Tampere University of Applied Sciences

The transformed products of Disulfoton contribute to Cholin-esterase inhibition activity on human

My Chu

BACHELOR'S THESIS
March 2020

Environmental and Energy Engineering
In collaboration with Environmental Risk Engineering Laboratory,
Hokkaido University

ABSTRACT

Tampereen ammattikorkeakoulu
Tampere University of Applied Sciences
Environmental and Energy Engineering

MY CHU:
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Bachelor's thesis 38 pages, appendices 5 pages
March 2020

The purpose of this study is to evaluate the toxicity (cholinesterase inhibition activity) of a solution containing a pesticide (disulfoton) during chlorination process and identify the transformation products contributing to the observed toxicity. This study was performed by preparing a batch of chlorinated samples, conducting assay test with cholinesterase from human blood and construct quantification analysis by LC/MS. As the result, the toxicity of the solution containing disulfoton increased by chlorination. Disulfoton transformed mostly into demeton-s-sulfone, with demeton-s-sulfoxide was the intermediate oxidation product. Other transformation product(s) than the oxon form contributed to the increased toxicity. In conclusion, possible future work should be implemented in order to observe a more precise and deeper results.

The thesis topic belongs to Environmental Risk Engineering laboratory, Hokkaido University. The permission of doing this thesis work was given by Professor. Matsushita – the associate professor of the laboratory. This research was supported and assisted by Environmental Risk Engineering laboratory, Hokkaido University.

Key words: disulfoton, chlorination, cholinesterase inhibition

CONTENTS

ABBREVIATIONS AND TERMS

A-buffer	Assay buffer
ACh	Acetylcholine
ADI	Acceptable daily intake
C	Concentration
Ch	Choline
ChE	Cholinesterase
DI	Daily intake/Value of measurement standard
DWQS	Drinking Water Quality Standards
GC/MS	Gas chromatography–mass spectrometry
LC/MS	Liquid chromatography–mass spectrometry
LD50	Lethal Dose, 50%
M	Molecular weight
m/z ratio	mass-to-charge ratio
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
mg/L	Milligram per liter
MHLW	Ministry of Health, Labour and Welfare
OPs	Organophosphates
P-buffer	Phosphate buffer
ppm	Parts per million
PTFE	Polytetrafluoroethylene
S	Solubility
TPs	Transformation products
µg/L	Microgram per liter

1 INTRODUCTION

Two million tons of pesticide are used worldwide annually, and it has been increasing continuously (Sharma et al, 2019). In particularly, the organophosphate pesticides (OPs) are the largest group of chemical insecticides and preferred for its low cost and effectiveness. For example, OPs take part of 70% of all insecticide's usage in the US for agriculture and pest control in livestock (Kiely, Donaldson & Grube 2004). Consequently, the application of OP pesticides has contaminated the water environment, threatening our drinking sources and leads to ecological and human health risks.

Concerning the situation, water samples were collected from tap water and rivers (Shinano and Agano) in 1995 and 2007, Japan. As the result, pesticides were found in both river and tap water throughout the year. (Narushima 2014.) To cope with such situational changes of the surrounding water quality management, the Ministry of Health, Labour and Welfare (MHLW) in Japan started to access the revision of Drinking Water Quality Standards (DWQS). New standards should be proposed to assure the ultimate safety of drinking water. All substances which have the likelihood of an adverse effect to human health are considered and measured on the locality. No specific pesticides were classified at that time; therefore, insecticide compounds were noted as Complementary Items to Set the Targets for Water Quality Management. The list consisted of 101 pesticides which need evaluation and monitoring under the target level. (Wakayaman 2005, 74-75.)

What is more, disulfoton, one of the systemic organophosphate pesticides (OPs) effective against insects, was included in the list. The pesticide was the target of this thesis. This insecticide could be oxidized into their oxon form during the chlorination process, which possess negative effect to the human nervous system. According to the EU Drinking Water Directive (1998), acceptable concentrations of pesticides are 0.1 µg/L separately and with related products were 0.5 µg/L in total.

1.1 Organophosphorus pesticide: Disulfoton

The organophosphorus compounds (OPs) are a diverse class of substances used largely in agricultural insecticides or chemical weapons. They can also be seen as drugs and nerve agents which inhibit acetylcholinesterase (AChE), the essential enzyme in neurotransmission.

During the early nineteen century, organophosphorus compound had been studied and used as an organophosphorus nerve gas for its miotic effect (tabun, sarin and soman) which almost served the German Army in the World War II. Linked to this militarily discovery, after the 1950s, these organic phosphates had been studied deeper. It appeared as a vital class of pest control agents that it was presented to be greatly insecticidal and effective against spider mites (Timperley 2014, 12-17.). Besides, some compounds could be useful for treatment of human filariasis or against tapeworms and flukes in animals (Sharma & Anand 1997, 124). However, until the 21st century, a vast number of OPs have been banned for use (parathion, chlorpyrifos, etc.) due to its toxicity to mammals and severe blood-AChE depression (Roberts & Reigart 2013, 43).

OPs can be exposed by countless occasions and pathways, generally the toxicity might vary depending on the dose, the host and the certain type of OPs. There are various of structures presenting the OPs. Yet, the most characterized structures that interact with esterase causing inhibition of AChE are shown in figure 1. The group R1 and R2 might be methyl or ethyl for pesticides. “X” represented different groups which indicate certain chemical function. (Mangas et al 2016, 31.)

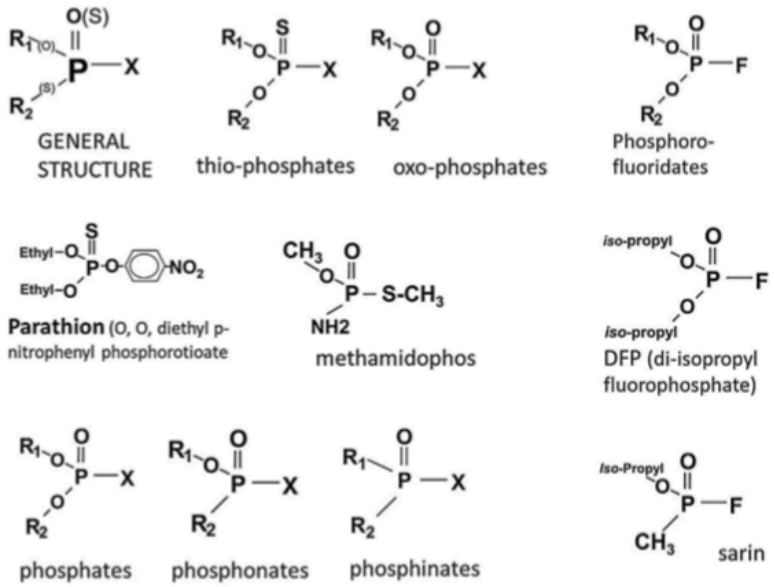


FIGURE 1. Some typical structures of OPs able to interact with esterases (Mangas et al 2016, 31)

The disulfoton, one of the systemic organophosphate insecticide, is the main object in this research. Disulfoton is a synthetic organic *thiophosphate* which has the formula as diethyl ester of S-[2-(ethylsulfanyl) ethyl] dihydrogen phosphorodithioate and its structure exhibits on figure 2. The pesticide is widely used in agriculture to prevent attack from harmful pests, for example, in Brazil's coffee crops (Faria et al 2016, 1). Since 1989, disulfoton had already been indicated as a *highly toxic* compound by Donald Morgan in the EPA manual. By mean of this, the 'highly toxic' chemical was listed oral LD50 values less than 50ppm for rat and possess vastly inhibition of neurotransmitter in human. Therefore, all products which contain greater than 2% of disulfoton will be classified as Restricted Use Pesticides (Greene & Pohanish 2005, 1580). More importantly, this organophosphate is seriously hazardous because it can be taken up by the plant and translocated into the vegetation and fruit that we consume (Morgan 1989, 1). Apart the exposure from chemical residues in/on food, the drinking water is another potential pathway for dietary contact.

Advanced water treatments are usually used to remove pesticides such as membrane technology. Chlorination is an important process in disinfecting water for drinking. Pre-chlorination or intermediate chlorine are solutions to prevent algae growth in sedimentation and filtration processes. As the result, chloride will come in contact with the pesticides and chlorination byproducts will be produced. (Ohno

et al 2008, 1753). According to Wu & Laird (2003, 261), by the oxidization of chloride, the OPs containing phosphorus-sulfur double bond (P=S) are transformed to their corresponding oxons with phosphorus-oxygen double bond (P=O). The stability of the oxon forms in water can differ from its structure. Subsequently, the oxidative metabolites are more toxic, at least 10 times higher than the parent pesticide (Usui 2012, 314).

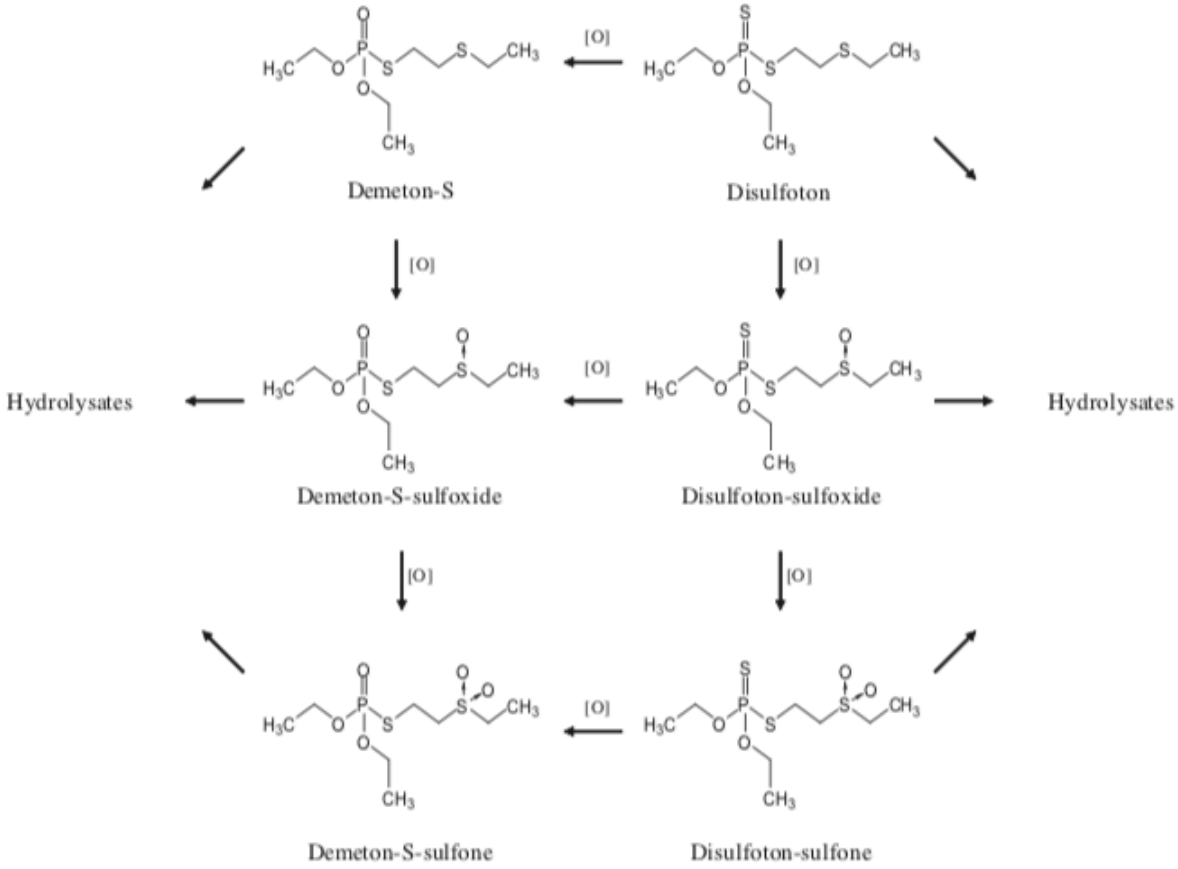


FIGURE 2. Proposed metabolic pathways of disulfoton (Usui et al 2012, 315)

Currently there are few detailed reviews on disulfoton toxicity toward human, nor its metabolites in the human blood. Based on Usui research (2012), there are five (5) proposed oxidative metabolites of disulfoton (disulfoton sulfone, disulfoton sulfoxide, demeton-S, demeton-S sulfoxide and demeton-S sulfone) in human whole blood. The oxon form of disulfoton is called demeton-S. All these chemicals have a physical state as a colorless to pale yellow oily liquid and it can get dark yellowish when it comes to technical product (Greene & Pohanish, 2005). The structures are exhibited in the figure 2 and their chemical and physical properties are recapitulated in table 1.

TABLE 1. Disulfoton and its metabolites properties

Name	Formula	M (g/mol)	CASRN	S(water) (mg/L)	Log Kow	Density (g/cm3)
Disulfoton (Greene&Pohanish, 2005)	$C_8H_{19}O_2PS_3$	274.40	298-04-4	12 at 20°C 25 at 22 °C	3.95	1.14
Disulfoton Sulfoxide (PubChem, 2020)	$C_8H_{19}O_3PS_3$	290.40	2497-07-6	2904 at 20 °C	1.73	1.27
Disulfoton Sulfone (EPA,1995)	$C_8H_{19}O_4PS_3$	306.39	2497-06-5	882 at 20 °C	1.87	1.29
Demeton-S (PubChem, 2020)	$C_8H_{19}O_3PS_2$	258.30	126-75-0	2000 at 20 °C	2.09	1.132
Demeton-S Sulfoxide (Alfachemistry, 2012)	$C_8H_{19}O_4PS_2$	274.34	2496-92-6	-	2.67 (Cheméo, 2016)	1.241
Demeton-S Sulfone (ChemSrc, 2020)	$C_8H_{19}O_5PS_2$	290.34	2496-91-5	-	3.4162	1.248

1.2 Cholinesterase inhibition activity

Acetylcholine (ACh) is a neurotransmitter that supports chemical interaction between a nerve cell and a target cell (another nerve cell, muscle fiber or gland). Acetylcholine binds to receptors and passes the signal onto the target. This stimulation of cholinergic pathways innerves virtually every organ in our human body. On the other hand, acetylcholinesterase (AChE) is a critical enzyme found in cholinergic neurons (the area of synapses) and in other non-neural tissues. This enzyme decomposes the neurotransmitter ACh into Choline (Ch) and acetic acids

and stops the transmission. (EPA 2000, 9-11.) When the transmission is not necessary, ChE binds with ACh.

Most oxidized thio-phosphates are transformed into oxo-phosphates that inducing toxicity to the ChE, behaving as an inhibitor. The mechanism of the process is shown in figure 3. The cholinesterase is one of serine hydrolases. From the reaction 1 and 2, the hydroxyl oxygen of the serine residue in the catalytic triad of the active centre, can attack OPs at the phosphorus atom, dislocating the primary leaving group 'X' and forming a covalent bond. Sometimes, reaction 3 might occur spontaneously by water or by stimulation from other ions. (Mangas et al 2017, 32)

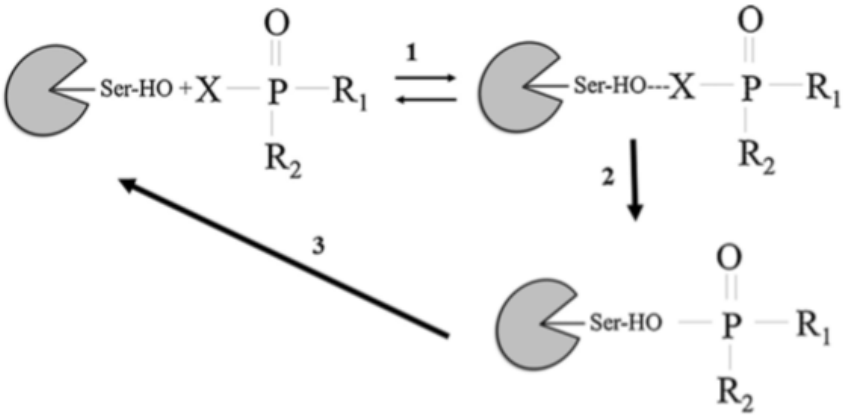


FIGURE 3. Reaction of inhibition of esterases by OPs (Mangas et al 2017, 32)

As the result, with the presence of the inhibitors, ChE binds with the OPs. The acetylcholine will not be broken down, which leads to the accumulation of acetylcholine. Consequently, serious health risk happens such as smooth muscle contractions, glandular secretions, flaccid paralysis, etc. and negatively affect the brain system. Based on the seriousness and half-life of exposure dose, the outcomes can be fatal. (EPA 2000, 11.)

1.3 Inhibition assay developed in the present study

According to Ohmori et al (2018), Environmental Risk Engineering Laboratory in Hokkaido University had developed a highly sensitive assay for evaluating ChE inhibition based on the direct measurement of choline.

$$\text{Acetylcholine} \xrightarrow{\text{ChE}} \text{Choline} \rightarrow \text{Quantification by LC/MS}$$

The experiment procedure is briefly presented in the flow diagram (figure 2). The inhibition of ChE activity is determined by tackling with three different kinds of samples. The first sample contains only pure water and ACh, choline is naturally released from ACh by hyrolysis even without ChE. The second one, which is enhanced ChE, will produce much more Ch by the enzyme activity. Finally, the last sample is suppressed by the addition of ChE inhibitor, as we could see the different from figure 3. The inhibition rate is calculated as below:

$$ChE\ inhibition\ rate = B/A \quad (1)$$

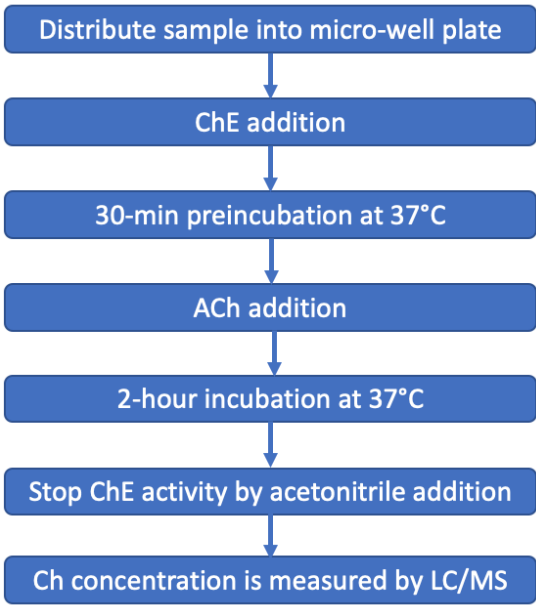


FIGURE 2. Flow diagram of ChE inhibition assay developed in the present study

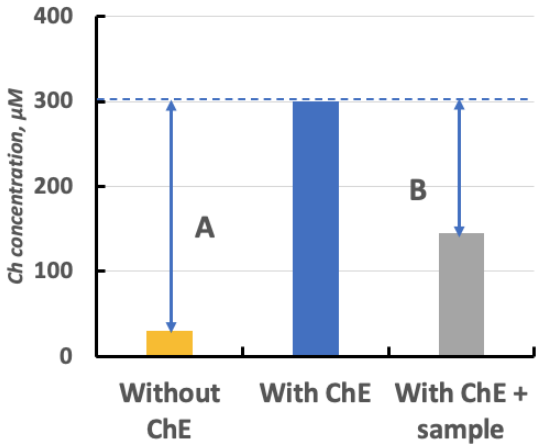


FIGURE 3. Determination of inhibition of ChE activity

Furthermore, the transformation products (PTs) of disulfoton was estimated by the its initial concentration. The original concentration of tested OPs should be roughly the same with the total concentration of leftover OPs, oxon form and other TPs.

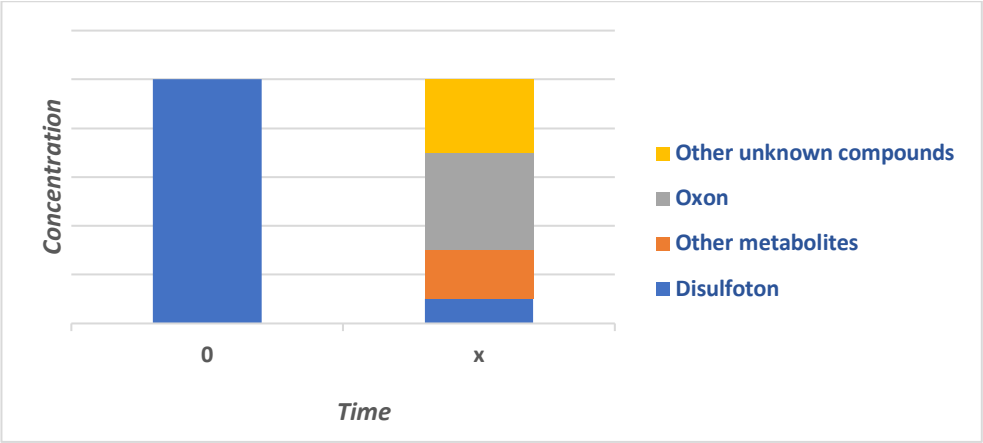


FIGURE 4. Risk assessment of TPs

According to MHLWJ (2003), DI value, the concept of a hazard index, are created to assess the total risk associated with exposure to multiple pesticides. The detection concentrations will be observed after the experiment and the desired/reference concentrations are based on the acceptable daily intake (ADI) of the pesticide. The target level should not be greater than 1 in order to meet the Drinking Water Quality Standards:

$$DI = \sum_{i} \frac{Detection\ concentrations}{Desired\ concentrations} \leq 1 \qquad (2)$$

2 SCOPE OF THE WORK

The aim of the current study was to use risk indicators for the selected pesticides disulfoton, to be comprised in drinking water quality regulations and guideline. To evaluate the toxicity, the change in the inhibition of cholinesterase (ChE) activity of solution containing disulfoton during disinfection phase was reviewed. The transformation products (TPs), which was ChE inhibitor in this case, should be identified and measured.

Currently, there are few researches concerning OPs water decomposition and their TPs in general and for disulfoton particularly. Concentration of this pesticide in drinking water are still not clear. Besides, there is no data regarding its ChE inhibition capability after the chlorination process towards human health. Therefore, further studies are required to accumulate information and knowledge on this substance.

3 MATERIALS AND METHODS

The method in the whole process is clarified in the diagram below. As can be seen, the underlined phases are the experiments' name and they will be explained in detail in the following sections.

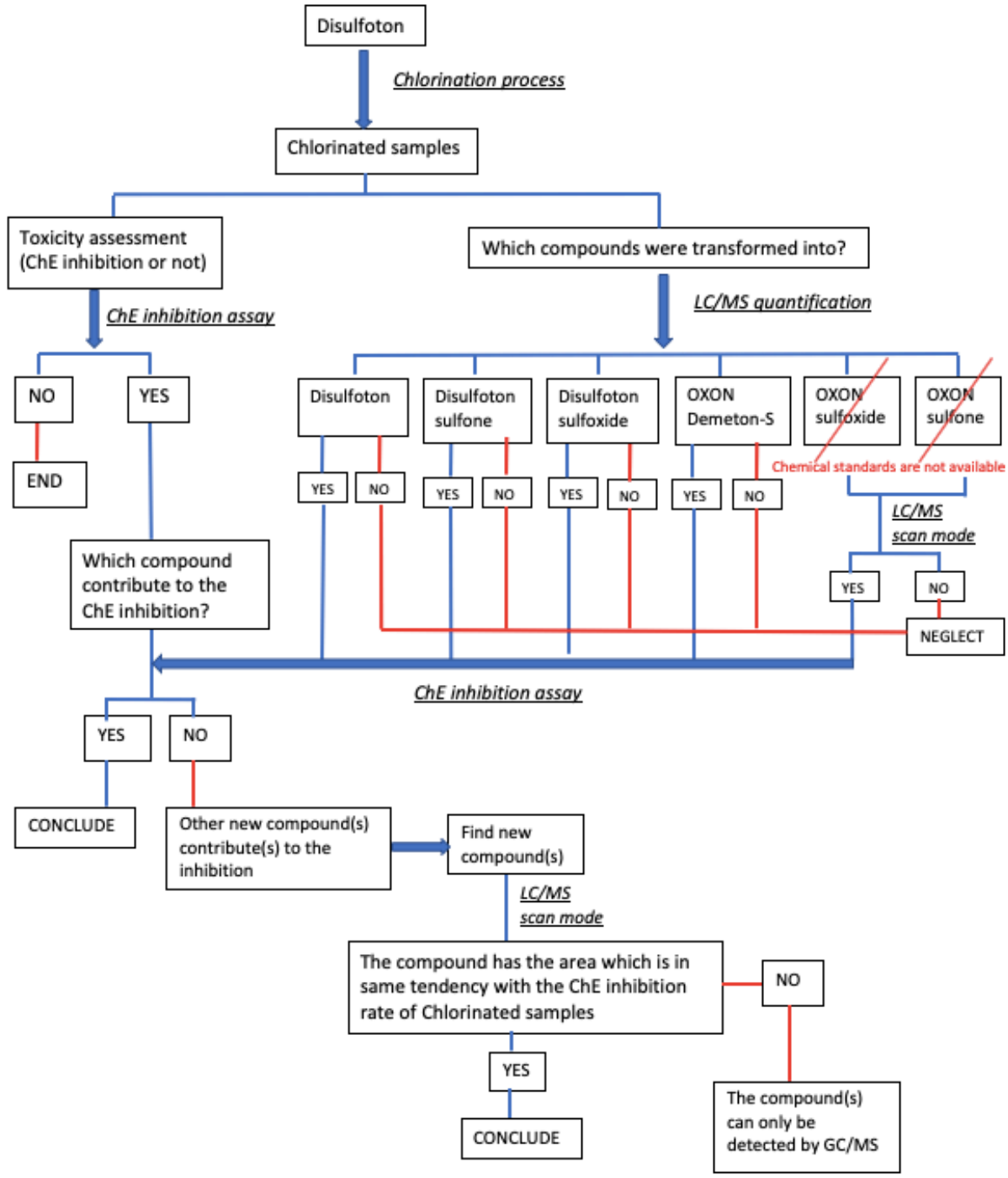


FIGURE 5. Research process and method

3.1 Materials

TABLE 2. Reagents and materials

<table>
<tr><td colspan="2"><u>Chlorination process</u></td><td rowspan="2">Material</td></tr>
<tr><td>Chemical</td><td>Properties</td></tr>
<tr><td>Disulfoton</td><td>Density = 1,14g/cm^3</td><td rowspan="20">- Mess flack
- Magnetic stirrer
- HACH chloride meter kit
- 10 sample bottles (62,5mL each)
- 100 mL flask
- Test tubes
- Stirring bath for test tube
- Vial bottles
- PTFE membrane 0,45μm
- Multiple pipette
- Pipettes
- Ice pack
- Polystyrene 96-well plate
- Glass 96-well plate
- LC/MS
- Incubation device (Tecan M200 Infinite Pro Microplate Reader)</td></tr>
<tr><td>NaClO</td><td>C = 10^5 mgCl_2/L</td></tr>
<tr><td>Na_2SO_3</td><td>C = 0,1M</td></tr>
<tr><td>P-buffer</td><td>$1\text{L}\begin{cases}1{,}1995\text{g of } \text{NaH}_2\text{PO}_4\\1{,}4196\text{g of } \text{Na}_2\text{HPO}_4\end{cases}$
Adjust to pH = 7</td></tr>
<tr><td colspan="2"><u>Transformation analysis</u></td></tr>
<tr><td>Chemical</td><td>Properties</td></tr>
<tr><td>Chemical standards</td><td>Disulfoton, disulfoton sulfoxide, disulfoton sulfone and demeton-S</td></tr>
<tr><td>Chlorinated samples</td><td>Diluted by organic solvent “1:1”</td></tr>
<tr><td>Organic solvent “1:1” (for dilution)</td><td>Methanol 100% : Ammonium formate 2mM (1:1)</td></tr>
<tr><td colspan="2"><u>ChE inhibition assay</u></td></tr>
<tr><td>Chemical</td><td>Diluting solvent</td></tr>
<tr><td>Neostigmine</td><td>P-buffer</td></tr>
<tr><td>Chlorinated sample</td><td>Organic solvent “1:1”</td></tr>
<tr><td>Disulfoton and its metabolites (dissolved in P-buffer)</td><td rowspan="3">A-buffer
(A) 2L of 150mM NaCl
(B) $\begin{cases}1\text{mM } \text{NaH}_2\text{PO}_4\ 500\text{mL}\\1\text{mM } \text{Na}_2\text{HPO}_4\ 1\text{L}\end{cases}$
Adjust the 2 solution (A) and (B) to pH = 7,4</td></tr>
<tr><td>Human blood ChE</td></tr>
<tr><td>Acetylcholine</td></tr>
<tr><td>Choline (dissolved in A-buffer)</td><td>P-buffer : Acetonitrile (1:1)</td></tr>
<tr><td>Acetone</td><td></td></tr>
</table>

3.2 Chlorination process

Chlorination process had been established by preparing a 1,5 liter of 30μM disulfoton solution to be reacted with chloride. Firstly, the appropriate concentration was defined based on the solubility. According to Bowman & Sans (1983),

$$\text{Solubility}_{\text{Disulfoton}} = 16{,}3\ \text{mg/L};\ \text{M}_{\text{Disulfoton}} = 274{,}392\ \text{g/mol} \quad (3)$$

$$\Longrightarrow \text{C}_{\text{max}} = \frac{\text{S}}{\text{M}} \approx 60\mu\text{M} \rightarrow \text{Suitable}\ \text{C}_{\text{Disulfoton}} = 30\mu\text{M} = 8{,}23\text{mg/L}$$

Secondly, a Phosphate buffer solution that maintains the constant pH of the solution was made by dissolving an exact amount of $\text{NaH}_2\text{PO}_4$and Na_2HPO_4 in ultrapure water, until reaching a pH of 7.

Finally, with the calculated amount, disulfoton was injected into the P-buffer solution, the solution should be stirred overnight (over 10 hours) with the magnetic plate and then filtered by PTFE 0,45μm before use. The mess flack was covered by aluminum foil to prevent the light impact.

3.2.1 Pre-experiment: suitable injection of Chloride

A pre-experiment was completed to find the exact amount of Chloride to be reacted in the process, so that in the final result, the free chloride remained under the control value.

Different concentrations of chloride were tested (6, 9, 12, 15, 18, 24 mg/L) through different time intervals from 0 to maximum 3 days. Based on previous experiments' results, the free chloride would dramatically decrease in the early stage and then remain nearly stable after 1-3 days. For each tested chloride concentration, a 100ml of Disulfoton solution was used and the free chlorine concentration was measured by HACH meter periodically. The process was modified according to the results so as to find a proper chloride concentration for the later process.

3.2.2 Batch preparation

Before the procedure, 10 sample bottles needed to be disinfected by NaClO (300 mgCl_2/L). The bottles were filled up to the cap, left overnight and then dried naturally upside down.

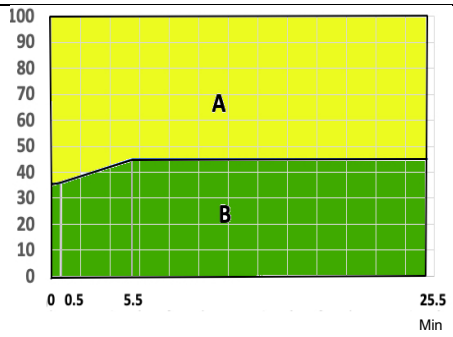
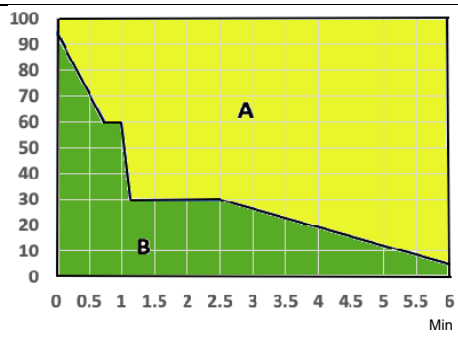
With the suitable concentration, chloride was injected into both of the 1,5 L filtered Disulfoton solution and an equivalent volume of 'zero' sample containing only ultrapure water. The free chloride should be managed during the whole process. The samples were then collected into 10 bottles representing time intervals and the chlorination process was stopped periodically by using Na_2SO_3 0,1M. The mole ratio of Na_2SO_3:Cl_2 was 2:1. After collecting all 10 samples (10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 1 day, 3 days, 5 days and 7 days), preservation was taken by aluminum foil to avoid light.

3.3 Transformation analysis

The quantitative equipment Liquid Chromatograph – Mass Spectrometer (Q Extractive, Thermo Fisher Scientific Inc., Waltham, MA, USA) was the main appliance using in the procedure. There were two main experiments using the device: to quantify the pesticide concentration in the chlorinated samples and to measure the choline concentration in the inhibition assay. The differences between the used columns, mobile phases and flow gradients were shown in the table below. More detailed settings of the LC/MS were presented in the appendix 1. The following methods were established according to Matsushita et al (2018), Usui et al (2012) and MacRitchie & Meadows (2012).

This section focused on the quantification of pesticide concentration, in order to analyse its transformation during chlorination.

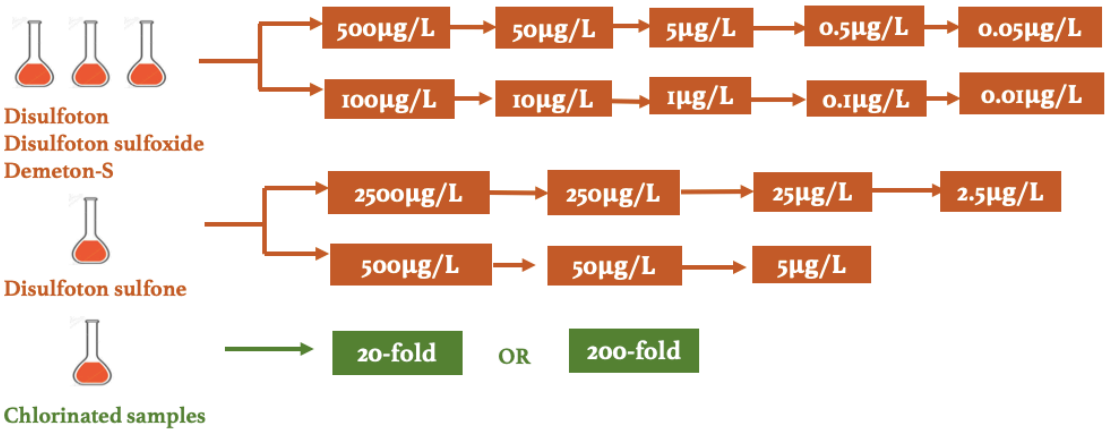
TABLE 3. LC/MS settings

	Pesticides	Choline
Column	Hypersil GOLD column 1.9 µm, 50mm×2.1mm	CORTECS R UPLC R HILIC 1.6 µm, 100mm×2.1mm
Mobile phase	A) Ammonium formate 2mM B) Methanol 100% C) Methanol 50% D) Methanol 50%	A) Ammonium formate 100nM B) Acetonitrile100% C) Methanol 50% D) Methanol 50%
Flow rate	0.2mL/min	0.2mL/min
Flow gradient		

3.3.1 LC/MS Quantification on Chlorinated samples

First of all, define if the analyte(s) would ionise in the mass spectrometer. The process was carried out by introducing a constant stream of analyte into the ion source using a syringe infusion pump. Disulfoton and its metabolites were prepared with high concentration of 1mg/L in an organic solvent “1:1”. Each sample was then injected through the syringe pump, which had to be thoroughly washed between use. The m/z ratio of the analyte(s) would be determined together with the suitable ionization mode.

To find the retention time, preparing a mixed solution of all compounds with concentration of 100mg/L equally in a solvent of P-buffer and Acetonitrile (ratio 1:1) and observe the results based on discovered m/z values. Therefore, instrument method was completed for further analysis. The corresponding values and pictures were displayed in the appendix 2.



FIRGURE 6. Disulfoton and its metabolites calibration lines

When analyzing the chlorinated samples, the samples should be diluted before the LC/MS measurement in order to decrease the impurities and the matrix in the solutions. Hence, dilution of 20-fold or 200-fold would be performed, in the organic solvent “1:1”. During the measurement, analytes were dissolved in acetone and diluted according to the calibration line figure 6 with the same diluting solvent with chlorinated samples. The calibration range was made according to the analyte solubility. The samples would then be transferred into vial bottles and taken into LC/MS. The sequence was arranged in a concentration-increasing order with injection volume shown in figure 7.

FIRGURE 7. Sequence arrangement

3.3.2 SCAN mode

From the raw files, peaks in the chromatogram of the samples which had m/z value from 50 to 500 would be taken into consideration. For example, oxon form sulfone and oxon form sulfoxide which the chemical standards were not available, it could be examined from the peaks if m/z values were varying around 3ppm from the references. The alternation of the peak areas through time should also be deliberated for further study.

3.4 Cholinesterase inhibition assay

The Cholinesterase inhibition assay experiment was conducted for the toxicity evaluation of the chlorinated samples and Disulfoton and its metabolites, Neostigmine was used as a positive control. Neostigmine was prepared in filtered phosphate buffer, stirred by shaking bath and then filtered with PTFE membrane 0,45µm. The calibration lines of choline, neostigmine tested samples were established as shown in the figure below. In this situation, the pesticides were prepared in P-buffer, which needed overnight stirring and prevention of light.

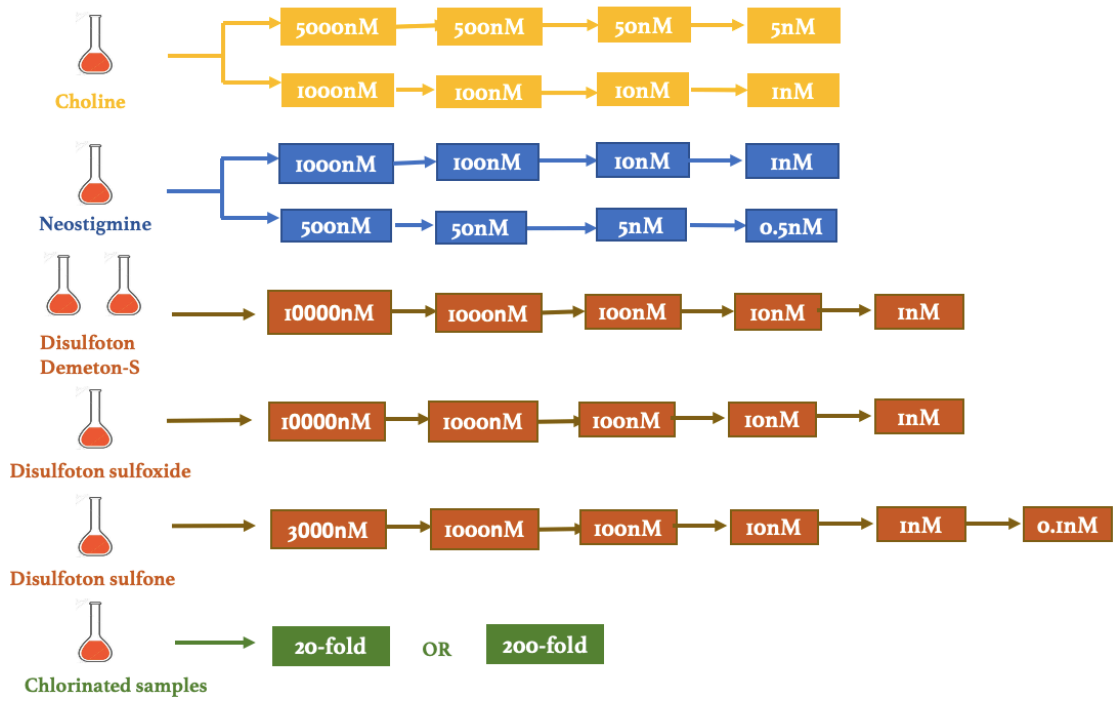


FIGURE 8. Choline calibration line and tested samples

The process of the inhibition assay was illustrated in the figure 9 below. All samples and solutions should be filtered by PTFE 0,45µm before use. The batch was injected respectively into the 96-well plate and kept cool before and during adding ChE and ACh, illustration was made in Table 5). The solutions were then mixed inside the wells by pipetting technique. After 1 hour of incubation, acetonitrile was used to stop the enzyme activity and the samples were 10-fold diluted in the final stage. To thoroughly dissolve the chemicals inside the micro-well plate, multiple pipette was used to mix the solution by pipetting technique.

TABLE 5. ChE and ACh injection into the well plate

	1	2	...9	10	11
A	(1)	(1)	(1)	(2)	(3)
B	(1)	(1)	(1)	(2)	(3)
C	(1)	(1)	(1)	(2)	(3)

(1)	ChE + ACh + Inhibitor (Sample)	With pesticide
(2)	ChE + ACh + 0 (P-buffer)	Without pesticide
(3)	0 + ACh + 0 (A-buffer) + (P-buffer)	Without pesticide and ChE

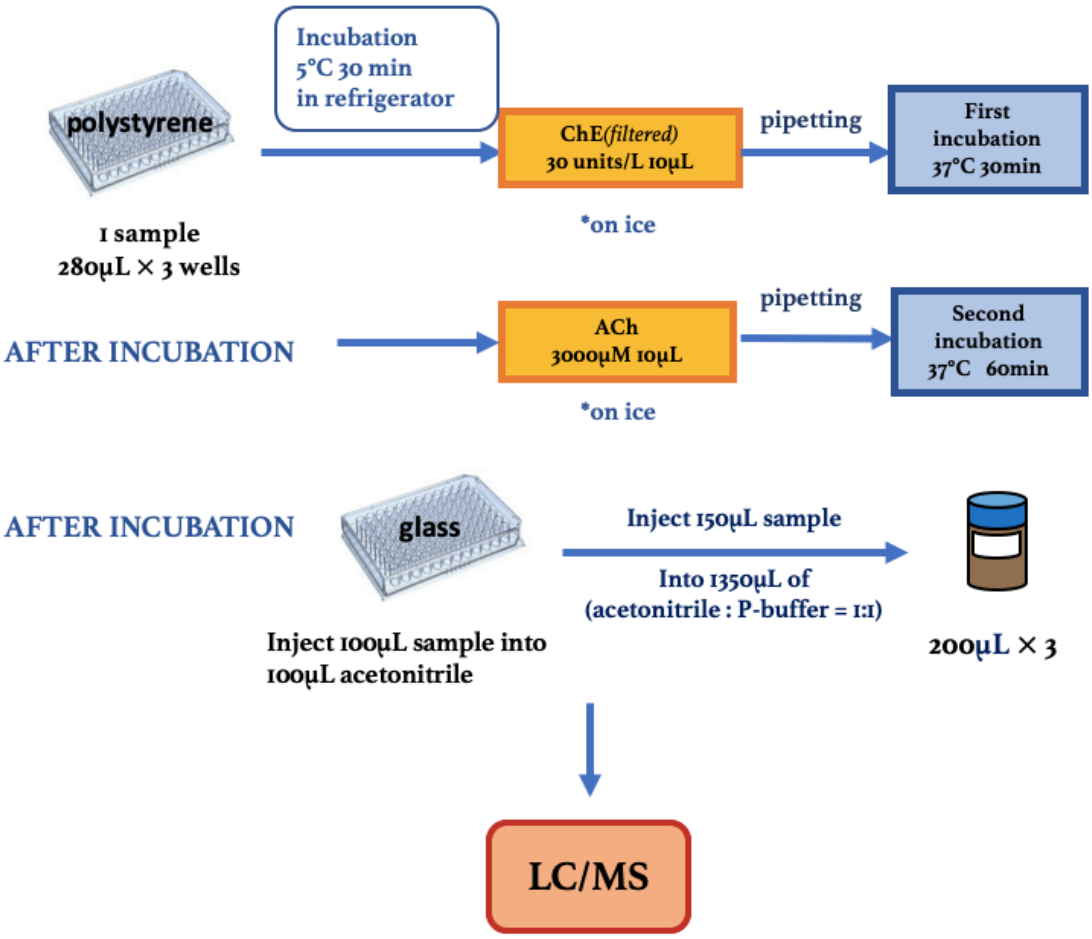
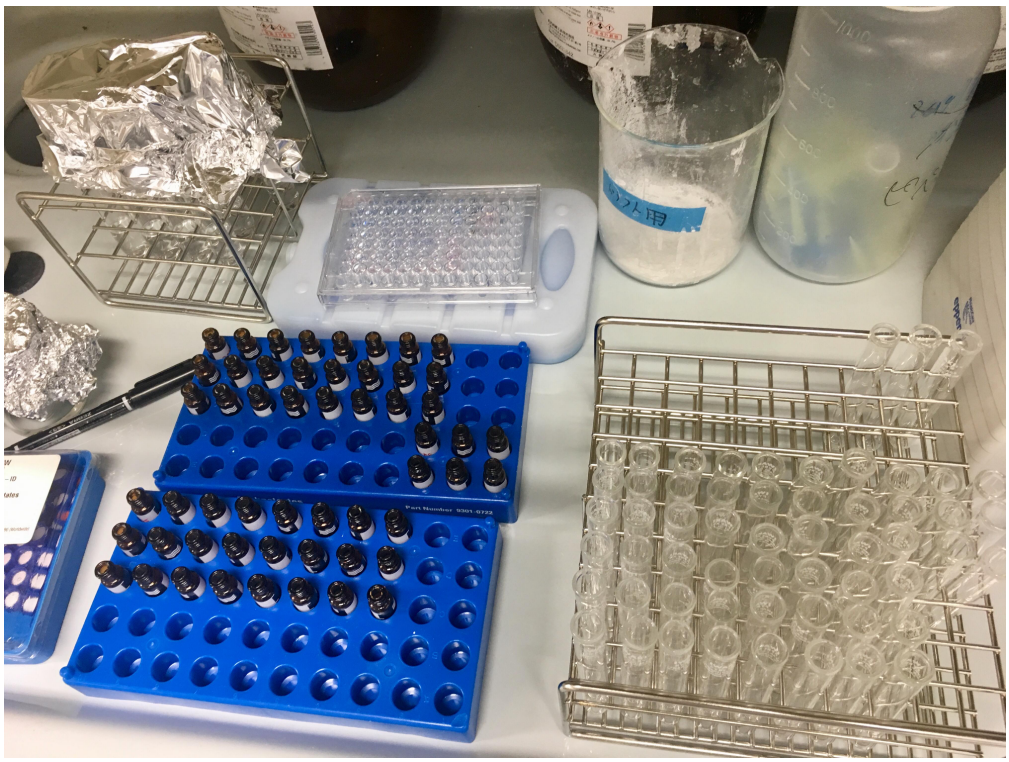


FIGURE 9. ChE inhibition assay process illustration

The arranged sequence with injection volume was displayed in figure 10 below. For chlorinated samples, concentration-increasing order was neglected.

FIGURE 10. Sequence arrangement



PICTURE 1. ChE inhibition assay experiment

4 RESULTS

4.1 Chlorination process

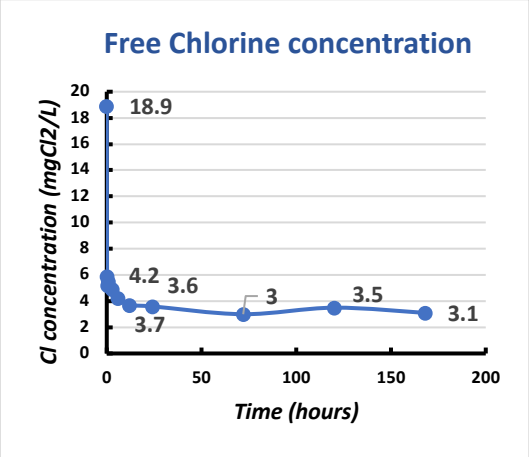


FIGURE 11. Free Cl_2 concentration

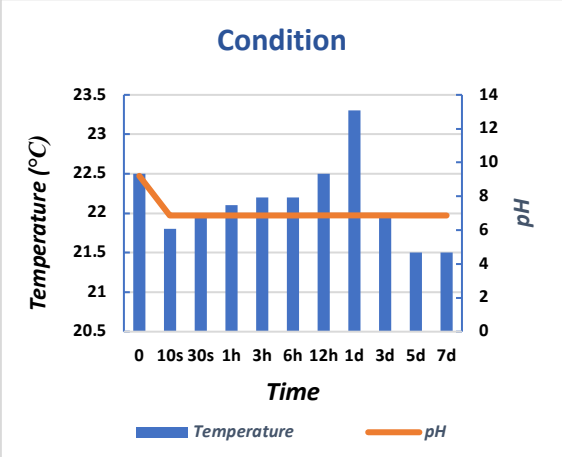


FIGURE 12. Temperature and pH

Due to the pre-experiment, suitable injected chlorination concentration was determined to be 20 mgCl_2/L. The free chloride dramatically dropped from 18,9 mg/L to 4,2 mg/L in the first 6 hours. After one day, the free chloride in the sample fluctuated at around 3 mg/L and 4 mg/L. At the result, the free chlorine remained at 3,1 mgCl_2/L after one week.

According to graph, the temperature was maintained at room temperature (about 22°C in average) and the pH was neutral at 7. The temperature was mainly affected by injected Na_2SO_3. The temperature and pH were monitored in order to successfully set up the real condition in water treatment process.

4.2 Toxicity test

The toxicity of the chlorinated samples was studied by the ChE inhibition assay experiment. Neostigmine was used as a positive control to make sure the inhibition test proceeds properly. The chlorinated samples were evaluated two times to perceive the stability.

4.2.1 Positive control with Neostigmine

The inhibition rates of neostigmine from different experiments were situated into the graph. All four (4) lines had the same trendline, that shown every experiment were carried out carefully with the same result.

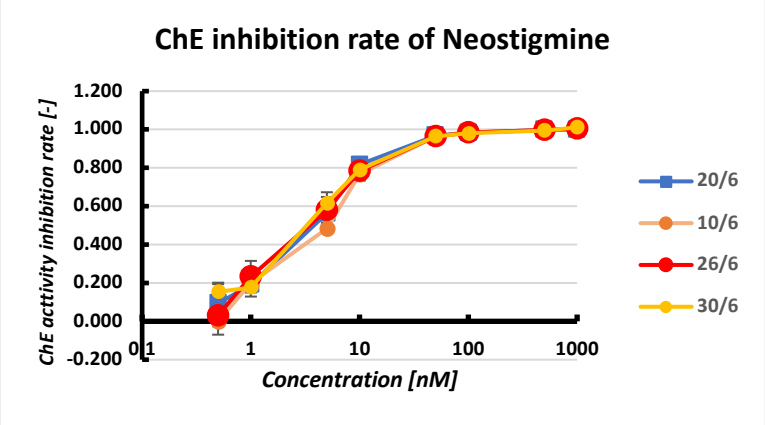
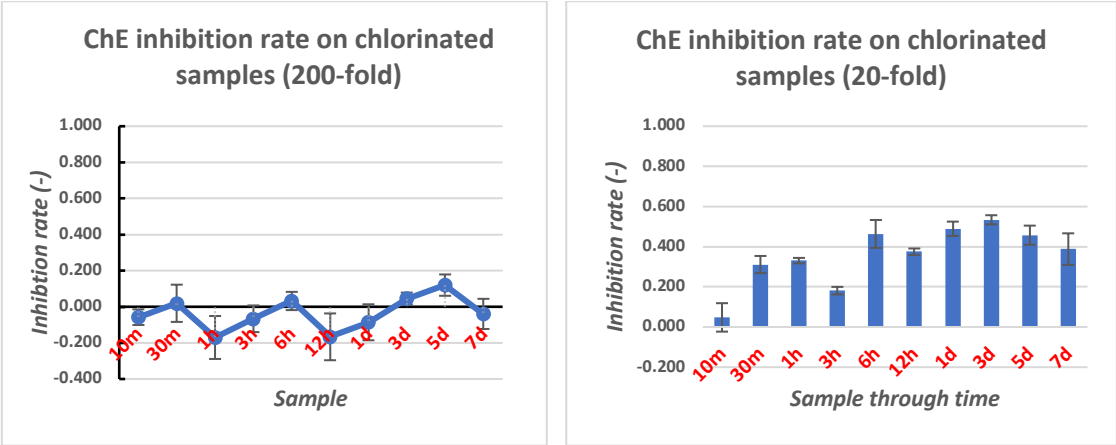


FIGURE 13. ChE inhibition rate of Neostigmine

With the smallest neostigmine concentration (0,5nM), there was nearly no inhibition activity. From 1nM to 10nM, the inhibition rate increased linearly from 20% to 80% then reached 100% at 50nM. The error between experiments were quite small and neglectable.

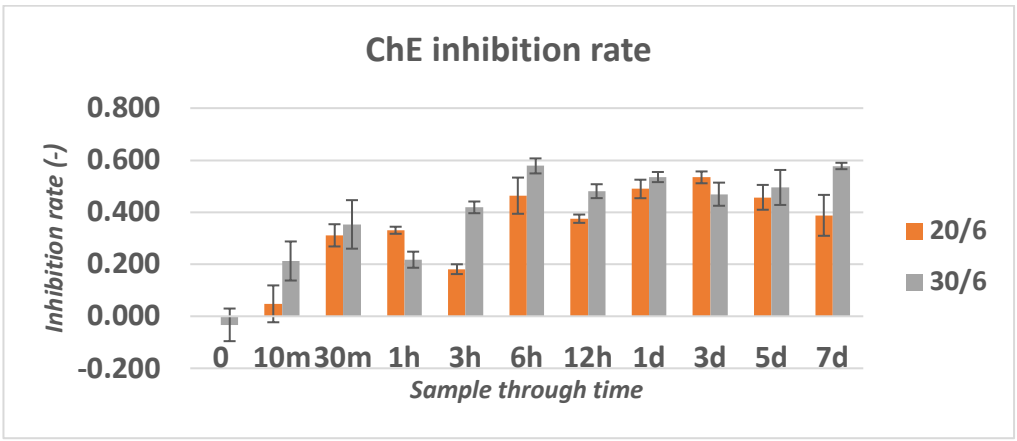
4.2.2 Chlorinated samples

The results of the cholinesterase inhibition test on chlorinated samples with 20-fold dilution were shown as more appropriate in comparing with 200-fold dilution. Since the inhibition rate oscillated between -20% to 20%, the higher dilution ratio caused in non-observative outcomes.



GRAPH 14. Cholinesterase inhibition test on the 20th of June 2019

With 20-fold dilution, we could evaluate that the samples gave significant effects to the cholinesterase inhibition activity. The rate was rising from the 10-minute sample (roughly 20%) and reached approximately maximum 60% after 7 days. The trendline increased through time in both experiments, on 20th of June and 30th of June 2019. The inhibition rates of different samples were not exactly similar due to errors in doing experiment.



GRAPH 15. Cholinesterase inhibition rate of chlorinated samples

4.2.3 Disulfoton and its metabolites

From the available chemical standards, the ChE inhibition assay tests were conducted on disulfoton, disulfoton sulfoxide and disulfoton sulfone. Disulfoton oxon form demeton-S was neglectable since it was not presented in the chlorinated samples. Neostigmine bromide was still used as a positive control.

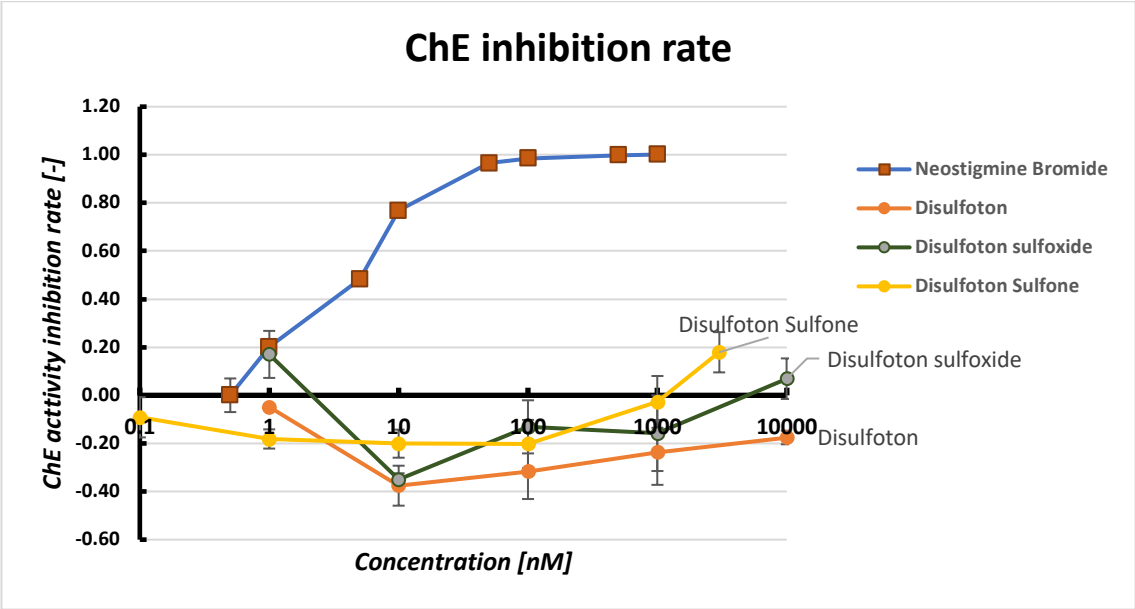


FIGURE 16. Cholinesterase inhibition rate of disulfoton and its metabolites

Disulfoton and disulfoton sulfoxide concentrations were prepared equivalently with the initial concentration of the chlorination process (30μM). In addition to the chemical reaction happened during the process, higher concentration than 10μM was not considered in this situation. Apart from this, disulfoton sulfone has lower solubility on water, therefore the maximum established concentration was 3μM. Apparently, there was no inhibition activity from the three compounds, as the inhibition rate did not rise beyond 20%. In conclusion, the contributor(s) to inhibition activity should be other unknown compound(s) or other unavailable chemical standards of disulfoton metabolites.

4.3 Transformation during chlorination process

The transformed products of disulfoton was detected and measured by using the LC/MS device and available chemical standards.

4.3.1 LC/MS quantification

Among the available chemical standards, disulfoton oxon form Demeton-S, disulfoton sulfoxide and disulfoton sulfone were effectively detected by the LC/MS device. The calibration line of the three compounds were shown in the graph.

Unfortunately, it was not appropriate to detect disulfoton by LC/MS but GC/MS. Disulfoton can only be detected by LC/MS with high concentration, which was over 500μg/L .The GC/MS setting for Disulfoton detection was shown in appendix 4, at the same time, solid phase extraction should be performed before the measurement (appendix 3). The chromatogram of disulfoton in the appendix 2 was captured by LC/MS machine with high concentration.

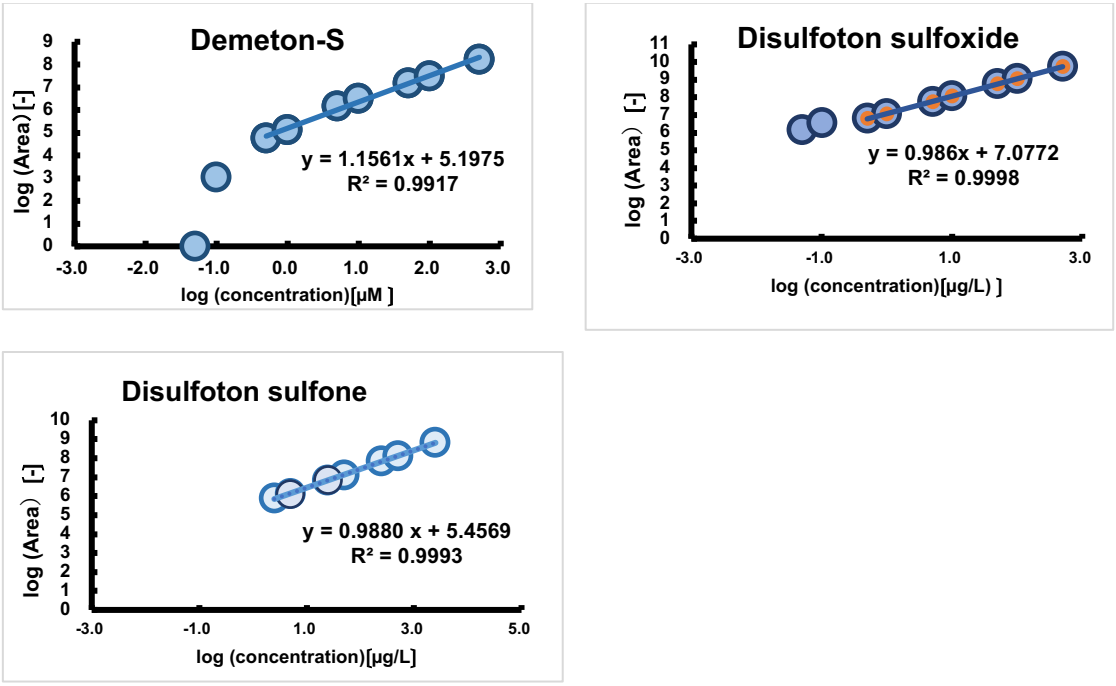


FIGURE 17. Calibration lines of Demeton-S, Disulfoton sulfoxide and Disulfoton sulfone

The concentrations of the three compounds were discovered. There was no peak appeared in the chromatograph for disulfoton sulfone, therefore disulfoton did not transform into disulfoton sulfone through the chlorination process. The concentrations of the two other metabolites were presented in the graph 17. As can be seen, the concentration of demeton-S was not stable during the process and could only be defined as a noise. The concentration of disulfoton sulfoxide (about 1μg/L) was considered too small comparing to the initial disulfoton concentration, which was 8,23 mg/L. Consequently, disulfoton should have changed into other compound(s) rather than demeton-S, disulfoton sulfoxide and disulfoton sulfone.

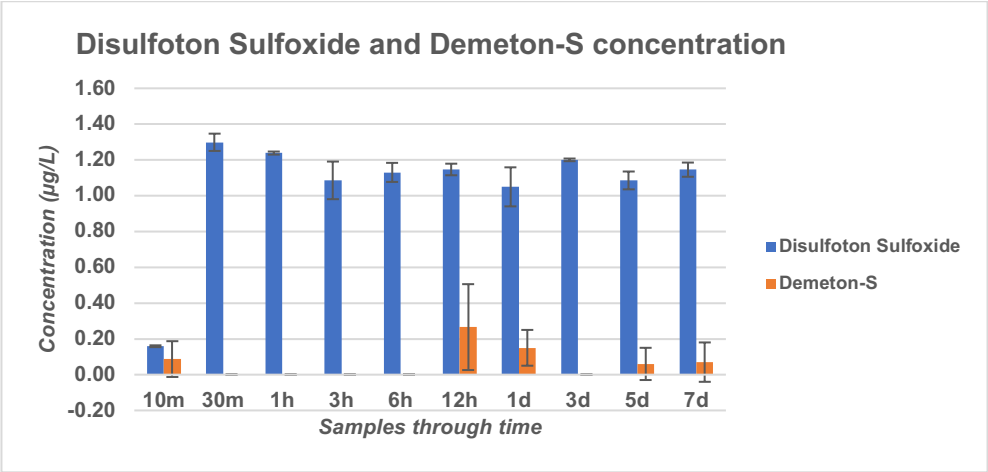


FIGURE 18. Disulfoton sulfoxide and demeton-S concentration

4.3.2 LC/MS SCAN MODE

New compounds in the chlorinated samples were discovered by scanning peak area of the m/z value from 50 to 500. Both positive and negative ionization mode were tested. The scanning process was performed two times for the positive scanning, which was 2 weeks apart. Extra comprehensive data will be shown in the appendix 5.

POSITIVE SCAN

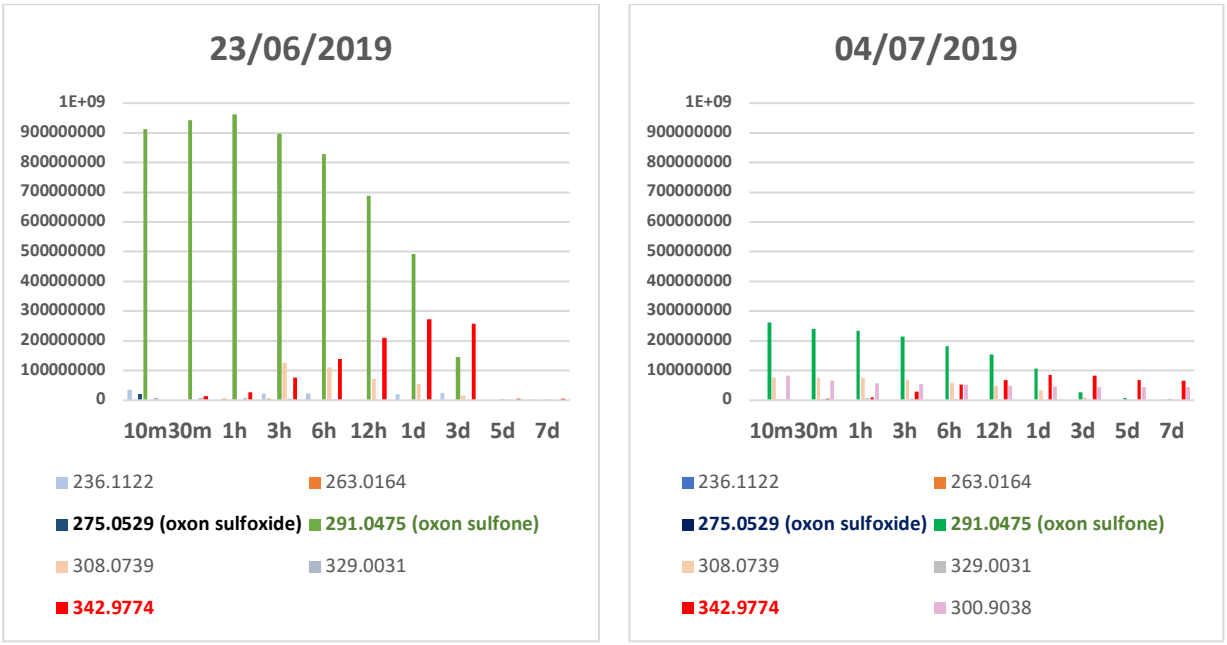


FIGURE 19. Positive scanning

As we can see from the graph, there were 8 peaks that could be plotted (236,1122; 275,0529; 263,0164; 291,0475; 308,0739; 342,9774; 329,0031 and 300,9038). From the result, oxon sulfoxide and oxon sulfone were presented in the chlorinated samples. However, oxon sulfoxide only appeared in the 10-minute sample and then disappeared. On the other hand, oxon sulfone had the most noticeable peak area among the discovered m/z value and shown up in nearly every sample. The trend line was declining by time. The other m/z values were unidentified and considered unknown compound.

There were differences between the two times. The peak area values decreased after 2 weeks. It could be concluded that the compounds got degraded through time in the solvent.

NEGATIVE SCAN

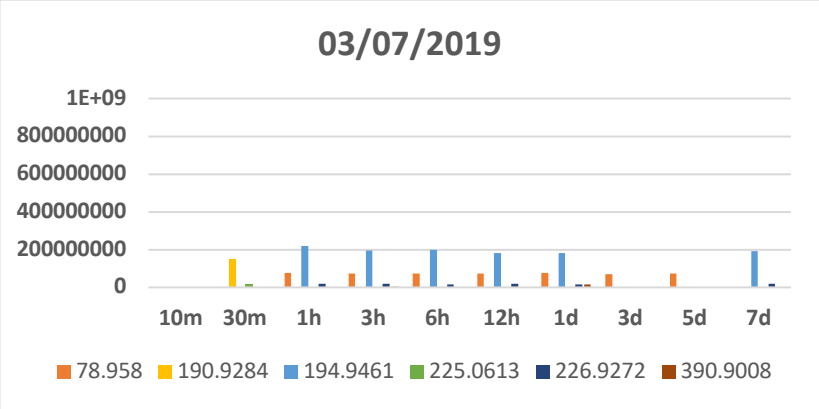


FIGURE 20. Negative scanning

Comparing with the positive scan results, negative scan did not seem to be suitable with the samples. There were total 6 peaks visible (78,968; 190,9284; 194,9461; 225,0613; 226,9272 and 390,9008). The peaks were unstable and unclear, which also caused the small peak areas. In consequence, the negative results might not be considered reliable for further assessment.

5 DISCUSSION

5.1 Transformation during chlorination process

The chlorination process was conducted successfully. The vial bottles had been covered by aluminium foil in order to avoid light. According to Tahara et al (2008, 147), another thiophosphate insecticide fenthion was exposed to UV light. In the research, three (3) more other metabolites were formed due to the irradiation. Even though the UV strength of sunlight is weak, the solar spectral intensity is naturally adequate to break down chemical bonds of the molecule. Therefore, it was necessary for the samples to prevent from light, undesired transformation of the compound should be avoided.

PICTURE 2. Covered vial samples.

The final chloride concentration residue was 3.1mg/L. According to National Drinking Water Regulations, MCLG and MCL is both acceptable below 4mg Cl_2/L (EPA, 2018). However, the target chlorine residue in Japan was set to be 1mg/L (Wakayama, 2007). In general, the result was merely satisfactory worldwide.

After the chlorination process, the transformation of disulfoton had been observed. Despite the fact the disulfoton could not be detected by LC/MS and the concentration of disulfoton throughout the process had not been perceived, Kamel et al research (2009, 528) revealed that disulfoton would disappear after one-hour interreacting with chlorine. Disulfoton sulfoxide and disulfoton sulfone were not transformed into, shown in the extremely minor concentration (that could

might be defined as noise) in the chlorinated samples. Demeton-S, the main oxon form of disulfoton could not detected during the process, which could be concluded that the pesticide did not transform into its oxon form. Alternatively, the scan results shown that demeton-s sulfone and demeton-s sulfoxide were present. The oxon sulfoxide was only found in the 10-minute sample, while oxon sulfone appeared in most sample. Based on figure 20, demeton-s sulfone peak area increased at the beginning and then reduced through time. For this reason, demeton-s sulfoxide was an intermediate oxidation product which was not stable and would be transformed into demeton-s sulfone. The outcome was similar with the result in Kamel et al article (2009, 530-532).

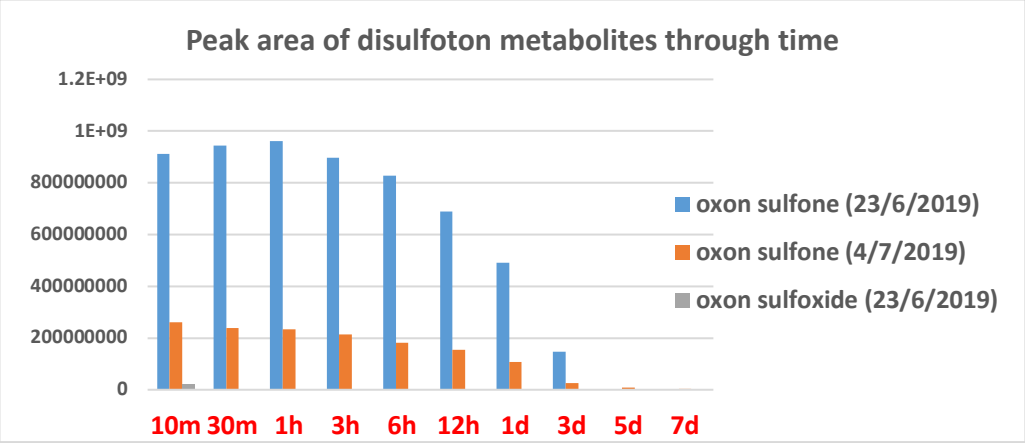


FIGURE 21. Peak area of demeton-s sulfone and demeton-s sulfoxide

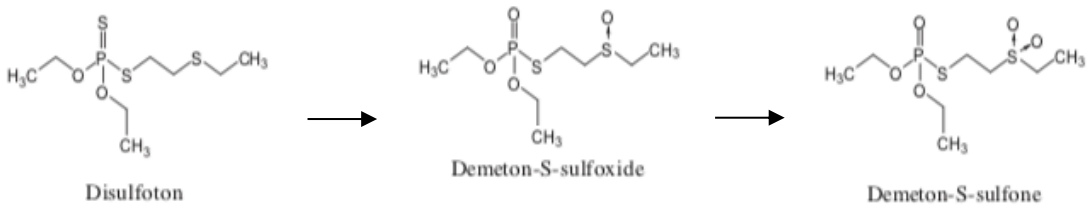


FIGURE 22. Observed disulfoton transformation pathway

However, as can be seen in the figure 20, there were differences in the scan results of oxon sulfone on 23th of June in compared with 4th of July. The peak area decreased significantly after 2 weeks, in addition, oxon sulfoxide could no longer be detected in the 10-minute sample on the 4th of July. It could be concluded that the compounds are easily degraded in the solvent.

Moreover, if comparing the pathway in figure 21 with figure 2 (proposed metabolic pathways of disulfoton) from Usui et al (2012, 315), it could be seen that disulfoton would be oxidized into demeton-S or disulfoton-sulfoxide before transformed into demeton-s-sulfoxide. Hence, the degradation of the pesticide and its related compounds happened quite rapid, that a smaller time-variation in collecting samples might help to gain a more detail outcome.

5.2 Contributors to the inhibitory activity

Since the toxicity of the chlorinated samples increased through time by chlorination, there should be ChE inhibitor(s) present in the solution. Disulfoton, disulfoton sulfoxide and disulfoton sulfone were tested for its toxicity with a reasonable concentration in relation to the initial disulfoton. As the result, none of them were the ChE inhibitor since the trendline was unstable and fluctuated only under 20%. Also, their inhibitory activities were quite trivial. From the table 6, the highest tested concentration of the three compounds in ChE inhibition test was vastly higher than the acceptable daily intake.

TABLE 6. Acceptable daily intake in relation with tested concentration

<table>
<tr><th>Compounds</th><th>ADI</th><th>Highest tested concentration</th></tr>
<tr><td>Disulfoton</td><td rowspan="3">$\sum_{i} C \leq 0.003\ mg/L$
For adult on daily basis (ATSDR, 2015)</td><td>2.74mg/L</td></tr>
<tr><td>Disulfoton sulfoxide</td><td>2.90mg/L</td></tr>
<tr><td>Disulfoton sulfone</td><td>0.92mg/L</td></tr>
<tr><td>Demeton-S</td><td colspan="2" rowspan="3">Need to be reviewed in the future</td></tr>
<tr><td>Demeton-S sulfoxide</td></tr>
<tr><td>Demeton-S sulfone</td></tr>
</table>

Following the method of Matsushita et al (2016), the TPs that might contribute to the observed ChE inhibition could be indicated by evaluating the correlation of the chlorinated samples' toxicity and its peak area. As we can see from figure 22, oxon sulfone peaks of area had a negative correlation with the induced toxicity and the coefficient of determination (R2) was quite small (0.371). Consequently,

oxon sulfone might not the contributor for the inhibition activity. Nonetheless, the concentration of each TP might not be reflected by its peak area since possible matrix effects can occur (Matsushita 2016, 257). Hence, the measurement of oxon sulfone concentration and its toxicity assessment should be performed in the future.

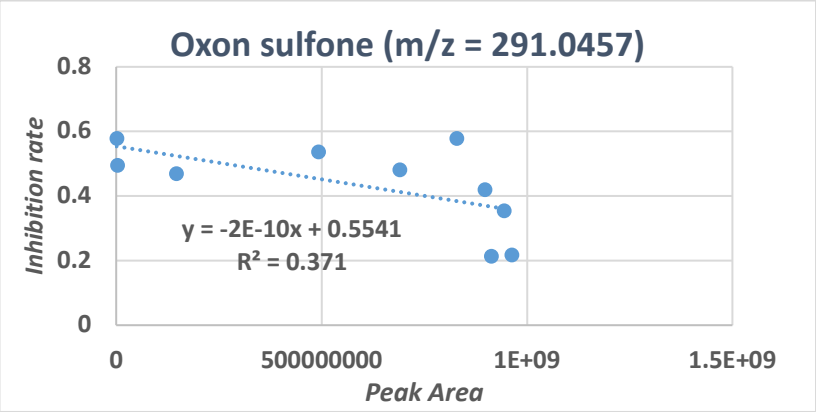


FIGURE 23. Relation of concentrations and toxicity of demeton-S sulfone

To look for more possible TPs that contributed to the obtained inhibition rate, every peak detected in the positive and negative ionization mode was considered. As the result, only one peak with the m/z value equalled to 342,9774 had the positive correlation (figure 23), also the coefficient of determination was high (0.78). Yet, the trendline did not start from the origin (0,0) which exhibited a 'shoulder' showing there should be more than one contributor in this situation. The inhibition rate was not only induced by this one compound but there should be several TPs that were not detected by LC/MS, had different trends in concentration with time and contributed equally to the ChE inhibitory activity

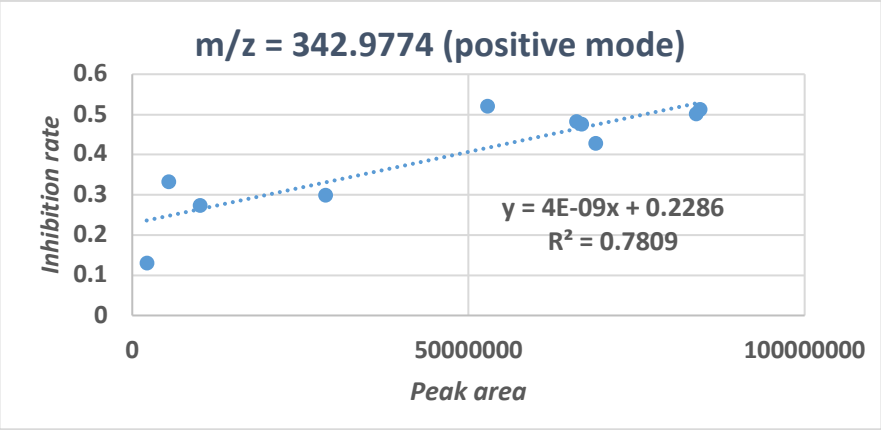


FIGURE 24. Relative of concentration and toxicity compound m/z=342.9774

However, the research procedure lacked some related chemical standards and equipment. Suggested work needs to be required in the future. The concentration of disulfoton should be indicated and measured through time by the GC/MS. More other transformed products might be detected by the gas chromatography device. The other ChE inhibitor(s) in the chlorinated samples could be signified. Finally, proper concentration of demeton-S sulfone formed during the chlorination process and its toxicity evaluation are required for a more precise result.

6 CONCLUSION

In this research, the toxicity and the transformation of disulfoton during chlorination process had been studied by carrying out ChE inhibition assay and the LC/MS quantitative analysis. Some valuable conclusions can be withdrawn.

Firstly, it was not suitable to measure disulfoton with concentration minor than 500μg/L by the LC/MS. Based on other references, disulfoton can be detected effectively by GC/MS. On the other hand, disulfoton sulfoxide, disulfoton sulfone and demeton-S can be detected by LC/MS successfully. Secondly, the toxicity of solution containing disulfoton increased by chlorination. Disulfoton, disulfoton sulfoxide and disulfoton sulfone did not contribute to the inhibition activity of the chlorinated solution and had insignificant inhibitory activity on human blood cholinesterase. Lastly, oxon form, which is well known to be a toxic transformation product of organophosphorus insecticides of disulfoton was not generated during chlorination. In contrast, demeton-S sulfone was formed, and demeton-S sulfoxide was an intermediate oxidation product of the transformation process. In conclusion, other transformation product(s) than the oxon form must contribute to the increased toxicity.

However, the study requires extra research in the future. With GC/MS device, disulfoton and other transformed products' concentrations during chlorination will be measured. Hence, other ChE inhibitor(s) could be indicated. Additionally, demeton-S sulfone concentration and its toxicity evaluation are needed for the final conclusion.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). 2015. Toxicological profile for disulfoton. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. Read on 18/3/2020. https://www.atsdr.cdc.gov/phs/phs.asp?id=976&tid=205

AlfaChemistry. 2012. Demeton-S Sulfoxide. Read on 15/3/2020. https://www.alfa-chemistry.com/cas_2496-92-6.htm

Bowman, B.T. & Sans, W.W. 1983. Determination of octanol-water partitioning coefficients (KOW) of 61 organophosphorus and carbamate insecticides and their relationship to respective water solubility (S) values. Journal of Environmental Science and Health, Part B, Volume 18, Issue 6. 667-683.

Cheméo (High Quality Chemical Properties). 2016. Chemical Properties of Demeton S-Sulfoxide (CAS 2496-92-6). Read on 15/3/2020. https://www.chemeo.com/cid/39-645-3/Demeton%20s-sulfoxide

ChemSrc. 2020. Demeton-S Sulfone. Read on 15/3/2020. https://www.chemsrc.com/en/cas/2496-91-5_443979.html

Council of the European Union. 1998. Drinking Water Directive: Council directive 98/83/EC on the quality of water intended for human consumption. Read on 15/3/2020. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:01998L0083-20151027&from=EN

EPA (United States Environmental Protection Agency). 2018. Edition of the Drinking Water Standards and Health Advisories Tables. EPA 822-F-18-001, p8. Read on 26/5/2019. https://www.epa.gov/sites/production/files/2018-03/documents/dwtable2018.pdf

EPA (United States Environmental Protection). 1995. Disulfoton sulfone 2497-06-5 | DTXSID8041901. DSSTox Substance Id. Read on 15/3/2020. https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID8041901#properties

EPA. 2000. The Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphorus and Carbamate Pesticides. Office of Pesticide Programs. Washington DC.

Faria, A. M., Noronha, L. M. S., Dardengo, R. P., Neves, A. A. & Queiroz, M. E., L. R. 2016. Mobility and degradation of disulfoton in coffee culture soil. Environmental Techonology and Innovation. Volume 5. 1-9.

Greene, S. A. & Pohanish, R. P. 2005. Sittig's Handbook of Pesticides and Agricultural Chemicals. 2nd edition. William Andrew Publisher.

Greene, S. A. & Pohanish, R. P. 2005. Sittig's Handbook of Pesticides and Agricultural Chemicals. William Andrew Publishing.

INCHEM. 1973. Disulfoton (WHO Pesticide Residues Series 3). International Programme on Chemical Safety. Read on 16/06/2019. http://www.inchem.org/documents/jmpr/jmpmono/v073pr13.htm

Kamel, A., Byrne, C., Vigo, C., Ferrario, J., Stafford, C., Verdin, G., Siegelman, F., Knizner, S. & Hetrick, J. 2009. Oxidation of selected organophosphate pesticides during chlorination of simulated drinking water. Water Research 43. Elsevier Ltd. 522-534.

Kiely, T., Donaldson, D. & Grube, A. 2004. Pesticide Industry Sales and Usage, 2000 and 2001 Market Estimates. EPA-733-R-04– 001. US Environmental Protection Agency, Washington, DC. http://www.epa.gov/oppbead1/pestsales/index.htm

MacRitchie, E. & Meadows, K. 2012. LC-MS/MS Method for the Analysis of Choline and Acetylcholine using a Syncronis HILIC 1,7μm Column. Thermo Fisher Scientific. Read on 12/2/2020. http://tools.thermofisher.com/content/sfs/brochures/AN-20498-LC-MS-MS-Method-Analysis-Choline-Acetylcholine-AN20498-E.pdf

Mangas, I., Estevez, J., Vilanova, E. & Franca, T. C. C. 2016. New insights on molecular interactions of organophosphorus pesticides with esterases. Toxicology. Volume 376. 30-43.

Matsushita, T., Hashizuka, M., Kuriyama, T., Matsui, Y. & Shirasaki, N. 2016. Use of urbitrap-MS/MS and QSAR analyses to estimate mutagenic transformation products of iopamidol generated during ozonation and chlorination. Chemosphere. Volume 148. 233-240.

Matsushita, T., Morimoto, A., Kuriyama, T., Matsumoto, E., Matsui, Y., Shirasaki, N., Kondo, T., Takanashi, H. & Kameya, T. 2018. Removals of pesticides and pesticide transformation products during drinking water treatment processes and their impact on mutagen formation potential after chlorination. Water Research 138. Elsevier Ltd. 67-76.

MHLWJ (Ministry of Health, Labor and Welfare, Government of Japan). 2003. Analytical method for the guideline values in the drinking water quality standard. Read on 16/6/2012. http://wwwhourei.mhlw.go.jp/hourei/doc/tsuchi/151014-h.pdf.

Morgan, D. P. 1989. Recognition and management of Pesticide Poisonings. U.S. Environmental Protection Agency. University of Iowa. College of Medicine.

Narita, K., Matsui, Y., Iwao, K., Kamata, M., Matsushita, T. & Shirasaki, N. 2013. Selecting pesticides for inclusion in drinking water quality guidelines on the basis of detection probability and ranking. Environment International. Volume 63. 114-120.

Narushima, T., Sato, T., Goto, Y. & Takahashi, Y. 2014. Pesticides in River and Tap Water in a Rice Production Area of Niigata, Japan. Water, Air and Soil Pollution. Volume 225, Issue 12. 225:2229.

Ohmori, K., Fujita, Y., Huang, Y., Matsushita, T., Matsui, Y. & Shirasaki, N. 2018. Toxicological contributions of transformation products derived from organophosphorus pesticides during chlorination. IWA World Water Congress & Exhibition.

Ohno, K., Minami, T., Matsui, Y. & Magara, Y. 2008. Effects of chlorine on organophosphorus pesticides adsorbed on activated carbon: Desorption and oxon formation. Water Research. Volume 42. Issues 6-7. 1753-1759.

PubChem. 2020. Demeton-S. Read on 15/3/2020. https://pubchem.ncbi.nlm.nih.gov/compound/Demeton-S

PubChem. 2020. Oxydisulfoton. Read on 15/3/2020. https://pubchem.ncbi.nlm.nih.gov/compound/Oxydisulfoton

Roberts, J. R. & Reigart, J. R. 2013. Recognition and Management of Pesticide Poisonings. 6th edition. EPA (United States Environmental Protection Agency).

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P. Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R. & Thukral, A. K. 2019. Worldwide pesticide usage and its impacts on ecosystem. SN Applied Sciences. Volume 1446.

Sharma, S. & Anand, N. 1997. Approaches to Design and Synthesis of Antiparasitic Drugs. Pharmacochemistry Library. Volume 25. 124-147

Tahara, M., Kubota, R., Nakazawa, H., Tokunaga, H. & Nishimura, T. 2008. The behavior and cholinesterase inhibitory activity of fenthion and its products by light and chlorination. Journal of Water Supply: Research and Technology – AQUA. IWA Publishing. 143-151.

Timperley, C. 2014. Best Synthetic Methods: Organophosphorus (V) Chemistry. 1st edition. Academic Press Publisher.

Usui, K., Hayashizaki, Y., Minagawa, T., Hashiyada, M., Nakano, A. & Funayama, M. 2012. Rapid determination of disulfoton and its oxidative metabolites in human whole blood and urine using QuEChERS extraction andliquid chromatography-tandem mass spectrometry. Legal Medicine 14. Elsevier Ireland Ltd. 309-316.

Wakayama, H. 2005. Revision of Drinking Water Quality Standards in Japan. Issue 264. 74-88.

Wakayama, H. 2007. Revision of Drinking Water Quality Standards in Japan. Water Supply Div., Health Service Bureau. Read on 26/5/2019. http://www.nilim.go.jp/lab/bcg/siryou/tnn/tnn0264pdf/ks0264011.pdf

Wu, J. & L, D. A. 2003. Abiotic transformation of chlorpyrifos to chlorpyrifos oxon in chlorinated water. Environmental Toxicology and Chemistry. Volume 22(2). 261-264.

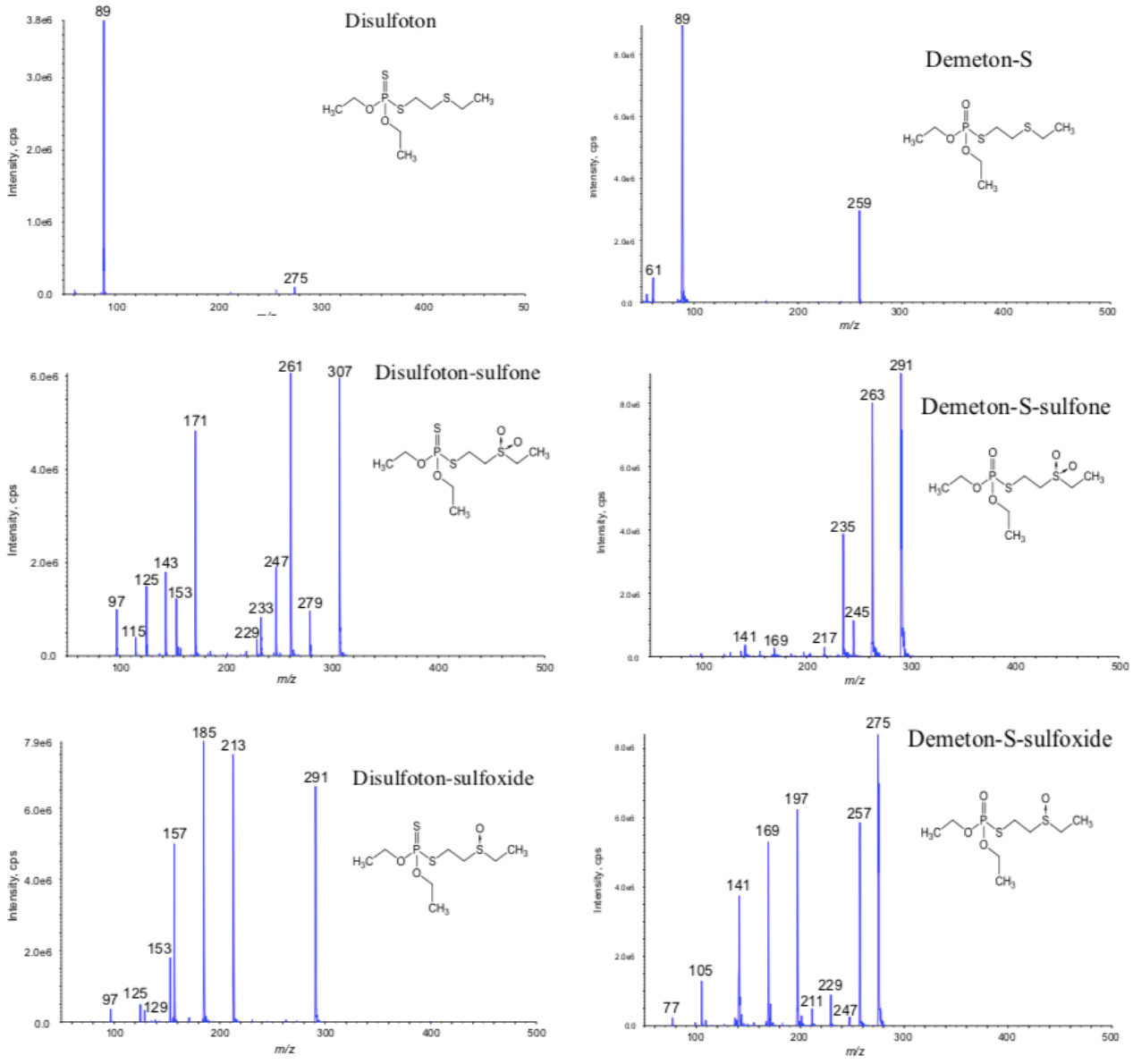
APPENDICES

Appendix 1. LC/MS setting

	Pesticides	Choline
Ionization conditions	HESI	HESI
Spray voltage (V)	3000 V	3000 V
Flow rate	0.2 mL/min	0.2 mL/min
Polarity	Positive	Positive
Sheath gas	50 units	Pressure: 50mTorr
Aux gas	50 units	Pressure: 30mTorr
Sweep gas	50 units	
Capillary Temperature (°C)	220°C	300°C
Aux gas heater temperature (°C)	450°C	
S-lens RF level	78	60

Appendix 2. Disulfoton and its metabolites ion spectra (Usui 2012)

Product ion spectra was obtained by MRM-EPI mode (500 ng/mL each). Collision energy, entrance and declustering potential were set at 10 V.



Appendix 3. Disulfoton solid phase extraction (Morimoto, 2012)

There are 2 types of cartridges used in order to proceed the experiment: PS2 (to capture hydrophilic compound such as pesticide) and AC2 (hydrophilic).

I. Elution pump conditioning:

1) Purge: dry inside the machine with Nitrogen gas for 5 minutes and then wash with Acetone 10ml/min for 1 minute 30 second
2) Cleaning cartridge: connect cartridge with connecting kits (male luer to male luer coupler) in the direction below, flow rate of Acetone is 1ml/min for 5 minutes and then use hand syringe to inject MiiliQ water in a reverse flow.

II. Concentrator (capture the compound)

1) Purge: wash with methanol 20ml/min for 3 minutes
2) Capture: change mobile phase to sample, with flow rate 1ml/min, the flow direction and cartridge position is shown in the figure

III. Elution (dissolve captured compound in organic solvent)

Connect cartridge to the elution pump following below figure, flow rate is 1ml/min. Remember to indicate the dilute ratio of the sample after.

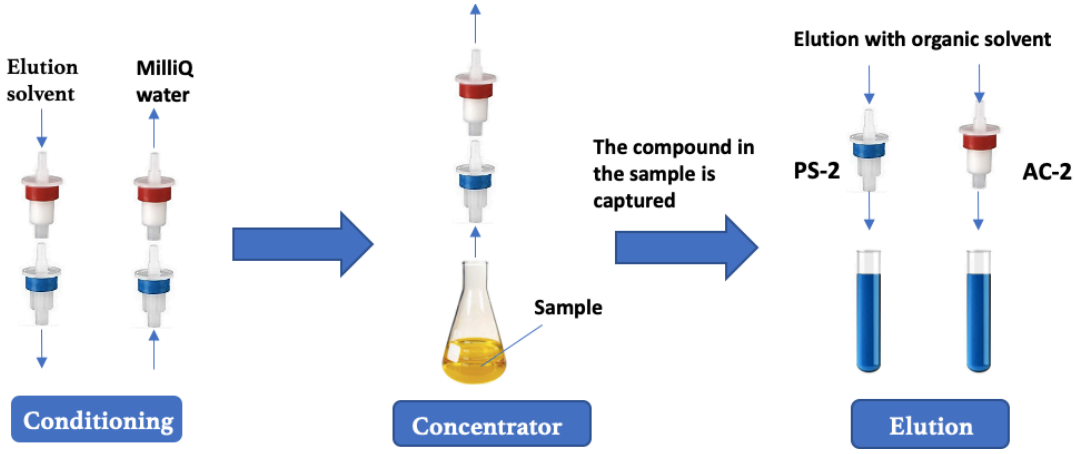


FIGURE. Solid phase extraction demonstration

IV. Dehydration (when the concentration is too small)

Sodium sulfate is used. Addition of the chemical powder is added to the test tube, then thoroughly dissolve it by vortex stirring until smooth. Wait until the particle settle down and take only the surface liquid.

Appendix 4. GC/MS setting (Morimoto, 2012)

Capillary column	J&W DB50-ms (30m x 0.25mm, 0.25μm)
Colum temperature	50°C (2min) →10°C/min→ 170(3min) →3°C/min →200°C(0min) →10°C/min → 300°C(10min)
Ion source temperature	300°C
Injector temperature	220°C
IF temperature	250°C
Injection mode	Spitless
Carrier gas	He, 20mL/min
Sample injection volume	1μL

Five-mixed internal standard solution (100 μg / mL nonane solution each):

- Naphalene-d8
- Phenanthrene-d10
- Anthracene-d10
- 9-Bromoanthracene
- Chrysene-d12

Appendix 5. SCAN results

POSITIVE SCAN

Result on 23/6/2019

m/z	10m	30m	1h	3h	6h	12h	1d	3d	5d	7d
236.1122	34775280			23031460	23484853		20548057	24021923		1593963
263.0164			5937983	5535812						
275.0529	21062147									
291.0475	9.13E+08	9.43E+08	9.62E+08	8.97E+08	8.29E+08	6.89E+08	4.92E+08	1.46E+08	2234859	772657.7
308.0739				1.27E+08	1.1E+08	71162116	53909614	16254988		
329.0031	6640165	6785226	6814303	5923196	4266509					
342.9774		13246342	26329700	75853951	1.38E+08	2.1E+08	2.73E+08	2.58E+08	5735584	5499945

Result on 4/7/2019

m/z	10m	30m	1h	3h	6h	12h	1d	3d	5d	7d
236.1122										
263.0164										
275.0529										
291.0475	2.62E+08	2.4E+08	2.33E+08	2.14E+08	1.83E+08	1.55E+08	1.08E+08	26307405	8563093	3655411
308.0739	76344596	76885106	75392478	68908324	60244783	49116932	33811675	9279285	3003429	1349880
329.0031	3618115	3618115	6814303	5923196	4266509					
342.9774	2143552	5360827	10020150	28715124	52815468	68864987	84440842	83791242	66792494	66017364
300.9038	82852771	65717901	57127971	54674553	52698831	48622993	46036616	43558027	44179369	43657321

NEGATIVE SCAN

Result on 4/7/2019

m/z	10m	30m	1h	3h	6h	12h	1d	3d	5d	7d
78.958			76758359	72015219	73895860	72661357	75238772	69196199	72928737	
112.9848		1.4E+09								
190.9284		1.52E+08								
194.9461			2.19E+08	1.96E+08	1.98E+08	1.83E+08	1.81E+08			1.91E+08
225.0613		20651930								
226.9272			19875005	17434159	16903740	17862006	14876113			17997624
390.9008				2568149			16391579			