

HAWASSA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE
SCHOOL OF POST GRAGUATE STUDIES
DEPARTEMENT OF CHEMISTRY



M.SC THESIS

**PHYSICOCHEMICAL CHARACTERIZATION, PROXIMATE ANALYSIS
AND DETERMINATION OF SELECTED HEAVY METALS IN COW'S
MILK AND COW'S FEED "ATELLA" SAMPLES COLLECTED FROM
ARSI NEGELLE TOWN, OROMIA REGION; ETHIOPIA.**

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NOVEMBER, 2024
HAWASSA, ETHIOPIA

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**A MASTER THESIS SUBMITTED TO DEPARTMENT OF CHEMISTRY,
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY**

**NOVEMBER, 2024
HAWASSA, ETHIOPIA**

DECLARATION

I do hereby declare that the thesis work entitled “**Physicochemical characterization, proximate analysis and determination of selected heavy metals in cow’s milk and cow’s feed “atella” samples collected from Arsi , Negelle town, Oromia region, Ethiopia**” is my original work and has not been submitted for a degree or diploma in any other universities by any other researchers or any students and that all sources of materials used for this thesis have been duly acknowledged.

Name:

Signature: _____

This thesis has been submitted for examination with my approval as thesis Advisor

Advisor: Alemayehu Paulos (PhD)

Signature: _____

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APPROVAL SHEET-1

This is to certify that the thesis entitled **“Physicochemical characterization, proximate analysis and determination of selected heavy metals in cow’s milk and cow’s feed “atella” samples collected from ArsiNegelle town, Oromia region, Ethiopia”** submitted in partial fulfillment of the requirements for the degree Master of Science in Chemistry of the graduate program of the Department of Chemistry, Hawassa University, and is a record of original research carried out by tajurausman (ID No PG Chem/013/10), under our supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged. Therefore, I recommend that it is accepted as fulfilling the thesis requirements.

ALEMAYEHU PAULOS (PhD)

Name of advisor

Signature

Date

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APPROVAL SHEET-2

We, the undersigned, members of the board of Examiners of the final defense by TajuraUsman have read and evaluated this thesis entitled **“Physicochemical characterization, proximate analysis and determination of selected heavy metals in cow’s milk and cow’s feed “atella” samples collected from ArsiNegelle town, Oromia region, Ethiopia”** examined the candidate. This is therefore to certify that, the thesis has been accepted for partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

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LIST OF ABBREVIATIONS

A DI	Accepted Daily Intake
AOAC	Analysis official Agricultural Chemists
BDL	Below Detection Limit
eV	Electromotive Volte
FAAS	Flame Atomic Absorption spectrophotometry
FAO	Food and Agriculture Organization of the United Nations
LOD	Limit of Detection
LOQ	Limit of Quantitation
MDL	Method Detection Limit
mg	Milligram
mL	Milliliter
ND	Not Detected
UV-VIS	Ultra Violate - Visible spectrophotometer
WHO	World Health Organization

Abstract

Arsi Negele, also known as NegeleArsi, is primarily known for its production of Areki, a traditional alcoholic beverage, and its byproduct atella, which is widely used to feed cows to enhance milk production. However, concerns about heavy metal contamination from agricultural practices associated with Areki's raw materials have emerged, highlighting potential health risks linked to cow's milk and feed in the area. This study focuses on physicochemical characterization, proximate analysis and determination of selected heavy metals (Cr, Zn, Cu, Ni, Fe, and Pb) in milk and atella samples. Samples of milk and atella were collected purposively from five sites: meja kiltotasefar, kiltu demasefar, melka shayitsefar, Agip sefar, and mobile sefar. In ArsiNegelle town, and homogenized. The physicochemical properties and proximate analyses were performed by following the AOAC method. The concentrations of the heavy metals were analyzed using Flame Atomic Absorption Spectrometry (FAAS). All the samples were analyzed in replicates, and the mean values are presented. The obtained results showed that the (temperature, pH) were $(20.3 \pm 0.26, 6.74 \pm 0.01)$ for the composite milk sample and $(20.7 \pm 0.01, 4.07 \pm 0.02)$ for atella sample. The results of proximate analysis such as moisture, dry matter, crude protein, crude fat, crude fiber, total ash, carbohydrate and gross energy contents of milk samples were $78.5 \pm 0.486 \%$, $3.84 \pm 0.03 \%$, $4.53 \pm 0.251 \%$, $3.35 \pm 0.02 \%$, $1.7 \pm 0.1 \%$, $0.436 \pm 0.015 \%$, $7.23 \pm 0.02 \%$ and 64.34 ± 0.023 kcal/g, respectively. On the other hand, the respective mean values of proximate analysis of atella sample were $11.36 \pm 0.20 \%$, $17.56 \pm 0.01 \%$, $14.7 \pm 0.2 \%$, $11.36 \pm 0.20 \%$, 17.56 ± 0.01 , $14.7 \pm 0.2 \%$, $42.36 \pm 0.23 \%$ and 276.71 ± 0.23 kcal/g respectively. From the analyzed metals Pb, Cr and Ni were not detected whereas the mean concentrations of Fe, Cu, and Zn were in the range of $(0.037 - 1.363)$, $(0.0983 - 0.853)$, and $(0.926 - 0.971)$ mg/L respectively for the (milk, atella) samples. While the physicochemical and proximate analysis reinforced the claims of the traditional community that atella is nutritious feed for cows as evidenced by the proximate composition of milk. Although the levels of the heavy metals in both atella and milk samples were in the permissible range as stipulated by WHO, the study further revealed implications of carryover of the heavy metals from the cow's feed atella to the milk.

Keywords: Arsi Negele, Atellam, Heavy metals, Milk, Physicochemical, Proximate analysis.

1. INTRODUCTION

1.1 Back Ground of the Study

Milk is a complex mixture of bioactive substances helpful to promote growth and development of infants in mammals [1]. Milk is widely consumed by children and adults as it is an important food item and beneficial for health and growth. Milk contains fat, proteins, carbohydrates, vitamins, and minerals [2]. Milk must be of satisfactory quality in order to protect the health of the community. It should be obtained from healthy animals. Milk quality and safety is a great concern in developing countries where production of milk and various milk products takes place under unhygienic conditions poor production and management practices. The potential chemical contaminants in milk products include pesticides, heavy metals, veterinary medicines, antibiotics, persistent organic pollutants (POPs), etc. They might enter the milk and dairy products reaching levels that are harmful to humans. They can be contaminated either through water, food, manufacturing and packing process or the packing materials. The presence of pesticides and heavy metals in dairy products may be attributed to the contamination of the original cow's milk which may be due to exposure of lactating cow to the environmental pollutants [3-5].

Heavy metal refers to any metallic chemical element that has a relatively high density and is toxic and poisonous at low concentration or quantity. Heavy metals represent a special group of food contaminants and the influx of heavy metals into the environment has significantly increased recently due to rapid industrialization, the use of new technologies that require heavy metals and decomposition of chemical fertilizers, pesticides etc. The metals can enter raw milk

and dairy products through the food chain. Heavy metals are persistent pollutants in the environment that can cause serious environmental and health hazards [5].

The toxic effect of heavy metals depends on the total amount absorbed, route of exposure, duration of exposure and age of the person. For example, young children are more susceptible to the effects of Pb exposure because they absorb several times more of the percent ingested and yet to develop excretory system compared with adults [6]. There is evidence that milk and other dairy products might contain varying amounts of different toxic contaminants. Causes for the presence of heavy metals in cow's milk are due to exposure of the cows to contaminated feed sources like atella, grass, drinking water, dust that settle on the grass, pharmaceutical medicines and their bioaccumulation process which potentially influence human Health [7].

Therefore, information on the level of heavy metals in milk and milk source of feed atella is important in assessing risk to human health. Currently, many analytical methods are being developed and validated to perform accurate measurements of pollutants such as heavy metals in environmental matrices in order to assess compliance with national and international legislations. Those legislations limit the allowable concentration of pollutants in the atmosphere to ultra-trace levels. When analytical methods are performed, validation is often supported by primary standards and certified reference materials at low concentrations, developed for the purpose of accurate calibration, with a traceable uncertainty statement [8].

1.2 Statements of the Problem

Heavy metal pollution in agricultural products possess significant health risks, particularly in regions like Arsi Negele, where atella, a byproduct of local brewery Areki production, is extensively used as cow feed. Given that the raw materials for Areki, wheat and maize, may contain heavy metals due to agricultural practices, these contaminants can accumulate in atella

and subsequently transfer to cow's milk. Agricultural activities (pesticides and fertilizers), use of new technologies, urban emissions and many other activities may contribute towards heavy metal pollution of the grains which then find their ways to enter the food chain, particularly the dairy products as a result of bioaccumulation [9]. They cause both acute and chronic effects for organisms especial human beings. Toxic heavy metals cause damages by enhancing the production of free radicals in several organs (brain, liver, kidney and heart) and interfering with cellular mechanisms against oxidation. Their toxicity is largely related to age, sex, and routes of exposure, daily intakes, duration of exposure and frequency of intake. These residues in milk are of particular concern even in low concentrations because milk is largely consumed by infants and children [10].to detect these heavy metals more sensitive and selective techniques are required such as FAAS . In this regard, research is important to provide recent information on the level of the contaminants in milk and milk source of feed atella, since there is no recent and enough information and no such research has been conducted on physicochemical and proximate analysis and determination of selected heavy metals in the current study area. Therefore, this study was aimed to assess the physicochemical properties and proximate composition analysis and determination of selected heavy metals in milk and milk source of feed atella sample collected from ArsiNegelle Town.

1.3. Objectives of the Study

1.3.1 General Objective

The main objective of this study was assessment of the physicochemical properties, proximate composition, and levels of selected heavy metals (Cr, Zn, Cu, Ni, Fe, and Pb) in milk and atella sample collected from Arsi Negele Town.

1.3.2 Specific Objectives

The specific objectives of this study were:

- To determine the physicochemical parameters such as temperature and pH
- To determine the proximate composition
- To determine the levels of selected metals (Cr, Zn, Cu, Ni, Fe, and Pb) of milk and atella samples
- To compare the results obtained with WHO

1.4. Significance of the Study

This study may have a significant contribution in recognizing, understanding and giving information about the level of physicochemical parameters, proximate analysis, and the level of selected metals in milk and atella and compares the identified result with recommended values. It also helps to recognize the people, the quality of the milk in the studied area. Finally, the study is buseful to the researchers to serve as starting information for further study.

1.5. Scope of the Study

This thesis work was taken place in milk and atella samples collected from arsinegelle town, and the sample was collected purposely based on the scale of use, and prepared and a composited representative samples were taken to determine the physicochemical properties, proximate composition and the determination of selected heavy metals (Cr, Zn, Cu, Ni, Fe, and Pb) using FAAS in the samples.

2 LITERATURE REVIEW

2.1. Milk

The main dietary factors that affect the bone mass are calcium and vitamin D, although potassium, zinc, vitamin A and K and protein play a role. From milk, calcium, phosphorus and magnesium are the most important minerals to bone health, of which calcium is the most abundant. Milk intake may be a marker for diet quality due to its high nutrient content. It contains macro and micro nutrients that are very important for the normal functioning of the body [11].

2.2. Physicochemical Composition of Milk

2.2.1. pH Value

Milk pH gives an indication of milk hygienic and it should not be ≤ 6.6 or ≥ 6.8 when milk temperature is 20°C, because cooling of milk reduces the risk of growth of bacteria while high milk temperature must be considered as favorable to the growth of bacteria in milk .The pH values higher than 6.8 indicates mastitic milk and pH values below 6.6 indicates increased acidity of milk due to bacterial multiplication [12].

2.2.2. Specific Gravity

Specific gravity is the ratio of density of the substance to the density of standard substance (water). The density of a substance varies with temperature, it is necessary to specify the temperature when reporting specific gravities or densities. The specific gravity of milk is influenced by the proportion of its constituents (e.g. Composition), each of which has different specific gravity approximately as follows; Water (1.000), Fat (0.930), Protein (1.346), Lactose (1.666), Salts (4.12) and SNF (1.616). The specific gravity of milk is decreased by addition of

water, addition of cream (fat), while removal of fat and reduction of temperature increase specific gravity of milk. Generally, normally milk has a specific gravity between 1.027 and 1.035 with an average value of 1.032 at 25°C [13]

2.2.3. Titratable Acidity

Titrateable acidity is a measure of freshness and bacterial activity in milk. The natural acidity of individual milk varies considerably, depending on species, breed, individuality, stage of lactation, physiological condition of the udder, etc. The higher the solid not fat contents of milk the higher the natural acidity and vice versa. When the milk is kept for some time, the bacteria will multiply and utilizes lactose and converts in to lactic acid, thereby increasing the acidity and decreasing the pH value. This acidity is known as developed or real acidity. The sum of natural acidity and developed acidity is known as titrateable acidity [14]. Titrateable acidity is expressed in terms of percentage lactic acid since lactic acid is the principal acid produced by fermentation after milk is drawn from the udder. Lactic acid is the primary acid in milk and milk products that is responsible for the lower pH. Fresh milk, however, does not contain any appreciable amount of lactic acid and therefore an increase in acidity is a rough measure of its age and bacterial activity [15].

2.4. Milk Production in Ethiopia

Ethiopia holds the largest livestock population in Africa. The total cattle production for the country is estimated to be 52.13 million and the total annual national milk production comes from about 10 million milking cows and is estimated 3.2 billion liter [16]. In Ethiopia, four major systems of milk production systems are distinguished and these are: pastoral, highland smallholder, urban and peri-urban and intensive dairy farming systems. Urban and peri-urban dairy production system is an important component of livestock production system in Ethiopia

[17]. Oromia region supplies more milk to the market compared to other regions. Even if Ethiopia has the largest milk producing cattle, it has low level of milk consumption compared to Kenya (90 lt/cap) and Uganda (50 lt/cap) with national per capita consumption of milk and milk products is estimated at 17 kg [18]. The southern nation nationalities people's region (SNNPR) is the second highly milk production potential area next to Oromia region in Ethiopia [19].

2.5. Sources of Heavy Metals

Heavy metals can be considered as deadly toxins that are accumulated (bioaccumulation) in plants and animals tissues through biological cycle. They are persistent contaminants in the environment; air, water and soil and are dangerous substances causing series health risks [20]. When heavy metals enter to human body, they disrupt normal cellular processes leading to toxicity in a number of organs. Their accumulation in different tissues is due to once if they are taken up in to the body and stored in a particular organ like liver and kidney, they will be excreted at low rate compared with their uptake [21]. All the sources of heavy metals can be either natural or anthropogenic sources [22]. An example of natural processes is geological deposition of metals by erosion in ground water, weathering of minerals, volcanic activities and forest fires are natural phenomena that release heavy metals to environment [23]. Human activities such as mining of minerals and ores, smelting of metals, exploration of energy resources like coal, oil and natural gas, waste disposal, waste incineration, urban effluents, traffic emissions, fertilizer and pesticides applications and long term application of waste water in agricultural lands, modern products like cosmetics, mercury amalgamation dental filling represent anthropogenic contribution to the environment [24].

Heavy metals are environmental unfriendly contaminants that can cause adverse effects on living organisms. They can enter to the human body either through inhalation or ingestion [25]. Due to

an increase in agricultural and industrial activities the concentration of heavy metals in the environment increases. When these metals are taken in by plants through absorption, they will be transfer to the plants. Animals that graze on such contaminated plants receive the toxic heavy metals that can result contaminations on milk and milk products and hence the cycle of these metals in the environment is linked with the food chain as soil-plant-animal-man [26]. Unlike organic pollutants which can be broken down, metals cannot be degraded or metabolize and will remain in the soil permanently [27].

2.6. Benefits and Harmfulness of the Essential and Non-essential Metals

Milk and dairy products are the most diverse natural food products in composition because they contain more than 20 trace elements. Most of these trace elements are essential and very important for human health, such as copper, zinc, manganese and iron. In addition, lead, cobalt, nickel, cadmium and chromium are some example of non-essential metals. Essential metals are cofactors in many enzymes and play an important role in many physiological functions of man and animals. Lack of these metals causes' disturbance and pathological conditions [30].Also, excess amount of these essential metals are harmful for human. The amount of metals in non-contaminated milk is admittedly minute, but their content may be significantly altered through manufacturing and packaging process. Also, metals that can contaminate different cattle feed and environment, such as lead, cadmium, chromium, nickel and cobalt, could be excreted into milk at various levels and cause serious problems [31].

2.6.1. Nickel

Little amount is needed by the human body to create red platelets [32]. Natural sources of nickel includes dusts from volcanic emission and weathering of rocks and soils, but in water it is derived from biological cycles and solubilization of nickel compounds from soil and from

sedimentation of nickel from the atmosphere . Nickel compounds are used for electroplating, NiCad alkaline batteries and catalysts. The most hazardous routes of nickel exposure are by inhalation, ingestion or skin contact in nickel and its alloy production plants. Inhalation of nickel causes irritation of nose and sinus and leads to loss of sense of smell. The most common adverse health effect of nickel in humans is an allergic skin reaction. Its toxicity leads to cancer and long term exposure damages the heart, lungs and nasal cavity and adversely harms the kidney, blood, liver and immune system [33].

2.6.2. Zinc

Zinc is essential for growth and development. It is a constituent of more than 200 metallo enzymes, many of which regulate carbohydrate, lipid and proteins. It plays vital roles in the synthesis of genetic material and regulation of gene expression and cell division, epithelial integrity, cellular immunity and sexual maturity. It is also used for the synthesis, storage and secretion of insulin. Infants need high zinc to support their rapid growth. Iron supplementation could decrease zinc absorption [34].

2.6.3. Copper

Copper is a component of essential enzymes (cuproenzyme) and is involved in respiration and synthesis of hemoglobin. It is an important metallic activator of several enzymes. Diets which contain excess copper have been reported to lead to toxicity and liver damage during childhood. The main anthropogenic sources of copper are pesticides, fertilizers, ore mining and smelting [35].

2.6.4. Chromium

Human activities that contribute chromium to the physical environments are tanneries, steel and industries fly ash. Long haul presentation can bring about kidney and liver harm, and harm excessively circulatory and nerve tissue [36]. Although Cr^{3+} has been proven to be essential for biological path ways like glucose metabolism, Cr^{6+} is the most toxic and carcinogen, allergen and acute irritation in humans. Its greater toxicity is due to its reduction to +3, +4 and +5 intermediates that induce free radical which can bind to intracellular macromolecules.

2.6.5. Lead

Lead is an ubiquitous heavy metal released in small amounts in to environments by natural processes. Young children undergo rapid development and consequently are more susceptible than adults to the effect of lead. It impairs central nervous system, especially in infants and young children during the critical stages of brain development. Lead exposure in children at minimal levels can be associated with intellectual and cognitive deficits characterized by reduction in intelligence quotient (IQ), short attention span, reduced short term memory, reading and learning disabilities [37]. Human exposure to lead comes from the main sources such as leaded gasoline (PbEt_4), lead based paints, having lead pipes in water supply system and exposure to lead mining, smelting and coal combustion [38]. The main uses of lead are industrial like lead storage batteries, alkyl lead production as an additive to petrol used as anti-knocking agent [39].

2.7. Analytical Techniques Used for Analysis of Heavy Metals

Different methods are used to determine the concentration of the metals in the environmental samples, which include FAAS, UV-VIS, monolayer voltammetry pulse, potentiometric, capillary electrophoresis, plasma emission spectroscopy, inductively linked plasma spectrometry, injection

flow spectrometer; Atomic Fluorescence Spectroscopy is an induction plasma mass spectrometer. Metals in milk are described by a number of techniques includes particle emissions from x-ray, semi-infrared spectroscopy, potentiometric method, titration capillary electrophoresis. The usual method for determining metals in milk by developing countries is a flame atomic absorption spectrophotometer. The benefits of spectrophotometer absorption are low cost, approximately quick detection and easy preparation operations. Obviously, inductively coupled plasma is also used and also studding by mass spectrometer, which detects more sensitivity of the elements than the usual one, but because of its high costs, it is most used in developed countries [40].

3 MATERIALS AND METHOD

3.1. Description of Study Area

The study was conducted around Arsis Negelle town. ArsiNegelle, also known as NegeleArsi, is a city located in the West Arsi Zone of the Oromia Region, located 250 km from the capital Addis Ababa to southeastern Ethiopia. Positioned at an elevation of 2,043 meters, it serves as the administrative center for the ArsiNegele District. The region is primarily known for its production of Areki, a traditional alcoholic beverage, and its byproduct atella, which is widely used as cow feed. The local economy is bolstered by agriculture and trade, with Areki production contributing significantly to the livelihoods of residents. The study area was selected based on the scale of production and use of atella and the availability of dairy farm producers who uses atella as main feed for cows.

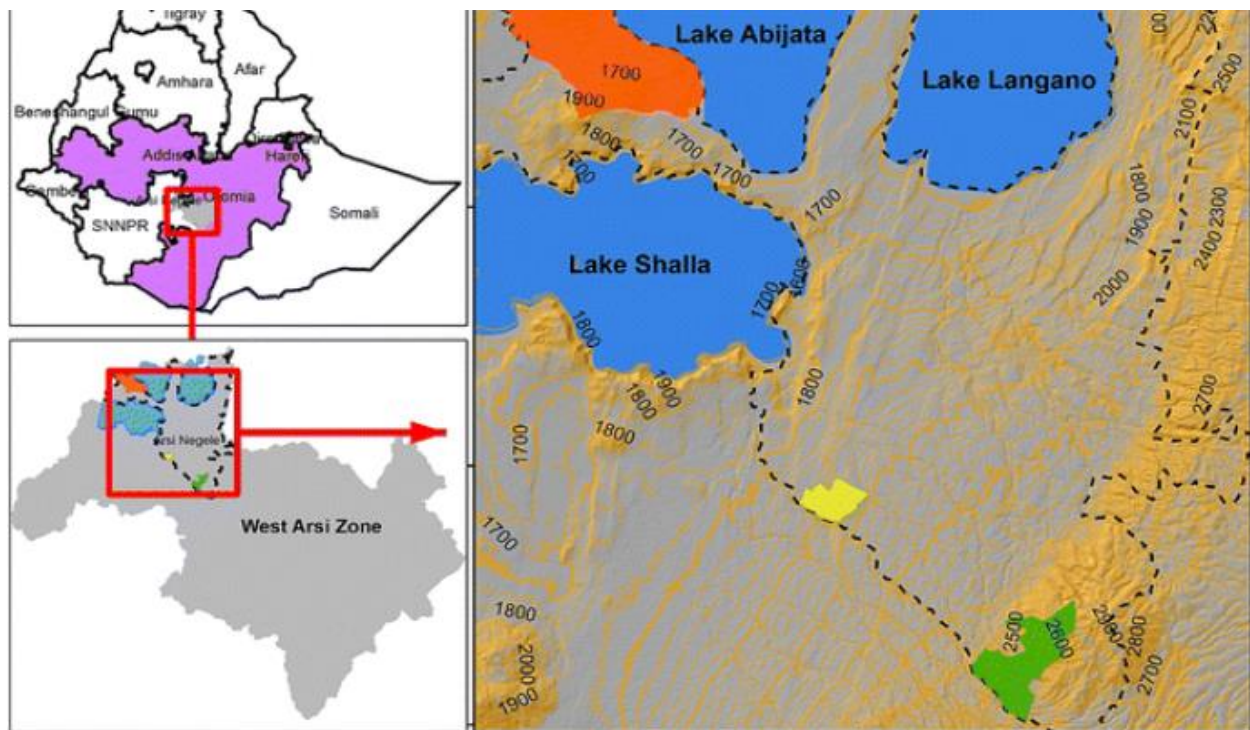


Figure 1: Map of the study area

3.2. Design, Sample Collection, Storages and Pretreatments

Samples of milk and atella were collected purposely from five sites: mejakiltotasefar, kiltudemasefar, melkashayitsefar, Agipsefar, and mobile sefar of ArsiNegelle town, and homogenized. Five lactate cows of urban dairy producers and milk source atella was purposely selected from the location and composite sample representatives was taken for the study. Milk sample of 500 mL was collected during morning milking time from each cow of urban dairy producers. The fresh cow's milk samples were collected in properly washed and cleaned sample bottles with tight fitting screw caps. The bottles were rinsed with milk samples, filled and sealed, in such a way that no air bubbles remained in the bottles. All milk and atella samples were labeled and transported to laboratory and stored in refrigerator at -4°C until analyses [41]. All analysis was done within 72 hours.

3.3. Instrument and Apparatus

The laboratory apparatus that was used during the study include: pipettes and micropipettes (Pyrex, USA) to measure different volumes of sample solution, acid reagents and metal standard solutions and dilute sample solutions, prepare standard solutions. Volumetric flasks (10, 25, 50 and 100 mL) were also used during dilution of sample and preparation of metal standard. Round bottom flasks 250 mL fitted with reflux condenser will be used in heating mantle (BI Barnstead Electro thermal, UK) to digest milk samples, spiked milk samples and blank solutions .A refrigerator (Hitachi, Tokyo, Japan) to keep the collected samples and digested samples until analysis. Digital analytical balance (Mettler Toledo, Model AG250, and Switzerland with ± 0.0001 g) to weigh milk samples.

Flame atomic absorption spectroscopy (BUCK SCIENTIFIC, Model 210VGP AAS, USA) equipped with deuterium background corrector and air-acetylene flame atomizer was used for analysis of the selected metals (Cr, Cu, Ni, Zn and Pb) in cow's milk samples and milk feed source atela.

3.4. Chemicals and Reagents

All Reagents and chemicals used in the analysis was Analytical Grade. A concentrated nitric acid (69-72% HNO₃, UNI-Chem. India) and 30% H₂O₂ (UNI-Chem. Chemical Reagent) was used in digestion of milk and milk source atella samples, blank and spiked solutions. Stock standard solutions (Buck Scientific purographics calibration standards, USA) containing 1000 mg/L of the metals Cr, Ni, Cu, Zn, and Pb from which 10 mg/L of intermediate standard obtained was used for preparation of calibration standards of each metal. 1,10 phenanthroline hydrate(99.8% ,Sigma Aldrich, Germany), sodium acetate Trihydrate(99%, Sigma Aldrich, Germany), ferrous ammonium sulfate hex hydrate (98%,Sigma Aldrich, Germany) was used for the determination of Iron metal in the milk sample. Throughout the experiment distilled water was used for sample preparation, dilution and rinsing apparatus before analysis.

3.5. Physicochemical Analysis of Milk and milk feed source atella Samples

3.5 .1. pH

The pH of the samples was measured using a digital pH meter after calibration using buffer solutions of pH 4, 7, and 10. Firstly, the sample was diluted with distilled water at a 1:1 (w/v) ratio. Then, the glass electrode of the pH meter was dipped into solution [42,43].

3.5.2. Temperature

The temperature of each sample was determined on-site by using a thermometer. Each sample was measured in replicates and the results were reported as mean \pm standard deviation (SD).

3.6 .Proximate analysis of milk and milk source atella sample

3.6.1. Moisture content

Moisture content was determined following the method described in AOAC, 2000 and N. Surayah Osman, et al., 2019 [44, 45]. A known amount of a sample was measured in a crucible and heated at 105 °C for about 3 h in the oven. The crucible was cooled in desiccators and weighed. The heating and cooling cycle was done until a constant weight was obtained. The amount of dry matter (DM) was calculated using Eq. 1. The moisture content can be calculated by subtracting the percentage of dry matter from 100.

$$\% \text{Moisture} = \frac{(W_1 - W_2)}{W_1} \times 100 \quad (1)$$

Where, W_1 = weight (g) of the sample before drying; W_2 =weight (g) of the sample after drying

3.6.2 Crude Protein (CP)

Kjeldahl wet digestion was used to determine the total N content of the composite samples. The percentage of the nitrogen and the corresponding crude protein (CP) content can be calculated using Eqns. 2 &3, respectively [45].

$$\%N = \frac{V_1 \times N \times 0.014}{W_1} \times 100 \quad (2)$$

Where, V_1 = volume of HCl, N =Normality of HCl, W_1 =Weight of sample.

$$\text{Crude protein (\%)} = \%N \times 6.25 \quad (3)$$

Where, %N is percent of nitrogen content in a sample; and 6.25 is the protein-nitrogen conversion factor [45].

3.6.3. Crude fat

The Soxhlet extraction technique was used to determine crude fat content using ether extract of samples according to AOAC, 2000 and T.E. Puwastien, et al., 2011 [45]. A known amount of sample was immersed in a clean and dry Soxhlet extraction apparatus. Then, n-hexane was placed in a round-bottomed flask and heated gently for 4 h, then the flask was cooled for 15 min. Concentrated crude fat was collected from Rota vapour at a reduced pressure of 40 °C. Then, the product was transferred into a beaker of known mass. Then, the amount of crude fat was calculated using Eq. 4.

$$\text{Crude Fat (\%)} = \frac{W_2 - W_1}{W_s} \times 100 \quad (4)$$

Where: W_2 = Weight of beaker with crude fat; W_1 = Weight of beaker; and W_s = Weight of sample

3.6.4. Ash

Ash content was determined following the protocol described in AOAC, 2000 [44]. A known powdered sample was placed on a cleaned, oven-dried and weighted crucible. First, it was heated on a hot plate for 30 min, then ashed in the muffle furnace at 550 °C for 8 h, cooled down in the desiccator, and weighed. The ash content of the samples (%) was calculated using Eq. (5).

$$\text{Ash (\%)} = \frac{(W_C + W_A) - (W_C)}{W_{DS}} \times 100 \quad (5)$$

Where: W_C is weight of crucible; W_{DS} weight of dry sample; and W_A is weight of ash.

3.6.5. Crude fiber content

The crude fiber was determined using the standard method mentioned in AOAC, 2000 [44], which involves digestion, filtration, washing, drying, and combustion. In brief, a 2 g powdered sample was transferred to a filter bag. Then, hot H₂SO₄ (1.25%) was added into Pyrex glass containing the sample and heated at 120 °C for 30 min. Next, the sample in the bag was boiled at 120°C for 30 min with hot NaOH (1.25 %). Then, the boiled solution was cooled, and the bag was washed with distilled water. The sample was transferred into a crucible and dried in an oven at 130°C for 2 h. The dried sample was kept in the furnace at 550 °C for 5 h. The mass of the crucible and the ash was recorded after cooling in the desiccator. Crude fiber content was then calculated using Eq. 6.

$$\text{Crude Fiber (\%)} = \frac{(W_C + W_{DST}) - (W_C + W_A)}{W_{ODS}} \times 100 \quad (6)$$

Where: W_C is weight of crucible; W_{DST} is weight of dry sample after acid and base treatment; W_A is weight of ash; and W_{ODS} is weight of original dry sample.

3.6.6. Carbohydrate by difference

Carbohydrate content was determined by subtracting the sum of moisture, ash, crude fat, crude fiber, and crude protein from 100 percentage as indicated by Eq. 7 [43].

$$\text{Carbohydrate (\%)} = 100 - [(M (\%) + A(\%) + CFa (\%) + CFb (\%) + Cp (\%))] \quad (7)$$

Where: M = moisture; A = ash; CFa = crude fat; CFb = crude fiber; and Cp = crude protein.

3.6.7. Gross Energy

Gross Energy value was estimated by using the Atwater factors for protein (4), fat (9), and carbohydrate (4) [43]. The gross energy was calculated using Eq. 8.

$$\text{Gross Energy (\%)} = [(Cp (\%) \times 4) + (CFa (\%) \times 9) + (CH (\%) \times 4)] \quad (8)$$

Where: Cp = crude protein; CFa = crude fat; and CH = carbohydrates

3.7. Sample Digestion and Preparation of the Sample for FAAS Analysis

3.7.1. Milk sample digestion

Optimized wet digestion procedure was employed for mineralization of the milk and milk source atela samples to obtain a clear solution. Optimum condition was obtained by making different trials using various volumes of solvents, times and temperatures of digestion. Collected milk and milk source atela samples were subjected to optimum wet digestion. Exactly 1 g of fresh cow milk sample was accurately weighed and quantitatively transferred to each 250 ml round bottomed digestion flask. To the milk samples, 6 ml of freshly prepared 4:2 (2:1, v/v) mixture of concentrated HNO₃ and H₂O₂ was added. Each mixture was then heated on a heating digester for 1 hr or 90 min by setting the temperature at 180°C. The digested solutions were then allowed to cool for 15 min before dismantling the condenser from the flask and further 15 min after removing the condenser. To the cooled solution, 10 ml distilled water was added to dissolve the precipitate formed on cooling and gently swirled for a while. The cooled digested samples were then filtered into a 50 ml volumetric flask using Whatman (No. 42) filter paper to remove any suspended or turbid residues. Subsequent rinsing of the filtrate with 10 ml distilled water was followed to dilute the resulting solution to the mark. For each milk sample, triplicate digestions were carried out. Blank digestions, consisting of deionized water and the reagents, were also subject to a similar digestion procedure [46]. Six reagent blank samples were prepared for the analysis of the milk and milk source atela samples. All the digested samples were stored in a refrigerator until analysis. All samples were digested in triplicates.

3.7.2 Digestion of atella sample

The wet ashing method was used to determine selected metals by FAAS [47]. Briefly, 20 mL of HNO₃ (69%) was placed into a conical flask containing 2g of powdered composite sample. Then, 10 mL of HClO₄ (70%) was added to digested flask using a pipette and heated at 120 °C for 1 h. Then, the digestate mixture was filtered using Whatman No. 1 filter paper into a cleaned 50 mL volumetric flask. The filter paper was rinsed with double distilled water until the mark of the volumetric flask. The blank sample was digested in the same way as the sample. Finally, at an appropriate wavelength of interested metals were determined by FAAS.

3.7.3. Operating Conditions of FAAS for Analysis of Metals

In this study a total of five metals for each sample was analyzed using FAAS with external calibration curve after the parameters such as burner and lamp alignment, slit width and wavelength adjustment were optimized for maximum signal intensity of the instrument. For each metal, the respective hollow cathode lamp was inserted in to the atomic absorption spectrophotometer, and the solution will be successively aspirated into the flame. Hollow cathode lamp for each metal, operated at the manufacturer's recommended conditions, and will be used at its corresponding primary source line. The acetylene and air flow rates will be managed to ensure suitable flame conditions. Six elements (Cr, Ni, Cu, Zn, and Pb) was analyzed by absorption mode of the instrument.

3.7.4. Instrument Calibration and Measurement of Selected Metals Concentration

Determination of trace metals concentration was done by using FAAS (BUCK SCIENTIFIC MODEL 210VGP) in Hawassa University Chemistry Laboratory. Intermediate standard solutions (10 mg/L) of metals of interest was prepared from the 1000 mg/L standard stock solutions. These solutions were diluted to the desired concentrations to calibrate the instrument

(A 10 ppm multi-element solution containing Cr, Zn, Co, Ni Fe and Pb was utilized to prepare elemental calibration solutions). This multi-element solution was diluted with 2% nitric acid to obtain working standards for each metal of interest). These intermediate standards were diluted with distilled water to obtain four working standards of each metal. After shaking and homogenizing the solutions, selected metals was analyzed after the instrumental operating conditions was optimized for maximum signal intensity of the instrument.

3.7.5. Method Detection Limit

Method detection limit is the smallest mass of analyte that can be distinguished from statistical variations in the blanks, which usually corresponds to the standard deviation of the blank solution times a constant or it is the amount of analyte that provides a signal equal to three times the standard deviation of the blank. Instrument detection limits were directly acquired from the instrument manual for each element under study. In this study, after the digestion of six blank solutions containing HNO₃ (69-72%) and H₂ O₂ (30%), six readings was taken from FAAS and the standard deviation will be calculated. The detection limits was obtained by multiplying the standard deviation of the reagent blank by three (MDL=3SD). The method detection limit for milk and milk source atella sample were listed in table one bellow.

Table 3.1 Method and Instrument Detection Limits Values for Each Metal Analyzed

Analyte	Pb	Cr	Cu	Ni	Zn	Fe
MDL(mg/l) for milk	0.067	0.089	0.009	0.081	0.044	0.08
MDL for atella	0.071	0.092	0.012	0.121	0.065	0.09
IDL for selected metal	0.040	0.040	0.005	0.020	0.005	

3.7.6. Method Validation

In present study due to the absence of certified reference materials for milk sample in our laboratory, the validity of the digestion procedure, precision and accuracy of FAAS was assured by spiking the raw cow's milk and milk source atela samples collected from sample site with standard of known concentration. The spiked and non-spiked milk samples were digested following the same procedure employed in the digestion of the respective samples and analyzed in similar condition. All the spiked samples was digested in triplicate following the optimized digestion procedure of milk and milk source atela samples. The digested spiked samples was analyzed for their respective metals content using FAAS and UV-VIS. Then the percentage recoveries of the analyte will be calculated by [48]:

$$\% Recovery = \frac{C(spiked) - C(nonspiked)}{C(added)} \times 100\% \dots \dots \dots \text{Equation (11)}$$

Where; C (spiked) is metal content of the spiked sample, C (non-spiked) metal content of non-spiked sample and C (added) is metal content of metal added.

3.7.7. Spiking Milk Sample

Known amount of each metal was added from the 1000 mg/L of stock solution in to flasks containing 1 g of raw milk sample to check the efficiency of digestion procedure. The procedure were as the following: 10 µL of 10 mg/L intermediate solution each metal (Cr, Pb, Zn, Ni, Fe and Cu) which are prepared from the intermediate 10 mg/L of the elements prior by taking 1 ml from the stock standard solutions containing 1000 mg/l of the metals was spiked at once in to triplicate round bottomed flasks containing 1 g of milk sample. Then, after the spiked samples was digested simultaneously with the unspiked samples based on the digestion method for milk samples, each sample was then determined for their respective metals (Cr, Pb, Zn, Ni, Co and

Cu) and Fe by FAAS and UV-VIS, respectively. The percentage recovery of metals determination was obtained as shown in the.

3.8 . Statistical Analysis

Microsoft Excel was used for statistical analysis. Statistical evaluations such as mean, standard deviation and relative standard deviation have done for all parameters of milk and milk source atela. All analyses were carried out in triplicate. Methodological precision was therefore evaluated with standard deviation (SD). Statistical analysis of the data was carried out using t-test to assess significant variation in the mean concentrations of selected metals and proximate analysis and physicochemical parameters with the standard values.

4. RESULTS AND DISCUSSION

4.1. Physicochemical parameters of Milk and milk source atella

Replicates of each milk and milk source atella sample were analyzed for each physicochemical property. Standard deviations and means of each triplicate were calculated using Microsoft Excel 2010 and the results were as indicated in Table 2 This table shows the values obtained for the physicochemical properties of milk and milk source atella samples. The mean values of physicochemical parameters such as temperature and pH is listed in Table 2 below. A slightly acidic nature of Atella was recorded.

Table 4.1 Physicochemical properties of milk and milk source Atella samples

Sample	Temperature		t-test (p)	pH		t-test (p)
	Recorded	Standard		Recorded	Standard	
Milk	20.3 ± 0.26	17.1	0.0008	6.74 ± 0.01	6.6 7	0.593
atella	20.7 ± 0.01	21.8	0.0003	4.07 ± 0.02	4.0	0.023

4.2. Proximate analysis of milk and atella samples

The results of the proximate analysis of milk and atella sample were indicated in Table 3. The mean values of the proximate analysis such as moisture, dry matter, crude protein ,crude fat, crude fiber, total ash, carbohydrate and gross energy contents of milk Samples were $78.5 \pm 0.486 \%$, $3.84 \pm 0.03 \%$, $4.53 \pm 0.251 \%$, $3.35 \pm 0.02 \%$, $1.7 \pm 0.1 \%$, $0.436 \pm 0.015 \%$, $7.23 \pm 0.02 \%$ and $64.34 \pm 0.023 \text{ kcal/g}$, respectively. On the other hand, the mean values of the proximate analysis such as moisture, dry matter, crude protein, crude fat, crude fiber, total ash, carbohydrate and gross energy contents of milk source atella were $11.36 \pm 0.20 \%$, $17.56 \pm 0.01 \%$, $14.7 \pm 0.2 \%$, $11.36 \pm 0.20 \%$, 17.56 ± 0.01 , $14.7 \pm 0.2 \%$, $42.36 \pm 0.23 \%$ and $276.71 \pm 0.23 \text{ kcal/g}$ respectively.

Table 4.2. Proximate analysis of milk and milk source atela samples

Parameters	Milk	Milk	t-test (p)	Atella	Atella	t-test (p)
	Recorded	Standard		Recorded	Standard	
Moisture %	78.5 ± 0.4	86.75	0.001	11.36 ± 0.20	11.18	0.1939
Dry matter (%)	3.84 ± 0.03	13	0.00017	17.56 ± 0.01	20.17	0.00014
Crude protein (%)	4.53 ± 0.251	2.6	0.00054	14.7 ± 0.20	18.72	0.00069
Crude fat (%)	3.35 ± 0.02	3.5	0.109	11.36 ± 0.20	5.43	0.00127
Crude fiber(%)	1.7 ± 0.1	1.1	0.00024	17.56 ± 0.01	19.73	0.00028
Total ash (%)	0.436 ± 0.015	0.65	0.000589	14.7 ± 0.2	4.4	0.00016 1
Carbohydrate (%)	7.23 ± 0.02	5	0.00057	42.36 ± 0.23	40.5	0.00086
Gross energy (kcal/g)	64.34 ± 0.023	78.25	0.000252	276.71 ± 0.23	271	0.00739

4.3. Discussion of Physicochemical Characteristics of milk and milk source atella

On the base of world health organization (WHO) standards and other scientific works, quality milk contains 6.6 pH. The pH values of the milk samples of the current study was 6.67 which complies with WHO standard. The pH is the parameter that determines the sample acidity and alkalinity and it is one of the parameter which is used to evaluate the quality of milk. At higher pH (up to 7.5) in cases of mastitis than in normal milk of mid –lactation [49].The pH range found in the current study was comparable with the findings in a previous investigation (6.38±0.60 to 6.77±0.88) [49]. In the other hand the pH of Atella was found in the range of 4, 07 which is

acidic. The acidic nature of Atella is due to acid formation during the fermentation process by acid-producing microorganisms (*Lactobacillus spp.*)[50]. Previous reports also showed that the pH of atella collected from different localities was found to be 3.80 ± 0.01 , which is in the range of the current finding for Atella[51]. The preferred pH levels of beverages are 3.5–4.0 [51].

4.4. Discussion of proximate analysis of milk and atella samples

The dry matter content was measured based on mass difference by drying at 100 °C [52]. The dry matter content of milk and milk feed source Atella samples were found in the range of 3.84 % to 17.56 %. This value agrees with the previous report that the dry matter of Atella (20-30%) [53]. The crude protein content of milk and milk feed source Atella was found to be varied from 4.53 % to 14.7 %, which is slightly lower than the study in [53]. Crude fat contents of milk and milk feed source atella varied from 1.7 % to 17.56 %. Fats are essential for the animal by yielding energy and enhancing the absorption and storage of vitamins (particularly lipid-soluble ones). However, high fats in the feed may decrease ruminal fermentation and digestibility of fiber, contribute to rumen fill, and decrease the rate of its passage [54]. Fiber is the measure of the indigestible (not fermentable) component in the diet [55]. Crude fiber contents of milk and milk feed source Atella samples ranged from 1.7 % to 17.56 %. Ash contents are the minerals or inorganic residues left after combustion at higher temperatures (> 500 °C) of the samples [56]. The ash content of the samples was in the range of 0.436 % to 14.7 %, and there was a significant difference among milk and milk feed source atella samples ($p < 0.05$). The carbohydrate contents of milk and milk feed source atella were found to be in the range of 7.23 % to 42.36 %.

Energy is a main requirement for living organisms. Gross energy represents the total energy Available in a substance; however, living organisms can't capture all the energy in the foods they

consume because of digestive and metabolic inefficiencies [27]. The gross energy contents of milk and feed atella were found to be from 64.34 kcal/g to 276.1 kcal.

4.6. Method Validation

The efficiency of the optimized procedure and analytical method were checked by adding known concentration of each metal in 1 g of cow's milk and milk source atela sample. As shown in Table 5 and Table 5 the results of percentage recoveries for the studied metals in cow's milk and milk source atela samples lies within the range 94.44 – 110.33 %, which is in the acceptable range (80 – 110%), this confirms that, the laboratory performance for each analyte is in control and the optimized wet digestion method is valid. Therefore, the optimized digestion procedure was valid for the cow's milk and milk source atella samples and is believed to remove metal fractions associated with organic matter.

Table 4.3. Recovery Values of Metals for the Analyzed Milk Sample

Metals	Conc. in sample (mg/L)	Amount added (mg/L)	Conc.in spiked sample (mg/L)	% Recovery	RSD	Method used
Pb	0.033± 0.002	0.03	0.061 ± 0.0015	94.44 ± 5.09	5.39	FAAS
Cr	0.029±0.003	0.03	0.062 ±0.003	110.03 ±10.0	9.08	FAAS
Ni	0.0035 ± 0.001	0.03	0.034 ± 0.001	101.4 4 ± 3.35	3.30	FAAS
Cu	0.0981 ± 0.001	0.1	0.197 ± 0.001	99.03 ± 1.05	1.06	FAAS
Zn	0.926 ±0.002	0.1	1.03 ±0.02	103.6 ± 10.96	10.58	FAAS
Fe	0.485± 0.028	0.1	0.136 ± 0.001	100.33 ± 2.08	2. 07	UV – VIS

Table 4.4.. Recovery Values of Metals for the Analyzed Milk source atella Sample

Metals	Conc. in sample (mg/L)	Amount added (mg/L)	Conc. in spiked sample (mg/L)	% Recovery	RSD	Method used
Pb	0.037± 0.0015	0.03	0.067 ± 0.002	99.97 ± 6.63	6.65	FAAS
Cr	0.033±0.002	0.03	0.065 ±0.001	107.96 ±10.5	9.73	FAAS
Ni	0.036 ± 0.001	0.03	0.067 ± 0.001	103.3 ± 3.3	3.19	FAAS
Cu	0.85 ± 0.03	0.10	0.95 ± 0.005	103.4 ± 5.77	5.58	FAAS
Zn	0.97 ±0.01	0.10	1.06 ±0.005	96 .66 ± 5.77	5.97	FAAS
Fe	1.36 ± 0.015	1.00	2.37 ± 0.012	100.66 ± 1.52	1.51	UV – VIS

4.7. The Level of Some Selected Metals in Milk and atella samples

Metals concentration (mg/L) in cow's milk and milk source atella samples collected from Arsi Negele town (average metals concentration and ± SD respectively are mentioned in Table 6. From Table 6, the results show that heavy metals such as, Pb, Cr, and Ni are below detection limit. On the other hand the concentration level of Cu in milk and milk source atella were found as 0.0983 ± 0.001 mg/L and 0.85 ± 0.030 mg/L respectively. The concentration level of Zn in the cow's milk samples and milk feed source atela are 0.926 ± 0.002 and 0.97 ± 0.011 mg/L, respectively. . Finally, the concentration level of Fe in the raw cow's milk and milk feed source atela are 0.037 ± 0.0015 mg/L and 1.363 ± 0.015 mg/L, respectively.

Table 4.5. The Levels of Metals in Cow's Milk and Milk source atela Sample (mean \pm SD, n = 3)

Metals	Mean concentration (mg/L)					
	Milk (Mean \pm SD)	WHO	t-test (p)	Atela (Mean \pm SD)	WHO	t-test (p)
Cr	BDL			BDL		
Pb	BDL			BDL		
Ni	BDL			BDL		
Cu	0.0983 \pm 0.0011	3	0.000126	0.853 \pm 0.030	30	0.073
Zn	0.926 \pm 0.0022	3	0.00069	0.97 \pm 0.011	43	0.000609
Fe	0.037 \pm 0.0015	0.5	0.000125	1.363 \pm 0.015	50	0.000456

4.8 Discussion of Some Selected Metals Contents in the Cow's Milk and milk feed source atella Samples

Amongst the sex metals analyzed, three metals (Fe, Cu and Zn) were detected whereas the other three metals (Pb, Cr and Ni) were below detection limit in milk and milk feed atella samples. This indicates that the feeds and the water which the cows use are also free from these toxic metals. The mean concentrations of metals detected in the milk samples in the present study were in the order of Zn > Cu > Fe for milk and Fe > Zn > Cu for milk feed atella samples.

4.9 Stastical analysis

Stastical analysis were done to assess the weather there is significance difference or not by using t- test and the analysis revealed that there is significance difference between temprataure measured and standard value at (p < 0.05) and no significance difference in pH of milk sample with standard. But there is significance difference in pH of atella sample and standard.

There was no significance difference in moisture, dry matter of milk and atella sample with standard at (p < 0.05). But there is significance difference in crude fiber, carbohydrate, and energy of milk and atella sample with standard at (p < 0.05).

5. Conclusion and Recommendations

5.1. Conclusion

The present study evaluated the physicochemical, proximate, and heavy metal analysis of milk and milk feed source atella samples. The finding of proximate analysis demonstrated that milk has a low total ash and relatively higher fat, protein, fiber, carbohydrate, ash, and energy and also milk feed atella has a low dry matter and relatively higher fat, protein, fiber, carbohydrate, ash, and energy. The optimized wet digestion method for the cow's milk and milk feed source atella analysis was found efficient for the metals studied and it was validated through the recovery test and a good percentage recovery was obtained (94.44 – 110.33 %). The mean values of metals of milk and milk feed source atella samples in the study area were found to follow the decreasing order; $Zn > Cu > Fe$ and $Fe > Zn > Cu$ for milk and milk feed source atella respectively. From six analyzed metals, three metals (Cr, Ni and Pb) were below detection limit. The quality and composition of cow milk depends on its physicochemical parameters that vary from milk to milk feed source. Moreover, the quality and compositions are affected by several factors such as type of breeds, forage consumption, feeding schemes, milking incidence, milking process, seasonal changes, lactation period and adulteration. The outcome of this study showed that physicochemical, proximate and heavy metals analysis was significantly correlated with FAO/WHO recommended.

5.2 Recommendations

Although the levels of the heavy metals in both atella and milk samples were in the permissible range as stipulated by WHO, the study further revealed implications of carryover of the heavy metals from the cow's feed atella to the milk. Therefore, it is important that the beverage producers use raw materials such as wheat and maize free of contaminations or originating from highly contaminated sites so that the safety of the dairy products is maintained. In addition, it is recommended that stakeholders consider further study at a larger scale with increased sampling sites analyzed individually to make more specific comments.

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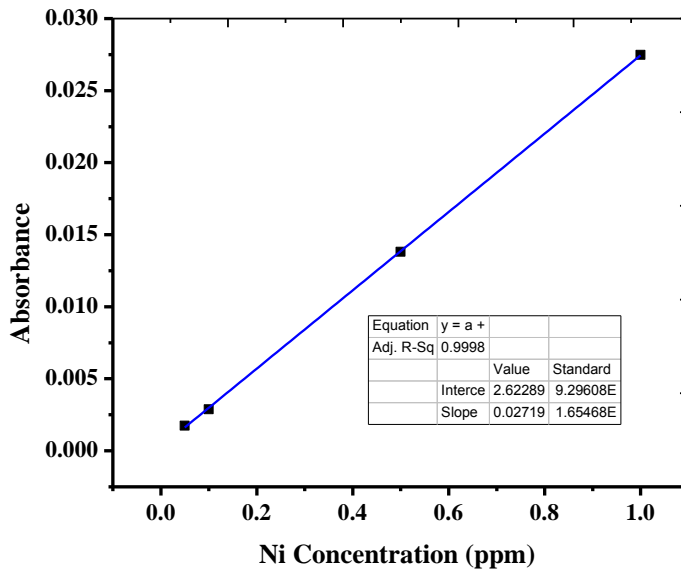
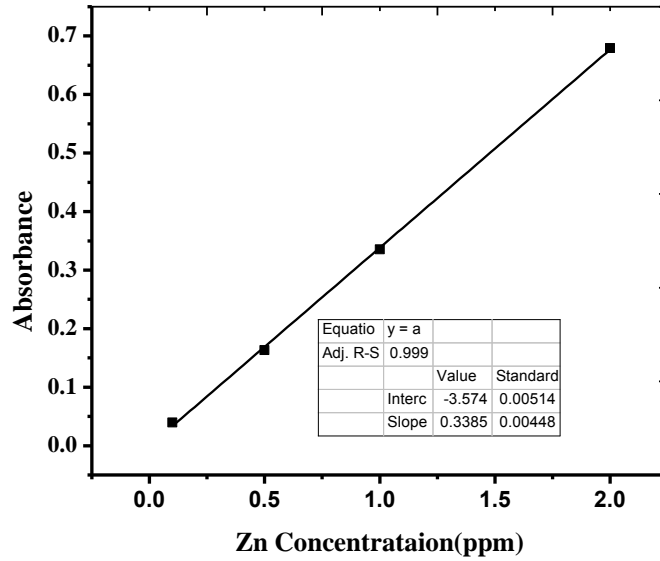
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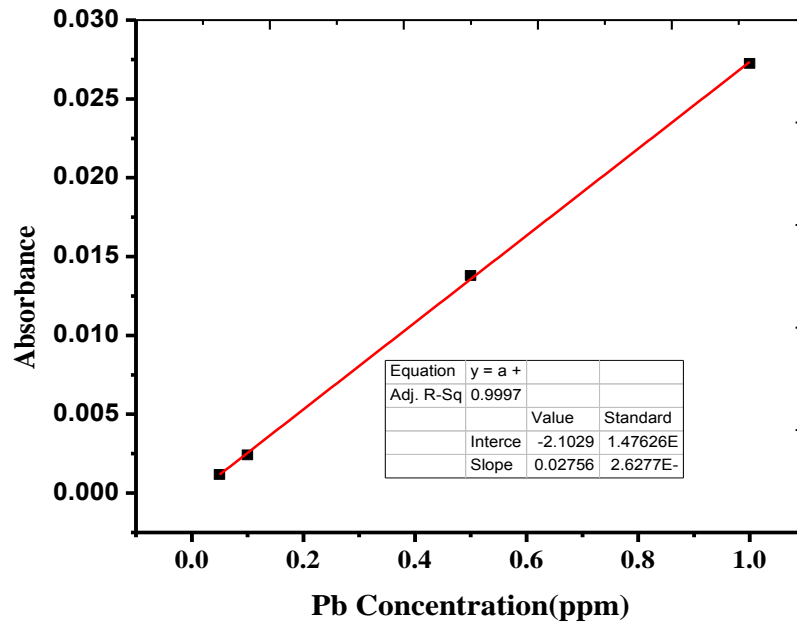
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APPENDICES

Calibration curve of selected metals





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