A NEW DERIVATIZING METHOD FOR THE SIMULTANOUS DETERMINATION OF 3-MONOCHLOROPROPANEDIOL AND 1,3-DICHLOROPROPANOL IN HEAT-PROCESSED FOOD SAMPLES

LEE BAI QIN

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

A NEW DERIVATIZING METHOD FOR THE SIMULTANOUS DETERMINATION OF 3-MONOCHLOROPROPANEDIOL AND 1,3-DICHLOROPROPANOL IN HEAT-PROCESSED FOOD SAMPLES

LEE BAI QIN

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **LEE BAI QIN**

Registration/Matric No: **SHC 130041**

Name of Degree: **DOCTOR OF PHILOSOPHY (EXCEPT MATHEMATICS & SCIENCE PHILOSOPHY)**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): **A New Derivatizing Method For The Simultaneous Determination Of 3-Monochloropropanediol And 1,3-Dichloropropanol In Heat-Processed Food Samples**

Field of Study: **ANALYTICAL CHEMISTRY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:
Name:
Designation:

Witness's Signature Date:
Name:
Designation:

ABSTRACT

3-Monochloro-1,2-propanediol (3-MCPD) and 1,3-dichloropropanol (1,3-DCP) are heat-produced contaminants that form during the acid hydrolysis of vegetable protein (acid-HVP), which is a process employed in the production of artificial soy sauces. Available laboratory study reports have shown that 3-MCPD is carcinogenic and toxic to the kidneys and reproductive organs, while 1,3-DCP is genotoxic, hepatotoxic and carcinogenic. On account of the carcinogenic properties reported, 3-MCPD and 1,3-DCP are listed as Proposition 65 (Prop. 65) and group 2B carcinogens. The Association of Official Analytical Chemists (AOAC) method of 3-MCPD detection requires high-temperature heating (60°C) and a costly derivatizing agent (61.10 USD mL^{-1}), heptafluorobutyrylimidazole (HFBI). The purpose of this study is to develop an alternative to the current methods of detecting and quantifying 3-MCPD and 1,3-DCP, especially in complex food matrices. Therefore, the focus of this study is on developing a cost and time-efficient procedure to detect 3-MCPD and 1,3-DCP simultaneously at room temperature (25°C). Silylation derivatizing agent Hexamethyldisilazane (HMDS) coupled with the catalyst Trimethylsilyl trifluoromethanesulfonate (TMSOTf) are used to derivatize 3-MCPD and 1,3-DCP. Box-Behnken simulation and point-to-point optimization are applied to optimize the developed derivatization process. An actual food sample analysis based on the Student's T-test analysis indicates there is no significant difference between the HFBI and HMDS derivatization methods used for 3-MCPD and 1,3-DCP quantification. The newly established derivatization method is applied to identify and quantify 3-MCPD and 1,3-DCP found in commercially available soy sauces, thick sauces and flavored seasonings sold in Malaysia. The determined concentrations are compared with the Malaysian Adult Nutrition Survey (MANS) to

investigate Malaysians' daily exposure to 3-MCPD and 1,3-DCP. In conclusion, this study introduces a cost-efficient derivatizing agent to quantify 3-MCPD and 1,3-DCP, and to establish a database of Malaysians' daily exposure to 3-MCPD and 1,3-DCP with a risk assessment.

ABSTRAK

3-Monochloro-1,2-propanediol (3-MCPD) dan 1,3-dichloropropanol (1,3-DCP) adalah bahan cemar yang terbentuk semasa hidrolisis asid protein sayur-sayuran (asid-HVP). Acid-HVP ialah proses untuk menghasilkan kicap soya buatan. Terdapat laporan makmal yang menunjukkan bahawa 3-MCPD adalah karsinogenik dan toksik kepada buah pinggang dan organ-organ pembiakan, manakala 1,3-DCP pula dilaporkan sebagai genotoksik, hepatoksik dan karsinogenik. Oleh sebab sifat-sifat karsinogenik yang dilaporkan, maka 3-MCPD dan 1,3-DCP telah disenaraikan sebagai Usul 65 (Prop. 65) dan kumpulan 2B karsinogen. Kaedah penentuan 3-MCPD oleh *Persatuan Official Analytical Chemists* (AOAC) memerlukan suhu yang tinggi (60°C) dan agen terbitan yang berkos tinggi (61.10 USD mL^{-1}), iaitu *heptafluorobutyrylimidazole* (HFBI). Tujuan kajian ini adalah untuk membangunkan satu pendekatan alternatif kepada pendekatan yang sedia ada dalam menentukan dan mengesan kewujudan 3-MCPD dan 1,3-DCP dalam sampel makanan yang kompleks. Oleh itu, fokus kajian ini adalah untuk membangunkan prosedur melibatkan kos dan kecakapan masa untuk mengesan 3-MCPD dan 1,3-DCP pada suhu bilik (25°C). Agen *silylation*, *Hexamethyldisilazane* (HMDS) bersama dengan pemangkin *trimethylsilyl trifluoromethanesulfonate* (TMSOTf) telah digunakan sebagai terbitan 3-MCPD dan 1,3-DCP. Simulasi *Box-Behkan* dan strategik pengoptimuman titik ke titik telah digunakan untuk mengoptimumkan proses terbitan. Dalam analisis sampel makanan, berdasarkan analisis *Student T-test*, penyelidik mendapati bahawa tidak ada perbezaan yang signifikan di antara terbitan HFBI dan HMDS bagi 3-MCPD dan 1,3-DCP. Dengan kaedah terbitan yang telah dibangunkan, penentuan dan kuatiti 3-MCPD dan 1,3-DCP telah dilakukan terhadap sampel-sampel makanan seperti kicap soya, kicap pekat dan serbuk perasa yang ada di pasaran Malaysia. Kepekatan yang

dilaporkan telah dibandingkan dengan survey Nutrisi Dewasa Malaysia (MANS) untuk menyiasat pendedahan harian rakyat Malaysia kepada 3-MCPD and 1,3-DCP. Sebagai kesimpulannya, kajian ini telah memperkenalkan penggunaan agen terbitan yang cekap dari segi masa dan kos untuk mementukan kuantiti 3-MCPD dan 1,3-DCP serta mewujudkan satu pangkalan data mengenai pendedahan harian terhadap risiko 3-MCPD dan 1,3-DCP kepada rakyat Malaysia.

ACKNOWLEDGMENTS

Completing a Ph.D. is not a road walked alone. I am grateful to numerous people who have assisted and accompanied me to the end of this journey. First, I would like to express my greatest gratitude to my main supervisor, Dr. Khor Sook Mei from Department of Chemistry, Faculty of Science. Thank you for all the advice given to improve my research, the motivation that kept me going to complete this study, and the financial support to initiate my university study. Next I would like to thank Associate Professor Dr. Che Wan Jasimah Bt Wan Mohamed Radzi from Department of Science and Technology Studies, Faculty of Science for the suggestions and advice provided throughout the study. Special thanks to all the members of University of Malaya High Impact Research Center (UM HIR) for the help to facilitate and maintain the instruments required to collect my data. I would like to take this opportunity to express my gratitude to the University of Malaya Fellowship Scheme for the scholarship in support of my study. In addition, I would like to thank University of Malaya for the Postgraduate Research Grant (PG030-2014A), University of Malay Research Grant (RP012C-14SUS) and Fundamental Research Grant Scheme (FP014-2013A) from Ministry of Higher Education Malaysia for assisting with the research needs of my project.

I am most grateful to my parents, Lee Thong Choy and Fong Soot Mooi for always being there to cheer me up. To my wife, Kee Yang Ling, thank you for always being there for me. Finally, special thanks to my friends and lab colleagues for the support, advice and motivation for me to reach this significant milestone in my life.

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
µ	:	Micro
1,3-DCP	:	1,3-Dichloropropanol
1,4-BD	:	1,4-Butanediol
1,5-PD	:	1,5-Pentadiol
2,3-DCP	:	2,3-Dichloropropane-1-ol
2-MCPD	:	2-Chloro-1,3-propanediol
3-MCPD	:	3-Monochloro-1,2-propandiol
Acid-HVP	:	Acid Hydrolyzed Vegetable Proteins
AOAC	:	Association of Official Analytical Chemists
BSTFA	:	Bis(trimethylsiyl)trifluoroacetamide
cAMP	:	Cyclic Adenosine Monophosphate
CE-ECD	:	Capillary Electrophoresis- Electrochemical Detection
CPN	:	Chronic Progressive Nephropathy
EB	:	Enumeration Block
EDI	:	Estimated Dietary Intake
EI	:	Electron Ionization
EU	:	European Union
FSANZ	:	Food Standard Australia New Zealand
g	:	Gram
GC	:	Gas Chromatography
GC-ECD	:	Gas Chromatography-Electrolytic Conductivity Detection

GC-MS	:	Gas Chromatography-Mass Spectrometry
H_2SO_4	:	Sulfuric Acid
HCl	:	Hydrochloric Acid
HFBA	:	Heptafluorobutyric Anhydride
HFBI	:	Heptafluorobutyrylimidazole
HHD	:	Halohydrin Dehalogenase enzyme
HMDS	:	Hexamethyldisiloxane
HPLC	:	High Performance Liquid Chromatography
HOAc	:	Acetic Acid
IARC	:	International Agency for Research on Cancer
IKU	:	Institute of Public Health
JECFA	:	Joint FAO/WHO Expert Committee on Food Additives
L	:	Liter
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
LQ	:	Living Quarters
MANS	:	Malaysian Adult Nutrition Survey
MIP	:	Molecular Imprinted Polymer
MSTFA	:	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
Na_2CO_3	:	Sodium Carbonate
NaOH	:	Sodium Hydroxide
NBS	:	N-Bromosuccinimide
PAH	:	Polycyclic Aromatic Hydrocarbons
PBA	:	Phenylboronic Acid
PKA	:	Protein Kinase A

PMTDI	:	Provisional Maximum Tolerable Dietary Intake
Prop. 65	:	Proposition 65
RSD	:	Relative Standard Deviation
S/N	:	Signal-to-Noise Ratio
SD rats	:	Sprague Dawley rats
SIM	:	Selected Ion Monitoring
TCCA	:	Trichloroisocyanuric Acid
TMS	:	Trimethylsilyl
TMSOTf	:	Trimethylsilyl Trifluoromethanesulfonate

CHAPTER 1: INTRODUCTION

Food safety has become a major concern worldwide in the past two decades. Consumers are more aware of the quality of food they consume and continuously seek better and safer food. Over the last decades, ignoring the aspect of food safety has led to foodborne disease outbreaks in society, resulting in chronic illness and sometimes death. Food producers and manufacturers have learned from outbreaks and are attempting prevention through production processes to lower the risk of food contamination. However, there are still producers and manufacturers out there who care less about food safety and more about profit margins and meeting market demand. With these negative objectives, alternatives are being researched and developed to replace traditional methods of food preparation.

This research focuses on process-induced intoxicants present in food. Briefly, such toxic compounds are formed post-harvest rather than pre-harvest, especially during food processing. During processing, certain chemicals or adulterants may form that are harmful to human health. An example is the formation of polycyclic aromatic hydrocarbons (PAH) during barbequing/smoking of meat (Chen & Lin, 1997). Among several process-induced food intoxicants, this research focuses on chloropropanols, which are chlorinated propanols that can be either mono-ol or diol. The chloropropanols studied in this research are 3-monochloro-1,2-propandiol(3-MCPD) and 1,3-dichloropropanol (1,3-DCP). The two mentioned chemicals are found abundantly in acid hydrolyzed vegetable proteins (acid-HVP) in processes applied to produce soy sauce artificially. Normal bacterial fermentation of soy sauce takes up to 3 months to complete, but with acid-HVP it is possible to produce artificial soy sauce in as little as 3

days. Acid-HVP can greatly increase soy sauce production, resulting in higher profits and the ability to meet market demand.

Industrially, 3-MCPD is a chemical used to reduce the freezing point of dynamite or as a dye intermediate in the coloring industry (NJDHSS, 1999). The United States Environmental Protection Agency also listed 3-MCPD as rodenticide under the term "alpha-chlorohydrin" (US EPA, 2013). Meanwhile, 1,3-DCP is a fumigant and has an important role in organic synthesis (OEHHA, 2005).

According to animal toxicology reports, 3-MCPD is reportedly harmful to kidneys and reproductive systems at medium and high-concentration exposure. In some cases, prolonged exposure to 3-MCPD has been reported to cause hyperplasia and tumors in the kidneys and reproductive organs, while 1,3-DCP is reported to have a carcinogenic effect on the kidneys, oral epithelium and liver. The toxicity of 1,3-DCP is generally higher than 3-MCPD. With the available toxicology reports, the International Agency for Research on Cancer (IARC) has classified 3-MCPD and 1,3-DCP as Group 2B carcinogens (possibly carcinogenic to humans) and OEHHA listed both toxicants under Proposition 65 (Prop. 65). With the available toxicology reports and classifications by the IARC and OEHHA, governments have established maximum tolerable limits for 3-MCPD in their respective countries. Maximum tolerable limits can generally be grouped into 3 concentrations: 1.00 mg kg^{-1}, 0.20 mg kg^{-1} and 0.02 mg kg^{-1}.

In general, methods of detecting 3-MCPD are divided based on the type of derivatizing agent used. Derivatization is a process of rendering polar chloropropanols to a neutral state, thus enabling their separation in a neutral gas chromatography column. Due to the tedious derivatizing agent sample preparation, high-temperature

treatment, high cost of operation and harsh storage environments, not all chloropropanol-contaminated foods on the market are monitored. To meet the demand of high-efficiency analysis, better and more efficient derivatizing agents are required.

Despite the increasing research on different types of derivatizing agent combinations with various catalysts, most combinations still require high temperature, long reaction time and high operation costs. To resolve matters associated with chloropropanol detection, this Ph.D. project is aimed at developing a derivatizing method that facilitates derivatization at room temperature and with a short derivatization period, and which can derivatize most compounds from chloropropanols and is inexpensive to measure chloropropanols in food samples. The objectives are:

i. To develop a combination comprising a derivatizing agent that is inexpensive and can derivatize chloropropanols extracted from various food sample types (solid, paste and liquid) and chromatography whereby the derivatized 3-MCPD and 1,3-DCP peaks are sharp and well-separated from background noise.
ii. To compare the efficiency and accuracy of the developed method with the standard AOAC method, and carry out statistical analysis testing to find any significant difference between the two derivatizing agents.
iii. To collect and quantify 3-MCPD and 1,3-DCP from commercially available soy sauces, dark soy sauces and chicken flavoring cubes in Malaysia according to the Malaysian Food Act's maximum tolerable limit of 0.02 mg kg^{-1}.
iv. To apply the 3-MCPD and 1,3-DCP quantified from objective (iii) to soy sauce consumption of Malaysians to determine their daily exposure to 3-MCPD and 1,3-DCP with a risk assessment.

In order to achieve the aforementioned objectives for this project, a derivatizing agent and catalyst combination is required, otherwise the derivatizing agent alone would be rendered inactive before the analysis. This also eliminates the need for harsh storage conditions, such as low temperature and dry storage environment for the derivatizing agents, which are moisture-sensitive. The developed combination must also be able to derivatize all the 3-MCPD and 1,3-DCP extracted from the various food sample types. Ideally, the developed derivatizing agent combination should be able to derivatize the hydroxyl group present in chloropropanols effectively.

The proposed derivatizing agent and catalyst combinations are hexamethyldisilazane (HMDS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf). HMDS is a weak silylating agent that replaces the hydroxyl group in 3-MCPD and 1,3-DCP with TMS derivatives, resulting in the final 3-MCPD-TMS and 1,3-DCP-TMS products. However, due to the low silylation activity of HMDS, the catalyst is required to "activate" the silylation agent. At present, several catalysts appear suitable for HMDS: sulfonic acid-functionalized nanoporous silica (Zareyee & Karimi, 2007), trichloroisocyanuric acid (TCCA) (Khazaei, *et al*., 2007), $InBr_3$ (Yadav *et al*., 2006), zirconyl triflate (Moghadam *et al*., 2008), K-10 montmorillonite (Zhang *et al*., 2006), H-β zeolite (Tillu, 2004), silica-supported perchloric acid (Shaterian *et al*., 2007), barbituric acid (Khazaei *et al*., 2007), iodine (Karimi & Golshani, 2000), $MgBr_2$ (Mojtahedi *et al*., 2006), N-bromosuccinimide (NBS) (Shaterian *et al*., 2008) and iron(III) trifluoroacetate (Firouzabadi *et al*., 2008). However, using the aforementioned catalysts yields harmful acidic by-products, and involves long derivatization periods, high-temperature treatment and large volumes of chemicals or toxic metal catalysts (Lee & Khor, 2015). TMSOTf is a very strong Lewis acid, whereby the compound readily reacts and increases the

reactivity of HMDS. The advantages of HMDS are that only a very small amount is required and no catalyst recovery procedure is necessary, as it can be injected together into the GC. This increases the developed method sensitivity since there is no loss of analytes during catalyst recovery.

In order to survey the presence of 3-MCPD and 1,3-DCP on the Malaysian market, a number of samples were collected from the Selangor and Kuala Lumpur commercial market and quantified with the developed methodology. This survey will offer insight on 3-MCPD and 1,3-DCP contamination in Malaysia. With the quantified data applied to the average soy sauce consumption, Malaysians' exposure to 3-MCPD and 1,3-DCP can be estimated. Data on daily soy sauce consumption and average body weight of the Malaysian population was obtained from the Institute of Public Health, Ministry of Health Malaysia. The data was extracted from the Malaysian Adult Nutritional Survey (MANS), where soy sauce consumption and average body weight are segregated by gender, weight, religion, income and education background. This data will provide insight on the exposure to 3-MCPD and 1,3-DCP according to different population groups. With the estimated dietary intake of 3-MCPD and 1,3-DCP, a risk assessment of Malaysians to 3-MCPD and 1,3-DCP can be done and evaluated.

CHAPTER 2: LITERATURE REVIEW

2.1 3-Monochloropropane-1,2-diol and 1,3-Dichloropropan-2-ol

3-MCPD and 1,3-DCP are part of the chloropropanol group and consist of 5 molecules that contain hydroxyl groups and chloride ions (**Figure 2.1**): 3-chloropropane-1,2-diol (3-MCPD), 2-chloro-1,3-diol (2-MCPD), 2,3-dichloropropane-1-ol (2,3-DCP), 1,3-dichloropropane-2-ol (1,3-DCP), 3-chloropropan-1-ol. 3-MCPD contains 2 hydroxyl groups and 1 chloride ion, which make it a very polar compound that is soluble in polar solvents such as water, alcohol, diethyl ether and acetone. 3-MCPD is in viscous liquid form and colorless but tends to turn to a yellow straw color (IARC, 2013). 3-MCPD has a molar mass of 110.54 g mol^{-1} and density of 1.32 g mL^{-1}. Industrially, 3-MCPD is utilized to lower the freezing point of dynamite, can serve as a dye intermediate, is used to dissolve cellulose acetate and is a rodent chemosterilant (NJDHSS, 1999). In the U.S. Environmental Protection Agency database 3-MCPD is known as alpha-chlorohydrin and is listed as a rodenticide (US EPA, 2013). In the food industry, 3-MCPD is a byproduct of acid-HVP, a method of producing soy sauce artificially. 3-MCPD is produced in the presence of chloride ions and lipids (**Figure 2.2**). Toxicology reports have shown that 3-MCPD in high and medium concentrations can cause kidney and reproductive organ failure. After being fed to rat models in high doses for prolonged periods, 3-MCPD was reported to cause hyperplasia and tumors in the kidneys and reproductive organs. Due to the toxic nature of 3-MCPD, it is categorized as a group 2B carcinogen (IARC, 2016) and listed as a proposition 65 (Prop. 65) compound (OEHHA, 2010). These listings characterize 3-MCPD as causing cancer, birth defects and other reproductive organ harms.

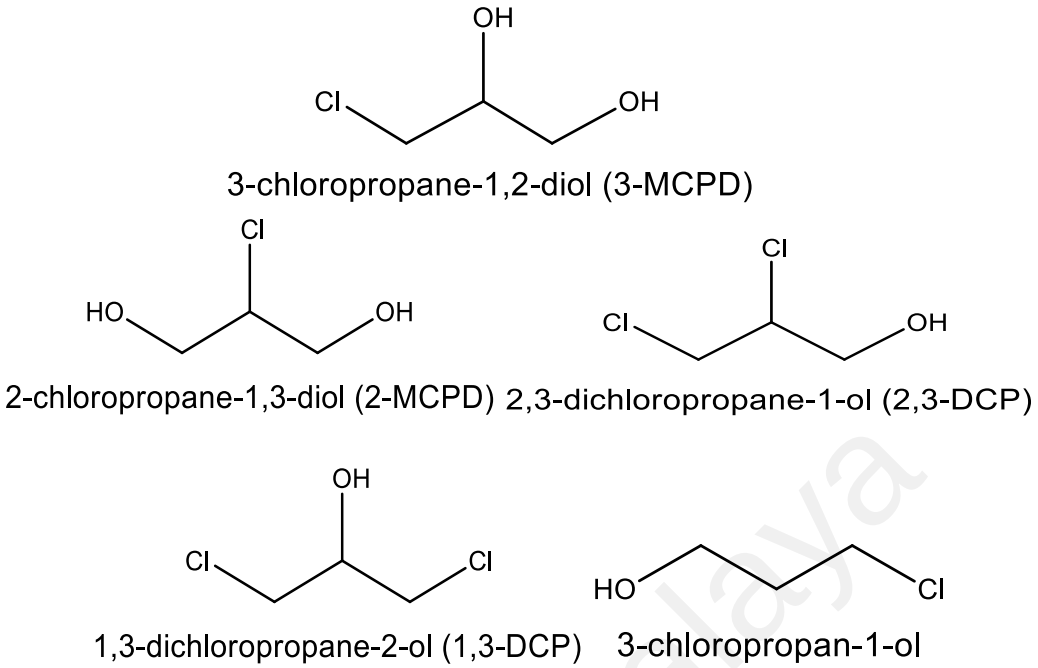

Figure 2.1: Chloropropanols.

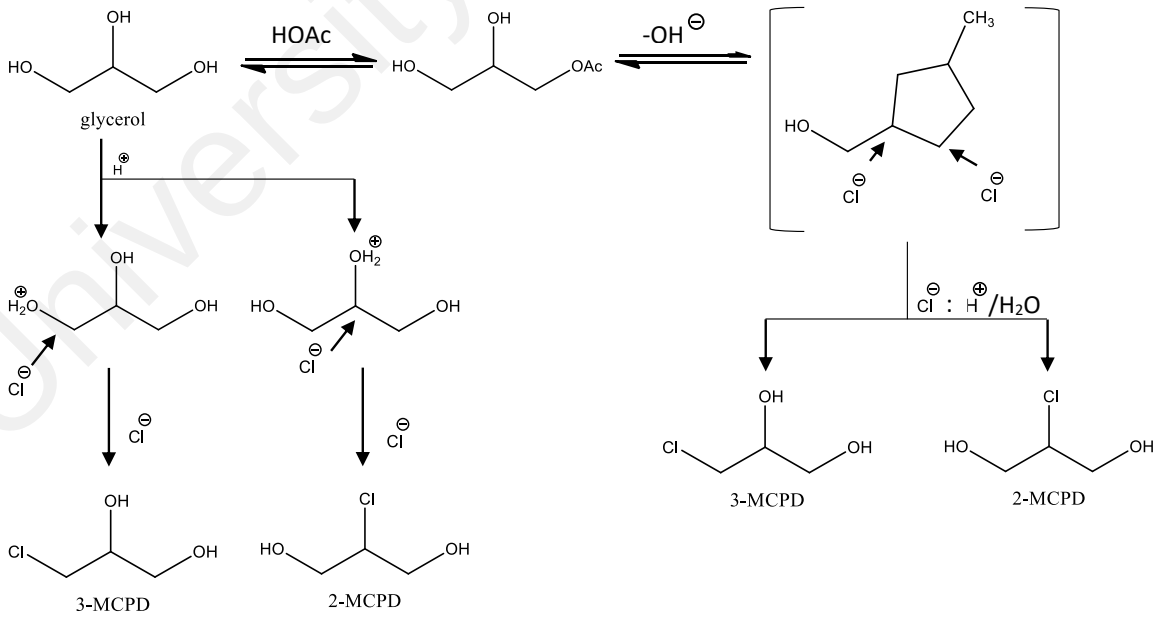

Figure 2.2: Formation of 3-MCPD and 2-MCPD from glycerol (Collier *et al*., 1991)**.**

1,3-DCP has 2 chloride ions and a hydroxyl group, and it is also a polar compound that can be dissolved in polar solvents. 1,3-DCP has a molecular mass of 128.99 g mol^{-1} and density of 1.35 g mL^{-1} at room temperature (25°C). 1,3-DCP is utilized in the synthesis of polymers, fumigants, and synthetic glycerol and a dye fixative in detergent (ILS, 2005; OEHHA, 2005). In acid-HVP, 1,3-DCP is generally found in lower concentrations than 3-MCPD (EU, 2004), because 1,3-DCP is formed in the presence of 3-MCPD and acetic acid (HOAc) (Collier *et al*., 1991). The ratio of 1,3-DCP to 3-MCPD is between 1:2 and 1:3630. Laboratory animal tests have indicated that 1,3-DCP has carcinogenic effects on the liver, kidneys, oral epithelium and tongue, and thyroid glands at intermediate and high doses (JECFA, 2002). Due to the genotoxicity, hepatotoxicity and cancer-inducing nature of 1,3-DCP, it is categorized under group 2B (IARC, 2016) and Prop. 65 (OEHHA, 2005).

2.2 Soy Sauce

There are currently 3 types of soy sauce available on the market: naturally fermented, acid-HVP and mixed, where naturally fermented soy sauce is mixed with acid-HVP soy sauce. 3-MCPD and 1,3-DCP are only detectable in acid-HVP soy sauce and acid-HVP soy sauce mixed natural soy sauce. In order to differentiate the various types of soy sauce, Japan has provided definitions and nomenclatures for different types of naturally fermented soy sauce (**Table 2.1**). Soy sauce is a clear liquid seasoning made by fermenting and maturing moromi, which is prepared by adding salt water or kiage (raw soy sauce) to shoyu koji with steamed rice followed by koji mold. These sauces include those made with the auxiliary use of enzymes, except proteolytic enzymes, such as cellulose in the manufacturing process, and are hereinafter referred to as "Soy sauce made by regular fermenting method." Another means involves adding shoyu koji, a

cultured mold, to soy beans or soy beans with grains, such as wheat, rice or wheat gluten prepared by steaming and/or another method.

The other soy sauce definition outlined by the Japanese government is a clear liquid seasoning made by fermenting and maturing moromi or kiage with added vegetable protein hydrolyzed by acid (vegetable protein of soy beans, etc. hydrolyzed by acid - hereafter referred to as such), or vegetable protein hydrolyzed by enzymes (vegetable protein of soy beans, etc. hydrolyzed by proteolytic enzymes - hereafter referred to as such), or vegetable protein hydrolyzed by fermenting (wheat gluten hydrolyzed by fermenting - hereafter referred to as such). These types will be referred to as "Soy sauce made by mixed and semi-fermenting method" in this study.

Table 2.1: Varieties, compositions and production of Japanese Shoyu according to the Japanese Agricultural Standard (JAS) (Leviton, 1980).

English name	Japanese name	NaCl % (W/V)	Total nitrogen % (W/V)	Reducing sugar % (W/V)	Alcohol % (V/V)	pH	Color
Regular shoyu	Koikuchi shoyu	1.55	3.8	3.8	2.2	4.6	Deep brown
Ligh-colored shoyu	Usukyuchi shoyu	1.17	5.5	5.5	0.6	4.8	Light brown
Tamari shoyu	Tamari shoyu	2.55	5.3	5.3	0.1	4.8	Dark brown
Clear shoyu	Shiri shoyu	0.50	20.2	20.2	Trace	4.6	Yellow to tan
Rich shoyu	Saishikomi shoyu	2.39	7.5	7.5	Trace	4.8	Dark brown

2.3 Natural Soy Sauce Production

Traditionally, soy sauce is produced through the bacterial fermentation of soy beans, wheat grain, water, and salt. The three major phases in soy sauce production through fermentation are: koji production, brine fermentation and refining (**Figure 2.3**) (Luh, 1995).

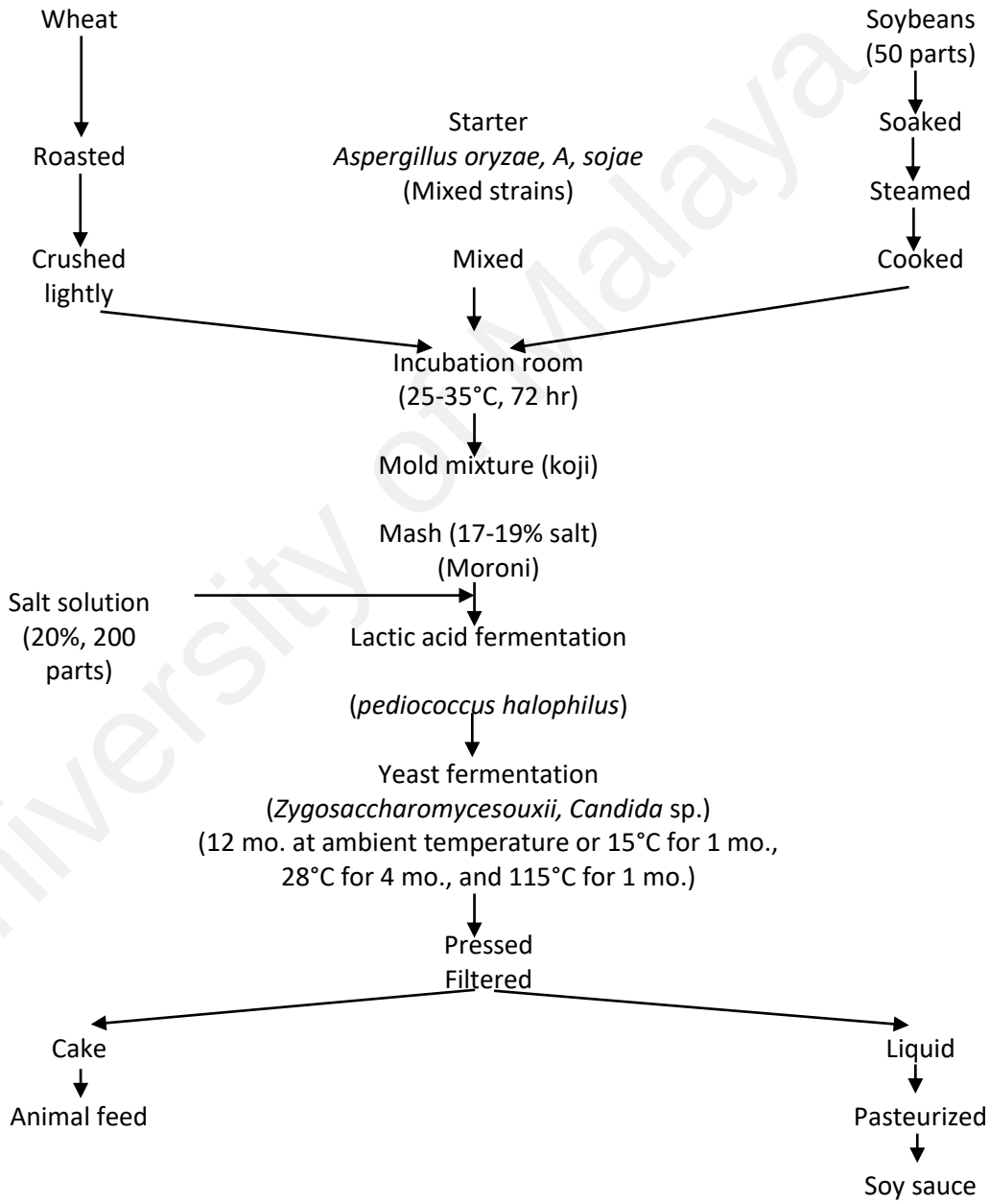

Figure 2.3: Traditional fermentation in soy sauce production (Luh, 1995).

Koji fermentation is the first production stage, where soy beans and roasted wheat grains are soaked in water and cooked to increase the moisture content. The ratios of soy beans and roasted wheat grains differ in various types of soy sauce. *Aspergillus oryza* or *Aspergillus sojae* is subsequently added to the mixture to initiate the fermentation process. It is important to select only the best bacteria to produce the finest quality soy sauce and increase the fermentation efficiency. Incubation occurs at 25˚C. The mixture is stirred continuously to remove the metabolic heat of the fermentation bacteria and regulate the fermentation temperature. Koji incubation lasts for 72 hours and results in a greenish yellow mass.

Following Koji fermentation is brine fermentation, where the lactic acid bacterium *Pediococcus halophiIus* and yeast species *Zygosaccharomyces rouxii* and *Candida* are added together in 20% brine. These microorganisms can tolerate a salt concentration of 20 g per 100 mL. The resultant mixture is called moromi mash. The high sodium content in moromi mash prevents the growth of microorganisms other than those which can tolerate high salt content environments.

The final step in natural soy sauce production is refinement, where the mash is pressed, filtered, pasteurized and packed. Pasteurization at 70-80°C for a few minutes ensures the inactivation of undesired enzymes and microorganisms.

2.4 Formation of 3-MCPD and 1,3-DCP in Acid-HVP Soy Sauces

The traditional production of soy sauce requires bacterial fermentation, whereby selected food-grade bacteria are added to raw mixtures consisting of soy beans, wheat gluten, and/or corn meal (**Figure 2.3**) (FAO, 2012). The bacteria will break down the proteins available, hence the aromatic flavor of soy sauce. The entire fermentation period requires approximately 4 months to complete, and such long fermentation time is the bottle neck that limits soy sauce production. Due to the high soy sauce demand on the market, especially in Asia, producers are coming up with faster and cheaper methods of producing soy sauce. Artificial soy sauces are made through the acid-HVP process. With acid-HVP soy proteins are broken down by acid at high temperature, which reduces soy sauce production time from 4 months to only 3 days. Unfortunately, due to the lack of microbial activity, artificial soy sauce has no aromatic flavor. Thus, artificial flavoring is added to increase the umami characteristic of soy sauce.

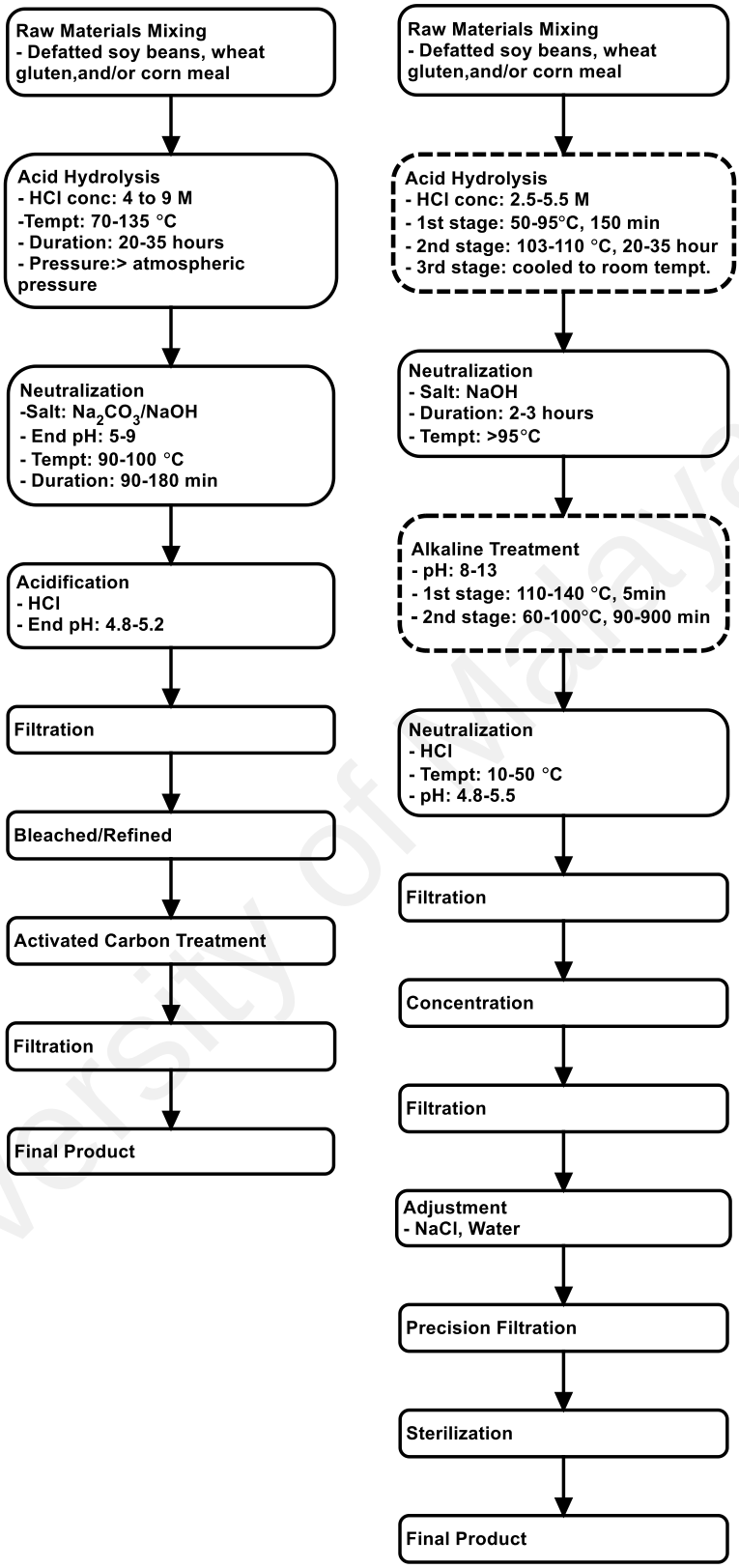

Figure 2.4: Conventional acid-HVP production (left); Dotted box - proposed modification for reducing 3-MCPD production time (right).

In acid-HVP soy sauce production, the process begins with the mixing of defatted soy beans, wheat gluten, and/or corn meal (**Figure 2.4**). Acid hydrolysis entails the next step, where hydrochloric acid (HCl) at a concentration between 4 and 9 M is added to the mixture, which is heated to 70-135°C for 20-35 hours. This is the process believed to cause the production of 3-MCPD and 1,3-DCP. A precursor for 3-MCPD production is the presence of glycerol, lecithin and other glycerides, which are likely present in soy beans and chloride ions from HCl. The mixture then undergoes neutralization with Na_2CO_3/ NaOH, when the mixture will attain a pH between 5 and 9. The production ends with filtration, refinement and activated carbon treatment. The formation of 3-MCPD during acid-HVP is found to be the highest when chloride reacts with lecithin, followed by diacylglycerols and glycerol (Velisek *et al*., 2003).

Since the procedure that promotes the formation of 3-MCPD and 1,3-DCP in acid-HVP can be identified, modification can be implemented to reduce this formation. FAO (2012) suggested 3 methods of 3-MCPD reduction during acid-HVP: careful control of the hydrolysis process, alkaline treatment after acid hydrolysis, and the substitution of HCl with H_2SO_4 for acid hydrolysis (**Figure 2.4**). Besides, the concentration of 3-MCPD can be further reduced in the final stage of soy sauce production through enzymatic removal. 3-MCPD formation is completely preventable if alkaline hydrolysis of soy proteins is selected over acid hydrolysis. The main concern is to reduce and/or remove 3-MCPD, since 1,3-DCP will only form in the presence of 3-MCPD and HOAc.

Careful hydrolysis is crucial to reduce 3-MCPD formation during acid hydrolysis, as this is the step that involves prolonged, high-temperature treatment. To reduce the formation of 3-MCPD, the concentration of HCl added to the mixture must be lowered, which will consequently reduce the concentration of extra chloride added to the

mixture. Evidently, upon acid molarity reduction, hydrolysis efficiency will diminish as well. To overcome this problem, the temperature can be increased gradually at specific holding times to optimize the hydrolysis process.

An alkaline treatment can further reduce the concentration of 3-MCPD formed during acid hydrolysis. 3-MCPD appears to be in an unstable state in alkaline pH environments (Reese, 2005), with pH higher than 6 causing the degradation of 3-MCPD. Thus, an alkaline treatment after careful acid hydrolysis of the soy bean mixture can effectively reduce the concentration formed during acid-HVP.

The last means of reducing the concentration of 3-MCPD in acid-HVP soy sauce is by enzymatic removal of 3-MCPD from the product. 3-MCPD can be removed by the enzyme halohydrin dehalogenase (HHD) extracted from *Arthrobacter sp.,* whereby 3-MCPD is converted to glycidol, and glycidol is further hydrolyzed to glycerol by epoxide hydrolase. However, according to pilot testing performed with a basic buffer at 30°C, 3-MCPD is not completely removed even after 24 hours. The technology is not mature and more research must be done to modify the HHD enzyme to effectively remove 3-MCPD on a larger industrial scale. Besides HHD extracted from *Arthrobacter sp.*, other HHD enzymes can be extracted from various bacteria to remove 3-MCPD. Bacteria that contain HHDs and their efficiency in removing 3-MCPD have been well-reviewed by You *et al*. (2013). In addition to 3-MCPD, HHDs are found to be effective in breaking down halogenated organic compounds such as 1,3-DCP. In fact, the HHD extracted from *Arthrobacter sp.* seems to be more efficient in removing 1,3-DCP than 3-MCPD.

The last alternative to prevent the formation of 3-MCPD is alkaline hydrolysis. As the terms suggests, this method involves a strong alkaline condition to hydrolyze the vegetable proteins in the soy mixture. Briefly, defatted soy beans, wheat gluten and corn meal are mixed and heated to dissolve the proteins. Alkaline minerals, such as calcium, sodium or potassium hydroxide are then added at heating temperatures between 27 and 54°C. Alkaline hydrolysis is carried out over several hours until the amino acid profile meets the requirements. However, this method is less likely to be applied in industry, as the procedure requires the cooking of soy protein, resulting in a partially racemized product (Borkenhagen, 1953). Alkaline-HVP also yields undesirable flavor characteristics and unbalanced amino acid profiles, resulting in unacceptable products. The only advantage of alkaline hydrolysis is the little or no humin formed at the end of the process (Hall, 1946).

2.5 Maximum Tolerable Limits of 3-MCPD

Due to the toxic nature of 3-MCPD, maximum tolerable limits are set in various countries for public health and global trading ease. The maximum tolerable limit is essentially categorized into 3 concentrations: 0.02 mg L^{-1}, 0.20 mg L^{-1} and 1.00 mg L^{-1} (**Table 2.2**). The lowest limit of 0.02 mg L^{-1} was set in the European Union (Europen Commission, 2001). Similar to the EU, Malaysia (*Malaysia Food Act 1983 and Regulations 1985*, 2012) and Singapore (Wong *et al*., 2006) enforce the same concentration regulation. 0.02 mg L^{-1} is also the minimum requirement for the limit of detection (LOD) and limit of quantification (LOQ) of the method developed in this study. As for Australia and New Zealand (*Australia New Zealand Food Authority Act*,

1991), the maximum tolerable limit is 0.20 mg L^{-1}. The highest maximum tolerable concentration limit is 1.00 mg L^{-1}, which is applicable to Canada (Canadian Standards, 2012) and the USA (FDA, 2008). The Provisional Maximum Tolerable Dietary Intake (PMTDI) recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is 2 µg (kg^{-1} bw) day^{-1} (JECFA, 2005). If the estimated dietary intake of 3-MCPD is below 2 µg (kg^{-1} bw) day^{-1}, the consumer will not sustain significant exposure to 3-MCPD that is harmful to health.

Regulatory control of 3-MCPD lowers the need for specific 1,3-DCP limits. This is because 1,3-DCP concentration is lower than 3-MCPD, as 1,3-DCP only forms after the formation of 3-MCPD. Nevertheless, some countries impose maximum limits for 1,3-DCP intake, for example Australia/New Zealand (0.005 mg/kg), Switzerland (0.05 mg/kg) and the USA (0.05 mg/kg).

Table 2.2: International maximum tolerable amounts of 3-MCPD in foods.

Country	Maximum Limit	Scope
Australia/New Zealand	0.20 mg kg^{-1}	Soy and oyster sauce (40% dry matter content)
Canada	1.00 mg kg^{-1}	Soy and oyster sauce
China	1.00 mg kg^{-1}	Acid-HVP seasoning
European Union (EU)	0.02 mg kg^{-1}	HVP and soy sauce
Malaysia	0.02 mg kg^{-1}	Liquid food with acid HVP
	1.00 mg kg^{-1}	Acid HVP
Singapore	0.02 mg kg^{-1}	Soy and related sauces
United States	1.00 mg kg^{-1}	Acid-HVP

2.6 Occurrence of 3-MCPD and 1,3-DCP

The occurrence of 3-MCPD and 1,3-DCP has been reported in the United Kingdom (Macarthur *et al*., 2000), Taiwan (Cheng *et al*., 2004), New Zealand (MAF, 2011), Hong Kong (Chung *et al*., 2008), the United States (Nyman *et al*., 2003), Singapore (Wong *et al*., 2006), Spain (León *et al*., 2008), Brazil (Vicente *et al*., 2011), and Belgium (Christova-Bagdassarian *et al*., 2013). In the EU, the Report of Experts participating in Scientific Cooperation Task 3.29 (EU, 2004) was published to inform on the occurrence of 3-MCPD and 1,3-DCP.

In Taiwan, Cheng *et al*. (2004) conducted a survey on 3-MCPD in soy sauce products during the 2002 fiscal year. In their study, 214 samples were collected from around Taiwan, consisting of 118 domestic and 26 imported soy sauce products. The LOD of detection method validation was 0.01 mg kg^{-1}, according to which, 87 domestic and 23 imported soy sauce products were found to contain undetectable concentrations of 3-MCPD. Meanwhile, 91 domestic soy sauce products contained 3-MCPD levels between 0.01 and 1.00 mg kg^{-1} and 10 samples contained 3-MCPD above 1.00 mg kg^{-1}, which exceeds the maximum tolerable limit set by the Taiwanese government. In contrast, only 3 imported soy sauce samples contained detectable 3-MCPD levels between 0.01 and 0.03 mg kg^{-1}.

Wong *et al*. (2006) conducted a survey on the 3-MCPD levels in soy and oyster sauces in Singapore. The reporting limit and lowest calibration point for the survey were 0.01 mg kg^{-1} and 0.05×10^{-1} mg kg^{-1}. From the total of 421 samples collected, 376 were found to contain detectable 3-MCPD amounts of 0.02 mg kg^{-1} or less, while 3 domestically produced and 42 imported samples contained 3-MCPD exceeding 0.02 mg

kg^{-1}. The highest concentrations detected were in soy and oyster sauces from Thailand and Taiwan, respectively.

3-MCPD in 55 soy sauces and related products in the United States was investigated in a case study by Nyman *et al*. (2003). The LOD for the method used was 0.05×10^{-1} mg kg^{-1}. In total, 19 samples were found to contain 3-MCPD levels over the recommended limit of 1.0 mg kg^{-1}. All soy sauce-related products manufactured locally met the maximum tolerable limits, while soy sauce products imported from the Asian region (Hong Kong, Vietnam, China, Philippines, and Thailand) did not meet the requirements. The highest quantified concentration of 3-MCPD was in soy sauce imported from Hong Kong.

Crews *et al*. (2003) conducted surveys of 3-MCPD in the United Kingdom in 2000 and 2003 to detect 3-MCPD concentrations using the AOAC method (AOAC, 2002). A total of 100 and 99 samples were collected in 2000 and 2002, respectively, from 5 areas in the UK. The detected 3-MCPD levels were compared between 2000 and 2002. In the year 2000, 32% of the collected samples contained 3-MCPD levels above 0.02 mg kg^{-1} (maximum tolerable limit in the UK), while 16 samples contained 3-MCPD above 1.00 mg kg^{-1}. The majority of samples collected contained 3-MCPD levels below 0.02 mg kg^{-1}. In 2002, samples containing more than 1.00 mg kg^{-1} 3-MCPD dropped to 8 compared to 16 samples in 2000 (50% reduction).

In Brazil, Vicente *et al*. (2011) conducted a survey on 3-MCPD and 1,3-DCP in soy sauces and similar products. The detection method employed had LOD of 0.02×10^{-1} mg kg^{-1}. Among 45 soy sauce samples collected, 7 contained detectable concentrations of 3-

MCPD, with the highest concentration being 4.405 mg kg^{-1}. The 16 soy sauce products did not contain any detectable 3-MCPD levels.

In Bulgaria, Christova-Bagdassarian *et al*. (2013) surveyed 3-MCPD. The method used to detect 3-MCPD levels had LOD of 0.23×10^{-2} mg kg^{-1}. From the 21 samples collected, the majority of non-compliant products were manufactured in Bulgaria, while soy sauces imported from China complied with EU regulations. This finding contrasts research from the United States (Nyman *et al*., 2003), where most soy sauces that did not comply with US standards were from the Asian region.

In addition to research journals, there are also technical survey reports prepared by governing bodies regarding 3-MCPD levels in soy sauces and related products. Food Standards Australia New Zealand (FSANZ) surveyed the occurrence of 3-MCPD in soy sauces and related products (FSANZ, 2003). The method utilized is AOAC with LOD of 0.01 mg kg^{-1}. A total of 39 samples were collected for the survey and 18 samples were tested for 3-MCPD content levels above the LOD. From the 18 samples, 14 contained 3-MCPD above 0.02 mg kg^{-1}. There were 8 soy seasoning sauce samples collected, of which 7 contained 3-MCPD levels above 0.02 mg kg^{-1}. The highest 3-MCPD level was quantified in soy sauce at up to 148.2 mg kg^{-1}. In Malaysia, no official technical reports exist on 3-MCPD in market products. However, according to a newspaper report, 22 sauces from 11 brands were recalled from the Malaysian market due to 3-MCPD concentrations detected exceeding 0.02 mg kg^{-1} (Sennyah, 2001).

In the European Union, collective data of 3-MCPD occurrence in 10 European countries was conveyed by the Report of Experts for Scientific Cooperation Task 3.2.9 (EU, 2004). For the report, Austria conducted a survey of 316 samples collected from

the Austrian market where 130 samples contained quantifiable levels of 3-MCPD, with the highest recorded concentration being 104 mg kg^{-1}. In Denmark, 316 samples were collected, of which 130 contained quantifiable levels of 3-MCPD. The highest 3-MCPD level was 104 mg kg^{-1}. 43 samples were tested for 3-MCPD in Denmark and 27 were found to contain quantifiable levels of 3-MCPD. The highest level recorded in Denmark was 90.0 mg kg^{-1}. In Finland, out of 163 samples 53 contained quantifiable levels of 3-MCPD and the highest level of 3-MCPD was 940 mg kg^{-1}. In France, out of 73 samples collected 39 contained quantifiable levels of 3-MCPD. Germany collected the highest number of samples (692) among the 10 participating countries, and 198 samples contained quantifiable concentrations of 3-MCPD. In Ireland, from 178 samples collected 47 contained quantifiable 3-MCPD, with the highest quantified level of 1779 mg kg^{-1}. From 273 samples collected and tested in the Netherlands, 77 were reported to contain quantifiable concentrations of 3-MCPD and the highest concentration found was 151 mg kg^{-1}. In Norway, out of 51 samples collected 47 contained quantifiable 3-MCPD with the highest being 146 mg kg^{-1}. Sweden reported that out of 76 samples collected, 31 contained quantifiable 3-MCPD. In the United Kingdom, 170 samples were collected and only 65 contained quantifiable 3-MCPD. The highest concentration recorded was 93.1 mg kg^{-1}. The occurrence of 3-MCPD in countries were summarized in **Table 2.3**.

Table 2.3: Occurrence of 3-MCPD in different countries.

Country	Instrument	LOD	Type of food analyzed	Number of Samples	Detected 3-MCPD	Range	Reference
Taiwan	GC-MS	0.01 µg mL^{-1}	Domestic soy sauce	188	101	0.01 - 10.00 mg kg^{-1}	(Cheng *et al*., 2004)
			Imported soy sauce	26	3	0.01 - 0.10 mg kg^{-1}	
Singapore	GC-MS	0.01 mg kg^{-1}	Soy sauce	317	44	>0.01 - >3.00 mg kg^{-1}	(Wong *et al*., 2006)
			Oyster sauce	104	18	>0.01 - 3.00 mg kg^{-1}	
United State	GC-MS	0.005 mg kg^{-1}	Domestic and Canadian soy sauce	9	-	-	(Nyman *et al*., 2003)
			Asian soy sauce	45	18	2.40 – 876.00 mg kg^{-1}	
			Unknown country of origin soy sauce	1	-	-	
UK	GC-MS	0.01 mg kg^{-1}	Soy sauce and related products	100	100	0.01 - 93.10 mg kg^{-1}	(Crews *et al*., 2003)
			Soy sauce and related products	99	99	0.01 - 21.20 mg kg^{-1}	
	-	-	Soy sauce and soy sauce-based products	170	65	LOD- 93.10 mg kg^{-1}	
Australia	GC-MS	0.01 mg kg^{-1}	Soy and oyster sauces	39	18	<0.01-150.00 mg kg^{-1}	(FSANZ, 2003)
Malaysia	-	-	Sauce and seasoning products	-	22	-	(Sennyah, 2001)
Brazil	GC-MS	0.9 µg kg^{-1}	Soy sauce	45	7	-	(Vicente *et al*., 2011)
			Special sauce containing soy sauce	16	-	-	
Bulgaria	GC-MS	2.3 µg kg^{-1}	Special sauce containing soy sauce	16	-	-	(Christova-Bagdassarian *et al*., 2013)
Austria	-	-	Soy sauce and soy sauce-based products	316	130	LOD- 104.00 mg kg^{-1}	(EU, 2004)
Denmark	-	-	Soy sauce and soy sauce-based products	43	27	LOD- 90.00 mg kg^{-1}	(EU, 2004)
Finland	-	-	Soy sauce and soy sauce-based products	163	53	LOD- 145.00 mg kg^{-1}	(EU, 2004)
France	-	-	Soy sauce and soy sauce-based	73	39	LOD- 145.00 mg kg^{-1}	(EU, 2004)

			products				
Germany	-	-	Soy sauce and soy sauce-based products	198	692	LOD- 158.00 mg kg^{-1}	(EU, 2004)
Ireland	-	-	Soy sauce and soy sauce-based products	178	47	LOD- 1779.00 mg kg^{-1}	(EU, 2004)
Netherlands	-	-	Soy sauce and soy sauce-based products	273	77	LOD- 108.00 mg kg^{-1}	(EU, 2004)
Norway	-	-	Soy sauce and soy sauce-based products	51	47	LOD- 146.00 mg kg^{-1}	(EU, 2004)
Sweden	-	-	Soy sauce and soy sauce-based products	76	31	LOD- 79.90 mg kg^{-1}	(EU, 2004)

The occurrence of 1,3-DCP is normally investigated together with 3-MCPD. However, not all detection methods are suitable for quantifying 1,3-DCP, as some of the derivatization methods developed can only derivatize diols in 3-MCPD. The detected concentrations of 1,3-DCP are found to be lower than 3-MCPD in the majority of reported studies. 1,3-DCP is identified mainly in soy sauce and soy-based products (**Table 2.4**), especially acid-HVP soy sauces. 1,3-DCP can also be found in meat products, mostly due to the processing procedures that involve prolonged high-temperature treatment such as smoking.

Table 2.4: Summary of 1,3-DCP in soy sauce and soy-based products, fish and seafood, meat and meat products and food ingredients. Data adapted from IARC (2013) and summarized from JECFA (2002).

Product	No.	LOQ (mg/kg)	Mean	n < LOQ	Highest conc.
Soy sauce and soy-based products	484	0.002-0.15	0.110	371	9.84
Fish and seafood	29	0.005	0.00025	26	0.0024
Meat and meat products	99	0.005	0.019	51	0.11
Food ingredients (including HVPs and malt extracts)	56	0.010	0.008	13	0.070

Aside from research papers and technical reports, 3-MCPD contamination has also caught media attention in various countries. BBC in the UK has warned the public on the health risks of consuming 3-MCPD contaminated soy sauces and stated what actions the government was taking to curb 3-MCPD contamination (BBC News, 1999). A news report also mentioned the severity of 3-MCPD contamination in the UK and named the brand with the highest 3-MCPD detected. In the Philippines, GMA news reported that soy sauce producers are urged to follow the Codex on the allowable concentration of 3-MCPD in soy sauce, as the country has not yet enforced standards on 3-MCPD in soy sauce (GMA News, 2007). According to Inquirer, the FDA director has advised the public to read soy sauce labels before purchasing and consuming. In the US, all imported soy sauces undergo safety evaluations including 3-MCPD monitoring before they can be sold on the market (Inquirer, 2011). However, some soy sauces are also sold on "underground markets" and do not pass safety evaluations. These soy sauces potentially contain 3-MCPD exceeding the permissible limits. The New Zealand Herald reported that the Ministry of Health in New Zealand carried out a series of reviews on the maximum tolerable limits established by other countries, and enforced the country's own limits. Subsequently, hundreds of sauces were voluntarily removed from the shelves (NZ Herald, 2001). The report also included soy sauce brands that had been removed. In Vietnam, the Ho Chi Minh City Health Department reported that locals rejected locally produced soy sauces due to 3-MCPD contamination. The Health Department made the contaminated soy sauce brands available in the report as well (Viet Nam News, 2001). According to Gulf News, the United Arab Emirates temporarily banned the import and sales of soy products from East Asia in the country and withdrew all available stocks nationwide (Gulf News, 2001). 3-MCPD contaminated soy sauces were reportedly being monitored after 3-MCPD was found to

be a suspected carcinogen. Public health enforcements were additionally set to update the public on the market monitoring carried out. The safety evaluation requirements for imported soy sauces would provide peace of mind for consumers regarding the safety of soy sauces being consumed. However, in Vietnam, a temporary high market demand for imported soy sauces arose, since no registration is required to sell locally made soy sauces.

2.7 Genotoxicity Studies

Genotoxicity refers to the damaging effect of compounds on the genetic material (DNA and RNA) of cells (Umang, 2012) that causes mutations, which may lead to cancer. IARC categorized 3-MCPD as a potentially carcinogenic compound (IARC, 2016). For this reason, it is important to evaluate the genotoxicity of 3-MCPD both *in vitro* and *in vivo*.

In vitro studies have been performed on bacteria based on the principle of reverse mutation. For 3-MCPD, *in vitro* studies have been done on *Salmonella* strains and *E.coli*. **Table 2.5** presents a summary of *in vivo* and *in vitro* genotoxicity studies on 3-MCPD. 3-MCPD reportedly exhibits a genotoxic influence on the *Salmonella* TA 100 and TA 1535 strains, with or without the presence of S9 fraction. The supernatant fraction is obtained from an organ, usually the liver, homogenated by centrifugation at 9000 g for 20 min in a suitable medium. This fraction contains cytosol and microsomes (Duffus *et al*., 2007) (Ohkubo *et al*., 1995; Silhankova *et al*., 1982; Stolzenberg & Hine, 1980; Zeiger *et al*., 1988). However, Stolzenberg & Hine (1979) reported that 3-MCPD is genotoxic to *Salmonella* TA 1535 and TA 100 only in the presence of S9 fractions.

In contrast, *Salmonella* strains TA 98, TA 1537, and TA1538 indicate that 3-MCPD is not a genotoxic compound, either with or without the presence of S9 fractions (Ohkubo *et al*., 1995; Silhankova *et al*., 1982; Stolzenberg & Hine, 1979). According to Silhankova *et al*. (1982) and Stolzenberg & Hine (1979) *Salmonella* TA98 does not exhibit genotoxicity, whereas Ohkubo *et al*. (1995) and Zeiger *et al*. (1988) found that the same strain only has a genotoxic effect without the S9 fraction. In the *E. coli* test end point for all strains (WP2, TM930, TM1080) with or without S9 fractions, Ohkubo *et al*. (1995) and Silhankova *et al*. (1982) reported negative genotoxicity findings. In short, the 3-MCPD bacterial test end appeared to be genotoxic to certain types and strains.

On the other hand, a yeast (*schizosaccharomyces pombe*) model showed that 3-MCPD is genotoxic without S9 fractions, while the genotoxic effect seemed to be reduced/removed in the presence of S9 fractions. Comparatively, this effect was observed in *Salmonella* strains TA98 and TA 677 (Ohkubo *et al*., 1995; Zeiger *et al*., 1988).

The genotoxicity of 3-MCPD to mammalian cells has been investigated *in vivo* and *in vitro*. Nonetheless, the findings regarding different types of cells and animal models are inconsistent. In an *in vitro* study, Henderson *et al*. (1987) reported that mouse lymphoma TK locus exhibited a positive genotoxicity effect with the presence of S9 fraction and a negative effect without the S9 fraction. Painter & Howard (1982) showed that with or without S9, 3-MCPD does not have a genotoxic effect on HeLa cells. In Chinese hamster V79 cells, 3-MCPD is reportedly genotoxic with or without the presence of S9 fractions (May, 1991).

Most findings from mammalian *in vivo* studies indicate that 3-MCPD is not a genotoxic compound (Epstein *et al*., 1972; Jaccaud & Aeschbacher, 1989; Jones *et al*., 1969; Jones & Jackson, 1976). Only one research showed a positive genotoxic effect, which was on Chinese hamster ovaries (CHO)-K1 cells without S9 fractions (El Ramy *et al*. 2007).

In summary, results from *in vitro* and *in vivo* 3-MCPD genotoxicity studies are generally inconsistent. *In vivo* results demonstrate that 3-MCPD is genotoxic to certain types of organisms and reacts differently with or without the presence of S9 fraction. In contrast, the majority of *in vivo* studies indicate that 3-MCPD has no genotoxic effects (**Table 2.5**).

Table 2.5: Genotoxicity of 3-MCPD, adapted from Lynch *et al*. (1998) and Schlatter *et al*. (2002).

Test End Point	Test Subjects	Dosage	Findings		Reference
			+S9	- S9	
Bacteria					
	Salmonella TA100	10 to 1000 µmol plate^{-1}	+	+	(Stolzenberg & Hine, 1980)
	Salmonella TA1535	2 to 200 µmol plate^{-1}	+	+	(Silhankova *et al*., 1982)
	Salmonella TA100, TA1535	100 to 10000 µg plate^{-1}	+	+	(Zeiger *et al*., 1988)
	Salmonella TA 100	10 to 1250 µg plate^{-1}	+	+	(Ohkubo *et al*., 1995)
	Salmonella TA 1535, TA100	100 to 1000 µmol plate^{-1}	+	-	(Stolzenberg & Hine, 1979)
	Salmonella TA98	10 to 10000 µg plate^{-1}	-	+	(Zeiger *et al*., 1988)
Reverse Mutation	*Salmonella*TA677, TA98	10 to 1250 µg plate^{-1}	-	+	(Ohkubo *et al*., 1995)
	Salmonella TA98	101 to 1000 µmol plate^{-1}	-	-	(Stolzenberg & Hine, 1979)
	Salmonella TA1537, TA1538, TA98	3 to 200 µmol plate^{-1}	-	-	(Silhankova *et al*., 1982)
	E.coli WP2, TM930, TM1080	2 to 200 µmol plate^{-1}	-	-	(Ohkubo *et al*., 1995)
	E.coli WP2, TM930, TM1080	2 to 200 µmol plate^{-1}	-	-	(Silhankova *et al*., 1982)
	Salmonella TA 100	Not Reported	-	nd	(Majeska & Matheson, 1983)
	Salmonella TA 97	10 to 10000 µg plate^{-1}	-	nd	(Zeiger *et al*., 1988)
Yeast					
Forward Mutation	*Schizosaccharomyces pombe*	100 to 300 mM	-	+	(Rossi *et al*., 1983)
Mammalian cells					
	Mouse Lymphoma TK locus	2 to 9 mg mL^{-1}	+	-	(Henderson *et al*., 1987)
In vitro (Mutation)	Related end point DNA synthesis inhibition (HeLa cell)	Not Reported	-	-	(Painter & Howard, 1982)
	Cell transformation (M2 fibroblasts)	100 to 2000 µg mL^{-1}	nd	+	(Piasecki *et al*., 1990)
	Chinese hamster V79 cells HPRT	0.3 to 70 mM	weak at >70 mM		(Gorlitz, 1991)

Table 2.5, continued

Table 2.5, continued: Genotoxicity of 3-MCPD, adapted from Lynch *et al*. (1998) and Schlatter *et al*. (2002).

In vitro (Sister Chromatid Exchange)	Chinese hamster V79 cells	700 to 2800 µg mL^{-1}	+	+	(May, 1991)
	Male Mice	5 to 10 mg kg^{-1} bw day^{-1}, 5 days (oral)	-		(Jones *et al*., 1969)
	Male ICR/Ha Swiss Mice	125mg kg^{-1} bw day^{-1} or 20mg kg^{-1} bw day^{-1}, 5 days (oral)	-		(Epstein *et al*., 1972)
In vivo (Dominant Lethal)	Male Wistar Rats	5, 10, 20 mg kg^{-1} bw day^{-1}, 5 days (oral)	-		(Jones & Jackson, 1976)
	Bone marrow micronucleus OF1 Mice	40 to 120 mg kg^{-1} bw	-		(Jaccaud & Aeschbacher, 1989)
	Chinese Hamster Ovary (CHO)-K1 cells	0.5-5mg mL^{-1}	nd	+	(El Ramy *et al*., 2007)

2.8 Metabolism and Biomarkers

Since genotoxicity studies are found to be inconclusive and organism-specific, the metabolism of 3-MCPD could provide insight into how 3-MCPD reacts with physiological systems. Two 3-MCPD metabolism pathways are suggested: the microbial metabolic pathway and the mammalian metabolic pathway (Jones, 1983). The final product of the microbial metabolic pathway is mercapturic acid and glycerol, while for the mammalian pathway it is oxalic acid (**Figure 2.5**).

There are no consistent results to demonstrate the physiology of the microbial metabolic pathway. It has been suggested that microbes utilize the enzyme HHD to oxidize 3-MCPD into glycidol. However, no specific types of microbes are involved, and only Gram positive bacteria are mentioned (Van Den Wijngaard *et al*., 1989). Glycidol is a class 2A genotoxic carcinogen (Lee *et al*., 2012), which can be further hydrolyzed into glycerol or deconjugated and acetylated into mercapturic acid (Lynch *et al*., 1998).

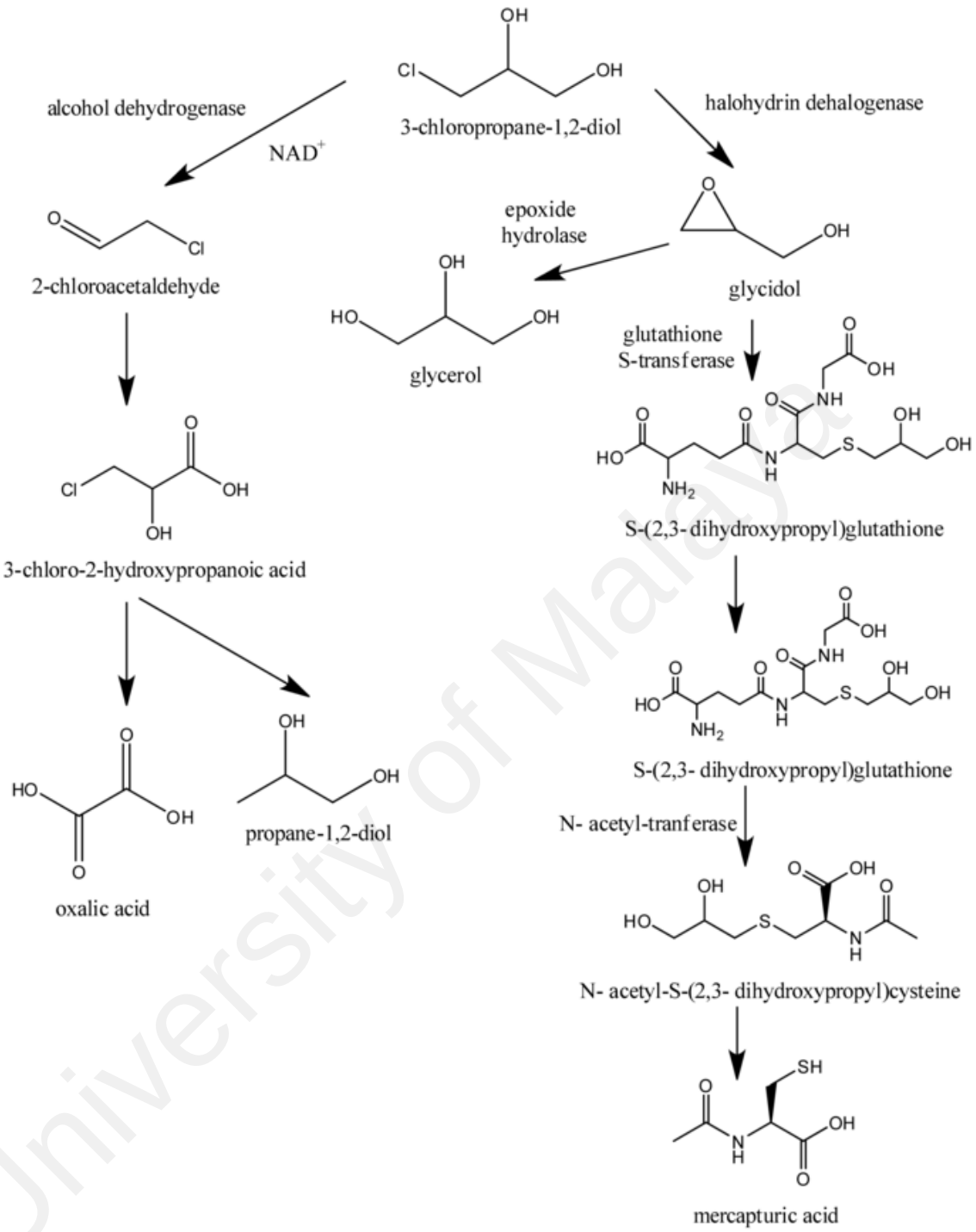

Figure 2.5: Proposed mammalian and microbial metabolic pathways for 3-MCPD (Lynch *et al*., 1998).

Jones (1975) suggested the mammalian and microbial metabolic pathways are the same in terms of the mercapturic acid end product, though not in the nephrotoxic and reproductive toxicity of 3-MCPD. Instead, 3-MCPD is oxidized into 2-chloroacetaldehyde by the alcohol dehydrogenase enzyme and is further converted to 3-chloro-2-hydroxypropanoic acid (chlorolactic acid). Chlorolactic acid is said to inhibit the respiratory and lactate metabolism mechanisms, which is significant in causing nephrotoxicity (Jones *et al*., 1981). Chlorolactic acid has been shown to have immunotoxic effects *in vitro* (Lee *et al*., 2005), and to suppress T- and B-lymphocytes as well as the production of cytokines. As a result, rats fed with medium to high doses of 3-MCPD showed signs of morbidity and mortality (Lee *et al*., 2005). To further reject the theory that mammals and microbes share the same metabolic pathway, Jones *et al*. (1978) proved that glycidol is not the major metabolite in the mammalian metabolic pathway *in vivo*. At the end of the mammalian metabolic pathway, oxalic acid has been shown to cause kidney failure through the formation of calcium oxalate. Calcium oxalate appears to be associated with focal necrosis, mineralization, and impaired kidney function (EAEMP, 2004). With the intermediate products that appear to cause nephrotoxicity and reproductive toxicity, the pathway ending with oxalic acid is a better explanation for the *in vivo* metabolism of 3-MCPD.

Biomarkers refer to a wide subcategory of medical signs in contrast to medical symptoms observed in a patient that can be measured accurately and are reproducible (Strimbu & Tavel, 2010). In short, biomarkers are biological indicators released when the body's physiological system is exposed to foreign compounds. Biomarkers are a reliable and accurate tool for disease detection, with therapeutic interventions proposed to countermeasure the response.

Biomarkers for 3-MCPD exposure are found in urine. In an investigation of 3-MCPD metabolism in rats and mice, Jones (1975) isolated and identified 2 biomarkers from urine: S-(2,3-dihydroxypropyl)cysteine (VII) and the corresponding mercapturic acid N-acetyl-S-(2,3-dihydroxypropyl)cysteine (VIII). With these findings, Jones (1975) suggested that the mammalian metabolic pathway is similar to the microbial metabolic pathway. However, Jones & Fakhouri (1979) later identified 1,3-DCP, N-acetyl-S-(2,3-dihydroxypropyl)cysteine and N,N-bis-acetyl-S,S'-(1,3-bis-cysteinyl)propan-2-ol in the urine of animals exposed to 3-MCPD. This further contradicts the theory that the metabolic pathway of mammals is similar to that of microbes. To accurately identify the metabolites in 3-MCPD metabolism [3-^{36}Cl]chloropropan-1,2-diol 3-MCPD was fed to male rats, and β-chlorolactic acid (IV) together with oxalic acid (V) was identified and isolated from the exposed rats' urine (Jones *et al*., 1978). However, since there is no conclusive metabolic pathway for 3-MCPD metabolism, the biomarkers found in these studies cannot be used to accurately measure physiological system exposure to 3-MCPD.

Li *et al*. (2003) studied the toxicological effects of 3-MCPD in rats and found increased N-acetyl-beta-D-glucosaminidase (NAG) activity in rats exposed to 3-MCPD. According to this finding, they suggested that NAG can serve as a biomarker in monitoring rat exposure to 3-MCPD. However, NAG is not a specific biomarker since it can also be detected when identifying renal illnesses (Skálová, 2005) due to injury or dysfunction caused by diabetes mellitus, nephrotic syndrome, inflammation, vesicoureteral reflux, urinary tract infection, hypercalciuria, urolithiasis, nephrocalcinosis, perinatal asphyxia, hypoxia, hypertension, heavy metal poisoning, and treatment with aminoglycosides, valproate, or other nephrotoxic drugs. To narrow down

specific biomarkers for 3-MCPD exposure, Li *et al*. (2010) applied metabonomic analysis to identify and isolate urine using ultra-performance liquid chromatography/mass spectrometry (UPLC-MS). According to the analysis, the compound galactosylglycerol is detectable as early as 10 days after exposure to 3-MCPD.

2.9 Toxicity Studies

Before 3-MCPD was identified as possibly carcinogenic, the FDA listed it as a rodenticide under the name alpha-chlorohydrin. 3-MCPD has an antifertility effect on rodents and is the main ingredient in the rodenticide Epibloc (Ericsson, 1982). Jones (1983) observed that the antifertility effect of 3-MCPD is species-specific, only effective in rats, rams, boars, guinea pigs, hamsters, rhesus monkeys and ejaculated human sperm but ineffective in mice and rabbits. 3-MCPD causes infertility by blocking the glycolysis pathway (Stevenson & Jones, 1984), which is responsible for sperm mobility as it compensates for the lack of oxidative phosphorylation (Miki, 2006; Mukai & Okuno, 2004). 3-MCPD impairs the 3'-5'-cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway in sperm, thus blocking glycolysis (Zhang *et al*., 2012) and reducing sperm mobility, which causes temporary or permanent infertility depending on dose exposure. Reduced production of progesterone in R2C rat Leydig cells following exposure to 3-MCPD was reported by Sun *et al*. (2013). 3-MCPD was observed to induce morphological changes and DNA damage to the Leydig cells, resulting in apoptotic cell death.

The immunotoxicity of 3-MCPD was investigated in female Balb/c mice (Lee *et al*., 2004, 2005). In both studies identical procedures were used, where 3-MCPD at doses of 0 (control), 25, 50 and 100 mg kg^{-1} were dissolved in water and administered to the female Balb/c mice. Lee *et al*. (2004) investigated the hematological changes, histopathological changes, antigen-specific immunity (response to sheep erythrocytes), proliferative potential of splenic lymphocytes (T- and B-cell mitogens), and natural killer (NK) cell activity (nonspecific immunity). In the latter study, Lee *et al*. (2005) evaluated the thymic subset, delayed-type hypersensitivity, mixed-lymphocyte reaction, and peritoneal macrophage activity. Both studies reported that female mice administered a high 3-MPCD concentration (100 mg kg^{-1}) exhibited immune system deterioration.

2.10 Carcinogenicity Studies on Mice

Since the genotoxicity of 3-MCPD seems to be organism and strain specific while antifertility is animal specific, it would be significant to identify the carcinogenic effect in different animal species as well. Hence, this section addresses the carcinogenicity of 3-MCPD in mice. So far, dermal, injection and drinking water studies have been done on CHR/HA (Van Duuren *et al*., 1974) and B6C3F1 animal models among others (Cho *et al*., 2008; Jeong *et al*., 2010).

In a dermal toxicology study, 19-month-old CHR/Ha Swiss mice served as the test animal model, where 50 female mice were exposed to 2 mg of 3-MCPD 3 times a week. The study lasted 19 weeks and no neoplastic findings were reported (Van Duuren *et al*., 1974). A study on the injection exposure to 3-MCPD was done on the same species of

mice (Van Duuren *et al*., 1974). Unlike the dermal study, the exposure dosage in the injection study was 1 mg once a week. After 19 weeks, no neoplastic findings were observed in the mice, although a local sarcoma was identified at the site of injection on 1 control mouse and dosed mice.

The sub-chronic toxicity of 3-MCPD in mice was investigated by Cho *et al*. (2008). In their study, 10 B6C3F1 male and female mice were exposed to 3-MCPD via drinking water for 13 weeks. The selected exposure doses were: 0 (control), 5, 25, 100, 200, and 400 ppm. By week 13, all the mice in the study survived. However, there was a significant weight difference in both male and female mice expose to 400 ppm 3-MCPD. As for the kidneys, the mice exposed to 200 ppm and 400 ppm recorded significantly higher weights compared to the kidneys of control mice. The male mice exposed to 400 ppm exhibited a significant decrease in sperm mobility, and there was exacerbated germinal epithelium degradation in the male mice exposed to 200 and 400 ppm. Likewise, the estrus cycle was significantly delayed in female mice exposed to 400 ppm. However, no histopathological change was observed in all mice exposed to 3-MCPD. In summary, 3-MCPD was found to target the kidneys, testes and ovaries.

In another drinking water study, 50 B6C3F1 male and female mice were exposed to 0 (control), 30, 100, and 300/200 ppm of 3-MCPD for 24 months (Jeong *et al*., 2010). The study was done according to the OECD and ICH requirements for a satisfactory carcinogenicity study. In the study, the exposure dosage of 300 ppm was reduced to 200 ppm due to the toxic effect of 3-MCPD. At the end of the study, significant weight differences as well as reduced water and food consumption were observed in mice exposed to high concentrations of 3-MCPD. There was no histopathological evidence to support the difference in hematology and serum findings. The researchers concluded

that 3-MCPD offers no evidence of carcinogenic potential from the carcinogenic study (**Table 2.6**).

Table 2.6: Carcinogenic potential of 3-MCPD in mice and rats.

2.11 Carcinogenicity Studies on Rats

Weisburger *et al*. (1981) investigated the carcinogenicity potential of 3-MCPD in Sprague Dawley (SD) rats. In this study, 26 male and female SD rats were exposed to 20/35 mg kg^{-1} and 60/70 mg kg^{-1} of 3-MCPD via oral gavage. The rats were fed with the initial 3-MCPD dosage, which was increased after 10 weeks of treatment. The treatment was given for 72 weeks and observation lasted 104 weeks. At the end of the study, the weights of the rats fed with higher concentrations of 3-MCPD significantly reduced. However, no neoplastic findings were reported in all rats fed with 3-MCPD.

As reported by Schlatter *et al*. (2002) in WHO, Sunahara, Perrin, & Marchesini (1993) conducted a study on 244 Fisher rats treated with 3-MCPD for 24 months. A total of 50 male and females were exposed to 3-MCPD in various concentrations (0, 20, 100 and 500 mg L^{-1}). A significant reduction in food and water intake for rats treated with 500 mg L^{-1} was reported, which resulted in greater weight loss than the control rat group. No mortalities were reported in this study, but 42% of the rats were terminated. The hematological and blood clinical parameters of all rats indicated no treatment-related signs. However, chronic progressive nephropathy (CPN) occurred in all rats exposed to 3-MCPD, with more serious cases reported in the male rats. Dose-related incidents of hyperplasia and/or tumors were observed, with an increase in the kidneys, testes, mammary glands and pituitary gland. As indicated above, there was a high incidence of renal and testicular Leydig-cell tumors, which was dose-dependent.

Cho *et al*. (2008) carried out a study on the carcinogenic effect of 3-MCPD in SD rats for 2 years. In the study, 4 groups of mice with 50 males and females per group were exposed to 3-MCPD through drinking water (0, 25, 100, and 400 ppm). At the end

of the study, the water intake of rats exposed to 400 ppm was significantly lower, contributing to substantial weight loss compared to the controls. There was no significant difference between the male and female rats. However, the survival rate of the rats in this study was less than 50%, which was due to the spontaneous pituitary tumors in both sexes. Similar to a research by Sunahara, Perrin & Marchesini (1993) on different rat species, CPN was also observed in this study. Renal tubular carcinomas were recorded in both males and females, and Leydig cell tumors in males, hence 3-MCPD was concluded to be a carcinogen.

Barocelli *et al*. (2011) conducted a 90 day toxicity study on Wistar rats exposed to 3-MCPD and 3-MCPD palmitic ester. The high, medium high and low 3-MCPD doses selected were 14.75 mg kg^{-1}, 3.68 mg kg^{-1}, and 0.92 mg kg^{-1}. Ten (10) control rats as well as 20 male and female rats were exposed to each 3-MCPD dose treatment that was administered through oral gavage. By the end of the study all male rats survived, while 7 females at high dose treatment and 1 female at medium high dose treatment were recorded dead. Nonetheless, all surviving rats showed signs of morbidity. The findings proved that 3-MCPD is lethal in high doses over short periods. Despite signs of morbidity, the rats in this study gained weight, which contrasts all aforementioned studies in this section that recorded lower weights. Given these points, 3-MCPD was deemed a cause of renal and testicular damage. Although the carcinogenicity study was found to be animal specific in line with the genotoxicity studies, the harm that 3-MCPD can cause to human physiological systems cannot be ignored. More toxicity studies on animals such as monkeys and mini pigs must be done to further confirm the toxicity and carcinogenic potential of 3-MCPD.

To examine the carcinogenic activity of 1,3-DCP (**Table 2.7**), 80 Wistar KFM/HAN male and female rats were administered low dosage (27 mg L^{-1}), mid dosage (80 mg L^{-1}), and high dosage (240 mg L^{-1}) via drinking water for a period of 104 weeks (Research & Consulting Co., 1986). In weeks 26, 52 and 78 of exposure, 10 rats from each sex group were killed. The toxicity results indicated that the mortality of male (32/50, $P < 0.05$) and female (27/50, $P < 0.05$) rats exposed to high doses of 1, 3-DCP was higher than the control group: male (18/50) and female (14/50) (no exposure). In male Wistar rats, the highest numbers of tumors observed in the liver were hepatocellular carcinomas while in the kidneys they were renal tubular adenomas. The report suggests that 1,3-DCP has a carcinogenic effect on the liver, kidneys, oral epithelium and tongue.

Table 2.7: Carcinogenicity study of 1,3-DCP in rats. Adapted from Research & Consulting Co. (1986).

Organ and finding	Males				Females			
	Exposure concentration (mg/L)				Exposure concentration (mg/L)			
	0	27	80	240	0	27	80	240
Liver (hepatocellular adenoma)	1/80	0/80	1/80	0/80	1/80	1/80	1/80	6/80
Liver (hepatocellular carcinoma)	0/80	0/80	2/80	11/80	0/80	0/80	1/80	44/80
Kidneys (Tubular adenoma)	0/80	0/80	3/80	10/80	0/80	0/80	0/80	1/79
Kidneys (Tubular carcinoma)	0/80	0/80	0/80	1/80	0/80	0/80	0/80	0/79
Tongue (Papilloma)	0/80	1/80	0/79	6/80	0/80	0/80	0/80	7/79
Tongue (Carcinoma)	0/80	0/80	1/79	6/80	0/80	1/80	1/80	4/79
Thyroid (Follicular adenoma)	0/80	0/80	3/80	3/78	1/79	0/80	3/80	4/79
Thyroid (Follicular carcinoma)	0/80	0/80	2/80	1/78	0/79	0/80	0/80	2/79

2.12 Methods of Detection

As mentioned in section 2.1, 3-MCPD is an organic polar compound. For this reason, it is detectable through gas-chromatography (GC), as high temperatures vaporize the compound and render it detectable by GC. Polar compounds are not easy to vaporize. Hence, because the boiling point of 3-MCPD is 213°C, the temperature of the GC inlet is set between 280 and 300°C. However, the polarity of 3-MCPD makes it difficult to separate in the GC column. Unless the column is polar, a non-polar column will give a negative result. However, this can be solved by derivatization, which changes 3-MCPD into its non-polar derivatives that are separable in a non-polar column. Derivatizing agents for chloropropanols can generally be divided into 2 major groups: diol functional group derivatization and hydroxyl functional group derivatization. Diol functional derivatization can only derivatize chloropropanols that have 2 adjacent hydroxyl groups, while hydroxyl functional derivatization can derivatize all hydroxyls present in the chloropropanols, including mono-ol and diol. Thus, only hydroxyl functional group derivatizing agents are able to derivatize both 3-MCPD and 1,3-DCP simultaneously. The simultaneous detection of 3-MCPD is vital, as 1,3-DCP can be present in acid-HVP soy sauce at lower concentration than 3-MCPD. At present, there are several derivatizing agents that can improve the detection of chloropropanols in a GC setting, such as phenylboronic acid (PBA), bis(trimethylsiyl)trifluoroacetamide (BSTFA), heptafluorobutyrylimidazole (HFBI), heptafluorobutyric anhydride (HFBA), and ketal/acetonide formation (**Table 2.8**). Beside GC, 3-MCPD can also be detected in HPLC-FLD with periodate derivatizing technique.

Table 2.8: Methods developed to quantify free 3-MCPD.

Derivatization Agent	Advantage	Disadvantage	Challenge	LOD	Instruments	References
-	No derivatization agent	High limit of detection	Determining a suitable polar column for better separation due to the high polarity of 3-MCPD	0.13 – 1.00 mg kg^{-1}	GC, CE-ECD	(Spyres, 1993; Xing & Cao, 2007)
Phenylboronic acid (PBA)	Works in aqueous and organic media, intensive characteristic ions	Requires GC with a sensitive detector, lack of confirmation ions	Determining a suitable alkaline to neutralize the acidity of the end product	$0.03x10^{-1}$-1.00 mg kg^{-1}	GC-MI-FIR, GC-FID, GC-MS-SIM, GC-MS/MS MRM,	(Anon, 1995; Divinová *et al*., 2004; Huang *et al*., 2005; IARC, 1994; Kuballa & Ruge, 2003; Plantinga *et al*., 1991; Rodman & Ross, 1986)
N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA)	Better heat stability of derivatized analytes	Requires an anhydrous derivatizing environment	Silicone residue can accumulate in the detector	$0.16x10^{-2}$ – $1.12x10^{-2}$ mg kg^{-1}	GC-FID, GC-MS-SIM, GC-MS	(Bodén *et al*., 1997; Gonzalez *et al*., 2011; Kissa, 1992)
N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)	Better stability of derivatized analytes	Requires an anhydrous derivatizing environment	Silicone residue can accumulate in the detector	$3.91x10^{-3}$ mg kg^{-1}	GC-MS	(Lee *et al*., 2007)
Acetone in toulene-4-sufonic acid	Reacts with diols in 3-MCPD specifically; intensive characteristic ions	Requires an anhydrous derivatizing environment; lack of confirmation ions	Normal acetone requires pre-treatment with Epsom salt; removal of acidic catalyst from the end product	$0.01x10^{-1}$ – $0.12x10^{-2}$ mg kg^{-1}	GC-MS	(Dayrit & Niñonuevo, 2004; Meierhans *et al*., 1998; Rétho & Blanchard, 2005)
Cyclohexanone	Applicable to free and bound 2- and 3-MCPD; solid catalyst can be removed easily	Requires an anhydrous derivatizing environment; lack of confirmation ions	Cyclohexanone requires pre-treatment to remove moisture	$0.01x10^{-1}$ - $0.03x10^{-1}$ mg kg^{-1}	GC-MS	(Becalski *et al*., 2013)

Heptafluorobutyryl imidazole (HFBI)	Wide range of samples	Requires an anhydrous derivatizing environment	Requires heat incubation; HFBI is expensive	$0.05x10^{-1}$ - 0.10 mg kg^{-1}	GC-ECD, GC-MS, GC-MS/MS MRM	(Bel-Rhlid *et al*., 2004; Brereton *et al*., 2001; Hamlet & Sutton, 1997; Robert *et al*., 2004; Van Bergen *et al*., 1992)
Heptafluorobutyric acid (HFBA)	Cheaper than HFBI	Requires an anhydrous derivatizing environment	Requires a catalyst for optimum derivatization and neutralization of the acidic product	$0.07x10^{-2}$ – $0.05x10^{-1}$ mg kg^{-1}	GC-MS, GC-ECD, GC-MS SIM	(Abu-El-Haj *et al*., 2007; Chung *et al*., 2002; Matthew & Anastasio, 2000)
Fluorescence derivatization	Fast alternative to GC	Requires periodate oxidation as pre-treatment	Requires color removal pre-treatment to quantify 3-MCPD in dark color samples	$0.36x10^{-3}$µg kg^{-1}	HPLC-FLD	(Hu *et al*., 2013)

3-MCPD, which is widely found in soy sauces, is a complicated sample matrix. Thus, a procedure to extract and clean the 3-MCPD prior to derivatization is required. The cleanup provides a cleaner extract, which helps improve the LOD of developed method validation, lowers background noise, increases recovery and enhances the chromatogram. 3-MCPD in food sample matrices must be extracted into aqueous solution, whereby it is loaded into a chromatography column. Chromatography columns follow the theory of solid-liquid extraction, which is similar to liquid-liquid extraction but has more advantages. Solid-liquid extraction prevents the possibility of emulsion formation between the organic and aqueous solutions, hence resulting in higher recovery and a cleaner extract. For the solid stationary phase to clean the 3-MCPD samples, diatomaceous earth is selected for its ability to absorb hydrophilic compounds. As for the mobile phase, a protic solvent such as acetone or dichloromethane can be used to extract 3-MCPD from the solid phase. Following solid phase extraction, the collected eluate is dried to near dryness under a gentle nitrogen flow and is subjected to derivatization.

Detection without derivatization often poses the limitation of high LOD. However, Spyres (1993) managed to quantify 3-MCPD with gas chromatography coupled with electrolytic conductivity detection (GC-ECD) and achieved LOD of 1 mg kg^{-1}. This LOD level is adequate for quantifying 3-MCPD levels in the United Stated and Canada where the maximum tolerable limit is 1 mg kg^{-1}. Xing & Cao (2007) attempted to detect 3-MCPD with capillary electrophoresis-electrochemical detection (CE-ECD) and achieved LOD of 0.13 mg kg^{-1}. Although the LOD is lower than the GC quantification method without derivatization, it is still not low enough the quantify 3-MCPD according to the EU's maximum tolerable limit. Leung *et al*. (2003) tried to quantify 3-MCPD

through molecularly imprinting polymer (MIP) as a potentiometric chemo-sensor but failed to obtain satisfactory results. The developed method still needs some improvements before it can be fully utilized as a chemo-sensor. However, the proposed MIP method can be applied as a stationary phase in the solid-liquid extraction of 3-MCPD from food matrices.

2.12.1 Acylation

Acylation is a reaction where an acyl group is introduced into a compound, resulting in the loss of the hydroxyl functional group from the compound and rendering it less polar or non-polar (Orata, 2012). The acylation derivatizing agents normally used to derivatize 3-MCPD are heptafluorobutyryl imidazole (HFBI) and heptafluorobutyric acid (HFBA). The heptafluorobutyrate method allows quantification in a wide range of samples (Rétho & Blanchard, 2005). Brereton *et al*. (2001) studied the HFBI derivatization of 3-MCPD extensively, which was later adopted into the European Standard (CEN, 2004) and AOAC (AOAC, 2002). The method utilizes deuterated 3-MCPD (3-$MCPD_{d5}$) as internal standard and requires chromatography column sample extraction. The derivatized 3-MCPD was then injected into GC-MS. However, the first chloropropanol derivatization by HFBI was reported by Van Bergen *et al*. (1992). GC-MS and GC-ECD were used to detect 3-MCPD-HFB and the reported LOD was 0.01 to 0.10 mg kg^{-1}. Hamlet & Sutton (1997) detected 3-MCPD-HFB with GC-MS/MS MRM and achieved LOD of 0.05 $\times 10^{-1}$ mg kg^{-1}, which is sufficiently low to quantify 3-MCPD in line with the EU limit. Later, Robert *et al*. (2004) and Bel-Rhlid *et al*. (2004) coupled 3-MCPD-HFB with GC-MS and obtained LOD of 0.05 $\times 10^{-1}$ mg kg^{-1}, which is the

same as the LOD achieved by Hamlet & Sutton (1997). The advantage of HFBI as a derivatizing agent is that it is not acidic, which means that at the end of derivatization neutralization is not required and the lifecycle of the GC column is shortened. In fact, excessive HFBI in the sample will protect the column, since it will react with co-extracted compounds that might otherwise harm the column lining. HFB-derivatized chloropropanols produce many characteristic ions, which are very helpful for compound confirmation in GC-MS (**Table 2.9**). However, HFBI is a very reactive derivatizing agent that requires harsh storage conditions (moisture-free and very low temperature) and an anhydrous condition for the derivatization procedure. Furthermore, HFBI is very expensive; in fact, it is the most expensive among derivatizing agents used to quantify 3-MCPD. Nonetheless, an alternative to HFBI is the cheaper HFBA.

Similar to HFBI, HFBA-derivatized 3-MCPD is based on the principle of acylation. Chung *et al*. (2002) combined HFBA derivatization with GC-MS detection and achieved LOD of 0.05 $\times 10^{-1}$ mg kg^{-1}. However, despite using a less sensitive GC-ECD, Matthew & Anastasio (2000) were able to quantify HFBA-derivatized 3-MCPD with LOD between 0.07 mg kg^{-1} and 0.17 mg kg^{-1}. Abu-El-Haj *et al*. (2007) developed HFBA derivatization with a suitable catalyst and attained LOD of 0.01 $\times 10^{-1}$ mg kg^{-1}. HFBA combined with a suitable catalyst can provide almost the same method LOD as HFBI. Nevertheless, comparable to HFBI, HFBA requires an anhydrous condition for the derivatization procedure. The acidic characteristic of HFBA is a concern as well, since it will react with the GC column. However, this can be solved by adding an alkali for neutralization prior to GC injection.

Table 2.9: Characteristic ions of HFB-derivatized 3-MCPD and 3-MCPD$_{d5}$ (Stadler & Lineback, 2008).

Analytes	MW	$[M-CH_2Cl]^+$	$[M-C_3F_7CO_2]^+$	$[M-C_3F_7CO_2CH_2]^+$	$[M-C_3F_7CO_2-HCl]^+$	$[M-C_3F_7CO_2-C_3F_7CO_2H]^+$
3-MCPD	502	453	289/291[a]	275/277[a]	253	75/77[a]
3-MCPD$_{d5}$	507	456	294/296[a]	278/280[a]	257	79/81[a]

[a] Isotopic chlorine cluster ion

2.12.2 Silylation

Silylation is the reaction that replaces the hydroxyl with a trimethylsilyl group, thus rendering the compound non-polar. The silylation derivatizing agents discussed herein are BSTFA and MSTFA. The first study to address silylation of 3-MCPD was published by Kissa (1992). In that study, 3-MCPD was derivatized by BSTFA and detected with GC-FID. The LOD achieved was 5.00 mg kg^{-1}. Later on, Bodén *et al*. (1997) detected BSTFA-derivatized 3-MCPD and quantified it with GC-MS. The LOD achieved was 0.04 mg kg^{-1}. Gonzalez *et al*. (2011) improved the method by implementing solid-phase extraction in the sample extraction procedure. The LOD of BSTFA-derivatized 3-MCPD was thus enhanced to 1.12×10^{-2} mg kg^{-1}, which met the 3-MCPD quantification requirements according to the EU's maximum tolerable limit. Racamonde *et al*. (2011) published a study where chloropropanols in food were quantified with BSTFA as a derivatizing agent and LOQ of 0.16×10^{-2} mg kg^{-1} was reported. In 2007, Lee *et al*. extracted 3-MCPD via solid-phase micro-extraction using 85-µm polyacrylate-coated fiber and derivatized it with MSTFA in headspace. BSTFA and MSFTA appeared superior to HFBI and HFBA derivatization, in the sense that 3-MCPD-TMS was more stable over time than 3-MCPD-HFB. A disadvantage of silylation is the potential accumulation of residual silicone on the GC detector in the long run, which might

reduce the sensitivity of the detector. Besides, the characteristic ions of BSTFA and MSTFA-derivatized 3-MCPD have low mass, which results in lower sensitivity.

2.12.3 Phenylboronic Acid (PBA)

Unlike silylation and acylation, PBA derivatization can be carried out in an aqueous condition. Divinová *et al*. (2004) and Breitung-Utzmann *et al*. (2003) derivatized 3-MCPD in aqueous condition. They extracted 3-MCPD from food samples and derivatized it with acetone/water-diluted PBA. The PBA derivative was then dissolved in hexane and injected into GC. However, PBA can only derivatize compounds that have diol but not single-hydroxyl groups. Initially, PBA was used to derivatize and quantify glucose in blood and food. The first PBA-derivatized 3-MCPD was reported by Rodman & Ross (1986), whereby 3-MCPD was extracted, derivatized and quantified with gas chromatography-matrix isolation-Fourier transform infrared spectrometry (GC-MI-FIR). Pesselman & Feit (1988) attempted to derivatize 3-MCPD with PBA in aqueous condition and successfully detected the dioxaborolane product with GC-ECD. On the other hand, Plantinga *et al*. (1991) and Anon (1995) detected PBA-derivatized 3-MCPD with GC-FID and the LOD established was between 0.5 and 1 mg kg^{-1}. In order to achieve a detection limit for quantification in line with the European Union's maximum tolerable limit, IARC (1994) derivatized 3-MCPD with PBA and quantified it with GC-MS SIM. With single-ion monitoring, the LOD improved to between 0.03 × 10-3 mg kg^{-1} and 0.01 mg kg^{-1}. Evidently, PBA-derivatized 3-MCPD requires GC coupled with a sensitive detector to achieve a low LOD. However, PBA-derivatized 3-MCPD produces intense characteristic ions but lacks confirmation ions (**Table 2.10**),

which consequently leads to some difficulties in compound confirmation. Besides, PBA derivatization requires high volumes of PBA, while excess PBA is detrimental to the GC column. Breitung-Utzmann *et al*. (2003) suggested that storing vials at -12°C for 12 hours prior to detection will precipitate excess PBA, since PBA cannot be removed through neutralization (PBA is a diol rather than acid).

Table 2.10: Characteristic ions of PBA-derivatized 3-MCPD and 3-MCPD$_{d5}$.

Analytes	MW	$[M]^{+-}$	$[M\text{-}CH_2Cl]^+$	Other Structurally Significant Ions
3-MCPD	196	196/198[a]	146/147[b]	103/104[b] $[Ph\text{-}BO]^{+-}$ 91 $[C_7H_7]^+$
3-MCPD$_{d5}$	201	201/203[a]	149/150[b]	103/104[b] $[Ph\text{-}BO]^{+-}$ 93 $[C_7H_5D_2]_+$

[a] Isotopic chlorine cluster ion
[b] Isotopic boron cluster ion

2.12.4 Ketal/Acetonide Formation

For ketal and cyclic acetal formation from 3-MCPD, 3-MCPD was extracted and derivatized with acetone in toluene-4-sulfonic acid monohydrate (Meierhans *et al*., 1998). The reaction must take place in an anhydrous condition because the acetals formed from derivatization are moisture-sensitive. Acetals will react with moisture and undergo hydrolysis into aldehyde or ketone and alcohol (Carey, 2000). Dayrit & Niñonuevo (2004) derivatized 3-MCPD with acetone in toluene-4-sulfonic acid monohydrate and achieved LOD of 1.20×10^{-3} mg kg^{-1}. Rétho & Blanchard (2005) modified the method by filtering the final products through a basic aluminum oxide cartridge but achieved LOD of 0.01×10^{-1} mg kg^{-1}. The catalyst toluene-4-sulfonic acid monohydrate must be removed prior to GC injection to prevent damage to the GC column. To simplify the derivatization procedure, Becalski *et al*. (2013) replaced the

aliphatic ketones with cyclic ketones (cyclohexanone) and the catalyst toluene-4-sulfonic acid monohydrate with solid catalyst (Nafion and Amberlyst). The advantage of ketone-derivatized 3-MCPD is the intense characteristic ions of the product analytes, which can produce a low LOD. However, the limited characteristic ions make it harder for compound identification (**Table 2.11**). The need for derivatization in an anhydrous environment is also a disadvantage of ketone derivatization.

Table 2.11: Characteristic ions of dioxolane/dioxane-derivatized 3-MCPD and 3-$MCPD_{d5}$.

Derivatizing Agent	Analytes	MW	$[M-C_nH_{2n+1}]+$	Other Structurally Significant Ions
Acetone	3-MCPD	150	135/137a	43$[C_2H_3O]^+$
	3-$MCPD_{d5}$	155	140/142a	43$[C_2H_3O]^+$
3-Pentanone	3-MCPD	178	149/151a	57$[C_3H_5O]^+$
	3-$MCPD_{d5}$	183	154/156a	57$[C_3H_5O]^+$
4-Heptanone	3-MCPD	206	163/165a	71$[C_4H_7O]^+$
	3-$MCPD_{d5}$	211	168/170a	71$[C_4H_7O]^+$
Cyclohexanone	3-MCPD	262	191/193a	99$[C_6H_{11}O]^+$
	3-$MCPD_{d5}$	267	196/198a	99$[C_6H_{11}O]^+$

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1 Reagents and Materials

3-MCPD (98%), 1,4-butanediol, 1,5-pentadiol, HMDS, and TMSOTf were purchased from Sigma Aldrich (St. Louis, MO, USA) and 1,3-Dcholoropropanol (98%) was purchased from Merck (Selangor, Malaysia). Aluminum oxide for column chromatography (neutral, 70-230 mesh size), sodium carbonate, sodium bicarbonate and sodium sulfate were purchased from R&M Chemicals (Selangor, Malaysia), while dichloromethane (HPLC grade) and HFBI (GC derivatization grade) were supplied by Fisher Scientific (Selangor, Malaysia). All the water used in this study was obtained through Millipore Milli-Q Plus water purification with 18.2 MΩ cm purity (Bedford, USA). All chemicals used were AR grade unless specified.

3.2 Sampling

Soy sauce, dark soy sauce, oyster sauce and chicken flavoring cube samples were selected randomly and sampled from local supermarkets around Kuala Lumpur and Selangor. The collected samples were stored at room temperature (25^{o}C) throughout the study. Prior to experimentation, each sample was mixed vigorously and thoroughly, and partitioned into 10 g in a sterile centrifuge tube.

3.3 Standard Solution Preparation

Stock solutions of 3-MCPD and 1,3-DCP were prepared by diluting the respective standards with ethyl acetate to achieve a final concentration of 200 mg L^{-1}. Seven calibration points were established for both analytes, with concentrations ranging between 0.001 and 10 mg L^{-1}. The calibration point concentrations were prepared through serial dilution from the 3-MCPD and 1,3-DCP stock solutions. The stock and calibration solutions prepared can be stored up to three months at 4°C prior to experimentation.

An internal standard is an important quality control measure that facilitates comparisons of the responses recorded with the instrument between the target analyte and internal standard. For this study, 1,4-BD was the internal standard selected. A stock solution of 1,4-BD was prepared by diluting it with ethyl acetate to a concentration of 200 mg L^{-1}. The internal standard concentration was set at 1.00 mg L^{-1}, so the stock solution was further diluted with ethyl acetate to 1.00 mg L^{-1}.

A surrogate standard was added to the experiment as part of the quality control to enable the calculation of recoveries. In this study, 1,5-PD was the selected surrogate standard. A stock solution of 1,5-PD was prepared by diluting 98% 1,5-PD to 2.00 mg L^{-1}. The stock solution was then further diluted to 1 mg L^{-1} with ethyl acetate according to standard.

3.4 Sample Cleaning

The sample cleaning procedure was adapted from the AOAC method with modifications done to the sample volume, stationary phase and extraction solvent. About 2 g of each sample (soy sauce, dark soy sauce, oyster sauce and chicken flavoring cube) were weighted and added to 8 g of aluminum oxide together with the surrogate standard (**Figure 3.1**). For the spiked samples, 1.00 mL of 1 mg L^{-1} 3-MCPD and 1-DCP were spiked. The mixture was then homogenized thoroughly. The aqueous sample was to be absorbed in the stationary phase. The sample was then loaded into a chromatography column (2cm internal diameter, 40cm length with 0porosity sintered disc). Prior to adding the sample, 1 g of cotton wool (pre-soaked with dichloromethane) and 1 g of anhydrous Na_2SO_4 were added to the chromatography column to remove additional moisture from the extraction solvent. In the process of adding the mixed aluminum oxide, the column was gently tapped to minimize the porous voids that tend to form in aluminum oxide. Another 1 g of Na_2SO_4 was added on top of the aluminum oxide. The chromatography column was eluted with 250 mL of dichloromethane at a flow rate of about 8 mL min^{-1}. In this process, 3-MCPD and other lipophilic substances were eluted together with the extraction solvent. The chosen extraction solvent was immiscible in water for this liquid-liquid phase separation. Eluate was collected and concentrated to near dryness under a gentle flow of purified nitrogen gas. 1.00 mL of ethyl acetate was then added immediately to the extract.

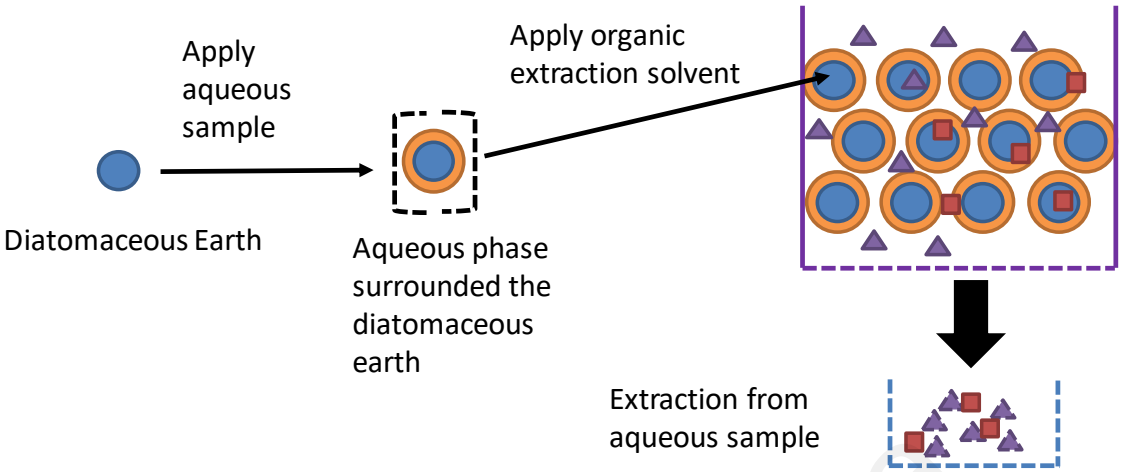

Figure 3.1: Column chromatography extraction procedure.

To determine the efficiency of the method developed to quantify 3-MCPD and 1,3-DCP in various types of foods, several food samples with different matrix types (solid: chicken flavoring, paste: oyster sauce and sweet sauce, liquid: soy sauce) were selected for the actual food analysis in this thesis.

3.5 Derivatization

For TMS derivatization, HMDS was the selected derivatizing agent and TMSOTf was the catalyst. 50 µL of HMDS was added to the ethyl acetate mixture prepared as explained in section 3.4. Then 10 µL of TMSOTf was added to the mixture. The vial was sealed and shaken by a vortex shaker. The entire derivatization process was carried out at room temperature. After 10 minutes of derivatization, 1 mL of water was added to the vial to stop the process. The vial was shaken in the vortex shaker for 30 seconds. The organic layer on top was transferred to a 2mL gas chromatography vial. A tip of Na_2So_4 was then added to the insert to remove moisture prior to analysis.

3.6 Instrumentation and operating conditions

The quantification of 3-MCPD-TMS and 1,3-DCP-TMS was performed using Shimadzu single quadrupole GC-MS-QP 2010 Plus gas chromatography-mass spectrometer (Kyoto, Japan). The capillary column used was DB-5MS, 30 m long, 0.23 mm in diameter and with 0.25µm thick film supplied by Agilent Technologies (Selangor, Malaysia). The quantification of 3-MCPD-HFB and 1,3-DCP-HFB was carried out with Agilent 7890A GC system with an inert mass spectrometry detector. The column was HP-5MS, 30m long, 0.23mm in diameter and with 0.25µm thick film, supplied by Agilent Technologies (Selangor, Malaysia). The instrumentation condition for HFBI derivatization was adopted from AOAC with slight modification (AOAC, 2002). Details of the operating conditions are tabulated in **Table 3.1**.

Table 3.1: GC operating conditions for HMDS and HFBI derivatives.

	HMDS derivative	**HFBI derivative**
Injection mode	Split-less	Split-less
Injection volume	1 µL	1.5 µL
Interference temperature	270	270
Carrier gas	Purified nitrogen	Helium
Flow rate	1 mL min^{-1}	1 mL min^{-1}
Oven program	60°C (2 min) Increase to 120°C at 5°C min^{-1} Increase to 300°C at 30°C min^{-1} (8 min)	50°C (1 min) Increase to 90°C at 2°C min^{-1} Increase to 270°C at 25°C min^{-1} (10 min)
Detection mode (Scan)	*m/z* 50 to 500	*m/z* 100-800
Detection mode (SIM)		
Signature ions	3-MCPD-TMS- *m/z* 116, 119, 147 1,3-DCP-TMS- *m/z* 93, 151, 154	3-MCPD-HFB- *m/z* 289, 291, 453 1,3-DCP-HFB- *m/z* 75, 111, 113
Quantifying ions	3-MCPD-TMS- *m/z* 239 1,3-DCP-TMS- *m/z* 185	3-MCPD-HFB- *m/z* 253 1,3-DCP-HFB- *m/z* 275

3.7 Sample Concentration Calculation

To determine the concentration of 3-MCPD and 1,3-DCP contaminants in the food samples, the following formula was employed:

$$\text{Concentration, mg L}^{-1} = (\text{Ratio of 3-MCPD or 1,3-DCP})\ (1/C) / \text{sample weight, g} \quad \text{(Eq. 3.1)}$$

Where C is the slope of the calibration curve.

The calibration curves were plotted using the response ratio of 3-MCDP/1,4 butanediol or 1,3-DCP/1,4 butanediol against the concentration of 1,3-DCP/3-MCPD in mg L^{-1}.

3.8 Method Optimization

Three important parameters (temperature, incubation time and catalyzer volume) must be optimized to generate a derivatization process that results in the lowest cost and shortest reaction time. To effectively optimize the parameters, two optimization strategies were utilized: point-to-point optimization and Box-Behnken statistical experimental design. Theoretical statistical optimization provides an estimated overview graph of the optimized parameters, while a point-to-point experiment complements the findings.

For the temperature optimization, the incubation temperature was set at 25°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C, and 95°C, the incubation period was fixed at 20 min and the catalyzer volume was 10 μL. Secondly, for each incubation period (5, 10, 15, 20, 25,

30, 35, 50 ,and 50 min), the temperature was fixed at 35°C and the catalyzer volume at 10 µL. Lastly, for the catalyzer volume optimization (5, 10, 15, 20, 25, and 30 µL), the incubation temperature and period were 35°C and 10 min, respectively.

3.9 Method Validation

Method validation LOD and LOQ were estimated according to the guidelines provided by EURACHEM (Magnusson & Örnemark, 2014). Briefly, target analyte concentrations (0.0005 mg L^{-1} for 3-MCPD and 0.0010 mg L^{-1} for 1,3-DCP) close to the GC quantification limit were prepared and derivatized. The concentration derivatizations (0.0005 mg L^{-1} for 3-MCPD and 0.0010 mg L^{-1} for 1,3-DCP) were then repeated 10 times. The mean and standard deviation of the obtained concentration were calculated and fitted into equations 3.2 and 3.3.

$$LOD \cong X_{M1} + 3_{SM1} \quad \text{(Eq. 3.2)}$$

$$LOQ \cong X_{M1} + 10_{SM1} \quad \text{(Eq. 3.3)}$$

Where X_{M1} is the mean concentration near the blank solution and $_{SM1}$ is the standard deviation for the concentration near blank solution.

Inter-day and intra-day precision evaluations were conducted to prove the ruggedness of the developed method. For inter-day precision (intermediate), three concentrations of 3-MCPD and 1,3-DCP (low: 0.005 mg L^{-1}; medium: 0.02 mg L^{-1}; and high: 1.00 mg L^{-1}) were selected for validation to be repeated 3 times (N=3) for 6 days. For intra-day precision (repeatability), the selected concentration derivatization was repeated 6 times a day with every experiment repeated 3 times.

1,4-Butanediol was selected as an alternative to the 3-$MCPD_{d5}$ internal standard. To validate 1,4-BD as an internal standard and 1,5-PD as a surrogate standard, half of the collected samples were extracted according to section 3.4 (without additional 1,4-BD and 1,5-PD), and derivatized and quantified with GC-MS. The absence of derivatized 1,4-BD-TMS and 1,5-PD-TMS from all extracted samples will confirm the suitability for selection as internal and surrogate standards for this study.

3.10 Dietary Intake Data

Dietary intake data on soy sauce consumption among Malaysians was obtained with permission from the Institute of Public Health (IKU), Malaysia. There are 13 states and 3 federal territories in Malaysia. To effectively cover a representation of the whole population, all states and federal territories were divided into enumeration blocks (EBs): 187 urban EBs and 150 rural EBs, for a total of 337 EBs in Malaysia. Each EB consists of 80-120 living quarters (LQs) and each LQ has 500-600 people on average. There were 4044 LQs in total upon survey completion. The respondents chosen for this survey were aged 18-59, with no pregnancies or currently breast-feeding status, and did not suffer from illnesses requiring special diets.

The estimated dietary intake (EDI) of soy sauce among Malaysians was calculated with the following formula:

$$\text{EDI} = \text{residual level of 3-MCPD or 1,3-DCP in soy sauces} \times \text{consumption of soy sauces/body weight.} \quad \text{(Eq. 3.4)}$$

3.11 Statistical Analysis

IBM SPSS Statistic version 23 was used to statistically analyze the data from the MANS survey. A graphic representation of the average soy sauce consumption by different population groups in Malaysia was prepared in Microsoft Office 2016. According to **Table 2.3**, 3-MCPD is found in very high concentrations in soy sauce and acid-HVP contaminated soy sauce. Thus, the soy sauces with average concentrations detected were selected to estimate the exposure of Malaysians to 3-MCPD and 1,3-DCP. Malaysians' soy sauce consumption was investigated through different types of independent variables among the entire population. The independent variables selected are age group, income level, education background, ethnicity, and gender. All responses collected were grouped according to different independent variables and one dependent variable (soy sauce consumption) and the means were investigated (T-test and one-way ANOVA). The calculated means were then compared to investigate the significant differences between each category of independent variables. To further investigate the soy sauce consumption trend in Malaysia, the collected data was also analyzed with regression and correlation analyses. These analyses should provide insight into the relationship between different independent variables affecting soy sauce consumption.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 3-MCPD-TMS and 1,3-DCP-TMS

Figure 4.1 illustrates the full chromatogram of 1,3-DCP-TMS, 1,4-BD-TMS, 3-$MCPD_{d5}$-TMS, 3-MCPD-TMS and 1,5-PD-TMS. Besides the deuterated 3-MCPD and 3-MCPD, all target compound peaks are sharp and well-separated. **Figure 4.2** (part a) displays the full electron ionization (EI) scan of 1,3-DCP-TMS and 3-MCPD-TMS with the scanning mass range of *m/z* 100-500 at 1 s $scan^{-1}$. No tailing, fronting, splitting, and rounded or negative peaks are observed for the target peaks in this study. This indicates that the correct oven temperature and GC column were selected to separate and detect 1,3-DCP-TMS and 3-MCPD-TMS.

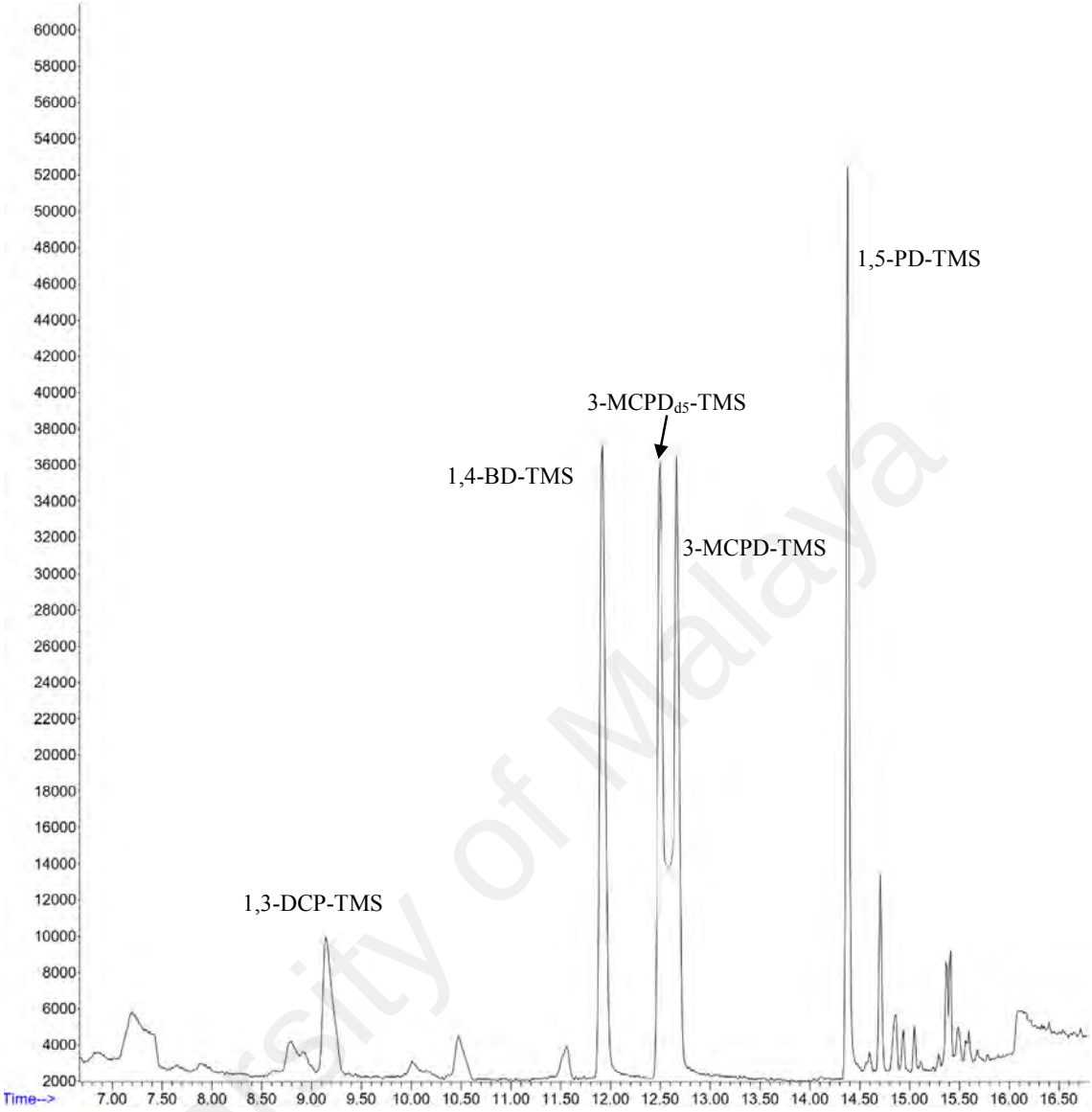

Figure 4.1: Full chromatogram of 1,3-DCP-TMS, 1,4-BD-TMS, 3-MCPD$_{d5}$-TMS, 3-MCPD-TMS and 1,5-PD-TMS.

Figures 4.2 (part b) and **Figure 4.2** (part c) represent the characteristic ions detected from the EI scan of 1,3-DCP-TMS and 3-MCPD-TMS. Few characteristic ions are shared by 1,3-DCP-TMS and 3-MCPD-TMS, such as *m/z* 55, 73, and 93. This is due to the similar molecular formula fractions shared by 1,3-DCP and 3-MCPD, as the derivatization process only replaces the hydroxyl group with a trimethylsilyl group. To

increase the sensitivity of the developed method, some ions were selected as specific characteristic ions and one ion as a quantification ion. With the selected ions, the SIM mode can be run in the GC program to specifically detect only the selected ions, thus lowering the LOD and LOQ of the developed method. For 1,3-DCP-TMS, *m/z* 93, 151, and 154 were selected as specific characteristic ions and the quantifying ion was *m/z* 186. For 3-MCPD-TMS, the selected characteristic ions were *m/z* 116, 119, and 147, while *m/z* 239 was selected as the quantifying ion.

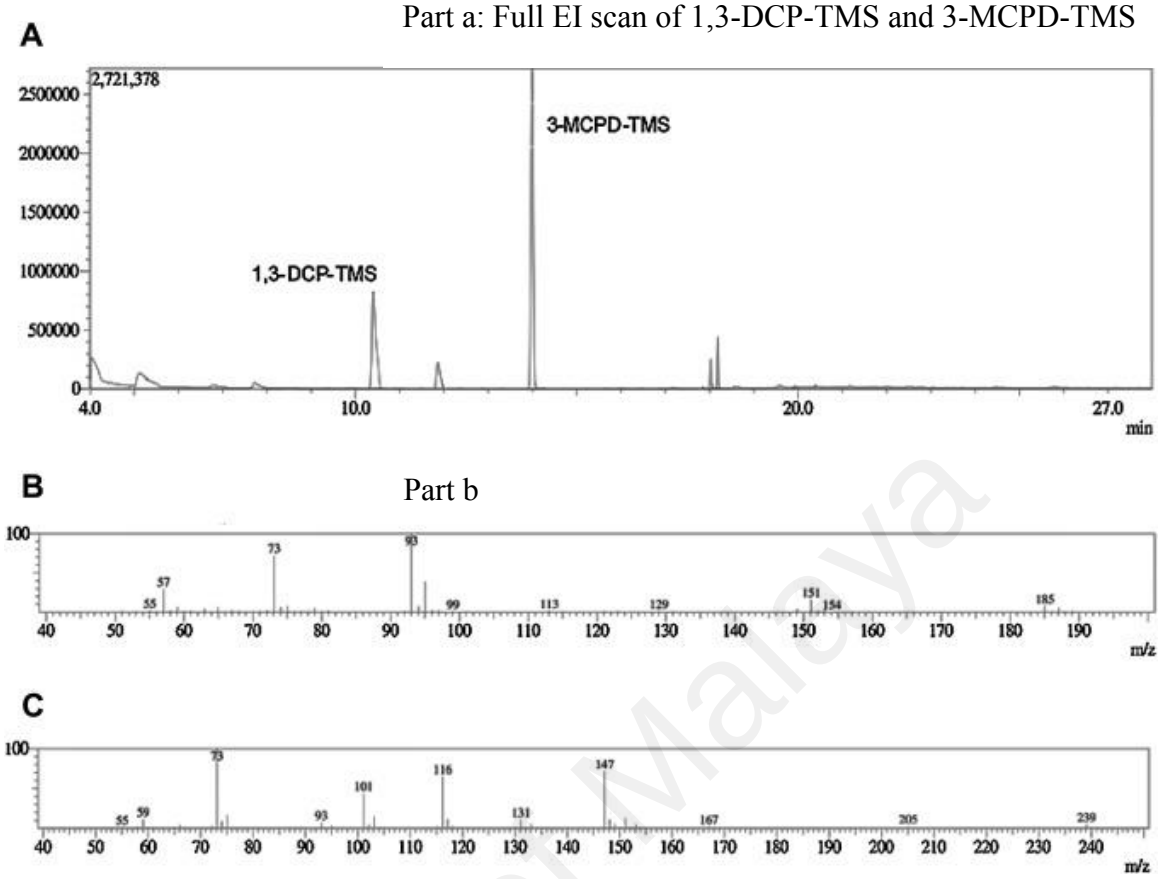

Figure 4.2: (a) Full EI scan of 1,3-DCP-TMS and 3-MCPD-TMS, (b) Characteristic ions of 1,3-DCP-TMS (*m/z* 93, 151, and 154), (c) Characteristic ions of 3-MCPD-TMS (*m/z* 116, 119, and 147).

Compared to other silylation agents (BSTFA and MSTFA), the HMDS-TMSOTf combination exhibits the additional advantage that derivatization can be performed at room temperature (25°C) in a short derivatizing period (10 min) (Lee et al., 2016). **Table 4.1** presents a detailed comparison of the advantages, disadvantages and challenges of HMDS, BSTFA, MSTFA, HFBI and HFBA. According to the **Table 4.1**, the end product from TMS silylation is thermally more stable then acylation, as HFB-derivatized chloropropanols will dissociate with time. Moreover, the HMDS derivatization agent is the only agent that permits conducting the procedure at room temperature (25°C). The other derivatizing agents require heating temperatures between 40 and 230°C. The storage condition for the silylation agent is generally less tedious than for acylation, with the latter requiring a dry, -20°C environment. The other advantage of HMDS is the very low cost of analysis, which is the lowest among all catalysts listed in **Table 4.1**, followed by BSTFA, MASTA, HFBA and HFBI. Generally, the silylation derivatizing agent costs less than the acylation agent. To further compare the cost per extraction, the assessed cost includes that of the derivatizing agent, extraction stationary phase, and extraction solvent (**Table 4.2**). The cost per sample with the current method is approximately 58% lower than the AOAC method. This signifies that the cost reduction for the currently developed method is not limited to the derivatizing agent but it includes the extraction stationary phase and solvent used.

Table 4.1: Comparison between various derivatizing agents used to derivatize 1,3-DCP and 3-MCPD simultaneously.

	HMDS	BSTFA	MSTFA	HFBI (AOAC Standard Method)	
ation mechanism	Silylation	Silylation	Silylation	Acylation	Acyl
ed end product storage at room ure)	Thermally more stable	Thermally more stable	Thermally more stable	Thermally less stable (end-products dissociate with time)	Ther prod time
ing agent storage s	Dry, room temperature	Dry, room temperature	Dry, 2-8°C	Dry, -20°C	Dry,
ation period	10 min	10 min-2 hrs	40 s	20 min	20 m
ation temperature	Room temperature	40-80°C	230°C	60°C	60°C
	1,3-DCP: 0.0028 mg L^{-1} 3-MCPD: 0.0008 mg L^{-1}	1,3-DCP: 1.4 ng mL^{-1} 3-MCPD:11.2-0.04 mg kg^{-1}	1,3-DCP:0.35 ng g^{-1} 3-MCPD: 3.91 ng g^{-1}	1,3-DCP: 10 µg kg^{-1} 3-MCPD: 5-100 µg kg^{-1}	1,3-I 3-MC
	1,3-DCP: 0.0043 mg L^{-1} 3-MCPD: 0.0011 mg L^{-1}	1,3-DCP: 4.8 ng mL^{-1} 3-MCPD: 34.5 ng mL^{-1}	1,3-DCP: NA 3-MCPD: NA	1,3-DCP: NA 3-MCPD: 10 µg kg^{-1}	1,3-I 3-MC
nts used for	GC-MS	GC-FID, GC-MS	GC-MS	GC-ECD, GC-MS, GC-MS/MS	GC-I
cts	Ammonia	Hydrogen fluoride	N-methyl trifluoroacetamide	No acid byproducts	Acid
erivatizing agent quoted by Sigma-	Very low (0.62 USD/mL)	Low (13.20 USD/mL)	High (14.62 USD/mL)	Very high (61.10 USD/mL)	High
	TMSOTf	TMCS	TMCS	-	Triet
es	This study	(Bodén *et al*., 1997; Gonzalez *et al*., 2011; Kissa, 1992)	(M. R. Lee *et al*., 2007)	(AOAC, 2002; Bel-Rhlid *et al*., 2004; Brereton *et al*., 2001; Hamlet & Sutton, 1997; Robert *et al*., 2004; van Bergen *et al*., 1992)	(Abu W. C Matt 2000

Comparison between various derivatizing agents used to derivatize 1,3-DCP and 3-MCPD simultaneously.

Table 4.2 Cost per sample extraction including derivatizing agent, stationary phase and extraction solvent costs.

Method	Chemicals	Usage (USD)	Total (USD)
Current Study	HMDS	0.25	18.64
	TMCS	0.12	
	Alumina	0.88	
	Dichloromethane	17.3	
	Ethyl acetate	0.09	
AOAC	HFBI	1.21	31.81
	Diethyl Ether	25.88	
	Extrelute	4.61	
	Iso-octane	0.11	

To fully understand the HMDS-TMSOTf combination derivatization mechanism, the derivatization of 3-MCPD is illustrated in **Figure 4.3** (Lee et al., 2016). Briefly, the addition of TMSOTf to HMDS will form a temporary compound (bis(trimethylsilyl) ammonio)trimethyl(((trifluoromethyl)sulfonyl)oxy)silicate(IV). This compound is very reactive and will react with the hydroxyl group in 3-MCPD to form 1-chloro-3-(trimethylsilyl)propan-2-ol, a partially silylated 3-MCPD. (bis(trimethylsilyl)ammonio) trimethyl(((trifluoromethyl)sulfonyl)oxy)silicate(IV) will turn into trimethyl(methyl (trimethylsilyl)ammonio)(((trifluoromethyl)sulfonyl)oxy) silicate(IV), which will further react with the second hydroxyl group in the partially silylated 3-MCPD to become (3-chloropropane-1,2-diyl)bis(trimethylsilane), a fully derivatized 3-MCPD–TMS. Following derivatization, (bis(trimethylsilyl)ammonio)trimethyl (((trifluoromethyl)sulfonyl)oxy) silicate(IV) will become N,N,1,1-tetramethyl-1-(((trifluoromethyl)sulfonyl)oxy)silan-aminium, with ammonia being a byproduct that turns into TMSOTf, which can be combined and activated by free HMDS in the derivatizing mixture. The generated ammonia gas as a byproduct is not a concern, since it will evaporate during oven heating in the GC and will not affect detector

performance. Unlike the byproducts from BSTFA derivatization, hydrogen fluoride will form hydrofluoric acid upon contact with water, which will deteriorate the GC column lining.

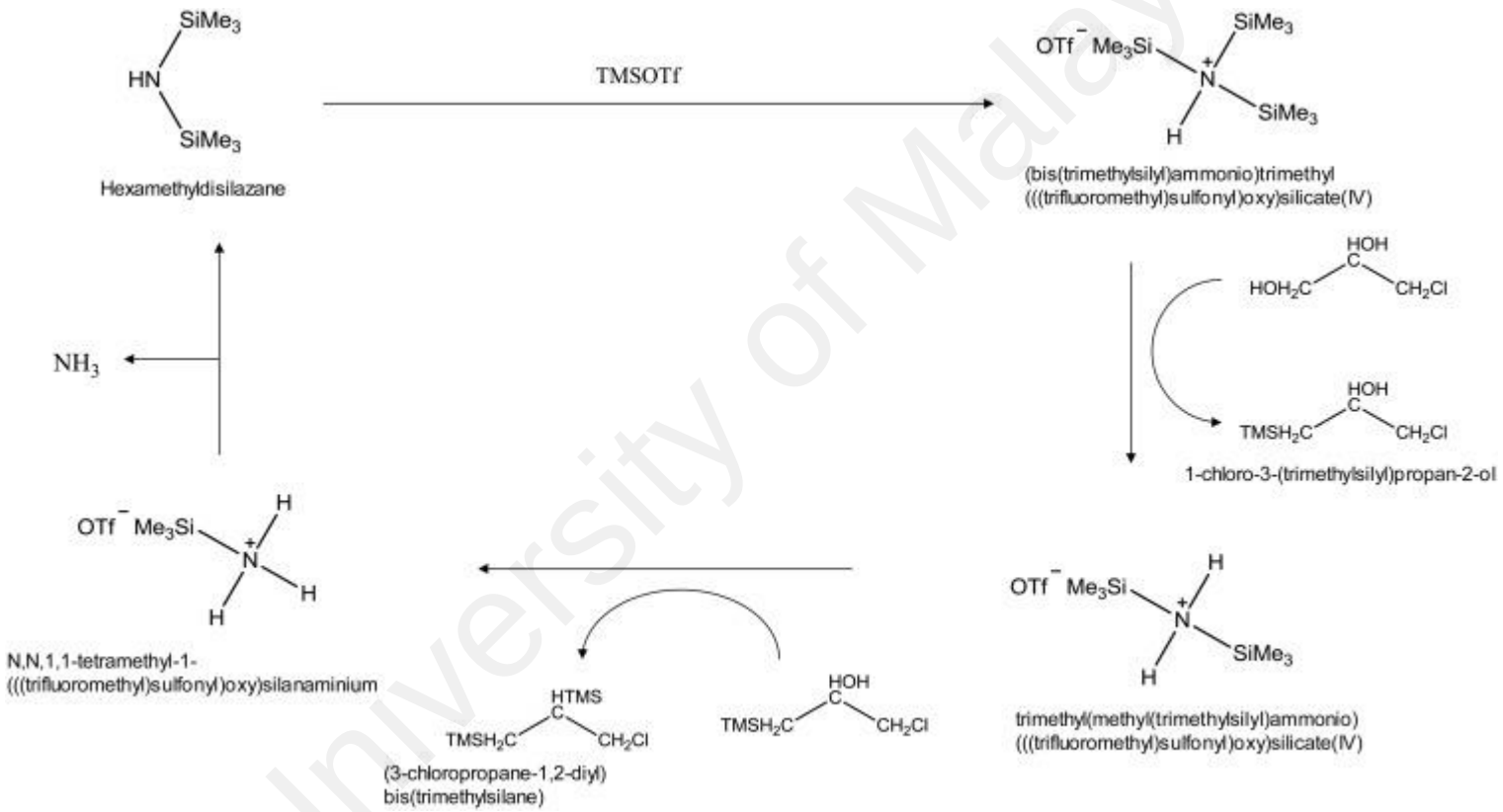

Figure 4.3: Silylation mechanism of HMDS-TMSOTf on 3-MCPD.

Recent studies have shown that 3-MCPD and 1,3-DCP not only occur in acid-HVP soy sauces but are also present in food products that contain acid-HVP contaminated raw ingredients, especially ready-to-eat foods, such as instant noodles and sausages. The wide spectrum of 3-MCPD and 1,3-DCP demands an effective sample cleanup for a lower limit of detection (LOD) and limit of quantification (LOQ). With this objective in mind, an effective sample extraction method that utilizes a minimal quantity of extraction solvent is required to reduce the cost and detection period of 3-MCPD and 1,3-DCP. The developed extraction method must also be able to extract 3-MCPD and 1,3-DCP from a wide range of sample matrices, from liquid to solid samples.

Two optimization strategies were used to determine the right combination amounts of derivatizing agent and catalyst required to derivatize 3-MCPD and 1,3-DCP. Beside Box-Behnken optimization, point-to-point optimization strategy was utilized to pinpoint the optimum derivatizing condition for the proposed derivatization mechanism. Not only is the derivatizing agent cheaper, but the developed procedure additionally reduces the cost of operation by employing a cheaper internal standard (1,4-BD) and surrogate standard (1,5-PD) for the concentration and method recovery calculation.

To effectively quantify the unknown concentrations of 1,3-DCP and 3-MCPD-TMS, a calibration curve with 7 calibration points was developed. The standardized concentrations selected as calibration points were: 0.01 mg kg^{-1}, 0.02 mg kg^{-1}, 0.05 mg kg^{-1}, 0.50 mg kg^{-1}, 1.00 mg kg^{-1}, 5.00 mg kg^{-1}, and 10 mg kg^{-1}. In this study, the R^2 values obtained for 1,3-DCP-TMS and 3-MCPD-TMS were both 0.999 (**Figure 4.4** part a). The 0.02 mg kg^{-1} calibration point was significant, since it is the lowest maximum tolerable limit established for 3-MCPD, while the LOD for 3-MCPD-HFB was 0.01 in line with the AOAC method to detect 3-MCPD (Brereton *et al*., 2001).

Figure 4.4 part b displays the calibration curve for 1,3-DCP-HFB and 3-MCPD-HFB. Both HFB derivatives of chloropropanols have an R^2 value of 0.999 as well. However, it is noticeable that the calibration curve slopes for 1,3-DCP-HFB and 3-MCPD-HFB are significantly different from each other, indicating a difference in terms of sensitivity. In contrast, the calibration curve slopes obtained for 1,3-DCP-TMS and 3-MCPD-TMS are nearly identical, since HMDS-TMSOTf derivatized 1,3-DCP and 3-MCPD with similar sensitivity and affinity. This is due to the difference between the HFBI and HMDS-TMSOTf reaction mechanisms. HMDS-TMSOTf reacted with both 1,3-DCP and 3-MCPD via the same S_N2 mechanism, resulting in a similar calibration curve (Orata, 2012). **Figure 4.5** presents the calibration curve for the 1,5-PD surrogate standard. 1,5-PD was derivatized with both HMDS-TMSOTf and HFBI derivatizing agents. It is not necessary to prepare a 1,4-PD internal standard calibration curve, because all calibration curves in **Figure 4.4** and **Figure 4.5** are relative to the 1,4-PD-TMS responses from MS.

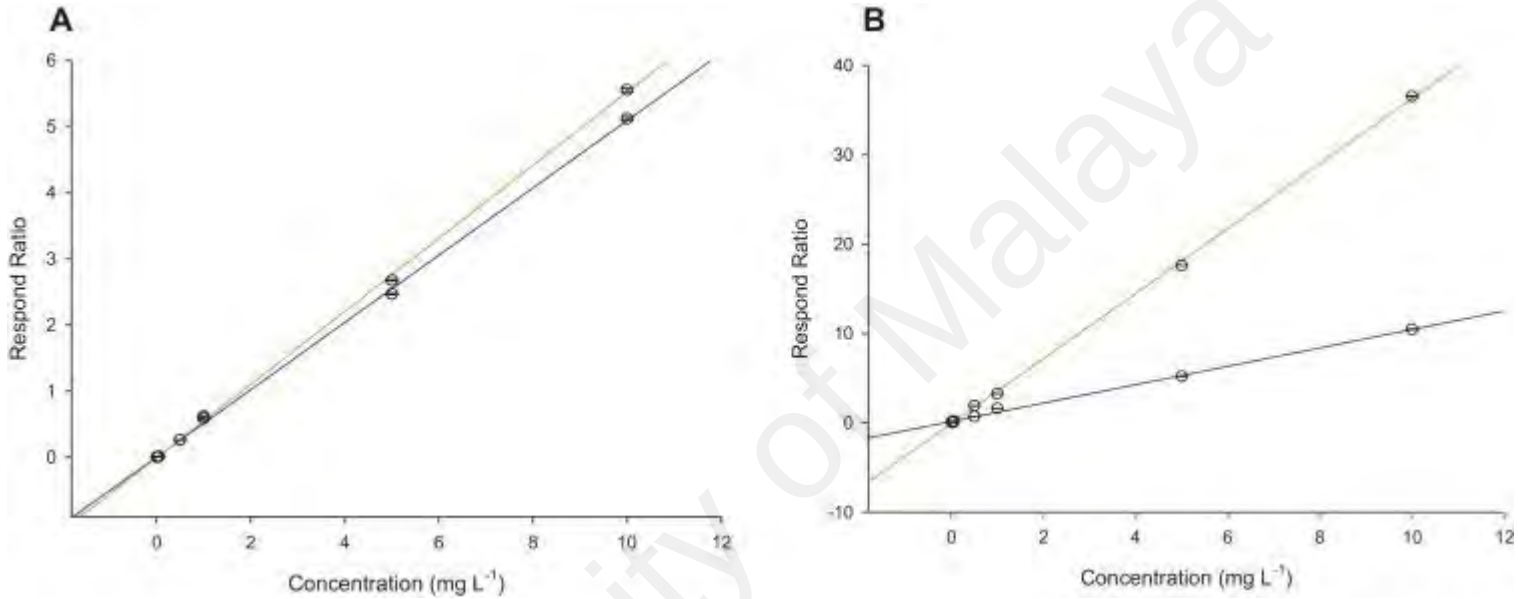

Figure 4.4: (a) Calibration curves of 1,3-DCP-TMS (blue line) and 3-MCPD-TMS (orange line). (b) Calibration curves of 1,3-DCP-HFB (blue line) and 3-MCPD-HFB (orange line). The error bars are the standard deviations from the triplicates performed for the calibration curves.

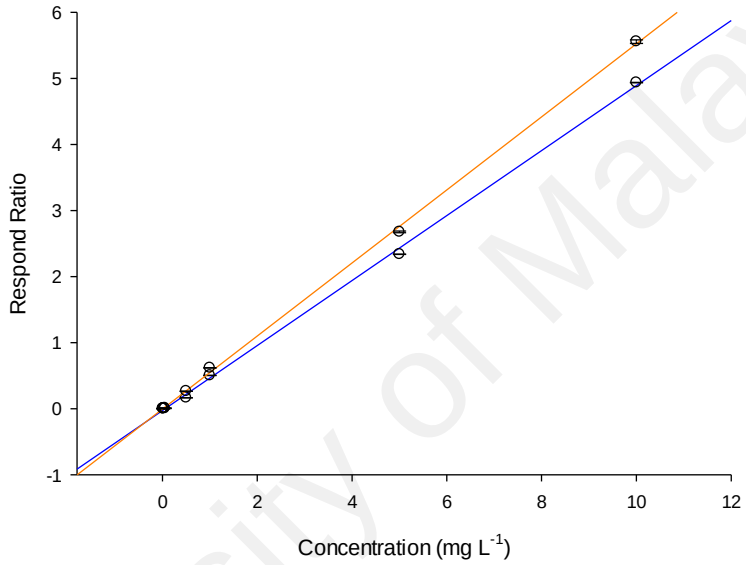

Figure 4.5: Calibration curves of 1,5-PD-TMS (blue line) and 1,5-PD-HFB (orange line). The error bars are the standard deviations from the triplicates performed for the calibration curves.

4.2 Optimization of HMDS-TMSOTf Derivatization

There were 3 parameters in HMDS-TMSOTf derivatization that required optimization: derivatization time, derivatization temperature, and TMSOTf volume. Among the 2 optimization strategies, point-to-point experimental derivatization complements the Box-Behnken experimental design optimization. With the 3 available parameters, Box-Behnken statistical analysis can be performed and the optimized derivatization condition can be predicted.

4.2.1 Box-Behnken Experimental Design Optimization

The effect and interactions of derivatization time, temperature, and TMSOTf volume were investigated using the Box-Behnken statistical experimental design. The ANOVA results from response surface quadratic model optimization for HMDS-TMSOTf derivatization are given in **Table 4.3** and the response surface plots are shown in **Figure 4.6**, **Figure 4.7**, and **Figure 4.8**. The second-order polynomial equation established for HMDS-TMSOTf derivatization is as follows:

Peak Area = −1.66058E + 0.05 + 294.04823 × Time + 27507.60568 × Temperature − 5022.48561 × Catalyst Volume − 20.21674 × Time × Temperature + 11.22133 × Time × Catalyst Volume − 21.39070 × Temperature × Catalyst Volume + 40.98592 × Time2 – 343.64132 × Temperature^2 + 233.84978 × Catalyst Volume^2

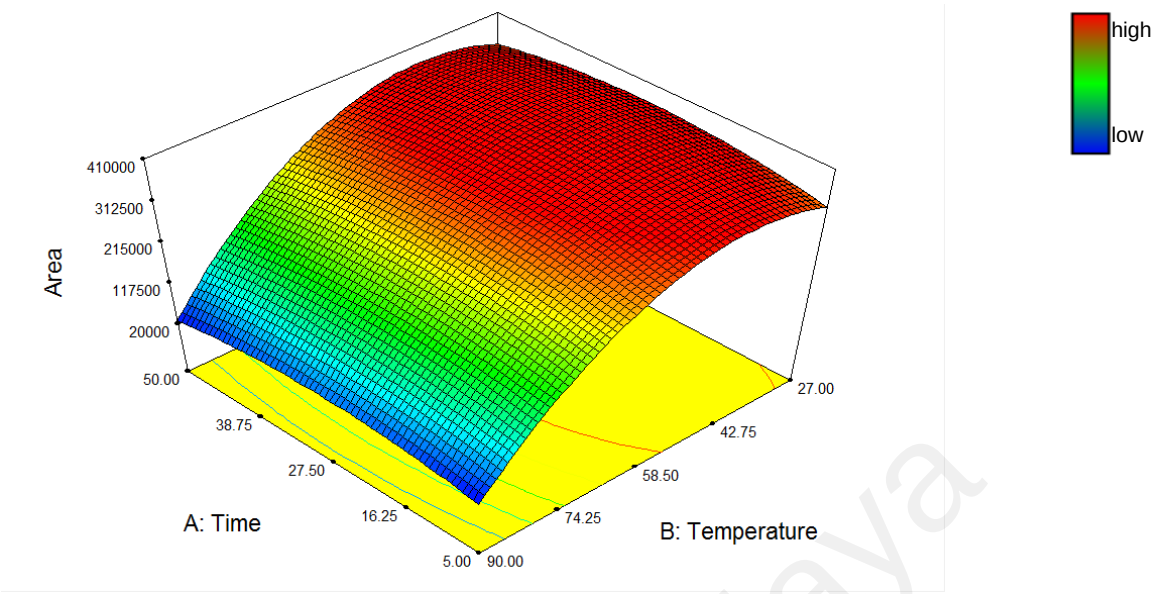

Figure 4.6: Response surface plot of response area between time and temperature.

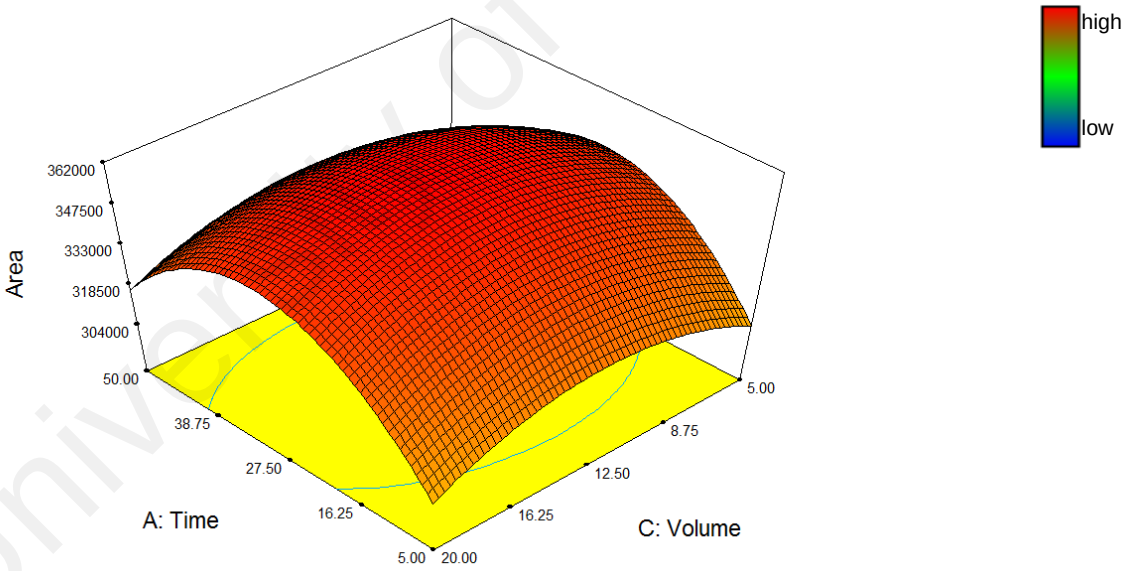

Figure 4.7: Response surface plot of response area between time and TMSOTf volume.

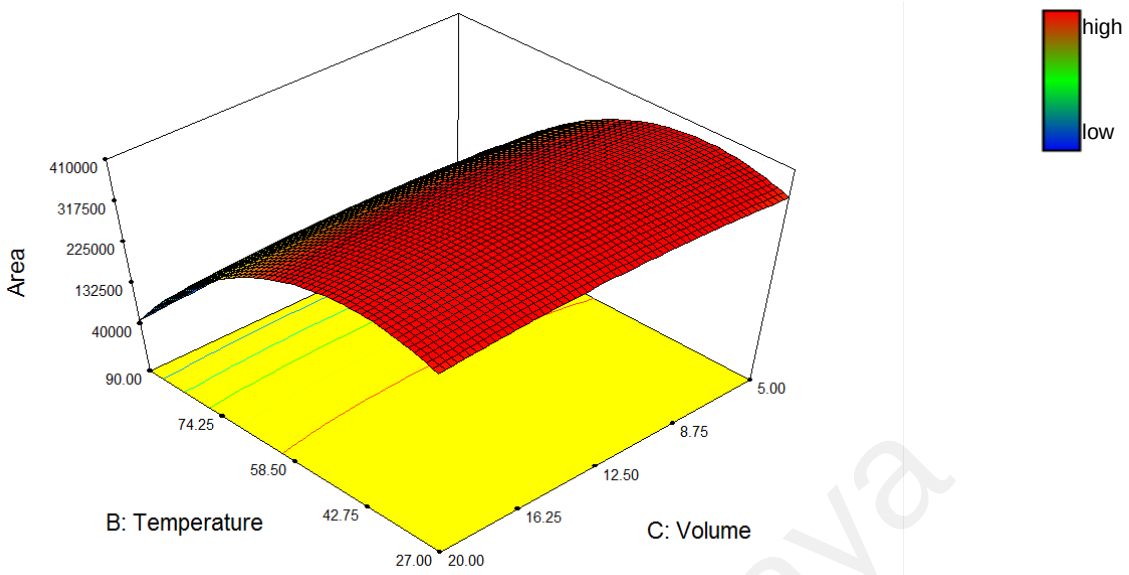

Figure 4.8: Response surface plot of response area between temperature and TMSOTf volume.

The ANOVA regression model indicated that the quadratic model was suitable for predicting the peak response for HMDS-TMSOTf derivatization, according to the Fisher F-test (Fmodal = 64.08) and a very low probability value (P-model <0.0001) (**Table 4.3**). The chance that the "Model F-value" would occur due to noise was only 0.01%. In a prediction model, if the predicted R^2 is closely related to the adjusted R^2, the model is accurate. If the difference between the predicted R^2 and adjusted R^2 is more than 0.20, then there is error in the data or the model. The R^2 value predicted for HMDS-TMSOTf derivatization was 0.9569, which is in reasonable agreement with the adjusted R^2 value of 0.9726. This adequate method precision was used to measure the signal-to-noise ratio. The ratio for the model established in this study was 20.114, and a ratio of >4 was desirable. The obtained ratio indicates that the model can be implemented to predict the optimum conditions for HMDS-TMSOTf derivatization to obtain the highest peak response levels. By substituting the desired derivatization

period, temperature and TMSOTf volume values, the prediction model was able to calculate the peak areas. Subsequently, the calculated peak area values were plotted to find the optimum conditions according to different laboratory and procedure settings.

Table 4.3: ANOVA for response surface quadratic model generated from the Box-Behnken experimental design.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2.87831E+11	9	31981226188	289.5068572	< 0.0001
A-Time	119127330.1	1	119127330.1	1.078388263	0.3336
B-Temperature	1.87038E+11	1	1.87038E+11	1693.14267	< 0.0001
C-Volume	12500000	1	12500000	0.113155002	0.7464
AB	53985756.25	1	53985756.25	0.488700669	0.5071
AC	25000000	1	25000000	0.226310004	0.6488
BC	0	1	0	0	1.0000
A^2	5287685329	1	5287685329	47.86624355	0.0002
B^2	89720383764	1	89720383764	812.1848169	< 0.0001
C^2	1158348782	1	1158348782	10.4858367	0.0143
Residual	773275581.3	7	110467940.2		
Lack of Fit	773275581.3	3	257758527.1		
Pure Error	0	4	0		
Cor Total	2.88604E+11	16			

4.2.2 Derivatization Time Optimization

Derivatization time optimization was carried out for 5 to 10 min (**Figure 4.9**). For this process, the derivatization temperature was set at a constant 35°C and TMSOTf volume of 10 µL. There were no significant changes in the recorded response area between 5 and 25 min. However, there was an increase in response area from 25 to 30 min and no significant change from 30 to 50 min derivatization period. Similar trends were observed in the response surface plots for Box-Behnken experimental design optimization (**Figure 4.6** and **Figure 4.7**). The Student's T-test was performed to calculate the significant difference between the response areas at 5 to 50 min of derivatization and no significant difference was found. HMDS-TMOSTf can be used for 5 min derivatization, which is shorter than the HFBI derivatization period (20 min).

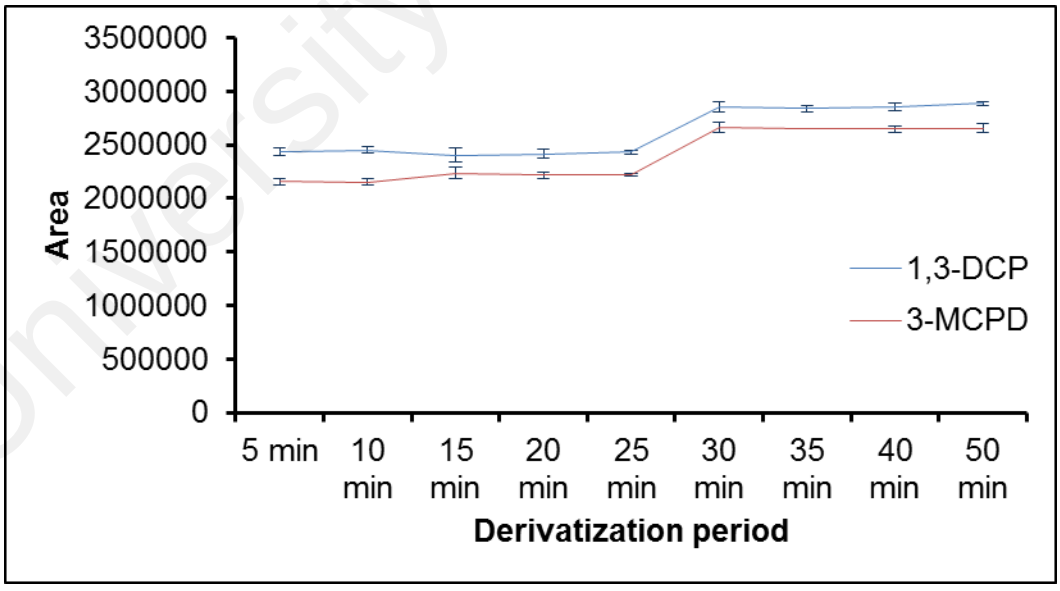

Figure 4.9: Experimental optimization of derivatization period.

4.2.3 TMSOTf Volume Optimization

The TMSOTf volumes employed for this optimization were from 5 to 30 µL (**Figure 4.10**). There were no significant changes in response area for the entire TMSOTf volume. A similar trend was observed for the Box-Behnken experimental design (**Figures 4.7** and **Figure 4.8**), where the addition of TMSOTf volume did not increase the GC-MS response area. This was due to the catalyst recovery at the end of derivatization (**Figure 4.3**), so a minimal catalyst volume was required to activate the derivatization process.

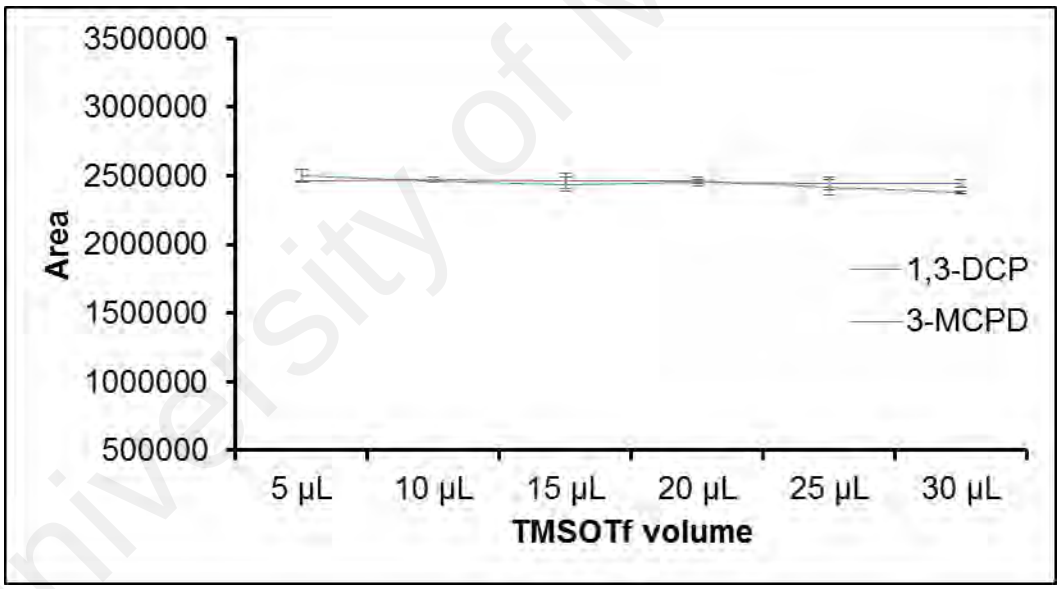

Figure 4.10: Experimental optimization of TMSOTf volume.

4.2.4 Temperature Optimization

Temperature optimization was carried out between 25 and 95°C with 10°C increments (**Table 4.11**). The response area increased from 25°C to 55°C. From 55°C, there was a reducing response peak area trend until 95°C. In the Box-Behnken surface plot, the same trend is observed, whereby the response peak area starts decreasing after 55°C. The decreasing response peak area at high temperature was due to the vaporization of 1,3-DCP-TMS and 3-MCPD-TMS. Meanwhile, temperatures above 77°C majorly decreased the response area, as the boiling point of TMSOTf is 77°C. A Student's T-test was carried out between 25°C and 55°C and no significant difference was found between the areas recorded for both derivatization temperature. Since there was no significant difference between 25°C and 55°C, the newly developed method of derivatizing 1,3-DCP and 3-MCPD can be applied at room temperature (25°C). This is an advantage over other derivatization techniques where hot temperatures are required for derivatization (**Table 4.1**) and if limited heating ovens are available in the laboratory. Derivatization can be done directly after the addition of HMDS-TMSOTf, without the need to wait for the oven to reach the required incubation temperature.

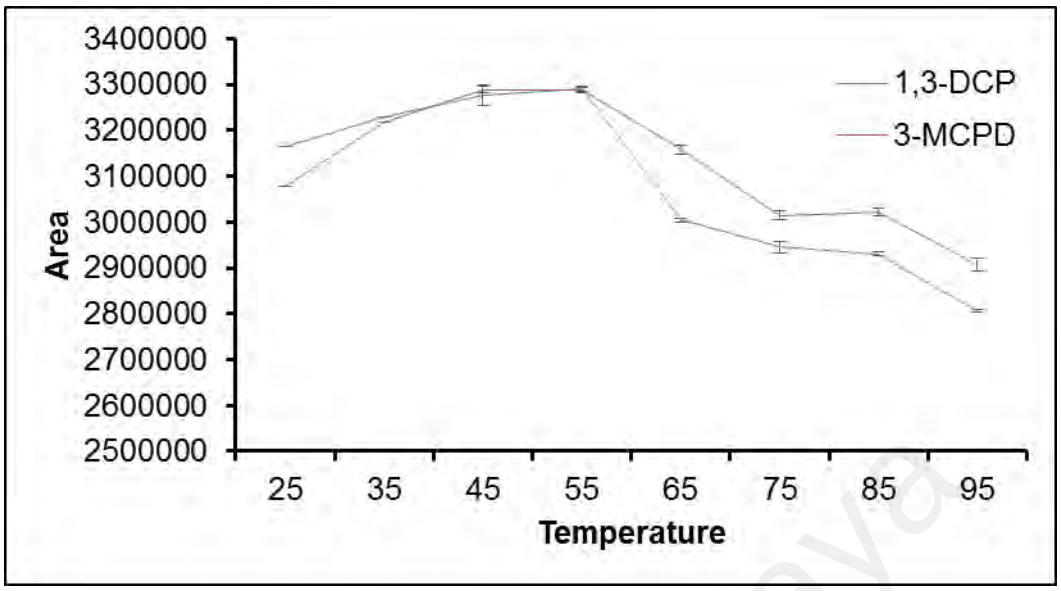

Figure 4.11: Experimental optimization of derivatization temperature.

4.3 Method Validation

To calculate the limit of detection (LOD) and limit of quantification (LOQ), the signal-to-noise (S/N) ratio of the peak signal was calculated based on the lowest concentration that was closest to the blank solution. Preparation and calculation details are available in section 3.9. Determining the LOD was based on an S/N ratio of 3, while LOQ determination was based on S/N of 10. The LOD of developed method validation in this study for 1,3-DCP-TMS and 3-MCPD-TMS was 0.0028 mg L^{-1} and 0.0008 mg L^{-1}, respectively. On the other hand, the LOQ for 1,3-DCP-TMS was 0.0043 mg L^{-1} and for 3-MCPD-TMS it was 0.0011 mg L^{-1}. Although the derivatization mechanism of HMDS was the same as BSTFA and MSTFA (silylation), the LOD and LOD obtained from this study was lower than the aforementioned derivatization agents. In other words, HMDS-TMSOTf has higher sensitivity compared to BSTFA and MSTFA. Although the final product of HMDS-TSOTf, BSTFA and MSTFA was the same, lower

LOD and LOD were reported in this study. This is due to the differences in detection instrument. Bodén et al. (1997) carried out method development using a Hewlet Packard (HP) GC coupled with a 5972 HP selective detector, while M. R. Lee et al. (2007) used the Varian Star 3400CX GC with Varium Saturn 2000 ion-trap MS. Compared to the MS used by Bodén et al. (1997), the more sensitive Agilent 5975 MS was employed in this research. Compared to the LOD and LOQ reported by M. R. Lee et al. (2007), the differences in GC manufacturer resulted in various sensitivities and logarithms utilized by the software for the signal-to-noise calculation and peak deconvolution. On the other hand, the LOD and LOQ of HMDS-TMSOTf were lower than the HFB-derivatized 1,3-DCP and 3-MCPD (**Table 4.1**), indicating that silylation was more sensitive than acylation in terms of derivatizing 1,3-DCP and 3-MCPD. The developed HMDS-TMSOTf derivatization can be used for 3-MCPD quantification according to the EU, since the LOD and LOQ were lower than the maximum tolerable limit of 0.02 mg kg^{-1}. The linearity range for 3-MCPD-TMS and 1,3-DCP-TMS in the current study was from 0.01 mg kg^{-1} to 10 mg kg^{-1}.

The selection of 1,4-BD as the internal standard and 1,5-PD as the surrogate standard was validated by the random selection of half of the samples, extraction and derivatization. All selected samples exhibited undetectable concentrations of 1,4-BD-TMS and 1,5-PD-TMS, signifying that they are suitable for implementation as internal standards. In fact, the recommendation for 1,4-BD as an internal standard in 3-MCPD quantification was also reported in the 3-MCPD quantification of powder condiments (Wang et al., 2008).

4.3.1 Intra-day and Inter-day Precision Validation

Intra-day and inter-day precision validation was done to investigate the reproducibility and repeatability of the developed method. The results were then compared with the AOAC method to identify the superior method (**Table 4.4**).

The percentage of RSD obtained from intra-day precision was 3% for both 1,3-DCP-TMS and 3-MCPD-TMS at 1.00 mg L^{-1}. As a similar trend, the intra-day precision for both HMDS-TMSOTf-derivatized 1,3-DCP and 3-MCPD was 4%. However, for the lowest concentration of 0.005 g L^{-1}, the RSD% for 3-MCPD-TMS was higher (5%) while for 1,3-DCP-TMS it was 4%. On the other hand, the intra-day precision for the highest concentration of 1,3-DCP-HFB recorded the highest RSD% (7%), while 3-MCPD-HFB had 1% RSD precision. For the intermediate concentration, HFBI-derivatized 1,3-DCP and 3-MCPD showed 2% and 5% RSD, respectively. 1,3-DCP-HFB and 3-MCPD-HFB were not detected at the lowest concentration (0.005 mg L^{-1}). The RSD% from HMDS-TMSOTf showed a more similar trend with 3 to 5%, while the RSD fluctuation for HFB derivatization was high at 1% to 7%.

Comparatively, the inter-day precision for the highest concentrations of HMDS-TMSOTf-derivatized 1,3-DCP and 3-MCPD produced the same RSD% (2%). The RSD% obtained from inter-day precision for 1,3-DCP-TMS and 3-MCPD-TMS was 5% in both cases. For the lowest concentration (0.005 mg L^{-1}), the RSD% for 1,3-DCP and 3-MCPD was 4% and 5%, respectively. Conversely, the RSD% for HFBI-derivatized 1,3-DCP and 3-MCPD was 4% and for intermediate concentration it was 0.02 mg L^{-1}. The RSD% was 2% for 1,3-DCP-HFB and 7% for 3-MCPD-HFB. Likewise, with intra-day precision, the lowest concentration of 0.005 mg L^{-1} was undetectable for HFBI-

derivatized 1,3-DCP and 3-MCPD. Similar to the intra-day precision test, the fluctuation in RSD% for HMDS-TMSOTf derivatization was lower than for HFBI derivatization.

All RSD% values obtained for the intra-day and inter-day precision tests on HMDS-TMSOTf were lower than 11% (% required by AOAC for precision and reliability (Committee, 1998)). These findings validate the developed method in terms of precision and reliability of the obtained results.

Table 4.4: Comparison of intra-day and inter-day precision of 1,3-DCP and 3-MCPD between HMDS-TMSOTf and HFBI derivatization.

% RSD	Concentration (mg L^{-1})	Number of replicates (N)	HMDS–TMSOTf		HFBI	
			1,3-DCP	3-MCPD	1,3-DCP	3-MCPD
Intra-day precision	0.005	3	4	5	ND	ND
	0.02	3	4	4	2	5
	1.00	3	3	3	7	1
Inter-day precision	0.005	3	4	5	ND	ND
	0.02	3	5	5	2	7
	1.00	3	2	2	4	4

ND: Not detected.

4.4 Analysis of Food Samples

To ensure that the method developed in this study is accurate, an actual food analysis was performed and compared between HMDS-TMSOTf and HFBI derivatizations. This should enable a comparison between the quantified concentrations and the efficiency of the newly developed method. For this stage, food samples were randomly purchased from the Malaysian market, consisting of solid, liquid and paste matrix samples. Due to the complexity of the food samples, a cleanup of the samples was required to remove

any hydrophilic compounds. The extraction procedure utilized is solid phase extraction, where alumina oxide is the stationary phase and dichloromethane is the mobile phase. The chromatography column applied is a self-packed column, in which the food sample matrices could be modified accordingly. This allowed the solid samples to be packed together in the column during column preparation. The sample cleanup facilitated a cleaner chromatogram background and less noise so it would be easier and more accurate to analyze the results later. With less interference, the amount of derivatizing agent required can be lowered as well.

The results of the comparison between HMDS-TMSOTf and HFBI derivatization in food samples are presented in **Table 4.5**. The concentrations were calculated based on a calibration curve, which was constructed based on the response ratio between the derivatized target analyte and 1,4-butanediol (1,4-BD). Unlike the AOAC method (AOAC, 2002) that utilizes deuterated 3-MCPD as an internal standard, 1,4-BD was selected as an internal standard in the current study. This is because the deuterated internal standard peak tends to stay very close to the target analyte peak and sometimes even overlap, making the result analysis part more difficult (**Figure 4.1**). The derivatized 1,4-BD peak was easily separated from the derivatized 3-MCPD peak, and the molecular structure of 1,4-BD was similar to that of 3-MCPD (with the presence of 2 hydroxyl groups). Hence, 1,4-BD is a suitable replacement for the expensive deuterated internal standard. To validate the methodology recoveries, the sample mixtures were fortified with surrogate standard during sample preparation. 1,5-Pentadiol (1,5-PD) was selected as the surrogate standard because it is molecularly quite similar to 3-MCPD and 1,3-DCP. Applying the surrogate standard can eliminate the need for matrix spike replication, consequently shortening the time required to analyze

and validate the results per sample analysis. For the data to be valid, the recovery percentage determined was between 80 and 120%. As seen in **Table 4.5**, all 1,5-PD recoveries are within the required limits, thus proving collected data validity.

Student's T-test was utilized to investigate the differences in levels of HMDS-TMSOTf and HFBI-derivatized 1,3-DCP and 3-MCPD in actual sample analysis. There was no significant difference between the concentration obtained from HMDS-TMSOTf and HFBI derivatization in all food samples tested ($p = 0.69$). Therefore, it can be said that the extraction procedure and derivatization method developed are accurate and precise according to the comparison with the AOAC analysis results.

Table 4.5: Comparison between HMDS-TMSOTf and standard HFBI derivatization method in actual food sample analysis (solid, liquid and paste)

Sample	**Soy Sauce**		**Sweet Sauce**		**Chicken Flavored Seasoning**		**Oyster Sauce**	
Derivatizing Agent	HMDS	HFBI	HMDS	HFBI	HMDS	HFBI	HMDS	HFBI
Recovery (%)	92 ± 5.77	94 ± 2.74	92 ± 1.88	98 ± 7.12	96 ± 0.61	93 ± 1.00	94 ± 3.37	83 ± 4.38
1,3-DCP (mg kg^{-1})	0.03 ± 3.32E-3	0.04 ± 3.00E-3	ND	ND	0.06 ± 5.18E-3	0.08 ± 2.78E-3	0.05 ± 7.61E-4	0.06 ± 5.87E-3
3-MCPD (mg kg^{-1})	0.01 ± 2.45E-4	0.01 ± 3.88E-4	ND	ND	0.01 ± 2.85E-4	ND	ND	ND

ND: Not detected

4.5 3-MCPD and 1,3-DCP Levels Identified in Soy Sauces, Dark Soy Sauces, Oyster Sauces and Chicken Cube Seasonings Sold in Malaysia

The occurrence of possible carcinogens (3-MCPD and 1,3-DCP) in soy sauce has been reported in many countries (**Table 2.3**). However, there is no detailed research report available on the occurrence of 3-MCPD and 1,3-DCP in soy sauce products sold in Malaysia. For this study, 16 soy sauce samples, 12 dark soy sauce samples, 9 oyster sauce samples and 6 chicken cube seasoning samples were collected from the States of Selangor and Kuala Lumpur and the levels of 3-MCPD and 1,3-DCP were quantified (**Figure 4.12**).

Among the collected samples, 58% of dark soy sauces, 60% of soy sauces, and 45% of oyster sauces did not contain detectable concentrations (below the LOD of HMDS-TMSOTf derivatization method validation) of 3-MCPD and 1,3-DCP. Generally, almost half of the soy sauces, dark soy sauces, oyster sauces, and chicken cube flavorings available in Malaysia are produced through the fermentation process. However, 5 soy sauce samples contained detectable concentrations of 3-MCPD, which are still below the Malaysian maximum tolerable limit of 0.02 mg kg^{-1}. These soy sauces might not be fully fermented but may be mixtures of traditional and artificial soy sauces (FAO, 2004). With the mixed mass production of soy sauces, it is still possible to carefully control the 3-MCPD in the final products. All chicken cube flavoring samples collected from the market contained detectable levels of 3-MCPS and 1,3-DCP. Among these, only 1 sample was found to contain 3-MCPD levels above 0.02 mg kg^{-1}. This study shows that the monitoring of 3-MCPD levels in Malaysia remains focused on soy sauces, dark soy sauces, and oyster sauces only. Beside these soy-related sauces, there is a risk that other food products are contaminated with 3-MCPD and 1,3-DCP, especially

when artificial soy sauces are one of the raw ingredients, such as in the chicken cube flavoring samples tested in this study. Food safety enforcers should extend the scope of 3-MCPD and 1,3-DCP monitoring and not only focus on soy sauce-related products but also on those containing soy sauce as a raw ingredient. Unmonitored products expose consumers to the risk of 3-MCPD and 1,3-DCP contamination.

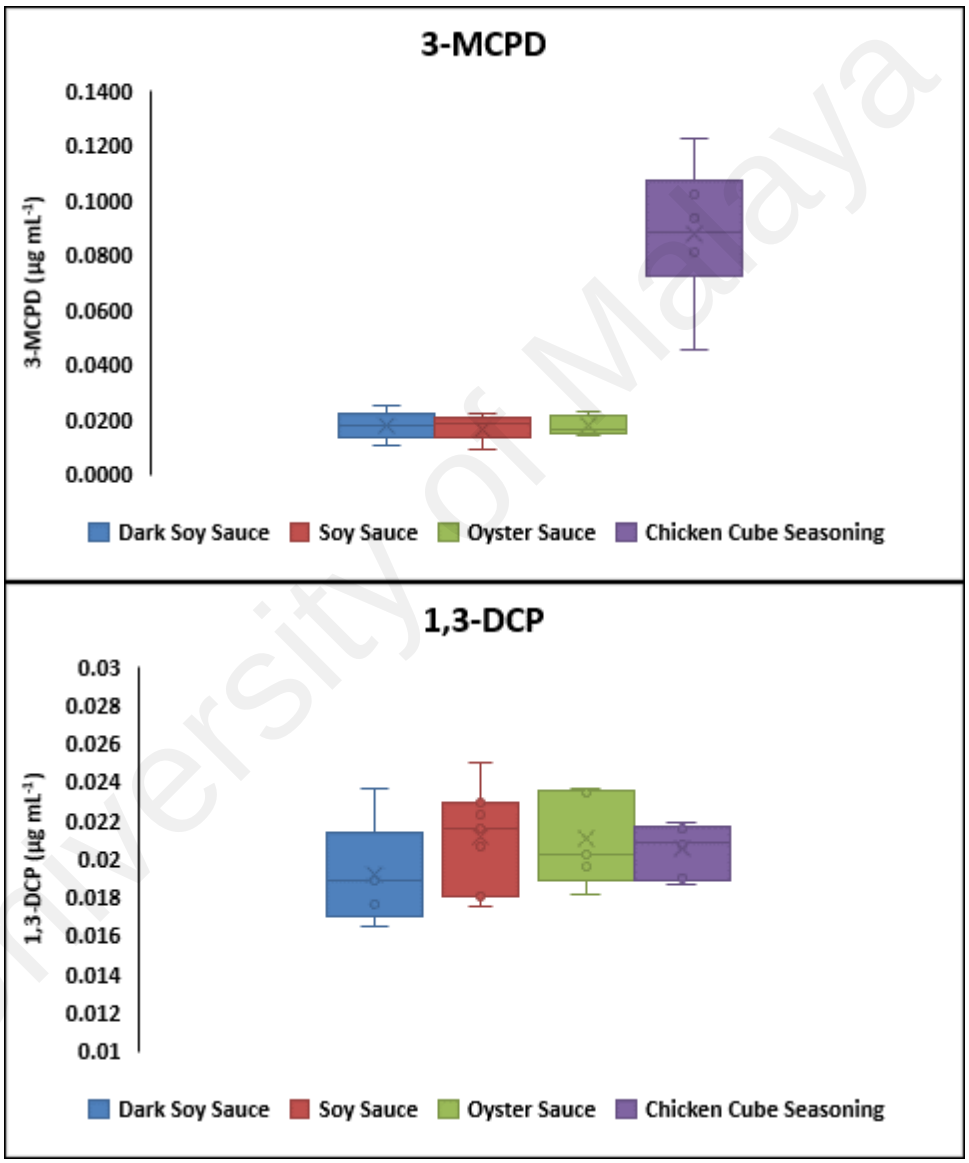

Figure 4.12: 3-MCPD and 1,3-DCP in dark soy sauce, soy sauce, oyster sauce and chicken cube seasoning. The box plot represents the median, interquartile range and standard deviation.

4.6 Dietary Intake of Soy Sauce in Malaysia

Dietary intake of soy sauce for this study was based on data provided by the Institute of Public Health Malaysia. A total of 3000 respondents participated from both Peninsular and East Malaysia, and this population is large enough to represent Malaysia in general. Data on soy sauce dietary intake in Malaysia is significant, as it depicts consumption patterns, which enables effective problem solving associated with public health crises, for example kidney failure and hypertension among certain population groups. The dietary intake of soy sauce in Malaysia was compared in terms of gender, education background, income group, age and ethnicity.

4.6.1 Gender

A gender comparison regarding soy sauce consumption is important, as it provides insight into which gender is more susceptible to health problems due to soy sauce consumption. In this survey, 1388 male and 1612 female respondents participated (**Figure 4.13**). The mean soy sauce consumption by a male per day is 6.26 g, while the female population in Malaysia consumes 6.03 g of soy sauce per day (**Figure 4.14**). Evidently, the male population consumes more soy sauce than the female population.

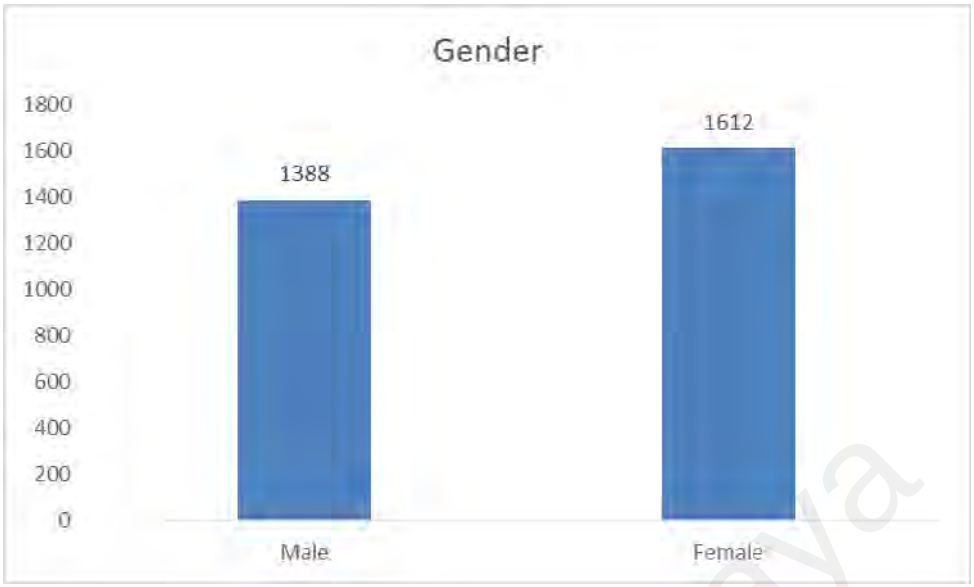

Figure 4.13: Respondent frequency based on gender.

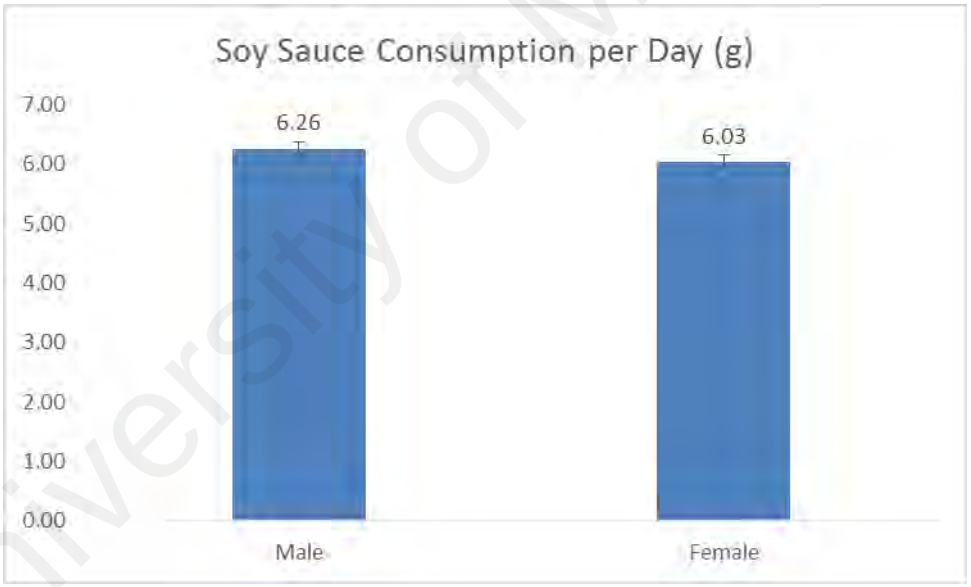

Figure 4.14: Soy sauce consumption based on gender.

An independent T-test was carried out to compare male and female soy sauce consumption patterns, and no significant difference was found: $t(2956) = 0.524$, $p = 0.600$. However, there was a significant difference in the prevalence of hypertension between males and females in Malaysia (Naing *et al*., 2016). Soy sauce intake is

normally associated with sodium intake, whereby lower soy sauce intake will contribute to lower sodium intake (Anderson *et al*., 2010). Although this may be true, other factors contribute to the risk of hypertension as well, for example family history, lack of physical activity, low potassium and low vitamin D diet, and alcohol abuse (Mayo, 2016).

4.6.2 Age

To identify the soy sauce consumption levels among different age groups in Malaysia, ages were grouped into 18-19, 20-29, 30-39, 40-49 and 50-59. These groups represent various generations in Malaysia. For the 18-19 age group, only a small number of respondents participated (132), which is 4.4% from all recorded respondents. For the other age groups the numbers of respondents were higher, with an average of 20% from the total percentage of respondents: 20-29 age group (689 respondents), 30-39 (832), 40-49 (757), and 50-59 (590) (**Figure 4.15**).

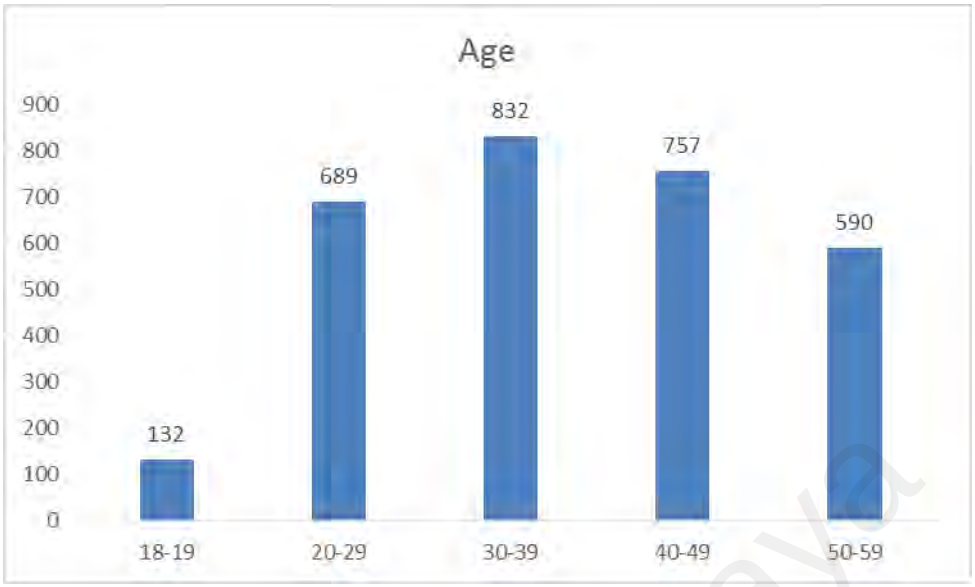

Figure 4.15: Respondent frequency based on age group.

One-way ANOVA showed no significant difference between various age groups ($t(2957) = 1.165$, $p = 0.324$). The lowest consumption of soy sauce was exhibited by the population aged 50 to 59 (5.54 mL day^{-1}), while the highest consumption was recorded for the population aged 20 to 29 (6.81 mL day^{-1}) (**Figure 4.16**). The decreasing soy sauce consumption in the elderly population may be due to the low sodium intake recommended to reduce the risk of hypertension (Anderson *et al*., 2010). On the other hand, the high consumption by those aged 20 to 29 may be due to the preference for having ready-to-eat meals over self-prepared meals, as these are working adults who often do not have time to cook themselves. This is the age group most vulnerable to 3-MCPD contamination, since ready-to-eat food contains lots of seasoning to make it more appetizing.

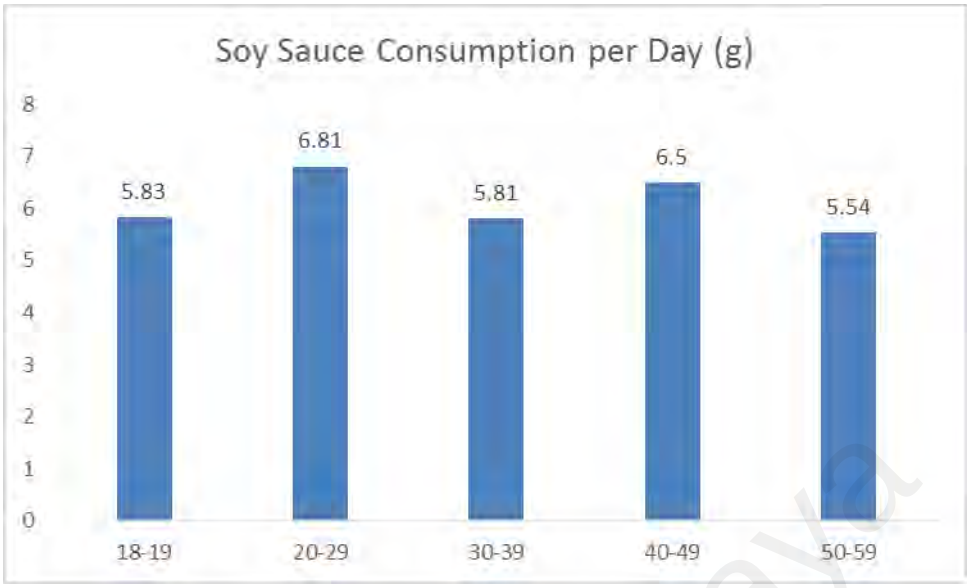

Figure 4.16: Soy sauce consumption based on age group.

4.6.3 Education background

This parameter was selected to study whether education background has an impact on soy sauce consumption in Malaysia. The education levels were roughly divided into "no formal education," "primary level," "secondary level" and "tertiary level." The number of respondents with a secondary level of education is the highest (1463), followed by tertiary education with 751 respondents and primary level with 623 respondents (**Figure 4.17**).

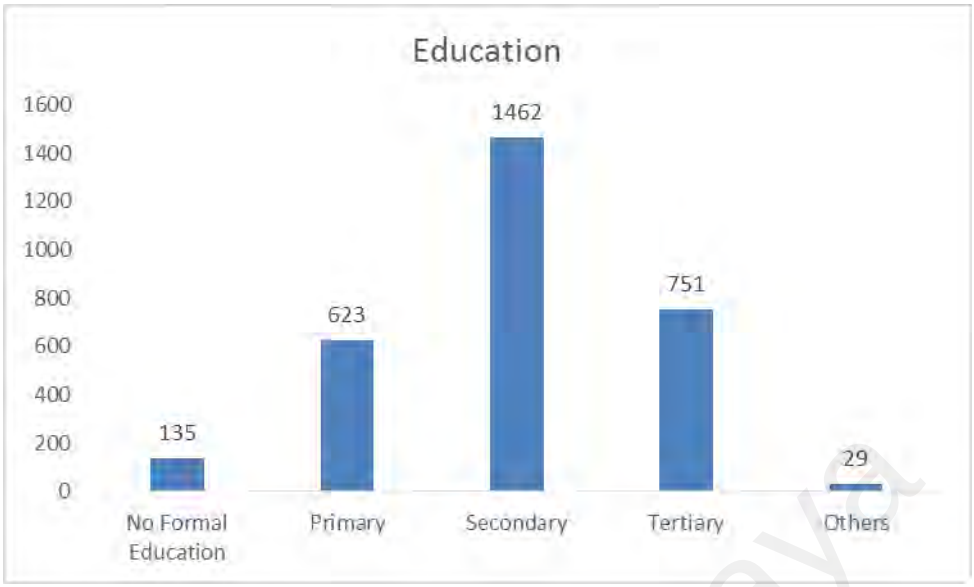

Figure 4.17: Respondent frequency based on education level

ANOVA indicted there is no significant difference in soy sauce consumption between various education levels ($p = 0.051$). The p-value obtained is 0.001, a little over the significant level of 0.050, meaning no significant difference was concluded. Malaysians with primary school level education exhibited the highest mean soy sauce consumption (7.19 mL day^{-1}) (**Figure 4.18**), followed by those with secondary school level of education (6.41 mL day^{-1}), and then participants with no formal education (5.83 mL day^{-1}). Despite no significant difference in soy sauce consumption among education levels, the population with a tertiary education background generally consumes less soy sauce compared to the other respondents. In reducing the risk of 3-MCPD exposure in Malaysia, government intervention is essential to educate the population on the importance of moderate soy sauce consumption.

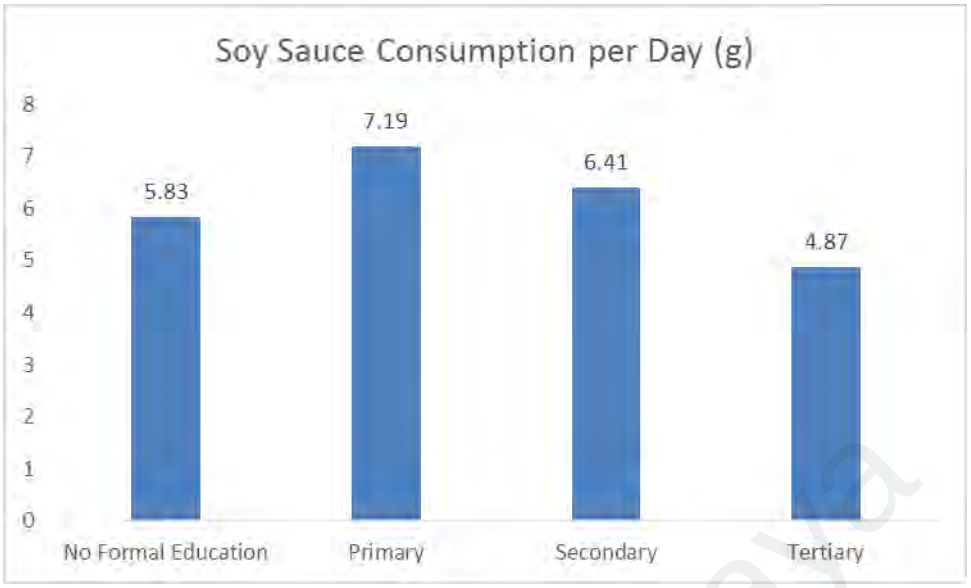

Figure 4.18: Soy sauce consumption based on education level.

4.6.4 Income groups

The income of Malaysians was grouped in 3 major categories based on monthly income, namely "low-income (⩽ RM2299.99)," "medium income (RM2300.00 – 5999.99)" and high income (⩾ RM6000.00)." The low-income group had the highest number of respondents (1836), the high-income group had the lowest number of respondents (333), and the medium income group had 801 respondents (**Figure 4.19**).

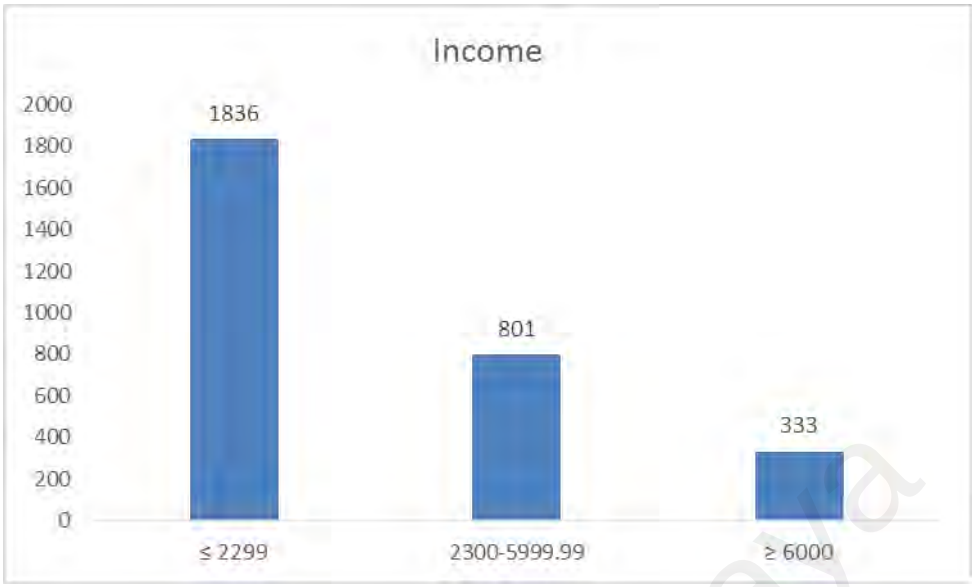

Figure 4.19: Respondent frequency based on income group.

One-way ANOVA showed a significant difference between all income groups ($t(2927) = 9.637$, $p = 0.000$). There is a noticeable decreasing trend with increasing income. The high-income group consumes the least soy sauce (4.18 mL day^{-1}) and the highest consumption of soy sauce is by the low-income group, who earn below RM2299.99 per month (6.85 mL day^{-1}) (**Figure 4.20**). Post hoc tests (Tukey HSD) demonstrated a significant difference in soy sauce consumption between the groups with ≤RM2299.99 and RM2300-5999.99 income ($p = 0.007$). There is also a significant difference between the ≤RM2299.99 and ≥RM6000.00 income groups ($p = 0.001$).

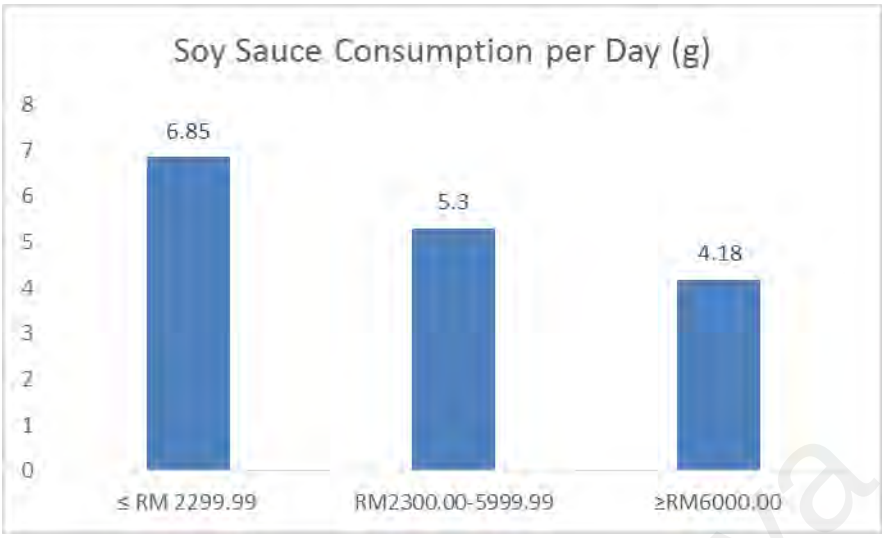

Figure 4.20: Soy sauce consumption based on income group.

4.6.5 Ethnicity

Malaysia is a unique country home to several ethnicities with diverse cultures and behaviors. The dominant ethnicities are Malay, Chinese and Indian. In this survey, 1477 Malay (49.2%), 519 Chinese (17.3%), and 133 Indian (4.4%) respondents were recorded. Other native races are found in Malaysia, which were grouped under "other natives" and consisted of 689 respondents (**Figure 4.21**).

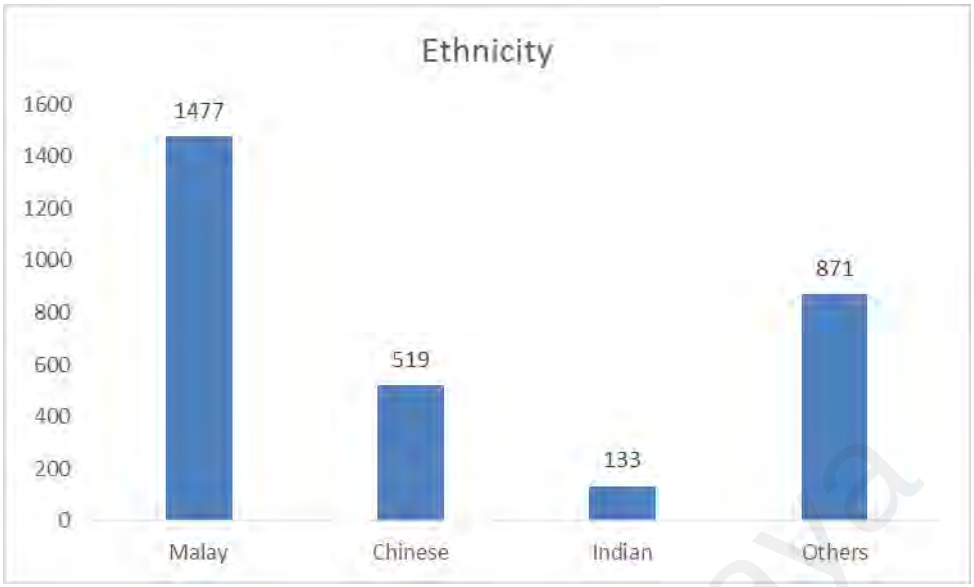

Figure 4.21: Respondent frequency based on ethnicity.

It is interesting to note there is a significant difference ($p = 0.04$) in terms of soy sauce consumption by the 3 main ethnicities, i.e. Malay, Chinese and Indian. Tukey's post hoc analysis showed a significant difference in soy sauce consumption by the Indian and Malay populations ($p = 0.017$). There is no significant difference between the Malay and Chinese populations ($p = 0.994$). Different ethnicities have their own unique ways of preparing food, which contributes to the difference in soy sauce consumption. The Malays consume the most soy sauce per day (6.51 mL), followed by the Chinese (6.26 mL) and lastly, the Indians (3.15 mL) (**Figure 4.22**). Numerous traditional Malay dishes require soy sauce for seasoning and coloring, thus contributing to the highest daily consumption of soy sauce. As for the Chinese, most of the time soy sauce is preferred as a dipping sauce at meal times. Indians tend to use less soy sauce in food preparation and at meal times, as they tend to use herbs for flavoring.

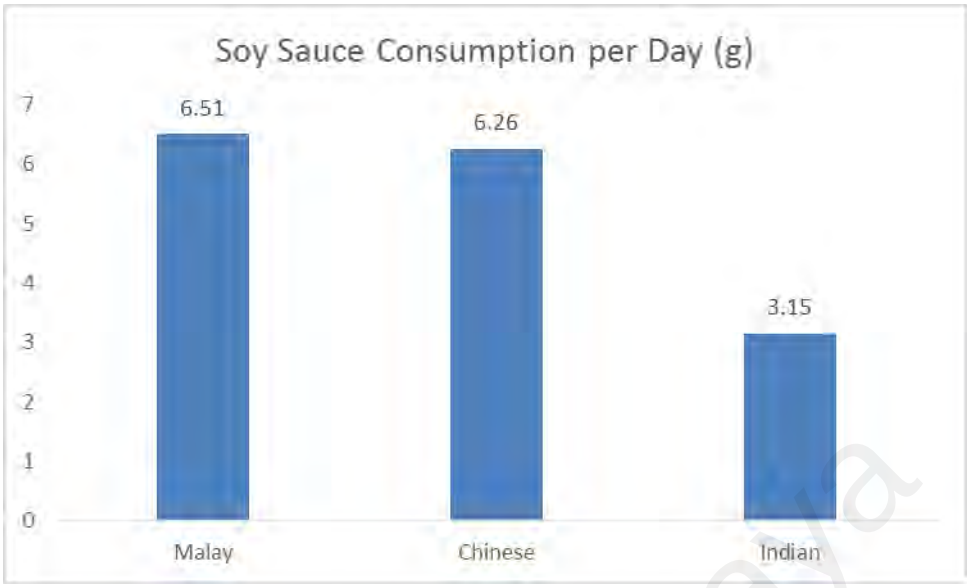

Figure 4.22: Soy sauce consumption based on ethnicity.

4.7 Regression and Correlation analyses of Soy Sauce Consumption by the Malaysian Population

The study of regression and correlation is divided into several stages. The relationship between age, education and income groups (independent variables) was initially investigated for the entire population of Malaysia. This shows the trend of soy sauce consumption in terms of age, education and income among all participating respondents. Then regression and correlation analyses were done on different population groups, for instance gender and ethnicity. Here, the independent variables were investigated according to a certain population in Malaysia to assess how the independent variables correlate with each other and with daily soy sauce consumption.

4.7.1 Regression and Correlation Analyses for All Respondents

The soy sauce intake of several Malaysian groups was examined based on age, education and income groups. The age and income groups are denoted by "years" and "RM" respectively, while education is a categorical variable. According to an initial analysis based on the Pearson correlation coefficient the independent variables are weakly related to each other, implying the absence of multicollinearity; meanwhile, the dependent variable has a relatively weak and negative correlation with the independent variables (**Table 4.6**). The regression analysis yielded a multiple correlation coefficient (R) of 0.085, meaning there is a weak relationship between the dependent variable and the set of predictors. The multiple coefficient of determination (R^2) derived was 0.007, where only 0.7% of variants in soy sauce intake per day are explained by the age, education level and income groups. In general, the regression analysis yielded a significance regression model with an F value of 7.155 and significance at the 0.01 level (p = 0.000). The derived model is:

Soy sauce intake per day (g) = 9.619 – 0.275*Age group – 0.277*Education level– 1.276*Income group

With a one-unit increase in age group, there is a 0.275unit decrease in soy sauce intake per day. Similarly, there is a 0.277unit decrease in soy sauce consumption with a one-unit increase in education level. Among all constant variables, the greatest reduction in soy sauce consumption is through the increase in income group, with a decrease of 1.276 units in consumption for a one-unit increase in income group.

Soy sauce intake for Malaysians appears to have an inverse relationship with the age, education level and income groups; the higher the age group, education level, or income

group, the lower the soy sauce intake per day is. As shown in **Table 4.7**, at the individual level, the age, education and income groups do not have a significant impact on soy sauce intake in Malaysia.

Table 4.6: Correlation matrix of variables.

	Soy Sauce Intake per Day (g)	Age Group	Education	Income Group
Soy Sauce Intake per Day (g)	1.000	-0.023	-0.040	-0.081
Age Group		1.000	-0.220	0.001
Education			1.000	0.370
Income Group				1.000

Table 4.7: Regression coefficients.

Independent Variable	Coefficient	T- value	Sig.
Age group	-0.275	-1.405	0.160
Education level	-0.227	-0.905	0.365
Income group	-1.276	-3.702	0.000

4.7.2 Gender

From section 4.6.1 it is evident that the average soy sauce consumption by the male population is higher than that by the female population. However, does the relationship between variables differ between individual populations? In the male population, the Pearson correlation coefficient indicates a weak, negative correlation between soy sauce intake and the age, education and income groups. A similar negative correlation was also observed in the female population. However, there is a slight difference in correlation patterns among independent variables for males and females (**Table 4.8**). In the male population, there is a positive correlation between the income and age groups: the higher the age group, the higher the income level recorded is. However, in the

female population the same correlation is negative, whereby the income group is inversely proportional to the age group. This means that the higher the age in the female population, the lower the income level recorded is.

The correlation trends among the male and female populations are as follows. There is a negative correlation between education level and age group, whereby the education level increases with the decrease in age group, as younger generations are graduating with higher education qualifications. There is also a positive correlation between income and education level, which is due to the growing educational opportunities offered by the government and private universities to younger generations in recent years. The increasing income with increasing education level is a norm, as higher education levels do provide more income.

Table 4.8: Correlation matrix of variables based on gender.

Population	Variables	Soy Sauce Intake per Day (g)	Age Group	Education	Income Group	Sig
Male	Soy Sauce Intake per Day (g)	1.000	-0.034	-0.038	-0.088	0.008
	Age Group		1.000	-0.148	0.062	
	Education			1.000	0.331	
	Income Group				1.000	
Female	Soy Sauce Intake per Day (g)	1.000	-0.012	-0.042	-0.075	0.02
	Age Group		1.000	-0.286	-0.052	
	Education			1.000	0.407	
	Income Group				1.000	

According to the regression analysis, the regression model for the male population is:

Soy sauce intake among the male population per day (g) = 9.853 – 0.310*Age group – 0.188*Education – 1.339*Income group

For the female population, the regression model is:

Soy sauce intake among the female population per day (g) = 9.374 – 0.231*Age group – 0.270*Education level – 1.210*Income group

The regression models show a similar trend between the male and female populations, whereby soy sauce consumption decreases as the constant variable unit increases. For example, for a one-unit increase in age group, there is a 0.310unit decrease in the male population and a 0.231unit decrease in the female population. Similarly, there is a 0.188unit decrease in males and a 0.270unit decrease in females with a one-unit increase in education level. The margin of decreasing soy sauce consumption is higher in the male population (1.339 unit) compared to the female population (1.210) when there is a one-unit increase in income group.

The multiple correlation (R) coefficient obtained for the male population is 0.094 and for the female population it is 0.079. The R values indicate there is a weak relationship between the independent and dependent variables. From **Table 4.9**, it can be concluded that the age group, education level and income group do not have a significant impact on soy sauce intake of the male and female populations.

Table 4.9: Regression coefficients based on gender.

	Independent Variable	Coefficient	T- value	Sig.
Male	Age group	-0.310	-1.152	0.249
	Education level	-0.188	-0.565	0.572
	Income group	-1.339	-2.780	0.006
Female	Age group	-0.231	-0.807	0.420
	Education level	-0.270	-0.719	0.472
	Income group	-1.210	-2.456	0.014

4.7.3 Ethnicity

As discussed in section 4.6.5, there is a significant difference in daily soy sauce consumption between various ethnicities in Malaysia. The correlation and regression analyses were intended to determine the correlation and regression between the independent variable and individual ethnicity populations (**Table 4.10**). The initial Pearson correlation analysis demonstrated the same trend for the Malay and Indian populations, with a decrease in soy sauce consumption with the increase in age group, education level and income group. However, for the Chinese population there is a positive correlation between soy sauce consumption and age group, with a trend of increasing soy sauce consumption with increasing age group. Regarding the correlation between independent groups, identical negative correlations were found between education level and age group, income level and age group, and income and education level, among the Chinese and Indian populations. Interestingly, for the Malay population there is a positive correlation between income and age group, whereby the level of income increases with age.

Table 4.10: Correlation matrix of variables based on ethnicity.

Population	Variables	Soy Sauce Intake per Day (g)	Age Group	Education level	Income Group	Sig.
Malay	Soy Sauce Intake per Day (g)	1.000	-0.042	-0.054	-0.086	0.008
	Age Group		1.000	-0.278	0.006	
	Education Level			1.000	0.372	
	Income Group				1.000	
Chinese	Soy Sauce Intake per Day (g)	1.000	0.009	-0.101	-0.115	0.029
	Age Group		1.000	-0.302	-0.080	
	Education Level			1.000	0.339	
	Income Group				1.000	
Indian	Soy Sauce Intake per Day (g)	1.000	-0.086	-0.070	-0.159	0.185
	Age Group		1.000	-0.333	-0.095	
	Education Level			1.000	0.389	
	Income Group				1.000	

The coefficients of multiple correlation (R) calculated from the regression analysis of soy sauce consumption by the Malay, Chinese and Indian populations are 0.102, 0.018, and 0.193 respectively. The regression model derived for the Malay population is:

Soy sauce intake among the Malay population per day (g) = 13.177 – 0.642*Age group – 0.779*Education level – 1.412*Income group

The regression model derived for the Chinese population is:

Soy sauce intake among the Chinese population per day (g) = 11.182 – 0.182*Age group – 0.766*Education level – 1.134*Income group

The regression model derived for the Indian population is:

Soy sauce intake among the Indian population per day (g) = 7.301 – 0.474*Age group – 0.318*Education level – 0.968*Income group

Similar to the entire Malaysian population, there is a weak relationship between age group, education level, income group and soy sauce consumption. However, among all independent variables in **Table 4.11**, the daily soy sauce consumption for all ethnicities is associated more with the age group. In the Malay population, there is only a 0.642 and 0.799 decrease in soy sauce consumption with a 1-unit increase in age group and education level, respectively. However, there is a 1.412unit decrease in soy sauce consumption with a 1-unit increase in income group. In the Chinese population, there is a 1.134unit decrease in soy sauce consumption with a 1-unit increase in income group. In the Indian population, the decrease in soy sauce consumption with a 1-unit increase in income group is the lowest (0.968 unit).

Table 4.11: Regression coefficients based on ethnicity.

Population	Independent Variable	Coefficient	T- value	Sig.
Malay	Age group	-0.642	-1.927	0.054
	Education level	-0.779	-1.435	0.152
	Income group	-1.412	-2.450	0.014
Chinese	Age group	-0.182	-0.467	0.641
	Education level	-0.776	-1.547	0.122
	Income group	-1.134	-1.919	0.056
Indian	Age group	-0.474	-1,256	0.211
	Education level	-0.318	-0.497	0.620
	Income group	-0.968	-1.588	0.115

4.8 Dietary Intake and Risk Assessment of 3-MCPD and 1,3-DCP

In dietary intake estimation, the calculated mean concentrations of 3-MCPD and 1,3-DCP in soy sauce were 0.017 μg mL^{-1} and 0.020 μg mL^{-1}, respectively (**Table 4.12**). Soy sauce consumption was then applied to Eq. 3.4 together with the average Malaysian body weight provided by the Ministry of Health, Malaysia. The mean soy sauce consumption alone does not represent Malaysians' exposure to 3-MCPD, as Eq. 3.4 takes into consideration body weight for exposure assessment.

According to **Table 4.12**, males consume more soy sauce than females. However, the female population's exposure to 1,3-DCP and 3-MCPD is higher than the male population due to the difference in mean body weight. Eq. 3.4 shows that the daily exposure to 3-MCPD and 1,3-DCP is divided by the mean body weight of the mentioned population. Thus, lower average body weight in the female population leads to a daily exposure of 0.0018 mg L^{-1} bw $day^{-1}$to 3-MCPD and 0.0016 mg L^{-1} bw day^{-1} to 1,3-DCP. This signifies that females are more susceptible to 1,3-DCP and 3-MCPD toxicity compared to the male population.

For the population aged 18 to 19 soy sauce consumption is the highest among other groups and the average body weight is the lowest. This directly makes this age group the population most susceptible to 3-MCPD (0.0017 mg L^{-1} bw day^{-1}) and 1,3-DCP (0.0020 mg L^{-1} bw day^{-1}) toxicity. In the age group comparison, the population that is exposed to the lowest doses of 3-MCPD and 1,3-DCP is the 50 to 59 age group, with exposure to 0.0014 mg L^{-1} bw day-1 of 3-MCPD and 0.0017 mg L^{-1} bw day^{-1} of 1,3-DCP. The lower sodium intake trend in the older group is a worldwide trend, as this

population is educated on the benefits of a low-sodium diet and the need to reduce the risk of blood pressure-related diseases (He & MacGregor, 2010).

The Indian population consumes the least soy sauce, not only among different ethnicities but also for all statistically independent variables in this study, with merely 3.15 g day^{-1}. The high average body weight of the Indian population (71.33 kg) additionally reduces their exposure to the toxicity of 3-MCPD (0.0008 mg L^{-1} bw day^{-1}) and 1,3-DCP (0.0009 mg L^{-1} bw day^{-1}). The Malay (3-MCPD: 0.0017 mg L^{-1} bw day^{-1}, 1,3-DCP 0.0019 mg L^{-1} bw day^{-1}) and Chinese (3-MCPD: 0.0016 mg L^{-1} bw day^{-1}, 1,3-DCP: 0.0019 mg L^{-1} bw day^{-1}) groups consume nearly the same daily soy sauce amounts and have similar average body weights, contributing to their comparable exposure to 3-MCPD and 1,3-DCP toxicity.

Population with tertiary education level recorded daily exposure to 3-MCPD of 0.0012 mg L^{-1} bw day^{-1} and to 1,3-DCP of 0.0014 mg L^{-1} bw day^{-1}. These exposure levels are the lowest among various education levels. The low exposure is due to the low soy sauce consumption. The group that is most susceptible to the toxicity of 3-MCPD and 1,3-DCP is the primary school education level, with 0.0019 mg L^{-1} bw day^{-1} and 0.0023 mg L^{-1} bw day^{-1} exposure respectively. The exposure to 3-MCPD and 1,3-DCP is seen to exhibit a reducing trend with increasing education level.

The lowest daily exposure to 3-MCPD and 1,3-DCP is experienced by those who earn more than RM6000, with 0.0010 mg L^{-1} bw day^{-1} of 3-MCPD and 0.0012 mg L^{-1} bw day^{-1} of 1,3-DCP. The low-income group is exposed to the highest level of 3-MCPD (0.0018 mg L^{-1} bw day^{-1}) and 1,3-DCP (0.0021 mg L^{-1} bw day^{-1}). The low-income group also consumes the most soy sauce daily and has the lowest average body weight.

Hence, exposure to 3-MCPD and 1,3-DCP increases with decreasing income level in Malaysia.

Although there is both high and low exposure to 3-MCPD and 1,3-DCP, the whole Malaysian population is generally not at risk of harmful exposure to 3-MCPD and 1,3-DCP. The percentile points estimated for Malaysians are lower than the JECFA-recommended PMTDI of 2 μg (kg^{-1} bw) day^{-1}. The highest recorded exposure to 3-MCPD is for the population with primary education level (0.0019 mg L^{-1} bw day^{-1}), far below the value recommended by JECFA. The maximum tolerable limit of 0.02 mg kg^{-1} is effective to prevent Malaysians from exposure to harmful doses of 3-MCPD through soy sauce consumption.

Table 4.12: Estimated dietary exposure to 3-MCPD and 1,3-DCP.

Variables	Parameters	Consumption (g day^{-1})	Average Body weight (kg) [b]	3-MCPD 0.017[a]		1,3-DCP 0.020[a]	
				Daily exposure (mg L^{-1} bw day^{-1})	% PMTDI[c]	Daily exposure (mg L^{-1} bw day^{-1})	% PMTDI[c]
Gender	Male	6.26	69.24	0.0015	0.08	0.0018	0.09
	Female	6.03	62.49	0.0016	0.08	0.0019	0.10
Age Group	18-19	5.83	59.17	0.0017	0.08	0.0020	0.10
	20-29	6.81	64.32	0.0018	0.09	0.0021	0.11
	30-39	5.81	66.82	0.0015	0.07	0.0017	0.09
	40-49	6.40	66.74	0.0016	0.08	0.0019	0.10
	50-59	5.54	65.59	0.0014	0.07	0.0017	0.08
Ethnicity	Malay	6.51	66.88	0.0017	0.08	0.0019	0.10
	Chinese	6.26	65.98	0.0016	0.08	0.0019	0.09
	Indian	3.15	71.33	0.0008	0.04	0.0009	0.04
Education Level	No Formal Education	5.83	60.22	0.0016	0.08	0.0019	0.10
	Primary	7.19	63.55	0.0019	0.10	0.0023	0.11
	Secondary	6.41	66.26	0.0016	0.08	0.0019	0.10
	Tertiary	4.87	67.35	0.0012	0.06	0.0014	0.07
Income Group	≤2299.99	6.85	63.96	0.0018	0.09	0.0021	0.11
	2300.00-5999.99	5.30	67.77	0.0013	0.07	0.0016	0.08
	≥6000.00	4.18	69.77	0.0010	0.05	0.0012	0.06

[a] Observed mean levels of 3-MCPD and 1,3-DCP in commercial soy sauces.
[b] Average body weight of the grouped population based on MANS data.
[c] For the Provisional Maximum Tolerable Daily Limit (PMTDI) of 3-MCPD and 1,3-DCP we used the JECFA recommended 2 µg kg^{-1} bw day^{-1}.

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

The simultaneous derivatization and quantification of 3-MCPD and 1,3-DCP at room temperature (25°C) was successfully developed. To date, this is the only derivatization procedure that can be carried out at room temperature and that takes a short time. 3-MCPD and 1,3-DCP were successfully extracted from various food sample types, ranging from solid, paste, and liquid samples. The procedure was optimized with 2 optimization strategies: The Box-Behnken experimental design optimization and experimental optimization. Both strategies provided similar optimization conditions: room temperature (25°C), 5 min and 5 μL TMSOTf derivatization. The polynomial equation generated from the Box-Behnken experimental design enables researchers to mix and match different derivatization temperature, derivatization period and catalyst volume values according to personal needs. All target compounds, including 3-MCPD-TMS and 1,3-DCP-TMS were separated clearly in the chromatogram and sharp undivided peaks were observed. The LOD and LOD of the method proposed in this study were 0.0008 and 0.0043 mg L^{-1}, respectively. These values are lower than with the derivatization technique recommended by AOAC. Furthermore, the inter-day and intra-day precision experiments showed low RSD percentages, indicating that the developed method is highly reproducible. The recoveries obtained from the actual food sample analysis were within 80-120%. According to the comparison of HMDS-TMSOTf and HFBI derivatizations in actual food sample analysis, both results show no significant differences in 3-MCPD and 1,3-DCP levels, indicating that the developed method is accurate and reliable. Compared to the amount of chemicals recommended by AOAC less chemicals were used in this study. The byproduct of HMDS-TMSOT derivatization is ammonia, which is not harmful to the gas chromatography (GC). The

procedure is operator-friendly and only minimal amounts of ammonia are released. In conclusion, the HMDS-TMSOTf derivatization procedure developed in this study is precise, accurate, reproducible, and highly sensitive.

From all collected food samples (soy sauce, dark soy sauce, oyster sauce, and chicken cube flavoring), only 1 sample contained 3-MCPD levels above 0.02 mg kg^{-1}. The survey conducted for this study demonstrated that almost half of all samples do not contain detectable 3-MCPD and 1,3-DCP concentrations, proving that most soy sauces, dark soy sauces, and oyster sauces are manufactured with the natural fermentation method. On the other hand, all chicken cube flavoring samples tested contained detectable concentrations of 3-MCPD and 1,3-DCP. The monitoring of 3-MCPD and 1,3-DCP should be extended beyond soy-related sauces to products that contain soy sauce as one of the ingredients. Such products may contain acid-HVP as an ingredient, resulting in 3-MCPD and 1,3-DCP contamination.

In terms of daily dietary intake of soy sauce, the various gender, education level, income and age groups exhibited no significant difference in soy sauce consumption. However, ethnicities in Malaysia displayed significant difference in soy sauce consumption, potentially due to the diverse means of food preparation and consumption according to ethnicity. Based on the estimated dietary exposure, Malaysians are not exposed to unacceptable levels of 3-MCPD through soy sauce consumption. The estimated daily exposure of Malaysians to 3-MCPD is below 2 μg (kg^{-1} bw) day^{-1} as recommended by JECFA. The highest exposure to 3-MCPD is only 0.0019 μg (kg^{-1} bw) day^{-1} based on this study.

As a future perspective, a sensing platform ought to be developed that enables direct 3-MCPD and 1,3-DCP detection without sample extraction and derivatization. Sample extraction is a very tedious task that necessitates skilled operators to obtain high and satisfactory recoveries. Although the derivatization procedure was greatly simplified by the findings from the present research, a method that facilitates direct detection will be more advantageous and user friendly. Sensing platforms, such as chemosensors and biosensors are good development candidates for the direct detection of 3-MCPD and 1,3-DCP. Up to now, only 1 chemosensor has been developed but is not yet fully applicable to sample detection, as the researchers have faced some difficulties with signal readings. The direct, simultaneous detection of 3-MCPD and 1,3-DCP without extraction and derivatization will increase food safety monitoring efficiency as 3-MCPD and 1,3-DCP levels can be determined at a faster rate.

REFERENCES

Abu-El-Haj, S., Bogusz, M. J., Ibrahim, Z., Hassan, H., & Al Tufail, M. (2007). Rapid and simple determination of chloropropanols (3-MCPD and 1,3-DCP) in food products using isotope dilution GC-MS. *Food Control*, *18*, 81–90.

Anderson, C. A. M., Appel, L. J., Okuda, N., Brown, I. J., Chan, Q., Zhao, L., … Stamler, J. (2010). Dietary sources of sodium in China, Japan, the United Kingdom, and the United States, women and men aged 40 to 59 years: The INTERMAP study. *Journal of the American Dietetic Association*, *110*(5), 736–745.

Anon. (1995). *Bestimmung von 3-Chlor-1,2-Propandiol (3-MCPD) in speisewürzen (Eiweißhydrolysate)*. *Amtliche sammlung von untersuchungsverfahren nach § 35 LMBG*. Berlin, Germany.

AOAC. (2002). Determination of 3-chloro-1,2-propanediol in foods and food ingredients, gas chromatography/mass spectrometric detection. *AOAC Official Method 2000.01.*, 48.1.06.

Australia New Zealand Food Authority Act (1991). New Zealand: https://www.dia.govt.nz//Pubforms.nsf/NZGZT/NZGazette149Nov01.pdf/$file/NZGazette149Nov01.pdf#page=42. Retrieved May 5, 2014 from https://www.dia.govt.nz//Pubforms.nsf/NZGZT/NZGazette149Nov01.pdf/$file/NZGazette149Nov01.pdf#page=42

Barocelli, E., Corradi, A., Mutti, A., & Petronini, P. G. (2011). Comparison between 3-MCPD and its palmitic esters in a 90-day toxicological study. *EFSA Supporting Publications*, *8*(9).

BBC News. (1999). Contaminated sauce warning. Retrieved May 7, 2017, from http://news.bbc.co.uk/2/hi/health/460900.stm

Becalski, A., Zhao, T., & Sit, D. (2013). Cyclohexanone/sulfonated polymer catalyst: a new simple derivatizing procedure for GC-MS determination of 2- and 3-monochloropropanediols. *Food and Energy Security*, *2*(2), 157–165.

Bel-Rhlid, R., Talmon, J. P., Fay, L. B., & Juillerat, M. A. (2004). Biodegradation of 3-Chloro-1,2-propanediol with Saccharomyces cerevisiae. *Journal of Agricultural and Food Chemistry*, *52*(20), 6165–6169.

Bodén, L., Lundgren, M., Stensiö, K.-E., & Gorzynski, M. (1997). Determination of 1,3-dichloro-2-propanol and 3-chloro-1,2-propanediol in papers treated with polyamidoamine-epichlorohydrin wet-strength resins by gas chromatography-mass spectrometry using selective ion monitoring. *Journal of Chromatography A*, *788*(1–2), 195–203.

Borkenhagen, L. K. (1953, October 27). Process for preparing amino acids. Google Patents. Retrieved May 4, 2014 from http://www.google.com/patents/US2657232

Breitung-Utzmann, C. M., Kobler, H., Herbolzheimer, D., & Maier, A. (2003). 3-MCPD: Occurrence in bread crust and various food groups as well as formation in toast. *Deutsche Lebensmittel-Rundschau*, *99*(7), 280–285. Retrieved May 5, 2014 from http://cat.inist.fr/?aModele=afficheN&cpsidt=14912319

Brereton, P., Kelly, J., Crews, C., Honour, S., Wood, R., & Davies, a. (2001). Determination of 3-chloro-1,2-propanediol in foods and food ingredients by gas chromatography with mass spectrometric detection: collaborative study. *Journal of AOAC International*, *84*(2), 455–65. Retrieved May 7, 2014 from http://www.ncbi.nlm.nih.gov/pubmed/11324611

Canadian Standards. (2012). Canadian Standards (Maximum Levels) for Various Chemical Contaminants in Foods. Retrieved May 10, 2014 from http://www.hc-sc.gc.ca/fn-an/securit/chem-chim/contaminants-guidelines-directives-eng.php#share

Carey, F. A. (2000). *Organic Chemistry 4th Edition* (4th ed.). United States: McGraw-Hill Higher Education.

CEN. (2004). *Foodstuffs: determination of 3-monochloropropane-1,2-diol by GC/MS (EN 14573)*. Brussels: European Committee for Standardization.

Chen, B. H., & Lin, Y. S. (1997). Formation of polycyclic aromatic hydrocarbons during processing of duck meat. *Journal of Agricultural and Food Chemistry*, *45*(4), 1394–1403.

Cheng, W. C., Chen, H. C., Lin, Y. P., Lee, H. F., Chang, P. C., & Chou, S. S. (2004). Survey on 3-monochloro-1,2-propandiol (3-MCPD) contents of soy sauce products during fiscal year 2002 in Taiwan. *Journal of Food and Drug Analysis*, *12*, 336–341.

Cho, W. S., Han, B. S., Lee, H., Kim, C., Nam, K. T., Park, K., … Jang, D. D. (2008). Subchronic toxicity study of 3-monochloropropane-1,2-diol administered by drinking water to B6C3F1 mice. *Food Chem Toxicol*, *46*(5), 1666–1673.

Cho, W. S., Han, B. S., Nam, K. T., Park, K., Choi, M., Kim, S. H., … Jang, D. D. (2008). Carcinogenicity study of 3-monochloropropane-1,2-diol in Sprague-Dawley rats. *Food and Chemical Toxicology*, *46*, 3172–3177.

Christova-Bagdassarian, V., Tishkova, J. A., & Vrabcheva, T. M. (2013). 3-Monochloro-1,2-propandiol (3-MCPD) in soy sauce from the Bulgarian market. *Food Additives & Contaminants: Part B*, *6*(3), 163–167.

Chung, S. W. C., Kwong, K. P., Yau, J. C. W., Wong, A. M. C., & Xiao, Y. (2008). Chloropropanols levels in foodstuffs marketed in Hong Kong. *Journal of Food Composition and Analysis*, *21*(7), 569–573.

Chung, W. C., Hui, K. Y., & Cheng, S. C. (2002). Sensitive method for the determination of 1,3-dichloropropan-2-ol and 3-chloropropane-1,2-diol in soy sauce by capillary gas chromatography with mass spectrometric detection. *Journal of Chromatography A*, *952*, 185–192.

Chung, W., Hui, K., & Cheng, S. (2002). Sensitive method for the determination of 1,3-dichloropropan-2-ol and 3-chloropropane-1,2-diol in soy sauce by capillary gas chromatography with mass spectrometric detection. *Journal of Chromatography. A*, *952*, 185–192.

Collier, P. D., Cromie, D. D. O., & Davies, A. P. (1991). Mechanism of formation of chloropropanols present in protein hydrolysates. *Journal of the American Oil Chemists Society*, *68*(10), 785–790.

Committee, A. P. V. M. A. (1998). *AOAC Peer Verified Methods Program–Manual on Policies and Procedures*. *AOAC International*.

Crews, C., Hasnip, S., Chapman, S., Hough, P., Potter, N., Todd, J., … Matthews, W. (2003). Survey of chloropropanols in soy sauces and related products purchased in the UK in 2000 and 2002. *Food Additives and Contaminants*, *20*, 916–922.

Dayrit, F. M., & Niñonuevo, M. R. (2004). Development of an analytical method for 3-monochloropropane-1,2-diol in soy sauce using 4-heptanone as derivatizing agent. *Food Additives & Contaminants*, *21*(3), 204–209.

Divinová, V., Svejkovská, B., Doležal, M., & Velíšek, J. (2004). Determination of free and bound 3-chloropropane-1,2-diol by gas chromatography with mass spectrometric detection using deuterated 3-chloropropane-1,2-diol as internal standard. *Czech J. Food Sci.*, *22*, 182–189.

Duffus, J. H., Nordberg, M., & Templeton, D. M. (2007). Glossary of terms used in toxicology, 2nd edition (IUPAC Recommendations 2007). *Pure and Applied Chemistry*, *79*(7), 1153–1344.

EAEMP. (2004). *European agency for the evaluation of medicinal products, oelxalic acid. Summary report EMEA/MRL/891/03-FINAL*. Retrieved May 5, 2014 from European agency for the evaluation of medicinal products, oelxalic acid. Summary report EMEA/MRL/891/03-FINAL

El Ramy, R., Ould Elhkim, M., Lezmi, S., & Poul, J. M. (2007). Evaluation of the genotoxic potential of 3-monochloropropane-1,2-diol (3-MCPD) and its metabolites, glycidol and beta-chlorolactic acid, using the single cell gel/comet assay. *Food Chem Toxicol*, *45*(1), 41–48.

Epstein, S. S., Arnold, E., Andrea, J., Bass, W., & Bishop, Y. (1972). Detection of chemical mutagens by the dominant lethal assay in the mouse. *Toxicology and Applied Pharmacology*, *23*(2), 288–325.

Ericsson, R. J. (1982). Alpha-chlorohydrin (Epibloc®): a toxicant-sterilant as an alternative in rodent control. Retrieved May 5, 2014 from http://digitalcommons.unl.edu/vpc10/13/

EU. (2004). *Reports on tasks for scientific cooperation*. Retrieved May 5, 2014 from http://ec.europa.eu/food/safety/docs/cs_contaminants_catalogue_mcpd_scoop_3-2-9_final_report_chloropropanols_en.pdf

Europen Commission. (2001). *Official Journal of the European Comminities*. Lexemborg: Office for Official Publication of the European Communites.

FAO. (2004). *Joint Fao/Who Food Standards Programme Codex Committee on Processed Fruits and Vegetables*. *Codex Standard for Soy Sauce* (Vol. 4).

FAO. (2012). *Reduction of 3-monochloropropane-1, 2-diol (3-MCPD) during the production of acid-hydrolyzed vegetable protein (Acid-HVPs) and products that contain acid-HVPs*. Retrieved May 12, 2014 from http://www.fao.org/input/download/standards/11024/CXP_064e.pdf.

FDA. (2008). Inspections, Compliance, Enforcement, and Criminal Investigations. Retrieved May 10, 2014 from http://www.fda.gov/iceci/compliancemanuals/compliancepolicyguidancemanual/ucm074419.htm

Firouzabadi, H., Iranpoor, N., Jafari, A. A., & Jafari, M. R. (2008). Iron(III) trifluoroacetate [Fe(F3CCO2)3] as an easily available, non-hygroscopic, non-corrosive, highly stable and a reusable Lewis Acid catalyst: Efficient O-silylation of α-hydroxyphosphonates, alcohols and phenols by hexamethyldisilazane (HMDS). *Journal of Organometallic Chemistry*, *693*(16), 2711–2714.

FSANZ. (2003). Chloropropanols in food: an analysis of the public health risk. Retrieved May 21, 2014 from www.foodstandards.gov.au

GMA News. (2007). Sauce producers urged to follow Codex. Retrieved May 7, 2017, from http://www.gmanetwork.com/news/money/content/41674/sauce-producers-urged-to-follow-codex/story/

Gonzalez, P., Racamonde, I., Carro, A. M., & Lorenzo, R. A. (2011). Combined solid-phase extraction and gas chromatography-mass spectrometry used for determination of chloropropanols in water. *J Sep Sci*, *34*(19), 2697–2704.

Gorlitz, B. D. (1991). *In Vitro Mammalian cell HPRT-test with 3-chloro-1,2-propanol*. Hannover, Germany: Fraunhofer-Insitute Fur Toxicologie und Aerosolforschung.

Gulf News. (2001). UAE bans soya products from East Asia. Retrieved May 7, 2017, from http://gulfnews.com/news/uae/general/uae-bans-soya-products-from-east-asia-1.421372

Hall, L. A. (1946). Protein hydrolysates; flavor ingredients for foods. *Food Industries*, *18*, 681-4-16. Retrieved May 21, 2014 from http://www.ncbi.nlm.nih.gov/pubmed/21025054

Hamlet, C. G., & Sutton, P. G. (1997). Determination of the chloropropanols, 3-chloro-1,2-propandiol and 2-chloro-1,3-propandiol, in hydrolysed vegetable proteins and seasonings by gas chromatography/ion trap tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *11*, 1417–1424.

He, F. J., & MacGregor, G. A. (2010). Reducing Population Salt Intake Worldwide: From Evidence to Implementation. *Progress in Cardiovascular Diseases*, *52*(5), 363–382.

Henderson, L. M., Bosworth, H. J., Ransome, S. J., Banks, S. J., Brabbs, C. E., & Tinner, A. J. (1987). *An assessment of the mutagenic potential of 1,3-dichloro-2-propanol, 3-chloro-1,2-propanediol and a cocktail of chloropropanols using the mouse lymohoma TK locus assay*. Huntingdon, Cambridgeshire England: Huntingdon Research Centre.

Hu, Z., Cheng, P., Guo, M., Zhang, W., & Qi, Y. (2013). A novel approach of periodate oxidation coupled with HPLC-FLD for the quantitative determination of 3-chloro-1,2-propanediol in water and vegetable oil. *Journal of Agricultural and Food Chemistry*, *61*, 6614–6621.

Huang, M., Jiang, G., He, B., Liu, J., Zhou, Q., Fu, W., & Wu, Y. (2005). Determination of 3-chloropropane-1,2-diol in liquid hydrolyzed vegetable proteins and soy sauce by solid-phase microextraction and gas chromatography/mass spectrometry. *Anal Sci*, *21*(11), 1343–1347.

IARC. (1994). *Monographs on the evaluation of carcinogenic risks to humans: some industrial chemicals* (Vol. 60). International Agency for Research on Cancer .

IARC. (2013). *Some Chemicals present in Industrial and Consumer Products, Food and Drinking Water*. *IARC Monographs on the Evaluation of Carcinogenic Risks to Human* (Vol. 101).

IARC. (2016). *Agents classified by the IARC monographs, Volumes 1–109*. Retrieved May 10, 2014 from https://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.pdf

ILS. (2005). *1, 3- Dichloro-2-propanol [CAS No. 96- 23- 1]. Review of toxicological literature* (Vol. Revision 1). U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER). Retrieved May 13, 2014 from http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/dichloropropanol_508.pdf

Inquirer. (2011). FDA to consumers: Don't buy unlabeled soy sauce. Retrieved May 7, 2017, from http://newsinfo.inquirer.net/21287/fda-to-consumers-don't-buy-unlabeled-soy-sauce

Jaccaud, E., & Aeschbacher, H. U. (1989). *Evaluation of 3-chloro-1,2-propanediol (3-MCPD) in the bone marrow and colonic micronucleus test in mice*. Lausanne, Swtizerland: Nestec Ltd, Research Centre.

JECFA. (2002). *1,3-dichloro-2-propanol. Safety evaluation of certain food additives and contaminants. In: WHO food addit ser 48*.

JECFA. (2005). *Joint FAO/WHO Expert COmmittee On Food Additives Sixty-fourth Meeting*. Rome. Retrieved May 3, 2014 from ftp://ftp.fao.org/es/esn/jecfa/jecfa64_summary.pdf

Jeong, J., Han, B. S., Cho, W. S., Choi, M., Ha, C. S., Lee, B. S., … Kim, C. Y. (2010). Carcinogenicity study of 3-monochloropropane-1, 2-diol (3-MCPD) administered by drinking water to B6C3F1 mice showed no carcinogenic potential. *Arch Toxicol*, *84*(9), 719–729.

Jones, A. R. (1975). The metabolism of 3-chloro-, 3-bromo-and 3-iodopropan-1, 2-diol in rats and mice. *Xenobiotica*, *5*(3), 155–165.

Jones, A. R. (1983). Antifertility actions of alpha-chlorohydrin in the male. *Aust J Biol Sci*, *36*(4), 333–350.

Jones, A. R., Davies, P., Edwards, K., & Jackson, H. (1969). Antifertility effects and metabolism of alpha and epi-chlorhydrins in the rat. *Nature*, *224*(5214), 83.

Jones, A. R., & Fakhouri, G. (1979). Epoxides as obligatory intermediates in the metabolism of α-halohydrins. *Xenobiotica*, *9*(10), 595–599.

Jones, A. R., Milton, D. H., & Murcott, C. (1978). The oxidative metabolism of α-chlorohydrin in the male rat and the formation of spermatocoeles. *Xenobiotica*, *8*(9), 573–582.

Jones, A. R., Porter, K., & Stevenson, D. (1981). The renal toxicity of some halogenated derivatives of propane in the rat. *Naturwissenschaften*, *68*(2), 98–99.

Jones, P., & Jackson, H. (1976). Antifertility and dominant lethal mutation studies in male rats with dl-alpha-chlorohydrin and an amino-analogue. *Contraception*, *13*(5), 639–646.

Karimi, B., & Golshani, B. (2000). Mild and Highly Efficient Method for the Silylation of Alcohols Using Hexamethyldisilazane Catalyzed by Iodine under Nearly Neutral Reaction Conditions. *The Journal of Organic Chemistry*, *65*(21), 7228–7230.

Khazaei, A., Zolfigol, M. A., Rostami, A., & Choghamarani, A. G. (2007). Trichloroisocyanuric acid (TCCA) as a mild and efficient catalyst for the trimethylsilylation of alcohols and phenols with hexamethyldisilazane (HMDS) under heterogonous conditions. *Catalysis Communications*, *8*(3), 543–547.

Khazaei, A., Zolfigol, M. A., Tanbakouchian, Z., Shiri, M., Niknam, K., & Saien, J. (2007). 1,3-Dibromo-5,5-diethylbarbituric acid as an efficient catalyst for the protection of various alcohols with HMDS under solvent-free conditions. *Catalysis Communications*, *8*(6), 917–920.

Kissa, E. (1992). Determination of 3-chloropropanediol and related dioxolanes by gas chromatography. *Journal of Chromatography A*, *605*(1), 134–138.

Kuballa, T., & Ruge, W. (2003). Nachweis und Bestimmung von 3-Monochlorpropan-1,2-diol (3-MCPD) mit GC-MS/MS. *Lebensmittelchem*, *57*, 57–58.

Lee, B. Q., & Khor, S. M. (2015). 3-Chloropropane-1,2-diol (3-MCPD) in Soy Sauce: A Review on the Formation, Reduction, and Detection of This Potential Carcinogen. *Comprehensive Reviews in Food Science and Food Safety*, *14*, 48–66.

Lee, B. Q., Wan Mohamed Radzi, C. W. J. B., & Khor, S. M. (2016). A simultaneous derivatization of 3-monochloropropanediol and 1,3-dichloropropane with hexamethyldisilazane-trimethylsilyl trifluoromethanesulfonate at room temperature for efficient analysis of food sample analysis. *Journal of Chromatography. A*, *1432*,

Lee, J. K., Byun, J. A., Park, S. H., Choi, H. J., Kim, H. S., & Oh, H. Y. (2005). Evaluation of the potential immunotoxicity of 3-monochloro-1, 2-propanediol in Balb/c mice: II. Effect on thymic subset, delayed-type hypersensitivity, mixed-lymphocyte reaction, and peritoneal macrophage activity. *Toxicology*, *211*(3), 187–196.

Lee, J. K., Byun, J. A., Park, S. H., Kim, H. S., Park, J. H., Eom, J. H., & Oh, H. Y. (2004). Evaluation of the potential immunotoxicity of 3-monochloro-1,2-propanediol in Balb/c mice: I. Effect on antibody forming cell, mitogen-stimulated lymphocyte proliferation, splenic subset, and natural killer cell activity. *Toxicology*, *204*, 1–11.

Lee, J. K., Ryu, M. H., & Byun, J. A. (2005). Immunotoxic effect of β-chlorolactic acid on murine splenocyte and peritoneal macrophage function in vitro. *Toxicology*, *210*(2), 175–187.

Lee, M. R., Chiu, T. C., & Dou, J. (2007). Determination of 1,3-dichloro-2-propanol and 3-chloro-1,2-propandiol in soy sauce by headspace derivatization solid-phase microextraction combined with gas chromatography-mass spectrometry. *Anal Chim Acta*, *591*(2), 167–172.

Lee, Y. J., Choi, I.-K., Sheen, Y. Y., Park, S. N., & Kwon, H. J. (2012). Moesin is a biomarker for the assessment of genotoxic carcinogens in mouse lymphoma. *Molecules and Cells*, *33*(2), 203–210.

León, N., Yusà, V., Pardo, O., Pastor, A., Leon, N., Yusa, V., … Pastor, A. (2008). Determination of 3-MCPD by GC-MS/MS with PTV-LV injector used for a survey of Spanish foodstuffs. *Talanta*, *75*(3), 824–831.

Leung, M. K. P., Chiu, B. K. W., & Lam, M. H. W. (2003). Molecular sensing of 3-chloro-1,2-propanediol by molecular imprinting. *Analytica Chimica Acta*, *491*, 15–25.

Leviton, R. (1980). *Soyfoods - Summer 1980: The Journal of the Soycrafters Association of North America*. Retrieved May 1, 2017 from https://books.google.com.vn/books?id=zCjRJ-0m3EEC

Li, N., Liu, Z., Jia, X., Cui, W., Wang, W., Zhang, X., … Wang, M. (2003). Study on the toxicological effect of chloropropanols on rats. *Wei Sheng Yan Jiu*, *32*(4), 349–352.

Li, Y., Liu, S., Wang, C., Li, K., Shan, Y. J., Wang, X. J., & Sun, C. H. (2010). Novel biomarkers of 3-chloro-1,2-propanediol exposure by ultra performance liquid chromatography/mass spectrometry based metabonomic analysis of rat urine. *Chemical Research in Toxicology*, *23*, 1012–1017.

Luh, B. S. (1995). Industrial production of soy sauce. *Journal of Industrial Microbiology*, *14*(6), 467–471.

Lynch, B. S., Bryant, D. W., Hook, G. J., Nestmann, E. R., & Munro, I. C. (1998). Carcinogenicity of monochloro-1,2-propanediol (alpha-chlorohydrin, 3-MCPD). *International Journal of Toxicology*, *17*, 47–76.

Macarthur, R., Crews, C., Davies, A., Brereton, P., Hough, P., & Harvey, D. (2000). 3-monochloropropane-1,2-diol (3-MCPD) in soy sauces and similar products available from retail outlets in the UK. *Food Additives and Contaminants*, *17*, 903–906.

MAF. (2011). *Survey of Chloropropanols in Soy Sauce: Imported Food Monitoring*. New Zealand: MAF NZ Standard. Retrieved May 4, 2014 from http://www.foodsafety.govt.nz/industry/importing/monitoring-and-review/surveys.htm

Magnusson, B., & Örnemark, U. (2014). Eurachem Guide: the fitness for purpose of analytical methods–a laboratory guide to method validation and related topics.

Majeska, J., & Matheson, D. W. (1983). Quantitative estimate of mutagenicity of tris-[1,3-dichloro-2 propyl]-phosphate (TCPP) and its possible metabolites in Salmonella. *Environ Mutagen*, *5*(3), 478 (abst.).

Malaysia Food Act 1983 and Regulations 1985. (2012). *Maximum permited proportion of 3- monochloropropane-1,2-diol (3- MCPD) in specific food*. Malaysia: International Law Book Services.

Matthew, B. M., & Anastasio, C. (2000). Determination of halogenated mono-alcohols and diols in water by gas chromatography with electron-capture detection. *J Chromatogr A*, *866*(1), 65–77.

May, C. (1991). *In Vitro Sister Chromatid Exchange Assay in Mammalian Cells*. Hannover, Germany: Fraunhofer-Insitute Fur Toxicologie und Aerosolforschung.

Mayo, C. (2016). High blood pressure (hypertension) Risk factors - Mayo Clinic. Retrieved November 9, 2016, from http://www.mayoclinic.org/diseases-conditions /high-blood-pressure/basics/risk-factors/con-20019580

Meierhans, D. C., Bruehlmann, S., Meili, J., & Taeschler, C. (1998). Sensitive method for the determination of 3-chloropropane-1,2-diol and 2-chloropropane-1,3-diol by capillary gas chromatography with mass spectrometric detection. *Journal of Chromatography A*, *802*(2), 325–333.

Miki, K. (2006). Energy metabolism and sperm function. *Society of Reproduction and Fertility Supplement*, *65*, 309–325.

Moghadam, M., Tangestaninejad, S., Mirkhani, V., Mohammadpoor-Baltork, I., Chahardahcheric, S., & Tavakoli, Z. (2008). Rapid and highly efficient trimethylsilylation of alcohols and phenols with hexamethyldisilazane (HMDS) catalyzed by reusable zirconyl triflate, [ZrO(OTf)2]. *Journal of Organometallic Chemistry*, *693*(11), 2041–2046.

Mojtahedi, M. M., Abbasi, H., & Abaee, M. S. (2006). MgBr2·OEt2 mediated protection of alcohols with hexamethyldisilazane: An efficient catalytic route for the preparation of silyl ethers under solvent-free conditions. *Journal of Molecular Catalysis A: Chemical*, *250*(1–2), 6–8.

Mukai, C., & Okuno, M. (2004). Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biology of Reproduction*, *71*(2), 540–547.

Naing, C., Yeoh, P. N., Wai, V. N., Win, N. N., Kuan, L. P., & Aung, K. (2016). Hypertension in Malaysia: An Analysis of Trends From the National Surveys 1996 to 2011. *Medicine*, *95*(2), e2417.

NJDHSS. (1999). *Glycerol-alpha-monochlorohydrin. Hazardous Substance Fact Sheet*. New Jersey. Retrieved May 7, 2015, from nj.gov/health/eoh/rtkweb/documents/fs/ 2453.pdf

Nyman, P. J., Diachenko, G. W., & Perfetti, G. A. (2003). Determination of 1,3-dichloropropanol in soy and related sauces by using gas chromatography/mass spectrometry. *Food Additives and Contaminants*, *20*, 903–908.

NZ Herald. (2001). Long wait for word on soy sauce. Retrieved May 7, 2017, from http://www.nzherald.co.nz/health/news/article.cfm?c_id=204&objectid=196478

OEHHA. (2005). *1,3-Dichloro-2-propanol:Review of Toxicological Literature*. Retrieved May 4, 2014 from https://ntp.niehs.nih.gov/ntp/htdocs/chem_ background/.../dichloropropanol_508.pdf

OEHHA. (2010). *3- MonoChloropropane-1,2-diol (3- MCPD; α-chlorohydrin)*. Califonia: Office of Environmental Health Hazard Assessment. Retrieved May 6, 2014 from https://oehha.ca.gov/media/downloads/crnr/123mcpd.pdf

Ohkubo, T., Hayashi, T., Watanabe, E., Endo, H., Goto, S., Endo, O., … Mori, Y. (1995). Mutagenicity of Chlorohydrins. *Nippon Suisan Gakkaishi*, *61*(4), 596–601.

Orata, F. (2012). *Derivatization reactions and reagents for gas chromatography analysis*. INTECH Open Access Publisher Rijeka.

Painter, R. B., & Howard, R. (1982). The Hela DNA-synthesis inhibition test as a rapid screen for mutagenic carcinogens. *Mutat Res*, *92*(1–2), 427–437.

Pesselman, R. L., & Feit, M. J. (1988). Determination of residual epichlorohydrin and 3-chloropropanediol in water by gas chromatography with electron-capture detection. *Journal of Chromatography A*, *439*(2), 448–452.

Piasecki, A., Ruge, A., & Marquardt, H. (1990). Malignant transformation of mouse M2-fibroblasts by glycerol chlorohydrines contained in protein hydrolysates and commercial food. *Arzneimittelforschung*, *40*(9), 1054–1055.

Plantinga, W. J. J., Van Toorn, W. G. G., & van der Stegen, G. H. D. H. D. (1991). Determination of 3-chloropropane-1,2-diol in liquid hydrolysed vegetable proteins by capillary gas chromatography with flame ionization detection. *Journal of Chromatography A*, *555*(1–2), 311–314.

Racamonde, I., González, P., Lorenzo, R. A., & Carro, A. M. (2011). Determination of chloropropanols in foods by one-step extraction and derivatization using pressurized liquid extraction and gas chromatography-mass spectrometry. *Journal of Chromatography A*, *1218*, 6878–6883.

Reese, P. (2005). *The origin and formation of 3-MCPD in foods and food ingredients.* Retrieved May 8, 2014 from http://www.foodbase.org.uk//admintools/ reportdocuments/43_84_FINAL_REPORT.pdf.

Research & Consulting Co. (1986). *104-week chronic toxicity and oncogenicity study with 1,3-dichlor-propan-2-ol in the rat. Report No. 017820*. Itingen, Switzerland.

Rétho, C., & Blanchard, F. (2005). Determination of 3-chloropropane-1,2-diol as its 1,3-dioxolane derivative at the μg kg−1 level: Application to a wide range of foods. *Food Additives & Contaminants*, *22*(12), 1189–1197.

Robert, M. C., Oberson, J. M., & Stadler, R. H. (2004). Model studies on the formation of monochloropropanediols in the presence of lipase. *J Agric Food Chem*, *52*(16), 5102–5108.

Rodman, L. E., & Ross, R. D. D. (1986). Gas—liquid chromatography of 3-chloropropanediol. *Journal of Chromatography A*, *369*(0), 97–103.

Rossi, A. M., Migliore, L., Lascialfari, D., Sbrana, I., Loprieno, N., Tortoreto, M., … Pantarotto, C. (1983). Genotoxicity, metabolism and blood kinetics of epichlorohydrin in mice. *Mutat Res*, *118*(3), 213–226.

Schlatter, J., Baars, A. J., DiNovi, M., Lawrie, S., & Lorentzen, R. (2002). *Safety Evaluation of Certain Food Additives and Contaminants: 3-Chloro-1,2-Propanediol*. *WHO Food Additives Series: 48*. Retrieved June 6, 2014 from http:// www.inchem.org/documents/jecfa/jecmono/v48je18.htm

Sennyah, P. (2001). Sauces withdrawn: foreign products found to contain excessive cancer-causing chemical. *The New Straights Times*. Retrieved June 11, 2014 from http://www.aboutsafety.com/article.cfm?id=1061.

Shaterian, H. R., Doostmohammadi, R., & Ghashang, M. (2008). Preparation of Silyl Ethers Using Hexamethyldisilazane in the Presence of N -Bromosuccinimide under Mild and Solvent-Free Conditions. *Chinese Journal of Chemistry*, *26*(9), 1709–1714.

Shaterian, H. R., Shahrekipoor, F., & Ghashang, M. (2007). Silica supported perchloric acid (HClO4–SiO2): A highly efficient and reusable catalyst for the protection of hydroxyl groups using HMDS under mild and ambient conditions. *Journal of Molecular Catalysis A: Chemical*, *272*(1–2), 142–151.

Silhankova, L., Smid, F., Cerna, M., Davidek, J., & Velisek, J. (1982). Mutagenicity of glycerol chlorohydrines and of their esters with higher fatty acids present in protein hydrolysates. *Mutat Res*, *103*(1), 77–81.

Skálová, S. (2005). The diagnostic role of urinary N-acetyl-beta-D-glucosaminidase (NAG) activity in the detection of renal tubular impairment. *Acta Medica-Hradec Kralove*, *48*(2), 75.

Spyres, G. (1993). Determination of 3-chloropropane-1,2-diol in hydrolysed vegetable proteins by capillary gas chromatography with electrolytic conductivity detection. *Journal of Chromatography A*, *638*(1), 71–74.

Stadler, R. H., & Lineback, D. R. (2008). *Process-induced food toxicants: occurrence, formation, mitigation, and health risks*. John Wiley & Sons.

Stevenson, D., & Jones, A. R. (1984). The action of (R)-and (S)-α-chlorohydrin and their metabolites on the metabolism of boar sperm. *International Journal of Andrology*, *7*(1), 79–86.

Stolzenberg, S. J., & Hine, C. H. (1979). Mutagenicity of halogenated and oxygenated three-carbon compounds. *J Toxicol Environ Health*, *5*(6), 1149–1158.

Stolzenberg, S. J., & Hine, C. H. (1980). Mutagenicity of 2- and 3-carbon halogenated compounds in the Salmonella/mammalian-microsome test. *Environ Mutagen*, *2*(1), 59–66.

Strimbu, K., & Tavel, J. A. (2010). What are biomarkers? *Current Opinion in HIV and AIDS*, *5*(6), 463.

Sun, J., Bai, S., Bai, W., Zou, F., Zhang, L., Su, Z., … Huang, Y. (2013). Toxic Mechanisms of 3-Monochloropropane-1,2-Diol on Progesterone Production in R2C Rat Leydig Cells. *Journal of Agricultural and Food Chemistry*, *61*(41), 9955–9960.

Sunahara, G., Perrin, I., & Marchesini, M. (1993). *Carcinogenicity study in 3-monochloropropane-1,2-diol (3- MCPD) adminstrated in drinking water to Fisher 344 rats*. Nestec Ltd, Research and Development submitted to WHO.

Sunahara, G., Perrin, I., & Marchessini, M. (1993). Carcinogenicity study on 3-monochloro propane 1, 2,-diol (3-MCPD) administered in drinking water to Fischer 344 rats. Report No RE-SR93003 Nestec Ltd. *Research and Development Switzerland*.

Tillu, V. H. (2004). Solvent free selective silylation of alcohols, phenols and naphthols with HMDS catalyzed by H-β zeolite. *Arkivoc*, *2004*(14), 83.

Umang, S. S. (2012). Importance of Genotoxicity & S2A guidelines for genotoxicity testing for pharmaceuticals. *Iosr Journal Of Pharmacy And Biological Sciences*, *1*(2), 43–54.

US EPA. (2013). Registration Review: Schedule for Beginning Reviews | Pesticides | US EPA. Retrieved June 11, 2014 from https://www.epa.gov/pesticide-reevaluation/registration-review-process

van Bergen, C. A., Collier, P. D., Cromie, D. D. O., Lucas, R. A., Preston, H. D., & Sissons, D. J. (1992). Determination of chloropropanols in protein hydrolysates. *Journal of Chromatography A*, *589*(1–2), 109–119.

van Den Wijngaard, A. J., Janssen, D. B., & Witholt, B. (1989). Degradation of epichlorohydrin and halohydrins by bacterial cultures isolated from freshwater sediment. *Microbiology*, *135*(8), 2199–2208.

Van Duuren, B. L., Goldschmidt, B. M., Katz, C., Seidman, I., & Paul, J. S. (1974). Carcinogenic activity of alkylating agents. *J Natl Cancer Inst*, *53*(3), 695–700.

Velisek, J., Calta, P., Dolezal, M. (Vysoka S. C. P. (Czech R. U. C. a A. P., Crews, C., & Hasnip, S. (2003). 3-chloropropane-1,2-diol in models simulating processed foods: precursors and agents causing its decomposition. *Czech Journal of Food Sciences - UZPI (Czech Republic)*.

Vicente, E., Arisseto, A. P., Monteiro, V., Furlani, R. P. Z., & Toledo, M. C. F. (2011). A survey of chloropropanols (3-MCPD and 1,3-DCP) in soy sauces and similar products from Brazil. *Toxicol Lett*, *205*, S145–S146.

Viet Nam News. (2001). Toxic soy sauce survives boycott. Retrieved May 7, 2017, from http://vietnamnews.vn/society/165105/toxic-soy-sauce-survives-boycott.html #UIZvK5BSMJStTACO.97

Wang, X., Song, G., Zhao, J., & Hu, Y. (2008). Determination of 3-MCPD in Powder Condiments by Dispersive Solid-phase Extraction. *Journal of Instrumental Analysis*, S1.

Weisburger, E. K., Ulland, B. M., Nam, J., Gart, J. J., & Weisburger, J. H. (1981). Carcinogenicity tests of certain environmental and industrial chemicals. *J Natl Cancer Inst*, *67*(1), 75–88.

Wong, K. O., Cheong, Y. H., & Seah, H. L. (2006). 3-Monochloropropane-1,2-diol (3-MCPD) in soy and oyster sauces: Occurrence and dietary intake assessment. *Food Control*, *17*, 408–413.

Xing, X., & Cao, Y. (2007). Determination of 3-chloro-1,2-propanediol in soy sauces by capillary electrophoresis with electrochemical detection. *Food Control*, *18*(2), 167–172.

Yadav, J., Reddy, B., Basak, A., Baishya, G., & Narsaiah, A. (2006). Indium Tribromide: An Efficient Catalyst for the Silylation of Hydroxy Groups by the Activation of Hexamethyldisilazane. *Synthesis*, *2006*(22), 3831–3834.

You, Z.-Y., Liu, Z.-Q., & Zheng, Y.-G. (2013). Properties and biotechnological applications of halohydrin dehalogenases: current state and future perspectives. *Applied Microbiology and Biotechnology*, *97*(1), 9–21.

Zareyee, D., & Karimi, B. (2007). A novel and highly efficient method for the silylation of alcohols with hexamethyldisilazane (HMDS) catalyzed by recyclable sulfonic acid-functionalized ordered nanoporous silica. *Tetrahedron Letters*, *48*(7), 1277–1280.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., & Mortelmans, K. (1988). Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. *Environ Mol Mutagen*, *11 Suppl 1*, 1–157.

Zhang, H., Yu, H., Wang, X., Zheng, W., Yang, B., Pi, J., … Qu, W. (2012). α-Chlorohydrin Inhibits Protein Tyrosine Phosphorylation through Blocking Cyclic AMP - Protein Kinase A Pathway in Spermatozoa. *PLoS ONE*, *7*(8), e43004.

Zhang, Z.-H., Li, T.-S., Yang, F., & Fu, C.-G. (2006). Montmorillonite Clay Catalysis XI 1 : Protection and Deprotection of Hydroxyl Group by Formation and Cleavage of Trimethylsilyl Ethers Catalysed by Montmorillonite K-10. *Synthetic Communications*, *28*(16), 3105–3114.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

LIST OF PUBLICATIONS

1. Lee, B. Q., & Khor, S. M. (2015). 3-Chloropropane-1, 2-diol (3-MCPD) in Soy Sauce: A Review on the Formation, Reduction, and Detection of This Potential Carcinogen. *Comprehensive Reviews in Food Science and Food Safety*, *14*(1), 48-66.
2. Lee, B. Q., Radzi, C. W. J. W. M., & Khor, S. M. (2016). A simultaneous derivatization of 3-monochloropropanediol and 1, 3-dichloropropane with hexamethyldisilazane–trimethylsilyl trifluoromethanesulfonate at room temperature for efficient analysis of food sample analysis. *Journal of Chromatography A*, *1432*, 101-110.

PRESENTATIONS

1. Simultaneous Detection of 3-MCPD and 1,3-DCP in Food Samples by Gas Chromatography with Mass Spectrometry – ICMIB, Mar 6-7, 2015, Malacca, Malaysia (Best Presentation Award)
2. One-Pot Derivatization of 3-Monochloropropanediol (3-MCPD) and 1,3-Dichloropropanol (1,3-DCP) at Room Temperature with HMDS – PACCON, Feb 9-11, 2016, Bangkok, Malaysia

SCIENTIFIC ARTICLES

1. University of Malaya researchers have developed a new, more cost-effective method for quantifying trace amounts of the carcinogen 3-monochloropropanediol, which has been detected in acid-hydrolyzed soy products. Retrieved from http://www.researchsea.com/html/article.php/aid/9559/cid/1/research/science/university_of_malaya/improving_the_analysis_efficiency_of_synthetic_soy_sauce.html