EXPLORING THE USE OF MSPE AS EXTRACTION METHOD FOR ANALYSIS OF URINARY DAP METABOLITES USING LC-MS

NURUL IFFAH BINTI AMIR SHAH RUDDIN

ADVANCED MEDICAL AND DENTAL INSTITUTE
(AMDI)
UNIVERSITI SAINS MALAYSIA

2023

EXPLORING THE USE OF MSPE AS EXTRACTION METHOD FOR ANALYSIS OF URINARY DAP METABOLITES USING LC-MS

By

NURUL IFFAH BINTI AMIR SHAH RUDDIN

Dissertation submitted in fulfilment of the requirements

for the degree of

Master of Science (Health Toxicology)

September 2023

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful.

All praises to Allah and His Blessing for the completion of this thesis. I thank God for all the times, trials and strength that have been given on me to finish this short project and this dissertation. I encountered so much in this journey. My humblest gratitude to the Holy Prophet Muhammad (peace be upon him) whose way of life has been a continuous guidance for me.

First and foremost, I would like to thank my supervisor, Dr. Maisarah Nasution bt. Waras, for her guidance, understanding, insightful comments, observations, and participation throughout the learning process. It has been a great pleasure and honour to have her as my supervisor. Furthermore, I would like to thank my co-supervisor, Dr. Vivien How and her team for their unwavering supports and contribution in this project.

In addition, I would want to express my gratitude to my loved ones especially my father, Encik Amir Shah Ruddin B. Md Sah, my mother, Puan Noor Zakiah bt. Ishak, and my sisters, Nurul Izzati and Nurul Anis, for their unwavering support throughout the process. I shall be eternally thankful for your kindness and understanding.

Also, I like to thank Muhammad Ariffuddin, who have willingly contributed his time and energy to share his knowledge and skills with me. I also want to extend my thanks to all my beloved friends, Goh Ngee Chuang, Thanussha and Hakimah who were with me and support me in the face of adversity. Last but not least, I am thankful to those who indirectly contributed in complete my works, your kindness means a lot to me.

May God bless the above cited personalities with success and honour in their life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

µ	micro
g	gram
L	Litre
mL	Millilitre
°C	Degree Celsius
&	And
%	Percent
=	Equals to
×	Multiply by
≤	Less than or equals to
≥	More than or equals to
n	Nano
<	Less than
>	More than
mm	Millimetres
V	Volt
M	Molar
mol	Moles
/	Per

LIST OF ABBREVIATION

DAP	Dialkyl phosphate
DBP	Dibutyl phosphate
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
ESI	Electrospray ionisation
et al.	And others (et alia)
GC-MS	Gas chromatography - mass spectrometry
LC-MS	Liquid chromatography - mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MNPs	Magnetic nanoparticles
MSPE	Magnetic solid-phase extraction
OP	Organophosphate
PEEK	Polyether ether ketone
ppb	Part(s) per billion
ppm	Part(s) per million
RSD	Relative standard deviation
SD	Standard deviation
SPE	Solid-phase extraction

ABSTRAK

Racun perosak organofosfat (OP) telah digunakan secara meluas dalam sektor pertanian. Kajian lepas telah menunjukkan bahawa metabolit dialkyl fosfat (DAP) dalam urin boleh dikurangkan melalui intervensi diet organik. Kajian kami memberi tumpuan kepada pembangunan kaedah untuk menganalisis metabolit DAP dalam menyiasat kesan intervensi diet organik terhadap pendedahan racun perosak OP dalam populasi luar bandar dan bandar. Objektif kami adalah untuk mengesahkan kaedah sedia ada menggunakan LC-MS dengan pengekstrakan SPE dalam menganalisis metabolit DAP dalam urin dan meneroka MSPE sebagai kaedah pengekstrakan untuk menganalisis metabolit DAP dalam urin. Kajian ini dilakukan menggunakan LC-MS dengan turus Agilent Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 µm. Oleh kerana batasan masa dan sampel, kami memilih untuk menganalisis dietil thiofosfat (DETP) dalam sampel urin kerana hasilnya yang lebih baik berbanding metabolit lain. Lengkung penentukuran telah dibina dengan spiking sampel urin pada kepekatan yang berbeza pada permulaan penyediaan sampel untuk kedua-dua kaedah SPE dan MSPE. Dari kajian kami, kaedah MSPE menghasilkan keputusan yang lebih baik berbanding kaedah SPE. Lineariti kaedah MSPE ialah $R^2 = 0.9818$ dan kaedah SPE ialah $R^2 = 0.9789$. LOD dan LOQ DETP melalui kaedah MSPE masing-masing adalah 0.51 dan 1.54 ppm dan SPE merekodkan LOD sebanyak 0.86 ppm dan LOQ sebanyak 2.62 ppm. Pengoptimuman masa pengekstrakan dalam kaedah MSPE menunjukkan bahawa 5 minit adalah yang terbaik. Kajian ini separa sah kerana kekangan masa dan lebih banyak penyelidikan diperlukan untuk mengoptimumkan dan mengesahkannya. Penggunaan MSPE, yang lebih mudah dan cepat dalam langkah penyediaan sampel boleh menjadi batu loncatan untuk penyelidikan masa depan untuk menganalisis metabolit DAP dalam air kencing.

ABSTRACT

Organophosphate (OP) pesticides have been widely used in agricultural sector. Past studies had shown that urinary dialkyl phosphate (DAP) metabolites can be reduced through organic diet intervention. Our study was focusing on developing an analytical method to analyse DAP metabolites in investigating the effect of organic diet intervention on OP pesticide exposure in rural and urban population. Our objectives were to validate the current existing method using liquid chromatography and mass-spectrometry (LC-MS) with solid phase extraction (SPE) extraction in analysing DAP metabolites in urine and to explore magnetic solid phase extraction (MSPE) as an extraction method for DAP metabolites analyses in urine. Analyses of DAP metabolites were performed using LC-MS with an Agilent Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 µm column. Due to time and sample limitation, we chose to analyse only diethyl thiophosphate (DETP) in urine sample because of its better yield compared to others. Calibration curve was constructed by spiking the urine sample at different concentration in the beginning of sample preparation for both SPE and MSPE methods. From our study, it was found out that MSPE method produced a better result compared to SPE method. The linearity of MSPE method was $R^2 = 0.9818$ while SPE method produced $R^2 = 0.9789$. The limit of detection (LOD) and limit of quantification (LOQ) of DETP through MSPE method were 0.51 and 1.54 part per million (ppm) respectively while SPE method recorded LOD of 0.86 ppm and LOQ of 2.62 ppm. Optimization of extraction time in MSPE method showed that 5 minutes was the best extraction time. This experiment was partially validated due to time constraint and more research is needed to optimize and validate it. The use of MSPE, which is simpler and faster in sample preparation step can be a steppingstone for future research in analysing DAP metabolites in urine.

CHAPTER 1

INTRODUCTION

1.1 Background

Organophosphates (OP) are chemical compounds synthesized by the process of esterification between phosphoric acid and alcohol. Organophosphates can go through hydrolysis with the liberation of alcohol from the ester bond. These chemicals are the primary elements of herbicides, chemicals, and insecticides in agriculture (Adeyinka et al., 2022). OPs and other pesticides are applied to protect the crops from undesirable organism, mainly to maintain quality and quantity of food production. However, application of pesticides come with unintended effects – the non-targeted organisms can be exposed and at risks to the adverse effects of these substances. OP pesticides are neurotoxicants and the signs and symptoms of acute, sublethal level of poisoning are presented as cholinergic syndrome (WHO, 1986; Eddleston, 2008; Adeyinka et al., 2022).

Table 1.1: Cholinergic syndrome of acute exposure to OPs (WHO, 1986; Eddleston, 2008; Jokanović, 2018; Adeyinka et al., 2022)

Muscarinic symptoms	Nicotinic symptoms
Salivation	Muscular weakness
Lachrymation	Tachycardia
Urination	Respiratory muscle weakness
Diarrhoea	Muscle twitching
Gastrointestinal cramps and pain	Muscle fasciculation
Emesis	Hypertension
Miosis	
Bradycardia	
Bronchospasm and bronchorrhea	

The health effects for chronic, low level of exposure of OPs exposure are still under investigation among the general population. However, prenatal exposure to OP has been associated with lower intelligence quotient (Bouchard et al., 2011), increases in attention problems and attention deficit hyperactivity disorder (ADHD) (Marks et al., 2010) and increase in abnormal reflexes (Engel et al., 2007). In occupational settings, exposure to OPs in occupational settings are associated with agriculture sector where chronic exposure might happen to farmers and insecticides applicators when they are exposed to it intentionally or unintentionally (CDC, 2017).

In biomonitoring studies, OPs exposure also has been detected in the general population of United States (Bradman et al., 2015; CDC, 2017; Hyland et al., 2019), Canada (Health Canada, 2021), Australia (Safe Work Australia, 2021), Spain (Cequire et al., 2015) and Denmark (Mørck et al., 2016). OP exposure in biomonitoring studies is detected from analysis of general metabolites and specific metabolites. Unfortunately, there is only a few studies conducted representing a few populations in Malaysia. A study by Ismail et al. (2011) was monitoring pesticide exposure to pesticide applicators in paddy fields had found that 2,3,6 trichloro 2-pyridinol (TCP), a chlorpyrifos metabolite was identified in the urine samples. Orang Asli studies have recorded that there was association between conventional fruits and vegetables intake and having fathers working in the farm (Sutris et al., 2017). The scarcity of pesticide residue data in Malaysia and human biomonitoring studies does not allow us to infer the extent of Malaysian exposure to this pesticide and not to mention the contribution of dietary pathway in Malaysian exposure to OP pesticides. Accordingly, there is not much to conclude with regards to the exposure of OPs pesticide in Malaysia.

The source of OPs exposure can be from the inhalation of polluted air, absorption from touching the contaminated surface and accidental ingestion. The main pathway of exposure in general population are through diet as proposed by several studies (Bradman et al., 2015; Lu et al., 2008; Tsuchiyama et al., 2022). Indeed, OPs are detected in food in many pesticides residue survey in many parts of the world (Jara & Winter, 2019; Ding et al., 2018; Wang et al., 2012). In Malaysia, vegetables grown in Cameron Highland have recorded a high amount of pesticide including OP pesticides residue especially in wet season (Munawar et al., 2021).

Organic diet is proposed to be one of the ways to reduce exposure in children, Organic food is produced by farmers who place a strong emphasis on the utilisation of renewable resources as well as soil and water conservation to improve environmental quality for future generations. Neither antibiotics nor growth hormones are administered to the animals used to produce organic meat, poultry, eggs, and dairy products. The majority of common pesticides, synthetic fertilisers, bioengineering, and ionising radiation are not used in the production of organic food (Somasundram et al., 2016). Organic diet intervention studies have been proved to reduce the dialkyl phosphate (DAP) metabolites concentration in urine of young children living in urban and agricultural communities (Bradman et al., 2015).

The available methods to assess OP pesticides exposure are through urinary metabolites. The most common is using solid phase extraction (SPE) and liquid-liquid extraction (LLE). However, these two methods are time consuming and require experties. This study intended to fill the following gaps:

a. Finding a sensitive and reliable method to analyse urinary DAP metabolites that can be used easily.

b. The exposure of OP pesticide in the general population in Malaysia have not been investigated as a result of conventional diet.

c. There is no data concluded on the effects of organic diet from food sold in Malaysia to reduce OPs exposure.

1.2 Problem statement

Organophosphate pesticides exposure has shown to affect the health of workers population in occupational setting and young children due to prenatal exposure. Dietary pathway is found to be a significant source of pesticide exposure particularly in general population. In Malaysia, the level of OP pesticides exposure as a result of consuming conventional diet is not known. In addition, the impact of organic diet intervention in reducing OP exposure is also not known. To date, the available methods that are usually used to analyse urinary metabolites are tedious and time-consuming. To address this research gap, we are looking to develop a new method of using magnetic solid phase extraction (MSPE) to analyse the DAP in urine sample.

Research question

Does MSPE can be used to analyse DAP metabolites in complex sample such as urine?

1.3 Objectives

General objective

To develop an analytical method to analyse DAP metabolites in investigating the effect of organic diet intervention on OP pesticide exposure in rural and urban population in Cheras/Kajang and Sepang, Malaysia.

Specific objectives

1. To explore the SPE and MSPE sample preparation methods for the analysis of DAP in urine sample.
2. To compare the analytical performance of the developed methods.

1.4 Significance of the study

This study will be a start-up for the use of magnetic solid phase extraction (MSPE) in analysing DAP metabolites in human urine. Developing a new and straightforward method can help in shorten the analysis time while maintaining its accuracy and precision. Measuring the DAP can give an overview on the exposure of the OP pesticides in the general population roughly and on the selected families specifically. Organic diet intervention study is carried out to test whether changing in dietary intake can reduce the exposure to pesticides especially OP. From this study, policy makers can make a proper intervention plan to reduce the exposure of OP pesticides to the people for a better health and life.

CHAPTER 2

LITERATURE REVIEW

2.1 Organophosphates

Organophosphates are likewise the primary elements of nerve gas. From World War I until the Gulf War, chemical weapons like nerve gas have been employed on several times during conflicts. The most recent deployment of nerve gas occurred in 2013 during the ongoing conflict in Syria. Sarin nerve gas was first employed as a terrorist weapon in Japan in 1994 and 1995. The first significant nerve gas catastrophe was a sarin attack in a Tokyo subway with 12 deaths and emergency medical evaluation of more than 5000 people (Tokuda et al., 2006; Harald et al., 2018; Adeyinka et al., 2022). The assassination of North Korea's half-brother in Malaysia also disclosed the use of VX (S-2 Diisopropylaminoethyl methylphosphonothioate) after further investigation (Latif & Chow, 2017).

According to Adeyinka et al. (2022), organophosphate exposure affects roughly 3 million individuals worldwide, with about 300,000 deaths. There are only a few deaths among the 8,000 Americans who are exposed to organophosphates. In some other reports, more than 200,000 people die each year from OP pesticide poisoning in rural areas of developing countries in the twenty-first century like Sri Lanka, Nepal, Morocco, and India (Eddleston et al., 2007; Shrestha & Shakya, 2013; Ezzouine et al., 2016; Fating et al. 2021)

2.1.1 Organophosphate as pesticides

Numerous formulations of hundreds of OP compounds have been produced and sold as insecticides all over the world since the late 1930s. When most commonly used organochlorine pesticides were phased out or outlawed in the 1970s, OP pesticides use peaked. Up until the year 2000, OPs made up 70% of all insecticides used in the U.S. However, that percentage has since been cut in half (Atwood & Paisley-Jones, 2017).

Food and Agriculture Organization of the United Nations, FAO (2021), mentioned that the use of insecticides increased worldwide by 36% between 2000 and 2019, reaching 4.2 million tonnes in 2019 (as in Figure 2.1). China, which consumed 1.8 million tonnes of pesticides in 2019—or 42 percent of the global total—surpassed the United States of America and Brazil by a wide margin (0.4 million tonnes each). In 2019, America saw the highest rates of pesticide application, followed by Asia, Oceania, Europe, and Africa. Between 2010 and 2019, the only region where pesticide consumption per acre of agricultural did not rise was Asia.

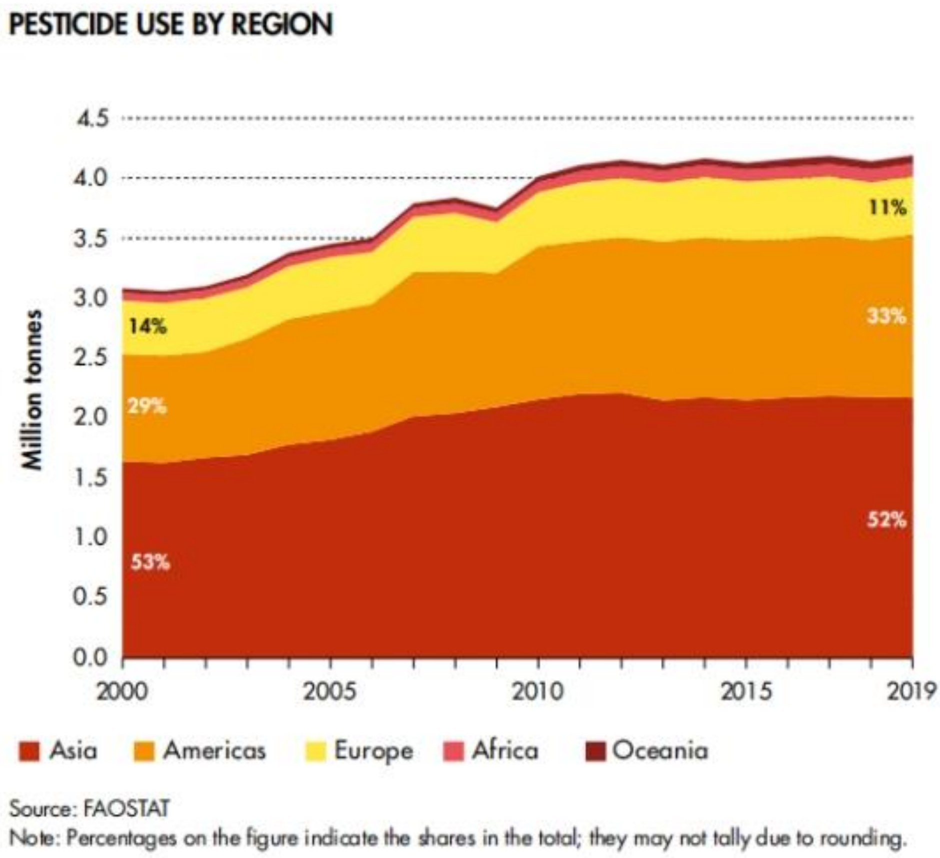

Figure 2.1: Pesticide use by region (Source: FAO, 2021)

Apart from that, local agricultural practice also contributes to pesticide exposure especially for those who lives near the agriculture area. FAO (2021) had appraised about 36076.8 tonnes of pesticides were used in Malaysia in 2020, which show a decreasing trend compared to previous year as shown in Figure 2.2.

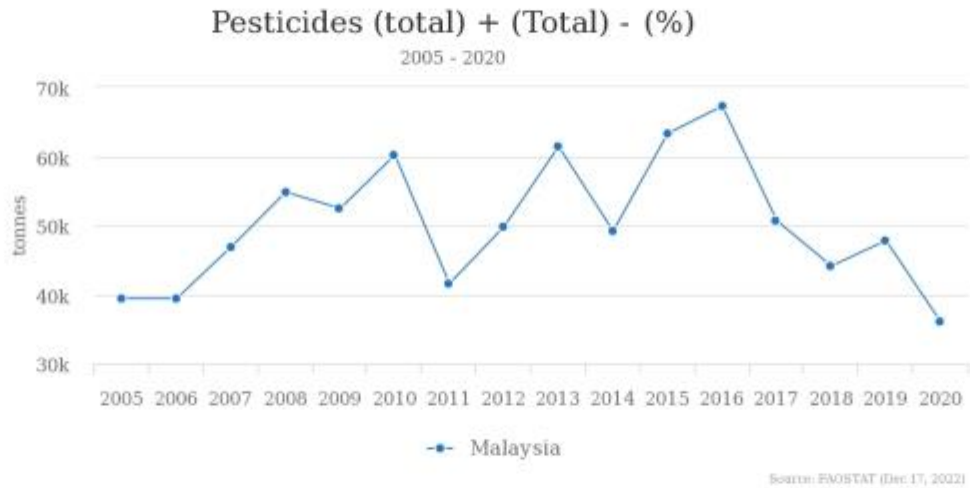

Figure 2.2: Total pesticides used in Malaysia from 2005-2020 (Source: FAO, 2021)

Organophosphates pesticides come in various types and brands that depends on its active ingredients. There are about 17 organophosphate pesticides that are available worldwide such as parathion, diazinon, malathion, fenitrothion, terbufos and others. However, the OP pesticides that are common in Malaysia are chlorpyrifos, malathion, diazinon, acephate, and phosmet (DOA, 2022). Malaysia is planning to ban the use of chlorpyrifos in agricultural sector starting from 1st May 2023 due to its severity impacts on human health compared to other types (Bernama, 2022).

2.1.2 Toxicokinetics of organophosphates

Exposure to OP can occur in many ways including inhalation, dermal and ingestion. When a molecule is absorbed, it attaches to an acetylcholinesterase (AChE) molecule in red blood cells, rendering the enzyme inactive. The inhibition of AChE leads to increasing acetylcholine in the synaptic junction that can cause overstimulation and disturbance in the neurotransmission in central and peripheral nervous system (Rubio et al., 2012).

Once in the human body, most OP pesticides are rapidly metabolised by hydrolytic cleavage and oxidative desulphurization which have biological half-lives of only a few hours (Barr et al., 2010). They break down into six dialkyl phosphate (DAP) metabolites: dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP), which are excreted in the urine within a few days (Garfitt et al., 2002; Barr and Angerer, 2006). These metabolites can be used for biomonitoring of insecticide exposure. The intake of foods that are contaminated with OP pesticides could be the prime source of DAP in urine in non-occupational urban and rural settings (Lu et al., 2008: Sokoloff et al., 2016; Oya et al., 2020; Nishihama et al., 2021).

The pathophysiological mechanisms behind the emergence of the three primary clinical symptoms associated with OP poisoning include cholinergic with anticholinesterase effects, membrane toxicity, and pro-oxidant (Sobolev et al., 2022). These interactions result in immunopathological illnesses, microcirculatory abnormalities, hypoxia, cytotoxic effects, and cell death. It is not well understood which molecular signalling mechanisms cause kidney cells exposed to OPs to live or die in determining its nephrotoxic effects of OPs (Sobolev et al., 2022).

2.1.3 Toxicology of organophosphates

Organophosphates are typically neurotoxicants and can affect the nervous system of the target organisms by poisoning them (Casida, 2009). Their species-selectivity is often minimal, therefore mammals—including people—are quite sensitive to their toxicity. Acute cholinergic toxicity and, occasionally, delayed neurotoxicity are the 2 primary kinds of neurotoxicity connected to acute OP exposure (Aldridge, 1981). In individuals with severe OP poisoning, an additional kind of toxicity known as the intermediate syndrome has been documented (Costa, 2013). When acute cholinergic symptoms have passed, it primarily manifests as muscle weakness; the specific mechanism is unknown (Costa, 2013). Acute or chronic exposure to organophosphates can create differing poisoning degrees in people, pets, plants, and bugs.

Some OP pesticides are also carcinogenic. The insecticides malathion and diazinon have been upgraded from Group 2B (a suspected carcinogen) to Group 2A (a probable carcinogen) by the International Agency for Research on Cancer (IARC), an organisation under WHO. Tetrachlorvinphos and parathion were designated as possible human carcinogens (Group 2B) (IARC, 2015).

Exposure to organophosphate pesticides or nerve gas can impacted organ systems. The clinical manifestation of these problems is caused by overstimulation of the nicotinic and muscarinic receptors (Peter et al., 2014). The organ system that can be affected are respiratory system, cardiovascular system, central nervous system, gastrointestinal and metabolic system, and renal system. This study is going to focus on the effect of OP pesticides to the renal system.

2.1.4 Long-term effects of organophosphate pesticides

The effects of OPs depend on the exposure levels, and these effects include excessive secretion of body fluids, cardiorespiratory depression and seizures that can be life threatening. Besides, acute exposure to OPs can also cause the survivors to suffer an array of long-term neurological and psychological effects. Among these effects are motor impairments, depressed mode, psychotic episodes, and reduction in signal detection and information processing, sustained attention, memory, sequencing and problem solving, cognitive flexibility and abstraction (Savage et al., 1988; Rosenstock et al., 1991; Steenland et al., 1994; Dassanayake et al., 2007; Pereira et al., 2014).

Chronic exposure to OPs can be linked with neurologic and psychiatry abnormalities such as motor dysfunction (extrapyramidal symptoms), depleting eye-hand coordination and reaction time, depression, anxiety, psychotic symptoms, reduce attention, information processing, learning and memory (Amr et al., 1997; Salvi et al., 2003; Stephens et al., 1995). These symptoms are recognized as chronic OP-induced neuropsychiatric disorders (COPIND) (Singh and Sharma, 2000).

Repeated exposure to OPs below the threshold for cholinergic toxicity also can cause various long-term effects. This is common in agriculture workers and pesticides sprayer. Persistent transformation in psychomotor speed, executive function, visuospatial ability, working and visual memory are among the associated effects (Ross et al., 2013).

Studies had been conducted to view the association between prenatal or postnatal exposure to OPs with behavioural abnormalities and intellectual disabilities (Jusko et al., 2019). Impaired cognition (Furlong et al., 2017; Tanner et al., 2020), attention-deficit disorder and behavioural problems (Chang et al., 2021; Hertz-Picciotto et al., 2018), autism spectrum disorder (Sagiv et al., 2018) and dyslexia (Xie et al., 2022) are among the problems connected to OPs exposure in children. Exposing the children to OPs in their early life stage could cause the cortical thickness and neuronal glial cell proportion in brain regions related to cognition to transform (Rauh et al., 2012).

2.2 Sources of OP exposure in Malaysia

Organophosphate pesticides can contaminate food, which makes them a significant source of human exposure. In a study by Ding et al. (2018) at a city in Eastern China, 10 OP pesticides residue were found in chicken, pork, fish, vegetables, tofu, eggs, milk, and cereals. The primary route of exposure for non-occupationally exposed people is through ingestion of food especially contaminated fruits and vegetables (Oates et al., 2014). People living near agriculture area can also be exposed to the pesticides that are present in drinking water, air and soil (ATSDR, 2008).

According to Department of Statistic Malaysia (2022), seven out of ten selected vegetables had self-sufficiency ratio (SSR) of greater than 100% in 2021. According to DOSM (2022), SSR indicates the degree that the nation's agricultural commodity supply (output) can satisfy local demand. The SSR of 100% or more implies that agricultural commodity output or supply is adequate to satisfy nation-wide demands, and vice versa. (DOSM, 2022). Tomato had the greatest SSR at 118.9%. Chilli continued to have the lowest SSR (29.3%). With a per capita consumption (PCC) of 6.6 kg/year, round cabbage continued to be the most popular vegetable, while brinjal had the lowest PCC of the vegetables chosen (1.2 kg/year). Imports of selected vegetables rose overall in 2021 compared to 2020. Round cabbage had the greatest overall imports among selected vegetables with 139.0 thousand tonnes, or 60.3% of the nation's supply. Chilli saw the largest overall rise, rising from 66.3 thousand tonnes to 73.7 thousand tonnes as in Figure 2.3.

Exhibit 2: Production, Export, Import and Per Capita Consumption (PCC) for Selected Vegetables, Malaysia, 2020 and 2021

Item	Year	Production (tonnes)	Export (tonnes)	Import (tonnes)	PCC (kg/year)
Tomato	2021	186,662.6	36,457.5	6,845.2	4.2
	2020	192,129.5	38,391.0	1,590.0	4.2
Mustard	2021	153,270.8	6,345.2	11,051.6	4.7
	2020	143,286.3	6,613.1	10,209.3	4.4
Cucumber	2021	96,353.1	22,644.7	12,523.2	2.6
	2020	101,482.0	19,709.7	8,752.8	2.7
Round cabbage	2021	91,389.1	3,547.0	139,035.7	6.6
	2020	80,641.2	2,293.6	136,747.9	6.2
Spinach	2021	80,404.4	9,904.7	1,274.0	2.1
	2020	74,250.8	8,826.8	891.3	2.0
Lettuce	2021	75,546.0	12,919.9	7,644.4	2.2
	2020	54,583.7	14,129.4	7,425.9	1.5
Lady's finger	2021	63,062.4	3,255.5	630.7	1.7
	2020	57,860.6	1,647.7	411.0	1.6
Long bean	2021	60,158.1	6,239.1	1,129.5	1.6
	2020	52,638.3	4,417.1	900.1	1.5
Brinjal	2021	42,731.6	7,690.5	3,945.8	1.2
	2020	39,785.7	6,875.2	2,524.9	1.1
Chilli*	2021	28,740.4	4,243.5	73,725.9	2.2
	2020	28,264.4	3,052.5	66,294.9	2.0

Note: *Excluding bird's eye chilli

Figure 2.3: Production, export, import and PCC for selected vegetables in Malaysia, 2020 and 2021. (Source: DOSM, 2022)

Malaysia imports most of its vegetables due to high local market's demand and the production is not enough to cover it. There are also problems with land scarcity and increasing price of labour and pesticides that resulted in high production costs. Besides, some vegetables are much cheaper when imported from China such as cauliflowers and broccoli (Ruban, 2016). Based on data from World Integrated Trade Solution (2022), Indonesia, China, Argentina, India, and the United States are Malaysia's major import partners in 2020 for vegetables. This practice raises a concern since we do not know which type of pesticides and how much pesticides were used on the vegetables in the farmland back in their home country.

2.3 Monitoring of OP pesticides exposure

OP pesticides like chlorpyrifos have been widely utilised, resulting in the contamination of many ecosystems, including soil, sediments, water, and air, as well as the disruption of biogeochemical cycles. Additionally, traces of chlorpyrifos have been found in sediments, soil, water, vegetables, food, and even human fluids. It has been proven that exposure to OP pesticides has caused health issues since it inhibits the cholinesterase enzyme, which has negative effects on the human nervous system, the immune system, and the brain, as well as the ecosystem. There are now several methods for detoxifying these pesticides, but microbial breakdown of chlorpyrifos, particularly by bacteria, has emerged as one of the most effective, affordable, and environmentally responsible methods (Dar et al., 2019).

Monitoring of ecological systems and food supply for OP pesticides concentration is crucial. Development of simple, quick, and extremely sensitive procedures to detect OP pesticide is required given their enormous use and toxicity. Even though there are several standard methods for detecting OP pesticides, the creation of portable sensors is necessary to make routine analysis much more practical. These sophisticated methods include molecular imprinted polymer-based sensors, surface plasmon resonance-based sensors, fluorescence sensors, colorimetric sensors, and others (Bhattu et al., 2021).

Malaysian government has established a guideline for pesticide residue in food known as maximum residue limits (MRL). Malaysia adopted the Codex ASEAN Crops MRLs (ASEAN, 2022) that follows WHO and FAO. Each pesticide has different MRL that depends on the type of vegetables itself. For example, MRL of chlorpyrifos for Chinese cabbage, lettuce and cauliflower are 1.0, 0.1 and 0.05 respectively (ASEAN, 2022).

To monitor the exposure of OP in human especially in occupational setting, urinary organophosphate metabolites such as dialkyl phosphate (DAP) levels should be tested. This can serve as a basic guideline in measuring urinary DAP in general population. These are the guide for urinary DAP as provided by Safe Work Australia (2021):

Urinary dialkyl phosphate: 100-1000 µmol/mol creatinine
— indicates medium level occupational exposure
Urinary dialkyl phosphate: greater than 1000 µmol/mol creatinine
— may be associated with a drop in the blood cholinesterase level
— indicates high occupational exposure

Urinary dialkyl phosphate: less than 100 µmol/mol creatinine
— indicates low occupational exposure or high non-occupational exposure

Urinary DAP levels greater than 100 µmol/mol creatinine indicate that work practices of that person may need to be reviewed. An assessment of erythrocyte or plasma cholinesterase activity should be carried out.

Acetylcholinesterase activity in erythrocytes:
70% of individual's baseline activity

An evaluation of the type of work, exposure pattern, toxicity of the pesticide(s) being handled or applied, and work procedures should be utilised to establish the frequency of regular screening. It is important to keep track of exposure episodes and usage patterns. If there is a considerable decrease in plasma or erythrocyte cholinesterase activity, the person should be watched for signs and symptoms of OP exposure, paying close attention to the neurological system.

2.4 Organic diet intervention

The global market for organic food items is expanding quickly (Brantsæter et al., 2017). These foods are produced, handled, processed, and distributed in accordance with certified organic standards. The use of synthetic pesticides, fertilisers, and genetically modified organisms is specifically prohibited. The belief that organic food is healthier and more environmentally friendly than food produced conventionally is a major factor in the growing demand. The overarching goal of organic agriculture is to maintain or improve the health of the soil and the ecosystem from the smallest soil creatures to humans. This goal applies to farming, processing, distribution, and consumption. On the authority of United Nations Food and Agricultural Organization (1999) "organic agriculture is a holistic production management approach which maintains and enhances agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity".

Recent studies suggested that dietary intake of vegetables and juices may contribute to relevant proportion of OP exposure among children and adult. From the studies, there is evidence that diet intervention studies can reduce the pesticides metabolites in excreted urine of young children when they consumed an organic diet (Lu et al., 2006, 2008). Curl et al. (2003) proposed that children in non-agricultural households with no residential pesticides use that consume mainly organic food have lower or no pesticide exposure. However, children from low-income background may encounter higher exposure to pesticides like pyrethroids due to poor housing quality, home pesticide use and associated pest infestations (Curl et al., 2003; Quirós-Alcalá et al., 2012; Whyatt et al., 2002). Children that live in agriculture area are exposed to a higher surrounding and residential contamination from drift or volatilization from neighbouring agricultural applications and take-home pesticide residue by parents that work in the farm (Bradman et al. 2011; Harnly et al. 2009; Lu et al. 2000; Quirós-Alcalá et al., 2011).

2.5 Dialkyl phosphates in human urine

A study conducted by Hyland, et al., (2019) showed that there was significant reduction in urinary metabolites of 13 pesticides including insecticides OP, nicotinoid, pyrethroid and herbicide 2,4-D after an introduction of organic diet. The measurement of these metabolites represents exposure that occurred recently, primarily within the last few days. Dialkyl phosphates (DAP) may also be released into the environment as a byproduct of the breakdown of organophosphorus insecticides (Lu et al., 2005), hence their presence in a person's urine may indicate direct exposure to DAP metabolite itself.

The National Biomonitoring Programme and other research studies typically measure six urinary DAP metabolites of organophosphorus insecticides: dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP). The six urine metabolites and the parent organophosphorus insecticides that produce them are listed in the table 2.1.

Table 2.1: Six urinary DAPs metabolites produced from the metabolism of more than one OP pesticides (Source: CDC, 2017)

Pesticide	DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Azinphos methyl	•	•	•			
Chlorethoxyphos				•	•	
Chlorpyrifos				•	•	
Chlorpyrifos methyl	•	•				
Coumaphos				•	•	
Dichlorvos (DDVP)	•					
Diazinon				•	•	
Dicrotophos	•					
Dimethoate	•	•	•			
Disulfoton				•	•	•
Ethion				•	•	•
Fenitrothion	•	•				
Fenthion	•	•				
Isazaphos-methyl	•	•				
Malathion	•	•	•			
Methidathion	•	•	•			
Methyl parathion	•	•				
Naled	•					
Oxydemeton-methyl	•	•				
Parathion				•	•	
Phorate				•	•	•
Phosmet	•	•	•			
Pirimiphos-methyl	•	•				
Sulfotepp				•	•	
Temephos	•	•				
Terbufos				•	•	•
Tetrachlorvinphos	•					

DMP - Dimethyl phosphate; DMTP - Dimethyl thiophosphate; DMDTP - Dimethyl dithiophosphate; DEP – Diethyl phosphate; DETP – Diethyl thiophosphate; DEDTP – Diethyl dithiophosphate

The finding of Oates et al. (2014) proved that adults urine DAP levels decreased after a week of eating predominantly organic food implies that OP pesticide exposure from conventionally produced meals is higher than that from organic foods and that eating organic food greatly lowers this exposure. Another indication that the bulk of ingested OP pesticides are metabolised and eliminated after a week of ingestion is the drop in total DAP levels by almost 90% after a week.

Lu et al. (2008) found that children continued to be exposed to OP pesticides both before and after the organic food substitution was conducted. However, their exposure to malathion and chlorpyrifos were significantly reduced over the 5-day organic eating period and eventually reached undetectable levels. The findings were convincing because, a) during the days when the organic diet intervention was in place, concentrations of urine metabolites of OP pesticides declined to below the level of analytic detection, and, b) neither the families nor the study participants had ever used OP pesticides. The outcome of the exposure assessment enables them to separate dietary exposures from overall exposures because an organic diet intervention program was incorporated into the study design.

In a review paper by Hurtado et al. (2018), organic farming appears to help people stay in the best possible health and reduce their risk of contracting chronic illnesses. This may be because organic meals of plant sources contain more bioactive components than traditional agricultural goods and less harmful substances like cadmium, synthetic fertilisers, and pesticides. Although numerous research has sought to link consumption of an organic diet with health-related biomarkers including homocysteine, low-density lipoprotein (LDL) oxidation, semen quality, and antioxidant activity and status, the results are still uncertain. As a result, pesticide levels are currently the most widely utilised biomarkers of consumption of conventional food.

From this literature review, it is evident that conventional foods can have higher amount of OP pesticides residue since conventional farming method consistently use chemicals substances like pesticides and fertilizers on their farm. The production of the farms might be abundant and have higher selling power due to the beautiful looking appearance. However, the long-term effect of chronic exposure to pesticide residue is still not known. So, we are encouraged to change our dietary behaviour to a healthier choice like consuming organic diet. Consuming organic diet have been proven to lower the amount of urinary DAP metabolites in a few studies conducted in America. By conducting this study, we are hoping that we can provide a satisfactory result that demonstrate the organic diet can lower the DAP metabolites in Malaysian population.

2.5.1 Analysis of urinary DAP metabolites

Table 2.2 References for urinary DAP metabolites analyses.

References	Method of extraction	Instrument	LOD (µg/L)	LOQ (µg/L)	Linearity
Ueyama et al. (2014)	SPE procedure	LC-MS/MS	DMP: 1.2 DMTP: 1.2 DEP: 0.6 DETP: 0.3	DMP: 0.4 DMTP: 0.4 DEP: 0.2 DETP: 0.1	DMP:0.999 DMTP:0.998 DEP: 0.997 DETP: 0.999
Fernández, et al. (2019)	LLE procedure	LC-MS/MS	-	0.5 for all analytes.	DMP: 0.997 - 0.999 DMTP: 0.990 - 0.997 DEP: 0.993 – 0.995

					DETP: 0.992 - 0.998
Sokoloff et al. (2016)	Hydrolysis procedure	GC-MS/MS	DMP: 1.00 DMTP:0.60 DEP: 1.00 DETP: 0.60	-	-
Barr et al. (2006)	LLE procedure	GC-MS/MS	0.1 in all analytes.	-	-
Bradman et al. (2015)	Hydrolysis procedure	GC-MS	DMP: 0.6 DMTP: 0.2 DEP: 0.2 DETP: 0.1	-	-
Oya et al. (2020)	SPE procedure	LC-MS/MS	DMP: 0.1 DMTP:0.05 DEP: 0.11 DETP: 0.02	-	0.98 for all analytes
Xie et al. (2022)	Dilute and shoot	LC-MS/MS	DMP: O.25 DMTP:0.10 DEP: 0.25 DETP: 0.10	DMP: 0.05 DMTP:0.02 DEP: 0.05 DETP: 0.02	-
Eskanazi et al. (2004)	SPE procedure	GC-MS	DMP: 0.08 nmol/L DEP: 0.05 nmol/L	-	-

DAPs are typically detected in human urine due to their highly polar characteristics given that they are present in blood at low concentrations (Abdeen et al., 2016; Berman et al., 2013; Lewin et al., 2017; Motsoeneng & Dalvie, 2015; Yan et al., 2009). The assessment of DAP concentrations offers the chance to gather important data regarding recent cumulative exposure to OP pesticides (1–3 days) (Koureas et al., 2014; Sokoloff et al., 2016).

To determine DAP metabolites in urine samples, several methods can be used. Gas chromatography and mass spectrometry (GC-MS) is a commonly used technique for the analysis of DAP metabolites (Eskenazi et al., 2004). This method involves separating the metabolites using gas chromatography and then detecting and quantifying them using mass spectrometry. Isotope dilution calibration is often employed to ensure accurate quantification of the metabolites (Eskenazi et al., 2004).

The DAP metabolites that are typically measured in urine samples include dimethyl phosphate, dimethyl dithiophosphate, dimethyl thiophosphate, diethyl phosphate, diethyl dithiophosphate, and diethyl thiophosphate (Eskenazi et al., 2004). These metabolites are commonly produced upon the metabolism of organophosphate pesticides (Ye et al., 2008). The presence and levels of these metabolites in urine can reflect exposure to organophosphate pesticides (Ye et al., 2008).

In order to analyse DAP metabolites in urine samples, the samples need to be prepared for analysis. This typically involves azeotropic distillation with methanol and evaporation under a nitrogen stream (Rothlein et al., 2006). The metabolites may also undergo chemical derivatization to enhance their detectability and quantification (Bravo et al., 2002).

It is important to note that the concentrations of DAP metabolites in urine can vary over time. Studies have shown temporal variability in the levels of these metabolites can impact the design of epidemiological studies (Meeker et al., 2013).

Factors such as exposure to organophosphate flame retardants and house dust concentrations have been found to be correlated with the levels of DAP metabolites in urine (Meeker et al., 2013).

Liquid chromatography and mass spectrometry (LC-MS) coupled with solid-phase extraction (SPE) or magnetic SPE also can be used to determine urinary DAP metabolites. Traditional LC detectors such as UV and fluorescence are not suitable for the direct determination of DAPs due to the lack of chromophore or fluorophore moieties in the analyte molecules (Hernández et al., 2002). However, the recent introduction of atmospheric pressure interfaces, particularly electrospray ionization (ESI), has made LC/ESI-MS a popular method for the determination of analytes that are unsuitable for conventional liquid chromatography (Hernández et al., 2002). The use of tandem mass spectrometry (MS/MS) in LC-MS/MS provides high selectivity for the analysis of DAP metabolites (Hernández et al., 2002).

Solid-phase extraction (SPE) is a common sample preparation method used in LC-MS/MS for the quantification of various analytes, including DAP metabolites (Zhao et al., 2022). SPE involves the extraction of analytes from the sample matrix onto a solid-phase sorbent, followed by elution and subsequent analysis by LC-MS/MS (Zhao et al., 2022). Magnetic solid-phase extraction (MSPE) is a variation of SPE that utilizes magnetic nanoparticles as the solid-phase sorbent, allowing for easy and efficient extraction of analytes (Hernández et al., 2002).

The combination of LC-MS/MS with SPE or MSPE has been successfully applied in the determination of various analytes in biological samples, including urinary metabolites (Zhao et al., 2022). For example, LC-MS/MS coupled with SPE has been used for the quantification of 18-hydroxycortisol in urine samples (Zhao et