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ACID LEACHING EXTRACTION TECHNIQUE FOR DETERMINATION OF METALS IN FISH AND MUSSEL BY ATOMIC SPECTROMETRY

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การใช้กรดเจือจางสกัดตะกั่ว โครเมียม แคดเมียม ทองแดง และสังกะสี จากอาหารทะเลเป็นวิธีการสกัดแบบของแข็ง-ของเหลวร่วมกับอะตอมมิกแอบซอร์บชันสเปกโตรเมตรี เทคนิคการสกัดแบบการชะด้วยกรดร่วมกับอัลทราโซนิคนี้ได้พยายามนำมาใช้สำหรับการวิเคราะห์หาพหุธาตุในอาหารทะเล (หอย และปลา) หลากหลายปัจจัย ได้แก่ ความเข้มข้นของกรดไนตริก ความเข้มข้นของกรดไฮโดรคลอริก ความเข้มข้นของไฮโดรเจนเปอร์ออกไซด์ ปริมาตรของตัวทำละลาย เวลาในการโซนิเคชัน และอุณหภูมิ ได้ถูกศึกษา พบว่าใช้เวลาในการโซนิเคชัน 30 นาที ที่อุณหภูมิประมาณ 56 องศาเซลเซียส ตัวทำละลายที่ใช้สกัดคือ 3.7 M กรดไนตริก/3.7 M กรดไฮโดรคลอริก/0.5 M ไฮโดรเจนเปอร์ออกไซด์ อัตราส่วน 1:1:1 ปริมาตร 6 มิลลิลิตร ผลการทดลองที่ได้โดยวิธีดังกล่าวเปรียบเทียบกับผลการทดลองโดยการย่อยสลายด้วยไมโครเวฟ ให้ค่าร้อยละโดยอัตราส่วนของผลการทดลองจากวิธีการชะ ต่อผลการทดลองจากวิธีการย่อยสลายสำหรับแคดเมียม ทองแดง และสังกะสี อยู่ในช่วงระหว่าง ร้อยละ 92-114 และ ร้อยละ 88-103 จากตัวอย่างปลา และหอย ตามลำดับ ในขณะที่โครเมียม และตะกั่วไม่สามารถชะออกมาจากตัวอย่างทั้งสองชนิดได้โดยสภาวะดังกล่าว ค่า MDL คือ 0.087 0.02 0.13 และ 0.63 มิลลิกรัม/กิโลกรัม สำหรับตะกั่ว แคดเมียม ทองแดง และสังกะสี ตามลำดับ การทดสอบความถูกต้องทำโดยการวิเคราะห์ DORM-2 (Dogfish muscle) พบว่าค่าจากการทดลองโดยวิธีการชะ และวิธีการย่อยสลายของแคดเมียมมีค่ามากกว่าค่ามาตรฐานรับรอง (Certified values) ส่วน %Recovery ของสังกะสี และทองแดงมีค่าอยู่ในช่วงร้อยละ 80-87

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KEY WORD: ULTRASONIC-ASSISTED ACID LEACHING/MICROWAVE-ASSISTED DIGESTION/SEAFOOD/ELEMENT

NIWAT MANUTSEWEE: ACID LEACHING EXTRACTION TECHNIQUE FOR DETERMINATION OF METALS IN FISH AND MUSSEL BY ATOMIC SPECTROMETRY THESIS ADVISOR: WANLAPA AEUNGMAITREPIROM, Ph.D., THESIS CO-ADVISOR: APICHAT IMYIM, Ph.D., 106 pp. ISBN 974-17-5998-3.

The use of diluted acid to extract lead, chromium, cadmium, copper and zinc from seafood was a solid-liquid extraction procedure with atomic absorption spectrometry. Ultrasonic-assisted acid leaching extraction technique was attempted for the multi-elemental analysis of seafood (mussel and fish) samples. The effects of several parameters: nitric acid concentration, hydrochloric acid concentration, hydrogen peroxide concentration, acid solvent volume, sonication time and temperature have been studied. A 30-minute sonication, 56 ^{o}C operating temperature and 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio as acid solvent were used. The results obtained by the proposed procedure were evaluated by comparison with the results obtained by microwave-assisted digestion. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique for cadmium, copper and zinc ranged from 92-114% and from 88-103% for fish and mussel samples, respectively. Whilst, chromium and lead were not leachable from both samples by this condition. The MDL were 0.08, 0.02, 0.13 and 0.63 mg/kg for lead, cadmium, copper and zinc, respectively. The accuracy of the developed method was test by analyzing DORM-2, Dogfish Muscle certified reference material. Found values from acid leaching technique and digestion technique of cadmium were higher than certified values. %Recoveries for zinc and copper were in the order of 80 to 87%.

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LIST OF ABBREVATIONS AND SYMBOLS

CRM	Certified Reference Material
FAAS	Flame Atomic Absorption Spectrometry
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
g	Gram
kg	Kilogram
LOD	Limit of detection
L	Liter
LDPE	Low density polyethylene
MDL	Method of detection
µg	Microgram
µL	Microliter
µm	Micrometer
mA	Milliampare
mg	Milligram
mL	Milliliter
mm	Millimeter
min	Minute
M	Molar
ng	Nanogram
nm	Nanometer
N.D.	Not detectable
ppb	Part per billion
ppm	Part per million
RSD	Relative standard deviation
rpm	Revolution per minute
sec	Second

LIST OF ABBREVATIONS AND SYMBOLS (continued)

S.D.	Standard deviation
UV/VIS	Ultraviolet-Visible
v/v	Volume by volume
W	Watt
w/v	Weight by volume

CHAPTER I

LITERATURE REVIEW

1.1 Metals in food

Trace elements and heavy metals are continuously released in the marine environment via natural and anthropogenic influx and they can contaminate the marine biota. The determination of these elements in seafood (such as fish and mussel) is interesting because some elements are toxic, on the other hand some elements are necessary for maintenance of optimum health. Furthermore organisms such as mussel and fish tend to accumulate metal pollutants, this cumulative behavior constitutes a potential hazard for the marine environment.

It is proposed that metal transportation in marine animal are in four ways [1]:

a) Metal ions are directly passed through the cell membrane (Ionophores).
b) Complex of metals are directly passed through cell membrane.
c) Complex of metals are rearranged, changed form and passed through cell membrane.
d) Particulate formed complex with metals and then passed through cytoplasm.

When human consume seafoods contaminated with metals, the metals are also taken into human body. For toxic elements, they can damage and have an effect to health. On the other hand, some elements are essential elements and component of functions in human. But when human consume them in excess level, they can affect to health.

Table 1-1 shows the examples of elements that can damage and affect to health. The symptoms are also described when these elements are taken into the body.

Table 1-1. The symptoms when elements are taken into body [2]

Elements	Symptoms	
	Acute health effect	Chronic health effect
Zinc (Zn)	Eating large amount of zinc can cause stomach cramps, nausea and vomiting.	Zinc can cause anemia and pancrease damage, also tend to lower the concentration of high-density lipoprotein (HDL) cholesterol.
Lead (Pb)	Lead may cause seizure, but usually symptoms from lead weeks to months of exposure.	Lead can cause reproductive hazard. It is a probable tetratogen in humans, and decrease fertility in males and females.
Copper (Cu)	Copper can damage vision and cause blindness when eyes contact with particles of copper metal. Dust or fumes of copper can also irritate the eyes, nose and throat. It may cause coughing and nose bleeds.	Copper can cause lung cancer and damage the liver when uptake the copper in very high levels.

Table 1-1. (continued)

Elements	Symptoms	
	Acute health effect	Chronic health effect
Cadmium (Cd)	Exposure to metal fume many result in flu-like symtomps: weakness, fever, headache, chills, sweating and muscular pain. Acute edema usually develops within 1-3 days.	Cadmium can cause lung and prostate cancer. Also, the chronic effect is kidney damage, manifested by excretion of excessive protein in the urine. In addition, cadmium can cause osteomalcia, osteoporosis, anemia, teeth discoloration and anosmia.
Chromium (Cr)	Chromium particles can irritate the eyes and its fumes can cause metal fume fever, a flu-like illness with metallic taste, fever, chills and muscle lasting about 24 hours.	Chromium, cancer-casing agent can cause lung and throat cancer and reproductive damage in human. Chromium can cause lung allergy, cough, wheezing and shortness of breath. Many scientists believe that there is no safe level of chromium exposure.

For the reason of toxicity of metals, WHO and Codex provide a list of contaminants and recommended maximum level for human uptake per day from Journal of Health Science [3] as illustrated in Table 1-2.

Table 1-2. List of contaminants and recommended maximum level for human consumption [3]

Contaminants	Daily maximum level for human consumption (mg of contaminants/ body weight 1 kg)
Arsenic	0.05
Cadmium	0.001
Copper	0.5
Iron	Not regulated*
Lead	0.0036
Mercury (total)	0.00071
Methyl mercury	0.00047
Tin	Not regulated*
Zinc	Not regulated*

*Regulated only value of maximum levels in food

Nowadays, many countries enforce regulations on contamination level of toxic metals in food and food products, especially seafood. These regulations require precise and accurate analytical method for the determination of metals. In general the determination of metals in food consists of two main steps: sample pre-treatment and determination steps.

1.2 Sample pre-treatment methods

Research concerning with elemental analysis in seafood samples were published continuously. Sample pre-treatment step is very important and has many approaches. Various sample pre-treatment methods will be selected for the best analytical performances.

Acid/wet digestion is widely used method for the preparation of solid samples. In this chapter, we will discuss two types of acid digestion: conventional acid digestion and microwave acid digestion. However, it is well known that concentrated acids or oxidizing reagents play a role to destroy the organic matter of samples. So the matrix effect can occur in determination step. A sample preparation method, called "acid leaching extraction method" will be considered to avoid this problem. [4-7]

1.2.1 Conventional wet digestion / conventional acid digestion [4]

Conventional acid digestion is a process of total oxidation and decomposition of organic constituents by liquid oxidizing reagents or concentrated acids. The mixture is heated by conductive heating (flames, hot plates, heating mantles, thermal ovens and heating blocks). The vessels used in conductive heating are usually poor conductor and only a small fraction of the liquid is maintained at the temperature of the vessel. Heating is slow, and boiling point solution, pressure and colligative properties limit the maximum achievable solution temperature.

Table 1-3 shows some demonstrative reagents and their properties that used with acid digestion method.

Table 1-3. Oxidizing reagents and their properties for acid digestion methods [4]

Oxidizing reagents	Properties
Hydrochloric acid (HCl)	- Excellent solvent for inorganic sample - Widespread use for dissolving many metal oxides
Nitric acid (HNO_3)	- A strong oxidant - Widely use for decomposing organic samples (convert organic samples to CO_2 and H_2O)
Sulfuric acid (H_2SO_4)	- An effective solvent - Many materials are decomposed by hot concentrated sulfuric acid - High viscosity (can not use in a large volume)
Perchloric acid ($HClO_4$)	A potent oxidizing agent
Hydrogen fluoride (HF)	It was used in conjunction with other acids (for difficult dissolution sample)
Hydrogen peroxide (H_2O_2)	- The addition H_2O_2 into mineral acids to promote solvent action and increase efficiency of decomposition process - Hasten the oxidation of organic materials in the samples
Oxidizing mixtures	To promote efficiency of decomposition process

Conventional acid digestion conditions as reported in previous works are summarized in Table 1-4.

Table 1-4. Summary of the conditions as reported in previous work for the conventional acid digestion

Reagent/ Volume (mL)	Sample weight	Temperature (^oC)	Time (hours)	Year/ Reference
conc. H_2SO_4 100 μL and 50% H_2O_2 1.5-2 mL	Dried sample 100-150 mg	Not specified	Not specified	1973/[8]
mixtures of conc. HNO_3/conc. $HClO_4$ (6:1) 3.5 mL or conc. HF/ conc. $HClO_4$ (3:1) 2.0 mL	Dried sample 125 mg	140	24	1978/[9]
conc. HF 4 mL, aqua regia 2 mL and conc. $HClO_4$ 2 mL	Dried sample 0.5 g	190	16	1980/[10]
conc. HNO_3 4 mL and conc. $HClO_4$ 2 mL	Dried sample 1-2 g	Not specified	Not specified	1982/[11]
conc. HNO_3 and conc. $HClO_4$ (for Pb, Zn, Cu and Cd)**, conc. HNO_3 and conc. H_2SO_4 (for Hg)**	Dried sample*	Not specified	Not specified	1985/[12]
conc. HNO_3 10 mL	Wet sample 2.0 g	More than 60	More than 72	1987/[13]
conc. HNO_3 5 mL and conc. $HClO_4$ 1 mL	Dried sample 2.0 g	60-70	3-4	1992/[14]

* Sample weight not specified

** Reagent volume not specified

Table 1-4. (continued)

.

Reagent/ Volume (mL)	Sample weight	Temperature (^oC)	Time (hours)	Year/ Reference
conc. HNO_3, conc. HCl and conc. HF**	Dried sample 0.125 g	Not specified	48	1996/[15]
conc. HNO_3 10 mL, conc. HF 1 mL and conc. $HClO_4$ 1 mL	Dried sample 0.5 g	40-180	More than 20	1999/[16]
conc. HNO_3**	Wet sample 1.0 g	160	Not specified	2000/[17]
mixtures of conc. HNO_3/ conc. $HClO_4$ (3:1) 10 mL	Dried sample 1.0 g	130-140	8	2001/[18]
conc. HNO_3 5 mL	Dried sample 1.0 g	130	Not specified	2003/[19]
conc. HNO_3 10 mL	Dried sample 1.0 g	90-150	More than 8	2004/[20]
conc. HNO_3 10 mL and conc. $HClO_4$ 5 mL	Dried sample 1.0 g	Not specified	Not specified	2004/[20]
conc. H_2SO_4 3.5 mL and 30% H_2O_2 4.5 mL	Dried sample 0.5 g	250	Not specified	2004/[20]

* Sample weight not specified

** Reagent volume not specified

The results indicate that acid digestion procedures are labor-intensive and have many drawbacks. The procedures are tedious, large time consuming (more than 10 hours), easily losses of most elements during the oxidation step and contamination can be occurred because it is carried out in an opened system. Large volumes of decomposition reagent(s) are used typically 15-100 mL and multiple addition. [5]

1.2.2 Microwave acid digestion [21]

Microwave digestion is a part of acid digestions. The use of microwave oven for the decomposition has become an important method for sample pre-treatment. Microwave digestion vessels are constructed from the low-cost materials and must also be thermal stable and resistant to chemical attack. Figure 1-1 is the example of microwave digestion unit and their accessories.

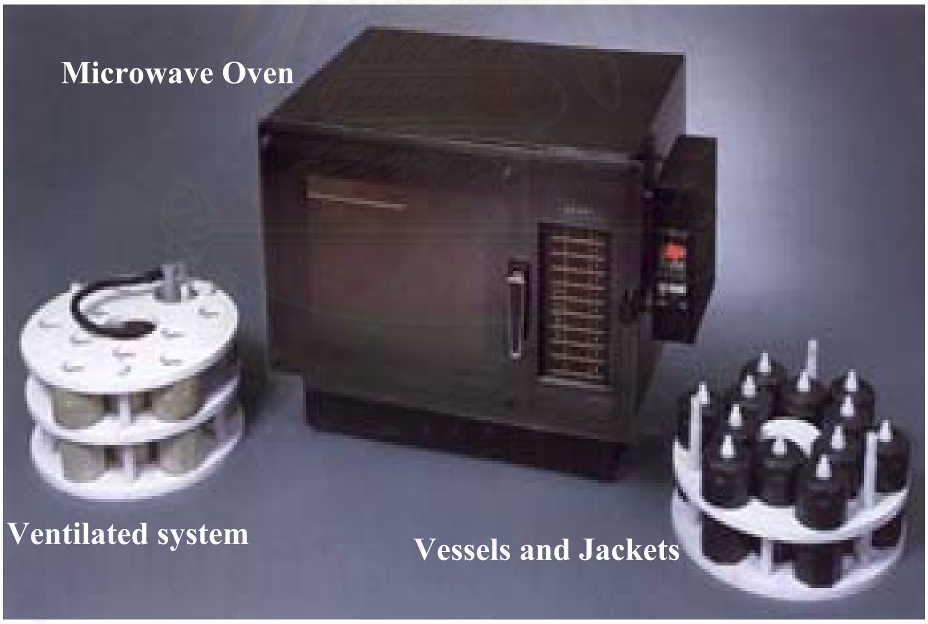


Figure 1-1. The microwave oven and their accessories

Later, some papers have been mentioned microwave acid digestions. Fish samples were digested with concentrated nitric acid, the mixture of nitric acid and hydrogen peroxide or concentrated nitric acid with vanadium pentaoxide for the determination of lead, cadmium, mercury, chromium, copper, zinc, iron, sodium, potassium, cobalt, molybdenum, nickel, calcium and magnesium. [22-30]

Analytical performance of microwave acid digestion using concentrated nitric acid and hydrogen peroxide were better than those of the conventional acid digestion using the mixture of concentrated nitric acid, concentrated sulfuric acid and vanadium pentaoxide for mercury determination, as shown in Table 1-5. [24]

Table 1-5. Analytical performances (mercury in fish samples) of microwave acid digestion and conventional acid digestion [24]

Parameters	Microwave digestion	Conventional acid digestion
Linearity	$R^2 = 0.998$	$R^2 = 0.996$
Detection limit	7.7 ng/g	19.4 ng/g
Accuracy (798 ± 74 ng/g)*	782 ± 73.8 ng/g	751 ± 98 ng/g

* Certified value

Mussel and biological samples were treated with concentrated nitric acid or concentrated nitric acid and hydrogen peroxide for cadmium and trace elements determinations. [25, 29].

Although microwave digestion method was the better method than conventional acid digestion, but some drawbacks were occurred. For instance in 1997, Sun *et al.* [31] determined aluminum in seafood and meat samples. They used the multi-steps of microwave digestion for proved the optimum results. First step, concentrated HNO_3 and concentrated HF were used for digested the samples. Then H_2O_2 and 4% (w/v) H_3BO_3 solution were used for eliminated the HF excess. Whilst, only concentrated HNO_3+H_2O_2 microwave digestion gave a low recovery of aluminum.

Table 1-6 summarized microwave digestion conditions of above researchers. [22-31]

Table 1-6. Summarized of microwave acid digestion conditions

Reagent/ Volume (mL)	Sample weight	Time	Reference
conc. HNO_3 2.5 mL and a few μg of vanadium pentaoxide	Wet sample 200 mg	90 seconds*	[22]
conc. HNO_3 1.8 mL	Wet sample 200 mg	10 minutes and cool down 25 minutes	[23]
conc. HNO_3 4 mL and 30%H_2O_2 0.2 mL	Dried sample 0.1 - 0.4 g	6 minutes*	[24]
conc. HNO_3 2 mL	Dried sample 0.2 g	2 minutes*	[25]
conc. HNO_3 5 mL and 30%H_2O_2 6 mL	Wet weight 1.0 g	60 seconds*	[26]
conc. HNO_3 and 30%H_2O_2 **	Dried sample***	Not specified	[27]
conc. HNO_3 7 mL	Dried sample 0.3 g	25 minutes*	[28]

*	Cooling time not specified
**	Reagent volume not specified
***	Sample weight not specified

Table 1-6. (continued)

Reagent/ Volume (mL)	Sample weight	Time	Reference
conc. HNO_3 5 mL and 30%H_2O_2 2.0 mL	Dried sample 250 mg	24 minutes*	[29]
conc. HNO_3 2 mL and 30%H_2O_2 0.5 mL	Dried sample 0.20 - 0.25 g	17 minutes*	[30]
conc. HNO_3 10 mL, conc. HF 0.5 mL, 30%H_2O_2 5 mL and 4% (w/V) H_3BO_3 solution 10 mL	Dried sample 0.5 - 1 g	Not specified	[31]

* Cooling time not specified

** Reagent volume not specified

*** Sample weight not specified

However, microwave digestion method requires high energy, the use of concentrated oxidizing acid and large cooling time. Other drawback is nitrous vapors formation (high carcinogenic) after organic matrix destruction stage.

1.2.3 Acid leaching extraction technique

Even through microwave digestion is better than conventional wet/acid digestion, but some disadvantages (e.g. nitrous vapors formation, the use of large time for cool down) of this method should be omitted. Acid leaching procedures can, therefore, be an alternative sample pre-treatment method.

Leaching is a term that has been applied to the extraction of metal from various samples. Acid leaching procedures do not the total matrix sample destruction but these procedures involve the breakdown of chemical bond between elements and the matrix sample constituents by acid mixture solution. Acid leaching is partial sample dissolution because the solid samples contact with a diluted acid solution (especially oxidizing reagent e.g. nitric acid, hydrochloric acid). The solid sample dissolution ratio increases when microwave energy or ultrasounds induce the leaching process. [7, 32-34]

A simple analyte separation without matrix decomposition is enough for many analytical techniques. Thus, atomic absorption spectrometry (mainly with the use of electrothermal atomization) allows analytical determinations to be carried out with minimum sample pretreatment owing to the low dependence of the analytical signal on the accompanying matrix as compared with other techniques for elemental analysis. [35]

When the mixture of acid solvent and solid sample are subjected to microwave energy, the mechanism through which metals reach the solution is also matrix decomposition. Microwave assisted-acid leaching procedure maybe called "pseudo-digestion". [32]

Kingston H.M. and co-workers [33, 36] found that the temperature was a key parameter for all leaching sample preparation methods. They compared the heating source between microwave oven and hot plate. The results showed that microwave heating with enhanced reaction control lead to improved precision compared to conventional heating source. In addition, they also found that hydrochloric acid was an important component of leaching solvent and it provided complexation and stabilization of some analytes by chloride species.

For ultrasound assisted-acid leaching procedure, ultrasonic energy has been used for a wide variety of applications in industry, medicine and science. In the analytical chemistry field, most applications lie in the ability of ultrasound to extract compounds from the solid matrix. An ultrasonic wave passes through a liquid, the wave oscillating pressure can cause a cavitation phenomenon which involves the generation, grown, osciollations, splitting and implosions of numerous of tiny gas bubbles called cavitation bubbles. As a result of cavitational bubble implosion, temperatures and pressures are generated at the center of the collapsed bubble. When a cavitating bubble collapses near the surface of a solid sample particle, microjets of solvent, propagated toward the surface at velocities greater than 100 m/s, cause pitting and mechanical erosion of the surface which leads to particle rupture, and consequently, to smaller particle size. Analyte (metal) are extracted into the liquid medium when the ultrasonic action is combined with acidic diluents. [37]

The advantages of acid leaching extraction method over microwave-assisted digestion are the following: [38]

1. Acid leaching extraction method is faster than microwave-assisted digestion. The time is much lower than that involved in the acid digestion procedure, but it is also necessary to take into account the time required for the reactor to cool before opening and the time needed for heating to dryness so that the excess of acid can be eliminated.
2. The consumption of reagents is diminished.
3. The procedure is safer than acid digestion as neither pressure nor high temperature are present during the extraction procedure. Furthermore, the use of corrosive concentrated acids is avoided.
4. The whole procedure is simpler since a less number of operations is involved that minimizes contamination risks.

Capelo J.L. *et al.* [37] summarized different characteristics of ultrasonic bath and ultrasonic probe in Table 1-7.

Table 1-7. Characteristics of ultrasonic bath and ultrasonic probe [37]

	ultrasonic bath	ultrasonic probe
Intensity (W/cm^3)	1-5	100
Distance dependant	Yes	No
Variable intensity	No	Yes
Direct application	No	Yes
Cost	Low	High
Sample Throughput	High	Low

More recently, ultrasound-assisted extraction has been applied to separate of inorganic compounds and metal ions from matrix, to facilitate their analytical determination, and to avoid traditional sample pretreatment methods such as dry ashing or wet digestion, which involve tedious and time-consuming treatments with corrosive reagents. [35]

Until now, only environmental samples were treated with these methods. Some researchers tried to develop methods for applying with other samples. Various parameters were adjusted. Finally, acid leaching extraction methods can be used for hair, biological tissue and food samples. Capelo J.L. *et al.* [37] summarized history of ultrasonic assisted-acid leaching in Table 1-8. They implied that, ultrasonic assisted-acid leaching was suitable method for variable samples.

Table 1-8. Ultrasonic assisted-acid leaching in variable samples [37]

Year	Matrix	Analyte (%extraction)
1989	SRM 2704 (sediment)	As (68), Fe (22), Mn (64), Pb (88)
1989	Fly ash	As (55), Ta (29), Pb (10), Se (100)
1990	SRM 1570 (spinach)	Mn (100), Zn (74), Fe (36), Cu (100)
1993	Sediment, spinach, coal and cement	Cu (60-98), Cr (2-74)
1993	Cabbage leaves and roots	Cd (86-89), Pb (1.5)
1994	Rice, sediment and soil	Cu (78-100), Cr (14-31), Pb (75)
1995	Polluted river sediment	Cr (26), Cu (62), Pb (62), Ni (63), As (79), Cd (82)
1995	2 sediments reference materials	Pb (75-90), Cr (30)
1996	9 biological reference materials [39]	Cd (100), Cu (100), Mn (100), Pb (100)
1996	Sediment and soil reference material	Cr (24-47), Ni (77-81)
1997	4 plant species	Co (87-97), Ni (77-81), Cu (70-86)
1998	Tea leaves	Ba (17), Cu (90), Fe (48), Pb (2), Zn (74)
1998	Sediment samples	Pb (88), Cu (85), Cd (106), Zn (82)
1998	Reference biological and sediment material [40]	Cd (56-102)
1998	Reference biological material	Cd (95), Cu (100)
1999	Coal	Pb (45)
1999	Reference sediment material	Pb (53)
1999	Reference biological and sediment materials	Cr (3-69), Pb (69-104)
1999	Mussel tissue [41]	Cd (100), Pb (100)
1999	Reference biological material	Cu (100)

Table 1-8. (continued)

Year	Matrix	Analyte (%extraction)
2000	UE-CRM 176 (ash), UK-135 WCR (sewage sludge), MURAT-ISSA 2 antartic Krill, UE-CRM 397 (human hair)	Cd (30-100)
2000	Human hair	Cd (100), Cr (100), Pb (100), Hg (100)
2000	Spruce seeds and plant reference material	Al (43-72), Cu (89-96), Li (48-100), Mn (91-101)
2000	Reference biological and sediment materials	Cd (89.5-105), Cu (90-101), Pb (88-98)
2000	Seafood samples	As (96-106)
2001	Reference environmental samples	As (84-89), Ba (35-101), Cd (79-100)
2001	Vegetable sample	Ca (100), Mg (100), Mn (100), Zn (100)
2001	Seafood samples [32]	As (106), Ca (105), Cd (100), Co (87), Cr (95), Cu (97), Mg (109), Mn (105), Pb (105), Zn (104)
2002	Seafood samples [42]	Se (100)

In the 1998, Azouri H.E. and co-workers [43] used acid leaching for leaching calcium, copper, iron, magnesium, zinc, manganese, cadmium and chromium in mussel samples. The effects of reagent, reagent concentration, sonication time and sample mass were studied. 3.9 M HNO_3/3.6 M HCl/0.3 M H_2O_2 of 1:1:1 ratio was used as leaching solvent for copper, manganese, iron, zinc, calcium and magnesium. 3.2 M HNO_3/ 0.1 M H_2O_2 was used as leaching solvent for cadmium. 200 mg of sample was used as sample mass and ultrasonic time was 120 minutes. The limit of detection were 0.081, 0.012, 0.059, 0.002 and 0.007 ppm for calcium, copper, iron, magnesium and zinc, respectively and relative standard deviation values from 0.6-7.1% as a function of the element concentration were obtained. Chromium was not leachable from mussel samples by using this condition.

In 2000, Filgueiras A.V. and his team [38] compared the ultrasound-assisted extraction and microwave-assisted digestion for determination of magnesium, manganese and zinc in plant samples. Six plant samples used in the human diet were analyzed, the concentration range of the three metals were approximately in the range of 1500–3000 mg/g for magnesium, 30–735 mg/g for manganese and 20–45 mg/g for zinc. Limit of detection corresponding to the ultrasound-assisted extraction method was 0.10, 1.26 and 0.65 mg/g for magnesium, manganese and zinc, respectively. The between-batch precision, expressed as %RSD, was about 0.5, 1.5 and 1% for magnesium, manganese and zinc, respectively. Analytical results for the three metals by ultrasound-assisted extraction and microwave-assisted digestion showed a good agreement, thus indicating the possibility of using mild conditions for sample preparation instead of intensive treatments inherent with the digestion method.

In 2001, Bermejo-Barrera P. *et al.* [34] studied ultrasonic assisted-acid leaching method as sample pre-treatment method. The performance of ultrasonic extraction was comparable to the microwave digestion procedure. It was found that a formula of acid solvent was not simultaneous leachable multi elements. Table 1-9 showed the suitable formula of acid solvent for the extraction of the specific elements.

Table 1-9. Mixture of oxidizing reagents for leached elements [34]

Elements	Nitric acid (M)	Hydrochloric acid (M)	Hydrogen peroxide (M)
Arsenic	0.5	3.0-3.5	0.5
Calcium	3.4-3.8	2.8-3.2	0.5
Cadmium	2.5	2.5	0.5
Cocalt	0.5	2.5	0.5
Chromium	3.8-4.0	2.4-2.7	0.5
Copper	3.6-4.0	3.0-3.5	0.5
Mercury	0.5	2.5	0.5
Magnesium	3.0-3.5	2.8-3.2	0.5
Manganese	3.4-3.8	2.5-3.0	0.5
Lead	2.5	2.0	0.5
Zinc	3.3-3.7	3.4-3.8	0.5
Iron	4.5	4.0	1.5
Selenium	1.2	0.5	0.5

Although, the results obtained from the procedure described were accurate and sufficiently precise, this method was complicate due to a formula of acid solvent could extract only the appropriate elements.

They also compared microwave assisted-acid leaching method and ultrasonic assisted-acid leaching method with microwave digestion method and slurry sampling method. Descriptions of sample pre-treatment methods were shown in Table 1-10. [7]

Table 1-10. Descriptions of sample pre-treatment methods [7]

Procedure	Acid reagents	Concentration	Time (min)
Microwave digestion	HNO_3, H_2O_2	70% v/v, 33% v/v	~ 60
Slurry sampling	HNO_3	1.0% v/v	~5
Microwave assisted-acid leaching method	$HNO_3/HCl/H_2O_2$	0.5 - 4.0 M/2.0 - 3.5 M/0.5 - 4.5 M	~30
Ultrasonic assisted-acid leaching method	$HNO_3/HCl/H_2O_2$	1.0 - 5.0 M/2.8 - 5.0 M/0.5 - 5.0 M	~27

Later, Nascentes C.C. and co-workers [44] found that ultrasonic assisted-acid leaching method was a fast and accurate method for the extraction of calcium, magnesium, manganese and zinc from vegetables. The nitric acid concentration, sonication time and particle size were optimized. The best extraction conditions were 0.14 M HNO_3, 10 min of sonication time and a particle size lees than 75 μm. %Ratio of obtained amount from leaching technique and obtained amount from digestion technique ranging from 96 to 102% was obtained under the optimum extraction conditions.

In 2002, Sastre J. and co-workers [45] determined cadmium, copper, zinc and lead in environmental samples. They reported the comparison of a microwave digestion with an aqua regia extraction and they also compared the method with a nitric acid leaching. The results showed that the aqua regia leaching method offered a good choice for monitoring low organic matter content samples as it was cheaper, and relatively faster than the microwave digestion method. %RSD was up to 15% for cadmium and 10% for lead, copper and zinc. For samples with a high organic matter content, nitric acid extraction method might be chosen. %RSD was 15% for lead and 12% for zinc, when as for cadmium and copper was lower than 8%.

In the same year, Al-Merey R. and co-workers [46] determined copper, lead and zinc in soil samples. Ultrasonic assisted-acid leaching method was used as sample pre-treatment method. They found that acid type, acid concentration, ultrasonic exposure time and sample matrix (chemical properties) were the main factors affecting the extracting process of lead, copper, and zinc from soil samples at ambient temperature (25 ^{o}C). The analytical performance of the ultrasonic assisted-acid leaching method is equal to the hot plate digestion method. The accuracy of the ultrasound method was validated using a certified reference soil sample (Soil-7). The variation of accuracy for lead and zinc was not more than 1.5%. The optimum experimental conditions for the extraction of copper, lead and zinc from some Syrian soil samples were found to be a small volume of concentrated HCl, and shorter exposure time was required. It has been also found that the extraction conditions depended on the sample matrix and the element to be determined.

In 2003, Moreno-Cid A. and Yebra M.C. [47] determined copper in mussel samples. They applied a flow injection technique with acid leaching procedure. The limit of detection was 0.06 ppm and a relative standard deviation was 2.7%.

In the same manner, Ruiz Jimenes J. and co-workers [48] used a flow injection technique with ultrasonic assisted-acid leaching method. They determined cadmium and lead from plants. The repeatability was 2.0 and 0.9% and the within-laboratory reproducibility was 7.1 and 2.8% for cadmium and lead, respectively.

In 2004, Aleixo, P.C. and co-worker [49] used ultrasonic-assisted acid leaching for the cadmium and lead determination in foods by beam injection flame furnace atomic absorption spectrometry (BIFF-AAS). Food samples were treated with 5 mL of 2.8 M nitric acid solution and sonication in an ultrasonic bath during 5–10 minutes. The detection limits were 0.03 μg/g for cadmium and 1.6 μg/g for lead. Relative standard deviations of measurements of cadmium and lead in food extracts were generally below 5 and 7%, respectively. For method validation, the certified reference materials Pig Kidney (BCR 186) and Rice Flour (NIES 10) were used. Quantitative cadmium and lead recoveries were obtained and no statistical differences were found at 95% level by applying the *t*-test.

Ultrasound extraction shows advantageous features for element speciation. Organometallic species can be extracted without changes in their integrity under suitable extraction conditions. Both organic and aqueous extraction media have been used for separation of organometallic and inorganic species from solid matrix, most applications used ultrasonic cleaning baths for extraction. [35]

A recent application of ultrasound-assisted extraction with the use of a probe-type sonicator has been reported for mercury speciation. Chromium speciation also could be extracted from industrial hygiene samples. The method have been successfully applied to the determination of arsenic and zinc and the estimation of heavy metal content in fractions and soluble metal in mud coming from soil sample. [50-52]

However, AOAC [53] referred standard methods for conventional acid digestion and microwave digestion. For conventional acid digestion, 50.0 g of wet samples are digested by 25 mL of concentrated HNO_3, 20 mL of concentrated H_2SO_4 and 1 mL of 50% H_2O_2. For microwave acid digestion, 0.2-0.5 g of dried samples are digested with 5 mL of concentrated HNO_3 and 2 mL of 30% H_2O_2 with a digestion time of about 45 minutes. Digestion program is shown in Table 1-11.

Table 1-11. AOAC digestion program [53]

Step	Power (watts)	Duration (min)
1	250	3
2	630	5
3	500	22
4	0	15

Whilst, AOAC did not report the standard method for food samples preparation by acid leaching.

Acid leaching extraction method may be used as an alternative to traditional sample preparation methods for elemental analysis. Acid leaching extraction methods usually involve the use of diluted acid to extract the element from solid samples. Moreover, acid leaching extraction methods provide a significant speeding up of those methods requiring long and tedious extractions. So far, analytical results obtained on applying acid leaching extraction method for sample preparation are very promising, and new developments are expected on the topics addressed in the present work.

1.3 Objective and scopes of the research

The main objective of this research is to study and develop the sample pre-treatment method by acid leaching extraction technique to investigate the simultaneous quantitative extraction of zinc, lead, copper, cadmium and chromium in fish and mussel. This study was divided into two parts. Firstly, use of fortified samples will perform the optimization of the extraction condition. The analytical results from acid leaching extraction technique were compared with results from microwave digestion. Secondly, the optimum method will be applied to a real sample. Finally, method validation will perform by using CRM.

CHAPTER II

EXPERIMENTAL

2.1 Chemicals

2.1.1 Nitric acid 65% (AR grade, Lab Scan, Ireland)
2.1.2 Hydrochloric acid 37% (AR grade, Lab Scan, Ireland)
2.1.3 Hydrogen peroxide 30% (Carlo Erba Reagenti, Italy)
2.1.4 Zinc(II) standard solution 1,000 ppm (Spectrosol grade, BDH, England)
2.1.5 Lead(II) standard solution 1,000 ppm (Spectrosol grade, BDH, England)
2.1.6 Copper(II) standard solution 1,000 ppm (Spectrosol grade, BDH, England)
2.1.7 Cadmium(II) standard solution 1,000 ppm (Spectrosol grade, BDH, England)
2.1.8 Chromium(III) standard solution 1,000 ppm (Spectrosol grade, BDH, England)
2.1.9 $Pd(NO_3)_2$ 10,000 ppm (Perkin Elmer Instrument, USA)
2.1.10 $Mg(NO_3)_2$ 10,000 ppm (Perkin Elmer Instrument, USA)
2.1.11 $NH_4H_2PO_4$ 10% (Perkin Elmer Instrument, USA)

2.2 Preparation of solutions

Deionized water was used for all preparation of solutions. Acid solvents were prepared from nitric acid, hydrochloric acid and hydrogen peroxide using volumetric glassware. Working standard solutions and soaking solutions were prepared from stock solutions of 1,000 ppm by diluting to the required concentration by 1% (v/v) nitric acid.

Matrix modifier solution for Cd and Pb analyses were prepared by diluting 1 mL of 10% $NH_4H_2PO_4$ and 0.1 mL of 10,000 ppm Mg stock solution to 10 mL with deionized water. Matrix modified solutions for Cu analyses were prepared by diluting 1 mL of 10,000 ppm Pd stock solution and 0.1 mL of 10,000 ppm Mg stock solution to 10 mL with deionized water.

To avoid metal contamination, all glassware and plasticware were soaked in 2% hydrochloric acid for 1 week, and then rinsed with deionized water three times. They were also soaked in 2% nitric acid for 1 week, then rinsed with deionized water three times before used.

2.3 Apparatus

2.3.1 Flame Atomic absorption spectrometer (Aanalyst 100, Perkin Elmer Instrument, USA)
2.3.2 Graphite Furnace Atomic absorption spectrometer (4110 ZL, Perkin Elmer Instrument, USA)
2.3.3 Microwave digestion unit and accessories (Multiwave, Paar Physica)
2.3.4 Centrifuge (Centaur 2, Sanyo, Japan)
2.3.5 Ultrasonic bath (Ney Ultrasonik, USA)
2.3.6 Analytical balance (TC-254, Denver Instrument Company, USA)

2.4 Sample fortification

Pb, Cd, Cu and Cr could not be determined directly by FAAS because the amounts of these elements in the real samples were lower than the detection limit. So elemental fortification was necessary for this study. The fortified samples were prepared by the following procedure.

Fresh fish and mussel tissues were carved to small pieces and soaked into 100 ppm Pb(II), Cu(II), Cd(II) for two days and into 100 ppm Cr(III) for seven days at 4 ^{o}C. All of soaked samples were washed with deionized water for 5-6 times, and homogenized. Samples were dried at 100 ^{o}C for 20 hours and grounded to small particle size. Then they were put into a LDPE bottle, and kept in a desicator.

2.5 Microwave digestion

Dried samples were weighted into quartz vessels about 0.20 g. 5.00 mL of concentrated HNO_3 and 0.50 mL of concentrated HCl were added into vessels (as recommended by manufacturer). Then teflon seals were capped and energy was applied follow program PAAR 002H (Table 2-1). After complete digestion, the vessels were incubated until the temperature of each vessel lower than 50 ^{o}C. The solution was transferred into a volumetric flask and made up to 10 mL with deionized water. This solution was kept in a LDPE bottle at 4 ^{o}C before measurement.

Table 2-1. Power program of PAAR 002H

Step	Power (W)	Time (mm:ss)	Power (W)	Fan
1	100	5	600	1
2	600	5	600	1
3	1000	10	1000	1
4	0	15	0	3

- **Method efficiency**

Spiking test was used to test the efficiency of microwave digestion method and the %recovery was investigated as following.

$$\%re\cos very = \frac{(A - B)}{C} \times 100$$

Where A = Amount of analyte (sample + added analyte)

B = Amount of analyte in sample

C = Amount of added analyte

2.6 Acid leaching extraction procedure

Dried fish and mussel samples were weighted into a plastic tube about 0.20 g and acid/oxidant solution was added. The used acid as HNO_3, HCl and H_2O_2 was proposed by Bermejo-Barrera, P. *et al.* and Azouzi, H.E. *et al.* [34, 43] for determination of many metals in mussel samples mentioned in Table 1-10. The appropriate formula of mixing acid solvent was investigated in this section. Mixture was sonicated in an ultrasonic bath for 15-60 minutes at ambient temperature and high temperature. The mixture was centrifuged at 3,000 rpm for 10 minutes. Liquid phase was transferred to a 10 mL volumetric flask. The solid residue was cleaned with 2 mL of deionized water, then centrifuged at 3,000 rpm for 15 minutes. Liquid phase was transferred to the same volumetric flask and the volume was adjusted to 10 mL with deionized water. This solution was kept in a LDPE bottle at 4 ^{o}C before measurement. The solid residue was undergone the microwave digestion as describe in section 2.5. Acid leaching steps were shown in Figure 2-1.

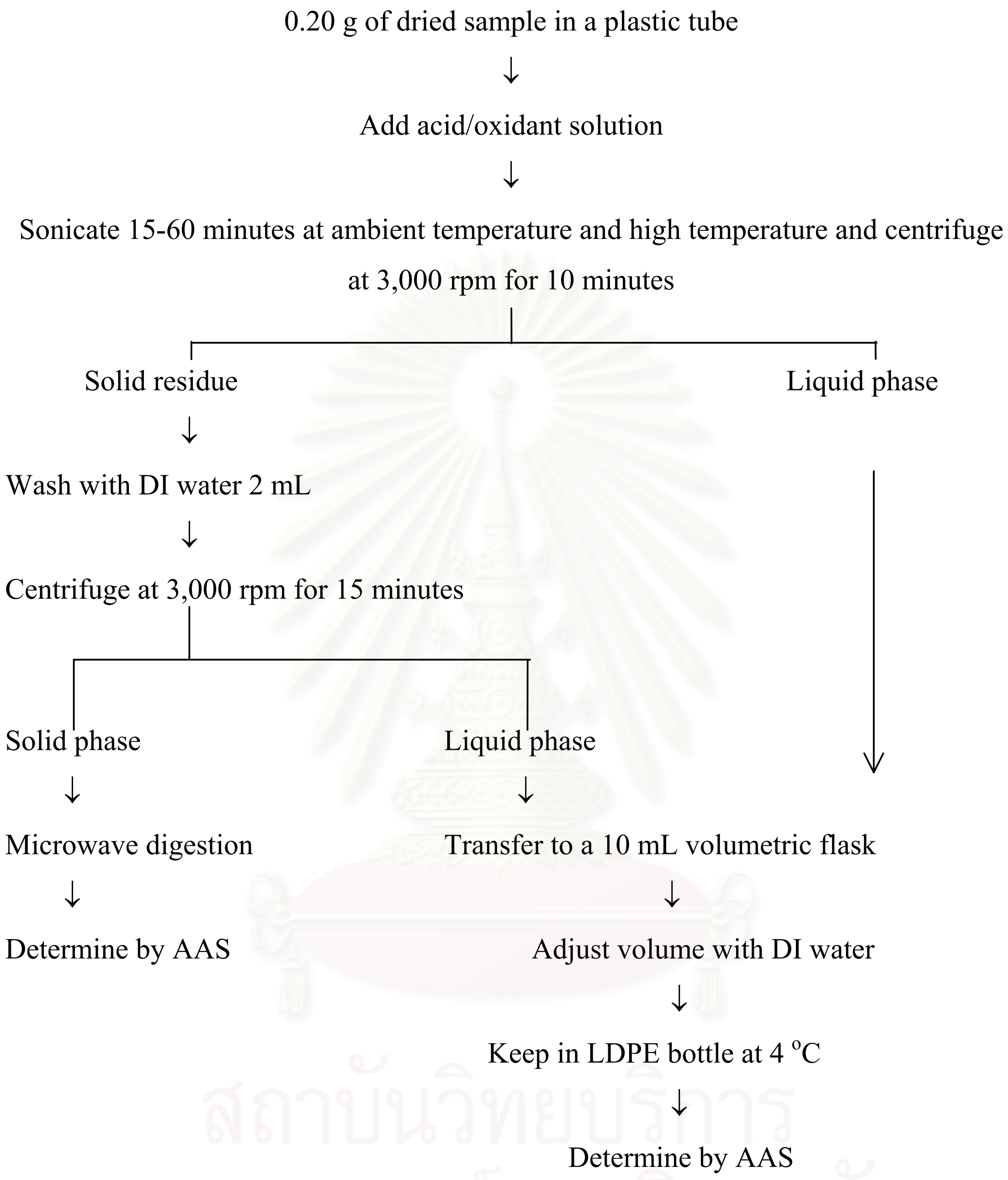


Figure 2-1. Schematic diagram of acid leaching extraction technique steps

Acquisitive results from acid leaching extraction technique were compared with results of microwave digestion. Subsequently a term "%ratio of metal amount obtained from leaching technique to amount determined by digestion technique" is employed. Percent ratio of obtained amount from leaching technique and obtained amount from digestion technique was investigated by the equation below.

$$\% M_{leach} \big/ M_{digest} = \frac{M_{leach}}{M_{digest}} \times 100$$

Where M_{leach} = Amount of analyte from acid leaching extraction technique (mg/kg of sample)

M_{digest} = Amount of analyte from microwave digestion technique (mg/kg of sample)

2.6.1 Optimization

Several parameters, such as concentrations of acid leaching solvent, acid solvent volume and sonication time, were varied to obtain the optimum conditions.

2.6.1.1 Fortified samples and real samples (for only Zn)

Forified samples were used for Pb, Cu, Cd and Cr determination. And real samples were used for Zn determination.

2.6.1.1.1 Leaching by deionized water

The main purpose of this study was to check weither the metals could be leached from fortified samples. And another purpose was to investigate the efficiency of deionized water as leaching solvent.

6 mL of deionized water without acid/oxidant solution was used as leaching solvent to leach elements in fish and mussel samples.

2.6.1.1.2 Effect of nitric acid concentration

The effect of nitric acid concentration was investigated by the following procedure.

Acid solvents were prepared by fixing concentration of HCl and H_2O_2 (3.7 M and 0.5 M, respectively) [43] and varying concentration of HNO_3 (0, 0.5, 1, 2, 3, 3.7 and 4 M). HNO_3, HCl, and H_2O_2 solutions were mixed with the ratio of 1:1:1. Acid solvents of each HNO_3 concentration were used for acid leaching extraction. Acquisitive results were compared for the optimized condition investigation.

2.6.1.1.3 Effect of hydrochloric acid concentration

The effect of nitric acid concentration was investigated by the following procedure.

Acid solvents were prepared by fixing concentration of HNO_3 and H_2O_2 (3.7 M and 0.5 M, respectively) [43] and varying concentration of HCl (0, 0.5, 1, 2, 3, 3.7 and 4 M). HNO_3, HCl, H_2O_2 solutions were mixed with the ratio of 1:1:1. Acid solvents of each HCl concentration were used for acid leaching extraction. Acquisitive results were compared for the optimized condition investigation.

2.6.1.1.4 Effect of hydrogen peroxide

Acid solvent without H_2O_2 (3.7 M HNO_3/3.7 M HCl/Deionized water of 1:1:1 ratio) was used as acid solvent to investigate the effect of hydrogen peroxide.

2.6.1.1.5 Effect of acid solvent volume

2, 4, and 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio were used for extraction. The results could be attributed to optimize the solvent volume used to leach the elements from fish and mussel samples.

2.5.1.1.6 Effect of sonication time

Fifteen minutes of sonication time for some elements were not enough. So these elements were remained in solid residue (Cd in fish and Cu in mussel). Increasing sonication time from 15 minutes to 30, 45 and 60 minutes may solve this problem. 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio were also used.

Leaching conditions for each experimental part were summarized in Table 2-2.

Table 2-2. Leaching condition for each experimental part (fortified and real samples)

Part	Sample weight (g)	Leaching solvent	Volume (mL)	Sonication time (min)
2.6.1.1.1	0.20	Deionized water	6	15
2.6.1.1.2	0.20	- 0 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 0.5 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 1 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 2 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 3 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 3.7M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 - 4M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1	6	15
2.6.1.1.3	0.20	- 0 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 0.5 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 1 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 2 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 3 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 3.7 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1 - 4 M HCl /3.7 M HNO_3/0.5 M H_2O_2 1:1:1	6	15
2.6.1.1.4	0.20	3.7 M HNO_3/3.7 M HCl/ Deionized water 1:1:1	6	15
2.6.1.1.5	0.20	3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1	2, 4, 6	15
2.6.1.1.6	0.20	3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1	6	15, 30, 45, 60

A Perkin-Elmer model Analyst 100 atomic absorption spectrometer equipped with an acetylene-air flame was used for Zn, Cu, Pb, Cd and Cr under conditions summarized in Table 2-3.

Table 2-3. FAAS conditions for the determination of Zn, Cu, Pb, Cd and Cr in acid digests and acid leachates from fish and mussel samples

Element	Wavelength (nm)	Slid width (nm)	Lamp current (mA)	Air flow rate (L/min)	C_2H_2 flow rate (L/min)
Zn	213.9	0.7	15	10	3.0
Cu	324.8	0.7	15	10	3.0
Pb	283.3	0.7	12	10	3.0
Cd	228.8	0.7	4	10	3.0
Cr	357.9	0.7	25	10	3.0

2.6.1.2 Real samples

The temperature during ultrasonic operation plays an important role on acid leaching efficiency [33]. When the samples were treated with high temperature, Pb, Cu and Cd might be more leachable from the samples. Similarly, the sonication time and acid/oxidant concentration might be effected the acid leaching efficiency.

2.6.1.2.1 Effect of temperature

Samples were treated with 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio. The temperatures during sonication were performed at ambient temperature and high temperature at about 52^oC-56^oC.

2.6.1.2.2 Effect of sonication time

Samples were treated with 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio at high temperature. Sonication time was increased from 15 minutes to 30 and 60 minutes, respectively. Acquisitive results were compared.

2.6.1.2.3 Effect of acid concentration

Pb could not be extracted from the samples. The use of stronger condition may solve this problem.

6 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio and 7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio [34] were used as acid solvent to leach Pb from the samples. Samples were treated with these solvents at ambient and high temperature for 15 minutes.

Leaching conditions for each experimental part were summarized in Table 2-4.

Table 2-4. Condition for each part of optimization (real samples)

Part	Sample weight (g)	Leaching solvent	Volume (mL)	Sonication time (min)	Temperature
2.6.1.2.1	0.20	3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1	6	15	Ambient and high temperature
2.6.1.2.2	0.20	3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1	6	15, 30, 60	High temperature
2.6.1.2.3	0.20	- 6 M HNO_3/6 M HCl/ 0.5 M H_2O_2 1:1:1 - 7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 1:1:1	6	15	Ambient and high temperature

Metal determinations were carried out by GFAAS under conditions summarized in Table 2-5.

Table 2-5. GFAAS conditions for the determination of Pb, Cd and Cu in acid digests and acid leachates from fish and mussel samples

Operating condition	Pb	Cd	Cu
Wavelength (nm)	283.3	228.8	324.8
Slit width (nm)	0.7	0.7	0.7
Injection volume (μL)	20	20	20

Element	Step	Temperature (^{o}C)	Ramp (s)	Hold (s)	Ar flow rate (mL/min)
Pb	Drying 1	110	1	30	250
	Drying 2	130	15	30	250
	Pyrolysis	850	10	20	250
	Atomization	1600	0	5	0
	Cleaning	2450	1	3	250
Cd	Drying 1	110	1	30	250
	Drying 2	130	15	30	250
	Pyrolysis	500	10	20	250
	Atomization	1500	0	5	0
	Cleaning	2450	1	3	250
Cu	Drying 1	110	1	30	250
	Drying 2	130	15	30	250
	Pyrolysis	1200	10	20	250
	Atomization	2000	0	5	0
	Cleaning	2450	1	3	250

2.6.2 Application of optimum conditions

The optimum conditions were applied with real samples, fish and mussel. The samples were prepared by the following procedure.

The fresh samples were homogenized and dried as described in section 2.4 without fortification. Dried samples were weighted in a plastic test tube about 0.20 g. And then 6.00 mL of acid solvent (3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio) was added. The mixture was sonicated at high temperature (56 ^oC) for 30 minutes. After that the mixture was centrifuged at 3,000 rpm for 10 minutes. The leachate was poured into a volumetric flask 10 mL. The residues were washed with 2 mL of deionized water and centrifuged at 3,000 rpm for 15 minutes. The supernatant was poured into the same volumetric flask and the volume was adjusted to 10 mL with deionized water. The solution was kept in a LDPE bottles at 4 ^oC before measurement. Cu and Cd were determined by GFAAS and Zn was determined by FAAS.

2.6.3 Method validation

2.6.3.1 Precision

The precision was studied by measurement 10 replicates of leachates. The precision is expressed in terms of the standard deviation (S.D.) and the relative standard deviation (%RSD), using the following formulas:

$$S.D. = \sqrt{\frac{\sum_{1}^{n} (x_i - \overline{x})^2}{n-1}}$$

$$\%RSD = \frac{S.D.}{\overline{x}} \times 100$$

where x_i = Concentration of analyte

x = Average concentration of analyte

n = Number of samples.

2.6.3.2 Limit of detection (LOD) and method detection limit (MDL)

The detection limit and quantification limits are define as:

$$LOD = 3S.D.$$
$$LOQ = 10\ S.D.$$

respectively, where S.D. is the standard deviation of 10 measurements of a reagent blank. In this study, the reagent blank was prepared by dilution 6.00 mL of acid solvent (3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1) to 10 mL with deionized water

The method detection limit is defined by:

$$MDL_{(mg/kg)} = \frac{LOD_{(mg/L)} \times V_{(L)}}{m_{(kg)}}$$

where V = Volume of leachates

m = Mass of samples.

2.6.3.3 Linearity

All working standard solutions were prepared from stock solutions 1,000 ppm. The calibration curves were run (n = 5) under the optimal condition. The lead, cadmium, copper and zinc concentrations ranged between 20-100 ng/mL, 2-10 ng/mL, 10-50 ng/mL and 0.5-2.5 μg/mL, respectively.

2.6.3.4 Accuracy

The accuracy was studied by using DORM-2 certified reference material (CRM) from National Council of Canada as sample. DORM-2 is dogfish (*Squalus acanthias*) muscle. 0.20 g of DORM-2 was treated with the optimum conditions.

Table 2-6 showed the elements for which certified values have been established for the dogfish reference material.

Table 2-6. Certified values of trace elements in dogfish reference material

Element	Concentration (mg/kg)
Aluminium	10.9 ± 1.7
Arsenic	18.0 ± 1.1
Cadmium	0.043 ±0.008
Cobalt	0.182 ± 0.031
Chromium	34.7 ± 5.5
Copper	2.34 ± 0.16
Iron	142 ± 10
Lead	0.065 ± 0.007
Mercury	4.64 ± 0.26
Nickel	19.4 ± 3.1
Selenium	1.40 ± 0.09
Silver	0.041 ± 0.013
Thallium	0.004
Tin	0.023
Zinc	25.6 ± 2.3
Methylmercury	4.47 ± 0.32
Arsenobetanine	16.4 ± 1.1
Tetramethylarsonium	0.248 ± 0.054

CHAPTER III

RESULTS AND DISCUSSION

In this chapter, we present the analytical performances. The effects of several parameters were discussed. Certified reference material (DORM-2) was used to prove the accuracy parameter.

3.1 Microwave digestion

The efficiency assessment measures the effectiveness of sample preparation method, care were taken to mimic the actual sample preparation as closely as possible. The efficiency of microwave digestion method was reported in term of %recovery by spiking test. The concentration covered the range of concern and should particularly included one concentration close to the quantitative limit. The AOAC manual for the Peer Verified Method Program showed in Table 3-1 with illustrated acceptable range of estimated recovery data as a function analyte condition. [53]

Table 3-1. Analyte recovery at different concentrations [53]

Concentration	Mean recovery (%)
100%	98-102
10%	98-102
1%	97-103
0.1%	95-105
100 ppm	90-107
10 ppm	80-110
1 ppm	80-110
100 ppb	80-110
10 ppb	60-115
1 ppb	40-120

Known amounts of analytes were added into the sample matrix (fish and mussel) and then the analytes were measured by FAAS. In fish samples, %recovery of Zn, Pb, Cu, Cr and Cd of 102.50 ± 1.09, 91.65 ± 5.80, 96.69 ± 1.33, 93.16 ± 2.23 and 90.80 ± 3.04 were obtained, respectively (n = 16).

%Recovery of Zn, Pb, Cu, Cr and Cd in mussel samples were 99.71 ± 1.64, 97.94 ± 2.01, 96.85 ± 1.24, 109.80 ± 2.56 and 91.17 ± 2.29 respectively (n = 16).

The average % recoveries of Zn, Pb, Cu, Cr and Cd from fish and mussel tissue were shown in Figure 3-1.

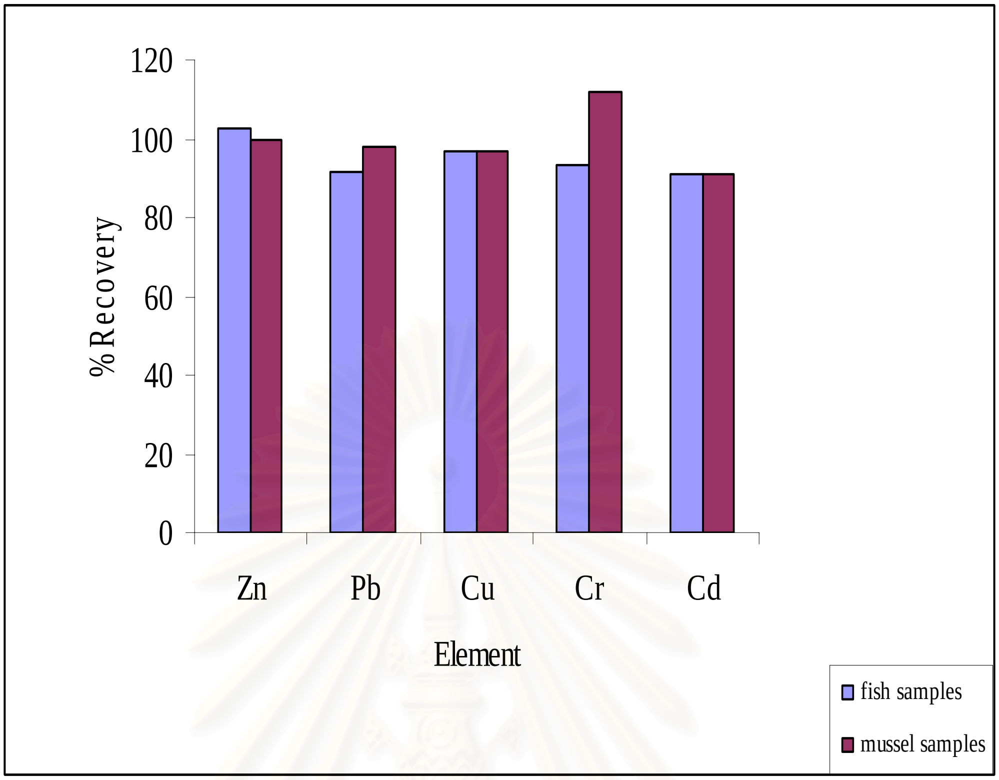


Figure 3-1. The average recoveries of Zn, Pb, Cu, Cd and Cr from fish and mussel samples

The analytical performances showed the acceptable %recovery values of 80-110% at 100 ppb-10 ppm. This indicated that, the microwave digestion method was a suitable method for treatment the samples. For this reason, the results from acid leaching extraction method could be compared with the results from the microwave digestion method.

3.2 Acid leaching extraction method

Samples (fish and mussel) were divided into 2 sets: (i) fortified samples (Pb, Cd, Cu and Cr), (ii) real samples. Metal determinations were carried out by FAAS for fortified samples (Pb, Cd, Cu and Cr) and real sample (Zn) and by GFAAS for real samples.

3.2.1 Fortified samples and real samples (for Zn only)

In this part, we studied the effects of nitric and hydrochloric concentration, effects of hydrogen peroxide in acid solvent, efficiency of deionized water, effects of acid solvent volume and effect of sonication time. The results of each parameter will be discussed.

3.2.1.1 Leaching by deionized water

In order to confirm the efficiency of acid solvent for leaching the real and fortified samples, the leaching by using deionized water was considered. In this study samples were treated with 6 mL of deionized water and the results were compared with the ones from 6 mL of 3.7 M HNO_3/ 3.7 M HCl/ 0.5 M H_2O_2 of 1:1:1 ratio.

Figure 3-2 showed that deionized water was not able to leach Pb and Cr from fortified fish samples. For Cd and Cu, the partial extractions by deionized water with %ratio of metal amount obtained from leaching technique to amount determined by digestion technique 7 and 14, respectively were found. It might be because some Cu and Cd adsorbed on surface of samples. These results indicated that deionized water was not sufficient to leach metal from fish tissue.

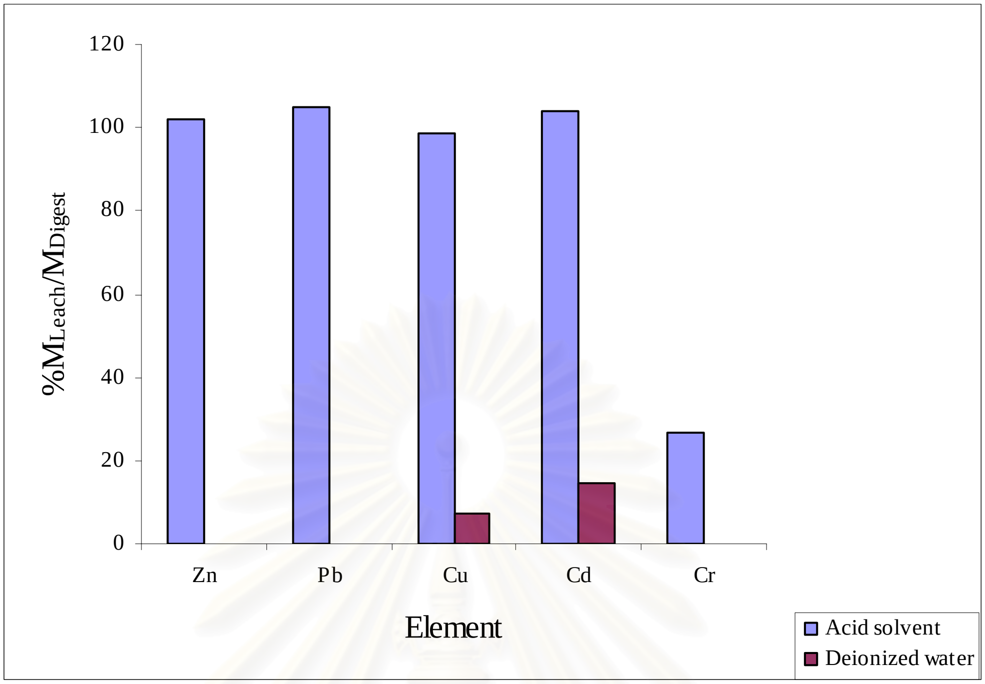


Figure 3-2. Comparison of acid solvent and deionized water to leach Zn, Pb, Cu, Cd and Cr in fish samples (0.20 g of samples were treated with 6 mL of acid solvent)

As can be seen in Figure 3-3, only small fractions of Pb, Cu and Cr were adsorbed on the surface of mussel samples. Deionized water could partially leach Pb, Cu and Cr from the samples (%ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Pb, Cu and Cr were less than 6, 12 and 15%, respectively). An amount of Cd was formed chemical bond with sample matrix. Whilst residues of Cd were adsorbed on surface samples. The obtained relation values were more than 60% when deionized water was used as medium.

For real samples, deionized water was not sufficient to extract Zn from the fish samples. Zn was partially leached from mussel samples by deionized water (%ratio of metal amount obtained from leaching technique to amount determined by digestion technique was less than 56%).

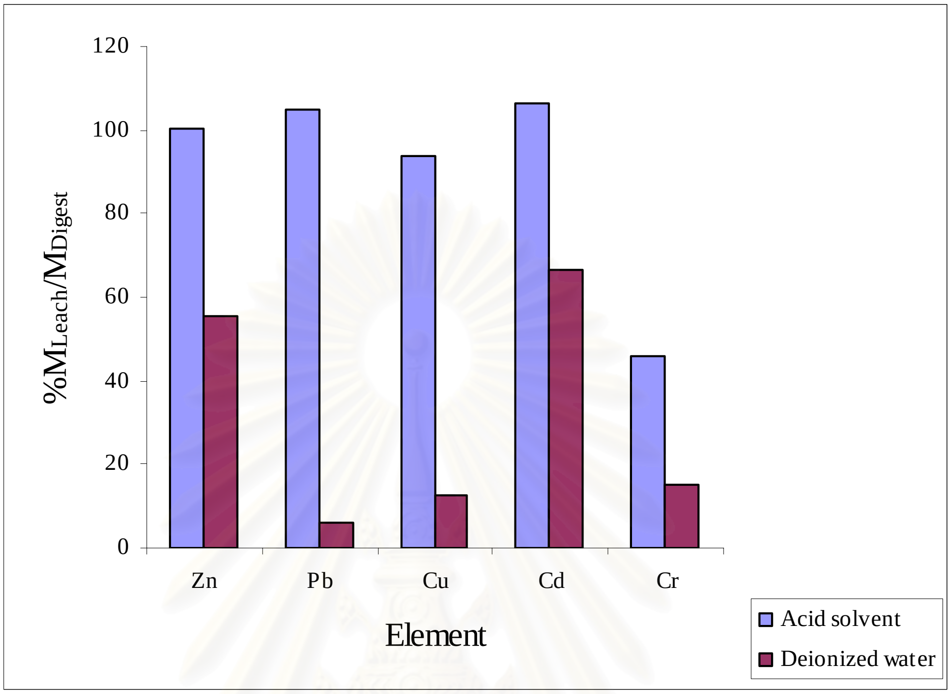


Figure 3-3. Comparison of acid solvent and deionized water to leach Zn, Pb, Cu, Cd and Cr in mussel samples (0.20 g of samples were treated with 6 mL of acid solvent)

3.2.1.2 Acid concentration dependence study

The main purpose of this study was the investigation for a general procedure suitable for maximum leachability of all metals from fish and mussel samples. In addition, P. Bermejo-Barrera *et al*. [7, 32, 33] reported that the most significant variables affecting the metal acid leaching from mussel tissue were the concentration of acids. For this reason, the concentrations of nitric and hydrochloric acid were optimized in order to provide quantitative relations of the elements.

3.2.1.2.1 Effect of nitric acid concentration

To investigate the effect of nitric acid concentrations, the concentrations of hydrochloric acid and hydrogen peroxide were constant, 3.7 M for HCl and 0.5 M for H_2O_2 (recommended by Azouzi H.E. and co-workers) [43] and the range of 0-4 M nitric acid was used.

%Ratio of metal amount obtained from leaching technique to amount determined by digestion techniques in function of nitric acid concentration were shown in Figure 3-4 and 3-5 for fish and mussel samples, respectively.

Figure 3-4 showed the constant relation of Pb, Cu and Cd (~100%) when 0-4 M nitric acid were used. But in case of Zn, nitric acid concentration showed a small influence on the leaching. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique was inferior of 90 when acid solvent consisted of 2 M nitric acid. For Cr, these acid solvents were not able to leach Cr from fish samples. These results indicated that the concentration of nitric acid was not significant variable for Zn, Pb, Cu and Cd.

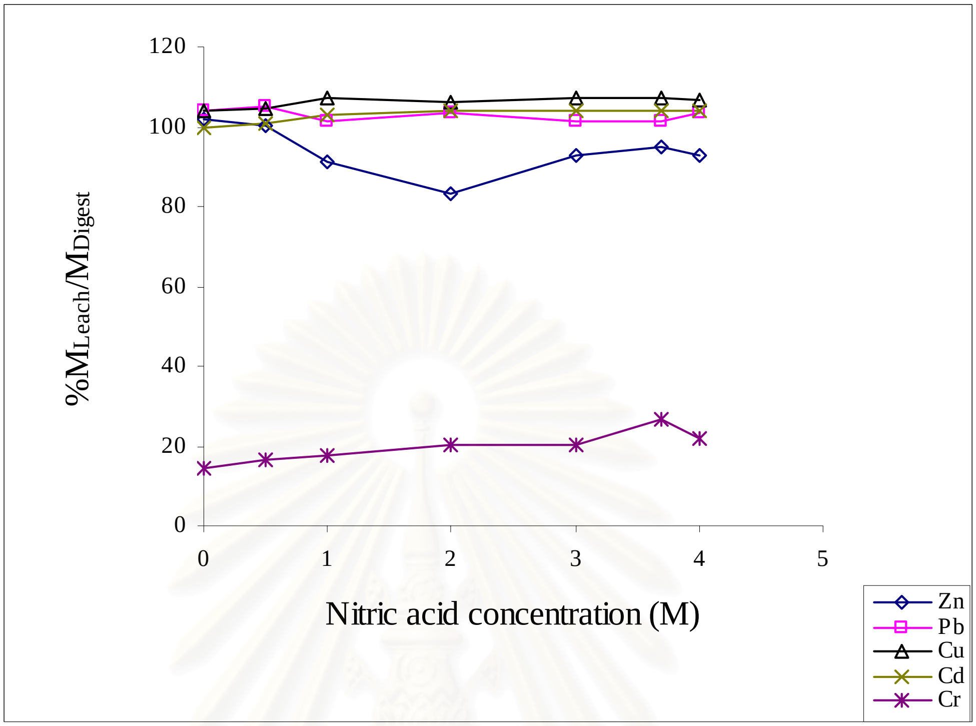


Figure 3-4. Effect of nitric acid concentration on the determination of Zn, Pb, Cu, Cd and Cr in fish samples (0.20 g of samples were treated with 6 mL of acid solvent, n = 4)

The results of mussel acid leaching extraction (Figure 3-5) showed the similarity as fish samples. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu and Cd were approached to 100% when 0-4 M nitric acids were used. In case of Cr, %ratio of metal amount obtained from leaching technique to amount determined by digestion techniques were slightly higher in comparison to fish samples.

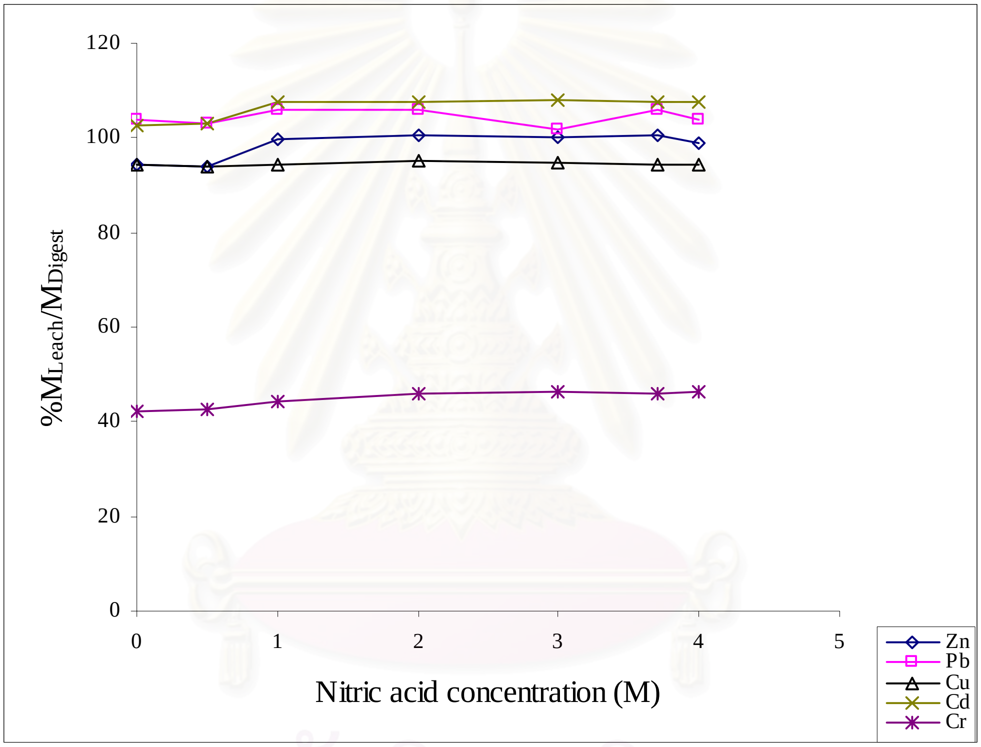


Figure 3-5. Effect of nitric acid concentration on the determination of Zn, Pb, Cu, Cd and Cr in mussel samples (0.20 g of samples were treated with 6 mL of acid solvent, n = 4)

3.2.1.2.2 Effect of hydrochloric acid concentration

Similar to the study of nitric acid concentration, the effects of hydrochloric acid concentrations were considered. In this case, concentrations of nitric acid and hydrogen peroxide were fixed at 3.7 M and 0.5 M, respectively (recommended by Azouzi H.E. and co-workers) [43], and the range for 0-4 M hydrochloric acid was used.

%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique in function of hydrochloric acid concentrations for fish and mussel samples are shown in Figure 3-6 and 3-7, respectively.

Figure 3-6 showed no significant effect of hydrochloric acid concentration for Cu and Cd leaching in fish samples. For Zn, the obtained %ratio of metal amount obtained from leaching technique to amount determined by digestion techniques were similar to the results of Zn leaching in Figure 3-2. But in case of Pb leaching, hydrochloric acid concentration appeared to be more important in comparison to nitric acid concentration effect. When hydrochloric acid concentrations were increased from 0.5 M to 3 M, %ratio of metal amount obtained from leaching technique to amount determined by digestion techniques decreased. However, when hydrochloric acid concentrations were 3.7 and 4 M, %ratio of metal amount obtained from leaching technique to amount determined by digestion technique closed to 100% were obtained.

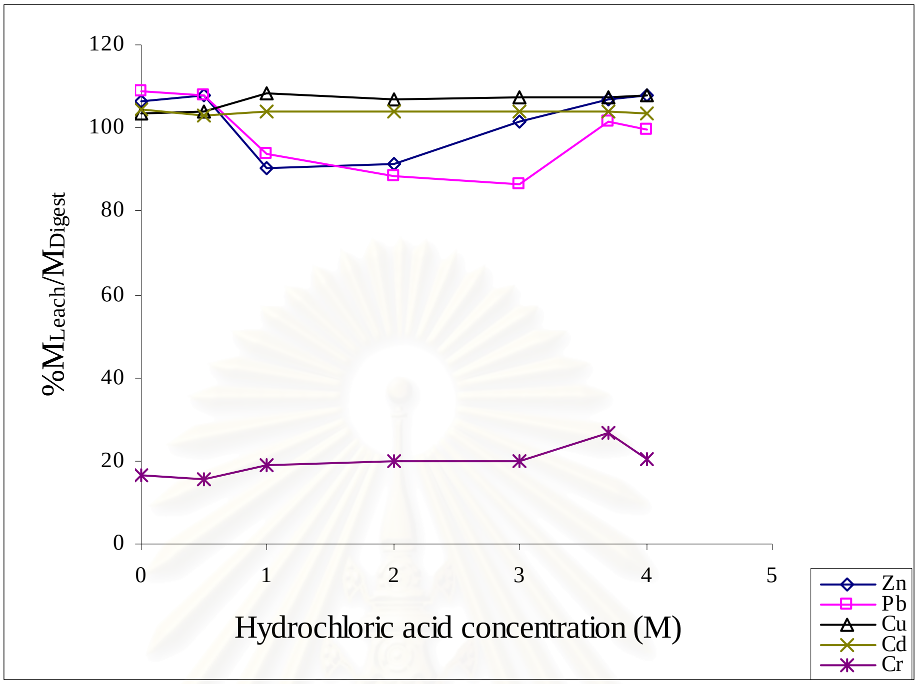


Figure 3-6 Effect of hydrochloric acid concentration on the determination of Zn, Pb, Cu, Cd and Cr in fish samples (0.20 g of samples were treated with 6 mL acid solvent, n = 4)

For mussel samples (Figure 3-7), the results showed that more than 90% relations were obtained for Zn, Pb, Cd and Cu leaching when acid solvents consisted of 0-4 M hydrochloric acid.

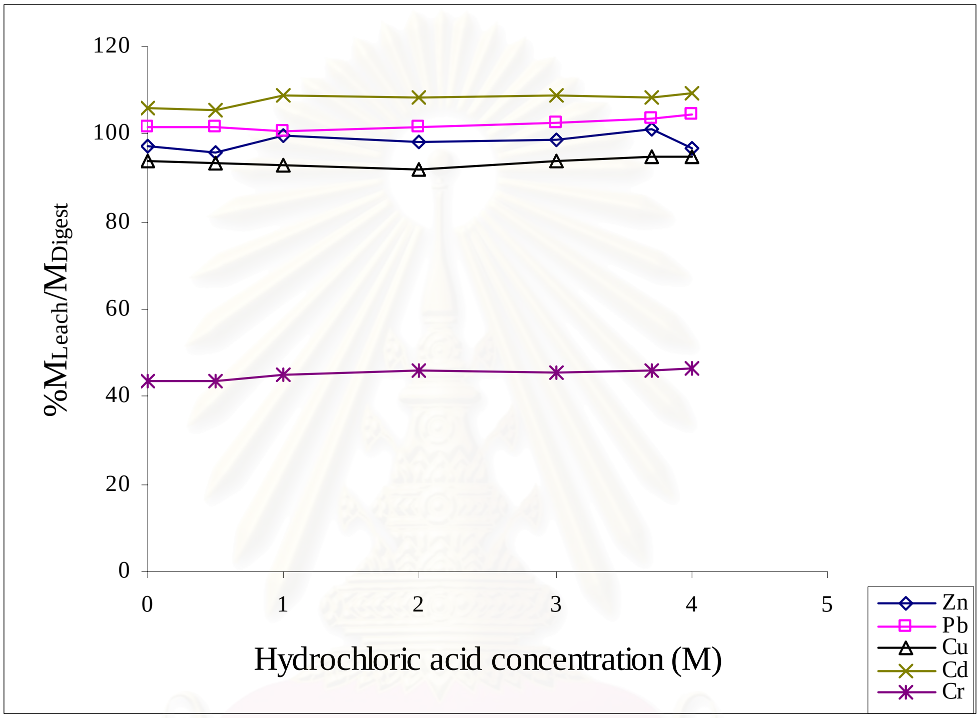


Figure 3-7. Effect of hydrochloric acid concentration on the determination of Zn, Pb, Cu, Cd and Cr in mussel samples (0.20 g of samples were treated with 6 mL acid solvent)

Similar to the results of nitric acid concentration effect study, the results showed no quantitative leaching of Cr. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of 16-27% and 43-47% were obtained for fish and mussel samples, respectively. This indicated that Cr leaching might be required stronger conditions than those employed. It might be because of the extreme strength chemical bond between Cr and sample matrix.

3.2.1.3 Effect of hydrogen peroxide

To test the effect of hydrogen peroxide on acid leaching efficiency, the acid leaching solvent without hydrogen peroxide (6 mL of 3.7 M HNO_3/3.7 M HCl/Deionized water of 1:1:1 ratio) was used. The results were summarized in Table 3-2 and 3-3 for fish and mussel samples, respectively. It was found that hydrogen peroxide was a significant variable effecting Cu leaching for both samples. %Ratio of metal amount obtained from leaching technique to amount determined by digestion techniques of 94.65 and 86.19 were obtained for fish and mussel samples, respectively.

Table 3-2. Effect of hydrogen peroxide on acid leaching efficiency[a] (fish samples)

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[b]	
	With 0.5 M H_2O_2	Without H_2O_2
Zn	98.24 ± 0.52	97.03 ± 2.47
Pb	101.11 ± 3.95	101.1 ± 3.95
Cu	107.32 ± 1.44	94.65 ± 1.58
Cd	104.22 ± 0.32	103.98 ± 0.59
Cr	26.78 ± 0.00	25.53 ± 0.66

[a] 0.20 g of samples were treated with 6 mL acid solvent

[b] mean %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

Table 3-3. Effect of hydrogen peroxide on acid leaching efficiency[a] (mussel samples)

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[b]	
	With 0.5 M H_2O_2	Without H_2O_2
Zn	103.17 ± 1.82	103.70 ± 1.76
Pb	103.13 ± 2.02	103.13 ± 2.02
Cu	95.16 ± 2.34	86.19 ± 0.72
Cd	109.40 ± 0.46	109.30 ± 0.46
Cr	46.12 ± 0.50	45.86 ± 0.66

[a] 0.20 g of samples were treated with 6 mL acid solvent

[b] mean %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

When applying the paired *t*-test [54, 55], it was found that significant difference of %ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Cu leaching (P = 95) occurred between acid leaching solvent with hydrogen peroxide. This indicated that organic matters (protein amino acids) had important role in controlling the mobility of Cu.

Hydrogen peroxide in acid solvent could dissociate to hydroxyl radical ($OH^{\bullet}$). The $OH^{\bullet}$ radical was most effective of all oxygen radical species. It could attack proteins, carbohydrates and polyunsaturated fatty acids. And it was believed that H_2O_2 interacted with Cu ions. Hence, it might be possible to improve the efficiency of the Cu extraction by using mixture of acid and hydrogen peroxide [43, 56, 57]. On the other hand, hydrogen peroxide was an insignificant variable for Zn, Pb and Cd leaching in fish and mussel samples. So hydrogen peroxide plays an important component for simultaneous quantitatively acid leaching extraction of Cu from fish and mussel samples.

Leaching of Cr from the fish and mussel samples were still not success by using 3.7 M HNO_3/3.7 M HCl/deionize water of 1:1:1 ratio as leaching solvent. So we had chosen 3.7 M HNO_3 for representative due to %ratio of metal amount obtained from leaching technique to amount determined by digestion technique of some elements in both samples were constant and inclined high. And we had also chosen 0, 0.5 and 3.7 M HCl for continual study because these concentrations gave high %ratio of metal amount obtained from leaching technique to amount determined by digestion technique and vicinity values.

The suitable conditions for Zn, Pb, Cu and Cd leaching were the use of solutions of 3.7 M HNO_3/0 M HCl/0.5 M H_2O_2, 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 and 3.7 M HNO_3/3.7 M HCl/0.5M H_2O_2. Contrary, Cr could be partially leached for every concentration of HNO_3 and HCl.

3.2.1.4 Effect of acid solvent volume

Two, four and six mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio were applied for fish and mussel samples. Analytical results were shown in Figure 3-8 and 3-9. For both samples, acid leaching efficiency for Zn, Pb, Cu and Cd were strongly affected by acid solvent volume, especially in case of Pb, Cu and Zn.

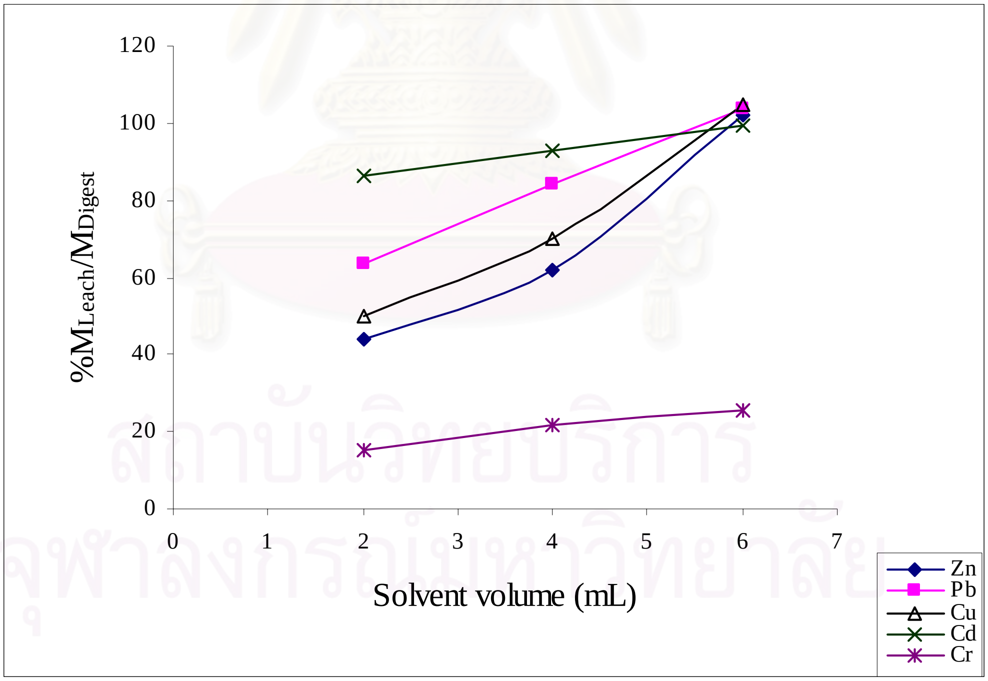


Figure 3-8. Effect of acid solvent volume to leach elements in fish samples (0.20 g of samples were treated with 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2)

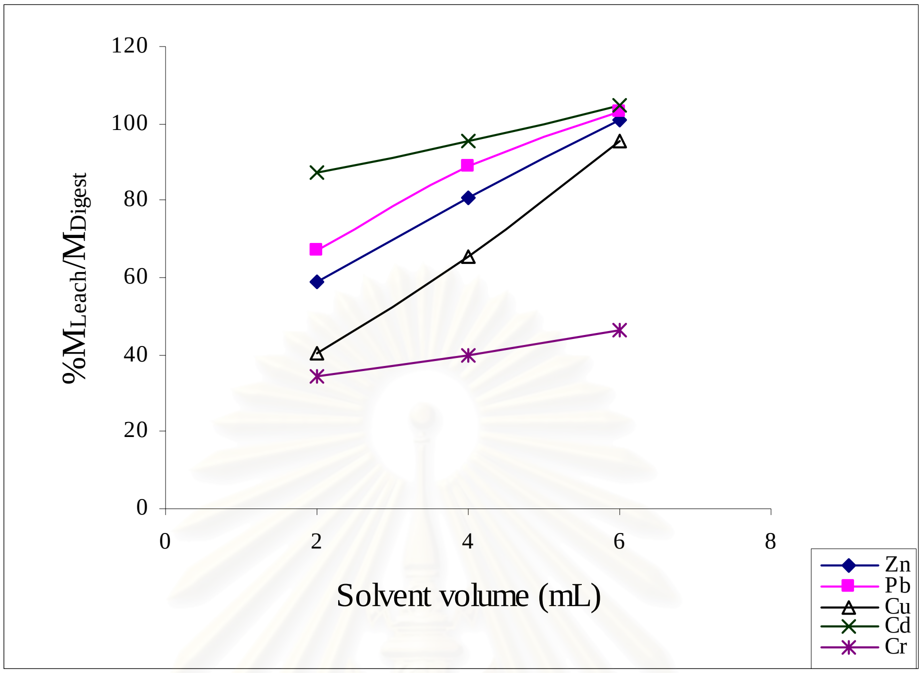


Figure 3-9. Effect of acid solvent volume to leach elements in mussel samples (0.20 g of samples were treated with 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2)

Figure 3.9 showed that increasing the solvent volume, Cr might be leached from the mussel tissues. But limit by limitation of instrument.

The different behavior offered by Cr could be related to the fact that acid solvent volume is not a significant parameter. Whilst acid solvent volume could be considered as a significant parameter for Zn, Pb, Cu and Cd leaching. Closed to 100% relation for Zn, Pb, Cu and Cd were found when 6 mL of acid solvent was used.

In conclusion, the optimum acid concentration of 3.7 M (nitric acid, hydrochloric acid), 0.5 M (hydrochloric acid), hydrogen peroxide concentration of 0.5 M (HNO_3: HCl: H_2O_2 = 1:1:1) and acid solvent volume of 6 mL were found. These proposed solvents were applied for fish and mussel samples. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr were presented in Table 3-4 and 3-5. These tables showed a good correlation between the results obtained for the use of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 and those obtained for the used of 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 except for Cr. The residual solids after acid leaching were digested by microwave digestion in closed vessels as mentioned in section 2.5 Chapter 2, and determined by FAAS in order to investigated the remained analytes concentration in residual solids.

Table 3-4. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr in fish and mussel samples by using 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1) mixed with 0.20 g of samples

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[a]	
	Fish	Mussel
Zn	100.67 ± 2.62	101.86 ± 1.83
Pb	103.76 ± 3.40	103.07 ± 2.27
Cu	101.93 ± 3.83	94.85 ± 1.38
Cd	100.01 ± 0.93	107.10 ± 0.97
Cr	26.00 ± 0.94	46.45 ± 0.58

[a] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 8)

Table 3-5. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr in fish and mussel samples by using 6 mL of 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 (1:1:1) mixed with 0.20 g of samples

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[a]	
	Fish	Mussel
Zn	101.12 ± 10.90	96.69 ± 1.71
Pb	103.96 ± 2.16	101.54 ± 1.78
Cu	103.13 ± 1.36	93.50 ± 0.21
Cd	103.67 ± 0.74	105.47 ± 0.36
Cr	5.71 ± 0.94	40.55 ± 0.50

[a] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

The results of remained concentration were shown in Table 3-6 and 3-7. Concentration found of Zn, Pb, Cu and Cd were not significant. This indicated that these elements were leachable from tissue. On the other hand, Cr could not be extracted by acid leaching extraction. However, acid solvent contained 3.7 M hydrochloric acid gave more Cr leaching efficiency than those contained 0.5 M hydrochloric acid.

Table 3-6. Concentrations of Zn, Pb, Cu, Cd and Cr that remained in the solid residues after treatment by 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1)

Elements	Fish			Mussel		
	Conc. of elements in initial solid sample (mg/kg)[a]	Conc. of elements in residues (mg/kg)	%M_{leach}/M_{digest}[b]	Conc. of elements in initial solid sample (mg/kg)[a]	Conc. of elements in residues (mg/kg)	%M_{leach}/M_{digest}[b]
Zn	26.85 ± 0.65	N.D.[c]	100.67 ± 2.62	54.54 ± 0.85	N.D.[c]	101.86 ± 1.83
Pb	129.41 ± 2.96	N.D.[c]	103.76 ± 3.40	270.59 ± 0.36	N.D.[c]	103.07 ± 2.27
Cu	395.26 ± 2.98	8.82	101.93 ± 3.83	420.59 ± 0.30	71.57	94.85 ± 1.38
Cd	240.20 ± 0.06	21.08	100.01 ± 0.93	186.76 ± 0.06	N.D.[c]	107.10 ± 0.97
Cr	465.69 ± 0.57	119.12	26.00 ± 0.940	890.20 ± 0.84	261.27	46.45 ± 0.576

[a] mean concentration (by microwave digestion) ± S.D. (n = 4)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 8)

[c] Not detectable

Table 3-7. Concentrations of Zn, Pb, Cu, Cd and Cr that remained in the solid residues after treatment by 6 mL of 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 (1:1:1)

Elements	Fish			Mussel		
	Conc. of elements in initial solid sample (mg/kg)[a]	Conc. of elements in residues (mg/kg)	$\%M_{leach}/M_{digest}$[b]	Conc. of elements in initial solid sample (mg/kg)[a]	Conc. of elements in residues (mg/kg)	$\%M_{leach}/M_{digest}$[b]
Zn	26.85 ± 0.65	3.43	101.12 ± 10.90	54.54 ± 0.85	12.74	96.69 ± 1.71
Pb	129.41 ± 2.96	N.D.[c]	103.96 ± 2.16	270.59 ± 0.36	N.D.[c]	101.54 ± 1.78
Cu	395.26 ± 2.98	4.41	103.13 ± 1.36	420.59 ± 0.30	73.04	93.50 ± 0.21
Cd	240.20 ± 0.06	14.22	103.67 ± 0.74	186.76 ± 0.06	N.D.[c]	105.47 ± 0.36
Cr	465.69 ± 0.57	254.41	5.71 ± 0.94	890.20 ± 0.84	574.51	40.55 ± 0.50

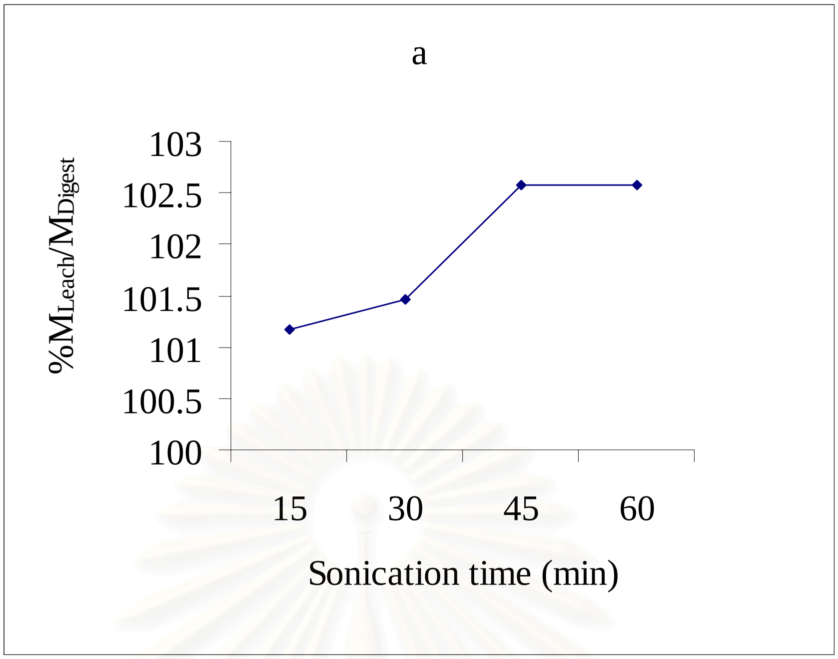
[a] mean concentration (by microwave digestion) ± S.D. (n = 4)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

[c] Not detectable

3.2.1.5 Effect of sonication time

To increase leaching efficiency for Cd (in fish) and Cu (in mussel) which slightly remained in solid residues, sonication time was evaluated for acid leaching extraction method. The exposure time was increased from 15 minutes to 30, 45 and 60 minutes. The solid residues were digested as described previously. It was found that increasing sonication time did not influence extraction efficiency for Cd and Cu although the amounts of Cu and Cd in residues were decreased but were not significant. Thus, 15 minutes of sonication time was recommended for further experiment. Figure 3-10 and 3-11 showed the results of Cd leaching from fish tissue and Cu leaching from mussel tissue, respectively.



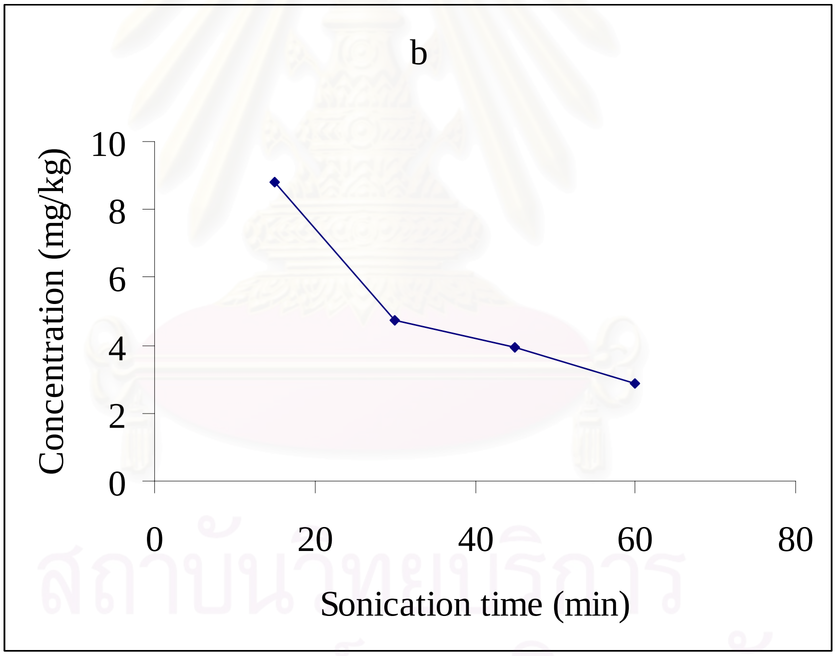
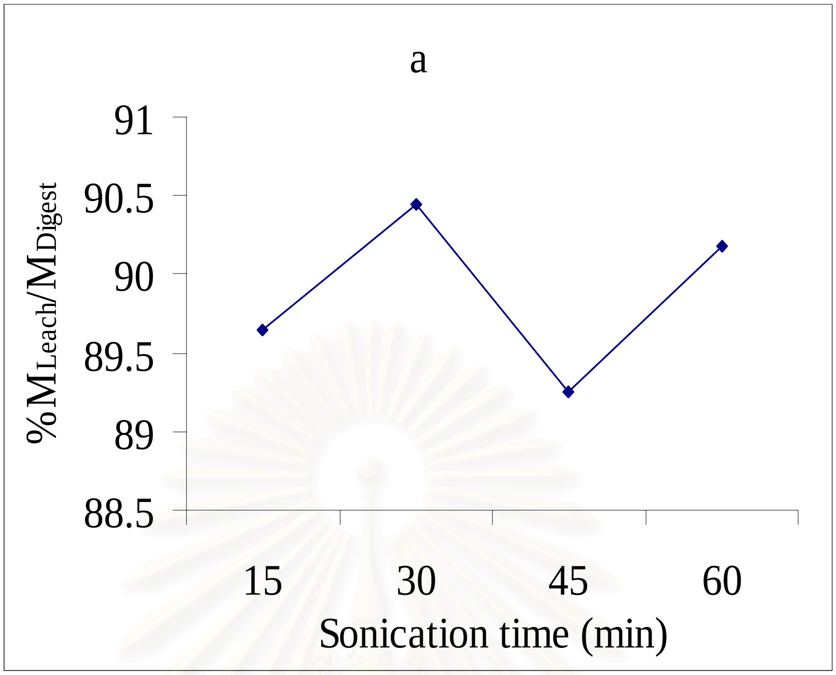


Figure 3-10. Effect of exposure time on the acid leaching extraction procedure (Cd in fish tissue)

a. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Cd in samples (0.20 g of samples were treated with 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2)

b. Concentration of Cd in residues



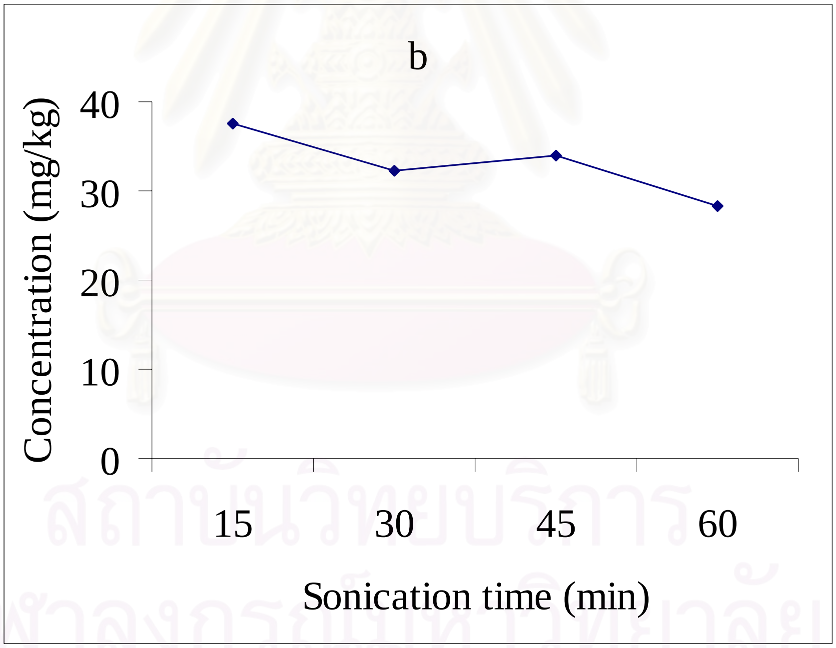


Figure 3-11. Effect of exposure time on the acid leaching extraction procedure (Cu in mussel tissue)

a. Concentration of Cu in samples (0.20 g of samples were treated with 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2)

b. Concentration of Cu in residues

3.2.2 Application to real samples

A solution of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1) and 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 (1:1:1) and a sonication time of 15 minutes provide quantitative leaching of Pb, Cd and Cu from 0.20 g of fortified samples. This procedure was used for real samples (fish and mussel) simultaneous preparation and analytical performances were shown in Table 3-8 and 3-9.

Table 3-8. Concentration of Pb, Cd and Cu from fish and mussel tissue, by microwave digestion method and acid leaching using 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1) mixed with 0.20 g of samples

Element	Concentration (mg/kg)[a]			
	Fish		Mussel	
	Microwave digestion	Acid leaching	Microwave digestion	Acid leaching
Pb	0.58 ± 0.01	N.D.[b]	0.44 ± 0.05	N.D.[b]
Cd	0.01 ± 0.01	N.D.[b]	0.62 ± 0.01	0.44 ± 0.04
Cu	1.28 ± 0.04	N.D.[b]	4.28 ± 0.48	2.56 ± 0.39

[a] mean concentration ± S.D. (n = 4)

[b] Not detectable

Table 3-9. Concentration of Pb, Cd and Cu from fish and mussel tissue, by microwave digestion method and acid leaching using 6 mL of 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 (1:1:1) mixed with 0.20 g of samples

Element	Concentration (mg/kg)[a]			
	Fish		Mussel	
	Microwave digestion	Acid leaching	Microwave digestion	Acid leaching
Pb	0.58 ± 0.01	N.D.[b]	0.44 ± 0.05	N.D.[b]
Cd	0.01 ± 0.01	N.D.[b]	0.62 ± 0.01	0.10 ± 0.45
Cu	1.28 ± 0.04	N.D.[b]	4.28 ± 0.48	1.41 ± 2.42

[a] mean concentration ± S.D. (n = 4)

[b] Not detectable

The results implied that these conditions were not able to leach metal from fish tissues but slightly leach from mussel tissues. It might be because of the difference of chemical bond between metal and sample matrix in fortified and real samples.

Although 3.7 M HNO_3/0 M HCl/0.5 M H_2O_2 (1:1:1) could be used as leaching solvent for the fortified samples, but this condition could not be applied for real samples. Because this condition was a weaker condition than 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1) and 3.7 M HNO_3/0.5 M HCl/0.5 M H_2O_2 (1:1:1). So we could omit this condition.

Variables such as temperature and sonication time could be considered. 6 mL of 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 (1:1:1) was chosen for further study.

3.2.2.1 Effect of temperature

Elke M.L. *et al.* [33] recommended that the temperature during sonication played an important role on acid leaching efficiency. It was possible to increase the dissolution of metal in acid solvent by increasing temperature.

Table 3-10 showed that concentration of Cu in leachates increased when high operating temperature was applied but not sufficient for quantitative extraction. The amounts of Pb and Cd in leachates were not affected by the temperature.

Table 3-10. Concentration of Pb, Cd and Cu from fish samples (operated at ambient and high temperature)[a]

Element	Concentration (mg/kg)[b]		
	Microwave digestion	Acid leaching	
		Ambient temperature	High temperature (~56 °C)
Pb	0.58 ± 0.01	N.D.[c]	N.D.[c]
Cd	0.01 ± 0.01	N.D.[c]	N.D.[c]
Cu	1.28 ± 0.04	N.D.[c]	0.40 ± 0.05

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

For mussel samples (Table 3-11), the amounts of Cu and Cd in leachates at high temperature were higher than ambient temperature. Cu concentration increased from 1.41 mg/kg to 3.64 mg/kg but standard deviation values were high.

Table 3-11. Concentration of Pb, Cd and Cu from mussel samples (operated at ambient and high temperature)[a]

Element	Concentration (mg/kg)[b]		
	Microwave digestion	Acid leaching	
		Ambient temperature	High temperature (~56 ^oC)
Pb	0.44 ± 0.05	N.D.[c]	N.D.[c]
Cd	0.62 ± 0.01	0.10 ± 0.45	0.48 ± 0.01
Cu	4.28 ± 0.48	1.41 ± 2.42	3.64 ± 0.04

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

However, no extraction of Pb in fish and mussel tissues was found. The analytical performances implied that operating temperature was an important parameter for Cu and Cd leaching.

3.2.2.2 Effect of sonication time

In this part, acid leaching at high temperature in function of sonication time was studied. The results were shown in Table 3-12 and 3-13 for fish and mussel tissues (real samples), respectively. As can be seen in table, an increasing in acid leaching efficiency for Cd and Cu with increasing the sonication time was found.

Table 3-12. Effects of sonication time (fish samples)[a]

Element	Concentration (mg/kg)[b]			
	Microwave digestion	Acid leaching		
		15 min	30 min	60 min
Pb	0.58 ± 0.01	N.D.[c]	N.D.[c]	N.D [c]
Cd	0.01 ± 0.01	N.D.[c]	0.06 ± 0.01	0.03 ± 0.01
Cu	1.28 ± 0.04	0.38 ± 0.02	1.16 ± 0.03	0.80 ± 0.02

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

Table 3-13. Effects of sonication time (mussel samples)[a]

Element	Concentration (mg/kg)[b]			
	Microwave digestion	Acid leaching		
		15 min	30 min	60 min
Pb	0.44 ± 0.05	N.D.[c]	N.D.[c]	N.D.[c]
Cd	0.62 ± 0.01	0.44 ± 0.01	0.55 ± 0.01	0.56 ± 0.02
Cu	4.28 ± 0.48	3.42 ± 0.07	4.25 ± 0.13	3.94 ± 0.03

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

Concentrations of Cd and Cu in samples were failed down slightly when sonication time was increased from 30 minutes to 60 minutes. This phenomenon might be occurred by re-adsorption (recommended by Mendez H. and co-workers) [47]. Cd and Cu in samples might be adsorbed on sample surface. On the other hand, Pb could not be leached from the sample tissue after 30 and 60 minutes treatment. Thus 30 minutes was chosen as operating time for Cu and Cd acid leaching.

3.2.2.3 Effect of acid solvent

As can be seen in previous results, Pb could not be leachable from the real samples. This indicated that Pb leaching might be required stronger conditions. In this study, the solution of 6 M (or 7.5 M) HNO_3/6 M HCl/0.5 M H_2O_2 (1:1:1) were used as acid solvents (recommended by Bermejo-Barrera P. *et al*) [34]. The results showed in Table 3-14 and 3-15.

Table 3-14. Pb concentration from fish and mussel samples by using 6 M HNO_3/6 M HCl/0.5 M H_2O_2 (1:1:1) and 7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 (1:1:1)[a] (operated at ambient temperature)

Condition	Pb concentration (mg/kg)[b]	
	Fish	Mussel
Microwave digestion	0.58 ± 0.01	0.44 ± 0.05
Acid leaching (6 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio)	N.D.[c]	N.D.[c]
Acid leaching (7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio)	N.D.[c]	N.D.[c]

[a] 0.20 g of samples were treated with 6 mL acid solvent

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

Table 3-15. Pb concentration from fish and mussel samples by using 6 M HNO_3/6 M HCl/0.5 M H_2O_2 (1:1:1) and 7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 (1:1:1)[a] (operated at 56 °C)

Condition	Pb concentration (mg/kg)[b]	
	Fish	Mussel
Microwave digestion	0.58 ± 0.01	0.44 ± 0.05
Acid leaching (6 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio)	N.D.[c]	N.D.[c]
Acid leaching (7.5 M HNO_3/6 M HCl/0.5 M H_2O_2 of 1:1:1 ratio)	N.D.[c]	N.D.[c]

[a] 0.20 g of samples were treated with 6 mL acid solvent

[b] mean concentration ± S.D. (n = 4)

[c] Not detectable

From the results, even if increasing the temperature and sonication time and the stronger acid solvents were used to promote the efficiency of Pb acid leaching technique. Pb still could not be leach from the samples.

3.2.3 Application of optimized condition

The optimized condition was shown in Table 3-16. This condition was used for Cd, Cu and Zn acid leaching extraction method. And real samples (fish and mussel) were treated with this condition.

Table 3-16. Optimized condition

Parameter	Optimum conditions
Acid solvent	3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio
Volume (mL)	6
Sample weight (g)	0.2
Sonication time (min)	30
Temperature (^{o}C)	~ 56

Cd, Cu and Zn were leached from fish and mussel samples with the optimum condition. The results were shown in Table 3-17.

Table 3-17. Concentrations of Cd, Cu and Zn from samples when optimized condition was used

Element	Concentration[a] (mg/kg)			
	Fish		Mussel	
	Microwave digestion	Acid leaching	Microwave digestion	Acid leaching
Cd[b]	0.01 ± 0.01	0.06 ± 0.01	0.62 ± 0.01	0.55 ± 0.01
Cu[b]	1.28 ± 0.04	1.16 ± 0.03	4.28 ± 0.48	4.25 ± 0.13
Zn[b]	28.82 ± 1.91	28.86 ± 0.87	56.58 ± 1.57	58.11 ± 0.98

[a] mean concentration ± S.D. (n = 4)

[b] Determination by GFAAS for Cd and Cu, Determination by FAAS for Zn

Moreover, the results from acid leaching were compared with the results from microwave-assisted digestion. Analytical performances were expressed in term of %ratio of metal amount obtained from leaching technique to amount determined by digestion technique and shown in Table 3-18.

Table 3-18. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Cd, Cu and Zn in fish and mussel samples

Element	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[a]	
	Fish	Mussel
Cd	114.12 ± 17.97	88.71 ± 0.40
Cu	92.02 ± 2.13	100.92 ± 3.22
Zn	100.42 ± 2.88	103.30 ± 4.69

[a] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

Concentration values of Zn from leaching method were closed to the values from microwave digestion method. Cd and Cu leachates from mussel samples were approached to concentrations from microwave digestion. Whilst Cd and Cu leachates from fish samples differed the concentrations from microwave digestion. But the difference was not significant. Paired *t*-test was used to confirm the assumption. [55]

3.2.4 Method validation

3.2.4.1 Precision

The relative standard deviation value (RSD) was used to define the precision. Under the optimum parameters, Cd and Cu were leached from samples. 6 independent batches and Zn was leached from samples 10 independent batches (Cd and Cu were measured by GFAAS and Zn was measured by FAAS). The precision of each analyte was shown in Table 3-19.

Table 3-19. %Relative standard deviation of Cd, Cu and Zn[a]

Elements	Fish		Mussel	
	Concentration (mg/kg)	%RSD	Concentration (mg/kg)	%RSD
Cd[b]	0.06	12.73	0.55	1.10
Cu[b]	1.16	2.85	4.24	3.04
Zn[b]	15.45	6.63	52.30	2.77

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 1:1:1 ratio

[b] Measured by GFAAS for Cd and Cu (n = 6), Measured by FAAS for Zn (n = 10)

For environmental and food samples, the precision is dependent on the samples matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20%.

The relative standard deviation values ranging from 3.23-13% for fish samples and 1.12-3.25% for mussel samples. They also indicated acceptable precision based on the AOAC standard. [53]

The acceptance criteria for precision were showed in Table 3-20.

Table 3-20. Estimated precision data as a function of analyte concentration [53]

Unit	RSD (%)
100%	1.3
10%	2.8
1%	2.7
0.1%	3.7
100 ppm	5.3
10 ppm	7.3
1 ppm	11
100 ppb	15
10 ppb	21
1 ppb	30

3.2.4.2 Limit of detection (LOD) and method detection limit (MDL)

The limit of detection and limit of quantification were investigated by determining the method blank solution. The solution was measured by GFAAS for Cd, Pb and Cu and by FAAS for Zn. The method detection limit established as the concentration (mg/kg) in fish and mussel samples, which provided an absorbance reading from the blank. The limit of detection was calculated by dividing three times the standard deviation of 10 absorbance readings of the reagent blank. The limit of detection and method detection limit of Pb, Cd, Cu and Zn were summarized in Table 3-21.

Table 3-21. MDL and LOD values (n = 10)

Element	MDL[a](mg/kg)	LOD[a](mg/L)
Pb	0.08	1.39
Cd	0.02	0.41
Cu	0.13	2.56
Zn	0.63	0.01

[a] Pb, Cd and Cu were measured by GFAAS and Zn was measured by FAAS

The calculated limit of detection and method detection limit of Pb, Cd, Cu and Zn were sufficiently low to allow the determination of these elements in real samples.

3.2.4.3 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in samples within a given range. The concentration range and R^2 were shown in Table 3-22.

Table 3-22. R^2 of Pb, Cd, Cu and Zn calibration curves (n = 5)

Element	Concentration range[a]	R^2
Pb	20-100 ng/mL	0.9988
Cd	2-10 ng/mL	0.9998
Cu	10-50 ng/mL	0.9988
Zn	0.5-2.5 μg/mL	0.9977

[a] Pb, Cd and Cu were measured by GFAAS and Zn was measured by FAAS

3.2.4.4 Accuracy

Certified reference material (CRM) was used to evaluate the accuracy of the method. The results were shown in Table 3-23.

Table 3-23. Analytical results obtained for the determination of Cd, Cu and Zn in a certified sample of dogfish muscle tissue, DORM-2 by AAS using acid leaching extraction method[a] and microwave-assisted digestion

Element	Certified values (mg/kg)	Found values (mg/kg)			
		Microwave digestion (n = 8)[b]	%Recovery	Acid leaching (n = 12)[b]	%Recovery
Cd [c]	0.043 ± 0.008	0.068 ± 0.02	158.14 ± 2.50	0.066 ± 0.01	153.49 ± 1.25
Cu [c]	2.34 ± 0.16	2.06 ± 0.077	88.03 ± 0.48	2.04 ± 0.15	87.18 ± 0.93
Zn [c]	25.6 ± 2.3	21.9 ± 2.2	85.55 ± 1.00	20.7 ± 0.89	80.86 ± 0.39

[a] 0.20 g of samples were treated with 6 mL 3.7 M HNO_3/3.7 M HCl/0.5 M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D.

[c] Cd and Cu were measured by GFAAS, Zn was measured by FAAS

Table 3-23 showed that, %recovery (leaching technique) of CRM for zinc and copper were ranged from 80-87%. From the Table 3-1, the obtained recovery values of zinc and copper were acceptable range. Whilst, found values from leaching technique of cadmium were higher than certified values. Thus indicating that cadmium could be contaminated from acid solvent. The use of high purity grade might solve this problem.

CHAPTER IV

CONCLUSION AND SUGGESTIONS FOR FURTHER STUDY

4.1 Conclusion

We are developing an acid leaching procedure for the multi-element extraction (lead, chromium cadmium, copper and zinc) provided a simple method for the simultaneous preparation of a number of samples without the need for careful control of the samples. The elemental in fish and mussel samples analysis was carried out using flame atomic absorption spectrophotometer (FAAS) and graphite furnace atomic absorption spectrophotometer (GFAAS). The experiments started with the use of fortified samples in order to obtain an optimized condition by means of the comparison with the results obtained from the microwave digestion in term of %Ratioof metal amount obtained from leaching technique to amount determined by digestion technique.

Successful acid leaching extraction involved the study of variables such as nitric acid, hydrochloric acid, hydrogen peroxide concentration, temperature, solvent volume and sonication time. The optimum condition was disclosed. 3.7 M HNO_3/3.7 M HCl/ 0.5 M H_2O_2 of 1:1:1 ratio was a suitable leaching solvent. Strength of the solvent was sufficienly to simultaneously extract zinc, copper and cadmium. Solvent volume was also an effective parameter. Increasing the volume allowed extraction of a large number of analytes. Caused by the limitation of instrument, the suitable volume was 6 mL. Zinc, copper and cadmium could be quantitatively recovered from 0.20 g of samples (fish and mussel). Another operating parameters; sonication time and operating temperature, were considered. Amounts of analytes increased continually in leachates when increasing sonication times. A 30-minute sonication time could be used due to a maximum %Ratioof metal amount obtained from leaching technique to amount determined by digestion technique. An operating temperature was a parameter promoting the efficiency of the acid leaching solvent. High temperature (56 °C) was applied with 30 minute ultrasonication. Whilst, this condition could not be used with chromium and lead extraction. The use of stronger condition might solve the problem.

The acid leaching extraction method had been demonstrated to be a rapid sample pre-treatment procedure. It could be carried out in one run in not over 50 minutes, 30 minutes for the ultrasonic process and 20 minutes for the centrifugation stage as compared with more than 1 hours for the microwave digestion method. Comparing with the procedure reported previously [34, 43], the present procedure lead to reduce the complexity of the leaching solution, avoiding the use of concentrated reagents (reduce the possibility of sample contamination) and reduce the sonication time from 120 to 30 minutes. Another advantage of the propose procedure is not to involve the use of high ultrasonic energy. In conclusion, the optimized condition of the acid leaching extraction procedure for determination of zinc, copper and cadmium in fish and mussel by FAAS/GFAAS:

- 0.20 g of homogenized sample
- 6 mL of acid leaching solvent (3.7 M HNO_3/3.7 M HCl/ 0.5 M H_2O_2 of 1:1:1 ratio)
- 30 minutes sonication time
- 56 °C of sonication temperature
- 10 mL final volume.

The method had been validated by determining zinc, copper and cadmium in DORM-2. %Recovery of CRM for zinc and copper were ranged from 80-87%. On the other hand, found values from leaching technique of cadmium were higher than certified values.

4.2 Suggestion for further work

This research provided some prospective points for the future work. Microwave assisted-acid leaching procedure is still awaited for further investigation. The application of this developed system should be verified. In addition, utrasound-assisted and microwave-assisted acid leaching extraction technique should be investigated for speciation analysis of some elements.

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APPENDICES

APPENDIX A

%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr

(Effect of nitric acid and hydrochloric acid concentration)

Table A1. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr for fish samples (Effect of nitric acid concentration)[a]

Element	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique						
	0 M	0.5 M	1 M	2 M	3 M	3.7 M	4 M
Zn	102.3 ± 1.16	100.13 ± 4.68	91.41 ± 2.95	83.43 ± 5.89	92.97 ± 2.69	94.93 ± 0.70	92.56 ± 2.42
Pb	104.17 ± 1.33	104.93 ± 1.33	101.41 ± 5.59	103.27 ± 4.46	101.40 ± 0.01	101.40 ± 0.01	103.27 ± 4.46
Cu	104.30 ± 0.83	104.57 ± 1.22	106.97 ± 1.16	106.10 ± 0.92	107.33 ± 0.85	107.32 ± 1.44	106.67 ± 0.93
Cd	100.01 ± 0.87	100.63 ± 0.83	103.10 ± 0.62	104.13 ± 0.16	104.10 ± 0.30	104.22 ± 0.32	103.77 ± 0.57
Cr	14.61 ± 0.59	16.32 ± 0.01	17.42 ± 0.58	20.43 ± 0.58	20.43 ± 0.58	26.78 ± 0.01	21.77 ± 0.01

[a] 0.20 g of samples were treated by 6 mL of acid solvent (concentration of hydrochloric acid and hydrogen peroxide was fixed at 3.7 M and 0.5 M, respectively)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n =4)

Table A2. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr for mussel samples (Effect of nitric acid concentration)[a]

Element	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique						
	0 M	0.5 M	1 M	2 M	3 M	3.7 M	4 M
Zn	94.36 ± 2.10	93.74 ± 2.61	99.57 ± 1.52	100.45 ± 1.41	100.34 ± 1.56	100.58 ± 1.24	98.78 ± 1.39
Pb	103.90 ± 0.01	102.87 ± 1.79	105.90 ± 1.91	105.90 ± 1.91	101.60 ± 0.01	105.90 ± 1.91	103.73 ± 1.85
Cu	94.40 ± 0.24	93.96 ± 1.37	94.43 ± 0.67	95.15 ± 1.12	94.86 ± 0.81	94.50 ± 0.64	94.28 ± 0.13
Cd	102.60 ± 0.35	102.87 ± 1.22	107.37 ± 0.29	107.70 ± 0.69	108.03 ± 0.99	107.37 ± 0.24	107.50 ± 0.60
Cr	42.31 ± 0.28	42.79 ± 0.27	44.42 ± 0.01	45.29 ± 0.30	46.51 ± 1.05	46.12 ± 0.50	46.34 ± 1.32

[a] 0.20 g of samples were treated by 6 mL of acid solvent (concentration of hydrochloric acid and hydrogen peroxide was fixed at 3.7 M and 0.5 M, respectively)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n =4)

Table A3. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr for fish samples (Effect of hydrochloric acid concentration)[a]

Element	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique						
	0 M	0.5 M	1 M	2 M	3 M	3.7 M	4 M
Zn	106.30 ± 1.85	108.73 ± 2.19	90.26 ± 1.86	91.41 ± 2.95	101.40 ± 0.69	106.93 ± 3.61	107.73 ± 3.23
Pb	108.00 ± 0.01	108.00 ± 0.01	93.97 ± 3.22	88.40 ± 6.43	86.55 ± 3.22	101.40 ± 0.01	99.54 ± 3.22
Cu	103.27 ± 1.17	103.77 ± 0.46	108.10 ± 0.69	106.80 ± 0.30	107.63 ± 0.34	107.32 ± 1.44	107.80 ± 0.35
Cd	104.27 ± 0.61	103.83 ± 1.10	104.20 ± 0.17	104.30 ± 0.41	103.83 ± 0.97	104.22 ± 0.32	103.70 ± 0.82
Cr	16.32 ± 0.01	15.63 ± 0.60	19.09 ± 0.58	20.09 ± 0.58	19.76 ± 0.01	26.78 ± 0.01	20.43 ± 0.58

[a] 0.20 g of samples were treated by 6 mL of acid solvent (concentration of nitric acid and hydrogen peroxide was fixed at 3.7 M and 0.5 M, respectively)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n =4)

Table A4. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr for mussel samples (Effect of hydrochloric acid concentration)[a]

Element	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique						
	0 M	0.5 M	1 M	2 M	3 M	3.7 M	4 M
Zn	97.27 ± 2.10	95.90 ± 0.81	99.85 ± 1.91	98.36 ± 1.61	98.74 ± 1.25	100.97 ± 0.26	96.91 ± 3.41
Pb	101.83 ± 1.79	102.87 ± 1.79	100.70 ± 0.01	101.67 ± 1.67	102.63 ± 1.67	103.60 ± 0.01	104.57 ± 1.67
Cu	93.97 ± 0.65	93.54 ± 0.24	92.69 ± 0.98	91.97 ± 0.37	93.66 ± 0.60	94.95 ± 0.61	94.95 ± 0.98
Cd	106.07 ± 0.67	105.63 ± 0.24	109.03 ± 0.74	108.17 ± 0.67	109.07 ± 0.35	108.60 ± 0.56	109.37 ± 0.07
Cr	43.31 ± 0.28	43.57 ± 0.10	44.94 ± 0.52	45.81 ± 0.61	45.29 ± 0.25	46.12 ± 0.50	46.51 ± 0.01

[a] 0.20 g of samples were treated by 6 mL of acid solvent (concentration of nitric acid and hydrogen peroxide was fixed at 3.7 M and 0.5 M, respectively)

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n =4)

APPENDIX B

%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr (Leaching by deionized water and Effect of acid solvent volume)

Table B1. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr from fish and mussel samples (Leaching by deionized water)[a]

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[b]			
	Fish samples		Mussel samples	
	DI water	Acid[c]	DI water	Acid[c]
Zn	N.D.[d]	101.78 ± 0.92	55.60 ± 0.34	100.44 ± 1.01
Pb	N.D.[d]	105.05 ± 2.50	6.21 ± 1.22	104.88 ± 1.25
Cu	7.07 ± 0.77	98.66 ± 1.39	12.53 ± 0.28	93.65 ± 0.78
Cd	14.36 ± 0.23	99.54 ± 0.32	66.66 ± 0.73	106.48 ± 1.06
Cr	N.D.[d]	26.78 ± 0.01	15.05 ± 0.74	46.12 ± 0.50

[a] 0.20x g of samples were treated by 6 mL of deionized water

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique ± S.D. (n = 4)

[c] 6 mL of 3.7 M HNO_3/ 3.7M HCl/ 0.5M H_2O_2 was used as medium

[d] Not detectable

Table B2. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr from fish samples (Effect of acid solvent volume)[a]

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[b]		
	2 mL	4 mL	6 mL
Zn	43.95 ± 3.86	61.76 ± 3.76	102.06 ± 2.82
Pb	63.35 ± 1.95	84.19 ± 1.12	103.77 ± 1.10
Cu	50.17 ± 0.94	69.97 ± 1.28	104.73 ± 0.91
Cd	86.14 ± 0.68	92.83 ± 1.13	99.52 ± 0.59
Cr	15.15 ± 0.58	21.91 ± 0.01	25.28 ± 1.18

[a] 0.20 g of samples were treated with 3.7 M HNO_3/3.7M HCl/0.5M H_2O_2 of 1:1:1 ratio

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique + S.D.[c] (n = 4)

Table B3. %Ratio of metal amount obtained from leaching technique to amount determined by digestion technique of Zn, Pb, Cu, Cd and Cr from mussel samples (Effect of acid solvent volume)[a]

Elements	%Ratio of metal amount obtained from leaching technique to amount determined by digestion technique[b]		
	2 mL	4 mL	6 mL
Zn	58.82 + 1.23	80.92 ± 1.03	100.93 ± 1.04
Pb	67.01 + 2.63	89.00 ± 1.52	103.07 ± 3.06
Cu	40.19 + 1.98	65.38 ± 1.42	95.23 ± 0.82
Cd	87.22 + 0.96	95.43 ± 0.89	105.00 ± 1.25
Cr	34.53 + 0.26	39.67 ± 0.27	46.21 ± 0.27

[a] 0.20 g of samples were treated with 3.7 M HNO_3/3.7M HCl/0.5M H_2O_2 of 1:1:1 ratio

[b] mean %ratio of metal amount obtained from leaching technique to amount determined by digestion technique + S.D.[c] (n = 4)

APPENDIX C

Concentration of Cd and Cu in leachates and solid residues
(Effect of sonication time)

Table C1. Effects of sonication time on acid leaching extraction technique[a]

Time (min)	Cd concentration in fish[b]		Cu concentration in mussel[b]	
	Concentration in leachate (ppm)	Concentration in residue (ppm)	Concentration in leachate (ppm)	Concentration in residue (ppm)
15	4.73 ± 0.01	0.18 ± 0.01	5.47 ± 0.03	0.77 ± 0.06
30	4.74 ± 0.01	0.10 ± 0.02	5.52 ± 0.01	0.65 ± 0.03
45	4.79 ± 0.03	0.08 ± 0.01	5.46 ± 0.06	0.69 ± 0.01
60	4.79 ± 0.03	0.06 ± 0.01	5.51 ± 0.03	0.57 ± 0.01

[a] 0.20 g of samples were treated with 6 mL of 3.7 M HNO_3/ 3.7M HCl/ 0.5M H_2O_2 of 1:1:1 ratio

[b] mean concentration ± S.D. (n = 4)

APPENDIX D

Raw data of method validation

Table D1. Concentration of cadmium and copper in fish and mussel samples[a] (precision)

Portion	Cd concentration (ppb)[b]		Cu concentration (ppb)[b]	
	Fish	Mussel	Fish	Mussel
1	1.17	11.30	23.61	91.72
2	0.87	11.33	23.30	85.81
3	1.19	11.09	24.52	86.66
4	1.24	11.36	24.19	84.45
5	1.17	10.96	23.19	85.90
6	0.99	11.24	22.90	88.75

[a] 0.20 g of samples were treated with 6 mL of 3.7 M HNO_3/ 3.7M HCl/ 0.5M H_2O_2 of 1:1:1 ratio

[b] Determined by GFAAS

Table D2. Concentration of zinc in fish and mussel samples[a] (precision)

Portion	Zn concentration (ppm)[b]	
	Fish	Mussel
1	0.031	1.06
2	0.30	1.04
3	0.32	1.05
4	0.32	1.03
5	0.28	1.08
6	0.32	1.04
7	0.34	1.03
8	0.32	1.04
9	0.28	1.04
10	0.30	1.05

[a] 0.20 g of samples were treated with 6 mL of 3.7 M HNO_3/ 3.7M HCl/ 0.5M H_2O_2 of 1:1:1 ratio

[b] Determined by FAAS

Table D3. Analytical performances of DORM-2 (by acid leaching)

Portion	Concentration (ppm)		
	Cd[a]	Cu[a]	Zn[b]
1	0.058	2.04	20.5
2	0.070	2.04	19.6
3	0.071	1.78	21.4
4	0.071	2.18	19.5
5	0.069	2.43	21.1
6	0.061	2.04	19.6
7	0.060	1.94	20.0
8	0.051	2.07	20.8
9	0.043	1.95	20.3
10	0.094	2.03	21.6
11	0.083	1.98	21.4
12	0.061	2.06	22.1

[a] Determined by GFAAS

[b] Determined by FAAS

Table D4. Analytical performances of DORM-2 (by microwave digestion)

Portion	Concentration (ppm)		
	Cd[a]	Cu[a]	Zn[b]
1	0.082	2.12	26.6
2	0.034	2.10	22.2
3	0.088	2.00	19.8
4	0.076	2.15	20.6
5	0.035	2.05	21.0
6	0.052	2.12	22.4
7	0.088	1.92	20.2
8	0.087	2.04	22.2

[a] Determined by GFAAS

[b] Determined by FAAS

APPENDIX E

Significance tests

Paired *t*-Test [54-55]

It frequently happens that two methods of analysis have to be compared by studying test samples containing substantially different amount of analyte. This difficulty is overcome by looking at the difference between each pair of results given by the two methods. Adopting the null hypothesis that there is no significant difference in the mean concentrations given by the two methods, we can test whether the mean of the differences differs significantly from zero.

$$t = \frac{\overline{x_d}}{s_d}\sqrt{n}$$

Where x_d = mean difference
s_d = standard deviation of the differences
n = number of data
t has (n-1) degrees of freedom

Data was rejected when the experimental values of *t* more than the critical values of *t*. Table E1 presented the critical values of *t*.

Table E1. The *t*-distribution

Value of *t* for a confidence interval of	90%	95%	98%	99%
Critical values of *t* for P values of	0.10	0.05	0.02	0.01
Number of degrees of freedom				
1	6.31	12.71	31.82	63.66
2	2.92	4.30	6.96	9.92
3	2.35	3.18	4.54	5.84
4	2.13	2.78	3.75	4.60
5	2.02	2.57	3.36	4.03
6	1.94	2.45	3.14	3.71
7	1.89	2.36	3.00	3.50
8	1.86	2.31	2.90	3.36
9	1.83	2.26	2.82	3.25
10	1.81	2.23	2.76	3.17
12	1.78	2.18	2.68	3.05
14	1.76	2.14	2.62	2.98
16	1.75	2.12	2.58	2.92
18	1.73	2.10	2.55	2.88
20	1.72	2.09	2.53	2.85
30	1.70	2.04	2.46	2.75
50	1.68	2.01	2.40	2.68
∞	1.64	1.96	2.33	2.58

Concentration of Cd, Cu and Zn in samples from acid leaching are not significant different with the results from microwave digestion. We prove all results by using paired *t*-test, as illustrated by the table E2-E7.

Table E2. The concentration of cadmium (ppb) in fish determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	1.06	1.17	-0.11
2	0.94	0.99	-0.05
3	0.77	0.87	-0.10
4	0.97	1.24	-0.27
		Mean	-0.13

The standard deviation of the differences is 0.08. Substituting in above equation gives t = 3.11. The critical value of t is 3.18 (P=0.05) and since the calculated value of t is more than this the null hypothesis is retained.

Table E3. The concentration of cadmium (ppb) in mussel determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	12.54	11.33	1.21
2	12.36	11.36	1.00
3	11.22	11.24	0.02
4	14.88	11.30	3.58
	$t_{\text{exp.}}$ = 1.89 less than $t_{\text{cri.}}$	Mean	1.44

Table E4. The concentration of copper (ppb) in fish determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	24.88	24.52	0.36
2	26.92	24.19	2.73
3	26.14	23.61	2.53
4	25.98	23.30	2.68
	$t_{\text{exp.}}$ = 2.03 less than $t_{\text{cri.}}$	Mean	1.40

Table E5. The concentration of copper (ppb) in mussel determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	87.20	91.75	-4.55
2	87.26	88.75	-1.49
3	89.26	85.90	3.36
4	85.23	85.81	-0.58
	$t_{\text{exp.}}$ = 0.50 less than $t_{\text{cri.}}$	Mean	-0.81

Table E6. The concentration of zinc (ppm) in fish determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	1.11	1.23	-0.12
2	1.11	1.12	-0.01
3	1.11	1.17	-0.06
4	1.15	1.16	-0.01
	$t_{\text{exp.}}$ = 3.10 less than $t_{\text{cri.}}$	Mean	-0.06

Table E7. The concentration of zinc (ppm) in mussel determined by two different methods for each of four-test portion

Portion	Microwave digestion	Acid leaching	Difference
1	0.62	0.59	0.03
2	0.54	0.60	-0.06
3	0.58	0.57	0.01
4	0.58	0.58	0.00
	$t_{\text{exp.}}$ = 0.12 less than $t_{\text{cri.}}$	Mean	-0.02

Application of paired *t*-test is also used to prove that hydrogen peroxide was significant variable effecting the Cu acid leaching from fish and mussel samples, as illustrated by the table A8-A9.

Table E8. The concentration of copper (ppm) in fish determined by two types of acid solvent for each of four-test portion

Portion	Add H_2O_2	No H_2O_2	Difference
1	7.52	6.65	0.87
2	7.73	6.92	0.81
3	7.75	6.77	0.98
4	7.67	6.71	0.96
		Mean	0.90

The standard deviation of the differences is 0.08. Substituting in above equation gives *t* = 22.50. The critical value of *t* is 3.18 (P=0.05) and since the calculated value of *t* is more than this the null hypothesis is rejected.

Table E9. The concentration of copper (ppm) in mussel determined by two types of acid solvent for each of four-test portion

Portion	Add H_2O_2	No H_2O_2	Difference
1	8.26	7.28	0.98
2	7.95	7.34	0.61
3	7.89	7.22	0.67
4	8.07	7.23	0.84
		Mean	0.78

The experiment value (9.17) more than the critical value of *t*. So the null hypothesis is also rejected.

APPENDIX F

Conventional acid digestion procedure for the Zn, Pb and Cu determination in mussel samples

F1 Chemicals and apparatus

F1.1 Nitric acid 65% (AR grade, Lab Scan, Ireland)
F1.2 Sulfuric acid 96-97% (AR grade, Merck, Germany)
F1.3 Hydrogen peroxide 30% (Carlo Erba Reagenti, Italy)
F1.4 Hot plate (Corning, USA)
F1.5 Sand bath
F1.6 Beaker 100 mL (Schott duran, Germany)
F1.7 Volumetric flask 25 mL (Witeg, USA)
F1.8 Plastic bottle (LDPE, Nalgene, Canada)
F1.9 Micropipette (SL 1000, RAININ, USA)

F2 Procedure

1.5 g of fresh sample was weighted into a 100 mL beaker. A 10 mL aliquot of mixture HNO_3 and H_2SO_4 1:1 ratio was added, and the sample was cautiously heated on a hot plate until any vigorous reaction subsided (heated at 120 ^{o}C for 5 hours). After cooling, 10 mL of HNO_3 was added and the sample solution was heated on the hot plate at 160 ^{o}C for 2 hours. After the solution was cooled, 2 mL of H_2O_2 was added and the solution was heated at 160 ^{o}C for an additional 1 hours. The cooled solution was brought into a 25 mL of volumetric flask. The solution volume was adjusted deionize water. The sample solution was kept in Nalgene bottle at 4 ^{o}C until analysis.

For spiking test, the procedure was similar as above with the following exception: an appropriate volume of analytes (Zn, Cu, Pb, Cd and Cr) that known exactly concentration were also added.

F3 Determination

The samples were run in atomic absorption spectrometry without further dilution. The calibration curve was made with 10 mL of mixture HNO_3 and H_2SO_4 1:1 ratio and deionize water. The concentrations of metals (Zn, Pb and Cu) were calculated from a linear regression equation.

VITA

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