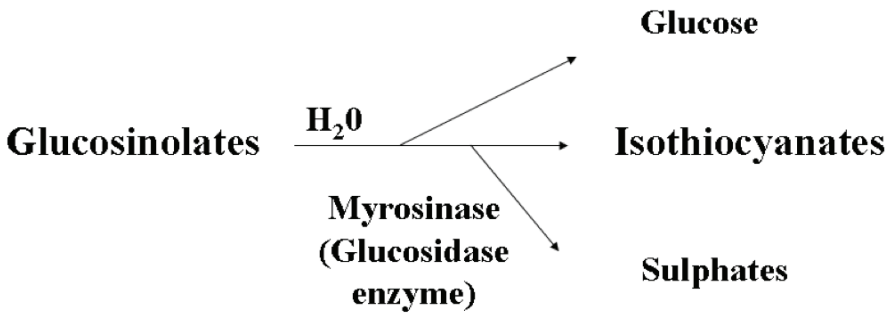


Figure 1. The Glucosinolate-Myrosinase System: Glucosinolate compounds are transformed to the allelotoxic isothiocyanates by hydrolysis, catalyzed by enzymes called Myrosinases

is the biologically active hydrolysis product of the pesticide metam-sodium (*metam; sodium methyl-dithiocarbamate*). Currently, large producers of potato, mint, carrots, onion and tree fruit in the Pacific Northwest rely on pre-plant metam sodium soil fumigation, with over 10 million pounds of metam-sodium being applied annually (PMSP 2002; NASS 2006). With its high vapor pressure (2.5-2.8 kPa at 20° C), substantial MITC surface emission and subsequent off-target air mass movement can occur (Lee et al., 2002; Li et al., 2006; Sullivan et al., 2004) and may exceed human exposure Levels of Concern (Merriman and Hebert, 2007). The naturally produced isothiocyanates from Brassicaceae green manures may in certain instances replace or can reduce the need for expensive, hazardous synthetic fumigants, supplying an additional tool for integrated pest management.

Section I: Brassicaceae, Glucosinolates and Isothiocyanates

"Biofumigation," the term coined by Kirkegaard and Sarwar (1998), describes the suppression of soil-borne pests and diseases arising specifically from glucosolinate (GSL) hydrolysis products released from incorporated tissues of cover crops such as mustard. These crops, from the Brassicaceae family, contain glucosinolate compounds that are hydrolyzed to the allelotoxic isothiocyanates (ITC), catalyzed by glucosidase enzymes known as myrosinases, as illustrated in Figure 1.

The Brassicaceae family (also known as Crucifereae) contains 350 genera and 2500 species (Rosa et al., 1997). A few examples are listed in Table 1. Many are grown as oilseed and vegetable crops, condiments, and forage, and their distinctive tastes are due to the glucosinolates and degradation products particular to the species. Plants in this family are usually fast growing, originally from temperate climates, and opportunistic. *Arabidopsis thaliana* (common name mouse ear cress) belongs to Brassicaceae as well as the cabbage, radish, cress, camelina, rapeseed, turnip, horseradish and mustard genera.

Glucosinolates are β-thioglucosides with a sulphonated oxime moiety and a variable side-chain derived from amino acids (Mithen, 2001; Figure 2). Glucosinolates are relatively nonreactive, stable, water soluble compounds stored in vacuoles. Hydrolysis is catalyzed by myrosinase enzymes that coexist in nearby myrosin cells that are distributed throughout the plant (Rosa et al., 1997; Rask et al., 2000; Fahey et al., 2001). Myrosin cells contain myrosin

grains that have been shown to form a continuous reticular system within the cytoplasm of the cell and denoted the myrosin body (Andreasson et al., 2001). As glucosinolate vacuoles do

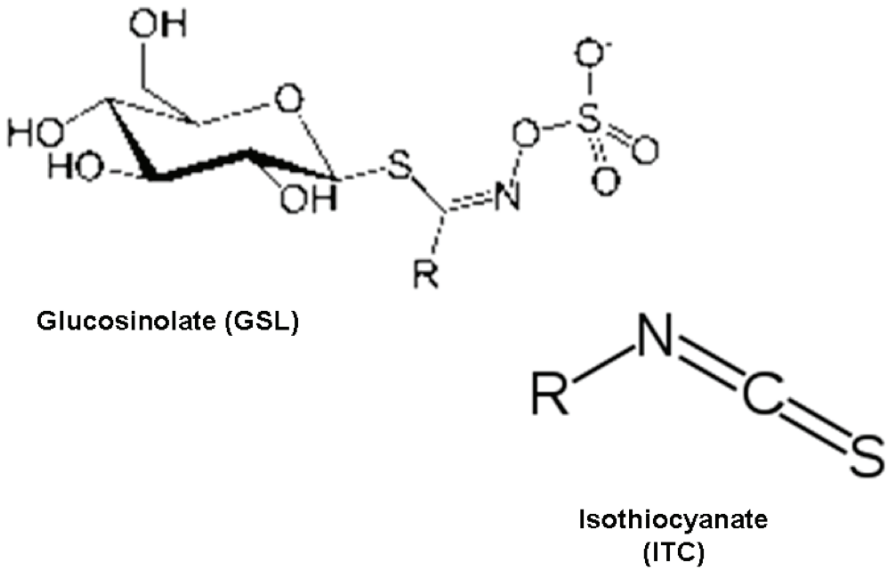


Figure 2: Glucosinolates are β-thioglucosides with a sulphonated oxime moiety and a variable side-chain. Isothiocyanates are the cognate hydrolysis product.

not appear to be present within myrosin cells, intercellular rather than intracellular separation occurs. Disrupting cellular tissues allows glucosinolates and myrosinase to mix, resulting in the catalyzed release of glucosinolate degradation products. It is only upon wounding, cell membrane rupture, herbivory, or other tissue damage, that the physical separation of the myrosinase from its glucosinolate substrate is overcome. Hydrolysis of glucosinolates results in release of glucose, sulfate and an unstable aglucone that spontaneously decomposes into different compounds of various toxicity including isothiocyanates, nitriles, epithionitriles,

oxazolidines and thiocyanates (Bones and Rossiter, 2006) as well as amines, depending on the reaction conditions (e.g. pH) and the nature of the glucosinolate side chain (Chew, 1988). Myrosinase enzymes cleave the sulfur-glucose bond regardless of either the enzyme or GSL substrate source. However, the particular enzyme and glucosinolate substrate influence reaction kinetics (MacLeod and Rossiter, 1985).

Fahey et al. (2001) identifies over 120 side chain structures for glucosinolates from 297 plants. The most numerous contain either straight or branched carbon chains, olefins, and many have hydroxyl or carbonyl or sulfur groups. The aliphatic glucosinolates are derived from methionine and chain-elongated homologues, and phenylalanine is the basis for aromatic glucosinolates. Alanine, leucine, isoleucine, valine, tyrosine and tryptophan are also precursors for glucosinolates (Fahey et al., 2001). The genetics of biosynthesis and modification of side-chain structure differ among the glucosinolate types (Raybould and Moyes, 2001). Aliphatic glucosinolate side-chain structure is determined by elongation of the initial side-chain and subsequent modifications, such as oxidation, desaturation and hydroxylation. (Mithen, 2001). Most glucosinolates are found in plants of the Order Capparales, which includes Brassicaceae and Capparaceae (Halkier and Gershenzon, 2006; Fahey et al., 2001). Methyl glucosinolate, the hydrolysis product of metam sodium, does not occur in Brassicaceae, although it is a characteristic component of the closely related Capparaceae. Glucosinolates with glycosylated R-groups appear in the families Resedaceae and Moringaceae, also of the Order Capparales. Glucosinolates also occur in Caricaceae (papaya) of the Order Violales (Fahey et al., 2001).

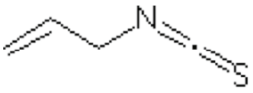
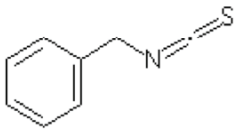
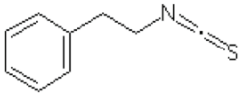
Among the degradation products, isothiocyanates are generally reported as the most biologically active and are recognized as broad-spectrum biocides (Brown and Morra, 1997). Only the aliphatic and aromatic glucosinolates form isothiocyanates upon hydrolysis (Bones and Rossiter, 1996; Rask et al., 2000). Conditions favoring isothiocyanate formation are roughly neutral pH, warm temperatures and presence of sufficient water. At a more acidic pH as in ferric soils, lower temperatures and drier conditions, the less biologically active nitriles predominate (Gil and MacLeod, 1980; Rosa et al., 1997). The quantity of GSLs is affected by aging and total GSL concentration decreases with plant age (Sarwar and Kirkegaard, 1998).

The structurally simpler aliphatic isothiocyanates have been shown to have greater biological activity under field conditions compared to the structurally more complex aromatic ITCs (Matthiessen and Kirkegaard, 2006). At 25 °C, the aliphatic allyl isothiocyanate and the aromatic benzyl- and 2-phenylethyl isothiocyanates (AITC, BITC and PEITC; Table 2) are 5, 1700 and 2300 times less volatile than methyl isothiocyanate, respectively (Boublik et al., 1984). Although many of the aromatic ITCs can have up to 70-fold greater contact toxicity than MITC, their lower volatility and greater soil partition coefficients renders these complex ITCs less biologically toxic in the soil substrate (Matthiessen and Kirkegaard, 2006). *Brassicas spp*. that can produce high amounts of short-chain aliphatic isothiocyanates will likely have the greatest potential for suppression of soil borne pathogens (McGuire, 2003),.However, Sarwar et al. (1998) noted aromatic ITCs were less effective than aliphatic analogs against mycelial growth of cereal root pathogens when added to headspace above a growing medium due to lower volatility, but more effective in the agar growing medium.

Both environment and ontogeny influence the plant related components of the

biofumigation potential. During flowering, there is a reduction in glucosinolate (GSL) concentration in the vegetative parts of the plant and also in the inflorescence which contains

Table 2: Physicochemical properties of Selected Isothiocyanates

Substance Name	2-Propenyl Isothiocyanate	Benzyl Isothiocyanate	2-Phenylethyl Isothiocyanate
Abbreviation	AITC	BITC	PEITC
Side Chain name	Allyl	Benzyl	Phenethyl
Glucosinolate Name	Sinigrin	Glucotropaeolin	Gluconasturtiin
Found In **	*B. juncea* (s, l, r)*	*S. alba* (l, r) *	*B. juncea* (l, s, r)* *S. alba* (r)
Structure			
CAS	57-06-7	622-78-6	2257-09-2
Formula	C_4H_5NS	C_8H_7NS	C_9H_9NS

* Plant part: s = seed, young seedling, or inflorescence l = leaf and/or stem r = root
** From Brown and Morra, 2005, pages 76-84

relatively large amounts of GSLs. In contrast, during seed maturation, GSLs are synthesised in large amounts in the siliques, and also accumulate in low levels in the root (Rask et al., 2000). Distribution of the glucosinolates varies among plant organs, with both quantitative

and qualitative differences among roots, leaves, stems and seeds within the plant (Fahey et al., 2001; Brown and Morra, 2005; van Dam et al, 2008).

B. juncea has AITC, *B. alba* has BITC, and PEITC was found in roots of both mustards (Brown & Morra, 1997; Sarwar and Kirkegaard, 1998; Smolinska et al., 1999). Smolinska (1999) analyzed AITC, BITC and PEITC produced from *B. juncea, B. napa, B. rapa* and *S. alba*, and found per gram of dry weight, 645 µg AITC, <16 µg each of BITC and PEITC were produced after thorough disruption of *B. juncea tissue. S. alba* tissue produced <16 µg of AITC and PEITC, but 43.5 µg of BITC.

Due to preferences for a less pungent, acrid taste, many Brassicaceae used for consumption have been selected for lower GSL content, and there is much variation within species in types and amounts of GSLs. However, the major GSLs and their relative proportions are generally stable and predictable for particular species and subspecies (Rosa et al., 1997; Kirkegaard and Sarwar, 1998). The distinctive taste of many horticultural cruciferous salad crops is due to their glucosinolate content. For example, watercress accumulates large amounts of phenylethyl glucosinolate, combined with low levels of 7-methylsulfinylheptyl and 8-methylsulfinyloctyl glucosinolates (Rosa et al., 1997). Rockets (*Eruca* and *Diplotaxis* species) possess 4-methylthiobutyl glucosinolate, and cress (*Lepidium spp.*) contains benzyl glucosinolate (Fahey et al., 2001).

For biofumigation, selecting a green manure that has the kinds and quantities of the GSLS and ultimately ITCs that act on the pathogens for the particular soil can be regionally important. The compounds found in a particular plant material depend on the specific glucosinolates in the plant, the genetic source, the treatment of the plant material prior to the

hydrolysis of the glucosinolates, and the conditions during that hydrolysis. Biological responses caused by the products from the glucosinolates thus depend on subtle differences in the treatment of the plant material (VanEtten et al., 1976). Matthiesen and Kirkegaard (2006) note that glucosinolate production and biofumigation potential has the capacity to vary significantly, citing studies with diverging results but also differences in experimental conditions. Cartea et al. (2008) studied seasonal variations in glucosinolate production and found temperature and day length affected amount and type of glucosinolate found in cabbage and kale. It is prudent to evaluate the ITCs from green manures where biofumigation is being adopted to determine if they are regionally relevant.

There have been a number of recent studies examining glucosinolate content in Brassicaceae, many analyzing compounds in the mustards examined in this thesis, which included *Brassica juncea* and *Sinapis alba*. Kirkegaard and Sarwar (1998) analyzed twenty eight Brassicaceae, including *Brassica juncea* and *Sinapis alba*, for glucosinolates in roots and shoots, and found shoots contained predominantly aliphatic glucosinolates, while roots contained more of the aromatic forms, predominantly 2-phenethyl GSL. Indolyl GSLs were ubiquitous, but present in relatively low concentrations. Results of the study indicate the difficulty with randomly using any *Brassica* (extending even to cultivars within a species) for targeting selected organisms for biofumigation purposes as composition and quantities of different ITCs vary. In a greenhouse study using rapeseed, chopped leaves and shoots were effective against root knot nematodes (*Meloidogyne chitwoodii*) but chopped roots were not (Mojtahedi et al., 1993). Since the quantity of GSLs in roots was higher than shoots and leaves, the authors suggest the roots decompose more slowly and would be a source of

prolonged soil pathogen control. Sarwar et al. (1998) showed that while a drop of AITC will volatilize within 5 minutes at room temperature, PEITC takes more than 72 hours. Perhaps the change in relative toxicity of the two ITCs was related to the slower volatilization and loss of PEITC from soil, so that while this compound is less toxic than AITC, it is able to exert its toxic effects longer. *B. juncea* roots release PEITC, which resulted in growth suppression of the take-all fungus (Angus et al., 1994).

According to Fahey (2001), at least 500 species of non-cruciferous dicotyledonous angiosperms have been reported to contain one or more of the over 120 known glucosinolates. Myrosinase is present in some soil organisms, cruciferous aphids, mammalian gut flora, some fungi and bacteria. In addition to plants, the enzymes have been discovered in the bacterium *Enterobacter cloacae* and the fungus *Aspergillus sydowi* (MacLeod and Rossiter, 1986). Myrosinase-like activity has also been observed in soils (Borek et al., 1996). ITCs are found in marine sponges and some fungi, but are not produced from GSL hydrolysis (Fahey et al., 2001). Sesquiterpene ITCs are found in marine sponges of the orders Axinellida and Halichondrida (Zubia et al., 2008).

GSLs signal a food source to some species. The larvae of the cabbage white butterfly (*Pieris sp*.) and related genera, which feed exclusively on plants containing the glucosinolate-myrosinase system, contain the gut protein NSP (nitrile specifier protein) which causes the glucosinolate-myrosinase reaction to yield nitriles istead of isothiocyanates (Agerbirk et al., 2008). Some specialized insects are able to sequester glucosinolates present in their food plants for use in their own defense against predators (Morant et al., 2008).

Herbivores have developed different strategies to counteract the presence of glucosinolates. The diamondback moth (*Plutella xylostella*; Ratzka et al., 2002) and the desert locust (*Schistocerca gregaria*) express a glucosinolate desulfatase in their guts, which rapidly desulfates the glucosinolates to yield desulfo-glucosinolates. Desulfo-glucosinolates cannot be hydrolyzed by the myrosinases and as a result no toxic degradation products are formed (Ratzka et al., 2002; Morant et al., 2008).

Glucosinolates are structurally and biosynthetically related to cyanogenic glucosides (for example, dhurrin, found in sorghum, is a cyanogenic glucoside) and are thought to have evolved from a cyanogenic glucoside predisposition (Rask et al., 2000; Morant et al., 2008). Agerbirk et al. (2008) found that glucosinolates derived from methionine were found in all *Sinapis clades*. The presence of glucosinolates and the enzyme myrosinase are chemotaxonomically significant in defining the order, however, specific variations of glucosinolates are not reliable taxonomic markers (Agerbirk et al., 2008).

Section II: Biocidal Activity of Isothiocyanates

Of concern to potato growers in Washington State is the nematode *Meloidogyne chitwoodii*. *Brassica napus* cultivar Jupiter (rapeseed) green manure has been shown to reduce the numbers of Columbia root-knot nematode by up to 90% (Mojtehedi et al., 1993). For two consecutive years, rapeseed green manure limited *M. chitwoodi* damage on potato tubers (*Solanum tuberosum*) in field experiments in Prosser, Washington. The data found a correlation between glucosinolate content (4-pentenyl isothiocyanate was the major GSL

detected) and the effectiveness of the green manure in controlling nematodes, and the rapeseed green manure was more effective than spring wheat, supporting a conclusion that biocidal chemistry was involved. However, the culled, unacceptable portion of the crop was 17% and 14% respectively for the two years of the study. Washington State processors may downgrade or reject fields with more than 10% cullage.

Studies have shown that ITCs vary in their effect on different organisms and that field conditions may affect toxic action as well (Bending and Lincoln, 1999; Papadopoulos and Alderson 2007). The improved soil properties and availability of nutrients such as nitrogen and phosphorus may be one reason for reduced pathogenic activity, and these effects may be additive to the glucosinolate degradation product effects. For example, nitrogen soil content did not correlate with reduced *Verticillium dahliae* (Davis et al., 1996). These studies leave open the possibility that the mechanism of soil pathogen control may be chemical, structural or it may be break crop effects, accomplished by growing a poor or non-host crop that accomplishes the reduction of soil pathogens (Mojtahedi et al., 1993; McGuire, 2003).

Olivier et al. (1999) tested 35 genotypes of *B. juncea* and 28 genotypes of *B. nigra*, selected from geographically diverse locations, against *V. Dahlia* and *Helminthsporum solani* (cause of silver scurf on potato tubers) and found all genotypes produced compounds that suppressed radial growth of both fungi, but results differed significantly. AITC, BITC and PEITC were present in most of the *B. juncea*, as well as the aliphatic 3-butenyl ITC. Four of the 35 genetypes of *B. juncea* contained little AITC, but were still inhibitory of radial growth of the fungi. AITC from *B. juncea* was more suppressive to five cereals than AITC from *B. napus* (Kirkegaard et al., 1996).

In studies conducted by Roubtsova et al., (2007), fifty centimeter (cm) polychlorovinyl tubes of soil infested with *M. incognita* were amended with broccoli tissue, either thoroughly mixed, or added to the first 10 cm. The PVC tubes were sealed and kept at 28° C for three weeks. The treatment was effective in reducing nematodes from 57-80% from controls. The tubes limited to 10 cm of broccoli tissue produced some control of nematodes throughout the tube, indicating the biocidal chemicals were volatile in the soil, however, control was superior with thorough mixing. This study suggests that volatile effects are weaker than contact effects, a conclusion supported by Mojtahedi et al. (1993).

Sarwar and Kirkegaard (1998) found the toxicity of aliphatic ITCs decreased with increasing length of the side chain although there was little difference between MITC and AITC in the headspace experiment. Fungi differed in sensitivity to the different ITCs; however, suppression of some fungi by AITC and PEITC was superior to that of the synthetic fumigant MITC, suggesting an important role for these compounds in the pest suppression potential of brassicas. There were 7-fold differences in the LD_H (lethal dose for fungi) values for some fungi to different ITCs, and 18 fold differences between fungal species in their sensitivity to particular ITCs. The study demonstrated the importance of identifying which GSL types release the most toxic hydrolysis products to particular target organisms.

Seeds of corn, soybeans, rapeseed, alfalfa, cucumber, and dandelion were exposed to commercial MITC and also volatiles from various Brassicacea, including AITC, BITC and PEITC from *S. Alba (Brassica hirta)* and *B. juncea* as well as other ITCs. I_{50} values (the mean of three replicates for 50% inhibition of seed germination) for both AITC and MITC were less

than 1 ppm headspace concentration in airtight glass containers for all species tested (Vaughn et al., 1997).

A study of GSL hydrolysis products on the resting stages of fungi was conducted by Smolinska et al. (1999) using tissue from *B. juncea, B. napus, B. rapa, S. alba* and *R. sativa* tissues against *Fusarium oxysporum, Sclerotium cepivorum* and *Sclerotinia sclerotiorum* chlamydospores. *B. juncea* and *S. alba*, at least for the first few days of the test, kept growth of the fungi to zero, and also contained the highest amounts of AITC, BITC, and PEITC when measured by Smolinska. The other Brassicaceae did not approach amounts of the three NITCs, and were not as effective in decreasing the density of resting fungal propagules (Smolinska et al., 1999).

Ochai et al. (2007) conducted field trials using green manures of Australian pea, Brassica oleracea, and sorghum sudangrass, and found that Verticillium wilt was reduced significantly, but potato tuber yields were not increased. Ramirez et al. (2009) found evidence to suggest that green manures may interfere with beneficial soil organisms, as measured in a test of two *B. juncea* genotypes against entomopathogenic nematodes.

Bhat and Subbareo (2001) recommend broccoli as a good rotation crop for its effect on *V. dahliae*, incorporating broccoli pieces into a field prior to sowing cauliflower.

Weed control is a benefit of fast-growing Brassica cover crops, which compete well for space and nutrients. After incorporation, biofumigation probably suppresses germination of small weed seeds (Boydston and Hang, 1995). Crops seeded too soon after the incorporation of a Brassica crop can also be damaged (McGuire, 2003).

III: Biofumigation, Emissions, and Soil Chemistry

In Washington State, the use of mustard as a green manure has steadily increased since 1999 (McGuire, 2003), and potato growers in the Columbia Basin are using high biomass-producing brassicas as part of an integrated pest management program. Green manuring is a traditional practice that provides general soil benefits in terms of nutrients, properties, organic matter, beneficial microorganism growth and break-crop effects, but it has been largely supplanted in modern agriculture by the advent of inorganic fertilizers. Interest in using mustard for green manure was piqued by findings that mustard and other *Brassica* crops and sorghum-sudangrass green manure soil amendments can suppress *Verticillium dahliae* (Davis et al., 1996), a major cause of the potato early dying complex (McGuire, 2003).

Studies have shown that ITCs vary in their effect on different organisms and that field conditions affect toxic action as well (Bending and Lincoln, 1999; Papadopoulos and Alderson, 2007). Bending and Lincoln (2000) investigated the effects of glucosinolate hydrolysis products on nitrifying bacteria in sandy loam and clay loam soils, and found ITCs reduced populations and inhibited growth of NH_4^+ (nitrosifying) oxidizing bacteria in both soils. ITCs had no apparent inhibitory effect on populations of NO_2^- oxidizing (nitrifying) bacteria in sandy-loam, but did reduce growth of these bacteria in clay-loam. After 42 days, mineralization of nitrogen in sandy-loam amended with PEITC was greater than in unamended soil, suggesting that this compound had a general fumigant effect on the soil microbiota. ITCs were more effective inhibitors of nitrification than intact GSLs or nitriles. PEITC was found to be the most toxic of the ITCs tested, but generally there were no

differences between the nitrifying inhibitory properties of aliphatic and aromatic ITCs in this study.

In a study using Indian mustard (*Brassica juncea*) tissue incorporated into a sandy-loam and a clay-loam soil, the GSL content in the incorporated plant material in both soils decreased significantly within a few days. 35% of the initial amount was lost after 2 days and only small amounts remained after 6 days (Bending and Lincoln, 1999). In a laboratory study of the disappearance of PEITC in soil, a half-life of 16 hours was estimated (Petersen et al., 2001). In another laboratory experiment, the release of isothiocyanates from plant residues from *Brassica, Cleome and Tropaeolum*[1] species incorporated into soil showed that the ITCs were detectable for 6 days, but the amounts decreased rapidly within the first 3 days (Papadopoulos and Alderson, 2007). In contrast to the glucosinolates, isothiocyanates are hydrophobic compounds, and the K_{ow} (octanol-water partition coefficient, a measure of lipophilicity) will depend partly on the side-chain structure. Isothiocyanates with longer hydrocarbon sidechains have higher K_{ow} than those with short sidechains and aromatic isothiocyanates have greater K_{ow} than the aliphatic (Schultz et al., 2005). For example the partition coefficient (log P) for AITC is 2.11 (NIOSH 1997) while for BITC and PEITC it is 3.16 and 3.47, respectively (Schultz et al., 2005). As a result of their hydrophobic nature, the isothiocyanates are sorbed mainly by the organic matter in the soil, and leaching losses are likely to be low compared with glucosinolates (Gimsing and Kirkegaard, 2009).

[1] Cleome (spiderflower) L. belongs to the Capparaceae family, and Tropaeolum belongs to the Tropaeolaceae (Nasturtium) family.

Gimsing and Kirkegaard (2006) found that both isothiocyanates and glucosinolates can remain in soil for several days after incorporation and both can be leached below the level of incorporation by irrigation. As part of this field study, a high GSL variety of *Brassica juncea* was grown in place and then pulverized to maximum 3 x 3 cm size pieces and tilled into the soil at late seed formation stage. Headspace ITC amounts from soil samples gave an isothiocyanate release efficiency for the high GSL variety of 56% at 30 minutes after application of the mustard pieces into the soil. By 6 hours, the proportion of GSLs detected had declined by about 50% in the high GSL variety. The concentration of ITCs declined rapidly during the first 4 days, was detected in the soil for up to 8 days after incorporation, with some trace amounts of ITCs detected at 12 days (Gimsing and Kirkegaard, 2006). In a laboratory study with a soil:water content of 1:1, glucosinolates were degraded very rapidly following logistic kinetics with half lives in the range 3.5–6.8 hours in a clay topsoil and 9.2–15.5 hours in a sandy topsoil (Gimsing and Kirkegaard, 2009).

To be effective, biofumigation requires plants with high glucosinolate content, maximum tissue disruption and soil mixing, water, and high soil temperatures (Gimsing and Kirkegaard, 2009). GSLs released from plants are very mobile in the soil environment and highly bioavailable. Residual glucosinolates are very weakly sorbed, readily leached and are microbially degraded and mineralized in soil. In contrast, isothiocyanates are strongly sorbed by the organic matter in soil, react strongly with nucleophilic groups present in soil, and are prone to volatilization losses in addition to microbial degradation and mineralization. The relatively rapid sorption and degradation of the isothiocyanates in the period of days after

incorporation minimizes the risks of persistence in the environment or leaching from the green manure incorporated soil (Gimsing and Kirkegaard, 2009). Post-incorporation irrigation at rates achievable in practice did not enhance ITC release in soil (Gimsing and Kirkegaard, 2006).

ITC persistence in soil has been studied by Papavizas et al. (1966), who reported that emergence of peas was not affected by high AITC mustards when planted at least seven weeks after incorporation of the tissue. While MITC toxicity reaches depths in the soil below the level of application, NITCs are less effective, and Mojtahedi et al. (1993) conclude green manures are most effective when thoroughly mixed into the soil.

Price et al. (2005) also showed that the air above soil with incorporated *Brassica juncea* tissue had a higher concentration of ITCs above a sandy soil low in organic carbon than above a clay soil with higher organic matter content. This result is supported by Bending and Lincoln (1999), who measured AITC in the headspace above sandy-loam and clay loam soils with incorporated *Brassica juncea* leaves. They found that less than 0.1% of the potential allyl GSL was found as AITC in the headspace, and methanethiol, sulfides and disulfides were found in the headspace at two orders of magnitude greater than the ITC. The authors proposed that in soils, most of the tissue disruption of the mustard occurs microbially and may be destroying or preventing enzymatic contact with GSLs, resulting in non-hydrolysis products in the headspace. This conclusion is supported by the finding that AITC formation peaked in both soils prior to peak microbial respiration. It is also conceivable that accumulating ITCs react with amines in plant tissue and soil (Bending and Lincoln, 1999).

Gimsing et al. (2008) and Matthiessen and Kirkegaard (2006) discuss microbial degradation of ITCs. In sterilized soils, extractable amounts of AITC and BITC, after an initial fast depletion, reduce slowly. NITCs in non-sterile soil showed decreased extractable amounts as a first-order reaction with half lives, indicating microbial degradation (Gimsing et al, 2008). Soils with enhanced biodegradation of MITC also demonstrated enhanced biodegradation of AITC in soil previously treated with metam sodium as compared with soils not treated, and sterilized soils (Warton et al., 2003). In this cross-enhanced biodegradation soil, 67% more plant tissue was required to cause 100% mortality to the test insect, instar weevils, as in the sterilized soil. Rumberger and Marschner (2003) showed that in non-sterile soil only traces of PEITC were left after 44 hours, whereas in a sterile soil the concentration of PEITC decreased very slowly within the 91 hour experimental time frame. NITC microbial degradation has not been studied in the field, but studies of the fumigant metam sodium show rapid elimination of MITC in soils where it has been used repeatedly. Microbes adapt and population growth is stimulated by utilization of the pesticide as a carbon or nitrogen source (Matthiessen and Kirkegaard, 2006; Stone and Hansen, 1993). Stone and Hansen (1993) found that *Brassica hirta* (*Sinapis alba*) cover crops incorporated as green manure resulted in increased opportunistic pathogen populations as compared to fields left fallow.

Environmental risks from biofumigation are presumed to be low because both glucosinolates and isothiocyanates are short lived in soil and do not accumulate. However, Gimsing and Kirkegaard (2006) showed GSLs and ITCs can migrate below the level of field incorporation.

Section IV: Hazards to Bystanders and Environment

Isothiocyanates are irritants and cause lachrymation, with severity of effects decreasing with length of side chain for aliphatic ITCs and from aliphatic to aromatic ITCs. For example, The NIOSH (National Institute of Occupational Safety and Health) International Safety Cards describe the effects of exposure to AITC as cough, sore throat, red skin and eyes, burning sensation in stomach, and can be absorbed through skin. Long term effects are "dermatitis, sensitization, and may have effects on liver, kidney, stomach, bladder, thyroid." Methyl isothiocyanate is the most toxic ITC and is produced when the synthetic pesticide, metam sodium (active ingredient sodium methyldithiocarbamate) is hydrolyzed in the field. 2-Propenyl Isothiocyanate (AITC; Table 2) is a federally registered active ingredient, the biologically active chemical in Oil of Mustard seed (OMS) used as a pesticide in pellet form. OMS is classified into toxicity category III for primary eye irritation and is considered to be a skin sensitizer. AITC was first registered in 1962 as a dog repellant (EPA R.E.D. 1993).

Unfortunately, there is scant information available for AITC that directly assesses potential human inhalation risks. Indirect estimates from experimentally derived oral and dermal lethal dose (LD_{50}) animal toxicological studies have been developed for AITC by the Department of Energy for setting Temporary Emergency Exposure Limits (TEELs[2], Department of Energy, 2008). These government estimates have been derived for establishing

[2] SCAPA (Subcommittee on Consequence Assessment and Protective Actions) developed TEELs so that DOE facilities could conduct appropriate emergency preparedness hazard analyses (EPHA) and perform consequence assessments. Toxicity parameters which have been experimentally derived, such as lethal dose 50% (LD_{50}) and lethal dose lowest (LD_{LO}), are used to set TEELs from mainly animal toxicology studies after making adjustments to extrapolate experimental results from animals to humans

if evacuation is necessary. An estimated threshold concentration of 350 ppb (TEEL-0) was established to represent a concentration in air below which most people should experience no appreciable risk or health effect. The reliability for using these conservative, evacuation-based estimates for acute human health risk assessment is questionable. For example, the TEEL-0 for MITC is 450 ppb (http://orise.orau.gov/emi/scapa/teels.htm). This air concentration is equivalent to two-times greater than the human inhalation no observable adverse effect level (NOAEL) of 220 ppb for this substance. The US Environmental Protection Agency's Office of Pesticide Programs acute inhalation level of concern is 22 ppb for bystander exposure (EPA, 2008). Considering the conclusion drawn by Mithen (2001) that side chain complexity is inversely related to toxicity, it is likely the inhalation toxicity values for allyl ITC and other NITCs will be much higher than 22 ppb.

There are many ITCs, including the ones identified in this study, that are approved for use as food additives and flavorings (Furia and Bellanca, ed., 1975), but they are also used as ingredients in contact pesticides. Brassicaceae such as mustard have traditional medicinal uses (wound poultices) and anticarcinogenic effects (Traka and Mithen, 2009); however, animal studies show AITC can have mutagenic effects.

Values for acute or chronic inhalation exposure for the NITCs monitored in this study have not been found in public databases, but there are studies for other exposure routes. AITC has a documented LD-50 oral rat 339 mg/kg that is often cited. The three NITCs are listed as mutagens and animal carcinogens at high, prolonged doses. Data from a long-term toxicity and carcinogenicity study on AITC in rats by oral gavage (NTP, 1982) determined a NOAEL for AITC at 12 mg/kg body weight per day, which corresponds to 720 mg/person/day.

Toxicological implications of glucosinolate hydrolysis must also include the possibility of oxazolidinethione (OZT) formation. The most common OZT, 5-vinyl-2-oxazolidinethione, is often referred to as "goitrin" because of its ability to induce goiter in the thyroid gland. High-protein rapeseed meal has limited use as a cattle feed, in part because the glucosinolate precursor of goitrin is present in large amounts. Other information on biological activity is relatively scarce (Brown and Morra, 2005).

Section V: Conclusion

There is abundant laboratory evidence and mounting field work to show that Brassicaceae green manures, by contributing glucosinolate degradation products such as isothiocyanates to the soil matrix, are providing pest and weed control. There are many combinations of factors, such as Brassicacea species and cultivar, stage of plant development at incorporation, field characteristics, repeated use and diminished effectiveness, and target soil pathogens that must be investigated and understood in order to make predictions about glucosinolate degradation products after green manure field incorporation. Addition of green manures to soil changes the biological balance therein, and there are many questions still to be answered.

A review of the literature did not uncover data that could be used to infer bystander exposure to isothiocyanates emitted at the site of mustard crop field incorporation, and inhalation toxicity values exist only for methyl isothiocyanate. Laboratory and field studies of NITC analysis in air headspace show that minute percentages of what could potentially be

formed from glucosinolates are emitted from soil media, and aliphatic ITCs are more prevalent than aromatic ITCs. As cultivars with higher GSL amounts are developed, and as techniques are implemented that improve yields of ITC formation from GSLs, increasing NITC exposure may be possible. A better understanding of the emission potentials and individual toxicities of NITCs will require greater investigative attention for assessing human inhalation risks, especially given different weather and soil moisture conditions at mustard incorporation.

Inhalation hazards that may be associated with biofumigation and off site effects have not been explored in the literature for ITCs, other than for MITC. Determining if there is a hazard to bystanders will require exposure data, and this thesis study is intended to contribute to that effort.

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Chapter 2: Manuscript

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Natural Isothiocyanates: Assessing Near Field Emissions after Incorporation of Green Manure Cover Crop

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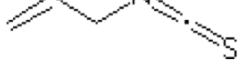
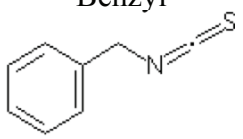
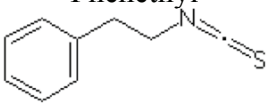
Introduction

In Washington State, an increasingly common practice is to use mustard, radish and other plants from the Brassicaceae family in rotational patterns with crops such as wheat and potatoes (McGuire, 2003). Green manures such as mustard provide nutrients and organic matter to improve soil tillage. Many Brassicaceae also offer the advantage of emitting volatile sulfur-based allelotoxins, notably isothiocyanates (ITCs), as a localized *de novo* response to plant tissue injury from predation or cultivation (Bones and Rossiter., 1996; Rosa et al., 1997). Many of the isothiocyanates produce a "biofumigation" activity effective against a wide range of soil-borne fungal and bacterial plant pathogens (Brown and Morra, 2005).

The NITCs of interest in this study are an aliphatic allylic (AITC), aromatic benzyl (BITC), and phenethyl (PEITC) isothiocyanates:

Substance Name	Abbreviation	Side Chain	CAS	Formula
2-Propenyl Isothiocyanate (allylic isothiocyanate)	AITC	Allyl 	57-06-7	C_4H_5NS
Benzyl Isothiocyanate	BITC	Benzyl 	622-78-6	C_8H_7NS
2-Phenethyl Isothiocyanate (phenethyl isothiocyanate)	PEITC	Phenethyl 	2257-09-2	C_9H_9NS

The simplest ITC, methyl isothiocyanate (MITC), has not been found as a GSL hydrolysis product in Brassicaceae (Fahey et al., 2001). The fumigant metam-sodium (sodium methyl-dithiocarbamate) can rapidly transform upon contact with soil and moisture to form MITC, the biologically active hydrolysis product. MITC is gaseous (2.5-2.8 kPa at 20° C; Leistra et al. 1976)) and has the potential for substantial surface emission and subsequent off-target air mass movement (Lee et al., 2002; Li et al., 2006; Sullivan et al., 2004). Recent air monitoring assessments in Washington State have shown MITC to exceed regulatory inhalation levels of concern to near-field bystanders and in near-by residential communities (Littke and Hebert, 2009; LePage et al., 2009; Merriman and Hebert, 2007).

Matthiessen and Kirkegaard (2006) reported that at 25^0 C , AITC, BITC, and PEITC are respectively 5, 1700 and 2300 times less volatile than methyl isothiocyanate. Although many of the aromatic ITCs can have up to 70-fold greater broad spectrum contact toxicity than MITC, their lower volatility and extensively greater soil partition coefficients render these complex ITCs less effective to soil borne pathogens in the soil substrate (Matthiessen and Kirkegaard, 2006). These investigators also noted that glucosinolate production and biofumigation potential has the capacity to vary significantly, citing regional studies with diverging results but also differences in experimental conditions. The structurally simpler aliphatic isothiocyanates such as AITC have been shown to have greater biological activity under field conditions compared to the more complex aromatic isothiocyanates (Matthiessen and Kirkegaard, 2006).

Isothiocyanates act as mucous membrane irritants and lacrimators. Their toxicity is associated with increased vapor pressure and is inversely related to greater structural

complexity (Mithin, 2001). The Relative Exposure Limit (REL) for MITC is 22 ppb (US EPA, 2008) based on irritation to eyes and mucous membranes. However, it has not been determined if NITCs pose an inhalation hazard to bystanders, agricultural workers and nearby residents during and after field incorporation. The types and quantities of isothiocyanates in air emissions from brassica during field incorporation have not been studied to our knowledge, nor has an inhalation toxicity value been determined.

In the fall of 2008, we sampled the air in and around a mustard field before, during, and four days following cultivation of two species of intermixed mustard, *Brassica juncea* and *Sinapis alba*. We developed an analytical method specifically to target multiple NITC compounds and selected three NITCs commonly known to be formed after green manure soil incorporation: AITC, BITC, and PEITC.

Of the above three NITCs, toxicity information is only available for AITC. AITC is a federally registered active ingredient, as the biologically active chemical in Oil of Mustard Seed (OMS) used as a pesticide in pellet form and first registered in 1962 as a dog repellant (EPA R.E.D. 1993). The NIOSH (National Institute of Occupational Safety and Health) describe the effects of exposure to AITC as cough, sore throat, red skin and eyes, burning sensation in stomach, and can be absorbed through skin. Long Term effects are "dermatitis, sensitization, and may have effects on liver, kidney, stomach, bladder, thyroid." Unfortunately, there is scant information available for AITC that directly assesses potential human inhalation risks. Indirect estimates from experimentally derived oral and dermal lethal dose (LD_{50}) animal toxicological studies have been developed for AITC by the Department of Energy for setting Temporary Emergency Exposure Limits (TEELs, Department of Energy,

2008). An estimated threshold concentration of 350 ppb was established to represent a concentration in air below which most people should experience no appreciable risk or health effect. The reliability for using these evacuation-based estimates for acute human inhalation health risk assessment should be viewed with some reservation since they are derived lethal dose values from other routes of exposure.

This study was designed to provide air concentrations for the above three selected NITCs after mustard field incorporation.. The emphasis of this study is to provide near-field NITC emissions after mustard green manure cover crop incorporation and preliminary air concentration data for understanding potential human risks may occur from inhalation exposure to naturally occurring isothiocyanates.

Materials and Methods

NITC air emission evaluations were conducted on a 102-acre center pivot-irrigated circle in Benton County, Washington starting October 25, 2008. This field was seeded in the summer with a mixture of oriental and yellow mustard (*Brassica juncea* and *Sinapis alba*) and routinely irrigated. At maturity (late flowering stage), the mustard was systematically flail chopped by tractor followed within approximately 20-minutes by disking. Sufficient residue of mustard was left on the field per Good Agricultural Practice to minimize surface erosion. Four activated charcoal air sampling masts (receptors) were positioned ca. 10-20 meters from the crop perimeter spaced evenly around the field. A fifth receptor with co-located activated charcoal cartridges was positioned at the center of the circle attached to the permanent

structure at the center pivot pad (Figure 1). The four perimeter receptors consisted of a single SKC air sampling unit (SKC Model 224-PCXR8) placed at the base of a ring-stand with a vertical crossbar set at 1 meter above ground. Tubing was connected to an SKC pump and

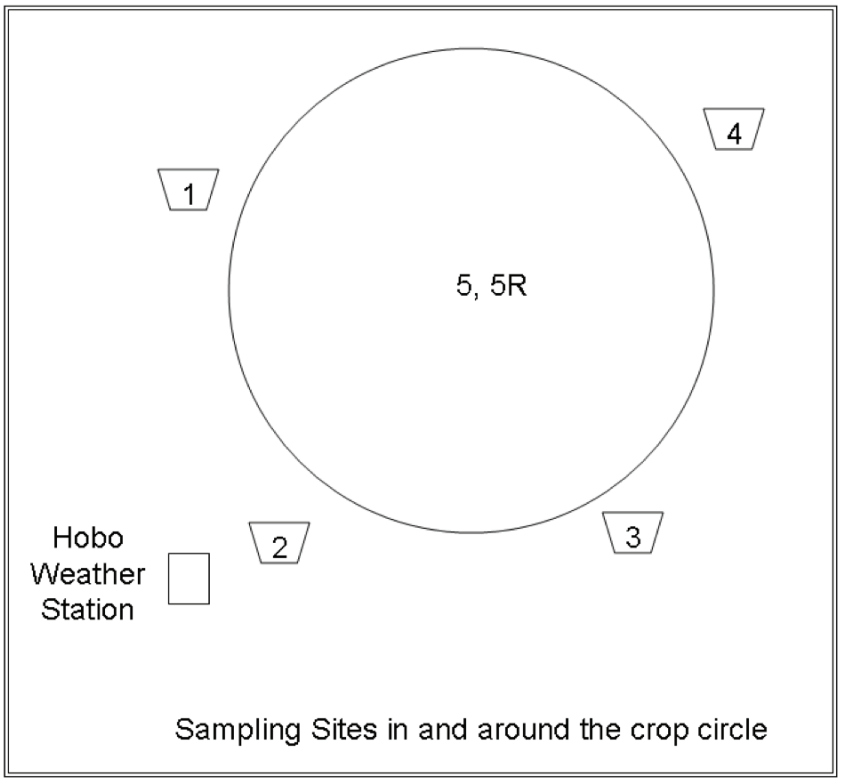


Figure 1: Sampling Sites in and around the Crop Circle. Four samplers were located around the circle, spaced about equally apart. A sampler with colocated sample were located at the center of the circle on the irrigation pivot pad.

to the 600 milligram activated charcoal-filled glass cartridge located horizontally on the crossbar, shielded from ultraviolet light and precipitation using a 1 inch polyvinyl chloride tubing sleeve. The receptors were operated at an air flow rate of ca. 2 L min^{-1} for intervals of approximately 4 hours on the day before mustard incorporation (-1 day), on the day of incorporation during the first approximately ¼ of the disking time period (A1), during the last approximately ¼ completion of disking time period (A2), immediately post disking (0), and once daily for 4-days following the green manure incorporation.

	Label	Time of Sampling Relative to Crop Incorporation	Date
BEFORE	-1	Prior to green manure incorporation	October 25
DURING	A1	First 2.5 hours during green manure incorporation	October 25
	A2	Last 6 hours during green manure incorporation	October 27
AFTER	0	Immediately following green manure incorporation	October 27
	1	First day following green manure incorporation	October 28
	2	Second day following green manure incorporation	October 29
	3	Third day following green manure incorporation	October 30
	4	Fourth day following green manure incorporation	October 31

At the start and end of all sampling collections, air flow measurements were taken and recorded. At the end of each sampling period, the collected charcoal cartridges were placed on blue ice and taken on the day of sampling to the WSU-Food and Environmental Quality Laboratory (WSU-FEQL) analytical facility where they were stored at -80 °C until analysis. Wind speed and direction and precipitation data were gathered from the WSU-AgWeatherNet

weather station located 3 miles from the study site in Benton City, WA. A Hobo weather station was sited near the field as well for comparison.

A trip blank (TB) was routinely shipped and stored with each set of air samples and later extracted with the sample set. On the last day of the study, a known concentration of a mixed AITC-BITC-PEITC working standard was applied to a charcoal cartridge accompanying the samples to the field. This trip spike was treated in a similar manner and extracted/analyzed as part of the data set. To assess trapping efficiency and potential breakthrough, a field spiked fortification set was performed to simulate sampling parameters at the field, but was conducted indoors at the WSU Tri-Cities campus. An unfortified cartridge control was run along with the field spiked fortification. A storage stability study for the three NITCs was conducted beginning February 2008.

The analytical method for charcoal extraction/quantitation of AITC, BITC, and PEITC was modified from an earlier method used for methyl isothiocyanate (Merriman and Hebert, 2007). The exposed charcoal cartridges were solvent extracted by sonication using a 1:1 mixture of carbon disulfide: ethyl acetate. The extract was filtered through a 0.45-um Whatman® Teflon® membrane, then placed in vials for analysis by gas chromatography. A Varian Star 3400CX gas chromatograph (Walnut Creek, CA) using thermionic-specific detection (TSD) with an 8200CX autosampler was used for residue detection and quantification. A 15-m x 0.53 mm, 1.2-μm-film-thickness EC-Wax chromatographic column was used for analyte separation, with ultrapure helium at ca 2-4 mL/min serving as the carrier gas. The initial column temperature of 55°C was ramped to 175°C by 20°C/min increments,

then to 225°C by 15° C/min increments, and held for 5 minutes. The injector port was set to 225°C the entire run, and the injection volume was 1 μL. The hydrogen, air and makeup gas flows were set at 3-4 mL/min, ca. 170 mL/min, and 25-30 mL/min, respectively. Retention times for AITC, BITC and PEITC were approximately 4.6, 9.6 and 10.4 min, respectively, verified against pure reference standards purchased from Sigma Aldrich. This method was validated, in triplicate, at three fortification levels covering the range of anticipated NITC concentrations in air. Stock solutions were prepared from reference solutions to use for spiking and working standards in appropriate dilutions. Concentrations of the selected NITCs from solvent-extracted charcoal cartridges were calculated by linear regression using a spreadsheet program (Microsoft Excel®, Redmond, Washington) from at least 4 external standards, and external calibration standards bracketed for every 2 or 3 samples in the analytical set. The method LOQ was established at 0.5 μg on activated charcoal for a total air volume of 480 L (2-L min^{-1}) over an averaged 4-hour air sampling period and adjusted for NITC air concentration (in ppb).

Results and Discussion

The analytical method developed for the measurement of multiple NITCs in single samples was found to be rugged with a limit of quantitation (LOQ) of 0.25 ppb for AITC, 0.16 ppb for BITC and 0.15 ppb for PEITC. Laboratory fortifications percent recoveries ran with each set of solvent-extracted field samples ranged from 84 to 118% for AITC, 73 to 97% for BITC, and 77 to 111% for PEITC. All combined laboratory method recoveries fell within

10% standard deviation. Linearity as measured by linear regression coefficient (R^2) of a minimum of 4 standards encompassing the range of encountered air concentrations for all analytical sets was ≥ 0.98.

Over the sampling period, NITC air concentrations, particularly for the AITC, were observed above the method's LOQ during and shortly after completion of field operations (Table 1, Table 2). The highest observed NITC concentrations occurred during tractor flail-chopping and disking with observed maximum single receptor air concentrations of 47.0 ppb for AITC and 1 ppb for BITC, as well as the PEITC maximum amount detected below the LOQ at 0.1 ppb (Figure 3). Since formation of NITCs result from tissue injury, it was not surprising to observe maximum air emission receptor AITC, BITC, and PEITC concentrations during flail chop and soil incorporation. The detection of PEITC below LOQ is reasonable considering it is found primarily in brassica roots that are less accessible during the flail chop and incorporation process. NITC emissions quickly attenuated after completion of field mustard incorporation and were below quantifiable levels at all receptors through the succeeding three days (Figures 2, 3 and 4). Conditions at incorporation and during 3-day post incorporation were dry and warm with light to moderate winds. Although not by design, sampling occurred during the warmest times of the day, with temperatures ranging from 4 to 19 ° C during each 4 hour sampling period and reaching at least 12.5 ° C at some point during each individual sampling period .

The only precipitation that occurred over the course of the study was on the last day, 4 days post green manure incorporation, measuring 0.25 mm and coinciding with a reoccurrence of AITC in samples from all 6 receptors at levels above the method LOQ.

Although appreciably less than during field mustard incorporation, recoveries were 4-fold greater than the receptor samples taken immediately after completion of flail chopping/disking. It is reasonable to suggest that increased surface moisture on the 4th day could enhance formation and emission of NITCs from previously unhydrolyzed glucosinolates residing on the tilled land surface. It is also possible that added soil moisture facilitated desorption of NITCs from soil organic matter and subsequent air emission. It was particularly evident in this case for the more volatile AITC.

Since air monitoring was not continuously performed, the observed within and near-field emissions may not represent the highest NITC air concentrations that can be encountered during and after mustard green manure field incorporation. Moreover, variation in NITC concentrations should be anticipated given different weather and soil moisture conditions at mustard incorporation.

There was substantial variation (ca. 12-fold) among the two exposed cartridges that were co-located at Site 5 (center pivot) during period A1. This was the period where we observed the highest concentrations for all NITCs. Pump failure cannot explain this difference in measured NITC concentrations. A possible source of variation is the orientation of the sampling cartridge on the receptor. The center field co-located cartridges air in-take orientation was ca. 90° relative to each other. Wind direction may have been a contributing factor in the discrepancy of air concentrations among these two cartridges at this receptor site. One possible explanation for this disparity among samples may lie in the activity around the sample sites that affected air flows and temperature, such as would occur from tractors

passing close to the sampler. On all other sampling events, the air concentrations from these two co-located samples were in agreement.

There is scant information available for directly assessing potential human inhalation risks to NITCs monitored in this field assessment. Indirect estimates from experimentally derived oral and dermal lethal dose 50% (LD_{50}) animal toxicological studies have been developed for AITC by the Department of Energy for setting Temporary Emergency Exposure Limits (TEELs, Department of Energy, 2008). This TEEL threshold was established for evacuation purposes to represent a concentration in air below which most people should experience no appreciable risk or health effect. The highest measured AITC concentration, 47 ppb, at the center of the field during flail chopping/disking operations, was ~7-fold lower than the TEEL estimated threshold concentration of 350 ppb.

Although hazards from the targeted NITCs are not well understood, these field emissions do not appear to pose an acute human inhalation exposure concern. The NITC air emissions from this single field assessment suggest that concentrations in air during field operations should not present an immediate acute human health inhalation concern to occupational workers or bystanders. However, this single regional study should not be viewed as definitive because NITC concentrations can be regionally variable and differences in replicated single receptor sampling orientation indicates substantial field concentration variation can occur.

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Table 1: Recovery Statistics for Samples

	AITC	BITC	PEITC
Total Number of Samples	48	48	48
Samples with detectable recoveries	24	9	3
Percent of Samples with Detectable Recoveries	50%	19%	6%
Percent of Samples With Recovery *Above the LOQ*	39.5%	6%	0%

Table 2: Statistics for Samples with Recoveries above the Limit of Quantitation (LOQ) in parts per billion (ppb)

	AITC	BITC	PEITC
Limit of Quantitation (LOQ) in ppb	0.25	0.16	0.15
Samples With Recovery *Above the LOQ*	19	3	0
Mean Average ppb recovery for samples with recoveries *above the LOQ*	4.5	0.44	n/a
Standard Deviation	10.54	0.46	n/a
Lowest ppb Recovery Above LOQ	0.26	0.17	n/a
Highest ppb Recovery Above LOQ	47.0	0.98	n/a

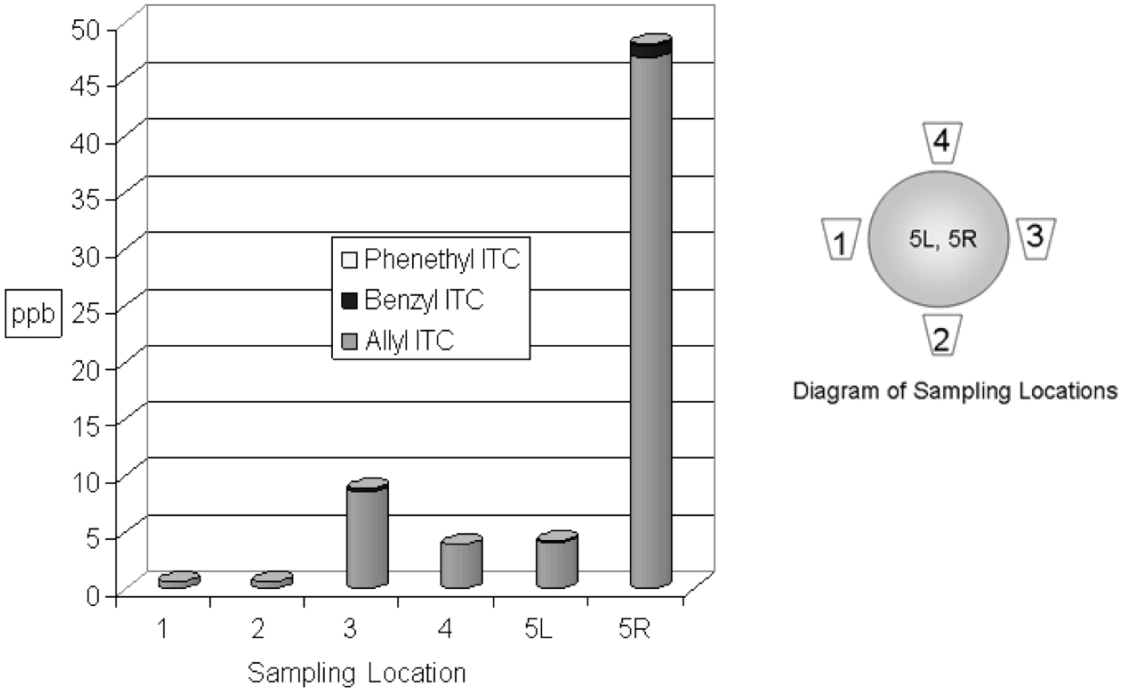

Figure 2: Time weighted average interval of air samples taken during the first ¼ of green manure incorporation (the A1 sampling period), showed the highest recoveries of the study.

Sampling Events: Key to Figures 3, 4 and 5

	Sampling Events	Time of Sampling Relative to Crop Incorporation	Date
BEFORE	-1	Prior to green manure incorporation	October 25
DURING	A1	First 2.5 hours of green manure incorporation	October 25
	A2	Last 6 hours of green manure incorporation	October 27
AFTER	0	Immediately following green manure incorporation	October 27
	1	First day following green manure incorporation	October 28
	2	Second day following green manure incorporation	October 29
	3	Third day following green manure incorporation	October 30
	4	Fourth day following green manure incorporation	October 31

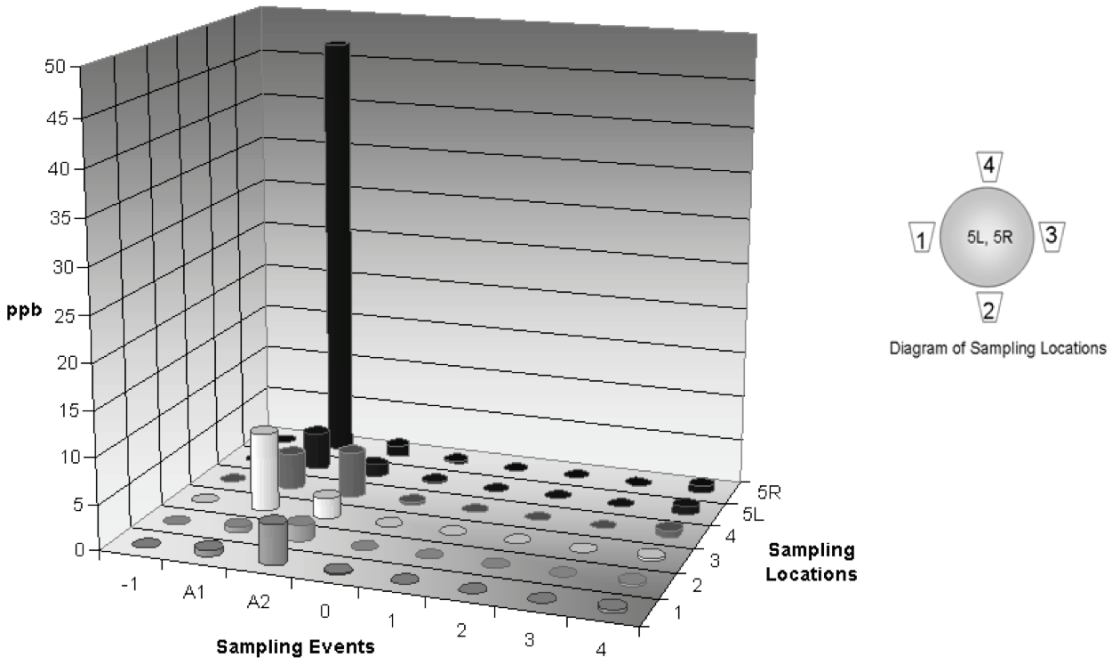

Figure 3: Allyl ITC. Time weighted average interval recoveries quantified up to 47 parts per billion (ppb). October 25 – 31, 2008

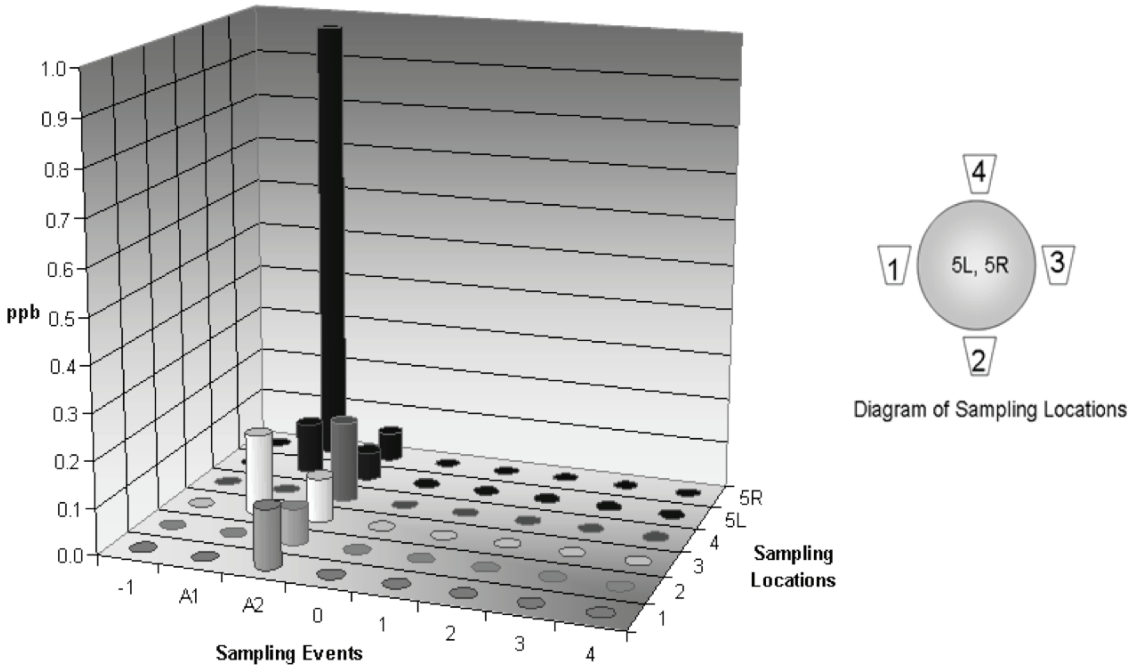

Figure 4: Benzyl ITC. Time weighted average interval recoveries quantified up to 0.98 parts per billion (ppb)**.** October 25 – 31, 2008

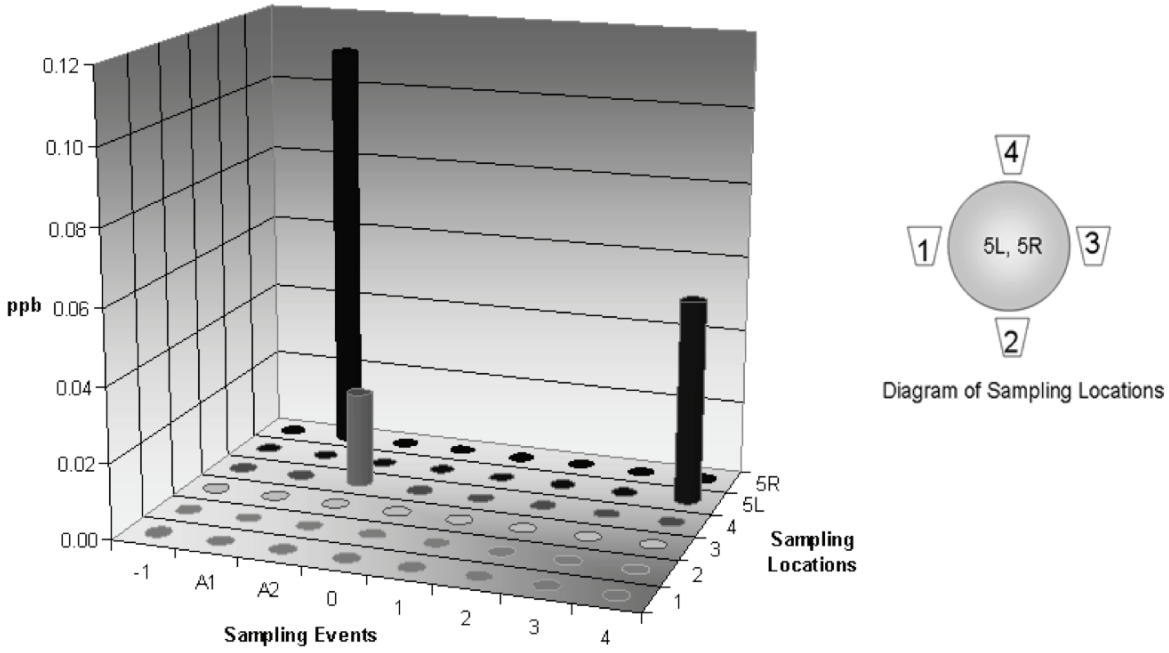

Figure 5: Phenethyl ITC. Time weighted average interval recoveries quantified below study LOQ up to 0.11 parts per billion (ppb). October 25 – 31, 2008

Chapter 3

Final Conclusions and Future Work

For biofumigation, selecting a green manure that has the kinds and quantities of glusinolates, and ultimately ITCs, that act on the pathogens for the particular soil can be regionally important. The characteristics of compounds found in a particular plant material depend on the specific glucosinolates in the plant, the genetic source, the treatment of the plant material prior to the hydrolysis of the glucosinolates, and the conditions during that hydrolysis, utilizing living green plants as fertilizer and pest control.

Although risks from the Natural Isothiocyanates (NITCs) such as those targeted in this study are not well understood, the field emissions monitored during our sampling do not appear to pose an acute human inhalation exposure concern. The NITC air emissions from this single field assessment suggest that concentrations in air during field operations should not present an immediate acute human health inhalation concern to occupational workers or bystanders. However, this single regional study should not be viewed as definitive because NITC concentrations can be regionally variable and differences in the replicated single receptor sampling due to orientation during this study indicates substantial field concentration variation can occur. There is potential for overall increased emissions in the future as we learn

more about NITCs as biofumigants. Cultivars will likely be developed for higher ITC-forming GSL content and agricultural practices that maximize biofumigant effects are implemented.

The potential for non-continuous, intermittent ITC emissions was demonstrated at the reappearance of AITC on the last day of the study. After 3 days with no detectable NITCs, AITC was again detected above the LOQ at all five sampling locations. The weather on this last day changed from the warm and mildly breezy weather pattern that held from the beginning of the study, to a cloudy sky with light (0.25mm) precipitation. It may be that the moisture percolating into the dry soil resulted in hydrolysis of glucosinolates remaining in the chopped pieces of mustard. It may be the reappearance would have occurred without the precipitation event as microbial degradation in the soil further disrupted the tissues of the mustard pieces that had been incorporated near the top of the soil. It is also possible that as soil water content increased, the hydrophobic AITC, having the lowest log P of the three NITCs, was desorbed from organic matter in the soil and volatilized to air emissions.

Much of the research quantifying ITC release from Brassicaceae has been conducted in the laboratory with macerated plant tissue, disrupting much more tissue than occurs in a field flail chop situation and exposing more plant tissue surface area resulting in reaction kinetics that are probably not reflective of agricultural field conditions. The nature of aliphatic and aromatic ITCs and varying volatility and hydrophobicity suggest potential for a more lengthy degradation process and perhaps longer lasting allelotoxic effects, and the reappearance of AITC at the end of the study supports this conclusion. Field studies are needed under varying conditions to determine the potential for continuous ITC effects over longer periods using Brassicaceae with more aromatic GSLs. Understanding of the dynamics

of ITC formation can help farmers “biofumigate” to maximum effect. It may be possible to select cultivars for green manure that can provide predetermined types and quantities of aliphatic and aromatic ITC-forming GSLs with desired short and long term toxic effects against targeted pathogens. Optimal timing could be determined for green manure incorporation, irrigation and coordination with other pest management strategies.

If trends continue, green manures will be employed as one aspect of an overall, integrated pest management system among agricultural enterprises. This will provide scientists further opportunities to conduct field studies, observing the natural*, in situ* result of green manure incorporation under varying weather conditions, soil hydration amounts, soil chemistry, species and cultivars of Brassicaceae, growth stage of the living plant, and other treatments, to understand and perhaps recommend particular green manure plants for unique regional needs. Long term studies of repeated green manure applications as affecting enhanced soil microbial degradation will be important.

There are numerous methods for extracting and analyzing glucosinolate content in plant and ITC content in soils, as well as ITC types and amounts needed to effectively control pathogens. It will be helpful to have a method of sampling, extracting and analyzing that is more universally practiced, so that results among different researchers can be fairly compared and conclusions drawn. This study developed a rugged and reliable method for analyzing NITC in air samples. It was our intention that this method facilitate more studies of glucosinolate hydrolysis products.

APPENDIX A

ANALYTICAL SUMMARY REPORT

Assessing Near–Field Naturally Occurring Isothiocyanate Emissions After Mustard Green Manure Cover Crop Incorporation

2008, Benton County, Washington

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FEQL Study No.: 1508

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Food & Environmental Quality Laboratory
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Study Timetable

Study Initiation Date	10/23/2008
Experimental Start Date	10/25/2008
Experimental Termination Date	4/28/2009
Report Date	6/03/2009

Abstract

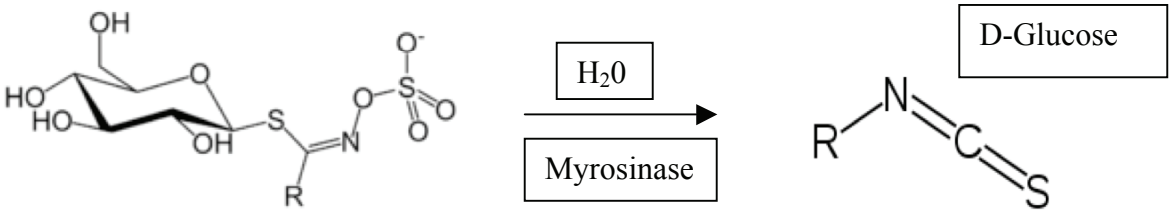
An assessment of near-field naturally occurring isothiocyanate (NITC) air emissions was conducted in Benton County, Washington, in the fall of 2008. The purpose of the study was to assess and quantify specific NITC air emissions after green manure mustard incorporation. A field air monitoring procedure and analytical method were developed specifically to target three NITC compounds commonly known to be formed after green manure soil incorporation: allylic-, benzyl-, and 2-phenethyl isothiocyanate. Air samples were collected using activated charcoal within and around the perimeter of a 102-acre crop circle before, during, and 4-days after mustard soil incorporation. The highest observed NITC concentrations occurred during tractor flail-chopping and disking field operations with maximum single receptor air concentrations of 47 ppb, 1 ppb, and 0.1 ppb, respectively, for the allylic-, benzyl-, and 2-phenethyl isothiocyanates. NITC measured air emissions appreciably attenuated shortly after soil incorporation activities ceased. Of particular note was an observed higher allylic ITC with detectable 2-phenethyl isothiocyanate air emissions 4-days post incorporation. This observation occurred on a day with light precipitation and may suggest increased surface moisture may have enhanced formation and emission of NITCs from previously unhydrolyzed glucosinolates residing on the incorporated land surface. Although risks to these targeted NITCs are not well understood, these NITC field emissions do not appear to pose an acute human inhalation exposure concern. For the allylic isothiocyanate, based on extrapolations from animal dermal lethal dose 50% data, the maximum observed air

concentration from this field assessment was ca. 7-fold less than the estimated threshold concentration below which it is anticipated that humans will experience no appreciable risk.

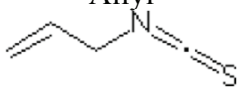
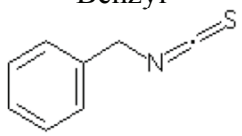
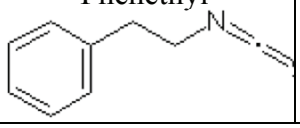
Introduction

Large producers of potato, mint, carrots, onion and tree fruit acreages in the Pacific Northwest rely on pre- plant soil fumigation to manage soil-borne nematodes and diseases (PMSP 2002). In Washington State, over 10 million pounds of metam-sodium, (*sodium methyldithiocarbamate*) are applied as a pre-plant fumigant in potato production annually (NASS 2006). When applied to soil and watered, metam sodium converts to biocidal methyl isothiocyanate, a strong irritant and considered a toxic air contaminant under the Code of California Regulations, Title 3, Section 6890(b).

An alternative approach to chemical fumigation, is the use of green manure cover crops containing naturally occurring isothiocyanate-producing compounds (NITCs) that can be incorporated into the soil (McGuire, 2003). This natural "biofumigation," the term coined by Kirkegaard and Sarwar (1998), relies on suppressing soil-borne pests and diseases from glucosolinate hydrolysis products, particularly isothiocyanates released from injured plant tissues when flail chopped then incorporated under the soil surface. Cover crops, from the family Brassicaceae and others, contain glucosinolate compounds that can be transformed to allelotoxic isothiocyanates by enzymatic hydrolysis.



The NITCs of interest in this study are an aliphatic allylic (AITC), aromatic benzyl (BITC), and phenethyl (PEITC) isothiocyanates:

Substance Name	Abbreviation	Side Chain	CAS	Formula
2-Propenyl Isothiocyanate (allylic isothiocyanate)	AITC	Allyl 	57-06-7	C_4H_5NS
Benzyl Isothiocyanate	BITC	Benzyl 	622-78-6	C_8H_7NS
2-Phenethyl Isothiocyanate (phenethyl isothiocyanate)	PEITC	Phenethyl 	2257-09-2	C_9H_9NS

The structurally simple aliphatic isothiocyanates such as AITC have been shown to have greater biological activity under field conditions compared to the structurally more complex aromatic isothiocyanates (Matthiessen and Kirkegaard, 2006). These researchers reported that at 25 ℃, the AITC, BITC, and PEITC are respectively 5, 1700 and 2300 times less volatile than methyl isothiocyanate. Although many of the aromatic ITCs can have up to 70-fold

greater contact toxicity than MITC, their lower volatility and extensively greater soil partition coefficients render these complex ITCs less effective to soil borne pathogens in the soil substrate (Matthiessen and Kirkegaard, 2006). These investigators also noted that glucosinolate production and biofumigation potential has the capacity to vary significantly, citing regional studies with diverging results but also differences in experimental conditions. Therefore the evaluation of naturally occurring isothiocyanates from green manures where biofumigation is being adopted is regionally relevant.

2008 NITC Field Emissions Field/Analytical Program

Field Study: NITC air emission evaluations were conducted on a 102 acre center pivot circle in Benton County, Washington starting October 25 2008 (Figure 1). This field was seeded in the summer with a mixture of oriental and yellow mustard and routinely irrigated. At maturity (late flowering stage), the mustard was systematically flail chopped by tractor followed within approximately 20-minutes by disking. Sufficient residue of mustard was left on the field per good agricultural practices to minimize surface erosion. Details for the field study can be found in Appendix I and Attachments A and B. More specific field information can be found in the field data book that accompanied this field emission study (FEQL-1508, 2008).

Air Monitoring: Four activated charcoal air sampling masts (receptors) were positioned ca. 10-20 meters from field edge of the crop circle perimeter. The receptors were positioned to

be accessible without interfering with field mustard green manure operations. The four perimeter receptors consisted of a single SKC air sampling unit (SKC Model 224-PCXR8), placed at the base of a ring-stand with a vertical crossbar set at 1 meter above ground. A fifth receptor location with co-located activated charcoal cartridges was positioned to the permanent structure at the center of the circle pivot. Tubing connected the personal sampler to the 600 milligram activated charcoal-filled glass cartridges located horizontally on the crossbar and shielded from ultraviolet light using a 1" PVC sleeve. The receptors were operated at an air flow rate of ca. 2 L min^{-1} for intervals of approximately 4 hours on the day before mustard incorporation (-1 day), on the day of incorporation at approximately ¼ completion of disking (A1), approximately ¾ completion of disking (A2), immediately post disking (0), and once daily for 4-days following the green manure incorporation. At the start and end of all sampling collections, air flow measurements were taken and recorded. Sampling flow rates, times, and dates were performed as described above. At the end of the sampling period, the collected charcoal cartridges were placed on blue ice and taken on the day of sampling to the WSU-Food and Environmental Quality Laboratory (WSU-FEQL) analytical facility where they were stored at -80°C until analysis. Wind speed and direction data together with precipitation data was gathered from the WSU-AgWeatherNet weather station located 3 miles from the study site in Benton City, WA (Attachment C).

Field and Laboratory QC: A trip blank (TB) was routinely shipped with each set of air samples and stored at -80° C and later extracted with the sample set (see Appendix II, Table 4). Four days after green manure incorporation, a known concentration of a mixed AITC-BITC-PEITC

working standard was fortified to a charcoal cartridge accompanying the samples to the field. This trip spike was treated in a similar manner and extracted/analyzed as part of this data set (see Appendix II, Table 2d). To assess trapping efficiency-potential breakthrough, a field spiked fortification set was performed under similar field, air sampling conditions but was conducted outdoors at the WSU Tri-Cities campus. Results are reported in Appendix II, Table 2c. An unfortified cartridge control was run along with the field spiked fortification. A storage stability study for the three NITCs was started in February 2008. Stability during freezer storage over this 408 day interval can be found in Appendix II Table 3.

Analytical Method: The analytical method for charcoal extraction/quantitation of AITC, BITC, and PEITC was modified from an earlier method used for methyl isothiocyanate (Merriman and Hebert, 2007). This method relied on a 80:20 ethyl acetate: carbon disulfide solvent mixture ratio. To improve extraction efficiency, the exposed charcoal cartridges were solvent extracted using a 1:1 mixture of carbon disulfide: ethyl acetate (i.e., 50% carbon disulfide in ethyl acetate) followed by sonication, filtration, and analysis by gas chromatography using thermal specific detection (TSD, see Attachment D). This modified method was validated, in triplicate, at three fortification levels covering the range of anticipated NITC concentrations in air (Appendix II, Table 2a). Stock solutions were prepared from reference solutions to use for spiking and working standards in appropriate dilutions. Concentrations of the selected NITCs from solvent extracted charcoal cartridges were calculated by linear regression from 4 external standards, and external calibration standards bracketed for every 2 or 3 samples in the analytical set. The method limit of

quantitation (LOQ) was established at 0.5µg on activated charcoal for a total air volume of 480 L (2-L min^{-1}) over an averaged 4-hour air sampling period and adjusted for NITC air concentration (in ppb).

Discussion of Results

The analytical method for the measurement of NITC was found to be rugged with LOQ of 0.25 ppb for AITC, 0.16 ppb for BITC and 0.15 ppb for PEITC. Laboratory fortifications percent recoveries ran with each set of solvent extracted field samples ranged from 84 to 118% for AITC, 73 to 97% for BITC, and 77 to 111% for PEITC. All combined laboratory method recoveries fell within 10% standard deviation (Appendix II, Table 2b). Linearity as measured by linear regression correlation (R^2) of a minimum of 4 standards among the range of encountered air concentrations for all analytical sets was $\geq$ 0.98.

Table 4 in Appendix II presents AITC, BITC, and PEITC concentrations from October 25 through October 31, 2008. Over the sampling period, NITC air concentrations, particularly for the AITC, were observed above the method's limit of quantitation during and shortly after completion of field operations. The highest observed NITC concentrations occurred during tractor flail-chopping and disking with observed maximum single receptor air concentrations of 47 ppb, 1 ppb, and 0.1 ppb, respectively, for AITC, BITC, and PEITC (see Figures 2 through 4). The quantity detected for PEITC was below the LOQ. Since formation of NITCs result from tissue injury, it was not surprising to observe maximum air emission receptor AITC, BITC, and PEITC concentrations during flail chop and soil incorporation. Since

PEITC is found primarily in root tissues that are less accessible to flail chopping and incorporation, it is understandable that the quantities detected were low. NITC emissions quickly attenuated after completion of field mustard incorporation and were below quantifiable levels at all receptors through the succeeding three days. Conditions at incorporation and during 3-days post incorporation were dry, warm, with light to moderate winds.

On the fourth day, post incorporation, a measurable 0.25 mm precipitation event (Appendix I, Figure 6) described as "a light rain, not enough to need a hat" occurred over the period of NITC air monitoring. During this period, AITC was observed among all receptors at levels above the method LOQ. These TWA AITC concentrations taken 4-days post green manure incorporation, although appreciably less than during field mustard incorporation, were however 4-fold greater than the receptor samples taken for the first 4-hours after completion of flail chopping/disking (Figure 2 and Appendix II, Table 4). It is reasonable to suggest that increased surface moisture on the 4^{th} day could enhance formation and emission of NITCs from previously unhydrolyzed glucosinolates residing on the incorporated land surface. This was particularly evident in this case for the more volatile AITC. Since air monitoring was not continuously performed, the observed within and near-field emissions may not represent the highest NITC air concentrations that can be encountered during and after mustard green manure field incorporation. Moreover, variation in NITC concentrations should be anticipated given different weather and soil moisture conditions at mustard incorporation.

There is scant information available for directly assessing potential human inhalation risks to NITCs monitored in this field assessment. Indirect estimates from experimentally derived

oral and dermal lethal dose 50% (LD_{50}) animal toxicological studies have been developed for AITC by the Department of Energy for setting Temporary Emergency Exposure Limits (TEELs, Department of Energy, 2008). The highest measured AITC concentration (47 ppb, see Figure 2 and Appendix II, Table 4) during flail chopping/disking operations was well below an estimated threshold concentration of 350 ppb. This TEEL threshold was established to represent a concentration in air below which most people should experience no appreciable risk or health effect.

Although risks to these targeted NITCs are not well understood, these field emissions do not appear to pose an acute human inhalation exposure concern. The NITC air emissions from this single field assessment suggest that concentrations in air during field operations should not present an immediate acute human health inhalation concern to occupational workers or bystanders. However, this single regional study should not be viewed as definitive since NITC concentrations can be regionally variable and differences in replicated single receptor sampling orientation indicates substantial field concentration variation can occur.

References

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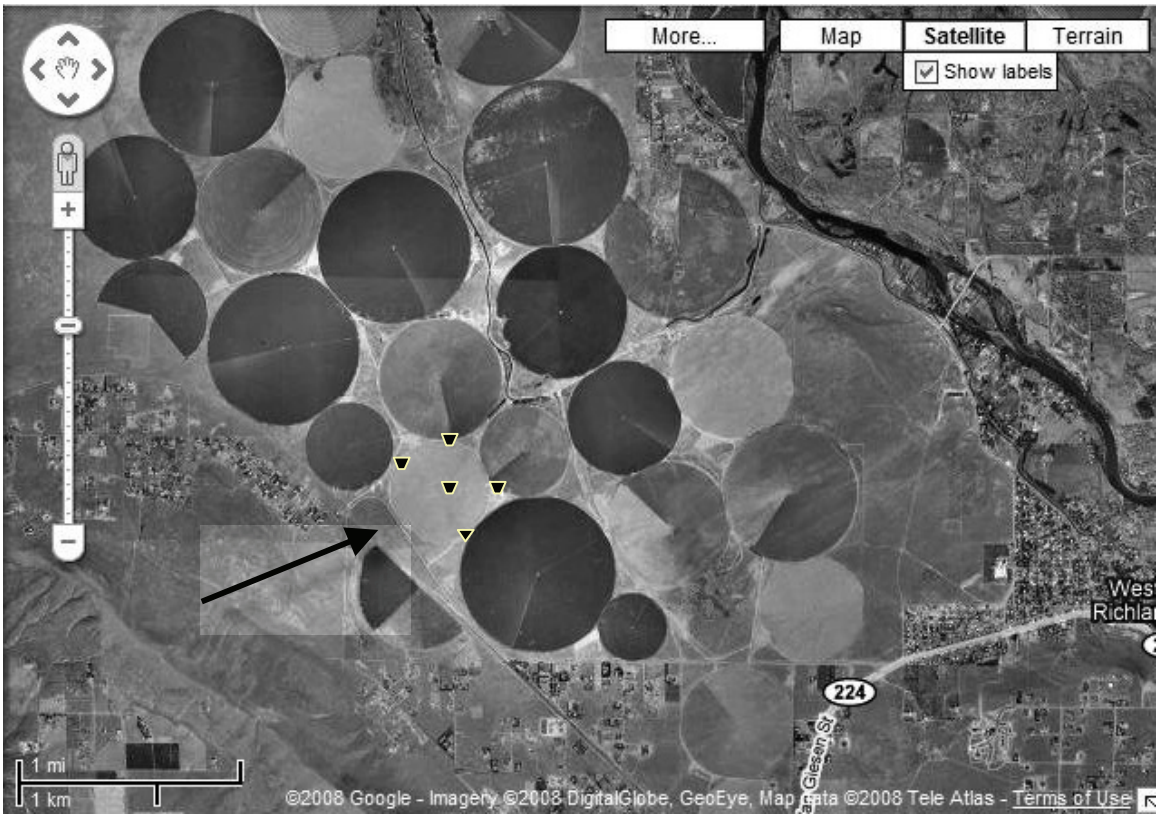
Figure 1
Site Map
Ruppert Road
Benton County, Washington State



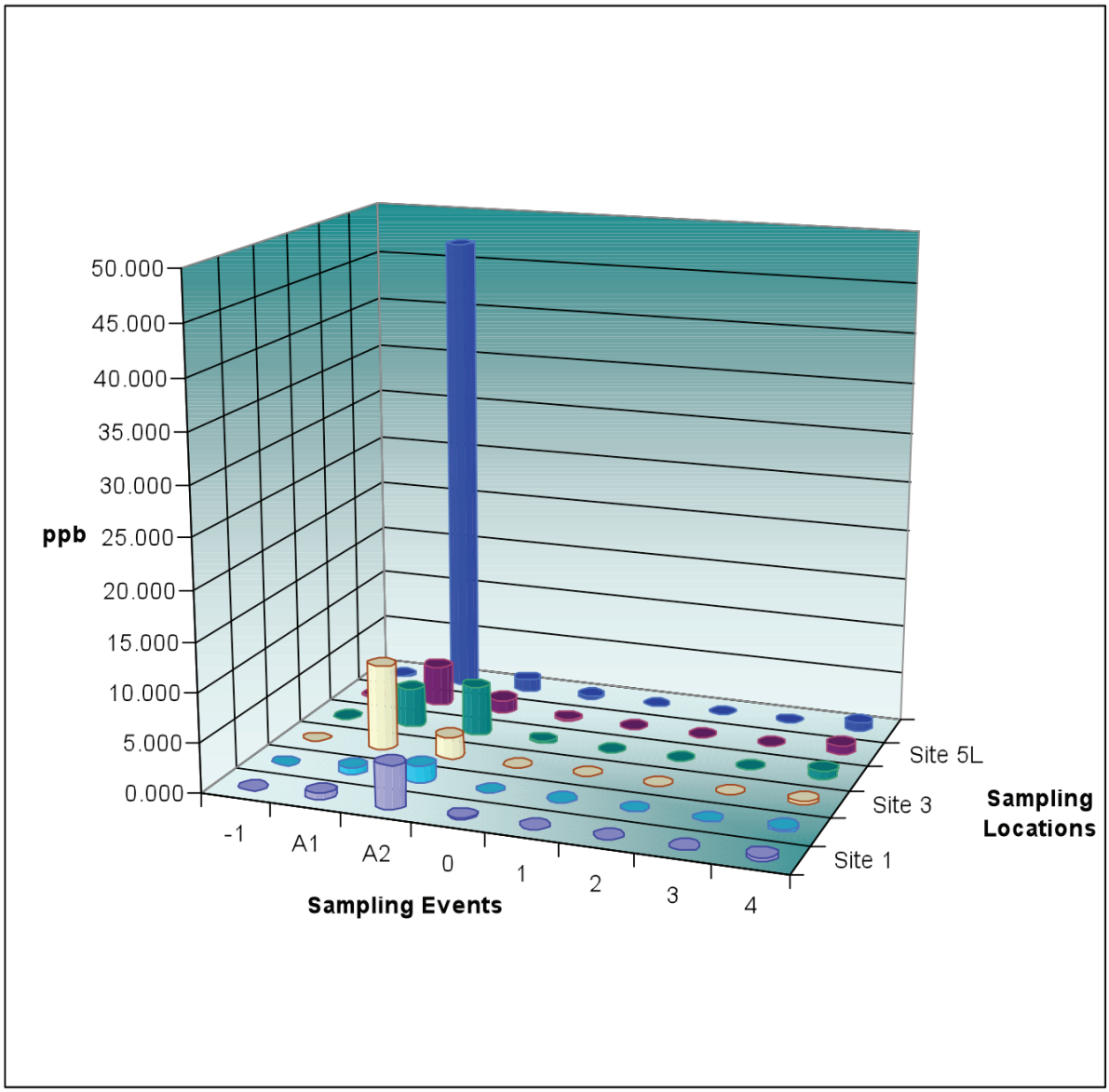
▼ Sampling Sites

46.312019, -119.421473

Township	T10N R27E
Meridian	Willamette
State	Washington
Source	USFS

Figure 2

TWA interval air monitoring for Allyl Isothiocyanate:
October 25 to October 31, 2008*



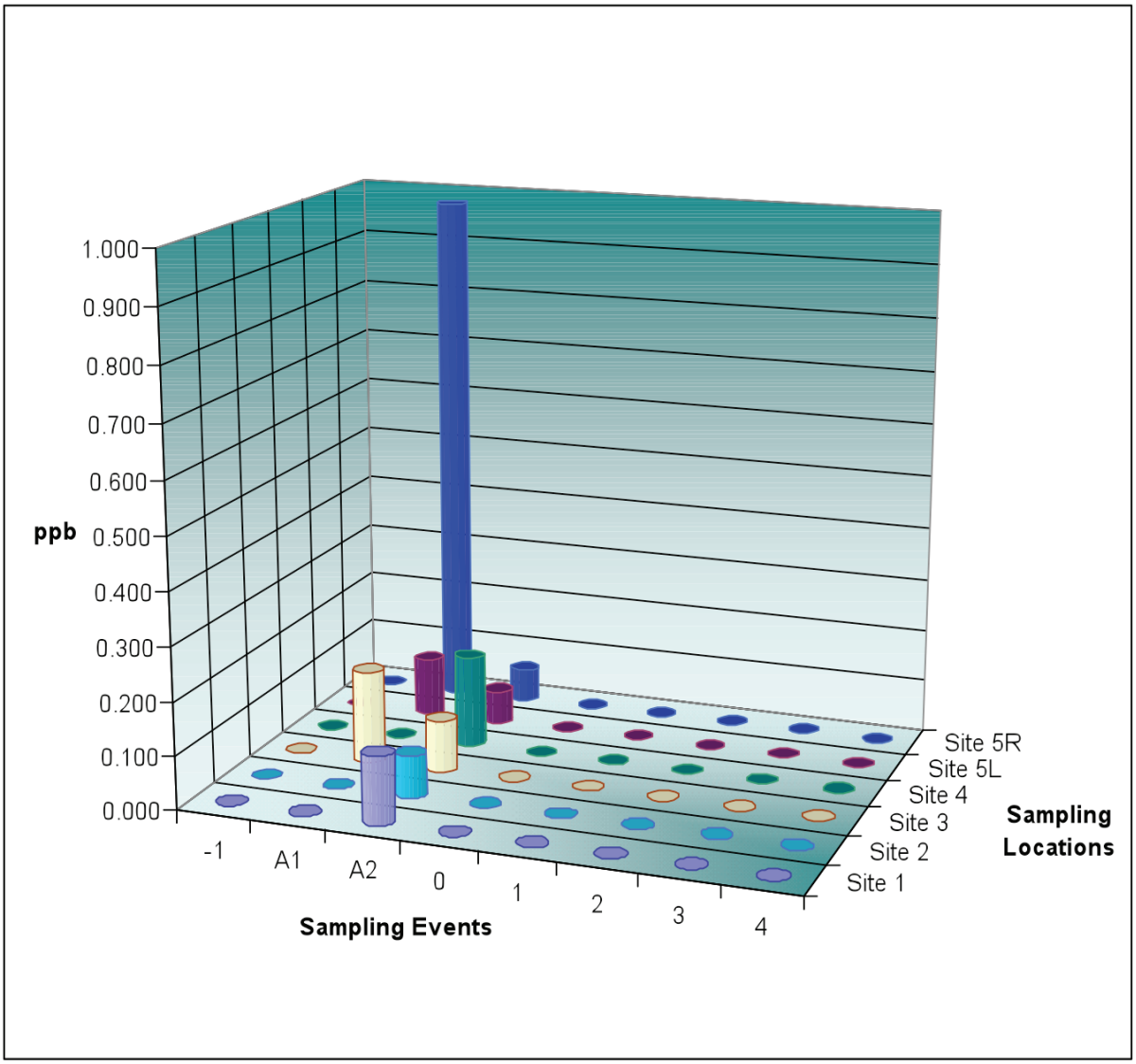
*** A1 = air monitoring at ca. ¼ of field mustard incorporation**

A2 = air monitoring at ca. ¾ field mustard incorporation

Figure 3

TWA interval air monitoring for Benzyl Isothiocyanate:

October 25 to October 31, 2008*



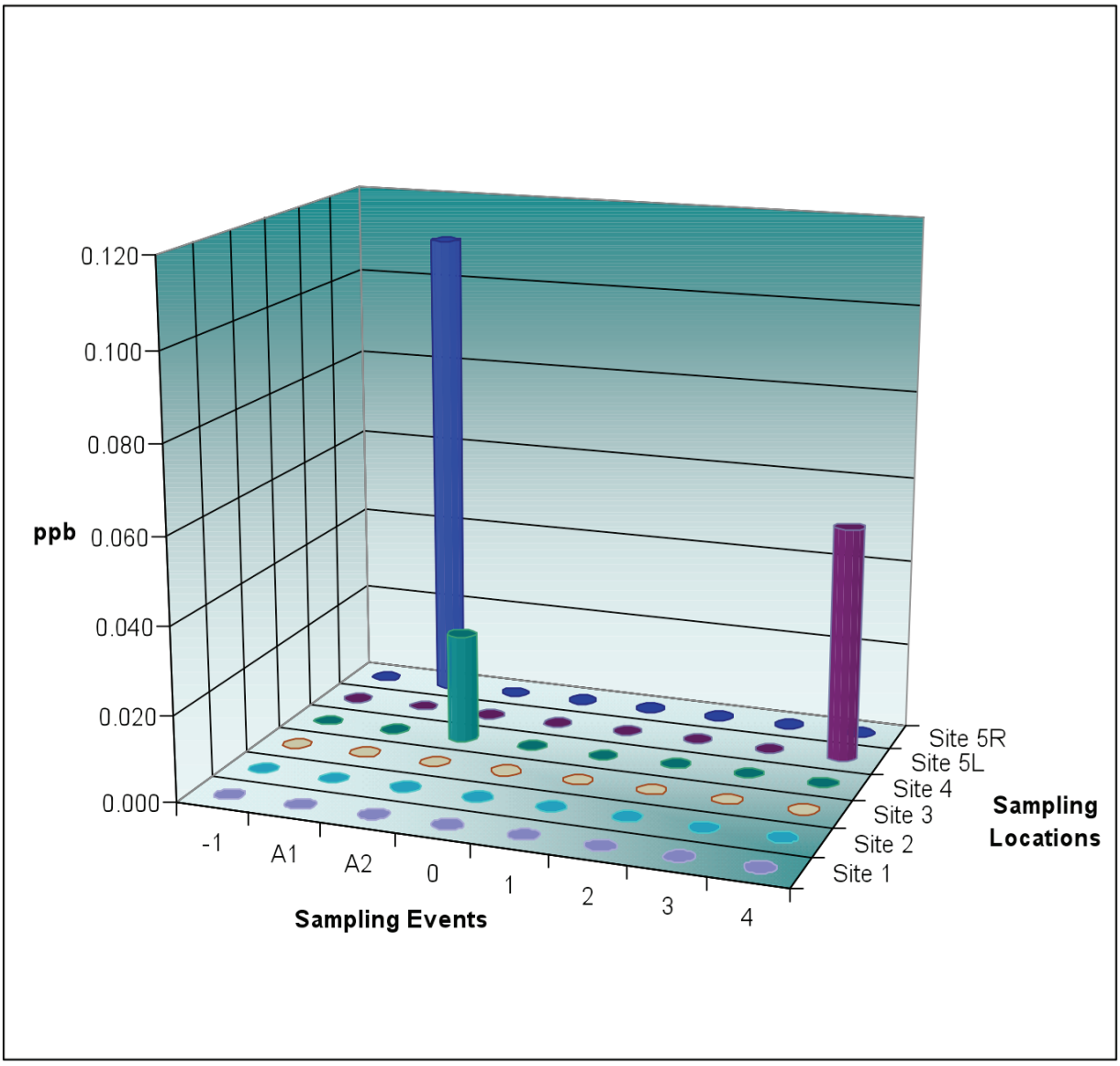
*** A1 = air monitoring at ca. ¼ of field mustard incorporation**

A2 = air monitoring at ca. ¾ field mustard incorporation

Figure 4

TWA interval air monitoring for 2-Phenethyl Isothiocyanate:

October 25 to October 31, 2008*



*** A1 = air monitoring at ca. ¼ of field mustard incorporation**

A2 = air monitoring at ca. ¾ field mustard incorporation

APPENDIX I. NEAR-FIELD NITC AIR MONITORING: FIELD SUMMARY

STUDY SCOPE

The purpose of this study was to estimate near-field NITC emissions after mustard green manure cover crop incorporation and to provide preliminary data for understanding if human risks may occur from inhalation exposure to naturally occurring Allylic and Aromatic ITCs.

A. FIELD PERSONNEL : Donna Trott, Graduate Student, Environmental Sciences WSU-TC

B. FIELD SITING

The center pivot circle was seeded in the summer of 2008 with a mixture of oriental and yellow mustard. Four activated charcoal air sampling masts (receptors) were positioned ca. 10-20 meters from field edge of the crop circle perimeter. The receptors were positioned to be accessible without interfering with field mustard green manure operations. The four perimeter receptors consisted of a single SKC air sampling unit (SKC Model 224-PCXR8), placed at the base of a ring-stand with a vertical crossbar set at 1 meter above ground. A fifth receptor location with co-located activated charcoal cartridges was positioned to the permanent structure at the center of the circle pivot. Tubing connected the personal sampler to the 600 milligram activated charcoal-filled glass cartridge located horizontally on the crossbar. The cartridge was shielded from ultraviolet light using a 1" PVC sleeve. The battery-powered units sampled air at ca. 2-L min^{-1}. Actual flows for each sampling cartridge were measured

by a rotometer at the start and end of each sampling period and recorded. The averaged two-point flow rate reading and sampling duration were used to calculate the total air volume. Additionally, an outdoor location at the WSU Tri-Cities campus served for conducting fortified NITC activated charcoal air evaluations.

C. NITC AIR MONITORING

The mustard cover crop was flail chopped on two separate days (October 25th and 27th). Flail chopping was followed within approximately 20 minutes by soil incorporated disking of the green chopped manure. NITC near-field monitoring was performed before, during, and 4 days post-green manure incorporation. There were 8 monitoring intervals/periods with 6 air samples taken per event for a total of 48 combined field receptor samples (see below).

(-1)	Prior to green manure incorporation	October 25
(A1)	First 2.5 hours of green manure incorporation	October 25
(A2)	Last 6 hours of green manure incorporation	October 27
(0)	Immediately following green manure incorporation	October 27
(1)	First day following green manure incorporation	October 28
(2)	Second day following green manure incorporation	October 29

(3)	Third day following green manure incorporation	October 30
(4)	Fourth day following green manure incorporation	October 31

Air was sampled mid-day for approximately 4 hours for each of the periods before and after cover crop incorporation. However, during flail chop and disking, the duration of the sampling events was modified to allow for field-related scheduling difficulties and safely accessing receptor locations. For example, due to a late afternoon start of green manure field incorporation on the first day, air monitoring during period A1 (the first ¼ of biofumigation), was limited to approximately 2.5 hours. The second "during application" A2 interval was delayed one additional day due to scheduling but began early in the morning on October 27th and continued until completion over a period of six hours. The dates and times for sample placement for all intervals are provided in Appendix B.

After each sampling event the activated charcoal sample tubes were removed from the receptor and transferred on blue ice to the Food & Environmental Quality Laboratory (FEQL), Washington State University, 2710 University Drive Richland, WA where they were placed in frozen storage at -80°C. Trip blanks routinely accompanied the sample shipment. Additionally, at the WSU-Tri-City campus, 600 mg blank activated charcoal cartridges was fortified and air sampled at a flow rate of

2-L min^{-1} for ca. six-hours to assess breakthrough and stability of evaluated NITCs. A control charcoal sample was run concurrently with these fortified field spikes.

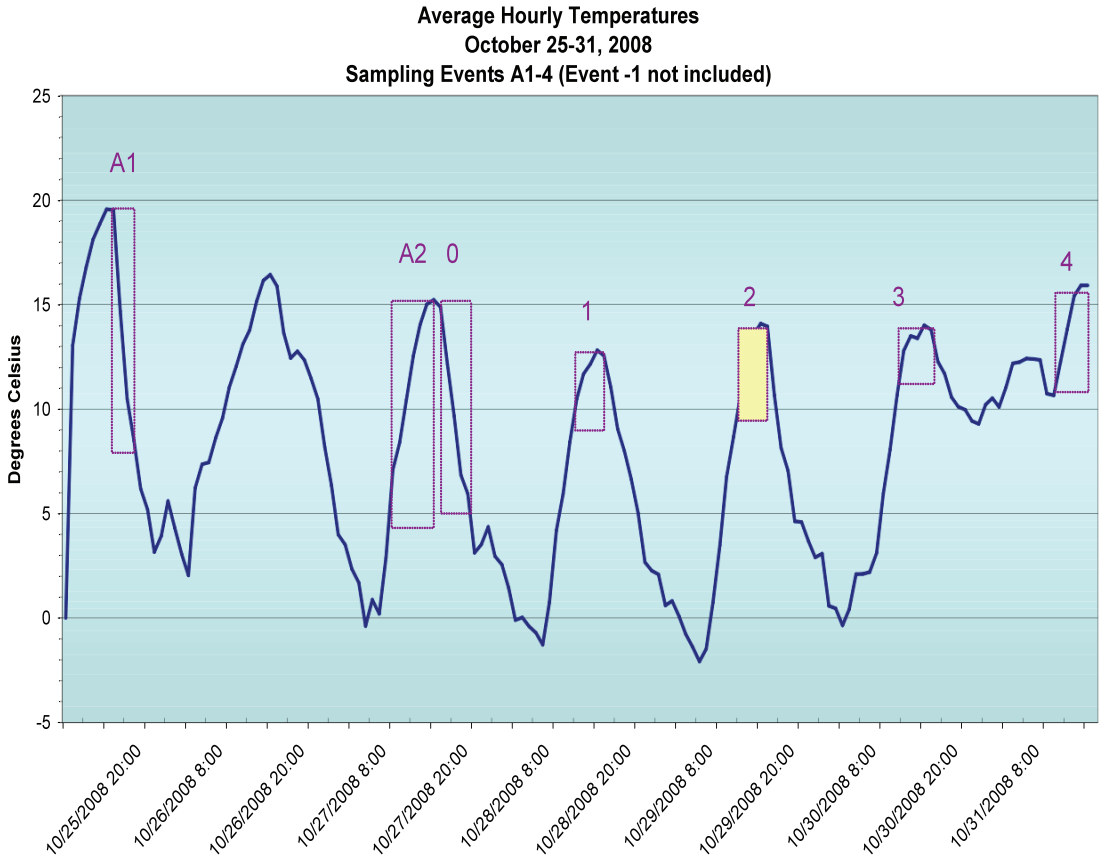
D. FIELD DOCUMENTATION AND RECORD KEEPING

All operations, data and observations appropriate to this study were recorded directly into the FIELD DATA BOOK (FEQL-1508). The data book for this study serves as an authentic record of fieldwork. All field data information will be archived with the project study file and summary report for a period of 5 years.

E. WEATHER DATA

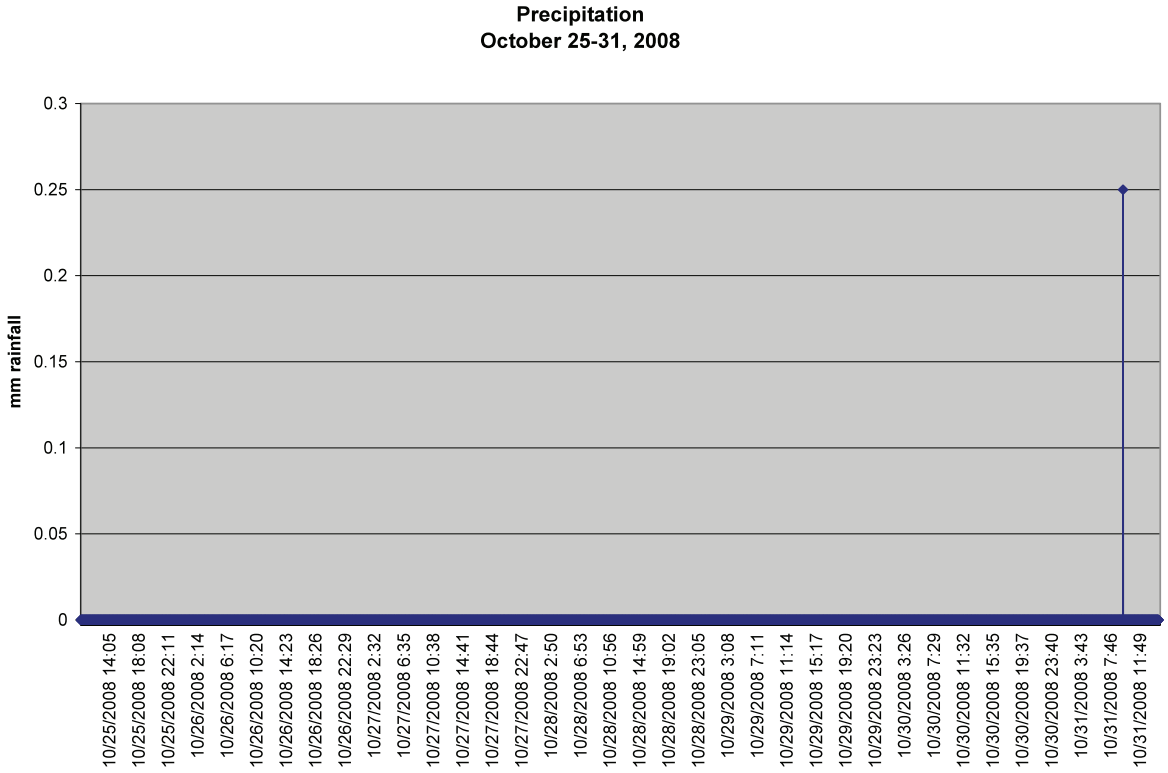
Weather data was collected by a WSU AgWeatherNet weather station at Benton City (Weather Station #75). This weather station was within 3 miles of the sampling site locations. Figures 5, 6, and 7 summarize air temperatures, precipitation, and wind velocity/direction, respectively, during the 6 day study period. An expanded data set (by hour) from the WSU AgWeatherNet weather station is provided in Attachment C.

Figure 5
Daily Air Temperature Data: Weather Station #75



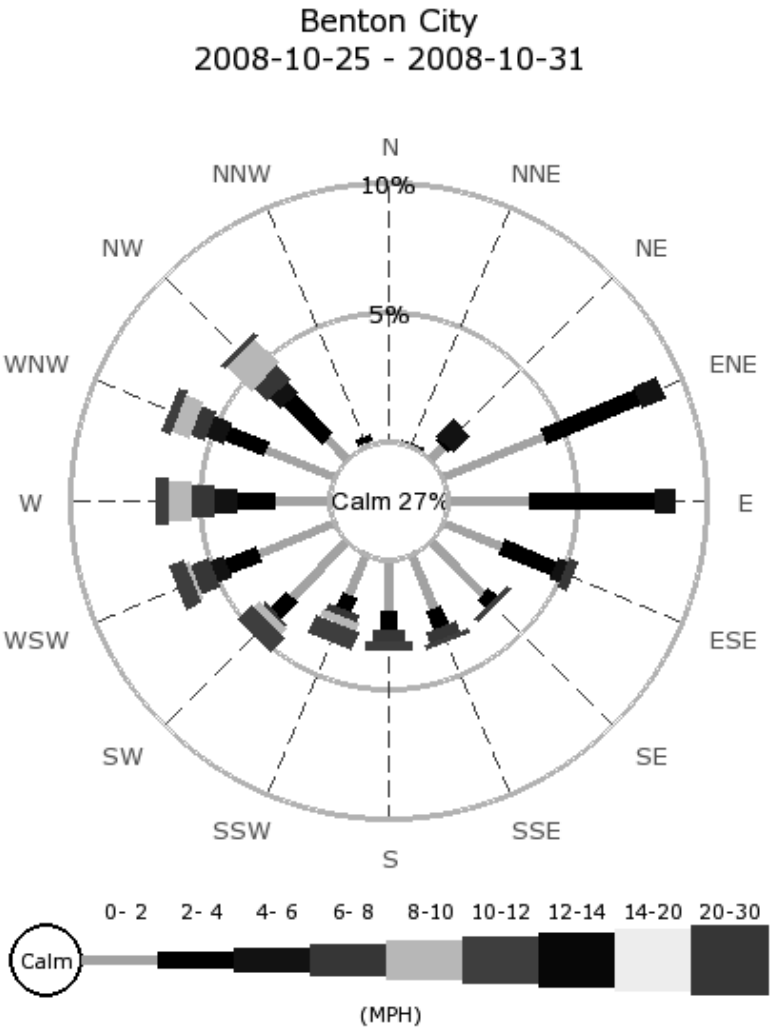
Source: On-site Hobo Weather station

Figure 6
Daily Precipitation Data: Weather Station #75



Source: On-site Hobo Weather station

Figure 7
Wind Rose Data: Weather Station #75



Source: AgWeatherNet http://weather.wsu.edu/

APPENDIX II: NEAR-FIELD NITC AIR MONITORING; ANALYTICAL SUMMARY

A. Introduction

An analytical method was developed and validated for extracting three natural isothiocyanates (NITC) from charcoal sampling cartridges. In 2005, FEQL validated a method for determining methyl isothiocyanate (MITC) from charcoal sampling tubes (Merriman and Hebert, 2007). This method was adapted from California Department of Pesticide Regulation "*Air Monitoring for Methylisothiocyanate During a Sprinkler Application of Metam-Sodium"* Report EH 94-02, 1994. The procedure involved extraction of the charcoal media using a 1:1 mixture of carbon disulfide:ethyl acetate followed by sonication, and filtration through a 0.45µm Teflon membrane. The sample extract was then analyzed by gas chromatography using thermionic specific detection (TSD). These earlier methods relied on a 80:20 ethyl acetate: carbon disulfide solvent mixture ratio. To improve NITC extraction efficiency, the air-exposed charcoal cartridges were solvent extracted using a 1:1 mixture of carbon disulfide: ethyl acetate (i.e., 50% carbon disulfide in ethyl acetate). This modified method was validated, in triplicate, at three fortification levels covering the range of anticipated NITC concentrations in air (Table 2a).

The method limit of quantitation (LOQ) was established at 0.5µg on activated charcoal for a total air volume of 480 L (2-L min^{-1}) over an averaged 4-hour air sampling period and adjusted for NITC air concentration (in ppb). The analytical method for the measurement of NITC was found to be rugged with LOQ of 0.25 ppb for AITC, 0.16 ppb for BITC and 0.15 ppb for PEITC. Laboratory fortifications percent recoveries ran with each set of solvent

extracted field samples ranged from 84 to 118% for AITC, 73 to 97% for BITC, and 77 to 111% for PEITC. All combined laboratory method recoveries fell within 10% standard deviation (Table 2b). Linearity as measured by linear regression correlation (R^2) of a minimum of 4 standards among the range of encounter air concentrations for all analytical sets was ≥ 0.98.

Working mix-stock solutions in appropriate dilutions were prepared from individual NITC reference standards for fortification and quantitation. Concentrations of the selected NITCs from solvent extracted charcoal cartridges were calculated by linear regression from 4 external standards. Instrument calibration standards bracketed for every 2 or 3 samples in the analytical set.

B. Materials and Methods

1. Equipment

The following equipment and/or its equivalent were used in this study:

Sartorius Micro M5P analytical balance

Sartorius LC3200D top-loading balance

Standard laboratory glassware and equipment

Ultrasonic bath (VWR brand)

Varian Star Chromatography Workstation

Varian Star 3400cx Gas Chromatograph

Varian 8200cx Auto Sampler

2. Reagents

The following reagents and/or equivalents were used in this study. All solvents were pesticide-analysis grade or better.

Analytical standards (Sigma-Aldrich, Inc.)

Carbon disulfide

Ethyl acetate

0.45 μm Teflon® membrane filter (Whatman®)

3. Standards

Standards were prepared to bracket the range of NITC concentrations expected in solvent extracted activated charcoal cartridges. The following test substances, standards, and standard dilutions were used for this study:

Table 1 Standards

Test substance

Compound	**Substance No.**	**Purity**	**Source**
Allylic isothiocyanate (AITC)	1347	95%	Aldrich
Benzyl isothiocyanate (BITC)	1346	98%	Aldrich
Phenethyl isothiocyanate (PEITC)	1348	99%	Aldrich

Mix Stock Solution

Compound	**Reference No.**	**Conc.**	**Solvent**
AITC-BITC-PEITC	M1347	10 mg/mL	EtOAc

AITC-BITC-PEITC	M1347-1	1 mg/mL	EtOAc
AITC-BITC-PEITC	M1347-2	100 µg/mL	EtOAc

Dilution of Stock Solution

Compound	**Reference No.**	**Conc.**	**Solvent**
AITC-BITC-PEITC	M1347-3	10 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-4	8 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-5	4 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-6	2 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-7	1 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-9	0.5 µg/mL	50% CS_2/EtOAc
AITC-BITC-PEITC	M1347-8	0.1 µg/mL	50% CS_2/EtOAc

Fortification Solutions

Compound	**Substance No.**	**Conc.**	**Solvent**
AITC-BITC-PEITC	M1347	10 mg/mL	EtOAc
AITC-BITC-PEITC	M1347-1	1 mg/mL	EtOAc
AITC-BITC-PEITC	M1347-2	100 µg/mL	EtOAc

All standard solutions were stored in the refrigerator at ca. 4 °C (I.D.Blitzen).

Dilutions are recorded in the FEQL Analytical Laboratory Standards Logbook.

4. Instrumentation

A Varian Star 3400CX gas chromatograph using thermionic specific detection (TSD) with 8200CX autosampler was used for NITC detection and quantification. Integration of chromatographic data was performed using Varian Star Chromatography Workstation software.

Column:	EC-WAX, 15m x 0.53mm, 1.2 μm film thickness
Carrier gas:	Ultrapure helium, column flow rate ca 2-4 mL/min.
Temperatures:	Detector: 300°C
	Injector port: 55 to 225°C (225°C/min), hold for 16 min.
	Oven program:
	Initial: 55°C, hold for 0.09min.
	Ramp 20°C/min to 175°C
	Ramp 15°C/min to 250°C, hold for 5 min.
Injection Volume:	1 uL
Retention Time:	NITC retention time is based on the observed retention times of external calibration standards in each set and dependent upon instrument used.

Detector Gases: Typical TSD detector gas flows were set at approximately 3-4 mL/min hydrogen, ca.170 mL/min air, and 25-30 mL/min makeup gas. The TSD bead current was adjusted as necessary from 3.1 to 3.25 A

5. Quantitation

The quantitation of the three NITCs was performed by electronic peak area measurement. AITC, BITC and PEITC concentrations were calculated by linear regression from a minimum of four external standards in the concentration range of the matrix-samples. For quality control during the GC operation, a laboratory matrix control and matrix fortified sample accompanied each analytical set. All samples were bracketed with external calibration standards. For each analytical set, at least four linearity standards were used in the calculation of the linear regression curve using a spreadsheet program (Microsoft Excel®). The estimated concentration of each NITC in the sample extract was corrected for dilution by multiplying by the final volume of extract. The NITC values (in µg) were calculated according to the following equations.

Eq 1: Total NITC (µg) = (x µg/mL detected concentration) (Final volume of extract, mL)

For example, sample set A1 included the preparation of air sample GM5(A1)102508 (sample date 10/25/08). The sample was processed for analysis to a final volume of 3 mL. The AITC linear regression line of best fit calculated from the 0.1-2 µg/mL calibration standards ($R^2 = 0.999$) of this set was:

$$Y = m X + B$$

Y (area counts) = 40408 X(detected concentration in µg/mL) - 775.46

The AITC-peak area count for this sample was 57738. Therefore, the concentration (in µg/mL) was:

$$X = \frac{(57738 + 775.46)}{40408} = 1.448 \text{ µg/mL}$$

The total Allyl NITC is then calculated according to Eq. 1:

Eq 1: 1.448 µg/mL x 3 mL = 4.344 µg AITC

Once the total micrograms per sample was obtained, the concentration per cubic meter was calculated by equation 2.

Eq 2: $\mu g/m^3$ = (x µg total AITC per sample)/ (total m^3 of air sampled)

From the example above, 0.26 m^3 air sampled:

$$\mu g/m^3 = 4.344\ \mu g\ AITC / 0.264\ m^3 = 16.455\ \mu g/m^3 \text{ or } 4.114\ ppb\ AITC$$

Each sample air concentration represents the amount of NITCs collected over the specific time interval of the sample. Cartridge sampling times, and beginning and ending flow rates, were recorded in the Field Data Book and used to calculate the total amount of air sampled for each individual cartridge.

To assess overall analysis precision and percent recovery a control sample was fortified with a known amount of NITC prior to extraction. For each analytical set, percent recovery for the fortified sample was calculated using peak areas according to the Equation 3.

Eq.3: $$\% \text{ Recovery} = \frac{(\text{Fortified Peak} - \text{Control Peak})\text{Calculated total NITC}}{\text{Fortification Amount}} \times 100$$

Example: The 600 mg-cartridge 1508-FS15, in sample set A1, was fortified with 0.5 µg of a AITC-BITC-PEITC mixed stock solution. The sample extract was prepared to a final volume of 3 mL for residue determination.

The AITC peak area count for this fortified sample was 6454. The corresponding control sample was none-detected. The fortified sample concentration was:

$$(6454\text{-}0) = 40408 \text{ X } \text{-}\text{-} 775.46$$

$$X = \frac{6454 + 775.46}{40408} = 0.179 \text{ μg/mL AITC}$$

The total concentration is then calculated according to *Eq. 1*:

$$0.179 \text{ μg/mL} \times 3 \text{ mL} = 0.537 \text{ μg AITC}$$

As there was no detected AITC in the control sample in this set, by *Eq.3*, the percent recovery for this fortified sample was:

$$\text{Percent Recovery} = \frac{(0.537 \text{ μg})}{0.5 \text{ μg}} \times 100 = 107\%$$

6. Confirmatory Techniques

Analytical standards were used to detect the presence of AITC, BITC, and PEITC in air samples by retention time. In the event that the GC did not confirm the presence of NITC, values were reported as "Not Detected" (ND). When NITC was detected but

the values per air volume sampled were lower than the calculated limit of quantitation but greater than the method limit of detection, concentrations were reported as parenthetical values.

7. Time Required For Analysis

The time required for an experienced person to work up a set of exposed activated charcoal cartridges with QC samples (6 samples plus 2 QC) for analysis was approximately 2 hours. The time required for the GC analysis of a single sample was approximately 16 minutes. The duration of the analysis of a sample set depended upon the number of samples in a set and was automated using the auto sampler associated with the instrument.

C. Results

Table 2a provides recovery information for the validation of AITC, BITC, and PEITC at 0.5, 5, and 25 µg on cartridge (in triplicate). Because of higher than anticipated NITC air concentrations, a series of three 60 µg on cartridge were additionally performed. All validations fell well within 70-120% recovery range with good precision among replicate samples. The laboratory fortifications ran with each set of solvent extracted field samples ranged from 84 to 118% for AITC, 73 to 97% for BITC, and 77 to 111% for PEITC. All combined laboratory method recoveries fell within 10% standard deviation (Table 2b).

Field-simulated fortifications were run outdoors at the WSU-Tri-City campus after completion of the green manure field study. Air was sampled at a rate of 2 L min^{-1} for ca. 6 hours, the longest field sampling time before cartridge collection. Percent recoveries for all three ITCs over this period were found to be quantitative (Table 2c).

Table 3 presents storage stability for the three ITCs. The maximum storage interval to solvent extraction/GC determination was 149 days. An earlier AITC/BITC/PEITC -80^{o}C storage stability was initiated in 2008 (Project # FEQL-1207C). Samples were assessed after a frozen storage interval of 408 days. The recovery results listed in Table 3 indicates that AITC and PEITC should be expected to be stable over this period with recoveries > 80%. However, BITC may be less stable (i.e, 64% recovery) over this storage period. Air concentration data was not adjusted for possible losses during frozen storage.

Table 4 reports air concentrations for all individually ran activated charcoal cartridges with their corresponding trip blank shipment cartridges. The highest observed NITC concentrations occurred during tractor flail-chopping and disking field operations with maximum single receptor air concentrations of 47 ppb, 1 ppb, and 0.1 ppb, respectively, for the allylic-, benzyl-, and 2-phenethyl isothiocyanates. NITC measured air emissions appreciably attenuated shortly after soil incorporation activities ceased.

Encountered Problems: There was substantial variation (ca. 12-fold) among the two exposed cartridges that were co-located at Site 5 (center pivot) during period A1. This was the period where we observed the highest concentrations for all NITCs. Pump failure cannot explain this difference in measured NITC concentrations. What may be a source of variation was orientation of the sampling cartridge on the receptor. The cartridge air in-take orientation

of was ca. 90°. Wind direction may have been a contributing factor in the discrepancy of air concentrations among these two cartridges at this receptor site. On all other sampling events (-1, A2, 0, 1, 2, 3, and 4-days post green mustard incorporation), the air concentrations from these two co-located samples were in agreement.

A single spiked trip sample was shipped with field treatment samples on the 4th day after mustard green manure incorporation. This field spike was prepared with a working standard from a previous study, and solvent effects may have occurred. The results reported in Table 2d ranged from 59% for BITC to 150% for PEITC. Although tabulated in this report, the use of an older working standard for fortification brings question as to the reliability of the AITC, BITC, and PEITC values reported for the fortified (spiked) trip sample.

Table 2a
Method Validation Results

Fortification (µg NITC)*	ITC	Recovery Range (%)	Average Recovery (%)	SD (%)	Number of Forts
0.5	Allyl	108 - 111	110	1.7	3
	Benzyl	90 - 91	91	0.9	
	Phenethyl	92 - 95	93	1.1	
5	Allyl	99 - 117	108	8.9	3
	Benzyl	77- 87	81	5.2	
	Phenethyl	81 - 94	86	6.5	
25	Allyl	113 - 117	115	2.0	3
	Benzyl	86- 90	88	1.9	
	Phenethyl	91 - 96	94	2.2	

Table 2b
Laboratory Fortifications for Allyl, Benzyl and Phenethyl ITC Analysis

Fortification (µg NITC)*	ITC	Recovery Range (%)	Average Recovery (%)	SD (%)	Number of Forts
0.5	Allyl	105.2 - 118.3	110	3.9	10
	Benzyl	90.0 - 96.8	94	2.5	
	Phenethyl	92.2 - 110.6	99	6.0	
5.0	Allyl	95.7 - 107.1	103	4.9	6
	Benzyl	84.9 - 94.1	90	3.2	
	Phenethyl	89.9 - 101.4	96	4.1	
60**	Allyl	83.5 – 97.6	93	4.7	7
	Benzyl	73.0 – 88.3	84	5.1	
	Phenethyl	77.4 – 91.9	88	5.1	

* in 3 mL extraction solvent (see Working Method, Appendix D)
** High fortifications and samples diluted for analysis

Table 2c
Summary of Field Spiked Fortification (FF) Recoveries

Fortification (µg NITC)*	ITC	Recovery Range (%)	Average Recovery (%)	SD (%)	Number of Forts
60	Allyl	89-93	91	2.6	2
60	Benzyl	80-86	83	3.9	2
60	Phenethyl	86-92	89	4.4	2

Table 2d
Trip Spiked Fortification(TS) Recovery
(ID: SRB103108)

Fortification (µg NITC)	ITC	Recovery Range (%)	Recovery (%)	SD	Number of Forts
50	AITC	N/A	77	N/A	1
50	BITC	N/A	59	N/A	1
50	PEITC	N/A	150	N/A	1

Table 3
Storage Stability

(408 days) 2/11/08 - 3/25/09

Sample ID	Days in storage	AITC Recovery (%)	BITC Recovery (%)	PEITC Recovery (%)
SS 2-11-08 MP1	408	91	65	90
SS 2-11-08 MP2	408	88	64	89
SS 2-11-08 MP3	408	88	63	88
Avg		**89**	**64**	**89**
std dev		2.0	0.9	1.0
min		88	63	88
max		91	65	90

Table 4
NITC Air Concentrations

			AITC		BITC		PEITC	
Sample Date	**Sample ID**	**Total Air volume sampled (m^3)**	**Results ($\mu g/m^3$)**	**Results (ppb)**	**Results ($\mu g/m^3$)**	**Results (ppb)**	**Results ($\mu g/m^3$)**	**Results (ppb)**
10/25/2008	GM1(-1)	0.33	ND	ND	ND	ND	ND	ND
	GM2(-1)	0.38	ND	ND	ND	ND	ND	ND
	GM3(-1)	0.35	ND	ND	ND	ND	ND	ND
	GM4(-1)	0.31	ND	ND	ND	ND	ND	ND
	GM5(-1)	0.38	(0.21)	(0.05)**	ND	ND	ND	ND
	GM5(-1)-R	0.41	(0.20)	(0.05)	ND	ND	ND	ND
	GMTB(-1)	0.00	ND	ND	ND	ND	ND	ND
10/25/2008	GM1(A1)	0.26	2.9	0.72	ND	ND	ND	ND
	GM2(A1)	0.25	2.6	0.64	ND	ND	ND	ND
	GM3(A1)	0.27	34.6	8.6	1.1	0.18	ND	ND
	GM4(A1)	0.26	16.0	4.0	ND	ND	ND	ND
	GM5(A1)	0.26	16.5	4.1	(0.69)	(0.11)	ND	ND
	GM5(A1)-R	0.27	188	47.0	6.5	1.0	(0.73)	(0.11)
	GMTB(A1)	0.00	ND	ND	ND	ND	ND	ND
10/27/2008	GM1(A2)	0.68	17.5	4.4	(0.81)	(0.13)	ND	ND
	GM2(A2)	0.66	7.2	1.8	(0.50)	(0.08)	ND	ND
	GM3(A2)	0.63	9.0	2.2	(0.60)	(0.10)	ND	ND
	GM4(A2)	0.67	20.0	5.0	1.1	0.17	(0.17)	(0.03)
	GM5(A2)	0.56	5.4	1.3	(0.39)	(0.06)	ND	ND
	GM5(A2)-R	0.52	5.0	1.2	(0.38)	(0.06)	ND	ND
	GMTB(A2)	0.00	ND	ND	ND	ND	ND	ND
10/27/2008	GM1(0)	0.48	(0.56)	(0.14)	ND	ND	ND	ND
	GM2(0)	0.48	ND	ND	ND	ND	ND	ND
	GM3(0)	0.47	ND	ND	ND	ND	ND	ND
	GM4(0)	0.49	(0.91)	(0.23)	ND	ND	ND	ND
	GM5(0)	0.50	(0.55)	(0.14)	ND	ND	ND	ND
	GM5(0)-R	0.26	1.0	0.26	ND	ND	ND	ND
	GMTB(0)	0.00	ND	ND	ND	ND	ND	ND
10/28/2008	GM1(1)	0.46	ND	ND	ND	ND	ND	ND
	GM2(1)	0.46	ND	ND	ND	ND	ND	ND
	GM3(1)	0.46	ND	ND	ND	ND	ND	ND
	GM4(1)	0.46	ND	ND	ND	ND	ND	ND
	GM5(1)	0.46	ND	ND	ND	ND	ND	ND

			AITC		BITC		PEITC	
Sample Date	**Sample ID**	**Total Air volume sampled (m^3)**	**Results ($\mu g/m^3$)**	**Results (ppb)**	**Results ($\mu g/m^3$)**	**Results (ppb)**	**Results ($\mu g/m^3$)**	**Results (ppb)**
	GM5(1)-R	0.39	ND	ND	ND	ND	ND	ND
	GMTB(1)	0.00	ND	ND	ND	ND	ND	ND
10/29/2008	GM1(2)	0.46	ND	ND	ND	ND	ND	ND
	GM2(2)	0.45	ND	ND	ND	ND	ND	ND
	GM3(2)	0.47	ND	ND	ND	ND	ND	ND
	GM4(2)	0.47	ND	ND	ND	ND	ND	ND
	GM5(2)	0.47	ND	ND	ND	ND	ND	ND
	GM5(2)-R	0.46	ND	ND	ND	ND	ND	ND
	GMTB(2)	0.00	ND	ND	ND	ND	ND	ND
10/30/2008	GM1(3)	0.45	ND	ND	ND	ND	ND	ND
	GM2(3)	0.45	ND	ND	ND	ND	ND	ND
	GM3(3)	0.46	ND	ND	ND	ND	ND	ND
	GM4(3)	0.45	ND	ND	ND	ND	ND	ND
	GM5(3)	0.45	ND	ND	ND	ND	ND	ND
	GM5(3)-R	0.45	ND	ND	ND	ND	ND	ND
	GMTB(3)	0.00	ND	ND	ND	ND	ND	ND
10/31/2008	GM1(4)	0.44	1.3	0.34	ND	ND	ND	ND
	GM2(4)	0.42	1.2	0.29	ND	ND	ND	ND
	GM3(4)	0.46	1.3	0.33	ND	ND	ND	ND
	GM4(4)	0.44	3.0	0.75	ND	ND	ND	ND
	GM5(4)	0.45	3.4	0.85	ND	ND	(0.36)	(0.05)
	GM5(4)-R	0.45	3.2	0.80	ND	ND	ND	ND
	GMTB(4)	0.00	ND	ND	ND	ND	ND	ND

* ND - not detected

** Values in parentheses are estimated concentrations that are above the method detection but below the method limit of quantitation

ATTACHMENT A: PROJECT PROTOCOL

Field and Analytical Protocol
Project No. FEQL-1508

PROJECT TITLE: **Assessing Near-field Naturally Occurring Isothiocyanate Emissions after Mustard Green Manure Cover Crop Incorporation**

PROJECT COORDINATOR	Vince Hebert: Laboratory Research Director
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Address:	2710 University Drive, Richland WA 99352
Telephone:	509-372-7393

PROJECT DURATION: October 2008 though March 31, 2009

PROJECT SUMMARY

The term 'biofumigation' describes the suppression of soil-borne pests and diseases by glucosinolate-containing plants (notably the *Brassicaceae*) arising specifically from the biocidal properties of the glucosolinate hydrolysis products, particularly isothiocyanates (ITCs), released from incorporated tissues of cover crops. One aliphatic (2-propenyl) and two aromatic isothiocyanates (benzyl and 2-phenylethyl) will be monitored near-field in air before, during, and possibly up to 10-days post cover crop ground incorporation. These three naturally occurring isothiocyanates exist at comparatively high concentration in readily available high biomass-producing brassicas used in the Columbia Basin (oriental mustard, *Brassica juncea* and yellow mustard, *Sinapis alba*). This preliminary near-field screening assessment just before after green manure ground incorporation will directly aid in assessing whether naturally occurring fumigant emissions can pose a near-field inhalation exposure concern to bystanders.

APPROACH

Field location and green manure incorporation: A center pivot circle in Benton County WA seeded in the summer of 2008 with a mixture of oriental and yellow mustard will be flail chopped followed immediately by disking in mid-to late October. Flail chopping and disking incorporates the green manure treatment into the top 6" of soil. Specific field information for this site (i.e, location, weather conditions during and post incorporation, original observations, etc.) will be documented in a FIELD DATA BOOK (see below for data book requirements).

Air Monitoring: Uniform siting procedures at the field plot will include positioning a minimum of four mast air samplers at ca. 90° around the periphery of the crop circle; a fifth mast will be placed in the center of the pivot circle (see Figure 1). Wind speed and direction data together with soil temperature and precipitation will be gathered using a portable weather station. Pre-incorporation (-1 day) air sampling for 2-propenyl, benzyl and 2-phenylethyl ITCs will be conducted at this site. These ITCs will be monitored during and through 4 days post-green manure incorporation.

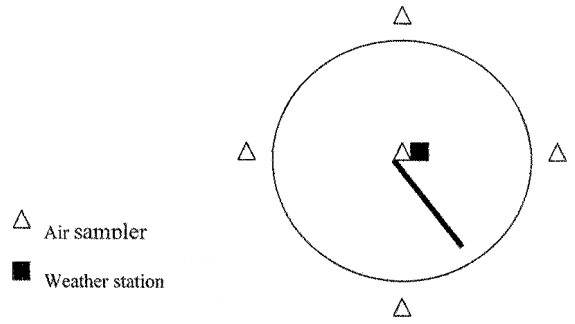


Figure 1: Center pivot air sampler locations

Sampling masts will consist of a post at approximately 1 meter height that can hold one charcoal sampling cartridge. One mast will hold a second co-located duplicate cartridge. Each cartridge will contain a 200/400 milligram coconut charcoal prepared by SKC West, Fullerton. Four-hour air sampling intervals will be conducted pre-incorporation, post application time ¼ and ¾ field incorporation, after completion of incorporation (time zero) and after days 1, 2, 3, and 4 days post-application. A battery-powered air pump (HiCheck Universal Sampler, SKC West) will be set at ca. 2 liters/minute, but actual flow will be measured at the start and end of each sampling period using calibrated flow meters. Field fortifications will be performed to monitor trapping efficiency over the application and post-application sampling interval period at the WSU Tri Cities campus.

Sampling Frequency and Duration: The sampling masts and meteorological equipment will be operated prior to application, during application, and over a number of 4 hour sampling intervals up to 4 days post application. Sampling intervals days may be rescheduled (+ or – 1 day) as a result of climatic conditions over the post-application period.

Proposed Number of Sampling Events
Number of samples: 1 plot x 5 stations x 1 sample/station x 8 sample intervals (-1 day, ¼ and 3/4 application, post application 0, 1, 2, 3, and 4 days). One field sampler will contain a co-located duplicate cartridge (48 total air monitoring samples are planned). A minimum of four field fortifications with respective controls will be conducted over the application-post application interval at the WSU Tri-Cities facility.

Sample Coding: The samples acquired from the field will be given a sample code that will be used to track each sample as it gathered. This code will be constructed so that each site, day,

collocation, time of day and trip blanks will have unique alphanumeric values that will be traceable. The coding will be as follows:

Station Site Code*	Interval Code (in days)**	Code***
Station 1 = GM1	-1, A1, A2, 0, 1, 2, 3, 4	Date of sampling
Station 2 = GM2	-1, A1, A2, 0, 1, 2, 3, 4	Date of sampling
Station 3 = GM3	-1, A1, A2, 0, 1, 2, 3, 4	Date of sampling
Station 4 = GM4	-1, A1, A2, 0, 1, 2, 3, 4	Date of sampling
Station 5 = GM5****	-1, A1, A2, 0, 1, 2, 3, 4	Date of sampling

* Station locations will be kept confidential.
** May be modified if sampling interval is delayed by rain; A1 is ¼ application and A2 is ¾ application sampling
*** Date of acquired field air sample
**** Station GM5 will have co-located replicate samples (GM5-R series)

Treatment blanks that will accompany each shipment and will receive a TB designation. A charcoal tube labeled **GM3-A1-102408** would uniquely identify the sample taken at station 3 one quarter way during soil incorporation of the green manure (GM). The code 102408 would indicate the date for this field air monitoring event.. A charcoal tube labeled **GMTB-1-102508** would indicate that the sample is a trip blank stored with the first day post application samples taken on October 25th, 2008. A sample labeled **GMF-4-102608** would indicate a field fortification (F) at the WSU-TC campus taken on the second day post application, October 26, 2008.

Sample Handling and Quality Control: At the end of each sampling period, the sampling media will be capped with labels uniquely identifying the individual sample. The bags will be transported daily to the WSU Food and Environmental Quality Laboratory where the samples will be stored at – 80° C prior to analysis. A trip blank (i.e., no 2-propenyl, benzyl, and 2-phenylethyl isothiocyanate) and chain of custody documentation will accompany each sample shipment. Fortified spikes will be made to the intakes of the air sampling tubes during the period of air monitoring. These tubes will be run outdoors for 4 hours during the air sampling period at the WSU-Tri Cities campus to verify quantitative field recovery of the vapor-trapped ITCs being examined.

Laboratory Analysis: The Food and Environmental Quality Laboratory (FEQL) is a regulatory science 40 CFR Part 160 Good Laboratory Practices (GLP) facility under the direction of Dr. Hebert. Extraction and analytical methods to be used in this evaluation will require development and validation before samples are analyzed. The selected ITCs in the solvent extract will be determined using gas chromatography with nitrogen-phosphorus thermionic specific detection or by GC/MS. The analytical method will be considered validated if recoveries from fortified field samples prepared at various spiking concentrations (in triplicate) range from 70 to 120%. All steps will be taken to insure sample integrity on an analytical set-by-set basis (i.e., controls, fortifications, calibrations, and linearities). The generated data will be expressed in units of mass per volume ($\mu g\ m^{-3}$) taken over the sampling interval for assessing near-field air residues.

Storage Stability: A frozen storage stability study will be initiated by fortifying 12 charcoal cartridges with a known amount of 2-propenyl, benzyl, and 2-phenylethyl isothiocyanate. Three cartridges will be evaluated at time zero (providing the method has been validated at the time of storage stability fortification), at an anticipated mid-point of storage, and after the longer storage interval of time before analysis.

Statistical Method: Criteria for acceptance of standard curve(s) or other statistical methods shall be determined by the Project Coordinator and documented in the raw data.

Field Documentation And Record Keeping: All operations, data and observations appropriate to this study should be recorded directly and promptly into the FIELD DATA BOOK. General instructions for completion of the field data book can be found in this book. This Data Book was designed for collecting field information and serves as an authentic record of fieldwork. It has six Parts containing the following information:

PART	SUBJECT
1	Personnel Log
2	Communications/ Field Chronological Log and Notes
3	Field Trial Site Information/Placement of Air Samplers
4	Air Sampler Calibration/Field Testing Data Sheets
5	Air Sampler Shipping Information
6	Meteorological Records

Laboratory Documentation and Record Keeping: All operations, data, and observations shall be recorded in the laboratory write-up sheets and laboratory logbooks, which must be signed and dated on date of entry. At a minimum, collect and maintain the following raw data:

- Analytical standard(s) receipt, use and disposition records
- Analytical standard(s) storage conditions
- Analytical standard(s) dilution calculations and preparation records
- Sample storage conditions and locations
- Calculation work sheets
- All chromatograms, including those which are not reported
- Chain of custody records
- Name of personnel conducting specific research functions
- Storage stability fortification records
- Concurrent recovery fortification records

A study file shall be developed and maintained by the FEQL Project Coordinator in conjunction with the analysis. It will contain a copy of the protocol, all pertinent raw data, documentation, records, correspondence, and the final analytical summary report. In addition, records of equipment maintenance and calibrations will be kept and periodically archived.

Analytical Summary Report: The analytical summary report shall contain, but not be limited to:

- Applicable method validation data
- Applicable storage stability data

- Residue levels from near-field air monitoring with concurrent fortified recoveries
- Meteorological data
- Complete copy of the analytical Working Method
- Clearly presented example calculations or statistical evaluations
- Discussion of results (including purpose of method modifications, sample storage conditions, etc.) -summary data associated with calibration standards (dilution and use records, calibration curves, etc.)

Laboratory Archives: When the final analytical summary report is completed the analytical report and all original field (Field Data Book) and analytical raw data will be retained at the FEQL Testing Laboratory. All original raw data shall be secured in the FEQL Testing Laboratory archives.

October 23, 2008

Vincent R Hebert
WSU-FEQL Project Coordinator

Date

ATTACHMENT B: SAMPLE INVENTORY

Sample ID	Air sampler START time	Air sampler END time	Total Air volume sampled (m3)	Extraction Date
GM1(-1)	10/25/08 8:50 AM	10/25/08 11:35 AM	0.33	3/23/2009
GM2(-1)	10/25/08 8:03 AM	10/25/08 11:25 AM	0.38	3/23/2009
GM3(-1)	10/25/08 8:20 AM	10/25/08 11:20 AM	0.35	3/23/2009
GM4(-1)	10/25/08 8:34 AM	10/25/08 11:14 AM	0.31	3/23/2009
GM5(-1)	10/25/08 7:47 AM	10/25/08 11:06 AM	0.38	3/23/2009
GM5(-1)-R	10/25/08 7:45 AM	10/25/08 11:05 AM	0.41	3/23/2009
GMTB(-1)	NA	NA	0.00	3/23/2009
GM1(A1)	10/25/08 3:10 PM	10/25/08 5:14 PM	0.26	3/21/2009
GM2(A1)	10/25/08 3:05 PM	10/25/08 5:11 PM	0.25	3/21/2009
GM3(A1)	10/25/08 2:45 PM	10/25/08 5:05 PM	0.27	3/21/2009
GM4(A1)	10/25/08 2:59 PM	10/25/08 5:08 PM	0.26	3/21/2009
GM5(A1)	10/25/08 2:48 PM	10/25/08 5:00 PM	0.26	3/21/2009
GM5(A1)-R *	10/25/08 2:48 PM	10/25/08 5:00 PM	0.27	3/21/2009
GMTB(A1)	NA	NA	0.00	3/21/2009
GM1(A2)	10/27/08 8:15 AM	10/27/08 2:15 PM	0.68	3/19/2009
GM2(A2)	10/27/08 8:12 AM	10/27/08 2:00 PM	0.66	3/19/2009
GM3(A2)	10/27/08 8:08 AM	10/27/08 1:40 PM	0.63	3/19/2009
GM4(A2)	10/27/08 8:06 AM	10/27/08 1:50 PM	0.67	3/19/2009
GM5(A2)	10/27/08 8:01 AM	10/27/08 1:14 PM	0.56	3/19/2009
GM5(A2)-R	10/27/08 8:04 AM	10/27/08 1:10 PM	0.52	3/19/2009
GMTB(A2)	NA	NA	0.00	3/19/2009
GM1(0)	10/27/08 2:10 PM	10/27/08 6:12 PM	0.48	3/20/2009
GM2(0)	10/27/08 2:05 PM	10/27/08 6:05 PM	0.48	3/20/2009
GM3(0)	10/27/08 1:43 PM	10/27/08 5:45 PM	0.47	3/20/2009
GM4(0)	10/27/08 1:52 PM	10/27/08 5:55 PM	0.49	3/20/2009
GM5(0)	10/27/08 1:20 PM	10/27/08 5:28 PM	0.50	3/20/2009
GM5(0)-R	10/27/08 2:55 PM	10/27/08 5:31 PM	0.26	3/20/2009
GMTB(0)	NA	NA	0.00	3/20/2009
GM1(1)	10/28/08 11:06 AM	10/28/08 2:55 PM	0.46	3/18/2009
GM2(1)	10/28/08 11:12 AM	10/28/08 3:04 PM	0.46	3/18/2009
GM3(1)	10/28/08 11:23 AM	10/28/08 3:14 PM	0.46	3/18/2009
GM4(1)	10/28/08 11:19 AM	10/28/08 3:09 PM	0.46	3/18/2009
GM5(1)	10/28/08 11:31 AM	10/28/08 3:21 PM	0.46	3/18/2009
GM5(1)-R	10/28/08 11:33 AM	10/28/08 3:23 PM	0.39	3/18/2009
GMTB(1)	NA	NA	0.00	3/18/2009
GM1(2)	10/29/08 10:52 AM	10/29/08 2:43 PM	0.46	3/17/2009
GM2(2)	10/29/08 10:59 AM	10/29/08 2:50 PM	0.45	3/17/2009
GM3(2)	10/29/08 11:11 AM	10/29/08 3:07 PM	0.47	3/17/2009

Sample ID	Air sampler START time	Air sampler END time	Total Air volume sampled (m3)	Extraction Date
GM4(2)	10/29/08 11:06 AM	10/29/08 3:02 PM	0.47	3/17/2009
GM5(2)	10/29/08 11:21 AM	10/29/08 3:16 PM	0.47	3/17/2009
GM5(2)-R	10/29/08 11:27 AM	10/29/08 3:17 PM	0.46	3/17/2009
GMTB(2)	NA	NA	0.00	3/17/2009
GM1(3)	10/30/08 11:05 AM	10/30/08 2:51 PM	0.45	3/16/2009
GM2(3)	10/30/08 11:12 AM	10/30/08 2:55 PM	0.45	3/16/2009
GM3(3)	10/30/08 11:22 AM	10/30/08 3:10 PM	0.46	3/16/2009
GM4(3)	10/30/08 11:16 AM	10/30/08 3:01 PM	0.45	3/16/2009
GM5(3)	10/30/08 11:32 AM	10/30/08 3:19 PM	0.45	3/16/2009
GM5(3)-R	10/30/08 11:33 AM	10/30/08 3:20 PM	0.45	3/16/2009
GMTB(3)	NA	NA	0.00	3/16/2009
GM1(4)	10/31/08 10:03 AM	10/31/08 1:47 PM	0.44	3/12/2009
GM2(4)	10/31/08 10:10 AM	10/31/08 1:52 PM	0.42	3/12/2009
GM3(4)	10/31/08 10:15 AM	10/31/08 2:05 PM	0.46	3/12/2009
GM4(4)	10/31/08 10:19 AM	10/31/08 1:58 PM	0.44	3/12/2009
GM5(4)	10/31/08 10:29 AM	10/31/08 2:13 PM	0.45	3/12/2009
GM5(4)-R	10/31/08 10:30 AM	10/31/08 2:14 PM	0.45	3/12/2009
GMTB(4)	NA	NA	0.00	3/12/2009

ATTACHMENT C: WEATHER DATA

WSU AgWeatherNet Daily Report
Station: Benton City
Date: 10-25-2008 to 2008-10-31-2008

Hourly Data Report.
Data Extracted: 2009-05-27.
Station: Benton City.
Lat: 46.3 Lng: 119.5 Elevation: 676.

Date (yyyy-mm-dd)	Hour PST	Air Temp (F)	Dew point (F)	RH (%)	Wind Speed (mph)	Wind Dir (Degree)	Solar Rad (W/m 2)	Precip (in)	Leaf Wet (Unity)	Soil Temp (F)	Soil Mois (%)
2008-10-25	0	42.8	36.2	77.4	2.7	225.4	0.0	0.0	0.0	56.5	1.2
10-25	1	42.1	36.6	80.7	1.9	192.8	0.0	0.0	0.0	56.2	1.1
10-25	2	45.0	37.6	74.9	2.3	271.1	0.0	0.0	0.0	56.0	1.1
10-25	3	47.3	37.4	68.3	4.3	295.3	0.0	0.0	0.0	55.7	1.1
10-25	4	47.6	36.8	66.1	3.5	219.7	0.0	0.0	0.0	55.5	1.2
10-25	5	47.4	36.4	65.4	4.5	241.2	0.0	0.0	0.0	55.3	1.1
10-25	6	49.5	35.2	57.7	8.0	270.2	1.3	0.0	0.0	55.1	1.1
10-25	7	50.6	35.6	56.3	4.9	261.4	37.6	0.0	0.0	54.9	1.1
10-25	8	51.8	37.2	57.3	5.6	259.7	116.1	0.0	0.0	54.7	1.2
10-25	9	54.6	38.6	54.6	7.2	255.9	215.3	0.0	0.0	54.6	1.2
10-25	10	59.8	40.1	48.1	7.3	255.1	389.4	0.0	0.0	54.5	1.2
10-25	11	62.3	40.9	45.3	7.8	276.1	374.6	0.0	0.0	54.5	1.2
10-25	12	63.4	40.4	42.8	4.5	263.1	335.1	0.0	0.0	54.6	1.2
10-25	13	64.5	38.8	38.6	2.2	158.5	333.1	0.0	0.0	54.8	1.2
10-25	14	65.9	37.1	34.4	3.7	237.2	354.3	0.0	0.0	55.3	1.2
10-25	15	66.4	36.4	32.9	1.5	200.6	225.4	0.0	0.0	55.8	1.2
10-25	16	63.4	35.9	35.8	1.8	103.6	71.8	0.0	0.0	56.4	1.2
10-25	17	51.3	35.1	53.8	1.8	82.4	1.7	0.0	0.0	57.1	1.2
10-25	18	42.9	32.2	65.6	1.2	139.8	0.0	0.0	0.0	57.6	1.2
10-25	19	40.3	31.8	71.4	0.8	149.5	0.0	0.0	0.0	58.0	1.2
10-25	20	38.4	31.3	75.5	0.4	202.5	0.0	0.0	0.0	58.2	1.2
10-25	21	34.8	30.0	82.4	1.5	101.5	0.0	0.0	0.0	58.1	1.2
10-25	22	36.5	31.4	81.6	1.7	103.0	0.0	0.0	0.0	57.9	1.2
10-25	23	39.4	31.8	73.9	4.0	90.2	0.0	0.0	0.0	57.6	1.2
10-26	0	42.2	32.0	67.1	3.3	152.1	0.0	0.0	0.0	57.2	1.2
10-26	1	36.1	30.5	80.0	1.6	209.3	0.0	0.0	0.0	56.8	1.1
10-26	2	38.0	31.5	77.3	1.6	203.5	0.0	0.0	0.0	56.3	1.2
10-26	3	43.4	29.6	59.2	4.7	67.5	0.0	0.0	0.0	55.9	1.1
10-26	4	47.3	27.0	45.1	7.2	155.8	0.0	0.0	0.0	55.4	1.1

Date (yyyy-mm-dd)	Hour PST	Air Temp (F)	Dew point (F)	RH (%)	Wind Speed (mph)	Wind Dir (Degree)	Solar Rad (W/m 2)	Precip (in)	Leaf Wet (Unity)	Soil Temp (F)	Soil Mois (%)
2008-10-26	5	47.8	23.4	37.9	7.5	163.4	0.0	0.0	0.0	55.0	1.1
10-26	6	48.5	20.9	33.2	8.2	179.2	3.1	0.0	0.0	54.6	1.1
10-26	7	49.7	19.7	30.3	10.0	229.8	79.0	0.0	0.0	54.2	1.1
10-26	8	52.2	19.3	27.1	11.4	234.8	214.7	0.0	0.0	53.9	1.1
10-26	9	53.9	20.1	26.3	11.9	204.8	341.3	0.0	0.0	53.6	1.1
10-26	10	55.9	21.1	25.6	11.9	221.1	441.7	0.0	0.0	53.4	1.2
10-26	11	57.4	19.3	22.4	12.2	172.4	484.9	0.0	0.0	53.3	1.2
10-26	12	58.9	21.2	23.0	12.3	233.6	473.1	0.0	0.0	53.3	1.2
10-26	13	60.7	21.7	22.0	11.3	285.3	445.6	0.0	0.0	53.6	1.2
10-26	14	61.8	21.5	21.0	11.0	270.0	360.1	0.0	0.0	54.0	1.2
10-26	15	61.3	18.7	19.0	10.7	305.2	216.2	0.0	0.0	54.6	1.2
10-26	16	59.1	16.9	19.0	9.4	322.6	71.8	0.0	0.0	55.2	1.2
10-26	17	56.7	15.9	19.8	8.7	320.6	1.6	0.0	0.0	55.8	1.2
10-26	18	56.6	15.2	19.3	9.0	314.1	0.0	0.0	0.0	56.3	1.2
10-26	19	57.1	16.4	20.0	10.2	279.7	0.0	0.0	0.0	56.7	1.2
10-26	20	55.5	16.7	21.4	8.7	285.4	0.0	0.0	0.0	56.8	1.2
10-26	21	54.2	16.8	22.6	4.4	278.6	0.0	0.0	0.0	56.9	1.2
10-26	22	50.4	18.4	28.2	3.2	277.2	0.0	0.0	0.0	56.8	1.2
10-26	23	40.1	19.1	42.5	0.8	218.3	0.0	0.0	0.0	56.7	1.2
10-27	0	36.7	19.3	49.0	1.7	271.7	0.0	0.0	0.0	56.6	1.1
10-27	1	37.9	21.3	50.8	2.8	305.0	0.0	0.0	0.0	56.3	1.2
10-27	2	34.5	20.4	55.9	1.1	193.7	0.0	0.0	0.0	56.1	1.2
10-27	3	30.6	19.0	62.4	1.3	127.1	0.0	0.0	0.0	55.7	1.1
10-27	4	29.7	19.1	65.2	0.5	153.7	0.0	0.0	0.0	55.3	1.1
10-27	5	29.8	19.0	65.0	0.0	172.3	0.0	0.0	0.0	54.9	1.1
10-27	6	29.7	19.7	67.3	0.8	98.7	3.1	0.0	0.0	54.5	1.1
10-27	7	33.2	21.1	61.1	1.1	218.5	73.1	0.0	0.0	54.0	1.1
10-27	8	43.5	23.0	44.3	3.5	103.3	206.5	0.0	0.0	53.6	1.1
10-27	9	48.3	21.3	34.0	4.6	91.6	332.8	0.0	0.0	53.2	1.1
10-27	10	51.1	22.3	32.0	3.6	104.0	428.6	0.0	0.0	52.8	1.1
10-27	11	53.8	23.5	30.5	3.1	152.1	480.1	0.0	0.0	52.5	1.2
10-27	12	56.2	23.4	27.8	4.0	192.0	482.7	0.0	0.0	52.5	1.2
10-27	13	58.4	24.3	26.7	5.5	75.6	435.1	0.0	0.0	52.7	1.2
10-27	14	59.6	25.0	26.3	6.4	69.0	341.0	0.0	0.0	53.2	1.2
10-27	15	59.1	25.6	27.5	6.1	56.6	207.3	0.0	0.0	53.9	1.2
10-27	16	55.1	26.0	32.4	3.4	80.5	49.4	0.0	0.0	54.7	1.2
10-27	17	46.3	25.3	43.5	1.9	136.1	0.9	0.0	0.0	55.4	1.1
10-27	18	41.5	24.4	50.5	0.1	149.8	0.0	0.0	0.0	56.0	1.1
10-27	19	37.7	23.8	57.1	0.8	151.3	0.0	0.0	0.0	56.4	1.1
10-27	20	36.7	23.5	58.5	0.6	216.8	0.0	0.0	0.0	56.6	1.2
10-27	21	34.8	24.3	65.0	0.0	183.5	0.0	0.0	0.0	56.5	1.2

Date (yyyy-mm-dd)	Hour PST	Air Temp (F)	Dew point (F)	RH (%)	Wind Speed (mph)	Wind Dir (Degree)	Solar Rad (W/m 2)	Precip (in)	Leaf Wet (Unity)	Soil Temp (F)	Soil Mois (%)
2008-10-27	22	37.3	26.1	63.8	1.9	290.3	0.0	0.0	0.0	56.3	1.2
10-27	23	35.3	25.9	68.4	0.7	211.4	0.0	0.0	0.0	56.0	1.2
10-28	0	38.2	26.7	63.0	3.1	307.7	0.0	0.0	0.0	55.7	1.2
10-28	1	37.6	27.0	65.1	3.3	316.3	0.0	0.0	0.0	55.3	1.2
10-28	2	37.0	26.6	65.8	1.8	238.4	0.0	0.0	0.0	54.8	1.1
10-28	3	35.5	26.1	68.1	1.7	304.1	0.0	0.0	0.0	54.4	1.1
10-28	4	35.3	26.3	69.3	2.8	294.0	0.0	0.0	0.0	54.0	1.1
10-28	5	32.7	25.2	74.1	1.2	159.5	0.0	0.0	0.0	53.6	1.1
10-28	6	28.7	23.7	83.0	0.7	157.4	3.1	0.0	0.0	53.1	1.1
10-28	7	31.3	25.9	80.7	1.4	156.3	50.4	0.0	0.0	52.7	1.1
10-28	8	37.8	28.2	68.2	0.7	228.2	168.9	0.0	0.0	52.3	1.1
10-28	9	42.7	27.5	54.7	2.6	288.1	242.0	0.0	0.0	51.9	1.1
10-28	10	47.9	25.7	41.8	1.7	235.0	321.9	0.0	0.0	51.6	1.1
10-28	11	52.5	29.0	40.3	3.2	130.5	443.8	0.0	0.0	51.3	1.1
10-28	12	53.9	28.9	38.0	3.5	161.2	336.6	0.0	0.0	51.3	1.1
10-28	13	54.1	29.6	38.8	3.3	88.8	224.5	0.0	0.0	51.4	1.1
10-28	14	55.9	30.1	37.1	2.0	108.9	279.9	0.0	0.0	51.8	1.1
10-28	15	55.5	30.2	37.8	3.8	75.9	134.0	0.0	0.0	52.3	1.1
10-28	16	52.7	30.2	41.9	3.1	85.4	32.4	0.0	0.0	52.8	1.1
10-28	17	47.5	28.8	48.2	1.4	141.7	2.4	0.0	0.0	53.4	1.1
10-28	18	43.9	29.5	56.7	0.0	162.3	0.0	0.0	0.0	53.9	1.1
10-28	19	38.9	28.3	65.4	0.0	122.1	0.0	0.0	0.0	54.2	1.1
10-28	20	36.1	28.0	72.1	0.1	99.4	0.0	0.0	0.0	54.4	1.1
10-28	21	34.1	27.0	74.9	2.0	98.7	0.0	0.0	0.0	54.4	1.1
10-28	22	33.5	26.9	76.5	0.4	153.6	0.0	0.0	0.0	54.3	1.1
10-28	23	32.4	26.3	78.4	0.6	141.4	0.0	0.0	0.0	54.1	1.1
10-29	0	32.2	26.4	79.1	0.3	106.3	0.0	0.0	0.0	53.8	1.1
10-29	1	32.6	27.7	82.0	1.0	210.7	0.0	0.0	0.0	53.5	1.1
10-29	2	29.5	25.5	86.7	1.3	115.5	0.0	0.0	0.0	53.2	1.1
10-29	3	30.0	26.9	89.7	0.7	226.6	0.0	0.0	0.0	52.8	1.1
10-29	4	29.2	26.8	92.4	0.7	93.8	0.0	0.0	0.0	52.4	1.1
10-29	5	29.0	26.8	93.2	0.2	140.8	0.0	0.0	0.0	52.0	1.1
10-29	6	29.9	27.1	90.5	1.3	272.8	2.4	0.0	0.0	51.6	1.1
10-29	7	32.4	28.2	85.4	0.5	100.5	91.6	0.0	0.0	51.2	1.1
10-29	8	39.6	31.1	71.3	3.3	96.5	143.7	0.0	0.0	50.8	1.1
10-29	9	43.3	32.1	64.6	1.5	177.3	312.4	0.0	0.0	50.5	1.1
10-29	10	47.5	32.7	56.2	2.2	173.7	291.4	0.0	0.0	50.1	1.1
10-29	11	51.0	32.9	49.8	2.8	182.1	420.3	0.0	0.0	50.0	1.1
10-29	12	54.9	32.9	43.1	3.4	175.6	518.0	0.0	0.0	50.0	1.1
10-29	13	56.7	33.3	41.1	4.4	97.5	415.4	0.0	0.0	50.2	1.1
10-29	14	57.4	33.0	39.6	4.7	166.6	317.9	0.0	0.0	50.6	1.1

Date (yyyy-mm-dd)	Hour PST	Air Temp (F)	Dew point (F)	RH (%)	Wind Speed (mph)	Wind Dir (Degree)	Solar Rad (W/m 2)	Precip (in)	Leaf Wet (Unity)	Soil Temp (F)	Soil Mois (%)
2008-10-29	15	57.1	32.7	39.4	3.9	181.9	186.9	0.0	0.0	51.3	1.1
10-29	16	55.0	32.2	41.8	1.0	161.2	59.1	0.0	0.0	52.1	1.1
10-29	17	46.5	30.5	53.8	0.0	71.4	0.7	0.0	0.0	52.9	1.1
10-29	18	40.7	30.4	66.4	0.0	97.7	0.0	0.0	0.0	53.5	1.1
10-29	19	36.9	28.9	72.6	0.0	87.3	0.0	0.0	0.0	54.0	1.1
10-29	20	35.6	28.0	73.8	0.5	134.2	0.0	0.0	0.0	54.2	1.1
10-29	21	33.1	27.8	80.4	2.1	73.1	0.0	0.0	0.0	54.2	1.1
10-29	22	35.2	28.6	76.6	2.4	89.6	0.0	0.0	0.0	54.1	1.1
10-29	23	35.9	29.0	75.6	0.3	163.7	0.0	0.0	0.0	53.8	1.1
10-30	0	33.8	28.8	81.7	0.0	170.2	0.0	0.0	0.0	53.5	1.1
10-30	1	31.4	28.1	88.0	0.0	98.0	0.0	0.0	0.0	53.2	1.1
10-30	2	30.9	28.1	90.5	0.7	212.3	0.0	0.0	0.0	52.8	1.1
10-30	3	32.2	29.9	91.3	0.0	132.8	0.0	0.0	0.0	52.5	1.1
10-30	4	32.5	30.3	91.4	1.0	111.8	0.0	0.0	0.0	52.1	1.1
10-30	5	35.2	31.7	86.8	1.1	209.5	0.0	0.0	0.0	51.7	1.1
10-30	6	34.6	31.6	88.8	1.2	88.9	1.3	0.0	0.0	51.4	1.1
10-30	7	35.7	32.0	86.0	0.0	206.2	52.4	0.0	0.0	51.1	1.1
10-30	8	43.1	34.3	70.9	1.0	288.1	169.5	0.0	0.0	50.8	1.1
10-30	9	46.3	34.8	63.9	1.3	218.2	173.0	0.0	0.0	50.5	1.1
10-30	10	51.6	35.6	54.4	2.2	187.3	346.4	0.0	0.0	50.3	1.1
10-30	11	55.3	35.8	47.7	2.5	118.4	399.1	0.0	0.0	50.3	1.1
10-30	12	57.0	35.6	44.4	4.5	88.1	331.4	0.0	0.0	50.3	1.1
10-30	13	57.3	35.5	43.8	3.6	84.0	180.9	0.0	0.0	50.6	1.1
10-30	14	57.8	35.5	43.1	1.3	108.1	177.9	0.0	0.0	51.0	1.1
10-30	15	57.9	35.9	43.5	2.7	116.1	78.3	0.0	0.0	51.6	1.1
10-30	16	55.2	35.4	47.1	1.5	90.6	22.0	0.0	0.0	52.1	1.1
10-30	17	51.6	35.4	53.8	2.9	59.9	0.2	0.0	0.0	52.7	1.1
10-30	18	52.7	37.3	55.5	2.1	66.6	0.0	0.0	0.0	53.1	1.1
10-30	19	52.0	36.3	54.9	3.9	82.1	0.0	0.0	0.0	53.5	1.1
10-30	20	49.1	36.3	61.2	2.3	89.7	0.0	0.0	0.0	53.7	1.1
10-30	21	48.4	37.3	65.4	3.3	80.0	0.0	0.0	0.0	53.8	1.1
10-30	22	48.3	38.1	67.7	4.2	95.0	0.0	0.0	0.0	53.9	1.1
10-30	23	50.7	38.0	61.5	4.4	168.3	0.0	0.0	0.0	53.9	1.1
10-31	0	52.1	39.3	61.7	5.5	308.7	0.0	0.0	0.0	53.9	1.1
10-31	1	50.7	39.9	66.2	3.2	192.5	0.0	0.0	0.0	53.9	1.1
10-31	2	50.6	40.4	67.9	4.6	282.9	0.0	0.0	0.0	53.8	1.1
10-31	3	50.4	40.2	68.0	3.9	292.9	0.0	0.0	0.0	53.8	1.1
10-31	4	50.8	39.7	65.6	3.2	291.6	0.0	0.0	0.0	53.7	1.1
10-31	5	50.0	39.9	68.0	0.6	266.9	0.0	0.0	0.0	53.7	1.1
10-31	6	49.5	40.2	70.1	1.8	266.1	0.2	0.0	0.0	53.7	1.1
10-31	7	50.7	41.6	70.8	1.6	142.8	5.8	0.0	0.0	53.6	1.1

Date (yyyy-mm-dd)	Hour PST	Air Temp (F)	Dew point (F)	RH (%)	Wind Speed (mph)	Wind Dir (Degree)	Solar Rad (W/m 2)	Precip (in)	Leaf Wet (Unity)	Soil Temp (F)	Soil Mois (%)
2008-10-31	8	50.7	46.1	84.2	1.2	245.0	29.7	0.0	0.7	53.6	1.1
10-31	9	51.0	49.2	93.5	0.5	151.1	54.6	0.0	1.0	53.5	1.1
10-31	10	53.1	48.9	85.7	1.5	201.2	117.0	0.0	0.6	53.5	1.1
10-31	11	56.8	46.7	69.0	2.8	256.1	291.6	0.0	0.0	53.5	1.1
10-31	12	60.8	45.2	56.5	2.3	230.9	387.5	0.0	0.0	53.5	1.2
10-31	13	63.0	45.5	52.7	1.2	181.7	332.8	0.0	0.0	53.7	1.2
10-31	14	66.3	46.5	48.8	1.6	201.0	273.6	0.0	0.0	53.9	1.2
10-31	15	64.2	47.5	54.8	1.9	223.7	86.8	0.0	0.0	54.3	1.2
10-31	16	60.9	47.8	62.0	1.0	144.0	39.4	0.0	0.0	54.8	1.2
10-31	17	51.9	45.9	79.7	0.3	75.4	0.5	0.0	0.0	55.2	1.2
10-31	18	49.7	46.1	87.4	0.8	103.1	0.0	0.0	0.0	55.6	1.2
10-31	19	49.9	47.1	89.8	0.9	207.6	0.0	0.0	0.0	55.9	1.2
10-31	20	46.9	45.1	93.4	1.9	89.2	0.0	0.0	0.2	56.0	1.2
10-31	21	47.7	46.3	94.6	2.9	120.0	0.0	0.0	0.3	56.0	1.2
10-31	22	47.2	45.6	93.9	1.6	129.8	0.0	0.0	0.4	55.9	1.2
10-31	23	44.2	43.8	98.6	3.4	93.3	0.0	0.0	0.7	55.8	1.1
11-01	0	43.3	43.1	99.5	4.4	70.8	0.0	0.0	0.8	55.6	1.1

ATTACHMENT D: WORKING METHOD

Food and Environmental Quality Laboratory
Washington State University FEQL Project Number: 1508

WORKING ANALYTICAL METHOD
DETERMINATION OF RESIDUES OF NITC
(Mixture of "Natural" 2-Propenyl-, Benzyl- and 2-Phenylethyl- Isothiocyanates)
IN CHARCOAL AIR SAMPLE CARTRIDGES BY GC-NPD

Introduction

This method is suitable for use with 600 mg charcoal cartridges. The charcoal is sonicated in extraction solvent and then filtered through a syringe filter for analysis by gas chromatography with nitrogen-phosphorus detection (NPD, also known as thermionic specific detector TSD).

The following extraction method is similar to the analytical method previously validated for use in MITC air sampling studies. Refer to the following projects:

- FEQL-NG-0605, MITC residential community air assessment; south Franklin County, WA,
- FEQL-1106, Optimizing fumigant efficacy while minimizing off-target volatile emissions
- FEQL-1207, MITC Residential Commnity Air Assessment for South Franklin County, WA

For this project, a different GC capillary column and chromatography program will be used. The revised method will be validated in triplicate at three levels of NITC concentration.

Method

1. Remove a set of charcoal air samples from the -80°C freezer. Immediately after taking the samples from the freezer, remove at least one cap from the end of the sample cartridge to prevent pressure build-up in the cartridge. Place the sample cartridge in a labeled Corex® tube to contain any spills and allow samples to warm to room temperature.

2. For each analytical set, prepare at least one fortified recovery sample by adding a known amount of NITC solution (in ethyl acetate) to an appropriate size cartridge. Fortification levels will range from the methods limit of quantitation (LOQ, 0.5 µg NITC) to concentrations that exceed the highest residues encountered.

3. For each analytical set, include a control blank cartridge of the appropriate size.

4. Carefully empty the entire contents of each cartridge (glass wool, plug, and charcoal) into labeled 25 mL screw-cap Corex® tubes.

5. Add 3 mL extraction solvent (50/50 mixture of ethyl acetate/carbon disulfide) to each tube by volumetric pipette, and then seal and place tube on ice (the solvent-charcoal interaction is exothermic). Prepare the extraction solvent in 500 milliliter batches by individually adding 250 mL of ethyl acetate to 250 mL carbon disulfide using graduated cylinders. Store the extraction solvent in a screw-cap dark glass bottle in the dark when not in use to avoid decomposition of carbon disulfide.

6. Sonicate the samples for ca. three minutes in a water-filled sonic bath (e.g. VWR

AquaSonic®) and vortex for ca 12 seconds. Maintain the samples in ice when not sonicating or vortexing, and until filtering.

7. Use a disposable glass Pasteur pipette to transfer an aliquot (~2-3 mL) to a plastic syringe fitted with a 0.45 µm Teflon membrane filter (Whatman®). Collect filtered sample in an appropriately labeled autosampler vial for analysis by gas chromatography (GC). (If possible, completely fill the vial to limit air space in the vial.) ~~added:~~ emc

8. The determination of NITC will be performed by gas chromatography with nitrogen-phosphorus thermionic detection (NPD). Conditions for determination are as follows:

   Instrument: A Varian Star 3400CX gas chromatograph (or equivalent) with nitrogen phosphorus detection (NPD) and 8200CX Autosampler will be used for residue detection and quantification. Integration of chromatographic data will be performed using Varian Star Chromatography Workstation software.

Column:	EC-WAX, 15m x 0.53mm, 1.2 µm film thickness
Carrier gas:	Ultrapure helium, column flow rate 2-4 mL/min.
Temperatures:	Detector: 300°C Injector port: 55 to 225°C (rate: 225°C per min), hold 16.09 min. Oven program Initial: 55°C, hold for 0.09min. Ramp 20°C/min to 175°C. Ramp 15°C/min to 225°C, hold 5 minutes
Injection volume:	1 µl
Retention time:	NITC retention time is based on the observed retention times of external calibration standards in each set and dependent upon instrument used.
Detector Gases:	Typical NPD detector gas flows will be set at approximately 3-4 mL/min hydrogen, ca. 170 mL/min air, and 25-30 mL/min makeup gas. The NPD bead current will be adjusted as necessary from 3.0 to 3.6 A.

9. NITC residue concentrations will be calculated using external NITC linearity standards dissolved in the 50/50 ethyl acetate/carbon disulfide solvent mixture. A standard curve will be generated for each analytical set and all samples will be bracketed with NITC calibration standards.

Submitted by:

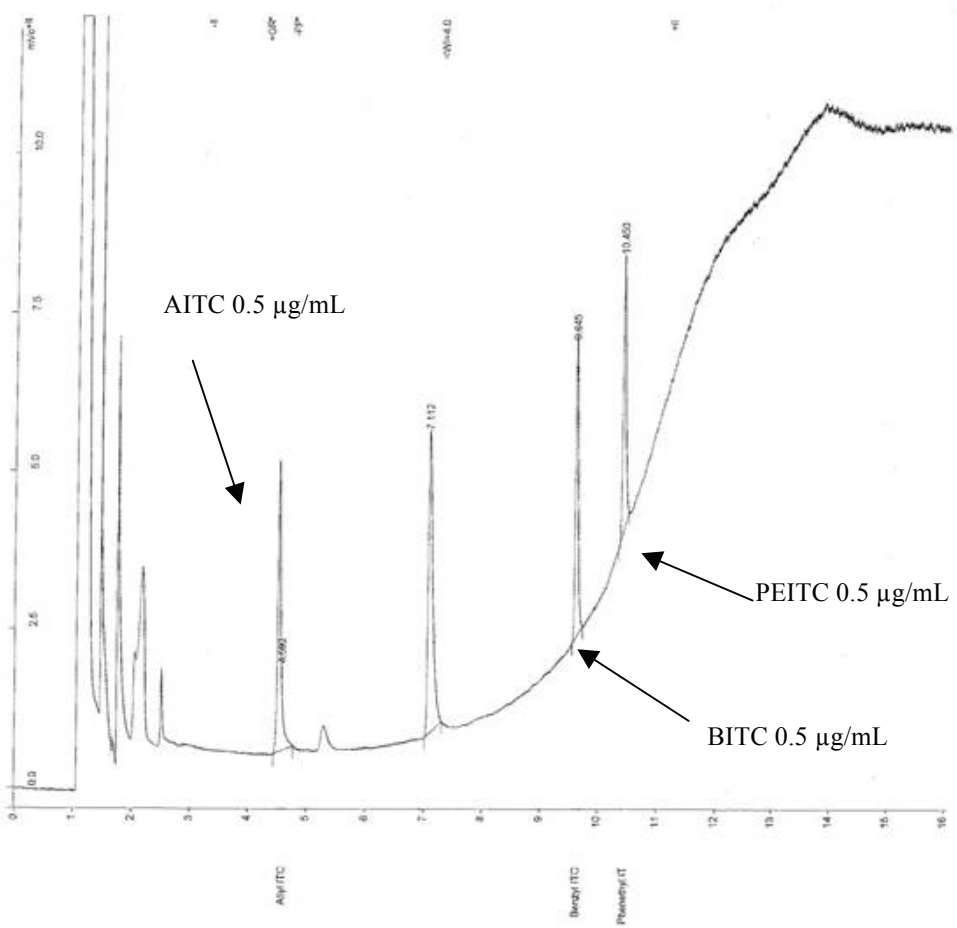
Donna Trott, Analyst — 3-12-09 Date

Approval:

Vincent R. Hebert
Project Coordinator — 3-12-09 Date

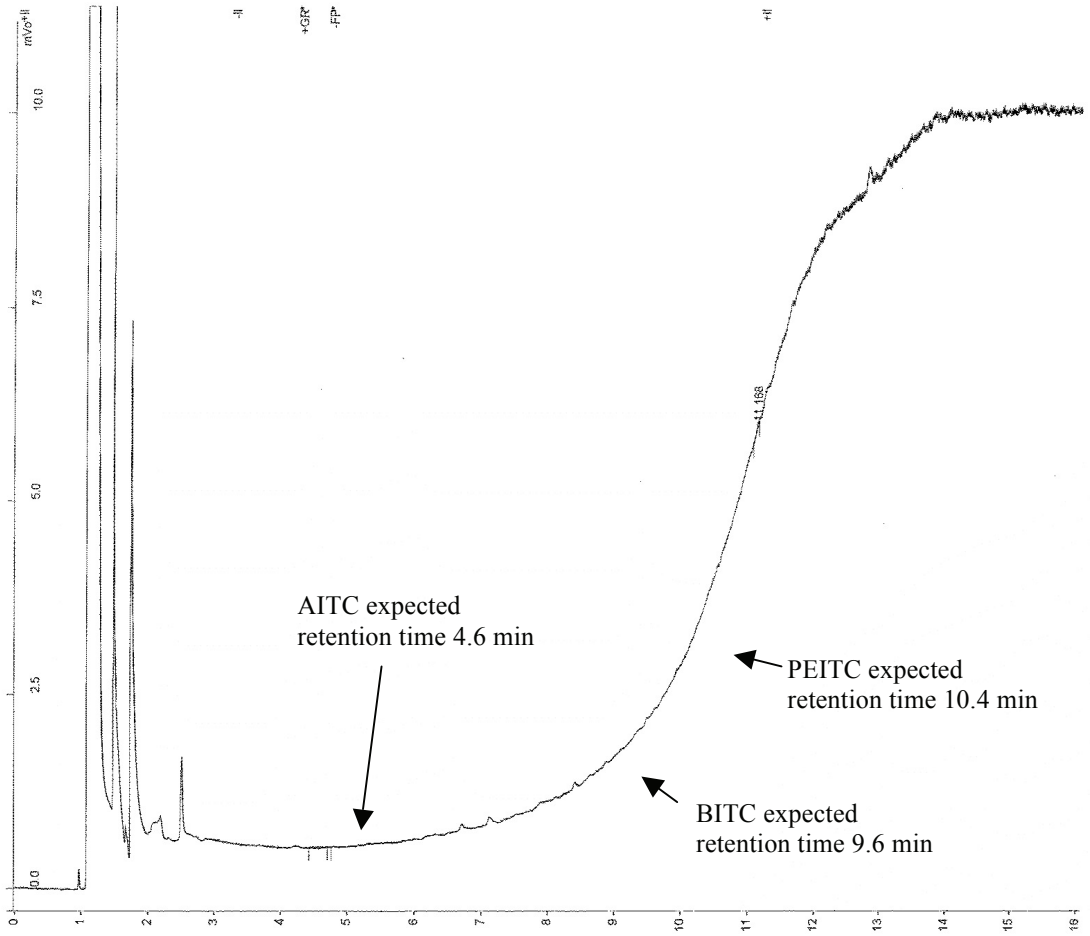
ATTACHMENT E: REPRESENTATIVE CHROMATOGRAMS

Figure 8
NITC Standard, 0.5 µg/mL
solution reference number M1347-9



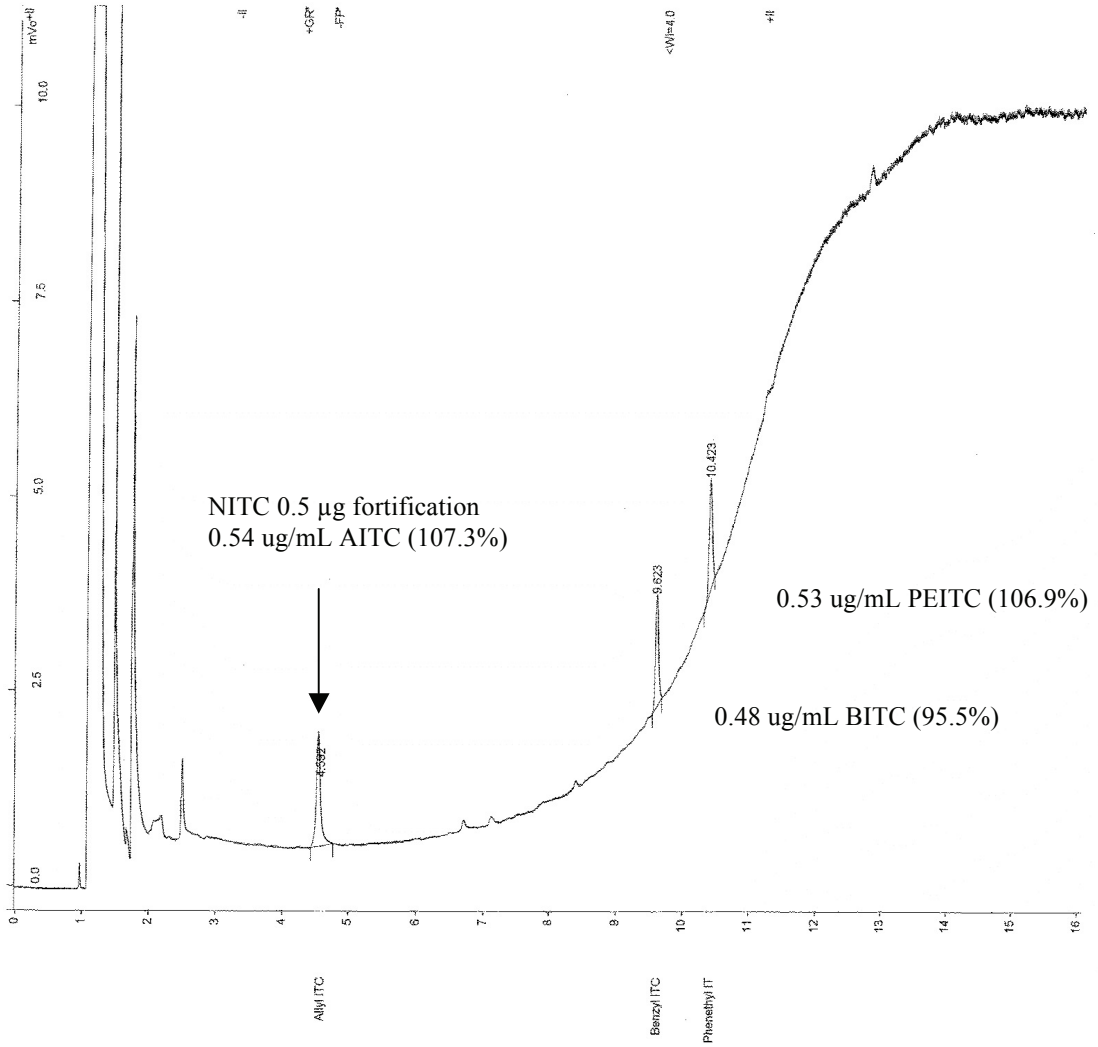
Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	Allyl ITC	24.7193	4.592	0.022	19170	GR	0.0	
2		34.2288	7.112	0.000	26545	BB	4.9	
3	Benzyl ITC	21.3485	9.645	-0.089	16556	BB	3.2	
4	Phenethyl IT	19.7033	10.450	-0.139	15280	BB	3.4	
	Totals:	99.9999		-0.206	77551			

Figure 9
Control, 600 mg cartridge
Sample ID: 1508-C8



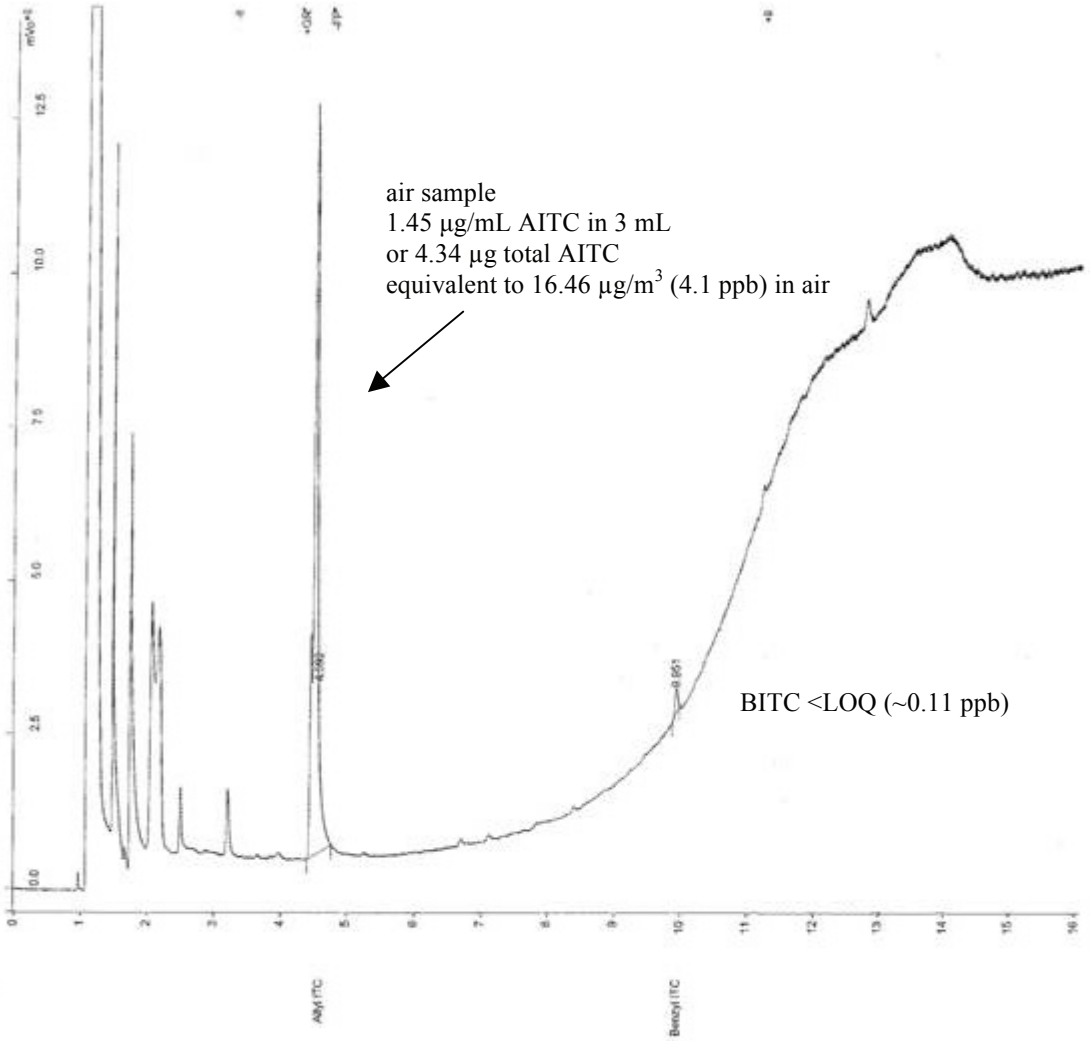
Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		100.0000	11.168	0.000	251	BB	4.8	
	Totals:	100.0000		0.000	251			

Figure 10
Fortified Sample, 600 mg cartridge
Sample ID: 1508-FS15



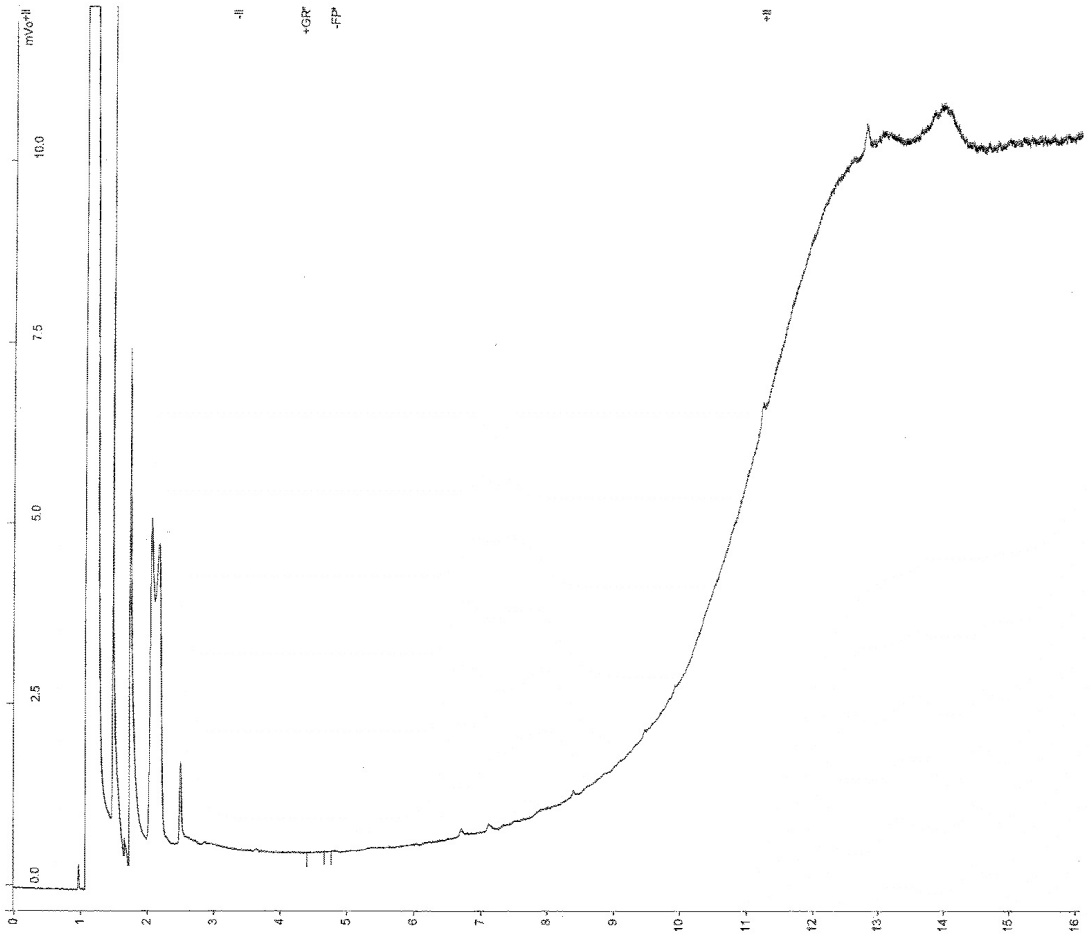
Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	Allyl ITC	39.4014	4.592	0.022	6454	GR	0.0	
2	Benzyl ITC	29.5099	9.623	-0.111	4834	BB	3.3	
3	Phenethyl IT	31.0887	10.423	-0.166	5093	BB	3.7	
	Totals:	100.0000		-0.255	16381			

Figure 11
Field Air Sample, 600 mg cartridge
Sample ID: GM5(A1)



Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	Allyl ITC	97.6940	4.592	0.022	57738	GR	0.0	
2	Benzyl ITC	2.3060	9.951	0.290	1363	BB	0.0	
	Totals:	100.0000		0.312	59101			

Figure 12
Trip blank, 600 mg cartridge
Sample ID: GMTB (A1)



Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
	Totals:	0.0000		0.000	0			