ADDIS ABABA UNVERSITY

SCHOOL OF GRADUATE STUDIES

Levels of Essential and Toxic Metals in Commercial Powdered Infant Formulas

BY

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By

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LIST OF ABBREVIATIONS

BF	Breast Feeding
AF	Artificial Feeding
SIDS	Sudden Infant Death Syndrome
AAP	American Academy of Pediatrics
PCBs	Polychlorinated Biphenyls
IgE	Immunoglobulin E
eHF	extensively hydrolyzed formulas
pHF	partially hydrolyzed formulas
βLG	β- Lactoglobulin
FF	Formula Fed

ID	Iron Deficiency
IDA	Iron Deficiency Anemia
WHO	World Health Organization
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
AAS	Atomic Absorption Spectrometry
FAAS	Flame Atomic Absorption Spectrometry
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
ETAAS	Electrothermal Atomic Absorption Spectrometry
FES	Flame Emission Spectrometry
MHz	Mega Hertz
SD	Standard Deviation
RSD	Relative Standard Deviation
X ± SD	mean plus or minus standard deviation
MDL	Method Detection Limit
BE	Bebelac
GU	Guigoz

RDA	Recommended Dietary Allowances

DRI	Dietary Reference Intakes
%	percentage
mg/L	milligram per liter
ng/mL	nanogram per liter
µg/g	microgram per gram
µg/kg	microgram per kilogram
mg/kg	milligram per kilogram
°C	degree centigrade
h	hour
min	minute
µL	microliter
mL	milliliter
g	gram

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Abstract

Levels of Essential and Toxic Metals in Commercial Powdered Infant Formulas

By: Adanech Alemu

Advisor: Dr. Merid Tessema

Samples of infant formulas were collected from different supermarkets. After preparation of samples, different digestion procedures were tested by varying reagent volumes, digestion time, temperature and amount of the sample to develop a procedure that consumes less regent volumes, short digestion time, low temperature of digestion and smaller mass of the sample. The optimal procedure required 4:00 hours and consumed 3 mL HNO_3 and 2 mL $HClO_4$ to completely digest 0.5 g of powdered infant formula samples. The accuracy of the optimized procedure was evaluated by analyzing the digest of the spiked samples with standard solution. Recoveries of the spiked samples varied from 91.33 % to 115.0 % for infant formula samples. Concentrations of essential and toxic metals in the samples were analyzed by flame atomic absorption spectrometer (FAAS) employing a four point external calibration curve. The observed average metals concentrations were (mean ± SD, µg/g and N = 9): Ca (2138.21 ± 13.35), Mg (274.70 ± 4.55), Fe (62.72 ± 1.22), Zn (32.53 ± 2.35), Cu (2.73 ± 0.10), Mn (0.65 ± 0.02) in NAN; Ca (2331.08 ± 20.67), Mg (310.58 ± 2.87), Fe (75.70 ± 2.55), Zn (42.98 ± 1.35), Cu (3.68 ± 0.30), Mn (0.84 ± 0.05) in S-26; Ca (2294.15 ± 19.85), Mg (295.24 ± 3.27), Fe (76.49 ± 1.77), Zn (42.21 ± 0.57), Cu (3.07 ± 0.27), Mn (1.01 ± 0.023) in Bebelac; Ca (2445.47 ± 24.49), Mg (298.35 ± 3.35), Fe (65.28 ± 4.09), Zn (39.41 ± 0.78), Cu (2.86 ± 0.15), Mn (0.56 ± 0.01) in Guigoz. This study showed that the metal contents of infant formulas vary with brands.

The toxic metals (Pb and Cd) were not quantified in all brands of infant formulas, which were below the method detection limit.

The daily intakes of the essential metals were calculated by using the feeding tables specified by the manufacturers of the various brands. The results were compared with recommended dietary allowances (RDA) and dietary reference intakes (DRI) for use in North America. The average daily intakes of most metals in powdered infant formulas were comparable with the recommended values, but showed lower or higher than the recommended values for some metals. The levels of metals in infant formulas are comparable with that of the manufacturers and literature values.

Key words: Infant formulas, Flame atomic absorption spectrometer (FAAS), Essential metals, Toxic metals

DECLARATION

I, the undersigned, declare that this is my original work and has not been for a degree in any other university and that all sources of material used for the project have been duly acknowledged.

Name: Adanech Alemu

Signature ____________

This project has been submitted for examination with my approval as a university advisor.

Name: Dr. Merid Tessema

Signature _______________

Place and date of submission: School of Graduate Studies
Addis Ababa University
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1. Introduction

Milk is a complete diet for children, adults and elderly people all over the world. It contains several essential metals, which play important and critical biochemical roles in the human body. Non-essential elements, on the other hand, are known to cause adverse health effects, ranging from cancer to chronic symptoms varying in intensity from simple irritation to extensive metabolic disturbances caused by the failure of various organs [1].
Milk should be controlled not only for fat and proteins as it is usual, but also for mineral content whenever possible. Milk is known as an excellent source of Ca, and it can supply moderate amounts of Mg, smaller amounts of Zn and very small amounts of Fe and Cu. Therefore the essential metals are interesting for determination of their adequate daily intake by the organism. On the other hand, due to the growing environmental pollution it is also necessary to determine and monitor the levels of toxic metals in milk, such as lead and cadmium, because they can significantly influence the human and animal health [2].

Infant feeding deserves top priority in any program aimed at sound child healthcare, irrespective of racial, communal and religious considerations. Optimal growth and development of infants can be guaranteed only when the intake of food and water provides the required doses of all essential elements and delivers only ineffectual doses of toxic elements [3]. It is estimated that proper infant feeding can prevent millions of deaths occurring from infantile gastroenteritis and malnutrition. Milk is the fundamental food for infants. The most natural and best source is from breast feeding and this is greatly encouraged for the first 6 months of life and should be continued for as long as 2 years [4]. Certain situations may warrant a special diet for metabolic reasons or in view of some pressing factors, artificial feeding may have to be resorted to.

Human milk is considered the best food for infants. It meets all their nutritional requirements and promotes infant health and development. Its composition is designed to provide the necessary energy and nutrients in appropriate amounts in a perfectly digestible and absorbable form [3, 5]. However, in some circumstances, e.g. insufficient milk syndrome, breastfeeding failure, social factors or for premature and low-birth-weight infants, it is necessary to substitute or fortify breast-feeding with specially designed formulas [6]. It is generally believed that breast-fed infants absorb adequate amounts of minerals and trace elements, whereas there is some concern about how well infants can utilize these nutrients from cow's milk formula and other infant diets. Therefore, most infant formulas contain much higher concentrations of minerals and trace elements than those of breast milk to ensure that formula fed infants receive the same nutritional benefits as breast fed infants in an optimal digestible and absorbable form [5]. Powdered milks are dried milk prepared by drying the concentrated liquid milks. These are stable and contain denatured proteins so that the curd is finer; they are available in a wide range to suit different ages. The amount of feeding milk depends on the variation in the energy needs and mineral requirements of the children at different ages under various conditions [7]. The basic ingredient in most baby milk preparation is cow's milk, which has been modified to make it as similar to mother's milk as possible. Most infant formulas are derived from animals or plants and therefore are mostly milk-based or soy-based formulations. Because of the nutritional properties of soy bean, diary-like products or infant formulas based on soybean are proposed as one of the most interesting alternatives for children who are allergic to animal proteins.

The adequate supply of nutrients through the provision of safe foodstuffs with a balanced composition is even more important for infants than for healthy children and adults during any other period of the life. The very rapid rate of growth of healthy infants born at full term, who doubles their weight in only 4-5 months after birth, results in relatively high requirements of energy and nutrients per kilogram body weight. In addition to meeting maintenance requirements, infants must cover the energy and substrate needs for the synthesis and deposition of newly formed tissues. The resulting large metabolic requirements contrast with the limited capacity of young infants to compensate for an unbalanced nutrient supply due to small body stores of nutrients and immature homeostatic mechanisms [8]. In addition to immediate consequences of infant feeding on growth, body composition, health and well-being, a number of recent studies have also provided indications that the quantity and quality of nutrient supply during infancy has important long-term consequences on organ development and function, health and disease risks as well as cognitive ability in later life. In consideration of the particular risk of infants to experience untoward effects by diets with providing either too low or too high supplies of specific substrates, and the fact that during the first months after birth usually one sole milk source must meet all the infant's dietary requirements, special efforts are required to secure an adequate dietary composition for infants [5, 6].

1.1. Benefits of Breast-Feeding

In most cases breast-feeding provides an adequate supply of nutrients to support healthy growth and development of infants, it provides anti-infective protection, and it forms a unique biological and emotional basis for the health and well-being of both mother and child [9]. Breast feeding (BF) offers anti-microbial, nutritional, contraceptive, hygienic, economic and psychological benefits to infants and their mothers. Indeed, these benefits of breast feeding protect against the mortality and morbidity associated with gastrointestinal and respiratory infection, and may have a lasting effect on health. For example, many studies suggest that artificial infant feeding (AF), *i.e.,* non-human-milk feeding, increases the risk for type-I diabetes mellitus in childhood [10]. There is also some evidence that AF increases the risk for Crohn's disease [11]. Breast milk composition varies with the age of the baby, time of day and point in the feeding thus adapting to the needs of the particular infant. Research continues to confirm the fact that breast milk is the optimal food source for developing babies. Breastfeeding completes the infant immune system. Breastfed infants have lower rates of insulin dependant diabetes, respiratory illness, otitis media, allergies, gastrointestinal illness, diarrhea and necrotizing enterocolitis which can be fatal in premature infants. Breastfed infants are also less likely than their formula fed counterparts to die of Sudden Infant Death Syndrome (SIDS). Studies indicate that breastfeeding may offer some protection against multiple sclerosis [12]. As well breastfeeding appears to confer some protection against certain childhood cancers. A number of published studies have examined the hypothesis that AF increases the risk for cancer in general or for a specific cancer or group of cancers [13]. Studies have also suggested that breastfed infants have better cognitive development. This is most profound in the case of preterm infants. Breastfeeding also provides many benefits for the mother. Benefits include lower risk of ovarian cancer and osteoporosis and pre-menopausal breast cancer. High frequency breastfeeding can serve also as a simple and free method of child spacing; one long used by traditional subsistence populations, although used alone, breastfeeding is not a birth control method. In addition to these physical benefits, breastfeeding brings with it a range of emotional benefits for both mother and child. The hormonal effects of breastfeeding enhance the desire for infant - mother proximity and lower anxiety levels in mothers [12].

Extensive and recent research shows that breastfeeding of infants provide advantages with regard to general health, growth and development, while significantly decreasing the risk of a large number of acute and chronic diseases. Older children and adults who were breastfed are protected against different syndromes, such as diabetes mellitus, allergic disease, obesity, and hypertension [5, 14].

1.2. Infant Formulas and Follow-on Formulas

Infants who cannot be fed at the breast, or should not receive breast milk, or for whom breast milk is not available, require breast milk substitutes of high quality for the first 4 to 6 months of life [5]. Standards for such breast milk substitutes, namely infant formulas manufactured from cows' milk or Soy, have been established in the European Union by the Infant Formulas Directive. According to the Infant Formulas Directive, "infant formulas" means foodstuffs intended for particular nutritional use by infants during the first four to six months of life and satisfying by themselves the nutritional requirements of this category of persons, or infant formulas means a breast-milk substitute specially manufactured to satisfy the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding. Whereas "follow-on formulas" means foodstuffs intended for particular nutritional use by infants aged over four months and young children and constituting the principal liquid element in a progressively diversified diet of this category of persons [15, 16].

Infant formulas are a product based on milk of cows or other animals or a mixture of other ingredients which have been proven to be suitable for infant feeding. The nutritional safety and adequacy of infant formula shall be scientifically demonstrated to support growth and development of infants. All ingredients and food additives shall be gluten-free [16]. Theoretically, infant formulas can be based on any appropriate blend of proteins, carbohydrates, fats, minerals and vitamins. However, infant milk products are based predominantly on cow's milk on the account of special properties of its proteins and lactose and its availability in Western countries. One of the major differences between cow's milk and human milk is the protein composition and content. Casein constitutes 80 % of the protein fraction in cow's milk and not more than 40 % in human milk. Thus, in most infant formulas cow's milk is enriched with whey or whey protein concentrate to attain usually a 40:60 casein/ whey protein ratio. The other major difference is the higher lactose content of human milk. In cow's milk based infant formulas the lactose content is increased by the addition of whey and/or lactose alone. In some cases, substitution or fortification of human milk with cow's milk based formulas is not a good choice as it can cause severe disturbances. Cow's milk allergy prevalence has been reported from 0.1 % to as high as 8 %, as all the major protein fractions of cow's milk are potentially antigenic and allergenic [6,8]. In some cases, formulas not based on cow's milk but made with soy protein or hydrolyzates of milk proteins are available.

Recently, and in the past, many authors have worked on infant formulations because of the need to maintain the good health of infants. Some research findings on infant formula report high aluminum content [6, 17], inadequate daily intakes of copper and zinc from infant formula consumption [4, 18] and low selenium content in infant formulations [19, 20]. Home-prepared bottles from powdered cow's milk can have substantial addition of metals due to the water supply, which can further distance the metal profile from that found in human milk. Although the contribution of water used in home-prepared formulas is seldom reported for essential elements, there is evidence that toxic metals, like lead and mercury, can be substantially added during infant feeding, and depending on the water and utensils used during reconstitution of powdered formulas [4].

Canned food, in comparison with fresh food, may cause drastic changes in the levels of essential and non-essential elements based on the environment with which they remain in touch, either during the growth period or the preservation period. It is thus imperative to establish an environmental check on the safe limits of concentration of toxic elements to ward off public health against possible toxicity.

1.2.1. Importance of Infant Formulas

Infant formulas are liquids or reconstituted powders fed to infants. They serve as substitutes for human milk. Apart from breast milk, infant formulas have a special role to play in the diets of infants because they are the major source of nutrients for infants and a unique source of food during the first months of life [21]. They are handy for urban women and many mothers in industrialized countries choose to use commercially manufactured formula to feed their newborn [7]. Despite the strong endorsement of breastfeeding by the American Academy of Pediatrics (AAP), most infants in the USA are fed some infant formula by the time they are 2 months old. Mothers are sometimes worried about or have been discouraged from breastfeeding because of concerns regarding toxins found in the body and often transferred to the baby through lactation [22]. Other reasons for mothers not to breastfeed their babies may include the demands of work or school, life circumstances, such as difficult home situations, and social problems with breastfeeding in public [23]. HIV-positive childbearing women are compelled to formula feed their children so as to minimize the risk of contamination of the virus [24, 25].

Despite the benefits of infant formulas as a major source of food for infants, the presence of contaminants, such as heavy metals, pesticides and polychlorinated biphenyls (PCBs) in infant formula may pose health risks to children. It has been reported that children are more susceptible to exposure because of their greater intestinal absorption than adults, and a lower threshold for adverse effects. These pollutants may arise from the raw materials used in production, poor quality production processes, adulteration of infant foods and bad practices by mothers as regards infant formulation preparation and handling [21].

1.2.2. Soy Protein-based Formulas

Although soy protein-based nutrition has been used during infancy for centuries in the Orient, the first use of soy formula feeding in Orient was in 1909. In 1929, Hill and Stuart proposed soy protein based feeding for infants with intolerance to cow milk-based feeding [26]. Before the 1960s, soy protein-based formulas used soy flour, which imparted a tan color and nutty odor to the formula, and infants consuming it often had diarrhea and excessive intestinal gas. These features and symptoms were attributed to residual indigestible carbohydrates in the soy. Since the mid-1960s, a soy protein isolate has been used, reducing these concerns and greatly increasing acceptance of the product.

The isolated soy protein-based formulas currently on the market are all free of cow milk-protein and lactose. All are iron-fortified and meet the vitamin, mineral, and electrolyte specifications addressed in the 1976 guidelines from the American Academy of Pediatrics for feeding full-term infants and established by the united state Food and Drug Administration [27]. Until 1980, mineral absorption from soy formulas was erratic because of poor stability of the suspensions and the presence of excessive soy phytates in the formula. Not surprisingly, conflicting results of studies addressing the adequacy of bone mineralization were reported. With the present formulations, bone mineralization, serum levels of calcium and phosphorus, and alkaline phosphatase levels in equivalent to those seen with cow milk-based formulas. Because soy protein isolate formulas still contain 1.5 % phytates and up to 30 % of the total phosphorus is phytate-bound, the total phosphorus and calcium content of the formulas is; 20 % higher than in cow milk-based formula, while still maintaining the mandated calcium to available phosphorus ratio (1:1 to 2:1). All soy-based formulas thus are iron-fortified and have proved as effective as iron fortified (12 mg/L) cow milk-based formulas in the prevention of iron deficiency in infants. With radio labeled zinc, the highest absorption of zinc is from human milk (41%) and the lowest is from soy formula (14 %). In 1996, the American Academy of Pediatrics issued a statement on aluminum toxicity in infants and children and discussed the relatively high content of aluminum in soy-based formulas [6]. Although the aluminum content of human milk is 4 to 65 ng/ mL that of soy protein-based formula is 600 to 1300 ng/mL [17]. The toxicity of aluminum is traced to increased deposition in bone and in the central nervous system, particularly in the presence of reduced renal function in preterm infants and children with renal failure. Because aluminum competes with calcium for absorption, increased amounts of dietary aluminum from isolated soy protein-based formula may contribute to the reduced skeletal mineralization (osteopenia) observed in preterm infants and infants with intrauterine growth retardation [28].

Soy-protein formulas, although different in carbohydrate and protein source, are similar in composition to cow's milk-protein formulas following the American Academy of Pediatrics, Committee on Nutrition, 1976 recommendations for nutrient levels in infant formulas [27]. Differences include a slightly higher protein level and slightly lower carbohydrate content [26]. Children who are allergic to cow milk are often given soy-based formulas. Like milk, large quantities of soy can result in a transient soy protein-induced gastroenteropathy with resultant chronic diarrhea and failure to thrive. Estimates of the number of children who have either Immunoglobulin E (IgE)-mediated reactions or gastroenteropathy from both milk and soy, range from 10 % to 30 % [29]. IgE-mediated milk allergy develops more frequently in babies with atopic dermatitis and may be triggered by trace amounts of cow milk antigen, which may be passed to the baby through the mother's breast milk from dairy products she has consumed, or from feeding cow's milk to the baby. Various milk proteins can cause an IgE-mediated reaction, which can result in hives, wheezing, asthma, and anaphylaxis. Extensively hydrolyzed casein formulas are often recommended for these children. At greatest risk are babies on soy formula because of their small size, critical developmental phase and the fact that formula is their main source of nutrient. The Swiss Federal Health Service, British Dietetic Association, and Israeli Health Ministry have warned parents and pediatricians that soy infant formula is so dangerous that it should be used only as a last resort [30]. These agencies are concerned because the plant estrogens in soy formula have been linked to premature puberty in girls, delayed or arrested puberty in boys, thyroid disease. Unfortunately, the plant estrogens are not all that is wrong with soy formula. Recently, another danger has come to light–manganese toxicity. Infants fed soy formula take in as much as 75 to 80 times more manganese per day than infants who are breast fed. Per liter, breast milk contains 3 to 10 μg manganese, cow's milk formula 30 to 50 μg, and soy formula a whopping 200 to 300 μg. Although manganese is a vital trace mineral, high levels are toxic to newborns [31].

1.2.3. Hypoallergenic Formulas

Feeding extensively hydrolyzed cow's milk formula to atopic children during the first 14 months of life can protect against the development of cow's milk allergy up to the age of four years. It has been suggested that this might be prevention and not just a postponement of the onset of symptoms, but more research is needed to verify this. Cross-reactivity between certain cow milk and goat milk proteins has been reported, suggesting that goat milk may not be a safe alternative to cow milk for many children with cow milk allergy. Occasionally, milk-allergic children may also develop a beef protein allergy. It should be noted that lactose, a sugar in milk that is commonly used ingredient in manufactured foods, may contain traces of casein and whey proteins, and has been reported to cause adverse reactions in individuals sensitive to milk proteins [32].

Food allergies are estimated to occur in 5 to 20 % of children. Allergy to cow's milk proteins is the most common food allergy in early childhood, occurring in 2 to 3 % of infants within the first year of life. Clinical manifestations of cow's milk protein allergy include gastrointestinal symptoms, urticaria, angioedema, atopic dermatitis, allergic rhinitis, asthma, or chronic cough. Gastrointestinal symptoms are chronic or acute vomiting, diarrhea, and colic. Colic appears to be a common symptom, but nearly always in combination with other symptoms [33]. Besides, allergy to cow's milk proteins can be a precursor to other, and sometimes, more serious diseases, such as other food allergies, respiratory allergies or, in the worst cases, even death. Currently, the only effective treatment for cow's milk protein allergy is avoidance of the allergen-containing food.

During infancy and early childhood a milk substitute is necessary for those children affected by cow's milk protein allergy. Hypoallergenic formulas, based on hydrolyzed cow's milk proteins, have been demonstrated to be the best option for children with cow's milk protein allergy [29]. However, despite the use of hydrolyzed formulas for child nutrition, cases of allergic reactions have been reported for extensively hydrolyzed formulas (eHF) and more commonly for partially hydrolyzed formulas (pHF) [29]. This indicates that minute amounts of residual intact proteins and/or peptides with a molecular weight high enough to be allergenic are still present in these formulas. More extensively hydrolyzed formulas result in lower residual antigenicity and a lower risk of allergic reactions. However, these formulas suffer from a bitter taste and, sometimes, the high concentration of amino acids present in these products can cause diarrhea in infants. Bovine ß-lactoglobulin (ßLG) is the main allergen present in cow's milk; even at concentrations as low as those present in hypoallergenic formulas it can generate allergic reactions. The allergenicity of ßLG is attributed to several amino acid sequences with a particular three-dimensional orientation within the protein structure [34, 35].

1.3. Growth of Breast-fed and Formula-fed Infants

It is well documented that the pattern of growth of formula-fed infants differs from that of breast-fed infants [19]. Breast-fed infants tend to gain less weight and usually are leaner than are formula-fed infants in the second half of the first year of life. This difference of growth pattern between breast- and formula-fed infants seems to be the result of differences of composition between the two diets, but may be also due to differences in infant self-regulation of energy intake. There is evidence that breast-fed infants self regulate their energy intake at a lower level than do formula-fed infants.

1.4. Effect of Infant Formula on Stool Characteristics of Infants

In the United States approximately 50 % of all newborns, and 87 % of 3-month-old infants, are fed a commercial formula either as their sole source of nutrition or as a supplement to breast milk. It is common for many formula-fed infants to be switched from one formula to another either by their parents or physicians. Although the reasons for such frequent formula switching are sometimes elusive, most of the changes occur because of perceived abnormalities in stooling patterns (too much/too little, too hard/too loose) or reports that the infant is uncomfortable while consuming a specific formula. Although there may be considerable variability in the frequency with which infants pass their stools, the effects of various formulas on stool characteristics is limited. Previous reports that have evaluated the impact of formula content on infant stool habits have been limited to cow's milk preparations with varying iron contents [36].

Parents are very concerned about their infant's tolerance to feedings. One of the primary concerns is whether an infant formula produces "constipation," a term that is used often to describe a condition in which stools are firm and perceived by parents to be passed with excessive effort and discomfort. Perceived intolerance also may be related to the fact that infants, whether breastfed or formula fed (FF), for unknown reasons are sometimes fussy, appear to have abdominal cramps, cry at inconvenient times, and regurgitate. Parents often switch their infant's formula as the result of these symptoms. The perceived presence of an allergy to milk protein may explain formula-switching as well. However, the incidence of true allergy to milk protein is far less common than the incidence of formula-switching. This suggests that other components of formulas produce symptoms in some infants that are viewed by parents as undesirable. Commercially available formulas differ from each other in processing and in sources and levels of protein, lipids, and micronutrients; there is reason to believe that these differences affect tolerance. Despite the great frequency of formula-switching, data comparing the tolerance to various infant formulas are limited. Whether infants who are initially breastfed differ in their reaction to infant formula from those who are FF from birth also is unclear [27].

1.5. Essential and Non Essential Elements

Trace elements play an important role in human biology, because they are either inadequately synthesized or not synthesized in the body. Trace amounts of some metals, manganese, copper, zinc, for example, are essential micro nutrients and have a variety of biochemical functions in all living organisms. While these elements are essential, they can be toxic when taken in excess. Both toxicity and necessity vary from element-to-element. In addition, some metals like lead do not occur naturally in the body, and their presence, usually as a result of occupational or pollution-related exposure, is detrimental to health and children are more sensitive to these metals than adults [37]. Metals such as aluminum, cadmium and lead are found throughout the environment and are present in virtually all food at extremely low levels. All of these contaminants are more likely to affect bottle-fed infants. Generally, infants fed formula made with tap water are at the highest risk from metals contaminating the water supply. Indeed some infant foods, such as commercial infant formulas, are deliberately fortified with essential elements such as zinc and copper to ensure that they provide infants' nutritional requirements for trace elements. Another important factor that must be borne in mind when assessing the exposure of infants to trace elements and heavy metals in their diets is that because infants grow and develop very rapidly in their first year of life, their energy requirements and hence their food consumption is on average much higher relative to their body weight than that of adults and older children [38].

The intake of heavy metals as a result of consumption of contaminated milk and milk products has toxic effects depending on contamination and absorption levels. Symptoms of heavy metal toxicity include dizziness, nausea, vomiting, diarrhea, sleeping disorders, loss of appetite and reduced conception rate. Heavy metals have also been linked to cardiovascular disease, depressed growth, impaired fertility, nervous and immune system disorders, increased spontaneous abortions, and elevated death rate among infants [39].

There is now growing evidence of the importance of trace elements in human nutrition, especially during infancy. Human milk and infant formula play an important role in infant nutrition, since they are the only source of food available to the majority of infants during the first year of life. In view of these facts, it is essential to know the contents of trace element in human milk and infant formula. It is also of importance to know the difference between human milk and cow's milk, since the preparation of infant formula is generally based on cow's milk and modified to resemble human milk [40].

1.5.1. Iron

Iron is essential for virtually every living organism. The dominating function of iron in the human body is as the oxygen-binding core of hemoglobin, the red pigment of blood transporting oxygen from the lungs to all tissues [41, 42]. During the progress of iron deficiency (ID), hemoglobin synthesis in the bone marrow is restricted resulting in anemia. Anemia caused by ID is called iron deficiency anemia (IDA), distinguishing this condition from other causes of anemia, such as infection, inflammation, hematological disorders and other nutritional deficiencies. Human milk has unique properties, making exclusive breast-feeding a superior form of nourishment for infants during the first half of infancy. In 1980, the world health organization (WHO) estimated the worldwide prevalence of anemia in children below 4 years of age to be 43 %, with a higher prevalence (51%) in developing regions as compared to (12 %) in industrialized countries. WHO communication reports for the same age group 20 % for children in industrialized countries and 39 % in non industrialized countries. In affluent populations, exclusive breast-feeding prevents IDA during the first half of infancy in healthy term infants. However, in socio-economically disadvantaged populations infants may be at risk of developing ID or even IDA if exclusively breast-fed beyond 4 months. In affluent societies, healthy term infants fed infant formulas unfortified with iron seldom develop IDA before 4 months of age, but are at higher risk than are breast-fed infants after that age. For this reason, most infant formulas are fortified with iron, but the optimal level of added iron is still an open question [8, 41, 43].

Iron plays an important role in many metabolic processes, including oxygen transport, oxidative metabolism, and cellular growth. During infancy, inadequate supply of iron resulting in iron-deficiency anemia is associated with morbidity, impaired growth, and decreased behavior and psychomotor development. Although iron requirements during infancy have become better defined, iron-deficiency anemia persists as one of the most common health problems worldwide, a condition that affects approximately 20–25 % of the world's infants. Today, much attention is being given to not only preventing iron-deficiency anemia but also to avoiding excessive iron supplementation. There is concern that excessive and widespread iron supplementation could lead to decreased resistance to infection and promotion of gastrointestinal illnesses. However, the literature on the relationship between iron status and chronic infection and disease contains conflicting viewpoints. Some investigators contend that mild iron-deficiency is beneficial for immunity whereas others argue that any deficits in iron status are detrimental. Iron absorption and metabolism are influenced by interactions between iron and other dietary nutrients. Many components of the diet act to inhibit or enhance iron absorption; this information is critical for food fortification programs designed to prevent iron-deficiency anemia worldwide [44].

1.5.2. Iron Requirements in Infancy

The term newborn infant has a total body iron content averaging about 75 mg/kg body weight, which can be compared with 55 mg/kg for an adult [44, 37]. In adult humans, there is little exchange of iron between the body and the environment, since body iron is recycled and physiological iron losses are small. In the infant, however, the recycling yields a deficit since a substantial part of iron turnover is diverted to growing tissues. In a 6-month old infant ~0.5 mg iron/day is needed for expansion of the blood mass and 0.1 mg/day for growth of muscle and other tissues. Since iron requirements excluding growth would equal iron losses (0.15 mg/day), infant growth increases iron requirements 5- fold to approximately 0.75 mg/day. As a consequence of reduced hemoglobin concentration and redistribution of iron to storage sites, the infant is able to double its birth weight without exhausting its reserves, independent of external iron. For the term, normal infant this occurs at about 5 months of age and, soon thereafter, iron utilization from the diet becomes critical for maintenance of iron balance. In proportion to body weight the need for dietary iron is greater during late infancy than during any other period of life. Based on certain assumptions, such as total body iron at birth and basal iron losses Oski estimated the average requirement for absorbed iron during the first year of life to be 280 mg, averaging 0.8 mg/day [45]. Such intake is virtually impossible to achieve with unmodified complementary foods, suggesting that additional iron is needed, either as iron addition to foodstuffs or as separate iron supplements to cover the estimated needs. Despite the documented preventive effect on ID of adding iron to infant formulas, IDA in infancy is still a major public health problem, not only in developing countries, but also in certain populations in industrialized countries, among whom non-fortified formulas and unmodified cows' milk are still widely used.

1.5.3. Calcium

At birth about 99 % of the calcium in the body is part of the structural matrix of bone, with the remainder being physiologically active as a free calcium pool within cells and the extra cellular fluid. Within cells, calcium acts as a second messenger, modulating the transmission of hormonal signals, and regulating enzyme function. It is involved in blood coagulation, nerve conduction, muscle contraction and reproductive functions. Although net calcium absorption and calcium retention may be related to the calcium intake, there are a range of other factors either in the diet, or related to metabolic regulation which influence the absorption, around 30 to 50 % of dietary calcium, and retention of calcium [8, 43]. Thus calcium status represents the integration of the absorption of calcium within the gastrointestinal tract, the deposition and resorption of calcium in bone and the excretion or retention of calcium in the kidney. Hormonal factors, such as calcitonin, parathyroid hormone and vitamin D, play a clear role in this integrated function, but the metabolic handling of other nutrients such as sodium, phosphorus, iron, zinc and magnesium are also known to exert an influence. In this regard the intake and metabolism of phosphorus is of particular importance, as effective calcium retention requires that adequate phosphorus is available. For this reason the ratio of calcium to phosphorus within formula should be specified.

1.5.4. Magnesium

Magnesium (Mg) is an essential mineral for humans which is necessary for the activation of >300 enzyme systems, especially in energy metabolism. In addition, Mg is an important structural component of bone and soft tissue cells. Low dietary intake of Mg and low Mg serum levels have been associated with major public health problems such as non-insulin dependent diabetes mellitus, osteoporosis and coronary artery diseases [43].

The metabolism of magnesium is tightly regulated. It is an integral constituent of bone mineral and as the second most abundant intracellular cation plays a fundamental role in many aspects of intermediary metabolism, as a cofactor for many different enzymes and as a modulator of physiological processes. Status is hard to define as plasma concentrations are maintained within narrow limits. It is absorbed in the distal ileum and colon, through a regulated process which may involve vitamin D and is influenced by the presence and body status of sodium, potassium and calcium. The plasma concentration and its availability to metabolism are regulated through a combination of gastrointestinal absorption, bone deposition and resorption and renal excretion. Gastro-intestinal absorption may be enhanced by lactose. High doses of magnesium are toxic, and untoward effects have been reported in newborn infants treated with antacids or whose mothers were treated with magnesium sulphate for hypertensive disorders of pregnancy. Magnesium toxicity from formulas has not been reported [8, 43].

1.5.5. Copper

Copper is required as an essential dietary trace element. It is required for cellular metabolism in enzymatic and non-enzymatic systems. Copper acts as an important metallic activator of several enzymes [46-48]. Copper status is not easy to determine, and the homeostatic mechanisms which control copper distribution and metabolism are incompletely understood. However, severe deficiency may manifest with abnormal collagen and bone development, sideroblastic anemia and neutropaenia. Newborns have a high liver content of copper which is drawn upon for growth during the first six months of life. The copper content of human milk is not directly influenced by maternal dietary intake of copper, and is generally higher than cows' milk. As cow milk presents a rather low concentration of copper, whereas infant formula powdered milks are fortified with this element. In the 1980s the concentration of copper in infant formulas was reported to vary very widely, with some milk having almost undetectable amounts [8, 43]. Copper interacts with other divalent cations, such as iron and zinc, for gastrointestinal absorption. Diets which contain excess copper have been reported to lead to toxicity and liver damage during childhood and the levels of copper in the tap water delivered through copper pipes has been reported to lead to toxicity, associated with levels in infant formula between 9 to 26.4 mg/L [18].

1.5.6. Zinc

Zinc is essential for growth and development. Zinc is a constituent of more than 200 metalloenzymes, many of which regulate the metabolism of carbohydrates, lipids and protein. It plays a key role in the synthesis of genetic material and the regulation of gene expression as well as in cell division, epithelial integrity, cellular immunity and sexual maturation. Zinc also plays a clear role in the synthesis, storage and secretion of Insulin. The young infant has a high zinc requirement to support the very rapid growth of early infancy. Symptoms of zinc deficiency include impaired growth and altered cognition in children, loss of appetite and taste sensitivity, eye and skin lesions, alopecia, diarrhea, susceptibility to infections, delayed healing of wounds and reproductive failure [4, 8, 43, 49]. Prolonged use of high doses of zinc results in a reduction of copper absorption, as it has been shown in patients with Wilson's disease [4, 43]. Iron supplementation could decrease zinc absorption. However, at levels present in food and at realistic supplementation levels, zinc absorption appears not be significantly affected by iron and copper [8].

1.5.7. Manganese

Manganese is an essential trace element known to be related particularly to reproductive function. It is involved in the formation of bone and in amino acid, cholesterol, and carbohydrate metabolism. Manganese is a component of many enzymes. Glycosyl transferases are specifically activated by manganese. Manganese deficiency is associated in animals with growth retardation, impaired glucose tolerance and skeletal abnormalities, brain damage, teratogenicity and abnormal metabolism of carbohydrates and lipids. Thus manganese should be an important pharmacodynamic component in the treatment of those diseases that are associated with its deficiency. For example, the content of manganese in Chinese medicinal plants with high therapeutic value for cancer is high. However, excessive manganese is toxic to humans and to animals: relatively high doses of manganese cause mutations in mammalian cells, damage to DNA and chromosome aberrations, and toxicity to the embryo and fetus [8, 31]. Manganese toxicity has been demonstrated for people who inhale manganese dust. The most prominent effect is central nervous system pathology, especially in the extra pyramidal motor system. Neurotoxicity can also be observed after ingestion of manganese. Manganese toxicity has been shown in children receiving long-term parenteral nutrition, and plays a role in cholestasis as well as in nervous system abnormalities in these children [43].

1.5.8. Cadmium

Cadmium (Cd) is a highly toxic metal with a natural occurrence in soil, but also spread in the environment due to human activities. Exposure to cadmium (Cd) occurs in many occupational settings, as well as in the general environment, especially in areas with industrial cadmium pollution or high natural cadmium content in the soil. Natural and anthropogenic sources such as industrial emissions, applications of fertilizers and sewage sludge from farming have led to the contamination of soils. As a result, an increased cadmium uptake occurs by crops and vegetables grown for human consumption [50]. It is easily taken up and accumulated in plants and crops through the root systems and present in all food. For the non-smoking population, food is the major source of cadmium exposure. About 50 % of our average dietary intake originates from cereal products. This may be of special concern to infants and young children since they are introduced to weaning diets, like liquid formulas or porridge, containing cereals at an early age. The formulas are usually prepared by adding drinking water, which could further increase the cadmium intake Studies on adverse health effects due to cadmium exposure are mainly focused on exposure and risk in adults. Numerous studies have shown that chronic exposure leads to selective accumulation of cadmium in the liver and kidneys. Cadmium has an extremely long biological half-life in man, up to 30 years, and tubular kidney damage occurs when a critical concentration is reached in the kidney cortex [51, 52]. However, studies in experimental animals indicate that the developing central nervous system, rather than the kidneys, is the critical organ after perinatal exposure in offspring. Neurotoxic effects like psychomotor disturbances, behavioral and cognitive disorders have been demonstrated in rat pups even after low-dose exposure to cadmium chloride and cadmium acetate during the gestation or lactation periods [52]. Bone is a target organ of Cd toxicity, and decreased bone density is found in the general population exposed to even low doses of Cd [53]. The International Agency for Research on Cancer concluded in 1993 that there was sufficient evidence to classify cadmium as a human carcinogen [50].

1.5.9. Lead

Lead occurs mostly in nature in the form of PbS and is used in accumulators, production of tetraethyl lead, ammunitions, solders and X-ray equipments [54]. Lead is a toxic metal, which accumulates in the vital organs of man and animals. Recent studies indicate that low levels of lead exposure are correlated with irreversible fetal brain damage, hypertension, cardiovascular disease, kidney dysfunction, impaired bone synthesis, impaired sperm production, and osteoporosis [55]. Dissipation of lead in the soils, natural waters, and atmosphere is due to deposition from emissions of vehicles that used leaded gasoline, discharge of industrial wastewaters, deterioration of lead-based paints and demolition or renovation of buildings with lead-based paint. From the atmosphere, waters and soils, the lead can arrive in the plants, and then introduces into the food chains of man and animals. Therefore the toxic effect of lead at low concentrations in the environment is a cause of increasing concern and explains the growing interest in determining its concentration in foodstuffs [56-59].

1.6. Analytical Methods for the Determination of Metals in Infant Formulas

Infant formulas are very important for infants by substituting breast milk, which are the main source of minerals. Therefore it is necessary to determine the levels of essential and non essential metals in infant formulas in order to maintain the health of infants.

A variety of analytical methods have been used for the elemental analysis in food and biological materials, including inductively coupled plasma mass spectrometry (ICP-MS) [3,5], inductively coupled plasma-atomic emission spectrometry (ICPAES) [60], polarized Zeeman atomic absorption spectrophotometry [40] and colourimetry [41]. The most frequently used analytical method to determine metals in foods and biological materials is atomic absorption spectrometry (AAS). This includes flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), eletrothermal atomic absorption spectrometry (ETAAS), and flame emission spectrometry (FES) [38, 54]. Many analytical methods including AAS for element determination in food materials require decomposition of the sample [38].

1.7. Sample Decomposition

The organic fraction must be separated from the mineral fraction to avoid interference in the signal readout. Different sample treatments, such as dry and wet digestion and decomposition with microwave heating in open and closed vessels have been used. Their efficiency is based on the time required and the completeness of the decomposition. Classical or traditional methods of solid sample digestion/dissolution are often referred to as wet decomposition, dry dissolution, or acid digestion and involve the use of mineral acids and oxidizing agents such as hydrogen peroxide to affect the dissolution of the sample.

1.7.1. Dry Decomposition

Dry decomposition or dissolution, often call “fusion” or “dry ashing,” is normally used for refractory materials or very difficult to digest samples such as geological materials, silicates, oxides, and alloys. Typically, a sample of less than 0.1 to as much as a few grams is mixed with a (typically) a four- to tenfold excess of the fusion reagent (most likely a metal alkaline hydroxides, carbonates, or borates). The most often favored is lithium metaborate. This occurs in a carbon or platinum crucible, which is then placed in a muffle furnace at 800 to 1000 ^{0}C for as little as 15 min to as much as 8 h and results in the formation of a molten salt. The melt is then added to a dilute solution of nitric acid and results in a solution for metal determination. This method has the advantage that it is almost universal and can be applied to just about every solid. Its disadvantages are (a) that volatile metals may be lost in the process, (b) dilution can cause a reduction in detection, (c) use of external reagents can increase blanks and, finally, (d) it can be time consuming and tedious [38, 54].

1.7.2. Wet Decomposition

Wet decomposition or acid digestion involves the use of oxidizing agents (hydrogen peroxide) and mineral acids to affect the dissolution of a sample. Hydrochloric acid (HCl) should be avoided in GFAAS due to the well-known interference from chloride. Sulfuric acid is used as a dehydrating agent and has a high boiling point of around 300^{0}C, which will increase rate of decomposition of some specific samples. While perchloric acid is a strong oxidizing agent, it is extremely hazardous. Hydrofluoric acid (HF) is useful for dissolving silicates. Aqua regia is widely used, but again, chloride interferences are to be avoided in GFAAS work [60, 61].

1.7.3. Microwave Digestion

Digestion with acids, which is necessary prior to instrumental proximate analysis, is time-consuming and is usually the slowest step in the analysis. Recently, several researchers have proposed the use of ultrasonic radiation to accelerate digestion of dairy products. The use of microwave energy as a heat source in acid digestion has been used in the last few years on a variety of food matrices. A microwave digestion system can reduce sample preparation time for proximate analysis by 80 % [61].

Microwave digestion involves the use of 2450 MHz electromagnetic radiation to dissolve samples. The microwaves interact with the polar molecules and induce alignment of the molecular dipole moment with the microwave field. The field changes constantly; causing rotation of the molecules and intermolecular collisions will produce heat. Consequently, the rate of microwave digestion is dependent on the coupling efficiency of microwaves with digestion acids. Microwave technology is often recommended for safety considerations. They are also programmable and can accommodate large numbers of samples [62].

1.8. OBJECTIVES

1.8.1. General Objective

The main objective of this project was to collect base line data on the levels of essential and toxic metals in infant formulas commercially available in Addis Ababa.

1.8.2. Specific Objectives

1. To develop suitable methods for the digestion of infant formulas samples.
2. To determine the levels of both essential (Ca, Mg, Fe, Cu, Mn and Zn) and toxic (Pb and Cd) metals in infant formulas by FAAS to be collected from supermarkets.
3. To compare the metal content in infant formula brands derived using feeding tables supplied by infant formula manufacturers.
4. To compare the levels of essential and toxic metals in infant formulas with literature data.

2. Experimental

2.1. Instrumentation and Apparatus

Analytical digital balance (SCIENTECH, SA120, USA) was used to weigh the infant formula sample. Micropipett (DRAGONMED, 100-1000 μL) was used for measuring different amounts of acid mixtures and standard solutions. A 250 mL round bottomed flasks fitted with reflux condensers were used in kjeldahl apparatus (Gallen hamp) hot plate to digest the powdered infant formula samples. 25, 50 and 100 mL volumetric flasks were used to dilute sample solutions and prepare standard solutions. A refrigerator (Hitachi, Tokyo, Japan) was used to keep the digested sample until analysis. Flame atomic absorption spectrometer (BUCK SCIENTIFIC MODEL 210 VGP, East Norwalk, USA) equipped with deuterium ark background corrector was used for analysis of the metals in the solutions of infant formulas (Ca, Mg, Fe, Cu, Mn, Zn, Pb and Cd) using air-acetylene flame.

2.2. Reagents and Chemicals

Analytical grade chemicals and reagents were used in the analysis of samples of the infant formulas. 69-72 % HNO_3 (Spectrosol, BDH, England), 70 % $HClO_4$ (A.C.S. Aldrich UK, reagent) were used for the digestion of infant formula samples. Lanthanum nitrate hydrate, La $(NO_3)_3.6H_2O$ (99 % Aldrich, USA) was used to prevent the interference of phosphates in the measurement of Ca and Mg. A stock standard solution of 1000 mg/ L, in 2 % HNO_3 of the metals Ca, Mg, Mn, Fe, Zn, Cu, Pb and Cd (BUCK SCIENTIFIC PURO-GRAPHIC^{tm}) were used for preparation of calibration standards and in the spiking experiments. Intermediate standard solutions of the metals were prepared by diluting the stock standard solutions to 10 mg/L, and then the working standard solutions were prepared from the intermediate solutions before analysis. Deionized water was used throughout the experiment for rinsing the apparatus and diluting of sample solutions.

2.3. Sample Collection and Preparation

Four different brands of commercially available infant formulas were used in this study. The four types of infant formulas used in the experiment were NAN, Bebelac, S-26 and Guigoz. 400 g of S-26 and Bebelac and 450 g of NAN and Guigoz were purchased from different supermarkets for the purpose of random sampling. For each of the infant formula brands three containers were used. From each container 50 g of the infant formulas powder was taken and mixed to prepare the bulk samples. From each bulk sample 0.5 g was used for digestion of samples.

Table 1. Characteristics of infant formulas purchased in triplicate from supermarkets in Addis Ababa. All samples were packaged in metal containers.

Brand	**Sample Information**
NAN (Netherlands)	Powder; iron-fortified; milk-based; suitable from birth.
Bebelac (Holland)	Powder; iron-fortified; milk-based; suitable from birth.
S-26 (Ireland)	Powder; iron-fortified; milk-based; suitable from birth.
Guigoz (Netherlands)	Powder; iron-fortified; milk-based; suitable from birth.

2.4. Procedures

2.4.1. Cleaning Apparatus

Apparatus such as volumetric flasks, measuring cylinders and digestion flasks were washed with detergents and tap water, then rinsed with deionized water, soaked in HNO_3 for 24 hours and again rinsed with deionized water.

2.4.2. Digestion of Infant Formula Samples

To prepare a clear colorless sample solution that is suitable for the analysis using AAS different infant formula digestion procedures were assessed using HNO_3, $HClO_4$ and H_2O_2 acid mixtures by varying parameters such as volume of the acid mixtures, digestion time and temperature. A 0.5 g of infant formula sample was weighed on a digital analytical balance and transferred quantitatively into a 250 mL round bottomed flask. To this flask 2.5 mL of HNO_3 (62-72 %) and 1.0 mL of $HClO_4$ (70 %) were added to the sample and the mixture were digested on a kjeldahl digestion apparatus by setting the Temperature dial at 8 (240 ^{0}C) for 2:30 h. Then the sample was cooled for 10 min, and after addition of 1.0 mL of HNO_3 and 1.0 mL $HClO_4$ the digestion was continued for 1:30 h. The digestion gave a clear colorless solution. After a total of 4:0 h digestion, the digested sample was allowed to cool for 15:0 min, and transferred quantitatively to a 25 mL volumetric flask and diluted with deionized water up to the mark. Each infant formula sample was digested in triplicate and hence a total of twelve digests were made for the samples of the infant formulas. Six reagent blank solutions were prepared by following the same digestion procedure as the sample. All the digested samples were stored in refrigerator, until the levels of all the metals in the sample solutions were determined by FAAS.

2.4.3. Determination of Metals in the Infant Formula Samples

For the determination of metals in the infant formulas, four series of working standard solutions were prepared from the 10 mg/L intermediate standard solutions of their respective metals, which were prepared by diluting the stock standard solutions of the metals with deionized water. Optimum acetylene and air flow rates were chosen to obtain suitable flame conditions. Other conditions such as slit width, wavelength, and lamp current were selected for each hollow cathode lamp according to the manufacturer's recommendation. Four point calibration curves were established by introducing the prepared working standard solutions in flame atomic absorption spectrometer. Immediately after calibration of the instrument, the sample solutions were aspirated in to the atomic absorption spectrometer and reading of the metal concentrations was recorded. Three replicate determinations were carried out on each sample. The same analytical procedure was employed in the determination of elements in each six digested blank. The operating conditions for the determination of metals by atomic absorption spectrometer are given in Table 2.

Table 2. Instrumental operating conditions for the determination of metals in infant formula samples using atomic absorption spectrometer.

Element	Wavelength (nm)	Slit width (nm)	Lamp current (mA)
Ca	422.7	0.7	2.0
Mg	285.2	0.7	1.0
Zn	213.9	0.7	2.0
Fe	248.3	0.2	7.0
Cu	324.7	0.7	1.5
Mn	279.5	0.7	3.0
Pb	283.2	0.7	2.0
Cd	228.9	0.7	2.0

2.4.4. Recovery Test

Recovery checks involve the addition of an aliquot of analyte to a real sample to evaluate the recovery efficiency of the method [63]. Recovery checks should be incorporated randomly in a sequence of analyzed samples. The level of spiked analyte may be equal to the expected level of analyte in order to evaluate differences between analyte from the sample or from the spike. On the other hand, minimal error in the recovery factor is obtained when the added analyte is several times larger than the native analyte.

The efficiency of the optimized procedure was checked by adding known concentration of each metal into 0.5 g sample. The procedure was as follows: 250 µL of Ca, 50 µL of Mg, and 15 µL of Fe from 1000 mg/L solutions and 500 µL of Zn from 10 mg/L were spiked at once into 0.5 g of infant formula sample and the remaining metals (75 µL of Cu, and 25 µL of Mn) from 10 mg/L solutions were spiked at once in to another round bottomed flask containing 0.5 g of powdered infant formula sample. After this the sample was digested according to the optimized method. Then the digests were transferred into 25 mL volumetric flask and diluted to the mark with deionized water. Finally the solutions were analyzed for each element that was spiked with atomic absorption spectrometer. As used for original samples triplicate spiked samples were prepared and triplicate readings were recorded.

2.4.5. Method Detection Limit

Limit of detection is the smallest mass of analyte that can be distinguished from statistical fluctuations in a blank, which usually corresponds to the standard of the blank solution times a constant. The limit of detection is most commonly defined as the mass of analyte that gives a signal equal to three times standard deviation of the blank [63]. Six reagent blank (HNO_3 and $HClO_4$) samples were digested following the same procedure as the samples and each of the samples were determined for the elements of interest (Ca, Mg, Fe, Zn, Cu, Mn, Cd, and Pb) by the atomic absorption spectrophotometer. The standard deviation for each element was calculated from the six reagent blank measurements to determine method detection limit.

3. Results and Discussion

3.1. Optimization of the Digestion Procedure

For infant formula samples different digestion procedures using the HNO_3, $HClO_4$ and H_2O_2 acid mixtures were assessed by varying volume of the acid mixtures, digestion time and temperature and the amount of the sample.

The optimum procedure was selected depending on, minimal reagent volume consumption, shorter digestion time, obtaining clear solution and simplicity. The optimal procedure chosen based on these criteria required 4.00 h for the complete digestion of 0.5 g powdered infant formula with 3.5 mL HNO_3 and 2.0 mL $HClO_4$. The digestion gave a clear colorless solution which is suitable for the analysis of metals by FAAS. This digestion procedure of infant formula samples were developed with some modification of literature procedure [1, 61].

The common drawbacks of other tested procedures were their higher chemical composition, observation of colors and formation of precipitate in some of the tested procedures. Due to the use of acid digestion procedure, the acid used for the digestion might add some amount of metals to the samples to be analyzed since most of the reagents have metals as impurities. So, it is necessary to prepare reagent blanks for the digestion performed. Thus, reagent blanks were prepared with the same reagents and subjected to the same digestion procedure as the samples.

3.2. Calibration of the Instrument

Calibration curves were prepared to determine the concentration of the metals in the sample solution. A series of working standard solutions were prepared from the 10 mg/L intermediate standard solutions of their respective metals, which were prepared prior by taking 1 mL from the stock standard solutions containing 1000 mg/L, in 2% HNO_3 of the metals. The correlation coefficients of the elements were determined using prepared standards versus their corresponding absorbances. The working standard solutions and the correlation coefficient of the calibration curve for each of the metals are presented in Table 3.

Table 3. Concentration of the standard solutions used to establish calibration graphs for the determination of metals in infant formula samples and their correlation coefficients.

Element	Concentrations of standard (mg/L)	Correlation coefficient
Ca	0.5, 1.0, 2.0, 4.0	0.99993
Mg	0.5, 1.0, 2.0, 4.0	0.99917
Fe	0.25, 0.5, 1.0, 2.0	0.99696
Zn	0.25, 0.5, 1.0, 2.0	0.99916
Cu	0.02, 0.2, 0.4, 0.8	0.9997
Mn	0.05, 0.1, 0.4, 0.8	0.99997
Pb	0.1, 0.2, 0.4, 0.8	0.99961
Cd	0.005, 0.02, 0.04, 0.08	0.99999

3.3. Method Validation

3.3.1. Precision

The precision of an analytical procedure expresses the closeness of agreement between a set of results. The precision of an analytical procedure is expressed as the variance, standard deviation, coefficient of variation, and relative standard deviation of a series of measurements. In this study the precision of the results were evaluated by the pooled standard deviation, and percentage relative standard deviation of the results of three samples (N = 9) and triplicate readings for each sample meaning that a total of 9 measurements for a given sample. These parameters are useful in estimating and reporting the probable size of indeterminate errors. The results of analysis were reported with corresponding pooled standard deviation of nine measurements for a sample and relative standard deviation. It can be seen that the values of percentage relative standard deviations (% RSD) are less than 10 % for all the mean concentrations (Table 6). This shows the precision of the results obtained by this method is good.

3.3.2. Method Detection Limit

Method detection limit is defined as the minimum concentration of analyte that can be measured by the analytical method with a given confidence limit [63, 64]. In this work, after digestion of six blank solutions, triplicate reading was obtained for each sample. Then the pooled standard deviation of the six blank reagents was calculated. The method detection limit of each element was obtained by multiplying the pooled standard deviation of the reagent blank by three ($3\sigma_{blank}$, n = 18), which is summarized in Table 4.

Table 4. Method detection limit for powdered infant formula samples (n = 18, and in µg/g).

Element	Ca	Mg	Fe	Zn	Cu	Mn	Pb	Cd
MDL (µg/g)	1.88	0.17	2.34	1.07	0.85	0.43	3.6	0.29

MDL: Method Detection Limit

3.3.3. Recovery Test

In this work the recovery of the method was checked by spiking standard solutions of elements with the sample. As shown in Table 5, the percentage recovery for powdered infant formula sample is between 91.33 to 115.0 %, which is within the acceptable range for all metals. The good recovery for most metals indicates the digestion method used for sample preparation is precise and reliable.

$$\% \text{ Recovery} = \frac{\text{(Amount after spike – Amount before spike)}}{\text{Amount Added}} \times 100$$

Table 5. Recovery test for powdered infant formula samples.

Element	[a]Amount before spike(µg/g)	Amount added (µg/g)	[a]Amount after spike (µg/g)	% Recovery
Ca	2294.15 ± 19.85	500	2818.45 ± 16.79	104.86
Mg	295.24 ± 3.27	100	394.63 ± 2.24	99.39
Fe	76.49 ± 1.77	30	106.25 ± 2.41	99.2
Cu	3.07 ± 0.27	1.5	4.44 ± 0.32	91.33
Mn	1.01 ± 0.023	0.5	1.585 ± 0.02	115.0
Zn	42.21 ± 0.57	10	52.09 ± 0.63	98.8

[a] values are mean ± SD of triplicate readings of triplicate analyses.

3.4. Concentration of Metals in Powdered Infant Formulas

Infant formulas are liquids or reconstituted powders fed to infants. They serve as substitutes for human milk. Apart from breast milk, infant formulas have a special role to play in the diets of infants because they are the major source of nutrients for infants and a unique source of food during the first months of life [21].

The concentrations of eight elements (Ca, Mg, Fe, Zn, Cu, Mn, Pb, and Cd) in the digested and diluted solutions of infant formulas were determined by flame atomic absorption spectrometer. Among the identified elements except Cadmium (Cd) and lead (Pb) which were below the method detection limit, all have been detected and quantified

(Table 6), and the % RSD was calculated. The most abundant metal among the element is Ca followed by Mg and Fe.

As it can be seen from Table 6 and Figure 1, 2, and 3, quantitatively there is a variation in concentration of essential metals within the infant formula brands. Except for Cd, and Pb, which were below the method detection limit in all brands, the selected elements were successfully determined. From the analyzed brands of infant formulas, NAN has lowest concentration for all elements: Ca (2138.21), Mg (274.7), Fe (62.72), Zn (32.53), and Cu (2.73) µg/g except for Mn 0.65 µg/g. The lowest concentration of Mn (0.56 µg/g) was found in Guigoz. The highest concentration of Ca (2445.47 µg/g) was found in Guigoz. The highest concentrations of Mg (310.58 µg/g), Zn (42.98 µg/g), and Cu (3.68 µg/g) were found in S-26. The remaining metals such as Fe (76.49 µg/g) and Mn (1.01 µg/g) were found in Bebelac. The percentage relative standard deviations (% RSD) for Ca is in the range of 0.62 – 1.0 %, for Mg is 0.92 – 1.66 %, for Fe is 2.0 – 6.27 %, for Zn is 1.35 – 7.22 %, for Cu is 3.66 – 8.79 % and for Mn is 1.79-5.95 %. The percentage relative standard deviations (% RSD) were less than 10 % for all the analyzed elements. These indicate that there is good precision in the measurements.

As it can be seen from Table 6 and Figure 4 and 5, the pattern of concentration of elements in all brands (NAN, S-26, Bebelac and Guigoz) was decreased as Ca > Mg > Fe > Zn > Cu >Mn. The concentrations of Ca and Mg are highest because they are macro elements. From the micro metals Fe is the highest followed by Zn.

Table 6. Mean concentration (X ± SD, N = 9, in µg/g) and percentage relative standard deviation (% RSD) of infant formula samples.

Type of sample	Concentration of metals (µg/g)							
	Ca	Mg	Fe	Zn	Cu	Mn	Pb	Cd
NAN	2138.21 ± 13.35	274.70 ± 4.55	62.72 ± 1.22	32.53 ± 2.35	2.73 ± 0.10	0.65 ± 0.02	Below MDL	Below MDL
%RSD	0.62	1.66	2.0	7.22	3.66	3.08	_	_
S-26	2331.08 ± 20.67	310.58 ± 2.87	75.7 ± 2.55	42.98 ± 1.35	3.68 ± 0.30	0.84 ± 0.05	Below MDL	Below MDL
%RSD	0.89	0.92	3.37	3.14	8.15	5.95	_	_
Bebelac	2294.15 ± 19.85	295.24 ± 3.27	76.49 ± 1.77	42.21 ± 0.57	3.07 ± 0.27	1.01 ± 0.023	Below MDL	Below MDL
%RSD	0.87	1.11	2.31	1.35	8.79	2.28	_	_
Guigoz	2445.47 ± 24.49	298.35 ± 3.35	65.28± 4.09	39.41 ± 0.78	2.86 ± 0.15	0.56 ± 0.01	Below MDL	Below MDL
%RSD	1.00	1.12	6.27	1.98	5.24	1.79	_	_

MDL: Method Detection Limit

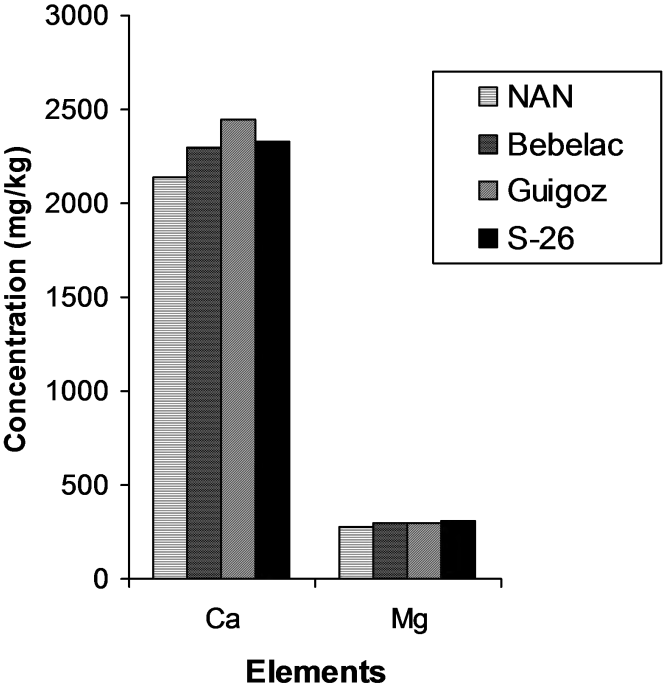

Figure 1. Average concentration of Ca and Mg in powdered infant formulas

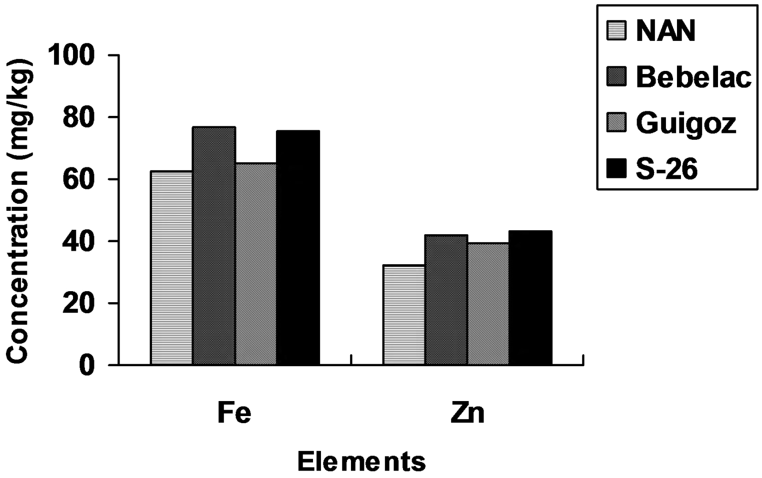

Figure 2. Average concentration of Fe and Zn in powdered infant formulas

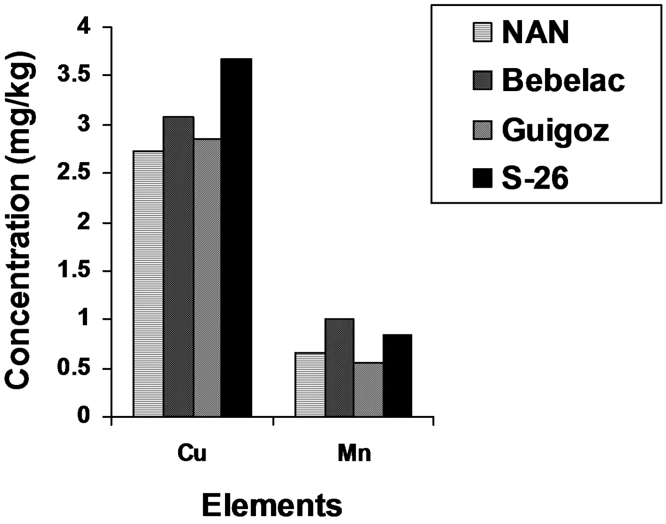

Figure 3. Average concentration of Cu and Mn in powdered infant formulas

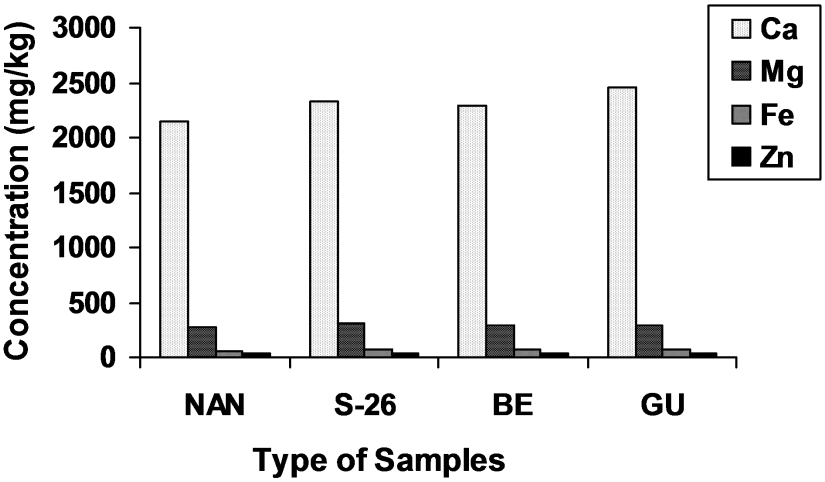

Figure 4. Concentration of metals Ca, Mg, Fe, and Zn within powdered infant formula samples (BE: Bebelac, and GU: Guigoz).

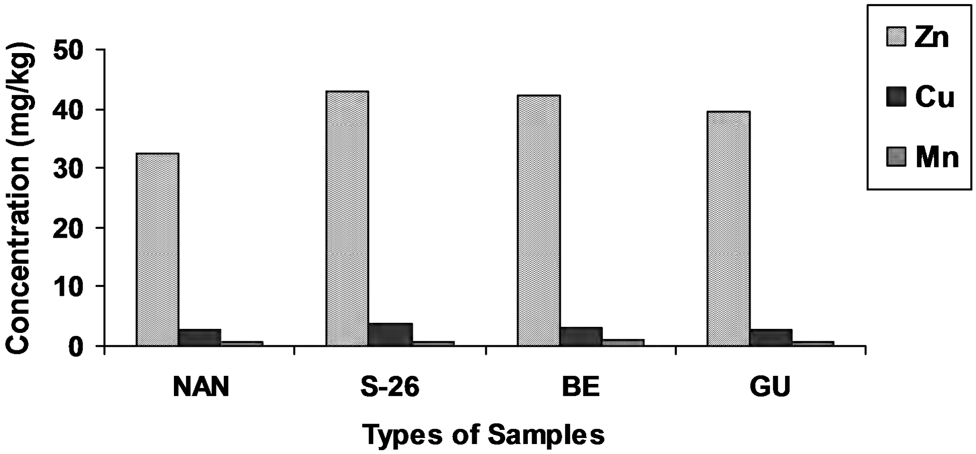

Figure 5. Concentration of metals Zn, Cu and Mn within powdered infant formula samples.

3.5. Comparison of Observed Metals Concentrations with Feeding Tables Supplied by the Infant Formula Manufacturers

Even though all the manufacturers recommended that Brest milk is best for babies, they suggested that preparing artificial feeding which substitutes breast milk is necessary for infants who cannot be fed at the breast, or should not receive breast milk, or for whom breast milk is not available. It is believed that the infant formulas prepared by the manufacturers provide all the known essential nutrients for growth and development of infants. The manufacturer of Bebelac infant formula was reported that the nutrient levels in Bebelac comply with the recommendations of the World Health Organization (WHO). Therefore, the amount of essential nutrients in NAN, Bebelac and Guigoz were given on the feeding table by the manufacturers, but the amount of essential nutrients in S-26 was not indicated on the feeding table. On the other hand the manufacturer recommended that Wyeth Formula S-26, a whey dominant infant formula, is close to human milk in its nutritional values and it meets the Codex Alimentarius standard for infant formula.

The comparison of essential metals in infant formula brands of this work with the feeding tables supplied by infant formula manufacturers is given in table 7. As it can be seen from Table 7, the mean concentrations of most metals in this study are comparable with the mean concentrations of metals given by the manufacturers in all brands, except for Ca and Mg. The concentration of Ca and Mg is lower than the concentration given by the manufacturers in all brands. The concentrations of Fe (76.49) and Zn (42.21) in Bebelac are higher than that of the manufacturers (48, 34) µg/g for Fe and Zn respectively. This difference may be arising from the difference in digestion method and analytical instrument used for analysis.

Table 7. Comparison of essential metals of the present study with the feeding tables supplied by infant formula manufacturers (in µg/g).

Metals	NAN[a]	NAN[b]	Bebelac[a]	Bebelac[b]	Guigoz[a]	Guigoz[b]	S-26[a]
Ca	2138.21	3200	2294.15	3680	2445.47	4250	2331.08
Mg	274.7	360	295.24	370	298.35	330	310.58
Fe	62.72	62	76.49	48	65.28	60	75.7
Zn	32.53	39	42.21	34	39.41	37	42.98
Cu	2.73	3.1	3.07	2.98	2.86	3	3.68
Mn	0.65	0.4	1.01	0.59	0.56	0.43	0.84

[a] stands for concentration of metals of present study.

[b] stands for concentration of metals from infant formula feeding tables.

3.6. Comparison of Observed Metals Concentrations with Literature Values

Determination of essential and toxic metals in infant formulas has received considerable attention in order to save the health of infants. Essential metals may be toxic for infants when taken in excess or small amount, and also some toxic metals found in infant formulas can affect the health of infants even at lower concentration. Therefore, many researchers have reported the concentration of metals in infant formulas. As it can be seen from Table 8, Ca and Mg contents of New Zealand infant formulas have been reported in the range of 3670 - 4920 µg/g and 381 - 451 µg/g, respectively. The concentration of Ca and Mg in present study is below the concentration of Ca and Mg reported in a New Zealand [60]. The concentrations of Fe and Zn in New Zealand infant formulas have been reported in the range of 25.5 – 80.9 µg/g and 13.7 - 42.3 µg/g, respectively. The amount of Fe and Zn of the present work is found in the range of the reported value. The concentration of Cu in the present study is also found within the range of concentration determined in New Zealand's infant formulas (1.94-4.53 µg/g).

The concentration of Mn in New Zealand infant formulas has been reported in the range of 0.73 - 4.04 µg/g. The concentration of Mn in the present work is almost found in the range of the reported value but some lower than the amount reported in New Zealand especially for Guigoz (0.56 µg/g). Iron value in infant formulas of Turkey has been reported in the range of 1.02 - 67.5 µg/g [38]. The concentration of Fe in the present study is almost comparable with the reported value, but some higher than the amount reported in Turkey especially for Bebelac (76.49 µg/g). Zn and Cu values in infant formulas of Turkey have been reported in the range of 21.9 – 29.8 µg/g and 1.48 – 2.63 µg/g, respectively. The amount of Zn and Cu is higher than the concentration reported in Turkey. The concentration of Mn in the present study is within the range (0.31 – 3.29) µg/g of concentration reported in Turkey [38].

Cu and Zn values in baby food of India have been reported in the range of 1.11-3.16 µg/g and 9.37-34.59 µg/g, respectively. The concentrations of Cu and Zn are almost comparable with the values reported in India [7], but some higher than the reported value especially for S-26 (3.68 and 42.98) µg/g for Cu and Zn respectively. The content of Zn in powder Italian infant formulas has been reported in the range of 13-110 µg/g [65]. The concentration of Zn in the present study is found within the range of the reported value in Italy. Iron value in infant formula of Spain has been reported in the range of 41.4 - 97.4 µg/g [66]. The amount of iron in the present study is found within the range of the reported value by Spain. Breastfeeding protects the young infant from iron deficiency. The level of iron in breast milk is relatively low (1.95 µg/g), but its bioavailability is in the range of 50 % to 80 %, probably because of the presence of lactoferrin, which enhances iron absorption. Exclusively breastfed infants have been shown to receive sufficient iron to sustain normal status until at least four to six months of age [1].

Pb and Cd values in baby food of India have been reported in the range of 0.0395 - 0.0777 µg/g and 0.00045 – 0.0177 µg/g, respectively. But the concentrations of Pb and Cd in present study were below the method detection limit.

Table 8. Comparison of metals concentrations of the present study with literature report (in µg/g).

Metals	* Present Study	New Zealand	Turkey	India
Ca	2138.21 - 2445.47	3670 - 4920	_	_
Mg	274.7 – 310.58	381 - 451	_	_
Fe	62.72 – 76.49	25.5 - 80.9	1.02 - 67.5	_
Zn	32.53 – 42.98	13.7 - 42.3	21.9 – 29.8	9.37 – 34.59
Cu	2.73 – 3.68	1.94 - 4.53	1.48- 2.63	1.11 – 3.16
Mn	0.56 – 1.01	0.73 - 4.04	0.31- 3.29	_

* Present Study: Ethiopia

3.7. Daily Intakes of Essential and Toxic metals from Infant Formulas

Estimates of the daily intakes of essential elements in infant formulas of the present study were calculated using the feeding tables specified by the manufacturers of the various brands. As it can be seen from Table 9, Guigoz has the highest daily intakes of Ca (273.28 mg/day). The highest daily intakes of Mg (34.69 mg/day), Fe (8.99 mg/day), Zn (4.96 mg/day), and Mn (0.12 mg/day) are recorded in Bebelac. S-26 has the highest daily intake of Cu (0.38 mg/day). The calculated daily intakes of essential metals from each infant formula brand were compared with the recommended dietary allowances (RDA) and dietary reference intakes (DRI) for use in North America [67]. The RDA or DRI for

0 – 6 month old bottle fed infants are as follows: Iron (6 mg/day), Znic (5 mg/day), Calicium (210 mg/day), and Magnesium (30 mg/day). The average daily intake of Ca in all brands of powdered infant formula is higher than its DRI value. The high amount of calcium intakes may induce adverse effects such as gastrointestinal and renal side effects. The average daily intake of Mg in NAN is almost the same as its DRI value, but for others little higher than the recommended value.

The average daily intake of Fe in all brands is almost comparable with the recommended value except for Bebelac which is higher than its DRI value. Even though, the DRI value of Zn is 5 mg/day, the daily dietary allowance of Zn is from 3 - 5 mg [7]. The calculated average daily intakes of Zn for all brands are found within the range of the recommended values.

The daily dietary allowance of Cu is between 0.5 – 1.0 mg. The calculated average daily intakes of Cu for all brands are below the recommended values. While there is no recommended dietary allowance for Mn, the National Research Council's "estimated safe and adequate daily dietary intake" is 2-5 mg [68]. The Institute of medicine recommends that intake of Mn from food; water and dietary supplements should not exceed the tolerable daily upper limit of 11 mg per day [38]. The calculated average daily intakes of Mn for all brands are below the recommended values. The average daily intakes of Pb and Cd were not calculated on the basis of feeding tables provided by the manufacturers because the concentrations of Pb and Cd were below the method detection limit. The recommended tolerable levels of Pb and Cd for infants and Children are 3.57 µg/kg body weight and 0.8 – 1.0 µg/kg body weight, respectively.

Table 9. Average daily intakes of metals from commercial infant formulas.

Intake (mg metal/day)	Infant formula brands			
	NAN	S-26	Bebelac	Guigoz
Ca	229.86	237.77	269.56	273.28
Mg	29.53	31.68	34.69	33.34
Fe	6.74	7.72	8.99	7.3
Zn	3.50	4.38	4.96	4.4
Cu	0.29	0.38	0.36	0.32
Mn	0.07	0.086	0.12	0.063

4. Conclusions

Infant formulas are a product based on milk of cows or other animals or a mixture of other ingredients which have been proven to be suitable for infant feeding. The nutritional safety and adequacy of infant formula shall be scientifically demonstrated to support growth and development of infants.

In this study commercially available powdered infant formulas (NAN, S-26, Bebelac and Guigoz) were analyzed for their contents of Ca, Mg, Fe, Zn, Cu, Mn, Pb, and Cd. The optimized wet digestion method for analysis was found efficient for the metals and it was evaluated through the recovery experiment and a good percentage recovery was obtained for the metals identified. From the analyzed powdered infant formulas NAN has the lowest content of essential metals than other brands of infant formulas. Pb and Cd were below the method detection limit in all powdered infant formulas. The concentrations of metals for this study were compared with metals concentrations with feeding tables supplied by the infant formula manufacturers. The concentrations of most of the metals determined are almost comparable with the feeding tables supplied by the infant formula manufacturers, except for Ca and Mg which are lower than that of the feeding tables. In addition, the observed results were also compared with the reported literature values analyzed from other parts of the world. The concentrations of most of the metals determined are found to be within the range of literature values, except for Ca and Mg which are lower than the literature values.

The daily intakes of essential elements in infant formulas were calculated using the feeding tables specified by the manufacturers of the various brands and were compared with the recommended dietary allowances (RDA) and dietary reference intakes (DRI) for use in North America. The essential metal contents of most metals were comparable with the recommended value, but some metals slightly lower or higher than the recommended value. Bottle fed infants consuming formulations with low or high levels of essential minerals may suffer nutritional excesses or deficiencies and consequent health problems.

Since infant formulas have a special role to play in the diets of infants, the nutritional safety and adequacy of infant formula should be scientifically demonstrated to support growth and development of infants. The present study will give some information about the mineral contents of imported powdered infant formulas. But to have a complete and general information further research should focus on the composition of infant formulas.

5. References

1. M. J. Munir, H. Shah, N. Shaheen, A. K. Saadia, R. Tariq, S. Manzoor, and M. Saqib. Pre- and post-expiry metal levels in canned dry milk, *Nutrition and Food Science,* **2004,** 34, 65-71.
2. M. Sikiric, and N. Brajenovi. Determination of metals in cow's milk by flame atomic absorption spectrophotometry, J*ournal of animal science, **2003*****,** 48, 481-486.
3. T. Prohaska, G. Kollensperger, M. Krachler, K.D. Winne, G. Stingeder, and L. Moens. Determination of trace elements in human milk by inductively coupled plasma sector field mass spectrometry (ICP-SFMS), *Journal of Analytical Atomic Spectrometry,* **2000**, 15, 335-340.
4. J. G. Dorea. Zinc and Copper in breast-milk and home-prepared milk fed to urban infants from low-income families, *The Journal of Trace Elements in Experimental Medicine,* **2002,** 15, 123–129.
5. N. Carrion, A. Itriago, M. Murillo, E. Eljuri, and A. Fernandez. Determination of calcium, potassium, magnesium, iron, copper and zinc in maternal milk by inductively coupled plasma atomic emission spectrometry, *Journal of Analytical Atomic Spectrometry,* **1994,** 9, 205-207.
6. A. S. Pereyra, G. B. Naranjo, L. S. Malec, and M. S. Vigo. Available lysine, protein digestibility and lactulose in commercial infant formulas, *International Dairy Journal,* **2003**, 13, 95–99.
7. R. M. Tripathi, R. Raghunath, V. N. Sastry, and T. M. Krishnamoorthy. Daily intake of heavy metals by infants through milk and milk products, *The Science of the total Environment,* **1999**, 227, 229-235.
8. D. K. Thompkinson, and S. Kharb. Aspects of infant food formulation, *Comprehensive Reviews in Food Science and Food Safety,* **2007**, 6, 1-12.
9. C. Agostoni, I. Axelsson, O. Goulet, B. Koletzko, K. F. Michaelsen, W. L. Puntis, J. Rigo, R. Shamir, H. Szajewska, D. Turck, Y. Vandenplas, and L. T. Weaver. Preparation and handling of powdered infant formula: A Commentary by the ESPGHAN Committee on Nutrition, ESPGHAN Committee on Nutrition: *Journal of*

Pediatric Gastroenterology and Nutrition, **2004**, 39, 320–322.

10. L. M. Gartner, and A. I. Eidelman. Breastfeeding and the use of human milk, *American Academy of Pediatrics*, **2005**, 115, 496-502.
11. L. Rees. Healthy digestion in infants, *SA Fam Pract,* **2005**, 47, 31-32.
12. Balancing and communicating issues related to environmental contaminants in breast milk position paper and resolution adopted by the Ontario Public Health Association (OPHA). *Ontario Public Health Association position paper and Resolution,* **2004**, 1-16.
13. M. K. Davis. Review of the evidence for an association between infant feeding and childhood cancer, *International journal of cancer,* **1998,** 29–33.
14. A. Oskarssona, I. P. Hallenb, J. Sundbergc, and K. P. Graw´ec. Risk assessment in relation to neonatal metal exposure**,** *Analyst,* **1998***,* 123, 19–23.
15. C. Mai. Draft revised standard for infant formula and formulas for special medical purposes intended for infants**,** *Codex Alimentaries Commission***, 2006**, 13-15.
16. Standard for infant formula and formulas for special medical purposes intended for infants, *Codex Standard*, **1998**, 72, 1-21.
17. C. Agostoni, I. Axelsson, O. Goulet, B. Koletzko, K. F. Michaelsen, J. Puntis, D. Rieu, J. Rigo, R. Shamir, H. Szajewska, and D. Turck. Soy protein infant formulae, and follow-on formulae: A Commentary by the ESPGHAN Committee on Nutrition, *Journal of Pediatric Gastroenterology and Nutrition*, **2006,** 42, 352-361.
18. B. Lönnerda. Copper nutrition during infancy and childhood, *American Journal of Clinical Nutrition*, **1998**, 67, 1046–1053.
19. M. A. Torres, J. Verdoy, A. Alegria, R. Barbera, R. Farreu, and M. J. Lagarda. Selenium contents of human milk and infant formulas in Spain. *The Science of the Total Environment,* **1999**, 228, 185-192.
20. A. Alegria, R. Barbera, R. Farre, M. J. Lagarda, R. M. Lorente, and A. Torres. Effectiveness of microwave based digestion procedures for the demineralization of human milk and infant formulas prior to fluorometric determination of selenium, *Nahrung*, **1996**, 40, 92-95.
21. A. Ikem, A. Nwankwoala, S. Odueyungbo, K. Nyavor, and N. Egiebor. Levels of 26 elements in infant formula from USA, UK, and Nigeria by microwave digestion and

ICP-OES, *Food Chemistry*, **2002**, 77, 439-447.

22. M. Walker. Known contaminants found in infant formula, *Mothering*, **2000**, 100, 67-70.
23. N. Guttman, and D.R. Zimmerman. Low- income mothers views on breastfeeding, *Social Science and Medicine*, **2000**, 50, 1457-1473.
24. P. Gaillard, E. Piwoz, and M. M. Farley. Collection of standardized information on infant feeding in the context of mother-to-child transmission of HIV, *Statistical Medicine,* **2001**, 20, 3525–3537.
25. A. Alioum, F. Dabis, L. Dequae-Merchadou, G. Haverkamp, M. Hudgens, J. Hughes, J. Karon, and V. Leroy. Estimating the efficacy of interventions to prevent mother-to-child transmission of HIV in breast-feeding populations: development of a consensus methodology, *Statistical Medicine,* **2001**, 20, 3539–3556.
26. Committee on nutrition, soy-protein formulas: Recommendations for use in infant feeding, *Pediatrics*, **1983**, 72, 359-363.
27. B. Lloyd, R. J. Halter, M. J. Kuchan, G. E. Baggs, A. S. Ryan, and M.L. Masor. Formula tolerance in post breast fed and exclusively formula-fed infants, *Pediatrics*, **1999**, 103, 1-6.
28. Committee on nutrition, soy protein-based formulas: Recommendations for use in infant feeding, *Pediatrics*, **1998**, 101, 148-153.
29. A. Høst, B. Koletzko, S. Dreborg, A. Muraro, U. Wahn, P. Aggett, J. L. Bresson, O. Hernell, H. Lafeber, K. F. Michaelsen, J. L. Micheli, J. Rigo, L. Weaver, H. Heymans, S. Strobel, and Y. Vandenplas. Dietary products used in infants for treatment and prevention of food allergy, *Archives of Disabled Children,* **1999**, 81, 80–84.
30. M. Salcines, and M. Mountford. In response to the NTP-CERHR expert panel meeting to discuss the draft reports on Genistein and Soy Formula, the International Formula Council (IFC), *Atlanta***, 2006**, 404, 252-254.
31. L. Shun-xing, Z. Feng-ying, L. Xian-liand, and C. Wen-lian. Speciation analysis and the assessment of bioavailability of manganese in phytomedicines by extraction with octanol and determination by flame atomic absorption spectrometry**,** *Phytochemistry Analytical,* **2005**, 16, 405–410.

32. M. Zarkadas, F. W. Scott, J. Salminen, and A. HamPong. FRCPC Common allergenic foods and their labeling in Canada a review, *Canadian Journal of Allergy and Clinical Immunology,* **1999**, *4*, 125-130.
33. L. M. Janas, M. F. Picciano, and T. F. Hatch. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula, *Pediatrics,* **1985,** 75, 775-783.
34. E. Garfield. Epidemiology and Etiology, *Essays of an Information Scientist*, **1985**, 8, 119-131.
35. M. Teresa, V. Mercedesde, F. Jose, and C. Diez-Masa. Analysis of trace amounts of bovine *b*-lacto globulin in infant formulas by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection*, Journal of Sepectroscopic Science,* **2005,** 28, 941–947.
36. J. S. Hyams, W. R. Treem, N. L. Etienne, H. Weinerman, D. MacGilpin, P. Hine, K. Choy, and G. Burke. Effect of infant formula on stool characteristics of young infants, *Pediatrics*, **1995**, 95, 50-54.
37. American Academy of Pediatrics, Committee on nutrition iron fortification of infant formulas, *Pediatrics*, **1999**,104, 119-123.
38. S. Saracoglu, K. O. Saygi, O. D. Uluozlu, M. Tuzen, and M. Soylak. Determination of trace element contents of baby foods from Turkey, *Food Chemistry,* **2007,** 105, 280– 285.
39. N. Yuzbasıy, E. Sezginz, M. Yıldırım, and Z. Yıldırım. Survey of Lead, Cadmium, Iron, copper and zinc in Kasar cheese, *Food Additives and Contaminants,* **2003**, 20, 464–469.
40. H. Gunshin, M. Yoshikawa, T. Doudou, and N. Kato. Trace elements in human milk, cow's milk, and infant formula, *Agricultural and Biological Chemistry,* **1985**, 49, 21-26.
41. F. C. Lima, C. Delerue-Mates, M. Carmo, and V. F. Vaz. Automation of iron and copper determination in milks using FIA systems and colourimetric detection, *Food Chemistry*, **1998**, 62,117-121.
42. Ora Laboratory Manual FDA Office of Regulatory Affairs, Division of Field Science, Section 11*, Nutrient Analysis*, **2005**, 4, 1- 34.

43. Scientific committee on food European Commission report of the scientific committee on food on the revision of essential requirements of infant formulae and follow-on formulae, **2003**, 127-152.
44. A. S. Ryan. Iron-deficiency anemia in infant development: Implications for growth, cognitive development, resistance to infection, and iron supplementation, *Yearbook of Physical Anthropology*, **1997**, 40, 25–62.
45. O. Hernell, and B. Lönnerdal. Iron status of infants fed low-iron formula: no effect of added bovine lactoferrin or nucleotides, *American Journal of Clinical Nutrition*, **2002,** 76, 858-864.
46. S. Tehrani, M. Rastegar, F. Parchehbaf, A. Rezvani, and Zolfaghar. Determination of copper by flame atomic absorption spectrometry after preconcentration with activated carbon impregnated with a new schiff base, *Chinese Journal of Chemistry*, **2005**, 23, 1437—1442.
47. A. Markevièius, and A.Dringelienë. Comparison of lead and copper exposure effect on immune cells in mice, *Acta Medica Lituanica*, **2004**, 11, 14-18.
48. K. A. Bauerly, S. L. Kelleher, and B. Lonnerdal. Effects of copper supplementation on copper absorption, tissue distribution, and copper transporter expression in an infant rat model, *AJP-Gastrointestinal Liver Physiology,* **2005**, 288, 1007–1014.
49. S. A. Tirmizi, M. H. Sarwar, M. Mazhar, F. H. Wattoo, A. N. Memon, and J. Iqbal. Analytical investigation of chromium and zinc in sweet, sour and bitter tasting fruits, vegetables and medicinal plants**,** *Quim. Nova,* **2007**, 30, 1573-1577.
50. N. Amina, S. Kanecoa, T. Suzukib and K. Ohtaa. Electrothermal atomic absorption spectrometric determination of cadmium in Bangladeshi vegetables with a metal tube atomizer and slurry sampling technique, *International Journal of Environmental Analaytical Chemistry*, 2003, 83, 1035–1044.
51. G. Eklund, and A. Oskarsson. Exposure of cadmium from infant formulas and weaning foods, *Food Additives and Contaminants*, **1999**, 16, 509 -519.
52. J. BoÈrjesson, L. Gerhardsson, A. SchuÈtz, R. Perfekt, S. Mattsson, and S. Skerfving. Kidney cadmium as compared to other markers of cadmium exposure in workers at a secondary metal smelter, *American Journal of Industrial Medicine*, **2001**, 39, 19-28.

53. R. Honda, K. Tawara , M. Nishijo , H. Nakagawa , K. Tanebe, and S. Saito. Cadmium exposure and trace elements in human breast milk. *Toxicology*, **2003**,186, 255- 259.

54. S. Tehrani, M. Rastegar, F. Parchehbaf, A. Khatamian, and Masoomeh. Determination of Pb(II) and Cu(II) by electrothermal atomic absorption spectrometry after preconcentration by a schiff base adsorbed on surfactant coated alumina, *Chinese Journal of Chemistry*, **2006**, 24, 765—769.

55. G. M. Scelfo, and A. R. Flegal. Lead in calcium supplements**,** *Environmental Health Perspectives*, **2000**, 108, 309-313.

56. C. Cabrera, Y. Madrid, and C. Camara. Determination of lead in wine, other beverages and fruit slurries by flow injection hydride generation atomic absorption spectrometry with on-line microwave digestion**,** *Journal of Analytical Atomic Spectrometry*, **1994**, 9, 1423-1424.

57. S. Dadfarnia, A. M. Haji, and Z. Dehghani. Immobilized stearic acid as a new sorbent for on-line pre concentration and determination of lead by flow injection flame atomic absorption spectrometry, *Journal of Brazilian Chemical Society,* **2006**, 17, 548-554.

58. M. S. Hosseini, and R. Hassan-Abadi. Flotation separation and flame atomic absorption spectrometric determination of trace amount of lead in some environmental samples**,** *Annali di Chimica*, **2007**, 97, 1269-1278.

59. S. Telisman, A. Pizent, J. Jurasovic, and P. Cvitkovic. Lead effect on blood pressure in moderately lead-exposed male workers, *American Journal of Industrial Medicine,* **2004**, 45,446–454.

60. P.J. McKinstrya, H.E. Indyka, and N.D. Kimb. The determination of major and minor elements in milk and infant formula by slurry nebulisation and inductively coupled plasma optical emission spectrometry (ICP-OES), *Food Chemistry*, **1999**, 65, 245- 252.

61. M. Angel, D. La Fuente, B. Carazo, and M. Jua´Rez. Determination of major minerals in dairy products digested in closed vessels using microwave heating, *Journal of Dairy Science*, **1997**, 80, 806–811.

62. J. Sneddon, C. Hardaway, K. K. Bobbadi, and A. K. Reddy. Sample preparation of solid samples for metal determination by atomic spectroscopy—an overview and selected recent applications*, Applied Spectroscopy Reviews*, **2006**, 41, 1–14.
63. D. J. Butcher, and J. Sneddon. A practical guide to graphite furnace atomic absorption spectrometry, *John Wiley and Sons,* **1998**, 147, 32-184.
64. L. Gazso, and G. Giorgi. Determination of trace metal pollution of various environmental samples using atomic absorption spectrometric methods, **2001**, 13-29.
65. P. M. Bertelli, and D. A. Monzani. Determination of aluminum and zinc in infant formulas and infant foods, *Journal of Food Composition and Analysis*, **1997**, 10, 36-42.
66. M. C. Yebra, A. Moreno-Cid, R. Cespon, and S. Cancela. Preparation of a soluble solid sample by a continuous ultrasound assisted dissolution system for the flow-injection atomic absorption spectrometric determination of iron in milk and infant formula, *Talanta*, **2004**, 62, 403-406.
67. National Academy of Sciences. Committee on dietary reference intakes, Dietary Reference intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride, Washington DC, *National Acadamy Press*, **1997**.
68. National Research Council. Recommended Dietary Allowances (10th ed.), Washington DC, *National Acadamy Press,* **1989**.