ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

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Retention of vitamin A and D_3 in fortified soybean oil during Ethiopian traditional cooking and shelf-stability during storage

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Declaration

I, the undersigned, declare that this is original work and has never been presented in this or any other University, as well as research center previously and all the sources materials used for this thesis, have been fully acknowledged.

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List of abbreviations

AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemist Society
ASLT	Accelerated Shelf life Testing
BCR	Benefit Cost Ratio
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
ESA	Ethiopian Standard Agency
FAO	Food and Agricultural Organization
FFA	Free Fatty Acid
FSA	Food Standard Association
GAIN	Global Alliance for Improved Nutrition
HPLC	High Performance Liquid Chromatography
LMIC	Low and Middle Income Countries
MNM	Micro Nutrient Malnutrition
PET	Poly Ethylene Terephthalate
RBDSBO	Refined, Bleached and Deodorized Soya Bean Oil
TFH	Tetra Hydro Furan
UVB	Ultra Violet B
VAD	Vitamin A Deficiency
WHO	World Health Organization

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Contents

Abstract

Retention of vitamin A and D_3 during cooking and shelf-life stability in fortified soya bean oil has been studied in other countries, but not yet in Ethiopia. Traditional cooking practice in Ethiopia is quite different from the cooking and food preparation of other countries. Yet, the impact of Ethiopian traditional food processing on the retention of the vitamins is not studied, as to our knowledge. And also most soya bean oil on other countries is produced from genetically modified soya seeds, but in Ethiopia, the oil is produced from organic (natural) soya bean seed. In recent years, fortified soya bean oil is widely available in Ethiopia. Since the oil is distributed throughout the country, its shelf stability should be studied as well. Therefore, this study was aimed to evaluate retention and shelf-life stability of vitamin A and D_3in fortified soya bean oil during Ethiopian traditional cooking and storage. The shelf-life stability of vitamin A and D_3 in refined, bleached and deodorized soya bean oil was assessed during six months of storage. The samples were stored at different storage temperatures; one sample was stored at room temperature (around 25°C) and another sample at 37°C. Accordingly, the stability of both vitamins A and D_3 was decreased with increasing storage time and temperature. But, as compared to vitamin A the stability of vitamin D_3 was highly reduced during the storage. The stability of vitamin A for oil3 (stored at room temperature) and oil4 (stored at37°C) during six months of storage was reduced by 5.42 and 8.77 % respectively. Whereas the stability of vitamin D_3 for oil3 and oil4 was reduced by 59 and 62% respectively. Retention of vitamin A and D_3 was evaluated by using the fortified oil for cooking of the food at 250°C for 80 minutes. At this cooking time and temperature, more than 60 and 97% of vitamin A and D_3 were retained respectively. From this it was concluded that vitamin A is sensitive to heat, a significant level of vitamin A can be lost during cooking and vitamin D_3 is sensitive to storage time, significant level of vitamin D_3 can be lost during storage. It was recommended that, the oil should cook at less than 200°C for better retention of vitamin A and the oil should not store for longer time for better stability of vitamin D_3.

1. Introduction

1.1. Background of the study

Food fortification refers to the practice of intentionally increasing contents of essential micronutrients in a food irrespective of whether the nutrients were originally in the food before processing or not. Apparently, the purpose is to improve the nutritional quality of the food and to provide a public health benefit with minimal risk (WHO and FAO, 2006). Malnutrition erodes the foundation of economic growth - people's strength, energy, creative and analytical capacity, initiative and entrepreneurial drive. Widespread poor health, lower learning capacity, diminished physical activity and depressing work performance place a heavy burden on prospects for national economic development (WHO and FAO, 2006).Worldwide there are over 2 billion people who suffer from a variety of micronutrient deficiencies. Under nutrition and nutrient deficiency are estimated globally to cause between 3 and 5 million deaths per year. The World Health Organization feels that micronutrient deficiency presents a huge threat to the health of the world's population.

Some common micronutrient deficiencies include iodine deficiency, vitamin A deficiency, Vitamin D deficiency, and iron deficiency. Vitamin-A deficiency (VAD) is the leading cause of preventable blindness in children--manifesting in a milder form as night blindness and progressing to permanent blindness in stronger cases (WHO, 2009). VAD also serves to exacerbate serious disease and illness, leading to increased rates of maternal and childhood mortality.VAD is a serious problem in developing countries, especially in Africa and South Asia (WHO, 2009). According to the report of (WHO, 2009) night blindness is estimated to affect 5.2 million preschool-age children and 9.8million pregnant women globally. In addition to VAD vitamin D deficiency is a global concern. Approximately 1 billion individuals, nearly 15% of the world’s population, are vitamin D deficient in which their serum has less than 20 ng/mlof 25(OH)D(Holick, 2010).

Micronutrient deficiency is one of the major public health problems in Ethiopia with women and children most at risk (EPHI, 2016). Dietary inadequacy of consumed nutrients, low bioavailability of key micronutrients from plant-based diets and infections are major contributing factors for micronutrient deficiencies in Ethiopia (EPHI, 2016). More than half of children and a quarter of adult women are anemic and nearly 40% of children are vitamin A deficient (Federal Ministry of Health Ethiopia, 2011). As a consequence, the nation's GDP is depressed by nearly half a billion dollars annually and each year. More than 50 thousand children die as a consequence of vitamin A, iron and folic acid deficiencies. These losses limit the capacity to meet national objectives for reducing mortality, poverty, and malnutrition as well as economic development. According to the report of WHO (2005), 41.6% of Ethiopian pregnant women are vitamin A deficient and 13.2% of preschool children are vitamin A deficient. Micronutrient survey reported by Ethiopian Public Health Institute, (2016) also indicated that 10.9%of Ethiopian school children are vitamin A deficient. According to the report of (FAO, 2010) 42%, Ethiopian children hospitalized with pneumonia also had rickets from severe vitamin D deficiency. Thus, vitamin A and D deficiency are a severe public health problem in Ethiopia with pregnant women and children most at risk.

The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have identified four main strategies for reducing micronutrient malnutrition. These are improving diet diversity, Food fortification, and bio-fortification, Supplementation and Disease control measures. Each of these strategies has a place in eliminating micronutrient malnutrition. To achieve maximum impact, the synergetic combinations of these strategies should be in place. According to WHO one of the best strategies to reduce vitamin A and D deficiency is through vegetable oil fortification (i.e. as oil is an ideal vehicle to vitamin A and D fortification).The most common fortified foods are cereals (and cereal based products), milk (and milk products), fats and oils, accessory food items, tea and other beverages, and infant formulas. Vegetable oil fortification is technologically feasible and cost effective.

1.2. Statement of the problem

Soya bean oil and palm oils are dominantly consumed in Ethiopia. More than 80% of these oils were imported from Malaysia and Indonesia for the past decades. Now a day the production of oil is highly increasing in Ethiopia from year to year deficient (Federal Ministry of Health Ethiopia, 2011). In Ethiopia, fortified soybean is produced largely by Health Care Food Manufacturers located in Addis Ababa.

Retention of vitamin A and D_3 during cooking on fortified soya bean oil has been studied in other countries, but not yet in Ethiopia. Traditional cooking practice in Ethiopia is quite different from the cooking and food preparation of other countries.

The shelf-life stability of vitamin A and D_3 in fortified soya bean oil during storage have been studied in Europe and America several years ago, but not yet in our country, even in Africa. Commonly soya bean oil in developed countries is produced from genetically modified seeds. Previous studies indicated that soya bean oil produced from genetically modified seed has a longer shelf life than oil produced from organic seed. This is because the genetically modified soya bean seeds have less free fatty acid composition (low linoleic acid and linolenic acid) as compared to organic soya bean (Neff and List, 1999).

Ethiopians use a relatively large amount of oil during cooking. Also, the oil is stored at open light and processed for a long time, which will affect its physicochemical properties. There are previous studies that support this idea. For instance, Zehara, (2018) reported the deterioration of niger seed oil and palm oil upon prolonged frying/cooking of potato chips. Youna*et al*., (2015) reported that retention of vitamin A and D_3 in soya bean oil was significantly reduced upon prolonged storage exposed to natural sunlight. Favaro, (1991) reported that 35 % of vitamin A can be lost during the frying of oil at a temperature of 180–200°C. As to our knowledge, there is no any study related to oil fortification and vitamin stability in Ethiopia. Therefore the main purpose of this research was to evaluate the retention and shelf-life stability of vitamin A and D_3 in fortified soya bean oil during cooking and storage.

1.3. Significance of the study

The significance of this study was:-

1. Providing information on the retention of vitamins A and D_3 upon processing of fortified soybean oil. This will ultimately guide optimizing our traditional food processing methods.

2. Providing information on the storage stability of these vitamins. Hence, guides for the future on how to store as such fortified products.

3. Guiding future oil fortification programs providing information on the physicochemical and nutrient retention properties of fortified oils during storage and processing

1.4. Objectives

1.4.1. General objective

- To evaluate the shelf life stability and retention of vitamin A and D_3 in fortified soybean oil during processing and storage

1.4.2. Specific objectives

- To evaluate the stability of vitamin A and D_3in fortified soya bean oil during storage
- To investigate retention of vitamin A and D_3in fortified soya bean oil upon Ethiopian common traditional food processing method
- To evaluate physicochemical characteristics and retention of vitamins A and D_3 of fortified soy bean oil during storage

2. Literature review

2.1. Worldwide micro nutrient deficiency

Micronutrient deficiencies – and the negative consequences of a diet lacking in essential vitamins and minerals/trace elements – continue to pose significant public health problems for many low- and middle-income country (LMIC) populations. This hidden hunger is more prevalent in vulnerable populations, including women of reproductive age and young children and female adolescents (Yang and Huffman, 2011). It is estimated that at least 1.6 billion people around the world suffer from anemia (McLean *et al*., 2009). Globally approximately 2 billion people suffer from chronic micronutrient deficiencies, with the most common deficiencies being caused by a lack of iron, folate, iodine, vitamin A, and zinc (Muthayya*et al*., 2013). Overall, micronutrient malnutrition has significant health and economic consequences (Black *et al*., 2013). Micronutrient deficiencies alone have been estimated to cost an annual GDP loss of (2-5) %in low and middle-income countries (LMIC), with direct costs estimated between US$ (20-30) billion every year (Dary, 2008). Consider anemia, which is estimated to cause a 17% reduction in productivity in heavy manual labor, as well as an estimated 2.5% loss of earnings due to lower cognitive skills (Ming, 2015). Annually, 40-60% of children 6-24 months of age in LMIC are at risk of impaired cognitive development due to iron deficiency, while anemia during pregnancy contributes to 20% of all maternal deaths, and reduced work productivity in adults (Black et al., 2013). Iodine deficiency causes some 35 million newborns to be born intellectually impaired as a result of poor maternal iodine status (UNICEF, 2015). The estimated intellectual losses for these newborns range from 7.4 to 15 IQ points.

2.1.1. Vitamin A deficiency and prevalence

Vitamin A is an indispensable nutrient that is required in small amounts by humans for the regular functioning of the visual system, the maintenance of cell function, for growth, epithelial cellular integrity, immune function, and reproduction. However, its deficiency leads to blindness and xerophthalmia, limits growth, weakens innate and acquired host defenses, exacerbates infection and therefore increases the risk of death (Sommer & West, 1996). Dietary requirements

for vitamin A are normally provided as a mixture of preformed vitamin A (retinol), which is present in animal source foods, and pro-vitamin A carotenoids, which are derived from foods of vegetable origin and which have to be converted into retinol by tissues such as the intestinal mucosa and the liver in order to be utilized by cells. Vitamin A deficiency is a global public health challenge. Worldwide, vitamin A deficiency affects 7 million pregnant women and about 3 million preschool-aged children, approximately one out of three children in sub-Saharan Africa (West, 2003). It is estimated that 254 million preschool-aged children throughout the world have low serum retinol levels and can, therefore, be considered to be clinically or subclinical vitamin A deficient (FAO, 2001). In the developing world, prevalence rates in this age group range from 15% up to as high as 60%, with Latin America, the Eastern Mediterranean, and the Western Pacific is at the low end of this range, and Africa and South-East Asia occupying the high end (FAO, 2001).

2.1.2. Vitamin D deficiency and prevalence

Vitamin D is a fat-soluble vitamin that is vital in immune system response, bone strength and muscular development. However, its deficiency exacerbates the severity of pneumonia, results in weak bones and teeth, and causes rickets, a bone-softening disease (WHO, 2011). Healthy adults obtain about 10-20% of vitamin D through dietary sources such as cheese, milk, or meat, and the remaining 80-90% through the conversion of UVB light from the sun. Vitamin D deficiencies are common in areas with little sunlight or among dark-skinned populations because the dark pigment in the skin can prevent absorption of the UVB light. It is estimated that 1 billion people are under the risk of vitamin D deficiency globally, infant, teenager, pregnant women and elderly are specifically more susceptible (Dror and Allen, 2010).

2.2. Food fortification

Food fortification refers to the addition of micronutrients to processed foods (WHO and FAO, 2005). In many situations, this strategy can lead to relatively rapid improvements in the micronutrient status of a population, and at a very reasonable cost, especially if advantage can be taken of existing technology and local distribution networks. Since the benefits are potentially

large, food fortification can be a very cost-effective public health intervention (Gera and Sachdev, 2012). However, an obvious requirement is that the fortified food(s) needs to be consumed in sufficient amounts by a large proportion of the target individuals in a population. It is also necessary to have access to and to use, fortificants that are well absorbed yet do not affect the sensory properties of foods. In most cases, it is preferable to use food vehicles that are centrally processed and to have the support of the food industry. Fortification of food with micronutrients is a valid technology for reducing micronutrient malnutrition as part of a food-based approach when and where existing food supplies and limited access fail to provide adequate levels of the respective nutrients in the diet foods (WHO and FAO, 2005). In such cases, food fortification reinforces and supports ongoing nutrition improvement programs and should be regarded as part of a broader, integrated approach to prevent MNM, thereby complementing other approaches to improve micronutrient status.

The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have identified four main strategies for reducing micronutrient malnutrition. Nutrition education leading to more diverse diets and better quality, Food fortification and bio-fortification, Supplementation and Disease control measures (FAO and WHO, 2006).Each of these strategies has a place in eliminating micronutrient malnutrition. To achieve maximum impact, the appropriate mix of these strategies should be in place simultaneously to promote the consumption and utilization of an adequate diet for all people in the world (FAO and WHO, 2006). Food fortification is considered a sustainable public health strategy because; it can reach wider at-risk populations through existing food delivery systems without requiring major changes in existing consumption patterns (Das *et al*., 2013).

2.2.1. Efficacy and effectiveness of fortification

The efficacy of food fortification has been demonstrated consistently and is now generally accepted (Bhutta*et al*., 2013).Recent systematic reviews suggest that micronutrient fortification of foods has the potential to significantly increase serum micronutrient concentrations and reduce deficiencies (Hennessy and Das, 2013). For instance, a recent systematic review of randomized and pseudo-randomized controlled trials included 60 acceptable trials on iron fortification and

iron biofortification. This review found that iron fortification of foods resulted in a significant increase in hemoglobin, serum ferritin and a reduced risk of anemia and iron deficiency (Gera and Sachdev, 2012). However, no effect was found on zinc concentrations, rate of infections, physical growth or mental and motor development (Gera and Sachdev, 2012). Vitamin A fortification efficacy has been established in the Philippines with fortified monosodium glutamate, margarine, and wheat buns trials (WHO and FAO, 2006). The efficacy of multiple micronutrient fortification has been demonstrated in several studies in Botswana, South Africa, and Tanzania. The effectiveness of fortification programs is not only determined by the efficacy of the fortified food but also by an effective implementation, monitoring, quality control and compliance and correction of identified issues. Starting in the early 20th century in the Americas and Europe, food fortification has made significant contributions to the elimination of deficiency diseases.

This has usually occurred when concurrent social changes and changes in the public health environment were in place to improve diets. For instance, marked declines in the prevalence of beriberi from thiamine deficiency have been observed in the southern US and Canada after voluntary and mandatory fortification of flours and bread with high-vitamin yeast (Fletcher et al., 2004). The mandatory addition of vitamin D to milk, which started in 1965 in Canada, has eliminated the wide spread problem with childhood rickets. Salt iodization has been, in placed since the 1920s in Switzerland and the USA, has reduced goiter prevalence globally and Universal Salt Iodization has led to the prevention of as many as 750 million cases of goiter in the past twenty-five years (Gorstein, 1989). After the introduction of vitamin-A fortified margarine in Denmark in 1917, the number of cases of xerophthalmia reported at Copenhagen hospital fell by more than 90% and had been eliminated by 1918 (Diosady and Mannar, 2013). A recent systematic evaluation of 76 studies and 41 contextual reports concluded that there is strong evidence of important and measurable improvements in micronutrient status and health outcomes in women and children after food fortification in wide geographic settings in LMIC. Fortifying with vitamin A was estimated to reduce the prevalence of deficiency in children under five from 33.3% to 25.7% globally.

2.2.2. Safety consideration of fortification

Ensuring safety requires that all partners – from industry to consumers - do their part in the fortification system. Ultimately, it is the responsibility of governments. Effective safety practices require the enforcement of legislation and regulations, as well as active and rigorous compliance to established standards. The possibility of over-consumption of nutrients in groups outside the target population, (and monitoring additional intakes and nutritional status associated with the consumption of fortified foods) should be actively and consistently monitored as an integral part of any fortification program (Dwyer *et al*., 2015).

2.2.3. Cost-effectiveness of food fortification

Although the cost-benefit ratio of fortification depends on local conditions and deficiency trends, resources, food vehicles and fortificants used, food fortification is generally recognized as one of the most cost-effective interventions (Horton *et al*., 2008). A review presented at the Future Fortified summit, (Horton *et al*., 2008) estimated that the benefit-cost ratio (BCR) of iron fortification in 10 countries with high levels of anemia is 8.7:1.0. For iodization of salt, a BCR of around 70:1 is demonstrated, while for folic acid a range extended from 11.8:1 for Chile to 30:1 in South Africa. Overall, after a thorough review of costs and benefits, the Copenhagen Consensus proposed micronutrient fortification – particularly iron fortification of staples and salt iodization – to be of the "best-buys" among the 30 interventions they considered for addressing the great challenges facing global development (Horton et al., 2008).

2.3. Types of fortification

Food fortification can take place in many forms. It is possible to fortify foods that are broadly consumed by the general population termed as universal (mass) fortification, to fortify foods designed for specific population subgroups, such as complementary foods for young children or rations for displaced populations known as targeted fortification and/or to allow food manufacturers to voluntarily fortify foods available in the market place called market-driven fortification (WHO, 2004). Mass (universal) fortification is nearly always mandatory, targeted fortification can be either mandatory or voluntary depending on the public health significance of

the problem it is seeking to address, and market-driven fortification is always voluntary, but governed by regulatory limits (WHO, 2004).

2.3.1 Mass fortification

Mass fortification is the form of a fortification in which one or more nutrients are added to foods commonly consumed by the general public, like cereals, condiments, and milk. It is frequently prompted, mandated and regulated by the government sector. Mass fortification is generally the best option when the majority of the population has an unacceptable risk, in terms of public health, of being or becoming deficient in specific micronutrients (WHO, 2004).

2.3.2 Targeted fortification

In targeted food fortification programs, foods intended at specific subgroups of the population are fortified, thereby increasing the intake of that particular group rather than that of the population as a whole. Examples include complementary foods for infants and young children, foods developed for school feeding programs, special biscuits for children and pregnant women, and rations (blended foods) for emergency feeding and displaced persons (WHO,2004).

2.3.3 Market-driven fortification

This type of fortification is a voluntary fortification applied to a situation whereby food manufacturer takes a business-oriented initiative to add specific amounts of one or more micronutrients to processed foods. Market-driven fortification is more widespread in industrialized countries, whereas in most developing countries the public health impact of market-driven food interventions is still rather limited (WHO, 2004).

2.4. Fortification of staple foods

2.4.1. Fortification of wheat flour

Since wheat flour is the primary staple food in a large number of countries in Europe, North America, the Middle East, and North Africa, and since consumption is increasing with the

globalization of diets, it is by far the most commonly used food vehicle in large-scale staple fortification programs. There are now 85 countries with legislation to fortify wheat flour produced in industrial mills. All the countries with mandatory legislation fortify wheat flour with iron and folic acid except Australia which does not include iron, and Congo, Nigeria, Philippines, UK and Venezuela, which do not include folic acid (Wirth *et al*., 2012). Five countries (Democratic Republic of Congo, Gambia, Namibia, Qatar, and United Arab Emirates) fortify at least half of their industrially milled wheat flour through voluntary efforts (Wirth *et al*., 2012).

Mandatory fortification of wheat flour has been reported as a key success in Morocco and Uzbekistan, with the latter having wheat flour enriched with iron and folic acid in half of the nation's flour mills (Wirth *et al*., 2012). Several studies have been conducted to determine the efficacy and effectiveness of wheat flour fortification with iron to reduce iron deficiency and iron-deficiency anemia (Zimmermann *et al*., 2005). A recent systematic review concluded that the effectiveness of flour fortification for reducing the prevalence of anemia is "limited" but evidence for reducing the prevalence of low ferritin in women was "more consistent" (*Pachon et al*., 2015). A recent study to determine whether anemia prevalence has been reduced among countries that fortify flour concluded that, anemia prevalence had, in fact, decreased significantly in countries that fortify flour with micronutrients, compared with countries that do not (Barkley et al., 2015). The study also found that countries that had been fortifying for a longer time were more likely to see reductions in anemia (Barkley *et al*., 2015).

2.4.2. Maize fortification

More than 200 million people rely on maize as a staple food, especially in sub-Saharan Africa, Southeast Asia, and Latin America (Pasricha*et al*., 2012). Estimates suggest that maize provides approximately 20% of the dietary energy (calories) consumed in the world (Pasricha*et al*., 2012). Sixteen countries have maize fortification programs in place. Mandatory maize flour fortification is happening in Brazil, Costa Rica, El Salvador, Kenya, Mexico, Namibia, Nigeria, Rwanda, South Africa, Tanzania, Uganda, the United States and Venezuela while Ghana, Malawi, and Mauritania. Although it is estimated that 48% of industrially milled maize flour is currently

fortified, one of the main challenges is that many people largely consume locally produced, unprocessed (and unfortified) maize meal milled at the village level or in small-scale hammer mills. Consequently, the number of small mills without fortification technology in a country will affect whether the fortification of maize flour is a feasible option (Pena-Rosas *et al*., 2014).

2.5. Vegetable oil fortification technology and feasibility

Fortification of vegetable oils and their derivatives (margarine, mayonnaise, etc.) with fat-soluble vitamins is technologically feasible (Nagy, 1995). Oil fortification consists of adding appropriate amounts of vitamin A concentrate to clarified, degassed oil at 45–50 °C. The solubility of commercially available vitamin A formulations in vegetable oils is excellent (Nagy, 1995). The most common commercial vitamin A formulation contains 1,000,000 IU vitamin A palmitate (300,000 mg/g) in a liquid form, stabilized with vitamin E ((alpha-tocopherol) or Butylated hydroxyanisole (BHA), Butylated hydroxyl toluene (BHT) mixture (Nagy, 1995)**.** To ensure that the vitamins are uniformly distributed, mixing takes place in vertical tanks that contain turbines or propeller agitators. Edible antioxidants (BHA and/or BHT) or natural antioxidants (e.g. alpha-tocopherol or ascorbyl palmitate) may be added to protect both the vitamin A and the oil.The stability of vitamin A in the oil depends greatly on the stability of the oil itself. Vitamin A oxidizes faster and loses its activity in the presence of oxidized oils (Bagriansky and Ranu, 1998).

To maintain vitamin A activity, fortified oil needs to be packaged in light-protected, sealed containers. Replacing the container headspace with inert gas will help retain the stability of both the oil and vitamin A before the container being opened. However, this is impractical in developing countries, so the micronutrient losses are compensated for by overage. The production and fortification of margarine-like semisolid products are carried out either in a batch or continuous process. The vitamin blend is premeasured according to the batch size of the margarine tanks and mixed with warm oil, in a ratio of 1:5, until a uniform solution is obtained (Bagriansky and Ranu, 1998). This premix is then incorporated into the margarine before the emulsifying process. This emulsion is chilled to partially crystallize the fat and packaged in continuously operating the equipment. Usually, the vitamin concentrate is supplied in containers,

which hold the specified amount of vitamin to be added per batch of oil. This avoids weighing errors by plant staff (Bagriansky and Ranu, 1998). The inclusion of vitamin A in vegetable oil does not require large investments in new technology. In continuous refining, investment is limited to piping, a small tank for pre-blending, a dosing pump, flow meters, and maybe an electronic control system. For batch production, a suitable tank should be equipped with an agitator and baffles to ensure an effective homogenization of the. Since vitamin A is readily soluble in edible oils, any degree of agitation of the oil that is being fortified is adequate to effect uniform distribution. Some agitation time must be allowed, depending on agitation intensity. Practically, a 30-min agitation is adequate, while intense agitation poses the risk of incorporation of air,(i.e. must be avoided to prevent vitamin A oxidation)(Diosady and Mannar, 2013).

2.5.1. Why fortifying edible oil?

Edible oils are widely consumed usually at uniform rates in particular regions (10-20g/day in African countries and up to 70-90g/capita/day in Asia), which makes oils attractive vehicle for fortification (Diosady and Mannar, 2013).Refined oils are consumed by an estimated 55% of the Ethiopian population in such quantities that an average woman could receive 47% of her recommended nutritional intake of vitamin A and an average child could receive 10-35% from fortified oil (FAO, 2010). Fortification programs for vitamin A in fats and oils are currently in place in 41 countries worldwide. Of these countries, well over half have mandatory fortification of margarine and/or oilsin general. It is important to note that approximately half of the countries with mandatory fortification are LMIC (Diosady and Mannar, 2013)**.** Edible oil is the number one recommended carrier of vitamin A and D, because, vitamin A and D are fat-soluble, they will readily dissolve in oil, thereby simplifying the fortification process.

2.5.2. The rationale for vitamin A and D fortification of oil

Fortification of food staples, including those provided in food aid programs can impact child health; directly by increasing children's vitamin A intake and indirectly by raising levels obtained by children from breast milk (Imdad*et al*., 2010). It may also reduce health problems associated with iron deficiency anemia and improve the mother's overall health. Oils and fats,

along with carbohydrates and proteins, are major components of the human diet. Oils provide energy, fat-soluble vitamins (vitamins A, D, and E), and essential fatty acids that are required for proper growth and development. The production of vegetable oils (canola, corn, cottonseed, coconut, olive, palm, peanut, safflower, soybean, and sunflower) is high throughout the world, and consumption is increasing, especially among lower socioeconomic groups ((Diosady and Mannar, 2013)**.** Consumption of vegetable oils over animal fats is preferable because vegetable oils typically contain much less saturated fat, and they contain no cholesterol. The rationale for oil fortification is based on the following criteria, which are commonly applied to assess vehicles for fortification.

A. Technical feasibility. Vegetable oils are suitable vehicles for fortification with the fat-soluble vitamins A, D, and E, as the production and refining of oils is a centralized process. These vitamins form a true solution and can be uniformly distributed in oil. The stability of vitamin A is greater in oils than in any other food, and oil facilitates the absorption of vitamin A by the body. Mixing is quite simple and can often be done with equipment readily available at mills, such as normal agitators and dosifiers. At low concentrations, vitamin A changes neither color, taste, and shelf life nor other product parameters of oils, which are highly relevant for producers and consumers. Stabilized vitamin A forms remain active in the end product, even when used for frying (Anon, 2009).

B. Human consumption levels. Vegetable oils are consumed by almost everyone; thus, it is possible to improve people's access to fat-soluble vitamins through oil fortification. In many countries, oil consumption is uniform: 10–20 g/capita/day in African countries and up to 70–90 g/capita/day in Asia. The near-universal consumption of oils ensures good coverage of populations, and the average consumption level allows moderate fortification levels for achieving public health impact. It is reasonable to assume that, ultimately, increased vitamin A intake from oils will result in a widely distributed, homogeneous improvement in the vitamin A status of affected populations (Anon, 2009).

C. Industry concentration. Oil milling is usually capital-intensive and thereby concentrated, with only a few oil mills serving the majority of national demand. Small-scale community

production is rare. Self-sufficient home growing and crushing of oilseeds, particularly in parts of Africa, constitute only a small fraction of oil use (Anon, 2009).

D. Cost-effectiveness. Oil fortification is very cost-effective. Since vitamin A can be added in a concentrated and stable form, the cost of equipment is moderate. Success requires widespread consumption of the fortified edible oil product, specific to each country. Where oil fortification is not yet possible or established, the addition of vitamin A to existing flour fortification programs can be technically feasible. Other vehicles are suitable for targeted fortification programs but do not have equivalent nationwide reach (e.g. milk) or are technically and economically more challenging (Anon, 2009).

2.6. Vitamin A and D stability and retention in oil during storage and food preparation

2.6.1. Stability of vitamin A during storage

The stability of vitamin A is key to the effectiveness of oil fortification. Vitamin A has to survive the supply chain; from its production to processing, storage, retail, and ultimately household use through the course of the whole shelf life of the product (often up to 24 months). Degradation of vitamin A cannot easily be countered by overages in dosage, as the variation in degradation is often unpredictable (Atwood *et al*., 1995). The quality of vitamin A used for fortification has the greatest impact on stability. Vitamin A is sensitive to light, oxygen, moisture, and to some extent heat, in sealed containers, while vitamin A is stable, as it is well protected from moisture and oxygen. Stability of vitamin A decreases significantly under direct UV light exposure, whereas indirect light, such as normal daylight hardly affects the stability (Atwood *et al*., 1995).

2.6.2. Effect of antioxidants on vitamin A in oil

Fats are oxidized by a free-radical-initiated chain reaction. Under normal conditions, the oils oxidize to form aldehydes, which have the characteristic rancid flavor (Mag and Diosady, 1995). Free-radical oxidation is catalyzed by metal ions. The rate of oxidation depends on the availability of free radicals through the initiation step of hydro-peroxide formation as well as on the presence of species that terminate the free-radical chain reaction. Thus, metal chelators that

remove metal ions from the system and free-radical scavengers such as phenolic compounds can dramatically retard the rate of fat oxidation. Since vitamin A gets oxidized by the same mechanism as the fat, vitamin A dissolved in oil will be greatly affected by the availability of free radicals and antioxidants in the system. Peroxide value is a measure of the oxidative stability of oil and is a critical parameter in ensuring stable fortified oil (Mag and Diosady, 1995).

Vitamin A competes for free radicals with the oil and acts as an antioxidant. Accordingly, free-radical scavengers, such as phenolic antioxidants, will protect both the oil and the added vitamin A from oxidative degradation. Tocopherols are good antioxidants for oils and vitamin A. When they supplement the naturally occurring tocopherol in crude vegetable oils or replace them after their reduction during refining, tocopherols are much more expensive than phenolic antioxidants. The most widely used synthetic antioxidants are BHA, BHT, and tertiary-butyl hydroquinone (TBHQ). BHA and BHT are allowed in food at levels up to 200 ppm in fats and oils. While TBHQ is more effective, its use is restricted in some jurisdictions (Mag and Diosady, 1995). Natural, organic antioxidants such as rosemary extract contain mixtures of similar phenolic compounds. All these compounds can be used in conjunction with metal chelating agents such as phosphates or citric acid. As the phenolic antioxidants are volatile at high temperatures, theyare less effective than tocopherols in protecting vitamin A at high frying temperatures (Mag and Diosady, 1995).

2.6.3. Stability of vitamin A during storage in sealed containers

In sealed and opaque containers that protect vitamin A and oil from light and air, losses of the vitamin are negligible for up to a year (Bagriansky and Ranum, 1998). Studies by Favaro*et al*., (1991) reported high retention after 9 months of storage in sealed containers at 23 °C but considerable losses at 18 months. Bauerenfeid et al. reported 91 % vitamin A retention after 6 months. Hoffman LaRoche reported that 90–95 % of vitamin A was retained in soybean oil after 6 months' storage at 20–25 °C—superior to retention in margarine over the same period (85–90 %). Studies of vitamin A retention during shipment (average 2–4 months) have shown values of 87–98 % (Atwood *et al*., 1995).

2.6.4. Stability of vitamin A during storage in open containers

Bagriansky and Ranum(1998), also reported on two studies that considered the stability of fortified oil in open cans. Studies by Favaro*et al*.,(1991)have shown no difference in stability between sealed cans and open cans for the first 6 months, even in the presence of light. However, after 3 additional months, only 48 % of original vitamin A level was retained in the opened cans exposed to light vs. 76 % in opened cans kept in the dark. Atwood *et al*.,(1995) reported an average of 70–88 % of the original vitamin A remaining after 30 days in open pails exposed to light, air, and temperatures of up to 35 °C (Atwood *et al*., 1995).

2.6.5 Retention of Vitamin A during food preparation

Mag and Diosady (1995), reviewed oil fortification technology for the Micronutrient Initiative. They reported that when vitamin A is used in foods that are subjected to severe heating, significant losses can occur, depending on temperature and time of heating. This is important in countries in which the practice of sautéing foods is widespread. Usual sautéing conditions involve heating the food with the oil to 150–160 °C for 5–10 min (Atwood et al., 1995). Synthetic antioxidants were found to have little effect on the retention of vitamins. The lack of protection from these compounds is well known, i.e. they are volatile at high temperatures. Tocopherols, however, have been shown to persist under sautéing conditions (Food Standards Association (FSA) and can therefore be expected to confer a measure of stability under these conditions. However, no direct evidence of this is a available. Commercial vitamin A palmitate is available with alpha-tocopherol (vitamin E) added and may need to be considered for oil fortification in some markets. Previous studies confirmed a wide variation in vitamin A stability during cooking, depending on time and temperature. The studies indicated that the deterioration of vitamin A in cooking oil is highly related to the treatment temperature. They grouped studies by temperature. Vitamin A is quite stable in Boiling, simmering, and stewing (100–120 °C) types of food preparation even when heated over an extended period (Favaro*et al*., 1991).When oils are subjected to light or deep frying (130–170 °C) vitamin A can lost at increasing rates (Favaro*et al*., 1991).

2.6.6. Toxicity considerations of vitamin A

Vitamin A is toxic in excessive amounts. Toxicity symptoms are a function of amount and length of time of excessive intake. Toxic symptoms have been reported from continued daily doses of 12,000–15,000 REs (40,000– 50,000 IUs) with adults and 7,500 REs with children (Anon, 1995). These are more than 10 times the recommended dosages. The only potential toxicity hazard is an overdose of highly concentrated pharmaceutical preparations of vitamin A. The pro vitamins (carotenes) are not toxic. A fortification program gains some additional safety from toxicity if a part of the vitamin A activity is supplied via beta–carotene (Anon, 1995). Edible oil fortification is generally considered safe. No incidents of intoxication have been reported. Even though vitamin A in pure form can be toxic in high dosage, in oil it is practically impossible for the consumer to exceed safety limits. For toxic effects, adults would need to consume more than a liter of edible oil daily. Accidental major over-fortification has not been observed so far (Favaro et al., 1991). Even though homogeneity in mixing the oil and vitamin A could be a technical challenge for some producers, the variation in vitamin A content in over-fortified batches remains far below safety levels. However, under-fortification—through technical challenges, unstable quality of vitamin A, or in a deliberate effort to cut costs—is observable in the absence of effective regulatory control systems (Favaro*et al*., 1991).

2.6.7. Stability of vitamin D in oil

Many researchers reported that vitamin D is moderately stable on many foods during storage and retain better on moderate heat processing. Denmark's research paper on the stability of vitamin D_3 and vitamin D_2 in oil, fish, and mushrooms after household cooking concluded that no significant difference was found between the retention of vitamin-D_3- and vitamin-D_2-spiked sunflower oil before and after household cooking.

2.6.8. Toxicity of vitamin D

Vitamin D is a fat-soluble vitamin that is naturally present in very few foods, added to others, and available as a dietary supplement. It is also produced endogenously when ultraviolet rays from sunlight strike the skin and trigger vitamin D synthesis (Institute of Medicine, Food and

Nutrition Board, 2010). Vitamin D obtained from sun exposure, food, and supplements is biologically inert and must undergo hydroxylation in the body for activation. It is one of the 24 micronutrients critical for human survival (Institute of Medicine, Food and Nutrition Board, 2010). Vitamin D promotes calcium absorption in the gut and maintains adequate serum calcium and phosphate concentrations to enable normal mineralization of bone and to prevent hypocalcemic tetany. It is also needed for bone growth and bone remodeling by osteoblasts and osteoclasts. According to the US, current guidelines consuming 400–800 IU of vitamin D should meet the needs of 97–98% of all healthy people. However, vitamin D is toxic at excess dosage. Long-term intakes above the UL increase the risk of adverse health effects. Most reports suggest a toxicity threshold for vitamin D of 10,000 to 40,000 IU/day and serum 25(OH)D levels of 500–600 nmol/L (200–240 ng/mL)(Cranney *et al*.,2006).

3. Materials and Methods

3.1. Study location

The studies were conducted in Addis Ababa University, Center for Food Science and Nutrition Laboratory and in Bless Agri Food Laboratory service PLC Laboratory.

3.2. Sample collection and sample size

The Refined, Bleached and Deodorized soya bean oil (RBDSBO) was collected from Kunifira Agro-Processing PLC. The samples were collected from different batches of production randomly on the first day of production. One sample for retention testing of vitamin A and D_3 during cooking, two samples for shelf-life stability testing across storage and one control, a total of four samples were collected. One liter of oil per sample was collected. Commercial Vitamin A (Retinol Palmitate) and Vitamin D_3 (cholecalciferol) were purchased from Global Alliance for Improved Nutrition (GAIN) and all standards were purchased from the local chemical supplier.

3.3. Chemicals and reagents

All reagents used in this study were of analytical grade.

3.4. Analytical method

3.4.1. Sampling technique

The collected samples were composited (mixed) in cleaned bowl. One sample was sealed in PET before adding of vitamin premix and three samples were sealed after adding of vitamin premix. The sealed oil samples were coded as Oil1, Oil2, Oil3 and Oil4 and stored in the Laboratory. Oil1 was used as a control, for chemical analysis of oil before fortification and for quantification of the amount of vitamin A and D_3in the oil before fortification. Oil2 was used for retention testing of vitamin A and D_3 during cooking. Oil3 and Oil4 were used for shelf-life stability testing across storage.

3.4.2. Chemical analysis of the oil

The chemical parameters of the oils were analyzed before and after fortification on the initial, third and sixth month of storage.

Determination of moisture and volatile matter content

The moisture content and volatile matter of the oil samples were determined by GEN LAB-OV/125/SS/FDIG/A-United Kingdom oven drying as described in ISO 662:2016. Briefly, the glass vessel was cleaned, dried in drying oven at 103 $\pm$ 2 ^{o}C for 30 minute, placed in desiccators to cool and weighed. Five gram of oil sample was weighed into the dried glass vessel and put in drying oven at 103 $\pm$ 2 ^{o}C for 1 hour. After cooling in desiccators to room temperature, the glass vessel with the sample was weighed again and again until a constant weight was obtained. Then the moisture and volatile matter content (%) were calculated using the following formula.

$$\text{Moisture content (\%)} = \frac{M1-M2}{M1-M0} * 100\% \quad \text{.....equation1}$$

Where:

$M_{0=}$the weight in gram of the glass vessel

$M_{1=}$ the weight in gram of the sample and the glass vesseland

$M_{2=}$ the weight in gram of the sample and residue.

Determination of peroxide value

The peroxide values of the oil samples as (meq O_2/kg) were determined using an official method of AOCS Cd 8–53 with slight modification. Based on the expected peroxide value of soya bean oil, 5g $\pm$0.1 g of oil was weighed into 250 mL of erlenmeyer flask. The sample was dissolved in 30 mL of glacial acetic acid-chloroform solution (3:2). After the addition of 0.5 mL of saturated potassium iodide solution; the mixture was kept in dark at ambient temperature for one minute.

After the addition of 30 mL of distilled water, 2mL of the saturated starch solution was added and the mixture had changed from dark purple to dark brown. The solution was titrated against $Na_2S_2O_3$ (0.01N) until the mixture was changed into white color. A blank determination was conducted and the concentration of peroxide value was calculated using the following formula.

$$PV = \frac{(Vs-Vb)*Nx1000}{W} \quad \text{.....equation2}$$

Where:

Vs = the volume of $Na_2S_2O_3$

Vb= the volume of $Na_2S_2O_3$ used in the blank sample

N = the normality of $Na_2S_2O_3$ (meq/mL used for titration) and

W = the weight of the cooking oil sample (g).

Determination of iodine value

The iodine value of the oil sample was measured using an official method of AOAC (2000). About 0.25 g of oil was weighed into 250 mL of a conical flask and dissolved in 10 mL of chloroform and 30 mL of hanus iodine solution and allowed it to stand in a dark for 30 minutes with occasional shaking. Then, 10 mL of 15% of potassium iodide was added and shaken thoroughly and 100 mL of freshly boiled and cooled water was added to wash down any free iodine from the stopper. The final solution was titrated against $Na_2S_2O_3$ (0.1N) until the yellow colorwas formed. Then 2-3 drops of the starch indicator were added and the mixture was changed into blue color and contained titration until the blue color completely disappeared. Then blank determination was conducted and concentration was calculated using the formula below.

$$\text{Iodine value}=\frac{(B-S)*N*0.127g/meq*100}{W} \quad \text{.....equation 3}$$

Where:

B= the volume of $Na_2S_2O_3$ for blank,

S = the volume of $Na_2S_2O_3$for sample

N = the normality of $Na_2S_2O_3$ and W = the weight of the sample

Determination of acid value

The acid value of the oil sample was measured using an official method of AOCS Ca 5a-40. The oil was mixed well before weighing. The mass of the test sample was taken based on the expected acid value listed in Table 1. According to WFP (2011) soya bean oil specification, the acid value must be below 0.6 mg maximum of KOH/g oil, which means less than one gram. Based on that, 20 g of oil sample was weighed into 250 mL of the conical flask. About 50 mL of 95% of ethanol containing 0.5 mL phenolphthalein (1%) was boiled at 70^{o}C and added to the flask. Then, the mixture was titrated against 0.1N of potassium hydroxide and shaken vigorously until changed from colorless to light pink color. A blank determination was conducted and the amount of acid value was calculated using the formula as follows.

Acid value $=\frac{56.1*V*N}{M}$…….…….equation 4

Where:

V = volume in (mL)of standard potassium hydroxide

N = normality of potassium hydroxide and

M= mass of test portion in g.

Determination of free fatty acid

The Free fatty acid value of the oil sample was measured using an official method of AOCS Ca 5a-40. The oil was mixed well before weighing. The mass of the test sample was taken based on the expected acid value listed in Table 1. According to WFP (2011) soya bean oil specification, the acid value must be below 0.6 mg maximum of KOH/g oil, which means less than one gram. Based on that, 20 g of oil sample was weighed into 250 mL of the conical flask. About 50 mL of 95% of ethanol containing 0.5 mL phenolphthalein (1%) was boiled at 70^{o}C and added to the flask. Then, the mixture was titrated against 0.1N of potassium hydroxide and shaken vigorously until changed from colorless to light pink color. A blank determination was conducted and the amount of free fatty acid was calculated using the formula written below.

Free fatty acid content (%) $=\frac{V*N*M}{10*m}$…….equation 5

Where:

V = volume in (mL) of the standard volumetric solution of potassium hydroxide

N = the normality of potassium hydroxide

M = the molar mass in (g per mole) of the acid chosen from Table 2 and

m = mass of the test portion.

Table1. Expected acid value and mass of test portion of fat and oil

Expressed acid value	Mass of test portion (g)	Accuracy of weighing of test portion(g)
Less than 1	20	0.05
1to 4	10	0.02
4 to 15	2.5	0.01
15 to 75	0.5	0.001
Greater than 75	0.1	0.0002

Sources: Ethiopian standard (ES ISO 660:2009)

Table 2. Fatty acids used to determine the free fatty acid levels in different types of oil

Types of fat/oil	Expressed as	Molar mass (g/mol)
Coconut oil, palm oil and similar other oils	Lauric acid	200
Palm oil	Palmitic acid	256
Oil from certain cruciferae	Erucic acid	338
All other fat	Oleic acid	282

Sources: Ethiopian standard (ES ISO 660:2009)

3.4.3. Fortification of soya bean oil with vitamin A and D_3

The vitamin premix, containing 1,000,000 IU/g of vitamin A (retinol palmitate), 100,000 IU/g of vitamin D3 (cholecalciferol) stabilized with vitamin E (alpha-tocopherol) purchased from GAIN was added to RBDSBO. 600 mg of vitamin premix was added to 3L of oil and homogenized using Omni international GLH-115, United State general laboratory homogenizer in a dark at room temperature for about 30 minutes. The oil was then blown with nitrogen gas and sealed on the PET bottle (Youna *et al*.,2015).

3.4.4. Storage of fortified oil samples

Oil2 was stored at room temperature and used for retention testing of vitamin A and D_3 during cooking on the first day of storage. Oil3 and Oil4 were stored at room temperature and 37°C respectively for six months for shelf-life testing. Addis Ababa room temperature which is closely 25°C can represent the middle cold area of whether condition and 37°C may represent the hottest areas of whether condition in Ethiopia.

3.4.5. Quantification of vitamin A and D_3in fortified soya bean oil before cooking

The amount of vitamin A and D_3 of the oil was quantified on the first week of fortification using HPLC UV–visible detector as described in the AOAC Official Method (2001.13) and AOAC Method (2002.05) with slight modifications.

3.4.6. Sample preparation

Saponification and extraction

Five gram of oil sample and standard were weighed appropriately in to flask, upon which 40 mL of 95 % of ethanol and 50 mg of pyrogallic acid were added to each sample and standard. This was followed by addition of 10 mL of 50% of KOH to each flask and standard. Then, the whole content was placed in water bath at 80°C for 45minutes. Then the flasks were cooled to room temperature using cooled water and 10 mL of glacial acetic acid was added into each flask to neutralize KOH. The sample and standard were extracted five times with hexane, the pooled extracts were then washed with 50 mL water until the aqueous layer appeared colorless when adding 2-3 phenolphthalein drops. Then, the mixture was filtered through a 0.45 μmMSÒNylon membrane filter and concentrated to dryness using rotary evaporator under nitrogen gas at 40 °C. The extract was reconstituted in 10 mLof pure methanol for vitamin A and in methanol–acetonitrile (4:1) for vitamin $D_3$and passed through 0.45 μmChromafil®Xtrafilter before HPLC injection (AOAC Official Method (2001.13) and AOAC Method (2002.05)).

Chromatographic determination of vitamin A

The HPLC system was started and allowed to warm up and equilibrated with mobile phase flow rate of 1mL/min. Then, vitamin A standard that has been saponified along with the sample was injected into HPLC and the mobile phase was adjusted to achieve a resolution of 1.5 or better for *cis* and *trans*forms. Then the sample was injected into the HPLC UV-visible detector and retinol was determined at a wavelength of 326 nm and 2.5 min retention time. Finally, the concentration of vitamin A on the sample was calculated by the following formula (AOAC Official Method (2001.13)).

Vitamin A mg/Kg (as retinol) $=\frac{A}{W} * 10$ ……..……equation6

Where:

A = the total test sample peak area of all *trans* and 13-*cis* retinol

10 = the dilution factor of the test portion and

W= weight of test portion (g)

Chromatographic determination of vitamin D_3

HPLC system was started and allowed to warm up and equilibrated with mobile phase flow rate of 1mL/min. Mobile, phase Methanol–acetonitrile (4:1 ratio, v/v) was prepared for quantitative analysis. The stability of the retention time was checked by injecting the working standard solution and the retention time was flocculated less than ±1%. The sample was injected into HPLC UV-visible detector and vitamin D_3 was determined at wavelength of 266 nm and the retention time 4.5 minutes. Finally, the concentration of vitamin D_3 on the oil sample was calculated using the following formula (AOAC Method (2002.05)).

Vitamin D3 mg/Kg$=\frac{AD3}{W} *10$…….……equation 7

Where:

AD3 = peak area of vitamin D_3

W = the weight of the test portion and

10 = dilution factor of test portion

3.4.7. Quantification of vitamin A and D_3 in cooked stew

Stew preparation

The most common type of stew in Ethiopia (Shiro) was prepared following the same commonly used traditional way of cooking. The entire ingredient used for the preparation of stew was weighed before cooking and the mixture also was weighed after cooking to know the mass fraction of the oil on the mixture. A known amount of oil was used to prepare the stew by heating at a temperature of 250°C, all the ingredients including water were added and the mixture was heated on standard laboratory hot plate till cooking was finished. During cooking the time and temperature were recorded. The whole content was cooked for about 1hour and 20 minutes at 250°C.

Saponification and extraction

Five gram of stew sample and standard was weighed appropriately into flask, 40 mL of 95 % of ethanol and 50 mg of pyrogallic acid were added to each sample and standard. Then, 10 mL of 50% of KOH was added to each flask and standard and placed on water bath at 80°C for 45minutes. The flasks were cooled to room temperature using cooled water and 10 mL of glacial acetic acid was added into each flask to neutralize KOH. The sample and standard were extracted five times with hexane. The pooled extracts were then washed with 50 mL water until the aqueous layer appeared colorless upon addition of2-3 phenolphthalein drops. Then, the mixture was filtered through a 0.45 μmMSÒNylon membrane filter and concentrated to dryness using rotary evaporator under nitrogen gas at 40 °C. The extracts were reconstituted in 10 mLof pure methanol for vitamin A and in methanol–acetonitrile (4:1) for vitamin $D_3$and passed through 0.45 μmChromafil®Xtrafilter before HPLC injection (AOAC Official Method (2001.13) and AOAC Method (2002.05)).

Chromatographic determination of vitamin A

HPLC system was started and allowed to warm up and equilibrated with mobile phase at a flow rate of 1mL/min. Then, vitamin A standard that has been saponified along with the sample was injected into HPLC and the mobile phase was adjusted to achieve a resolution of 1.5 or better for *cis*and*trans*forms. Then, the sample was injected into the HPLC UV-visible detector and retinol was determined at wavelength of 326 nm and with retention time of 2.5 minutes. Finally, the concentration of vitamin A on the stew sample was calculated by the following formula (AOAC Official Method (2001.13)).

Vitamin A mg/Kg (as retinol) $=\frac{A}{W} * 10$……equation 8

Where:

A = total test sample peak area of all *trans* and 13-*cis* retinol

10 = dilution factor of test portion in and

W= weight of test portion in (g).

To know the concentration of vitamin A on the oil, the mass fraction of the oil was calculated by dividing the mass of oil to the total mass of the mixture and multiplied by the weight of the test portion. Then the concentration of vitamin A on the oil was calculated using the following formula.

Vitamin A mg/Kg (as retinol) $=\frac{W*C}{Mf}$……equation 9

Where

W = mass of the test portion in (g), C = the concentration of vitamin on the mixture in ppm and Mf= the mass fraction of the oil in (g)

Chromatographic determination of vitamin D_3

HPLC system was started and allowed to warm up and equilibrated with mobile phase flow rate of 1mL/min. Mobile, phase Methanol–acetonitrile (4:1 ratio, v/v) was prepared for quantitative analysis. The stability of the retention time was checked by injecting the working standard solution and the retention time was flocculated less than ±1%. The sample was injected into HPLC UV-visible detector and vitamin D_3 was determined at wavelength of 266 nm and the retention time 4.5 minutes. Finally, the concentration of vitamin D_3 on the oil sample was calculated using the following formula (AOAC Method (2002.05)).

$$\text{Vitamin D3 mg/Kg} = \frac{AD3}{W} * 10 \quad \text{……equation 10}$$

Where:

AD3 = peak area of vitamin D_3

W =the weight of the test portion and

10 = dilution factor of test portion.

To know the concentration of vitamin D_3 on the oil, the mass fraction of the oil was calculated by dividing the mass of oil to the total mass of the mixture and multiplied by the weight of the test portion. Then the concentration of vitamin D_3 on the oil was calculated using the following formula.

$$\text{Vitamin D3 mg/Kg} = \frac{W * C}{Mf} \quad \text{…… equation 11}$$

Where:

W = mass of the test portion in (g), C = the concentration of vitamin D_3 on the mixture in ppm and Mf= a mass fraction of the oil in (g)

3.4.8. Shelf life stability testing of Vitamin A and D_3in fortified soya bean oil across storage

The shelf life of oil was determined using normal shelf life testing (NSLT). Oil3 (stored at room temperature and Oil4 (stored at37°C) were stored for six months. The vitamin A and D_3 content were analyzed in the initial, third and sixth months. The sample preparation and chromatographic determination were done following the same mentioned procedure described above. Percent of vitamin A and D_3 retention were calculated at the third and six months of storage using the following formula (Youna *et al*.,2015).

$$\% \text{ of retention} = \frac{(\text{vitamin})t}{(\text{vitamin})0} * 100 \quad \text{.....equation12}$$

Where:

$(\text{Vitamin})^0$=the initial content of vitamin

Vitamin)$t^=$the final content of vitamin

3.4.9. Method validation

To evaluate the performance of the analytical instrument; the LOD, LOQ, precision, recovery, linearity and the working range were primarily identified.

Identification

Identification of vitamins was determined by retention time of individual vitamin A and D3 injecting at the same condition and its precision was determined by percent of relative standard deviation (%RSD).

Limit of detection

The limit of detection was defined as the minimum quantity of vitamin substance giving a signal-to-noise ratio of ≥ 3. The Limit of Detection was measured for vitamin A (Retinol),and D_3, by injecting standard solutions of different concentrations and measuring for each substance of the amount injected on the column and the S/N values.

Limit of quantification

The limit of quantification was defined as the lowest concentration of vitamins that can be determined with acceptable accuracy and precision. This also corresponds to the lowest concentration of the calibration curve which was set at 0.15 µg/mL for vitamin D_3 and 5µg/mL for vitamin A to give a signal-to-noise ratio of ≥ 10.

Precision

The precision of the method was evaluated through the repeatability of the method by assessing ten replicate injections of vitamin standard at the same concentration during the same day under the same experimental conditions to obtain an acceptable %RSD.

Standard curve and linearity

Standard curves were prepared for both vitamins by using the standard solutions covering the concentration range from 5-60 µg/mL for vitamin A and 0.15-3.6 µg/mLfor vitamin D_3. Peak areas of the different vitamins were plotted against the concentrations and linear regression analysis (un-weighted; regression line forced through zero) was used to calculate the equation and the correlation coefficient of the standard curves.

Recovery

Recovery was determined by spiking the oil sample with the known concentration of vitamin standard. The sample was initially analyzed without spiking and then twice each with spiking in the lower and upper working ranges. Besides, a reagent blank was measured.

Specificity and selectivity

The specificity/selectivity of the method was assessed by the visual inspection and comparison of chromatograms of blank samples and samples containing different amounts of vitamins (CRM, PT, and spiked Samples). Because of the separation power of the chromatographic column and the selectivity of the Uv detector, no interfering peaks were observed near the retention times of the vitamin in the blank samples.

Linear Working Range

The linear working range was found to be between 5μg/mL and 60μg/mL for vitamin A and between 0.15μg/mL and 3.6μg/mLfor vitamin D_3 with the method showing a strong correlation within these concentrations. As validation practices for the various parameters above showed, measurements within these linear working ranges were done with a suitable level of precision and accuracy.

Standard preparation

Stock standard solution (1 mg/mL) was prepared separately by dissolving 10 mg of the individual vitamin in 10 mL pure methanol for vitamin A and in methanol–acetonitrile (80+20) for vitamin D_3 and stored in darkness at 4°C. Working standard solutions were prepared daily by methanol dilution of the stock standard solutions in appropriate proportions into the concentrations of 20 μg/mL for A and 10 μg/mL for D_3.

3.5. Chemical analysis of soya bean oil across storage

The chemical property of the oil stored at room temperature and 37°C were analyzed before and after fortification. Also, these chemical properties of the oil were analyzed across storage at the third and sixth month of storage period. The method of analysis was the same with retention testing described above.

3.6. Experimental design and statistical analysis

The experimental design used in this study was Completely Randomized Design (CRD) and data were analyzed by analysis of variance (ANOVA) using statistical software SPSS version 20 and significance differences were tested at ($p < 0.05$).All laboratory analyses were performed in triplicate, and averages were presented.

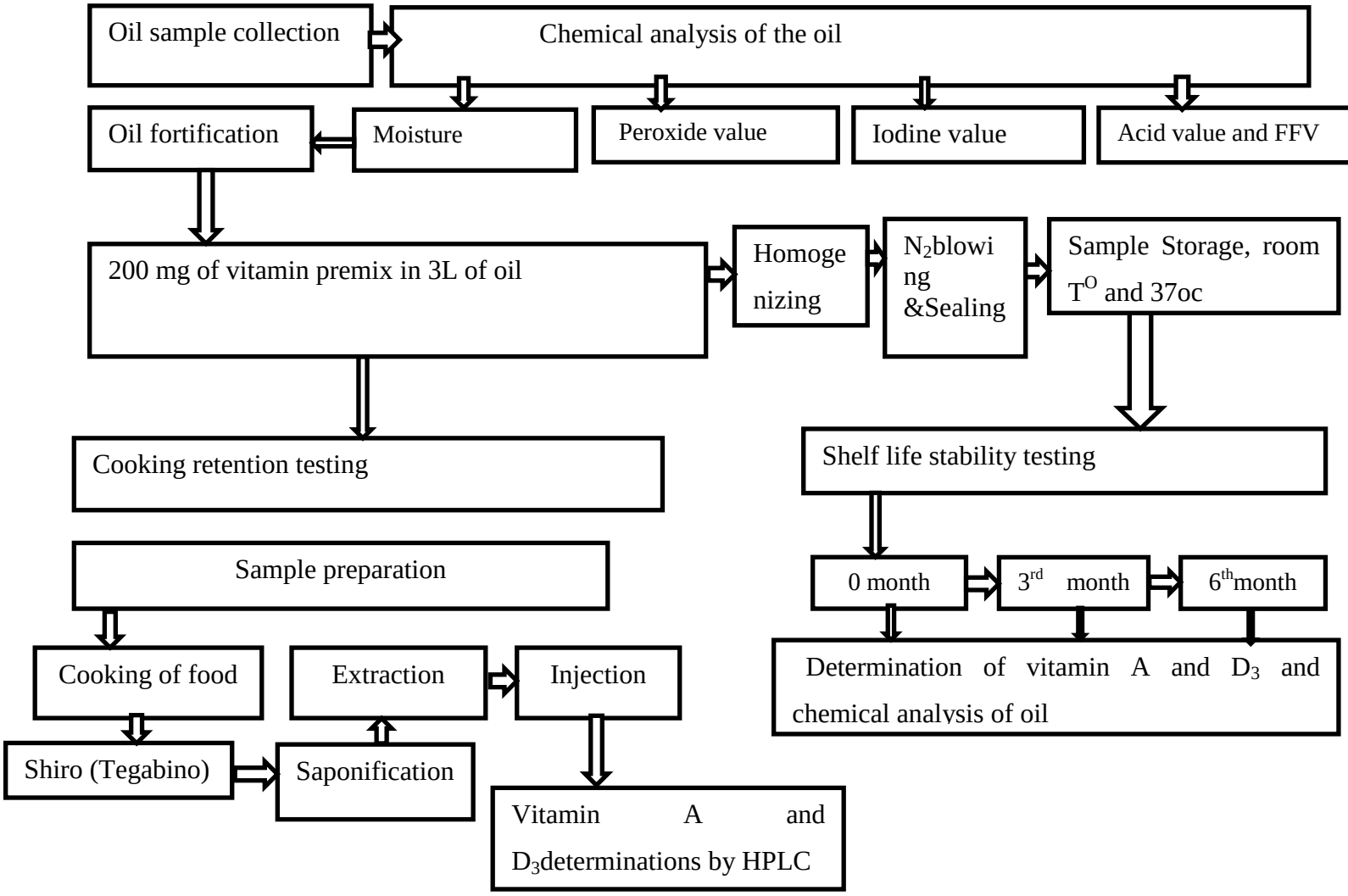

Figure 1. Experimental frame work of the study

4. Results and discussion

4.1. Chemical characteristics of soya bean oil before and after fortification

The chemical characteristics of the soya bean oil before and after fortification was reported in Table 3. There was no significant difference in all of the chemical parameters except free fatty acid value before and after fortification. The shelf-life stability of fortified vitamin in the oil depends upon the shelf-life stability of the oil, which depends on the chemical characteristics of the oil like peroxide value, acid value, moisture, iodine value and level of free fatty acid. For instance, low moisture content of oil is advantageous in terms of storage stability (Orhevba&Efomah, 2012).With this regard, the percentage moisture and volatile matter in both fortified and unfortified soya bean oil in this study were 0.1%, which is below the set maximum limit (i..e. < 0.2%) by World Food Program (2011).

Similarly, peroxide value (PV) is used as an indicator of deterioration of edible oils. In this study, the peroxide value of the unfortified and fortified soya bean oil were 1.0 and 1.2 meqO_2/Kg respectively (Table 3). Fresh oils have peroxide value below 10meqO_2/Kg (Vidrih*et al*.,2010). According to the recommendation by WFP (2011), the maximum peroxide value of fresh refined and fortified soya bean oil must not exceed 2 meqO_2/Kg. Also, as per Ethiopian standard edible oils should contain a PV below 10 meqO_2/Kgoil (Compulsory Ethiopian Standard CES-16, 2014; Compulsory Ethiopian Standard CES-19, 2014).Iodine value (IV) which indicates the degree of un-saturation in oils was found to be 118g of iodine absorbed/100g of oil in both fortified and unfortified soya bean oil (Table 3). The higher the IV, the more unsaturated fatty acid composition of fat and/or oil (Fakhri&Qadir, 2011; Scrimgeour, 2005).

Another key shelf life-determining factor of oil is the level of free fatty acid. Oils and fats are well characterized/distinguished mainly by their fatty acid composition.The levels of free fatty acid in unfortified and fortified oil were 0.087 and 0.098 respectively (Table 3). According to WFP (2010), the maximum FFA (%) in fresh refined and fortified soya bean oil must be 0.1%. As reported in Table3the chemical characteristics of the soya bean oil before and after

fortification had satisfied the set requirements by both WFP and ESA. Furthermore, this study indicated that fortification had no effect on the chemical parameter of oil.

Table 3. Chemical properties of soya bean oil before and after fortification at initial storage period

Parameters	Type of s**oya bean oil**	
	Unfortified oil	Fortified oil
Moisture &volatile mater (%)	0.10 ± 0.01[a]	0.10 ± 0.01[a]
Peroxide value (meqO_2**/Kg oil)**	1.00 ± 0.09[a]	1.20 ± 0.09[a]
Iodine value (g/100g oil)	118.00 ± 2.30[a]	118.00 ± 3.05[a]
Free fatty acid (%)	0.087± 0.001[b]	0.098± 0.001[a]
Acid value (mg KOH/goil)	0.17±0.02	0.20 ±0.06

Data were expressed as mean± SE. All analyses were done in triplicate. Mean values in the same row with different superscripts are statistically significant at $p < 0.05$.

4.2. Retention of vitamin A and D_3 during cooking

The chromatographic method of determination of vitamin A and D_3 was validated ahead of the sample analysis. All the quality control protocols were detailed in materials and methods section. In brief, the LOD for the vitamin A and D_3 determinations with S/N ≥3 were 1.83 and 0.09 respectively. The LOQ for vitamin A and vitamin D_3 were 5.5 µg/mL and 0.27 µg/mL respectively with S/N ≥ 10.The precision of the method was evaluated through the repeatability of the method by assessing ten replicate injections of vitamin standard at the same concentration during the same day under the same experimental conditions to obtain an acceptable %RSD. The % RSD for peak area of vitamin A and D_3 were 4.5 and 5.2 respectively. Standard curves were prepared for both vitamins by using the standard solutions covering the concentration range from 5-60 µg/mL for vitamin A and 0.15-3.6 µg/for vitamin D_3. Peak areas of the different vitamins were plotted against the concentrations and linear regression analysis (un-weighted; regression

line forced through zero) was used to calculate the equation and the correlation coefficient of the standard curves. The recovery was assessed by spiking the oil with standard solutions. Following this, the recovery rate was determined for the added quantity of standard and the mean recovery rate was recorded as 100 and 100.06% for vitamin A and D_3 respectively. The specificity/selectivity of the method was assessed by the visual inspection and comparison of chromatograms of blank samples and samples containing different amounts of vitamins (CRM, PT, and spiked Samples). Linear working range was found to be between 5μg/mL and 60 μg/mL for vitamin A and between 0.15μg/mL and 3.6μg/mL for vitamin D_3.

Table 4 showed the concentration of vitamin A and D_3 in fresh unfortified, fresh fortified and fortified cooked soya bean oil. There was a significant difference in vitamin A content among the three oil samples ($p<0.05$). Since vitamin A (retinol) is not found in plant based foods, the fresh soya bean oil had no vitamin A. After fortification, the vitamin A content of the fresh oil was 65.78mg/Kg. Meanwhile, after the fortified oil was used to cook Ethiopian tradition stew (Shiro) at 250º C for 1hr and 20min, the vitamin A concentration was reduced to 39.25mg/Kg. Apparently, this reduction might be due to the high temperature processing for prolonged time. Vitamin A is quite stable during boiling, simmering, and stewing (100-120 ºC), even when heated over an extended period (Favaro*et al*., 1991). Favaro*et al*. (1991) found that 99% of vitamin A was retained when fortified oil was added to rice and cooked for 15 minutes at 100-120 ºC. The same authors reported that 88% of vitamin A was retained when oil was added to beans and boiled for 90 minutes at 100-120 ºC. In this study, the oil was added to Shiro (commonly made of beans and spices) and boiled at 250º C for 1hr and 20 min. At this cooking time and temperature 60.04%vitamin A was retained. Previous study also reported that 64% of vitamin A was retained when the soya bean oil, corn oil, and safflower oil were added in different foods and cooked at more than 200 ºC for 30 minutes.

Whereas there was no significant difference in vitamin D_3 content between fresh and cooked oil, because vitamin D_3 is less sensitive to heat and light (Favaro*et al*., 1991. Many researchers reported that both vitamin D_2 and vitamin D_3 are moderately stable in many foods during storage and retain better on moderate heat processing. A study in Denmarkconcluded that no significant difference was found between the retention of vitamin-D_3- and vitamin-D_2-spiked sunflower oil

before and after household cooking. In the present study also there was no significant difference in vitamin D_3concentrationbefore and after household cooking. Another reason for acceptable retention of both vitamin A and D_3 in cooked food is that due to the presence of antioxidants. Some study has been documented the use of antioxidants to enhance the stability of vitamin A in oil (Favaro*et al*., 1991). Steenhoek, (1997) tested the stability of vitamin A with and without BHA in Indonesian vegetable oil during double frying conditions at temperatures up to 193º C. In the first test with 200 ppm of BHA, 77% vitamin A was retained. In the second test without BHA, 37 % of vitamin A was retained. Gopal et al, (1956) also found that, after 5 minutes of frying of Vanaspati (vegetable gee) at 200°C, retention of vitamin A was 71% with BHA and 60% without BHA. The gap between the two studies stated above was due to frying time. In our study vitamin E (alpha-tocopherol) was added to soya bean oil as an antioxidant and the cooking temperature and time were 250 º C and 1hour and 20 minutes correspondingly. In this scenario, more than 60 % of vitamin A was retained.

So the gap with the previous study could be due to the cooking time, temperature, types of oil and the type of antioxidant. Steenhoek and Gospal used antioxidant BHA and Indonesian oil and Vanaspati respectively for their experiment. Several sources suggested that TBHQand BHA might offer superior protection, particularly in soybean oil (Bauernfeind*et al*., 1953). Retention of vitamin A and D_3 during cooking on fortified soya bean oil has been studied in other countries, but not yet in Ethiopia. Traditional cooking practice in Ethiopia is quite different from the cooking and food preparation of other countries. As explained above most of the Ethiopian mothers have developed a culture of cooking the food at high temperatures for a long time to make the food delicious, which harms the retention of heat-sensitive nutrients, mostly vitamins. And also most of the Ethiopian foods are very spicy and ingredient rich, which have an effect on vitamin retention during cooking. Having this postulation, we inspired to evaluate the retention of vitamin A and D_3 in fortified soya bean oil during Ethiopian traditional cooking style to provide concrete input to edible oil producers, government stakeholders, and society.

The government of the Federal Democratic Republic of Ethiopia ratified that fortification of vegetable oil with vitamin A and D_3 is voluntary starting from 2011 E.C to reduce the deficiency of vitamin A and D_3 in the country. Because many studies indicated that the prevalence of

vitamin A and D_3 is severe in Ethiopia. So this study may give information on, how much vitamin can we add to oil and how much vitamin can be retained in Ethiopian traditional household cooking.

The retention in (%) of both vitamins was calculated using the following formula (Youna *et al.*, 2015).

% retention of vitamins=$\frac{(\text{vitamin})t}{(\text{vitamin})0} * 100$……..equation 13

Where:

$(\text{vitamin})^0$=vitamin content before cooking and

$(\text{Vitamin})^t$= vitamin content after cooking time

% retention vitamin A=$\frac{39.25}{65.78} * 100$=60.04%

% retention vitamin D_3 =$\frac{4.1}{4.21} * 100$=97.39%

Table4.Concentration of vitamin A and D_3in unfortified fresh, fortified fresh and fortified cooked soya bean oil (mg/Kg)

Type of soya bean oil	**Concentration of vitamins((mg/Kg)**	
	Vitamin A	Vitamin D_3
Unfortified fresh	0.00 ± 0.00^c	0.00 ± 0.00^b
Fortified fresh	65.78 ± 0.01^a	4.21 ± 0.01^a
Fortified cooked	39.25 ± 0.01^b	4.10 ± 0.01^a

Data were expressed as mean ± SE, all analyses were done in triplicate. Mean values in the same column with different superscript are significantly different at $p < 0.05$.

4.3. Chemical characteristics of fortified soya bean oil after3 months of storage at room temperature and 37°C

The chemical characteristics of unfortified fresh, fortified fresh, fortified stored at room temperature and at 37^{o}C of soya bean oil after 3 months of storage period was reported in Table 5. The peroxide value and free fatty acid level had significantly increased during storage, while no significant change was observed in moisture content and acid value. Even though peroxide was increased during storage, it was an acceptable value as recommended by WFP (2011)(i.e.<10meqO_2/Kg. Similarly, the free fatty acid level of the fortified soya bean oil significantly increased during the 3rdmonth of storage.

There was also a significant difference in PV between the fortified oil stored at room temperature and 37^{o}C, apparently the later with higher PV. The stability of vitamins on oil depends upon the physicochemical characteristics, storage condition and the packaging material of the oil. One of the important parameters used to assess the quality of soya bean oil is the peroxide value, which is an indicator of the level of lipid oxidation (Codex Alimentarius, 2013). According to the recommendation of Codex 210 Codex Alimentarius (2013) and WFP (2011) the PV of fortified soya bean oil must not exceed the upper limit (10meq O_2 /Kg during storage.

In the present study, the PV had increased with storage time and temperature. Another key parameter of oil quality assessment is free fatty acid value, which should be <1.15% in fortified soya bean oil as perWFP (2011) recommendation. The FFA% in all the oil samples in the present study was within the st limit. However, as compared to the value at the first month of storage, there was significant increment on 3rd month of storage. Moreover, the increment was also observed as the storage temperature increased. In fact, increased PV and FFA in edible oils with storage temperature and time was supported by previous studies too (Andarwulan et al., 2014).

Table 5. Chemical properties of unfortified fresh, fortified fresh, fortified stored at room temperature and at 37^{o}C of soya bean oil after 3 months of storage period

Parameters	**Type of soya bean oil**			
	Oil 1	Oil 2	Oil 3	Oil 4
Moisture &volatile mater	0.10 ± 0.01^{a}	0.10 ± 0.01 ^{a}	0.10 ±0.02 ^{a}	0.20 ±0.06 ^{a}
Peroxide value (meqO_2/**Kg oil)**	1.00 ± 0.09^{c}	1.20 ± 0.09^{c}	4.17±0.02^{b}	5.50 ±0.25^{a}
Free fatty acid **(%)**	0.09 ± 0.00^{b}	0.10 ± 0.00^{b}	1.13±0.01^{ab}	1.15±0.02^{a}
Acid value (mg KOH/goil)	0.17±0.02 ^{a}	0.20 ±0.06 ^{a}	0.27±0.01 ^{a}	0.30 ±0.12 ^{a}

Data were expressed as mean ± SE. All analyses were done in triplicate. Mean values in the same row with different superscripts are significantly different at p<0.05.Oil 1-control (unfortified oil); Oil 2: fortified fresh oil; Oil 3: fortified oil stored at room temperature; Oil 4: fortified oil stored at 37 ^{o}C.

4.4. Shelf life stability of the vitamin A and D_3during 3rd month of storage

The vitamin A and D_3 contents of fortified soya bean oil after 3 months of storage period was reported in Table 6 (p<0.05).Accordingly, the vitamin A content of the oil had significantly decreased after 3 months of storage period. In fact, the vitamin A content was more reduced in the oil stored at 37^{o}C.The amount of vitamin A lost was 4.36 and 4.42 % respectively. Favaro et al. (1991) reported that, in sealed and opaque containers losses of vitamin Aare negligible for a year time. Similarly, Hoffman and LaRoche (1999) reported that vitamin A in soybean oil stored at 20-25ºC retained 95to 100% of its original content over 3 months. Hence, keeping fortified soya bean oil in cool place better to reduce the possible loss of vitamin A due to exposure to temperature. Also incorporation of antioxidants in fortified oils will protect the loss of vitamins during storage (Favaroet al., 1991).For instance, with BHA the stability of vitamin A in fortified soya bean oil was 100-93% after 3 months of storage at 32 ^{o}C (Favaroet al., 1991). This is a similar finding with the present study, in which 96% of vitamin A was retained after 3 months of storage at both room temperature and 37^{o}C. In the present study vitamin E was added in the oil as an antioxidant.

As reported in Table 6,vitamin D_3 content of the oil had significantly decreased after 3 months of storage period ($p<0.05$). In both storage temperatures the loss was around 50%. Apparently, the more pronounced reduction during storage was mainly due to storage time. Many researchers reported that both vitamin D_2 and vitamin D_3 are moderately stable in many foods during storage. Hoffman and LaRoche (1999) reported that reduction of vitamin D_2 and D_3in oil and fat with increasing storage time. As compared to vitamin A, vitamin D_3 was highly reduced during storage. This indicated the higher stability of vitamin A than vitamin D_3 during storage.

The shelf-life stability of vitamin A and D_3 in fortified soya bean oil during storage have been studied in Europe and America several years ago, but not yet in our country, even in Africa. Commonly soya bean oil in developed countries is produced from genetically modified seeds. Previous studies indicated that soya bean oil produced from genetically modified seed has a longer shelf life than oil produced from organic seed. This is because the genetically modified soya bean seeds have less free fatty acid composition (low linoleic acid and linolenic acid) as compared to organic soya bean. Neff and List (1999) looked at how soya bean lines that were genetically modified for high C16:0 and high C18:0, changed the oxidative stability of natural oils. The stability of vitamins is dependent on the stability of the oil; if the oil is oxidized the vitamin concentration will be reduced upon storage(Morales and Przybylski, 2013). Soya bean oil in Ethiopia is produced from natural (organic) soya seed. Yet, as to our knowledge there was no study on the shelf stability of soya oil in Ethiopia. Thus, this study was aspired to estimate the shelf-life stability of vitamins A and D_3 in fortified soya bean oil (produced from organic seed) during storage. Accordingly, the retention (%) of both vitamin A and D_3 on 3^{rd} month of storage was (95.64, 47.27)% and (95.58, 46.56)% at room temperature and 37^{o}C, respectively. At both storage temperatures highest retention was observed in vitamin A concentration (Table 6).

Table 6. Concentration of vitamin A and D_3 (mg/Kg) of fresh fortified and unfortified soya bean oil stored at room temperature and at 37 ^oC for 3 months

Type of soya bean oil	Concentration of vitamins (mg/Kg)	
	Vitamin A	Vitamin D_3
Fresh, unfortified oil	0.00 ± 0.00^d	0.00 ± 0.00^c
Fresh, fortified oil	65.78 ± 0.01^a	4.21 ± 0.01^a
Fortified oil stored at RT	$62.91\pm0.0b^b$	1.99 ± 0.01^b
Fortified oil stored at 37^oC	62.87 ± 0.01^c	1.96 ± 0.02^b

Data were expressed as mean ± SE, all analyses were done in triplicate. Mean values in the same column with different superscript are significantly different at $p < 0.05$.Oil 1-control (unfortified oil); Oil 2: fortified fresh oil; Oil 3: fortified oil stored at room temperature; Oil 4: fortified oil stored at 37 ^oC.

4.5. Chemical characteristics of the oil during 6thmonth of storage

As reported in Table 7, there was a significant difference in chemical characteristics of fortified soya bean oil during six month of storage period. The moisture/volatile matter content, PV and FFA had increased during six months of storage significantly. Moreover, the higher storage temperature (37^oC) had an obvious effect in deteriorating the quality of the oil as compared to room temperature storage. However, though the values had increasing trend, still the PV and AV during six months of storage were within the acceptable limit. But the value of moisture and free fatty acid for oil4 was unacceptable value (i.e. 0.25 and 2)% respectively. Free fatty acids (FFA) are produced by the hydrolysis of oils and fats. The level of FFA depends on time, temperature and moisture content of the oil. Since FFA is less stable in neutral oil, they are more prone to oxidation and rancidity. Thus, FFA is a key feature linked with the quality and commercial value of oils and fats. The remarkable rise of free fatty acid in the fortified soya bean oil stored at 37 ^oC might be linked with the increased moisture content. This might have accelerated the hydrolysis process in the oil (Nielsen, 1994).

Table 7.Chemical properties of unfortified fresh, fortified fresh, fortified stored at room temperature and at 37^{o}C of soya bean oil after 6 months of storage period

Parameters	Type of soya bean oil			
	Oil 1	Oil2	Oil 3	Oil4
Moisture &volatile mater	0.10 ± 0.01^{c}	0.10 ± 0.01^{c}	0.15±0.01^{b}	0.25±0.02^{a}
Peroxide value (meqO_2/Kg oil)	1.00 ± 0.09^{c}	1.20 ± 0.09^{c}	5.17±0.01^{b}	7.00 ±0.04^{a}
Free fatty acid value (%)	0.09 ± 0.00^{c}	0.10 ± 0.01^{c}	1.15±0.02^{b}	2.00 ±0.01^{a}
Acid value (mg KOH/goil)	0.17±0.02^{b}	0.20 ±0.06^{b}	0.30 ±0.11^{b}	0.40 ±0.02^{ab}

Data were expressed as mean ± SE. All analyses were done in triplicate. Mean values in the same row with different superscripts are significantly different at p<0.05.Oil 1-control (unfortified oil); Oil 2: fortified fresh oil; Oil 3: fortified oil stored at room temperature; Oil 4: fortified oil stored at 37 ^{o}C.

4.6. Shelf life stability of the vitamin A and D_3 during 6th month of storage

In this study, at six months of storage the vitamin A concentration in the oil had decreased significantly (p<0.05). Also, at 37^{o}C storage temperature the loss was significantly higher than room temperature storage (Table 8).Briefly, the vitamin loss in the fortified oil stored at room temperature and 37 ^{o}C was 5.42 and 8.77 % respectively. Similarly, a minimal/negligible loss of vitamin A in fortified oil kept in sealed and opaque containers that protected light and air for a year, was reported by (Favaro*et al.*, 1991).In the present study, the fortified soya bean oil was sealed in PET packaging and stored at room temperature and 37°C for 6 months and retained 94.58% and 91.23% vitamin A, respectively**.** Hoffman and Roche (1999) reported retention of vitamin A in margarine to be (90-95) % and (85-90) % after 3 months and 6 months of storage respectively, in sealed can container at 25º C.

Similarly, at six months of storage the vitamin D_3 concentration in the oil had decreased significantly (p<0.05) (Table 8). Also, at room temperature and 37^{o}C storage the vitamin D_3loss was 59 and 62% respectively. Compared with vitamin A, apparently the vitamin D_3 loss upon the same storage period and temperature was higher. In fact, many researchers reported that both vitamin D_2 and vitamin D_3 are moderately stable in many foods during storage(Hoffman and LaRoche, 1999).

Table 8 .Concentration of vitamin A and D_3 (mg/Kg) in fresh fortified and unfortified soya bean oil stored at room temperature and at 37 ^oC for 6 month

Type of soya bean oil	Concentration of vitamins (mg/Kg)	
	Vitamin A	Vitamin D_3
Fresh, unfortified oil	0.00 ± 0.00^d	0.00 ± 0.00^d
Fresh, fortified oil	65.78 ± 0.01^a	4.21 ± 0.01^a
Fortified oil stored at RT	62.11 ± 0.01^b	1.71 ± 0.02^b
Fortified oil stored at 37^oC	60.01 ± 0.01^c	1.60 ± 0.02^c

Data were expressed as mean $\pm$ SE. All analyses were done in triplicate. Mean values in the same column with different superscripts were significantly different at $p<0.05$. RT: Room Temperature

5. Conclusion and recommendation

5.1. Conclusion

There was no significant difference in all of the chemical parameters except free fatty acid value before and after fortification. The peroxide value of the unfortified and fortified soya bean oils were within the set limit by WFP. Thus, this study supported the idea that fortification had no significant effect on the chemical characteristics of the oil. The fortified soya bean oil was used to cook Ethiopian tradition stew (Shiro) at 250^0C for more than one hour. Upon which, significant amount of vitamin A was lost, whereas the vitamin D_3 loss was not significant.

After 3 and 6 months of storage, the peroxide value and free fatty acid level of the fortified oil had significantly increased. At both storage periods, the higher storage temperature (37^oC) had a more deteriorating effect on the quality of the oil as compared to room temperature storage. Both vitamin A and D_3concentration of the oil had significantly decreased after 3 months of storage period, loss at higher temperature storage being more. At both storage temperatures highest retention was observed in vitamin A concentration. Similarly, upon six months of storage the vitamin A and D_3concentration in the oil had decreased significantly. Compared with vitamin A, yet again the vitamin D_3 loss upon the same storage period and temperature was higher. In conclusion, keeping fortified soya bean oil in cool place was better to reduce the possible loss of the fortifying vitamins. Also, Ethiopian traditional Shiro preparation method should be optimized to obtain maximum retention of the vitamins keeping the sensory quality acceptable.

5.2. Recommendation

- Since vitamin A was heat sensitive, it is advisable to cook the fortified oil in less than $200^{O}C$**.** Also, further study should be conducted to optimize most of high temperature Ethiopian food processing methods to have maximum retention of the vitamins.
- Similarly, since vitamin D_3 is sensitive to storage time, it is not recommended to store the fortified oil for a long time.
- Awareness creation to the consumer is very important on proper cooking and storage of fortified edible oils.
- From this study, it is recommended that adding 65-85 ppm of vitamin A to soya bean oil can compensate more than 50 % the Recommended Dietary Allowance (RDA) for adult men, women and almost 100% for children with an estimation of 50% loss and 15g/day oil consumption.

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7. Annex

Validation of the chromatographic method

To evaluate the performance of analytical instrument; the LOD, LOQ, precision, recovery, linearity and the working range were primarily identified and the following results were obtained.

Identification

Identification of vitamins was determined by retention time of individual vitamin A and D_3 injecting at the same condition and its precision was determined by percent of relative standard deviation (%RSD).The %RSD values in Table 9 indicate high precision and good reproducibility of the method which is less than (AOAC Official Method (2001.13) and AOAC Method (2002.05)).

Table 9.Summary of retention time (RT) for vitamin A and D_3 standards

Vitamins	**Retention time (min)** **Mean**	**N**	**SD**	**%RSD**
Vitamin A	2.50	6	0.034	1.36
Vitamin D3	4.53	6	0.023	0.51

Limit of detection and quantification

The limit of detection was determined based on the signal/noise ratio. Standard solutions of different concentrations were injected to determine the limit of detection. The concentration at which the peak area was still at least 3 times higher than the noise was defined as the limit of detection. The limit of quantification was determined based on the signal/noise ratio. Standard solutions of different concentrations were injected to determine the limit of quantification. The

concentration at which the peak area was still at least 10 times higher than the noise was defined as the limit of detection.

Table 10. Limit of detection and quantification for vitamin A and D_3

Vitamins	LOD (ppm)	LOQ (ppm)
Vitamin A	1.83	5.5
Vitamin D_3	0.09	0.27

Accuracy and precision

The precision of the method was evaluated through the repeatability of the method by assessing ten replicate injections of vitamin standard at the same concentration during the same day under the same experimental conditions to obtain an acceptable %RSD. The % RSD for peak area of vitamin A and D were 4.5 and 5.2 respectively. The %RSD values indicate high precision and good reproducibility of the method which is less than 6 (AOAC Official Method (2001.13) and AOAC Method (2002.05)).

The accuracy was assessed by spiking the oil with standard solutions. The sample was initially analyzed without spiking and then twice each with spiking in the lower and upper working ranges. Besides, a reagent blank was measured. Following this, the recovery rate was determined for the added quantity of standard and the mean recovery rate was recorded as 100 and 100.06% for vitamin A and D3 respectively.

Extraction recovery

Recoveries were determined by spiking the oil sample with the known concentration of vitamin standard.

Table 11.Results of recovery

Vitamins	Added concentration (ppm)	Final concentration (ppm)	Recovery (%)
Vitamin A	22.25	22.25	100%
Vitamin D_3	16.25	16.26	100.06%

The recovery acceptance criteria for this concentration range were between 70% and 120% (AOAC Official Method (2001.13) and AOAC Method (2002.05)). The recovery calculated ranged from 100% to 100.06% which was within the acceptable range showing the extraction recovery capacity of the method.

Linear working range

As indicated in table 10 and 11the linear working range was found to be between 5 μg/mL and 60 μg/mL for vitamin A and between 0.15 μg/mL and 3.6 μg/mL for vitamin D3 with the method showing a good correlation within these concentrations. As validation practices for the various parameters above showed, measurements within these linear working ranges can be done with a suitable level of precision and accuracy.

Table 12. Linear working range for vitamin A

Level	Ppm	Peak area
1	5	10852733
2	10	21386702
3	15	31732006
4	20	41437742
5	30	63166338
6	40	83683596
7	60	124496636

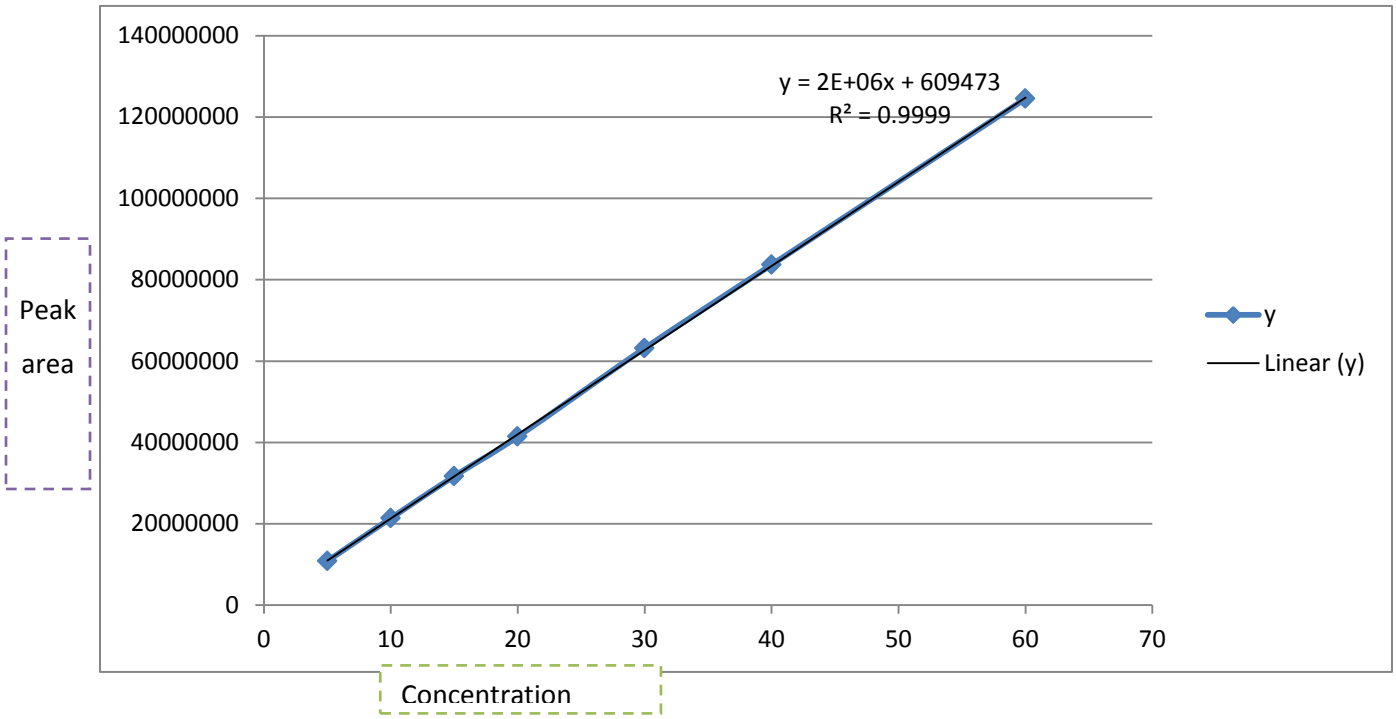

Figure 2.Calibration curve of standard vitamin A

Table 13. Linear working range of vitamin D_3

Level	ppm	Peak area
1	0.15	351563
2	0.30	617003
3	0.45	1028029
4	0.90	1916598
5	1.35	2754933
6	1.80	3707115
7	2.70	5483604
8	3.60	7337512

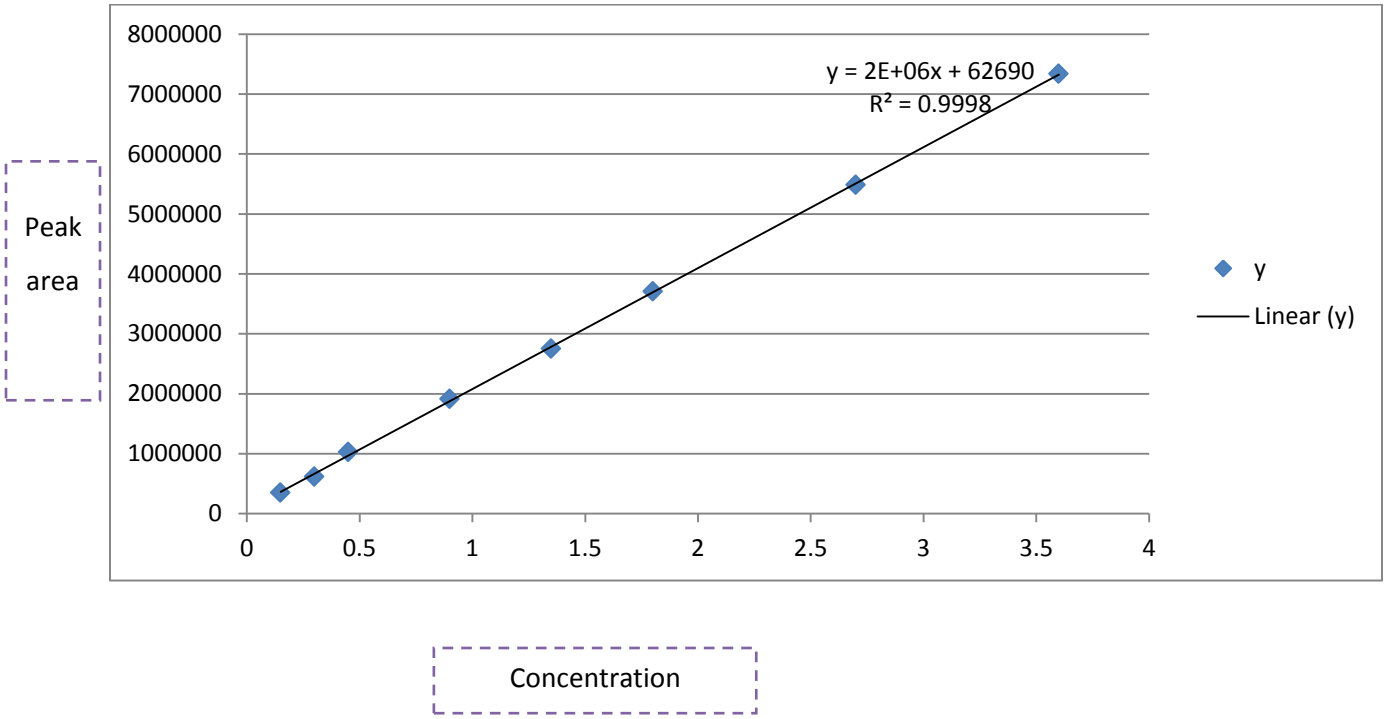

Figure 3.Calibration curve of vitamin D_3 standard

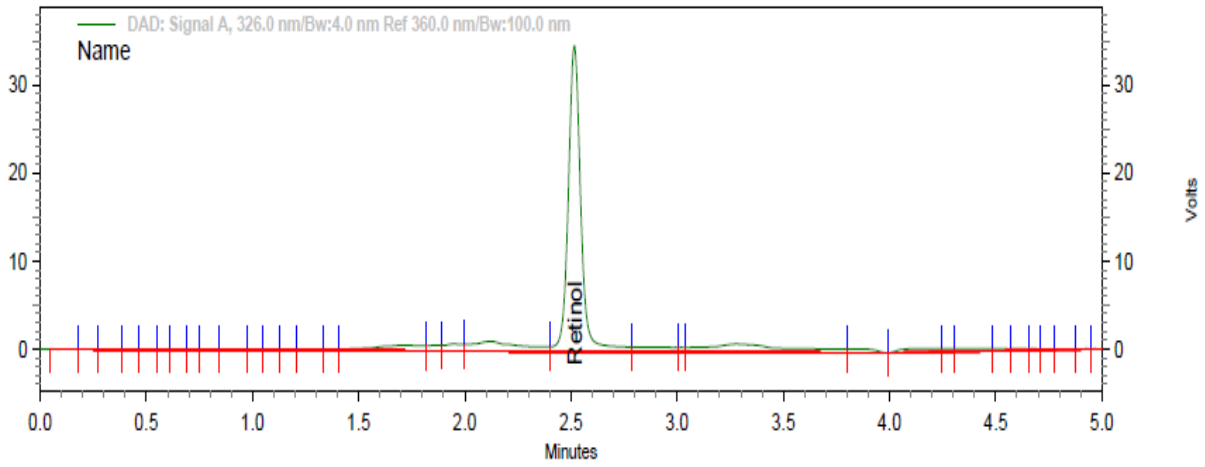

Figure 4.Chromatogram of vitamin A standard

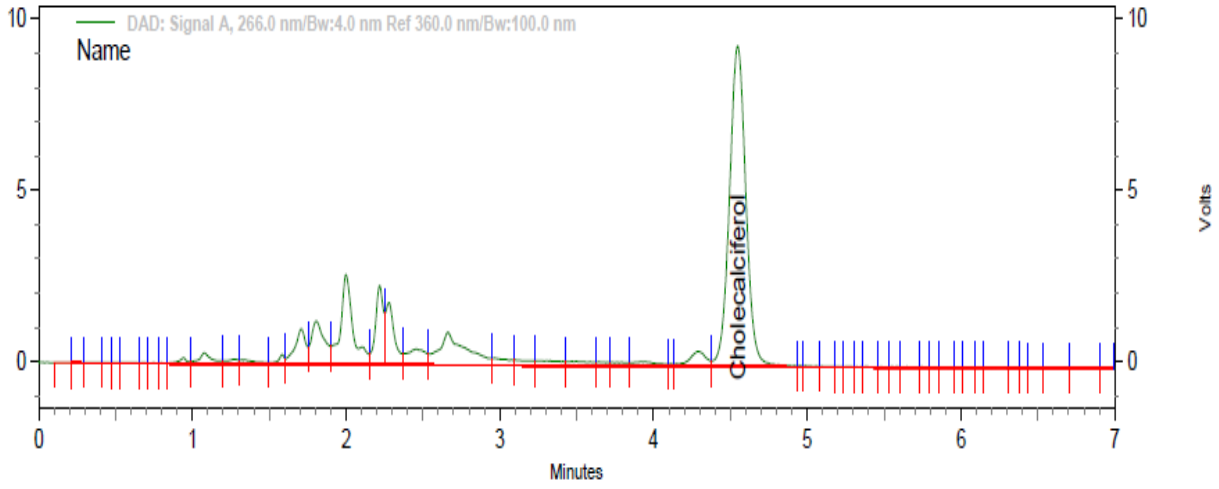

Figure5.Chromatogram of vitamin D_3 standard

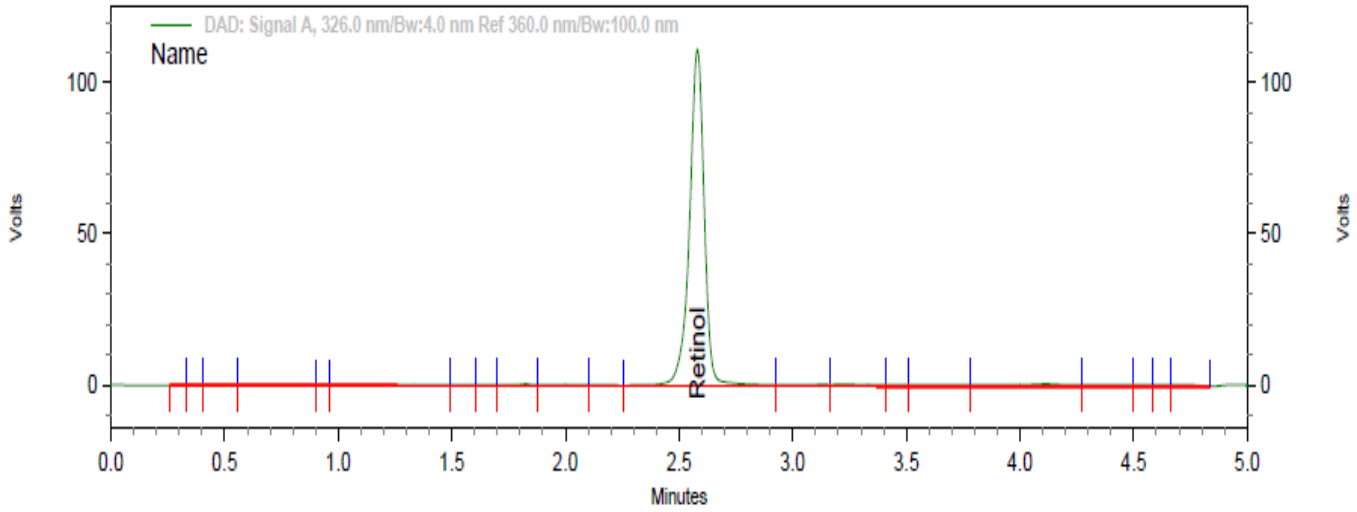

Figure 6.Chromatogram of vitamin A determination in oil sample

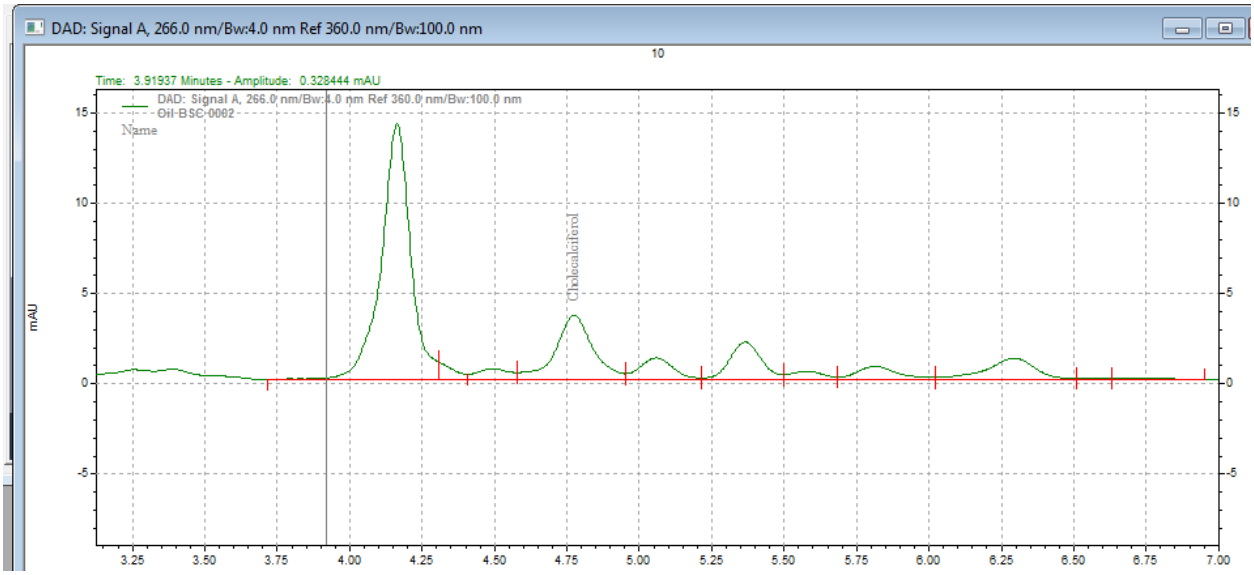

Figure7.Chromatogram of vitamin D_3 determination in oil sample