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Determination of heavy metals in fish and seafood

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science in Forensic Science

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ABSTRACT

The aim of this study was to determine concentrations level of heavy metals (zinc, copper, cadmium and lead) in edible parts of fish and seafood. The levels of these metals in fish (6) and seafood (4) purchased from the Kelantan markets were measured. The conventional wet acid digestion method was used for the mineralization of these samples prior to the quantitative determination by graphite furnace atomic absorption spectrometry (AOAC International, 2002). The heavy metal concentrations in the fish ranged from (0.020 ± 0.01 to 0.900 ± 0.12 µg/g ww) for Cu, (2.593 ± 0.36 to 9.610 ± 0.08 µg/g ww) for Zn, (0.025 ± 0.0025 to 0.150 ± 0.0014 µg/g ww) for Cd and (0.004 ± 0.014 to 0.110 ± 0.026 µg/g ww) for Pb. Meanwhile, the heavy metal concentrations in seafood ranged from (0.440 ± 0.06 to 9.300 ± 0.10 µg/g ww) for Cu, (1.669 ± 0.46 to 9.800 ± 0.23 µg/g ww) for Zn, (0.340 ± 0.0032 to 0.930 ± 0.0023 µg/g ww) for Cd and (0.006 ± 0.017 to 0.180 ± 0.029 µg/g ww) for Pb. In average, all heavy metals in seafood were higher than in fish. This study also showed that the heavy metals concentration in demersal fish and seafood were higher compared to pelagic species. The metal concentrations in the pelagic species ranged from (0.020 ± 0.01 to 0.672 ± 0.24 µg/g ww) for Cu, (2.593 ± 0.36 to 5.693 ± 0.16 µg/g ww) for Zn, (0.025 ± 0.0025 to 0.041 ± 0.0023 µg/g ww) for Cd, and (0.004 ± 0.014 to 0.005 ± 0.022 µg/g ww) for Pb. Meanwhile, the metal concentrations in demersal species ranged from (0.440 ± 0.06 to 9.300 ± 0.10 µg/g ww) for Cu, (1.669 ± 0.46 to 9.800 ± 0.23 µg/g ww) for zinc, (0.130 ± 0.0023 to 0.930

± 0.0023 µg/g ww) for Cd and (0.006 ± 0.017 to 0.180 ± 0.029 µg/g ww) for Pb. Comparison of heavy metals concentration between pelagic and demersal species were difficult due to the lack of literature information from other studies at more global scale. However, all the average values of these metals from all populations were lower than guidelines and regulations stipulating maximum permissible levels set by the Malaysian Food Act 1983 and Food Regulations 1985 Fourteen Schedule. These metals concentration found in fish and seafood were also considered to be lower compared with literature in other studies. Thus, these concentration levels should result in no acute toxicities of heavy metals since they are lower than the limit. It was concluded that heavy metals concentration level in commercial fish and seafood must be monitored comprehensively and periodically with respect to the consumer health.

ABSTRAK

Tujuan kajian ini adalah untuk menentukan paras kepekatan logam berat (zink, kuprum, kadmium dan plumbum) yang terkandung dalam ikan dan makanan laut. Paras kepekatan logam berat dalam ikan (6) dan makanan laut (4) yang dibeli dari pasar-pasar di Kelantan telah ditentukan. Kaedah konvensional penghadaman asid basah (wet acid digestion) telah digunakan sebelum penentuan kuantitatif menggunakan graphite furnace atomic absorption spectrometry (AOAC International 2002). Kepekatan logam berat dalam ikan adalah dalam lingkungan (0.020 ± 0.01 hingga 0.900 ± 0.12 µg/g ww) bagi Cu, (2.593 ± 0.36 hingga 9.610 ± 0.08 µg/g ww) bagi Zn, (0.025 ± 0.0025 hingga 0.150 ± 0.0014 µg/g ww) bagi Cd dan (0.004 ± 0.014 hingga 0.110 ± 0.026 µg/g ww) bagi Pb. Sementara, kepekatan logam berat dalam makanan laut adalah dalam lingkungan (0.440 ± 0.06 hingga 9.300 ± 0.10 µg/g ww) bagi Cu, (1.669 ± 0.46 hingga 9.800 ± 0.23 µg/g ww) bagi Zn, (0.340 ± 0.0032 hingga 0.930 ± 0.0023 µg/g ww) bagi Cd dan (0.006 ± 0.017 hingga 0.180 ± 0.029 µg/g ww) bagi Pb. Secara purata , kepekatan logam berat dalam makanan laut lebih tinggi daripada ikan. Kajian ini turut menunjukkan kepekatan logam berat dalam ikan dan makanan laut demersal adalah lebih tinggi jika dibandingkan dengan spesis pelagik. Kepekatan logam dalam spesis pelagik adalah dalam lingkungan (0.020 ± 0.01 hingga 0.672 ± 0.24 µg/g ww) bagi Cu, (2.593 ± 0.36 hingga 5.693 ± 0.16 µg/g ww) bagi Zn, (0.025 ± 0.0025 hingga 0.041 ± 0.0023 µg/g ww) bagi Cd, dan (0.004 ± 0.014 hingga 0.005 ± 0.022 µg/g ww) bagi Pb. Sementara, kepekatan logam dalam

spesis demersal adalah dalam lingkungan (0.440 ± 0.06 hingga 9.300 ± 0.10 µg/g ww) bagi Cu, (1.669 ± 0.46 hingga 9.800 ± 0.23 µg/g ww) bagi zinc, (0.130 ± 0.0023 hingga 0.930 ± 0.0023 µg/g ww) bagi Cd dan (0.006 ± 0.017 hingga 0.180 ± 0.029 µg/g ww) bagi Pb. Perbandingan kepekatan logam berat di antara spesis pelagik dan demersal adalah sukar disebabkan kekurangan informasi mengenainya daripada literasi kajian lain di skala yang lebih global. Bagaimanapun, nilai purata bagi kesemua logam berat bagi kesemua populasi adalah rendah jika dibandingkan dengan garis panduan dan peraturan limit maksimum yang telah ditetapkan oleh Malaysian Food Act 1983 dan Food Regulations 1985 Fourteen Schedule. Kepekatan logam berat yang dijumpai dalam ikan dan makanan laut ini juga adalah rendah jika dibandingkan dengan literasi daripada kajian lain. Oleh yang demikian, paras kepekatan ini seharusnya tidak menyebabkan toksik logam berat yang teruk kerana nilainya adalah rendah daripada limit. Secara kesimpulannya, paras kepekatan logam berat dalam ikan dan makanan laut komersial mestilah dipantau dari masa ke semasa bagi kesihatan pengguna.

CHAPTER I

INTRODUCTION

1.0 Fish and Seafood Controversial Issues.

Fish is widely consumed in many parts of the world by humans because it has high protein content, low saturated fat and also contains omega fatty acids known to support good health (US EPA, 2004). Fish also contains advantageous fatty acid profile, resulting from the consistent content of essential polyunsaturated fatty acids, such as eicosapentaenoic and docosahexaenoic acid, known to support good health (Usydus *et al.,* 2009). It is estimated that the consumption of one portion of fatty fish, daily, delivers about 900 mg/day of n-3 acids, with consequent reduction of mortality in patients with coronary diseases (Kris-Etherton *et al.,* 2002).

Nutritionists also consider these products to be an important source of high-quality proteins, minerals, vitamin D and essential fatty acids such as omega 3 (Me´dale *et al.,* 2003), although only half the population follows the recommendation of the National Nutrition and Health Program (PNNS) to consume fish at least twice a week (Ministe`re Franc- ais de l'Emploi et de la Solidarite´ and Ministe`re de´ le´gue´ a` la Sante´, 2001).

Meanwhile, seafood such as scallops, shrimps, crab, and other crustaceans and mollusk contain quite high concentrations of carotenoids (Britton *et al.* 2009), low levels of fat, essential amino acids (Holland *et al.* 1993) and also possess a relatively

higher content of vitamins and minerals (Caballero, 2009). For an instance, bivalve mollusks have much higher concentration of vitamins such as folate, and cobalamin, whereas minerals such as magnesium, phosphorus, iron, selenium and iodine (Caballero, 2009).

Today it is generally accepted that fish and seafood is important in a healthy and balanced omnivorous human diet (World Health Organization, 2003). However, the evaluation of risks and benefits of the consumption of these products has been particularly controversial. Even though, fish and seafood are very important human foods, but they exposed to chemicals in polluted and contaminated waters (Ikem *et al.,* 2005). Toxicologists tend to regard fish and seafood as a major vector for toxic substances such as metal trace elements and persistent organic pollutants (Guérin *et al.*, 2011). The bioaccumulation of heavy metals by fish and seafood make these food items can be a rich source of metal (Buzina *et al.,* 1989; Piotrowski *et al.,* 1981). The interactions of heavy metals with usual elements from diet have an important role in acute and chronic toxicity (Leblanc *et al.,* 2005). The most important forms of aquatic pollution are heavy metals since they accumulate in aquatic organisms and may transfer to humans in the food chain (Ashraf *et al.,* 2006).

Make thing worst, heavy metals are non-biodegradable inorganic chemicals, so it cannot be metabolized and will not break down into harmless forms (Kromhout *et al.,* 1985). For an instance the study done by Filov *et al.,* (1993) showed that, the elimination rate of cadmium is so slow (an average 2.00 mg/day) and prolonged excessive cadmium ingestion will cause cadmium accumulation inside the human body. By that, population can be contaminated with heavy metals by ingestion these

contaminated fish and seafood. Thus, exposure to heavy metals is an important problem of environmental toxicology since most of these metals are toxic to humans, animals and plants.

Because of a greater awareness of this problem, this present study has been interested in determining the heavy metals in marine fish and seafood. Even if the waters around the study area are not yet polluted, still we need to find the existing levels of heavy metals in commercial fish and seafood to know whether these metals concentrations are within the safety limits that had been set by the country or otherwise. For the above reasons, the concentration of heavy metals cadmium (Cd), copper (Cu), zinc (Zn) and lead (Pb) in common species of fish and seafood were determined in this present study. The elements were determined in samples of edible muscle tissue of each species.

CHAPTER II

LITERATURE REVIEW

2.0 Heavy Metals in Fish and Seafood

According to Lawley *et al.,* (2008), the term "heavy metal" refers to any relatively high-density metallic element that is toxic or poisonous even at low concentrations. These elements have no known bio-importance in human biochemistry and physiology, and consumption, even at very low concentrations, can cause toxic effects, because they tend to accumulate in the human body over time. Heavy metals are natural components of the earth's crust and cannot be destroyed.

Based on National Research Council (U.S.), Committee on Diet and Health (1989), heavy metal contaminants with major potential for toxicity in the committee's view are antimony, arsenic, cadmium, chromium, lead, mercury and nickel. Contaminants with a modest potential for toxicity include copper, iron, manganese, selenium and zinc. Those of minor or no toxicity are aluminum, silver, strontium, thallium and tin. This classification is based, among other parameters, on potency for producing effects and accessibility of the toxicant.

Because of these heavy metals potential toxicity, regulatory bodies throughout the world have set a limit on the acceptable amounts of these contaminations in certain foods. As in our country, a safeguard for human health, guidelines and regulations

stipulating maximum permissible levels of cadmium, lead, zinc and copper in fish and seafood have been set by Malaysian Food Act 1983 and Food Regulations 1985 Fourteen Schedule to limit dietary exposure of consumers to toxic metals.

Evidence exists that heavy metals in the environment has increased during the past 200 years and it is not surprising that it can be found as a contaminant of aquatic animals (Shukla *et al.,* 1973). This statement is supported by some researchers who have found heavy metals including mercury, lead, cadmium (Sharif *et al.,*1991, 1993), chromium, nickel, silver, arsenic, zinc (Edwards *et al.,* 2001), and copper (Cohen *et al.,* 2001) in fish. Recently, in a study performed in Catalonia (Spain) they found that among 11 analyzed food groups, fish and seafood showed the highest concentrations of arsenic, cadmium, mercury, and lead (Llobet *et al,* 2003).

2.1 Sources of Heavy Metals

According to Lawley *et al.,* (2008), heavy metals can be present in marine environment naturally. As in seawater itself, it contains trace quantities of most of the elements. Among the main toxic heavy metals in seawater, arsenic (2.6 ppb in seawater), selenium (0.0 ppb), manganese (0.4 ppb), chromium (0.2 ppb), mercury (0.15 ppb) and cadmium (0.11 ppb) are of concern. Sediments are considered the ultimate sink for metals in aquatic environments including bays, estuaries and the ocean (Clements, 1991) or come from geological process such as volcanos and erosion (Turekian, 1968).

Heavy metals can also be present in marine environment as a result of human activities, such as mining, irrigation, energy extraction, agricultural practices, incineration, industrial emissions and car exhausts. They may also originate from contamination during manufacturing, processing and storage or from direct addition (Lawley *et al.*, 2008). Phase of industrialization and other anthropogenic activities in the vicinity of coastal areas are always positively associated with elevated level trace metals in marine biota (Eisler *et al.,* 1978; Philips, 1978). Solid and liquid wastes emanating from the industrial activities such as semiconductors, wood preservatives, and also from oil-based products are the inevitable by products of manufacturing process (Alkarkhi *et al.,* 2008). These wastes contain toxic chemicals such as chromium salts, sulfides and other substances including heavy toxic trace metals (Tariq *et al.,* 2006). These heavy metals are stable elements and persist for long periods in the environment (Lawley *et al.*, 2008).

The discharge of effluents and associated toxic compounds into aquatic systems represents an ongoing environmental problem due to their possible impact on communities in the receiving aquatic water and a potential effect on human health (Canivet *et al.,* 2002). Especially in highly polluted and industrial areas, point and non-point sources of anthropogenic chemicals and metals have polluted rivers with highly complex mixtures of chemicals and other anthropogenic perturbations to degree where life in rivers is severely impacted (Smolders *et al.,* 2004). The use of coastal waters as a convenient receptacle for domestic and industrial wastes threatens the quality of seafood rivers and coastal waters are presently exposed not only to increasing quantities of natural materials such as metals and nutrients, but

also to cocktails of industrial derived contaminants, many of which exhibit significant persistence and capabilities for bioaccumulation (Sawyer *et al.,* 1994). In addition, the increasing in population density and the intensification of agricultural activities in certain area are among the main causes of water pollution (Department of Environment, Annual Report 1991).

Ultimately, heavy metals found in the environment gets concentrated in bodies of water are eventually absorbed by fish in the water (Jacob *et al.,* 2008). As Clement, (1991) state that fish accumulate metals from their food, as well as water that passes over their gills and through their gut, and some laboratory studies have shown that water is the primary route of exposure. However this finding is controversial. Other studies indicate that food is the primary route of exposure (Douben, 1989).

2.2 Heavy Metals and Health Effects

Excessive levels of heavy metals in marine organisms pose the main risk to people. In study done by Guérin *et al.,* (2007); Sirot *et al.,* (2008b, 2008c, 2009), found that the highest consumers of fish and seafood in their study population present a non-negligible probability of exceeding the reference toxicological values for heavy metals consumed. However, not all people are equally susceptible to the effects of heavy metals that can occur in seafood. Fetus and young children are the most sensitive to the effects of metals, but adults can be affected as well (Gochfeld, 2003; Hightower *et al.,* 2003). Even within life stages (fetus, child adult), there are differences in sensitivity or susceptibility to contaminants. Cases of acute metal poisoning, epidemiological studies, and animal models have all indicated that there can be

severe effects from exposure to high levels of these heavy metals. The toxic effects of these elements will be briefly summarized here.

2.2.1 Cadmium (Cd) and Health Effects

As Lawley *et al.,* (2008), pointed out, in humans, long-term exposure of cadmium may lead to kidney damage, as cadmium tends to accumulate in the kidneys. Clinically, patients suffer tubular dysfunction resulting in aminoaciduria, proteinuria and glucosuria. Other adverse health effects include diarrhea, stomach pains and sickness, bone defects, immune-system damage, possible infertility, possible damage to DNA and carcinogenic effects. This finding was supported by Kjellström *et al.,* (1977), the cadmium, in humans, accumulates throughout life, mainly in the kidney, with a slight decline in concentration in old age. Cadmium is carcinogenic, but the main documented effects have been on the kidney tubule and through the loss of calcium, it impacts the bone. Cadmium toxicity in Japan resulted in itai-itai (ouch-ouch) diseases, a painful disease characterized by weakened bones and pathologic fractures. It is chronic osteoporosis and osteomalacic condition that primarily affects multiparous females (Kobayashi, 1978). Cadmium is readily absorbed from food, particularly in women who may have low iron saturation (Agency for toxic Substances and Disease Registry [ATSDR], 1999), which increased the expression of a divalent cation transporter, inadvertently increasing cadmium uptake. Cadmium may damage cells by its activity in the plasmalemma where it reacts with phosphate groups of the lipid bilayer to alter permeability in the nucleus where it is mutagenic on lysosomal membranes, and as inhibitor of mitochondrial activity (Viarengo, 1985).

2.2.2 Lead (Pb) and Health Effects

According to Lawley *et.al* (2008), lead is very damaging to health, particularly for infants, children and the developing fetus. Its adverse effects include disruption of hemoglobin synthesis, kidney damage, increased blood pressure, miscarriage, nervous-system disruption, reduced fertility and learning disabilities and behavioral problems in children. Lead can cross the placenta and may damage the nervous system and brain of the developing fetus. Symptoms of chronic lead poisoning occur following daily ingestion of 2 to 4 mg for several few months, whilst acute poisoning will occur after daily doses of 8 to 10 mg for a few weeks. Lead in humans causes neurobehavioral and cognitive dysfunction (Needleman *et al.,* 1990) and retarded psychomotor development (Schwartz *et al,,* 1987). The effects of lead on cognition are even evident in middle-aged and elderly people (Payton *et al.,* 1998). Lead also causes hypertension (Schwartz, 1991). According to Hutton (1987), the major health problems of Pb are manifested in three organ systems namely the haematological, nervous and renal systems. In the haematological system, Pb interferes with the last stage of haem synthesis, the incorporation of Fe into protoporphyrin, catalyzed by haem synthetase. Acute effects of Pb on the central nervous system are generally seen in children and are manifested by severe encephalopathy that can culminate in coma and death.

2.2.3 Zinc (Zn) and Health Effects

Excessive doses of zinc (>40 mg/day) can impair HDL-cholesterol levels, thus presenting increased risk for developing cardiovascular disease, cause gastrointestinal disturbances, and depress immune system functioning (Walter *et al.,* 2003). Eating large amounts of zinc, even for a short time, can cause stomach cramps, nausea and vomiting and when taken longer it can cause anemia, pancreas damage (Cunningham *et al.,* 1975). In the other hand, it is also known if high levels of zinc affect human reproduction or cause birth defects. Rats that were fed large amounts of zinc became infertile or had smaller babies (Webber, 1972). In addition, irritation was also observed on the skin of rabbits, guinea pigs and mice when exposed to some zinc compounds. Skin irritation will probably occur in people (Singh, 2005).

2.2.4 Copper (Cu) and Health Effects

Copper has been shown to cause oxidative damage in biological biosystems. Copper-accelerated lipid peroxidation in human erythrocytes has been reported (Hochtein *et al.,* 1980). Dougherty *et al.,* (1982) found that intraperitoneal administration of copper to vitamin E and selenium-deficient rats caused an increased in ethane production and acute mortality in rats. These findings were supported by the study of Dillard *et al.,* (1984), which showed that copper fed rats showed elevated penthane exhalation, increased TBA reactants in blood, kidney and liver and increased lipofuscin fluorescence in splenic extracts in vitamin E-deficient condition compared to vitamin E-deficient control. Since copper can generate highly

reactive .0H, it may have carcinogenic effects. It is reported that copper disturbed DNA synthesis *in vitro* (Sideris *et al.,* 1988). In the presence of hydrogen peroxide and DNA, both copper produced potent mutagenic effects (Tkeshelashvili *et al.,* 1991). Also clastogenic effects of copper on the bone marrow chromosomes of mice have been reported (Agarwal *et al.,* 1980).

2.3 Aim

The aim of this study was to determine concentrations level of heavy metal (Cd, Cu, Pb and Zn) in edible parts of fish and seafood.

2.4 Objectives

The objectives of this study were:-

1. To know concentrations level of heavy metal (Cd, Cu, Pb and Zn) in edible parts of fish and seafood.
2. To compare heavy metal concentrations in fish and seafood with guidelines and regulations stipulating maximum permissible levels set by the Malaysian Food Act 1983 and Food Regulations 1985 Fourteen Schedule.
3. To investigate whether these metals are within the permissible limits for human consumption.

CHAPTER III

MATERIALS AND METHODS

3.0 Sample Species of Fish and Seafood

Species fish and seafood that were analyzed for four (4) heavy metals (Cu, Cd, Pb and Zn) were listed in (**Table 3.1**).

Table 3.1: List of species fish and seafood samples.

No.	ISSCAAP code	Types	Local name	English name	Scientific name
1.	33	D	Kikek	Ponyfish	*Leiognathus spp.*
2.	38	D	Pari	Rays	*Myliobatis spp.*
3.	34	P	Cincaru	Hardtail scad	*Megalaspis cordyl*
4.	34	P	Selayang	Round scad	*Decapterus spp.*
5.	37	P	Kembung	Indian mackerel	*Rastrelliger spp.*
6.	34	P	Selar pucat	Yellowtail scad	*Alepes spp.*
7.	56	D	Lala	Bay scallop	*Aequipecten irradians*
8.	57	D	Sotong	Common squid	*Loligo spp.*
9.	42	D	Ketam biru	Blue swimming crab	*Portunus pelagicus*
10.	45	D	Udang putih	Small white prawn	*Metapenaeus lysianassa*

Notes:

ISSCAAP = International Standard Statistical Classification of Aquatic Animals and Plants (Annual Fisheries Statistics, 1989); **D** = Demersal types (fish and seafood which live on or near the bottom of the sea or lakes) (Walrond, 2009); **P** = Pelagic types (fish and seafood which occupies the surface layers of both inshore and offshore oceanic waters) (Walrond, 2009).

3.1 Chemicals and Reagents

The chemicals and reagents that had been used in the laboratory were listed in the (**Table 3.2**). All chemicals and reagents were analytical grade.

Table 3.2: List of chemicals and reagents.

No.	Chemicals and reagents
1.	Concentrated HNO_3 (AnalaR grade, BDH 69%) (Merck, Darmstadt, Germany)
2.	Deionised water,resistivity ≥ 18.2 Mohm (Milli-Q^{TM})
3.	Copper 1000 mg/L Perkin Elmer®
4.	Lead 1000 mg/L Perkin Elmer®
5.	Zinc 1000 mg/L Perkin Elmer®
6.	Cadmium 1000 mg/L Perkin Elmer®
7.	Matrix modifier: Magnesium nitrate hexahydrate, $Mg(NO_3)_2.6H_2O$ 0.2% w/v (M8636 Sigma-Aldrich).
8.	Matrix modifier: Ammonium dihydrogen phosphate, $NH_4H_2PO_4$ 0.2 % w/v (Suprapur Merk, Darmstadt, Germany)
9.	Matrix modifier: Palladium, N_2O_6Pd 0.2% w/v (76040 Sigma-Aldrich)

3.2 Apparatus

The apparatus that had been used in the laboratory were listed in the (**Table 3.3**). In this present study the apparatus were handled and measured carefully to produce the actual and precise results.

Table 3.3: List of laboratory apparatus.

No.	Apparatus
1.	Volumetric flask 10 and 50 mL (SCHOTT DURAN)
2.	Pipette 5µL, 1000 µL (Gilson, France)
3.	Weighing machine (DRAGON 3002)
4.	Graphite Furnace Atomic Absorption Spectrometer (Perkin Elmer ® Aanalyts 800 Atomic Absorption Spectrometry)
5.	WinLab 32 ^{TM} AAS computer software.
6.	Hollow Cathode Lamp : Cd, Pb, Cu and Zn (Perkin Elmer®)
7.	Test tube (Pyrex ® UNDER LIC CORNING U.S.A)
8.	Digital Heating Block (2048 × 1536 - Grant UBD2)
9.	Glass funnel (SCHOTT DURAN)
10.	Polypropylene containers with tightly fitting lids 10 mL (SCHOTT DURAN)
11.	0.45mm PTFE filter (Whatman ®)
12.	Refrigerator (HITACHI)
13.	Stainless steel spatula
14.	Ice compartment

3.3 Preparation of Reagent Blank Solutions

The reagent blank solutions were prepared by using 3 ml of concentrated HNO_3 (AnalaR grade, BDH 69%), then diluted to a 50 ml volume with deionised water.

3.4 Preparation of Standard Calibration Solutions

Concentration of standard stock solutions (Cu, Zn, Cd and Pb) available in laboratory was 1000 mg/L respectively. From this concentration the standard calibration solution of 100 µg/L for each metal was prepared. The preparations were as follow:-

i. Standard calibration solution for Cu.

   5 µL standard stock solutions of Cu was diluted with 40 µL of 0.5 % v/v nitric acid in 50 mL volumetric flask and diluted to volume with deionised water. Then, standard calibration solution concentrations of 5, 35, 55, 65 and 75 µg/L were set in GF-AAS method respectively.

ii. Standard calibration solution for Zn.

   5 µL standard stock solutions of Zn was diluted with 40 µL of 0.5 % v/v nitric acid in 50 mL volumetric flask and diluted to volume with deionised water. Then, standard calibration solution concentrations of 5, 35, 55, 65 and 75 µg/L were set in GF-AAS method respectively.

iii. Standard calibration solution for Pb.

   5 µL standard stock solutions of Pb was diluted with 40 µL of 0.5 % v/v nitric acid in 50 mL volumetric flask and diluted to volume with deionised water. Then, standard calibration solution concentrations of 5, 10, 20, 40 and 50 µg/L were set in GF-AAS method respectively.

iv. Standard calibration solution for Cd.

5 µL standard stock solutions of Cd was diluted with 40 µL of 0.5 % v/v nitric acid in 50 mL volumetric flask and diluted to volume with deionised water. Then, standard calibration solution concentrations of 10, 20, 30, 40 and 50 µg/L were set in GF-AAS method respectively.

3.5 Preparation of Quality Control (QC) Solutions

The reagent control concentration solutions of 10 µg/L for Cu, Zn, Cd and Pb were prepared respectively. The preparations were as follow:-

i. Quality control solution for Cu.

1 ml from standard calibration solution that previously prepared in (3.4[i]) was diluted with 40 µL of 0.5 % v/v nitric acid in 10 mL volumetric flask and diluted to volume with deionised water.

ii. Quality control solution for Zn.

1 ml from standard calibration solution that previously prepared in (3.4[ii]) was diluted with 40 µL of 0.5 % v/v nitric acid in 10 mL volumetric flask and diluted to volume with deionised water.

iii. Quality control solution for Pb

1 ml from standard calibration solution that previously prepared in (3.4[iii]) was diluted with 40 µL of 0.5 % v/v nitric acid in 10 mL volumetric flask and diluted to volume with deionised water.

iv. Quality control solution for Cd

1 ml from standard calibration solution that previously prepared in (3.4[iv]) was diluted with 40 µL of 0.5 % v/v nitric acid in 10 mL volumetric flask and diluted to volume with deionised water.

3.6 Preparation of Matrix Modifier Solutions for GF-AAS

Matrix modifiers were used for modification of ashing furnace temperatures, resulting in elimination of the matrix with no loss of the analyte (or atomisation of the analyte, but not the matrix). The preparations of matrix modifier solutions were as follow;-

i. Matrix modifier solution for Pb and Cd

1:1 of 0.2% w/v $Mg(NO_3)_2.6H_2O$ in 0.5% v/v nitric acid and 0.2 % w/v $NH_4H_2PO_4$ in 0.5% v/v nitric acid were mixed.

ii. Matrix modifier solution for Cu.

1:1 of 0.2% w/v $Mg(NO_3)_2.6H_2O$ in 0.5% v/v nitric acid and 0.2%w/v Palladium, N_2O_6Pd in 0.5% v/v nitric acid were mixed.

iii. Matrix modifier solution for Zn.

1 ml of 0.2% w/v $Mg(NO_3)_2.6H_2O$ was make up to 10 ml with 0.5% v/v nitric acid.

3.7 Preparation, Sampling and Storage of Samples

Fishes (6) and seafood (4) were collected from five big markets located at province of Kelantan were selected and analyzed for heavy metals. Since these products were purchased raw, not cook before analysis, the collected samples were immediately put into an ice compartment and transported to the laboratory for further analysis. In the

laboratory, the edible portion of the primary samples was mix, ground and remix to obtain a single homogeneous composite sample of the product. For shellfish, only the soft content is ground. The mixing equipment used is made of stainless steel and the hardware used to prepare the composite samples was thoroughly washed between each preparation in order to avoid cross contamination between species and composite samples. After preparation, the composite samples were stored at -20°C in polypropylene containers until the time of analysis (Sirot *et al.* 2008).

3.8 Sample Analysis for Determination of Total Heavy Metal Concentrations by GF-AAS.

All samples were accurately weighted to approximately 0.5g directly in test tubes. Then, they were digested in 3 ml of concentrated HNO_3 (AnalaR grade, BDH 69%). They were placed in a digital heating block digester first at low temperature for 1 hour and then they were fully digested at high temperature (140 °C) for 3 hours. The digested samples were then diluted to a 50 ml volume with double-distilled water (Yap *et. al,* 2003). After filtration, the prepared samples were determined for Cd, Cu, Pb and Zn by using graphite furnace atomic absorption spectrophotometry (GF-AAS). The data were presented in µg/L (w/v). The w/v was converted into wet weight (ww) by using a conversion formula (AOAC International, 2002). The latter was then used for comparative purposes. To avoid possible contamination, all glassware and equipment used were acid-washed. To check for contamination, procedural blanks were analyzed in every five samples. Quality control samples, made from standard solutions of Cd, Cu, Pb and Zn, were analyzed in every five samples to check for the metal recoveries.

3.9 Measurement of Sample

The standard calibration solutions in (3.4) were injected into the GF-AAS at the specified condition parameter (see **Table 3.4**). The appropriate wavelength, gas mixture/temperature program and other instrumental parameters for each metal that applied in this current study were getting from the manual provided with the instrument. The responses (peak area) versus concentration of each standard solution were plotted. Then, quality control and samples solutions were injected into GF-AAS. The response and concentration (µg/L) of Cd, Pb, Cu and Zn in quality control and sample solution were recorded. Next the µg/g of Cd, Pb, Cu and Zn in samples was calculated by using formula (see 3.10).

Table 3.4: Instrumental parameter for GF-AAS

Metal	Wavelength nm	Temperature (°C)/ramp hold (s)		Cleaning out step (°C)	Injection volume (µL)
		Ashing step	Atomization step		
Pb	283.3	650/15-10	1900/0-4	2500	20
Cd	228.8	350/15-10	1200/0-4	2500	20
Zn	213.9	800/3-10	2300/0-2	2500	20
Cu	324.8	700/3-10	1300/0-4	2500	20

3.10 Calculations and Evaluation of Results (AOAC International, 2002).

The concentrations (C) of metal in the test sample were calculated according to this formula:

$$C = \frac{(a-b) \times V}{m \times 1000}$$

Where, C = concentration in the test sample (µg/g); a = concentration in the test solutions (µg/L); b = average concentration in the blank solutions (µg/L); V = volume of the test solution (mL); m = weight of the test portion (g).

Meanwhile, for the limits of detection (LOD) for each metal were calculated as:

LOD = 3 X standard deviation of the average of the blank determinations.