DEBRE BERHAN UNIVERSITY

COLLEGE OF SCIENCE POSTGRADUATE PROGRAM

DEPARTMENT OF CHEMISTRY

DETERMINATION OF LEVELS OF TOXICICTY METALS LIKE Ni, Pb, Cr & Cd IN TWO BRANDS OF CHOCOLATES AND TWO BRANDS CANDIES MARKETED IN DEBRE BERHAN CITY, ETHIOPIA

MSc THESIS

By

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Advisor: Tesfaye Demissie (Ph.D)

FEBRUARY 2021

Debre Berhan, Ethiopia

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A MASTER THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY IN PARCIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

By

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FEBRUARY 2021

Debre Berhan, Ethiopia

ThesisApproval Sheet

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CERTIFICATION SHEET

A thesis research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance by Aychiluhim Belachew entitled "determination of level of toxicity metals like Ni, Pb, Cr & Cd in two brands chocolates and two brands candies marketed in Debre Berhan city, Ethiopia" and Recommended that it be submitted as fulfilling the thesis requirement.

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Name of Co- advisor	signature	Date

As mentioned of the Board of Examiners of the MSc. thesis open defense examined. We certified that we have read and evaluate the thesis prepared by Aychiluhim Belachew and the examined the candidate. We recommended that thesis be accepted as fulfilling the thesis requirements for the degree of masters of science in Chemist

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Declaration

I, Aychiluhim Belachew the undersigned, declare that this work entited“determination of level of toxic metals like Ni, Pb, Cr & Cd in two brands chocolates and two brands candies marketed in Debre Berhan city, Ethiopia is my own work. It is reported in my own words under the supervision of my advisor except for the information that is taken from some sources as references. In compliance with internationally accepted practices, I have duly acknowledged and referenced all source of materials used for the study. This study has not been submitted for any degree in this university or other university. It is offered for the partial fulfillments of the degree of master of scince (MSc.) in chemistry.

AYCHILUHIM BELACHEW

Signature--

date of submission-------------------------------------

Acknowledgments

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Special thanks go to my father Belachew and wife Hina mulatu that supports me morally love during my work.

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Abstract: *In present study four toxic metals, cadmium (Cd), nickel (Ni),chromium(Cr) and lead (Pb) were determined in chocolates and candy samples available in local markets of Debre Berhan city. Concentrations of understudy toxic metals (TMs) were determined by inductively coupled plasma optical emission spectroscopy (ICP−OES).* Validation of the methodology was performed by standard addition method and conventional acid digestion on electric hot plate to obtained TMs concentration, for comparative purpose to obtain results within the 95% confidence level. Excel computer system, Statistical analysis of independent t-test (SAIT). The SAIT result showed that there is no significance difference in the mean concentration of toxic metals $(P > 0.05)$. The origin software were used for data analysis. The concentration of Ni ranged from 0.245 ± 0.043 mg/Kg to 0.363 ± 0.076 mg/Kg in chocolate samples and from 0.197 ± 0.013mg/kg to 0.233 ± 0.032mg/kg in candy samples. It was the highest among all other toxic metals determined. Lead was ranged from 0.03 ± 0.006mg/kg to 0.046 ± 0.006mg/kg in chocolates and from 0.004 ± 0.004mg/kg to 0.023 ± 0.018mg/kg in candy samples. It was the lowest among all other toxic metals determined.Chromium was ranged from 0.148 ± 0.013mg/kg to 0.208 ± 0.014mg/kg in chocolates and from 0.17 ± 0.003mg/kg to 0.181 ± 0.007mg/kg in candy samples. Cadmium was ranged from 0.0495 ± 0.007 mg/Kg to 0.0525 ± 0.002 mg/Kg in chocolates and from 0.0485 ± 0.001 mg/Kg to 0.0495 ± 0.001 mg/kg in candy samples. The mean concentration of Ni (found in ChB-2) and Pb (found in ChB-1&ChB-2) were above the limit;Pb &Cd (found in CB-1&CB-2) and Cd (found in ChB-1) were below the limit and also the rest toxic metals were safe the limit. Hence, clear information should be set about the presence of toxic metals in chocolates and candies by accepted WHO.

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List of Acronyms and Abbreviations

AAS	Atomic absorption spectroscopy
AFS	Atomic fluorescence spectroscopy
CDM	Conventional wet-acid digestion method
DNA	Deoxyribonucleic acid
FAO	Food and agricultural organization
IARC	International Agency for Research on Cancer
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IQ	Intelligence quotient
JECFA	Joint FAO/WHO Expert Committee on Food Additives
PMTDI	Provisional Maximum Tolerable Daily Intake
SAIT	Statistical analysis of independent t-test
SD	Standard deviation
RSD	Relative standard deviation
TMs	Toxic metals
SPSS	Statistical Package for Social Science
WHO	World health organization

CHAPTER ONE

1.INTRODUCTION

1.1 Background of the study

Chocolate products are among the most commonly purchased and globally inspired items. In addition to being heavily advertised to children (most targeted population), they are also used in a variety of foods recipes, especially cakes, tarts and cookies. Chocolate is composed of solid particles, whose normal solid concentration is about 60 to 70% sugar, cocoa and milk. Beside these main solid constituents, other ingredients like hydrogenated vegetable oil, salts, buffering agents, permitted emulsifier and cocoa butter could be source of metals, [1]. As chocolates and cocoa powder are consumed all over the world, consumers are usually concern not only about their nutritional status but for its health safety viewpoint as well.

Essential trace metals are beneficial when present below the limit of tolerance, but can be toxic if taken in excess. This transition between essentiality and toxicity varies from element to element. Substantial evidence supports the importance of trace elements in human nutrition. These trace elements play a crucial role in various biochemical functions of the body as some of these forms are integral enzyme cofactors.

The possibility of the presence of lead (Pb) and cadmium (Cd) in chocolate is a matter of health consideration because the chemical composition of cocoa allows strong binding of Pb and Cd[2]. The presence of Pb and Cd in chocolate products could be natural or due to processing [3]. These can be absorbed directly by the the obroma (cocoa tree), can be introduced during the preparation process or result from contamination via utensils, environmental pollution or transportation and storage, but, regardless, they may be found in the final product [4].

The American Environmental Safety Institute (AESI) claimed that the chocolate manufacturers neither take appropriate measures to remove potentially dangerous levels of lead and cadmium from their chocolate products nor notified consumers of their health risks. On the contrary, world chocolate manufacturers have dismissed claims that their products pose any health hazard due to Pb and Cd.

Chocolates are also considered an important source of nickel, as this element is used as catalyst in hydrogenation process. Moreover, cocoa butter that is used in chocolates may also contain nickel. Ni contamination in chocolates could also be due to the type of containers used for storage and transportation[5,6]. As chocolate matrix is very complex and has high levels of organic compounds very specific digestion method that restricts the losses of the analyte of interest but doesn't introduce any contamination is very important. In this study, the wet digestion method was used, followed by analysis with Atomic Absorption Spectrophotometry of chocolate samples.

This study identified not only the levels of toxic and essential metals in chocolate samples but also discusses the Dietary Reference Intake (DRI) and the percentage contribution of observed elements in chocolate samples for nutritional adequacy. Provisional tolerable daily intake (PTDI) for Pb and Cd has also been calculated.

The flavour of chocolate differs depending on the ingredients used and the preparation method of chocolate. Since chocolates are more popular among children, manufacturers are very concern about their competitors in the market. Due to this reason the chocolates and other confectioneries are sold in a very attractive manner, wrapped in colourful packaging materials. These packaging materials may contain non-food grade substances, printing inks which can contain toxic substances. The major danger is with the colourful pigments used in packaging materials. PbCrO4 is an inorganic pigment used in paints and inks. However, most of the countries have prohibited the use of PbCrO4 in food packages [7].

These toxic substances could have a potency to migrate into the food. Due to the frequent hand-to-mouth behavior, children can be easily ingested by toxic metals. Regular consumption of contaminated chocolates results in accumulation of toxic metals in human organs and may results in serious health problems. Therefore the aim of this study was to estimate the levels of toxic metals present in the chocolate confectionery wrappers and the degree of migration of these metals during the storage conditions acute, chronic or sub-chronic, neurotoxin, carcinogenic, mutagenic or teratogenic [8].

The mineral content of this ingredient has great value for nutritional control of the foods. However, the determination of metals in such samples involves a difficult step of digestion, considering that its matrix contains high contents of organic compounds [9].

Chocolate is a vastly nutritious energy source, with a fast metabolism and good digestibility. The presence of cocoa, milk and sugar in its composition can be the warranty of proper ingestion of proteins, carbohydrates, fats, minerals and vitamins [10]. It is therefore necessary to monitor human exposure to TMs present in the food chain [11].

Toxicity of Cd came in the headlines after the Itai-Itai disease was found to be caused by high intake of Cd in Japan. When Cd is ingested in excess amounts, it induces toxicity symptoms like gastrointestinal pains, nausea, respiratory distress, diarrhoea, impaired reproductively, kidney damage and hypertension [12-14].

Lead contamination in chocolates and candies is a very old problem that has evolved with time. Since the middle of the 19th century, various measures including regulations and public education were implemented to minimize the contamination of chocolates and candies from such sources [15]. Nowadays, industrial activities dominate the globalflux of lead in the environment [16, 17] and have become the predominant sources of lead in many food items, including candies [18]. Nickel occurs naturally more in vegetables than in animal flesh [19]. However, nickel toxicity in humans is not a very common occurrence because the intestinal absorption of nickel is very low [20].

Apart from environmental contamination sources of nickel in foods, this metal may also be derived in foods from processing activities such as drying, cooking and canning in nickel-containing vessels [21]. Different complex matrices of the analytical sample require prior mineralization for most analytical methods, and this step is critical in the whole analytical procedure for the determination of metal concentration. Analysis of metals in food samples has conventionally been performed by atomic absorption spectrometry [22, 23]. Sample digestion techniques, such as microwave, and conventional acid digestion method (CDM) for TMs determination have been used widely for the dissolution of target elemental analytes. These digestion techniques, however, require the use of concentrated mineral acids and high temperatures whereas high pressures are required in the case of microwave applications [24].

The current study is aimed to determine the concentration of Ni, Cd, Pb, and Cr in two brands of candies and two brands chocolates available in the local markets of Debre Berhan city. Due to the contamination of toxic metals present.

1.2 .Statement of the problem

Chocolates and candies are among the sweet food items because they contain significant amounts of sugar. Chocolate bars are the most consumed cocoa-derived product worldwide. The regular and moderate consumption of chocolate bars and other cocoa derivatives can benefit health due to their antioxidant activity, high carbohydrate content and presence of some essential metallic elements such as Ca, Cu, Fe, K, Mg, Mn,etc. These elements are frequently added to commercial foodstuffs designed for domestic animals [23, 24].

These groups of food contribute about 10% of the daily energy intake. Sweets supply about half of the total sugar intake for all groups of age and may reach 90% in the group of too heavy children and teenagers [22].

Recent studies have been conducted about the determination of toxic metals in chocolate confectionery wrappers used by the chocolate manufacturers in Sri Lanka and it is migration to chocolates. The toxicity of lead, chromium, antimony, and nickel has been studied. In addition to this, the determination of metal contents in various chocolate samples has been studied in Herzegovina [24]. Seven metals (Mn, Cu, Zn, Cd, Cr, Fe, and Pb) are determined by this study using flame atomic absorption spectrometry (F-AAS).

The assessment of heavy metal contamination (Ni and As) in local brand chocolates and candies is also studied in Tiruchirappalli, India [25]. Similar researches have been conducted in various countries abroad. The toxicity of Cd, Ni, Pb, and Cr causes several health effects like gastrointestinal pains, nausea, respiratory distress, diarrhea, kidney damage, impaired reproductively and hypertension [12, 13, and 14].

However, there were no attentions given to those toxic metals that may present in these foods. Toxic metals like Cd, Ni, Pb, and Cr marketed in Debre Berhan town, there is no evidence concerning the assessment, and determination of toxic metals in different brands of chocolates and candies, there were no published researches in the form of journal articles. Since, to the best of our knowledge, the determination of such toxic metals in chocolate and candies marketed in Debre Berhan city is not studies even though a number of people and children are consuming them day to day because of their sweetness.Therefore, this study was intended to determine the concentration level of different brands of chocolates and candies marketed in Debre Berhan town.

The study will also compare the concentration level of such metals with the international tolerable limit, which was accepted by FAO/WHO.

1.3. Objectives of the study

1.3.1. General objective

- The main objective of this study was to investigation the concentration of toxicicity of metals in different brands of chocolates and candies marketed in Debre Berhan city.

1.3.2. Specific objectives

- The following were the specific objectives of the study:-

  - To determine the concentration level of toxicicity of metals (Cd,Ni,Pd,and Cr)) in different brand of chocolates and candies that are marketed from Debre Berhan city,
  - To compare the concentration level of toxiciccity of metals that can be present in these city different brands, and
  - To compare the concentration of toxic metals with international tolerable limit values accepted by joint FAO / WHO expert committee on food additives (JECFA).

1.4. Research question

- The following were the research question of the study:-

  - ✓ In what amount of toxic metals like Cd, Ni, Pb and Cr exist in different brands of chocolates and candies that are sold in the market of Debre Berhan city?
  - ✓ In what extent concentration of each metal are varied among them in their brands of such food items?
  - ✓ Are their amounts of concentration agreed with the international limit value accepted by JECFA?

Hypotheises

1. There is no significant difference each toxic metal between ChB-1 and ChB-2, CB-1 and CB-2 (H_o=Null hypothesis).
2. There is a significant difference each toxic metal between ChB-1 and ChB-2, CB-1 and CB-2 (H_1=Alternative hypothesis).

1.5. Significance of the study

Nowadays, people because of their sweetness and nutritional value frequently consume chocolates and candies. This is true for the case in Debre Berhan city.

A. It enables to recognize the concentration level of toxic metals in the samples under study, which is used to provide basis for assessing their importance for health;

B. It will show the level of potential health risks that can result from feeding chocolates and candies;

C. Help as a benchmark for other researchers to study the concentration level of toxic metals in different brands of chocolates and candies other than Cd, Ni, Pb and Cr.

D. It will provide adequate information to the society whether toxic metals are present in different brands of chocolates and candies or not.

E. It will find information for other researchers to determine the concentration of toxic metals in other various brands of chocolates and candies marketed in the city.

1.6. Limitation of the study

As the area of the researcher is remote, connection problem is one factor in order not to run the researcher work on time and effectively. The other problem was used to the material determine the concentration of toxic heavy metals for the effectiveness of the researcher completion. In addition, inefficient light/sustainable electric power was used to the other problem to wrote, send and receive the feedback from my advisor.More over, other factor in order not to run effectively the research work on time is covid-19 (corona).

CHAPTER TWO

2.LITRATURE REVIE

2.1.Toxic metals in chocolate wrappers and their colorful packages

Safefood packaging is very important. The EU Framework Directive 89/109/EEC states that “food contact materials shall be safe and must not transfer constituents in quantities that could endanger human health or induce an unacceptable change in the foodstuffs composition”. In the production process of thermoplastic polymers, such as polyethylene (PE),polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephtalate (PET), polycarbonate (PC), acrylonitrile-butadiene-styrene (ABS) and polyamide (PA),different catalysts may be used which can contain low levels of heavy metals. The sources for Cd and Pb are impurities originating from inorganic pigments and stabilizers. Antioxidants can contain Ni, while thermal stabilizers can contain Ni and Pb [26]. Today industries are using different colourful packaging materials to make their products attractive to the consumers without considering about the risk of food contamination. Especially food products such as candies that are likely to be consumed frequently by small children are wrapped in colorful packages in order to induce them to purchase the products. Candies and its packages that hold heavy metals, such as lead and chromium, can be put onto the children’s hands that pose danger [27,28], due to their frequent hand to-mouth behavior, and children are likely to be posed by ingesting these heavy metals. There is a general understanding that the package surface in contact with the food should be free of printing ink, that is, the package itself should form an effective barrier between the printed surface and the food, and the unintentional transfer of components of printing inks from the outer printed surface onto the food contact surfaces should be avoided. Use of printing inks only on the outer surface of the package does not ensure that it will not contaminate the food, although the inner surface of the package is coated with films such as polyethylene or polypropylene [28].

2.2.Toxicity Metal and their factors

Toxicity of any metal is governed by several factors. There are interaction with essential metal, formation of metal protein complexes, chemical form of the metal element, immune status of the host and age and stage of development of the host. Its electrochemical character and oxidation state, its absorption and transport in body tissues, the stability and solubility of its compounds in body fluids, its case of excretion and reaction with functioning tissues and organelles and with essential metabolites[29]. Interaction of toxic metals and essential metals occur when metabolism of toxic metals is similar to that of the essential metal. Lead and Cadmium interact with Calcium in nervous system and skeletal system respectively. Lead replaces the zinc on heme enzymes. Metal protein complexes are formed due to detoxification of toxic metals in the body i.e. Metallothionin form complexes with Cd[30]. Metal can interact with protein leading to an allosteric effect, or with DNA or RNA to stop normal metabolism or with unknown compounds leading to a change in physiologic process [29]. Contamination of food products with heavy metals may cause a serious risk for human health because of the consumption of even a small amount of metals can lead to considerable concentrations in human body leading to biotoxic effects. The biotoxic effects of heavy metals refer to the harmful effects of heavy metals to the body when consumed above the bio-recommended limits. The nature of effects could be acute, chronic or sub-chronic, neurotoxin, carcinogenic, mutagenic or teratogenic [31]

2.3.Health effects of some toxicmetals

2.3.1. Lead and Chromium Toxicity

Lead absorbed from the food and the atmosphere is retained in tissues like lungs, liver, kidney, and bones. The short term and long term exposure to high level of lead can cause brain damage, paralysis, abdominal pain, anemia, renal disease, memory loss, damages to kidneys, reproductive and immune systems [32]. Chromium can exist as Cr (III) or Cr (VI). Cr (VI) form is highly toxic.Cr (VI) exposure has been known to be associated with cancer induction in humans, especially bronchial carcinoma and lung cancer [33,36]. The mechanism(s) of Cr (VI)-induced carcinogenicity is within the cell may result from damage to cellular components during the hexavalent to trivalent chromium reduction process, by generation of free radicals, including DNA damage [34]. The water-insoluble Cr (VI) compounds of particulate forms are more potent carcinogens than the watersoluble ones [35,36]

2.3.2.Nickel and cadmnium Toxicity

Nickel, is a well known carcinogen to humans, by altering the DNA functions. Even though their DNA-damaging potentials are rather weak, they interfere with the nucleotide and base excision repair at low, nontoxic concentrations. For example, both water-soluble Ni (II) and particulate black NiO greatly reduced the repair of DNA. Ni (II) disturbed the very first step of nucleotide excision repair [37].

Cadmium is a very toxic metal. All soils and rocks,including coal and mineral fertilizers, contain some cadmium. It is the most toxic heavy metal. Humans may be exposed to this metal primarily by inhalation and ingestion and can suffer from acute and chronic intoxications. Cadmium has the capability to bind with cysteine, glutamate, histidine and aspartate ligands and can lead to the deficiency of iron.Cadmium toughens the tissues and hardens the arteries. It also hardens the personality. Unfortunately, it is also a deadly toxic metal associated with heart disease, cancers of all kind, kidney disease, diabetes and other serious health problems [38,39].

2.4. Sample digestion methods

2.4.1. Wet-digestion method

This method involves the destruction of organic matter through decomposition of the sample by an acid or mixture of acids.Ittakes place in open vessels, intubes, on hot plate or in aluminum heating block or in closed vessels at elevated pressure with thermal or microwave heating. Applicability of wet digestion technique is dependent on the type of food. Carbohydrates easily mineralize with nitric acid at 180℃, while fats, proteins, and amino acids cause incomplete digestion due to the relatively low oxidation potential of nitric acid at 200℃.These materials require addition of H_2SO_4 and / or $HClO_4$ with all the problems related to their use at high temperature and pressure [19,20].

Wet digestion with HNO_3, $HClO_4$ and H_2O_2 is recommended because of better recovery. The type of acid used in the preparation procedure can have important consequences in the measurement step. It is commonly known that in all atomic spectrometric techniques HNO_3 is the most desirable reagent. In spite of occasionally observed signal suppression in its presence, no sever analytical problems are encountered in practice with HNO_3at concentrations up to 10%,sometimes higher, in all atomic spectrometric techniques as long as its concentration is similar in calibration and sample solutions. $HClO_4$, added in most mineralization procedures, many organic compounds (decompose, dissolved, oxidzed) and also rarely responsible for analytical problems [20, 40].

CHAPTER THREE

3.Materials and Methods

3.1 Apparatus and Instrument

The materials and apparatus used to study this research were:

Polyethylene plastic bags were used for storade of chocolate and cand samples. Drying oven (GALLENKAP) was used for drying samples. Mortar and pestle and Electric Blast Drier were used respectively to grind and homogenize, dried and chocolate and candy samples. Electronic analytical balance (Witeg, MSH−20D) was used for weighting the samples.

Volumetric flasks (25ml and 50ml,100ml, 500ml, and 1000ml were used for preparing working standard solutions and dilute the final digested blanks, spiked samples, un spiked samples, and stock solution. Micro-pipette was used for measuring volumes of standard solutions. Funnel used for transfer solutions. Stirrer was used for stirring the solutions. ICP-OES is the instrument that used to analysis selected toxicicty metals in chocolate and candy samples. Other apparatuses used in the study includes; digestion flasks , watch glass, volumetric flasks, filter paper (what man #102), parafilm for covering the volumetric flasks containing the solution, microwave oven for drying the sample were used, samples were kept in the refrigerator (LEC, Refrigerator, PLC, England) prior to characterization and Electronic beam balance (Nimbus,NBL−254i,UK)for mass measurement . Desiccators, crucible, Soxhelet apparatus, distillation apparatus, reflux setup, digestion unit, distillation unit, titration unit, Erlenmeyer flask,and beakers, glassware plastic bottles, reagent bottles and thermometer during the laboratory work, Inductively coupled plasma optical emission spectrometer (ICP−OES) was applicable for the analysis of toxic metals in the different brands of chocolates and candies.ICP-OES used for metal determination from aqueous solutions obtained through food and plant digestion are mainly based on inductively coupled plasma optical emission spectrometry (ICP−OES). ICP−OES has the advantages of high samples throughout due to the multi-elemental determination. This method has a wide working range [42]. Due to its advantages, ICP−OES has become one of the most used techniques for elemental determination. Many studies have been conducted to validate this method for metal analysis in a large variety of sample types. Dry, wet and microwave digestion procedures are employed for the determination of chemical elements using

ICP−OES technique, obtaining satisfactory recovery, detection limits and standard deviation for trace metal determination [43]. For this particular study, the conventional wet acid digestion method has been used.

3.2.Chemicals and Reagents

The reagents are: -

- HNO_3(69% Spectrosol, BDH, UK), $HClO_4$ (70%, Blulux, Laboratories, PLTD, India) were used for sample digestion

The chemicals are:

Stock standard solutions ($NiCl_2.6H_2O$,$Cd(NO_3)_2$, $Pb(NO_3)_2$, and $Cr_2(SO_4)_3$)

3.4.Description of the study areas

The study area is Debre Berhan city, which is the capital of North Shewa Zeon. It is found about 130km away from Addis Ababa. The study area was selected because of the availability of the samples in the local markets in the city.

3.5.Sample Collection and Handling

The level of toxicicty metals in, two different brands of chocolate and candy samples collected from Debre Berhan city July/2020 districts were carried out using ICP-OES. The study was particularly focused on chocolate and candy as these sampling sites are main traffic density Debre berhan city and surrounding north Shewa Amhara. The results were analyzed and compared for selective determined toxicicty metal concentration among two brands chocolate and candy samples.

However, to obtain detailed information and draw strong conclusions on the level of toxicicty metals may need collection of samples from some parts of the shopes.

Table3.1: Characteristics of samples purchased from the local markets.

Sample Type	BrandName/Labeling	Nature	Main ingredients	Made in
Chocolate	Ricco/CHB-1	Milk	Hydrogenated vegetable fat, Cacao powder,milk solids, sugar	UAE
	Take/CHB−2	Cacao Powder	Sugar, hydrogenated vegetable fat, cacao powder, milk solids	UAE
	Juicy/CB-1	Strawberry fruit flavour	Liquid Glucose, sugar and artificial flavoring substance	Ethiopia
Candy	Royal/CB-2	Strawberry fruit and sugar	Sugar, glucose syrup and citric acid	Ethiopia

3.3. Treatment of the apparatus

The polyethylene sampling bottle, reagent bottles, volumetric flask, measuring cylinder, beakers, porcelain mortar and pestle and any other materials and apparatus that were used during the laboratory work were glass materials soaked for 24 hrs with diluted HNO_3 (10%), regularly washed with tape water, using detergent and rise with distilled water.After washing, all the apparatus were rinsed with distilled water

3.4 Determination of toxicicty Metals

The term heavy metal refers to toxicicty metals that has a relatively high density (Cd, Pb, Cr and Ni). They were determined by analysis of the digested sample using ICP-OES.

3.5.Preparation of standard solutions and Analysis of samples

All working standards were prepared by diluting stock standard solutions (1000ppm) of the metals to be analyzed. Determination of the metal concentration in experimental solution would be based on the calibration curve. Plotting the calibration curve stock solutions of 1000mg/L was prepared. The calibration standard solutions were used to calibrate instrument response with respect to analyte concentration followed standard procedure [41]. Calibration curves were prepared for each of the metals by running a range of concentration of freshly prepared standard solution in their respective linear ranges. In this study, for the linear dynamic range, the calibration sample of Ni, Pb, Cr, and Cdwere prepared from their sock standard solutions containing 1000mg/L for each metals HNO_3 69% and distilled water . Working solutions were prepared from the standard solutions of metals by appropriate dilution of the intermediate solution (10mg/L).

3.6. Procedure

3.6.1. Sample Digestion

Conventional digestion method (CDM) About 1.5 gm of three replicates samples were taken separately in pyrex flasks (50 mL in capacity), the contents of flasks were treated with 20 mL and 4ml mixture of acid and oxidant (69% HNO_3 and 70% $HClo_4$ respectively) for decomposition of organic matter. The contents of flasks were heated on electric hotplate at 80℃, for 3–4 hrs, till clear. After cooling the resulted clear solution for about 10 minutes, 15 ml of distilled water added and mixed. Then, each replicate samples were transferred into 50ml volumetric flasks using a funnel and WhatmanNo.102 filter paper. The resulted solution was diluted to 50 ml with distilled water. After dilution, the solutions were kept in the refrigerator until analysis.

The determination of Cd, Ni and Pb by ICP-OES. Blanks (without sample) were carried through the complete procedure of both methods. The concentrations were obtained directly from calibration graphs after correction of the intensity for the signal from an appropriate reagent blank. The blanks were always prepared in same manner as samples. The digests obtained by methods were subjected to ICP-OES.

The samples collected from the local markets in Debre Berhan city were dried using microwave oven (GALLENKAP) for about three hours at 70^0C operating temperature and then grinded in to pieces. Around 15.0g of each sample kept until digestion. From this mass of sample, about 1.50g of triplicate chocolate samples and 1.50g of candy samples were measured and kept for digestion.

(a) RICCO (b) TAKE

Figure3.1: Photograph of chocolate samples;(a) milk-based chocolate (b) Cacao powder-based chocolate

(C) ROYAL (d) JUICY

Figure3.2: Photograph of candy samples; (c) Strawberry fruit flavor candy and (d)Strawberry and sugar candy

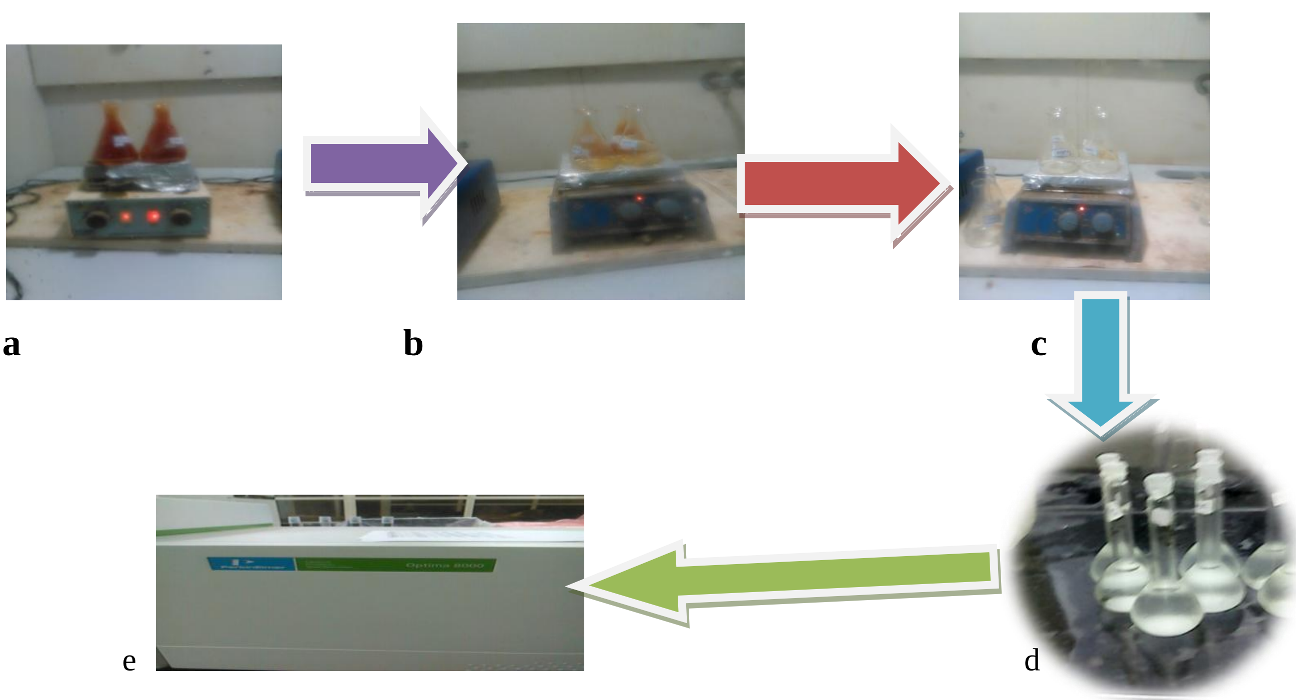


Figure3.3: Sample solutions at different stages of digestion (a) at the moment when digestion has brown color begun 30 minute (b) while digestion, after 1:30 hour (c) obtain clear solution, after 3:30 hours and (d)solutions after diluting the digested sample (after 4:00 hours)(E) determination of TMs in ICP-OES

3.6.2. ICP-OES operating analysis

Metal contents of the final solution were determined by using ICP−OES.The operating conditions of ICP−OES are given in the table below.

Table3.2: Operating conditions of ICP−OES

S/No.	Operating conditions	Value
1	RF power	1300W
2	Plasma flow	15 L min^{-1}
3	Auxiliary flow	2.0 L min^{-1}
4	Nebulizer flow	0.8 L min^{-1}
5	Sample uptake rate	1.5 ml min^{-1}

3.7. Instrumental Calibration

All working standards were prepared by diluting stock standard solutions (1000ppm) of the metals to be analyzed. Determination of the metal concentration in experimental solution would be based on the calibration curve. Plotting the calibration curve stock solutions of 1000mg/L was prepared. The calibration standard solutions were used to calibrate instrument response with respect to analyte concentration followed standard procedure.

3.7.1 Precision and Accuracy

3.7.1.1.Precision

Precision means the values gathered from repeated measurements are close to each other in cluster, when the procedure will apply repeatedly to multiple aliquots of a single homogeneous volume of sample matrix. Precision of analytical procedure is usually expressed as a relative standard deviation (RSD) of the three replicate results and spiked samples are subjected to the same digestion procedure like actual mass[44]. The percentage relative standaer deviations (%RSD) of the samples were calculated as;

$$\%RSD= \frac{SD}{X} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . . [1]$$

Where RSD= relative standard deviation; X = mean; SD = Standard deviation

Relative average deviation was parameter of choice for expressing precision level should not exceed 15% of the relative standard deviation (RSD).

3.7.1.2 Accuracy

Accuracy is an analytical method describes the values gathered from repeated measurements are close to the target (true value concentration of the analyte). Accuracy was determined by replicate analysis of samples containing known amounts of the analyte. Accuracy was expressed as matrix spike recovery and percent recovery results were calculated by the following equation [45]. The spiked samples are then subjected to the same digestion procedure like actual sample.

$$\%R \; = \frac{CM\,spiked - CMunspiked}{CMAmount\ of\ add} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. [2]$$

Percentage recovery of the spiked concentrations for Ni, Pb, Cr and Cd in chocolate and candy samples was calculated by using the following equation.

Where % R = percentage recovery (in %)

CM spiked= concentration amount found in spiked sample (in mg/L)

CM un spiked= concentrationamount found in unspiked sample(in mg/L)

CMA spike = concentration amount added as a spike (in mg/L)

CM = metal concentration

3.7.2 Method of Detection Limit (LOD) and Limit of Quantification (LOQ)

3.7.2.1 Method of Detection Limit (LOD)

Method of detection limit is a minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, and is determine from analysis of sample in a given matrix containing the analyte [46]. The concentration at which we can decide whether an element is present or not. The limit of detection (LOD) is a measure of how sensitive the analytical method is and is the lowest concentration or weight of analyte that can be measured at a specific confidence level. For the determination of limit of detection of the analytical method (LOD), triplicate ten blanks were prepared in parallel and analyzed for their metal contents.

The standard deviation (SD) of the ten blanks was calculated and multiplied by three to determine the method detection limit. Hence, method of detection limit for each metal would be estimated by digesting ten replicates of method banks with the optimum procedure for chocolate and candy samples.

$$LOD = \frac{3SD}{m} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.. [3]$$

3.7.2.2. Limit of Quantification (LOQ)

Limit of quantification is the lowest concentration of analyte that can be determined with acceptable level of uncertainty. The limit of quantification (LOQ) is the smallest quantity of analyte that can be measured with acceptable accuracy and precision and obtained from analysis of ten replicate blank which is digest, in the sample digestion procedure as actual samples. In this study limit of quantification will obtain from analysis of ten reagents blanks which are digests in the same digestion procedure as actual sample. Limit of Quantification is ten times of the standard deviation can be calculated;

$$LOQ = \frac{10SD}{m} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. [4]$$

3.8. Statistical Analysis

All the samples analysis in the study were carried out in triplicate and the results were reported as mean and standard deviation.

Mean concentration of metals in samples was calculated by using Microsoft Excel 2007 computer package and reported as mean$\pm$SD. Finally, the results of this study were compared with other literatures and JECFA limit.

Mean significance difference was checked by independent t-tests within different brands of samples using the principles of statistical analysis of independent t-test. Statistical analysis of independent t-test was used to determine the significance variation of toxic metals between samples.

Origin software (8.0)was also used to draw intensity versus concentration calibration curves and to know the correlation coefficients for each sample.

Table3.3: Standard conditions used in the determination of different toxic metals and their detection limit using ICP−OES.

Toxic Metals	Wave length(nm)	Detection Limit(mg/L)	Intensity(W)
Ni	231.604	0.015	620.0
Pb	220.353	0.042	150.0
Cr	267.716	0.0071	2200.0
Cd	228.802	0.0027	1400.0

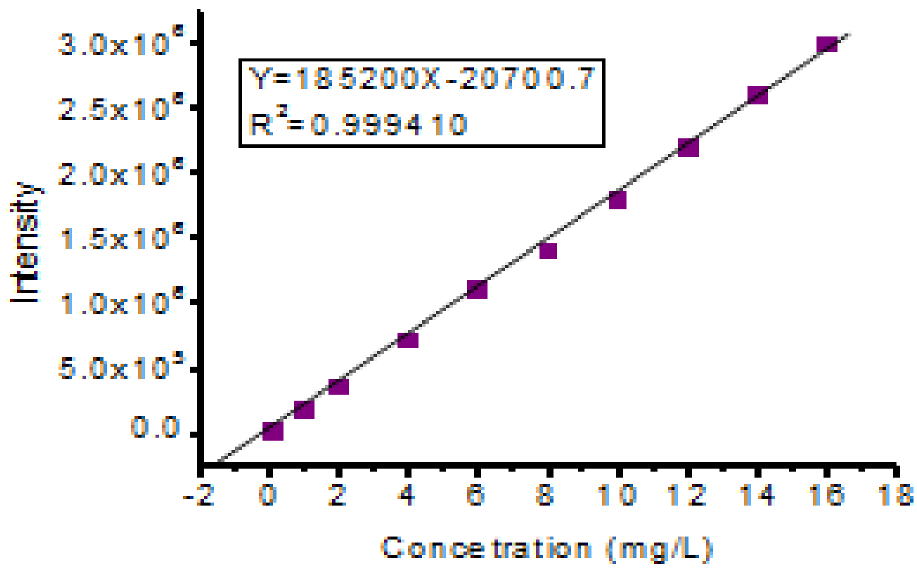
CHAPTER FOUR

4.RESULT AND DISCUSSION

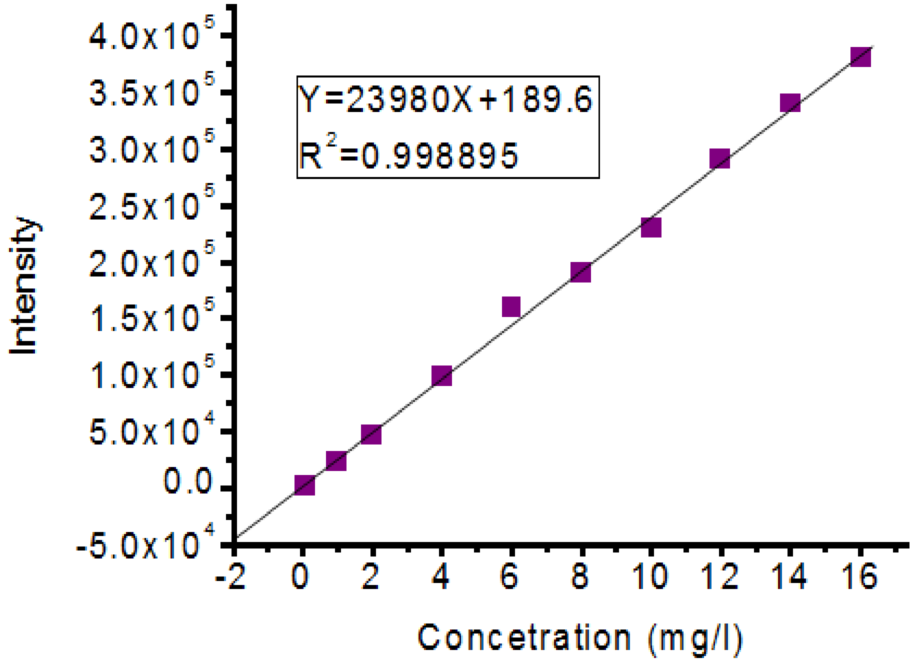
4.1. Concentration of the selecte toxic metals in chocolate and candy

To analyse samples by the ICP-OES techniques, the calibration curve of each TMs must be determined first with good linear regression. Figures 4.1 and figure4.2 show the obtained calibration curves by ICP-OES measurements of the interested metals. These elements include iron Ni, Pb, Cr and Cd respectively.

4.2. Calibration curve for standard solutions

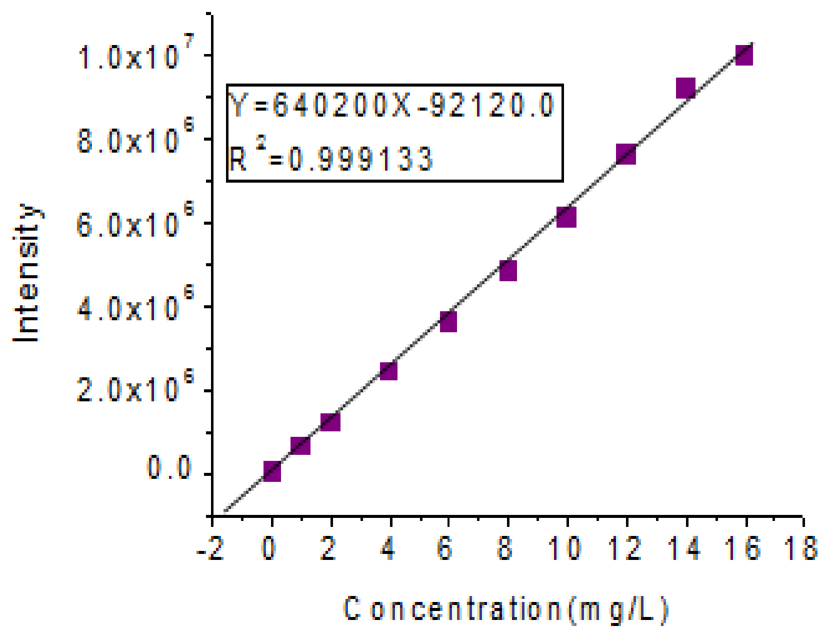


(a) Ni

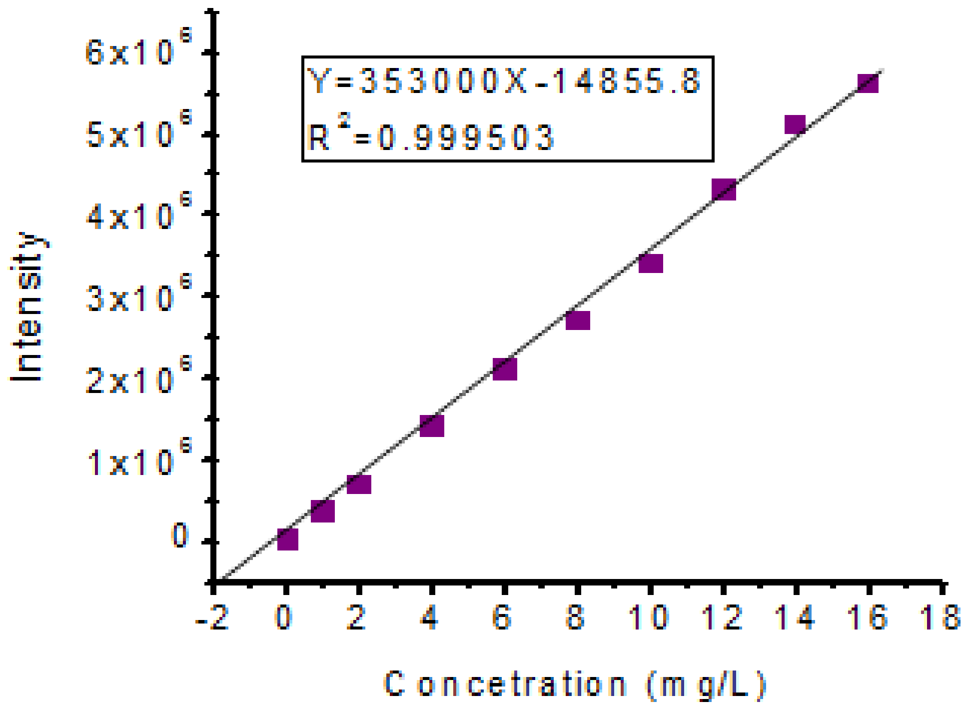


(b) Pb

Figure 4.1: Calibration curves for (a) Nickel (b) Lead standard solutions



(C) Cr



(d) Cd

Figure 4.2: Calibration curves for (c) Chromium and (d) Cadmiumstandard solutions

The correlation coefficient values that listed below in table 4.1 indicate the degree of linear association between the concentration of standards and intensity of the radiation. The Calibration Correlation factor on a regular base falls between for rest TMs 0.998895(for Pb) and 0.999503 (for Cd) as follow.

So that the accuracy and precision of our method was the R^2 of the Calibration, the spike test and the recovery of our control sample which is the concentration limit.

Table4.1: The correlation coefficients of the obtained calibration curves from the ICP-OES measurements.

Toxic metals	Concentration of standard solutions(mg/L)	Correlation coefficient
Ni	0.05,1, 2,4, 6,8,10,12,14,16	0.999410
Pb	0.05, 1, 2,4, 6,8,10,12,14,16	0.998895
Cr	0.05, 1, 2,4, 6,8,10,12,14,16	0.999133
Cd	0.05, 1, 2, 4, 6,8,10, 12,14,16	0.999503

4.3. Evaluation of results

4.3.1. Limit of Detection (LOD) and Limit of Quantification (LOQ)

In this study, LOD and LOQ for each metal were determined from analysis of triplicates of laboratory control samples (method of blank) which were digested in the same digestion procedure as actual samples. Therefore, ten blank samples were digested for chocolate abd candy samples and the blank were analyzed for the contents of Ni, Cd, Pb and Cr by ICP-OES. The standard deviation for each element was calculated from blank measurement. Below Limit of Detection (LOD), the analyte is not detected and above limit of quantification it is measured. In present study, method of detection was determined from the standard deviation obtained from the ten blank samples.

Hence, LOD is $\frac{3SD}{m} blank$ and LOQ is $\frac{10SD}{m} blank$. From (Table) the LOD values for all TMs that were analyzed by ICP-OES wheat samples were ranged from 0.099-0.122mg/l and LOQ values were also ranged from 0.330-0.408mg/l and Whereas IDL ranged from0.0027-0.042mg/l

Table4.2 : Method detection limit, limit of quantification and instrument detection limit of chocolate and candy samples determined by using blank solutions

Toxicicty metals	IDL (mg/L)	LOD(mg/L)	LOQ(mg/L)
Ni	0.015	0.113	0.376
Pb	0.042	0.099	0.330
Cr	0.0071	0.122	0.408
Cd	0.0027	0.112	0.374

4.3.2 Accuracy and Precision of Analytical Results

The %RSD did not differ by more than 15% of the mean which indicate that the analytical method used was precise and reliable. In brief, accuracy measured by percentage of the recovery and precision is also measured by percentage of relative standard deviation. In this study, triplicates of each sample was used to evaluate analytical method for accuracy and precision and the obtained percentage recovery varied in the two different brands chocolate and candy samples which were almost in acceptable ranges.

Percentage recovery of the spiked metals ranged from 87% to 98.95%for chocolate samples and from 86.56 to 99.0% for candy samples. This indicates that the method applied to determine toxic metals was valid.

The %RSD in present study did not differ by more than 10% of the mean which indicate that the analytical method used was also precise and reliable.

$$\%R \;=\frac{CM\,spiked-CMunspiked}{CMAmount\;of\;add}\times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [5]$$

Percentage recovery of the spiked concentrations for Ni, Pb, Cr and Cd in chocolate and candy samples was calculated by using the following equation.

Where % R = percentage recovery (in %)

CM spiked= concentration amount found in spiked sample (in mg/L)

CM un spiked= concentrationamount found in unspiked sample(in mg/L)

CMA spike = concentration amount added as a spike (in mg/L)

CM = metal concentration

Table 4.3: Recovery test results for toxicicty metal determination in chocolate and candy sample (mean & SD)

Samples	Toxic metals	concentrationamount found in unspiked sample (mg/L)	concentration amount added as a spike (mg/L)	concentration amount found in spiked sample (mg/L)	% Recovery
Chocolate	Ni	0.188	1.25	1.425	98.95
	Pb	-0.005	1.25	1.077	87.0
	Cr	0.166	1.25	1.316	92.08
	Cd	0.049	1.25	1.286	98.96
Candy	Ni	0.070	1.25	1.307	99.0
	Pb	0.012	1.25	1.094	86.56
	Cr	0.018	1.25	1.169	92.0
	Cd	0.002	1.25	1.239	98.96

Concentrations in mg/Kg of each toxic metal in the digested samples were also calculated using the following expression.

$$[M]=\frac{C}{W}\times V \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . [6]$$

[M] = total metal concentration (in mg/Kg)

C = concentration of the metal in the digested samples (in mg/L)

W=weight/mass/ of the digested sample

V=final volume of the digested sample solution (ml)

Table 4.4: Shows the concentration of(mean $\pm SD$) selected toxicicty metals in different brands of chocolate and candies using ICP-OES in mg/L

Sample	Brand	Concentration(mg/Kg)			
		Ni	Pb	Cr	Cd
Chocolate	Ricco/ChB-1	0.245±0.043	0.03±0.006	0.184±0.013	0.0495±0.007
	Take/ChB-2	0.363±0.076	0.046±0.006	0.208±0.014	0.0525±0.002
Candy	Juicy/CB-1	0.233±0.032	0.023±0.018	0.181±0.007	0.0495±0.001
	Royal/CB-2	0.197±0.013	0.004±0.004	0.17±0.003	0.0485±0.001

The concentration of four toxicicty metals Ni, Pb, Cr and Cd were determined by ICP−OES in two brands of chocolates and candies. Replicate solutions of each sample were measured. The concentrations were calculated using the mean of each value with standardization of the instrument by using the standard solutions and blank reagent. Toxic metal concentrations were based on the samples' dry weight and the mean results are the average values of the replicates.

The results indicate that the mean concentration of Ni,Pb, Cr, and Cd in chocolate samples was greater than that of their mean concentration in candy samples.The ChB-1 and ChB-2 main ingredients cacao, milk and sugar and the CB-1 and CB-2 main ingredients sugar, glucose and Strawberry fruit. The concentration toxic metals was found higher in cocoa based chocolates as compared to milk and sugar based chocolates in the order cacao$>$milk$>$sugar. The observed values are in good agreement with the reported values in cacao, milk and sugar based on chocolate and candies Cd (0.352, 0.132 & 0.099), Pb (2.48, 2.25 & 1.11) and Ni (4.33, 3.55 & 1.45) µg/g respectively [69].

Nickle (Ni)

Nickel is the 24^{th} most abundant element and naturally combined with other elements in the earth's crust and found in all soil types. In the environment, it is primarily found combined with oxygen or sulfur as oxides or sulfides. Nickel is one of the trace heavy metals found in the environment occurring at very low level[72]. Nickel was found to have the highest concentration in ChB-2sample analyzed (Table 4.5). The result obtained from ChB-2 sample was 0.363±0.076mg/L and >0.245±0.043mg/L from ChB-1, 0.233±0.032mg/Lfrom CB-1and0.197±0.013mg/L from CB-2 samples

Lead (Pb)

Lead is a naturally occurring metal. It can also rarely found in its elemental form as well as in compound form.Lead was foun to have the highest concentration in ChB-2 sample analyed(table4.5). The result obtained from ChB-2 sample was 0.046±0.006mg/L and >0.03±0.006mg/L from ChB-1, 0.023±0.018/Lfrom CB-1and 0.004±0.004/L from CB-2 samples.

Cadmium (Cd)

Cadmium is the 48^{th} element and a member of group 12 in the Periodic table of elements. The most common oxidation number of cadmium is +2. The toxicity of cadmium relates to smelting where the main route of exposure is through the lungs [12]. Cadmium was foun to have the highest concentration in ChB-2 sample analyed(table4.5). The result obtained from ChB-2 sample was 0.208 ± 0.014mg/L and > 0.0495 ± 0.007mg/L from ChB-1, 0.0495±0.001mg/Lfrom CB-1and 0.0485±0.001mg/L from CB-2 samples.

Chromium(Cr)

Chromium is the most abundant element on earth. It occurs in several oxidation states in the environment ranging from Cr (II) to Cr (VI) [12]. The most commonly occurring forms of Cr are trivalent Cr (III) and hexavalent Cr (VI), with both states being toxic to animals, humans and plants [12, 23]. Cadmium was foun to have the highest concentration in ChB-2 sample analyed(table4.5). The result obtained from ChB-2 sample was 0.208±0.014mg/L and >0.184±0.013mg/L from ChB-1, 0.181±0.007mg/Lfrom CB-1and 0.17±0.003mg/L from CB-2 samples.

4.4.Comparison of toxicicty Metals in Present Study with other Reported Results literature and permisible values

Lead was found in the concentration range of $0.03 \pm 0.006\text{mg/kg} - 0.046 \pm 0.006\text{mg/kg}$ in the chocolate brand of samples. Pb was found in the concentration range of $0.004 \pm 0.004\text{mg/kg} - 0.023 \pm 0.018\text{mg/kg}$ in the candy brand of samples. This concentration of Pb is below the concentration reported from this site 1.11-2.48mg/kg[51]. However, its concentration is agreement in the chocolate the concentration reported from this site(0.03-0.04mg/kg)[24]. Furthermore, it is in both chocolate concentration was found to be above the provisional maximum tolerable daily intake limits of permisible and in both candy concentration was found to be below the PMTDI limits of permisible this site(0.025mg/kg)[48].

.Chromium was found in the concentration range of 0.184 ± 0.013 mg/kg − 0.208 ± 0.014 mg/kg in the chocolate brand of samples and the concentration range of 0.17±0.003mg/kg to 0.181±0.007mg/kg in the candy brand of samples. Its concentration in chocolate and candy brands of this study was found to be lower than the report from this site(1.14mg/kg)[67] and agreement the report from this site (ND-5.37mg/kg) [11]. However,its content was agreement the JECFA limits for chocolate samples and Candy samples were found to be safe for Chromium risks compared to the permissible limit value(0.1-1.2mg/kg)[48].

Cadmium in analyzed samples from this study was below,agreement and greater the reported literature values for chocolate and candy samples. It was also below and safe the permissible limits set by permissible limit value (0.05-0.2mg/kg) [48].

For chocolate samples, the concentration of Cd was found to be in the range of 0.0495 ± 0.007mg/Kg − 0.0525 ± 0.002mg/Kg. For candy samples, its concentration 0.0485±0.001mg/Kg − 0.0495±0.001mg/kg that is, the mean concentration agreement in the chocolate and candy concentration reported from this site(0.001-0.03mg/kg)[24,11].

It was also concentration in chocolate and candy brands of this study was found to be lower than the report from this site (0.099-0.353mg/kg)[11,51]. However, its concentration in chocolate and candy brands of this study was found to be greater than the report from this site(0.001mg/kg)[53]. Furthermore, it is in CB-1, CB-2 and ChB-1 concentration was found to be below the provisional maximum tolerable daily intake limits of JECFA and in ChB-2 concentration was found to be agreement the PMTDI limits of WHO (0.05-

0.2mg/kg)[48].This indicates that the chocolate and candy samples under study are relatively safe for cadmium toxicity.

The concentration of Ni in chocolate and candy brands was in the range of 0.245 ± 0.043mg/Kg – 0.363 ± 0.076mg/Kg & 0.197 ± 0.013mg/kg – 0.233 ± 0.032mg/Kg, respectively. This concentration range of Ni is in below with the literature value reported in this site(1.45-4.33mg/kg)[51,11] chocolate and candy brands and its concentration is agreement in the two samples the concentration reported from this site(0.041-8.29mg/kg)[24].

However, the concentration was above the provisional maximum tolerable daily intake limits of JECFA in ChB-2 this site(0.2-0.3mg/kg)[48]. The concentration range of Ni is in agreement with PMTDI limits of WHO in ChB-1 and both candy brands. .

4.5. Evaluation of the mean toxicicty metal concentrations among the two brands of chocolates and candies

From each the determined toxicicity metals like, Ni, Pb, Cr, and Cd were found in all chocolate and candy brands.

4.5.1.Chocolate samples

As shown in the above Table 4.4 from all the determined TMs, Ni was found in the greatest concentration. Its concentration ranged from 0.245mg/Kg for ChB−1 to 0.363 mg/Kg for ChB−2.The lowest determined toxic metal was Pb whose concentration range is from 0.03 mg/Kg for ChB-1 to 0.046 mg/Kg for ChB-2.Cd was found in a concentration of 0.0495 mg/Kg for ChB-1 to 0.0525 mg/kg for ChB-2.

On the other hand, the content of Cr concentration ranged from 0.184 mg/kg for ChB-1 to 0.208 mg/Kg for ChB-2. All TMs, Ni, Pb, Cr and Cd were detected in all chocolate brands. The concentration of toxic metals in both ChB−1 and ChB−2 was found in the order of Ni > Cr > Cd >Pb.

Figure 4.3 shows the comparison of concentration of TMs in two different brands of chocolates. The mean concentrations of Ni (found in ChB-2) and Pb(found in ChB-1 and ChB-2) were found above the provisional tolerable daily intake limit (PTDIL). The mean concentrations of Ni(found in ChB-1), Cd(found in ChB-2), and Cr(found in ChB-1 and ChB-2) were found good agreement the provisional tolerable daily intake limit (PTDIL), whereas mean concentrations of Cd in ChB-1 were found below the provisional tolerable daily intake limit (PTDIL) as defined by FAO/WHO [48].

4.5.2.Candy samples

As shown in the above Table 4.5, two different brands of candies were subjected to toxic metal determination. From all the determined TMs, Ni was found in the greatest concentration. The concentration of Ni ranged from 0.197 mg/kg for CB−2 to 0.233 mg/Kg for CB−1. The lower determined toxic metal was Pb whose concentration range is from 0.004 mg/kg for CB-2 to 0.023 mg/kg for CB-1. The concentration of Cr ranged from 0.17 mg/kg for CB-2 to 0.181 mg/kg for CB-1.

On the other hand, the content of Cd was found in the range of 0.0485 mg/kg for CB-2 to 0.0495 mg/Kg for CB-1. The concentration of toxic metals in both CB−1 and CB−2 was found in the order of Ni > Cr > Cd >Pb. Figure 4.4 shows the comparison of concentration of toxic metals in the two candy brands. The mean concentrations of both brand candy the Ni and Cr good agreement the provisional tolerable daily intake and the mean concentrations of Pb and Cd were found below the provisional tolerable daily intake limit as defined by FAO/WHO [48].

From the toxic metals, in all chocolate and candy brands, the concentration of Pb was found to be relatively the first lower than any other toxic metal determined. Its concentration is some what varied in candy samples. However, it has comparable concentrations in chocolate samples.Its content was in the range is from 0.03 mg/Kg to 0.046 mg/Kg and range is from 0.004 mg/kg to 0.023 mg/kg in chocolate and candy samples, respectively.

When we see the case for Cd, it was the second lower detected in chocolate and candy samples. However, it has comparable concentrations in chocolate and candy samples. Its content was in the range of 0.0495 mg/kg to 0.0525 mg/kg and 0.0485 mg/kg to 0.0495 mg/kg in chocolate and candy samples, respectively.

On the other hand, the content of Ni and Cr were detected in chocolate and candy samples, they have comparable concentration. Ni content was range of 0.245 mg/kg to 0.363 mg/kg and 0.197 mg/kg to 0.233 mg/kg in chocolate and candy samples, respectively. Cr content was range of 0.184 mg/kg to 0.208 mg/kg and 0.170 mg/kg to 0.181 mg/kg in chocolate and candy samples, respectively.

The findings of this study indicate that our body can accumulate toxicicity metals like Ni, Pb, Cr and Cd when we consume food items like chocolates and candies.This higher concentration of toxic metals in candies and chocolates may be due to the raw materials such as cocoa beans, cocoa solids, hydrogenated vegetable oils and cocoa butter as well as their colorful packages.

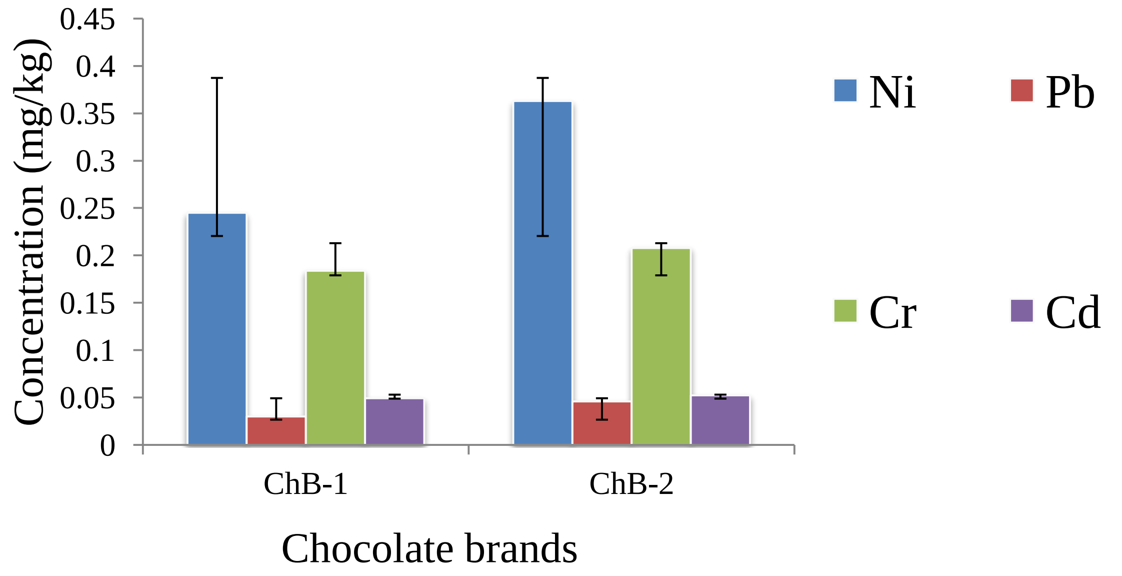


Figure4.3:mean concentration of TMs in the two brands of…

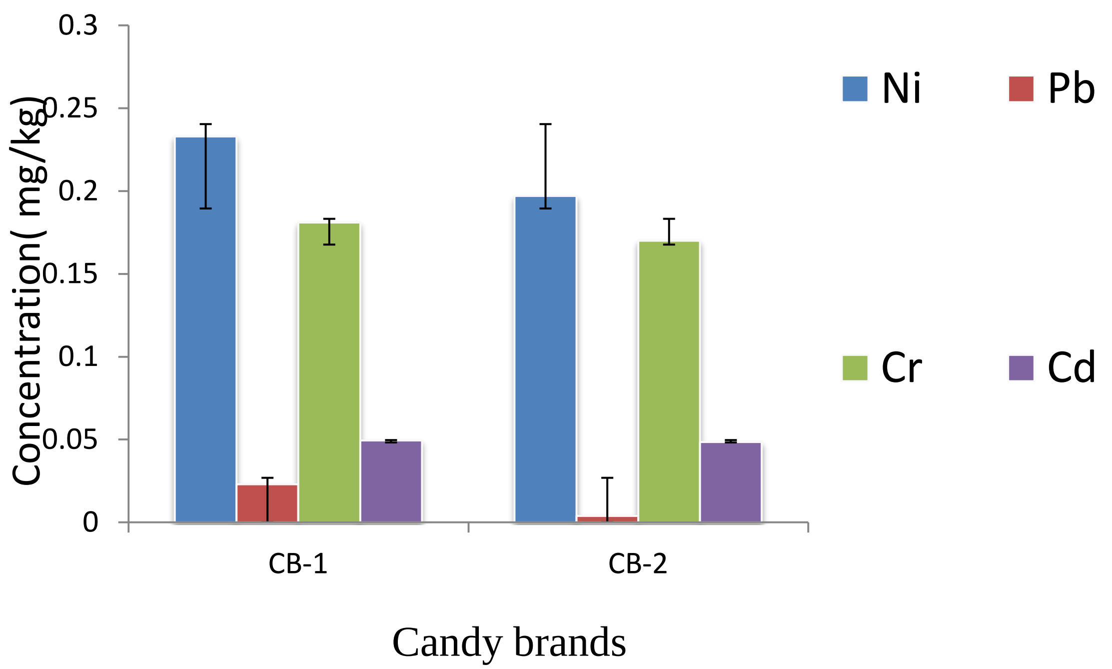


Figure4.4: mean concentration of TMs in the two brands of candies

The error bars most often represent the standard deviation the data. Small SD bars = low spread, data are clumped around the mean (i.e more reliable), it indicate that the method applied to determine toxic metals was valid. Error bars on the graph can be used to get for each toxic metals have not a significant difference between both chocolate and candy brands in the above figure 4.3 and figure 4.4 respectively. They have overlap between the standard deviation error bars, almost all each toxic metals small standard deviation bars were observed in good agreement with the reported these site [72].

The concentration of toxicity metals in two brands of chocolates and two brands candies in Debre berhan city is relatively enough and its amount is safe . The concentration of toxicicity metals may be present in chocolate and candy mainly ingredients and packaging wrappers used by the manufacturers. In this study chocolate samples were found to contain higher concentration of toxic metals than candy samples.

Previous researcher Pb contamination in chocolates and candies may be mainy associated with atmospheric emission, confectionery wrappers, colourful packaging and prepare container [25,77,73,55 and 74).

Cd naturally found from the soil cocoa beans [57,58 and 59]. As a result, its concentration could vary significantly in candies and chocolates. The FAO/WHO (2015) data, the level of Cd for chocolate brands ranges from 0.20mg/Kg−2.00mg/Kg [68].

The natural Ni content of various food items varies from NDmg/Kg−10mg/Kg [69]. The observed concentration for Ni in the present study is in agreement with this value, ranged from 0.245−0.363mg/Kg for chocolate samples and from 0.197mg/kg−0.233mg/Kg for candy samples. Nickel content in this present study may be the high content of hydrogenated vegetable oil and cocoa butter which are the main ingredients used in the production of chocolates.

Chromium is another detected toxic metal in both of the samples. ChB−1,ChB−2 and also CB-1, CB-2, it was detected of chocolate and candy brands respectively, for this study. A study conducted in Herzegovina showed the concentration of Cr in the range of ND−5.37mg/Kg for chocolate samples of which the highest concentration belongs to cocoa-based chocolates [11]. In this present study,Cr was found to have an average concentration of 0.20mg/Kg (ranged from 0.184mg/kg − 0.208mg/Kg) for chocolate samples. For candy samples, its average concentration was 0.18mg/Kg (ranged from 0.17mg/kg −0.181mg/Kg) which indicates that this much concentration of Cr in the present study is below the value determined in Herzegovina[11].

The presence of Pb and Cr in chocolate and candy samples can linked with the colored packages (wrappers) of samples can be contaminated, may be toxic metals could be migrate from the package adhere the inner coverage of package.

4.6.Statistical analysis of independent t-test

To identify which assumption is accepted or rejected, look at the Levene's Test for Equality of Variance. This test determines if the two conditions have about the same or different toxic metals amount of variability between ChB-1and ChB-2, CB-1 and CB-2.If the P value in the sig column is not significant the two conditions are the same, if not they are different and viceversal.

According to independent sample t-test, t- test for Equality of Means which is significant at P value indicates that, the actual value of independent sample t-test where as the Levene's Test for Equality of Variance show that the null hypothesis (Ho) which says there is no difference has been rejected or accepted at P value which implies the alternative hypothesis (H1) which says there is a significant difference has been accepted or rejected respectively. Independent t-tests had been performed for the mean concentration of toxic metals between different brands of chocolates and candies.

Independent t-test is powerful statistical technique that can be used for the separation and estimation of the different causes of variation of more than one means for different measurements. The possible sources of variation are due to the random error in measurement, which causes a different result to be obtained each time a measurement is repeated under the same conditions. As the means vary from one sample to another, independent t- test whether there is significance difference between the samples means and thus enabling to explain the cause of error [71] .

For this study, the significance of variation in mean concentration of toxic metals between chocolate and candy samples was analyzed using independent t-test and SPSS (Version 23) software to know the presence or absence of significant differences in mean concentration of toxic metal within the analyzed samples. The independent sample t- test was applied to identify each toxic metal which different brands of chocolate and candy samples.

Table 4.5: P values within the two-chocolate brands samples at 95% confidence interval.

Toxic metals	Chocolate				ChB-1 with ChB-2		
	ChB-1		ChB-2				
	Mean	Std. D	Mean	Std. D	T	Df	Sig.(2-tailed)
Ni	0.2445	0.0431	0.363	0.0757	1.916	4	0.195
Pb	0.0300	0.0057	0.0460	0.0057	2.828	4	0.106
Cr	0.1840	0.0127	0.2080	0.0141	1.897	4	0.198
Cd	0.0495	0.0071	0.0525	0.0021	1.784	4	0.216

Note: Not significant at $P > 0.05$ means no difference b/n groups

As the finding of this study indicated in the above Table 4.6, there is a statistically difference between each toxic metal the mean number of ChB-1 and ChB-2.

As the group statistics box revealed that, toxic metals the mean of ChB-1,Ni(0.2445 ± 0.0431), Pb(0.0300 ± 0.0057), Cr(0.1840 ± 0.0127) and Cd(0.0495 ± 0.0071) was less than the mean of ChB-2,Ni(0.363 ± 0.0757),Pb(0.0460 ± 0.0057),Cr(0.2080 ± 0.0141) and Cd(0.0525 ± 0.0021) respectively and the null hypothesis (Ho) which says there is no significant difference has been accepted and the alternative hypothesis (H1) which says there is a significant difference has been rejected at $P > 0.05$, $Df = 4$, toxic metals like, Ni, Pb, Cr and Cd t and P value $t =$1.916,2.828, 1.897, 1.784 and $P =$0.195, 0.106, 0.198, 0.216 respectively.

This implies that, the mean concentration of each toxic metals have not a significant difference between chocolate. From this, we can conclude that the ChB-1 and ChB-2 the level of toxic metals were able to the same.

Table 4.6: P values within the two-candy brands samples at 95% confidence interval.

Toxic metals	Candy				CB-1 with CB-2		
	CB-1		CB-2				
	Mean	Std. D	Mean	Std. D	T	Df	Sig.(2-tailed)
Ni	0.233	0.03182	0.1970	0.01273	1.465	4	0.281
Pb	0.023	0.01839	0.0040	0.00424	1.424	4	0.290
Cr	0.181	0.00707	0.1700	0.00283	2.043	4	0.178
Cd	0.0495	0.00071	0.0485	0.00071	1s.414	4	0.293

Note: Not significant at $P > 0.05$ means no difference b/n groups

As shown in Table 4.7 in the candy each toxic metal of CB-1 and CB-2, regarding to the finding of independent sample t-test result showed that, there is a statistically difference between the mean number of CB-1 and CB-2.

As the group statistics box revealed that, toxic metals the mean of CB-1 Ni (0.233 ± 0.03182), Pb(0.023 ± 0.01839), Cr(0.181 ± 0.00707) and Cd(0.0495 ± 0.00071) was greater than the mean of CB-2 Ni(0.1970 ± 0.01273), Pb(0.0040 ± 0.00424), Cr(0.1700 ± 0.00283) and Cd(0.0485 ± 0.00071) respectively and the null hypothesis (Ho) which says there is no significant difference has been accepted and the alternative hypothesis(H1) which says there is a significant difference has been rejected at $P > 0.05$, $Df = 4$,toxic metals like, Ni, Pb, Cr and Cd t and P value $t =$1.465, 1.424, 2.043, 1.414 and $P = 0.281$, 0.290, 0.178, 0.293 respectively.

This implies that,the mean concentration of each toxic metals have not a significant difference both candy. From this, we can conclude that the CB-1 and CB-2 the level of toxic metals were able to the same.

Ingeneral TMs, the independent t-test result indicates that the mean concentration of each toxic metals have not a significant difference between both chocolate and candy brands $(P > 0.05)$, and the same that the standard deviation error bars on the graph can be used to get for each toxic metals have not a significant difference between both chocolate and candy brands in the above figure 4.3 and figure 4.4 respectively.

They have overlap between the standard deviation error bars, almost all each toxic metals small standard deviation bar, so more reliable and it indicate that the method applied to determine toxic metals was valid.

CHAPTER FIVE

5.CONCLUSION AND RECOMMENDATION

5.1. Conclusion

TheICPOES technique has been successfully employed here for the determination toxic metals in a complex matrix like chocolate and candy.Two chocolate and two candy brands marketed in the city of Debre Berhan, were analyzed to their toxic metal content. The analysis was done after digesting the samples using conventional wet acid digestion method.

The mean concentration of Ni (found in ChB-2) and Pb (found in ChB-1 and ChB-2) were higher than the JECFA limit, while the mean concentration of Ni (found in CB-1, CB-2 and ChB-1), Cd (found in ChB-2) and Cr found in both brand chocolate and candy samples were safe the JECFA limit. The concentration mean of Pb (found in CB-1 and CB-2) and Cd(found in ChB-1, CB-1 and CB-2) were below the JECFA limit. However, their concentration value agrees with most of other literatures [7, 16 and 44]. The concentration of Ni was found in the range of $(0.245 \pm 0.043 - 0.363 \pm 0.076)\mathrm{mg/Kg}$ for chocolate samples and from $(0.197 \pm 0.013 - 0.233 \pm 0.032)\mathrm{mg/Kg}$ for candy samples and the concentration of Pb was found in the range of $(0.03 \pm 0.006 - 0.046 \pm 0.006)\mathrm{mg/kg}$ for chocolate samples and from $(0.004 \pm 0.004 \ - 0.023 \pm 0.018)\mathrm{mg/kg}$ for candy samples.

Another toxic metal determined was Cr whose concentration ranged from $(0.184 \pm 0.013 - 0.208 \pm 0.014)\mathrm{mg/Kg}$ in chocolates and from $(0.17 \pm 0.003 - 0.181 \pm 0.007)\mathrm{mg/Kg}$ in candies. In general, the Cd content in this study was below its values reported by other literatures as well as the JECFA provisional maximum tolerable daily intake limits. Its concentration ranged from $0.0495 \pm 0.007\mathrm{mg/Kg} - 0.052 \pm 0.002\mathrm{mg/Kg}$ in chocolate and from $(0.0485 \pm 0.001 - \ 0.0495 \pm 0.001)\ \mathrm{mg/kg}$ in candy samples.

In chocolatesthe mean concentrations of Pb, Cd, Ni and Cr were found to be higher in cocoa powder and cocoa-based products than in milk and sugarbased chocolates. The frequent use of cocoa-based chocolates and confectionaries containing cocoa powder should be reduced, otherwise intake of lead and nickel will cross the limits prescribed by the JECFA. Raw materials should properly be handled and if necessary be assessed for metal contents before use. These research findings will hopefully create consciousness towards frequent utilization of chocolates.

5.2. Recommendation

This study was the first to the areas on the determination of some selected TMS; Ni, Pb, Cd and Cr effect of chocolates and candies. chocolates and candies are existences of essential elements Magnesium, Copper, potassium…etc and polyphenol compounds class of antioxidants, flavonoids, commonly found in such foods, have attracted great interest in potentially lowering risk. Since cocoa products contain greater antioxidant capacity and greater amounts of flavonoids. It is important to explore chocolate's potential effects. Since ancient times, chocolate has long been used as a medicinal remedy and been proposed in medicine today for preventing various chronic diseases [78].

Nevertheless, some metals like Ni, Pb, Cr and Cd that can exist in chocolates and candies are toxic for the human body if excessively ingested. Based on the results of this study,it is recommended that:-

The results showed that from the entire understudy toxic metals Ni (found in ChB-2) and Pb (found in ChB-1 and ChB-2) were higher than the JECFA limit, therefore there should be great care for Debre Berhan city and surrounding people in feeding chocolate.

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