ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND
COMPUTATIONAL SCINCES
DEPARTMENT OF CHEMISTRY

STUDIES ON THE DETERMINATION OF FLUORIDE CONTENT IN LEAFY VEGETABLES CULTIVATED IN RIFT VALLEY AND NON RIFT VALLEY AREAS OF ETHIOPIA

BY
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DECLARATION

I, the undersigned, declare that this thesis is my original work and has been submitted in partial fulfillment of the requirements for the degree of masters of Science at Addis Ababa University. All sources of materials used for this thesis have been duly acknowledged. This paper has never been submitted to and/or presented in any other university, college or institution in candidature of any other degree, diploma, or certificate.

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Dedication

This research work dedicated to my mother Sewunet Smuneh Dejenie for her tiredness and bloody path on behalf of me to uplift me on this stage and my father Abeble Dagnaw Desta, my sisters, and brothers, to all my family and friends who moves ups and downs because of me.

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List of abbreviations and acronyms

CSA	Central Statistical Agency
EIA	Ethiopian Investment Agency
EPA	Environmental Protection Agency
WHO	World Health Organization
FAO	Food and Agricultural Organization
EFSA	European Food Safety Authority
MCLG	Maximum Contaminant Level Goal
USA	United States of America
UK	United Kingdom
AAU	Addis Ababa University
EDTA	Ethylene Diamine Tetra Acetic acid
TISAB	Total Ionic Strength Adjustment Buffer
ISE	Ion Selective Electrode
LOD	Limit of Detection
LOQ	Limit of Quantification
UNODC	United Nation Office of Drug and Crime
RSD	Relative Standard Deviation
SD	Standard Deviation
CRM	Certified Reference Material
SRM	Standard Reference Material
Fw	Fresh weight of vegetables
Dw	Dry weight of vegetables
ANOVA	Analysis of Variance
Df	Degree of freedom
$F_{cal.}$	F calculated
$F_{crit.}$	F critical
mV	milivolt
SPSS	Statistical Program for Social Sciences
SNNPR	Southern Nations Nationalities and Peoples Region

Abstract

Fluorosis is considered as a public health problem in different parts of the world including Ethiopia via exposure of fluoride from drinking water, alcohol and beverage, food stuff and other sources. In Ethiopia, this problem is mainly observed in Rift Valley Region. Many researches have been conducted on water bodies, cereals and beverages. However, the fluoride contents in leafy vegetable have not been studied so far in the country. The main objective of this study was to determine the fluoride content in selected leafy vegetables lettuce, Swiss chard, cabbage and Abyssinian cabbage cultivated in Rift Valley and non Rift Valley area of Ethiopia. Fluoride contents of water, soil and leafy vegetables were determined using alkali fusion – ISE technique from six irrigation farms in the study area. The fluoride contents (mg/kg, dry wt.) of leafy vegetable ranges were: lettuce 2.95−5.76; Swiss chard 2.74−5.40; cabbage 2.12−2.70 and Abyssinian cabbage 2.08−2.59. The fluoride levels in irrigation water bodies (mg/L) ranges: 0.43−7.66 in the study areas. The soluble and total fluoride levels in the studied farmland soil (mg/kg) ranges were: 4.30−23.4 and 133−802 in the sample sites, respectively. Higher fluoride levels were observed in the vegetables cultivated in Rift Valley farms compared to non Rift valley farms. Similarly higher fluoride levels were also observed in the irrigation water and the farmland soil in the Rift Valley area. The statistical analysis, ANOVA shows that the significant difference between mean fluoride contents of vegetables at ($p < 0.05$) confidence level. The Pearson correlation shows variable (weak, moderate or strong) relationship between the fluoride levels in water and vegetables and between fluoride levels in soil and vegetables. The fluoride contents in leafy vegetable were comparable with values reported in literature. In general, leafy vegetables contribute significant amount to total intake of fluoride to those who regularly consumes these vegetables.

Key words: Fluoride, Leafy vegetables, Soil, Water, Rift Valley, Ethiopia.

1. INTRODUCTION

1.1. Background

Ethiopia has diverse climate and altitude conditions which are conducive to various agricultural activities. There are several lakes and perennial rivers that have great potentials for agriculture irrigation and about 2.6 billion cubic meters ground water potential. The potentially land area for irrigation is estimated at 10 million hectares, out of which only about 1% is currently under irrigation (Ethiopian Investment Agency, 2012).

Most of the soil type in the country ranges from light clay to loam, favorable weather, altitude, adequate water and availability of suitable soils, the potential to develop horticultural crops, such as fruits, vegetables, root crops and diversified agro-ecology are well suited for horticultural production in the country (Ethiopian investment agency, 2012; Amsalu *et al*., 2014).

As a result these, various types of vegetable crops are grown in Ethiopia under rain-fed and/or irrigation systems (Alemayehu *et al.*, 2010). The major economically important vegetables include hot and sweet peppers (*Capsicum spp.*), Ethiopian mustard/cabbage (*Brassica carinata*), onion (*Allium cepa*), tomato (*Solanum lycopersicum*), chili (*C. chinense*), carrot (*Daucus carota*), garlic (*A. sativum*) and cabbage (*B. oleracea* var. c*apitata*).

Vegetables are important ingredient of human diet since they contain carbohydrates, proteins, as well as vitamins, minerals, trace elements and fibers, and also have beneficial anti oxidative effects. In the past, they were not considered as a major part of the Ethiopian diet, except during the fasting period. Recently, however, the consumption of vegetables increases in urban area as a result of exposure of different culture and awareness of the food value of vegetables (Asfaw *et al*, 2013).

Vegetable production in Ethiopia ranges from home gardening, smallholder farming to commercial farms owned both by public and private enterprises which is important for food security, for human nutrition and health (source of vitamins, minerals, antioxidants, dietary fiber

and for having anti-carcinogenic properties), raw material for agro-industries, a source of employment as it needs intensive labor and foreign currency. Vegetables serve as suitable crops for farming systems diversification and land intensification (maintaining ecological balance as the diverse species), particularly with recent increases in the establishment of small and medium scale irrigation schemes in the country with diverse agro-ecologies suitable for the production of vegetables in Tropical, sub-tropical and temperate climatic zones (Amsalu *et al*., 2014).

Smallholder irrigation vegetable production in the Central Rift Valley region of Ethiopia is instrumental in ensuring in the year round availability of fresh vegetables in the local market in the country (Etissa *et al*., 2014).

In Ethiopia, Vegetables took up (160,050 ha) about 1.18% of the area under all crops at national level and (7,557,282 quintal) of lettuce, head cabbage, Ethiopian cabbage, tomatoes, green peppers, red peppers and Swiss chard vegetable production. However, of the total estimated area under vegetables, the lion share which is about 67.98% and 19.86% was under red peppers and Ethiopian cabbage, respectively. As to production of vegetables contribute 2.78% to all crops production total, conversely, of the total production of vegetables, the same crops have the lion share, i.e. about 31.69% and 42.76%, in that order (CSA, 2012). These vegetable production shows geometrical increase compared with the production of vegetables and fruits and production area covered in double reported in 2010/2011(EIA, 2012).

The excessive intake of fluoride has adverse effect for human health resulting dental, skeletal and non skeletal fluorosis, chronic and endemic disease in the rift valley of Ethiopia. The food chain has significant contribution to the total fluoride exposure of animals and human. Literature survey revealed that the level of fluoride in leafy vegetables in Ethiopia has not been determined so far. Hence it is worthwhile to determine the fluoride content of common leafy vegetables cultivated and consumed in Ethiopia.

1.2. Objective

1.2.1. General objective

The main objective of this study was to determine the fluoride content in selected leafy vegetables lettuce, Swiss chard, cabbage and Abyssinian cabbage cultivated in rift valley and non rift valley area of Ethiopia.

1.2.2. Specific objectives

- To determine the fluoride contents of irrigation water, farmland soil and selected leafy vegetables cultivated in Ethiopia.
- To compare the fluoride content of selected leafy vegetables in the study area.
- To compare fluoride content of leafy vegetables cultivated in Ethiopia with values in the literature.
- To correlate the levels of fluoride in the vegetable, irrigation water, and farmland soil.

1.3. Significant of the study

There is no study conducted on the fluoride content of leafy vegetables in Ethiopia. The results of this study will provide useful baseline data on the levels of fluoride in selected leafy vegetables for further studies and quantification of vegetable fluoride contribution for estimation of total fluoride intake by an individual.

2. LITERATURE REVIEW

2.1. Selected leafy vegetables in this study

2.1.1. Lettuce

Lettuce belongs to the Asteraceae family (formally Compositae). It is a self-pollinating annual vegetable which produces a dense rosette of leaves early in the season, followed by flower stalk initiation whereby the central cylindrical stem elongates and indeterminate flowering may last for up two months.

Lettuce originated in a region occupying parts of Iran and Turkey and is likely a descendent of a wild lettuce (*Lactuca serricola*). Lettuce is the most important salad vegetables. Today for organic producers, lettuce represents one of the most common and highest grossing products for fresh, local markets and have different classes of lettuce, distinguished by their morphologies and end use (Jared, 2010).

2.1.2. Swiss chard

Swiss chard has been cultivated since 300 B.C. and roots of the wild chard were used as medicine. The wild form is found in the Canary Islands, Mediterranean region, and East to Southern Asia. The first records of Swiss chard cultivation suggest the Mediterranean area, perhaps Italy as the centre of origin.

Swiss chard is basically a beet grown for its tops. It is probably the ancestor of our common beet. Swiss chard does well as a transplant and should be on a 12 inch spacing if frequently cropped or on 18 inches if you want it to spread more. It is available in red, pink, yellow or green leaf forms and is one of our winter garden staples. The green leaf forms tend to be hardier. Slender petioled varieties hold up better in severe cold than do the broad ones. Many older varieties have a vaguely soapy flavor, but the varieties Swiss chard of Geneva, French Swiss chard, Perpetual and Dorat are quite mild as are most of the red varieties when young. An exciting newer mix is

Bright Lights. The heirloom of it is Rainbow Chard. Its flavor is good, but not exceptional. Older leaves are always stronger flavored. Swiss chard stands summer heat quite well if adequately watered. It can be harvested by pulling outer leaves as needed or by cutting it off just above the crown and letting it resprout? The latter method usually gives more tender leaves and less stem. This can be a very ornamental plant and a good source of winter greens in mild climates (Patricia, 2013).

2.1.3. Cabbage

Cabbage has been cultivated at least 2,000 years. The cabbage started as a loose head in the Middle East, but moved to Europe and Asia thousands of years ago. It developed its firm head only in the 16th century. When starting, try to keep the temperature at 60 degrees or less to avoid leggings. For fall and winter harvest, plant 2-3 months before the first frost. It can be intensively planted at 12 x 12 inches for small-head types to 18 x 18 inches for large types. Ornamental cabbages look like giant roses. When the head is firm as a softball, you can harvest. Cut the head carefully, leaving the bottom leaves and extra bonus heads will grow. If the cabbage is ready for harvest, but you are not, twist it enough to crack the roots and it will store in the garden without splitting for quite a while. It can be stored in a root cellar environment for months if harvested with the roots attached. Red cabbages seem to be more insect and disease resistant. They are also very winter hardy. Over watering may increase club root. Cabbage is a heavy user of P, K and S. Sometimes this whole family requires extra boron (Patricia, 2013).

Cabbages have low carbohydrates, fats, calories. Good source of protein (balanced), minerals, vitamin A, vitamin C, other vitamins and known anticancer properties includes antioxidants, ascorbic acid, tocopherols, carotenoids, isothiocyanates, indoles, flavanoids.

2.1.4. Abyssinian cabbage

Abyssinian cabbage belongs to Brassicaceae family and a fairly hardy, flavoursome, nutritious greens type originating from Ethiopian Mustard. Fast growing and popular for salad leaves if cut young and tender. Texsel is particularly adapted to temperate climates.

Cultivated for its edible leaves in some areas, plants that are given some protection from the cold can supply edible leaves all through the winter.

Edible uses

Leaves and young stems are used raw or cooked. Used when up to 30 cm tall. A mild and pleasant cabbage flavour, the young growth can be cut finely and used in mixed salads, whilst older leaves are cooked like cabbage leaves.

Immature flowering stems - cooked. Used like broccoli, they make a nice vegetable. Edible oil is obtained from the seed. Oil from the wild species is high in erucic acid, which is toxic, though there are some cultivars that contain very little erucic acid and can be used as food (http://practicalplants.org/wiki/Brassica_carinata).

Cabbage Abyssinian cabbage Swiss chard Lettuce

Figure 1: leafy vegetable pictures sampled for the study and cultivated in different part of Ethiopia.

Table 1: different naming system of selected leafy vegetables in the study

Scientific name	English name	Amharic name
Lactuca sativa	Lettuce	*Selata*
Beta vulgaris var. cicla	Swiss chard	*Kosta*
Brassica oleracea var. capitata	Cabbage	*Tikile gomen*
Brassica carinata	Abyssinian cabbage	*Abesha gomen*

2.2. Fluoride

Fluorine is a pale yellow gas, has a strong and characteristic odor that can be detected in very small amounts, as low as 20 parts per billion. This property is very helpful to those who work with fluorine. It means that the gas can be detected and avoided in case it leaks into a room.

2.2.1. Sources and occurrences of fluoride in the environment

Fluoride is the thirteen most abundant and the most reactive elements that exist in different form of complex compounds on the earth's crust, as a result of strong affinity for calcium and other metals. It combines easily with every other element except helium, neon, and argon. It reacts with most compounds, often violently. It must be handled with extreme care in the laboratory.

Fluorine never occurs as a free element in nature. The most common fluorine minerals are fluorspar, fluorapatite, and cryolite. Apatite is a complex mineral containing primarily calcium, phosphorus, and oxygen, usually with fluorine. Cryolite is also known as Greenland spar. (The island of Greenland is the only commercial source of this mineral.) It consists primarily of sodium aluminum fluoride (Na_3ALF_6).

2.2.2. Exposure of human to fluoride

The major sources of internal exposure of human beings to fluorides are the diet (food, water, beverages) and fluoride-containing dental products (toothpaste, fluoride supplements). Internal

exposure to fluorides can also occur from inhalation (cigarette smoke, industrial emissions), dermal absorption (from chemicals or pharmaceuticals), ingestion or parental administration of fluoride-containing drugs, and children also exposed through ingestion of fluoride-containing soil (EPA, 2006).

2.2.2.1. Water

The major dietary source of fluoride for most people from drinking water, including water consumed directly, food and beverages prepared at home or in restaurants from drinking water, and commercial beverages and processed foods (EPA, 2006); in Ethiopia from hot spring, lakes, shallow wells and boreholes reported as higher source and lower source in springs and rivers (Kloos and Tekle-Haimanot, 1999). Fluoride exposure of human beings and animals mainly depend on the water quality and fluoride concentration in water depends on several contributing factors such as pH, total dissolved solids, alkalinity and hardness (Viswanathan *et al.*, 2009).

Lithology and soil are the main factors that control the quality of water. The amount of fluoride occurring naturally in groundwater is governed principally by climate, composition of the host rock and hydrogeology and some anthropogenic activities such as use of phosphate fertilizers, pesticides, sewage and sludge for agriculture, depletion of groundwater, etc (Pal *et al.*, 2012). And the others, when fluorides added intentionally to the drinking water body as supplements. The prescribed norm for fluoride limit in drinking water is 0.8 - 1.5 mg/L (EPA, 2006).

2.2.2.2. Food and beverages

The availability of fluoride for consumption is not only from drinking water but also from other sources such as diet (dairy products like milk, fruits and vegetables, beverages like tea and coffee and soil and crops like wheat, rice, and potato) (Arora and Bhateja, 2014). Intake of fluoride ion into roots is largely dependent on the concentration of fluoride ion in the soil, the type of soil, soil pH. F^- is taken up by plants passively through a process which is dominantly diffusion controlled. At neutral and acidic pH, F^- is usually bound to soil surfaces and not available to plants. In an acidic environment (pH < 6) both F^- and Al^{3+} can leach into water and form $(AlF)^{2+}$

and $(AlF_2)^+$ complexes (Hem, 1989; Wenzel and Blum, 1992; Haidouti, 1995; Neal, 1995); whereas under alkaline conditions, F^- exists predominantly as the free F ion, which is available for passive uptake by the plant (Barrow and Ellis, 1986; Wenzel and Blum, 1992) (Battaleb-Looie et al, 2013). Fluoride is more soluble in acid soils due to which its uptake by plants is enhanced and mainly accumulated to the leaf. Most foods whether derived from plant or animal life, contain fluoride ion at least in minute amounts. The excess accumulation of fluorides in vegetation leads to visible leaf injury, damage to fruits, changes in the yield (Yadav *et al.*, 2012). The fluoride levels of food specifically vegetables depend upon the nature of soil and quality of irrigation water and varies from place to place (Saxena and Sewak, 2015).

Many water sources in Ethiopia mainly in Rift Valley contain F^- at elevated concentrations of up to 33 mg/L. All foodstuffs contain at least some traces of F^-. The dietary intakes of F^-, in water, beverages, and foods, vary widely according to the various sources of exposure, i.e. soil, water, atmospheric pollution, dietary (vegetables, grains and other staples, salt, drinks (tea)) (Zerabruk *et al.*, 2010; Atlabachew *et al.*, 2011; Tegegne *et al.*, 2013; Gizaw *et al.*, 2014; Embiale *et al.*, 2014; Asayehegn *et al.*, 2014; Mustofa *et al.*; 2014; Syume and Chandravanshi, 2015; Nigus and Chandravanshi, 2016), leads developmental dental and skeletal fluorosis.

Vegetables and fruits normally contain fluoride at low concentration (0.1-0.4 mg/kg) and particularly, fluoride concentration in spinach has reported, i.e. 25.70 µg/g (Gautam *et al.*, 2010), 29.15 µg/g (Bhargava and Bhardwaj, 2009) and *(*Saxena and Sewak, 2015), in cabbage 2-20 mg/kg (EFSA, 2010). The recommended daily fluoride intake (3 mg/day for 60 kg body weight) of food (EFSA and ANAD, 2013).

2.2.2.3. Air

Fluoride (either as hydrogen fluoride, particulate fluorides, or fluorine gas) is released to the atmosphere by natural sources such as volcanoes and by a number of anthropogenic sources. In North America, anthropogenic sources of airborne fluoride include coal combustion by electrical utilities and other entities, aluminum production plants, phosphate fertilizer plants, chemical production facilities, steel mills, magnesium plants, and manufacturers of brick and structural

clay. Estimated airborne releases of hydrogen fluoride in the United States in 2001 were 67.4 million pounds (30.6 million kg; TRI, 2003), of which at least 80% was attributed to electrical utilities (ATSDR, 2003). Airborne releases of fluorine gas totaled about 9,000 pounds or 4,100 kg (TRI, 2003). Anthropogenic hydrogen fluoride emissions in Canada in the mid-1990s were estimated at 5,400 metric tons (5.4 million kg or 11.9 million pounds), of which 75% was attributed to primary aluminum producers (CEPA, 1996; EPA, 2006).

2.2.3. Fluoride metabolism

Fluoride mostly enters the body via the gastrointestinal tract and is absorbed quickly in the stomach without the need of specialized enzymatic systems from different sources. It crosses epithelia in the form of undissociated acid (hydrogen fluoride) which is dominant at low pH (<3.5) and at higher pH the ionized form dominates (Whitford GM, 1994 and 1996).

The rate of fluoride absorption from the stomach is depends on the acidity of its contents. However, several other factors influence the rate of absorption, including the solubility of the ingested fluoride compound. More soluble compounds such as sodium fluoride (NaF) and hydrogen fluoride would result in faster absorptions, whereas less soluble fluoride compounds, such as calcium fluoride (CaF_2) and magnesium fluoride (MgF_2) would slow absorption (Ekstrand and Ehrnebo, 1983).

Once fluoride reaches plasma, it is rapidly deposited in the calcified skeleton (dental and bone) or excreted via the kidneys. Fluoride skeletal uptake is also modified by factors such as the activity of bone modeling and remodeling and age. The degree of fluoride retained in the bone depends on age and pH. The fluoride that is not stored in bone is excreted mainly via the kidneys, with a minimal quantity excreted through feces. Both urinary flow and pH are involved in regulating renal clearance of fluoride from plasma. The proportion of ingested fluoride excreted through urine, like absorption depends on pH and other factors. Diets with a high proportion of vegetable and fruit intake lead to urinary pH on the alkaline side, whereas protein-rich diets lead to acidification of urine (Martı´nez-Mier, 2011).

Once fluorides are absorbed to plasma, some amounts of fluorides are excreted via the kidneys with urine, whereas 90% of fluoride rapidly associated with calcified tissues (bone and teeth) due to the combination of fluorides with calcium ions of teeth and bone and form calcium fluorophosphates (fluorapatite) crystal. This results stiffness of tissues and joints and finally leads to fluorosis in later stage (Tewari and Dubey, 2009).

2.2.4. Fluoride health risk

Fluoride is a common element in the earth's crust and is highly soluble in water. Major fluoride exposure comes from drinking water and minor amounts from food staffs and water based beverages and contribute a significant fluoride concentration for total dietary intake (Liu *et al*., 2014). Fluoride exposure from different sources has both acute and chronic effects for human beings.

In the past some year's different scholars suggest that fluoride has both beneficial and detrimental effects on human health. Particularly, for the formation and development of dental and skeletal parts of human body (WHO, 2004 and 2006; Meenakshi and Maheshwari, 2006; Viswanathan *et al*, 2009; Cordeiro *et al*, 2012; Battaleb-Looie *et al*, 2013;). On the other hand, this scenario was changed, the exposure of fluoride totally have health risks for human beings (SCHER, 2011).

Fluoride beyond desirable amount (0.5-1.5 mg/L) in ground water is a major problem in many parts of the world (Yadav *et al.*, 2012). This elevated intake of fluoride leads to dental, skeletal and non skeletal fluorosis which is a worldwide problem. In addition, exposure to excessive fluoride can also cause metabolic, structural and functional damages of organs like kidney, liver, nerve system, endocrine gland and reproductive system (Liu *et al*., 2014).

The public health problem of fluorosis is prevalent in the high water F^- levels found in the Rift Valley region of Ethiopia, which is characterized by relatively high volcanic activity (Kloos and Tekle-Haimanot, 1990, 1999). Particularly, drinking water has been considered the main reason for the development of fluorosis, but dietary intake may also be a contributor in areas with high

concentration of fluoride in water, soil, and biota (Dessalegne and Zewge, 2013). The onset of fluorosis and the severity of symptoms are governed by chronic fluoride ingestion, the most important being quantity of fluoride ingested and duration of exposure, 70% from drinking water and 30% from other food source. Fluoride and calcium interact in a negative manner and a study has also indicated that calcium intake through diet decreases the retention of fluoride in bones. The percentage of calcium in vegetables, cereals and fruits varies considerably, while milk is known to be the richest source of calcium, therefore these food items considerable decreases the effect of fluorosis (Bhargava and Bhardwaj, 2009).

Fluoride that is located in soils absorbed, translocated and accumulated in plants. The accumulation of fluoride in the leaf occurs through absorption from soil and adsorption from the air. The amount of uptake by plants depends upon the type of plant and the type of soil and the amount and type of fluoride found in the soil. With plants that are sensitive for fluoride exposure even low concentrations of fluoride can cause leave damage and a decline in growth (Gautam *et al*, 2010).

Animals including human being that eat fluoride-containing plants accumulate large amounts of fluoride in their bodies. Fluoride primarily accumulates in bones. Consequently, animals that are exposed to high concentrations of fluoride suffer from dental decay and bone degradation, i.e. fluorosis (EFSA, 2010).

The formation of fluoroaptite from hydroxyapitite causes the change in structural and functional characteristics of calcified tissue mainly teeth and bones. When the teeth and bones exposed with elevated amount of fluoride for a long time, fluorosis risk observed due to the following chemical reactions (Kanyora *et al.*, 2014).
Fluoroaptite forms according to the reaction:

$$Ca_{10}(PO_4)_{6(}OH)2 + 2F^- \rightarrow Ca_{10}(PO_4)_6F_2 + 2OH^- \quad (1)$$

Or hydroxyfluorapatite according to the reaction:

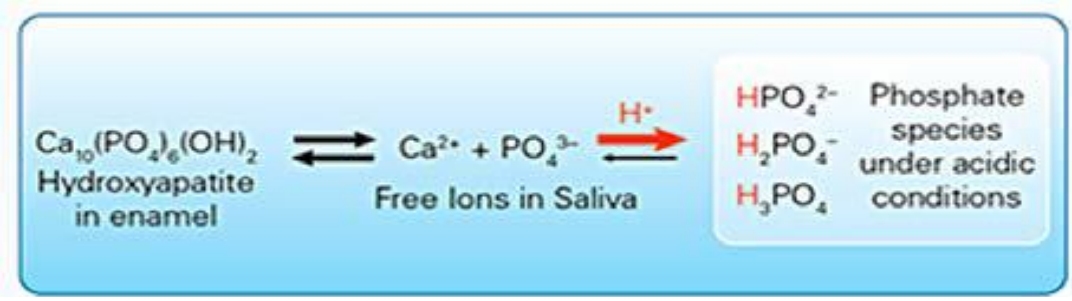
$$Ca_{10}(PO_4)_6(OH)_2 + F^- \rightarrow Ca_{10}(PO_4)_6FOH + OH^- \quad (2)$$

2.2.4.1. Dental fluorosis

Many of the studies, from various parts of the world reported the development of dental fluorosis. Even if the people consume drinking water with fluoride less than 1.0 mg/L, which implies that the optimal fluoride dose level in drinking water may vary with various features like local climatic conditions, methods of food processing and cooking, amount of food and water intake and its fluoride and other nutrients level in dietary habits of the community (Viswanathan *et al.*, 2009).

Enamel fluorosis is a dose-related mottling of enamel that can range from mild discoloration of the tooth surface to severe staining and pitting. The condition is permanent after it develops in children during tooth formation, a period ranging from birth until about the age of 8. Severe enamel fluorosis results in tooth loss; loss of tooth function; or psychological, behavioral, or social problems. Severe enamel fluorosis is characterized by dark yellow to brown staining and discrete and confluent pitting, which constitutes enamel loss (EPA, 2006).

Dental caries is an infectious disease caused by the complex interaction of cariogenic (caries-causing) bacteria with carbohydrates (i.e., sugars) on the tooth surface over time. Cariogenic bacteria metabolize carbohydrates for energy, and produce organic acids as by products. The acids lower the pH in the plaque biofilm. The hydroxyapatite of tooth enamel is primarily composed of phosphate ions (PO_4^{3-}) and calcium ions (Ca^{2+}). Under normal conditions, there is a stable equilibrium between the calcium and phosphate ions in saliva and the crystalline hydroxyapatite that comprises 96% of tooth enamel. When the pH drops below a critical level (5.5 for enamel, and 6.2 for dentin), it causes the dissolution of tooth mineral (hydroxyapatite) in a process called demineralization. When the pH is elevated by the natural buffer capacity of saliva, mineral gets reincorporated into the tooth through the process of remineralization (Fejerskov, 1997; Silverstone, 1980; Featherstone, 2004). This is shown in Scheme 1.



Scheme 1. Demineralization/remineralization cycles.

Fluoride ions (F^-) replace hydroxyl groups (OH^-) in the formation of the apatite crystal lattice. In fact, the presence of fluoride increases the rate of remineralization (Curly, 2009).

Fluorapatite is inherently less soluble than hydroxyapatite, even under acidic conditions.When hydroxyapatite dissolves under cariogenic (Koenigs *et al*., 2013).

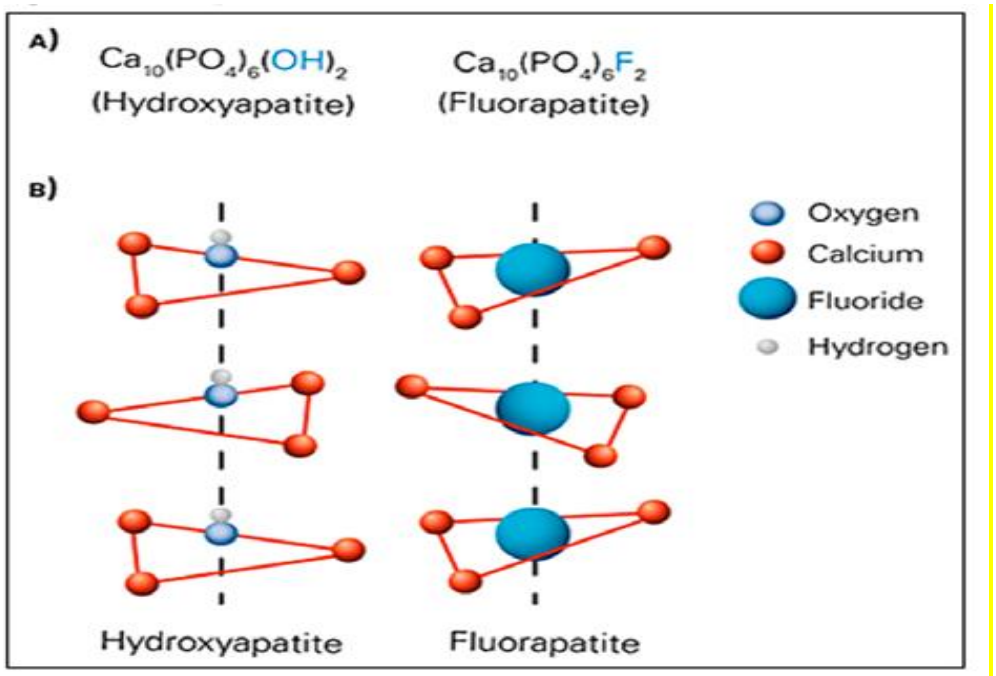


Figure 2: Fluorapatite Formation. (A) Fluoride ions (F^-) replace hydroxyl groups (OH^-) in hydroxyapatite to form fluorapatite in the tooth enamel. (B) A portion of the apatite crystal lattice is depicted showing the replacement of hydroxide for fluoride (Posner, 1985).

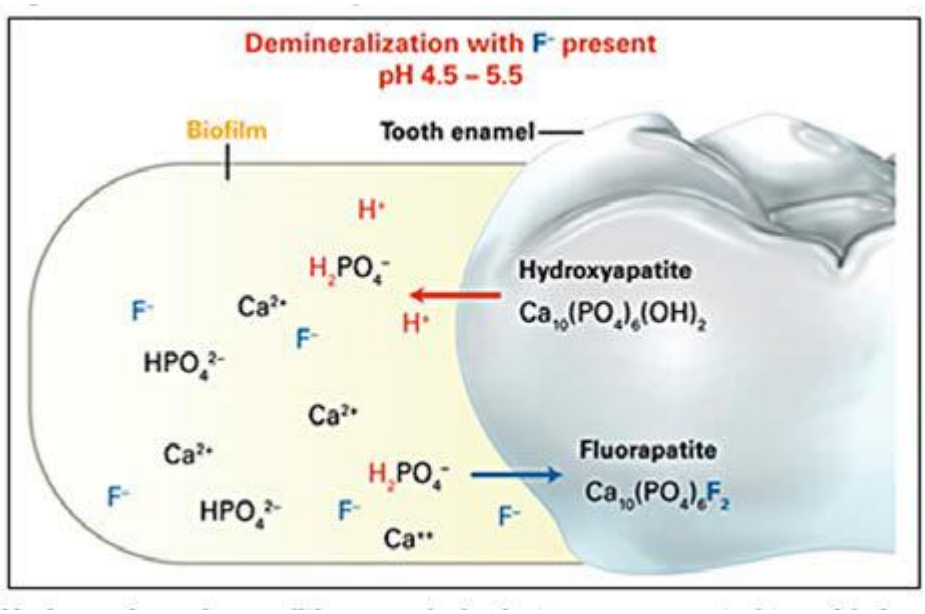


Figure 3: Fluoride reactivity. Under cariogenic conditions, carbohydrates are converted to acids by bacteria in the plaque biofilm. When the pH drops below 5.5, the biofilm fluid becomes undersaturated with phosphate ion and enamel dissolves to restore balance. When fluoride (F^-) is present, fluorapatite is incorporated into demineralized enamel and subsequent demineralization is inhibited (Clarkson, 1993 and Cury, 2009).

Figure 4: Picture showing dental fluorosis.

2.2.4.2. Skeletal fluorosis

Skeletal fluorosis is a bone and joint condition associated with prolonged exposure to high concentrations of fluoride. Fluoride increases bone density and appears to exacerbate the growth of osteophytes present in the bone and joints, resulting in joint stiffness and pain. The condition is categorized into one of four stages: a preclinical stage and three clinical stages that increase in severity. The most severe stage (clinical stage III) historically has been referred to as the "crippling" stage. At stage II, mobility is not significantly affected, but it is characterized by chronic joint pain, arthritic symptoms, slight calcification of ligaments, and osteosclerosis of the cancellous bones. EPA's MCLG of 4 mg/L protects against these precursors to more serious mobility problems (EPA, 2006).

Figure 5: Picture showing skeletal fluorosis.

3. EXPERIMENTAL

3.1. The study area

Ethiopia is one of the East African countries which are bordered with Sudan in the Western, Kenya in the Southern, Djibouti and Somalia in the Eastern and Eritrea in the Northern Africa. The great African Rift Valley extends from Syria and Jordan in the Middle East to Mozambique. It is associated with high fluoride levels in groundwater. The Ethiopian part of the African Rift Valley bisects the country in a south western direction. This seismically active area contains active volcanoes in the Danakil Depression in the North and near-surface, young volcanic rocks in the middle and Southern parts of the Rift Valley. The Rift Valley lies mostly in one of the three major climatic zones of Ethiopia, the hot, arid lowland zone (locally known as kolla), below 1500 m. The two other zones, the temperate zone (woyna dega) (1500–2400 m) and the cool, humid highland zone (dega) (above 2400 m) cover most of the remaining area of Ethiopia (Tekle-Haimanot *et al*., 2006).

The selection of the study area in the Rift Valley were based upon observation of the severity of dental fluorosis through literature review of related works, accessibility for sampling, availability of selected leafy vegetables during sampling, common part of the diet for the community and fluoride level in the water body. The non-Rift Valley areas have the same selection criteria, except selecting areas on which no dental fluorosis observed in the community.

A total of six areas were selected for the study. These areas were Hawassa (Hawassa Zuria Woreda), Ziway (Dugda Woreda) and Wonji Shoa (Adama Woreda) in the Rift Valley and other three areas Akaki, Kera and Pecock Park for non Rift Valley areas were selected from SNNPR, Oromia Regional State and Addis Ababa Administration, respectively.

3.2. Sample collection

The selected leafy vegetable samples (lettuce, Swiss chard, cabbage, Abyssinian cabbage), soil and irrigation water samples were collected using random sampling technique from three sub

sites nearly 2-3 km apart for each study area and thoroughly mixed for each sample type and size.

The leaves of selected vegetable types collected from five matured vegetables randomly from the center and four corners for each sub site and thoroughly mixed for each vegetable type and for each study area independently. The samples were collected with polyethylene bag and transported to the laboratory for further preparations and treatments of a total of 24 vegetable samples from the six sampling sites (Chitambar, 2003).

Six composite farmland soil samples were also collected from surface soil where leafy vegetables samples grown and sampled. Five spots were collected from the center and four corners for each sub site and thoroughly mixed on plastic tray. These spots were taken from the root zone, i.e. 15-20 cm depth in a random fashion with recommended auger. Quartering method was used to get homogeneous and representative 1 kg soil samples. The soil samples were stored in the polyethylene plastic bag and transported to the laboratory for preparations (USDA – NRCS, 2007 and OSU, 2012).

A total of six irrigation water samples were collected from the main water body, i.e. rivers and lakes from the respective study area: Hawassa and Ziway lakes, Awash, Akaki, Kera and Bulbula rivers used for irrigation of leafy vegetables at the point of diversion in the study area in the pre-cleaned and labeled 500 mL polyethylene bottle and transported to the laboratory for further treatment and preparation.

Table 2: Geographical location of the sample areas.

Sample area	Longitude	Latitude	Altitude in m	Distance from Addis Ababa in km
Hawassa	38°29′E	7°03′N	1715	275
Ziway	38°43′E	7°55′N	1671	160
Wonji Shoa	39°13′E	8°27′N	1547	120
Akaki	38°47′E	8°53′N	2129	Addis Ababa
Kera	38°45′E	8°59′N	2318	Addis Ababa
Pecock Park	38°46′E	9°00′N	2347	Addis Abab

(Source http://www.4shared.com/rar/gy2VBcEV/Google_Earth_Pro_7111871_Final.html)

3.3. Materials and chemicals

3.3.1. Apparatus and instruments

Polyethylene plastic bags and 500 mL polyethylene plastic bottle were used for collecting vegetable leaves, soil and water samples. An oven (Digitheat, J.P. Selecta, Spain) was used to dry samples and an electronic blending device (Geepas electric coffee grinder, Mainland, China) was used for grinding and homogenizing the samples. Weighing balance (Sartorius Group, Model VIC 303, USA), with precision of 0.001 g was used for weighing of vegetable and soil samples. Muffle furnace (Audiotronics, Wagtech International Ltd., UK) was used for fusion of samples within nickel crucibles (50 mL). A pH/ISE meter (Orion model, EA 940 Expandable Ion Analyzer, USA) equipped with combination fluoride ion selective electrode (Orion Model 96-09, USA) was employed for the determination of fluoride in the samples and standards solutions. A pH meter (HANNA instrument, HI 9025, Malaysia) equipped with glass electrode was used to measure the pH values of sample solutions. Borosilicate volumetric flasks (1000 mL) were used for preparation of 8 M NaOH solution. Hot plate with magnetic stirrer was used for dissolution of soluble fluoride in the soil sample and fusion cake. Measuring cylinders (Duran, Germany), pipettes (Pyrex, USA); micropipettes (0.5-10.5 µL, 1-100 µL, 100-1000 µL Dragonmed, Shangai, China) were use during measuring of different volumes of samples solutions and fluoride standard solutions. 50 mL plastic centrifuge tubes were used for the storage of sample solutions. Plastic funnels were also used for sample filtration. Different types of volumetric

flasks (50, 100, 500 and 1,000 mL) and 50 mL plastic beakers were used sample and standard preparation during the determination of fluoride.

3.3.2. Chemicals and reagents

The reagents that were used in the analysis were all of analytical grade. De-ionized water was used throughout the experiment. Nitric acid (69%, Research Lab Fine Chemical Industries, Mumbai, India) was used for cleaning purpose and sodium fluoride (99%, Analar, NaF, BDH Chemicals Ltd, England) used to prepare standard solutions. The pH standard buffers (pH of 4, 7 and 10) were used for pH calibration purpose. Sodium chloride (Fisher Scientific UK), glacial acetic acid (100%, Sigma-Aldrich Laborchemikalien, Germany), trisodium citrate (BDH Laboratory Supplies, poole, England), and EDTA (Scharlau Chemie S.A., Barcelona, Spain) were used to prepare Total Ionic Strength Adjustment Buffer (TISAB) solution. Sodium hydroxide (Scharlau Chemie S.A., Sentmenat, Spain) solution was used to dissolve vegetables samples homogeneously before alkali fusion and also used to adjust the pH of TISAB solution to pH of 5.3. Hydrochloric acid (36%, Fisher Scientific UK Limited) was used for neutralization of dissolved fusion cake. TISAB was prepared by dissolving 58 g sodium chloride, 57 mL glacial acetic acid, 7 g of trisodium citrate and 2 g EDTA in 500 mL de-ionied water into 1000 mL beaker and its pH was adjusted to 5.3 with 5 M sodium hydroxide. The solution was then transferred to 1000 mL volumetric flask and diluted to the mark with de-ionized water.

3.4. Sample preparation

3.4.1. Sample pretreatment

The collected vegetable samples, lettuce, Swiss chard, cabbage and Abyssinian cabbage were washed with tap water and then with distilled water, air dried for 20 days to constant weight, chopped/grinded with electrical blender (g-pass), sieved (1.4 mm sieve size, stainless steel) and stored in polyethylene bag for further preparation.

The soil samples collected from the six sample sites were air dried to constant weight for ten days, the air dried samples were ground using pre-cleaned mortar and pestle and sieved through a 1.4 mm polyethylene sieve to make the sample uniform and remove plant materials which is not completely changed into soil, such as stones, gravels and other materials. The part of the sample which passes through the sieve was collected in to leveled plastic bag and stored for the fusion and analysis.

The collected six irrigation water samples were filtered with Whatman no. 42 filter paper (125 mm diameter) and stored in 500 mL polyethylene bottle for fluoride determination.

3.4.2. Alkali fusion

3.4.2.1. Fusion of vegetable samples

The fusions of selected leafy vegetables were done by the reported method (Malde *et al.,* 2001) in triplicate. A 0.5 g dry weight basis accurately weighted for each vegetable sample with 50 mL nickel crucible and 5 mL of 8 M NaOH added and thoroughly mixed. Then the crucible was subjected to 150 ^{o}C oven until dryness and transferred to muffle furnace with 200 ^{o}C for 2 hours and the temperature raised to 525 ^{o}C for about 3 hours. Then the fusion cake was cooled to room temperature and added 14 mL de-ionized water. The crucible was kept on a hot plate in order to aid the dissolution of the fused cake. After dissolution was completed, the sample solution transferred into 50 mL plastic beaker. The sample solution were neutralized using concentrated HCl drop wise to decrease the pH of the solution 13-12 to 8-8.5 and then diluted HCl up to the final pH of 7.0-7.4 with continuous stirring and pH control. The sample solutions were then transferred to 50 mL plastic volumetric flask and diluted with de-ionized water to the volume by rinsing the beaker. Then the sample solutions were filtered with Whatman no. 42 filter paper with 50 mL volumetric flask and subjected to fluoride measurement.

3.4.2.2. Fusion of soil samples

Total soil fluoride was determined by the reported method (McQuaker *et al.*, 1977) in triplicate fusion for each soil samples. 0.50 g of prepared soil samples were weighed directly into 50 mL nickel crucibles and moistened with 1 mL de-ionized water. To the moistened sample, 6.0 mL of a 17 M sodium hydroxide solution was added and the contents placed in an oven (150 ^{o}C) for 2 h until the sodium hydroxide had solidified. The crucible containing the dry sample was removed from the oven and transferred in to a muffle furnace for fusion at 600 ^{o}C for 30 min. After cooling for 1 h, 15 mL of de-ionized water was added to the sample and the contents heated on a hot plate for approximately 3 h to facilitate the dissolution of the fusion cake. About 7 mL of concentrated hydrochloric acid was added drop wise to decrease the pH from 12.0-13.0 to 8.0-8.5 with continuously stirring and pH control. Subsequently the samples were transferred to a 50 mL plastic volumetric flask. The crucible was rinsed successively with de-ionized water until the final volume reached 50 mL and all the washings were mixed and filtered with Whatman no. 42 filter paper (125 mm, diameter) in pre-cleaned and rinsed 50 mL plastic volumetric flask and subject to fluoride determination.

The chemical reaction takes place during the fusion of samples is:

$$\text{Sample-F} + \text{NaOH} \rightarrow \text{NaF} + \text{Sample-OH} \quad (3)$$

3.5. Instrument calibration

3.5.1. Calibration of fluoride ion selective electrode and pH meter

Different working standard solutions were prepared from 1000 mg/L NaF stock solution through serial dilution. Fluoride stock solution was prepared by dissolving 2.21 g of anhydrous sodium fluoride (99.0% NaF, BDH Chemicals, England) in 500 mL de-ionized water into 1000 mL volumetric flask and diluted to the mark with de-ionized water. The calibration curve was prepared using fluoride concentration 0.05, 0.5, 1, 5 and 10 mg/L for fluoride determination in vegetable samples. The calibration curve was also prepared using fluoride concentration 0.5, 1.0, 5.0, 10 and 20 mg/L for determination of fluoride in irrigation water and soil samples. The slope

and coefficient of determination values of −58 mV/decade and 0.9999, respectively, were obtained. The ISE was placed in a beaker containing 5 mL of standard solution, along with 5 mL of TISAB (1:1) at room temperature (25 ^{o}C).

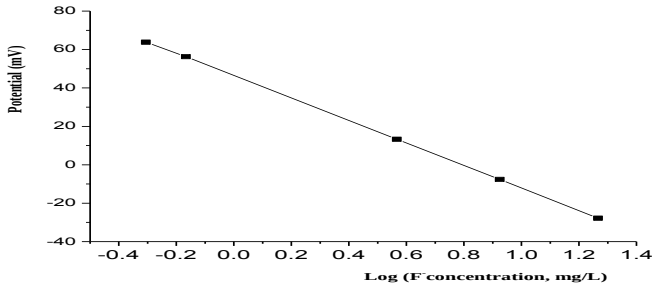


Figure 6: calibration curve for fluoride determination in vegetables, soil and water samples.

The potential developed in ISE and the concentration of F^- in the solution correlated using Nernst equation:

$$E = E^o - (2.303RT/zF) \log [F^-] \quad (4)$$

Where: R is the gas constant (8.314 Joules/degree/mole); T is the absolute temperature (K); z is the charge of fluoride ion in the solution; E is measured electrode potential in a solution; E^o is standard potential of reference electrode; F is the Faraday's constant (96,500 coulombs/mole); and $[F^-]$ is the concentration of fluoride ion in a particular sample solution.

The pH meter was calibrated using pH 4, pH 7, and pH 9 buffer solutions before preceding the pH measurement of reagent blank and sample solutions.

3.5.2. Reagent blank

A reagent blank can be defined as a solution without the interest of analyte and consists of reagent water and all reagents that normally are in contact with a sample during the entire analytical procedure. The reagent blank is used to determine the contribution of the reagents and

the preparative analytical steps to error in the measurement (www.thermoscintific.com). In this study, one reagent blank was prepared for each set (batch) of fusion and determination.

3.6. Fluoride determination

Fluoride ion in solutions can be determined by using different methods. Potentiometric and chromatographic methods are the main fluoride ion determining methods. Colorimetric methods are also available, but more time consuming and lack of sensitivity than the other methods (Kakabades and Venkateswarlu *et al*, 1971). Other methods that have been used include fluorometric, enzymatic and proton activation analysis (Rudlph *et al*, 1973). The latter technique is sensitive to trace amounts of sample, and requires minimal sample preparation. The most accurate method of sample preparation is microdiffusion techniques, such as the acid-hexamethyldisiloxane (HMDS) (EFSA, 2004).

3.6.1. Spectrophotometry

Spectrophotometric methods are widely used in the determination of fluoride because of advantages such as simplicity, convenience, accuracy and reproducibility (Zolgharnein *et al.*, 2009). They are based on the reaction of fluoride with coloured metal chelate complexes, producing either a mixed-ligand ternary complex or replacement of the ligand by fluoride to give a colourless metal-fluoride complex and the free ligand with a colour different to the metal-ligand complex (Barghouthi and Amereih, 2012).

3.6.2. Chromatography

Ion chromatography (IC) utilizes anion exchange resins as a stationary phase to separate fluoride ions from other species. In most cases, conductivity detectors are used to detect the ions in the eluent. Both the stationary phase and the eluent must be chosen to separate fluoride from overlapping ions. This method is generally applied to the separation of weak organic acids and its use for fluoride determinations is based on the fact that fluoride is an anion of a weak acid, hydrogen fluoride, with a pK_a of 3.19, similar to that of weak organic acids. The acids elute in

order of increasing pK_a. At low pH, anions of strong acids remain disassociated and are excluded from the resin and are rapidly eluted. Hydrogen fluoride exists primarily in the molecular form, and interacts with the resin, delaying its elution. In this way, fluoride is sufficiently separated from ionic interferences to be reliably quantified. Interfering anions, such as chloride, emerge as one peak before the fluoride elutes. Resolution can be controlled by adjusting the pH (Carolyn *et al*., 2003).

3.6.3. Potentiometry

Potentiometry is an electroanalytical method, in which the concentration (activity) of ionic species is measured using an electrochemical cell consisting of two special electrodes. Potentiometric determination by using fluoride ion selective electrode is the most commonly used F^- determining method since it is easy to use, relatively cheap, sufficiently sensitive, selective, accurate and very small concentrations of fluoride ions (up to 10^{-6} M) can be determined by fluoride selective electrode (Rajković and Novaković, 2007). The levels of fluoride in samples were determined by fluoride ion selective electrode by using direct measurement technique rather than incremental techniques (standard or known addition, sample addition, and sample subtraction), and potentiometric titration techniques. Because, direct measurement technique is a simple and fast technique for analyzing large number of samples and measurements can be made over a wide range of concentration.

Addition of total ionic strength adjustment buffer (TISAB) provides constant ionic strength and adjusts pH values of solutions to pH of 5.2−5.5. This pH adjustment helps to avoid the interferences of cations with fluoride ions since the complexing agent called ethylene di-amine tetra acetic acid (EDTA) which is the component of TISAB make complexes with polyvalent cations (Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, etc.) to generate free fluoride ion in solutions. Adjustment of pH also avoids the interferences of hydroxide ion (OH^-) with fluoride electrode in basic solutions and that of hydronium (H_3O^+) ion with fluoride ion in acidic solutions.

In this study, all the determination were made in triplicate at room temperature and the concentration of fluoride was recorded in the unit of mg/L directly from the instrument reading for each solution.

3.6.1. Total fluoride determination in selected leafy vegetables

The total vegetable fluoride was determined from prepared and ready sample solutions for determination through alkaline fusion by the reported methods (Malde *et al.*, 2001). Equivalent amount of TISAB and aliquot, i.e. 5 mL total ionic strength adjustment buffer (TISAB) was added to 5 mL filtrate into 50 mL plastic beaker and measured the concentration of fluoride with Orion ISE meter combined with expandable electrode against the concentration of NaF standard solutions in triplicate with continuous stirring.

3.6.2. Fluoride determination in soils

3.6.2.1. Total fluoride in soil

The total soil fluoride was determined using already prepared and ready sample solutions for determination through alkaline fusion by the reported methods (McQuaker *et al.*, 1977). Equivalent amount of TISAB and aliquot, i.e. 10.0 mL total ionic strength adjusting buffer (TISAB) was added to 10.0 mL filtrate into 50 mL plastic beaker and measured the concentration of fluoride with Orion ISE meter combined with expandable electrode through continuous stirring against the concentration NaF standard solutions in triplicate.

3.6.2.2. Soluble fluoride in soil

Soluble fluoride in soil samples were determined after weighing 5 g soil in 50 mL plastic beaker and mixed with 10 mL de-ionized water. Then it was stirred for about 1 hour to dissolve fluoride in the soil and filtered with Whatman no. 42 filter paper (125 mm, diameter) in 50 mL volumetric flask. After this, the filtrate was diluted to the volume with de-ionized water. 10 mL aliquot solution of the filtrate was mixed with 10 mL TISAB solutions in 50 mL plastic beaker. The concentration of fluoride in the solution was measured using fluoride ISE with continues stirring against NaF standard solution.

3.6.3. Fluoride determination in irrigation water

The fluoride contents of water were determined by mixing an aliquot of 5 mL filtered water sample with 5 mL of TISAB solution into 50 mL plastic beaker with continuous stirring in triplicate analysis for each water sample using ISE combined with glass electrode.

3.7. Method validation

3.7.1. Detection limit

This is the lowest analyte concentration that can be detected and identified with a given degree of certainty. The limit of detection (LOD) is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence.

There are several methods of estimating the LOD, all of which depend on the analysis of blank specimens and examination of the signal to noise ratio. A minimum requirement for signal to noise of 3 is widely accepted and can be calculated 3 time average standard deviation of reagent blank. The LOD is not a robust or rugged parameter and can be affected by minor changes in the analytical system (e.g. temperature, purity of reagents, matrix effects, and instrumental conditions). It is therefore important that this parameter is always verified by laboratories adopting previously validated methods (UNODC, 2009). The limit of quantification (LOQ) is the lowest concentration of the interest of analyte at which a measurement is quantitatively meaningful and calculated as 10 times the average standard deviation of reagent blank solutions (Malde, *et al*. 2001).

In this study, the LOD of fluoride ISE was evaluated with ten reagent blank solution measurements and the lower detection limit of fluoride ISE was 0.02 mg/L.

3.7.2. Precision and accuracy

The precision of an analytical procedure is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. In this study, the precision of fluoride determination in vegetables, soil and water expressed in terms of standard deviation or relative standard deviation (SD or RSD).

The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentrations (for example, a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. In this study, the accuracy expressed in terms of percent difference (Huber, 2007).

3.7.3. Validation of analytical procedure

Most commonly validation of analytical procedures performed using certified reference material (CRM) or standard reference material (SRM) and recovery test. Even though, the former is very important for validation, it is very expensive and not available as the interest of analyst. But the later technique is the most preferred one due to its accessibility, availability and having cheap costs. Recovery is similar to accuracy but includes the extraction efficiency of an analytical method. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. The recovery result clearly shows the presence or absence of interfering agents in the analytical procedure.

In this study, the validation of analytical procedures for the determination of fluoride using fluoride ISE was evaluated with recovery test. This is the most popular and simple method validation technique under economic point of view. In the recovery test 25%, 50% and 100% of fluoride measured in the original vegetables were spiked in to 0.5 g vegetable samples from 20 mg/L fluoride standard solution in triplicate. Lettuce, Swiss chard, cabbage and Abyssinian cabbage was spiked with 36, 72, 144 µL; 33.8, 67.5, 135 µL; 16.4, 32.8, 65.5 µL and 16.2, 32.4, 64.8 µL of 20 mg/L standard F^- solution, respectively.

The spiking experiments were also done in irrigation water and farmland soil samples. Similar percentage of fluoride standard solutions with vegetables was spiked by considering different volume of water and mass of soil in the recovery of water soluble fluoride. The recovery of fluoride in water, soluble soil and total soil were spiked with 478.8, 957.5, 1915 µL; 1.44, 2.88, 5.75 mL; 5, 10, 20 mL of 20 mg/L standard fluoride solution, respectively.

The percent of recovery of fluoride in the sample was calculated by comparing the spiked sample with unspiked one using the following formula (Thompson *et al*, 1995 and Burns *et al*, 2002).

$$\% \text{ recovery} = (C/C_{ref}) \times 100 \quad (4)$$

Where: C is the concentration of fluoride recovered and C_{ref} is the fluoride concentration spiked or added in the sample.

4. RESULTS AND DISCUSSION

4.1. Recovery test for fluoride determination

In this study, the analytical procedure used for the determination of fluoride in vegetables, farmland soil and irrigation water samples were validated by spiking experiment. The recovery test was conducted for all vegetables, soil and water samples. Recovery test results of fluoride in vegetable sample are given in (Table 3). The percentage of recovered fluoride in vegetable sample ranges from 89–108% which is within the accepted range (AOAC, 2002) for percent of recovery. This recovery indicates that there was random error in the fluoride determination methods.

The recovery test results of water samples are given in (Table 4).The percentage recovery of irrigation water samples were 105 ± 8, 108 ± 2 and 97 ± 2 which is within the accepted range of percent recovery. This confirms that there was no interference during the measurement of fluoride in irrigation water sample in this analytical procedure.

The recovery test results of water soluble and total fluoride in soil are given in (Table 5). The percentage recovery of both water soluble and total fluoride in soil ranges from 94–110% which is also within the accepted range of percent recovery and confirms that presence of random error during the fluoride measurement procedures.

The percentage recovery of all the vegetables, water and soil samples confirm the absence of systematic error during sampling and fluoride determination procedure. The variation of percent recovery may have only arise from random error. Therefore, the selected analytical methods for this study were reliable and effective for the determination of fluoride.

Table 3: Recovery test result for leafy vegetables.

Vegetables	Concentration of F^- in un spiked sample (mg/kg)	Concentration of F^- added in un spiked sample (mg/kg)	Concentration of F^- in spiked sample (mg/kg)	Percent of recovery (%)
Lettuce	5.76 ± 0.03	1.44	7.05 ± 0.01	89 ± 9
	5.76 ± 0.03	2.88	8.53 ± 0.008	96 ± 3
	5.76 ± 0.03	5.76	11.2 ± 0.03	94 ± 5
Swiss chard	5.40 ± 0.03	1.35	6.81 ± 0.01	105 ± 10
	5.40 ± 0.03	2.70	7.85 ± 0.006	91 ± 2
	5.40 ± 0.03	5.40	10.4 ± 0.005	92 ± 1
Cabbage	2.62 ± 0.02	0.66	3.33 ± 0.004	108 ± 5
	2.62 ± 0.02	1.31	3.81 ± 0.007	91 ± 5
	2.62 ± 0.02	2.62	5.42 ± 0.03	107 ± 11
Abyssinian cabbage	2.59 ± 0.02	0.65	3.19 ± 0.003	92 ± 5
	2.59 ± 0.02	1.30	3.97 ± 0.01	106 ± 5
	2.59 ± 0.02	2.59	5.21 ± 0.009	101 ± 4

Table 4: Recovery test for water samples.

Types of sample	Concentration of F^- in un spiked sample (mg/L)	Concentration of F^- added in un spiked sample (mg/L)	Concentration of F^- in spiked sample (mg/L)	Percent recovery (%)
Irrigation water	7.66 ± 0.18	1.92	9.67 ± 0.06	105 ± 8
	7.66 ± 0.18	3.83	11.8 ± 0.06	108 ± 2
	7.66 ± 0.18	7.66	15.1 ± 0.15	97 ±2

Table 5: Recovery test for soil samples.

Types of sample	Concentration of F^- in un spiked sample (mg/kg)	Concentration of F^- added in un spiked sample (mg/kg)	Concentration of F^- in spiked sample (mg/kg)	Percent recovery (%)
Water soluble fluoride in soil	23.4 ± 0.1	5.85	28.7 ± 1	94 ± 4
	23.4 ± 0.1	11.7	34.8 ± 0.3	97 ± 2
	23.4 ± 0.1	23.4	45.7 ± 3	94 ± 3
Total fluoride in soil	802 ± 59	200	1062 ± 37	110 ± 5
	802 ± 59	401	1278 ± 37	107 ± 3
	802 ± 59	802	1572 ± 56	96 ± 7

4.2. Fluoride distribution in vegetable samples

The fluoride concentrations of selected leafy vegetables cultivated in different part of Ethiopia are given in (Table 6). The distribution of fluoride in lettuce, Swiss chard, cabbage and Abyssinian cabbage across the study area shows almost similar patterns. The highest and lowest fluoride concentration both in lettuce and Swiss chard were 5.76, 5.40 mg/kg and 2.95, 2.74 mg/kg recorded in Hawassa and Kera, respectively; in cabbage the highest was 2.70 mg/kg in Ziway and the lowest 2.12 mg/kg in Kera; in Abyssinian cabbage the highest was 2.59 mg/kg in Hawassa and the lowest was 2.08 mg/kg in Kera.

The fluoride concentration of lettuce, Swiss chard, cabbage, and Abyssinian cabbage was higher in Rift Valley compared with non Rift Valley areas in this study. But fluorides of Abyssinia cabbage in Wonji Shoa is comparable with non Rift Valley areas, this might be arise from nature of vegetables, other micro or macro nutrient interaction in the agro – ecological zone. Lettuce and Swiss chard was highly sensitive for fluoride accumulation compared with that of cabbage and Abyssinian cabbage. This vegetables grown in high fluoride source, accumulates high fluoride concentration and at low fluoride source, accumulates low fluoride concentration. In the case of fluoride concentration in cabbage and Abyssinian cabbage has observed relatively

smaller variation and comparable results in all the study areas. This indicates that cabbage and Abyssinian cabbage has lower sensitivity to fluoride.

Table 6: Leafy vegetables fluoride content grown in Ethiopia dry weight bases (mean ± SD, mg/kg, n = 9).

Sample site	F^- concentration (mg/kg)			
	Lettuce	Swiss chard	Cabbage	Abyssinian cabbage
Hawassa	5.76 ± 0.03	5.40 ± 0.03	2.62 ± 0.02	2.59 ± 0.02
Ziway	4.61 ± 0.05	3.18 ± 0.02	2.70 ± 0.03	2.40 ± 0.01
Wonji Shoa	4.96 ± 0.10	4.58 ± 0.02	2.48 ± 0.02	2.18 ± 0.03
Akaki	3.26 ± 0.02	3.03 ± 0.02	2.40 ± 0.01	2.23 ± 0.01
Kera	2.95 ± 0.01	2.74 ± 0.02	2.12 ± 0.01	2.08 ± 0.01
Pecock Park	3.21 ± 0.02	3.14 ± 0.01	2.47 ± 0.02	2.25 ± 0.01

2.1. Comparison of fluoride levels in vegetable samples in the study areas

The fluoride distribution in vegetables across the sampling site and within a particular site is clearly shown in Figure 7. The distribution pattern of fluoride in lettuce and Swiss chard have highest fluoride concentration across the study area, except the fluoride concentration of lettuce in Pecock Park have smaller value than Akaki and vice versa in Swiss chard.

The fluoride concentrations in cabbage and Abyssinian cabbage have all most closer value both in Rift Valley and non Rift Valley areas. This small difference in accumulation of fluoride might depend on the nature of vegetables and agro ecological zone.

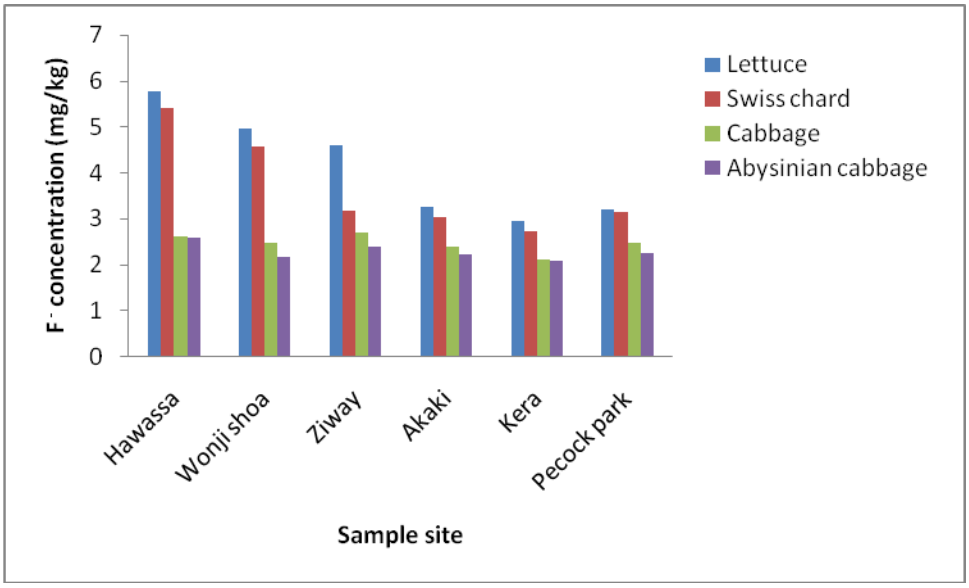


Figure 7: Comparison of fluoride level in lettuce, Swiss chard, cabbage and Abyssinian cabbage in the study area.

2.2. Comparison fluoride concentration in this study with literature values

Many researchers determine the concentration of fluoride in selected leafy vegetables in different parts of the world. The result of this study was compared with literature values (Table 7). The fluoride concentration of lettuce reported (Saxena and Sewak, 2015) was 5.6 mg/kg which is comparable to fluoride content of lettuce in Hawassa and higher value compared with the other site in this study.

The fluoride concentration of cabbage was reported in 2011 in Nigeria and 2009, 2012 and 2015 in India. The concentration of fluoride in this study was from 2.70–2.12 mg/kg which are higher than the value reported by (Paul *et al*., 2011) and (Pal *et al.*, 2012). The fluoride of cabbage in this study also has lower values compared with the values reported by (Bhargava and Bhardwaj, 2009) and within the range reported by (Saxena and Sewak, 2015). The fluoride concentration in spinach reported by (Radha Gautam *et al*., 2010) is the highest in leafy vegetables compared with the present leafy vegetable fluoride concentration.

To the best of my knowledge there is no report on the concentration of fluoride in Swiss chard and Abyssinian cabbage in literature. As a result of this, the present study on fluoride contents of Swiss chard and Abyssinian cabbage was not compared with values in literature.

Table 7: Comparison of fluoride concentration in leafy vegetable samples with literature values.

Vegetable type	F^- Concentration (mg/kg, dry wt.)	Origin	Reference
Lettuce	5.7	India	Saxena and Sewak, 2015
	2.95–5.76	Ethiopia	Present study
Swiss chard	2.74–5.40	Ethiopia	Present study
Spinach	15.98–25.7	India	Radha Gautam et al., 2010
Cabbage	0.022–0.047	Nigeria	Paul *et al*., 2011
	3.91–11.30	India	Bhargava and Bhardwaj, 2009
	1.28–11.30	India	Saxena and Sewak, 2015
	1.25 ± 0.07	India	Pal *et al.*, 2012
	2.12–2.70	Ethiopia	Present study
Abyssinian cabbage	2.08–2.59	Ethiopia	Present study

4.3. Fluoride distribution in irrigation water samples

The distribution of fluoride in different irrigation water body is given in (Table 8). The water bodies found in the Rift Valley have high fluoride concentration compared with non Rift Valley rivers. The fluoride distribution in water was higher than the fluoride concentration in soil both in water soluble and total fluoride in soil. This might be via the movement of fluoride from soil and environment to the water body through different natural and anthropogenic factors; like runoff, rain, industrial waste disposal, toothpaste to the water body, etc. Fluoride more than 1.5 mg/L in drinking water and 10 mg/L in irrigation waters (WHO, 1984), becomes toxic to animals

and human beings, and toxic to some crops and animals. In this study, the fluoride levels in irrigation water bodies were below the permissible limit which is safe for life.

Table 8: Fluoride distribution in selected irrigation water body (mean ± SD, mg/L, n = 3).

Sample site	Water body	F^- concentration (mg/L)
Hawassa	Hawassa lake	7.66 ± 0.18
Ziway	Ziway lake	2.06 ± 0.08
Wonji Shoa	Awash river	2.77 ± 0.08
Akaki	Akaki river	0.78 ± 0.02
Kera	Kera river	0.43 ± 0.04
Pecock Park	Bulbula river	1.48 ± 0.03

4.4. Fluoride distribution in soil samples

4.4.1. Soluble fluoride distribution in soil samples

The water soluble fluoride distribution in soil is given in (Table 9). The order of water soluble fluoride distribution in the study area was comparable with the fluoride distributions observed in the irrigation water. The highest fluoride distribution was 23.4 mg/kg in Hawassa soil and the lowest fluoride was 4.30 mg/kg in Kera soil. This extreme value and the other order of distribution confirm the relationship of soluble fluoride concentration in soil and water. The highest water soluble fluoride distribution in Hawassa might be from the presence of water soluble fluoride complex compounds, application of phosphate fertilizers in the area and other agricultural pesticides. On the other hand, the lowest values in Kera soil might be from the presence of insoluble fluoride complex compounds in the area or absence of fluoride source in the soil and low anthropogenic effects.

Table 9: Soluble fluoride distribution in soil (mean ± SD, mg/kg, n = 9).

No.	Sample site	F^- concentration (mg/kg)
1	Hawassa	23.4 ± 0.1
2	Ziway	15.1 ± 0.5
3	Wonji Shoa	19.7 ± 0.5
4	Akaki	8.77 ± 0.5
5	Kera	4.30 ± 0.3
6	Pecock Park	7.53 ± 0.05

4.4.2. Total fluoride distribution in soil samples

Total fluoride distribution in soil is given in (Table 10). The highest fluoride was 802 mg/kg recorded in Hawassa and the lowest was 133 mg/kg in Akaki soil. This difference might be depend on the availability of soluble and in soluble fluoride complex compound, the presence or absence of different anthropogenic activities like industries wastes and swage, application of phosphate fertilizers, agricultural pesticides, insecticides and fungicides in the areas.

In general, the fluoride distributions in vegetables, soil and water were highest in Hawassa, because Hawassa is one of place found in the main Ethiopian rift valley region which was characterized by high fluoride distribution in different water bodies, rocks and soils and high fluorosis risk area in the country.

Table 10: Total fluoride distribution in soil (mean ± SD, mg/kg, n = 9).

No.	Sample site	F^- concentration (mg/kg)
1	Hawassa	802 ± 59
2	Ziway	595 ± 33
3	Wonji Shoa	553 ± 44
4	Akaki	133 ± 8
5	Kera	263 ± 12
6	Pecock Park	336 ± 13

4.5. Fluoride distribution in fresh vegetables

All vegetables selected for this study; lettuce, Swiss chard, cabbage and Abyssinian cabbage consumed in fresh weight based in Ethiopia. The fluoride levels in this study reported in dry weight and fresh weight based. This fluoride levels in each vegetables can be calculated from the fluoride level of dry weight basis under percent moisture content consideration.

The percentage moisture content of each vegetable was calculated using the following formula.

$$\% \text{ moisture} = [(Fw-Dw) / Fw] \times 100 \quad (5)$$

Where: Fw = Fresh weight, Dw = dry weight of vegetable samples.

In this study, 500 g of lettuce, Swiss chard, cabbage and Abyssinian cabbage fresh weight vegetables were air dried for 20 days until constant weight obtained for all sample sites. The measured (dry weight, % moisture) of lettuce: (57.23 g, 88.6%); Swiss chard: (73.6 g, 84.7%); cabbage: (65.16 g, 87%) and Abyssinian cabbage: (93.88 g, 81.2%) for Hawassa; lettuce (52.04, 89.5%); Swiss chard (67.71, 86.4%); cabbage (93.29, 81.3%) and Abyssinian cabbage (88, 82.4%) for Ziway; lettuc (48.57, 90.2%); Swiss chard (56.56, 88.6%); cabbage (82.54, 83.4%) and Abyssinian cabbage (70.22, 85.9%) for Wonji Shoa; lettuce (50.06, 89.9%); Swiss chard (87.79, 82.4%); cabbage (58, 88.4%) and Abyssinian cabbage (70.11, 86%) for Akaki; lettuce

(45.51, 90.8); Swiss chard (72.12, 85.5%); cabbage (65, 87%) and Abyssinian cabbage (67.19, 86.5%) for Kera; lettuce (44.79, 91%); Swiss chard (72.15, 85.6%); cabbage (60, 88%) and Abyssinian cabbage (61.54, 87.6%) for Pecock Park.

The fresh weight basis of fluoride levels in lettuce, Swiss chard, cabbage, and Abyssinian cabbage were calculated and reported in (Table 11). Because these leafy vegetables consumed in fresh weight based in the country. This conversion was performed using the following formula.

$$\text{Fresh weight fluoride} = \text{Dry weight fluoride} \times (100 - \%\ \text{moisture})/100 \quad (6)$$

Table 11: Fluoride concentration in fresh vegetables.

Sample site	F^- concentration			
	Lettuce	Swiss chard	Cabbage	Abyssinian cabbage
Hawassa	0.657	0.826	0.341	0.487
Ziway	0.484	0.432	0.504	0.422
Wonji Shoa	0.486	0.522	0.412	0.307
Akaki	0.329	0.533	0.278	0.312
Kera	0.271	0.397	0.275	0.289
Pecock Park	0.289	0.452	0.296	0.279

4.7. Statistical analysis

4.7.1. Analysis of variance (ANOVA)

All the measurements were done in triplicate except total fluoride determination in vegetable and soil samples which were determined ninth times and reported as mean ± SD. The statistical analysis was done using SPSS version 22 soft ware. The mean concentration variability of fluoride in vegetables were analyzed using analysis of variance in one way (ANOVA) which is the most common and popular statistical methods for the comparison of between and within

sample means at 95% confidence level for the significance difference test. This statistical tool tells us weather the variation comes from measurement variability or sampling heterogeneity. The magnitude and direction of correlation of fluoride concentration in vegetables with water and soil samples were also tested using Pearson correlation.

Table 12: Analysis of variance (ANOVA) between and within leafy vegetable samples at 95 % confidence level.

Vegetables	Comparison	Df	F_{cal}	F_{crit}	Remark
Lettuce	Between samples	5	36.9	2.41	Significant difference between sample means
	Within samples	48			
Swiss chard	Between samples	5	182	2.41	Significant difference between sample means
	Within samples	48			
Cabbage	Between samples	5	9.38	2.41	Significant difference between sample means
	Within samples	48			
Abyssinian cabbage	Between samples	5	9.68	2.41	Significant difference between sample means
	Within samples	48			

Df = degree of freedom, F_{cal} = F calculated, F_{crit} = F critical

A one-way ANOVA between and within groups analysis of variance (Table 12) was conducted to explore the impact of sample site on fluoride concentration of vegetables and impacts of vegetable types. Sample sites were Hawassa, Ziway, Wonji Shoa, Akaki, Kera, and Pecock Park.

There was a statistically significant difference between and within the group at ($p < 0.05$) level in mean fluoride concentration of leafy vegetable samples.

4.7.2. Pearson correlation

The Pearson correlation shows weak, moderate or strong relationship among variables in the positive or negative direction. The Correlation of fluoride in vegetables with soil and water given in (Table 13 and 14). The concentration of fluoride in lettuce have very weak positive correlation in all irrigation water body except Awash and Bulbula irrigation water sample, strong negative and positive correlation observed, respectively. In Swiss chard, strong and moderate positive correlation observed except Akaki irrigation water sample which has negative moderate correlation. In cabbage, no correlation with Ziway lake irrigation water sample. In Abyssinian cabbage strong positive and negative correlation observed in Ziway lake, Bulbula, Akaki and Kera rivers. Moderate negative and positive correlation observed in Hawassa and Akaki river water samples. On the other hand, fluoride in Ziway lake and Bulbula river have positive correlation with all vegetable types.

Table 13: Correlation of fluoride in irrigation water and vegetable samples.

Vegetables	Hawassa lake	Ziway lake	Awash river	Akaki river	Kera river	Bulbula river
Lettuce	0.135	0.145	-0.897	0.112	0.165	0.957
Swiss chard	0.921	0.641	0.616	-0.377	0.486	0.451
Cabbage	-0.329	0.031	0.185	-0.795	0.839	0.450
Abyssinian cabbage	-0.505	1.00	0.422	-0.988	-0.922	0.720

The correlations of fluoride between vegetable and soluble fluoride in farmland soil were also show variable relationship like vegetable and irrigation water sample. The concentration of fluoride in lettuce have strong correlation with soluble fluorides in Hawassa, Ziway, Kera and Pecock Park farmland soil. Moderate negative correlation with Wonji Shoa and Akaki farmland.

The fluoride concentration in Swiss chard shows positive strong correlation with Pecock Park and no correlation with Wonji Shoa farmland soluble fluoride. In cabbage very weak negative correlation was observed in Akaki and Kera farm land soil and strong correlation was observed in the other sample sites. In Abyssinian cabbage strong negative correlation was observed in Akaki and weak and moderate correlation observed in the other sites. In another perspective, positive correlation was observed in Pecock Park and negative correlations in Kera farmland soils across all vegetable samples.

Table 14: Correlation of soluble fluoride in farmland soil and vegetables.

Vegetables	Hawassa	Ziway	Wonji Shoa	Akaki	Kera	Pecock Park
Lettuce	0.771	0.950	-0.440	-0.568	-0.785	0.621
Swiss chard	0.413	-0.400	0.008	0.323	-0.531	0.880
Cabbage	-0.882	0.908	0.746	-0.202	-0.074	0.879
Abyssinian Cabbage	0.214	0.436	-0.218	-0.646	-0.104	0.195

In general variable correlation between leafy vegetable and irrigation water and between leafy vegetable and soil fluoride was observed across the studied areas. This may be due to variation of pH, minerals, agro ecology and different anthropogenic activities.

5. CONCLUSION

The contents of fluoride in four vegetables, the respective farmland soil and irrigation water body from six sample sites were determined. The fluoride contents in leafy vegetables (mg/kg) were 2.95–5.76 in lettuce, 2.74–5.40 in Swiss chard, 2.12–2.70 in cabbage and 2.08–2.59 in Abyssinian cabbage in all six irrigation farms. The fluoride level in irrigation water (mg/L) was 0.43–7.66 and the farmland soil fluoride contents (both soluble and total, mg/kg) were 4.30–23.4 and 133–802, respectively.

One way analysis of variance (ANOVA) shows that there was significant difference in the mean fluoride concentrations of vegetables at ($p < 0.05$) confidence level. This means a single vegetable type across the six sampling site have different fluoride concentration. This may be due to variation of fluoride source in the soil, irrigation water and different anthropogenic activities. The Pearson correlation shows variable (weak, moderate or strong) relationship between the fluoride levels in water and vegetables and between fluoride levels in soil and vegetables.

The fluoride concentrations of vegetables found in this study are comparable with values in the literature and safe for life. However, consumption of these vegetables has significant contribution to the total intake of fluoride depending on the amount and regularity of consumption.

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