Development and validation of a methodology for the determination of persistent organic pollutants in human milk from Western Australia

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M. Sc.

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Abstract

Human milk (HM) contains a variety of nutrients and bioactive compounds that are required for both optimal infant growth and development of newborn’s immune system to protect against a diverse array of diseases and infections. However, due to historical extensive use of persistent organic pollutants (POPs) in Western Australia, the presence of these pesticides in HM is of great concern due to their potential health effects for breastfed infants. There has been little information regarding these pesticides in West Australian women’s milk since the 1990s, thus it is essential to measure the current contamination status of HM. To do this, there is a critical need for an accurate and effective analytical method to measure these pesticides in HM.

Typically, POPs are lipophilic therefore pesticide concentrations are standardized to the fat content of HM. The methods used to measure HM fat have not been compared in a systematic manner. In order to accurately calculate the concentrations of POPs in HM, I compared the gravimetric method to creamatocrit and esterified fatty acid (EFA) methods with HM of varying fat content (29.5–89.9 g/L). Whilst correlations between the methods ($r^2=0.99$) were extremely good, the creamatocrit method was found to provide values closer to that of the reference method than the EFA. The simplicity and dramatic reduction of milk volume required by the creamatocrit method (< 0.1 mL) also make it a reasonable alternative to the gravimetric method (> 2.0 mL) of fat measurement.

Integral to accurate POPs measurement, it is necessary to address the matrix enhancement, as matrix-match calibration is not possible due to the unavailability of
pesticide-free HM. This is the first study that investigated and measured the extent and variability of the matrix enhancement of 88 pesticides in HM by GC-MS/MS. Strong matrix enhancement (16–9434%) was observed for these pesticides in HM. Various methods such as analyte protectants (APs), pulsed splitless and large volume injection were optimized to address the matrix enhancement of the pesticides. A mixture of ethylglycerol, gulonolactone and D-sorbitol (20, 2 and 2 mg/mL, respectively) together with pressure pulsed injection (30 psi + 2 μL) was demonstrated to be the most effective and useful method to address the matrix enhancement in GC-MS/MS analysis of various pesticides in HM.

Building on these results, a novel sample preparation method-Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS), was systematically developed, optimized and validated for 88 pesticides in HM. In addition, the milk volume was scaled down from 10 mL to 1 mL, resulting in satisfactory recoveries (70–120%). This new methodology miniaturizes the sample extraction and significantly reduces the requirement of HM as compared to widely used extraction techniques.

Application of these refined methods was then applied to a cross sectional (n=40) and longitudinal study of West Australian mothers (n=16) where HM samples were collected during the first 12 months of lactation. The creatatocrit method and the validated QuEChERS method with GC-MS/MS were used to measure the milk fat and the pesticides in HM respectively. Results showed 4-fold decrease of total DDTs since 2003 and the calculated dietary intake (CDI) observed in this study was much lower (59–117 fold) than the tolerable daily intake (TDI) proposed by FAO/WHO.
The longitudinal study is the first to investigate changes of HM POPs over the first year of lactation. *p,p'*-DDE, *p,p'*-DDT and β-HCH were the only pesticides found in HM and their concentrations decreased significantly throughout the first year of breastfeeding. No significant association was observed between *p,p'*-DDE in HM and maternal characteristics. No significant association was found between the detected pesticides and infant growth and development during the breastfeeding.

In summary, this thesis presents a validated QuEChERS methodology for pesticide residues analysis using GC-MS/MS, which is up to 10 times more sensitive than current reported methods for HM. This method can be used for the routine monitoring of different classes of pesticides in HM from Western Australia and other countries.
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Abbreviations

ACN: Acetonitrile

CDI: Calculated daily intake

APs: Analyte protectants

BMI: Body mass index

C18: Octadecyl-bonded silica

CDI: Calculated daily intake

DDT: Dichlorodiphenyltrichloroethane

DG: Diacylglycerol

DGAT: Diacylglycerol acyltransferases

ED: Enhancement difference (solvent vs. human milk)

EDI: Estimated daily intake

EFA: Esterified fatty acid

ME: Matrix enhancement

MFG: Milk fat globule

MRM: Multiple reaction monitoring

RER: Rough endoplasmic reticulum

FAO: Food and Agriculture Organization of the United Nations

FFA: Free fatty acids

GC-ECD: Gas chromatography electron capture detector

GC-MS/MS: Gas chromatography tandem mass spectrometry

GCB: Graphitized carbon black

GPC: Gel permeation chromatography
Abbreviations

HCB: Hexachlorobenzene
HCH: Hexachlorocyclohexane
HM: Human milk
HMO: Human milk oligosaccharides
HMBANA: Human milk banking association of North America
LC-MS/MS: Liquid chromatography tandem mass spectrometry
IS: Internal standard
LCLD: Lowest calibrated level for detection
LCLQ: Lowest calibrated level for quantitation
LLE: Liquid liquid extraction
LOD: Limit of detection
LOQ: Limit of quantitation
LDs: Lipid droplets
MG: Monoacylglycerol
MRM: Multiple reaction monitoring
OCPs: Organochlorine pesticides
OPPs: Organophosphate pesticides
PAHs: Polycyclic aromatic hydrocarbons
PCBs: Polychlorinated biphenyls
PCDD/Fs: Polychlorinated dibenzodioxins/polychlorinated dibenzofurans
PLE: Pressurized liquid extraction
POPs: Persistent organic pollutants
PSA: Primary secondary amine
QuEChERS: Quick, easy, cheap, effective, rugged and safe
tr: Retention time
SPE: Solid phase extraction
Abbreviations

TAGs: Triacylglycerols
TPP: Triphenylphosphate
TDI: Tolerable daily intake
WA: Western Australia
WHO: World Health Organization
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**Poster presentation**


Statement of candidature contribution

The author, Jian Du, performed majority of the work presented in this thesis, while other individuals also contributed to each chapter and the publications arising from this thesis. The contribution of the each manuscript authors is provided below.

**Chapter 1**

The author reviewed the literature and wrote this chapter as a literature review. Dr. Melvin Gay, E/Professor Peter Hartmann, A/Professor Robert Trengove and Dr. Donna Geddes critically reviewed this chapter.

**Chapter 2**

The author designed the study and performed all the laboratory work, interpreted and analyzed the results and wrote the manuscript for publication. Dr. Melvin Gay and Dr. Ching Tai Lai supported the statistical analysis and reviewed the manuscript. E/Professor Peter Hartmann, A/Professor Robert Trengove and Dr. Donna Geddes critically reviewed the manuscript.

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Chapter 7

The author wrote this chapter. Dr. Melvin Gay, E/Professor Peter Hartmann, A/Professor Robert Trengove and Dr. Donna Geddes critically reviewed this chapter.
Chapter 1 Persistent organic pollutants and human milk

1.1 Introduction

Human milk (HM) is a product of evolution that specifically meets the need of human infants and with a composition markedly different to cow’s milk and the milk of other mammals (Williams, 1961; Hartmann, 2008; Ballard & Morrow, 2013; Lönnerdal, et al., 2017). HM contains a variety of nutrients (proteins, fat, lactose, oligosaccharides, minerals, vitamins and amino acids), growth factors and immunologically components (secretory immunoglobulins, lysozyme, lactoferrin, cellular components (T cells, stem cells, macrophage, neutrophils, lymphocytes and leucocytes) and glycoconjugates) that are essential for both optimal infant growth and the development of the immune system to protect against a diverse array of diseases (Newburg, 2001; LaKind, et al., 2004; Hale & Hartmann, 2007; Chatterton, et al., 2013; Lönnerdal, 2017). HM, in comparison to substitutes such as artificial formula and related baby food products, is important to infants, women, society and the economy, and there is convincing evidence about the benefits of breastfeeding for human health in both developed and developing countries (Leaf & Winterson, 2009; Quigley, et al., 2012; Rollins, et al., 2016; Victora, et al., 2016).

1.1.1 Benefits of human milk for infants

HM contains a vast array of bioactive components that act in a synergistic way to protect infants from pathogens. The bioactive components (such as free fatty acids and lactoferrin) also ensure efficient nutritional requirements of infants’ optimal growth,
which improves the survival, health and development of the breastfed infants (Victora, et al., 2016). HM has marked benefits for the breastfed infants, having both short-term and long-term health consequences. Early nutrition via breastfeeding can induce lifetime programming effects on metabolism, growth and neurodevelopment and in turn diminishing the risk of non-communicable diseases (Barker, 1992). Breastfed infants have decreased risk of developing a number of diseases such as hypertension, heart disease and diabetes mellitus in later life (Osmond & Barker, 2000; Das, 2007). For the breastfed infants, the bioactive components of HM stimulate growth, development and maturation of specific organs especially the gut (Goldman, et al., 1996; Musilova, et al., 2014; Walker & Iyengar, 2015). Other components including lactoferrin and free fatty acids, not only contribute significantly to the nutrients that are digested and absorbed by the breastfed infants, but also serve to protect the infants from a wide range of pathogens (Newburg, 2001; Marincola, et al., 2012). HM also includes a high concentration of complex HMOs that have many important roles in health and development of the infants. These HMOs have probiotic activities that prompt various anti-infective properties as well as growth of the harmless bacteria (Bode, 2012; Musilova, et al., 2014). In general, breastfed infants have remarkably lower incidences of diarrhea, otitis media, intestinal and respiratory disease and several times less mortality than those who are breastfed for shorter periods or formula fed (Newburg & Walker, 2007). Breastfed infants have optimum neurological, cognitive (language, intelligence and speech) and brain development and higher IQ than their formula fed counterparts (Morrow-Tlucak, et al., 1988; Lucas, et al., 1992; Lanting, et al., 1994; Quigley, et al., 2012). It is likely that breastfeeding ensures adequate nutrition and polyunsaturated fatty acids (PUFAs) for brain growth and development. The benefits of HM are realized not only in the childhood but also in their later life (Das, 2006; Innis, 2008). Long-term benefits include reduced cholesterol levels and disease in adulthood,
including hypertension, obesity, diabetes and cardiovascular disease (Jones, et al.; LaKind, et al., 2004; Horta, et al., 2007). Studies have shown that adolescent and adult obesity rates could be reduced by up to 30% if infants received any breastfeeding in their infancy compared with no breastfeeding (Owen, et al., 2005; Ip, et al., 2009). Up to 30% and 40% reduction in the incidence of type 1 and type 2 diabetes mellitus have been reported respectively for infants who exclusively breastfed for at least three months (Das, 2007). Overall, more than 820 000 deaths in children (<5 years) worldwide could be prevented annually if the breastfeeding practices are conducted by mothers for all newborn babies (Walker, et al., 2013).

HM has also been shown to confer benefits to preterm infants because of its bioactive components, such as fat, protein, vitamins, minerals, cellular components, oligosaccharides, nucleic acids and glycoconjugates. Fortified HM supports the preterm infant’s rapid growth rate and also contributes to the development of the preterm infant’s immature host defense systems and gastrointestinal function (Schanler, 1995). There is compelling evidence that the use of HM for preterm infants is associated with lower mortality rates, better neurodevelopment and better health in adulthood (Schanler, et al., 1999; Leaf & Winterson, 2009). Whereas, preterm formula carries a much greater risk with a several fold higher incidence of necrotizing enterocolitis, late onset sepsis and mortality rates compared to those fed with fortified HM (Lucas & Cole, 1990; Schanler, 2007). While HM has conferred numerous benefits to the infant in association to infant health, these correlations may not prove causation due to other underlying factors such as environment and lifestyle in later life.
1.1.2 Benefits of breastfeeding for mothers

Studies have shown that breastfeeding not only benefits the breastfed infants, but also has short- and long-term benefits on maternal physical and mental health (Leaf & Winterson, 2009). In the short-term, breastfeeding results in more rapid maternal weight loss and is associated with faster maternal uterine involution (Negishi, et al., 1999; Baker, et al., 2008). Maternal weight loss after birth has been shown to be attributed to the increased energetic cost of lactation where mothers require approximately 525–625 calories per day for milk production (Baker, et al., 2008). By contrast, mothers that do not breastfeed weighted around 1.4 kg more than those who exclusively breastfed for more than 6 months (Krause, et al., 2010). In the long-term, breastfeeding has positive effects such as a reduction in risk for cardiovascular disease and type 2 diabetes (Stuebe, et al., 2005; Schwarz, et al., 2009). Furthermore another study has shown that women with a cumulative breastfeeding history of more than 12 months had significant reduction in developing cardiovascular disease, hypertension and diabetes than those who have never breastfed (Schwarz, et al., 2009). The cumulative breastfeeding history also has an inverse association with the incidence of both breast and ovarian cancer (Ip, et al., 2009; Stuebe, et al., 2009). Each additional year of breastfeeding is associated with a 4.3% reduction in breast cancer (Collaborative Group on Hormonal Factors in Breast, 2002), and each additional month of breastfeeding is associated with a reduction of 2% in epithelial ovarian cancer (Danforth, et al., 2007). In addition to the physical health, breastfeeding also has significant impact on mother’s mental health. Many studies have shown that mothers who exclusively breastfed for 6 months have better outcomes in regulating and dealing with stresses generated by exercise, mathematical challenges and public speaking compared to formula-feeding mothers (Altemus, et al., 1995; Light, et al., 2000; Mezzacappa, et al., 2005). Breastfeeding mothers are also more likely to associate with positive moods, increased sleep duration, less anxiety and
depression compared to formula-feeding mothers (Carter & Altemus, 1997; Mezzacappa & Katlin, 2002; Doan, et al., 2007; Mohamad Yusuff, et al., 2016).

1.1.3 Social and economic benefits of breastfeeding

It is well established that HM and breastfeeding provide both short- and long-term benefits to both mothers and infants. However, the social and economic benefits of HM and breastfeeding are often unrecognized particularly by mothers as formula advertisements portray formula milk to be as good as or even better than HM (Piwoz & Huffman, 2015). HM substitutes, such as artificial formula and related baby food products, reap annual profits for multinational companies in the realms of billions of dollars. It has been projected that if all the mothers exclusively breastfed for 6 months, there would be a savings of $58 billion related to the global sales of all HM substitutes (based on figures from 2013), and a minimum of $2 billion per year in Australia (Smith & Ingham, 2001; Piwoz & Huffman, 2015). In contrast, it is predicted if suboptimal breastfeeding practices persist, the associated global economic losses will be projected to reach $71 billion by 2019 (Rollins, et al., 2016). These losses are conservative as are not inclusive of treatment costs related for infant illness and infectious diseases such as otitis media, diarrhea and pneumonia (Smith & Ingham, 2001; Bartick & Reinhold, 2010; Rollins, et al., 2016). Estimates of the reduction of treatment costs of childhood disorders are at least $351.6 million in four countries (e.g., USA, UK, China and Brazil) if the prevalence of exclusive breastfeeding up to six months or continued breastfeeding up to 1 year or 2 years was increased by 10% based on current levels (Rollins, et al., 2016). In Australian, it was estimated that a minimum of $11.5 million could be saved annually if increasing the exclusive breastfeeding at the first three months from 60% to 80% (Drane, 1997). These costs do not include numerous other chronic or common
illness and out-of-hospital health care costs (Smith, et al., 2002). Many of the potential social and economic costs related to adulthood diseases and maternal diseases are difficult to measure, thus these figures discussed are likely to be a gross underestimate of the total cost savings.

Given the enormous benefits of breastfeeding to both infants, mothers and social economy, World Health Organization (WHO) and American Academy of Pediatrics (AAP) recommend exclusive breastfeeding for about six months followed by continued breastfeeding up to first year of life and beyond (WHO, 2003).

1.2 Bioactive components of human milk

HM contains enormous protective components, such as secretory immunoglobulins, lysozyme and lactoferrin, that protect the infant from infections and diseases. HM also contains a wide variety of nutritional and development components that safeguard infant’s growth and development (Kulski & Hartmann, 1981; Newburg, 2001). These components are dynamic and change with length of gestation, during lactation, within feeding, within and between individual mothers (Anderson, et al., 1981; Ballard & Morrow, 2013). The specific protective and nutritional compositions of HM during the lactation period are ideally suited to the needs of the growth and development rate of the infants.

HM in the first few days (<5 days) postpartum is colostrum, which contains higher levels of immunomodulatory components, such as secretory immunoglobulins, lactoferrin, lysozyme and human milk oligosaccharides (HMO), but contains relatively lower concentrations of macronutrients such as lactose and fat (Nagasawa, et al., 1972; Mickleson & Moriarty, 1982; Newburg, 2001). Lysozyme catalyzes the lysis of
bacterial cell walls, lactoferrin and secretory immunoglobulins protects infants from specific infectious agents by attaching itself to the mucosal epithelium of the intestine (Quan, et al., 1992). The high concentration of immunological components and lower concentration of macronutrients in colostrum indicates that the primary functions of colostrum are to protect vulnerable newborn against a diverse array of infection and to help the development of the premature infant’s own immune system. The colostrum also make it physiologically advantageous for the premature infant that has undeveloped immune and immature digestive systems (Anderson, et al., 1981). The colostrum is rich in maximum immunological compositions such as cellular components, secretory immunoglobulins, lysozyme and lactoferrin, that protect infants from pathogens, but also induce the build-up of infant’s immune system (Godhia & Patel, 2013).

The protective and macronutrients change from colostrum to transitional milk (5 days - 2 weeks postpartum), with protective components decreasing and macronutrients increasing. The progressive increases in macronutrients, such as fat and lactose, support the nutritional and developmental needs of the rapidly growing infants (Ballard & Morrow, 2013). Production of transitional milk generally lasts for approximately two weeks, after which HM is consider as mature milk. Mature HM contains different amounts of protective and nutritional components than does colostrum and transitional HM. Mature HM remains relatively similar in composition over the course of lactation until weaning, with higher concentrations in fat and lactose to support the fast growth and development rate of the infant. The most three abundant components in mature HM are lactose, fat and protein, contributing approximately 40%, 50% and 10%, respectively to the total calories of HM (Emmett & Rogers, 1997; Robenek, et al., 2004). There are also numerous enzymes (such as lipase, alkaline phosphatase, xanthine oxidase, glutathione peroxidase and lysozyme) in HM, which play multiple roles in
influencing the nutritional digestion, gastrointestinal function and the delivery of 
nutrition to infants (Shahani, et al., 1980; Khaldi, et al., 2014). The vitamins and growth 
factors found in milk are important for infant growth and development. Deficiencies of 
vitamins, such as already identified vitamin A and pyridoxine (B6) have adverse effects 
on the growth and health of the infants (Ballard & Morrow, 2013). The components of 
HM are either synthesized by lactocytes in the mammary gland or are transported from 
plasma via the lactocytes to milk.

HM confers enormous benefits to the optimal growth and development of the infants, to 
the health of the breastfeeding dyad, and to the social economy, thus breastfeeding 
should be encouraged in both developing and developed countries. However, there has 
been an increasing concern about the presence of persistent organic pollutants (POPs) in 
HM since dichlorodiphenyltrichloroethane (DDT) was first found in HM in 1951 
(Hemminki, et al., 1982). Since then, more chemicals have been detected in HM despite 
many POPs being banned for decades (Mueller, et al., 2008; Hassine, et al., 2012; 
Luzardo, et al., 2013; Mannetje, et al., 2013). These POPs tend to bio-accumulate in 
human body due to their lipophilic and persistent properties. There are concerns that 
exposure to POPs in HM may carry some potential adverse health effects on the 
children (Kim, et al., 2013), therefore more concentrated effort is needed to monitor and 
characterize the potential adverse effects of POPs in HM.

1.3 Persistent organic pollutants

1.3.1 Background

Persistent organic pollutants (POPs), such as organochlorine pesticides (OCPs), 
organophosphate pesticides (OPPs), pyrethroid pesticides (pyrethroids) and carbamate
pesticides (carbamates) are widely used globally to protect agriculture and households after DDT was first introduced in 1940s (Köhler & Triebskorn, 2013). However, it has since been recognized that some POPs, particularly the DDT and its metabolites, are detrimental to human health. When POPs are spilled into the environment, they can percolate into the soil, ground water and atmosphere. As these pesticides are resistant to chemical, physical and biological degradation, they are transported over long distances through atmospheric circulation and water movement, making them ubiquitous environmental chemicals. POPs also have long half-lives and lipophilic properties, therefore they tend to biomagnify in wildlife, especially in species at the top of food chain, such as animals and humans (Tanabe, 2002).

Inhalation, ingestion and dermal contact are routes of entry for POPs into the human body (Van Oostdam, et al., 1999; Needham & Wang, 2002). After entering the blood, these xenobiotic chemicals form a distribution equilibrium by dissolving in plasma and binding to plasmas proteins such as human blood albumin (HBA), globulins and lipoproteins (Maliwal & Guthrie, 1981). The equilibrium rate and stability of the resultant complex varies between pesticides, and is related to the octanol-water partition coefficient (Kow) of each compound and other factors, such as plasma protein content and lipid content (Skalsky & Guthrie, 1978; Li, et al., 2011). The pesticides then distribute and concentrate in the high fat tissue compartments, such as adipose tissue, liver, kidney, brain and breast.

1.3.1.1 Organochlorine pesticides (OCPs)

OCPs refer to a wide range of organic chemicals, which contain at least one covalently bonded atom of chlorine as the dominant functionality. In Australia, DDT was first
introduced in the 1940s to deter insects and pests from agricultural practices, commercial buildings and general households (Reid, et al., 2013). Other OCPs, such as aldrin, dieldrin, heptachlor, chlordane, hexachlorobenzene (HCB) and hexachlorocyclohexane (HCH), were also widely used to eradicate pests and termites in Australia as well as in many other parts of the world (Stacey & Tatum, 1985).

OCPs registration and usage increased during the 1950s and peaked in the 1970s. Detailed information regarding the uses of OCPs is shown in Table 1.1. OCPs are especially resistant to environmental degradation, therefore OCPs and their metabolites (e.g. DDT-DDE and Lindane - β-HCH) have very long environmental half-lives ($t_{1/2}$: the time taken for half of the quantity of pesticide to be degraded) of up to 6 years (Norén & Meironyté, 2000). As OCPs were discovered to be toxic even at low concentration to humans and aquatic life, their use in Australia had decreased dramatically between the middle-1970s and early 1980s. Most of these pesticides were banned for agricultural use by 1985 and were completely eliminated for termite control in most states and territories in Australia by the 1990s (Harden, et al., 2005; Reid, et al., 2013). Currently, OCPs and products that contain OCPs are not permitted to be either used or imported into Australia.

Table 1.1 Organochlorine pesticides (OCPs) use and year banned in Australia.

<table>
<thead>
<tr>
<th>OCPs</th>
<th>Examples of registered uses in Australia</th>
<th>Year of banned in Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCH</td>
<td>Control plant insects and insect pests in stored seed, control white grubs and symphylids in food.</td>
<td>Deregistered for general use in 1985</td>
</tr>
<tr>
<td>Aldrin</td>
<td>Control ants and subterranean termites in the farming, industry and domestic gardens. Soil treatment for crops.</td>
<td>Deregistered by 1985</td>
</tr>
</tbody>
</table>
## Chapter 1 Literature Review

### 1.3.1.2 Organophosphate pesticides (OPPs)

OPPs are esters of phosphoric or phosphorothioic acids that contain either a phosphoryl bond (-P=O) or thiophosphoryl bond (-P=S), and the general structures of OPPs are shown in Figure 1.1. Similar to OCPs, OPPs are widely used for pest control in both domestic households and a wide range of agriculture processes.

<table>
<thead>
<tr>
<th>OCPs</th>
<th>Examples of registered uses in Australia</th>
<th>Year of banned in Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCB</td>
<td>Small quantities were used to protect grains against fungus, and were also used in the production of rubber, dyes, wood preservation and fireworks.</td>
<td>Deregistered between 1985 and 1987</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>Control a broad spectrum of insects on crops and in agricultural and industrial premises.</td>
<td>Deregistered in 1987</td>
</tr>
<tr>
<td>DDT</td>
<td>Control insects and large quantities were used to control mosquitoes.</td>
<td>Banned in 1987</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>Control funnel ants, grey-black beetle and beetle borer in crops such as crane growing areas and banana plantations.</td>
<td>Phased out by 1997</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>Control locusts and argentine ants as well as soil treatment on farms. Control of termites in buildings.</td>
<td>Phased out by 1997</td>
</tr>
<tr>
<td>Mirex</td>
<td>Control of giant termite (<em>Mastotermes darwiniensis</em>).</td>
<td>Ceased after 2006</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>Control a variety of insects and mites in agricultural and horticultural crops.</td>
<td>Deregistered in 2010</td>
</tr>
</tbody>
</table>
However, unlike OCPs, OPPs tend to degrade faster in the environment with half-lives in soil ranging from a few hours (<10 hours) to as long as a few months after application, which makes them as an attractive alternative to the more persistent OCPs (Giesy, et al., 2014). Subsequently, OPPs use has dramatically increased and is now the most commonly used insecticides in Australia (Immig, 2010). Approximately 700 commercially available products containing OPPs, such as dichlorvos, ethion, fenthion, phosmet, profenofos, prothiofos and terbufos, are approved for registration and use in Australia (Immig, 2010). Currently, around 5 000 tons of active ingredients are used annually on fruits, vegetables, livestock and domestic animals, and for pest control in buildings (Oglobline, et al., 2001; Radcliffe, 2002).

![Generic structure of Organophosphate pesticides (OPPs).](image)

**Figure 1.1** Generic structure of Organophosphate pesticides (OPPs).

### 1.3.1.3 Pyrethroids and carbamates

Pyrethroids are synthesized and are structurally similar to natural pyrethrins, that are originally produced by the flowers of pyrethrum (*Chrysanthemum cinerariaefolium*) (Feo, et al., 2012). The general structure of pyrethroids is shown in **Figure 1.2**. Pyrethroids were derived by modifying the active sites (R₁- and R₂-) in order to improve
the specificity and activity of pyrethrins, while maintaining the high knockdown and low toxicity (Schleier & Peterson, 2011). Pyrethroids are synthetic insecticides with high hydrophobicity (log $K_{ow}$ ranging between 5.7 and 7.6) and low water solubility and volatility (Feo, et al., 2010). They also have short half-lives (<100 days) in soils after application with oxidation and hydroxylation forming the major degradation pathways (Laskowski, 2002). In humans, they are rapidly metabolized to non-toxic metabolites, such as phenoxybenzoic acid, and are easily eliminated from the body, thus their toxicity to humans is low (Corcellas, et al., 2012).

![Figure 1.2 Generic structure of pyrethroids.](image)

Pyrethroids use has increased substantially over the past few decades due to their effectiveness against many pests, relatively low mammalian toxicity and low environmental persistence. Currently, there are almost 300 insecticidal products containing pyrethroids, such as bifenthrin, cypermethrin, flumethrin and permethrin that are registered for use in Australia in the forms of powders, sprays and shampoos. Currently, they are widely used in industrial and residential areas for pest control, in the field treatment for insect control and in commercial products for animal wellbeing (Corcellas, et al., 2012).
Carbamates are derivatives of carbamic acid (HOCONH₂) and the general structure of the carbamates is shown in Figure 1.3. Carbamates are relatively unstable and can be broken down in the environment within a few hours (<5 hours) to a few months (<6 months) in soils after application (Immig, 2010). Due to their relatively low mammalian toxicity, carbamates such as bendiocarb, carbaryl, methiocarb, methomyl, pirimicarb and propoxur are registered in Australia for use in a wide range of agricultural and domestic situations for the control of wireworm, slugs and snails (Immig, 2010).

![Figure 1.3 Generic structure of carbamates.](image)

1.3.2 Transfer of POPs into HM

During pregnancy, the mammary gland enters the final phase of development under the control of hormones, such as estrogen, prolactin, growth hormone, progesterone, epidermal growth factor and parathyroid hormone related proteins, that accelerate the growth as pregnancy develops (Berry, et al., 2007). At this stage, the blood flow to the breasts increases due to the demand of necessary nutrients, components and precursors for the production of HM components (Hale, 2004). These precursors pass through a ductal luminal wall that separates the capillaries from the single layer of lactocytes
(secretory epithelial cells), and into the lactocytes as depicted in Figure 1.4. The lactocytes are responsible for the synthesis and secretion of milk components, including fat, lactose, lactoferrin and caseins (Hartmann, 2008). As lactocytes can only synthesize short and medium chain fatty acids (C $\leq$ 14:0) for triacylglycerols (TAGs) formation, long chain fatty acids (C $>$ 14:0) are imported from the circulation into HM (Czank, et al., 2007). Over 80% of fat in HM originates from blood lipids (Neville, et al., 1983). Similarly, POPs in the blood can also pass through the ductal luminal wall and enter the lactocytes during pregnancy and lactation. The transfer efficiency of POPs into the HM depends on several factors such as pesticide lipophilicity, pesticide-plasma protein binding, etc. (Skalsky & Guthrie, 1978; Maliwal & Guthrie, 1981). Several postulations of the transport mechanisms are depicted in Figure 1.4. POPs can transfer into HM via the large intercellular gap during the first lactation stage (secretary differentiation) when the lactocytes secrete unique milk (colostrum) constituents, such as lactose, and the transcellular pathway throughout the second lactation stage (secretary activation), when the lactocytes secrete copious milk (Hale, 2004; Pang & Hartmann, 2007).

**Intercellular Transport:** During the secretory differentiation stage, the intercellular junctions between the lactocytes are open and increase permeability of the epithelium. Thereby, it permits easy transfer of lipids, proteins, immunoglobulins and even higher levels of electrolytes (sodium and potassium) into the early milk. Similarly, POPs diffuse into milk at slightly higher concentrations via the intercellular junctions to milk compartment (McManaman & Neville, 2003; Hale, 2004).

**Transcellular Transport:** As lactation progresses, the growth of the lactocytes results in the closure of the intercellular junctions, thereby markedly reduces the likelihood of
intercellular transport of POPs into HM. However, POPs can still enter the milk by the following mechanisms during the secretory activation stage.

1) The transport proteins in human blood such as HBA, globulins and lipoproteins can carry the toxic POPs into HM by passing through the lactocyte membranes (Skalsky & Guthrie, 1978).

2) Specific receptors in lactocyte can engulf the POPs and transport them into alveolus.

3) Caveolae invaginations (50-100 nm) on the membrane of adipocytes, endothelia cells and muscle cells, can be induced by POPs, suggesting the involvement of caveolae in the endocytosis and transport of POPs into the alveolus (Bourez, et al., 2012). A significant fraction of caveolin (an essential components of caveolae) was found in the rough endoplasmic reticulum (RER) membranes where the milk fat globule (MFG) is produced, indicating that POPs in the caveolae can be incorporated into the MFG (Robenek, et al., 2004).

**Figure 1.4** Diagrams of mammary alveolar show milk secretion and pathways of POPs entering in and out of the milk compartments. Milk fat globule (MFG) is secreted by lactocytes into lumen (red color arrow), while the passage of POPs into and out of lactocytes via intercellular gaps (blue color arrow) and the transcellular spaces (green color arrow) during secretary differentiation and secretory activation stage of lactation.
1.3.3 Interaction of POPs with MFG

MFG is composed of a neutral lipid core consisting mainly of TAGs and cholesterol esters surrounded by a phospholipid monolayer including primarily phospholipids and sterols as shown in Figure 1.5 (Ducharme & Bickel, 2008). MFG is predominantly synthesized in RER of the lactocyte and electron micrographs have also shown that the MFG are tightly connected with both RER and mitochondria (Farese & Walther, 2009). RER contains many enzymes, such as diacylglycerol acyltransferases and Acetyl-CoA carboxylase, which are essential for MFG synthesis (Farese & Walther, 2009; Walther & Farese Jr, 2009). Mitochondria provides energy and necessary precursors, such as fatty acids and nicotinamide adenine dinucleotide phosphate (NADPH), for the synthesis of TAGs and biogenesis of MFG (Vance, 2003).

![Structure of a milk fat globule (MFG). Shown are the nonpolar lipids of the core (e.g., TAGs, cholesterol esters and sterol ester), the polar surface lipids of the monolayer (e.g., phospholipid and sterols) and the postulated locations of POPs in the MFG.](image)

**Figure 1.5** Structure of a milk fat globule (MFG). Shown are the nonpolar lipids of the core (e.g., TAGs, cholesterol esters and sterol ester), the polar surface lipids of the monolayer (e.g., phospholipid and sterols) and the postulated locations of POPs in the MFG.
The interaction between POPs and the MFG during milk synthesis and the localization of the POPs on the MFG remain unknown. However, studies have hypothesized several interaction mechanisms between POPs and MFG described below (Hale, 2004; Robenek, et al., 2004).

1) If POPs are inserted into the endoplasmic leaflet of RER membranes, the high affinity of TAGs of the MFG for POPs causes POPs to be extracted out of the endoplasmic leaflet and are transferred into the core of the MFG (Figure 1.6A). An accumulation of neutral lipids within endoplasmic leaflet and cytoplasmic leaflet of ER drives a budding of a nascent MFG from the RER membrane and bulges out of the membrane.

**Figure 1.6** Schematic diagrams showing two possible pathways that POPs are interacting with milk fat globule (MFG) during milk synthesis modified based on Robenek et al. (Robenek, et al., 2004)
2) If POPs are inserted into the cytoplasmic leaflet of RER membranes, POPs could diffuse freely in the cytoplasmic leaflet and are transferred along with this leaflet to the surface of the MFG during MFG biogenesis in RER (Figure 1.6B).

The growth of MFG involves the addition of large amounts of TAGs to its core and phospholipids to its surface. POPs could also be incorporated to the surface of MFG during MFG growth in RER. Therefore, POPs could be transferred to the alveolar lumen through intercellular and transcellular transport, and secreted out of lactocyte with MFG in HM.

1.3.4 Adverse effects of POPs

The presence of POPs in HM is of great concern due to the potential health effects on the breastfed infants, as these pesticides can interfere with the functions of normal endocrine system by mimicking, blocking, modulating or altering the synthesis, metabolism or transport of hormones (Soto, et al., 1995). Due to their immature defense systems and low levels of enzymes in the metabolism and detoxification of POPs, the fetus and infants are considered to be at higher risk for pesticide toxicity compared to adults (Holland, et al., 2006). Therefore, potential adverse effects by these POPs on fetus and infant are anticipated.

Exposure to these POPs has been associated with a wide range of unfavorable human infant outcomes (Perera, et al., 2003; Eskenazi, et al., 2004; Whyatt, et al., 2004; Dallaire, et al., 2012; Boucher, et al., 2013). Many epidemiologic studies based on POPs concentrations in maternal biofluids, such as serum, cord blood and urine, suggest that prenatal exposure to POPs is associated with negative birth outcomes (e.g., weight,
body length and head circumference) (Whyatt, et al., 2004), delayed neurodevelopment, poor cognitive performances and growth retardation during early childhood (<3 years) (Eskenazi, et al., 2006b; Eskenazi, et al., 2007; Boucher, et al., 2013). Marks et al. examined the relationship between maternal blood levels of OPPs and their metabolites and infant attention-related outcomes at age of 3.5 and 5 years, and found significant association between high prenatal OPPs exposure and attention deficit hyperactivity disorder at the age of 5 (Marks, et al., 2010). In a longitudinal prenatal exposure study of 329 children at 6 months through to 7 years of age, also demonstrated that the maternal OPPs concentrations significantly affect intellectual development and cognitive performances (e.g., poorer memory, processing, comprehension and reasoning) in 7-year-old children (Bouchard, et al., 2011).

Epidemiological studies on infant growth outcomes are scarce, and almost all the current studies have normally chosen anthropometrical data at birth as a proxy measure of in utero development (Eskenazi, et al., 2004; Wolff, et al., 2007). Little is known about the effects of the pesticides in HM on infant growth outcomes. No epidemiological study has been conducted yet to investigate long-term relationship between POPs in HM and infant growth and development. Studies based on the concentrations of OCPs in serum have found adverse effects of POPs on adult human fertility via modulating the steroid sex hormones, decreasing semen quality and altering menstrual and ovarian cycles (Buck Louis, et al., 2013). Several studies also demonstrate significant associations between the detected pesticides (dieldrin, oxychlordane, HCH, etc.) in serum and increased risk of various cancers (e.g. breast cancer and prostate cancer) (Calle, et al., 2002; Xu, et al., 2010).


1.3.5 Importance of monitoring POPs in HM

With rising in concerns over the potential risks of POPs in HM, the safety of HM is increasing being recognized as HM is the main food source for the infant particularly at the early age and these pesticides could impair infant growth and development. Therefore, studies about the possible influence of pesticides in HM on infant growth outcomes are warranted. HM is an ideal biological matrix for monitoring POPs, as it is non-invasive compared to others such as blood and adipose tissues (Jaraczewska, et al., 2006; Noakes, et al., 2006; Reid, et al., 2013). Monitoring POPs in HM also has several advantages (Johnson-Restrepo, et al., 2007; Ennaceur, et al., 2008):

1) POPs concentrations in HM reflect the actual maternal body burden from all exposure sources (e.g., inhalation, ingestion and dermal contact).

2) HM accurately determines the daily intake of POPs by infants and also examines for any potential effects of the POPs in HM on the infant growth and development during the breastfeeding period.

3) HM reflects the exposure of POPs in a general population over time, and the maternal body burden reflects the environment the infants will likely grow and develop after the breastfeeding period.

1.4 Factors that can influence POPs levels in HM

Many factors have been associated with the levels of POPs in HM such as milk sampling and handing, the fat content of HM and maternal characteristics such as age, parity, lactation stage, body weight and body mass index (BMI), along with other external factors (Harris, et al., 2001).
1.4.1 Sampling of HM

In order to obtain accurate POPs measurements in HM, a representative HM sample for analysis is required. However, most published studies do not have consistent sampling protocols. No account has been made for the wide variation in milk composition (e.g. fat content) with regard to the length of gestation, lactation stage, within day and between mothers (Atkinson, et al., 1980; Anderson, et al., 1981). For example, the fat and lactose levels increase rapidly, while protein declines gradually from colostrum to transitional milk and to mature milk, and then remains relatively constant during exclusively breastfeeding. The milk composition then changes around the introduction of complementary food and resembles to that of colostrum at the final stage of weaning (Emmett & Rogers, 1997; Moltó-Puigmartí, et al., 2011; Ballard & Morrow, 2013). During a 24-hour period, concentrations of lactose and protein remain relatively stable. However, the fat content varies around 3-fold (22.3-61.6 g/L) within a day (Khan, et al., 2013). The difficulties encountered in obtaining representative milk samples centre around the high variation of milk fat. WHO recommends that sampling for POPs analysis can be carried out between 3 weeks and 2 months after delivery once breastfeeding is well established (WHO, 2007a). However, it provides no guidance on when to obtain a single sample that might be representative. Here, a summary of the methods that have been employed previously to collect a representative milk sample is shown in Table 1.2 (Prentice, et al., 1981; Saint, et al., 1986; Arthur, et al., 1987; Kent, et al., 2006). Since at least 100 mL of HM may be required for many methods of analysis, considerations should be made that the volume and process should neither pose undue maternal burden nor compromise the infant’s nutritional status. In order to reflect individual maternal burden of POPs, and determine the potential associations between POPs concentrations in HM and both maternal characteristics and infant growth outcomes, HM samples should be collected from each mother and analyzed
individually instead of pooling samples from different mothers. This allows for identification of individuals with high levels of POPs enabling determination of the exposure source.

<table>
<thead>
<tr>
<th>HM collection</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low volume (0.25 mL) milk samples</strong></td>
<td>Collecting milk from both breasts before and after every feed in a 12 h period (Prentice, et al., 1981).</td>
</tr>
<tr>
<td><strong>Avoid interruption of normal feeding</strong></td>
<td>Collecting small amount (0.5 mL) of pre- and postfeed milk at each feed for a 24 h period (Saint, et al., 1986).</td>
</tr>
<tr>
<td><strong>WHO</strong></td>
<td>Collecting either postfeed milk or dripping milk (milk from one breast while the infant feeds at the other) (WHO, 2007a).</td>
</tr>
</tbody>
</table>

### 1.4.2 HM handing

After the collection of HM, appropriate handing of the sample is critical to maintain the composition of milk as close as possible to that of the fresh milk. This is because inappropriate HM handing, such as storage conditions (e.g., container and temperature), freezing and thawing of HM during fat and POPs analysis, may lead to reduction of nutritional and immunological components (Goldblum, et al., 1981). Fat is more likely to be affected by these conditions. Significant fat loss would often accompany with a reduction of liposoluble POPs levels in HM and this can occur at multiple points of handing of the sample as well as choice of storage container.
Milk fat adheres to the walls of the storage containers especially to soft plastic containers, such as polyethylene bags (Arnold, 1995; Chang, et al., 2012). Several studies have compared the effect of multiple storage containers on milk composition and the results show that glass containers with solid lid would prevent degradation of some nutrients, while polyethylene bags exhibited the greatest fat and liposoluble vitamins loss (Arnold, 1995; Arslanoglu, et al., 2010). However, the glass containers pose a potential risk for operators and there is possibility of introduction of micro particles of glass into the HM. Therefore, hard plastic containers made of polypropylene or polycarbonate are preferable to glass containers.

Storage temperature also impacts milk composition and should be stored at a temperature that minimizes the lipolysis of fat. Bile salt-stimulated lipase (BSSL) actively converts TAGs to free fatty acids (FFA), monoacylglycerol (MG) and diacylglycerol (DG) at high temperature (4-37°C) (Bitman, et al., 1983). TAGs content was shown to decrease by 10% after storing the HM at -20°C for 5 months, while FFA, MG and DG increased by 10% 1.3% and 0.5%, respectively as compared to that in fresh HM (Berkow, et al., 1984). TAGs loss was more serious at the storage of 4°C and 37 °C for longer than 4 hours. However, fat lipolysis was not so pronounced at -70°C, because BSSL remains inactivity at this temperature (Bitman, et al., 1983; Groer, et al., 2014). Therefore, to ensure accurate fat content measurement for POPs analysis, milk samples should be stored at -70°C for long-term storage.

Freezing and thawing have been shown to impact HM composition as well. Thawing HM at a higher temperature results in greater fat loss and enhanced lipase activity compared to a lower temperature. For example, thawing of frozen milk in a 40°C water bath for 10 min will cause a 7.8% of fat loss, while relatively no lipolysis occurs during
thawing at 4°C (Berkow, et al., 1984; Vieira, et al., 2011). Thawing with heat or microwaves facilitates the lipolysis of fat and also enhances the adherence of fat to the sides of storage containers, leading to greater fat loss. Repeated freezing and thawing HM also increases fat lipolysis due to the enhanced BSSL activity. Results have shown that multiple freezing and thawing causes up to 20% fat loss (Bitman, et al., 1983).

Currently, it is not known in detail how HM samples have been stored and treated. However, it is clear that collection and handing process may collectively result in the reduction of fat content in HM, which thereby could potentially affect the POPs concentrations in HM as the POPs are reported based on HM fat. In conclusion, HM collected for POPs analysis should ideally be stored in a glass or hard plastic containers at -70°C. HM samples should then be thawed once at 4°C prior to fat content and POPs measurements.

### 1.4.3 Fat content of HM

HM contains an average fat content of 40 g/L and approximately 98% of the fat comprises of TAGs and the remainder consists of DG, FFA, phospholipids and sterols (Czank, et al., 2007). Fat is the second largest solid component and also the major source of energy for infants, contributing to over half of the total energy of the milk (Jensen, 1999). In addition to its nutritional role, the digestion products of fat such as FFA and MG actively protect infants against multiple pathogens, protozoa and viruses (Newburg, 2001). Fat, however, is the most variable component of HM, and changes with gestational age, circadian rhythm, within a feed, between feeds, with stage of lactation and between mothers as shown in Table 1.3.
Table 1.3 Factors affecting the fat content of HM

<table>
<thead>
<tr>
<th>Factor affecting the fat content of HM</th>
<th>Fat content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation age</td>
<td>Higher fat content in Preterm milk (32.4 ± 1.6 g/L) than Term milk (30.7 ± 2.1 g/L) (Lemons, et al., 1982).</td>
</tr>
<tr>
<td></td>
<td>Higher fat content during the Day (42.8 ± 9.1 g/L) and Evening (43.2 ± 9.1 g/L) than Morning (37.1 ± 10.1 g/L) and Night (37.2 ± 10.3 g/L) (Kent, et al., 2006).</td>
</tr>
<tr>
<td>Circadian rhythm</td>
<td>Higher fat content in Postfeed milk (56.0 ± 17.0 g/L) than Prefeed milk (32.0 ± 12.0 g/L) (Khan, et al., 2013).</td>
</tr>
<tr>
<td>Within a feed</td>
<td>Gradual increase of fat content from Colostrum (26.0 ± 14.8 g/L), Transitional milk (31.1 ± 15.3 g/L) to Mature milk (30.6 ± 12.9 g/L) and Wean milk (36.0 ± 8.0 g/L) (Garza, et al., 1983; Moltó-Puigmartí, et al., 2011).</td>
</tr>
<tr>
<td>Lactation stage</td>
<td></td>
</tr>
</tbody>
</table>

As discussed in Section 1.2.3, POPs are thought to be incorporated into either the core (TAGs) or the surface (Phospholipids) of the MFG during milk synthesis. Higher levels of POPs (e.g., total DDT, HCB, Lindane and Dieldrin) have been observed in postfeed samples than prefeed samples due to the significantly higher fat content in postfeed milk than prefeed milk (Stacey, et al., 1985). Therefore, to eliminate the high variation of fat content, the POPs concentration in HM should be normalized to the fat content of HM. This then allows for direct comparison of POPs residues in HM from various regions around the world and enables researchers to explore the current magnitude and trends of POPs.

Unfortunately, the correlation of POPs to fat varies across studies due to variable lipid measurement methods used by investigators. Several techniques including gravimetric method, esterified fatty acid (EFA) and creamatocrit method have been employed to measure the fat as shown in Table 1.4 (Stern & Shapiro, 1953; Bligh & Dyer, 1959;
Meier, et al., 2002). Each of the methods has inherent advantages and disadvantages. Based on the different principles of measurements, it is not unexpected to record differences of fat content measured according to the method employed. However, surprisingly no comprehensive comparison of these three methods has been carried out yet.

**Table 1.4** Comparison of different methods for HM fat measurement.

<table>
<thead>
<tr>
<th>Method for fat measurement</th>
<th>Procedures</th>
<th>HM volume</th>
<th>Usage of chemical</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric method</td>
<td>Measurement of fat mass after drying the liquid-liquid extraction of HM to constant weight (Bligh &amp; Dyer, 1959).</td>
<td>&gt;2 mL</td>
<td>Large consumption of organic solvent (&gt;50 mL)</td>
<td>1 sample/1 h</td>
</tr>
<tr>
<td>EFA</td>
<td>Conversion of fatty acid esters to hydroxamic acids, which forms red complexes with ferric chloride, followed by spectrometry analysis (Stern &amp; Shapiro, 1953).</td>
<td>&lt; 0.1 mL</td>
<td>&lt;0.1 mL</td>
<td>10 samples/1 h</td>
</tr>
<tr>
<td>Creamatocrit method</td>
<td>Calculation of the percentage of cream layer based on total milk column after centrifuge (Meier, et al., 2006).</td>
<td>&lt;0.1 mL</td>
<td>Reagent-free</td>
<td>60 samples/1 h</td>
</tr>
</tbody>
</table>

1.4.4 Maternal Characteristics

1.4.4.1 Age

Food consumption is the primary source of human exposure to POPs and many of these POPs such as majority of OCPs and some OPPs are resistant to metabolism in the body (Albers, et al., 1996). Therefore, POPs are not easily eliminated via either urine or feces.
As many of these POPs have long half-lives (particularly for OCPs) and lipophilic properties, they tend to accumulate in maternal adipose tissues (LaKind, et al., 2004). Therefore, with the high fat content of HM, POPs levels in HM are expected to increase with an increase of maternal age. In a large study of 511 primipara mothers in Germany, significant positive associations were observed between the maternal age (<24 to >38) and POPs levels (e.g., DDT, β-HCH and HCB) in HM (Zietz, et al., 2008). These relationships between maternal age and POPs levels in HM have been confirmed in multiple studies (Minh, et al., 2004; Chao, et al., 2006; Sudaryanto, et al., 2006; Ennaceur, et al., 2008). However, a small number of studies have had conflicting results that could be attributed to several factors, such as the small number of participants and narrow range of maternal age (Kunisue, et al., 2004b; Sudaryanto, et al., 2005). External factors, such as the continuous use of particular POPs over long periods in the studied area could also contribute to the non-significant association (Azeredo, et al., 2008).

1.4.4.2 Parity

Due to high lipophilic nature of POPs, breastfeeding is a major pathway for their elimination and reduction of maternal body burden. Adipose tissues provide around 50 - 60 % of the lipid in HM and POPs are mobilized with lipid mobilization from adipose tissue and transported into HM (Koppe, 1995). A mother is estimated to depurate around 10–30% of POPs during a 3-month period of breastfeeding, thus multiparous mothers could excrete more POPs through multiple pregnancies and lactations than primiparous mothers (Zietz, et al., 2008). Consequently, POPs levels in HM tend to decrease with increased maternal parity provided maternal POPs exposure is reduced or eliminated over time. Many studies have reported negative associations between POPs levels in HM and increased parity (Kunisue, et al., 2004b; Kunisue, et al., 2006;
Azeredo, et al., 2008; Ennaceur, et al., 2008; Zietz, et al., 2008). Azeredo et al. found that the total DDTs (sum of DDT, DDE and DDD) were 2.4-fold lower for the multiparae (346.7 ng/g fat) than the primiparae (838.7 ng/g fat), suggesting that the first breastfed infant is exposed to higher levels of POPs (Azeredo, et al., 2008). As with maternal age, however some studies failed to find the relationship between POPs levels and parity. This may be due to either the small number of sample for each parity group or recent exposure of POPs in the studied area (Behrooz, et al., 2009). In addition, certain cofactors, such as mother’s age (older primipara vs. younger multipara) and diet habits could also obscure the significant correlation typically not accounted for in the data analysis (Behrooz, et al., 2009; Bedi, et al., 2013).

1.4.4.3 Stage of lactation

POPs detected in HM primarily come from maternal body depots, such as hepatic lipid and adipose tissues (Koppe, 1995; Bergkvist, et al., 2008). Provided there was no recent exposure of POPs in the studied area, one would expect a decrease in POPs concentrations in HM as lactation processes. Many studies have observed the decline of POPs and decrease of maternal body burden during the course of lactation: from high levels in colostrum to lower levels in transitional milk and even lower levels in mature milk (Harris, et al., 2001; Ribas-Fitó, et al., 2005; Yu, et al., 2007). Yu et al. compared OCPs concentrations in colostrum (day 4 or 5) and mature milk (week 2), and reported that the average decline of HCB, \( p,p'\)-DDE and \( p,p'\)-DDT in colostrum to mature milk was 2%, 4% and 10%, respectively (Yu, et al., 2007). Ribas-Fitó et al. observed a greater decrease of HCB (31%), \( p,p'\)-DDE (22%) and PCBs (36%) in colostrum (day 3) to week three milk (Ribas-Fitó, et al., 2005). An even higher decrease of \( p,p'\)-DDE (62.7%) and \( p,p'\)-DDT (40%) was observed when week one milk was compared with
week five milk (Chávez-Almazán, et al., 2016). However, few of these studies have sampled beyond four months nor have followed the mothers over a longer time period (Chávez-Almazán, et al., 2016). This is due to multiple challenges, such as low milk production, cessation of breastfeeding and unexpected reasons (e.g., lost contacts and relocations), resulting in limited data to study HM POPs longer than 4 months despite WHO recommendations of exclusively breastfeeding for about 6 months with continuation of breastfeeding for 1 year and beyond (WHO, 2003).

Sampling time of the lactation stages is important for POPs measures in HM due to the compositional changes (e.g. fat) between the different lactation stages. WHO protocol recommends the sampling can be carried out between 2 weeks to 2 months after delivery. However, more studies will be required to evaluate the changes of POP levels in HM between various lactation stages and so that interpretations are relevant to the full breastfeeding period.

1.4.4.4 Body composition and body mass index (BMI)

Body composition (BC) measurements are able to differentiate between fat mass and fat-free mass, making infant BC more accurate in assessing body fat as compared to body mass index (BMI) (Bemben, et al., 1998). There are many techniques, such as dual-energy X-ray absorptiometry, computed tomography, magnetic resonance imaging and air displacement plethysmography, that have been used to measure body composition (Ellis, 2007). The limitations of these measurements include expensive and invasive, and even expose infants to low levels of radiation even though they provide more accurate measurements. On the other hand, anthropometric measurements such as bioelectrical impedance spectroscopy (BIS) and ultrasound are inexpensive and
noninvasive methods for measuring body composition. However, the accuracy of both BIS and ultrasound measurements are highly rely on the validated age-matched prediction equations (Lingwood, et al., 2012; Gridneva, et al., 2016). Maternal body composition or BMI can change dramatically during pregnancy and lactation, which may mobilize the POPs originally stored in the fat depots of the adipose tissues by lipolysis during weight loss (Hue, et al., 2006; Kim, et al., 2011a). Both human and animal studies have shown that the release of POPs from adipose tissues is an important source of blood POPs. Several studies have demonstrated a significant increase of POPs levels in blood with weight loss (Hue, et al., 2006; Kim, et al., 2011a). During weight loss, the POPs stored in maternal fat are mobilized and transferred to the milk via the maternal circulation as described in Section 1.3.2. A few studies have reported significant associations between POPs (e.g., total DDTs) in HM and maternal adipose tissues (Waliszewski, et al., 2001; Shen, et al., 2007). In a large cohort study of 355 Swedish mothers, a 0.61% median weight increase of the initial weight per week during pregnancy and 9.4% weight loss between delivery and sampling have been described (Lignell, et al., 2009). This study found significant positive associations between POPs (e.g., most PCBs and PCDD/Fs congeners) in HM and weight loss after delivery, and significant negative associations with weights gain during pregnancy. In addition, higher BMI correlated to lower levels of PCBs in HM (Wang, et al., 2008; Mannetje, et al., 2013). This is probably because higher BMI or weight gain allows for extra adipose tissues to store the POPs and “dilute” the maternal body burden of POPs. As with other factors influencing POPs levels in HM, some studies could not find a relationship between maternal BMI and POPs levels in HM (Someya, et al., 2010; Asante, et al., 2011; Devanathan, et al., 2012). Most of the studies attribute the insignificant results to insufficient sample size and limited BMI data. In addition, difference in diet and
metabolic activity of POPs between the mothers may also influence the relationship and have not been measured in these studies.

1.4.5 Other external factors

Dietary intake is the major source of human exposure to POPs especially for the more lipophilic and persistent OCPs (Behrooz, et al., 2009; Leng, et al., 2009; Zhou, et al., 2011; Shen, et al., 2012). It has been demonstrated that human exposure to POPs via the diet is much higher than other exposure routines, such as inhalation and dermal exposure (Behrooz, et al., 2009). In general, POPs levels in food are ranked as follows: cereals < vegetables < dairy products < meat < aquatic food. The role of dietary habits in the control of POPs in HM has been supported by many studies, which demonstrated relationships between the levels of these POPs and dietary habits. Tue et al. and Hassine et al. have associated higher POPs levels in HM, such as p, p’-DDE, β-HCH, HCB and some PCBs with frequent consumption of meat, dairy products and seafood (Tue, et al., 2010; Hassine, et al., 2012). Many studies have also correlated higher levels of OCPs, PCDD/Fs and PCB in HM with the amount and frequency of fish consumption (Kim, et al., 2011b; Sun, et al., 2011; Shen, et al., 2012; Lu, et al., 2015). As such studies that have not found relationships between maternal diet and HM POPs are likely lack of detailed and/or reliable information about the diet (Dahl, et al., 2010; Mishra & Sharma, 2011).

As discussed, many factors such as milk sampling and handing, HM fat content, maternal characteristics and other external factors can affect the POPs concentrations in HM. Various method have been developed to ensure accurate determination of POPs in HM.
1.5 Methods for the analysis of POPs in HM

Due to the trace levels of POPs in HM and the coexistence of numerous bioactive components, POPs cannot be analyzed directly without prior sample preparation. Thus, optimization of the sample preparation process is critical for successful POPs analysis, which is also the most time consuming and labor-intensive part of the analysis. As hypothesized in Section 1.3.3, POPs are either incorporated in the core (TAGs) or bounded to surface of MFG. An effective and efficient extraction system is required to mitigate the interference of the high fat and protein in HM. These components interfere with the chromatographic performance of the analytical instruments, such as gas chromatography electron capture detection (GC-ECD), gas chromatography tandem mass spectrometry (GC-MS/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS), limiting the accurate detection and quantitation of POPs (Schenck & Lehotay, 2000; Schenck, et al., 2002; Balnova, et al., 2007; Luzardo, et al., 2013). In order to minimize the co-extracted interferences, multiple sample cleanup steps are required to remove coextractives in the HM extract prior to the analytical step (Zhou, et al., 2012; Kim, et al., 2013; Abdallah & Harrad, 2014). At least three steps, including extraction, purification (cleanup) and detection, are required regardless of the choice of analytical method.

1.5.1 Sample extraction

To effectively extract POPs, disruption of the MFG membrane with strong solvents to expose the TAGs and POPs is required (Jensen, et al., 1997). Various analytical methods have been developed to extract POPs, and common sample extraction techniques include liquid liquid extraction (LLE) (Mahmoudi, et al., 2007; Rojas-Squella, et al., 2013), solid phase extraction (SPE) (Jaraczewska, et al., 2006; Tue, et
al., 2010), Soxhlet extraction (Abballe, et al., 2008; Zhou, et al., 2011) and pressurized liquid extraction (PLE) (Sun, et al., 2005). Each extraction technique has its limitations and shortcomings as detailed below.

1.5.1.1 Liquid liquid extraction (LLE)

LLE is one of the most commonly used techniques to extract POPs in HM due to its simplicity, good reproducibility and free of complex instrumentation. The extraction process transfers the POPs from HM to a water immiscible organic solvent. Methanol, ethanol or isopropanol is often added into the HM to break the MFG and expose the TAGs allowing for a more effective extraction (Jensen, et al., 1997; Song, et al., 2013). The optimal extraction solvent should allow maximum partitioning of the POPs and minimum matrix components into the organic phase. Various extraction solvents have been employed including non-polar solvents such as hexane (Zhou, et al., 2012), pentane (Çok, et al., 2012) and diethyl ether (Yao, et al., 2005), or a mixture of non-polar and polar solvents such as hexane/diethyl ether (Thomsen, et al., 2010), hexane/acetonitrile (Ennaceur, et al., 2008), hexane/dichloromethane (Erdoğru, et al., 2004), diethyl ether/petroleum (Costopoulou, et al., 2006; Mishra & Sharma, 2011), cyclohexane/acetone (Eggesbø, et al., 2009). The mixture of non-polar and polar solvents results in higher extraction efficiency compared to a single solvent, especially for the extraction of multiclass analytes with a wide range of polarities. Multiple extractions of the same sample are required to improve the extraction efficiency for LLE. Sonication, hand mixing and vortexing have been introduced to increase the contact between extraction solvents and HM in order to improve the extraction efficiency (Chao, et al., 2006; Azeredo, et al., 2008). LLE has been successfully used to extract various POPs including OCPs (Mishra & Sharma, 2011; Ennaceur & Driss,
2013; Kim, et al., 2013), OPPs (Rashmi Sanghi, 2003; Shivi Srivastava, 2011) and pyrethroids (Zehringer & Herrmann, 2001; Bouwman, et al., 2006).

However, the main drawbacks of LLE are the requirement of large volume of HM (10-100 mL) and the toxic solvents (~250 mL), along with laborious solvent evaporation. Further, multiple cleanup steps are often required to remove the coextractives due to poor selectivity of the extraction solvents for POPs.

1.5.1.2 Solid phase extraction (SPE)

SPE is another frequently used extraction technique, which involves the partitioning of analytes between the HM loaded on sorbents and the extraction solvent. The SPE usually comprises the following steps (Berrueta, et al., 1995; Zwir-Ferenc & M. Biziuk, 2006):

1) Activation/condition of sorbent
2) Sorption/retention of both analytes and matrix impurities onto the sorbent
3) Removal of impurities with the wash solvent
4) Desorption/elution with the target compounds

Diatomaceous earth is the most frequently used sorbent due to its unique physical and chemical characteristics, where various types of silanol groups of the diatomaceous earth can adsorb many HM polar matrix components (Al-Ghouti, et al., 2003; Malarvannnan, et al., 2009). Many commercially available SPE sorbents, such as C18, magnesium silicate and OASIS HLB, are also used to remove the matrix components of HM (Jaraczewska, et al., 2006; Azeredo, et al., 2008; Croes, et al., 2012). Selection of solvents is an important aspect of SPE, because the solvents should elute the POPs from the sorbents while retaining the matrix components of HM on the sorbent. Diethyl ether
and dichloromethane have been employed as the elution solvents (Sudaryanto, et al., 2006; Bedi, et al., 2013). SPE have been used in the extraction of POPs such as OCPs (Sudaryanto, et al., 2005; Sudaryanto, et al., 2006), OPPs (Bedi, et al., 2013), pyrethroids (Di Muccio, et al., 1997) and PCBs (Minh, et al., 2004; Tue, et al., 2010) with satisfactory recovery (60 – 120 %).

SPE requires less volume of HM (10-40 mL) and organic solvent (~ 100 mL) than LLE, and the SPE technique also has the potential for automation. However, SPE is still a complex method requiring a volume of HM that is still challenging for mothers with low milk production or preterm infants who are likely to be more vulnerable to the effect of POPs.

1.5.1.3 Soxhlet extraction

Soxhlet extraction, developed by Soxhlet in 1879, is often used to extract POPs from HM (Hui, et al., 2008; Dahl, et al., 2010). Soxhlet extraction maintains a favorable analyte concentration gradient between HM and extraction solvent, which allows for a continuous extraction of POPs (Luque de Castro & Priego-Capote, 2010; Zhou, et al., 2011). Soxhlet extraction is automated under high temperature in a continuous manner. The solubility and diffusion rates of POPs increase with the application of higher extraction temperature and longer extraction time (~ 24 hours), resulting in better extraction efficiency. Similar to LLE, a wide range of extraction solvents, such as hexane/acetone (Zhu, et al., 2009; Shi, et al., 2013), hexane/dichloromethane (Johnson-Restrepo, et al., 2007; Zhou, et al., 2011) and ethanol/toluene (Hui, et al., 2008; Dahl, et al., 2010), have been employed for the Soxhlet extraction to analyze POPs including OCPs, PCBs, polycyclic aromatic hydrocarbons (PAHs) and PCDD/Fs in HM.
Similar to LLE, the main drawbacks of Soxhlet extraction are the requirement of large volumes of HM (20-40 mL) and harmful organic solvents (>250 mL), and the long extraction time (~ 24 hours).

1.5.1.4 Pressurized liquid extraction (PLE)

PLE has been used for the extraction of POPs due to its superiority over other extraction techniques, such as LLE, SPE and Soxhlet, in terms of extraction time and consumption of HM and extraction solvent (Ramos, et al., 2002; She, et al., 2007; Lacorte & Guillamon, 2008). PLE comprises stainless-steel cells used to load the solid samples, where high temperature and pressure are introduced during the extraction. In order to obtain solid HM, HM samples are either freeze-dried or mixed with dispersive agents (e.g., diatomaceous earth and anhydrous sodium sulfate) before loading into the PLE cells (Sun, et al., 2005; She, et al., 2007; Lacorte & Guillamon, 2008). Optimization of PLE factors, such as static time and cycles, purge time and flush time, is necessary to obtain high extraction efficiency of targeted POPs in HM (Lacorte & Guillamon, 2008). In addition, a wide range of high temperature (70 - 110 °C) and pressure (1000 - 1500 psi) has been used for the determination of POPs residues in HM (She, et al., 2007; Weldon, et al., 2011; Toms, et al., 2012). The use of high temperature and pressure can decrease the viscosity and surface tension of HM, and increase the diffusion rates of POPs, which allows for better extraction efficiency and shorter extraction time. PLE only requires a much smaller volume of HM (~ 1 mL) and extraction solvent (10 - 20 mL) compared to other extraction techniques mentioned.

PLE, however is not suitable for the extraction of thermal-labile compounds due to thermal degradation resulting in low recovery (Mustafa & Turner, 2011).
1.5.2 Purification

Due to the limited selectivity of extraction solvent, a large amount of matrix components (e.g., fat, sugars and proteins) can be extracted simultaneously with POPs from HM. Since POPs are in low abundance in HM, the co-extracted matrix components can interfere and suppress the analytes, thus resulting in poor chromatographic performance and poor sensitivity. Therefore, a purification step is necessary for complex matrices like the HM extracted by LLE, SLE, Soxhlet and PLE (Minh, et al., 2004; Sun, et al., 2005; Mahmoudi, et al., 2007; Abballe, et al., 2008; Rojas-Squella, et al., 2013). In addition, this purification step can also reduce instrumental contamination and the deterioration of the chromatographic column, thus minimizing instrument downtime and extending the lifespan of the column. Several purification methods, such as sulfuric acid treatment, SPE cleanup and gel permeation chromatography (GPC) have been used for HM before the instrument analysis.

1.5.2.1 Sulfuric acid treatment

It is common to use concentrated sulfuric acid to remove the HM co-extractives such as fat and proteins, as these matrix components are hydrolyzed by the concentrated sulfuric acid to form water-soluble amino acids and free fatty acids (Çok, et al., 2003; Rojas-Squella, et al., 2013). This is followed by partition of POPs into the organic solvent, while leaving the hydrolyzed interferences in the aqueous layer. This is an attractive purification method as only a few milliliter (~ 2 mL) of sulfuric acid is used followed by centrifugation. Sulfuric acid treatment has been successfully employed for the determination of certain OCPs (Karakaya, et al.; Rojas-Squella, et al., 2013) and some PCBs congeners (Çok, et al., 2003; Szyrwińska & Lulek, 2007) with good recovery (75-125 %) and LOQ (0.05-17 ng/g fat).
Sulfuric acid treatment however is not suitable for the sulfuric acid-labile POPs such as dieldrin, endrin, endosulfan, heptachlor and dicofol, because these pesticides can be sulphonized completely when in contact with the sulfuric acid (Chung & Chen, 2011). Thus, this acid treatment method can only be applied to a limited number of POPs that are relatively stable to sulfuric acid.

### 1.5.2.2 Gel permeation chromatography (GPC)

GPC has been widely used in the purification step for removing HM matrix interferences, such as fat, proteins and other compounds of large molecular mass. GPC separation is based on the difference in molecular mass between POPs (< a few hundreds Dalton) and the co-extractives (600-1500 Dalton) in HM (Fujii, et al., 2011; Devanathan, et al., 2012). This is because the small mass of the analytes can be retained in the pores of the GPC stationary phase and will be washed out slowly, while the large molecular-mass interferences are not retained and are washed out rapidly. This method is very effective in the removal of fat, where Garrido Frenich et al. found that <1% of the initial fat remained in the food sample after the GPC cleanup (Garrido Frenich et al., 2006). The most commonly used stationary phase for GPC cleanup in HM is the Bio-Beads S-X3, which is a series of porous cross-linked polystyrene polymers with molecular-mass exclusion limit up to 2000 Dalton (Mathur, et al., 1980; Cajka & Hajšlová, 2003). Different ratio of dichloromethane/hexane have been chosen as mobile phase for eluting various POPs in HM, including OCPs, some PCBs congeners and pyrethroids (Di Muccio, et al., 1997; Kunisue, et al., 2004a; Kunisue, et al., 2006; Subramanian, et al., 2007; Someya, et al., 2010). Other eluting solvents, such as ethyl acetate/cyclohexane (Cajka & Hajšlová, 2003; Raab, et al., 2008), have also been used.
for the determination of OCPs, obtaining the recovery of 85-130% and LOD of 0.5-30 ng/g fat (Zhou, et al., 2011).

However, the fraction collected from GPC usually requires further purification with SPE columns to enhance cleanup efficiency (Hui, et al., 2008; Tue, et al., 2010; Zhou, et al., 2011).

1.5.2.3 SPE cleanup

SPE is another common purification method used to remove coextractives from HM. Both conventional glass columns packed with sorbent(s) and commercial cartridges have been used as mentioned in Section 1.5.1.2 (Hassine, et al., 2012; Ennaceur & Driss, 2013). Usually, the HM extracts needs to be concentrated and re-dissolved in small amount of solvent before loading it on the sorbent(s). Purification is based on that the unretained fat and other lipophilic impurities will be first eluted by non-polar solvent (e.g., hexane), leaving the POPs bind onto the sorbent (Zwir-Ferenc & M. Biziuk, 2006). Various solvents with different eluotropic strength will then be choose to elute the target analytes according to their polarity (Erdoğrul, et al., 2004; Schuhmacher, et al., 2009). The SPE cleanup method has been successfully applied to a wide range of POPs in HM including OCPs, OPPs, pyrethroids, PCBs, PCDD/Fs and PAHs with low LOD (0.001-0.09 ng/g fat) and a wide recovery range (25-130%) (Darnerud, et al., 2011; Weldon, et al., 2011; Çok, et al., 2012). SPE cleanup can be used directly after sample extraction (Bergkvist, et al., 2012; Croes, et al., 2012) or as an additional cleanup step after sulfuric acid treatment (Škrbić, et al., 2010; Mishra & Sharma, 2011) and GPC (Malarvannan, et al., 2009) to further remove the remaining coextractives. Some studies have employed multiple SPE cleanup columns simultaneously for the
purification in order to remove maximal matrix interferences in HM extract and have achieved better results (Costopoulou, et al., 2006; Kumar, et al., 2006; Çok, et al., 2012). However, the drawbacks of the SPE cleanup are the requirement of time-consuming optimization of the sorbent(s) and eluting solvent(s). Some POPs will also be lost during the cleanup step due to the strong adherence to the sorbent(s), resulting in low recovery (Panseri, et al., 2013).

1.5.3 QuEChERS

Even though the traditional extraction techniques, such as LLE, SLE, Soxhlet and PLE coupled with purification step such as sulfuric acid treatment, GPC and SPE cleanup, are still widely used for the extraction of POPs from HM, these techniques are time-, labor- and solvent-intensive procedures. To overcome these challenges, a “Quick, Easy, Cheap, Effective, Rugged and Safe” (QuEChERS) approach was developed for the analysis of pesticide residues in fruits and vegetables (Anastassiades & Lehotay, 2003). The original QuEChERS method involves the extraction with a small volume of acetonitrile (ACN) followed by addition of salts (MgSO₄ and NaCl) to induce partitioning. Samples are then subjected to a small amount of a mixture of MgSO₄ and primary secondary amine (PSA) to remove co-extractives before instrument analysis. Figure 1.7 shows the main steps in the QuEChERS method for the determination of pesticides in food matrices (Anastassiades & Lehotay, 2003; Wilkowska & Biziuk, 2011).
Figure 1.7 The general steps of the QuEChERS method for determining pesticides in food matrices (fruits and vegetables) (Anastassiades & Lehotay, 2003; Wilkowska & Biziuk, 2011).

Since then, the original QuEChERS method has been modified with either acetate buffers (AOAC Official method 2007.01) or citrate buffers (European Standard Method EN 15662) to cater for pH-dependent analytes (e.g., captan, folpet and pymetrozine), achieving good recoveries for the base-labile and acid-labile pesticides without impairing the cleanup performance (Lehotay, et al., 2005a; Anastassiades, et al., 2007).

Many studies have also modified the cleanup step with various sorbents, such as PSA, ocadecyl-bonded silica (C18) and graphitized carbon black (GCB) to remove matrix components like sugars, fatty acids, organic acids and some pigments (Anastassiades & Lehotay, 2003; Lehotay, et al., 2005b; Yang, et al., 2012; Chamkasem, et al., 2013; Li, et al., 2013; Zheng, et al., 2013).
The QuEChERS method simplifies the laborious and time-consuming extraction and cleanup steps, which significantly reduces the consumption of both sample and solvent. The QuEChERS approach is very flexible and it serves as a template for modification depending on the matrix composition and analyte properties. The QuEChERS method has been widely applied to a wide range of commodities, such as low fat food (e.g., fruits and vegetables) (Lesueur, et al., 2008; Kwon, et al., 2012; Kaewsuya, et al., 2013) and high fat food (e.g., olive oil, egg, meat, animal milk and avocado) (García-Reyes, et al., 2007; Selvi, et al., 2012; Chamkasem, et al., 2013; Li, et al., 2013). Much work has been carried out to determine pesticide residues (e.g., OCPs, OPPs, pyrethroids and carbamates) as well as other chemicals such as mycotoxins (Aguilera-Luiz, et al., 2011; Vaclavik, et al., 2013) and veterinary drugs (Kinsella, et al., 2009; Parab & Amritkar, 2012).

The QuEChERS method has had limited application to HM sample preparation. Where the method has been employed, the milk was pretreated “according to” the modified QuEChERS (AOAC Official method 2007.01 or European Standard Method EN 15662) without details of appropriate optimization and validation of method (Kinsella, et al., 2009; Brondi, et al., 2011; Jeong, et al., 2012).

1.5.4 Instrument analysis

Several instruments, such as gas chromatography coupled with various detectors have been used to separate and detect POPs in HM. However, accounting for the complexity of the HM extract and the low abundance of POPs in HM, it is essential to use the instrument techniques with the highest capability to provide high selectivity and
sensitivity for POPs detection and quantitation (Bergkvist, et al., 2012; Çok, et al., 2012; Croes, et al., 2012; Hassine, et al., 2012).

Gas chromatography coupled with electron capture detector (GC-ECD) is frequently used for multiresidue analysis of HM POPs. Due to its relatively low running cost, GC-ECD has often been employed to determine OCPs in HM, achieving satisfactory recovery (61-127%) and LOD (0.02-204 ng/g fat) (Johnson-Restrepo, et al., 2007; Ennaceur, et al., 2008). However, the drawbacks of the GC-ECD are its robustness and the lack of selectivity, where the detector is unable to differentiate between the chromatographic interference and POPs, and to identify co-eluting compounds (e.g., chiral isomers of DDT, DDE and DDD). Therefore, GC coupled to mass spectrometry (GC-MS) is often required to confirm the peak identification and quantification obtained by GC-ECD (Bedi, et al., 2013). In order to increase the selectivity and minimize the co-elution analytes, two capillary columns of different polarity are employed simultaneously to the GC-ECD for pesticide analysis in HM (Romanić & Krauthacker, 2006; Szyrwińska & Lulek, 2007). Excellent recovery (90–100%) and LOQ (1-5 ng/g fat) have been reported with two capillary columns for OCPs analysis in HM (Raab, et al., 2008). Similarly, two-dimensional gas chromatography (GC × GC) coupled to ECD has also been applied for the analysis of POPs in HM due to its high-resolution power from the use of two columns of different separation properties (Cajka & Hajšlová, 2003). However, GC × GC conditions are difficult to optimize and the cost of GC × GC-ECD is much higher compared to traditional GC-ECD, therefore there has been limited study reporting of HM POPs with GC × GC separation.

Compared to GC-ECD, mass spectrometry (MS) is considered to be a more selective and sensitive detection technique, which allows for simultaneous detection and
identification of co-eluting compounds. Therefore, GC-MS in electron ionization (EI) mode with selected ion monitoring (SIM) for the target analysis of multiclass POPs has become a routine monitoring tool. GC-MS has been used widely for the detection of multiclass POPs (e.g., OCPs, OPPs, pyrethroids and PCBs) in HM (Erdoğru, et al., 2004; Poon, et al., 2005; Weldon, et al., 2011). GC-MS has also been used to confirm the peak identification of pesticides that are determined and quantified by GC-ECD (Bouwman, et al., 2006; Mahmoudi, et al., 2007; Bedi, et al., 2013). However, for GC-MS with SIM analysis, the matrix interferences can have the same mass as the targeted analytes, which can cause false positive and inaccurate quantitation.

Recently, with the stricter regulations and lower POPs levels present in samples, GC coupled with tandem mass spectrometry (GC-MS/MS) (Feo, et al., 2012) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have been used to provide higher sensitivity and selectivity (Shi, et al., 2013; Hernik, et al., 2014). MS/MS with multiple reaction monitoring (MRM) acquisition method provides a higher selectivity and sensitivity. In MS/MS acquisition, the precursor ion of the targeted analyte is selected and energy is introduced to the precursor ion to induce fragmentation. The fragmentation pattern (product ions) is unique to the compound of interest, and then the specific product ions are selected for quantitative and qualitative analysis. MS/MS greatly enhances the detection sensitivity and selectivity as compared to GC-MS-SIM. The difference between SIM and MRM is shown in Figure 1.8. Multiresidue compounds can be monitored with GC-MS/MS using MRM within a single analysis (Holmes, et al., 2015). This is because both the precursor and product ions of the target analyte are monitored, which decreases the influence of HM matrix and other interference and increases the confidence for the identification of the detected compounds. Therefore, GC-MS/MS also has been widely used to determine the POPs
residues (e.g., pyrethroids) in HM, resulting in lower LOD (0.0023-1.1 ng/g fat) (Corcellas, et al., 2012; Feo, et al., 2012).

Figure 1.8 Principle of the two acquisition modes in mass spectrometry: (A) Selected ion monitoring (SIM) and (B) Multiple reaction monitoring (MRM).

However, despite all the advances in instrumentation, an appropriate sample preparation is still critical as the matrix co-extractives can contribute greatly to peak enhancement, which significantly impacts the accuracy and the quantitation of POPs (Poole, 2007).
1.6 Matrix-induced chromatographic response enhancement

1.6.1 Background

One of the main factors affecting pesticide analysis in GC analysis is the matrix-induced chromatographic response enhancement (matrix enhancement), which was first explained by Erney and co-workers (Erney, et al., 1993). Improved chromatographic peak shapes and increased peak responses for pesticides are observed in matrix samples as compared to that in matrix-free solvent. This phenomenon could be due to the presence of matrix components, such as lipids, waxes, pigments and other non-volatile compounds that are still present in the extract (Hajšlová & Zrostlíková, 2003; Poole, 2007). These components tend to deactivate and block the active sites (e.g. free silanols and metal ions) in the GC inlet and column, thus protecting the analytes from absorption by the active sites and thermal degradation by the hot inlet (Georgakopoulos, et al., 2007; Poole, 2007; Hercegová, et al., 2010; Kwon, et al., 2012). Whereas in the absence of matrix, the active sites can only interact with the analytes and a greater thermal stress is imposed on these analytes, thus significantly reducing the injected molecules eventually being detected (Anastassiades, et al., 2003; Maštovská, et al., 2005; Poole, 2007). This leads to a higher LOD, lower response and poorer chromatographic peak shape of the affected pesticides in pure solvent as compared to matrix standards (Poole, 2007).

1.6.2 Factors that affect the matrix enhancement

To date, many studies have looked at the matrix enhancement of various classes of pesticides in food matrices (e.g., tea, honey, rice wine, vegetables and fruits) (Sánchez-Brunete, et al., 2005; Georgakopoulos, et al., 2007; González-Rodríguez, et al., 2008;
Xu, et al., 2009; Hercegová, et al., 2010; Chen, et al., 2012; Li, et al., 2012). Matrix enhancement has been found to be influenced by many factors, such as the matrix types, chemical structure of analytes and the analyte concentration: matrix components ratio. The more non-volatile components (e.g., pigments and lipids) that remain in the sample extract, the greater the matrix enhancement will be (de Sousa, et al., 2012). Sugitate et al. found that the mean matrix enhancement of the pesticides was 129%, 146%, 171%, 191% and 225% in potato, soybean, orange, spinach and brown rice, respectively (Sugitate, et al., 2012). The matrix enhancement of brown rice is 18 times stronger than white rice due to the higher amount of lipid content in the brown rice (Kwon, et al., 2012). Thus, the influence of matrix enhancement needs to be carefully investigated for each matrix to ensure accurate quantification.

Various classes of pesticides can also exert different degrees of matrix enhancement. Pesticides with carboxyl (-COOH), hydroxyl (-OH), amino groups (R-NH-), carbamates (-O-CO-NH-), phosphate (-P=O), benzimidazole (C7H6N2-) and urea (-NH-CO-NH-) are the most susceptible type of analytes to the matrix enhancement (Anastassiades, et al., 2003; Poole, 2007). Stronger matrix enhancement is observed in analytes with multiple susceptible chemical groups than those with either less or no susceptible chemical group (Hajšlová, et al., 1998; Schenck & Lehotay, 2000).

The analyte concentration: matrix concentrations ratio also affects the degree of matrix enhancement (Schenck & Lehotay, 2000; Georgakopoulos, et al., 2007; Kwon, et al., 2012). Lower concentrations of analytes are subject to stronger matrix enhancement than the higher concentrations. This is because the active sites in the GC system are limited, which allows less of the analytes to be detected for the lower concentrations as
compared to the higher concentrations. Therefore, significantly distorted peaks occur for low concentration of analytes (Georgakopoulos, et al., 2007).

### 1.6.3 Matrix enhancement of POPs in HM

Although the existence of matrix enhancement is well recognized, incredibly no study on the matrix enhancement in HM has been carried out. Sugitate et al. had identified that MG and cholesterol were two of the matrix components that caused the matrix enhancement (Sugitate, et al., 2012). The fat fraction of HM consists of TAGs, FFA, cholesterol, phospholipids, DG and MG. All these components have chemical groups, which could bind to the active sites in the GC system and reduce the thermal stress on the thermal labile pesticides in the inlet (Sugitate, et al., 2012). Therefore, strong matrix enhancement of POPs in HM would be expected, making it critical to evaluate the variability and the extent of the matrix enhancement for each pesticide in HM in order to determine the uncertainty introduced by the matrix enhancement.

### 1.6.4 Methods used to eliminate/compensate for the matrix enhancement

Matrix enhancement results in overestimation of recoveries of analytes in matrix standards and the concentrations of pesticide residues in samples, when calibration standards in solvent are used to make the calculation (Maštovská, et al., 2005). In order to minimize the discrepancy, various methods have been employed to either compensate or eliminate the matrix enhancement such as matrix-matched calibration, isotopically labeled standards, reducing matrix components (either extensive cleanup or dilution of the final extract), using different injection techniques and introduction of analyte protectants (APs) (Erney, et al., 1997; Godula, et al., 1999; Schenck & Lehotay, 2000;
Chapter 1 Literature Review

Anastassiades, et al., 2003; Poole, 2007; Garrido Frenich, et al., 2009; Stahnke, et al., 2012).

1.6.4.1 Matrix-matched calibration

Matrix-matched calibration is the most widely used method to address the matrix enhancement in food by preparing the standards in blank extracts to provide the same matrix enhancement as the analyzed sample extracts (Erney, et al., 1997; Kwon, et al., 2012). Matrix-matched calibration method works well in compensating for the matrix enhancement. However, this method requires laborious and time-consuming preparation of matrix-matched standards for each sample type. The main drawback of this method is that the availability of appropriate blank (free of target analyte) may not be accomplished. The matrix-matched calibration is not suitable for POPs analysis in HM as the nonexistence of a POPs free HM sample.

1.6.4.2 Isotopically labeled standards

The use of isotopically labeled standards is regarded the best method to compensate for the matrix enhancement as these isotopically labeled standards are added directly to the HM sample (Malarvannan, et al., 2009; Mannetje, et al., 2013). The isotopically labeled standards represent the actual targeted analyte with identical retention time and also undergo the same analytical process. Therefore, isotopically labeled standards are used directly instead of the calibration standards in solvent for the quantification of the analytes in the sample. However, each labeled standard is only representative of the specific compound, making it impracticable for routine multiresidue analysis of POPs in HM. Further, commercially available isotopically labeled standards are limited and expensive (Hajšlová & Zrostlíková, 2003).
1.6.4.3 Reducing matrix components

Since the matrix enhancement is mainly induced by the matrix components remaining in the injected sample, several studies have tried either extensive cleanup or dilution of the final extract in order to reduce the matrix enhancement (Schenck & Lehotay, 2000; Balinova, et al., 2007; Stahnke, et al., 2012). With extensive cleanup, more matrix components are removed from the final extract, reducing the matrix enhancement. The dilution results indicate a linear correlation between matrix enhancement and the logarithm of matrix concentration, and the matrix enhancement is reduced up to 1000-fold with 10 levels of dilution of the final extract. But for stronger matrix enhancement, a higher dilution is required (Stahnke, et al., 2012). Dilution of matrix components in the final extract also dilutes the target analyte, which increases LOD of the target analyte. This is not desirable for HM where POPs concentrations are expected to be very low. Further, both the extensive cleanup and dilution of the final extract do not completely eliminate the matrix enhancement. In practice, complete removal of the matrix components is not possible and is labor- and time-intensive. Moreover, the presence of matrix components that do not interfere in the analysis can improve peak quality and decrease LOD (Anastassiades & Lehotay, 2003; Anastassiades, et al., 2003). Matrix enhancement should thereby be used rather than be eliminated.

1.6.4.4 Different injection techniques

It is well established that the matrix enhancement occurs mainly in the GC inlet using the classic splitless injection technique and is due to long residence time of the analyte in the hot inlet (Wylie, et al., 1991; Poole, 2007). Reduction of residence time in the hot inlet can therefore decrease absorption and degradation of the analyte. Injection
techniques that introduce larger amounts of the analyte into the inlet can also decrease the matrix enhancement due to the limited number of active sites in the GC system (Müller & Stan, 1990a; Godula, et al., 2001). Thus, instead of concentrating on reducing the matrix components, many studies have employed different injection techniques to minimize thermal degradation and to improve peak response of the analytes (Godula, et al., 1999; Godula, et al., 2001; Zrostliková, et al., 2001). Compared with other injection techniques, pulsed splitless injection rapidly sweeps the analyte from the inlet into the column, thus reducing interaction with the active sites on the inlet surface and thermal degradation of the analyte (Wylie, et al., 1991; Wylie, et al., 1992). Several studies in solvent have demonstrated that pulsed splitless injection could reduce adsorption and/or thermal degradation of thermal-labile compounds (e.g., Endrin and p,p'-DDE) (Wylie, et al., 1991; Wylie, et al., 1992). Recently, pulsed splitless injection with GC or GC/MS has been used for the analysis of contaminants in propofol (Peng, et al., 2013) and methamphetamine crystal (Sasaki & Makino, 2006), and higher peak response along with lower LOD (0.2-5.6 μg/g) have been observed in these studies. Despite the advantages of pulsed splitless injection, no study has investigated the performance of pulsed splitless injection on the matrix enhancement of POPs in HM.

### 1.6.4.5 Analyte protectants

The introduction of analyte protectants (APs) to both solvent and matrix standards can also effectively address the matrix enhancement (Erney & Poole, 1993; Anastassiades, et al., 2003; Wang, et al., 2011; Li, et al., 2012). APs are compounds with multiple hydroxyl/amino groups that strongly interact with the active sites in the GC system, reducing the thermal stress on the susceptible analyte and thereby protecting the analyte from degradation. The effect of APs were systematically examined by Anastassiades et
al. in fruits and vegetables and they found for the first time that a combination of APs (ethylglycerol, gulonolactone and sorbitol) was more effective in compensating for the matrix enhancement of entire pesticide elution range than using individual APs (Anastassiades, et al., 2003; Maštovská, et al., 2005). So far, many studies have successfully applied APs to normalize the matrix enhancement issues in various samples, such as tea (Li, et al., 2012), Chinese herbs (Wang, et al., 2011) and other vegetables and fruits (González-Rodríguez, et al., 2008; Kirchner, et al., 2008; González-Rodríguez, et al., 2009; Przybylski & Bonnet, 2009), and have achieved better recovery, more accurate quantitation, lower LOD and LOQ than that in pure solvent without the addition of APs.

González-Rodríguez et al. demonstrated that a mixture of ethylglycerol, gulonolactone and sorbitol at 10, 1 and 1 mg/mL in the final solutions could equalize the matrix enhancement in leafy products (chard, spinach and lettuce) (Anastassiades, et al., 2003; González-Rodríguez, et al., 2008). By preparing calibration standards in solvent with the APs, González-Rodríguez et al. also reported better recovery values and LOD for 11 fungicides in grapes and wines than in pure solvent without APs (González-Rodríguez, et al., 2009).

Kirchner et al. compared the performance of APs (ethylglycerol, gulonolactone and sorbitol) with matrix-matched standards for the analysis of 16 pesticides in apples and they found that the APs method provided comparable linearity, repeatability and LOQ to the matrix-matched standards (Kirchner, et al., 2008). Furthermore, lower signal-to-noise was observed in APs than the matrix-matched standards as greater matrix components in blank extracts were injected to the analytical instrument, which also demonstrate the superiority of using APs.
There are many advantages of using APs to address the matrix enhancement compared to other methods, such as lower LOD, more accurate quantitation, less maintenance of the GC system, inexpensive and convenient. However, no study has ever reported the performance of APs on the matrix enhancement of POPs in HM and applied the APs for the determination of POPs in HM.

Overall, there is clear evidence that HM confers enormous benefits to the breastfeeding dyad and to the social economy. However, there is widespread concern about the potential health effects of HM POPs on infant growth and development particularly during the early neonatal period. Surprising, no study has examined whether the HM POPs could adversely affect infant growth and development during the breastfeeding period, when the infant undergoes rapid growth and development of vital organs. In addition, few studies have investigated the long-term relationship between POPs in HM and lactation. In Western Australia, there is little information about the POPs in women’s milk since the most recent study conducted in 2002/2003, which is based on a single pooled HM samples (Mueller, et al., 2008). However, current methods for POPs analysis are time-, labor, solvent- and HM-intensive. Therefore, an accurate and effective analytical method is critically needed in order to accurately identify and determine the POPs in HM and to understand the current magnitude of POPs in Western Australia. The daily intake of HM POPs during breastfeeding has not yet been reported in Western Australia. This valuable information can provide insight into the actual daily dose of POPs delivered to the infant.
1.7 Aims

In order to understand the current POPs concentrations in HM of Western Australian women and evaluate the adverse health effects of the HM POPs on infant development, a simple and effective methodology based on QuEChERS and GC-MS/MS needs to be established for detection and quantitation of HM POPs. This validated method can then be applied to determine POPs in a cross-sectional and a longitudinal study throughout the first year of lactation carried out in Western Australia. A cross-sectional study will describe the current levels of POPs in HM and to compare the POPs concentrations observed in this study with other studies reported both in Australia and other countries. A longitudinal study can more accurately measure changes of HM POPs during the first year of lactation, and allow calculation of daily intake of HM POPs for the individual breastfed infant. It also allows investigation for the first time the potential adverse health effects of the HM POPs on infant growth and development throughout the first year of life. Furthermore, the relationship between maternal characteristics on HM POPs will also be examined in the longitudinal study to better understand the factors that can affect POPs concentrations.

The specific aims of this thesis were:

1. To compare HM fat measurement methods in order to accurately determine the fat content of HM. Creamatocrit and EFA will be systematically compared with the gravimetric method (Reference method) for fat measurement in HM of varying fat content. (Chapter 2)

2. To investigate and measure the extent and variability of the matrix enhancement of 88 POPs in HM with GC-MS/MS. Comparison of the peak quality and evaluation of the matrix enhancement of these POPs in pure solvent and HM extract will be carried out. (Chapter 3)
3. To investigate the performance of APs, pulsed splitless injection and large volume injection to address the matrix enhancement of 88 POPs in HM. (Chapter 3)

4. To systematically develop, optimize and validate the QuEChERS method for HM preparation. This validated QuEChERS method will be employed to detect and quantify 88 POPs in HM. (Chapter 4)

5. To carry out a cross-sectional study (n=40) in Western Australian mothers to compare with those reported in Australia and other countries in order to understand current magnitude of POPs levels in HM in Western Australian. (Chapter 5)

6. To carry out a longitudinal study (n=16) to overcome the limitations of the cross-sectional study. Accurate daily intake of HM POPs by infant through the first year of lactation will be measured. Further investigation of the potential effects of HM POPs on infant growth and development over the first year of life will be explored. Associations between maternal characteristics on HM POPs will also be investigated. (Chapter 6)
Chapter 2 Comparison of gravimetric, creamatocrit and esterified fatty acid methods for determination of total fat content in human milk

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2.1 Abstract

The gravimetric method is considered the gold standard for measuring the fat content of human milk (HM). However, it is labor intensive and requires large volumes of HM. Other methods such as creamatocrit and esterified fatty acid assay (EFA) have also been used widely in fat analysis. However, these methods have not been compared concurrently with the gravimetric method. Comparison of the three methods was conducted with HM of varying fat content. Correlations between these methods were high ($r^2=0.99$). Statistical differences ($P<0.001$) were observed in the overall fat measurements and within each group (low, medium and high fat milk) using the three methods. Overall, stronger correlation with lower mean (4.73 g/L) and percentage differences (5.16%) was observed with the creamatocrit method than the EFA method.
when compared to the gravimetric method. Furthermore, the ease of operation and real-
time analysis make the creamatocrit method preferable.

### 2.2 Introduction

Human milk (HM) contains a variety of nutrients and immunologically active
components that are required for both optimal growth and the development of a
newborn’s immune system against an array of diseases and infections (LaKind, et al.,
2004). Milk fat is the major source of energy for infants, contributing over half of the
total energy of HM (Hamosh, et al., 1985). However, fat is the most variable nutritional
component in HM, changing substantially within and between feeds, between breasts,
and amongst mothers as well as with stage of lactation (Kent, et al., 2006; Czank, et al.,
2009). Despite the importance of milk fat for the rapidly growing human infant and the
multiple methods of analysis of fat content available, no extensive comparative studies
have been conducted on fat analysis of HM.

It is standard in biological fluids such as urine, to use urinary creatinine as
normalization in the comparison of studies between different populations. In HM,
lipophilic compounds, such as persistent organic pollutants (POPs), bind to the central
core of the milk fat globules. Therefore, when making comparisons, values should be
normalized to the fat content of HM. For example, when estimating POPs dosage,
precise measurement of fat will reflect more accurately the maternal-infant environment
and associated risks. Unfortunately the vast array of components in milk, such as
proteins, hydrophilic components, micellar casein and fat globules, which are dispersed
in the liquid colloid, make accurate measurement of fat challenging.
Several techniques have been employed to measure fat in milk. The gravimetric or reference method is based on the measurement of fat mass in a sample after liquid-liquid extraction of the milk fat (Bligh & Dyer, 1959). The esterified fatty acid (EFA) assay has been adapted from analysis of total fatty acids in blood and works on the principle of breaking the ester linkages (–COO-R-) in lipid species such as triacylglycerols, which constitute approximately 98% of the fat in milk, followed by spectrometry analysis (Stern & Shapiro, 1953; Jensen, 1995). Creamatocrit method has been developed as a rapid and feasible tool for use in the clinical setting (Lucas, et al., 1978; Meier, et al., 2006). Whole milk is centrifuged and measurements are made of the skim milk and cream layer to calculate the cream content of the milk.

While differences in recorded fat content resulting from the detected methods employed are not unexpected, these differences have not been critically examined. Differences in measurement might lead to errors in the calculation of the caloric content in milk. This is important in situations where infant growth is paramount, such as in preterm infants. Similarly, estimation of fat-soluble contaminant is not possible without determination of fat content. In this study, we compared three methods, specifically the gravimetric, EFA and creamatocrit methods for the analysis of fat content in HM.

### 2.3 Material and Methods

#### 2.3.1 Sample

This study was approved by the Ethics Committee of The University of Western Australia. Term milk was collected from one mother and stored at -20°C in a plastic container. The term milk was thawed at 37°C for one hour and was divided into four 100 mL aliquots. The first 100-mL aliquot was sub-divided into aliquots of 5 mL.
(medium fat content, n=20). 50 mL from the second 100-mL aliquot was diluted 2-fold with 50 mL of double deionized (DDI) water. It was then divided into aliquots of 5 mL (low fat content, n=20). The remaining two 100-mL aliquots were centrifuged at 750 x g for 5 min (Eppendorf 58410R, Hamburg, Germany) and 50 mL of skim milk was removed from each of the sample. The remaining content (containing fat and skim milk) in each tube were combined and divided into 5 mL aliquots (high fat content, n=20).

A total of 60 samples were prepared and stored at -20°C. Prior to analysis, each 5 mL aliquot was thawed at 37°C for 30 min and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 s.

2.3.2 Reagents and Standards

Chloroform and methanol were purchased from Chem-Supply (Gillman, SA, Australia). Absolute ethanol was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (32%, w/w) was purchased from Scharlau (Barcelona, Spain). Hydroxylamine hydrochloride, sodium hydroxide, trichloroacetic acid, triolein standard stock solution, hydrochloric acid and ferric chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). DDI water used in the experiments was generated by Ibis Technology Ultrapure Water purification system (Perth, WA, Australia).

2.4 Determination of total fat content in HM

2.4.1 Gravimetric method (FOL extraction)

The gravimetric method used is based on the modified method of Folch et al. (Folch, et al., 1957). Briefly, 2 mL of HM was mixed with 40 mL of chloroform/methanol (2:1, v/v). The mixture was homogenized thoroughly and centrifuged at 1509 x g for 10 min.
The clear homogenate was transferred to a separating funnel. Subsequently, 7.8 mL of water was mixed with the homogenate and allowed to stand until phase separation was observed. The proportion of water to homogenate was 2:10 (v/v) to ensure that no interfacial fluff was formed in the biphasic system obtained. The lipid layer (lower layer) was collected. The aqueous layer (top layer) was rinsed with chloroform/methanol mixture (2:1, v/v) and was allowed to stand until phase separation. The ratio between the aqueous layer and the rinsing solvent was around 1:1 (v/v) to prevent interfacial fluff. The lipid layer was collected and combined with the previous collection. The combined lipid fraction was then evaporated to dryness in a rotary evaporator and dried to constant weight under vacuum and the lipid content was determined gravimetrically.

2.4.2 Esterified fatty acids (EFA)

The EFA method used is modified based on the method of Stern and Sharpiro (Stern & Shapiro, 1953; Atwood & Hartmann, 1992). Samples (2.5 µL) and standards (triolein, 0-200 mM, 2.5 µL) were pipetted in duplicate into a deep-well plate followed by addition of 400 µL of absolute ethanol and mixed well. Then, 100 µL of 2 M hydroxylamine hydrochloride and 100 µL of 3.5 M sodium hydroxide were added, mixed well and allowed to stand for 20 min at room temperature. The samples were acidified by addition of 100 µL of 4.08 M HCl. Color change from dark yellow to brown was observed after the addition of 100 µL of a ferric chloride/trichloroacetic acid solution (3.75 g TCA in 5 ml 0.37 M FeCl₃). Due to the hygroscopic nature of hydroxylamine hydrochloride and FeCl₃-TCA, they were freshly prepared. The mixture was thoroughly mixed and duplicate aliquots of 100 µL were pipetted into a flat bottom
96-well plate. The plate was then analyzed using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 540 nm.

### 2.4.3 Creamatocrit

The creamatocrit method used is based on the modified method of Lucas et al. (Lucas, et al., 1978). The milk sample was drawn into two 75 μL micro-hematocrit capillary tubes (Kimble, TN, USA) and one end of the capillary was sealed with critocaps (Kimble, TN, USA). The tubes were then centrifuged in a micro-hematocrit centrifuge (BHG Hermle, USA) at 12 000 x g for 10 min. The creamatocrit (%) was measured using Creamatocrit Plus™ (Medela AG, Switzerland), which was based on the ratio of cream layer and total milk volume. The creamatocrit (%) was converted to fat content (g/L) based on the following formula: fat content = 3.968 + (5.917 x creamatocrit (%)) (Meier, et al., 2006).

### 2.4.4 Data analysis

Statistical analysis was carried out using R 3.2.0 using the package nlme for the linear mixed models (Pinheiro, et al., 2009) and the package Lattice for Bland-Altman plots (Sarkar, 2009). Linear mixed effects were used to determine the relationship between the fat content and the three different methods. The fixed effect factor was the method. The random effects were the group (low, medium and high fat) and individual aliquot. Differences were considered to be significant if $P<0.05$. Results were expressed as mean and standard deviation (SD). Bland-Altman plots were used to investigate if there were systematic effects of the measured fat content on the difference between the methods.


2.5 Results

Overall, the fat content measured was statistically different ($P<0.001$) between the different analytical methods and also within each of the sample groups (low, medium and high fat). However, excellent correlations ($r^2>0.99$) were found between the methods (Figure 2.1). The fat content measured by the gravimetric method was significantly higher ($P<0.001$) than that measured by both EFA and the creamatocrit methods in all the three different sample groups of low, medium and high fat milk (Table 2.1).

The intra-assay precision in each sample group (low, medium and high fat) within each method was also tested. The gravimetric method gave a mean coefficient of variation (CV) of 1.74%. The largest CV was observed in medium fat milk (2.89%) followed by low (1.40%) and high fat milk (0.94%). The EFA method gave a mean CV of 5.71% with the highest CV observed in low fat milk (10.95%) followed by medium (4.34%) and high fat milk (1.84%). The creamatocrit method followed a similar pattern to the EFA method with a mean CV of 3.94% and the highest CV observed in low fat milk (6.58%) followed by medium (3.48%) and high fat milk (1.75%).

When comparing the three methods, the largest mean difference was observed between the gravimetric and the EFA methods in low, medium and high fat milk (Table 2.1). A smaller difference was observed between the gravimetric and the creamatocrit methods in low, medium and high fat milk (Table 2.1).

The box plots (Figure 2.2) show the percentage mean difference in low, medium and high fat milk in gravimetric-EFA methods (36.45%, 19.13% and 8.49%, respectively), gravimetric-creamatocrit methods (6.68%, 4.58% and 4.26% respectively) and creamatocrit-EFA methods (27.80%, 11.01% and 1.95% respectively). The correlations
between the methods were: gravimetric-EFA ($r^2 = 0.994$); gravimetric-creamatocrit ($r^2 = 0.995$) and EFA-creamatocrit ($r^2 = 0.988$). The Bland-Altman plots showed that the differences between these methods were within 2SD (Figure 2.3).

**Figure 2.1** Linear correlation between the fat content (g/L) measured by gravimetric and EFA methods (A), gravimetric and crematocrit methods (B), and EFA and crematocrit methods (C).
Table 2.1 Fat content of the sample groups measured by gravimetric, EFA and creamatocrit.

<table>
<thead>
<tr>
<th>Fat content (g/L)</th>
<th>Sample size</th>
<th>Mean fat content (g/L) (SD)</th>
<th>Mean difference a</th>
<th>Mean difference b</th>
<th>Mean difference c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gravimetric</td>
<td>EFA</td>
<td>Creamatocrit</td>
<td></td>
</tr>
<tr>
<td>Low fat milk</td>
<td>20</td>
<td>29.49 (0.42)</td>
<td>18.75 (2.11)</td>
<td>26.11 (1.76)</td>
<td>10.73 (1.89)\textsuperscript{d}</td>
</tr>
<tr>
<td>Medium fat milk</td>
<td>20</td>
<td>53.80 (1.60)</td>
<td>43.47 (1.94)</td>
<td>48.94 (1.75)</td>
<td>10.32 (2.52)\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.10-52.70</td>
<td>40.65-47.62</td>
<td>45.60-52.40</td>
<td></td>
</tr>
<tr>
<td>High fat milk</td>
<td>20</td>
<td>89.76 (0.87)</td>
<td>82.14 (1.55)</td>
<td>83.81 (1.50)</td>
<td>7.62 (1.71)\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.84-90.90</td>
<td>79.71-85.52</td>
<td>81.70-87.40</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean difference of fat content measured by gravimetric and EFA presented as mean (SD).

\textsuperscript{b} Mean difference of fat content measured by gravimetric and creamatocrit presented as mean (SD).

\textsuperscript{c} Mean difference of fat content measured by creamatocrit and EFA presented as mean (SD).

\textsuperscript{d} P < 0.001
Figure 2.2 Differences of fat content (%) between methods for each sample group: gravimetric and EFA (A), gravimetric and creamatocrit (B) and EFA and creamatocrit (C).
2.6 Discussion

In this study, we observed excellent correlations between the reference gravimetric method for measuring milk fat content and both EFA and creamatocrit methods. However, the linear mixed model analysis demonstrated a significant difference between these three methods. These differences can be partly explained by the differing principles of each of the methods. Since the gravimetric method has been designated the reference method for measuring fat in HM, we have compared both the EFA and the creamatocrit methods, which are simpler techniques for fat measurement, to the gravimetric method.

Despite a strong correlation ($r^2 = 0.994$; Figure 2.1A) between the EFA and gravimetric methods, which is consistent with previous literature (Atwood & Hartmann, 1992), we found that the EFA method tended to underestimate the fat content by 7.62 to 10.73 g/L (Table 2.1) with the percentage difference of 8.49 to 36.45% compared to the gravimetric method (Figure 2.2A). Underestimation of fat content in sow milk was also observed by Atwood & Hartmann (Atwood & Hartmann, 1992). Underestimation of fat content may be due to the fundamental principles underpinning the EFA method. The EFA method disrupts the ester linkages of the triacylglycerols, which account for 98% of the total fat in milk, whereas the gravimetric method partitions the fat and measures its mass, essentially measuring total fat. Therefore, we should observe small differences (0.59 to 1.79 g/L, based on the measured value using the gravimetric method in this study) between these two methods. However in reality, absolute reaction of the triacylglycerols in the EFA method is impossible leading to further underestimation (Casadio, et al., 2010). On the other hand, the gravimetric method could potentially overestimate the fat as the partitioning step is selective toward all hydrophobic and hydrophilic compounds in milk and is not specific to only the lipid compounds.
Cerbulis and Custer have reported that casein is also soluble in the extraction solvent (chloroform/methanol), thus further accentuating the difference between the gravimetric and the EFA methods (Cerbulis & Custer, 1967).

The creamatocrit and the gravimetric methods showed an excellent correlation (Figure 2.1B). However, the creamatocrit underestimated the fat content by 3.38 to 5.96 g/L (Table 2.1) with a smaller difference of 4.24 to 6.68% compared to the gravimetric method (Figure 2.2B). As the creamatocrit uses centrifugal force to separate the skim and cream layer, some fat is retained in the skim layer (Kent, et al., 2006; Czank, et al., 2009). Therefore, it is expected that the measured value by the creamatocrit would be lower compared to the gravimetric method. As with any sample handling, the milk fat globule can also undergo degradation into free fatty acids, which occupy less space than cream (Lucas, et al., 1978), compounding the underestimation of fat by the creamatocrit method. Consistent with our finding, Ganguli et al. also observed that the creamatocrit method underestimated the fat content (by about 2%) in sow and rat milk whilst providing good correlation (Table 2.2) when compared to the gravimetric method (Ganguli, et al., 1969). A recent paper compared the creamatocrit method with mid infrared spectroscopy and concluded that the creamatocrit overestimated the fat content (O’Neill, et al., 2013). However, methodological concerns exist regarding centrifugation of the HM samples. The samples were centrifuged for 15 min at 1315 x g instead of the standard 15 min at 12000 x g used in all other studies (Fleet & Linzell, 1964; Lucas, et al., 1978; Meier, et al., 2002). This would result in a lower compaction of the cream layer, and would account for the higher creamatocrit values observed and therefore higher fat content calculated as compared to the mid infrared spectroscopy.
When the creatocrit method was further compared with the EFA method, there was an excellent correlation ($r^2=0.988$, Figure 2.1C). However, compared with the creatocrit method the EFA method underestimated the fat content by 1.67 to 7.36 g/L (Table 2.1) with the percentage difference of 1.99 to 28.19% (Figure 2.2C). The underestimation of the fat content by the EFA method could again be due to differences in the principles of the two methods. Our findings are similar to that observed by Meier et al. (Meier, et al., 2006), where they also reported a mean difference of 6.80 g/L and good correlation ($r^2=0.95$) between the creatocrit and the EFA.

The relationships were further analysed by Bland-Altman plots, which showed no systematic error in the relationship between the fat content measured by gravimetric-EFA (Figure 2.3A), gravimetric-creatocrit (Figure 2.3B) and creatocrit-EFA methods (Figure 2.3C).

Each of the methods investigated here have inherent advantages and disadvantages. The gravimetric method requires the largest volume of milk (>2 mL) among the methods compared, and is also labor-, time- and solvent-intensive. Whilst this method is precise (CV=1.7%), due to its complicated procedures more than 15 hours are required to conduct a normal batch of 15 samples. The EFA method on the other hand only employs a small amount of milk (<0.1 mL) and chemicals (<0.1 mL). However, the EFA is also labor-intensive requiring at least 1.5 hour to process a normal batch of 15 samples. Among the three methods investigated, the EFA has the lowest precision (CV = 5.7%).
Figure 2.3 Bland-Altman plots showing the differences between gravimetric and EFA methods (A), gravimetric and creamatocrit methods (B), and EFA and creamatocrit methods (C). The dotted line is the mean and the solid lines are ± 2SD of the mean.
Table 2.2 Comparison of correlation coefficient by gravimetric, EFA and creamatocrit methods with previous studies.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sample</th>
<th>Sample size</th>
<th>Correlation coefficient ($r^2$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric-EFA</td>
<td>Human milk</td>
<td>60</td>
<td>0.99</td>
<td>Current study</td>
</tr>
<tr>
<td>Gravimetric-EFA</td>
<td>Sow milk</td>
<td>33</td>
<td>0.99</td>
<td>(Atwood &amp; Hartmann, 1992)</td>
</tr>
<tr>
<td>Gravimetric-creamatocrit</td>
<td>Human milk</td>
<td>60</td>
<td>0.99</td>
<td>Current study</td>
</tr>
<tr>
<td>Gravimetric-creamatocrit</td>
<td>Cow milk</td>
<td>16</td>
<td>0.99</td>
<td>(Ganguli, et al., 1969)</td>
</tr>
<tr>
<td>Gravimetric-creamatocrit</td>
<td>Rat milk</td>
<td>4</td>
<td>0.99</td>
<td>(Ganguli, et al., 1969)</td>
</tr>
<tr>
<td>Creamatocrit-EFA</td>
<td>Human milk</td>
<td>60</td>
<td>0.99</td>
<td>Current study</td>
</tr>
<tr>
<td>Creamatocrit-EFA</td>
<td>Human milk</td>
<td>37</td>
<td>0.95</td>
<td>(Meier, Engstrom, Zuleger, Motykowski, Vasan, Meier, et al., 2006)</td>
</tr>
</tbody>
</table>
Both the gravimetric and EFA methods require the use of laboratory equipment, and are not suitable for real-time analysis in a hospital setting. The creamatocrit method on the other hand is a reagent-free technique requiring only a small amount of milk (<0.1 mL).

In this study, the creamatocrit method has good precision (CV= 3.9%) and the highest throughput (15 min for a normal batch of 15 samples). Besides being an inexpensive analysis, it also does not require a skilled operator. Therefore, real-time analysis can be performed by clinicians in the hospital. Furthermore, this study has shown closer correlation of the creamatocrit measured fat content with the reference method (gravimetric) than the EFA method.

2.7 Conclusions

This is the first study that has systematically compared three different methods of measuring fat content in human milk: gravimetric, EFA and creamatocrit. Both the EFA and creamatocrit methods showed excellent correlation with the gravimetric method. There were differences between methods in measured fat content, which can be explained by the different principles of the methods. The fat content measured by the creamatocrit method had values closer to that of the gravimetric method than the EFA method. Significant underestimation using the EFA method could be clinically relevant for low fat milk. The choice of method should take into account whether the measurement is performed in the laboratory or clinical setting and the requirements for accuracy and precision.
2.8 Acknowledgements

J. Du sincerely appreciates the International Postgraduate Research Scholarships (IPRS), Australian Postgraduate Award (APA) and UWA Safety-Net Top-up Scholarships for financial support. J. Du also wishes to thank G.Z. Zhao for his active help and cooperation. This work was also supported by an unrestricted research grant from Medela AG.
Chapter 3 Evaluation of matrix enhancement of 88 pesticides in HM and comparison of various methods to address the matrix enhancement

This chapter describes the matrix enhancement of 88 pesticides in HM and compares the performance of APs, pulsed splitless and large volume injection to address the matrix enhancement. The performance of APs mixture (ethylglycerol, gulonolactone and D-sorbitol at 20, 2 and 2 mg/mL, respectively) with high pressure pulse (30 psi) and larger volume injection (2 µL) was found to be an effective method to address the matrix enhancement of the pesticides in HM. This chapter includes three parts:


3.2 Analyte protectant compensation for matrix-induced chromatographic peak enhancement in the gas chromatography tandem mass spectrometry analysis of 88 pesticides in human milk.

3.3 Evaluation of pulsed splitless injection to address matrix enhancement with and without analyte protectants in the analysis of 88 pesticides in human milk.
3.1 An evaluation of matrix-induced chromatographic enhancement effect of 88 pesticides in HM using gas chromatography tandem mass spectrometry

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3.1.1 Abstract

The matrix-induced chromatographic enhancement effect of 88 pesticides, including OCPs, OPPs, carbamates, pyrethroids, fungicides and other pesticides was evaluated in HM. QuEChERS method was used to prepare the HM. A concentration range (10-100 ng/mL) of the 88 pesticides was prepared in pure solvent and in HM extract and analyzed using GC-MS/MS. 99% of the investigated pesticides in pure solvent were found to suffer from thermal degradation and absorption, causing distorted and broadened peaks. In contrast, the same concentration of pesticides in HM exhibited strong peak enhancement and improved peak shape. The matrix components in HM provided strong protection for these susceptible analytes. The influence of matrix
Chapter 3.1 Evaluation of Matrix Enhancement

enhancement varied substantially across the pesticide groups, where OCPs were less affected by matrix enhancement than other pesticides groups. The pesticides with susceptible chemical groups were more likely to be affected by the matrix enhancement. More significant peak enhancement was observed in pesticides at lower concentration. In this study, we found that the matrix enhancement of the pesticides in HM was stronger than other matrices. This paper provided the fundamental information about the variability and intensity of the matrix enhancement of selected pesticides in HM.

3.1.2 Introduction

Human milk (HM) is a product of evolution that specifically meets the need of human infants. It contains a variety of nutrients and bioactive compounds that are required for optimal infant growth and the development of newborn’s immune system to protect against a diverse array of diseases and infections (Newburg, 2001; LaKind, et al., 2004; WHO, 2007b). However, with the extensive use of pesticides in agriculture for pest control, these pesticides could enter the human body and be detected in HM (Kowalski, et al., 2007). The presence of pesticides in HM is of great concern because of the potential health effects to the breastfed infant, such as neurotoxicity, delayed development, immune deficiency, abnormal behavior and growth retardation (Pratt, et al., 2012; Eskenazi, et al., 2013). The pesticide concentrations in HM can be used as unique biomarkers to reflect pesticides in environment and to assess human exposure. Over the years, sophisticated analytical methods have been developed to analyze pesticide residues with a wide range of physic-chemical properties within a single run. However, the ability to monitor and quantitate these pesticides at trace levels is challenging due to distorted peak shapes and low signal responses of standards in solvent (Needham & Wang, 2002; Hajšlová & Zrostliková, 2003).
One of the main factors affecting pesticide analysis using gas chromatography (GC) is the “matrix-induced chromatographic response enhancement effect (matrix effect)”, which was first described by Erney and his co-workers (Erney, et al., 1993). Improved gas chromatographic peak shapes and increased detector responses for pesticides are observed in matrix samples as compared to the same concentrations of analytes in matrix-free solvent. This phenomenon could be due to the presence of matrix components, such as lipids, waxes, pigments and other non-volatile compounds that are still present in the extract (Hajšlová & Zrostlíková, 2003; Poole, 2007). These components tend to deactivate and block the active sites (e.g. free silanols and metal ions) in the GC inlet and column, thus protecting the analytes from absorption by the active sites and thermal degradation caused by the hot inlet (Georgakopoulos, et al., 2007; Poole, 2007; Hercegová, et al., 2010; Kwon, et al., 2012). Whereas in the absence of matrix, the active sites can only interact with the analytes and a more serious thermal stress is imposed on these analytes, thus significantly reducing the injected molecules eventually being detected (Anastassiades, et al., 2003; Maštovská, et al., 2005; Poole, 2007). Therefore, poorer chromatographic peak shapes with lower responses, resulting in higher detection limit are generally observed in pure solvent as compared to that in matrices. This can result in the overestimation of pesticides concentrations in real samples, when standards in solvent are used in the quantification.

To date, many studies have looked at the influence of matrix effect of multiresidue pesticides in various food matrices, such as tea (Li, et al., 2012), honey (Sánchez-Brunete, et al., 2005), Chinese herbs (Wang, et al., 2011), rice wine (Chen, et al., 2012), vegetables and fruits (Maštovská, et al., 2005; González-Rodríguez, et al., 2008; Xu, et al., 2009). Matrix effect was found to be affected by the type of matrices. Sugitate et al. found that the mean matrix effect of pesticides was 129%; 146%; 171%; 191% and
225% in potato, soybean, orange, spinach and brown rice, respectively (Sugitate, et al., 2012). The matrix effect of brown rice was 18 times more than white rice due to the higher lipid content in brown rice (Kwon, et al., 2012). More distinct matrix effect is also observed when higher amounts of non-volatile components, such as pigments and lipids, are present in the extract (de Sousa, et al., 2012). The degree of matrix enhancement is also influenced by the chemical structure of the analytes. Pesticides with carboxyl (-COOH), hydroxyl (-OH), amino groups (R-NH-), carbamates (-O-CO-NH-), phosphate (-P=O), benzimidazole (C7H6N2-) and urea (-NH-CO-NH-) are the most susceptible types of analytes to this effect (Anastassiades, et al., 2003; Poole, 2007).

Although the existence of matrix effect is well recognized, no studies on the matrix effect of pesticides in HM have been carried out to date. Various methods have been employed to either compensate or eliminate the matrix effect (Erney, et al., 1997; Godula, et al., 1999; Schenck & Lehotay, 2000; Poole, 2007; Garrido Frenich, et al., 2009; Stahnke, et al., 2012). The most commonly used methods to address matrix effect are: (1) Matrix matched calibration. This is not suitable for HM, because pesticide-free HM is impossible to obtain (Bedi, et al., 2013), and (2) Isotopically labeled standards. This is not practicable in routine analysis of multiclass and multiresidue pesticides as commercially available isotopically labeled standards are rather limited and expensive. Therefore, it is important to evaluate the variability and extent of the matrix effects for each pesticide in HM in order to determine the uncertainty caused by the matrix effect.

In this study, 88 representative pesticides frequently reported in monitoring studies were selected. They cover a wide range of chemical classes, such as organochlorine (OCPs), organophosphate (OPPs), carbamates, pyrethroids, fungicides, and other pesticides.
The aim of this study was to evaluate the matrix effect in pesticide residue analysis of HM by gas chromatography tandem mass spectrometry (GC-MS/MS). This research intends to measure the extent and variability of the matrix effect that different chemical classes of pesticides exert at different concentrations in HM.

### 3.1.3 Materials and Methods

#### 3.1.3.1 Materials

The pesticides used in this study are listed in Table 3.1.1. The pesticide standard solutions at 100 µg/mL, all 95% or higher purity, were obtained from Ultra Scientific (North Kingstown, RI, USA). LC-MS grade water and acetonitrile (ACN) were from Thermo Fisher Scientific (Waltham, MA, USA). Sodium acetate (NaOAc) and magnesium sulfate (MgSO₄) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotopically labeled quality control (QC) standards, acenaphthene-D₁₀, phenanthrene-D₁₀ and chrysene-D₁₂ were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The pesticide standard solutions (100 µg/mL) were mixed and diluted with ACN to prepare the stock standard solution (1 µg/mL) containing all the pesticides. IS (TPP) and QC standards were also prepared in ACN. For pesticides composed of several isomers, such as permethrin (1, 2), phenothrin (1, 2), cyfluthrin (1, 2, 3,4), cypermethrin (1, 2, 3,4), difenoconazole (1, 2), propiconazole (1, 2), methiocarb (1, 2) and fenvalerate (1, 2), the concentration in the stock standard solutions is the sum of the individual isomers. The stock standard solutions were then stored at −80°C.
3.1.3.2 Instrumentation

Chromatographic separation and determination of the pesticides were carried out on a Bruker Daltonics 450 GC equipped with a Bruker Daltonics Scion TQ triple quadrupole mass spectrometer, a Bruker 1177 Split/Splitless injector (Billerica, MA, USA) and a Combi-Pal autosampler (CTC Analytics AG, Switzerland). The inlet temperature was kept constant at 250°C. Sky 4.0 ID precision inlet liners with glass wool from Restek (Bellefonte, PA, USA) were used for all experiments. Injection was performed in splitless mode with an injection volume of 1 µL.

For the GC separation, a Restek Rtx-5MS with Integra-Guard column (10 m + 30 m x 0.25 mm x 0.25 µm) (Bellefonte, PA, USA) was used. The initial oven temperature of the column was 80°C for 3 min and ramped at 30°C/min to 150°C and then at 10°C/min to 300°C, where it was held for 10 min. The total run time was 31 min. Helium (6.0 GC grade) was used as carrier gas at a constant flow of 1.1 mL/min.

The mass spectrometer was operated in EI mode. The transfer line and ion source temperature were 270°C and 200°C, respectively. Argon was used as the collision gas. Identification and quantification were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. The optimized MRM transitions and collision energies for each compound are listed in Table 3.1.1.

3.1.3.3 Sample preparation

Term milk was collected from one mother and stored at -20°C in a plastic container and the HM matrix extract was prepared using an acetate buffered QuEChERS method with slight modifications (Lehotay, et al., 2005a; Lehotay, et al., 2005b). 10 mL of ACN (1% HOAc) was added to 10 mL of HM in a 50 mL centrifuge tube. Then, extraction reagents (4 g MgSO₄ and 1 g NaOAc) were added, and the tube was shaken
immediately. The tube was placed in an ice bath to prevent possible thermal degradation of some pesticides during the salting out process, and the tube was centrifuged at 3993 x g for 10 min. The extract (organic layer) was then transferred into a new tube and kept at -20°C for 2 hours. Freezing is critical for fatty samples in order to remove coextractives with limited solubility in ACN (e.g. lipid, wax and sugars) from the extract (Norli, et al., 2011; Anagnostopoulos, et al., 2013). For the cleanup step, 150 mg MgSO₄, 50 mg C18 and 50 mg PSA per 1 mL of the extract (organic layer) was used. The mixture was shaken vigorously for 1 min and centrifuged at 3993 x g for 10 min. The final extract was transferred into an amber vial and stored in the freezer at -80°C until analysis.

### 3.1.3.4 Preparation of standards in ACN

A set of pesticide solutions at 10, 50 and 100 ng/mL was prepared by appropriate dilution of the stock standard solution (1 µg/mL) with ACN.

### 3.1.3.5 Preparation of standards in HM extract

To evaluate the matrix enhancement of the pesticides, a series of pesticide solutions at 10, 50 and 100 ng/mL were prepared by appropriate dilution of the stock standard solution (1 µg/mL) with HM extract obtained in Section 3.1.3.3. The extract was analyzed under the same chromatographic conditions and the levels of any detected pesticides were deducted when calculating the matrix effect.

All solutions contained final concentration of 50 ng/mL TPP (IS) and 100 ng/mL QC standards.
3.1.3.6 Matrix effect

The matrix effect was evaluated by comparing the response from pure solvent with that from the same amount of pesticide in HM. The matrix effect (ME%) was determined using the following formula:

\[ ME\% = \left( \frac{B}{A} - 1 \right) \times 100\% \]

A. represents the average peak area of each pesticide in solvent.
B. represents the average peak area of each pesticides in HM.

ME=0 indicates no matrix effect. The values 0-20%, 20-50% and above 50% represent weak, medium and strong matrix effect, respectively. Negative values represent suppressions of the analyte signal.

3.1.4 Results and Discussion

3.1.4.1 Chromatographic analysis

The described GC-MS/MS conditions resulted in good separation and simultaneous analysis of 88 pesticides of various classes, such as OCPs, OPPs, fungicides, carbamates, pyrethroids and herbicides in both pure solvent and HM at 100 ng/mL as shown in Figure 3.1.1. The chromatogram of the spiked milk extract showed higher peak intensity and improved peak shape compared to that in solvent only, which demonstrated significant response enhancement of the pesticides provided by the matrix components in HM.
3.1.4.2 Matrix effect

Figure 3.1.2 shows representative compounds from different pesticide classes that are enhanced by the matrix effect (matrix enhancement) at a concentration of 100 ng/mL.

Table 3.1.1 summarizes the matrix effect of individual pesticides at three different concentrations. It is observed that the degree of matrix effect varies significantly between the different classes of pesticides at the same concentration and also for the same pesticide at different concentrations. Figure 3.1.3 shows the number of compounds that exhibit weak, medium and strong matrix effect at different concentrations. 77 out of 88 pesticides at 100 ng/mL exhibited strong matrix effects and the number slightly increased with the decrease of pesticide concentrations.
Figure 3.1.2 Peak response between the pesticides at 100 ng/ml in pure ACN (a) and HM extract (b).
Increase in the average matrix enhancement for majority of the pesticide classes is observed when the pesticide concentration decreases from 100 ng/mL to 50 ng/mL. However, the degree of enhancement cannot be fully determined at 10 ng/mL, as only OCPs are detected at this concentration in pure solvent, as shown in Table 3.1.2. This significant peak enhancement for low concentrations of the pesticides was also observed in other studies on fruits and vegetables (Schenck & Lehotay, 2000; Georgakopoulos, et al., 2007). The interaction between the analytes and the analytical instrument (active sites and thermal stress) was so serious that almost all OPPs, carbamates, pyrethroids, fungicides and herbicides could not be detected at 10 ng/mL in pure solvent. However, the matrix effect for OCPs was not as notable as the other groups, such as OPPs and fungicides at the same concentrations. The peak enhancement of OPPs and fungicides was over 8-fold and 10-fold higher than that of OCPs at 100 ng/mL and 50 ng/mL, respectively. The response enhancement variability between the individual pesticides within its class will be discussed.

Table 3.1.1 Retention time and MRM transitions for the 88 pesticides selected in this study. The influence of matrix enhancement of individual pesticides differed at three different concentrations (10, 50 and 100 ng/mL).

<table>
<thead>
<tr>
<th>CAS Number</th>
<th>Pesticides</th>
<th>t_R (min)</th>
<th>Transition ions* (Collision Energy, eV)</th>
<th>Dwell time (ms)</th>
<th>ME (%)a</th>
<th>ME (%)b</th>
<th>ME (%)c</th>
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<td>Alpha-HCH</td>
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<td>44.37</td>
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<td>31.50</td>
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<td>284.0&gt;249.0 (15) 286.0&gt;214.0 (30)</td>
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<td>146.40</td>
<td>15.72</td>
<td>27.03</td>
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<tr>
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<td>20.05</td>
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<tr>
<td>CAS Number</td>
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<td>ME (%)</td>
<td>ME (%)</td>
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<td>ME (%)</td>
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<td>Dwell time (ms)</td>
<td>ME (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ME (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ME (%)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Dwell time (ms)</td>
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<td>ME (%)(^b)</td>
<td>ME (%)(^c)</td>
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<td>314.0&gt;271.0 (10) 314.0&gt;162.0 (20) 226.0&gt;186.0 (18)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 95737-68-1</td>
<td>Pyriproxifen</td>
<td>18.63</td>
<td>226.0&gt;157.0 (30) 226.0&gt;105.0 (10) 342.0&gt;300.0 (15)</td>
<td>88</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 13642-54-5</td>
<td>Fluquinconazole</td>
<td>19.88</td>
<td>342.0&gt;315.0 (18) 342.0&gt;288.0 (20)</td>
<td>53</td>
<td>-</td>
<td>897.02</td>
<td>863.81</td>
</tr>
<tr>
<td>C 119446-68-3</td>
<td>Difenconazole 1</td>
<td>22.09</td>
<td>323.0&gt;265.0 (15) 323.0&gt;202.0 (30)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 119446-68-3</td>
<td>Difenconazole 2</td>
<td>22.19</td>
<td>344.0&gt;329.0 (10) 344.0&gt;156.0 (40)</td>
<td>66</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C 131860-33-8</td>
<td>Azoxystrobin</td>
<td>22.93</td>
<td>168.0&gt;153.0 (10) 168.0&gt;109.0 (15) 166.0&gt;96.0 (15)</td>
<td>133</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>D 2032-65-7</td>
<td>Methiocarb 1</td>
<td>9.43</td>
<td>168.0&gt;153.0 (10) 168.0&gt;109.0 (15) 166.0&gt;83.0 (18)</td>
<td>133</td>
<td>-</td>
<td>422.93</td>
<td>376.30</td>
</tr>
<tr>
<td>D 23103-98-2</td>
<td>Pirimicarb</td>
<td>12.40</td>
<td>168.0&gt;153.0 (10) 168.0&gt;109.0 (15) 166.0&gt;83.0 (18)</td>
<td>88</td>
<td>-</td>
<td>137.19</td>
<td>110.60</td>
</tr>
<tr>
<td>D 2032-65-7</td>
<td>Methiocarb 2</td>
<td>13.36</td>
<td>168.0&gt;153.0 (10) 168.0&gt;109.0 (15)</td>
<td>38</td>
<td>-</td>
<td>4567.3</td>
<td>1853.12</td>
</tr>
<tr>
<td>D 28434-01-7</td>
<td>Bioresmethrin</td>
<td>17.36</td>
<td>171.0&gt;143.0 (10) 171.0&gt;128.0 (10) 181.0&gt;165.0 (18)</td>
<td>24</td>
<td>-</td>
<td>667.40</td>
<td>489.13</td>
</tr>
<tr>
<td>D 82657-04-3</td>
<td>Bifenthrin</td>
<td>17.88</td>
<td>181.0&gt;166.0 (10) 181.0&gt;160.0 (10) 181.0&gt;115.0 (40)</td>
<td>44</td>
<td>-</td>
<td>219.38</td>
<td>218.12</td>
</tr>
<tr>
<td>D 26002-80-2</td>
<td>Phenothrin 1</td>
<td>18.29</td>
<td>183.0&gt;168.0 (15) 183.0&gt;151.0 (15) 183.0&gt;127.0 (15)</td>
<td>53</td>
<td>-</td>
<td>90.83</td>
<td>41.68</td>
</tr>
<tr>
<td>D 26002-80-2</td>
<td>Phenothrin 2</td>
<td>18.38</td>
<td>183.0&gt;153.0 (150) 183.0&gt;152.0 (20)</td>
<td>-</td>
<td>-</td>
<td>379.95</td>
<td>330.45</td>
</tr>
<tr>
<td>D 52645-53-1</td>
<td>Permethrin 1</td>
<td>19.64</td>
<td>183.0&gt;168.0 (10) 183.0&gt;152.0 (20)</td>
<td>53</td>
<td>-</td>
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<td>Permethrin 2</td>
<td>19.76</td>
<td>183.0&gt;168.0 (10) 183.0&gt;152.0 (20)</td>
<td>-</td>
<td>-</td>
<td>990.60</td>
<td>857.35</td>
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<tr>
<td>D 52315-07-8</td>
<td>Cypermethrin 1</td>
<td>20.47</td>
<td>181.0&gt;152.0 (20) 181.0&gt;115.0 (40)</td>
<td>-</td>
<td>-</td>
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<td>487.24</td>
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<tr>
<td>D 52315-07-8</td>
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<td>439.01</td>
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<tr>
<td>CAS Number</td>
<td>Pesticides</td>
<td>$t_R$ (min)</td>
<td>Transition ions* (Collision Energy, eV)</td>
<td>Dwell time (ms)</td>
<td>ME (%)$^a$</td>
<td>ME (%)$^b$</td>
<td>ME (%)$^c$</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------</td>
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<td>-----------</td>
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</tr>
<tr>
<td>D</td>
<td>Cypermethrin 3</td>
<td>20.56</td>
<td>225.0&gt;119.0 (18) 225.0&gt;147.0 (10)</td>
<td>133</td>
<td>-</td>
<td>1837.7</td>
<td>1470.29</td>
</tr>
<tr>
<td>D</td>
<td>Cypermethrin 4</td>
<td>20.66</td>
<td>150.0&gt;123.0 (18) 150.0&gt;150.0 (5)</td>
<td></td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Fevalerate 1</td>
<td>21.52</td>
<td>253.0&gt;172.0 (10) 253.0&gt;199.0 (25)</td>
<td>66</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Fevalerate 2</td>
<td>21.77</td>
<td>394.0&gt;266.0 (10) 394.0&gt;238.0 (35)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Indoxacarb</td>
<td>22.43</td>
<td>44</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Deltamethrin</td>
<td>22.56</td>
<td>306.0&gt;264.0 (10) 306.0&gt;159.0 (30)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Trifluralin</td>
<td>10.59</td>
<td>201.0&gt;172.0 (15) 201.0&gt;138.0 (10)</td>
<td>44</td>
<td>-</td>
<td>422.79</td>
<td>790.35</td>
</tr>
<tr>
<td>E</td>
<td>Simazine</td>
<td>11.29</td>
<td>215.0&gt;172.0 (15) 215.0&gt;138.0 (10)</td>
<td></td>
<td>-</td>
<td>309.93</td>
<td>235.39</td>
</tr>
<tr>
<td>E</td>
<td>Atrazine</td>
<td>11.37</td>
<td>215.0&gt;172.0 (15) 215.0&gt;138.0 (10)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Propanil</td>
<td>12.67</td>
<td>375.0&gt;316.0 (10) 375.0&gt;288.0 (20)</td>
<td></td>
<td>-</td>
<td>369.57</td>
<td>283.12</td>
</tr>
<tr>
<td>E</td>
<td>Pendimethalin</td>
<td>14.38</td>
<td>383.0&gt;254.0 (30) 383.0&gt;282.0 (10)</td>
<td>44</td>
<td>-</td>
<td>805.58</td>
<td>457.26</td>
</tr>
<tr>
<td>E</td>
<td>Haloxyp methyl</td>
<td>14.75</td>
<td>44</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Fluazifop-p-butyl</td>
<td>15.83</td>
<td>253.0&gt;162.0 (15) 340.0&gt;253.0 (10)</td>
<td></td>
<td>-</td>
<td>1444.9</td>
<td>518.54</td>
</tr>
<tr>
<td>E</td>
<td>Diclofop-methyl</td>
<td>17.22</td>
<td>394.0&gt;266.0 (10) 394.0&gt;238.0 (35)</td>
<td>24</td>
<td>-</td>
<td>412.29</td>
<td>418.58</td>
</tr>
</tbody>
</table>

A: Organochlorine pesticides (OCPs); B: Organophosphate pesticides (OPPs); C: Fungicides; D: Carbamates and pyrethroids; E: Herbicides and other pesticides.

$t_R$: retention time.

ME (%)$^a$: matrix enhancement of pesticides at 10 ng/mL; ME (%)$^b$: matrix enhancement of pesticides at 50 ng/mL; ME (%)$^c$: matrix enhancement of pesticides at 100 ng/mL.

*: The first transition ion was used for quantification, while the other transition(s) was used as qualifying ion(s).

- : Matrix enhancement effect cannot be calculated. This is due to the poor peak shape of the pesticides in solvent alone, which does not allow accurate integration.
3.1.4.3 Matrix effect of OCPs

OCPs (Group A in Table 3.1) do not have functional groups like hydroxyl (-OH), carboxyl (-COOH), phosphate (-P=O) and carbamate (-O-CO-NH-). Therefore, OCPs usually show either mild or medium matrix effect (Wang, et al., 2011; Li, et al., 2012). However, in this study, some of the OCPs such as heptachlor, endrin, chlordane, methoxychlor, endosulfan sulphate, o,p'- and p,p'- DDE, o,p'- and p,p'- DDD, o,p' - and p,p' - DDT exhibit strong matrix enhancement in HM (see Table 3.1).

HM is a complex matrix containing numerous bioactive components, such as fat, lactose and proteins, some of which could still remain in the extracted milk. The presence of these matrix components protects the OCPs from either being absorbed or interacting with the active sites within the instrument. Therefore, the affected OCPs in HM exhibited remarkable peak enhancement as compared to those in pure solvent. Li et al. also found that these OCPs exhibited strong matrix effect in three tea matrices.

Figure 3.1.3 Distribution of matrix enhancement of the pesticides at different concentrations in HM.
(green, oolong and black tea) (Li, et al., 2012). As some of the affected OCPs are thermally susceptible, such as DDT and endrin, they are easily degraded in pure solvent due to the high temperature of the GC injection port, column and MS ion source. However, the matrix components in milk extract reduce the thermal stress on these analytes. Stronger peak enhancement and improved peak shape were observed for the same concentrations of OCPs in matrix standards. Response enhancement increased from 95.3% to 106.2% when the concentrations decreased from 100 ng/mL to 50 ng/mL, demonstrating increased matrix effect at lower analyte concentrations (Table 3.1.2). Some OCPs like heptachlor epoxide, endrin, beta-endosulfan and endosulfan sulphate were not detected at regulatory concentrations (10 ng/mL) prepared in pure solvent. This will limit the ability to accurately identify and quantify these compounds at trace concentrations in samples, resulting in false negatives.

3.1.4.4 Matrix effect of OPPs

OPPs (Group B in Table 3.1.1) are found to be subject to notable matrix effect, because they share the similar phosphate (-P=O/-P=S) functional group, which is susceptible to matrix effect (Erney, et al., 1993; Schenck & Lehotay, 2000). Sugitate et al. found that the average matrix effect of OPPs was remarkably high in potato, spinach, orange, brown rice and soybean (Sugitate, et al., 2012). In this first study of matrix enhancement of OPPs in HM, strong matrix effects were observed at both high (100 ng/mL; ME %: 788.8%) and low concentrations (50 ng/mL; ME %: 1128.7%) as shown in Table 3.1.2. Most of the OPPs could hardly be detected at 10 ng/mL in pure solvent and fenitrothion could not even be detected at 50 ng/mL (Table 3.1.1).
Table 3.1.2 Average matrix enhancement of different pesticide groups at various concentrations.

<table>
<thead>
<tr>
<th>Pesticide groups</th>
<th>Average matrix enhancement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ng/mL</td>
</tr>
<tr>
<td>OCPs</td>
<td>153.1</td>
</tr>
<tr>
<td>OPPs</td>
<td>-</td>
</tr>
<tr>
<td>Fungicides</td>
<td>-</td>
</tr>
<tr>
<td>Carbamates and pyrethroids</td>
<td>-</td>
</tr>
<tr>
<td>Herbicides and other</td>
<td>-</td>
</tr>
</tbody>
</table>

* : Exclude pesticides in solvent that cannot be detected or accurately integrated.
- : The matrix enhancement cannot be calculated, as the pesticides in solvent cannot be detected or accurately integrated.

This is similar to the results reported by Georgakopoulos et al., who investigated the matrix effect of four OPPs (chlorpyrifos, dimethoate, methamidophos and methidathion) in 18 matrices (Georgakopoulos, et al., 2007). Among the selected OPPs in this work, dichlorvos, phosmet, fenitrothion and profenofos are more affected by the matrix enhancement. It is also observed that OPPs with P=O bonds rather than P=S bonds are more affected by matrix enhancement (Hajšlová, et al., 1998). As compounds containing the P=O functional group are more polar than those with P=S groups, a greater matrix enhancement is observed (Schenck & Lehotay, 2000). This is observed by comparing structural similar pesticides such as profenfos, which has a P=O bond, and exhibited ME% of 821.4% at 100 ng/mL as compared to prothiofos, which has a P=S bond, and exhibited ME% of 212.9% at the same concentration (Table 3.1.1). The largest matrix enhancement among the OPPs was phosmet, which was also observed by
Schenck and Lehotay, and could be due to the two P-O and one P=S functional groups (Schenck & Lehotay, 2000).

### 3.1.4.5 Matrix effect of fungicides

Nearly half of the selected fungicides (Group C in Table 3.1) have the susceptible chemical groups, for example triadimenol, flutriafol, hexaconazole, cyproconazole, and tebuconazole contain the hydroxyl group (-OH), and propyzamide and iprodione contain the amino group (R-NH-). These fungicides are either too polar or too thermolabile to be analyzed by GC when in solvent alone due to the thermal stress imposed on these analytes and the adsorption by the system. Therefore, these fungicides suffer from peak tailing and severe deterioration of peak shape. In this study, pyriproxifen, azoxystrobin, iprodione and difenoconazole were not detected at 100 ng/mL in pure solvent, but are detectable in HM extract. Other fungicides such as chlorothalonil and tebuconazole, which were hardly detectable by GC-MS/MS at 100 ng/mL in pure solvent only, were easily identified in HM with several-fold increase in signal intensities. Matrix enhancement increased significantly, when the fungicide concentration decreased from 100 ng/mL to 50 ng/mL, for flutriafol, expoxiconazole and cyproconazole respectively (Table 3.1). De Sousa et al. also observed the significant increase of the matrix enhancement for chlorothalonil when the concentration decreases from 500 ng/mL to 100 ng/mL in five different matrices (grape, pineapple, tomato, potato and soil) (de Sousa, et al., 2012).

The degradation and adsorption are also more pronounced in this pesticide group as none of the fungicides (except for triadimefon) could be integrated at 10 ng/mL in pure solvent.
3.1.4.6 Matrix effect of carbamates and pyrethroids

Carbamate pesticides (Group D in Table 3.1.1) share the same carbamate functional group (−O−C=O−NH−), which is prone to be absorbed by the active sites during sample injection, separation and detection (Poole, 2007). It is also known that more polar analytes have a stronger interaction with the active sites (Georgakopoulos, et al., 2007). The matrix effect of methiocarb is up to 10-fold more than that of pirimicarb at the concentration of 50 ng/mL and 100 ng/mL. The main structural difference between them is that methiocarb has a −O−C=O−NH− rather than a −O−C=O−N−, which demonstrated that the -NH group was more likely to be affected by the matrix effect. We found that deltamethrin, which was not detected at 100 ng/mL in pure solvent, was more prone to the matrix effect than cypermethrin and cyfluthrin, which was detected at 100 ng/mL in pure solvent. De Souse et al. also found that deltamethrin was more affected by matrix effect than cypermethrin in four different fruit matrices (de Sousa, et al., 2012). These selected carbamates and pyrethroids are so susceptible to the strong matrix effect that all the compounds were not detected at the concentration lower than 10 ng/mL. Therefore, there are limited reports of these insecticides in HM.

3.1.4.7 Matrix effect of herbicides

Most compounds in this group (Group E in Table 3.1.1) have a -NH group, such as propanil, pendimethalin and diflufenican, whereas compounds like simazine and atrazine have more than one -NH group. This group of herbicides suffers from strong peak enhancement, which is attributed to the strong interaction between the amino groups and the active sites in the GC system (Hajšlová, et al., 1998; Georgakopoulos, et al., 2007). These interactions were so strong that propanil at 100 ng/mL in solvent was not detectable. However, good chromatographic peak shape and intensity were observed in propanil in HM due to the masking of the active sites by the matrix components in
HM matrix (Figure 3.1.2). None of these compounds except trifluralin can be detected at 10 ng/mL in solvent. Overall, the average matrix enhancement for all the pesticides in Group E increased from 385.6% to 555.0% when the concentration decreased from 100 ng/mL to 50 ng/mL.

As shown in this study almost all 88 pesticides experienced strong peak enhancement in HM as compared to pure solvent. Pesticides with the functional groups discussed above are the most susceptible to matrix effect in the HM extract, which is similar to that reported in existing literature (Hajšlová, et al., 1998; Schenck & Lehotay, 2000; Poole, 2007). However, the matrix enhancement observed in HM was substantially stronger than that observed in other matrices such as Chinese herbs, teas, fruits and vegetables (Schenck & Lehotay, 2000; Wang, et al., 2011; Li, et al., 2012; Sugitate, et al., 2012). This could be attributed to the relatively high fat content of HM and matrix components remaining in HM even after the cleanup. Sugitate et al., has identified that MG and cholesterol were two of the matrix components that can cause peak enhancement (Sugitate, et al., 2012). As the fat fraction of HM consists of TAGs, non-EFA, cholesterol, phospholipids, di- and mono-acylglycerols (Newburg, 2001). All these components have chemical groups, which could interact with the active sites in the GC system and also reduce thermal stress experienced by thermally labile pesticides. This is the first paper to elaborate the matrix effect of different groups of pesticides in HM. This study exhibits that the matrix components present in the milk extract can be used to improve the peak quality and decrease the LOD of problematic pesticides. Based on current findings, future studies will focus on maximizing the matrix effect with various methods in order to develop a routine analysis method for detecting multiclass pesticides in HM.
3.1.5 Conclusions

In this study, matrix effect of 88 pesticides from a wide range of chemical classes, such as OCPs, OPPs, carbamates, pyrethroids, fungicides, herbicides and other pesticides at three different concentrations, was first investigated in HM using GC-MS/MS. We found that all the pesticides in HM had remarkable peak enhancement as compared to solvent standards due to the presence of components like cholesterol and monoacylglycerols in HM extract. OCPs are less affected by matrix enhancement as compared to other groups of pesticides. Some pesticides, such as dichlorvos, iprodione, pyriproxifen, difenoconazole, azoxystrobin, indoxacarb, deltamethrin and propanil, could hardly be detected at 100 ng/mL in solvent due to the strong absorption by the active sites and the thermal stress in the GC system. A common trend is observed where larger matrix enhancement is experienced when the concentration of pesticides decreases. This study provides valuable information about the matrix enhancement experienced by different groups of pesticides in HM. The benefits of the matrix components in the milk extract can be used to improve the peak quality and improve the detection and quantitation limits of these pesticides.

3.1.6 Acknowledgements

J. Du sincerely appreciates the International Postgraduate Research Scholarships (IPRS) and Australian Postgraduate Award (APA). This work was also supported by an unrestricted research grant from Medela AG. The authors gratefully acknowledge Bruker for the support for this project with the provision of the SCION GC-MS/MS system used for all the measurements. The authors gratefully acknowledge the support of the NCRIS scheme through Bioplatforms Australia and Metabolomics Australia for pesticide research.
3.2 Analyte protectants compensation for matrix-induced chromatographic peak enhancement in the gas chromatography tandem mass spectrometry analysis of 88 pesticides in human milk

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3.2.1 Abstract

Strong matrix-induced chromatographic response enhancement was observed in 88 pesticides spiked into human milk (HM). Various analyte protectants (APs) were investigated to evaluate matrix enhancement compared to pure solvent. A mixture of ethylglycerol, gulonolactone and D-sorbitol at 20 mg/mL, 2 mg/mL and 2 mg/mL, respectively was found to induce stronger peak enhancement than individual APs. The peak shape and intensity of matrix susceptible pesticides in solvent were improved significantly with the addition of APs. Compared to pesticide standards in HM, the addition of APs to standards in solvent provided similar response enhancement, and
showed good agreement between the solvent and the HM calibration curves. This demonstrated that APs could be effectively employed to address matrix enhancement and can substantially decrease the limit of detection of the affected pesticides. This is the first reported study, where APs were demonstrated as an effective and useful method to match the matrix enhancement in GC-MS/MS analysis of pesticides in HM.

### 3.2.2 Introduction

Pesticides are synthetic chemicals that are released into the environment and can percolate into the soil and ground water. These pesticides are resistant to chemical, physical and biological degradation, and can accumulate in the fat tissues of humans via inhalation, ingestion, or dermal adsorption (Needham & Wang, 2002; Van Oostdam, et al., 2005). During milk synthesis, the pesticides are mobilized from the fat tissues into the breast milk either by adhering to the surface or enclosed within the milk fat globules (Jensen, et al., 1997). The presence of pesticides in HM is of great concern due to the potential health effects for the breastfed infant, such as detrimental effects on neurodevelopment, immune deficiency, endocrine disruption, abnormal behavior and growth retardation (Edwards, et al., 2010; Pratt, et al., 2012). Therefore, the need for effective analytical methods to analyze a wide array of pesticides in HM is critical. Gas chromatography coupled to mass spectrometry (GC-MS) has been shown to be a powerful technique for the measurement of pesticide residues due to its reliable identification and quantification (Hernandez, et al., 2013).

However, one of the main factors affecting GC-MS based analysis of the pesticides is the matrix-induced chromatographic response enhancement effect, also known as matrix effect, which was first explained by Erney and co-workers (Erney, et al., 1993).
This phenomenon is caused by the presence of matrix components (e.g. lipids, waxes, pigments and other non-volatile compounds) remaining in the extract (Hajšlová & Zrostlíková, 2003; Poole, 2007). These compounds can mask the active sites (e.g. free silanols and metal ions) in the GC system, thus protecting the analytes in the matrix extract from absorption by the active sites and degradation by high thermal stress in the inlet (Georgakopoulos, et al., 2007; Hercegová, et al., 2010; Kwon, et al., 2012). In the absence of matrix components, the analytes bind to the inlet’s active sites and significantly reduce the amount detected. This leads to a higher limit of detection, lower response and poorer chromatographic peak shape of the affected pesticides in pure solvent as compared to matrix standards (Poole, 2007). In our previous research, we have found that most of the pesticides in HM experienced strong response enhancement and pesticides with hydroxyl (-OH), amino groups (R-NH-), carbamates (-O-CO-NH-), phosphate (=P=O) and urea (-NH-CO-NH-) functional entities were more susceptible to the enhancement (Du, et al., submitted). The most commonly used method to overcome the matrix enhancement in HM is by isotopically labeled standards. However, this method is not practicable in routine multi-residue analysis due to the limited commercially available isotopically labeled standards and the cost (Hajšlová & Zrostlíková, 2003). Another widely used approach to address the matrix enhancement is matrix-matched calibration (Anastassiades & Lehotay, 2003; Poole, 2007; Kwon, et al., 2012). But unlike the availability of pesticide-free fruits and vegetables, it is difficult to obtain pesticide-free HM. An alternative is the standard addition technique, however, this requires many analysis runs per sample and increases cost and decreases throughput. Therefore, the need to develop an effective method to address matrix enhancement of multi-residue pesticides in HM is required.
The introduction of analyte protectants (APs) to solvent standards is another effective method to model or counteract the matrix enhancement (Erney & Poole, 1993). Similar to matrix components, APs can mask the active sites in the GC inlet and column, and also alleviate the thermal stress imposed on some of the pesticides. Thus, peak tailing and analyte losses are significantly reduced in the presence of APs. The effect of APs were systematically reviewed by Anastassiades et al. in fruits and vegetables (Anastassiades, et al., 2003), where a combination of APs (ethylglycerol, gulonolactone and sorbitol) was observed to be more effective in compensating for matrix enhancement than using a single AP (Maštovská, et al., 2005). In recent years, many studies have successfully employed APs to normalize the matrix enhancement caused by the matrix components in various samples, including soil, juice and honey (Sánchez-Brunete, et al., 2005), apple, grapes and wines (Kirchner, et al., 2008; González-Rodríguez, et al., 2009). However, no study has been reported on the effect of APs on matrix enhancement of pesticides in HM.

Therefore, in this study we evaluate the performance of various concentrations of APs (ethylglycerol, gulonolactone and D-sorbitol) on peak enhancement of 88 pesticides, including organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), carbamates, pyrethroids, fungicides and other pesticides. This is the first study that evaluates the performance of APs to address the matrix enhancement of pesticides in HM.
3.2.3 Materials and Methods

3.2.3.1 Materials

Pesticide standard solutions (100 µg/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingstown, RI, USA). The pesticide standard solutions were mixed and diluted with acetonitrile (ACN) to a stock standard solution (1 µg/mL) containing all the pesticides. Isotopically labeled quality control (QC) standards, acenaphthene-D\textsubscript{10}, phenanthrene-D\textsubscript{10} and chrysene-D\textsubscript{12} were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). IS (TPP) at 1 µg/mL and QC standards at 10 µg/mL were prepared in ACN.

LC-MS grade acetonitrile (ACN) and LC-MS water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylglycerol (98% purity), gulonolactone (95% purity) and D-sorbitol (≥ 98% purity) were from Sigma-Aldrich (St. Louis, MO, USA). Ethylglycerol (100 mg/mL) was prepared in ACN, while gulonolactone and D-sorbitol were prepared in 80:20 (v/v) ACN/water. Sodium acetate (> 99.0%) and magnesium sulfate (99.5% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). C18 and primary and secondary amine exchange (PSA) were obtained from Agilent (Little Falls, DE, USA).

3.2.3.2 Instruments

This part is same as described in 3.1.3.2. Identification and quantification were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. The optimized MRM transitions and collision energies for each compound are listed in the Table 3.1.1. Data collection and processing were performed using Bruker MSWS 8 Software.
3.2.3.3 Sample preparation of HM

The study was approved by the Human Research Ethics Committee of the University of Western Australia. Term milk was collected from one mother and stored at -20°C in a plastic container and 10 mL of HM was extracted with 10 mL ACN (1% HOAc) in a 50 mL centrifuge tube. Then, extraction reagents (4 g MgSO₄ and 1 g NaOAc) were added, and then the tube was shaken immediately. The tube was placed in an ice bath to prevent possible thermal degradation of some pesticides during the salting out process, and the tube was centrifuged at 3993 x g for 10 min. Afterwards, 6 mL of extract was transferred to a clean centrifuge tube and stored in a freezer at -20°C for 2 hours. Freezing is critical for fatty samples to remove coextractives with limited solubility in ACN (e.g. lipid, wax and sugars) (Norli, et al., 2011; Anagnostopoulos, et al., 2013). Then, the extract was centrifuged at 3993 x g (0°C) for 10 min and 5 mL was transferred to the cleanup tube. For the cleanup step, 150 mg MgSO₄, 50 mg C18 and 50 mg PSA per 1 mL of the extract (upper layer of ACN) was used. The mixture was shaken vigorously for 1 min and centrifuged at 3993 x g for 10 min. The final extract was transferred into an amber vial and stored in the freezer at -80°C until analysis.

3.2.3.4 Preparation of APs solutions for evaluation

Ethylglycerol solutions at 5, 10, 20 and 50 mg/mL were prepared in ACN. Gulonolactone and D-sorbitol solutions at 0.5, 1, 2 and 5 mg/mL were prepared in ACN/Water. For evaluations of APs, various compositions and concentrations of individual APs were investigated based on peak enhancement of the pesticides as compared to the pesticides in pure solvent.
3.2.3.5 Preparation of pesticide standards in ACN with and without APs

A set of pesticide solutions at 0, 5, 10, 50 and 100 ng/mL was prepared by appropriate dilution of the stock standard solution (1 µg/mL) with ACN. For the preparation of solvent standards with APs, the optimized amount of APs (with a final concentration of 20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol) was added to the pesticide solutions. All solutions contained 50 ng/mL of TPP (IS) and 100 ng/mL of the QC standards.

3.2.3.6 Preparation of pesticide standards in HM with and without APs

The same set of pesticide solutions at 0, 5, 10, 50 and 100 ng/mL was prepared by appropriate dilution of the stock standard solution (1 µg/mL) with HM extract obtained in Section 3.2.3.3. For the preparation of matrix standards with APs, the same amount of the optimized APs was added to the HM extract.

3.2.4 Results and Discussion

3.2.4.1 Compounds susceptible to the matrix enhancement

The matrix-induced peak enhancement (matrix enhancement) of the 88 pesticides was extensively evaluated in our previous study, and we found that almost all these pesticides experienced strong matrix enhancement in HM compared to standards in solvent. Also, matrix enhancement was more significant for pesticides at lower concentration. The matrix enhancement of OCPs was not as notable as other groups such as OPPs and fungicides. OPPs and fungicides experienced over 8-fold and 10-fold higher peak enhancement than that of OCPs at 100 ng/mL and 50 ng/mL, respectively.
Some pesticides in pure solvents, such as dichlorvos, iprodione, pyriproxifen, difenoconazole, azoxystrobin, indoxacarb, deltamethrin and propanil, were so greatly affected by thermal degradation and interaction with the active sites in the liner and that they were not detected at levels of 100 ng/mL. Whereas, higher peak intensity and improved peak shapes were observed in pesticide-spiked HM extract. Table 3.2.1 shows the compounds in HM that are strongly affected by matrix enhancement.

### 3.2.4.2 Optimization of individual analyte protectants

The substantial peak enhancement observed in HM is most likely induced primarily by the high fat content, as fat is the second largest component in the HM which consists of TAGs, cholesterol, phospholipids, di- and mono-acylglycerols (Hamosh, et al., 1985). These compounds can bind to the active sites in the GC system and allow more analytes to be transferred from the inlet onto the GC column. It has been reported that monoacylglycerol and cholesterol are the two dominant matrix components that cause peak enhancement (Sugitate, et al., 2012). Due to the significant peak enhancement provided by the matrix components in HM extract as compared to other matrices, such as herbs, teas, fruits and vegetables (Przybylski & Bonnet, 2009; Wang, et al., 2011; Li, et al., 2012), an optimal combination of APs that can compensate for the enhancement was needed for HM.

According to previous studies, no notable peak enhancement was observed when low concentrations of ethylglycerol (<5 mg/mL) were used (Anastassiades, et al., 2003; Maštovská, et al., 2005). Therefore, higher concentrations of ethylglycerol (5, 10, 20 and 50 mg/mL) were evaluated (shown in Table 3.2.2).
### Table 3.2.1 Pesticides that are affected by matrix enhancement in human milk.

<table>
<thead>
<tr>
<th>Pesticide type</th>
<th>Compound name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organochlorine pesticides (OCPs)</td>
<td>Heptachlor, heptachlor epoxide a, trans-chlordane, cis-chlordane, ( o,p'-DDT ), ( o,p'-DDD ), ( o,p'-DDE ), ( p,p'-DDE ), ( p,p'-DDD ), ( p,p'-DDT ), endrin, beta-endosulphan, endosulphan sulphate, methoxychlor, mirex</td>
</tr>
<tr>
<td>Organophosphate pesticides (OPPs)</td>
<td>Dichlorvos, ethoprophos, terbufos, diazinon, chlorpyrifos methyl, fenitrothion, malathion, fenthion, chlorpyrifos, prothiofos, profenofos, piperonyl butoxide, phosmet</td>
</tr>
<tr>
<td>Fungicides</td>
<td>Etridiazole, propyzamide, chlorothalonil, metalaxyl-m, triadimefon, penconazole, fipronil, triadimenol, flutriafol, hexaconazole, cyproconazole, propiconazole, tebuconazole, tebuconazole, epoxiconazole, iprodione, pyriproxifen, Fluquinconazole, difenoconazole, azoxystrobin</td>
</tr>
<tr>
<td>Carbamates and pyrethroids</td>
<td>Methiocarb, pirimicarb, bioresmethrin, bifenthrin, phenothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate, indoxacarb, deltamethrin</td>
</tr>
<tr>
<td>Herbicides and other pesticides</td>
<td>Trifluralin, simazine, atrazine, propanil, pendimethalin, haloxyfop methyl, fluazifop-p-butyl, diclofop-methyl, diflufenican</td>
</tr>
</tbody>
</table>
The largest peak enhancement was observed for most of the pesticides (75%) with 20 mg/mL of ethylglycerol, and also showed improved peak quality, especially for the early-eluters, such as dichlorvos, ethoprophos, trifluralin, HCB and lindane. This is in good agreement with the results by Maštovská et al., where ethylglycerol induced strong signal enhancement of early-eluting analytes (Maštovská, et al., 2005). The ethylglycerol concentrations below 5 mg/mL provided limited protection to the affected pesticides.

Gulonolactone was demonstrated to be an effective single additive, covering a wide range of analytes, especially for the middle-eluting compounds (Maštovská, et al., 2005). In this study, the largest enhancement was observed for most of the pesticides (77%) with 2 mg/mL of gulonolactone (Table 3.2.2). No significant increase in matrix enhancement was observed when higher gulonolactone concentration (5 mg/mL) was used. D-sorbitol, which has similar volatility and properties to that of late-eluting pesticides, provided better protection to the late elutors (Maštovská, et al., 2005). Limited peak enhancement was observed when low concentrations (0.5 and 1 mg/mL) of D-sorbitol were used. However, 72% of the pesticides experienced the largest peak enhancement when 2 mg/mL of D-sorbitol was used.

It was observed that a further increase of APs concentrations did not significantly increase the peak enhancement of these pesticides, thus demonstrating that there are limited active sites in the GC system and this is in consistent with the findings by other researchers (Anastassiades, et al., 2003; Sánchez-Brunete, et al., 2005; Li, et al., 2012). In this study, 20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol provided the optimal peak enhancement for most of the investigated pesticides in pure solvent (Table 3.2.2).
**Table 3.2.2** Influence of each analyte protectants (APs) on peak enhancement, and the number of compounds with the largest peak enhancement provided by various concentrations of APs (n = 3).

<table>
<thead>
<tr>
<th>APs</th>
<th>Concentration (mg/mL)</th>
<th>Number of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylglycerol</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gulonolactone</td>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

**3.2.4.3 Comparison of peak enhancement provided by APs mixture and individual APs**

According to previous studies, no single APs was able to address the peak enhancement for all the analytes due to the wide range of chemical and physical properties of pesticides and matrices (Erney & Poole, 1993; Anastassiades, et al., 2003; Maštovská, et al., 2005). Therefore, the performance of APs mixture (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol) on the peak enhancement of the 88 pesticides was compared with individual APs. Figure 3.2.1 shows a comparison of the
peak response of the selected OCPs, OPPs, fungicides, carbamates and pyrethroids, herbicides and other pesticides at 100 ng/mL. The APs mixture provided better peak enhancement as compared to ethylglycerol, gulonolactone and D-sorbitol alone. Peak quality of compounds like dichlorvos, ethoprophos, triadimenol, indoxacarb and propanil were significantly improved in the APs mixture, as the synergistic effect of the APs mixture protects the entire eluting range and induce stronger peak enhancement for the analytes.

Figure 3.2.1 Comparison of peak enhancement. (Continues)
Figure 3.2.1 Comparison of peak enhancement. (Continues)
Chapter 3.2 Evaluation of Analyte Protectants

3.2.4.4 Performance of the optimized APs mixture on the matrix enhancement

To evaluate the effectiveness of the APs mixture (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol) on the matrix enhancement, solvent standards and HM standards with APs were prepared as detailed in Section 3.2.3.5 and 3.2.3.6. The peak area of the matrix standards with APs was assigned as 100% and the relative abundance of the solvent standards with APs was quantified using the following formula (Li, et al., 2012):

![Figure 3.2.1](image_url) Peak response of different pesticide types (A: OCPs, B: OPPs, C: Fungicides, D: Carbamates and pyrethroids and E: Herbicides and other pesticides) in ACN with the presence of the APs mixture (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol) and individual APs (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol, respectively).
Chapter 3.2 Evaluation of Analyte Protectants

Normalization ratio (\%) = \frac{A_{\text{std}}/A_{\text{IS}}}{A_{\text{mstd}}/A_{\text{mIS}}} \times 100

Where $A_{\text{std}}$ is the peak area of pesticide in solvent with APs and $A_{\text{IS}}$ is the peak area of IS in solvent with APs; $A_{\text{mstd}}$ is the peak area of pesticide in matrix with APs and $A_{\text{mIS}}$ is the peak area of IS in matrix with APs.

Normalization ratios of 80-120\% (corresponding to ± 20\% solvent standards with APs vs. matrix standards with APs) represent acceptable quantitative agreement, showing no significant difference between signal response of solvent standards and human milk standards with APs. This means that the matrix enhancement can be quantitatively reproduced using APs at the optimized concentrations.

**Figure 3.2.2** shows the peak shapes and intensities of pesticides in solvent standards and HM standards at 100 ng/mL with and without APs mixture (ethylglycerol, gulonolactone and D-sorbitol at 20 mg/mL, 2 mg/mL and 2 mg/mL, respectively). The selected pesticides represent compounds that display different levels of matrix enhancement: aldrin and heptachlor being less susceptible, whereas fenthion, $p,p'$-DDT, ethoprophos, fenitrothion and phosmet are more susceptible to the enhancement. Pesticides such as chlorothalonil, propanil and iprodione were highly susceptible to the matrix enhancement to such an extent that serious peak distortion was observed in pure solvent. It has been demonstrated that pesticides containing chemical groups like hydroxyl (-OH), phosphate (-P=O), amino groups (-R-NH-), carbamates (-O-CO-NH-) and urea (-NH-CO-NH-) are very susceptible to the matrix enhancement.

The addition of the APs mixture to both pesticide standards in solvent and HM extract significantly improved both the quality and intensity of the peaks for the affected
Chapter 3.2 Evaluation of Analyte Protectants

pesticides as compared to that in solvent alone (Figure 3.2.2). Furthermore, the presence of the APs mixture also minimizes the response difference observed between solvent standards and matrix standard.

Figure 3.2.2 Comparison of peak shapes and intensities of pesticides. (Continues)
Figure 3.2.2 Comparison of peak shapes and intensities of selected pesticides in HM (a) and solvent (b) with and without optimized APs mixture.

A normalization ratio was used to investigate the efficiency of the APs mixture to reproduce the matrix-induced response enhancement. Figure 3.2.3 shows the normalization ratio of the affected pesticides at 100 ng/mL in solvent and HM standards with APs. For the majority of the pesticides (86%), the normalization ratio lies within the acceptable range (80-120%), indicating that the addition of APs practically eliminates the differences between solvent and HM extract. The normalization ratio of all selected herbicides were within the range. However, the ratios of profenofos and methiocarb 2 were slightly over 120%, while the ratio of $p,p'$-DDT, endosulfan sulfate,
indoxacarb, cyfluthrin and cypermethrin isomers were lower than 80%. The peak enhancement effect was compensated for most of the analytes (see Figure 3.2.3 F).

Figure 3.2.3 Normalization of different pesticides. (Continues)
Figure 3.2.3 Normalization of different pesticides. (Continues)
Figure 3.2.3 Normalization ratio of different pesticide types (A: OCPs, B: OPPs, C: Fungicides, D: Carbamates and pyrethroids and E: herbicides and other pesticides) in ACN and HM extract with the presence of APs mixture (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol). Dotted lines represent the acceptable range of 80% and 120%, respectively. F. Shows the number of each pesticide types that lies within and out of the acceptable range (80-120%).
In order to understand the effectiveness of using APs in solvent for pesticides quantification, the calibration curves of several pesticides in solvent were compared to pesticides in HM with and without APs (Figure 3.2.4). Lower slopes and intercepts were observed for the affected pesticides in pure solvent without the introduction of APs as compared to the pesticides in HM extract, while the calibration curves of pesticides, such as chlorothalonil and propanil could not even be generated in pure solvent. Similar calibration slope and intercepts were observed between both the HM extract and the solvent with APs, and is within the deviation (± 15 %).

**Figure 3.2.4** Comparison of calibration curves (based on peak response normalized to IS TPP) of aldrin, chlorpyrofos, chlorothalonil and propanil obtained by injection of solvent (ACN) standards and HM extract with and without the addition of APs mixture (ethylglycerol, gulonolactone and D-sorbitol at 20, 2 and 2 mg/mL, respectively in the injected sample). Dashed lines represent peak area tolerance for values (±15%) obtained in solvent standards with APs mixture. (Continues)
Figure 3.2.4 Comparison of calibration curves (based on peak response normalized to IS TPP) of aldrin, chlorpyrofos, chlorothalonil and propanil obtained by injection of solvent (ACN) standards and HM extract with and without the addition of APs mixture (ethylglycerol, gulonolactone and D-sorbitol at 20, 2 and 2 mg/mL, respectively in the injected sample). Dashed lines represent peak area tolerance for values (±15%) obtained in solvent standards with APs mixture.
This indicates that the addition of APs can be effectively employed to protect susceptible pesticides from degradation and improve peak quality, thus reducing limit of detection (such as azoxystrobin and deltamethrin). APs can also be used as a ‘matrix equivalent’ for the quantification of pesticides in HM. In this study, the use of APs mixture to compensate for the matrix enhancement of the pesticides in HM has been successfully demonstrated. As the availability of pesticide-free HM is not feasible, this alternative facilitates accurate quantification of pesticides in HM without the need for matrix-matched samples, or the need to use the standard addition technique for quantification.

3.2.5 Conclusions

In this study, we investigated the matrix enhancement of 88 pesticides in HM, covering a wide range of pesticide groups, including OCPs, OPPs, Fungicides, carbamates and pyrethroids, Herbicides and other pesticides, and evaluated the chromatographic peak enhancement induced by various concentrations of ethylglycerol, gulonolactone and D-sorbitol using GC-MS/MS. A mixture of 20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol was found to be the optimal for counteracting the matrix enhancement in HM. Peak intensity and shape of the susceptible pesticides were significantly improved in the presence of APs in pure solvent. The addition of APs mixture to solvent and HM yielded acceptable normalization ratios for the affected pesticides and more than 86% of the investigated pesticides were within the acceptable range, providing both qualitative and quantitative possibilities. For the first time, a mixture of ethylglycerol, gulonolactone and D-sorbitol was demonstrated to be an effective alternative to matrix-matched standard or the standard addition methods to address the matrix enhancement of pesticides analysis in HM using GC-MS/MS.
3.2.6 Acknowledgements

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3.3 Evaluation of pulsed splitless injection to address matrix enhancement with and without analyte protectants in the analysis of 88 pesticides in human milk

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\textsuperscript{c}Metabolomics Australia, Western Australia

3.3.1 Abstract

The performance of pulsed splitless injection to address the matrix enhancement of 88 pesticides in human milk (HM) with and without analyte protectants (APs) was first investigated here. We demonstrated that larger volume injection (2 µL) at high pulsed pressure (30 psi) induced the strongest peak enhancement and also significantly improved peak shape of thermally labile pesticides. Even though the enhancement difference (ED) between solvent standards and HM standards was reduced for some pesticides, the response difference was still pronounced for majority of the pesticides. With the introduction of analyte protectants (APs) mixture to the solvent and HM, the ED was reduced significantly for all the pesticides. Further analysis with spiked pesticides (10 to 100 ng/mL) showed that the normalization ratio was within the acceptable range (70-120%), demonstrating that the ED between the solvent and HM
was negligible. Here, we have demonstrated that the use of APs together with pressure pulsed injection can be an effective alternative to matrix matched standard or standard addition methods, to address the matrix enhancement of pesticides in HM.

### 3.3.2 Introduction

Matrix-induced chromatographic response enhancement (matrix enhancement) is an inevitable problem in pesticide residue analysis and was first described by Erney et al. (Erney, et al., 1993). Pesticides that are susceptible to matrix enhancement are compounds with functional groups such as hydroxyl (-OH), carboxyl (-COOH), amino groups (R-NH-), phosphate (-P=O/-P=S), carbamates (-O-C=O-NH-), urea (-NH-CO-NH-) and benzimidazole (C7H6-N=N-). These compounds are more likely to be absorbed by the active sites (e.g. free silanols and metal ions) in the GC inlet and column. Some compounds, such as endrin, aldrin, chlorothalonil and DDE, suffer from serious degradation due to high thermal stress imposed by the inlet (Wylie, et al., 1992; Anastassiades, et al., 2003; Poole, 2007). Therefore, analyte losses and peak tailing are commonly observed when these compounds in solvent are injected into the GC system (Hajšlová & Zrostlíková, 2003; Maštovská, et al., 2005). On the other hand, matrix components, such as waxes, pigments, lipids and other non-volatile compounds are present in food and HM. These matrix components can bind with and deactivate the active sites on the GC flow path and protect the analytes from interacting with the active sites (Erney, et al., 1997; Georgakopoulos, et al., 2007; Hercegová, et al., 2010). This results in larger peak response and improved peak shape of the analytes in matrix-containing solutions as compared to that in pure solvent. Thus, overestimation of the affected analytes in matrix can occur when analytes in pure solvent are used for quantitative analysis (Poole, 2007; Kwon, et al., 2012). Matrix-matched calibration is
commonly used to address the matrix enhancement in the food industry (Erney, et al., 1997; Poole, 2007; Kwon, et al., 2012). However, this method is not suitable for HM analysis as it is difficult to obtain pesticide-free HM. Another widely used method is isotopically labeled standards, however this is not feasible in routine multi-residue analysis due to limited commercially available standards and the cost involved (Hajšlová & Zrostlíková, 2003).

Several studies have tried extensive sample cleanup (Schenck & Lehotay, 2000; Balinova, et al., 2007), and dilution of the final extract (Stahnke, et al., 2012) in order to reduce the matrix components. However, in practice, complete removal of the matrix components is neither possible nor cost-effective. Moreover, the presence of matrix components that do not interfere in the analysis can improve peak quality and improve detection limits (Anastassiades & Lehotay, 2003; Anastassiades, et al., 2003). Therefore, instead of concentrating on reducing the matrix components, many studies have employed various injection techniques to minimize thermal degradation and to improve peak response of pesticides (Godula, et al., 1999; Godula, et al., 2001; Zrostlíková, et al., 2001). It is well known that the matrix enhancement occurs mainly in the GC inlet when using the classic splitless injection technique (Wylie, et al., 1991; Poole, 2007). Therefore, reducing the residence time of compounds in the hot inlet will decrease their absorption and degradation. Also introducing larger amounts of the compounds into the hot inlet will decrease the response differences between matrix and matrix-free solvent as more compounds, even in matrix-free solvent, can be transferred from the inlet to the GC column (Müller & Stan, 1990a; Godula, et al., 2001).

2001; Zrostlíková, et al., 2001; Mašťovská, et al., 2004; Čajka, et al., 2005; Štajnbaher & Zupančič-Kralj, 2008), pulsed splitless injection involves increasing the column head pressure, and column flow, for a short duration during sample injection. The high head pressure rapidly sweeps the analytes from the inlet into the column, thus reducing interaction with the active sites on the inlet surface and thermal degradation of the analytes (Wylie, et al., 1991; Wylie, et al., 1992). Pulsed splitless injection also allows larger injection volume (up to 5 µL) without the risk of backflash, where lower volume expansion is observed when the inlet pressure is high as compared to classic splitless injection. Injection of larger volume (>1µL) in a classic splitless inlet would lead to notable peak deformations and peak broadening (Wylie, et al., 1992; Godula, et al., 1999). Several studies have demonstrated that the pulsed splitless injection could reduce adsorption and/or thermal degradation of labile compounds (Wylie, et al., 1991).

Recently, pulsed splitless injection with GC or GC/MS has been used for the analysis of contaminants (Wylie, et al., 1992; Sasaki & Makino, 2006; Peng, et al., 2013), where higher peak response and lower limit of detection have been observed in these studies. However, there is limited study about the extent of matrix enhancement using pulsed splitless injection and the response difference of the analyte in pure solvent and matrix.

Therefore, we have evaluated the performance of pulsed splitless injection on peak enhancement of 88 pesticides, such as OCPs, OPPs, carbamates, pyrethroids, Fungicides, Herbicides and other pesticides in human milk. The performance of pulsed splitless injection to reduce the enhancement difference between the pesticides in pure solvent and in HM was investigated. This is the first study, where a combination of APs and pulsed splitless injection to address the matrix enhancement was investigated.
3.3.3 Materials and Methods

3.3.3.1 Materials

This part is described in 3.2.3.1.

3.3.3.2 Instrumentation

This part is described in 3.2.3.2.

3.3.3.3 Sample preparation of HM

This part is described in 3.2.3.3.

3.3.3.4 Preparation of pesticide standards in ACN with and without APs

This part is described in 3.2.3.5.

3.3.3.5 Preparation of pesticide standards in HM with and without APs

This part is described in 3.2.3.6.

3.3.3.6 Evaluation of different pressure pulses

The performance of pulsed splitless injection at various pressure pulses from 0 to 55 psi (excluding the actual base head pressure of 14 psi) and different injected volumes on the matrix enhancement was investigated as shown in Table 3.3.1.
Table 3.3.1 Parameters of different injected volume at various pressure pulses that are used in this study (n=3).

<table>
<thead>
<tr>
<th>Pressure (psi)*</th>
<th>Injected volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (14)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>4</td>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>15 (29)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>30 (44)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>45 (59)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>55 (69)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

*Splitless injection column head pressure is 14 psi and the actual pressures used are in the parentheses.

The evaluations were based on comparing the peak response of pesticides at different pressure pulses against the peak response at no pulsed injection. The effect of the pressure pulse on the peak enhancement of each pesticide was calculated using the following formula:
3.3.4 Results and Discussion

3.3.4.1 Influence of different pulsed pressure on peak enhancement

This is the first study that investigates the effectiveness of pulsed splitless injection on the matrix enhancement of pesticides in HM. Substantial peak enhancement was observed when pulsed pressure from 15–55 psi was used as compared to that without pressure pulse (Figure 3.3.1). For analytes that are greatly affected by matrix enhancement, such as propanil, chlorothalonil, iprodione, fluquinconazole, difenoconazole, indoxacarb, deltamethrin and azoxytrobin, improved peak shape and increased peak response were more notable with pulsed pressure. Meanwhile, these compounds when in pure solvent experienced either degradation or serious interaction with the active sites in the inlet, thus they are only detectable at 100 ng/mL using the classic hot splitless injection. This shows that by using pressure pulsing, less analytes are degraded as compared to classic splitless injection. However, no significant increase in responses was observed for most of the analytes when higher pulsed pressures (45 - 55 psi) were applied. This phenomenon was also reported by Godula et al. where distinct loss of volatile OPPs was observed when high head pressure (80 psi) was used (Godula, et al., 1999). The sweeping of the carrier gas probably causes these losses, where excessive head pressure interferes with the mass spectrometer resulting in
significant reduction of peak response which prevails over the peak sharpening effect (Maštovská, et al., 2004).

![Graphs showing influence of different pulsed pressures on peak enhancement.](image)

**Figure 3.3.1** Influence of different pulsed pressures on peak enhancement. (Continues)
Figure 3.3.1 Influence of different pulsed pressures on peak enhancement. Peak enhancement of representative compounds at 100 ng/mL that are injected at different pulsed pressures (a-e: 0, 15, 30, 45 and 55 psi).
As different classes of pesticides react differently to the pressure pulses, the enhancement ratio (ER) of each pesticide was evaluated, where higher ER represents stronger peak enhancement. All the pesticides in this study observed peak enhancement as the pressure pulse increased from 15 to 55 psi, with the largest ER for OPPs (578.8 - 1653.0%), followed by Fungicides (415.8-1215.2%) and carbamates and pyrethroids (244.3-928.9%). Lastly, OCPs (86.7-214.4%) were least influenced by pressure pulse injections (as shown in Figure 3.3.2). This suggested that by reducing the residence time of the pesticides in the hot injection inlet would greatly reduce the decomposition and adsorption of these thermal labile pesticides. For compounds that were found to be more susceptible to matrix enhancement in HM from our previous studies, pressure pulse greatly enhanced the peak response of compounds such as heptachlor (267.3%), endrin (307.0%), profenofos (532.6%), epoxiconazole (1106.7%), diflufenican (1106.7%), simazine (1139.3%) and phosmet (8952.6%) as well as p,p'-DDT (453.3%) and methoxychlor (753.3%) not shown in Figure 3.3.2. Endrin, p,p’- DDT and methoxychlor are known to suffer from thermal-degradation in the liner, therefore with the introduction of pressure pulse, higher signal response and better peak shapes were observed. Analytes that have chemical structures such as hydroxyl (-OH), amino groups (R-NH-), carbamates (-O-CO-NH-), phosphate (-P=O), benzimidazole (C\textsubscript{7}H\textsubscript{8}N=N-) and urea (-NH-CO-NH-) are more like to interact with the active sites in the GC system, resulting in lower signal response in solvent standards as compared to matrix standards. However, with the shorter inlet residence of the analytes with the pressure pulse mode, enhanced peak response was observed for these groups of pesticides. However, no distinct increase of ER for most of the pesticides were observed, when the head pressure greater than 45 psi was applied.
Chapter 3.3 Evaluation of Pressure pulse with Analyte Protectants

Figure 3.3.2 Influence of different pressure pulses on the enhancement ratio (ER) of different classes of pesticides. (Continues)
**Figure 3.3.2** Influence of different pressure pulses on the enhancement ratio (ER) of different classes of pesticides. (Continues)
### 3.3.4.2 Evaluation of pressure pulse on the address of the matrix enhancement between solvent and HM

To further evaluate the performance of pressure pulse on the matrix enhancement, peak enhancement difference (ED) between solvent standards and HM standards at various pressures were calculated using the following formula:

$$\text{ED} (\%) = \frac{A_3 - A_2}{A_2} \times 100$$  \hspace{1cm} (2)

Where $A_3$ and $A_2$ are the peak areas of HM standard with pulsed injection and solvent standards with pulsed injection, respectively. ED of ±20% was used to represent minimum difference between signal responses of solvent standards and matrix standards, which indicated that the matrix enhancement could be neglected.

**Figure 3.3.2** Influence of different pressure pulses on the enhancement ratio (ER) of different classes of pesticides (A: OCPs, B: OPPs, C: Fungicides, D: Carbamates and pyrethroids and E: Herbicides and other pesticides) at the pulsed pressure of 15 psi, 30 psi, 40 psi and 55 psi, respectively.
Figure 3.3.3A shows the average ED of different classes of pesticides at various head pressures. The average ED for OCPs (95.3%) was smaller as compared to the other pesticide classes, such as carbamates and pyrethroids (550.5%), OPPs (788.8%), Fungicides (735.54%), Herbicides and other pesticides (823.3%) when no pressure pulsed was used. A similar trend was observed in all these pesticide classes, where the ED decreased as the pulsed pressure increased from 15 to 45 psi. This suggested that pressure pulse could be used to decrease matrix enhancement caused by the matrix components in HM. For the 88 pesticides investigated, all the pesticides except for heptachlor epoxide, showed strong matrix enhancement when no pressure pulse was used (Figure 3.3.3B). Whereas, 38% of the pesticides (33 out of 88) were within the acceptable ED range (± 20%) when pressure pulse of 55 psi was applied. More than half of the compounds within the ED acceptable ranges were OCPs. Whereas, thermal labile pesticides or the pesticides with susceptible functional groups were still outside the acceptable range.

3.3.4.3 Evaluation of large volume injection on the matrix enhancement

In this study, an injection volume of up to 4 µL can be used at 15 psi without peak deformation. However, serious peak broadening and peak splitting were observed for most of the pesticides when a larger volume (> 1 µL) at a higher pressure (> 30 psi) was employed (Figure 3.3.4). Slight peak broadening was also observed when 4 µL of sample was introduced at 30 psi. Godula et al. reported that the large volume injection was possible at 60 psi head pressure by using a retention gap (Godula, et al., 1999). However, the use of retention gap would introduce new active sites in the system, which can cause uncertainty during the analysis (Godula, et al., 1999). Therefore, in this study
only large volume injection at 15 psi (1-4 µL) and 30 psi (1-3 µL) on ED was investigated.

**Figure 3.3.3** Average enhancement difference (ED) of different classes of pesticides (A: OCPs, B: OPPs, C: Fungicides, D: Carbamates and pyrethroids and E: Herbicides and other pesticides) between solvent standards and HM standards at the pressure pulse of 0, 15, 30, 45 and 55 psi (A). Distribution of the pesticides that is within the acceptable ED range (±20%) between solvent and HM standards (B).
Among the parameters investigated, the injection volume (2 µL) at 30 psi showed the least ED between the solvent standards and HM standards, where 45% (40 out of 88) of the pesticides were within the acceptable range. This was higher than that observed (38%) when only pressure pulse (55 psi) was used (Figure 3.3.3B). However, over 55% of the studied pesticides were still affected by the matrix enhancement even after the application of larger volume injection and higher pressure pulse.

**Figure 3.3.4** shows the performance of large volume injection (a-d: 1 µL-4 µL) on the peak enhancement of fipronil at 100 ng/mL with different pulsed pressure injections.
3.3.4.4 Performance of APs mixture with pulsed splitless injection on the matrix enhancement

The introduction of analyte protectants (APs) to solvent standards has shown to be an effective method to equalize the response difference caused by the matrix components in the sample extracts (Erney & Poole, 1993; Anastassiades, et al., 2003). A mixture of APs (20 mg/mL ethylglycerol, 2 mg/mL gulonolactone and 2 mg/mL D-sorbitol) has been demonstrated to induce similar peak enhancement for most of the pesticides in both solvent and HM in our previous study. However, there is limited information on the synergistic performance of APs and pulsed splitless injection to address the matrix enhancement. Therefore, the performance of the APs mixture on the matrix enhancement with large volume injection (2 µL) and high pressure pulse (30 psi) was evaluated using the following formula (Li, et al., 2012):

\[
\text{Normalization ratio (\%)} = \frac{A_{\text{std}}}{A_{\text{mstd}}} \times \frac{A_{\text{IS}}}{A_{\text{mIS}}} \times 100
\]

Where \( A_{\text{std}} \) is the peak area of pesticide in solvent with APs and \( A_{\text{IS}} \) is the peak area of IS in solvent with APs; \( A_{\text{mstd}} \) is the peak area of pesticide in HM and \( A_{\text{mIS}} \) is the peak area of IS in HM.

Normalization ratio of 70-120% is used as the tolerable limits, which indicates that there is no significant difference in peak response between solvent and matrix standards (Maštovská, et al., 2005). The normalization ratio of the 88 pesticides at the concentration of 10 to 100 ng/mL both in solvent and HM with APs mixture was investigated (as shown in Figure 3.3.5). 99% and 98% of the pesticides spiked at 100 and 50 ng/ml respectively fell within the acceptable range. Even at the concentration of 10 ng/mL, 89% of these pesticides were in the acceptable range.
Figure 3.3.5 Normalization ratio of the pesticides at different concentrations.

(Continues)
In our previous study, we had found that the normalization ratio of several pesticides, such as profenofos, \( p,p' \)-DDT, endosulfan sulfate, indoxacarb, cyfluthrin and cypermethrin isomers, were still outside the acceptable range even after the combination of APs with classic hot splitless injection. Meanwhile with this newly developed method, the majority of these pesticides are now within the acceptable range. Here, we demonstrated the synergistic effect of APs with larger volume injection and pressure pulse to address the matrix enhancement.
3.3.5 Conclusions

This is the first study that evaluates the performance of pulsed splitless injection to address the matrix enhancement of 88 pesticides in HM. The largest response enhancement was observed in pesticides with susceptible functional groups using large volume injection (2 µL) and high pulsed pressure (30 psi). However, this is still inadequate with enhancement different of over half of the 88 pesticides still residing out of the acceptable range between solvent and HM. However, this can be overcome with the addition of APs mixture to normalize the peak enhancement difference between the pesticides in solvent and HM with more than 89% of the investigated pesticides at 10 ng/mL falling within the acceptable range (70–120%). This is the first paper that describes the synergistic effect of APs, pressure pulse and large volume injection to address the matrix enhancement of pesticides in HM. Future work will include the application of this method to detect HM samples collected from different areas of Western Australia.

3.3.6 Acknowledgements

J. Du sincerely appreciates the International Postgraduate Research Scholarships (IPRS), Australian Postgraduate Award (APA) and UWA Safety-Net Top-up Scholarship for financial support. This work was also supported by an unrestricted research grant from Medela AG. The authors gratefully acknowledge Bruker for the support for this project with the provision of the SCION GC/MS/MS system used for all the measurements. The authors gratefully acknowledge the support of the NCRIS scheme through Bioplatforms Australia and Metabolomics.
Chapter 4 Development and validation of a scalable method for the determination of 88 pesticide residues in human milk using QuEChERS and gas chromatography tandem mass spectrometry

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4.1 Abstract

In this study, a scalable QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method with gas chromatography tandem mass spectrometry (GC-MS/MS) was first established to determine 88 different pesticides in human milk (HM). The extraction and cleanup reagents of QuEChERS method were optimized for these pesticides in HM. The optimized method was validated using HM of varying fat content (26.1–83.8 g/L) in order to cover the typical physiological HM fat range in the general population and to ensure the accuracy of the QuEChERS method. Consistent recoveries were obtained for HM with different fat content spiked at different levels of pesticides (5–50 ng/mL). This method was then validated by post-spiking with a wide concentration range of
0.5-100 ng/mL in medium fat milk, and the average recoveries were 64.1-126.7% (RSD: 3.6-33.9%), 68.4-117.7% (RSD: 2.2-17.4%) and 76.4-115.3% (RSD: 2.5-13.5%) for the spiked concentrations at 10, 20 and 100 ng/mL respectively. The lowest calibrated level for detection (LCLD) and lowest calibrated level for quantitation (LCLQ) were 0.2-2 ng/mL and 0.2-5 ng/mL for most of the pesticides except cyfluthrin and cypermethrin. The validated method will be applied to the analysis of 88 targeted pesticide residues in HM from Western Australian women in a cross-sectional and longitudinal study.

### 4.2 Introduction

Persistent organic pollutants (POPs) are widely used in agriculture and in domestic household all over the world to control weeds and pests (Köhler & Triebskorn, 2013). Inhalation, ingestion and dermal contact are routes of entry to the human body for these pesticides (Van Oostdam, et al., 1999; Needham & Wang, 2002). These pesticides are circulated via carrier proteins and then concentrated in the high fat tissue compartments, such as adipose tissue, liver and human milk (HM) (Maliwal & Guthrie, 1981; Needham & Wang, 2002). There has been rising concerns over the pesticides in HM as it is the main food source for infants, particularly at the early age, when many vital processes, such as cell differentiation and development of organ systems occur (Eskenazi, et al., 1999). Infants, due to their immature immune systems and low levels of enzymes involved in the detoxification of the pesticides, are more vulnerable to the potential effects of the pesticides (Holland, et al., 2006; Eskenazi, et al., 2007). Many epidemiologic studies suggest that prenatal exposure to POPs is associated with negative birth outcomes (e.g., weight, body length and head circumference), delayed neurodevelopment, poor cognitive performances and growth retardation during early childhood (Whyatt, et al., 2004; Eskenazi, et al., 2006b; Eskenazi, et al., 2007).
Therefore, to ensure the safety of HM, more studies about the pesticides in HM are warranted.

Due to the low abundance of POPs in HM and the coexistence of numerous nutritional and immunologically active components such as fatty acids, sugars and proteins (Hale & Hartmann, 2007), POPs cannot be analyzed directly without prior sample preparation. Therefore, pesticides analysis in HM usually involves sample pretreatment and analytical determination by using gas chromatography-electron capture detector (GC-ECD) (Kim, et al., 2013; Raab, et al., 2013), gas chromatography-tandem mass spectrometry (GC-MS/MS) (Çok, et al., 2012; Croes, et al., 2012) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Shi, et al., 2013; Hernik, et al., 2014). Sample preparation is the most time consuming part of this analysis as multiple cleanup steps are required to remove matrix interferences and coextractives from the initial HM extract prior to the analytical step (Zhou, et al., 2012; Kim, et al., 2013; Abdallah & Harrad, 2014). Various methods have been developed to extract POPs from HM, including liquid liquid extraction (LLE) (Mahmoudi, et al., 2007; Rojas-Squella, et al., 2013), solid phase extraction (SPE) (Minh, et al., 2004; Sudaryanto, et al., 2005), pressurized liquid extraction (PLE) (Sun, et al., 2005), and Soxhlet extraction (Abballe, et al., 2008; Zhou, et al., 2011), and followed by cleanup steps using gel permeation chromatography (GPC), SPE and/or sulfuric acid treatment. However, these techniques are not only time- and labor intensive, but also require large volume of solvent (10-250 mL) and HM (10–40 mL) (Devanathan, et al., 2012; Ennaceur & Driss, 2013; Kim, et al., 2013). The HM required for these extraction methods increases the pressure for HM collection in particular from preterm mothers whose milk production is often low. It calls for a method that is more efficient and requires a smaller amount of HM for routine analysis of POPs.
The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach has become the method of choice for rapid extraction and cleanup of various matrices for multiresidue analysis since its initial development in 2003 (Anastassiades & Lehotay, 2003; Lehotay, et al., 2005a; Anastassiades, et al., 2007). However, as the original QuEChERS method was developed mainly for the analysis of pesticide residues in fruits and vegetables, extraction of fatty food such as egg, milk and avocado, resulted in lower recoveries for certain lipophilic pesticides such as hexachlorobenzene (HCB), chlordane and dichlorodiphenyldichloroethylene (DDE) (Lehotay, et al., 2005b; Chamkasem, et al., 2013). Several studies have modified the extraction method to tailor for fatty food and also pH-sensitive pesticides using either acetate or citrate buffers (Lehotay, et al., 2005a; Anastassiades, et al., 2007). The extraction step is usually followed by a dispersive solid-phase extraction (dSPE) cleanup step, which uses various sorbents with specific applications, such as MgSO₄ (to remove excess water), primary secondary amine (PSA: to remove matrix components like fatty acids, organic acids and various sugars) and octadecyl-bonded silica (C18: to remove fat residues and lipophilic interferences) (Anastassiades & Lehotay, 2003; Lehotay, et al., 2005b; Chamkasem, et al., 2013).

While several studies have reported pesticides in HM using the modified QuEChERS methods (AOAC Official method 2007.01 or European Standard Method EN 15662) (Kinsella, et al., 2009; Brondi, et al., 2011; Jeong, et al., 2012), no study has optimized and validated the QuEChERS method specifically for HM.

The aim of this study is to develop and validate a scalable QuEChERS-based method for the determination of 88 different classes of pesticides, such as OCPs, OPPs, Cabamates, pyrethroids, Fungicide, Herbicides and other pesticides in HM. This
validated method will then be applied to determine the multiresidue pesticides in HM collected from Perth, Western Australia (WA).

4.3 Materials and Methods

4.3.1 Materials and Reagents

Pesticide standard solutions (100 µg/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingstown, RI, USA). The pesticide standard solutions were mixed and diluted with acetonitrile (ACN) to a stock standard solution (1 µg/mL) containing all the pesticides. LC-MS grade ACN and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylglycerol (98% purity), gulonolactone (95% purity) and D-sorbitol (≥ 98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylglycerol (100 mg/mL) was prepared in ACN, while gulonolactone and D-sorbitol were prepared in 80:20 (v/v) ACN/water. Sodium acetate (> 99.0%) and magnesium sulfate (99.5% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecyl-bonded silica (C18) and primary secondary amine (PSA) were purchased from Agilent (Little Falls, DE, USA). Isotopically labeled quality control (QC) standards, acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12} were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). IS (TPP) at 1 µg/mL and QC standards at 10 µg/mL were prepared in ACN.
4.3.2 GC-MS/MS analysis

Chromatographic separation and determination of the pesticides were carried out on a Bruker Daltonics 450 gas chromatography (GC) with a Bruker Daltonics Scion TQ triple quadrupole mass spectrometer (MS) (Billerica, MA, USA), a Bruker 1177 Split/Splitless injector and a Combi-Pal autosampler (CTC Analytics AG, Switzerland). Sky 4.0 ID precision inlet liners with wool from Restek (Bellefonte, PA, USA) were used. Injection was performed in pulsed splitless mode (30 psi) with an injection volume of 2 µL. For GC separation, a Rtx-5MS with Integra-Guard column (10 m + 30 m x 0.25 mm x 0.25 µm) (Bellefonte, PA, USA) was used. The inlet temperature was kept constant at 250°C. The initial oven temperature of the column was 80°C for 3 min and then ramped at 30°C/min to 150°C and at 10°C/min to 300°C, where it was held for 10 min. The total run time was 31 min. Helium (6.0 GC grade) was used as carrier gas at a constant flow of 1.1 mL/min.

The mass spectrometer was operated in EI mode. Transfer line and ion source temperature were at 270°C and 200°C, respectively. Argon was used as the collision gas. Identification and quantification of 88 pesticides were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. The optimized MRM transitions and collision energies for each compound are listed in Table 3.1.1. Data collection and processing were performed using Bruker MSWS 8 Software.

4.3.3 Human milk treatment

The final preparation method was optimized and validated using spiked HM, as described under Results and Discussion. Term milk was collected from one mother and stored at -20°C in a plastic container. In the final method, 1 mL of HM was placed into a 15 mL centrifuge tube and 1 mL ACN containing 1% HOAc and 100 ng/mL QC
Chapter 4 Optimization and validation of QuEChERS methodology

standards (acenaphthene-D\textsubscript{10}, phenanthrene-D\textsubscript{10} and chrysene-D\textsubscript{12}) was added. Extraction reagents (0.4 g MgSO\textsubscript{4} and 0.1 g NaOAc) were added and shaken immediately to avoid formation of lumps, and the tube was then placed in an ice bath. The extraction tube was centrifuged at 3993 x g for 10 min. 0.6 mL of extract was transferred to a clean 15 mL centrifuge tube and stored at \(-20^\circ\text{C}\) for 2 hours. The extract was then centrifuged at 3993 x g for 10 min, and 0.5 mL was transferred to a cleanup tube (157 mg MgSO\textsubscript{4}, 9 mg C18 and 9 mg PSA). The tube was vortexed and centrifuged at 3993 x g for 10 min. The final extract was transferred into a screw cap amber vial and kept at \(-80^\circ\text{C}\) until analysis.

### 4.3.4 Preparation of working standard solutions

The pesticide standard solutions (100 \(\mu\text{g/mL}\)) were mixed and diluted with acetonitrile (ACN) to prepare the stock standard solution (1 \(\mu\text{g/mL}\)) of all the pesticides. The working calibration standard solutions were prepared by appropriate dilution of the 1 \(\mu\text{g/mL}\) stock standard solution with ACN (1% HOAc). The concentrations of the working calibration standards were 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL. For the recovery study, HM were spiked with the corresponding volume of the standard solution and left standing overnight in the fridge (4°C) before extraction.

A combination of APs mixture (ethylglycerol, gulonolactone and D-sorbitol) containing internal standard TPP (IS) was added to the final extract of HM extract and all the working standard solutions for GC-MS/MS analysis. As demonstrated in Chapter 3.3, the addition of the APs mixture and pressure pulse injection (30 psi + 2 \(\mu\text{L}\)) can equalize the enhancement difference between the pesticides in pure solvent and in HM extract. The final concentrations of ethylglycerol, gulonolactone and D-sorbitol were
20, 2 and 2 mg/mL, respectively. All solutions contained final concentration of 50 ng/mL TPP (IS) and 100 ng/mL QC standards.

4.3.5 Evaluation of the method using HM with different fat content

Pooled term HM (700 mL) was thawed at 37°C for 1 hour and 100 mL was diluted 2-fold with 100 mL of double deionized (DDI) water (Low fat milk: LFM). 400 mL of the term milk was centrifuged at 750 x g for 5 min (Eppendorf 58410R, Hamburg, Germany) and 200 mL of skim milk was removed. The remaining content (containing fat and skim milk) was mixed (High fat milk: HFM). The remaining 200 mL of the term milk was used as it is (Medium fat milk: MFM). Figure 4.1 presents the schematic diagram for the evaluation of QuEChERS performance in LFM, MFM and HFM. The fat content of these HM (LFM, MFM and HFM) was measured using the creamatocrit method (Meier, et al., 2002). The QuEChERS method was then scaled down from 10 mL to 1 mL of HM.

4.3.6 Validation of the method

The performance of the optimized QuEChERS method and GC-MS/MS was further validated by spiking various concentrations of pesticide standards at 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL in MFM. The GC-MS/MS was first conditioned with 8 injections of pesticide standard at 20 ng/mL in solvent. The method validations were then run in blocks, where each block contained 9 calibration standards (0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL analyzed in random orders) in APs, 9 spiked HM samples and a pesticide standard (20 ng/mL) for quality control. A total of seven blocks were analyzed in this validation study.
Figure 4.1 Schematic diagram of validation of QuEChERS method in HM with different fat content.
4.3.7 Quality control and Statistical analysis

To minimize quantitation bias and possible sources of contamination from the instrument and sample preparation procedures, solvent blank, solvent standards and HM extract were ran simultaneously with the HM samples to ensure accurate instrument performance and quantification reproducibility. Isotopically labeled quality control (QC) standards, acenaphthene-D$_{10}$, phenanthrene-D$_{10}$ and chrysene-D$_{12}$ were employed to evaluate the efficiency of the extraction and cleanup steps, while TPP (IS) was used to evaluate the performance of the instrument throughout the entire analytical procedure. The LOD and LOQ for this validated QuEChERS method were determined experimentally with the lowest standards in APs mixture providing the signal-to-noise ratio (S/N ratio) greater than 3 and 10, respectively. All the pesticides were quantified using the working standard solutions with APs. Accuracy and precision, based on the recovery experiments (n=7) of HM samples spiked at 10, 20 and 100 ng/mL, were expressed as relative standard deviation (RSD, %). Statistical analyses were carried out using SPSS software (SPSS, version 19.0 for windows, SPSS, Inc, IL, USA).

4.4 Results and Discussion

4.4.1 Optimization of QuEChERS extraction procedure

Currently, there is no validated extraction method for POPs in HM. Even though previous studies have employed the original QuEChERS method for the extraction in milk, the extraction efficiency was not investigated (Kinsella, et al., 2009; Brondi, et al., 2011; Jeong, et al., 2012). The objective of the sample preparation is to increase the extraction efficiency of the analytes and to minimize the coextractives. The QuEChERS method involves two major steps: sample extraction and cleanup. Therefore, a systematic approach was designed to optimize the QuEChERS extraction for POPs
analysis using 10, 5 and 1 mL of HM (Figure 4.2) and the cleanup step using 1 mL of the crude extract obtained from the optimized QuEChERS extraction (Figure 4.3).

Figure 4.2 Schematic diagrams of the optimization of QuEChERS extraction procedures using 10, 5 and 1 mL human milk. (Continues)
Chapter 4 Optimization and validation of QuEChERS methodology

Figure 4.2 Schematic diagrams of the optimization of QuEChERS extraction procedures using 10, 5 and 1 mL human milk.

Figure 4.3 Schematic diagrams of the optimization of QuEChERS cleanup procedures using 1 mL of human milk and the optimized QuEChERS extraction method.
Firstly in the extraction step, based on the liquid partitioning of analytes between ACN and HM, the ratio of partitioning salts (anhydrous MgSO$_4$ and NaOAc) was investigated as the extraction efficiency of pesticides is affected by the salts composition (Anastassiades & Lehotay, 2003). Optimization of the partitioning salts was primarily based on the distinction of the phase separation between the layers, and followed by the peak response of the spiked pesticides using GC-MS/MS, as shown in Figure 4.4 and Figure 4.5 respectively. It is observed that as the amount of NaOAc increases, the separation layers (from top to bottom: ACN layer, lipid layer, aqueous layer and salts layer) became more apparent and clear. It is also observed that a thicker lipid layer was formed when the ratio of NaOAc increases to greater than 20% of total salts. Similar phenomenon was also observed when the volume of HM was scaled down from 10 mL to 1 mL as shown in Figure 4.4. The thick lipid layer could significantly retain more lipophilic pesticides, which resulted in poor recovery of those pesticides in the ACN layer (Chamkasem, et al., 2013). A higher ratio of NaOAc also reduces the saturation of MgSO$_4$, which is essential to induce phase separation and partitioning of the analytes from the aqueous matrix into the ACN layer.

In terms of the peak response of the spiked pesticides, MgSO$_4$: NaOAc ratio of 4:1 gave the highest overall peak response for the selected pesticides than other ratios throughout the three volumes (10, 5 and 1 mL) of HM investigated (Figure 4.5). This optimal salts ratio (4 MgSO$_4$: 1 NaOAc) worked acceptable well to induce the pesticides partition into the ACN layer (top layer). This could be due to the properties of the salts, where when the ratio of MgSO$_4$: NaOAc is greater than 80% (> 4:1), the excessive MgSO$_4$ ensure adequate removal of water from the sample and induce partitioning, thus minimizing the retention of lipophilic pesticides in the thick fat layer.
Figure 4.4 Phase separation of human milk in 10, 5 and 1 mL with different ratios of salts (MgSO₄ and NaOAc). The ratios of MgSO₄ and NaOAc are 4:0 (A), 4:1(B); 2:1(C) and 1:1 (D).
Whereas, if a lower ratio of MgSO₄ and NaOAc (< 4:1) is used (Figure 4.4D), the increased fat layer could retain more lipophilic pesticides, thus leading to the decreased peak response of some pesticides, such as lindane, \( p,p'-\)DDE, \( p,p'-\)DDD and phosmet (Figure 4.5). These findings are similar to the studies that also reported the low recoveries (< 50%) for these lipophilic pesticides in fatty matrices, such as flaxseed, peanut and avocado (Lehotay, et al., 2005b; Koesukwiwat, et al., 2010).

Overall, this optimized ratio of MgSO₄ and NaOAc (4:1) gave the highest peak response for most of the pesticides (85%) investigated in this study, as shown in Figure 4.5. The similar results were observed when the volume of HM was scaled down from 10 mL to 5 mL and 1 mL. This is the first study that demonstrated the scalability of the QuEChERS extraction method, from 10 mL of HM and 5 g salts to 1 mL of HM and 0.5 g salts, while maintaining excellent extraction recovery and peak response.

**Figure 4.5** Peak response of the selected pesticides spiked at 100 ng/mL in human milk using various ratios of MgSO₄/ NaOAc. The volume of human milk is 10 mL (A), 5 mL (B) and 1 mL (C). (Continues)
Figure 4.5 Peak response of the selected pesticides spiked at 100 ng/mL in human milk using various ratios of MgSO₄/NaOAc. The volume of human milk is 10 mL (A), 5 mL (B) and 1 mL (C).
4.4.2 Optimization of QuEChERS cleanup procedure

Matrix components including lipids, sugars and proteins are coextracted to some extent by ACN. Since POPs are in low abundance in HM, the co-extracted matrix components can interfere and suppress the analytes, thus resulting in poor chromatographic performance and poor sensitivity. Therefore, the cleanup of the HM extract is required to achieve good GC-MS/MS performance. Sorbents, such as PSA and C18, are often used to remove pigments, lipids, fatty acids and various sugars, while MgSO$_4$ is used to absorb the remaining water in the ACN extract (Anastassiades & Lehotay, 2003; Lehotay, et al., 2005b; Koesukwiwat, et al., 2010; Chen, et al., 2013). GCB is not investigated in this study as it has a strong affinity for planar pesticides, such as HCB and chlorothalonil (Kinsella, et al., 2009; Zheng, et al., 2013).

A wide combination of cleanup sorbents (MgSO$_4$, PSA, C18) was used to optimize the cleanup step (Figure 4.3). Before addition of the cleanup sorbents, the HM extract was kept at -20°C for 2 hours. We observed that a proportion of the coextracted materials precipitated and settled to the bottom of the HM. Freezing is critical for fatty samples to remove coextractives that have limited solubility in ACN (e.g. lipid, wax and sugars) (Koesukwiwat, et al., 2010; Norli, et al., 2011; Anagnostopoulos, et al., 2013). Based on the peak response of the pesticides previously spiked in HM, the ratio of MgSO$_4$: PSA: C18 (1: 0.05: 0.05) gave the highest response in all the three cleanup groups (75mg, 125mg and 175mg), as shown in Figure 4.6. The three best combinations for each total weight group were then compared with the AOAC Official method 2007.01 (75 mg MgSO$_4$/25 mg PSA/25 mg C18), and we found that the cleanup sorbent with 157 mg MgSO$_4$, 9 mg PSA and 9 mg C18 provided the largest peak response for most of the investigated pesticides (Figure 4.6D).
Figure 4.6 Peak response of selected pesticides spiked at 20 ng/mL in human milk with various amounts and ratios of cleanup sorbents (MgSO₄/PSA/C18). The total amount of sorbents used in the cleanup was 75 mg (A), 125 mg (B), 175 mg (C) and comparison between the three best combinations and the AOAC method (D). (Continues)
Figure 4.6 Peak response of selected pesticides spiked at 20 ng/mL in human milk with various amounts and ratios of cleanup sorbents (MgSO₄/PSA/C18). The total amount of sorbents used in the cleanup was 75 mg (A), 125 mg (B), 175 mg (C) and comparison between the three best combinations and the AOAC method (D).
Overall, I have developed for the first time that partitioning salts (0.4 g MgSO₄ and 0.1 g NaOAc) and a cleanup sorbent mixture (157 mg MgSO₄, 9 mg PSA and 9 mg C18) provided the best peak response for the extraction of different pesticides using 1 mL of HM. This optimized QuEChERS method is further evaluated and validated in the following sections.

### 4.4.3 Evaluation of the optimized method using HM with different fat content

In reality, HM contains a wide range of fat, it is therefore essential to investigate the efficiency and robustness of the optimized method for the extraction of pesticides in HM samples with varying fat content. Pooled HM was processed into three aliquots (LFM, MFM and HFM) with the fat content of 26.1 g/L, 48.9 g/L and 83.8 g/L respectively. Preliminary evaluation on the influence of milk fat using the QuEChERS method was based on the recoveries of the spiked pesticides (50 ng/mL) in 10 mL of HM. It was observed that 85% of the pesticides fell within the recovery range of 70-120% regardless of the milk fat content (Figure 4.7A). However, a slight decrease in the recovery for some pesticides, such as HCB, beta-HCH, lindane, p,p'-DDE and phenothrin was observed with the increase of HM fat content, which was consistent with the findings in other high fatty food (Lehotay, et al., 2005b; Chamkasem, et al., 2013). When the optimized method was used to extract MFM spiked at varying concentrations of pesticides (5–50 ng/mL), it was observed that the recovery of most pesticides (81%) was within the range of 70–120% (Figure 4.7B). This demonstrates that this method is robust enough to extract various classes and concentrations of pesticides in 10 mL of HM.
The evaluation of this method was further examined by scaling down the volume of HM to only 1 mL, and we found that the recoveries of these pesticides were similar to that observed in 10 ml of HM (Figure 4.7C). For some pesticides, such as HCB, aldrin, cis-chlordane, p,p'-DDE, marginally better recoveries are observed in the LFM than that in HFM and MFM. Overall the RSD% for most of the 88 pesticides was lower than 20%, except for HCB, malathion, propanil, diclofop-methyl, cyfluthrin and cypermethrin. In the case of QC standards (acenaphthene-D$_{10}$, phenanthrene-D$_{10}$ and chrysene-D$_{12}$) added at 100 ng/mL to all samples in the study, their average recoveries were 96% (8%, RSD), 99 % (10%, RSD) and 84% (9%, RSD), respectively.

These studies demonstrate that this optimized QuEChERS method is reproducible and robust for pesticide analysis using 1 mL of HM of varying fat content that is commonly observed in the population. In order to ensure that this developed method was suitable for routine analysis, the analytical performance parameters, such as linearity, recovery (trueness) and precision as well as LCLD and LCLQ were determined.

### 4.4.4 Method validation

As demonstrated in our previous study (Chapter 3), solvent standards with APs mixture can be used to establish the calibration curves for pesticide quantitation in HM. Linearity of the calibration was confirmed from analysis of seven replicates of the pesticides in the range of 0.2–100 ng/mL. The majority of the pesticides gave linear regression ($R^2$) values greater than 0.99 except dichlorvos, propyzamide, methiocarb, propiconazole, fevalerate and deltamethrin (Table 4.1).
Figure 4.7 Recovery of the pesticides spiked in human milk with different fat content (LFM, MFM and HFM) using the optimized QuEChERS method. (A) Influence of fat content on the pesticides spiked (50 ng/mL) in 10 mL of HM; (B) Influence of different spiked concentrations (5–50 ng/mL) in 10 mL of HM; (C) Influence of fat content on the pesticides spiked (50 ng/mL) in 1 mL of HM; (D) Influence of different spiked concentrations (5–50 ng/mL) in 1 mL of HM. (Continues)
Figure 4.7 Recovery of the pesticides spiked in human milk with different fat content (LFM, MFM and HFM) using the optimized QuEChERS method. (A) Influence of fat content on the pesticides spiked (50 ng/mL) in 10 mL of HM; (B) Influence of different spiked concentrations (5–50 ng/mL) in 10 mL of HM; (C) Influence of fat content on the pesticides spiked (50 ng/mL) in 1 mL of HM; (D) Influence of different spiked concentrations (5–50 ng/mL) in 1 mL of HM.
Table 4.1 Method validation data. Pesticides, Linear ranges, Linear regression ($R^2$), Recoveries ($n = 7$), Relative standard deviations (RSD), LCLD and LCLQ of the 88 pesticides (except cypermethrin and cyfluthrin) obtained by GC-MS/MS analysis of human milk.

<table>
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<tr>
<th>Groups</th>
<th>Pesticide</th>
<th>Linear range (ng/mL)</th>
<th>$R^2$</th>
<th>10 ng/mL spiked level</th>
<th>20 ng/mL spiked level</th>
<th>100 ng/mL spiked level</th>
<th>LCLD (ng/mL)</th>
<th>LCLQ (ng/mL)</th>
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<td>LCLQ (ng/mL)</td>
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## Table 1

### Linear range, Recovery and RSD for QuEChERS methodology

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### Groups of Pesticides

A: Organochlorine pesticides (OCPs).
B: Organophosphate pesticides (OPPs).
C: Fungicides.
D: Carbamates and pyrethroids
E: Herbicides and other pesticides.

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The recovery study of this method was validated by spiking HM with three different concentrations at 10, 20 and 100 ng/mL in MFM (n = 7 replicates per level). As shown in Table 4.1, the average recoveries for most of the 88 pesticides quantified by GC-MS/MS were in the range of 64.1-126.7%, 68.4-117.7% and 76.4-115.3% for the spiked concentrations of 10, 20 and 100 ng/mL, respectively, which were within the acceptable ranges of 70–120%. For the troublesome lipophilic pesticides, such as HCB and p,p’-DDE, that have been reported previously with extremely low recoveries (< 50%) in fatty matrices (Lehotay, et al., 2005b; Koesukwiwat, et al., 2010), satisfactory recoveries were obtained in this study. The precision was investigated based on the reproducibility of seven replicates and reported in terms of RSD (%), and the RSDs were 3.6-33.9%, 2.2-17.4% and 2.5-13.5% for the spiked concentrations at 10, 20 and 100 ng/mL. While the validation was ran over a course of four continuous days, the QC standards (acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12}) added at 100 ng/mL to all samples were also monitored, and their RSDs were 15%, 12% and 21% throughout the entire injections.

The LCLD and LCLQ for each pesticide were also evaluated experimentally by injecting various concentrations of pesticides (0.2, 0.5 1, 2, 5 and 10 ng/mL) with APs mixture. LCLD and LCLQ were assessed based on the lowest experimental concentrations. The LCLD and LCLQ range for the various classes of pesticides such as OCPs (LCLD: 0.2–0.5 ng/mL; LCLQ: 0.2–1.0 ng/mL), OPPs (LCLD: 0.2–1.0 ng/mL; LCLQ: 0.2–2.0 ng/mL), fungicides (LCLD: 0.2–1.0 ng/mL; LCLQ: 0.2–2.0 ng/mL), carbamates and pyrethroids (LCLD: 0.2–2.0 ng/mL; LCLQ: 0.5–5.0 ng/mL), herbicides and other pesticides (LCLD: 0.2–1.0 ng/mL; LCLQ: 0.2–2.0 ng/mL) were observed (Table 4.1). These LCLD and LCLQ values are lower than currently reported method using traditional extraction techniques in HM (Kumar, et al., 2006; Bordajandi, et al.,
2008; Zhou, et al., 2012). In addition, our method also significantly reduces the requirement of solvent and HM, simplifies the sample preparation, and improves the recovery of multiresidue lipophilic pesticides.

4.5 Conclusions

In this study, a rapid and efficient QuEChERS method with GC-MS/MS has been optimized and validated for the simultaneous analysis of 88 different classes of pesticides in HM. The validate method using extraction salts (0.4 g MgSO$_4$ and 0.1 g NaOAc) and a cleanup sorbent mixture (157 mg MgSO$_4$, 9 mg PSA and 9 mg C18) gave the best recovery with just 1 mL of HM. This method was demonstrated to be robust and reproducible in extracting HM of varying fat content and pesticide concentrations. The recoveries, linearity and LOQs were satisfactory for simultaneous identification and quantitation of the pesticides in HM. The current method will be used to monitor the pesticides in HM from Western Australian women in a cross-sectional (Chapter 5) and longitudinal study (Chapter 6).

4.6 Acknowledgements

J. Du sincerely appreciates the International Postgraduate Research Scholarships (IPRS) and Australian Postgraduate Award (APA). This work was also supported by an unrestricted research grant from Medela AG. The authors gratefully acknowledge Bruker for the support for this project with the provision of the SCION GC/MS/MS system used for all the measurements. The authors gratefully acknowledge the support of the NCRIS scheme through Bioplatforms Australia and Metabolomics Australia for pesticide research.
Chapter 5 Low concentrations of pesticides in human milk of Western Australian women and its influence on infant growth outcomes: a cross-sectional study

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5.1 Abstract

Persistent organic pollutants in human milk (HM) at high levels are considered to be detrimental to the breastfed infant. To determine the pesticide concentration in HM, a cross-sectional study of 40 Western Australian (WA) women was carried out. Gas chromatography tandem mass spectrometry (GC-MS/MS) with a validated QuEChERS was used for the analysis of 88 pesticides in HM. \( p,p' \)-dichlorodiphenyldichloroethylene (\( p,p' \)-DDE) with a mean concentration of 62.8±54.5 ng/g fat was found, whereas other OCPs, OPPs, carbamates and pyrethroids were not detected in HM. Overall, no association was observed between HM \( p,p' \)-DDE concentrations and maternal age,
parity, body mass index and percentage fat mass. Furthermore, for the first time no significant association was found between \( p,p' \)-DDE concentrations in HM and infant growth outcomes, such as weight, length, head circumference and percentage fat mass. The calculated daily intake was significantly different to the estimated daily intake of total DDTs and was well below the guideline proposed by WHO. The DDTs levels in WA have also significantly decreased by 42-fold since the 1970s and are currently the lowest in Australia. Future research, such as a longitudinal study, is needed to investigate the long-term influence of these pesticides in HM on infant growth outcome.

5.2 Introduction

Persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), pyrethroids and carbamate pesticides are widely used in agricultural practice (Köhler & Triebskorn, 2013). Many of these pesticides are resistant to chemical, physical and biological degradation, thus they are ubiquitously found in the environment despite restrictions on their use. Besides being effective in eradicating pests, many of the pesticides are also harmful to human health. Thus, there is a widespread of concern about the potential health effects of POPs in HM on infant growth and development. To date, most studies have used prenatal pesticides exposure and infant anthropometrical size at birth as a proxy measure of \textit{in utero} development, and have failed to yield consistent results (Mazdai, et al., 2003; Wu, et al., 2010; Stasinska, et al., 2014). To the best of our knowledge, little is known about the effects of the pesticides in HM on the infant growth outcomes during breastfeeding.

In Australia, dichlorodiphenyltrichloroethane (DDT) was first introduced in the 1940s and other pesticides such as heptachlor, aldrin, dieldrin, chlordane,
hexachlorocyclohexane (HCH) and hexachlorobenzene (HCB) were registered for use in the 1970s (Reid, et al., 2013). All new houses in Western Australia (WA) were treated with pesticides during the early stages of construction in order to eradicate termites (Stacey & Tatum, 1985). Since the 1970s, production and application of DDT and most pesticides were restricted and prohibited in WA (Stacey, et al., 1985). However, as many of these POPs have long half-lives and high fat solubility properties, they tend to bio-magnify in wildlife, especially in species at the top of food chain, such as animals and humans (Radcliffe, 2002; Tanabe, 2002). As the presence of POPs can interfere with the function of normal endocrine system and the immature defense mechanism of the developing fetus and infants, making them more vulnerable to these pesticides than the adults (Soto, et al., 1995). POPs exposure prenatally via the placenta and postnatally via breastfeeding may result in delayed development, immune deficiency, abnormal behavior and growth retardation (Eskenazi, et al., 2006a; Chao, et al., 2007; Koureas, et al., 2012). Surprisingly, there has been no investigation into the relationship between the POPs concentrations in HM and the breastfed infant growth outcomes. As HM is the sole natural food for infants particular at the early age, studies about the possible influence of POPs in HM on infant growth outcomes are warranted. Previous studies have detected POPs in HM from WA, and have observed a decline trend of POPs concentrations in HM (Stacey & Thomas, 1975; Stacey, et al., 1985; Stevens, et al., 1993). The most recent study of POPs concentrations in WA was based on a single pooled HM sample from 11 women in 2003, which could not represent the POPs concentrations for individual mothers and the current concentration in the general population (Mueller, et al., 2008). Since then, no further studies of these levels have been reported. Based on the public concern about the current status of the contamination and safety of HM, it is essential to continue monitoring the POPs in WA.
The aims of this study were to (1) describe the current pesticide concentrations in HM from WA mothers and the changes in these pesticide concentrations during the first year of lactation in a cross-sectional cohort; (2) investigate associations between the detected pesticides and maternal and infant characteristics and anthropometrics; (3) calculate the daily intake of the pesticides by individual infants and evaluate the risk to the infant.

5.3 Materials and methods

5.3.1 Chemicals

The pesticide standard solutions (100 µg/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingstown, RI, USA). The pesticide standard solutions (100 µg/mL) were mixed and diluted with acetonitrile (ACN) to prepare a stock standard solution (1 µg/mL) of all the pesticides. LC-MS grade acetonitrile (ACN) and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylglycerol (98%), gulonolactone (95%) and D-sorbitol (≥98%) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (>99.0%) and magnesium sulfate (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecyl-bonded silica (C18) and primary secondary amine (PSA) were obtained from Agilent (Little Falls, DE, USA). Isotopically labeled quality control (QC) standards, acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12} were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).
5.3.2 Study population and sample collection

The study was approved by the Human Research Ethics Committee of The University of Western Australia. Breastfeeding dyads (n=40) were recruited in WA between 2013 and 2015. These mothers volunteered to participate in this study and provide a milk sample in one of the following months of lactation: 2 (n=11), 5 (n=9), 9 (n=10) or 12 months (n=10). Milk samples (1-5 mL) were collected in glass containers before and after feeding from each breast. The fat content of HM was measured immediately using the Creamatocrit method (Meier, et al., 2002), and the remaining milk was stored at -20°C. All participants provided informed written consent and completed a questionnaire including relevant demographic data.

5.3.3 HM sample treatment

HM samples (n=40) were thawed at room temperature for 3 hours and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 s. 1 mL of HM was placed into a 15 mL centrifuge tube and 1 mL ACN containing 1% HOAc and 100 ng/mL QC standards (acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12}) was added. The validated acetate buffered QuEChERS method was employed to extract the HM. Extraction reagents (0.4 g MgSO_{4} and 0.1 g NaOAc) were added and shaken immediately, and the tube was then placed in an ice bath. The extraction tube was centrifuged at 3993 x g for 10 min. 0.6 mL of the extract was transferred into a clean 15 mL centrifuge tube and stored at -20°C for 2 hours. The extract was then centrifuged at 3993 x g (0°C) for 10 min and 0.5 mL was transferred to a cleanup tube (157 mg MgSO_{4}, 9 mg C18 and 9 mg PSA). The tube was vortexed and centrifuged at 3993 x g for 10 min. The final extract was transferred into a screw cap amber vial and kept at -80°C until analysis.
5.3.4 Preparation of working standard solutions

The working standard solutions (0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL) were prepared by appropriate dilution of the stock standard solution (1 µg/mL) with ACN. A combination of APs mixture (ethylglycerol, gulonolactone and D-sorbitol) containing internal standard TPP (IS) was added to the final extract of each HM sample and to all working standard solutions for GC-MS/MS analysis. The final concentrations of ethylglycerol, gulonolactone and D-sorbitol were 20, 2 and 2 mg/mL, respectively. As demonstrated in our previous study, the addition of APs mixture can equalize the enhancement difference between the pesticides in pure solvent and in HM extract, which can then be used for the quantification of the pesticides in HM.

5.3.5 GC-MS/MS analysis

Chromatographic separation and determination of the pesticides were carried on a Bruker Daltonics 450 gas chromatography (GC) with a Bruker Daltonics Scion TQ triple quadrupole mass spectrometer (MS) (Billerica, MA, USA), a Bruker 1177 Split/Splitless injector and a Combi-Pal autosampler (CTC Analytics AG, Switzerland). Sky 4.0 ID precision inlet liners with wool from Restek (Bellefonte, PA, USA) were used. Injection was performed at pulsed splitless mode (30 psi) with an injection volume of 2 µL. For GC separation, a Rtx-5MS with Integra-Guard column (10 m + 30 m x 0.25 mm x 0.25 µm) (Bellefonte, PA, USA) was used. The inlet temperature was kept constant at 250°C. The initial oven temperature of the column was 80°C for 3 min and ramped at 30°C/min to 150°C and then at 10°C/min to 300°C, where it was held for 10 min. The total run time was 31 min. Helium (6.0 GC grade) was used as carrier gas at a constant flow of 1.1 mL/min.
The mass spectrometer was operated in EI mode. Transfer line and ion source temperature were at 270°C and 200°C, respectively. Argon was used as the collision gas. Identification and quantification of 88 pesticides were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. Data collection and processing were performed using Bruker MSWS 8 Software.

5.3.6 Quality control

Isotopically labeled quality control (QC) standards were employed to evaluate the efficiency of the extraction and cleanup steps, while TPP (IS) was used to evaluate the performance of the instrument throughout the entire analytical procedure. A QC mixture containing pesticide standards (20 ng/mL) was analyzed between sample batches to check for any interferences and cross-contaminations. The recoveries for the pesticides were within the range of 70–120% with the relative standard deviations (RSD, n=7) of 3.6-33.9%, 2.2-17.4% and 2.5-13.5% for spiked concentrations of 10, 20 and 100 ng/mL, respectively. Limit of detection (LOD) and limit of quantitation (LOQ) were determined experimentally with the lowest standards containing APs.

5.3.7 Maternal and infant anthropometric measurements

Maternal body weight was measured using an electronic scale (Seca, California, USA, accuracy 0.1 kg). The height, age and parity were self-reported by participants. Maternal BMI was calculated according to the following formula: BMI = kg/(m²).

Single measurements of infant weight, length and head circumference were performed at each time point when the milk sample was collected. Infant weight was determined by weighing before breastfeeding using electronic scales (±2.0 g; Medela Electronic Baby Weigh Scales, Medela AG, Switzerland). Infant crown-heel length was measured,
to the nearest 0.1 cm, on a hard surface with perpendicular to the surface headpiece and footpiece and a non-stretch tape. Infant head circumference was measured with non-stretch tape.

24 – hour infant milk intake was determined by test weighing (Arthur, et al., 1987). Mothers weighed their infants before and after each feed from each breast using electronic scales (±2.0 g; Medela Electronic Baby Weigh Scales, Medela AG, Switzerland) for a period of 24 hours plus 2 extra breastfeeding sessions. All measurements of milk intake are expressed in grams, which are considered to be equivalent to mL (density of milk: 1.03 g/mL). The daily calculated POPs intake for each infant in this study was calculated based on these data where available, otherwise previous 24 – hour milk intake values (900, 899, 520 and 218 mL at 2, 5, 9 and 12 months, respectively) by Kent et al. were used instead (Kent, et al., 1999).

5.3.8 Maternal and infant body composition measurements

Whole body bioimpedance (wrist to ankle) of the participants were measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia).

Mothers’ whole body bioimpedance was measured according to the manufacturer’s instructions.

Infants’ whole body bioimpedance was measured using an adult protocol (wrist to ankle) and analyzed with settings customized for each infant according to Lingwood et al. (Lingwood, et al., 2012). Resistance at 50 kHz was used in percentage fat mass equations developed for infants (Bocage, 1988; Lingwood, et al., 2012).

Infants’ ultrasound skinfold measurements (triceps and subscapular) were carried out using Aplio XG (Toshiba, Japan) machine, PLT-1204BX 14-8 MHz transducer and
sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). The double skinfold thickness, measured directly from images using the on screen electronic calipers, was used in percentage fat mass equations developed for skinfolds measured with skinfold calipers (Slaughter, et al., 1988).

### 5.3.9 Statistical analysis

Statistical analyses were carried out either using SPSS software (SPSS, version 19.0 for windows, SPSS, Inc, IL, USA) or RStudio 0.99.896 (Pinheiro, et al., 2009). The results were expressed as mean ±SD unless stated otherwise. Pesticides that were below the LOD were considered as not detected and were not included in the calculations. Detected pesticides were normalized to the fat content in the milk (ng/g fat). Overall interactions between \( p,p' \)-DDE and both maternal and infant characteristics were carried out using linear model and Pearson correlation coefficients were used to determine the correlation between \( p,p' \)-DDE and the individual maternal and infant characteristics. Differences was considered to be significant if \( P < 0.05 \).

Estimated daily intake (EDI) (µg/kg body wt./day) of the pesticides for each infant was calculated based on the pesticides concentration in HM (µg/g fat; \( C_{\text{pesticide}} \)), fat content in HM (g/mL; \( C_{\text{fat}} \)), average daily consumption of HM (mL; \( V_{\text{milk}} \)) and body weight of infant (kg; \( M_{\text{infant}} \)) using the following equation:

\[
\text{EDI} = \frac{C_{\text{pesticide}} \times C_{\text{fat}} \times V_{\text{milk}}}{M_{\text{infant}}}
\]

One-way ANOVA and Tukey’s all pair comparison tests were used to compare differences in pesticides concentration, EDI and calculated daily intake (CDI) at the
different lactation months (Hothorn, et al., 2008). A paired t-test was used to compare the differences between the EDI and the CDI by the infant at various lactation months.

### 5.4 Results and Discussion

#### 5.4.1 Participant demographics

The demographic characteristics of the study participants (n=40) are presented in Table 5.1. The average age (±SD) of the mothers was 33.3±3.8 years old and the median parity was 2.0. The maternal characteristics, such as HM fat and maternal BMI measured in this study is similar to previous HM POPs studies enabling comparison between different populations (Polder, et al., 2009; Bedi, et al., 2013).

#### 5.4.2 Pesticide residues in HM

A total of 88 pesticides, including OCPs, OPPs, carbamates and pyrethroids, were analyzed in this study. Overall, only p,p′-DDE was detected in 87.5% of the HM samples in this cohort with a mean concentration (±SD) of 1.9±1.9 ng/mL (range: 0.2-10.1 ng/mL) and 62.8±54.5 ng/g fat (range: 6.3-209.2 ng/g fat). The distribution of p,p′-DDE in HM at different lactation months is shown in Figure 5.1. The large variation in p,p′-DDE levels observed between months in this cohort could be due to variation in lifestyles, dietary habits and metabolic activity between mothers (Waliszewski, et al., 2001). The dynamic distribution of the pesticides through the blood circulatory system and selective partitioning from plasma to the breast could also contribute to the observed variation (Shen, et al., 2007). These factors may account for the lack of significant decrease of p,p′-DDE in HM observed between the lactation months in this cohort (Table 5.2).
Table 5.1 Characteristics of participants in a cross-sectional study measuring the concentrations of pesticides in human milk collected from women at 2, 5, 9 and 12 months postpartum in Western Australia.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Months</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers (n = 40)</td>
<td>Age</td>
<td>31.8 ± 4.3</td>
<td>33.9 ± 2.2</td>
<td>34.4 ± 4.0</td>
<td>33.2 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>Parity⁵</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>HM fat content (g/L)</td>
<td>24.6 ± 8.9</td>
<td>26.2 ± 14.5</td>
<td>36.7 ± 17.4</td>
<td>45.0 ± 17.9</td>
</tr>
<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td>26.3 ± 5.7</td>
<td>22.8 ± 5.5</td>
<td>23.4 ± 5.4</td>
<td>26.0 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>Percentage fat mass (BIS⁶)</td>
<td>35.5 ± 3.5</td>
<td>31.5 ± 5.7</td>
<td>27.8 ± 8.8</td>
<td>34.0 ± 7.4</td>
</tr>
</tbody>
</table>

Infant

| Gender (M/F) | 7/4 | 6/3 | 9/1 | 4/6 |
| Infant weight (kg)* | 5.2 ± 0.7 | 7.4 ± 0.9 | 9.0 ± 0.8 | 9.6 ± 0.6 |
| Body length (cm)* | 57.1 ± 2.4 | 64.6 ± 2.0 | 72.1 ± 1.6 | 73.3 ± 1.9 |
| Head circumference (cm)* | 39.3 ± 1.3 | 43.4 ± 1.0 | 45.7 ± 1.3 | 46.4 ± 1.3 |
| Percentage fat mass (BIS⁶)** | 19.9 ± 3.8 | 27.5 ± 3.7 | 23.7 ± 2.4 | 23.3 ± 4.3 |
| Percentage fat mass (ultrasound skinfolds) | 25.6 ± 4.2 | 27.2 ± 4.2 | 25.3 ± 6.1 | 27.4 ± 5.2 |

⁵ Expressed as median,
⁶ BIS: bioimpedance spectroscopy

* Significant difference \( (P < 0.05) \) observed between months 2-5, 2-9, 2-12, 5-9 and 5-12.

** Significant difference \( (P < 0.05) \) observed between months 2-5.
**Figure 5.1** Distribution of \( p,p' \)-DDE concentrations (ng/g fat) in human milk between 2, 5, 9 and 12 months of lactation in Western Australian women. Values of \( p,p' \)-DDE are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o).

**Table 5.2** Summary of detected \( p,p' \)-DDE in human milk collected at 2, 5, 9 and 12 months postpartum. No significant difference was observed of \( p,p' \)-DDE between lactation months*.

| Lactation months | No. Of Samples | Frequency of detection (%) | Mean ± SD (ng/g fat)  
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Range)</td>
</tr>
<tr>
<td>2 months</td>
<td>11</td>
<td>9 (81.8 %)</td>
<td>79.7 ± 72.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(14.6 - 209.2)</td>
</tr>
<tr>
<td>5 months</td>
<td>9</td>
<td>8 (88.9 %)</td>
<td>79.9 ± 60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(19.1 - 188.3)</td>
</tr>
<tr>
<td>9 months</td>
<td>10</td>
<td>8 (80.0 %)</td>
<td>51.5 ± 52.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6.3 - 165.0)</td>
</tr>
<tr>
<td>12 months</td>
<td>10</td>
<td>10 (100 %)</td>
<td>43.0 ± 27.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(17.5 - 108.5)</td>
</tr>
</tbody>
</table>

* \( P \)-values for \( p,p' \)-DDE in HM between each lactation months 2–5 (1.00), 2–9 (0.71), 2–12 (0.47), 5–9 (0.72), 5–12 (0.49) and 9–12 (0.9).
5.4.3 Associations between pesticide residues in HM and both maternal and infant characteristics

Many studies have collected HM in the first 6 months postpartum (Zietz, et al., 2008; Bouwman, et al., 2012). To our knowledge, this is the first study that had collected HM throughout the first year of lactation and investigated the relationship between POPs levels in HM and both maternal and infant characteristics. The interactions between HM $p,p'$-DDE and both maternal and infant anthropometrics were evaluated and no significant associations ($P = 0.44$) were observed, therefore all the samples were pooled and analyzed as a whole ($n = 40$).

There was no significant association between HM $p,p'$-DDE and both maternal age ($P = 0.42$) and parity ($P = 0.36$). Further no significant association was also observed between $p,p'$-DDE levels and maternal BMI ($P = 0.64$). BMI does not differentiate between fat mass and fat-free mass, but measurement of maternal body composition (BC) or percentage fat mass using bioimpedance spectroscopy (BIS), is more accurate in assessing body fat (Bemben, et al., 1998). As pesticides are related to body fat, this is the first study that investigates the relationship between pesticides and maternal percentage fat mass. No significant association was observed between $p,p'$-DDE levels and maternal percentage fat mass ($P = 0.67$) (Table 5.3). There was no significant difference in percentage fat mass at different lactation months ($P = 0.07$) in this cross-sectional cohort. This is the first study that investigated the possible relationships between the detected pesticides in HM and maternal BC.
Table 5.3 Relationship between *p,p*-DDE concentrations in human milk and both maternal and infant characteristics, expressed as the correlation coefficient (r) and *P* value. Correlation is significant at the *P* value < 0.05.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Correlation coefficient r (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.14 (0.42)</td>
</tr>
<tr>
<td>Parity</td>
<td>-0.18 (0.36)</td>
</tr>
<tr>
<td>Maternal body mass index (BMI) (kg.m⁻²)</td>
<td>-0.08 (0.64)</td>
</tr>
<tr>
<td>Percentage fat mass</td>
<td>0.09 (0.67)</td>
</tr>
<tr>
<td><strong>Baby demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>-0.14 (0.43)</td>
</tr>
<tr>
<td>Body length (cm)</td>
<td>-0.20 (0.26)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>-0.11 (0.54)</td>
</tr>
<tr>
<td>Percentage fat mass (BIS*)</td>
<td>0.25 (0.16)</td>
</tr>
<tr>
<td>Percentage fat mass (ultrasound skinfolds)</td>
<td>0.09 (0.63)</td>
</tr>
</tbody>
</table>

*BIS: bioelectrical impedance spectroscopy*

### 5.4.4 Adverse effects of pesticide residues on infant growth outcomes

No significant associations were observed between *p,p*-DDE concentrations in HM and infant weight (*P* = 0.43), body length (*P* = 0.26), head circumference (*P* = 0.54) and percentage fat mass (ultrasound: *P* = 0.63; BIS: *P* = 0.16) during breastfeeding. No significant associations were observed between the detected *p,p*-DDE and infant growth outcomes in this cohort, even with more specific body fat measurements, using BIS and ultrasound (Table 5.3). To the best of our knowledge, this is the first study that has assessed possible effects of POPs in HM on the infant growth outcomes during breastfeeding. It is crucial that no adverse effects were observed between HM POPs and
infant growth outcomes as several studies have associated smaller head circumference during the first year of life to poorer cognitive abilities and school performance at the age of 8 (Hack, et al., 1991; Perera, et al., 2003).

Few studies have examined the relationship between HM POPs and infant growth due to the difficulties in collecting both HM and infant growth data at each lactation months. However, current studies have examined infant birth outcomes, such as birth weight, length and chest circumferences with prenatal POPs exposure, which failed to yield consistent results (Eskenazi, et al., 2004; Whyatt, et al., 2004; Wolff, et al., 2007). As HM is the sole food source for an exclusively breastfed infant for the first 4 to 6 months of life, detected pesticides in HM will more accurately reflect the influence of these pesticides on the infant growth.

However, due to the lack of interaction and associations between HM p,p’-DDE and both maternal BC and infant anthropometrics in this study, the drawbacks of the cross sectional study are highlighted where the concentration of these pesticides and the infant anthropometrics are not determined at different time points within infants. Thus, it is essential that a longitudinal study be performed to monitor the influence of these pesticides on the individual infant throughout the breastfeeding.

5.4.5 Infant health risk assessment

The most direct concern with pesticides in HM is the potential detrimental exposure to the infant, which ideally requires knowledge of dose to the infant rather than concentration in isolation. Studies to date have used constant values for $C_{fat}$, $V_{milk}$ and $M_{infant}$ in the calculation of EDI, which is based on the assumption that a 5 kg infant will
Ingest 700 mL of HM per day containing 3% fat (Van Oostdam, et al., 1999; Behrooz, et al., 2009; Bedi, et al., 2013). However, these studies did not consider the large variations of $C_{fat}$, $V_{milk}$ and $M_{infant}$ between individual infants at different lactation months. In this study, the calculated daily intake (CDI) of DDTs (sum of DDT and its metabolites, DDE and DDD) is based on the actual measured values ($C_{fat}$, $C_{pesticide}$ and $M_{infant}$) for each infant. A larger decrease of ingested DDTs was observed in the CDI, with a significant difference ($P = 0.03$) between 2 and 12 months, as compared to the EDI ($P = 0.47$). The overall CDI and EDI for DDTs by the breastfed infants throughout the first year of lactation were 0.17 µg/kg body wt./day (range: 0.01-1.04 µg/kg body wt./day) and 0.26 µg/kg body wt./day (range: 0.03-0.88 µg/kg body wt./day) respectively (Table 5.4). A significant difference ($P < 0.01$) was observed between the overall EDI and CDI, where EDI overestimated the intake value by 52%. Smaller overestimations (10–22%) were observed at 2 and 5 months between CDI and EDI, while the later 9 and 12 months showed more dramatic overestimations (110-350%) for EDI.

These results clearly demonstrate the significant difference between CDI and EDI, which indicates that CDI should be used where possible to reflect the actual pesticide intake by the infants, particularly in the later months. The overall CDI of DDTs by an infant in this study is 59- and 117- fold below the recommended tolerable daily intake (TDI) by FAO/WHO (FAO/WHO, 2000) and Health Canada (Van Oostdam, et al., 1999), respectively. Low level of DDTs detected in HM from WA poses minimal risk to the infant, which also further confirms the findings between HM $p,p'$-DDE and infant growth outcomes in this study.
Table 5.4 Calculated daily intake (CDI) and estimated daily intake (EDI) of DDTs by infants in Western Australia in comparison to tolerable daily intake (TDI) proposed by Health Canada and FAO/WHO.

<table>
<thead>
<tr>
<th></th>
<th>CDI (µg/kg body wt./day)</th>
<th>EDI (µg/kg body wt./day)</th>
<th>P value *</th>
<th>TDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Range)</td>
<td>(Range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data</td>
<td>0.17</td>
<td>0.26</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.01 - 1.04)</td>
<td>(0.03 – 0.88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 months</td>
<td>0.30</td>
<td>0.33</td>
<td>0.49</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>(0.09 - 0.65)</td>
<td>(0.06 - 0.88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 months</td>
<td>0.27</td>
<td>0.33</td>
<td>0.34</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>(0.09 - 1.04)</td>
<td>(0.08 - 0.79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>0.10</td>
<td>0.21</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.01 - 0.29)</td>
<td>(0.03 - 0.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>0.04</td>
<td>0.18</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.01 - 0.09)</td>
<td>(0.03 - 0.88)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


b Health Canada (Van Oostdam, et al., 1999).

c Paired t-test was used to compare the differences between the EDI and the CDI by the infant at various lactation months. Differences are considered to be significant if P < 0.05.

5.4.6 Temporal trends of pesticides in HM

Historically the extensive use of DDT resulted in extraordinary high DDTs concentrations in HM from WA (Table 5.5) (Miller & Fox, 1973; Stacey & Thomas, 1975; Stacey, et al., 1985; Stacey & Tatum, 1985; Stevens, et al., 1993; Smith, 1999). A significant downward trend of DDTs (42-fold decrease) in HM can be observed since 1970, from 2660 ng/g fat to 63 ng/g fat in this study (Figure 5.2). When compared with the most recent data of DDTs in WA conducted in 2002/03 (Mueller, et al., 2008), the levels of DDTs have decreased by 4-fold. c
This declining trend reflects the decrease of human exposure to DDT in WA which is consistent with the worldwide decreasing trend in DDT body load (Smith, 1999). Our findings are also in agreement with the trace levels of \( p,p' \)-DDE observed in maternal plasma (1.05 ng/mL) (Reid, et al., 2013), blood (0.5 ng/mL) and abdominal tissues (70 ng/g fat) of WA participants. Other studies have also found trace (< 0.001 ng/g) or no detectable pesticides in sediment, mussels and in the effluent or associated biota in WA’s three treated ocean outfalls (Chegwidden, 1979). All these findings demonstrated the effectiveness of strict prohibition of these pesticides in WA leading to the decrease of human exposure to these pesticides over time.

![Figure 5.2](image-url)  

**Figure 5.2** Historical trend of mean total DDTs levels in human milk of WA mothers between 1970s and 2010s (Stacey & Thomas, 1975; Stacey, et al., 1985; Stevens, et al., 1993; Mueller, et al., 2008), while 2010s are results from this study.
### Table 5.5 Comparison of DDTs, β-HCH and HCB residue levels (ng/g fat) in human milk in Australia.

<table>
<thead>
<tr>
<th>Region</th>
<th>Year of sampling</th>
<th>Mothers (N)</th>
<th>Human milk samples</th>
<th>DDT</th>
<th>DDE</th>
<th>DDE/DDT</th>
<th>DDD</th>
<th>Total DDT</th>
<th>β-HCH</th>
<th>HCB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Victoria</td>
<td>1970</td>
<td>39</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>4630a</td>
<td>na</td>
<td>na</td>
<td>(Newton &amp; Greene, 1972; Smith, 1999)</td>
</tr>
<tr>
<td>Western Australia</td>
<td>1970/71</td>
<td>22</td>
<td>23</td>
<td>10b</td>
<td>61b</td>
<td>6</td>
<td>na</td>
<td>2660c</td>
<td>na</td>
<td>25b</td>
<td>(Stacey &amp; Thomas, 1975; Smith, 1999)</td>
</tr>
<tr>
<td>Queensland (Urban)</td>
<td>1971/72</td>
<td>20</td>
<td>20</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>8600</td>
<td>na</td>
<td>na</td>
<td>(Miller &amp; Fox, 1973)</td>
</tr>
<tr>
<td>Queensland (Rural)</td>
<td>1971/72</td>
<td>20</td>
<td>20</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>16900</td>
<td>na</td>
<td>na</td>
<td>(Miller &amp; Fox, 1973)</td>
</tr>
<tr>
<td>Western Australia (Urban)</td>
<td>1979/80</td>
<td>45</td>
<td>130</td>
<td>12b</td>
<td>30b</td>
<td>3</td>
<td>na</td>
<td>1277a</td>
<td>1b</td>
<td>8b</td>
<td>(Stacey, et al., 1985; Smith, 1999)</td>
</tr>
<tr>
<td>Western Australia (Rural)</td>
<td>1979/80</td>
<td>95</td>
<td>137</td>
<td>14b</td>
<td>27b</td>
<td>2</td>
<td>na</td>
<td>1281a</td>
<td>na</td>
<td>7b</td>
<td>(Stacey, et al., 1985; Smith, 1999)</td>
</tr>
<tr>
<td>Western Australia</td>
<td>1980</td>
<td>14</td>
<td>14</td>
<td>7b</td>
<td>34b</td>
<td>5</td>
<td>na</td>
<td>1680a</td>
<td>1b</td>
<td>9b</td>
<td>(Stacey &amp; Tatum, 1985; Smith, 1999)</td>
</tr>
<tr>
<td>Western Australia</td>
<td>1990/91</td>
<td>31</td>
<td>128</td>
<td>Na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>800c</td>
<td>na</td>
<td>na</td>
<td>(Stevens, et al., 1993)</td>
</tr>
<tr>
<td>Victoria</td>
<td>1993</td>
<td>17</td>
<td>60</td>
<td>225</td>
<td>960</td>
<td>4</td>
<td>na</td>
<td>1185c</td>
<td>345</td>
<td>411</td>
<td>(Quinsey, et al., 1995)</td>
</tr>
<tr>
<td>Western Australia</td>
<td>2002/03</td>
<td>na</td>
<td>8</td>
<td>250</td>
<td>33</td>
<td>0.1</td>
<td>0.1</td>
<td>258e</td>
<td>21</td>
<td>17</td>
<td>(Mueller, et al., 2008)</td>
</tr>
<tr>
<td>All regions</td>
<td>2002/03</td>
<td>na</td>
<td>17</td>
<td>9</td>
<td>310</td>
<td>37</td>
<td>0.2</td>
<td>320f</td>
<td>21</td>
<td>18</td>
<td>(Mueller, et al., 2008)</td>
</tr>
<tr>
<td>Western Australia</td>
<td>2013/15</td>
<td>40</td>
<td>40</td>
<td>na</td>
<td>62.8</td>
<td>na</td>
<td>na</td>
<td>63</td>
<td>na</td>
<td>na</td>
<td>Present study</td>
</tr>
</tbody>
</table>

na: not applicable.

Data from Smith et al. 1999.

Whole milk basis (ng/mL milk).

Pooled milk.
5.4.7 National and international comparisons

Residue levels in HM were compared with those reported in Australia as well as those reported from different countries in order to evaluate the current magnitude of DDTs in HM in WA. There is limited recent data about the pesticides in HM in Australia (Noakes, et al., 2006; Mueller, et al., 2008). When compared with national data, the HM concentration of \( p,p' \)-DDE in current study is 2.4-7.5 times lower than corresponding levels in HM from other States/Territories such as Tasmania, Adelaide and Melbourne (Figure 5.3).

![Figure 5.3](image)

**Figure 5.3** Comparison of \( p,p' \)-DDE levels in HM from this study with various regions and territories of Australia conducted in 2002/2003 (data adapted from Mueller et al. 2008).

This spatial variation in Australia could be due to the inconsistent legislation to pesticides control in various states before the 1990s. In addition, differences in dietary habits and living environments between the States may also contribute to the variations. When compared with international data, residue levels of DDTs in WA milk are similar
to those reported for Norway (Polder, et al., 2009), Denmark (Shen, et al., 2007), Belgium (Croes, et al., 2012) and USA (Johnson-Restrepo, et al., 2007), and are a few orders of magnitude lower than those found for some Asian and Africa countries such as India (Bedi, et al., 2013), Iran (Behrooz, et al., 2009), South Africa (Darnerud, et al., 2011) and Ethiopia (Gebremichael, et al., 2013) (Table 5.6).

Due to their close proximity, New Zealand and Australia have adopted similar legislations and regulations. However, the concentration of DDTs observed in New Zealand is 5-fold higher than the levels found in the current study, suggesting that more extensive historical DDT use in New Zealand (Mannetje, et al., 2013). In some developing countries, such as India and African countries, limited quantity of DDT is still allowed to be used indoor for vector control (e.g. malaria) (Mandavilli, 2006). Thus, DDTs levels of HM from these countries, such as South Africa, Ethiopia, Iran and India were obviously much higher (Table 5.6). In general, DDTs levels in HM appear to be higher in developing countries than those in developed countries.

Table 5.6 Concentrations of DDTs (ng/g fat) in human milk from various countries.

<table>
<thead>
<tr>
<th>Country/region</th>
<th>Year of sampling</th>
<th>Mothers (N)</th>
<th>Human milk samples</th>
<th>Fat content (g/L)</th>
<th>DDTs (ng/g fat)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>2006/07</td>
<td>60</td>
<td>60</td>
<td>31</td>
<td>583&lt;sup&gt;a, *&lt;/sup&gt;</td>
<td>(Leng, et al., 2009)</td>
</tr>
<tr>
<td>India</td>
<td>2011</td>
<td>53</td>
<td>53</td>
<td>32</td>
<td>1914&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Bedi, et al., 2013)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>2000/01</td>
<td>36</td>
<td>36</td>
<td>31</td>
<td>333&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Chao, et al., 2006)</td>
</tr>
<tr>
<td>Korean</td>
<td>2008</td>
<td>&gt;50</td>
<td>50</td>
<td>22</td>
<td>225&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Kim, et al., 2013)</td>
</tr>
<tr>
<td>Japan</td>
<td>2008/09</td>
<td>90</td>
<td>9&lt;sup&gt;#&lt;/sup&gt;</td>
<td>32</td>
<td>119&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(Fujii, et al., 2011)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>2003</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>1600&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(Sudaryanto, et al., 2005)</td>
</tr>
<tr>
<td>Philippine</td>
<td>2004</td>
<td>33</td>
<td>33</td>
<td>22</td>
<td>170&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Malarvannan, et al., 2009)</td>
</tr>
<tr>
<td>Turkey</td>
<td>2009</td>
<td>47</td>
<td>47</td>
<td>36</td>
<td>338&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(Çok, et al., 2012)</td>
</tr>
<tr>
<td>Iran</td>
<td>2006</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>3560&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(Behrooz, et al., 2009)</td>
</tr>
<tr>
<td>Country/region</td>
<td>Year of sampling</td>
<td>Mothers (N)</td>
<td>Human milk samples</td>
<td>Fat content (g/L)</td>
<td>DDTs (ng/g fat)</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------</td>
<td>-------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>----------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Australian and New Zealand</td>
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<td></td>
<td></td>
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<td></td>
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<td>New Zealand</td>
<td>2007/2010</td>
<td>39</td>
<td>37</td>
<td>39</td>
<td>385&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(Mannetje, et al., 2013)</td>
</tr>
<tr>
<td>Australia/WA</td>
<td>2013/15</td>
<td>40</td>
<td>40</td>
<td>33</td>
<td>63&lt;sup&gt;g&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Poland</td>
<td>2002/05</td>
<td>28</td>
<td>28</td>
<td>14</td>
<td>1621&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Hernik, et al., 2011)</td>
</tr>
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<td>2003</td>
<td>12</td>
<td>12</td>
<td>27</td>
<td>665&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(Yu, et al., 2007)</td>
</tr>
<tr>
<td>Norway</td>
<td>2002/06</td>
<td>377</td>
<td>377</td>
<td>36</td>
<td>53&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(Polder, et al., 2009)</td>
</tr>
<tr>
<td>Latvia</td>
<td>2002/04</td>
<td>15</td>
<td>15</td>
<td>na</td>
<td>267&lt;sup&gt;h&lt;/sup&gt;</td>
<td>(Bake, et al., 2007)</td>
</tr>
<tr>
<td>Denmark</td>
<td>1997/01</td>
<td>43</td>
<td>43</td>
<td>na</td>
<td>82&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(Shen, et al., 2007)</td>
</tr>
<tr>
<td>Belgium</td>
<td>2009/10</td>
<td>84</td>
<td>84</td>
<td>44&lt;sup&gt;*&lt;/sup&gt;</td>
<td>60d&lt;sup&gt;h&lt;/sup&gt;</td>
<td>(Croes, et al., 2012)</td>
</tr>
<tr>
<td>Germany</td>
<td>2007/08</td>
<td>516</td>
<td>516</td>
<td>36</td>
<td>125&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(Raab, et al., 2013)</td>
</tr>
<tr>
<td>Croatia/Zagreb</td>
<td>2009/10</td>
<td>20</td>
<td>29</td>
<td>na</td>
<td>278&lt;sup&gt;h&lt;/sup&gt;,&lt;sup&gt;*&lt;/sup&gt;</td>
<td>(Klinčić, et al., 2014)</td>
</tr>
<tr>
<td>UK</td>
<td>2001/03</td>
<td>54</td>
<td>54</td>
<td>na</td>
<td>157&lt;sup&gt;h&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(Kalantzi, et al., 2004)</td>
</tr>
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<td>North and South Americas</td>
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<td></td>
<td></td>
<td></td>
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<td>2004</td>
<td>38</td>
<td>38</td>
<td>22</td>
<td>65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(Johnson-Restrepo, et al., 2007)</td>
</tr>
<tr>
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<td>2001/02</td>
<td>69</td>
<td>69</td>
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<td>493&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Azeredo, et al., 2008)</td>
</tr>
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<td>Africa</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>2001</td>
<td>28</td>
<td>28</td>
<td>na</td>
<td>6320&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Darnerud, et al., 2011)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>2010</td>
<td>39</td>
<td>29</td>
<td>na</td>
<td>14460&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Gebremichael, et al., 2013)</td>
</tr>
</tbody>
</table>

na: not data available

* Values represented as median value.

<sup>a</sup>Pooled sample

<sup>b</sup>Sum of o,p<sup>′</sup>-DDE + p,p<sup>′</sup>-DDD + p,p<sup>′</sup>-DDT

<sup>c</sup>Sum of p,p<sup>′</sup>-DDE + p,p<sup>′</sup>-DDD + p,p<sup>′</sup>-DDT

<sup>d</sup>Sum of p,p<sup>′</sup>-DDE + p,p<sup>′</sup>-DDT

<sup>e</sup>Sum of o,p<sup>′</sup>-DDE + p,p<sup>′</sup>-DDD + o,p<sup>′</sup>-DDD + o,p<sup>′</sup>-DDT + p,p<sup>′</sup>-DDT

<sup>f</sup>Sum of o,p<sup>′</sup>-DDE + o,p<sup>′</sup>-DDD + o,p<sup>′</sup>-DDD + o,p<sup>′</sup>-DDT + p,p<sup>′</sup>-DDT

<sup>g</sup>p,p<sup>′</sup>-DDT only

<sup>h</sup>Sum p,p<sup>′</sup>-DDE + o,p<sup>′</sup>-DDT + p,p<sup>′</sup>-DDT
5.5 Conclusions

Since the prohibited use of selective pesticides in WA, out of the 88 targeted pesticides, only \( p,p' \)-DDE was detected at trace levels in this cohort. The levels observed in this study were also among the lowest reported in Australia and worldwide. No association was established between the detected \( p,p' \)-DDE levels and both the maternal characteristics and infant growth outcomes. Using the more accurate CDI, the daily intake by the infant is at least 59 fold below the TDI recommended guidelines, thus indicating that WA infants are not at risk from the pesticides in mother’s milk. Therefore, breastfeeding should continue be encouraged due to its enormous immunological and nutritional benefits for optimal infant growth and development. However, to understand the prolonged impact of these pesticides on individual dyads during the first year of lactation, a longitudinal study with a larger cohort to track the infant development is essential.

5.6 Acknowledgements

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Chapter 6 Longitudinal study of pesticide residue levels in human milk during the first year of lactation: Risk assessment for infants

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3Metabolomics Australia, Western Australia

6.1 Abstract

The presence of pesticides in human milk (HM) is of great concern due to the potential health effects on the breastfed infants. To determine the influence of these pesticides on infant growth and development, a longitudinal study was first conducted in Australia. HM samples (n=99) were collected from 16 mothers throughout the first year of lactation. A validated QuEChERS method and gas chromatography tandem mass spectrometry were used for the analysis of 88 pesticides in HM. \( p,p' \)-DDE, \( p,p' \)-DDT and \( \beta \)-HCH were detected with a mean concentration (± SD) of 52.25 ± 49.88 ng/g fat, 27.67 ± 20.96 ng/g and 48.00 ± 22.46 ng/g respectively, whereas other pesticides (n = 85) were not detected. The concentrations of the detected pesticides decreased significantly throughout the first year of lactation. No significant adverse effects of HM
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*p,p’*-DDE on infant growth outcomes such as weight, length, head circumference and percentage fat mass, were first reported during breastfeeding. The average calculated daily intake of total DDTs in this cohort was 62-125 times lower than the threshold reference, and we also observed for the first time that the daily intake of DDTs by infant decreased significantly throughout lactation.

6.2 Introduction

Persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), carbamates and pyrethroids are synthetic chemicals that are released into the environment (Kowalski, et al., 2007; Köhler & Triebskorn, 2013). When introduced into the environment, these chemicals can percolate into the soil and ground water, and can also be transported over long distances via atmospheric circulation and water movement. These pesticides can be absorbed by inhalation, ingestion and dermal contact (Needham & Wang, 2002; Van Oostdam, et al., 2005). As these xenobiotics enter the human body, they bind to transport proteins such as human blood albumin (HBA), globulins and lipoprotein in the plasma (Maliwal & Guthrie, 1981). The chemicals are then redistributed and accumulated in tissue compartments with high fat content, such as adipose tissue, the liver, kidneys, brain and breasts (Needham & Wang, 2002). In lactating women, the pesticides can be transferred from the blood to the human milk (HM) with other necessary nutrients and precursor for the production of HM components (Skalsky & Guthrie, 1978). However, the mechanisms underlying the transfer of the pesticides in HM are not yet known.

The presence of pesticides in HM is of great concern due to the potential health effects on the breastfed infants, as these pesticides can interfere with the function of normal
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endocrine systems (Soto, et al., 1995). Exposure to these xenobiotics has been associated with a wide range of adverse effects, such as delayed neurodevelopment, poor cognitive performance and growth retardation during early childhood (Eskenazi, et al., 2006b; Eskenazi, et al., 2007; Boucher, et al., 2013). Marks et al. reported significant association between high prenatal exposure to OPPs and its metabolites and attention deficit/hyperactivity disorder in children at the age of 5 (Marks, et al., 2010). A longitudinal prenatal exposure study of 329 children through to 7 years of age also demonstrated that high maternal OPPs concentrations can significantly affect intellectual development and cognitive performances (e.g., poorer memory, processing, comprehension and reasoning) of the child (Bouchard, et al., 2011).

However, epidemiological studies on infant growth outcomes are scarce, where almost all the current studies have used prenatal pesticide exposures and infant anthropometrical size at birth such as birth weight, length, head and chest circumference as a proxy measure of in utero development, which have failed to yield conclusive results (Mazdai, et al., 2003; Wu, et al., 2010; Stasinska, et al., 2014). Pirera et al. have reported a significant inverse association between chlorpyrifos concentrations in prenatal plasma and infant birth length and weight, but not with head circumference (Perera, et al., 2003). While Wolff et al. found an inverse association between DDE concentrations in prenatal urine and infant birth weight and head circumference, but not with birth length (Wolff, et al., 2007). To the best of our knowledge, little is known about the effects of the pesticides in HM on the infant growth and development. The infants on the early lactation stages are more vulnerable to the potential effects of the pesticides due to their immature nervous systems and low levels of enzymes involved to detoxify the pesticides (Holland, et al., 2006). Surprisingly, no studies have been conducted yet to examine the relationship between POPs in HM and infant growth and
development during breastfeeding. Therefore, studies about the possible influence of pesticides in HM on infant growth outcomes are warranted.

Pesticides have been extensively used in Western Australia (WA) in the past to protect agricultural products, buildings and households against insects and pests (Reid, et al., 2013). High concentrations of certain pesticides, such as DDT and its metabolites, HCB and dieldrin have been detected in Western Australia (WA) women’s milk from previous studies (Stacey & Thomas, 1975; Stacey, et al., 1985; Stevens, et al., 1993). The most recent study was conducted in 2002/03 (Mueller, et al., 2008), however, this result for WA was based on a single pooled milk from 11 women, making it a conservative estimate for the individual and not allowing for investigation of factors that might influence HM POPs. Since then little information about these pesticides in women’s milk in WA has been reported.

Therefore, the aims of this study were to (1) describe the current pesticide concentrations in WA mothers’ milk and changes in pesticide concentrations during the first year of lactation in a longitudinal cohort; (2) investigate associations between pesticide concentrations and maternal characteristics, and evaluate the potential effects of the detected pesticides on infant growth outcomes; (3) calculate the daily intake of the pesticides by each infant throughout the first year of lactation and compare with current guidelines.

### 6.3 Materials and methods

#### 6.3.1 Chemicals and reagents

The pesticide standard solutions (100 µg/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingstown, RI, USA). The pesticide standard solutions
(100 µg/mL) were mixed and diluted with acetonitrile (ACN) to prepare a stock standard solution (1 µg/mL) of all the pesticides. LC-MS grade ACN and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylglycerol (98%), gulonolactone (95%) and D-sorbitol (≥ 98%) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (> 99.0%) and magnesium sulfate (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecyl-bonded silica (C18) and primary secondary amine (PSA) were obtained from Agilent (Little Falls, DE, USA). Isotopic labeled quality control (QC) standards, acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12} were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 6.3.2 Ethics, sample collection and processing

This study was approved by the Human Research Ethics Committee of The University of Western Australia, and the methods were carried out according to the approved guidelines. Breastfeeding dyads (n=16) were recruited in Western Australia between 2013 and 2015. All participants provided informed consent form and completed a confidential questionnaire including relevant demographic data. These mothers volunteered to participate in this study and provide a milk sample at 2, 5, 9 and 12 months of lactation. Milk samples (1-5 mL) were collected in glass containers before and after feeding from each breast. Fat content of HM was measured immediately using the Creamatocrit method (Meier, et al., 2002), and the remaining milk was stored at -20°C. HM samples (n=99) were thawed at room temperature for 3 hours and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 s.
1 mL of HM was put into a 15 mL centrifuge tube and 1 mL of ACN containing 1% HOAc and 100 ng/mL QC standards (acenaphthene-D_{10}, phenanthrene-D_{10} and chrysene-D_{12}) was added, and the tube was vortexed for 1 min. The validated QuEChERS method was employed to extract the HM. Extraction reagents (0.4 g MgSO_{4} and 0.1 g NaOAc) were added to the mixture and shaken immediately. The tube was then placed in an ice bath to prevent thermal degradation of some pesticides during the salting out process. The extraction tube was centrifuged at 3993 x g for 10 min. 0.6 mL of the extract was transferred into a clean 15 mL centrifuge tube and stored in a freezer (-20°C) for 2 hours. The extract was centrifuge at 3993 x g (0°C) for 10 min and 0.5 mL was transferred to a cleanup tube (157 mg MgSO_{4}, 9 mg C18 and 9 mg PSA). The tube was vortexed for 1 min and centrifuged at 3993 x g for 10 min. The final extract was transferred into a screw cap amber vial and kept at –80°C until analysis.

### 6.3.3 Maternal and infant anthropometric measurements

All maternal and infant anthropometric measurements were made at 2, 5, 9 and 12 months at the time of milk sampling. Maternal body weight was measured using an electronic scale (Seca, California, USA, accuracy 0.1 kg). The height, age and parity were self-reported by participants. The maternal BMI was calculated according to the following formula: BMI = kg/(m^{2}).

Infant weight was determined by weighing before breastfeeding using electronic scales (± 2.0 g; Medela Electronic Baby Weigh Scales, Medela AG, Switzerland). Infant crown-heel length was measured, to the nearest 0.1 cm, on a hard surface with perpendicular to the surface headpiece and footpiece and non-stretch tape. Infant head circumference was measured with non-stretch tape.
6.3.4 Maternal and infant body composition measurements

Whole body bioimpedance (wrist to ankle) of the participants were measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia).

Mothers’ whole bioimpedance was measured according to the manufacturer’s instructions.

Infants’ whole bioimpedance were measured using an adult protocol (wrist to ankle) and analyzed with settings customized for each infant according to Lingwood et al (Lingwood, et al., 2012). Resistance at 50 kHz was used in percentage fat mass equations developed for infants (Bocage, 1988; Lingwood, et al., 2012).

Infants’ ultrasound skinfold measurements (triceps and subscapular) were carried out using Aplio XG (Toshiba, Japan) machine, PLT-1204BX 14-8 MHz transducer and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). The double skinfold thickness, measured directly from images using the on screen electronic calipers, was used in percentage fat mass equations developed for skinfolds measured with skinfold calipers (Slaughter, et al., 1988).

6.3.5 24 – hour infant milk intake

24 – hour milk intake was determined by the testing weighing procedure (Arthur, et al., 1987). Briefly, mothers weighted their infants before and after each feed from each breast for a period of 24 hours. The difference in weight in grams is considered equivalent to mL (density of milk: 1.03 g/mL). If the data was unavailable, the data from previous 24 – hour milk intake study (900, 899, 520 and 218 mL at 2, 5, 9 and 12 months, respectively) was used (Kent, et al., 1999).
6.3.6 GC-MS/MS analysis and Quality control

Chromatographic separation and determination of the pesticides were carried on a Bruker Daltonics 450 gas chromatography (GC) with a Bruker Daltonics Scion TQ triple quadrupole mass spectrometer (MS), a Bruker 1177 Split/Splitless injector (Billerica, MA, USA) and an Combi-Pal autosampler (CTC Analytics AG, Switzerland). Sky 4.0 ID precision inlet liners with wool from Restek (Bellefonte, PA, USA) were used. For the GC separation, a Rtx-5MS with Integra-Guard column (10 m +30 m x 0.25 mm x 0.25µm) (Bellefonte, PA, USA) was used. The inlet temperature was kept constant at 250°C. The initial oven temperature of the column was 80°C for 3 min and ramped at 30°C/min to 150°C and then ramped at 10°C/min to 300°C, where it was held for 10 min. The total run time was 31 min. Helium (6.0 GC grade) was used as carrier gas at a constant flow of 1.1 mL/min. The injection was performed at pulsed splitless mode (30 psi) with an injection volume of 2 µL. The mass spectrometer was operated in EI mode. Transfer line and ion source temperature were 270°C and 200°C, respectively. Argon was used as the collision gas. Identification and quantification of 88 pesticides were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. Data collection and processing were performance using Bruker MSWS 8 Software.

Working standard solutions (0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL) were prepared by appropriate dilution of the stock standard solution (1 µg/mL) with ACN. A combination of APs mixture (ethylglycerol, gulonolactone and D-sorbitol) containing internal standard TPP (IS) was added to the final extract of each HM sample and all the working standard solutions for GC-MS/MS analysis. The final concentrations of ethylglycerol, gulonolactone and D-sorbitol were 20, 2 and 2 mg/mL, respectively. Isotopically labeled quality control (QC) standards were employed to evaluate the
efficiency of the extraction and cleanup steps, while TPP (IS) was used to evaluate the performance of the instrument throughout the entire analytical procedure. A QC mixture containing pesticide standards was analyzed between sample batches to check for any interferences and cross-contaminations.

### 6.3.7 Statistical analysis

Statistical analyses were carried out using SPSS software (SPSS, version 19.0 for windows, SPSS, Inc, IL, USA) and R 3.2.0 using the package nlme for the linear mixed models (Pinheiro, et al., 2009). The results were expressed as mean ± SD unless stated otherwise. Pesticides that were below the LCLD were considered as not detected and were not included in calculations. The detected pesticides were reported based on the HM fat (ng/g fat). Linear mixed models were used to investigate associations between pesticide concentrations in mother’s milk and infant anthropometrics and maternal anthropometrics throughout the first year of lactation in this study. Calculated daily intake (CDI) (µg/kg body wt./day) of the pesticides for each infant is calculated based on the pesticides concentration in HM (µg/g fat; \( C_{\text{pesticide}} \)), fat content in HM (g/mL; \( C_{\text{fat}} \)), average daily consumption of HM (mL; \( V_{\text{milk}} \)) and body weight of infant (kg; \( M_{\text{infant}} \)) using the following equation:

\[
\text{CDI} = \frac{C_{\text{pesticide}} \times C_{\text{fat}} \times V_{\text{milk}}}{M_{\text{infant}}}
\]

One-way ANOVA and Tukey’s all pair comparison tests (Hothorn, et al., 2008) were used to compare differences in pesticides concentration, estimated daily intake (EDI) and CDI at the different lactation months. Paired samples t-test was used to compare the daily intake of the detected pesticides by each infant using the EDI and CDI. Differences were considered to be significant if \( P < 0.05 \).
6.4 Results

6.4.1 Fat content and pesticide residues in HM

The demographic characteristics of the study participants (n = 16) are presented (Table 6.1). The average fat content of all the HM samples was 38.9 ± 21.6 g/L (11.6 to 106.3 g/L), which was taken during postfeed (left: n = 28; right: n = 11) and prefeed (left: n = 38; right; n = 22). However, the fat content in postfeed milk (left: 55.1 ± 21.4 g/L; right: 54.9 ± 24.0 g/L) was significantly higher ($P < 0.01$) compared to prefeed milk (left: 28.8 ± 15.3 g/L; right: 27.6 ± 10.0 g/L) (Figure. 6.1). Only 3.4% of the targeted pesticides (3 of 88 pesticides), namely $p,p'$-DDE, $p,p'$-DDT and $\beta$-HCH, were detected in our HM cohort (Table 6.2). Other pesticides, such as OPPs, Fungicides, carbamates and pyrethroids were not detected in the milk. The most frequently detected and abundant pesticide in HM is $p,p'$-DDE. It was detected in 83% of the cohort (83 of 99 samples). The mean concentration ($\pm$ SD, range) of $p,p'$-DDE was $1.56 \pm 1.22$ ng/mL (range: 0.21 - 6.21 ng/mL) and when normalized to the fat content of HM was $52.25 \pm 49.88$ ng/g fat (range: 5.67 - 278.48 ng/g fat). The level of $p,p'$-DDE was higher in the postfeed milk (left: $2.12 \pm 1.36$ ng/mL; right: $1.57 \pm 0.95$ ng/mL) compared to the prefeed milk (left: $1.44 \pm 1.32$ ng/mL; right: $1.08 \pm 0.78$ ng/mL). $p,p'$-DDT was detected in the milk of two mothers with a mean concentration ($\pm$ SD) of $27.67 \pm 20.96$ ng/g fat (range: 6.08 - 69.55 ng/g fat) (Table 6.2). Among the mothers who participated in this study, the insecticide, $\beta$-HCH, was only detected in one mother, with an average concentration of $48.00 \pm 22.46$ ng/g fat (range: 14.95 - 65.08 ng/g fat) (Table 6.2).
Table 6.1 Characteristics of participants in a longitudinal study measuring the concentration of pesticides in human milk collected from women at 2, 5, 9 and 12 months postpartum in Western Australia.

<table>
<thead>
<tr>
<th>Mother (n = 16)</th>
<th>Mean ± SD (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>33.9 ± 5.1 (24 - 43)</td>
</tr>
<tr>
<td>Parity</td>
<td>2.3 ± 1.1 (0 - 4)</td>
</tr>
<tr>
<td>Infant gestational age (weeks)</td>
<td>39.4 ± 1.4 (38 - 43)</td>
</tr>
<tr>
<td>Lactation stage (Months)</td>
<td>2 5 9 12</td>
</tr>
<tr>
<td>Maternal body mass index (BMI) (kg.m(^{-2}))</td>
<td>24.9 ± 4.6 (20.4 - 35.5) 24.3 ± 5.3 (19.0 - 35.2) 24.5 ± 5.9 (17.9 - 37.2) 23.7 ± 6.5 (18.2 - 37.2)</td>
</tr>
<tr>
<td>Percentage fat mass (BIS(^b))</td>
<td>34.4 ± 5.3 (25.7 - 44.7) 32.8 ± 6.2 (25.1 - 47.2) 32.6 ± 6.9 (20.0 - 44.3) 30.2 ± 7.6 (19.4 - 44.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infant (Male/Female= 9/7)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Months)</td>
<td>2.0 ± 0.1 (1.9 - 2.2)</td>
<td>5.1 ± 0.2 (1.8 - 5.4)</td>
<td>9.2 ± 0.3 (8.8 - 9.8)</td>
<td>12.2 ± 0.3 (11.6 - 12.7)</td>
</tr>
<tr>
<td>Infant weight (kg)*</td>
<td>5.6 ± 0.9 (4.4 - 7.4)</td>
<td>7.3 ± 0.8 (6.2 - 8.7)</td>
<td>8.5 ± 0.9 (6.7 - 10.1)</td>
<td>9.3 ± 0.7 (8.3 - 10.6)</td>
</tr>
<tr>
<td>Body length (cm)*</td>
<td>57.9 ± 1.8 (54.5 - 60.0)</td>
<td>64.5 ± 2.4 (60.5 - 69.5)</td>
<td>70.0 ± 2.3 (66.0 - 74.0)</td>
<td>73.6 ± 2.4 (69.0 - 76.3)</td>
</tr>
<tr>
<td>Head circumference (cm)*</td>
<td>39.6 ± 1.5 (37.0 - 42.0)</td>
<td>42.8 ± 1.9 (40.0 - 45.0)</td>
<td>45.4 ± 1.8 (43.0 - 48.5)</td>
<td>46.3 ± 1.5 (44.3 - 49.5)</td>
</tr>
<tr>
<td>Percentage fat mass with ultrasound skinfolds</td>
<td>25.6 ± 4.7 (20.9 - 37.9)</td>
<td>26.1 ± 3.5 (19.7 - 33.2)</td>
<td>25.7 ± 4.7 (15.0 - 34.7)</td>
<td>24.8 ± 4.2 (18.2 - 30.9)</td>
</tr>
<tr>
<td>Percentage fat mass with BIS(^b)</td>
<td>22.1 ± 2.0 (19.2 - 24.1)</td>
<td>27.6 ± 2.8 (21.7 - 30.9)</td>
<td>25.1 ± 5.3 (16.3 - 33.8)</td>
<td>25.2 ± 3.8 (19.6 - 30.6)</td>
</tr>
</tbody>
</table>

\(^b\) BIS: bioimpedance spectroscopy.

* Significant difference (\(P < 0.05\)) was observed between months 2-5, 2-9, 2-12, 5-9, 5-12 and 9-12.
Figure 6.1 Distribution of fat content in pre- and postfeed milk collected from each breast from 2 to 12 months (n = 99 samples from 16 mothers). The fat contents are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). *Indicates significant difference (P < 0.05).

Table 6.2 Summary of detected pesticides in HM samples (n = 99) collected from 16 Western Australian mothers.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Frequency of detection (%)</th>
<th>Mean ± SD (ng/mL) (Range)</th>
<th>Mean (ng/g fat) (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p,p'-DDE</td>
<td>83</td>
<td>1.56 ± 1.22 (0.21 - 6.21)</td>
<td>52.25 ± 49.88 (5.67 - 278.48)</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>9</td>
<td>0.70 ± 0.42 (0.26 - 1.55)</td>
<td>27.67 ± 20.96 (6.08 - 69.55)</td>
</tr>
<tr>
<td>β-HCH</td>
<td>4</td>
<td>2.17 ± 1.69 (0.59 - 4.55)</td>
<td>48.00 ± 22.46 (14.95 - 65.08)</td>
</tr>
</tbody>
</table>
Significant differences ($P < 0.01$) were observed in the $p,p'$-DDE concentration (ng/mL) between pre- and postfeed milk from each breast (Figure. 6.2A). However, no significant difference in $p,p'$-DDE concentration was observed in both pre- and postfeed milk ($P = 0.24$) and the left and right breast ($P = 0.37$) after the concentration of $p,p'$-DDE was normalized to the fat content of HM (Figure. 6.2B).

**Figure 6.2** Distribution of $p,p'$-DDE in pre- and postfeed milk collected from each breast before (A) and after (B) normalized to fat content of HM. Values of $p,p'$-DDE are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). *Indicates significant difference ($P < 0.05$).
6.4.2 Changes of pesticide concentrations during the first year of lactation

In this study, the \( p, p' \)-DDE concentrations in HM declined from 70.90 ± 70.58 ng/g fat at 2 months to 57.54 ± 47.32 ng/g fat at 5 months, 45.16 ± 31.71 ng/g fat at 9 months and to 22.35 ± 13.96 ng/g fat at 12 months. An overall 68% decrease of \( p,p' \)-DDE concentrations was observed over the lactation period of the first 12 months (Figure 6.3). A significant difference \((P < 0.05)\) was observed between the \( p,p' \)-DDE concentrations in HM at 2 and 12 months postpartum with no significant differences observed between months 2 - 5, 2 - 9, 5 - 9, 5 - 12 and 9 - 12. Similarly, the concentrations of \( p,p' \)-DDT and \( \beta \)-HCH in this study also decreased by 45% and 73% respectively over the lactation period of 2 - 12 months.

![Graph showing changes of pesticide concentrations during lactation](image)

**Figure 6.3** The average concentration of \( p,p' \)-DDE (ng/g fat) in HM collected during lactation period from 2 to 12 months. Values of \( p,p' \)-DDE are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). Insert is the mean plot of \( p,p' \)-DDE levels from this cohort. Significant difference \((P < 0.05)\) is observed between 2 and 12 months.
Associations between pesticide concentrations and maternal demographics, and the potential influence of the detected pesticides on infant growth were investigated. No significant association was observed between \( p,p' \)-DDE concentrations in HM and maternal age \((P = 0.06)\) and parity \((P = 0.65)\). No significant associations were also observed between \( p,p' \)-DDE and both maternal BMI \((P = 0.27)\) and percentage fat mass \((P = 0.70)\). Significant increase in infant weight \((P < 0.01)\), length \((P < 0.01)\) and head circumference \((P < 0.01)\) was observed from 2 to 12 months (Figure 6.4). However, no significant associations were observed between \( p,p' \)-DDE and the infant growth outcomes, such as weight \((P = 0.40)\), length \((P = 0.13)\), head circumference \((P = 0.07)\) and percentage fat mass (ultrasound skinfolds: \(P = 0.34\); bioimpedance spectroscopy (BIS): \(P = 0.11\)). However, mothers with male infants were found to have significantly higher \((P = 0.03)\) concentrations of \( p,p' \)-DDE in their milk \((55.18 \pm 45.41 \text{ ng/g fat})\) as compared to mothers with female infants \((29.34 \pm 18.90 \text{ ng/g fat})\) (Figure 6.5).

Figure 6.4 Infant growth outcomes during breastfeeding. (Continues)
Figure 6.4 The changes of infant weight (A), body length (B) and head circumference (C) during breastfeeding from 2-12 months. Values are shown by box plots illustrating range (error bars), quartiles (box) and median (indicated by bold line). ** Indicates significant difference ($P < 0.01$).
6.4.3 Health risk assessment of POPs for infant

The average calculated daily intake (CDI) of DDTs (sum of DDT and its metabolites, DDE and DDD) throughout the first year of lactation was 0.16 µg /kg body wt./day, which was much lower than the tolerable dietary intake (TDI) proposed by FAO/WHO (FAO/WHO, 2000) and Health Canada (Van Oostdam, et al., 1999) at 10 and 20 µg /kg body wt./day, respectively (Table 6.3). This study also demonstrated that the CDI of DDTs by the infants decreased significantly ($P < 0.01$) during the first year of lactation from 0.33 µg /kg body wt./day at 2 months to 0.03 µg /kg body wt./day at 12 months, which is in conjunction with the infants' rapid development and also the decrease of the maternal bioburden.
Table 6.3 Comparison between calculated daily intake (CDI) and estimated daily intake (EDI) of DDTs by infants during the first year of lactation in comparison to tolerable daily intake (TDI) proposed by Health Canada and FAO/WHO.

<table>
<thead>
<tr>
<th></th>
<th>CDI (µg/kg body wt./day)</th>
<th>EDI (µg/kg body wt./day)</th>
<th>TDI (µg/kg body wt./day)</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Range)</td>
<td>(Range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data</td>
<td>0.16</td>
<td>0.23</td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.02 - 0.69)</td>
<td>(0.03 - 0.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 months</td>
<td>0.33</td>
<td>0.30</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>(0.07 - 0.69)</td>
<td>(0.08 - 0.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 months</td>
<td>0.18</td>
<td>0.27</td>
<td></td>
<td>10.00 ^a</td>
</tr>
<tr>
<td></td>
<td>(0.06 - 0.52)</td>
<td>(0.08 - 0.72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>0.09</td>
<td>0.23</td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.02 - 0.22)</td>
<td>(0.03-0.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>0.03</td>
<td>0.10</td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.02 - 0.05)</td>
<td>(0.03-0.25)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


^bHealth Canada (Van Oostdam, et al., 1999).

* P value reflects the difference between CDI and EDI, and the difference is significant if P < 0.05.

6.5 Discussion

In this study, the fat content in postfeed milk was significantly higher (P < 0.01) as compared to prefeed milk (Figure 6.1), which reflects the higher degree of emptying of the breast after breastfeeding as compared to that before breastfeeding (Daly, et al., 1993). HM p,p'-DDE concentrations were also significantly higher (P < 0.01) in postfeed milk than the prefeed milk for each breast (Figure. 6.2A). These findings suggested an association between lipophilic POPs such as p,p'-DDE, and the fat content of HM, which support the current hypothesis that the POPs are encapsulated either in the core (TAGs) or adhere to the surface (phospholipids) of HM fat during milk synthesis (Robenek, et al., 2004; Farese & Walther, 2009). This hypothesis was further supported when no significant difference in p,p'-DDE was found in pre- and postfeed
milk after the concentration of \( p,p' \)-DDE was normalized to the fat content of HM (Figure. 6.2B). Therefore, either pre- or postfeed milk from any breast can be used in the assessment of pesticide exposure provided the pesticides concentrations are normalized to the fat content of HM. Overall, we found a large variation of \( p,p' \)-DDE concentrations (5.67–278.48 ng/mL) in this cohort, which could be due to different lifestyle, dietary habits and metabolic activity between mothers (Waliszewski, et al., 2001).

As \( p,p' \)-DDE is a metabolite of \( p,p' \)-DDT, the two mothers who had detectable \( p,p' \)-DDT in their milk, also have higher \( p,p' \)-DDE concentrations of 125.34 ng/g fat and 80.50 ng/g fat respectively, which were higher than the average level, 52.25 ± 49.88 ng/g fat, observed in this cohort. Previous studies have shown that the half-life of DDE and DDT in human body was approximately 4 and 6 years, respectively. The ratio between DDE and DDT is used as an indicator of DDT exposure history, where DDE/DDT ratio (> 5) represents historical exposure and low DDE/DDT ratio (< 5) suggests recent exposure (Mishra & Sharma, 2011; Zhou, et al., 2012). The DDE/DDT ratio of the two mothers with DDT detected was 3.4 and 8.1 respectively. Both mothers had different exposure characteristics, where one mother (DDT/DDE ratio of 3.4) frequently visited a country that still had documented high levels of DDT, while the other (DDT/DDE ratio of 8.1) grew up on a farm and was involved in the harvesting process during the period from 2004 to 2010. These could be the possible exposure sources of DDT in their HM, however this was based on maternal recollection and further investigation is required.

Among the mothers who participated in this study, only one mother had \( \beta \)-HCH, while other isomers of HCHs (\( \alpha \)-HCH, \( \gamma \)-HCH and \( \delta \)-HCH) were not detected in HM.
Among the isomers of HCHs, \( \beta \)-HCH is more metabolic stable and persistent, accounting for over 90% of the total HCH detected in HM (Malarvannan, et al., 2009; Polder, et al., 2009). Similar to DDE/DDT exposure history, \( \beta \)-HCH/\( \alpha \)-HCH ratio is used as an indicator of HCH exposure history (Devanathan, et al., 2009). \( \alpha \)-HCH and \( \gamma \)-HCH were not detected in this study, which suggests that the mother in whom we detected \( \beta \)-HCH had historical exposure to HCH.

The long-term trend of the POPs in HM for individual mothers was also investigated. While a steady decline of HM \( p,p' \)-DDE concentrations was observed in majority (n = 10) of the mothers during the lactation period of 2 to 12 months, fluctuations of HM \( p,p' \)-DDE between the months were also observed in 6 mothers. The different change patterns between the mothers could be due to their different lifestyle, diet habits and metabolic activity. However, we observed an overall decline of \( p,p' \)-DDE, \( p,p' \)-DDT and \( \beta \)-HCH by 68%, 45% and 73%, respectively during the first year of lactation. As HM POPs primarily come from maternal body storage such as hepatic lipid and adipose tissues (Koppe, 1995; Bergkvist, et al., 2008), thus the significant decrease of these pesticides in HM throughout the lactation months also indicates that the mother’s body burden is also decreasing through the lactation. The decrease of pesticides in HM has also been observed in other studies. Yu et al., reported that the average decline of \( p,p' \)-DDE and \( p,p' \)-DDT in colostrum (day 4/5) to week 2 milk was 4% and 10% respectively (Yu, et al., 2007). However, to the best of our knowledge, all previous studies have been carried out at the early lactation stages of less than 4 months due to the difficulties in recruiting mothers and collecting HM for longitudinal study (Yu, et al., 2007; Zietz, et al., 2008; Takekuma, et al., 2011; Bouwman, et al., 2012). Other challenges such as low milk production, cessation of breastfeeding and unexpected reasons (e.g., lost contacts and relocations) also result in limited data for lactation study.
longer than 4 months. These short lactation studies can only reflect the earliest and highest level of pesticides in HM. Therefore, our longitudinal study more accurately reflects the long-term trend of the detected pesticides in HM throughout the first year of lactation in a larger cohort (n = 16).

Besides monitoring changes of POPs in HM, this is the first study that also evaluates the potential influence of these pesticides in HM on the infant growth throughout the first year of lactation. No significant relationships were observed between HM \( p,p' \)-DDE and infant growth outcomes, such as weight, length and head circumference during the first 12 months of lactation. To the best of our knowledge, there are no studies that have assessed the pesticides in HM with infant growth based on infant body composition (BC) using both ultrasound and BIS. Body composition (BC) measurements are able to differentiate between fat mass and fat-free mass, making infant BC more accurate in assessing body fat as compared to body mass index (BMI) (Bemben, et al., 1998). As pesticides are accumulated in the high fat tissue compartments after exposure, it is essential to investigate the relationship between HM pesticides and infant BC. No significant association was observed between \( p,p' \)-DDE concentrations in HM and infant percentage fat mass measured by either ultrasound skinfolds or BIS. This absence of significant associations could be due to the low levels of pesticides present in HM in this study. For the first time, we have clearly demonstrated that the detected pesticides in HM have no significant effects on the infant growth outcomes during the first year of lactation.

No significant association was observed between HM \( p,p' \)-DDE and maternal age (\( P = 0.06 \)) in this study. While previous studies have found the significant association (Minh, et al., 2004; Chao, et al., 2006), as the pesticides tends to bioaccumulate in
maternal body and increases with maternal age. Some studies have also reported strong associations between pesticides concentration in HM and maternal parity as more POPs are excreted through multiple pregnancies and lactations for multiparous mothers than primiparous mothers (Kunisue, et al., 2006; Azeredo, et al., 2008). However, no significant association was observed between $p,p\prime$-DDE and parity ($P = 0.65$) in this study, which could be due to the small sample size (16 mothers) of this cohort. No significant association was also observed between $p,p\prime$-DDE and maternal BMI ($P = 0.27$). As the pesticides originally stored in maternal body fat can be mobilized and transferred to HM by lipolysis during weight loss, surprising no study has ever evaluated the association between maternal BC and the pesticides in HM. We did not observe significant association between maternal percentage fat mass and $p,p\prime$-DDE throughout the first year of lactation. We found that there was no significant change of the maternal BC ($P = 0.08$) during the breastfeeding period of 2 to 12 months, which could probably explain the absence of the significant association between maternal BC and $p,p\prime$-DDE in HM.

Even though no adverse effect was observed between the detected POPs in HM and infant growth outcome during the breastfeeding. In order to ensure that the detected $p,p\prime$-DDE in HM poses no risk to the infants and understand the actual POPs dose to the infant rather than HM POPs concentrations in isolation. Therefore, calculated daily intake (CDI) of POPs by the infants was calculated based on the concurrent actual measurements, such as HM fat content ($C_{fat}$), infant weight ($M_{infant}$) and 24-hour milk intake ($V_{milk}$) through the first year of lactation. Whereas in previous studies, the estimated daily intake (EDI) has been calculated using constant values for $C_{fat}$ (0.03 g/mL), $M_{infant}$ (5 kg) and $V_{milk}$ (700 mL) (Van Oostdam, et al., 1999; Bedi, et al., 2013), which did not account for the large variations of these values between individual infants
at different lactation stages. Overall, significant difference ($P < 0.01$) was observed between CDI and EDI. Where, the EDI was found to underestimate the pesticide concentrations by 10% for 2 months and overestimate the concentrations by 50%, 155% and 233% at 5, 9 and 12 months postpartum respectively (Table 6.3). This is due to the actual weight gain of the infant from $5.6 \pm 0.9$ kg (2 months) to $9.3 \pm 0.7$ kg (12 months) and actual reduction of milk intake from $890 \pm 57$ mL (2 months) to $256 \pm 81$ mL (12 months).

We also demonstrate for the first time that the CDI of $p,p'$-DDE decreases significant ($P < 0.01$) during the breastfeeding period of 2 to 12 months (Figure 6.6), which is in conjunction with the significant infant weight gain ($P < 0.01$) and significant decrease of $p,p'$-DDE in HM ($P < 0.05$) throughout the breastfeeding. However, no significant decreases of the EDI were observed between 2 and 12 month ($P = 0.17$) (Figure 6.6). The results clearly demonstrate that CDI rather than EDI should be used to reflect the actual pesticides intake by the infants throughout the lactation months. The CDI values ($0.02 - 0.69 \mu g /kg$ body wt./day) for all the infants during the entire breastfeeding period in this cohort were 14-500 and 28-1000 times lower than the tolerable daily intake (TDI) currently set at 10 and 20 $\mu g /kg$ body wt./day by and FAO/WHO (FAO/WHO, 2000) and Health Canada (Van Oostdam, et al., 1999) respectively, suggesting that the maternal HM in this cohort poses no risk to individual infants in WA.
Figure 6.6 Difference between calculated daily intake (CDI) and estimated daily intake (EDI) of DDTs by individual infants throughout the first year of lactation in WA. ** Indicates significant difference $P < 0.01$. 
In order to understand the current magnitudes of POPs in HM in WA, the results obtained in this study were compared with those reported in WA. We found that the total DDTs in this study decreased by 93.4% and 79.9% as compared to previous study conducted in WA in 1990s (800 ng/g fat) (Stevens, et al., 1993) and the most recent study in Australia in 2000s (257.6 ng/g fat) (Mueller, et al., 2008), respectively. Our findings are also consistent with the low pesticide concentrations reported in maternal blood, cord blood, placenta and abdominal tissues in WA (Noakes, et al., 2006; Reid, et al., 2013), and demonstrate the low concentrations of these pesticides in the environment and continuing decrease of human exposure to the pesticides over time in WA.

As compared to other countries (Table 6.4), the concentrations of DDTs observed in this study are similar to those reported for Norway (Polder, et al., 2009) and USA (Johnson-Restrepo, et al., 2007), but are a few orders of magnitude lower than that observed in malaria-prone countries such as Vietnam (Minh, et al., 2004), Malaysia (Sudaryanto, et al., 2005) and India (Bedi, et al., 2013) where DDTs was widely used to combat mosquito borne malaria and was only recently banned in the 1990s and 2000s. However, WHO have recently allowed limited use of DDT for indoor control of malaria vectors in malaria endemic countries (Breman, et al., 2007). Therefore, concentrations of DDTs in South Africa (Darnerud, et al., 2011) and Ethiopia (Gebremichael, et al., 2013) are 122 and 330 times higher than that in WA. In generally, DDTs concentrations in HM appear to be higher in developing countries than those in developed countries. The HCHs concentrations observed in this study are 4 - 63 times lower than the concentrations in other countries such as India (Bedi, et al., 2013), Malaysia (Sudaryanto, et al., 2005) and Iran (Behrooz, et al., 2009), where HCHs are still extensively used.
**Table 6.4** Comparison of DDTs and HCHs (ng/g fat) in HM from various countries.

<table>
<thead>
<tr>
<th>Country/region</th>
<th>Year of sampling</th>
<th>Mothers (N)</th>
<th>HM samples</th>
<th>Fat content (g/L)</th>
<th>DDTs</th>
<th>HCHs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran</td>
<td>2006</td>
<td>23</td>
<td>23</td>
<td>22</td>
<td>2685&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3005&lt;sup&gt;A&lt;/sup&gt;</td>
<td>(Behrooz, et al., 2009)</td>
</tr>
<tr>
<td>Indonesia</td>
<td>2003</td>
<td>15</td>
<td>15</td>
<td>na</td>
<td>1100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>(Sudaryanto, et al., 2006)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>2000/01</td>
<td>36</td>
<td>36</td>
<td>31</td>
<td>333&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>(Chao, et al., 2006)</td>
</tr>
<tr>
<td>India</td>
<td>2011</td>
<td>53</td>
<td>53</td>
<td>32</td>
<td>1914&lt;sup&gt;B&lt;/sup&gt;</td>
<td>199&lt;sup&gt;B&lt;/sup&gt;</td>
<td>(Bedi, et al., 2013)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>2000/01</td>
<td>42</td>
<td>42</td>
<td>23</td>
<td>2100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58&lt;sup&gt;C&lt;/sup&gt;</td>
<td>(Minh, et al., 2004)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>2003</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>1600&lt;sup&gt;d&lt;/sup&gt;</td>
<td>230&lt;sup&gt;C&lt;/sup&gt;</td>
<td>(Sudaryanto, et al., 2005)</td>
</tr>
<tr>
<td>Philippine</td>
<td>2004</td>
<td>33</td>
<td>33</td>
<td>22</td>
<td>170&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6&lt;sup&gt;A&lt;/sup&gt;</td>
<td>(Malavannan, et al., 2009)</td>
</tr>
<tr>
<td>Slovakia</td>
<td>2003</td>
<td>12</td>
<td>12</td>
<td>27</td>
<td>665&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20&lt;sup&gt;C&lt;/sup&gt;</td>
<td>(Yu, et al., 2007)</td>
</tr>
<tr>
<td>Norway</td>
<td>2002/06</td>
<td>377</td>
<td>377</td>
<td>36</td>
<td>53&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;C&lt;/sup&gt;</td>
<td>(Polder, et al., 2009)</td>
</tr>
<tr>
<td>USA</td>
<td>2004</td>
<td>38</td>
<td>38</td>
<td>22</td>
<td>65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19&lt;sup&gt;D&lt;/sup&gt;</td>
<td>(Johnson-Restrepo, et al., 2007)</td>
</tr>
<tr>
<td>South Africa</td>
<td>2001</td>
<td>28</td>
<td>28</td>
<td>na</td>
<td>6320&lt;sup&gt;f&lt;/sup&gt;</td>
<td>12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>(Dannerud, et al., 2011)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>2010</td>
<td>33</td>
<td>33</td>
<td>na</td>
<td>17170&lt;sup&gt;h&lt;/sup&gt;</td>
<td>na</td>
<td>(Gebremichael, et al., 2013)</td>
</tr>
<tr>
<td>New Zealand</td>
<td>2007/2010</td>
<td>39</td>
<td>37</td>
<td>39</td>
<td>385&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8.7&lt;sup&gt;A&lt;/sup&gt;</td>
<td>(Maanetje, et al., 2013)</td>
</tr>
<tr>
<td>Australia/WA</td>
<td>2013/15</td>
<td>16</td>
<td>99</td>
<td>39</td>
<td>52&lt;sup&gt;e&lt;/sup&gt;</td>
<td>48&lt;sup&gt;C&lt;/sup&gt;</td>
<td><strong>Present study</strong></td>
</tr>
</tbody>
</table>

na: data not available

* Values represented as median value.

<sup>a</sup> Sum of $p,p'$-DDE + $o,p'$-DDE + $p,p'$-DDD + $p,p'$-DDT

<sup>b</sup> Sum of $p,p'$-DDE + $p,p'$-DDD + $p,p'$-DDT

<sup>c</sup> Sum of $p,p'$-DDE + $p,p'$-DDD + $p,p'$-DDT + $o,p'$-DDT

<sup>d</sup> Sum of $p,p'$-DDE + $p,p'$-DDT

<sup>e</sup> $p,p'$-DDE only

<sup>f</sup> Sum of $p,p'$-DDE + $o,p'$-DDE + $p,p'$-DDD + $o,p'$-DDD + $p,p'$-DDT + $o,p'$-DDT

<sup>A</sup> Sum of α-HCH + β-HCH + γ-HCH

<sup>B</sup> Sum of β-HCH + γ-HCH

<sup>C</sup> β-HCH only

<sup>D</sup> Sum of α-HCH + β-HCH + γ-HCH + δ-HCH
6.6 Conclusions

This study highlights that only trace amount of \( p,p'-\text{DDE}, \) \( p,p'-\text{DDT} \) and \( \beta-\text{HCH} \) pesticides were detected in HM collected in Western Australia and their concentrations decreased significantly throughout the first year of lactation. For the first time, a longitudinal study investigating the influence of the detected pesticides on the infant growth outcomes, such as weight, length, head circumference and body composition were conducted. No significant associations have been observed between the detected pesticides in HM and infant growth during the first year of lactation. Here, we have found that CDI is more accurate to reflect the daily POPs intake by infant during breastfeeding than the commonly used EDI. The CDI of the pesticides by individual infant decreased significantly during the first year of lactation, and was at least 14 to 500 times lower than current recommended guidelines. Therefore, breastfeeding should continue to be encouraged due to its enormous immunological and nutritional benefits for optimal infant growth and development.

6.7 Acknowledgements

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Chapter 7 General discussion and future perspectives

7.1 General discussion

HM contains a vast collection of bioactive components that have multiple functional biological roles that provide nutritional requirements for optimal growth, growth factors that program the infant development for later health, and activated immune components that provide protection as well as maturing infant immune system (Emmett & Rogers, 1997; Hale & Hartmann, 2007; Innis, 2007; Ballard & Morrow, 2013; Lönnerdal, et al., 2017). HM contains thousands of beneficial bioactive components to attain these outcomes and these components or the necessary substrates are either imported from maternal circulation or produced in the lactocytes. Unfortunately, environmental contaminants, in particular the pesticides, can enter HM via maternal circulation and lactocytes. The present of pesticides in HM is of great public concern due to their potential detrimental health effects for the breastfed infant. The infant, particularly the preterm infant, is more vulnerable to the potential effects of the pesticides due to their immature systems and low levels of enzymes involved in the detoxification of the pesticides (Eskenazi, et al., 2007). To determine the presence and levels of 88 POPs in HM from Western Australian women and to evaluate the adverse effects of HM POPs on the infant throughout the first year of lactation. HM was collected during the period from 2013 to 2015. A simple and accurate QuEChERS method with GC-MS/MS was then systematically developed and applied to a cross-sectional study and a longitudinal study. New methodologies were developed and these solved challenges, such as matrix enhancement, the best approach to address the matrix enhancement and dosage of POPs
by infant. We believe this method is state of the art with detection levels well below established methods and will allow regular accurate monitoring of HM POPs not only in Australia but also worldwide.

Reporting of HM POPs is currently standardized to the fat content as HM exhibits large fat variations in HM with and among mothers. Depending on sampling of the milk, such as pre- and postfeed, where fat content increases significantly for postfeed HM, the fat content may vary from 28.1 g/L to 61.8 g/L (Khan, et al., 2013). The reported values of HM POPs are standardized to HM fat in order to overcome this variability and allow comparison of HM POPs between different studies. The three widely used analytical techniques to measure milk fat, include gravimetric method, EFA and creamatocrit. However, there is no existing comprehensive examination of the difference between the methods to enable interpretation of various studies. I subsequently systematically evaluated the accuracy and efficiency of each of the fat measurement techniques. The creamatocrit method (mean difference: 4.2–6.7%; CV: 3.9%) was more accurate than EFA method (mean difference: 2–28%; CV: 3.9%) compared to gravimetric method, which is often regarded as the gold standard. The simplicity and robustness along with the absence of chemicals and rapid analysis make the creamatocrit method an attractive option (Meier, et al., 2002; Meier, et al., 2006). Recently, mid-infrared is being explored for analysis of HM, and has been employed mainly in the dairy industry but requires both an expensive and complex mid-infrared analyzer and validated calibration for HM (Smilowitz, et al., 2014). Furthermore, most mid-infrared analyzers require large volume samples of milk (60–200 mL). Smilowitz et al. found that the mid-infrared spectroscopy measured significantly higher fat values than the gravimetric method (Smilowitz, et al., 2014). Recently, O’Neill et al. also claimed that the creamatocrit fat analysis was less accurate than mid-infrared spectroscopy analyzer (O’Neill, et al.,
However, major methodological concerns exist regarding centrifugation of the HM samples. The samples were centrifuged for 15 min at 1315 x g instead of the standard 15 min at 12 000 x g used in all other studies (Fleet & Linzell, 1964; Lucas, et al., 1978; Meier, et al., 2002), which accounts for the higher creamatocrit values observed as compared to the mid infrared spectroscopy. Creamatocrit currently requires less milk volume (< 0.1 mL) compared to mid-infrared analyzer (60–200 mL), and also is much more economical and efficient. In addition, the creamatocrit can also be used as a rapid and feasible tool in the clinical setting for estimating the fat and caloric concentration of HM particular for the premature infants in the neonatal intensive care unit.

When measuring POPs in HM, it is integral to investigate matrix enhancement of the targeted 88 pesticides with GC-MS/MS due to the unavailability of pesticide-free HM and limited commercially available isotopically labeled standards. It has been demonstrated that all of the selected 88 pesticides are strongly affected by the matrix enhancement in HM as compared to that in pure solvent (Table 3.1.1). These results provide base variability and intensity of the matrix enhancement of the 88 pesticides in HM. As compared to the matrix enhancement of similar pesticides reported in other studies (Schenck & Lehotay, 2000; Sugitate, et al., 2012), It has been found that the matrix enhancement of these pesticides is to be much stronger in HM than the other matrices (Table 7.1). This could be attributed to the relatively higher fat content of HM and matrix components remaining in HM. Therefore, solvent standards should not be used in the quantification of pesticides in HM as this will result in overestimation of pesticide concentrations in HM samples. Despite this, many studies continue to quantify the pesticides in HM using solvent standards without considering the matrix enhancement (Bedi, et al., 2013; Chávez-Almazán, et al., 2016).
Table 7.1 Comparison of the matrix enhancement of selected pesticides in different sample matrices.

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Matrix enhancement (%) at 100 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potato*</td>
</tr>
<tr>
<td>Phosmet</td>
<td>30</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>43</td>
</tr>
<tr>
<td>Fipronil</td>
<td>13</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>30</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>18</td>
</tr>
<tr>
<td>Diazinon</td>
<td>14</td>
</tr>
<tr>
<td>Terbufos</td>
<td>na</td>
</tr>
<tr>
<td>Alpha-endosulfan</td>
<td>10</td>
</tr>
<tr>
<td>Lindane</td>
<td>6</td>
</tr>
<tr>
<td>na: not available.</td>
<td></td>
</tr>
</tbody>
</table>

*Data from (Sugitate, et al., 2012).
$Data from (Schenck & Lehotay, 2000).
*The results in this thesis.

As it is essential to address the matrix enhancement, we also choose to thoroughly investigate APs, pulsed splitless and large volume injection to identify an effective method to address the matrix enhancement in HM rather than reducing matrix enhancement components. A mixture of ethylglycerol, gulonolactone and D-sorbitol at 20, 2 and 2 mg/mL was found to induce similar peak enhancement for the selected pesticides in solvent as that in HM (Figure 3.2.3). This agrees with findings that APs mixtures can mask the active sites in the GC system and alleviate the thermal stress imposed on the pesticides, thus resulting in higher peak intensity and improved peak shapes as compared to those pesticides without APs (Anastassiades, et al., 2003; Maštovská, et al., 2005). The above mentioned APs mixture addressed the matrix enhancement for 86% of the 88 pesticides investigated in this study, indicating that the
addition of APs mixture can practically eliminate the differences between the solvent and HM extract.

Besides using APs mixture, the performance of different pulsed pressures and injection volumes on the matrix enhancement were also investigated in HM for the first time. Improved peak intensity and shape have been found for all the pesticides when higher pulsed pressures (15–55 psi) were used compared to that without pressure pulse. High pressure pulse allows the use of larger volume injection without notable backlash and peak deformations as compared to the classic splitless injection (Wylie, et al., 1992; Godula, et al., 1999). The injection volume (2 µL) at 30 psi showed the least ED between solvent standards and HM standards, where 45% (40 out of 88) of the pesticides were within the acceptable range. This is higher than when only pressure pulse at 55 psi (38%) was used. From these results we can conclude that using higher pressure pulse and larger volume injection reduces the matrix enhancement, but does not fundamentally address the matrix enhancement of all 88 pesticides tested for HM.

Despite high pressure pulse and larger volume injection not fully addressing the matrix enhancement for the pesticides, they can greatly increase the peak response and peak quality (Figure 3.3.1). Based on these findings, All the optimized factors: the APs mixture, high pressure pulse (30 psi) and larger volume injection (2 µL), were combined and the results shown that up to 99% of the pesticides spiked at 10–100 ng/mL fell within the acceptable range (70–120%). This not only demonstrates the synergistic effect of APs mixture with pressure pulse and larger volume injection to address matrix enhancement of the selected pesticides in HM, but also presents a simple, effective and reliable method using a mixture of commercially available reagents to address the matrix enhancement of pesticides in HM. This optimized method also overcomes the
limitations of commonly used methods such as matrix-matched calibration and isotopically labeled methods. Here, I have demonstrated that this method can be used to accurately quantify multi-class pesticide residues in HM.

With respect to the method of POPs analysis, currently, there are limited publications that have used QuEChERS method for HM sample preparation. Many of these extractions were carried out “according to” the modified QuEChERS methods (AOAC Official method 2007.01 or European Standard Method EN 15662), without details of appropriate optimization and validation of the method (Kinsella, et al., 2009; Brondi, et al., 2011; Jeong, et al., 2012). In this thesis, the QuEChERS method including both the partitioning salts (MgSO₄/NaOAc) and cleanup sorbents (MgSO₄/PSA/C18) was first systematically optimized for 88 pesticides in HM. The optimized QuEChERS method was then validated using HM of varying fat content (29.5–89.8 mg/mL) spiked with a range of pesticide concentrations (10–100 ng/mL). The QuEChERS method was then scaled down from 10 mL to 1 mL and further validated (Table 4.3.3). This new QuEChERS method provides good recoveries in the range of 64.1-126.7%, 68.4-117.7% and 76.4-115.3% with the relative standard deviations (RSD, n=7) of 3.6-33.9%, 2.2-17.4% and 2.5-13.5% for the spiked concentrations at 10, 20 and 100 ng/mL, respectively. The LOD and LOQ of this QuEChERS are also lower than some traditional HM extraction techniques (Kumar, et al., 2006; Zhou, et al., 2011; Zhou, et al., 2012).

This validated QuEChERS method substantially reduces the volume of HM required to just 1 mL, which greatly alleviates the pressure for the collection of HM samples in particular from preterm mother whose milk production is often low, compared to the commonly used extraction techniques such as LLE (Rojas-Squella, et al., 2013), SPE
(Minh, et al., 2004), PLE (Sun, et al., 2005) and Soxhlet extraction (Zhou, et al., 2011). Therefore, our method with reduced HM requirements makes it easier to sample and monitor the POPs concentrations, particularly for the longitudinal studies. In addition, this QuEChERS method also greatly simplifies the HM process and reduces the requirement of solvent, glassware and time (Table 7.2). Currently, limited data is available for lactation studies longer than 6 months mainly due to the requirement of large volumes of HM for the traditional extraction techniques (Ribas-Fitó, et al., 2005; Zietz, et al., 2008).

The validated QuEChERS enabled us to analyze 88 different classes of pesticide residues in HM in a single injection as compared to most of the current studies that can only target a limited number of pesticides in HM (Chao, et al., 2006; Malarvannan, et al., 2009; Mishra & Sharma, 2011). This newly validated QuEChERS method was next utilized for the extraction of pesticides from HM samples in both a cross-sectional and longitudinal cohort of mother and infants from WA.

There has been an increasing concern over the presence of POPs in HM as it is the main food source to breastfed infant during a time of rapid development, involving processes such as cell differentiation, body growth, development of vital organ systems and metabolic pathways (Eskenazi, et al., 1999). Few studies have assessed the potential adverse effects of HM POPs on the human infant throughout the lactation, and no information of the pesticides in HM have been reported since the study by Mueller et al., in WA (Mueller, et al., 2008). This study was performed in 2003, and was based on a single pooled HM from 11 women, making it a conservative estimate for the individual and not allowing for investigation of factors that might influence on POP concentrations in HM.
Table 7.2 Comparison between the validated QuEChERS and traditional HM extraction techniques.

<table>
<thead>
<tr>
<th>HM preparation technique</th>
<th>Requirement of HM and solvent</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Reference</th>
</tr>
</thead>
</table>
| QuEChERS                 | 1 mL HM 1 mL solvent         | 1. Only small quantities of solvent and HM required.  
2. Simplified HM extraction and cleanup steps.  
3. Short extraction time with high outcome. | Lower recoveries for highly nonpolar pesticides such as chlordane, HCB, cyfluthrin and pyrethrin. | This study |
| Liquid-liquid extraction (LLE) | 10 - 100 mL HM ~ 250 ml solvent | Easy to operate and simple instrument. | 1. Large volume of HM and toxic organic solvent.  
2. Laborious solvent evaporation and complicated cleanup steps. | (Costopoulou, et al., 2006; Ennaceur & Driss, 2013) |
| Solid phase extraction (SPE) | 10 - 40 mL HM ~ 100 ml solvent | 1. Reduced volume of HM and solvent.  
2. Potential for automation. | 1. Relatively expensive instrument and longer extraction time.  
2. Not suitable for thermal-labile compounds | (Di Muccio, et al., 1997; Croes, et al., 2012) |
| Soxhlet                  | 20 - 40 mL HM > 250 ml solvent | Automated at high temperature in a continuous manner | 1. Relatively expensive instrument and longer extraction time.  
2. Not suitable for thermal-labile compounds | (Tsang, et al., 2011; Shi, et al., 2013) |
| Pressurized liquid extraction (PLE) | ~ 1 mL HM 10 – 20 mL solvent | 1. Reduced volume of HM and solvent.  
2. Extraction can be automated. | 1. Relatively expensive instrument and longer extraction time.  
2. Not suitable for thermal-labile compounds | (Ramos, et al., 2002; She, et al., 2007; Lacorte & Guillamon, 2008; Mustafa & Turner, 2011) |

To determine the current status of POPs in HM in women from WA, a cross-sectional cohort of 40 lactating mothers at either 2, 5, 9 or 12 months of lactation was recruited to provide milk samples. Fewer pesticides were detected and the detected concentrations were much lower than previous studies conducted in WA (Stacey & Thomas, 1975; Stacey, et al., 1985; Stevens, et al., 1993). p,p’-DDE in HM was up to 60-fold lower than that observed in previous studies (Table 5.5). This decreasing trend
is consistent with decreasing levels worldwide due to banning and prohibition of these pesticides (Norén & Meironyté, 2000; Zietz, et al., 2008; Vukavić, et al., 2013). These findings are also consistent with the low pesticide concentrations reported in maternal blood, cord blood, placenta and abdominal tissues in WA (Noakes, et al., 2006; Reid, et al., 2013), and demonstrate the low concentrations of these pesticides in the environment and continuing decrease of human exposure to the pesticides over time in WA. Further, there were no significant associations between maternal characteristics, such as age, parity and lactation months and the detected HM \( p,p' \)-DDE, nor were there any adverse influences of \( p,p' \)-DDE on infant growth measured as weight, body length, head circumference and percentage fat mass at any of the time points. However, due to the inherent shortcomings of a cross-sectional study, a smaller longitudinal study over the first year of lactation was carried out to confirm these findings.

Sixteen mothers and infants participated in the 12-month longitudinal study and provided milk samples at 2, 5, 9 and 12 months. Similar to the cross-sectional study, \( p,p' \)-DDE was also detected in 83% of the longitudinal HM samples. Overall, \( p,p' \)-DDE, \( p,p' \)-DDT and \( \beta \)-HCH in HM decreased by 68%, 45% and 73%, respectively during the first year of lactation. Several studies have shown that the pesticides in HM are imported from maternal adipose stores such as hepatic lipid and adipose tissues (Koppe, 1995; Bergkvist, et al., 2008), thus this decrease of HM pesticides throughout the lactation indicates the reduction of the maternal body burden through the breastfeeding. We also found that CDI by the infant decreased significantly during breastfeeding, so that they were receiving fewer doses as lactation progressed. Almost all previous longitudinal studies have been carried out at the early lactation stages of less than 4 months and can only reflect the earliest and highest level of pesticides in HM (Yu, et al., 2007; Zietz, et al., 2008). Therefore, this longitudinal study more accurately reflects the
long-term trend of the detected pesticides in HM and the infant exposure throughout the first year of lactation, which can provide invaluable information of the influence of POPs on breastfed infants growth according to WHO’s recommendation of in the first year of breastfeeding.

Most of the studies that have associated detrimental outcomes with POPs exposure have been performed using prenatal pesticides exposure. Indeed these environmental effects have been linked to fetal programming and developmental origins of disease paradigms. The “Fetal programming” hypothesis proposed by Barker suggests that impaired fetal growth could lead to permanent deficits in childhood development and could also be the origins of a number of chronic diseases in adulthood, such as heart disease, diabetes and hypertension (Osmond & Barker, 2000). Subsequently, POPs exposure with infant birth outcomes such as birth length, weight and chest circumference has been investigated. However, these studies have used prenatal pesticides exposure during pregnancy and infant anthropometrical size at birth as a proxy measure of *in utero* development, which have failed to yield consistent results (Eskenazi, et al., 2004; Whyatt, et al., 2004; Wolff, et al., 2007). To the best of our knowledge, little is known about the effects of these pesticides in HM on the infant growth outcomes during breastfeeding despite the first 1000 days of life being considered the most plastic period of human programming and development (Copper, et al., 2014). In general, most studies have been hampered by their inability to sample HM and collect infant growth data throughout the lactation. Infant growth whilst an unsophisticated measure is still considered a good determinant of perinatal and postnatal health. Several studies have reported that poorer cognitive abilities and school performance in childhood are related to smaller infant head circumferences at birth or during the first year of life (Hack , et al., 1991; Perera, et al., 2003). To investigate the influence of POPs on infant growth and development in early
life, accurate measure of POPs in HM is critical and HM POPs are more closely associated to the accurate POPs exposure than prenatal pesticides exposure. Our longitudinal study for the first time demonstrated that there were no significant relationships between \( p,p' \)-DDE concentrations in HM and infant growth outcomes such as weight, body length, head circumference and body composition throughout the first year lactation. However, the innate differences in size between each infant would have introduced considerable variance into the relationship, SD-scored for infant growth variables would be more informative and easier to visualize the associations between POPs concentrations and infant growth. This should be examined in the future work.

In order to ensure that the detected, \( p,p' \)-DDE in HM poses no risk to the infants and understand the actual POPs dose to the infant rather than HM POPs concentrations in isolation, both cross-sectional and longitudinal studies calculated the daily intake (CDI) of \( p,p' \)-DDE based on the concurrent actual measurements, such as milk fat concentration (\( C_{fat} \)), infant weight (\( M_{infant} \)) and milk intake (\( V_{milk} \)) throughout the first year of lactation. Whereas in previous studies, the estimated daily intake (EDI) that has been calculated using constant values for \( C_{fat} \) (0.03 g/mL), \( M_{infant} \) (5 kg) and \( V_{milk} \) (700 mL) (Van Oostdam, et al., 1999; Bedi, et al., 2013), did not account for the large variations between individual infants at different lactation months. In general, most studies have been hampered by the inability to collect \( C_{fat} \), \( M_{infant} \) and \( V_{milk} \) data for individual infants. We found that the EDI significantly differed from CDI, where the EDI underestimates the pesticide concentrations by 10% at 2 months and overestimates the concentrations by 50%, 155% and 233% at 5, 9 and 12 months postpartum respectively (Table 6.3). These results clearly demonstrate that CDI rather than EDI should be used to calculate the accurate POPs dosage to the infant. CDI values for all
the infants in this study were up to 500 times lower than the TDI, which further demonstrates that maternal HM in WA poses no health risk to individual infants.

In summary, I have designed a protocol for the accurate determination of 88 different POPs in HM with GC-MS/MS, where a new QuEChERS method was optimized and validated specially for POPs in HM. This thesis also systematically evaluated the matrix enhancement of these targeted POPs in HM, and investigated various methods such as APs, pressure pulse and large volume injection to address the matrix enhancement. This new methodology was applied to both cross-sectional and longitudinal studies for the detection and quantification of POPs in HM from women in WA. A significant decrease of HM POPs was found throughout the first year of lactation in the longitudinal study. Also for the first time, no significant association between HM POPs and infant growth and development was reported. This thesis can serve as a protocol and be used to analyze POPs in regular monitoring of not only in term HM but also for preterm HM in Australia and beyond in the future.

The limitations of the study include a) Sample size is relatively small and more mothers should be recruited and included in the future study; b) Information about mother’s demographics (such as diet, lifestyle, living locations, occupation, etc.) should be collected alongside the HM collection to better understand the sources of each detected pesticides.

7.2 Future perspectives

Future work is required to clarify the exposure sources and pathways of the detected pesticides in Australia. This will require the collection of more detailed maternal
characteristics such as place of birth, relocations, diet composition before and after pregnancy, social status and any other habits, such as drug abuse, smoking or drinking. This information will provide valuable information regarding interaction between maternal environment and HM POPs. The environment in which the individual lives in their first 18 years of life will have the greatest influence on programming for health later in life. Potentially parts of the population will be exposed to a greater extent of POPs due to their geography. Therefore, future studies should also focus on rural areas as well as metropolitan areas.

Although this work covered a large number of pesticides, metabolites of those pesticides such as OPPs, carbamates and pyrethroids, need to be examined. It will be essential to identify any metabolites of these POPs not only in HM but also in maternal blood and urine collected one month before and after birth. This provides information related to infant prenatal and postnatal exposure to the pesticides and their metabolites, which will assist in the evaluation of potential influences of these prenatal and postnatal exposures on infant birth and growth outcomes in the first 2 years of life. If possible collection of infant biofluids to determine the presence of any metabolites of these POPs will demonstrate that the metabolized pesticides can also be transferred from mother to infant through HM. Other analytical techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) can also be utilized to assist the analysis of the thermal labile POPs and the metabolites.
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Comparison of gravimetric, creamatomcrit and esterified fatty acid methods for determination of total fat content in human milk

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ABSTRACT
The gravimetric method is considered the gold standard for measuring the fat content of human milk. However, it is labor intensive and requires large volumes of human milk. Other methods, such as creamatomcrit and esterified fatty acid assay (EFA), have also been used widely in fat analysis. However, these methods have not been compared concurrently with the gravimetric method. Comparison of the three methods was conducted with human milk of varying fat content. Correlations between these methods were high ($r^2 = 0.99$). Statistical differences ($P < 0.001$) were observed in the overall fat measurements and within each group (low, medium and high fat milk) using the three methods. Overall, stronger correlation with lower mean (4.73 g/l) and percentage differences (5.18%) was observed with the creamatomcrit than the EFA method when compared to the gravimetric method. Furthermore, the ease of operation and real-time analysis make the creamatomcrit method preferable.

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1. Introduction

Human milk contains a variety of nutrients and immunologically active components that are required for both optimal growth and the development of a newborn’s immune system against an array of diseases and infections (LaKind, Amina Wilkins, & Berlin, 2004). Milk fat is the major source of energy for infants, contributing over half of the total energy of human milk (Hammoh, Bitman, Wood, Hamosh, & Mehta, 1985). However, fat is the most variable nutritional component in human milk, changing substantially within and between feeds, between breasts, and among mothers and as well as with stage of lactation (Czank, Simmer, & Hartmann, 2009; Kent et al., 2006). Despite the importance of milk fat for the rapidly growing human infant and the multiple methods of analysis of fat content available, no extensive comparative studies have been conducted on fat analysis of human milk.

It is standard in biological fluids, such as urine, urinary creatinine is normally used for comparison in the comparison of studies between different populations. In human milk, lipophilic compounds, such as persistent organic pollutants (POPs), bind to the central core of the milk fat globules and, therefore, when making comparisons, values should be normalized to the fat content of human milk. For example, when estimating POPs dosage, precise measurement of fat will reflect more accurately the maternal-infant environment and associated risks. Unfortunately, the vast array of components in milk, such as proteins, hydrophobic components, micellar casein and fat globules, which are dispersed in the liquid colloid, make accurate measurement of fat challenging. Thus, total fat determination in milk requires a quantitative extraction of all lipid compound classes (Kumar, Lindley, & Mastana, 2014).

Several techniques have been employed to measure fat in milk. The gravimetric reference method is based on measurement of fat mass in a sample after liquid-liquid extraction (Bligh & Dyer, 1959). The esterified fatty acid (EFA) assay has been adapted from analysis of total fatty acids in blood and works on the principle of breaking ester linkages (−COO−R−) in lipid species, such as triacylglycerols, which constitute approximately 98% of the fat in milk, followed by spectrometry analysis (Jensen, 1995; Stern & Shapiro, 1953). Creamatomcrit method has been developed as a rapid and feasible tool for use in the clinical setting (Lucas, Gibbs, Lyster, & Baum, 1978; Meier et al., 2006). Whole milk is centrifuged and measurements are made of the skim milk and cream layer to calculate the cream content of the milk.

Whilst differences in recorded fat content resulting from the detection methods employed are not unexpected, these differences have not been examined. Differences in measurements might lead
to errors in the calculation of the caloric content. This is important in situations where infant growth is paramount, such as in preterm infants. Similarly, estimation of fat-soluble contaminants is not possible without determination of fat content.

In this study, we compared three methods, specifically the gravimetric, EFA and creatamotrit methods for the analysis of fat content in human milk.

2. Material and methods

2.1. Sample

This study was approved by the Ethics Committee of The University of Western Australia. Term milk from the mother was thawed at 37 °C for one hour and was divided into four 100 mL aliquots. The first 100 mL aliquot was sub-divided into aliquots of 2 mL (medium fat content, n = 20), 50 mL from the second 100 mL aliquot was diluted 2-fold with 50 mL of deionized water. It was then divided into aliquots of 5 mL (low fat content, n = 20). The remaining two 100 mL aliquots were centrifuged at 750 g for 5 min (Eppendorf 5804R, Hamburg, Germany) and 50 mL of skim milk was removed from each of the sample. The remaining content (containing fat and skim milk) in each tube were combined and divided into 5 mL aliquots (high fat content, n = 20).

A total of 60 samples were prepared and stored at −20 °C. Prior to analysis, each 5 mL aliquot was thawed at 37 °C for 30 min and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 s.

2.2. Reagents and standards

Chloroform and methanol were obtained from Chem-Supply (Gillman, SA, Australia). Absolute ethanol was supplied by Merck (Darmstadt, Germany). Hydrochloric acid (32%, w/v) was obtained from Scharla (Barcelona, Spain).

Hydroxyamine hydrochloride, sodium hydroxide, trichloroacetic acid, trilein standard stock solution, hydrochloric acid and ferric chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water used in the experiments was generated by Ibis Technology Ultrapure Water purification system (Perth, WA, Australia). All chemicals were of analytical grade and were used as received without further purification.

2.3. Determination of total fat content in human milk

2.3.1. Gravimetric method (FOL extraction)

The gravimetric method used is based on the modified method of Folch, Lipid, and Sloane-Stanley (1957). Briefly, 2 mL human milk was mixed with 40 mL of chloroform/methanol (2:1, v/v). The mixture was homogenized thoroughly and centrifuged at 1500 g for 10 min. The clear homogenate was transferred to a separating funnel. Subsequently, 7.8 mL of water was mixed with the homogenate and allowed to stand until phase separation was observed. The proportion of water to homogenate was 2:10 (v/v) to ensure that no interfacial fluid was formed in the biphasic system obtained. The lipid layer (lower layer) was collected. The aqueous layer (top layer) was rinsed with chloroform/methanol mixture (2:1, v/v) and was allowed to stand until phase separation. The ratio between the aqueous layer and the rinsing solvent was around 1:1 (v/v) to prevent interfacial fluid. The lipid layer was collected and combined with the previous collection. The combined lipid fraction was then evaporated to dryness in a rotary evaporator and dried to constant weight under vacuum and the lipid content determined gravimetrically.

2.3.2. Esterified fatty acids (EFA)

The EFA method used is modified based on the method of Stern and Shaprio (Atwood & Hartmann, 1992; Stern & Shapiro, 1953). Samples (2.5 μL) and standards (trilinolein, 0–200 mM, 2.5 μL) were pipetted in duplicate into a deep-well plate followed by addition of 400 μL of absolute ethanol and mixed well. Then, 100 μL of 2 M hydroxylamine hydrochloride and 100 μL of 3.5 M sodium hydroxide were added, mixed well and allowed to stand for 20 min at room temperature. The samples were acidified by addition of 100 μL of 4.08 M HCl. Color change from dark yellow to brown was observed after addition of 100 μL of a ferric chloride/trichloroacetic acid solution (3.75 g TCA in 5 mL 0.37 M FeCl₃). Due to the hygroscopic nature of hydroxylamine hydrochloride and FeCl₃-TCA, they were freshly prepared. The mixture was thoroughly mixed and duplicate aliquots of 100 μL were pipetted into a flat bottom 96-well plate. The plate was then analyzed using an EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 540 nm.

2.3.3. Creatamotrit

The creatamotrit method used is based on the modified method of Lucas et al. (Lucas et al., 1978). The milk sample was drawn into two 75 μL micro-hematocrit capillary tubes (Kimble, TN, USA) and one end of the capillary was sealed with cellophane (Kimble, TN, USA). The tubes were then centrifuged in a micro-hematocrit centrifuge (BRH Hermle, USA) at 12,000 g for 10 min. The creatamotrit (%) was measured using Creatamatrit Plus™ (Medela AG, Switzerland), which was based on the ratio of cream layer and total milk volume. The creatamotrit (%) was converted to fat content (g/L) based on the following formula: fat content = 3.968 × (5.917 × creatamotrit (%)) (Meier et al., 2006).

2.4. Data analysis

Statistical analysis was carried out using R 3.2.0 using the package lme4 (Bailey, Bates, DebRoy, Sarkar, 2009) and the package Lattice for Bland-Altman plots (Sarkar, 2009). Linear mixed effects were used to determine the relationship between the fat content and the three different methods. The fixed effect factor was the method. The random effects were the group (low, medium and high fat) and individual aliquot. Differences were considered to be significant if P < 0.05. Results were expressed as mean and standard deviation (SD). Bland-Altman plots were used to investigate if there were systematic effects of the measured fat content on the difference between the measurement methods.

3. Results

Overall, the fat content measured was statistically different (P < 0.001) between the different analytical methods and also within each of the sample groups (low, medium and high fat). However, excellent correlations (r² > 0.99) were found between the methods (Fig. 1).

The fat content measured by the gravimetric method was significantly higher (P < 0.001) than that measured by both EFA and the creatamotrit methods in all three sample groups of low, medium and high fat milk (Table 1).

The intra-assay precision in each sample group (low, medium and high fat) within each method was also tested. The gravimetric method gave a mean coefficient of variation (CV) of 1.74%. The largest CV was observed in medium fat milk (2.89%) followed by low (1.40%) and high fat milk (0.84%). The EFA method gave a mean CV of 5.71% with the highest CV observed in low fat milk (10.9%) followed by medium (5.34%) and high fat milk (1.84%). The
creamotocrit method followed a similar pattern to the EPA method with a mean CV of 3.94% and the highest CV observed in low fat milk (6.58%) followed by medium (3.48%) and high fat milk (1.75%).

When comparing the three methods, the largest mean difference was observed between the gravimetric and the EPA methods in low, medium and high fat milk (Table 1). A smaller difference was observed between the gravimetric and the creamotocrit methods in low, medium and high fat milk (Table 1).

The box plots (Fig. 2) show the percentage mean difference in low, medium and high fat milk in gravimetric-EPA methods (36.45, 19.13 and 8.94%, respectively), gravimetric-creamotocrit methods (6.68, 4.58 and 4.26% respectively) and creamotocrit-EPA methods (27.80, 11.01 and 5.55% respectively).

The correlations between the methods were: gravimetric-EPA ($r^2 = 0.984$); gravimetric-creamotocrit ($r^2 = 0.995$) and EPA-creamotocrit ($r^2 = 0.988$). The Bland-Altman plots showed differences between these methods were within 1.25D (Fig. 3).

4. Discussion

In this study, we observed excellent correlations between the reference gravimetric method for measuring milk fat content and both EPA and creamotocrit methods. However, the linear mixed model analysis demonstrated a significant difference between these three methods. Since the gravimetric method has been designated the reference method for measuring fat in human milk, we have compared both the EPA and the creamotocrit methods, which are simpler techniques for fat measurement, to the gravimetric method.

Despite a strong correlation (Fig. 1A) between the EPA and gravimetric methods, which is consistent with previous literature (Atwood & Hartmann, 1992), we found that the EPA method tended to underestimate the fat content by 7.62–10.73 g/L (Table 1) with the percentage difference of 8.49–36.45% compared to the gravimetric method (Fig. 2A). Underestimation of fat content in raw milk was also observed by Atwood & Hartmann (Atwood & Hartmann, 1992). Underestimation of fat content may be due to the fundamental principles underpinning the EPA method. The EPA method disrupts the ester linkages of triacylglycerol, which account for 98% of the total fat in milk, whereas the gravimetric method partitions the fat and measures its mass, essentially measuring total fat. Therefore, we should observe small differences (0.55–1.79 g/L, based on the measured value using the gravimetric method in this study) between these two methods. However, in reality, absolute reaction of the triacylglycerol in the EPA method is impossible leading to further underestimation (Casado et al., 2011). On the other hand, the gravimetric method could potentially overestimate the fat as the partitioning step is selective.

Table 1

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Sample size</th>
<th>Mean fat content (g/L)</th>
<th>Mean difference £</th>
<th>Mean difference ¥</th>
<th>Mean difference £#</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Gravimetric</td>
<td>EPA</td>
<td>Creamotocrit</td>
</tr>
<tr>
<td>Low fat milk</td>
<td>20</td>
<td>29.49 (0.63)</td>
<td>18.75 (2.11)</td>
<td>26.11 (1.79)</td>
<td>10.73 (1.89)</td>
</tr>
<tr>
<td>Medium fat milk</td>
<td>20</td>
<td>51.80 (1.66)</td>
<td>43.47 (1.94)</td>
<td>48.94 (1.75)</td>
<td>16.32 (2.32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.10–52.70</td>
<td>40.85–47.62</td>
<td>45.60–52.40</td>
<td></td>
</tr>
<tr>
<td>High fat milk</td>
<td>20</td>
<td>85.76 (0.87)</td>
<td>72.14 (1.55)</td>
<td>83.81 (1.50)</td>
<td>7.62 (1.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.84–90.90</td>
<td>79.71–85.52</td>
<td>81.70–87.40</td>
<td></td>
</tr>
</tbody>
</table>

* Mean difference of fat content measured by gravimetric and EPA presented as mean (SD).
* Mean difference of fat content measured by gravimetric and creamotocrit presented as mean (SD).
* Mean difference of fat content measured by creamotocrit and EPA presented as mean (SD).
* $P<0.001$.

Fig. 1. Linear correlation between the fat content (g/L) measured by gravimetric and EPA methods (A), gravimetric and creamotocrit methods (B), and EPA and creamotocrit methods (C).
Fig. 2. Differences of fat content (%) between methods for each sample group: gravimetric and EFA (A); gravimetric and creamatocrit (B) and EFA and creamatocrit (C). Toward all hydrophobic and hydrophilic compounds in milk and is not specific to only the lipid compounds. Cerbulis and Custer have reported that casein is also soluble in the extraction solvent (chloroform/methanol), thus further accentuating the difference between the gravimetric and the EFA methods (Cerbulis & Custer, 1967).

Fig. 3. Bland-Altman plots showing the differences between gravimetric and EFA methods (A), gravimetric and creamatocrit methods (B) and EFA and creamatocrit methods (C) for all sample groups. The dotted line is the mean and the solid lines are ±2SD of the mean.

The creamatocrit and the gravimetric methods showed an excellent correlation (Fig. 1B). However, the creamatocrit underestimated the fat content by 3.38-5.96g/1 (Table 1) with a small percentage difference of 4.24-6.68% compared to the gravimetric method (Fig. 2B). As the creamatocrit uses centrifugal force to separate the skin and cream layer, some fat is retained in the skin.
layer (Czanki et al., 2009). Therefore, it is expected that the measured value by the creamatocrit would be lower compared to the gravimetric method. As with any sample handling, the milk fat globule can also undergo degradation into free fatty acids, which occupy less space than cream (Lucas et al., 1978), compromising the underestimation of fat by the creamatocrit method. Consistent with our finding, Ganguil et al. also observed that the creamatocrit method underestimated the fat content (by about 25%) in sow and rat milk whilst providing good correlation when compared to the gravimetric method (Ganguil, Smith, & Hanson, 1969). A recent paper compared the creamatocrit method with mid infrared spectroscopy and concluded that the creamatocrit overestimated the fat content (O‘Neill, Radomacher, Sparks, & Adamkin, 2013). However, methodological concerns exist regarding centrifugation of the human milk samples. The samples were centrifuged for 15 min at 1315g instead of the standard 15 min at 12000g used in all other studies (Fleet & Linsell, 1984; Lucas et al., 1978; Meier et al., 2002). This would result in a lower compaction of the cream layer, and would account for the higher creamatocrit values observed and, therefore, higher fat content calculated, as compared to the mid infrared spectroscopy.

When the creamatocrit method was compared with the EFA method, there was an excellent correlation (Fig. 1C). However, compared with the creamatocrit method the EFA underestimated the fat content by 1.67–7.36 g/l (Table 1) with the percentage difference of 1.98–28.19% (Fig. 2C). The underestimation of the fat content by the EFA method could again be due to differences in the principles of the two methods. Our findings are similar to that observed by Meier et al. (2008), where they also reported a mean difference of 6.80 g/l and good correlation (Table 2) between the creamatocrit and the EFA.

The relationships were further analyzed by Blund-Altman plots, which showed no systematic error in the relationship between the fat content measured by gravimetric-EFA (Fig. 3A), gravimetric-creammatocrit (Fig. 3B) and creamatocrit-EFA methods (Fig. 3C).

Each of the methods investigated here have inherent advantages and disadvantages. The gravimetric method requires the largest volume of milk (>2 ml) among the methods compared, and is also labor-, time-, and solvent-intensive. Whilst this method is precise (CV = 1.7%), due to its complicated procedures only one milk sample can be processed in an hour. The EFA method on the other hand only employs a small amount of milk (<0.1 ml) and chemicals (<0.1 ml). However, the EFA is also labor-intensive allowing only 10 samples to be processed per hour. Among the three methods investigated, the EFA has the lowest precision (CV = 5.7%). Both the gravimetric and EFA methods require the use of laboratory equipment, and are not suitable for real-time analysis in a hospital setting. The creamatocrit method on the other hand is a reagent-free technique requiring only a small amount of milk (<0.1 ml). In this study, the creamatocrit method has good precision (CV = 3.9%) and has the highest throughput (60 samples per hour). Besides being an inexpensive analysis, it also does not require a skilled operator. Therefore, real-time analysis can be performed by clinicians in the hospital. Furthermore, this study has shown closer correlation of the creamatocrit measured fat content with the reference method (gravimetric) than the EFA method.

5. Conclusions

This is the first study that has systematically compared three different methods of measuring fat content in human milk: gravimetric, EFA and creamatocrit. Both the EFA and creamatocrit methods showed excellent correlation with the gravimetric method. There were differences between methods in measured fat content, which can be explained by the different principles of measurement behind the methods. The fat content measured by the creamatocrit method had values closer to that of the gravimetric method than the EFA method. Significant underestimation using the EFA method could be clinically relevant for low fat milk. The choice of method should take into account whether the measurement is performed in the laboratory or clinical setting and the requirements for accuracy and precision.

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J. Du sincerely appreciates the International Postgraduate Research Scholarships (IPRS), Australia Postgraduate Award (APA) and UWA Safety-Net Top-up Scholarship for financial support. J. Du also wishes to thank C.Z. Zhao for his active help and cooperation. This work was also supported by an unrestricted research grant from Medela AG.

Reference


Pesticides in human milk of Western Australian women and their influence on infant growth outcomes: A cross-sectional study

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c Metabolomics Australia, Western Australia, Australia

HIGHLIGHTS

- Cross-sectional study of 88 POPs in human milk over first year of lactation.
- p,p'-DDE was detected in 87.5% of the human milk samples.
- No significant associations between p,p'-DDE and infant growth outcomes.
- Estimated daily intake overestimates human milk POP concentration.
- Human milk infant intake of DDTs is below the recommended daily intake guidelines.

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ABSTRACT

Persistent organic pollutants in human milk (HM) at high levels are considered to be detrimental to the breastfed infant. To determine the pesticide concentration in HM, a pilot cross-sectional study of 40 Western Australian (WA) women was carried out. Gas chromatography-tandem mass spectrometry (GC-MS/MS) with a validated QuEChERS was used for the analysis of 88 pesticides in HM. p,p'-dichlorodiphenyltrichloroethane (p,p'-DDE) with a mean concentration of 62.8 ± 54.5 ng/g fat was found, whereas other organochlorines, organophosphates, carbamates and pyrethroids were not detected in HM. Overall, no association was observed between HM p,p'-DDE concentrations and maternal age, parity, body mass index and percentage fat mass. Furthermore, the first time no significant association was found between p,p'-DDE concentrations in HM and infant growth outcomes such as weight, head circumference and percentage fat mass. The calculated daily intake was significantly different to the estimated daily intake of total DDTs and was well below the guideline proposed by WHO. The DDTs levels in WA have also significantly decreased by 42 - fold since the 1970s and are currently the lowest in Australia.

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1. Introduction

Persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), pyrethroids and carbamate pesticides are widely used in agricultural practice (Köhler and Treibsorn, 2013). Many of these pesticides are resistant to chemical, physical and biological degradation, thus they are ubiquitously found in the environment despite restrictions on their use. Besides being effective in eradicating pests, many of the pesticides are also harmful to human health. Thus, there is widespread concern about the potential health effects of POPs in HM on infant growth and development. To date, most studies have used prenatal pesticides exposure and infant anthropometrical size at birth as a proxy measure of in utero development, and have failed to yield consistent results (Mazdiyasni et al., 2005; Wu et al., 2010; Sarin et al., 2014). To the best of our knowledge, little is known about the effects of the pesticides in HM on the infant

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growth outcomes during breastfeeding.

In Australia, dichlorodiphenyltrichloroethane (DDT) was first introduced in the 1940s and other pesticides such as heptachlor, aldrin, dieldrin, chlordane, hexachlorocyclohexane (HCH) and hexachlorobenzene (HCB) were registered for use in the 1970s (Keil et al., 2013). All new houses in Western Australia (WA) were treated with pesticides during the early stages of construction in order to eradicate termites (Stacey and Tatum, 1985). Since the 1970s, production and application of DDT and most pesticides were restricted and prohibited in WA (Stacey et al., 1985). However, as many of these POPs have long half-lives and high fat solubility properties, they tend to bio-accumulate in wildlife, especially in species at the top of food chain, such as animals (e.g. seals and dolphins) and humans (Backiffe, 2002; Tanabe, 2002). As the presence of POPs can interfere with the function of normal endocrine system, the immature defense mechanism of the developing fetus and infants makes them more vulnerable to these pesticides than adults (Bruckner, 2000). POPs exposure prenatally via the placenta and postnatally via breastfeeding may result in delayed development, immune deficiency, abnormal behavior and growth retardation (Eslizenzi et al., 2006; Chao et al., 2007; Kourea et al., 2012). Surprisingly there has been no investigation into the relationship between POP concentrations in HM and the breastfed infant growth outcomes. As HM is uniquely tailored for human infant and constitute a major portion of the infants’ diet particularly in early life, studies about the possible influence of POPs in HM on infant growth outcomes are warranted. Previous studies have detected POPs in HM from WA, and have observed a decline in POP concentrations in HM (Stacey and Thomas, 1975; Stacey et al., 1985; Stevens et al., 1991). The most recent study of POP concentrations in WA was based on a single pooled HM sample from 11 women in 2003, which could not represent the POP concentrations for individual mothers and the current concentration in the general population (Mueller et al., 2008). Since then, no further studies of these levels have been reported. Based on the public concern about the current status of the contamination and safety of HM, it is essential to continue monitoring the POPs in WA.

The aims of this study were to (1) describe the current pesticide concentrations in HM from WA mothers and the changes in these pesticide concentrations during the first year of lactation in a cross-sectional cohort; (2) investigate associations between the detected pesticides and maternal and infant characteristics and anthropometrics; (3) calculate the daily intake of the pesticides by individual infants and evaluate the risk to the infant.

2. Materials and methods

2.1. Chemicals

The pesticide standard solutions (100 µg/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingstown, RI, USA). The pesticide standard solutions (100 µg/mL) were mixed and diluted with acetonitrile (ACN) to prepare a stock standard solution (1 ng/mL) of all the pesticides. LC-MS grade acetonitrile (ACN) and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylglycol (98%), galactonate (95%) and D-sorbitol (≥98%) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (≥98.5%) and magnesium sulfate (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecylsilyl (C18) and primary secondary amine (PSA) were obtained from Agilent (Little Falls, DE, USA). Isotopic labeled quality control (QC) standards, acenaphthene-D₃, phenanthrene-D₁₀ and chrysene-D₁₂ were purchased from Restek (Bellefonte, PA, USA), and the internal standard (IS), triphenylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Study population and sample collection

The study was approved by the Human Research Ethics Committee of The University of Western Australia. Breastfeeding dyads (n = 40) were recruited in metropolitan WA between 2013 and 2015. The mothers whom volunteered were predominantly of Caucasian background, married and with a college degree and provided a milk sample in one of the following months of lactation: 2 (n = 11), 5 (n = 9), 9 (n = 10) or 12 months (n = 10). Milk samples (1–5 mL) were collected in glass containers before and after feeding from each breast. The fat content of HM was measured immediately using the Creamatocritt method (Meser et al., 2002), and the remaining milk was stored at −20 °C. All participants provided informed written consent and completed a questionnaire including relevant demographic data.

2.3. HM sample treatment

HM samples (n = 40) were thawed at room temperature for 3 h and then homogenized with a mixer (ELM Ltd, Riga, Latvia) for 15 s. 1 mL of HM was placed into a 15 mL centrifuge tube and 1 mL ACN containing 1% HAC and 100 ng/mL QC standards (acenaphthene-D₃, phenanthrene-D₁₀ and chrysene-D₁₂) was added. A validated acetate buffered QuEChERS method was employed to extract the HM (Lehezy et al., 2005a, 2005b). Extraction reagents (0.4 g MgSO₄ and 0.1 g NaCl) were added and shaken immediately, and the tube was then placed in an ice bath. The extraction tube was centrifuged at 3993 g for 10 min. 0.6 mL of the supernatant was transferred into a clean 15 mL centrifuge tube and stored in −20 °C for 2 h. Freezing is critical for fatty samples to remove coextractives with limited solubility in ACN (e.g. lipid, wax and sugars). The supernatant was then centrifuged at 3993 g (0 °C) for 10 min and 0.5 mL was transferred to a cleanup tube (157 mg MgSO₄, 9 mg C18 and 9 mg PSA). The tube was vortexed and centrifuged at 3993 g for 10 min. The final extract was transferred into a screw cap amber vial and kept at −80 °C until analysis.

2.4. Working standard solutions preparation

The working standard solutions (0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL) were prepared by appropriate dilution of the stock standard solution (1 µg/mL) with ACN. A combination of anlyte protectants (APs) mixture (ethylglycol, galactonolate and D-sorbitol) containing internal standard TPF (IS) was added to the final extract of each HM sample and to all working standard solutions for GC-MS/MS analysis. The final concentrations of ethylglycol, galactonolate and D-sorbitol were 20, 2 and 2 ng/mL, respectively and the IS was 100 ng/mL. As demonstrated in our previous study, the addition of APs mixture can equalize the enhancement difference between the pesticides in pure solvent and in HM extract, which can then be used for the quantification of the pesticides in HM.
USA) was used. The inlet temperature was kept constant at 250 °C. The initial oven temperature of the column was 80 °C for 3 min and ramped at 30 °C/min to 150 °C and then ramped at 10 °C/min to 300 °C, where it was held for 10 min. The total run time was 31 min. Helium (6.0 GC grade) was used as carrier gas at a constant flow of 1.1 ml/min.

The mass spectrometer was operated in electron impact (EI) mode. Transfer line and ion source temperature were at 270 °C and 200 °C, respectively. Argon was used as the collision gas. Identification and quantification of 88 pesticides were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. The optimized MRM transitions and collision energies for each compound are listed in Table S1. Data collection and processing were performed using Bruker MSWDS 8 Software.

2.6. Quality control

Isotopically labeled quality control (QC) standards were employed to evaluate the efficiency of the extraction and cleanup steps, while TIP (S) was used to evaluate the performance of the instrument throughout the entire analytical procedure. A QC mixture containing pesticide standards (20 ng/ml) was analyzed between sample batches to check for any interferences and cross-contaminations. The recoveries for the pesticides were within the range of 70–120% with the relative standard deviations (RSD, n = 6) of 3.6–33.9%, 2.2–17.4% and 2.5–13.5% for spiked concentrations of 10, 20 and 100 ng/ml, respectively. Limit of detection (LOD) and limit of quantitation (LOQ) were determined experimentally with the lowest standards containing AIs providing signal-to-noise ratio (S/N ratio) greater than 3 and 10, respectively. The LOD and LOQ range for the various classes of pesticides such as OCPs (LOD: 0.2–0.5 ng/ml; LOQ: 0.5–1.0 ng/ml), OPPs (LOD: 0.2–1.0 ng/ml; LOQ: 0.5–4.0 ng/ml), fungicides (LOD: 0.2–1.0 ng/ml; LOQ: 0.5–2.0 ng/ml), carbamates and pyrethroids (LOD: 0.2–2.0 ng/ml; LOQ: 0.5–5.0 ng/ml) and herbicides and other pesticides (LOD: 0.2–1.0 ng/ml; LOQ: 0.5–2.0 ng/ml) were observed.

2.7. Maternal and infant anthropometric measurements

Maternal body weight was measured using an electronic scale (Seca, California, USA, accuracy 0.1 kg). The height, age and parity were self-reported by participants. Maternal BMI was calculated according to the following formula: BMI = kg/m².

Single measurements of infant weight, length and head circumference were performed at each time point when the milk sample was collected. Infant weight was determined by weighing before breastfeeding using electronic scales (±0.2 g; Medela Electronic Baby Weigh Scales, Medela AG, Switzerland). Infant crown-heel length was measured, to the nearest 0.1 cm, on a hard surface with perpendicular to the surface headpiece and footpiece and a non-stretch tape. Infant head circumference was measured with non-stretch tape. The daily calculated POP intake for each infant in this study was calculated based on the 24 – hour milk intake values reported by Kent et al. (Kent et al., 1999).

2.8. Maternal and infant body composition measurements

Whole body biopsies (wrist to ankle) of the participants were measured using the Impedimed SF87 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia).

Mothers’ whole body biopsies was measured according to the manufacturer’s instructions.

Infants’ whole body biopsies was measured using an adult protocol (wrist to ankle) and analyzed with settings customized for each infant according to Lingwood et al. (Lingwood et al., 2012).

Resistance at 50 kHz was used in percentage fat mass equations developed for infants (Bocage, 1989; Lingwood et al., 2012).

Infants’ ultrasound skinfold measurements (triceps and sub-scapular) were carried out using Apilo XC (Toshiba, Japan) machine, PLT-1204BX 14–8 MHz transducer and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). The double skinfold thickness, measured directly from images using the on-screen electronic calipers, was used in percentage fat mass equations developed for skinfolds measured with skinfold calipers (Slaughter et al., 1988).

2.9. Statistical analysis

Statistical analyses are carried out either using SPSS software (SPSS, version 19.0 for windows, SPSS, Inc., IL, USA) or RStudio (RStudio, 2010). The results were expressed as mean ± SD unless stated otherwise. Pesticides that were below the LOD were considered as not detected and were not included in the calculations. Detected pesticides were normalized to the fat content in the milk (ng/g fat). Overall interactions between pg′-DDE and both maternal and infant characteristics were carried out using linear model and Pearson correlation coefficients were used to determine the correlation between pg′-DDE and the individual maternal and infant characteristics. Differences were considered to be significant if P < 0.05.

Estimated daily intake (EDI) (µg/kg body wt/day) of the pesticides for each infant was calculated based on the pesticides concentration in HM (µg/g fat; C_HM). Fat content in HM (g/ml; C_fat) average daily consumption of HM (ml; V_{milk}) and body weight of infant (kg; M_{infant}) using the following equation:

\[
\text{EDI} = C_{\text{pesticide}} \times C_{\text{fat}} \times V_{\text{milk}} \times M_{\text{infant}}
\]

One-way ANOVA and Tukey’s all pair comparison tests (Hothorn et al., 2008) were used to compare differences in pesticides concentration, EDI and calculated daily intake (CDI) at the different lactation months. A paired t-test was used to compare the differences between the EDI and the CDI by the infant at various lactation months.

3. Results and discussion

3.1. Participant demographics

The demographic characteristics of the study participants (n = 40) are presented in Table 1. The average age (±SD) of the mothers was 33.3 ± 3.8 years old and the median parity was 2.0. The maternal characteristics, such as HM fat and maternal BMI measured in this study is similar to previous HM POP studies enabling comparison between different populations (Polder et al., 2009; Bedi et al., 2013).

3.2. Pesticide residues in HM

A total of 88 pesticides, including OCPs, OPPs, carbamates and pyrethroids, were analyzed in this study. Overall, only pg′-DDE was detected in 87.5% of the HM samples in this cohort with a mean concentration (±SD) of 1.9 ± 1.0 ng/ml (range: 0.2–10.1 ng/ml) and 62.8 ± 54.5 ng/g fat (range: 6.3–209.2 ng/g fat). The distribution of pg′-DDE in HM at different lactation months is shown in Fig. S1. The large variation in pg′-DDE levels observed between months in this cohort could be due to variation in lifestyles, dietary habits and metabolic activity between mothers (Waliszewski et al., 2001). The dynamic distribution of the pesticides through the blood circulatory system and selective partitioning from plasma to the breast.
could also contribute to the observed variation (Shen et al., 2007). These factors may account for the lack of significant decrease of p,p'-DDE in HM observed between the months of lactation, especially in the 9th and 12th months, in this cohort (Table 2).

3.3. Associations between pesticide residues in HM and maternal characteristics

Many studies have collected HM in the first 6 months postpartum (Zietz et al., 2008; Bowesman et al., 2012). To our knowledge, this is the first study that collected HM throughout the first year of lactation and investigated the relationship between POP levels in HM and both maternal and infant characteristics. The interactions between HM p,p'-DDE and both maternal and infant anthropometrics were evaluated and no significant interactions (P > 0.44) were observed, therefore all the samples were pooled and analyzed as a whole (n = 40).

There was no significant association between HM p,p'-DDE and both maternal age (P = 0.42) and parity (P = 0.56). Further no significant association was observed between p,p'-DDE levels and maternal BMI (P = 0.14). BMI does not differentiate between fat mass and fat-free mass, but measurement of maternal body composition (BC) or percentage fat mass using bioimpedance spectroscopy (BIS), is more accurate in assessing body fat (Bembon et al., 1998). As pesticides are related to body fat, this is the first study that investigates the relationship between pesticides and maternal percentage fat mass. No significant association was observed between p,p'-DDE levels and maternal percentage fat mass (P = 0.07) (Table 3). There was no significant difference in percentage fat mass at different lactation months (P > 0.07) in this cross-sectional cohort. This is the first study that investigated the possible relationships between the detected pesticides and maternal BC.

3.4. Adverse effects of pesticide residues on infant growth outcomes

No significant associations were observed between p,p'-DDE concentrations in HM and infant weight (P = 0.63), body length (P = 0.26), head circumference (P = 0.54) and percentage fat mass (ultrasound: P = 0.63; BIS: P = 0.16) during breastfeeding (Table 3). No significant associations were observed between the detected p,p'-DDE and infant growth outcomes in this cohort, even with more specific body fat measurements, using BIS and ultrasound. To the best of our knowledge, this is the first study that has assessed possible effects of POPs in HM on the infant growth outcomes during breastfeeding. It is crucial that no adverse effects were observed between HM POPs and infant growth outcomes as several studies have associated smaller head circumference during the first year of life to poorer cognitive abilities and school performance at the age of 8 (Black et al., 1991; Perosa et al., 2003). Few studies have examined the relationship between HM POPs and infant growth due to the difficulties in collecting both HM and infant growth data at each lactation month. However, current studies have examined infant birth outcomes, such as birth weight, length and chest circumference with prenatal POP exposure, which failed to yield consistent results (Eskenazi et al., 2004; Wyhait et al., 2004; Wolff et al., 2007). As HM is the sole food source for an exclusively breastfed infant for the first 4–6 months of life, detected pesticides in HM will more accurately reflect the influence of these pesticides on the infant growth.

However, due to the lack of interaction and associations between HM p,p'-DDE and both maternal BC and infant anthropometrics in this study, it would be pertinent to conduct a longitudinal study that includes women from different geographical areas in WA to be able to determine the differences between metropolitan and rural environment.

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**Table 1** Characteristics of participants in a cross-sectional study measuring the concentrations of pesticides in human milk collected from women at 2, 5, 9 and 12 months postpartum in Western Australia.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Months</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers (n = 40)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>31.8 ± 4.3</td>
<td>33.0 ± 2.2</td>
<td>34.4 ± 4.0</td>
<td>33.2 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Parity*</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HM fat content (g/L)</td>
<td>24.0 ± 8.9</td>
<td>26.2 ± 14.5</td>
<td>36.7 ± 17.4</td>
<td>45.0 ± 17.9</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.3 ± 5.7</td>
<td>22.8 ± 5.5</td>
<td>23.4 ± 5.4</td>
<td>26.0 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Percentage fat mass (BIS)</td>
<td>35.5 ± 3.5</td>
<td>31.5 ± 5.7</td>
<td>27.8 ± 8.8</td>
<td>34.0 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>Infant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>7/4</td>
<td>6/3</td>
<td>9/1</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td>Infant weight (kg)**</td>
<td>5.2 ± 0.7</td>
<td>7.4 ± 0.9</td>
<td>9.0 ± 0.8</td>
<td>9.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Body length (cm)**</td>
<td>57.1 ± 2.4</td>
<td>64.6 ± 2.9</td>
<td>72.1 ± 1.6</td>
<td>73.3 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Head circumference (cm)**</td>
<td>39.3 ± 1.3</td>
<td>43.4 ± 1.0</td>
<td>45.7 ± 1.3</td>
<td>46.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Percentage fat mass (ultrasound skinfolds)</td>
<td>19.0 ± 3.8</td>
<td>27.5 ± 3.7</td>
<td>23.7 ± 2.4</td>
<td>22.3 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Percentage fat mass (ultrasound skinfolds)</td>
<td>25.6 ± 4.2</td>
<td>27.2 ± 4.2</td>
<td>25.3 ± 6.1</td>
<td>27.4 ± 5.2</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference (P < 0.05) observed between months 2–5, 2–9, 2–12, 5–9 and 5–12.
**Significant difference (P < 0.05) observed between months 2–5.
*Expressed as median.
**BIS: bioimpedance spectroscopy.

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**Table 2** Summary of detected p,p'-DDE in human milk collected at 2, 5, 9 and 12 months postpartum. No significant difference was observed of p,p'-DDE between lactation months*.

<table>
<thead>
<tr>
<th>Lactation months</th>
<th>Sample</th>
<th>Frequency of detection (%)</th>
<th>Mean ± SD (ng/g) [Range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>11</td>
<td>9 (81.8%)</td>
<td>70.7 ± 72.4 (149–2002)</td>
</tr>
<tr>
<td>5 months</td>
<td>9</td>
<td>8 (88.8%)</td>
<td>75.9 ± 90.8 (181–1833)</td>
</tr>
<tr>
<td>9 months</td>
<td>10</td>
<td>8 (80.8%)</td>
<td>51.5 ± 25.5 (13–165)</td>
</tr>
<tr>
<td>12 months</td>
<td>10</td>
<td>10 (100%)</td>
<td>45.8 ± 27.1 (17.5–106.5)</td>
</tr>
</tbody>
</table>

*P-values for p,p'-DDE in HM between each lactation months 2–5 (1.00), 2–9 (0.71), 2–12 (0.47), 5–9 (0.72), 5–12 (0.49) and 9–12 (0.09).
3.5. Infant health risk assessment

The most direct concern with pesticides in HM is the potential detrimental exposure to the infant, which ideally requires knowledge of dose to the infant rather than concentration in isolation. Studies to date have used constant values for $C_{ini}$, $V_{ini}$, and $M_{ini}$ in the calculation of EDI, which is based on the assumption that a 5 kg infant will ingest 700 mL of HM per day containing 3% fat (Van Oostdam et al., 1999; Bezewor et al., 2000; Bedi et al., 2013). However, these studies did not consider the large variations of $C_{ini}$, $V_{ini}$, and $M_{ini}$ between individual infants at different lactation months. In this study, the calculated daily intake (CDI) of DDTs (sum of DDT and its metabolites, DDE, and DDD) is based on the actual measured values ($C_{ini}$, $V_{ini}$, and $M_{ini}$) for each infant. A larger decrease of ingested DDTs was observed in the CDI, with a significant difference ($P = 0.03$) between 2 and 12 months, as compared to the EDI ($P = 0.47$). The overall CDI and EDI for DDTs by the breastfeeding infant throughout the first year of lactation are 0.17 $\mu$g/kg body wt./day (range: 0.01–1.04 $\mu$g/kg body wt./day) and 0.26 $\mu$g/kg body wt./day (range: 0.03–0.88 $\mu$g/kg body wt./day) respectively (Table 4). A significant difference ($P < 0.01$) is observed between the overall CDI and EDI, where EDI overestimated the intake value by 52%. While smaller overestimations (10–22%) were observed at 2 and 5 months ($P = 0.49$ and 0.34 respectively) between CDI and EDI, the later months (9 and 12 months, $P = 0.08$ and < 0.01 respectively) show more dramatic overestimations (110–350%) of daily POP intake. These results clearly demonstrate the significant difference between CDI and EDI, which indicates that CDI should be used where possible to reflect the actual pesticide intake by the infant throughout the lactation months, particularly in the later months. The overall CDI of DDTs by an infant in this study is 59–117 fold below the recommended tolerable daily intake (TDI) by PAG/WHO (PAG/WHO, 2000) and Health Canada (Van Oostdam et al., 1999) respectively. Thus indicating that low level of DDTs detected in HM from WA poses minimal risk to the infant, which also further confirms the findings between HM $\mu$g/$\mu$g$\cdot$DDE and infant growth outcomes in this study.

3.6. Temporal trends of pesticides in HM

Historically the extensive use of DDT resulted in extraordinary high DDTs concentrations in HM from WA (Table S2) (Miller and Fox, 1973; Stacey and Thomas, 1975; Stacey et al., 1985; Stacey and Tatum, 1985; Stevens et al., 1993; Smith, 1999). A significant downward trend of DDTs (42-fold decrease) in HM can be observed since 1970, from 2660 ng/g fat to 63 ng/g fat in this study (Fig. 1). When compared with the most recent data of DDTs in WA

Table 4

<table>
<thead>
<tr>
<th></th>
<th>CDI (μg/kg body wt./day) [Range]</th>
<th>EDI (μg/kg body wt./day) [Range]</th>
<th>$P$-value $^*$</th>
<th>TDI (μg/kg body wt./day) [Range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>All data</td>
<td>0.17 (0.01–1.04)</td>
<td>0.26 (0.03–0.88)</td>
<td>&lt;0.01</td>
<td>10.00 $^*$</td>
</tr>
<tr>
<td>2 months</td>
<td>0.30 (0.09–0.85)</td>
<td>0.33 (0.06–0.88)</td>
<td>0.49</td>
<td>20.00 $^*$</td>
</tr>
<tr>
<td>5 months</td>
<td>0.27 (0.05–1.04)</td>
<td>0.33 (0.08–0.79)</td>
<td>0.34</td>
<td>11.00</td>
</tr>
<tr>
<td>9 months</td>
<td>0.10 (0.01–0.25)</td>
<td>0.21 (0.03–0.09)</td>
<td>0.08</td>
<td>20.00 $^*$</td>
</tr>
<tr>
<td>12 months</td>
<td>0.04 (0.01–0.05)</td>
<td>0.18 (0.03–0.08)</td>
<td>&lt;0.01</td>
<td>20.00 $^*$</td>
</tr>
</tbody>
</table>

$^*$ Health Canada (Van Oostdam et al., 1999).
$^*$ Paired t-test was used to compare the differences between the EDI and the CDI by the infant at various lactation months. Differences are considered to be significant if $P < 0.05$. 

Fig. 1. Historical trend of mean total DDTs levels in HM of WA mothers between 1970s and 2010s (Stacey and Thomas, 1975; Stacey et al., 1985; Stevens et al., 1993; Mueller et al., 2008), while 2010s are results from this study.
conducted in 2002/03 (Mueller et al., 2008), the levels of DDTs have decreased by 4-fold. This declining trend reflects the decrease of human exposure to DDT in WA which is consistent with the worldwide decreasing trend in DDT body load (Smith, 1999). Our findings are also in agreement with the trace levels of p,p'-DDE observed in maternal plasma (1.05 ng/ml) (Bedi et al., 2009), blood (0.5 ng/ml) and abdominal tissues (70 ng/g fat) of WA participants. Other studies have also found trace (<0.001 ng/g) or no detectable pesticides in sediment, mussels and in the effluent or associated biota in WA’s three treated ocean outfalls (Chegwidden, 1979). All these findings demonstrated the effectiveness of strict prohibition of these pesticides in WA leading to the decrease of human exposure to these pesticides over time.

3.7. National and international comparisons

Residue levels in HM were compared with those reported in Australia as well as those reported from different countries in order to evaluate the concentrations of DDTs in HM in WA. There is limited recent data about the pesticides in HM in Australia (Noakes et al., 2006; Mueller et al., 2008). When compared with national data, the HM concentration of p,p'-DDE in current study is 2.4–7.5 times lower than corresponding levels in HM from other States/Territories such as Tasmania, Adelaide and Melbourne (Fig. 5). This spatial variation in Australia could be due to the inconsistent legislation to pesticides control in various states before the 1990s. In addition, differences in dietary habits and living environments between the States may also contribute to the variations.

When compared with international data, residue levels of DDTs in WA milk are similar to those reported for Norway (Polder et al., 2009), Denmark (Shen et al., 2007), Belgium (Cros et al., 2012) and USA (Johnson-Restrepo et al., 2007), and are a few orders of magnitude lower than those found for some Asian and Africa countries such as India (Bedi et al., 2013), Iran (Behrouz et al., 2009), South Africa (Dannerud et al., 2011) and Ethiopia (Gebremiriam et al., 2013) (Table 5). Due to their close proximity, New Zealand and Australia have adopted similar legislations and regulations. However, the concentration of DDTs observed in New Zealand is 5–fold higher than the observed level in current study, suggesting that more extensive historical DDT use in New Zealand (Manerje et al., 2013). In some developing countries, such as India and Africa countries, limited quantity of DDT is still allowed to be used indoor for vector control (e.g. malaria) (Mandavilli, 2006). Thus, DDTs levels of HM from those countries, such as South Africa, Ethiopia, Iran and India were obviously much higher. In general, DDTs levels in HM appear to be higher in developing countries than those in developed countries (Table 5).

4. Conclusions

Since the prohibited use of selective pesticides in WA, out of the 88 targeted pesticides, only p,p'-DDE was detected at trace levels in

<table>
<thead>
<tr>
<th>Country/Region</th>
<th>Year of sampling</th>
<th>Mothers (%)</th>
<th>Human milk samples</th>
<th>Fat content (g/l)</th>
<th>DDTs (ng/g fat)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>2006/07</td>
<td>60</td>
<td>60</td>
<td>31</td>
<td>583^1,2</td>
<td>Leng (2009)</td>
</tr>
<tr>
<td>India</td>
<td>2011</td>
<td>53</td>
<td>53</td>
<td>32</td>
<td>1914^3</td>
<td>Bedi et al. (2009)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>2000/01</td>
<td>36</td>
<td>36</td>
<td>31</td>
<td>333^4</td>
<td>Chao et al. (2001)</td>
</tr>
<tr>
<td>Korean</td>
<td>2008</td>
<td>&gt;50</td>
<td>50</td>
<td>22</td>
<td>225^5</td>
<td>Kim et al. (2013)</td>
</tr>
<tr>
<td>Japan</td>
<td>2008/09</td>
<td>90</td>
<td>90</td>
<td>17</td>
<td>179^6</td>
<td>Fujii et al. (2011)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>2003</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>160^7</td>
<td>Suzuki (2005)</td>
</tr>
<tr>
<td>Philippine</td>
<td>2004</td>
<td>33</td>
<td>33</td>
<td>22</td>
<td>170^8</td>
<td>Malavas (2009)</td>
</tr>
<tr>
<td>Turkey</td>
<td>2009</td>
<td>47</td>
<td>47</td>
<td>36</td>
<td>338^9</td>
<td>Co (2012)</td>
</tr>
<tr>
<td>Iran</td>
<td>2006</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>350^10</td>
<td>Behrouz et al. (2009)</td>
</tr>
<tr>
<td>Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td>2013/15</td>
<td>40</td>
<td>40</td>
<td>33</td>
<td>65^12</td>
<td>Present study</td>
</tr>
<tr>
<td>Europe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poland</td>
<td>2002/05</td>
<td>28</td>
<td>28</td>
<td>14</td>
<td>191^13</td>
<td>Hetnik et al. (2011)</td>
</tr>
<tr>
<td>Slovak</td>
<td>2003</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>383^14</td>
<td>Yu et al. (2007)</td>
</tr>
<tr>
<td>Norway</td>
<td>2002/06</td>
<td>377</td>
<td>377</td>
<td>36</td>
<td>53^15</td>
<td>Polder et al. (2009)</td>
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<tr>
<td>Latvia</td>
<td>2002/04</td>
<td>15</td>
<td>15</td>
<td>82</td>
<td>287^16</td>
<td>Bake et al. (2007)</td>
</tr>
<tr>
<td>Denmark</td>
<td>2002/01</td>
<td>43</td>
<td>43</td>
<td>44</td>
<td>60^17</td>
<td>Shen et al. (2007)</td>
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<tr>
<td>Belgium</td>
<td>2003/10</td>
<td>84</td>
<td>84</td>
<td>36</td>
<td>125^18</td>
<td>Croes et al. (2012)</td>
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<tr>
<td>Germany</td>
<td>2007/08</td>
<td>516</td>
<td>516</td>
<td>29</td>
<td>278^19</td>
<td>Kudr et al. (2013)</td>
</tr>
<tr>
<td>Croatia/Greece</td>
<td>2008/10</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>175^20</td>
<td>Olten et al. (2014)</td>
</tr>
<tr>
<td>UK</td>
<td>2001/03</td>
<td>54</td>
<td>54</td>
<td>15</td>
<td>65^21</td>
<td>Kalieni et al. (2004)</td>
</tr>
<tr>
<td>North and South America</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>2004</td>
<td>38</td>
<td>38</td>
<td>22</td>
<td>65^22</td>
<td>Johnson-restrepo (2007)</td>
</tr>
<tr>
<td>Brazil</td>
<td>2001/02</td>
<td>69</td>
<td>69</td>
<td>69</td>
<td>493^23</td>
<td>Azevedo et al. (2008)</td>
</tr>
<tr>
<td>Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>2001</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>620^24</td>
<td>Dannerud et al. (2011)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>2010</td>
<td>39</td>
<td>39</td>
<td>29</td>
<td>1440^25</td>
<td>Gebremiriam et al. (2013)</td>
</tr>
</tbody>
</table>

Note: not data available.

^1 Values represented as median value.
^2 Pooled sample.
^3 Sum of µg/g DDE = µg/p,p'-DDE + µg/p,p'-DDT.
^4 Sum of µg/p,p'-DDE = µg/p,p'-DDE + µg/p,p'-DDT.
^5 Sum of µg/p,p'-DDD = µg/p,p'-DDD + µg/p,p'-DDT.
^6 Sum of µg/p,p'-DDD = µg/p,p'-DDD + µg/p,p'-DDT.
^7 Sum of µg/p,p'-DDD = µg/p,p'-DDD + µg/p,p'-DDT.
^8 Sum of µg/p,p'-DDD = µg/p,p'-DDD + µg/p,p'-DDT.
^9 µg/p,p'-DDD only.
^10 µg/p,p'-DDD only.
Appendix

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terminated and polyethylene glycol-bisphenol A bisphenol A E and factors that may predict the level of contamination. Sci. Total Environ. 407, 4904–4909.
Longitudinal study of pesticide residue levels in human milk from Western Australia during 12 months of lactation: Exposure assessment for infants

Jian Du, Zoya Gridneva, Melvin C. L. Gay, Ching T. Lai, Robert D. Trengove, Peter E. Hartmann & Donna T. Geddes

The presence of pesticides in human milk (HM) is of great concern due to the potential health effects for the breastfed infant. To determine the relationships between HM pesticides and infant growth and development, a longitudinal study was conducted. HM samples (n = 99) from 16 mothers were collected at 3, 5, 9 and 12 months of lactation. A validated QuEChERS method and Gas chromatography tandem mass spectrometry (GC-MS/MS) were used for the analysis of 88 pesticides in HM. Only p,p’,DDT, p,p’DDE, endo-HCH were detected with a mean concentration (±SD) of 52.25 ± 49.88 ng/g fat, 27.47 ± 20.96 ng/g fat and 48.00 ± 22.46 ng/g fat respectively. The concentrations of the detected pesticides decreased significantly throughout the first year of lactation. No significant relationships between HM p,p’DDE and infant growth outcomes: weight, length, head circumference and percentage fat mass were detected. The actual daily intake (ADI) of total DDTs in this cohort was 14–1000 times lower than the threshold reference and significantly lower than the estimated daily intake (EDI). Further, the ADI decreased significantly throughout the first 12 months of lactation.

Persistent organic pollutants (POPs), such as organochlorine pesticides (OCPs), organophosphate pesticides (OPs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzop- dioxins (dioxins), are synthetic chemicals that are present in the environment. When introduced into the environment, these chemicals can percolate into the soil and ground water, and can be transported over long distances through atmospheric circulation and water movement. These chemicals are absorbed by inhalation, ingestion and dermal contact. As these xenobiotics enter the body, they bind to transport proteins such as human blood albumin (HBA), globulins and lipoprotein in the plasma. The more lipophilic chemicals, such as OCPs, are then redistributed and accumulated in tissue compartments with high fat content, such as adipose tissue, the liver, kidneys, brain and breasts. Whereas the more hydrophilic xenobiotics, such as carbanute, are more easily metabolized in the liver and then excreted. In lactating women, these xenobiotics can be transferred from the blood to milk together with other necessary nutrients, precursors for the production of human milk (HM) components. However, the mechanisms underlying the transfer of these chemicals in HM are not yet known. This paper will focus on the persistent organic pollutants in HM with respect to pesticides.

The presence of pesticides in HM is of great concern due to the potential health effects for the breastfed infant, as many of these pesticides are known to interfere with the function of normal endocrine systems. Exposure to these xenobiotics has been associated with a wide range of adverse effects, such as delayed neurodevelopment, poor cognitive performance and growth retardation during early childhood. High prenatal exposure to OPs and its metabolites has been associated with attention deficit/hyperactivity disorder in children at 5 years of age.

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Table 1. Participant characteristics of a longitudinal study measuring the concentration of pesticides in human milk collected from women (n = 16) at 2, 5, 9, and 12 months postpartum in Western Australia. BIS: biodeimpedance spectroscopy. Significant difference (P < 0.05) was observed between months 2–5, 2–9, 2–12, 5–9, 5–12 and 9–12.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother (n = 16)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>33.9 ± 5.1 (24–43)</td>
</tr>
<tr>
<td>Purity</td>
<td>2.3 ± 1.1 (0–4)</td>
</tr>
<tr>
<td>Infant gestational age (weeks)</td>
<td>39.4 ± 1.4 (38–43)</td>
</tr>
<tr>
<td>Lactation stage (Months)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Maternal body mass index (BMI) (kg m(^{-2}))</td>
<td>24.9 ± 4.6 (20.4–35.5)</td>
</tr>
<tr>
<td>Percentage fat mass (BMI)</td>
<td>34.4 ± 5.3 (25.7–44.7)</td>
</tr>
<tr>
<td>Infant (Male/Female = 7/9)</td>
<td></td>
</tr>
<tr>
<td>Age (Years)</td>
<td>2.0 ± 0.1 (1.9–2.2)</td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>5.6 ± 0.9 (4.4–7.4)</td>
</tr>
<tr>
<td>Body length (cm)</td>
<td>57.9 ± 1.8 (54.5–60.0)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>39.6 ± 1.3 (37.0–42.0)</td>
</tr>
<tr>
<td>Percentage fat mass (ultrasound skinfolds)</td>
<td>25.6 ± 4.7 (20.9–37.9)</td>
</tr>
<tr>
<td>Percentage fat mass (BIS)</td>
<td>22.1 ± 2.6 (19.2–24.1)</td>
</tr>
</tbody>
</table>

Similarly, a longitudinal study of 329 children through to 7 years of age confirmed that high maternal OPs concentrations were associated with poor intellectual development and cognitive performance. In terms of infant growth or size, high levels of chlorpyrifos in maternal prenatal plasma has been associated with smaller infant birth length and weight and high concentrations of DDE in maternal prenatal urine with smaller infant birth weight and head circumference.

With respect to recent study postnatal exposure to pesticide, Liu (2016) reported an association between smaller newborn head circumference and the metabolites of OPs, such as diallylphosphate (DAP) and diallylphosphate (DEP) which more pronounced in the male infant. Follow-up assessment of these infants at 2 years of age showed that prenatal exposure of the fetus to OPs was associated with delayed adaptive skills whilst postnatal exposure to OPs were associated with delayed social and motor development skills. Caution must be taken in interpretation of these studies as it is difficult to differentiate between prenatal and postnatal exposure to these pesticides. Further unraveling postnatal infant exposure due to HM or other sources such as food and the environment is even more difficult due to lack of measurement of both volume of HM consumed as well as reliable dietary records. Epidemiological studies of pesticides in HM in relation to postnatal infant growth outcomes are non-existent therefore we lack understanding of the effects of the pesticides in HM on the infant growth and development despite the infant in being more vulnerable to the potential effects due to their immature biological systems and the high levels of enzymes required to detoxify these pesticides.

Similarly, OPs have been extensively used in Western Australia (WA) in the past to protect agricultural products, buildings and households against insects and pests, and high concentrations of pesticides, such as DDT and its metabolites, HCH and dieldrin, have been detected in Western Australian (WA) women’s milk previously. The most recent study was conducted in 2002/03, however this result was based on a single pooled milk, making it a conservative estimate for the individual and not allowing for investigation of factors that might influence on HM POPs. Since then little information about these pesticides in women’s milk in WA has been reported.

The aim of this study was to describe the current pesticide concentrations in HM and changes during the first year of lactation in a longitudinal cohort. Associations between pesticide concentrations between infant growth outcomes were investigated along with maternal characteristics. The daily intake of the pesticides was calculated rather than estimated and tracked throughout the first year of lactation.

Results

Participants. The demographics and characteristics of the study participants are presented in Table 1. No significant differences were observed in maternal BMI (P > 0.96), maternal fat mass (P > 0.46) and infant fat mass measured by ultrasound skinfolds (P > 0.87) and biodeimpedance spectroscopy (BIS: P > 0.24) throughout the first year of lactation. Whereas significant differences (P < 0.01) were observed in infants’ weight, body length and head circumference throughout the first year of life.

HM fat content and pesticide concentrations. The mean fat content of all HM samples (postfeed: n = 39; prefeed: n = 60) was 38.9 ± 21.6 g/L (1.16 to 106.3 g/L). Fat content of postfeed milk (left: 55.1 ± 21.4 g/L; right: 54.9 ± 24.0 g/L) was significantly higher (P < 0.01) than prefeed milk (left: 28.8 ± 15.3 g/L; right: 27.5 ± 10.0 g/L) (Fig. 1).

Only 3 of the 88 pesticides (3.4%), p,p’-DDE, p,p’-DDT and p,p’-HCH, were detected (Table 2). Other pesticides, such as OPs, fungicides, carbamates and pyrethroids, were not detected. The most frequently detected and abundant pesticide in HM was p,p’-DDE, detected in 83 of the 99 samples (83%). The mean concentration of p,p’-DDE was 1.56 ± 1.22 ng/mL (range: 0.21–6.21 ng/mL) and when normalized to the HM fat content was 52.25 ± 49.88 ng/g fat (range: 5.67–278.48 ng/g fat). The level of p,p’-DDE was significantly higher in the postfeed
Appendix

Figure 1. Distribution of fat content in pre- and postfeed milk collected from each breast from 2 to 12 months (n = 99 samples from 16 mothers). The fat contents are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). *Indicates significant difference (P < 0.05).

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Frequency of detection (%)</th>
<th>Mean ± SD (ng/mL) (Range)</th>
<th>Mean (ng/g fat) (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p,p’-DDE</td>
<td>83</td>
<td>1.56 ± 1.22 (0.21–4.21)</td>
<td>52.25 ± 49.88 (5.37–274.40)</td>
</tr>
<tr>
<td>p,p’-DDT</td>
<td>9</td>
<td>0.70 ± 0.42 (0.06–1.55)</td>
<td>27.67 ± 20.96 (6.08–69.55)</td>
</tr>
<tr>
<td>β-HCH</td>
<td>4</td>
<td>2.17 ± 1.69 (0.59–4.55)</td>
<td>46.40 ± 22.46 (14.95–65.08)</td>
</tr>
</tbody>
</table>

Table 2. Summary of detected pesticides in HM samples (n = 99) collected from 16 Western Australian mothers.

Milk (left: 2.12 ± 1.36 ng/mL; right: 1.57 ± 0.95 ng/mL) compared to the prefeed milk (left: 1.44 ± 1.32 ng/mL; right: 1.08 ± 0.78 ng/mL) (P < 0.01; Fig 2A). After the normalization of p,p’-DDE to HM fat content there were no significant differences between the pre- and postfeed p,p’-DDE concentrations (left: P = 0.24; right: P = 0.37; Fig 2B).

P,p’-DDT was detected in the milk of 2 mothers with a mean concentration of 27.67 ± 20.96 ng/g fat (range: 6.08–69.55 ng/g fat) (Table 2). The insecticide, β-HCH, was detected in 1 mother, with a mean concentration of 46.40 ± 22.46 ng/g fat (range: 14.95–65.08 ng/g fat) (Table 2).

Changes in pesticide concentrations during the first year of lactation. The overall HM p,p’-DDE concentrations declined from 70.00 ± 70.58 ng/g fat at 2 months to 57.54 ± 47.32 ng/g fat at 5 months, 45.16 ± 31.71 ng/g fat at 9 months and to 22.35 ± 13.86 ng/g fat at 12 months. There was an overall 68% decrease of p,p’-DDE concentrations over the first 12 months of lactation (Fig. 3). A significant difference (P = 0.03) was observed in p,p’-DDE concentrations between 2 and 12 months only. Similarly, the concentrations of p,p’-DDT and β-HCH in this study also decreased by 45% and 73% respectively over the lactation period of 2–12 months.

Pesticide concentrations, maternal and infant characteristics. There were no significant relationships between HM p,p’-DDE concentrations and maternal age (P = 0.06), parity (P = 0.65), maternal body mass index (BMI) (P = 0.27) and percentage fat mass (P = 0.08). However, mothers of male infants had significantly higher (P = 0.03) concentrations of p,p’-DDE in their milk (55.18 ± 45.41 ng/g fat) compared to mothers of female infants (29.34 ± 18.90 ng/g fat) (Supplementary Fig. S1). Significant increases in infant weight (P < 0.01), length (P < 0.01) and head circumference (P < 0.01) were observed throughout the first year of life (Supplementary Fig. S2). No significant associations were observed between p,p’-DDE and the infant growth outcomes; weight (P = 0.40), length (P = 0.13), head circumference (P = 0.07) and percentage fat mass (ultrasound skinfolds: P = 0.34; BIS: P = 0.11).
Appendix

Figure 2. Distribution of p,p'-DDE in pre- and postfeed milk collected from each breast before (A) and after (B) normalized to fat content of HM. Values of p,p'-DDE are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). *Indicates significant difference (P < 0.05).

Figure 3. The average concentrations of p,p'-DDE (ng/g fat) in HM collected during lactation period from 2 to 12 months. Values of p,p'-DDE are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line) and outliers (o). Insert is the mean plot of p,p'-DDE levels from this cohort. Significant difference (P < 0.05) is observed between 2 and 12 months.

Infant exposure to HM DDT. The average calculated actual daily intake (ADI) of DDTs (sum of DDT and its metabolites, DDE and DDD) throughout the first year of lactation were 0.16 µg/kg body wt./day and 0.23 µg/kg body wt./day, respectively (Table 3). The ADI of DDTs by the infants decreased significantly during the first year of lactation from 0.33 µg/kg body wt./day at 2 months to 0.03 µg/kg body wt./day at 12 months (P < 0.01; Fig. 4), which is in conjunction with the infants’ rapid development and also the significant decrease of the maternal bioburden (P = 0.03; Fig. 3). No significant difference was observed in the EDI of DDTs by the infants over the 12-month period. The average EDI of DDT at 2 and 5 months (0.27–0.39 µg/kg body wt./day) is comparable to that observed in ADI and drastically overestimates at 9 and 12 months (0.10–0.23 µg/kg body wt./day; P < 0.01; Table 3).
Table 3. Average calculated daily intake (ADI) and estimated daily intake (EDI) of DDTs by infants during the first year of lactation in comparison to tolerable daily intake (TDI) proposed by FAO/WHO and Health Canada.

<table>
<thead>
<tr>
<th>Lactation months</th>
<th>ADI (µg/kg body wt/day) (Range)</th>
<th>EDI (µg/kg body wt/day) (Range)</th>
<th>P value*</th>
<th>TDI (µg/kg body wt/day) (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All data</td>
<td>0.14 (0.02-0.69)</td>
<td>0.23 (0.03-0.90)</td>
<td>P&lt;0.005</td>
<td>0.50 (0.05-0.90)</td>
</tr>
<tr>
<td>2 months</td>
<td>0.33 (0.07-0.89)</td>
<td>0.10 (0.08-0.60)</td>
<td>0.45</td>
<td>0.50 (0.20-0.10)</td>
</tr>
<tr>
<td>5 months</td>
<td>0.18 (0.06-0.52)</td>
<td>0.27 (0.09-0.72)</td>
<td>0.36</td>
<td>0.50 (0.20-0.10)</td>
</tr>
<tr>
<td>9 months</td>
<td>0.09 (0.02-0.22)</td>
<td>0.13 (0.03-0.45)</td>
<td>P&lt;0.005</td>
<td>0.50 (0.20-0.10)</td>
</tr>
<tr>
<td>12 months</td>
<td>0.03 (0.02-0.05)</td>
<td>0.10 (0.03-0.25)</td>
<td>P&lt;0.005</td>
<td>0.50 (0.20-0.10)</td>
</tr>
</tbody>
</table>

Discussion

This is the first longitudinal study to examine pesticide concentrations in HM and infant growth parameters over the first 12 months of lactation. Only p,p'-DDE, p,p'-DDT and β-HCH in HM were detected outside of the 88 pesticides tested with a sensitive and validated QuEChERS ("Quick, Easy, Cheap, Effective, Rugged and Safe") method. This microscale extraction method is based on the liquid-liquid partitioning of acetonitrile and dispersive solid-phase extraction (dSPE), such as MgSO4, sodium acetate, primary secondary amine (PSA), and the pesticides are partitioned into the acetonitrile layer. After normalization of the concentrations to the fat content of HM, the levels of pesticides decreased substantially throughout the first year of lactation and p,p'-DDE levels were not related to measures of infant growth such as infant weight, length, head circumference and adiposity.

The HM samples in this cohort were analyzed for pesticides with an optimized QuEChERS methodology. This method had excellent recoveries (70 - 120%) and detection limits (0.2 - 2.0 ng/mL) lower than that of previous studies, which ensured we did not underestimate the levels of pesticides in HM. The GC-MS/MS method was also optimized for HM which allows the simultaneous screening of 88 different pesticides within a single injection.

The fat content of HM is the most variable component in HM and is known to be higher in postfeed milk as we have demonstrated. HM p,p'-DDE concentrations were also significantly higher in postfeed milk than prefeed milk (Fig. 2A), suggesting an association between lipophilic pesticides (e.g. p,p'-DDE) and the fat content of HM. These results support the current hypothesis that POPs are encapsulated either in the core (triaclyglycerols) or adhere to the surface (phospholipids) of HM fat during milk synthesis. Thus, after normalization of p,p'-DDE to the HM fat content, the significant difference of p,p'-DDE concentration between pre- and postfeed milk disappeared (Fig. 2B). Therefore, either pre- or postfeed milk can be collected for the measurement of pesticides provided the concentrations are normalized to the fat content of HM.

We detected large individual variation in HM pesticide concentrations, for example p,p'-DDE concentrations ranged from 5.67 to 278.48 ng/mL, and the types of pesticides (e.g. p,p'-DDT, p,p'-DDE and β-HCH), which likely reflect the differences in exposure, lifestyle, dietary habits, travel and metabolic activity between mothers.

As p,p'-DDE is a metabolite of p,p'-DDE, the 2 mothers with detectable HM p,p'-DDT also had higher p,p'-DDE concentrations of 125.34 ng/g fat and 80.50 ng/g fat respectively as expected. The ratio between DDE and DDT is commonly used as an indicator of DDT exposure history, a ratio of high DDE/DDT ratio (4.4) suggests recent exposure. However, the ratio of DDE/DDT ratio of the two mothers with detectable DDT was 3.4 and 8.1 respectively. Both mothers had different exposure characteristics, where one mother frequently visits a country that still has documented high levels of DDT (DDT/DDT ratio of 3.4), while the other grows up on a farm and was involved in the harvesting process during the period from 2004 to 2010 (DDT/DDT ratio of 8.1). These are possible exposure sources of DDT are however based on maternal recollection and further investigation is required including sampling of lactating women living in agricultural regions.

Hexachlorocyclohexane (HCH), is widely used in Australia for insect control. Among its isomers (α-HCH, β-HCH, γ-HCH and δ-HCH), α-HCH is the most metabolic stable and persistent, and accounts for over 90% of the total HCH detected in HM. Similar to DDE/DDT exposure history, the β-HCH/α-HCH ratio was used as an indicator of HCH exposure history. However, the δ-HCH/α-HCH ratio were not detected in this study, suggesting that the mother in whom we detected δ-HCH had historical exposure to HCH (β-HCH/α-HCH ratio > 5).

Over the first 12 months of lactation, there was an overall trend for the detected POPs in HM to steadily decline for p,p'-DDE (68%), p,p'-DDT (45%) and β-HCH (73%) suggesting a reduction in maternal body burden. However, we only observed significant differences between the time points 2 and 12 months for HM p,p'-DDE concentrations. This may be attributable to the fact that 10 women showed a decline in HM POPs, while the other 8 showed fluctuations possibly due to change in diet or metabolic activity. Increasing the sample size may increase the ability to detect differences between the months. These results are consistent with previous studies that have shown reductions of pesticide concentration in HM over shorter periods of time such as a decrease in p,p'-DDE and p,p'-DDT in colostrum (day 4/5) to week 2 by 4% and 10% respectively. Similarly a substantial decrease of p,p'-DDE between colostrum (day 3) and week 3 of 22% has been documented. Extensive studies have been carried out on dioxins and furans, PCBs and polybrominated diphenyl ethers (PBDEs). There are limited studies that have investigated pesticide levels in HM which extend to a period of more than 6 months of lactation and have a sufficient number of participants. This is one of the more extensive studies which measured pesticides in HM from 16 mothers, longitudinally over 12 months, providing information relevant to the longer term development of the infant in accordance to the WHO recommendation of breastfeeding for up to 1 years and beyond.
Many factors, such as maternal age, parity and BMI, have been associated with HM pesticide concentrations. Previous studies have reported associations between HM p,p’-DDE and maternal age, suggesting increased bioaccumulation of pesticides with increasing age. Further, increased maternal parity has been associated with lower HM pesticide concentration, which is attributed to the increased excretion of body stores through multiple pregnancies and lactations, whereas increased BMI has been associated with lower HM pesticide concentration. In this study we found no association between HM p,p’-DDE and maternal age (P = 0.06) or parity (P = 0.65) although maternal age is borderline. The absence of a relationship between maternal age and HM POPs may be due to the small number of participants (n = 16) in this study. As body composition measurements, such as BIS, are better measures of fat mass we hypothesized that we might find a relationship between fat mass and HM pesticides. However, we were unable to find a relationship between HM p,p’-DDE and maternal BMI (P = 0.27) and maternal fat mass (P = 0.08). These results are similar to that observed by Utrisch et al. where pesticide levels in maternal serum were investigated with respect to BMI and fat mass. A greater sample size may determine whether or not maternal adiposity is associated with HM pesticides content.

Many studies have reported the adverse effect of pesticides by associating prenatal exposures with infant growth and development. However, none has evaluated the potential influence of these pesticides in HM on the infant growth throughout the first year of lactation. In this cohort, we saw significant increases in infant growth outcomes, such as weight, length and head circumference, over the first 12 months as expected. However, none of these measures were associated with HM levels of p,p’-DDE. Further utilizing 2 different measures of infant adiposity (ultrasound skinfolds and BIS), we found no relationship with HM p,p’-DDE. These findings may be influenced by the fact that the levels of pesticides detected in HM in this study were at trace levels and the small numbers of participants (n = 16). Whilst associative relationships do not indicate causation, it is reassuring that the low levels of pesticides detected in WA women’s milk do not appear to be of concern.

Whilst the levels of the detected pesticides in HM in this study were very low, HM is the sole food source for a breastfed infant in the first 6 months of life and contributes substantially to the infant diet in the following 6 months. Thus it is important to consider the dose of ingested pesticides. To get a more accurate measure of the dose, we measured both the fat content of the milk and the 24 hour intake of the infant at 2 to 5 months, where intake has been shown not to change during this time period, and 9 and 12 months and substitute the estimated values with actual values in the formula for estimated daily intake. This provides a more accurate calculation of the actual daily intake (ADI) by the infant as it is well documented that both the fat content of
milk and the daily volume of milk vary 3-fold between breastfeeding infants\(^a\). A more accurate quantification for the daily intake of pesticides in infant is essential as studies assessing the effects of pesticides in HM are currently inaccurate and have the potential of not detecting an effect or producing an effect that is not real. Further effects of pesticide must be carefully monitored before implementing interventions to reduce both maternal and infant exposure. As expected, we found a significant difference between ADI and EDI. The EDI, which is based on constant values, underestimated the pesticide intake by 10% at 2 months and overestimated intake by 50 to 233% in the later months (Table 3). The differences are largely due to the overestimation of milk intake at 9 and 12 months of lactation (9 months: 482 ± 76 mL; 12 months: 256 ± 81 mL) for the EDI where a constant of 700 mL is used. Interestingly, the ADI decreased significantly from 2 to 12 months of lactation (Fig 4), but EDI did not, clearly demonstrating that EDI is not an accurate measure of infant dose. The ADI for several infants exceeded the TDL\(^b\) (0.35 g/kg body weight/day) at 2 and 5 months. However, as the breastfeeding progresses, the ADI at 9 and 12 months are 2 to 25 times lower than the TDL, suggesting that maternal HM pesticides in this cohort poses minimal risk to individual infants in WA. Whilst concern about the effects of pesticide exposure to infants is warranted as one must take into consideration the relatively short period of exposure via breastfeeding relative to a lifetime. Further, HM serves as an important environmental indicator of population exposure.

In order to understand the current magnitude of POPs in HM in WA, the results obtained in this study were compared with those reported in Australia as well as those reported from other countries. The total DDTs in this study have decreased by 93% and 80% as compared to previous studies conducted in WA (1990)\(^a\) and in Australia (2000)\(^a\) respectively. Our findings are also consistent with the trace pesticides levels found in bovids (e.g. maternal blood and cord blood) and in tissues (e.g. placenta and abdominal) in WA\(^a\), which demonstrate the low presence of environmental pesticides and the continuing decline of human exposure to these pesticides over time in WA. As compared to other countries, the concentrations of DDTs observed in this study are similar to those reported for Norway\(^a\) and USA\(^a\), but are a few orders of magnitude lower than that observed in malaria-prone countries such as Vietnam\(^a\), Malaysia\(^a\) and India\(^a\) where DDTs was widely used to combat mosquito borne malaria and have only recently been banned in the 1990s and 2000s (Table 4). WHO, however, have recently allowed limited use of DDT for indoor control of malaria vectors in malaria endemic countries\(^a\).

Therefore, concentrations of DDTs in South Africa\(^a\) and Ethiopia\(^a\) are 122 and 330 times higher than that in WA. Concurrent with these DDTs findings, the HCHs concentrations measured in this study are 4 to 63 times lower than the concentrations recorded in countries such as India\(^a\), Malaysia\(^a\) and Iran\(^a\), where HCHs are still extensively used and are commensurate with levels observed in Vietnam\(^a\), Slovakia\(^a\) and USA\(^a\).

The results regarding the levels of pesticides in HM from women in WA, Australia are encouraging however development of analytical methods should continue to focus on reducing detection limits thereby making associative studies more meaningful.

Conclusions

In this study, out of the 88 targeted pesticides, we have detected trace amounts of \(\beta\)-HCH, \(\beta\)-HCH and \(\beta\)-HCH in HM collected from Western Australian women, and the levels of these pesticides decreased substantially throughout the first year of lactation. This is the first longitudinal study to investigate the relationships of detected pesticides on the infant growth outcomes such as weight, length, head circumference and body composition of which we found none. Further, EDI dramatically overestimates infant dose after 2 months of lactation while the more accurate ADI show reduced dose to infants. The ADI of pesticides for individual infants in Western Australia decreased significantly during the first year of lactation, and was 2 to 17 times lower than current recommended guidelines.

Materials and Methods

Chemicals and reagents. The pesticide standard solutions (100 \(\mu\)g/mL) at 95% or higher purity were obtained from Ultra Scientific (North Kingston, RI, USA). Pesticide standard solutions (100 \(\mu\)g/mL) were mixed and diluted with acetonitrile (ACN) to prepare a stock standard solution (1 \(\mu\)g/mL) of all the pesticides. LC-MS grade acetonitrile (ACN) and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethylene glycol (98%), glycerol (99%) and D-sorbitol (\(\geq 98\%\)) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (\(\geq 99.0\%\)) and magnesium sulfate (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecylsil (C18) and primary secondary amine (PSA) were obtained from Agilent (Little Falls, DE, USA). Isotopic labeled quality control (QC) standards, acenaphthene-D10, phenanthrene-D10, and chrysene-D12, were purchased from Resek (Belfonte, PA, USA), and the internal standard (IS), tripheylphosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ethics, sample collection and processing. This study was approved by the Human Research Ethics Committee of The University of Western Australia, and the methods were carried out in accordance with the approved guidelines. Western Australian breastfeeding mothers (n = 16) were recruited between 2013 and 2015. All participants provided informed consent and completed a questionnaire including relevant demographic data. Milk samples were collected (1–5 mL) at 2, 5, 9 and 12 months of lactation into glass containers before and after feeding from each breast. HM fat content was measured immediately using the Drematocrit method\(^a\), and the remaining milk was stored at \(-20^\circ\)C. HM samples (n = 98) were thawed at room temperature for 3 hours then homogenized with a mixer (ELMI Lab., Riga, Latvia) for 15 seconds. 1 mL of HM was put into a 15 mL centrifuge tube and 1 mL of ACN containing 1% acetic acid and 100 ng/mL QC standards (acenaphthene-D10, phenanthrene-D10, and chrysene-D12) was added, and the tube was vortexed for 1 min. A validated method based on acetone buffered QuEChERS was employed to extract the HM\(^a\). Extraction reagents (0.4 g MgSO4 and 0.1 g NaAc) were added to the mixture and shaken
### Table 4. Comparison of DDEs and HCHs (ng/g fat) in HM from various countries. na data not available.

<table>
<thead>
<tr>
<th>Country/region</th>
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<th>Mothers (N)</th>
<th>HM samples</th>
<th>Fat content (g/g)</th>
<th>DDEs</th>
<th>HCHs</th>
<th>References</th>
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<td>39</td>
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<td>Present study</td>
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*Values represented as median value. 1Sum of p,p'-DDE + α,α'-DDE + p,p'-DDD + p,p'-DPT + p,p'-DDE. 2Sum of p,p'-DDE + α,α'-DDE + p,p'-DDD + α,α'-DDE + α,α'-DPT. 3Sum of p,p'-DDE + α,α'-DDE + p,p'-DDD + α,α'-DDE + α,α'-DPT + α,α'-DPT. 4Sum of α-HCH + β-HCH + γ-HCH. 5Sum of β-HCH + γ-HCH. 6Sum of γ-HCH + δ-HCH. 7Sum of α-HCH + β-HCH + γ-HCH + δ-HCH.

Immediately, the tube was then placed in an ice bath to prevent thermal degradation of some pesticides during the salting out process. The extraction tube was centrifuged at 3993 g for 10 min. 0.6 mL of the supernatant was transferred into a clean 15 mL centrifuge tube and stored in a freezer (−20°C) for 2 hours. The supernatant was centrifuged at 9932 g (0°C) for 10 min and 0.5 mL was transferred to a cleanup tube (157 mg MgSO4, 9 mg C18 and 9 mg PSA). The tube was vortexed for 1 min and centrifuged at 9932 g for 10 min. The final extract was transferred into a screw cap amber vial and kept at −80°C until analysis.

### Maternal and infant anthropometric measurements.

All maternal and infant anthropometrics measurements were made at 2, 5, 9 and 12 months at the time of milk sampling. Maternal body weight was measured by an electronic scale (Seca, California USA, accuracy 0.1 kg). The height, age and parity were self-reported by participants. Maternal BMI was calculated as BMI = kg/m².

Infant weight was determined by weighing before breastfeeding using electronic scales (±0.2 g; Medela Electronic Baby Weigh Scales, Medela AG, Switzerland). Infant crown-heel length was measured, to the nearest 0.1 cm, on a hard surface with between a head and footpiece with non-stretch tape. Infant head circumference was measured with non-stretch tape.

### Maternal and infant body composition measurements.

Whole body biopimedance (wrist to ankle) was measured with Impedimed SBF7 biopidealpdesrpam analyzer (Impedimed, Brisbane, Queensland, Australia) according to the manufacturer’s instructions however the infant’s whole biopimedance were analyzed with settings customized for each infant according to Longwood et al. Resistance at 50 kHz was used in infant percentage fat mass equations. Infants’ ultrasound skinfold measurements (triceps and subscapular) were made using Apilo 8G (Toshiba, Japan) machine, PLT-1200RX 14–90 MHz transducer and Parker ultrasonic gal (Fairfield, NJ, USA). The double skinfold thickness, measured directly from images using the on screen electronic calipers, was used in percentage fat mass equations developed for skinfolds measured with skinfold calipers.

### 24-hour infant milk intake.

24-hour milk intake was determined by the testing weighing procedure as previously documented. Briefly mothers weighed their infants before and after each feed from each breast for a period of 24 hours. The difference in weight in grams is considered equivalent to mL (density of milk: 1.03 g/mL). If the data was unavailable, the data from previous 24-hour milk intake study were used.

### GC-MS/MS analysis and Quality control.

Chromatographic separation and determination of the pesticides were carried on a Bruker Daltonics 450 gas chromatography (GC) with a Bruker Daltonics Scion TQ triple quadrupole mass spectrometer (MS), a Bruker 1117 Split/Splittless injector (Billericia, MA, USA) and a PAL Combi autosampler (CTC Analytics AG, Switzerland). Sky 4.0 1D precision inlet liner with wool from Restek (Bellefonte, PA, USA) was used. For the GC separation, a Rtx-5MS with Integra-Guard column (10 m + 30 m × 0.25 mm × 0.25 μm) (Bellefonte, PA, USA) was used. The inlet temperature was kept constant at 250°C. The initial oven temperature of the column was 80°C for 3 min and ramped at 30°C/min to 150°C and then ramped at 10°C/min to 300°C, where it was held for 10 min. The total run time was 31 min. Helenium (6.9 GC grade) was used as carrier gas at a constant flow of 1.1 mL/min. The injection was performed at pulsed splitless mode (head pressure: 44 psi) with an injection volume of 2μL. The mass spectrometer was operated in electron
Appendix

Impact (EI) mode. Transfer line and ion source temperature were 276 °C and 200 °C, respectively. Argon was used as the collision gas. Identification and quantification of 88 pesticides were carried out by tandem MS using scheduled multiple reaction monitoring (MRM) mode. The optimized MRM transitions and collision energies for each compound are listed in Supplementary Table 1. Data collection and processing were performed using Bruker MSWSS 8 Software.

Working standard solutions (0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL) were prepared by appropriate dilution of the stock standard solution (1 ng/mL) with ACN. A combination of APs mixture (ethylglycerol, gulonolactone and D-sorbitol) containing internal standard TPP (15) was added to the final extract of each HM sample and all the working standard solutions for GC-MS/MS analysis. The final concentrations of ethylglycerol, gulonolactone and D-sorbitol were 20, 2 and 2 ng/mL, respectively and the IS was 100 ng/mL. Isotopically labeled quality control (QC) standards were employed to evaluate the efficiency of the extraction and cleanup steps, while TPP (IS) was used to evaluate the performance of the instrument throughout the entire analytical procedure. In the absence of a true "blank" HM matrix, the use of APs mixture was investigated to counteract the matrix effect in HM. Similar peak responses were observed between the pesticides spiked (10–100 µg/L) in APs mixture and in HM samples with APs mixture. A QC mixture containing pesticide standards was analyzed by sample batches to check for any interferences and cross-contaminations. The recoveries of majority (88.6%) of the 88 pesticides were within the range of 70–120%. Limit of detection (LOD) and limit of quantitation (LOQ) were assessed experimentally with the lowest standards spiked in APs mixture providing signal-to-noise ratio (S/N ratios) of less than 3 and 10, respectively. The LOD and LOQ of the 88 pesticides spiked in APs mixture were 0.2–2.0 ng/mL and 0.5–5.0 ng/mL, respectively.

Infant daily intake. The ADI (µg/kg body wt./day) of the pesticides for each infant was calculated based on the pesticides concentration in HM (µg/g fat, C_pasteit) fat content in HM (g/mL, C_fat), average daily consumption of HM (mL, V_hum) and body weight of infant (kg, M_infant) using the following equation:

\[
\text{ADI} = \frac{C_{\text{pasteit}} \times C_{\text{fat}} \times V_{\text{hum}}}{M_{\text{infant}}}
\]

The estimated daily intake (EDI) is based on the same formula as ADI, but was calculated using constant values for C_fat, 0.03 g/mL, M_infant (5 kg) and V_hum (700 mL) based on previous studies44–46.

Statistical analyses. Statistical analyses were carried out using SPSS software (SPSS, version 19.0 for Windows, SPSS, Inc, IL, USA) and R 3.2.0 using the package nlme for linear mixed models which account for intra- and inter-individual variation47. Results were expressed as mean ± SD unless stated otherwise. Pesticides that were below the LOD were considered as absent and were not included in the calculations. Detected pesticides were reported based on the HM fat (mg/g fat). Linear mixed models were used to investigate associations between HM pesticide concentrations and both maternal and infant anthropometrics. One-way ANOVA and Tukey’s all pair comparison tests48 were used to compare differences in pesticides concentration, EDI and ADI at the different lactation months. Paired samples t-test was used to compare the daily intake of the detected pesticides using the EDI and ADI. P < 0.05 was considered significant.

References
Appendix


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Author Contributions

I.D. collected samples, conducted experiments and data analyses, interpreted data and results, wrote and reviewed the manuscript; Z.G. collected samples and reviewed the manuscript; M.C.L.G. conducted experiments, interpreted data, wrote and critically reviewed the manuscript; C.T.L. conducted statistical analyses and reviewed the manuscript; R.D.T. designed experiments and reviewed the manuscript; P.E.H. reviewed the manuscript; D.T.G. designed the study, wrote and critically reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/strep

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