EXTRACTION OF DIETARY FIBER FROM CASSAVA PULP AND ASSESSMENT OF THEIR MERCURY BIOACCESSIBILITY INHIBITION AND INTESTINAL UPTAKE FROM FISH USING AN *IN VITRO* DIGESTION/ CACO-2 MODEL

Natta Kachenpukdee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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การสกัดใยอาหารจากกากมันสำปะหลัง และการประเมินการยับยั้งชีวภาพพร้อมใช้, การดูดซึมของปรอทจากปลาในลำไส้ด้วยเส้นใยอาหารที่ได้โดยใช้วิธี *In vitro* Digestion/Caco-2 Model

นางสาวนัฏฐา คเชนทร์ภักดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2558

EXTRACTION OF DIETARY FIBER FROM CASSAVA PULP AND ASSESSMENT OF THEIR MERCURY BIOACCESSIBILITY INHIBITION AND INTESTINAL UPTAKE FROM FISH USING AN *IN VITRO* DIGESTION/CACO-2 MODEL

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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นัฏฐา คเชนทร์ภักดี : การสกัดใยอาหารจากกากมันสำปะหลัง และการประเมินการยับยั้งชีวภาพพร้อมใช้, การดูดซึมของปรอทจากปลาในลำไส้ด้วยเส้นใยอาหารที่ได้โดยใช้วิธี *In vitro* Digestion/Caco-2 Model (EXTRACTION OF DIETARY FIBER FROM CASSAVA PULP AND ASSESSMENT OF THEIR MERCURY BIOACCESSIBILITY INHIBITION AND INTESTINAL UPTAKE FROM FISH USING AN *IN VITRO* DIGESTION/CACO-2 MODEL) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รัชฎาพร อุ่นศิวิไลย์, 178 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อหาสภาวะที่เหมาะสมในการสกัดใยอาหารจากกากมันสำปะหลังและประเมินประสิทธิภาพในการลดปริมาณปรอทในเนื้อปลา ด้วยการศึกษาชีวภาพพร้อมใช้ (bioaccessibility) และการดูดซึมทางลำไส้ (intestinal uptake) ในกระบวนการสกัดใยอาหารต้องการแยกแป้งออกจากใยอาหารด้วยการประยุกต์ใช้เอนไซม์ การหาปริมาณของเอนไซม์ที่เหมาะสมโดยใช้วิธีการพื้นผิวตอบสนอง ตัวแปรตามที่ใช้ในการพิจารณาคือ ร้อยละของใยอาหารที่สกัดด้วยสารละลายที่เป็นกลาง จากผลการศึกษาพบว่า ปริมาณของใยอาหารสูงสุดที่ได้ คือ ร้อยละ 79.68% จากการใช้ปริมาณแอลฟา-อะไมเลส 0.1% (w/v) อะไมโลกลูโคซิเดส 0.1% (v/v) และนิวเทรส 1% (v/v) โดยใยอาหารที่ได้จากกระบวนการนี้เรียกว่า crude dietary fiber (CDF) จากนั้นจึงดัดแปลงใยอาหารที่ได้ด้วยวิธี etherification เรียกใยอาหารที่ได้จากกระบวนการนี้ว่า modified dietary fiber (MDF)

การประเมินชีวภาพพร้อมใช้และชีวปริมาณการออกฤทธิ์ของปรอทในสภาวะที่มีใยอาหารหยาบและใยอาหารดัดแปลงมีส่วนช่วยในการประเมินค่าความเสี่ยงต่อสุขภาพมนุษย์ได้ ใช้แบบการจำลองสภาวะการย่อยในระบบย่อยอาหารและการสะสมใน Caco-2 cell เพื่อศึกษาการลดค่าชีวภาพพร้อมใช้และชีวปริมาณการออกฤทธิ์ของปรอทด้วยใยอาหารหยาบและใยอาหารดัดแปลง ปริมาณของปรอทในปลาและชนิดของใยอาหารหยาบ ผลการศึกษาพบว่าใยอาหารทั้งสองชนิดสามารถลดปริมาณชีวภาพพร้อมใช้ของปรอทในลำไส้ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยที่ใยอาหารหยาบลดปริมาณชีวภาพพร้อมใช้ได้ 3-57% และใยอาหารดัดแปลงสามารถลดได้ 34-85% เมื่อเปรียบเทียบกับตัวอย่างควบคุม นอกจากนั้นยังพบว่าเมื่อเติมใยอาหารหยาบปริมาณ 500 mg ในระบบย่อยสามารถลดปริมาณชีวภาพพร้อมใช้ของปรอทในลำไส้ได้ 23-62% และใยอาหารดัดแปลงลดได้ 71-84% เมื่อเปรียบเทียบกับตัวอย่างควบคุม ในลำดับสุดท้ายได้ทำการศึกษาการสะสมของปรอทใน Caco-2 cell จากการศึกษาพบว่า ในตัวอย่างที่ไม่มีการเติมใยอาหารปรอทสะสมในเซลล์ 5.97-9.07% ส่วนตัวอย่างที่มีการเติมใยอาหารหยาบ ปรอทสะสมในเซลล์ 4.02-6.54% และตัวอย่างที่เติมใยอาหารดัดแปลงปรอทสะสมในเซลล์ 5.09-7.13% จึงกล่าวโดยสรุปใยอาหารจากกากมัน-

สำปะหลังสามารถลดปริมาณชีวภาพพร้อมใช้ของปรอทในลำไส้ (bioaccessibility) และการดูดซึมทางลำไส้ (intestinal uptake) ได้ โดยการยับยั้งการถ่ายเทสารปรอทไปสู่ส่วนใสหลังการย่อยในโมเดลระบบย่อยอาหาร และอาจสามารถประยุกต์ใช้ในอาหารเสริมสุขภาพและผลิตภัณฑ์เสริมอาหาร

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

NATTA KACHENPUKDEE : EXTRACTION OF DIETARY FIBER FROM CASSAVA PULP AND ASSESSMENT OF THEIR MERCURY BIOACCESSIBILITY INHIBITION AND INTESTINAL UPTAKE FROM FISH USING AN *IN VITRO* DIGESTION/CACO-2 MODEL.
THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 178 PP.

CASSVA PULP/DIETARY FIBER/MODIFIED CELLULOSE/ENZYMATIC DIGESTION PROCESS/CELL/BIOACCESSIBILITY/BIOAVAILABILITY/ *IN VITRO* DIGESTION/CAC0-2

The objectives of this study were to determine the optimal extraction conditions of cassava pulp and their effects on mercury bioaccessibility and intestinal uptake. The extraction process requires the starch to be separated from the fiber by enzyme application. The enzyme reaction conditions for the solubilization were optimized via a response surface methodology (RSM). The selected dependent variable was a percentage of the neutral detergent fiber (NDF). The highest NDF (79.68%) of crude dietary fiber obtained from enzymatic digestion conditions was 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v), and 1% of neutrase (v/v). These dietary fibers were then modified using an etherification method.

The estimation of mercury (Hg) bioaccessibility and bioavailability with crude dietary fiber (CDF) and modified dietary fiber (MDF) is useful for the risk assessments of human health. Using an *in vitro*/Caco-2 cell model, the reduction of fish mercury bioaccessibility and bioavailability by CDF and MDF were studied. The

effect of both the fish mercury and the fiber quantity were also determined. The results indicated that both fiber types significantly reduced Hg bioaccessibility ($p < 0.05$). CDF reduced fish mercury bioaccessibility to 3-57% and 34-85% for MDF compared with the control in a dose-dependent manner. The quantity of fish mercury was reduced to 23-62% (CDF) and 71-84% (MDF) when 500 mg of fiber was added in a digestion model test (fish tissue amount 0-4 g) compared with the control (the control lacks fiber). Furthermore, Caco-2 cells from the digestion model test were utilized for intestinal cell accumulation and bioavailability evaluation. The results showed that the Hg transferred to the intracellular compartment was in the range of 5.97-9.07% for control, 4.02-6.54% for CDF and 5.09-7.13% for MDF. In conclusion, it is suggested that these fibers prepared from cassava pulp can decrease intestinal uptake by inhibiting mercury transfer to the aqueous fraction in the digestion model test and that they can be used in functional food and dietary supplement products.

School of Food Technology Student's Signature_____

Academic Year 2015 Advisor's Signature_____

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Natta Kachenpukdee

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LIST OF ABBREVIATIONS

AAS	=	Atomic Absorption Spectroscopy
ADF	=	Acid Detergent Fiber
ADL	=	Acid Detergent Lignin
Al^{3+}	=	Aluminum
ANOVA	=	Analysis of Variance
BAL	=	2,3-dimercaptopropanol
BCA	=	Bicinchoninic Acid
BET	=	Brunauer-Emmett-Teller
°C	=	Degree Celsius
Cd	=	Cadmium
CDF	=	Crude Dietary fiber
CH_3Hg	=	Methyl Mercury
CH_3HgCl	=	Methylmercury Chloride
Co	=	Cobalt
CO_2	=	Carbon Dioxide
CVAAS	=	Cold Vapor Atomic Absorption Spectroscopy
CVAFS	=	Cold Vapor Atomic Fluorescence Spectroscopy
Cu	=	Copper
DHA	=	Docosahexaenoic Acid
DMEM	=	Dulbecco's Modified Media

LIST OF ABBREVIATIONS (Continued)

DMPS	=	2,3-Dimercapto-1-propanesulfonic Acid
DMSA	=	Dimercaptosuccinic Acid
DMSO	=	Dimethylsulfoxide
DPBS	=	Dulbecco's Phosphate Buffered Saline
DTPA	=	Diethylenetriaminepentaacetic Acid
EDTA	=	Ethylenediaminetetraacetic Acid
EPA	=	Eicosapentaenoic Acid
FBS	=	Fetal Bovine Serum
FTIR	=	Fourier Transform Infrared
H_2	=	Hydrogen
HCl	=	Hydrochloric Acid
HEPES	=	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid
Hg	=	Mercury
$HgCl_2$	=	Mercuric Chloride
HgO	=	Mercuric Oxide
HgS	=	Mercuric Sulphide
H_2O_2	=	Hydrogen Peroxide
ICP/MS	=	Inductive Coupled Plasma Spectrometer Mass Spectrometer
IUPAC	=	International Union of Pure and Applied Chemistry
GI tract	=	Gastrointestinal Tract
Kg	=	Kilograms
kHz	=	Kilohertz

LIST OF ABBREVIATIONS (Continued)

KJ	=	Kilojoule
L	=	Liter
M	=	Molar
MDF	=	Modified Dietary
MeHg	=	Methyl Mercury
mg	=	Milligrams
ml	=	Milliliter (10^{-3}l)
mM	=	Millimolar (10^{-3}mol l^{-1})
MSG	=	Monosodium Glutamate
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCl	=	Sodium Chloride
NAD	=	Nicotinamide Adenine Dinucleotide
NaOH	=	Sodium Hydroxide
NCP	=	Noncellulosic Polysaccharides
NDF	=	Neutral Detergent Fiber
Ni	=	Nickel
n-3 PUFAs	=	Omega-3 Polyunsaturated Fatty Acids
OH^-	=	Hydroxyl Radical
PAHs	=	Polycyclic Aromatic Hydrocarbons
Pb	=	Lead
PCBs	=	Polychlorinated Biphenyls
ppm	=	Part per million

LIST OF ABBREVIATIONS (Continued)

rpm	=	Revolutions per minute
TDA/AAS	=	Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer
TC	=	Total Cholesterol
TG	=	Triglycerides
ZN	=	Zinc
µg	=	Microgram

CHAPTER I

INTRODUCTION

1.1 Introduction

Mercury (Hg) is a toxic element that is a naturally occurring element from a volcanoes and is released into the environment when a volcanoes erupts, or when coal is burned, which is the main source of mercury emission into the environment (Bernard et al., 2001). It is found in various forms: elemental or metallic mercury, inorganic mercury, and organic mercury (US EPA, 2003). Elemental mercury can remain for more than a year in the air, where it can be transported to water or land. It is deposited in the sediment, soil, land, water where it is transformed into methylmercury (MeHg) via bacteria by methylation process, absorbed by phytoplankton and accumulates in food chains of as zooplankton, fish and other sea animals (WHO, 2005). Humans can be exposed to the mercury in methyl mercury by eating fish or aquatic animal contaminated with mercury which is the main cause of methyl mercury exposure (US EPA, 2004). The highest levels of mercury can be found in swordfish, shark, king mackerel and tilefish (Bose-O'Reilly et al., 2010).

The toxicity of Hg depends on the method of entry, the form of the Hg and the level of exposure. The nervous system is highly sensitive to mercury. MeHg and elemental mercury can cause severe damage to the central nervous system. Inorganic mercury such as metallic mercury does not pass easily through the blood brain barrier in adults. However, it can pass easily through the blood brain barrier in fetuses and

children and through the placenta (Bernhoft, 2011). Embryos and fetuses are more sensitive to MeHg than children and adults. MeHg exposure could cause acute and chronic toxicity that affect the neurological system, such as Parkinson's disease and post-polio syndrome. If Hg exposure occurs at higher levels, the damage would appear sooner compared with lower levels. It can accumulate in the body and would eventually appear (Weiss et al., 2002).

Fish is an important source of high-quality protein, vitamin D, selenium, omega-3 fatty acids, and other nutrients. Recently, omega-3 fatty acids, including eicosapentaenoic acid (EPA, C22:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), were found to play a role in brain and retina development and function (Allen and Harris, 2001). However, it is the main cause of exposure to MeHg in humans. Metallic mercury is slowly absorbed by the gastrointestinal tract (approximately 0.01%) while it is absorbed by the lungs quickly. Gastrointestinal absorption of MeHg is about 90%. Humans excrete about 90% Hg in urine and feces that is ingested (Jewett and Duffy, 2007). As a result, the US Environmental Protection Agency (US EPA) has established a reference dose (RfD) for methylmercury of 0.1 μg Hg/kg body weight per day (US EPA, 2001).

Chelating agents could decrease the bioaccessibility of heavy metal to be absorbed or reabsorbed in the gastrointestinal tract. Ethylenediaminetetra acetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) 2,3-dimercaptopropanol (BAL), D-penicillamine (D-β, β-dimethylcysteine), deferoxamine dimercaptosuccinic acid (DMSA), penicillamine and 2, 3-dimercapto-1-propane sulfonate (DMPS) are used as chelation agents for the removal of mercury through inhibition of mercury bioaccessibility or intestinal re/absorption, however, many of these treatments have

reported side effects and are thus not suitable for long term application (Nogueira et al., 2003). Recently, many researchers have reported applying dietary fibers as adsorbents of heavy metal because of their nontoxic properties (Nawirska, 2005; Hu et al., 2010; Shim et al., 2009; Sembries et al., 2004; Zhang et al., 2011; Idouraine et al., 1996). Two possible mechanisms to bind/absorb mercury are chemical sorption and physical sorption. Firstly, chemical sorption is a positive charge of metal cation connected with carboxyl anion, and secondly, physical adsorption has the characteristics of absorbents, such as surface area and porosity (Zhang et al., 2011). The advantage of dietary fiber is that is can be absorbed/bound with mercury or other materials and carries them through the gastrointestinal tract because it is resistant to digestion by the human alimentary enzymes (Dhingra et al., 2012).

Cassava pulp is a by-product of cassava starch factory processing. Cassava (*Manihot esculenta* Crantz.) is a main crop of Thailand, especially in the northeast of Thailand. The total cassava root crops of Thailand in 2011 were approximately 21,060,903 tons (Thailand Tapioca starch, 2011) and they are used mainly for cassava starch production. The residual after the starch has been separated is called cassava pulp. It still contains 60% of starch and 30% of fiber (cellulose) (Apiwatanapiwat et al., 2011). Cassava pulp contains approximately 60% starch, and 30% fiber (Chatakanonda et al., 2003), therefore, the concept of the extraction process of dietary fiber is treating starch and proteins with hydrolysis enzymes. The product from the extraction process is Neutral Detergent Fiber (NDF), which is the most abundant structural component in plant cells (lignin, hemicellulose, and cellulose). This is because cassava pulp mainly contains insoluble fiber such as cellulose (Jung, 1997). Cellulose has a low adsorption capacity. So, chemical modification gives cellulose

new properties, such as hydrophilic or hydrophobic character, improved elasticity, water sorbency, ion-exchange capability, and thermal resistance (Saliba et al., 2005).

Bioaccessibility is the maximum concentration of chemicals or nutrients that are released from the food matrix into aqueous fraction following simulated digestion, which are then available for absorption by the intestinal mucosa (Laparra et al., 2003). The combination of *in vitro* digestion model and Caco-2 cell line is useful tool to measure bioaccessibility and bioavailability. In addition, this method can gives information related to *in vivo* experiments (Courraud et al., 2013).

The couples *in vitro* digestion/Caco-2 cell model was the first method to evaluate chemical risks to humans by offering a tool to approach bioavailability (O'Sullivan et al., 2007). *In vitro* digestion is a simulated system that is related to the human digestive system and is used for measuring bioaccessibility (Intawongse and Dean, 2006). Caco-2 cell can be used to evaluate intestinal cell uptake and help to estimate bioavailability (Glahn et al., 2002). In addition, the *in vitro* method is less expensive, faster and easier to control than with the use of animals and humans (Courraud et al., 2013). However, *in vitro* studies cannot be substituted for *in vivo* studies. Confirmations should be obtained via *in vivo* studies (Donhowe et al., 2014).

Some studies have suggested that particular dietary factors, including fiber and phytochemicals, can similarly impact on MeHg bioavailability. For example, co-consumption of wheat bran with MeHg simultaneously increased fecal mass and decreased the time for absorption. For this reason, it can decrease MeHg levels in the blood and brain (Shim et al., 2009; Tashiro and Shimura, 1982). Thiol-containing compounds in garlic are believed to act as metal-chelating or complexing agents that can increase mercury excretion (Block, 1985). Dietary fiber functional cookies have

no toxic or harmful actions on animals or humans, and the dietary fiber food is able to decrease total cholesterol (TC) and triglycerides (TG) concentrations to some extent in serum and increases excretion of Cd^{2+} and Pb^{2+} in feces (Hong et al., 2012).

Green tea extract (31-2000 mg), black tea extract (31-2000 mg), and soy protein (50-100 mg) significantly reduced mercury bioaccessibility by 82-92%, 88-91%, and 44-87%, respectively. Grapefruit juice (0.5-10 ml) did not reduce mercury in the aqueous phase. Wheat bran (50-1000 mg) decreased mercury bioaccessibility by 84%, and oat bran and psyllium reduced bioaccessibility (greater than 500 mg by 59-75% and 15-31%, respectively. This study suggests that co-consumption of foods containing phytochemicals simultaneously with fish that contain mercury may potentially reduce mercury absorption. Phytochemicals present in tea, including catechins (green tea) and theaflavins and thearubegins (black tea) are all well known chelators of metals. It is plausible that these phytochemicals may form insoluble complexes with mercury and reduce bioaccessibility. Isolated soy protein and wheat bran also decrease bioaccessibility of mercury from fish tissue following *in vitro* digestion. Grapefruit juice had little effect on the reduction of mercury which may be due to the fact that interactions between mercury and phytochemicals might be disrupted by other constituents (sugar, vitamin C, and citric acid) in grapefruit juice (Shim et al., 2009).

Unfortunately, only a few studies have investigated the reduction in bioaccessibility and bioavailability of heavy metals with dietary fiber, thus, information about the specific effects of dietary fiber on human intestinal absorption of methylmercury is lacking.

1.2 Research objectives

The objectives of this study were:

1. To optimize crude dietary fiber extraction condition which focuses on the neutral detergent fiber (NDF) and the etherification method of crude dietary fiber (MDF). The chemical composition and functional properties of both fibers were investigated as following:

1.1 Chemical composition : crude protein, moisture, ash, fat, carbohydrate, crude fiber, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL).

1.2 Functional properties:water holding capacity (WHC), oil holding capacity (OHC), swelling, solubility, COOH content, surface area, pore volume, pore diameter and by testing chemical compounds using Fourier Transform Infrared (FTIR).

2. To study the effect of CDF and MDF on mercury bioaccessibility by using the *in vitro* digestion model.

3. To evaluate the absorption reduction of methyl mercury by CDF and MDF in Caco-2 cell lines.

1.3 Research hypothesis

Crude dietary fiber obtained from the digestion enzymatic method show a poor mercury absorption capacity because cassava pulps mainly contain insoluble fiber (lignin, hemicellulose and cellulose). Therefore using the etherification method to modify crude dietary fiber leads to the adsorption of effective heavy metal/ or lead to the adsorption of the materials in heavy metal. Both types of dietary fiber can be

used in the reduction of mercury biaccessibility in humans by the inhibition of mercury transfer to the aqueous fraction using couple *in vitro* digestion and Caco-2 cell model.

Our main hypotheses are the following: (1) dietary fibers (insoluble fibers) will bind mercury in the gut, making it insoluble and unavailable for absorption by intestinal mucosa, and (2) dietary-containing foods will bind mercury in a dose-dependent manner.

1.4 Scope

The dried cassava pulp used as a source of dietary fiber in this study was obtained from Sanguan Wongse Starch Co., Ltd. First of all, the study optimized the extract condition of crude dietary fiber using response surface methodology (RSM). Independent variables such as the quantity of α-amylase, amyloglucosidase and nutrase were optimized using a 3-factors, 3-level Box-Behnken statistical design. The selected dependent variables are percentages of the NDF. The chemical composition and functional properties of the dietary fiber will be investigated. These crude dietary fibers will then be modified by the etherification method with acrylonitrile and hydroxylamine. In addition, both dietary fibers affecting the mercury bioaccessibility will be estimated by using *in vitro* digestion and the Caco-2 human intestinal cell model system.

1.5 Expected results

CDF) and MDF from dried cassava pulp is capable of decreasing mercury bioavailability by inhibition of the mercury transfer to the aqueous fraction and could be applied in functional food and dietary supplement products.

Results from this research will lead to the full utilization of waste from cassava starch processing, and increase the value of these wastes. In addition, more knowledge will be obtained about physicochemical, functional properties, binding capacity *in vitro* and applications of dried cassava pulp in food models. This research will also lead to a greater understanding of the efficacy of dietary fiber to reduce heavy metal toxicity. The resulting dietary fiber from dried cassava pulp should provide a promising functional food or dietary supplement.

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CHAPTER II

LITERATURE REVIEW

2.1 Mercury

Mercury is a toxic element (Hg) with the atomic number 80 and has no biological functions like other metals, such as iron, copper, and zinc. It is liquid at room temperature and its density is 13.456 g/cm^3 (López et al., 1999). It has 3 forms: elemental mercury, inorganic mercury and organic mercury (e.g., methylmercury). The elemental mercury can be transformed into inorganic mercury in combination with other chemicals, such as chlorine, sulfur, or oxygen and it also combines with carbon to become organic mercury (ATSDR, 1999). Methylmercury is the most commom form of organic mercury, which is produced via bacteria in water and soil by methylation process (Isabelle Ji, 2011).

2.1.1 Forms of mercury

2.1.1.1 Elemental mercury

Elemental or metallic mercury is a shiny, silver-white, odorless liquid. It is a colorless, odorless gas, if heated (UNEP, 2002). The symbol in this form is Hg(0) or Hg^0 (uncharged), where the 0 means the oxidation state of the mercury atom. Elemental mercury can become mercury vapour when it is emitted into the air. Rates of evaporation depend on temperature: It can evaporates at the high temperature.

Mercury vapour is highly toxic (Chrystall and Rumsby, 2009). Inhalation of elemental mercury is the main cause of exposure to humans (80%). Absorbtion via the lungs is rapid and it passes directly to the target organ (brain) which affects the nervous system causing neurological damage (Bernhoft, 2011). After exposure to elemental mercury, it can be transformed to inorganic mercury by oxidation process and accumulated in the body, for example, in the brain or kidney (ATSDR, 1999). It can also be found in thermometers, dental amalgams, electrical switches and preservatives. Recently it commonly found in lamps, electronics, and skin lightening creams (Chrystall and Rumsby, 2009).

2.1.1.2 Inorganic mercury

Inorganic mercury compounds are more commonly found in nature than the elemental form of mercury. Mercury is a natural compound of the Earth's crust and exists in all rocks, soils and sediments, usually appear in inorganic form (Organization, 1991). Cinnabar is inorganic mercury, which occurs naturally in mercuric sulfide (HgS). Other inorganic mercury include mercuric oxide (Hg^0) and mercuric chloride ($HgCl_2$) (Young et al., 2002). Mercuric mercury is a single mercury atom with 2 charges, which bind with negatively charges to form salt. It can bind with sulphur and organic chemicals to form organic mercury compounds (Chrystall and Rumsby, 2009). Some forms of mercuric mercury are soluble in water, such as mercurous (Hg_2^{2+}) and mercuric (Hg^{2+}) but mercury oxide (HgO) and mercury sulphide (HgS) compounds have low solubility in water (Authority, 2012). In humans, absorption of inorganic mercury through the gastrointestinal tract ~10%, are unable cross the blood-brain barrier in adult. In contrast, absorption of elemental mercury by inhalation is rapid and goes directly to the all major organs (Park and Zheng,

2012). Babies can obtain in organic mercury via breast milk, which is the main cause to exposure of mercury in infants (Abadin et al., 1997).

In addition, humans can be exposed to inorganic mercury via dental amalgams and skin lightening creams, and industrial activities, such as mining and concrete and steel manufactured products. Inorganic mercury was used as a fungicide, but this has been discontinued in many countries (Chrystall and Rumsby, 2009).

2.1.1.3 Organic mercury

Organic mercury is formed when a mercury atom or inorganic mercury is bound to a carbon molecule. The most common form is methylmercury (CH_3Hg^+) that is found in fish and seafood. It can bind with chloride such as methylmercury chloride (CH_3HgCl) (Cornelis et al., 2006). In water inorganic mercury transforms to organic mercury via bacteria by methylation process and accomulates in fish or sea animals (Wang and Wong, 2003). The concentration of organic mercury is related to order in the food chain, with larger fish usually containing higher concentrations of organic mercury than smaller fish (Chemicals, 2002). Absorption of organic mercury through the gastrointestinal tract is greater than by any other pathway. However, accumulation is longest in the brain. Mercury can remain in the body for weeks or months by accumulating in the kidneys and it is then excreted through the urine and feces. This causes damage to humans by interrupting the intracellular thiol processes, and the mercury leads to oxidative stress, lipid peroxidation, mitochondrial dysfunction, and changes in heme metabolism; mercuric chloride has been shown to depolarize the mitochondrial inner membrane and increase the formation of hydrogen peroxide (Chemicals, 2002). The methyl mercury compound is dangerous to humans. It is absorbed from gut into the bloodstream and goes to all tissues where it

accumulates in the organs. In the brain methyl mercury can be transformed to inorganic mercury and passes through the blood brain barriers and placental barriers especially in fetuses (Toimela et al., 2004).

Humans are mainly exposed to organic mercury (in particular methylmercury), through the dietary consumption of fish. The older predatory fish such as swordfish and tuna are usually contain high levels of mercury (Authority, 2012).

2.1.2 Mercury cycle in the environment

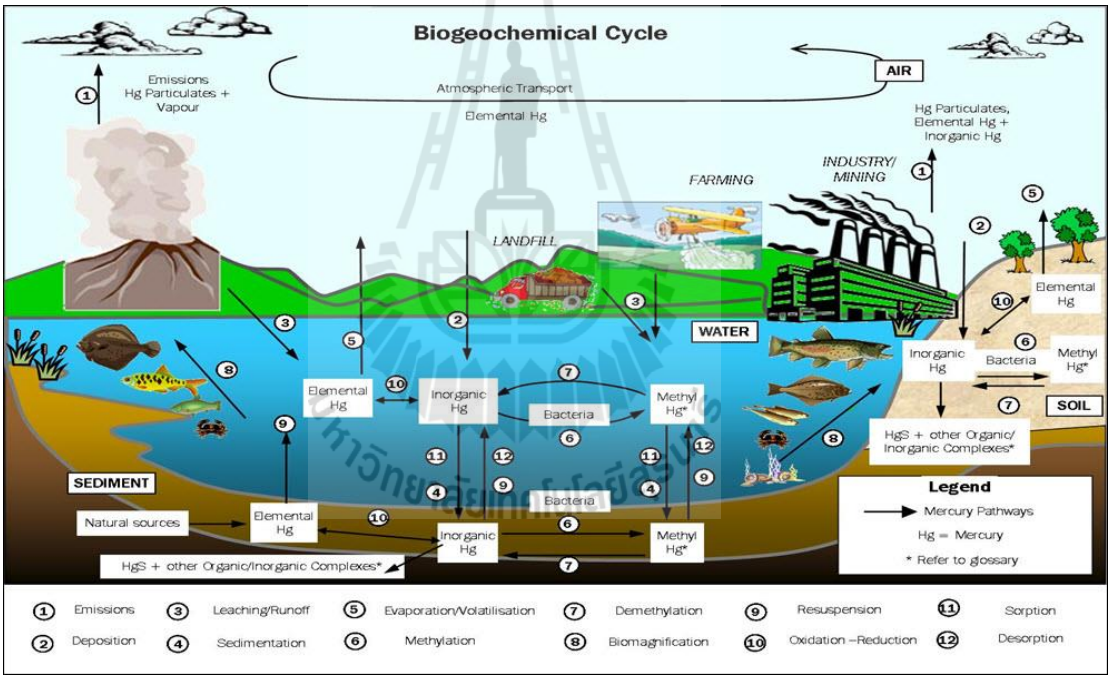


Figure 2.1 Shows a volcano, a landfill, farming, industry/mining, trees, soil, a body of water with fish and sediment and mercury flowing between them.

Source: Ministry for the Environment (2008).

Air: Mercury emissions occur from volcanoes and industry in elemental mercury form, and it is released by mining into the air and enters into the water and the land through rain.

Land: mercury comes from volcanos, landfills, farming and industry, and mining allows it to enter the water through leaching or runoff.

Soil: Elemental mercury moves to inorganic mercury by anoxidation-reduction process. Inorganic mercury moves to organic mercury via bacteria in the soil by a methylation process and back to inorganic mercury via bacteria by a demethylation process.

Water: In the water, there is a process of transformation between methylmercury and inorganic mercury via bacteria by a methylation or a demethylation process. Elemental mercury becomes inorganic mercury by an oxidation-reduction process. Biomagnification of mercury in water occurs through fish and other aquatic animals. Mercury transfers to the water from land by leaching/runoff and from the air by deposition.

Sediment: elemental mercury enters water by resuspension. Inorganic mercury moves to elemental mercury through oxidation-reduction and from inorganic mercury to methylmercury by methylation and from methylmercury to inorganic mercury by demethylation.

Most of the mercury emissions in the environment come from human activity, particularly from coal-fired power stations including heating systems and waste incinerators (Chemicals, 2002). The mining of gold is a main cause of the accumulation of mercury in the environment and in other metals, such as copper, zinc and silver. Approximately 5% of all mercury released in wastewater comes from

healthcare facilities. Dental amalgam is the main cause of exposure to mercury in humans because it contains up to 50% elemental mercury (Organization, 2007). Metallic mercury is used to produce chlorine gas and caustic soda, and is also used in many products such as thermometers, dental fillings, and batteries. Mercury salts are sometimes used in skin lightening creams and as antiseptic creams and ointments (Kumar et al., 2000).

Since July 1999, the mercury-based vaccine preservative thiomersal (sodium ethylmercurythiosalicyclate or thimerosal) has come under public and professional scrutiny because of concern about the cumulative amount of mercury in infants and children. In 2006 the WHO Global Advisory Committee on Vaccine Safety reported that there were no reasons to change current immunization practices. However, WHO continues to review the evidence for pre-term and malnourished infants (WHO, 2006). In addition, the use of mercury in skin-lightening creams products are increasingly popular in dark-skinned women. Its use can cause serious health problems in the long term (Al-Saleh et al., 2004).

2.1.3 Mercury bioavailability

Mercury bioavailability is the amount of mercury available to enter biological functions. Increasing bioavailability is very dangerous because the mercury can be transformed via bacteria by methylation/demethylation processes that cause health effects (Isabelle, 2005).

Aquatic organisms can accumulate MeHg from smaller organisms. In higher organisms, mercury was ingested from CH_3Hg contaminated food. Bioavailability processes within the gastrointestinal tract (GI tract), is amount of MeHg released from food metrix to digesta. The amount of MeHg that enters the GI tract does not

necessarily access the bloodstream or reach critical target organs. The amount of MeHg which enters the bloodstream depends on bioavailability and absorption. Absorption and bioavailability depend on types of mercury, age, frequency of meals and other dietary variables. Absorption of liquid elemental mercury from the GI tract is <1%. In contrast, MeHg is readily absorbed from the lungs 50-100% (Gochfeld, 2003). Metallic mercury in the vapor form is readily absorbed through the lungs. Because of its soluble properties, elemental mercury can pass through cell membranes as well as the blood-brain and placental barriers to reach target organs. In the bloodstream, mercury undergoes catalase and peroxidase-mediated oxidation in red blood cells and tissues and is transformed into inorganic mercuric mercury (Hg^{++}) and mercurous mercury (Hg^{+}), and these processes that limit its absorption. Inorganic mercury has low lipophilicity and has a limited ability to cross cell membranes (Fernandes Azevedo et al., 2012). These processes depend on the amounts of mercury exchanged between the blood compartment and organs, including the brain (Gochfeld, 2003).

Table 2.1 Absorption of mercury species by routes.

	Elemental (Hg^0)	**Organic (Hg_i)**	**Organic**
Lungs	Almost complete	Variable	Almost complete
GI tract	Negligible	Variable	Almost complete
Dermal	Negligible	Negligible	Moderate to high

Source: Gochfeld, 2003.

2.1.4 Health effects of methylmercury

Humans can be exposed to MeHg by eating fish that contain MeHg (Skerfving et al., 1970). The toxiciry of Hg depends on the dose, the composition of the mercury compound and the route of entry (Tchounwou et al., 2003). Recently, there have been concerns about the effects of Hg on the health in children because MeHg easily passes through the blood-brain barrier and causes damage to the central nervous system, especially in fetuses. When mothers eat fish and shellfish that contain MeHg they can affect the development of the brains and nervous systems of their children (Counter and Buchanan, 2004). Exposure to MeHg in fetuses can cause effects on neurodevelopment. The prenatal period is a susceptible stage of life. MeHg will inhibit fundamental processes in brain development, such as neuronal cell division and migration (Myers et al., 2003). As a result, fetuses are a high-risk group for MeHg exposure becuase the developing brain in the fetuses is highly sensitive to MeHg, and it accumulates in cord blood rather than in the mother's blood system. Thus, exposure to MeHg during pregnancy is a very serious problem, especially in mothers who consume large amounts of fish or sea food The evaluation of elementary or mercuric Hg exposure can be used for plasma or serum as a biomarker, especially red blood cells (RBC-Hg) which are the best biomarker of MeHg exposure (Sakamoto et al., 2004). In addition to the symptoms of methylmercury poisoning such as impairment of peripheral vision, numbness and loss of feeling, tingling sensations along the limbs, lack of coordination of movement, the impairment of speech and hearing, muscle weakness, dramatic mood swings, memory loss, and mental disturbance (Navarro, 2006).

The toxicity of inorganic mercury such as mercuric ions it can be connected with sulfhydryl groups of amino acids, which are building blocks for enzymes. The

most toxic form of mercury is organic mercury because it can be absorbed via the GI tract more easily than in other ways (Bose-O'Reilly et al., 2010). The toxicity of inorganic mercury depends on dosage, acute exposure to mercury vapor, erosive bronchitis and bronchiolitis potentially leading to respiratory failure which may also be accompanied by CNS symptoms such as tremors or erethism. Chronic exposure usually produces neurological dysfunction. Low-level exposure causes weakness, fatigue, anorexia, weight loss and gastrointestinal disturbance. Higher exposure levels are associated with fine muscle fasciculations punctuated every few minutes by coarse shaking and may result in emotional changes, loss of memory, insomnia, depression, fatigue, and in severe cases even delirium, hallucinations, gingivitis and copious salivation (Bernhoft, 2011).

2.1.5 Human exposure to methylmercury

Methylmercury is one of the the toxic elements in aquatic food chains in the long term and can affect humans who eat fish. It is the primary source of human mercury poisoning (Bizily et al., 2003). Mercury occurs naturally in the earth's crust and oceans, and is caused by the burning of industrial products and the combustion of coal. Mercury becomes more toxic from bacteria in water called methylmercury. Fish absorb methylmercury as water passes through their gills. They increase the amount of MeHg which accumulates when they feed on smaller organisms or smaller fish. MeHg can bind with the protein in fish tissue and cooking (eg, deep-frying, boiling, baking, pan-frying) and does not degrade MeHg or remove it (Saulo, 2004).

Methyl mercury is absorbed through the GI tract and accumulates in many tissues, but does not cross the blood-brain barrier as elemental mercury does. However, it can be transformed to elemental mercury, such as salt mercury, that

makes it insoluble, relatively stable, and poorly absorbed (Bernhoft, 2011). Feng et al. (2007) have reported that the toxicity of methylmercury (MeHg) has caused widespread concerns for human health. MeHg is highly toxic, especially in the nervous system. Although the main cause of exposure to MeHg is through contaminated fish. Qiu et al. (2008) have reported that 98 persons from the Wanshan Hg mining area (China), showed high levels of MeHg in their hair because they consume rice, the staple food of the local inhabitants which shows high levels of Hg and MeHg.

The level of Hg in the hair and blood is used to evaluate the accumulation of Hg in humans (Akagi and Naganuma, 2000). Human exposure to methyl mercury in the long-term and frequent intake of seafood with high mercury levels, especially in pregnant women, causes high concentrations of mercury in the blood and hair. These concentrations have been associated with an increase in DNA damage in blood cells (Renzoni et al., 1998). The fetus is sensitive to MeHg exposure and has effects on infant development due to levels of exposure (Gilbert and Grant-Webster, 1995).

MeHg is found in seafood and freshwater fish throughout the world (Grandjean et al., 1997). High levels of MeHg are found in shark, swordfish, king mackerel and tilefish so people are recommended not to consume these fish (Saulo, 2004). The regulatory level of methylmercury exposure is 1.0 ppm, which provides enough protection for young children, and for a significant number of consumers who exceed the acceptable daily intake (Tollefson and Cordle, 1986). The level of MeHg in blood is related to the intake of MeHg from fish. A daily intake of 1 microgram of methylmercury would produce an Hg concentration in the blood of 0.8 micrograms/kg (Sherlock et al., 1984).

2.1.6 Regulation of mercury

Many government agencies have issued guidance that is designed to limit mercury exposure from fish consumption and increasingly discriminates between organic and inorganic forms (Table 2.2).

2.1.7 Methods of analyzing mercury levels

Most of the analytical methods are used to analyze the components of wastewater, drinking water, sediment, and other environmental samples. The analytical methods for metals require acid digestion before analysis. The digestion solubilizes particulate matter in the sample and removes the potential interferences within the sample matrix. Inductively coupled plasma mass spectrometry (ICP/MS) is a tool for mercury determinations. The detection limits are of less than 100 pg of Hg (Hintelmann et al., 2002).

An HPLC detector usually gives poor sensitivity with inorganic and organic mercury. In recent years, analytical methods with element-specific detection methods, such as atomic absorption spectrometry (AAS) coupled with plasma mass spectrometry (ICP-MS) have been used as routine techniques (Liang et al., 2003).

The ICP-MS method has been extensively developed due to new high performance compact equipment. Metals are ionized at a high temperature (8000 K) and focused on a mass detector. In clinical and forensic toxicology, Analyzing the composition of metals is most important for monitoring heavy metal exposure, such as lead, mercury, cadmium or bismuth. Some other metals are of clinical or forensic interest, such as beryllium, boron, lithium, aluminum, vanadium, manganese, chromium, cobalt, nickel, copper, selenium, molybdenum, silver, tin, antimony, tellurium, barium, and platinum (Goullé et al., 2005).

Table 2.2 Limited mercury exposure from fish consumption.

Country	Food product	Substance	Levels
USA	All fish(fresh/saltwater, finfish, crustaceans, molluscs)	Methylmercury	1.0 ppm.2 NOTE: An older FDA requirement had a 0.5 ppm maximum for specific fish, such as tuna. This requirement is still listed in some US State regulations.
Canada	Retail fish (with exceptions) Exceptions: fresh/frozen tuna, shark, swordfish, escolar, marlin, and orange roughy	Total Mercury	0.5 ppm 1.0 ppm
Oceania (Australia & New Zealand)	Crustacea, fish and molluscs: Gemfish, billfish (including marlin), southern bluefin tuna, barramundi, ling, orange roughy, rays and all species of shark, and Fish for which insufficient samples are available to analyse in accordance with clause 6	Mercury	Mean 0.5 mg/kg Mean 1.0 mg/kg
China	Seafood	Methylmercury	0.5 mg/kg
Taiwan	Seafood	Methylmercury	0.5-2 ppm NOTE: MRL varies by seafood variety.
Europe	Fishery products and muscle meat of fish, except specific exclusions* * Exclusions	Mercury	0.5 mg/kg 1.0 mg/kg

Source: US EPA (2003).

Total mercury levels are usually determined with the cold vapor atomic absorption (CVAA) method. The elemental mercury was observed at 253.7 nm. The instrumentation can detect a limit of 0.002 ppb mercury. These methods are used for determining concentrations of inorganic mercury and organic mercury (methylmercury, methylmercury, phenylmercury). This method separates the organic mercury species by primarily using gas chromatography followed by electron capture, plasma emission, mass spectrometer, or CVAA detection. Extraction of the organic mercury species into an organic solvent is required and also provides preconcentration. Inorganic mercury must be converted to an organic mercury compound for its determination (Oda and Ingle Jr, 1981).

The advantage of cold-vapor atomic fluorescence spectrometry (CVAFS) is that is simple, sensitive and cost-effective, but it is not popular as a detector for mercury compounds. Many reseachers use atomic fluorescence spectrometry as a specific detector for HPLC for mercury speciation, which adopts post column ultraviolet (UV) irradiation to oxidize organic mercury to inorganic mercury (Liang et al., 2003).

Thermal Decomposition Amalgamation/Atomic Absorption Spectrophotometry (TDA/AAS) is an analytical method for determining the concentration of mercury in samples. The DMA-80 Direct Mercury Analyzer instrument is a type of TDA/AAS which was developed to determine Hg (Butala et al., 2006). The principle of this system is to weigh a portion of sample (fresh fish) and then to combust it. The mercury is released and is selectively trapped in a gold amalgamator. Upon heating, the mercury is desorbed from the amalgamator, an atomic absorption measurement is performed and the mercury concentration is calculated (Torres et al., 2012).

2.1.8 Fish consumption

The most common form of mercury in fish is methylmercury, which binds/connects with sulfhydryl groups of protein in muscle tissue. Ingestion of fish muscle is an important exposure pathway for mercury to humans (VAN et al., 1994). Fish are an important source of protein and polyunsaturated fatty acids (n-3 PUFA) in humans, especially in mothers. Most mothers believe fish consumption leads to better fetus development. However, fish also contain toxic elements, such as methyl mercury (MeHg). Eating fish that is contaminated with MeHg is the main cause of exposure to MeHg in humans. The concentrations of Hg in red blood cells (RBC-Hg) are the best biomarker of MeHg exposure. Docosahexaenoic acid (DHA, C22:6n-3), which is important fatty acids for normal brain development and function, is also derived from fish consumption (Sakamoto et al., 2004). Hg levels in hair below 5 and 10 ppm are considered as acceptable to protect fetuses and adults (Boischio and Henshel, 2000). MeHg is absorbed and distributed to the brain, where it accumulates. The concentraion of MeHg in the brain is about five times higher than that in blood. MeHg is also distributed in human hair. The hair-to-blood concentration ratio, expressed as μg Hg/g hair to μg Hg/ml blood, is about 250:1 (Castoldi et al., 2008).

Fish consumption advisory bodies are increasingly concerned about toxic elements in water. Most states in the country issue fish consumption advisories. These advisories introduce the dangers of consuming fish. The toxicity levels which are considered safe meal sizes, safe meal frequencies, and species fit for consumption (Gibson, 2005). The advisories may suggest that people avoid eating some kind of fish that contain high MeHg. Some warnings apply to specific water types (like lakes). Some may focus on groups of particularly sensitive people, such as pregnant women and children). Some warnings tell us that certain fish are safe to eat. These

warnings are obtained from the monitoring of contaminated fish and establishing waters where it is safe to eat fish which are increasing (Golchin, 2012; Joseph, 2003).

2.1.9 Nutritional aspects of fish consumption

The consumption of fish has long been associated with health benefits. Fish is an important source of many nutrients, including protein and omega-3 polyunsaturated fatty acids (PUFAs) that are beneficial for human health. In addition, fish contains minerals (calcium, iron, selenium, zinc, etc.), and vitamins, namely A, B3 (nicotinamide), B6 (pyridoxine), B12 (cobalamine), E (d-tocopherol), and D in fish tissues (Sidhu, 2003). The public have become confused about the risks and benefits of fish intake. Fish consumption could reduce rates of death from coronary heart disease (CHD), that might be because of long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in fish. DHA is important for neurodevelopment during gestation and infancy. However, it contains toxic elements such as mercury, dioxins, and polychlorinated biphenyls (PCBs) that are present in some fish species (Mozaffarian and Rimm, 2006). Thus, the easiest way to avoid concerns about contaminants is simply to eat a variety of fish and other seafood (Meyers et al., 2003).

Consumption of fish (1-2 servings/week), especially species with high n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduces the risk of coronary death by 36% and mortality by 17% and may favorably affect other clinical outcomes. An intake of 250 mg/day of EPA and DHA appears sufficient for primary prevention (Mozaffarian and Rimm, 2006). The intake of fish containing Hg at high levels lead to brain development damage in babies. But low intake of omega-3 fats from fish is also dangerous. Several observational studies of

the fish intake among pregnant women, who ate less than two servings of fish perweek and children born to mothers who ate fish at least twice a week found that it has similar benefits for mother and their babies' brain development. Thus, women should avoid seafood consumption (Meyers et al., 2003).

2.1.10 Chelating agent of methylmercury

Chelating agents are used to reduce the toxicity of heavy metal, including Hg poisoning but the potential preventive role of chelating agents against heavy metal poisoning has not been explored much (Guan and Dai, 2009). Typically, ethylenediaminetetraacetic acid (EDTA), Diethylenetriaminepentaacetic acid (DTPA) 2,3-dimercaptopropanol (BAL), D-Penicillamine (D-β, β-dimethylcysteine), Deferoxamine dimercaptosuccinic acid (DMSA), penicillamine and 2, and 3-dimercapto-1-propane sulfonate (DMPS) (Tandon et al., 2001) are used as chelation agents for the removal of systemic mercury through inhibition of mercury bioaccessibility or intestinal re/absoprtion, however, many of these treatments have reported side effects (Karlsson et al., 2010) and are thus not suitable for long term application.

During the Second World War, BAL was developed as a chelating agent to reduce toxicity of arsenic based war gases. However, it has high toxicity and side effects. The characteristics of an ideal chelator are greater affinity for toxic metal, low toxicity, ability to penetrate cell membrane, rapid elimination of metal and higher water solubility (Flora et al., 2008).

EDTA and dimercaprol (British anti-lewisite, BAL) are becoming outmoded and can be replaced by meso-2,3-dimercaptosuccinic acid (DMSA, succimer) for treatment of lead intoxication and by sodium salt of 2,3-dimercapto-1-propanesulfonic acid (DMPS, DimavalR) for treating lead, mercury or arsenic intoxication (Aposhian

et al., 1995). Ethylenediaminetetraacetic acid (EDTA) is a very effective chelating agent for the removal of the toxicity of heavy metal, but it is quite persistent in the environment due to its low biodegradability (Tandy et al., 2004).

2.1.11 Diet and methylmercury

The toxicity of methylmercury from fish is high with 90-95% absorbed through the intestinal cell (Janle et al., 2014). Most mercury is bound with a sulphydryl group of proteins, approximately 1-10% of the absorbed mercury is found in the blood and 90% of the blood burden are in the red blood cells bound to the cysteine residues of hemoglobin (Ramos, 2013).

It has been suggested that interaction between diet and methylmercury may affect methylmercury absorption, metabolism and excretion (Rowland et al., 1984). Factors affecting absorption are interactions between nutrients and food components, the effects of luminal condition, food preparation and processing practices, and the nature of the food matrix.The effects are on micronutrient absorption ability (bioavailability) or on the qualtity of nutrient to be absorbed (bioaccessibility) (Etcheverry et al., 2011). Recently, many researchers have reported applying nutrient as adsorbents of mercury, such as green tea extract, black tea extract, soy protein, oat bran, psyllium and wheat bran which significantly reduced mercury bioaccessibility. This study suggests that eating phytochemicals with fish tissue that could inhibit mercury transfer to aqueous fraction results in the reduction of mercury bioaccesibility (Shim et al., 2009). Water-soluble dietary fibers from apple peel and water-insoluble dietary fibers from wheat bran and soybean-seed hulls were used to evaluate their binding capacities for four toxic elements (Pb, Hg, Cd, and dietary fibers can adsorb heavy metals and act as a potential “functional food” (Zhang et al., 2011).

Dietary fiber is the main nutrient having the effect of binding or absorbing mercury or other heavy metal by using chemisorption and physical absorption which carries them through the gastrointestinal tract (GI tract). Thus, dietary fiber can reduce absorption in intestinal cells (Kay, 1982). Garlic appears as a potential chelating agent which can increase the excretion of methylmercury (Janle et al., 2014). Dietary bran may reduce the levels of mercury in the brain after methylmercury exposure and may therefore reduce the neurological damage. Dietary bran may reduce toxicity of mercury and may therefore reduce the neurotoxic effects of the organomercurial. Feeding with 15-30% bran in the diet of mice can decrease the mercury in the brain, blood and small intestine. The fibres have little effect on mercury levels in other tissues (Roland et al., 1986).

2.2 Cassava and cassava pulp

2.2.1 Cassava starch industry, will betes and utilization

Cassava (*Manihot esculenta* Crantz.) is the third most important crop in Thailand. The root crop is known by many names in Thailand, but cassava and tapioca are the most widely used terms (Thailand Tapioca starch, 2011). Cassava was introduced into the southern part of Thailand from Malaysia during 1786-1840 and was gradually distributed throughout the country within a few years (Cenpukdee et al., 1992). The main area of the crop is found in the northeast of Thailand, especially in Nakhon Ratchasima. Cassava has excellent drought tolerance properties and can be planted in almost all types of soil. So, it has rapidly increased in this area which is frequently subject to drought conditions (Ekasingh et al., 2007). Cassava root in Thailand is grown by a large number of farmers (0.5-2 ha/farm). The total acreage of cassava, which peaked at about 1.6 million in 1988/89 is now reduced to 1.2 million

(1998/99). The national agricultural policy promoted reductions in the planting areas and increase in yields. However, total production in 1998/99 was only 17 million tons or less than 70% of the peak of 24 million tonnes in 1988/89 (Sriroth et al., 2000).

Table 2.3 Planting area, production and yield of cassava in Thailand from 1994/95 to 2004/2005.

Year	Planting area (ha)	Total production (tones)	Yield (t/ha)
1994/95	1,245,157	16,217,378	13.02
1995/96	1,228,114	17,387,780	14.16
1996/97	1,230,381	18,083,579	14.70
1997/98	1,119,096	15,968,474	14.27
1998/99	1,172,374	17,315,554	14.77
1999/00	1,095,631	16,930,190	15.45
2000/01	1,106,880	18,396,000	17.53
2001/02	999,040	16,868,000	17.66
2002/03	1,029,600	19,718,000	19.29
2003/04	1,081,120	21,440,000	20.27
2004/05	1,043,840	16,938,000	17.18

Source: Sriroth et al., 2000.

Cassava roots are utilized for native starch, modified starch, MSG, sweeteners and alcohol, glucose, fructose, sorbitol, sago, citric acid, paper, textile, and for plywood (Howeler, 2003).

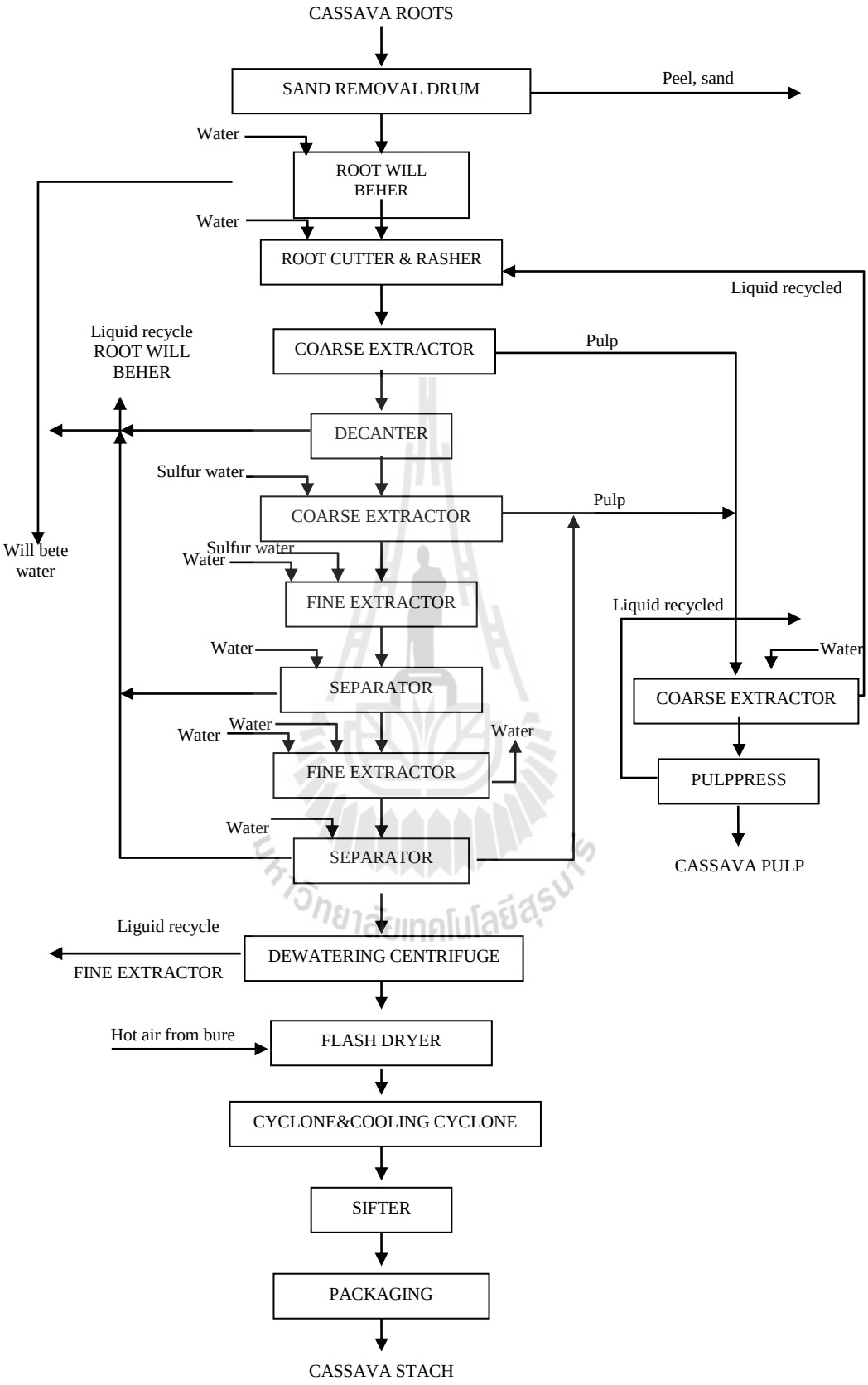


Figure 2.2 Example of the cassava starch manufacturing process in Thailand.

Dried cassava pulp is a by-product of cassava starch factory processing and it contains a large quantity of starch. Thus it can be used as a source of dietary fiber (Khempaka et al., 2009). It acts as a polymer matrix with variable physicochemical properties within the gastrointestinal tract. Therefore, it can bind/absorb for the removal of heavy metal (Kay, 1982).

2.3 Dietary fibers

Dietary fiber is the main component of plant cells, resistant to the digestion by human alimentary enzymes. During digestion in gastrointestinal tracts, dietary fiber forms a matrix with both fibrous and amorphous characteristics (Kay, 1982). The properties of dietary fiber are important to determine the nutritional value, which depend on the physio-chemical characteristics of the fiber itself (Staffolo et al., 2012). Dietary fibers occurs in 3 forms in the small intestine, for example, soluble polymer chains in solution, insoluble macromolecular assemblies and sponge-like networks passing along the intestine. Their physical properties are viscosity, water holding, cation exchange, organic acid adsorption, gel filtration, and partide-size distribution that influence the functions of the gastrointestinal tract. The surface properties of water-insoluble components, and the network properties of the swollen, hydrated components are important for the biological effects of dietary fiber in the intestine for absorption and increase in stool weight (Eastwood and Morris, 1992). The main physiological effect of dietary fiber in the small intestine is to reduce the amount of nutrients are released from structure (Brownlee et al., 2006). Dietary fiber can link with other molecules by ionic bonds, hydrogen bonds, and weaker hydrophobic and dispersion forces. These interactions may have an effect on absorption. In

addition, changes in the surrounding pH and osmolality also affects matrix structure and function. (Kay, 1982).

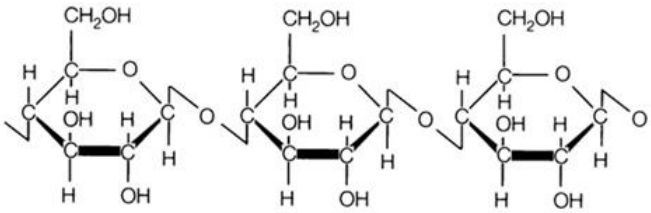
2.3.1 Classification of dietary fiber

The two major classes of dietary fiber are cellulose and lignin that include noncellulosic polysaccharide (NCP) (Powers, 1996).

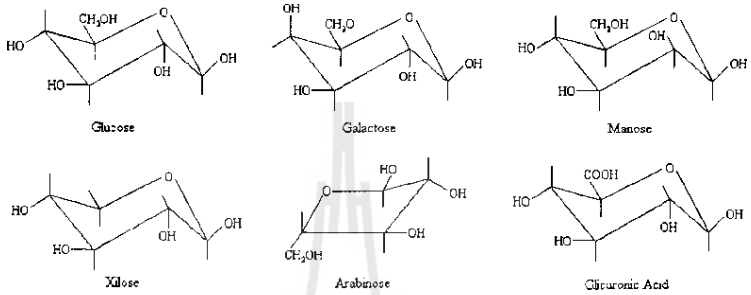
2.3.1.1 Polysaccharide

Cellulose, the most abundant molecule in nature, is composed of anhydrous-d-glucose unit with 1,4 & linked glucose units with up to 10,000 β-d-glucose residue are linked in a long chain. Hydrogen bonds link sugar residues in microfibril structure (Powers, 1996). Non-cellulosic polysaccharide (NCP) includes those polysaccharides like inulin and guar and the plant gums and mucilages (Silk, 1989). Hemicelluloses are polysaccharides in cell walls, solubilized by aqueous alkali after removal of water soluble and pectic polysaccharides. The nature of bonds are backbones of β- 1,4-1inked pyranoside sugars, but differ from cellulose in that they are smaller in size (often less than 200 sugar residues) (Inglett, 2012). The hemicelluloses are subclassified on the basis of the monomer sugar residue (Riccioni et al., 2012). Pectin is one of the most complex classes of plant polysaccharides and is found in all higher flowering plants, which are the large group of glycanogalacturonans, acidic plant polysaccharides in which the backbone consists of 1,4-linked α-D-galacturonic acid residues (Ovodov, 2009).

(a)



(b)



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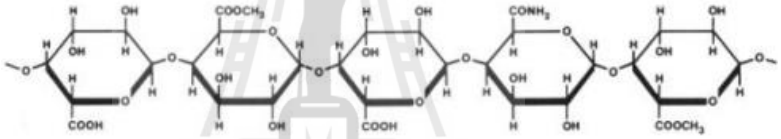

(d)

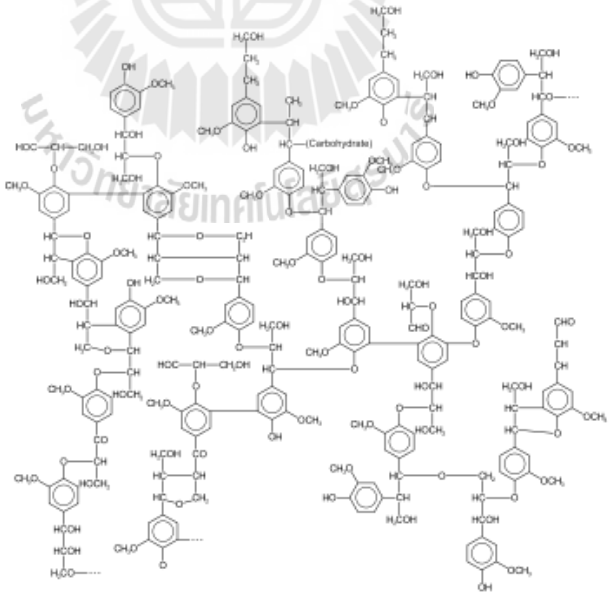

Figure 2.3 Structure of dietary fiber (a) cellulose (b) major component sugars of hemicelluloses (c) pectin and (d) lignin.

Source: Anderson et al. (2009).

2.3.1.2 Lignin

Lignin is a polymeric natural product including coniferyl, sinapyl, and p-coumaryl alcohols that have undergone a complex dehydrogenative polymerization process (Lawoko, 2005). It is a large group of aromatic polymers resulting from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids. These polymers are found in cell walls making them thick and rigid (Vanholme et al., 2010).

2.3.2 Physicochemical and functional properties

Dietary fibre can increase faecal output from the gastrointestinal tract and the time rate of passage through it (Van Dokkum et al., 1983). The bulk of the fiber passes through the GI tract and the capacity of the fibre to retain water is important for its functional properties (McConnell et al., 1974). It acts as a polymer matrix within the gastrointestinal tract, making variable physicochemical properties including susceptibility to bacterial fermentation, water-holding capacity, cation-exchange, and adsorptive functions (Kay, 1982).

2.3.2.1 Cation exchange properties

"Cation exchange capacity" (CEC) is the ability to attract and bind hydrogen ions. Hydrogen ions have a positive charge. Therefore, plant cells containing negative charges on their surface can bind with hydrogen ions. The negative charges in plants cell are different depending on the kind of plant (McBurney et al., 1983). The number of free carboxyl groups on the sugar residues are related to the functional proporties of dietary fiber. Acidic polysaccharides formed with cation complex show the effects on mineral balance, electrolyte absorption, and heavy metal toxicity (Kay, 1982).

2.3.2.2 Water-holding capacity of dietary fiber

The water-holding capacity of dietary fiber has important physiological effects on the intestine (Kay, 1982). The particle size of dietary fiber has an effect on water-holding capacity because it determines the volume of the space within the fiber matrix that is available for water entrapment (Căpriţă et al., 2010). The physical structure of fiber is an important determinant of hydratability. The method of fiber preparation has more effect on the water holding capacity of fiber than on its chemical composition. In the upper intestine, the water-holding capacity of fiber may affect nutrient absorption, postprandial satiety, and intestinal motility (Robertson and Eastwood, 1981). Dietary fiber can absorb water with surface adsorption of water molecules. The presence of sugar residues with free polar groups confers a significant hydrophilic capacity to polysaccharides (Kay, 1982).

2.3.2.3 Susceptibility to bacterial enzyme degradation

Dietary fiber is resistant to the digestion of human intestinal enzymes. However, fermentation occurs in the large intestine. Fermentation in the colon is significant because it eliminates certain actions of fiber observed *in vitro* or in the upper intestine (Kay, 1982). Biodegradation of dietary fiber involves chemical degradation by the action of bacteria and fungi via enzymatic action in the colon (Mohan, 2011). Fiber degradation in the colon is due to bacterial enzyme in the colon, the transit time through the colon, and the physical and chemical composition of the fiber (Schweizer and Edwards, 2013). The susceptibility to fermentation in the human colon depends on the size of the dietary fiber. In addition, the surface area of dietary fiber is influenced by the breakdown of cellulose by bacteria (Bjorck et al., 1984). Bacterial degradation of dietary fiber in the colon occurs in two stages. Extracellular

hydrolysis of polysaccharides into component mono- and disaccharides is followed by intracellular anaerobic glycolysis (Pool-Zobel et al., 1993). During degradation, enzymes from microorganisms break down complex polymers. The products from fermentation are short chain volatile fatty acids, acetate, proprionate, and butyrate, which are readily absorbed and utilized for energy and lipid synthesis. Other products are lactic and formic acids, ethanol, and CO_2. Therefore, fermentation of dietary fiber can be a major source of energy (Gu, 2003; Kay, 1982). Digestion of polysaccharides varies between 30 to over 90%. Pectin and hemicellulose are almost completely lost during digestion and absorbtion as they pass through the colon. Most of cellulose is retained after being digested. Lignin is resistant to bacterial degradation and is almost completely recovered in the stool (Kay, 1982). In general, the fiber of fruit and vegetables are much more fermentable than cereal brans because the cell walls of cereals are thicker and have a high degree of lignification (Căpriţă et al., 2010).

2.3.2.4 Adsorption of organic materials

Organic materials such as bile acids, other steroids, various toxic compounds, and bacteria may be bound to fiber as it passes along the gastrointestinal tract (Ekvall, 2005). *In vitro* studies reveal that adsorption of bile acids is dependent on the composition of the fiber, the chemistry of the sterol and pH (Kay, 1982). The adsorption capacity of bile acids in wheat, corn, oat, barley and rice fibers is favored by an acidic pH, a large hydrophobic surface area and greater hydrophobicity of the bile acid. A low level of pH would block ionization of carboxyl groups and hydroxyl groups on lignin's phenyl propane units suggesting a hydrophobic bonding mechanism (Zhang et al., 2011). Dietary fiber can reduce hypocholesteremic by adsorption/binding of bile acids in the structure of fiber, making increased fecal

excretion of bile acids, thus lowering serum cholesterol levels. Soluble fibers such as pectin, psyllium, guar and oat bran are effective serum cholesterol-lowering agents (Theuwissen and Mensink, 2008). There is a relationship between cereal fiber consumption and the prevention of colorectal cancer (Truswell, 1993). Free carboxyl groups of fiber bind with di or trivalent metal anions. The positive charge of trivalent cations such as Al^{3+} is not fully neutralized by the carboxyl anion, so free valences are available to bind with anions (Kay, 1982).

Ou et al. (1999) reported that water-soluble dietary fiber, water-insoluble dietary fiber from wheat bran, and the carboxymethylated product of dietary fiber, the binding capacity of dietary fibers for heavy metals will be slightly affected by amino acids, calcium, iron, and zinc, but significantly affected by copper. Colon fermentation releases part of the heavy metal ions from dietary fibers. From the results it can be concluded that dietary fibers from wheat bran can effectively bind all three tested metal ions to prevent the body from being affected by their toxicity.

Nawirska (2005) reported that the dietary fibers of pomace, a will bete product from fruit pressing, have the potential for binding with heavy metal ions. The quantity of metal ions bound varies from one fiber component to another. As it can be inferred from the results of the study, pectin will be characterized by a particularly high capacity for metal ion binding. The hemicellulose fraction ranked second with respect to metal ion binding capacity. Binding of heavy metals to lignin will be found to be generally poor.

Hu et al. (2010) reported that rice bran hemicellulose (RBHA), hemicellulose (RBHB) and hemicelluloses (RBHC) have the potential for binding heavy metal ions. The quantity of metal ions bound varies from one rice bran fiber to

another. As can be inferred from the results of the study, RBHB will be characterized by the highest capacity for metal ion (Pb, Cu and Cd) binding, followed by RBHC and RBHA. Binding of heavy metals to insoluble dietary fiber (RBDF) and cellulose from rice bran will be found to be poor. Lignin from rice bran will be the least active fraction for binding heavy metal ions.

2.3.2.5 The BET surface area, pore volume and pore diameter

Surface area is one of the physical properties of porous materials. BET analysis is the method for determining surface areas from nitrogen adsorption isotherms (Walton and Snurr, 2007), which is one of its important properties, are commonly reported as BET surface areas obtained by applying the theory of Brunauer Emmett and Teller to nitrogen adsorption isotherms measured at 77 K. This is a standard procedure that allows for comparisons among different materials (Tan et al., 2012). The method described is based on gas adsorption and use of the BET equation, but the adsorption measurements are entirely different (Haynes, 1973). The method uses a sample chamber of known volume with an adsorbent to be analyzed. The amount of the adsorptive gas adsorbed, at the measured pressure is determined. Then, a relative pressure in the sample chamber and the quantity of the adsorptive gas at the relative pressure is correlated and the BET equation is used to determine the quantity of surface area, the pore volume and the pore diameter (Wenman, 1995).

The surface area of a solid includes both the external surface and the internal surface of the pores. Due to the weak bonds involved between gas molecules and the surface (less than 15 KJ/mole), gas physical absorption is considered non-selective, thus filling the surface layer by layer depending on the available solid surface and the relative pressure (Niazi, 2009). Filling the surface area provides a

measurement for the surface area, because the amount of gas adsorbed when the mono-layer is saturated is proportional to the entire surface area of the materials. The adsorption/desorption analysis is called an adsorption isotherm (Niazi, 2009). The six IUPAC standard adsorption isotherms are shown below. They differ because the systems demonstrate different gas/solid interactions.

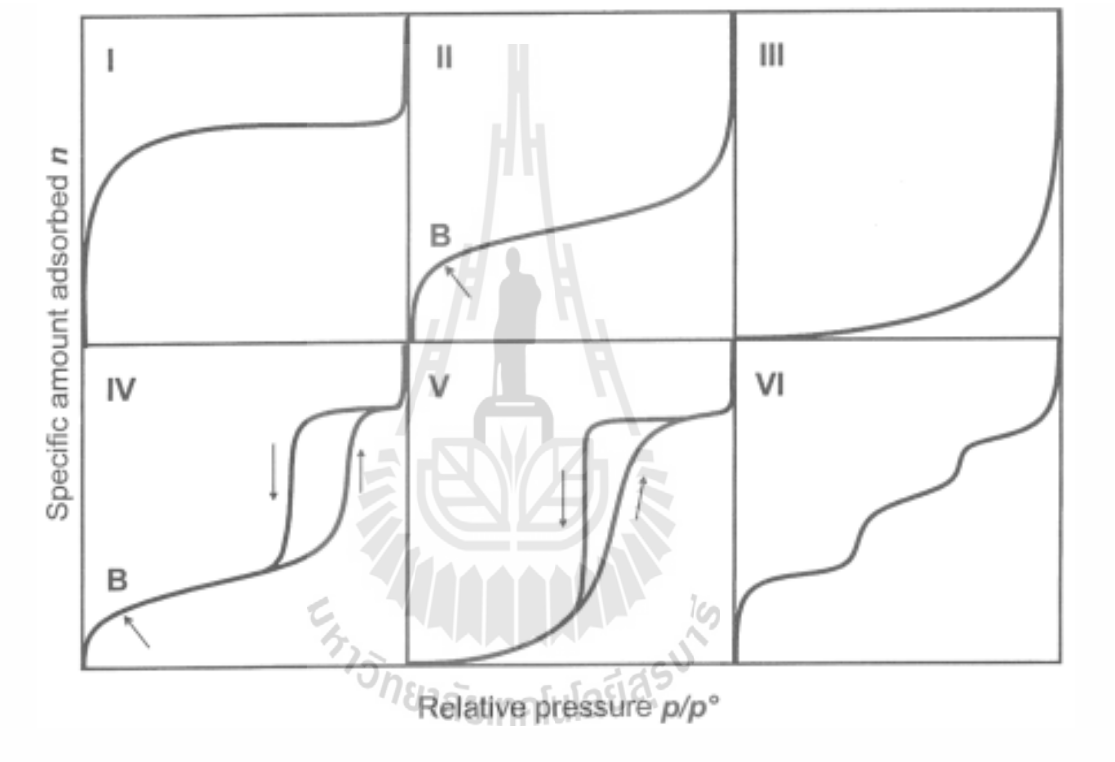

Figure 2.4 Different types of physisorption isotherms as observed for different adsorbents: type I: microporous; type II: non-porous or macroporous; type III: non-porous or macroporous with weak interaction; type VI: mesoporous; type V: mesoporous with weak interaction; and type VI: layer-by-layer adsorption.

Source: Lowell and Shields (1991).

The Type I isotherm is typical of microporous solids and chemisorption isotherms. Type II is shown by finely divided non-porous solids. Type III and Type V are typical of vapor adsorption (i.e. water vapor on hydrophobic materials). Type V and VI feature a hysteresis loop generated by the capillary condensation of the adsorbate in the mesopores of the solid. Finally, the rare type VI step-like isotherm is shown by nitrogen adsorbed on special carbon. Once the isotherm is obtained, a number of calculation models can be applied to different regions of the adsorption isotherm to evaluate the specific surface area or the micro and mesopore volume and size distributions (Li, 2007).

2.3.2.6 Fourier transform infrared FTIR

Fourier transform infrared (FT-IR) spectroscopy is used for the study of carbohydrates due to its ability to identify the main functional groups and the chemical bonds of plant sugars and complex carbohydrates. This method is sensitive, rapid and an inexpensive and only requires a small number of samples. FTIR is best for homogeneous samples or those composed of only a few materials (Lammers et al., 2009). IR radiation is passed through a sample. Then, the sample absorbs some of the infrared radiation (Lungu et al., 2014). The spectrum shows the molecular absorption and transmission, creating a molecular fingerprint for each specific sample, making infrared spectroscopy useful for several types of analysis (Eranna, 2014).

2.4 Enzymatic digestion process

Nonresistant starch is solubilized and hydrolyzed to glucose by the two enzymes, combined action of pancreatic α-amylase and amyloglucosidase (Mir et al., 2013). Because starch consists of two types of linkages, the alpha-1,4 and the alpha-

1,6, which are the different structures for starch molecules. An alpha-1,4 glucosidic bonds is called amylose which has a single chain polymer of 500 to 2000 glucose subunits. On the other hand, alpha-1,6 glucosidic linkages result in a branched glucose polymer called amylopectin (Albani, 2007).

α-amylase (EC 3.2.1.1) is an enzyme that catalyzes the endohydrolysis of 1 ,4-α-D-glucosidic linkages in polysaccharides containing three or more (1→4) -α-linked D-glucose units, such as starch. This enzyme may also be called glycogenase, endoamylase, Taka-amylase A, or 1 ,4-α-D-glucan glucanohydrolase. Amyloglucosidase (EC 3.2.1.3) is an enzyme that hydrolyzes of terminal (1→4) -linked α-D-glucose from non-reducing ends of the chains of polysaccharides, such as starch. This enzyme also hydrolyses 1 ,6-α-D-glucose links. This enzyme may also be referred to as glucoamylase, v- amylase, lysosomal a-glucosidase, acid maltase, exo-1 ,4-α-glucosidase, glucose amylase, Y-1 ,4-glucan glucohydrolase, 1 ,4-α-D-glucan glucohydrolase, or glucan (Bijl and Pelenc, 2012).

2.5 Modified dietary fiber

Cellulose itself has a low ion-exchange or a poor adsorption capacity (Sahni and Reedijk, 1984). The chemical modification of dietary fiber is the most important route to change the properties of dietary fiber and it is a renewable resource in the industry and it can improve human health (Habibi and Lucia, 2012). So, chemical modification should be carried out for the cellulose to achieve efficient ion-exchange capacity. This method give new cellulose properties such as hydrophilic or hydrophobic character, improved elasticity, water sorbency, ion-exchange capability, and thermal resistance (Kamel et al., 2006).

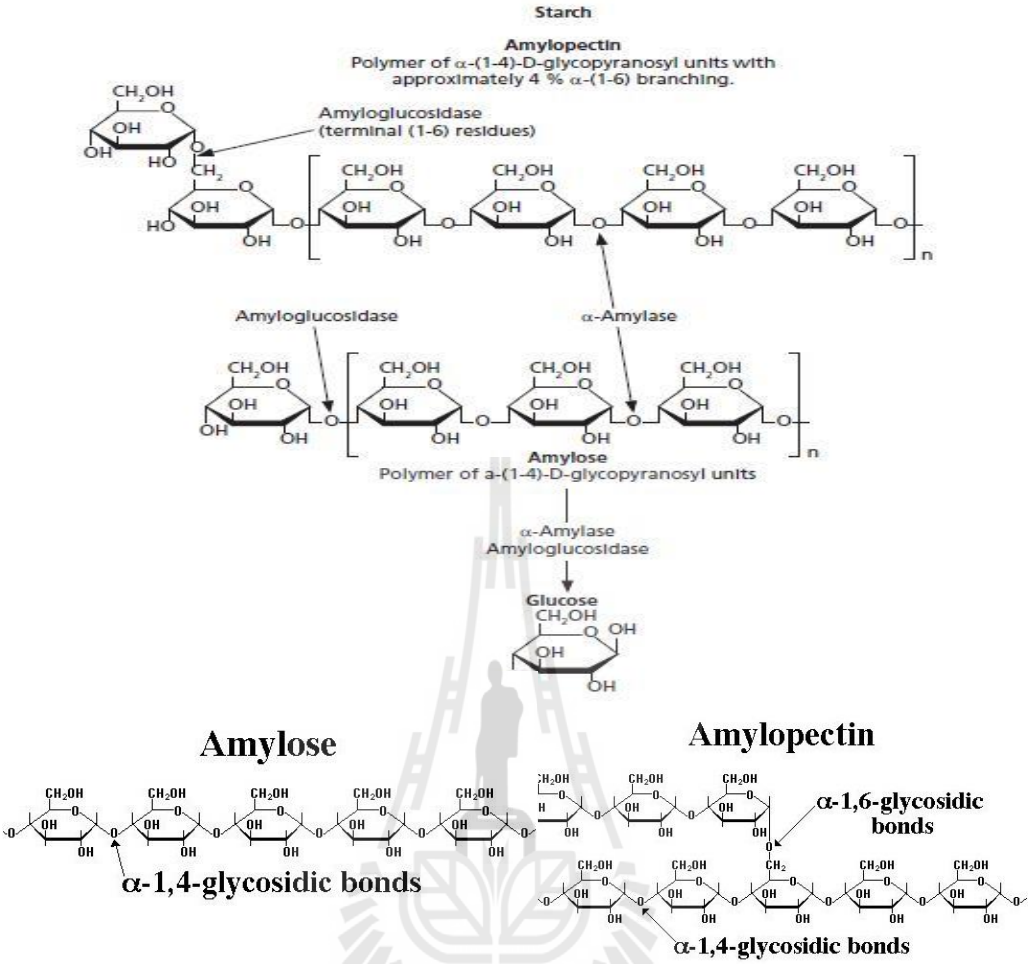

Figure 2.5 Structure of starch contain different 2 type of linkage, an unbranched, single chain polymer with the α 1,4 glucosidic bond called amylose and α 1,6 glucosidic bond in a branched glucose polymer called amylopectin. The hydrolysis of starch to glucose is catalysed by α-amylase and amyloglucosidase.

Source: Siegrist (2013).

2.5.1 Esterification

Dietary fiber was modified with citric acid at high temperature to convert citric acid into citric acid anhydride which combines with cellulosic hydroxyl groups

to form an ester linkage and thus introduces carboxyl functional groups to the fiber (Thanh and Nhung, 2009). Esterification of carboxyl groups in acidic aqueous solution increases the absorption capacity of these materials with positively charged metal ions. Heat treatment could result in cross-linking between two molecules. Thus, modification with citric acid would improve the metal binding capacity due to cross-linking (Qiu and Hu, 2013). The esterification process increases the carboxylic content of the wood fiber surface leading to a corresponding increase in the sorption of divalent metal ions. This modified wood pulp has Cu(II) and Pb(II) binding capacities of 24 mg g^{-1} and 83 mg g^{-1}, respectively (Thakur and Singha, 2015). The esterification reaction of wood pulp with succinic anhydride also leads to the introduction of carboxyl groups (Doczekalska et al., 2014).

2.5.2 Halogenation

Halogenation is a cellulose modification technique, which is a chemical reaction that involves the reaction of an organic compound with a halogen (O'Connell et al., 2008). Tashiro and Shimura (1982) synthesize chlorodeoxycellulose by reacting cellulose powder with thionyl chloride in a dimethylformamide solvent. The chlorodeoxycellulose was functionalised with ethylenediamine, thiourea, thiosemicarbazide, thioacetamide, hydroxylamine and hydrazine. However, these syntheses have low reactivity.

Aoki et al. (1999) synthesised 6-deoxy-6-mercaptocellulose and its S-substituted derivatives from 6-bromo-6-deoxycellulose. Their metal ions adsorption capacity was increased because carboxyl, amino, isothiouronium, mercapto and additional hydroxyl groups were introduced to cellulose. The derivatives containing carboxyl groups due to the reaction with 2-mercaptobutanedioic acid had an

adsorption capacity for Cu(II), Ni(II) and Pb(II) of 36 mg g^{-1}, 9 mg g^{-1} and 104 mg g^{-1} respectively. The derivatives from the amino and carboxyl groups are the result of the reaction with cysteine which has an adsorption capacity for Cu(II), Ni(II) and Pb(II) of 22 mg g^{-1}, 8 mg g^{-1} and 28 mg g^{-1} respectively.

2.5.3 Oxidation

Oxidation is a method used for the chemical modification of cellulose, which is prepared by oxidation of the oxidised cellulose (O'Connell et al., 2008). In the first step, prepared of dialdehyde cellulose by periodate oxidation of cellulose. This dialdehyde cellulose was then oxidised using mildly acidified sodium chlorite. The 2,3-dicarboxy cellulose oxidised to nearly 100% oxidation level was completely soluble in water, but the 2,3-dicarboxy cellulose of 70% oxidation was largely insoluble. These methods can modify functional properties by increasing the heavy metal adsorption capability and uptake levels of 184 mg g^{-1} and 236 mg g^{-1} achieved for Ni(II) and Cu(II), respectively (Maekawa and Koshijima, 1984). Finally, synthesised cellulose-hydroxamic acid derivatives from dialdehyde cellulose were obtained by the previous periodate oxidation method. This modified cellulose were capable of adsorbing 246 mg g^{-1} Cu(II) (Maekawa and Koshijima, 1990).

2.5.4 Etherification

The etherification method of cellulose is achieved by initially reacting the cellulose carrier with sodium methylate to form alkali cellulose, which subsequently reacts with organic halide, epichlorohydrin, yielding reactive epoxy groups for further functionalisation with polyethyleneimine as a chelating agent. These materials had a metal absorbtion rate for Co(II), Cu(II) and Zn(II), respectively, of 2.5 mg g^{-1}, 38 mg g^{-1} and 12 mg g^{-1} (Navarro et al., 1996).

Saliba et al. (2005) modified sawdust by reacting sawdust with acrylonitrile to add cyano groups to the cellulose structure. These cyano groups were then amidoximated by reaction with hydroxylamine. This amidoximated sawdust had a high adsorption capacity for Cu(II) of 246 mg g^{-1} and for Ni(II) of 188 mg g^{-1}.

2.6 *In vitro* method

2.6.1 *In vitro* digestion model

In vitro digestion models are used to provide a tool to study the effects from digestive factors such as enzyme, pH, time, amount of nutrient released from food components and reaction between food and nutrients during digestion. However, the results of *in vitro* digestion models are often different to *in vivo* models because of the difficulties in accurately simulating the physicochemical conditions occurring in animal and human digestive tracts (Hur et al., 2011).

This model is conducted to simulate the human digestive system via a two or three steps that include a mouth, gastric and intestinal digestion (Calsamiglia and Stern, 1995). Gastric digestion was started by adding pepsin to pH 2 (adults) or to pH 4 (infants). Acidification of the samples to pH 2 or 4 is important, because pepsin begins to denature itself and thus will lose its activity at pH $\geq$ 5. For the intestinal digestion, the samples are neutralized to pH 5.5-6 prior to the addition of pancreatin (which consists of a cocktail of pancreatic enzymes such as pancreatic amylase, lipase, ribonuclease, and proteases such as trypsin) and bile salts (which are emulsifiers), and finally re-adjusted to pH 6.5-7. The final digestion step is the digestion by lingual alpha-amylase, which is an enzyme that breaks apart the

glycosidic bonds of starch molecules, i.e., amylose and amylopectin (Etcheverry et al., 2011).

2.6.2 TC7 clone of Caco-2 cell

Caco-2 cell line is widely used to study intestinal absorption of nutrients at a screening step. Several clones have been isolated from Caco-2 cell line and characterized for their activities (Turco et al., 2011). The clones from Caco-2 cells, such as TC-7, were isolated from the late passage of parental Caco-2 cells to obtain a more homogeneous population, thus improving reproducibility and developing intercellular junctions (Sambuy et al., 2005). The TC7 clone had a high expression of glucose transporters and showed minimal differences in terms of paracellular transport and passive diffusion properties compared with the parental Caco-2 cell line. However, P-glycoprotein (P-gp)- mediated the active efflux of cyclosporin which was found to be higher in TC7 cells compared with the Caco-2 parental line (Turco et al., 2011).

2.6.3 Combination of *in vitro* digestion and Caco-2 cell

The principle of *in vitro* methods is for measuring bioaccessibility including solubility, dialyzability, or a gastrointestinal model for bioaccessibility, and the Caco-2 models for bioavailability (Nimalaratne, 2015). *In-vitro* methods for the measurement of the bioaccesibility of metals are important approaches to assess the chemical risks to humans (Wragg and Cave, 2003). The study of bioaccessibility is the first step in the assessment of bioavailability, which is the maximum amount of nutrient or other substance in food that is released from its matrix in the GI tract (bioaccessible fraction) that is available for intestinal absorption and transport to the blood stream. The bioaccessibility and bioavailability are affected by the sample

matrix and the experimental conditions applied, including gastric and intestinal pH, incubation temperature and residence time (Moreda-Piñeiro et al., 2011).

In vivo studies using animal models (e.g., primates, swine, dogs, rabbits, and rodents) have been developed to determine bioavailability. Monkeys are the first choice of study because they are closely related to human (Rees et al., 2009). However, testing with animals is very expensive, difficult to perform and ethically controversial (Moreda-Piñeiro et al., 2011) .

Moreda-Piñeiro et al. (2011) describe several *in vitro* method approaches to measurement of biaccessibility and bioavailability.

(1) Bioaccessible fraction is the maximum concentration of the target compound that is released in the simulated GI solution after digestion.

(2) The bioaccessible fraction is achieved by using human GI microbiota (Simulator of the Human Intestinal Microbial Ecosystem).

(3) The dialyzable fraction of the compound, which can be dialyzed through a semi-permeable membrane with a specified pore size considered as (dialysate) bioavailable fraction.

(4) The fraction of the compound capable of being retained or transported through a solid or microporous supports (bioavailable fraction) in which human Caco-2 cells are grown can be incorporated in the intestinal epithelial model.

Some *in vitro* procedures also simulate digestion with saliva. In the mouth, samples are ground to be broken down into smaller fragments to increase the surface area. Saliva contains buffers maintaining the acid–base balance and salivary amylase. This process takes a few seconds to minutes because the pH of saliva is close to neutral. It is therefore usually assumed that saliva has only a few effects on the

in vitro method. However, it is dependent on various kinds of materials (Intawongse and Dean, 2006).

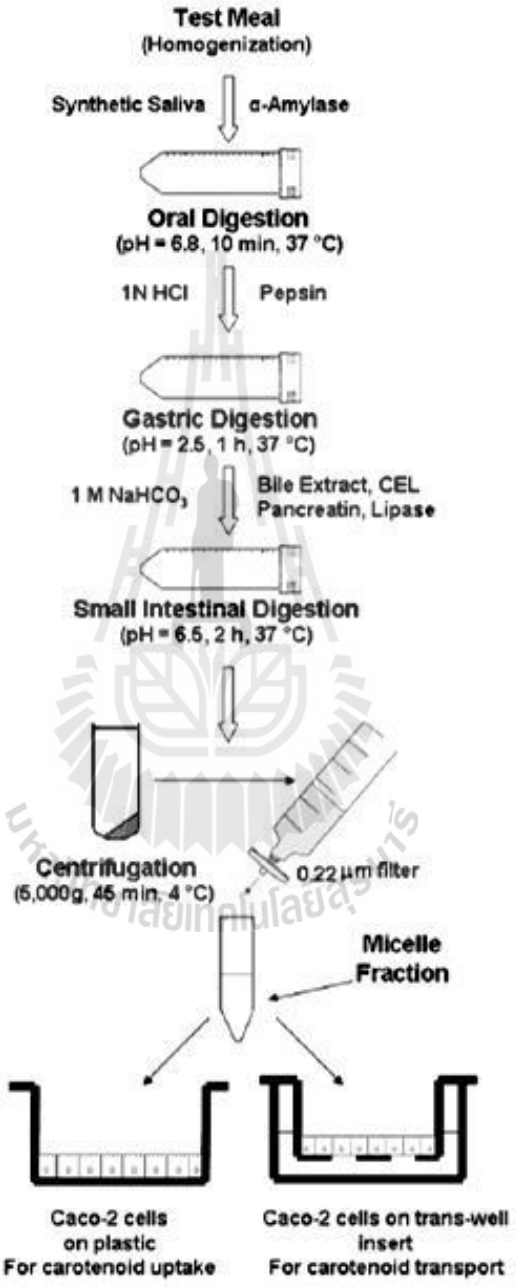

Figure 2.6 Graphical representation of coupled *in vitro* digestion/ Caco-2 cell uptake model to assess carotenoid bioaccessibility.

Source: Failla et al. (2008).

2.7 References

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CHAPTER III

ENZYMATIC DIGESTION OPTIMIZATION OF DIETARY FIBER FROM CASSAVA PULP AND THEIR MODIFICATION WITH THE ETHERIFICATION METHOD

3.1 Abstract

The objectives of this study were to determine the optimal extraction condition of cassava pulp and to modify it using etherification methods to improve its physical properties. The major part of cassava pulp consists of tuber plant cell material which is particularly rich in starch. The extraction process requires the starch to be separated from the fiber by enzyme application. The enzyme reaction conditions for the solubilization were optimized via a response surface methodology (RSM). Independent variables such as quantity of α-amylase, amyloglucosidase and neutrase were optimized using a 3-factor, 3-level Box-Behnken statistical design. The selected dependent variable was the percentage of neutral detergent fiber (NDF). The highest NDF (79.68%) of the crude dietary fiber (CDF) was obtained from enzymatic digestion condition of 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v). In addition, the result indicates that enzymatic optimization of dried cassava pulp extraction process is indeed a possible route for obtaining dietary

fiber for food supplementation. Modification of CDF could be developed with improving physiochemical and functional properties for binding with mercury.

Keyword: cassava pulp, dietary fiber, extraction, modified, mercury

3.2 Introduction

Some foods might contain high levels of heavy metals which humans can be exposed to through various pathways (Tasrina et al., 2015). Water irrigation, solid waste disposal, sludge applications, vehicular exhaust, and industrial activities are the major sources of contamination by heavy metals in soil (Harmanescu et al., 2011). There are many reports concerning the transfer of heavy metals from polluted soils to various sources of foods, such as vegetables (Zhang et al., 2011; Bahemuka and Mubofu, 1999), rice (Fu et al., 2008), wheat (Huang et al., 2008) and chicken (ZHUANG et al., 2009). Heavy metals such as lead, cadmium, and mercury are toxic at very low concentrations due to their nonbiodegradable nature and prolonged biological half-life (Khanna, 2011). Humans can be exposed to heavy metal contamination via inhalation, ingestion, skin absorption and the gastrointestinal tract (Singh, 2005).

Mercury (Hg) is a highly toxic element, the accumulation of which in humans can cause severe liver damage (hepatotoxicity) and damage to the central nervous system (neurotoxicity), DNA (genotoxicity), and kidney (nephrotoxicity) in animals and humans (Sharma et al., 2014). The toxicity depends on the amount of exposure (Hong et al., 2012). Humans can be exposed to Hg though various pathways. Eating fish contaminated with methylmercury (MeHg) is the main pathway of human exposure (Keating et al., 1997). Chelating agents are commonly used for removing

mercury from heavy metal, however, side effects have been reported (Tandon et al., 2001). Recently, many researchers have reported the application of dietary fibers as adsorbents for heavy metal because of their nontoxic properties (Nawirska, 2005; Hu et al., 2010; Shim et al., 2009; Sembries et al., 2004; Zhang et al., 2011; Idouraine et al., 1996).

Dietary fiber is a component of plant foods that is resistant to the digestion of human alimentary enzymes though the gastrointestinal tract (GI tract). Fiber may be divided into two classes including non-α-glucan polysaccharides (cellulose, hemicelluloses, and pectins) and lignins (Kay, 1982). In the GI tract, dietary fiber acts as a polymer with physicochemical properties, including bacterial fermentation, water-holding capacity, cation exchange, and adsorptive functions (Căpriţă et al., 2010). Physical and chemical composition are important to determine the physiological actions of fiber (Kay, 1982). Consequently, for over a decade, dietary fibers from various food products including wheat bran (Ou et al., 1999), rice bran, oat bran and fruit fibers have been reported to be used as chelating agents because of their non toxicity (Idouraine et al., 1996). In recent years, natural polymeric materials have been gaining interest as potential adsorbents of heavy metals because of their nontoxic properties (Chang and Juang, 2004).

Two possible mechanisms of binding between dietary fiber and heavy metal are chemisorption and physical sorption. Chemical adsorption is the main mechanism: it frees the carboxyl group from uronic acids/phenolic groups from lignin and forms a coordination complex with di/trivalent metal anions or the positive charge of trivalent cations which is connected with carboxyl anion (Zhang et al., 2011). Physical adsorption is used to characterize the surface, surface area and pore

size of fiber (Thommes, 2010). Thus, dietary fiber can be absorbed and bound together which carries them through the digestive tract because it is resistant to digestion by human enzymes (Kay, 1982).

Cassava (*Manihot esculenta* Crantz.) is one of the most important crops in Thailand, especially in the northeast of Thailand. This root crop is known by many names in Thailand, such as cassava or tapioca (Sriroth et al., 2000). The total amount of cassava root crops in Thailand in 2011 was 21,060,903 tonnes. The main production of the crop is in the northeast of Thailand, especially in Nakhon Ratchasima, which can produce about 5,003,878 Tonnes (Thailand Tapioca starch, 2011)

Dried cassava pulp is a by-product of cassava starch factory processing, which contains a large amount of starch (Khempaka et al., 2009). Cassava is used as food for humans and for feeding animals. In Thailand in 1998, the total amount of fresh root crops from cassava was 15,440,000 tonnes producing 6,980,000 tonnes of starch (Thai Tapioca Trade Association, 1999). Starch extraction is carried out in general starch factories, where cassava tubers are separated into starch granules and fibrous residual during manufacturing by centrifugation. The fibrous residual, called cassava pulp (Koatdoke et al., 2011), accounts for approximately 10-30% (w/w) of the original tubers. Therefore, cassava pulp in Thailand is estimated to produce at least one million tons from 10 million tons of fresh tubers (Khempaka et al., 2009).

Cassava pulp accounts for approximately 10-30% by weight (wet) of the original tubers (Thailand Tapioca starch, 2011) and contains approximately 60% starch and 30% fiber (Apiwatanapiwat et al., 2011). Most cassava pulps are rich in fiber and thus hold potential as bioactive absorbants for heavy metal binding to

prevent toxicity. The concept in the extraction process of dietary fiber is to treat them with starch and proteins with enzymes for hydrolysis and the result is Neutral Detergent Fiber (NDF), which is the most abundant structural component in plant cells (lignin, hemicellulose and cellulose) (Hohmann et al., 2012).

The primary goal of this study was to determine the optimal extraction condition of crude dietary fiber from cassava pulp focusing on the neutral detergent fiber (NDF). Then the modified crude dietary fiber will be treated by the etherification method and the physicochemical and functional properties of both fibers will be investigated in order to apply dietary strategies to reduce the potential of mercury absorption in high risk foods.

3.3 Materials and methods

3.3.1 Preparation of dried cassava pulp

The dried cassava pulp leaves used as a source of fiber in this study were obtained from Sanguan Wongse Starch Co., Ltd. They were dried at 60°C (UM500, Memmert, Germany) for 8-12 hours. The leaves were ground with a grinder (High speed grinder, 3500 w, Simon, Inc., Foodmachine, China) into fine powder. The cassava pulp powder was then kept in a sealed container until required for further treatment.

3.3.2 Optimization crude dietary fiber extraction

In order to find factors affecting the optimized yield of MDF, the Response Surface Methodology was used to get the optimum points. Independent variables such as the amount of *X1*: α-amylase (EC 3.2.1.1, Merk, Darmstadt, Germany), *X2*: neutrase (EC 3.4.24.28, Novozymes Co., Bagsvaerd, Denmark),) and *X3*:

amyloglucosidase (EC 3.2.1.3, Bray, Co. Wicklow, Ireland) were optimized using 3-factors with 3-levels. Each level of α-amylase was at 0.1%, 0.2% and 0.3% (w/v), and underwent a 30 min treatment at pH 6 and 95°C and was adjusted to pH 7.5 with sodium hydroxide solution (NaOH) (Merck Ltd.) prior to adding neutrase. The concentrations of neutrase were at 0.5%. 1.0% and 1.5% (v/v) and the solution was treated for 30 min at a temperature of 60°C and it was adjusted to pH 4.5 with hydrochloric acid solution (Carlo). Amyloglucosidase was added to the samples. The concentrations of amyloglucosidase were 0.1%, 0.3% and 0.5% (v/v) for 30 min at a temperature of 95°C. The Box-Behnken design requires 15 fewer runs in a 3-factor experimental design. The resulting hydrolysate was separated by centrifugation (Hettich, Universal 32R, DJB labcare Ltd.) at 10000xg for 10 minutes to separate the supernatant from the fiber-enriched sediment. The sediment was washed with doubly distilled water, re-centrifuged for 10 min at 10000xg and dried at a temperature of 50°C in a hot air oven. The selected dependent variables were a cumulative percentage of NDF (*Y1*).

Table 3.1 Variables in the Box-Behnken design.

Variables		Levels Used		
		Low	**Medium**	**High**
Independent variables:	X1 = alphaamylase	0.1	0.2	0.3
	X2 = neutrase	0.5	1.0	1.5
	X3 = amyloglucosidase	0.1	0.3	0.5
Dependent variables:	Y1 = Neutral detergent fiber (NDF)			

3.3.3 Determination of the physicochemical properties of the crude dietary fiber

3.3.3.1 Proximate analysis

The recommended methods of the Association of Official Analytical Chemists (AOAC, 2005) was adapted to determine the quantification of crude protein, moisture, ash, fat and carbohydrate in the crude dietary fiber which was prepared as above.

3.3.3.2 Determination of neutral detergent fiber (NDF)

The neutral detergent fiber (NDF) was analyzed according to the method of Van Soest et al. (1991). 1 g of sample was weighed (W0, precision ±0.0001 g) in a clean dried crucible. The crucibles were placed in a hot extraction unit. 100 ml of neutral detergent solution and 50 µl of heat resistant amylase were added and heated to boiling point for 60 minutes (moderate and constant degree of boiling). Then the solution was filtered and washed with boiling distilled water twice. Then it was transferred in crucibles to the cold extraction unit and washed with acetone at least twice. It was oven-dried in the crucibles at 103°C for 6 hours minimum. Finally, the crucibles were transferred to a desiccator and weighed (W1) after cooling (Van Soest et al., 1991).

3.3.3.3 Determination of acid detergent fiber (ADF)

Acid detergent fibers (ADF) were analyzed according to the AOAC Official Method 973.18 (AOAC, 2005). the NDF residue was dispersed in the crucible (with distilled water) then 100 ml of acid detergent solution were added and boiled. The heat was adjusted and the residue was boiled for 60 minutes (moderate and constant degree of boiling). The solution was filtered with boiling and with

distilled water twice. The crucibles were then transferred to the cold extraction unit and washed with acetone twice. The crucibles were placed in a dry oven at 103°C for 6 hours minimum. Finally, the crucibles were transferred to a desiccator and weighed (W2) after cooling.

3.3.3.4 Determination of acid detergent lignin (ADL)

Acid detergent lignin (ADL) was analyzed according to the AOAC Official Method 973.18 (AOAC, 2005). The crucibles were placed on a plate and filled (3/4) with ADL solution and the solution was stirred every hour and refilled with the ADL solution as necessary. Three hours after the initial addition of ADL solution, it was filtered and the solution was washed with hot distilled water at least 6 times until the pH was neutral. After that the crucibles were oven dried at 103°C overnight. Next, the crucibles were transferred to a desiccator and weighed (W3) after cooling. The sample residues were added to the crucibles at 550°C for 3 hours minimum. While the samples in the crucibles were still hot (but under 250°C) they were put into an oven (103°C) for 1 hour then cooled down in a desiccator and weigh (W4) after cooling.

Calculation for sequential procedure

$$NDF\ (\%) = ((W1 - W4)/ W0) \times 100$$

$$ADF\ (\%) = ((W2 - W4)/ W0) \times 100$$

$$ADL\ (\%) = ((W3 - W4)/ W0) \times 100$$

The components that are determined by these tests are summarized below:

NDF = Cellulose + Hemicellulose + Lignin + Mineral Ash

ADF = Cellulose + Lignin + Mineral Ash

ADL = Lignin + Mineral Ash

Cellulose content = ADF - ADL

Hemicellulose content = NDF - ADF

3.3.4 Determination of the functional properties of the crude dietary fiber

3.3.4.1 Water holding capacity (WHC)

The water holding capacity of the dietary fiber was determined using the method of Jasberg et al. (1989) with some modifications. A dried sample (3 g) was mixed with an excess of deionized and distilled water and allowed to hydrate for 2 h. The excess water was then removed by allowing the wet sample to drain through a fine-meshed wire screen. A portion of the wet sample on the screen was carefully removed, weighed and dried to constant weight (±0.05 mg) in a hot-air oven (110°C). WHC was defined as follows: (Jasberg et al., 1989)

$$\text{WHC} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \quad \text{(i)}$$

3.3.4.2 Oil binding capacity (OBC)

The oil binding capacity (OBC) was determined by the method of Caprez et al. (1986). A dried sample (5 g) was mixed with soybean oil in a centrifugal tube and left for 1 h at room temperature (25°C). The mixture was then centrifuged at 1500 g for 10 min, the supernatant was decanted and the pellet recovered by filtration through a nylon mesh. OBC was expressed as follows:

$$\text{OBC} = \frac{\text{pellet weight} - \text{dry weight}}{\text{dry weight}} \quad \text{(ii)}$$

3.3.4.3 Water solubility index (WSI)

The water solubility index (WSI) was determined by AACC, method No. 44-19 (AACC, 2000). The powder (S1, g) was dispersed in a centrifuge tube by adding water with a powder/water ratio of 0.02/1 (w/w) at ambient temperature. Then the dispersion was incubated in a water bath (WB22, Memmert,Germany) at 80°C for 30 min, followed by centrifugation at 6000 rpm for 10 min. The supernatant was carefully collected in a pre-weighed evaporating dish (S2, g) and was dried at 103±2°C, and the evaporating dish with the residue was weighed again (S3, g). WSI was calculated using the following formula:

$$\text{WSI (\%)} = \frac{S3 - S2}{S1} \times 100 \qquad \text{(iii)}$$

3.3.4.4 Swelling capacity (SC)

The swelling capacity (SC) was determined on the fiber fractions in accordance with the method of Robertson et al. (1999) with modification. The sample (250 mg) was weighed in a 10 ml graduated cylinder (10.5 mm ID). Distilled water was added to the graduated cylinder in 2 ml increments (to a total of 10 ml) with mixing to prevent lump formation. After equilibrium (18 h), the bed volume was recorded. SC was expressed as the volume occupied by the hydrated sample per gramme of dry sample (ml/g DM). All measurements were performed in triplicate.

3.3.4.5 COOH content analysis

COOH content analysis was performed as described in the United States Pharmacopoeia (USP, 1990). 1.05 g of the sample was accurately weighed and dispersed in 50 ml of a 2% (w/w) solution of calcium acetate (Carlo) for 30 min. The suspension was then titrated with standardized 0.1 N NaOH solution using

phenolphthalein as an indicator. The volume of NaOH solution consumed was corrected for the blank. The carboxylic content in the sample was calculated from the following relationship:

$$\text{COOH content} = \frac{N \times V \times M_w \text{ COOH}}{\text{Weight of the sample (mg)}} \times 100 \qquad \text{(iv)}$$

Where N is the normality of NaOH, and V is the volume of NaOH in ml consumed in titration, after correcting for the blank.

3.3.5 Preparation of modified fiber with the etherification method

Crude dietary fiber was reacted with CS_2 (Carlo) and NaOH (17.5% w/v) at a ratio of 1:3:6, respectively. Samples were placed in a 100 ml reaction bottle at 30°C for 1 h; the bottle was shaken for another 1 h at room temperature. The samples were homogenously mixed with acrylonitrile (AN) at a ratio of 1:3 (crude fiber:AN) at 30°C for 2 h with occasional shaking (Acm-42303-U, Universal shaking machine, India). The sample was then precipitated with dilute HCl, as a precipitating agent and then filtered and washed with distilled water until neutrality following drying at a temperature of 60°C for 24 h (Kamel et al., 2006).

The sample was treated either by hydroxylamine or by hydroxylamine hydrochloride (Carlo) 1% (w/v), whose pH is adjusted to between 9 and 10 by using sodium carbonate (Carlo). After treatment at a constant temperature (from 60 to 80°C) for a given time (from 30 min to 3 h), the fiber was filtered, washed with deionized water, and dried under a vacuum to get the modified dietary fiber (MDF) (Saliba et al., 2000; Saliba et al., 2005).

3.3.6 Fourier transform infrared spectrophotometer (FTIR)

FTIR spectroscopy analysis is the method used for identifying functional groups and to acquire more information about materials and products. Two mg of samples were prepared in tube. IRspectra (4000-400 cm^{-1}) were recorded using a Bruker T27/HYP 2000 (Germany) spectrometer with a resolution of 4 cm^{-1} and 64 scans per sample (Oh et al., 2005).

3.3.7 Determination of surface area and porosity

Before the adsorption analyses, 1 g of samples were degassed at 150°C in vacuum for 3 hours (Lu and Chung, 2001). N2 adsorption-desorption isotherm of all samples (dried cassava pulp, crude dietary fiber and modified dietary fiber) were measured by BELL SOLF (Japan) at 77° K. The surface area was determined by the Brunauer-Emmett-Teller (BET) method. The BET analysis is the standard method for determining surface area, porosity/ the volume of the porous area and pore diameter (Lu and Chung, 2001).

3.3.8 Scanning electron microscopy (SEM)

SEM analysis was conducted on the surfaces of injected composite samples freeze fractured in liquid nitrogen. The sample surfaces were sputter coated with fine layers of gold in an Edward Sputter Coater and analyzed by JEOL 6010LV scanning electron microscopes.

3.3.9 Statistical analysis

The results are presented as representative data from at least two independent sets of experiments. Data are expressed as the mean±standard error. For cellular uptake studies, a sample size of n = 3 was used. Statistical analysis for each parameter assessed was performed using analysis of variance (ANOVA) followed by Tukey's

post hoc test (SAS, Gary, NC). Differences between means were considered statistically significant at $p < 0.05$.

3.4 Results and discussion

3.4.1 Chemical composition of dried cassava pulp

Dried cassava pulp was determined for crude protein, ash, moisture, fat, carbohydrate, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose, and hemicellulose. The results are shown in Table 3.2.

Table 3.2 Chemical composition of dried cassava pulp.

Component	%Content (dried basis)
Crude protein	2.60±0.06
Ash	2.10±0.03
Moisture	4.11±0.23
Fat	0.13±0.03
Starch	59.39±0.02
Neutral detergent fiber (NDF)	31.67±1.78
Acid detergent fiber (ADF)	28.21±0.10
Acid detergent lignin (ADL)	2.44±0.27
Cellulose[a]	25.78±0.17
Hemicellulose[b]	3.46±0.37

Note: [a] ADF -ADL, [b] NDF-ADF.

Table 3.2 shows the chemical composition of dried cassava pulp of which starch is the main substance at 59.39±0.02% followed by NDF and ADF at 31.67±1.78 and 28.21±0.10%, respectively. The chemical composition of dried

cassava pulp contains more than 50% starch because the starch granules are not completely separated from the cassava pulp during the processing of cassava starch in the starch factory (Saengchan et al., 2015). When starch is extracted from cassava tubers during manufacturing, cassava tubers are separated into starch granules and fibrous residual material. After extraction, starch granules remain in the cassava pulp because separation of both materials is quite difficult (Koatdoke et al., 2011). The chemical composition of dried cassava pulp was similarly reported in previous research with the amounts of starch, ash, protein fat and fiber in cassava pulp as 69.89, 1.70, 1.55, 2.12, and 27.75% (dry basis), respectively (Sriroth et al., 2000). The fiber content of dried cassava pulp was previously reported in the form of insoluble fiber, and the percentages of fiber in neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) were 36.7, 9.8, and 3.9%, respectively (Suksombat et al., 2006). The major substance of dried cassava pulp is starch (about 60% of dry basis) together with cellulose (~25% of dry basis) which is the main fibrous component of plants and is responsible for providing the rigidity of the cell walls. (Rubin, 2008). Thus, cellulose remains in cassava pulp after the separation of the starch granules from the fibrous residue. The results show a large amont of neutral detergent fiber (NDF) (~31% of dry basis) which is insoluble fiber in plant cells including hemicellulose, cellulose, and lignin (Kaczmarczyk et al., 2012). Insoluble fiber usually decreases the accumulation of toxic metal due to its absorption of heavy metal in the small intestine because of their binding and/or sequestering effects whereas soluble fiber usually increases retention of toxic metals (Mertens, 2002). In addition, the form of insoluble fiber does not dissolve in water, thus increasing the bulk of faecal matter and promoting regular bowel movements. Insoluble fibre could

decrease the transit time through the gastro-intestinal tract, reducing the accumulation of toxic elements and helping to prevent risk to humans (Jacobs Jr et al., 1998; Mertens, 2002).

Dried cassava pulp shows a small amount of crude protein (2.26% of dry basis), as having relatively low protein content compared with other sources of energy (such as maize and wheat) (Ceballos et al., 2006). The cassava starch contains about 3.6% protein and the peels about 5.5% (Iyayi and Losel, 2004). In contrast, cassava leaves are a good source of protein such as lysine (Eggum, 1970) with an average value of 21% (Iyayi and Losel, 2004). The differences in composition of cassava pulp depend on several factors, such as geographic location, variety, age of the plant, environmental conditions and the extraction process used to separate starch granules and fibrous residual (Montagnac et al., 2009).

These results indicate that the dried cassava pulp prepared in this study has high fiber content and that it is possibile to prepare crude dietary fiber from dried cassava pulp to reduce the toxicity of mercury. Even though the fiber contents of dried cassava pulp are problematic, its content is also composed of a high amount of starch and small amount of crude protein effecting crude dietary fiber chemical composition and functional properties. Therefore, it is necessary to digest starch with α-amylase and amyloglucosidase and digest protein with neutrase in the next step. It is certainly worthwhile to investigate the application of crude dietary fiber for binding with heavy mercury metal. Biosorption with dietary fiber is becoming a potential alternative to the existing technologies for the removal of toxic metals in the gastrointestinal tract. The major advantages of using polymeric material to removal

heavy metal are that it is inexpensive and highly effective to reduce the heavy metal ions to very low levels (Kratochvil and Volesky, 1998).

3.4.2 Optimization of crude dietary fiber extraction

From the optimization with response surface methodology using Box-Behnken design with 3 independent factors at 3 levels, the NDF is presented in Table 3.3.

Table 3.3 Effects of variables on the response Y (%NDF).

Exp. No[a]	α-amylase (%w/v) X_1	Nuetrase (%v/v) X_2	Amyloglucosidase (%v/v) X_3	%NDF
control	0	0	0	31.67 ± 1.78^{h}
1	0.2	0.5	0.5	58.53 ± 0.25^{fg}
2	0.3	0.5	0.3	54.22 ± 1.49^{g}
3	0.3	1.0	0.1	69.49 ± 1.96^{bc}
4	0.3	1.0	0.5	69.44 ± 2.26^{bc}
5	0.2	1.5	0.5	65.15 ± 2.62^{cde}
6	0.2	0.5	0.1	68.45 ± 1.10^{bcd}
7	0.2	1.0	0.3	57.50 ± 1.95^{fg}
8	0.2	1.5	0.1	63.06 ± 2.01^{def}
9	0.3	1.5	0.3	61.39 ± 1.51^{ef}
10	0.2	1.0	0.3	64.74 ± 1.51^{cde}
11	0.1	0.5	0.3	62.19 ± 4.95^{ef}
12	0.2	1.0	0.3	60.60 ± 4.44^{ef}
13	0.1	1.0	0.5	70.95 ± 0.42^{b}
14	0.1	1.5	0.3	70.86 ± 0.59^{b}
15	0.1	1.0	0.1	79.68 ± 0.55^{a}

Note: [a] Experiments were conducted in a random order.

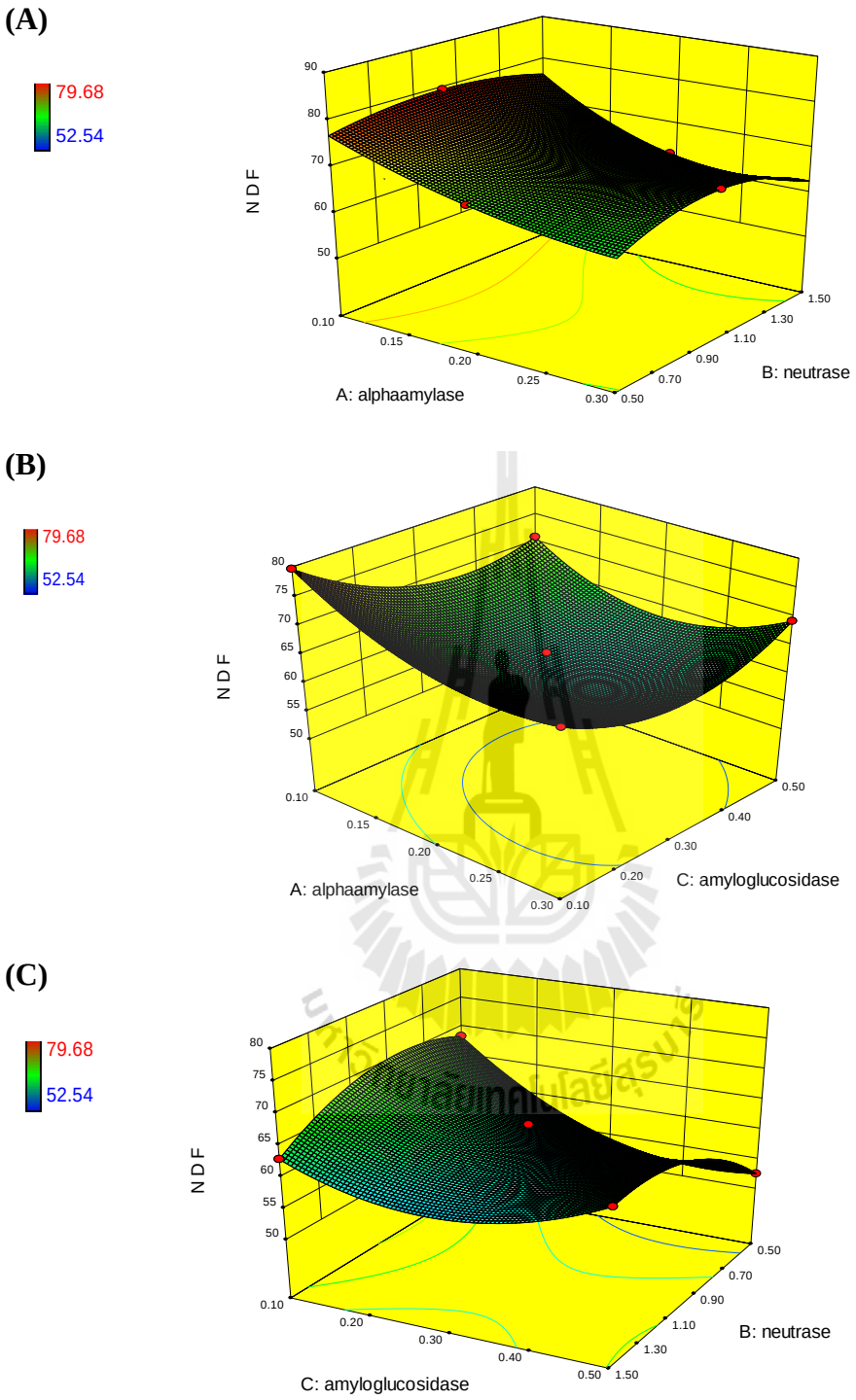


Figure 3.1 Surface plot showing, the interaction effect between (A) α-amylase and neutrase, (B) α-amylase and amyloglucosidase, (C) neutrase and amyloglucosidase.

The highest NDF shown under the condition of heating temperature is at 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v) (Figure 3.1 and Table 3.3). Optimization $Z = 38.14 + 34.61X_1 + 21.18X_2 - 29.15X_3 - 21.56X_1X_2 - 22.22X_1X_3 - 31.45X_2X_3 + 28.18X_1^2 + 24.41X_2^2 + 21.06X_3^2$. The different amount of NDF yields were obtained in response to varying the reaction conditions for the enzymatic digestion process. Response Surface Methodology used 3-factors (α-amylase, neutrase with 3-levels significantly affected the yields of NDF (insoluble fibers). The optimal levels of the 3 factors, resulting in an optimal yield of ~80% NDF by weight of dry matter were 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v) (Table 3.3 and Figure 3.1). The maximal yields obtainable by varying the levels of the 3 factors in the model revealed a response optimum of ~80% NDF. After the digestion process NDF showed increasing range from 31.67±1% to 79.68±0.55% due to the digestion starch and protein, thus helping to increase the percentage of NDF in the raw material. This result confirms that NDF of crude dietary fiber as a percentage of total dry matter content was obtained. This yield corresponded to ~31% of the original raw material.

The crude dietary fiber preparation with starch and protein enzymatic digestion. Starch is a polymer of glucose linked to another molecule with glycosidic bonds. This glycosidic bond is stable at high pH but hydrolyzes at low pH. Two types of glucose polymers are present in starch including amylose and amylopectin. Therefore, it is necessary to digest them with 2 types of enzyme (El-Fallal et al., 2012). In these steps, a couple of enzymes were used for starch digestion as α-amylase for hydrolysis of alpha-1,4-glycosidic linkages of polysaccharides yielding dextrins, oligosaccharides, maltose and D-glucose (Vujičić-Žagar and Dijkstra, 2006)

and amyloglucosidase for decomposing starch into glucose by removing glucose units from the non-reduced end of the polysaccharide chain (Sengupta and Dasgupta, 2008). The enzymes were chosen on the basis of an initial component of raw material (dried cassava pulp). The process was accomplished in three steps. The conditions, (temperature and pH), were applied based on optimum conditions for each enzyme. The first steps were to hydrolyze at a very high temperature, 95°C, to gelatinize the starch and assist viscosity lowering. This step was then hydrolysis protein, and decomposing starch into glucose with amyloglucosidase (Meyer et al., 2009). These products from starch digestion were then removed by washing with distilled water due to solubility in water because they contain a lot of hydroxyl groups (Szejtli, 1998). Nautrase is a protease which can break down protein into amino acids, forming an amino group, $-NH_2$ (Eng et al., 1994). Amino acids are generally soluble in water and insoluble in non-polar organic solvents such as hydrocarbons (Fleck and Petrosyan, 2014). These properties were used for the removal of amino acid by washing with distilled water.

After the removal of starch and protein, pretreatments using varying equal dosages of the enzyme α-amylase, protease, and amyloglucosidase. The relative levels of dietary fibers increased significantly when the enzymatic starch and protein removal was introduced (Table 3.3) compared with control which increased from 31.67±1% to 79.68±0.55%. These results suggest that the enzymatic digestion process with enzymes α-amylase, protease, and amyloglucosidase can assist with crude dietary fiber (CDF) preparation.

3.4.3 Chemical composition and functional properties of CDF

Table 3.4 Chemical composition of crude dietary fiber (CDF).

Component	%Content (dried basis)
Crude protein	1.06±0.01
Ash	3.92±0.04
Moisture	5.41±0.07
Fat	0.33±0.03
Starch	9.60±0.61
Neutral detergent fiber (NDF)	79.68±0.55
Acid detergent fiber (ADF)	78.26±0.64
Acid detergent lignin (ADL)	4.09±0.05
Cellulose[a]	74.16±0.78
Hemicellulose[b]	1.42±0.76

Note: [a] ADF -ADL, [b] NDF-ADF.

Table 3.4 presents in percentages the physicochemical properties of CDF. From the analysis, CDF contained crude protein, ash, moisture, fat, starch, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose, and hemicelluloses by 1.06, 3.92, 5.41, 0.33, 9.6, 79.68, 78.26, 4.09, 74.16, and 1.42%, respectively. After extraction with an enzymatic digestion, CDF showed an increase in the chemical composition of ash, moisture, fat, NDF, ADF, and ADL. For crude protein, starch and hemicellulose were decreased (Table 3.4), which might be due to the removal of starch and protein from the dried cassava pulp also gave high quantity of other compositions, when calculated in percentage forms. Figure 3.4 shows the removal of the starch granules from the fiber structure. This result suggests that this method can remove 83.87% of starch and 40.76% of protein from the structure of the CDF and also gave a high quantity of NDF at 79.68% including

74.16% cellulose because NDF is the main component in plant cells (Lounglawan et al., 2011).

CDF was reported in the form of insoluble fiber as a percentage of high neutral detergent fiber (NDF), which included cellulose, hemicellulose and lignin. It has also been reported that insoluble fiber can bind with heavy metal better than soluble fiber (Mertens, 2002). It is certainly worthwhile to investigate their application as a source for the binding of mercury.

Table 3.5 Functional properties of the crude dietary fiber (CDF).

Functional properties	Content
Water holding capacity (WHC) (%)	6.83±0.38
Oil binding capacity (OBC) (g oil/g sample)	3.96±0.87
Water solubility index (WSI) (%)	3.79±0.65
Swelling capacity (SC) (ml/g DM)	8.40±1.21
COOH content (%)	5.44±0.76

As shown in Table 3.5, it was found that the water holding capacity is 6.83%, oil binding capacity 3.96 g oil/g sample, water solubility index 3.79%, swelling capacity 8.40 ml/g DM and COOH content 5.44%. The functional properties of dietary fiber affect the nutrient absorption in the colon and stool weight. These differences in action are more likely due to differences in physical characteristics along the gastrointestinal tract. These results could be used to evaluate the potential of dietary fiber to bind/absorb heavy metal and modify them for improving their functional properties in the next step. Dietary fiber can passing along the intestine. The functional properties that influence function along the gastrointestinal tract are a combination of water holding capacity, oil binding capacity, water solubility, swelling

capacity and carboxyl group (-COOH) content. The biological effects of dietary fiber along the intestine and colon may be improved by absorption in the gut and an increase in stool weight (Eastwood and Morris, 1992).

In Table 3.5 the CDF shows high values of WHC (6.83%) and SW (8.40 ml/g) due to the main constituents of CDF which are polysaccharides, such as cellulose, which seem to be formed in chain segments as in insoluble fibers but they are interconnected, so that they can increase hydration and swelling (Rees et al., 1982). These properties might be attributed to its lower density. A fiber preparation having a low density would result in a greater surface area, polar groups, and uronic acid groups in the surrounding water, leading to an increase in its swelling volume (Gordon, 1989; Bao and Chang, 1994). Furthermore, from the COOH capacities (5.44%), the stronger ion binding activity of these dietary fiber relative to cellulose might be due to the presence of uronic acids. Perhaps this is because high uronic acid contents can bind with heavy metal leading to a decrease in the absorption of heavy metal (Furda, 1990). The WHC and OBC properties related to retaining water or lipids in the fiber structureassist heavy metal or mercury absorption in the fiber structure. Differences in the water holding properties could be important in determining fibre activity in the gut (Gourgue et al., 1992).

Bao and Chang (1994) reported that carrot pulp contained 37-48% of total dietary fiber which had a high WHC range from 9.42 to 10.52 g water/g organic matter. With regard to carotenes, WHC, and fiber content, carrot pulp products are a good source of dietary fiber (Bao and Chang, 1994).

Some previous studies indicate that the functional properties and physiological effects of fibers are related to the source, variety, processing method, and analytical

method of dietary fiber (Yangilar, 2013). A good understanding of these properties is essential for the utilization of dietary fibers.

Chau et al. (2004) reported that insoluble dietary fiber were isolated from carrot pomace. This study revealed that carrot pomace was rich in insoluble dietary fiber (50.1-67.4 g/100 g), which were mainly composed of pectic polysaccharides, hemicellulose, and cellulose. These insoluble dietary fibers were found to have high functional properties, adsorption capacity, and amylase-inhibition activity. It was shown that the yield, composition, and functional properties of dietary fiber are affected by their preparation methods.

3.4.4 Modified crude dietary fiber using the etherification method

Table 3.6 Chemical composition of modified dietary fiber (MDF).

Component	%Content (Dried basis)
Crude protein	0.87±0.23
Ash	6.76±0.57
Moisture	3.54±0.45
Fat	0.12±0.08
Starch	1.13±0.32
Neutral detergent fiber (NDF)	87.58±1.32
Acid detergent fiber (ADF)	80.21±2.34
Acid detergent lignin (ADL)	5.67±1.08
Cellulose[a]	74.54±1.38
Hemicellulose[b]	7.37±0.48

Note: [a] ADF -ADL, [b] NDF-ADF.

Table 3.6 shows the chemical constituents of MDF in percentages. Crude protein, ash, moisture, fat, starch, neutral detergent fiber (NDF), acid detergent fiber

(ADF), acid detergent lignin (ADL), cellulose and hemicelluloses were 0.87, 6.76, 3.54, 0.12, 1.33, 87.58, 80.21, 5.67, 74.54, and 7.37, respectively. The chemical composition of MDF shows more neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicelluloses and less crude protein, moisture, fat and starch than is contained in CDF (Table 3.4). This suggests that chemical modification of cellulose by grafting long branch polymer chains from its surface enables an improvement in the chemical composition of cellulose and could potentially introduce other functional properties (Bruce, 2014).

Table 3.7 Functional properties of modified dietary fiber (MDF).

Functional properties	Content
Water holding capacity (WHC) (%)	7.12±0.21
Oil binding capacity (OBC) (g/g sample)	5.87±0.76
Water solubility index (WSI) (%)	6.13±1.11
Swelling capacity (SC) (ml/g DM)	10.40±1.03
COOH content (%)	6.86±0.97

The functional properties of the modified dietary fiber shown in Table 3.7 are that water holding capacity is 7.12%, oil binding capacity 5.87 g oil/g sample, water solubility index 6.13%, swelling capacity 10.40 ml/g DM and COOH content 6.86%. The functional properties of modified dietary fiber show a greater water holding capacity, oil binding capacity, water solubility index, swelling capacity and COOH content than CDF (Table 3.5).

The crude dietary fiber shows a high percentage of cellulose with ~79% (Table 3.4). However, cellulose itself has poor physical properties and also has a low absorption capacity. For this reason, chemical modification by using the etherification

method, copolymerization, or cross linking should be carried out for the cellulose to achieve efficient ion-exchange capacity. This method gives new properties of cellulose such as hydrophilic or hydrophobic characteristics, improved elasticity, water sorbency, ion-exchange capability, and thermal resistance (McDowall et al., 1984). These properties can increase cellulose absorption capacity (Saliba et al., 2005). Modifications of cellulose by grafting of polymers is the main method of attaching polymers to cellulose. This method produced new cellulose by directly modifying cellulose, which can be modified to cellulose by grafting a second polymer as a long branch on the cellulose molecule (Bruce, 2014; O'Connell et al., 2008).

CDF has a low quantity of functional properties (Table 3.5) compared with MDF (Table 3.7) due to cellulose being a polar molecule simply because it has carbon positions containing an OH group. However, its β 1,4 linkage makes it insoluble in water. So, modification of cellulose is essential to take advantage of its benefits. Furthermore, its hydrophilic character will most probably lead to moisture absorption and swelling of the cellulose. However, the hydroxyl groups of cellulose can be changed by increasing the number of available hydroxyl groups by breaking the hydrogen bonds, resulting in swelling of the cellulose structure and, thus, increased surface area and strength is achieved (Bruce, 2014). Accordingly, these factors can increase WHC and SC in MDF.

These data were similar to the findings of Saliba et al. (2005) whose method produced amidoximated sawdust sorbent by directly modifying cellulose that improved its functional properties. This amidoximated cellulose had a high adsorption capacity for direct modification of cellulose, leading to heavy metal adsorbent materials.

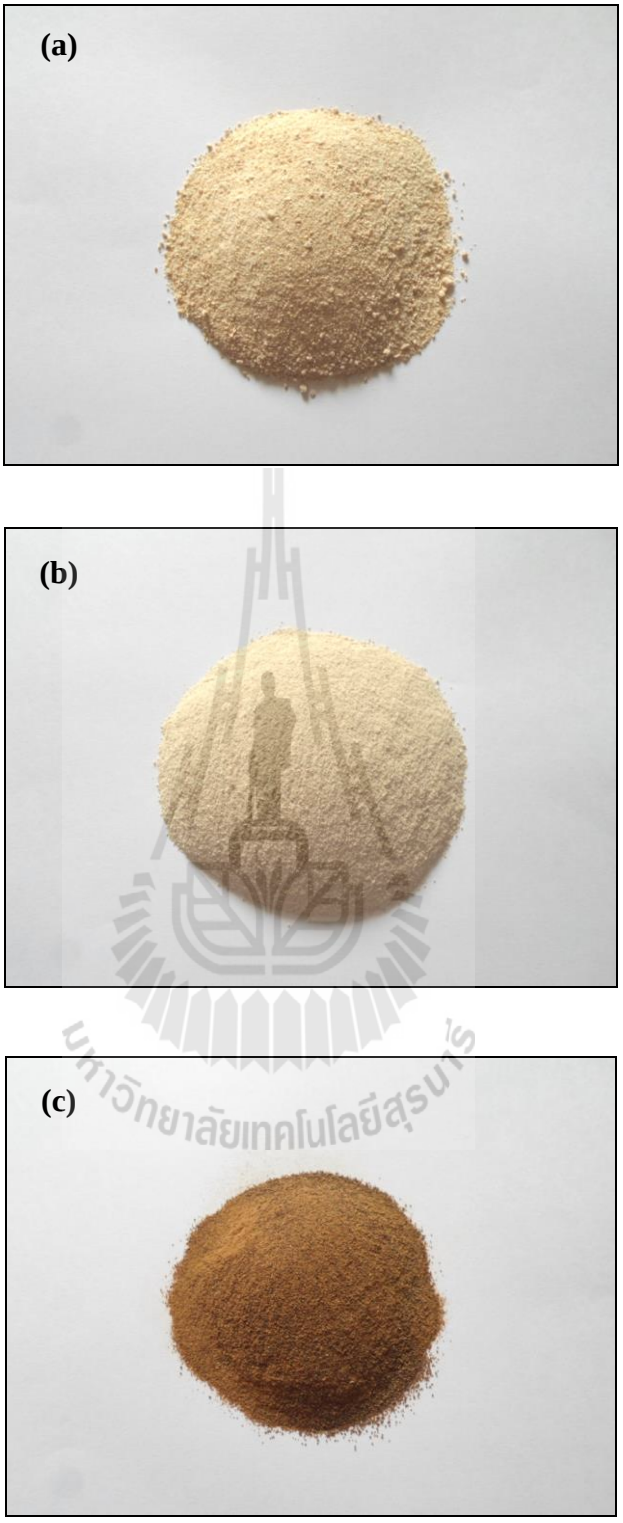
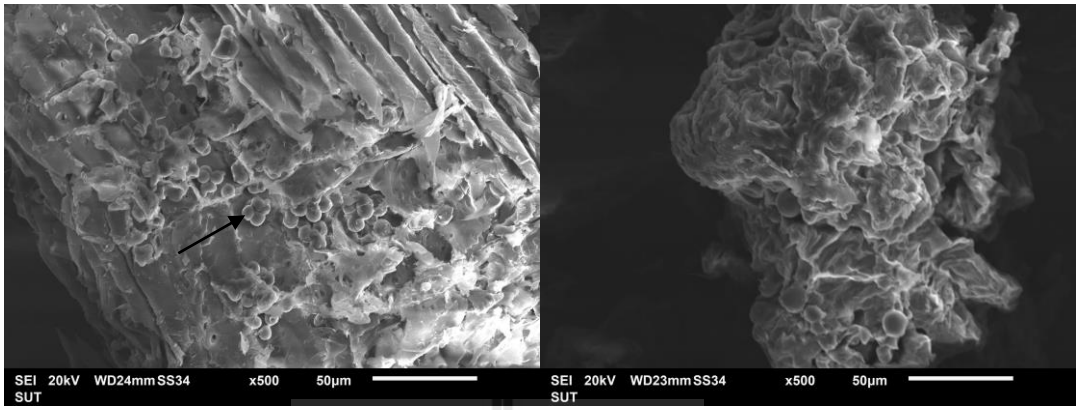
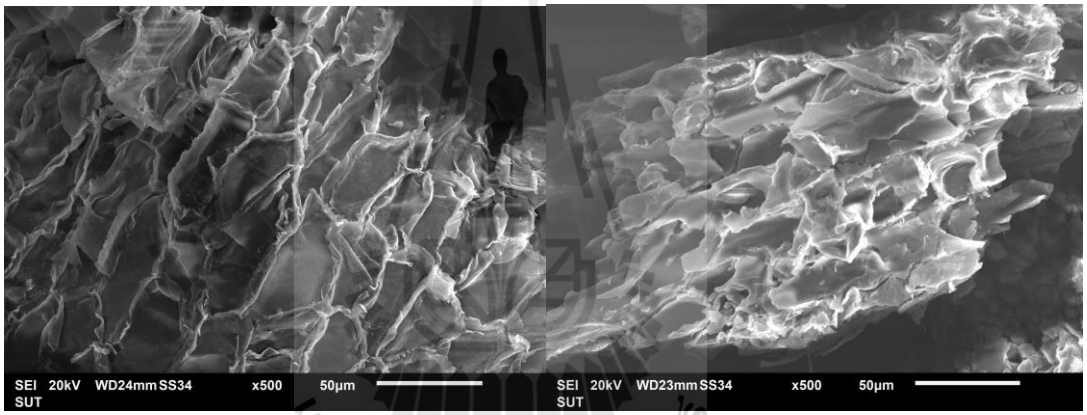


Figure 3.2 Photographs of (a) dried cassava pulp, (b) crude dietary fiber and (c) modified dietary fiber.

(a)



(b)



(c)

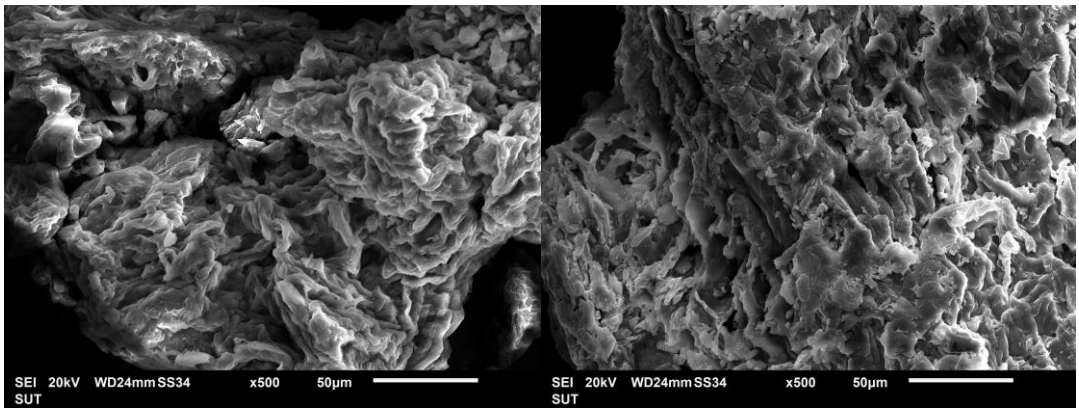


Figure 3.3 Scanning electron micrographs (SEM) of (a) dried cassava pulp, (b) crude dietary fiber and (c) modified dietary fiber.

3.4.5 Component content determination (FTIR)

The main goal of the study was to monitor fiber changes occurring during extraction and modification. A study of the dried cassava pulp, crude dietary fiber and modified dietary fiber using FTIR microspectroscopy technique showed differences in individual structure of fibers (Yan et al., 2009). The results of the FTIR analysis are shown in Table 3.8 and Figure 3.4.

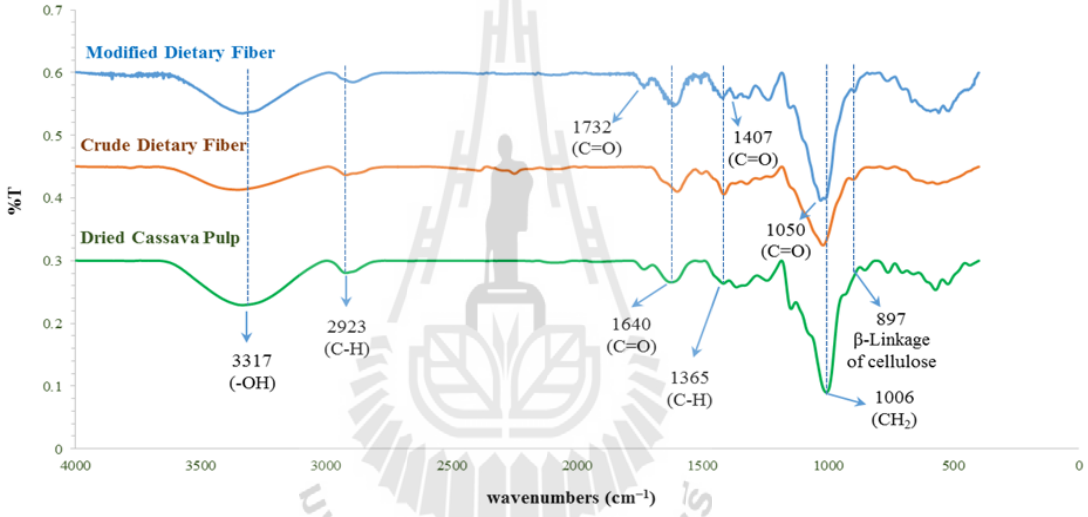


Figure 3.4 FTIR spectra of dried cassava pulp, crude dietary fiber (CDF) and modified dietary fiber (MDF). The IR region is between 500-3500 cm^{-1}.

Table 3.8 IR assignments of the main vibrations in the FTIR spectra.

IR region (cm^{-1})	Vibrations (cm^{-1})	Assignments
3700-3000	3317	Hydrogen bonded OH bond stretching vibrations of α cellulose OH Hydroxyl group
3000-2800	2923	C-H stretching of lignocellulosic components
1800-1500	1732	C=O stretching
	1640	C=O stretching vibrations in conjugated carbonyl of lignin
1500-1200	1365	C-H bending
	1407	C=O stretching in carboxamide functional groups
1200-650	1006	CH_2 stretching vibrations
	1050	C=O stretching of cyano group
	897	β-linkage of cellulose

- IR region 3700-3000 cm^{-1}

The broad band in this region is due to the OH-stretching vibrations arising from hydrogen bonding in cellulose (Oh et al., 2005). The vibrations at 3317 cm^{-1} (Figure 3.4) were assigned to the hydroxyl groups (Atef et al., 2014). The natural fiber or polymer composites due to the hydroxyl groups (-OH) appearedin the cellulose and lignin structure of the fiber molecule (Soom et al., 2009). Dried cassava pulp, crude dietary fiber and modified dietary fiber show high contain of cellulose in the structure. The hydroxyl groups of cellulose were available to bind with water. Therefore, it can also affect binding or absorption with mercury (Brígida et al., 2010).

- IR region 3000-2000 cm^{-1}

The characteristic absorption peak at 2923 cm^{-1} suggests C-H stretching vibrations in lignocellulosic components such as cellulose, hemicellulose and lignin (Ibrahim et al., 2011). This absorption is associated with lignocellulosic components such as cellulose, hemicellulose and lignin. The structure of cellulose is formed by glycosidic linkages and the hydroxyl group with a small amount of carboxyl, while hemicellulose and lignin are formed by ether bonds (Ibrahim et al., 2011; Chen et al., 2011; Colom et al., 2003; Castellano et al., 2004).

- IR region 1800-1500 cm^{-1}

The spectrum of the acetyl group is observed by the C=O band at 1732 cm^{-1} of fiber, corresponding to hemicellulose (Himmelsbach et al., 2002; Proniewicz et al., 2001). Hemicelluloses contain backbones of p- 1,4-1inked pyranoside sugars, but they are different from cellulose, because they are smaller in size (often less than 200 sugar residues), and they contain a variety of sugars and are branched.

The band at 1640 cm^{-1} C=O shows the stretching vibrations in conjugated carbonyl of lignin. Lignin contains 2 main types of acid including carboxylic and phenolic groups on its surface. Both groups bind with metal ions (Mormann and Michel, 2002). The adsorption capacity of metal ions is dependent on pH and ionic strength. When the pH value increases, the carboxyl group (COOH) which are dissociated from the carboxyl anions (RCOO-) show stronger interaction with the toxic cations resulting in higher binding capacity of the dietary fiber (Ilharco and Brito de Barros, 2000).

-IR region 1500-1200 cm^{-1}

The band at 1365 cm^{-1} can be assigned to C-H, which presents characteristic peaks of cellulose nanofibrils (Robert et al., 2004). CH_2 bending vibrations are related to the structure of cellulose and aromatic skeletal vibrations (Rosu et al., 2010; Penttilä et al., 2013; Marques et al., 2006; Kizil et al., 2002).

- IR region 1200-650 cm^{-1}

The band at 1006 cm^{-1} was attributed to CH_2 stretching vibrations (You et al., 2009). The band at 897 cm^{-1} can be assigned to the β-linkage of cellulose. The band at 1050 cm^{-1} of modified dietary fiber is related to C=O stretching of the cyano group (Jiang et al., 2011). The band approximately 700 cm^{-1} has been assigned to the out-of-plane vibrations of O–H groups or to the rotational vibrations of the whole H_2O molecule (Kizil et al., 2002).

Moreover, a different band was observed for the fiber. The result indicated that the molecular structure of the MDF had changed. The new peak at 1732 cm^{-1} of the MDF corresponded to the -C=O group (Himmelabach et al., 2002), which confirmed the introduction of the MDF side chain into the cellulose backbone by graft copolymerization.

These data were similar to those found in the studies of Saliba et al. (2005) in which the FTIR results show cyanoethylation or grafting of cellulose is accompanied with the formation of a new band at 1050 cm^{-1} of MDF due to the cyano group being involved in a decreasing of asymmetry index and also showing hydrogen bond strength while the crystallinity index increased.

Ekbafe et al. (2011) reported that the FTIR spectra of the physical mixture and the resulted hydrogel arenew peaks at 1407 cm^{-1} which can be attributed to C=O

stretching in carboxamide functional groups and symmetric and asymmetric stretching modes of the carboxylate groups. This peak appears in MDF, which confirms the modification of fiber by grafting of cellulose.

3.4.6 Surface area, pore volume and pore diameter

The surface area is important for the evaluation of the characteristics of porous materials. The BET analysis is the standard method for measuring surface areas by using nitrogen adsorption isotherms and by calculating quantities of gas adsorption onto the material surfaces. The surface areas were calculated according to the theory of Brunauer, Emmett, and Teller by using nitrogen adsorption isotherms measured at 77 K. This is a standard procedure that allows for comparisons between different materials (Walton and Snurr, 2007). This method of analysis is based on gas adsorption use of the BET equation and allows one to calculate the quantities of the surface area (Nelsen and Eggertsen, 1958).

Table 3.9 Properties of samples.

Type of fiber	Surface area (m^2/g)	Pore diameter (mm)	Pore volume (cm^3/g)
Dried cassava pulp	4.47×10^{-2}	64.58	7.65×10^{-3}
Crude dietary fiber	4.79×10^{-1}	72.10	8.13×10^{-3}
Modified dietary fiber	5.73×10^{-1}	119.58	8.67×10^{-3}

Table 3.9 shows that The BET surface area, pore volume and pore diameter were determined by N adsorption at 77 K using the ASAP 2010 analyzer produced by Micromeritics. Before the adsorption analyses, the samples were degassed at 150°C in vacuum for 3 h. Modified dietary fiber shows the highest surface area, pore diameter and pore volume followed by crude dietary fiber and dried cassava pulp respectively.

These results suggest that when starch and protein are separated from fiber by the enzymatic digestion process, it results in an increase in surface area, pore diameter and pore volume. In addition, there are other methods for direct modification. As an alternative, valuable properties deficient in native cellulose can be imparted to cellulose by grafting a second polymer as a long branch on the cellulose molecule leading to heavy metal adsorbent materials (Lu and Chung, 2001; Walton and Snurr, 2007).

Table 3.9 shows that modification of cellulose increases the properties of fiber after modification, and these properties are important to heavy metal absorption. The main factor which influences heavy metal absorption is surface area. Surface modification of cellulose can be performed by chemical modifications. Cellulose can be modified by grafting a small molecule on the surface. This technique is most commonly used to modify functionalities on the material surface which canchange functional groups on the fiber surface. Cellulose ethers are the most common method of cellulose modification. Modification of cellulose by grafting polymer chains on its surface results in the alteration of the physical and chemical properties or other functionalities that could improve mercury binding capacity (Bruce, 2014).

Chau et al. (2004) reported that the surface characteristics of natural fibers and cellulose were investigated using scanning electron microscopy (SEM) and the BET-surface area was studies for the water adsorption.

3.5 Conclusion

The results obtained indicate that enzymatic optimization of dried cassava pulp extraction process is indeed a possible route for obtaining dietary fiber for food supplementation. In addition, modification of crude dietary fiber from dried cassava

pulp could improve the physiochemical and functional properties for binding or absorption with mercury or heavy metals.

3.6 References

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CHAPTER IV

DIETARY FIBER FROM CASSAVA PULP AND ASSESSMENT OF MERCURY BIOACCESSIBILITY USING AN *IN VITRO* DIGESTION

4.1 Abstract

The estimation of mercury (Hg) bioaccessibility in crude dietary fiber (CDF) and modified dietary fiber (MDF) is useful for human health risk assessment using an *in vitro* digestion model and the reduction of fish mercury bioaccessibility by CDF and MDF was studied. The CDF and MDF were prepared from cassava pulp, which was obtained by an enzymatic digestion process (CDF) and then modified with the etherification method (MDF). The effect on the quantity of fish mercury and fibers (CDF and MDF) were also determined. The results indicate that the CDF reduced fish mercury bioaccessibility to 2-57% and 34-85% for MDF compared with control (0-1000 mg of NDF in 1 g of fish tissue) in a dose-dependent manner. The quantity of fish mercury was reduced to 21-39% (CDF) and 70-84% (MDF) when 500 mg of fiber were added in a digestion model test (fish tissue amount 0-4 g) compared with control (the control lacks fiber). In conclusion, it is suggested that CDF and MDF prepared from cassava pulp decreases mercury bioavailability by inhibiting mercury transfer to the aqueous fraction in the digestion model test.

Keyword: bioaccessibility, *in vitro* method, *in vitro* digestion model

4.2 Introduction

Mercury (Hg) is a natural element released from the Earth's crust through a variety of industrial processes including combustion of coal, which is considered the largest source of mercury emission into the air (Bernard et al., 2001). It is found in various forms: elemental and metallic mercury, inorganic mercury compounds, and organic mercury compounds (US EPA, 2003). Methyl mercury (MeHg) is a form of Hg commonly found in water generated through metabolism by bacteria which makes its way up the food chain to accumulate in fish, particularly predatory fish (Bose-O'Reilly et al., 2010).

Hg is highly toxic to humans impacting several aspects of health including: direct impacts to the nervous system, heart, kidneys, lungs, and immune system and it can eventually lead to death (US FDA, 2004). Humans are primarily exposed to MeHg through consumption of fish or shellfish (US EPA, 2003). Fish consumption is popular due to its nutrient density including omega-3 fatty acids including eicosapentaenoic acid (EPA, C22:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), that play a role in brain and retina development and function (Allen and Harris, 2001). However, several fish species have also been identified as the main source of MeHg exposure in humans including several commonly consumed species such as bass (striped), bluefish, Chilean sea bass, golden snapper, walleye, king mackerel, tile fish, swordfish, shark and tuna that have the highest levels of Hg ranging from 0.2 to 1 ppm (US EPA, 2007). In general, more than 90% of the mercury in fish is found to be methylmercury (Jewett and Duffy, 2007). Following absorption, MeHg accumulates in the brain, muscle, and kidney, presumably because of its strong

affinity for sulfhydryl groups (US EPA, 1997). Additionally, MeHg can cross the placental-blood and blood-brain barriers (Bernhoft, 2011).

Children are more susceptible than adults to Hg toxicity due, in part, to differences in stages of brain development that occur during fetal development as well as through normal development during infancy and childhood periods. Excessive prenatal exposure can result in developmental delays (Rogers, Emmett, Ness, and Golding, 2004). As a result, the US Environmental Protection Agency (US EPA) has established a reference dose (RfD) for MeHg of 0.1 μg Hg/kg body weight per day (US EPA, 2001). In addition, the U.S. EPA conducted a national statistical survey of fish fillet tissue with a sample size of 541 sites on boatable rivers in 2008-2009. Sample sites were identified as being urban or non-urban. All sample Hg concentrations were above the 3.33 μg kg^{-1} (ppb) quantitation limit, and an estimated 25.4% (±4.4%) of the 51,663 river miles assessed exceeded the U.S. EPA 300 μg kg^{-1} fish-tissue based water quality criterion for Hg, representing 144±181.8 river miles (Wathen, Lazorchak, olsen, and Batt, 2015).

In order to mitigate exposure to Hg, chelation agents have been applied for the systematic removal of Hg through inhibition of Hg bioaccessibility or intestinal re/absorption (Sears, 2013). This includes compounds such as ethylenediamine-tetraacetic acid (EDTA), Diethylenetriaminepentaacetic acid (DTPA) 2,3-mercaptopropanol (BAL), D-Penicillamine (D-β, β-dimethylcysteine), Deferoxamine dimercaptosuccinic acid (DMSA), and penicillamineand 2, 3-dimercapto-1-propane sulfonate (DMPS). While promising, many of these treatments have reported side effects and are thus not suitable for long term application, particularly in children (Tandon and Singh, 2000; George et al., 2004; Wang et al., 1999). Recently, many

researchers have reported applying dietary strategies to reduce the potential for Hg absorption from high risk foods (Nawirska, 2005; Hu et al., 2010; Zhang et al., 2011). Some studies have suggested that particular dietary factors, including fiber and phytochemicals, can similarly impact MeHg bioavailability (Shim et al., 2009). For instance, 15-30% of wheat bran decreased the total Hg concentration in the brain, blood and small intestine and may therefore reduce the neurotoxic effects of the organomercurial in animal studies (Rowland et al., 1986). Thiol compounds in garlic act as metal-chelating or complexing agents that can increase mercury excretion (Block, 1985). Dietary fiber functional cookies have no toxic or harmful actions on animals or humans, and the DF food was able to decrease TC and TG concentrations to some extent in serum and increased excretion of Cd^{2+} and Pb^{2+} in feces (Hong et al., 2012).

Dietary fibers in particular have demonstrated promise as heavy metal adsorbents because of their nontoxic and non-biodegradable nature (Kay, 1982). Cassava pulp is a by-product of cassava starch factory processing. Cassava (*Manihot esculenta* Crantz.) is the main crop of Thailand, especially in the northeast of Thailand. Total cassava root crops of Thailand in 2011 were approximately 21,060,903 tons. Cassava pulp accounts for approximately 10-30% by weight (wet) of the original tubers (Thailand Tapioca starch, 2011) and contains approximately 60% starch and 30% fiber (Apiwatanapiwat et al., 2011). Most cassava pulps are rich in fiber and thus hold potential as bioactive absorbants for heavy metal binding to prevent toxicity.

By virtue of high throughput and lower cost, this *in vitro* technique has been used to assess the bioaccessibility of nutrients, phytochemicals and contaminants. For

instance, bioaccessibility has been used as a surrogate to assess the bioavailability of cadmium from vegetables, aflatoxin B1 from peanuts and ochratoxin A from buckwheat (Versantvoort and Rompelberg, 2004). Shim, Ferruzzi, Kim, Janle, and Santerre (2009) previously reported that green tea extract (31-2000 mg), black tea extract (31-2000 mg), and soy protein (50-100 mg) were able to reduce mercury bioaccessibility from fish tissues by 82-92%, 88-91%, and 44-87%, respectively. However, inclusion of grapefruit juice (0.5-10 ml) did not reduce mercury in the aqueous phase. Fiber sources including wheat bran (50-1000 mg) oat bran and psyllium have also been reported to reduce bioaccessibility of mercury from the same fish tissue at amounts greater than 500 mg of fiber. This study suggests that co-consumption of foods containing dietary fibers could bind with Hg. To expand on these efforts, the objective of this study was to determine the effects of dietary fiber prepared from cassava pulp on Hg bioaccessibility using a coupled *in vitro* digestion/Caco-2 human intestinal cell model.

In this study, the crude dietary fiber was used for the observation of mercury in fish tissue obtained from cassava pulp, which is a byproduct of cassava starch factory processing. After extraction with the enzymetic digestion process, approximately 79% of Neutral Detergent Fiber (NDF) was obtained, which is the most abundant structural component in plant cells (lignin, hemicellulose and cellulose), including 74% of cellulose (Chen, 2014). Therefore, chemical modifications with the etherification method are necessary to improve new properties of cellulose for reducing Hg in the gastrointestinal tract (GI tract) (Saliba et al., 2005).

Two possible mechanisms of binding are chemisorption and physical sorption. Chemisorption is the main one; a free carboxyl group from uronic acids/phenolic groups from lignin form a coordination complex with di/trivalent metal anions or the positive charge of trivalent cations is connected with carboxyl anion (Zhang et al., 2011; Kay, 1982). Physical adsorption is used to characterize the surface and pore features of fiber. Thus, dietary fiber can be absorbed/bound and carries them through the digestive tract because it is resistant to digestion by human elementary enzyme (Camire et al., 2001).

In recent years, *in vitro* screening methods have been developed and used for the evaluation of nutrient bioaccessibility and bioavailability from foods (Etcheverry et al., 2011). *In vitro* methods are useful to provide knowledge of factors that affect absorbtion such as the chemical composition of the nutrient in food and/or nature of the food matrix, and interactions between nutrients and other organic components during digestion (Gibson et al., 2006). The main advantage of *in vitro* methods are that they are less expensive, faster than *in vivo* studies, and it is easier to control the experimental variables than in human or animal studies (Johnson, 1988). However, *in vitro* studies cannot be substituted for *in vivo* studies, and should be done via *in vivo* studies (Etcheverry et al., 2011).

The couples *in vitro* digestion/Caco-2 cell model are useful to estimate human health risk assessment (Fu and Cui, 2013). *In vitro* digestion is conducted to simulate the human GI tract. These methods are fast, reproducible and can be used to measure the bioaccessible fraction, which is an important parameter for the evaluation of bioaccessibility and bioavailability (Van de Wiele et al., 2007). Caco-2 cell can be used to evaluate intestinal cell uptake and the estimation of bioavailability (Britton

et al., 2009). Bioaccessibility is the first step of bioavailability, which is the fraction of a compound that is released from the food matrix in the gastrointestinal lumen and which is available for absorption into the intestinal mucosa (Rein et al., 2013).

The *in vitro* digestion model can be divided into two or three steps including gastric and intestinal digestion that simulate the human digestive system (Chiang et al., 2008). For the gastric phase, pepsin is added to the samples for adjusting pH to 2 or 4. Acidification of the samples is important, because pepsin begins to denature and lose its activity at pH ≥ 5. The samples are adjusted to pH 5.5-6 prior to the addition of pancreatin (which consists of a cocktail of pancreatic enzymes such as pancreatic amylase, lipase, ribonuclease, and proteases such as trypsin) and bile salts (emulsifiers), before the start of the intestinal phase and finally adjusted to pH 6.5-7. The third digestion step is digestion by salivary enzyme alpha-amylase, which is an enzyme that breaks the glycosidic bonds of starchmolecules (Etcheverry et al., 2011).

4.3 Materials and methods

4.3.1 Sample preparation

Crude dietary fiber (CDF) and modified dietary fiber (MDF) were used as a source of fiber in this study and prepared by the enzymatic digestion method and modified by the etherification method (see Chapter III) The fiber was kept in a sealed container until used for further treatment.

Swordfish was obtained from the Gulf of Mexico and shipped frozen from the Department of Nutrition Science, Purdue University, West Lafayette, Indiana, USA. From the sample mercury analysis, it contained a total of 1.17 ppm mercury. The fish tissue was thawed and homogenized in a blender. Replicate samples (1 g of

homogenized fish tissue) were weighed into a 50 ml polypropylene centrifuge tube with a screw cap. One milliliter of saline (0.9% NaCl, Sigma-Aldrich) was added to the test tube and was homogenized twice by a cell disruptor at 20 kHz and 150-500 watts for 30 s and was then mixed with CDF/MDF (0, 50, 100, 500, and 1000 mg) prior to the intiation of the digestions.

4.3.2 *In vitro* digestion

The 2-stage *in vitro* digestion model used in the present study was originally described by Garrett, Failla, and Sarama (1999) with modification. The gastric phase was initiated with additional porcine pepsin (3 mg/ml, Sigma Chemical Co., St. Louis, MO) and adjustment of the pH to 2-2.5 with 0.1 M HCl (Analytical grade, Sigma Chemical Co.). Samples were vortexed and flushed to the top of the tube with nitrogen gas (99.99%, Air Gas, Indianapolis, IN) and were then incubated at 37ºC for 1 h in a shaking water bath at 150 rpm (VWR, Cornelius, OR). The intestinal phase was initiated by pH adjustment to 5.3 with 100 mM sodium bicarbonate solution (Sigma Chemical Co.) and with an addition of 9 ml of a bile extract/pancreatin/lipase mixture: pancreatin (0.4 mg/ml, Sigma Chemical Co., St. Louis, MO), lipase (0.2 mg/ml, Sigma Chemical Co.) and porcine bile extract (2.4 mg/ml, Sigma Chemical Co.); then, the pH was adjusted to 6.5-7.0 with 0.1 M NaOH (Analytical grade, Sigma Chemical Co.), and the solution was made up to 30 ml with 0.9% saline (pH 7). Samples were vortexed and flushed to the top of the tube with nitrogen gas and were incubated at 37ºC for 1 h in a shaking water bath at a speed of 150 rpm. One sample tube was separated for digesta, and the other 3 sample tubes were centrifuged at 167,000 g for 35 min (Beckman L8-70M, Beckman Coulter, San Antonio, TX).

Aliquots of raw materials, digesta, aqueous phases, and residual pellets were collected and stored at -80ºC prior to analysis.

Data analysis

$$\text{Relative bioaccessibility (\%)} = \frac{\text{µg/kg of Hg in aqueous}}{\text{µg/kg of Hg in digesta}} \times 100$$

$$\text{Absolute bioaccessibility (µg/g)} = \frac{\text{\%bioaccessibility} \times \text{starting material}}{100}$$

4.3.3 Determination of mercury

Cells were centrifuged at 200 rpm for 10 min at room temperature (20-25°C) (Eppendorf Centrifuge 5415 D, Hamburg, Germany), and the supernatant was discarded. An aliquot of cells was analyzed for total Hg using a Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer (TDA/AAS) Mercury Analyzer (DMA-80, Milestone Inc., Pittsburgh, PA) as described by Shim et al. (2009). Total Hg in the aqueous fraction and pellet was also determined. Total Hg data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

4.3.4 Statistical analysis

The results are presented as representative data from at least two sets of experiments. Data are expressed as the mean±standard error. For cellular uptake studies, a sample size of n = 3 was used. Statistical analysis for each parameter assessed was performed using analysis of variance (ANOVA) followed by Tukey's post hoc test (SAS, Gary, NC). Differences between means were considered statistically significant at $p < 0.05$.

4.4 Results and discussion

4.4.1 Effect of fiber amount on mercury bioaccessibility and absolute bioaccessibility

In this study, the Hg in fish tissue was assumed to be primarily in the MeHg form because it is assumed that the majority (95-99%) of the mercury present in fish tissue has already been converted to the methyl form (Wiener and Spry, 1996). Bioaccessibility of Hg from fish tissue digested with or without differing amounts and types of MDF was determined by *in vitro* digestion. This method is designed to assess the release and solubilization of MeHg from gastric and small intestinal digestion of a test meal. Bioaccessibility is the amount of Hg released from fish tissue and transferred to the aqueous fraction (following centrifugation). This fraction is defined as available for subsequent intestinal absorption. This approach does not account for chemical changes or release from the fiber which can occur in the human gut. The bioaccessibility was evaluated by varying the amounts of both fibers (0, 50, 100, 500, and 1000 mg). The fiber obtained from the enzymatic digestion process (CDF) and the modified methods (MDF) was used. The aim of this study was to evaluate the effect of the amount of fiber on Hg bioaccessibility and to determine a suitable amount of fiber for use in the *in vitro* digestion in the next step.

The Hg concentrations found in the raw material (swordfish) are 1.17 mg/kg. European legislation limits the maximum concentration of total Hg in seafood products to 0.5 mg/kg ww or 1 mg/kg ww, depending on the fish species considered (EC, 2008). The CDF and MDF were assessed for their effect on mercury bioaccessibility from fish tissue effect using the *in vitro* digestion model. The percentage of Hg in digesta released from the fish tissue (bioaccessibility) was

defined as absorbed/bound with fibers. The Hg that remained in the aqueous fraction was defined as evaluate potential of fiber to reduce Hg.

The first step involved studying the effects of the amounts of CDF and MDF on mercury bioaccessibility and absolute bioaccessibility from 1 g of fish tissue. Table 4.1 and Figures 4.1-4.2 show the effects of fiber in the form of CDF and MDF from cassava on Hg bioaccessibility from co-digested fish tissue. Both forms of dietary fiber showed significantly reduced Hg bioaccessibility in a dose dependent manner from 0-1000 mg of CDF and MDF ($p < 0.05$). Hg bioaccessibility was decreased by 2-57% for CDF and 35-85% for MDF compared to control (fish only) with inclusion of CDF and MDF up to 1000 mg per digestive reaction. MDF 1000 mg showed the strongest effect on mercury bioacessibility (8.60±0.56 %) (Table 4.1). A comparison of both fibers suggests that MDF showed significantly more mercury inhibition than CDF ($p < 0.05$). MDF decreased mercury bioaccessibility to 35-85% and 2- 57% for CDF compared to control for all the amounts used.

The present results suggest that the addition of dietary fiber may impact on Hg bioaccessibiilty potentially by the binding of Hg as it is released from digested fish or through alteration of the digestibility of the fish matrix itself. The actual mechanism for Hg reduction is likely to be a combination of physical and chemical adsorption. Physical sorption is nonspecific, which does not include the sharing or transfer of electrons. Therefore, these mechanisms are the absorption of heavy metal in the fiber matrix while the adsorbed molecules of heavy metal are free to cover the surface of the adsorbent (dietary fiber). Chemisorption is the connection between the fiber matrix of phenolic groups from lignin and carboxyl groups from uronic acid (Zhang et al., 2011; Kay, 1982), which is specific and dependent on the formation of covalent

bonds (sharing of electrons) between the adsorbate and a specific fiber surface site. The binding capacity is dependent on the activity of the adsorbent. Chemisorption is generally considered to be irreversible, while physical adsorption is considered to be reversible (Sengupta, 2001). The carboxyl group content involves interaction with the toxic cations. In addition, when the pH value increases, the carboxyl group was dissociated to carboxyl anions (RCOO-) which showed stronger interaction with the toxic cations and which resulted in higher binding capacity of the dietary. A low pH would block or suppress ionization of carboxyl group (Kay, 1982). For this reason, MDF showed more mercury inhibition than CDF due to the carboxyl group content (COOH) in the structure molecule (Tables 3.5 and 3.7).

MDF showed that significantly stronger binding with Hg than CDF might be due to the chemical composition of MDF. MDF showed more neutral detergent fiber; NDF (Cellulose, Hemicellulose, Lignin), acid detergent fiber; ADF (lignin, cellulose), acid detergent lignin, and ADL (lignin) than CDF (Tables 3.5 and 3.7). These are insoluble fibers (cellulose, hemicellulose and lignin). It has been reported that insoluble fibers binds more easily with Hg than soluble fibers (Mertens, 2002).

The main mechanism for reducing Hg of cellulose is physical sorption, which is used to characterize the surface and pore volume of the fiber structure. Both fibers show a high percentage of cellulose at 79%. However, cellulose has low adsorption capacity and poor physical stability (Saliba et al., 2000). Therefore, chemical modification (etherification) give new cellulose properties such as surface area, pore diameter and pore volume. As a result, MDF showed a greater reduction in Hg than CDF. These physical properties as the adsorption potential for fiber. However, the mechanism of Hg reduction is a combination of physical and chemical adsorption for

metal removal (Liu et al., 2011). In addition, hemicellulose and lignin can bind with Hg by using chemisorption because they are composed of carboxyl group in the fiber structure.

There areany parameters which include: heavy metal content, fiber content, pH, amount of adsorbent, initial concentration of metal, contact time, the distribution ratios of the metals, inter-competition among metals, surface physical and chemical characteristics and processes such as ion exchange, chemical reaction, etc. (Ngoh, 2006). As a result, mercury bioaccessibility was significantly reduced with higher amounts of co-digested fibers and likely increased interactions between dietary fiber and Hg .

Bioaccessibilities can vary depending on factors such as the composition of the food matrix, pH, shaking time and enzyme conditions (Biehler et al., 2011; Calatayud et al., 2012). However, the bioaccessibilities can be very different (38-83%) with the same method and fish species (Torres-Escribano et al., 2010). These results suggest that the total mercury in food is not necessarily bioavailable.

These results were similar to those found in a previous report by Shim et al. (2009) that showed that fiber sources including wheat bran (50-1000 mg), oat bran and psyllium also reported reduced bioaccessibility of mercury from the same fish tissue at amounts greater than 500 mg of fiber. This study suggests that co-consumption of foods containing phytochemicals or select dietary fibers with mercury. This study suggests that the relative bioaccessibility of Hg in dietary-containing foods will bind mercury in a dose-dependent manner. This would suggest that increasing the amount of fiber could increase Hg inhibition to aqueous fraction.

The optimal amount of dietary fiber to be used in the next step is 500 mg, even though 1000 mg shows the highest reducing mercury bioaccessibility due to the sticky nature of the experiment. Polymeric components of the diet (eg, proteins and gelatinized starch) affect digesta viscosity in the GI tract. Particulate material such as insoluble fiber will also contribute to overall viscosity or be directly proportional to the fraction of the total volume of digesta (Eastwood and Morris, 1992).

Table 4.1 Fiber comparisons of types (CDF and MDF) and amounts (0-1000 mg) of 1 g of fish tissue.

Type	Amount of fiber (mg)	Total Mercury (µg/g)		Relative bioaccessibility[1] (%)	Absolute bioaccessibility[2] (µg/g)
		Digesta	Aqueous		
CDF	0	$0.80\pm0.02^{b,3}$	$0.45\pm0.02^{b,45}$	$56.25\pm1.72^{e,5}$	$0.66\pm0.08^{e,5}$
	50	$0.75\pm0.07^{a,1}$	$0.41\pm0.01^{b,45}$	$54.67\pm2.56^{d,5}$	$0.64\pm0.07^{d,5}$
	100	$0.90\pm0.03^{d,6}$	$0.43\pm0.01^{b,45}$	$47.78\pm1.11^{c,45}$	$0.56\pm0.09^{c,45}$
	500	$0.96\pm0.10^{e,7}$	$0.30\pm0.01^{b,345}$	$31.25\pm1.09^{b,234}$	$0.38\pm0.07^{b,234}$
	1000	$0.84\pm0.05^{c,4}$	$0.20\pm0.04^{a,123}$	$23.80\pm0.98^{a,123}$	$0.28\pm0.12^{a,123}$
MDF	0	$0.80\pm0.08^{b,3}$	$0.46\pm0.03^{c,5}$	$57.50\pm1.21^{e,5}$	$0.67\pm0.13^{e,5}$
	50	$0.78\pm0.10^{a,2}$	$0.29\pm0.10^{b,234}$	$37.17\pm1.02^{d,345}$	$0.44\pm0.08^{d,345}$
	100	$0.87\pm0.09^{c,5}$	$0.21\pm0.04^{ab,123}$	$24.14\pm0.87^{c,123}$	$0.28\pm0.04^{c,123}$
	500	$0.90\pm0.04^{d,6}$	$0.14\pm0.03^{a,12}$	$15.55\pm0.68^{b,12}$	$0.18\pm0.03^{b,12}$
	1000	$0.93\pm0.04^{e,2}$	$0.08\pm0.01^{a,1}$	$8.60\pm0.56^{a,1}$	$0.10\pm0.02^{a,1}$

Note: [1]Relative bioaccessibility is defined as the % of mercury recovered in digesta.

[2]Absolute bioaccessibility is the amount of mercury from the fish recovered in digesta.

*Data represent mean +/- SEM from n = 3 independent *in vitro* digestion experiment.

*Presence of different letters indicate significant differences between treatments as determined by a Tukey's post hoc test ($p < 0.05$) a, b, c... is fiber dose effect and 1, 2, 3...is fiber type effect.

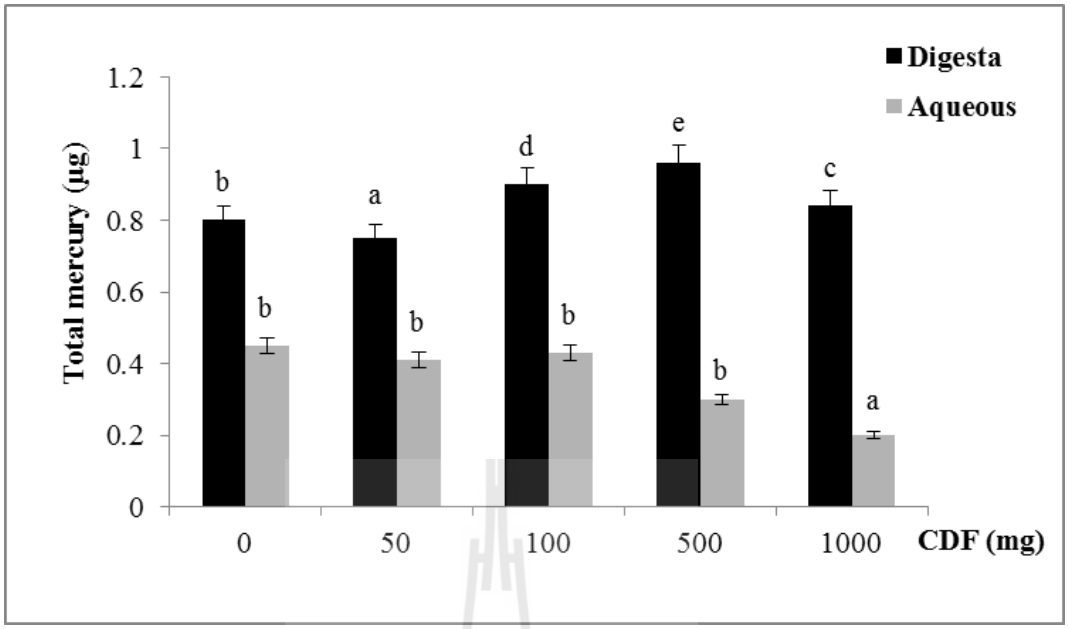

Figure 4.1 Total mercury in digesta and aqueous fraction fron *in vitro* digestion of 1 g of fish tissue in the presence of increasing amounts of CDF. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

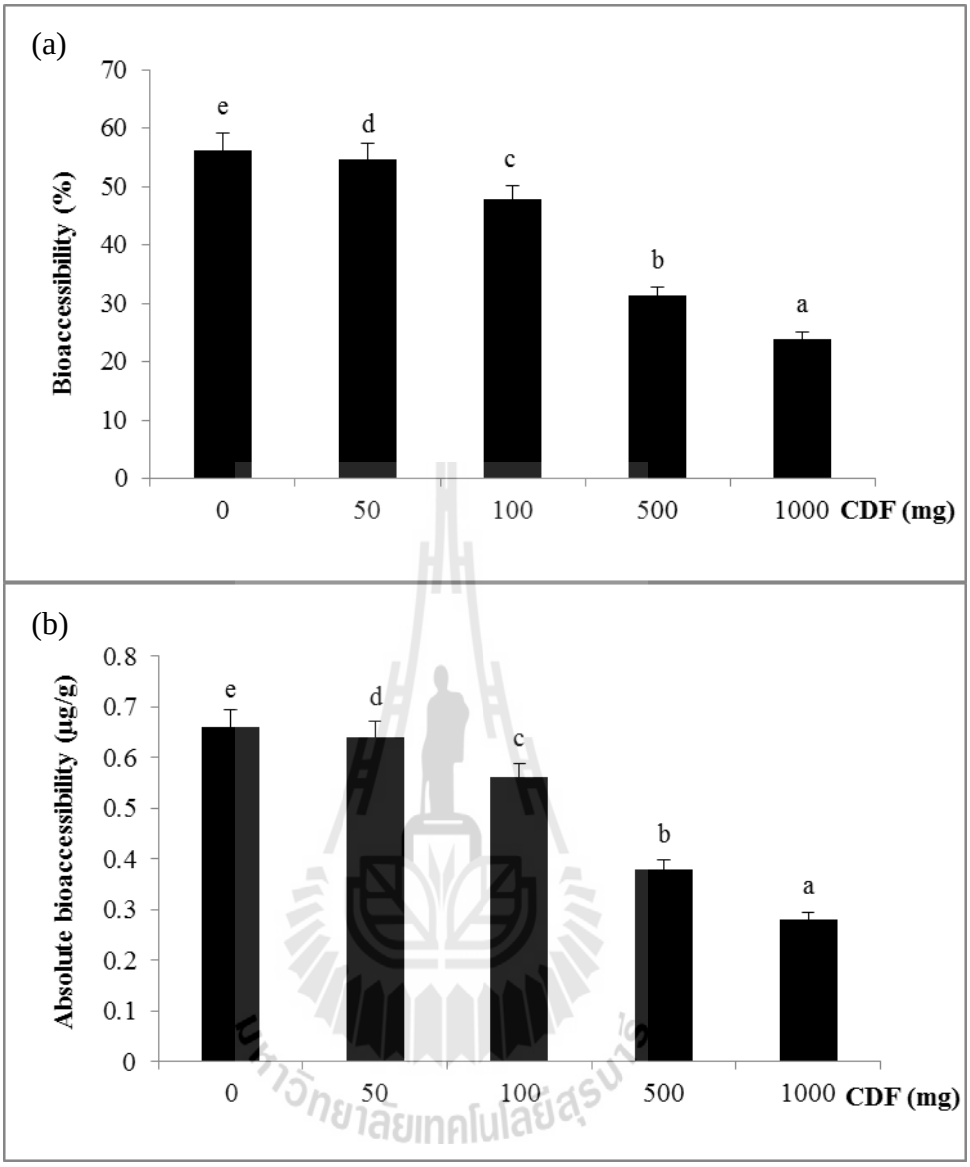

Figure 4.2 Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 1 g fish tissue in the presence of increasing amounts of CDF. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

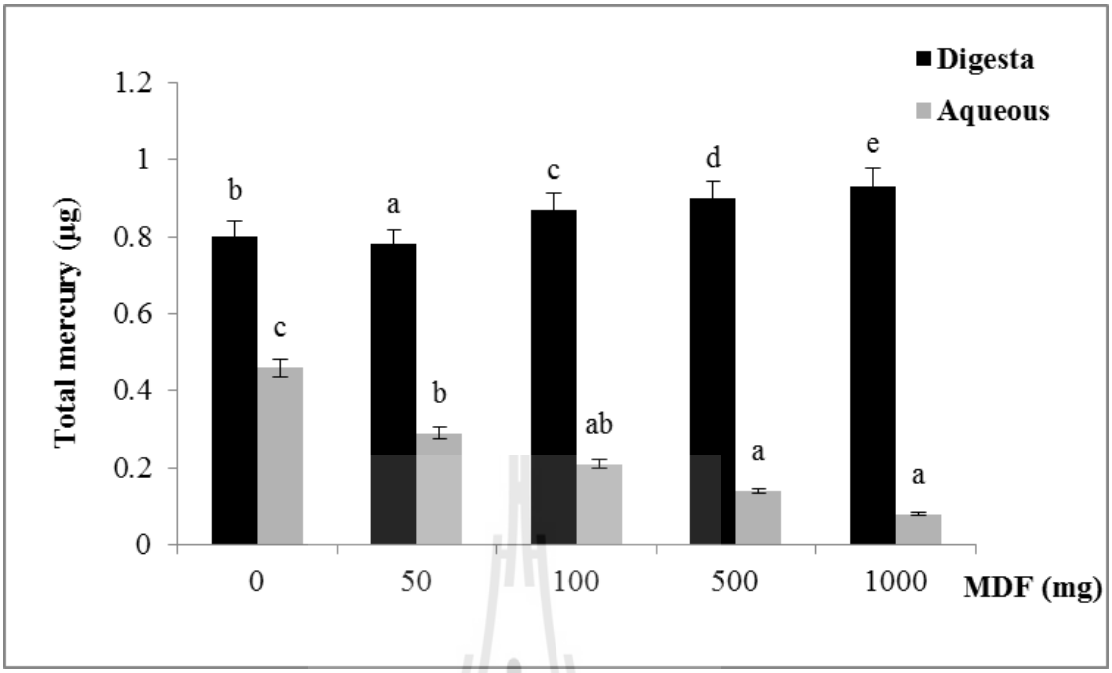

Figure 4.3 Total mercury in digesta and aqueous fraction of MDF from *in vitro* digestion of 1 g fish tissue in the presence of increasing amounts of CDF. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

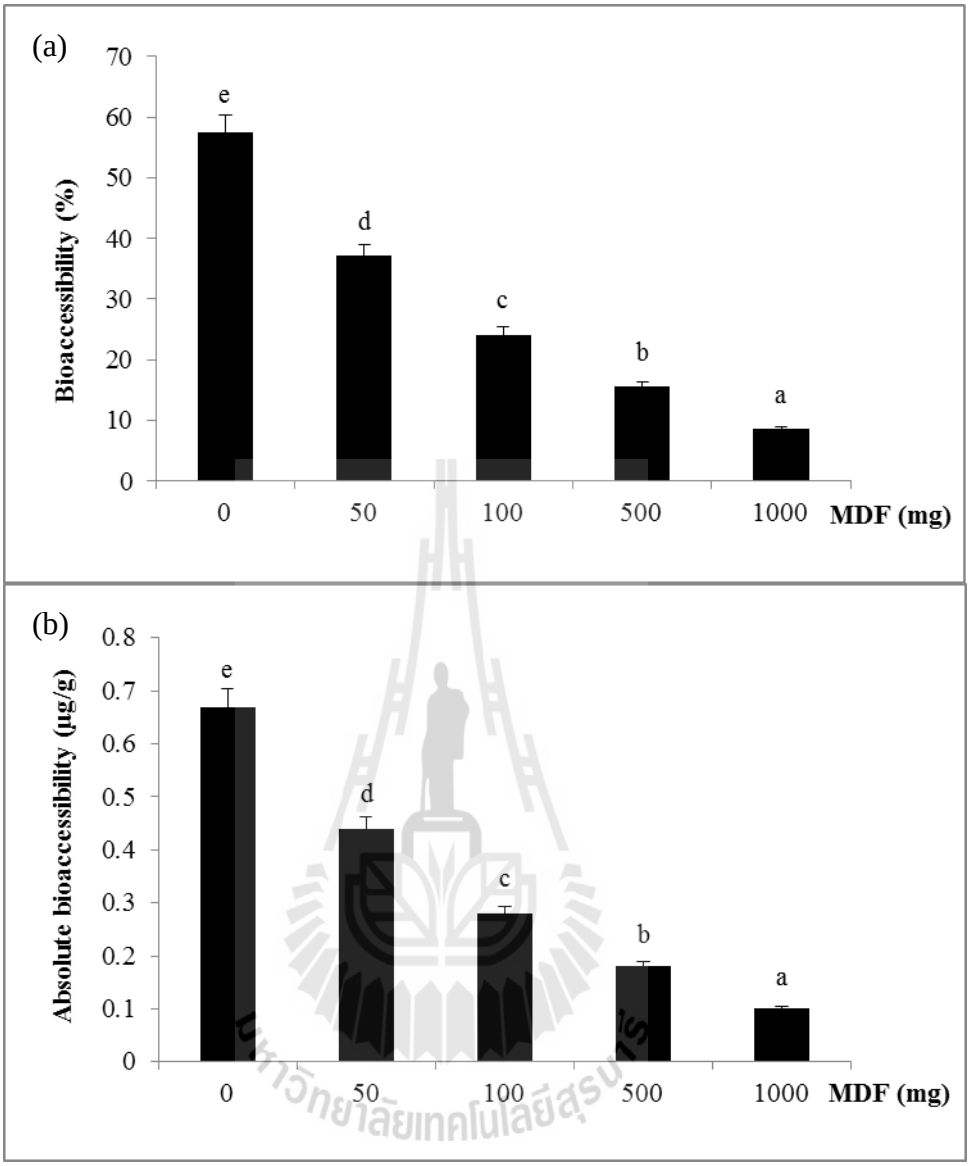

Figure 4.4 Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 1 g fish tissue in the presence of increasing amounts of MDF. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

4.4.2 Effect of amounts of fish on mercury bioaccessibility and absolute bioaccessibility

After the evaluation of the effects of different amounts of fiber on mercury bioaccessibility, the effects of the amounts of fish on mercury bioaccessibility were evaluated for different amounts of fish tissue (0, 1, 2, 3, and 4 g). The results show the percentage of total mercury present in digested fish tissue without additional fiber in the *in vitro* digestion model. The aim of this study was to evaluate the effect of the amounts of fish on Hg bioaccessibility and the results of this study will be applied as the control for a comparison with the results in the next step (with fiber). The results are shown in Table 4.2 and Figure 4.3.

The amount of mercury in the aqueous fraction of different amounts of fish tissue (0, 0.5, 1, 2, and 4 g by not used fiber) range from 0.24±0.01 to 0.81±0.01 µg/g and the bioaccessibility of Hg increased between 0.5-1 g of fish tissue range from 75.00±3.57% to 87.50±2.78% of total mercury in digesta. After 1 g of fish tissue, the bioaccessibility of Hg becomes lower and quite stable (range from 38.21±2.13 to 27.45±2.64) as shown in Table 4.2 and Figure 4.3.

These results are similar to those previously reported by Shim et al. (2009) that showed that the relative bioaccessibility of Hg is dependent on the fish tissue content, and that the bioaccessibility of Hg becomes lower as the amount of fish tissue increases. This would suggest that increasing fish tissue is not necessarily increasing bioaccessibility. On the contrary opposite, the amount of mercury released from fish tissue becomes less. Thus, it seems that that increasing fish tissue does not necessarily increase bioaccessibility. On the contrary, the Hg released from fish tissue is proportionally lower, suggesting a saturation of the digestion model. Because most of

the inorganic Hg and MeHg in seafood are bound to sulfhydryl groups of proteins, the proteins may not be completely hydrolyzed in the gastrointestinal tract. Thus, the Hg bound to them would not be soluble in the aqueous fraction (Calatayud et al., 2012). The concentration of Hg depends on the fish type analyzed, and there are reports that Hg is higher in swordfish than tuna and sardine. In addition, the concentration of Hg depends on the fish's age, size, sex, metabolism and feeding habits (Cabañero, Carvalho, Madrid, Batoreu, and Cámara, 2005). However, bioaccessibilities could be different depending on factors such as the composition of food matrix, pH, shaking time and enzyme conditions (Carbonell-Capella et al., 2014).

The enzyme used in the in vitro digestion such as pepsin, which cleaves C-terminal linkages of Phe, Leu and Glu, does not cleave at Val, Ala or Gly linkages. Pancreatin contains amylase, lipase, ribonuclease and trypsin. Therefore the amino acid composition of fish affects bioaccessibility and makes differences in bioaccessibility in the same seafood or the same *in vitro* digestion method (Calatayud et al., 2012).

After the evaluation of the effects of fish amounst (0, 1, 2, 3, and 4 g) on the bioaccessibility of mercury, then the effects of both fiber types were studied (500 mg). The aim of this study was to evaluate the potential of both fibers to inhibit Hg transfering to the aqueous fraction. The potential of fibers was compared with control in the previous step. The results are shown in Table 4.3 and Figures 4.4-4.5.

Table 4.2 Total mercury in each phase (µg/g wet weight), relative bioaccessibility and absolute bioaccessibility following *in vitro* digestion of different amounts of fish tissue (0-4 g) in control (no fiber). Data represent the absolute bioaccessibility.

Fish (g)	Total Mercury (µg/g)			Relative bioaccessibility[1] (%)	Absolute bioaccessibility[2] (µg/g)
	Starting Material	Digesta	Aqueous		
0	0	0[a]	0[a]	0[a]	0[a]
0.5	0.58	0.32±0.02[b]	0.24±0.01[b]	75.00±3.57[d]	0.43±0.03[b]
1	1.17	0.56±0.01[c]	0.49±0.01[c]	87.50±2.78[e]	1.02±0.06[d]
2	2.34	1.57±0.04[d]	0.60±0.02[d]	38.21±2.13[c]	0.89±0.03[c]
4	4.68	2.95±0.03[e]	0.81±0.01[e]	27.45±2.64[b]	1.28±0.05[e]

Note: [1]Relative bioaccessibility is defined as the % of mercury recovered in digesta.

[2]Absolute bioaccessibility is the amount of mercury recovered from the fish in digesta.

*Data represent mean +/- SEM from n = 3 independent *in vitro* digestion experiment.

*Presence of different letters indicate significant difference between treatments as determined by Tukey's post hoc test ($p < 0.05$).

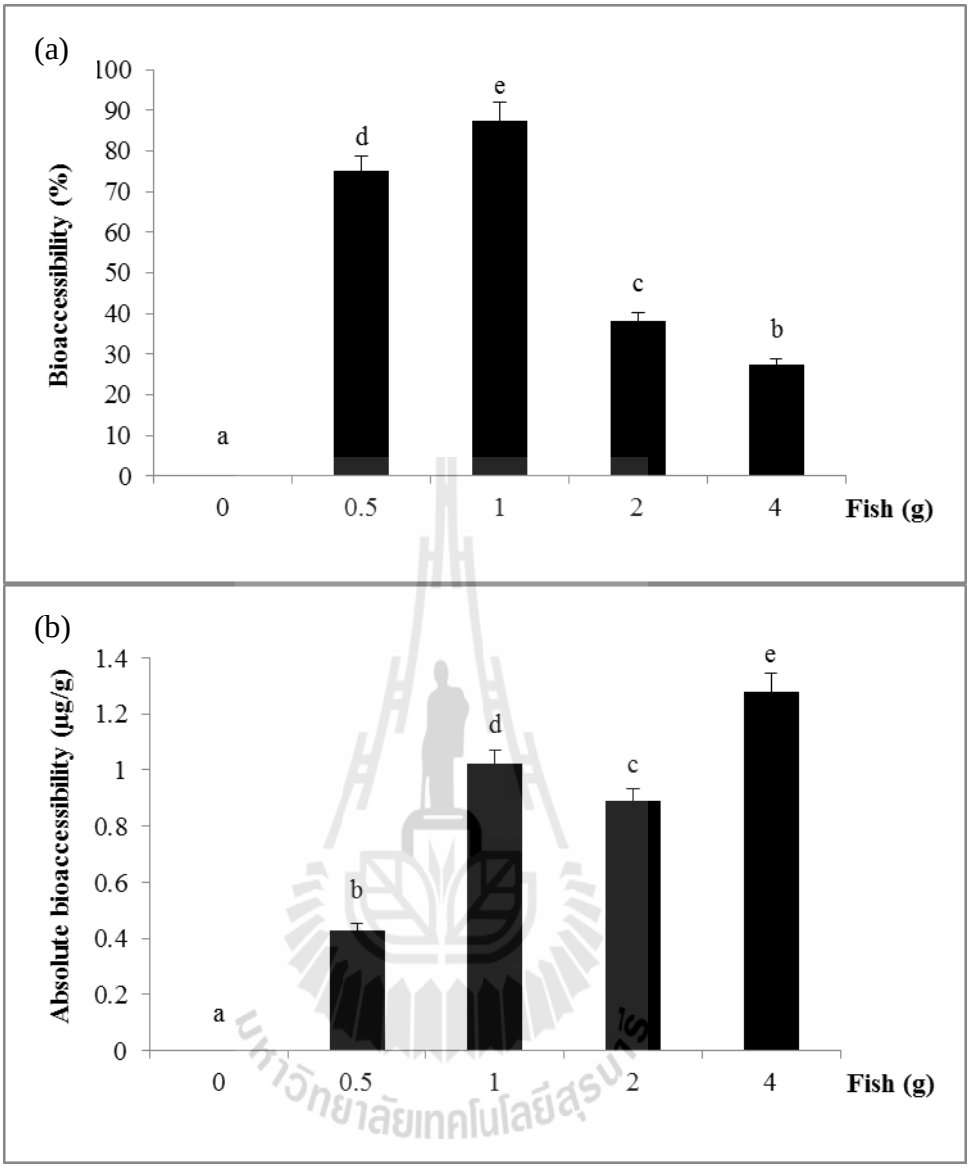

Figure 4.5 Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 0-4 g fish tissue by unused fiber (control). Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

Table 4.3 shows the effects of both dietary fibers (500 mg) on different amounts of fish tissue (0-4 g). Both dietary fibers significantly reduced Hg bioaccessibility when the amount of fish tissue was in the range of 0-4 g ($p < 0.05$) when compared with control (no dietary fiber added). When CDF contained 0.5 g of fish tissue, the mercury bioaccessibility decreased by approximately 48% when compared with control and increased to 62% for 1 g and decreased to 30% for 2 g and 23% for 4 g of fish tissue. For MDF with 0.5 g of fish tissue, the mercury bioaccessibility decreased by approximately 79% when compared with control and increased to 86% for 1 g and decreased to 71% in 2-4 g of fish tissue. The results show that the bioaccessibility of Hg becomes lower and quite consistent above 1 g of fish tissue. For control, the amount of mercury in the aqueous fraction from different amounts of fish tissue (0, 0.5, 1, 2, and 4 g) ranges from 0.24±0.01 to 0.81±0.01 µg/g and the bioaccessibility of Hg when the amount of fish tissue is increased from 0.5 to 1 g of fish tissue ranges from 75.00±3.57% to 87.50±2.78% of total mercury in the digesta. Above 1 g of fish tissue per digestive reaction, the bioaccessibility of Hg was observed to decrease e (range from 38.21±2.13 to 27.45±2.64). A comparison of both fibers suggests that MDF showed significantly more mercury inhibition than CDF ($p < 0.05$).

It is possible that these fibers may form insoluble complexes with Hg, which reduce Hg bioaccessibility as wheat bran and oat bran showed similar significant decreases in bioaccessibility of Hg from fish tissue following *in vitro* digestion (Idouraine et al., 1996; Ou et al., 1999). Therefore, dietary fibers may be more efficient to reduce Hg toxicity than synthetic chelating agents (e.g. DMPS) for long-term chronic methylmercury exposure in fish-eating populations by inhibiting the

transfer of Hg to aqueous fraction and thus reducing mercury bioavailability (Shim et al., 2009). However, CDF were moderately effective at reducing the bioaccessibility of Hg from fish tissue. MDF shows greater reductions in Hg following the *in vitro* digestion model because chemically modified cellulose with amidoxime groups by reacting acrylonitrile with the fiber through an etherification reaction in order to add cyano groups to the cellulose structure. These cyano groups were then amidoximated by reaction with hydroxylamine. This amidoximated sawdust had a high adsorption capacity, leading to heavy metal adsorbent materials (Saliba et al., 2005; Saliba et al., 2000). These data are similar to previous studies, in which amidoximated wood sawdust and wood flour were prepared using the etherification method. In comparison to the untreated material, amidoximated wood sawdust and wood flour increased heavy metal adsorption capacity from the aqueous solution. The factors which were affected by the quantity adsorbed are pH, initial metal ion concentration and process time (Saliba et al., 2005).

These results suggest that CDF and MDF decreased mercury bioavailability by inhibition of mercury transfer to the aqueous fraction. Adsorption or binding of MeHg is dependent on the ratio of fiber and fish tissue, composition and physiological function of the fiber and luminal factor. The most important component of dietary fiber is cellulose. So, the main mechanism of MeHg inhibition is the positive charge of Hg which is not fully neutralized by carboxyl anion. Thus, free valences would be available to bind external anions.

Figures 4.6 and 4.7 show the effect of both dietary fiber (500 mg) on different amounts of fish tissue (0-4 g) when compared with control (no dietary fiber added). When the Hg concentration increased, adsorption by dietary fiber increased and then

decreased after 1 g of fish tissue. This is due to the adsorption sites of dietary fiber and Hg concentration. These suggest that the quantity adsorbed corresponds to the ratio between Hg concentration (amount of fish) and dietary fiber content. The ratio complex with a 1:0.5 (Hg concentration:dietary fiber content) shows the highest reduction in Hg bioaccessibility. Therefore, it should be noted that the adsorption capacities of dietary fiber depend on the concentrations of Hg and of the dietary fiber content. CDF are readily converted into amidoximated material (MDF) that supports physical and functional properties suitable adsorbents for heavy metal ions presented in aqueous solution. Due to the low cost of cassava pulp together with their ease of disposal, their use as adsorbents for the removal of Hg from fish in gastrointestinal tract would seem to be very useful.

By virtue of high throughput and lower cost, this *in vitro* technique has been used to assess the bioaccessibility of nutrients, phytochemicals and contaminants. For instance, bioaccessibility has been used as a surrogate to assess the bioavailability for cadmium from vegetables, aflatoxin B1 from peanuts and ochratoxin A from buckwheat (Versantvoort et al., 2005). Shim et al. (2009) previously reported that green tea extract (31-2000 mg), black tea extract (31-2000 mg), and soy protein (50-100 mg) were able to reduce mercury bioaccessibility from fish tissues by 82-92%, 88-91%, and 44-87%, respectively. However, inclusion of grapefruit juice (0.5-10 ml) did not reduce mercury in the aqueous phase. Fiber sources, including wheat bran (50-1000 mg), oat bran and psyllium havae also been reported to reduce the bioaccessibility of mercury from the same fish tissue at amounts greater than 500 mg of fiber. This study suggests that co-consumption of foods containing dietary fibers could bind with mercury. Unfortunately, only a few studies have investigated

the reduction in bioaccessibility and bioavailability of heavy metals with dietary fiber; information about the specific effects of dietary fiber on human intestinal absorption of methylmercury is lacking (Ou et al., 1999).

In general the main effects of insoluble fiber to reduce Hg bioaccesiibility in the GI tract, is to bind/absorb with the Hg ion before they can be absorbed through the gut wall. Moreover, reduction in the time for absorption and digesta viscosity are an important mechanism to reduce Hg ion. Insoluble fiber appears to speed pass through the stomach and intestine that can help reduce the intestinal transit time. For digesta viscosity, the principal physiological effect of dietary fiber in the small intestine is to reduce the rate of release of nutrients. This mechanism increases the viscosity by an inhibition mixing process that promotes transport of enzymes to their substrates (Eastwood and Brydon, 1985).

Ou et al. (1999) reported that water-insoluble dietary fiber from wheat bran and the carboxymethylated product can effectively bind Hg, Cd, and Pb to prevent the body from being affected by their toxicity. The pH value significantly affected the binding capacity for heavy metals.

Calatayud et al. (2012) studied the Hg concentrations and bioaccessibility of fish in Spain and reported that the concentration of Hg varied between 10.2-1004 ng/g in wet weight with bioaccessibility in the range of 35-100%.

Nawirska (2004) reported that the dietary fibers of pomace, which is a product from fruit pressing, have the potential for binding heavy metal ions. The quantity of metal ions bound varies from one fiber component to another. As inferred from the results of the study, pectin was characterized by a particularly high capacity for metal ion binding. The hemicellulose fraction was ranked second with respect to

metal ion binding capacity. Binding of heavy metals to lignin was found to be generally poor.

Hu et al. (2010) reported that rice bran hemicellulose A (RBHA), hemicellulose B (RBHB) and hemicelluloses C (RBHC) have the potential for binding heavy metal ions. The quantity of metal ions bound varies from one rice bran fiber to another. As inferred from the results of the study, RBHB was characterized by the highest capacity for metal ion (Pb, Cu, and Cd) binding, followed by RBHC and RBHA. Binding of heavy metals to insoluble dietary fiber (RBDF) and cellulose from rice bran was found to be poor. Lignin from rice bran was the least active fraction for binding heavy metal ions.

Table 4.3 Fish comparisons (0-4 g) with 500 mg of fiber. Total mercury in each phase (µg/g wet weight) and relative bioaccessibility following *in vitro* digestion. Data represent the absolute bioaccessibility.

Type	Fish (g)	Total mercury (µg/g)			Relative bioaccessibility (%)	Absolute bioaccessibility (µg/g)
		Starting material	Digesta	Aqueous		
CDF	0	0	$0^{a,1}$	$0^{a,1}$	$0^{a,1}$	$0^{a,1}$
	0.5	0.58	$0.33\pm0.04^{b,2}$	$0.13\pm0.01^{b,2}$	$39.39\pm0.10^{e,8}$	$0.22\pm0.01^{b,8}$
	1	1.17	$0.86\pm0.05^{c,4}$	$0.28\pm0.01^{c,34}$	$32.50\pm0.08^{d,7}$	$0.38\pm0.01^{c,7}$
	2	2.34	$1.84\pm0.04^{d,6}$	$0.49\pm0.01^{d,5}$	$26.60\pm0.07^{c,6}$	$0.62\pm0.03^{d,6}$
	4	4.68	$3.90\pm0.11^{e,8}$	$0.82\pm0.04^{e,6}$	$21.02\pm0.08^{b,5}$	$0.98\pm0.01^{e,5}$
MDF	0	0	$0^{a,1}$	$0^{a,1}$	$0^{a,1}$	$0^{a,1}$
	0.5	0.58	$0.39\pm0.03^{b,3}$	$0.06\pm0.01^{b,12}$	$15.38\pm0.03^{e,4}$	$0.09\pm0.01^{b,4}$
	1	1.17	$0.93\pm0.04^{c,5}$	$0.13\pm0.01^{c,2}$	$13.98\pm0.03^{d,34}$	$0.16\pm0.01^{c,34}$
	2	2.34	$2.10\pm0.03^{d,7}$	$0.23\pm0.01^{d,3}$	$10.95\pm0.02^{c,23}$	$0.25\pm0.02^{d,23}$
	4	4.68	$4.25\pm0.07^{e,9}$	$0.29\pm0.05^{e,4}$	$6.82\pm0.02^{b,2}$	$0.32\pm0.01^{e,2}$

Note: [1]Relative bioaccessibility is defined as the % of mercury recovered in digesta.

[2]Absolute bioaccessibility is amount of mercury from the fish recovered in digesta.

*Data represent mean +/- SEM from n = 3 independent *in vitro* digestion experiment.

*Presence of different letters indicate significant differenced between treatments as determined by Tukey's post hoc test ($p < 0.05$) a, b, c... is the fiber dose effect and 1, 2, 3...is the fiber type effect.

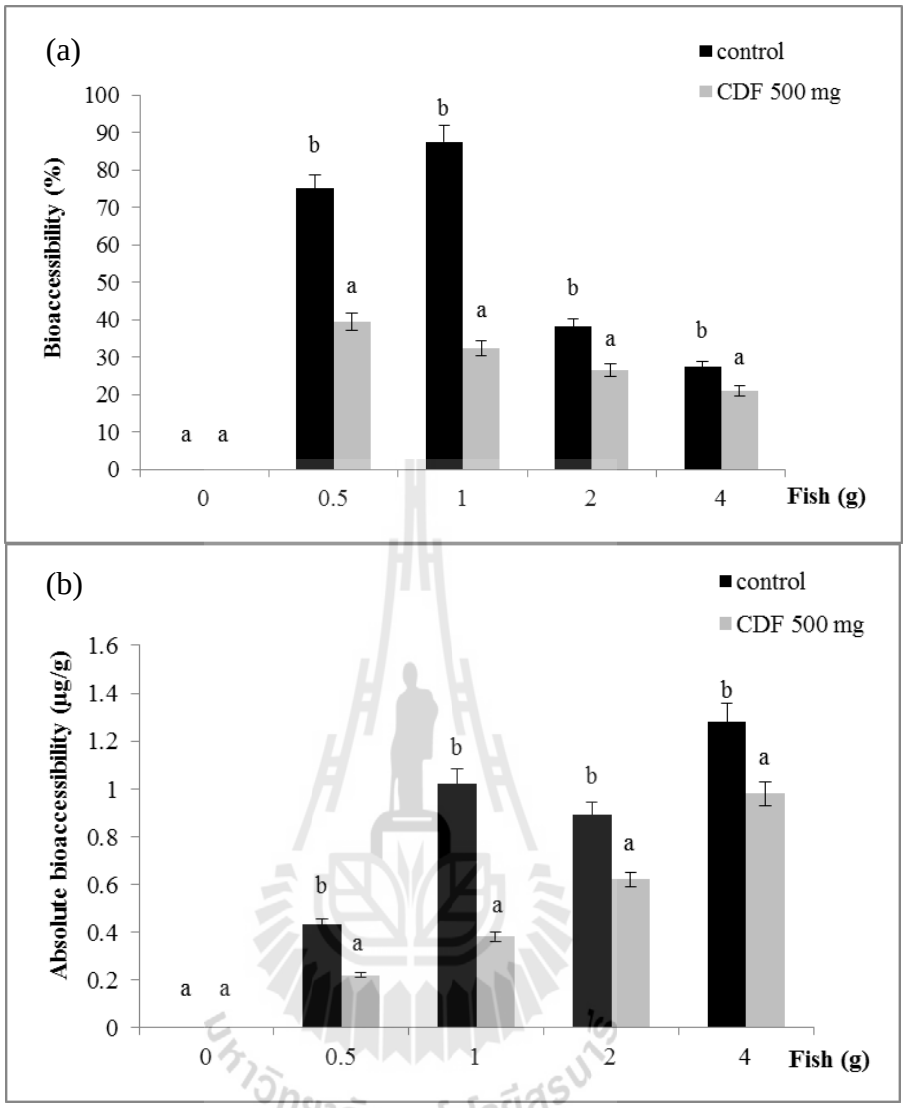

Figure 4.6 Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 0-4 g fish tissue with 500 mg of CDF in the presence of increasing amounts of dietary fiber. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. The presence of different letters indicate significant differences within treatments as determined by Tukey's post hoc test ($p < 0.05$).

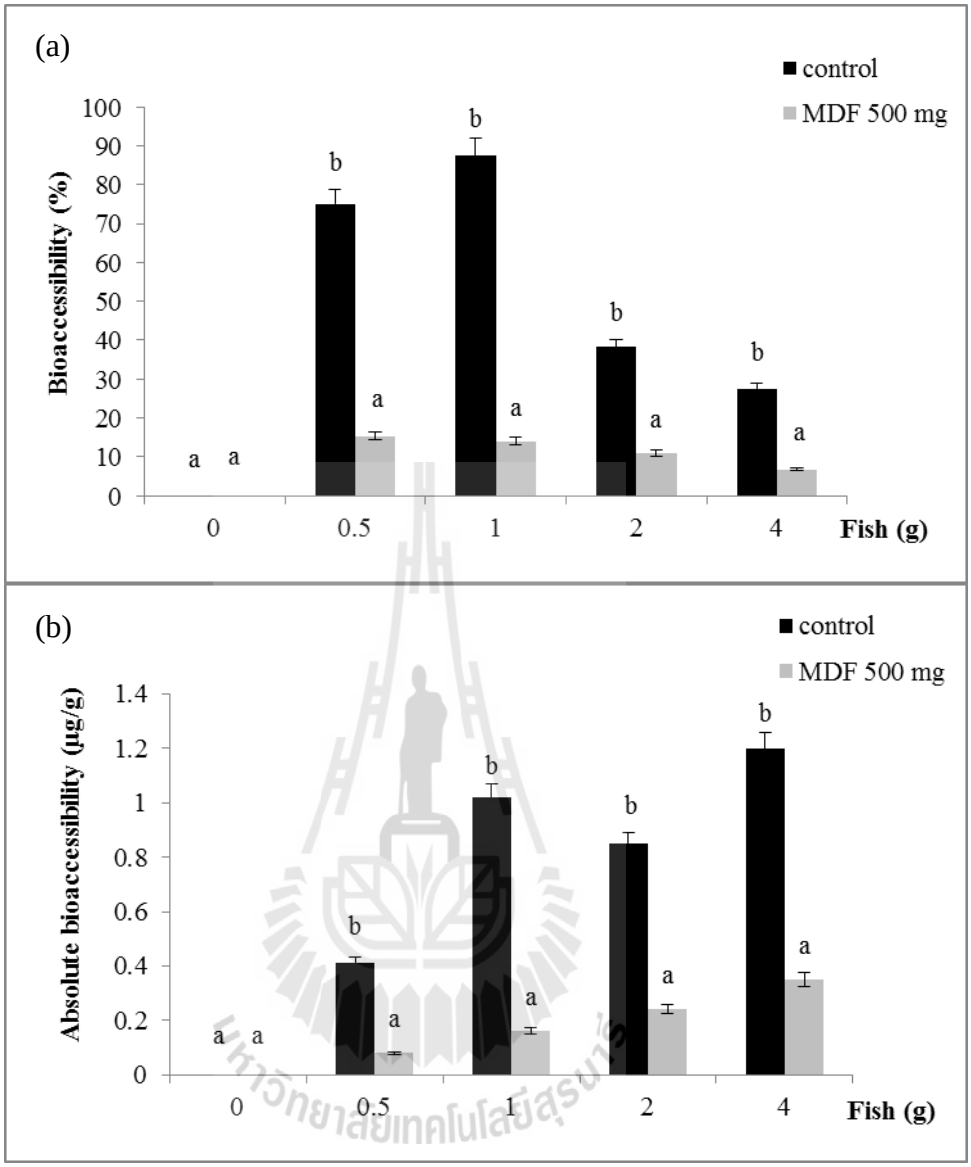

Figure 4.7 Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 0-4 g fish tissue with 500 mg of MDF in the presence of increasing amounts of dietary fiber. Data represent mean±SEM from n = 3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. The presence of different letters indicate significant differences within treatments as determined by Tukey's post hoc test ($p < 0.05$).

4.5 Conclusion

In conclusion, this study suggests that dietary fiber might act as a chelating agent for reducing mercury bioavailability. It also shows the potential of dietary fiber prepared from cassava pulp for decreasing Hg bioavailability. However, confirmations with *in vivo* systems should also be carried out.

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CHAPTER V

MERCURY UPTAKE AND ACCUMULATION BY TC7 CLONE OF Caco-2 CELL

5.1 Abstract

The effects of CDF and MDF on bioaccessibility of Hg from fish tissue by an *in vitro* digestion with a TC7 clone of Caco-2 cells were investigated. Hg in aqueous fraction following *in vitro* digestion was incubated with TC7 clone of Caco-2 cell to investigate for intestinal cell accumulation and bioavailability evaluation. The results showed that the Hg transferred to the intracellular compartment was in the range of 5.97 to 9.07% for the control, 4.02-6.54% for CDF and 5.09 to 7.13% in media with 500 mg of MDF. In conclusion, it was suggested that fiber prepared from cassava pulp could decrease mercury bioavailability by inhibiting mercury transfer to the aqueous fraction in the digestion model test.

Keyword: bioaccessibility, *in vitro* method, *in vitro* digestion model

5.2 Introduction

In vitro screening methods have been developed and improved for the estimation of nutrient bioaccessibility and bioavailability from foods in the gastrointestinal tract. This method is useful to obtain information of nutrient absorption in the gut (Carbonell-Capella et al., 2014). Nevertheless, *in vitro* methods

can not be used to measure bioavailability completely. This is because there are many factors that affect nutrient absorption such as nutrient status, age, genotype, physiological state (e.g., pregnancy, lactation, and obesity), chronic and acute infectious disease states, secretion of hydrochloric acid, gastric acid, and/or intrinsic factors (Etcheverry et al., 2011). *In vitro* bioaccessibility or bioavailability methods are useful to obtain information about nutrient absorption, including the interaction between nutrients and/or food components. Factors that can possibly influence nutrient absorption are, for example, pH and enzymes, food preparation, and the nature of the food matrix. These factors are important for nutrient absorbtion (Sandberg, 2005). The advantage of *in vitro* methods are that they are less expensive, faster, and it is easier to control the experimental variables than in *in vivo* methods, including human or animal studies. However, although *in vitro* studies cannot be substituted for *in vivo* studies, they can be used to screen and evaluate the nutrient absorption in the gut wall (Johnson, 1988).

Bioaccessibility, which is the amount of nutrient that is released from the food matrix before absorption in the gut, is only dependent on digestion and release from the food matrix (Heaney, 2001). Bioavailability, which is the amount of nutrient that is absorbed and available for physiological functions, is dependent on digestion, release from the food matrix, absorbtion by intestinal cells, and transport across the intestinal epithelium in the body (Versantvoort and Rompelberg, 2004; Heaney, 2001).

Oral bioaccessibility and bioavailability, also known as *in vivo* and *in vitro* gastrointestinal extractions, are important methods to assess chemical risks to humans (Dean and Ma, 2007). Human health-risk assessment depends on the fraction of

substance from the gastrointestinal tract that reaches the systemic circulation and is available to promote its action in the exposed organism (Schoof, 2003). The first step in the assessment of bioavailability is the study of bioaccessibility, that is the maximum amount of a substance that is released from the food matrix into the aqueous fraction and its available for absorption by intestinal cells and enters into the blood stream (Versantvoort and Rompelberg, 2004). Bioavailability and bioaccessibility are affected by the digestion process, bioactive compounds and their stability (Carbonell-Capella et al., 2014). The study of element bioavailability in food can be estimated by *in vivo* (humans or animals) or *in vitro* methods. However, human experiment provides better results for estimating the bioavailability than *in vivo* animal and *in vitro* methods (Zia et al., 2011). Among various animals used in *in vivo* models such as primates, swine, dogs, rabbits, and rodents, monkeys are the first choice for *in vivo* bioavailability studies due to their similarities to humans. However, there have been few studies using monkeys due to the costs and legal implications associated with their use (Vilahur et al., 2011).

Testing animals is expensive, difficult to perform, and ethically controversial, and it provides limited data in each experiment (Preedy, 2015). *In vitro* methods involve conditions during digestion such as temperature, agitation, pH, and enzyme and chemical composition that are similar to the human body. This method can be used for bioavailability measurement (de la Guardia and Garrigues, 2015). The factors affecting bioavailability are the maximum concentration of interested compounds that are released to the aqueous fraction (bioaccessible fraction) and the fraction of interested compounds that are available for absorption in the gut using Caco-2 cells (intestinal epithelial model) (Moreda-Piñeiro et al., 2011). These methods are useful

to provide bioavailability studies with information because of their simplicity, rapidity, ease of control, low cost, high precision and good reproducibility. Some *in vitro* procedures include a saliva phase (simulate digestion with saliva in the mouth) while some other procedures do not include it. Because the process in the mouth will last from a few seconds to minutes and the pH of saliva is close to neutral, some products such as protein or fat can be digested in 2 steps (gastric and intestinal phase) without the saliva step. (Intawongse and Dean, 2006). It is therefore usually assumed that saliva has few effects on the *in vitro* digestion process (Dufailly et al., 2008).

Both methods (*in vivo* and *in vitro*) can be used to study Hg absorption. With *in vivo* studies, it is difficult to determine the relative importance of the different factors, which is not the case for *in vitro* studies (Ismail, 1999). The advantage of the Caco-2 cell line is that one can: 1) assess nutrient absorption easily and rapidly, 2) explain the pathways of nutrient transport, 3) determined the diffussion of the nutrients and 4) assess the toxic effects (Meunier et al., 1995).

Caco-2 cells have been widely used as an *in vitro* intestinal epithelial model for predicting drug absorption in humans (Yuan et al., 2009). This cell model also has been used in pharmaco-toxicology and food research, particularly in the study of absorption, bioavailability and cell uptake in nutrients, minerals and heavy metals (Mazzoleni et al., 2009). The Caco-2 cell model has been used to assess contaminant bioavailability in food for evaluation of human health risks since food is a major route of exposure. The total amount of an ingested contaminant is not absorbed and transferred to the body (Versantvoort and Rompelberg, 2004).

Many researchers have isolated the Caco-2 parental cell line. The TC7 clone is one of these clones, which was isolated from a late passage of the parental Caco-2 line. These clones have shown a more homogeneous population and developed intercellular junctions. Therefore, it is suitability for the evaluation of intestinal absorption and transport (Turco et al., 2011). TC7 clones are used for the screening of compounds of interest because they shows morphological characteristics of brush borders, microvilli and tight junctions similar to the Caco-2 monolayer (Balimane and Chong, 2005). However, the TC7 clones can increase transport time compared with the Caco-2 cell line (Le Ferrec et al., 2001). The accumulation of Caco-2 cells and TC7 clones are similar with the cells accumulating 20-40% and 5-10% of micellarized carotenoid and chlorophyll derivatives, respectively (Ferruzzi et al., 2001). This is a good alternative to using TC7 clones to study intestinal absorption in humans. In addition, TC7 clones have an advantage over using Caco-2 cells because they can express high levels of CYP3A4 enzymes (Awortwe et al., 2014).

Bioaccessibility can be evaluated by the *in vitro* digestion method, generally simulating gastrointestinal digestion and uptake by Caco-2 cells (Courraud et al., 2013). Recently, studies of bioavailability in foods usually use the coupled methods with the Caco-2 cell model and the *in vitro* gastrointestinal digestion model. It has been suggested that *in vitro* digestion following Caco-2 cell nutrient uptake are reliable predictors for the estimation of nutrient absorption in humans (Hur et al., 2011).

By virtue of high throughput and lower costs, this *in vitro* technique has been used to assess the bioaccessibility of nutrients, phytochemicals and contaminants. For instance, bioaccessibility has been used as a surrogate to assess the bioavailability of

cadmium from vegetables, aflatoxin B1 from peanuts and ochratoxin A from buckwheat (Versantvoort and Rompelberg, 2004). Shim and others (2009) have previously reported that green tea extract (31-2000 mg), black tea extract (31-2000 mg), and soy protein (50-100 mg) were able to reduce mercury bioaccessibility from fish tissues by 82-92%, 88-91%, and 44-87%, respectively. However, inclusion of grapefruit juice (0.5-10 ml) did not reduce mercury in the aqueous phase. Fiber sources, including wheat bran (50-1000 mg) oat bran and psyllium, have also been reported to reduce the bioaccessibility of mercury from the same fish tissue at amounts greater than 500 mg of fiber. This study also suggests co-consumption of foods containing dietary fibers with mercury could reduce Hg bioaccessibility. To expand on these efforts, the objectives of this study were to determine the effects of dietary fiber prepared from cassava pulp on Hg bioaccessibility using a coupled *in vitro* digestion/Caco-2 human intestinal cell model. Our main hypotheses are the following: (1) dietary fibers (insoluble fiber) will bind Hg in the gut, making it insoluble and unavailable for absorption by intestinal mucosa, and (2) dietary-containing foods will bind Hg in a dose-dependent manner.

5.3 Materials and methods

5.3.1 Caco-2 human intestinal cell culture

The procedure for cellular uptake described by Ferruzzi and others (2002) was applied with slight modifications. Accumulation of mercury by human intestinal cells was investigated using the TC7 clone of the Caco-2 human intestinal cell culture model between passages 85 and 90. Cells were seeded in 6-well plastic dishes (Costar Coming, New York, NY) and cultured in Dulbecco's Modified Eagle's Medium

(DMEM, BioWhittaker, Lonza) with 4.5 g/L glucose and L-glutamine. The medium was supplemented with 1% v/v autoclaved HEPES (10 mM, Sigma-Aldrich, St. Louis, MO), 1% v/v non-essential amino acids (0.1 mM, BioWhittaker, Lonza), 1% v/v P/S (penicillin/streptomycin, 100 U/L/100 U/L, BioWhittaker, Lonza), 0.1% v/v gentamicin (50 µg/L, Sigma-Aldrich) and 10% v/v fetal bovine serum (FBS, Atlanta Biologicals), and cells were incubated in a humidified atmosphere of air/CO_2 (95%/5%) at 37°C. Uptake experiments were performed as a monolayer 11-14 days post-confluent. Prior to the initiation of the experiments, a monolayer was washed twice with 1 ml of phosphate-buffered saline. Two ml of test media were prepared by diluting the filtered aqueous fraction from *in vitro* digestion and basal DMEM at a ratio of 1:3 were then added to the apical surface of the cells. Cells were incubated at 37°C for 6 h. Following incubation, the medium was removed by aspiration and the cells were washed twice with 1 ml of PBS. Cells were collected by scraping into 0.75 ml of ice-cold PBS and were stored at -80°C until analysis. Each experimental treatment was performed in triplicate.

Data analysis

$$\text{Uptake efficiency (\%)} = \frac{\text{accumulation of Hg in cell (ng/well)}}{\text{Hg content in test media (ng/well)}} \times 100$$

5.3.2 Assessment of toxicity and cellular viability

Cellular viability was determined using methylthiazoletetrazolium-(MTT, [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Sigma Chemical Co., St. Louis MO). The method was initiated by adding MTT solution (5 µl MTT solution/300 µl DMEM with no phenol red) to each well in a 24-well plate (the

monolayer was added to the test media and incubated at 37ºC for 6 h and was then washed twice with PBS), followed by incubation at a temperature of 37ºC for 2 h. The purple product was dissolved with 300 µl of dimethylsulfoxide (DMSO). The purple solution (50 µl) was loaded in a 96-well plate, and 50 µl of DMSO was added. The absorbance was read at 570-630 nm with a 96-well plate reader (Bio-Tek Instruments. Inc. Tustin, CA). Each experiment was performed in duplicate. Cellular viabilities in all treatments were generally between 90 and 95%.

5.3.3 Protein assay

Cells were homogenized by sonic disruption, and cell protein was measured using a BCA (Bicinchonic Acid) protein assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Rockford, IL).

5.3.4 Determination of mercury

Cells were centrifuged at 200 rpm for 10 min at room temperature (20-25°C) (Eppendorf Centrifuge 5415 D, Hamburg, Germany), and the supernatant was discarded. An aliquot of cells was analyzed for total Hg using a Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer (TDA/AAS) Mercury Analyzer (DMA-80, Milestone Inc., Pittsburgh, PA) as described by Shim and others (2009). Total Hg in the aqueous fraction and pellets was also determined. Total Hg data obtained from each well of cells were normalized to the corresponding concentration of cellular proteins.

5.3.5 Statistical analysis

The results are presented as representative data from at least two sets of experiments. Data are expressed as the mean±standard error. For cellular uptake studies, a sample size of n = 3 was used. Statistical analysis for each parameter

assessed was performed using analysis of variance (ANOVA) followed by Tukey's post hoc test (SAS, Gary, NC). Differences between means were considered statistically significant at $p < 0.05$.

5.4 Results and discussion

5.4.1 Mercury (Hg) uptake by Caco-2 cells

Determination of bioaccessibility is the first step towards measuring bioavailability; bioaccessibility is the amount of ingested nutrients of aqueous fraction materials that is available for absorption into the intestinal mucosa. This study used a Caco-2 cell line as a model of the intestinal epithelium to evaluate intestinal cell accumulation and to support a reliable estimation of bioavailability (Etcheverry et al., 2011; Carbonell-Capella et al., 2014).

The objective of this study is a comparison the capability of Hg accumulation between dietary fiber (CDF and MDF). The intestinal of mercury uptake was conducted by exposing TC7 clone of the Caco-2 cell with media containing aqueous fraction from *in vitro* digestion (unused fiber). The samples from a previous study were diluted 1:3 with basal DMEM (test mode) and incubated at a temperature of 37°C for 6 h with different amounts of fish tissue (0-4 g). Cells were harvested after incubation at the times indicated and the Hg content was determined. This system was used to evaluate the level of cellular uptake and to make an approximation to the *in vivo* situation (DelRaso et al., 2003). The total amount of mercury in the test media ranged from 5.78±0.3 to 21.84±0.3 ng for no fiber added (control) and appeared to increase in proportion to the increase in the amount of fish tissue. After 6 h, the cells contained 0.44±0.1 to 1.42±1.6 ng mercury/mg cellular protein. The uptake efficiency

ranged from 5.97±0.54% to 9.07±0.57 and appeared to be related to the increase in the amount of fish tissue (Table 5.1). The difference in uptake efficiency depends on the mercury concentration in the test media (Calatayud et al., 2012). With high concentrations (21.84 ng) of Hg, the efficiency of the uptake is low (5.97%), whereas with low concentrations (5.78 ng) of Hg, the efficiency of the uptake increases (9.07%). This result implies that the bioaccesssible but not necessarily bioavailability (Wang et al., 2012) and the concentration of mercury (Hg) in the gut lumen uptake may be linear (Cardinali et al., 2011). Methylmercury (MeHg) is quickly and readily absorbed in the gastrointestinal tract and can be absorbed in larger amounts than for inorganic forms (de la Guardia and Garrigues, 2015). Previous studies have shown that approximately 95% of MeHg in fish ingested by humans or about 95% of methylmercuric nitrate given orally to volunteers is absorbed from the gastrointestinal (GI) tract. Most of the MeHg is absorbed by ingestion through the GI tract. In addition, exposure to MeHg by inhalation is believed to be high (Aberg et al., 1969).

When ingested fish contain MeHg, MeHg is absorbed into the bloodstream via the gut and enters the red blood cells. Most of the MeHg (90%) in the blood is bound with hemoglobin, which binds to cysteine residue number 104 of the α chain and the numbers 93 and 112 of the β chain in hemoglobin. Numbers 104 and 112 appear in the contact junction of the hemoglobin molecule. Number 93 is on the surface of the hemoglobin molecule, therefore, it can bind to MeHg easily (Goyer et al., 2000). The total amount of Hg in humans is in inorganic form because MeHg (organic mercury) is slowly transformed to mercuric ion. The percentage of inorganic Hg in whole blood, plasma, breast milk, liver, and urine was 7%, 22%, 39%, 16-40%, and 73%, respectively (IPCS 1990).

Most of the Hg is accumulated in the scalp hair (about 20%) and in whole blood (about 5%) (Schoeny, 1996). The transport of MeHg in the human body is not due to lipid solubility, because MeHg ion is hydrated in aqueous solutions. MeHg is transported across the intestinal cell monolayer in an absorptive direction through passive transcellular diffusion. Many studies have reported the mechanism of MeHg transportation in other organs. For example, MeHg is bound to thiols, such as glutathione (GSH), or to different forms of cysteine (cysteine (Cys), homocysteine (Hcys), and N-acetylcysteine (NAC)) (Vázquez et al., 2014). The information on MeHg transportation to target organs is still limited. The studies of intestinal absorption is of great interest because the intestinal mucosa are the first barrier to the distribution of MeHg. Therefore, this process may affect Hg toxicity (Bridges and Zalups, 2010). The only studies of this process reported that Na^+ -independent neutral amino acid transporters (LATs) participate in the transport of CH_3Hg-Cys in intestinal cells but not in the transport of CH_3HgCl (Mori et al., 2012). These transporters are located in the basolateral membrane of the enterocytes, but the information is not clear. However, the basolateral membrane contains other transporters such as organic anion transporter (OAT) or LATs that could participate with CH_3Hg (Zalups and Ahmad, 2005).

The transportation of Hg across the blood-brain barrier, associated to form the coordinated complexes in MeHg-L-cysteine form between amino acids containing SH groups are polar and CH_3Hg species are nonpolar. Therefore, the cysteine contained C-Hg-S to form with Hg. These actions may affect MeHg transport (Goyer et al., 2000). Ingestion of MeHg is quickly absorbed in the GI tract and this form was absorbed better than the inorganic forms (Tsutomu et al., 1990). Elemental mercury

and MeHg in the brain are slowly transformed to mercuric ions that readily cross the placenta barrier, which cannot cross to the adult blood brain barrier. Therefore, children are more sensitive to toxicity of mercury than adults due to differences in the stages of their brain development (Rogers et al., 2004). MeHg is very stable in the human body compared to the other forms. Although it changes to mercuric ion in tissue, intestinal flora, and the fetal liver. These sites of action are known, but the enzymes in mammalian tissues that support the transformation have not yet been identified. MeHg can be transformed to ionic mercury at a rate of about 1%/day (Goyer et al., 2000).

The MeHg can be excreted from the body in bile and feces, which is secreted into bile, and reabsorbed for some part and returned to the liver. The Hg was excreted in the mercuric form after transformation (~90% in feces as mercuric Hg). These mechanisms occur in adults, but not in nursing infants due to incomplete development. The half-life elimination of Hg lasts approximately 45-90 days. However, it might be about 5 half-lives (~1year) for those who are exposed regularly to MeHg (Goyer et al., 2000).

Although many studies have reported on the absorption rate of MeHg, many mechanisms related to the absorption process remain unclear, for example, the factors that affect absorption and the mechanisms that transfer MeHg across the basolateral membrane of the gill epithelium, including a carrier mediated pathway. Therefore, it seems MeHg can cross the gill epithelium through specific pathways (Vázquez et al., 2014).

The accumulation of mercury intestinal uptake by using the TC7 clone of the Caco-2 cell with media containing aqueous fraction from *in vitro* digestion (with fiber

500 mg). The samples from previous studies were diluted 1:3 with basal DMEM (test media) and incubated at temperature 37°C for 6 h with different amounts of fish tissue (0-4 g) and different types of dietary fiber (CDF and MDF 500 mg).

The total mercury in the test media ranges from 3.17±0.2 to 18.41±0.3 and 1.12±0.2 to 7.01±0.3 ng for CDF and MDF respectively. The amount of Hg transferred to the cell ranges from 6.54±0.39 to 4.65±0.40 and 5.09±1.18 to 7.13±2.06 % of the mercury in the test media for CDF and MDF respectively. The cellular mercury uptake in MDF is lower than that for CDF, thus MDF significantly reduces mercury uptake by Caco-2 cell with 500 mg of fiber ($p < 0.05$). The results show low cellular accumulation, so it can be assumed that the cell monolayer simply acts as a barrier (Calatayud et al., 2012; Vázquez et al., 2014). However, this concept can lead to misinterprations when working with compounds like MeHg, which presents a low percentage in cellular content (~5-9%) as can be seen in Table 5.1.

Table 5.1 Accumulation of mercury by TC7 clone of Caco-2 human intestinal cells incubated for 6 h at 37ºC.

Fish (g)	No fiber			CDF			MDF		
	Test media (ng)	ng mercury/ mg protein	Uptake Efficiency (%)	Test media (ng)	ng mercury/ mg protein	Uptake Efficiency (%)	Test media (ng)	ng mercury/ mg protein	Uptake Efficiency (%)
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0
0.5	$5.78\pm0.3^{b,3}$	$0.44\pm0.01^{b,3}$	9.07 ± 0.57^{c}	$3.17\pm0.02^{b,2}$	$0.26\pm0.01^{b,2}$	6.54 ± 0.03^{c}	$1.12\pm0.1^{b,1}$	$0.06\pm0.1^{b,1}$	5.09 ± 0.18^{ns}
1	$11.44\pm0.9^{c,3}$	$0.85\pm0.01^{bc,2}$	8.66 ± 0.43^{c}	$5.19\pm0.01^{c,2}$	$0.38\pm0.01^{bc,2}$	4.96 ± 0.02^{bc}	$2.96\pm0.1^{c,1}$	$0.18\pm0.1^{bc,1}$	6.58 ± 0.77^{ns}
2	$14.72\pm1.6^{d,3}$	$0.95\pm1.02^{c,2}$	6.85 ± 0.17^{bc}	$12.55\pm0.06^{d,2}$	$0.87\pm0.03^{c,2}$	4.02 ± 0.03^{b}	$5.32\pm0.1^{d,1}$	$0.37\pm0.1^{c,1}$	7.13 ± 0.06^{ns}
4	$21.84\pm0.3^{e,3}$	$1.42\pm1.06^{d,2}$	5.97 ± 0.54^{b}	$18.41\pm0.03^{e,2}$	$0.98\pm0.01^{d,2}$	4.65 ± 0.03^{bc}	$7.01\pm0.1^{e,1}$	$0.48\pm0.1^{d,1}$	6.68 ± 0.30^{ns}

Note: *Data represent mean +/- SEM from n = 3 independent Caco-2 uptake experiment.

*Presence of different letters indicate significant difference between treatments as determined by Tukey's post hoc test ($p < 0.05$).

a, b, c…. is fiber dose effect and 1, 2, 3….is fiber type effect and ns = non-significant ($p > 0.05$).

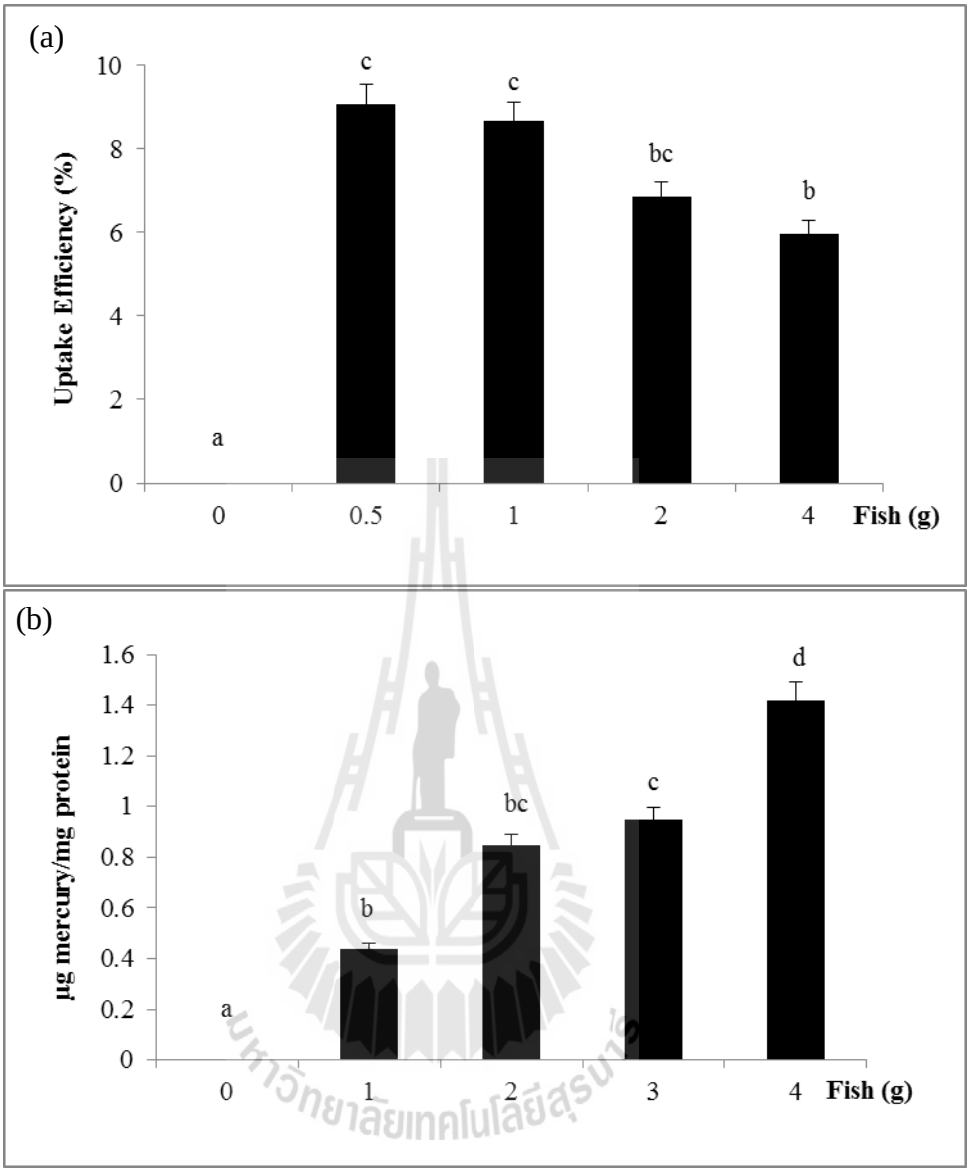

Figure 5.1 Uptake efficiency (a) and accumulation (b) of MeHg by Caco-2 human intestinal cells from test media containing MeHg from aqueous fraction of *in vitro* digestion (No dietary fiber). Data represent mean±SEM from n = 3 independent Caco-2 uptake experiment. Presence of different letters indicate significant differences between treatments as determined by Tukey's post hoc test ($p < 0.05$).

The concentrations used in the experiments in this study relate to *in vitro* digestion by using aqueous fraction, and the different concentrations of mercury added to the Caco-2 cells did not affect the integrity of the cell monolayer. In turn, viability was over 80% for all of the concentrations assayed. The differences in the accumulation varied according to the Hg concentrations added to the cell (concentration in test media). The results were similar to the control, with high concentration of Hg efficiency the uptake was low ,whereas with low concentration of Hg, efficiency of the uptake increased. Although an increase in ng mercury/mg protein correlated to increasing total Hg in the test media, the % uptake efficiency decreased (Table 5.1). Probably epithelial cells have limited absorpion (Calatayud et al., 2012). The % uptake efficiency of Caco-2 cell from *in vitro* digestion, which was treated with MDF 500 mg was not significant ($p > 0.05$). This is because the concentration of Hg in the test media was very low. So, the different concentrations did not affect uptake efficiency.

These data are similar to those of previous studies that reported the fiber from wheat, soy protein, and phytochemical such as catechins (green tea) and theaflavins (black tea) reduced the bioavailability of Hg which might be more efficient than synthetic chelating agents (e.g., DMPS) (Shim et al., 2009).

Moreda-Piñeiro et al. (2011), who studied Hg retention and transport in Caco-2 cells obtained for standards and the bioaccessible fraction of seafood samples. The Hg bioaccessible fraction in swordfish has a high cell retention. In contrast, transport to the basal compartment was low (<14%). Transportation of Hg depends on the Hg concentration, with high exposures (249 ng) the apical-basal transport is low (3%), whereas with lower levels of exposure (30 ng) the transport level increases (14%).

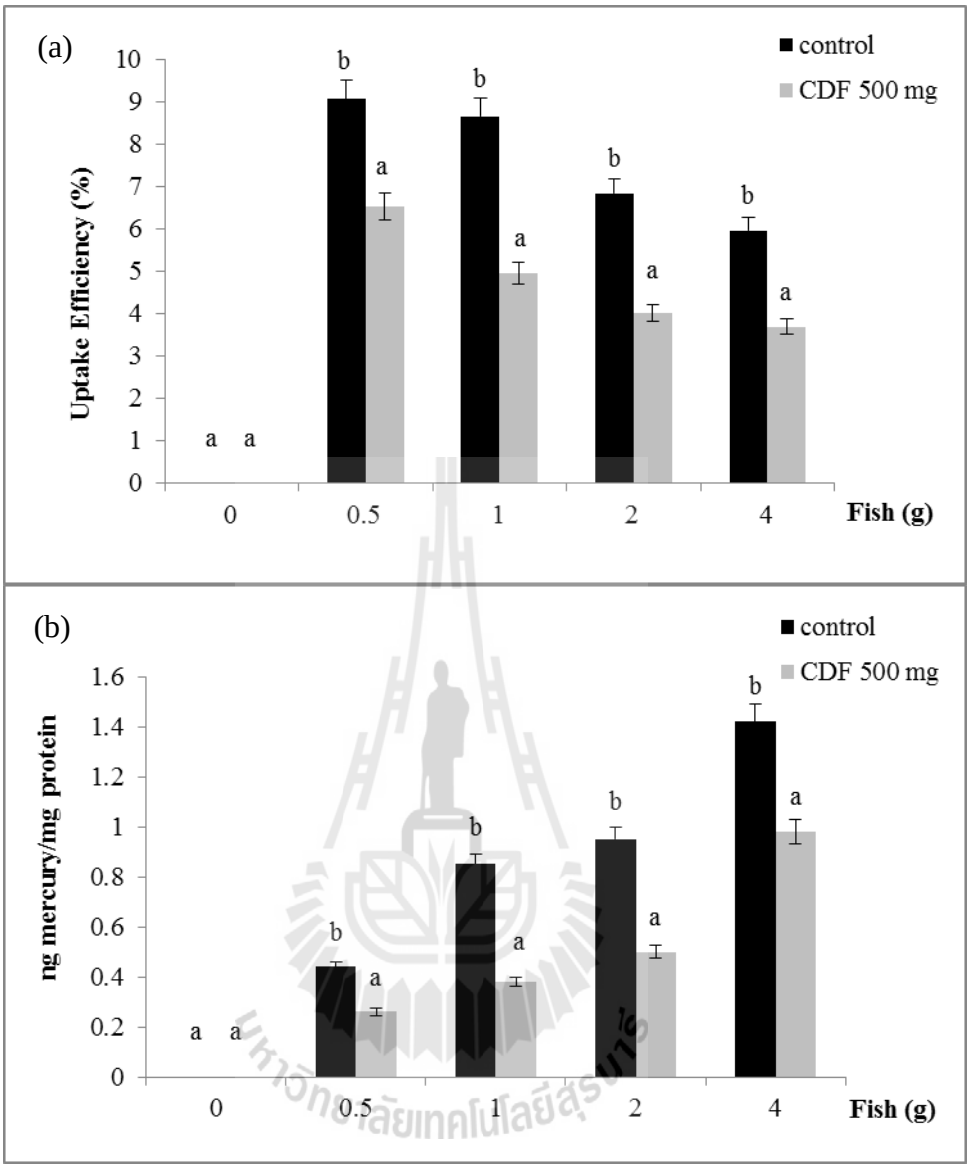

Figure 5.2 Uptake efficiency (a) and accumulation (b) of MeHg by Caco-2 human intestinal cells from test media containing MeHg from aqueous fraction of *in vitro* digestion with CDF. Data represent mean±SEM from n = 3 independent Caco-2 uptake experiment. Presence of different letters indicate significant differences within treatments as determined by Tukey's post hoc test ($p < 0.05$).

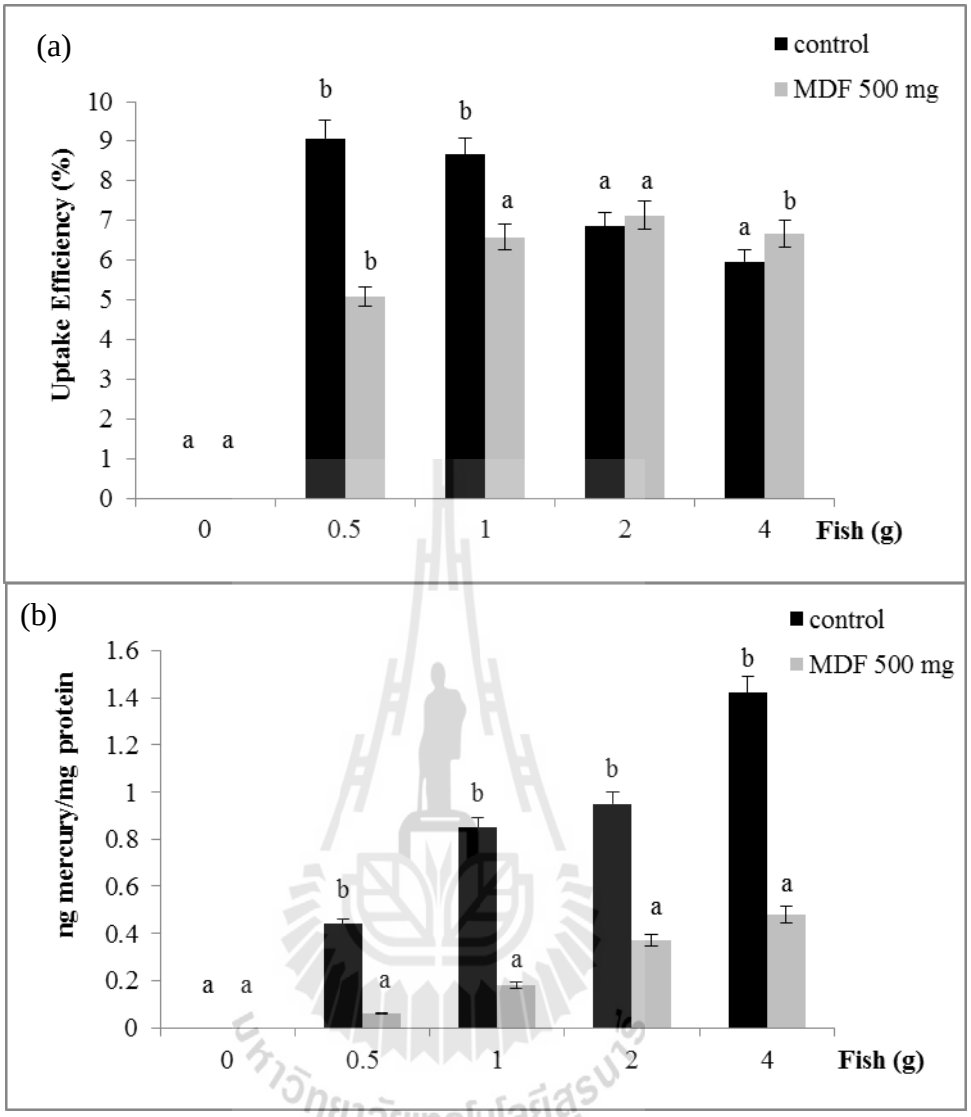

Figure 5.3 Uptake efficiency (a) and accumulation (b) of MeHg by Caco-2 human intestinal cells from test media containing MeHg from aqueous fraction of *in vitro* digestion with MDF. Data represent mean±SEM from n = 3 independent Caco-2 uptake experiment. The presence of different letters indicates significant differences within treatments as determined by Tukey's post hoc test ($p < 0.05$).

5.5 Conclusion

This study confirmed the positive effects of crude dietary fiber and modified dietary fiber on inhibiting mercury absorption by using an *in vitro* digestion model coupled with a human intestinal cell model. These results suggest that both types of fiber can reduce methylmercury exposure to the human body. To evaluate the toxicological significance of methylmercury exposure by fish consumption, the results confirm that *in vitro* digestion model coupled with an intestinal cell model may be a rapid and cost-effective alternative for evaluating the bioavailability of methylmercury from fish.

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CHAPTER VI

SUMMARY

Cassa pulp is a potential source for the production of dietary fiber. This study prepared crude dietary fiber (CDF) for investigation by using the enzymatic digestion process. CDF was obtained from enzymatic digestion condition of 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v). CDF were then modified by the etherification method which is properly known as modified dietary fiber (MDF). Then the physicochemical and functional properties of both fibers were investigated. The results from the test revealed that MDF showed more neutral detergent fiber: NDF (Cellulose, Hemicellulose, Lignin), acid detergent fiber; ADF (lignin, cellulose), acid detergent lignin; ADL (lignin), surface area, pore diameter and pore volume than CDF. The Fourier transform infrared (FT-IR) spectra shows 3 types of fiber (cassava pulp, CDF and MDF). The absorptions show the highest peaks were due to cellulose and hemicellulose. Moreover, a different infrared spectrum was observed for the fiber. This indicated that the molecular structure of the fiber had changed. The new peak at 1732 cm^{-1} corresponded to the –C=O group (Himmelabach et al., 2002) of the modified dietary fiber, which confirmed the introduction of the modified dietary fiber side chain into the cellulose backbone by graft copolymerization. These properties had an effect on the potential of Hg reduction. A study of the effect of fiber on Hg bioaccessibility by using *in vitro* digestion was conducted to simulate the human digestive system for measuring bioaccessibility. An comparison of both fibers suggests that MDF show significantly

more mercury inhibition than CDF. Furthermore, Caco-2 cells from the digestion model test were utilized for intestinal cell accumulation and bioavailability evaluation. Caco-2 cells can be used to evaluate intestinal cell uptake and the extent of its reliability for the *in vivo* situation in estimating bioavailability. The results show a low cellular accumulation (~5-9%). However, the cellular mercury uptake in MDF is lower than for CDF. This suggests that MDF shows a greater reduction in mercury uptake by the Caco-2 cells. In conclusion,these results confirm that dietary fiber from cassava pulp might act as a chelating agent for reducing mercury bioavailability.

APPENDIX

SUPPORTING MATERIALS FOR CHAPTER III, IV, AND V

Table 1A Analysis of variance of Y_1 (enzymatic digestion process).

Source	Sum of Squares	df	Mean Square	F Ratio	p-value
X_1	34.22	1	34.22	2.59	0.2486
X_2	13.03	1	13.03	0.99	0.4250
X_3	7.75	1	47.75	3.62	0.1975
$X_1 X_2$	0.56	1	0.56	0.043	0.8555
$X_1 X_3$	18.84	1	18.84	1.43	0.3546
$X_2 X_3$	81.00	1	81.00	6.14	0.1315
X_1^2	118.04	1	118.04	8.95	0.0960
X_2^2	72.65	1	72.65	5.51	0.1435
X_3^2	123.75	1	123.75	9.38	0.0921
$X_1^2 X_2$	9.29	1	9.29	0.7	0.4898
$X_1^2 X_3$	3.18	1	3.18	0.24	0.6723
$X_1 X_2^2$	4.12	1	4.12	0.31	0.6326
Total error	26.39	2	13.19	13.19	
Total (corr)	707.37	14			

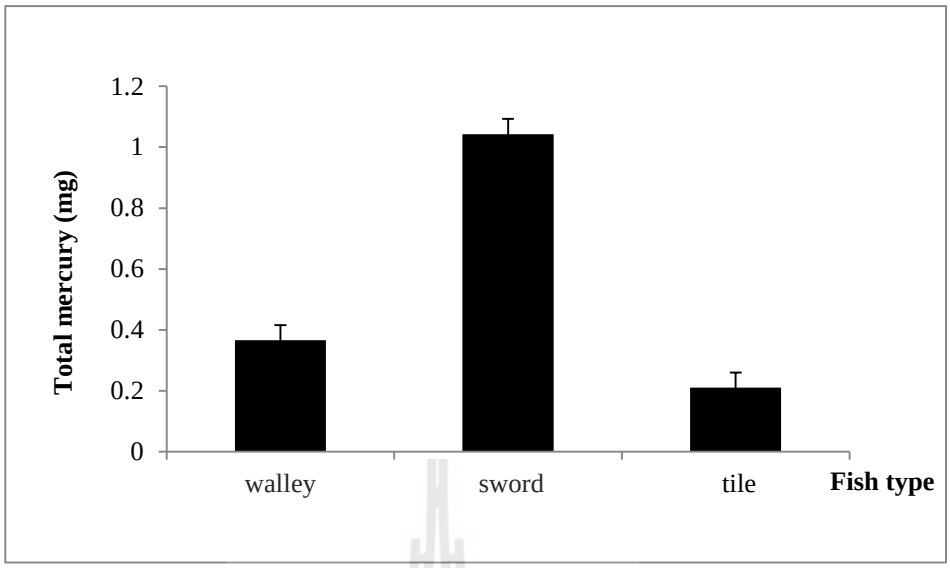

Figure 1A Total mercury (Hg) in starting materials.

Note: Walleye: fresh water fish, Sword and Tile fish: salt water fish.

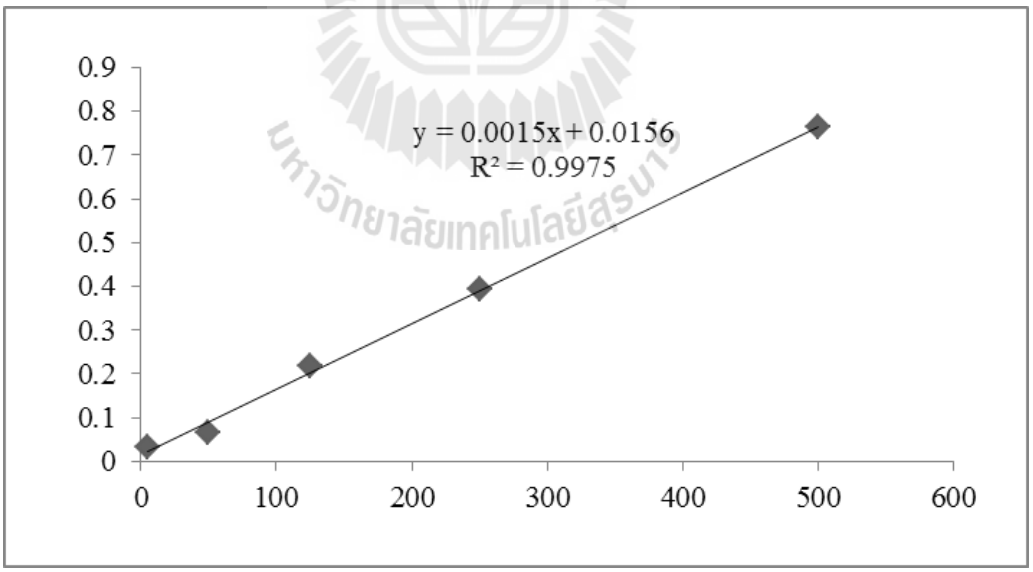

Figure 2A Standard curve for determining cell protein.

Preparation of samples

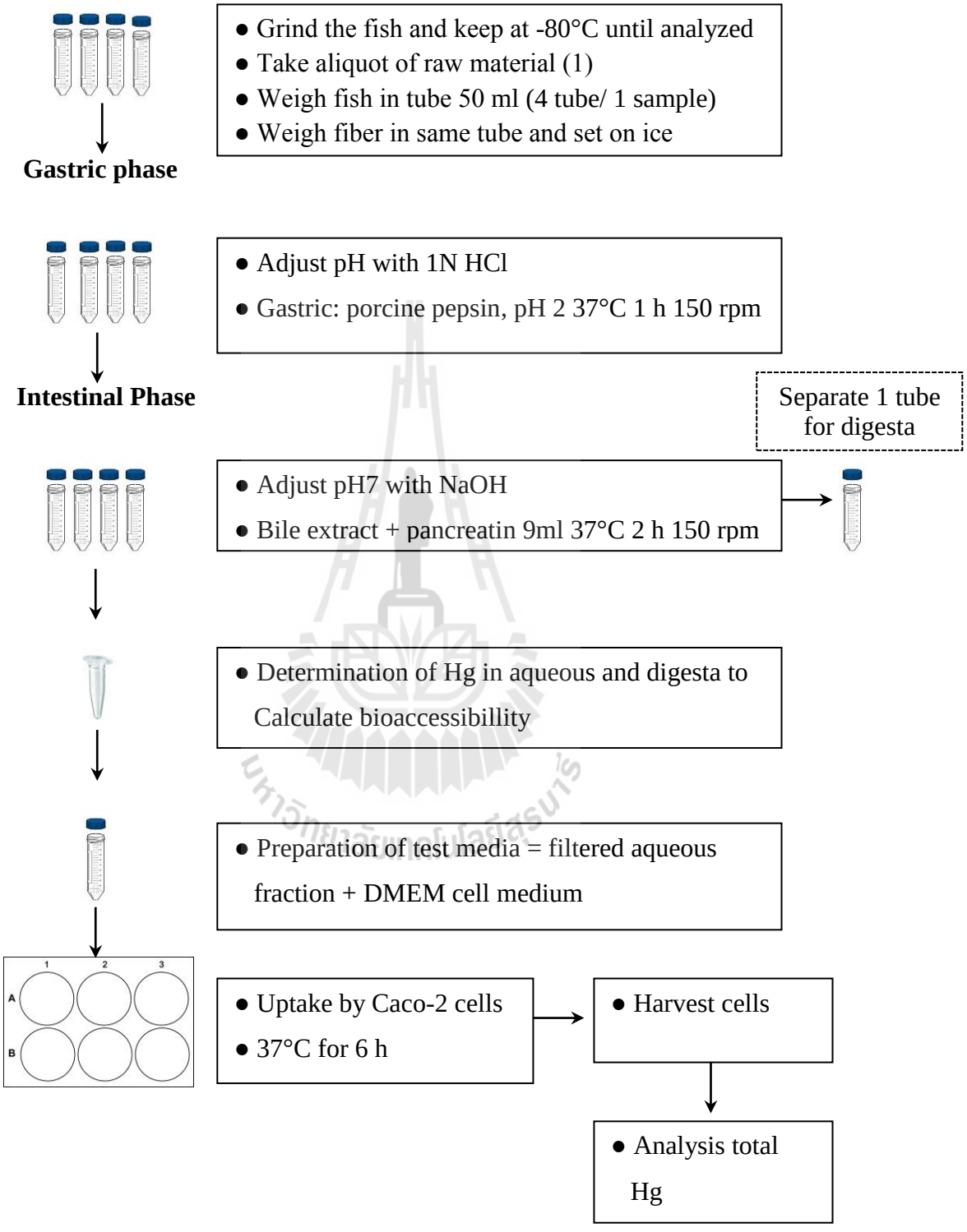

Figure 3A Diagram of *in vitro* digestion/Caco-2 cell model.

BIOGRAPHY

Natta Kachenpukdee was born on July 8th, 1981 in Nakhon Si Thammarat, Thailand. She studied at High School at Kanlayaneesithammarat School (1993-1999). In 2003, she received the degree of Bachelor of Science (Fisheries Industry) from Rajamangala Institute of Technology, Trang campus, Thailand. In 2004-2006, she received the degree of Master of Science (Fisheries Product) from Kasetsart University. In 2006-2010, she worked at Rajamangala University of Technology Srivijaya, Trang campus. In 2010-2015, she received a scholarship from Rajamangala University of Technology Srivijaya to study for the degree of Doctor of Philosophy in Food Technology at Suranaree University of Technology. During her graduate study, she obtained opportunities to present her research work at the ICAAI International Conference on Agriculture and Agro-Industry 2014 (Chiang Rai, Thailand, 20-21th November, 2014), the IBCELC International Biotechnology, Chemical Engineering and Life Science Conference 2014 (Okinawa, Japan, 4-6th September, 2014) and at the MTCHM Macrotrend Conference on Health and Medicine 2014 (Paris, France, 20-21th December, 2014). She also published her research work under the title of " Enzymatic digestion optimization of Dietary Fiber from Cassava Pulp and Their Effect on Mercury Bioaccessibility and Intestinal Uptake from Fish Using an *In vitro* Digestion/Caco-2 Model" in Internatonal Food Research Journal (Vol. 23, No.2, page 660-666) in 2016.