

Technical Requirements for the Fortification of Wheat Flour in Ethiopia

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Foreword

The purpose of this document is to provide a comprehensive overview on the dangers of micronutrient deficiencies (MNDs) as well as the rationale behind using fortification as an effective means of prevention. It is aimed primarily at building the technical capacities of flour processing facilities producing on both the large and the small scale. While it addresses the regulatory framework created by the Ethiopian Federal Ministry of Health (MOH) and its sub-bodies, it can be used as a guide for all nations who are currently fortifying or hoping to fortify wheat flour.

A primary goal of this document is to inform manufacturers of the technical requirements surrounding the implementation of a food fortification program. It addresses options for types of equipment to be added as well as encourages ways to produce such equipment using local labor and materials. It further discusses laboratory methods for analysis of the vitamin and mineral concentration in the fortified food. Overall, it is hoped that this document can be used as a manual for flour factories to smoothly and successfully implement their own fortification programs according to the guidelines set by the MOH and its legislative branch of the Food, Medicine and Health Care Administration and Control Authority (FMHACA).

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Abbreviations

MOH	Ministry of Health
EHNRI	Ethiopian Health and Nutrition Research Institute
MOI	Ministry of Industry
FMHACA	Food, Medicine and Health Care Administration and Control Authority
GAIN	Global Alliance for Improved Nutrition
MI	Micronutrient Initiative
MaNHEP	Maternal and Newborn Health in Ethiopia Partnership
MND	Micronutrient Deficiency
VAD	Vitamin A Deficiency
WHO	World Health Organization
IDD	Iodine Deficiency Disorder
NNP	National Nutrition Program
NNS	National Nutrition Strategy
RNI	Recommended Nutrient Intake
SS	Stainless Steel
Al	Aluminum
HP	Horsepower
kW	Kilowatt
ETB	Ethiopian Birr
USD	U.S. Dollar
FAO	Food and Agriculture Organization of the United Nations
EAR	Estimated Average Requirement
RDA	Recommended Daily Allowance
UL	Tolerable Upper Intake Level
MSDS	Material Safety Data Sheet
PPE	Personal Protective Equipment
HPLC	High Performance Liquid Chromatography
QA	Quality Assurance

Glossary

Micronutrient	A chemical element of substance required in trace amounts for the normal growth and development of living organisms.
Vitamin	An organic substance that is essential in minute quantities to the nutrition of animals, especially with respect to coenzymes and metabolic processes, but do not provide energy or serve as building units.
Mineral	An inorganic substance that occurs naturally in certain foods and is needed for good health.
Supplementation	The provision of relatively large doses of micronutrients, usually in the form of pills, capsules, or syrups.
Fortification	The addition of micronutrients to processed foods.
Fortificant	The vitamin or mineral added to a fortified food.
Restoration	Adding the same amount of micronutrients back to a processed food that was lost during milling.
Mandatory Fortification	Government-run program that legally obliges certain food producers to fortify dictated foods or food groups with specified micronutrients.
Mass Fortification	Fortification programs aimed at the general public by stipulating fortification of a food commonly consumed across all sub-populations.
Targeted Fortification	Fortification programs aimed at increasing micronutrient consumption of a particular sub-population, such as children under 24 months or pregnant and lactating women.
Voluntary Fortification	The free choice of a food manufacturer to fortify its products, with or without government encouragement.
Volumetric Feeder	A device that automates feeding by depositing consistent volumes of a substance.
Gravimetric Feeder	A device that automates feeding by comparing key measured variables (loss or gain in weight) with set point values, and sending a signal to the controller for corrections.

Gear Box	An enclosed system of assembled gears that transmits mechanical energy from the motor to the agitator.
Motor Controller	Devices that, via an electric connection, govern the performance of an electric motor, from starting to stopping the motor, selecting forward or reverse motion, selecting and regulating the speed, and protecting against overloads and faults.
EAR cut-point method	A method used by the WHO for estimating the concentration of a particular fortificant that should be added to a specified food matrix.
Recommended Nutrient Intake	The daily dietary intake level necessary to meet the nutrient requirements of almost all (i.e. 97-98%) healthy individuals within a particular age, gender, and physiological status group.
Recommended Daily Allowance	Equivalent to the recommended nutrient intake.
Estimated Average Requirement	The average dietary intake level needed to meet the nutrient requirement of healthy individuals within a certain sub-group of the population. Usually it is about two standard deviations lower than the RNI.
Tolerable Upper Intake Level	The highest average daily nutrient level unlikely to pose risk of adverse health effect to almost all (97.5%) of healthy individuals in an age and sex specific population group.
Feasible Fortification Level	The level of fortification that will provide the greatest number of at-risk individuals with an adequate intake without causing an unacceptable risk of excessive intake in other population groups.

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Chapter 1:

Introduction

1.1 Background

A diet adequate in the essential vitamins and minerals is necessary for proper growth and development. While minute amounts are required, on the order of micrograms to milligrams a day, deficiencies in such micronutrients may have serious consequences for reproduction, immune system response, physical and mental growth, and energy metabolism. In many developing nations, including Ethiopia, malnutrition coupled with high rates of disease results in elevated instances of micronutrient deficiencies. Micronutrient deficiencies (MNDs) are responsible for an estimated 7.3% of the global burden of disease. (1) The 2002 *World Health Report* ranked deficiencies in iodine, iron, vitamin A, and zinc among the world's most serious health risk factors. (2) The roles and required amounts of vitamins and minerals are seen in Tables A-1 and A-2 of Appendix A.

Micronutrient deficiencies are responsible for 7.3% of the global burden of disease, with vitamin A, iodine, iron and zinc deficiencies being among the most severe.

There are three main strategies to combat micronutrient deficiencies, which can and should be used in conjunction with each other:

1. Maintenance of a balanced diet rich in essential vitamins and minerals
2. Supplementation
3. Fortification

While preferred, **maintenance of a balanced diet** is most difficult to attain, as it necessitates a transformation in both the accessibility and affordability of a wide range of food products, from cereals to fruits and vegetables to animal products. Moreover, it requires the establishment and propagation of nutrition education programs and as well as increased marketing of micronutrient-rich foods. Thus, for vulnerable and at risk populations, activities to promote lasting food security should be coupled with supplementation or fortification initiatives.

Supplementation is defined as the provision of relatively large doses of micronutrients, usually in the form of pills, capsules, or syrups. (1) It has the advantage of being the fastest way to distribute needed amounts of vitamins and minerals to already deficient or at risk populations and is proven to be a relatively cost effective strategy for reducing cases of MND. However, it is

generally thought of as a short-term strategy to compliment a long-term solution. Supplementation has been used widely in Ethiopia through the Health Extension Worker Program, which provides doses of vitamin A to children under 5, folic acid to pregnant woman, and zinc to those suffering from diarrheal disease. Because vitamin A stores in the body for 4-6 months, a single high dose of vitamin A twice a year helps reduce rates of deficiencies.

Food Fortification, the practice of adding vitamins and minerals to processed foods, has helped eliminate MNDs in both developed and developing nations.

Lastly, **fortification** is a term used to describe the addition of micronutrients to processed foods. Fortification has been used in the developed nations since the 1930's with fortification of cereals, flours, margarine, and milk. However, fortification is particularly suited to developing countries as the cost of fortified food is less than 2% more than the cost of unfortified food. (1) For instance, fortification of sugar with vitamin A has been used extensively in Latin America since 1970, resulting in the near eradication of vitamin A deficiency (VAD) in the Western Hemisphere. Through the UN's Global Alliance for Improved Nutrition (GAIN), food fortification is now common in many Sub-Saharan African countries, as shown through the flour fortification programs displayed in **Table 2** and **Table B-1**.

1.2 The Situation in Ethiopia

Ethiopia is one of the poorest and least developed countries in the world, ranking 173rd out of 187 countries on the 2013 United Nation Programme's Human Development Report, with 29.6% of the population living below the poverty line. (3; 4) 70% of the average rural diet is cereal-based, which, combined with a history of drought and famine, insufficient agricultural production, and infectious disease, contributes to a reported 46% of Ethiopians that are undernourished, the second highest rate in sub-Saharan Africa. (5) The four most prevalent forms of malnutrition are acute and chronic malnutrition, iron deficiency anemia, iodine deficiency disorder, and vitamin A deficiency. (6) Other common micronutrient deficiencies include folic acid, zinc, and vitamins B and D. Table 1 shows levels of common MNDs within Ethiopia.

Table 1. Deficiency rates in Ethiopia (7)

Study Subjects	Indicators	Prevalence (%)
Vitamin A		
Children, 6 – 59 months	Night Blindness	4.3 – 7.3
	Bitot’s Spot	2.2
	<0.7 SRC $\mu\text{mol/L}$	37.6
Iron Deficiency - Anemia		
Children, 6 – 59 months	Hb <11.0 g/dL	54.0
	Hb <4.0 g/dL	4.0
Women, 15 – 49 years	Hb <11.0 g/dL	30.4
	SF <50 $\mu\text{g/L}$	50.1
Folic Acid		
Women of child bearing age	SFA <2 ng/mL (severe)	46.0
	SFA <4.9 ng/mL (marginal)	21.2
Iodine		
Children	Goiter rate	35.8
Women	Goiter rate	27.0

SRC = Serum Retinol Concentration; Hb = Hemoglobin; SF = Serum Ferritin; SFA = Serum Folic Acid

1.2.1 Vitamin A Deficiency

Vitamin A is a fat soluble vitamin found in green, leafy vegetables, orange vegetables such as carrots and pumpkins, or animal products. Vitamin A is found in animal products in the form of retinols and in vegetables in the form of pro-vitamin A β -carotene, which must be converted to retinol before absorption by the body. Vitamin A is essential for the development of the eyes and immune system response.

An estimated 40% of Ethiopian children are deficient in vitamin A. Studies indicated that improving the vitamin A status of deficient populations could improve childhood mortality by 23% and pregnancy-related mortality among women by 40% as well as raise the national GDP by 1.23%. (8) Vitamin A deficiency (VAD) is the leading cause of childhood blindness in Ethiopia. In some areas, the prevalence of Bitot’s Spot reaches over 6 times that of the World Health Organization’s (WHO) cutoff for defined public health problem. Studies show that

giving vitamin A to children with diarrhea or measles reduces rates of illness by 15% and 50%, respectively. (9)

Surveys conducted between 1957 and 1959 in various Ethiopian towns first revealed VAD as a serious national public health problem. Since then, the Ministry of Health along with various local and international supporters have attempted a series of intervention methods, ranging from small-scale projects to national supplementation programs. First implemented in 1989, vitamin A supplements were originally distributed as part of the primary health care system. Over the years, the program was modified to distribute capsules through the Extended Program on Immunization, National Immunization Days, or as part of health packages including deworming, measles vaccines, mosquito nets, etc. Now, vitamin A capsules are distributed twice a year to children under the age of 5 through the Health Extension Worker Program, which trains and employs two female health care providers per kebele. While supplementation programs have shown to be effective and coverage rates are reported at 80%, problems with patient tracking, adequate supply, or reduced potency have kept VAD rates high. (10)

1.2.2 Iron Deficiency Anemia

Iron, an essential mineral, is an important component of hemoglobin, the substance in red blood cells responsible for transporting oxygen from the lungs to the rest of the body. Without enough iron, the amount of oxygen-carrying red blood cells will diminish, resulting in a condition called iron-deficiency anemia. (11) Without an adequate supply of oxygen in all parts of the body, people feel exhausted and worker productivity plummets. Iron deficiency is especially common in women, as they lose iron through blood during menstruation. A diet low in foods of animal origins is the main cause of iron deficiency, but it is compounded by high incidence of malaria and other parasitic diseases.

*Vitamin A Deficiency is responsible for **Bitot's Spot** [a characteristic clouding of the eye caused by a buildup of karatin debris in the conjunctiva] and **xerophthalmia**, [a condition where the eye produces no tears] both of which can result in permanent blindness. 40% of Ethiopian children are deficient in vitamin A.*

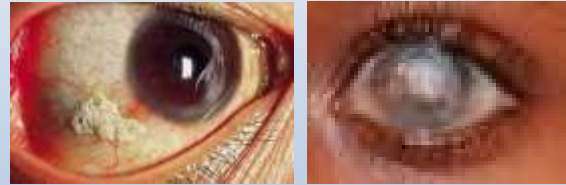


Figure 1. Bitot's spot and conjunctival xerosis, caused by VAD

Iron is present in meat and fish in the form of heme iron, of which up to 40% can be absorbed by the body. Other sources such as teff or other grains, nuts, or vegetables contain non-heme iron, which is only about 10-20% absorbed by the body. Additionally, commonly consumed phenolic compounds (i.e. coffee), oxalates (i.e. tea), and foods high in phytates (i.e. lentils or dried beans) inhibit iron adsorption. (12) In Ethiopia, more than half of children under five as well as over a quarter of women are anemic. (13) Iron deficiency anemia depresses a country's GDP by resulting in a less effective workforce and lowering concentration levels in school children, thus preventing maximum progress through education. Iron deficiency also hinders the immune system response.

Consumption of foods such as coffee, tea, lentils, and beans inhibits iron absorption.

1.2.3 Zinc and Folic Acid Deficiencies

Zinc deficiencies are widespread in areas where diets lack animal-based foods. Zinc is crucial for immune system response; case studies show that zinc supplements can reduce duration of diarrhea by 20% and stool frequency by 62%. (14) In Ethiopia, zinc supplements are distributed free of charge to those with diarrhea through the Health Extension Worker Program.

Folic acid is necessary for proper development of the fetus. Nearly two-thirds of Ethiopian women are deficient in folate, a condition which results in birth defects such as spinal bifida, anencephaly and encephalocele. Folate capsules may be distributed to pregnant women through the Health Extension Worker Program. However, as with vitamin A and zinc, problems with supply, potency, and coverage plague the system.

1.2.4 Iodine Deficiency Disorder

Iodine deficiency disorder (IDD) has severe consequences, resulting in impaired thyroid hormone synthesis or enlargement, also known as goiter. IDD is responsible for mental retardation, cretinism, and infant mortality. While very minute amounts of iodine are required by the body, on the order of micrograms, the only natural source of iodine is from vegetables grown in iodine-containing soil or fish from seawaters. As such, fortification of

Existing structures in Ethiopia for the prevention of MNDs include the provision of free vitamin A, zinc, and folate supplements as well as mandatory salt iodization.

salt with iodine is recognized as the most cost effective solution to treating Iodine deficiency. At the 1990 World Summit on Children, leaders of most countries pledged to universally iodize all salt. (15) Ethiopia began its own salt fortification program in 2009. (16)

1.2.5 Government Response

Despite the abovementioned supplementation efforts, food fortification in Ethiopia remains constricted to only a few private companies, such that Ethiopia is one of only 27 sub-Saharan African countries without a legal program. (17) Programs and food vehicles chosen by other countries are shown in Table B-1.

In the 2011 National Nutrition Program (NNP), the MOH outlined a National Nutrition Strategy (NNS) for the elimination of VAD and other micronutrient deficiencies by 2015. The

Ethiopia's 2011 National Nutrition Program outlines food fortification as a key strategy for the elimination of VAD and other MNDs.

NNS listed food fortification as a key strategy, identifying flour, oil, and sugar as promising food vehicles for fortification. After the publication of the NNP, the MOH mobilized a task force to investigate matters concerning food fortification. This task force included its subordinate bodies at the Food, Medicine, and Health Care Administration and Control Authority (FMHACA)

and the Health and Nutrition Research Institute (HNRI) as well as stakeholders at the Ministry of Industry, Ministry of Trade, Ministry of Commerce, and several non-governmental organizations. From there, FMHACA, the branch of the MOH responsible for passing legislation concerning food and drugs, initiated an investigation into the capabilities of various food processing facilities chosen semi-randomly from all regions of Ethiopia. The investigation helped those involved determine the current feasibility of the fortification program as well as what resources, whether educational, financial, or technological, were needed. Further specifics involving government regulations are included in the chapter 2 of this paper.

1.3 Choosing the Proper Food Vehicle

An effective vehicle for food fortification fulfills the following criteria:

1. Is consumed by a majority of the targeted population in quantities large enough to provide at least a substantial percentage of the added fortificant,
2. Is processed in a centralized facility,
3. Is unaltered (taste, smell, chemical composition), by the fortificant,
4. Is technically feasible to fortify, in terms of cost and available technology.

Commonly fortified foods around the world include:

- Flours (i.e. wheat, maize)
- Fats (i.e. margarine) and oils
- Milk and dairy products
- Sugar
- Salt

1.4 Why Fortify Flour?

Since 2005, wheat flour has had the second largest percent share of national grain consumption in Ethiopia (Figure 2). Commercially processed flours are consumed by 28% of Ethiopians, while an estimated 58% of the population consumes wheat flours and grains. At an estimated average per capita intake of 64 kg/year, at recommended addition values and accounting for losses in cooking, processing, or storage and transportation, an adult female could receive 64% RNI (recommended nutrient intake) for folic acid, 64% RNI for vitamin A, 26% for iron, and 81% for vitamin B₁₂, and all zinc requirements. The cost is estimated by the Ethiopian Ministry of Health to be just

Wheat flour is an ideal vehicle for fortification because it can be processed centrally, is consumed by a large percentage of the population, and is technically easy to fortify.

\$0.10/person/year to fortify. (7) As stated above, and shown below in Table 2 and Appendix B, flour is a common vehicle chosen for fortification by many African nations.

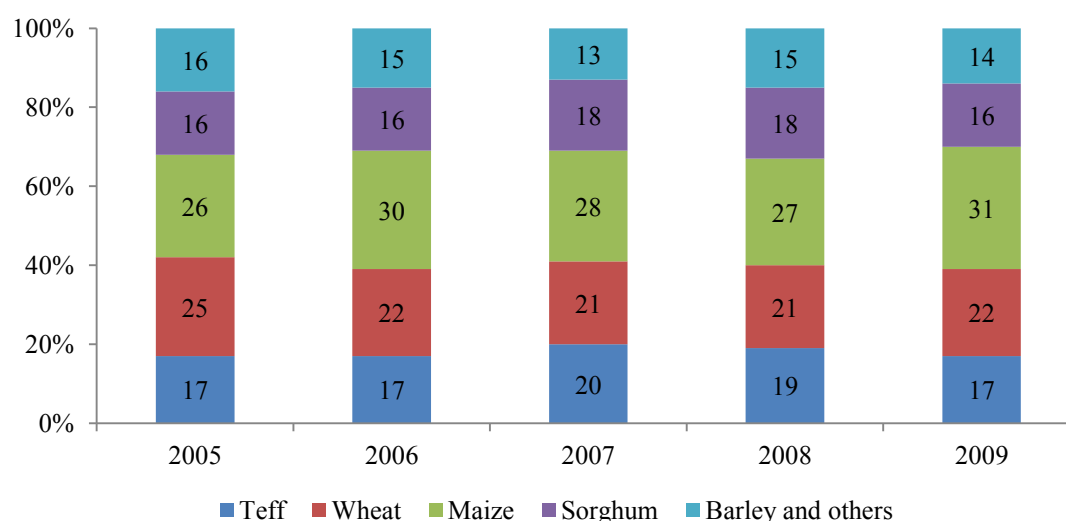


Figure 2. National grain consumption patterns

Table 2. Wheat Flour Fortification Programs in Sub-Saharan Africa

Country	M,V,S*	Dosage (g/t flour)	Vitamin and Minerals
Nigeria	M	250	Vitamin A, vitamin B1 and B2, niacin, folate, iron
Morocco	M	90-120	Vitamin B1, vitamin B2, niacin, folate, iron
Cote d'Ivoire	M	100	Iron and folate
South Africa	M	200	Vitamin A, vitamin B1, vitamin B2, vitamin B6, niacin, folate, iron, zinc
Ghana	M	350	Vitamin A, vitamin B1, vitamin B2, niacin, folate, vitamin B12, ferrous fumerate, zinc
Sudan	S	200	Ferrous sulphate and folate
Congo	V	250	Iron and folate
Uganda	M	350	Vitamin A, vitamin B1, vitamin B2, vitamin B6, vitamin B12, niacin, folate, ferrous fumerate, zinc
Egypt	S	200	Ferrous sulphate and folate
Zambia	M	200	Vitamin A, vitamin B2, vitamin B6, vitamin B12, niacin, folate, sodium iron EDTA, zinc

- *Mandatory (all), voluntary, or some mandatory

As seen in Table 3, milling of cereals such as wheat or maize, depletes the grain of its original nutrient content. **Restoration** of nutrient levels back to their original value is one type of fortification process that is encouraged in every nation.

Table 3. Effect of Milling on Vitamin and Mineral Content of Maize (18)

Vitamin (mg/kg)	Whole Maize	Dehulled	Degermed
Vitamin A	0	-	-
Thiamine (B1)	4.7	4.4	1.3
Riboflavin (B2)	0.9	0.7	0.4
Niacin	16.2	13.9	9.8
Pyridoxine (B6)	5.4	5.4	1.6
Vitamin E	0	-	-
Folate	0.3	0.2	0.1
Biotin	0.073	0.055	0.014
Mineral			
Calcium	30.8	26.7	14.5
Phosphorus (mg/g)	3,100	2,500	800
Zinc (mg/kg)	21.0	17.1	4.4
Iron (mg/kg)	23.3	19.7	10.8

While the grain **teff**, used to make the national bread *injera*, is another commonly consumed food in Ethiopia, it is less suitable for fortification because:

- It is more dense in essential vitamin and minerals, such as iron,
- It is milled mostly by small farmers or individuals,
- It is more costly than wheat flour,
- It is consumed in higher percentages by the wealthy and urban populations.

Similarly, **maize flour**, which has the highest consumption patterns in Ethiopia, is currently not suitable for fortification in Ethiopia because of the lack of central maize processors.

Chapter 2:

Regulatory Framework

Successful and sustainable fortification programs require the support of governing bodies. In order to ensure fortified foods are safe and reliable for consumption, the government is responsible for setting regulations and quality control and assurance stipulations concerning the addition of vitamins and minerals to processed foods. Fortification programs can be classified into one of two groups: mandatory or voluntary. (1)

1. **Mandatory fortification** programs legally oblige food producers to fortify particular foods or food categories with certain micronutrients. The government stipulates regulations that must be adhered to by all food processing companies. Such stipulations include the types of vitamins and minerals to be added, minimum and maximum levels of vitamin addition, packaging notations, and methods and frequency of measuring the concentration of vitamins in the food matrix. Mandatory programs are recommended for countries, such as Ethiopia, that have high levels of micronutrient deficiencies or public health risks but low levels of public awareness concerning fortified foods. As shown in Table 2 and Table B-1, the majority of flour fortification programs in sub-Saharan Africa are mandatory. There are two types of mandatory fortification programs:

- a. **Mass fortification** programs are desired to reach the mass public by dictating foods to fortify that are commonly consumed by all sub-populations.

Targeted fortification programs focus on food intended for consumption by specific sub-populations, such as children under 24 months or pregnant and lactating women.

2. **Voluntary fortification** occurs when a food manufacturer freely chooses to fortify its products, with or without government encouragement. However, government may exercise a level of control over such programs in order to prevent misleading of the public. For instance, companies that do choose to fortify may have to follow similar

Mandatory fortification is preferred over voluntary fortification in countries with high levels of MNDs and low levels of awareness. FMHACA is responsible for setting legislation concerning food fortification within Ethiopia.

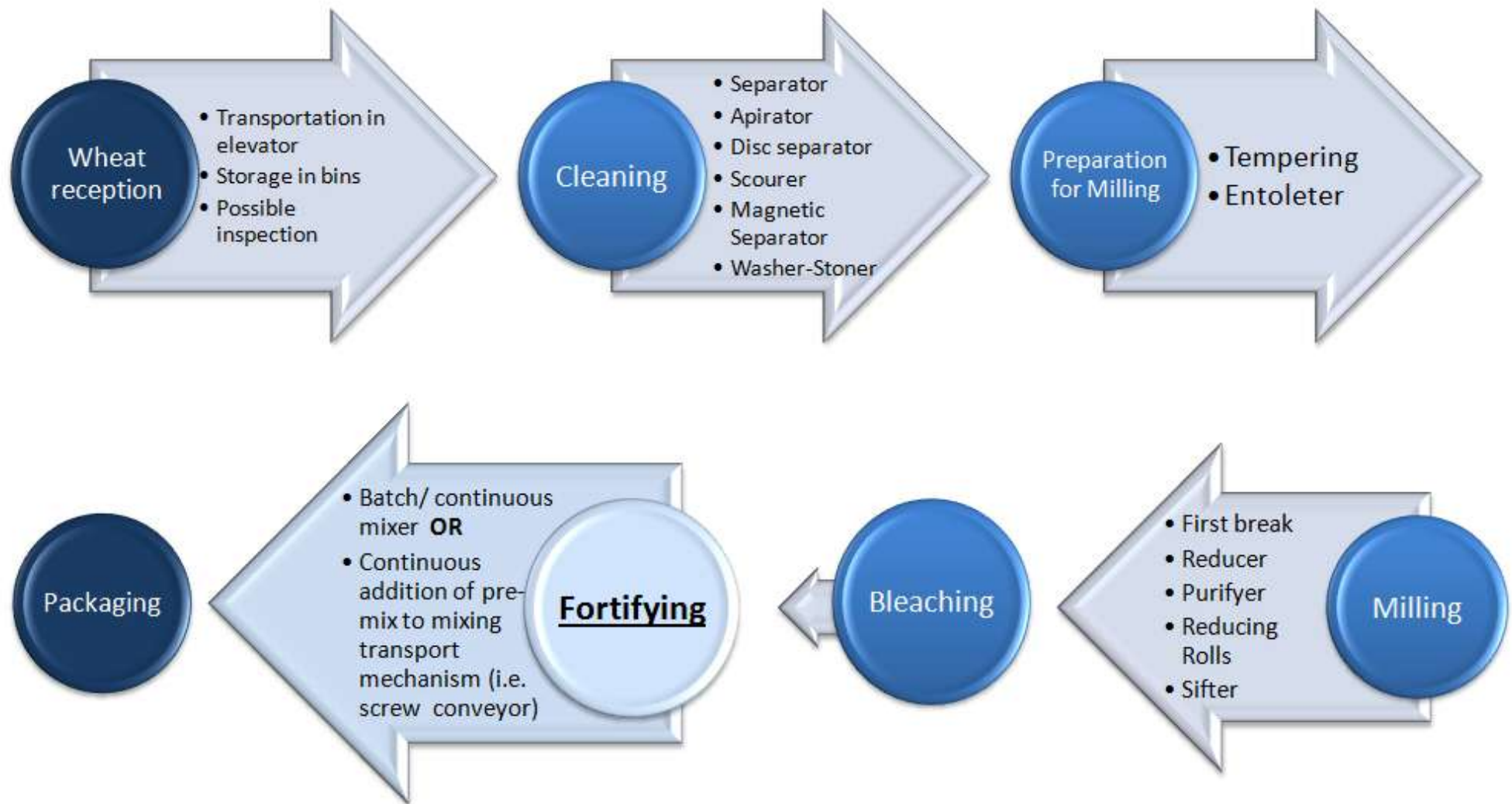
government-set regulations concerning minimum and maximum nutrient levels, quality assurance and control, and identification of fortified goods. Voluntary programs work well in societies with a specific demand for fortified goods, such that the company itself chooses to fortify in order to satisfy the market demand.

FMHACA is the branch of the Federal Ministry of Health responsible for passing legislation regarding the fortification of wheat fours.

Chapter 3:

How to Implement

3.1 Simplified Milling Process Introducing Fortification

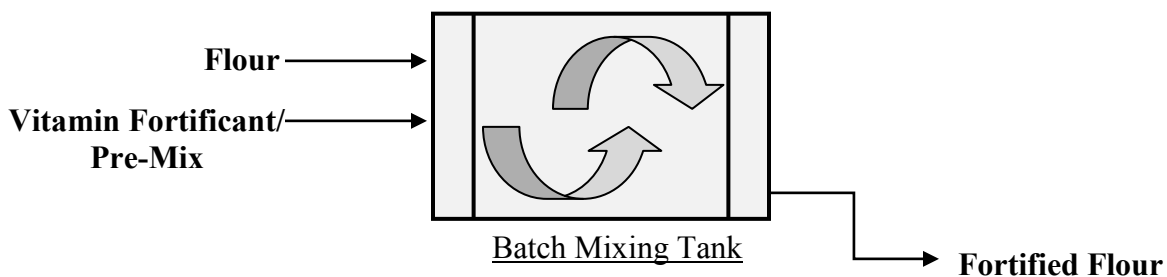


3.2 Fortification Methods

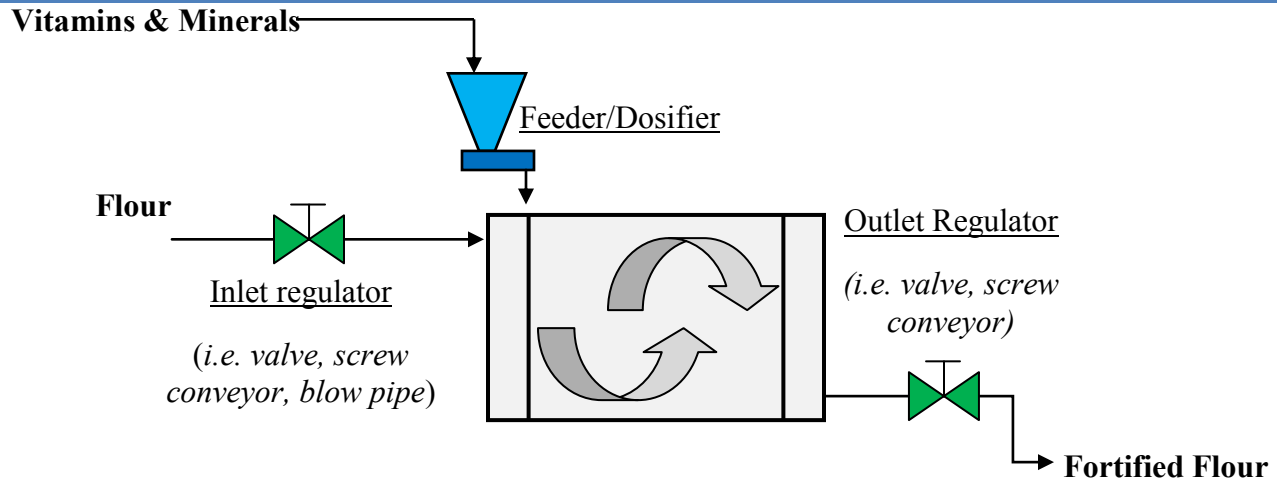
Table 4 lists several common ways for achieving uniformly fortified flour in the mill. Methods are applicable for small to large size faculties. Several other methods exist for fortifying, such as adding the vitamin pre-mix during the actual milling and reducing of the flour, but such methods are not as established. Refer to the literature to learn about alternate methods.

Table 4. Factory methods for flour fortification

Method	Description	Equipment Needed	Recommended For...
<u>3.2.1. Batch Mixing</u>	Flour and vitamin pre-mix are mixed together in a batch operation.	Simple Agitation Tank for solid-solid mixing. Mixer may be manual or electric .	Small companies who chose low cost over mixing time or automation.



<u>3.2.2. Continuous Mixing</u>	Flour and vitamin pre-mix are mixed together under continuous flow of both ingredients.	<ol style="list-style-type: none"> Method of continuous addition of flour and vitamin pre-mix, i.e. feeder or dosifier. However, this feeder does not have to be as precise as those needed for continuous metering. Agitation Tank for solid-solid mixing fitted with inlet and outlet valves that can be controlled either manually or through PID controls. 	Companies willing to pay a higher price to process flour at a higher rate while ensuring accuracy.
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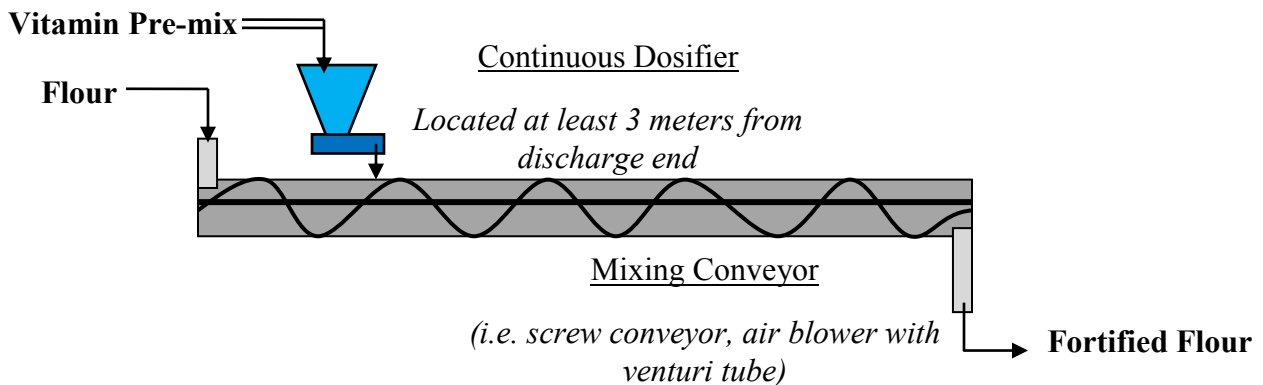


3.2.3 Continuous Metering

Premix is continuously metered or fed into the flour flow using a precision microingredient powder feeder (dosifier). Mixing occurs as the flour flows along a screw conveyor, or similar mechanism.

1. A **feeder or dosifier** to meter out the pre-mix
2. A means to **agitate the pre-mix** so it feeds without bridging or clumping.
3. **Mechanical or electronic way to adjust the flow rate** of the pre-mix.
4. Some **level of mixing after the pre-mix has been added** to the flour (i.e. **screw conveyor**)

This method is fast, easy, and reliable. It is relatively low cost for factories that already have some sort of screw conveying mechanism installed.



**3.2.4 Two-Stage
Mixing**

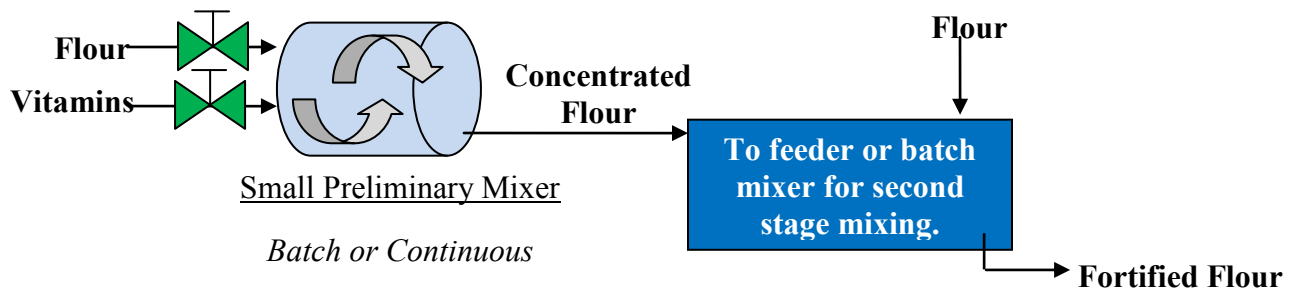
Two-stage mixing can be incorporated into any of the above three processes. Because the vitamin pre-mix is very concentrated, a **pre-mixing step to mix the flour and the vitamin for a first dilution** may help reduce the mixing time of the second mixing stage and ensure uniformity.

Agitator/Mixer, batch or continuous, that is smaller in size than second mixer. Output from the mixer will feed into the input of the second mixer or dosifier.

Companies with variable flour flow that desire high accuracy.

Feed Regulators

Optional (for continuous



Chapter 4:

Fortification Equipment and Control Options

4.1 Dosifiers

Feeders/dosifiers allow for the controlled, continuous addition of vitamins to the flour matrix. Several types of feeders exist including volumetric, gravimetric and pneumatic. Only a few are discussed here.

Volumetric feeders operate under the principle that, given a media with constant density, adding a consistent volume of that media is the same as adding a constant mass. The **screw feeder**, mentioned below, is the most common type; other types of volumetric feeders include a **revolving disk feeder** and a **drum feeder**.

Gravimetric feeders add an additional level of precision to the feeding process by comparing discharge weights to set-point values, and sending a signal to a controller to make any needed adjustments. Examples include **loss-in weight** or **gain-in-weight feeders**, which measure the weight loss or the weight gain, respectively, of the substance being added. This weight sensing device is coupled with a basic feeding mechanism, such as the volumetric screw feeder.

Gravimetric and volumetric feeders should be placed *above* the mixing device. In factories already equipped with a screw conveyor for transporting the flour, the screw conveyor can be used as the mixing mechanism, provided the feeder is added at least 3 meters upstream from the final product deposition.

If the factory is equipped with an air blower as a conveying device, a **pneumatic feeder** can be placed in any location. A **venturi tube** should be placed between the feeder and the air blower to control the flow rate of the vitamins.

4.1.1 Screw Feeder

The most commonly used feeder for controlled additions of vitamin premixes is the screw feeder. The screw feeder deposits consistent volumes of vitamins that are carried along a rotating screw.

The flow rate of the screw feeder is defined as:

$$Q = 60 \left(\frac{\pi}{4} \right) D^2 \cdot S \cdot n \cdot \rho \cdot C \cdot \varphi$$

Where:

- Q = flow rate
- D = hydraulic diameter = $D_o - D_i$
- S = pitch (i.e. D , $\frac{3}{4} D$, $\frac{1}{2} D$, etc.)
- n = rotation speed
- ρ = density
- C = inclination factor (= 0 for horizontally oriented feeders)
- φ = loading efficiency

Proper steps should be carried out to calibrate the screw feeder. X-Y plots of fortificant mass deposited (y) vs. rpm (x) as well as vitamin mass deposited (y) vs. time running (x) should yield straight lines. Follow manufacturer's instructions provided with the feeder.

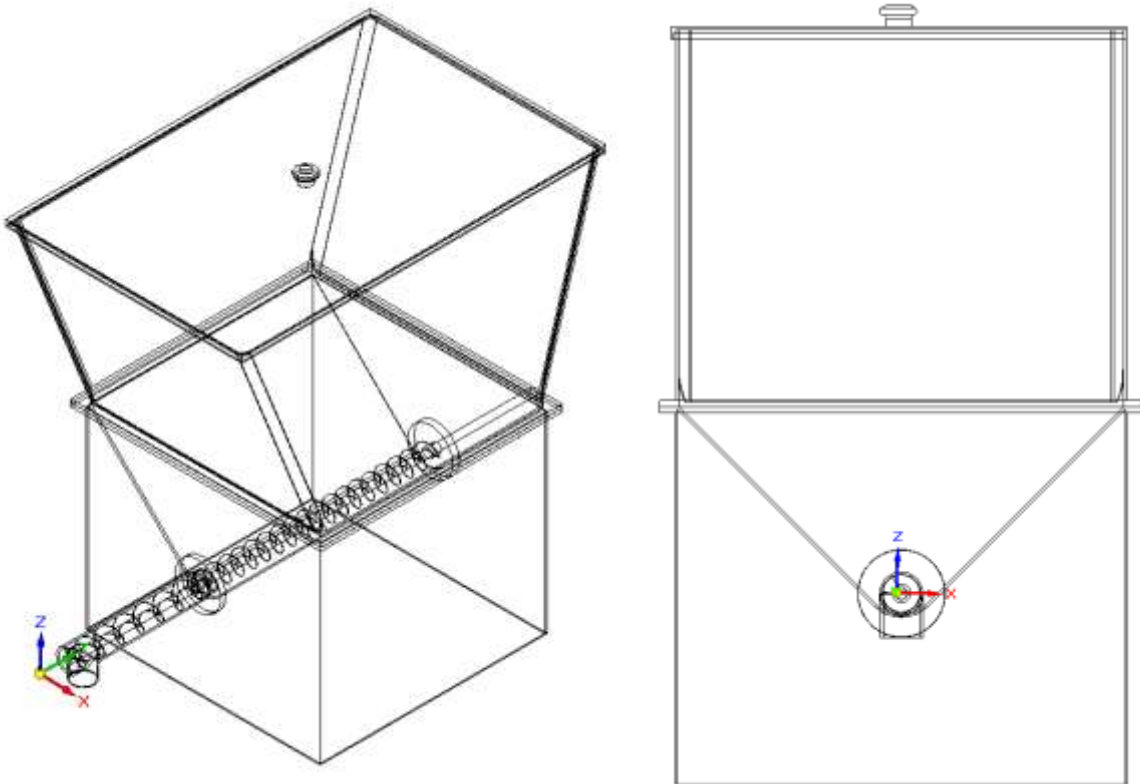


Figure 3. Screw feeder angled and front views

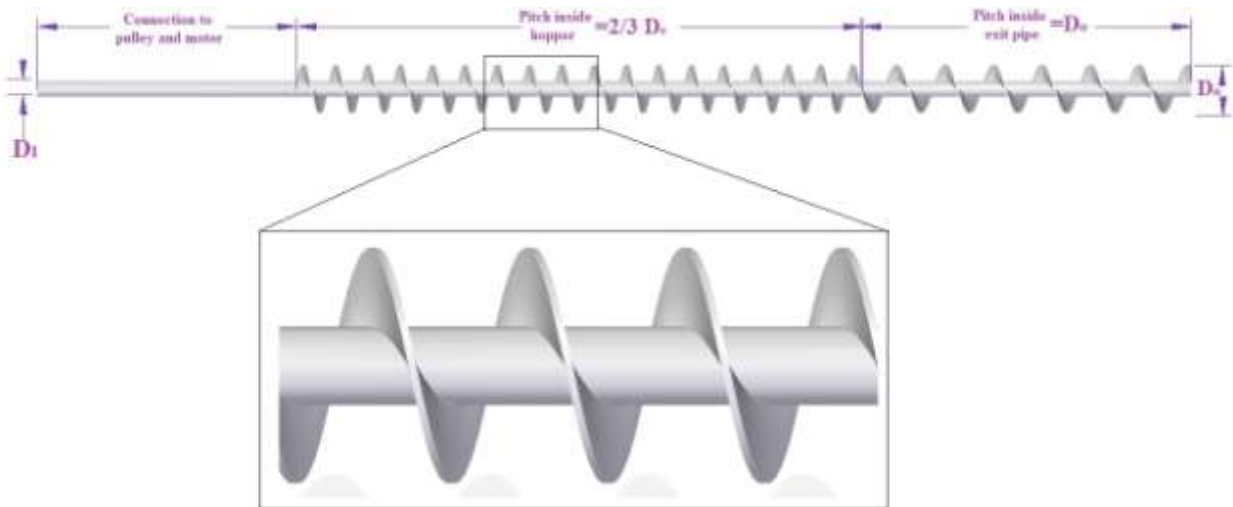


Figure 4. Example screw dimensions for screw feeder

4.1.2 Rotating Cup Feeder

Factories desiring locally produced machinery at a low cost should consider the rotating cups feeder. It is less precise than the screw feeder, so should be coupled with a mixer or blender such as those shown in section 4.2. The cups scoop up small amounts of vitamin premix and dump them out as they are turned upside down. It is a cost effective device to consider to make one of the mixers in section 4.2 operate by continuous flow instead of batch flow.

The shaft of the rotating disk should be attached to a motor to provide the mechanical energy for rotation. The top half of the tank can be removed and the disk extracted for easy filling, cleaning and maintenance. There should be a tight fitting between the top and bottom of the tank. The tank should be filled less than half way up with vitamin premix. A hopper can also be fitted to the tank for easy refill.

The flow rate of the rotating cups feeder is defined as:

$$Q = N \cdot [\pi r^2 h] \cdot n$$

Where:

- N = number of cups on the disk
- r = radius of the cup
- h = height of the cup
- n = rotation speed

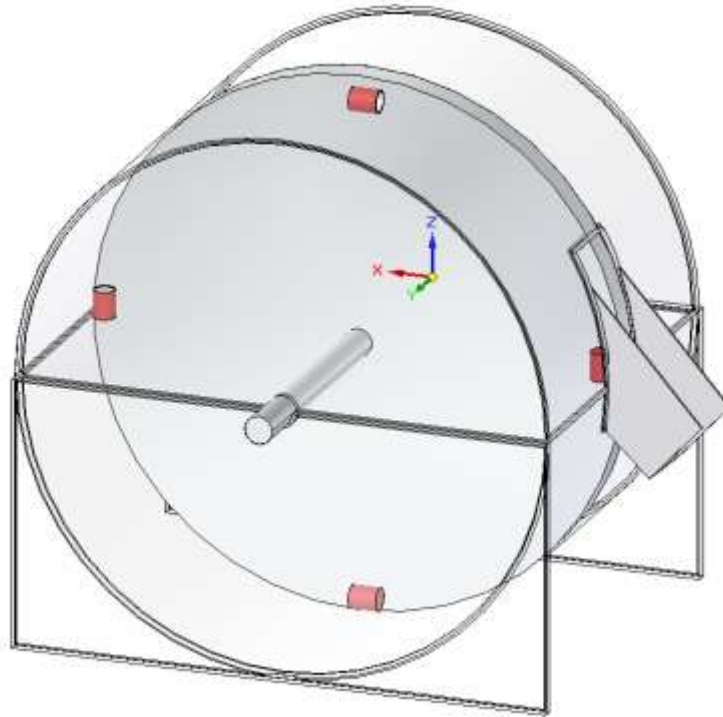


Figure 5. Rotating cup feeder

4.2 Mixers and Blenders

4.2.1 Built in-country with manual controls

The following designs are based off a small-scale stainless steel and aluminum machines manufactured in country at Mebratu Araya and Son's Engineering in Mekelle, Tigray. The machines were tested and proved to be possible for local manufacturers to produce and capable of uniformly mixing the vitamin premix with the food vehicle.

Flour factories may wish to consider purchasing similar designs from local metal workshops. Other designs exist for purchase internationally.

4.2.1.1 Batch-fed V-Mixer for dry flour mixing

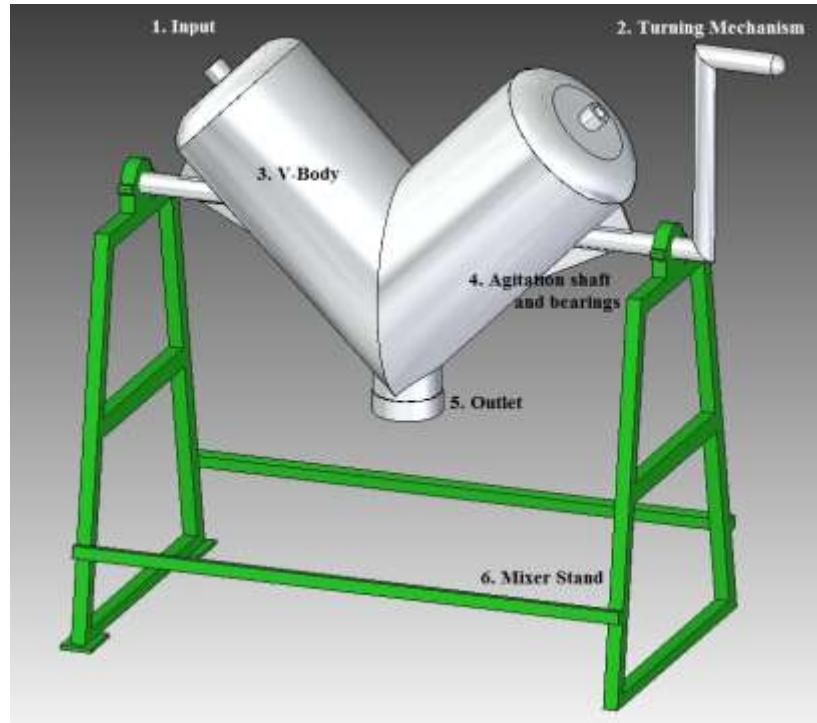


Figure 6. Manual V-Mixer for Flour Fortification

Table 5. Parts and descriptions of a V-Mixer

No.	Part Name	Description
1	Input	<p>Flour can be added through one leg of the V-body, while vitamins and minerals can be added through the opposite leg. The lids should be tightly fitted to the main body frame either through latches, screw caps, or valved openings.</p> <p>For small scale operations without automation, latched lids or screw caps are sufficient. Very small operations may find a tightly fitting cap sufficient, but leaking may occur. For larger, automated systems, the lids may be welded to the main body and fit with a small opening controlled by a valve. The operation of this valve will be controlled by an external automation system, such as a PID controller.</p> <p>The lids should be rounded for better mixing.</p>
2	Turning mechanism	<p>For small scale operations, the V-body can be turned manually through a hand crank (shown here). The hand crank is welded to the end of the agitation shaft.</p> <p>For larger operations, the handle will not be added; instead the end of the agitation shaft can be fitted with a pulley (not shown here) and connected to a motor.</p>

3	V-Body	The main body consists of two cylinders rolled out of stainless steel or aluminum sheet metal and attached at a 90° or smaller angle.
4	Agitation shaft and bearing	<p>The V-body turns via the rotation of the bar. The bar may either pass through the body of the V or be attached to the sides. In the latter case, external attachments or supports (such as the ones shown above) are recommended.</p> <p>In the case of an aluminum body with a stainless steel shaft, the bar may be bolted to an aluminum hub which is welded to the main body.</p> <p>Two bearings support the agitator shaft and allow fluid rotation of the bar.</p>
5	Outlet	<p>The fortified flour will exit through the vertex of the V-shape. The opening should be closed during mixing by a valve or a tightly fitting lid with a latch or screw cap.</p> <p>Valves, such as gate valves or butterfly valves, are the preferable opening and closing mechanism. The valve can be opened and closed manually or fitted with automatic controls.</p> <p>Because large valves can be expensive, very small operations may find a tightly fitting cap adequate. A pin can be inserted through the diameter of the cap to prevent the cap from falling off during rotation. Leaking of the flour may occur.</p>
6	Mixer stand	The mixer stand can be made of any strong metal, such as iron or steel. In the case of motorized rotation, the stand should be modified to support the motor and house the motor and pulley assembly.

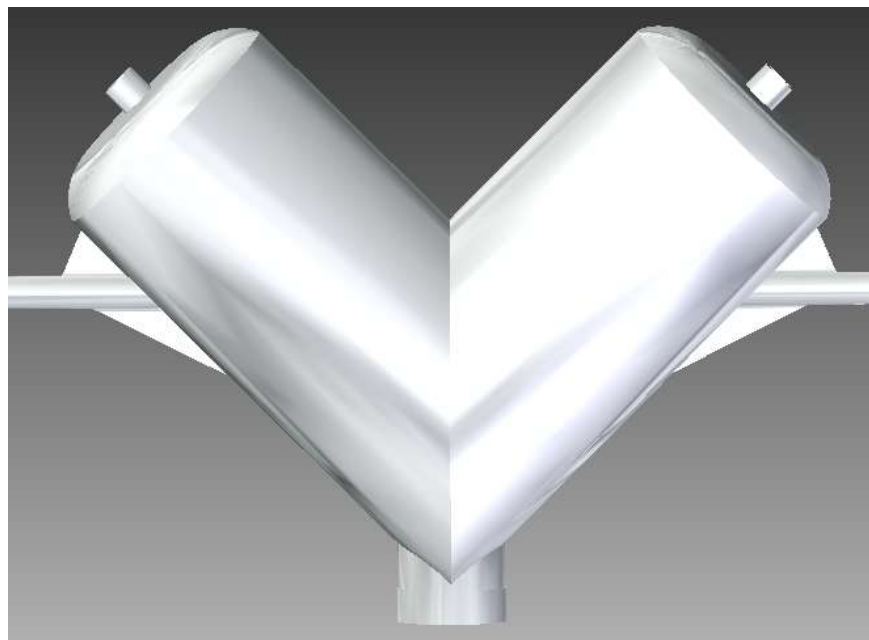


Figure 7. V-Mixer main body

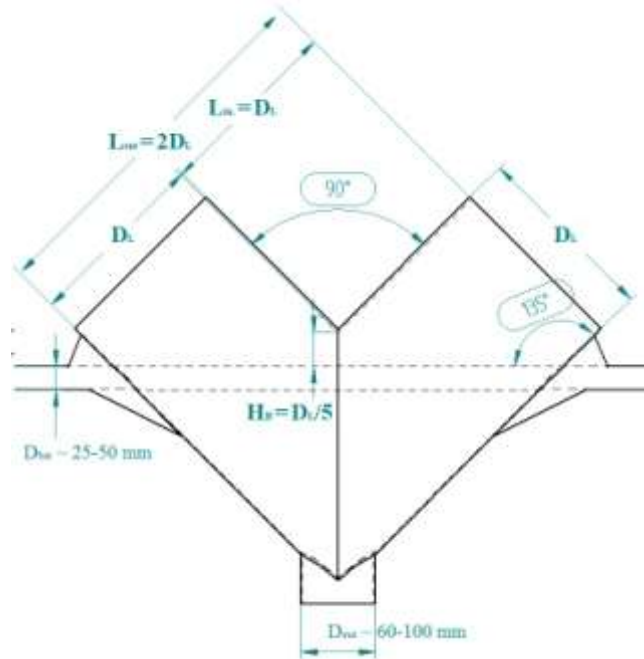


Figure 8. Dimensions for body of V-mixer

4.2.1.2 Batch or continuous horizontal mixer with pin-type agitator for wet flour mixing

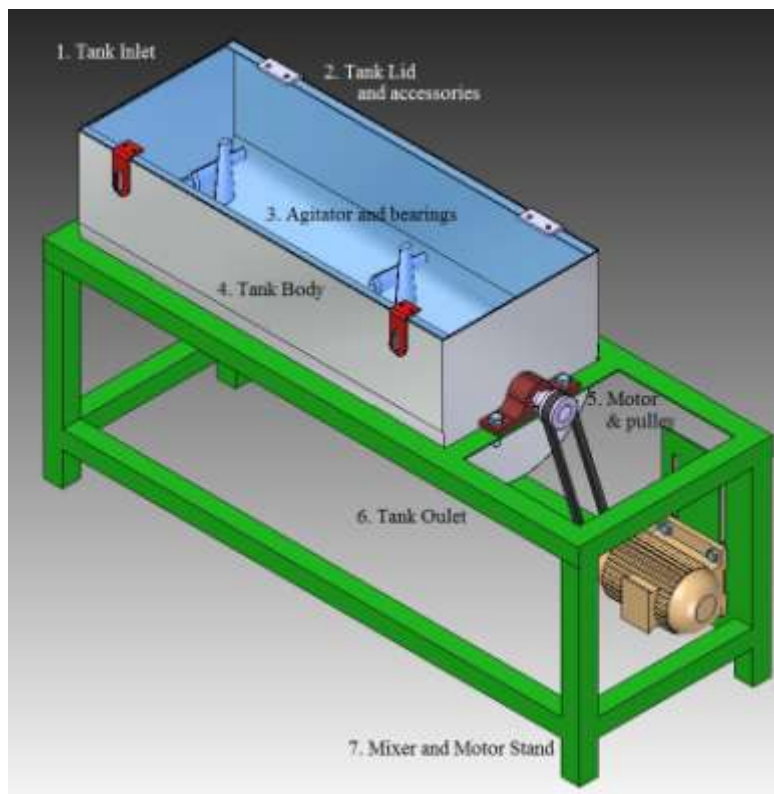


Figure 9. Sample system for water-flour mixer for pasta or bread making.

Table 6. Parts and descriptions for wet flour mixer

No.	Part Name	Description
1	Inlet	<p>For batch operations, the water and powders may be added through simple openings in the lid.</p> <p>For continuous operations, the powders should be added via a continuous feeder or dosifier, such as a screw feeder or rotating cups apparatus (see section 4.1). The water will flow through a valve and the flow rate may be controlled by a rotometer or other control.</p>
2.	Tank lid and accessories	<p>The tank lid should fit firmly to the tank body to avoid leaking. In this photo it is held down by hinges and latches. Also in the photo, the lid is constructed from a thick, transparent plastic to allow visual inspection of the mixing process.</p>
3.	Agitator and Bearings	<p>The agitator shaft is supported by a housed ball bearing, which allows for smooth rotation of the agitator, eliminates extraneous movement of the shaft, and holds the agitator.</p> <p>The agitator consists of tapered blades that alternate through the four main orientations: up, right, down, left. The blades are positioned such that the narrow side is the one that cuts through the dough.</p> <p>At the manufacturing workshop in Mekelle, the shape of the agitator blades was produced by cutting segments of a 25 mm diameter stainless steel pipe, and cutting each side at tapered angles using an electric hand saw, where the cuts began 2 inches up the length of the pipe. The cut edges and top were welded shut and the whole blade was lightly pressed to flatten. A small semi-circle was shaved into the bottom of the blade for a snugger direct weld to the agitator shaft.</p>
4.	Tank Body	<p>The tank body can be fabricated from either stainless steel or aluminum sheet metal. For large mixers, horizontal supports beams that transverse the top may be added.</p>
5.	Motor and Pulley	<p>The motor turns the agitator via a connection between pulleys attached to both the motor and agitator shaft. A standard pulley and belt is adequate, but a pulley-belt pair with teeth or notches is preferable to avoid any slipping due to torque upon start up.</p> <p>The motor assembly should be housed (not shown here) for safety and longevity of the assembly. At the very least, a metal guard should be built surrounding the pulley and pulley belts.</p> <p>To control the speed of the agitator shaft, a combination of pulleys with different sized diameters may be employed, where increasing the pulley diameter on the agitator shaft slows the agitation speed. Another alternative is a gear box, which uses different sized gears to the same effect. A final alternative</p>

		is a motor drive, such as an inverter, which connects electrically to the motor which regulates speed electronically. This last option provides the greatest operating flexibility. Moreover, the inverter may be controlled automatically, by a PID controller, for example.
6.	Outlet	For batch operations, the final dough must be cleaned out manually. For continuous operations, a rectangular cutout should be added to the tank bottom at the opposite end from where the flour is added. Under the outlet hole, a screw conveyor can be placed. As the mixed dough is pressed out the hole, it will be carried away by the screw conveyor.
7.	Mixer and Motor Stand	The tank and the motor assembly should be supported externally to prevent unnecessary load on the mixer itself and to promote stability. The stand can be fabricated from any steel or iron.

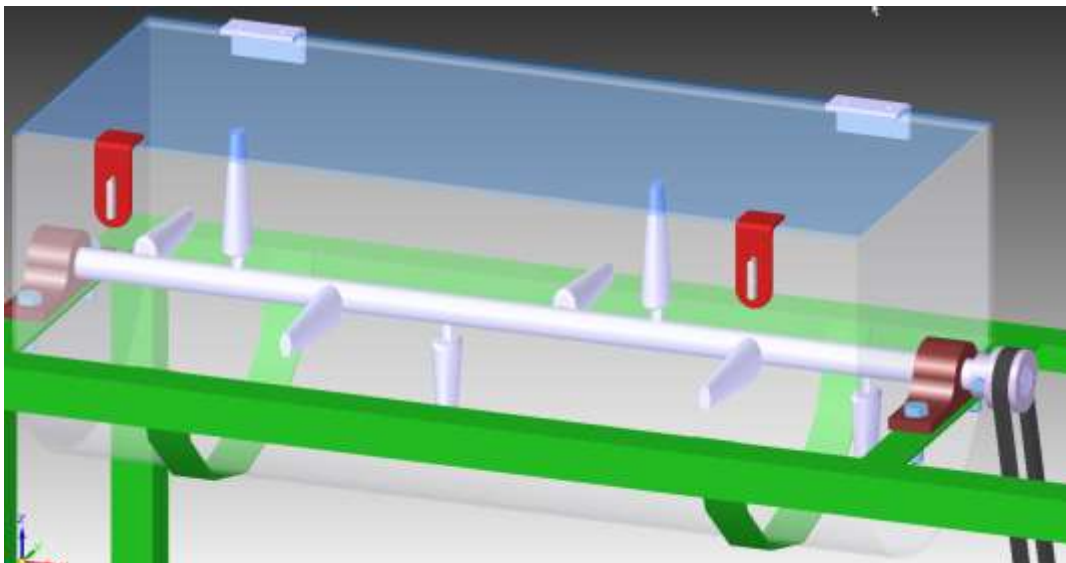


Figure 10. Example of water-flour mixer

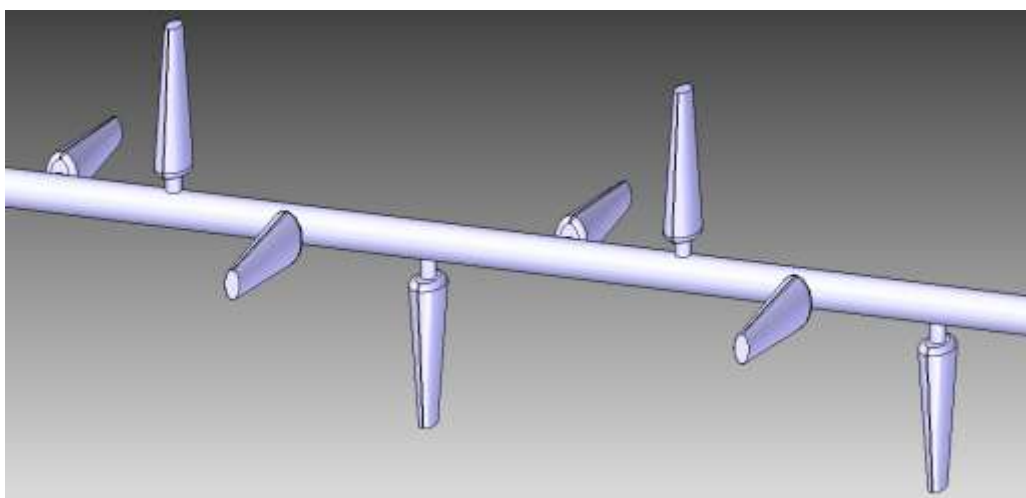


Figure 11. Example agitator for flour-water mixing in pasta or bread making

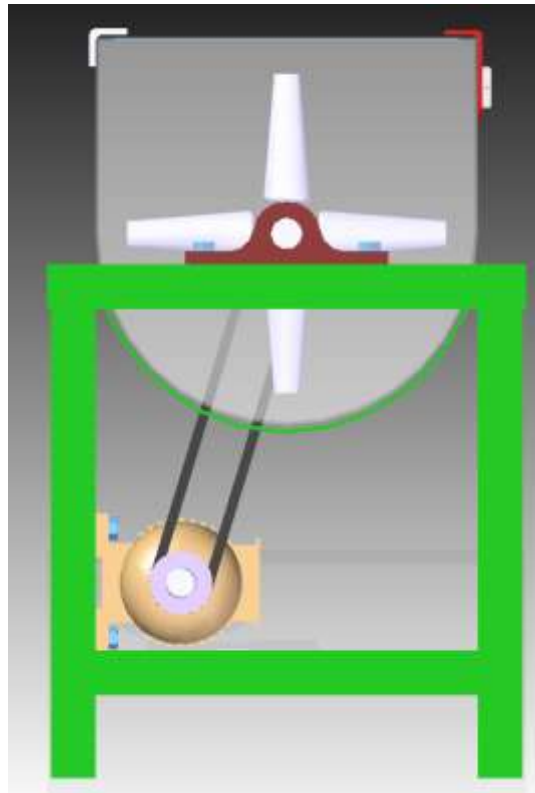


Figure 12. Side view of water-flour mixer

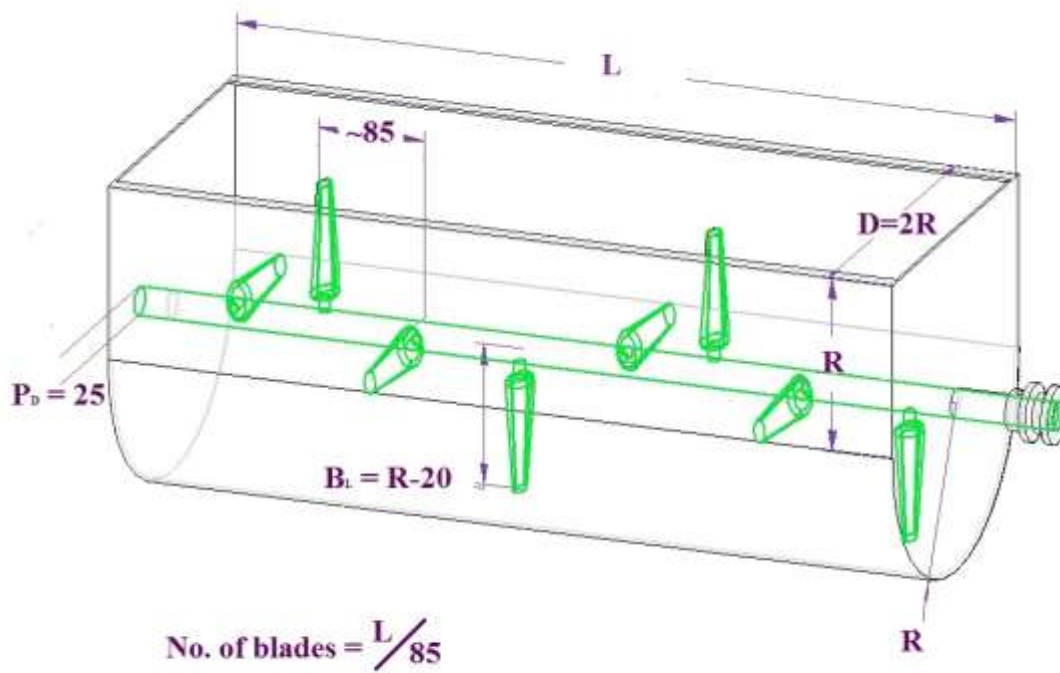


Figure 13. Dimensions for horizontal tank; numbers in millimeters (mm).

4.2.1.3 Horizontal mixer with paddle-type agitator for dry flour mixing

A horizontally oriented cylindrical tank with a paddle-type agitator is good for mixing of dry flour. Shown in Figure 14, the tank opens in the middle such that the agitator can be easily removed for cleaning or maintenance. There should be a tight seal between the two halves to prevent flour from spraying from the tank. For batch operations, the tank can be built to rotate, or tip, in order to empty its contents.

The paddles are oriented at **45° angles** in each of the four cardinal directions. For large tanks, **two paddle agitators** rotating side by side are recommended.

Please note, this design is common in industry, but, unlike previous designs, was not built locally and tested. However, it is believed to be possible for local metal workers to manufacture.

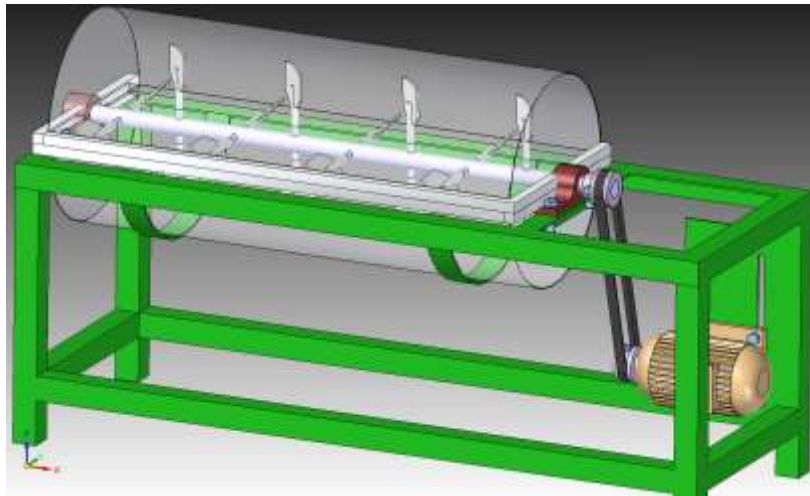


Figure 14. Example set up for paddle-type agitator tank

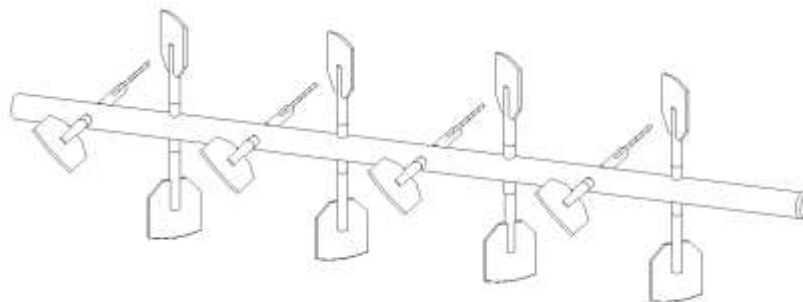


Figure 15. Paddle-type agitator for dry powder mixing

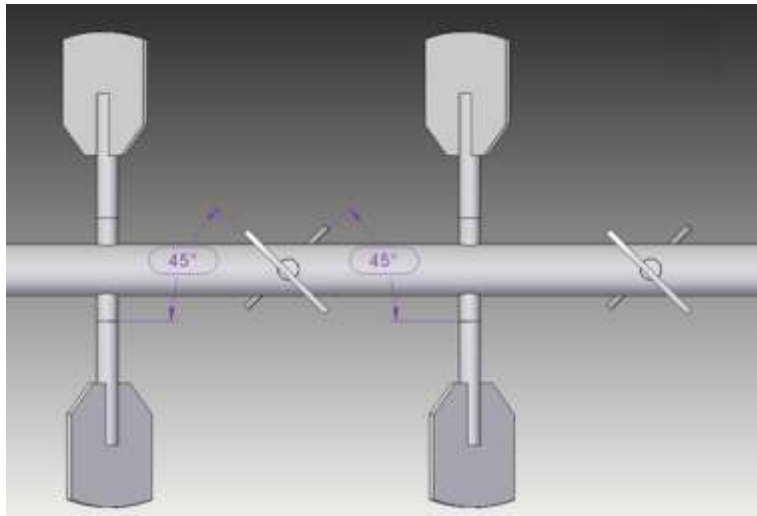


Figure 16. Top/side view of paddle-type agitator

4.2.1.4 Horizontal mixer with plow-type agitator for dry or wet flour mixing

A horizontally oriented cylindrical tank with a plow-type agitator is another good option for mixing of dry or wet flour. Like the previous tank shown Figure 14, the tank opens in the middle such that the agitator can be easily removed for cleaning or maintenance.

The plows are oriented each of the four cardinal directions and should almost scrape the edges of the tank.

Please note, this design is common in industry, but, unlike previous designs, was not built locally and tested. However, it is believed to be possible for local metal workers to manufacture.

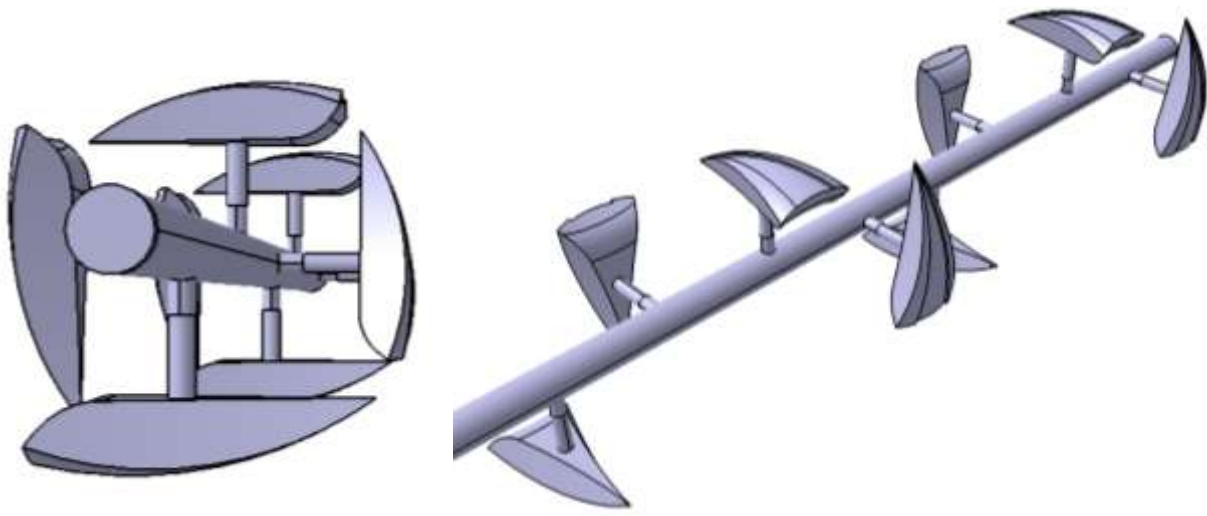


Figure 17. Front and angled views of plow-type agitator, respectively

4.2.1.5 Horizontal mixer with double ribbon/helical agitator for dry flour mixing

This design is very popular and effective for mixing dry flour. The helical blades should almost scrape the edges of the cylindrical tank shown in Figure 14. One screw is right handed, while the other is left handed, thereby pushing flour sections in opposite directions for efficient mixing. This twisted shape of the blades is very precise and may be difficult to replicate using local manufacturing techniques.

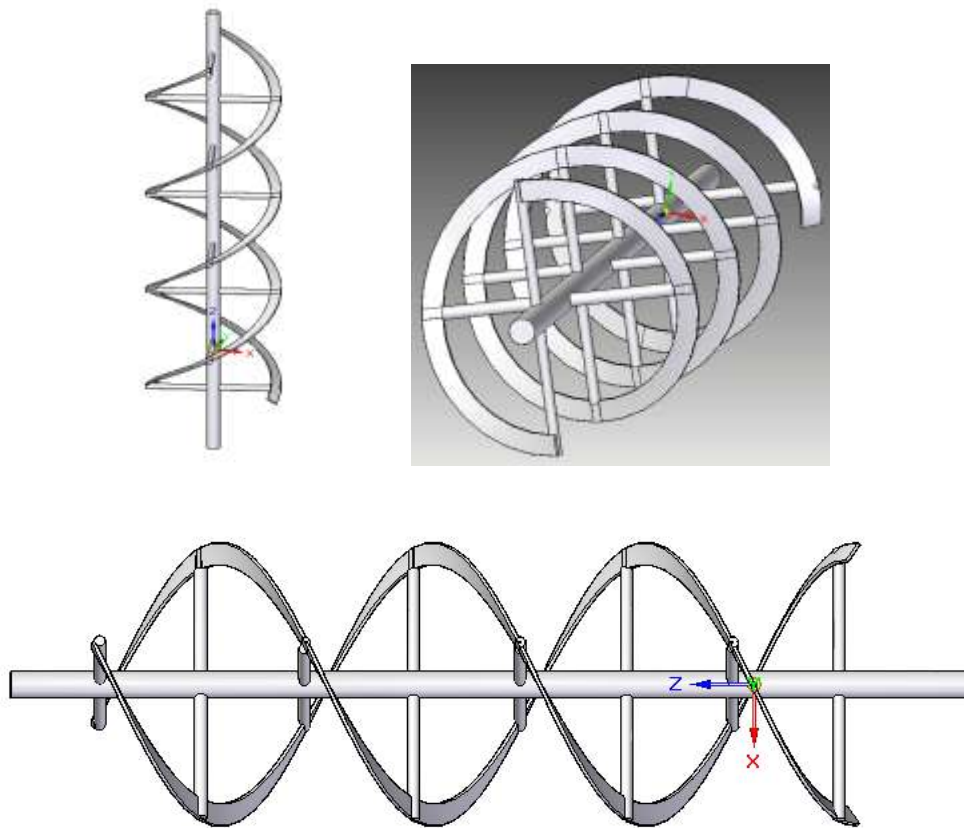


Figure 18. Various views of double ribbon/helical agitator.

4.2.1.6 Additional designs

Several other agitator types with stationary shell tanks exist and are mentioned in the literature, including z-type, mullers, or a variety of single or double arm “kneaders”. Other tumbler shapes are possible as well, including the double cone shape.

4.2.2 Built in-country with automatic controls

For a fully automated system, the following variables must be controlled:

- Inlet flow rate of all inputs (*for continuous system*)
 - For **solids**, will be dictated by a feeder, such as those shown above, or blower.
 - For **liquids**, will be dictated by a controlled valve or rotometer. A pump may be used to provide flow at the inlet, while gravity will be the main force for exit flow.
- Outlet flow rate of all outputs (*for continuous system*)
- Residence time
 - For **continuous systems**, will be determined indirectly by tank size and inlet and outlet flow rates.
 - For **batch systems**, will be set by mixer operator/automated control system
- Mixing speed (*for both batch and continuous*)

Therefore, it is possible to retrofit locally made designs with automatic controls given that an experienced engineer and technician trained in automatic controls are involved. The following equipment is needed:

1. Motor Drive (i.e. inverter) to determine speed and run time of the motor
2. Feeders with controllable flow rates (for solids)
3. Valves with controllable flow rates (i.e. rotometer; for liquids)
4. Pump with controllable rate (for liquid transport)

4.2.3 Out of country equipment suppliers

For locally-made equipment, contact local metal workshops to ask about capabilities. Follow advices given in next section on use of food grade metals. VonallCo in Mexico, Addis Ababa (contact information in section 4.3.2.2) is another option for a local company skilled in equipment for food mixing

ABC Hansen Africa

PO Box 25354
Pretoria, South Africa
Mills, microfeeders

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Batch or continuous mixers for flours or oil, in-line powder-liquid mixers with feeder

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4.3 Food Grade Metal Requirements

4.3.1 Stainless Steel vs. Aluminum

Stainless steel and **aluminum** are the two metals safe for food processing units. Table 7 provides a direct comparison between stainless steel and aluminum, which may be useful in choosing equipment material. In general, facilities operating at a large capacity may require stainless steel for its greater strength and resistance to wear. Smaller facilities may find aluminum more suitable for its lower cost and greater availability. Aluminum may also be chosen for specialized shapes, since aluminum is soft and can be shaved into the proper shape, or melted and poured into a mold. Melting stainless steel requires far more energy and sophisticated equipment.

Table 7. Comparison of Stainless Steel and Aluminum

Stainless Steel	Aluminum
Corrosion resistant; weld lines must be treated to avoid rusting	Corrosion resistant; weld lines will not rust
Relatively easy to weld	Difficult to weld; requires high amperage
High strength	Light weight
Lower strength to weight ratio	High strength to weight ratio
Surface more resistant to wear	Easily scratched or dented; surface requires polishing to smooth
More expensive	Less expensive
Good conductor of heat	Poor conductor of heat
Available in Addis Ababa	Available in regional cities

4.3.2 Stainless Steel

4.3.2.1 Grades

Stainless Steel is classified based on its relative percentages of nickel and chromium, which both provide strength and rust resistance. There are 150 grades of stainless steel, but only 304 and 316 are recommended for use in food equipment:

Table 8. Options for food grade stainless steel

Grade	Description
304	There are two types of 304 grade stainless steel, 18/8 and 18/10, which correspond to the percentages of chromium and nickel, respectively. 18/10 has a slightly higher nickel content (10% compared to 8%) so is slightly more durable and shiny than 18/8. 304 grade stainless steel must also contain less than 0.8% carbon and at least 50% iron. 304 grade is easier to roll and weld.
316	316 grade contains a slightly higher nickel content than 304, so is more resistant to rust, especially in chloride environments. It is generally more expensive than 304 and therefore less common.

4.3.2.2 Suppliers

Stainless Steel (304) metal and accessories such as welding electrodes and pickling and passivating (cleaning and preserving) agents may be purchased from VonallCom PLC., located near the Coffee and Tea Authority in the Mexico, Addis Ababa.

Vonall Com PLC

Tel: 251 (0)55 116 59/69

Fax: 251 (0)55 116 47

Email: info@vonall.com

Web: www.vonall.com

Mexico, south of Coffee and Tea Authority

4.3.2.3 Proper Welding Techniques and Treatment

Stainless Steel must be welded with a proper stainless steel electrode which is equal to or higher in grade than the metal to be welded. For instance, a 308 SS electrode is appropriate for welding 304 SS, but would not be appropriate for welding 316 SS. Using the wrong electrode could damage the surface of the SS sheet metal, or cause rusting along the weld.

Stainless steel is known for its corrosion-resistant properties, which are due to the relatively high percent (16-36%) of chromium (Cr). However, many are surprised to know that stainless steel does rust, and certain steps must be taken to avoid this phenomenon. The concentration of chromium in stainless steel is so high that it forms a thin, tightly adhering, and

Stainless steel does rust, and certain steps must be taken to avoid this phenomenon.

impervious layer of mostly chromium oxide (CrO) when exposed to oxidizing surfaces such as air, water, or caustics. The formation of this protective layer is called passivation, and it occurs naturally over the course of over 14 days. However, the process may be disrupted by the presence of dirt; inorganic compounds such as oils and grease, ferrous metals, copper, etc.; and other impurities. (19) When the passivation process is impeded, the CrO layer does not fully form, leaving the surface open and vulnerable to rusting.

Furthermore, stainless steel may rust if any iron-containing particles rub off on the surface of the metal. This may occur if any equipment (i.e. grinders, cutting blades) used on the stainless steel had been previously used on regular steels or iron alloys. In this situation, the iron particles on the surface will rust. However, after oxidation of these surface particles, no further rusting will occur.

It is therefore recommended to undertake the following treatments:

- 1) Cleaning/Pickling
- 2) Passivation Encouragement

These two steps can be carried out either mechanically or chemically, as represented by Table 9.

Table 9. Stainless steel treatment techniques

	Chemical Treatment	Mechanical Treatment
Cleaning	<p>Involves the use of pickling agents such as hydrochloric or hydrofluoric acid. Because these are very strong, caustic acids, they must be dealt with by trained personnel wearing protective equipment.</p> <p>Cleaning shines the surface, removes darkening of the surface due to weld burns, and eliminates rust or scale from ferrous, copper, or aluminum alloys, inorganic compounds such as oils/grease, and dirt.</p> <p>Pickling sludge is a hazardous waste, so must be neutralized prior to disposal. It is imperative to wash off all acidic residues with ample amounts of water.</p> <p>Pickling agents on the market include brands such as Antox or WonderGel.</p>	<p>The surface may be cleaned through mechanical abrasion, which removes surface particles and weld burns.</p> <p>A stainless steel brush is one acceptable tool for mechanical abrasion. The brush should be applied only to weld lines to avoid scratching or damaging the main surface.</p> <p>The other alternative for mechanical abrasion is to use a grinder that has not been previously used on any iron-containing metals, such as regular steel.</p>
Encouraging Passivation	<p>Involves the use of passivation agents, such as nitric acid or citric acid to encourage the formation of the chromium oxide layer. Passivation agents available on the market include brands such as Antox or WonderGel.</p> <p>Passivation can also be encouraged by submerging parts into a heated dilute acid bath, (i.e. citric or nitric acid).</p>	<p>Mechanical abrasion techniques such as the ones described above will help passivation occur naturally by removing any inorganic or dust particles that may have inhibited the formation of the chromium oxide layer. However, it will not actively speed up the passivation process as chemical treatment does.</p>

4.3.3 Aluminum

4.3.3.1 Grades

The following grades are commonly used for food processing equipment.

Table 10. Common grades of aluminum and aluminum alloys used in food processing industries.

Grade	Description
1100	This grade is pure aluminum and is easiest to work with because it is soft, ductile, and has lower welding requirements than the other alloys. It is not heat-treatable and ideal for molding into intricate shapes because it hardens less quickly than other types of aluminum. It has excellent corrosion-resistance properties.
3003	The most widely used of all aluminum alloys, the addition of manganese to this aluminum increases its strength by 20% as compared to 1100 grade. It has excellent corrosion resistance and workability and it is possible to be welded, drawn, spun, or brazed.
5005	This grade is an improved version of grade 3003. It has similar characteristics, but is less likely to streak or discolor.

4.3.3.2 Suppliers

Vonall Com PLC

Tel: 251 (0)55 116 59/69

Fax: 251 (0)55 116 47

Email: info@vonall.com

Web: www.vonall.com

Mexico, south of Coffee and Tea Authority

Yared Building Materials Shop

Downtown Mekelle, near Tea-Chat Café

4.3.3.3 Welding and Shaping Techniques

Aluminum is more difficult to weld than steel because it has a lower melting point, higher thermal conductivity, and doesn't change color before it begins to melt. Because aluminum is a good conductor of heat, it requires a lot of heat to be welded. It is helpful to heat the aluminum

before welding to achieve a stronger weld that is less susceptible to cracking. It is also crucial to remove the aluminum oxide layer that may have formed over time on the surface of the aluminum because the oxide has a melting point three times as high as the melting point of aluminum (higher even than its boiling point!) so may sink into the aluminum layer during welding.

Before contracting an aluminum design, check with the metal workshop to ensure they have aluminum welding capabilities. If not, they may be able to contract the work out to another local workshop with capabilities. Machines for welding aluminum are also available for rent in the regions. There are several methods for welding aluminum. TIG welding, using Tungsten gas is most common, followed by MIG welding or Gas Metal Arc Welding. However, if such gases are not commonly available (as is often the case in Ethiopia), stick welding can be used.

Compared to stainless steel, aluminum is more difficult to weld, but easier to mold and shape.

An alternative to aluminum welding is **aluminum molding**, by which raw aluminum is melted at high temperatures and poured into a pre-fabricated mold. This process is common around Ethiopia and, while it may be costly or technically difficult to mold large shapes (i.e. tanks), it is a good option for lids, pulleys, or agitator blades. Such items may also be formed from **aluminum shaping**, by which an aluminum bar is cut into shape using a variety of mechanical or hand tools, such as a lathe.

4.4 Additional Equipment Materials

4.4.1 Motors and Motor Drives

For many factories, electrical agitation is preferred. Motors may be acquired in country through several whole-sellers. Table 11 provides a summary of power requirements and estimated cost.

Table 11. Motor Requirements and Costs

Motor Required (HP/kW)¹	Cost (ETB/USD)²
0.75/ 0.56	2,850/ \$146 ³
1.0/ 0.75	3,200/ \$164
1.5/ 1.12	3,700/ \$190
2.0/ 1.49	3,980/ \$204
3.0/ 2.24	4,550/ \$234
4.0/ 2.98	5,250/ \$270
5.5/ 4.10	6,200/ \$318
7.5/ 5.59	6,980/ \$358
10/ 7.46	7,990/ \$410
15/ 11.49	12,550/ \$640
20/ 14.91	13,900-19,900/ \$714-1,068
30/ 22.37	20,800-26,900/ \$1,068-1,381
50/ 37.28	47,200/ \$2,424
75/ 55.93	79,100/ \$4,062
100/ 74.57	114,990/ \$5,905
150/ 111.85	116,750/ \$5,996
180/ 134.23	194,500/ \$9,988
215/ 160.33	224,500/ \$11,529

Ideal motor speed for dry flour mixing is generally less than 100 rpm. Motor speed may be reduced through the addition of a gear box or a motor drive.

A **gear box** is an enclosed system of assembled gears that transmits mechanical energy from the motor to the agitator. A gearbox can be designed to change speed, direction, or torque of the mechanical energy through mechanical methods. There is less flexibility in setting speed and direction than is provided with motor controllers, but it is a lower cost alternative.

¹ A ratio of diameter to height of tank of 0.8 was used. To lower power requirements as volume increases, lower the diameter to height ratio, while keeping the tank diameter to impeller diameter the same.

² Cost estimates are provided by **Jimma Enterprises** (located in Piassa, Addis Ababa) for normal speed, copper wired motors reaching up to 1500 rpm. Reduced speed motors have somewhat higher costs, while aluminum wired motors have lower costs. It is generally cheaper to purchase a motor from Addis Ababa than regional cities.

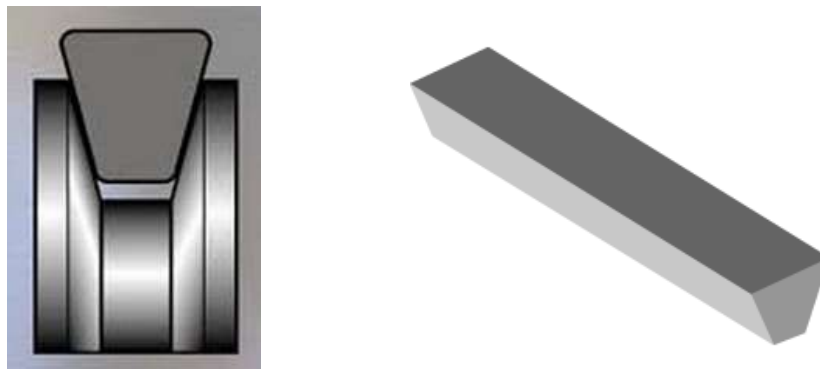
³ Exchange rate from May 24, 2014.

Motor controllers are devices that, via an electric connection, govern the performance of an electric motor, from starting to stopping the motor, selecting forward or reverse motion, selecting and regulating the speed, and protecting against overloads and faults. Types include motor starters (simple starting and stopping of the motor), adjustable-speed drives (start and stop as well as set speed and rotation), and intelligent controllers (uses a microprocessor to match motor torque to load for energy efficiency). An inverter is an example of an adjustable-speed drive.

4.4.2 Power Transmission: *Pulleys and Pulley Belts*

Vee Belts/ Wedge Rope

The V-belt is the basic belt for power transmission. Its cross-sectional shape is trapezoidal, such that the belt cannot slip off the pulley. Slippage is low but can occur.



Multi Groove Belts/ Poly V Belts

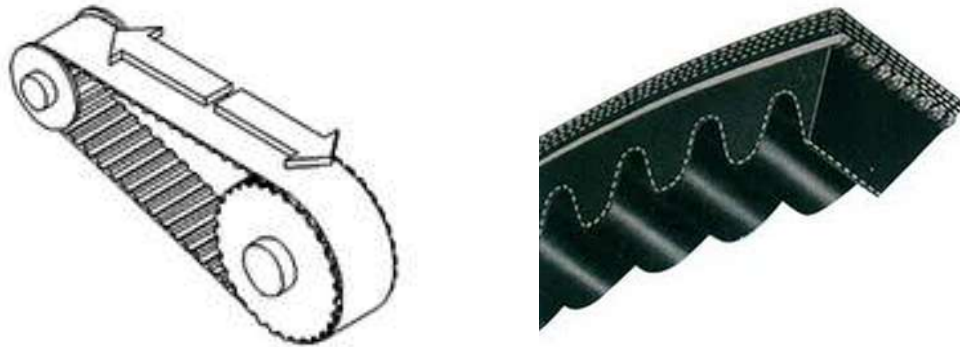
These belts consist of 5 or 6 V shapes alongside each other for greater belt flexibility. This belt may last longer than the single v-belt.



Timing Belts

(aka toothed, notch, cog, synchronous belts)

These belts have teeth that fit into a matching toothed pulley. If correctly tensioned, they have no slippage, run at constant speed, and are often used to transfer direct motion for indexing or timing purposes.



4.4.3 Additional Supplies

Additional supplies for processing equipment may include:

- Pulley and motor belt with proper safety shield
- Aluminum or stainless steel screws, bolts or nuts
- Steel or iron metal for stand
- Bearings for smooth rotation of agitator shaft
- Automatic controls, as desired

4.5 Mixing Tank Sizing Requirements

- Step 1: Determine daily production rate
- Step 2: Determine actual operating time per day
- Step 3: Decide if batch or continuous operation is desired
- Step 4: If batch, decide how many times per day (batch) or total minutes per day you want to run the tank. If continuous, flow rate of the tank should be the same as oil production flow rate.

Step 5: Guess a tank size and assume a reasonable mixing/residence time for complete mixing

Step 6: Calculate initial tank size needed using residence time from step 5.

$$\text{Batch tank volume} = (\text{production rate}) / \text{desired batches}$$

$$\text{Total batch mixing time} = (\text{desired batches})(\text{residence time})$$

$$\text{Continuous tank volume} = (\text{production rate})(\text{residence time})$$

Step 7: Guessed and calculated volume should be similar. If not, guess a new tank size (and thus new residence time) and recalculate tank volume.

Note: To size a feeder, follow equations and guidelines given in sections 4.1 and 5.1.1.

Scaled up values for the as-built V-Mixer and horizontal stationary tank can be found in Appendix D.

4.6 Cost Comparisons

Table 13 and Table 15 shows estimated costs for a V-Mixer and stand-still agitation tank scaled according to the dimensions presented in Figure 8 and Figure 13, respectively. Table 12 and Table 14 list some of the scaled dimensions for both tank types.

Please see

Appendix D: Cost Estimates from Local Manufacture Scale Up for a complete breakdown of costs. Note that numbers shown in the cost tables are calculated assuming each machine is made individually and no reduction in material costs or labor is given by buying in bulk. Please note that the cost listed does not include motor and motor drive.

Motor costs are found in Table 11. Adjustable speed drives can be purchased from Jimma Enterprises in Piassa, Addis Ababa. A 1.5 HP inverter costs about 10,600 Ethiopian Birr (ETB). Gear boxes will be slightly cheaper.

Table 12. Scale-up values for V-Tumbler

Empty Volume (L)	20	50	100	300	500	1000	2000	3000	4000
Production Values									
Empty Volume (ft ³)	0.71	1.77	3.53	10.59	17.66	35.31	70.63	105.94	141.26
Production Capacity (kg/batch) ⁴	4	10	20	60	100	250	600	1000	1350
Mixer Dimensions									
Leg Diameter (mm)	211	287	361	521	618	778	981	1123	1236
Outer Leg Length (mm)	391	530	668	964	1143	1440	1814	2077	2286
Inner Leg Length (mm)	180	244	307	443	525	662	834	954	1050

Table 13. Estimated Costs for Locally Built V-Mixer

Empty Mixer	Estimated Price			
Volume (L)	SS Body (ETB)	SS Body (USD)	Al Body (ETB)	Al Body (USD)
20	5,997	308	4,671	239
50	8,016	411	6,195	317
100	10,098	518	7,768	398
300	14,439	740	10,924	560
500	17,553	900	13,244	679
1,000	22,687	1,163	16,896	866
2,000	29,673	1,522	21,648	1,110
3,000	35,245	1,807	25,360	1,300
4,000	40,140	2,058	28,573	1,465

Table 14. Scale-up values for stand-still agitation tank

Empty Volume (L)	50	100	200	300	500	1000	2000	3000	4000
Production Capacity (kg/batch), est. ⁵	11.2	22.4	44.9	67.3	112	224	449	673	898
Diameter (m)	0.30	0.38	0.48	0.55	0.68	0.86	1.19	1.37	1.50
Length (m) ⁶	0.70	0.89	1.12	1.28	1.37	1.72	1.79	2.05	2.25
No. agitator blades	8	10	13	15	16	20	21	24	27

⁴ Loading ranges between 40-60%, increasing with tumbler sie.⁵ Estimated to be 40% loading⁶ Length to diameter ratio is variable. For calculation purposes, we range between L=1.5*D – 2.3*D

Table 15. Estimated costs for locally built stand-still agitation tank

Empty Mixer Volume (L)	Estimated Price			
	SS Body (ETB)	SS Body (USD)	AI Body (ETB)	AI Body (USD)
50	7,834	402	7,046	361
100	10,052	515	8,273	424
200	13,271	681	10,693	548
300	16,531	848	13,332	684
500	21,447	1,100	17,267	885
1,000	30,253	1,551	31,004	1,590
2,000	44,275	2,271	50,222	2,575
3,000	55,497	2,846	69,407	3,559
4,000	66,254	3,398	91,060	4,670

Chapter 5:

Premix Options

5.1 Addition Amounts

The dietary goal of fortification, as defined by the World Health Organization, is to provide 97.5% of individuals in the population group(s) at greatest risk of deficiency with an adequate intake of micronutrients, without causing a risk of excessive intakes in other

The World Health Organization (WHO) recommends an approach to calculating the amount of fortificant that should be added to each food matrix, called the **EAR-cut point method**. It assumes that the distribution of nutrient intake among a population can be approximated by a bell curve. In deficient populations, there is a large proportion of the population consuming below the **Recommended Nutrient Intake** (RNI) level. The WHO together with the Food and Agriculture Organization (FAO) define RNI as “*the daily dietary intake level necessary to meet the nutrient requirements of almost all (i.e. 97-98%) healthy individuals within a particular age, gender, and physiological status group.*” In order that the requirements of nearly *every* person in the group are met, it is set about two standard deviations higher than the **Estimated Average Requirement** (EAR) of the subpopulation. The EAR is the “*average amount required by a population group.*” As shown in Figure 19, the principle of the EAR-cut point method is to shift the bell curve such that only about 2-3% of the population consumes less than his/her respective EAR value.

It is important to note that the curve shifts based on the EAR and not the RNI. This is to avoid risk of the population exceeding the **tolerable upper intake level** (UL), which is the “*highest average daily nutrient level unlikely to pose risk of adverse health effect to almost all (97.5%) of healthy individuals in an age and sex specific population group.*” Once it is determined how far the curve can be shifted, the **Feasible Fortification Level**, the level that satisfies the above defined goal of fortification programs, is set. How far the curve shifts is based upon data collected for populations concerning consumption and purchasing habits. Such habits for Ethiopia were listed in the chapter 1 of this paper.

Values of RNI, EAR, and UL for select micronutrients and subpopulations are listed in Appendix A.

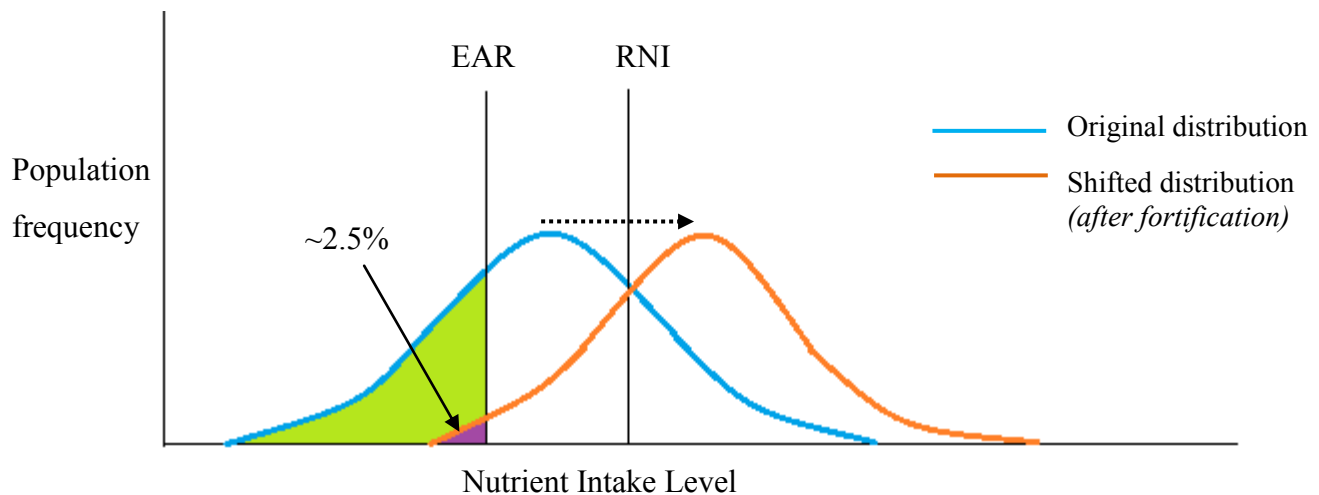


Figure 19. The EAR-cut point method for determining feasible fortification levels

5.1.1 Calculations

Refer to the guidelines set by FMHACA to determine the desired vitamin concentration in the fortified food matrix.

1. Batch Operation

Amount of premix to be added

$$= \frac{(\text{Desired vitamin conc.}) \times (\text{Vol. of food matrix in tank})}{(\% \text{ vitamin in premix})}$$

Ex: We have a tank that holds 5,000 L of oil. We wish to fortify the oil such that the final product has 20 ppm vitamin A. How much mix should we add, if our premix is 40% vitamin A?

$$\left(20 \frac{\text{mg vitamin A}}{\text{L oil}} \right) (5,000 \text{ L oil}) \div \left(0.4 \frac{\text{g vit.A}}{\text{g mix}} \right) = 250 \text{ g mix}$$

2. Continuous Operation

Flow rate of vitamin premix

$$= \frac{(\text{Desired vitamin conc.}) \times (\text{Flow rate of food matrix})}{(\% \text{ vitamin in premix})}$$

Ex: Under normal operating conditions, our factory produces 5 L oil per minute. We wish to fortify the oil such that the final product has 15 ppm vitamin A. At what rate should we set our pre-mix feeder, if our premix is 50% vitamin A?

$$\left(15 \frac{\text{mg vitamin A}}{\text{L oil}}\right) \left(5 \frac{\text{L oil}}{\text{min}}\right) \Big/ 0.5 \frac{\text{g vit. A}}{\text{g mix}} = 150 \frac{\text{mg mix}}{\text{min}} = \mathbf{9 \text{ g mix/s}}$$

3. Two-Stage Mixing

Outlet vitamin concentration from tank 1

$$= \frac{(\text{Amt. Premix added}) \times (\% \text{ vitamin in premix})}{\text{Vol. of food, in tank 1}}$$

Final vitamin concentration from tank 2

$$= \frac{(\text{Vitamin Conc., tank 1}) (\text{Vol. or flow rate, tank 1})}{(\text{Vol. or flow rate, tank 2})}$$

Ex: We desire to produce oil with a concentration of 20 ppm using a two-stage batch tank setup. The tanks hold 500 L and 5,000 L of oil, respectively and our premix contains 50% vitamin A. How much premix should we add to the first tank?

$$\text{Conc. of vit. A, tank 1} = \frac{\left(20 \frac{\text{mg vit. A}}{\text{L oil}}\right) (5,000 \text{ L oil})}{500 \text{ L oil}} = 200 \text{ ppm vitamin A}$$

$$\frac{\left(200 \frac{\text{mg vit. A}}{\text{L oil}}\right) \times 500 \text{ L oil}}{0.5 \text{ g vit. A/g mix}} = \mathbf{200 \text{ g mix}}$$

Note: either of the above equations for one tank operation could have been used with the same result. These set of equations are useful only if the intermediate concentration is desired.

5.2 Cost Comparison

Table 16. Costs of Select Premix Options (20)

Core Package Premix	
Premix Composition	Active Ingredient
<ul style="list-style-type: none"> • Ferrous Fumarate • Folic Acid 	60 ppm as iron 2.6 ppm
Addition Rate, g/MT flour	200
Premix Cost, US\$/kg	\$6.58
Fortification Cost, US\$/MT flour	\$1.32
Restoration Package Premix	
Premix Composition	Active Ingredient
<ul style="list-style-type: none"> • Ferrous Fumarate • Folic Acid • Thiamine Monomitate (vitamin B₁) • Riboflavin (vitamin B₂) • Niacin (vitamin B₃) 	60 ppm as iron 2.6 ppm 6.0 ppm 5.0 ppm 45 ppm
Addition Rate, g/MT flour	300
Premix Cost, US\$/kg	\$12.48
Fortification Cost, US\$/MT flour	\$2.50
WHO Package Premix	
Premix Composition	Active Ingredient
<ul style="list-style-type: none"> • Ferrous Fumarate • Folic Acid • Vitamin A • Vitamin B₁₂ • Zinc Oxide 	60 ppm as iron 2.6 ppm 3.0 ppm 0.02 ppm 55 ppm as Zinc
Addition Rate, g/MT flour	300
Premix Cost, US\$/kg	\$12.48
Fortification Cost, US\$/MT flour	\$2.50

Table 17. Cost Estimate of Select Fortificants (1)

	Adult EAR	Nutrient content of fortificant (%)	Cost of fortificant (US\$/kg)	Overage (%) ⁷	Annual cost of fortificant (US\$) ⁸
Vitamin A					
• Vitamin A (SD-250)	429 µg	7.5	42	50	0.136
• Vitamin A palmitate, 1 million IU	429 µg	30	52	30	0.042
Vitamin D, water soluble	5 µg	0.25	33	20	0.035
Vitamin E	8 mg	67	26	20	0.163
Vitamin C	37 mg	100	10	250 ⁹	0.567
Thiamine (vitamin B₁)	1.0 mg	81	24	40	0.018
Riboflavin (vitamin B₂)	1.1 mg	100	38	30	0.024
Niacin (vitamin B₃)	12 mg	99	9	10	0.053
Folic acid	188 µg ¹⁰	100	90	50	0.011
Vitamin B₁₂, 0.1% watersoluble	2.0 µg	0.1	38	30	0.043
Iron¹¹					
• NaFeEDTA	7.0 mg	13	15.45	5	0.383
• Ferrous bisglycinate	7.0 mg	20	25	5	0.402
• Ferrous fumarate	10.5 mg	33	5.12	5	0.075
• FeSO ₄ , dried	10.5 mg	33	2.35	5	0.034
• FeSO ₄ , encapsulated	10.5 mg	16	12.28	5	0.371
• Electrolytic iron	21.1 mg	97	5.76	5	0.058
Zinc (as oxide)	6 mg ¹²	80	3.35	5	0.012
Calcium (as phosphate)	833 mg	39	2.7	5	2.652
Iodine (as potassium iodate)	107 µg	59	20	25	0.002

⁷ Overages is the additional amount that is added to account for losses in storage, production, and distribution.

⁸ The cost of supplying enough micronutrient to meet 100% of the EAR of an adult male, daily for one year. It includes a +20% overage to account for variability in the fortification process.

⁹ Vitamin C is one of the least stable fortificants and a high overage is needed. However, if the fortified food is not subject to heat of oxidation, the overage can be much lower.

¹⁰ Folic acid is 1.7 times more bioavailable than naturally-occurring food-folates, so the EAR is divided by 1.7.

¹¹ The EAR for iron depends on its bioavailability from the diet. If the diet contains large amounts of iron absorption inhibitors, it may need to be multiplied by 2. The value given here assumes a food matrix of wheat flour.

¹² Assuming a moderate bioavailability of zinc.

5.3 List of Suppliers

The **GAIN** premix facility works with 18 premix blenders and 40 vitamin and mineral manufacturers. Contact the GAIN office in Ethiopia to learn more about acquiring vitamins or premixes from one of their many partners.

Ethiopia GAIN contact info:

TK International Building, across from the line taxi station in Bole

Addis Ababa

Ethiopia Branch Manager, Alem Abay

Email: aabay@gainhealth.org

Web: gpf.gainhealth.org

List of GAIN Premix Blenders

- AQC Chem Lab Pvt Ltd
- Bio-Organics Nutrient Systems Ltd
- Caravan Ingredients
- DSM Nutritional Products LTd
- Eurogerm
- Fortitech
- Glambia Nutritionals Duetschland Gmbh
- Granotec Chile SA
- Hexagon Nutrition
- LycoRed
- Muhlenchemie/ SternVitamin
- Piramal Enterprise Ltd
- Pristine Organics Pvt Ltd
- Research Products Company
- Shree Additives (Pharma and Foods) Ltd.
- The Wright Group
- Ufuk Kimya Llac San. Tic. Ltd. Stl

List of Vitamin and Mineral Manufacturers

- Ajay Europe, SARL
- Aland (Jiangsu) Nutraceuticals Co Ltd.
- Akzo Nobel Functional Chemicals Pte Ltd
- BASF SE
- Beijing Vita Sci-Tech Co Ltd
- Calibre Chemicals
- Canton Laboratories Pvt Ltd
- Chemische Fabrik Budenheim KG
- Crown Technology, Inc
- Dr. Paul Lohman GmbH KG
- DSM Nutritional Products Ltd
- Industrial Metal Powders Pvt Ltd
- Israel Chemical Ltd
- Jiangsu Brother Vitamins Co Ltd
- Jiangxi tianxin Phamaceutical Co Ltd
- Jubilant Organosys Ltd
- K&S Kali and Eire Ltd
- **ETC.**

Select premix blenders are shown below.

BASF Corp.

Offices in Europe, Africa (Kenya), America,
and Asia
www.basf.com

Tel: +49 (0)621 60-0
+1 800 526-1072 (Toll free)
Fax: +49 (0)621 60-42525

DSM Nutritional Products

Offices in Europe, Africa (Egypt, South
Africa), America, and Asia
www.dsm.com

Tel: +31 (0)45 578 8111 (Netherlands)
+27 11 398 6900 (South Africa)

Fortitech Co.

Offices in Europe, Africa (South Africa),
America and Asia
www.fortitechpremises.com

Tel: +1 518 372 5155 (USA, headquarters)
+27 11 398 6900 (South Africa)

Hexagon Chemical Corporation

1335 G. Araneta Avenue, SMH
Quezon City, Philippines, 1100
Hexagonchemical.com.ph

Tel: +632 781-5835/781-1447
+632 740-7604/731-4875
Email: info@hexagonchemical.com.ph

Watson Inc.

301 Herrernan Drive
West Haven CT 06516 USA
Several international distributors
www.watson-inc.com

Tel: +1-800-388-3481
Email: info@watson-inc.com

The Wright Group

P.O. Box 821
Crowley, LA 70526 USA
Several international distributors
www.thewrightgroup.net

Tel: +1 (337) 783-3096 (customer service)
+1 (337) 783 3096 ext. 158 (sales)
+1 (800) 201-3096 (toll free)

Chapter 6:

Chemical Analysis

6.1 Semi-Quantitative Method

These methods are fast and relatively easy to carry out in a laboratory setting. They are not sufficient for determining exact concentration of added micronutrients, but may be useful for periodic or routine checks. Care should be taken to adhere to all safety warnings listed on the chemical reagents Material Safety Data Sheets (MSDS) and proper personal protective equipment (PPE) should be worn.

6.1.1 Iron Spot Test¹³

Principle

In an acidic medium, ferric iron and potassium thiocyanate (KSCN) react and form an insoluble red pigment. Other types of iron, such as elemental iron or ferrous iron, will act similarly after they are oxidized to the ferric form through reaction with hydrogen peroxide (H₂O₂). The spot formed will be proportional in size to the concentration of iron in the flour. This concentration can be measured semi-quantitatively by a visual comparison against a series of known standards.

Safety Precautions

Hydrochloric acid is a strong acid and may cause severe burns. Gloves, eye protection, and lab coats should be worn when using HCl. If in contact with eyes or hands, wash the affected areas thoroughly. If in contact with clothing, remove all clothing and wash skin that may have been affected.

Always add acid to water, not the other way around. Read the MSDS of all chemicals involved.

Materials

1. Filter paper Whatman #1
2. Manual sieve

¹³ Sources: (23; 24; 25)

3. Watch glass

Chemical Reagents

1. 2N Hydrochloric acid solution (HCl)
 - To a 500 mL beaker, add 100 mL distilled water. Slowly pour 17 mL of concentrated HCl followed by 83 mL more of water.
2. 10% Potassium Thiocyanate solution (KSCN)
 - Dissolve 10 g of KSCN in 100 mL of water.
3. 3% Hydrogen Peroxide solution (H₂O₂) – *required only if using an elemental iron or ferrous salt*
 - Add 5 mL concentrated 30% H₂O₂ to 45 mL distilled water. Prepare daily and discard after completing the analysis.

Methods

1. Place the filter paper over the watch glass.
2. Wet the surface of the filter paper with the solution of KSCN. Let the liquid penetrate the paper fibers.
3. Using a hand sieve, sift a portion of the flour in order to load a thin layer over the entire wet area. Scrape off any excess flour.
4. Mix 10 mL of the 2N HCl solution with 10 mL of the 10% KSCN solution. Add a small amount of this solution over the flour.
5. Let it stand for a few minutes for the reaction to occur. *If using elemental iron or ferrous salts, add a small amount of the 3% H₂O₂ solution to the flour. Let it stand for a few minutes for the oxidation to Fe(III) to occur.*
6. Red color spot will indicate the presence of iron from any form.

Interpretation

The number and distribution of spots indicate the relative concentration and homogeneity of all forms of iron in the sample. Perform this test using flour containing different, known concentrations of iron in order to provide a relative comparison, as demonstrated in Figure 20.

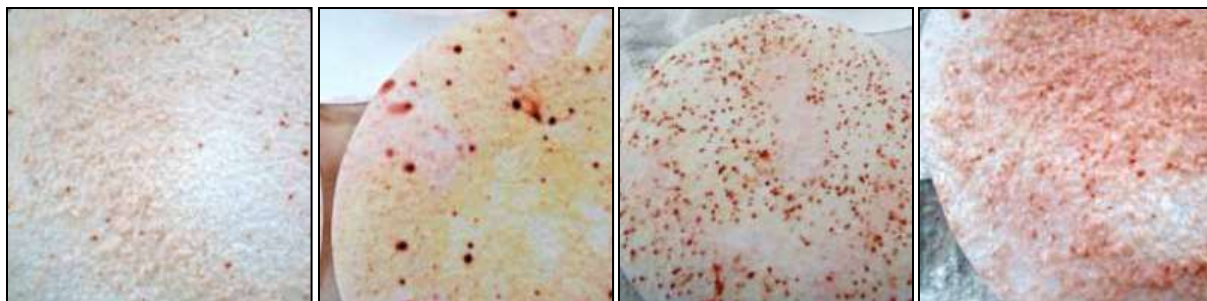


Figure 20. Sample results from an iron spot test

6.1.2 Vitamin A Test¹⁴

Principle

Retinol and its esters of palmitate or acetate forms anhydretinol in the presence of the chromogenic reagent of trifluoroacetic acid (TFA) dissolved in dichloromethane (DCM). The compound has a transient blue color, whose color intensity is proportional to the concentration of the retinol. This concentration can be measured semi-quantitatively by a visual comparison against a reference scale of standard copper sulfate solutions.

Safety Precautions

The chromogenic reagent is corrosive, and proper protective clothing, gloves, and eyewear should be worn by trained personnel.

Critical Points

The chromogenic reagent is unstable and should be prepared frequently and stored in an amber bottle. It can last 5 days unrefrigerated or 14 days in the refrigerator. If acetic anhydride is added to the solution, the chromogenic reagent is stable for 18 days at room temperature. If refrigerated, the reagent should be removed from the refrigerator for 2-3 hours prior to use.

To verify the quality of the reagent, a control with a known concentration of vitamin A in oil should be analyzed at the same time, and the intensity of the blue color should match the expected intensity on the reference scale.

¹⁴ Sources: (23; 24; 26)

The color of the blue solution is transient, so the comparison of the color with the reference scale should be made within the first 10 seconds of reaction. A syringe rather than a pipette is used for the addition of the reagent to ensure the addition is vigorous and rapid.

Materials

1. Balance
2. Glass test tube
3. Glass syringe (5-10 mL)
4. Plastic Pasteur pipettes
5. Fibre glass filter disks (3 per test tube)
6. Glass rod
7. Colorimetric scale similar to that shown in Figure 21 **OR** standard copper sulfate solutions

Chemical Reagents

1. Distilled water
2. Copper Sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
 - Only needed if color cards are not available
 - Prepare a 300 g/L stock solution by dissolving 15 grams $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 50 mL distilled water
3. Chromogenic reagent: Trifluoroacetic acid (TFA)/Dichloromethane (DCM)
 - Mix 30.0 mL TFA (FW: 114.03, 99.5%) in 60 mL DCM (FW: 84.93, 99.5%)
 - Store properly in amber bottle

NOTE: While TFA has proved to be easier to handle and will not run cloudy due to moisture absorption, alternative chromogenic reagents (shown below) may be used, such as trichloroacetic acid (TCA) and antimony trichloride (Carr Price Solution). TCA is more readily available and cheaper than TFA, but is more corrosive and its complexes are less stable. While DCM is the preferred solvent, other solvents such as hexane or chloroform may be used.

- a. Trichloroacetic acid (TCA): Dissolve 25 g of TCA (FW: 163.39) in 35 mL of DCM and heat gently to dissolve. Make up the solution to 50 mL with the

solvent. 15mL of acetic acid may be added to increase the stability of the solution by reducing effects due to atmospheric moisture. Store in a brown bottle.

- b. Antimony trichloride (Carr Price Reacion): Dissolve 100 g antimony trichloride (SbCl_3 , FW: 228.11) in 300 mL chlorogorm. 15mL of acetic acid may be added to increase the stability of the solution by reducing effects due to atmospheric moisture. Keep the reagent as dry as possible and away from light.

Methods

Preparation of Standard Solutions (if color cards not available)

1. Prepare the following solutions from the 300 g/L stock solution:

Volume (mL) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ – 300 g/L to prepare 10 mL	Concentration of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (g/L)	Approximate Concentration of Retinol (mg/kg)
1	30	10
2	60	20
3	90	30
4	120	40

2. Make up to volume (10 mL) with distilled water
3. Add 5 mL of the standard solutions into labeled test tubes. The test tubes should be the same type as that used for the samples. Close the tubes tightly with a rubber stopper or screw cap.
4. The tubes may be kept indefinitely at room temperature.

Analysis of Samples

1. Add 1 gram of fortified flour to a test tube.
2. Add 0.5 mL of distilled water and shake the tube vigorously to wet the flour.
3. With the syringe, rapidly add 3 mL of the chromogenic reagent to the tube, and shake the tube for a few seconds to ensure mixing.
4. Place 3 fiber glass discs into the mouth of the tube and gently press down using the glass rod. Depress the discs gently and evenly so as to press the solid material down.

5. Withdraw the glass rod and compare the solution with the color card or previously made standards.

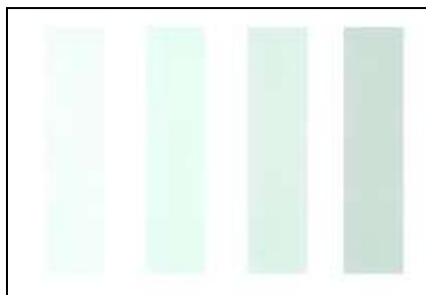


Figure 21. Sample color chart depicting concentration of vitamin A (mg/kg) in flour

6.2 Quantitative Methods

Quantitative methods provide exact concentrations of chemicals and require more sophisticated chemicals and equipment, such as spectrophotometers and High Performance Liquid Chromatography (HPLC). See Appendix C for selected procedures and refer to the literature for alternate variations of the procedures.

6.3 Local Laboratory Suppliers

FarMed PLC

Pastor, in front of St. Paul's Hospital
Addis Ababa

Micron International Trading House PLC

Tel: 251-11-551 43 34
Fax: 251-11-551 14 93
Email: micron@ethionet.et
Mexico, near Coffee and Tea Authority
Addis Ababa

Neway PLC

Piazza, near St. George church
Addis Ababa

Star Avenue Chemicals

Tel: 251-11-663-12 54/251-91-163 76 84
Web: www.staravchem.com
Bole, Jema Blvd. Suite 401
Addis Ababa

Volumetric Educational Supply

Tel: 251-11-840 04 89/251-91-151 44 67
Email: volumetricplc@aol.com
Web: www.volumetric.com.et
Kirkos, in Cherkos Trading Center
Addis Ababa

6.4 Rapid Testing Kits

A variety of testing kits exists for fast, reliable analysis of certain chemicals, and can be good alternatives given equipment limitations (i.e. no HPLC, spectrophotometer). Kits range from quantitative to semi-quantitative.

1. **BASF semi-quantitative kits** for *vitamin A concentrations in oil, flour, or sugar*.

This kit uses a colorimetric method to determine vitamin A concentration. It operates off the principle that retinol in an organic solution can be reacted with a chromogenic solution to produce a blue color whose intensity is proportional to the concentration of the retinol in the sample. There are three main steps: saponification, extraction, and reaction with chromogenic solution. Saponification converts any ester forms to the free alcohol form of retinol. This is done in the kit with water and 2-propanol, but it can be done in the lab with a variety of reagents (i.e. NaOH, etc.). Next, the retinol is extracted from the food matrix using an organic non-polar solvent. n-Hexane is used in the kit, which is common in most laboratory methods. Salt can be added to improve the

separation. After the separation, the solution reacts with a solution of trichloroacetic acid in dichloromethane (the chromogenic agent), similar to the Carr-Price Method. The blue color of the sample is compared with the swatch of colors given in the kit to yield a semi-quantitative result. The blue color is fleeting so the comparison must be made within the few seconds of reaction with the chromogenic reagent. The blue color typically shows only if the vitamin A concentration is above 0.5 mg/kg.

This kit is very affordable, at about 3 U.S. cents per sample.

2. **iCHECK FLUORO** by BioAnalyt: *quantitative kit for vitamin A in dairy, sugar, flour, or blood.*

This is a trusted kit that gives quantitative results, proven to be similar to those obtained by HPLC methods. It operates off the principle that retinol absorbs UV light at 325 nm and fluoresces at 460 nm when excited. This fluorescence is enhanced when retinol is bound to the retinol binding protein (RBP), present in blood serum. The portable fluorometer measures the level of fluorescence, which is proportional to vitamin A concentration.

The kit costs about 9.50 Euro/sample.

3. **iCHECK™ – iEX™ IRON** by BioAnalyt: *quantitative kit for iron in vitamin premixes, flour, beverages, and fish and soy sauce.*

Another trusted and validated kit by BioAnalyt, this kit measures exact ferrous and ferric iron concentration in food matrices. Samples are reacted with pre-made reagents and iron concentration is determined using a portable photometer.

4. **iCHECK™ – iEX™ ZINC** by BioAnalyt: *quantitative kit for zinc in vitamin premixes and flour.*

Samples are reacted with pre-made reagents and total zinc concentration (zinc oxide, zinc chloride, or zinc sulfate) is determined using a portable photometer.

Contact Information

BASF Corporation:

Tel: + 49 (0)621 60-0 (Germany headquarters)
+ 1-973-245-6000 (North America Branch)
+ 1-800-526-1072 (Toll free)

Web: www.basf.com

→ *For more BASF rapid testing kits, visit:*

<http://www.food-fortification.com/Videos.aspx>

BioAnalyt:

Tel: +49 (0)3328-35150-00

Email: contact@bioanalyt.com

Web: www.bioanalyt.com

Rheinstraße 17, 14513, Teltow, Germany

→ *For more BioAnalyt testing kits, visit:*

http://www.bioanalyt.com/images/content/downloads/products/BioAnalyt_Product_Catalogue_June_2013_EN.pdf

6.5 External Analysis

In the case of limited laboratory equipment, supplies, or personnel, samples may be sent to external labs for analysis. In this case, the manufacturer must legally wait for test results before the product may be shipped out for sale to ensure quality assurance of the product. The following is a non-comprehensive list of labs specializing in food testing.

Domestic Laboratories

Bless Agri Food Laboratory Services, PLC

Tel: +251 (0)913 04 75 55

Web: www.blessagri.com

Legetafo, Addis Ababa

Ethiopian Conformity Assessment Enterprise

Tel: +251 (0)11 646 05 69

Web: www.eca-e.com

Bole, Addis Ababa

Branch offices in Adama, Hawassa, Bahir Dar, Dessie, Dire Dawa, Mekelle, and Jimma

International Laboratories

Campden BRI

Tel: +44 (0)1386 842000

Email: information@campdenbri.co.uk

Web: www.campdenbri.co.uk

Campden, United Kingdom

Council for Scientific and Industrial Research (CSIR)

Tel: +27 (0)82 896 2314

Email: mwaldner@csir.co.za (Michael Waldner)

Web: www.csir.co.za/food_and_beverage

Offices in Cape Town, Kwalazulu Natal and Pretoria, South Africa

InterTek Worldwide

Tel: +49 421 65727 390

Email: web.food@intertek.com

Web: www.intertek.com

Offices in Europe, the Middle East and Africa

Muriex NutriSciences (MicroChem Siliker)

Tel: +1-312-938-5151 (Headquarters)

Email: info@msnx.com

Web: www.merieuxnutrisciences.com

70 offices in 18 countries, including France, India, Italy, South Africa, and Turkey

Chapter 7:

Packaging

As is the case in all industrially processed foods, fortified foods must be correctly identified in appropriate packaging. Such packaging must include clear information such as:

- Product brand name
- Address of manufacturer
- List of ingredients
- Minimum levels of vitamins and minerals
- Date of expiry

Certain vitamins and minerals are sensitive to environment conditions such as light or heat. Packaging should be designed to avoid degradation of the vitamins due to external influences, such as light. For instance, vitamin A in the form of retinyl palmitate, is unstable under UV light, so dark or opaque packing is recommended for food containing vitamin A. Several B vitamins as well as folic acid are also labile to light.

Chapter 8:

Further Reading

Guidelines on Food Fortification with Micronutrients

Produced by: World Health Organization/ Food and Agriculture Organization of the U.N.

Edited by: Lindsey Allen, Bruno de Benoist, Omar Dary, Richard Hurrall

This document explains the basic principles of food fortification and how to implement successful fortification programs, from both the public to the private level. It gives detailed discussions of each vitamin and mineral with its related deficiencies as well as the properties of each vitamin and mineral that may be used in fortification. It may be useful for government bodies seeking to choose which type of fortification program to implement, levels of fortificants to mandate, the type of fortificant, and an estimated cost of the program. It also instructs governments how to properly monitor and evaluate implemented programs. It is a very comprehensive discussion of fortification in general.

Assessment of Feasibility and Potential Benefits of Food Fortification

By: The Ethiopian Federal Ministry of Health

This document is useful for Ethiopian consumers and producers alike in understanding the government rationale for fortification. It provides a justification for fortification in Ethiopia as presents several options for pre-mixes and technology. It provides country-specific information with respect to the choice of a proper food vehicle as well as an estimated cost for the new program.

Fortification Handbook: *Vitamin and Mineral Fortification of Wheat Flour and Maize Meal*

By: The Micronutrient Initiative

This document provides a helpful overview of the rational behind fortifying wheat flours as well as helpful hints for how companies wishing to fortify flours should proceed. While it is tailored toward flour producers, its discussion of micronutrient deficiencies or recommended pre-mixes may be useful for factories wishing to fortify other foods (i.e. oil).

Capacity Building of Small Millers: Training Manual

Produced by: Health (South Africa), GAIN, Micronutrient Initiative, UNICEF

This manual provides a comprehensive overview for millers looking for fortify from social, business, and technical perspectives. It is well designed and easy to read. Topics include benefits of fortification, regulations, the fortification process and equipment, quality assurance, proper packaging, and health and safety.

In addition, many textbooks exist dealing solely with fortification. Titles include:

- *Handbook of Food Fortification and Health: from Concepts to Public Health Applications* by Victor Preedy, Rajaventhana Srirajaskanthan and Vinood B. Patel
- *Food Fortification: Food fortification with micronutrients* by Habtamu Fekadu Gemedo
- *Food Fortification and Supplementation: Technological, Safety, and Regulatory Aspects* by Berry Ottaway

Chapter 9:

Conclusions

Final Guidelines for Wheat Millers:

- ➔ Food fortification of wheat flour, a practice frequently carried out in other African and international nations, is a promising intervention method to reduce rates of vitamin A, iron, folic acid and other micronutrient deficiencies in Ethiopia, a country with the 2nd highest rates of malnutrition in sub-Saharan Africa.
- ➔ The Federal Ministry of Health, the Food, Medicine and Health Care Administration and Control Authority, the Federal Ministry of Industry, the Federal Ministry of Trade, and the Federal Ministry of Commerce are working together to pass legislation mandating fortification of edible oils and wheat flour within Ethiopia.
- ➔ The wheat flour fortification process is simple: the powdered vitamin pre-mix is mixed evenly with the milled flour through one of two steps:
 1. Batch or continuous mixing by a agitation tank before packaging, or
 2. Continuous metering of the vitamin pre-mix via a dosifier into a mixing transport mechanism (i.e. screw feeder, air bower).
- ➔ Wheat flour products, such as biscuits or pasta, may also be fortified.
- ➔ All equipment (mixing tank and/or feeder) may be imported or built locally. If the latter, companies should consider the types of materials and designs appropriate as well as the level of automation needed. Locally built designs offer lower prices at the expense of greater sophistication. Very precise feeders are not yet available locally.
- ➔ Vitamin pre-mixes can be obtained through many suppliers. The GAIN office in Addis Ababa can help in finding suppliers. The amount of vitamins to be added is dictated by regulations set by FMHACA.

- ➔ Quality assurance is necessary to determine levels of vitamins in fortified foods. Nutrient analysis can be measured quantitatively in well-equipped laboratories, and select vitamins may be measured quantitatively through BioAnalyt kits. Samples may be outsourced to external laboratories for complete analysis. Several semi-quantitative methods exist for fast, routine checks of fortificant levels.

- ➔ Follow all guidelines set by FMHACA with respect to QA and packaging. More guidelines with respect to fortification can be found in literature.

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Appendix

Appendix A: About vitamin and minerals

A-1: Role of vitamin and minerals

Table A- 1. Role of Vitamins

Vitamin	Chemical Form	Soluble In..	Role	Effects of Deficiency	Range of RNI (varies w/ age/sex)	Natural Sources	Toxicity
Vitamin A	Retinols (retinyl esters or palmitates); Pro-vitamin A carotenoids (converted to retinols in body)	Fat	Function in visual cycle in eye retina; function in body tissues to maintain growth and soundness of cells	Blindness or xerophthalmia; increased severity of infections, measles, diarrhea; increased mortality	375-850 µg/d	Animal products (retinols); green leafy vegetables, yellow vegetables, yellow and orange fruits (carotenoids)	Liver damage, bone abnormalities, joint pain, headaches, vomiting; at concentrations above 900 mg/day or 60,000 mg/4-6 mos.
<i>B Vitamins</i>							
Thiamin (B₁)		Water	Co-enzyme functions in metabolism of carbohydrates and branched chain amino acids	Beriberi (affected cardiovascular system-wet, or affected nervous system – dry; may be accompanied by trouble speaking, walking, or lower leg paralysis); polyneuritis, Wernicke-Korsakoff	0.1-1.5 mg/d	Pork, organ meats, whole grains, legumes	No

				Syndrome, affected nervous system);			
Riboflavin (B₂)		Water	Co-enzyme functions in redox reactions	Stunted bone growth, angular cheilitis or stomatitis (mouth inflammations), dermatitis	0.3-1.6 mg/d	Milk and dairy, meats, green vegetables	No
Niacin	Nicotinic acid; Nicotinamide	Water	Needed for hydrogen transfer in numerous dehydrogenases	Pellagra (chronic wasting disease), diarrhea, dementia, dermatitis	2-17 mg NE/d	Liver, lean meats, whole grains, legumes	No
Vitamin B₆	Pyridoxine, Pyridoxamine, Pyridoxal	Water	Reacts to riboflavin to form PLP, needed for metabolism of amino acids, glycogen, and sphingoid bases	Nasolateral seborrhea (skin inflammation), glossitis (tongue inflammation) peripheral neuropathy, infantile convulsions	0.1-2.0 mg/d	Meats, vegetables, whole grain cereals	At concentrations above 100 mg/day
Pantothenate		Water	Needed for fatty acid metabolism, component of CoA	Fatigue, sleep disturbances, impaired coordination, nausea	1.7-7.0 mg/d	Animal tissues, whole grain cereals, legumes	No
Bitoin		Water	Co-enzyme functions with bicarbonate-dependent carboxylations	Fatigue, depression, nausea, dermatitis, muscular pains	5-35 mg/d	Liver, yeast, egg yolks, soy flour, cereals	No
Vitamin B₁₂	Cobalamin and derivatives	Water	Needed for folate cycle; needed for conversion of propionate and amino acids to CoA	Demyelination of peripheral nerves and spinal column	0.4-2.8 mg/d	Synthesized by microorganisms that are ingested through foods of animal origin	No

Vitamin C	Ascorbic acid, Ascorbate	Water	Electron donor for 11 enzymes, promotes iron absorption, stabilizes folate in food, good antioxidant	Scurvy (can cause death, pseudo-paralysis, hemorrhages, or gingivitis), anemia	30-80 mg/d	Citrus fruits and veggies. Vitamin C easily lost in heating, transporting, and storage	Risk of gastric cancer, diarrhea, and hemolysis above 2-3 g/day
Vitamin D	Cholecalciferol (D ₃); Ergocalciferol (D ₂)	Fat	Maintains blood levels of calcium and phosphate, needed for transcription of cell cycle proteins	Problems with bone mineralization, muscle contractions, nerve conduction, and cellular function	5-15 µg/d	Synthesized in the skin from UV exposure	Hypercalcuria or hypercalcemia
Vitamin E	8 forms (--tocophenol); synthesized by plants from homogentisic acid	Fat	Antioxidant; protects polyunsaturated fatty acids, proteins, and DNA from free radical oxidation	Rare; Oxidative stress, damage to cell membranes, leaking of cell contents to external fluids, cardiac or skeletal myopathies, neuropathies, liver necrosis, muscle and neurological problems	Unknown (estimated around 7mg TE/d)	Plants and animal products	Low
Vitamin K	Phylloquinone (K ₁ – plant source); Menaquinone (K ₂ – bacteria source)	Fat	Maintenance of blood coagulation,; chemical modification of proteins with calcium binding properties	Rare but serious; hemorrhagic disease in infants (Vitamin K Bleeding Disease) causes death and brain damage	5-55 µg/d	Green leafy vegetables, liver, fermented foods (i.e. cheeses)	Low; fear of liver damage or neonatal hemorrhages

Table A- 2. Role of Minerals

Mineral	Purpose	Deficiency	Range of RNI (varies w/ age and sex)	Natural Sources	Toxicity
Calcium	5 th most prevalent element in human body, 99% found in skeleton, 1% found in teeth and soft tissues; ion form has role in most metabolic processes; stores in bone mineral	Osteroperosis	300-1000 mg/day	Milk and dairy product; is easily lost through the hair, skin, and nails	Low, some calcium deposits
Iodine	Needed for synthesis of thyroid hormones by the thyroid gland; role in growth and development of brain and central nervous system	<i>Fetus:</i> abortions, stillborns, mental deficiency/cretinism, spastic diplegia, dwarfism, psychomotor defects <i>Neonate:</i> goiter, hypothyroidism <i>Child/adolescent:</i> goiter, impaired mental function or physical development <i>Adult:</i> impaired mental function, goiter, hypothyroidism	2-30 µg/kg/day	Food grown in soils rich in iodine or in seawater (fish)	Iodine-induced hypothyroidism' levels above 40-150 µg/kg/day
Iron	Carries oxygen from the lungs to the tissues by red blood cell hemoglobin; part of enzyme systems in tissues	Most common deficiency; growth stunting, lowered work ability, decreased brain function, anemia, depressed immune system, mood changes, impairment of memory and learning ability	5-50 mg/day (assuming a 10% iron bioavailability of food)	Absorption increased by vitamin C and inhibited by phenolic compounds (tea, coffee, coca) and phytates (cereals, legumes, roots, nuts); heme iron	Possible

				(up to 40% absorption): red meat, poultry, fish; non-heme iron (about 10-20% absorption): cereals, pulses, legumes, vegetables	
Magnesium	Co-factor in enzymes needed for energy metabolism, protein synthesis, RNA and DNA synthesis and maintenance of electrical potential of nervous tissues and cell membranes. 60-70% of magnesium stored in skeleton, 30-40% in muscles and soft tissues	Infrequent; nausea, anorexia, muscular weakness, lethargy, staggering, weight loss, neurologic and neuromuscular defects	26-224 mg/day	Green vegetables, legume seeds, peas, beans, nuts; inhibited by fiber	Above 380 mg/day; hypermagnesium, nausea, hypertension, diarrhea
Selenium	Protects body tissues against oxidative stress; maintains defenses against infection; needed for growth and development; needed for thyroid hormone metabolism	Muscular weakness, myalgia, congestive heart failure; Kaschin-Beck Disease (bone and joint disease: shortening of fingers and long bones with growth retardation); Keshan Disease (fatigue, cardiac arrhythmia, loss of appetite, cardiac insufficiency, heart failure); increased mortality due to AIDs	13-42 µg/day	Food grown in selenium-rich soils	Above 400 µg/day; hair loss, structural changes in keratin of hair and nails, icteroid skin, gastrointestinal disturbances
Zinc	Central role in immune system; component of a large number of enzymes needed for synthesis	Growth retardation, delayed sexual and bone maturation, skin lesions, diarrhea, impaired	1.0-1.4 mg/day	Lean red meat whole-grain cereals, pulses,	Above 28-45 mg/day rare instances of nausea, vomiting,

	and degradation of carbohydrates, lipids, proteins, nucleic acids and metabolism of micronutrients; stabilizes molecular structure of cell components and cell membranes; needed for genetic expression	appetite, increased susceptibility to infections, behavioral changes		legumes	diarrhea, fever, lethargy; real concern of affecting copper status
Folate and Folic Acid	Essential for DNA and biosynthesis cycle; needed along with B ₆ and B ₁₂ for methylation cycle	Neural tube defects (improper closure of spinal cord and cranium resulting in spinal bifida, anencphaly, etc.) in pregnant women	400 µg/day	Present in a low density in most foods; high density in liver; adequate in green leafy vegetables (if consuming three servings/day)	Above mg amounts. Concerns of masking levels of pernicious anemia (result of Vitamin B ₁₂ deficiency)

Source: derived from (12)

A-2: Notable micronutrient levels among select subpopulations

Table A- 3. Tolerable Upper Intake Levels for Select Micronutrients.

Nutrient (unit)	1-3 years	4-8 years	9-13 years	19-70 years
Vitamin A (µg RE)¹	600	900	1,700	3,000
Vitamin D (µg)²	50	50	50	50
Vitamin E (mg α-tocopherol)	200	300	600	1,000
Vitamin C (mg)	400	650	1,200	1,000
Niacin (vitamin B₃) (mg NE)	10	12	20	35
Vitamin B₆ (mg)	30	40	60	100
Folic acid (µg DFE)³	300	400	600	1,000
Choline (mg)	1,000	1,000	2,000	3,500
Iron (mg)	40	40	40	45
Zinc (mg)	7	12	23	45
Copper (mg)	1	3	5	10
Calcium (mg)	2,500	2,500	2,500	3,000
Phosphorus (mg)	3,000	3,000	4,000	4,000
Manganese (mg)	2	3	6	11
Molybdenum (µg)	300	600	1,100	2,000
Selenium (µg)	90	150	280	1,100
Iodine (µg)	200	300	600	1,100
Fluoride (µg)	1,300	2,200	10,000	10,000

Source: (1) The FAO/WHO recommends the following UL's for vitamins A, B₃, B₆, C, D, and E, and calcium, selenium, and zinc. The remaining values are recommended by the United States Food and Nutrition Board of the Institute of Medicine.

¹ 1µg RE = 3.33 IU vitamin A

² As calciferol, where 1µg = 40 IU vitamin D

³ Refers to folic acid derived from fortified foods, or supplemental folic acid

Table A- 4. Estimated Average Requirement (EAR¹) for Select Subpopulations

Nutrient (unit)	1-3 years	4-6 years	19-50 years, female	Pregnant women, second trimester	Lactating women, 0-3 months	19-50 years, male
Vitamin A (µg RE)²	286	321	357	571	607	429
Vitamin D (µg)³	5	5	5	5	5	5
Vitamin E (mg α-tocopherol)	4	4	6	6	6	8
Vitamin C (mg)	25	25	37	46	58	37
Thiamine (vitamin B₁) (mg)	0.4	0.5	0.9	1.2	1.3	1.0
Riboflavin (vitamin B₂) (mg)	0.4	0.5	0.9	1.2	1.3	1.1
Niacin (vitamin B₃) (mg NE)	5	6	11	14	13	12
Vitamin B₆ (mg)	0.4	0.5	1.1	1.6	1.7	1.1
Folate (µg DFE)⁴	120	160	321	480	400	320
Vitamin B₁₂ (mg)	0.7	1.0	2.0	2.2	2.3	2.0
Iron (mg)						
15% bioavailable	3.9	4.2	19.6	>40.0	7.8	7.2
10% bioavailable	5.8	6.3	29.4	>40.0	11.7	10.8
5% bioavailable	11.6	12.6	58.8	>40.0	23.4	21.6
Zinc (mg)						
High bioavailability	2.0	2.4	2.5	3.5	4.8	3.5
Moderate bioavailability	3.4	4.0	4.1	5.8	7.9	5.8
Low bioavailability	6.9	8.0	8.2	11.7	15.8	11.7
Calcium (mg)	417	500	833	833	833	833
Selenium (µg)	14	17	22	23	29	28
Iodine (µg)	64	64	107	143	143	107

¹ RNI is set 2 standard deviations above the EAR.

² 1µg RE = 3.33 IU vitamin A

³ As calciferol, where 1µg = 40 IU vitamin D

⁴ Refers to folic acid derived from fortified foods, or supplemental folic acid

A-3: Regional and demographic data for vitamin A deficiency within Ethiopia

Table A- 5. Regional Rates of VAD.

Regional State	Predominantly Urban or rural	Main Foods	% VAS*	% VAD**
Afar	Rural - pastoral	Livestock products, cereals (sorghum, maize)	21.5	57.3
Tigray	Rural - agriculture	Cereals (teff), legumes (peas, lentils, beans)	79.2	14.3
Amhara	Rural - agriculture	Cereals (teff), legumes (peas, lentils, beans)	16.0	40.7
Addis Ababa	Urban	Cereals (teff), legumes (peas, lentils, beans)	15.0	29.3
Oromiya	Rural – diverse geography	Cereals, root crops (yam, potatoes, cassava, etc.)	10.2	56.0
SNNPR	Rural – diverse geography	Cereals, root crops (yam, potatoes, cassava, etc.), fruits and vegetables	33.9	11.3
Beneshengul-Gumuz	Rural – diverse geography	Cereals, root crops (yam, potatoes, cassava, etc.)	7.3	27.8
Harari	Urban	Cereals (teff), legumes (peas, lentils, beans)	1.1	35.8
Dire Dawa	Urban	Cereals (teff), legumes (peas, lentils, beans)	11.6	48.0
National			22.6	37.7

* VAS = has received vitamin A supplementation within the past 6 months

** VAD = shows subclinical deficiency, with retinol serum levels >0.7µmol/L

Source: (21; 10)

Table A- 6. Demographic Distribution of VAD

Variable	% VAD Deficient
Sex	
Female	33.5
Male	34.8
Age (months)	
24 and below	33.9
25-48	34.0
49-72	35.0
Vaccination Status	
None/incomplete	39.3
Complete	29.7
Vit. A supplementation in last year	
At least once	28.2
Not at all	38.2
Overall illness in last 15 days	
None	31.2
At least one illness	38.0
Residence	
Rural	32.8
Urban	37.3
Religion	
Christian	25.6
Muslim	47.5
Household Size	
5 and below	31.7
6 and above	38.2
No. under 5 children	
1 or none	30.4
2 and above	38.6
Literacy of mothers	
Illiterate	37.4
Literate	29.6
Parity	
2 and below	29.2
3 and above	38.1
Knowledge of vit. A	
Not at all	37.3
At least one fact	23.4
Age (years) of mothers	
24 or below	36.2
25-35	33.7
Above 35	33.9

Source: (21; 10)

Appendix B: Food Fortification Programs

B-1: Fortification Programs across Sub-Saharan Africa

Table B- 1. Fortification Programs across Sub-Saharan Africa

	Country	Wheat Flour	Vegetable Oil	Sugar	Maize Flour	Year (in effect)
1	Benin	A, M	A, M			2008
2	Burkina Faso	A, V	S, V			2008
3	Burundi					NA
4	Cameroon	A, M	A, M			2012
5	Chad					NA
6	Congo	S, V				
7	Congo, Dem. Rep.	S, V				
8	Cote d'Ivoire	A, M	A, M			2007
9	Ethiopia					NA
10	Ghana	A, M	A, M			2009
11	Guinea	A, M	A, M			2007
12	Guinea Bissau					NA
13	Kenya	A, M	A, M	S, V	S, M	2013
14	Liberia	A, M	A, M	A, M		2013
15	Madagascar					NA
16	Malawi	A, M	A, M	A, M	S, M	2014
17	Mali	A, M	S, V		S, M	2007
18	Mauritania	A, M	A, M			2010
19	Mozambique	Decree Pending	Decree Pending			NA
20	Niger	A, M	A, M		S, V	
21	Nigeria	A, M	A, M	A, M	S, M	2002
22	Rwanda	A, M	A, M	A, M	S, M	2014
23	Senegal	A, M	A, M			2009
24	South Africa	S, M			S, M	2003
25	South Sudan					
26	Tanzania	A, M	S, M	S, M	S, M	2012
27	Uganda	A, M	A, M		S, M (size criterion)	2013
28	Zambia	S, V		A, M	S, V	1998

A: All; S: Some; M: Mandatory; V: Voluntary

*Year may vary by vehicle; recorded year indicated year of first legislation/ regulation

Source: (17)

B-2: Impact of Folic Acid Fortification

Table B- 2. Impact of Folic Acid Fortification

Country	NTD ¹ prevalence pre-fortification per 1,000 births	NTD prevalence post-fortification per 1,000 births	Percent decrease in NTD prevalence
Argentina			
Spinal bifida ²	1.27	0.66	48
Anencephaly	³ 0.86	0.37	57
Brazil			
Spinal bifida	1.45	1.42	2 ⁴
Anencephaly	1.12	0.69	38
Canada			
Spinal bifida	0.86	0.40	53
Anencephaly	0.52	0.32	38
Chile			
Spinal bifida	1.02	0.46	55
Anencephaly	0.63	0.37	41
Costa Rica			
Spinal bifida	0.73	0.29	60
Anencephaly	0.37	0.12	68
Iran (all NTD's)			
	3.16	2.19	31
Saudi Arabia (all NTD's)			
	1.9	0.76	60
South Africa			
Spinal bifida	0.93	0.54	42
Anencephaly	0.41	0.37	10
United States			
Spinal bifida	0.50	0.35	30
Anencephaly	0.26	0.18	31

Source: (22)

¹ NTD = Neural Tube Defect

² Spinal bifida is malformation of the spine.

³ Anencephaly is a fatal malformation of the brain.

⁴ This study was conducted over three months only. The authors concluded they needed more time.

Appendix C: Photos from Local Manufacture



Figure 22. As-built V-Mixer



Figure 23. As-built horizontal agitator



Figure 24. Power transmission for horizontal mixer



Figure 25. Pin-type blades for wet flour mixing

Appendix D: Cost Estimates from Local Manufacture Scale Up

Table D- 1. Breakdown of estimated scale up costs for V-Mixer

Mixer Capacity (L)	Price (ETB)/Unit	Unit	Price (ETB)	Price (USD)
304 Stainless Steel Sheet Metal - m²				
20	1750.00	1.35	2,358.15	120.93
50	1750.00	1.85	3,240.50	166.18
100	1750.00	2.37	4,147.53	212.69
300	1750.00	3.57	6,254.37	320.74
500	1750.00	4.38	7,667.92	393.23
1,000	1750.00	5.89	10,308.54	528.64
2,000	1750.00	8.16	14,283.09	732.47
3,000	1750.00	10.05	17,593.56	902.23
4,000	1750.00	11.76	20,585.85	1,055.68
Aluminum Sheet Metal - m²				
20	766.67	1.35	1,033.09	52.98
50	766.67	1.85	1,419.65	72.80
100	766.67	2.37	1,817.01	93.18
300	766.67	3.57	2,740.01	140.51
500	766.67	4.38	3,359.28	172.27
1,000	766.67	5.89	4,516.12	231.60
2,000	766.67	8.16	6,257.35	320.89
3,000	766.67	10.05	7,707.66	395.26
4,000	766.67	11.76	9,018.56	462.49
Welding electrodes - 90 sticks				
20	2500	1.00	2,500.00	128.21
50	2500	1.36	3,393.02	174.00
100	2500	1.71	4,274.94	219.23
300	2500	2.47	6,165.53	316.18
500	2500	2.92	7,310.05	374.87
1,000	2500	3.68	9,210.08	472.31
2,000	2500	4.64	11,603.98	595.08
3,000	2500	5.31	13,283.24	681.19
4,000	2500	5.85	14,620.09	749.75
SS welding rod - m				
20	431.03	0.79	338.46	17.36
50	431.03	0.89	382.38	19.61

100	431.03	0.99	425.75	21.83
300	431.03	1.20	518.72	26.60
500	431.03	1.33	575.01	29.49
1,000	431.03	1.55	668.44	34.28
2,000	431.03	1.82	786.17	40.32
3,000	431.03	2.02	868.75	44.55
4,000	431.03	2.17	934.49	47.92

Labor

20 -		800	800.00	41.03
50 -		1000	1,000.00	51.28
100 -		1250	1,250.00	64.10
300 -		1500	1,500.00	76.92
500 -		2000	2,000.00	102.56
1,000 -		2500	2,500.00	128.21
2,000 -		3000	3,000.00	153.85
3,000 -		3500	3,500.00	179.49
4,000 -		4000	4,000.00	205.13

Total

	Using SS (ETB)	Using SS (USD)	Using AI (ETB)	Using AI (USD)
20	<i>5,996.61</i>	<i>307.52</i>	<i>4,671.55</i>	<i>239.57</i>
50	<i>8,015.90</i>	<i>411.07</i>	<i>6,195.05</i>	<i>317.69</i>
100	<i>10,098.21</i>	<i>517.86</i>	<i>7,767.70</i>	<i>398.34</i>
300	<i>14,438.63</i>	<i>740.44</i>	<i>10,924.26</i>	<i>560.22</i>
500	<i>17,552.97</i>	<i>900.15</i>	<i>13,244.33</i>	<i>679.20</i>
1,000	<i>22,687.07</i>	<i>1,163.44</i>	<i>16,894.65</i>	<i>866.39</i>
2,000	<i>29,673.24</i>	<i>1,521.70</i>	<i>21,647.50</i>	<i>1,110.13</i>
3,000	<i>35,245.55</i>	<i>1,807.46</i>	<i>25,359.64</i>	<i>1,300.49</i>
4,000	<i>40,140.43</i>	<i>2,058.48</i>	<i>28,573.15</i>	<i>1,465.29</i>

Table D- 2. Breakdown of estimated scale-up costs for stand still mixing tank with agitator.

Mixer Capacity (L)	Price (ETB)/Unit	Unit	Price (ETB)	Price (USD)
304 Stainless Steel Sheet Metal - m²				
50	1750.00	0.80	1,401.94	71.89
100	1750.00	1.28	2,241.00	114.92
200	1750.00	2.03	3,557.36	182.43
300	1750.00	2.66	4,661.46	239.05
500	1750.00	3.66	6,407.59	328.59
1,000	1750.00	5.81	10,171.41	521.61
2,000	1750.00	8.94	15,647.70	802.45
3,000	1750.00	11.72	20,504.29	1,051.50
4,000	1750.00	14.19	24,839.17	1,273.80
Aluminum Sheet Metal - m²				
50	766.67	0.80	614.18	31.50
100	766.67	1.28	981.77	50.35
200	766.67	2.03	1,558.46	79.92
300	766.67	2.66	2,042.16	104.73
500	766.67	3.66	2,807.13	143.96
1,000	766.67	5.81	4,456.05	228.52
2,000	766.67	8.94	6,855.18	351.55
3,000	766.67	11.72	8,982.83	460.66
4,000	766.67	14.19	10,881.92	558.05
Welding electrodes - 90 sticks¹				
50	2500	0.33	833.33	42.74
100	2500	0.53	1,332.08	68.31
200	2500	0.85	2,114.55	108.44
300	2500	1.11	2,770.85	142.09
500	2500	1.73	4,316.61	221.36
1,000	2500	2.74	6,852.19	351.39
2,000	2500	5.27	13,176.77	675.73
3,000	2500	6.91	17,266.45	885.46
4,000	2500	8.37	20,916.81	1,072.66
SS turning rod - m²				
50	431.03	1.90	818.97	42.00
100	431.03	2.78	1,198.92	61.48
200	431.03	4.22	1,819.52	93.31
300	431.03	5.38	2,318.63	118.90

¹ Based on ratios of tank circumference

² Assumed to be tank length plus the number of agitator blades the length of the tank radius

500	431.03	6.83	2,943.03	150.92
1,000	431.03	10.32	4,449.59	228.18
2,000	431.03	14.32	6,170.35	316.43
3,000	431.03	18.44	7,946.19	407.50
4,000	431.03	22.55	9,717.69	498.34
Labor				
50 -		4000	4,000.00	205.13
100 -		4500	4,500.00	230.77
200 -		5000	5,000.00	256.41
300 -		6000	6,000.00	307.69
500 -		7000	7,000.00	358.97
1,000 -		8000	8,000.00	410.26
2,000 -		8500	8,500.00	435.90
3,000 -		9000	9,000.00	461.54
4,000 -		10000	10,000.00	512.82
Parts and Accessories				
Bearings, all sizes	260.00	2	520.00	26.67
hinges/latches, all sizes	30.00	1	60.00	3.08
Pulley	100	2	200	10.26
Total				
	Using SS (ETB)	Using SS (USD)	Using AI (ETB)	Using AI (USD)
50	7834.23	401.76	7,046.48	361.36
100	10052.00	515.49	8,272.78	424.24
200	13271.44	680.59	10,692.54	548.34
300	16530.94	847.74	13,331.64	683.67
500	21447.24	1099.86	17,266.78	885.48
1,000	30253.20	1551.45	31,004.31	1,589.96
2,000	44274.81	2270.50	50,221.55	2,575.46
3,000	55496.93	2846.00	69,407.27	3,559.35
4,000	66253.67	3397.62	91,059.86	4,669.74

Appendix E: Quantitative Methods for QA

Spectrophotometric Method for Determining Total Iron Concentration¹

Principle

Combustion of organic materials removes all but the mineral parts of food and converts all iron present to the oxidized form of Fe(III). The oxidized form can be reduced to the Fe(II) through reaction with hydroxylamine hydrochloride. Fe(II) concentration can be determined spectrophotometrically using reagents that react with Fe(II) to form colored complexes under certain pH conditions. Addition of sodium acetate reduces competition by hydronium ions (H_3O^+) for the ligand.

Critical Points

Water used must be distilled or deionized in order to avoid interference by iron in the water. For some chromogenic reagents, it is necessary to maintain the pH between 5 – 6.

Materials

1. Analytical balance
2. Spectrophotometer UV-VIS
3. Furnace
4. Freezer or refrigerator
5. Volumetric flasks (25, 100, 250 mL)
6. 250 mL Erlenmeyer flask
7. Volumetric and graduate pipettes
8. Porcelain crucibles
9. Graduated cylinders

Chemical Reagents

All chemicals listed are of analytical grade

¹ Source: (24; 27)

1. Sodium acetate trihydrated ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$), 99%
2. Nitric acid, 65%
3. Hydrochloric acid, 37%
4. 1,10-phenanthroline-monohydrate*
5. Hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$)
6. Acetic acid
7. Standards solutions for iron, such as Ammoniacal Ferrous Sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$)

*Alternative chromogenic reagents include:

- Bathophenanthroline, disulphonic salt of 4,7-dyphenyl – 1,10 phenanthroline, or
- α,α – dyridyle ($\text{C}_{16}\text{H}_8\text{N}_2$), or
- Ferrozine, acid [3-(2-pyridyle)-5,6-bis-(4-phenylsulphonic)-1,2,4-triazine dysodic salt]

Methods

I. Preparation of Solutions

1. **Chromogenic Solution: 1,10-phenanthroline***

- Dissolve 0.1 g 1,10-phenanthroline in about 80 mL H_2O . Let it cool down and dilute to 100 mL. Store in dark bottle in a refrigerator. Discard if solution turns slightly pink, indicating presence of iron.

2. **Acetate Buffer – 2M**

- In a 500 mL beaker, add 68 g sodium acetate trihydride and dissolve in approximately 100 mL H_2O . Add 60 mL galacial acetic acid and dilute to 500 mL. Transfer the solution to a glass flask. The solution is stable indefinitely.

3. **Hydroxylamine Hydrochloride – 10%**

- Add 10 g of hydroxylamine hydrochloride to a beaker and dissolve with 100 mL water. Transfer the solution to a glass flask. The solution is stable indefinitely.

*Alternative chromogenic solutions, each stable for 3-4 months, should be stored in a transparent plastic bottle, and discarded if the solution turns pink:

a. **Bathophenanthroline**

- Create a 0.05% solution with 2M sodium acetate as solvent. Use heat as needed.

b. **α,α - dipyridyle**

– Create a 0.1% solution with 2M sodium acetate as solvent. Use heat as needed.

c. **Ferrozine**

– Create a 0.05% solution with 2M sodium acetate as solvent. Use heat as needed.

II. Standard Solutions

1. Create a stock solution of 1000 mg Fe/L using chosen standard (i.e. Ammoniacal Ferrous Sulfate) and distilled water. Add a few drops of HCl to the solution. The solution is stable for about 6 months.
2. Create a second stock solution of 10 mg Fe/L from previous solution. Add 2 mL HCl. The solution is stable for about 6 months.
3. From the stock solutions, create standards ranging from 0 – 5.0 mg/L (0.0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0)
4. Pipet 10 mL of the standard solutions in 25 mL flasks.
5. Add 5.0 mL acetate buffer and 4.0 mL of 1,10-phenanthroline to the flask. Mix well and color will start developing.
6. Let it stand for 30 minutes, and then make up to volume (25 mL) using deionized water.
7. Turn on the spectrophotometer and for 15-20 minutes to warm up. Adjust the wavelength to 510 nm and the mode to absorbance. Set the absorbance of the instrument to zero with a blank of deionized water.
8. Record the absorbance of all standards and plot absorbance versus known concentration to achieve a straight line.

III. Determination of Total Iron

1. Ashing the sample
 - a. Grind about 100 g of flour in mortar and pestle and mix to homogenize.
 - b. Weigh 1.00 g of flour and place into crucible. The crucible should be cool and dry.
 - c. Place crucible into the furnace at 550°C for 6 hours.
 - d. If a white or grayish ash is obtained, the process is complete. If not, heat until the sample is white or gray ash only.

- e. Let the samples cool at room temperature for 5 minutes and then place in a desiccator for 1 hour. The samples should reach room temperature.
2. Preparation of the ash sample
 - a. Add 5 mL of concentrated HNO_3 to the crucible
 - b. Evaporate the acid by heating the crucibles on top of a hot plate at low temperature. The solution should not boil.
 - c. Dissolve the remaining residue by adding 2 mL of concentrated HCl and heating for a few minutes.
 - d. Let the crucible cool and transfer to a 25 mL volumetric flask. Wash crucible with distilled water and bring solution to volume (25 mL) with deionized water.
 3. Determination of iron content
 - a. Pipette 10.0 mL of the sample solution into a 25 mL volumetric flask. Add 1.0 mL hydroxylamine hydrochloride solution, mix well, and let stand for 5 minutes.
 - b. Add 5.0 mL acetate buffer and 4.0 mL of 1,10-phenanthroline to the flask. Mix well and color will start developing.
 - c. Let stand for 30 minutes and make up to volume with deionized water.
 - d. Record the absorbance of the sample using the spectrophotometer at 510 nm. Calculate the concentration using the calibration curve generated in the previous section.

Determination of Vitamin A by High Performance Liquid Chromatography¹

Principle

The process of saponification converts fats to fatty acids and retinyl esters to retinol and its corresponding fatty acids. Retinol can be quantified using HPLC with UV detection at 328 nm.

Safety Precautions

Potassium hydroxide is caustic and causes severe burns and should be dealt with by trained personnel wearing proper PPE. Flammable liquids should be worked with behind a fume hood.

¹ Source: (24; 28; 29)

When heating flammable liquids, leave ample headroom in the flask and add boiling chips before beginning.

Critical Points

Due to the labile nature of retinol, it is important to saponify samples under a nitrogen atmosphere and in the presence of pyrogalllic acid.

Materials

1. HPLC system
 - a. Pump operating continuously at 1.0-2.0 mL/min
 - b. Injector with a 20 μ L fixed loop
 - c. Reverse phase C18 column, 10 μ m, capable of separating *cis* and *trans* isomers
 - d. Photometric detector monitoring absorbance at 328 nm
 - e. Data collection system or integrator
2. Erlenmeyer flask (125 mL) with neck adapted for connecting reflux condenser
3. Hot plate
4. Reflux condensers
5. Volumetric flasks (10, 100, 500 mL)
6. Nitrogen blanket apparatus

Chemical Reagents

All liquids should be of analytical grade.

1. Certified vitamin A concentration (USP) **OR** Retinyl palmitate, all-trans
2. Acetic acid, glacial
3. Methanol, HPLC grade
4. Ethanol, 95%
5. Tetrahydrofuran (THF)
6. n-Hexane, HPLC grade, 95% – *only if using retinyl palmitate as standard*
7. Pyrogalllic acid, crystal – *only if using retinyl palmitate as standard*
8. 2-propanol – *to check purity of retinyl palmitate*
9. Sodium hydroxide (KOH) pellets

Methods

I. Preparation of Solutions

1. **Mobile Phase for HPLC**

- Combine 860 mL methanol with 140 mL distilled water. Mix well. Stir overnight and degas prior to use

2. **THF-methanol (50-50)**

- Combine 500 mL tetrahydrofuran with 500 mL 95% ethanol. Mix well.

3. **Potassium hydroxide solution – 50%**

- In a fume hood, add 500 g KOH pellets to 500 mL water in a 2 L Erlenmeyer flask. The solution will give off substantial heat, so add the KOH in 100 g increments, while cooling the flask with cold water. Swirl to aid mixing.
- Store in glass container with cork stopper

4. **Vitamin A working standard**

a. Using USP standard

- Create a 500 mg/L stock solution using ethanol as the solvent. Use a small amount of acetone (less than 3 mL) to aid dissolution of the retinyl acetate concentration before adding ethanol.
- Store solution at 4°C in dark conditions. Solution is stable for two weeks.

b. Using retinyl palmitate

- Dissolve 55 mg retinyl palmitate in 100 mL hexane. Add a pea-sized piece of pyrogalllic acid.
- Pipette 5 mL solution to second 100 mL flask and dilute to volume with 95% ethanol.
- Store solution at 4°C in dark conditions. Solution is stable for two weeks.

c. Check purity of standard

- Create solution with concentration of 10 mg/L of retinyl palmitate in 2-propanol.
- Measure absorbance of this sample using UV-VIS spectrophotometer at 325-328 nm, using 2-propanol as the blank.
- Calculate purity as follows:

$$Purity (\%) = \frac{A_{max} \times (5 \times 10^6)}{960 \times w}$$

Where A_{max} equals absorbance maximum and w equals weight of sample in mg.

II. Preparation of samples

1. Grind solid samples to pass through a 40 mesh sieve.

III. Saponification and extraction of sample

1. Arrange the reflux system as shown in Figure 26. Turn on the hot plate to preheat

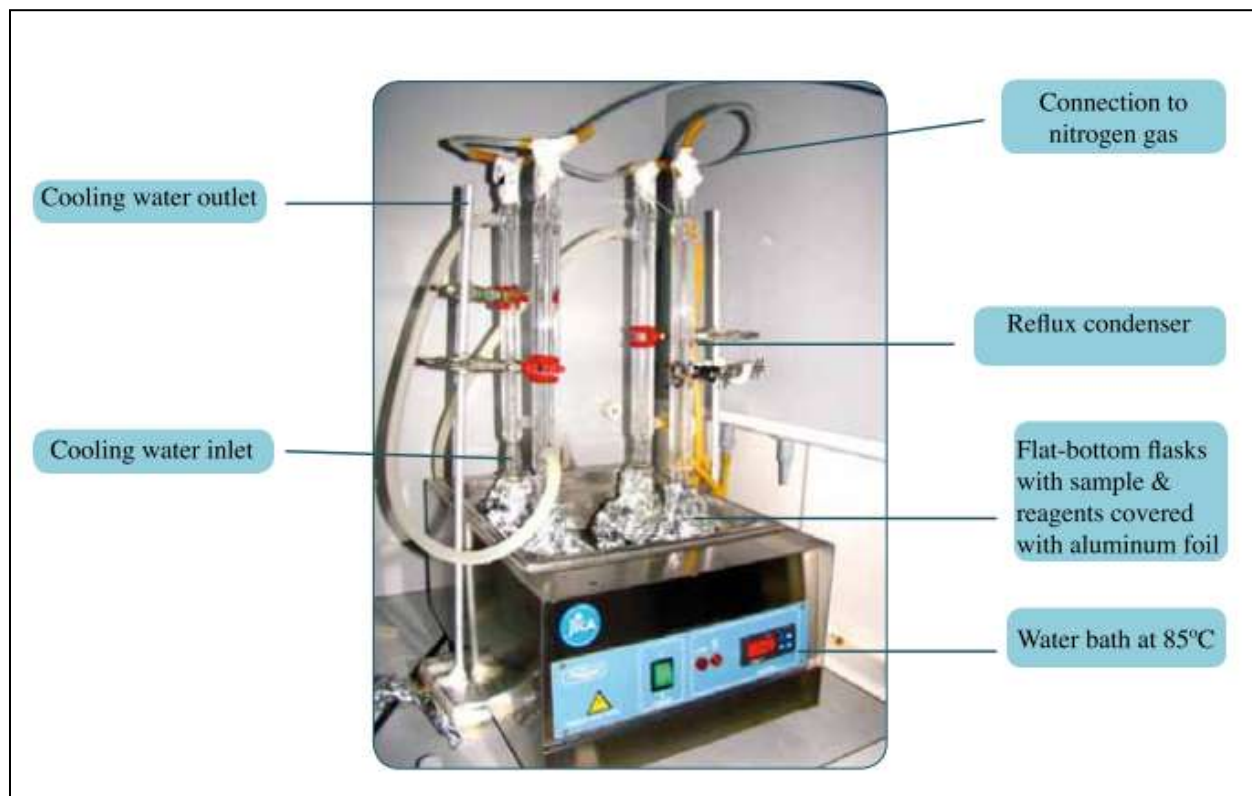


Figure 26. Setup of reflux system for quantitative vitamin A analysis

2. Preparation of standards

- a. High standard – add 33 mL 95% ethanol to 3 mL vitamin A working standard
- b. Intermediate standard – add 35 mL 95% ethanol to 2 mL working standard
- c. Low standard – add 37.5 mL 95% ethanol to 1.0 mL working standard
- d. Proceed to step 4.

3. Preparation of samples
 - a. Weigh 10 g of wheat flour
 - b. Add 40 mL ethanol
4. Add a pea-sized piece of pyrogalllic acid to each standard and sample flask. Add a glass bead to promote even boiling.
5. Swirl all flask to ensure even mixing.
6. Turn on nitrogen flow. Keep it on for the remainder of the refluxing.
7. Pipette 10 mL of 50% KOH solution to each flask and immediately place flasks on hot plate under reflux condenser.
8. Reflux for 45 minutes. Swirl flasks every 10 minutes.
9. Remove flasks from heat and stopper. Quickly cool to room temperature with cold water.
10. Pipette 10 mL acetic acid into each flask to neutralize the KOH. Mix well and let flasks cool to room temperature.
11. Transfer the solution in each flask to a 100 mL volumetric flask. Dilute to volume with the 50:50 THF:ethanol solution.
12. Stopper and invert flasks ten times
13. Allow samples to set a room temperature for at least 1 hour or overnight in a refrigerator to allow fatty acid salts formed during saponification to precipitate. Centrifugation may be used to help solids settle.

III. HPLC Determination

1. Optimum conditions:
 - Mobile Phase: Methanol:water (89:11)
 - Flow rate: 1.3 mL/min
 - Wavelength: 323 nm
 - Retention time: around 11-12 min
2. Start HPLC system and adjust flow rate to 1.3 mL/min. Equilibrate for at least 30 minutes with mobile phase running.

3. Inject vitamin A standard into HPLC system. Adjust mobile phase to achieve a resolution of 1.5 or better for *cis* and *trans* isomers. All *trans* retinol should elute in 6 minutes or longer.
4. Inject standards. Adjust detector sensitivity as needed. Repeat until peaks are reproducible.
5. Inject sample solutions. To ensure consistent performance of HPLC, add known standard solution after every nine samples to verify peak height.

IV. Calculations

1. Plot area under peak (y) vs. concentration (mg/L) (x) for the three standards inject. Find the equation of the straight line.
2. Calculate the retinol concentration in the injected samples using the equation of the calibration curve.
3. Find the concentration in the original flour sample through the following equation:

$$\text{Vitamin A} \left(\frac{\text{mg}}{\text{kg}} \right) = \frac{(\text{Retinol concentration in sample})(\text{Initial volume of sample})}{(\text{sample weight})}$$

Determination of Thiamin (Vitamin B1) by High Performance Liquid Chromatography¹

Principle

Thiamin is extracted from the flour matrix in an autoclave with diluted sulfuric acid. After enzyme hydrolysis, thiamin is oxidized with potassium ferricyanide in sodium hydroxide to form a fluorescent thiochrome which can be measured using HPLC with excitation at 370 nm and emission at 430 nm.

Critical Points

Thiamin is more heat-resistant in pH media below 5.5. Samples and standard solutions of thiamin must be protected from light and parafilm is added during autoclaving to protect from heat.

¹ Source: (29)

The oxidizing agent must be freshly prepared, and the thiochrome should be analysed with HPLC shortly after creation.

Rinse the HPLC column thoroughly after use to remove any salts. Rinse first with water, then with methanol.

Materials

1. Autoclave (121-123°C)
2. Agitator Vortex
3. Analytical balance
4. Water bath (40°C)
5. HPLC system
 - Pump operating continuously at 1.0-2.0 mL/min
 - Injector with a 100 µL fixed loop
 - Reverse phase C18 column, 5µm
 - Fluorescent detector
6. Volumetric flasks (25, 100, 1000 mL)
7. Beakers (25, 100, 1000 mL)
8. Glass funnels
9. Amber glass vials
10. Volumetric pipettes
11. Graduated cylinders
12. 10 mL test tubes
13. Glass rods
14. Filter paper Whatman No. 41

Chemical Reagents

1. Galacial acetic acid
2. Liquid paraffin (mineral oil)
3. Methanol, HPLC grade
4. Potassium ferricyanide
5. Sodium acetate, 99.5%

6. Sodium hexanosulfonate, 98%
7. Sodium hydroxide
8. Sulfuric acid, 95-97%
9. Thiamin monomitate or hydrochloride, Standard
10. Triethylamine, >99%
11. α Amylase

Methods

I. Preparation of Solutions

1. **Amylase – 5%**
 - Weigh 2.5 g amylase and add around 5 mL distilled water. Let it stand until it is fully hydrated. Make up volume to 50 mL with distilled water and mix thoroughly.
2. **Potassium ferricyanide – 1%**
 - Dissolve 1 g potassium ferricyanide in water and dilute to 100 mL. Prepare fresh daily.
3. **Oxidizing Reagent**
 - Mix 4.0 mL 1% potassium ferricyanide solution with 96 mL 15% sodium hydroxide solution. Use this reagent within four hours after preparation.
4. **Sodium acetate – 2.5M**
 - Dissolve 205 g of sodium acetate anhydrous in 1L distilled water.
5. **Sodium hydroxide – 15%**
 - Dissolve 15 g sodium hydroxide in distilled water. Let the solution cool and bring volume up to 100 mL
6. **Sulfuric Acid – 0.1M**
 - Prepare 1 L of a 1:10 dilution of concentration sulfuric acid (97-98%) using water as the solvent. Mix well. Add acid to water for safety.
7. **Mobile phase (HTAA:Methanol, 83:17)**
 - Prepare HTAA: Dissolve 0.9602 g sodiumhexanosulfonate in 25 mL HPLC grade water. Transfer to a 1-L volumetric flask. Add 1.3 mL triethylamine and 10 mL acetic acid. Make up to volume with HPLC grade water.

- Mix 83 mL of the HTAA solution with 17 mL methanol. Filter the solution through a 0.45µm filter.
 - Degas solution prior to use.
8. **Thiamine stock standard solution – 100 mg/L**
- Dry Thiamine mononitrate USP reference standard for 1-2 hours at 60-70°C in a vacuum oven until it reaches constant weight.
 - Dissolve the amount of Thiamine mononitrate equivalent to 50 mg Thiamine in a 500 mL flask using 0.1M sulfuric acid.
 - Store the solution in an amber flask in a refrigerator for up to one month.
9. **Thiamine standard solution – 10 mg/L**
- Prepare a 1:10 dilution of the above solution using 0.1M sulfuric acid to dilute.
10. **Thiamine working standard solutions**
- Prepare working standards with concentrations of 0.05 mg/L, 0.1 mg/L, and 0.2 mg/L. Use sulfuric acid as the solvent.

II. Extraction

1. Weigh accurately 10 g flour. Add to 100 mL beaker
2. Add 10-20 mL 0.1 M sulfuric acid. Agitate sample with glass rod. Add 0.1 M sulfuric acid to bring total volume to around 50 mL and add 1-2 mL paraffin oil.
3. Cover the beaker with foil or a watch glass and sterilize in autoclave for 15 minutes at 121-123°C.
4. Let the solution cool and transfer to a 100 mL volumetric flask containing 8 mL of 2.5 M sodium acetate. pH should be 4.5.
5. Add 5 mL of the 5% amylase suspension.
6. Incubate at 40°C for 20 minutes. Cool the solution and make up to volume with distilled water without taking into account the paraffin layer.
7. Filter the solution through a glass funnel with filter paper. Discard the first 5-10 mL of the filtrate.

III. Thiochrome reaction

1. Add 5 mL of the oxidizing solution to 10 mL of the filtrate. Agitate and immediately neutralize the solution with about 1.5 mL acetic acid to bring the pH to 4.5.
2. Filter the solutions with 0.45 μ m filter and put into an autosampler vial.

IV. HPLC Analysis

1. Optimum Conditions
 - Column: XBridge C18 Column, 5 μ m, 4.6 x 150 mm
 - Flow: 1.0 mL/min
 - Fluorescence Detector: excitation wavelength = 370 nm; Emission wavelength = 430 nm
 - Injection volume: 100 μ L
2. Start HPLC system and allow to equilibrate for about 1 hour with the mobile phase flowing at 1.0 mL/min.
3. Inject the working standard solutions in duplicate to prepare the calibration curve, plotting area vs. concentration.
4. Inject samples under the same operating conditions, interspersing with a known standard every 9 samples to ensure consistent performance.
5. Determine the thiamine concentration in the injected sample solution using the regression equation determined above.
6. Calculate the thiamine in the flour sample by multiplying the concentration in the injected sample by the sample's initial volume over mass (about 100 mL/10g)

Determination of Riboflavin (Vitamin B2) by High Performance Liquid Chromatography¹

Principle

Riboflavin is extracted from the fortified flour sample in an autoclave with dilute sulfuric acid. An amylase suspension breaks down the starch in the flour so the flour suspension can be

¹ Source: (29; 31)

filtered. Methanol is added to the filtrate to precipitate any unwanted compounds. The purified riboflavin is analyzed by HPLC with fluorimetric detection.

Critical Points

Riboflavin is labile to light and must be protected from light at all times. Prepare the stock solution in a separate room from the standards to avoid contamination.

Sample extracts should be analyzed on the same day of preparation as they tend to lose 9% riboflavin per day. Stock solution is stable up to 1 month, but the concentration decays gradually, so it is best to prepare fresh solutions.

Riboflavin concentration is calculated in flour samples using peak height, not area. Using area overestimates riboflavin concentration.

After using the HPLC, rinse the column to eliminate all salt residues from the mobile phase and then wash the column with methanol. Never leave the mobile phase in the column.

Materials

1. Autoclave (121-123°C)
2. Agitator Vortex
3. Analytical balance
4. Water bath (40°C)
5. Centrifuge (3000 rpm)
6. HPLC system
 - Pump operating at 1.0-2.0 mL/min
 - Injector with 50 µL fixed loop
 - Data collection system or integrator
 - Reverse phased C18 column with fluorimetric detection
7. Volumetric flasks
8. Beakers
9. Glass funnels
10. Amber glass vessels
11. Volumetric pipettes

12. Graduated cylinders
13. Centrifuge tubes (50 and 10 mL)
14. Test tubes (10 mL)
15. Glass rods
16. Filter paper Whatman No. 41

Chemical Reagents

1. Acetic acid, 99.8%
2. Amylase
3. Sodium acetate, 99.5%
4. Sulfuric acid, 95-97%
5. Methanol, HPLC grade
6. Sodium hexanosulfonate, 98%
7. Triethylamine, >99%
8. Riboflavin USP reference standard

Methods

I. Preparation of Solutions

1. Acetic Acid – 0.02 M

- Add 1.2 mL glacial acetic acid to about 500 mL distilled water. Make up volume to 1L.

2. Amylase – 5% w/v

- Weigh 1.25 g amylase and add around 5 mL distilled water. Let stand until completely hydrated. Make up volume to 25 mL with distilled water and agitate thoroughly. Prepare only the amount needed.

3. Sodium acetate – 2M

- Dissolve 164 g sodium acetate anhydrous in 1 L distilled water.

4. Sulfuric acid – 0.1 M

- Add 10 mL concentrated sulfuric acid to about 600 mL water and make up volume to 1 L.

5. Mobile phase (HTAA: Methanol, 83:17)

- Prepare HTAA: Dissolve 0.9602 g sodiumhexanosulfonate in 25 mL HPLC grade water. Transfer to a 1-L volumetric flask. Add 1.3 mL triethylamine and 10 mL acetic acid. Make up to volume with HPLC grade water.
- Mix 83 mL of the HTAA solution with 17 mL methanol. Filter the solution through a 0.45µm filter.
- Degas solution prior to use.

II. Preparation of Standards

1. Riboflavin stock standard solution – 100 mg/L

- Dry riboflavin USP reference standard for 1-2 hours at 60-70°C in a vacuum oven. Store dried standard in a dessicator.
- Weigh 50 mg of dried standard and place into a 500 mL volumetric flask. Dissolve in 0.02 M acetic acid and place in an ultrasonic bath for 30 minutes or until the riboflavin is completely dissolved. Make up to volume with 0.02 M acetic acid.
- Store the solution in an amber flask in a refrigerator for up to one month.

2. Riboflavin standard solution – 1 mg/L

- Prepare a 1:100 dilution of the stock solution using distilled water as the solvent. Prepare solution every time samples are run and discard after use.
- The actual concentration can be calculated by UV absorbance at 266 nm and the extinction coefficient of 870.

3. Riboflavin working standard solutions

- Prepare 25 mL dilutions of the 1 mg/L solution with concentrations of 0.04, 0.08, 0.12 mg/L. Use water as the solvent. Prepare every time samples are run and discard after use.

III. Procedure

1. Add 10 g flour to a 100 mL beaker. Prepare samples in duplicate.

2. Add 10-20 mL 0.1 M sulfuric acid. Agitate the sample with a glass rod and add more 0.1 M sulfuric acid so final volume is about 50 mL. A slurry without lumps should be obtained.
3. Cover the beaker with foil or a watch glass and sterilize in an autoclave for 15 minutes at 121-123°C.
4. Transfer the hot solution to a 100 mL volumetric flask containing 8 mL of 2 M sodium acetate.
5. Let the solution cool and add 5 mL of the 10% amylase suspension.
6. Incubate at 40°C for 20 minutes. Cool and make up to volume with distilled water.
7. Filter the solution through a glass funnel with filter paper Whatman No. 41. Discard the first 5-10 mL of the filtrate.
8. Pipette 4.0 mL of the filtrate to a centrifuge tube containing 4.0 mL methanol. Mix and centrifuge.
9. Pipette 4.0 mL of the clear supernatant to a test tube. Dilute with 2.0 mL water and mix on the vortex. Filter the solution through a 0.45 µm membrane. This is the solution for HPLC analysis.

IV. HPLC Determination

1. Optimum conditions:
 - Colum: C18, Waters. 150 mm x 4.1 mm ID
 - Flow rate: 1.5 mL/min
 - Detector: Fluorescence: excitation wavelength = 423 nm; Emission wavelength = 525 nm
 - Injection volume: 50 µL
2. Start HPLC system and adjust flow rate to 1.0 mL/min. Equilibrate for at least 1 hour with mobile phase running.
3. Adjust flow to 1.5 mL/min. Inject the standards in duplicate from low to high concentrations (0.04-0.12 mg/L).
4. Inject sample solutions. To ensure consistent performance of HPLC, add known standard solution after every nine samples to verify peak height.

5. Determine the riboflavin concentration in the injected sample solution using the regression equation determined above.
6. Calculate the riboflavin in the flour sample by multiplying the concentration in the injected sample by the sample's initial volume over mass (about 100 mL/10g) and by the sample dilution over initial volume (about 3 over 100 mL)

Determination of Niacin (Vitamin B3) by High Performance Liquid Chromatography¹

Principle

Niacin is extracted from the flour matrix through alkaline digestion with calcium hydroxide. The latter is then precipitated out via acid addition. The sample extract is purified when niacin is extracted from a C18 cartridge while other colored compounds and impurities are retained. Finally, the purified niacin sample is analyzed via HPLC.

Critical Points

Maintaining proper pH level during different steps is critical. For instance, the pH must be decreased to precipitate the calcium hydroxide. It then must be made neutral because niacin will not be retained in the C18 column at a neutral pH, but many other color compounds will be retained, thus obtaining a cleaned extract. Finally, the pH of the extracted niacin must be reduced again to match that of the mobile phase.

Because the filtration process is slow, it may be more time efficient to centrifuge extracts prior to filtration.

The reagents used in the mobile phase of the HPLC, sodium dodecyl sulfate and acetonitrile, must be very pure to obtain a stable baseline. With pure reagents, the separation is very good.

Materials

1. Autoclave, 121-123°C

¹ Source: (29; 30)

2. Agitator Vortex
3. Analytical balance
4. Centrifuge (optional)
5. HPLC System
 - Pump operating at 1.0-2.0 mL/min
 - Manual or auto-sampling injector with a 100 μL fixed loop.
 - Reverse phase C18 column, 5 μm (4.1x250 mm)
 - UV detector
6. C₁₈ cartridges (Sep-pak)
7. Erlenmeyer flasks
8. Volumetric flasks
9. Beakers
10. Volumetric pipettes
11. Graduated cylinders
12. Glass rods
13. Filter paper Whatman No. 42 and 2

Chemical Reagents

1. Acetonitrile, HPLC grade
2. Calcium hydroxide
3. Ethanol
4. O-Phosphoric acid, 85% purity
5. Oxalic acid
6. Sodium dodecyl sulfate, >99% purity for ion-pair chromatography

Methods

I. Preparation of Solutions

1. Mobile Phase

- Solution A: dilute 1.00 mL o-phosphoric acid in 600 mL water and filter through a 0.45 μm filter

- Solutions B: In a beaker, mix 120 mL water and 230 mL acetonitrile. Dissolve 1.00 g sodium dodecyl sulfate in the solution. Bring total volume to 400 mL with water. Filter through a 0.45 μ m filter.
- Mix the 600 mL of solution A with the 400 mL of solution B to make 1 L of mobile phase. Mix well.

2. Niacin standard solutions (2000 mg/L, 2 mg/L)

- Dissolve 200 mg niacin in a 1L mixture of 1:1 ethanol:water. The solution is stable for 2 weeks at room temperature. This is the stock solution.
- Create a working standard solution of 2 mg/L by preparing a series of 2 dilutions (1:20 and 1:50). Use water to dilute.

II. Procedure

1. Weigh 2 g niacin-fortified flour in a 500 mL blender jar.
2. Add 198mL water and 10 g calcium hydroxide.
3. Prepare three standard solutions with different concentrations using the working standard (2 mg/L niacin solution).
 - a. Low standard: 10 mL working standard with 190 mL water
 - b. Intermediate standard: 20 mL working standard with 180 mL water
 - c. High standard: 40 mL working standard with 160 mL water.
4. Add 10 g calcium hydroxide to each standard. From now on, treat the standards and the samples identically.
5. Blend approximately 30 seconds at high speed with a vertical stirrer or blender.
6. Autoclave 15 min at 121°C.
7. Cool in ice bath for at least 30 minutes.
8. Transfer extracts to a 250 mL volumetric flask and bring to volume with water.
9. Filter the cold solution through Whatman paper No. 2V, filtering only the supernatant. Centrifuge beforehand to help obtain a clear filtrate.
10. With a volumetric pipette, transfer 100 mL of the filtrate to a 250 mL Erlenmeyer flask containing 300 mg oxalic acid.
11. Mix well and adjust final pH to 6.5-7.0 by dropwise addition of the filtrate or by adding a few crystals of oxalic acid.

12. Centrifuge the filtrate at 5,000 rpm for 10 minutes. Filter slowly through 1 or 2 pieces of Whatman No. 42 paper.
13. Condition C18 cleanup cartridge with 10 mL ethanol and then pass 10 mL water.
14. Slowly pass 10 mL clear sample filtrate through the cartridge. Discard the first 6 mL and collect the next 3.5 mL in a vial.
15. Add 1 drop 85% phosphoric acid (H_3PO_4) and mix well.

IV. HPLC Analysis

7. Optimum Conditions

- Column: C18. Waters. 250 mm x 4.1 mm ID
- Flow: 1.5 mL/min
- Detector: UV at 254 nm
- Injection volume: 100 μL
- *Note:* may modify mobile phase to change retention time and peak shape with 95% mobile phase and 5% acetonitrile.

8. Start HPLC system and allow to equilibrate
9. Inject the working standard solutions to prepare the calibration curve, plotting area vs. concentration to obtain the regression equation.
10. Inject samples under the same operating conditions, interspersing with a known standard every 9 samples to ensure consistent performance.
11. Determine the niacin concentration in the injected sample solution using the regression equation determined above.
12. Calculate the niacin in the flour sample by multiplying the concentration in the injected sample by the sample's initial volume over mass (about 250 mL/2g)

Determination of Folic Acid (Vitamin B₉/pteroylglutamic acid) by Microbiology¹

Principle

Folic acid is measured indirectly by tracking microorganism growth as folic acid is the limiting factor for microorganism growth. Prior to measuring bacteria growth, the optimum growth time must be determined and the bacteria must be propagated and maintained. Turbidity is measured in solutions and used to calculate concentration in samples.

Critical Points

- This method is not applicable when extraneous turbidity or color interferes with the turbidimetric measurements.
- Protect solutions from unnecessary light exposure.
- Media is highly hygroscopic and should be stored in cool, dry places or refrigerated.
- When preparing media, cool down rapidly after sterilization to avoid pH changes, unwanted precipitation, loss of nutrients, or darkening of the media due to Maillard reaction.
- If using media brands other than HiMedia, bacteria growth may vary and the method may need to be standardized again.
- Glassware used must be cleaned with a neutral soap that does not stimulate bacterial growth. Glassware should be thoroughly rinsed in pure water and heated in the oven to eliminate traces of contamination.
- Water used in the analysis must be ultrapure and treated with activated charcoal to remove any color impurities,
- Use of inoculated and non-inoculated blanks is essential as controls to check contamination.
- Periodically, check the purity of the inoculums by culturing some cells in Blood Agar and verifying that only *Enterococcus hirae* has grown. A Gram stain is recommended.

¹ Source: (29; 32)

Materials

1. Analytical balance
2. Autoclave
3. Automatic pipette, 1 mL and 50 μ L
4. Blender
5. Bunsen burner
6. Centrifuge (5000 rpm)
7. Dessicator
8. Freezer (-10 to 20 $^{\circ}$ C)
9. Glass or disposable cuvettes for readings in visible light
10. Hot plate with agitator
11. Incubator (35-37 $^{\circ}$ C)
12. pH meter
13. Quartz cuvettes, 1 mL for reading in UV light
14. Refrigerator (4-8 $^{\circ}$ C)
15. UV-Vis Spectrophotometer
16. Thermometer
17. Vortex mixer
18. Water bath for incubation (35-40 $^{\circ}$ C)
19. Beakers
20. Erlenmeyer and volumetric flasks
21. Glass sterile vials of 5 mL
22. Membranes of 0.45 μ m
23. Parafilm
24. Plastic sterile tubes for centrifuge
25. Filter paper Whatman No. 1 and No. 5
26. Test tubes 20 mm x 150 mm with caps
27. Volumetric pipettes

Chemical Reagents

1. Bacteria, *Enterococcus hirae*, ATCC 8042
2. α -Amylase from *Bacillus* sp.
3. Hydrochloric acid, 37%
4. Folic acid, USP standard
5. Broth for inoculums preparation for microbiological assays of vitamins (Micro vitamin test inoculums broth, Hi Media M133)
6. Powdered activated carbon
7. Potassium chloride
8. Sodium chloride
9. Sodium lauryl sulfate or Nuetral Extran
10. Potassium dihydrogen phosphate
11. Sodium dihydrogen phosphate
12. Sodium hydroxide
13. Medium for folic acid analysis (Folid acid medium AOAC, HiMediaM126)
14. Medium for strain maintenance in microbiological assays of vitamins (Mico vitamin test culture agar, HiMedia M132D)
15. Medium for strain propagation Brain Heart Infusion or Trypticase Soy Broth Pancreatin
16. Toluene

Methods

I. Preparation of Solutions

1. Hydrochloric acid – 0.1N

- Dilute 8.3 mL concentrated hydrochloric acid in 1 L solution using distilled water as solvent. Always add acid to water for safety. Work in a fume hood. Mix well. Store in a glass container in a cool, dark place.

2. Distilled water treated with activated carbon

- Add 10 mg activated carbon to 1 L distilled water. Agitate and let settle for one day. Filter the solution through Whatman No. 1 filter paper. Prepare solution every time it will be used.

3. Phosphate buffer – 0.1 M, pH=7

- Dissolve 9 g sodium chloride, 0.2 g potassium chloride, 1.44 g Sodium dihydrogen phosphate and 0.24 g potassium dihydrogen phosphate in 500 mL distilled water treated with activated carbon.
- Adjust pH to 7 with 4N potassium hydroxide. Transfer the solution to a 1-L volumetric flask and bring to volume with treated water.
- Prepare a fresh buffer every time. If stored, sterilize in an autoclave at 121-124°C for 30 minutes and keep in a refrigerator.

4. Sterile glycerin – 15% v/v

- Add 17 mL glycerine 97% to a glass bottle with a screw cap containing isotonic saline solution. Sterilize in an autoclave at 121-123°C for 30 minutes and cool to room temperature. Prepare fresh each working day.

5. Ammonium hydroxide, around 40% v/v

- Bring 40 mL concentrated ammonia to 100 mL total volume with distilled water. Add the ammonia to about 30 mL water first for safety. Store in a plastic container, away from acids. Solution is stable indefinitely.

6. Ammonium hydroxide – 0.1 M

- In a 100 mL volumetric flask containing about 50 mL distilled water, add 1.4 mL concentrated ammonia and bring to volume with distilled water.

7. Sodium hydroxide – 0.1 N

- Dissolve 4 g sodium hydroxide with 300 mL distilled water. Cool the solution in a bath. Bring the total solution to 1-L volume with distilled water. Store the solution at room temperature in a polyethylene container. Solution is stable indefinitely.

8. Sodium hydroxide – 0.01 N

- Perform a 1:10 dilution of the above solution with water as the solvent. Store as above. The solution is stable indefinitely.

9. Sterile isotonic saline solution – 0.9% w/v

- Dissolve 9 g sodium chloride in 100 mL distilled water. Bring total volume up to 1-L with distilled water. Sterilize in an autoclave at 121-124°C for 30 minutes.

II. Preparation of Folic Acid Standard Solutions

1. Stock solution – 100 mg/L

- Dry about 1 g standard folic acid, USP, in a crucible using an oven or a moisture analyzer to constant weight.
- Weigh 50 mg dried standard and dissolve with phosphate buffer. Bring volume to 500 mL. Store the solution in a refrigerator in a dark container with a 2-mm toluene layer. Solution is stable for 2 months.

2. Concentration readings

- Transfer 10 mL stock standard to a 100 mL volumetric flask and bring to volume with phosphate buffer 0.1 M.
- Read the absorbance of the solution in a UV-spectrophotometer with 1-cm light path cuvette at wavelengths of 282 nm and 346 nm. Use the phosphates buffer as the blank.
- Read the absorbencies three times and take the average. Multiply the results by 160 for the absorbencies at 282 nm and by 613.33 for the readings at 346 nm.

3. Intermediate Standard 1 – 1 mg/L

- Prepare a 1:10 dilution of the stock solution using distilled water as solvent. Prepare about 100 mL total. Adjust final pH to 7-8 using HCl or 0.1N NaOH.
- Store in a dark container with a 2m toluene layer. Solution is stable for about two weeks.

4. Intermediate Standard 2 – 100 ng/mL

- Prepare a 1:10 dilution of the intermediate standard 1 using water as solvent. Prepare fresh each time it is needed.

5. Working standard – 1 ng/mL

- Prepare a 1:100 dilution of the intermediate standard 2 using water as solvent. Prepare fresh each time it is needed.

III. Culture medium for HiMedia brand

NOTE: only the amount of medium needed should be prepared each time

1. Trypticase soy broth

- Add 30 g broth to 1L distilled water and heat to dissolve.
- Adjust pH to 7.3 ± 0.2 with 0.1N HCl or 0.1N NaOH.
- Sterilize in an autoclave at 121-124°C for 15 minutes.
- Add to tubes and incubate for 24 hours at 35-17°C.
- If no microbial growth is observed, use or store it. For storage, seal the tubes with parafilm and store at 2-8°C. It is stable for two months.

2. Medium for maintenance of strains used in vitamin microbiological assays (M132D)

- Add 11.1 g agar to 1 L distilled water and bring to a boil for 2-3 minutes. Cool rapidly in a cold bath.
- The medium should be at pH = 6.7 ± 0.2 ; if not, adjust it.
- Add 10 mL medium to tubes and sterilize in autoclave at 121-124°C for 5 minutes. Cool to room temperature on top of an inclined surface to get a slant.
- Incubate for 24 hours at 35-17°C. If no microbial growth is observed, use or store it. For storage, seal the tubes with parafilm and store at 2-8°C. It is stable for two months.

3. Inoculum broth for vitamin microbial assays (HiMedia 133)

- Add 52.1 g medium to 1 L distilled water. The pH should be at 6.7 ± 0.2 .
- Add 10 mL medium to tubes and sterilize in autoclave at 121-124°C for 5 minutes. Cool to room temperature on top of an inclined surface to get a slant.
- Incubate for 24 hours at 35-17°C. If no microbial growth is observed, use or store it. For storage, seal the tubes with parafilm and store at 2-8°C. It is stable for two months.

4. Medium for folic acid analysis (HiMedia M126)

- Add 11.1 g medium to 1 L distilled water and bring to a boil for 2-3 minutes. Cool rapidly in a cold bath.
- The medium should be at pH = 6.7 ± 0.2 ; if not, adjust it.
- Prepare the amount needed for each run only.

IV. Procedure

1. Strain propagation
 - a. In aseptic conditions, take a minimum quantity of the freeze-dried strain and inoculate in Trypticase soy broth. Incubate 24 hours at 37°C. The growth will look as a precipitate at the bottom of the tube.
 - b. After the bacteria have been transferred to the agar for maintenance (part IV 2), discard the vial.
2. Strain maintenance (Stock culture)
 - a. Bacteria grown in this section are the source of inoculums for the analysis. Do not use bacteria grown in part 1 for the inoculum if it is more than one week old.
 - b. Take a portion of the reconstituted strain and cultivate it in a tube with agar for strain maintenance used in the microbiological assay for vitamins (HiMedia 132D) and incubate 24 h at 37°C.
 - c. Store at 4-8°C. Repeat this procedure weekly. Before using a new culture in assay, make several successive transfers of culture in 1-2 week period. From the maintenance tube, prepare the inoculum for the analysis of folic acid in food.
3. Preserving the strain for a long time
 - a. Through this procedure, the strain will be preserved for over three months. This is useful for having a stock culture that can be used at any time.
 - b. Inoculate *Enterococcus hirae* ATCC 8042 in 1 mL Trypticase soy broth. Incubate 24 hours at 35-37°C.
 - c. Add 1 mL glycerine-15% and store at -70°C.
 - d. Repeat this procedure during preservation of the culture in Trypticase soy broth with glycerol-15% from the transference of the strain.
4. Inoculum Preparation
 - a. Transfer cells from the stock culture in part 3 to the sterile tube containing 10 mL broth for inoculums (HiMedia 133).
 - b. Incubate 16-18 h at 35-37°C.

- c. Under aseptic conditions, centrifuge culture at 3000 rpm for 5 minutes and decant the supernatant. Wash cells with 10 mL sterile 0.9% saline solution, resuspending the cells in the solid pellet by agitation. Repeat this procedure two more times and re-suspend the pellet in 10 mL sterile 0.9% saline solution. This suspension is the stock inoculum.
 - d. To obtain the work inoculum, take 10 μ L of the stock inoculums and add it to 10 mL sterile isotonic saline solution.
 - e. A larger quantity of inoculums might be prepared following the proportion of stock inoculums/isotonic saline solution. Store it in 5-mL sterile vials in the refrigerator.
 - f. Use the amount of inoculums needed at room temperature the day of the analysis.
5. Sample Preparation
- a. Grind solid samples and homogenize them.
 - b. In a 250-mL Erlenmeyer flask, weigh 2 g wheat flour.
 - c. Add 50 mL distilled water treated with activated carbon and mix well.
 - d. Add 5 mL 10% amylase to the samples. Cover with a beaker or aluminum and incubate at 37°C overnight.
 - e. Sterilize the solution in an autoclave at 121-123°C for 15 minutes.
 - f. Mix well and cool flasks to room temperature in a water bath.
 - g. Transfer solutions to a 200 mL volumetric flask and bring to volume with distilled water treated with activated carbon. Agitate the solution.
 - h. Centrifuge 25 mL of the solution at 3,000 rpm for 5 minutes.
 - i. Take 5 mL supernatant and transfer to a 100 mL volumetric flask with distilled water treated with activated charcoal. Agitate.
 - j. If the solution is turbid, filter through filter paper Whatman No. 5. If turbidity persists, filter through a 0.45 μ m membrane.
6. Microbiological assay
- a. Prepare the following tubes with screw cap:
 - i. 3 empty tubes for uninoculated blanks (sterilization controls),
 - ii. 3 empty tubes for inoculated blanks (controls to check contamination and purity),

- iii. 12 tubes containing 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mL folic acid working standard solution – 1ng/mL. Prepare each in duplicate.
 - iv. 10 tubes containing 0.5, 1.0, 2.0, 3.0, and 5.0 mL sample final extract, in duplicate.
- b. Add distilled water treated with activated carbon to the tubes to get a final volume of 5mL.
 - c. Add 5 mL agar for folic acid assays (HiMedia 126) to all the tubes and close them
 - d. Sterilize in autoclave for 5 minutes at 121-124°C.
 - e. When the tubes are out of the autoclave, cool them down as rapidly as possible to keep color formation at a minimum. An ice water bath is useful. Take precautions to keep sterilizing and cooling conditions uniform throughout the assay.
 - f. Aseptically inoculate each tube by adding 25 μ L inoculums with an automatic pipette and sterile tips, except to the blanks without inoculation.
 - g. Incubate 16 hours at 35-37°C in a water bath.
 - h. After incubation, check for growth in the blanks. If there is growth, contamination might have occurred and this invalidates the assay. Growth is indicated by a cloudy solution.
 - i. Before reading each tube, agitate for 10 seconds in a vortex mixer.
 - j. Warm the spectrophotometer up and set the wavelength to 550 nm.
 - k. After 15-30 minutes, adjust the instrument to 100% transmittance with the blanks without inoculation.
 - l. Read the % transmittance of the inoculated blanks..
 - m. Set 100% transmittance with the inoculated blanks and read the % transmittance of the rest of the tubes.
 - n. Discard the tubes and wash the glassware as described in the glassware cleaning procedure.

V. Calculations

1. Calculate the %T for each standard concentration

- Plot the average %T on the y axis, versus the volume (mL) of standard solution- 1 ng/mL in each tube on the x axis. A curve of the following form should be obtained:

$$y = ax^2 + bx + c$$

- For each of the sample assay solutions, solve for “x” in the above equation, where “x” is the *equivalent volume of folic acid standard in each tube*. Do not use the values for 0.5 and 5 mL.
- Divide each value obtained by the quantity, in mL, of the sample extract added to each tube, from 1 to 4 mL. Calculate the average for each sample.
- Discard any %T values that are not within $\pm 10\%$ of the calculated average for each sample. Calculate new averages without the discarded values. If more than 3 values are discarded, the assay is invalid and must be repeated.
- Calculate the concentration of folic acid with the following equation:

$$\text{Folic acid} \left(\frac{mg}{kg} \right) = \frac{\bar{X}}{1000} \times \frac{V_3}{V_4} \times \frac{V_1}{W_s} \times C_{std}$$

Where:

\bar{X} is the new average after discarding outliers,

W_s is the sample weight, in grams

V_1 is the initial volume (200 mL)

V_2 is the aliquot volume (5mL)

V_3 is the volume of the second dilution (100 mL), and

C_{std} is the actual concentration of the stock solution of folic acid

V. Optimum incubation time

- Prepare ten tubes with 5 mL distilled water only and ten tubes of 5 mL folic acid standard solution, 1 ng/mL.
- Add 5 mL medium for folic acid analysis (HiMedia 126). Sterilize, cool, and add 25 μ L inoculums. Incubate at 37°C.
- Take out two water only tubes and two tubes containing folic acid standards at times of 16, 18, 22, and 24 hours.
- Mix and set the wavelength at 55 nm. Set the 100% transmittance with the tube with only water. Read the %T of the tubes containing the standard.

- When the change in %T between two different incubation times is lower than 3 units, that is the optimum incubation time.

VI. Spectrophotometer verification

- Wash and dry a crucible in an oven until it reaches constant weight. Weigh before using.
- Prepare the inoculums as described in above procedure.
- In an Erlenmeyer flask containing 300 L medium for folic acid acid (HiMedia 126), aseptically add 1 mL standard folic acid solution -100µg/mL. Sterilize in autoclave at 121°C and cool.
- Add 1 mL working inoculums suspension and incubate for 16 hours at 37°C.
- Centrifuge at 3,000 rpm for 5 minutes and wash with 50 mL 0.9% sterile saline solution. Repeat three times and resuspend cells in 25 mL isotonic sterile solution.
- Pour solution in crucible. evaporate this aliquot in a water bath at 100°C. Dry to constant weight in a vacuum oven at 110°C.
- Weigh the crucible with the dried cells. From this value, subtract the weight of the empty crucible and subtract 0.09 g(weight of sodium chloride in the saline solution) to find the weight of the cells.
- Prepare 50 L bacteria solution, where every mL is equivalent to 0.5 mg dry cells. Take an aliquot of solution prepared in step 4 and calculate the volume of the aliquot with the following equation:

$$Aliquot\ volume\ (mL) = 50\ mL \times \frac{10\ mL}{Weight\ cells\ (g)} \times \frac{1g}{1000\ mg} \times \frac{0.5\ mg}{1\ mL}$$

Use this volume to determine the total weight of dry cells per each tube in step 9.

- In different tubes, add 0 (blank), 1, 1.5, 2, 2.5, 3, 4, and 5 mL diluted aliquot from step 8 in duplicate.
- Add 0.9% sterile saline solution to the tubes as necessary to complete the volume to 5 mL.
- Add 5 mL medium for folic acid assays (HiMedia 126).

12. Agitate and read % transmittance in UV spectrophotometer at 550 nm, setting 100% transmittance to the tubes with 0 mL.
13. Plot %T (y axis) vs. weight of dry cells per each tube(x axis). The points should form a straight line with a correlation coefficient higher than 0.99.
14. Repeat verification procedure three times to ensure spectrophotometer can be used in assay. After, develop composite curve of the three trials that can be used to calculate cell weight for any future tests.

VII. Glassware cleaning procedure

1. Autoclave the glassware which had been in contact with the bacteria for thirty minutes at 121°C.
2. Rinse the glassware three times with tap water.
3. Soak in sodium lauryl sulfate for 24 hours.
4. Clean test tubes with a brush.
5. Rinse the glassware 8 times with tap water and twice with distilled water.
6. Dry in an oven at 250°C.

