Debra Berhan University

College of Natural and Computational Science

Department of Chemistry

Determination of levels of some essential and non-essential elements in apple fruits in Amhara region Lay Gayint District by flame atomic absorption spectrophotometer (FAAS)

By

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Advisor Approval sheet

This is to certify that the thesis entitled with**Determination of levels of some essential and non-essential elements in apple fruits in Amhara region Lay Gayint District by flame atomic absorption spectrophotometer (FAAS)** submitted in partial fulfillment of the requirements for the Degree of Master of Science in Chemistry, in the graduate program of the Department of Chemistry which has been conducted byWondemagegneTesfaye**were** done under our supervision. Therefore, I recommend that the student has fulfilled the requirements and hence here can submit the thesis to the department.

HulugirgeshDegefu(Ph.D) _____ _____

Name of Advisor **Signature** **Date**

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Signature of Board of Examiners

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Declaration

I the under signed, declare that this thesis is my original work and has not been presented for degree in any other university and that all source of materials used for the thesis have been duly acknowledged.

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Abstract

The levels of four essential metals (Ca Mg, Fe, and Zn) and two non-essential metals (Pb and Cd) were determined in Anna and BR-64 apple fruit varieties cultivated in Lay Gayint district, Ethiopia. Wet digestion method using a mixture of 5mL of concentrated HNO_3 and 1mL $HClO_4$ was used for digestion of the samples. The determination processes were done by flame atomic absorption spectrometry for all essential and non-essential metals. The results obtained revealed that the concentrations of metals in the Anna sample varieties in mg/kg dry weight were: Ca (137.306), Fe (3.8266), Mg (129.81),Zn (0.3433), Pb (0.0059) and Cd (0.0018) and BR-64 apple sample varieties in mg/kg dry weight were: Ca(138.796),Fe (3.25),Mg (131.046),Zn (0.2866), Pb (0.0029) and Cd (0.0018). The results showed that the levels of metals were slightly higher in the Anna than BR-64 apple sample except magnesium and Cadmium. In addition, the content of metals in both the analyzed samples variety were found below the FAO/WHO maximum permissible limit and hence these are safe for human consumption and can be considered as a good source of essential nutrients.

Keywords:*Anna apples, BR-64 apples, Essential metals, FAAS, Non-essential metals,Wet digestion.*

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1. Introduction

1.1 Background of the study

Fruits and vegetables play a number of important roles in human health because they contain a wide range of compounds including the antioxidants such as vitamin C and E, phenolic and carotenoids that are important in neutralizing free radicals known to cause cancer, cataracts, heart disease, hypertension, stroke and diabetes. In addition, fruits and vegetables are important source of essential elements, which play vital roles in the proper development and good health of human body (1). They are also the most important sources of vitamin A, a nutrient important for several metabolic activities in the body, in addition to its role as antioxidant. They provide foliate and potassium that are known to prevent birth defects, cancer, heart disease, hypertension and stroke. They are also good sources of minerals such as iron, zinc, calcium, potassium, and phosphorus and contain ample fiber, important for digestion and bowel movements. In general, WHO places low fruit and vegetable consumption among its twenty risk factors in global mortality, just behind the better-known killers such as tobacco use and high cholesterol levels(2,3).

Apples are well-known and widespread fruits of the genus MalusDomestica belonging to the family Rosaceae. They were introduced into Ethiopia some 60 years ago by missionaries, Ralph Wiegand, southwestern Ethiopia (>2700 m.a.s.l.), At such high altitudes in the tropics, average temperatures are lower, which allows easier reaching of chilling conditions, but seasonal amplitudes remain low (4) . Unfortunately, systematic observations have been carried out only once in the Ethiopian highlands, on apple cultivars introduced in 1976 (5).Some productive low-chill apple trees have been mainly restricted to areas with a humidtropical mountain climate in the southern Ethiopia until the past 20 years. As a result, there islittle knowledge available about the physiological responses of apple trees to the otherhighland areas in the country, these having rich potential for apples and other temperatefruits, zespecially sub-humid central and northern highlands were apple production.Is becoming popular at present Ethiopian highlands are endowed with a mosaic of soils and climate which are suitable for the production of many temperate fruits and nut crops.

The country is characterized by having diverse topography and agro-ecological zones, of which over 50% of the total area is highland at elevation between2000 – 4500 m.a.s.l. with adequate

water resources and low temperature during winter that can favor many temperate fruit crops to grow(6).

Apples are among the most widely consumed fruits in various countries and are consumed fresh or the processed forms such as juices and dried Apple. They make very significant part of the diet in humans and represent a good source of dietary fiber, pectin, potassium, and vitamins A and C(7).The major part of the edible portion of fresh apples contains 75–95% water. Also, apples contain a significant number of different classes of phenolic compounds, which can protect the human body against oxidative stress by scavenging oxygen free radicals (8). They also contain essential and non-essential elements. Non-essential elements are priority pollutants that pose potential risks to human health and the environment (9). An element is essential when it is consistently determined to be present in all healthy living tissues and when its deficiency symptoms are noted, with depletion or removal, which disappears when the elements are provided to the tissues (10). The apple plants can absorb these metals and store them in the roots and then transport into the shoots and fruits. On reaching the apple fruits, these metals form the vehicles of contamination for humans inducing adverse health effects. The existence of these various elements in apple fruits depending on the characteristics of climate, soliland composition of irrigation water. However, the concentration of these essential and non-essential elements present in apple fruits is not determined until now in the study area.

There are different techniques so far reported for the determination of metals in plant products. These analytical techniques were direct current argon plasma optical emission spectroscopy (DCP-OES), flame atomic absorption spectrometry (FAAS) (11) ,graphite furnace atomic absorption (GFAA), inductively coupled argon plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS)(12). These methods are most commonly used for the determination of metals in environmental samples because of their inherent selectivity, sensitivity, precision and accuracy.

In this study, flame atomic absorption spectrometry (FAAS) technique will be used to determine the concentration of essential elements (Ca,Mg, Fe,Zn) and toxic metals (Pb and Cd) in apple fruits due to its low cost, fast analysis time, friendly operation and wide application. The results also will be compared with literature values.

(a) Anna **(b)BR-64**

Figure 1: Image of Anna and BR 64

1.2 Statement of the problem

Apple fruits should be safe enough for general human consumption and should not represent any significant risk to health over a lifetime of consumption. Essential and non-essential metal concentrations in soils and irrigation water of apple plants are the major component in the determination of apple quality. Apparently, the various nutrient compositions of apple fruits in many countries are affected by industrialization. However, in developing countries like Ethiopia, particularly in Amhara RegionLaye Gayint district,the concentrations and kinds of essential and non-essential metals in apple fruits is not studied until now. Mostheavy metals generated fromagricultural and other human activities will have pollution effects on the soils and irrigation water and then consequently to that of apple fruits quality, here byposing a great danger to human lives. The metals in apple fruits most often will include calcium, manganese, nickel, lead, iron, cadmium, zinc and copper. Thus, the toxic metals like cadmium and lead, and even when the concentration of essential metals like calcium, manganese, nickel, iron, zinc and copper become above or below the WHO permission level, they are believed to have adverse effects on the central nervous system, the cardiovascular system, kidneys and the immune system. Therefore, due to these adverse health effects of metals, the detection of these metals in apple

fruit sample is very important to give attention for the commercialization of quality of apple fruits in the study area.

1.3 Objectives of the Study

1.3.1 General objective

The general objectives of the study is to determine the concentrations and kinds of some essential and non-essential elements in Anna and BR-64apple fruits samples by using flame atomic absorption spectrophotometer (FAAS) .

1.3.2 Specific objectives

- To detect the levels of some selected essential metals (Ca, Mg, Fe, and Zn) in the two varieties (Anna, BR-64) of apple fruits by flame atomic absorptionspectrometer.
- To determine the levels of some selected non-essential metals (Pb, Cd) in the two varieties (Anna, BR-64) of apple fruits by flame atomic absorptionspectrometer.
- To compare the levels and kinds of these some selected essential and non-essential metals in the two varieties (Anna, BR-64) of apple fruits.

2. REVIEW OF RELATED LITERATURES

2.1 Chemical ingredients of apple

Apples, rich source of phytochemicals, are widely consumed and epidemiological studies have linked the consumption of apples with reduced risk of some cancers, cardiovascular disease, asthma, and diabetes. In the laboratory, apples have been found to have very strong antioxidant activity, inhibit cancer cell proliferation, decrease lipid oxidation, and lower cholesterol. The existence of various nutrient compositions among species of apple fruits depending on the characteristics of the farm land soil, climate, cultivation conditions and composition of irrigation water (13, 14 and 15).

2.2. Essential and non-essential elements

Metals can be classified as either essential elements (important for life) or non-essential elements. Non-essential metals (with no known physiological functions to humans) are prioritypollutants that pose potential risks to human health and the environment (9). An element isessential whenit is consistently determined to be present in all healthy living tissues and whenits deficiency symptoms are noted, with depletion or removal, which disappears when these elements are provided to the tissues. There are 20 mineral elements essential for plant growth. Carbon (C), hydrogen (H) and oxygen (O) are supplied by air and water. The six macronutrients, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S), are required by plants in large amounts. The rest of the elements are required in trace amounts (micronutrients). Essential trace elements include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), sodium (Na), zinc (Zn), molybdenum (Mo), andnickel (Ni). Studies have also shown that a number of other mineral elements are beneficial tothe growth of plants and are required for some plants (16).

Heavy metals are metals having a density of 5 g/cc. These metals occur in all foods as naturalor inherent components of plant and animal tissues and fluid and also may be present as a result of contamination or deliberate addition (17). They have important positive and negative roles inhuman life. Some of heavy metals are considered essential including iron, zinc and copper while some metal ions like cadmium, lead and mercury are non-essential metals which havetoxic roles in biochemical reactions in our body(18).Although only required in small amounts, trace

elements (or micronutrients), are essential for plant growth. These nutrients often act as catalysts in chemical reactions.

Trace element plays an important role in chemical, biological, biochemical, metabolic, catabolic and enzymatic reactions in the living cells of plants, animals and human beings. Trace elements have great significance due to their tendency to accumulate in the vital human organs over prolong period of time. It is possible to have toxicities of trace elements, as well as deficiencies. In fact, some of the trace element deficiencies in plants can cause nutrient deficiencies in the animals that graze those plants(19).Metal accumulation in plants depends on the plant species, types of soil, environment andagricultural practice. Some plants may accumulate toxic metals at high levels which may beharmless to the plant but could be harmful to humans if ingested. The total metal content in soil is the result of parent materials, fertilizers, atmospheric deposition, agrichemicals, and organic wastes(14). In addition, they could be contaminated from various species include in trace metals as farmers wash them with waste water before bringing them to market (10).Keeping in mind the potential toxicity and persistent nature of heavy metals, and the frequentconsumption of vegetables and fruits, it is necessary to analyze these food items to ensure thelevels of these contaminants meet agreed international requirements.

Cadmium (Cd) is a well-known environmental hazard, exerts a number of toxic effects in humans andanimals (20). Cadmium is water soluble and can be transferred efficiently from soil toplants, which may affect human health if there is excessive intake from a contaminated foodsource. Increased concentrations of Cd in agricultural soils are known to come from humanactivities such as the application of phosphate fertilizer, sewage sludge, wastewater, andpesticides, mining and smelting of metallifer us ores with high Cd content and traffic (21, 22).It accumulates mainly in the kidney and liver and high concentrations have been found to leadto chronic kidney dysfunction(23, 24). The basis of Cd toxicity is its negative influence oneenzymatic systems of cells, resulting from substitution of other metal ions in metalloidenzymesand its very strong affinity to biological structures containing –SH groups, such as proteins, enzymes, and nucleic acids. Many effects of Cd^{2+} action result from interactions with necessary micro-and macro elements.

Lead (Pb) is a toxic, bio-accumulative heavy metal with no known biological function. Its sources may be natural, as it is found in the earth's crust and thus enters the food and water

supply. It is absorbed by food stuffs (particularly green leafy vegetables) growing on soil where lead is present. Lead is absorbed by plants to varying degrees depending on the form of the metal present, and the pH and the temperature of the soil. It can adversely affect many organs systems and numerous conditions such as high pressure, anemia, kidney damage, impairedwearing and mental retardation elevated levels in women may result in a shortened gestationperiod, while young children are considered at great risk because of their ability to effectivelyabsorbed lead and thereby suffer mental and physical development retardation(25).

Zinc (Zn) is the fourth important micronutrient after vitamin A, iron and iodine, and is nowreceiving increasing global attention and is particularly necessary in cellular replication, thedevelopment of the immune response and as a cofactor for many enzymes of the body (26).Although Zn has been found to have low toxicity to human, prolonged consumption of largedoses can result in some health complications such as fatigue, dizziness and neutropenia.

Freeradicals and prevent cell structure damage. It is regulated in the body via absorption, excretionand a combination of processes. However, very large single or daily intakes of copper can harmyour health. Long term exposure to copper dust can irritate your nose, mouth and eyes, andcause headaches, dizziness, nausea and diarrhea. Higher level of copper ausea (27).

Magnesium (Mg) is the health status the digestive system and the kidneyssignificantly influence magnesium status. Magnesium absorbed in the intestine and theirs transported through the blood to the cells in the tissues. Approximately one-third dietary magnesium is absorbed in the body.Magnesium is co-factor for manyco-enzymes, also affects metabolism of K and Ca. Its deficiency is related to high blood pressure, pregnancyproblems, and poor heart function.Magnesium is active component of several enzyme systems in which thymine pyrophosphate is co-factor.Oxidative phosphorylation is greatly reduced in the absence of magnesium. Magnesium is also an essential activator for the phosphate-transferring activator enzyme myokinase,diphophopyridinenucleotide kinase,and creativekinase (28*).*

Calcium (Ca) needs in human body for numerous functions, such as building and maintaining thebones in water mayexperience vomiting, diarrhea, stomach cramps and teeth, blood clotting, transmitting of the nerve impulses and regulating heart's rhythm. Ninety nine percent of calcium in a human body is stored in bones and teeth. Theremaining one percent is found in the blood

and other tissues. The body gets calcium by pullingit from the bones when blood levels of calcium drop too low, usually when quite a longtimepassessince having taken calcium with meal. Calcium can also be found in dark green leafyvegetables, dried beans and legumes, calcium fortified juice in addition to milk powder and dairy products are a convenient source of calcium for many people(29).

Iron (Fe) is one of the most abundant metals in the universe. It is a mineral necessitates the red blood cell formation and also one of the most useful in biochemical functions. In the humanbody, iron is present in all cells and has several vital functions as a carrier of oxygen to the tissues from the lungs in the form of hemoglobin (Hb), as a facilitator of oxygen use andstorage in the muscles as myoglobin, as a transport medium for electrons within the cells in the cytochromes, and as an integral part of enzyme reactions in various tissues. Iron in generahas poor availability from foods derived from plant sources compared to foods from animalsources. Iron deficiency can be a very difficult diagnosis, since the symptoms such as lethargy, tiredness and dizziness, are non-specific can be found in a variety of ailments (30, 31).

3. MATERIALS AND METHOD

3.1 Description of the study area

3.1. 1 Geographical location

Lay Gayint is one of the woredain the Amhara Region of Ethiopia, Part of the South Gondar Zone. It is bordered on the south by Tach Gayint and Simada, on the southwest by Misraq Este, on the west by Farta, on the north by Ebenat, and on the east by the SemienWolloZone. The altitude of this woreda varies from 1,500 to 3,100 meters above sea level. The woreda is mostly found in Dega and Woina-Dega climatic zone with the mean annual temperature ranging8℃-20℃ and the annual rainfall is erratically distributed and varies from 400 to 1,100 mm.The administrative center is NefasMewcha which has an altitude and longitude of 11^{0}44'N38^{0}28'E coordinates of 11^{0}44'N38^{0}28'E and an elevation of 3120 meters above sea level. It is 735km away from Addis Ababa and it is 175 km awayfromBahir Dar and 75 km from Debre Tabor town.

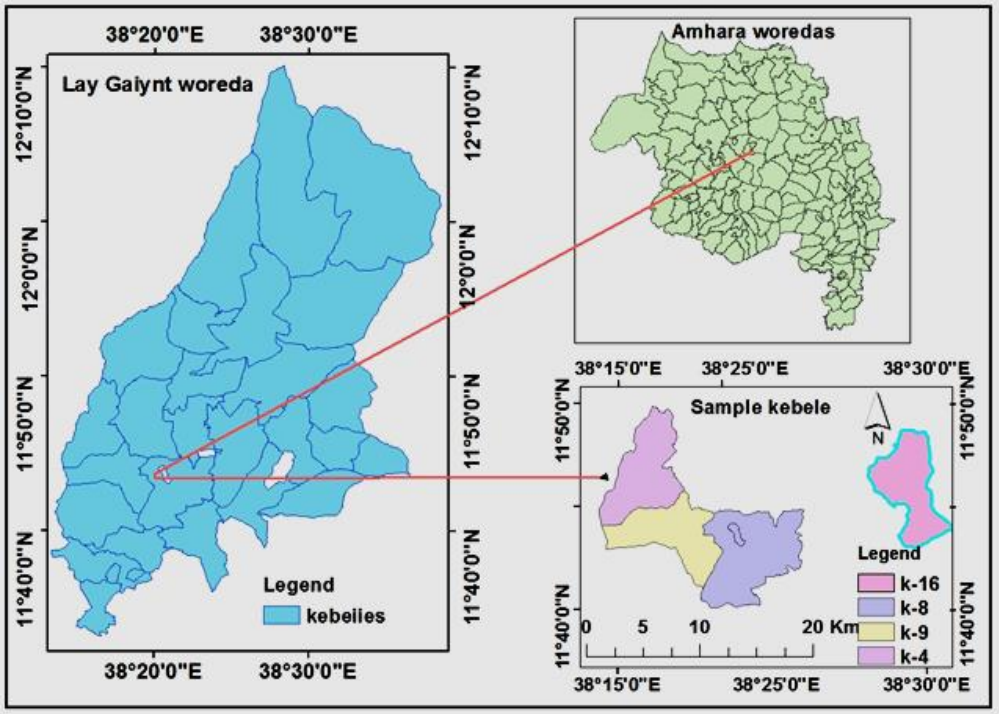


Figure 2: Map of study area in Lay Gayint woreda GIS

3.1.2 Population

Based on the 2007 national census conducted by the Central Statistical Agency of Ethiopia (CSA), this woreda has a total population of 206,499 of whom 104,401 are men and 102,098 women. It has 31 kebeles and among them, 18 kebeles have a great potential for apple production which is mainly supplied to DebreTabore and other parts of the Region.

3.1.3 Climate

The district is characterized by three climate climate zones ,Dega(highland),WoynaDega(mind land) and kola(low land) .Based on Laygint district Agricultural and Rural Development Office LDARDO report of 2015, the highest and the lowest daily temperature generally record are 20 and 8 respectively , and the annual average temperature is14 degree centigrade.The annual rangebetween 600-120mm.

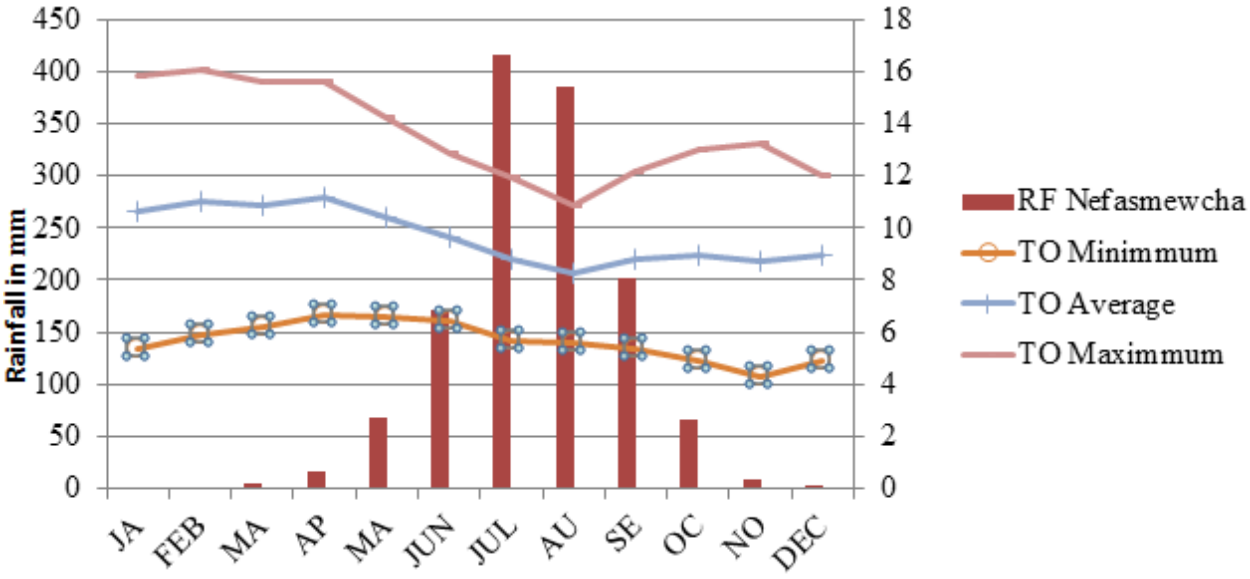


Figure 3: Mean monthly Rain fall distribution of the study area. (2013-2017) Source: National Metrological Agency (NMA, 2017)

3.2 Equipment and reagents

3.2.1Equipment.

Stainless steel and Teflon (PTFE) knife wereused to cut the fruit samples in to pieces while air-circulating oven was used for drying the apple fruit samples placed on porcelain. Blending device, ceramic pestle and mortar was used to grind and homogenize the fruit samples. Digital analytical balance will be used for weighing the fruit samples and a refrigerator was used to keep the digested sample until analysis. A 100 mL round bottom flasks fitted with reflux condenser was used in Kjeldahl apparatus. Micropipette(1-10 μL and 100-1000 μL) wasused to measure the volume of reagents and standards. Atomic absorption spectrophotometer equipped with deuterium arc background corrector was used for analysis of the metals (Ca, Mg, Fe,Zn, Pb and Cd) in apple fruitsamples using air acetylene flame.

3.2.2 Reagents and chemicals

Deionized water was used throughout the experiments for all dilutions and rinsing purposes. 70 % HNO_3, 70 % $HClO_4$ and 37 % HCLwasused as received for the digestion of apple fruit samples. Stock standard solutions of the metals (1000 mg/L) calibration standards, prepare as nitrates for each element in 2 % HNO_3, was used for the preparation of calibration curves for the determinant of metals in applefruit samples.

3.3 Procedures

3.3.1 Collection and preparation of apple samples

3.3.2 Sample Collection

For this study two different homogenized apple varieties were taken from four local apple cultivar or farmers in Lay Gayint district. These apple sample varieties were Anna and BR-64 apple fruits. The four sub-sites (Titera, Sale, WuhaMider and Amba Maryam) in Lay Gayint district were chosen for this study because they are the main apple fruit producers, highly demanded and also supplied to the market in Debre Tabor and other parts of the small town. 500gm of the homogenized and fully matured Anna and BR-6 apple fruits were separately collected.

Figure 4 Sample collection Area

3.3.3 Sampling and pretreatment

Anna and BR-64 apple fruits were collected randomly from the respective varieties of apple plants grown in four sub-sites of the district. They were placed in ice-box to keep them as fresh as possible with polyethylene sheet and transported to the laboratory within 1 day after collection. Surface contaminants of the fruits were washed with tap water, rinsed with deionized water and dried with tissue paper. Each apple fruit samples were separately dissected into pieces along the equatorial plane with plastic knife. Seeds and large particles of cellular materials were removed to represent the edible portion. The apple fruit samples were homogenized separately in a blender and stored in polyethylene bags until the time of analysis.

3.3.4 Digestion of samples

For the digestion of apple fruit samples, different conditions such as: digestion time, volume ratio of reagents and digestion temperature were tried to obtain optimum conditions as indicated in (Table 1). The optimum conditions for apple sample digestion are Nitric acid andperchloric acid mixture with (5:1 v/v), digestion temperature 340^{o}c and digestion duration of 3:00 hours.

Applying the optimized procedure, 0.3gm of dried and homogenized apple sample was transferred into a 100 mL round bottomed flask. Then 6 mL mixture of HNO_3 (69- 72%) and $HClO_4$ (70%) (5:1 v/v) was added and the mixture was digested on the Kjeldahl digestion apparatus by setting the temperature first at 120^0c for 30 minutes and then, increased to 340 ^{0}c for the remaining 3:00 hours. Then after, the digested solution was allowed to cool for 10 min without dismantling the condenser from the flask and for 5 min after removing the condenser. In the cooled solution, deionized water was added to dissolve the precipitate formed on cooling. Then the solution was filtered in to 100 mL volumetric flask with 125mm diameter of Watmann No. 41 filter paper. Subsequently, the round bottom flask was rinsed with some deionized water and filtered into the volumetric flask. After adding 1% lanthanum chloride solution, the volumetric flask was filled to the mark with deionized water. The digests were prepared in triplicates and for each sample three repeated measurements were performed by the flame atomic absorption spectrometer. Preparation of a reagent blank was also performed in parallel with the fruit samples keeping all digestion parameters the same (32).

3.4 Analysis of samples for metals content study

Standard solutions containing 100 mg/L were prepared in 100 mL volumetric flask from the atomic absorption spectroscopy standard stock solutions that contained 1000 ppm. Five working standards for Mg and Zn and four working standards for Fe, Ca,Pb and Cd metals were prepared from these secondary standards. These working standards were prepared freshly for each element from the secondary standards by appropriately diluting with deionized water for calibration purpose (Table 2). Then, Ca, Mg, Fe, Zn,Pb and Cd were analyzed with FAAS (BUCK SCIENTIFIC MODEL novAA 400P) equipped with deuterium arc background corrector and standard air-acetylene flame system using external calibration curve after the parameters were optimized for maximum signal intensity of the instrument. For each element, their respective hallow cathode lamp was inserted in to the atomic absorption spectrophotometer, and the solution was successively aspirated into the flame. The acetylene and air flow rates were managed to ensure suitable flame conditions. Three replicate determinations were carried out on each sample. The elements were determined by absorption/concentration mode and then, the instrument readout was recorded for each solution.

The same analytical procedure was employed for the determination of elements in the six digested blank solutions.

3.5 Method Validation

The proposed method was validated by evaluating different parameters such as linearity, accuracy (in terms of recovery) and precision (in terms of repeatability).Limit of detection (LOD) and limit of quantification (LOQ).

3.5.1. Accuracy and Precision

The accuracy and precision of the proposed procedure were evaluated by the analysis of matrix spike samples. Accuracy was evaluated through recovery studies of sample spikes. Precision was evaluated regarding repeatability by estimating the relative standard deviation (RSD) of the recovery percentage for each spiked level (33). In this study, the recovery test was done by spiking a suitable known quantity of metal standard solution into a test portion of the sample. For doing so, each sample was spike in triplicates at a calibration concentration 2.5 mg/L of Ca; 0.5 mg/L of each Mg, Fe and Zn; 0.25 mg/L of Cu and 1.5 mg/L ofPb and Cd.

The spiked and non-spiked samples were digested and analyzed using the same analytical procedure as the Anna and BR-64 apple samples. The recoveries of the analyses were calculated by using equation 1 (34).

$$\%\,\text{Recovery} = \frac{\text{Concentration of spiked result} - \text{Concentration of unspiked result}}{\text{Amount added}} \times 100 \qquad (1)$$

The relative standard deviation for replicate analyses of the same sample was obtained as dividing the 3standard deviation by the mean value of the data.

3.5.2 Limit of Detection

The limit of detection (LOD) is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. In this study, the LOD was obtained from triplicate analysis of reagents blanks which were digested in the same digestion procedure as the actual samples. It is the same as a concentration, which gives a signal 3 times the standard deviation of the blank. The LOD for each analyze was calculated and the results are presented in Table 3 (35).

3.5.3 Limit of Quantization

The limit of quantization (LOQ) is the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy under the stated conditions of test. In this study, LOQ was obtained from triplicate analysis of reagents blanks which were digested in the same digestion procedure as the actual samples. The quantification limit of each element was calculated as ten times the standard deviation of the blank (10S blank) and the results are indicated in Table 3 (35).

3.6. Statistical analysis

All analyses were carried out in triplicates and the data were presented as mean ± standard deviations. T-test was used to compare one set of measurements with another to test for statistically significant differences at $P < 0.05$. Other statistical calculation such as regression line, errors, intercept and slope were calculated.

4. RESULTS AND DISCUSSION

4.1 Optimization of digestion of apple samples

A series of procedures involving some changes in reagent volume, reagent composition, digestion temperature and time were tested. Accordingly, nine procedures were tested for digestion of the apple samples. The optimized procedure and conditions indicated under number 8 (Table 1) were used throughout the analysis.

Table 1: Attempted digestion procedure for 0.3gm of digested apple samples.

Trial No.	**Reagent used**	**Volume ratio (mL)**	**Temp (^{0}c)**	**Digestion time (hrs)**	**Observations**
1	HNO_3:$HClO_4$	5:10	180	4:40	Clear light yellow
2	HNO_3:$HClO_4$	4:10	200	4:40	Clear light yellow
3	HNO_3:$HClO_4$	5:2	220	4:45	deep yellow
4	HNO_3:H_2O_2	10:1	240	3:20	Clear yellow
5	HNO_3:H_2O_2	5:10	240	4:15	Clear light yellow
6	HNO_3:$HClO_4$:H_2O_2	3:2:1.5	260	4:00	Clear light yellow
7	HNO_3:$HClO_4$:H_2O_2	3:2:1	260	4:35	Clear light yellow
8	HNO_3:$HClO_4$	5:1	340	3:00	Clear and color less (optimized)
9	HNO_3:$HClO_4$	2.5:2	320	3:15	Clear light

The optimized procedure was selected depending upon: clarity of digests, minimal reflux time/digestion time, minimal reagent volume consumption, absence of undigested apple samples, simplicity and acceptable use of masses of apple samples. Based on these criteria, the optimal digestion procedure chosen was the one that requires 3 hrs, for complete digestion of 0.3g of digested apples with 5 mL HNO_3 (69-72 %) and 1 mL $HClO_4$ (70 %) (Table1). However, the other tested procedures have some limitation. They require higher reagent volume and longer digestion time. In addition, they result in the formation of turbid digests and colored digest solutions.

4.2. Instrument calibration

The qualities of results obtained for essential and non-essential metals analysis using FAAS are seriously affected by the calibration and standard solution preparation procedures. The instrument was calibrated using five series of working standards for Mg and Zn, and four series of working standard so of Ca, Fe ,Pb and Cd. For all analytes, the analytical curves showed correlation coefficients (*R*) values higher than 0.997, indicating a good linear correlation between the analytical signal and the analyte concentration.

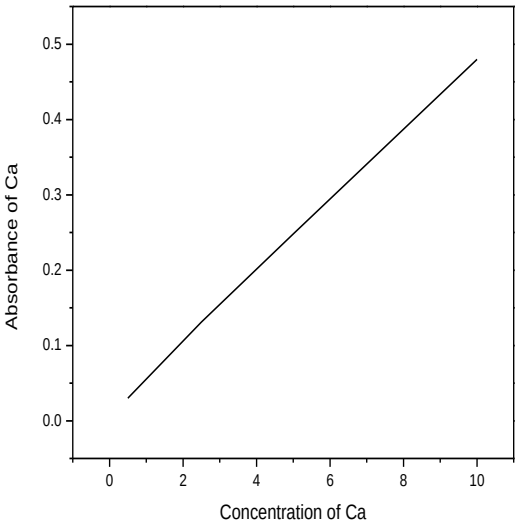
Table 2:ThestandardConcentrationof metals and their Absorbance.

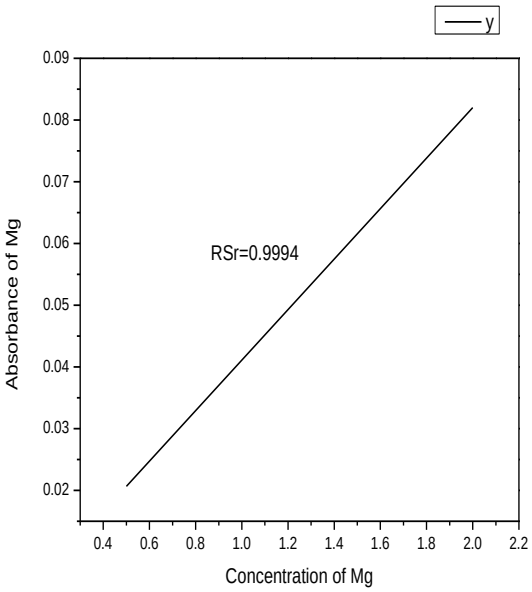
Ca		**Mg**		**Fe**		**Zn**	
Conc.(ppm)	**Abs**	**Conc.(ppm)**	**Abs**	**Conc.(ppm)**	**Abs**	**Conc.(ppm)**	**Abs**
0	0.005	0	0.0002	0.5	0.0196	0	0.002
2.5	0.153	0.5	0.0206	1	0.0372	0.25	0.0224
5	0.311	1	0.0411	2	0.0724	0.5	0.0433
10	0.627	1.5	0.0615	4	0.1428	1	0.0852
-	-	2	0.082	-	-	2	0.1689

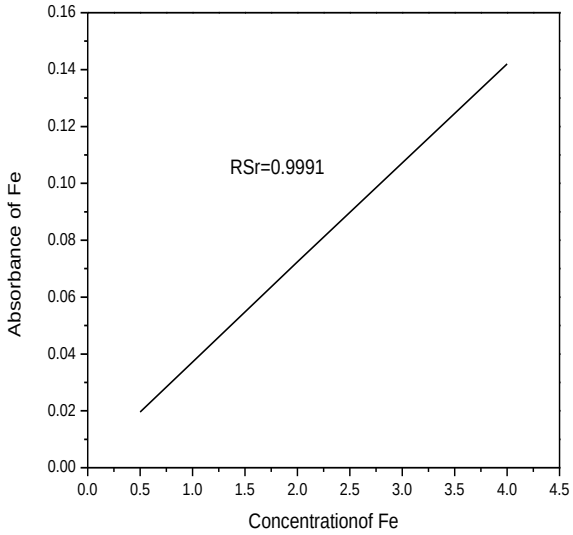
Pb		**Cd**	
Conc.(ppm)	**Abs**	**Conc.(ppm)**	**Abs**
0.5	0.107	0	0.0015
1	0.2155	0.5	0.0697
1.5	0.324	1.5	0.2061
2	0.4325	2	0.2743

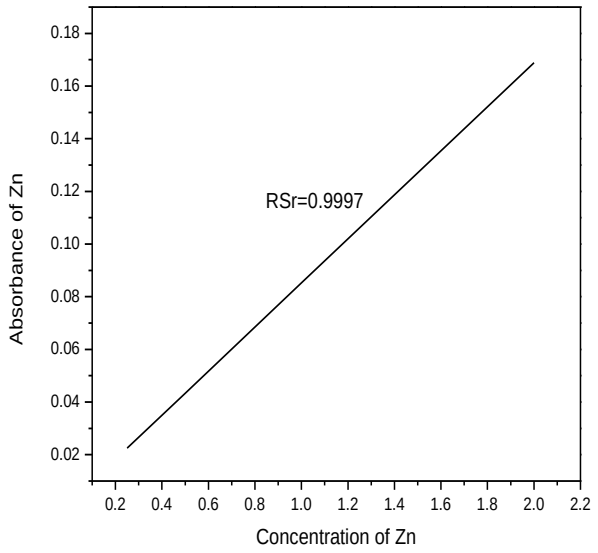
Table 3: Instrumental operating conditions for determination of metals using FAAS

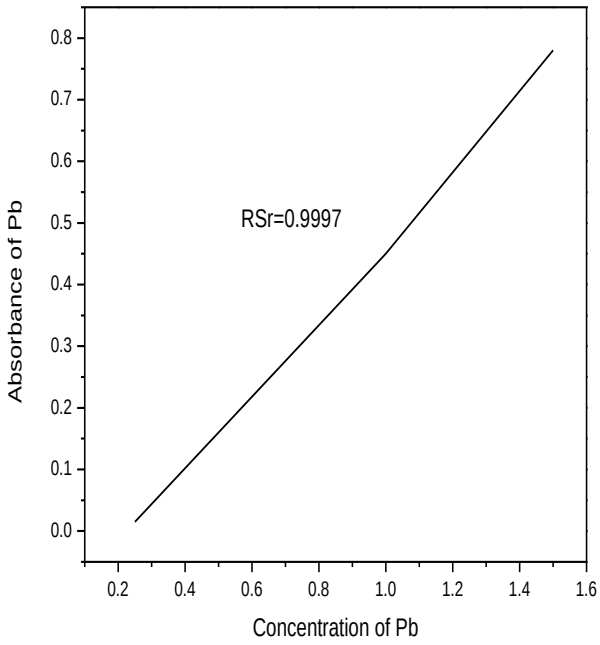
Element	**λmax (nm)**	**Slit width (nm)**	**Current (mA)**	**Energy Set (J)**	**Flame type**
Fe	248.3	0.2	4	458.00	Air, acetylene
Mg	285.21	0.8	2	266.00	Air, acetylene
Zn	324.8	0.7	2	432.00	Air, acetylene
Ca	422.7	0.8	3	277.00	Air, acetylene
Pb	283.3	0.2	1.2	330.00	Air, acetylene
Cd	228.8	0.2	1 .2	325.00	Air, acetylene


Absorbance of Ca
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
Concentration of Ca


y
Absorbance of Mg
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
RSr=0.9994
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Concentration of Mg


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance of Fe
RSr=0.9991
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Concentrationof Fe


Absorbance of Zn
Concentration of Zn
RSr=0.9997
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2


Absorbance of Pb
Concentration of Pb
RSr=0.9997
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

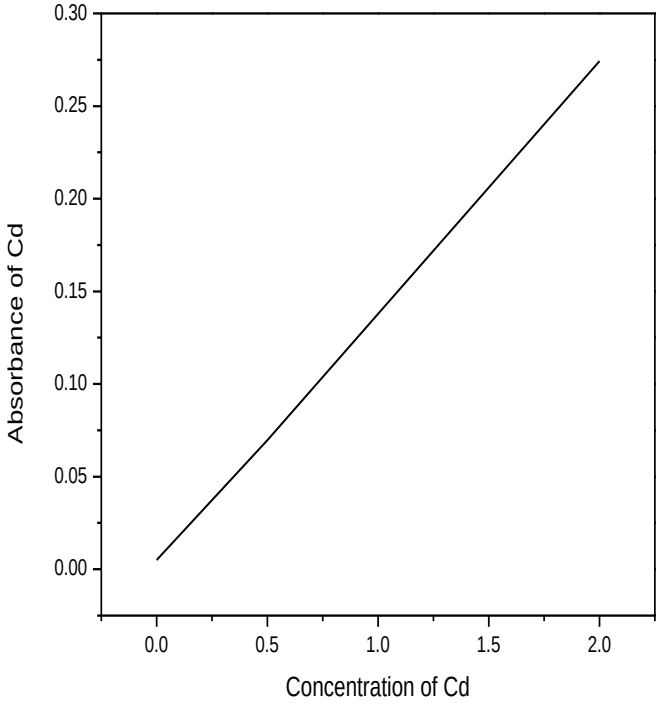


Figure 5: Calibration lines of the analyzed metals

4.3 Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) values for all metals analyzed ranged from 0.001–0.78 mg/kg and 0.0033–2.57 mg/kg respectively. The LOD and LOQ method obtained were low enough to detect the presence of metals of interest at trace levels in both samples.

Table 4: Limits of Detection and Quantitation values of metals.

Metals	**Ca**	**Fe**	**Mg**	**Zn**	**Pb**	**Cd**
LOD	0.78	0.520	0.001	0.03	0.0012	0.001
LOQ	2.57	1.72	0.035	0.099	0.004	0.0033

4.4 Precision and Accuracy

The precision and accuracy of the proposed method were evaluated by means of matrix spike recovery tests. The recovery values of triplicate analysis of the matrix spike Anna and BR-64 apple varieties and the RSD values were calculated and the results were presented in Table 5 and 6 respectively.

Table 5: Recovery and precision test results of metals for Anna apple matrix spike sample

Metals	**Conc.in sample (mg/kg)**	**Amount added.**	**Conc.in spiked sample (mg/kg)**		**Recovery (%)**
	(Mean ± SD)		**(Mean ± SD)**	**RSD**	
Ca	137.306 ± 2.61	2.5	139.603 ± 2.529	1.811	92.00
Fe	3.826 ± 1.060	0.5	4.2966 ± 1.058	0.246	94.00
Mg	129.81 ± 0.6323	0.5	130.273 ± 0.6351	0.4875	92.6
Zn	0.3433 ± 0.2640	0.5	0.82 ± 0.1322	0.161	95.34
Pb	0.0059 ± 0.036	1.5	1.295 ± 0.0055	0.42	129.10
Cd	$0.0018 \pm 1x10^{-8}$	1.5	1.3643 ± 0.0458	3.3570	90.83

Table 6: Recovery and precision test results of metals for BR-64 apple matrix spike sample.

Metals	**Conc.in sample (mg/kg)**	**Amount added**	**Conc.in spiked sample (mg/kg)**		**Recovery (%)**
	(Mean ± SD)		**(Mean ± SD)**	**RSD**	
Ca	138.796 ± 2.4029	2.5	141.133 ± 3.5512	2.5162	93.49
Fe	3.25 ± 0.9430	0.5	3.7233 ± 0.9374	25.1765	94.66
Mg	131.046 ± 7.748	0.5	131.5166 ±7.7286	5.8765	90.12
Zn	0.2866 ± 0.1067	0.5	0.75 ± 0.1044	13.92	92.68
Pb	0.0029 ± 0.0018	1.5	1.3323 ± 0.0277	2.079	133.036
Cd	0.0018 ± 0.0412	1.5	1.3673 ± 0.0734	5.3682	91.033

As it can be seen in Table 5, the percentage recovery of all the metal analysis in the Anna apple samples ranges from 90.83-129.10 and the RSD values ranged between 0.161-3.57. From Table 6, the percentage recovery of all metal analysis in the BR-64 apple samples were ranged from 90.12–133.036% and the RSD values ranged from 2.516– 5.876 % in the acceptable range except Fe (25.176 %)and Zn (13.92%).

The matrix spike recovery obtained in both apple varieties fall within the acceptable range of 90.12–133.036% for a good recovery. This high percentage recovery obtained from current study validates the accuracy of the method and the reliability of the levels of metal concentration. The RSD values of most metals in both samples were < 10%, indicating that the proposed method was precise

4.5. Results of the determination of selected essential and non-essential metals.

4.5.1. Levels of metals in Anna and BR-64 apple samples.

Table 7: Average metals concentration and relative standard deviation of Anna and BR-64 apple samples. (Mean ± SD, n=3

Metal	**Anna apple**		**BR-64 apple**	
	Conc.(mean ± SD) (mg/kg)	**RSD (%)**	**Conc.(mean ± SD) (mg/kg)**	**RSD (%)**
Ca	137.306 ± 2.61	1.9008	138.796 ± 2.4029	1.731
Fe	3.8266 ± 1.0603	0.8168	3.25 ± 0.943	29.61
Mg	129.81 ± 0.6323	0.4870	131.0466 ± 7.748	7.748
Zn	0.3433 ± 0.2640	76.90	0.2866 ± 0.1067	37.22
Pb	0.0059 ± 0.0036	61.016	0.0029 ± 0.0018	62.068
Cd	0.0018 ± $1x10^{-8}$	$5.5x10^{-4}$	0.0018 ± 0.0412	22.905

As it can be seen in Table 7, the relative standard deviation of Pb in the Anna apple sample was 61.106 and the relative standard values of Pb and Cd in BR-64 apple fruit were 62.068 and 22.905.These high relative standard deviation might be due to contamination.

4.5.2 Comparison of level of metals in Anna and BR-64 apple varieties.

Calcium is needed in human body for numerous functions such as building and maintaining the bones and teeth, blood clotting, transmitting of the nerve impulses and regulating heart's rhythm. The body gets calcium by pulling it from the bones when blood levels of calcium drop too low, usually when quite a long time passes since having taken calcium with meal. It can also be found in dark green leafy vegetables, fruits, dried beans and legumes. As can be seen from Table 7, the average concentration of calcium in both Anna and BR-64 apple fruit samples were higher than all the metals analyzed since it is macro elements of plants. The level of calcium metal in BR-64apples (138.796mg/kg) was slightly higher than that of Anna apple fruit varieties (137.306mg/kg).

Iron necessitates the red blood cell formation and it is one of the most useful in biochemical functions. In this study, iron was the second most accumulated essential metal next to calcium in both Anna and BR-64 apple fruit varieties. The Anna apple iron content was slightly higher than BR-64 apple variety. The results obtained in both varieties of apples were lower than the FAO/WHO maximum permissible limit which is 425 mg/kg (36).

Zinc is now receiving global attention and is particularly necessary in cellular replication, the development of the immune response and as a cofactor for many enzymes of the body. The results in Table 7 reveal that the concentrations of Zinc in the Anna and BR-64apple fruit varieties were 0.3433 mg/kg and 0.2866 mg/kg respectively. The concentration difference may be ascribed to variation of cultivation condition and sample treatment. Its amount in this study was much lower than WHO recommended maximum limit which is 100 mg/kg (36).

Magnesium is the fourth most abundant cation in the body and the second most abundant cation in intercellular fluid. It a cofactor for 350 cellular enzymes may of which are involved in energy metabolism. It is also involved in protein and nucleic acid synthesis and is needed for normal vascular tine and insulin sensivetey. In the study area Anna and BR-64 apple sample, the result Mg concentration were 129.81mg/kg and 132.0466mg/kg respectively Table(7) .The (37) recommended limit of Mg is 200mg/kg and then the level of magnesium found in these study were much lower than this limit.

Lead is a toxic metal and it is absorbed by plants to varying degrees depending on the form of the metal present, the pH and the temperature of the soil. In the studied Anna and BR-64 apple

sample, the results of lead concentrations were 0.0059mg/kg and 0.0029 mg/kg respectively (Table 7).The (37) recommended maximum limit ofPb is 0.3mg/kg and then the levels of lead found in this study were much lower than this limit.

Cadmium is a highly toxic metal with a natural occurrence in soil, but it is also spread in the environment due to human activities. It is easily taken up and accumulated by plants and crops through root system. As can be seen from table 7, the mean concentrations of cadmium in the Anna and BR-64 apple fruit samples were 0.0018 mg/kg and 0.0015 mg/kg respectively. The concentrations of cadmium obtained in both apple samples were almost the same and they are much lower than the WHO recommended maximum limit is 0.2 mg/kg (37).

According to the results as obtained, the major part of elements in the two apple varieties consist of Ca followed by Mg and other minor elements, such as Fe, Zn, Pb and Cd (Figure 6).

In general, the mean concentrations of metals in both Anna and BR-64 bulk apple samples collected from all sampling site decreased in the order of: Ca > Mg > Fe > Zn > Pb > Cd and Ca > Mg > Mn > Fe > Cd > Pb respectively.

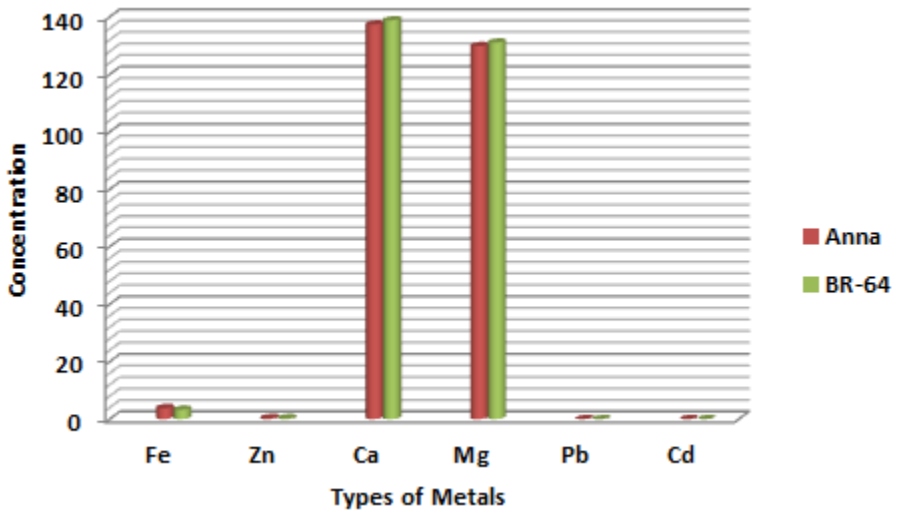


Figure 6: Average concentration of essential and non-essential metals in Anna and BR-64 apple types**.**

4.6 Statistical analysis

In this study, samples were collected from four different site areas where they are commercially available. Each sample was mixed thoroughly and one representative bulk sample was taken for

each apple type. During theseprocesses a number of random errors may be introduced in each digest and replicate measurements. T-test was used to compare levels of metals in Apple with BR-64 apple varieties to test for statistically significant differences at $P < 0.05$.

Table8: Statistical analysis of investigated element ($t_{4,0.05}$=2.78)

Elements	**Anna apple**		**BR-64 apple**		**$c_{calculated}$**	**P value**	**Remark**
	Mean	**SD**	**Mean**	**SD**			
Ca	137.306	2.61	139.0796	2.529	0.551	0.637	NS
Fe	3.826	1.060	3.25	1.115	0.770	0.522	NS
Mg	129.81	0.6322	131.0466	0.718	0.294	0.796	NS
Zn	0.3433	0.2640	0.2866	0.1067	0.693	0.566	NS
Pb	0.0059	0.0036	0.029	0.0018	1.550	0.261	NS
Cd	0.0018	$1x10^{-8}$	0.0018	0.0412	0.234	0.837	NS

DF-degree of freedom, SD-standard deviation, S- Significant, NS – No significant difference ($t_{4,0.05}$=2.78) -means t-critical value at 4 degree of freedom ($p<0.05$).

As indicated from table 8, there was a significant difference ($P < 0.05$) in the mean concentrations of metals in the two apple varieties while no significant difference in MagnesiumCalcium, Zinc, Irion, lead and cadmium metals.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Apple fruit is consumed locally as fresh and also it is an important source of income. However, no literature values discovered the levels of metals in apple fruits in the study area. In this study, the concentrations of six metals (Ca,Fe,Mg, Zn, Cd and Pb) have been analyzed by flame atomic absorption spectrometry (FAAS). Wet digestion method using a mixture of 5 mL of concentrated HNO_3 and 1 mL of concentrated $HClO_4$ was used as an effective method for the fruit sample digestion. This was revealed by the good recoveries (90.1 -133.03 %) obtained which were found in the acceptable range for the analyzed metals. From both the Anna and BR-64 apple samples, the amount of the analyzed metals were found to follow decreasing order; Ca > Mg > Fe>Zn > Pb > Cd and Ca > Mg > Fe > Zn >Cd >Pbrespectively.

5.2 Recommendation

It recommended that findings of this study indicted the level of calcium in both homogenized in both Anna and BR-64 samples variety were higher than the other analyzed metals and hence apple fruit are advisable source of calcium metal.Moreover use of representative number of sample separately from different geographical source has not been made in this study. Thus, the upcoming researchers are recommended to analyze metals in representative number of samples separately based on their agro climate zones and use the result as stepping ladder for further investigation and more elaborative mineral analysis on the apple fruits.At last continuous monitoring of apple fruits for heave metals accumulation is necessary because trace elements are not bio-degradable andhavetendency to accumulate on soil surface which increase availability for up take by fruit.

6.References

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Sr. No.	Lab. No.	Client Code	Ca	Mg	Fe	Zn	Pb	Cd
			mg/kg					
1	1125/20	Anna R1	140.25	129.08	4.25	0.39	0.007	0.0018
2	1126/20	Br 64 R1	139.14	124.14	4.32	0.41	0.005	0.0021
3	1127/20	Anna R2	135.26	130.16	4.61	0.45	0.009	0.0019
4	1128/20	Br 64 R2	142.16	139.42	2.54	0.23	0.0021	0.0014
5	1129/20	Anna R3	136.41	130.19	2.62	0.19	0.0019	0.0017
6	1130/20	Br 64 R3	135.09	129.58	2.89	0.22	0.0016	0.0021
7	1131/20	Blank	0.25 ,0.21 , 0.22	0.14,0.12 ,0.11	0.05, 0.04,0.03	0.02,0.03,0.02	0.0010	0.004

Spiked Sample Analysis of Laboratory Report

Lab. No.	Client Code		Ca	Mg	Fe	Zn	Pb	Cd
			mg/kg					
		Added conc. Ppm	2.5	0.5	0.5	0.5	1.5	1.5
1125/20	Anna R1		142.46	129.54	4.73	0.87	1.299	1.382
1126/20	Br. R1		141.46	124.62	4.79	0.87	1.309	1.282
1127/20	Anna R2		137.65	130.63	5.07	0.92	1.289	1.312
1128/20	Br. R2		144.51	139.87	3.03	0.70	1.363	1.409
1129/20	Anna R3		138.70	130.65	3.09	0.67	1.298	1.399
1130/20	Br. R3		137.43	130.06	3.35	0.68	1.325	1.411

Instrument Calibration

Metal	ave length (n	conc. Of Standard	Silt width (nm)	Current (mA)	Energy set or PMT	R^2 regration	Equation for calibration curve	Dl	Flame
Ca	422.7	0 ,2.5 , 5 ,10	0.8	3	277	0.9992	y=0.0632x-0.005	0.002	air acetylene
Mg	285.21	0 ,0.5 , 1 ,1.5 , 2.0	0.8	2	266	0.9994	y= 0.0409x+0.0002	0.001	air acetylene
Fe	248.3	0 ,0.5 ,1, 2 ,4	0.2	4	450	0.9991	Y= 0.0352x+ 0.002	0.003	air acetylene
Zn	324.8	0 , 0.25 ,0.5 1, 2	0.7	2	432	0.9997	Y= 0.0837x- 0.006	0.001	air acetylene
Cd	228.8	0 , 0.5 ,1.5 ,2	0.2	1.2	325	0.9994	Y= 0.1364x + 0.0015	0.001	air acetylene
Pb	283.3	0.5 ,1 ,1.5 ,2	0.2	1.2	330	0.9997	Y= 0.217x-0.0015	0.002	air acetylene

Where Y = absorbance

X = Concentration of analyte

Dl = Detectin limit

PMT = photo multiplier tube

The standard Concentration of metals and their Absorbance.

Ca		Mg		Fe		Zn	
Conc.(ppm)	**Abs**	**Conc.(ppm)**	**Abs**	**Conc.(ppm)**	**Abs**	**Conc.(ppm)**	**Abs**
0	0.005	0	0.0002	0.5	0.0196	0	0.002
2.5	0.153	0.5	0.0206	1	0.0372	0.25	0.0224
5	0.311	1	0.0411	2	0.0724	0.5	0.0433
10	0.627	1.5	0.0615	4	0.1428	1	0.0852
		2	0.082			2	0.1689

Pb		Cd	
Conc.(ppm)	**Abs**	**Conc.(ppm)**	**Abs**
0.5	0.107	0	0.0015
1	0.2155	0.5	0.0697
1.5	0.324	1.5	0.2061
2	0.4325	2	0.2743