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Xianyu Chen Date

I. Development of a passive sampler and static sampling chamber to measure personal exposure to gaseous PAHs. II. Method development for determination of current-use and persistent pesticides in cow milk, human breast milk, baby formulas, and human serum using gas chromatography tandem mass spectrometry.

By

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Doctor of Philosophy

Chemistry

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Date

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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2012

Abstract

I. Development of a passive sampler and static sampling chamber to measure personal exposure to gaseous PAHs. II. Method development for determination of current-use and persistent pesticides in cow milk, human breast milk, baby formulas, and human serum using gas chromatography tandem mass spectrometry.

By Xianyu Chen

Exposure to polycyclic aromatic hydrocarbons (PAHs) is regarded as an important environmental risk factor for humans. A passive sampler made of 80 sections of 2 cm long GC columns was tested in our study. The main purpose of this passive sampler is to detect the amount of gaseous PAHs people breathe in every day. Since the sampling rate is a critical parameter that evaluates how well a passive sampler works, we developed a “static chamber” to determine this rate. Ultimately, we will use these results to design a sensitive, reliable, simple, economical and user-friendly passive sampler to measure personal exposure to gaseous semi-volatiles.

An analytical method to determine organochlorine (OC), organophosphate (OP), carbamate, and pyrethroid insecticide residues in cow milk, human milk, and baby formulas was developed. This method involves a liquid-liquid extraction, freezing-lipid filtration, and solid-phase extraction procedure followed by gas chromatography tandem mass spectrometry (GC-MS/MS) for the identification and quantification of targeted pesticides.

The sample preparation method for pesticides analysis in milk proposed above was tested and modified to analyze OC, OP, pyrethroid, and carbamate pesticides in serum. However, during the method validation process, this method and its modified ones were both proved to yield unsatisfying recoveries and fail to clean-up serum matrices. Afterwards, a different method was adapted, modified, and validated for pesticides analysis in pooled serum samples. This sample preparation method started with C18 SPE cartridges and was followed by florisil SPE procedures. Extracted samples were analyzed in GC-MS/MS for the identification and quantification of targeted pesticides.

In the GC-MS/MS analysis, ionization of pesticide molecules was achieved by electron ionization in positive mode. Multiple reaction monitoring (MRM) was the acquisition mode used for the monitoring of two MS/MS transitions for each analyte.

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To all the people in my lab, thanks for make our lab like a big happy family. It was such a pleasure to work with you guys. I couldn't ask for better lab mates. I also need to give especial thanks to Geneva C Bernoudy and Albert Sang-Kyu Lee. Geneva, thanks for making sure our lab has all the supplies on time and I don't need to worry about doing experiment without proper lab supplies. Albert, thanks for your hard work and helping me do all these tedious and time-consuming extractions without any complains.

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Lastly to my dearest family, you guys are the best things that have ever happened in my life. Thanks for the endless support and encouragement. You are there for every single step I have taken, every small success I have achieved, and every mistake I have made. Thanks for loving me the way I am and constantly making sure I am on the right track. Without you, none of this will become true. Thanks you!

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List of Acronyms

1-MET	1-Methylnaphthalene
2-MET	2-Methylnaphthalene
ACET	acenaphthene
ACET-d10	acenaphthene-d10
ACEY	acenaphthylene
ACN	acetonitrile
ant	anthracene
atr	atrazine
azm	azinphos-methyl
ben	bendiocarb
chlm	chlorpyrifos-methyl
cpy	chlorpyrifos
CV	coefficient of variation
cyf	cyfluthrin
cyp	cypermethrin
DCM	dichloromethane
DDE	dichlorodiphenyldichloroethane
ddeop	o,p-DDE
ddepp	p,p-DDE
DDT	dichlorodiphenyltrichloroethane
ddtop	o,p-DDT
del	deltamethrin
dia	diazinon
dic	p,p-dicofol
EC	endcapped
EC	endcapped
EI	electron ionization
EI	electron ionization
endoA	endosulfan-α
endoB	endosulfan-β
EPA	Environmental Protection Agency
fen	fenobucarb
fev	fenvalerate
FID	flame ionization detector
FLU	fluorene
FLUT	fluoranthene
fon	fonofos
GC	gas chromatography
GCB	graphitized carbon black
GPC	gel permeation chromatography

GPC	gel permeation column
hcb	hexachlorobenzene
hep	heptachlor epoxide
HLB	hydrophilic–lipophilic balanced copolymer
ICH	International Conference for Harmonization
IS_chlm	chlorpyrifos-methyl D6
IS_cpy	chlorpyrifos D10
IS_cyp	13C-cypermethrin
IS_ddepp	13C-p,p-DDE
IS_par	parathion D10
ISTD	internal standard
LOD	limits of detection
MW	molecular weight
NAP	Naphthalene
OC	organochlorine
OCPs	organochlorine pesticides
OP	organophosphate
PAH	polycyclic aromatic hydrocarbon
par	parathion
PBDE	polybrominated diphenyl ether
pbo	piperonyl butoxide
PCB	polychlorinated biphenyl
PCBs	polychlorinated biphenyls
per	permethrin
PHE	phenanthrene
PHE-d10	phenanthrene-d10
pral	prallethrin
PSA	primary and secondary amine
PYR	pyrene
res	resmethrin
RR	response ratio
RT	retention time
S/N	signal-to-noise
SD	standard deviation
SPE	solid phase extraction
SPE	solid phase extraction
TS	time segment
ACh	acetylcholine
AChE	acetylcholinesterase
CI	Chemical Ionization

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Chapter 1 Exposure, human health, and exposure assessment

1.1 A short history of exposure assessment

With the development of science and technology, numerous chemicals and toxicants have been used in daily life. It is almost impossible for normal people to totally avoid exposure to these toxicants. Throughout human history, there have been many incidents that human were exposed to contaminants and toxicants in both occupational and community settings. In 1600s, the London "Fumifugium" (sulfurous smog) was a famous example.[1] In 1832, the drinking water in East London was contaminated with infected sewage, causing the British cholera epidemic and 6536 victims in London.[2] Methyl isocyanate, a toxic petrochemical normally used in the production of rubber and adhesives, was responsible for killing thousands of people in Bhopal, India during the 1984 chemical spill.[3]

Despite the mortality behind these numerous incidents, they evoked a response in society and greatly contributed to the development of public health. In the 20th century, the systematic analysis of occupational exposure and the corresponding health response began with the work done by Alice Hamiltion and many others.[4] However, the analysis of community exposure began much later this. This is because in occupational settings, the potential sources of exposure are usually easy to identify, ranging from one to multiple chemicals, while in community settings, the potential sources of exposure are not so easy to identity and the exposures are generally much lower than in occupational settings.

In the late 1930s, many organizations began to collect information on the toxicity of contaminants, exposure routes, and human health effects, which helped publish the first set of occupational exposure limits.[5] This also helped push the idea of exposure limit to the industrial hygiene community. In the late 1980s, threshold limit values (TLVs) for over 600 chemicals were available from the American Conference of Governmental Industrial hygienists (SCGIH), and federal standards personal exposure limits (PEL) from Occupational Safety and Health Administration (OSHA) were also reauthorized.[6,7] Around the same time period, Food and Drug Administration lowered the allowable content of toxicants and biological agents in food, consumer products, and drinking water. All these regulations and exposure limits indicated the public, academic, industrial, and governmental awareness of chemical pollution.

Two steps are involved when a chemical entering the human body: contact (exposure), followed by actually entry (crossing the boundary). In order to establish the relationship of a toxicant to a biological effect, the quantitative measure of human exposure to this toxicant must be obtained first. Exposure to toxicants and chemicals can occur through three main routes: ingestion, inhalation, and dermal absorption. In 1997, The US EPA Exposure Factors Handbook explains these exposure routes in details. People can be exposed to toxicants through ingestion route when they consume drinking water, fruit and vegetable, meat and dairy, breast milk, fish and shellfish, soil, and grain. While for contaminants in air, people are mainly exposed through inhalation. Dermal exposure can happen through various activities in different environment. Such as activities

involving water (swimming, bathing, etc.), soil (gardening, construction, etc.), and indoors (carpets, floors, countertops, etc.).

1.2 Inhalation route and its exposure assessment

The respiratory system contains three main parts: nasopharyngeal, tracheobronchial, and pulmonary.[9] Each part can remove some of pollutants from the inhaled air. The nasopharyngeal part filters out large particles inhaled. The tracheobronchial part helps move particles from deep areas of the lung to the oral cavity where these particles can be swallowed and excreted later. The pulmonary part can engulf particles entering certain part of the lung. Despite all the mechanisms of removal found in each of the part, pollutants can still deposit in various regions of the lung.

Unlike large particles in the air, gaseous pollutants are more evenly dispersed in the air and more easily to enter the human body. According to Integrated Risk Information System (IRIS), the default inhalation rate of people is 20 m^3/day.[9] By measuring the concentration of a certain pollutant in the air, the amount of this pollutant entering into human body during certain amount of time can be estimated.

Before the 1980s, the most common way to estimate personal exposure in community settings was from stationary monitors located outdoors or from emissions measured as they exit from a process.[10] The results were used to make health related ambient air standards. However, during that time, more and more people realized that this kind of

measurement was very rudimentary and could not reflect the real personal exposure very accurately. With the development of technology in this area, more approaches were developed to do personal exposure assessment in community settings, such as personal monitoring, indoor air measurements, and outdoor activity related air monitoring.[11]

More details will be discussed in Chapter 2 of this dissertation.

1.3 Pesticides, toxicity, and their exposure routes

Pesticides are any substance or mixture of substances which are intended for preventing, destroying, repelling, or mitigating any pests. They can also be used as plant regulators, defoliants or desiccants. Pesticides can be either man-made or natural. Since nowadays, pesticides have been so widely used in different areas, it is almost impossible for people to totally avoid pesticides exposure. Therefore, pesticides exposure and assessment have become a hot topic throughout the past few decades.

Usually, pesticides can be divided into two different categories: persistent pesticides and non-persistent pesticides. Persistently pesticides are stable in in the environment and resist being broken down, while non-persistent pesticides are compounds that break down quickly in the environment. Persistent pesticides mainly consist of organochlorine pesticides (OCs). Because OCs are persistent in the environmental and inexpensive to make, they were widely used in agriculture and insect control in the early

and mid-20th century. Non-persistent pesticides include several different classes of pesticides such as organophosphates, carbamates, chlorinated phenols, pyrethroids, selected herbicides and fungicides.

Among all these pesticides, we will focus on four main classes of neurotoxic insecticides, which are organophosphates (OPs), organochlorines (OCs), pyrethroids, and carbamates. Figure 1-1 shows the target sites of these insecticides. OPs and carbamates can inhibit acetylcholinesterase (AChE), which is an enzyme that regulates neurotransmitter levels at the synapse. OCs and pyrethroids can interfere with impulse transmission along the axon.

Some type of OCs has significantly toxicity to plants or animals, including humans. One of the well-known examples is dichlorodiphenyltrichloroethane (DDT), which was widely used in the mid-20th century, can accumulate in food chains and cause reproductive problems in certain bird species by interfering with calcium metabolism in birds and the eggshells that are using calcium compounds for structural integrity.[12]

Pyrethroids usually interact with neurons and slow the kinetics of sodium channels by slowing both the activation and inactivation phases of channel gating, causing neurons to become hyper-excitable and even spontaneously active.

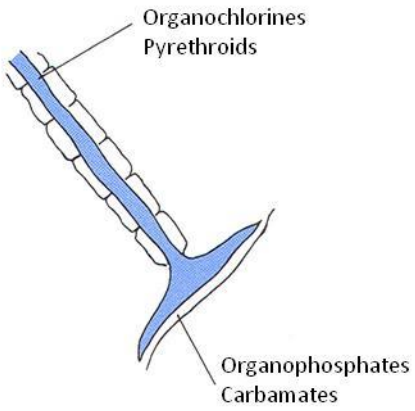


Figure 1-1 Potential action sites of the four classes of insecticides on the axon and terminal portions of the nerve.[13]

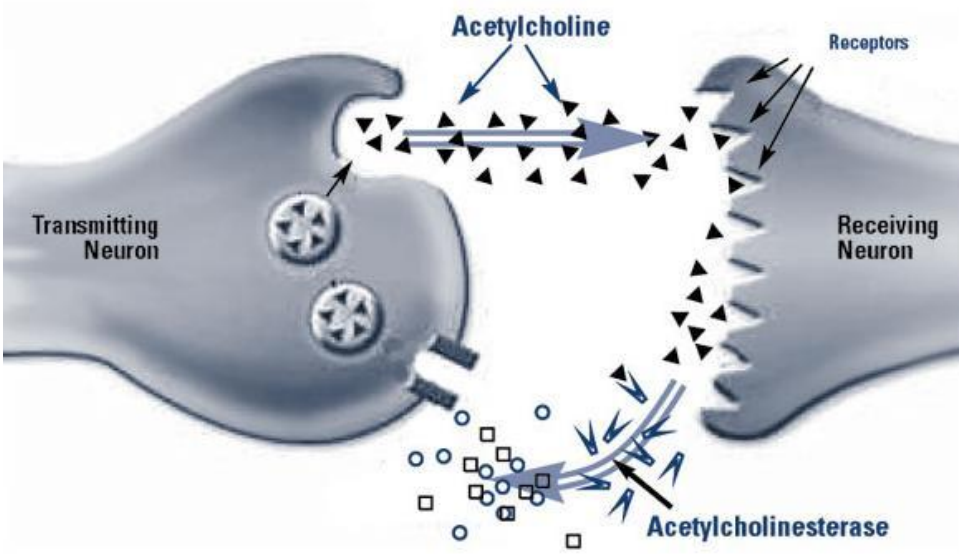


Figure 1-2 Normal neurotransmission involving acetylcholinesterase.[14]

During a normal neurotransmission, after ACh (acetylcholine) is released from the nerve into the synaptic cleft, it binds to ACh receptors on the post-synaptic membrane, which relays the signal from the nerve. Afterwards, AChE, which locates on the post-synaptic

membrane, terminates the signal transmission by hydrolyzing ACh (Figure 1-3 (A)). The free choline from the previous process is then taken up by the pre-synaptic nerve and ACh is synthesized through choline acetyltransferase. If a cholinergic neuron wants to receive another impulse, ACh must be released from the ACh receptors, which only happens when the concentration of ACh in the synaptic cleft is very low. Therefore, inhibition of AChE causes accumulation of ACh in the synaptic cleft and results in impeded neurotransmission.[15]

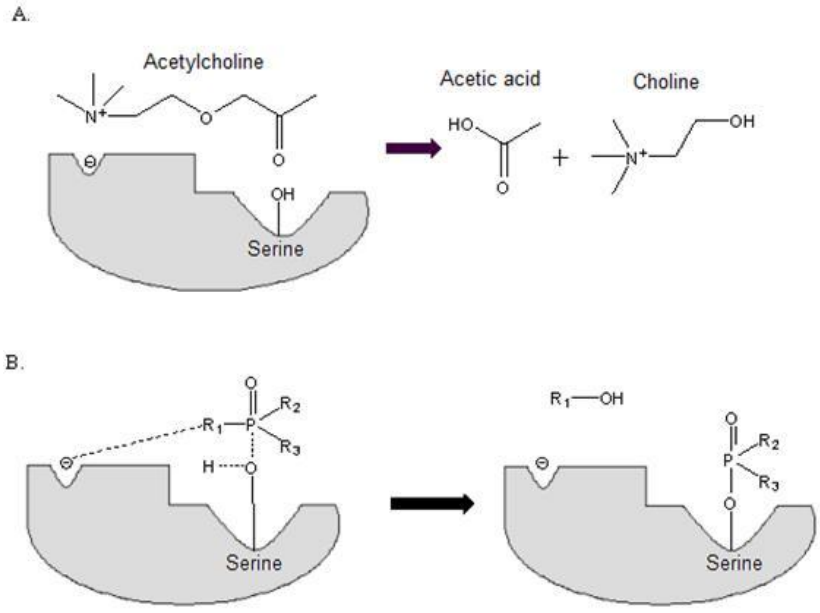


Figure 1-3 Choline Esterase Inhibitors[16]

OPs belong to a class of irreversible AChE inhibitors. As shown in Figure 1-3 (B), OP can leave a phosphoryl group in the esteratic site of AChE, which is slowly hydrolyzed in days and can become covalently bound, interrupting the hydrolysis process of ACh. Unlike

OPs, carbamates belong to reversible AChE inhibitors, which only occupy the esteratic site of AChE for a short period and can be hydrolyzed from minutes to hours.[18]

People can be exposed to pesticides through all three different routes: ingestion, inhalation and dermal absorption. However, for people with different professions and different ages, the main routes of exposure might vary. For instance, farmers are mainly exposed through dermal absorption while applying pesticides in the field; adults who do not contact with pesticides professionally are exposed to pesticides mainly through their diet; children at their early stage usually crawl on the ground and may put their hands in the mouths, which increase their chances to be exposed to pesticides through dermal absorption and ingestion.

1.4 Breast milk

Understanding the toxicity of the above pesticides is important when studying neurodevelopment in the developing fetus and in infants. In general, the period of developing an organ is more vulnerable than the period before or after an organ is developed, which means exposure happening during the development of an organ is more likely to cause adverse effects. Therefore, it is important to understand the time lines of normal neural development in humans, which is shown in Figure 1-4, with the prenatal period scaled in months and the postnatal period scaled in years.

The central nervous system, brain and spinal cord, begin to develop in the early stage of prenatal period. The nervous system continues to develop until the later stage of the postnatal period. Interruption of neural development during this period can result in severe abnormalities. Some people think that the placental barrier could help block the pesticides exposure from mothers because it is composed of structures that separate the maternal and the fetal blood. However, when it comes to pesticides exposure, these compounds can penetrate the placenta and impact the developing embryo and fetus.

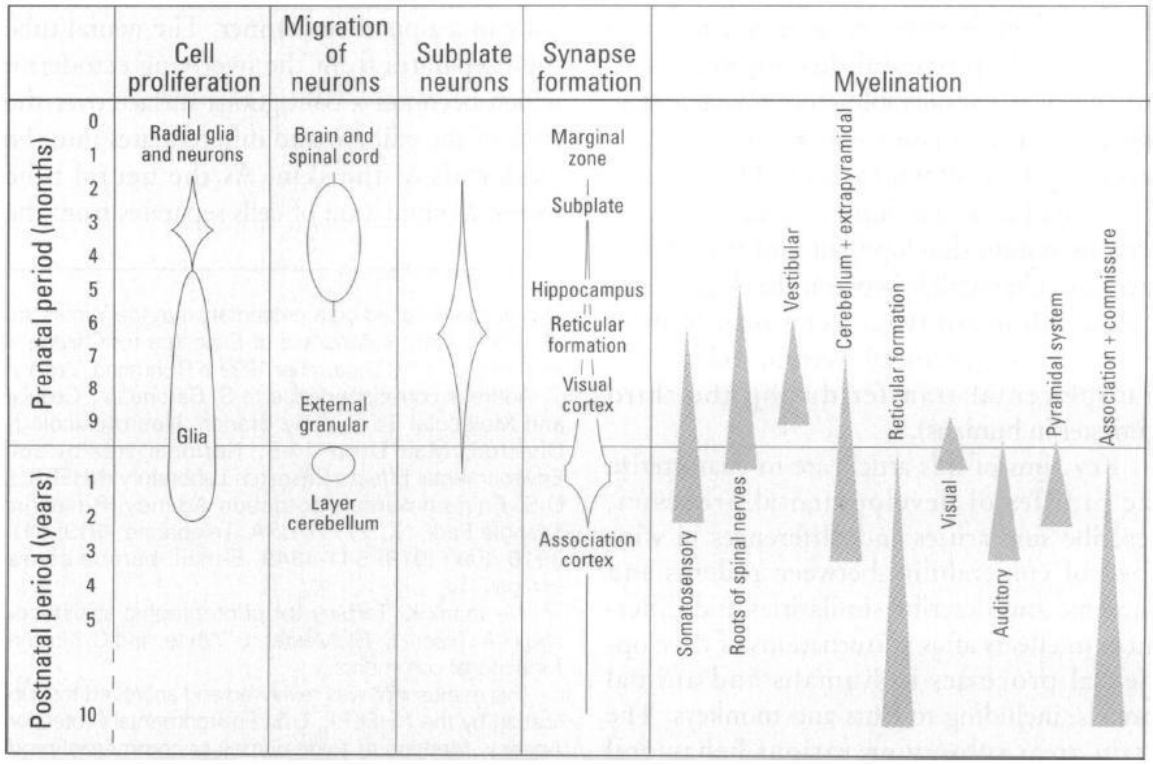


Figure 1-4 Time lines for developmental process in the early stage of humans.

Most epidemiologic studies nowadays are mainly focused on pesticides exposure and the effects on neurodevelopment during prenatal period. However, as we can see from

Figure 1-4, many critical neurodevelopmental processes continue from birth to the infancy and does not stop until several years later. Despite these vulnerabilities, the lack of pesticides exposure during infancy is a fact that is in an urgency to be addressed.

One of the reasons for this scientific knowledge gap is the difficulty to collect biological samples from infants. It is easier to collect maternal blood, urine, and umbilical cord blood from mothers for prenatal pesticides exposure study and collect blood and urine from elder children. In epidemiologic studies, pesticides measurement in biological samples is preferred over environmental samples because the results from these biological samples are more straightforward and directly reflect the absorbed dose. Usually, blood or serum is preferred for assessing OCs exposure because the non-polarity of OCs makes them retain in blood and fat and excrete in breast milk. On the other side, because human body produces water soluble metabolites for OPs, carbamates, and pyrethroids, urine is preferred in analysis of the exposure to OPs, carbamates, and pyrethroids.

It is not recommended to collect blood and can be very challenging to collect urine from infants. However, since infants have relatively homogeneous diets, breast milk can be a great indicator of pesticides exposure for neonates and pre-weaning infants.

Chapter 3 of this dissertation will focus on developing and validating methods for assessing neonates' and infants' dietary pesticides exposure using breast milk.

1.5 Serum

While the research project with breast milk focuses on pesticides exposure during infancy, the serum methods developed in Chapter 4 can evaluate exposures to the fetus during gestation by measuring the serum content of mothers.

With the increased supply of fresh fruits and vegetables, as well as various pesticides used for pest control, pesticides exposure-related diseases are continuously affecting human health. The four classes of pesticides mentioned earlier are either acutely toxic at high doses or have the potential to exert more subtle toxicity at lower levels. Therefore, serum can also be a great biomarker to indicate pesticides exposure of elder children and adults.

1.6 Objective and significance

This dissertation contains three different research projects covering different techniques to measure personally exposure to environmental pollutants and toxicants, which provides useful supplement data for future reference in exposure assessment. The passive sampler and static sampling chamber project provides data for inhalation exposure assessment. Breast milk project provides a useful and easy way to assess ingestion pesticides exposure of neonates and infants. Finally, the serum project provides way to measure pesticides exposure of fetuses, elder children, and adults.

Chapter 2 Development of a passive sampler and static sampling chamber to measure personal exposure to gaseous PAHs

2.1 Introduction

PAHs and human health concerns

PAHs have at least two fused benzene rings and only contain carbon and hydrogen, whose pure chemicals are usually colorless, white, or pale yellow-green solids and sometimes with a weak but pleasant odor. They are a group of over 100 different chemicals that mainly come from the incomplete burning of fossil fuels, wood, garbage, tobacco, or other organic substances[20]. Some PAHs are contained in asphalt that is used in road construction, and some others can be used to make dyes, plastics and pesticides or even be used in medicines[22].

Eighteen PAHs were selected in our research projects, which are naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, and benzo(g,h,i)perylene. Comparing to the other PAHs, these 18 PAHs were chosen because (1) more information is available; (2) there is higher chance that human beings will be exposed to; (3) it is suspected that they are either more harmful or their harmful effects are representative of the other PAHs; and (4) within all the identified PAHs, they are among the highest concentration levels in some of hazardous waste sites.[23]

Some PAHs are known or suspected carcinogens and may be related with other health problems.[24] Although some harmful effects have not been seen in people, animal studies have shown that PAHs can be harmful to the skin, body fluid, and ability to fight diseases after either short or long term exposure. PAHs will enter the human body when people breathe in contaminated air that contains PAHs. When exposed to PAHs, some other compounds presenting in the environment at the same time can affect the rate that PAHs enter the body. PAHs can enter all the tissues of the body that contain fat and can be stored in the kidneys, liver, and fat. After PAHs enter the body, they are changed into many different substances. Some PAHs have the ability to bind with DNA and thus cause cancer. Fortunately, most PAHs will not stay in the body for a long time and will leave primarily in the feces and urine. The federal government has set regulations to protect people from the possible health effects of eating, drinking, or breathing PAHs. For example, based on the data from the EPA, taking in 0.3mg of anthracene, 0.06mg of acenaphthene, 0.04mg of fluoranthene, 0.04mg of fluorene, or 0.03mg of pyrene per kilogram of the body weight each day will not harm the health.

Personal sampling

Indoor, outdoor and occupational air concentrations of PAHs can be monitored by either stationary or personal monitors. A stationary sampler is usually placed at a height that is the same as the breathing zone in different areas, such as living rooms, fields, and workplaces. A personal sampler is portable and should be placed as close to the breathing zone as possible. Research shows that there are differences between results

obtained by stationary samplers and personal samplers. In particular, stationary samplers may underestimate the true personal exposure to pollutants in the air.[28] Therefore, personal samplers may be a better choice for monitoring the exposure to gaseous PAHs. For this, there are two approaches: active samplers and passive samplers.

Active samplers

Active samplers are the most common method used today by the CDC and EPA.[29] Active samplers usually contain two parts. One part is a glass, quartz fiber, or Teflon filter, which is used to collect particle associated materials. The other part is a solid adsorbent located on line downstream from the filter to collect gaseous compounds that pass through the filter. The solid adsorbent is usually a polyurethane foam plug or XAD resins. It can also retain compounds that volatilize from the filter during sampling. Active samplers need a pump to pull air passing through the sampling module, and a flow meter to precisely measure the volume or flowing rate of the air that passes through the sampling module. Both the pump and the flow meter need an outside power supply. Different active samplers work under quite different flowing rates, and the sampling time is typically from several hours to one day. Peaks, ceilings, and weighted average concentrations of pollutants during the sampling time can be known from the result.[30] The denuder sampler is another type of active samplers. It uses a denuder tube to collect gaseous compounds that pass from downside to upside of the filter.[31]

Passive samplers

Passive samplers, which were introduced 30 years ago, rely on diffusion for mass transport and therefore do not require any types of pumping apparatus. The first passive sampler was a tube-type diffusive sampler for sulfur dioxide and nitrogen dioxide in occupational environments in 1973.[32] A variety of passive samplers have been described since then. They rely on diffusion through an air gap, permeation through a membrane, or both. Various passive samplers are used to measure air pollutants such as volatile organic compounds (VOCs), nitrogen dioxide (NO_2), and sulfur dioxide (SO_2) in indoor, occupational, and outdoor settings. However, their use is far less common than active samplers because of sensitivity problems, and to my knowledge, only one passive sampler has been used as a personal monitor in community settings.[33] Although passive samplers have not been widely used in occupational and indoor air monitoring, they are more frequently used outdoors. Passive samplers vary in designs, sizes, shapes, and adsorbents. There are different types of adsorbents, such as polymer resins, chemical reagents, and porous adsorbents. The mechanism of passive samplers is based on diffusion. Most passive samplers have a high capacity to sequester pollutants but need a long sampling time, and have a linear uptake over the sampling period. Therefore, it is critical to know the sampling rate.

Generally, passive samplers are based on the free flow of analytes from the atmosphere to the adsorbent. It is assumed that the adsorbent is uniform and traps chemicals through gaseous diffusion and adsorption. The analytes can attach on the adsorbent

either chemically or physically. For physical adsorption, it can capture analytes via $\pi - \pi$ interaction, Van der Waal's force, or both. The sampling process continues until experimenters stop the experiment. Generally, experimenters want the capacity of the sampler to greatly exceed the amount captured during the sampling period, which limits the so-called "back diffusion" to a negligible level.

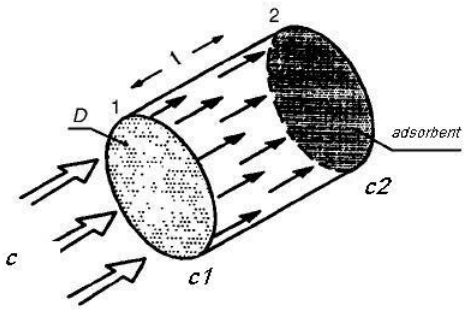


Figure 2-1 Diagram of diffusion process (Brown et al. 2000) [34]

In Figure 2-1, position 1 is the beginning of the diffusion path of an analyte whose concentration is c_1. In ideal cases, the adsorbent at position 2 will reduce the concentration of the analyte c_2 to zero because of adsorption. The concentration gradient is the driving force for the diffusion. The diffusive passive samplers rely on the principles of Fick's first law, which can be described as

$$J = -D\frac{\partial c}{\partial x} \qquad (1)$$

Where J = the diffusion flux, D = the diffusion coefficient, c = the concentration, and x = the position.

Assuming the concentration is zero at the surface of the absorbing medium, the concentration gradient is linear, and there is no back diffusion, the mass of the analyte that is absorbed by diffusion based on equation 1 can be determined as

$$m_s = \frac{ADctk}{l} \qquad (2)$$

Where A = the cross-sectional area of the diffusion path, c = the concentration of the given analyte in the air outside the sampler, l = the length of the diffusion path, and k = the correction factor that is equal to 1.0 for the ideal system. In ideal cases, the diffusive sampling rate can be described as

$$Q = \frac{m_s}{ct} = D\frac{A}{l} \qquad (3)$$

Comparison between active samplers and passive samplers for personal sampling

Active samplers give high accuracy and precision if correctly calibrated. They are good at providing information for an incidental point exposure since their sampling time is short. However, active samplers require a pump, which may give errors due to flow variability. A pump requires electricity or a battery and is usually bulky and noisy. Furthermore, it should only be used by trained people. All these disadvantages make active samplers less preferable than passive samplers in personal sampling. Compared with active samplers, passive samplers, which do not need a pump, are easier to handle and less expensive. Generally, passive samplers are small and light, and do not produce noise, which means that they do not affect people's daily life when sampling. However, the

disadvantages are that the sampling rates are usually very low compared to active samplers and can be affected by various external factors such as temperature, wind, and back diffusion.

A newly developed passive sampler

Fan (Fan et al. 2006) has developed a sensitive, simple, and cost-effective passive sampler (Figure 2-2) to measure personal exposure to gaseous PAHs in community settings.[33] It contains 4 units each consisting of 80 sections of 1cm long SPB-5 GC columns [poly (5% diphenyl with 95% dimethylsiloxane)] (0.75mm i.d., 7 μ m film thickness), which are tied together as a unit and placed in a stainless steel tube. Both ends of the sampler are exposed to the air when sampling.

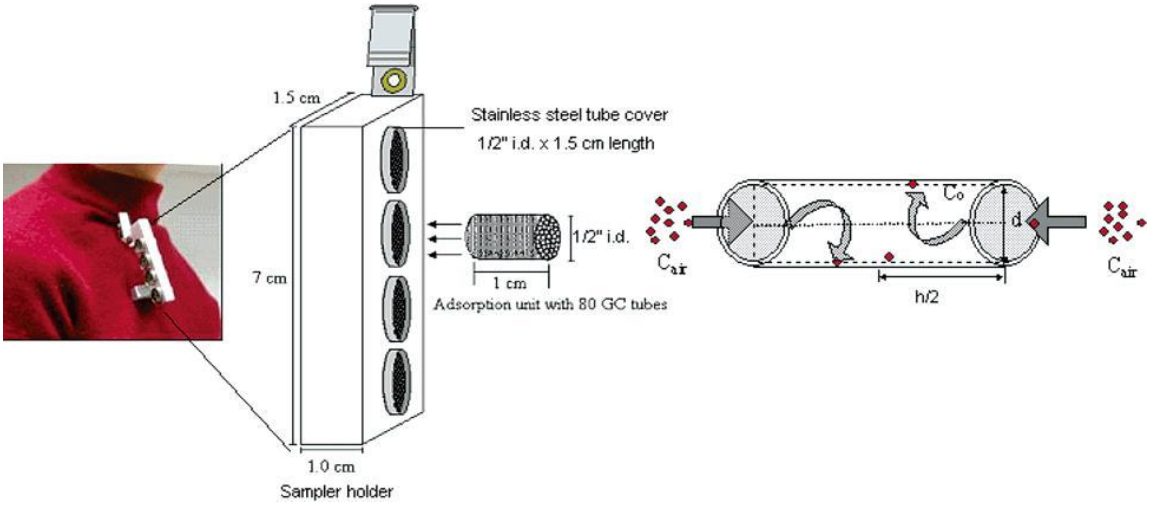


Figure 2-2 Schematic diagram of the passive sampler and its diffusion process (Fan et al. 2006)[33]

The maximum diffusion length L and the maximum adsorption area A are presented as

$$L = \frac{d+h}{2},\ A = \pi dhN \qquad (4)$$

Where d = the diameter of the column, h = the tube length, and N = the total number of tubes. From equation 3 and equation 4, the ideal sampling rate can be calculated, and it is easy to see that a large N with a large diameter will give a large sampling rate. Based on the analytical detection limits and the PAH concentrations (10-2000ng/m^3 for naphthalene, and even smaller for the other compounds) in the testing system, Fan found that a 320 1cm long SPB-5 GC column with a 750 µm internal diameter and a 7 µm film thickness provided a high enough sampling rate (~30 mL/min) for measuring PAHs in the tests.[33] Besides the geometry of the sampler, Brown has described other parameters that will affect the sampling rate, such as the physical and chemical property of the adsorbent, sampling duration, PAHs concentration, humidity, face velocity, and temperature.[34] Since there is no way to avoid influences from these parameters, the ideal sampling rate may not be realized when sampling. The actual sampling rate needs to be determined and evaluated under different sampling conditions. Therefore, it is necessary to develop a well characterized chamber to test the actual sampling rate.

Fan generated a dynamic dilution system providing a controlled test atmosphere in the laboratory to evaluate this passive sampler's real sampling rate (Figure 2-3).[33] Each pure solid PAH standard was placed in a diffusion vial in oven #2, which could be set at a desired temperature to produce the required emission rate. The PAH gas mixture generated in the diffusion vial was diluted with a purified air stream and introduced into

the mixing ball, where all the PAHs and humidity-controlled air stream were well mixed, to provide a continuous supply of gaseous PAHs at designated concentrations. The humidifier, mixing ball, and passive sampling house were placed in oven #1 where the temperature could be controlled. The active sampler was used to collect parallel samples. The sampling rate of the passive sampler was calculated as

$$Q = Q_a \frac{M_p}{M_a} \qquad (5)$$

Where M_p and M_a are the PAH masses collected on the passive sampler and the active sampler, respectively; Q_a is the sampling rate of the active sampler.

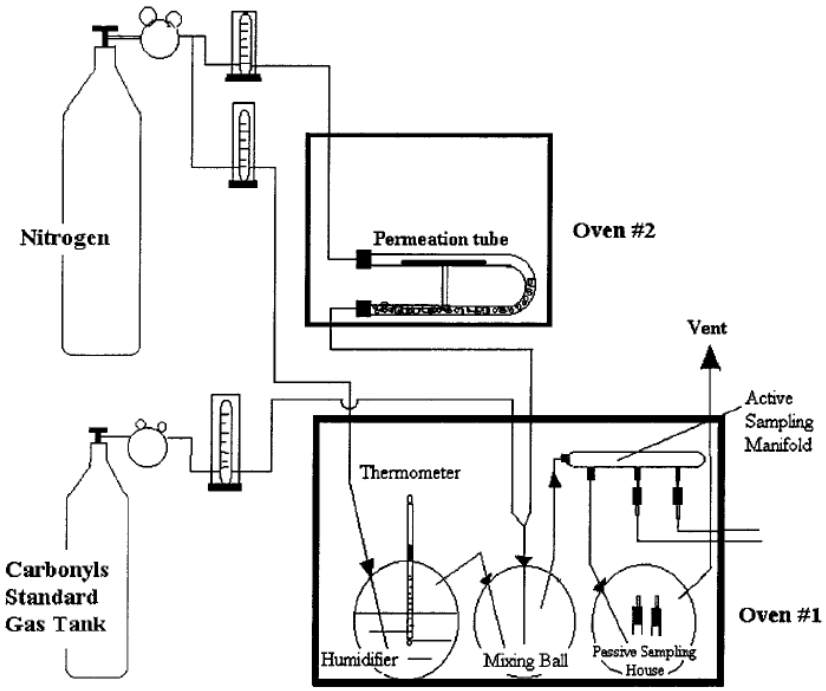


Figure 2-3 Dynamic dilution system for generation of a controlled test atmosphere (Zhang et al. 2000)[35]

Similar to the system above, most chamber work requires the development of a constant source of a contaminant in a flow chamber. Such chambers are expensive to construct since they require a source of the contaminant, which is normally a diffusion tube or similar apparatus, a pump, sampling ports, etc.

In our study, we are attempting to develop a so-called “static chamber” to evaluate the passive sampler. In a static chamber, a known amount of material is introduced into the chamber as an initial concentration, and the concentration is reduced exponentially by both the sampling itself and the leakage of the chamber.

2.2 Chemicals, standard and internal standard preparation

EPA method 8310 PAH mixture (500 µg/mL in acetonitrile) containing the 18 PAHs mentioned above was purchased from Restek (Bellefonte, PA). Acenaphthene-D10 (2000 µg/mL in methanol) was purchased from Protocol Analytical LLC (Metuchen, NJ). Phenanthrene-D10 (1000 µg/mL in methylene chlride) was purchased from SPEX CertiPrep. 1-Methylnaphthalene (100 µg/mL in toluene), 2-Methylnaphthalene (100 µg/mL in methanol), naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene were purchased from Chem Service (West Chester, PA). All solvent used were of analytical grade. We obtained acetonitrile from Fischer Scientific (Phillipsburg, NJ, USA), hexane and dichloromethane from Sigma Aldrich.

Individual stock solutions of the 10 native standards (naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene) were prepared for each analyte in acetonitrile. The mix stock solution and ten calibration standard solutions of varying concentrations ranging from 1-2000 ppb were prepared by adding EPA method 8310 PAH mixture in acetonitrile. An internal standard working solution containing the two labeled analytes (acenaphthene-D10 and phenanthrene-D10) was prepared at 400ppb in acetonitrile. All the solutions were at -20^{o}C.

2.3 Chamber Development and Characterization

In a static chamber (Figure 2-4), a known amount of analytes was put on the watch glass and introduced into the chamber. After evaporation, the gaseous analytes was collected by passive samplers in the chamber. The concentration of the analytes was chosen to be similar to that found in the environment in field study. A desiccator cabinet made from acrylic (inside dimensions: 30.5 cm $\times$ 30.5 cm $\times$ 30.5 cm) was used as the static chamber in our research to provide a sealed system. A small fan was placed inside the chamber to accelerate the evaporation of the analytes and make sure the gaseous analytes inside the chamber were well mixed. Passive samplers were hung on the shelf.

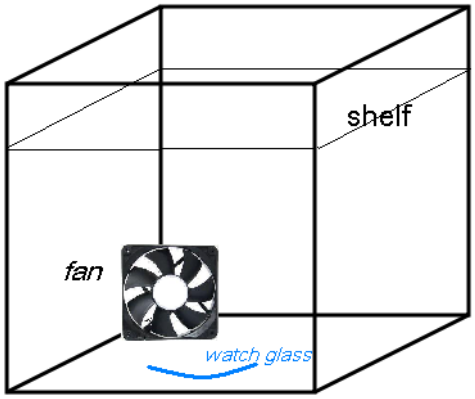


Figure 2-4 Schematic diagram of the static chamber

The mathematics inside the chamber while sampling could be described as

$$\frac{dA}{dt} = -kA,\ k = \frac{(S+L)}{V} \rightarrow A(t) = Ae^{-kt}$$

Where A = initial amount of analytes in the chamber, t = time, S = sampling rate of passive samplers in the chamber, L = leakage rate of the chamber, and V = volume of the chamber.

Total amount of analytes collected on samplers together with these leaked out of the chamber within T time period (M_T) can be calculated as

$$M_T = A - A(T) = A - Ae^{-kT} = A\left(1 - e^{-kT}\right)$$

Since $\frac{S+L}{S} = \frac{M_T}{M}$ (where M = amount of analytes collected on samplers), with some unit conversion and the known volume of the chamber in our case (28.3 L), the sampling rate of the passive sampler could be calculated as

$$\left(1+\frac{\mathrm{L}}{\mathrm{S}}\right)\mathrm{M}=\mathrm{A}\left(1-\mathrm{e}^{-2.143\times10^{-3}(\mathrm{L+S})\mathrm{T}}\right)$$

Where L = leakage rate of the chamber (mL/min), S = sampling rate of passive samplers in the chamber (mL/min), A = initial amount of analytes in the chamber (ng), M = amount of analytes collected on samplers (ng), and T = sampling time (hour).

Therefore, in order to determine the sampling rate of the passive sampler, it was necessary to know the leakage rate of the chamber. A solvent was put in the chamber to saturate its space and the loss of the solvent within a time range was recorded. The leakage rate can be calculated as

$$R=\frac{V_{loss}\times V_{chamber}}{V_{saturation}\times T}$$

Where T = the amount of time that the solvent was left in the chamber (in min), V_{loss} = the volume of the solvent that was lost in the time range (in mL, already deducting the volume needed to saturate the chamber), $V_{saturation}$ = the volume of the solvent needed to saturate the chamber (in mL), and $V_{chamber}$ = the volume of the chamber (in mL).

Five different solvents varying in vapor pressure, acetonitrile, acetone, ethyl acetate, hexane, and water, were used in this experiment. If water was used, since there is water molecules in the air, the equation for the leakage rate was a little different, which can be described as

$$R=\frac{V_{loss}\times V_{chamber}}{V_{saturation}\times(1-x)\times T}$$

Where x = the relative humidity in the external environment (in fraction of saturation).

At standard pressure and room temperature, 5.8 mL of acetonitrile, 20.7 mL acetone, 11.37 mL ethyl acetate, 26.4 mL hexane, and 0.456 mL water are needed to saturate the chamber respectively; the values reflect the vapor pressure of each compound.

Acetonitrile was put in a cylinder and placed in the chamber to check the leakage first, but the loss of acetonitrile in 24 hours was so small that it was difficult to record the data precisely. The same thing happened when we used water. For this reason, the more volatile compound, acetone and hexane, were used. However, restricted by the volume capacity of the cylinder, acetone and hexane were too volatile and could not provide enough volume loss data in a single experiment.

Therefore, ethyl acetate, whose volatility is between acetonitrile and acetone, was used in two independent but parallel experiments that provided two sets of data (Figure 2-5).

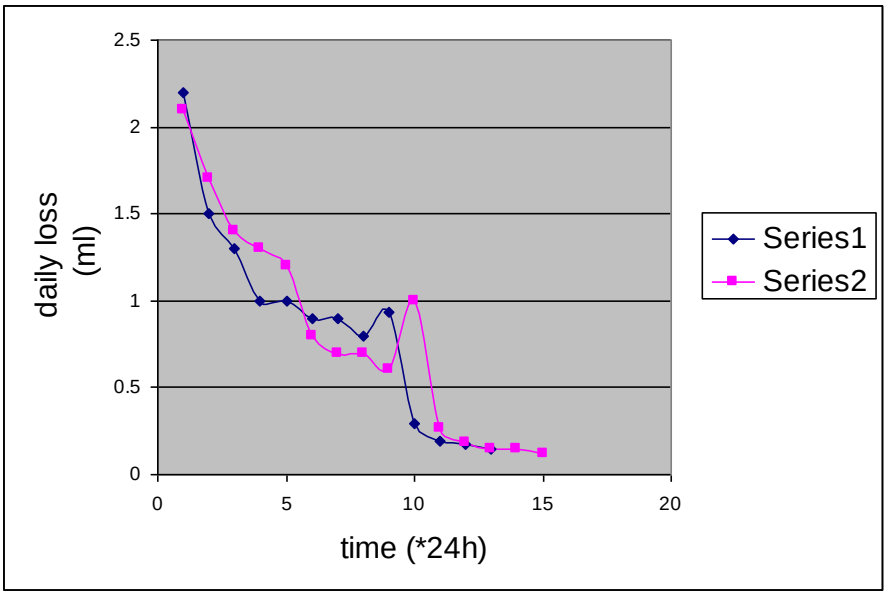


Figure 2-5 Daily loss of ethyl acetate to check the leakage rate of the static chamber

These two lines dropped quickly at the beginning, and became smooth in the end. Theoretically, if the leakage rate of the chamber is consistent, there should be two flat lines on the graph.

With further research, we found out that the sudden jump in the middle of each curve was due to the shape of the cylinder we used to fill ethyl acetate and was placed in the chamber afterwards. In the gas exchange process between two phases, a larger cross sectional area has smaller resistance to diffusive flow, while a smaller one has larger resistance.[36] The cylinder we used in the experiment has a larger cross sectional area on the top and a smaller one in the bottom. Since the cross sectional area at the bottom of the cylinder was small, the gas exchange across this surface manifested a larger resistance. The evaporation rate of ethyl acetate in the bottom of the cylinder was not fast enough to compensate the leakage loss, resulting in what the figure presented.

Therefore, the data in Figure 2-5 was influenced by the resistance between two phases and could not reflect the real situation.

To overcome the flow resistance between two phases, two types of beakers with different cross sectional areas (A: 17.1cm^2 and B: 32.6cm^2) were used, which both have larger cross sectional areas than the cylinder. Since larger cross sectional areas were available to reduce the flow resistance, less volatile solvent than ethyl acetate could be used. Therefore, a beaker filled with acetonitrile was put in the chamber and the remaining volume of acetonitrile was recorded every 24 hours. The data was summarized in Table 2-1. Although there is no straightforward mathematic relationship between the cross sectional areas and the daily loss of the solvent, beaker B with larger cross sectional area generally had more daily loss of acetonitrile than that of Beaker A, which is consistent with flow resistance theory.

Table 2-1 Daily loss of acetonitrile in beaker A and B in the chamber

Daily loss		
Beaker A (mL)		Beaker B (mL)
#1	#2	
47.1	48.8	65.2
30.0	31.4	50.5
24.0	21.8	43.0
		37.2
		33.9

However, it was almost impossible for the chamber, which was manufactured to provide a relatively sealed environment as a desiccator cabinet, to lose large amount of

acetonitrile each day. One possible reason is that acetonitrile reacts with acrylic, which is used to make the chamber. This is confirmed by Nalgene. There are limited solvents that will not harm acrylic, including hexane and water. Due to the low volatility of water, results from water are usually not very accurate since the daily loss of water is very small. After considering the volatility and the safety of acrylic, hexane became the only choice.

In order to make the cross sectional area as large as possible within the acceptable dimension to overcome the flow resistance between the liquid and gas phases, a culture dish (cross sectional area: 176.6cm^2) with hexane was placed in the bottom of the chamber. After several hours, severe condensation was observed. It might be caused by two reasons: (1) the high volatility of hexane; and (2) the air movement from the fan accelerated the evaporation of hexane and caused over-saturation in the chamber. We successful stopped the condensation after placing the culture dish filled with acetonitrile onto the shelf in the chamber to avoid the air movement from the fan. The chamber, which had a culture dish filled with acetonitrile on its shelf, was kept in an environment with relatively stable temperature to conduct the leakage rate checking experiment. Four sets of parallel experiments were conducted and the leakage rate of the chamber was about 28.3 ± 1.7 mL/min (Table 2-2). This leakage rate of the chamber was close to the sampling rate of the passive samplers in Fan's paper (~30 mL/min)[33].

Table 2-2 Leakage Rate of the Chamber

Experiment Number	Volume of the loss (mL)	Time (hours)	Leakage Rate (mL/min)
# 1	111.60	66.00	29.89
# 2	113.60	69.00	29.10
# 3	110.60	69.00	28.33
# 4	98.60	67.00	26.01
Average leakage rate			28.34
SD			1.67

In order to install the fan inside the chamber, we drilled a hole on the wall of the chamber to let the power wire go through. Although we sealed the hole with clay, it might still be responsible for the high leakage rate of the chamber. The original idea to include the fan was to mix the air in the chamber and ensure that every corner of the chamber is saturated at all times. The next experiment was conducted to check if the fan is necessary.

A culture dish filled with water was placed on the shelf with the fan off. A digital humidity recorder in the low corner of the chamber showed that the chamber reached saturation in 30 minutes without the fan. Since the volatility of hexane is much higher than water, it is reasonable to assume that hexane needs less than 30 minutes to saturate the chamber without the fan. Comparing with the experimental time of more than 60 hours, the time for the chamber to reach saturation of hexane vapor is

negligible. Therefore, a brand new chamber without the hole and the fan was introduced to conduct the leakage rate checking experiment, in which a culture dish filled with hexane was placed on the shelf. Results were shown in Table 2-3. The final leakage rate of the chamber was about 4.86 mL/min, which was reduced remarkably. The leakage rate of the chamber was updated periodically.

Table 2-3 Leakage of the chamber using hexane as checking solvent in a long time period

Volume of the loss (ml)	Time (hours)	Leakage Rate (mL/min)
179.5	504	4.89
171.3	480	4.83

2.4 GC Method

A Hewlett-Packard Model 5890A Series II GC equipped with an Agilent Technologies (Santa Clara, CA) model flame ionization detector (FID) and a 7683B Series Injector autosampler (Agilent Technologies, Santa Clara, CA) was used.

Between the DB-5 GC column (5% diphenyl - 95% dimethyl polysiloxane, 30 m, 0.25 mm i.d. and 0.25 µm film thickness) and HP-1 GC column (100% dimethylpolysiloxane, 30 m, 0.32 mm i.d. and 0.52 µm film thickness), HP-1 column gave us better chromatogram for PAHs on GC-FID.

We started with the temperature programming in Table 2-4 and got the GC-FID chromatogram as shown in Figure 2-6 (A). As we can see, two sets of peaks, benz(a)anthracene and chrysene, as well as benzo(b)fluoranthene and

benzo(k)fluoranthene, did not get enough baseline resolution. After adjusting the temperature programming to the one in Table 2-5, baseline resolution, minimal peak broadening and tailing were achieved as was shown in Figure 2-6 (B). Retention time of each PAHs was used to accurately identify its peak. With better baseline resolution, the area count of each compound could be measured more accurately.

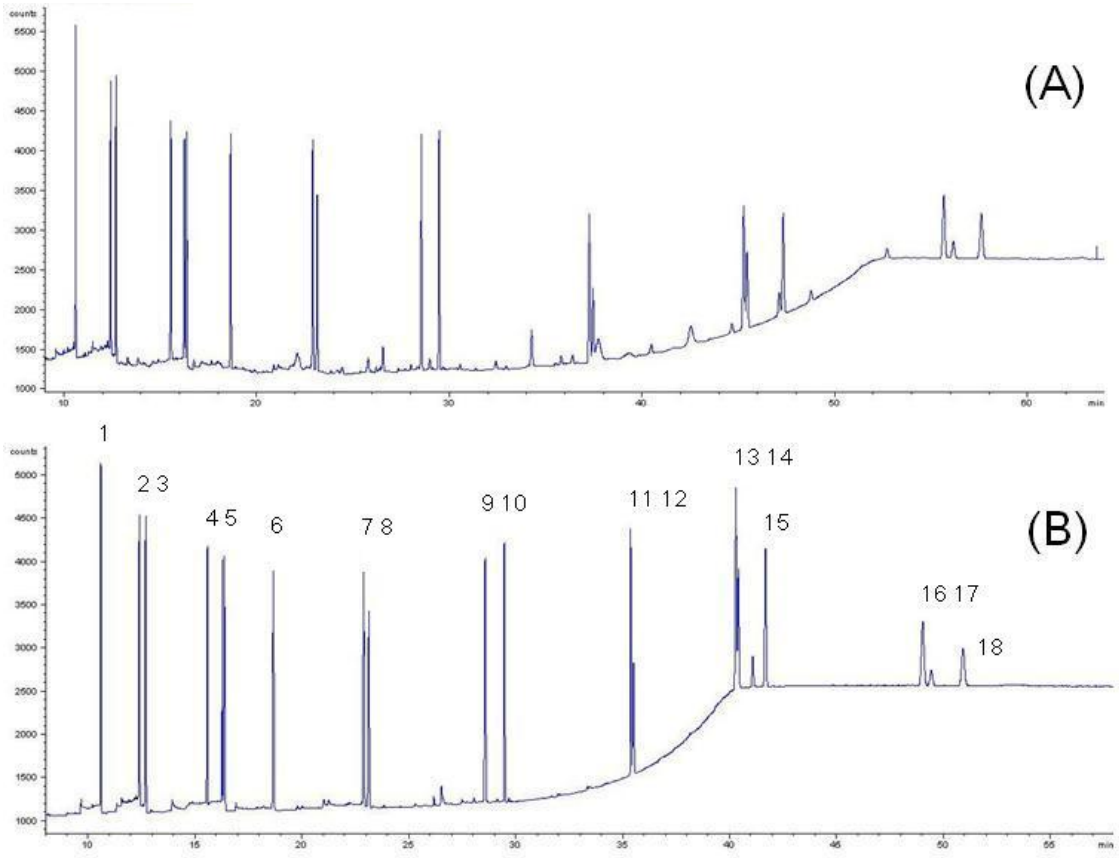


Figure 2-6 GC-FID chromatogram of the 18 PAHs. X-axis = time (min). Y-axis = area counts. 1, naphthalene; 2, 1-methylnaphthalene; 3, 2-methylnaphthalene; 4, acenaphthylene; 5, acenaphthene; 6, fluorene; 7, phenanthrene; 8, anthracene; 9, fluoranthene; 10, pyrene; 11, benz(a)anthracene; 12, chrysene; 13,

benzo(b)fluoranthene; 14, benzo(k)fluoranthene; 15, benzo(a)pyrene; 16, indeno(1,2,3-cd)pyrene; 17, dibenz(a,h)anthracene; 18, benzo(g,h,i)perylene.

Table 2-4 Temperature ramping parameter for Figure 2-6 (A)

	Rate (^{0}C /min)	Final Temp (^{0}C)	Final Time (min)
Level 1	10	130	2
Level 2	5	210	2
Level 3	2.5	260	12.5

Table 2-5 Temperature ramping parameter for Figure 2-6 (B)

	Rate (^{0}C /min)	Final Temp (^{0}C)	Final Time (min)
Level 1	10	130	2
Level 2	5	260	14.5

After adjusting the gas flow rate and split/splitless ratio, in the finalized GC-FID method, all the samples were injected into GC-FID with a HP-1 GC column (100% dimethylpolysiloxane, 30 m, 0.32 mm i.d. and 0.52 µm film thickness). The temperate grogramming began at 35 ^{0}C, held for 2 min, 35-130 at 10 ^{0}C/min to 130 ^{0}C, held for 2 min, 130-260 at 5 ^{0}C/min to 260 ^{0}C, and then held for 14.5 min. The helium carrier gas was at a constant flow of 3-4 mL/min. Nitrogen makeup gas, air and hydrogen flow rates were 30, 350 and 35 mL/min, respectively. The injection was 1.0 µL with a split/splitless ratio of 1/30. Other relevant analytical parameters included 2 mm i.d. single taper injection liner, injection port temperature of 260 ^{0}C, and FID temperature of 270 ^{0}C.

2.5 The characterizations of passive samplers made from GC capillary columns

Each passive sampler used in our research projects was constructed from 80 sections of 2cm long gas chromatography (GC) capillary columns, which were tied together with a stainless steel wire. The sampler was hung on the shelf in the static chamber to sample for 24 hours before taken out for extraction. The extract from the solvent extraction was injected in GC-FID for analysis. Passive samplers were re-used after baking at 250 ^{0}C for two hours. The amount of PAHs samplers collected during the 24 hours sampling time was quantified through a multi-point calibration curve (Figure 2-7) derived from the GC-FID analysis of 10 calibration standard solutions.

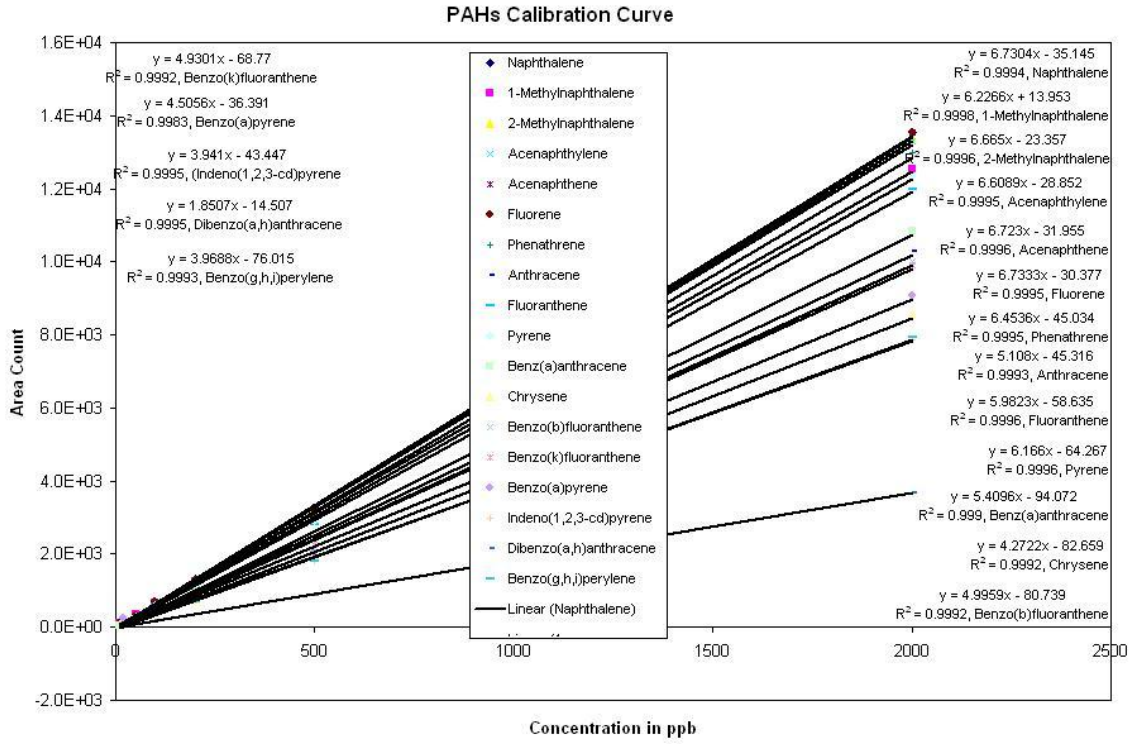


Figure 2-7 PAHs Calibration Curve

In our experiments, three types of GC Columns with column coating materials varying in polarity were used (Table 2-6).

Table 2-6 Parameters of HP-1, DB-5 and DB-17 GC columns

Column Name	Inner Surface Coating Materials	i.d. (mm)	Film Thickness (μm)
HP-1 GC column	100% Dimethylpolysiloxane	0.32	0.52
DB-5 GC column	5% diphenyl - 95% dimethyl polysiloxane	0.25	0.25
DB-17ms GC column	50% phenyl - 50% methyl polysiloxane	0.32	0.25

Solvent Extraction for passive samplers after sampling

After sampling in the chamber for 24 hours, passive samplers were taken out and the flexible wires binding the column pieces together were removed. The column pieces from each sampler were transferred into a clean and hexane-rinsed 15ml disposable centrifuge tube for solvent extraction. The solvent extraction procedure was listed below.

Tube 1 with 80 column pieces from a sampler + 5 mL Hexane
→ Vortex for 2 min
→ Hexane Extract into Tube 2
→ Tube 1 + 5 mL 1:1 Hexane/Dichloromethane
→ Sonicate Tube 1 for 20 min
→ Extract into Tube 2
→ TurboVap Extract in Tube 2 to 300 µl at 30 ^{0}C and 10 psi
→ Transfer to a GC auto-sampler vial and inject into GC-FID

2.6 Results and Discussion

The cutting-ends of newly made samplers were usually very rough, while the ones of used samplers were usually smooth, which might influence the diffusion length L and the adsorption area A, and thus could lead to different sampling rates.

Twelve used DB-5 passive samplers with smooth cutting-ends sampled in the chamber with 100 µL PAHs working solution (10 ppm in acetonitrile) on the watch glass for 24 hours. Every 3 out of the 12 samplers were put together as a group for extraction. The same procedure was repeated with 12 newly made DB-5 samplers with rough cutting-ends. Because of the low vapor pressures of the high molecular weight compounds, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene,

benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene and benzo(g,h,i)perylene, no useful data was obtained for these compounds. Therefore, Table 2-7 only shows the data for the ten low and medium molecular weight PAHs, which were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene. All the later experiments were also only based on these 10 PAHs.

Table 2-7 Comparison of newly made DB-5 samplers and used ones

PAHs Name	Area Counts from the GC-FID chromatogram							
	Newly made samplers				Used samplers			
	1	2	3	4	5	6	7	8
naphthalene	359.5	348.5	241.9	244.2	241.7	142.5	196.1	210.5
1-methylnaphthalene	513.3	599.1	547.4	530.2	533.6	559.9	546.4	577.6
2-methylnaphthalene	78.5	130.6	152.7	113.5	60.3	90.6	92.6	139.7
acenaphthylene	114.7	86.7	91.4	115.9	94.3	133.3	92.5	186.8
acenaphthene	687.7	231.2	218.4	218.9	200.9	295.5	200.5	120.4
fluorene	668.2	440.0	396.9	412.0	525.0	392.9	409.5	450.5
phenanthrene	69.3	943.3	88.3	1297.8	240.7	319.8	206.1	101.6
anthracene	238.4	68.2	81.8	175.2	69.9	319.8	90.0	215.4
fluoranthene	215.7	241.0	254.3	122.3	460.7	400.5	383.4	84.7
pyrene	169.4	85.1	72.2	58.8	103.3	75.8	82.4	208.4

Table 2-8 Two Way Analysis of Variance of the data from Table 2-7

Source	DF	ANOVA SS	Mean Square	F Value	Pr > F
PAHs	9	1802533.568	200281.508	7.42	<0.0001
Sampler	1	43543.112	43543.112	1.61	0.2090

PAHs*Sampler	9	344479.950	38275.550	1.42	0.2010

The ANOVA results in Table 2-8 confirmed that the sampling rate of each PAH was different. However, there was no significant difference between the newly made samplers and the used ones. We could also avoid the rough cutting-ends with better and correct cutting techniques.

Individual PAH was introduced into the chamber with two DB-5 samplers each time for 24 hours. The experiment for each PAH was conducted in duplicate. The data shown in Table 2-9 was the average of the duplicate.

Table 2-9 Amount of PAHs collected on two DB-5 samplers in 24 hours and their sampling rates

PAHs	Column type	Number of samplers	Initial amount of PAH in the chamber (μg)	Amount of PAHs collected (μg)	Sampling rate (mL/min)
naphthalene	DB-5	2	12900	3.101	0.018
1-methylnaphthalene	DB-5	2	10	0.065	0.504
2-methylnaphthalene	DB-5	2	10000	20.209	0.155
acenaphthylene	DB-5	2	1270	2.395	0.145
acenaphthene	DB-5	2	1640	2.202	0.103
fluorene	DB-5	2	1320	0.924	0.054
phenanthrene	DB-5	2	28.2	0.083	0.226
anthracene	DB-5	2	1.26	0.037	2.342
fluoranthene	DB-5	2	2.21	0.057	2.026
pyrene	DB-5	2	7.76	0.02	0.198

In the field, we usually expect to collect only 8-40 ng PAHs, while as shown in Table 2-9, each sampler could capture much more PAHs than 40 ng. This means that these passive samplers can act as “infinite” sinks for PAHs in the field.

The sampling rates in Table 2-9 were small in general. Eight of them were even less than 1mL/min, while the ones in Fan’s paper were around 30ml/min. The i.d. of the column we used (0.25 mm) was much smaller than the one in Fan’s paper (0.75 mm), and we only used 2 samplers instead of 4.[33] These two differences could result in lower sampling rates in some extend according to Equation (3) and (4), but would not cause the sampling rates drop so severely.

Majority of the sampling rates in Table 2-9 increased with decreasing initial PAHs concentrations in the chamber, which suggested that the samplers possibly reached their saturation points before 24 hours, resulting in the low sampling rates when we did the calculation based the experimental time of 24 hours.

A sampler was put in the chamber with 5ml 25.39ppm acenaphthylene for 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, and 24 hours respectively to assess our assumption. We chose acenaphthylene because it gave clear peaks on the GC chromatogram, and had more consistent retention time and lower toxicity than the other 9 PAHs compounds. If the sampler did not reach the saturation point, we would get an increasing relationship if we used the mass collected by the sampler and the sampling time to plot a graph. However, from 4 hours to 24 hours, the mass collected by the sampler was the same, which meant that the sampler reached its saturation point in

less than 4 hours. Since the sampler reached its saturation point in less than 4 hours, the calculated sampling rates in Table 2-9 were smaller than the actual ones. Therefore, in the later experiments, we lowered the initial PAHs concentrations (2-20 μg in the chamber for each trial) to avoid saturation.

After lowering the initial PAH concentration levels in the chamber, we re-conducted some previous experiments with DB-5 passive samplers, whose results were shown in Table 2-10. Almost all of them had improved sampling rates comparing with the ones in Table 2-9, noting that the sampling rates in Table 2-10 were based on one DB-5 sampler while the ones in Table 2-9 were based on two DB-5 samplers.

Table 2-10 Sampling rates of single DB-5 sampler with lower initial PAHs concentrations in the chamber in 24 hours

PAHs	Column type	Number of samplers	Initial PAH concentration in the chamber (ng/mL)	Sampling Rate (mL/min)
acenaphthylene	DB-5	1	0.02214	1.405
acenaphthene	DB-5	1	0.02729	0.677
fluorene	DB-5	1	0.03179	0.211
phenanthrene	DB-5	1	0.02857	0.740
anthracene	DB-5	1	0.02943	0.950
fluoranthene	DB-5	1	0.02671	1.356
pyrene	DB-5	1	0.02993	0.846

After lowering the initial concentration levels of PAHs in the chamber, the amount of naphthalene, 1-methylnaphthalene and 2-methylnaphthalene collected on the sampler

was too low to detect on the GC-FID under the current settings. This indicated that we were losing these three low molecular weight PAHs. There are two reasons for this. First, because of their high volatility, we might lose the low molecular weight PAHs in the TurboVap step of the solvent extraction. This is the intrinsic property of the molecules, and we are unable to change it. Second, due to their volatility, they did not adsorb to the column materials of the sampler very well and might diffuse back into the air in the chamber. Therefore, column materials of samplers that can preferentially absorb low molecular weight PAHs were needed.

We used DB-5 GC columns (cross-linked/surface bonded 5% phenyl with 95% methylpolysiloxane, 0.25mm i.d., 0.25µm film thickness) to make passive samplers for all the experiments mentioned above. This type of columns has both π-π interaction and Van der Waal's force. It may be impossible to increase the Van der Waal's force, but if we pick a more polar column, such as a DB-17ms GC column (cross-linked/surface bonded 50% phenyl with 50% methylpolysiloxane, 0.32mm i.d., 0.25µm film thickness), it might provide stronger π-π interaction to capture the low molecular weight PAHs. However, with the limited data in Table 2-11, we could not prove that DB-17 passive sampler could capture more volatile PAHs better. Further experiments were conducted to do the comparison.

Table 2-11 Sampling rates of three DB-17 passive samplers for low molecular weight PAHs in 24 hours

PAHs	Column	Number of	Initial PAH	Sampling

	type	samplers	concentration in the chamber (ng/mL)	rate (mL/min)
Naphthalene	DB-17	3	0.12250	0.105
1-Methylnaphthalene	DB-17	3	0.10714	0.106
2-Methylnaphthalene	DB-17	3	0.10893	0.225

Comparing with the chamber leakage rate (~5 mL/min), the sampling rates of DB-5 and DB-17 samplers were lower (< 2mL/min), which meant that small errors in determining the leakage rate of the chamber would greatly influence the sampling rate calculation of passive samplers. In order to increase the sampling rate, more samplers were used in each sampling trial for later experiments.

Based on all the previous data, the modified experimental conditions for later experiments were (1) initial amount of PAHs in the chamber (2-20 μg), (2) five samplers were used in the chamber for each sampling trial, and (3) time for each trial remained 24 hours.

In order to better compare DB-17 and DB-5 samplers, we conducted experiments under the modified experimental conditions (Table 10). No useful data was obtained for naphthalene, 1-methylnaphthalene and 2-methylnaphthalene. However, for the medium molecular weight PAHs in Table 2-12, DB-5 sampler had higher sampling rate than DB-17 sampler.

Table 2-12 Sampling rates of five DB-5 and DB-17 samplers

	$S_{DB\text{-}17}$ (mL/min)	$S_{DB\text{-}5}$ (mL/min)	$\Delta S = S_{DB\text{-}5} - S_{DB\text{-}17}$ (mL/min)

acenaphthylene	0.326	1.606	1.280
acenaphthene	0.686	2.756	2.070
fluorene	0.846	0.927	0.081
phenanthrene	2.177	2.536	0.359
anthracene	2.581	4.027	1.446
fluoranthene	2.344	4.98	2.636
pyrene	2.191	3.551	1.360

The next experiment was designed to check how much PAHs we lost during the TurboVap step, especially for the three low molecular weight PAHs we could not get useful data from. 1 mL analyte solution with known concentration was mixed with 6.5 mL Hexane and 2.5 mL Dichloromethane. This mixture was concentrated to 300 µL using a TurboVap at 30^0C and 10psi of air and injected into GC for analysis. We did both separate and mix trials. In separate trials, only one compound was tested each time using its individual stock solution, while in mix trials, the 1 mL analyte solution contained all 18 PAHs. The percent error in Table 2-13 was calculated as

$$\text{Percent Error} = \frac{\text{Calbulated Moles from GC data} - \text{Initial Moles in 1 mL analyte solution}}{\text{Initial Moles in 1 mL analyte solution}} \times 100\%$$

Table 2-13 Percent Error of PAHs in separate and mix trials

PAHs Name	Percent Error (%)	
	Separate trials	Mix trials
Naphthalene	-9.24	32.53
1-Methylnaphthalene	32.96	81.49
2-Methylnaphthalene	38.33	81.09
acenaphthylene	-8.66	5.10
acenaphthene	-7.62	15.12
fluorene	-4.22	8.72
phenanthrene	-8.26	14.72
anthracene	-21.88	24.78

fluoranthene	-19.63	54.56
pyrene	-11.89	62.10

Instead of giving negative percent errors to reflect the loss from TurboVap step, all the percent errors in mix trials and two of them in separate trials were positive. Some of the percent errors in mix trials were even higher than 50%.

The most likely reason for the results in Table 2-13 was that the response of GC varied slightly from run to run. For instance, gas flow rate might vary by few percent in a chromatography experiment, which cause the change of the detector response. Sometimes, a calibration curve is only accurate for the one set of samples running in the same batch under the same conditions. In order to overcome these problems, internal standards are usually introduced, which are widely used in chromatography. If signal from the internal standard increases by 5% due to the change of the gas flow, signal from the analytes usually also increases by 5%. Since the area under each peak on the chromatogram is proportional to the concentration of that analyte injected into the GC, response factor F can be calculated as[38]

$$\frac{\text{Area of analyte signal}}{\text{Concentration of analyte}} = \text{F}\left(\frac{\text{Area of standard signal}}{\text{Concentration of standard}}\right)$$

With the known concentration of the internal standard and the known response factor F of the detector, the more accurate concentrations of analytes can be calculated. Most of the time, the relative response of an instrument to the analyte and internal standard

remains the same over a range of concentrations. However, in some critical cases, the assumption of constant response factor can lead to errors up to 40%.[39]

Two internal standards (acenaphthene-D10 & phenanthrene-D10) were introduced into our later experiments in order to increase the accuracy of GC analysis.[40] Experiments to get response factor F were conducted in two different concentrations (1000 ppb and 2000 ppb) repeatedly using PAHs working solution. The ANOVA analysis of the experimental results were shown in the Table 2-15, which indicated that each PAH had a different F and the F might also be different between the two concentration levels (1000 ppb and 2000 ppb) for each PAH. Table 2-14 shows the average of relative response F of the FID detector to each PAH under the two different concentration levels, which was used in the later calculations of experimental results to get more accurate PAHs concentrations.

Table 2-14 the average relative response F of the FID detector to each PAH under 1000ppb and 2000ppb concentration levels

PAHs Name	Internal Standard	Response Factor F
Naphthalene	acenaphthene-D10	0.8549
1-Methylnaphthalene	acenaphthene-D10	0.8724
2-Methylnaphthalene	acenaphthene-D10	0.9449
acenaphthylene	acenaphthene-D10	1.0163
acenaphthene	acenaphthene-D10	1.0311
fluorene	acenaphthene-D10	1.1046
phenanthrene	phenanthrene-D10	1.1131
anthracene	phenanthrene-D10	0.8648
fluoranthene	phenanthrene-D10	1.1484
pyrene	phenanthrene-D10	1.1918

Table 2-15 ANOVA results of response factor F for the 10 PAHs under two different concentration levels

Source	DF	Pr > F
PAHs	9	<0.0001
Concentration	1	0.017

As we discussed earlier, PAHs loss could occur during TurboVap step of the extraction procedure prior to GC analysis. If a known amount of internal standard was added the extract before the TurboVap step, we could assume the ratio of standard to analyte remained constant because the same fraction of each was lost in TurboVap step. Since the PAHs collected by 5 passive samplers in previous experiments were usually in the range of 10-200 ng, 80 ng of each internal standard (acenaphthene-D10 & phenanthrene-D10) were added to samples right before the TurboVap step.

In some previous experiments, we tried to compare the sampling rates of DB-5 and DB-17 samplers, but did not get enough data. The nest experiments were designed to do the comparison among DB-5, DB-17 and HP-1 samplers and assess if PAHs will interfere with each other during the sampling process. In the separate trial, each PAH was tested individually and the sampling rate showed in Table 2-16 came from a single trial. In the mix trial, a PAH mixture working solution containing all the 10 PAHs was introduced into the chamber for sampling and their sampling rates showed in Table 2-16 were the

average of the triplicate. Table 2-17 and Figure 2-8 to Figure 2-12 were derived from the results in Table 2-16. The results of paired t test in Table 2-17 confirmed that DB-5 sampler worked better than DB-17 sampler in the mix trial. As shown in Figure 2-8 and 1-9, generally, DB-5 sampler worked better than HP-1 sampler, while HP-1 sampler worked better than DB-17 sampler. This is because DB-5 sampler has both π-π interaction and Van der Waal's force with the PAHs, which make this type of semi-polar sampler captures PAHs better. Since most PAHs are non-polar and have aromatic rings, the non-polar HP-1 sampler also captures PAHs pretty well with its Van der Waal's force. However, for the polar DB-17 sampler, it does not provide strong Van der Waal's force. Since most of the PAHs are non-polar, DB-17 sampler cannot capture PAHs well with only π-π interactions. As we can see from Figure 2-10 to Figure 2-12, sampling rates from the separate trial were generally faster than the ones from the mix trial, which indicated the possible existence of interfere among PAHs.

Table 2-16 Sampling rates of HP-1, DB-5 and DB-17 passive samplers in the separate trial and mix trial

PAHs Name	Separate Trial (single trial)			Mix Trial (Triplet)		
	S_{HP-1} (ml/min)	S_{DB-5} (ml/min)	S_{DB-17} (ml/min)	S_{HP-1} (ml/min)	S_{DB-5} (ml/min)	S_{DB-17} (ml/min)
Naphthalene	0.087	2.254	0.294	1.189	0.503	0.298
1-Methylnaphthalene	2.256	0.531	0.830	0.344	0.662	0.347
2-Methylnaphthalene	2.521	0.451	1.227	0.377	0.943	0.409

acenaphthylene	0.216	1.997	0.290	0.089	0.393	0.101
acenaphthene	1.134	0.820	1.127	0.200	0.757	0.333
fluorene	0.722	0.598	0.464	2.112	2.083	2.060
phenanthrene	0.580	2.123	1.167	0.663	0.834	0.268
anthracene	0.859	0.834	0.450	1.097	1.360	0.390
fluoranthene	1.631	1.224	0.313	0.931	1.048	0.350
pyrene	4.116	1.334	0.779	0.513	1.328	0.632

Table 2-17 Paired t test results of the data in Table 2-16

P-value (α=0.05)	S DB-5	S DB-17	M HP-1	M DB-5	M DB-17
S HP-1	0.7199	0.0813	0.2090		
S DB-5		0.0943		0.5066	
S DB-17					0.4600
M HP-1				0.0962	0.0830
M DB-5					0.0004

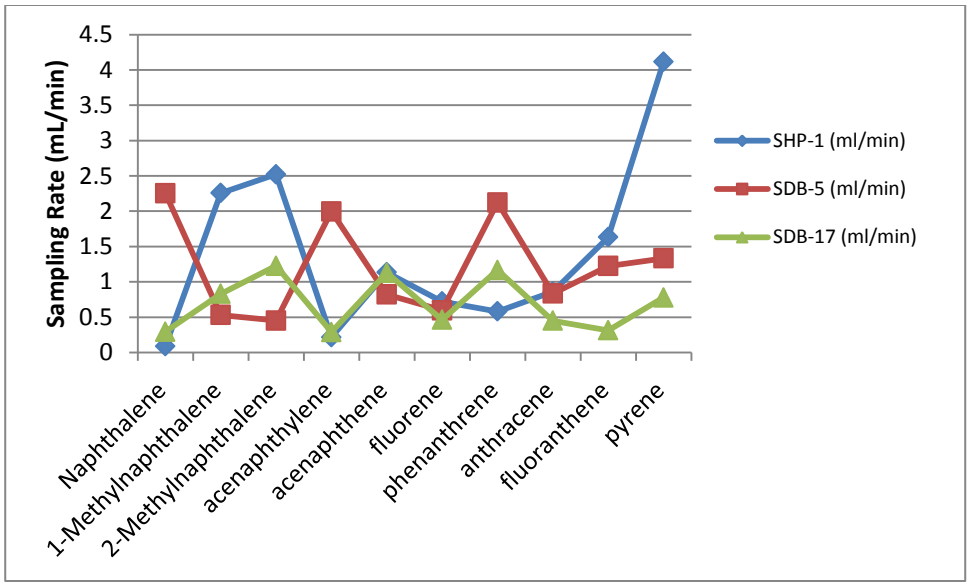


Figure 2-8 Sampling rate of HP-1, DB-5 and DB-17 passive samplers in separate trial

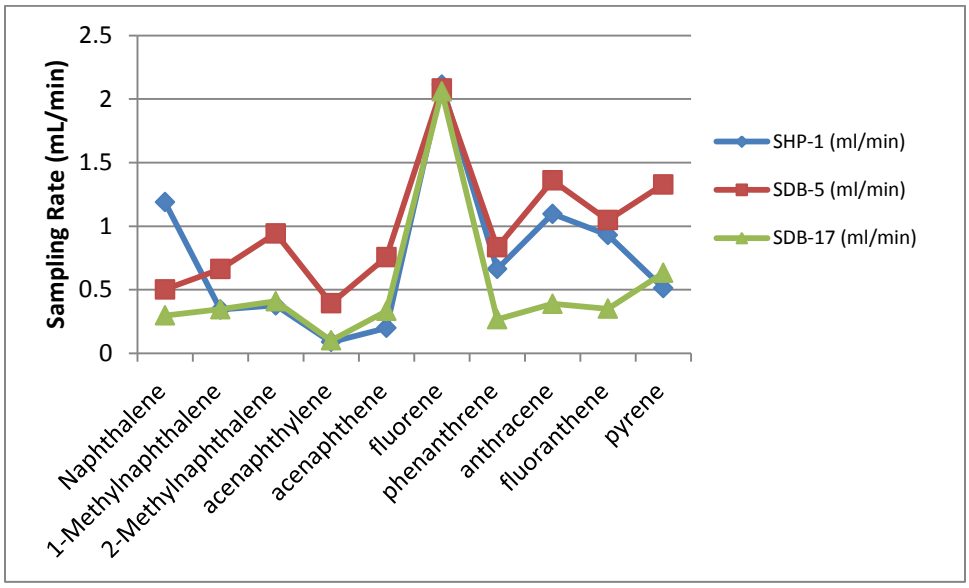


Figure 2-9 Sampling rate of HP-1, DB-5 and DB-17 passive samplers in mix trial

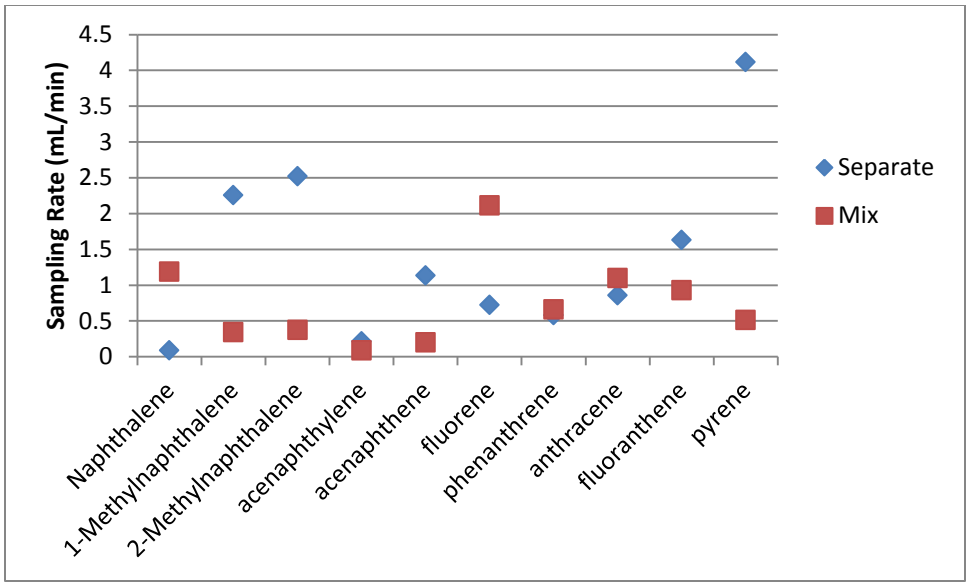


Figure 2-10 Sampling Rate of HP-1

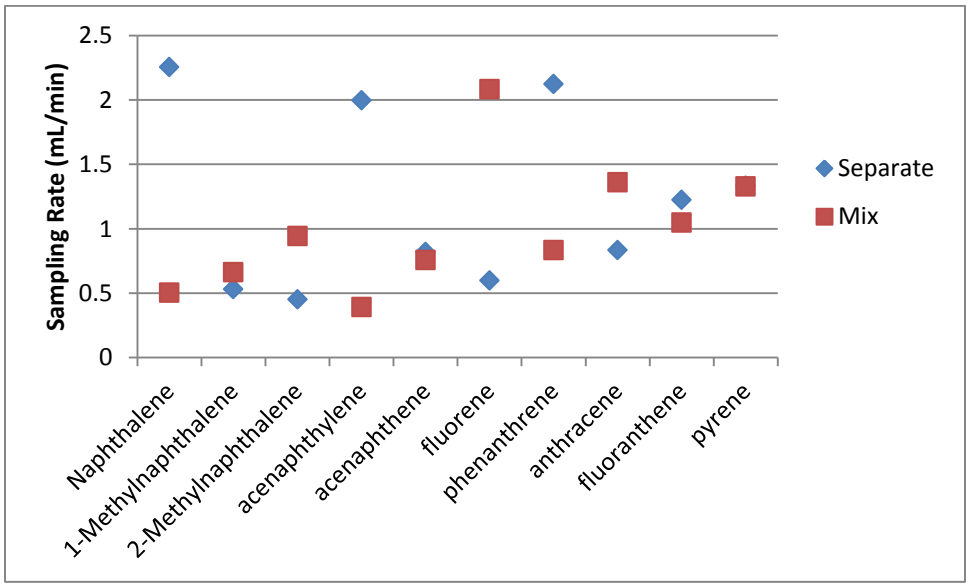


Figure 2-11 Sampling rate of DB-5

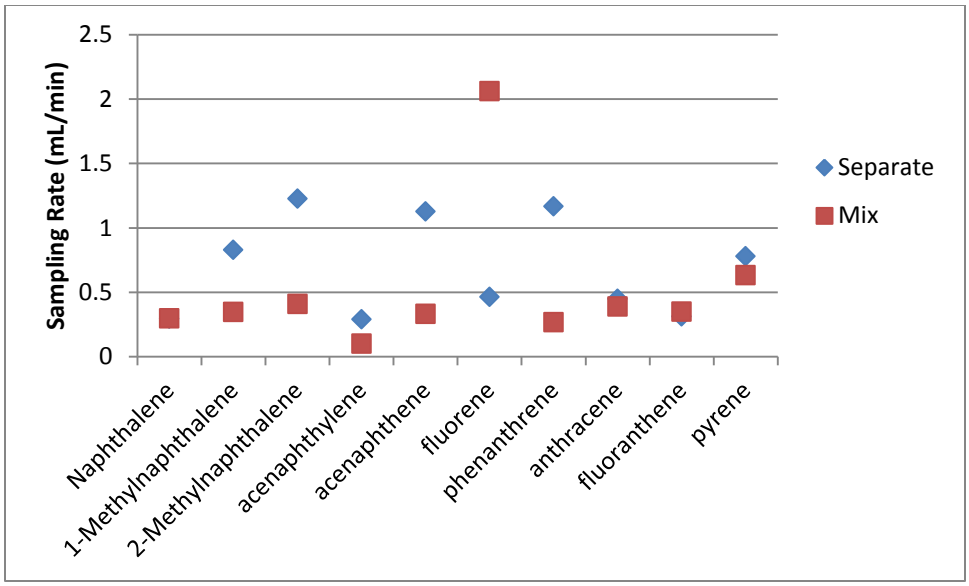


Figure 2-12 Sampling rate of DB-17

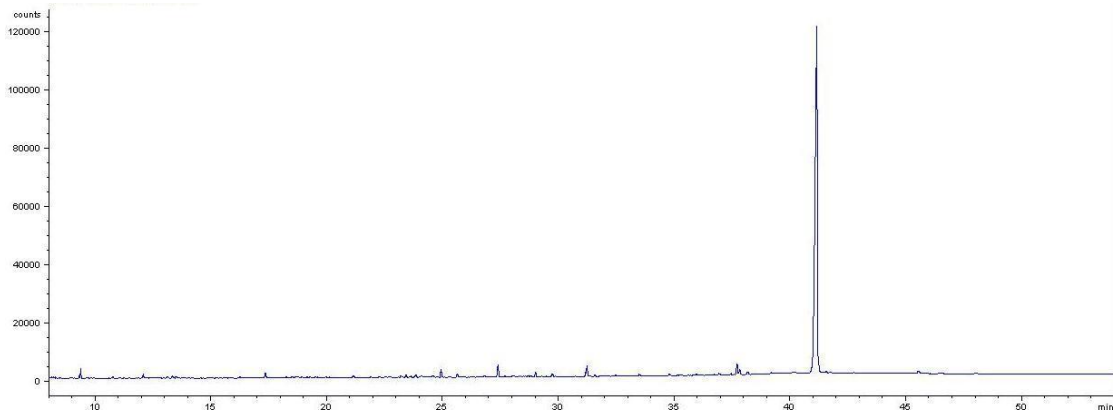


Figure 2-13 GC-FID chromatogram of the 18 PAHs with the unidentified peak. X-axis = time (min). Y-axis = area counts.

As shown in Figure 2-13, at some point of this PAHs research projects, an unidentified giant peak with the consistent retention time of about 41 minute appeared on every GC-FID chromatogram except for solvent blanks. Since this peak was so big that we could

barely see the PAHs peaks on the same chromatogram. A series of experiments were conducted to identify this peak.

(1) The peak disappeared if we injected solvent blanks, individual PAH stock solutions, calibration standard solutions or the internal standard working solution directly into the GC, which means this peak did not come from the GC.

(2) After sampling in the chamber, samplers were extracted without adding internal standard before TurboVap step. Even without internal standard, this peak still appeared which means that it was not caused by internal standards.

(3) Samplers were extracted without sampling in the chamber. This failed to eliminate the peak means that the peak did not come from the chamber materials.

(4) If we extracted the sampler without sampling in the chamber but with more polar solvent acetonitrile instead of hexane and dichloromethane, a smaller peak appeared comparing with the one in (3).

(5) No unidentified peak if we extracted the newly made sampler from a new purchased GC column after sampling in the chamber.

These series of experiments confirmed that this unidentified peak came from the coating materials on the inner surface of the GC column used to make these passive samplers. This peak could be used to determine if the sampler began to lose its coating materials because of multiple extraction process. We did not keep tracking how many times we extracted each sampler, but based on the time that this unidentified peak

appeared, each sampler might be able to extract at least 4 or 5 times before losing its coating materials. More experiments will be needed to confirm this prediction.

2.7 Conclusions

Exposure to polycyclic aromatic hydrocarbons (PAHs) is regarded as an important environmental risk factor for humans. The federal government has set regulations to protect people from the possible health effects of eating, drinking, or breathing PAHs. This brings requirements for monitoring human exposure to PAHs. A passive sampler made of 80 sections of 2cm long GC columns has been tested in our research. The main purpose of this passive sampler is to detect the amount of gaseous PAHs people breathe in every day. Since the sampling rate is a critical parameter that evaluates how well a passive sampler works, we developed a “static chamber” to determine this rate. In this chamber, a known amount of material is introduced. Assuming complete mixing, the initial concentration will be reduced exponentially with time by both the sampling process of the passive sampler and the leakage of the chamber. A simple leakage rate checking experiment can be conducted to find the leakage rate of the chamber and the sampling rate of the passive sampler can be calculated. Three types of columns with similar dimension but different column coating materials varying from non-polarity to medium-polarity were tested.

Chapter 3 Method development for determination of current-use and persistent pesticides in cow milk, human breast milk and baby formulas using gas chromatography tandem mass spectrometry

3.1 Introduction

Organochlorines (OCs), organophosphates (OPs), carbamates, and pyrethroids

Some of organochlorine insecticides are known as environmentally persistent pollutants and tend to bio-accumulate in fatty tissues due to their lipophilicity[41 42]. Although banned in most countries, organochlorines (OCs) are still routinely detected in umbilical cord blood and breast milk worldwide. They can block the chloride-ion uptake and some of them, such as dichlorodiphenyltrichloroethane (DDT), can also interfere with sodium channels. Dichlorodiphenyldichloroethane (DDE), a metabolite of DDT, is one of the most studied OCs due to its potential human neurodevelopmental toxicity.[43]

Due to the developmental neurotoxicity, the organophosphates (OPs) are among those pesticides that are the most widely studied and are possibly responsible for many pesticide poisonings around the world[44]. Their toxicity is exerted mainly through acetylcholinesterase (AChE) inhibition and subsequent hyper-excitation of post-synaptic cholinergic receptors. Because rat pups are more sensitive to OP effects than adult rats, they are commonly used as models to study neurodevelopment in human neonates and infants[45]. Studies have shown that some OPs can cause long-term neurochemical and behavioral changes in rats exposed both prenatally and postnatally to levels producing no measureable toxicity[46]. Rauh et al. conducted a study to investigate the neurotoxic effects of prenatal exposure to chlorpyrifos in 254 children from New York City through the first 3 years of their life. Comparing with lower exposure group, at the age of three,

children exposed to higher chlorpyrifos concentration levels were significantly more likely to experience Psychomotor Development Index and Mental Development Index delays, attention problems, attention-deficit/hyperactivity disorder problems, and pervasive developmental disorder problems.[47] Whyatt et al. found out that in a sample size of 314 mother-newborn pairs in New York City, prenatal organophosphate chlorpyrifos exposures had impaired fetal growth among these samples and organophosphate diazinon exposures might have contributed to the effects as well[48].

Carbamates are also potent AChE inhibitors. Whyatt et al. conducted a study among 314 minority mother-newborn pairs in New York City and proved that the carbamate propoxur metabolite 2-isopropoxyphenol in cord plasma was inversely associated with birth length while controlling for chlorpyrifos and diazinon[48].

With the discontinuation of many organophosphorus and organochlorine insecticides, pyrethroid insecticides become one of the most widely used domestic and agricultural pesticides. They comprised a quarter of the world market as early as 1995.[49] The US Environmental Protection Agency (EPA) withdrew the residential registrations for two commonly used organophosphates chlorpyrifos and diazinon in 2000-2001[50 51]. Since then, pyrethroids have become a top choice for household pest control because they are potent insecticides with relatively low mammalian toxicity. By prolongation of the kinetics of voltage-gated sodium channels, pyrethroids exert their acute toxicity through pharmacological actions upon the central nervous system.[52]

Because of the relatively non-volatility of pyrethroids, some people believed that there was minimal inhalation exposure if they were used for residential pest control[53]. However, recent studies have shown that despite of their relatively low volatility, children are still exposed to pyrethroids during development. Whyatt et al. found trans-permethrin and piperonyl butoxide in pregnant women's urine[54]. Morgan et al. found that 67% of a cohort of preschool children had detectable levels of the pyrethroid metabolite 3-phenoxybenzoic acid in their urine[55]. Lu et al. also found pyrethroid metabolites in the urine of elementary age children resulting from residential exposure[56]. These studies have proved that developing fetus and children are exposed to measurable levels of pyrethroids. Because some animal studies have shown that some pyrethroids are more acutely toxic to developing animals than adults[57], people are concerned about the potential developmental neurotoxicity of pyrethroids exposure.

Human breast milk, cow milk, and baby formulas

Human breast milk remains the best sole nutritious food for infants around the world, especially in the early stage of infancy and is recommended for the entire first year[25]. It is a complex biological fluid composed in several main compartments including an aqueous phase with true solutions (87%), colloidal dispersions of casein molecules (0.3%), emulsions of fat globules (4%), fat globule membranes, and live cells. This composition varies among and within women and is influenced by many factors such as genetic individuality, maternal nutrition and the stage of gestation and lactation. The protein content of human break milk is high in early secretions (15.8 g/L) and slowly

decreases with the establishment of lactation (8.0-9.0 g/L). The total fat content is within the range of 30-50 g/L[58] or 3.5-4.5%[59].

Persistent liposoluble pesticides such as organochlorine insecticides can bio-accumulate in the fatty tissues suggesting that human breast milk contains lipids originating directly from the adipose tissue, a good biological fluid for the development of exposure biomarkers[60]. Because of the neurodevelopmental and endocrine health concerns posed by current-use and historically used pesticides[21 61 62 63], concentrations of pesticide residues in human breast milk are an important factor to measure maternal contamination and to estimate the total chemical intake by infants through breast feeding.

Although the American Academy of Pediatrics and the American Academy of Family Physicians recommend breast milk for optimal infant nutrition, formulas that approximate the composition of human breast milk are still widely used for infants in their early stage of life. Despite of many varieties of baby formulas, they can be classified based on three basic criteria including caloric density, carbohydrate source, and protein composition. It is recommended that formulas should provide 7-16% calories from protein, 30-55% calories from fat, at least 1% calories from linoleic acid, and the rest from carbohydrates. Baby formulas usually contain relatively uniform lipid composition and 3.3-3.8% fat.[64]

Table 3-1 General composition (%) of human milk, cow milk and infant formulas [65]

Component	Milk		Formulas				
	Human (36days)	Cow	1	2	3	4	5
Protein	1	3.4	1.52	1.5	1.5	1.6	1.5
Casein, % protein	40	82	40	82	40	0	40
Fat	3.9	3.3	3.8	3.6	3.6	3.3	3.4
Lactose	6.8	4.8	7	7.2	7.2	7.2	7.6
Kilocalories/dL	72	75	68	68	68	65	67

Method development

Usually, the determination of pesticides in biological samples involves three steps: extraction, clean-up and chromatographic analysis.

Extraction

Liquid-liquid extraction is widely used in pesticides analysis of human breast milk. In the study of Minh et al., persistent organochlorine residues were extracted from 10 g human breast milk with the limit of detection (LOD) of about 0.1 ng/g. The main solvent used in this extraction was 10 mL hexane/dichloromethane (DCM) (1:1, v/v).[27] In the study of Alle et al., OCs were extracted from 2-5 g breast milk. Each sample was added to 20 g anhydrous sodium sulfate (Na_2SO_4) and then extracted twice with 150 mL petroleum benzene/DCM (4:1, v/v) in a glass column containing 20 g florisil. The LOD expressed on fat basis was about 5 ng/kg.[66] In the study of Bouwman et al., 10 mL milk

was deproteinised with 30 mL acetone and extracted with *n*-hexane for DDT and pyrethroids analysis. The limits of quantification (LOQ) for the individual pesticide in whole milk (~4% fat) was within the range of 0.042-0.349 µg/L.[67] Eight OCs were measured in human breast milk in the study of Damgaard et al. Milk samples (10 mL) were extracted wit 250 mL acetone/*n*-hexane (2:1, v/v)[68]. Thirteen OCs were measured in breast milk from 87 Tunsian mothers throughout their lactation periods in the study of Ennaceur et al. Milk samples (2-10 mL) were extracted three times with 26 mL *n*-hexane/acetonitrile/ethanol (20:5:1, v/v/v). The LOD expressed on fat basis for the 13 OCs was about 1 ng/g.[69] Persistent OCs were measured in the study of Devanathan et al. Each milk sample (10 mL) was added onto a glass column packed with 10 g diatomaceous earth and extracted with 200 mL diethyl ether. After removing the lipids by gel permeation chromatography (GPC), the extract was passed through a florisil column for further procedure.[70] OCs were measured in human milk from central Taiwan in the study of Chao et al. Each milk sample (2 g) was extracted with 2 mL glacial acetic acid and 2 mL methanol for further procedure. The LOD of OCs ranged from 1 to 4.83 ng/g lipid.[71] In the study of Ntow et al., 2 g human milk was added to 10 g anhydrous sodium sulfate and extracted twice with 50 mL acetone/hexane (1:1, v/v) for further analysis of OCs. Most of the OCs analyzed by GC-MS in this study had an LOQ at or below 0.01 ng/g.[72] In a study of Sanghi et al., OCs and OPs in breast milk were measured with the LOD of 0.001 mg/kg and 0.01 mg/kg for OCs and OPs respectively. After adding 2-5 drops of 10% sodium chloride and 15% DCM to each milk sample, hexane was used to extract analytes. The organic layer was extracted twice with *n*-hexane, while the

aqueous phase was extracted twice more with the same solvents. DCM was removed by hexane. Samples were analyzed after combining the organic phases.[73] PCBs and OCs were measured in human milk in a study of Zhao et al. Milk samples were denatured by equal volumes of ethanol before extracted with 125 mL *n*-hexane/acetone (3:1, v/v).[74]

Sample clean-up

In the extraction of pesticides from biological tissues, lipids might be co-extracted with targeted pesticides because of their potential solubility in organic solvents. While injecting these samples into the GC for analysis, these lipids in samples can retain on the injection port, ionization source and/or the GC column, which causes poor GC chromatographic performance. The clean-up can be the most laborious step in many analytical procedures since the pesticides need to be accurately separated from the co-extracted fatty matrices. Different methods have been developed to clean-up samples and eliminate the interferes from the co-extracted lipids, such as liquid-liquid partitioning[75], column chromatography with gel permeation[76], florisil[77], alumina, silica or their combination[78 79], multiple cleanup methods[80], sulphuric acid[81], and supercritical fluid extraction[82]. However, some of these methods will degrade certain pesticides[83], while the others require large amount of organic solvents and multiple steps and thus can be time and labor consuming. Therefore, some simple but effective clean-up procedures have been developed recently.

In Hong's paper, they introduced a simple and effective clean-up method.[84] Since there is a significant difference of melting points between lipids (below about 40 ^{0}C) and

chlorinated pesticides (above about 260 ^{0}C), in Hong's paper, 90% of the lipids extracted from the fish samples were easily removed by freezing-lipid filtration method. After extraction, lipids in extract were precipitated as frozen form in a -20 ^{0}C freezer, while chlorinated pesticides were still dissolved in the organic solvent used to do the extraction. The frozen lipids were then removed by filtering.

Solid phase extraction (SPE) is another simple way to clean-up samples. As one of the most important techniques in sample preparation, SPE can offer a significantly more rapid, simple and easy way to extract pesticide residues and clean-up fatty matrices[85]. The difficult part of using SPE is choosing the right SPE product and elution protocol. Although glass beads, florisil, alumina, silica, C18, polymers and charcoal SPE columns are sufficient enough for certain classes of pesticides, they do not work very well to capture diverse pesticides and clean-up complex matrices.[86]

Graphitized carbon black (GCB) adsorbents are preferred in removing chlorophyll of green vegetable extracts, but perform poorly in eliminating fatty acid matrices.[87] Since 100% organic solvents such as acetonitrile are often used in extraction and elution, C18 cannot retain hydrophobic interferences from fatty matrices very well either. The chemical structure of silica-based primary and secondary amine (PSA) is shown in Figure 3-1, which has two ion-exchange sites (pKa 10.1 and 10.9). PSA has significantly high ion-exchange capacity for removing fatty acids and becomes one of the most powerful solid phase extraction adsorbents for cleaning-up fatty matrices and analysis of multi-

pesticide residues. He et al. proved that if toluene, hexane and/or acetone were used for elution, the capacity of PSA for removal of fatty acids would be severely reduced.[86]

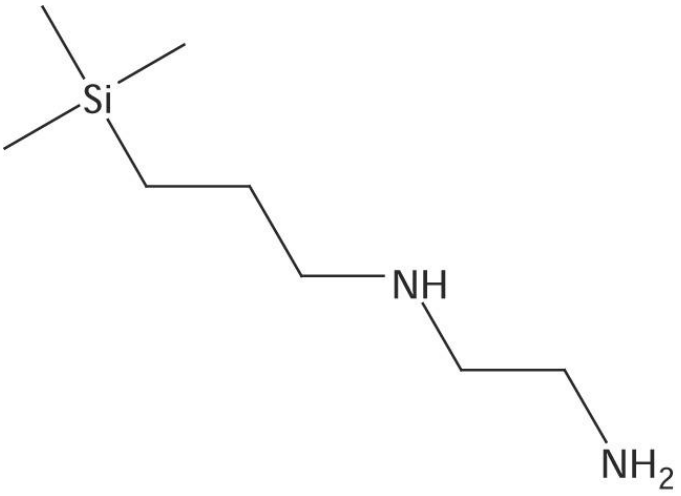


Figure 3-1 the chemical structure of silica-based PSA

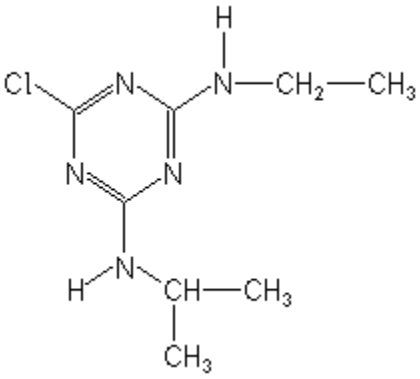
Purposes of this study

Many recent studies are focused only on OC levels in breast milk, while few non-US studies measure both OP and pyrethroid levels. Although a number of different methods have been developed to analyze pesticides in breast milk, especially organic chlorine and organophosphorus insecticides, as well as some other persistent organic pollutants, such as polychlorinated biphenyls, methods for identifying current use pesticides, i.e., pyrethroids and carbamates are less well developed for human milk. There are also a limited number of methods available to measure multiple classes of pesticides in a single breast milk sample.[66 27 67 68 69 70 89 71 90 37 72 91 73 92 74] To our

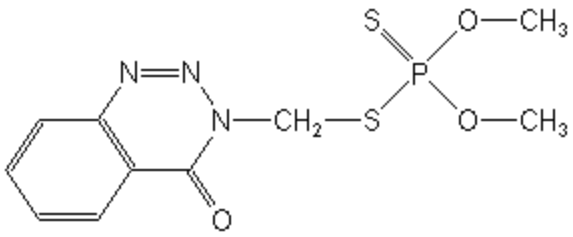
knowledge, no studies measure levels of all four classes (OCs, OPs, pyrethroids, and carbamates) of pesticides in human breast milk in a single sample.

Therefore, improvement of existing methods and the development of new, efficient, high-throughput methods for all the four classes of pesticides is of great importance in evaluating exposures experienced by infants through breast feeding. Thus, the aim of this study was to develop a highly selective and sensitive analytical method to eliminate the interferences from the complicated milk matrices, improve separation of targeted pesticides, and both identify and quantify the concentration levels of four different classes of pesticides in human breast milk.

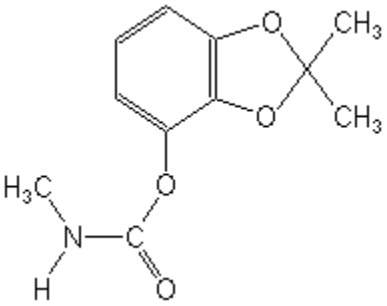
Our focus is on four pesticides classes including organochlorine (OC), organophosphate (OP), carbamate, and pyrethroid insecticides. The chemical structures of the representative pesticides from the four classes of pesticides in this study are shown in Figure 3-2.



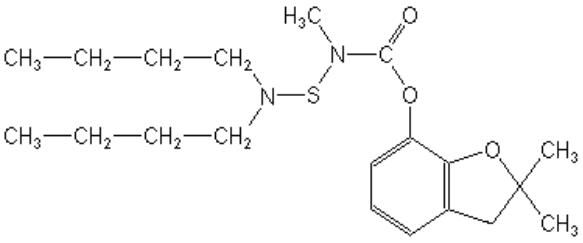
atrazine (mw 215.7 g/mol)



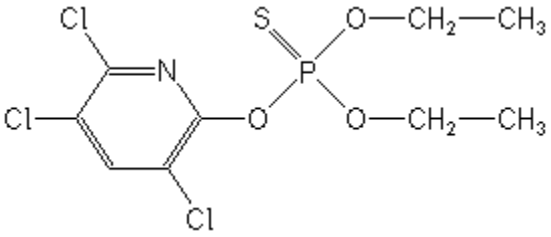
azinphos-methyl (mw 317.3 g/mol)



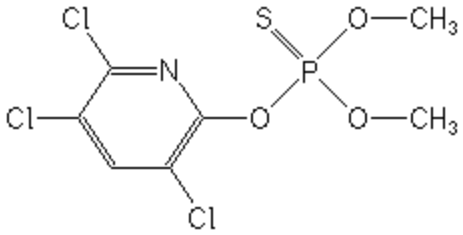
bendiocarb (mw 223.2 g/mol)



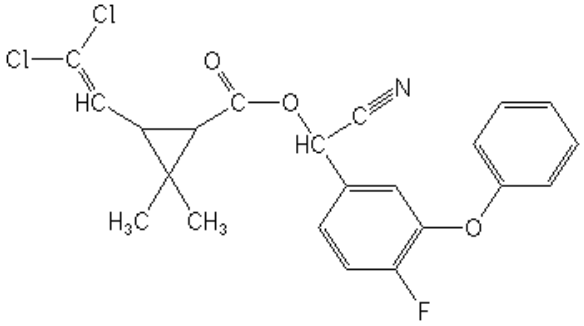
carbosulfan (mw 380.2 g/mol)



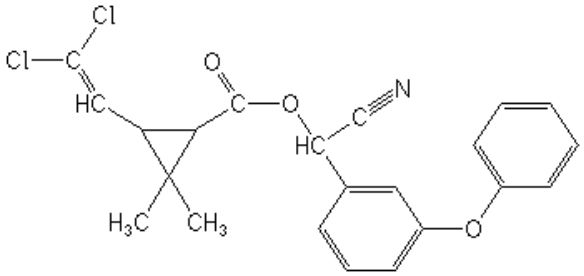
chlorpyrifos (mw 350.6 g/mol)



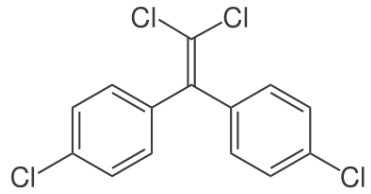
chlorpyrifos-methyl (mw 322.6 g/mol)



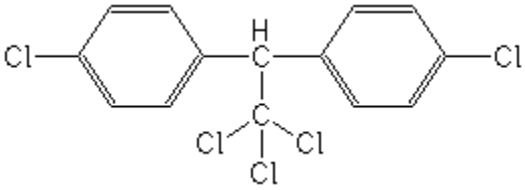
cyfluthrin (mw 434.3 g/mol)



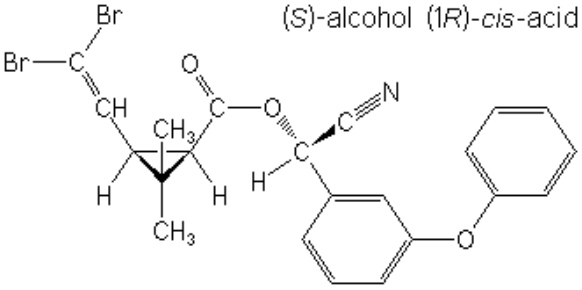
cypermethrin (mw 416.3 g/mol)



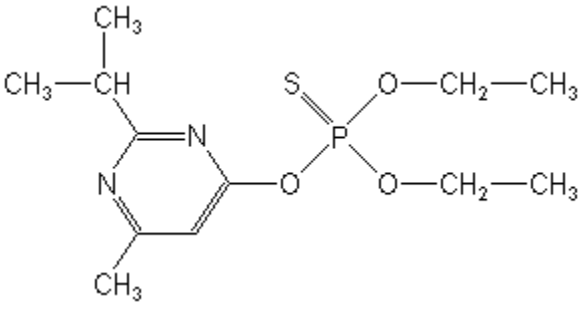
DDE (mw 318.0 g/mol)



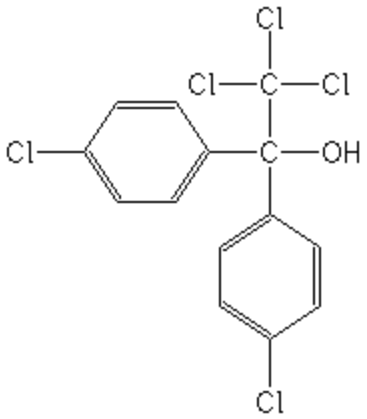
DDT (mw 354.5 g/mol)



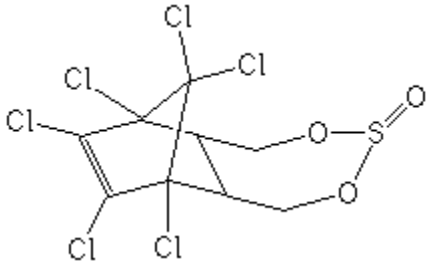
deltamethrin (mw 505.2 g/mol)



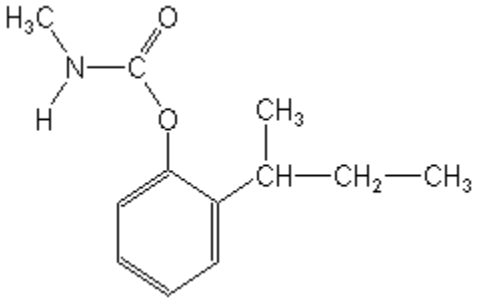
diazinon (mw 304.3 g/mol)



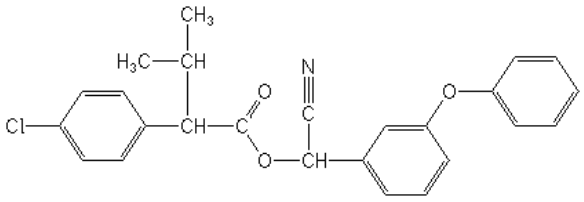
dicofol (mw 370.5 g/mol)



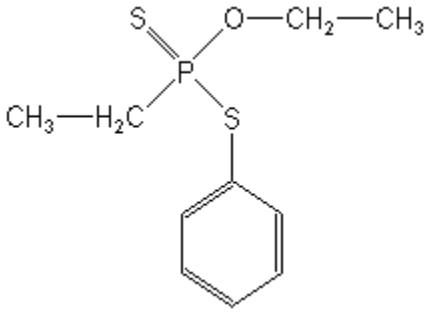
endosulfan (mw 406.9 g/mol)



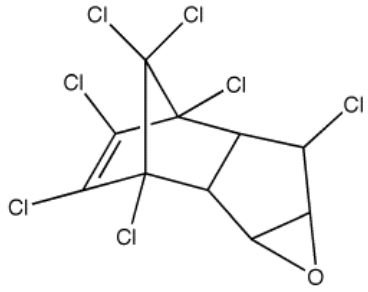
fenobucarb (mw 207.3 g/mol)



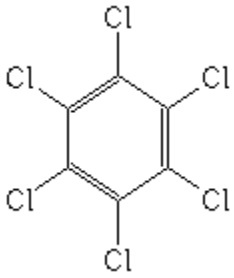
fenvalerate (mw 419.9 g/mol)



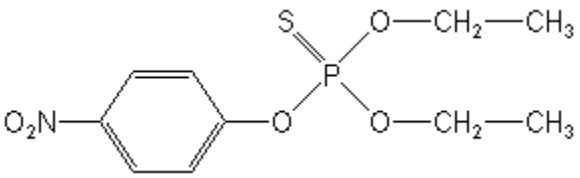
fonofos (mw 246.3 g/mol)



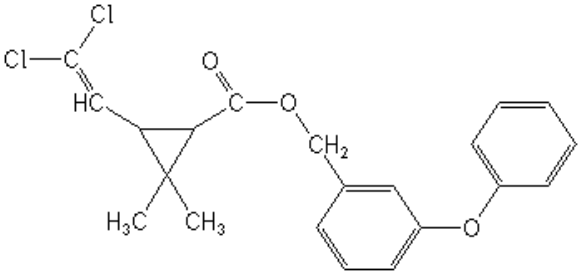
heptachlor epoxide (mw 389.3 g/mol)



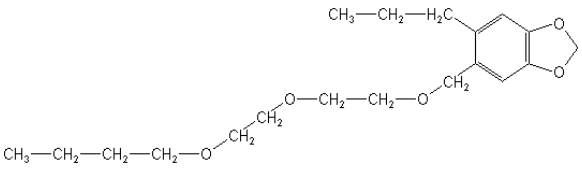
hexachlorobenzene (mw 284.8 g/mol)



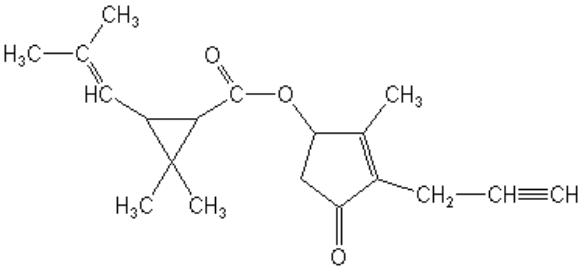
parathion (mw 291.3 g/mol)



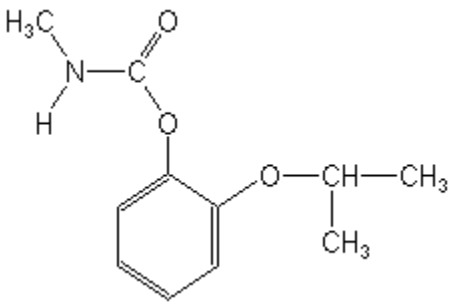
permethrin (mw 391.3 g/mol)



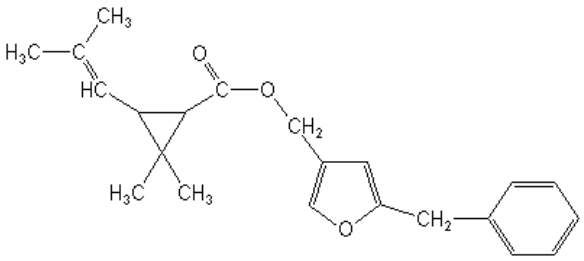
piperonyl butoxide (mw 338.4 g/mol)



prallethrin (mw 300.4 g/mol)



propoxur (mw 209.2 g/mol)



resmethrin (mw 382.5 g/mol)

Figure 3-2 Chemical structures of pesticides investigated in this study

3.2 Materials and methods

Sample Collections

Human breast milk samples were collected during the year 2010 and 2011. These samples were stored at -20 ^{o}C until analysis. Different brands of cow milk samples and baby formula powder were purchased from local grocery stores. Baby formula samples

were made from baby formula powder based on manufacture instructions. All the samples were divided into appropriate aliquots before storage.

Chemicals

The native standard of fenobucarb (fen) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The native standards of fonofos (fon), p,p-dicofol (dic), and heptachlor epoxide (hep) were purchased from Ultra Scientific. The native standards of bendiocarb (ben), parathion (par), o,p-DDE (ddeop), prallethrin (pral), p,p-DDE (ddepp), o,p-DDT (ddtop), piperonyl butoxide (pbo), and cypermethrin (cyp) were all purchased from Chem Service. The native standards of hexachlorobenzene (hcb), atrazine (atr), diazinon (dia), chlorpyrifos-methyl (chlm), chlorpyrifos (cpy), endosulfan-α (endoA), resmethrin (res), azinphos-methyl (azm), permethrin (per), and deltamethrin (del) were purchased from Supelco Analytical. The native standard of endosulfan-β (endoB) was purchased from Crescent Chemical Co. The native standard of cyfluthrin (cyf) was purchased from Los Almos. The internal Standards of chlorpyrifos-methyl D6, chlorpyrifos D10, parathion D10, 13C-*p,p*-DDE, and 13C-cypermethrin were purchased from Cambridge Isotope Laboratories.

All solvent used were of analytical grade. We obtained acetonitrile, hexane and toluene from Fischer Scientific (Phillipsburg, NJ, USA), Sigma Aldrich and Macron, respectively. Acetic acid (Glacial) was purchased from Avantor Performance Materials (Phillipsburg, NJ). Bondesil-PSA (40µm) was purchased from Agilent Technologies. Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tubes and PSA bonded silica 500mg 6mL SPE tubes

were both purchased from Supelco (Bellefonte, PA). Oasis HLB Extraction Cartridges (3 cc/60 mg) were purchased from Waters Corporation (Milford, MA).

Native Standards and Internal Standards Preparation

Individual stock solutions of the native standards were prepared for each analyte in acetonitrile. Stock solutions were stored at -20^{o}C. Ten working standard solutions of varying concentrations ranging from 1-1000 ppb were prepared by adding individual stock solutions of analytes in acetonitrile/toluene (3:1, v/v). The working standard solutions were stored at -20^{o}C.

Individual stock solutions of the labeled internal standards were prepared in acetonitrile. Stock solutions were stored at -20^{o}C. An internal standard working solution including all the five labeled analytes was prepared at 400ppb in acetonitrile/toluene (3:1, v/v) and stored at -20^{o}C.

The calibration standards were made freshly by adding 100 µL native standard working solution and 50 µL internal standard working solution into 1mL organic cow milk followed by a complete sample preparation procedure.

Preparation and extraction procedure for milk samples

Materials: Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE cartridges, empty labeled test tubes, pipettes and matching tips, beakers, 6 mL capacity reservoir from Varian,

frits (6ml, ½", 20µm) from Agilent, filter paper from Whatman, Zymark TurboVap LV Evaporator (Framingham, MA), and GC vials, inserts and caps.

Solutions: acetonitrile, hexane, acetonitrile/toluene (3:1, v/v).

Sample preparation procedure:

Cow milk purchased from grocery stores and human milk collected from participates were taken from the -20 ^{0}C freezer to thaw at room temperature. They were vortexed to reach homogenization before sample preparation.

Matrix blank samples

- Spike with 50 µL of 400 ppb ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 1 mL cow milk
- Vortex 30 seconds to mix

Calibration standards

- Spike with 50 µL of ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 100 µL of the native standard (S1-S10: 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000ppb) to the corresponding test tube (e.g. S1 native standard in S1 test tube)
- Add 1 mL cow milk
- Vortex 30 seconds to mix

Unknown samples (label test tubes with appropriate unknown sample ID)

- Spike with 50 µL of ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 1 mL milk from each unknown sample
- Vortex 30 seconds to mix

For the other working samples, depending on their purposes (recovery studies, stability studies, etc.), 50 µL of 400 ppb ISTD and the selected level of 100 µL native standard were spiked into each sample at different steps of the extraction procedure.

Extraction procedure:

- Add 4 mL acetonitrile to each sample (test tube 1), vortex at 1000rpm for 3min, and sonicate for 10min
- Add 300 mg NaCl to test tube 1, vortex at 1000 rpm for 2 minutes, and centrifuge at 2500 rpm and 10 ^{0}C for 8 minutes
- Transfer the 4 mL acetonitrile extract to test tube 2
- Add 4 mL hexane to test tube 1, vortex at 1000 rpm for 3 minutes, and centrifuge at 2500 rpm and 10 ^{0}C for 8 minutes
- Transfer the 4 mL hexane extract to test tube 3
- Evaporate the 4 mL hexane extract in test tube 3 to dryness under 45 ^{0}C and 15-20 psi, reconstitute with 1 mL acetonitrile, centrifuge for 2 minutes, and decant the 1 mL acetonitrile to test tube 2

- Place test tube 2 in -20 ^{0}C fridge for overnight
- Quickly filter the solvent in test tube 2 in an empty SPE cartridge with a filter paper and frit in the bottom. Air might be needed to push the solvent through the filter paper and frit. Collect the filtered solvent in test tube 4
- Add 100 mg PSA and 300 mg Na_2SO_4 to test tube 4, vortex at 1000 rpm for 3 minutes, centrifuge for 2 minutes, and decant the 5 mL solvent to test tube 5
- Evaporate the 5 mL solvent in test tube 5 to about 2 mL under 45 ^{0}C and 20 psi
- Precondition each Carb/PSA SPE cartridge with 5 mL acetonitrile/toluene (3:1, v/v)
- Load the 2 mL solvent in test tube 5 to the preconditioned cartridge and discard the eluent
- Elute cartridge with 10 mL acetonitrile and collect the eluent in test tube 6
- Elute cartridge with 10 mL toluene and collect the eluent in test tube 7
- Combine eluent in test tube 6 and 7, and evaporate to dryness under 45 ^{0}C and 15-20 psi
- Reconstitute with 50 µL acetonitrile/toluene (3:1, v/v) for GC analysis

Baby formula solutions were made from baby formulas powder based on manufacture instructions. After dissolving the powder in suitable amount of distilled water, solutions were sonicated at 37 ^{0}C for 30 minutes before stored at the -20 ^{0}C freezer. When extracted with acetonitrile, samples were vortexed for 15 minutes instead of 3 minutes. After adding NaCl, they were vortexed for another 5 minutes instead of 2 minutes.

When extracted with hexane, samples were also vortexed for 15 minutes instead of 3 minutes. After adding disperse PSA, they were vortexed for 5 minutes instead of 3 minutes.

Instrumental Analysis

The sample analysis was carried out with an Agilent Model 7000 gas chromatography tandem mass spectrometry, which was operated in multiple reactions monitoring (MRM) mode for mass analysis of positive ions generated by electron ionization (EI^+). MS was auto-tuned periodically to obtain optimum sensitivity. A HP-5MS column was used (30 m, 0.25 mm ID, 0.25 µm film) with a GC temperature program to optimize the separation. A 2 µl injection was used with an injector temperature of 250 ^{0}C under pulsed splitless mode. The temperature program began at 100 ^{0}C and was held for 2 min, increased at 10 ^{0}C/minutes to 205 ^{0}C and held for 3 min, increased at 10 ^{0}C/minutes to 280 ^{0}C and held for 4 min, and finally increased at 25 ^{0}C/minutes to 310 ^{0}C and held for 12 min. The total run time was 40.2 min. The flow rate of carrier gas helium began at 1.2 mL/minutes and was held for 28 min, and was increased at 1 mL/minutes per minutes to 1.8 mL/minutes and held until the end of the run. Quantification and confirmation ions were monitored for each native pesticide and its respective isotopically labeled internal standard. Masses for each ion monitored and its time segment (TS) for analysis are shown in Table 3-2. They were selected by monitoring the intensity, peak shape, signal to noise ratio, and potential interferences in milk samples.

A dummy transition was added after the final time segment (time segment 15) to ensure deltamethrin in time segment 15 get enough scans.

Table 3-2 Quantification and confirmation ions monitored for the analytes in this study

Compounds		RT (min)	TS	MW	Fragmentation	
Name	Abbreviation				Quantification Ions	Confirmation Ions
Native Pesticides						
fenobucarb	fen	10.51	1	207.3	121.2→103.1 @20	121.2→51.2@40
hexachlorobenzene	hcb	11.75	2	284.8	284.0→249.1@25	284.0→214.2@40
atrazine	atr	12.09	2	215.7	200.3→104.1@20	200.3→122.2@10
fonofos	fon	12.48	3	246.3	246.2→137.2@5	109.1→63.1@15
bendiocarb	ben	12.65	3	223.2	151.2→84.1@15	151.2→68.2@25
diazinon	dia	12.65	3	304.3	304.3→179.3@15	179.3→121.0@40
chlorpyrifos-methyl	chlm	13.76	4	322.6	286.2→93.2@26	288.2→93.0@20
chlorpyrifos	cpy	15.19	5	350.6	314.2→258.0@25	314.2→286.1@5
parathion	par	15.22	5	291.3	291.3→81.0@40	291.3→90.9@35
p,p-dicofol	dic	15.26	5	370.5	139.1→111.1@15	139.1→75.1@30
heptachlor epoxide	hep	16.28	6	389.3	353.1→263.1@10	353.1→282.1@15
o,p-DDE	ddeop	17.18	7	318.0	246.2→176.2@35	248.2→176.3@30
prallethrin	pral	17.18	7	300.4	123.2→87.1@15	123.2→105.2@20
endosulfan-α	endoA	17.38	7	406.9	241.1→206.1@20	239.1→204.1@15
p,p-DDE	ddepp	18.08	8	318.0	246.2→176.2@35	248.2→176.2@30
endosulfan-β	endoB	18.93	9	406.9	241.1→206.1@15	239.1→204.1@20
o,p-DDT	ddtop	19.25	9	354.5	235.2→199.1@15	235.2→165.1@25
piperonyl butoxide	pbo	20.63	10	338.4	176.2→103.1@30	176.2→91.1@40

resmethrin	res	20.67	10	382.5	171.2→143.2@5	123.2→95.2@6
azinphos-methyl	azm	22.00	11	317.3	160.2→77.2@20	132.2→77.1@15
permethrin	per	23.22	12	391.3	183.2→77.0@40	183.2→153.2@15
cyfluthrin	cyf	23.95	13	434.3	163.1→127.2@5	206.2→151.1@25
cypermethrin	cyp	24.36	13	416.3	163.1→127.1@15	181.2→152.2@25
fenvalerate	fev	25.78	14	419.9	125.2→89.0@20	167.2→89.2@40
deltamethrin	del	26.78	15	505.2	253.1→93.2@20	181.2→152.2@30
Internal Standards						
chlorpyrifos-methyl D6	IS_chlm	13.70	4	328.6	291.9→99.0@25	291.9→274.0@30
chlorpyrifos D10	IS_cpy	15.05	5	360.6	324.0→260.0@20	324.0→292.0@10
parathion D10	IS_par	15.09	5	301.3	301.0→115.0@15	-
13C-*p,p*-DDE	IS_ddepp	18.07	8	330.0	258.0→188.0@40	-
13C-cypermethrin	IS_cyp	24.47	13	422.3	170.0→98.0@15	170.0→134.0@10

* For compounds having isomers and multiple peaks on GC chromatogram, the retention time (RT) in this table represents the retention time for the first peak. TS = time segment.

Quantification method

Concentrations of targeted compounds were determined with respect to their corresponding labeled internal standards (chlorpyrifos-methyl D6, chlorpyrifos D10, parathion D10, 13C-*p,p*-DDE, or 13C-cypermethrin). The concentrations of permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin were determined by individual isomers.

Limit of Detection

The LOD was calculated as the lowest concentration of an analyte giving a signal of three-times the base line noise on the GC chromatogram. In our cases, The LOD value was estimated based on a lower standard concentration displaying a minimum signal to noise (S/N) ratio of three when injecting milk-matrix samples spiked with native standard and internal standard solutions.

Extraction efficiency

The extraction efficiency of the method was determined by analysis of replicate 1 mL milk samples spiked with two different concentration levels of pesticides (25 ppb and 10 ppb). In the recovery study, samples were separated into two groups (Group A and Group B) with the same spiking level. Replicate milk samples in Group A were spiked with the designated native standard before extraction and internal standard right before the final evaporation step, while replicate milk samples in Group B was spiked with the designated native standard and internal standard both before the final evaporation step. The extraction recovery was calculated by comparing the response ratio of Group A to that of Group B.

Accuracy

The method accuracy was determined by calculating repeated measurements of milk samples spiked at two different concentration levels (5ng/mL and 25ng/mL). We

calculated the percent deviation of the observed mean concentrations from the nominal spiked concentrations.

Precision

The method precision was determined by calculating the relative standard deviation (RSD) of repeated measurements of milk samples spiked with native standard pesticides at two different concentration levels (5ng/mL and 25ng/mL). Replicate samples were prepared and analyzed daily during a 3-day period to determine the inter-day precision.

3.3 Results

Recoveries

Milk samples (1 mL/sample) spiked with 100 µL 100 ppb or 250 ppb native standard were extracted, cleaned-up and analyzed. As shown in Figure 3-3, the recoveries between two different spiking levels were relatively consistent. Recoveries ranged from 34-102%, with about 75% of recoveries between 60 and 80%. For the concentration level of 25 ppb, the standard deviation ranged from 1.2% to 12.4% for all the pesticides. However, at the concentration level of 10ppb, especially for hexachlorobenzene and deltamethrin, their standard deviation was close to 50%. The concentration level of 10ppb was close to the LOD of deltamethrin, which was partially responsible for its high

SD. The transitions we picked for hexachlorobenzene sometimes did not respond very well on the GC and thus might cause its high SD.

The recoveries present in Figure 3-3 are satisfactory because optimizing the performance for every individual compounds from different classes of pesticides is challenging, especially in complicate matrices like milk. Because of the diverse chemical and physical properties of these pesticides, some analytes must be somewhat compromised for the overall performance of the method. The proposed method in this study was the best compromise to obtain the satisfactory extraction efficiency.

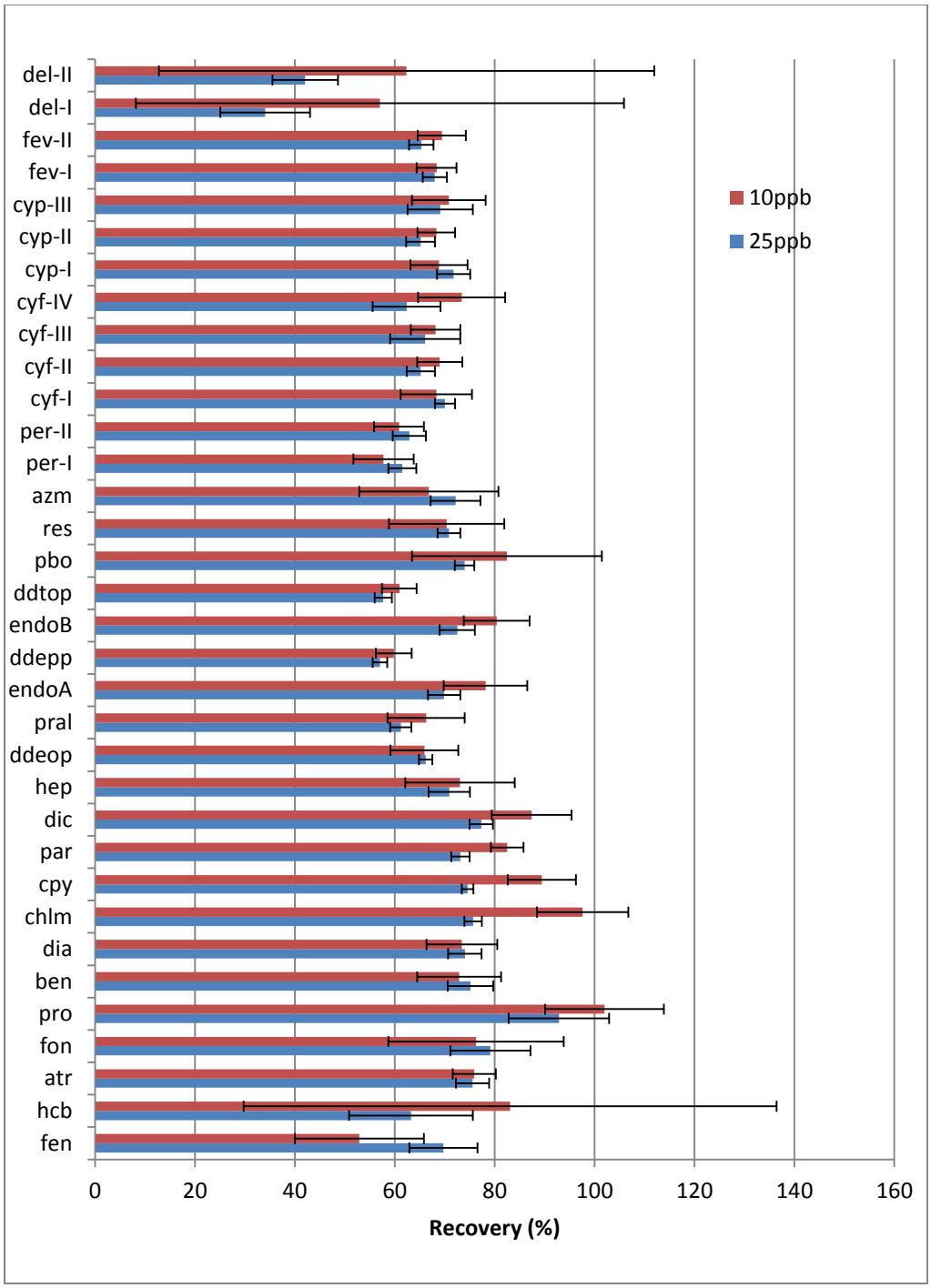


Figure 3-3 Recoveries of cow milk under two different concentration levels (25 ppb and 10 ppb)

The recoveries of baby formulas were not as good as the ones for cow milk, but most of them were still within the range of 50-80%. For azinphos-methyl, the recovery was 151.9% with a standard deviation of 56.1%, which was caused by the matrix effects of baby formulas on the GC. Both of the two transitions for azinphos-methyl were influenced heavily by matrix effects in our method.

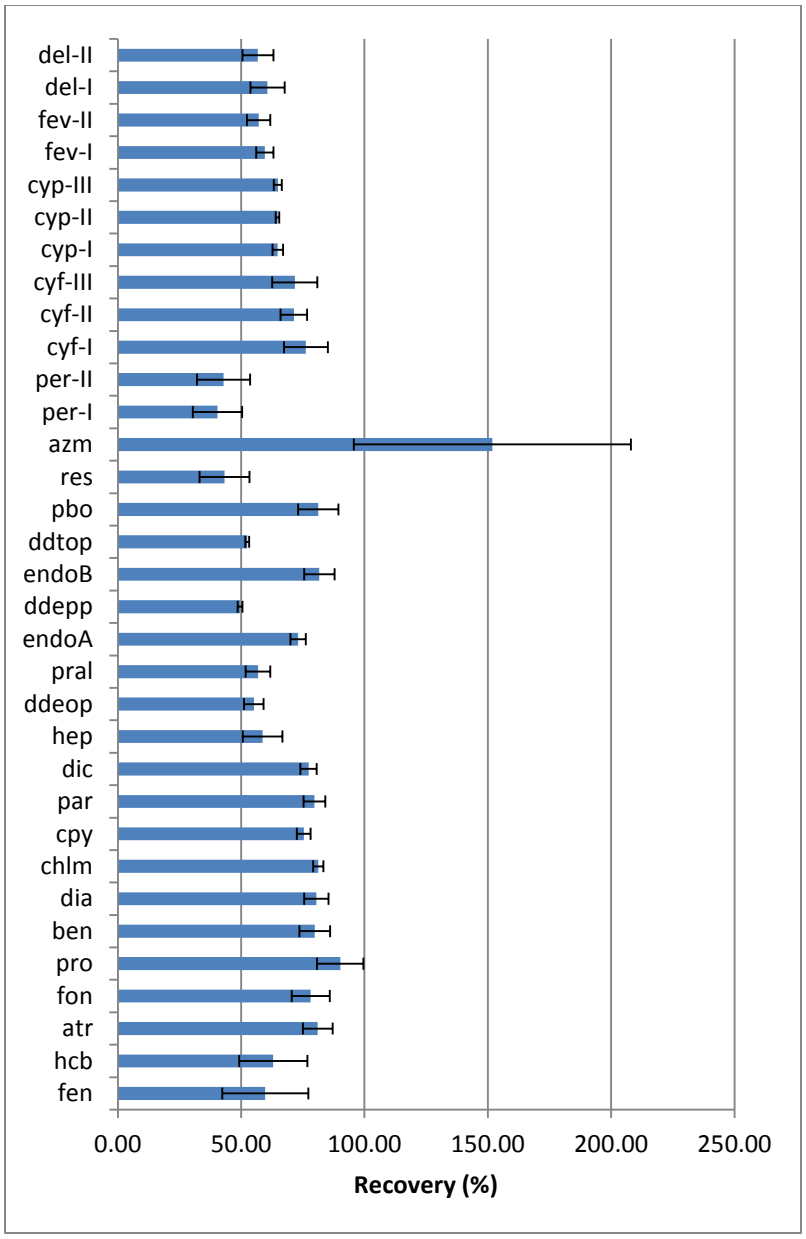


Figure 3-4 Recoveries of baby formulas under the concentration level of 25 ppb

Limits of Detection (LOD)

Five procedural matrix blanks were analyzed and three pesticides were consistently detected in both cow milk and baby formulas: hexachlorobenzene, *p,p*-DDE, and

resmethrin. For these three pesticides, the LOD was determined from the blank value variability: LOD=3×SD, where SD is the standard deviation of the analyte in 10 blank samples.

For compounds with no observable existence in matrix blanks, the LOD values were estimated based on a lower standard concentration displaying a minimum signal to noise (S/N) ratio of three when injecting milk-matrix samples spiked with native standard and internal standard solutions. This reflected the lowest concentration level we can confidently identify and claim the peak for a certain compound on the GC.

Table 3-3 shows that except for deltamethrin, for all the other pesticides, our sample preparation method allows the detection of them in cow milk samples at concentration levels lower than 1ppb. This was satisfactory results since the volume of milk used in this proposed was only 1 mL comparing with other methods mentioned in the introduction part of this chapter.

Table 3-3 limit of detection in cow milk

	Milk	
Name	S/N=3 (ppb)	$3\times SD_{matrix\ blanks}$ (ppb)
fenobucarb	0.1749	
hexachlorobenzene	0.0010	0.0675
atrazine	0.0145	

fonofos	0.0135	
bendiocarb	0.0101	
diazinon	0.0051	
chlorpyrifos-methyl	0.0060	
chlorpyrifos	0.0076	
parathion	0.6431	
p,p-dicofol	0.0099	
heptachlor epoxide	0.0096	
o,p-DDE	0.0068	
prallethrin	0.3408	
endosulfan-α	0.0060	
p,p-DDE	0.0005	0.0138
endosulfan-β	0.0123	
o,p-DDT	0.0033	
piperonyl butoxide	0.0194	
resmethrin	0.1064	0.2430
azinphos-methyl	0.2119	
Permethrin-I	0.1292	
Permethrin-II	0.2263	
cyfluthrin-I	0.0735	
cyfluthrin-II	0.0771	

cyfluthrin-III	0.2963	
cyfluthrin-IV	0.2736	
cypermethrin-I	0.0829	
cypermethrin-II	0.0984	
cypermethrin-III	0.7745	
fenvalerate-I	0.0309	
fenvalerate-II	0.0572	
deltamethrin-I	1.6692	
deltamethrin-II	1.6428	

Precision and Accuracy

The results are shown in Table 3-4. For majority of the analytes, the accuracy and precision fall within the range of 80-120% and 0-15% respectively. These results indicate that our sample preparation and GC method produced acceptable accuracy and precision.

Table 3-4 Accuracy and precision for analysis of targeted compounds from replicate samples at two different concentration levels (5ppb and 25ppb) in cow milk

Name	Accuracy (%)	Precision (%)	
		Within-day	Between-day

	5ppb	25ppb	5ppb	25ppb	5ppb	25ppb
fenobucarb	82.07	86.31	17.70	14.19	21.37	12.64
hexachlorobenzene	84.33	89.77	14.03	13.63	14.04	1.17
atrazine	84.60	102.19	3.98	6.47	8.76	14.13
fonofos	90.80	97.47	9.64	8.22	27.76	25.62
bendiocarb	92.33	98.89	5.32	6.14	11.58	10.69
diazinon	89.80	103.37	6.11	5.85	11.27	11.53
chlorpyrifos-methyl	87.73	95.73	2.44	1.64	2.93	3.67
chlorpyrifos	106.80	101.56	3.55	1.93	20.74	4.55
parathion	105.47	96.73	5.54	3.76	10.89	1.06
p,p-dicofol	95.73	104.96	3.76	3.57	0.79	11.24
heptachlor epoxide	101.13	108.64	5.04	4.42	8.93	3.93
o,p-DDE	96.07	105.33	3.75	2.56	1.51	2.64
prallethrin	108.60	100.24	5.87	2.14	12.22	8.38
endosulfan-α	93.67	103.36	3.56	2.90	6.90	1.45
p,p-DDE	92.13	101.28	1.93	1.36	9.44	4.58
endosulfan-β	86.80	93.55	5.63	2.43	6.00	8.80
o,p-DDT	82.13	90.2	2.32	3.89	6.05	6.10
piperonyl butoxide	85.93	90.8	4.12	3.10	0.27	1.75
resmethrin	119.00	109.69	7.95	5.75	9.19	17.71
azinphos-methyl	107.53	100.56	8.42	9.01	17.26	5.33

Permethrin-I	91.80	92.31	4.50	8.49	11.32	9.72
Permethrin-II	90.20	94.68	6.37	5.07	9.79	4.06
cyfluthrin-I	91.60	102.04	3.90	2.75	5.79	9.45
cyfluthrin-II	89.73	101.44	5.47	2.38	3.25	6.45
cyfluthrin-III	92.13	100.51	7.63	4.42	3.15	10.84
cyfluthrin-IV	74.53	100.27	8.48	7.04	10.27	8.25
cypermethrin-I	90.07	95.21	5.39	5.18	8.60	3.26
cypermethrin-II	90.07	98.52	2.79	2.38	4.74	4.97
cypermethrin-III	98.67	103.21	7.47	3.68	4.06	4.56
fenvalerate-I	97.33	99	2.87	3.45	13.57	11.38
fenvalerate-II	95.33	100.93	3.60	4.40	11.66	11.78

Analysis of human breast milk, cow milk and baby formulas

The concentration levels of those pesticides that are detectable in human breast milk, cow milk, and baby formulas are shown in Table 3-5. For cow milk and baby formula samples, each category contains 10 different samples. The 10 human breast milk samples came from 6 different participants. Multiple samples were collected from each participant at different breastfeeding stage.

Table 3-5 concentration levels (ppb) of analytes in cow milk, human breast milk and baby formulas

		hcb	atr	fon	dia	ben	chlm	cpy	dic
Cow milk	1	0.277	0.205	0.278	0.209	0.199	0.128	0.220	0.230
	2	0.152	0.091	0.165	0.139	0.154	0.084	0.156	0.127
	3	0.156	0.093	0.191	0.160	0.170	0.120	0.205	0.122
	4	0.161	0.108	0.148	0.127	0.160	0.072	0.146	0.149
	5	0.200	0.067	0.070	0.077	0.127	0.042	0.124	0.098
	6	0.095	0.053	0.074	0.070	0.089	0.050	0.101	0.073
	7	0.154	0.056	0.045	0.066	0.059	0.030	0.088	0.084
	8	0.058	0.039	0.087	0.046		0.035	0.049	0.061
	9	0.073	0.032	0.049	0.040		0.030	0.043	0.043
	10	0.054		0.065	0.045	0.043	0.027	0.035	0.033
Human milk	1	1.119	0.071		0.069			0.711	0.180
	2	0.900	0.105		0.100	0.106		0.319	0.239
	3	0.579			0.028			0.060	0.103
	4	0.502			0.032			0.067	0.116
	5	0.349						0.035	0.031
	6	1.208	0.028					0.082	0.073
	7	1.852						0.132	1.115
	8	0.244	0.033					0.027	0.132
	9	0.731			0.044			0.038	0.061

	10	0.224						0.022	0.029
Baby formulas	1	0.224	0.066	0.014	0.023	0.044	0.027	0.062	0.080
	2	0.212	0.042	0.008	0.019		0.021	0.051	0.071
	3	0.362	0.077		0.021			0.035	0.096
	4	0.229	0.056		0.019		0.016	0.045	0.076
	5	0.165	0.040				0.013	0.045	0.047
	6	0.186	0.030					0.034	0.048
	7	0.244					0.046	0.000	0.046
	8	0.133						0.033	0.035
	9	0.135						0.032	0.029
	10	0.218						0.038	0.039

		hep	ddeop	endoA	ddepp	endoB	ddtop	pbo	azm
Cow milk	1	0.270	0.169	0.179	0.164	0.165	0.143	0.204	0.166
	2	0.097	0.093	0.092	0.089	0.078	0.075	0.124	
	3	0.095	0.093	0.084	0.090	0.089	0.070	0.205	
	4	0.139	0.100	0.103	0.111	0.094	0.083	0.154	0.068
	5	0.074	0.057	0.063	0.064		0.050	0.168	0.751
	6		0.043	0.042	0.044		0.033	0.086	
	7		0.044	0.037	0.061		0.038	0.042	
	8		0.053	0.042	0.073		0.044	0.054	

	9		0.040	0.041	0.060		0.031	0.055	
	10		0.026		0.036		0.023	0.041	
Human milk	1	1.778	0.050		1.924		0.062		
	2	2.182	0.070		1.828		0.076	0.150	
	3	0.925	0.020		0.675		0.021	0.009	0.420
	4	1.010	0.021		0.705		0.020		
	5	0.602	0.010		0.879		0.027		0.386
	6	2.722	0.033		3.454				0.620
	7	3.670	0.058		5.644				
	8	0.661	0.022		0.866	0.008	0.039	0.018	0.173
	9	0.356	0.028		4.002			0.022	0.184
	10	0.366	0.007		0.902		0.017		
Baby formulas	1		0.057	0.052	0.057		0.045		
	2		0.043	0.029	0.044		0.040		
	3		0.041	0.044	0.043		0.046		
	4		0.043	0.042	0.041				
	5		0.024		0.022		0.017		
	6		0.020	0.020	0.022		0.016		
	7		0.011	0.028	0.016				
	8		0.014		0.017		0.014		
	9		0.011		0.012		0.008		

	10		0.015		0.024		0.012		

		per-I	per-II	cyf-I	cyf-II	cyf-III	cyp-I	cyp-II	cyp-III	fev-I	fev-II
Cow milk	1	0.255	0.348	0.297	0.172	0.189	0.198	0.229	0.233	0.173	0.170
	2	0.090	0.118	0.152	0.103	0.089	0.078	0.093	0.119	0.105	0.089
	3	0.103	0.159	0.106	0.081	0.074	0.094	0.082	0.115	0.133	0.097
	4	0.115	0.144				0.134	0.152	0.171	0.108	0.093
	5	0.116	0.137								
	6	0.055	0.070								
	7	0.063	0.101		0.060	0.051	0.066	0.078	0.068		
	8	0.062	0.099		0.056	0.047	0.083	0.125	0.131		
	9	0.042	0.073		0.049	0.056	0.085	0.085	0.097		
	10				0.034	0.030		0.030	0.033		
Human milk	1			0.488	0.352	0.276	0.356	0.393	0.431		
	2										
	3										
	4										
	5										
	6						0.258	0.087	0.150		
	7										
	8										
	9										
	10										
Baby formulas	1						0.056	0.086	0.076		
	2						0.061	0.104	0.092		

	3						0.083	0.117	0.119		
	4						0.128	0.190	0.164		
	5						0.106	0.148	0.144		
	6						0.058	0.055	0.075		
	7						0.065	0.078	0.072		
	8						0.076	0.069	0.065		
	9										
	10										

*Blank means no detectable concentration levels

Some of organochlorine and organophosphorus insecticides such as hexachlorobenzene, chlorpyrifos, p,p-dicofol, o,p-DDE and p,p-DDE exist in all the samples we analyzed. There are detectable levels of permethrin, cyfluthrin and fenvalerate in some of the cow milk samples, but not in human breast milk and baby formula samples. Some of the pesticides, such as piperonyl butoxide, azinphos-methyl and heptachlor epoxide can only be detected in some of the cow milk and human breast milk samples but not in baby formula samples. This is expected because most pesticides have higher LOD in baby formulas than in cow milk and human breast milk.

3.4 Discussion

Liquid-liquid extraction

Based on different extraction methods listed in the introduction part, several solvents are commonly used to denature and/or extract pesticides from milk samples: methanol, formic acid, acetonitrile, acetone, ethanol, diethyl ether, DCM, benzene, cyclohexane, and *n*-hexane, which are listed in the order of polarity from polar (polarity index 5.1) to nonpolar (polarity index 0.0)[94]. The polarity of the pesticides in this study also varies from polar to nonpolar. After comparing the polarity and ruling out solvents with relatively high toxicity, hexane and acetonitrile (ACN) were chosen to extract relatively nonpolar and polar pesticides respectively.

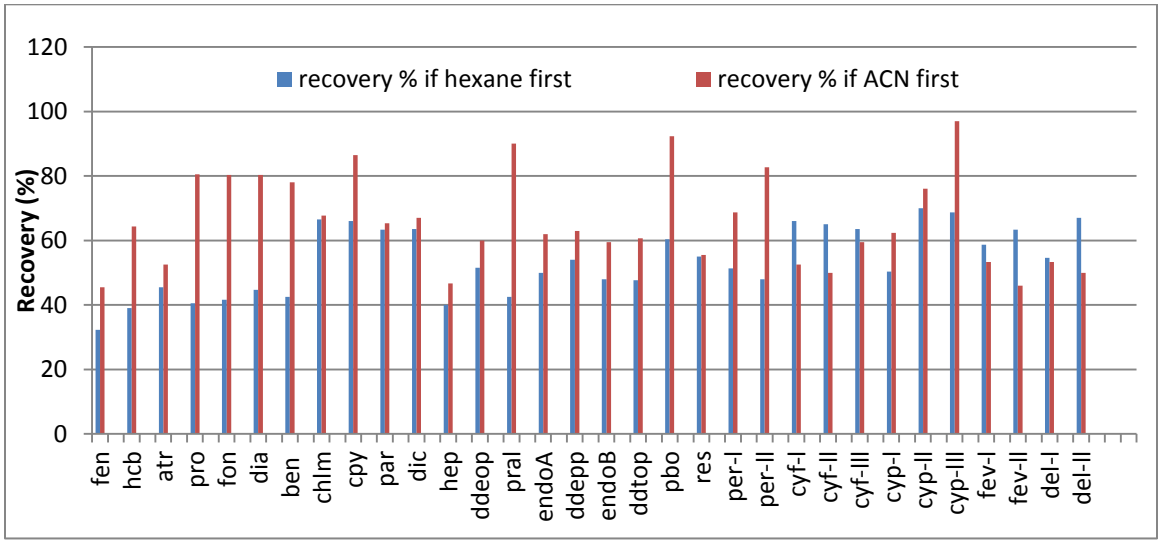


Figure 3-5 the comparison of recoveries (%) between two methods. Blue bars represent the method that first extracting with hexane and followed by ACN extraction. Red bars represent the method that first extracting with ACN and followed by hexane extraction.

The results in Figure 3-6 show that for majority of the pesticides, if the milk samples were extracted with ACN first and followed by hexane extraction, better recoveries were achieved.

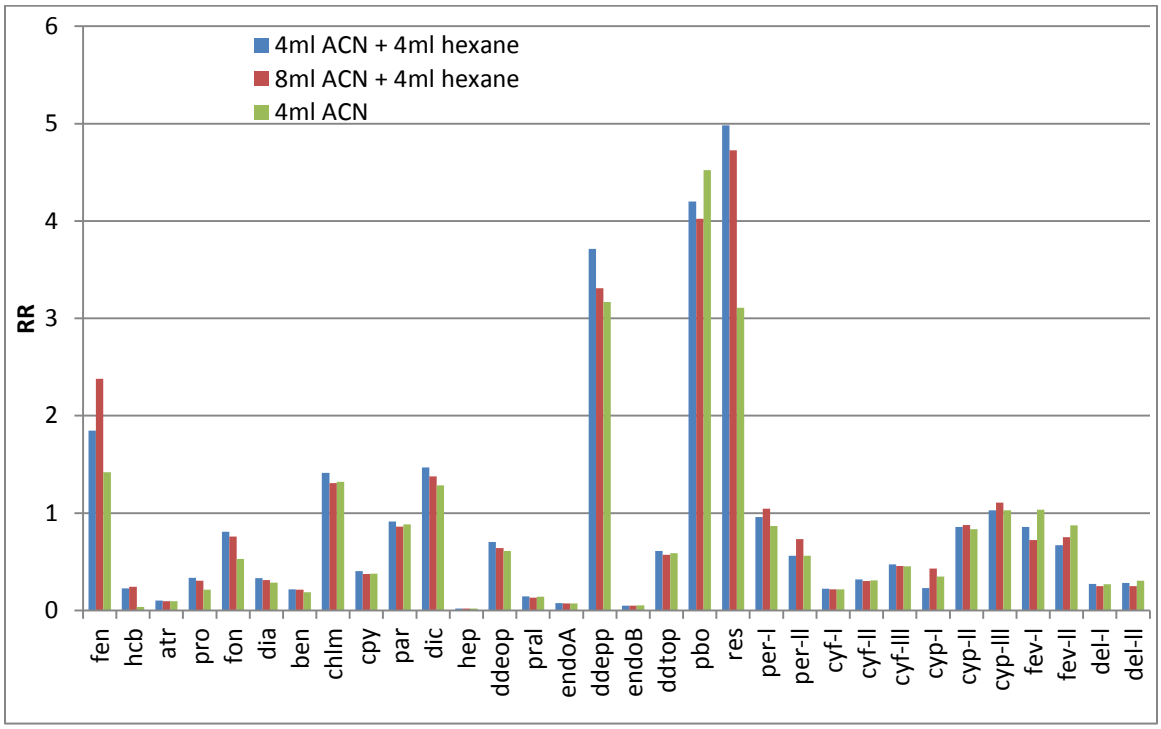


Figure 3-6 Comparison of RR among 3 different liquid-liquid extraction methods. Blue, red, and green bars represent method (1) extract with 4 mL ACN first and then 4 mL hexane, method (2) extract with 8 mL ACN first and then 4 mL hexane, and method (3) extract with 4 mL ACN only, respectively.

Figure 3-6 shows that hexane was needed for better results, while increasing the volume of the extraction solvent acetonitrile did not necessarily give better results. Therefore, method (1) liquid-liquid extraction was chosen for later studies and the modified liquid-liquid extraction procedure was shown in Figure 3-7.

Figure 3-7 Flow diagram of the liquid-liquid extraction procedure followed by SPE and GC analysis

Sample clean-up

In order to develop a SPE procedure for cleaning-up and concentrating targeted pesticides from milk extracts, different SPE tubes and eluting organic solvents were tested.

The Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tube was tested first with the SPE procedure adapted from Hunter et al.[95] After preconditioning a cartridge with 5 mL acetonitrile/toluene (3:1, v/v), about 5 mL extract from the procedure in Figure 3-7 was loaded to it. After loading, the cartridge was eluted twice with 10 mL acetonitrile/toluene (3:1, v/v). The eluent was collected, combined, evaporated to dryness, and reconstituted with 50 µL acetonitrile/toluene (3:1, v/v) for GC/MS analysis. The results from the corresponding recovery studies are shown in Figure 3-8.

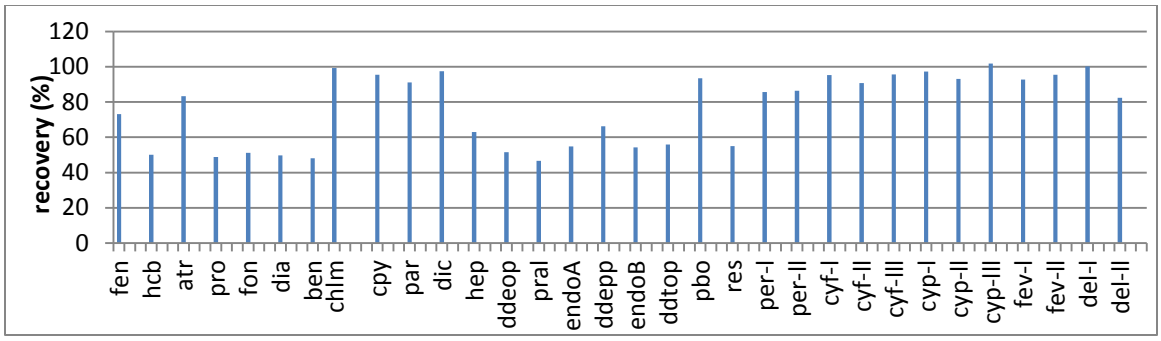


Figure 3-8 Recoveries (%) with the Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tubes.

Some of the recoveries in Figure 3-8 were lower than 50%. We could either lose them in the liquid-liquid extraction part or during the SPE procedure. The next experiment was designed to check if we lost some of the pesticides during the SPE procedure. About 5 mL extract from Figure 3-7 procedure was loaded to a preconditioned Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tube. The extract passing through the cartridge was collected to check the breakthrough of the cartridge. Different combinations of eluting solvents were applied: (1) elute twice with 10 mL acetonitrile/toluene (3:1, v/v), (2) elute with 10 mL acetonitrile first and then 10 mL toluene, and (3) elute with 5 mL acetonitrile first and then 15 mL toluene. Each combination was tested in replicate samples and the results are shown in Figure 3-9. Results in Figure 3-9 show that different eluting solvents combinations worked better with different groups of pesticides, but generally, eluting solvents combination (2) with 10 mL acetonitrile and 10 mL toluene worked better for most of the compounds, which was selected for later experiments.

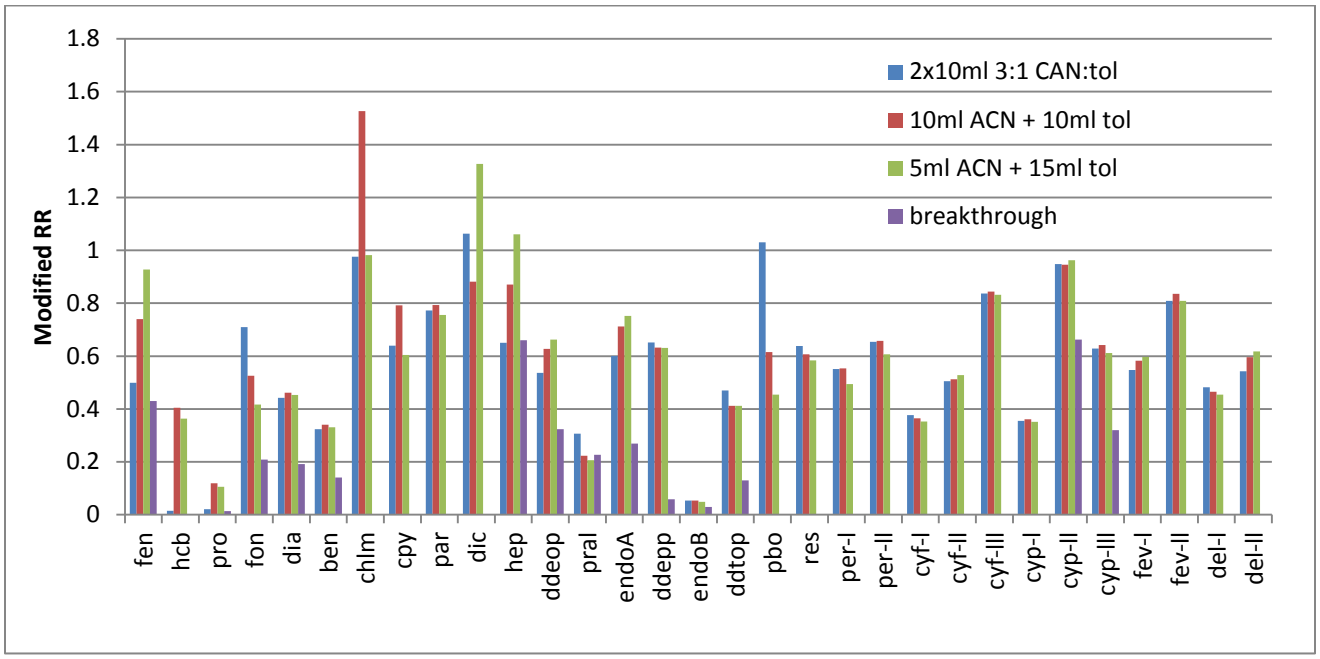


Figure 3-9 the comparison of modified response ratio (RR) among 3 different combinations of eluting solvents and the breakthrough of the carb/PSA cartridges. Blue, red, and green bars represent combination (1), (2), and (3), respectively. Purple bars represent the breakthrough of the cartridge with 5 mL sample loading. In order to fit the graph in the same scale for better visualization and comparison, the RR for an individual pesticide of each solvent combination was divided by the average RR of the 3 different combinations for that pesticide to get the modified RR.

Analytes which had breakthrough in Figure 3-9 also showed lower recoveries in Figure 3-8. These consistent results indicate that the breakthrough of the cartridge was one of the reasons that caused low recoveries for some of the analytes in Figure 3-8. The breakthrough could be either caused by the large loading volume of the extract and/or

the nature of the loading solvent diethyl ether. After blowing hexane extract to dryness, diethyl ether was originally used to reconstitute the pesticides extracted by hexane because of its polarity and that it is miscible with acetonitrile. Since diethyl ether might compromise the integrity of carb/PSA cartridges, acetonitrile was used to replace it for reconstitution.

The next experiment was designed to assess if switching loading solvent and reducing loading volume could eliminate the breakthrough of the cartridge. After spiking 100 µL 250 ppb native standard to 1 mL organic milk, group 1 samples were prepared following the procedure in Figure 3-10 and group 2 samples were also prepared the same way but without TurboVap the 5 mL extract to 2 mL before loading to preconditioned SPE cartridges. Results were shown in Figure 3-11, which indicate that after reducing the loading volume from 5 mL to 2 mL, the recoveries were greatly improved for these pesticides having relatively low recoveries in Figure 3-8 and breakthrough in Figure 3-9.

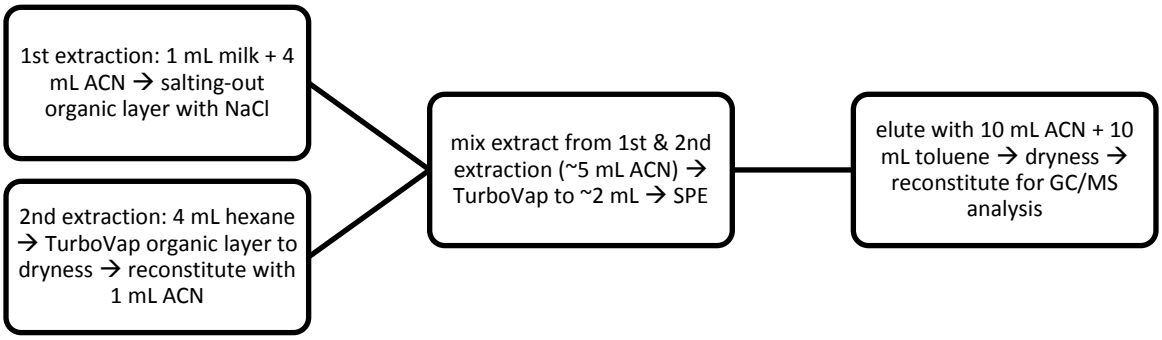


Figure 3-10 Flow diagram of the modified liquid-liquid extraction and SPE procedure

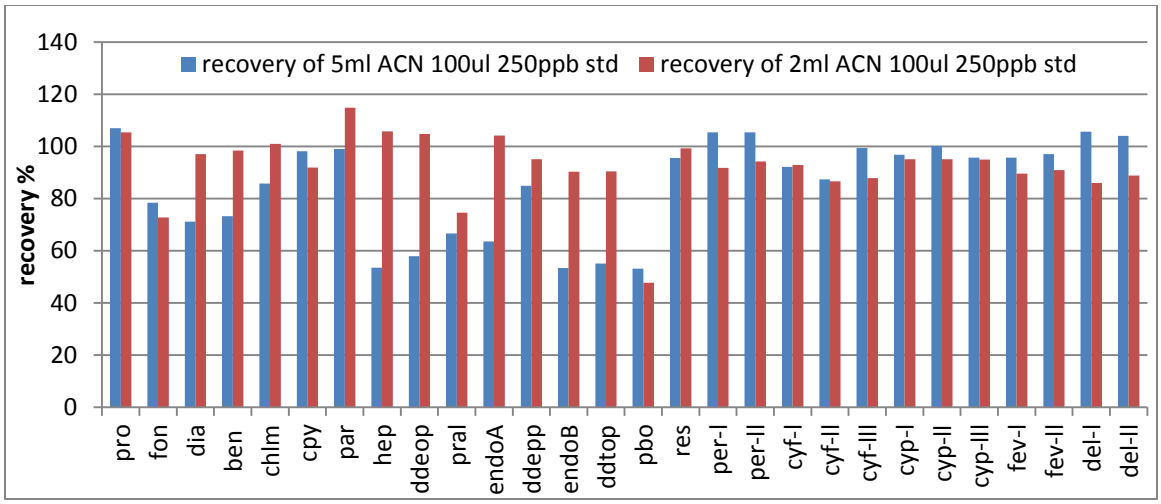


Figure 3-11 the comparison of recoveries from two different groups of samples. Blue and red bars represent group 2 and 1 samples respectively.

As we mentioned earlier, graphitized carbon black (GCB) adsorbents are usually preferred in removing chlorophyll of green vegetable extracts, but perform poorly in eliminating fatty acid matrices[87]. However, PSA is one of the most powerful solid phase extraction adsorbents for clean-up fatty matrices and multi-pesticide residue analysis[86]. Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tubes are more expensive than PSA bonded silica 500mg 6mL SPE tubes. Since in milk samples, the main matrix interferences come from fatty acids, PSA is preferred. If we could use PSA SPE cartridges to replace the carb/PSA SPE cartridges in our samples preparation, this method overall would be more economically friendly.

This experiment was designed to assess how PSA cartridges work with multi-pesticide residues. Acetonitrile (2 mL) was spiked with 100 µL 250 ppb native standard before loading to the PSA cartridge preconditioned with 5 mL acetonitrile/toluene (3:1, v/v).

Two different combinations of eluting solvents were tested: (1) elute twice with 10 mL acetonitrile/toluene (3:1, v/v) and (2) elute with 10 mL acetonitrile first and then 10 mL toluene. Due to poorly adjusted time segments on GC/MS when these samples were run, we did not obtain the complete data for all the pesticides. The partially obtained data were shown in Figure 3-12. It is obvious that PSA only did not work as well as carb/PSA cartridges.

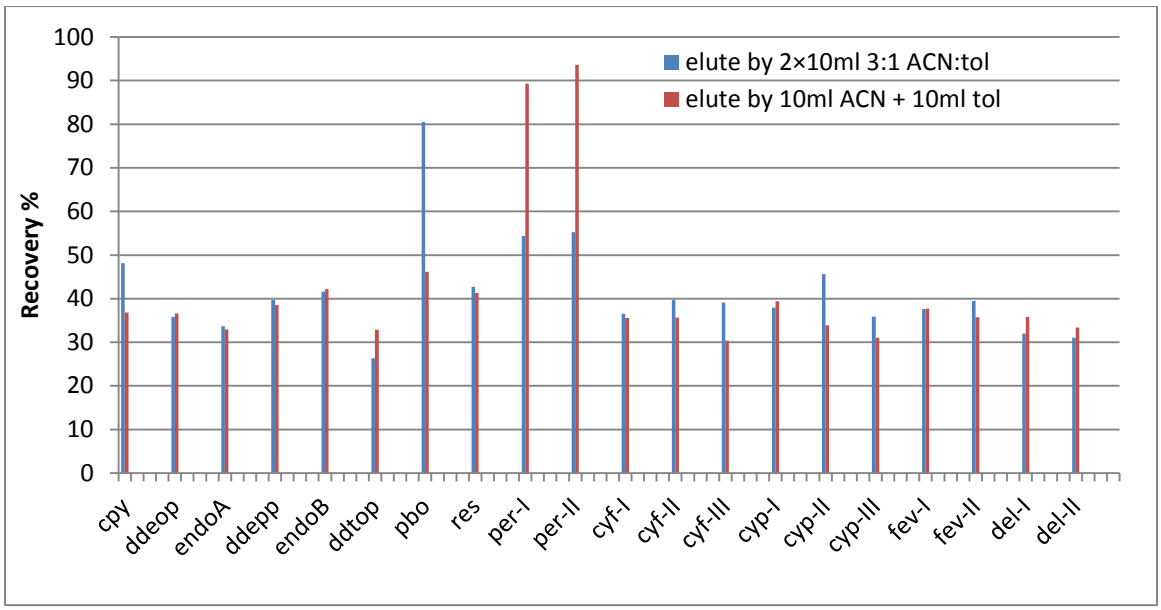


Figure 3-12 Recoveries of 500mg 6ml PSA cartridges without matrices

Milk samples (1 mL/sample) spiked with 100 µL 100 ppb or 250 ppb native standard were prepared following the procedure in Figure 3-10 with Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tubes. As shown in Figure 3-13, the recoveries between two different spiking levels were pretty consistent and almost all the recoveries were within the range of 70-90%.

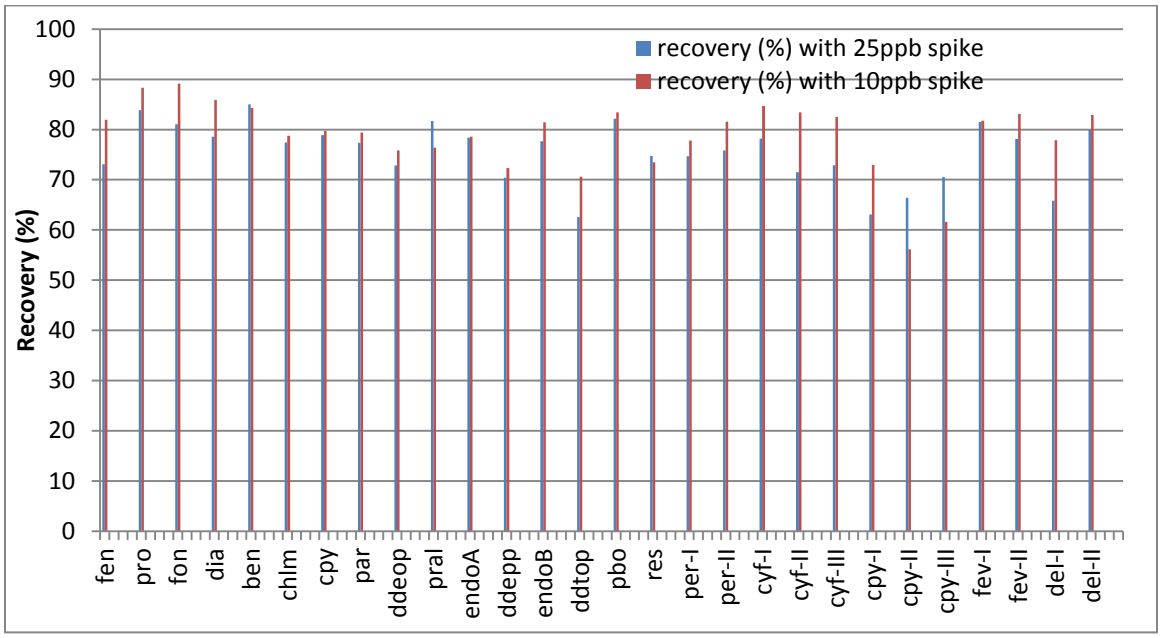


Figure 3-13 Recoveries at two different spiking levels

Although this latest sample preparation procedure gave acceptable recoveries, it failed to deliver samples that were clean enough, which resulting in high LOD. After injecting about 50 samples prepared by this procedure into the GC/MS, peaks on chromatogram began to tailing. If more samples were injected into the GC, dark residues would be observed at the bottom of the liner located at the injection port, in the GC capillary column near the injection end, and on the electron ionization (EI) source of the GC. Also, for majority of the pesticides, no peaks could be identified below 0.5 ppb on GC chromatograms. Therefore, several approaches were tested to clean-up samples.

Acetonitrile with 1% acetic acid was used to replace acetonitrile for the first extraction while preceding the remaining steps as usual. The original idea was using acetonitrile

with 1% acetic acid to denature the protein in milk at the beginning of the sample preparation and reduce their chances to be carried all the way to the end of the procedure, which would help further clean the samples. Milk samples (1 mL/sample) spiked with 100 µL 250 ppb native standard were prepared using acetonitrile or 1% acetic acid acetonitrile as the first liquid-liquid extraction. The other steps remained the same. Results are shown in Figure 3-14. Recoveries using 1% acetic acid acetonitrile ranged from less than 25% to more than 200%. Also, large error bars indicate that data within triplicate samples were extremely inconsistent. Therefore, using 1% acetic acid acetonitrile for the first extraction failed to deliver consistent and useful results.

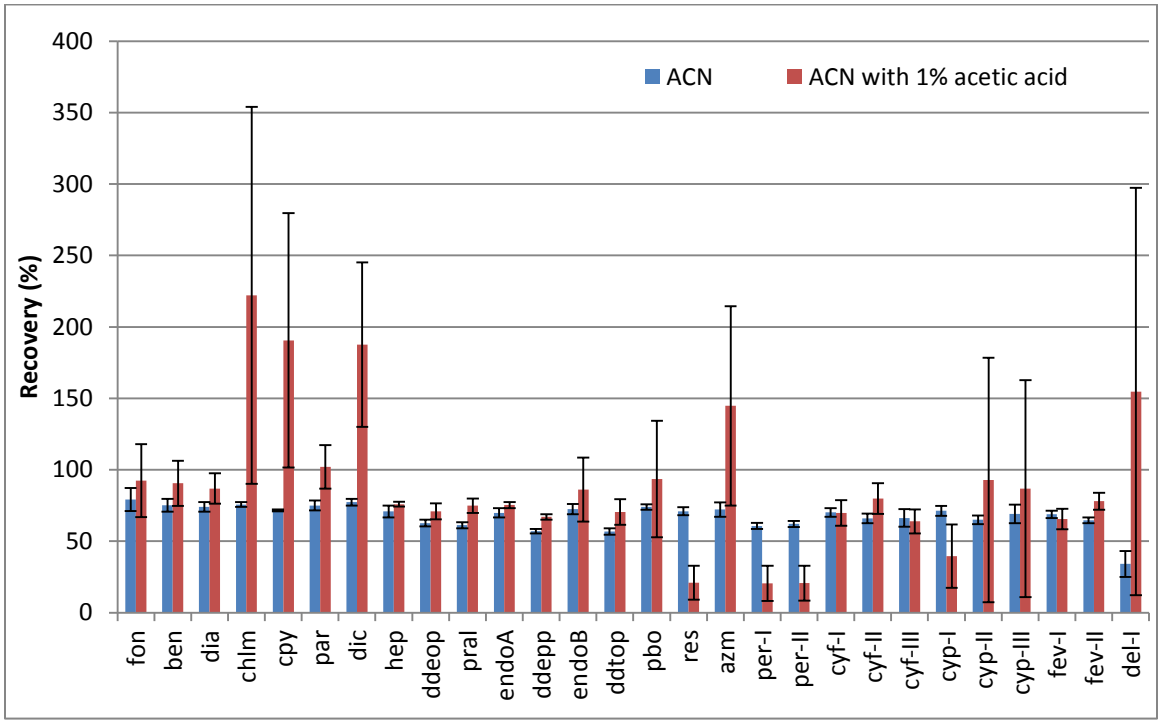


Figure 3-14 Recoveries of two sample preparation methods. Blue and red bars represent the first extraction with acetonitrile and 1% acetic acid acetonitrile respectively.

Instead of using Dual-Layer Envi-Carb II/PSA 500/300mg 6mL SPE tubes, Oasis HLB Extraction Cartridges (3 cc/60 mg) were tested. The following SPE procedure was adapted from Olsson et al.[96] and modified in our lab. After combining the extract from the first and second liquid-liquid extraction, it was TurboVap to 1 mL. This 1 mL extract was mixed with 5 mL H_2O before loading to the cartridge that was preconditioned with 3 mL methanol and 3 mL 1% acetic acid in H_2O. The cartridge was then eluted twice with 2.5 mL methanol after loading and the eluent was collected. However, the test tube used to collected eluent had yellow residues after evaporating the collected eluent, which indicating dirty samples. We did not inject these samples into the GC for analysis since they were not clean enough.

Disperse PSA was introduced and worked well in further cleaning-up samples, which helped finalize the sample preparation procedure in this study.

Some previous experiments were re-conducted with this new procedure that adding disperse PSA extraction step. Cow milk samples (1 mL) spiked with 100 µL 250 ppb native standard and 50 µL 400 ppb internal standard were used. The experimental results are shown in Figure 3-15.

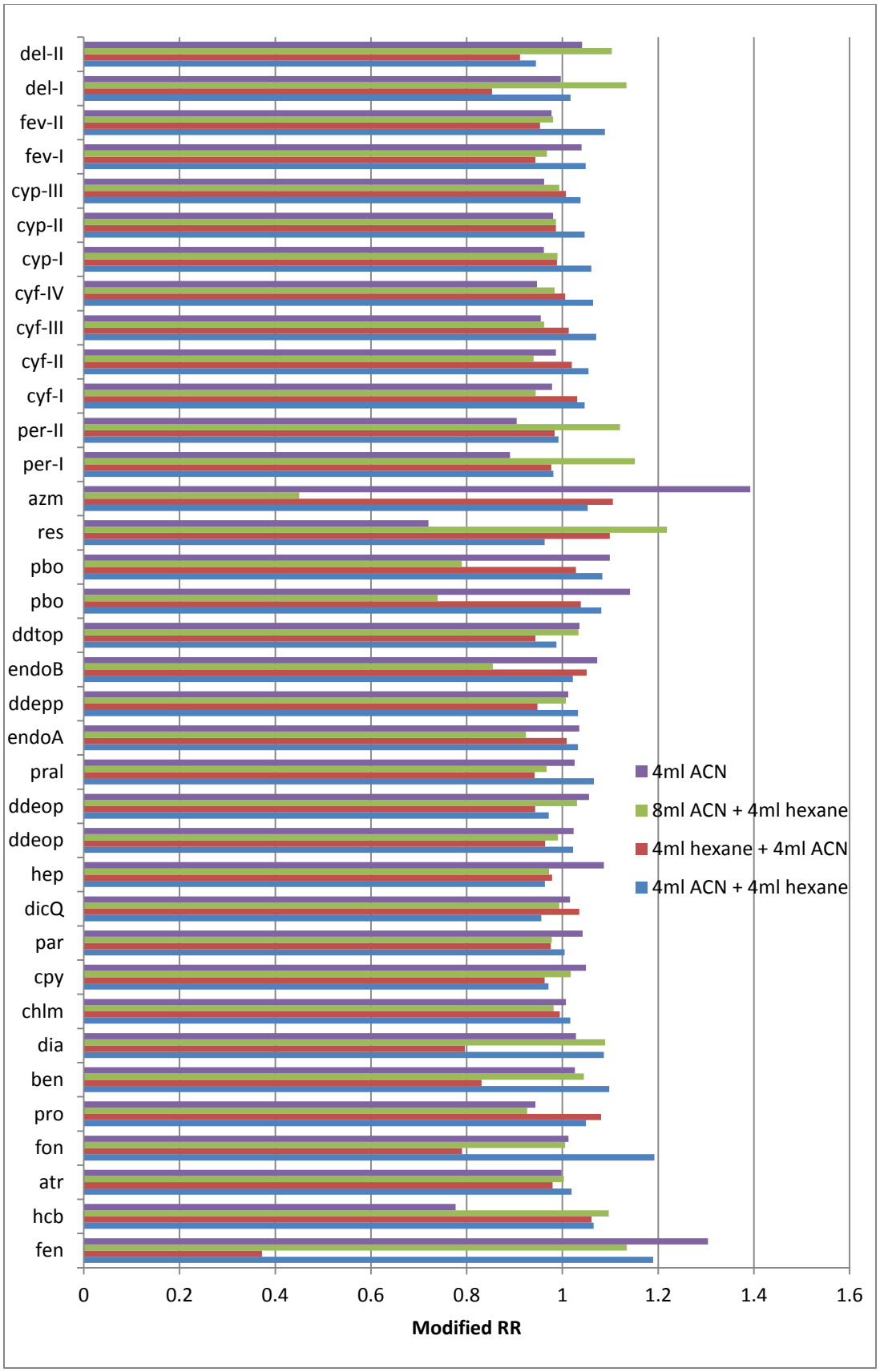

del-II
del-I
fev-II
fev-I
cyp-III
cyp-II
cyp-I
cyf-IV
cyf-III
cyf-II
cyf-I
per-II
per-I
azm
res
pbo
pbo
ddtop
endoB
ddepp
endoA
pral
ddeop
ddeop
hep
dicQ
par
cpy
chlm
dia
ben
pro
fon
atr
hcb
fen
4ml ACN
8ml ACN + 4ml hexane
4ml hexane + 4ml ACN
4ml ACN + 4ml hexane
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Modified RR

Figure 3-15 the comparison of modified response ratio (RR) among 4 methods with different combination of extraction solvents and their volumes. Blue bars represent the method that first extracting with 4 mL ACN and followed by 4 mL hexane. Red bars represent the method that first extracting with 4 mL hexane and followed by 4 mL ACN. Green bars represent the method that first extracting with 8 mL ACN and followed by 4 mL hexane. Purple bars represent the method that only extracting with 4 mL ACN without hexane. In order to fit the graph in the same scale for better visualization and comparison, the RR for an individual pesticide of each method was divided by the average RR of the 4 different methods for that pesticide to get the modified RR. Sample size n=3.

The comparison of blue and red bars in Figure 3-15 further confirm that if hexane and acetonitrile were both used to extract milk samples, extracting with acetonitrile followed by hexane extraction worked better than the reverse way. The comparison of blue and green bars in Figure 3-15 indicate that increasing the volume of acetonitrile during the liquid-liquid extraction step did not necessarily give better results. The comparison of blue and purple bars in Figure 3-15 show that, most of the time, especially for relatively nonpolar compounds such as cyfluthrin and cypermethrin, hexane was needed to achieve better results. This result is also consistent with all the previous results.

Method development for baby formulas

We tried to directly adapt sample preparation method for milk to baby formulas, whose recovery results are shown in Figure 3-16 (red bars). The recoveries were much lower than those of milk samples. Comparing with milk, which was in emulsion form, baby formula solutions, which were made from powder based on manufacture instructions, contained some relatively large particles. These particles might contain pesticides molecules inside, which probably affected the recovery.

In order to achieve better results for baby formula samples, they were sonicated to reach homogenization before extraction and longer vortex and sonication time was needed during extraction step comparing to cow milk and human milk samples. The results of recovery study for baby formulas with sonication at the beginning and longer vortex time are shown in Figure 3-16 (blue bars). Especially for these pesticides with relatively low recoveries, this new procedure significantly improved the recovery.

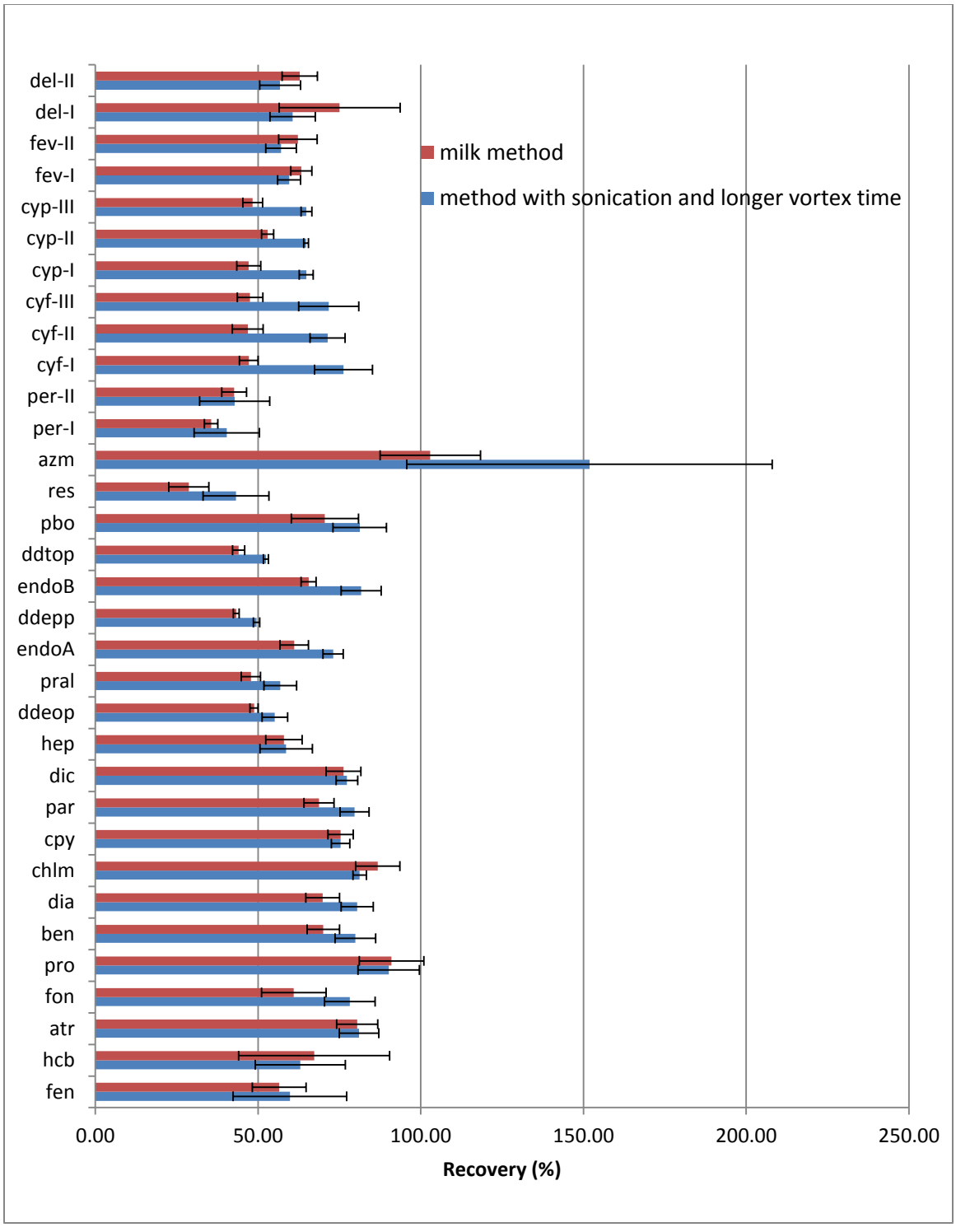


Figure 3-16 recoveries of two different methods for baby formulas at the concentration level of 25 ppb

GC-MS and high sensitive electron ionization (EI) MS analysis

Ions were selected based upon the relative abundance observed in EI spectra and the S/N ratio for that specific ion. For each ISTD compound, the selected fragment ion must also retain the label to distinguish from its corresponding native analyte. For the analyte having its corresponding labeled ISTD, the selected fragment ion must have its naturally occurring isotope peak.

Table 3-6 lists the retention time and fragmentation including the best three transitions for generally purposes in our lab. These parameters were based on an Agilent Model 7000 gas chromatography with triple quadruple tandem mass spectrometric detection. A HP-5MS column was used (30 m, 0.25 mm ID, 0.25 μm film) with a GC temperature program #1 (Table 3-7) that began at 100 ^{0}C and was held for 2 min, and was then increased at 10 ^{0}C/minutes to 310 ^{0}C and held for 2 min. The total run time was 25 min. The flow rate of carrier gas helium was 1.2 mL/minutes.

Table 3-6 retention time and fragmentation for the pesticides in this study

Pesticide	RT (minutes)	MW	Fragmentation from our Experiment (the 3 best transitions)								
			Q1	Q3	CE	Q1	Q3	CE	Q1	Q3	CE
metolcarb (met)	9.20	165.2	108.2	108.1	10	108.2	107.1	20	108.2	77.9	20
fenobucarb (fen)	10.98	207.3	121.2	77.1	20	121.2	103.1	20	121.2	51.2	40
hexachlorobenzene (hcb)	11.71	284.8	284.0	283.7	10	284.0	249.1	25	284.0	214.2	40
atrazine (atr)	12.15	215.7	200.3	200.1	5	200.3	104.1	20	200.3	94.0	30

propoxur (pro)	12.43	209.2	110.2	110.1	5	110.2	66.1	20	110.2	51.2	40
fonofos (fon)	12.46	246.3	109.1	109.1	5	109.1	81.0	5	109.1	63.1	15
bendiocarb (ben)	12.63	223.2	151.2	84.1	15	151.2	151.2	5	151.2	68.2	25
diazinon (dia)	12.67	304.3	179.3	179.2	5	179.3	137.4	20	179.3	121.0	40
carbofuran (car)	12.68	221.3	164.2	148.1	25	164.2	163.4	10	164.2	149.2	10
chlorpyrifos-methyl (chlm)	13.60	322.6	286.2	286.1	10	286.2	93.0	20	125.1	79.1	10
chlorpyrifos (cpy)	14.57	350.6	314.2	258.0	25	314.2	285.9	10	314.2	194.2	30
parathion (par)	14.62	291.3	291.3	109.1	10	291.2	81.1	10	291.3	90.9	35
dicofol, p,p- (dic)	14.63	370.5	139.1	111.1	15	139.1	139.1	5	139.1	75.1	30
heptachlor epoxide (hep)	15.20	389.3	353.1	262.8	20	353.1	352.7	5	353.1	281.9	15
DDE, o,p- (ddeop)	15.74	318.0	246.2	246.1	5	246.2	176.2	35	246.2	211.1	25
prallethrin (pral)	15.81	300.4	123.2	123.1	5	123.2	55.3	20	123.2	87.1	15
endosulfan-α (endoA)	15.90	406.9	239.1	239.0	5	241.1	241.0	5	241.1	206.1	20
DDE, p,p- (ddepp)	16.33	318.0	246.2	176.2	35	246.2	246.1	10	246.2	211.1	20
endosulfan-β (endoB)	17.02	406.9	237.1	165.2	35	237.1	199.1	20	237.1	237.2	10
DDT, o,p- (ddtop)	17.11	354.5	235.2	165.1	25	235.2	235.0	10	235.2	199.1	15
DDT, p,p- (ddtpp)	17.80	354.5	235.2	165.2	25	235.2	235.0	5	235.2	199.2	20
piperonyl butoxide (pbo)	18.18	338.4	176.2	103.1	30	176.2	77.2	40	176.2	91.1	40
resmethrin (res)	18.25	382.5	171.2	143.2	4	123.2	81.2	20	-	-	-
EPN (epn)	18.76	323.3	169.2	141.1	5	323.3	157.1	20	-	-	-
azinphos-methyl (azm)	19.49	317.3	132.2	77.1	15	132.2	51.2	35	160.2	77.2	20
permethrin (per)	20.44	391.3	183.2	183.1	10	183.2	168.2	20	183.2	115.2	40
cyfluthrin (cyf)	21.03	434.3	163.1	163.2	5	163.1	91.2	20	163.1	127.2	5
cypermethrin (cyp)	21.34	416.3	163.1	91.0	20	165.1	91.2	20	163.1	93.2	15
fenvalerate (fen)	22.24	419.9	167.2	89.2	40	125.2	125.1	5	125.2	89.0	20
deltamethrin (del)	22.76	505.2	181.2	181.1	10	181.2	152.2	30	253.1	93.2	20

Because cyfluthrin and cypermethrin both have isomers, they usually have four peaks on GC chromatogram[97]. However, with the above temperature program, all the three transitions for either cyfluthrin or cypermethrin only have 3 peaks. The third and fourth

peak had no resolution and looked like one peak on chromatogram. For compounds that usually have two peaks, such as permethrin and fenvalerate, good baseline resolution could not be achieved. Also, because the retention time for some pesticides was so close that we could not be separated these pesticides into different time segments. If a time segment contains too many analytes and transitions, the intensity and scan points of each peak in that time segment will be reduced, which will resulting in bad peak shape and errors in peak area counts. However, the linear temperature program #1 was a starting point, which provided information on the retention characteristics of the analytes. The next steps were to adjust the temperature programs to obtain adequate resolution and suitable analysis time.

Changing the ramp rate can alter the resolution of the peaks eluting in the middle of the chromatogram. When decreasing the ramp rate or adding mid-ramp hold, better resolution of later eluting peaks usually can be achieved. A mid-ramp hold is a several minute isothermal portion somewhere during a temperature ramp, which is usually used 20-30 ^{0}C below the temperature that the peak of the interest is eluting. The time of a mid-ramp hold is usually 2-5 minutes, because if it is too short or too long, there will be either no effect or a detrimental effect on peak resolution.[98] Temperature program was adjusted gradually based on these rules and all the programs we tested are listed in Table 3-7. Temperature program #4, the final program we used in our project, provided adequate resolution and acceptable analysis time considering the number of analytes for each run. Because of the matrices, extracted samples often contain compounds that elute after the last analyte of interest. The long final hold time (12 minutes) was to

ensure all the other compounds from milk or baby formulas matrices elute from the GC column for every run and avoid column contamination.

Table 3-7 four different temperature programs for the GC/MS

Temperature Program	Rate (^{0}C/minutes)	Temp (^{0}C)	Hold Time (minutes)
#1		100	2
	10	310	2
#2		100	2
	10	205	1
	10	223	1.5
	10	310	2
#3		100	2
	10	205	3
	10	310	2
#4		100	2
	10	205	3
	10	280	4
	25	310	12

After achieving good resolution for each analyte, they were placed into different time segments to achieve better sensitivity. Analytes having relatively close masses and retention times were placed into the same time segment (Table 3-2) to avoid significant reductions in the accelerating voltage that might lead to the loss of sensitivity.

Table 3-6 lists the best three transitions for generally purposes in our lab. After repeatedly injecting milk samples spiked with high concentration level of pesticides, quantification and confirmation ions for these native pesticides and isotopically labeled standards were selected by monitoring the intensity, peak shape, signal to noise ratio, and potential interference in milk samples (Table 3-2). For propoxur, because the peak from the interference of the milk matrices overlapped with the peak from its fragment ions and we could not distinguish these two peaks, it was not analyzed in the method validation samples and unknown samples. Propoxur is not included in Table 3-2.

Furthermore, dwell time, which is the product of sample number and integration time, were adjust to ensure enough data points for each peak and thus achieve ideal peak shape for each analyte. Replicate samples were injected into GC multiple times under different dwell time settings. The responses for each individual analyte were recorded and analyzed. There statistic results are shown in Table 3-8. The dwell time, which gave higher response but lower coefficient of variation (CV) in a specific time segment, was selected for that specific time segment.

Table 3-8 comparison of different dwell time with GC responses

	Dwell Time	fen	hcb	atr	pro	fon	ben	dia	chlm	cpy
Average of GC response	HD	1775	2505	1407	103	2382	812	3460	2566	1578
	MD	2014	2558	2699	114	2534	848	3597	2862	1717
	LD	2054	2522	2632	102	2556	860	3611	2803	1741
CV	HD	0.140	0.031	0.339	0.052	0.042	0.034	0.031	0.104	0.029
	MD	0.079	0.051	0.049	0.140	0.057	0.078	0.048	0.048	0.045
	LD	0.091	0.065	0.065	0.097	0.077	0.087	0.073	0.081	0.074

	Dwell Time	par	dic	hep	pral	ddeop	endoA	ddepp	endoB	ddtop
Average of GC response	HD	556	13263	361	324	5571	1218	33649	754	8634
	MD	585	14289	376	329	5500	1315	35581	782	9454
	LD	551	14052	367	327	5479	1255	34725	751	9238
CV	HD	0.114	0.044	0.051	0.034	0.036	0.030	0.021	0.031	0.060
	MD	0.054	0.039	0.051	0.096	0.051	0.047	0.042	0.063	0.047
	LD	0.041	0.079	0.081	0.084	0.089	0.055	0.071	0.075	0.102

	Dwell Time	pbo	res	azm	per-I	per-II	cyf-I	cyf-II	cyf-III	cyf-IV
Average of GC response	HD	10979	7101	1367	1366	850	1218	1583	1105	962
	MD	11595	7550	1899	1457	879	1380	1775	1228	1178
	LD	11353	7384	1885	1443	912	1354	1761	1172	1176
CV	HD	0.021	0.018	0.550	0.038	0.031	0.133	0.150	0.188	0.073
	MD	0.038	0.039	0.228	0.062	0.044	0.079	0.079	0.077	0.077
	LD	0.068	0.065	0.195	0.066	0.071	0.096	0.119	0.106	0.108

	Dwell Time	cyp-I	cyp-II	cyp-III	cyp-IV	per-I	per-II	del-I	del-II
Average of GC response	HD	2267	1967	1946	1159	6086	4011	806	1919
	MD	2602	2232	2123	1466	6895	4562	910	2235
	LD	2550	2238	2108	1417	6703	4425	925	2195
CV	HD	0.174	0.169	0.159	0.113	0.160	0.152	0.121	0.217
	MD	0.061	0.099	0.102	0.084	0.084	0.091	0.091	0.106
	LD	0.082	0.109	0.145	0.112	0.121	0.112	0.098	0.140

*CV = coefficient of variation, HD = high dwell time, MD = medium dwell time, and LD = low dwell time. Results were based on quantification ions.

Limit of detection (LOD)

Method validation is a major concern for method development, which includes specificity or selectivity, linearity of calibration, repeatability, accuracy, precision, recovery, proof of applicability, and limit of detection and quantitation. Generally, the limit of detection is the smallest quantify of analyte that is “significantly different” from the blank. Since the term “significantly different” can be defined in so many different ways, there are many ways to define the LOD, which is a very ambiguous point.[99 100 101 102]

The International Conference for Harmonization (ICH), whose purpose is to bring together the regulatory authorities of Europe, Japan and the US to discuss scientific and technical aspects of product registration, defines the LOD as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOD is frequently confused with the sensitivity of the method. The sensitivity

of an analytical method is its ability to distinguish small differences in concentration or mass of the compound of interest, which is sometimes reflected by the slope of a calibration curve that is obtained by plotting the response from some instrumentation against the amount of an analyte.[103] ICH defines the limit of quantitation (LOQ) as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. There are several ways to determine the LOD and LOQ, but samples used for this purpose must contain analytes whose levels are across the two regions.

In our cases, we focused on LOD instead of LOQ. The ICH[104] described several approaches to determine the LOD: (1) visual evaluation, (2) signal-to-noise, and (3) the standard deviation of the response and the slope. In the visual evaluation approach, the LOD is determined by analyzing samples with known concentration levels and establishing the minimum concentration level that the analyte can be reliably detected. This method is more commonly used for non-instrumental methods. The signal-to-noise approach can only be applied to analytical methods that exhibit baseline noise. In the GC analysis, the instrumental LOD is the ability of GC to distinguish between signal and noise. A signal-to-noise ratio of 3 is generally considered acceptable for estimating the LOD. The LOD is this case is more applied to methods that are entirely instrumental related with no involvement of chemical or other procedural steps, because the variance in the other steps is usually much larger than the one related with instrumentation.

Another common way to determine the LOD is more from a statistical angle and the LOD depends on the standard deviation of blanks (*s*) and two risk values α and β that are associated with type I and II errors respectively.[105] Figure 3-17 shows the meaning of limit of detection using this calculation method.[99] Each curve is a *t* distribution, which is broader than a Gaussian distribution because it applies to a smaller population. If we use 98% confidence and $LOD = t \times s$, there is only 1% chance that the signal from a blank will exceed the limit of detection. Therefore, we are 99% confident that signal above LOD is from the analyte instead of the blank. If the degree of freedom is within certain range, the values of Student's *t* are all very close to 3. Therefore, sometimes 3*s* can be used to calculate the LOD, in which *s* is the standard deviation of blanks.

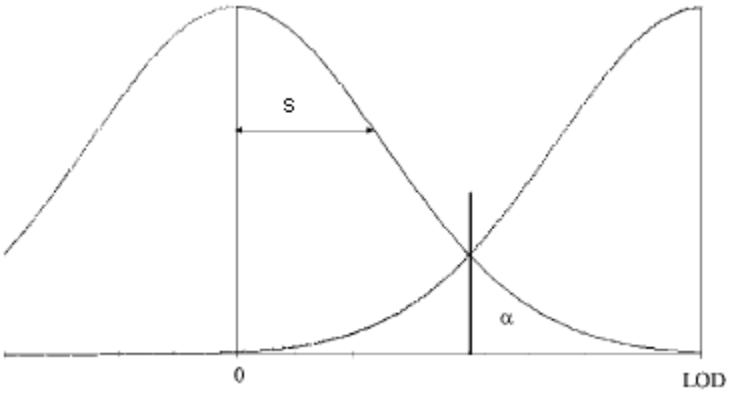


Figure 3-17 Graphical representation of the limit of detection [99]

Researchers also calculate the limit of detection based on the ability of a method to determine an analyte in a sample matrix without considering its source of origin, which is also known as Taylor method[106]. A number of samples (n>7) at each of 3

concentration levels are analyzed to calculate the standard deviation for each level. The calculated standard deviations are plotted against concentration levels and s_0 is obtained through extrapolation, which is equivalent to the *y*-intercept of the plotted regression line. The limit of detection is defined as $3s_0$ at 95% confidence. If the lowest concentration level used in this method is not close enough to zero, the extrapolation distance may be excessive and cause negative values. Figure 3-18 is an example of getting negative values through extrapolation for resmethrin. Seven samples at each of the 4 concentration levels were analyzed to obtain the data in Figure 3-18. Usually in Taylor method, if a negative value is obtained, the limit of detection can be calculated as 3*s* instead, where s is the standard deviation of the lowest concentration level.

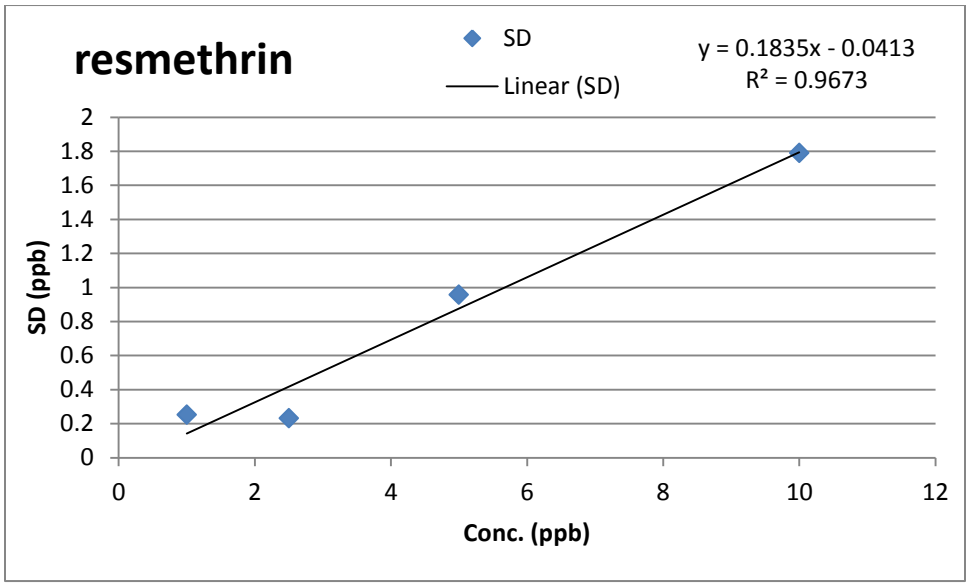


Figure 3-18 Taylor method with 4 concentration levels for resmethrin

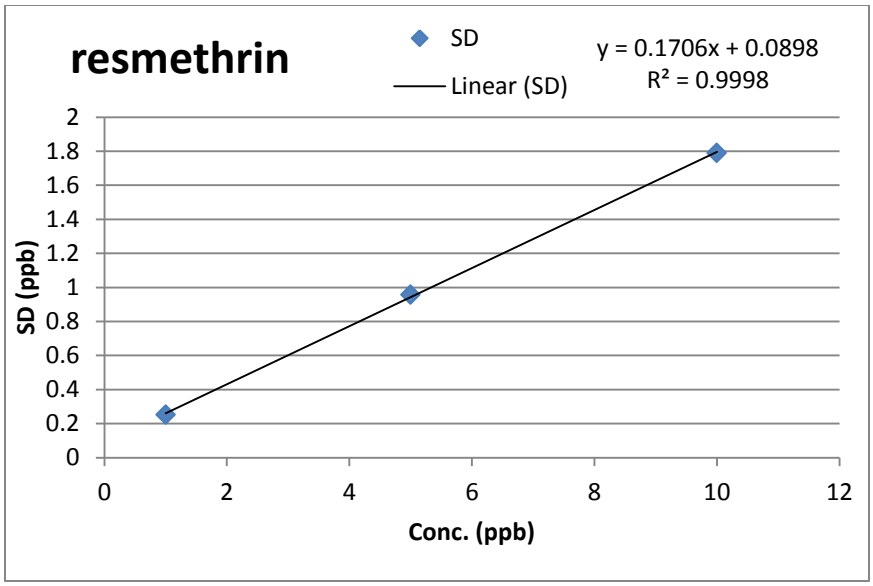


Figure 3-19 Taylor method with 3 concentration levels for resmethrin

Figure 3-19 was obtained by excluding the data point at 2.5 ppb in Figure 3-18, which also gave a positive intercept for s_0 instead of the negative one in Figure. This is a good example to show one of the disadvantages using Taylor method. The intercept and resulting LOD can be significantly different based upon the concentration levels used in calculating the intercept. Furthermore, this method is also highly dependent on instrumental conditions. A small absolute error in a lower concentration level is likely to produce odd values for the associated standard deviation. Similarly, variations such as a small difference in injection volume and matrix effects can lead to large absolute deviation for a high concentration level, which can significantly affect the slope of the standard deviation/concentration curve.

Table 3-9 list the LOD calculated from both Taylor method and Signal-to-Noise method. For the Signal-to-Noise method, the LOD value was estimated based on a lower

concentration level displaying a minimum signal to noise (S/N) ratio of three on the injection of replicate cow milk samples spiked with standard and internal standard solutions. For the Taylor method, the LOD was calculated from 7 cow milk samples spiked with native standards at 4 lower concentration levels and internal standards.

Table 3-9 LOD from the Taylor method and Signal-to-Noise method in cow milk samples

Names of Pesticides	LOD (ppb)	
	Taylor Method	Signal-to-Noise
fen	0.1464	0.1749
hcb	0.0288	0.0010
atr	0.0075	0.0145
fon	0.1593	0.0135
ben	0.3366	0.0101
dia	0.1749	0.0051
chlm	0.1110	0.0060
cpy	0.2628	0.0076
par	0.9003	0.6431
dic	0.0843	0.0099
hep	0.0273	0.0096
ddeop	0.0564	0.0068
pral	1.3095	0.3408

endoA	0.0669	0.0060
ddepp	0.0069	0.0005
endoB	0.0969	0.0123
ddtop	0.0189	0.0033
pbo	*0.2019	0.0194
res	*0.7579	0.1064
azm	*0.8572	0.2119
per-I	0.0360	0.1292
per-II	0.0957	0.2263
cyf-I	0.0276	0.0735
cyf-II	0.0819	0.0771
cyf-III	0.0483	0.2963
cyf-IV	1.2582	0.2736
cyp-I	0.0696	0.0829
cyp-II	0.0423	0.0984
cyp-III	0.0810	0.7745
fev-I	*0.0545	0.0309
fev-II	0.1653	0.0572
del-I	6.8604	1.6692
del-II	6.2517	1.6428

* The starred data means their intercepts from Taylor method were negative, and thus were calculated as 3s, where s was the standard deviation of the lowest concentration level.

Because of all the drawbacks we mentioned for using Taylor method, Signal-to-Noise method was preferred in our study.

Among all these pesticides, 3 of them, which are hexachlorobenzene, *p,p*-DDE, and resmethrin, had fairly high detectable concentration levels in matrix blanks of cow milk samples, Signal-to-Noise method was not a very good choice because the signal-to-noise values for these compounds in matrix blanks were much higher, or sometimes two magnitudes higher than 3. The concentration levels of these three compounds in matrix blanks were calculated based on moving the intercept of the RR/concentration calibration curve to zero. After properly calculating the concentrations for each compound, the LOD was determined from one of the methods we discussed earlier with a formula of $LOD = 3 \times s$, where s is the standard deviation of the concentrations in replicate matrix blank samples. For the data in Table 3-3, 10 matrix blank samples were used.

3.5 Conclusions

An analytical method for the determination of organochlorine (OC), organophosphate (OP), carbamate, and pyrethroid insecticide residues in cow milk, human milk, and baby

formulas was successfully developed. This method involves a liquid-liquid extraction, freezing-lipid filtration, and solid-phase extraction procedure followed by gas chromatography tandem mass spectrometry (GC-MS/MS) for the identification and quantification of targeted pesticides. Ionization of pesticides molecules was achieved by electron ionization in positive mode. Multiple reactions monitoring (MRM) was the acquisition mode used for the monitoring of two MS/MS transitions for each analyte. The average recoveries obtained in cow milk, at two different fortification levels (10 ppb and 25 ppb), had a range of 34-102% with about 75% of the recoveries between 60% and 80%. The estimated limit of detection was lower than 1ppb, which was low enough for detection of targeted pesticides that are generally expected in these types of samples. For majority of the analytes, the accuracy and precision fell within the range of 80-120% and 0-15% respectively. The application of this method was investigated in 10 human milk samples collected from the field, 10 cow milk samples and 10 baby formula samples purchased from local grocery stores. Some of organochlorine and organophosphorus insecticides such as hexachlorobenzene, chlorpyrifos, p,p-dicofol, o,p-DDE and p,p-DDE exist in all the samples we analyzed. There are detectable levels of permethrin, cyfluthrin and fenvalerate in some of the cow milk samples, but not in human breast milk and baby formula samples. Some of the pesticides, such as piperonyl butoxide, azinphos-methyl and heptachlor epoxide can only be detected in some of the cow milk and human breast milk samples but not in baby formula samples.

To our knowledge, this is the first breast milk method to include the OCs, OPs, carbamates, and pyrethroids.

Chapter 4 Method development for determination of current-use and persistent pesticides in human serum using gas chromatography tandem mass spectrometry

4.1 Introduction

As we mentioned in last chapter that pesticides are used extensively all over the world in agriculture and for residential pest control. Pesticides exposure happens mainly through the skin, eyes, inhalation, and ingestion. The fat-soluble pesticides and relatively water-soluble ones can be easily absorbed through intact skin.

Chronic exposure to pesticides might potentially harm human health. Studies have shown that pesticides exposure is associated with endocrine, immune and neuropsychological disorders, and neurodegenerative diseases, such as Parkinson's and Alzheimer. [107] [108] [109]

Some other studies have also shown that some pesticides can induce certain types of cancers. Mathur et al. found that the concentration levels of organochlorine pesticides in blood were significantly higher in breast cancer patients than those in normal women despite of their age, diet, and geographic distribution[110]. The study of Clary et al. suggested that there was increased pancreatic cancer mortality among long-term residents in the areas of high application rates of 1,3-dichloropropene, captafol, pentacholoronitrobenzene, and dieldrin[111]. A case-control study of multiple myeloma among males was conducted by Cantor et al. They found that in the countries where pesticides were more heavily used, the risk for multiple myeloma was greater for farmers than those in the countries where pesticides were less heavily used.[112] Blair et al. conducted a study to evaluate the mortality experience of a cohort of 3827 white men licensed to apply pesticides in Florida to assess the health effects of chronic

pesticides exposure. They found that some pesticides might be carcinogenic in humans.[113]

Therefore, monitoring the concentration levels of these pesticides in human bodies can help assess their adverse health effects due to chronic exposure. The best way to measure human exposure to pesticides is to measure pesticides and their metabolites in biological samples including serum, fat, urine, blood, or breast milk.

Comparing with other biological samples such as urine, measuring concentration levels of pesticides in blood products, such as whole blood, plasma, or serum, has its own advantages: (1) concentrations of the parent compounds can be monitored directly; (2) do not require detailed information on the metabolism; (3) provide much more accurate information as to which pesticides people were exposed; and (4) no corrections for dilution are necessary since blood is a regulated fluid.[114] Therefore, among these biological samples, blood products analysis is considered as one of the simplest method for assessing body burden.[115] Jack et al. also found that there were close correlations between the concentrations of pesticides in blood and fat even in non-occupationally exposed individuals, and concentrations of organochlorine pesticides in the blood was a good way to estimate body burdens and exposure.[116] Many studies have been done to determine the concentration levels of pesticides in biological samples, especially in blood products.

Due to the complexity of serum matrices, their sample preparation methods usually include one or more clean-up steps to remove interferences from matrices and increase

the accuracy and limit of detection. In the next few paragraphs, some representative sample preparation methods for pesticides analysis in serum using LC/MS, LC-MS/MS, GC/MS, or GC-MS/MS were listed.

Frías et al. developed a method to analyze organochlorinated compounds in human serum using GC-MS/MS. Four mL of serum was mixed with 2 mL of methanol for 1 min. Five mL of *n*-hexane/ethyl ether (1:1, v/v) was added to the mixture and mixed for 5 min. After centrifuging, organic layer was collected, and aqueous phase was extracted twice more with another 5 mL of *n*-hexane/ethyl ether (1:1, v/v). The extract was further cleaned-up through 0.5 mL of H_2SO_4 and high-performance liquid chromatography with photodiode-array detector. The LOD ranged from 0.01 to 0.62 μg/L.[117 118]

SPE method was used to determine polychlorinated biphenyls (PCBs) and selected organochlorine pesticides (OCPs) in the study of Conka et al. Serum (5 g) was mixed with 5 mL water/1-propanol (85:15, v/v) and sonicated for 5 min before loading. C18 (EC) SPE column (1 g/6 mL) was preconditioned with 5 mL methanol followed by 5 mL water/1-propanol (85:15, v/v). After loading the sample mixture, matrix residues were washed out with 5 mL water/1-propanol (85:15, v/v) and the SPE column was dried by aspiration of ambient air for 1 hour. Analytes were eluted with 5 mL of *n*-hexane/DCM (1:1, v/v). The collected eluent was further cleaned-up with florisil-silica gel column treated with sulphuric acid. The recoveries were 99-120% for PCBs and 88-115% for OCPs. The values of LOQ were 0.01-0.02 ng/mL_{serum} for PCBs and 0.01-0.16 ng/mL_{serum} for OCPs.[119]

Garí et al. developed another method to analyze organochlorine compounds in human serum. Serum sample (1 mL) was added to 3 mL of *n*-hexane and 2 mL of conc. H_2SO_4, mixed, and centrifuged. After taking out the supernatant *n*-hexane layer, the remaining mixture was extracted twice with 2 mL *n*-hexane. Two mL of conc. H_2SO_4 was added to the 7 mL *n*-hexane extract. The organic layer was transferred, evaporated to dryness, and reconstituted with 25 µL isooctane for GC analysis.[115]

Lacassie et al. developed a rapid, specific and sensitive method to determine 29 OPs in serum by GC/MS. Serum sample (2 mL) was deposited on an Oasis hydrophilic–lipophilic balanced copolymer (HLB) cartridge (3 mL/60 mg) that was preconditioned with 2 mL methanol followed by another 2 mL of deionized water. After loading the sample, deionized water (2 mL) was added to the cartridge for washing. Three mL ethyl acetate was added to the cartridge as eluting solvent, and the eluent was collected, evaporated to dryness, and reconstituted with 100 µL of ethyl acetate for GC/MS analysis. Extraction recoveries were 40-108%, and the LOD for individual OP was within the range of 80-100 µg/L.[120]

Pérez et al. developed a method to measure pyrethroid, OP, carbamate and fipronil pesticides and the synergist piperonyl butoxide in human plasma. The plasma sample (2 mL) was loaded to a Varian ABS ELUT-Nexus 60 mg/3 mL SPE cartridge that was preconditioned with 2 mL methanol followed by 2 mL deionized water. The cartridge was washed twice with 2 mL deionized water first and then twice with 2 mL of 40% methanol in water. After drying the cartridge, toluene (2 × 1 mL) was used to elute the

cartridge. The eluent was collected, evaporated, and reconstituted for GC analysis. The extraction recoveries ranged from 20% to 98% and the LOD was in the range of 10-158 pg/mL.[121]

Pitarch et al. developed a rapid method for the multiresidue determination of OCs and OPs in human serum by SPE and GC-MS/MS. One mL serum was diluted with 4 mL water and passed through a 500 mg C18 cartridge that was previously conditioned with 5 mL methanol, 5 mL methyl *tert*-butyl ether and 3 mL deionized water. After loading the sample, the cartridge was washed with 3 mL deionized water and dried before eluting with 5 mL *tert*-butyl ether. The eluent was collected, evaporated to dryness, and reconstituted with 0.5 mL *n*-hexane for GC analysis. The LOD was in the range of 0.05-0.5 ng/mL for most of the analytes.[122]

Sundberg et al. also developed a simple and fast extraction method for OCs and PCBs in avian serum. One mL of fortified serum sample was amended with 500 mg solid urea (~8M) before loading. The denatured serum-lipoprotein-analyte complex was loaded to the Oasis hydrophilic-lipophilic-balanced (HLB) SPE cartridge for the SPE procedure. The recoveries were 90-101% for PCBs and 74-101% for OCs.[123]

In a study of Barr et al., the serum proteins were denatured with 4 mL of saturated ammonium sulfate before the clean-up steps.[114]

In the paper of Keller et al., they compared five extraction methods for measuring PCBs, polybrominated diphenyl ethers (PBDEs), OCs, and lipid content in serum. Although

these five methods varied in liquid-liquid extraction steps, for the clean-up steps, they either used alumina columns followed by gel permeation columns (GPC) or acidified silica columns.[124]

The aim of this study was to develop an analytical method using gas chromatography tandem mass spectrometry for the determination of OC, OP, pyrethroid, and carbamate insecticide residues in serum. The amount of each serum sample that is collected in the field is usually very small because serum samples are invasive. Therefore, a method that can analyze various classes of pesticides in a single serum sample with limited sample volume is preferred to fully assess pesticides exposure. The application of this method was investigated in unknown serum samples collected from Thai farmers.

4.2 Materials and Methods

Serum Samples

Pooled serum samples were used as blank matrices. They were properly aliquoted and stored at -20 ^{o}C until analysis. Unknown serum samples were collected from Thai farmers and also stored at -20 ^{o}C until analysis.

Chemicals

The native standard of fenobucarb (fen) was purchased from Sigma-Aldrich (St. Louis, MO). The native standards of fonofos (fon), *p, p*-dicofol (dic), and heptachlor epoxide

(hep) were purchased from Ultra Scientific. The native standards of bendiocarb (ben), parathion (par), *o, p*-DDE (ddeop), prallethrin (pral), *p, p*-DDE (ddepp), *o, p*-DDT (ddtop), piperonyl butoxide (pbo), and cypermethrin (cyp) were all purchased from Chem Service. The native standards of hexachlorobenzene (hcb), atrazine (atr), diazinon (dia), chlorpyrifos-methyl (chlm), chlorpyrifos (cpy), endosulfan-*α* (endoA), resmethrin (res), azinphos-methyl (azm), permethrin (per), and deltamethrin (del) were purchased from Supelco Analytical. The native standard of endosulfan-*β* (endoB) was purchased from Crescent Chemical Co. The native standard of cyfluthrin (cyf) was purchased from Los Almos. The internal Standards of chlorpyrifos-methyl D6, chlorpyrifos D10, parathion D10, 13C-*p, p*-DDE, and 13C-cypermethrin were all purchased from Cambridge Isotope Laboratories.

All solvent used were of analytical grade. We obtained acetonitrile and methanol from Fischer Scientific (Phillipsburg, NJ), hexane from Sigma Aldrich, and toluene from Macron. Ethyl acetate and propanol were purchased from EMD. Acetic acid (Glacial) was purchased from Avantor Performance Materials (Phillipsburg, NJ). Bondesil-PSA (40 µm) was purchased from Agilent Technologies. Oasis HLB Extraction Cartridges (3 cc/60 mg) were purchased from Waters Corporation (Milford, MA). Fisher PrepSep 1 g florisil 6 mL SPE columns, Fisher PrepSep 500 mg C18(EC) 6 mL SPE columns, PSA bonded silica 500 mg 6 mL SPE tubes, Supelclean LC-18 500 mg 6 mL SPE tubes, and Dual-Layer Envi-Carb II/PSA 500/300 mg 6 mL SPE tubes were Supelco (Bellefonte, PA). Isolute 500 mg C18(EC) 6 mL SPE columns were purchased from Biotage. HyperSep 1000 mg florisil 6

mL SPE columns were purchased from Thermo (Bellefonte, PA). Strata-XL 100 µm polymeric reversed phase 200 mg 6 mL tubes were purchased from Phenomenex.

Native Standards and Internal Standards Preparation

Individual stock solution of the native standards was prepared for each analyte in acetonitrile. Stock solutions were stored at -20^{o}C. Ten working standard solutions of varying concentrations ranging from 1-1000 ppb were prepared by adding individual stock solution of analytes into acetonitrile/toluene (3:1, v/v). The working standard solutions were stored at -20^{o}C.

Individual stock solution of the labeled internal standards was prepared in acetonitrile. Stock solutions were stored at -20^{o}C. An internal standard working solution including all the five labeled standards was prepared at 400 ppb in acetonitrile/toluene (3:1, v/v) and stored at -20^{o}C.

The calibration standards were made freshly by adding 200 µL native standard working solution and 50 µL internal standard working solution into 2mL pooled serum followed by the whole sample preparation procedure.

Preparation and Extraction Procedure for Serum Samples

Materials: Isolute 500 mg C18(EC) 6 mL SPE columns from Biotage, HyperSep 1000 mg florisil 6 mL SPE columns from Thermo, empty glass test tubes, pipettes and matching

tips, beakers, Zymark TurboVap LV Evaporator (Framingham, MA), and GC vials, inserts and caps.

Solutions: 5% Na_2SO_4 in H_2O/propanol (85:15, v/v), methanol, H_2O/propanol (85:15, v/v), hexane/ethyl acetate (1:1, v/v), acetonitrile/toluene (3:1, v/v).

Sample Preparation Procedure:

Matrix blank samples

- Spike with 50 µL of 400 ppb ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 2 mL of pooled serum
- Vortex 30s to mix
- Add 2 mL of 5% Na_2SO_4 in H_2O/propanol (85:15, v/v)
- Vortex at 1000 rpm for 10 min

Calibration standards

- Spike with 50 µL of 400 ppb ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 200 µL of the native standard (S1-S10: 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000ppb) to the corresponding test tube (e.g. S1 native standard in S1 test tube)
- Add 2 mL of pooled serum
- Vortex 30s to mix

- Add 2 mL of 5% Na_2SO_4 in H_2O/propanol (85:15, v/v)
- Vortex at 1000 rpm for 10 min

Unknown samples

- Spike with 50 µL of 400 ppb ISTD
- Visually confirm the correct volume of ISTD has been added to each test tube
- Add 2 mL of serum from unknown samples
- Vortex 30s to mix
- Add 2 mL of 5% Na_2SO_4 in H_2O/propanol (85:15, v/v)
- Vortex at 1000 rpm for 10 min

For the other samples, depending on their purposes (recovery studies, stability studies, etc.), 50 µL of 400 ppb ISTD solution and the selected level of 200 µL native standard solution were spiked into each sample at different steps of the extraction procedure.

<u>Extraction Procedure:</u>

- Condition C18 cartridges with 3 mL methanol
- Condition C18 cartridges with 3 mL H_2O/propanol (85:15, v/v)
- Load samples carefully
- Wash cartridges with 3 mL H_2O/propanol (85:15, v/v) and do not collect eluent
- Elute cartridges twice with 5 mL hexane/ethyl acetate (1:1, v/v) and collect eluent

- Pipette out and discard the aqueous layer in the bottom
- Add 500 mg Na_2SO_4
- Vortex at 1000 rpm for 5 min
- Centrifuge at 2500 rpm and 10 ^{0}C for 5 min
- Decant extract into a new test tube
- Evaporate extract to about 2 mL at 20 psi and 45 ^{0}C
- Condition florisil cartridges with 5 mL hexane/ethyl acetate (1:1, v/v)
- Load samples and collect eluent
- Elute cartridges twice with 5 mL hexane/ethyl acetate (1:1, v/v) and collect eluent
- Evaporate collected eluent to dryness
- Reconstitute with 50 µL of acetonitrile/toluene (3:1, v/v)

Quality Control (QC) Samples

Two quality control spiking solutions, a low level (QCL) and a high level (QCH), were prepared by serial dilution of the individual stock solutions of the native pesticides. The concentrations for individual native pesticides are listed in Table 4-1. QC samples were prepared by spiking 200 µL of QC solution and 50 µL of ISTD solution into pooled serum samples followed by the whole extraction and clean-up steps

Table 4-1 Concentrations of QCH and QCL spiking solutions

	QCH (ppb)	QCL (ppb)
fen	100.0	25.0
hcb	25.1	6.3
atr	249.7	62.4
pro	250.1	62.5
fon	24.9	6.2
ben	100.4	25.1
dia	99.9	25.0
chlm	25.4	6.4
cpy	50.2	12.6
par	249.6	62.4
dic	25.4	6.4
hep	249.8	62.4
ddeop	24.8	6.2
pral	21.7	5.4
endoA	25.0	6.3
ddepp	24.9	6.2
endoB	24.5	6.1
ddtop	24.5	6.1
ddtpp	25.9	6.5
pbo	50.2	12.6

res	249.2	62.3
azm	499.9	125.0
per	49.9	12.5
cyf	99.7	24.9
cyp	100.2	25.1
fen	49.9	12.5
del	247.7	61.9

Instrumental Analysis

Refer to the Instrumental Analysis section in Chapter 2. Propoxur, prallethrin and azinphos-methyl were not analyzed in this study because they did not response very well on the GC-MS/MS due to the interferences from serum matrices.

Quantification Method

Concentrations of analytes were determined with respect to their corresponding labeled internal standards (chlorpyrifos-methyl D6, chlorpyrifos D10, parathion D10, 13C-*p, p*-DDE, or 13C-cypermethrin). Matrix-based calibration curve was prepared with the described extraction method. In the calibration curve, the area counts of the native pesticide quantification ion divided by the area counts of the internal standard quantification ion was plotted against 10 different concentration levels: 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ppb. The concentration levels of the calibration curve covered

the entire linear range of the analysis and most of the r^2 values were larger than 0.99. The concentrations of unknown samples were calculated based on the slope and intercept provided by a linear regression analysis of the calibration plot. The concentrations of permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin were determined by individual isomers.

Limit of Detection

The LOD was determined as the smallest amount of the analyte in serum matrices that gave a signal-to-noise (S/N) ≥ 3.

Extraction Efficiency

A recovery study for each pesticide in the serum matrices was carried out in order to assess the extraction efficiency of the described method. Replicate pooled serum samples (2 mL) were spiked at two different concentration levels of pesticides (25 ppb and 10 ppb). These two concentration levels were picked because they were at intermediate points on the calibration curve. Samples were separated into two groups (Group A and Group B) under each spiking level. Replicate serum samples in Group A were spiked with the designated native standard before extraction and internal standard right before the final evaporation step, while replicate serum samples in Group B were spiked with the designated native standard and internal standard right before the final evaporation step. The extraction recovery was calculated by comparing the response ratio of Group A to that of Group B.

Accuracy

The method accuracy was determined by repeatedly measuring pooled serum samples spiked at two different concentration levels (QCL & QCH), in which *n* = 15 at each concentration level. We calculated the percent deviation of the observed mean concentrations from the nominal spiked concentrations.

Precision

The method precision was determined by calculating the relative standard deviation (RSD%) of repeated measurements of serum samples spiked with native standard pesticides at two different concentration levels (QCL & QCH). Replicate samples were prepared and analyzed daily during a 5-day period to determine the within-day, and between-day precision for each analyte.

Sample Storage Stability

The stability of analytes in serum was determined by repeatedly analyzing pooled serum samples spiked at two different concentrations (QCL & QCH) and stored at -20 ^{0}C. The fortified serum samples were extracted and analyzed at days 0, 30, 60, and 90. Percent reduction in the concentration will be calculated for each concentration level when we obtain the data. This experiment is still undergoing with no known results yet.

4.3 Results

Recoveries

As shown in Figure 4-1, the extraction recoveries of the analytes are 48.5-88.5% at 10 ppb and 50.7-95.7% at 25 ppb. These recovery results are consistent between the two different concentration levels. The recoveries for pyrethroid insecticides are relatively lower than the other classes of pesticides. The standard deviations of the recoveries are 1.2-13.4% at 10 ppb, while the ones at 25 ppb are 3.1-12.7% except for fen at 39.7% and hcb at 53.7%. The large variations for fen and hcb were partially because the GC did not respond very consistently to their corresponding fragment ions.

The recoveries are satisfactory because optimizing the performance for every single compound from four different classes of pesticides is challenging, especially in complicate matrices like serum. Because of the diverse chemical and physical properties of these pesticides, some analytes must be somewhat compromised for the overall performance of the method. The described extraction method in this chapter was the best compromise to obtain the satisfactory extraction efficiency in serum.

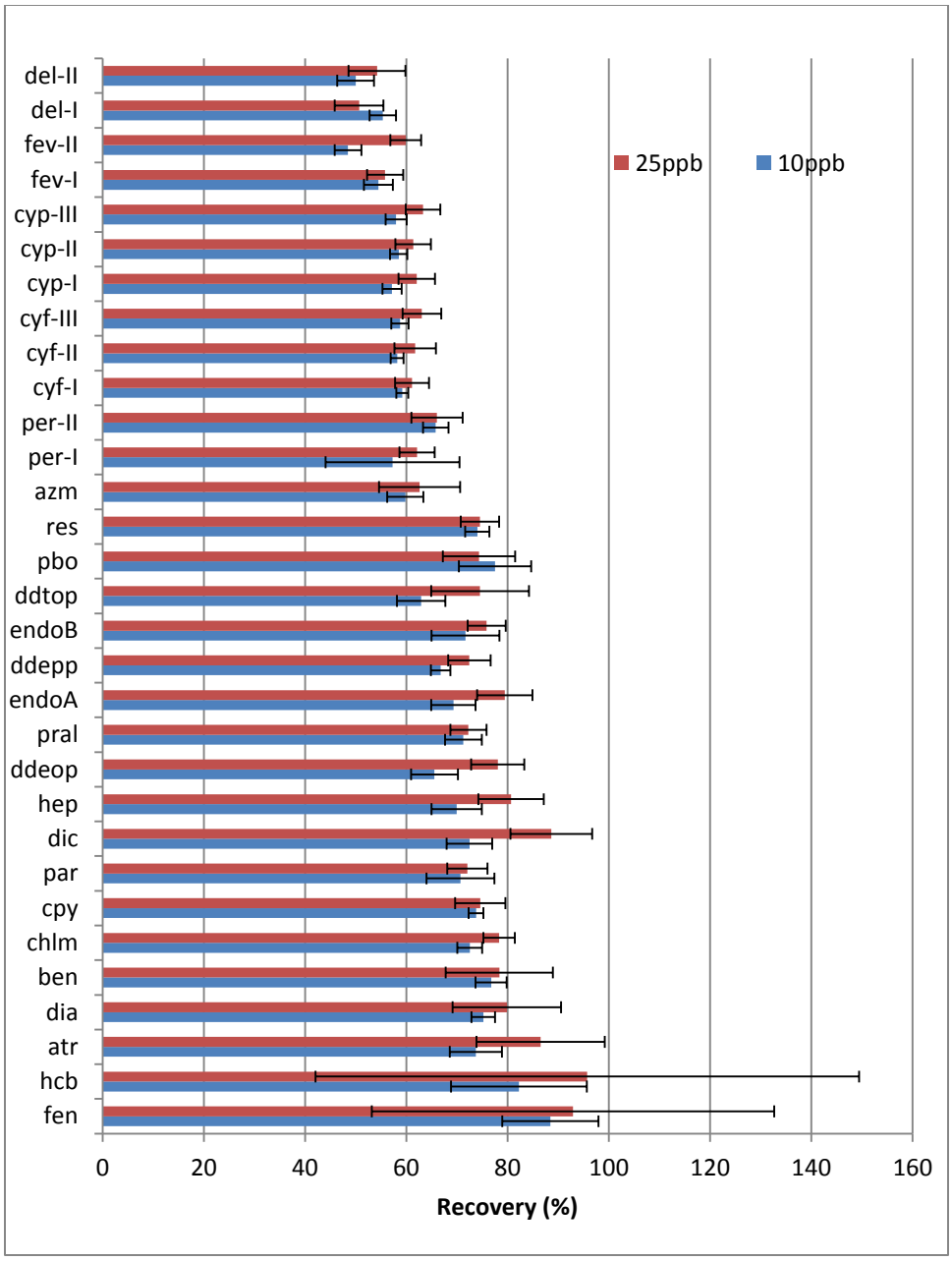


Figure 4-1 Recoveries of described method at 25 ppb and 10 ppb

Limit of Detection (LOD)

The limit of detection for all the analytes ranged from 0.0009 to 0.3697 ppb, with the majority of them below 0.1 ppb. As shown in Table 4-2, OCs have relatively lower LOD, while pyrethroids have relatively higher LOD. OPs and carbamates are in the middle.

Table 4-2 LOD for individual pesticides with the proposed extraction method

	LOD (ppb)
fen	0.0077
hcb	0.0084
atr	0.0189
fon	0.0019
dia	0.0017
ben	0.0039
chlm	0.0035
cpy	0.0037
par	0.0422
dic	0.0089
hep	0.0048
ddeop	0.0019
pral	0.0774
endoA	0.0041

ddepp	0.0009
endoB	0.0061
ddtop	0.0154
pbo	0.0023
res	0.1040
azm	0.1844
per-I	0.0084
per-II	0.0130
cyf-I	0.0107
cyf-II	0.0086
cyf-III	0.0062
cyp-I	0.0109
cyp-II	0.0061
cyp-III	0.0240
fev-I	0.0115
fev-II	0.0151
del-I	0.3697
del-II	0.2524

Accuracy and Precision

The accuracy for majority of the pesticides was within the range of 80-120%. Although the remaining ones did not fall into this range, they were still very close to 80% or 120% except for hcb. The accuracy for hcb was 167.7% at QCL and 155.1% at QCH, which was consistent with its recovery and corresponding standard deviation results in Figure 4-1. The within-day precision for individual analyte was 2.87-10.74% at QCL and 1.42-7.92% at QCH, while the between-day precision for individual analyte was 2.90-10.66% at QCL and 1.44-7.52% at QCH. They were all within the satisfactory range.

Table 4-3 Method precision and accuracy

	Accuracy (%)		Precision (%)			
	QCL	QCH	QCL		QCH	
			Within-day	Between-day	Within-day	Between-day
fen	119.4	91.0	8.41	8.48	7.81	7.27
hcb	167.7	155.1	10.74	10.66	7.92	7.52
atr	92.9	99.7	6.78	6.38	6.69	6.74
fon	103.9	95.5	7.39	7.61	5.43	5.11
ben	89.9	86.6	4.70	4.88	3.55	3.40
dia	84.1	84.1	4.23	4.36	4.58	4.54
chlm	116.0	110.9	4.62	4.59	1.48	1.48
cpy	92.5	87.4	4.33	4.50	3.12	3.16

par	83.4	92.9	5.14	5.13	4.62	4.44
dic	108.4	100.0	8.13	7.87	6.86	6.94
hep	88.4	89.6	7.47	7.48	4.00	4.11
ddeop	90.5	86.1	7.87	8.24	2.48	2.55
endoA	85.2	83.3	8.54	8.57	4.57	4.82
ddepp	102.0	88.5	3.60	3.62	1.87	1.93
endoB	97.9	92.7	4.97	5.01	4.24	4.30
ddtop	107.4	114.7	7.09	7.13	5.17	5.04
pbo	81.8	79.0	8.04	9.00	3.18	3.23
res	113.3	96.0	8.04	8.29	3.83	3.90
per-I	118.0	110.6	7.35	7.34	4.22	4.43
per-II	121.4	113.2	6.05	6.26	4.55	4.67
cyf-I	86.8	83.3	4.48	4.41	2.10	2.12
cyf-II	80.7	79.0	3.15	3.10	1.53	1.53
cyf-III	84.8	85.3	3.65	3.66	2.63	2.69
cyp-I	79.1	77.7	3.52	3.51	1.59	1.60
cyp-II	81.2	78.3	2.91	2.91	1.42	1.44
cyp-III	94.2	89.9	2.87	2.90	3.15	3.14
fev-I	79.4	74.1	3.90	4.00	2.86	2.93
fev-II	99.5	94.1	5.93	5.94	1.95	1.93
del-I	96.1	98.1	4.97	5.02	4.05	3.97

del-II	74.0	76.5	4.38	4.60	2.62	2.64

4.4 Discussions

The original idea of this study was to adapt the method for pesticides analysis in milk we developed in chapter 2 and use it in serum. The proposed method in chapter 2 was to analyze representative OP, OC, pyrethroid, and carbamate insecticides in cow milk and human milk. Experiments were conducted in serum samples (2 mL) at the concentration level of 25 ppb. Replicate serum samples were separated as two groups: (1) acetonitrile was used for the first liquid-liquid extraction, and (2) 1% acetic acid acetonitrile was used for the first extraction. The remaining steps were the same as described in chapter 2. Results are summarized in Figure 4-2. Recoveries were satisfactory for both of the two extraction solvents. Overall, group (1) provided relatively better recoveries, especially for fen, hcb, fon, ben, and dia. However, group (2) provided relatively smaller standard deviations among replicate samples and thus had better consistency.

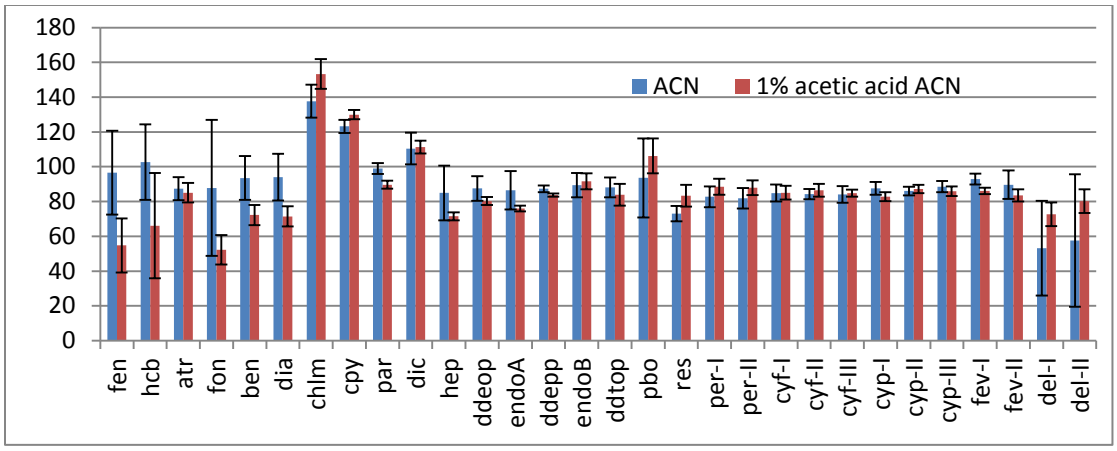


Figure 4-2 Recoveries of using acetonitrile and 1% acetic acid acetonitrile for extraction. Y-axis represents percent recovery (%).

Two complete calibration curve standards in serum matrix were prepared using the extraction solvents, acetonitrile and 1% acetic acid acetonitrile, respectively. However, for majority of the pesticides listed in this study, the lowest concentration level could be confidently detected on the GC was above 1 ppb. Since the concentration levels of pesticides in serum are usually very low, the detectable level above 1 ppb was not acceptable.

In the hope of reducing the interferences from serum matrix and increasing limit of detection, we further modified the proposed method in chapter 2 by adding 1 mL saturated ammonium sulfate to denature the serum proteins before the extraction. However, this modification did not work. The recoveries were low with large standard deviations among replicate samples, and the lowest concentration level can be confidently detected on the GC was still above 1 ppb.

Since the proposed method in chapter 2 did not work in serum and neither of the modified ones worked, we decided to adapt the method from Conka et al.[119] The adapted method from Conka et al. was modified in several different ways, such as slightly different sample fortification and preparation steps, as well as different combinations of SPE cartridges. Detailed information is listed in Figure 4-3.

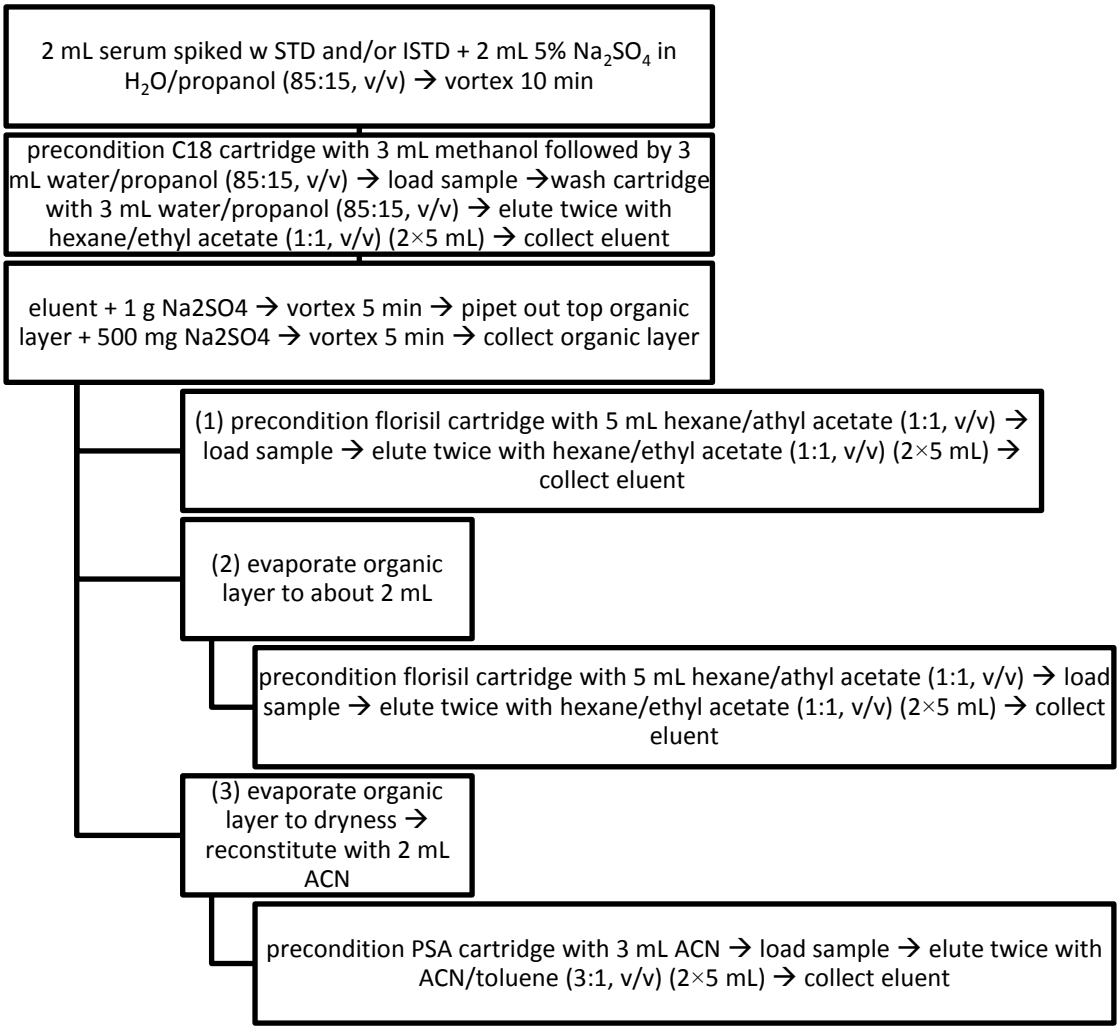


Figure 4-3 Flow diagram of three extraction methods that were modified from the one in Conka et al. [119]

Recovery studies were conducted for these three methods with replicate serum samples at the concentration level of 25 ppb. Cartridges used in this study were Fisher PrepSep 1 g florisil 6 mL SPE columns, Fisher PrepSep 500 mg C18(EC) 6 mL SPE columns, and PSA

bonded silica 500 mg 6 mL SPE tubes, which were all purchased from Supelco (Bellefonte, PA). Results are shown in Figure 4-5. The recoveries of method (3) and (1) were below 30%. Apparently, method (2) provided best recoveries among these three methods.

Florisil (magnesium silicate) is a registered trademark of U.S. Silica Company, whose structure is shown in Figure 4-4. Because florisil is extremely polar in nature, it is ideal for the isolation of polar compounds from nonpolar matrices. As we mentioned in Chapter 2, PSA is a weak anion exchanger with a pKa of 10.1 and 10.9, and the bi-dentate nature of its ligand allows for chelation. Therefore, PSA also has strong affinity and high capacity for removing fatty acids, some types of polar pigments, and sugar to clean-up samples and reduce matrix effects for GC analysis. Florisil and PSA cartridges are both normal phase packing. However, in this study, florisil cartridges worked better than PSA cartridges.

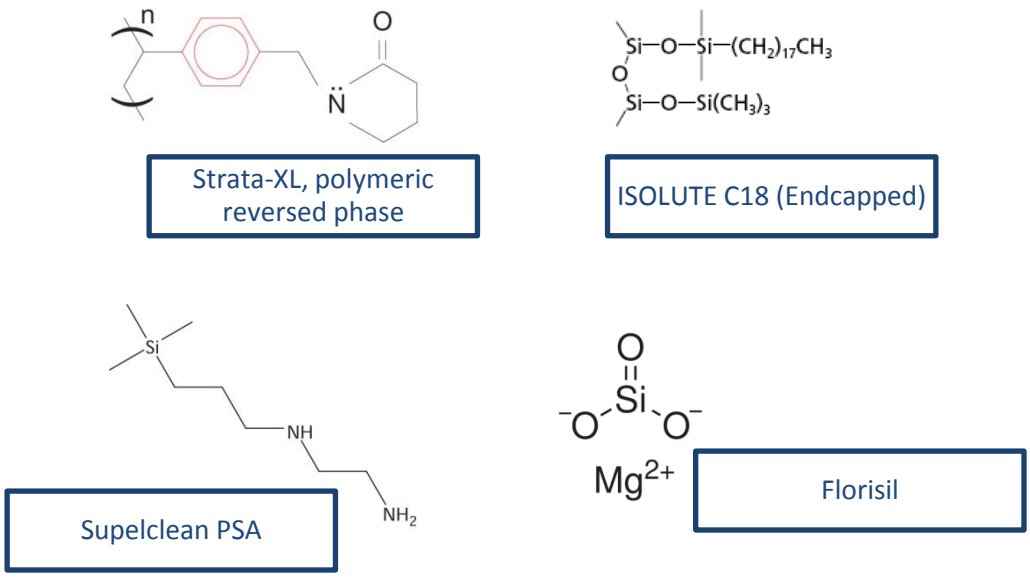


Figure 4-4 Chemical structures of four representative SPE packing in this study

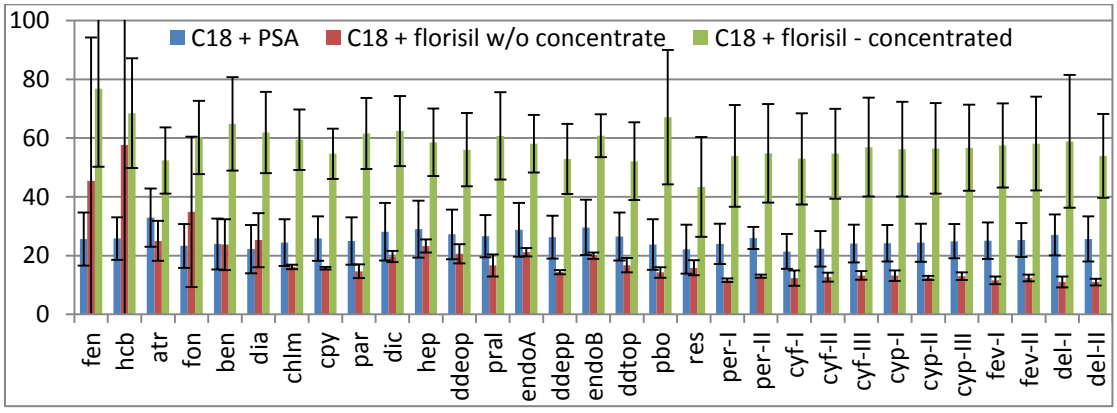


Figure 4-5 Recoveries of three extraction methods. Blue, red, and green bars represent extraction methods (3), (1), and (2) described in Figure 4-3. Y-axis represents the percent recovery (%).

A complete calibration curve standards in serum were prepared using method (2). Except for par, pral, endoB, res, azm, fev, and del, all the other pesticides could be detected and quantified at the lowest concentration level (0.1 ppb) of the calibration curve. Par, endoB, res, fev, and del could also be detected and quantified at two or three concentration levels higher than the lowest one, which were still below 1 ppb. These results were satisfactory.

Because the C18 cartridges contain silica-based packing, one should always keep about 1 mm of solvent above the packing bed to prevent the SPE packing from drying throughout the whole SPE clean-up steps. Since the conditioning, loading, washing, and eluting steps with C18 cartridges in this method took hours, extra attention should be paid during these hours to avoid the packing from drying out, which was labor-consuming. In order to make the SPE steps easier, a polymer-based SPE cartridge, Strata-XL was introduced. Since Strata-XL is polymer-based, there is no need to concern the problems of drying out the packing. C18 and Strata-XL are both reverse packing cartridges, and their chemical structures of the parking are shown in Figure 4-4. C18 cartridges are packed with the strong non-polar phase. The clean-up mechanism of C18 (EC) is based on tri-functional silane chemistry, and the residual silanols on the silica surface are end-capped to minimize secondary silanol interactions. On the other hand, the functionalized polymeric sorbent of Strata-XL provides strong retention of neutral and aromatic compounds, which should work well with the analytes of this study in serum.

Replicate serum samples fortified to 10 different concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ppb) as well as replicate serum matrix blanks were extracted and cleaned-up with method (2) in Figure 4-3. Samples in the comparison group were prepared with the same method, but Strata-XL cartridges were used to replace C18 cartridges. Results are compared in several different ways as shown in Figure 4-6 and 3-7 and Table 4-4 and 3-5. For chlorpyrifos-methyl D6, chlorpyrifos D10, parathion D10, and 13C-*p, p*-DDE, Strata-XL provided slightly higher area counts with much larger standard deviations (SD) than C18. For 13C-cypermethrin, C18 provided better data than Strata-XL. For hcb, Strata-XL provided slightly higher RR than C18, while for the other 10 pesticides that had detectable concentration levels in serum matrix blanks, C18 provided higher response ratio (RR) than Strata-XL. At concentration levels of 0.1, 0.25, 0.5, 1, and 2.5 ppb, C18 provided better RR for some of the pesticides and Strata-XL provided better RR for the others. Generally, there was no significantly advantage over one another for these two cartridges. However, it was pretty obvious that C18 provided much better linearity than Strata-XL, especially for permethrin, cyfluthrin, and cypermethrin. Therefore, the C18 cartridges were continually used for later experiments.

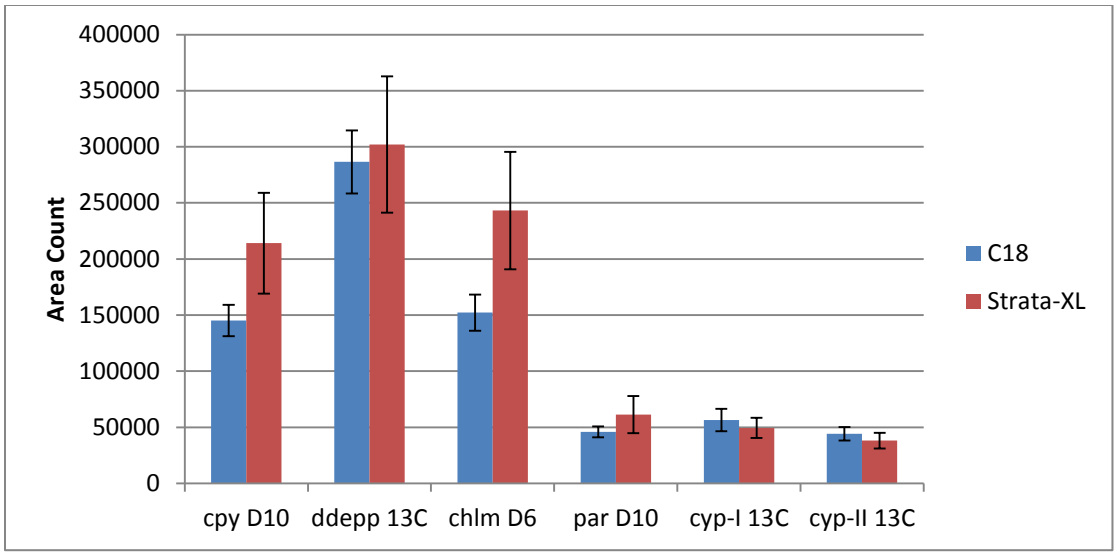


Figure 4-6 Area counts of the 5 internal standards. Blue and red bars represent samples cleaned-up with C18 and Strata-XL, respectively.

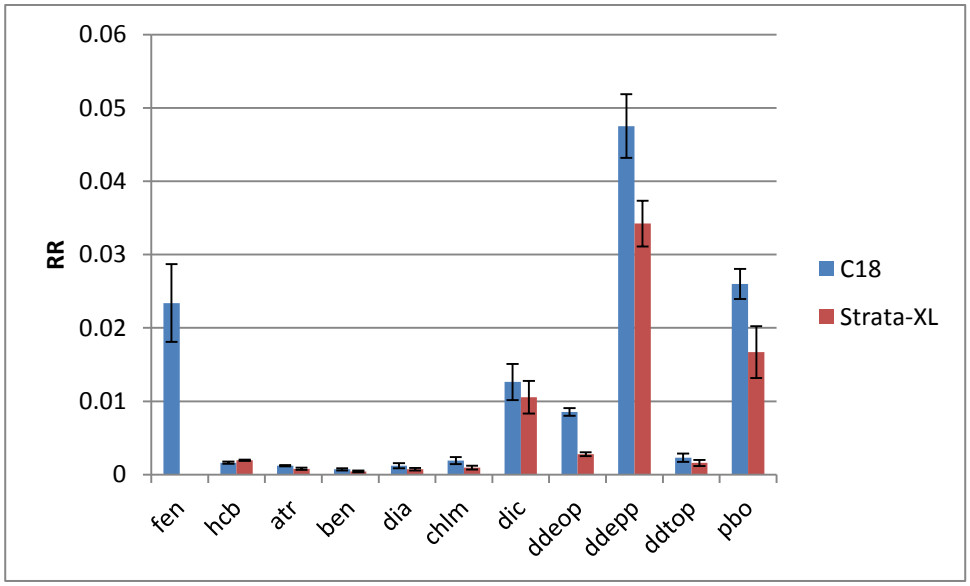


Figure 4-7 RR of 11 pesticides that had detectable levels in pooled serum matrix blanks. Blue and red bars represent samples cleaned-up with C18 and Strata-XL, respectively.

Table 4-4 RR of native pesticides under 5 different concentration levels with 2 different clean-up cartridges. S-XL represents Strata-XL.

	0.1 ppb		0.25 ppb		0.5 ppb		1 ppb		2.5 ppb	
	C18	S-XL	C18	S-XL	C18	S-XL	C18	S-XL	C18	S-XL
fen	0.061	0.068	0.083	0.089	0.127	0.136	0.207	0.187	0.424	0.445
hcb	0.003	0.005	0.005	0.009	0.011	0.016	0.020	0.029	0.050	0.077
atr	0.002	0.003	0.004	0.005	0.008	0.011	0.013	0.018	0.030	0.051
ben	0.002	0.002	0.005	0.005	0.010	0.010	0.019	0.018	0.044	0.048
dia	0.004	0.005	0.008	0.009	0.017	0.018	0.032	0.031	0.080	0.085
chlm	0.006	0.006	0.013	0.012	0.027	0.027	0.049	0.048	0.118	0.120
cpy	0.004	0.004	0.007	0.007	0.016	0.015	0.027	0.026	0.065	0.066
par	-	-	-	-	-	-	0.038	-	0.092	0.093
dic	0.032	0.044	0.061	0.109	0.134	0.211	0.236	0.369	0.577	0.980
hep	0.000	0.001	0.001	0.001	0.001	0.002	0.003	0.003	0.006	0.009
ddeop	0.013	0.008	0.018	0.016	0.033	0.033	0.053	0.057	0.122	0.141
pral	-	-	-	-	0.015	0.007	0.022	0.012	0.043	0.030
endoA	0.001	0.002	0.003	0.004	0.005	0.007	0.009	0.013	0.021	0.032
ddepp	0.082	0.075	0.123	0.118	0.235	0.222	0.384	0.365	0.903	0.901
endoB	-	0.002	0.001	0.003	0.003	0.005	0.005	0.009	0.011	0.023
ddtop	0.007	0.008	0.013	0.013	0.027	0.027	0.045	0.048	0.107	0.126
pbo	0.065	0.061	0.104	0.119	0.218	0.245	0.355	0.405	0.843	1.067

res	-	-	-	0.249	-	0.351	-	0.454	0.632	0.830
azm	-	-	0.018	0.016	0.042	0.039	0.070	0.065	0.160	0.167
per-I	0.027	0.046	0.034	0.042	0.063	0.068	0.103	0.103	0.242	0.267
per-II	0.018	0.048	0.021	0.031	0.039	0.047	0.062	0.063	0.141	0.170
cyf-I	0.014	0.044	0.023	0.034	0.049	0.054	0.084	0.083	0.202	0.203
cyf-II	0.026	0.057	0.043	0.054	0.076	0.090	0.131	0.129	0.306	0.323
cyf-III	0.029	0.069	0.046	0.061	0.090	0.092	0.146	0.154	0.356	0.377
cyp-I	0.020	0.054	0.034	0.046	0.071	0.073	0.122	0.117	0.295	0.297
cyp-II	0.023	0.055	0.036	0.045	0.072	0.082	0.121	0.121	0.289	0.299
cyp-III	0.038	0.098	0.063	0.066	0.129	0.144	0.217	0.223	0.536	0.528
fev-I	-	0.021	0.018	0.031	0.040	0.052	0.071	0.073	0.167	0.198
fev-II	-	0.012	0.011	0.018	0.022	0.030	0.040	0.044	0.094	0.112
del-I	-	-	-	-	-	-	-	-	0.020	0.027
del-II	-	-	0.012	0.019	0.026	0.035	0.044	0.054	0.113	0.148

Table 4-5 r^2 of the calibration curve for each pesticide with 2 different clean-up cartridges

	C18	Strata-XL
fen	0.992618	0.951655
hcb	0.993972	0.983262

atr	0.992641	0.988974
ben	0.995992	0.993974
dia	0.99741	0.99207
chlm	0.998316	0.997653
cpy	0.994138	0.996605
dic	0.995971	0.996307
hep	0.992115	0.995913
ddeop	0.995558	0.997128
endoA	0.994548	0.996238
ddepp	0.996518	0.996135
ddtop	0.994363	0.993915
pbo	0.993894	0.99547
per-I	0.98272	0.762251
per-II	0.96489	0.304011
cyf-I	0.993744	0.581256
cyf-II	0.993449	0.817326
cyf-III	0.991778	0.788188
cyp-I	0.99358	0.742918
cyp-II	0.994015	0.76464
cyp-III	0.995796	0.720913

So far, the best recoveries we obtained were the ones in Figure 4-5 using sample preparation method (2). The recoveries were within the range of 50-70% for majority of the pesticides. The next experiments were designed to check if we were losing them in the florisil SPE steps. Replicate samples of hexane/ethyl acetate (1:1, v/v, 2 mL) were fortified to two concentration levels (10 ppb and 50 ppb) of native pesticides. They were loaded to florisil cartridges preconditioned with 5 mL hexane/ethyl acetate (1:1, v/v). After sample loading, cartridges were eluted with 2 × 5 mL hexane/ethyl acetate (1:1, v/v). Eluent were collected, evaporated to dryness, reconstituted, and injected to GC for analysis. Results are shown in Figure 4-8, which were consistent with the recoveries in Figure 4-5. This indicates that the relatively low recoveries in Figure 4-5 are mainly caused by the low recoveries of florisil cartridges.

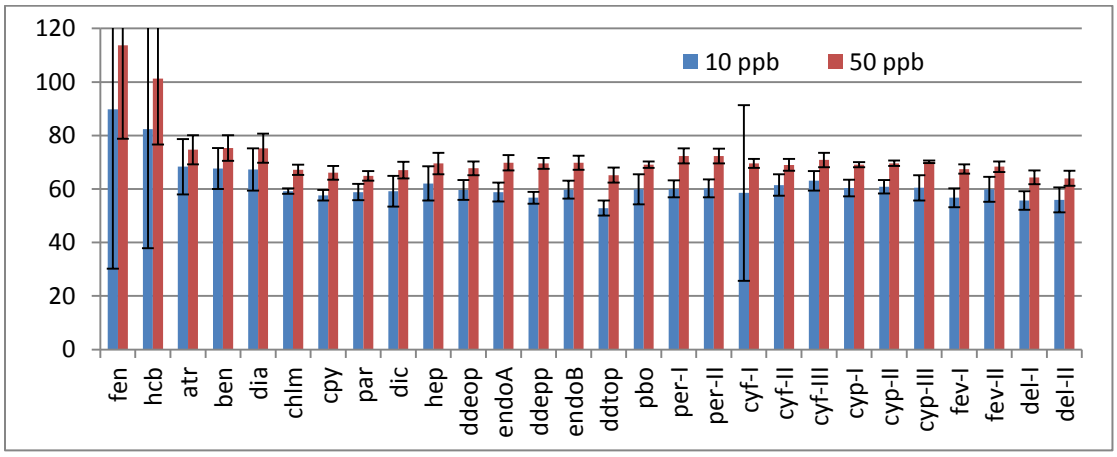


Figure 4-8 Recoveries of Florisil SPE cartridges. Y-axis represents percent recovery (%).

The next experiment was designed to examine the cause of the low recoveries from florisil cartridges. Replicate 2 mL of pooled serum samples were fortified to the

concentration level of 10 ppb for extraction. All the steps were the same as the ones described in method (2) until the sample loading step of florisil cartridges. The breakthrough during the sample loading was collected in separate test tubes for GC analysis. After sample loading, one group of cartridges were eluted twice with 5 mL hexane/ethyl acetate (1:1, v/v), another group of cartridges were eluted with 5 mL ethyl acetate followed by another 5 mL of hexane. Results indicated that the low recoveries from florisil cartridges were mainly caused by the breakthrough during the sample loading step, and the 2 × 5 mL hexane/ethyl acetate (1:1, v/v) elution worked much better than the 5 mL ethyl acetate + 5 mL of hexane elution. If we could collect the breakthrough and combine it with the eluent, recoveries of the florisil cartridges would be greatly improved.

This experiment was conducted to examine if the breakthrough from florisil cartridges is clean enough to collect. Replicate blank serum samples were prepared using method (2). The collected breakthrough (~2 mL) in the florisil loading steps was spiked with native standards to the concentration level of 25ppb and ISTD, evaporated to dryness, reconstituted with 50 µL acetonitrile/toluene (3:1, v/v), and injected into GC. After evaporated the breakthrough in test tubes to dryness, the bottom of the test tube walls were clean through visual observations. After repeatedly injecting replicate breakthrough samples into the GC, the response of native pesticides and ISTD remained the same without decreasing throughout these injections. Therefore, the breakthrough was clean enough to collect and combine with eluent for GC analysis. By collecting the

breakthrough, we could avoid losing pesticides in loading steps and thus increase the recoveries of the whole method.

Since we ran out of the Fisher PrepSep 1 g florisil 6 mL SPE columns and Fisher PrepSep 500 mg C18(EC) 6 mL SPE columns from Supelco (Bellefonte, PA) used in method development period and they were also discontinued, their replacements, Isolute 500 mg C18(EC) 6 mL SPE columns from Biotage and HyperSep 1000 mg florisil 6 mL SPE columns from Thermo were used in method validation and the extraction of unknown samples. The finalized method is described in the Materials and Methods section of this chapter (Chapter 3).

The recovery study was conducted in replicate pooled serum samples fortified at two different concentration levels, 10 ppb and 25 ppb. Results are shown in Figure 4-1. The recoveries were lower than we expected. One of the most important reasons might be that we used different C18 cartridges. As shown in Table 4-6, because of the difference in carbon loading, there might be secondary interactions that were responsible for the low recoveries. Although the carbon loading of ISOLUTE C18 cartridges was the closest to 10% we could find in the market, there were still 8.6% differences.

Table 4-6 Parameters of ISOLUTE C18 and PrepSep C18 cartridges

	Carbon Loading (%C)	Particle Size (µm)	Pore Size (Å)	Surface Area (m^2/g)
ISOLUTE C18	18.6	59	52	524

(EC)				
PrepSep C18 (EC)	10	40-50	60	500

The finalized sample preparation method was examined in unknown serum samples. The amount of individual unknown serum sample collected from Thai farmers was limited. Although our method validation was conducted in 2 mL of serum samples, we would like to know if this method is also applicable to 1 mL of serum samples. Two groups of 10 unknown serum samples (20 different unknown samples in total) were analyzed. The first group of 10 unknown samples (Sample ID 1-10) was extracted with 1 mL of serum and the second group of 10 unknown samples (Sample ID 11-20) was extracted with 2 mL of serum. Table 4-7 only lists the pesticides with detectable concentration levels in unknown samples. The frequency of each pesticides being detected in the two groups indicates that the finalized method works well for 1 mL of serum too.

Table 4-7 Concentrations (ppb) of individual pesticide with detectable concentration levels in 20 unknown serum samples collected from Thai farmers

Sample ID	hcb	atr	fon	dia	chlm	cpy	dic	hep
	1 mL serum per sample							
1	0.0353	0.0145	0.0056	0.0059	0.0047	0.0085	0.0720	-

2	0.0393	0.0095	0.0036	-	0.0039	0.0082	0.0265	0.0527
3	0.0246	0.0150	0.0022	-	-	-	0.0205	-
4	0.0220	-	-	-	-	0.0214	0.1485	-
5	0.0396	-	-	-	-	0.0203	0.0606	-
6	0.0214	-	-	0.0051	-	-	0.0145	-
7	0.0400	-	-	-	-	0.0047	0.0978	-
8	0.0276	-	-	-	-	-	0.0280	0.0590
9	0.0309	-	-	-	-	-	0.0181	-
10	0.0340	-	-	-	-	0.0083	0.0278	-
11	0.0376	-	-	-	-	-	0.0122	-
12	0.0275	-	-	-	-	-	0.0300	-
13	0.0373	-	-	-	-	-	0.0342	0.1403
14	0.0286	-	-	-	-	-	0.0292	-
15	0.0461	0.0398	-	-	-	-	0.0314	0.4724
16	0.0360	-	-	-	-	-	0.0301	0.0936
17	0.0236	-	-	-	-	-	0.0127	-
18	0.0242	-	-	-	-	0.0044	0.0087	-
19	0.0270	-	-	-	-	0.0061	0.0162	-
20	0.0259	0.0056	-	-	-	0.2543	0.0091	-

Sample ID	ddeop	ddepp	pbo	res	cyp-I	cyp-II	cyp-III	
	2 mL serum per sample							
1	0.0061	0.0850	0.0121	0.6806	0.0279	0.0334	0.0368	
2	0.0087	1.9171	0.0150	2.1524	-	-	-	
3	0.0031	0.1769	0.0115	1.2190	-	-	-	
4	0.0093	4.4653	0.0103	0.9152	-	-	-	
5	0.0046	2.6115	0.0087	0.4258	-	-	-	
6	0.0031	0.3845	0.0111	2.1027	-	-	-	
7	0.0065	2.0138	0.0093	1.7110	-	-	-	
8	0.0037	2.2895	0.0064	0.7451	0.0080	0.0085	0.0122	
9	0.0022	0.9396	0.0087	2.2903	-	-	-	
10	-	0.2307	0.0102	4.7877	-	-	-	
11	0.0052	1.9571	0.0074	4.8259	-	-	-	
12	-	1.8185	0.0108	1.4823	-	-	-	
13	0.0046	3.2963	0.0091	15.3761	0.0497	0.0616	0.0948	
14	-	1.6009	0.0089	2.1731	0.0436	0.0487	0.0661	
15	0.0079	7.5169	0.0072	2.0623	0.0190	0.0322	0.0400	
16	-	7.6255	0.0086	1.3731	-	-	-	

17	0.0030	1.1394	0.0084	1.3217	-	-	-	
18	-	0.2166	0.0086	1.0309	-	-	-	
19	-	0.9058	0.0095	2.1886	0.0157	0.0291	0.0387	
20	-	0.1689	0.0119	2.3036	0.0080	0.0226	0.0122	

4.5 Conclusions

The sample preparation method for pesticides analysis in milk proposed in Chapter 2 was tested and modified to analyze OC, OP, pyrethroid, and carbamate pesticides in serum matrices. However, during the method validation process, this method and its modified one were both proved to yield unsatisfying recoveries and fail to clean-up serum matrices. Afterwards, a different method was adapted, modified, and validated for pesticides analysis in pooled serum samples. This sample preparation method started with C18 SPE cartridges and was followed by florisil SPE procedures. Extracted samples were analyzed in a gas chromatography tandem mass spectrometry (GC-MS/MS) for the identification and quantification of targeted pesticides. Ionization of pesticide molecules was achieved by electron ionization in positive mode. Multiple reactions monitoring (MRM) was the acquisition mode used for the monitoring of two MS/MS transitions for each analyte. Extraction recoveries were satisfactory and ranged from 49% to 96% in pooled serum. The limit of detection in serum matrices ranged from 0.0009 to 0.3697 ppb, with the majority of them below 0.1 ppb. The accuracy for

majority of the pesticides was within the range of 80-120%. The within-day and between-day precision were in the range of 1.42-10.74%. The 3 months stability study was conducted to assess the degradations of these pesticides in serum and the results will be on their way shortly. Finally, this validated method was applied to 20 unknown serum samples that were obtained from Thai farmers. In conclusion, the adapted sample preparation method of PCBs and OCs analysis in serum was successfully modified to measure concentration levels of OC, OP, pyrethroid, and carbamate insecticides in 1 mL of serum to assess human exposure to persistent and current-use pesticides.

Chapter 5 Conclusions and future development

In this research, we designed a simple static sampling chamber that is much more cost efficient than that of commonly used apparatus to accurately measure sampling rates of passive sampling devices to assess air pollution. However, more future work is still needed.

Besides checking the leakage rate of the chamber, there are also several other rate constants needed to be determined, including the loss of analytes to the chamber wall. If this static chamber is developed properly, it will be a contribution to the passive sampler research field since the whole static chamber setup costs less than $300 compared to the several thousand dollars needed to setup a flow chamber.

In future experiments, the number of times each sampler extracted must be recorded. After the large peak in Figure 2-13 appears, we can trace back to the recorded number of extractions for the sampler to determine when the sampler begins to lose its coating materials. GC/MS analysis may also help us identify the coating materials if there are any. We can also check if losing coating materials will affect the sampling rate and to what level.

There are still many unknown parameters, and the data are not yet good enough to see how the passive sampler works. In the future, more columns with different coating materials will be tested to determine the optimum coating materials for a specific type of compounds. All the data presented here were obtained from GC-FID analysis, GC-MS will be used to confirm these results. Furthermore, a different extraction mechanism

might be developed that can better preserve the integrity of column coating materials and give passive samplers longer life time.

Other experiments need to be done including designing a simple way to effectively control the humidity of the chamber, assessing how temperature, face velocity, PAHs concentrations, and sampling time affect sampling rates, and determine if it is possible to use this type of passive samplers to measure other semi-volatile compounds such as pesticides.

Our ultimate goal is to design a sensitive, reliable, simple, economical, and user-friendly passive sampler to measure personal exposure to gaseous semi-volatiles and their concentration levels in the atmosphere. We are in the process of developing a table similar to Table 5-1 in which we use our knowledge of analytical procedures and our chamber to aid us in determining sampling rates. If we could successfully develop a table like this, other researchers can develop their own sampling strategies using this table as a reference.

Table 5-1 (Dummy table – the entries are examples of what we might discover)
Performances of Passive Samplers Made from Different Types of Columns When Sampling Different Pollutants

Name of Pollutants	Types of Different Columns Used To Make Passive Samplers				
	DB-1	HP-5	Rtx-35	OV-225	……
Low Molecular Weight PAHs	Poor	Good	Adequate	Poor	……
High Molecular Weight PAHs	Good	Poor	Good	Adequate	……
Pesticides	Good	Adequate	Adequate	Poor	……
Phthalates	Good	Poor	Adequate	Poor	……
……	……	……	……	……	……

We only studied for gaseous PAHs at this stage, but in the future, passive sampling devices made of various capillary columns will be assessed, and different gaseous air pollutants will be tested. After completion, this work will provide a more economic and simpler way to assess human exposure through the inhalation route.

We developed analytical methods for the analysis of over 20 pesticides covering four main classes of neurotoxic insecticides in various complicated matrices including human breast milk, cow milk, baby formula, and human serum. This work, focusing on assessing pesticides exposure in the postnatal period and infancy, overall addresses the scientific knowledge gap between prenatal pesticides exposure measurement and pesticides exposure measurement for elder children. As a primary source of nourishment of infants, breast milk provides a great way to assess pesticides exposure of neonates and infants through ingestion route. By using serum as the biomarker, it also provides useful data to measure personally exposure to pesticides for adults through ingestion,

inhalation, and dermal absorption routes. Despite all these promising results, more work is still in need.

A 90-day storage stability study and a 48-hour auto sampler stability study are need to determine if these pesticides degrade during the storage process and the sample analysis process on the GC.

Moreover, all the samples related with pesticides presented in this dissertation were all analyzed by GC tandem mass spectrometry with electron ionization (EI). In the future, these samples will also be analyzed by GC-MS/MS with chemical ionization (CI), which is often considered as a lower energy alternative to EI for volatile analytes. In CI, ionization is caused by proton transfer, so it is a much lower energy process, resulting in less residual energy being processed by the protonated molecules. Therefore, the fragmentation is largely reduced. CI is generally considered a “softer” ionization than EI, and a better way to analyze smaller molecules. Due to the nature of CI, hopefully, it will give more sensitivity to our results than EI. Moreover, if CI works, it will not only give us comparable results but also provide an alternative way to analyze our samples.

We will also test the developed pesticides analysis methods in milk and serum samples collected from several current studies in our lab, including milk samples collected by our Chiang Mai University collaborators for a pilot birth cohort study, milk samples shipped to our laboratory from Texas A&M University, and serum samples collected from Thai farmers.

Usually, when performing an exposure assessment, it involves 5 steps: (1) determining the exposure routes, (2) identifying the environmental media that transports the contaminants, (3) determining the concentration of contaminants, (4) determining the exposure time, frequency, and duration, and (5) identifying the population exposed.[9]

Our currently work mainly focuses on step (3) to determine the concentration of contaminants. In the future, we will try to corporate more with other laboratories and different studies to move my research also towards the other steps of the exposure assessment.

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