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Degradation of pesticides in food and beverages: Implications for risk assessment

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Degradation of insecticides in food and beverages: Implications for risk assessment

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An Abstract of
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Abstract

Degradation of insecticides in food and beverages: Implications for risk assessment
Samantha A. Radford

Insecticide metabolites have been used for years as biomarkers of exposure to parent insecticide compound under the assumption that there is a one-to-one correlation between urinary metabolite output and insecticide exposure. However, if insecticides degrade in food before it is eaten, the degradates produced are likely to be chemically equivalent to urinary insecticide metabolites excreted by the human body. Therefore, the degradates produced could be mistaken for metabolites of parent compound produced in the body. In this case, insecticide exposure would be overestimated. For this reason, we have studied the degradation of insecticides in food via two methods. In the first experiment, beverages were fortified with insecticide and extracted for both parent and degradation products. Degradation of insecticides in the beverages was suggested both by the loss of the parent compound and the production of degradates. As further evidence that the loss of insecticide was actually degradation and not some other mechanism such as insecticide adsorption to glass storage jars, it was shown that there was no statistical difference in insecticide concentrations from samples containing insecticide stored in standard amber glass jars, silanized amber glass jars, or vortexed amber glass jars after seven days of storage. In the second study, a sample of fruit and vegetable baby foods was collected and analyzed for both insecticides and their degradation products. The insecticides and their degradation products were found in many of the baby food samples. Further, these analytes were found in baby foods labeled as organic as well as in conventional baby foods. Together, these studies demonstrate the need for better understanding of the

relationship between insecticide degradation products and urinary insecticide metabolites so better estimations of the population's exposure to insecticides may be made.

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List of Acronyms

3-PBA	3-phenoxybenzoic acid
Ach	acetocholine
AChE	acetocholinesterase
ACN	acetonitrile
ALS	amyotrophic lateral sclerosis
CDC	Centers for Disease Control and Prevention
CE	collision energy
chlm	chlorpyrifos methyl
cpy/chlor	chlorpyrifos
cyf/cyflu	cyfluthrin
cyp/cyper	cypermethrin
DAP	dialkyl phosphate
DCCA	cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid
DDA	2,2-bis(4-chlorophenyl)acetic acid
DDD	tetrachlorodiphenylethane
DDE	dichlorodiphenyldichloroethane
DDT	dichlorodiphenyltrichloroethane
DEDTP	diethyldithiophosphate
del/delta	deltamethrin
DEP	diethylphosphate
DETP	diethylthiophosphate
dia/diaz	diazinon
dic	dicofol
DMDTP	dimethyldithiophosphate
DMP	dimethylphosphate
DMTP	dimethylthiophosphate
DNQ	did not quantify
ECD	electron capture detection
endo A	endosulfan α
endo B	endosulfan β
EPA	Environmental Protection Agency
ESI	electrospray ionization
FDA	Food and Drug Administration
fev	fenvalerate
FQPA	Food Quality Protection Act
GABA	gamma aminobutyric acid
GC	gas chromatography or gas chromatograph
HCl	hydrochloric acid

hep	heptachlor epoxide
HLB	hydrophilic-lipophilic balance
IQ	intelligence quotient
ISTD	internal standard
LC	liquid chromatography or liquid chromatograph
LLE	liquid-liquid extraction
LOD	limit of detection
m/z	mass-to-charge ratio
mal	malathion
MDA	malathion dicarboxylic acid
MeOH	methanol
MRL	minimal risk level
MRM	multiple reaction monitoring mode
MS/MS	triple quadrapole mass spectrometry
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NOAEL	no observable advers effect level
OC	organochlorine
OP	organophosphorus
par	parathion
PBPK	physiologically based pharmacokinetic modeling
PDP	Pesticide Data Program
per/perm	permethrin
ppb	parts per billion
pral	prallethrin
PSA	primary-secondary amine
Q/C	quantification/confirmatory ions
QCH	quality control high
QCL	quality control low
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe
RSD	relative standard deviation
RT	retention time
S/N	signal-to-noise ratio
SIM	single ion mode
SPE	solid phase extraction
TCPy	3,5,6-trichloro-2-pyridinol
USDA	United States Department of Agriculture
VSSC	voltage sensitive sodium channel

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CHAPTER 1: INTRODUCTION AND BACKGROUND

Can anyone believe it is possible to lay down such a barrage of poisons on the surface of the earth without making it unfit for all life? They should not be called "insecticides" but "biocides."
- Rachel Carson

Current insecticide use and history of insecticide regulation in the United States

Insecticides are one of the few compound classes designed to kill. These compounds are used worldwide in both agricultural and residential settings.[1, 2] In 2007, $11.2 billion was spent on insecticides and 892 million pounds of insecticide was used worldwide.[3] In particular, insecticides are widely used in the United States, where $4.3 billion was spent on 93 million pounds of insecticide active ingredients.[1, 3, 4] Insecticides are used largely in agricultural settings; 65 million pounds of active insecticide ingredients were used on agriculture alone in the United States in 2007.[3]

Beginning in 1939, *p,p*-dichlordiphenyltrichlorethane (DDT), an organochlorine (OC) insecticide, was used to control insects both in the United States and worldwide.[5] The United States used great quantities of DDT to control vector-borne diseases such as malaria and to control insects on crops.[6] As it became apparent that insects were developing resistance to DDT and as the public became more concerned about health effects, DDT use decreased. Even as early as 1948, other organochlorines such as methoxychlor were being registered as substitutes for DDT.[7] Public concern came to a head in 1962 with the publication of Rachel Carson's *Silent Spring*,[8] which discussed case studies of the toxicity of DDT and other organochlorine insecticides. The book also explained how pesticides and other toxic chemicals applied to one area may spread through the environment and eventually to the human population, comparing this spread

to the nuclear fallout that her audience was familiar with.[8] According to the biographer Linda Lear,

> After *Silent Spring* caught the attention of President John F. Kennedy, federal and state investigations were launched into the validity of Carson's claims. Communities that had been subjected to aerial spraying of pesticides against their wishes began to organize on a grass-roots level against the continuation of toxic pollution. Legislation was readied at all governmental levels to defend against a new kind of invisible fallout.[8]

After these investigations of the environmental effects of organochlorine insecticides, the USDA indeed canceled more and more DDT registered uses. By 1971, the EPA had canceled all registered uses of DDT in the United States.[6]

While DDT is the most well-known OC insecticide, there were others used in the United States that were eventually banned as well. Industrial Formulation Chlordane, a mixture of over 140 chemicals including heptachlor epoxide, nonachlor, and chlordane, was used beginning in 1948 as both a fumigant and as termite control. As time went on, people began to learn about chlordane's potential adverse health effects and its ability to bioaccumulate in adipose tissue.[9] In 1983, it was banned for any use in the United States except as termite control applied underground around homes' foundations, and in 1988, its use in the United States was cancelled completely.[9] Methoxychlor, an OC used to control insects such as cockroaches and mosquitoes as well as to protect crops, was deregistered in 2003 because of concerns about its effects as an endocrine disruptor and its ability to bioaccumulate.[7] Endosulfan, an OC introduced into the United States in 1954 for use on vegetables and other crops, will not be completely deregistered until 2016.[10, 11, 12] While the EPA asserts that endosulfan "does not present a risk to human health through dietary exposure," the department is removing it from use due to concerns

for agricultural workers who apply the insecticide and due to its persistence in the environment.[12]

Of course, while organochlorine insecticide use was waning, other insecticides, such as organophosphorus (OP) insecticides and pyrethroids, were being produced and used.[13] The first organophosphorus insecticide, which is similar to nerve gases designed in World War II Germany, was developed alongside these compounds designed to kill humans.[14] While OPs are more acutely toxic to humans than OCs are, their lack of persistence led to them being favored as insecticides.[14]

Pesticide registration for uses on crops was controlled by the FDA until 1970 when the EPA was established. From 1958 until 1996, pesticides in processed foods were regulated by the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act of 1938.[15] This amendment contained the Delaney Clause, which stated:

> …That no additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animal, or if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animal…[15,16]

Therefore, even when a pesticide was deemed to have a *de minimis* risk of carcinogenic activity, it was still considered unsafe in processed foods. As more research became available concerning chronic exposure to insecticides, it was found that many widely-used pesticides were potential carcinogens. Further, as analytical methodologies improved, researchers began to discover that low levels of carcinogens were ubiquitous in food. For this reason, complying with the Delaney Clause became impractical at best and impossible at worst, and modernized legislation was needed.[15]

In 1996, the Food Quality Protection Act (FQPA) was passed, which excluded pesticides in both processed and raw foods from the Delaney Clause.[17] Under this new law, pesticide concentrations for which there is "…reasonable certainty of no harm…from aggregate exposure to pesticide chemical" are considered safe.[15] Therefore, since pesticides of the same class often have a similar mechanism of toxicity, the United States Environmental Protection Agency (US EPA) is required to "consider the cumulative effects of exposure" to these classes of pesticides as a whole.[17]

Between the deregulation of DDT and the enactment of the FQPA, OP insecticides became favored because of their shorter half lives in both the environment and in the body and because of their lesser environmental effects.[13] However, OPs have been found to have much higher mammalian toxicity than OCs.[13, 17-19] For this reason, the US EPA began phasing out the use of OPs in residential areas in the late 1990's. For example, chlorpyrifos and diazinon, two OP insecticides, were restricted for use in residential areas in the United States in 2001 and 2004, respectively.[20] Instead, two new insecticide classes, pyrethroids and carbamates, are now largely used in the residential setting, although a few OPs that have been deemed less toxic, such as malathion and acephate, are still used.[4, 20-22] Restrictions on pyrethroids have been tightened over the past twenty years as well. While products containing lower concentrations of pyrethroids are still acceptable for home use, use of granular or emulsified concentrates have been restricted to field use since 1995.[19]

Health outcomes associated with insecticide exposure

Effects on nervous system

Although different classes may work through very different mechanisms, insecticides work by targeting the nervous system. [5, 9, 19, 23] There are reports that acute or long-term exposure to these compounds leading to nerve damage. For example, some studies have shown that OP poisoning may have lingering effects that can last for years after a major exposure event, such as decreased vibration sensitivity and impaired nerve conduction. Many studies show that lower level, chronic pesticide exposure may also have cognitive and psychomotor neurobehavioral effects.[2, 24] For example, farmers who used sheep dip with OP insecticides and were therefore chronically exposed to low levels of the pesticide had significant motor peripheral nerve axonal dysfunction including increase in vibration, increased cold perception threshold, and increases in distal motor, shortest F-wave, and sensory latencies (time required for an electrical impulse to travel down a nerve).[25] Exposure to pyrethroids, another insecticide class, may also cause nerve damage. In mammals, pyrethroids have been less suspected in the past of chronic health effects than OCs and OPs.[19] While many argue that not enough is known about pyrethroids to be sure of their lower toxicity, others deem them "relatively non-toxic and harmless" when used at low levels.[26] However, there have been some reported cases of chronic exposure causing ill effects. One interesting report was of a woman who used pyrethroids daily in an unventilated room and developed motor neuron disease indistinguishable from amyotrophic lateral sclerosis (ALS-Lou Gehrig's disease). While she recovered somewhat two months after cessation of exposure, she still experienced

weakness in her upper limbs and atrophy of her tongue. By seven months later, she was completely recovered. [27]

Endocrine disruption

With chronic exposure, organochlorines have reproductive and endocrine effects in the human population, because many OCs have secondary estrogenic or antiandrogenic mechanisms of toxicity.[5, 28] In particular, the target system for methoxychlor in humans is the reproductive system, not the neurological system because when methoxychlor is demethylated in the body, it is activated for estrogenic activity.[29] While DDT does primarily target the neurological system, it too is known for steroidal activity. It has been found that women's exposure to *p,p'*-DDT before 14 years of age increased risk of breast cancer by a factor of five.[30] In 2004, a study showed that exposure of fathers to high levels of DDT increased risk of children's birth defects such as congenital malformations of the nervous and osteomuscular system.[31] Perhaps most alarming, mothers' exposure to DDT and subsequent prenatal exposure of daughters has been shown to affect daughters' fecundability much later in life.[32]

Toxicological studies have indicated that pyrethroid insecticides may also have endocrine-disrupting effects. Zhou et al., who used an E-Screen assay to several insecticides, found that permethrin, fenvalerate, cypermethrin, and deltamethrin induced MCF-7 cell proliferation significantly (a marker of estrogenic activity).[33] Studies in mice given *cis*-permethrin orally for 6 weeks showed that male mice displayed adverse reproductive system effects, including reduced sperm count, motility, testicular

testosterone production, and plasma testosterone levels. The reductions in these levels were dose dependent.[34]

Effects on children's neurodevelopment

Due to the FQPA, there is a new focus specifically on health effects from children's exposure to pesticides.[15] This change in focus has occurred partially because of the growth and neurodevelopment that takes place during childhood, which leads to concerns that insecticide exposure may have more far-reaching effects on children than adults (Figure 1).[35] Children's high metabolic rates and surface-to-volume ratio lead to a higher concentration of insecticides in their bodies than in adults.[36-37] Finally, children's behaviors also put them at risk for greater insecticide exposure. For example, some foods preferred by younger children, such as fresh fruit juices, tend to have a higher concentration of insecticides than many other foods.[38] Children's tendencies, such as more hand-to-mouth activity and more time outdoors, also lead to a higher risk for insecticide exposure.[36-39]

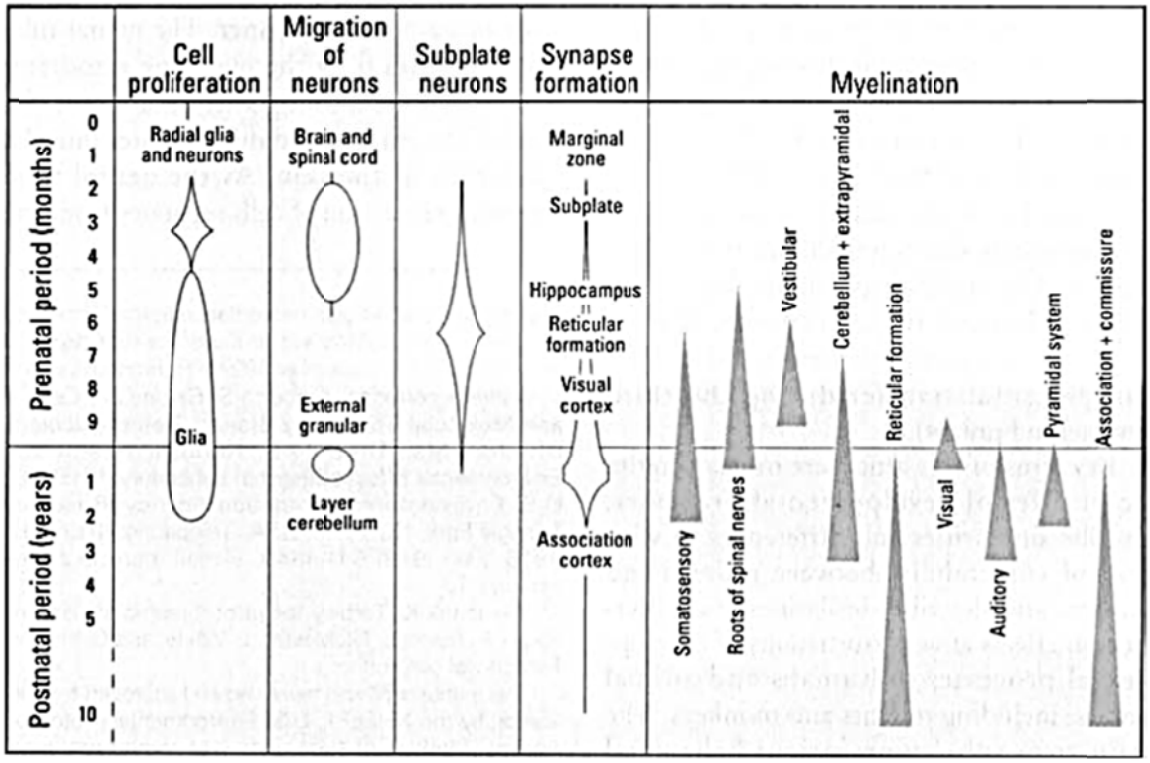


Figure 1: Timelines for neurodevelopment in humans. Reprinted from Rice and Barone, *Environmental Health Perspectives*.[35]

There have been studies showing that there are adverse cognitive effects produced from children's exposure to insecticides. In one of the most compelling examples, two groups of Yaqui children in Mexico, who had similar genetic makeup, diets, and cultural traditions were compared for cognitive ability. The only apparent difference in environment was pesticide exposure due to the children's location in either foothills or a valley. The group that lived in a valley had parents who largely worked in agriculture and were involved in the spraying of crops with multiple pesticides. As a result, high levels of OC pesticides (which although banned in the US, are readily available in Mexico) were found in both the children's cord blood and the mother's breast milk. The other group of children lived in the foothills where parents were ranchers and who avoided pesticide exposure other than annual DDT sprayings by the government to

control mosquitoes. However, there were marked differences in the children's stamina, hand-eye coordination, 30-minute memory, and ability to draw a person, an indication of cognitive ability (Figure 2).[40]

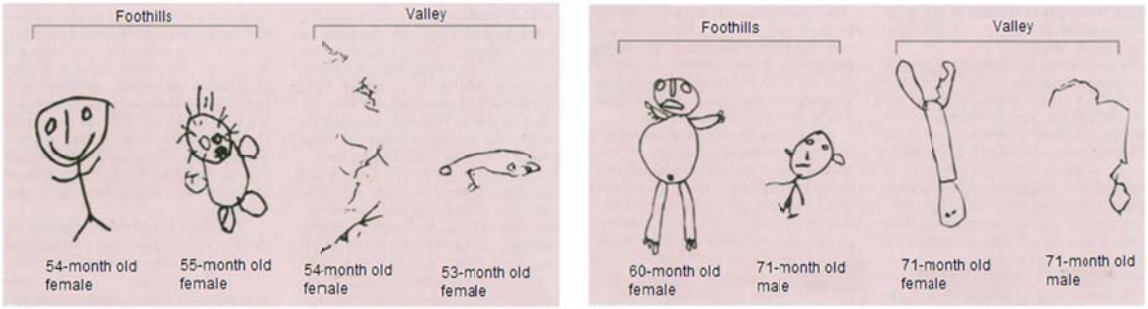


Figure 2: Representative drawings from 4- and 5-year old Yaqui children from the foothills and valley of Sonora, Mexico. Children in the valley had higher pesticide exposures both *in utereo* and through breast milk. Reprinted from Guillette et al., *Environmental Health Perspectives*. [40]

While the primary mechanism of OP insecticides is acetocholineesterase inhibition, it is known that some OPs target other neurological pathways, including growth factors and other neurotransmitter systems. These pathways may be compromised at lower OP concentrations than those needed for acute acetocholineesterase inhibition, and seem to be affected at levels currently found in the US population.[41] Several recent studies have connected prenatal OP exposure to lower IQ and cognitive development in children.[41-43] In one example, maternal urinary OP metabolite (dialkyl phosphates, DAPs) concentrations were associated with poorer scores for working memory, processing speed, verbal comprehension, perceptual reasoning, and IQ (full-scale intelligence quotient) when children were 7 years old. However, the children's urinary DAP concentrations did not correlate with lowered IQ or cognitive ability. While the maternal DAP levels from this study were somewhat higher than that

of the average US population of pregnant women, they were within the range of distribution levels found in pregnant women in the US.[41] A similar study in a different population found that prenatal exposure to chlorpyrifos was associated with adverse effects on IQ and working memory of seven-year old children. While residential use of chlorpyrifos was still permitted when the mothers were recruited, and chlorpyrifos exposure was likely higher for these women than on current pregnant women, the population is still exposed to chlorpyrifos through diet.[42]

Use of biomarkers to monitor human exposure to insecticides

Because of the large number of potential environmental and metabolic pathways, using concentrations of insecticides in food and the environment is a difficult way to assess exposure to insecticide. Therefore, as discussed above, biomarkers are used to quantify insecticide exposure more accurately.[20] Physiologically based pharmacokinetic modeling (PBPK), which describes absorption, distribution, metabolism, and excretion of chemicals, is used to understand what biological matrix is most likely to contain a biomarker.[44] Since there is a one-to-one ratio between pesticide molecules metabolized and metabolites formed, pesticide metabolites in urine have been used for years as biomarkers of exposure to pesticides. For example, Bradman et al[37] did a study using urinary DAPs to measure OP pesticide exposure of children. Lu et al[4] have also used urinary 3-phenoxybenzoic acid (3-PBA), a metabolite of several pyrethroids, as a biomarker of pyrethroid exposure in children. Metabolites corresponding to specific insecticides, such as 3,5,6-trichloro-2-pyridinol (TCPy) for chlorpyrifos and malathion dicarboxylic acid (MDA) for malathion have also been used to assess human exposure.[36,

[45] However, what if the assumption of a one-to-one relationship between intake of pesticide and urinary output of the metabolite is wrong? For example, depending upon urinary organochlorine metabolites to quantify OC exposure would likely lead to underestimation of exposure, since OCs and their metabolites tend to bioaccumulate in adipose tissues. On the other hand, it is possible that people are exposed to insecticide degradates as well as parent compound. If this is the case, and the degradation products are not further metabolized beyond conjugation, overestimation of insecticide exposure based on urinary metabolites is possible.

Metabolites as biomarkers

The immediate question, then, is what makes a good biomarker? According to the Agency for Toxic Substances and Disease Registry, biomarkers are "indicators signaling events in biologic systems or samples."[23, 39, 46] Good biomarkers must show "that a direct exposure to the compound of interest has occurred."[47] For example, the concentration of an analyte in someone's personal breathing space would not be considered a biomarker, since there is no guarantee that the person exhaled all the analyte found in that volume of air, nor that they would absorb the entire dose of analyte from the air. Biomarkers also need to be easily collected, causing the subject as little discomfort as possible.[47] For example, hair, fingernail clippings, blood, and urine are all good materials from which to obtain biomarkers. Bone marrow, a tissue that requires an invasive procedure to obtain, would be a poor biomarker source. The biomarker found in the sample should also be both measurable with good sensitivity and specific to the event studied.[47]

It is certainly true that the urinary insecticide metabolites are good biomarkers in that they are found in an easily collected source and their concentration is easily measured. However, it is questionable whether they are truly specific to the "event" being studied, namely, exposure to insecticides. This is because insecticides may be degraded in the environment through various pathways, such as hydrolysis or photolysis. For example, malathion tends to hydrolyze best in basic environments, while diazinon is more likely to be hydrolyzed in acidic conditions due in part to its lower electronegativity.[39, 46, 48] Both conditions may be found in the environment. Pyrethrins and pyrethroids are susceptible to photolysis or hydrolysis in soil or water.[19] When these pesticides degrade in the environment, they tend to break down into the same metabolites found in urine. So how could one tell if DAPs, 3-PBA, or other "metabolites" found in urine are really from pesticides actually ingested by the subject? As one might expect, the difference can't be seen (Figure 3).

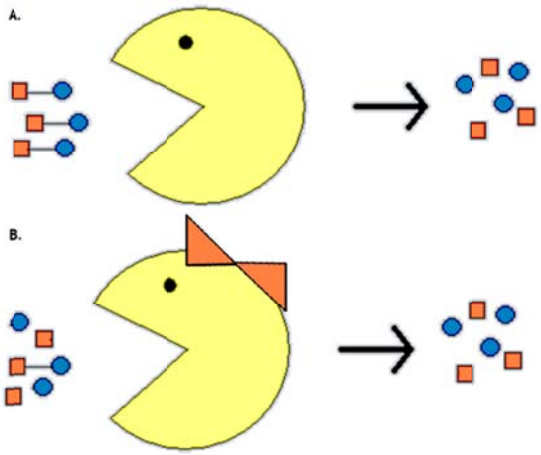


Figure 3: Simplified comparison of urinary metabolites with differing parent and degradate nonpersistent insecticide exposures. In A, the subject is exposed to 3 molecules of parent compound, resulting in 3 molecules of blue metabolites and 3 molecules of red metabolites. In B, the subject is exposed to 1 molecule of parent compound and 2 molecule of each type of degradates. However, Subject B's urinary metabolite output is equivalent to that of Subject A.

Complexities of measuring human insecticide exposure

While indirect measurement of OP and pyrethroid exposure through metabolites is currently necessary due to the short half-life of these compounds in the human body, previous studies have suggested that use of urinary insecticide metabolites may lead to overestimation of parent exposure.[20] In 2001, an article was published discussing dietary exposure to chlorpyrifos and TCPy levels in urine.[49] Duplicate diets were analyzed for chlorpyrifos, and chlorpyrifos levels in food were also estimated based on a semi-quantitative food questionnaire. Both of these values were compared with TCPy levels in urine. While there was a positive association between estimated chlorpyrifos concentrations in food and creatinine-corrected TCPy concentrations, dietary intake of chlorpyrifos only accounted for 7% of TCPy output.[49] In addition to this study, there have been others that have suggested that insecticide degradates are indeed found in food.

While there is no hard evidence in this area yet, it is also possible that some insecticide degradates have toxic properties in and of themselves. For example, the first metabolite of many OPs, including chlorpyrifos, is formation of an oxon, the toxic form of the compound.[23] It has been assumed in the past that there is little to no toxicity from insecticide degradation product exposure. However, there are currently few data on the adsorption, further metabolism, and potential toxicity of many insecticide degradates.[36, 38] Therefore, it is difficult to separate the health effects of insecticide degradates from that of the parent compounds. Given that these degradates inherently contain functional groups similar to those of their parent compounds, it would not be surprising if they also have cause similar adverse effects on the human body. Further research is needed to understand the health effects of insecticide degradates more completely.

Past research on insecticide degradation in food

Despite the importance of understanding insecticide degradation in the environment and its ability to cause exposure misclassification bias, there have been only a handful of studies relating to the subject. The following are summaries of studies either related to the use of insecticide metabolites as biomarkers of exposure or related to degradation of insecticides in food.

In 2004, Lu et al[38] analyzed the DAP concentrations of fresh orange juice and apple juice. A subset of juices was also intentionally fortified with OP insecticides to discover whether they would hydrolyze and produce more DAPs. Dialkyl phosphates were found both in organic and conventional juices, although the concentrations in conventional juices tended to be higher. There tended to be more dimethyl DAPs in apple juice and more diethyl DAPs in orange juice, which is consistent with the fact that dimethyl OPs such as azinphosmethyl are sprayed on apples, while diethyl OPs such as chlorpyrifos are often used on citrus. Juices fortified with OP pesticides showed degradation of insecticides into DAPs. Overall percent degradation of OP pesticides into dimethyl DAPs, formed from azinphosmethyl was found to be about 12.0% in 3 days at 4ºC. Percent degradation to diethyl DAPs, formed from diazinon and chlorpyrifos, was 36.2% under the same conditions.[38]

In 2005, Morgan et al[36] published an article examining the exposures of preschoolers to both chlorpyrifos and its specific metabolite TCPy. At the time of the study, chlorpyrifos was still legal for residential and daycare use in the United States. Samples collected for 48 hours included food duplicates, indoor and outdoor air, urine, indoor dust, outdoor soil, transferable residues collected with a polyurethane foam roller, and hand, food preparation area, and hard floor wipes. Both chlorpyrifos and TCPy were

detected in 100% of indoor dust samples. Chlorpyrifos was also detected in 100% of indoor air samples. Finally, TCPy was detected in >95% of indoor air samples, solid food, and hard floor surface wipes. In most samples, chlorpyrifos levels were higher than TCPy levels, but in food from homes and day-care centers, TCPy levels were 12 and 29 times higher respectively than chlorpyrifos levels.[36]

In 2007, the effect of antioxidants on organophosphorus and carbamate insecticide degradation rate was studied.[50] In this article, red grape juice, water acidified to pH 3.5 (near that of grape juice), and acidified water fortified with quercetin, a flavonol found in red grapes, were used. Each matrix was fortified with aldicarb, methiocarb, demeton-S-methyl, and fenamiphos, and aliquots were analyzed for parent compound and oxidative degradation products for twenty days. The addition of quercetin to water slowed oxidation of all insecticides except methiocarb, and red grape juice showed even slower oxidation of insecticides, apart from methiocarb. Analytic degradation, or degradate production due to degradation of sample during analysis, was not accounted for, severely limiting the usefulness of the study.[50]

In 2008, an article was published discussing the effect of storage and processing of semolina wheat on its organophosphorus insecticide content.[51] Grain treated with malathion, fenitrothion, chlorpyrifos methyl, and primiphos methyl at approved doses was stored in a small-scale shed for up to five months. Samples were taken periodically and the concentration of insecticides was analyzed. Some of the grain was also taken periodically and processed into semolina, in which the bran and germ have been removed, and the flour was also analyzed for insecticide content. Finally, some of this flour was used periodically to make spaghetti, and the pasta was also analyzed for

insecticide content. The five-month storage period was not long enough to reduce pesticide content below minimal risk levels (MRLs) recommended by the Codex Alimentarius Commission for wheat, [51] although this might be partially due to the small scale storage shed, since the main mechanism of insecticide loss being studied was volatilization. As one might expect, both processing the wheat into flour, which removes the outer layers of the grain, and subsequently processing the flour into pasta greatly reduces insecticide levels. The only exception to this rule is that initially, malathion and fenitrothion residue levels were greater in flour than in wheat. This effect is likely because the somewhat lipophilic insecticides penetrated the seed coat, which was discarded when flour was made, into the bran and germ. While degradation products were not analyzed in this study, it was hypothesized that the disappearance of insecticides was due to a combination of volatilization and degradation.[51]

Also in 2008, an article was published measuring both DAPs and OPs in produce.[52] While specific produce types were not named because pesticide application data was not available, only produce containing at least one OP was used in the study. Of these samples, 60% had a molar ratio of DAPs:OPs greater than one. Another aim of the study was to follow malathion degradation on strawberries. Strawberries were collected several days following routine malathion application on strawberry fields. After collection, strawberries were analyzed for malathion, malaoxon (the oxidation product of malathion), DMP, DMTP, and DMDTP. While malaoxon was not detected, the molar sum of malathion and DAPs stayed constant throughout the study, and as malathion concentration decreased, DAP concentration increased. This phenomenon suggests hydrolysis as the main route of malathion degradation in this environment (Figure 4).

Again, these samples were not controlled for analytic degradation, or degradate production due to degradation of sample during analysis, calling this study into question as well.[52]

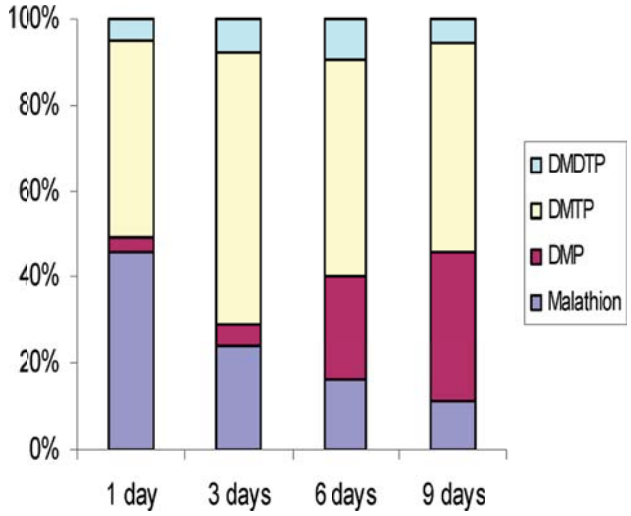


Figure 4: Degradation of malathion in strawberries into dimethyl phosphate, dimethylthiophosphate, and dimethyldithiophosphate. *Used with permission.*[52]

Background information on insecticide classes

Mechanisms of organochlorine insecticides

Organochlorine (OC) insecticides, as the name suggests, are compounds containing chlorine (Figure 5). The most well-known OC insecticide, 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (*p,p*-dichlordiphenyltrichlorethane, DDT), was first synthesized in 1874 but was not used as an insecticide until 1939.[5-6] DDT was banned for use in the United States in 1972. DDT is only slightly soluble in water (0.085mg/L at 25°C) and has a very low vapor pressure.[5] DDT has two common degradation products, 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethane (*p,p*-dichlorodiphenydichloroethane, DDE) and 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene (dichlorodiphenyldichloro-

ethane, DDD), that are often detected in the environment alongside DDT (Figure 6), and the *o,p*- isomers of each compound are often detected as well, although at lower concentrations.[5] Because of the persistence and lipophilicity of DDT and its degradation products, it tends to both bioaccumulate and biomagnify.[5]

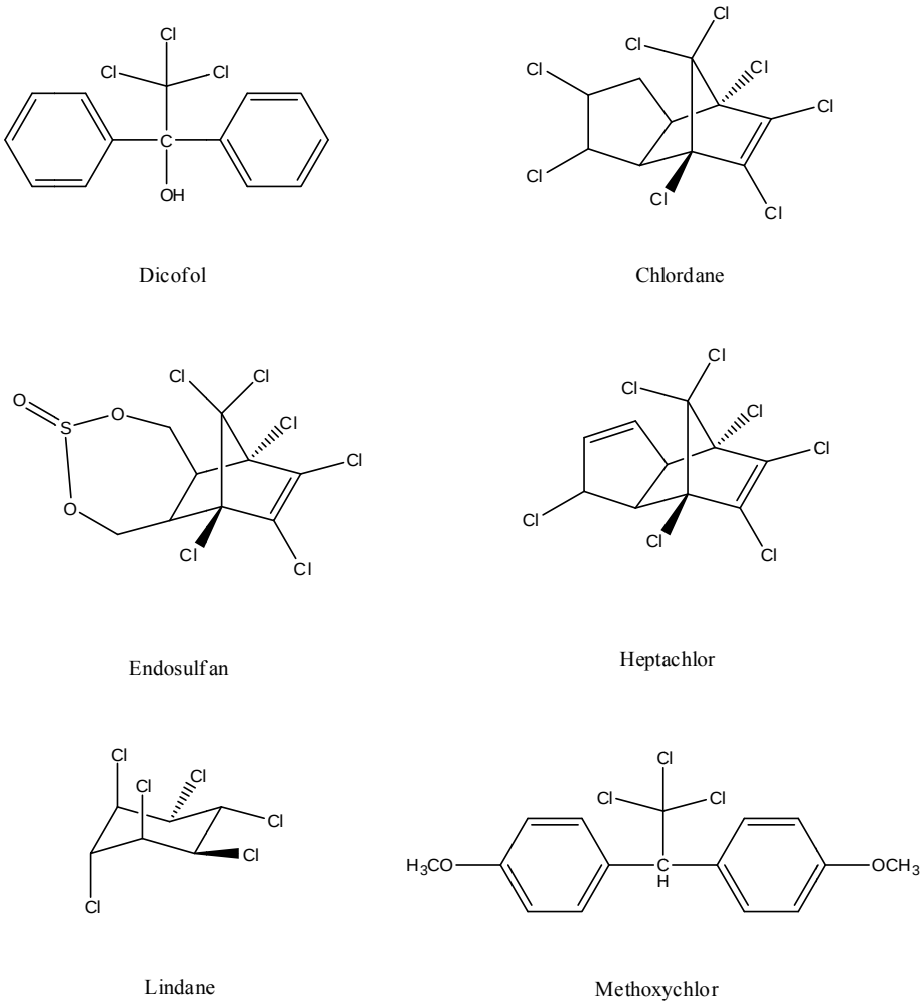


Figure 5: Structures of organochlorine insecticides.[53]

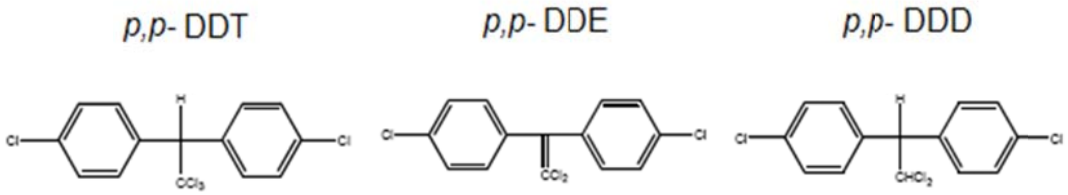


Figure 6: Structures of DDT and its degradates DDE and DDD.[5]

Organochlorines are designed to affect the nervous system.[54] DDT accomplishes this goal by disrupting voltage sensitive sodium channels (VSSCs), proteins found within lipid membranes that allow sodium to flow into nerve cells.[18, 54] Normally, depolarization opens voltage sensitive sodium channels (VSSCs), which causes a sharp rise in action potential and allows sodium ions to flow into the cell. The membrane potential of the cell increases, inducing more VSSCs to open. Before the sodium equilibrium potential is reached, potassium ion channels open, while VSSCs close. This causes a quick drop in cell membrane potential below that of the resting potential, approaching the K^+ equilibrium. The potential slowly rises back to the resting point, and the VSSC is ready to operate again (Figure 7).[18, 55-56] DDT inhibits this action by holding one of two "gates" of the VSSC open, allowing a surplus of sodium ions to flow in. Because of this, the depolarizing after-potential is increased and there are repetitive discharges. Acute poisoning by DDT leads to headaches, dizziness, nausea and vomiting, and convulsions and/or tremors in more severe cases.[53]

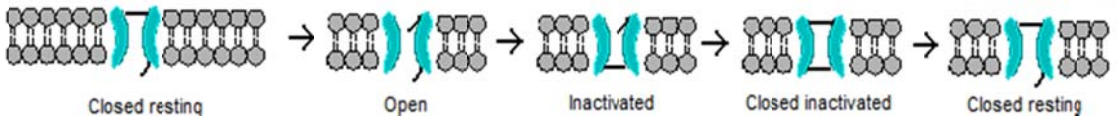


Figure 7: Normal action of voltage sensitive sodium channels. Opening of VSSCs is prompted by depolarization of the nerve cell membrane, and subsequent closing is triggered when potassium channels open.[56]

Other OC insecticides may affect the nervous system in a different manner. While the mechanism of neurotoxicity of chlordane is not well known, it is hypothesized that the compound may inhibit γ-aminobutyrid acid (GABA) or inhibit membrane permeability to Ca^{2+}.[9] Endosulfan is also suspected to affect GABA as a noncompetitive

antagonist. When GABA binds to its receptor, chloride ion channels open, allowing chloride to flow into the neuron and inhibiting neuron firing. Therefore, when GABA is inhibited from binding, neurons may be overstimulated.[10]

Metabolism of DDT and DDE

In humans, DDT is reductively dechlorinated to tetrachlorodiphenylethane (DDD), which is then degraded to 2,2-bis(4-chlorophenyl)acetic acid (DDA), and possibly conjugated before finally excreted in urine.[5, 57, 58] However, DDT is only eliminated at a rate of about 1% of DDT stored in the body per day, not including DDT sequestered in adipose tissue, which is overturned even more slowly.[59] People may also be exposed to DDE, or some DDT in the body may be converted to DDE, but further metabolism of DDE to DDA is slow, so this metabolite to bioaccumulate in the adipose tissue.[5, 58] DDE may also be excreted directly through breast milk in lactating mothers.[60]

Mechanism of organophosphorus insecticides

Organophosphate (OP) insecticides are esters of phosphoric acid (see Figure 8). These insecticides were developed from nerve gas manufactured during World War II.[14] OPs are now one of the most used classes of insecticides in the United States, particularly in the agricultural sector. [20, 61] In 2006, there were 32 different types of OPs licensed for use in the United States by the EPA, and in 2007, 15 million kilograms of OP insecticides were used in the United States making up 35% of total US insecticide use. [3,62]

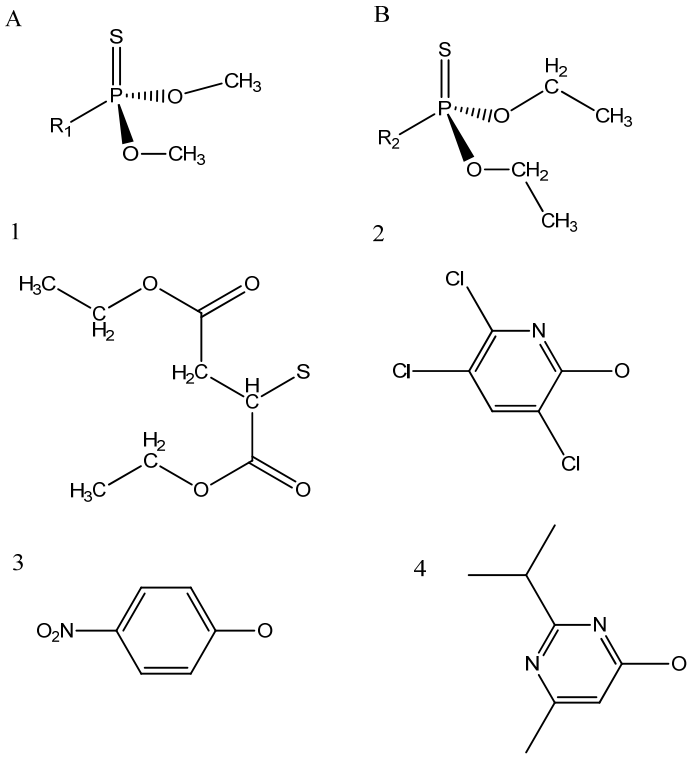


Figure 8: Examples of organophosphorus pesticides.[63] A is part of dimethyl OPs with the substituents shown: [1]malathion, [2]chlorpyrifos methyl, [3]parathion methyl. B is part of diethyl OPs with the following substituents: [2]chlorpyrifos, [3]parathion, [4]diazinon. Note that although substituent structures are markedly different, all OPs are esters of phosphoric acid.

Organophosphorus insecticides are bioactivated by cytochrome P450 in human liver microsomes to its oxon form (Figure 9).[64] Once activated, the pesticide becomes neurotoxic.

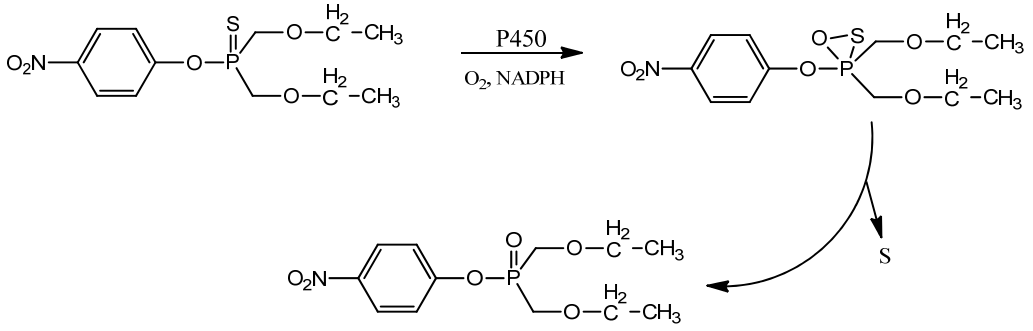


Figure 9: Bioactivation of parathion to its oxon form. The sulfon form will not bind to acetocholineesterase.[64]

In normal nerve conduction, an electrical signal will travel from the brain down a nerve to the synapse. At this point, the electrical signal will open voltage-gated calcium ion channels, which are sensitive to changes in membrane potential. Opening of these channels causes acetylcholine (ACh) to be released. These signal molecules will travel across the synapse and bind to a receptor on the next nerve, causing the electrical impulse to continue down its length. Once the signal has been received, the ACh molecules must be removed within a few milliseconds before another impulse can arrive. The serine esterase acetylcholinesterase (AChE) removes ACh and degrades it into acetic acid and choline.[18]

The primary mechanism of OP toxicity is serine binding in the active site of AChE.[20] Because ACh is blocked from binding to AChE, the signal molecule accumulates at the receptor, causing the neurotransmitter to act continually (Figure 10).[46]

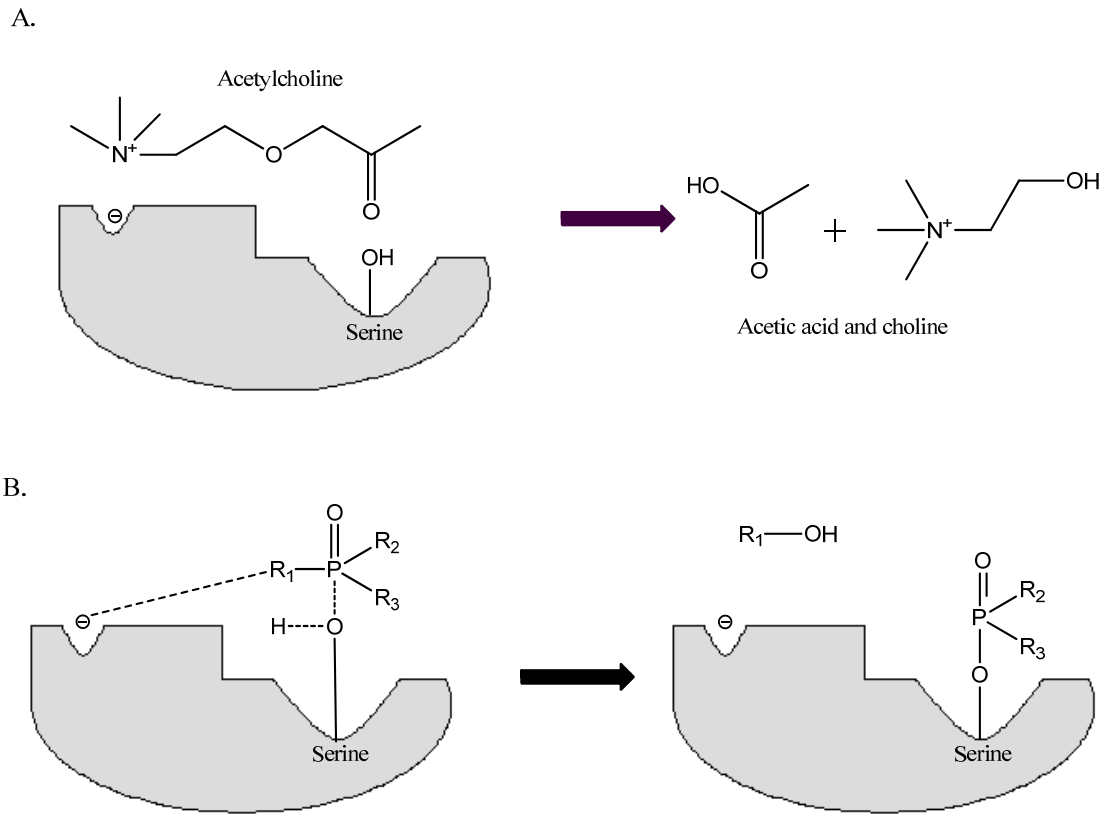


Figure 10: A. Degradation of ACh by AChE. B. Inhibition of AChE by OP pesticide. The insecticide binding is reversible, but several hours pass before the serine-phosphorus bond is broken.[65]

The target organ system for OP pesticide poisoning is the nervous system.[66] There are many symptoms of acute OP pesticide poisoning, but the symptoms which show are dependent upon which nerves have AChE bound by the oxons. Some symptoms of OP pesticide poisoning include respiratory distress, low heart rate, abdominal cramping or incontinence, drowsiness, blurred vision, tearing of eyes, pinpoint pupils, tremors, and twitching. Fortunately, only a high pesticide exposure would lead to the dramatic symptoms listed above. For example, based on rat studies, the no observable adverse effect level (NOAEL) for acute oral exposure to diazinon is 0.6mg/kg/day, and the lowest

observable adverse effect level (LOAEL) is 1.2mg/kg/day. Therefore, based on this data, a 70kg man would have to ingest 0.084g diazinon before adverse effects would be seen. The LOAEL for acute oral exposure to chlorpyrifos in men has also been set to 0.1mg/kg/day.[23]

Metabolism of organophosphorus insecticides

Despite the efficient absorption of organophosphate insecticide, unless an individual is exposed to a high dose of OPs, the insecticide will not be found in the individual.[46] Instead, metabolites of the pesticide will be found, since it is quickly metabolized inside the body. Once an OP pesticide is absorbed, either through inhalation, the skin, or the gastrointestinal tract, it may be bioactivated to its oxon form. The pesticide or oxon will usually then be hydrolyzed at the ester linkage. This reaction is performed by a cytochrome P450, the same type of enzyme that activates the insecticide (Figure 11).[64]

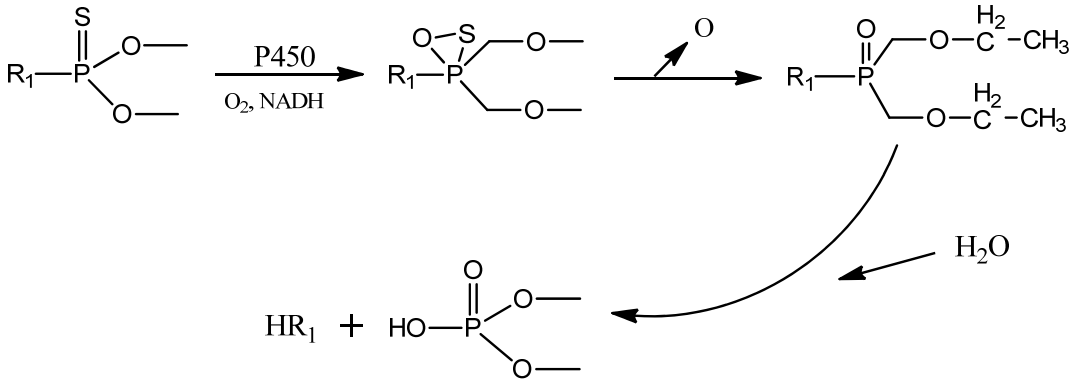


Figure 11: Metabolism of an OP.[14] While a dimethyl OP is shown producing a specific metabolite and dimethyl phosphate, diethyl OPs would react the same way. Also note that oxidation at the double bond is not required before hydrolysis and thiophosphates may also be produced.

After hydrolysis, two molecules are formed: a molecule unique to the metabolized pesticide and a more general dialkylphosphate (DAP).[23, 39, 46] There are six different DAPs derived from OP pesticides: dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP) (Figure 12). Most of OP pesticides may produce more than one type of DAP.[62] The metabolites of the pesticide are more polar than the pesticide itself, and they can be excreted through the urine. [39] Some of the specific metabolites, such as 3,5,6-trichloropyridinol (TCPy) from chlorpyrifos, are conjugated prior to excretion.[23] Unlike in OCs, this metabolism/excretion process requires only days or even hours. [36]

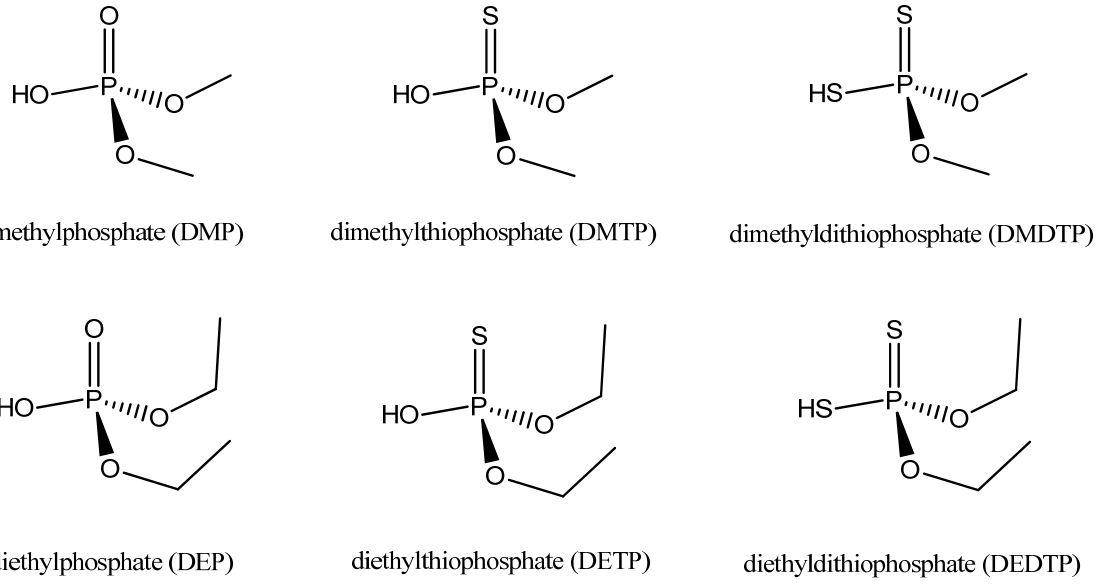


Figure 12: The six dialkyl phosphates.[1] Pesticides that have been bioactivated, in which the double bonded sulfur is exchanged for an oxygen, may produce the oxon DAPs.

Mechanism of pyrethroid insecticides

Pyrethrins are insecticides extracted from chrysanthemum flowers, possibly used as long ago as 400 BC in Persia.[67] The flower's extract, pyrethrum powder, may have been brought to Europe from Caucasia in the early 19th century.[67] The powder is unstable in light and air, an undesirable property for a compound used outdoors.[17] Therefore, the synthetic pyrethroids were designed to be more stable to photolysis without affecting their potency as insecticides.[19] Pyrethroids, unlike OPs, do not work by affecting cholinergic synapses.[18] Since cholinergic synapses are much more common in vertebrates than invertebrates, these synthetic compounds, still toxic to insects, are minimally toxic to mammals.[17-19] Because of this, pyrethroids are very commonly used in the United States, especially since the US EPA began phasing out the use of OP pesticides in residential areas in the late 1990s.[4, 21, 22]

As shown in Figure 13, all pyrethrins have both an alcohol moiety and a carboxylic acid moiety. The alcohol moiety contains a cyclopentenolone ring, while the acid moiety contains a cycolopropane ring. Historically, pyrethroids tended to conserve at least one of these groups. As more of these compounds were synthesized, though, developers tended to depart more from these templates.[17]

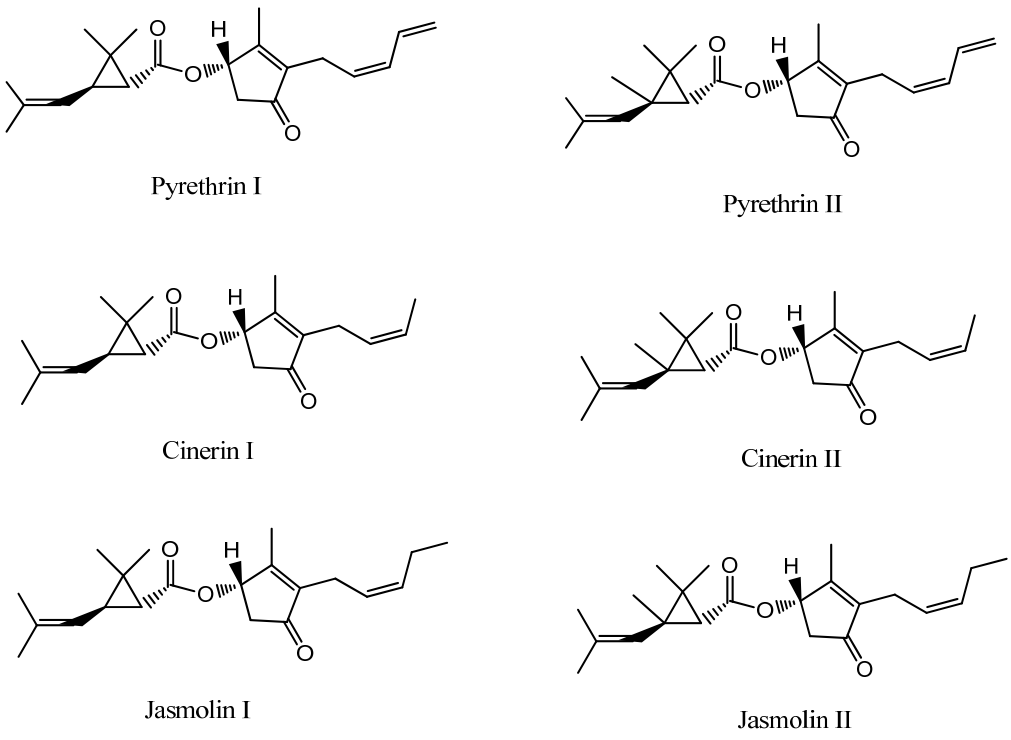
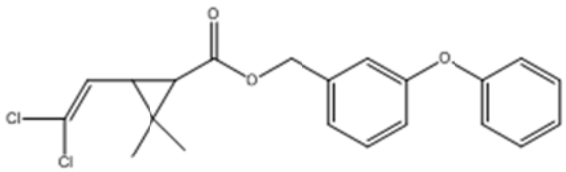
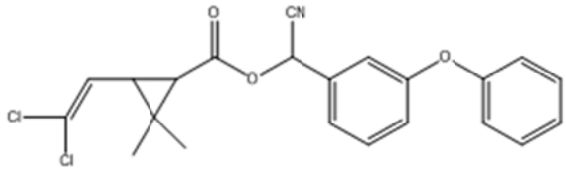


Figure 13: The six natural pyrethrins, which are extracted from chrysanthemums.[17]

Like OC and OP pesticides, pyrethrins and pyrethroids mainly affect the nervous system. Similarly to OCs, pyrethroids disrupt VSSCs found within lipid membranes that allow sodium to flow into nerve cells.[18, 54] There are two main classes of pyrethroids: Type I and Type II, which are distinguished by the absence or presence, respectively, of a cyano group alpha to the ester linkage (Figure 14). Type I pyrethroids cause repetitive firing of the nerve by depolarizing the membrane above the potential needed for action potential generation, while Type II pyrethroids cause even more depolarization so that the repeated action potentials have diminished amplitude (Figure 15). In insects, these effects lead to paralysis and death.[19]



Permethrin



Cypermethrin

Figure 14: Examples of Type I (permethrin) and Type II (cypermethrin) pyrethroids.[17] The rigidity of the cyclopropane ring causes a *cis*-/*trans*-effect. There is also a chiral center on the α-carbon on the other side of the ester linkage on Type II pyrethroids.[19] Type I compounds are found as a doublet on chromatograms, while Type II compounds create a quartet, which may not be fully resolved.

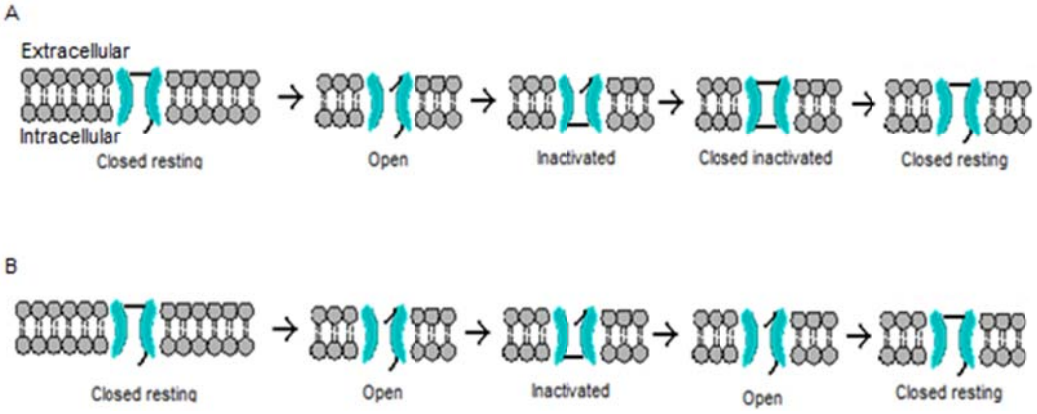


Figure 15: Perturbation of action potential of VSSCs affected by pyrethroids.[55] A: Normal action of VSSCs. Sodium is able to flow into the cell only while the channel is open. B: Pyrethroid modified action of VSSCs. Sodium continues to flow into the cell when it should not.

The presence or absence of the cyano group on pyrethroids changes the toxic effects of the molecules, likely due to the difference in how long the compounds affect sodium channel action. Type I pyrethroids cause a sodium tail current lasting 6-150ms, while Type II pyrethroids cause tail currents lasting over 290ms. The elevated after potential caused by Type I pyrethroids causes repetitive nerve discharge, while the longer sodium tail current from Type II pyrethroids causes summation of after-potentials, leading to depolarization of the nerve and action potential suppression.[19]

These differences in action on the neuron also change how an animal reacts to treatment with Type I or Type II pyrethroids. Generally, when mice are given a large dose of Type I pyrethroids, they display aggressive behavior, increased sensitivity to external stimuli, followed by fine tremor, coarse whole body tremor, an elevated body temperature, and finally coma and death. Type II pyrethroid poisoning leads to pawing and burrowing behavior, profuse salivation, increased startle response, abnormal hind leg movements, coarse whole body tremors, sinous writhing, then seizures and death.[19]

Metabolism of pyrethroids

As with OP pesticides, pyrethroids are quickly detoxified through hydrolysis of the ester linkage. This process forms a carboxylic acid and an alcohol (Figure 16). Like the metabolism of OP pesticides, this reaction also takes place in the liver, and is performed by a cytochrome P450 or carboxylesterase.[68] The metabolite 3-phenoxybenzyl alcohol is then oxidized to 3-phenoxybenzoic acid. The metabolites of pyrethroids are then turned into the glycine, sulfate, glucuronide, or glucoside conjugates before being filtered out by the kidneys to be excreted in urine.[19]

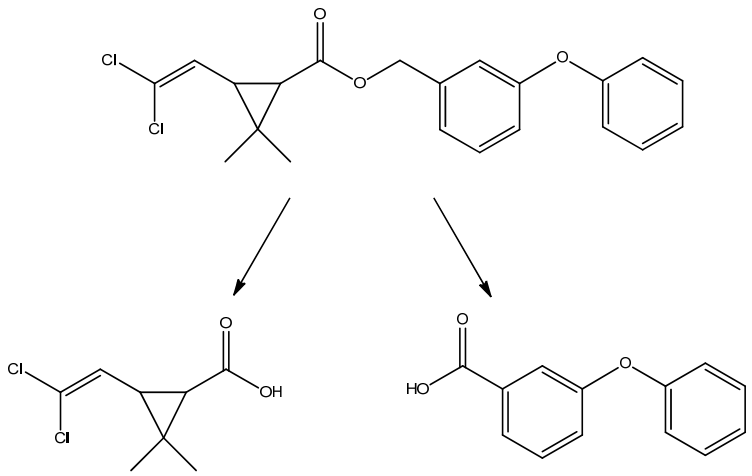


Figure 16: Hydrolysis of permethrin results in 3-phenoxybenzoic acid, which is common to many pyrethroids, and cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid, which is specific to permethrin.[69, 70]

Proposed investigations

Little has been studied on the degradation of insecticides in food. All studies mentioned above are focused only on organophosphorus insecticides, with the exception of one article which mentions carbamates. Little, if any work, of this nature has been done relating to pyrethroid insecticides. Given that pyrethroids are now one of the most used classes of insecticides in the United States, a systematic study of the degradation of pyrethroids in food is needed. Also, only a few studies have been done to follow the rate of degradation of insecticides over time, and all of the studies shown here look at one specific food (wheat, grape juice, strawberries). Of those that do follow the degradation of pesticide in food, only two use both the insecticide and its degradation product to do analyses. Of these studies, only one focuses on the insecticide exposure of children, a population segment especially vulnerable to insecticides.[36] Finally, only one of the studies observing insecticide degradates controls for analytic degradation.[38] The absence of this control severely limits the ability to interpret these studies with confidence. More

information is needed on the relationship between insecticides and their degradation products in food in order to understand human exposure to insecticides and to avoid exposure misclassification bias. This research is particularly important concerning foods that children favor because of their greater susceptibility to long-term effects from insecticide exposure.

For these reasons, I have chosen to study the degradation of insecticides in several beverage matrices, largely including juices. Children from the ages of 3-12 years tend to drink at least one serving of fruit juice daily.[38] Therefore, it is especially important that we understand more about the degradation kinetics of insecticides in juices.

Secondly, while research is ongoing to discover insecticide concentrations in commonly purchased commercial baby food, no research has been done to learn more about the insecticide degradation product concentration of such baby foods. For this reason, both insecticide and insecticide degradation residues in a sample of several vegetable and fruit baby foods have been analyzed. While the insecticide extraction and gas chromatographic analysis is based on a method developed previously by our laboratory, the degradation product extraction and liquid chromatography analysis were developed from a modified version of a urine metabolite extraction method developed at the CDC. [71-73]

Finally, my goal after graduation is to teach at the undergraduate level. I want to instill my own love of science and learning into future graduates. In particular, I am interested in introducing students to food chemistry and exposure assessment. Therefore, a simplified version of the insecticide extraction method has been developed.

In summary, the following are the specific aims I have achieved:

I. Development of a method for the analysis of malathion and insecticide degradation products in food by liquid chromatography with LC-MS/MS detection.
II. Analysis of insecticide degradation in beverages both by following the loss of insecticide and the production of insecticide metabolites.
III. Analysis of the concentration of insecticides and insecticide metabolites in baby foods.
IV. Development of a simplified method for insecticide analysis for use in undergraduate laboratories equipped with gas chromatography.

CHAPTER 2: METHOD DEVELOPMENT FOR THE EXTRACTION AND SEPARATION OF INSECTICIDE DEGRADATION PRODUCTS FROM BABY FOOD

Hypothesis

Insecticide degradation products may be extracted from food using a procedure based on current urinary insecticide metabolite extraction methods.

Introduction

Urinary organophosphorus (OP) and pyrethroid insecticide metabolites are commonly used as biomarkers of insecticide exposure.[20, 36, 37, 74-76] The use of these metabolites as biomarkers of exposure assumes that for every one molecule of urinary metabolite output specific to an insecticide, a person has absorbed and metabolized one molecule of that parent insecticide.[20] However, degradates of some insecticides, including OPs, have been found in food.[36, 38, 52] Since these degradates are often the same compounds as urinary insecticide metabolites, use of these compounds as biomarkers of exposure may lead to overestimation of insecticide exposure.[77]

There are relatively few studies of the degradation of insecticides in food, either by examination of the kinetics of insecticide degradation in food matrices or by the detection of degradates present in food.[36, 50-52, 78] Some of these examinations only consider loss of parent compound,[51, 78] while others only observe insecticide degradates.[38] However, only one of these studies are concerned with pyrethroid degradation, and it does not observe production of degradation products.[78] Pyrethroid and OPs are both used on crops both in the United States and abroad.[3] Further, both classes may be used on the same crop, leading to the possibility of contamination by degradation product residues from either class. Finally, understanding of human exposure to pyrethroids is particularly

important since use of this insecticide class has increased in the United States due to the declining use of OPs.[79]

Due to the Food Quality Protection Act of 1996, there is a new focus specifically on health effects from children's exposure to pesticides.[15] This change in focus has occurred partially because of the growth and neurodevelopment that takes place during childhood, which leads to concerns that insecticide exposure may have more far-reaching effects on children than adults.[35] Children's high metabolic rates and surface-to-volume ratio lead to a higher concentration of insecticides in their bodies than in adults.[37, 36] There have been studies showing that there are adverse cognitive effects produced from children's exposure to insecticides.[40-43] Recently, it has been found that these effects be caused by OPs even at concentrations normally found in the US population.[41] While similar results found for pyrethroid expousure have not been found, there has been little to no research done on the effects of pyrethroids on cognitive development. Therefore, it is important to have accurate understanding of children's exposure to insecticides. Since prepared baby foods comprise a large part of most infants' diets in the United States, the three most popular fruits and three most popular vegetables were chosen for method development.[80]

In order to perform complete analyses of both OP and pyrethoid insecticide degradation, methods for the extraction and analysis of both parent insecticide compounds and their degradates are required. A suitable method for the extraction of insecticides from foods and the susequent analysis of these analytes by gas chromatography has previously been developed.[71] However, there are few methods developed for the analysis of insecticide degradates in food. [38, 52, 81] Maloxon, an

oxidation product of malathion, has been extracted from strawberries.[52] DAPs, non-specific hydrolysis products from OPs, have been extracted from produce and juice. [38, 52, 81] Oxidative degradation products of OPs and carbamates have been extracted from grape juice.[50] Methods to analyze (TCPy), a specific degradate of chlorpyrifos, in food have also been developed.[36, 82] However, none of these methods combine analysis of pyrethroid and OP degradation products, and only one of these method has been tested for analytic degradation of parent insecticide into analytes,[38] which renders the other methods nearly useless. For these reasons, a method for the extraction of the insecticide degradates malathion dicarboxylic acid (MDA), 3,5,6-trichloro-2-pyridinol (TCPy), cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA) and 3-phenoxybenzoic acid (3-PBA) from baby food was developed.

Method

Standards

A native stock standard containing 10 ng/g MDA, TCPy, DCCA, and 3-PBA in acetonitrile (ACN) was used to create standard dilutions from 5-2000 ng/g. Isotopically labeled standards (MDA-D_6, DCCA-$^{13}C_3$, and 3-PBA-$^{13}C_6$) of 1000 ng/g were made in ACN.

Baby foods

Baby foods were purchased from local grocery stores. Three fruits and three vegetables were chosen based upon the most frequently purchased baby foods in the United States.[80]

Sample preparation

The method is based on an extraction of urinary insecticide metabolites developed at the CDC, but with substantial modification.[72, 73] One gram (1.0g) of baby food, 50µL labeled ISTD, and 2mL water filtered by a Milli-Q system (Millipore, Massachusetts, US) were added to a trace-cleaned conical centrifuge tube. The tube was vortexed at 1000rpm for 4 min and centrifuged at 1200rpm for 7 min. An Oasis HLB cartridge was set up on a vacuum manifold and preconditioned with 3mL methanol (MeOH) and 3 mL 1% acetic acid in H_2O. The water extract was filtered through a Bond Elut Reservoir cartridge and then loaded onto the HLB cartridge. After the cartridge was loaded, it was washed twice with 2 mL 1:5:94 acetic acid:MeOH:H_2O. The cartridge was then dried under vacuum. A test tube was placed under the cartridge in the vacuum manifold and the sample was eluted twice with 2.5 mL methanol. Because the cartridge had been dried completely, vacuum was required to start the elution process. Once eluate began to flow through the cartridge, the vacuum was broken. The eluted sample was evaporated to dryness using a Turbovap LV (Zymark, Massachusetts, US) under air at 15 psi and 45°C and were then reconstituted with 100 µL of 30:70 MeOH:H_2O. The sample was then vortexed briefly and centrifuged for 3 min before being transferred to a LC vial for analysis. Any residual particulate matter was left behind.

Calibration curves ranging from 0.25-100 ng/g were created in matrix using 50µL standard dilutions of native standard. This range was based on amounts of insecticide degradation products found previously in foods and juices.[38, 36] Matrix calibration samples were then extracted as described above.

Analysis by LC-MS/MS

An Agilent 6460 Triple Quad LC-MS/MS equipped with a negative mode electrospray ionization (ESI) interface was used to analyze samples. A Zorbax Eclipse Plus Phenyl-Hexyl column (3.0 × 100mm, 3.5µm particle size, Agilent, USA) was used for separation and kept at 45°C. Solvent A was H_2O with 1% acetic acid and solvent B was MeOH with 1% acetic acid, and the flow rate was as shown in Table 1. The following parameters were used: the source temperature was 250°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 35psi, and the corona voltage was 3500V. Each metabolite was matched to its own isotopically labeled internal standard except for TCPy, whose internal standard was isotopically labeled DCCA. Ions analyzed in MRM mode and their optimized fragmentor and collision energies are shown in Table 2. Mass Hunter Quantitative software (Agilent, Santa Clara, CA) and Microsoft Excel (Redmond, WA) were used for data analysis.

minute	% B	flow rate (mL/min)
0	30	0.8
1.5	35	0.8
3	50	0.8
8	60	0.8
8.8	100	1
10	100	1
11	80	1
13	80	1

Table 1: Flow rate through column for metabolite analysis.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)	RT (min)
MDA-ISTD	280	147	80	1	3.3
MDA	273	141	80	1	3.9
MDA	273	157	80	12	3.9
TCPy	198	198	96	0	7.3
TCPy	196	196	96	0	7.3
DCCA-ISTD	210	210	90	0	6.7
DCCA	207	207	90	0	8.2
DCCA	209	209	90	0	8.2
3-PBA-ISTD	219	99	98	20	7.4
3-PBA	213	93	122	16	9.0
3-PBA	213	169	122	8	9.0

Table 2: Instrument parameters for optimized parent and daughter ions of insecticide degradation products with fragmentor energies, collision energies, and retention times. The quantification ions (listed first for each native pair) are more abundant than the confirmatory ions.

Results

Method performance

Two sets of matrix-based calibration curves were created, one in vegetables and one in fruits. Calibration curves were linear and resulted in R^2 <0.98 (Figure 1).

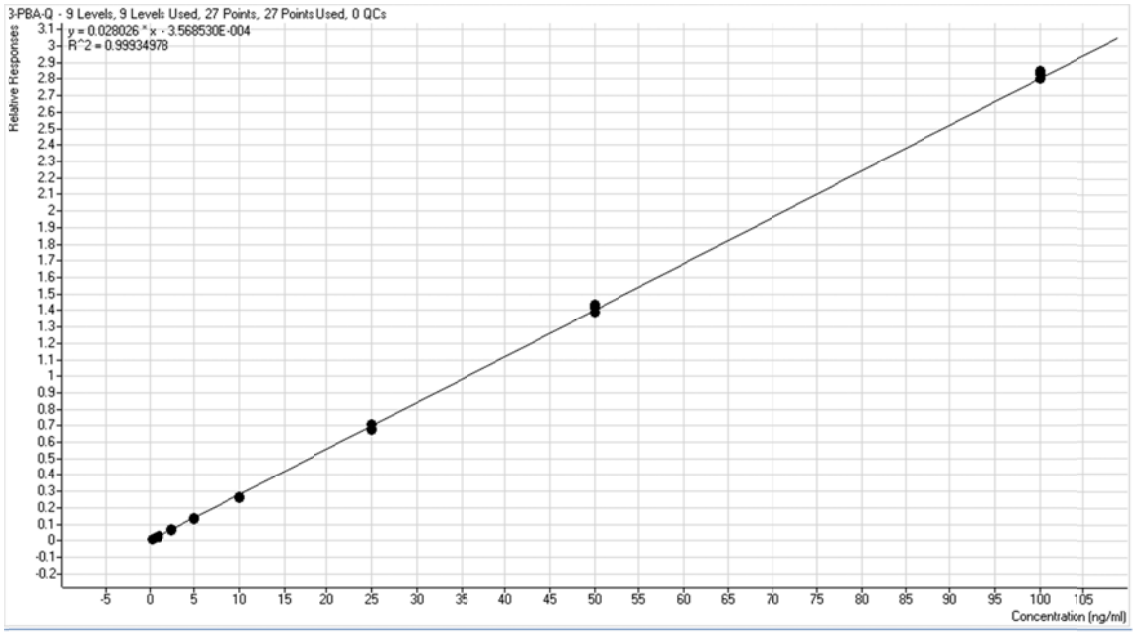


Figure 1: Calibration curve for 3-PBA in vegetables. All calibration points were injected in triplicate.

Method accuracy for fruits and vegetables was determined using relative recoveries of spiked samples at two concentrations. For each matrix, 1.0g of each type of food was fortified with either parent or metabolite compounds to either 10 or 25ng/g. Fortified samples were then treated the same as other samples. Within-run precision, presented as relative standard deviation (RSD), was also calculated for fruits and vegetables based on these fortified samples. Finally, limits of detection were calculated using replicates of blanks and low spiked samples as the lowest analyte concentration at which reliable detection is feasible. If analyte was detected in the blank samples, the following procedure was used to determine the limit of detection. Ten replicate samples were injected and the mean blank concentration was determined. The standard deviation (SD_{Blank}) of the blank concentrations was also determined. The LOD, defined here as the lowest concentration acceptable as a measured quantity is defined by the equation:

$$LOD = mean_{blank} + 3(SD_{blank})$$

If no analyte was detected in the sample, a different procedure was used to determine the LOD. MassHunter software affords an estimate of the signal-to-noise ratio (S/N) for each sample taken. The reported LOD is determined by noting when S/N drops below 3 among the standards. If the S/N for the lowest standard is greater than three, we report the LOD as the concentration that would have produced S/N=3 based on extrapolation. For example, if the lowest standard were 0.1 ng/g and the reported S/N was 6, we would report the LOD as 0.05 ng/g since it would be expected that such a concentration would result in S/N =3 given linear extrapolation of response versus concentration and equal noise.

As shown in Table 3, relative recoveries were between 80-120% for all samples except TCPy in fruit. Table 4 summarizes method performance by showing average relative recovery, relative standard deviation, and limits of detection stratified by fruits and vegetables. All RSDs are under 15% with the exception of MDA.

	MDA	TCPy	DCCA	3-PBA
Green Beans				
QCL	85.8	119.5	100.0	94.5
QCH	93.1	95.4	93.4	97.8
Peas				
QCL	109.8	93.0	104.6	91.0
QCH	106.8	84.5	110.2	94.1
Carrots				
QCL	79.6	116.6	104.1	92.9
QCH	80.2	108.2	97.7	88.1
Apples				
QCL	76.6	133.1	103.1	114.5
QCH	86.1	123.7	102.3	112.6
Banana				
QCL	107.3	109.3	109.7	98.3
QCH	127.1	110.2	106.0	93.3
Pears				
QCL	92.0	123.5	93.2	98.9
QCH	109.1	132.3	100.5	101.5

Table 3: Relative recoveries for degradation products in baby food expressed as percentages. QCL is at 10 ng/g, while QCH is at 25 ng/g.

	low spike	hi spike	LOD (ng/g)
vegetable			
MDA	91.7±15.9	93.4±13.3	2.3
TCPy	109.7±14.5	96.0±11.9	2.7
DCCA	102.9±2.6	100.4±8.7	0.87
3-PBA	92.8±1.7	93.3±4.9	0.23
fruit			
MDA	92.0±15.4	107.5±20.6	0.18
TCPy	122.0±12.0	122.1±11.1	0.76
DCCA	102.0±8.3	102.0±8.3	0.87
3-PBA	103.9±9.2	102.5±9.7	0.24

Table 4: Results for recovery of analytes from fruits and vegetables. Relative recoveries, RSDs, and LODs are presented. Low spike is at 10ng/g, and high spike is at 25ng/g.

Test for analytic degradation

A major limitation of some environmental hydrolysis product analyses is that hydrolysis of the parent compound during extraction and/or separation could lead to overestimation of analyte.[83] To test for analytic degradation in baby food fruits and vegetables and to refute the argument that degradation products were not present in samples until insecticides were hydrolyzed during extraction and/or analysis, six samples of 1.0g carrots and six samples of 1.0g apples were obtained. For each matrix, three of the samples were fortified with 25ng/g pesticide sample, and all samples were fortified with metabolite ISTD. The samples were then extracted and analyzed according to the insecticide metabolite procedure. It was determined that there was no significant difference between insecticide degradate concentrations between samples that were or were not fortified with parent compound using a two-tailed *t*-test ($p = 0.05$).

Discussion

Solid phase extraction

This method is based on a urinary insecticide metabolite procedure developed at the Centers for Disease Control and Prevention.[72] Since Oasis HLB cartridges are compatible with aqueous analytes and water has been used as the extraction solvent for insecticide degradates in food previously, water was chosen for the extraction solvent in this procedure.[52]

MDA (pK_a = 5.64, 4.00), TCPy (pK_a = 4.55), DCCA (pK_a = 3.89), and 3-PBA (pK_a 3.95) are all acidic compounds.[84-86] However, using acidified water (pH = 2.8) to condition the column and to wash after loading helps to suppress ionization of acidic compounds and allows them to be better retained by hydrophobic interactions with the sorbent.[87] Washing cartridges before elution with the acidified methanol/water mix removes salts and proteins. As methanol is a more nonpolar solvent, it is able to elute the analytes off the column.[87]

The divinylbenzene component of the Oasis HLB cartridge allows for π- π interactions. This interaction causes pigments, which are often include vinyl compounds, to be retained on the cartridge. While visual observation of the cartridge allows observation of pigments on the cartridge after elution, some pigments are eluted with the analyte, and extracts are somewhat dirty. However, liquid chromatographic analysis is more able to allow for dirty samples than gas chromatography, so pigmented samples are still able to be analyzed.

Method performance

All recoveries were between 80-120% except TCPy in fruit (122%). However, TCPy is the only analyte in this method without a matching isotopically labeled standard; its comparative internal standard is DCCA. Therefore, if DCCA interacted differently with the matrices than TCPy, for example, if TCPy were preferentially adsorbed by proteins or other matrix surfaces over DCCA, those differences could lead to the high recoveries seen in this study. Ion abundances in the mass spectrometer may change due to change in solvent composition which in turn shifts retention time. Such changes that are not matched by ISTD may also lead to less accurate recoveries.

Recoveries for TCPy could possibly be corrected if an extraction efficiency experiment was done. In such an experiment, some samples would be fortified with both native standard and ISTD before extraction. Others would be only spiked with ISTD before extraction, but would be spiked with native standard after extraction but before analysis. This would allow analysis of analyte lost during the extraction procedure. If less DCCA were lost during extraction than TCPy, that observation could partially explain the high recovery of TCPy.

Imprecision was under 15% for all analytes except MDA. RSDs under 15% are preferred for analytical analyses.[88] MDA tends to produce less precise results because it is the only analyte which has a deuterated ISTD instead of one containing ^{13}C or other less easily exchanged isotopes.

Green beans and peas produced acceptable recoveries (between 80-120%) for all analytes. Carrots and bananas produced acceptable recoveries for all analytes except MDA, apples produced acceptable recoveries for all analytes except TCPy, and pears for

all analytes except TCPy and MDA. However, only one low spike and one high spike for each matrix was analyzed. A larger number of samples (*N*) would allow more statistical analyses and would likely lead to more accurate and precise results.

This method could easily be expanded to analyze produce or other foods for insecticide degradation products. If other foods were analyzed, prehomogenization using a blender or food processor before the extraction step would be required to insure maximum recovery of analyte.

Analytic degradation

High concentrations of insecticides which degrade into the target analytes were spiked into baby foods before extraction of degradates to test for analytic degradation. Even when using as much as 25ng/g insecticide, which is 100x the lowest degradate calibration point used, significant amounts of degradation products were not produced. This fact allows us to use this method to analyze the degradation of insecticides in food without creating false positive detection of insecticide degradate.

Conclusions

The specific OP degradation products MDA and TCPy and pyrethroid degradates DCCA and 3-PBA has been analyzed using one chromatographic method. Relative recoveries are generally between 80-120%, while imprecisions are generally under 15%. While more analyses need to be done to evaluate the between-day precision of this method, it is suitable for the analysis of insecticide degradates in baby food. Use of this method in actual baby food samples will allow more complete understanding of

insecticide degradation product concentrations in food, and this knowledge in turn will allow more accurate estimation of human insecticide exposure.

APPENDIX A: DEVELOPMENT OF LIQUID CHROMATOGRAPHY METHODS FOR INSECTICIDE DEGRADATES, MALATHION, AND DAPS

Separation of insecticide degradation products by LC-MS/MS

In order to study the degradation of insecticides in food and beverages, a new LC-MS/MS method to separate and quantify four insecticide metabolites (MDA, TCPy, DCCA, and 3-PBA) using LC-MS/MS was desired. The parent and daughter fragmentations were previously optimized in our laboratory; results are shown in Table 1. Fragment structures for 3-PBA and MDA are shown in Figures 1 and 2. TCPy and DCCA are not shown because they did not fragment at a sufficient intensity; therefore, their chlorine patterns are instead used for confirmation.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)	RT (min)
MDA-ISTD	280	147	80	1	3.3
MDA	273	141	80	1	3.9
MDA	273	157	80	12	3.9
TCPy	198	198	96	0	7.3
TCPy	196	196	96	0	7.3
DCCA-ISTD	210	210	90	0	6.7
DCCA	207	207	90	0	8.2
DCCA	209	209	90	0	8.2
3-PBA-ISTD	219	99	98	20	7.4
3-PBA	213	93	122	16	9.0
3-PBA	213	169	122	8	9.0

Table 1: Instrument parameters for optimized parent and daughter ions of insecticide degradation products with fragmentor energies, collision energies, and retention times. The quantification ions (listed first for each native pair) are more abundant than the confirmatory ions.

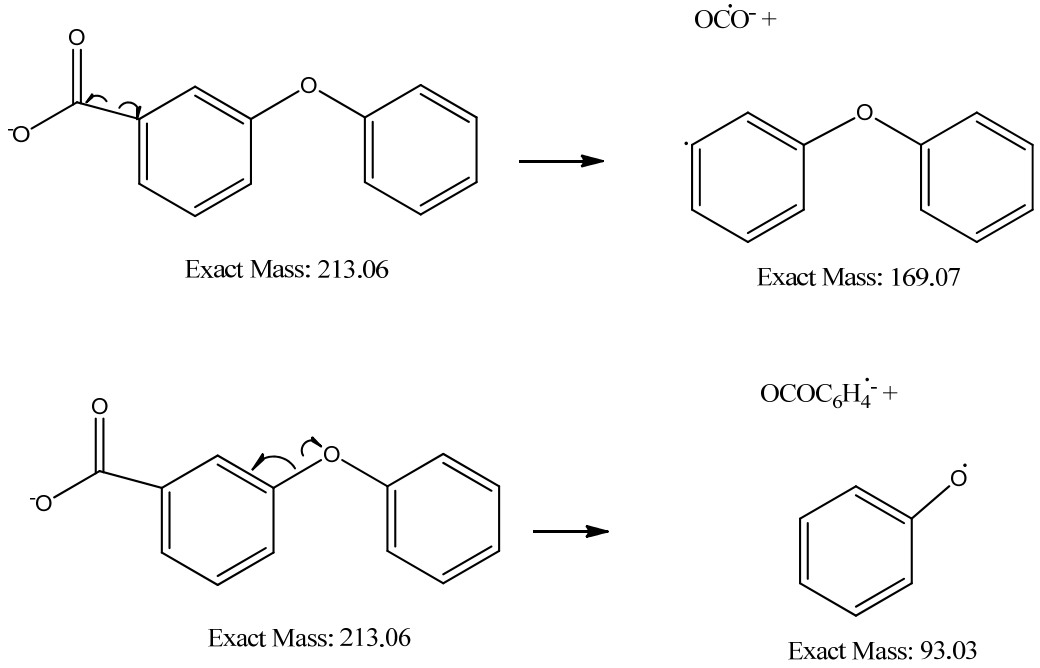


Figure 1: Fragmentation of 3-phenoxybenzoic acid (3-PBA).

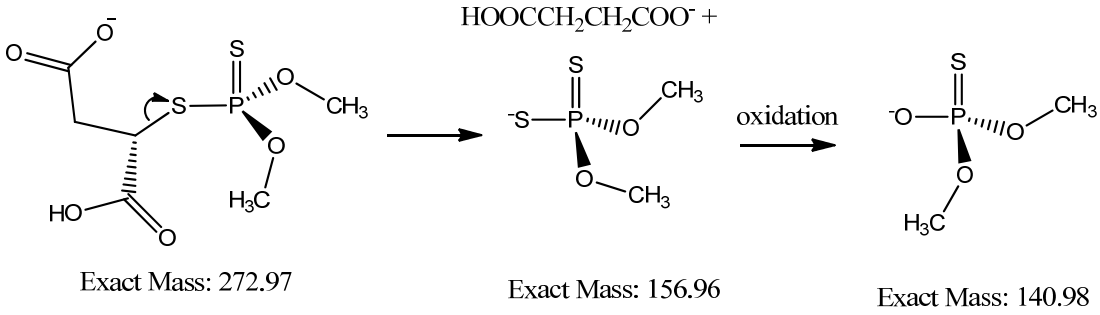


Figure 2: Fragmentation of malathion dicarboxylic acid (MDA).

Method

The chromatography profile was optimized for metabolite separation on a Zorbax Eclipse Plus Phenyl-Hexyl column (3.0 × 100mm, 3.5µm particle size, Agilent, USA). Several trials using different solvent profiles were tested. In each trial, solvent A is 0.1% acetic acid in water, while solvent B is 0.1% acetic acid in methanol. At the end of each trial, 100% solvent B is allowed to flow through the column for at least 2 min to rinse

any organic residue out of the column, then 30% solvent B is run through the column for at least 2 min to equilibrate the column for the next run.

Results and discussion

In Figure 3, a mixture of the four compounds was injected into the instrument with the solvent profile shown below. MDA eluted first, followed by TCPy, DCCA, and 3-PBA. Ideally, at least 45 sec between each elution is desired in order to completely separate the compounds.

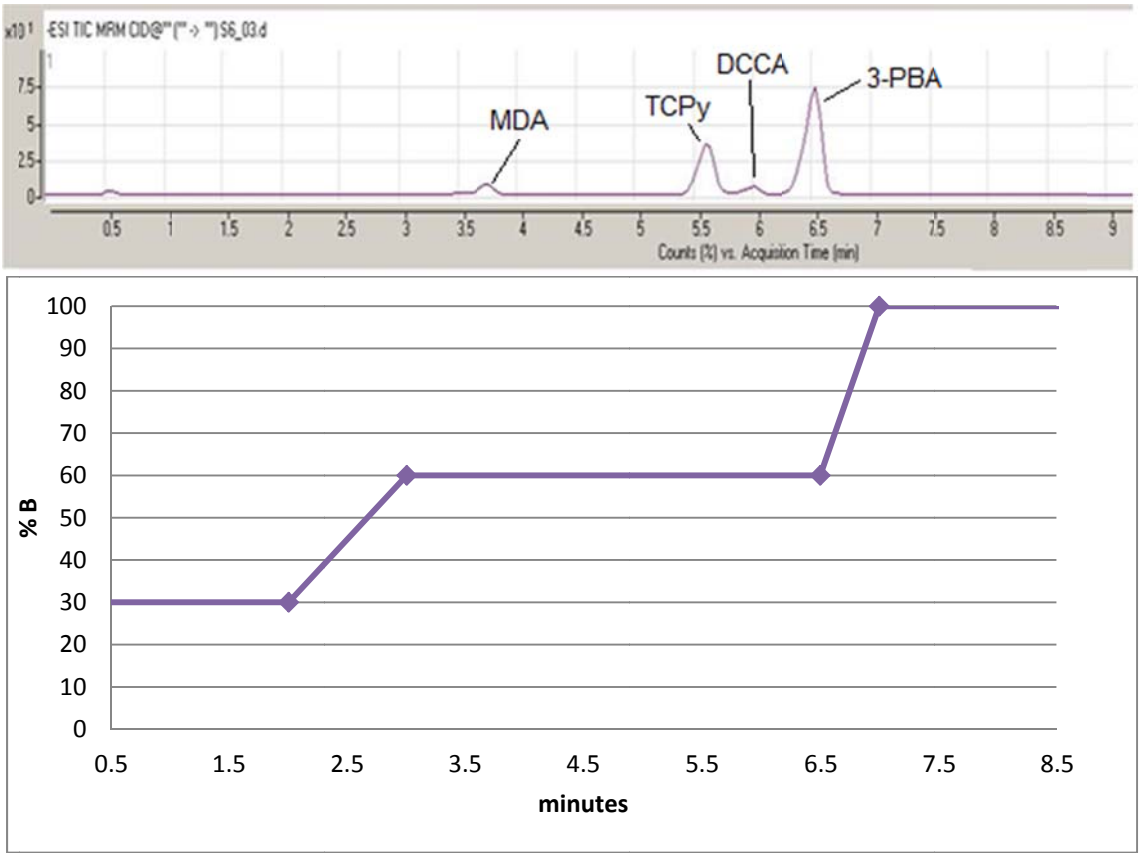


Figure 3: Trial 3 for insecticide degradate separation. Trials 1-2 are not shown. The first small peak is the solvent front.

In Figure 4, the increase in % MeOH from 2-3 minutes is less sharp than in Figure 3 because a slow increase in % MeOH is allowed from 0-2 min and from 3-6.5 min. Although it was hypothesized that the lack of isocratic chromatography during compound elution would negatively impact peak shape, the goal was to separate the compounds better. While the compounds were better separated, peak shape was unacceptable due to front tailing and broadness.

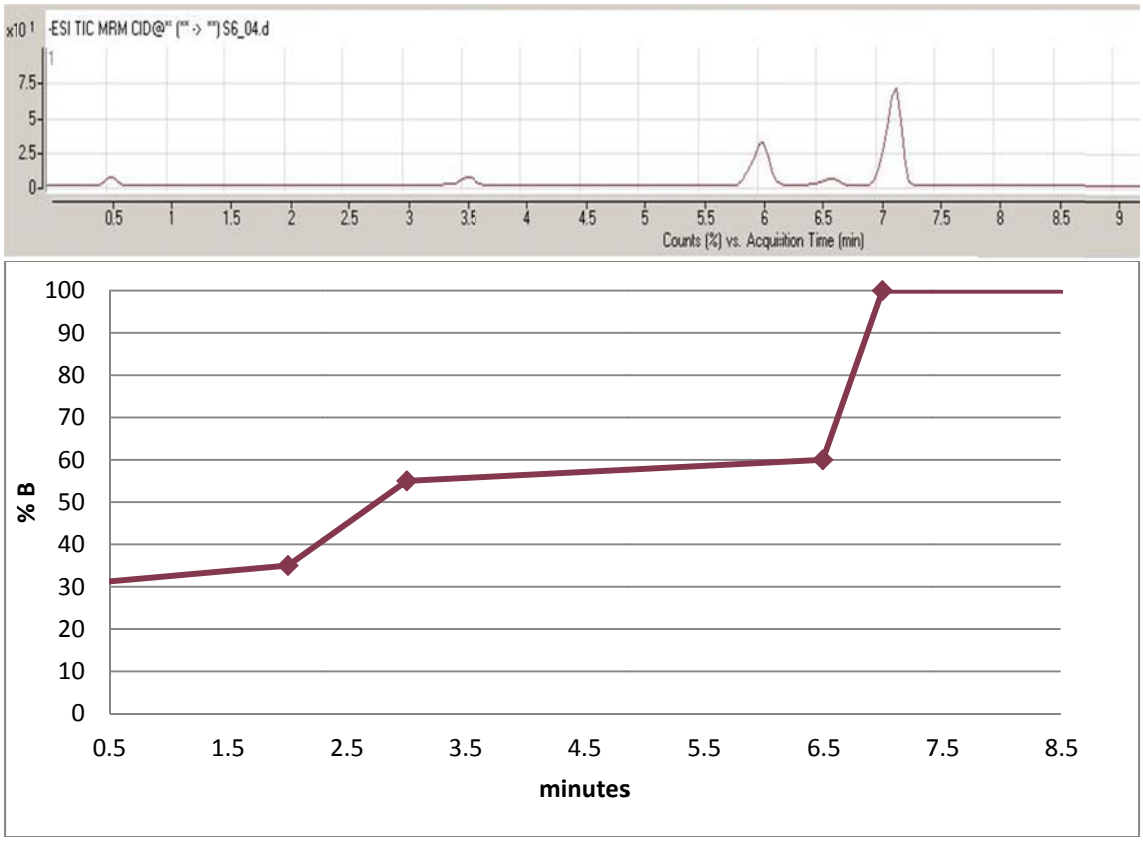


Figure 4: Trial 4 for separation of insecticide degradates.

In Figure 5, % B increases from 60-70% from 3-6.5 minutes, rather than from 55-60% as in Figure 4. This change to a higher concentration of methanol causes TCPy and DCCA to coelute. Therefore, this type of change was abandoned, and solvent profiles similar to the first shown were used.

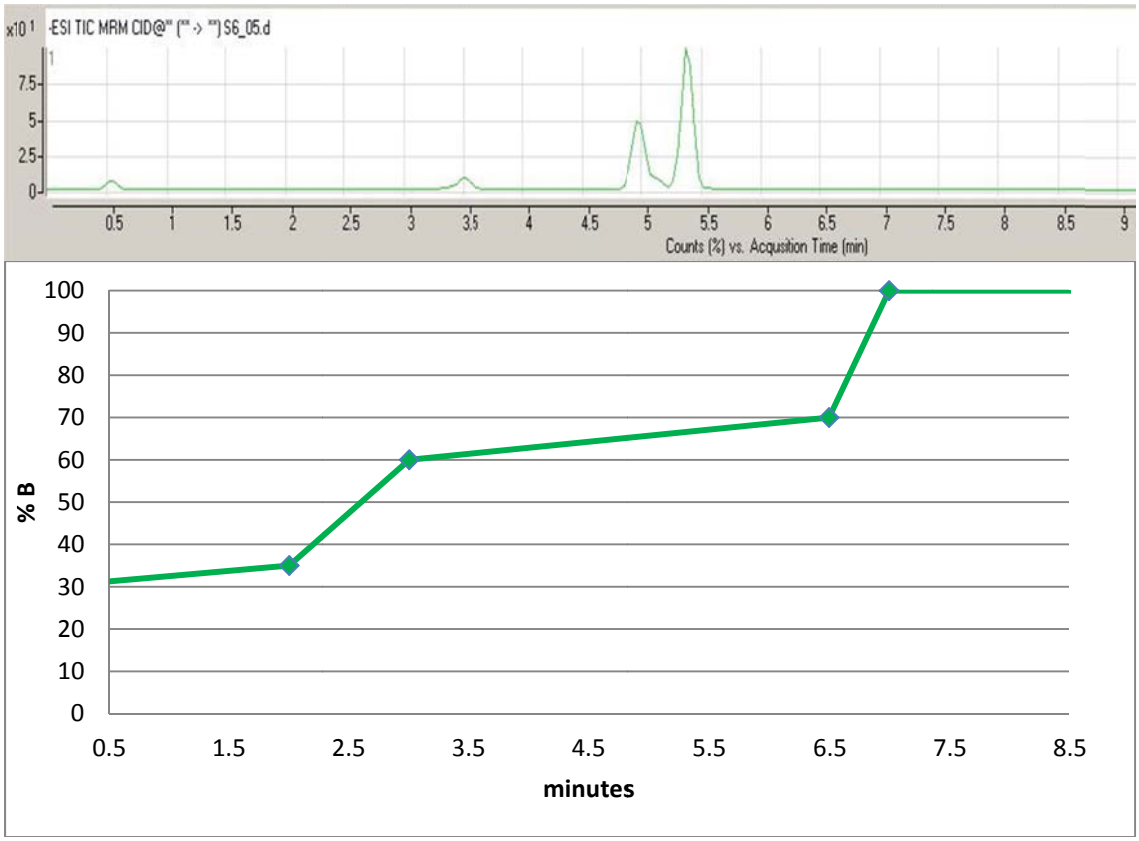


Figure 5: Trial 5 for separation of insecticide degradates.

In Figure 6, the solvent profile is identical to that in Figure 3, except that second and fourth transitions are moved back 30 sec each. This way, time of isocratic elution is shorter. The compounds elute slightly sooner, but seperation is not any better. Also, peak shape is preferable in Figure 3.

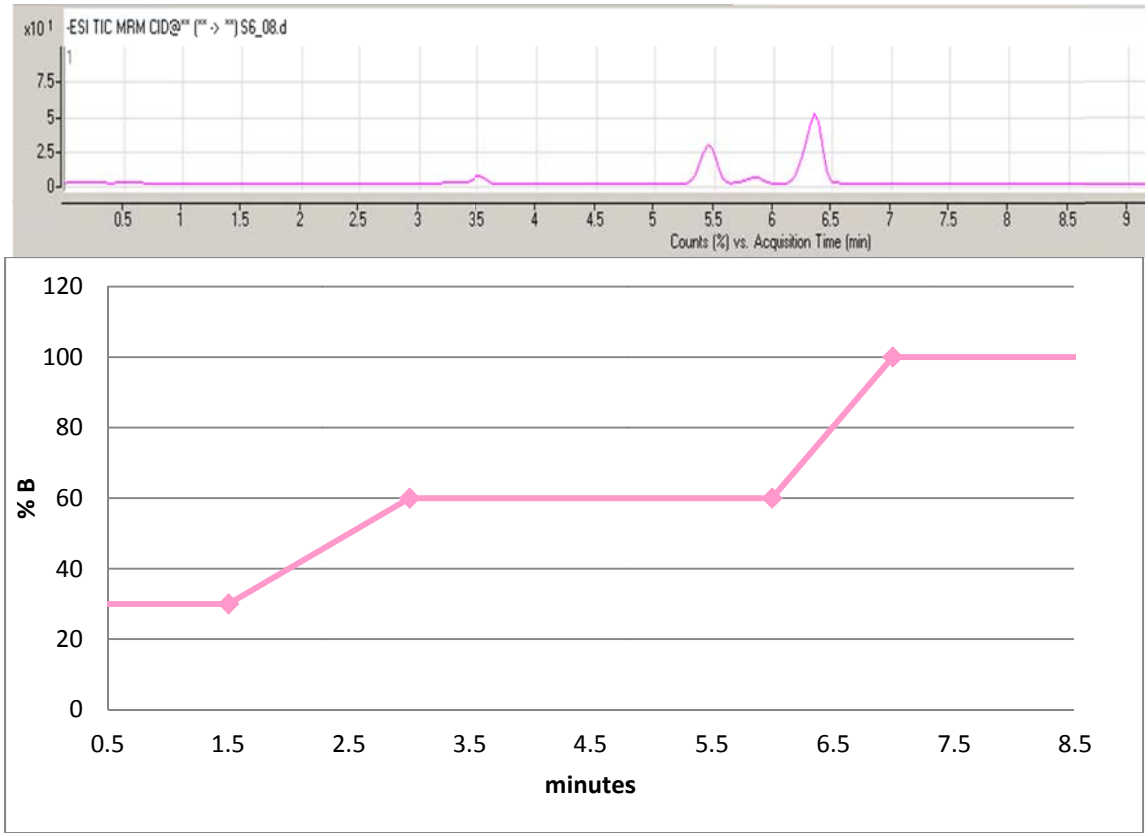


Figure 6: Trial 8 for separation of insecticide degradates.

Finally, a profile with a more gradual build of % B was chosen (Figure 7). This profile allowed separation of over 45 sec between each peak, and peak shape was also much sharper and more symmetrical.

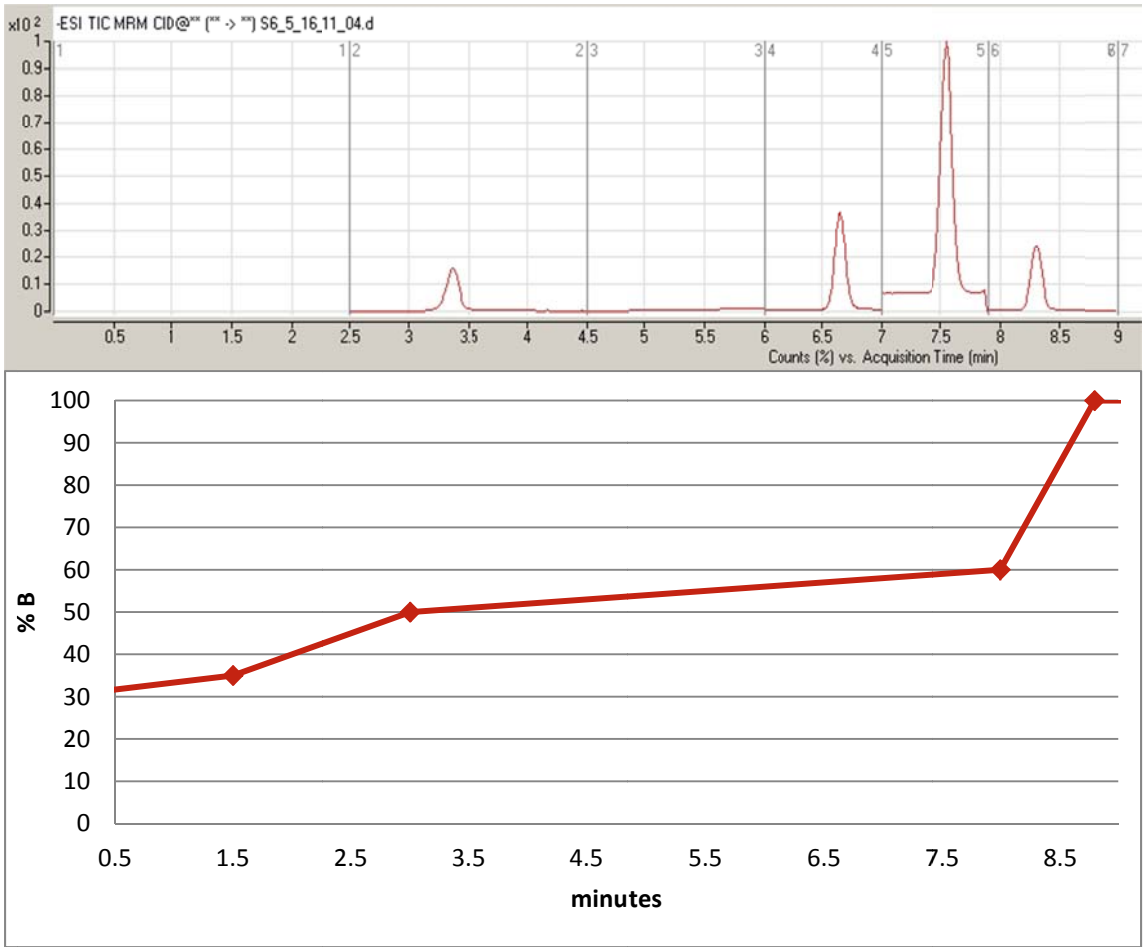


Figure 7: Final Trial for separation of insecticide degradates. The segmenting contributes to the higher signal-to-noise ratio and the baseline shift and will be explained below.

The mass spectrometer allows time segmentation so that all mass transitions don't need to be followed simultaneously. Instead, only the desired transitions are followed in a given time frame. It is hypothesized that using the segmentation feature of the instrument creates less background noise. After the chromatography solvent profile was optimized, the mass spectrometry profile was segmented. Figure 8 demonstrates segment development. During the first trial, DCCA was included in Segment 2. However, Segment 2 ended before DCCA was detected, so it is not observed. In the second trial, DCCA was included in the third segment instead. As seen in the last trial, all four compounds may have their own segment. While this segmentation decreases noise, it also increases the risk that a peak is split between segments. If a peak split occurs, the peak not completely detected and is therefore unable to be analyzed For this reason, DCCA is included in a section with 3-PBA during analysis, as shown in the second trial.

It should be noted that the hypothesis that segmentation reduces background signal is confirmed by the segmentation optimization. Segments that contain two compounds, such as Segment 2 in Trial 1 and Segment 1 in Trial 2, have a higher background signal than segments containing only one compound. However, the background signal for DCCA, even when it is alone in Trial 3, is significantly higher than the signal for MDA or 3-PBA. This is likely because DCCA is recognized only by chlorine patterns and is not fragmented (Q *m/z* =207 → 207, C *m/z* = 209 → 209). Therefore, it does not use true tandem mass spectrometry, and its detection is much less specific. This decreased specificity leads to higher background signal from matrix contamination.

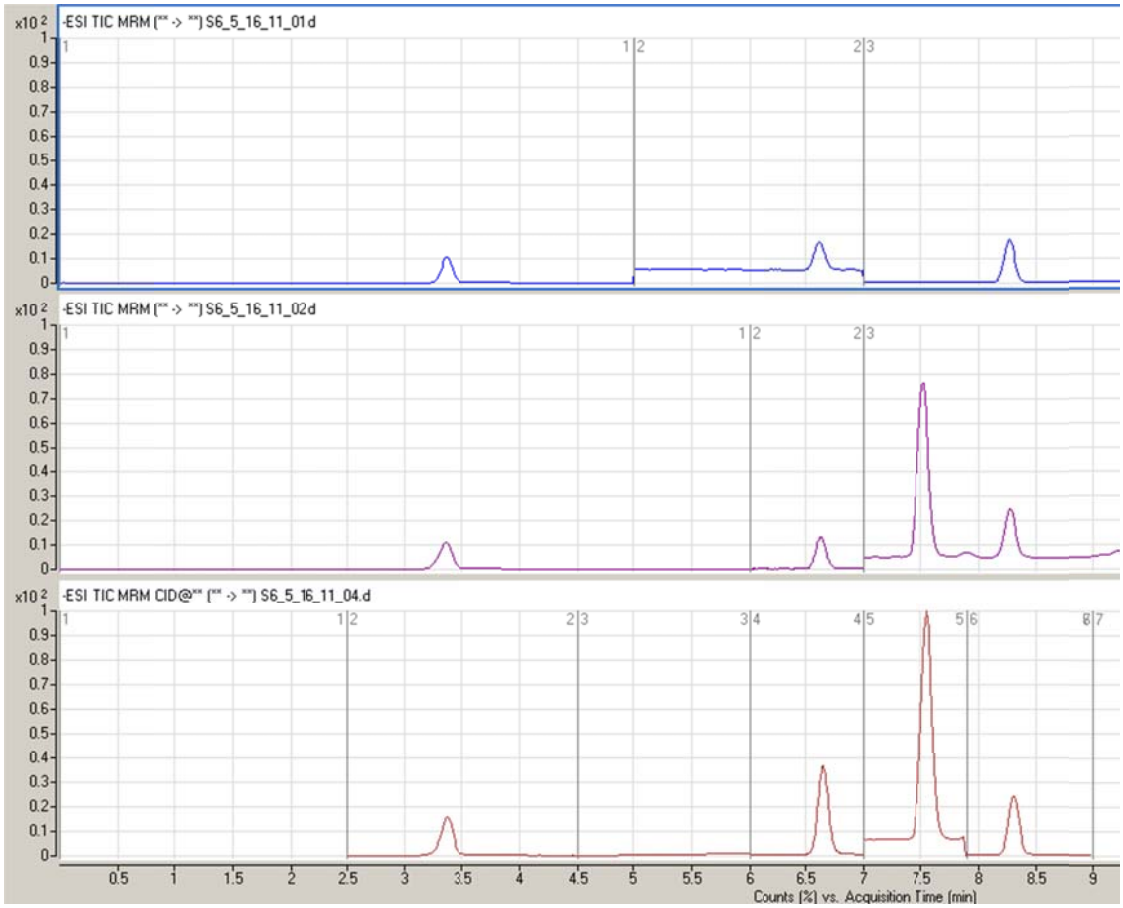


Figure 8: MS/MS segmentation development. The first analyte is MDA, the second analyte is TCPy, the third is DCCA (not shown in Trial 1), and the fourth is 3-PBA.

Conclusions

This chromatographic method effectively separates insecticide degradation products. Combining this method with the extraction method explained previously in this chapter will allow the analysis of insecticide degradation products in food and beverages, which will allow better assessment of dietary insecticide exposure.

Separation of malathion by LC-MS/MS

While analysis of malathion by GC-MS/MS has been attempted in our laboratory in the past, results were problematic because of difficulty obtaining consistent results with quantification and confirmatory ions. This was likely due to the thermal lability of malathion, causing it to degrade in the injection port.[89] Since LC-MS/MS does not require volatilization of the analyte and does not introduce it the high temperatures necessary for GC analysis, a method for malathion analysis extended from previous. LC-MS/MS methods.[90, 91]

Methods

Positive mode ESI was used to analyze malathion with the following parameters: the source temperature was 300°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 45psi, and the corona voltage was 3500V.

The standard was analyzed by SIM mode to confirm the compound's presence (*m/z* = 331). Next, the mass spectrometry was optimized using Agilent's MassHunter Optimization software (Agilent Technologies, Santa Clara, CA). Finally, a solvent profile was optimized (Table 2) using a BetaSil C18 column (3.0 × 100mm, 3.0µm particle size, Thermo Scientific).

minute	% B	flow rate (mL/min)
0	30	0.7
4	100	0.7
6	100	0.7
6.5	30	0.7
8.5	30	0.7

Table 2: Solvent profile for malathion, in which solvent A is 0.1% acetic acid in water, and solvent B is 0.1% acetic acid in methanol.

Once the LC method was optimized, an extraction method developed by Hunter et al.[71] was used to create two sets of matrix-based calibration curves in baby food fruits and vegetables, and QC samples at concentrations of 2 and 5 ng/g were tested for accuracy.

Results

Malathion was found in single ion mode. It was possible to fragment the analyte, and results of daughter ion optimization are in Table 3. Figures 9 and 10 depict resulting daughter ions and schemes which would produce them. These results for parent and daughter ions have been confirmed by comparison with Garcia-Reyes.[92]

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)
Mal ISTD	341	100.1	90	21
Mal-Q	331	127	60	5
Mal-C	331	99	60	21

Table 3: Precursor and product ions for malathion.

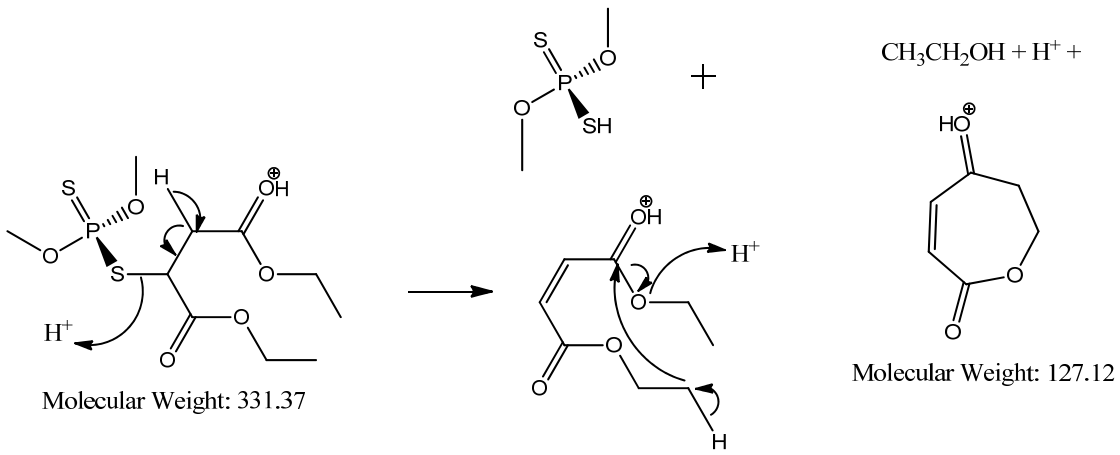


Figure 9: Fragmentation of malathion into *m/z* = 127.

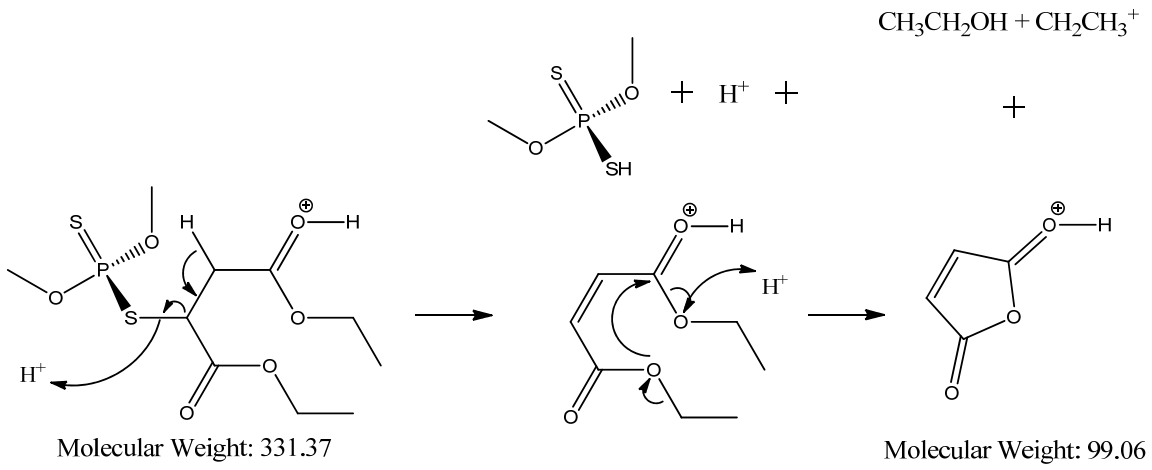


Figure 10: Fragmentation of malathion to *m/z* = 99.

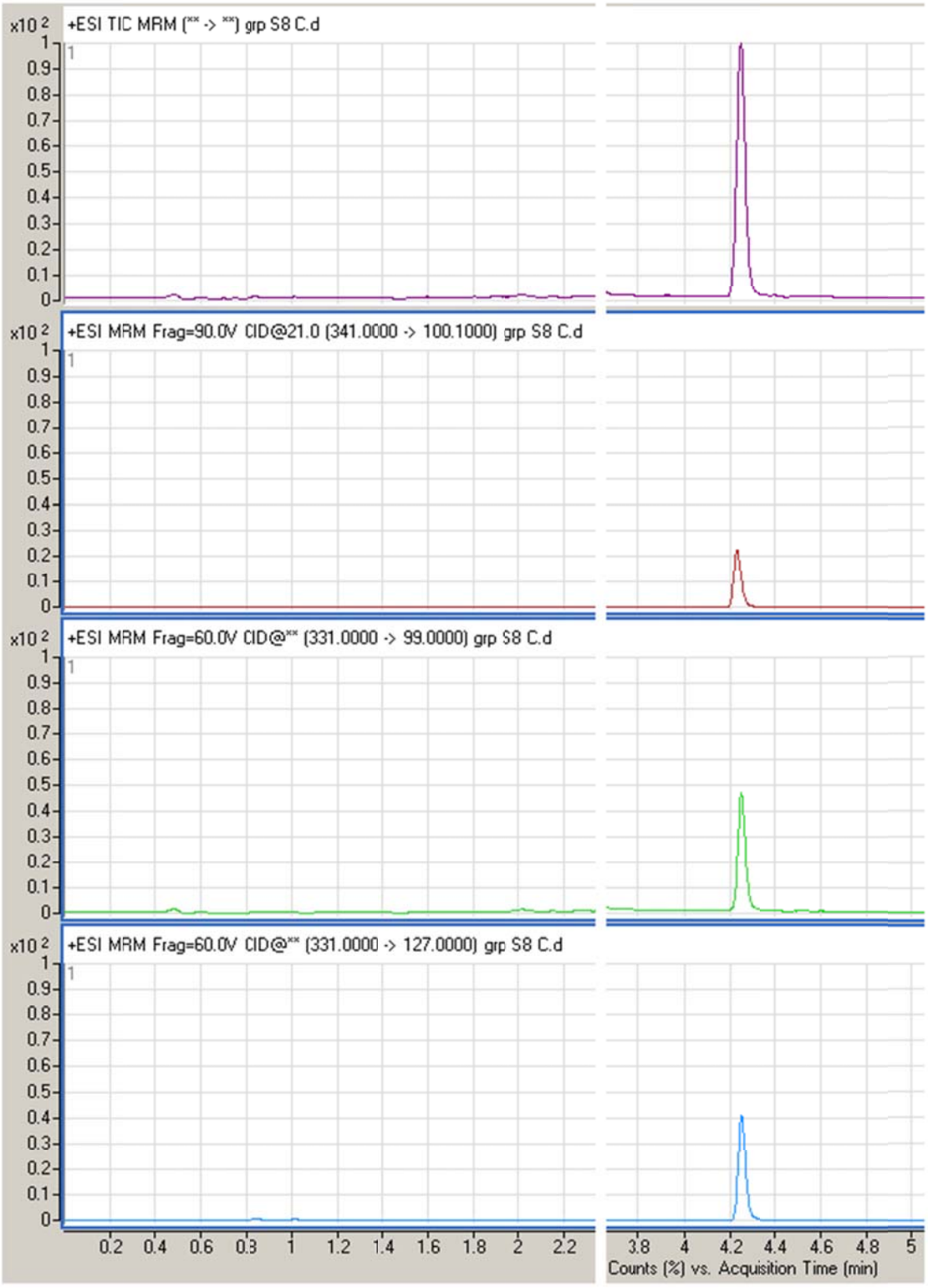


Figure 11: Extracted chromatograph for malathion. The 341 → 100 transition represents the ISTD, malathion D_{10}, which contains deuterium atoms on the two ethyl groups past the two ester linkages.

Figure 11 shows the extracted chromatogram of both the quantification and confirmatory ions for the final solvent profile. While solvent profile optimization was simplified by the fact that there was only one compound, isocratic elution was not used for two reasons. Beginning chromatography with a higher concentration of water concentrates the analyte, allowing for a sharper peak. Also, the eventual increase in methanol concentration washes organic residues off the column, allowing for cleaner chromatography over extended periods of time.

Calibration curves in baby food fruits and vegetables are linear with a correlation coefficient > 0.99. Table 4 shows extraction method performance in fruits and vegetables. Recoveries are between 92-104%, and RSDs are under 16%.

	QC low	QC high	LOD (ng/g)
vegetables	92.1±15.2	103.6±15.2	2.8
fruits	100.9±4.5	95.0±4.5	3.6

Table 4: Method performance for malathion in baby food vegetables and fruits. Low spike is at a concentration of 2.0ng/g, while high spike is at a concentration of 5.0ng/g.

Conclusions

This chromatographic method allows the rapid and accurate quantification of malathion in baby foods and juices at the low ppb level.

Method development for liquid chromatographic separation of dialkyl phosphates

Although dialkylphosphate (DAP) analysis was not used in the studies included in this dissertation, the use of these compounds as biomarkers of OP exposure make them useful for both future degradation studies and urinary metabolite studies.

Methods

First, each individual standard was injected onto the column using single ion mode (SIM) detection for confirmation of analyte presence. All these analytes were optimized to find which daughter ions would give the greatest signal intensities using the MassHunter Optimizer software (Agilent Technologies, Santa Clara, CA). Electrospray ionization (ESI) with jet stream technology was used. Daughter ions were confirmed by setting up multiple reaction monitoring (MRM) mode using the parameters produced by the optimization software for each compound and then testing individual analytes for daughter ion detection. Finally, MRM mode was used to determine the best chromatography to separate the compounds. Figure 12 shows results of different chromatography profiles, where Solvent A is 0.1% acetic acid in water and Solvent B is 0.1% acetic acid in methanol. A 2µL injection was used on a C18 column with a bore of 2.1mm and 3µm particle size. Figure 13 shows solvent profiles tested using mass spectrometry conditions indicated in Table 5.

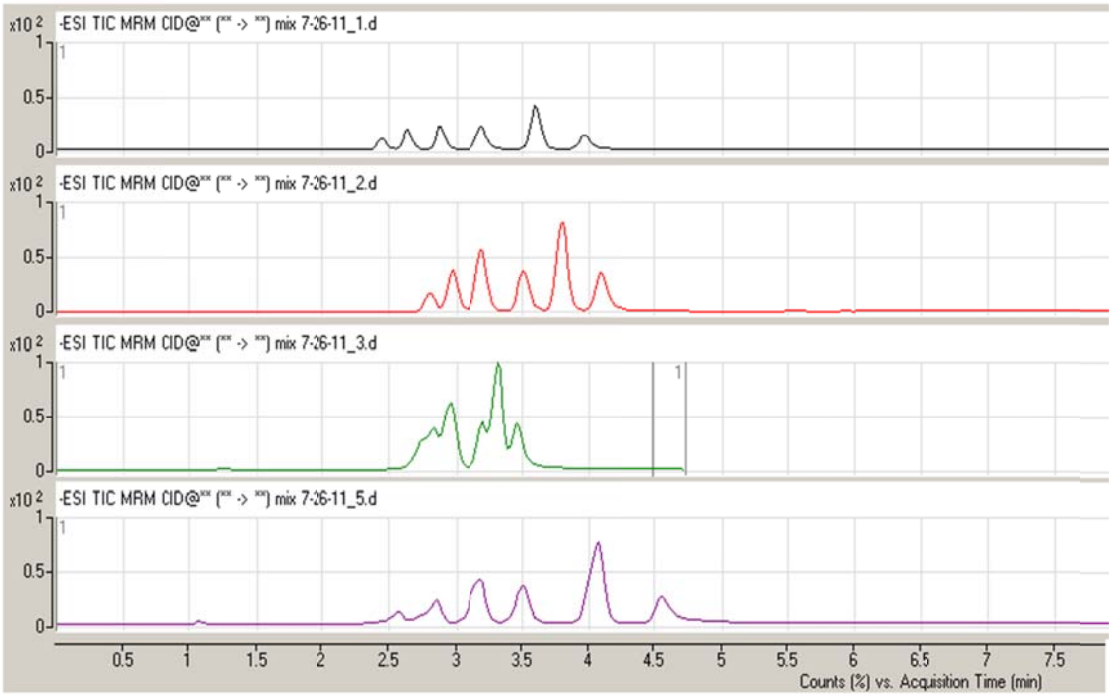


Figure 12: Examples of chromatograms produced using solvent profiles in Figure 9. The order of retention, from first to last, is dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP).

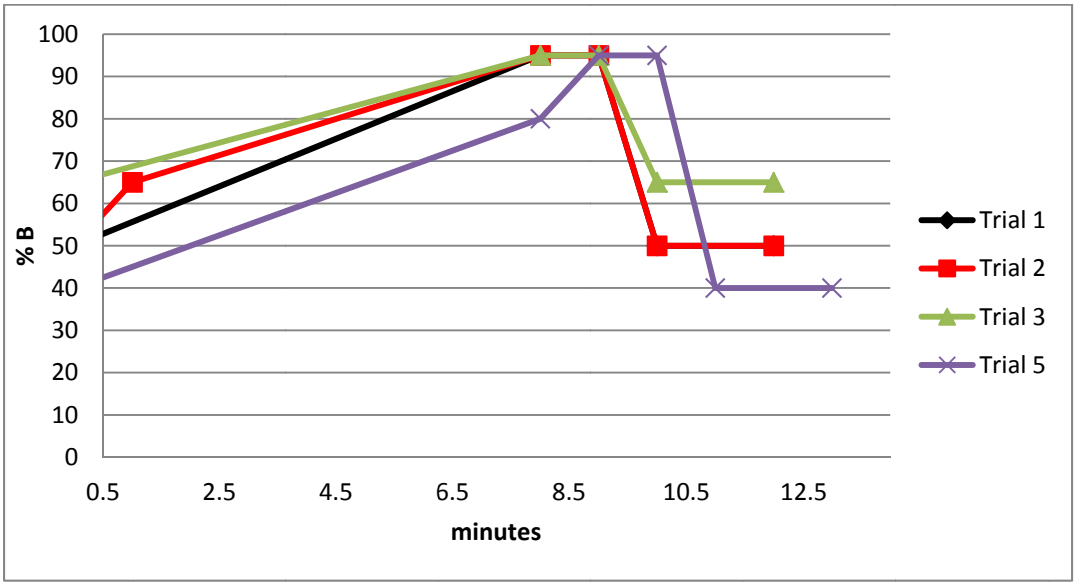


Figure 13: DAP solvent profiles corresponding to chromatograms shown in Figure 12. Solvent profiles are shown on a different time scale than chromatograms to demonstrate the complete profile.

gas temperature	300°C
gas flow	5 L/min
nebulizer	45 psi
sheath gas temperature	250°C
sheath gas flow	11 L/min
capillary voltage	3500 V
nozzle voltage	500 V

Table 5: Mass spectrometry conditions for DAP analysis.

Results

Each parent ion was found using single ion mode. Example SIM results for dimethylthiophosphate (DMTP, MW 142g/mol) are shown in Figure 6. It was possible to fragment each analyte, and results of daughter ion optimization are in Table 14. Figures 3-8 depict possible results daughter ions and schemes which would produce them. These results for parent and daughter ions have been confirmed with those previously obtained by Dularent and by Hernández.[93-94]

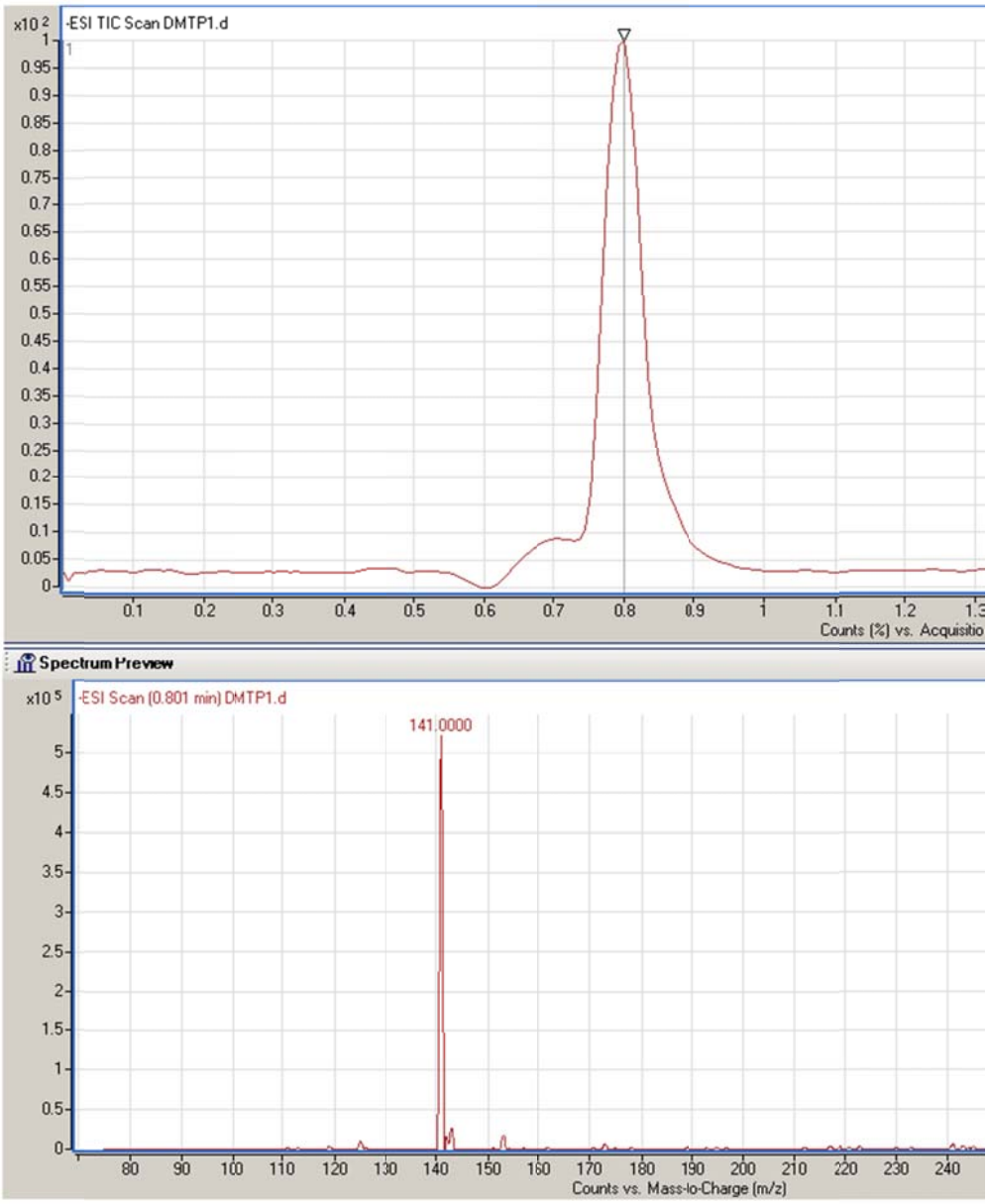


Figure 14: SIM results for dimethylthiophosphate. Negative ion mode is used.

Compound	parent	daughter	dwell time	fragmentor	CE	polarity
DEDTP	185	156.9	50	60	9	negative
	185	110.9	50	60	5	negative
DETP	169	141.0	50	60	9	negative
	169	94.9	50	60	17	negative
	169	79.0	50	60	50	negative
DMDTP	157	141.9	50	60	13	negative
	157	111.9	50	60	21	negative
	157	97.0	50	60	45	negative
DEP	153	125.0	50	60	4	negative
	153	79.0	50	60	25	negative
DMTP	141	125.9	50	60	13	negative
	141	94.9	50	60	21	negative
DMP	125	110.0	50	70	13	negative
	125	79.0	50	70	25	negative
	125	63.0	50	70	13	negative

Table 6: Fragmentation of DAPs using electrospray ionization. Parent ions and daughter ions are shown in order of detection intensities with fragmentor and collision energies in volts.

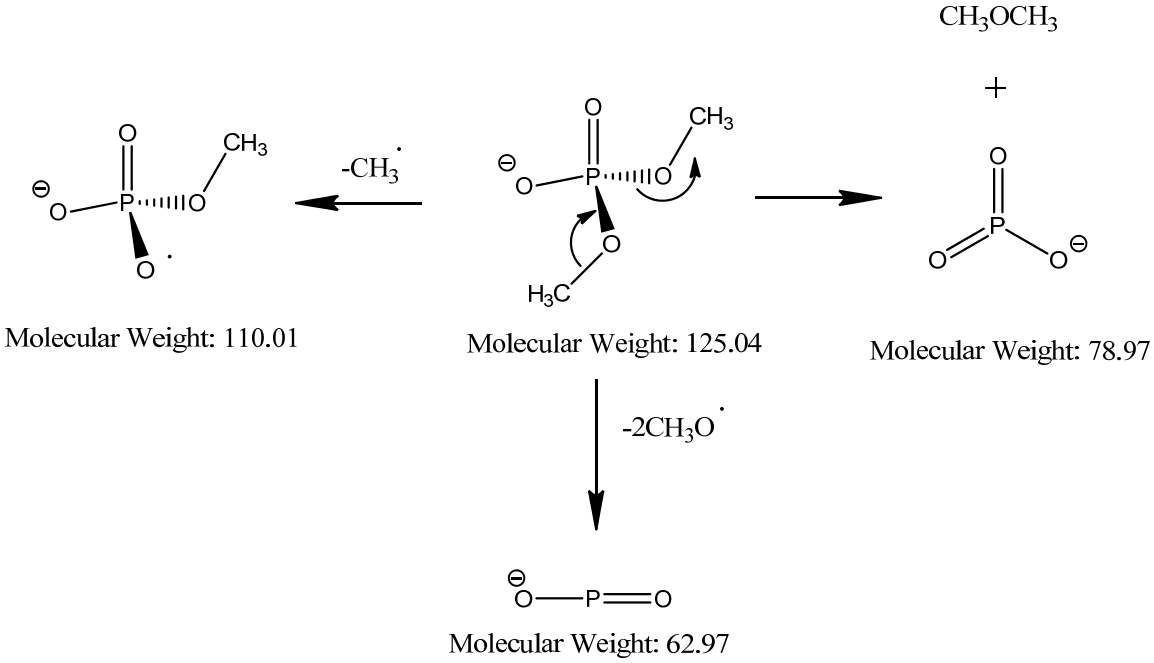


Figure 15: Fragmentations for dimethyl phosphate (DMP).

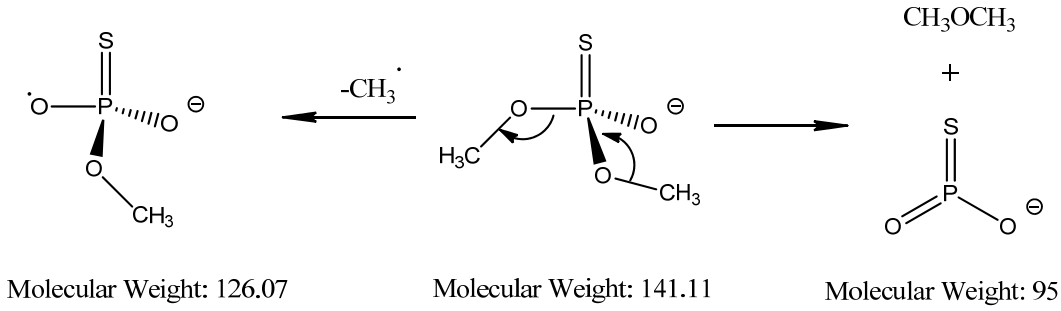


Figure 16: Fragmentations for dimethylthiophosphate (DMTP).

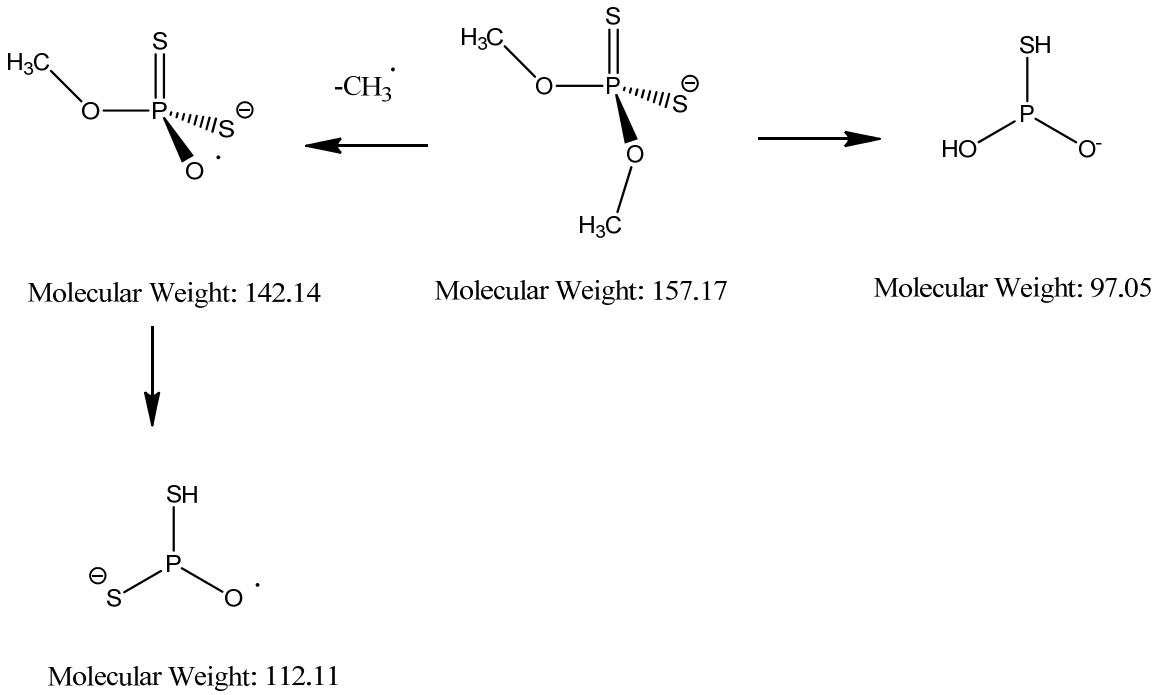


Figure 17: Fragmentation for dimethyldithiophosphate (DMDTP).

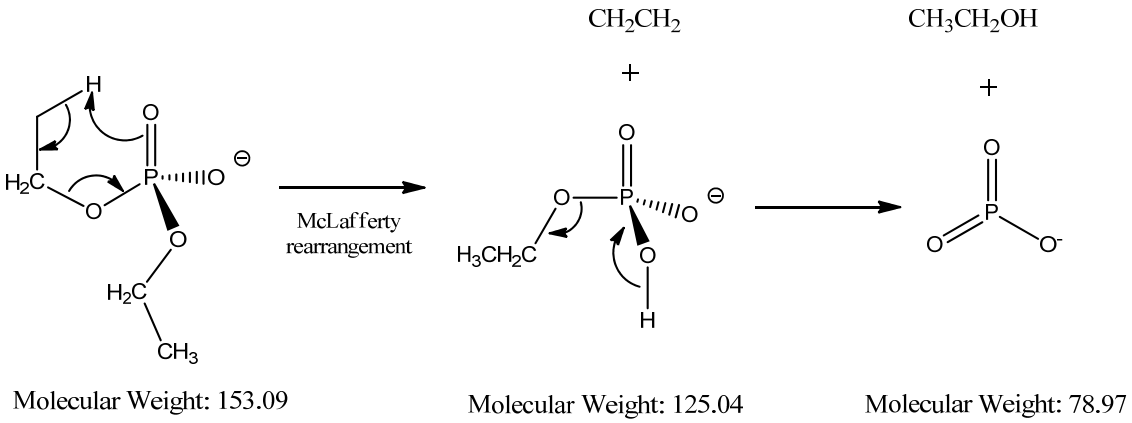


Figure 18: Fragmentation for diethylphosphate (DEP).

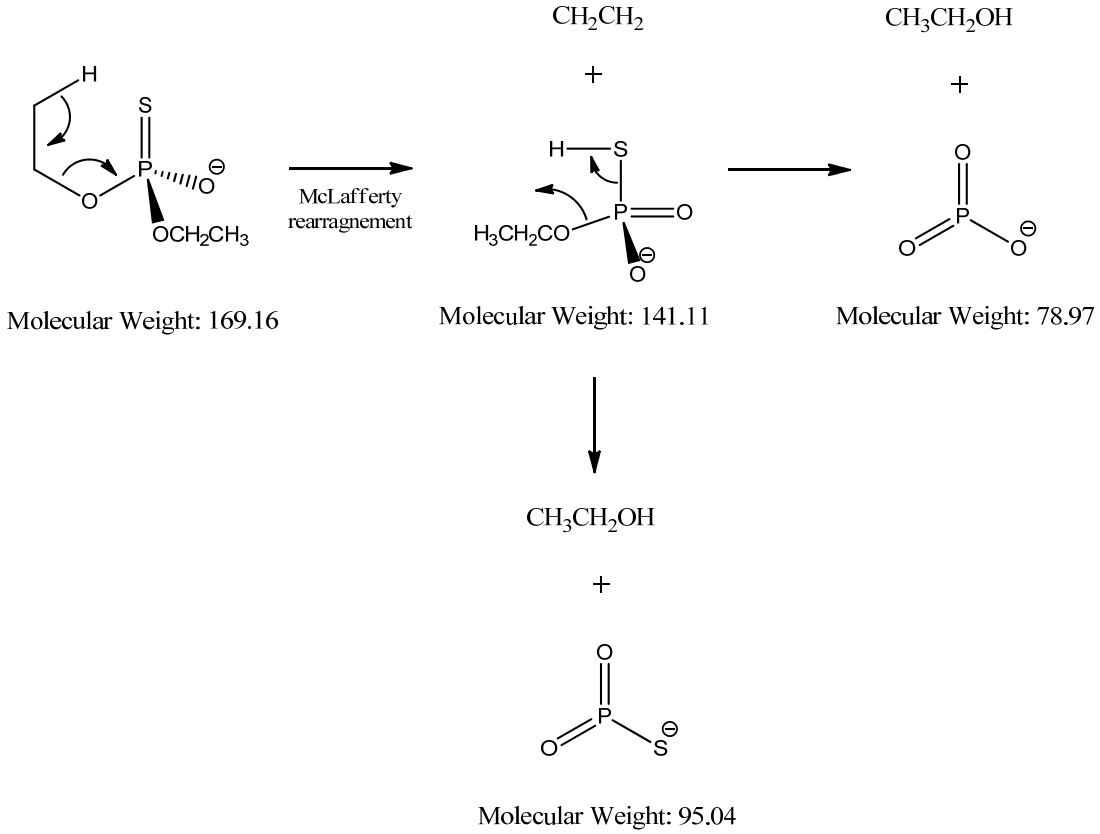


Figure 19: Fragmentation for diethylthiophosphate (DETP). Rearrangement of parent ion electrons to put the negative charge on the sulfur, thereby causing the McLafferty rearrangement to protonate the oxygen, would lead to the *m/z* = 95 daughter ion.

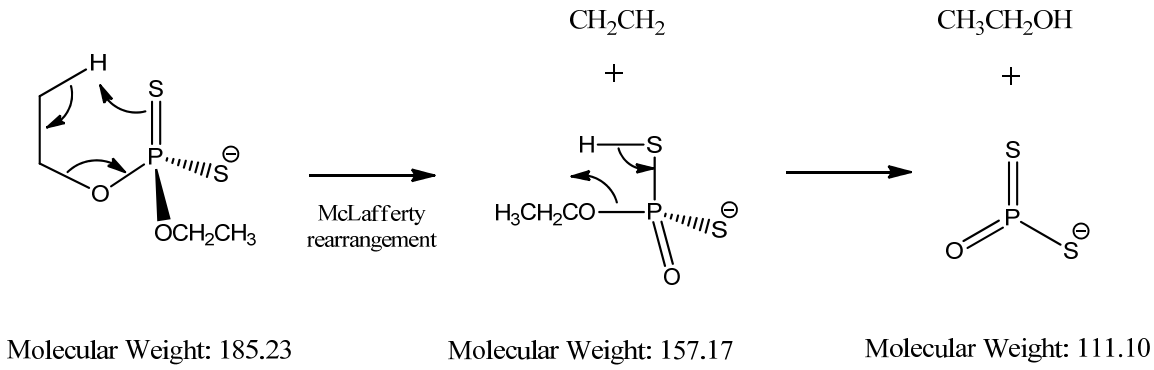


Figure 20: Fragmentation for diethyldithiophosphate (DEDTP).

Concerning chromatography solvent profiles (Figures 12 and 13), Trial 1 produces optimum results. Trial 2 increased the speed at which % MeOH was increased for the first minute. This change caused DMP to elute more slowly, but it subsequently decreased the time between each compound's elution. Trial 3 simply started with more acidified methanol, but this change increased coelution until there was no separation between dimethyl DAPs and diethyl DAPs. Finally, starting with a lower percentage of methanol in Trial 5 increased separation, but the peaks lost definition. However, because a highly concentrated standard was used for these tests, there is the possibility that the front tailing is caused by overloading of the column. Regardless, Trial 1 was chosen as the best solvent profile.

Conclusions

This chromatographic method allows sufficient separation of all six dialkyl phosphates.

CHAPTER 3: DEGRADATION OF INSECTICIDES IN BEVERAGES

Hypothesis

Insecticides degrade in fruit-based beverages, and this degradation may be observed by following both the loss of parent insecticide and by the production of insecticide degradate.

Introduction

While insecticide use on crops has contributed to increased crop yields and variety in the American diet over the past sixty years, it has also exposed people to new environmental toxicants.[95] Children are at particular risk when exposed to insecticides because of their physical and behavioral differences from adults; children's neurological systems are not completely developed, any damage done may permanently inhibit maturation.[95] Therefore, it is important to understand children's exposure to insecticides.

Urinary insecticide metabolites have been used for years as biomarkers of exposure to insecticides. For example, Bradman et al.,[37] used urinary dialkyl phosphate (DAP) concentrations to estimate OP insecticide exposure of children. Lu et al.,[4] have also used urinary 3-phenoxybenzoic acid (3-PBA), a metabolite of several pyrethroids, as a biomarker of pyrethroid exposure in children However, the assumption that there is a one-to-one correlation between insecticide dose and urinary metabolite output, central to this exposure quantification method, may be incorrect. Insecticides may be degraded in the environment through various pathways. In particular, OPs and pyrethroids are both susceptible to hydrolysis because they are esters.[4, 96] Pyrethrins, pyrethroids, and some OPs are susceptible to photolysis or hydrolysis in both soil and water.[19, 39] When these insecticides degrade in the environment, they tend to break down into the same metabolites found in urine. Therefore, it is difficult, if not impossible, to assess whether

insecticide metabolites found in urine are due to pesticide metabolism in the body or intake of the metabolite itself.

Few studies have been done concerning the degradation of organophosphorus insecticides in food,[38, 50-52] and none have been carried out on the degradation of pyrethroids in food. Further, only two of these studies follow the concentration of both parent and degradation product in food simultaneously.[50, 52]

For this project, the degradation of three OPs (diazinon, malathion, and chlorpyrifos) and four pyrethroids (permethrin, cyfluthrin, cypermethrin, and deltamethrin) are studied in several beverages using gas chromatography with electron capture detection (GC-ECD) (Figures 1 and 2). Electron-capture detection has been used for the analysis of insecticides previously.[97-100] ECD is a highly sensitive detection method with equal or better sensitivity for halogenated compounds than mass spectrometry detection for chromatographic separation. GC-ECD is still used in many EPA standard operating procedures for water testing.[101] Finally, GC-ECD is much more cost effective for the determination of halogenated compounds than GC-MS. The addition of an electron capture detector to a currently owned GC will cost approximately $2,500, but adding an MSD to a GC could cost $30,000. While ECD is selective in that it preferentially detects halogenated compounds, confirmation of the method with GC-MS, if possible, is still suggested to eliminate the possibility of erroneous identification of interfering peaks as insecticides.[97] The method used here has already been confirmed using GC-MS.[71]

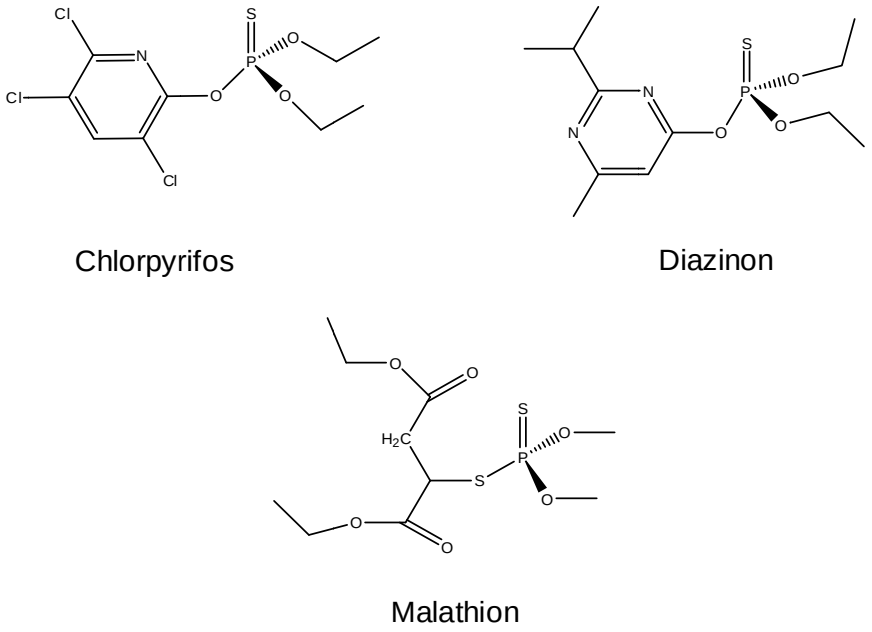


Figure 1: Organophosphorus insecticides analyzed for degradation in this study.

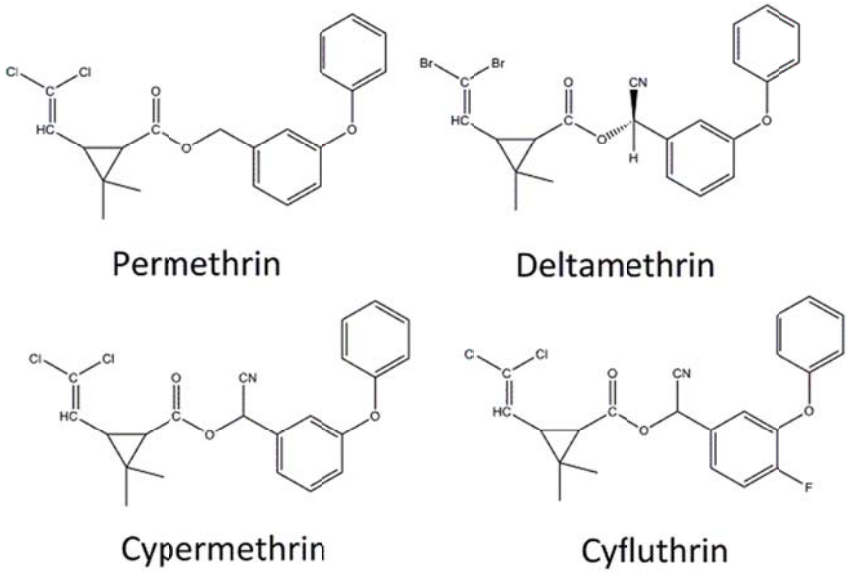


Figure 2: Pyrethroids analyzed for degradation in this study.

Milli-Q (Millipore, Billerica, MA, USA) water, which has been filtered to a resistance of 18.2 MΩ·cm, was chosen as a simple matrix free from enzymes or other interferences. Orange juice, apple juice, white grape juice, and concord grape juice were

all chosen for analysis due to children's preference for fruit juices (see Appendix for studies using apple juice and concord grape juice).[38] Red wine and white wine, because of their similarity to grape juices, were also considered suitable matrices.

A subset of insecticides with members from these two classes (chlorpyrifos, malathion, and permethrin) were chosen for analysis using MS/MS detection. These three compounds are of particular interest because of their current frequency of detection in fruits, vegetables, and grains.[102] In the 2009 USDA Pesticide Data Program annual summary, chlorpyrifos was found in twelve different types of produce, while malathion was found in six. Of the pyrethroids studied by ECD in this work, permethrin was found in the most types of produce.[102] During this experiment, chlorpyrifos and permethrin were analyzed by GC-MS/MS, while malathion was analyzed by LC-MS/MS. Here, the formation of insecticide degradation products was also followed by LC-MS/MS. Malathion dicarboxylic acid (MDA), the specific metabolite of malathion, 3,5,6-trichloro-2-pyridinol (TCPy), the specific metabolite of chlorpyrifos, cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), a specific metabolite of permethrin, and 3-phenoxybenzoic acid (3-PBA), a degradation product that is formed from several pyrethroids, are all used to follow degradation of malathion, chlorpyrifos, and permethrin (see Figure 3).

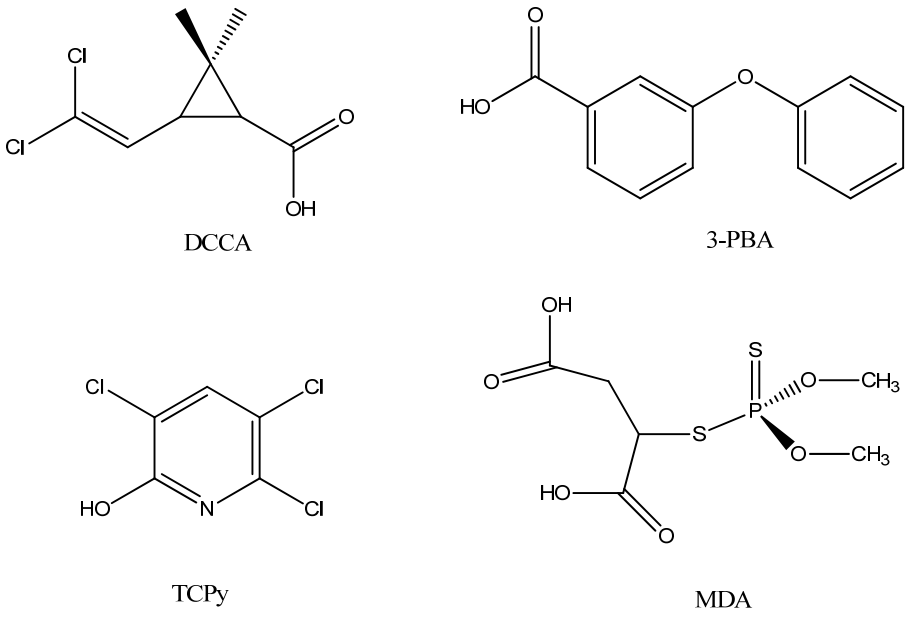


Figure 3: Metabolites of permethrin (3-PBA and DCCA), chlorpyrifos (TCPy), and malathion (MDA).

Methods

Reagents and materials

Acetonitrile (HPLC grade), toluene (Chromosolv grade), methanol (HPLC grade), and glacial acetic acid were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). NaCl (ACS grade) was obtained from EMD (Gibbstown, NJ, USA). Water used was purified in-house to 18.2 MΩ·cm with a Milli-Q® water system (Millipore, Billerica, MA, USA). Supelclean™ ENVI-CARB-II/PSA SPE cartridges (Bed A: 500mg ENVI-CARB; Bed B: 300mg primary secondary amine, PSA) were purchased from Sigma-Aldrich, Inc (Bellefonte, PA, USA). ENVI-CARB is graphitized carbon black, which has a strong affinity for organic polar and non-polar compounds in reversed-phase conditions. In particular, the hexagonal ring structures retain planar compounds, such as pigments and sterols, from fruits and vegetables.[103] Supelclean PSA is a polymerically bonded phase

containing primary and secondary amines and has a strong affinity for more polar sugars, fatty acids, and organic acids.[103] Oasis HLB extaction cartridges (200mg, 6mL) were purchased from Waters Corporation (Millford, MA). For GC-ECD operation, helium and nitrogen (both zero grade and with 99.999% ultra-high purity) were obtained from Nexair Gases, Inc (Suwanee, GA, USA).

Standards

For ECD work, three OP insecticides (diazinon, malathion, and chlorpyrifos) and four pyrethroids (permethrin, cyfluthrin, cypermethrin, and deltamethrin) were analyzed. The insecticide standards were obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) and/or Chem Service, Inc (West Chester, PA, USA). A stock solution containing all seven insecticides at 10 ppm was prepared in acetonitrile.

For MS/MS work, a stock standard containing 10mg/mL malathion, permethrin, and chlorpyrifos in ACN was used to fortify juices. The insecticide standards were obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) and/or Chem Service, Inc (West Chester, PA, USA). Standard dilutions of this stock from 5-4000ng/mL were used to create calibration curves. Isotopically labeled standards (malathion D_{10}, cypermethrin $^{13}C_4$, and chlorpyrifos D_{10}) at 1000ng/mL were made in ACN to serve as internal standards for parent compounds. A stock containing 10ng/g MDA, TCPy, DCCA, and 3-PBA in ACN was used to create standard dilutions from 5-2000ng/mL. These dilutions were used to create calibration curves. Isotopically labeled standards (MDA-D_6, DCCA-$^{13}C_3$, and 3-PBA-$^{13}C_6$) of 1000ng/mL were made in ACN and used as internal standards for degradate analysis.

Identification and quantification of pesticides

Standards for GC-ECD work were prepared the following way. Standards of the seven insecticides in acetonitrile were made in increasing concentrations (1, 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, and 1000ng/mL) and used to create a calibration curve. Method detection limits were calculated using a power regression curve due to the non-linear response of the ECD. Peaks were manually integrated using ChemStation software (Agilent Technologies, Santa Clara, CA) and data was analyzed using Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA).

Standards for MS/MS work were prepared using in matrix using increasing concentrations of analyte. Parent compound calibration curves ranged from 0.50-200ng/mL and were calculated using a linear curve, and degradate calibration curves ranged from 0.5-100ng/mL and were calculated using a linear curve. Peaks were manually integrated using the Agilent Quantitative software (Agilent Technologies, Santa Clara, CA) and data was analyzed using Microsoft Excel 2007 and Microsoft Excel 2011 (Microsoft, Redmond, Washington, USA).

Fortification and extraction protocol

Beverages were obtained from a local grocery store. For ECD work, 50mL of water, white grape juice, red wine, and orange juice were each fortified to an initial concentration of 500ng/mL of the seven insecticides and stored at 2.5 ºC in an amber glass jar. Extraction and clean-up were performed immediately after insecticide fortification, and then 12hr later, 1, 2, 4, 7, 14, and 15 days later (n = 3 for each matrix each day). Blanks were also analyzed on day 0 in the same fashion (n = 2 per matrix).

During the MS/MS degradation study, 50mL of water, white grape juice, white wine, and red wine were fortified to 200 ng/mL malathion, chlorpyrifos, and permethrin. Fortified beverages were then stored in amber glass jars in a refrigerator at 2.5°C. Extraction and clean-up of parent insecticides were performed immediately after insectide fortification, and then 0.5, 1, 3, 7, 11, 14, and 15 days later (n = 3 for each matrix each day). For metabolite extraction, extraction and clean-up were performed immediately after fortification and then 1, 3, 7, 11, 14, and 15 days later (n = 3 for each matrix each day). Blank juices were extracted for both parent insecticide and degradation products (n = 2 per matrix).

Insecticide extraction

The extraction procedure is based on that of Hunter et al.[71] A 1.0mL sample was added to a trace-cleaned 15mL conical centrifuge tube. For MS/MS work, 50µL parent compound ISTD was added to the test tube at this time. Then, ~0.5g NaCl and 5.0mL acetonitrile (ACN) was added to the sample. The tube was vortexed for 3 minutes and centrifuged for 6 minutes. Next, an ENVI-CARB-II/PSA cartridge was preconditioned with 5 mL of a 25% solution of toluene in ACN. After preconditioning, 2.0mL of supernatant from the extracted, centrifuged sample was loaded onto the cartridge. Then, 10mL of the ACN/toluene solution was eluted through the cartridge, and the eluate was collected in a trace-cleaned 15mL centrifuge tube. The sample was evaporated at 20 PSI and 38°C to near-dryness. The original cartridge was eluted a second time with 25% toluene in ACN, and collected in the same tube. The sample was evaporated again at 20 PSI and 38°C to dryness. ECD samples were reconstituted with 1.0mL ACN and stored

in the refrigerator until analysis when they were transferred to a GC vial. MS/MS samples were reconstituted with 100µL ACN, briefly vortexed, and then evaporated to dryness. The samples were then stored dry in a refigerator until analysis, at which point they were reconstituted with 50µL 25% toluene in ACN and transferred to a GC vial.

Metabolite extraction

For the metabolite extraction, a 1.0mL sample of beverage and 50µL degradate ISTD were added to a trace-cleaned conical centrifuge tube and vortexed briefly. An Oasis HLB cartridge was set up on a vacuum manifold and preconditioned with 3mL methanol (MeOH) and then 3mL 1% acetic acid in H_2O. After the HLB cartridge was loaded with the sample, it was washed twice with 2 mL 1:5:94 acetic acid:MeOH:H_2O. The cartridge was then dried under vacuum for at least 10 min. A test tube was placed under the cartridge in the manifold and the sample was eluted twice with 2.5mL methanol. Because the cartridges were completely dry, vacuum was required to begin elution. Once the first drop of eluate passed through the cartridge, the vacuum was broken. After elution, the sample was evaporated in a TurboVap LV (Zymark, Massachusetts, US) at 20 psi and 45°C to ~100µL and 100µL ACN was added to the test tube to redissolve any analyte dried onto the tube wall. The sample was briefly vortexed then evaporated to dryness. Samples were capped and stored dry until analysis, when the sample was then reconstituted with 100µL 30:70 MeOH:H_2O. Samples were then vortexed briefly before being transferred to a LC vial for analysis.

Instrumental analysis

Parent compound separation by GC-ECD: For GC-ECD separation, the gas chromatograph used was a Hewlett-Packard Model 5890A Series II equipped with an Agilent Technologies electron capture detector and 7683B Series Injector autosampler (Santa Clara, CA, USA). A DB-5 column (Agilent Technologies, Inc., Santa Clara, CA, 30 m, 0.25 mm i.d., 0.25 μm film thickness [5% phenyl, 95% dimethylpolysiloxane]) was used, and a 2 mm i.d. single-taper injection liner was used to prolong column life. Injection volume was 1.0 μL (1:30 split). The helium carrier gas was at a flow rate of 0.88 mL/min, while the nitrogen make up gas flow was 13 mL/min. The injector temperature was 260ºC. The temperature program started at 80°C and stayed at that temperature for 2 min before being heated linearly by 10ºC/min to a final temperature of 280ºC which was held for 13 min. The ECD temperature was 280ºC.

Parent compound separation by GC-MS/MS: For GC-MS/MS separation of chlorpyrifos and permethrin, an Agilent 7890 GC with MS/MS detection equipped with positive mode electron impact (EI) ionization was used. The column used was an Agilent HP-5MS [(5% phenyl)-methylpolysiloxane, 30m x 250 μm x 0.25μm]. The injection port was held at 250°C, and injection was 1μL (splitless), and a gooseneck injection liner with glass wool and 1m deactivated silica guard column was used. The carrier gas was He at a flow rate of 50mL/min, and the column temperature profile was as follows: The column was held at 100°C for 2 min before linear heating by 10°C/min to 205°C. This temperature was held for 3 min before linear heating by 10°C/min to 280°C. This temperature was held for 4 min before linear heating by 25°C/min to a final temperature

of 310°C. This final temperature was held for 12 min. Analysis was performed in MRM mode, monitoring two characteristic parent ions for each compound (Table 1).

Compound	Precursor ion	daughter ions (Q, C)	CE(V) (Q, C)	RT (min)
native				
chlorpyrifos	314.2	286.1, 258	5, 25	15.44
permethrin	183.2	153.2, 77	15, 40	22.44, 23.59
ISTD				
chlorpyrifos	324	292, 260	10, 20	14.4
cypermethrin	170	134, 98	10, 15	23.45

Table 1: Precursor and daughter ions (*m/z*) for chlorpyrifos and permethrin. Permethrin has two retention times listed because it is a duplet peak due to its chiral center. The first peak from isotopically-labeled cypermethrin is used as the ISTD. Collision energy (CE) is measured in electron volts.

Malathion analysis by LC-MS/MS: Because the potential for thermal degradation makes malathion difficult to analyze by GC,[104] an Agilent 6460 Triple Quad LC-MS/MS equipped with a positive mode electrospray ionization (ESI) interface was used to analyze samples for malathion in MS/MS work. A BetaSil C18 column (3.0 × 100mm, 3.0µm particle size, Thermo Scientific) was used for separation and kept at 45°C. Solvent A was H_2O with 1% acetic acid and solvent B was MeOH with 1% acetic acid, and the flow rate was 0.7mL/min (Table 2). The following parameters were used: the source temperature was 300°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 45psi, and the corona voltage was 3500V. Ions analyzed in MRM mode and their optimized fragmentor and collision energies are shown in Table 3.

minute	% B	flow rate (mL/min)
0	30	0.7
4	100	0.7
6	100	0.7
6.5	30	0.7
8.5	30	0.7

Table 2: Solvent profile for malathion analysis.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)
Mal ISTD	341	100.1	90	21
Mal-Q	331	127	60	5
Mal-C	331	99	60	21

Table 3: Parent and daughter ions for malathion.

Degradate separation by LC-MS/MS: An Agilent 6460 Triple Quad LC-MS/MS equipped with a negative mode ESI interface was used to separate degradation products. A Zorbax Eclipse Plus Phenyl-Hexyl column (3.0 × 100mm, 3.5µm particle size, Agilent, USA) was used for separation and kept at 45°C. Solvent A was H_2O with 1% acetic acid and solvent B was MeOH with 1% acetic acid, and the flow rate was as shown in Table 4. The following parameters were used: the source temperature was 250°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 35psi, and the corona voltage was 3500V. Each metabolite was matched to its own isotopically substituted internal standard except for TCPy, whose internal standard was isotopically substituted 3-PBA. Ions analyzed in MRM mode and their optimized fragmentor and collision energies are shown in Table 5.

Linear calibration curves were made individually in matrix, although grape juices were mixed to create one calibration curve for all grape matrices. The curve contained 9 points from 0.25 ng/g to 100 ng/g.

minute	% B	flow rate (mL/min)
0	30	0.8
1.5	35	0.8
3	50	0.8
8	60	0.8
8.8	100	1
10	100	1
11	80	1
13	80	1

Table 4: Flow rate through column for metabolite analysis.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)	RT (min)
MDA-ISTD	280	147	80	1	3.3
MDA	273	141	80	1	3.9
MDA	273	157	80	12	3.9
TCPy	198	198	96	0	7.3
TCPy	196	196	96	0	7.3
DCCA-ISTD	210	210	90	0	6.7
DCCA	207	207	90	0	8.2
DCCA	209	209	90	0	8.2
3-PBA-ISTD	219	99	98	20	7.4
3-PBA	213	93	122	16	9.0
3-PBA	213	169	122	8	9.0

Table 5: Parent and daughter ions for insecticide metabolites.

Statistical analysis

Analyte concentrations were determined using the calibration curves described. Each concentration was logarithmically transformed, and these transforms were averaged

for each day's sample. If possible, linear trend lines relating log concentrations and time in days were determined, and error bars denote standard deviation of the log transform. P-values were determined from the linear regression results and half-lives were determined using the slope (m) and the relationship $t_{1/2} = -log(2)/m$. The criterion for significance was predetermined to be a p-value < 0.05 for the regression slope. R^2 represents the fraction of the variance accounted for by the linear model. The balance of the variance, i.e., $(1-R^2)$ is the unaccounted-for variance of the error in the model.

Study of glass/insecticide interactions by GC-ECD

First, 100mL water was fortified with 500ng/mL of each of the seven pesticides used previously. In order to test for interactions with the polar glass wall, one amber glass jar (identical to the jars used to store fortified liquids in the above study) was silanized using dimethyldichlorosilane in order to cap exposed hydroxyl groups. Next, this jar and two other unsilanized jars were used to hold 25mL each of the fortified water. Three 1.0mL aliquots of the leftover fortified water were immediately extracted using the protocol described in the previous section to confirm initial concentration, and the three jars of water were put into a 2.5ºC refrigerator for a week. After the week had passed, three 1.0mL aliquots were taken from the silanized jar and from one of the unsilanized jars for extraction. The other jar was vortexed for one minute to test for physical adsorption to the glass wall before three 1.0mL aliquots were also taken from it for extraction and analysis. The extracted samples were separated by GC-ECD using the method described above, and a two-tailed t-test was used to determine significant differences in sample concentrations ($p < 0.05$).

Results

Study of parent degradation by GC-ECD

Each insecticide was clearly separated from one another during analysis (Table 6). Chromatograms were generally clean and did not have interfering peaks except near malathion (Figure 4). The interfering peaks near malathion likely affected quantification since they caused the baseline to be difficult to determine. No insecticide was found in blank aliquots of samples.

Insecticide	Retention time (min)
diazinon	16.038
malathion	17.879
chlorpyrifos	18.157
permethrin	24.539
cyfluthrin	25.172
cypermethrin	25.528
deltamethrin	27.378

Table 6: Retention times of insecticides.

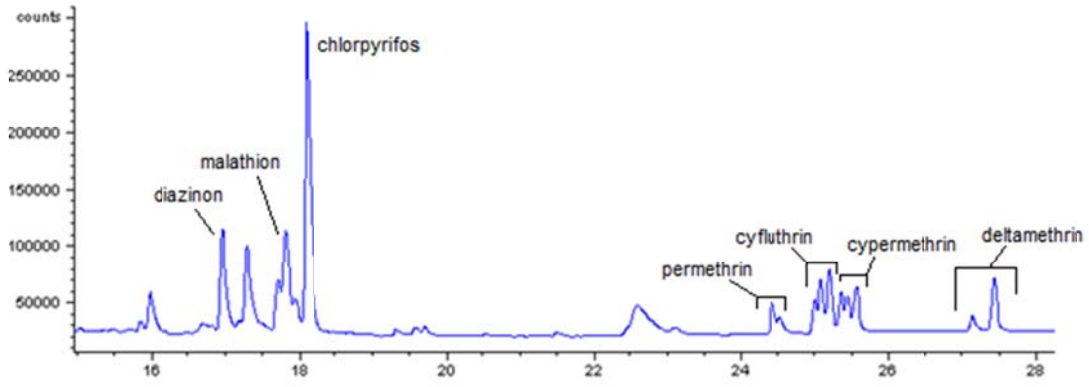


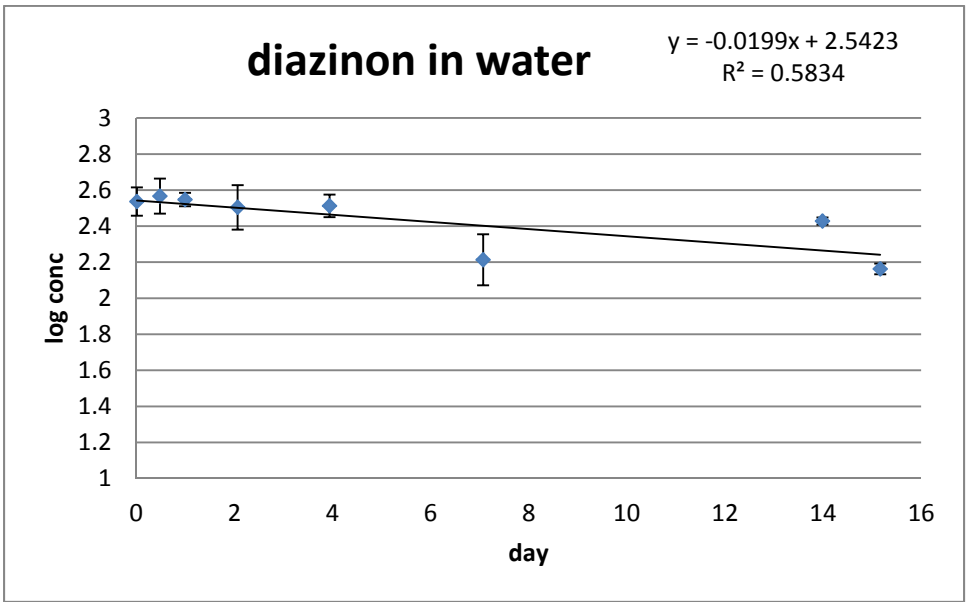
Figure 4: Chromatogram of seven insecticides in orange juice, the dirtiest matrix, from day 0. Pyrethroids show multiplet peaks because of stereoisomers.

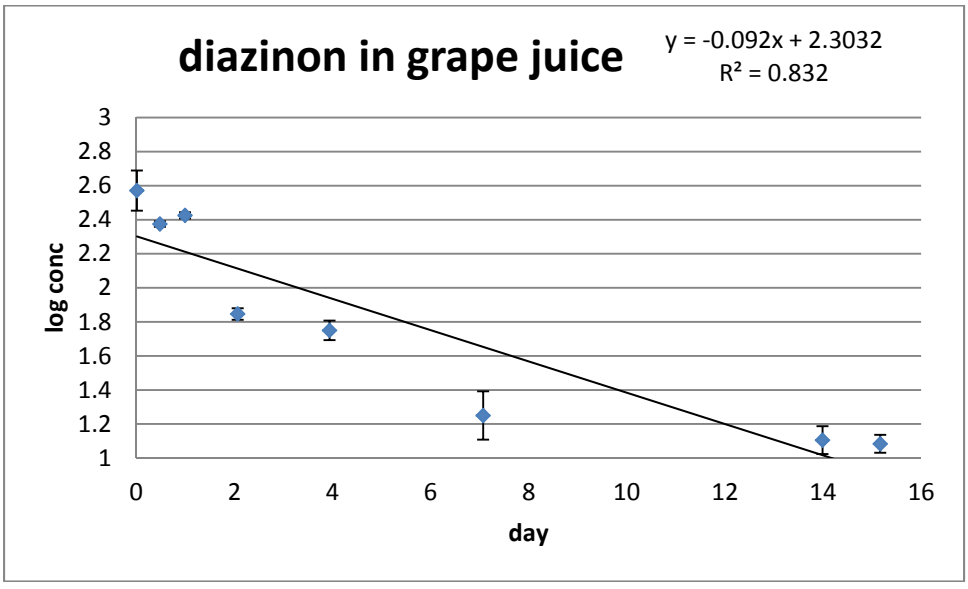
	% recovery[a]	rate constant[b]	std error[b]	p-value	half life[c]
water					
diazinon	69.5%	1.99E-02	6.85E-03	0.0274	15.2
malathion	125.2%	6.75E-03	1.85E-03	0.0107	44.6
chlorpyrifos	77.4%	8.92E-03	3.30E-03	0.0355	33.8
permethrin	105.3%	3.70E-03	1.24E-03	0.0244	81.5
cyfluthrin	97.8%	6.75E-03	2.13E-03	0.1284	44.6
cypermethrin	104.0%	8.12E-03	8.58E-04	0.0001	37.1
deltamethrin	104.4%	6.13E-03	2.48E-03	0.0480	49.1
grape juice					
diazinon	76.3%	9.20E-02	1.69E-02	0.0016	3.3
malathion	137.1%	4.61E-03	2.82E-03	0.1535	65.3
chlorpyrifos	85.3%	1.62E-02	5.14E-03	0.0199	18.6
permethrin	90.8%	1.49E-02	1.97E-03	0.0003	20.2
cyfluthrin	80.5%	4.61E-03	2.65E-03	0.0002	65.3
cypermethrin	89.2%	1.80E-02	3.06E-03	0.0011	16.7
deltamethrin	73.6%	1.72E-02	5.28E-03	0.0174	17.5
red wine					
diazinon	40.7%	1.29E-01	1.14E-02	0.0003	2.3
malathion	111.4%	8.90E-03	3.86E-03	0.0606	33.8
chlorpyrifos	68.4%	1.25E-02	5.84E-03	0.0766	24.1
permethrin	106.9%	9.99E-03	2.78E-03	0.0114	30.1
cyfluthrin	105.1%	1.18E-02	3.57E-03	0.0161	25.5
cypermethrin	119.3%	1.04E-02	3.17E-03	0.0167	28.9
deltamethrin	109.9%	1.32E-02	4.02E-03	0.0170	n/a
orange juice					
diazinon	97.0%	2.34E-02	1.15E-02	0.0881	12.9
malathion	140.2%	6.33E-03	3.64E-03	0.1331	47.6
chlorpyrifos	93.0%	8.06E-03	6.89E-03	0.2865	37.3
permethrin	88.1%	6.97E-04	2.34E-03	0.7761	432.1
cyfluthrin	78.7%	3.20E-03	3.07E-03	0.3377	94.1
cypermethrin	82.1%	4.82E-03	1.62E-03	0.0250	62.5
deltamethrin	69.6%	-1.82E-03	3.74E-03	0.6442	n/a

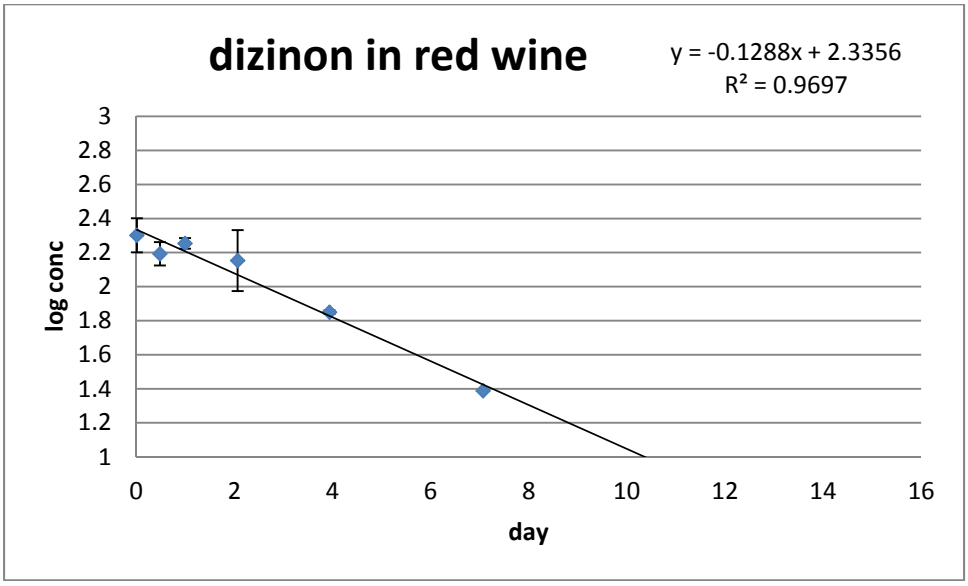
Table 7: Insecticide degradation results by GC-ECD. [a]Calculated using average concentration from day 0. [b]In $^{1}/_{day}$. [c]In days.

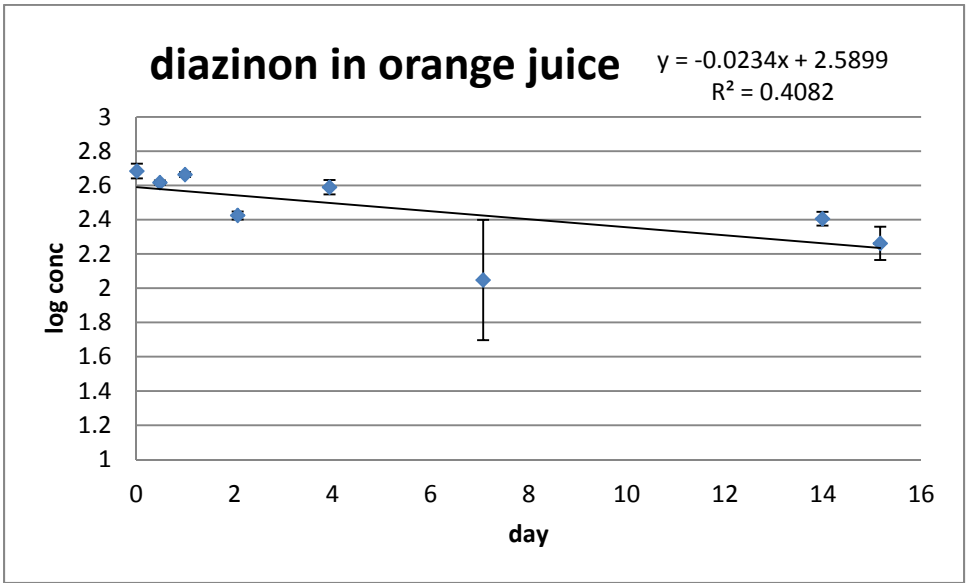
For each day's data set, the concentration was log transformed, and the average of these points was used to determine regression. According to the criterion for significance being a p-value < 0.05 for the regression slope, all insecticides except cyfluthrin degraded significantly in water, all but malathion degraded significantly in grape juice, all but malathion and chlorpyrifos degraded significantly in red wine, and none but cypermethrin degraded significantly in orange juice (see Table 7). However, all insecticides had a positive rate constant (defined as the negative of the slope of average log concentration plotted against day) except deltamethrin in orange juice, which implies that some statistically insignificant degradation was perhaps occurring. Figures 5-32 display graphs of degradation of each insecticide in each matrix. Error bars denote standard deviation for the mean of each day's log transformed concentration.

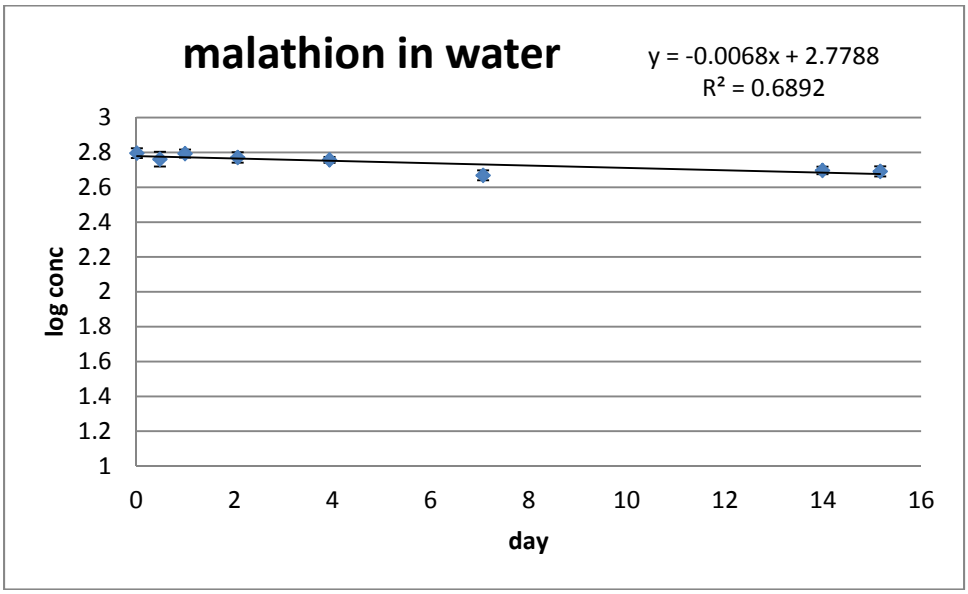
Figures 5-32: Degradation shown by GC-ECD analysis. Bars indicate standard deviation.

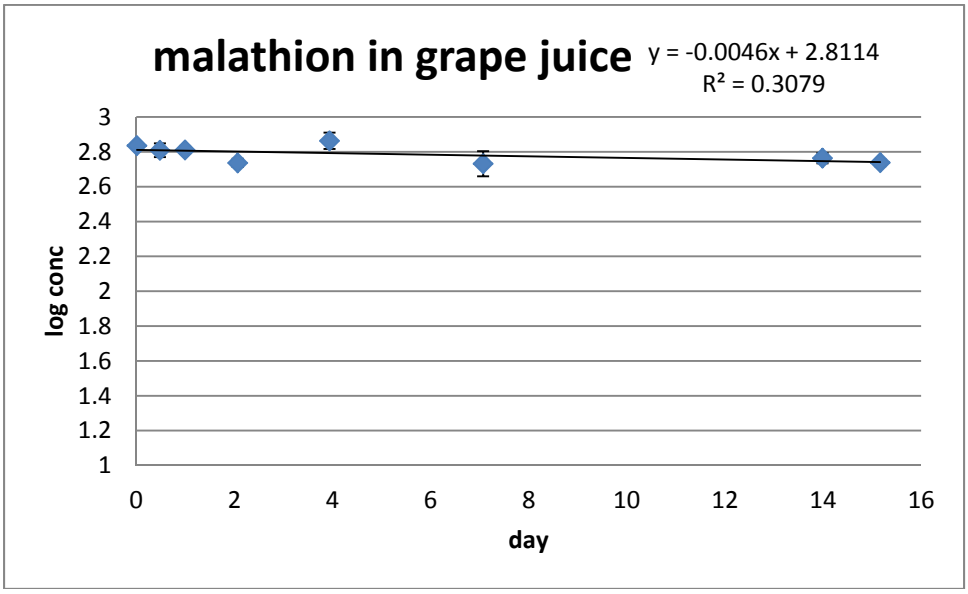


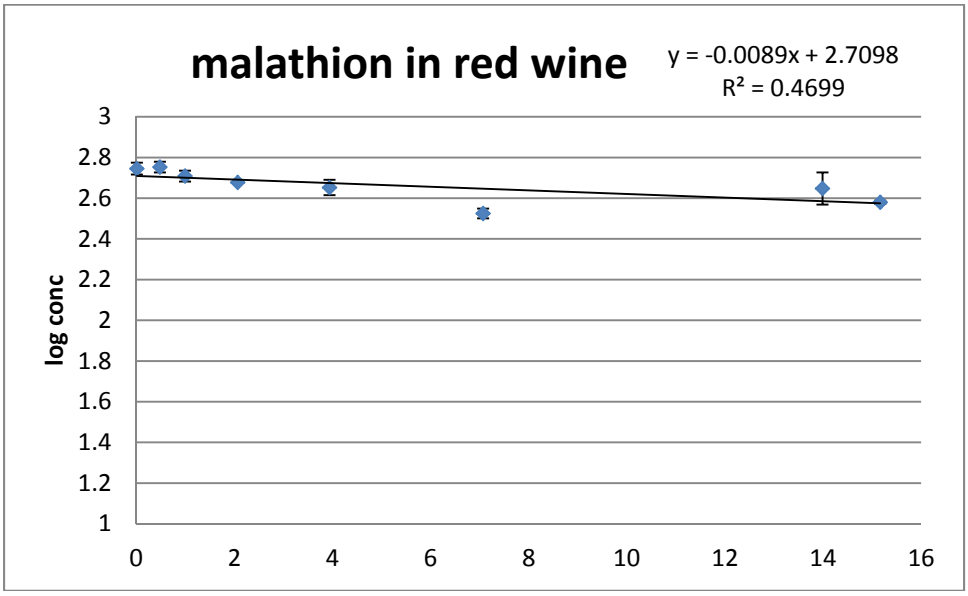


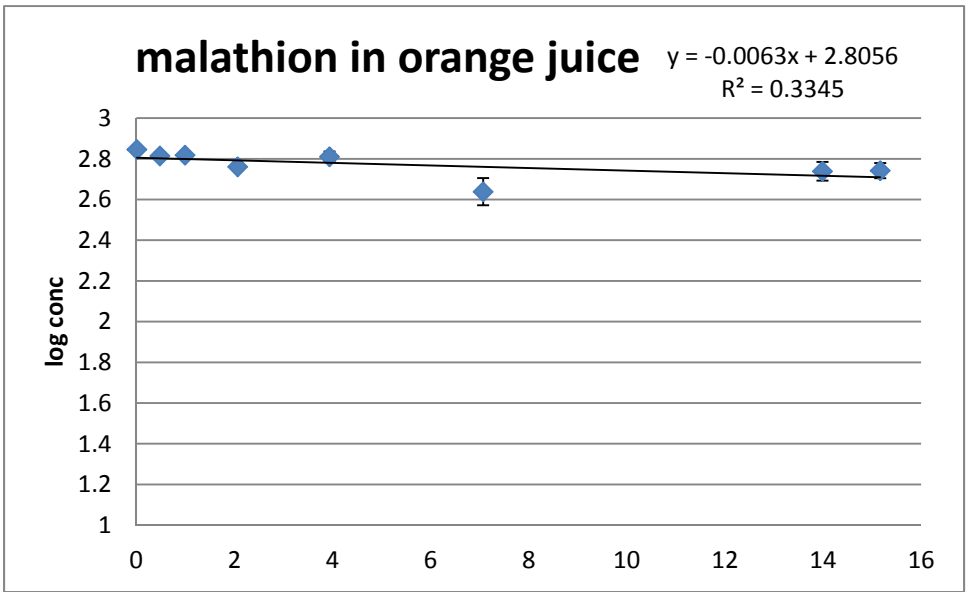

dizinon in red wine
y = -0.1288x + 2.3356
R² = 0.9697
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

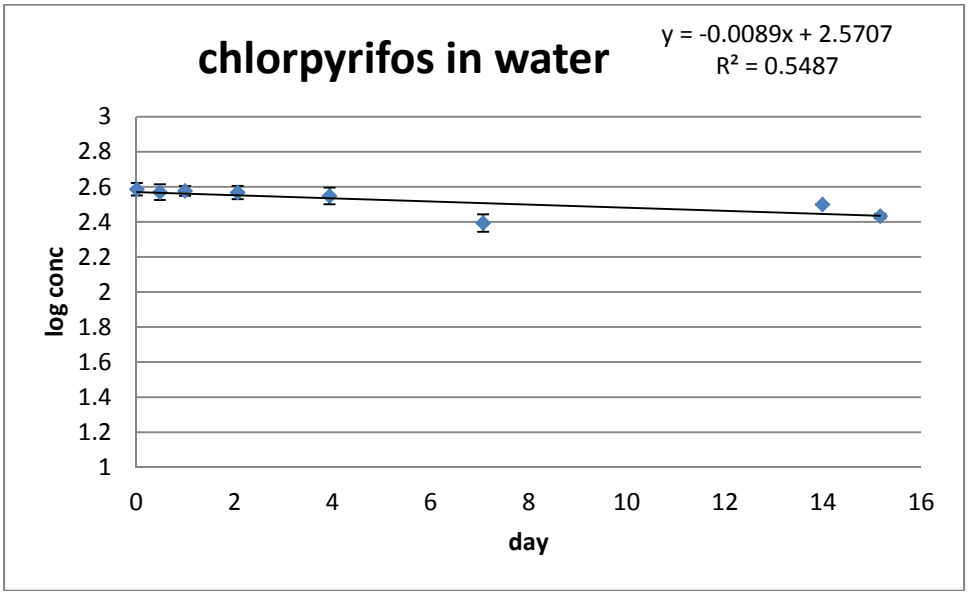

diazinon in orange juice
y = -0.0234x + 2.5899
R² = 0.4082
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

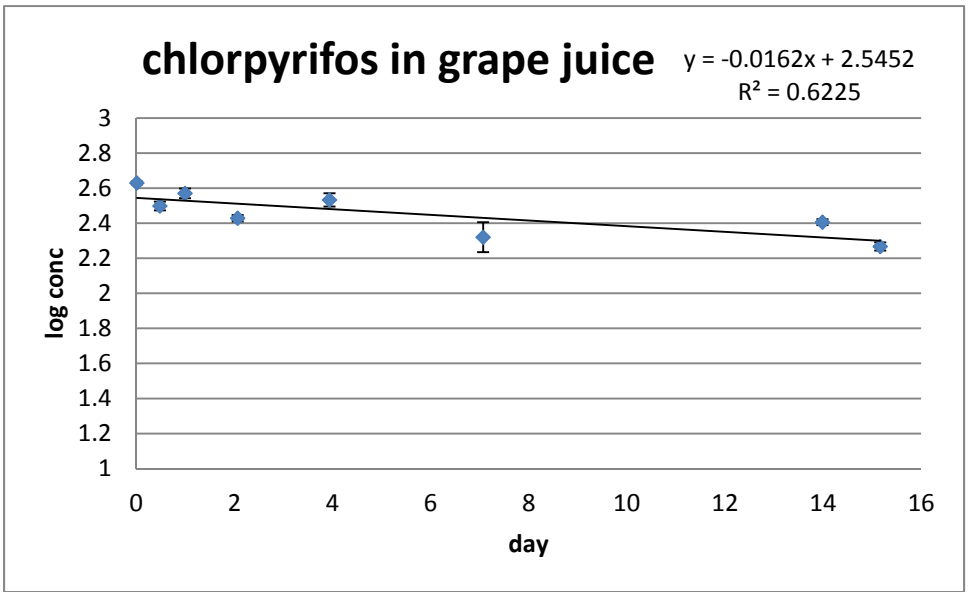

malathion in water
y = -0.0068x + 2.7788
R² = 0.6892
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

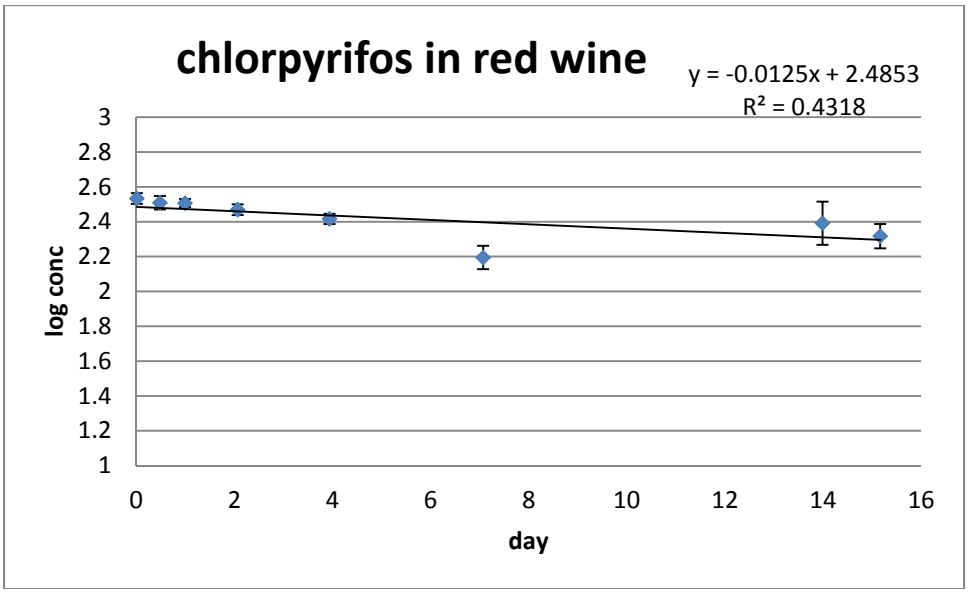

malathion in grape juice
y = -0.0046x + 2.8114
R² = 0.3079
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

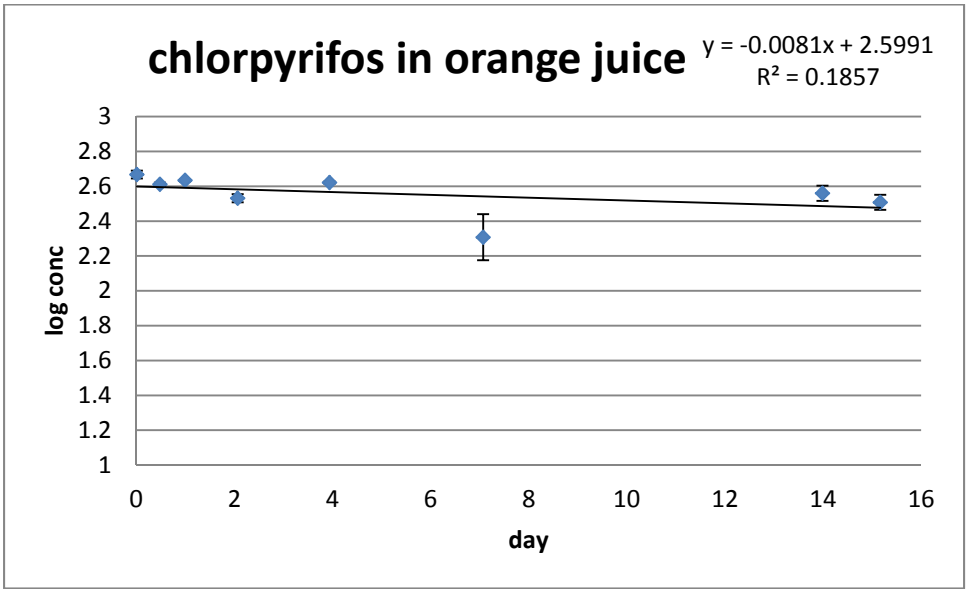

malathion in red wine
y = -0.0089x + 2.7098
R² = 0.4699
log conc
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

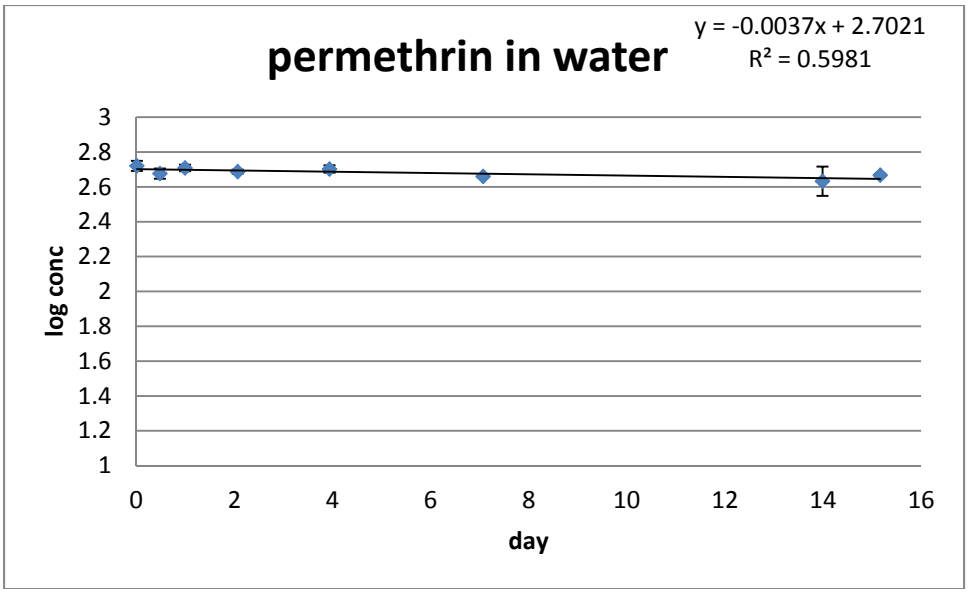

malathion in orange juice
y = -0.0063x + 2.8056
R² = 0.3345
log conc
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

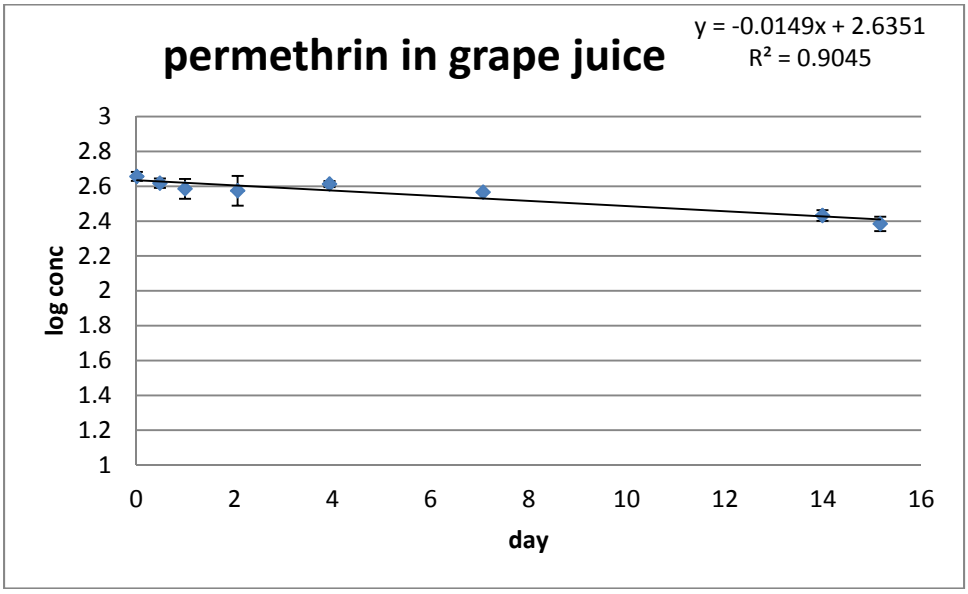

chlorpyrifos in water
y = -0.0089x + 2.5707
R² = 0.5487
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

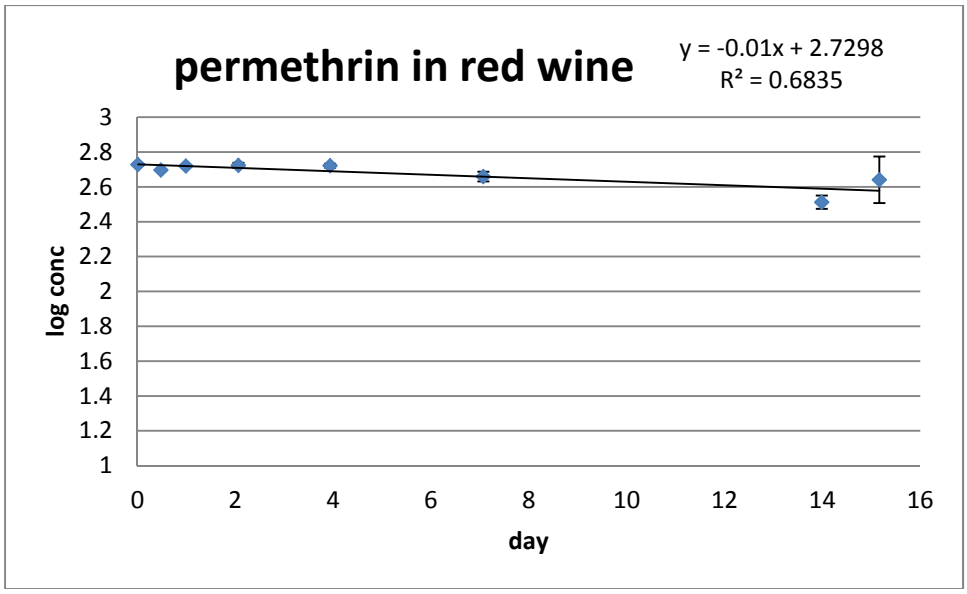

chlorpyrifos in grape juice
y = -0.0162x + 2.5452
R² = 0.6225
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

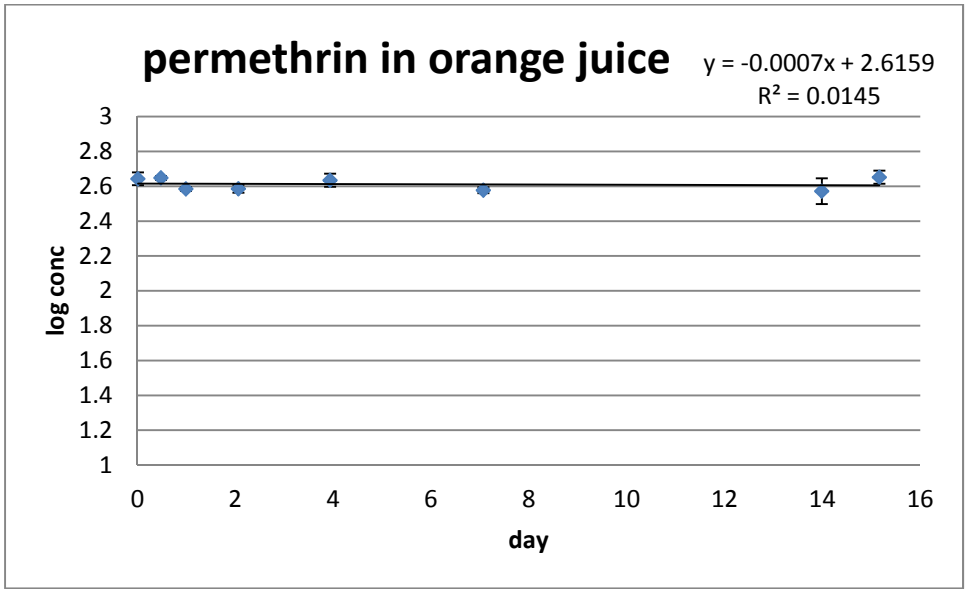

chlorpyrifos in red wine
y = -0.0125x + 2.4853
R² = 0.4318
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

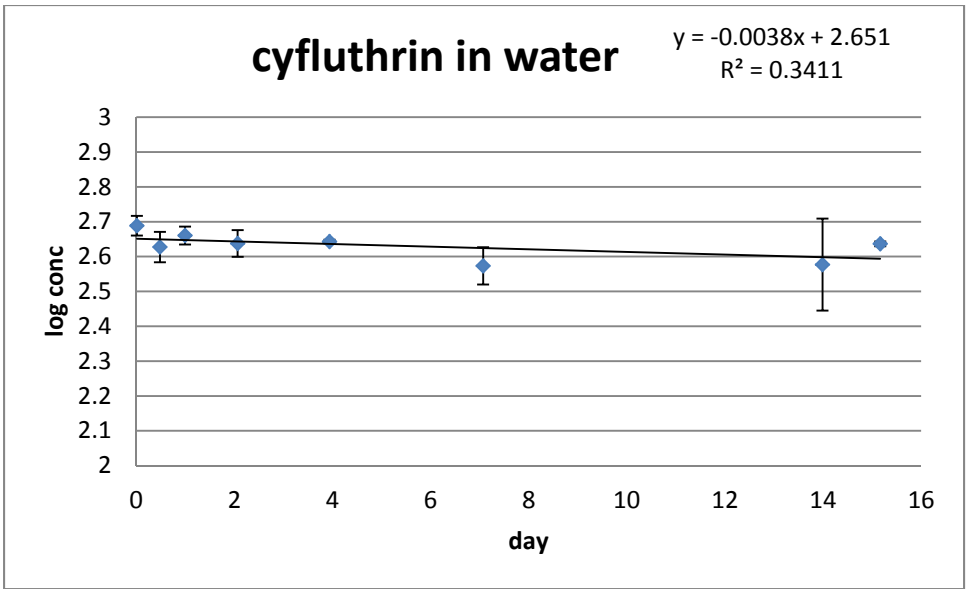

chlorpyrifos in orange juice
y = -0.0081x + 2.5991
R² = 0.1857
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

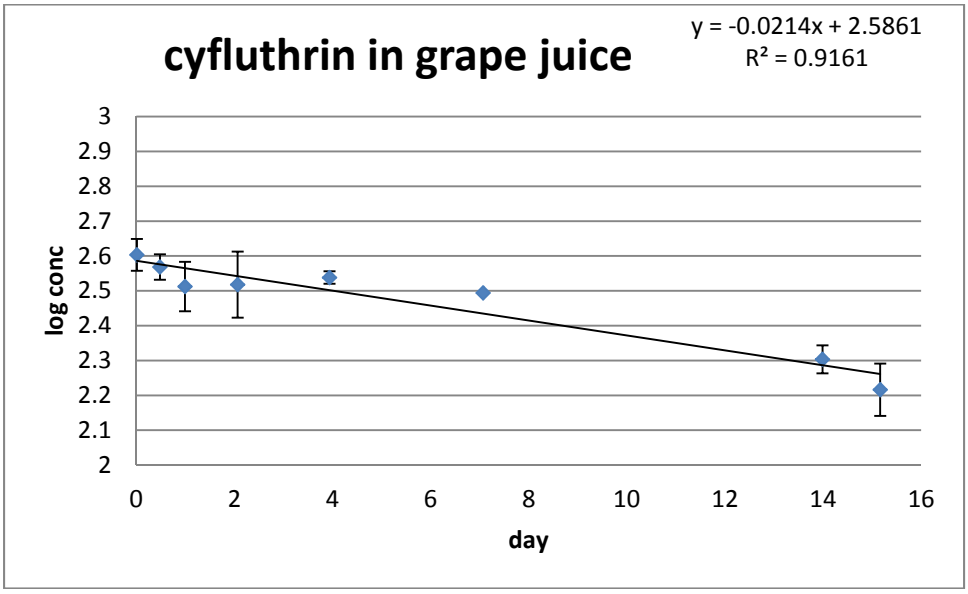

permethrin in water
y = -0.0037x + 2.7021
R² = 0.5981
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

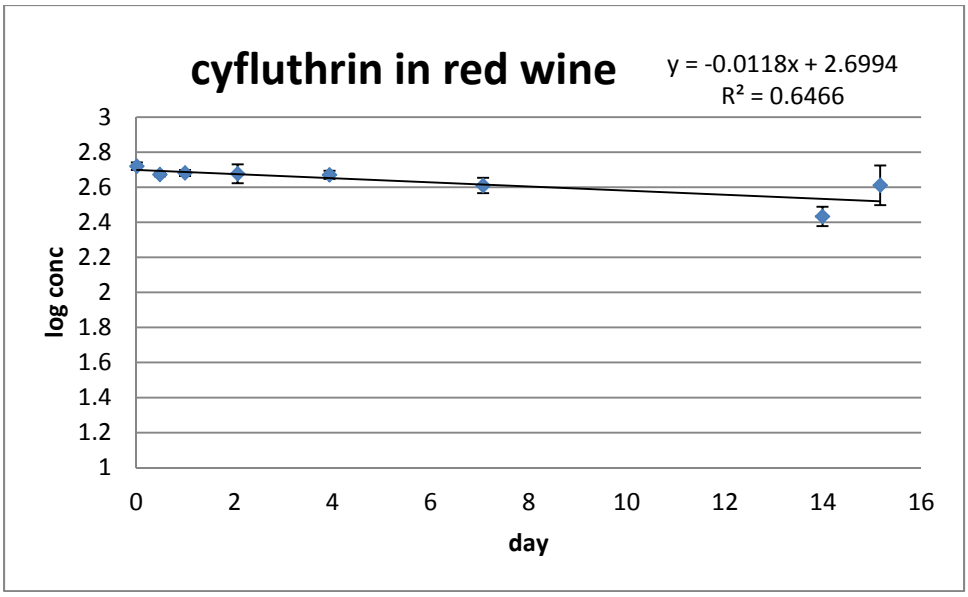

permethrin in grape juice
y = -0.0149x + 2.6351
R² = 0.9045
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

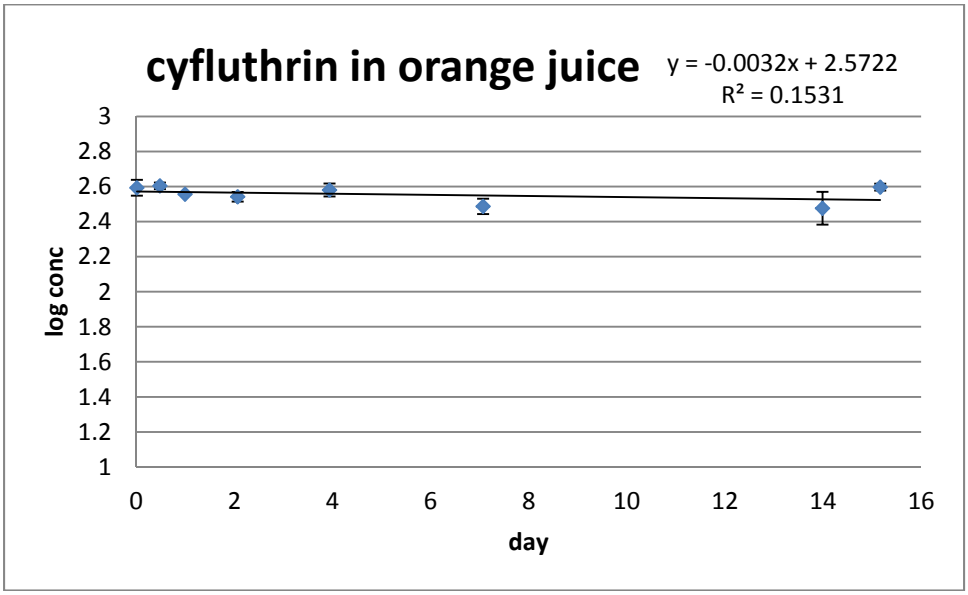

permethrin in red wine
y = -0.01x + 2.7298
R² = 0.6835
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

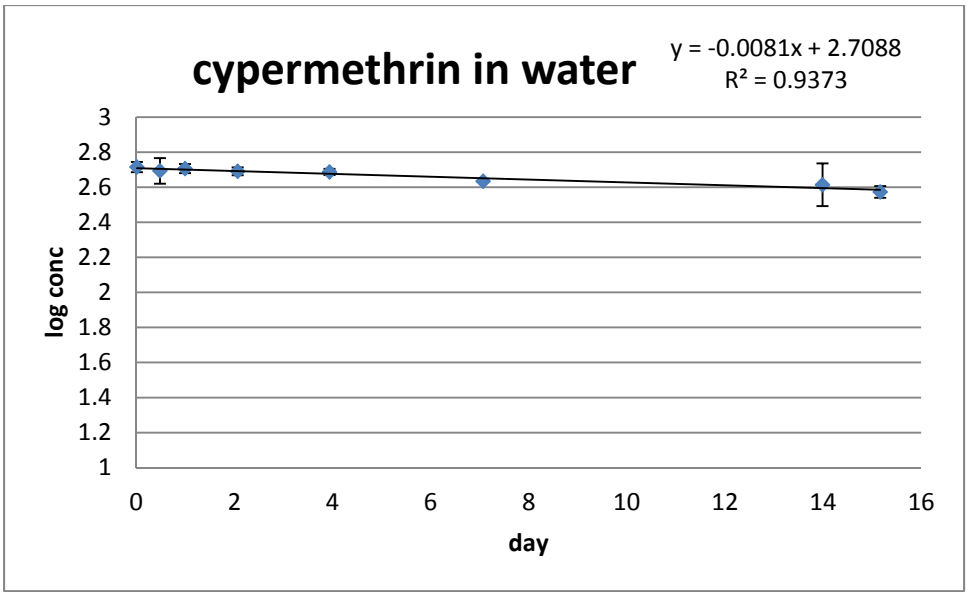

permethrin in orange juice
y = -0.0007x + 2.6159
R² = 0.0145
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

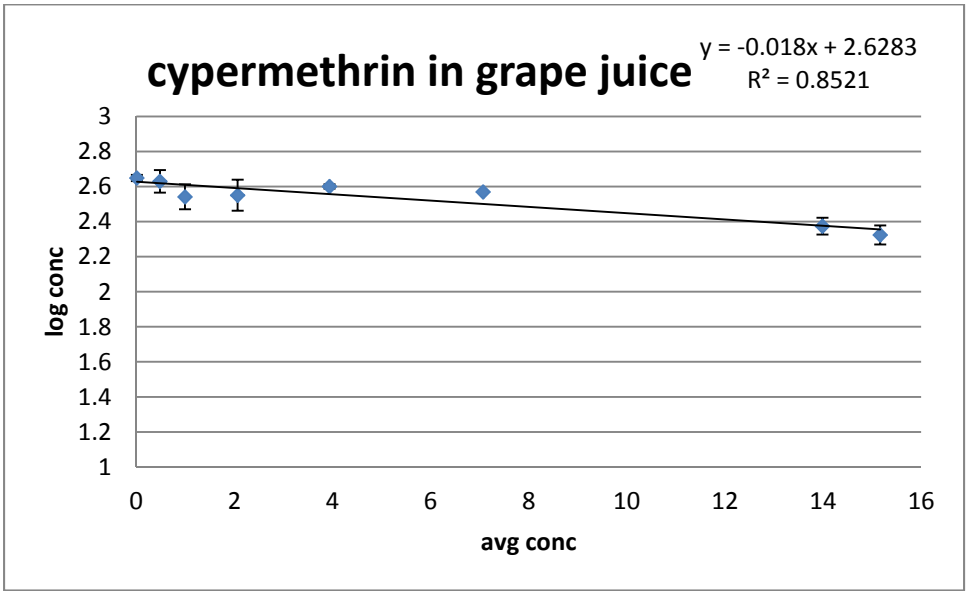

cyfluthrin in water
y = -0.0038x + 2.651
R² = 0.3411
log conc
day
3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16

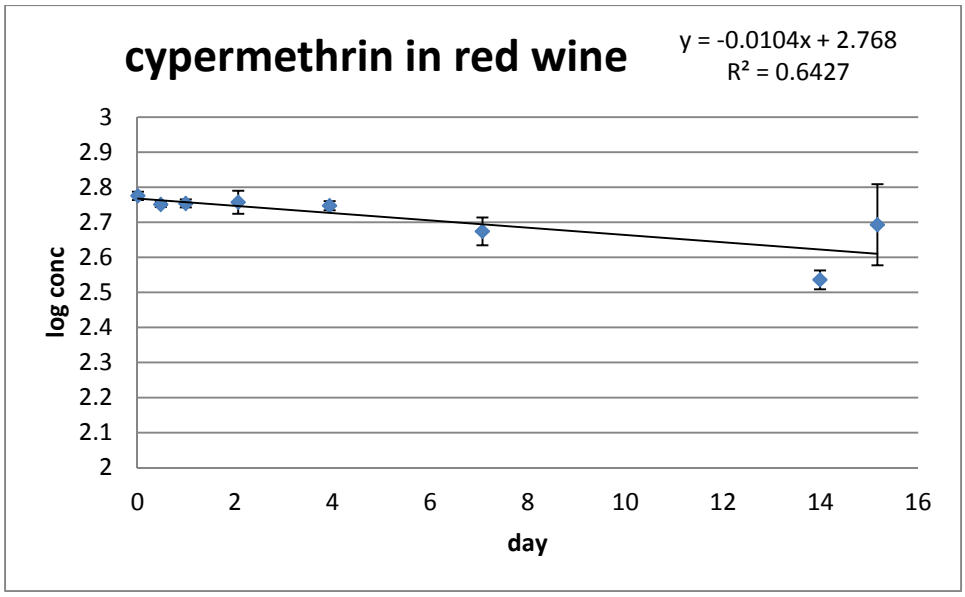

cyfluthrin in grape juice
y = -0.0214x + 2.5861
R² = 0.9161
log conc
day
3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16

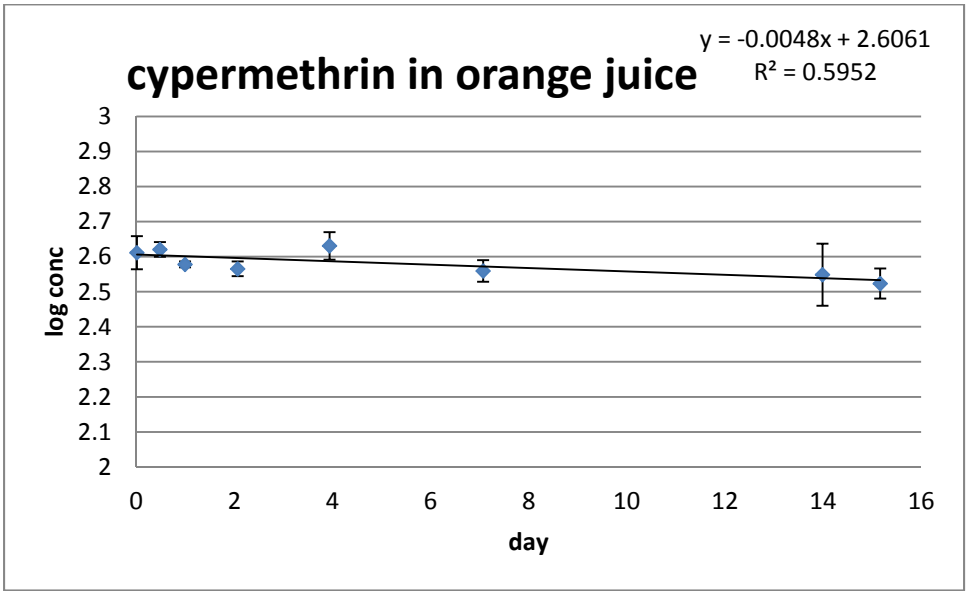

cyfluthrin in red wine
y = -0.0118x + 2.6994
R² = 0.6466
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

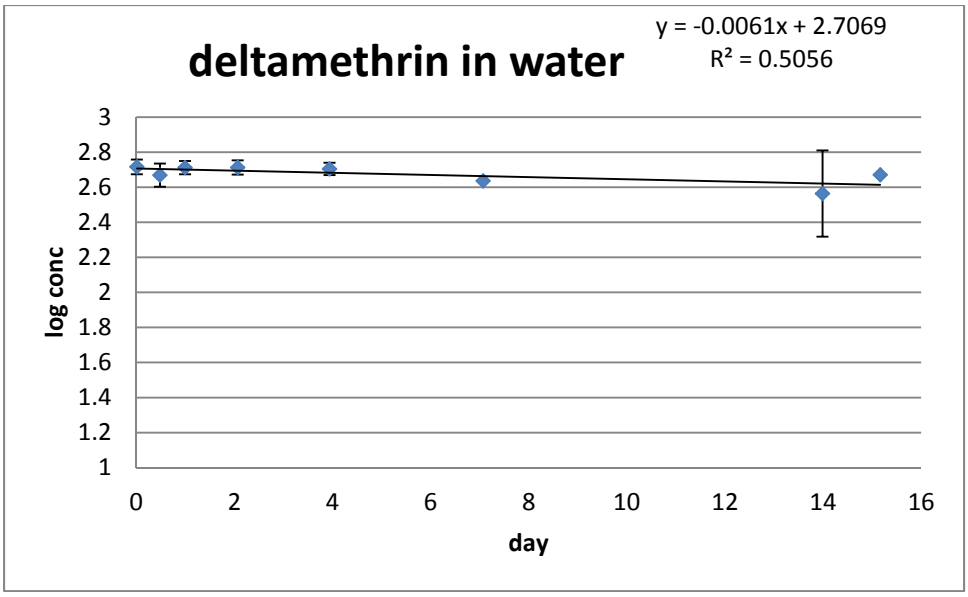

cyfluthrin in orange juice
y = -0.0032x + 2.5722
R² = 0.1531
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

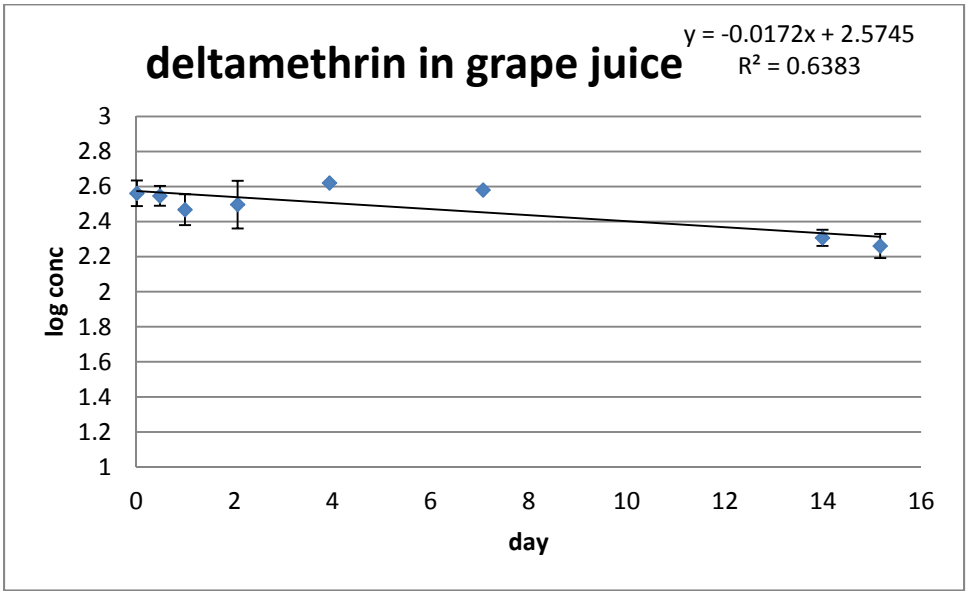

cypermethrin in water
y = -0.0081x + 2.7088
R² = 0.9373
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

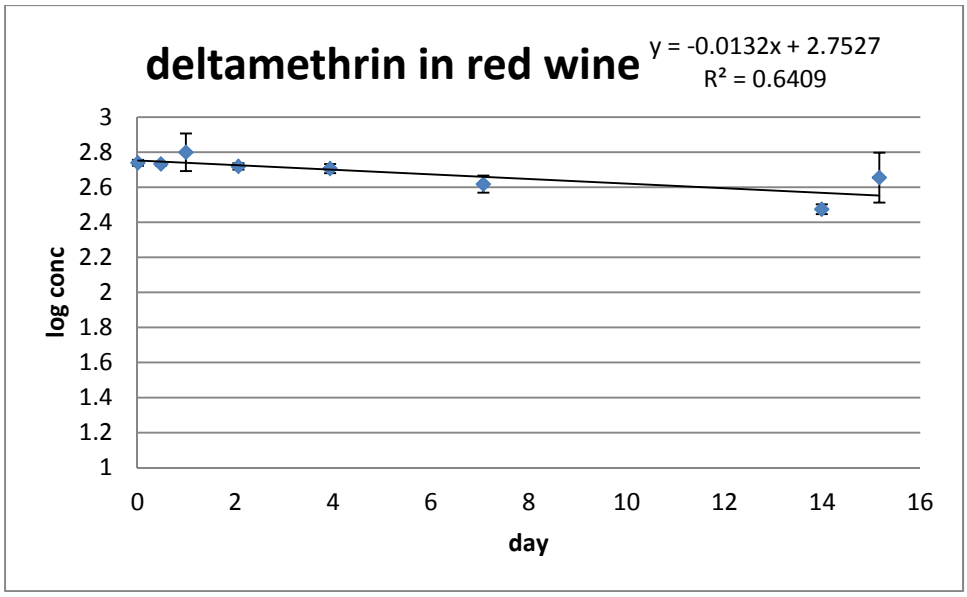

cypermethrin in grape juice
y = -0.018x + 2.6283
R² = 0.8521
log conc
avg conc
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16

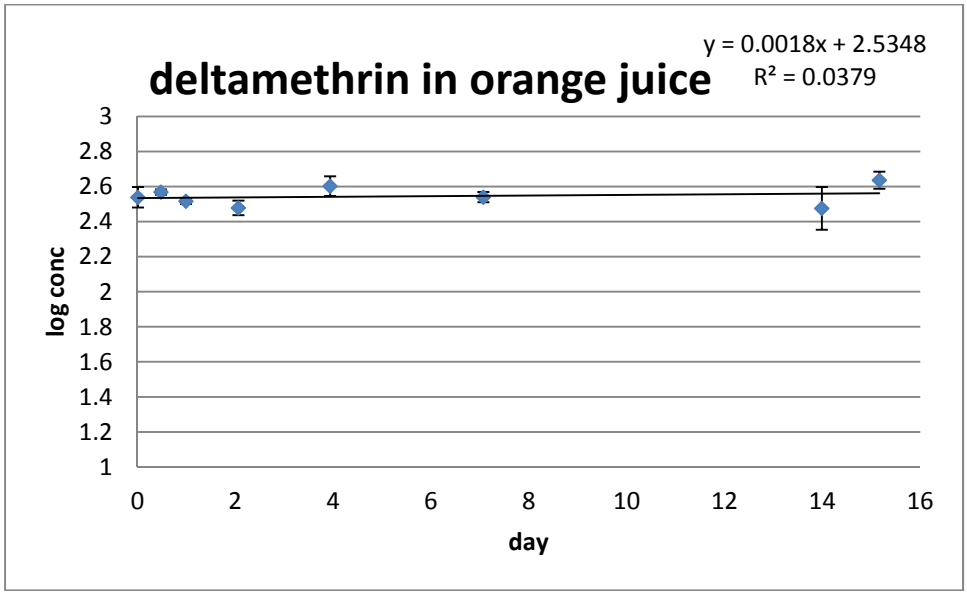

cypermethrin in red wine
y = -0.0104x + 2.768
R² = 0.6427
log conc
day
3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16


cypermethrin in orange juice
y = -0.0048x + 2.6061
R² = 0.5952
log conc
day
3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16


deltamethrin in water
y = -0.0061x + 2.7069
R² = 0.5056
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16


deltamethrin in grape juice
y = -0.0172x + 2.5745
R² = 0.6383
log conc
day
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
2
4
6
8
10
12
14
16



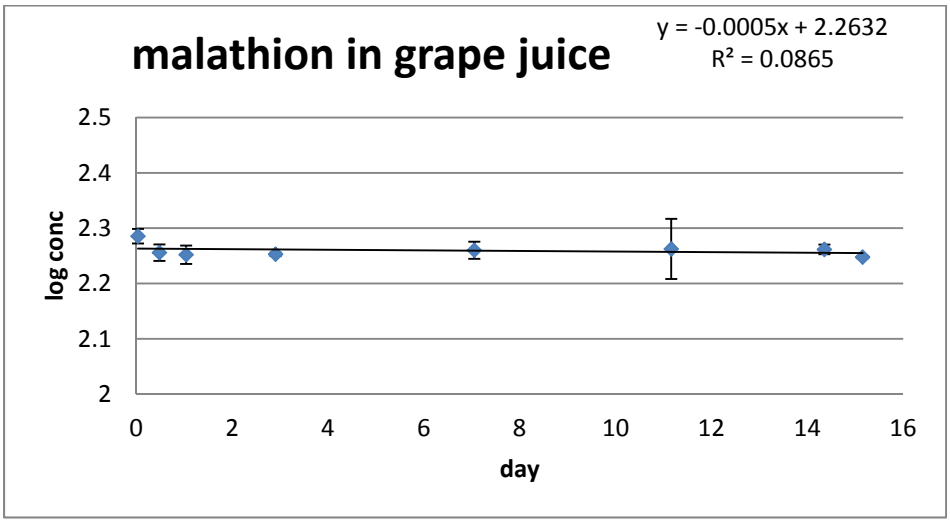


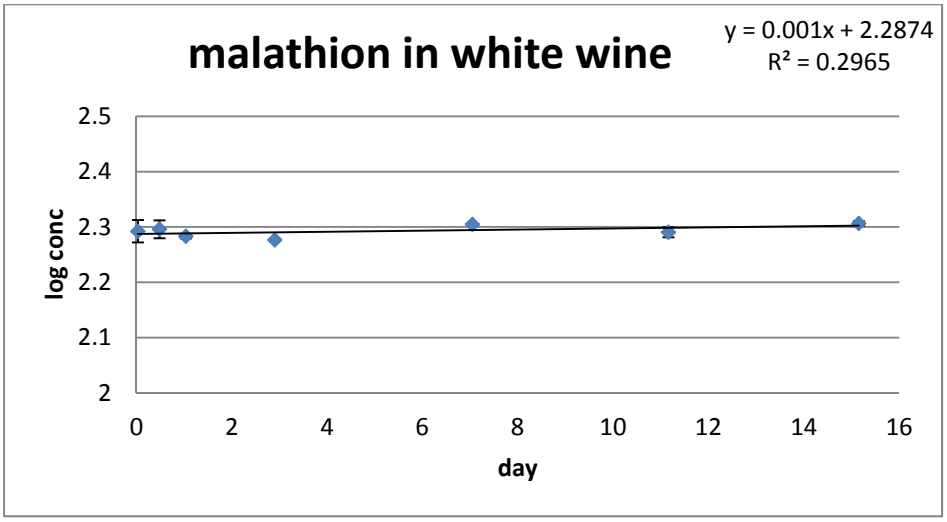
Results are shown for insecticides analyzed by MS/MS in Table 8. No parent compound was found in any blank samples. Figures 33-41 present graphical representations of insecticide degradation. Degradation analysis was not completed for the water matrix.

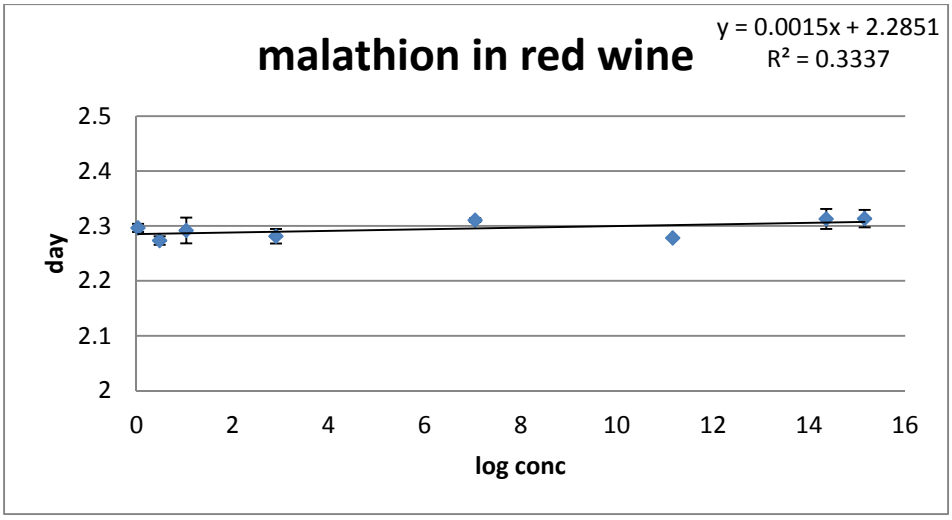
	% recovery[a]	rate constant[b]	std error[b]	p-value	half life[c]
grape juice					
malathion	88.45%	5.40E-04	7.16E-04	0.48	558
chlorpyrifos	74.71%	4.21E-03	1.76E-03	0.06	71
permethrin	72.74%	8.15E-03	8.86E-04	9.3E-05	37
white wine					
malathion	101.22%	-9.90E-04	6.82E-04	0.21	n/a
chlorpyrifos	77.62%	5.40E-04	9.17E-04	0.58	557
permethrin	69.47%	5.18E-03	1.84E-03	0.03	58
red wine					
malathion	102.87%	-1.48E-03	8.55E-04	0.13	n/a
chlorpyrifos	86.69%	1.21E-05	1.01E-03	0.99	24836
permethrin	80.36%	2.47E-04	1.99E-03	0.91	1220

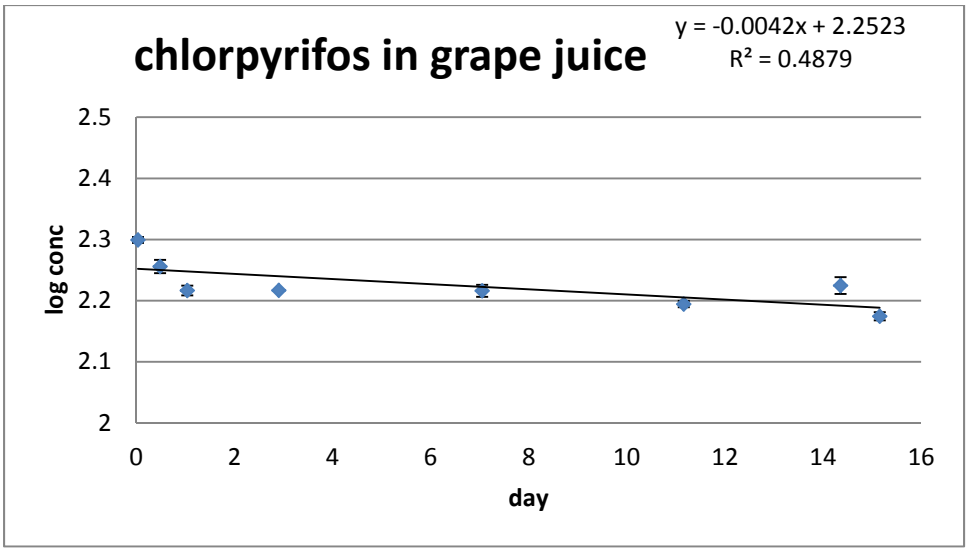
Table 8: Degradation of insecticides in grape-based beverages. [a]Recovery is based on concentration found in day 0 samples. [b]Units of $^{1}/_{day}$. [c]Units of days.

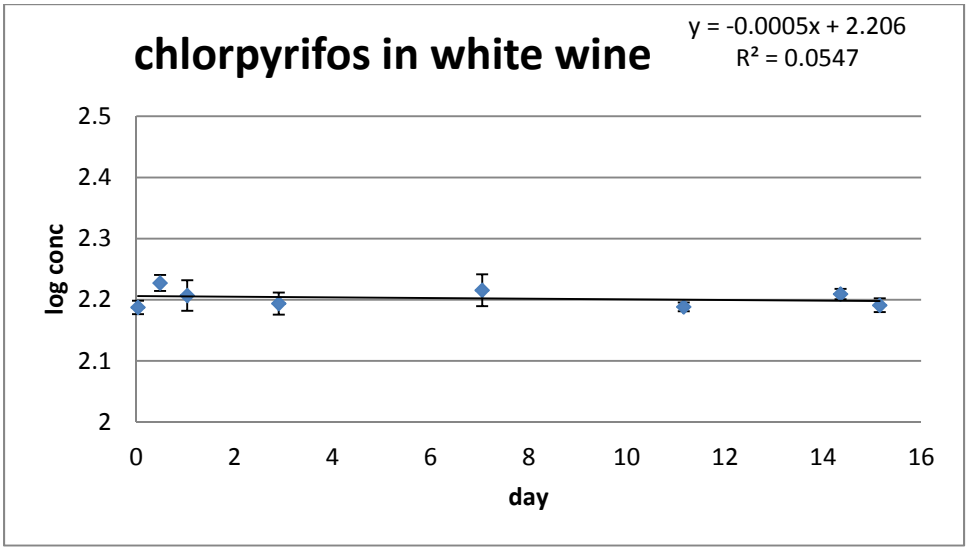
Figures 33-41: Analysis of insecticide degradation by GC-MS/MS and LC/MS/MS. Bars indicate standard deviation.

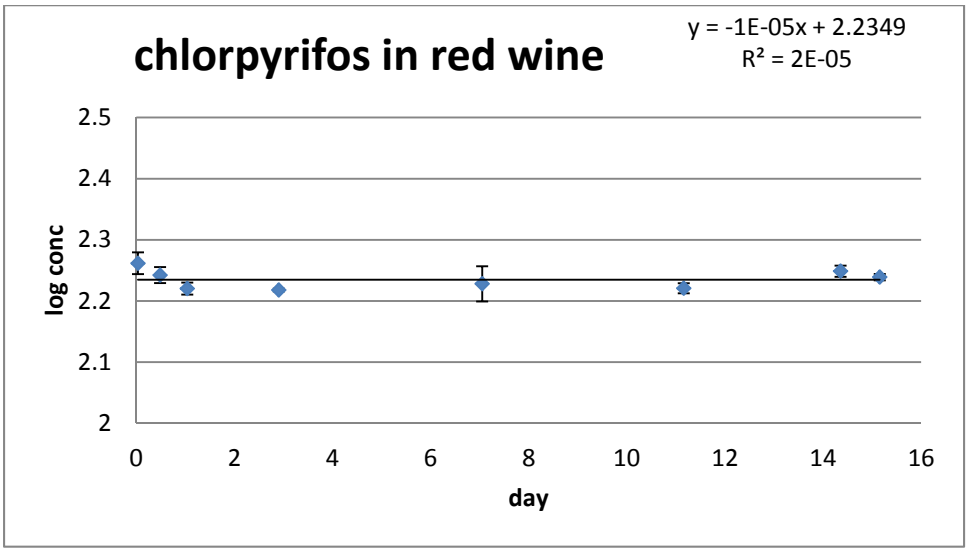


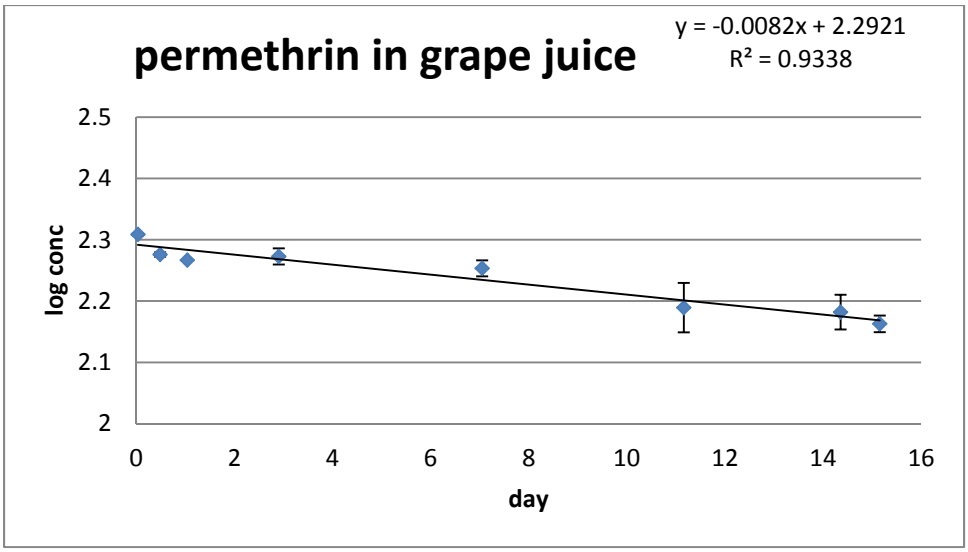


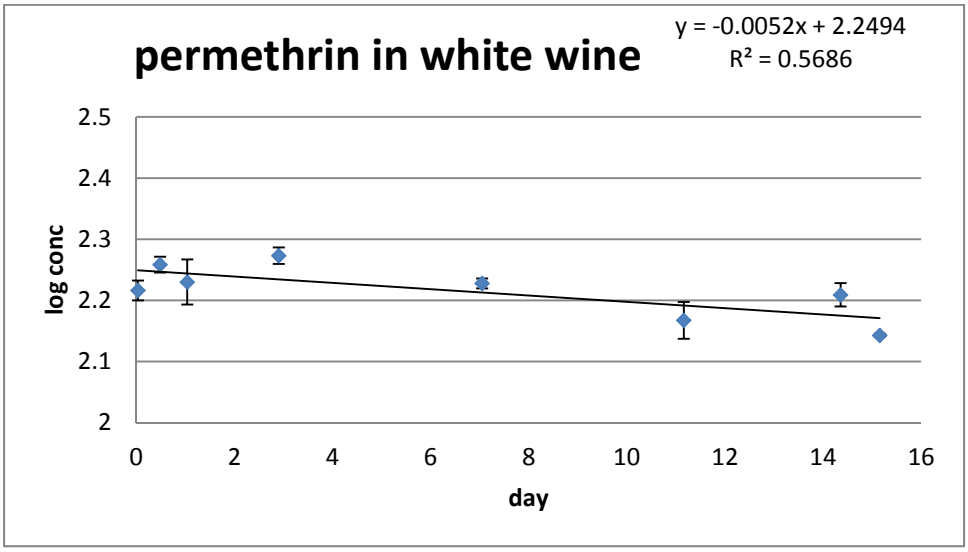


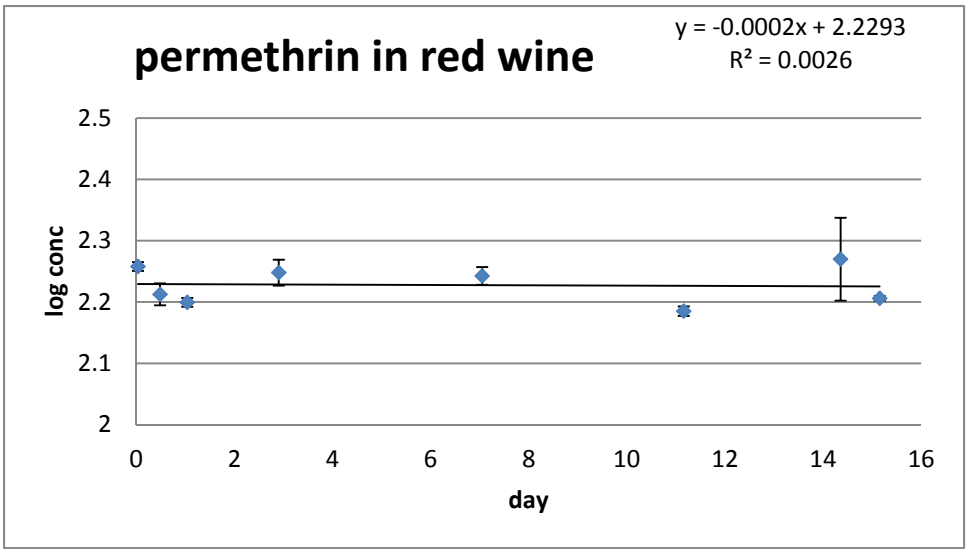

chlorpyrifos in grape juice
y = -0.0042x + 2.2523
R² = 0.4879
log conc
day
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16


chlorpyrifos in white wine
y = -0.0005x + 2.206
R² = 0.0547
log conc
day
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16


chlorpyrifos in red wine
y = -1E-05x + 2.2349
R² = 2E-05
log conc
day
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16


permethrin in grape juice
y = -0.0082x + 2.2921
R² = 0.9338
log conc
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16
day


permethrin in white wine
y = -0.0052x + 2.2494
R² = 0.5686
log conc
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16
day


permethrin in red wine
y = -0.0002x + 2.2293
R² = 0.0026
log conc
2.5
2.4
2.3
2.2
2.1
2
0
2
4
6
8
10
12
14
16
day

Blank concentrations of degradation products are shown in Table 9, and results for MDA are presented in Table 10. DCCA could not be analyzed because of an interfering peak with the quantification ion. Figures 42-53 present graphical representations of metabolite production.

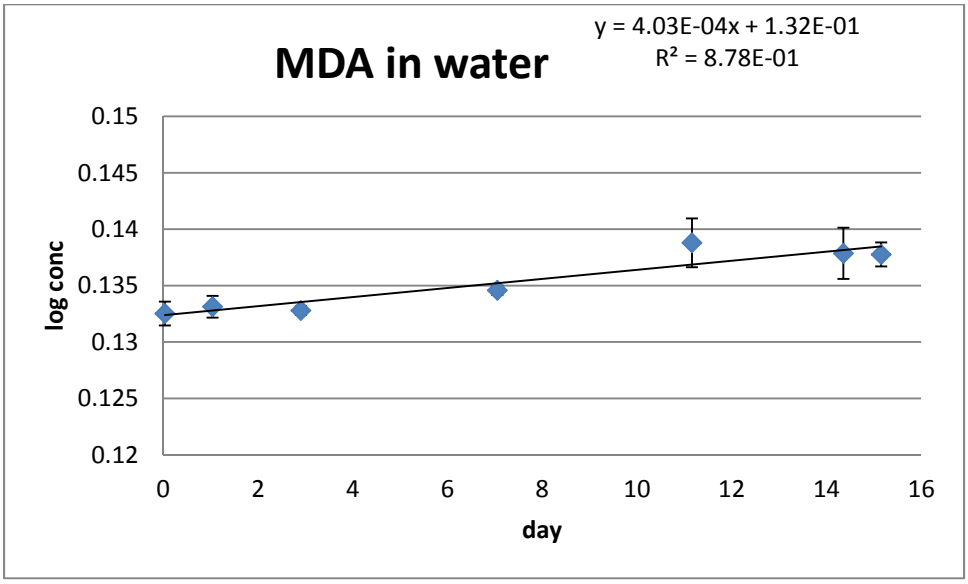
	MDA	TCPy	3-PBA
water	1.36	0.00	0.16
grape juice	1.34	0.00	0.12
white wine	1.35	0.00	0.11
red wine	1.35	0.00	0.10

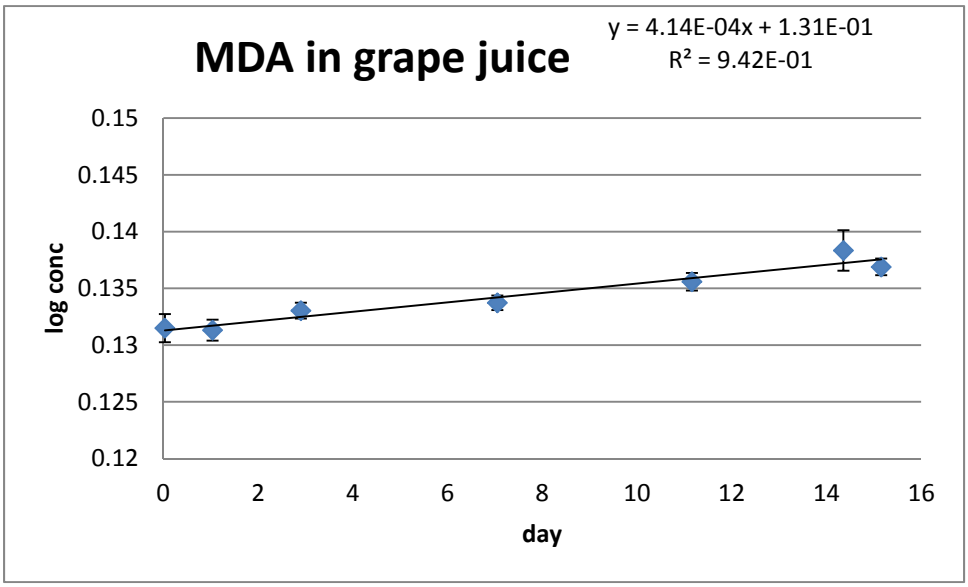
Table 9: Concentrations of degradation products in blank samples in ng/g. Blanks were extracted in duplicate.

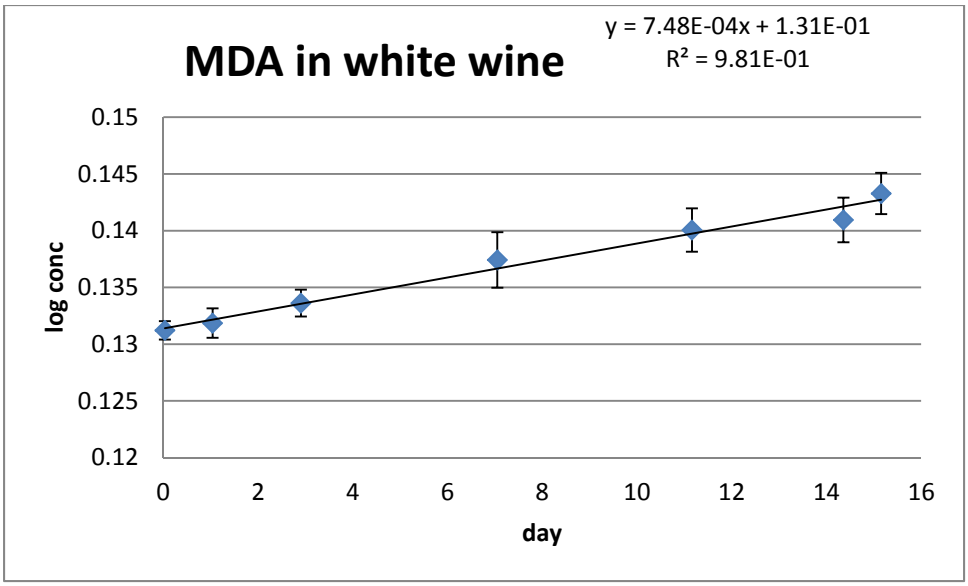
	blank conc[a]	rate constant[b]	std error[b]	p-value
water	1.36	4.03E-04	6.73E-05	1.86E-03
grape juice	1.34	4.14E-04	4.58E-05	2.76E-04
white wine	1.35	7.48E-04	4.60E-05	1.61E-05
red wine	1.35	7.71E-04	4.81E-05	1.73E-05

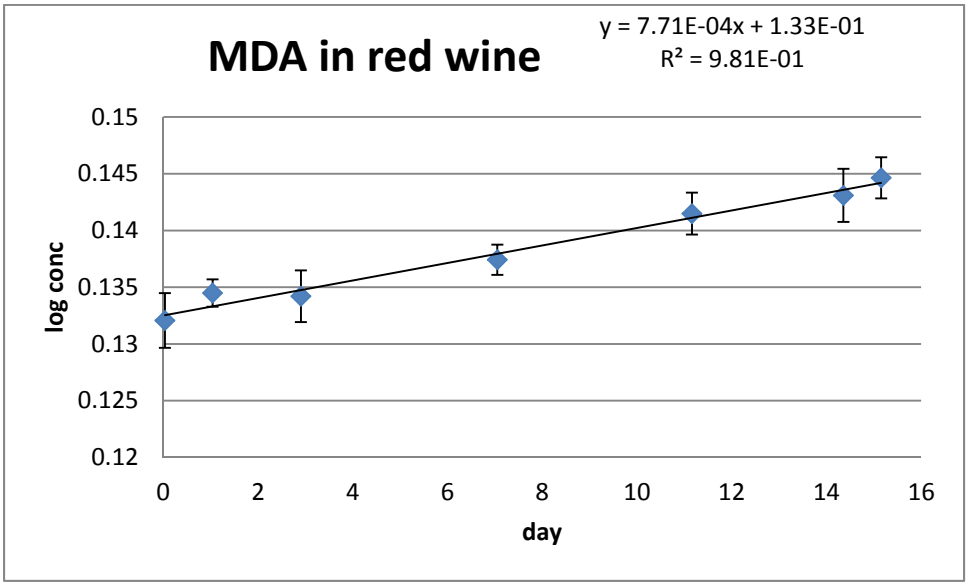
Table 10: Production of MDA over 15 days in samples initially fortified with 200ng/g malathion. [a]Concentration in units of ng/g. [b]Units of $^{1}/_{day}$.

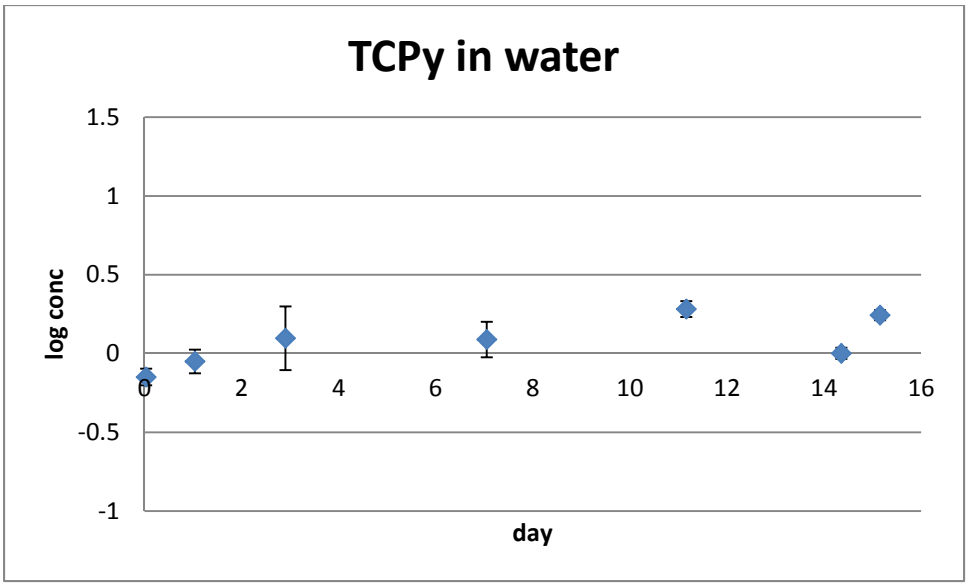
Figures 42-53: Production of insecticide degradation products over fifteen days. Trendlines and statistics for TCPy are not displayed due to its more complicated changes in concentration.

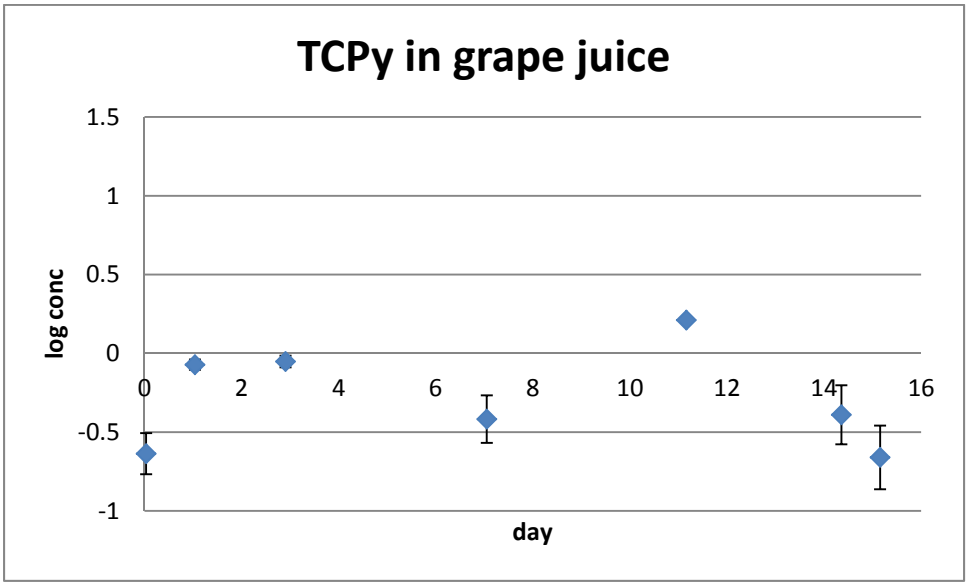


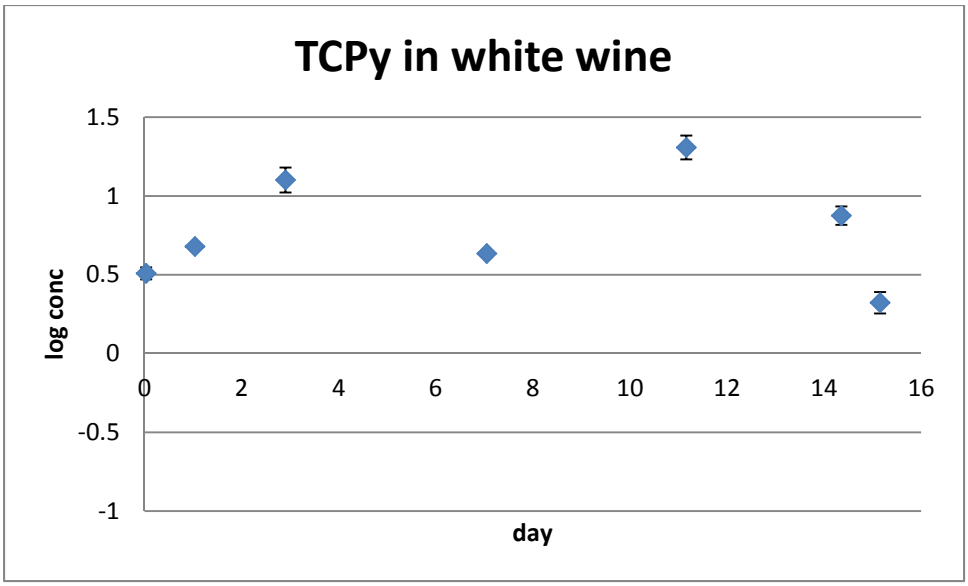


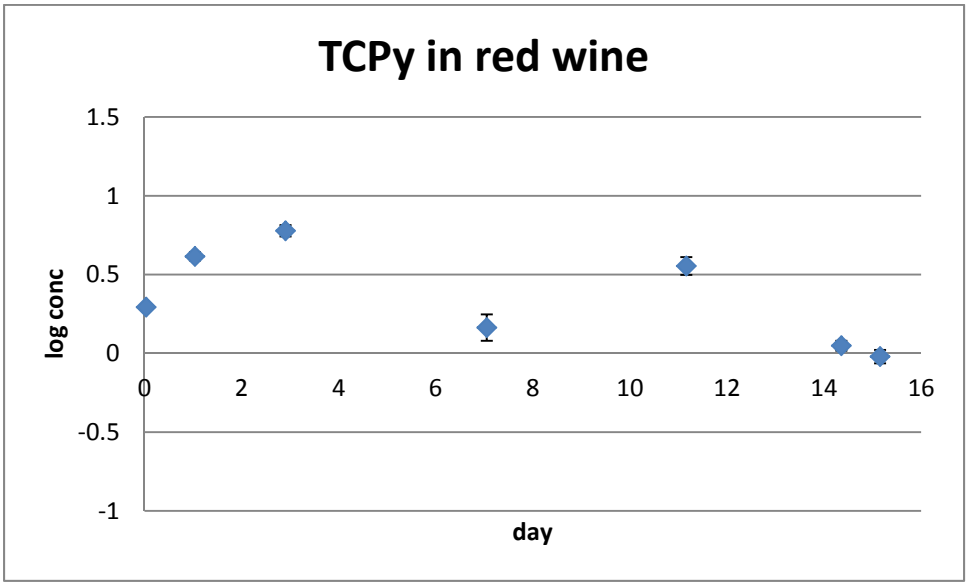

MDA in white wine
y = 7.48E-04x + 1.31E-01
R² = 9.81E-01
log conc
0.15
0.145
0.14
0.135
0.13
0.125
0.12
0
2
4
6
8
10
12
14
16
day

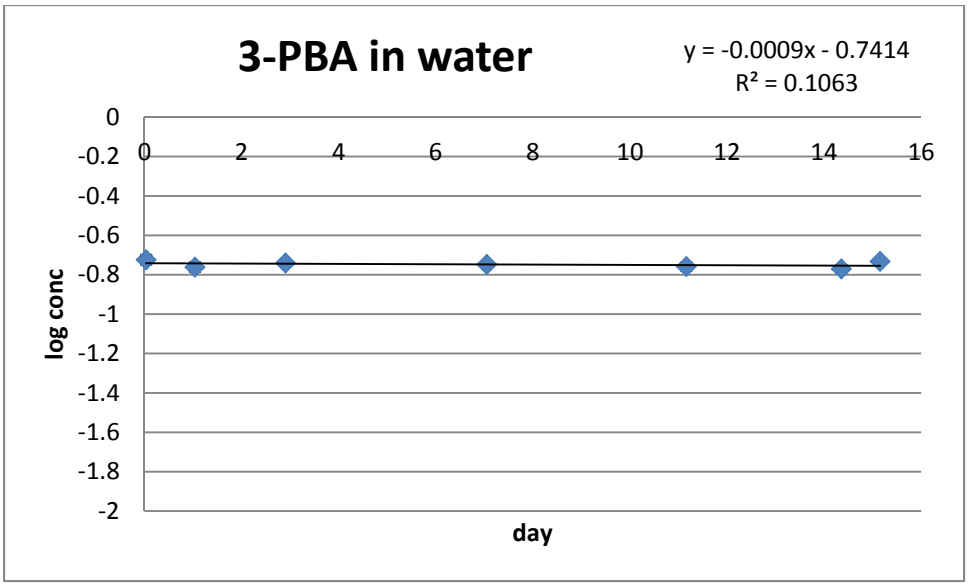

MDA in red wine
y = 7.71E-04x + 1.33E-01
R² = 9.81E-01
log conc
0.15
0.145
0.14
0.135
0.13
0.125
0.12
0
2
4
6
8
10
12
14
16
day

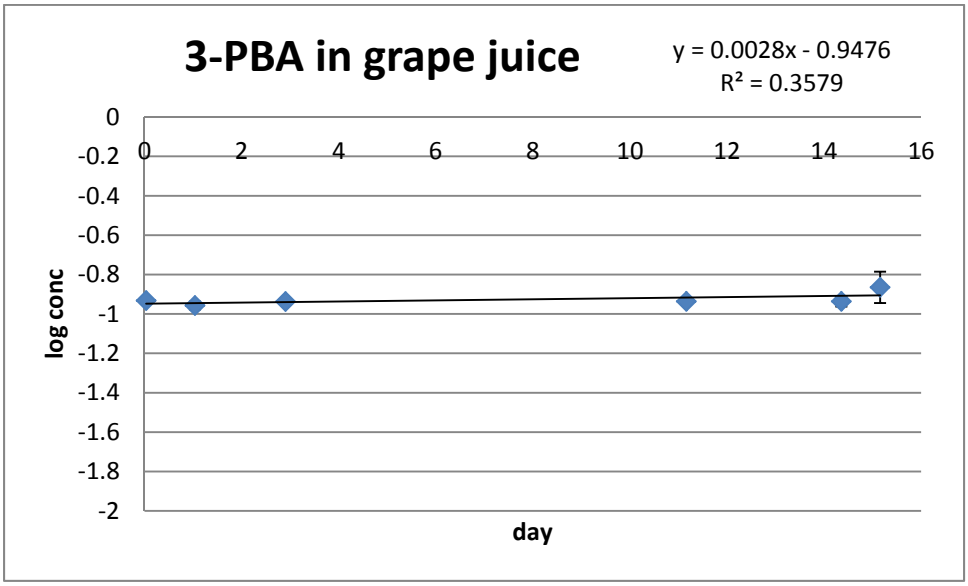

TCPy in water
1.5
1
0.5
0
-0.5
-1
log conc
0
2
4
6
8
10
12
14
16
day

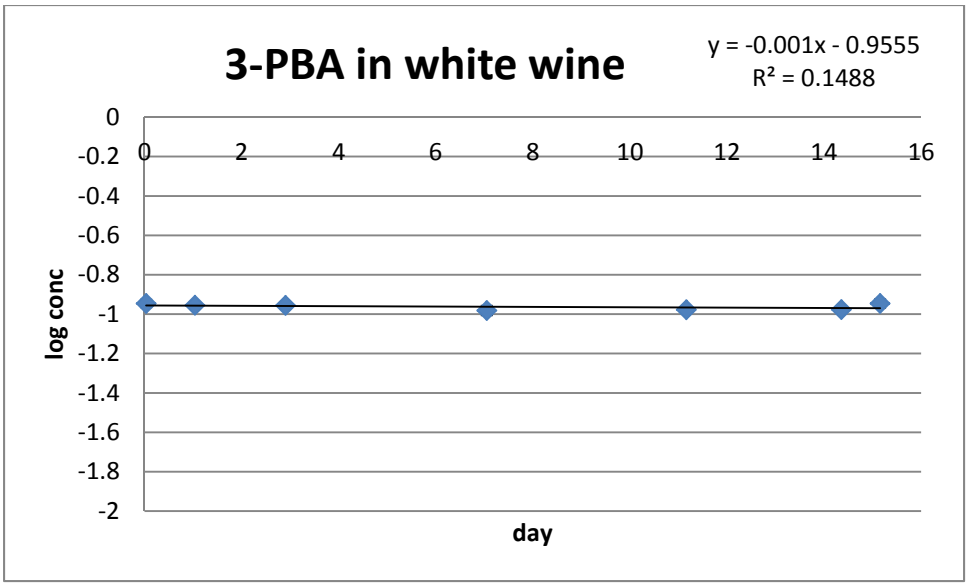

TCPy in grape juice
1.5
1
0.5
0
-0.5
-1
log conc
0
2
4
6
8
10
12
14
16
day

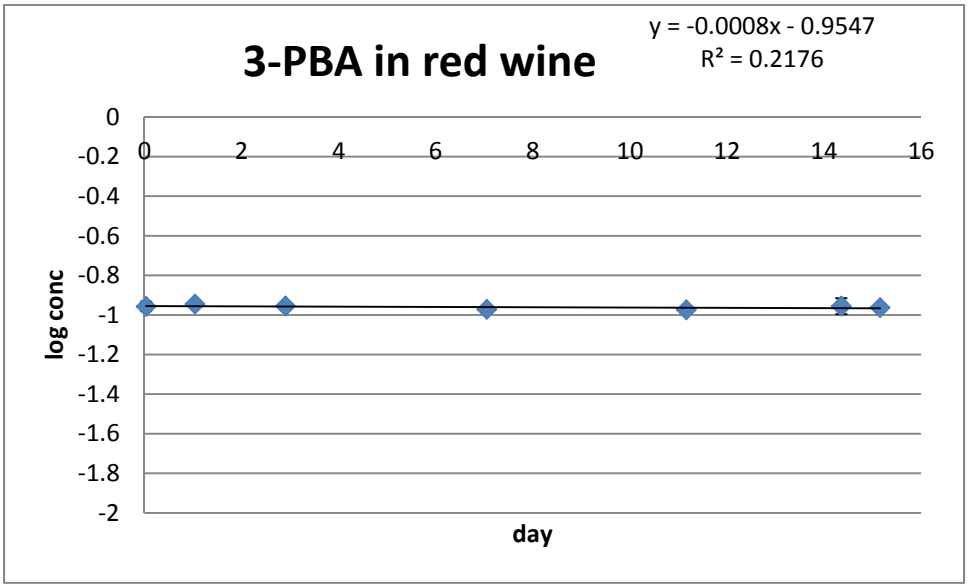

TCPy in white wine
log conc
1.5
1
0.5
0
-0.5
-1
0
2
4
6
8
10
12
14
16
day


TCPy in red wine
log conc
1.5
1
0.5
0
-0.5
-1
0
2
4
6
8
10
12
14
16
day


3-PBA in water
y = -0.0009x - 0.7414
R² = 0.1063
log conc
day
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.8
-2
0
2
4
6
8
10
12
14
16


3-PBA in grape juice
y = 0.0028x - 0.9476
R² = 0.3579
log conc
day
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.8
-2
0
2
4
6
8
10
12
14
16





Study of glass/insecticide interactions by GC-ECD

As seen in the previous ECD study in water, diazinon and chlorpyrifos showed poor recovery (Figure 54). Neither diazinon nor malathion showed significant degradation in water over seven days, but the chlorpyrifos in the untreated jar did. Using a two tailed t-test, all pyrethroids showed significant loss ($p < 0.05$) with the untreated jar

and the silanized jar, and all pyrethroids except permethrin showed marginally significant loss ($p < 0.10$) in the vortexed jar. For each insecticide, there was no significant difference in concentration among Day 7 samples except for the silanized cyfluthrin sample.

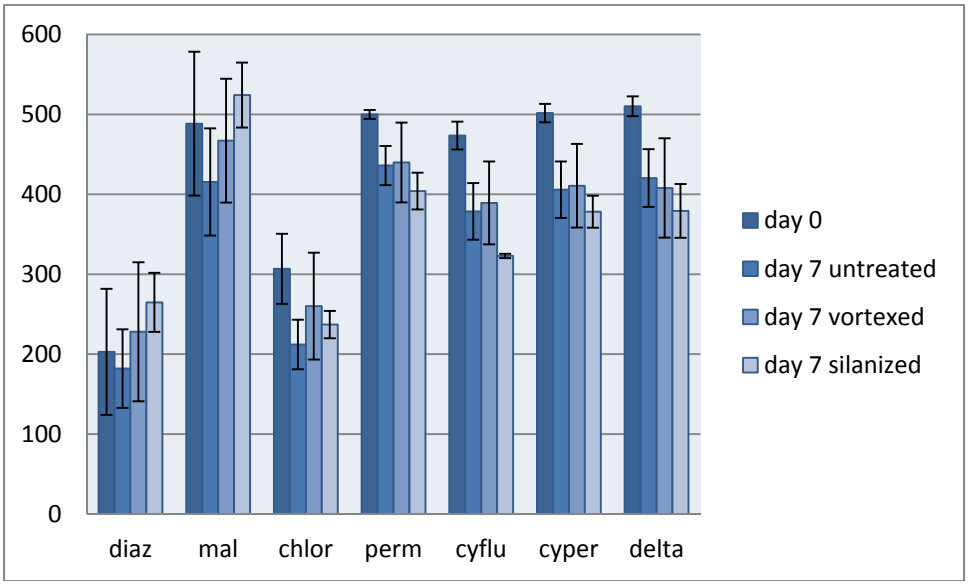


Figure 54: Effect of jar treatment on insecticide degradation. There is no significant difference among day 7 samples except for the silanized cyfluthrin samples, indicating that jar adsorption plays an insignificant role in the disappearance of insecticide.

Discussion

By half-life, diazinon displayed quickest degradation (2.3-15.2 days according to matrix). In fact, diazinon became undetectable after day 7 in red wine. This phenomenon is likely due to the fact that while the other insecticides used in this study are base hydrolyzed, diazinon generally degrades more quickly in acidic matrices.[38, 96] This hypothesis is supported by the fact that diazinon showed the longest half-life in water, the only non-acidic matrix tested. The quick degradation of diazinon may also

explain the low recovery of diazinon in each matrix (40.7% in red wine, 69.5% in water). If some of the insecticide degraded in a near-instantaneous fashion upon addition to matrix, recovery would then be low.

Orange juice showed the least degradation of insecticides, both by regression significance and half-life. While half-life and significance are often related, as a larger slope generally is correlated with a greater probability of significance, the variability about the line may reduce significance of data. For example, diazinon has a half-life of 15.2 days in water and a *p*-value of 0.03, but diazinon in orange juice has a half-life of 12.9 days and a *p*-value of 0.09. This greater variability found for orange juice samples may be due to the greater complexity of this matrix, i.e. analyte interactions with the solid pulp in the juice. This complexity may also explain the generally longer half-lives for insecticides in orange juice. Pulp may give insecticides surfaces to interact with and adsorb to, thereby delaying degradation. Other experiments of insecticide degradation in solid food have been performed with inconclusive results (see Appendix 2), so it is not surprising that insecticides degrade less in orange juice than the translucent matrices.

Without observation of degradate production, loss of insecticide does not guarantee that these compounds are actually degrading. The loss may be due to some other mechanism. Volatilization is not a suspected mechanism of loss because neither OPs nor pyrethroids are particularly volatile. However, adsorption of the insecticides to the wall of the glass storage container is a more likely alternative mechanism of loss. To confirm that the disappearance of insecticide observed was due to degradation and not simply adsorption to the glass container storing the samples, an additional study of parent insecticide loss by GC-ECD was conducted. Adsorption was controlled by both chemical

and physical methods, namely jar silanization and vortexing of a non-silanized jar. Although the pesticides from the vortexed jar generally showed a slightly higher concentration of pesticide than the silanized or untreated jars, they also produced the largest standard deviations for the triplicate samples. Especially noteworthy, there was no significant difference between the concentrations of pesticide in any of the three Day 7 samples except for cyfluthrin. Overall, the evidence suggests that there may be some pesticide adsorption by the jar wall, but the amount of insecticide found seems to indicate that loss of insecticides was largely due to some other mechanism, which is hypothesized to be degradation.

While insecticide loss over time had been observed using GC-ECD, and the loss was shown to not be due to simple adsorption of the insecticides to the glass wall, the best way to tell if insecticide loss is truly due to degradation would be to observe production of degradates as well as loss of parent insecticide. For this reason, another study which followed both the loss of parent compound and the production of insecticide degradation using chromatography with tandem mass spectrophotometric detection was conducted.

For MS/MS work, significant degradation ($p < 0.05$) occurs for permethrin in grape juice and white wine, and degradation of chlorpyrifos has a p-value of 0.058 in grape juice. Earlier experiments (see Appendix) had strongly suggested degradation of malathion. However, malathion results are suboptimal because of poor recovery of analyte in some samples due to the evaporation used for solvent exchange. In future studies, separate samples may be extracted for malathion analysis on LC-MS/MS so the solvent exchange step is not needed.

Significant production of MDA, the metabolite of malathion, is seen for all four matrices. Unfortunately, the amount of MDA seen throughout the experiment falls below the limit of detection (LOD), so results are semi-quantitative. However, the fact that there is a significant increase in MDA for every matrix every time this degradation experiment is conducted (see Appendix A also) strongly supports the conclusion that MDA is being produced from malathion, albeit at very low quantities. Given the low concentrations of MDA and the very small, yet significantly significant, change in concentration over time, it is possible for MDA to be synthesized, yet observable malathion degradation to be statistically insignificant. Several possible solutions may be suggested for future studies. First, assuming malathion degrades in these beverages, higher initial concentration of malathion should lead to higher concentrations of MDA. Second, a larger aliquot of sample could be taken each day for degradate analysis, leading to concentration of analyte. Finally, background concentrations of native compounds may be partially due to contamination of ISTD, as evidenced by background native compounds in solvent blanks spiked only with ISTD. The current ISTD concentration used is somewhat high (50ng/g). Lowering its concentration could also lower the background concentration, allowing for lower LODs.

MDA is produced almost twice as quickly in the wines than in water and grape juice. White wine generally has an alcohol content of 11-13% (mass percent), while red wine has an alcohol content of 12-14%. It has been shown that, in some situations, esters hydrolyze more quickly in aqueous solutions with low ethanol content than in pure water.[105] While this is surprising chemically since hydrolysis is usually more likely in more aqueous solvents since there is simply more water available for reaction,[106, 107] this

fact may simply be because it is difficult to dissolve the ester in pure water, which keeps it from reacting.[105]

While TCPy is not present in any blank matrix samples, it appears in the Day 0 samples for each matrix, implying a nearly instantaneous hydrolysis of a fraction of the chlorpyrifos. From that point, though, the picture becomes more complicated. In water, TCPy continues to be produced until about day 4, at which point it seems to reach equilibrium. For the grape matrices, though, production reaches a peak at day 4, but then there is a drop in concentration at day 7. TCPy concentration increases again at day 11, and then decreases for days 14 and 15. A pattern of one cycle of production then loss would make sense, as it would imply production of TCPy accompanied by degradation of this degradation product. This pattern, consisting of production, loss, then more production, is more puzzling. Initially, it was thought that an error in the data was evident, so the experiment was repeated. Yet in the second experiment, this pattern was repeated, suggesting a two-phase production-degradation-production process. For this reason, the experiment was repeated and it was made sure that samples were not knocked over (see Appendix), but the TCPy pattern was duplicated. Given the complexity of food matrices, even of liquid foods, it is possible and even likely that multiple interactions are occurring between both chlorpyrifos and matrix and TCPy and matrix, for example, analyte being adsorbed by polyphenols or other food components. These interactions, each with their own kinetics and equilibria, could cause the pattern seen for TCPy over two weeks. The fact that TCPy does not show such a complicated pattern in pure water, which is obviously a much simpler matrix that would not be able to adsorb analytes,

supports the hypothesis of more complicated action in the more complicated grape matrices.

While there is no reaction rate available for TCPy, greater amounts of TCPy are present in wine matrices than in water and grape juice. As with MDA, this fact may be because hydrolysis is faster in aqueous solutions with lower concentrations of ethanol than in pure water alone. Also like MDA, a significant increase in TCPy content occurs between blank matrix samples and samples fortified with insecticide, despite the fact that are no statistically significant examples of chlorpyrifos degradation.

The opposite occurrence seems to happen for permethrin and 3-PBA, in that while both grape juice and red wine show significant degradation of the parent compound, there is no difference between the amount of 3-PBA in blank samples versus samples fortified with permethrin, nor does the 3-PBA concentration change over time. One explanation for this phenomenon is that when permethrin hydrolyzes, it should not be 3-phenoxybenzoic acid that forms, but 3-phenoxybenzyl alcohol (see Figure 2). The acid form of the compound is typically analyzed in urinary metabolite studies because the alcohol is oxidized to the acid in the body.[19] However, this oxidation may not be happening in these matrices. Oxidation of alcohols to carboxylic acids is particularly not likely in pure water, where no enzymes or even other catalysts are available.

As mentioned in the results section, DCCA was not analyzed because of a broad interfering peak on the chromatograph. While the other analytes had both a parent and daughter ion for both quantification and confirmation ion pairs, DCCA did not fragment cleanly in the MS. Therefore, its chlorine pattern was used for confirmation instead, and the parent ion was the same as the daughter ion for both quantification and confirmatory

ion pairs. Since there are no true parent-daughter ion pairs to confirm identity of the DCCA peak, there is a greater probability for interfering peaks in the chromatography. For this reason, if the confirmation ion alone may be analyzed, it is not specific enough to confidently be quantified. While the interfering quantification peak may have been a component of the grape products, the fact that it was also seen in water samples leads one to believe it is related to a chromatography shortcoming. While no information was found about *m/z* = 207 for liquid chromatography, a common background peak of *m/z* = 207 was found in gas chromatography in the form of hexamethylcyclotrisiloxane.[108] Even though silica based reverse-phased liquid chromatography columns are generally stable under acidic conditions, some hydrolysis at the Si-O bonds occurs over the column life time, and production of SiO_2 from may be observed.[109] The slightly elevated temperature (45°C), while lowering solvent viscosity and allowing higher solvent flow, may also contribute to column degradation.[109] While the formation of hexamethylcyclotrisiloxane seems less likely from the Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column, which is made by tightly packing dimethylphenylhexylsilane chains onto a silica base,[110] particularly without the high heat that is used in gas chromatography, silica chains and methyl groups are available to form this interfering compound.

For matrices in which chlorpyrifos and malathion degradation is seen, regardless of statistical significance, there is a large difference between amount of parent compound lost and degradation product produced. For example, for malathion in grape juice, about 16ng/g is lost from day 0 to day 15, but less than 1ng/g MDA is produced. As another example in grape juice, nearly 50ng/g chlorpyrifos is lost over the two weeks, but the highest TCPy concentration ever seen is about 1.6ng/g. While it may be that

simultaneous production and degradation of the degradation products themselves are occurring, causing a smaller amount of hydrolysis products to be seen, it is also possible that oxidation of insecticides (for example, exchange of sulfur for oxygen in the phosphate group) is also occurring.[50] In such a case, the analysis of the single degradation product would give an incomplete picture of the loss of parent compound over time. Further research on other possible degradation products, including the direct oxidation products such as the oxon of chlorpyrifos, would be of interest.

There are some limitations to the MS/MS portion of this study. First, current methods offer insufficient sensitivity to quantify 3-PBA and MDA concentrations observed at the spiking levels studied. Three solutions to these issues are proposed. First, higher initial parent insecticide concentrations could lead to higher degradate concentrations. Second, higher sample volumes for the degradation product analysis would allow concentration of analytes. Finally, background concentrations of native compounds are partially due to contamination of ISTD, as evidenced by background native compounds in solvent blanks spiked only with ISTD. For example, the concentration of native 3-PBA in ACN spiked with ISTD is 0.159±0.003ng/mL, but native 3-PBA is not found in ACN not spiked with ISTD. The current ISTD concentration used is somewhat high (50ng/g). Lowering its concentration would also lower the background concentration, allowing for lower LODs.

A second possible issue for the MS/MS portion of this study is the analysis of 3-phenoxybenzoic acid instead of 3-phenoxybenzyl alcohol. It would be interesting to add the alcohol to the method to see if it is formed instead of the acid. Even if this is the case, it would still be relevant to the use of the acid as a biomarker of insecticide exposure

because the alcohol is converted in the human body to the acid before conjugation and excretion.[19] While it seems likely that exposure to 3-phenoxybenzyl alcohol rather than 3-phenoxybenzoic acid would still lead to conversion to the acid before excretion, there is a possibility that the alcohol alone would follow a different metabolic pathway than 3-phenoxybenzyl alcohol produced by the body from permethrin. In that case, perhaps urinary 3-phenoxybenzyl alcohol levels could be compared to 3-PBA, and conclusions about exposure to the degradate could be made.

Direct comparisons between ECD and MS/MS data cannot be made because initial insecticide concentrations were different (500ng/g vs 200ng/g). While rates are pseudo-first order, implying that initial concentration should not affect degradation rates, the effect of initial concentration on degradation rate has not yet been studied. Despite the difference in initial concentrations, some similarities between studies may be seen. While there is no data for parent compounds in water by GC-MS/MS as of yet, there was significant degradation of malathion, chlorpyrifos, and permethrin in water in the ECD data. Degradation product data for MDA and TCPy in water supports the hypothesis that these two compounds degrade in water. For both methods, grape juice shows statistically significant degradation of permethrin, but not for malathion. Results for chlorpyrifos in the MS/MS data give a *p*-value of 0.058, while chlorpyrifos in the ECD set degrades significantly. Finally, while data on white wine is not available for the ECD data set, results from red wine also suggested significant degradation of permethrin. The appendix contains a set of data analyzed by GC-MS/MS and LC-MS/MS which follows the degradation of an initial concentration of 500ng/g insecticides in four juices. The GC data in this appendix study should be considered semi-quantitative in that some

experimental parameters, e.g. column condition, were not optimal. Particularly in light of the LC work, the data are, nonetheless, of interest and are included as part of hypothesis generation.

Urinary insecticide metabolites are often used as biomarkers of exposure to parent insecticide.[37, 38] Multiple studies, including this one, have shown that the same compounds used as urinary metabolites are often produced in food before insecticide metabolism in the body.[38, 52, 77] Some insecticide degradates, such as dialkyl phosphates, may be further degraded after ingestion.[38] However, other research concerning both dialkyl phosphates and TCPy in animal models suggests that these compounds are largely adsorbed by the body and then excreted unchanged in the urine.[77] If ingested insecticide degradates truly are excreted unchanged in urine, observation of these analytes would lead to overestimation of insecticide exposure.

While the primary mechanism of OP insecticides is acetocholineesterase inhibition, it is known that some OPs target other neurological pathways, including growth factors and other neurotransmitter systems. These pathways may be compromised at lower concentrations than those needed for acute acetocholineesterase inhibition.[41] Several recent studies have connected prenatal OP exposure to lower IQ and cognitive development in children.[41-43] Similar studies have not been done with pyrethroids yet; however, there is the chance that these compounds also have secondary mechanisms of toxicity that occur at low-level exposures. Children's exposure to insecticides are of particular interest since they are in the process of neurodevelopment and the potential for permanent damage from insecticides is greater.[38, 41] Furthermore, children have lower activity of paraoxonases, which are known to detoxify some OP

insecticides.[41-43] Finally, foods that children prefer, such as fruit juices, tend to have higher insecticide concentrations.[38] Given this information, even if insecticide exposure is overestimated, the amount of exposure actually occurring may still give cause for concern.

It has been assumed in the past that there is little to no toxicity from insecticide degradation product exposure. However, there are currently few data on the adsorption, further metabolism, and potential toxicity of many insecticide degradates.[38, 36] Therefore, it is difficult to separate the health effects of insecticide degradates from that of the parent compounds. Further research is needed to better understand the health effects of insecticide degradates.

Conclusions

Statistically significant degradation of OP and pyrethroid insecticides is seen in several liquid beverage matrices. The fact that loss of insecticides over time is due to degradation and not some other mechanism, like adsorption onto the glass wall of the matrix container, is supported through examination of insecticide interactions with the glass jar. There was no significant difference in degradation of insecticides among regular glass jars, silanized glass jars, and jars vortexed before extraction. The degradation of insecticides in these food matrices is further supported by the fact that degradation products of these insecticides are produced after spiking of a matrix with the parent compound. These data support the contention that more should be understood about people's, particularly children's, exposure to insecticide degradation products and

its relation to the use of urinary insecticide metabolites as biomarkers of exposure to insecticides.

APPENDIX B: OTHER STUDIES OF INSECTICIDE DEGRADATION

Liquid-liquid extraction

Standards

Standards for all GC-ECD studies listed here were prepared the following way. Standards of the seven insecticides in acetonitrile were made in increasing concentrations (1, 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, and 1000 ng/mL and used to create a calibration curve. Method detection limits were calculated using a power regression curve due to the non-linear response of the ECD. Peaks were manually integrated using ChemStation software (Agilent Technologies, Santa Clara, CA) and data was analyzed using Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA).

Sample preparation

A 10mg/g stock solution of seven insecticides (diazinon, malathion, chlorpyrifos, permethrin, cyfluthrin, cypermethrin, and deltamethrin) in ACN was used to create 50mL beverage fortified with 1000ng/g insecticides. A 1000mg/g solution of pentchornitrobenzene (PCNB) was also used to fortify juices to 1000ng/g as an internal standard. Samples were stored in amber glass jars in a refrigerator at 2.5°C.

Plan

Samples were extracted in triplicate immediately after juice fortification and 1, 2, and 7 days thereafter. Blanks were fortified to 1000ng/g ISTD and also extracted in duplicate.

Extraction procedure

The following method is based on a method for the extraction of DAPs out of orange and apple juice, but the derivitization step was unnecessary and therefore deleted.[111] Two grams NaCl was added to a clean test tube. Then, 2.0 mL of sample was added. The sample was vortexed with the salt for two minutes. Next, 0.5mL 6M HCl was added to each test tube for acidification. 2.0 mL ACN was also added to the test tube. Tubes were vortexed for 5 min and centrifuged for 4 min. The supernatant was moved into a new test tube containing 10mg potassium carbonate, a drying agent. 1.0mL ACN and 1.0mL ethyl ether was added to the first tube. The sample was vortexed and centrifuged as before, and the supernatant was added to the other extract. This process of extraction was repeated once more with 2.0mL ethyl ether and once again with 1.0mL ACN and 1.0mL ethyl ether. The total extract (in the second test tube) was centrifuged 4 min to force out any water and the supernatant was transferred to a new test tube with 5mg potassium carbonate. This extract was centrifuged and the supernatant was transferred to a clean test tube. The sample was evaporated to dryness and reconstituted with toluene for analysis.

Analysis by GC-ECD

All studies in this appendix used the following GC-ECD settings. The gas chromatograph used was a Hewlett-Packard Model 5890A Series II equipped with an Agilent Technologies electron capture detector and 7683B Series Injector autosampler (Santa Clara, CA, USA). A DB-5 column (Agilent Technologies, Inc., Santa Clara, CA, 30 m, 0.25 mm i.d., 0.25 μm film thickness [5% phenyl, 95% dimethylpolysiloxane]) was

used, and a 2 mm i.d. single-taper injection liner was used to prolong column life. Injection volume was 1.0 μL (1:30 split). The helium carrier gas was at a flow rate of 0.88 mL/min, while the nitrogen make up gas flow was 13 mL/min. The injector temperature was 260ºC. The temperature program started at 80°C and stayed at that temperature for 2 min before being heated linearly by 10ºC/min to a final temperature of 280ºC which was held for 13 min. The ECD temperature was 280ºC.

Statistical analysis

All parent insecticide samples in this appendix are analyzed the following way. Concentrations were determined using the calibration curves described. Each concentration was logarithmically transformed, and these transforms were averaged for each day's sample. Linear trend lines relating log concentrations and time in days were determined, and error bars denote standard deviation of the log transform. P-values were determined from the linear regression results and half-lives were determined using the slope (m) and the relationship $t_{1/2} = -log(2)/m$. R^2 represents the fraction of the variance accounted for by the linear model. The balance of the variance, i.e., $(1-R^2)$ is the unaccounted-for variance of the error in the model.

Results and discussion

The averages of each day's logarithmic transform of concentration were plotted against time. Results are as shown in Table 1. At p =0.05, significant degradation of at least one insecticide may be observed in each matrix over the course of a week.

Recovery is generally acceptable (between 80-120%) in all matrices except orange juice, where solids such as pulp may interfere with recovery.

As demonstrated in Figure 1, many sample averages showed a relatively high standard deviation of concentration from day 7. However, loss of insecticide is still apparent. For example, even the sum of the last day's concentration and its standard deviation may be lower than the difference between first day's concentration and its standard deviation.

PCNB was added as an internal standard. However, there were three problems associated with the ISTD. First of all, it was added at the beginning of analysis to the 50mL of stock juice rather than before each extraction, so if it also degraded throughout the week of the experiment, it would give unreliable results. Second, the ISTD coeluted with diazinon, making analysis of both difficult. Finally, PCNB did not produce consistent area counts, making it an unreliable internal standard. Therefore, the internal standard was ignored and samples were analyzed without it.

Chromatograms of all matrices except red wine were clean. There were many extraneous peaks in red wine, although they did not interfere with analysis. However, these peaks demonstrated need for possible further clean-up. Also, despite use of potassium carbonate and multiple sample transfers, water was often left behind in the samples, resulting in extended evaporation times.

	% recovery[a]	rate constant[b]	std error[b]	p-value	half life
water					
diazinon	87.2%	1.61E-01	1.98E-02	0.01	1.9
malathion	93.7%	3.57E-02	9.11E-03	0.06	8.4
chlorpyrifos	93.1%	1.02E-01	5.13E-03	0.00	3.0
permethrin	74.3%	1.18E-02	4.17E-03	0.11	25.6
cyfluthrin	82.8%	4.86E-02	1.07E-02	0.05	6.2
cypermethrin	78.1%	3.27E-02	6.92E-03	0.04	9.2
deltamethrin	107.6%	-1.51E-02	5.01E-03	0.09	n/a
orange juice					
diazinon	120.3%	4.93E-02	2.84E-02	0.22	6.1
malathion	76.2%	-4.79E-03	1.88E-02	0.82	n/a
chlorpyrifos	125.3%	2.20E-02	8.91E-03	0.13	13.7
permethrin	82.4%	2.27E-02	5.14E-03	0.05	13.3
cyfluthrin	71.3%	8.15E-03	2.26E-03	0.07	37.0
cypermethrin	70.6%	2.77E-02	5.39E-03	0.04	10.9
deltamethrin	59.8%	3.32E-03	2.30E-02	0.90	n/a
red wine					
diazinon	136.0%	1.72E-01	6.06E-02	0.22	1.8
malathion	117.4%	3.21E-02	1.23E-02	0.12	9.4
chlorpyrifos	98.7%	1.05E-01	6.41E-02	0.24	2.9
permethrin	76.5%	2.77E-02	4.33E-03	0.02	10.9
cyfluthrin	81.0%	1.49E-02	1.55E-03	0.01	20.3
cypermethrin	86.2%	1.63E-02	5.99E-03	0.11	18.5
deltamethrin	108.2%	1.84E-02	6.17E-03	0.10	16.3
white wine					
diazinon	69.7%	1.31E-01	1.49E-02	0.01	2.3
malathion	100.3%	6.37E-03	7.57E-03	0.49	47.3
chlorpyrifos	35.4%	-1.35E-03	2.22E-02	0.96	n/a
permethrin	72.4%	-4.56E-04	3.90E-03	0.92	n/a
cyfluthrin	87.4%	-1.11E-03	4.74E-03	0.84	n/a
cypermethrin	91.3%	-1.34E-03	4.66E-03	0.80	n/a
deltamethrin	115.9%	-3.71E-03	3.03E-03	0.35	n/a

Table 1: Study of degradation of insecticides in four matrices using a liquid-liquid extraction method. [a] Recovery based on average concentration from day 0. [b] Units of $^{1}/_{day}$.

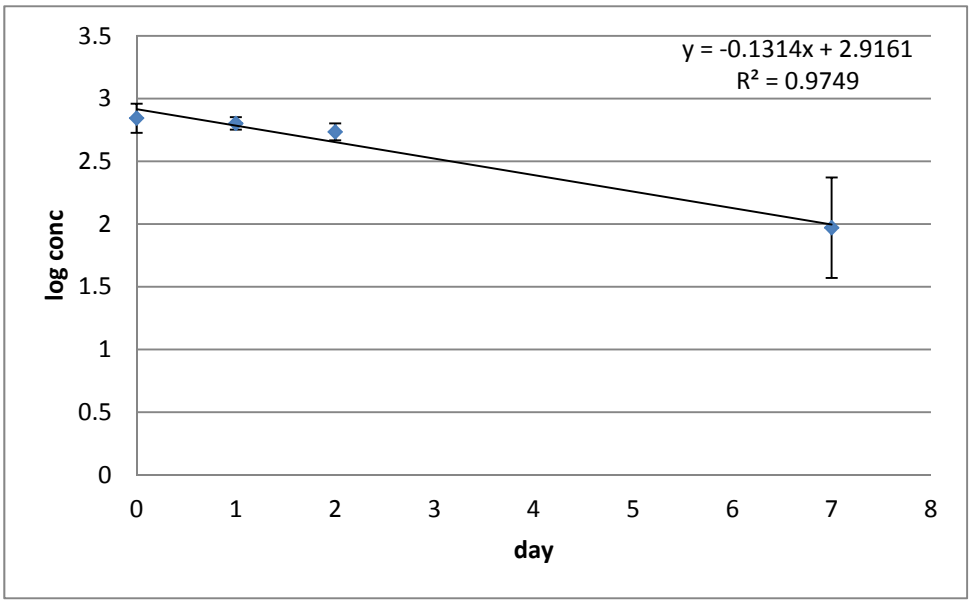


Figure 1: Degradation of diazinon in white wine by LLE.

QuEChERS analysis

In the first degradation analysis, there was no sample clean-up, leading to noisy chromatograms in red wine and dirtying of the GC column. To prevent these shortcomings, a different method known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was attempted.[112] A primary-secondary amine (PSA) clean-up of the sample was also used to reduce matrix enhancement effect by removing any fatty acids.[113]

Sample preparation

A 10mg/g stock solution of seven insecticides (diazinon, malathion, chlorpyrifos, permethrin, cyfluthrin, cypermethrin, and deltamethrin) in ACN was used to create 75mL beverage fortified with 1000ng/g insecticides. Samples were stored in amber glass jars in a refrigerator at 2.5°C.

Plan

Samples were extracted in triplicate immediately after matrix fortification and then 1, 3, 4, 5, 6, and 7 days thereafter. Solvent based calibration curves (1-1000ng/g) were used.

Extraction Procedure

2.5mL of sample and 2.5mL 1% acetic acid in ACN were added to a centrifuge tube. ACN was acidified to stabilize insecticides sensitive to base-catalyzed hydrolysis. Next, 2.0g anhydrous magnesium sulfate (a drying agent) and 0.5g sodium acetate (a buffer) were added to the tube, and tubes were gently vortexed for 1 min. The tube was then centrifuged, and the supernatant was transferred to a clean test tube. Next, 100mg PSA and 150mg anhydrous magnesium sulfate was added to the test tube and it was vortexed 1 min. The extract was then moved to a new tube, and samples were evaporated dry and reconstituted with 1mL toluene before analysis by GC-ECD as explained in the previous section.

Results and discussion

Samples from day 0 had a concentration of about half that of day 1. Further, in the pyrethroids, day 1 tended to produce an approximate 200% recovery. From that point, pseudo-first order kinetics are followed (see Figure 2). It is very likely that the samples were initially fortified, aliquots were extracted for day 0, and then samples were accidentally refortified before day 1 samples were extracted. Therefore, recovery as

shown in Table 2 is based on recovery of 2000ng/g on day 1, and regression analysis is based on days 1-7.

	% recovery[a]	rate constant[b]	std error[b]	p-value	half life
water					
diazinon	17.7%	-7.73E-03	2.43E-02	0.77	n/a
malathion	37.9%	1.48E-01	4.03E-02	0.02	2.0
chlorpyrifos	20.3%	-5.61E-02	2.54E-02	0.09	n/a
permethrin	134.7%	3.98E-02	5.12E-03	1.5E-03	7.6
cyfluthrin	131.0%	1.12E-01	1.72E-02	2.9E-03	2.7
cypermethrin	130.7%	5.21E-02	2.38E-02	0.08	5.8
deltamethrin	87.0%	9.71E-02	1.82E-02	0.01	3.1
white wine					
diazinon	10.9%	4.63E-03	5.29E-02	0.93	65.0
malathion	28.6%	7.56E-02	2.62E-02	0.04	4.0
chlorpyrifos	22.0%	-5.21E-02	4.58E-02	0.32	n/a
permethrin	106.6%	3.48E-02	1.32E-02	0.06	8.6
cyfluthrin	87.4%	5.37E-02	1.79E-02	0.04	5.6
cypermethrin	89.1%	2.46E-02	2.09E-02	0.29	12.2
deltamethrin	62.5%	6.33E-02	1.64E-02	0.02	4.8
red wine					
diazinon	12.77%	-5.47E-02	3.41E-02	0.18	n/a
malathion	27.99%	5.42E-02	1.05E-02	0.01	5.5
chlorpyrifos	21.28%	-6.50E-02	3.35E-02	0.12	n/a
permethrin	88.89%	1.52E-02	6.36E-03	0.08	19.8
cyfluthrin	66.11%	2.44E-02	5.73E-03	0.01	12.3
cypermethrin	62.69%	8.48E-03	8.30E-03	0.35	35.5
deltamethrin	41.73%	2.68E-02	7.28E-03	0.02	11.2
orange juice					
diazinon	30.25%	5.83E-02	3.18E-02	0.16	5.2
malathion	44.41%	2.02E-01	4.10E-02	0.02	1.5
chlorpyrifos	51.91%	6.26E-02	2.67E-02	0.10	4.8
permethrin	102.32%	7.22E-02	2.80E-02	0.08	4.2
cyfluthrin	84.86%	8.48E-03	8.30E-03	0.35	35.5
cypermethrin	85.96%	1.48E-01	4.09E-02	0.04	2.0
deltamethrin	113.25%	1.35E-01	3.71E-02	0.04	2.2

Table 2: Study of degradation of insecticides in four matrices using QuEChERS extraction method. [a]Recovery based on average concentration from day 1. [b]Units of $^{1}/_{day}$.

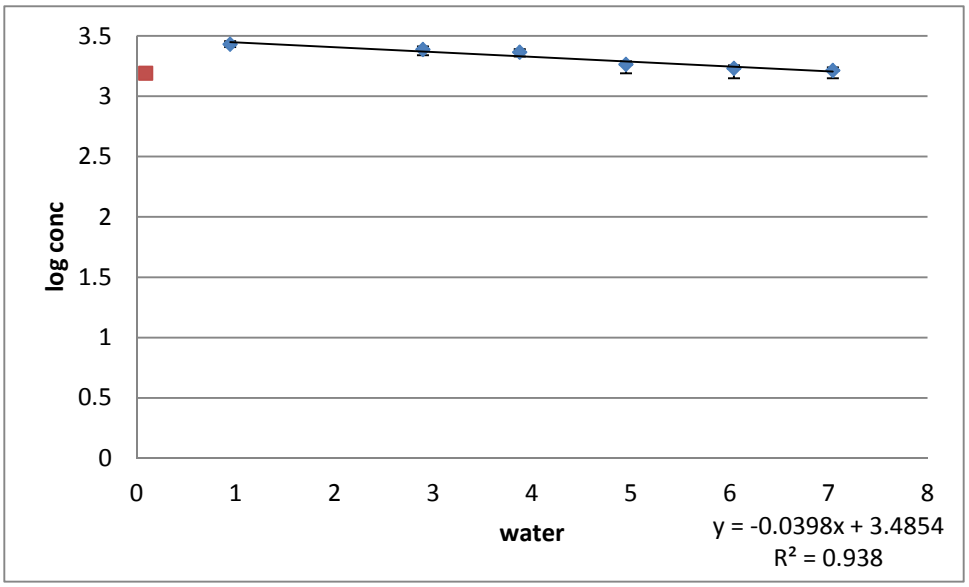


Figure 2: Degradation of permethrin in water. The data point from Day 0 is shown seperately because the beverages were erroranously refortified before extraction on Day 1.

Given the corrections explained above, recovery of all three OPs is quite low using this method (12.8-51.9%). Recovery of OPs assuming 1000ng/g on day 0 is also very low, so this phenomenon does not contridict the idea that matrices may have been fortified twice.

Significant degradation was found in all matrices for malathion and deltamethrin. All other insecticides showed significant degradation in at least one matrix except for diazinon. While this might seem suprising given diazinon's quick degradation in other experiements and the fact that is is hydrolyzed through acid catalyzation, its poor recovery likely contributes to the inability to see any trend.

Chromatograms were cleaner than with the liquid-liquid extraction method tried previously. However, the low OP recovery was unacceptable, so a method developed previously in our laboratory and optimized for use with GC-ECD was used for insecticide extraction from this point forward.[71]

Degradation of insecticides in solid food

A method previously developed in our laboratory is able to extract the seven insecticides used in this study with high recoveries across all food groups except fats (butter, oils, etc).[71] Since loss of insecticide over time had already been confirmed in liquid foods, this method was selected to study insecticide degradation in solid food. Baby food specifically was chosen because it is prehomogenized, making it easier to fortify with insecticide than less processed food. Chicken and carrots were chosen as representative meat and vegetable matrices for analysis.

Sample preparation

A 10mg/g stock solution of six insecticides (malathion, chlorpyrifos, permethrin, cyfluthrin, cypermethrin, and deltamethrin) in ACN was used to create 25g food fortified with 500ng/g insecticides. Samples were stored in amber glass jars in a refrigerator at 2.5°C.

Plan

Samples were extracted in triplicate immediately after matrix fortification and then 0.5, 1, 2, 4, 7, and 14 days thereafter. Solvent based calibration curves (1-1000ng/g) were used.

Sample extraction

A sample of 1.0g food was taken and exact weight was recorded to 0.1mg. Samples were then extracted and analyzed by GC-ECD as explained above.

Results and discussion

While recovery was acceptable for all insecticides in carrots, it was low in nearly all chicken samples (Table 3). This makes sense in light of the fact that chicken contains more lipids, which makes extraction of these somewhat non-polar insecticides more difficult.

There was no statistically significant degradation in any samples. In fact, significant gain of all pyrethroids occurs in chicken. While this is surely not due to synthesis of the insecticides, it likely that the insecticides interact with the proteins in chicken and are too strongly adsorbed to be extracted. As the proteins in the chicken also break down, they release the insecticides, which are then extractable. This hypothesis is also supported by the initial low recovery of insecticide in chicken.

	% recovery[a]	rate constant[b]	std error[b]	p-value	half life
carrots					
malathion	85.8%	-4.64E-03	3.24E-03	0.212	n/a
chlorpyrifos	61.2%	8.56E-05	7.72E-03	0.992	3516
permethrin	107.0%	-1.42E-03	4.40E-03	0.760	n/a
cyfluthrin	96.8%	-1.78E-03	3.86E-03	0.665	n/a
cypermethrin	93.6%	-2.03E-03	3.18E-03	0.552	n/a
deltamethrin	97.4%	-1.10E-03	3.68E-03	0.776	n/a
chicken					
malathion	67.5%	-9.17E-03	5.88E-03	0.180	n/a
chlorpyrifos	51.7%	-9.97E-03	7.00E-03	0.214	n/a
permethrin	80.3%	-6.65E-03	2.40E-03	0.040	n/a
cyfluthrin	80.9%	-7.18E-03	1.97E-03	0.015	n/a
cypermethrin	79.1%	-8.74E-03	2.13E-03	0.009	n/a
deltamethrin	69.3%	-8.08E-03	1.89E-03	0.008	n/a

Table 3: Study of degradation of insecticides in solid foods using Hunter SPE extraction method. [a]Recovery based on average concentration from day 0. [b]Units of $^{1}/_{day}$.

First test of degradation using MS/MS detection

While the experiment testing the adsorption of insecticides to matrix container jars helped support the fact that insecticides are truly degrading in liquid foods (see Chapter 2), the strongest support would be to observe both the degradation of insecticides and the production of degradation products. When our laboratory obtained new instrumentation, namely, a GC-MS/MS and a LC-MS/MS, new research pathways were opened. LC-MS/MS can be used to analyze insecticide metabolites in urine without derivatization of analyte.[76] This urinary method may be used to extract insecticide degradation products from beverages as well. The incubation step with *β*-glucuronidase/sulfatase is removed since there should not be in conjugation of degradants in juices. Therefore, both insecticide degradation and metabolite production may be followed simultaneously.

The insecticide metabolite stocks available in our laboratory include MDA, a metabolite of malathion, TCPy, a metabolite of chlorpyrifos, and DCCA and 3-PBA, metabolites of several pyrethroids. Dialkyl phosphates (DAPs) are also available, but they are non-specific metabolites of OP insecticides. In order to follow the degradation of each parent compound individually, malathion, chlorpyrifos, and permethrin were chosen for analysis.

Sample preparation

To allow simultaneous observation of parent compound and degradation products, 50mL of grape juice and 50mL of Milli-Q water were fortified to 200ng/g malathion,

chlorpyrifos, and permethrin using a 10ng/g stock in ACN. Samples were then stored in amber glass jars in a refrigerator at 2.5°C.

Plan

Samples were analyzed in triplicate for parent compound immediately after fortification and 0.5, 1, 2, 4, 7, 10, 14, and 15 days thereafter. Calibration curves for parent compounds were created in ACN. Similarly, samples were analyzed in triplicate for degradation products immediately after fortification and 1, 4, 7, 10, 12, 14, and 15 days thereafter. Matrix matched calibration curves were made for insecticide degradation products in water and grape juice.

Sample extraction

Parent samples were extracted and analyzed as described in the MS/MS section of Chapter 3 with one crucial difference: ISTD was not added to samples until after extraction. However, malathion was never analyzed, for reasons described in the Results and Discussion section.

Degradation product samples were extracted and analyzed the same way as described in the GC-MS/MS section of Chapter 3 with a slight difference. Instead of taking three 1.0mL samples in three test tubes and spiking them each with 50µL ISTD before loading sample, 3.0mL sample was added to a test tube and spiked with 150µL ISTD. The sample was vortexed, and then 1.05mL was added to each HLB cartridge during the loading step.

Results and discussion

Results for parent compounds are shown in Table 4. While both peaks for permethrin are quantified together through ChemStation, used in the ECD work, each peak is analyzed individually in the MassHunter software (Agilent Technologies, Santa Clara, CA, USA) for the GC-MS/MS. Recoveries for insecticides in both matrices are low, particularly for chlorpyrifos. There are two possible reasons for this problem. First, ISTD for the parent compounds was added after extraction rather than before, preventing correction for any loss during the extraction process. The need for ISTD to correct for inconsistencies in extraction is evidenced by both the scatter and standard deviation of this work (compare Figure 3 to Figure 38 in Chapter 2 for the most direct comparison with the same matrix and initial concentrations). compared to that seen in other degradation studies shown here. Second, the calibration curve for parent compound was made in solvent rather than in matrix. While this method seems acceptable for ECD work, inclusion of matrix seems to strongly effect ionization of analytes in the mass spectrometer. For this reason, future insecticide analysis by GC-MS/MS uses matrix-matched calibration curves.

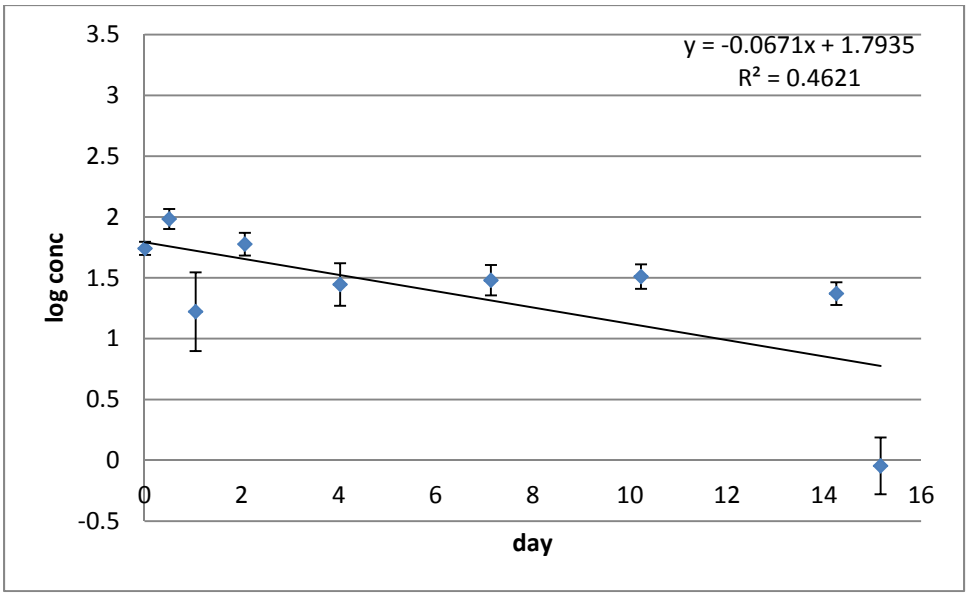


Figure 3: Degradation of chlorpyrifos in grape juice as examined by GC-MS/MS.

Half lives of both chlorpyrifos and permethrin in both water and grape juice are fairly short, particularly compared to data from other degradation studies examined here. However, it is likely that the observed rate is affected by the problems with parent analyte quantification mentioned above, therefore, these rates are likely unreliable. From a semi-quantitative point of view, though, it may be said that significant degradation of both chlorpyrifos and permethrin occur in both water and grape juice.

	% recovery[a]	rate constant[b]	std error[b]	p-value	half life
water					
chlor	22.1%	0.0578	0.0219	0.034	5.2
perm I	66.3%	0.0245	0.0028	4.59E-05	12.3
perm II	60.1%	0.0170	0.0019	4.45E-05	17.7
grape juice					
chlor	27.6%	0.0671	0.0274	0.044	4.5
perm I	45.6%	0.0235	0.0092	0.037	12.8
perm II	42.3%	0.0263	0.0084	0.016	11.5

Table 4: Study of degradation of insecticides in water and grape juice using GC-MS/MS. [a]Recovery based on average concentration from day 0. [b]Units of $^{1}/_{day}$.

Results for degradation products are shown in Table 5. Because it was already decided that these data could not be used for anything but an appendix, blank samples of water and grape juice unfortunately were not tested for insecticide degradation product. Therefore, it cannot be determined whether there is a difference between blank insecticide degradation values and those after fortification with parent insecticide. However, significant production of TCPy is seen in both water and grape juice. Interestingly, this correlates with the fact that the shortest half life seen in this data set is for chlorpyrifos.

	rate constant	std error	p-value
water			
MDA	0.0010	0.0019	0.627
TCPy	0.0265	0.0058	0.004
3-PBA	0.0088	0.0057	0.171
grape juice			
MDA	0.0021	0.0025	0.433
TCPy	0.0230	0.0056	0.006
3-PBA	n/a	0.0096	0.766

Table 5: Study of degradation of insecticides in water and grape juice using LC-MS/MS. Units for the rate constant and standard error are $^{1}/_{\text{day}}$.

Repeat test of degradation using MS/MS detection

While the data set obtained previously to this one is examined in Chapter 2, it was decided that one more degradation analysis should be done multiple reasons. Initially, it was thought that an error in the data was evident with TCPy, so the experiment was repeated. In the second experiment, this pattern was repeated, suggesting a two-phase degradation-production-degradation process. Secondly, given that our laboratory is particularly interested in the risks of children's exposures to insecticides, it seemed to

make sense to test degradation of insecticides in a wider variety of juices rather than in wines. These data should be considered semi-quantitative in that some experimental parameters, e.g., GC column conditions, were not optimum. They are, nonetheless, of interest, and are included as part of hypothesis generation. However, before this degradation study was undertaken, the LC-MS/MS analysis of degradation products in orange juice was tested to confirm that presence pulp does not adversely affect final concentration results.

Comparison of recoveries in orange juice with washed vs. unwashed pulp

The calibration curves for pesticide metabolites (MDA, TCPY, cis/trans DCCA, and 3-PBA) seem to have different slopes in orange juice than in other liquid beverage matrices. To assure that degradate adsorbed to orange juice pulp did not affect results, a test was done to compare results when pulp was washed with Milli-Q water and loaded onto the column.

Four test tubes were filled with 1mL orange juice (low pulp) and spiked to 5 ng/mL of metabolite standard and 50 ng/mL ISTD, while four other test tubes were filled with 1mL orange juice and spiked to 50 ng/mL of metabolite standard and 50 ng/mL ISTD. Of each spiking level, two tubes were designated to be washed and two were designated not to be washed. Each tube was vortexed briefly then centrifuged for 5 minutes. The unwashed samples were processed as previously described previously, with the orange juice supernatant being loaded onto the Oasis column. For the samples designated for washing, 2 mL water was added to tubes after loading of supernatant. The pulp pellet was gently pipeted up and down to facilitate mixing into the water before the

tubes were vortexed 3 min. The tubes were then centrifuged for 5 min again and then the liquid was added to the cartridge. The method then continued as normal.

As shown in Table 2, there are no significant differences between washed and unwashed samples (p = 0.05). While this may be because nearly all analyte is in the liquid part of the orange juice rather than the pulp, it is more likely due to the presence of ISTD correcting any loss of metabolite during the extraction process. For example, TCPy has the lowest p-value (largest difference between washed and unwashed samples), likely because it is the only analyte without a matched isotopic ISTD. Significant concentrations of metabolites were not found in blanks. Recoveries were between 80-120% except in the cases of the 3-PBA at the high spike concentration.

		washed	not washed	p-value
MDA-Q	low	4.45	4.43	0.979
	high	52.54	53.12	0.798
TCPY-Q	low	4.62	4.06	0.091
	high	40.83	45.23	0.104
DCCA-Q	low	4.12	4.76	0.077
	high	42.89	45.06	0.165
3-PBA-Q	low	3.83	3.76	0.577
	high	34.91	36.2	0.418

Table 6: Affect of washing orange juice pulp on metabolite recoveries. Concentrations are presented in ng/g. Low fortification is at 5 ng/mL; high fortification is at 50 ng/mL. A t-test assuming unequal variances was used.

Sample preparation

To allow simultaneous observation of parent compound and degradation products, 50mL each of white grape juice, red grape juice, apple juice, and orange juice were fortified to 500ng/g malathion, chlorpyrifos, and permethrin using a 10ng/g stock in ACN. Samples were then stored in amber glass jars in a refrigerator at 2.5°C.

Plan

Samples were analyzed in triplicate for parent compound immediately after fortification and 0.5, 1, 3, 7, 11, 14, and 15 days thereafter. Blanks and matrix matched calibration curves for parent compounds were extracted on day 4. Similarly, samples were analyzed in triplicate for degradation products immediately after fortification and 1, 4, 7, 10, 12, 14, and 15 days thereafter. Blanks and matrix matched calibration curves for degradation products were extracted on day 5. For both parent compound and degradation products, red and white grape juices were combined for one grape calibration curve.

Sample extraction

Both parent insecticides and degradation products were extracted and analyzed as explained in the MS/MS section of Chapter 2.

Results and discussion

Parent compound results are show in Table 7. Unfortunately, sub-optimal parameters, e.g. poor column conditioning which produced tailing, led to less reliable results for both recoveries and rate constants. While chlorpyrifos demonstrated significant degradation in all matrices except red grape juice, malathion degraded significantly in only apple juice, and permethrin actually showed a significant increase in concentration in both red grape juice and orange juice. Again, these results may be related to peak tailing and larger variances in calibration points. However, the pulp in

orange juice may also initially adsorb permethrin and then release it over time, allowing it to be extracted.

	% recovery[a]	rate constant[b]	std error[b]	p-value	half life
apple juice					
mal	93.50%	1.88E-03	6.51E-04	0.03	160.3
chlor	104.34%	1.08E-02	1.09E-03	6.06E-05	27.8
perm I	86.76%	-4.01E-03	2.37E-03	0.14	n/a
perm II	86.13%	-1.16E-03	2.57E-03	0.67	n/a
white grape juice					
mal	93.50%	-1.01E-03	7.71E-04	0.24	n/a
chlor	118.88%	1.14E-02	1.78E-03	6.67E-04	26.3
perm I	124.43%	-1.45E-04	2.97E-03	0.96	n/a
perm II	120.07%	-4.74E-04	2.92E-03	0.88	n/a
red grape juice					
mal	97.66%	-8.26E-04	7.92E-04	0.34	n/a
chlor	111.65%	2.07E-03	1.21E-03	0.14	145.5
perm I	67.53%	-6.99E-03	2.39E-03	0.03	n/a
perm II	64.39%	-5.99E-03	2.35E-03	0.04	n/a
orange juice					
mal	87.31%	-4.85E-03	4.11E-03	0.29	n/a
chlor	120.59%	2.90E-03	1.10E-03	0.04	103.6
perm I	104.96%	-2.07E-02	2.90E-03	3.86E-04	n/a
perm II	115.44%	-2.16E-02	3.41E-03	7.32E-04	n/a

Table 7: Study of degradation of insecticides in juices using GC-MS/MS. [a]Recovery based on average concentration from day 0. [b]Units of $^{1}/_{day}$.

Concentrations of insecticide degradation products in juices are seen in Table 8. While a background level of 3-PBA was seen in all samples, likely due at least in part to contamination of native compound in the ISTD, no TCPy is seen in any samples, and MDA is found only in white grape juice.

	MDA	TCPy	3-PBA
apple juice	0	0	0.12
white grape juice	0.56	0	0.13
red grape juice	0	0	0.16
orange juice	0	0	0.17

Table 8: Blank concentrations of degrdates in juices in ng/mL.

As seen in other degradation analyses including degradates, significant production of MDA is seen in all juices except for orange juice, where MDA could not be analyzed because of interfering peaks (Table 9). As also seen in other studies, there is no statistical difference between 3-PBA concentrations in blank samples or those fortified with permethrin.

	blank conc[a]	rate constant[b]	std error[b]	p-value
apple juice	0	1.65E-03	1.91E-04	3.44E-04
white grape juice	0.564395	1.85E-03	3.11E-04	1.92E-03
red grape juice	0	1.67E-03	3.86E-04	7.53E-03

Table 8: Kinetics for production of MDA in juices. Concentration in ng/mL.

Perhaps the most interesting part of this study is the fact that the unusual production-loss-production pattern happens for TCPy, just as seen in the data used in the MS/MS section of Chapter 2 (Figures 4-6). While each of these three matrices produce this pattern for TCPy, they each occur at different ranges of concentration. TCPy concentrations are highest in red grape juice (~21ng/mL), followed by apple juice and then white grape juice (~3ng/mL).

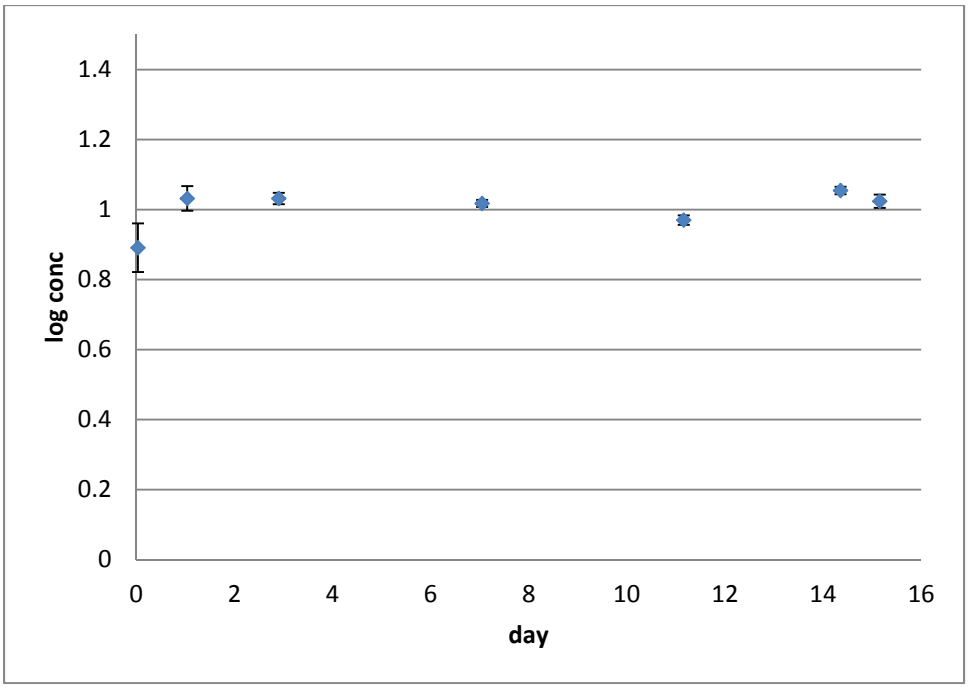


Figure 4: TCPy in apple juice.

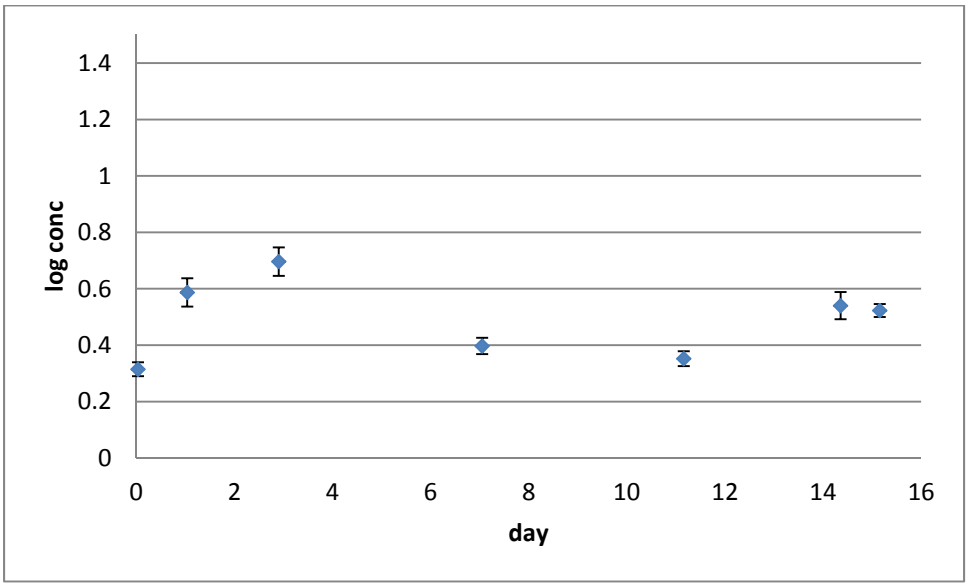


Figure 5: TCPy in white grape juice.

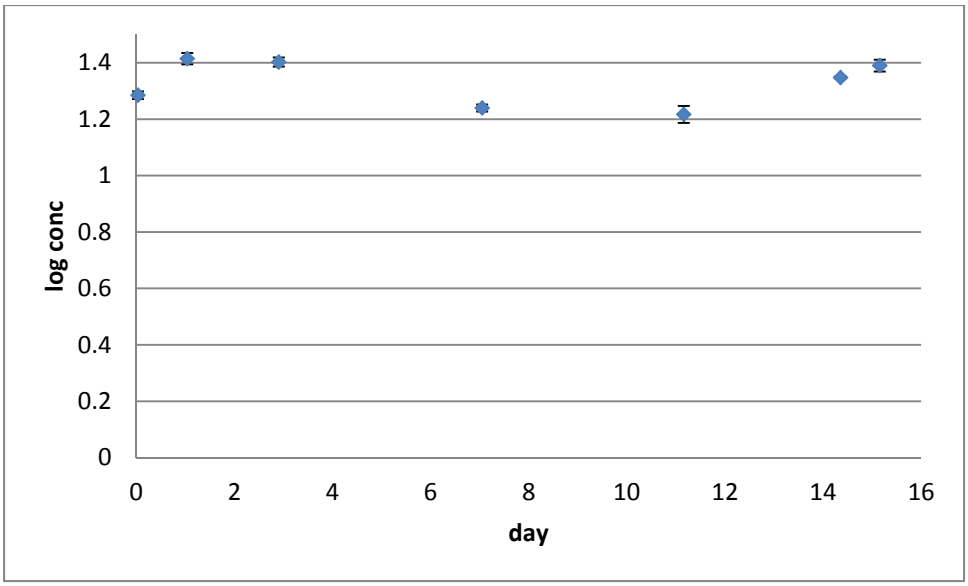


Figure 6: TCPy in red grape juice.

This "production-loss-production" pattern of TCPy concentration is not observed in orange juice. This fact is somewhat surprising given that orange juice is the most complex of the matrices studied; therefore, one might expect it to show the most complex kinetics. However, TCPy in orange juice demonstrates statistically significant (p = 3E-4) pseudo-first order production instead (Figure 7).

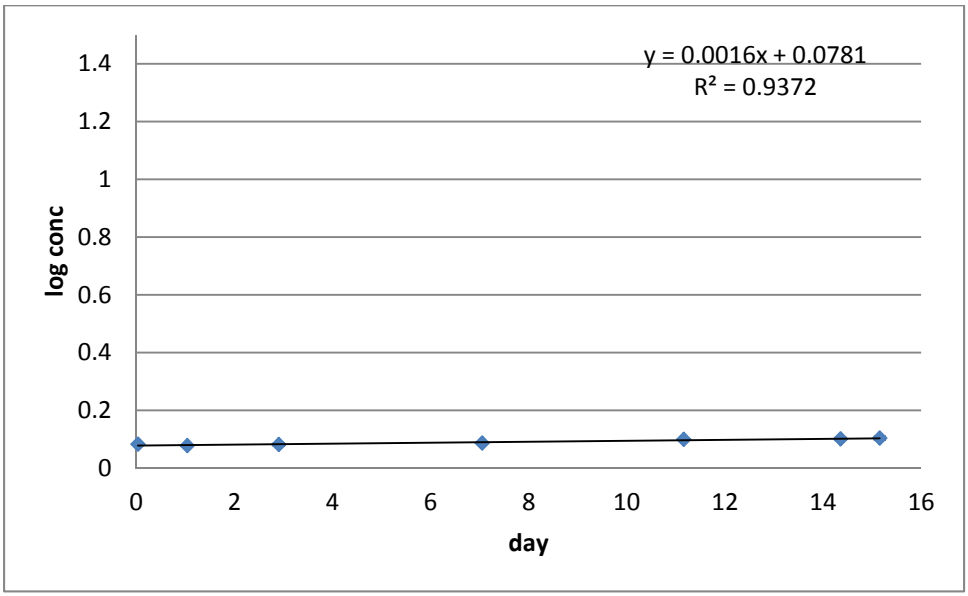


Figure 7: TCPy in orange juice. Note the lack of "production-loss-production" pattern.

Conclusions

Many extraction and chromatographic procedures have been used to study the degradation of insecticides in food. The method developed by Hunter[71] seems to be optimal for insecticide extraction and clean-up, particularly for GC-ECD based chromatography. While solvent-based calibration curves without the use of ISTD result in acceptable (80-120%) recoveries of insecticides for GC-ECD work, matrix-matched calibration curves and use of ISTD are needed for GC-MS/MS.

While degradation seems difficult to follow in solid food, there is support for the hypothesis that many OP and pyrethroid insecticides degrade in fruit juices and wines. Not only is the statistically significant degradation of insecticides followed over time, but the production of two degradation products, MDA and TCPy, are also followed. These data support the contention that more should be understood about people's, particularly children's, exposure to insecticide degradation products and its relation to the use of urinary insecticide metabolites as biomarkers of exposure to insecticides.

CHAPTER 4: STUDY OF INSECTICIDES AND INSECTICIDE DEGRADATES IN BABY FOOD

Hypothesis

Both insecticides and their degradates area found in processed foods such as baby foods.

Introduction

Urinary insecticide metabolites have been used for years as biomarkers of insecticide exposure.[37, 57, 70, 75] When these metabolites are used, particularly for non-persistent insecticides, it is assumed that there is a one-to-one ratio between insecticide ingested and metabolite output. If insecticides degrade on food, though, they would likely produce degradates identical to urinary metabolites. If these degradates are absorbed by the body and then excreted through the urine unchanged, there is a chance that insecticide exposure will be overestimated.

There are few studies on whether there are insecticide degradation residues in food, but most of them seem to center on organophosphorus (OP) insecticides. As part of the study on OP degradation in juices, Lu et al also tested blank fruit juice for dialkyl phosphates (DAPs), nonspecific OP degradates.[38] The DAP and OP content of fresh produce has also been evaluated.[52] Some specific OP degradates, such as 3,5,6-trichloro-2-pyridinol (TCPy) and 2-isopropyl-6-methyl-4-pyrimidinol, degradation products of chlorpyrifos and diazinon respectively, have been analyzed in duplicate diets of preschoolers.[36, 77] Degradation products were found in all of these media, affirming the need for more research in this area.

Although pyrethroids have become the preferred insecticide class since OP use was limited after the Food Quality Protection Act of 1996 (FQPA), there has been almost no research done on the degradation of these insecticides in food. The only such research

found examines the dissipation of pyrethroids in vegetables by observing loss of parent compound.[78] No data is offered concerning production of degradation products. Again, the lack of data points to a need for more research in this area.

As recognized in the FQPA, children's particular risk factors make understanding of their insecticide exposure particularly important. Children's physiological traits, such as their high metabolic rate, immature neurological system, lower detoxifying enzymatic activity, and small stature (which places them closer to the ground where pesticides may settle) put them at higher risk for long-term damage from insecticide exposure. [36, 37, 41] Their behavioral tendencies, such as preference for foods which tend to contain higher amounts of insecticides, hand-to-mouth activity, and length of time spent outdoors, also puts them at risk for greater insecticide exposure.[36, 39] There have been many studies of children's exposures to insecticides.[4, 37, 75, 114-115] These studies often measure amount of insecticide in the environment and compare this quantity to amount of insecticide metabolite in children's urine. However, few studies were found that addressed the possibility that children may be exposed to the degradation product itself, possibly confounding correlations between insecticide exposure and urinary metabolite output.[36, 77]

For these reasons, we carried out a study of insecticides and their degradation products in baby food. Pyrethroid, organochlorine (OC), and OP insecticides were analyzed by GC-MS/MS, and malathion, another OP, was analyzed by LC-MS/MS. OC degradation products were also analyzed by GC-MS/MS, while two OP and two pyrethroid degradation products were analyzed by LC-MS/MS.

While data specifically correlating to insecticide content in baby food were not found, data was found for five of the chosen six fruits and vegetables from the United States Department of Agriculture Pesticide Data Program (PDP) (Table 1).[102, 116] The PDP was implemented in 1991 to test foods for pesticide residues, and it has been used as a dietary assessment tool for the FQPA since 1996.[102]

	Apples	*Bananas*	*Pears*	*Green Beans*	*Carrots*
chlorpyrifos	0.9	0.4	0.9	1.1	0.1
cypermethrin	0	0	0	0.9	0
diazinon	8.5	0	0.2	0	0
DDE *p,p*	0	0	0	1.4	28.1
DDT *p,p*	0	0	0	0	0.3
dicofol	0.1	0	0.1	0	5.2
endosulfan α	6.9	0	0.3	15.2	0
endosulfan β	12.7	0	0.7	7.8	0.4
endosulfan sulfate	0	0	0	22.2	1.6
heptachlor	0	0	0	0	100
malathion	0	0	0.5	0	0
permethrin	0.1	0	0.1	0	0.5
piperonyl butoxide	0.1	0	0	0	0

Table 1: Frequency of detection in percentage of selected insecticides in fruits and vegetables found by the Pesticide Data Program. [102, 116] LODs were on the order of ng/g. Data on apples and pears were obtained in 2009, while other data were obtained in 2007. It should be noted that only one sample was tested for heptachlor in carrots, thus yielding the 100% recovery.

Methods

Reagents and materials

Acetonitrile (HPLC grade), toluene (Chromosolv grade), methanol (HPLC grade), and glacial acetic acid were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). NaCl (ACS grade) was obtained from EMD (Gibbstown, NJ, USA). Water used was purified in-house to 18.2 MΩ·cm with a Milli-Q® water system (Millipore, Billerica, MA,

USA). Supelclean™ ENVI-CARB-II/PSA SPE cartridges (Bed A: 500mg ENVI-CARB; Bed B: 300mg primary secondary amine, PSA) were purchased from Sigma-Aldrich, Inc (Bellefonte, PA, USA). ENVI-CARB is graphitized carbon black, which has a strong affinity for organic polar and non-polar compounds in reversed-phase conditions. In particular, the hexagonal ring structures retain planar compounds, such as pigments and sterols, from fruits and vegetables.[103] Supelclean PSA is a polymerically bonded phase containing primary and secondary amines and has a strong affinity for more polar sugars, fatty acids, and organic acids.[103] Oasis HLB extaction cartridges (200mg, 6mL) were purchased from Waters Corporation (Millford, MA).

Purchase of baby food

Three of the most popular baby food vegetables (peas, green beans, and carrots) and the three most popular baby food fruits (apples, bananas, and pears) were obtained from local grocery stores.[80] Two brands were tested, but both conventional and organic versions of one brand were used.

Standards

Pesticide and pesticide metabolite standards were obtained from the National Center for Environmental Health, Center for Disease Control and Prevention (CDC, Atlanta, GA) or Crescent Chemical (Islandia, NY). Standards were made from 1-500 ng/g of insecticides listed in Table 2. Isotopically labeled internal standards (cypermethrin $^{13}C_4$, diethyl parathion D_{10}, chlorpyrifos D_{10} chlorpyrifos methyl D_6, and

p,p-DDE $^{13}C_{12}$) at 200 ng/g were made in 25% toluene in ACN for compounds analyzed by GC-MS/MS.

A stock containing 10mg/g malathion in ACN was used to create standard dilutions from 5-1000 ng/g, and these diluted standards were used to create calibration curves. An isotopically labeled standard containing 1000 ng/g malathion D_{10} in ACN was used as ISTD.

A stock containing 10 mg/g MDA, TCPy, DCCA, and 3-PBA in ACN was used to create standard dilutions from 5-1000 ng/g. These dilutions were used to create calibration curves. Isotopically labeled standards (MDA-D_6, DCCA-$^{13}C_3$, and 3-PBA-$^{13}C_6$) of 1000 ng/g were made in ACN.

Parent compound and DDE extraction method

Each baby food was extracted as follows. Malathion was extracted in the same manner as the other parent compounds, but a separate set of extracts were used so samples wouldn't require splitting or solvent exchange for LC analysis. First, 5.0g baby food was weighed to the nearest 0.1mg and added to a trace-cleaned 15mL conical centrifuge tube. Next, 50µL ISTD was added. The tube was vortexed briefly before the addition of ~2g NaCl. Then, 5 mL acetonitrile (ACN) was added to the sample. The tube was shaken by hand before being vortexed for 5 minutes and centrifuged for 10 minutes at 1200rpm. An ENVI-CARB-II/PSA cartridge was preconditioned with 5 mL of a 25% solution of toluene in ACN. After preconditioning, the sample supernatant was loaded onto the cartridge. Then, 10 mL of the ACN/toluene solution was eluted through the cartridge, and the elutant was collected in a trace-cleaned 15 mL centrifuge tube. The

sample was evaporated at 20 PSI and 38°C to near-dryness. After evaporation, the cartridge was eluted into the tube again using 10 mL of the ACN/toluene solution. The sample was evaporated again at 20 PSI and 38°C to dryness. Samples were capped and stored dry until analysis. Parent compounds analyzed by GC-MS/MS were reconstituted with 50µL 25% toluene in ACN, while malathion was reconstituted with 100µL 30% methanol in water.

Degradation product extraction

Each baby food was extracted as follows. First, 1.0mL of baby food (weighed to the nearest 0.1mg) and 50µL ISTD were added to a trace-cleaned conical centrifuge tube and vortexed briefly. Then, 2.0mL Milli-Q water was added to the test tube. The sample was vortexed 3 min and centrifuged for 10 min at 1200rpm. During this time, an Oasis HLB cartridge was set up on a vacuum manifold and preconditioned with 3.0mL methanol (MeOH) and then 3.0mL 1% acetic acid in H_2O. The water extract from the sample was filtered through a Bond Elut Resivoir cartridge (Agilent Technologies, Santa Clara, CA) and then loaded onto the cartridge. It was then washed twice with 2 mL 1:5:94 acetic acid:MeOH:H_2O. The cartridge was then dried under vacuum. A test tube was placed under the cartridge in the manifold and the sample was eluted twice with 2.5mL methanol. Vacuum was required to begin to pull down the sample, but it was removed as soon as the cartridge started eluting. The sample was evaporated to dryness at 15 psi and 45°C and then capped and stored dry until analysis. The sample was reconstituted with 100 µL of 30:70 MeOH:H_2O. The reconstituted sample was then

vortexed briefly and centrifuged for 3 min before being transferred to a LC vial for analysis, leaving behind any residual particulate matter.

Quantification of compounds in food samples

Linear matrix-matched calibration curves were made for malathion, degradation products, and compounds analyzed by GC-MS/MS. Fruit calibration curves were made using a mixture of bananas, apples, and pears, while vegetable calibration curves were made with a mixture of carrots, peas, and green beans. All calibration curves were made using 1.0g food. The insecticide calibration curve contained 9 points from 0.1ng/g to 50ng/g (which became equivalent to 0.02ng/g-10ng/g when compared to samples extracted from 5g food), while the degradation product calibration curve contained 9 points from 0.25ng/g to 50ng/g.

GC-MS/MS analysis of parent compounds

For GC-MS/MS analysis of parent insecticides, an Agilent 7890 GC with MS/MS detection equipped with positive mode electron impact (EI) ionization was used. An Agilent HP-5MS [(5% phenyl)-methylpolysiloxane, 30m x 250 μm x 0.25μm] column was used for separation. The injection port was held at 250°C, injection was 1μL (splitless), and a gooseneck injection liner with glass wool and 1m deactivated silica guard column was used. The carrier gas was He at a flow rate of 50mL/min, and the column temperature profile was as follows: The column was held at 100°C for 2 min before linear heating by 10°C/min to 205°C. This temperature was held for 3 min before linear heating by 10°C/min to 280°C. This temperature was held for 4 min before linear

heating by 25°C/min to a final temperature of 310°C. This final temperature was held for 12 min. Analysis was performed in MRM mode, monitoring two characteristic parent ions for each compound.

Compound	Precursor ion	daughter ions (Q, C)	CE(V) (Q, C)	RT (min)
native				
diazinon	304.3, 179.3	179.3, 121	15, 40	12.79
chlorpyrifos methyl	288.2, 286.2	93, 93.2	20, 26	13.95
chlorpyrifos	314.2	286.1, 258	5, 25	15.44
parathion	291.3	90.9, 81	35, 40	15.46
dicofol *p,p-*	139.1	111.1, 75.1	15, 30	15.52
heptachlor epoxide	353.1	282.1, 263.1	15, 10	16.52
DDE *o,p-*	248.2, 246.2	176.3, 176.2	30, 35	17.41
endosulfan α	241.1, 239.1	206.1, 204.1	20, 15	17.61
DDE *p,p-*	248.2, 246.2	176.2, 176.2	30, 35	18.31
endosulfan β	241.1, 239.1	206.1, 204.1	20, 15	19.17
DDT *o,p-*	235.2	199.1, 165.1	15, 25	19.46
prallethrin	123.2	87.1, 105.2	15, 20	19.47
permethrin	183.2	153.2, 77	15, 40	22.44, 23.59
cyfluthrin	163.1, 206.2	127.2, 151.1	5, 25	24.19, 24.31, 24.46
cypermethrin	163.1, 181.2	127.1, 152.2	5, 25	24.62, 24.75, 24.87
fenvalerate	167.2, 125.2	89.2, 89	40, 20	26.09, 26.46
deltamethrin	253.1, 181.2	93.2, 152.2	20, 30	27.10, 27.49
ISTD				
chlorpyrifos methyl	291.9	274, 99	30, 25	13.2
chlorpyrifos	324	292, 260	10, 20	14.4
parathion	301	115	15	15.46
DDE *p,p-*	258	188	40	16.0

Table 2: GC-MS/MS parent and daughter ions. Permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin were detected as multiple peaks because of stereoisomers.

LC-MS/MS analysis of malathion

Because the potential for thermal degradation makes malathion difficult to analyze by GC, an Agilent 6460 Triple Quad LC-MS/MS equipped with a positive mode electrospray ionization (ESI) interface was used to analyze samples. A BetaSil C18 column (3.0 × 100mm, 3.0µm particle size, Thermo Scientific) was used for analysis and kept at 45°C. Solvent A was H_2O with 1% acetic acid and solvent B was MeOH with 1% acetic acid, and the flow rate was 0.7mL/min (Table 3). The following parameters were used: the source temperature was 300°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 45psi, and the corona voltage was 3500V. Ions analyzed in MRM mode and their optimized fragmentor and collision energies are shown in Table 4.

minute	% B	flow rate (mL/min)
0	30	0.7
4	100	0.7
6	100	0.7
6.5	30	0.7
8.5	30	0.7

Table 3: Solvent profile for malathion analysis.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)
Mal ISTD	341	100.1	90	21
Mal-Q	331	127	60	5
Mal-C	331	99	60	21

Table 4: Parent and daughter ions for malathion.

Metabolite analysis by LC-MS/MS

An Agilent 6460 Triple Quad LC-MS/MS equipped with a negative mode ESI interface was used to analyze samples. A Zorbax Eclipse Plus Phenyl-Hexyl column (3.0 × 100mm, 3.5µm particle size, Agilent, USA) was used for separation and held at 45°C. Solvent A was H_2O with 1% acetic acid and solvent B was MeOH with 1% acetic acid, and the flow rate was as shown in Table 5. The following parameters were used: the source temperature was 250°C, the vaporizer gas flow (N_2) was 5 L/min, the nebulizer gas flow was set to 35psi, and the corona voltage was 3500V. Each metabolite was matched to its own isotopically labeled internal standard except for TCPy, whose internal standard was isotopically labeled DCCA. Ions analyzed in MRM mode and their optimized fragmentor and collision energies are shown in Table 6.

minute	% B	flow rate (mL/min)
0	30	0.8
1.5	35	0.8
3	50	0.8
8	60	0.8
8.8	100	1
10	100	1
11	80	1
13	80	1

Table 5: Flow rate through column for LC-MS/MS degradate separation. Solvent A is 0.1% acetic acid in water, while solvent B is 0.1% acetic acid in methanol (MeOH).

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)	RT (min)
MDA-ISTD	280	147	80	1	3.3
MDA-Q	273	141	80	1	3.9
MDA-C	273	157	80	12	3.9
TCPy-Q	198	198	96	0	7.3
TCPy-C	196	196	96	0	7.3
DCCA-ISTD	210	210	90	0	6.7
DCCA-Q	207	207	90	0	8.2
DCCA-C	209	209	90	0	8.2
3-PBA-ISTD	219	99	98	20	7.4
3-PBA-Q	213	93	122	16	9.0
3-PBA-C	213	169	122	8	9.0

Table 6: Parent and daughter ions for degradates. Fragmentor energies, collision energies, and retention times are listed.

Method Validation

Limits of detection. Limits of detection were calculated using replicates of blanks and low spiked samples as the lowest analyte concentration at which reliable detection is feasible. If analyte was detected in blank samples, the LOD was calculated according to the following equation: $LOD = mean_{blank}+3(SD_{blank})$ where at least 10 replicate injections were used. If no analyte was found in blank samples, then the LOD was extrapolated based on a signal-to-noise ratio of three.

Accuracy and precision. Method accuracy and precision were determined by measurements of fortified samples at two concentrations (10 and 25ng/g for degradates by LC-MS/MS, 2 and 5ng/g for all others). Spiked samples were then extracted and analyzed alongside other samples. Percent recovery was calculated based on the fraction of observed concentrations to nominal spiked concentrations.

Test for analytic degradation. To test for analytic degradation in baby food fruits and vegetables and to refute the argument that degradation products were not present in

samples until insecticides were hydrolyzed during extraction and/or analysis, six samples of 1.0g carrots and six samples of 1.0g apples were obtained. For each matrix, three of the samples were fortified with 25ppb pesticide sample, and all samples were fortified with metabolite ISTD. The samples were then extracted and analyzed according to the insecticide metabolite procedure.

Results

Tables 7 and 8 show percent recoveries of compounds in vegetables and fruits, respectively, analyzed by GC-MS/MS and of malathion, and Table 9 shows both recovery precision and LODs stratified into fruits and vegetables.

	Green Beans		*Peas*		*Carrots*	
	QCL	**QCH**	**QCL**	**QCH**	**QCL**	**QCH**
dia	113.4	82.9	68.5	86.9	153.7	143.8
chlm	87.5	92.6	239.7	60.7	92.4	95.8
cpy	103	102.1	105.8	103.4	103.8	105.7
par	104.6	101.6	95	100	105.4	107.6
mal	98.9	106.1	72.3	82.3	124.8	117.4
dic	99.5	81.7	73.8	79.7	101.1	98.8
hep	112.9	98.1	83.4	101	142.9	125.5
DDE op	101.5	102.5	94.4	102.5	114.8	107.7
endo A	113.8	98.6	90.7	102.8	116.7	107.3
DDE pp	99.3	95.3	102.7	97.8	97.7	91.7
endo B	104.3	121.2	142.5	130.2	112.3	105.2
DDT op	89.2	102.1	103.5	93.8	90.9	89.2
pral	116.8	134.2	168.7	164.7	123.4	123.6
per I	46.2	43.9	65.3	62.1	44.2	34.3
per II	60.5	62.5	75.9	71.8	57.9	58.9
cyf I	55	49.9	31.2	17.4	71.5	74.5
cyf II	82.9	79.1	77.4	78.8	80.6	79.3
cyf III	80.5	86.5	82.7	82.9	89.8	81.8
cyp I	75.8	75.1	31.2	17.4	81.1	80.2
cyp II	94.6	90.8	93.2	91.3	91.5	89.9
cyp III	94.8	92.1	93.5	93.4	93.9	88.5
fev I	125.5	128.5	123.5	124.2	123.4	127.8
fev II	130.8	134.8	139.2	142.6	145.2	151.8

Table 7: Percent recovery of spiked analytes in vegetables. Low spike is 2ng/g; high spike is 5ng/g. Permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin are detected as multiple peaks due to multiple stereomers. Abbreviations match full names in Table 2 with the exception of mal = malathion.

	Apples		*Bananas*		*Pears*	
	QCL	**QCH**	**QCL**	**QCH**	**QCL**	**QCH**
dia	85.4	94.1	76.2	104.1	92.8	79
chlm	58	64.2	101.6	61.5	62.5	62.6
cpy	41.4	46	80.9	47.9	47.3	46.7
par	59.1	64.4	79.6	69.9	66.2	67.1
mal	100.6	93.9	102.6	99.9	68.3	85.8
dic	88.6	92.9	100.2	107.1	104.8	97.8
hep	76.7	81.1	169.8	83.1	83.9	82.6
DDE op	108.7	119.3	109.3	119.5	115.7	114.6
endo A	49	53.9	110.5	55.4	57.3	57.3
DDE pp	78.9	83.5	96.8	88.1	85.7	84
endo B	60.4	69.8	115.5	50.9	59.6	64.7
DDT op	92.5	107.5	112.1	89.5	88.1	92.6
pral	103	123.7	96.6	120.1	105.2	108.1
per I	153.7	156.9	86.9	292.7	88.8	85.6
per II	143.3	152.6	83.4	338.8	140.1	131.9
cyf I	91.1	100.2	96.5	88.8	92.6	88.5
cyf II	90.8	99.6	96.4	72.6	93.9	92.5
cyf III	97.5	110.6	104.7	98.4	104	102.6
cyp I	95.6	105.8	96.5	88.8	108.8	105.6
cyp II	88.4	96.3	94.3	94.2	97	93.2
cyp III	91.3	104.2	97.7	115.6	99.4	105.5
fev I	95.3	105.8	100.5	221.8	119.8	114.3
fev II	100.9	117.1	106.4	235.5	135.5	132.2

Table 8: Percent recovery of spiked analytes in fruits. Low spikes are 2ng/g; high spikes are 5ng/g.

	Vegetables			Fruits		
	QCH	**QCL**	**LOD**	**QCH**	**QCL**	**LOD**
dia	38.10%	32.60%	0.145	9.90%	13.70%	0.046
chlm	61.80%	23.40%	0.126	32.40%	2.20%	0.106
cpy	1.40%	1.70%	0.281	37.70%	2.00%	0.176
par	5.70%	3.90%	0.403	15.20%	4.10%	0.656
mal	26.60%	17.60%	2.8	21.30%	7.60%	3.6
dic	16.70%	12.10%	0.010	8.60%	7.30%	0.292
hep	26.30%	13.90%	0.455	47.00%	1.30%	0.870
DDE op	10.00%	2.90%	0.031	3.50%	2.30%	0.129
endo A	13.30%	4.30%	0.042	46.10%	3.10%	0.039
DDE pp	2.60%	3.20%	0.020	10.40%	3.00%	0.020
endo B	16.80%	10.70%	0.064	40.80%	15.90%	0.040
DDT op	8.30%	6.90%	0.018	13.10%	10.00%	0.027
pral	20.70%	15.10%	3.246	4.40%	7.00%	0.436
per I	22.40%	30.20%	0.076	34.60%	59.00%	0.118
per II	15.00%	10.30%	0.222	27.60%	54.90%	0.074
cyf I	38.50%	60.60%	0.051	3.00%	7.20%	0.044
cyf II	3.50%	0.30%	0.113	3.00%	15.90%	0.068
cyf III	5.70%	2.90%	0.029	3.90%	5.90%	0.029
cyp I	43.70%	60.50%	0.04	7.30%	9.70%	0.040
cyp II	1.60%	0.80%	0.094	4.70%	1.70%	0.078
cyp III	0.70%	2.80%	0.152	4.40%	5.80%	0.054
fev I	0.90%	1.80%	0.026	12.30%	43.90%	0.067
fev II	5.20%	6.00%	0.033	16.30%	39.90%	0.066

Table 9: Precisions of GC-MS/MS recoveries in vegetables and fruits presented as RSDs and LODs presented in ng/g.

As shown in Table 10, relative recoveries were between 80-120% for all LC-MS/MS samples except TCPy in fruit. Table 11 summarizes LC-MS/MS method performance by showing average relative recovery, relative standard deviation, and limits of detection stratified by fruits and vegetables. All RSDs are under 15% with the exception of MDA. Concerning the possibility of analytic degradation, it was determined that there was no significant difference between insecticide degradate concentrations

between samples that were or were not fortified with parent compound using a two-tailed *t*-test ($p = 0.05$, data not shown).

	MDA	TCPy	DCCA	3-PBA
Green Beans				
QCL	85.8	119.5	100.0	94.5
QCH	93.1	95.4	93.4	97.8
Peas				
QCL	109.8	93.0	104.6	91.0
QCH	106.8	84.5	110.2	94.1
Carrots				
QCL	79.6	116.6	104.1	92.9
QCH	80.2	108.2	97.7	88.1
Apples				
QCL	76.6	133.1	103.1	114.5
QCH	86.1	123.7	102.3	112.6
Bananas				
QCL	107.3	109.3	109.7	98.3
QCH	127.1	110.2	106.0	93.3
Pears				
QCL	92.0	123.5	93.2	98.9
QCH	109.1	132.3	100.5	101.5

Table 10: Relative recoveries for degradation products in baby food expressed as percentages. QCL is at a level of 10 ng/g, while QCH is at a level of 25 ng/g.

	low spike	hi spike	LOD (ng/g)
vegetable			
MDA	91.7±15.9	93.4±13.3	2.3
TCPy	109.7±14.5	96.0±11.9	2.7
DCCA	102.9±2.6	100.4±8.7	0.87
3-PBA	92.8±1.7	93.3±4.9	0.23
fruit			
MDA	92.0±15.4	107.5±20.6	0.18
TCPy	122.0±12.0	122.1±11.1	0.76
DCCA	102.0±8.3	102.0±8.3	0.87
3-PBA	103.9±9.2	102.5±9.7	0.24

Table 11: Results for recovery of degradation products from fruits and vegetables. Relative recoveries, RSDs, and LODs are presented. Low spike is at 10ng/g, and high spike is at 25ng/g.

Actual concentrations of insecticides in vegetables are shown in Table 12, concentrations of insecticides in fruits are show in Table 13, and concentrations of degradates for all matrices are shown in Table 14. Analytes whose QCs did not meet the criterion of 80-120% recovery are not quantified and are displayed as DNQs. If a data point is above 0 but below the LOD, a value of LOD/√2 is assigned to it as recommended by Hornung and Reed.[117] Figure 1 summarizes detection frequencies in vegetables and fruits, and Figure 2 stratifies detection frequencies by organic versus conventional foods.

	Green Beans			***Peas***			***Carrots***		
	Brand 1	**Brand 2**	**Brand 2 Org.**	**Brand 1**	**Brand 2**	**Brand 2 Org.**	**Brand 1**	**Brand 2**	**Brand 2 Org.**
dia	0	0	0	0	0	0	0	DNQ	0
chlm	0	0	0	0	0	0	0	0	0
cpy	0	0	0	0	0	0	0	0	0
par	0	0	0	0	0	0	0	0	0
mal	0	0	0	0	0	0	0	0	0
dic	0	0	0	0	0	0	0	0	0
hep	0	0	0	0	0	0	0	0	0
endo α	0	0	0	0	0	0	0	0	0.045
endo β	0.045	0	0	DNQ	DNQ	DNQ	0.045	0.045	0.045
DDT op	0.038	0.021	0	0.031	0.027	0.022	0.048	0.033	0.036
pral	0	0	0	0	0	0	0	0	0
per I	0	0	DNQ	DNQ	0	0	0	0	DNQ
per II	0	0	0	0	0	0	0	0	0
cyf I	DNQ	2.364	DNQ	0	0	0	DNQ	DNQ	0
cyf II	0	0	0	0	0	0	0	0	0
cyf III	0	0	0	0	0	0	0	0	0
cyp I	DNQ	DNQ	DNQ	DNQ	0	0	0.028	0.028	0
cyp II	0	0.866	0	DNQ	0	0	0	0	0
cyp III	0	1.264	0	0	0	0	0	0	0
fev I	0	0	0	0	0	0	0	0	0
fev II	0	0	0	0	0	0	0	0	0

Table 12: Concentration of parent compounds in vegetables (ng/g). Analytes are marked as DNQ (did not quantify) if QC recoveries were not between 80-120%. A value of LOD/√2 is assigned to concentrations below the LOD but greater than zero and QCs.

	Apples			***Bananas***			***Pears***		
	Brand 1	**Brand 2**	**Brand 2 Org.**	**Brand 1**	**Brand 2**	**Brand 2 Org.**	**Brand 1**	**Brand 2**	**Brand 2 Org.**
dia	0.033	0.033	0.048	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ
chlm	0	0	0	0.075	0.075	0.075	0	0	0
cpy	0	0	0	0	0.124	0	0	0	0
par	0	0	0	0	0	0	0	0	0
mal	0	0	0	0	2.54	2.54	0	0	0
dic	0	0	0	0	0.207	0.207	DNQ	0	DNQ
hep	0	0	0	0	0	0	0	0	0
endo α	0	0	0	0	0	0	0	0	0.028
endo β	0.087	0	0.028	0	0.028	0	0	0.028	0
DDT op	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ
pral	DNQ	0	0	0	DNQ	0	0	0	0
per I	0	DNQ	0	DNQ	DNQ	DNQ	0	DNQ	0
per II	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ
cyf I	0.048	0	0	0.031	0.031	0	0	0	0.031
cyf II	0	0	0.048	0.048	0.048	0	0.048	0	0.048
cyf III	0.046	0	0	0	0.032	0	0	0	0.021
cyp I	0.028	0	0.028	0.028	0.028	0	0.028	0	0.028
cyp II	0	0	0.055	0	0.055	0.055	0	0	0.055
cyp III	0	0	0.038	0	0.025	0	0	0	0.038
fev I	0	0.047	0.047	0	0.047	0	0.067	0.047	0
fev II	0.033	0.212	0.023	0.023	0.023	0.023	0.023	0.023	0.023

Table 13: Concentration of parent compounds in fruits (ng/g). Analytes are marked as DNQ (did not quantify) if QC recoveries were not between 80-120%, and a value of LOD/√2 is assigned to concentrations below the LOD but greater than zero.

	Green Beans			*Peas*			*Carrots*		
	Brand 1	Brand 2	Brand 2 Org.	Brand 1	Brand 2	Brand 2 Org.	Brand 1	Brand 2	Brand 2 Org.
MDA	0	0	0	3.42	2.72	3.63	0	0	0
TCPY	0	2.85	0	0	0	0	0	0	0
DCCA	0	0	0	0	0	0	0	0	0
3-PBA	0	0	0	0	0	0	0	0	0
DDE-*o,p*	0	0	0	0	0	0	0	0	0
DDE-*p,p*	0.014	0	0	0.014	0	0	0.042	0.014	0.023
	Apples			*Bananas*			*Pears*		
	Brand 1	Brand 2	Brand 2 Org.	Brand 1	Brand 2	Brand 2 Org.	Brand 1	Brand 2	Brand 2 Org.
MDA	0	0	0	DNQ	0	0	1.81	0	0
TCPY	0	0	0	0	0	0	0	0	0
DCCA	0	0	0	0	0	1.64	0	0	0
3-PBA	0	0	0	0	0	0	0	0	0
DDE-*o,p*	0	0	0	0	0.027	0	0	0	0
DDE-*p,p*	0.013	0.013	0.013	0.013	0.023	0.013	0.013	0.013	0.013

Table 14: Concentration of insecticide degradation products in fruits and vegetables (ng/g). Analytes are marked as DNQ (did not quantify) if QC recoveries were not between 80-120%, and a value of LOD/√2 is assigned to concentrations below the LOD but greater than zero.

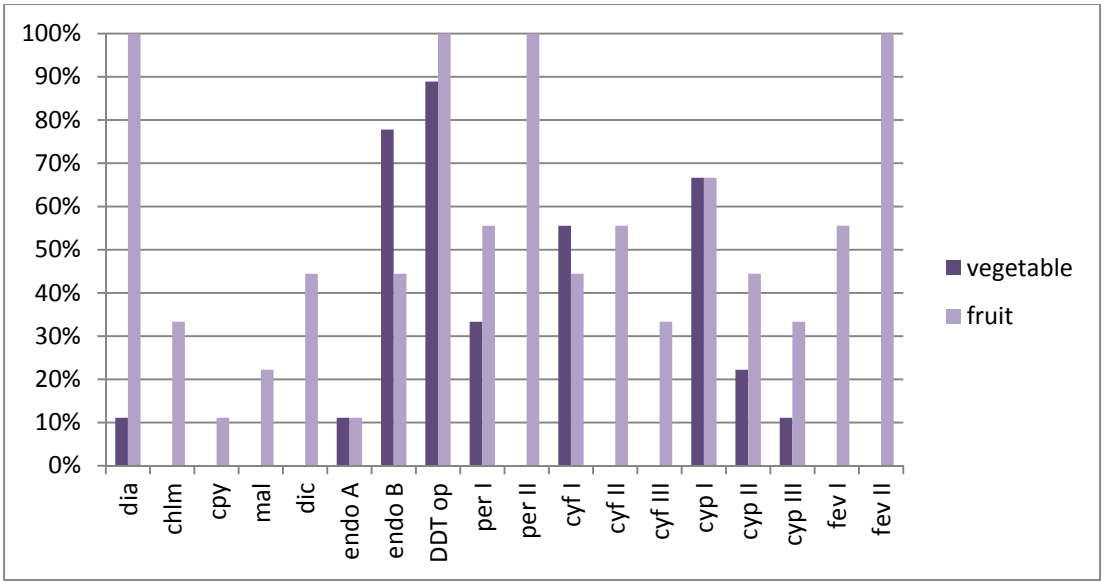


Figure 1: Frequency of insecticide detection in vegetables and fruits. Pyrethroids emerge from the column in multiplets because of the presence of multiple stereomers, and each peak is quantified individually.

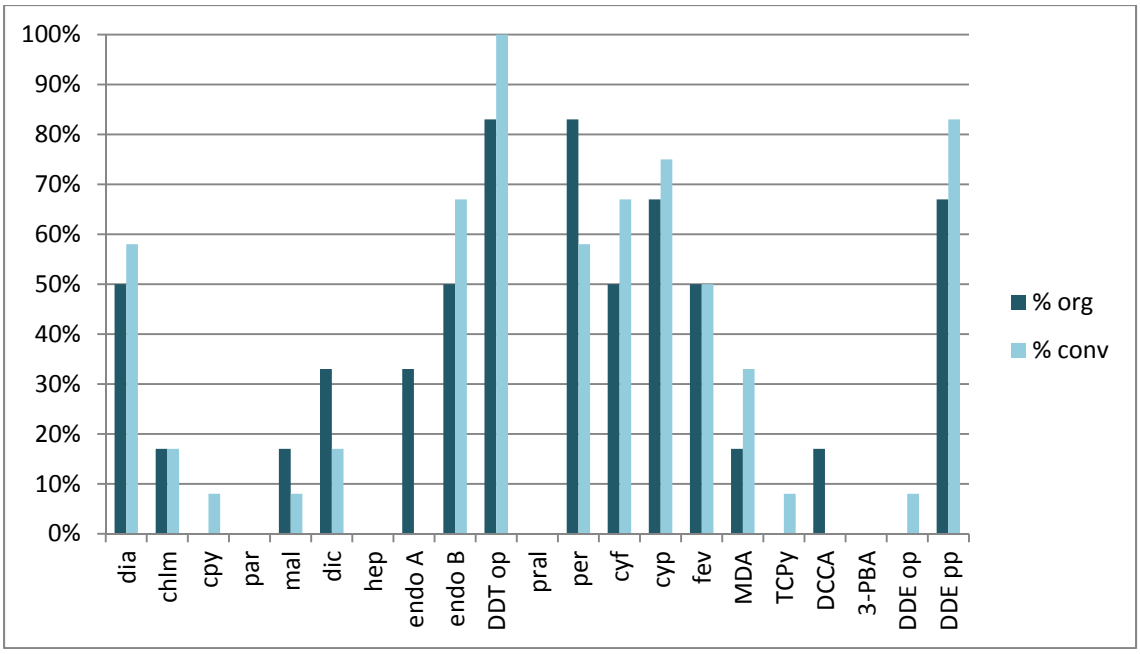


Figure 2: Frequency of detection in organic and conventional baby foods.

Discussion

Most recoveries of compounds separated by GC-MS/MS and malathion are within 80-120% which is ideal for analytical analyses.[118] For degradates separated by LC-MS/MS, all recoveries were between 80-120% except TCPy in fruit (122%).

As a whole, pyrethroids were the most frequently detected insecticide class. This observation makes sense as pyrethroids are one of the most used classes of insecticides in the United States. However, the pyrethroid degradates DCCA and 3-PBA were not frequently detected. DCCA was only found in one vegetable sample, and 3-PBA was not found in any samples. Although there was not much difference in pyrethroid detection frequency between organic and conventional samples (Figure 2), organic samples tended to contain lower concentrations of insecticide residues than conventional samples. This is to be expected since pyrethroids should not be applied to organic foods.

As might be expected due to their environmental persistence, organochlorine insecticides and degradation products, particularly DDT and DDE, are frequently encountered analytes in the samples. There is little difference in OC concentrations between organic and conventional samples. This observation makes sense, as application of many OCs has been banned for decades, so OC residues are due to past application of these persistent compounds. Therefore, a difference in OC concentration would not be expected in organic versus conventional samples. DDT was detected in all fruit samples, including organic, and was detected in eight out of nine vegetable samples (missing only from the organic green bean sample). The major DDT metabolite DDE, which is also environmentally persistent, was also frequently found in baby foods. This fact is particularly interesting in light of the fact that DDT was banned from use in the United States in the 1970s.[6] While DDE is not used as a urinary biomarker of DDT exposure due to its own lipophilicity and persistence,[5, 32] the fact that it is also a toxic compound makes it a degradate of interest. Endosulfan β is also detected frequently in fruits and vegetables. Again, this makes sense as endosulfan will not be deregistered for use in the United States until 2016.[12]

Of the three insecticide classes studied, organophosphorus insecticides were the least frequently detected, which makes sense given the decrease in OP use since 1996. Diazinon was found in all fruit samples, but only one vegetable sample of carrots. Malathion was only found in two fruit samples, while MDA is found in five total samples. Interestingly, malathion and MDA are not found concomitantly. Similarly, chlorpyrifos and TCPy are each found in only one sample, but they are each in different samples.

Insecticides and their degradation products are found more frequently in fruits than in vegetables (Figure 1). While the 2009 USDA PDP data doesn't seem to indicate a higher detection frequency of the analyzed insecticides on fruits than on vegetables, it is still possible that there are more insecticides used on fruits than vegetables. The PDP LODs tend to be at least fivefold higher than that of this study, so their detection frequency is subsequently lower as well. Most insecticides found in one food in the PDP data are also found in the same food in this study. In a particularly striking example, for the five food types studied both here and in the PDP data, malathion is only found in bananas for both this data and that from the PDP.

While relatively few degradates are found in the baby foods, this may be partially due to the fact that degradate extraction and separation method generally produces higher LODs than the method leading to GC separation. There are a few ways these LODs may be lowered in the future. First, the ISTD for LC analytes is highly concentrated (50ng/g in sample). In the case of 3-PBA, native compound is found in solvent spiked only with ISTD (at about 0.1ng/g). This background concentration affects the 3-PBA LOD. Second, a larger mass of baby food may be used for degradation product extraction in the future in order to concentrate more analyte. While the smaller sample size was initially chosen due to the difficulty of completely separating the solid baby food from the water solvent, longer centrifugation could be used to avoid clogging the filtering cartridge frit in the future.

It is not certain whether 3-phenoxybenzoic acid, 3-phenoxybenzyl alcohol, or both compounds would be produced from the degradation of pyrethroids such as permethrin, cypermethrin, cyfluthrin, and deltamethrin. Hydrolysis of these compounds

sould lead to 3-phenoxybenzyl alcohol, not 3-phenoxybenzoic acid. However, in the mammalian body, the alcohol moiety is transformed to the acid by an oxidase.[19] This oxidation may not happen in fruits and vegetables. In the future, it would be of interest to add 3-phenoxybenzyl alcohol to the extraction and chromatographic methods to see if it is found in any baby food samples.

Urinary insecticide metabolites are often used as biomarkers of exposure to parent insecticide.[37, 38] Multiple studies, including this one, have shown that the same compounds used as urinary metabolites are sometimes found in food before insecticide metabolism in the body.[38, 52, 77] Some insecticide degradates, such as dialkyl phosphates, may be further degraded after ingestion.[38] However, other research concerning both dialkyl phosphates and TCPy in animal models suggests that these compounds are largely adsorbed by the body and then excreted unchanged in the urine.[77] If ingested insecticide degradates truly are excreted unchanged in urine, observation of these analytes would lead to overestimation of insecticide exposure.

While the primary mechanism of OP insecticides is acetocholineesterase inhibition, It is known that some OPs target other neurological pathways, including growth factors and other neurotransmitter systems at lower concentrations than those needed for acute acetocholineesterase inhibition.[41] Several recent studies have connected prenatal OP exposure to lower IQ and cognitive development in children.[41-43] Similar studies have not been done with pyrethroids yet; however, there is the chance that these compounds also have secondary mechanisms of toxicity that occur at low-level exposures. Children's exposure to insecticides are of particular interest since they are in the process of neurodevelopment and the potential for permanent damage from

insecticides is greater.[38, 41] Furthermore, children have lower activity of paraoxonases, which are known to detoxify some OP insecticides.[41, 43] Finally, foods that children prefer tend to have higher insecticide concentrations.[38] Given this information, even if insecticide exposure is overestimated, the amount of exposure actually occurring may still give cause for concern.

It has been assumed in the past that there is little to no toxicity from insecticide degradation product exposure. However, there are currently few data on the adsorption, further metabolism, and potential toxicity of many insecticide degradates.[38, 36] Therefore, it is difficult to separate the health effects of insecticide degradates from that of the parent compounds. Further research is needed to better understand the health effects of insecticide degradates.

Conclusions

This study clearly demonstrates the presence of insecticides in baby food. In addition, insecticide degradation products are found in all baby foods analyzed, although these degradates were not usually found to be concomitant with their parent insecticide. These facts seem to imply that urinary metabolite output does not guarantee insecticide exposure. Overall, these data support the need for greater understanding both for the absorption, excretion, and potential toxicity of insecticide degradates as well as the relationship between insecticide degradation products found in food and urinary biomarkers of insecticide exposure in order to more accurately quantify the populations exposure to insecticides.

CHAPTER 5: SIMPLIFICATON OF INSECTICIDE EXTRACTION METHOD FOR UNDERGRADUATE EXPERIMENTS

Hypothesis

Undergraduate chemistry students are capable of performing simplified solid-phase extractions of insecticides with reasonable accuracy and precision, and allowing them to do so gives them the opportunity to learn more about real-world applications of quantitative analysis.

Introduction

Pesticides are used worldwide in both agricultural and residential settings.[1-2] In particular, organophosphorus insecticides and pyrethroid insecticides are widely used in the United States. [1, 119] These classes of insecticides have been used extensively since the 1970s after organochlorine pesticides, such as DDT, were banned in the United States.[119]

For these reasons, researchers analyze thousands of samples annually for these insecticides for a variety of purposes including regulatory enforcement and surveillance monitoring using an assortment of methods.[71, 120] In the United States, insecticides are regularly monitored in domestically-grown and imported foods and juices to ensure compliance with residue limits or tolerances set by the U.S. Environmental Protection Agency.[71] The U.S. Department of Agriculture's International Maximum Residue Limit Database includes U.S. tolerance limits for various foods as well as maximum acceptable levels in 70 other countries for a range of insecticides.[3] However, there are still countries with limited or no control over insecticide residues in food.[121-122]

Children's exposure to insecticides is of particular interest because much growth and neurodevelopment happens during childhood, raising concern of long-lasting effects.[15, 38-39] Further, their behaviors and food preferences, particularly for fruit juices,

lead to a higher risk for insecticide exposure. These factors suggest the need for methods to assess dietary exposures by quantifying insecticide residues in various foods.

Although analytical chemistry textbooks explain sample extraction/clean-up and gas chromatography procedures,[39, 123] students may retain the idea that samples may be directly injected into analytical instrumentation with little to no prior preparation. The purpose of this lab is to present the student to one of the many customized sample preparation procedures based upon the commonly used QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)[124] extraction methods for the rapid, high-throughput, inexpensive multiresidue determination of insecticides using baby food as a matrix. In addition, the student will be introduced to general analytical procedure using gas chromatography with mass spectrometry or electron-capture detection. In addition, the student may use UV/VIS spectrophotometry to discover the difference in pigment concentration before and after sample clean-up.

Methods

Experimental procedure

Reagents:

1. Acetonitrile: extraction solvent
2. Clean-up solvent. 25% v/v toluene in acetonitrile
3. Solid phase extraction (SPE) cartridges: Supelclean™ ENVI-Carb-II/PSA (500/300 mg, 6 ml) Supelco cartridges, Florisil, Bondesil PSA (primary-secondary amine), or C_{18} (500mg/6mL) cartridges may be used. C_{18} cartridges are not recommended for heavily pigmented matrices.

4. NaCl
5. Juice without pulp (white or red grape, cranberry, etc). Wine may also be used but may not be preferable in classes with students under 21 years of age. Samples given to students for GC analysis should be fortified to a given concentration between 25-100 ng/mL of pesticides mentioned in results. Samples used for UV/VIS analysis should not be fortified with pesticide.

Juice Extraction Procedure:

1. To a labeled 15-mL, trace-cleaned, glass, disposable centrifuge tube, add 1.0 mL of juice, 5.0 mL of acetonitrile, and ~0.50g of NaCl. Sodium chloride is added to make water immiscible with acetonitrile.
2. Vortex this mixture for 3 minutes.
3. Centrifuge the sample for 5 minutes in order to separate the acetonitrile and aqueous layers.
4. Save these samples as 2.0 mL of the supernatant (organic layer) will be used in the cleanup procedure.

Cleanup Procedure:

1. Pre-condition a clean-up cartridge by wetting it with 5 ml of the 3:1 acetonitrile:toluene solution. (**Caution!** Use toluene in hood only!)
2. Load 4.5 mL of the organic extract (supernatant) onto the cartridge. Be careful not to load any of the aqueous (bottom) layer. Do not collect the liquid that comes out of the cartridge.

3. Elute the cartridge with 5 mL of 3:1 acetonitrile:toluene solution, collecting the eluant in a labeled 15-ml trace-cleaned, glass, disposable centrifuge tube. Repeat elution with 5mL into same tube.
4. Reduce eluate by evaporation by Turbo-Vap II (Biotage) using nitrogen at 10 PSI and 45°C for 15 minutes, then continue evaporation at 20 PSI and 45°C for 30 minutes. Other options include evaporation with a gentle stream of nitrogen or air. If an evaporator is not available, let the samples evaporate overnight under a fume hood with a Kimwipe covering tube to limit contamination.
5. Reconstitute in 1 mL of acetonitrile if using electron capture detection, *vortex briefly (~10 seconds)* before transferring to GC vial and putting into tray. If analyzing by GC-MS, reconstitute with 50µL 25% toluene in acetonitrile.
6. Store in the refrigerator/freezer until ready to analyze the sample.

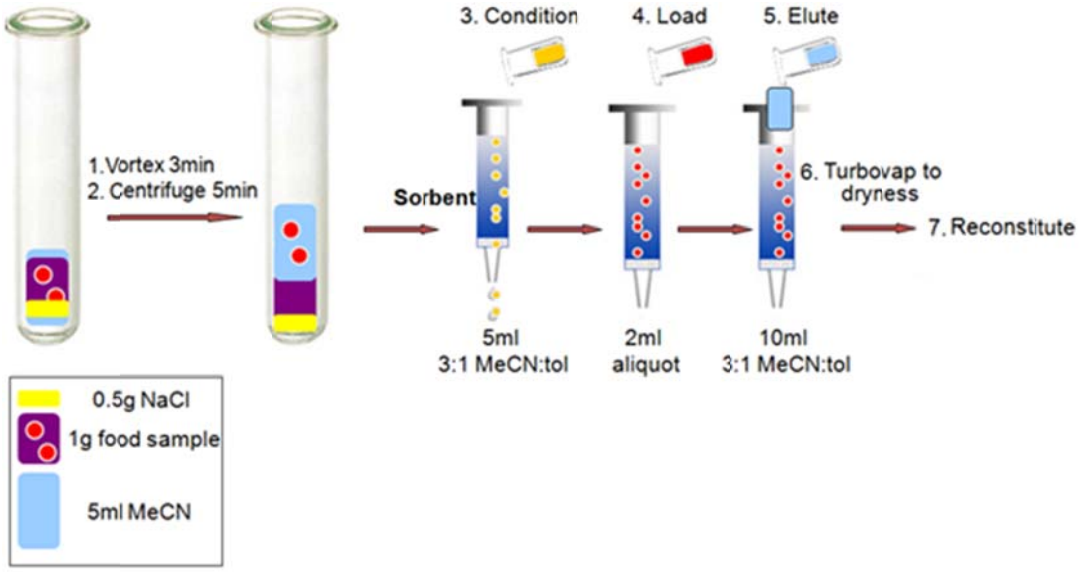


Figure 1: Scheme for pesticide extraction and elution.

Separation by GC-ECD: ECD works best for halogenated compounds. For GC-ECD separation, the gas chromatograph used was a Hewlett-Packard Model 5890A Series II equipped with an Agilent Technologies electron capture detector and 7683B Series Injector autosampler (Santa Clara, CA, USA). A DB-5 column (Agilent Technologies, Inc., Santa Clara, CA, 30 m, 0.25 mm i.d., 0.25 μm film thickness [5% phenyl, 95% dimethylpolysiloxane]) was used, and a 2 mm i.d. single-taper injection liner is recommended to prolong column life. Injection volume was 1.0 μL (1:30 split). The helium carrier gas was at a flow rate of 0.88 mL/min, while the nitrogen make up gas flow was 13 mL/min. The injector temperature was 260ºC. The temperature program started at 80°C and stayed at that temperature for 2 min before being heated linearly by 10ºC/min to a final temperature of 280ºC which was held for 13 min. The ECD temperature was 280ºC.

Separation by GC-MS/MS: For GC-MS/MS separation of chlorpyrifos and permethrin, an Agilent 7890 GC with MS/MS detection equipped with positive mode electron impact (EI) ionization was used. The column used was an Agilent HP-5MS [(5% phenyl)-methylpolysiloxane, 30m x 250 μm x 0.25μm]. The injection port was held at 250°C, and injection was 1μL (splitless), and a gooseneck injection liner with glass wool and 1m deactivated silica guard column was used. The carrier gas was He at a flow rate of 50mL/min, and the column temperature profile was as follows: The column was held at 100°C for 2 min before linear heating by 10°C/min to 205°C. This temperature was held for 3 min before linear heating by 10°C/min to 280°C. This temperature was held for 4 min before linear heating by 25°C/min to a final temperature of 310°C. This final temperature was held for 12 min. Analysis was performed in MRM mode, monitoring

two characteristic parent ions for each compound (Table 1). Isotopically labeled internal standard is not required but is listed below for reference.

Compound native	**Precursor ion**	**Product ions (Q, C)**	**CE(V) (Q, C)**	**RT (min)**
chlorpyrifos methyl	288.2, 286.2	93, 93.2	20, 26	13.76
chlorpyrifos	314.2	286.1, 258	5, 25	15.19
parathion	291.3	90.9, 81.0	35, 40	15.22
dicofol *p,p-*	139.1	111.1, 75.1	15, 30	15.26
DDE *o,p-*	248.2, 246.2	176.3, 176.2	30, 35	17.18
DDE *p,p-*	248.2, 246.2	176.2, 176.2	30, 35	18.08
DDT *o,p-*	235.2	199.1, 165.1	15, 25	19.25
cyfluthrin	163.1, 206.2	127.2, 151.1	5, 25	23.95, 24.07, 24.17, 24.23
cypermethrin	163.1, 181.2	127.1, 152.2	5, 25	24.36, 24.50, 24.61
fenvalerate	167.2, 125.2	89.2, 89.0	40, 20	25.78, 26.14
ISTD				
chlorpyrifos-methyl	291.9	274, 99	30, 25	13.20
chlorpyrifos	324	292, 260	10, 20	14.40
parathion	301	115	15	
DDE *p,p-*	258	188	40	16.00
cypermethrin	170	134, 98	10, 15	23.45

Table 1: Precursor/product ions with optimized collision energies. Internal standards are isotopically labeled. Pyrethroids have multiple retention times because of chiral centers.

Optional: UV/VIS analysis before and after cleanup:

1. Use UV/VIS spectrophotometry to find λ_{max} for the juice used by scanning across wavelengths and choosing the wavelength with maximum absorbance (use juice not fortified with pesticide for this part of the experiment).

2. Assuming the pure juice is defined to have 100% pigmentation, and use volumetric flasks to dilute juice with distilled water to make 50%, 10%, 5%, and 1% pigmentation. For example, 50% pigmentation would be 1:1 juice:water, 10% 1:9 juice/water, etc.
3. Create a Beer-Lambert plot by finding the absorbance of each sample, and include a distilled water sample on the plot (set to A=0 or use reagent blank) if using a single beam apparatus.
4. Extract two samples of juice (without pesticide) as written in the "Juice extraction procedure" above.
5. From one sample, carefully pipette to transfer all supernatant (5.0mL) into a new test tube *without transferring any of the aqueous layer*. Reduce this organic solvent by evaporation to dryness. Add 1.0mL distilled water to reconstitute and vortex briefly (~10 seconds).
6. Using the second sample from step 4, add all 5.0mL supernatant to a preconditioned cartridge and process the sample using the "Clean-up procedure" above. Evaporate the eluant to dryness and reconstitute with 1.0mL distilled water, vortexing briefly.
7. Find out the absorbance at λ_{max} for both the extracted sample and the extracted/cleaned sample and use the calibration curve to discover what percent of pigment is left. **Caution!** *Do not* put samples reconstituted with water into the gas chromatograph!

Pilot study with undergraduates

To insure that this protocol is indeed feasible for undergraduate students, a cohort of students currently enrolled in Quantitative Analysis at Emory University was recruited to test the procedure using either PSA or C_{18} cartridges. Students were offered bonus points in their class to complete the experiment. Before the experiment, students were given a short lecture concerning the purpose and significance of the experiment and were taught how solid-phase extraction works before they completed the extraction. They were then given the procedure to read before the experiment (Procedure and background information given to students is shown in Appendix below. Students were advised to load 4.5mL of extract rather than 2.0mL to increase sensitivity). Students were required to answer three short pre-laboratory questions to gain entrance to the experiment in order to ensure at least cursory reading beforehand. Each student was given one apple juice sample with an unknown concentration of insecticide (all samples actually contained 50ng/mL insecticide mix). Trained researchers created matrix-based calibration curves with both PSA and C_{18} cartridges, and the students' samples will be separated by GC-MS/MS, and results will be analyzed by trained researchers as well.

Results

Trained researcher results

Triplicate samples of red wine and grape juice were fortified to 25 or 100 ng/g (parts per billion; ppb) insecticide for extraction. Percent recoveries for C_{18} cartridges and Florisil cartridges were determined using a pre-spike/post-spike experiment using GC-MS/MS. In this experiment, one set of samples was fortified in triplicate to either 25

or 100 ng/g and then extracted and cleaned using the procedure explained in the supplemental material, while the other set was extracted and cleaned up prior to pesticide fortification. The ratio of pre-spike to post-spike recovery was used to determine percent recovery to account for any matrix effects. Percent recoveries between 80-120% were deemed acceptable.[125] According to results, C_{18} cartridges are preferred for OCs such as dicofol and DDE, while Florisil cartridges are preferred for pyrethroid insecticides, such as permethrin, cyfluthrin, cypermethrin, and fenvalerate. Both cartridges give acceptable results for several organophosphorus insecticides, such as chlorpyrifos, chlorpyrifos-methyl, and parathion. Supelclean™ ENVI-Carb-II/PSA cartridges work for any of the pesticides listed in Table 1.

Qualitative UV-Vis results

Concerning the UV/VIS study, students should see a dramatic reduction in pigment absorbance and concentration after the clean-up step. In particular, Florisil eliminates pigments from samples much better than C_{18} cartridges, so absorbance will show a greater decrease with Florisil cartridges than with C_{18} cartridges. When students are led to understand that pigments will dirty GC columns and may cause poor results, this experiment will demonstrate to them the need for matrix clean-up.

Student results

Although students have gone through this laboratory experiment as described in the methods section, results have not been obtained yet. Students seemed to be able to

perform the experiment with little trouble other than being unfamiliar with the type of pipets used.

Hazards and notes

Insecticides chosen for this experiment may be adapted to those available to the laboratory but should have the same general properties.

Students should wear gloves, goggles, and any other appropriate personal protective equipment during this experiment to avoid contact with insecticides. Students should also work in a hood to avoid inhalation of organic solvents. Pregnant or nursing women should not participate in this experiment as *in utero* insecticide exposure or infant insecticide exposure through breast milk may affect children's neurodevelopment.[40-41]

A solvent (acetonitrile or 3:1 acetonitrile:toluene) calibration curve should be made for each insecticide used in the experiment. While the detection limit for these insecticides is ≤1 ng/g, the calibration curve should be made between 10-350 ng/g, thereby ensuring 80-120% recovery may be seen. It may be more practical for this calibration curve to be made up by the instructor as long as he or she explains what has been done to the students.

A ring stand may be set up with a clamp to hold a cartridge with a 450-mL beaker underneath. The beaker will catch preconditioning and loading solvent. The test tube may then be set up inside the beaker in a way that the eluate will be collected.

It should be noted that the recommended fortification levels (25-100 ppb) are well above insecticide concentrations found in fruit juices; but these concentrations are used to make sure insecticides may be easily quantified by students.

Conclusions

It is possible to simplify a pesticide extraction method in such a way that the procedure costs less money and time, yet still retains its value as a way to quantitatively assess insecticides in juice. This simplified method is easy enough to be followed by undergraduate chemistry students and allows them an opportunity to learn about real-world application of the concepts they learn in both their chemistry lecture and laboratory classes.

APPENDIX C: ANALYSIS OF PESTICIDES BY GAS CHROMATOGRAPHY

Introduction

Insecticides

Pesticides are one of the few compound classes designed to kill. Insecticides, or pesticides designed to target insects, work by affecting the nervous system. Since people have nervous systems that work in very similar ways to those of insects, we too can be harmed by insecticides. For this reason, it is very important for us to understand human exposures to these compounds.

In the United States, several government agencies are involved in pesticide regulation and exposure studies, including the Centers for Disease Control and Prevention, the Environmental Protection Agency, and the Food and Drug Administration. While these groups are concerned about people's exposure to pesticides through use in residential settings or through working in agriculture, the main way most people are exposed to pesticides is through their diet. For this reason, many extraction and separation methods have been developed to monitor pesticide residues in food. The procedure you will follow is based on a method developed here at Emory University.

Extraction methods for gas chromatographic separations

A sample usually may not be run through an analytical instrument as is, because doing so would damage the instrument. For example, water will destroy some gas chromatography detectors such as electron capture detectors. Fats or pigments in matrices will dirty GC columns as well, quickly rendering the column useless. For this reason, an *extraction* is done on the sample. The extraction process uses the chemical equilibriums such as you have discussed previously in Quantitative Analysis to move the

analyte from one solvent to another (Figure 1). If the solvents are immiscible (will not dissolve into one another), the analyte may then be removed from the original matrix, leaving behind many unwanted compounds.

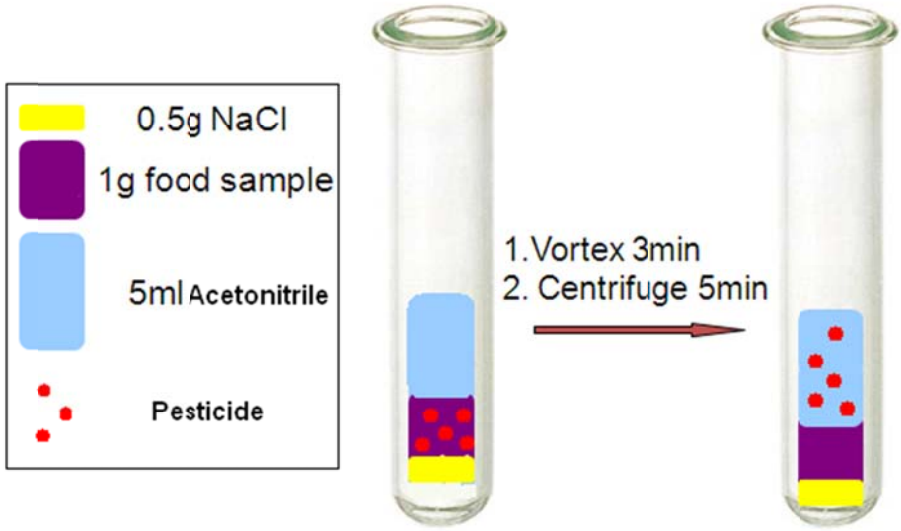


Figure 1: Pesticide extraction. The addition of sodium chloride causes the acetonitrile (ACN) to be immiscible in water. The pesticide prefers to be in acetonitrile (the top layer), so the *supernatant* may be removed and analyzed.

In an ideal world, an analytical chemist would be able to take a sample, extract the compound of interest, and leave all interfering compounds behind (Figure 2). Of course, this isn't usually the case. For example, acetonitrile in food will dissolve pigments such as chlorophyll as well as pesticides. For this reason, a clean-up step is needed. A solid phase extraction (SPE) cartridge is often used for this clean up process. The SPE cartridge contains a layer of sorbent such as C-18, primary-secondary amine, or other compounds which are able to trap interfering compounds. The supernatant from the extraction step explained above may be loaded onto the cartridge, and a different solvent

may be used to wash the pesticide off the cartridge while leaving behind the pigments and fats.

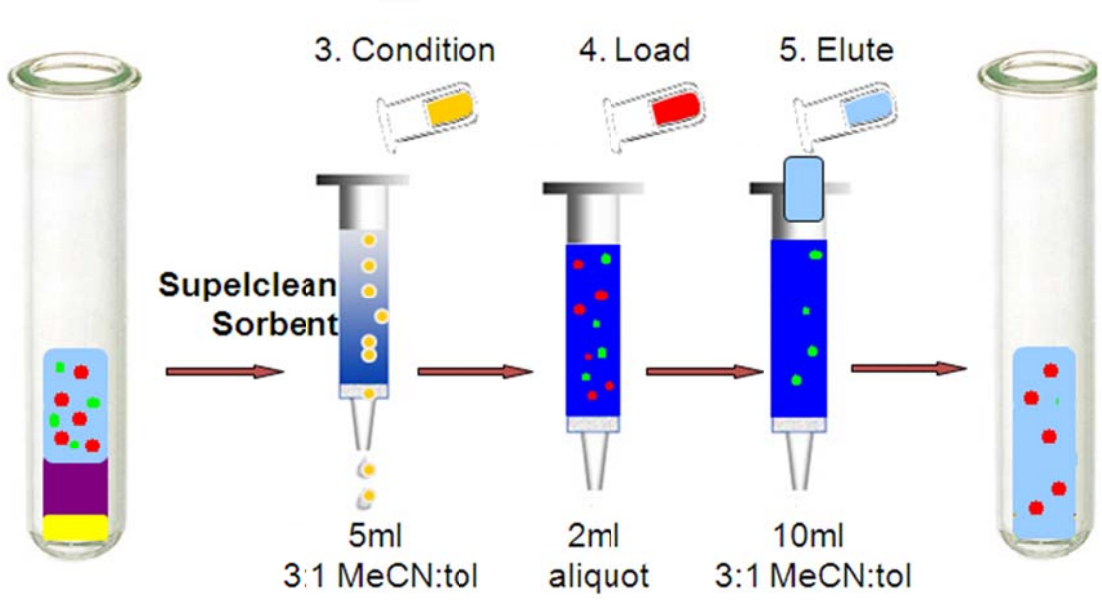


Figure 2: Clean-up step. An aliquot from the supernatant of the first tube is loaded in step 4. The green particles represent unwanted contaminants.

Procedure

YOU WILL BE WORKING WITH PESTICIDES AND ORGANIC SOLVENTS. IT IS ESSENTIAL THAT YOU WEAR GLOVES AND GOGGLES THROUGHOUT THE PROCEDURE.

1. Add 1.0 mL of juice, 0.5g NaCl, and 5.0mL acetonitrile (ACN) to test tube. Vortex the tube for 3 min and centrifuge 6 min at 3100rpm (make sure centrifuge is balanced).

2. During centrifugation, set up a cartridge using a ring stand with a beaker underneath. Record the type of cartridge you are using. Precondition cartridge with 5mL 3:1 v/v ACN:toluene solution. Allow solution to drain completely

through cartridge into a beaker. WARNING: Do not take toluene out of the hood!

3. Load 2.0mL of sample supernatant (top layer) onto column. Do not get any of the aqueous layer into the column. Allow sample to drain completely through the cartridge into a beaker.

4. Place a clean, labeled test tube underneath the cartridge. Load 5.0mL of 3:1 ACN:toluene onto cartridge and allow to elute (drain completely) into the test tube. Load 5.0mL of 3:1 ACN:toluene onto cartridge again and allow it to drain into same test tube.

5. Place test tube into evaporator at 38°C and 15psi and reduce volume to dryness. Give sample to instructor to store in the refrigerator until GC analysis. When the time comes for the instructor to analyze the sample, the sample will be reconstituted with 100µL 3:1 ACN:toluene.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

In this research, the degradation of insecticides in food was analyzed multiple ways. In order to carry out the analysis, liquid chromatographic methods with triple quadrupole detection were developed for DAPs, malathion, and insecticide degradation products. A method for the rapid, high-throughput, and accurate extraction of MDA, TCPy, DCCA, and 3-PBA was also developed. The procedure involved adding Milli-Q water to a 1.0g sample of baby food, vortexing, and centrifuging solid residue down. The supernatant of this sample was then extracted and cleaned by solid-phase extraction using an HLB cartridge. Instrumental analysis was carried out by LC-MS/MS. To evaluate the method, fortification studies were carried out in six baby food matrices at a high (25ng/g) and low (10ng/g) concentration, and 70% of recoveries were between 80-120%.

Once suitable methods were developed, the degradation of insecticides in juices was studied both by GC-ECD and by GC-MS/MS and LC-MS/MS. In the GC-ECD study, statistically significant ($p < 0.05$) loss of insecticide by pseudo-first order kinetics over the course of two weeks was observed for 64% of observations. Insecticide loss was seen least in the most complex matrix, orange juice, where more interactions with matrix could occur. Loss was confirmed to not be simply adsorption of insecticide onto the glass container wall by experiments involving both silanization and vortexing of the jar.

A smaller subset of insecticides (malathion, chlorpyrifos, and permethrin) was analyzed for degradation in juices using GC-MS/MS and LC-MS/MS. In this study, both the loss of parent compound and the production of degradation product were followed over two weeks. Statistically significant degradation of permethrin in grape juice and and white wine was observed, although production of 3-PBA did not occur. Conversely, while degradation of malathion and chlorpyrifos was not deemed statistically significant,

significant production of their degradates, MDA and TCPy, occurred. For all matrices, there was statistically significant production of MDA that occurred with pseudo-first order kinetics. While there was no background TCPy in blank juice samples, TCPy was found in samples after chlorpyrifos fortification and followed a production-loss-production trend in all samples but water, where TCPy followed a production-loss trend.

In the other study, the concentration of both insecticides and their degradation products in three baby food vegetables and three baby food fruits was analyzed. Out of the five OPs studied, four of them were found in at least one sample, and diazinon was found in four out of six food types. Malathion was found only in bananas, but its degradation product, MDA, was found in peas, bananas, and pears. Chlorpyrifos and chlorpyrifos methyl were also only found in bananas, but TCPy was found in pears. Out of the four OCs analyzed, only heptachlor epoxide was not seen in any samples. Endosulfan was in all types of vegetables and fruits. DDT was found in all vegetable and fruit types, and its degradation product DDE was also found in all vegetable and fruit types. Of the six pyrethroids analyzed, only deltamethrin was not found in any samples. Permethrin and cypermethrin were observed in all vegetable and fruit types, while cyfluthrin observed in all food types except peas. Prallethrin was in both apples and bananas, and fenvalerate was seen in all fruit types. Of the two general pyrethroid degradates analyzed, only DCCA was found in bananas.

As with any research, improvements may be made. First, the precision and accuracy of the insecticide degradate extraction method is poor for TCPy in most matrices, particularly peas. While this fact may be due to differences in matrix interactions between this analyte and its ISTD isotopically labeled 3-PBA, the cost of

isotopically labeled TCPy ISTD is prohibitively expensive (>$10,000), so buying a matching labeled ISTD is unfortunately not an option. Two sets of calibration curves had been made, one in a mixed vegetable matrix and one in a mixed fruit matrix. Perhaps calibration curves should be made individually for each fruit and vegetable to produce better accuracy and precision, particularly for this compound. Between-run precision for the method may also be found in the future.

A few improvements could also be made in the analysis of insecticide degradation in beverages. While MDA demonstrated statistically significant production in all matrices, all concentrations were below the LOD, making results only semi-quantitative. Similarly, all concentrations found for 3-PBA were below the LOD, and no production was observed. If the LOD for these compounds were lowered, perhaps more quantitative data could be produced. This may be accomplished in multiple ways. First, the sample size used for degradate extraction could be increased. This way, analyte would be more highly concentrated, leading to larger signal-to-noise ratios. Secondly, metabolite ISTD concentration, which is currently 50ng/mL, could be lowered, which would lead to a reduction of background native standard concentrations. However, even if the LOD was reduced enough to observe actual 3-PBA concentrations, this analyte may still not be produced by the degradation of permethrin, since hydrolysis alone would lead to the alcohol moiety instead of the acid. The addition of 3-phenoxybenzyl alcohol to the extraction and chromatographic method would allow observation of this degradation product in the future.

Improvements possible for the study of parent insecticide and degradates in baby food are similar to those mentioned for the study of insecticide degradation in beverages.

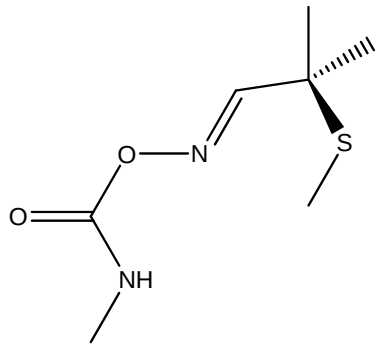
LODs for the compounds analyzed by LC-MS/MS are higher, generally by approximately an order of magnitude, than those for GC-MS/MS. This fact leads to lowered sensitivity for MDA, TCPy, DCCA, 3-PBA, and malathion. In particular, the concentration of ISTD in malathion is five times that of the compounds analyzed by GC-MS/MS. Lowering of ISTD concentration and thereby reducing interference by native standard contamination from ISTD would possibly allow lowering of the LOD and greater sensitivity. Increasing sample size for degradate extraction could also increase sensitivity for these analytes.

In summary, the hypothesis that insecticides degrade in foods is studied in two ways, by both following degradation of insecticides in beverages fortified with parent insecticide and by analyzing both the insecticide and degradate concentration in a variety of baby food. Both methods of study suggest that insecticide degradation occurs in food before it is ever consumed. The presence of these degradation products may cause overestimation of the population's exposure to insecticides, since urinary metabolites are used as biomarkers of insecticide exposure and these compounds are often the same as degradates found on food. For this reason, there should be ongoing study of the degradation of insecticides in food and of the metabolism of these degradation products.

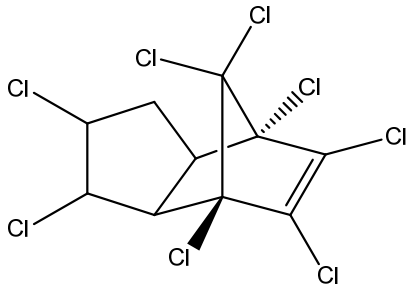
APPENDIX D: STRUCTURES OF REFERENCED INSECTICIDES

Alphabetic listing of insecticides by common name

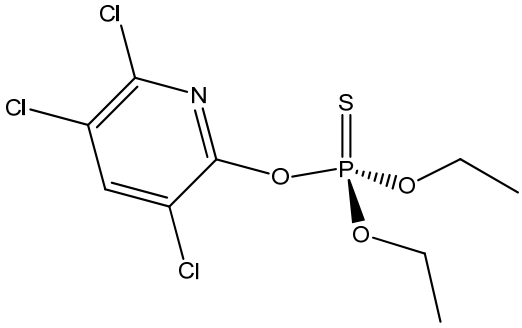
1. Aldicarb (carbamate): (EZ)-2-methyl-2-(methylthio)propionaldehyde O-methylcarbamoyloxime



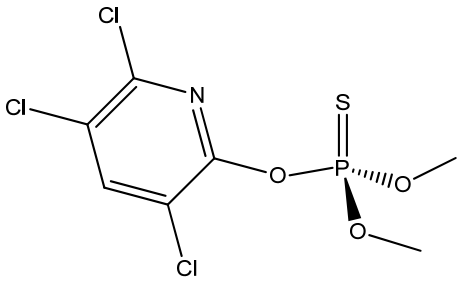
2. Chlordane (OC): 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene



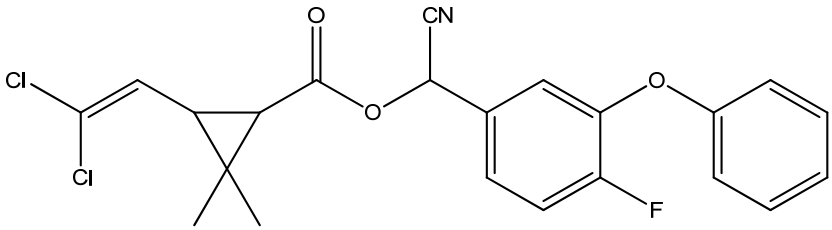
3. Chlorpyrifos (OP): O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate



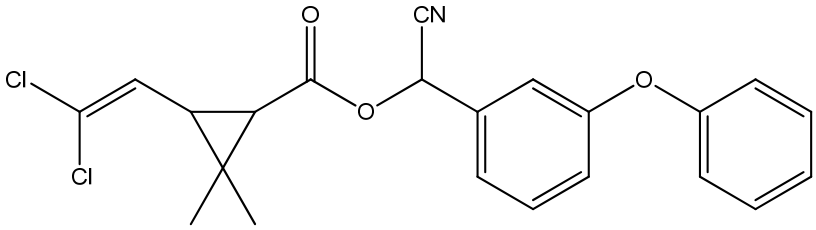
4. Chlorpyrifos methyl (OP): O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate



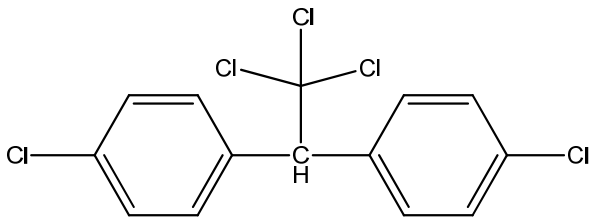
5. Cyfluthrin (pyrethroid): (RS)-α-cyano-4-fluoro-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate



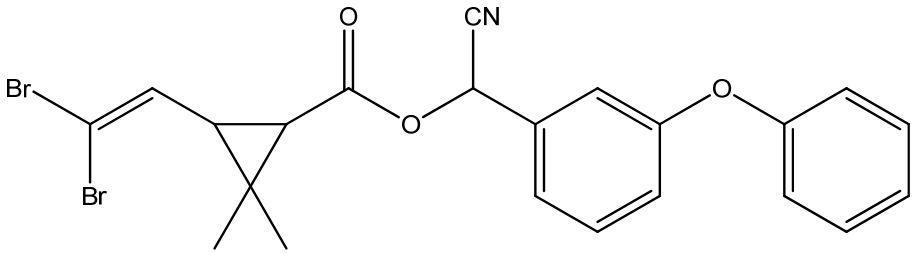
6. Cypermethrin (pyrethroid): (RS)-α-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate



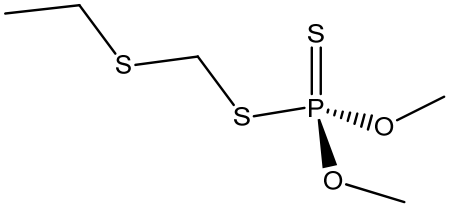
7. *o,p*-DDT (OC): 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane



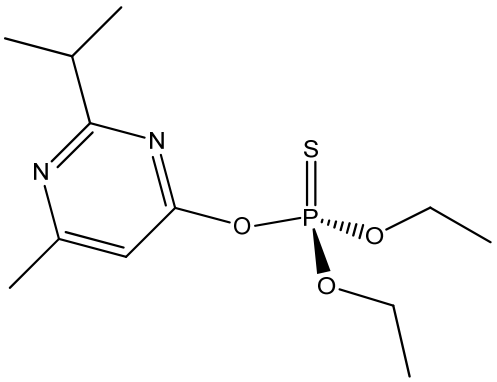
8. Deltamethrin (pyrethroid): (S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate



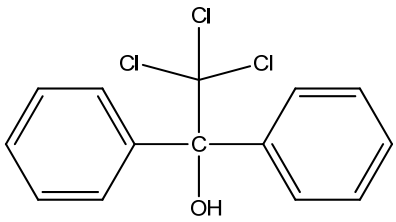
9. Demton-S-methyl (OP): *S*-2-ethylthioethyl *O,O*-dimethyl phosphorothioate



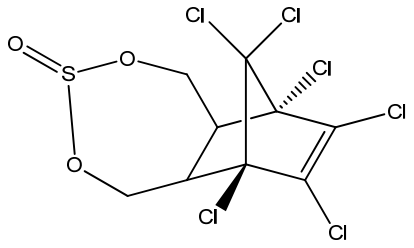
10. Diazinon (OP): O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate



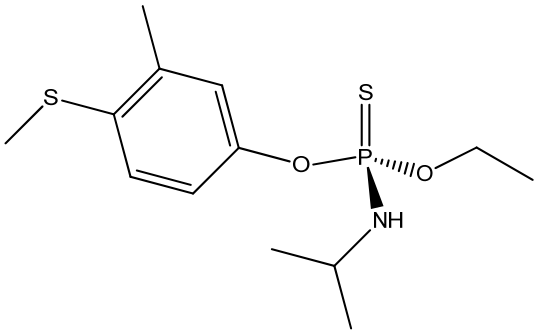
11. *p,p*-dicofol (OC): 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol



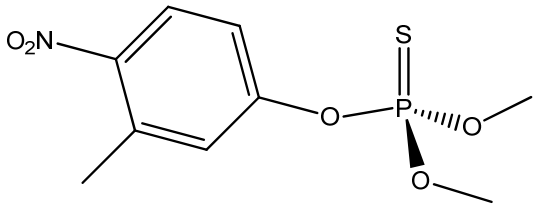
12. Endosulfan (OC): 1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenebismethylene sulfite



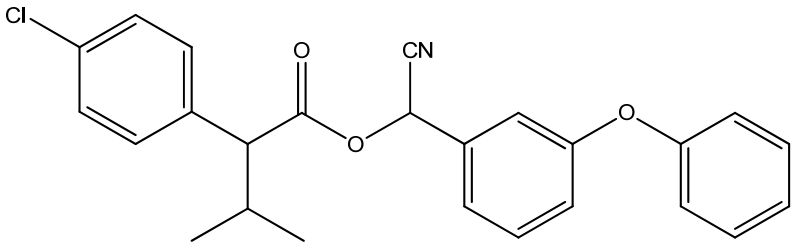
13. Fenamiophos (OP derivative): (*RS*)-(ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate)



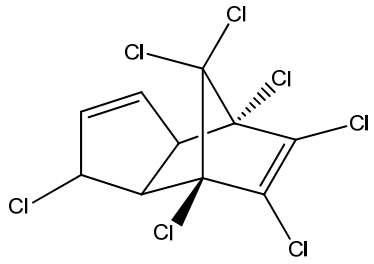
14. Fenitrothion (OP): *O,O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate



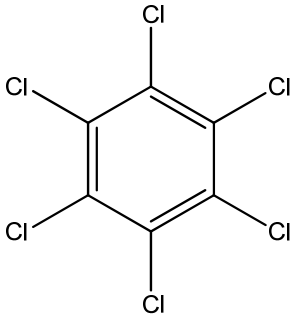
15. Fenvalerate (pyrethroid): (α*RS*)-α-cyano-3-phenoxybenzyl (2*RS*)-2-(4-chlorophenyl)-3-methylbutyrate



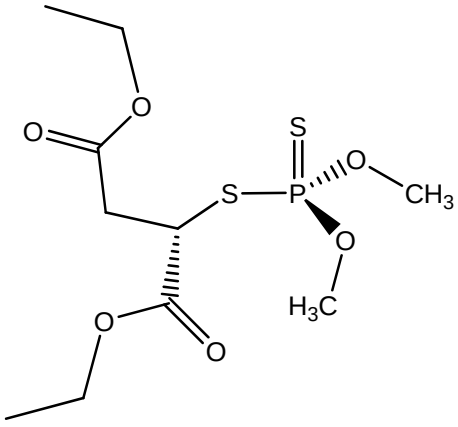
16. Heptachlor epoxide (OC): 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene



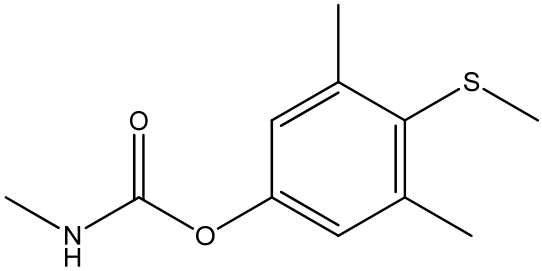
17. Hexachlorobenzene (OC): hexachlorobenzene



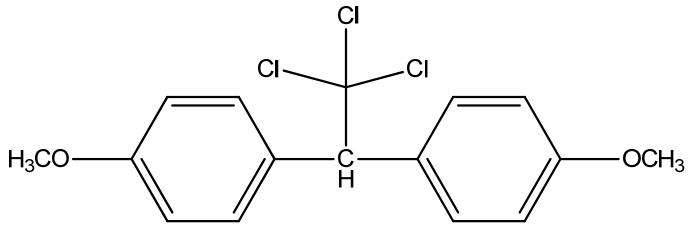
18. Malathion (OP): diethyl (dimethoxyphosphinothioylthio)succinate



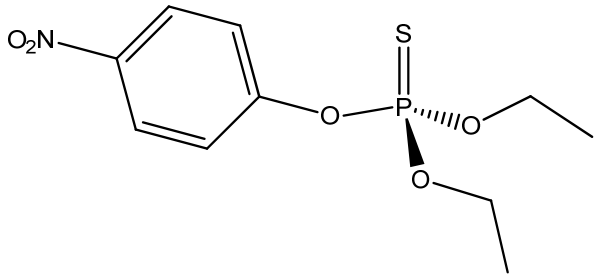
19. Methiocarb (carbamate): 3,5-dimethyl-4-(methylthio)phenylmethylcarbamate



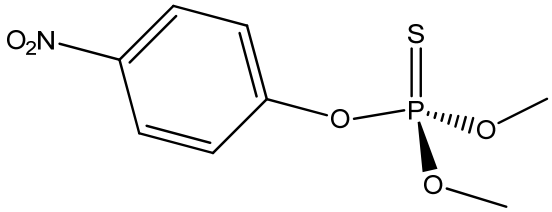
20. Methoxychlor (OC): 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane



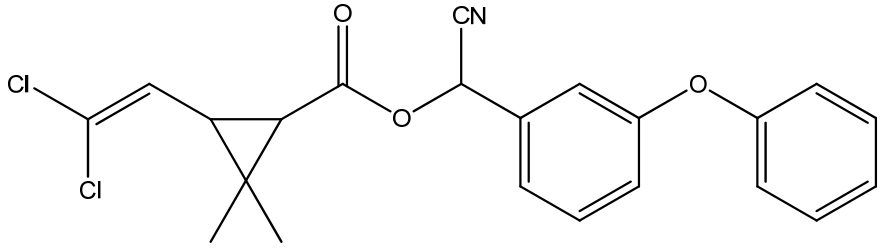
21. Parathion (OP): O,O-diethyl O-4-nitrophenyl phosphorothioate



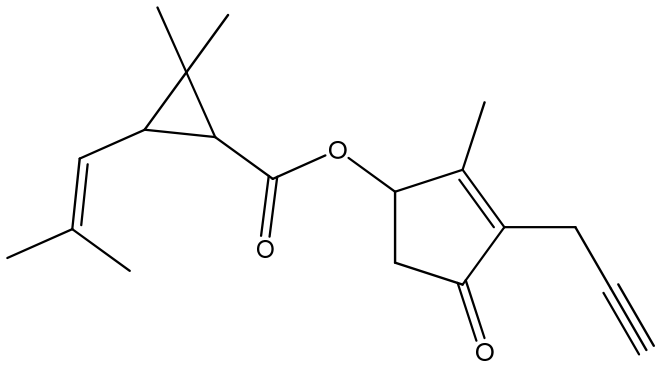
22. Parathion methyl (OP): O,O-dimethyl O-4-nitrophenyl phosphorothioate



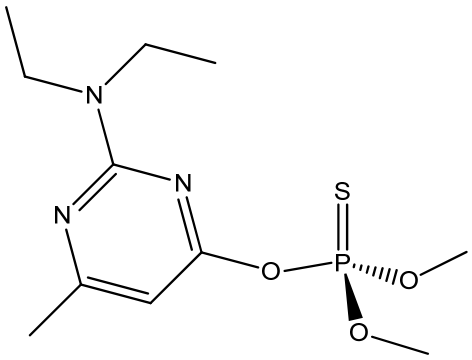
23. Permethrin (pyrethroid): 3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate



24. Prallethrin (pyrethroid): (RS)-2-methyl-4-oxo-3-prop-2-ynylcyclopent-2-enyl (1RS,3RS;1RS,3SR)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate

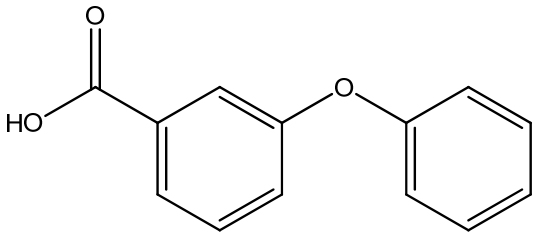


25. Pirimiphos methyl (OP): O-[2-(diethylamino)-6-methylpyrimidin-4-yl] O,O-dimethyl phosphorothioate

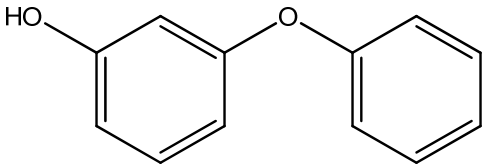


Alphabetic listing of degradates by common name – parent compound in parentheses)

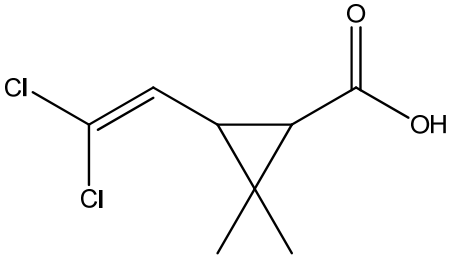
1. 3-phenoxybenzoic acid (nonspecific pyrethroid)



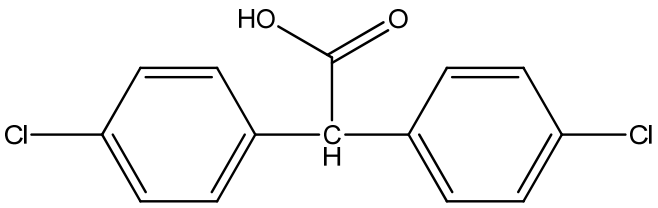
2. 3-phenoxybenzyl alcohol (nonspecific pyrethroid)



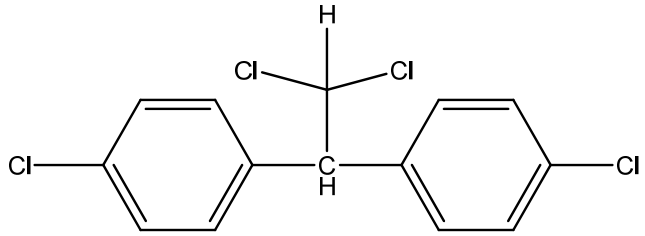
3. DCCA: cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (nonspecific pyrethroid)



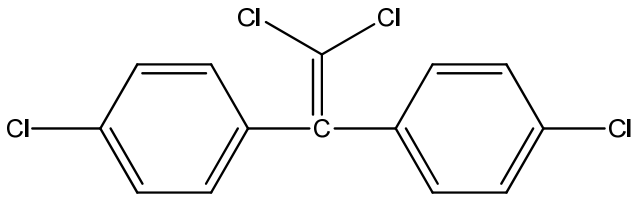
4. DDA: 2,2-bis(4-chlorophenyl)acetic acid (DDT)



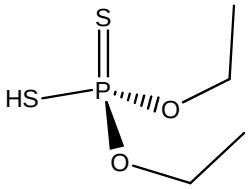
5. DDD: 1,1-dichloro-2,2-bis(4-ethylphenyl)ethane (DDT)



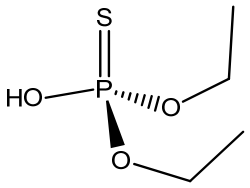
6. DDE: 1,1-bis-(4-chlorophenyl)-2,2-dichloroethane (DDT)



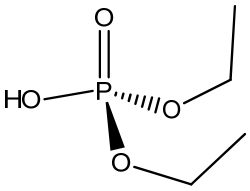
7. DEDTP: diethyldithiophosphate (nonspecific OP)



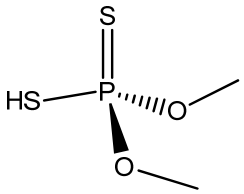
8. DETP: diethylthiophosphate (nonspecific OP)



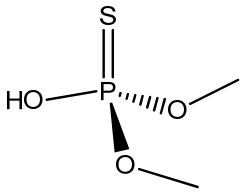
9. DEP: diethylphosphate (nonspecific OP)



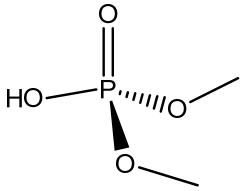
10. DMDTP: dimethyldithiophosphate (nonspecific OP)



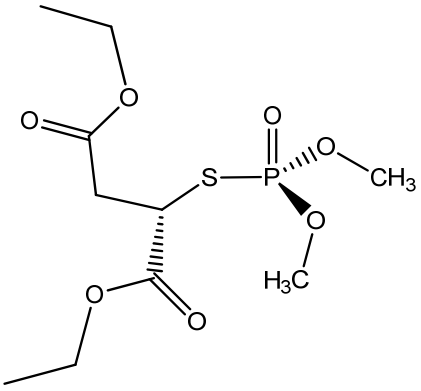
11. DMTP: dimethylthiophosphate (nonspecific OP)



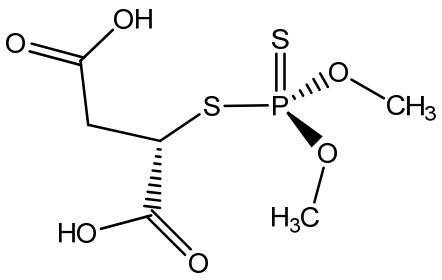
12. DMP: dimethylphosphate (nonspecific OP)



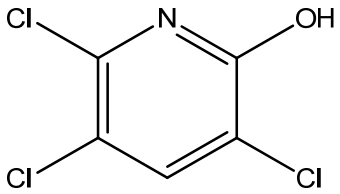
13. Maloxon (malathion)



14. MDA: malathion dicarboxylic acid (malathion)



15. TCPy: 3,5,6-trichloro-2-pyridinol (chlorpyrifos, chlorpyrifos methyl)



CHAPTER 7: REFERENCES

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