Process Development for Quadruple Fortification of Salt

By

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Abstract

This process was developed to simultaneously deliver iron, iodine, folic acid, and vitamin B_{12} for holistic prevention of anaemia and other risk factors for prenatal complications in vulnerable populations. Salt was chosen as the 'vehicle' for the micronutrients because 'doses' of the micronutrients can be predicted. The process was based on a cold forming extrusion-based microencapsulation (for making premix) that ensures that added micronutrients are stable, indistinguishably mixed with salt, do not segregate, and do not cause changes to the sensory characteristics of salt and foods.

The dusting of the micronutrient premix with titanium dioxide masked its brown colour while its coating with soy stearin prevented the adverse moisture aided interaction between iron and iodine. The hydrophobic nature and the initial amount of soy stearin used for coating caused the micronutrient premix to float; contamination of TiO_2 due to its recycling caused dark spots on the surface premix. Double coating with 5% hydroxypropyl methylcellulose and 5% soy stearin solved the floating problem; coating with a mixture of 15% TiO_2 and 10% soy stearin solved both problems.

The required pH ($\geq$ 8) for full dissociation of folic acid, its solubility, and stability caused vitamin B_{12} instability; hence, both micronutrients cannot be added to salt through a solution. In salt, they were lost through oxidative degradation. The coextrusion of folic acid and vitamin B_{12} with ferrous fumarate (a reducing agent) minimized the oxidative degradation of the micronutrients. The colour masking and coating of the premix ensured that the micronutrients did not affect the colour of the salt. Also, the coextrusion prevented the micronutrients from photodegradation.

The salt samples can deliver 50-200% recommended dietary allowances of the micronutrients based on the consumption of 10g of salt per day. The micronutrients were very stable in the salt and met the set target (70% retention after 6-month storage). Over 85% of the micronutrients were retained in salt after 6-month storage, even at 45 ^{o}C/60-70% RH. The micronutrients were stable in cooking and did not cause any changes to the sensory properties of the salt or food.

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‘To everything there is a season, and a time to every purpose under the heaven’

Ecc. 1:1

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'We are the clay and You are the potter' (Isaiah 64:8). Lord, I am grateful for what You have molded me into out of the miry clay. Through it all, I have learned to depend upon Your word!

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List of Abbreviations

RH: Relative humidity

TiO_2-Titanium dioxide

B_{12}- Vitamin B12

FA- Folic Acid

I- Iodine

Fe- Iron

Co-Cobalt

DFS -Double Fortified Salt

TFS- Triple Fortified Salt

QFS- Quadruple Fortified Salt

CNCbl- Cyanocobalamin

MeCbl- Methylcobalamin

HOCbl-Hydroxycobalamin

CN- Cyano functional group

CH_3- Methyl functional group

HO- Hydroxo functional group

HCl- Hydrochloric acid

HNO_3- Nitric acid

Na_2EDTA- disodium ethylenediaminetetraacetate

FeNaEDTA- Ferric sodium ethylenediaminetetraacetate

SHMP- Sodium hexametaphosphate

HPMC- hydroxypropyl methylcellulose

SS- Soy stearin

DNA- Deoxyribonucleic acid

H- Hydrogen

CO-Carbon monoxide

MPT- 6-Methylpterin

PABA-Glu - p-Aminobenzoyl-L-glutamic acid

pABGA - p-Aminobenzoyl-l-glutamic acid

PCA - Pterine-6-carboxylic acid

THF- Tetrahydrofolate

RO- Reverse osmosis

MS-Mass spectrum

SEM- Scanning electron microscopy/microscope

nm- Nanometer

µm- Micrometer

µg – Microgram

µL-Microliter

mL- Milliliter

mM-Millimolar concentration

mg- Milligram

cm^3- Cubic centimeter

g-Gram

kg-Kilogram

M- Molar concentration

N- Normal concentration

ppm-Part per million

A_T - Amount of TiO_2 used for colour masking (% $^{w}/_{w}$)

ρ- Density

ρ_B -Bulk density in g cm^{-3}

ρ_P - Particle density in g cm^{-3}

ρ_w - Water density = 1 g cm^{-3}

ρ_H - Hexane density = 0.66 g cm^{-}

T-Temperature/thickness

T_{HPMC} – Thickness of hydroxypropyl methylcellulose layer

T_{SS-} Thickness of soy stearin layer

r-Radius

v-Volume

m- Mass

t- Time

$^{w}/_{w}$- Weight per weight

$^{w}/_{v}$- Weight per volume

k- Degradation constant

[x]- Concentration of x

ΔL- Change in the degree of whiteness

pKa- Acid dissociation constant

E_a- Activation energy

R- Gas constant (8.314 J/K·mole)

ICP-OES- inductively coupled plasma optical emission spectrometry

UHPLC- Ultra-high-performance liquid chromatography

HPLC- High-performance liquid chromatography

TIC- Total ion current

IR- Infrared

UV-Vis- Ultraviolent-visible

RDA- Recommended Dietary Allowance

RDI- Recommended Dietary Intake

UL- Tolerable Upper Intake Level

NTDs - Neural tube defects

USD- United States dollar

ACS-American Chemical Society

USP- The United States Pharmacopeia

WHO-World Health Organization

UofT- University of Toronto

IGN- The Institute for Global Nutrition

FAO- The Food and Agriculture Organization

NIN - The National Institute of Nutrition, India

ETH-The Swiss Federal Institute of Technology

CDC- Centers for Disease Control

UNICEF- The United Nations Children's Fund

1.0 Introduction

Micronutrient deficiencies can lead to permanent adverse effects on growth and development; they may even cause death. Anaemia is a major contributing factor to 200,000 annual maternal deaths and more than a million annual infant and neonatal deaths, most of which occur in developing countries (Smith *et al.*, 2019). While the primary cause of anemia is iron deficiency, the lack of iron in the diet is exacerbated by vitamin B_9 and B_{12} deficiencies, which also leads to adverse perinatal outcomes such as neural tube defects (Sukla, Nagar and Raman, 2014; Turner, 2018).

Changing lifestyles to include healthy balanced diets is the ultimate solution to micronutrient deficiency disease; it is a long-term goal that cannot be achieved in the foreseeable future (Nair, Augustine and Konapur, 2016). Food fortification can be an inexpensive, rapid, effective intervention against specific micronutrient deficiencies that is sustainable (Dollimore, 1996). Therefore, a comprehensive multiple micronutrient fortification would be the most effective preventive intervention for anemia and other debilitating conditions that adversely affect the economic well-being and health of approximately one-third of the world population.

Critical to successful food fortification is finding a staple food that is regularly consumed by all the affected population, at a predictable "dose." Salt could be an ideal vehicle, as it is universally consumed at a nearly constant level independently of socioeconomic status (Mannar, 2018). With the advent of salt iodization, infrastructure for adding some micronutrients is widely available. Unfortunately, salt is an aggressive chemical, and the interaction of added micronutrients with it, each other, and food components must be prevented.

The technology for the double fortification of salt with iodine and iron was developed here at the University of Toronto. The technology is based on the physical separation of the reactive component (iron) by microencapsulation (Li, Diosady and Wesley, 2010). The technology has been successfully scaled and introduced in India, where some 50 million consumers have benefitted – with an estimated 1 million children cured of anemia (Diosady, Mannar and Krishnaswamy, 2019). It has been shown that iron deficiency is often coupled with deficiencies of folic acid and vitamin B_{12} that lead to severe cases of anemia and maternal and infant mortality, as well as preventable congenital disabilities (Goonewardene, Shehata and Hamad, 2012).

Based on the technology for the double fortification of salt and to holistically solve the problem of anaemia and associated maternal and infant health problems, a process was developed for

simultaneous delivery of iodine, iron, folic acid and vitamins B_{12} through salt (Quadruple Fortified Salt). The process allows for physical separation of the added micronutrients, preventing their interaction and loss during processing and distribution. Also, it improved the organoleptic properties of the Quadruple Fortified Salt. Over 85% of the added micronutrients were retained after six months of storage at 45 ^{o}C and 60-70% RH. The process was demonstrated on a pilot scale in India. The formulation will be tested in India for its efficacy in a major study by the Cornell University, funded by the Center for Disease Control starting this fall.

2.0 Research Objectives and Scope

The overall objective of the research project was to develop a process for the quadruple fortification of salt with iodine, iron, folic acid, and vitamin B_{12} based on the understanding of the kinetics of the breakdown of folic acid, and the interaction between iron and iodine. The fortified salt must be affordable, generally acceptable, with minimal alteration to the sensory properties of the salt.

In achieving the overall objective, several sub-objectives had to be met.

Optimize the Coating Procedure in Iron Premix Production

While the technology for double fortification is well established in the laboratory, there were two challenges encountered during scale-up and field testing. The iron premix float, and there were dark spots on the iron premix. The possible factors responsible for these problems were elucidated, and solutions were proffered to the problems.

Develop Analytical Methods

There are established analytical methods for quantifying iodine and iron in salt. One of the objectives of this thesis was to develop the analytical methods for the other micronutrients, folic acid, and vitamin B_{12}. The possibility of using a simple spectrophotometric method that relies on a distinct absorption wavelength for folic acid, which is selective for folic acid in the presence of products of degradation of folic acid was evaluated for quantifying folic acid in the spray solution and salt. After several failed attempts, the prospect of using UHPLC-MS to quantify vitamin B_{12} and folic acid in premix was evaluated.

Understand the chemistry of degradation of folic acid and vitamin B_{12}

The prospect of using the total ion current (TIC) chromatogram obtained from UHPLC-MS, Compound Discoverer™ Software, and Chem3D to predict the products of folic acid degradation and possible mechanism degradation were evaluated. Also, the possible impact of pH and ascorbate on the degradation of vitamin B_{12} was evaluated.

Formulate Triple Fortified Salt (Iron + Iodine + Folic Acid)

Formulating Triple Fortified Salt (TFS) was the first step towards quadruple fortification of salt. The possibility of formulating TFS by adding folic acid and iodine (potassium iodate) as a solution and iron as an extruded and microencapsulated ferrous fumarate (iron premix) to salt was

evaluated. The prospect of co-extruding iron and folic acid to solve the colour problem (yellow, caused by spraying folic acid on the salt) and improve folic acid stability with the reductive potential of ferrous iron was evaluated. Ultimately, the TFS should deliver 100% of RDA for folic acid, 30-56% of RDA for Fe, and 200% of RDA for iodine.

Formulate Quadruple Fortified Salt (Iron + Iodine + Folic Acid + Vitamin B_{12})

By adopting the process for the triple fortification of salt, Quadruple Fortified Salt (QFS) was formulated. The possibilities of making Quadruple Fortified Salt by spraying a solution that contained potassium iodate, folic acid and vitamin B_{12}, potassium iodate and folic acid or just potassium iodate on salt, while the corresponding micronutrients (iron, iron and vitamin B_{12} or iron, vitamin B_{12} and folic acid) were added as an extruded and microencapsulated premix were evaluated for stability of the micronutrients and their impact on the sensory properties of the salt. Ultimately, the QFS should deliver 100% of RDA for folic acid, 30-56% of RDA for Fe, 200% of RDA for iodine, and 100% of RDA for vitamin B_{12}.

Develop Kinetic Model for Predicting Stability of Micronutrients in the Fortified Salt

The possibility of using kinetic data derived from the stability studies to develop a kinetic model for predicting the stability of micronutrients in the fortified salt was evaluated.

Evaluate the Impact of Some Cooking Techniques on the Stability of Folic Acid and Vitamin B_{12}

The likely impact of boiling on the stability of folic acid and vitamin B_{12} in cooked rice was evaluated.

Carryout a Small-Scale Sensory Survey for Food Prepared with QFS

The possible impact of the micronutrients in the QFS on the sensory properties of cooked rice was determined using a small-scale sensory panel.

2.1 Research Hypothesis

The thesis explored the hypothesis that based on the understanding of the kinetics of folic acid degradation and iodine loss, a process for producing stable salt containing more than 30% of Recommended Dietary Allowances (RDAs) of iron, iodine, folic acid, and vitamin B_{12} without significantly impacting the sensory properties of the salt and food cooked with the salt can be developed.

3. Background

3.1. Micronutrient Deficiency

Micronutrients are nutrients needed in small quantities in the human diet, comprised mostly of trace metals (minerals) and vitamins. They are involved in almost all human cellular and molecular functions. Hence, their deficiencies or excessive intake can be detrimental to health, which may ultimately lead to death if the situation is unchecked (Ferrari, 2002). Micronutrient deficiencies are widespread, affecting more than 2 billion people but are more profound in the developing regions of the world. While young children and women of reproductive age are the ones most at risk, its effects cut across all age groups (Allen, 2006). Iron, iodine, and vitamin A deficiencies are the most prevalent micronutrient deficiencies; they have profound effects on human health (Ahmed, Hossain and Sanin, 2013). In the recent past, multiple micronutrient malnutrition has become a significant public health concern precisely because of their effect on maternal and infant mortality, congenital disorders, and the health of women and infants,(Black, 2003; Siekmann *et al.*, 2003). The impact of micronutrient deficiencies includes; congenital disorders, impaired or reduced mental and physical capacity or development, increased risk for infections and diseases, reduced productivity, and increased mortality rate (Bailey, West Jr and Black, 2015). These effects follow a vicious intergenerational cycle (*Figure 1*).

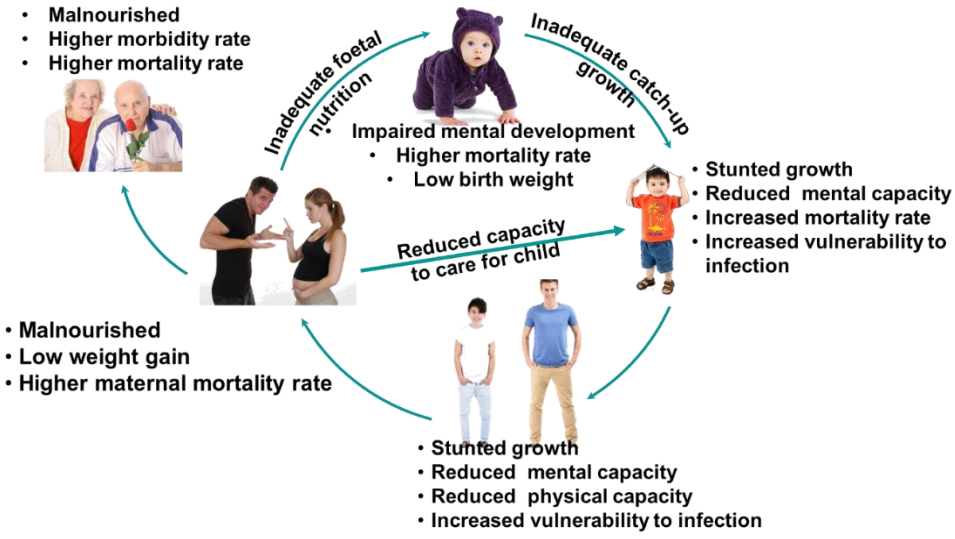


Figure 1:The effect of micronutrient inadequacies across the life span

3.2 Prevention and Control of Micronutrient Deficiencies

The WHO has highlighted dietary diversification, food fortification, supplementation with vitamin and minerals, and global public health and disease control as the measures to prevent and control micronutrient deficiency (FAO, 1996). Infection and diseases worsen the impacts of micronutrient deficiencies. Some disease conditions are a direct result of micronutrient deficiencies, and some infections are a risk factor for the incidence of micronutrient deficiencies (Katona and Katona-Apte, 2008). The control of infections and diseases reduce the effects of micronutrient deficiencies.

Micronutrient deficiencies result mainly from inadequate intake of micronutrients, while eating foods from different sources that are rich in various micronutrients can solve the problem of micronutrient deficiencies (Nguyen *et al.*, 2018). While most of the approaches for combating micronutrient deficiencies can only improve the intake of micronutrients, food diversification also enhances the consumption of other food constituents such as plant metabolites that function as antioxidants and provitamins. This approach is the most sustainable and desirable; however, it takes the longest time to implement. Also, the lack of funds to purchase varieties of food in an impoverished population is a barrier to this approach (Allen *et al.*, 2006b).

Micronutrient supplements can meet the daily requirements of micronutrients. This approach can supply an adequate amount of specific vitamins and minerals in a very short time. Iron, folic acid, vitamin B_{12}, and A supplements are available in the market. While the supplements containing fat-soluble vitamins are less frequently administered (usually 2-3 times in a year), the supplement for the water-soluble micronutrients and vitamins are more regularly used (Soren and Biswas, 2020). The lack of adequate distribution and supply, poverty, and poor compliance to prescription impedes the successful implementation of this approach to a population.

Finally, staple foods fortified with micronutrients can supply an adequate amount of micronutrients to a population (Li, Diosady and Jankowski, 2011; Li, Diosady and Wesley, 2011). Fortifying a food that is widely consumed by a large proportion of a population can effectively and rapidly improve the micronutrient status of the population at a reasonable cost (Allen *et al.*, 2006b). It is a cost-effective approach for meeting the micronutrient need of a large population. Food fortifications are easily implemented if the chosen food is centrally produced and if the fortification is supported by the food industry such that the existing distribution channels can be leveraged.

Most of these approaches require educating the population about accepting the change in their traditional norms that these approaches may cause.

3.3 Food Fortification

The addition of essential micronutrient(s) to food to boost its nutritional value for meeting the nutrient needs of a population is termed food fortification or enrichment(Codex Alimentarius, 1987). The efficacy of food fortification in the reduction of the global prevalence of micronutrient deficiencies is well established (Darnton-Hill and Nalubola, 2002). Food fortification started with the iodization of salt and has expanded to different staple foods (cereals, milk, grains, flour, rice, and many other foods) and other micronutrients (iron, calcium, folic acid, and vitamins A and D). Fortification can either be voluntary or mandatory (*Figure 2*). Fortification may be for a general population or target population and maybe market-driven, in which case the addition of micronutrient(s) to food gives the food an advertising advantage. In most cases, mass fortification is mandatory, while the market-driven fortification is voluntary but guided by regulations.

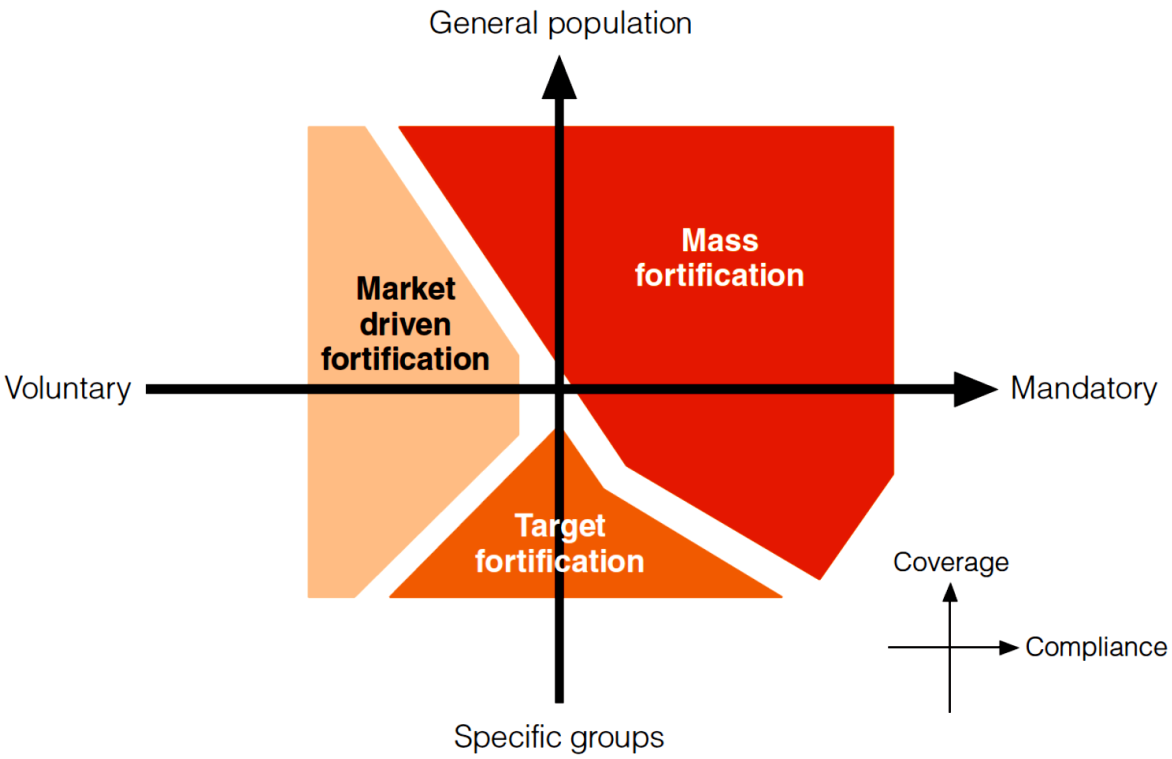


Figure 2:The different types of fortification (Allen et al., 2006a)

3.3.1 Principles of Food Fortification

The following are the principles of food fortification:

1. The addition of essential nutrient(s) to food may prevent, reduce the risk of, or correct inadequate essential nutrient(s) of a population such that the required or recommended intake of the nutrient(s) is met or that the health of the population is improved or maintained and the nutritional value of the food is maintained or improved.
2. A national or regional authority should decide on the mandatory or voluntary addition of nutrient(s) based on scientifically proven gravity and extent of public health need. The authority should make specific standards, regulations, and guidelines for the type of food(s), the nutrient(s) that can be added, the amount of nutrient(s), the labeling and advertisement of the fortified food.
3. The upper level of intake (UL) should guide the amount of nutrient(s) that should be added to food; where UL is not available, it must be scientifically proven that the amount to be added will not result in adverse health effects.
4. The intake of nutrient(s) from all sources must be considered when deciding the amount of nutrient to prevent the risk of excessive intake; hence, an adequate analytical method for determining the quantity of nutrient(s) is essential.
5. The selection of food should be based on the intended purpose of adding the nutrient(s) and should consider the dietary pattern, socioeconomic situation, and any risk to the health of the population. Alcoholic beverages are not to be used as nutrient vehicles.
6. The food selected must be habitually, adequately, and uniformly consumed by the target population.
7. The added nutrient can be natural or synthetic but must be pure, safe, stable (during processing, packaging, distribution, and storage) and bioavailable.
8. The cost-effectiveness of the approach must be considered (FAO, 1996).

3.3.2 Food Fortification Technology

Once the suitable fortificant(s) and food vehicle(s) are selected, the typical techniques used in food fortification involve mixing processes (Lotfi *et al.*, 1996). Depending on the nature of the fortificant and food vehicle, the mixing can be either a solid-solid, solid-liquid, or liquid-liquid mixing. In solid-solid mixing factors such as size, hygroscopic, and electrostatic properties, shape,

density, and proportions of the components must be considered for effective mixing and to prevent segregation of components of the fortified food during processing and handling. In solid-liquid mixing, fortificant in liquid form or solid form but dissolved in a suitable solvent is added to a solid food vehicle. The liquid or solution must be added to the moving powder and not the mixer surface. Spray mixing or drip-feeding are commonly used techniques. In liquid-liquid, the fortificant in liquid form or solid form but dissolved in a suitable solvent is mixed with liquid food. Mixability of the food and fortificant, flowing characteristics, proportions, and viscosity are factors usually considered for liquid-liquid mixing.

Encapsulation helps to enhance the handling, bioavailability, potency, and stability of nutrients in food. In practice, encapsulation may occur before or after mixing operation. Mixing of the components of iron premix used for salt fortification is before encapsulation, while mixing of iron premix with the iodized salt occurs after the encapsulation (Modupe, Krishnaswamy and Diosady, 2019). In the process described, solid-solid and solid-liquid were employed. Iodine solution (in some cases with additional micronutrients) is mixed with salt. Iron (in some cases coextruded with other micronutrients) is mixed with salt.

3.3.3 Microencapsulation Technology Used in Food Fortification

Microencapsulation is the process of entrapping substances (active substances, in this case, micronutrients) within a shell or coating material, resulting in particles with diameters from few micrometers to few millimeters (Srivastava, Semwal and Sharma, 2013). There are many problems associated with the addition of micronutrients to food because these micronutrients are active chemical moieties, and so are the constituents of food. Such problems include a change in natural colour, flavour, and texture of food, and instability of nutrients, which results from the chemical and physical interaction among the chemical constituents of food and the micronutrients (Diosady, Alberti and Venkatesh Mannar, 2002). Microencapsulation is one of the traditional methods for preventing such interactions. Also, it helps to improve the handling, protect, and control the release of micronutrients (Srivastava, Semwal and Sharma, 2013). However, these benefits must not significantly increase the cost of the food.

Based on morphology, microencapsulates can be categorized mainly into two types: the reservoir and the matrix types. With the reservoir type, the coating material encapsulates pure

micronutrient(s). It is also called a core-shell encapsulate. The other types have micronutrient(s) embedded and uniformly distributed within coating material. Since the micronutrient(s) are usually exposed to the surface of the microencapsulates, an additional coating is usually applied. Both reservoir and coated matrix type may have additional layers of material which may serve several functions. For instance, in the double fortification of salt, the coated matrix type has additional layers – TiO_2 to mask the colour of ferrous fumarate, hydroxypropyl methylcellulose to hold the TiO_2 in position and to form a physical barrier, and soy stearin to prevent moisture penetration and further form a physical barrier between iron and iodine. These additional layers may help to compartmentalise micronutrients within the premix.

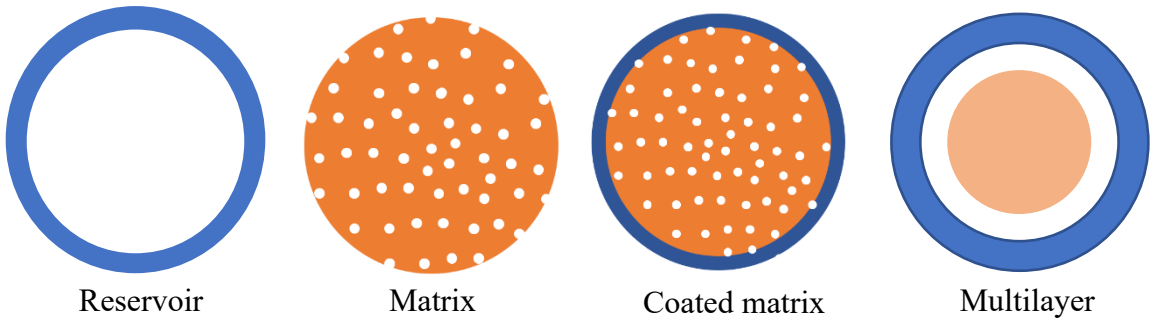


Figure 3:Types of microencapsulation

Table 1: Coating Materials for Microencapsulation of Functional Food Additives (Desai and Jin Park, 2005; Madene et al., 2006)

Category	Coating Materials	Widely used methods	Interest
Carbohydrate	Starch, maltodextrins, chitosan, ethylcellulose, cellulose acetate phthalate, simple sugars cellulose acetate butyrate, corn syrup solids, dextrin, modified starch, cyclodextrins, methylcellulose, ethylcellulose, carboxymethyl cellulose, dried glucose syrup	Spray- and freeze-drying, extrusion, coacervation, inclusion complexation, and edible films	Film-forming, excellent emulsifier, encapsulant,

Gum	Gum arabic, agar, alginates, carrageenan, pectins	Spray-drying, syringe dropping method	Emulsifier, film-forming
Lipids	Tristearic acid, wax, paraffin, beeswax, natural fats and oils, lecithin, glycerides	Water-resistant film, liposomes, emulsion, extrusion	A barrier to oxygen and water
Protein	Gluten, casein, gelatine, albumin, zein, soy protein, whey protein	Emulsion, spray-drying	Good emulsifier

There are different techniques and encapsulants available for microencapsulation (Table 1). The choice of method and material depends on the intended function of the microcapsule, the nature of active substance and encapsulant, the expected physical characteristics (size, shape, colour, density, and structure) of the microcapsule and the cost (Desai and Jin Park, 2005). Ideally, encapsulant should be non-reactive; have excellent rheological properties, especially at high concentration; chemically stable; neutral taste and odour; have excellent film-forming, gelling, and barrier properties; non-hygroscopic (Augustin and Sanguansri, 2008). It is usually challenging to find an encapsulant with all these properties; hence in practice, a combination of encapsulants is used (Desai and Jin Park, 2005). A few of the techniques used for encapsulation are discussed in the following.

A. Coacervation/Phase Separation

Coacervation is one of the oldest encapsulation methods used for industrial applications (Jyothi *et al.*, 2010). It is based on aqueous-phase separation process of a homogeneous polymer solution into two phases. Due to the electrostatic interaction between the two components of the homogeneous polymer solution, a complex is formed. An associative complexation and phase segregation result such that one phase has a high concentration (coacervate) and the other phase has a low concentration of the polymer (Dong *et al.*, 2011). Given the role played by electrostatic interaction, pH is a critical factor in this process (Park and Yeo, 2007). The microcapsules obtained by this process are usually stabilized by crosslinking or thermal treatment (Jyothi *et al.*, 2010). Encapsulation by this method usually involves three steps (*Figure 4*).

The first step involves mixing an aqueous solution of a polymer with a homogenized emulsion of oil and solution of another polymer. The second step involves lowering of the pH to the point that opposite electric charges in the system cause the formation of a complex. The complex in the liquid phase is distributed over the oily surface to form the shell- microcapsules. The final step involves the stabilization and hardening of the microcapsules with the addition of crosslinking agents. This method has been used for coating essential oils, fragrances, dyes, vitamin C, and drugs. Chitosan, ethylcellulose, gelatin, and gum arabic are some of the polymers used for coacervation (Dong *et al.*, 2011).

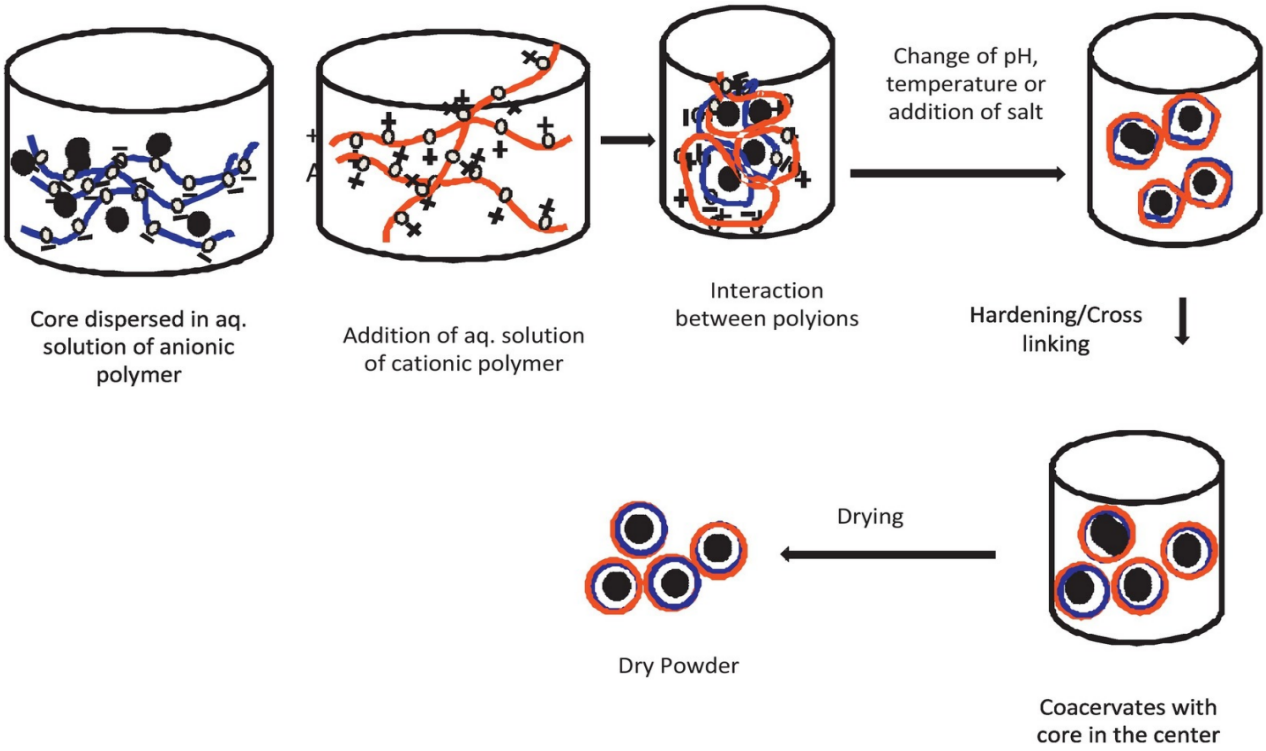


Figure 4:Schematics of the coacervation process (Timilsena et al., 2019)

B. Spray Drying

"Spray drying is the transformation of feed from a fluid state into a dried particulate form by spraying the feed into a hot drying medium." (Masters, 1991). It was first designed to produce milk powder but has since been adapted for many applications, including microencapsulation (Anandharamakrishnan, 2015). The working principle of spray drying involves convection heating wherein moisture is removed from a liquid fed into a heated and humidity-controlled

chamber. Spray drying consists of four steps: feed solution atomization, atomized solution contact with the hot gas, moisture evaporation, and particle separation (*Figure 5*) (Ishwarya, Padma and Anandharamakrishnan, 2017).

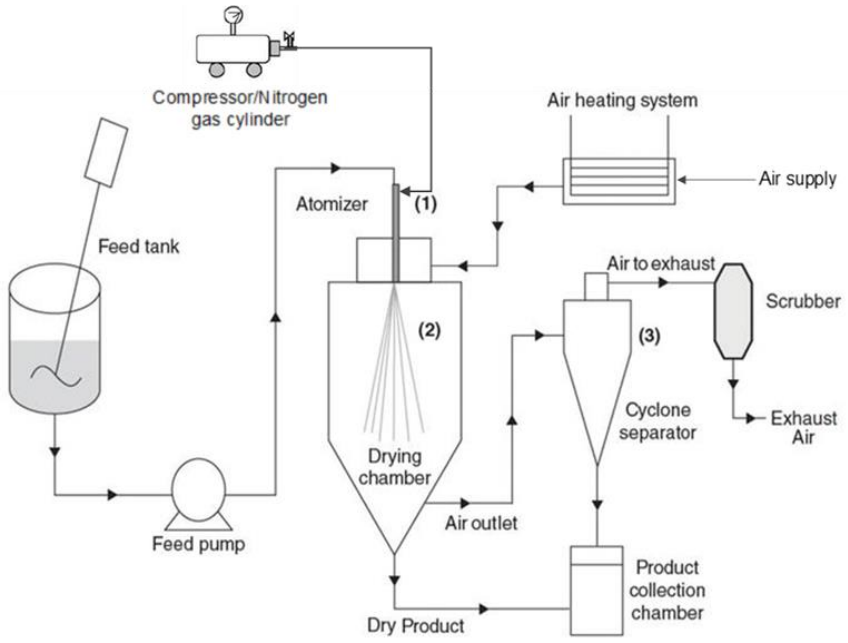


Figure 5: Operating steps of a spray dryer: (1) atomization, (2) feed and heat contact, and (3) product collection. Adapted from Ishwarya, Padma and Anandharamakrishnan (2017)

At the core of spray drying is the atomization of the feed solution (a stable emulsion, suspension, or solution of an active ingredient in the coating material solution). The atomization increases the surface area of the particles to increase the rate of evaporation of liquid. There are several types of atomizers-pneumatic atomizer, pressure nozzle, spinning disk configurations, two-fluid nozzle, and sonic nozzle. The nature of feed and the desired properties of the product dictate the choice of an appropriate atomizer. Those with higher energies produce fine particles; the particle sizes can also be controlled by adjusting the viscosity and surface tension of the feed. The feed can either be in contact with co-current or countercurrent hot airflow. The temperature sensitivity of the product guides the choice of the current of hot air. Product from a co-current hot air design is usually exposed to a lower temperature compared to the countercurrent hot air design. However, the countercurrent hot air design is more energy efficient.

Aerosol droplets of the solution of starting material(s) are formed by the atomizer. The solvent is removed from aerosol droplets as they come in contact with the hot air flow, achieved through evaporation and solute condensation. Diffusion is key to this process; as the solvent evaporates at the surface, the 'dissolved' solids diffuse back into the core. These heat and mass transfer processes are driven by the difference between the solvent's vapor pressure and its partial pressure toward a gas phase. The heat and mass transfers lead to loss of liquid from the droplets to form fine powders. The products are then collected in a cyclone that aids the separation of the product from the humid air. There are reports on the coating of flavors and iron compounds with gum arabic, starch derivatives, and dextrinized and hydrolyzed starches (Pegg and Shahidi, 2007; Romita, Cheng and Diosady Levente, 2011; Singh, Siddiqui and Diosady, 2018).

C. Lyophilization/Freeze Drying

This technique, also known as cryodesiccation, is usually used for drying expensive heat-sensitive materials like proteins. Its high cost and long processing time limit its commercial applicability (Marques, Silveira and Freire, 2006). It involves drying of the core dissolved or suspended in the wall material solution that is frozen. The drying is under a vacuum and by sublimation - direct conversion of the frozen liquid to gas. Three main stages are involved: freezing, primary drying by sublimation, and the secondary drying by desorption (Fang and Bhandari, 2012).

The core material dissolved or suspended in a solution of the wall material is frozen to a temperature below the material triple point, usually between -20 ^{o}C and -40 ^{o}C. This stage is the most critical of the whole process. Slow freezing is essential for large ice crystals, which aid fast and efficient freeze-drying (Fang and Bhandari, 2012). For the primary drying, the pressure of the containers that hold the material is lowered, and just enough heat is supplied to sublime the ice. The pressure is controlled by a vacuum system that also speeds up the drying. About 95% of the moisture content sublimes at this stage. The remaining moisture is removed at the secondary drying stage. The temperature is slightly increased at this stage to about 0^{o}C to dissociate water from the frozen material that is then evaporated (Fang and Bhandari, 2012).

An advancement in this technology now exists - spray freezing drying. Spray freezing drying involves the use of an atomizer to form droplets which are frozen. Frozen droplets are dried at low temperature and pressure (Costantino *et al.*, 2000). Some of the common wall materials used

include proteins, maltodextrins, disaccharides, poly(vinyl alcohol), poly(ε-caprolactone), and gums (Abdelwahed *et al.*, 2006).

D. Centrifugal Extrusion

This technique is a liquid coextrusion process that uses a concentric orifice nozzle located on the head, which is an outer circumference of the rotating (encapsulating) cylinder (Figure 6). The core material and encapsulants in liquid forms are pumped separately through the different orifices of the concentric nozzle. The liquid core material is pumped through the inner orifice and a liquid encapsulating agent through the outer orifice to form a co-extruded rod of core material surrounded by the wall material (Desai and Jin Park, 2005). The extruded rods are broken into droplets by the centrifugal force generated by the rotating device. Due to surface tension, the coating material encloses the core material. The droplets fall on a moving bed of fine starch, which not only helps with moisture absorption but also serves as a cushion for the microcapsules (Desai and Jin Park, 2005). Usually, the sizes of the microcapsules formed range from 150µm to 2000 µm. The speed of rotation of the device controls the magnitude of the centrifugal force, which dictates the capsule size. About 20-80% loading capacity is achievable with this method.

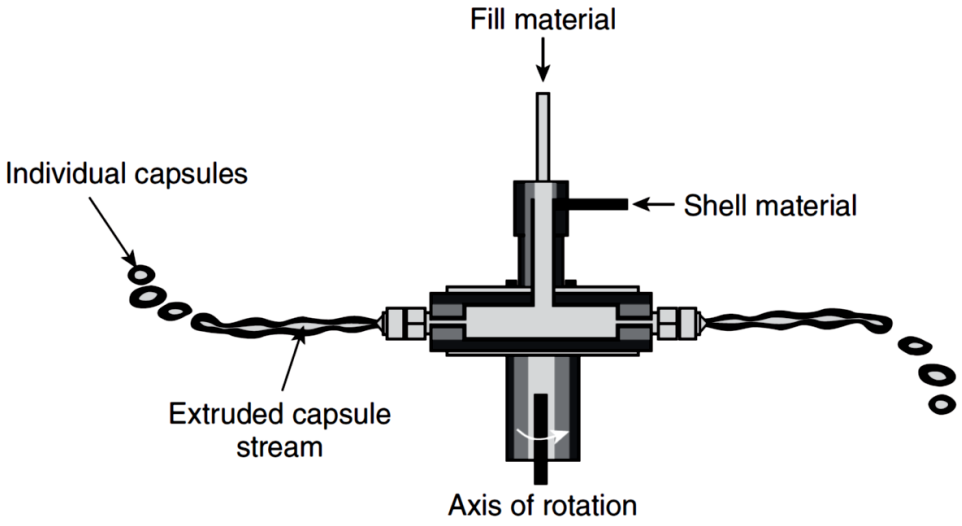


Figure 6:Schematic of centrifugal extrusion equipment (Oxley, 2012)

The core and the coating material must be in a pumpable state, and the viscosity must be low enough to allow for the breakage of the extrudate into droplets by centrifugal force. The coating material must harden rapidly. The typical coating materials used in this method include fats,

sodium alginate, waxes, starches, cellulose derivatives, polyethylene glycol, gelatin, fatty acids, carrageenan, and gum acacia. A blend of two or more coating materials may be used (Gibbs *et al.*, 1999). Vitamins, seasonings, and flavour oils are among the common materials coated with this method. The capsule produced usually has slow-release properties (Solanki *et al.*, 2013).

E. Centrifugal Suspension Separation

Encapsulation by this technique is based on suspending core particles in the solution of the coating material. The suspension is pumped through a rotating disk atomizer fixed on a drying or cooling apparatus (*Figure 7*). The centrifugal force causes microcapsules to form at the edge of the rotating disk (Sparks and Mason, 1987). Some of the core particles are usually uncoated, while some of the coating material ended up not being used in every batch. The coating material, which is smaller in size than the core particles, is usually recovered by sieving (Sparks, Jacobs and Mason, 1993).

The process is inexpensive and has a high throughput; it takes seconds to minutes to coat a core material. It coats a wide range of materials having a diameter from 30μm to several millimeters. The coating can be 1-200μm thick (Barbosa-Cánovas *et al.*, 2005). However, the process produces uncoated particles. The uncoated particles are difficult to separate by size screening. Materials with low melting points (solid at room temperature) such as fats and diglycerides are usually used as the coating material. Active materials are applied as a suspension or solution. This method is used to coat materials that are sensitive to moisture like aspartame, methionine, and vitamins. Spray-dried flavors are also coated with this method (Barbosa-Cánovas *et al.*, 2005).

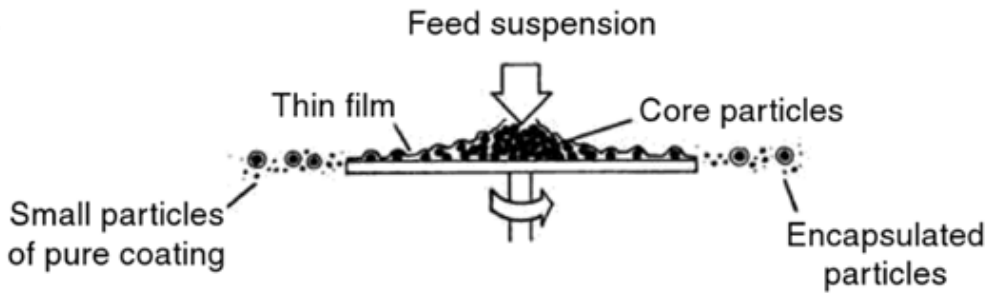


Figure 7:Schematics of centrifugal suspension-separation equipment (Barbosa-Cánovas et al., 2005)

F. Spray Cooling and Spray Chilling

This method of encapsulation is like spray drying; both involve atomizing a homogenous suspension of core material into a liquefied coating material through a nozzle into a temperature-controlled environment (Barbosa-Cánovas *et al.*, 2005). Spray cooling or chilling, however, uses a heated nozzle, cold air, and waxes, fats, lipids, or gelling hydrocolloids as the coating material (Figure 8). The cold air in the atomizing chamber causes the fat to solidify around the core material; hence the microcapsules formed by this method are not soluble in water. As a result, this method is frequently used to coat hydrophilic material (such as minerals and some vitamins). The downside of this coating method is the interaction between fat and the core material, which may lead to the rancidity of the fat. Meltable solids or liquid fats with a melting point of 45-122 ^{o}C are used as the encapsulating material. The use of mono and diacylglycerides with melting points of 45-65 ^{o}C is also common (Risch, 1995b).

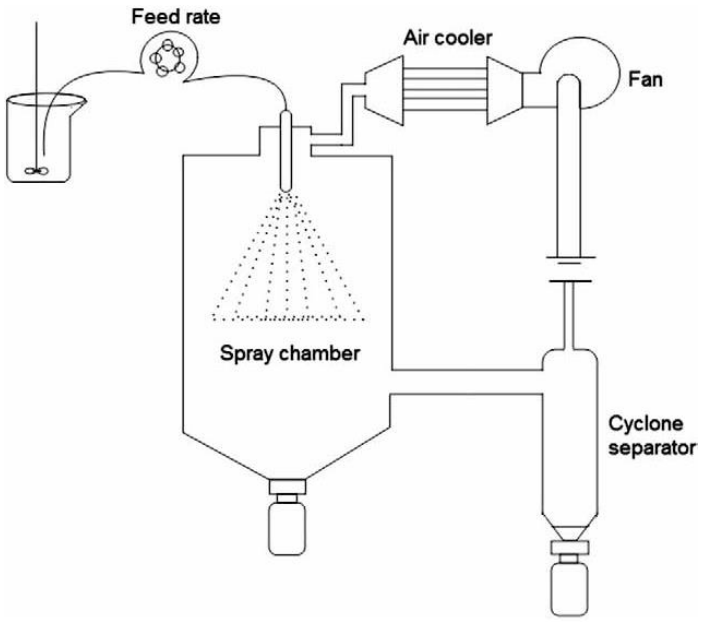


Figure 8:Schematics of spray chilling equipment (Okuro, de Matos Junior and Favaro-Trindade, 2013)

G. Co-crystallization

This technique incorporates core material into the matrix of a carbohydrate (Figure 9). The core material is added to a supersaturated solution of sucrose maintained by heating just enough not to induce crystallization. The mixture is vigorously agitated to aid nucleation. As the temperature is

increased (above 120 ^{o}C), crystallization begins as a substantial amount of heat is emitted, which initiate cooling (Desai and Jin Park, 2005). The agitation continues until agglomerated crystals are completely formed. The agglomerated crystals trap core material in their void spaces. The microcapsules are then dried, and size screened. The rate of nucleation and crystallization must be carefully controlled (Desai and Jin Park, 2005).

This technique improves the solubility, wettability, homogeneity, dispersibility, hydration, anticaking, stability, and flowability of the core material. This technique can dry liquid core materials without an additional drying step. The technique has been used to encapsulate yerba mate and orange peel oil (Beristain *et al.*, 1996).

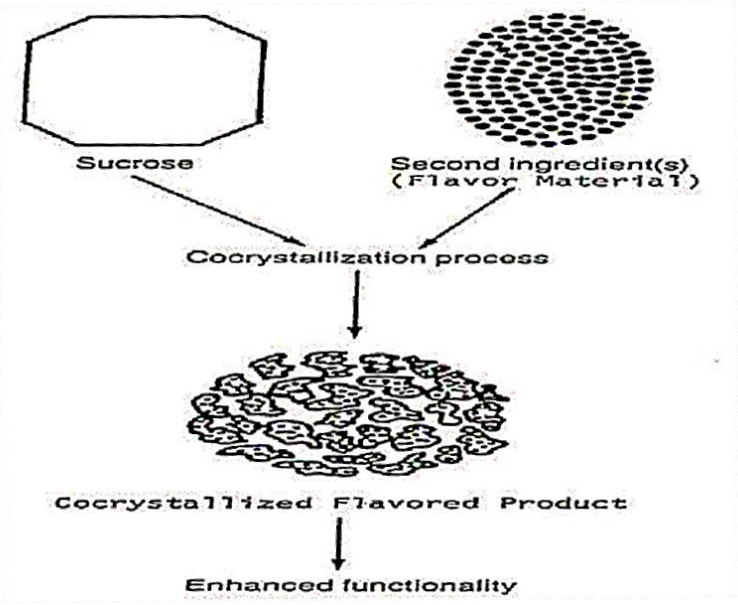


Figure 9:Schematics of cocrystallization process (Abd El-Kader and Hashish, 2019)

H. Fluidized-Bed Coating

This technique applies a coating material to air-suspended particles (fluidized bed). Hot air blown through the base of the chamber, which initially holds core particles, keeps the particles suspended and aids the drying of the coating solution on the surface of the particles. The coating material in liquid form sprayed through a nozzle dispenses over the suspended particles. The partially coated particle gains weight and moves to the bottom of the bed. Once the particle is dried, it moves upwards. The coating and drying cycles are repeated until the desired amount of coating material (which corresponds to the desired thickness) is applied. Maintaining this recirculation requires that

the blown hot air rate must be between the particle minimal fluidization velocity (rising) and the pneumatic transport velocity. The size, shape, density of the particle, the viscosity and density of the blown air, and the porosity of the particle bed dictate the appropriate airflow rate (Guignon, Duquenoy and Dumoulin, 2002). The operating temperature, the flow rate of spray solution, and hot air are vital parameters that dictate the quality of encapsulation and whether particles agglomerate.

The nozzle atomizes the coating solution. Different nozzles are used based on the required size of the droplet and viscosity of the spray solution. Ultrasound nozzle produces 1-10µm droplet size; the pneumatic nozzle produces 10-100 µm droplet size; a hydraulic nozzle produces 80-350 µm droplet size. The hydraulic nozzle can handle spray solutions with high viscosity. They have different pressure operation ranges and spray solution flow rates capacities. Adjusting these parameters dictates not only the size of the droplets but also the size of the zone of atomization. An appropriate size of the droplet is essential in preventing the problems associated with ineffective drying. As a rule of thumb, the particle size to droplet size ratio must be at least 10 (Guignon, Duquenoy and Dumoulin, 2002).

The coating solution droplets touch the surface of the core particles and glue to it. The contact occurs in the atomization zone. Droplet- particle contact is followed by rapid drying. The rate of drying depends on the size of the droplet, airflow rate, particle size distribution, operating temperature, and humidity. The tiny droplets are dried before they come in contact with the core particle and are lost unless they touch a wet surface.

Aside from the coating of particles, a fluidized bed operation can also be used for the simultaneous agglomeration and coating of small particles (Oshinowo *et al.*, 2012; Diosady, Alberti and Venkatesh Mannar, 2002). A variety of materials, both hydrophilic and hydrophobic, can be used for coating. They are either be in a molten state or dissolved in evaporable solvents. These options increase the versatility of a fluidized bed. Also, many configurations of fluidized bed now exist-tangential-spray, bottom-spray, and top-spray (Figure 10). They differ in the position of introducing the coating solution into the system (Desai and Jin Park, 2005). The method can only encapsulate solid particles, and the particle size of the final products cannot be less than 10 µm (Gouin, 2004).

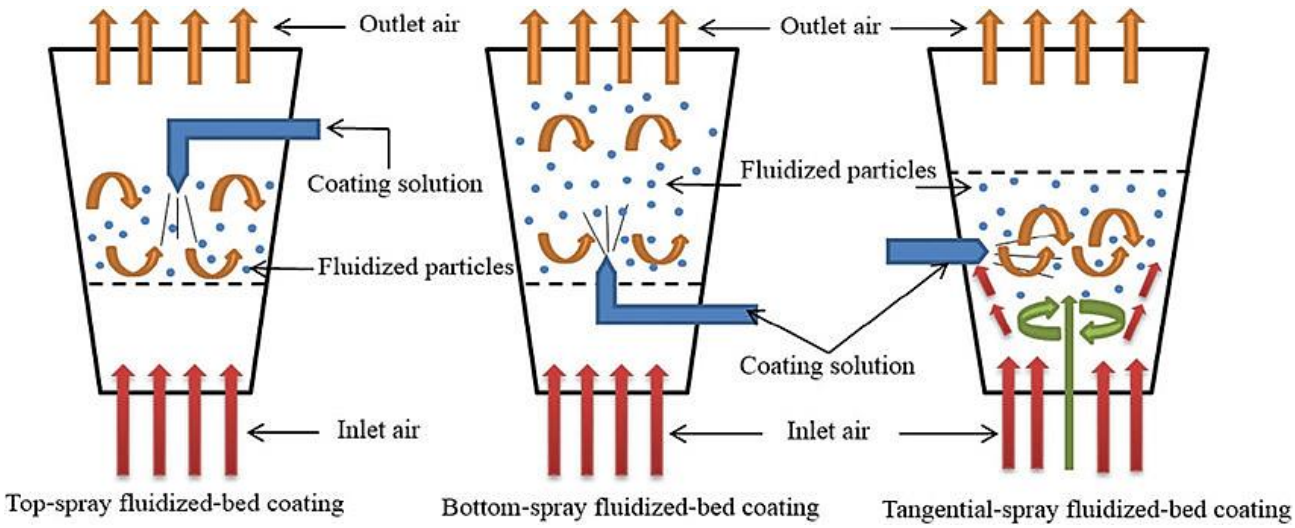


Figure 10:Schematics of Fluidized-Bed Coating Operations (Bakry et al., 2016)

I. Liposome Entrapment

This encapsulation technique depends on the amphiphilic nature of phospholipids-structurally having a hydrophilic head and a hydrophobic tail. Due to this nature, phospholipid spontaneously forms liposomes when dispersed in water (Risch, 1995a). A liposome is a phospholipid bilayer that encloses a lipid or aqueous compartments. So, this technique can encapsulate either hydrophilic or hydrophobic substances, but not a mixture of both. A hydrophilic material is entrapped in the aqueous phase in the core, while a hydrophobic material is incorporated into the lipid phase. There are three types of liposomes: multilamellar, small unilamellar, and large unilamellar vesicles (*Figure 11*). Although the multilamellar vesicles are not uniform in size and have low loading capacity, the core material is not subjected to harsh treatments when they are formed. The small unilamellar vesicles have a more uniform size, but their small size limits their capture volume. The large unilamellar vesicle is usually used because it is more homogenous in size and has a higher encapsulation capacity (Shahidi and Han, 1993).

The multilamellar vesicle is formed by hydrating an evaporated solution of phospholipids in chloroform. High-intensity ultrasonication or pumping multilamellar vesicles through a French pressure cell device produces small unilamellar vesicles. Also, it can be produced by injecting a solution of phospholipids in ethanol into an aqueous solution. The large unilamellar is formed by infusion, reverse-phase evaporation, and detergent dilution (Shahidi and Han, 1993). The obtained liposomes are dried by freeze-drying. Generally, the permeability, stability, surface activity, and

affinity are dependent on the size and lipid composition (Gibbs *et al.*, 1999). This technique encapsulates flavours, vaccines, hormones, enzymes, and vitamins. Commonly phospholipids used are phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine (Bozzuto and Molinari, 2015).

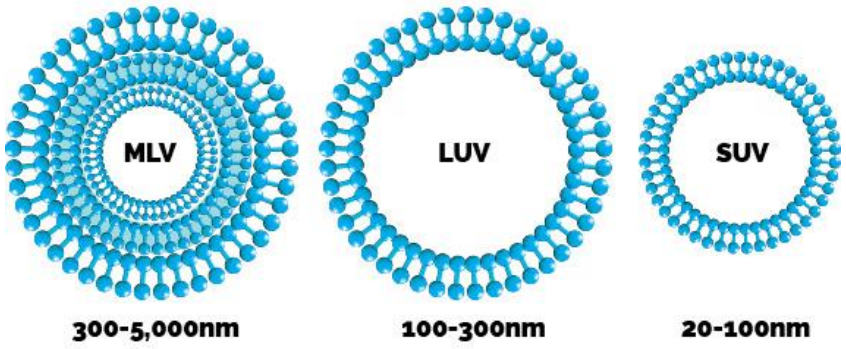


Figure 11: Schematic representation of the different types of liposomes (Pandey, Rani and Agarwal, 2016)

J. Inclusion Complexation

This technique of encapsulation takes place at the molecular level, unlike every other method of encapsulation. The cyclic structure of β-cyclodextrin is the basis of this technique. β-cyclodextrin is a glucose oligomer with several glucopyranose units joined with α-1→4 bonds to form a cyclic structure. β-cyclodextrin has a hydrophobic cavity and a relatively hydrophilic surface. In aqueous solution, the hydrophobic centre of β-cyclodextrin is filled with water; this is not energetically favourable. A molecule that is less polar than water and fits the dimension of the cavity of the β-cyclodextrin readily are energetically favoured to replace the water molecule in the hydrophobic cavity. Hence, the molecule is entrapped in the cavity of the cyclodextrin. (Shahidi and Han, 1993). In general, the loading capacity of this method is dependent on the molecular properties of the core material. It is an excellent coating method for highly volatile material. There are several methods for forming inclusion complexes (*Figure 12*):

Kneading

This method uses a slurry of cyclodextrin. A liquid or dissolved solid of the core material is added to the slurry and kneaded in a mortar. The paste is dried and washed to remove the core material that is not complexed with the cyclodextrin. This method is used for poorly water-soluble core

material like ibuprofen, omega-3-fatty acids, and essential oils (Pereva *et al.*, 2016; Cheirsilp and Rakmai, 2016).

Co-precipitation

In this method, the core material is dissolved in an organic solvent, and mixed with an aqueous solution of cyclodextrin. The mixture is cooled to induce crystallization. The crystals are washed and dried. This method is used to encapsulate water-insoluble substances like oxaprosin and some essential oils (Mennini *et al.*, 2016).

Freezing Drying

This is used for thermolabile and water-soluble core material. A mixture of cyclodextrin and core material in water is stirred, then freeze-dried. The powder obtained is washed and vacuum dried. This method produces good yield and can be scaled up. A modified cyclodextrin, hydroxypropyl-β-cyclodextrin, is usually used (Michalska *et al.*, 2017). Chloramphenicol and essential oil from cinnamon, clove, basil, tarragon, black pepper, thymol, and thyme are encapsulated with this method (Aiassa *et al.*, 2016; Cheirsilp and Rakmai, 2016).

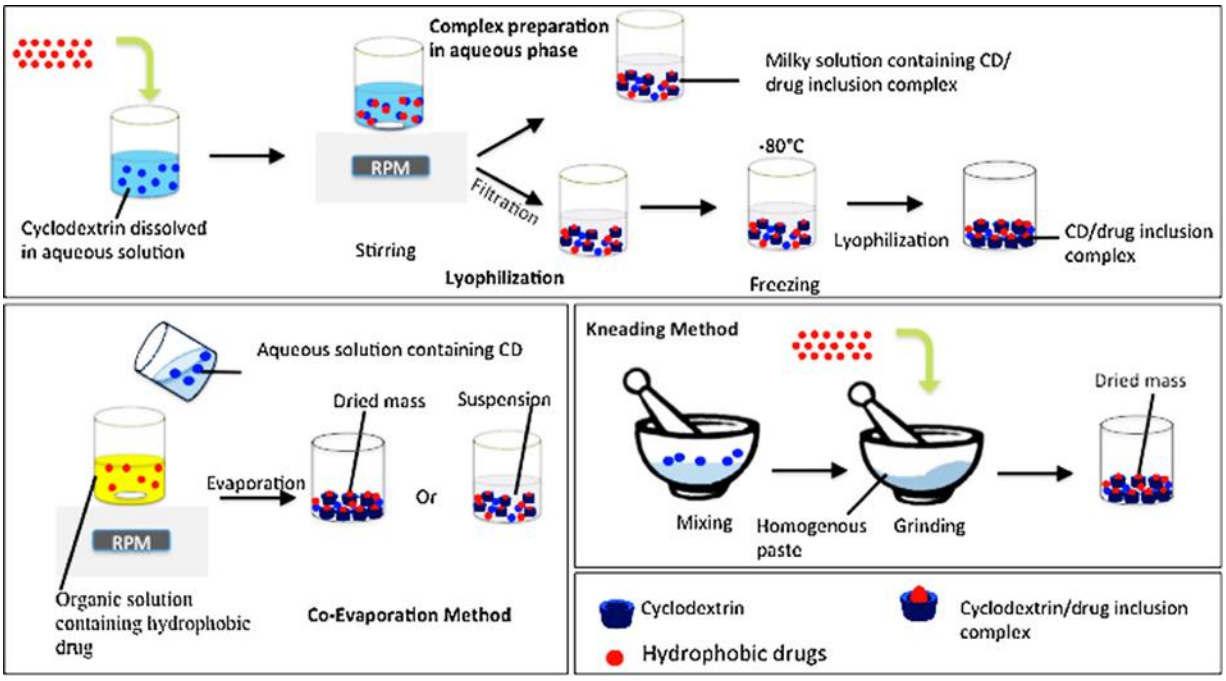


Figure 12:Methods for making inclusion complexes (Gharib et al., 2015)

K. Extrusion

'Extrusion can be simply defined as the process of forming a new material (the extrudate) by forcing material through an orifice or die under controlled conditions' (*Mollan*, 2003). This process usually results in the change of physical properties of the initial material. It combines several operations, such as mixing, cooking, kneading, shearing, shaping, and forming (Bordoloi and Ganguly, 2014). It uses high-temperature short-time equipment for cooking and texturizing cereal-based food.

However, the extrusion discussed in this section is not in this context; it is a method for encapsulation at a relatively low temperature (Desai and Jin Park, 2005). It uses a temperature, less than 115 ^{o}C, and a pressure less than 100 psi. The method uses carbohydrates or a mixture of protein and carbohydrates to form a hard, dense, glassy pellet that protects active agents embedded in its matrix from oxidation and evaporation.

In the extrusion process described by van Lengerich (2010), a mixture of carbohydrates and protein was used to encapsulate omega-3 fatty acids. Glycerol and antioxidant were used as a plasticizer, and to prevent rancidity, respectively. An emulsion of omega-3 fatty acid and water was added to the matrix material in an extruder. Antioxidant solubilized in glycerol was also added. All the materials were thoroughly mixed to form a homogenous dough (melt) with even dispersal of the oil droplets with the dough. Evaporation within the extruder setup aids cooling, which helps to keep low operating temperatures. The added liquids aid lubrication that promotes low shear, pressure, and temperature operation. The plasticized dough was cooled before it was extruded through the die. In the process described, a separate cutter apparatus was added to cut the extrudate. In some other described extrusion microencapsulation processes, the dough is extruded into a dehydrating liquid such as isopropyl alcohol, which aids solidification and impact breaking of the extrudates (Desai and Jin Park, 2005).

This method is used for encapsulating enzymes, flavour, and essential oil. It produces large particles, typically larger than 500µm. The method is limited by its loading capacity, which is usually 10-15%. Also, the free active agent at the surface of the extrudate is lost by evaporation (Harrington and Schaefer, 2014).

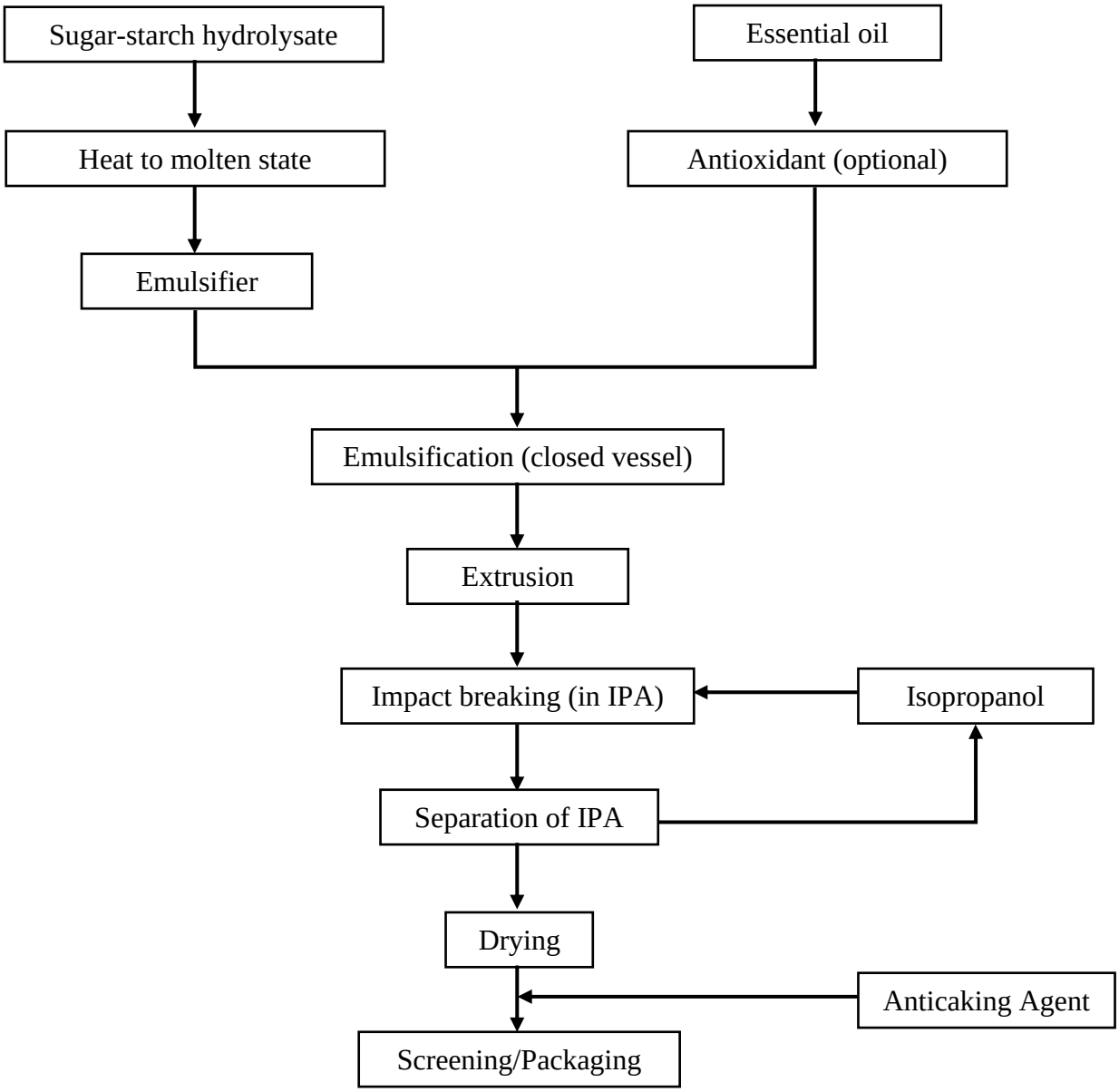


Figure 13:Flow diagram of microencapsulation of *essential oil* by extrusion method described by Swisher (1957)

L. Pan Coating

Pan coating is one of the oldest encapsulation methods. There are two methods for achieving pan coating (Jyothi *et al.*, 2012). The first one involves the mixing of the core material with a coating material that has a relatively low melting point (for example, fat). The mixture is tumbled in a pan to which heat is applied. The melting coating material covers the surface of the core material; this is cooled when the core material is completely encapsulated. In the second method, the coating

material dissolved or suspended in a volatile solvent is sprayed through a nozzle on the surface of core materials as they are tumbling in the pan. The suspension or solution of the coating material is applied until the desired level of encapsulation is achieved. The residual solvent is removed by passing warm air over the encapsulated particles or by drying in an oven (Bansode *et al.*, 2010). Also, a melted coating material (fats) can be sprayed on the tumbling core material. Unlike pan coater, the use of a volatile solvent is not necessary as the temperature of the coating chamber of a drum coater is adjustable to suit the evaporation of water. Pan coating is a relatively easy coating technique. However, there is a limit to the size of the particles that can be encapsulated, usually larger than 600 µm (Bansode *et al.*, 2010). This method was used for applying carbohydrate, and fat-based coats on titanium dioxide masked iron extrudate (Modupe, Krishnaswamy and Diosady, 2019).

Most of these encapsulation techniques are not compatible with our process that aims to have a microcapsule that has similar size and colour as salt and has a sufficient coat that prevents moisture penetration. The ideal micronutrient microcapsule must be white, 300-700µm diameter having a fat coating that does not cause the microcapsule to float. The desired particle size, which will prevent the segregation of micronutrients from salt, limits the use of some microencapsulation techniques. Coacervation, spray drying, molecular inclusion, cocrystallization, and spray chilling produce microcapsules that are smaller than the desired size. Some of the techniques (like extrusion, spray drying, spray cooling, cocrystallization) cannot mask the reddish-brown ferrous fumarate, turning it to white, because they can only form matrix microcapsules. For some techniques (coacervation, liposome entrapment, molecular inclusion), they cannot accommodate the use of titanium dioxide, commonly used as the whitening agent. The final products of molecular inclusion, liposome entrapment, centrifugal extrusion, and coacervation require an additional drying step to dry the liquid or the slurry state of the starting material. The final products from cocrystallization, fluidized bed, and freeze-drying are usually irregular in shape. The porosity of final products limits the use of cocrystallization, spray drying, and fluidized bed. With centrifugal suspension separation, some particles are not coated and are difficult to separate from the coated particles (Pegg and Shahidi, 2007).

There is no single microencapsulation method that can agglomerate ferrous fumarate, colour mask it with a whitening agent, and coat the agglomerate with a wall material that resists moisture.

Several attempts to use fluidized bed and spray drying in a one-step approach to achieve an ideal ferrous fumarate microcapsule were not successful because of cost, irregular shape, high porosity and insufficient colour masking (Romita, Cheng and Diosady Levente, 2011; Oshinowo *et al.*, 2016; Oshinowo *et al.*, 2012). For the fortification of salt, the adopted technology is cold extrusion-based microencapsulation that has three distinct steps- agglomeration, colour masking, and coating. With this technology, each of these steps can be optimized independently for the formation of an ideal ferrous fumarate microcapsule. The size of the extrudate, the colour of the microcapsule, and the coating step are independently adjustable. This approach combines extrusion for agglomeration and fluidized bed and pan coating for encapsulation. The technology is relatively easy to scale-up.

Table 2:Summary of the Processes Involved in the Microencapsulation Techniques (Desai and Jin Park, 2005)

Microencapsulation technique	Major steps in encapsulation
Centrifugal extrusion	Prepare the solution of core material Prepare the solution of coating material Co-extrude of the solutions through nozzles
Centrifugal suspension separation	Mix the core in a solution of the coating material Obtain encapsulated tiny particles by pouring the mixture over a rotating disc Dry the encapsulated particles
Coacervation	Form three immiscible chemical phases Deposit the coating around the core material Solidify the coating
Cocrystallization	Prepare a supersaturated sucrose solution Add the core into the supersaturated solution Reduce the heat once crystallization is achieved
Extrusion	Mix matrix material, plasticizer and core material Extrude the molten mixture through a die Cool or pass the extrudate through dehydrating liquid

Fluidized-bed coating	Fluidize the core particles. Spray on coat
Inclusion complexation	Prepared complexes by mixing or grinding or spray-drying
Liposome entrapment	Microfluidization Ultrasonication Reverse-phase evaporation
Lyophilization	Prepare a mixture of core material in the coating solution Freeze-dry the mixture
Spray-cooling/chilling	Prepare the dispersion (a mixture of coating and core material) Homogenize the dispersion Feed the dispersion into a chamber of cooled or chilled air through an atomizer
Spray-drying	Prepare the dispersion (a mixture of coating and core material) Homogenize the dispersion Feed the dispersion into a hot chamber through an atomizer

3.3.4 Importance of Cold Extrusion-Based Microencapsulation in Fortification of salt

A. Size Matching

Segregation is always a problem when a composite is made up of particles of different sizes and densities. The size and density particles of salt and the micronutrients are seldom the same. Several studies reported the segregation of micronutrient(s) from salt due to differences in sizes and densities (Diosady *et al.*, 2002; Andersson *et al.*, 2008; Li, Diosady and Wesley, 2009). For iodate, which is a white and soluble substance, adhesion of the particle to the surface of the salt particles solves the problem of segregation. The adhesion is achieved by dissolving potassium iodate in water and spraying it onto the salt. However, some fortificants cannot be added with this approach because they are coloured, insoluble, or very reactive. For example, ferrous fumarate is a reddish-brown compound, insoluble, and destabilizes iodine in double fortified salt. It cannot be added by spraying its solution unto salt. Hence, agglomerated ferrous fumarate, which matches the size of the salt particle, was used in double fortification of salt. The agglomeration is achieved by extrusion. Yusufali, in his thesis, hypothesized that agglomerating iron to match the size of salt

particles would minimize the segregation of iron from salt (Yusufali, 2001). Li et al. (2009) further hypothesized that applying a glossy polymer coat on agglomerated iron would improve the particle surface properties and density of the iron agglomerate, which would minimize the segregation of iron in fortified salt.

B. Colour Matching

Some of the micronutrients are coloured; hence, they may impact the organoleptic characteristics of salt and reduce the acceptability of the fortified salt. Colour coating can effectively solve the problem of the reddish-brown ferrous fumarate. One of the downstream processes of cold extrusion-based microencapsulation is colour coating, a unit operation after the cutting of the extrudate to match the size of salt. The cut extrudate is dusted with titanium dioxide. The choice of titanium dioxide is due to its inert nature that prevents its interaction with other components of the premix, while its white colour matches the colour of salt.

The titanium dioxide is held on the surface of the extrudate by a weak adhesion force. Van der Waals force, the formation of solid or liquid bridges, electrostatic force, and mechanical interlocking due mainly to the surface roughness, is thought to have been responsible for the adhesion (Yadava, 2008). These forces are too weak to withstand other processes required for making the fortified salt and the agitation during handling and distribution of the salt. Hence, the colour coated extrudate is further coated with a carbohydrate-based coat and or soy stearin. The percentage composition of titanium dioxide used is 20-30% depending on the particle size (Yadava *et al.,* 2012; Li *et al.,* 2011).

C. Formation of a Physical Barrier

The addition of two or more micronutrients to salt poses the challenge of interaction among the micronutrients. The interaction between iron and iodine in Double Fortified Salts was reported in several studies (Diosady *et al.*, 2002; Diosady, Alberti and Venkatesh Mannar, 2002). The coating ensures that the reactive or incompatible micronutrients are physically separated. More so, it prevents moisture penetration into the coated extrudate; moisture aids interaction among reactive and incompatible components of the salt (McGee, Sangakkara and Diosady, 2017). Finally, it ensures that the colour masked agent is held tightly on the surface. The coating disintegrates during

cooking. Extrusion-based microencapsulation process developed for potassium iodate and ferrous fumarate was part of the unit operations of the double fortification of salt (Diosady, Alberti and Venkatesh Mannar, 2002; Li *et al.*, 2011; Li, Diosady and Wesley, 2010). The extrudate was encapsulated with fluidized-bed using a carbohydrate-based polymer as the coating material. The efficacy of the process to prevent interaction between iron and iodine and yet produce a premix with a high iron bioavailability was reported (Yadava *et al.*, 2012; Li, Diosady and Wesley, 2010).

3.3.5 Cold Extrusion Based Microencapsulation

The popularity of extrusion has grown in the food and other industries in recent decades because of its versatility, low cost of operation, high productivity, and being environmentally friendly. In the food industry, extrusion is used for mixing or degassing ingredients, homogenization, grinding, shearing, starch cooking (gelatinization), protein denaturation and texturization, texture alteration, enzyme inactivation, pasteurization and sterilization of food to prevent spoilage and pathogenic microorganisms, thermal cooking, shaping products, expansion, puffing, agglomerating ingredients, dehydration, and unitizing.

In our laboratory, cold extrusion-based microencapsulation is used for agglomerating ferrous fumarate with ~50μm diameter to larger particles with ~500μm diameter (Li, Diosady and Wesley, 2009). The operating temperature is less than 100^{o}C, hence, the term cold extrusion. Unlike the cooking extrusion that relies on additional heat and shear generated heat for gelatinization, the cold extrusion relies on the shear generated heat for gelatinization. Extrusion combines mixing, cooking, kneading, shearing, shaping, and forming to force a material through a die opening of the desired cross-section. The extrusion process can be summarized thus:

(1) feeding of the extruder barrel,

(2) mixing and sometimes kneading,

(3) pumping,

(4) extrusion through the die, cutting and

(5) air drying

Specifically, extrusion of ferrous fumarate requires the addition of a binder, water, and lubricant to ferrous fumarate to ease the process. Li et al. (2011) reported the use of wheat flour, rice flour, durum wheat flour, cornmeal, potato starch, and dextrin as binders. The study found that potato

starch, dextrin, and cornmeal failed to bind ferrous fumarate irrespective of the amounts of water and oil added in the dough. Ultimately, durum wheat flour was used as the binder because it allowed for the extrusion of the dough with the highest proportion of ferrous fumarate (Li *et al.*, 2011).

With a KitchenAid mixer, the ferrous fumarate and the binder were mixed with a previously melted shortening, a partially hydrogenated soybean oil. The shortening was used as the lubricant to reduce friction, and the shear generated heat. Water (used a plasticizer) was added to the mix. Before extrusion, the mix was kept in an airtight container for preconditioning for 2-3 hours. Some of the downstream processes after extrusion include cutting, colour masking, fluidized bed and/or pan coating (Li *et al.*, 2011; Yadava *et al.*, 2012).

3.3.6 Downstream Processes After Extrusion

A. Cutting and Size Matching

One essential goal of cold extrusion-based microencapsulation is to match the size of the ferrous fumarate premix particles with that of salt. However, the product of extrusion is noodle-like ferrous fumarate cylindrical strands. These are then cut into small sizes to match the size of salt particles. Ideally, the extruder has a cutting unit attached to it, but in the laboratory, the control on the rotor is limited. Also, the flow rate of the extrudate from the die is somewhat challenging to control. Hence, the extrudate noodles from the die are collected and allowed to dry for a few hours. The air-dried extrudate is then cut using a knife or an electric blender, and then size screened to 300-700 μm.

B. Colour Masking

Colour and appearance are ranked first among the sensory variables that affect food acceptability (McFetridge, 1997). Colour-masking is used to change the appearance of foods to improve their acceptability. In salt fortification, it provides a means for masking the reddish-brown colour of the iron extrudate so that it looks white like salt. Hence, improving the colour of the premix and the appearance of the fortified salt. Colour-masking is either achieved by dusting the ferrous fumarate extrudate with a whitening agent before coating or by simultaneously agglomerating ferrous fumarate and coating with a suspension of a whitening agent in soy stearin. Some of the commonly used substances in the food industry include titanium dioxide, talc, and zinc oxide (Marmion, 1991). Its inert nature and bright white colour made titanium dioxide the preferred whitening agent (Li, Diosady and Wesley, 2010).

C. Fluidized Bed Coating

This technique is an effective way to apply a uniform layer of shell materials to solid particles. It has the advantage of being a faster process than the pan coating (A.D. Salman, 2007). Top sprayer is typically used because it is cost-efficient (Dewettinck and Huyghebaert, 1999). Diosady et al. (2002) made the first attempt to use fluidized bed technology in salt fortification. The study reported simultaneous agglomeration and coating of potassium iodate and potassium iodide with fluidized bed technology. The process was scaled up to large industrial scale (Oshinowo et al. (2012). The process used a suspension of titanium dioxide in molten soy stearin for coating. Subsequently, Li et al. (2011) and Yadava et al. (2012) developed a process for agglomerating ferrous fumarate by cold extrusion and coating the extrudate with a fluidized bed. Before coating, the extrudate was dusted with titanium dioxide. A carbohydrate-based material (HPMC) was preferred for coating the extrudate (Yadava *et al.*, 2012). The process developed by Li et al. (2011) and Yadava et al. (2012) improved on the density and surface properties of the iron premix previously produced by Oshinowo et al. (2012).

D. Pan coating

In laboratory ferrous fumarate premix production, pan coating is the final coating step if a fat-based coat is to be applied. The fluidized bed coated extrudate was placed in an inclined rotating pan. A motor rotates the stainless-steel pan inclined at 45°, and the rotation speed was adjusted to 60-70 rpm so that the coated extrudate loaded in the pan could freely fall from the top rim of the pan. As the particles rotate inside the pan, a coating solution (5% soy stearin) was sprayed onto the particles. The soy stearin (hydrophobic material) was applied as a solution in dichloromethane as molten soy stearin could not be sprayed in the laboratory (Li, Diosady and Wesley, 2009).

3.3.7 Challenges in Multiple Micronutrient Food Fortification

A. Sensory Problems

There is a tendency for micronutrients added at the intended fortification level to change the traditional taste, colour, texture, odour of the fortified food. For example, coloured micronutrients may impact the acceptability of the food. Minerals may aid the oxidation of fat in the food, which may result in undesirable taste and odour. Some micronutrients have unpleasant sensory properties. For example, sulphates have an awful taste. This will impact the food to which they are added. Generally, sensory properties drive customer acceptance of food. Hence, a change in the

traditional sensory properties of food caused by the addition of micronutrients may affect the acceptance of fortified food.

B. Chemical Interaction

Minerals may be reactive chemicals; most vitamins have reactive functional groups; foods, the vehicle of the micronutrients, are also made up of reactive chemicals. Hence, there is a potential for chemical interaction among these chemical moieties in fortified food. These interactions can lead to a change in sensory properties of the food as experienced with mineral aided oxidation of fat. More importantly, the functional group responsible for the potency of a vitamin may be involved in and lost with the reactions.

C. Incompatibility

Due to physical and chemical properties, and interference in absorption and metabolic functions, some micronutrients may not be compatible in a matrix. The impact of pH on the stability of some micronutrients may affect their compatibility in a liquid matrix. For example, while folic acid is only soluble at high pH, vitamin B_{12} is only stable at a lower pH. Competitive inhibition of micronutrients for absorption may prevent the simultaneous addition of some micronutrients. Iron and zinc are a well-known example: zinc significantly inhibits the absorption of iron in the gastrointestinal tract (Rossander-Hulten *et al.*, 1991). Hence, it can be challenging to deliver both micronutrients through food simultaneously.

D. Cost

The microencapsulation technology that will prevent interaction among micronutrients; that will solve their potential sensory problem; that will deal with their incompatibilities; that will cause a differential release of micronutrients such that the problem of the competitive absorption is solved will impact the cost of producing fortified foods. It is a challenge to make the cost affordable for the target population, which is the rural poor in developing countries in our case.

E. Social Acceptance

The above challenges are tied to the acceptance and the affordability of the fortified food. If any of the challenges are not adequately tackled, the fortified food may not be able to achieve its intended purpose.

3.4 Advances in Salt Fortification Technologies

3.4.1 Technology developed for Double Fortification of Salt at University of Toronto

Iodization of salt is one of the greatest inventions in terms of its impact on humans. The research on salt fortification in Prof. Diosady's Food Engineering Lab at the Department of Chemical Engineering and Applied Chemistry, University of Toronto, started with the assessment of iodine stability in iodized salt. Salts obtained from different countries were iodized with potassium iodate (Diosady and Mannar, 2000; Diosady *et al.*, 1997; Diosady *et al.*, 1998). The effects of impurities, humidity, and packing materials on the stability of iodine in the salt were investigated. Hygroscopic impurities and high humidity aggravated the loss of iodine in iodized salts. Low-density polyethylene bags provided an effective moisture barrier and significantly reduced the loss of iodine. About 85-90% of iodine was retained in the iodized salt stored with low-density polyethylene bags for six months. These studies highlighted the importance of impurities and moisture content in the stability of iodine. Lastly, the application of iodine as a solution resulted in a uniform distribution of iodine in the salt.

Given the impact of iodized salt on the reduction of the global prevalence of iodine deficiency, salt became recognized as an attractive vehicle for the combined iodine and iron fortification program. The main challenge is the interaction between iron and iodine, which leads to loss of iodine. Three strategies were used to prevent this possible interaction: the use of stabilizers, encapsulation of iron, and encapsulation of iodine.

Diosady et al. (2002) investigated the impact of stabilizers on iodine stability in Double Fortified Salt (DFS) made by adding iron and iodine to the salt in solid and solution forms, respectively. Potassium iodate and potassium iodide, and ferrous fumarate and ferrous sulphate were sources of iodine and iron, respectively. The effect of sodium hexametaphosphate (SHMP) and calcium carbonate as stabilizers and magnesium chloride as hygroscopic impurities on iodine stability were studied. SHMP is an iron chelator and may prevent iron from interacting with iodine in the salt. The use of stabilizers marginally improved the stability of iodine only in pure salt as most of the iodine was lost when magnesium chloride, a common impurity of salt, was added. Hence, Diosady et al. (2002) suggested the physical separation of iodine and iron as a measure to prevent the loss of iodine in DFS. They showed that ferrous fumarate was better than ferrous sulphate, and potassium iodate was better than potassium iodide in terms of iodine stability.

In the first attempt of physical separation of iron and iodine in DFS, Diosady et al. (2002) simultaneously agglomerated and encapsulated potassium iodide or iodate with dextrin, SHMP, and gelatin by spray drying and in a fluidized bed. Ferrous ammonium citrate, ferrous sulphate, and ferrous fumarate were used as sources of iron. Again, ferrous fumarate was better than the other two compounds in terms of iodine stability and organoleptic changes in the salt. The sticky nature of iodine encapsulated with gelatin prevented its further use. Dextrin was a better coating material than SHMP in terms of iodine stability in the salt. Although microencapsulation of iodine was a promising approach, agglomeration of the ferrous fumarate to match the size of salt particles was a way forward. This choice was due to two essential reasons. The encapsulation of iodine is not the traditional way of applying iodine to salt; hence, encapsulating iodine would mean that the established iodization infrastructure, which is worth millions of USD, will be abandoned. Also, ferrous fumarate, the preferred source of iron, will cause organoleptic changes in the salt if it is not encapsulated.

Hence, ferrous fumarate was agglomerated and encapsulated in a two-step process using fluidized bed technology, as described by Oshinowo et al. (2016). In this process, ferrous fumarate, hydroxypropyl methylcellulose (HPMC), titanium dioxide, and sodium hexametaphosphate (SHMP) were used to form agglomerated iron particles which were then coated with a suspension of TiO_2 in molten soy stearin (Figure 14). Oshinowo et al. (2004), Diosady et al. (2006), and Oshinowo et al. (2007) showed that the technology was promising for preventing the interaction of iron and iodine in salt samples from several sources. Diosady et al. (2006) compared other iron compounds (reduced iron, ferrous sulphate, and FeNaEDTA) with ferrous fumarate as a source of iron using the technology. Also, the study compared separate encapsulation of iodine and iron compared with just the encapsulation of iron. Like previous studies, ferrous fumarate was better than the other sources of iron in terms of iodine stability, and encapsulation of only iron was observed to be sufficient to physically separate iron and iodine.

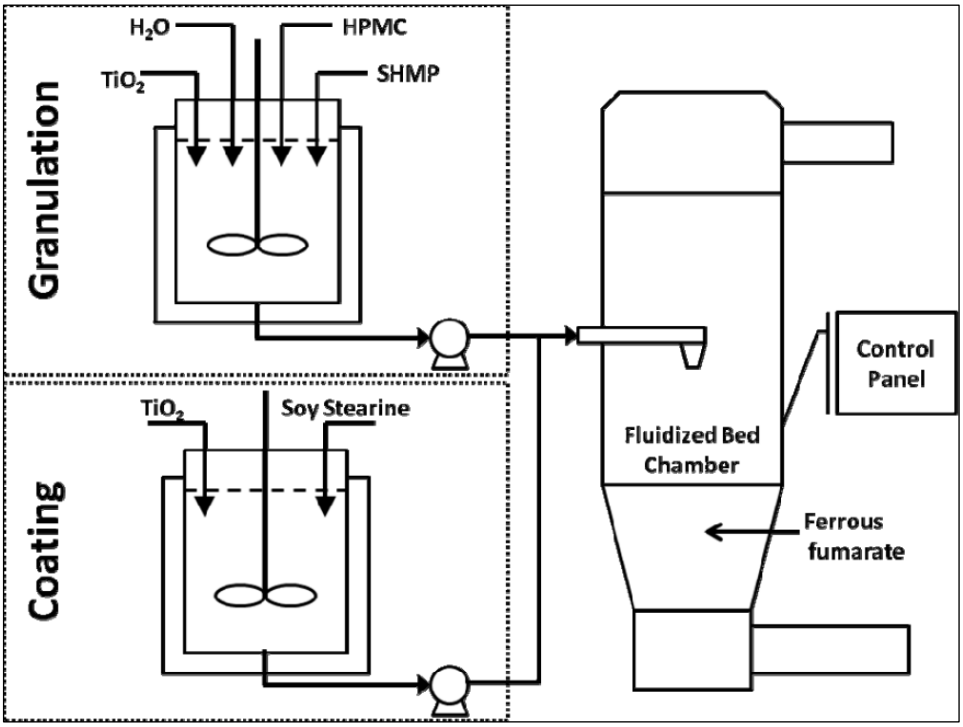


Figure 14: Fluidized bed set-up for agglomeration and coating of ferrous fumarate (Oshinowo *et al.*, 2012)

The challenges of required costly equipment, high porosity, low density, surface defects, and marginally acceptable colour of the granular iron formed with the technology necessitated a change in the technology. Hence, Li et al. (2009) introduced a new technology (extrusion-based microencapsulation, initially suggested by Rizwan Yusufali) for making granular iron for salt fortification (Figure 15). The technology, described by Li et al. (2010), Li et al. (2011), and Yadava et al. (2012), combines extrusion, colour coating, and encapsulation. A mixture of ferrous fumarate, binder, and fat was extruded; the extrudate was cut to the desired size (400-700µm), the cut extrudate particles were colour masked with TiO_2 and coated with HMPC using a fluidized bed. The technology uses a forming extruder for agglomeration, replacing the fluidized bed used in the previous technology. In the new technology, the colour masking step was separated from encapsulation. Also, HPMC was used for coating instead of the stearin used in the previous technology. The operational details of the technology were described by Li et al. (2011) and Yadava et al. (2012). They used a robust experimental design to arrive at the operational details.

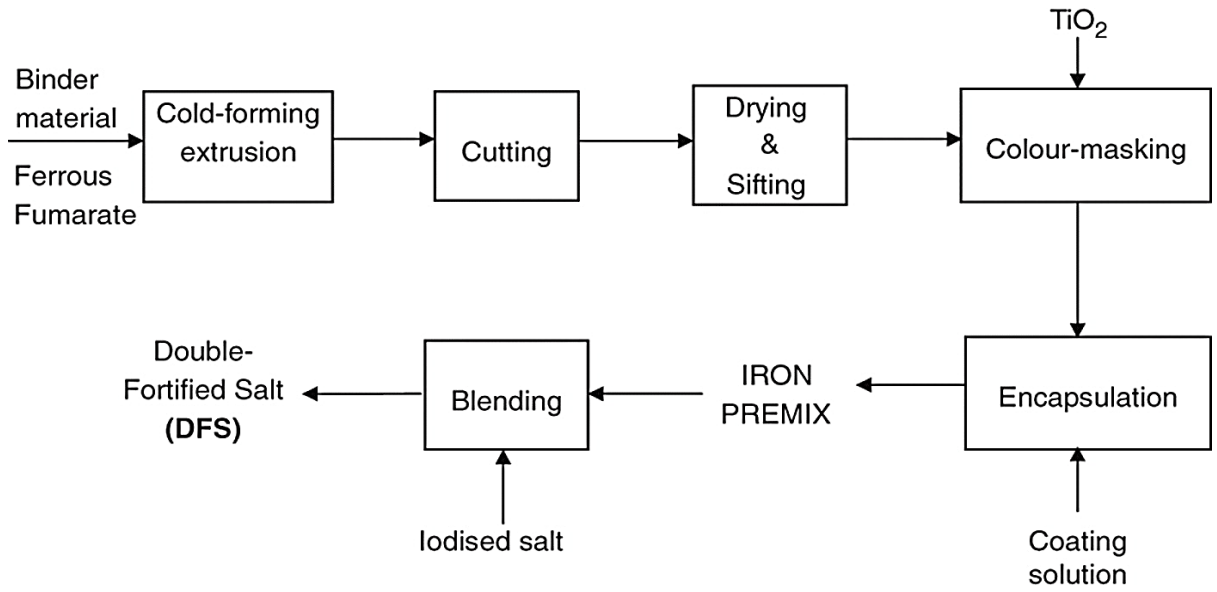


Figure 15: Flowchart for cold extrusion-based microencapsulation of ferrous fumarate (Li *et al.*, 2011)

The durum semolina was the best binder for the extrusion of ferrous fumarate. The ratio of ferrous fumarate to the binder was 4:1. Shortening (2.5% $^{w}/_{w}$ of the mixture of ferrous fumarate and semolina) eased the extrusion of the iron as a lubricant. The extrudate obtained was cut into the desired size (300-700 µm) and colour masked with 25-30% $^{w}/_{w}$ TiO_2. HPMC was the best of all the encapsulating materials used, and 10% $^{w}/_{w}$ coating material was enough to form a good coat. The coating material was applied as a 2.5 % $^{w}/_{v}$ solution in ethanol and water solvent system in a fluidized bed.

This technology was transferred and scaled-up in India (Diosady, Mannar and Krishnaswamy, 2019). At scale-up, a drum coater was used for colour masking and coating. Also, soy stearin was used instead of the HPMC with the idea that its hydrophobic properties will be useful in preventing moisture from penetrating the core of the iron premix. Li et al. (2010) and Modupe et al. (2019) showed the effectiveness of the technology in preventing interaction between iron and iodine on salt.

The extrusion-based technology can only work with a salt of similar particle size as the iron premix, 300-700µm, else, the premix may segregate from salt in the package. Iron premix with a much smaller size could adhere to the surface of salt particles. Hence, Romita et al. (2011) developed a single-step spray drying encapsulation process for iron. The process aimed to reduce

the particle size of iron premix. With a robust experimental design, Romita et al. (2011) found that 9% $^{w}/_{w}$ ferrous fumarate, 6% $^{w}/_{w}$ HPMC (E15), 63% $^{w}/_{w}$ sodium fumarate, and 22% $^{w}/_{w}$ TiO_2 was the optimal spray-drying mixture. The colour masking agent was TiO_2. While this was a viable process, salt processing plants are incapable of handling very fine particulates, and this approach was not pursued on a large scale.

3.4.2 Technology developed for Double Fortification of Salt by other Research Groups

Other research groups - the Human Nutrition Laboratory, Swiss Federal Institute of Technology, Zürich, and the National Institute of Nutrition, India (NIN) are also involved in the development of technology for the iron fortification of salt. Unlike the University of Toronto Group, NIN started with fortifying salt with iron. The bioavailability of iron from several iron compounds was studied; iron from ferrous salts was more bioavailable than iron from ferric salts in humans (Rao, Prasad and Apte, 1972). The bioavailability of ferric pyrophosphate was boosted with sodium hydrogen sulphate (Rao and Vijayasarathy, 1975). However, due to cost, ferrous sulphate was preferred to iron pyrophosphate. The high chance for the oxidation of ferrous sulphate resulted in the use of orthophosphoric acid and ferrous sulphate- orthophosphoric acid serving as a stabilizer (Rao and Vijayasarathy, 1975). Narasinga Rao and Vijayasarathy (1978) developed an alternative formulation, wherein salt was fortified with ferrous sulphate, sodium hydrogen sulphate, and orthophosphoric acid. The sodium hydrogen sulphate served as an absorption promoter while orthophosphoric acid served as a stabilizer. Organoleptic changes observed during scale-up led to further change in the formulation. In the new formulation, ferrous sulphate and sodium hexametaphosphate were added to salt. There was no change in the organoleptic properties of the salt, and iron bioavailability was significantly improved (Ranganathan, 1992). Iodine was added to this formulation to make a DFS (Narasinga Rao, 1994; Nair *et al.*, 1998).

The Swiss Federal Institute of Technology (ETH) Research Group started with DFS as an improvement over the technology developed by NIN. The organoleptic changes caused by ferrous sulphate prompted the encapsulation of ferrous sulphate with hydrogenated fat using a fluidized bed technology (Zimmermann *et al.*, 2003). Zimmermann et al. (2004) changed the source of iron to micronized ferric pyrophosphate. The choice was due to the colour and taste of the ferrous sulphate. The ferric pyrophosphate was micronized to improve its iron bioavailability (Wegmüller,

Zimmermann and Hurrell, 2003; Zimmermann *et al.*, 2004; Fidler *et al.*, 2004). The group compared the DFS prepared with micronized ferric pyrophosphate with the encapsulated ferrous fumarate prepared by the group at the University of Toronto for consumers' acceptance, iron bioavailability, and iodine stability. The formulation developed by the group at the University of Toronto was better in all of these respects (Andersson *et al.*, 2008).

3.4.3 The Challenges with the Scale-up of the Technology for Double Fortification of Salt developed at the University of Toronto

The three research groups have scaled up their technology and evaluated the efficacy of the fortified salt to reduce the prevalence of iodine and iron deficiency. DFS produced by the three research groups significantly reduced the prevalence of iodine and iron deficiency in the studied populations (Nair *et al.*, 1998; Andersson *et al.*, 2008; Haas *et al.*, 2014). As with all the other research groups, the scale-up of the technology developed at the University of Toronto met with some challenges that called for some iterations of the technology. This led to making some changes to the technology to suit the industrial application. This thesis explains some of the problems encountered and the solutions proffered.

Specifically, two issues that called for modification of the technology developed at the University of Toronto Laboratory- the floating of the premix and observed black spots on the iron premix. The floating resulted from the coating with soy stearin. The floating problem resulted in washing away most of the iron premix during cooking. A probe into the reason(s) for the dark spots observed on the premix samples revealed that at the pilot-scale, the brown extrudates were tumbled in excess TiO_2 inside the drum coater while coating with HPMC or soy stearin. The excess amount of TiO_2 not used was sieved out before the final layer of the coat was applied. The TiO_2 sieved out was used in the next batch. The contamination of the TiO_2 by iron particles during this process was responsible for the black spots observed on the surface of the premix. The black spot was due to the oxidation of the iron. In the formulation, 30-35% $^{w}/_{w}$ TiO_2 was used for colour masking.

3.4.4 Technology developed for the Fortification of Salt with Multiple Micronutrients

The success of DFS proved that salt is an ideal vehicle for multiple micronutrients. The ETH research group and our group at the University of Toronto had developed technology for fortifying

salt with other micronutrients in combination with iodine and/or iron. The ETH group developed a novel spray-cooling technique for making a microcapsule that contained iron, iodine, and vitamin A for formulating a fortified salt. A suspension of micronized ferric pyrophosphate and potassium iodate in a mixture of molten palm fat, lecithin, and retinyl palmitate was spray cooled (Wegmüller *et al.*, 2006). Zimmermann et al. (2004) showed that although the salt formulated with this microcapsule significantly improved the iron, iodine, and vitamin A profile of a population in Morocco; the salt had some problems. There was a significant change in the colour of the salt formulated with the microcapsule as the colour difference (ΔE) between iodized salt, and the fortified salt was approximately 9. Also, iodine and vitamin A were not stable. About 40% iodine was lost due to the encapsulation process; an additional 20% was lost due to storage (6 months). For vitamin A, 30% was lost due to the encapsulation process; an additional 15% was lost in salt stored for 6 months. Hence, they recommended an improvement in technology to minimize the change in colour and the loss of iodine and vitamin A.

The UofT group attempted the addition of vitamin A to the DFS by using a different approach: pan coating. Different iron sources were screened for the stability of vitamin A. In terms of vitamin A stability, ferric sodium ethylenediaminetetraacetic acid, was observed to be better than other sources of iron. Also, the granulation and encapsulation of the iron and vitamin A was better than the granulation and encapsulation of iodine, iron and vitamin A (Raileanu and Diosady, 2006). A significant loss of vitamin A was observed irrespective of the source of iron, vitamin A or iodine used (Raileanu and Diosady, 2006; Rutkowski and Diosady, 2007).

Fortification of salt with micronutrients was expanded with the addition of folic acid to salt by UofT. The fortification of salt with folic acid was attempted to reduce the prevalence of iron deficiency independent anaemia and congenital disabilities, primarily neural tube defects. The first attempt was the formulation of salt fortified with iodine and folic acid. Sangakkara(2011) and McGee et al. (2017) developed a stable solution containing iodine and folic acid, which was sprayed on and mixed with salt. A solution of folic acid (1% $^{w}/_{v}$) and iodine (3% $^{w}/_{v}$) sodium carbonate buffer system (adjusted to pH 9) was stable. The technology can be easily adapted into the traditional salt iodization technology. McGee (2012) combined the technology developed by Romita et al. (2011) and McGee et al. (2017) to develop a Triple Fortified Salt (TFS) containing iron, iodine, and folic acid. Iodine and folic acid were added as a solution sprayed onto the salt,

while the iron was added as encapsulated particles made with spray drying technology (McGee, 2012). There was a significant loss of iodine in the Triple Fortified Salt due to high moisture and defective coating of the iron capsules. The study proposed oxidative stress as the cause of the loss of folic acid in the TFS. The addition of folic acid changed the colour of the salt to a yellow hue, dependent on folic acid concentration.

3.5 Micronutrients of Interest

3.5.1 Iodine

The early appreciation of the significance of micronutrients to human health began with iodine, as it became clear that preventable mental retardation and brain damage in childhood is mainly due to iodine deficiency (Trumpff *et al.*, 2013). Iodine is key to the synthesis of thyroid hormone (triiodothyronine (T3) and thyroxine (T4)). This hormone regulates human growth, development, and metabolism. In concert with selenium, it is vital to initiate foetal brain development (Schomburg and Köhrle, 2008).

Therefore, iodine deficiency negatively impacts human growth and development as a direct effect of impaired thyroid hormone synthesis. Fifty percent of about 1.57 billion people affected with iodine deficiency have enlarged thyroids, known as goitre (Kennedy, Nantel and Shetty, 2003). Goitre is the common and visible symptom of iodine deficiency (van der Reijden, Zimmermann and Galetti, 2017). Iodine deficiency is a risk factor for spontaneous abortions, stillbirths, congenital anomalies, infant mortality, cretinism, impaired mental function, increased susceptibility to nuclear radiation, spastic diplegia, delayed physical development, hypo-and hyperthyroidism (Li and Eastman, 2012). Lactating women, infants, pregnant women, and fetuses are the groups most affected by iodine deficiency end (WHO, 2007). Iodized salt now reaches about 90% of the world population, causing a drastic reduction in the global prevalence of iodine deficiency (IGN, 2019).

The recommended dietary intake (RDI) of iodine ranges from 90-290 µg depending on the stage of life (Li and Eastman, 2012). The amount of iodine in natural food is dependent on the source of food. For instance, foods of marine origin are rich in iodine. The amount of iodine in fruits and vegetables is dependent on the iodine content of the soil, which is dependent on the nature of the soil and the type of fertilizer used. Generally, the iodine content of food ranges from 20 µg/kg to

20 mg/kg dry weight. The foods from places close to the seas are usually rich in iodine, unlike foods from places with higher altitudes (Kapil, 2007). For example, an average Swiss food can only contribute 140 µg/day. This contribution from food is short of the average 150 µg/day recommended (Haldimann *et al.*, 2005).

Iodine Fortificants

Generally, iodates or iodides of calcium, sodium, and potassium are used as sources of iodine for salt fortification. The sodium salts are more soluble and have a higher percentage of iodine than the potassium salts. However, they are more reactive and less stable. The prominently used sources of iodine are potassium salts. They have been used as food additives for about eighty years (WHO/FAO, 2006). Iodates are less soluble in water than the iodides (Preedy, Srirajaskanthan and Patel, 2013). *Table 3* summarizes the properties of some of the sources of iodine. Potassium iodate is more expensive than potassium iodide, but its advantages over potassium iodide offset the cost. Comparatively, potassium iodate is more resistant to reduction under hot and humid climatic conditions. It does not require the addition of stabilizers. Hence, potassium iodate is preferred, especially in hot and humid countries.

Table 3: Iodine Fortificants *(WHO/FAO, 2006)*

Fortificants	% Iodine	Solubility in water (g/L)		
		0°C	20°C	30°C
Iodine	100	-	-	0.3
Calcium iodide	86.5	646	676	690
Calcium iodate	65		1.0	4.2
Potassium iodide	76.5	1280	1440	1520
Potassium iodate	59.5	47.3	81.3	117
Sodium iodide	85	1590	1790	1900
Sodium iodate	64	-	25.0	90.0

No matter the source of iodine used, the main problem with the iodization of salt is the loss of iodine. The stability of iodine in potassium iodate and potassium iodide is affected by oxidizing and reducing agents. While potassium iodate is affected by reducing agents, potassium iodide is affected by oxidizing agents. In both cases, iodate and iodide dissociate to iodine, which sublimes and is lost. Equations (1 & 2) represent the chemical equations for the reduction of iodate and loss of iodine.

$2IO_3^- + 12H^+ + 10e^- \rightarrow I_{2(S)} + 6H_2O$ $E_o = 1.194$ V Equation 1

$I_{2(S)} \rightarrow I_{2(g)}$ Equation 2

3.5.2 Iron

Iron is a moderately soft, ductile, malleable, and dense metal. Its physical attributes depend on the level of carbon and silicon it has. It has three allotropies (alpha, delta, and gamma) and four naturally occurring isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe, ^{58}Fe), of which ^{56}Fe is the most abundant and stable nuclear configuration. It has oxidation states -1 to +8, but these are generally classified as +2 or +3. However, Fe^{4+} is of importance in the living system; it is a transient species of iron involved in the physiological redox reaction of Fe^{2+} and Fe^{3+}. The interchange of Fe^{2+} and Fe^{3+} oxidation states is responsible for most of the function of iron in living systems. This property allows it to mediate metabolic redox reactions in cytochrome, haemoglobin, myoglobin, and other enzymes. The interchange of Fe^{2+} and Fe^{3+} oxidation state allows it to play a crucial role in energy generation, cell proliferation, respiration, and DNA synthesis (Camaschella, 2015). The same property allows for the involvement of iron in the generation of free radicals, which are detrimental to cell membranes and DNA (Andrews, 2000).

Since iron is crucial to numerous metabolic processes, its deficiency is manifested as a complex systemic disease with anaemia as its primary manifestation and diagnostic marker for iron deficiency(Modupe and Oladiji, 2016). Aside from anaemia, iron deficiency also has a profound effect on work capacity and motor and mental development (CDC, 2002). A lot of maternal mortality, premature delivery, low birth weight, and increased infant mortality is associated with iron deficiency during pregnancy (Allen, 2000; Scholl, 2011). With over 2 billion people affected globally, iron deficiency remains the most prevalent micronutrient deficiency and one of the leading risk factors for mortality, causing about 800,000 deaths annually (StoltzFus, Mullany and Black, 2004). Developing countries are more affected, where the effect of iron deficiency is aggravated by diseases and infections.

The RDA for iron varies between 8-18mg based on gender, age, and other physiological demands. There are two forms of iron obtainable from food, non-haem and haem iron. The latter is sourced from animal products (meat, poultry, and seafood) and former from non-animal products (black

tea, cacao, cereals, green leafy vegetables, and dried fruit). The haem iron is more bioavailable than the non-haem iron (UNICEF, 2001; Lopez *et al.*, 2016).

Iron Fortificants

The ideal choice of iron fortificant remains a compromise among cost, bioavailability, and reactivity that can cause undesirable organoleptic changes. The reactive nature of iron makes it the most challenging micronutrient to add to food. There are several iron fortificants to choose from (*Table 4*).

Iron fortificants can be categorized as:

- water-soluble;
- poorly water-soluble but soluble in dilute acid; and
- water-insoluble and poorly soluble in dilute acid.

Being water-soluble and relatively cheap makes ferrous sulphate the most frequently used iron fortificant. It remains a reference for judging other iron fortificants. As desirable as it is for iron fortificants to be soluble to aid their bioavailability, most soluble iron fortificants are very reactive. Their reactive nature poses a significant risk of organoleptic changes in food (WHO/FAO, 2006). However, microencapsulation and the use of stabilizers may reduce organoleptic changes (Nair *et al.*, 1998; Rao, 1994). The iron fortificants that fall into the category of poorly water-soluble but soluble in acid categories are bioavailable in humans because the gastric juice is acidic. The bioavailability of iron in this group is reduced in babies because they produce little gastric juice and adults with an illness that affects the amount of gastric juice their stomach produces. This category of iron fortificants has the advantage of causing little or no undesired organoleptic changes. The water-insoluble iron fortificant are rarely used because they are poorly bioavailable. When used, an increased amount is added to account for their low bioavailability. Sometimes, micronization of the fortificant is used to increase bioavailability. Novel iron fortificants such as sodium iron EDTA and ferrous bisglycinate are sometimes used. The conjugated EDTA and glycine increase their bioavailability. *Table 4* provides a summary of the commonly used iron fortificants.

Table 4: Possible Iron Fortificants *(WHO/FAO, 2006)*

Compound	Iron content (%)	Relative bioavailability	Relative cost (per mg iron)
Water-soluble			
Ferrous sulfate. $7H_20$	20	100	1.0
Ferrous sulfate, anhydrous	33	100	1.0
Ferrous gluconate	12 8	9	6.7
Ferrous lactate	19	67	7.5
Ferrous bisglycinate	20	>100	17.6
Ferric ammonium citrate	17	51	4.4
Sodium iron EDTA	13	>100	16.7
Poorly water-soluble, soluble in dilute acid			
Ferrous fumarate	33	100	2.2
Ferrous succinate	33	92	9.7
Ferric saccharate	10	74	8.1
Water-insoluble, poorly soluble in dilute acid			
Ferric orthophosphate	29	25–32	4.0
Ferric pyrophosphate	25	21–74	4.7
Elemental iron – – –			
H-reduced	96	13–148	0.5
Atomized	96	(24)	0.4
CO-reduced	97	(12–32)	<1.0
Electrolytic	97	75	0.8
Carbonyl	99	5–20	2.2
Encapsulated forms			
Ferrous sulphate	16	100	10.8
Ferrous fumarate	16	100	17.4

Ferrous Fumarate

Ferrous fumarate is the iron fortificant of interest in this project. Ferrous fumarate is a ferrous salt of trans-butene dioic acid with the chemical formula $C_4H_2FeO_4$ (*Figure 16*). The percentage composition of iron in its pure form is approximately 33%. It is a reddish-orange powder. It is sparingly soluble in water with a solubility of 0.14 % $^{w}/_{v}$, with even lower solubility in alcohol (less than 0.01 % $^{w}/_{v}$) but soluble in mild acidic solution (Kapor *et al.*, 2012). The IR-spectroscopy analysis showed ferrous fumarate has polyhedron coordination in the shape of an elongated octahedron.

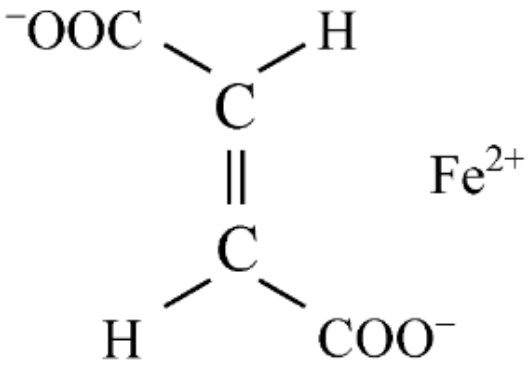


Figure 16: The Chemical Structure of Ferrous Fumarate

Ferrous fumarate has been used in many studies as iron fortificant (Li, Diosady and Jankowski, 2008; Oshinowo *et al.*, 2004). It is usually encapsulated when used as a fortificant to mask its colour and to prevent its potential interaction with the components of food. Aside from being used as a fortificant, ferrous fumarate is used for supplement preparations. Its extensive usage is due to its good adsorption affinity, low toxicity, and bland taste (Geisser and Burckhardt, 2011). The absorption and low toxicity properties are due to the ligand, fumarate ion, which is an intermediate of the citric acid cycle, and its presence in humans.

3.5.3 Folate

Folate is one of the water-soluble vitamins which is present naturally in some foods. It is composed of three moieties - glutamate(s), pteridine ring, and p-aminobenzoyl group. Two of the three moieties are involved in its metabolism. The glutamate moiety controls its transport in and out of the cell, and its affinity for enzymes. The reduction of the pteridine ring is essential for its involvement in one-carbon metabolism. The reduction occurs at 5, 6, 7, and 8 positions (Preedy,

2012). The one-carbon metabolism is essential for phospholipids, nucleic acids, and amino acids metabolism (Cossins, 2014).

The fortification of food with folic acid became mandatory in Canada and the USA because folate deficiency is a risk factor for many chronic diseases and congenital disabilities. It is a major risk factor for neural tube defects (NTDs) in newborns (Williams *et al.*, 2015; De Wals *et al.*, 2007). About 260,000 NTDs affected birth outcomes were estimated in 2015 (Blencowe *et al.*, 2018). Folate deficiency causes high plasma homocysteine concentration, a risk factor for stroke and cardiovascular disease - one of the leading causes of death in the United States (Wald, Law and Morris, 2002). Also, high plasma homocysteine level is associated with a higher risk of impaired cognitive function in adults, and many abnormal pregnancy outcomes. Folic acid deficiency also causes a form of anaemia, known as megaloblastic anaemia. Although there is not enough data on the global prevalence of folic acid deficiency, in most of the countries that have national data, the national prevalence is higher than 20% in low-income countries but less than 5% in high-income countries (Rogers *et al.*, 2018).

The RDI for folate is 0.4 mg for adults. Folates are naturally present in fruits, green vegetables, yeast, and liver. However, folates in food are quickly degraded, and there is limited access to folic acid-rich foods in some countries (Crider, Bailey and Berry, 2011). More so, it is difficult, if not impossible, to find any food that can supply the RDI of folate per serving (Exler, 2012). Synthetic folate, folic acid, is more stable and readily absorbed in the GIT.

Folic Acid

Folic acid, a fully oxidized and synthetic form of folate, is pteroyl monoglutamic acid. It consists of three moieties: a pteridine ring, p-aminobenzoate, and glutamic acid (

Figure 17). The p-aminobenzoate moiety links the pteridines ring and the glutamate moiety. It has a chemical formula, $C_{19}H_{19}N_7O_6$. The presence of just one molecule of glutamate increases its absorption relative to natural folate that has more than one glutamate molecules. Folic acid is practically insoluble in water (0.01 mg/mL); its solubility is improved in acid and alkaline solution; however, it is more stable in alkaline solution.

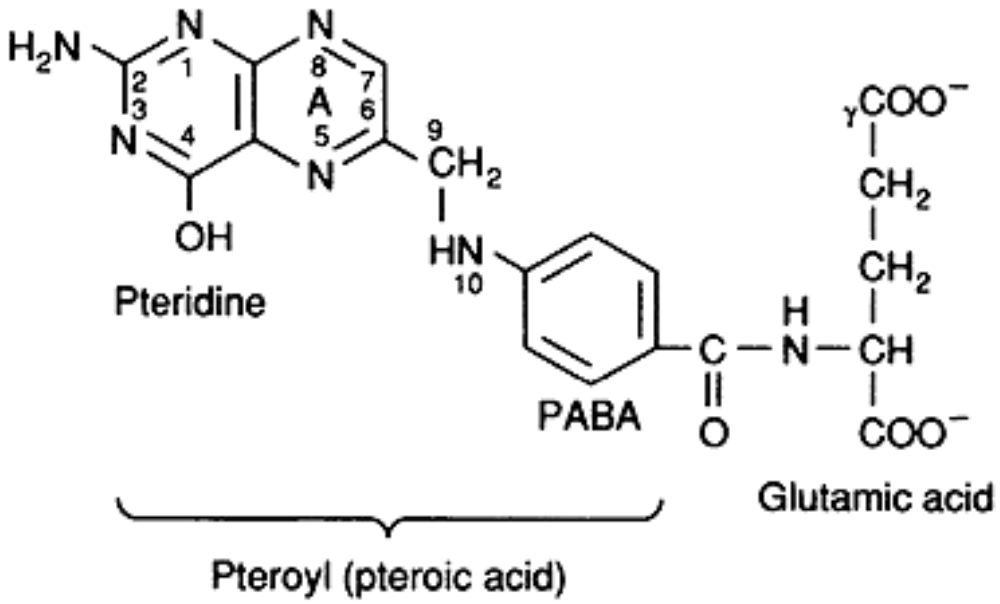


Figure 17: The Chemical Structure of Folic Acid

Aside from pH, the stability of folic acid is affected by temperature, light, oxidizing, and reducing agents. Except for the temperature, other factors follow the same mechanism for the degradation of folic acid. Folic acid is cleaved at the C^9-N^{10} to produce 6-methylpterin (MPT) and p-aminobenzoyl-L-glutamic acid (PABA-Glu) (Gazzali *et al.*, 2016). In the case of thermal degradation, glutamate is first cleaved at 180^{o}C, followed by degradation to 6-methylpterin and para-aminobenzoic acid (PAGA) (Vora, Riga and Alexander, 2002; Vora *et al.*, 2002; Vora *et al.*, 2004). The amine functionalities were completely lost at 195^{o}C. These studies on thermal degradation were conducted within a few hours; a longterm exposure to heat may impact the stability of folic acid (McGee, Sangakkara and Diosady, 2017). The concentration of folic acid also influences the stability of folic acid via the concentration of MPT and PABA-Glu present, both of which are activators for folic acid degradation (Gazzali *et al.*, 2016).

None of the three moieties can singly elicit the vitamin function of folic acid; they work in synergy to function as a vitamin. A study on the folate receptor binding showed that the glutamate moiety, responsible for many of folic acid functions, is exposed outside of the receptor pocket while the other parts are responsible for the binding of folic acid to the receptor (Gazzali *et al.*, 2016). Its structural integrity is thus essential to its function as a vitamin. Hence, the potency of folic acid is

lost with the cleavage of any of its three moieties. Aside from this, the cleavage of the amino group attached to the pteridine ring may also lead to the loss of folic acid potency. Amines are known to react with a reducing sugar (Maillard reaction), the products of which have many impacts such as browning, flavour formation, and antioxidative activity (Khalifah *et al.*, 1996; Schneider *et al.*, 2002). The possible mechanism of formation of N^2-[1-(carboxyethyl)]folic acid from the reaction between folic acid and reducing sugar had been shown (Schneider *et al.*, 2002). Hence, the structural integrity of folic acid must be maintained in fortified salt, so that the intention for adding folic acid to salt can be achieved.

3.5.4 Vitamin B_{12}

Vitamin B_{12} is one of the water-soluble vitamins. It shares its structural tetrapyrrole ring with coenzyme F430 and chlorophyll, but it is distinguished from them by its central cobalt ion. The cobalt-carbon bond gives vitamin B_{12} its ability to act as a coenzyme. Cyanocobalamin is a common form of vitamin B_{12}; there are other forms of vitamin B_{12}. While the R group in cyanocobalamin is cyanide, other forms – methylcobalamin, aquacobalamin, hydroxycobalamin, and adenosylcobalamin- have methyl, water, hydroxyl and 5'-deoxyadenosy, respectively, as their R groups (Preedy, 2012). The methyl and adenosylcobalamin are the biologically active vitamin B_{12}. The R groups in the cobalamins are interchangeable. The importance of vitamin B_{12} in humans is tied to its being a cofactor for two enzymes - methionine synthase and methylmalonyl CoA mutase. These enzymes are involved, directly or indirectly, in the synthesis of metabolites involved in red blood cell formation, neurological function, and DNA synthesis (Stabler, 2012). Vitamin B_{12} interacts with folic acid in one-carbon metabolism (involved in red blood cell formation and DNA synthesis) (Selhub, 2002).

Vitamin B_{12} deficiency may lead to a methyl-folate trap, which leads to having complications of folic acid deficiency (Combs, 2012). The deficiency of B_{12} causes megaloblastic aneamia, demyelination of the skeletal architecture that supports the spinal cord, demyelination of cranial and peripheral nerves, and white matter in the brain. Hence, neurological disorders such as neuropathy, myelopathy, dementia, neuropsychiatric abnormalities, and optic atrophy linked with B_{12} deficiency (Ralapanawa *et al.*, 2015). Also, glossitis, malabsorption, infertility, and thrombosis are associated with B_{12} deficiency (Stabler, 2013). In high-income countries, the leading cause of

B_{12} deficiency is autoimmune diseases that affect its absorption mechanism, while in low-income countries, its deficiency is mainly due to low intake (Green *et al.*, 2017). The prevalence of vitamin B_{12} deficiency is higher in South America, Africa, and Asia than in North America and Europe (Green *et al.*, 2017).

The RDI of vitamin B_{12} is 2.4 µg for an adult (Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference, 1998). It is naturally found in animal-based diets - fish, meat, poultry, eggs, milk, and milk products. Unlike some nutrients whose amounts in breastmilk are relatively constant irrespective of the mother's nutrient status, the amount of vitamin B_{12} in breastmilk is significantly affected by the vitamin B_{12} status of the mother (de Benoist, 2008). Hence, there is a need for nursing mothers to have an adequate level of vitamin B_{12}. Vitamin B_{12} is one of the few nutrients that cannot be obtained from plants. This fact makes strict veganism a risk factor for vitamin B_{12} deficiency (Stabler and Allen, 2004).

Cyanocobalamin

Cyanocobalamin is the synthetic form of vitamin $B_{12,}$ usually used for fortification. Cyanocobalamin is a cyanide derivative of cobalamin (5, 6-dimethylbenzimidazolyl cobamide). Like other cobalamins, cyanocobalamin has corrin ring with a central cobalt atom, coordinated to nitrogen of the four pyrrole groups, the nitrogen of the 5,6-dimethyl benzimidazole, and cyanide. In other cobalamins, the cyanide is substituted with other ligands (Figure 18). It is a tasteless, odourless reddish crystal (Shaw and Bessell, 1960; Smith, 1952). It is stable in the dry state at 100 ^{o}C, begins to decompose at 200 ^{o}C, and melts above 300 ^{o}C (Smith, 1952; Bonnett, 1963). Even in solutions, cyanocobalamin in generally a stable compound (Hartley, Stross and Stuckey, 1950). Unlike most organic compounds, it is not soluble in many nonpolar solvents but soluble in water, alcohols with lower molecular weight, acetic acid, acetone cyanohydrin, phenols, dimethyl sulfoxide, and dimethylformamide (Smith, 1952; Bonnett, 1963).

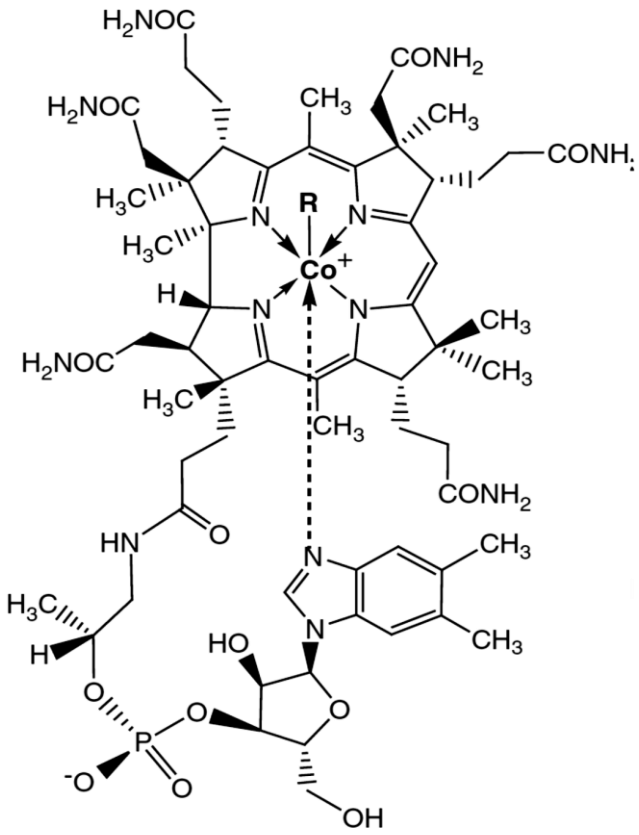


Figure 18: The General Structure of Cobalamin: vitamin B12 (CNCbl, R=CN), Coenzyme B12 (R=5'-deoxy-5'-ado), Methylcobalamin (MeCbl, R=CH3), Aquacobalamin (R=H2O+), Hydroxocobalamin (HOCbl, R=HO) (Kräutler, 2009)

Despite being a stable compound, cyanocobalamin is photolabile and decomposes under the action of reducing agents, heavy metals, and oxidants (Shchavlinskii *et al.*, 1995). It is also susceptible to pH-dependent hydrolysis (Connors, Amidon and Stella, 1986; Armitage *et al.*, 1953) and photolytic degradation. It forms hydroxycobalamin at pH 9.3 - 14 and aquacobalamin at below pH 2. These degradative products are harmless as they can be activated to methyl-, and deoxyadenosyl cobalamins (Shchavlinskii *et al.*, 1995). However, some products of degradation due to acid or alkaline hydrolysis (produced from deamination of the side chains, cyclization at the B ring, and amidation of the carboxylic group) may have their vitamin activity lost, even have antivitamin activities in some cases (Schneider and Stroinski, 1987; Schrauzer, 1972).

3.6 Possible Chemical Interactions

Two of the significant challenges in adding multiple micronutrients to food is the chemical interaction among the micronutrients and between the micronutrient and chemical constituents of the food. In most cases, such interactions lead to loss of potency of the micronutrients, unpleasant sensory properties of food, ultimately, consumer rejection. Hence, it is imperative to understand the potential chemical interactions among the micronutrients of interest and interaction between them and the fortificant vehicle (food). Iodine, iron, folic acid, and vitamin B_{12} are of interest in this thesis, and the vehicle for the fortificants is table salt (sodium chloride).

Iodine is usually added to salt in the form of iodate or iodide of potassium. The two sources of iodine are very stable in salt. However, iodate is less soluble than iodide but has more resistance to oxidation, heat, and humidity. It is more stable than iodide (Diosady and Mannar, 2000). Hence, it is commonly used as an iodine fortificant. In the presence of reducing agents such as ferrous compounds, iodate is reduced to iodine, which sublimes. The reduction of iodate is accelerated by moisture, heat, and impurities in foods. In the case of salt, the presence of magnesium, a hygroscopic impurity in salt, accelerates the rate of degradation of iodate in iodized salt (Diosady *et al.*, 2002). Iron accelerates the loss of iodine in fortified salt. The loss of iodine in salt that contained iron followed the stoichiometry ratio of 5:1 iron to iodate, as shown in Equation 3 (Li, Diosady and Wesley, 2010). There is no interaction between the salt (NaCl) and iodate.

$$10Fe^{2+} + 2IO_3^- + 12H^+ \rightarrow 10Fe^{3+} + 6H_2O + \uparrow I_2 \qquad Equation\ 3$$

Although not as reactive as ferrous sulphate, the ferrous ion in ferrous fumarate still has a relatively high reducing power. The ferrous moiety may react with other components of the salt that are susceptible to reducing agents. An example is iodate, the iodine fortificant, that is reduced to iodine (Li, Diosady and Wesley, 2010). However, ferrous iron seems to enhance the stability of folic acid against thermal degradation (Day and Gregory, 1983; Gazzali *et al.*, 2016; Modupe, Krishnaswamy and Diosady, 2019). Also, ferrous iron can improve the stability of vitamin B_{12} (Zuck and Conine, 1963). These imply that ferrous fumarate should dictate how other micronutrients will be added to salt. For double fortified salt, the reductive tendency of ferrous iron was reduced or prevented by the addition of stabilizers (orthophosphoric acid and sodium hexametaphosphate) or microencapsulation (Rao and Vijayasarathy, 1975; Diosady *et al.*, 2002)

Folic acid, the folate fortificant, is relatively stable. Folic acid is sparingly soluble in water, but it is very soluble in sodium hydroxide and carbonate by forming sodium folate. The stability of folic acid in solution is pH-dependent; it is more stable at higher pH (Liang, Zhao and Hao, 2013). High pH favours the oxidation of ferrous to ferric iron, which limits iron bioavailability(Mitra and Matthews, 1985). Also, vitamin B_{12} is not stable at high pH. The defined degradation pathways for folic acid is through oxidation and thermal degradation. The oxidative pathway, which is aided by photolysis, has 6-formylpterin and p-aminobenzoyl-l-glutamic acid as its products while the thermal pathway has glutamic acid and pteroic acid as its products (Gazzali *et al.*, 2016). The reductive power of ferrous fumarate may enhance the stability of folic acid (Modupe, Krishnaswamy and Diosady, 2019).

Cyanocobalamin, the commonly used vitamin B_{12} fortificant, is very stable. However, in a strong acidic solution the seven side amide groups may be lost; and in strong alkaline solutions, a lactam ring is formed by cyclization of c and d amide groups attached to the B ring. The Co-C bond that is responsible for most of its biological functions is very stable in cyanocobalamin. The Co-C bond is very difficult to break. Although this bond is very strong, cobalt can be reduced in the presence of sodium tetrahydridoborate in a two-step reaction from Co^{3+} to Co^{1+} with Co^{2+} as intermediate. Other reducing agents that aid this reaction include zinc, sodium formate, and chromium (II)acetate. The Co^{1+} is spontaneously oxidized to Co^{3+}. Ascorbic acid is a mild reducing agent and may be used to prevent oxidative degradation of folic acid. However, ascorbic acid has a negative impact on the stability of vitamin B_{12} (Ahmad *et al.*, 2014). Also, B_{12} is not stable in the pH (greater than 7) required for the desired solubility (1% $^{w}/_{v}$) and stability of folic acid.

3.7 Metabolic interdependence of micronutrients of interest

The absorption and metabolic function of micronutrients are sometimes enhanced or inhibited by other micronutrients. The importance of iodine is due to its involvement in thyroid metabolism. Iron deficiency negatively impacts thyroid metabolism-reducing plasma thyroxine and triiodothyronine concentrations, reducing the peripheral conversion of thyroxine to triiodothyronine and increasing the circulation of thyroid-stimulating hormone (Hess, 2010; Hess and Zimmermann, 2004). Zimmermann et al. (2002), Hess (2010), and Eftekhari et al. (2006) showed the negative impact of iron deficiency on iodine prophylaxis in some randomized

population studies. The involvement of iron in thyroid metabolism, among other reasons, justifies the addition of iron to iodized salt. Other micronutrients that significantly impact iodine metabolism include selenium, vitamin A, and zinc (Hess, 2010).

The other micronutrients of interest did not affect the absorption of iron. The absorption of iron is negatively affected by zinc, calcium, phytates, and phenolic compounds (Sandström, 2001). Ascorbic acid, meat, fish, seafood, citric, lactic, malic, and tartaric acids enhance the absorption of iron (Zijp, Korver and Tijburg, 2000; Dasa and Abera, 2018).

There are three types of anaemia linked to micronutrient deficiencies - iron deficiency, megaloblastic, and pernicious anaemia. The last type is due to the inability of the body to absorb vitamin B_{12} while the others are due to inadequate micronutrient status. Megaloblastic anaemia is due to folic acid and vitamin B_{12} deficiencies. Therefore, a practical nutritional approach to solving the problem of anaemia must include not just iron but also folic acid and vitamin B_{12}.

Aside from considering a formulation that combines both folic acid and vitamin B_{12} for reducing the prevalence of anaemia, folic acid, and vitamin B_{12} metabolism intertwines in the one-carbon metabolism of humans, so are the symptoms of folic acid and vitamin B_{12} deficiencies that revolves around the one-carbon metabolism. The deficiency of vitamin B_{12} results in the trap of folate as methyl-folate, a functional folate deficiency (Das and Herbert, 1976). Vitamin B_{12} is a coenzyme for methionine synthase that catalyses the methylation of homocysteine to methionine. The methyl group required for the reaction is supplied by methyl-folate that is derived by the irreversible reduction of methylene-folate to methyl-folate by methylene-THF reductase. In the absence of vitamin B_{12}, folate becomes increasingly trapped as methyl-folate. Hence, there is a lack of the nonmethylated folates needed for serine-glycine interconversion and the synthesis of purines and pyrimidines (Selhub *et al.*, 2009). The interdependency of folate and vitamin B_{12} also explains why deficiencies of either folate or vitamin B_{12} result in hyperhomocysteinemia. Finally, there is historical evidence that suggests that excessive consumption of folate/folic acid may mask the symptoms of vitamin B_{12} deficiency and negatively impact the early diagnosis of vitamin B_{12} deficiency (Selhub *et al.*, 2009; Israels and Wilkinson, 1949; Chodos and Ross, 1951). Hence, Chodos and Ross (1951) and Oakley (1997) suggested a simultaneous addition of folic acid and vitamin B_{12} to food.

The metabolic interdependence of folic acid and vitamin B_{12} and the contribution of both micronutrients to anaemia and other prenatal and postnatal complications gave birth to the idea of developing a process for the addition of iodine, iron, folic acid, and vitamin B_{12} to salt - quadruple fortification of salt. Based on the consumption of 10g of salt per day in India (Vinodkumar and Rajagopalan, 2009), the fortified salt will deliver 56-200% RDA of iron, iodine, folic acid, and vitamin B_{12}. The process will ensure that the micronutrients are very stable, designed to prevent adverse chemical interactions and changes to the sensory properties of salt or foods containing the salt.

4.0 Experimental Materials and Methods

4.1 Experimental Materials

In Table 5 are the material used for this research work

Table 5:List of Chemicals used

Purpose	Material	Supplier	Grade/Description
Salt	Refined non-iodized Canadian salt	Sifto Canada Corp.	Fine grain, clean, dry
	Indian salt	TATA, (India)	Fine grain, clean, dry
Micronutrients	Iron premix	JVS Food Pvt, (India)	Granular
	Folic acid	Bulk Pharmaceuticals Inc., (Toronto, Ontario, Canada)	USP grade
	Potassium iodate	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	ACS reagent grade
	Ferrous fumarate	Dr Paul Lohmann Chemicals (Emmerthal, Germany) & JVS Food Pvt, (India)	Food-grade (mean diameter ~10μm)
	Vitamin B_{12}	JVS Food Pvt, (India)	Food-grade
Buffer Solution	Sodium carbonate, anhydrous	J. T. Baker (Stratford, Prince Edward Island, Canada)	Reagent grade (99.9%)
	Sodium bicarbonate	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS Reagent grade
Extrusion & Encapsulation	Soy stearin (SS)	JVS Food Pvt, (India)	Food grade
	Hydroxypropyl methylcellulose (HPMC)	Dow Chemicals Co., (Midland, MI, United States)	HPMC E15
	Semolina	Walmart (Toronto, Canada) & JVS Food Pvt, (India)	Food-grade
	Vegetable shortening	Walmart (Toronto, Canada)	Food grade
	Zinc oxide	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	Food grade

	Titanium(IV) oxide	ACROS Organics, USA JVS, (India)	Anatase powder (98+%)
	Lecithin	Archer Daniels Midland Co. USA	
	Dichloromethane	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	
	Maltodextrin	Cerestar USA INC	D.E 7.0
Folic acid analysis	Hydrochloric acid	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	3-Aminophenol	Alfa Aesar (MA, USA)	For use in research and development 98+%
	Sodium hydroxide	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Sodium nitrite	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Sulfamic acid	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	ACS reagent grade
	Zinc granules	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Sodium carbonate	J. T. Baker (Stratford, Prince Edward Island, Canada)	Reagent grade (99.9%)
	Formic acid	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	HPLC grade
Iodine analysis	Potassium iodide	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Potassium iodate	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	ACS reagent grade (99.5%)

	Sulfuric acid	EMD Chemicals Inc. (Oakville, Ontario, Canada)	ACS grade
	Sodium thiosulfate solution	VWR International, (Mississauga, Ontario, Canada)	ACS grade, 0.1N
	Starch indicator, 1.0%	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	For laboratory and manufacturing use
Iron and zinc analysis	Nitric acid	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Hydrochloric acid	Caledon Laboratory Ltd, (Georgetown, Ontario, Canada)	ACS reagent grade
	Sodium EDTA	BioShop Canada Inc.	ACS reagent grade
	Multielement standard	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	Certified reference material
Vitamin B_{12} analysis	Methanol	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	HPLC grade
	Acetonitrile	Sigma–Aldrich Chem (Oakville, Ontario, Canada)	HPLC grade

4.2 Formulation of Iron Premix

4.2.1 Dough Preparation

In the case of iron premix, ferrous fumarate was mixed with durum wheat at ratio 4:1 by weight using a Kitchen Aid mixer (Figure 19). The durum wheat served as a binder that helps to agglomerate the ferrous fumarate powder through a cross-linked gluten system. Crisco vegetable shortening (0.25% $^{w}/_{w}$) was melted, added, and thoroughly mixed with the mixture of ferrous fumarate and durum wheat. The shortening serves as a lubricant, which eases the movement of the dough through the extruder. The dough was formed by adding water (120-160 mL/kg dough). The components were thoroughly mixed until the required dough consistency was attained. The dough

was preconditioned for 3 hours by keeping it in an airtight Ziploc bag. The 3 hours of rest allow for the formation of the cross-linked gluten system.

Figure 19:Ultra Power® Series KitchenAidTM Stand Mixer

4.2.2 Agglomeration of Ferrous Fumarate

The agglomeration of ferrous fumarate was carried out with a La Monferrina P12 pasta extruder. It is a cold-forming single screw extruder with two tanks: one for dough preparation and the other for extrusion (Figure 20). The machine operates at low screw speeds and low shear. At the end of the screw flight is a die holder that holds the die in position. The screw is made with high-grade steel. It has a screw length-to-diameter ratio of 15:1 and a constant root diameter. The die and its holder are made of bronze. The orifices of the die are constructed with Teflon. The diameter of the orifice defines the diameter of the iron filament that is obtained from the extruder.

The extruder was prewarmed before the micronutrient dough extrusion. Durum wheat dough was run through the extruder for about 20 minutes to prewarm it. For this step, an 8mm flat die was installed in the extruder. Once the extruder was warm enough, the remaining durum wheat dough was pushed through the extruder with the micronutrient dough. After the durum wheat had been completely pushed out of the extruder, the die was changed, and the micronutrient(s) dough was extruded through a 0.6mm angel hair die. A combination of several kneading and extruding operations was usually essential to warm up the dough for a smooth extrusion through the die. The configuration of the screw (the volume per turn is decreased toward the die) allowed kneading

operation to compress the dough before forced through the die. This increased the density by making the extrudate less porous.

Figure 20:La Monferrina P12 pasta extruder, screw, and angel hair die

4.2.3 Cutting and Particle Size Screening

Before cutting, the extrudate was dried in open-air for several hours until the extrudate was brittle enough for the cutting step. The dried extrudate was hand crushed and cut with a blender or a manual pressing cutter (pasta machine, Figure 21). The cutting and particle size screening operations were running intermittently to minimize loss. A series of Canadian Standard Sieves with sieve sizes of 1000, 700, 600, 500, and 300 μm were used for size screening. Extrudate collected from sieve sizes 1000, and 700 μm were further cut. Extrudates from sieve sizes 500 and 600 μm were collected for further processing; extrudates outside this size range were discarded.

Figure 21:Ferrous Fumarate Noodle (left), Cutting of the Noodle (middle) and Black & Decker Power Crush Countertop Blender

4.2.4 Colour-masking with TiO_2

A known amount of the size screened extrudate particles were placed in a glass beaker, a known amount of titanium dioxide powder (5-30% $^{w}/_{w}$ of extrudate)was applied by contacting the TiO_2 powder with the surface of the extrudate. Specifically, the adhesion is aided by a plastic spatula for approximately 10 minutes until all the TiO_2 powder uniformly attached to the surface of the iron particles. The resulting particles were off-white with a greyish tint. The colour masking step was followed by coating in either a fluidized bed or drum (rotating pan) coater.

4.2.5 Fluidized Bed Coating

Uni-Glatt top spray fluidized bed was used as described by Yadava et al. (2012). The coating solution was prepared by wetting the hydrophilic polymers, HPMC, with 5mL hot water. Ethanol was then added to make a 2.5% ($^{w}/_{v}$) solution of the coating material. This concentration allows for a proper viscosity that is good enough for coating without clogging the spray nozzle. The solution was stirred with a magnetic stirrer to ensure that the coating material was completely dissolved. Before the coating operation, the coating chamber was warmed up for approximately 10 minutes. The colour masked extrudate was added into the coating chamber once the chamber was warmed up to about 60 ^{o}C. The airflow rate was varied by the air-flap to maintain fluidization. The coating solution was delivered through the spraying nozzle by compressed air (at ~2 bars) on the fluidized extrudates. The coating material formed a uniform and thin film around the fluidized particles. The amount of coating material used was 5% $^{w}/_{w}$ of the particles. The operating temperature was maintained at 70-80 ^{o}C to ensure rapid evaporation of the ethanol-water solvent from the fluidized particles, resulting in a thin polymer film on the particle surface.

Figure 22:Uni-Glatt top spray fluidized bed assembly

4.2.6 Pan Coating

Ferrous fumarate extrudate that has been colour masked and fluidized bed coated were further coated with a pan coater (Figure 23a). The pan is inclined at an angle (usually about 45^{o}) and ran at a speed that allows the particles to tumble in the pan. A hairdryer is attached to the base of the pan coater to aid the evaporation of the solvent. The base of the pan coater completely covered the air outlet grille of the hairdryer so that the sprayed coating solution was not in direct contact with the dryer. The solution of coating material was sprayed on the tumbling particles with an Aldrich flask-type sprayer (Figure 23b). The fluidized bed precoated extrudate was coated with a solution of soy stearin in dichloromethane or soy stearin and lecithin in dichloromethane. Pan coating completes the process of making iron premix.

In the pilot plant, the drum coater was preferred to fluidized bed due to cost. In the laboratory, a pan coater was preferred to the fluidized bed because it can easily be used to model the coating in

a drum coater (Figure 24). Hence, a pan coater was subsequently used to apply both HPMC and soy stearin.

Figure 23: Pan coater set up with a hairdryer (a) and Aldrich flask-type sprayer (b)
Caution: the hairdryer was completely covered by the base of the pan coater so that the solvent does not have contact with it.

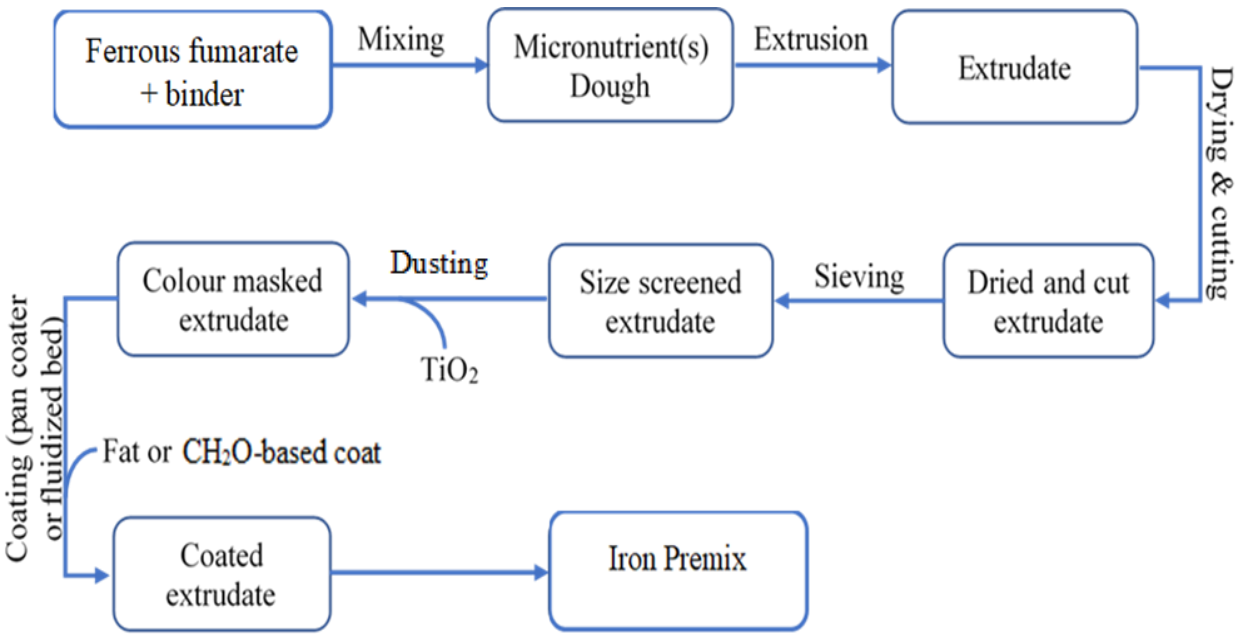


Figure 24:Overall process for premix formulation

4.3 Scale-Up and Optimizing the Iron Premix formulation

4.3.1 Scale-Up of Iron Premix Production in India

The technology described by Li et al. (2011) and Yadava et al. (2012) was scaled up at JVS Food Pvt, Jaipur India. The ratio of ferrous fumarate to durum semolina was kept as recommended (80:20) except in batch 1; the amount of TiO_2 for colour masking was varied from 25-35% $^{w}/_{w}$; the type of coating material and amount used was also varied. In batches 1-3, three different coating materials were used to compare the performance between hydrophilic (HPMC) and hydrophobic (soy stearin) coatings, and Sepifilm which is a commercial product, i.e., ready-to-use gastrosoluble film-coating agent, is primarily designed for pharmaceutical applications with a high cost. In batches 4-6 and 7-9, HPMC was used for coating with a repeated increase from 5% to 7.5% and then 10%, whereas the colour-masking of TiO_2 was set at 35% and 30%, respectively for two groups. The two groups of batches 4-6 and batches 7-9 were designed to evaluate the combined effect amount of TiO_2 and hydrophilic coating material HPMC that can effectively prevent interaction between iron and iodine in the fortified salt. The mixture of ferrous fumarate and binder was extruded. A spheronizer was used to cut and polish the extrudate from cylindrical strands into spheres with a particle size in the range of 300-700µm. The spherical microcapsules were dusted with titanium dioxide and coated inside a drum coater.

4.3.2 Effect of Coating Materials on the Density of Iron Premix

Two sets of experiments were carried out to determine the factor(s) that is/are responsible for the floating of the premix made at scale in India. The premix that floats was coated with 10% soy stearin. In the first experiment, a wide range of coating materials and their mixture were used. In this experiment, extrudate obtained from the JVS Food Pvt, the pilot plant in India, was colour masked with 30%$^{w}/_{w}$ TiO_2. The carbohydrate-based coats (HPMC, Maltodextrin) were applied with a fluidized bed as described by Li et al. (2011) while the soy stearin, lecithin, or a blend of soy stearin and carbohydrate coat were applied with a pan coater.

In the second set of experiments, iron extrudate obtained from JVS Food Pvt India was colour masked with 25% TiO_2 and coated with several coating materials (better materials from the first experiment). The impact of the change in the coating materials on the sinking/floating property of the iron premixes was evaluated. Since the floating problem was not observed in the initial iron premix coated with only 5% $^{w}/_{w}$ soy stearin and given the emulsifying property of lecithin (plant-based), two approaches were attempted in the laboratory for solving the floating problem of iron

premix- coating with a mixture of lecithin and soy stearin, and dual coating with 5% $^{w}/_{w}$ HPMC and 5% $^{w}/_{w}$ soy stearin. Also, 10% $^{w}/_{w}$ HPMC or soy stearin (positive and negative control samples) was applied to the colour masked extrudate. An HMPC solution (2.5% $^{w}/_{v}$) in the solvent system (1:1 absolute ethanol: dichloromethane) was sprayed on the colour masked extrudate in an inclined and rotating pan using a glass sprayer at about 3 mL/min. A solution of soy stearin and or lecithin (5% $^{w}/_{v}$) in dichloromethane were applied to colour masked extrudate using the same method. A hairdryer was attached to the base of the pan coater to aid the quick evaporation of the solvents. The sinking property, particle, and bulk density of the iron premix samples were studied.

4.3.3 Effect of the Amount of TiO_2 and the Method of Applying TiO_2 and Coating Material on the Colour of the Premix

At the pilot plant, the reuse of titanium dioxide recovered from previous batches caused the formation of dark spots of uncoated ferrous fumarate on iron premix. In order to prevent the use of an excessive amount of TiO_2 and its reuse, the minimum amount of TiO_2 required for effective colour masking was determined. The iron extrudate obtained from JVS Foods Pvt. India was used. A pan coater, which is a representative model of drum coaters, was used to apply the coating material. It was used instead of the fluidized bed used initially in the laboratory because the fluidized bed has a suction compartment that removes the loose TiO_2. Hence, in the fluidized bed, the quantification of the actual amount of TiO_2 adhering to the ferrous fumarate was impractical. Four different amounts of TiO_2 were dusted (5, 10, 15, and 20% $^{w}/_{w}$) on the surface of the iron extrudate, after which 5% $^{w}/_{w}$ HPMC and 5% $^{w}/_{w}$ soy stearin were applied by pan coating, as earlier described. Three control batches were made for these batches. Two of the batches were made by dusting the extrudate with 12.5 or 25% $^{w}/_{w}$ TiO_2; then coated with 5% $^{w}/_{w}$ HPMC using a fluidized bed (as described by Yadava et al. (2012)) and 5% $^{w}/_{w}$ soy stearin using a pan coater. The third control batch was made by coating the extrudate with HPMC and soy stearin by pan coating without colour masking with TiO_2. The optimal amount of TiO_2 required for colour masking iron agglomerate was then determined based on the whiteness of the premix.

4.3.4 Effect of Coating with a Mixture of TiO_2 and Coating Material

Even when pan and drum coater was used, a small amount of TiO_2 was still blown away from the surface of the extrudate. The feasibility of applying TiO_2 and coating material together as a mixture was evaluated to prevent the blowing away of loosely attached TiO_2. The brown iron extrudate

obtained from JVS Foods Pvt. India was coated with a mixture of TiO_2 and HPMC or soy stearin to ultimately coat the extrudate with 5-20% $^{w}/_{w}$ TiO_2 and 10% $^{w}/_{w}$ HPMC or soy stearin. The extrudate was also coated with just 10% HPMC or soy stearin without TiO_2 to determine the contribution of the coating materials to the whiteness of the premix. The colour of these premix samples was compared to those made by having separate colour masking and coating unit operations.

4.4 Formulation of Fortified Salt

4.4. 1 Formulation of Iodized Salt

Refined salt (about 400 µm diameter) obtained from *Sifto Canada Corp* was crushed in a ribbon blend to remove any lumps of salt. A solution of potassium iodate (3.37 % $^{w}/_{v}$), which translated to 2% $^{w}/_{v}$ iodine, was prepared in a 100mL volumetric flask. The iodine solution (2.5mL/ kg salt) was sprayed onto the salt as it was being mixed inside the ribbon blender. This corresponds to 50ppm iodine in the salt. The mixing operation was stopped intermittently to remove the iodine solution stuck to the blade of the blender. The salt was mixed with the iodine solution for about 30 minutes. The iodized salt was then collected and spread on aluminum foil for air drying.

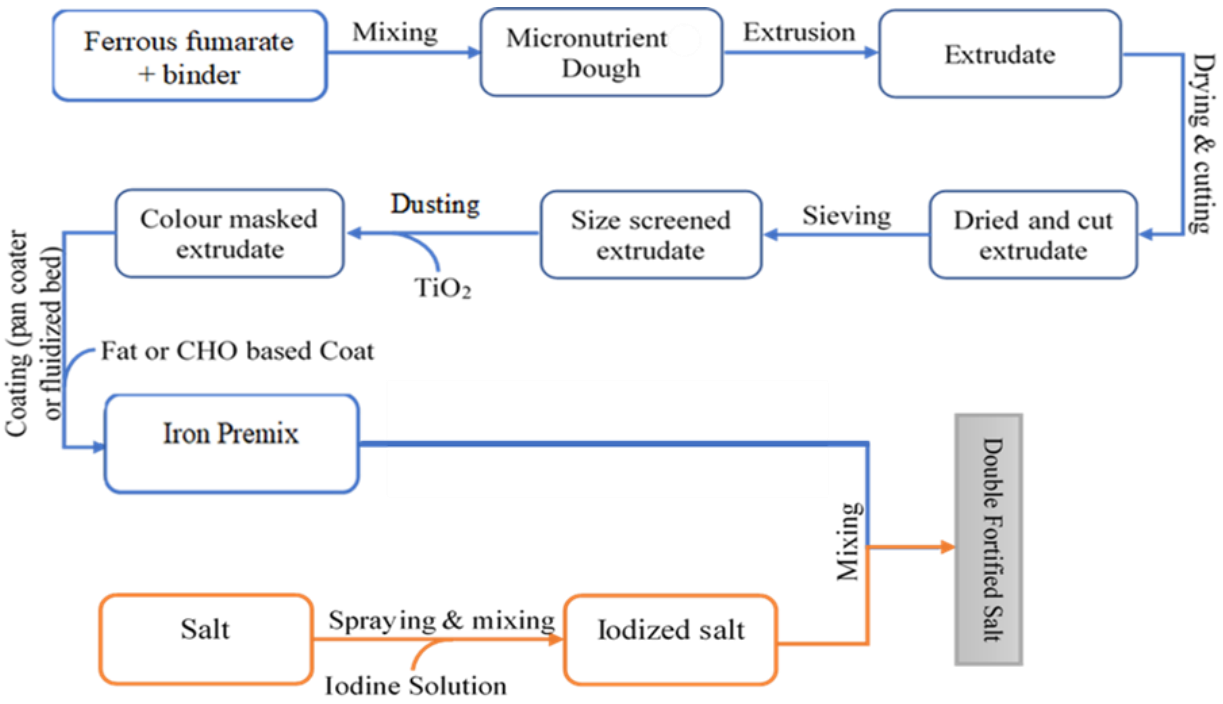


Figure 25:Schematic process flow for making Double Fortified Salt

4.4.2 Formulation of Double Fortified Salt (DFS)

The air-dried iodized salt was mixed with extruded and microencapsulated ferrous fumarate (iron premix). Specifically, for a 2kg batch, 10g of the iron premix was mixed with 1990g of iodized salt in the ribbon blender (*Figure 25*). The mixing ensured uniform dispersion of 0.5% $^{w}/_{w}$ iron premix in iodized salt. The concentration of iron in the iron premix was about 20% $^{w}/_{w,}$ such that the formulated DFS contained 1000ppm iron and 50ppm iodine.

4.4.3 Triple Fortification of Salt with Iodine Iron and Folic Acid

4.4.3.1 Formulation of Spray Solution for Triple Fortification of Salt

McGee (2012) formulated a fortified salt with 0.1% $^{w}/_{v}$ folic acid and iodine solution; found that high moisture content of the salt accelerated the loss of folic acid and iodine in fortified salt. She proposed that using a solution that contains a higher concentration of folic acid, and iodine will reduce the moisture added to salt and enhance the stability of folic acid and iodine in the fortified salt. Hence, solutions with higher concentrations (1-3 % $^{w}/_{v}$) of folic acid and iodine were prepared. The solutions were prepared with sodium carbonate buffers (0.1-0.3M); the impact of the concentration of sodium carbonate buffer on the solubility of folic acid in the solutions was investigated. Given the solubility problem encountered in the first sets of solutions, the perceived importance of pH in the stability of folic acid, and to make the preparation of the solution more straightforward, a different set of solutions was prepared. In the new set of solutions, the concentration of folic acid was 1% $^{w}/_{v,}$ while that of iodine was 2% $^{w}/_{v}$. There was a switch from the carbonate buffer to just sodium carbonate solution (0.1M). This solution was used to solubilize folic acid and to adjust the pH of the solution (7-10).

Due to the precipitation of folic acid in the solution below pH 9 and to evaluate the impact of citrate (a mild antioxidant) on the stability of folic acid, a new set of solutions of folic and iodine were formulated. In some of the solutions, the pH was adjusted to 9 with 0.1M sodium carbonate. In others, the pH was not adjusted; the solutions were prepared with 0.1M sodium carbonate (pH= 11.2). As with the previous set of solutions, the concentration of folic acid was pegged at 1% $^{w}/_{v}$, which supplies about 100% RDI of folic acid through salt. However, a 50% reduction of folic acid (0.5% $^{w}/_{v}$) was evaluated for its effect on the colour of the salt.

4.4.3.2 Storage and Stability Study of Micronutrients in Spray Solution for Triple Fortification of Salt

The spray solutions were tightly secured in scintillation vials and were stored in temperature-controlled incubators (25, 35, 45 ^{o}C). The stability of iodine and folic acid in the spray solutions were monitored for two months. Specifically, the concentrations of micronutrients were determined for the freshly prepared solutions and at 0.5, 1, and 2 months of storage.

4.4.3.3 Formulation of Triple Fortified Salt (TFS)

Some of the spray solutions formulated for the triple fortification of salt were used. Three of the iron premix samples were used; they were coated with 10% soy stearin, 10% HPMC or dual 5% HPMC, and 5% soy stearin. In addition to the three premix samples, a premix sample obtained from the JVS Foods Pvt., India, similarly double-coated with 5% HPMC and 5% soy stearin, was used. The process schematic is presented in Figure 26

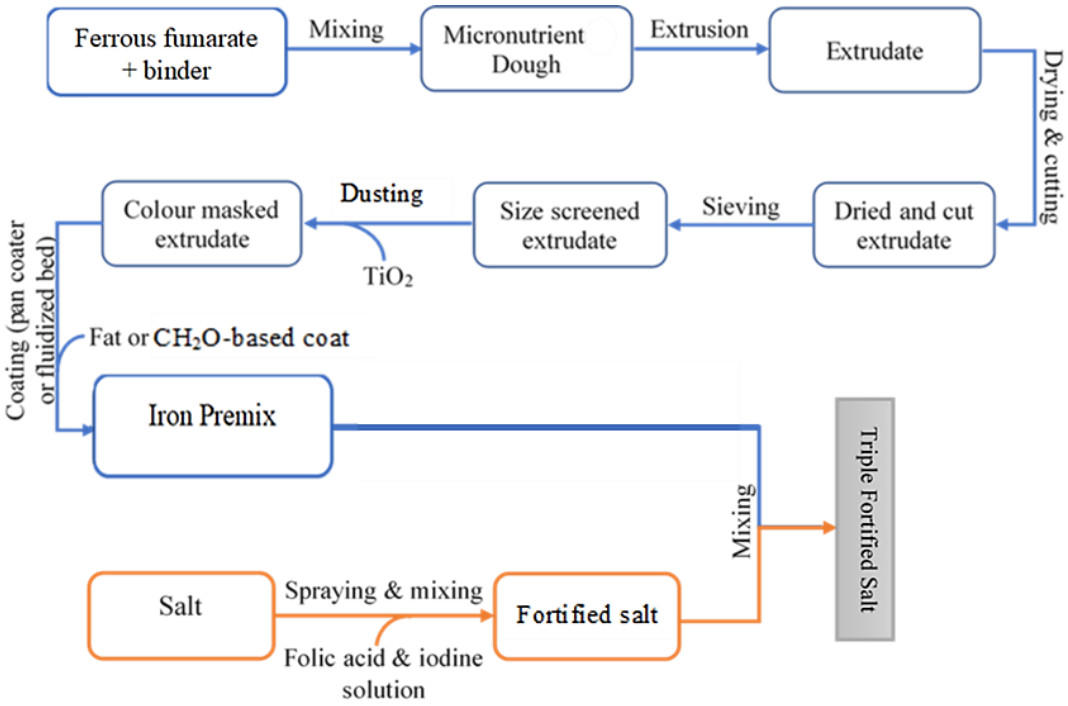


Figure 26:Schematic process flow for making Triple Fortified Salt

Refined salt (~400 µm diameter) obtained from *Sifto Canada Corp* was mixed in a ribbon blender to remove the lumps of salt. The solution (5 mL) (prepared for the triple fortification of salt) was sprayed on the salt (2kg) while the salt was being mixed in the ribbon blender. A spray bottle was

used to apply the solution. The mixing operation was stopped intermittently to remove the solution that was stuck to the blade of the blender. After a homogenous mixing of the salt with the solution, the salt was collected on an aluminum foil. It was air-dried overnight.

The air-dried folic acid and iodine fortified salt was mixed with the iron premix samples. Specifically, for a 2kg batch, 10g of the iron premix is added to 1990 g of the fortified salt in the ribbon blender. This ensures a uniform and indistinguishable dispersion of 0.5% $^{w}/_{w}$ iron premix in fortified salt. The concentration of iron in the iron premix was about 20% $^{w}/_{w,}$ such that the formulated TFS had 1000ppm iron, 50ppm iodine, and 12.5 or 25ppm folic acid.

4.4.3.4 Formulation of Iron-Folic Acid Premix

Due to the potential colour problem that results from spraying folic acid (yellow) on salt, the process for the formulation of Triple Fortified Salt was optimised such that folic acid was no longer added through solution but through the premix. Since there is a paucity of knowledge on the interaction between iron and folic acid, two premix designs were made- one has the iron and folic acid in the core, and the other has folic acid separated from the iron core by a thin layer of TiO_2.

In the first design, the same process for the formulation of iron premix was followed (
Figure 27a). The only difference in the process is the addition of folic acid (7g) to the ferrous fumarate dough. To ensure a homogenous mixture, the 7g of folic acid was added and thoroughly mixed with about 10 g of ferrous fumarate. This mixture was mixed with 90g of ferrous fumarate. The mixture was further diluted with ferrous fumarate until 800g ferrous fumarate (required amount per batch of extrusion) contained the 7g folic acid.

In the second design, the process of making iron premix was followed until the colour masking step. There were two colour masking steps intermitted with the coating steps- the first colour masking step was to create a thin layer between iron extrudate and the folic acid layer, and the second colour masking step was to mask the yellow colour of folic acid(
Figure 27b). After the first colour masking step, a homogenous suspension of folic acid (0.52% $^{w}/_{v}$) in a 2.5% $^{w}/_{v}$ HPMC was sprayed on the TiO_2 masked iron extrudate tumbling in a pan coater. The folic acid-coated iron extrudate was colour masked with TiO_2 and coated with HPMC and the soy stearin.

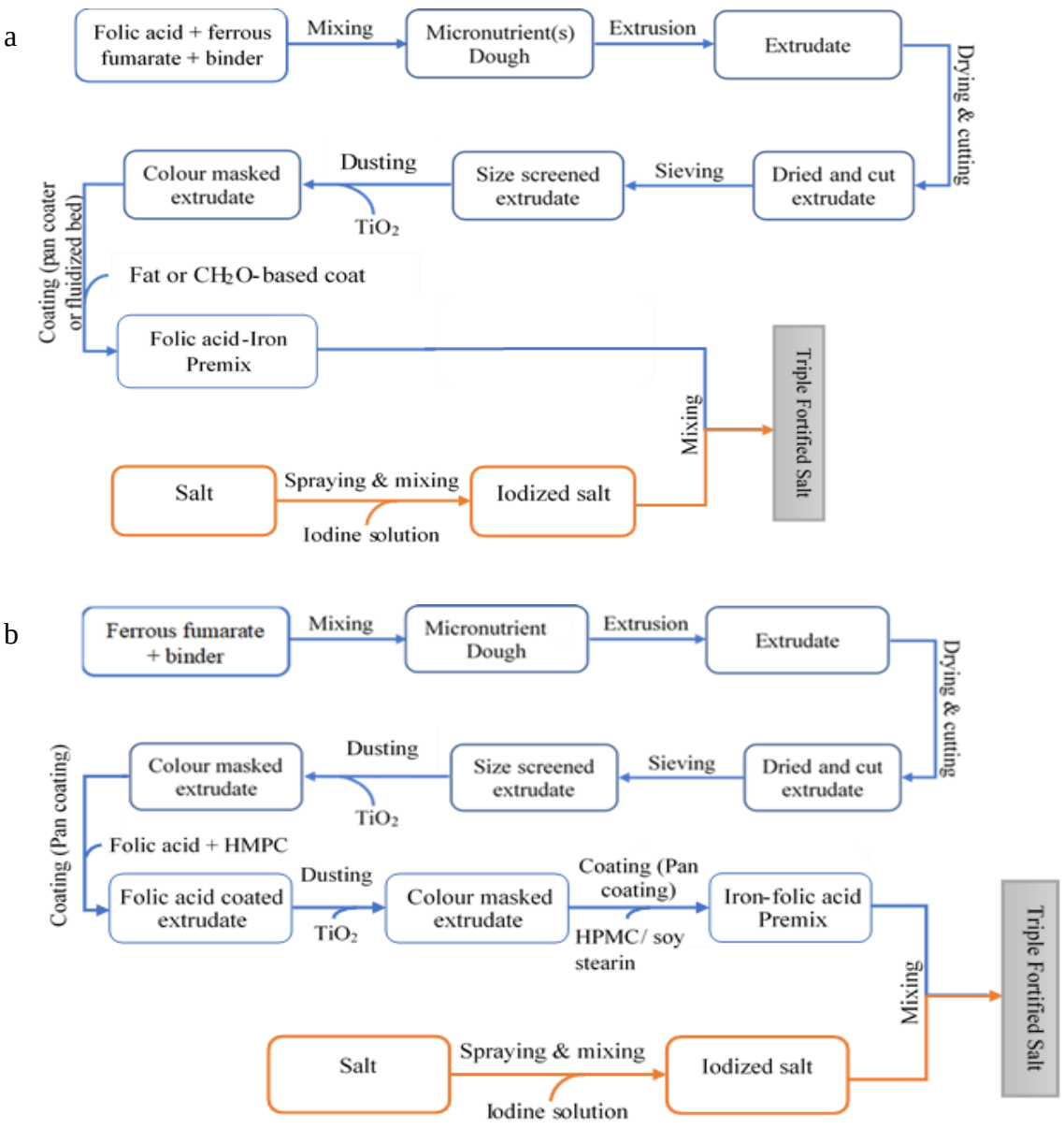


Figure 27:Schematic process flow for making Iron-Folic Acid Premix

(a) Formulation of Fe-FA premix which has iron and folic acid at the core of the premix
(b) Formulation of $Fe_{extrudate}$-FA premix which has just iron at the core of the premix, while folic acid was sandwiched between titanium dioxide layer.

The two iron-folic acid premix samples made were used to formulate TFS. They were added to iodized salt that was prepared as described in the 'Formulation of Iodized Salt' Section. The TFS contained 1000ppm iron, 50ppm iodine, and 25ppm folic acid. The colour (L*a*b) of the optimized TFS was evaluated, as described later in 'Section 4.5.2.7'.

4.4.3.5 Handling and Storage of Triple Fortified Salt

TFS samples were divided first into four portions by a glass divider; the fourth portion was shared over the other three portions. This ensured a randomized division of the salt into the three portions. They were packed in Zip-LockTM polyethylene bags and stored in incubators with controlled temperature and humidity (25, 35, and 45 ^{o}C; 60-70% RH). Also, the incubator was in complete darkness so that the impact of light was minimized. In some cases where the impact of light was studied, the salt samples were placed on a bench where they were exposed to the lights in the laboratory.

4.4.3.6 Stability Study of TFS

The stability of micronutrients in some of the premix samples and each salt sample was monitored. Immediately after the samples were prepared, the amounts of micronutrients in the salt samples were determined. After six months and or one year of storage, the amount of micronutrients remaining was determined and was reported as the percentage of the amount in the freshly prepared samples.

4.4.4 Formulation of Quadruple Fortified Salt (QFS)

4.4.4.1 Formulation of Spray Solution for Quadruple Fortification of Salt

The addition of iodine, folic acid, and vitamin B_{12} as a solution and the addition of iron as agglomerated and encapsulated ferrous fumarate seemed an easy route to making QFS. Hence, solutions containing iodine (3% $^{w}/_{v}$), folic acid (2% $^{w}/_{v}$), and vitamin B_{12} (0.015% $^{w}/_{v}$) were made with a 0.2M carbonate buffer solution as the first step towards the formulation of QFS. The effect of erythorbate, ascorbate, and citrate on the stability of the micronutrients in the solution was evaluated.

Given the problem of precipitation encountered with the first set of spray solution, a second set of solutions was formulated. The new solutions contained several combinations of micronutrients with concentration relatively lower than the first set: iodine (2% $^{w}/_{v}$), folic acid (1% $^{w}/_{v}$), and vitamin B_{12} (0.01% $^{w}/_{v}$); iodine (2% $^{w}/_{v}$) and vitamin B_{12} (0.01% $^{w}/_{v}$) or just vitamin B_{12} (0.01% $^{w}/_{v}$). Some of the solutions had their pH adjusted (to 2.8, 8, or 9) with citric acid, ascorbic acid, or sodium carbonate, while the pH of others was not adjusted. Also, the effect of pH on the stability

of iodine, folic acid, and vitamin B_{12} solutions was evaluated. For this, four solutions containing the three micronutrients had their pH adjusted to 7, 8, 9, and 10 with 0.1M sodium carbonate.

4.4.4.2 Storage and Stability Study of Micronutrients in Spray Solution Prepared for Quadruple Fortification of Salt

Some of the spray solutions were tightly secured in scintillation vials and were stored in conditioned incubators (25, 35, 45 ^{o}C). The stability of iodine and folic acid in the spray solutions was monitored for 2 months because most of the small-scale salt processing plants stores their spray solution for about a month. In specifics, the concentrations of micronutrients were determined in the freshly prepared solutions, and at 0.5, 1, and 2 months of storage.

4.4.4.3 Formulation of Quadruple Fortified Salt with Spray Solution containing Folic Acid, Iodine, and Vitamin B_{12}

Although the low stability of B_{12} in the solutions discourage the further use of the solution containing iodine, folic acid, and vitamin B_{12} in the formulation of QFS, however, some of the freshly prepared solutions were used to formulate QFS. Spray solution that contained iodine (2%), folic acid (0.5-1%), and vitamin B_{12} (0.01%) was sprayed on refined salt. The fortified salt was air-dried overnight, and the dried salt was mixed with iron premix to make the QFS. Ultimately, the salt contained 1000ppm iron, 50ppm iodine, 0.25ppm vitamin $B_{12,}$ and 12.5 or 25ppm folic acid. Only the stability of iodine and folic acid in salt was studied because of the technical issue of extracting and quantifying vitamin B_{12} in the salt. This was due to the difficulty of getting a solution that can differentially extract vitamin B_{12} from the fortified salt.

4.4.4.4 Formulation of Iron-B_{12} premix

The incompatibility and the comparative stability of folic acid and vitamin B_{12} in solutions that contained iodine and folic acid or B_{12} necessitate the moving of B_{12} from the spray solution to the iron premix (iron-B_{12} premix). Four of the unit operations for the formulation of iron premix were identified as potential places for the integration of B_{12} into the premix making process, as described in *Figure 28*. Specifically, B_{12} was either added at the point of making the micronutrient dough, sprayed on the size screened extrudate before colour masking, added with $TiO_{2,}$ or added with the coating material. Per 1375g batch, 71mg of B_{12} was added to the iron premix.

For the addition to the dough (core), 71mg cyanocobalamin was added to the ferrous fumarate before the addition of the wheat flour. To ensure homogenous mixture, the 71 mg cyanocobalamin was added and thoroughly mixed with about 10g of ferrous fumarate. This mixture was mixed with another 90g of ferrous fumarate. The 71mg cyanocobalamin was further diluted with ferrous fumarate until the required 800g ferrous fumarate per batch was added. For the addition before colour masking, a solution of B_{12} was sprayed on the iron extrudate (SPRAY). Also, vitamin B_{12} was added to TiO_2 used for colour masking (TiO_2), or HPMC used for coating (HPMC).

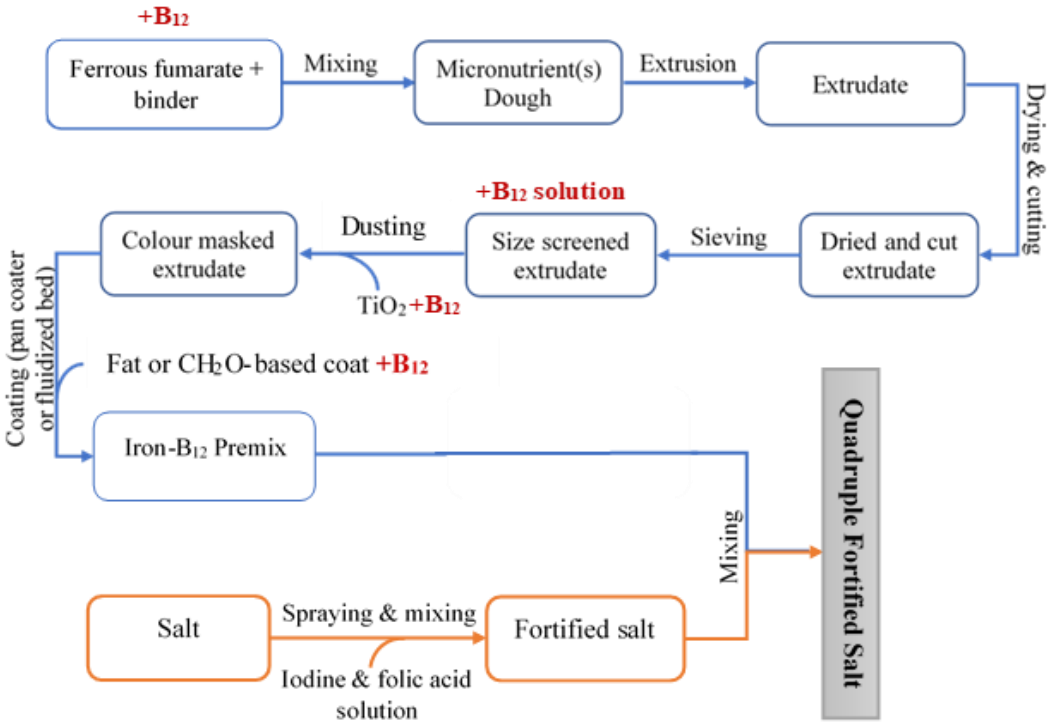


Figure 28:Schematic process flow for making Iron-B_{12} Premix

4.4.4.5 Formulation of Iron-B_{12}-Folic acid Premix

Given the potential problem of colour that may result from spraying folic acid directly on salt in formulating Quadruple Fortified Salt, folic acid was moved to the iron-B_{12} premix. Given that folic acid and B_{12} are not compatible in liquid forms and that there is a paucity of knowledge on the

interaction between folic acid and B_{12}, two designs of the premix were formulated, as described in Figure 29.

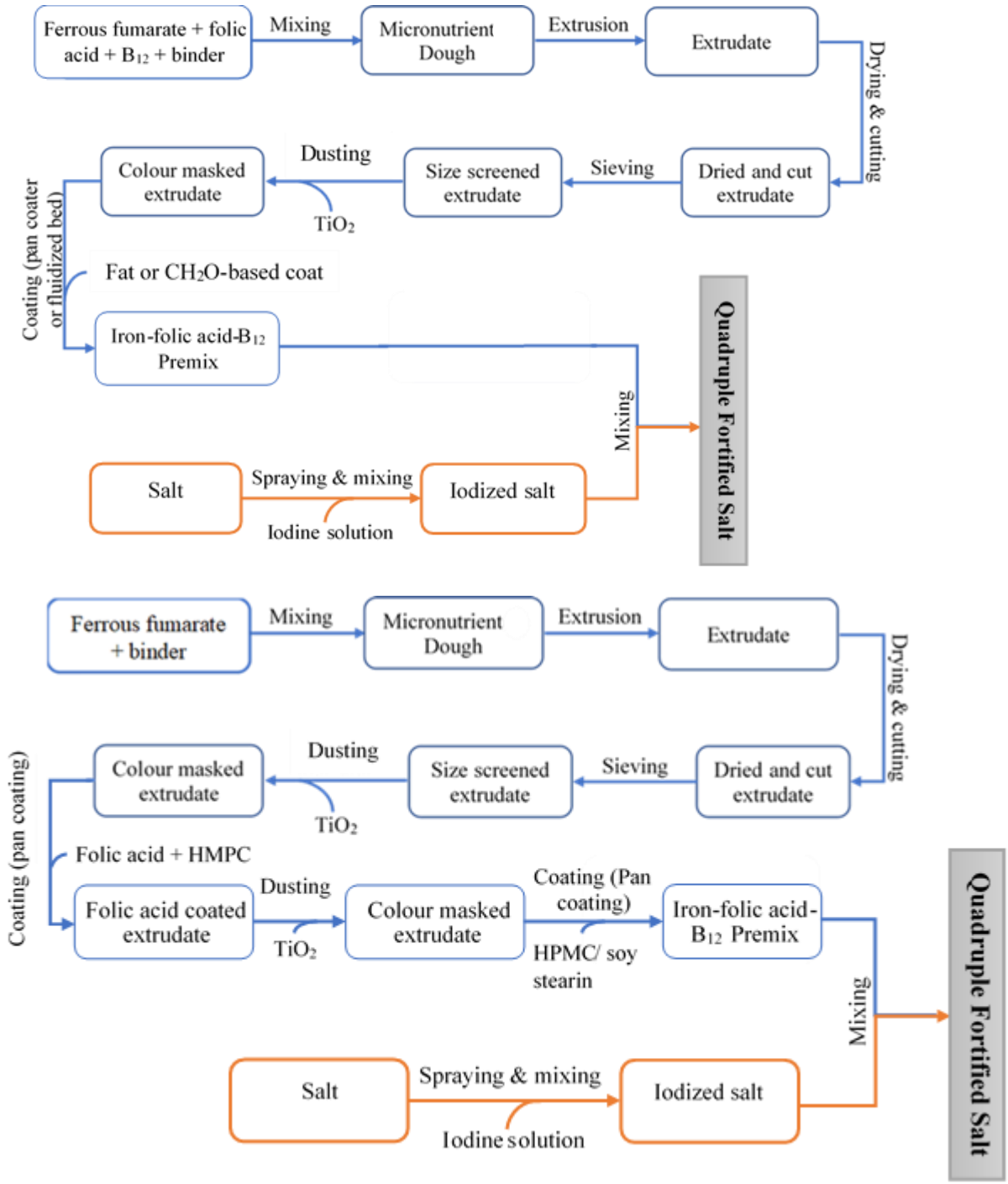


Figure 29:Schematic process flow for making Iron-B_{12}- Folic acid Premix

(a) Formulation of Fe-B_{12}-FA premix which has iron, vitamin B_{12} and folic acid at the core of the premix

(b) Formulation of $(Fe\text{-}B_{12})_{extrudate}$-FA premix which has just iron and vitamin B_{12} at the core of the premix, while folic acid was sandwiched between titanium dioxide layer.

In the first design, iron, $B_{12,}$ and folic acid were inside the core of the premix. There was not much change made to the process of making iron premix. All the three micronutrients were added to the durum wheat; this mixture was used to make the micronutrient dough. All other processes of making iron premix were followed.

In the second design, folic acid was separated from the iron premix by a thin layer of TiO_2. This was to study if the interactions of B_{12} and folic acid in the premix have a negative effect on the stability of both micronutrients. Only iron and B_{12} were extruded. The process of making iron premix was followed until the colour masking step. There were two colour masking steps in between which a homogenous suspension of folic acid (0.52% $^{w}/_{v}$) in a 2.5% $^{w}/_{v}$ HPMC was sprayed on the initially colour masked iron-B_{12} extrudate. The folic acid-coated extrudate was colour masked with TiO_2 and coated.

4.4.4.6 Formulation of Quadruple Fortified Salt with the Optimized Premix

Depending on the types of premix used – iron-B_{12} or iron-folic acid-B_{12} premix, corresponding spray solution – iodine and folic acid or iodine solution, was used. Just as with the method previously described used for formulating fortified salt, 5 mL solution was sprayed on 2kg salt and air-dried overnight. The premix was then mixed with the semi fortified salt at a 1:200 ratio. The salt contained 1000ppm iron, 50ppm iodine, 25ppm folic acid, and 0.25ppm vitamin B_{12}.

4.4.4.7 Handling and Storage of Fortified Salt and Stability study

Formulated salt was divided first into 4 portions by a glass divider; the fourth portion was shared over the other three portions. This ensured a randomized division of the salt into the three portions. They were packed in Zip-LockTM polyethylene bags and stored inside incubators with controlled temperature and humidity (25, 35, and 45 ^{o}C; 60-70% RH). Also, the incubator was in complete darkness so that the impact of light is minimized. In some cases where the impact of light was studied, the salt samples were exposed to natural lights in the laboratory.

The stability of micronutrient in some of the premix samples and all the salt samples were monitored. Immediately after the samples were prepared, the amounts of micronutrients in the salt samples were determined. After six months and or one year of storage, the amount of

micronutrients remaining in the salt samples was determined and was reported as the percentage of the amount in the freshly prepared samples.

4.4.4.8 Laboratory scale - sensory survey for Quadruple Fortified Salt

A sensory survey was carried on rice cooked with some of the QFS samples. Ten members of our laboratory participated in the survey.

4.5 Analytical Methods

4.5.1 Determination of Iron Content in Premix Samples

4.5.1.1 Total Iron

The method described by Moldoveanu and Papangelakis (Moldoveanu and Papangelakis, 2013) was adapted for quantifying total iron content in the premix. The premix samples (100mg) obtained from JVS, India, were added into digestion vials. Aqua regia (15 mL) was then added to the vial, and its cap tightened. The samples were digested using microwave digester (ETHOS EZ, Milestone Inc.) for 2 hours. The resulting solutions were poured into 50 mL volumetric flasks and made to the mark with 5% HNO_3. The resulting solution was diluted at a ratio of 1:10 with 5% HNO_3. The iron content in the sample was analyzed using inductively coupled plasma optical emission spectrometry (Agilent Dual View 720). The concentration of iron in the sample was calculated using equation (4).

$$\%\ concentration\ of\ iron\ = \frac{concentration\ of\ iron\ in\ the\ working\ solution}{concentration\ of\ sample\ in\ the\ working\ solution} \times 100 \quad Equation\ 4$$

4.5.1.2 In vitro Iron Bioaccessibility Approximation

The iron premix will disintegrate with cooking. However, the release of iron from some of the iron premix into a 0.1M HCl (pH1) was investigated. The premix (100 mg) was added into 500 mL Erlenmeyer flasks, and then 250 mL HCl solution was added to the tube. The flasks were placed inside a Cole-Parmer StableTemp Water bath (EW-14575-12) coupled with Cole-Parmer Polyscience Dual Action Shaker, set at 37^{o}C and 160 rpm for 2 hours. In all premix samples, 1 mL of the solution was taken from the digestion tube at 30-minute intervals. The withdrawn solution (1mL) was mixed with 9 mL 5% $^{v}/_{v}$ nitric acid. The solutions were filtered with a 45 µm syringe filter. The concentration of the iron in the solutions was measured using ICP-OES. The amount was presented as a percentage of the total amount of iron in the premix.

4.5.1.3 Determination of the Amount of Iron on the Surface of the Premix

4.5.1.3.1 Quantification of the Amount of Iron Released into pH 4 HCl Solution

The iron particle integrity was evaluated using a dissolution test, on the basis that ferrous fumarate, the iron fortificant, has a high solubility in acid, and the fat coating will not be solubilized at pH≥4. The rate of dissolution indicates the efficiency of the film coating. The test is only applicable to coating materials that are insoluble at pH 4. The iron premix (100mg) was added into digestion tubes; the appropriate amount of 0.0001 M HCl solution was added. The solution was collected from the tube after 2 hr. The solution was filtered (0.45 µm) before being analyzed with ICP-OES.

4.5.1.3.2 Quantification of the Amount of Iron Released into EDTA Solution

The first method for quantifying iron on the surface premix with 0.0001N HCl is limited depending on the coating material used. Hence, the use of a solution of Na_2EDTA, a good iron chelator, and a mild solution. Na_2EDTA (20 mL of 5% $^{w}/_{v}$ adjusted to pH 7 with sodium hydroxide) was added to 100 mg of some of iron premix samples into a 40 mL beaker. The mixture was stirred on a magnetic stirrer for 5 minutes. The solution was filtered with a syringe filter (0.45 µm). The filtrate was diluted at a ratio 1:9 with 5% $^{v}/_{v}$ HNO_3. The iron content in the resulting solution was then analyzed with ICP-OES.

4.5.2 Determination of Physical Properties of the Premix Samples

4.5.2.1 Evaluation of the Surface Morphology of the Premix

The scanning electron microscope (SU-3500 VP SEM, Hitachi High-Technologies) was used to determine the surface morphology of the premix. The premix was attached to an SEM specimen stub with a carbon conductive double-coated adhesive tape. Air was blown over the attached premix to remove any loose premix. Samples were examined, and micrographs were recorded at an acceleration voltage of 1.5 kV, with a working distance of 51 mm, under a high vacuum as described by Singh et al. (2018). However, the sample was not gold-coated, as described by Singh et al. (2018), to make the surface defects more visible.

4.5.2.2 Bulk Density

An empty 25mL scintillation vial was weighed (W_1), then filled with the premix samples and tapped until no apparent volume change was observed. The weight of the sample-filled vial was

then recorded (W_2). The vial was emptied and filled with water and weighed (W_3). The bulk density of the sample was then calculated according to Equation (5):

$$\rho_B = \frac{W_2 - W_1}{W_3 - W_1} \times \rho_W \qquad Equation\ 5$$

where ρ_B is the bulk density in g cm^{-3};
ρ_w is the water density =1 g cm^{-3}

4.5.2.3 Particle Density

After bulk density was determined as described, the void volume in the sample-filled flask was determined by a dropwise addition of hexane. The weight of the flask was measured (W_4), and equation (6) was used to calculate the particle density

$$\rho_P = \frac{(W_2 - W_1)}{\left(\frac{W_3 - W_1}{\rho_W}\right) - \left(\frac{W_4 - W_2}{\rho_H}\right)} \qquad Equation\ 6$$

where ρ_p is the particle density in g cm^{-3};
ρ_w is the water density = 1 g cm^{-3};
ρ_H is the hexane density = 0.66 g cm^{-3}

4.5.2.4 Evaluation of Sinking Properties of the Premix

Water in a 1000 mL beaker was stirred with a magnetic bar stirrer. Thirty individual premix particles were counted and gently added to the stirred water in the beaker. The number of premix particles still floating after 2 minutes was counted. If more than 2 premix particles still float after 2 minutes, the floating was considered to be significant.

4.5.2.5 Estimation of the Thickness of Layers of Materials in the Premix

The impact of the amount of TiO_2 used for colour masking on the thickness of each layer of coating materials and TiO_2 that make up the premix samples was estimated. The estimation considered the proportion of each of the materials that made up the premix, their densities (ρ), and the average weight of a particle (m). From the density and mass, the volume (v) of the particle was calculated (Equation 7). The radius (r) was estimated by using Equation 8. The thickness of the material (T)

was estimated by subtracting the radius of the inner material from the radius of outer material (Equation 9).

$$v = \frac{m}{\rho} \qquad Equation\ 7$$

$$r = \left(3 \times \frac{v}{(4 \times \pi)}\right)^{\frac{1}{3}} \qquad Equation\ 8$$

$$T = r_b - r_a \qquad Equation\ 9$$

4.5.2.6 Evaluation of the Effect of the Loading Capacity of Pan Coater on Effective Coating

The optimal ratio of the amount of premix to pan coater size was determined. Four different amounts (5,10, 15, and 20g) of premix were coated in a pan coater with a fixed size (Diameter=16 cm). A mixture of 15% TiO_2 and 10% soy stearin was used for coating the premix. The colour and uniform distribution of the blend of the coating material and TiO_2 were examined. An iPhone 8 Camera was used to capture the pictures of the premix samples that was placed inside a photo lightbox. The pictures were used to evaluate the distribution of the coating mixture on the extrudate.

4.5.2.7 Evaluation of the Colour of the Premix

For some of the iron premix samples, the image of the iron premixes prepared in the laboratory was captured under the same lighting condition and then analyzed using ImageJ software to capture the RGB colour coordinates, which was then translated to Hunter-L a* b* colour coordinates. For other iron premix samples due to availability of equipment, the L*a*b* colour properties of premix samples were determined using a colorimeter (Chroma Meter CR-400/40, Konica Minolta Photo Imaging U.S.A., Inc., Mahwah, NJ) as described by Modupe et al. (2019). The L*a*b colour analysis was used to assess the whiteness of the premix as a yardstick for the impact of the amount of TiO_2 and the method of coating used on the effectiveness of colour masking.

For Fe-B_{12} premix, the impact of the point of addition of vitamin B_{12} to the iron premix process on the colour of the premix was evaluated by physical examination.

4.5.3 Determination of Iodine Content in Spray Solution and Salt Samples

The iodine content in the spray solution and salt samples were determined by iodometric titration Method 33.149, described by the Association of Official Analytical Chemists (1984) and McGee (2012).

4.5.4 Development of an Analytical Method for Folic Acid in Spray Solution and Salt

The method developed by Nagaraja et al. (2002) was initially tried for quantifying folic acid in the spray solution and fortified salt. The method was based on the reductive cleavage of folic acid and the formation of the colour complex of the product of folic acid degradation. This method was not reproducible in our salt matrix. Hence, the need to develop a simple spectrophotometric method.

4.5.4.1 Preparation of Folic Acid Solution

A solution of sodium carbonate (0.1M) was prepared using reverse osmosis (RO) water. The solution was used to prepare 250 µg/mL folic acid (stock solution). The stock solution was then reconstituted to 125, 100, 75, 25 and 10, 5, 2.5, and 1 µg/mL with the sodium carbonate solution. Sodium carbonate was used because it is one of the very few solutions that can dissolve folic acid, and it is part of the iodine and folic acid solution that is sprayed on salt when TFS is formulated.

4.5.4.2 Scanning the Wavelength for Folic Acid

The absorbance of the 25µg/mL folic acid solution was scanned between 240-800 nm, using Cary-50 UV⁄Vis spectrophotometer (Varian Inc. CA, USA). The sodium carbonate solution was used as the baseline solution. For accuracy and precision of the scanning protocol, a 25µg/mL folic acid solution was scanned three times, and three 25µg/mL folic acid solutions were scanned.

4.5.4.3 Evaluation of the Specificity of the Method

The method described by Nagaraja et al. (2002) was used to reductively cleave folic acid to pterine-6-carboxylic acid (PCA) and p-aminobenzoyl-l-glutamic acid (pABGA). The reaction was designed such that the starting concentration of folic acid was 25µg/L. Three reaction setups were made - the first was scanned (240 -500 nm) after 30 minutes, the second after 60 minutes, and the third after 120 minutes of degradation reaction. Also, 10µg/mL pteroic acid, 10µg/mL para-aminobenzoic acid, 10µg/mL glutamic acid, 10µg/mL cyanocobalamin and 10µg/mL folic acid

solutions were scanned (230-700nm). The distinct wavelength for folic acid determined using the data obtained from all these scans was 285nm.

Also, the selectivity of the analytical method for folic acid spray solution in the presence of other components of spray solution and salt was evaluated. The absorbance of solutions containing 10 µg/mL folic, 10 µg/mL folic acid + 20 µg/mL iodine, and 10 µg/mL folic acid + 20 µg/mL iodine + 10 µg/mL citrate were measured. The concentrations were based on the proportion of the constituents that will be extracted from salt.

The selectivity of the analytical method for folic acid in the presence of the product of reductively cleaved folic acid was probed. Folic acid solutions (2.5 µg/mL and 25 µg/mL) were subjected to reductive cleavage, using 5N hydrochloric acid and zinc (Nagaraja et al., 2002). The absorbance of the solutions of reacted and unreacted 2.5 µg/mL and 25 µg/mL was measured at the chosen wavelength of 285nm.

4.5.4.4 Extraction of Folic Acid from Spray Solution and Salt

For salt, 5g of salt was weighed into a 50mL falcon tube, and 10mL of 0.1M sodium carbonate solution was added. The mixture is thoroughly mixed for 1minute. To have a clear solution, the supernatant was filtered with a 0.45µm syringe filter. For spray solution, 100 µL of the solution was measured into a 100 mL volumetric flask. The volumetric flask was filled to the mark 0.1M sodium carbonate. This was then thoroughly mixed. The amount of folic acid extracted from the spray solution and salt was quantified by measuring the absorbance of the extract at 285nm (Modupe, Maurras and Diosady, 2020).

4.5.5 Determination of vitamin B_{12} in Spray Solution and Salt

The use of a simple spectrophotometric method for B_{12} was not successful due to two reasons-very low concentration of B_{12} in salt and possible interference with folic acid absorbance at 365nm, which was the maximum absorption wavelength of vitamin B_{12} in the solution. The direct quantification of B_{12} by HPLC did not work either due to a high concentration of salt. This will cause the salt to precipitate in the column, damaging it. These reasons led to using HPLC-MS coupled with solid-phase extraction.

Vitamin B_{12} was analyzed with HPLC-MS. Before the analysis, solid-phase extraction was used to desalt and preconcentrate B_{12}. A 5g sample of QFS was dissolved in 5 mL of RO water in a 50 mL falcon tube. The mixture was vortex mixed for 5 minutes. The mixture was centrifuged for 5 minutes. The supernatant was then injected through a 1mL solid-phase extraction column, which had been preconditioned with methanol (1 mL) and RO water (2 mL). The column was washed with water (2 mL) and B_{12} eluted with methanol (2 mL). The eluate was vacuum evaporated. The resulting B_{12} solid was then reconstituted with RO water (20 µL). Lack of instruments to adequately regulate the pressure during the solid-phase extraction affected the reproducibility of the method. Hence, B_{12} was not quantified directly from salt but only from the premix.

In the case of the premix in salt, water was used to wash out salt from the premix before the micronutrients were extracted. Salt containing premix (10 g) was added into a porcelain Buchner funnel with a filter paper (Whatman Grade 4). Water was added to salt in the funnel until only the premix was retained on the filter paper. The premix was collected and ground with a mortar and pestle. RO water (20 mL) was added to the premix as it was ground. The solution was filtered (45µm; syringe filter) to remove solid particles. The amount of B_{12} in the solution was determined using HPLC-MS. Acetonitrile was used as the mobile phase.

The same method was used for quantifying folic acid in the premix. The simple spectrophotometric method developed for folic acid cannot accurately quantify folic acid in the premix as there seems to be a component of the premix that interferes with the absorbance of folic acid at 285nm. In the case of folic acid, formic acid is used as the mobile phase.

4.5.6 Determination of Folic Acid and Vitamin B_{12} in Food

4.5.6.1 Extraction of Micronutrients from the Food

Folic acid and vitamin B_{12} were extracted from rice boiled with QFS (Figure 30). After cooking, a known amount of HPLC water was added to the cooked food and homogenized with a KitchenAid KHB1231 2-Speed Hand Blender. The paste was centrifuged for 20 minutes at 6000rpm, with the temperature set at 25 ^{o}C. The oil from the supernatant was removed by liquid-liquid extraction using analytical grade hexane. A known amount of celite and extracted aqueous solution was added into a 50 mL falcon tube. This was thoroughly mixed and centrifuged at 1000rpm for 10 minutes. The supernatant was filtered with a 0.45 µm syringe filter.F

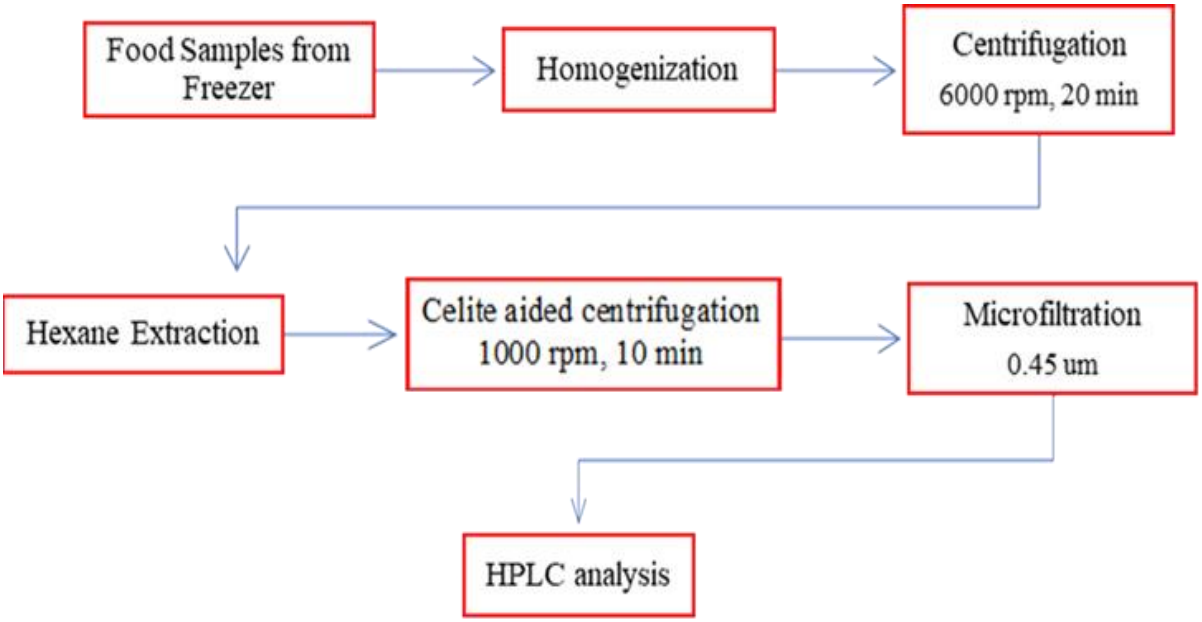


Figure 30:Schematic process flow for extraction of micronutrients from the cooked foods

4.5.6.2 Quantification of Folic acid and Vitamin B_{12} in Cooked Food

The amount of folic acid and vitamin B_{12} in food was determined by HPLC, as described in Table 6. A gradient eluant system with 20mM phosphate buffer pH 3 and acetonitrile was used.

Table 6:HPLC conditioned for Folic Acid and Vitamin B12 analysis

Column	Kinetex, 2.6 µm, C18, 100 Å, LC column 100 x 4.6 mm
Dimensions	100 x 4.6 mm ID
Elution Type	Gradient
Elution A	Acetonitrile
Elution B:	20mM Phosphate buffer pH 3
Gradient Profile	Step No. | Time (min) | Sol. A | Sol. B 0 | 0.5 | 5 | 95 1 | 5.0 | 25 | 75 2 | 5.00 | 5 | 95 3 | 5.00 | 75 | 25 4 | 5.00 | 5 | 95
Flow Rate	1 mL/min
Col. Temp	ambient
Detection	UV-Vis Abs.-Variable Wave.(UV) @ 278 & 360 nm (22°C)
Run time	20 min
Injection volume	20µL

4.6 Evaluation of the Colour of Fortified Salt

The L*a*b* colour properties of fortified salt samples (DFS, TFS, and QFS) were determined by colorimeter (Chroma Meter CR-400/40, Konica Minolta Photo Imaging U.S.A., Inc., Mahwah, NJ) as described by Modupe et al. (2019). The L*a*b colour analysis was used to assess the whiteness and yellowness of the salt samples. This was used to judge the impact of folic acid on the colour (a potential driver of acceptance) of the salt samples.

4.7 Elucidation of the Mechanism of Degradation of Folic Acid in Triple Fortified Salt

The products of folic acid degradation in salt after its storage for six months were evaluated. Using uHPLC-MS, the total ion chromatogram of a freshly prepared Triple Fortified Salt and after its storage for six months was determined and subjected to differential analysis using a software (Compound Discoverer) to detect the newly formed compounds in the stored salt. The software was configured to detect any compound, whose concentration in the stored sample was at least 200% higher than its concentration in the salt when it was freshly prepared, as a newly formed compound; these compounds were considered as the products of folic acid degradation in the salt. Molecular analysis, using Chem3D 17.1 Software, was carried out on one of the detected products of degradation of folic acid in salt to estimate some of its bond length, partial atomic charges, and bond angle. This analysis was used to predict the compound's molecular structure. The structure of the compound obtained from the ChemACX database was used for the molecular analysis.

4.8 Kinetic Modeling of Degradation of Micronutrients in the Different Fortified Salts

For some of the salt samples, the amounts of micronutrients were determined at 0.5, 1, 2, 4, and 6 months of storage. The data obtained from these studies were used to extrapolate kinetic data of the degradation of micronutrients in the salt over the period. The expression of zero (Equation 10) and first-order kinetics (Equation 11) were used. The best fit was selected.

$$[A]_t = -kt + [A]_0 \qquad \text{Equation 10}$$

$$ln[A]_t = -kt + ln[A]_0 \qquad \text{Equation 11}$$

The kinetic data obtained were verified with data obtained from a 12-month stability study. The same data was used to extrapolate the equation to predict the long-term stability of micronutrients in the salt.

4.9 Effect of Boiling on the Stability of Folic Acid and Vitamin B_{12}

A known amount of folic acid and vitamin B_{12} was added to boiling water. Samples were taken from the boiling water at 5, 10, 15, and 20 min. The amount of B_{12} and folic acid in the solution were quantified by spectrophotometry and HPLC-MS, respectively, as described below.

The degradation of B_{12} and folic acid in rice and lentils cooked with salt fortified with B_{12} and folic acid was determined. A known amount of unfortified and fortified salt was used for cooking rice. The rice was boiled for 20 minutes. Folic acid and vitamin B_{12} were extracted and quantified by HPLC, as described in 'Section 4.5.6'.

5. Results and Discussion

5.1 Scaling Up of the Technology for Making Iron Premix

The cold-forming extrusion-based microencapsulation of ferrous fumarate has four primary operations - particle agglomeration, cutting and size matching, colour masking, and coating. The technology ensures three main functions in Double Fortified Salt (DFS): it prevents chemical interaction between iron and iodine, it masks the brown colour of ferrous fumarate and prevents segregation in salt by matching the size of the iron premix to the size of salt. Overall, it achieves a uniform and visually indistinguishable dispersion of 0.5% $^{w}/_{w}$ iron in iodized salt. The challenges of the scale-up of the particle agglomeration for size matching had been solved. The extruder was mechanically adjusted, and a spheronizer was used for cutting and shaping the extrudate into 300-600µm spheres. There were challenges with the colour masking and coating that were also overcome.

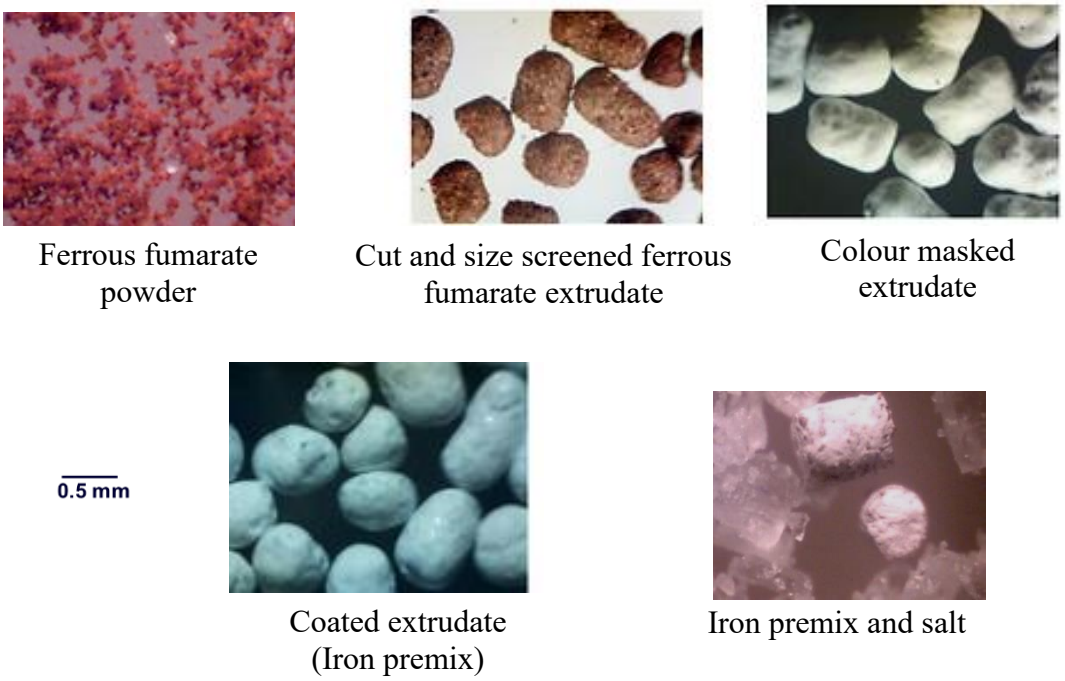


Figure 31:Ferrous fumarate through the development stages in Double Fortified Salt

In the laboratory, the colour masking was done by rubbing TiO_2 on the surface of the extrudate in a beaker; the coating was then carried out in a fluidized bed. There were some modifications to colour masking, and coating steps at the pilot plant. At the pilot plant, the colour masking and coating steps were carried out in a drum coater. The TiO_2 was dusted on the surface of the extrudates by the tumbling in the drum coater as the desired amount of coating material (HPMC

or Sepiflim solution (5% $^{w}/_{v}$ for desirable viscosity) or melted soy stearin) was sprayed on the extrudate. TiO_2 and coating material were applied intermittently, allowing it to stick the TiO_2 powder in place on the surface of the extrudates. These steps resulted in white iron premix samples that were barely distinguishable when mixed with salt (*Figure 31*).

5.1.1 Effect of Coating Composition on the Iron Content and Iron Bioaccessibility from the Premix Obtained from Pilot Plant

Based on the results of work at the University of Toronto, 25-35% $^{w}/_{w}$ TiO_2 was used for colour masking, and 5-10% $^{w}/_{w}$ of coating materials were used for coating in the pilot plant (Li et al., 2011; Yadava et al., 2012). Both hydrophilic and/or hydrophobic coats were tested. The total iron in the premix samples (1-9) obtained from the pilot plant was analysed; the result was used to calculate the percentage composition of iron in the premix. There was very little difference in the iron content of the nine premix samples. The premix contained 18-20% Fe (Figure 32). The result is consistent with previous studies (Li et al., 2011; Yadava et al., 2012) and the estimate from the material balance of the premix formulation. Given the percentage of iron in the premix, 1000ppm iron in the fortified salt is achievable by adding 5g of iron premix to 1kg of salt.

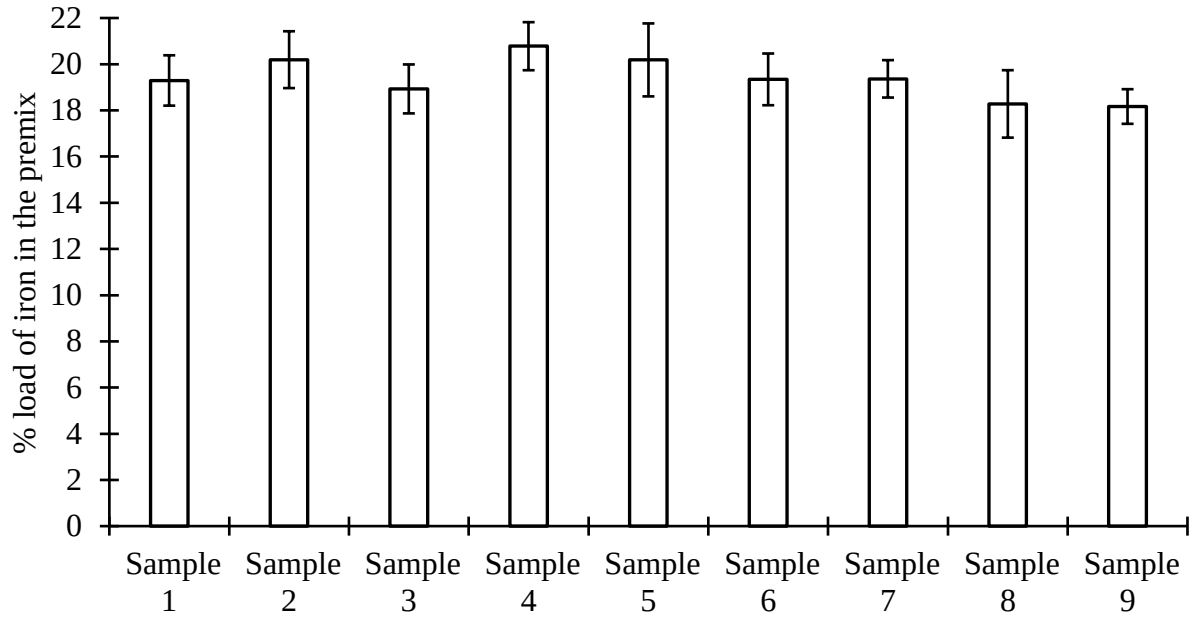


Figure 32: Effect of the coating composition on the iron content of the premix samples

Sample 1-9 varied in the amount of TiO_2 and coating materials used for making the premix. Samples 2-3 and 7-9 were colour masked with 30% TiO_2 while samples 1 and 4-6 were colour masked with 25% and 35% TiO_2, respectively. Sample 1 was coated with 10% HPMC, sample 2 was coated with 10% Sepifilm, sample 3 was coated with 5% soy stearin, samples 4 and 7, 5 and 8, and 6 and 9 were coated with 5, 7.5 and 10% HPMC, respectively. Values are average of four replicates ± standard deviation.

While it is essential to have adequate encapsulation of iron to prevent the interaction of iron with iodine, this must not prevent the bioavailability of iron. The premix and its coating are designed to disintegrate with most of the cooking methods; however, a few cooking methods may only soften but may not disintegrate the premix and the coating. Hence, it is imperative to study the bioaccessibility of iron from the premix. The bioaccessibility experiment was developed on the premises that once the coating is digested in the stomach, the iron from the premix should be bioavailable. The result (*Figure 33*) showed that the coating material in the amount used should be digestible in the stomach (even if the cooking method did not disintegrate the premix) as more than 50% of the iron in the premix was dissolved in the simulated gastric solution. Hence, the iron in the premix should be bioavailable for metabolic use, given that enzymatic actions will cause an increased iron bioaccessibility in the stomach. There was no clear-cut difference in terms of bioaccessibility between coating with 10% HPMC or 5% soy stearin. The bioaccessibility of iron from premix coated with either of the materials was higher than in premix coated with a costly Sepifilm.

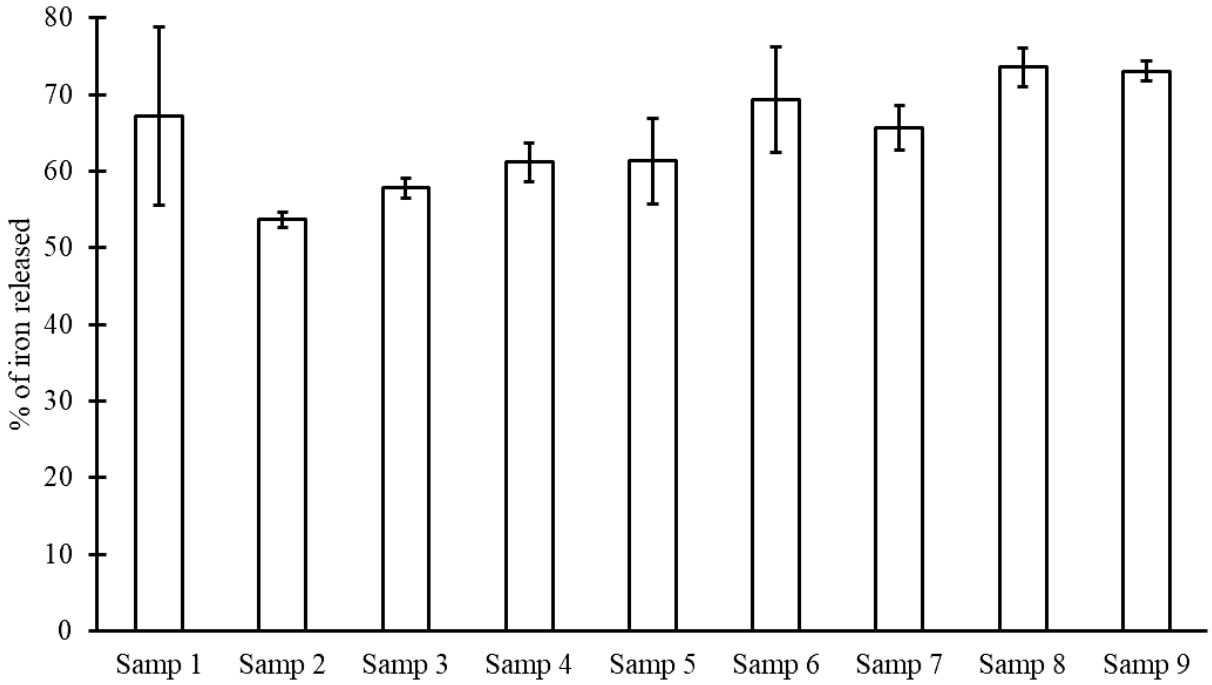


Figure 33:Effect of the coating composition on the iron bioaccessibility from premix at pH1 (0.1N HCl)

Sample 1-9 varied in the amount of TiO_2 and coating materials used for making the premix. Samples 2-3 and 7-9 were colour masked with 30% TiO_2 while samples 1 and 4-6 were colour masked with 25% and 35% TiO_2, respectively. Sample 1 was coated with 10% HPMC, sample 2 was coated with 10% Sepifilm, sample 3 was coated with 5% soy stearin, samples 4 and 7, 5 and 8, and 6 and 9 were coated with 5, 7.5 and 10% HPMC, respectively. Values are average of four replicates ± standard deviation.

5.1.2 Effect of Coating Composition on the Amount of Exposed Iron on the Surface of Iron Premix

The premix coating performs two functions - it holds the whitening agent in place and prevents the chemical interaction between iron and iodine, which may lead to the loss of the iodine in the fortified salt(Yadava *et al.*, 2012). Therefore, an ideal coating must result in a premix with little or no iron exposed on its surface. The amount of iron exposed on the surface of the premix samples can characterize the integrity of the coating. Two methods were used to determine the amount of exposed iron in this study. The first relied on the use of sodium EDTA, a good iron chelator(Romita, 2011). The second method described by Yadava et al. (2012) was based on the solubility of ferrous fumarate in 0.0001N HCl and the inability of this solution to dissolve the coating material used in the formulation of the premix.

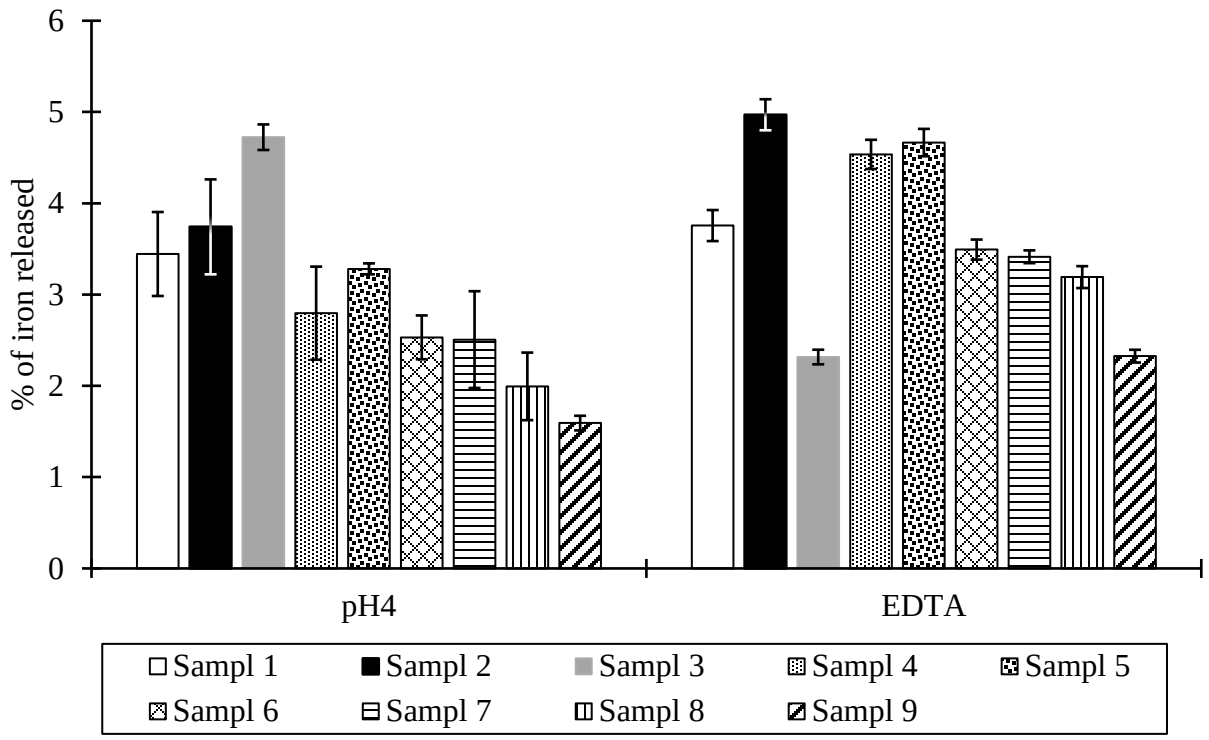


Figure 34:The amount of iron exposed on the surface of iron premix determined by dissolution in HCl solution pH4 and Na_2EDTA.

Sample 1-9 varied in the amount of TiO_2 and coating materials used for making the premix. Samples 2-3 and 7-9 were colour masked with 30% TiO_2 while samples 1 and 4-6 were colour masked with 25% and 35% TiO_2, respectively. Sample 1 was coated with 10% HPMC, sample 2 was coated with 10% Sepifilm, sample 3 was coated with 5% soy stearin, samples 4 and 7, 5 and 8, and 6 and 9 were coated with 5, 7.5 and 10% HPMC, respectively. Values are average of four replicates ± standard deviation.

With both methods, 5% or less of the iron in the premix was exposed (*Figure 34*). This value is close to those (< 10%) reported by Yadava et al. (2012) and implies a good coating. A good coat will prevent iodine loss from DFS. The amount of iron dissolve in EDTA from the surface of the premix seems to correlate better with the iodine lost from DFS after two months of storage; hence, it is more accurate than the other method (Figure 35a). Comparatively, coating with 5% soy stearin (fat) was better than coating with 5% HMPC for retaining iodine in salt (premix sample 3 vs. 7 in *Figure 34*). The amount of iron exposed on the surface of the premix samples (premix sample 4 vs. 7, 5 vs. 8 and 6 vs. 9 in *Figure 34*) showed that increasing the amount of TiO_2 (30 to 35%) used for colour masking may negatively affect the effective coating of the premix with HPMC. The use of 10% HPMC (premix sample 9 in *Figure 34*) resulted in less iron on the surface of the premix when 30% TiO_2 was used for colour masking. Hence, the use of 10% coating material and colour masking with 30% TiO_2 were subsequently adopted. From the result, the use of an excessive amount of TiO_2 as colour masking agent may not be necessary, as it will lead to a larger particle size requiring more coating material for effective coating.

5.1.3 Effect of Coating Composition on the Stability of Iodine in Double Fortified Salt

The interaction between iron and iodine in Double Fortified Salt that led to the loss of iodine was minimized by microencapsulation of the iron. Else, this will lead to the displacement of iodine from the iodate, which is then lost by sublimation. The impact of the coating composition of the premix obtained from JVS Foods Pvt. on iodine stability in DFS was investigated. In all the salts stored for two months, at least 75% iodine was retained irrespective of the conditions of storage (Figure 35a). The retention of iodine in DFS at the 6^{th} month of storage at $45^{\circ}C$ (Figure 35b) clearly showed that 10% $^{w}/_{w}$ coating was adequate if only 30% of TiO_2 is used for colour masking (salt sample 6 vs. 9 in Figure 35b). The iodine retention in DFS formulated with iron premix colour masked with 30% TiO_2 and coated with 10% HPMC (salt sample 9) was comparable to the iodine retained in the iodized salt (salt sample 10). Hence, the premix colour masked with 30% TiO_2 and coated with 10% HPMC was the best formulation in this test series.

The results were consistent with observations from the coat integrity evaluation, which showed that iron premix coated with soy stearin (sample 3) and 10% $^{w}/_{w}$ HPMC (sample 9) had the least

exposed iron. The Double Fortified Salts formulated with these premix samples were among the three salts with the highest iodine retention after 6-month storage at 45˚C.

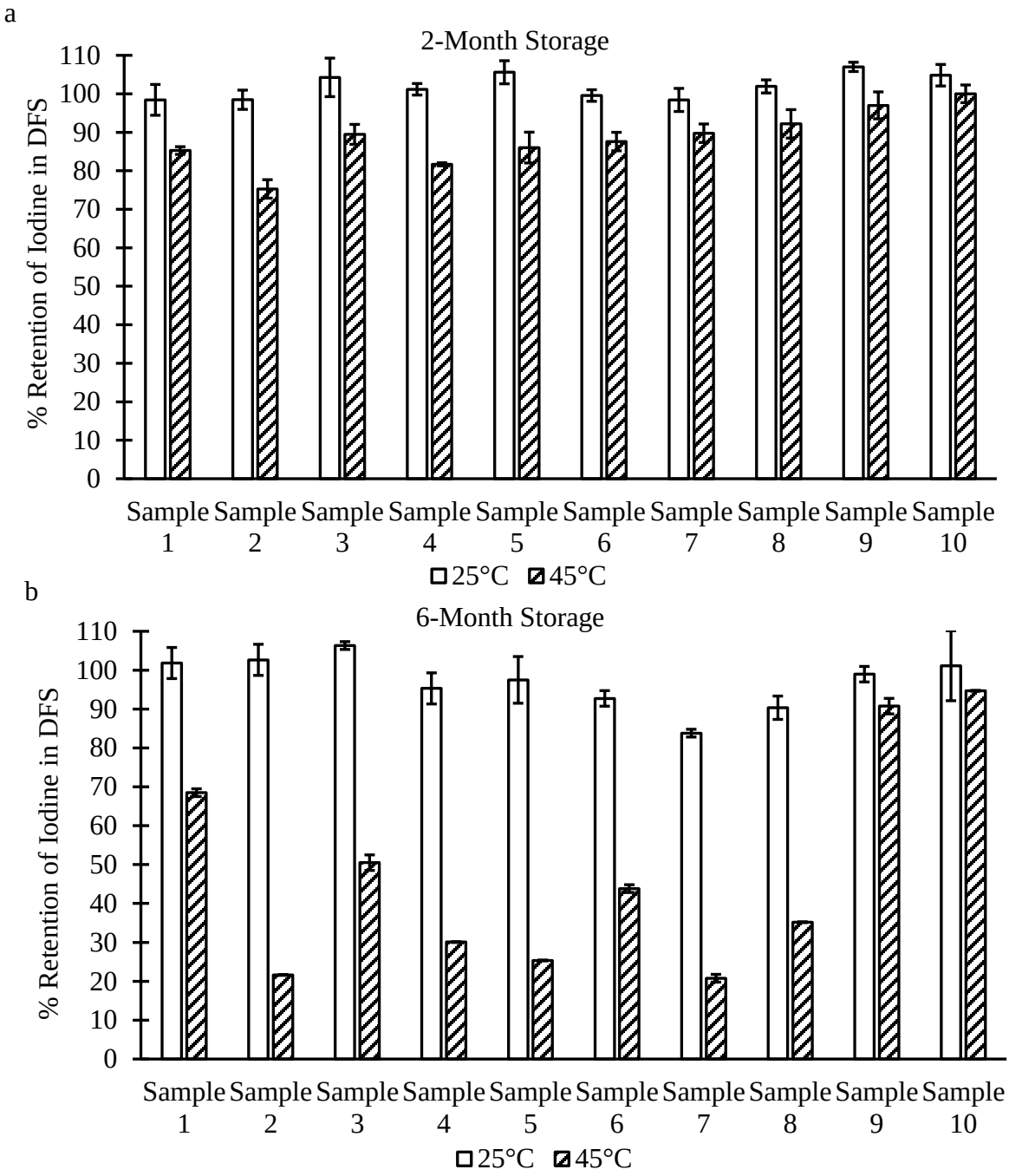


Figure 35:The Retention of Iodine in Double Fortified Salt at 2nd Month (a) and 6th Month (b) of Storage

Salt samples 1-9 were DFS samples made with corresponding premix sample 1-9, while sample 10 was an iodized salt. Values are average of four replicates ± standard deviation

Four factors impacted the loss of iodine from the salt - the amount of TiO_2 used to mask the brown colour of the extrudate, the properties of the material used to coat the premix, the amount of coating material used, and storage temperature. The use of more than 30% $^{w}/_{w}$ TiO_2 (as seen with salt sample 6 vs. 9 in Figure 35 b) does not result in effective coating with 10% $^{w}/_{w}$ of the coating material. The use of more of TiO_2 will require more coating material to hold the TiO_2 in place on the surface of the premix and to form an effective physical barrier between iron and iodine in a DFS. The use of 10% coating material resulted in a better physical barrier between iron and iodine than when 5% or 7.5% was used. Soy stearin being hydrophobic was a better coat than HPMC. When the same amount of TiO_2 and coating material is used (30% and 5%, respectively), soy stearin prevented the interaction between iron and iodine better than HPMC (as seen with salt sample 3 vs. 7). Its hydrophobicity impedes moisture penetration to the iron core. The increase in the storage temperature of the salt accelerated the impact of these factors.

5.2 Problems Encountered During the Scale-Up of Double Fortification Technology

The two problems were encountered during the scale-up of the technology for the double fortification of salt: floating of the premix in cold water and dark spots on the iron premix. These have the potential of negatively impacting the consumer acceptance of DFS. The iron premix that float can be lost when salt is washed before cooking. The dark spots are unsightly. This section covers the factors that may be responsible for the problem and the solutions proffered.

5.2.1 Floating of Iron Premix

Field tests of DFS in India indicated that premixes coated with soy stearin float in water. Two factors may be responsible for this – low particle density and hydrophobicity due to soy stearin. The densities of a premix coated with 10% soy stearin that floats and a premix coated with 10% HPMC that does not float in water were determined. Soy stearin has a density of ~ 0.9, so large amounts of coating material can reduce the particle density to less than 1.0, causing particles to float. This was the case with particles made previously by fluidized bed technology, where up to 25% of the particle was soy stearin (Oshinowo *et al.*, 2016). It was found that the particle and bulk densities of premixes coated with 10% $^{w}/_{w}$ HPMC and those coated with 10% $^{w}/_{w}$ soy stearin made

in the laboratory (Table 7) were more than 1.0g.cm^{-3}. Hence, surface tension due to the hydrophobicity of soy stearin but not density was responsible for the observed floating.

Two approaches were evaluated for solving the floating problem - dual coating with 5% HPMC and 5% soy stearin and coating with 11% mixture (10: 1) of soy stearin and lecithin. The two approaches significantly solved the floating problem (Table 7). The fact that lecithin, an emulsifier, solved the sinking problem confirmed that the hydrophobicity of soy stearin was the factor responsible for the observed floating. The lecithin wets the surface of the premix so that it sank in water.

Table 7: Impact of coating material on the particle and bulk density and floating properties of the iron premix

Coating materials	Particle density (g/cm^3)	Bulk density (g/cm^3)	Floating
10% SS	2.1±0.1	1.1±0.1	Significant
10% HPMC	2.2±0.2	1.1±0.1	Insignificant
10% SS + L	2.1±0.2	1.2±0.2	Insignificant
5% HPMC, 5% SS	**2.1±0.1**	**1.1±0.0**	**Insignificant**

Values are average of four replicates ± standard deviation; SS: soy stearin; HPMC: hydroxypropyl methylcellulose; L: lecithin

The iron release profile from premix favoured all the premix samples except the one coated with 10% soy stearin (*Figure 36*). All premixes, except the one coated with 10% soy stearin, released about 80% of their iron content before 60 minutes. The release profile is not in any way a disadvantage to premix coated with 10% soy stearin, as most cooking methods melt the fat. Even in the cases of mild cooking methods that would not melt the fat, the gastric lipases will digest the coat to make the iron bioavailable. From the microscopic imaging, there was no significant difference in the surface morphology of the four premix samples (*Figure 37*). The coating with 5% HPMC and then 5% soy stearin was adopted since the soy stearin will provide significant resistance to moisture, which the lecithin and soy stearin blended coat may not provide. While the dual coating increased the premix formulation by one step at the laboratory scale, this will have no significant impact at the pilot plant as drum coater can be used to apply both HPMC and soy stearin.

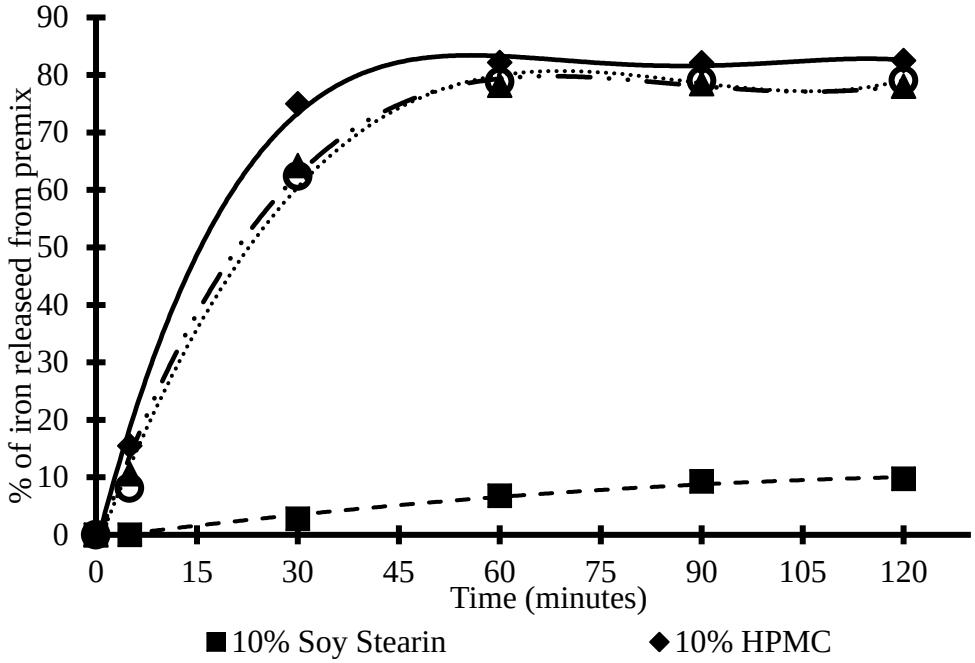


Figure 36: The effect of the optimized coating material on the time profile of iron release from the premix at pH 1

HPMC: hydroxypropyl methylcellulose; Values are average of four replicates; the standard deviations of the data points were so small that they did not show when inserted in the graph

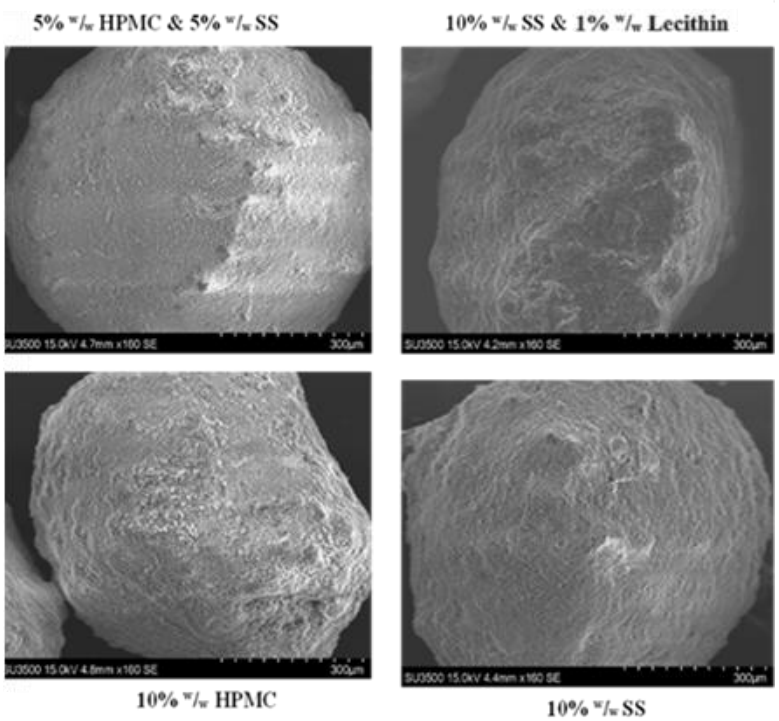


Figure 37: SEM images (magnification on the image) of iron premix coated with different materials. HPMC: hydroxypropyl methylcellulose; SS: soy stearin

5.2.1.2 The Effect of the New Coating Materials on the Stability of Iodine in DFS

All the premix samples except the one coated with soy stearin and lecithin were used to formulate DFS. The premix sample coated with soy stearin and lecithin was not used because a preliminary study showed that the coat did not provide an effective physical barrier between iron and iodine in the salt. In addition to the three premix samples made in the laboratory, another premix obtained from the pilot plant (coated with 5% $^{w}/_{w}$ HPMC and 5% $^{w}/_{w}$ soy stearin, as one of the premix made in the lab) was used to formulate DFS. The results showed that 10% $^{w}/_{w}$ coating material provided an effective barrier between iron and iodine in DFS. After 1-year storage, only 30% loss of iodine was observed in one of the salt samples at 45°C, and less than 10% of the iodine added was lost in all the other salts after 6-month storage. After one year at 45°C/60-70% RH, having soy stearin as the outer coat was marginally better than having HPMC as the outer coat. Also, the result supported our initial hypothesis that having 5% HPMC and 5% soy stearin will be as good as having a 10% soy stearin coat in terms of iodine stability in the salt (*Figure 38*). Hence, iodine should be very stable in DFS stored at a relatively high temperature (about 45 °C) if stored in a moisture-proof container/environment.

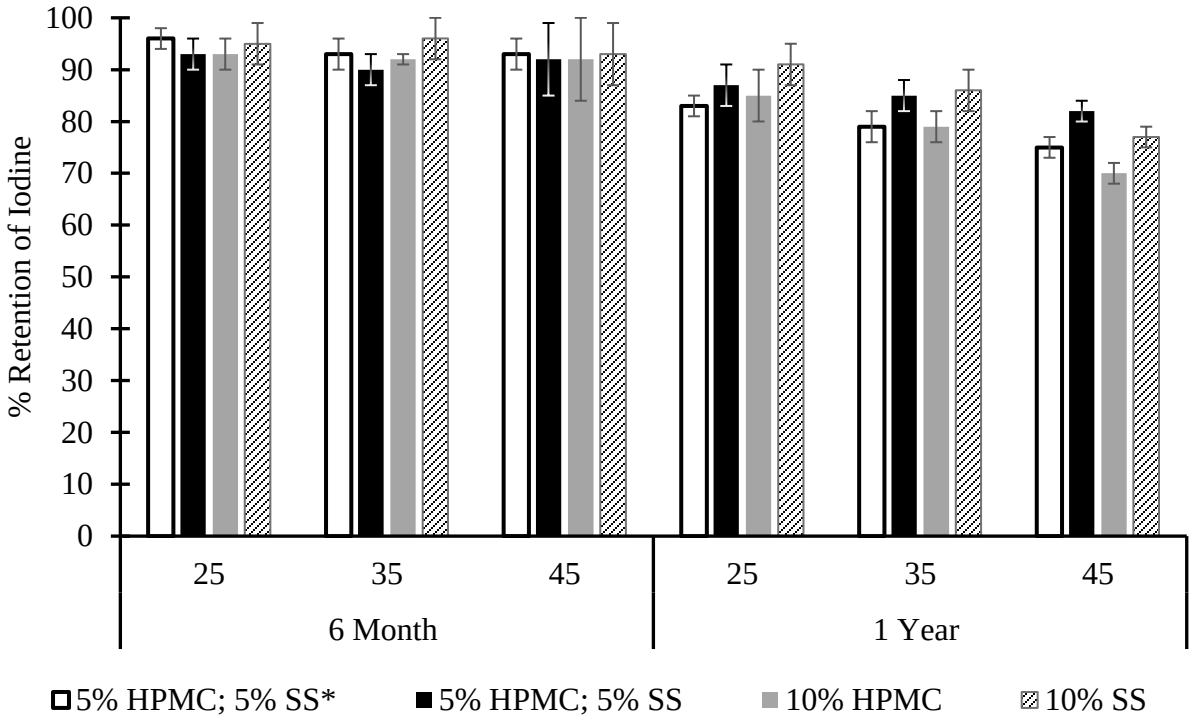


Figure 38:Stability of Iodine in DFS for 6 Months and 1 Year

*Sample was obtained from pilot plant in India; HPMC: hydroxypropyl methylcellulose; SS: Soy stearin. Values are average of four replicates ± standard deviation

5.2.2 Black Spots on the Surface of Iron Premix Particles

During commercial-scale production of iron premix, black spots were noted on the surface of some premix particles. In the plant, TiO_2 was dusted on the surface of the iron extrudate by tumbling in a drum coater while HPMC was sprayed. The HPMC ensured that TiO_2 adhered to the surface of the iron extrudates. However, the compressed air used to apply the HMPC solution blows away the TiO_2. So, an excess of TiO_2 was usually added to achieve the required whiteness. Before soy stearin is applied, the excess TiO_2, which contained some ferrous fumarate particles, was sieved out and reused in subsequent batches. The contamination of TiO_2 with iron was responsible for the black spot observed. Thus reducing the amount of excess TiO_2 added and eliminating the recycling of contaminated TiO_2 provides a solution to this problem.

5.2.2.1 Effect of the Amount of Titanium Dioxide on Iron Premix Colour

The first step was to determine the optimal amount of TiO_2 that is required for colour masking using a pan coater that simulates the drum coater used in the pilot plant. The fluidized bed was not used because it blows away loose TiO_2 so that the actual amount adhering to the surface was not known. Unlike the drum coater that was used to perform both the colour masking and coating functions, the pan coater was only used for coating. Before coating with pan coater, TiO_2 was dusted on the surface of the iron extrudate in a beaker. The whiteness of the premix coated with the pan coater was compared with those coated with a fluidized bed.

Table 8: L*a*b* Colour of Iron Premixes

TiO_2 (%) $^{w}/_{w}$	**Method**	**L***	**a***	**b***	**ΔL***
12.5	Fluidized Bed	75.6	0.7	-0.2	NA
25	Fluidized Bed	76.2	0.7	0.0	0.6
5	Pan Coater	73.6	0.5	-0.2	NA
10	Pan Coater	83.5	-0.4	-0.1	9.9
15	**Pan Coater**	**89.1**	**-0.6**	**-0.2**	**5.6**
20	Pan Coater	90.6	-0.5	-0.3	1.5

L^* (+ = lighter; - = blacker); a^* (+ = red; - = green); b^* (+ = yellow; − = blue); ΔL* was calculated subtracting the L* value of a premix coated with a less amount of TiO_2 from the other that is colour masked by the next level of TiO_2 (for example 10% -5%), the values are averages of 3 replicates.

The whiteness of the premix samples after they have been coated with HPMC and soy stearin showed that almost 50% of the TiO_2 is probably blown away in a fluidized bed (Table 8). The amount of TiO_2 lost was higher with the use of fluidized bed than with pan coater. With the pan coater, the whiteness of the premix increases with the increase in the amount of TiO_2 used for colour masking. However, the incremental increase was lower when the amount of TiO_2 used exceeded 15%. Hence, 15% $^{w}/_{w}$ was the optimal amount of TiO_2 required for colour masking as any additional TiO_2 does not significantly impact the whiteness of the premix.

5.2.2.2 Impact of the amount of titanium dioxide on the size and coating of the premix

Since the amount of coating material used in the premix was kept at 10% $^{w}/_{w}$ as established by Yadava et al. (2012), the change in the amount of TiO_2 used for colour masking will have an impact on the thickness of the coating material layer around the premix. The concentric volumes of the materials that encapsulated the iron core were extrapolated from the amounts and densities of material. The thickness of the layer was derived from the radii of the volumes. Three assumptions were made for the estimation – that the particles were spherical, uniformly distributed, and there was no loss of material during premix formulation.

The thickness of each layer of the materials that made up the premix is a function of the amount of the material used and the diameter of the premix before the material is applied onto the surface of the premix. Hence, by increasing the amount of material applied onto the premix, provided the prior dimension of the premix is kept constant, the thickness of the material in the premix would increase. However, if material is applied on the surface of a bigger premix, the thickness of the layer of the material applied would decrease compared to a smaller premix, if the same amount of material is applied.

Titanium dioxide, being denser than the other materials used in formulating iron premix, significantly impacts the density of the premix. The amount of TiO_2 used has a linear relationship with the density of the premix (Equation 12). Increasing the amount of TiO_2 used for colour masking increased the thickness of its layer in the premix. The increased thickness of the TiO_2 layer increased the size of the premix particle (*Table 9*). Since the amount of HMPC and soy stearin used was kept constant, the thickness of their layers was reduced as the amount of TiO_2 used was

increased(*Figure 39*). More so, the increase will reduce the thickness of the HPMC and soy stearin layers, which may reduce the effectiveness of the coating.

$$\rho_p = 0.0168(A_t) + 0.911 \quad \text{Equation 12}$$

$$T_{ss} = -0.0848A_t + 13.1 \quad \text{Equation 13}$$

$$T_{HPMC} = -0.0641A_t + 9.4 \quad \text{Equation 14}$$

Where ρ_p = weight of premix (mg)

A_t = amount of TiO_2 used for colour masking (% $^w/_w$)

T_{SS} = thickness of soy stearin layer (µm)

T_{HPMC}= thickness of HPMC layer (µm)

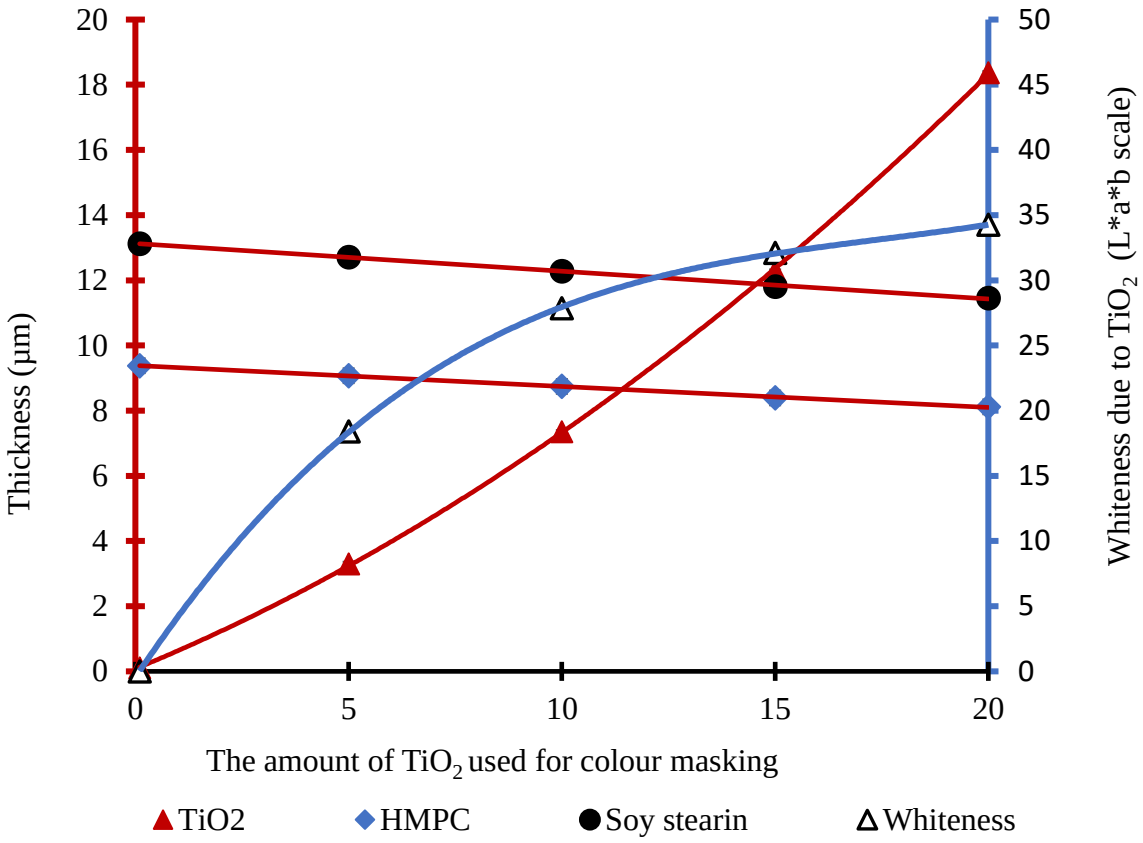


Figure 39: The effect of the amount of TiO_2 used for colour masking on the whiteness of premix and thickness of HPMC and soy stearin layer.

The whiteness is against the right Y-scale while the others are against the left Y-Scale. Whiteness due to TiO_2 was calculated by subtracting L* of premix coated without colour masking with TiO_2 (L*=56.66) from other premix coated and colour masked with varying amounts of TiO_2. Values are average of four replicates; the standard deviations of the data points were so small that they did not show when inserted in the graph

Table 9: The effect of the amount of titanium dioxide used for colour masking on the radius of iron premix, particle, and bulk densities

TiO_2 used (% $^{w}/_{w}$)	0	5	10	15	20
Particle radius µm	360.9	361.4	363.1	366.0	374.8
Particle density(g/cm^3)	-	2.0±0.0	2.1±0.0	2.1±0.0	2.18±01
Bulk density (g/cm^3)	-	1.0±0.0	1.1±0.0	1.2±0.0	1.24±0.0

5.2.2.3 Feasibility of Suspending Titanium Dioxide in the Coating Material

In the pilot plant, the drum coat operator uses an excessive amount of TiO_2 for colour masking. This is to quickly achieve the desired whiteness because TiO_2 is blown by the compressed air used to apply the HPMC that glues it to the surface of the premix. The excess TiO_2 is recovered and reused in the next batch. The use of 15% TiO_2 (the optimal amount for colour masking) will not prevent the compressed air required for applying the coating material from blowing away the loosely attached TiO_2 from the surface of the iron extrudate. The operators will still be forced to use an excessive amount of TiO_2. Applying a suspension of TiO_2 in the coating material will eliminate this possibility. The feasibility of coating premix with a suspension of TiO_2 in HPMC or soy stearin solution was evaluated as a potential route to permanently solving the reuse of TiO_2. The whitening and coating agents will be applied together, like white paint on the surface of the extrudate.

5.2.2.3.1 Effect of the Amount of Extrudate in a Pan Coater (Diameter=16cm) on Coating

For the suspension of TiO_2 in HPMC or soy stearin, the loading capacity of the drum/pan coater (the amount of extrudate per unit size of pan coater) will affect the quality of coating (Pandey *et al.*, 2006). The iron extrudate (5-20g) was coated with a suspension of 15% TiO_2 in 10% soy stearin. Figure 40 shows the distribution of extrudate in the pan coater. There were spaces at the base of the pan coater uncovered by the extrudate when 5g and 10g extrudate were added. The base of the pan coater was sufficiently covered by 15g extrudate. The additional 5g added to make 20g extrudate formed an additional layer of the extrudate in the pan coater.

For the pan coater used (diameter = 16cm), 15g extrudate is the optimal amount. A lower loading capacity resulted in most of the coating blend falling on the base of the pan coater and not the extrudate, while higher loading resulted in few of the extrudates particles not effectively coated.

For a lower loading capacity, the coated extrudate has a uniform but lower degree of whiteness. For a higher loading capacity, there was no uniform degree of whiteness (Figure 40). The result shows that the amount of extrudate per pan coater is a critical factor. Hence, the optimal loading capacity of the drum coater used at the pilot plant must be determined to achieve a high-quality coating.

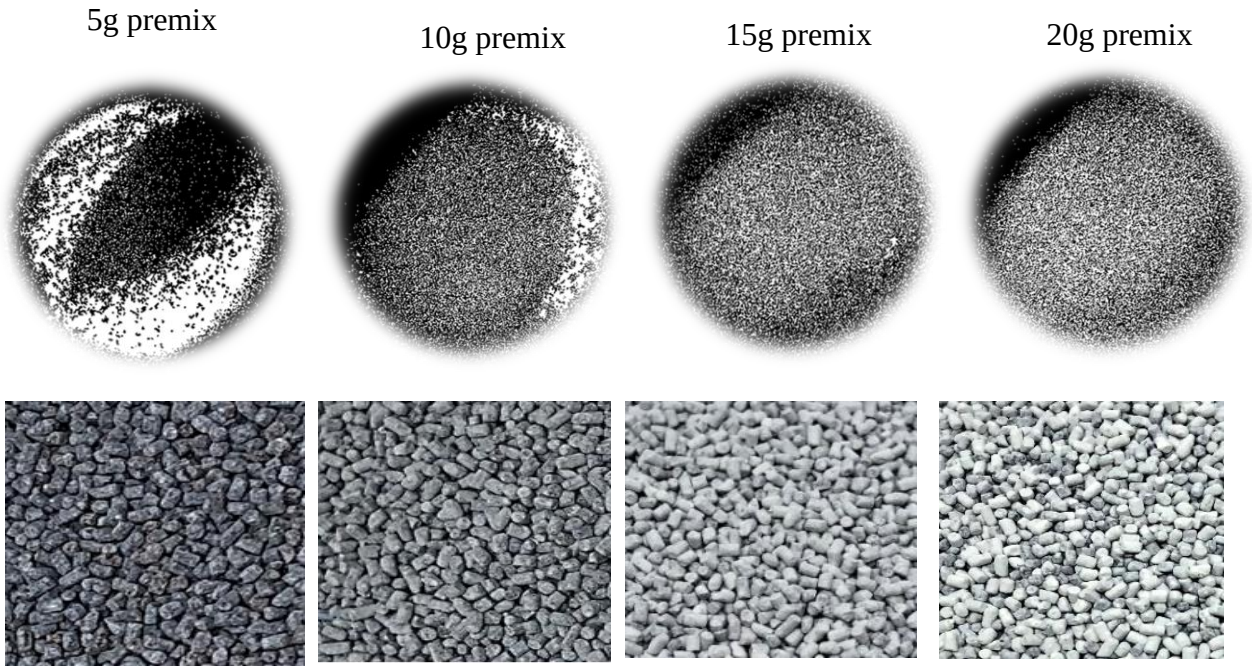


Figure 40: The effect of the amount of extrudate in a pan coater (diameter =16cm) on a uniform and effective coating of the iron extrudate. The L* value for the premix is 66.46, 79.48, 86.37, and 90.83, respectively.

5.2.2.3.2 Effect of coating with a mixture of Titanium Dioxide and Coating Material on Whiteness and Other Properties of Iron Premix

By using the optimal amount of extrudate (15g) per pan coater, the impact of coating with a suspension of TiO_2 in HPMC or soy stearin solution on the whiteness of premix was evaluated. First, the contribution of either HPMC or soy stearin to the whiteness of iron premix was determined without colour masking with TiO_2. From the colour analysis of the coated extrudate (Table 10), soy stearin contributes more to the whiteness of the premix than HPMC.

Coating with TiO_2 suspended in soy stearin resulted in a whiter premix than coating with TiO_2 suspended in HPMC. Hence, the suspension of TiO_2 in soy stearin was used in subsequent sets of the coating. Next, the adequate amount of TiO_2 required for the mixture was determined; the use

of 10% coating material was maintained. The amount of TiO_2 used corresponded to the degree of whiteness of the premix. However, the ΔL* decreases as the amount of TiO_2 increases. The premix was stirred in cold water to evaluate the effectiveness of the method to hold TiO_2 in position on the premix surface. There was no noticeable TiO_2 that fell off from the premix or exposed iron on the surface of the four premix samples, except in premix coated with a suspension of 20% TiO_2 in 10% soy stearin solution (Table 11). The capacity of the amount of soy stearin used to tightly stick the titanium dioxide (20%) on the surface of the premix may have been overwhelmed. Although colour masking with 15% TiO_2 then coating with 10% SS resulted in the highest degree of whiteness, it was not selected, as the optimal approach, because it will cause premix to float in water.

Table 10:Impact of coating with a blend of coating material and titanium dioxide on the colour of the premix

Coating Material	L*	a*	b*
No colour masking and coating	33.0	14.8	22.6
10% HPMC	37.4	11.5	15.9
10% Soy stearin	56.7	5.0	7.9
5% TiO_2 + 10% SS Blend	64.4	0.3	-4.2
10% TiO_2 + 10% SS Blend	76.8	-1.2	-3.7
15% TiO_2 + 10% SS Blend	86.4	-1.5	-0.7
20% TiO_2 + 10% SS Blend	91.9	-1.7	-1.8
10% TiO_2 + 10% HPMC Blend	60.3	1.7	0.8
15% TiO_2 + 10% HPMC Blend	74.0	-0.5	-2.5
15% TiO_2 colour mask, then 10% SS coat	94.0	-0.1	2.7
15% TiO_2 colour mask, then 10% HPMC coat	88.7	-0.5	1.8
15% TiO_2 colour mask, then 5% HPMC and 5% SS coat	89.1	-0.57	-0.2

L* (+ = lighter; - = blacker); a* (+ = red; - = green); b* (+ = yellow; − = blue); the values are averages of 3 replicates; HPMC: hydroxypropyl methylcellulose; SS: Soy stearin; the premix colour masked with 15% TiO_2 then coated with 10% SS has the highest degree of whiteness, but it was not selected because it floats in water.

These observations were confirmed with the scanning electron microscope (SEM) image of the premix (Figure 41). The 2700 × magnified SEM images clearly showed the dispersed distribution of TiO_2 in premix coated with a suspension that has the lowest amount of TiO_2. The dispersed

distribution is responsible for the lower degree of whiteness observed. The distribution of TiO_2 became denser as the amount of TiO_2 in the coating mixture increased. The 35× magnified SEM images showed that TiO_2 fell off the from the premix coated with a mixture of 20% TiO_2 and 10% soy stearin. This was not observed in the other three samples.

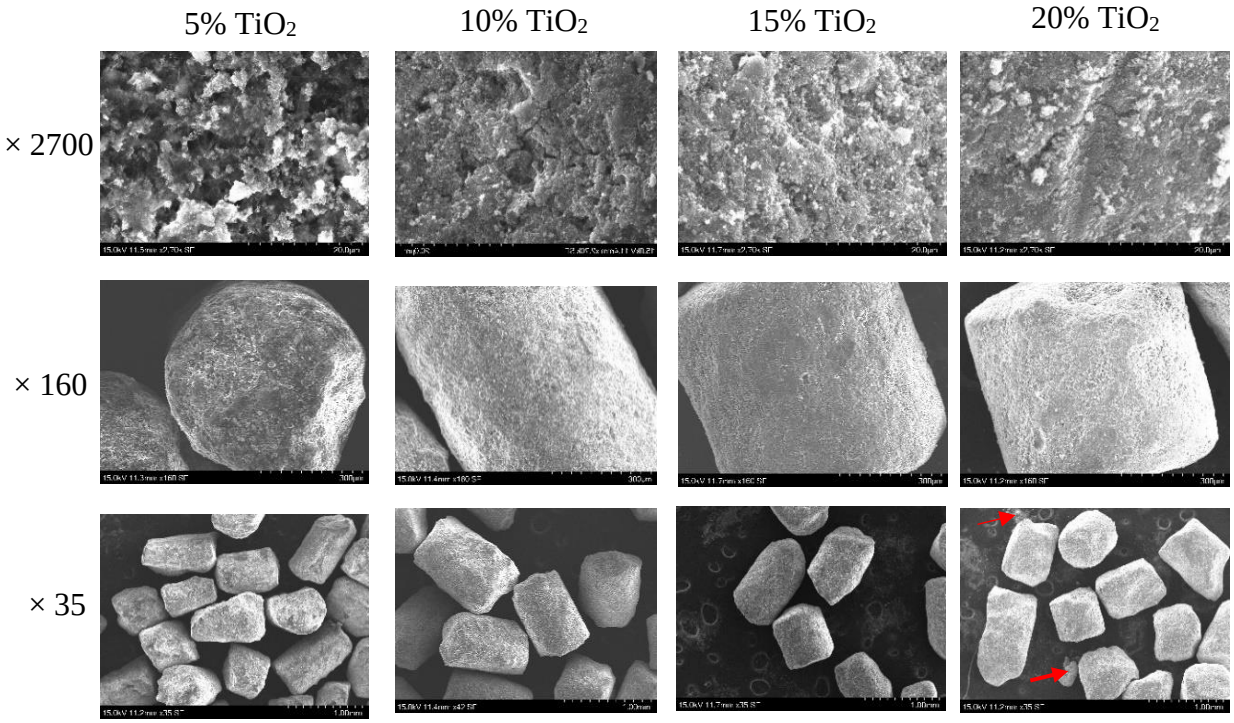


Figure 41:The impact of the amount of TiO_2 on the surface morphology of premix coated with a mixture of TiO_2 and soy stearin.

Table 11:Physicochemical properties of premix coated with a suspension of TiO_2 in soy stearin

Coating Mixture	Bulk Density (g/cm^3)	Particle Density (g/cm^3)	TiO_2 Falling Off	Amount of Iron Released (%)
5% TiO_2 + 10% SS	1.0±0.0	2.0±0.0	Not observed	0
10% TiO_2 + 10% SS	1.1±0.0	2.0±0.0	Not observed	0
15% TiO_2 + 10% SS	1.2±0.0	2.1±0.0	Not observed	0
20% TiO_2 + 10% SS	1.2±0.0	2.1±0.0	Observed	7.5±0.1

SS: soy stearin; the TiO_2 was suspended in a solution of soy stearin in dichloromethane

A solution of Na_2EDTA (5% $^{w}/_{v}$) was used to check for surface integrity. The solution being an iron-chelating agent will dissolve any iron it touches. Since iron is in the core of the premix, the Na_2EDTA solution will only have iron if it penetrates through the coat to the iron core. The solution did not penetrate the premix samples except the one coated with a suspension of 20% TiO_2 in 10% soy stearin (Table 11). The amount of TiO_2 used in the suspension tends to impact the particle and bulk density of the premix. There was a significant increase in the bulk and particle density as the amount of TiO_2 increases. However, the difference in the density was small and did not affect the floating of the premix. All the premixes sank when dropped into water.

The time-release profile of iron from these premix samples was different from the premix that was first colour masked and dual coated with HPMC and soy stearin (Figure 36 vs. Figure 42). For the latter, most of the iron was released before 30 min. For the premix coated with a suspension of 15% TiO_2 in soy stearin, 80% of the iron content was released after 2 hours. The sharp difference will have no impact on the bioavailability of iron from the premix, as most of the cooking methods will melt fat. Even with some mild cooking methods, the gastric lipases will digest the fat coat.

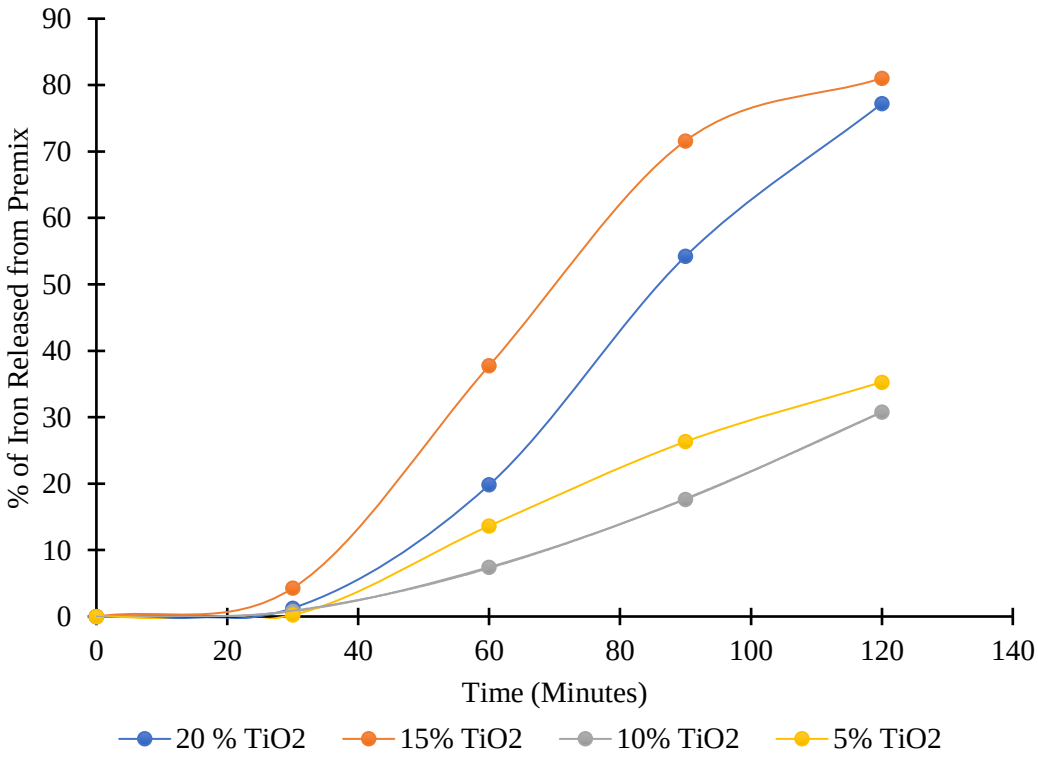


Figure 42:Bioaccessibility of iron from premix coated with a mixture of TiO_2 and soy stearin.
The amount of soy stearin used was the same in all the samples. The standard deviations of the data points were so small that they did not show when inserted in the graph

These observations support the use of a suspension of 15% TiO_2 in 10% soy stearin for coating iron extrudate. In terms of the whiteness of the premix, there was no significant difference between coating with a suspension of 15% TiO_2 in 10% soy stearin and colour masking first (with 15% TiO_2) before dual coating with 5% HPMC and 5% soy stearin. This premix did not float in water, and there was no noticeable TiO_2 falling off from the premix coated with this suspension. More so, the premix coated with the suspension can withstand the mechanical friction of mixing with a ribbon blender, as the integrity of the coating was intact after mixing with salt in the ribbon blender.

The configuration of the drum coater used at the pilot plant can allow for applying this suspension. Coating with the suspension solves the fundamental problem that led to the use of an excessive amount of TiO_2- the blowing away of TiO_2 from the surface of extrudate due to the use of compressed air. It will enhance the adequate adhesion of the TiO_2 powder by the soy stearin on the surface of the dark iron spheres. If this can be achieved at the pilot plant, recycling of contaminated TiO_2 will no longer occur. Hence, the ultimate solution to the black spots observed on the premix may be coating with a suspension of TiO_2 in soy stearin. However, the impact on the retention of iodine in DFS still needs to be tested.

5.3 Understanding the Chemistry of Interaction among Micronutrients in the Fortified Salt

The understanding of the chemistry of interaction among micronutrients is vital for developing a robust process for adding micronutrients to salt. The loss of iodine in the fortified salt is through a redox reaction between ferrous iron and iodate that leads to the production of elemental iodine from potassium iodate (Diosady *et al.*, 2002). The iodine is then lost via sublimation. Li et al. (2010) reiterated that the loss of iodine in salt when in contact with iron followed the stoichiometry ratio of 5:1 iron to iodate. Hence, the use of stabilizers which chelate iron so that it is not available for reaction with iodate and ultimately microencapsulation (the physical separation of iron from iodine) prevent the interaction of iron and iodine in fortified salt (Diosady *et al.*, 2002; Li, Diosady and Wesley, 2009). The microencapsulation of the extruded ferrous effectively separated the iron and iodine, such that most iodine was retained even at 45 ^{o}C and 70% RH (Li, Diosady and Wesley, 2010; Modupe, Krishnaswamy and Diosady, 2019).

Unlike iodine and iron, there is a paucity of understanding of how folic is lost and how it can be prevented in the salt. The three molecules that make up folic acid can dissociate, so can the amide

group on C2. The loss of any of these molecules or functional groups will lead to the loss of the vitamin potency. Folic acid is relatively stable at high temperatures; in a solid state, it starts to degrade at 180 ^{o}C with the cleavage of folic acid to glutamic acid and pteroic acid. At 200 ^{o}C, it degrades to 2-amino-6-formyl-1H-pteridin-4-one (6-methyl pterin), 4- aminobenzoic acid, and glutamic acid (Gazzali *et al.*, 2016). Nagaraja et al. (2002) indicated that oxidative cleavage of folic acid yields the same products as thermal degradation. The same authors also described the products of reductive cleavage as p-amino-benzoyl glutamic acid and 6-methyl pterin. Although the thermal stability of folic acid suggested that it can be extruded, there is no definite knowledge of its compatibility with iron. Hence, a simpler route (making a solution of folic acid and iodine) was first explored.

Folic acid is sparingly soluble in water and requires sodium hydroxide or carbonate to be very soluble. Carbonate was chosen because it is more compatible with food applications (McGee, Sangakkara and Diosady, 2017). The targeted amounts of folic acid and iodine were very stable and soluble in 70 mM sodium carbonate (Modupe, Krishnaswamy and Diosady, 2019). In the spray solution, the sodium salt of folic acid was observed. The molecular weight from MS spectra obtained suggests that there are two types of sodium salt- mono- and disodium salts of folic acid (Figure 43).

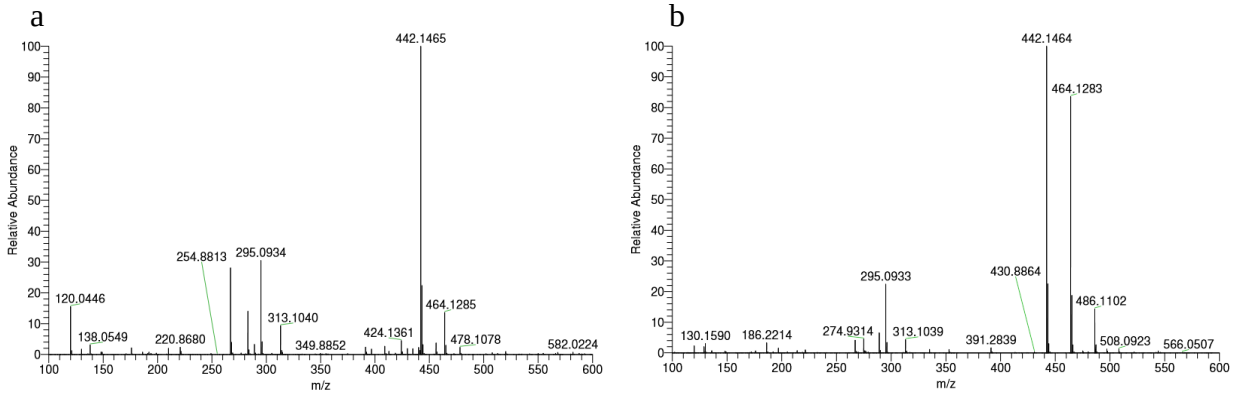


Figure 43:MS Spectra of Folic Acid in Sodium Carbonate Solution (a) without Potassium Iodate (b) with Potassium iodate

The m/z=464 and 486 indicate the formation of sodium salts of folic acid

Given that folic acid would have fully dissociated at the pH of the solution (pH 9), the two carboxyl groups attached to the glutamic moiety of the folic acid may have participated in the formation of the sodium salts. The presence of potassium iodate (iodine fortificant) in the solution seems to have favoured the formation of the disodium salt of folic acid as well as increasing the abundance

of the monosodium salt of folic acid. The formation of the sodium salts of folic acid is responsible for the solubility of folic acid in the sodium carbonate solution. Also, the increased abundance of the sodium salts of folic acid may be responsible for the relatively higher stability of folic acid in the spray solution that contained potassium iodate and folic acid than those that contained just folic acid. The sodium salts of folic acid may be more stable than folic acid (Blum, 1990).

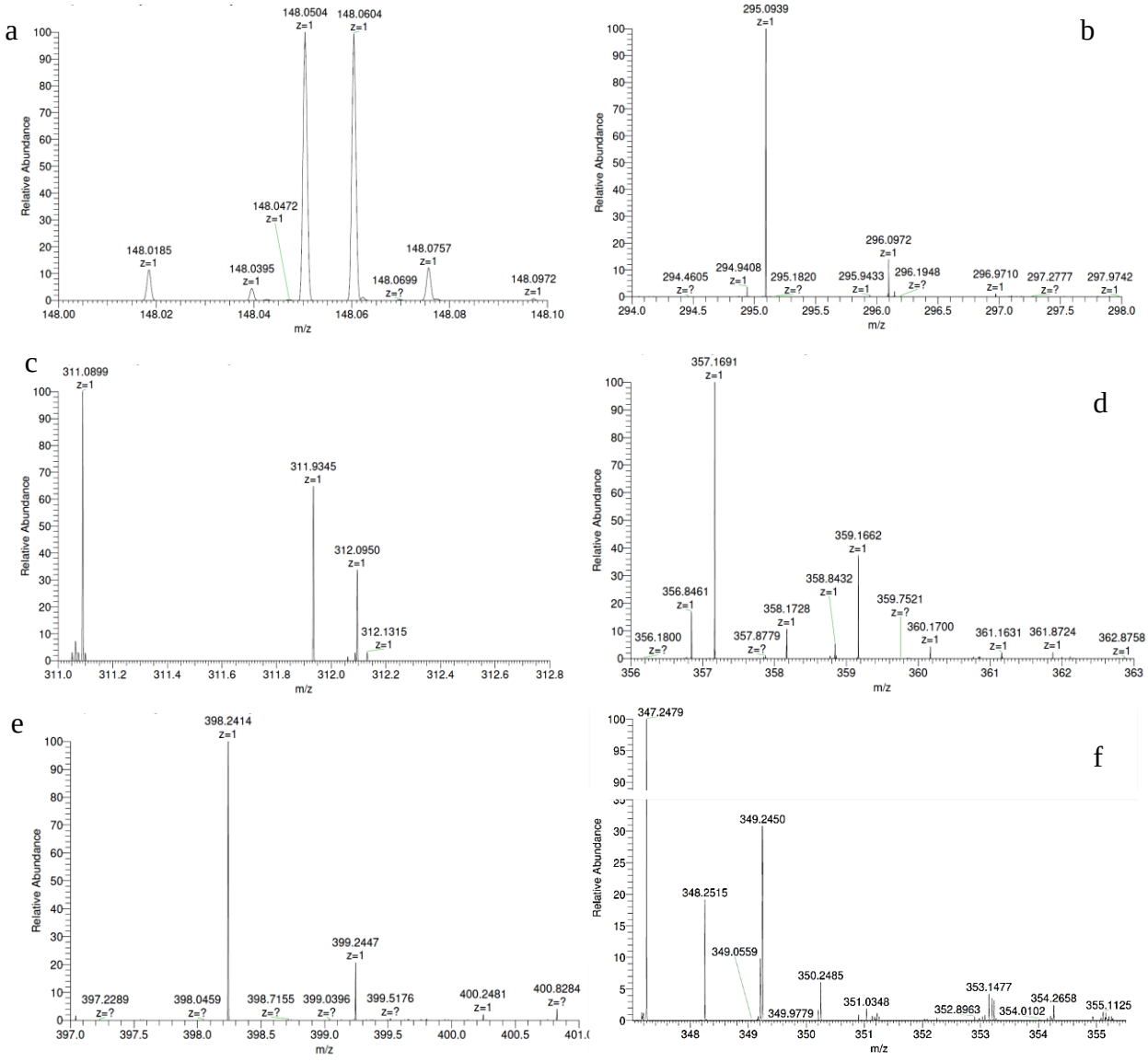


Figure 44:MS Spectra of the Candidates of Folic Acid Degradation Products

a) Glutamic acid; b) aldehyde of pteroic acid; c) pteroic acid; d) & e) decarboxylated folic acid; f) chloride additive of pteroic acid

The mono and disodium salts of folic acid in the solution have been identified and found to enhance the stability of folic acid in the solution. Hence, the solution was used to formulate Triple Fortified

Salt (TFS). The next step was to identify the products of degradation of folic acid in the salt. The identification is a feasible route for elucidating the impact of the chemistry of the interaction among the micronutrients on folic acid. The total ion chromatogram (TIC) spectra of freshly prepared TFS and after its storage for six months were obtained and subjected to differential analysis using the Compound Discoverer Software. This analysis reliably provided information on the products of degradation of folic acid and iodine.

The differential analysis on the TIC spectra found a compound (348 g/mol) as a valid product of degradation of folic acid. The fingerprint of the spectra of the product showed that it is a chloride compound (*Figure 44*f). The subtraction of the atomic mass of chlorine (35.5) from the molecular weight of the compound (348 g/mol) suggested that the compound detected is a chloride additive of pteroic acid (Modupe, Krishnaswamy and Diosady, 2019). Also, compounds with molecular weights 148, 295, 311, 357, and 398 g/mol were found on the TIC analysis of the TFS sample stored. These are probably glutamic acid, aldehyde of pteroic acid, pteroic acid, and decarboxylated folic acid. Most of these compounds, especially the glutamic acid and pteroic acid, further confirm the postulation that the compound identified by the software is a chloride additive of pteroic acid.

There are four known major routes for folic acid degradation: reductive degradation, radio/photolysis, thermal degradation, and oxidative cleavage. Only the thermal degradation and oxidative cleavage are likely under the conditions of storage and the constituents of the fortified salt. Also, the products of degradation observed on the TIC of the degraded folic acid in the salt sample suggested both thermal and oxidative degradation.

Although the formation of the sodium salt of folic acid seems to mitigate the oxidative degradation, ferrous fumarate (the iron fortificant) will inhibit the oxidative degradation better than the formation of the sodium salt of folic acid. More so, adding the folic acid directly to salt as a solution would affect the colour of the salt. Hence, a premix of folic acid and ferrous fumarate was formulated. In this premix, the reductive potential of the ferrous fumarate stabilized folic acid. The stability study carried out on the premix supported the hypothesis of oxidative degradation for folic acid. In the study, the stability of folic acid was higher when ferrous fumarate and folic acid were coextruded than in other samples. Also, from the study, it was observed that TiO_2 aided the photocatalytic degradation of folic acid. Thus, further supporting the coextrusion of ferrous fumarate and folic acid so that folic acid is hidden from direct light.

The loss of folic acid in the salt samples was temperature dependent. So, thermal degradation was involved. Although there are studies that showed that folic acid in the solid-state is stable to temperatures below 180 ^{o}C, in these studies, folic acid was only subjected to high temperatures for only a few hours (Vora *et al.*, 2004). So, prolonged exposure to high temperatures may have caused the observed degradation. More so, the decarboxylation products of folic acid degradation support the idea that oxidation and thermal degradation were the routes for the loss observed. Barrett and Lund (1989) suggested that both oxidation and thermal degradation of folic acid can occur in concert.

Given the predicted products, the logical pathway for the degradation of folic acid in the salt is illustrated in *Figure 45*. Folic acid was decarboxylated in two steps. In parallel, some folic acid degraded to glutamate and 4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzaldehyde. The latter was oxidized to 4-(((2-amino-4-oxo-4,8-dihydropteridin-6-yl)methyl)amino)benzoic acid (pteroic acid). The pteroic acid was chlorinated.

Chem3D Software was used to predict the structure of the chloride. By using the software, the Huckel charges on the atoms of pteroic acid and the bond lengths and angles between the atoms were estimated. N8 has the maximum positive partial charge (0.50) of the possible atoms that were available for chlorine interaction. Also, the bond length of C5-N8 and N8-C12 and the bond angle of C5-N8-C12 favours N8 as the atom likely to interact with a chlorine atom. Hence, the chloride was determined to be 4-(((2-amino-4-oxo-8-chloro-4,8-dihydropteridin-6-yl) methyl) amino) benzoic acid.

The understanding of the mechanism of degradation of folic acid in the salt and the colour problem of folic acid guided the choice of the right process for the formulation of TFS. Also, the detected products of degradation of folic acid guided the development of a robust analytical method that is selective for folic acid in the presence of the products of degradation. More so, analytical method can be developed to target the products of folic acid degradation in salt. This may lead to having a robust quality control for the process of triple fortification of salt. With this understanding, TFS was formulated with a premix that contained ferrous fumarate and folic acid and a solution of potassium iodate.

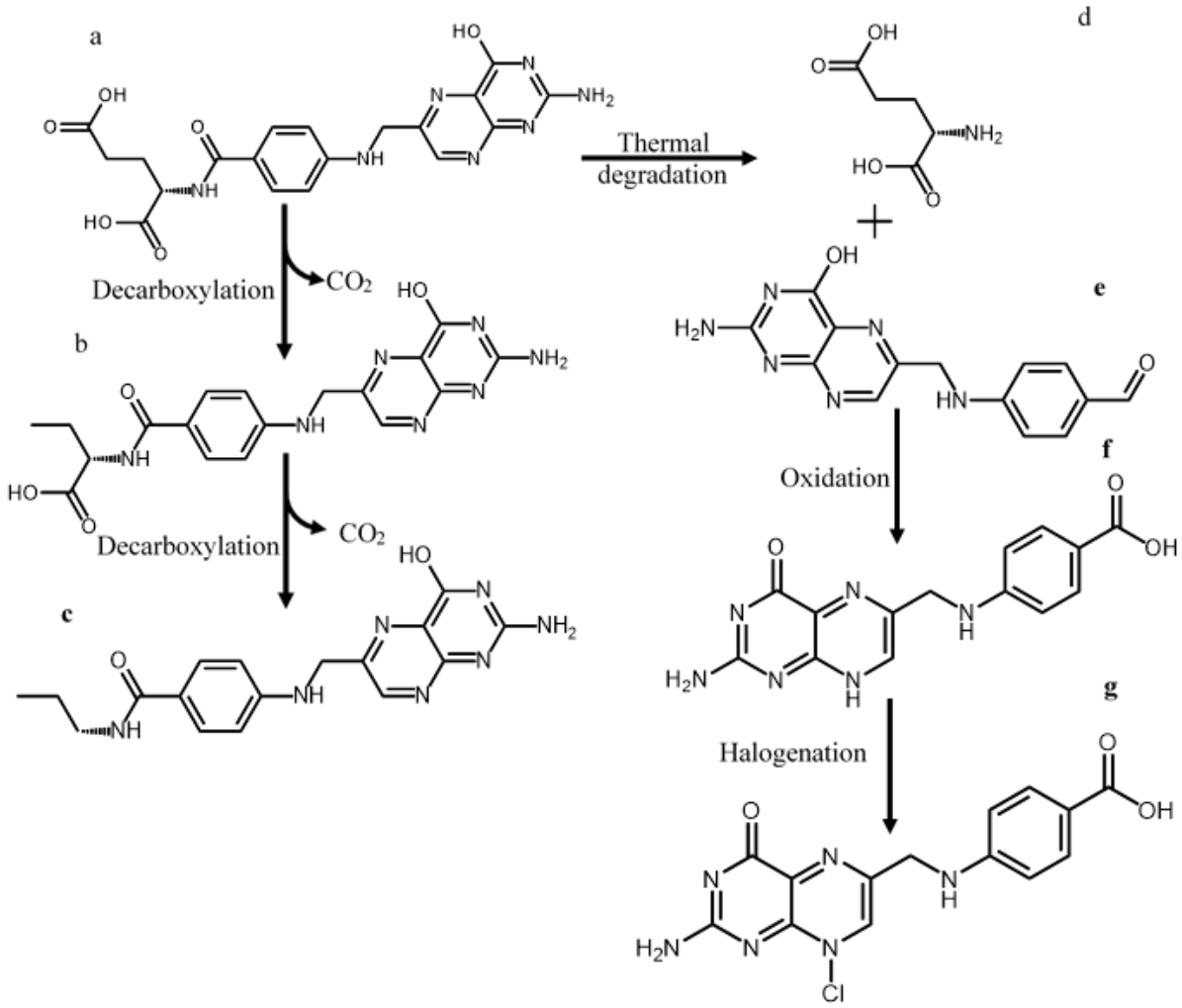


Figure 45:Proposed degradation pathway for folic acid in Triple Fortified Salt

a) (4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzoyl)-L-glutamic acid (folic acid)
b) 2-(4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzamido)butanoic acid (decarboxylated folic acid)
c) 4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)-N-propylbenzamide (decarboxylated folic acid)
d) 2-aminopentanedioic acid (glutamic acid)
e) 4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzaldehyde (aldehyde of pteroic acid)
f) 4-(((2-amino-4-oxo-4,8-dihydropteridin-6-yl)methyl)amino)benzoic acid (pteroic acid)
g) 4-(((2-amino-4-oxo-8-chloro-4,8-dihydropteridin-6-yl)methyl)amino)benzoic acid (chloride additive of pteroic acid)

Just as with the triple fortification of salt, it was initially thought that formulating Quadruple Fortified Salt (QFS) by adding ferrous fumarate premix to salt fortified with a solution of iodine, folic acid, and vitamin B_{12} would be well compatible with the established technology for the double fortification of salt. Hence, a solution of iodine, folic acid, and vitamin B_{12} was formulated. Given the experience with the triple fortification of salt, 70 mM sodium carbonate was used to formulate the solution. All the micronutrients were stable in the solution, except vitamin B_{12}. pH was thought to be the factor responsible. Hence, solutions of iodine, folic acid, and vitamin B_{12} with adjusted

pH (8,9,10 and 11) were formulated. The stability of vitamin B_{12} had a negative correlation with the pH of the solution. Even at pH 8, the stability of vitamin B_{12} did not meet the set target (at least 70% retention after 2 months). Attempts to stabilize vitamin B_{12} in the solutions with antioxidants proved abortive. Instead, ascorbate, one of the antioxidants used, subjected vitamin B_{12} to photolytic degradation through protonation; it decolorised vitamin B_{12} on exposure to natural light (See Section 'Quadruple Fortification of Salt' for details).

This understanding of the incompatibility of vitamin B_{12} in solution guided the development of process for the quadruple fortification of salt. Vitamin B_{12} was first removed from the spray solution and added to the iron premix. The full understanding of folic acid degradation (as explained above), and its impact on the colour of the salt when added as a solution, ultimately led to the formulation of a premix of iron, folic acid, and vitamin B_{12}. The premix was added to iodized salt to form a QFS.

5.4 Development of Analytical Method for Folic Acid

An analytical method that is accurate and selective for folic acid in the presence of other analytes (especially products of degradation of folic acid and other components of salt) is crucial for the process developed for adding folic acid to salt. For quality control and to monitor the stability of folic acid in the salt, it is essential to have a method for quantifying folic acid in the fortified salts. The gold standard method for quantifying vitamins, especially the water-soluble vitamins, consist of microbial assay (Yin *et al.*, 2018). In the recent past, a lot of HPLC based methods have been developed for quantifying folic acid (Arcot and Shrestha, 2005). Neither the microbial assay nor the HPLC methods are compatible with quantifying folic acid in fortified salt. Given the very low concentration of folic acid in the salt and the difficulty in differentially extracting folic acid from salt, there are concerns that the microbes used in the standard assay may not survive under a high salt concentration environment, or they may not perform optimally. Also, there were concerns that the salt will precipitate in the HPLC column, especially in in the case where a large number of samples are expected.

A method developed by Nagaraja et al. (2002) could have been used. This method is based on the catalytic and reductive cleavage of folic acid with zinc granules and hydrochloric acid, diazotization of the cleaved product with nitrite, and coupling with 3-amino phenol. However, the method was not reproducible in the salt matrix. Moreover, the method involves many reaction

steps, which exposed the method to many errors. Also, the method is tedious and time-consuming. Hence, a straightforward and fast spectrophotometric method was developed. It is based on the distinct absorption wavelength of folic acid at the UV/Vis range.

5.4.1 Determination of the Absorption Wavelength for Folic Acid

From the scanning procedure, three maximum absorptions were observed at **256nm, 283nm, and 366nm** (*Figure 46*). Although the wavelengths observed were slightly different from those observed by Matias et al. (2014), they also reported three wavelengths for folic acid in a phosphate buffer. The scanning procedure was precise and accurate. The same spectra were obtained when two solutions of 25 µg/mL folic acid were scanned, and a solution of 25 µg/mL folic acid was scanned twice (*Figure 46*).

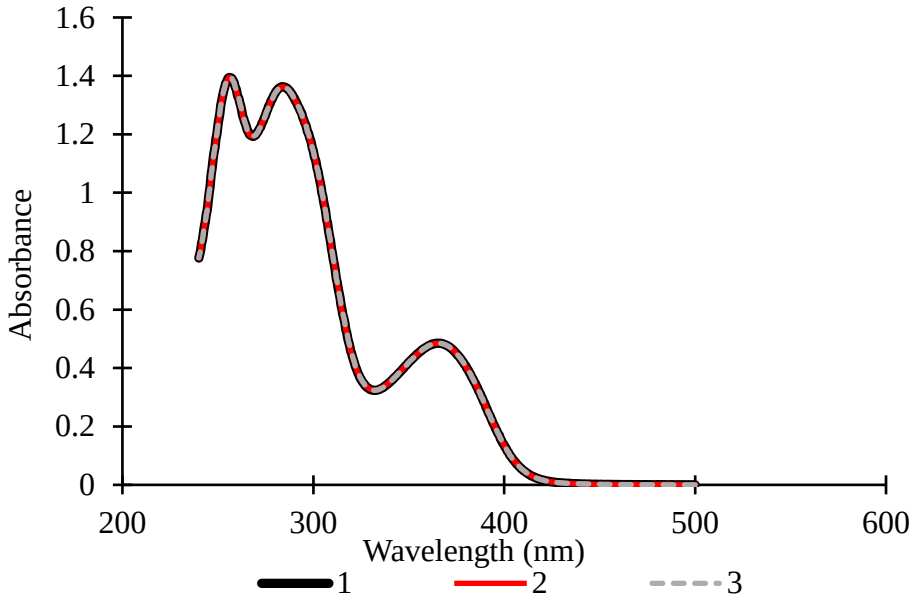


Figure 46:The absorption spectra from scanned absorbance of 25 µg/mL folic acid

The choice of one of the three wavelengths was based on which wavelength is selective for folic acid in the presence of potential degradation products of folic acid and other components of salt. In making this choice, folic acid degraded by the method described by Nagaraja et al. (2002) was scanned. Also, the solutions (10 µg/mL) of the potential products of degradation of folic acid (pteroic acid, para-aminobenzoic acid, and glutamic acid) and vitamin B_{12} were scanned. At 285nm, the maximum absorption of the degraded folic acid was significantly reduced; the

maximum absorption decreases with the time of the degradation (*Figure 47*a). This result is consistent with the study reported by Gazzali et al. (2016). Folic acid shared similar maximum absorption with pteroic acid at 256 and 365nm with vitamin B_{12} at 365nm. Aminobenzoic acid had a maximum absorption at 266nm, which was like one of the maximum absorptions observed with the degraded folic acid (*Figure 47*b). This led to the choice of 285nm as the distinct wavelength for folic acid

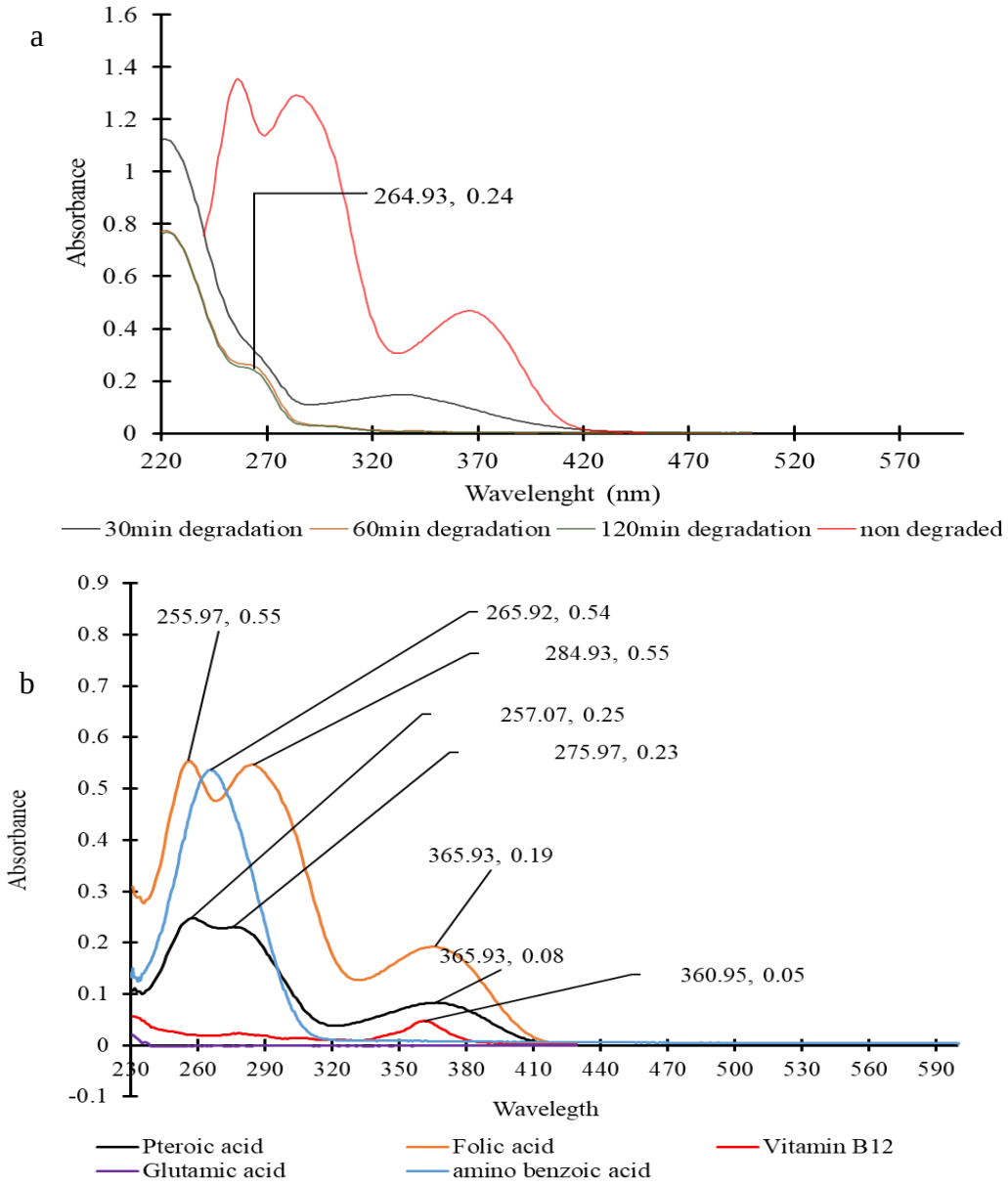


Figure 47:Comparative absorption spectra of folic acid and its potential products of degradation (a) folic acid degraded for 30, 60 & 120 minutes by the method described by Nagaraja et al. (2002) (b) solution of the pure potential product of degradation of folic acid

5.4.2 Selectivity of the Absorption Wavelength for Folic Acid

The selectivity of the chosen wavelength (285nm) to distinguish the degradative product of folic acid was evaluated (Table 12). The absorbance of 2.5 and 25 µg/mL of folic acid degraded by the method described by Nagaraja et al. (2002) was measured. No absorbance was observed when the 2.5 µg/mL folic acid was degraded. Even with a higher concentration (25 µg/mL), only negligible absorbance was observed (0.25±0.00) compared with an absorbance of 1.32±0.00 observed for the absorbance of non-degraded folic acid (25 µg/mL). This absorbance may be attributed to remaining folic acid, not cleaved in the reaction condition. Still, this showed that the method could distinguish between folic acid and the product of the reductive cleavage of folic acid.

Table 12:Selectivity of the Analytical Method to the Product of Degradation of Folic Acid

Concentrations of Folic Acid (µg/mL)	**Absorbance at 285nm**	
	Folic Acid	**Reductively Cleaved Folic Acid**
2.5	0.13±0.00	-0.03
25	1.32±0.00	0.25±0.00

The selectivity of the wavelength for folic acid in the presence of potential constituents of salt was evaluated. The levels of the other components in the solution represented their ratio to folic acid in spray solution or salt. There was no significant change in the absorbance of folic acid in the presence of citrate, iodine, and or vitamin B_{12} (Table 13). This showed that the other constituents would not interfere with the absorbance of folic acid at 285nm.

Table 13:Selectivity of the 285nm to folic Acid in the presence of other components of salt

Absorbance at 285nm			
Folic Acid	**Folic Acid + Iodine**	**Folic Acid + Iodine + B_{12}**	**Folic Acid + Iodine + B_{12} + Citrate**
0.55± 0.01	0.56± 0.02	0.56 ± 0.00	0.55 ± 0.00

The concentration of folic acid in all the solutions was 10µg/mL. The concentration of other component reflects their proportions to folic acid in spray solutions

5.4.3 Determination of the Calibration Range

A scatter plot of the concentrations and absorbance confirmed a linear relationship between absorbance and folic acid concentration in the range of 1-125 µg/mL. The relationship between the absorbance and concentration of folic acid was linear between 1-50µg/mL. Given the limitation

of Beer's law and that a double beam spectrophotometer is used, the calibration range was narrowed to 1-25 µg/mL (*Figure 48*). This corresponded to having the maximum absorbance less than 1.5 (Burgess, 2007). The linear regression equation for calculating the amount of folic acid in salt and spray solution based on the absorbance at 285nm was obtained thus;

$$A_{285nm} = 0.0539 \times [\text{folic acid}] \qquad \text{Equation 15}$$

with a correlation coefficient (R^2) =1

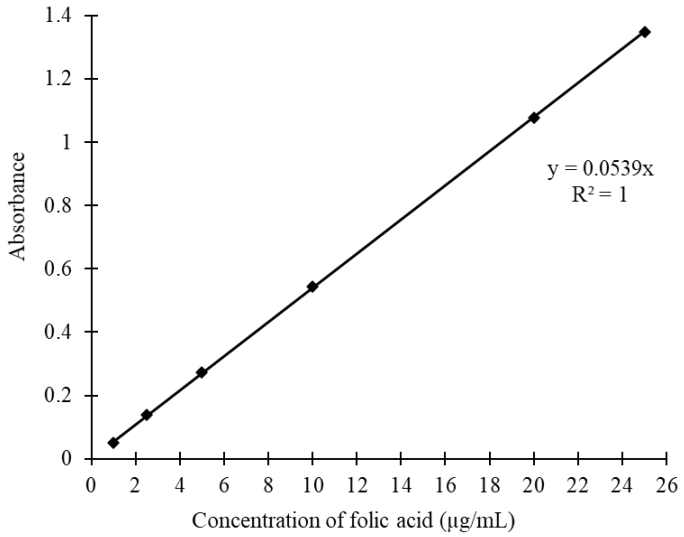


Figure 48:Absorbance - concentration relationship of folic acid solution in 0.1M sodium carbonate.
Values are average of six replicates; the standard deviations of the data points were so small that they did not show when inserted in the graph

The concentration of folic acid in fortified salt (12.5 or 25µg/g) falls within the linear range. Although the concentration of folic acid in the spray solution used was higher than this range, it could be easily diluted to fall within this range. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the ICH Guideline (2005); they are 0.01 and 0.03 µg/mL, respectively (Table 14). The LOD and LOQ observed in this study were close to that reported by Matias et al. (2014) despite that a different solvent was used. The ranges of the recoveries of folic acid from salt and spray solutions were 99-101.7% and 98.2-104.1%, respectively. The ranges were within 80-120% as prescribed by ICH (2005). Hence, the analytical method has acceptable accuracy. Since the data were obtained from experiments carried out on different days and at different laboratories, the analytical method may be said to be precise. The analytical parameters are summarized in Table 14.

Table 14: Analytical Parameters

Parameters/Characteristics	Values
Colour	Slightly yellow to colourless depending on the folic acid concentration
λ_{max} (nm)	285
Stability (h)	20hr
Beer's law range (µg/ml)	1–25
Limit of detection (µg/ml)	0.01
Limit of quantification (µg/ml)	0.03
Percentage recovery (salt) (%)	100±1.2
Percentage recovery (spray solution) (%)	100±1.9
Regression Equation	
Slope	0.0539
Intercept	0.00
Correlation coefficient (*R*)	1.00

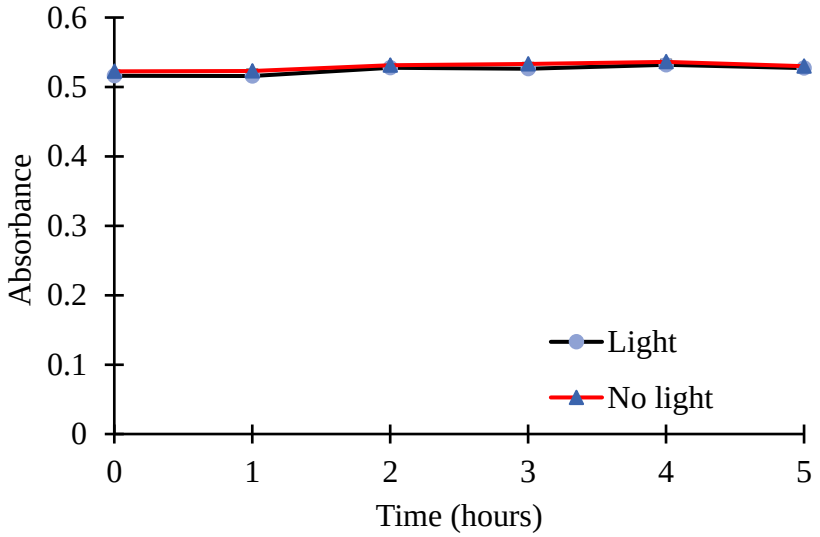


Figure 49: Stability of the absorbance at 285nm of the extracted folic acid upon the exposure to light
Values are average of four replicates; the standard deviations of the data points were so small that they did not show when inserted in the graph

The folic solution extracted from salt were exposed to lights in the laboratory; the impact of the exposure for 5hr was evaluated. The absorbance at 285nm remained constant throughout this period (*Figure 49*). Hence, a brief exposure to light as it may occur during sample preparation should not impact the analysis results.

This simple spectrophotometric method is accurate, precise, and easy to use. This method could not be used for quantifying folic acid in Fe-FA or Fe-FA-B12 premix, as some component(s) of the premix interfered with the absorbance of folic acid at 285nm. The component(s) was not identified. From premix samples, the amount of folic acid was determined by UHPLC-MS.

5.5 Triple Fortification of Salt

The positive result from efficacy studies on Double Fortified Salt to reduce iron deficiency (Ramírez-Luzuriaga *et al.*, 2018; Diosady, Mannar and Krishnaswamy, 2019) showed that a fortified salt is a cost-effective approach for providing iodine and iron for impoverished populations. However, in many areas, there is a need to provide folic acid to prevent congenital disabilities and other forms of anemia. Salt is a logical vehicle for adding folic acid to the diet of rural poor without access to folic acid fortified foods. This section covers the process developed for adding folic acid to DFS to form a Triple Fortified Salt (TFS).

5.5.1. Preliminary Studies: Increase in Concentration of Micronutrients in the Spray Solution

As explained before, DFS was made by spraying a solution of iodine on salt, while iron was added as extruded and microencapsulated ferrous fumarate (iron premix). Adding folic acid to the iodine solution was the easiest route to formulating TFS. This route was tested earlier in our group.

McGee (2012)and McGee, Sangakkara, & Diosady (2017) prepared a spray solution containing iodine and folic acid (0.35% $^{w}/_{v}$ each) for the formulation of a salt containing folic acid and iodine. High moisture content in the salt accelerates the loss of iodine in the salt (Allen *et al.*, 2006a). Excess moisture added while spraying solution of iodine weakened the coat on the iron premix in the resultant salt, which allowed the interaction of iron and iodine (McGee, 2012). Hence, they increased the concentration of folic acid and iodine to 1-3% $^{w}/_{v}$. The increased concentrations reduced the volume of spray solution, consequently lowering the moisture content of the salt, minimising the loss of iodine from the salt fortified with folic acid and iodine (McGee, Sangakkara and Diosady, 2017).

Building on the work of McGee (2012), the impact of the concentration of sodium carbonate buffer on the solubility of folic acid in the spray solution was evaluated. The solubility of folic acid in

the solution responded to the concentration of the carbonate buffer; at least 0.2M sodium carbonate buffer was required to dissolve 3% folic acid in the solution. The MS spectra of folic acid in the solution showed the formation of mono- and disodium salt of folic acid. The sodium salt is responsible for the solubility of folic acid and explains why a higher concentration of sodium carbonate buffer is required to dissolve 3% folic acid (as it serves as the source of sodium).

Subsequently, three spray solutions were formulated, two of which were similar to those prepared by McGee, Sangakkara, & Diosady (2017), and the third contained 1.8% folic acid and 3% iodine. The stability studies carried out on the spray solution showed that both iodine and folic acid were very stable in the solution. Less than 20% of the added micronutrients were lost after 2-month storage, even at 45 ^{o}C (Table 15). This result was consistent with those reported by McGee(2012). While increasing the concentration of the iodine resulted in a significant increase in the stability of iodine and folic acid, increasing the concentration of folic acid did not impact the stability of iodine and folic acid in any particular pattern. At the point of preparation, 0.2M sodium carbonate buffer dissolved folic acid, irrespective of the amount of folic acid added (1, 1.8 or 2% folic acid). However, in the solutions that contained more than 1% $^{w}/_{v}$ folic acid, some of the folic acid precipitated out from the solution, which can be a significant problem for small salt plants that store their spray solutions for up to a month.

Table 15:Stability of Folic Acid and Iodine in Spray Solution (2-Month Preliminary Study)

Types of Solution	Folic Acid			Iodine		
	25 ^{o}C	35 ^{o}C	45 ^{o}C	25 ^{o}C	35 ^{o}C	45 ^{o}C
1% FA + 2% I	86.7±0.4	87.9±2.9	82.3±0. 5	88.7±0.9	86.1±0.7	84.6±7.1
2% FA + 2% I	91.2±6.5	87.0±10.45	85.8±5.6	93.5±0.6	93.5±1.0	89.7±0.3
1.8% FA + 3% I	99.5±9.5	97.5±13.2	94.9±10.7	93.8±3.2	93.8±0.6	95.0±0.8

FA=folic acid, I= iodine; the solutions were formulated with 0.2M sodium carbonate buffer; the pH was adjusted to pH 9

5.5.2 Stability of Iodine and Folic Acid in the Newly Formulated Spray Solution for Triple Fortification of Salt

The observed folic acid precipitation in the initial solutions led to the reformulation of the spray solution. Of the three concentrations of folic acid initially used, 1% folic acid, was chosen; it will supply 100% of folic acid RDA when used to formulate fortified salt. The possibility of reducing

the concentration to 0.5% while still meeting 50% RDA of folic acid in fortified salts was investigated. In order to be consistent with industrial practice, the concentration of iodine was maintained at 2% in the spray solution. Also, the buffer solution used in the previous method was replaced with a sodium carbonate solution (0.1M). The solution of sodium carbonate itself is a buffer as it dissociates to sodium and bicarbonate ions. The sodium carbonate solution was used to dissolve folic acid and to adjust the pH of some of the spray solutions. The change from the use of the buffer (prepared with sodium carbonate and sodium bicarbonate) reduced the number of steps required for making the spray solution. In all the samples, 70-100% of folic acid and iodine were retained after two months of storage (Table 16). The subsequent sections (5.3.2.1 - 5.3.2.3) cover the impact of pH, folic acid concentration, and citrate on the stability of folic acid and iodine in the spray solution.

Table 16: Stability of Folic Acid and Iodine in the Optimized Spray Solution

Types of Solution	Folic Acid			Iodine		
	25 ^{o}C	35 ^{o}C	45 ^{o}C	25 ^{o}C	35 ^{o}C	45 ^{o}C
pH adjusted to 9						
0.5% FA	82.4±3.4	82.0±1.9	68.8±6.7			
1% FA	92.1±2.3	84.4±6.9	71.5±3.4			
0.5% FA + 2% I	91.1±2.1	88.4±4.9	74.4±2.2	94.7±4.7	92.7±0.6	89.4±4.6
1% FA + 2% I	88.7±4.7	89.3±2.6	80.2±5.2	96.5±4.0	94.4±1.0	88.6±2.7
0.5% FA + 1% C	80.4±8.2	73.0±5.4	60.0±9.0			
1% FA + 1% C	76.2±9.9	71.9±4.1	79.5±7.1			
0.5% FA + 2% I + 1% C	85.6±4.5	84.8±3.6	68.3±2.7	93.3±4.8	92.9±1.0	89.2±4.7
1% FA + 2% I + 1% C	90.2±3.2	88.9±4.4	91.5±6.7	96.0±6.1	94.7±3.2	92.3±5.0
Unadjusted pH						
2% I				100.0±1.0	96.5±5.1	93.1±1.2
2% I + 1% C				102.1±1.8	100.4±3.4	92.7±3.6
0.5% FA	100.1±5.0	95.3±0.3	93.9±6.5			
1% FA	97.3±3.3	91.5±3.2	90.1±2.1			
0.5% FA + 2% I	101.6±2.3	97.3±5.5	95.4±7.9	94.4±3.9	91.0±1.3	90.3±3.2
1% FA + 2% I	98.8±0.4	94.3±3.5	91.5±7.8	96.7±1.6	94.0±4.0	84.9±4.3

FA=folic acid, I= iodine, C= citrate; for some solution, just enough amount of 0.1M that can dissolve folic acid was added to folic acid and iodine, water and additional 0.1M was added to adjust their pH; for others, iodine and folic acid were just dissolved in 0.1M sodium carbonate.

5.5.2.1 Impact of pH on the Solubility and Stability of Folic acid and Iodine in the Spray Solution

pH had a significant effect on the solubility of folic acid in the solutions. After a few weeks, folic acid precipitated out of the solutions at pH 7 and 8 (Figure 50). This observation was consistent with the study of Taub and Lieberman (1953), who found that folic acid solution at pH 6 turned cloudy after a few days. Folic acid in solutions adjusted to pH 9 and 10 did not precipitate even after a few months. Hence, the pH of the new solutions was at least maintained at pH 9. In order to obtain an approximately pH 9 solution, 0.742g sodium carbonate, 3.37 g potassium iodate, and 1 g folic acid were dissolved in 100 mL of water.

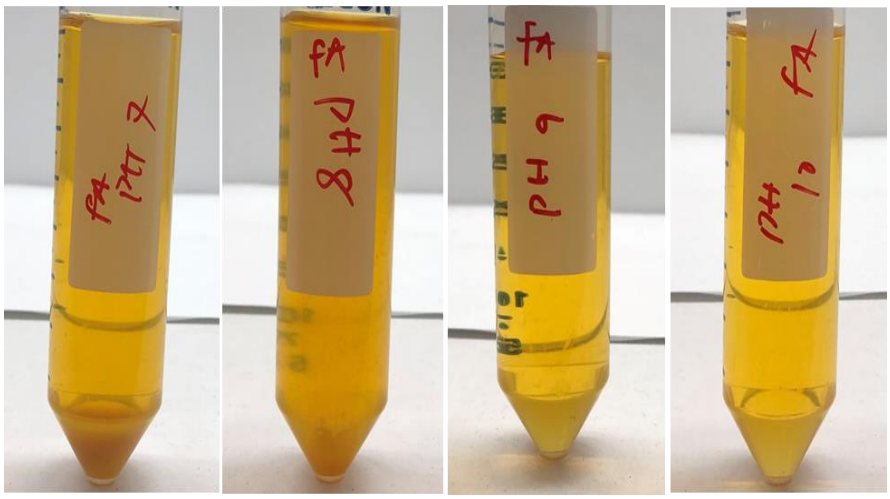


Figure 50:Effect of pH on the solubility of folic acid

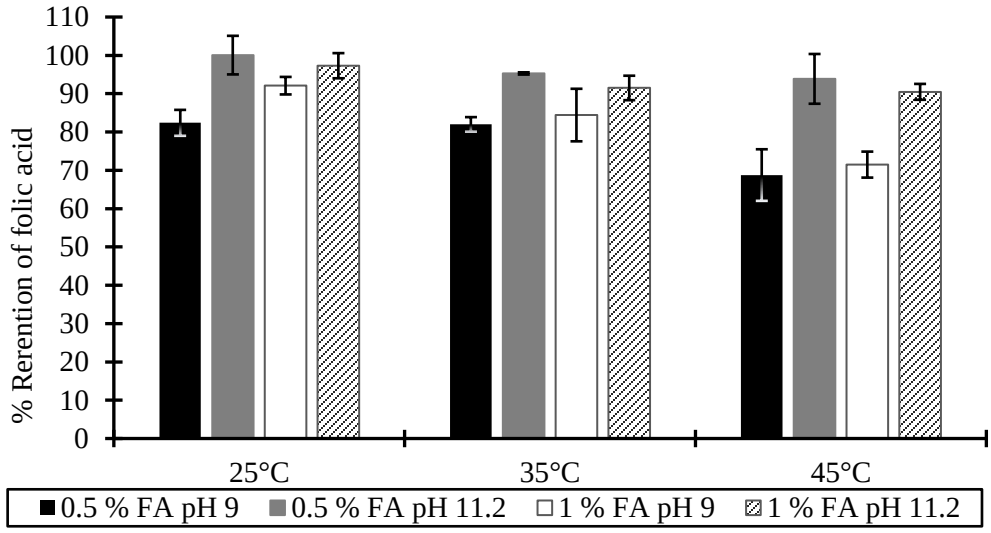


Figure 51:Effect of pH on folic acid stability in the spray solutions at three different temperatures of storage (2-month)
FA: Folic acid

The pH of the spray solution significantly impacted the stability of folic acid. For 0.5% folic acid, as pH decreased, the stability of folic acid in the solution decreased. For 1% folic acid, pH only significantly impacted the solution stored at 45 ^{o}C (Figure 51). Change in pH did not affect the stability of iodine in the solution. Although a higher pH favoured the solubility and stability of folic acid, a higher pH may not be desirable if the addition of vitamin B_{12}, a micronutrient that is essential in the metabolism of folic acid, is contemplated; vitamin B_{12} is not stable in alkaline solution.

5.5.2.2 Effect of Folic Acid Concentration on the Stability of Iodine and Folic Acid in Spray Solutions

The concentration of folic acid in the spray solution did not significantly affect the stability of folic acid and iodine in the spray solutions, except at 25°C (Figure 52). The percentage of folic acid retained in the spray solution containing 0.5% folic acid was significantly reduced compared to other solutions. This trend was also observed in the earlier spray solutions; however, we do not understand what is responsible for the trend. Iodine seems to have stabilized folic acid in some of the solutions. The same trend was reported by McGee et al. (2017). The presence of potassium iodate accelerated the formation of sodium salts of folic acid in the solution. This may be responsible for the improved stability of folic acid in the solution that contained potassium iodate.

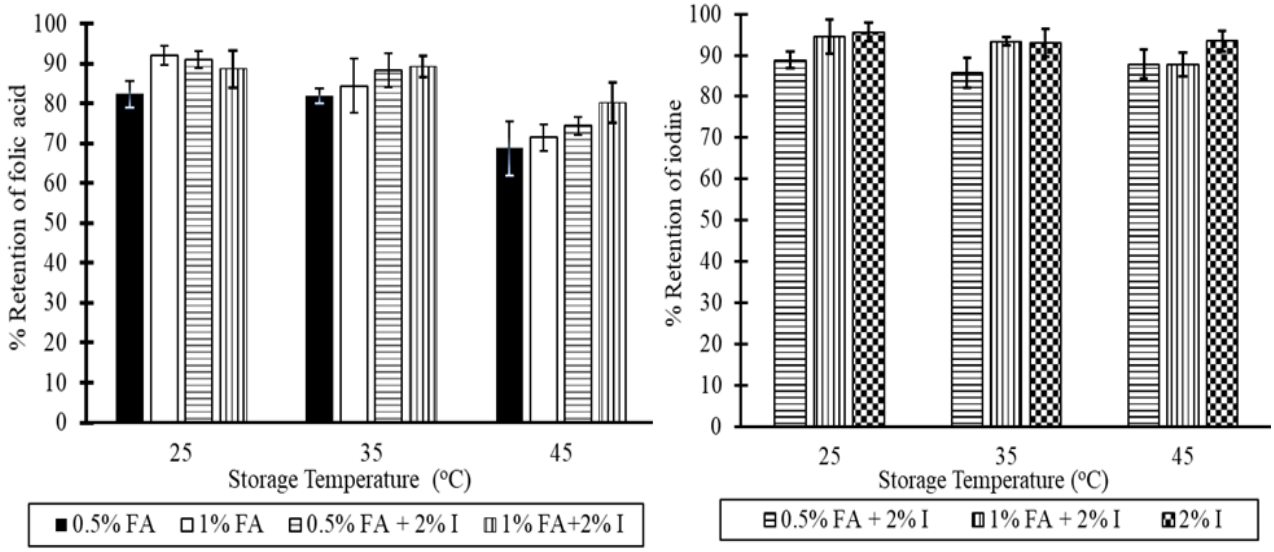


Figure 52: Effect of folic acid concentration on folic acid and iodine stability in the spray solution at three different temperatures of storage (2-month). FA: folic acid; I: Iodine

Colour is one of the factors that affect the acceptability of fortified food (Mejia, 1994). The reduction of folic acid concentration in the solution has a high potential for improving the colour of salt and maintaining the stability of both iodine and folic acid, while still meeting the target concentration of folic acid in the fortified salt. Moreover, this may reduce the cost of salt fortification.

5.5.2.3 Effect of Citrate on the Stability of Folic Acid in Spray Solutions

Folic acid is susceptible to degradation by oxidizing agents (Fennema, 1996). Hence, we investigated citrate as a folic acid stabilizer. Citrate did not significantly improve the stability of folic acid in the spray solution; it only slightly improved the stability of folic acid at 45°C (*Figure 53*). This result confirmed that citrate is not needed in the spray solution formulation, reducing the potential impact of its cost on the overall cost of making TFS. Given the results from the stability study carried out on the spray solution and future opportunity to accommodate vitamin B_{12} into the spray solution, the optimal spray solution for the triple fortification of salt consisted of 0.5-1% folic acid and 2% iodine at pH 9.

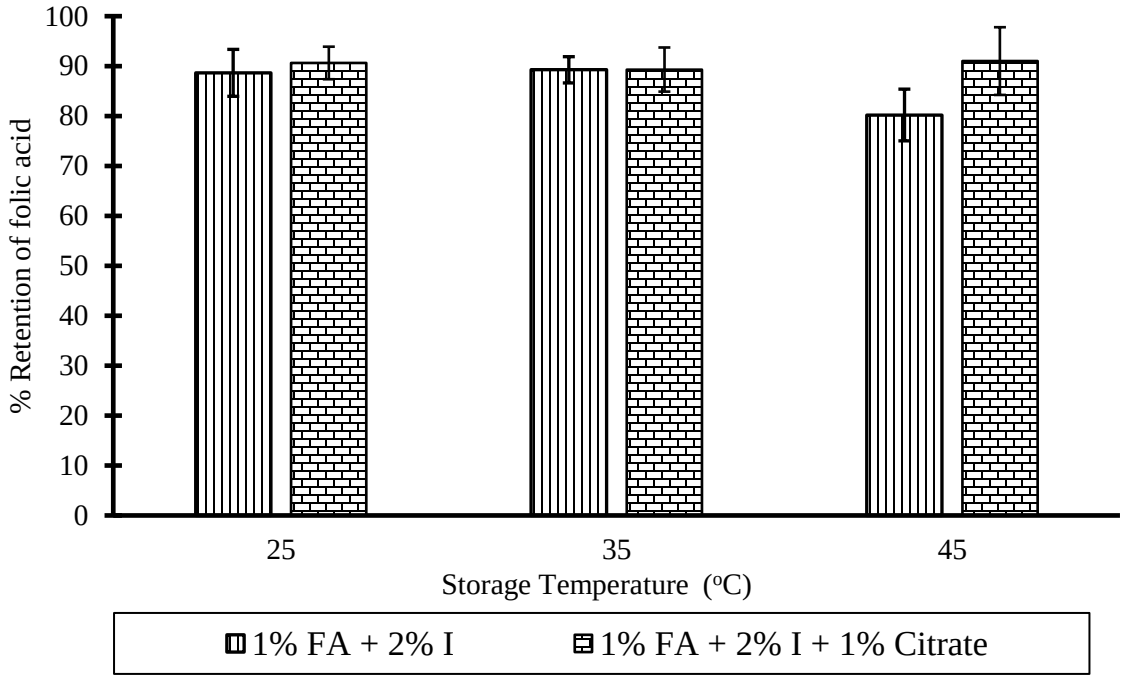


Figure 53:Effect of citrate on folic acid stability in the spray solutions at three different temperatures of storage (2-month)
FA: Folic acid; I: Iodine

5.5.3 Stability of Iodine and Folic Acid in Triple Fortified Salt (TFS) after 6-Month Storage

The first attempt on triple fortification of salt was made by McGee (2012). She used formulated TFS by spraying 0.1% iodine and folic acid to salt and adding iron premix prepared by a one-step agglomeration and encapsulation using spray drying (Romita, Cheng and Diosady Levente, 2011). The idea was that the relatively smaller iron premix particle would adhere to the surface of the salt particles. The use of a lower concentration of folic acid and iodine in the spray solution led to adding more liquid to salt (30mL/kg salt); hence, increasing the moisture content of the salt. The high moisture content of the salt accelerated the rate of loss of iodine in the salt. Also, the colour of the iron premix was not acceptable as it forms grey spots in the salt (Romita, Cheng and Diosady Levente, 2011).

In contrast to the process developed by McGee (2012), a higher concentration of folic acid and iodine (05-1% and 2%, respectively) was used. The volume of spray solution added was drastically reduced from 30mL per kg salt to 2.5mL per kg salt. The moisture content of the fortified salt was 0.06% compared to 2.9% of the fortified salt formulated by McGee (2012). One of the TFS samples was formulated with a solution that contained 1% folic acid, 2% iodine, and 1% citrate. With this sample, the effect of citrate on the stability of folic acid in the TFS was investigated. Also, the premix similar to that developed by Li et al. (2011) using forming extrusion and microencapsulation technique was used in place of the premix formulated by spray drying. In the new process for formulating TFS, iron premix particle with similar particle size and density like salt is aimed. The size and density will ensure that the iron premix does not segregate from the salt.

Salt stays on the distribution channels for an average of 2 months (Diosady *et al.*, 2006). Also, the target population buys a small amount of salt, typically consumed within two months. Hence, the goal is to have at least 70% of the micronutrients retained in the fortified salt after 6-month storage. After 6-month storage, 70-85% of folic acid and 85-95% of iodine were retained in all the samples, even at 45^{o}C and 60-70% RH (Table 17). This result confirmed that the process could be used to deliver iron, iodine, and folic acid simultaneously through salt. Given the traditional distribution channel of salt, Triple Fortified Salt has the potential of reaching millions of vulnerable households that otherwise may not have access to diets with sufficient iron, iodine, and folic acid. The impact of the factors (such as the material used for coating the premix, concentration of folic, and citrate) on the stability of folic acid and iodine in the TFS will be covered in subsequent sections

Table 17:Stability of Folic Acid and Iodine in TFS after 6-Month Storage

Types of		Retention of Folic Acid (%)			Retention of Iodine (%)		
Sol.	Prem.	25 ^{o}C	35 ^{o}C	45 ^{o}C	25 ^{o}C	35 ^{o}C	45 ^{o}C
C	-	82.3±6.1	80.4±5.9	78.9±4.5	-	-	-
D	-	81.1±1.2	77.3±0.8	72.9±3.3	87.5±1.5	86.9±2.1	80.2±1.4
I	H&S*	-	-	-	95.6±2.3	93.0±3.4	93.4±2.5
I	H&S	-	-	-	93.2±2.9	90.3±3.9	92.3±6.8
I	S	-	-	-	95.3±3.8	95.5±3.7	92.6±7.0
I	H	-	-	-	92.6±2.7	92.3±3.7	91.9±8.0
C	H&S*	78.7±2.7	72.8±0.9	79.3±4.8	88.8±2.1	85.7±3.7	87.8±3.4
D	H&S*	73.8±2.2	69.1±2.0	68.8±0.4	94.4±4.1	93.4±1.1	87.8±2.9
C	H&S	83.5±0.5	82.2±2.9	83.5±0.5	89.6±3.2	89.7±3.9	87.4±1.8
D	H&S	80.6±1.5	77.8±1.2	73.2±1.5	93.1±1.5	94.0±4.3	93.0±1.4
C	S	86.4±2.2	82.9±4.9	80.2±4.3	88.7±4.5	86.0±3.7	87.3±2.3
D	S	77.5±6.1	76.2±4.2	71.2±4.	89.1±2.2	90.7±1.3	90.0±2.4
C	H	76.1±2.9	83.1±4.0	80.9±1.7	91.6±3.5	91.1±3.7	90.9±2.1
E	H&S*	72.3±1.9	82.7±3.9	71.1±0.6	93.6±3.0	90.1±2.6	88.1±2.6

Sol.=solution: C=0.5% folic acid + 2% iodine, D=1% folic acid + 2% iodine, E= 1% folic acid + 2% iodine + citrate and I= 2% iodine. Prem.=iron premix: H&S= iron premix coated with 5% HPMC and 5% soy stearin, H= iron premix coated with 10%, S= iron premix coated with 10% soy stearin, and * iron premix obtained from India.

5.5.3.1 Effect of Iron Premix Coating on Stability of Micronutrients in Salt

The SEM images of the premix used for preparing TFS samples showed rough surfaces on the premix coated with 10% HPMC and double-coated with 5% HPMC and 5% soy stearin (formulated in the lab) (Figure 55). The rough surface was similar to that of the uncoated iron extrudate; hence, it may indicate the imperfect coating of the premix samples because the colour masking and the coating material should have filled up the rough edges. Although the impact of the coating material on the stability of folic acid in the TFS did not follow any trend, TFS prepared with imperfectly coated premix samples retained marginally more folic acid. This trend was most noticeable in samples stored at 35 ^{o}C. Iron may be stabilizing folic acid in the TFS samples. This trend had been observed in rice fortified with iron and folic acid (Li, Diosady and Jankowski, 2011). The apparent imperfection of the iron premix did not impact the stability of iodine in the

salt (Figure 54). Folic acid may be playing a stabilizing role in the salt; it may have shielded iodine from the effect of the exposed iron.

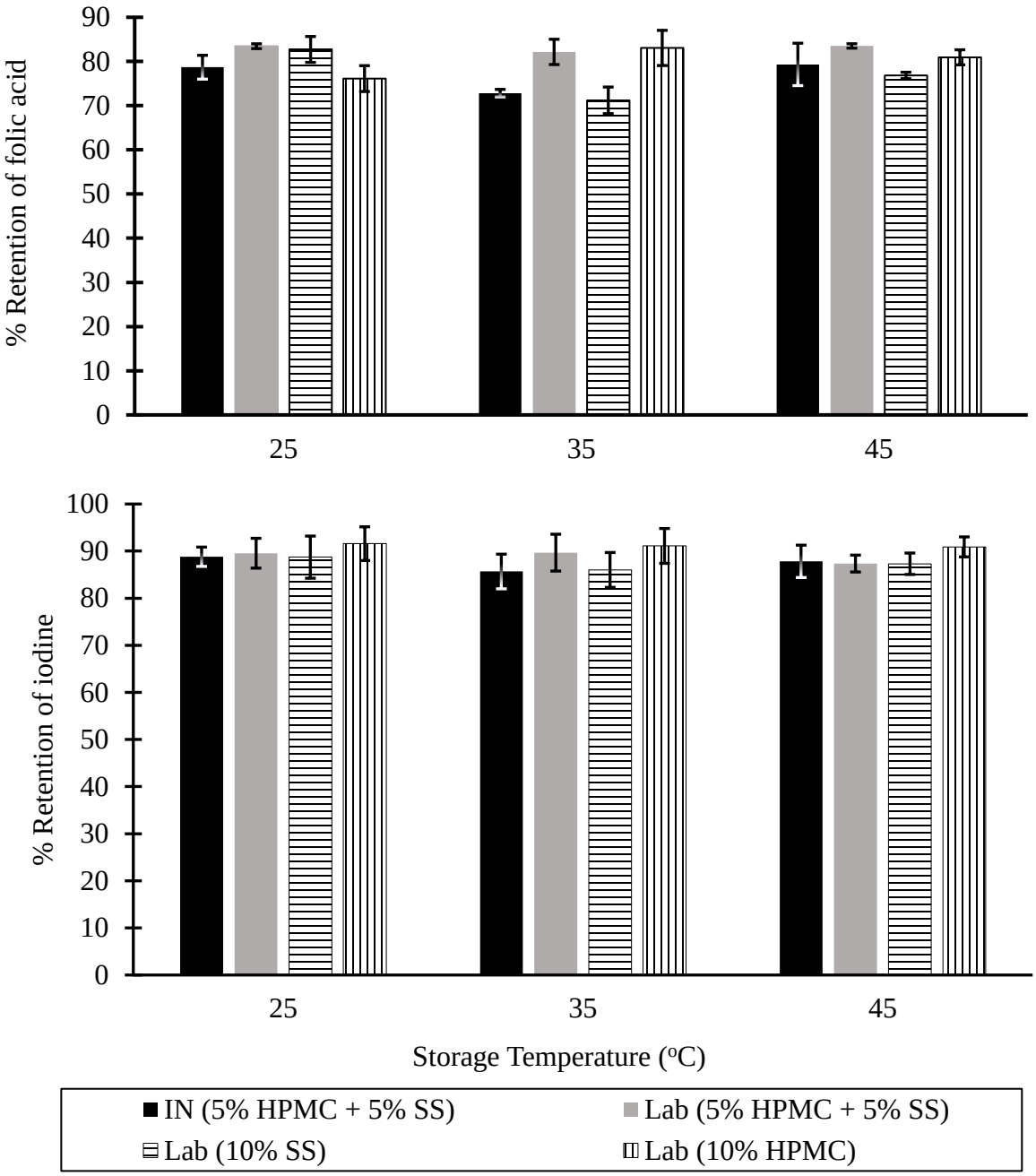


Figure 54: Effect of premix coating materials on iodine and folic acid stability in TFS after 6 months at different storage temperatures

Two of the premix samples, IN (5% HPMC + 5% SS and Lab (10% SS), had a perfect coat, the others did not. FA: folic acid; HPMC: hydroxypropyl methylcellulose; and SS: soy stearin. IN=premix produced by JVS Foods Pvt. India and Lab=premix produced in the laboratory.

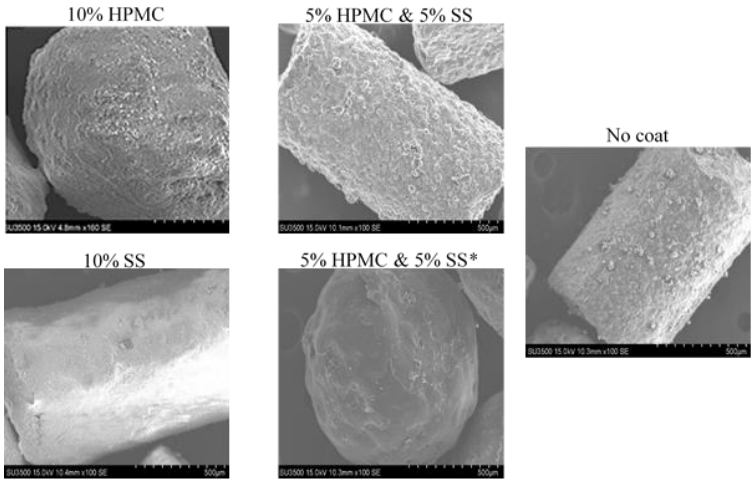


Figure 55:SEM images (magnification on the image) of the different iron premix coated used for formulating TFS. HPMC: hydroxypropyl methylcellulose; SS: soy stearin; * obtained from India

5.5.3.2 Effect of Citrate on the Stability of Folic Acid and Iodine in the Premix

Folic acid is susceptible to reductive and oxidative degradation; hence, the effect of citrate, a metal ion chelator, on the stability of folic acid in the TFS was investigated. Citrate had no significant impact on the stability of folic acid or iodine in the TFS samples (Figure 56), just as it had no impact on folic acid and iodine in the spray solution. The cost that would have been incurred for the addition of citrate can be ignored, since citrate did not significantly improve the stability of folic acid. The 'avoided' cost of citrate is good for the cost-effectiveness of the technology to deliver iodine, iron, and folic acid simultaneously to the vulnerable population.

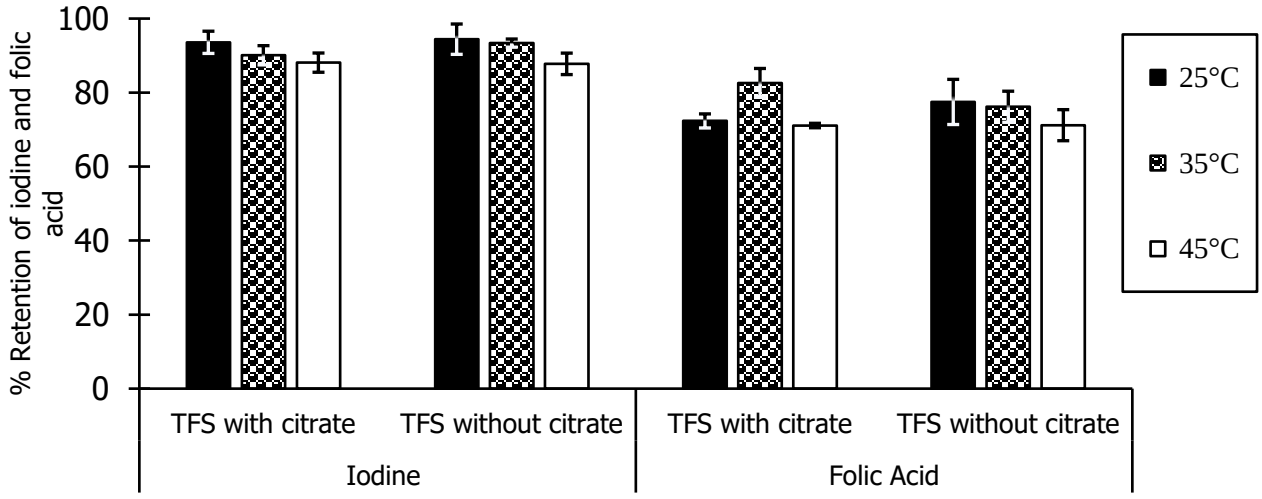


Figure 56:Effect of the addition of citrate to TFS on the stability of iodine and folic acid in the salt after 6 months at different storage temperatures

5.5.3.3 Effect of Folic Acid Concentration on Stability of Folic Acid and Colour of Salt

In the tested range, folic acid concentrations (12.5 -25 ppm) did not significantly affect the stability of folic acid in TFS except at 35°C (Figure 57). At this temperature, the retention of folic acid in 12.5 ppm folic acid salt was significantly higher than that in the salt containing 25 ppm folic acid. Lower concentration of folic acid (12.5 ppm) seems to enhance the stability of folic acid in the TFS samples. Also, reducing the concentration of folic acid in the salt (from 25 ppm to 12.5 ppm) improved the colour of TFS (Table 18). The yellow colour of the salt due to folic acid was significantly reduced in TFS with a lower concentration of folic acid. The reduction in folic acid concentration will improve the acceptance of the salt. Moreover, the salt fortified with 12.5 ppm folic acid can still deliver the target concentration of folic acid (50% RDA of folate). Although reducing the concentration of folic acid improved the colour of TFS, there was still concern about the slight yellow colour of the TFS.

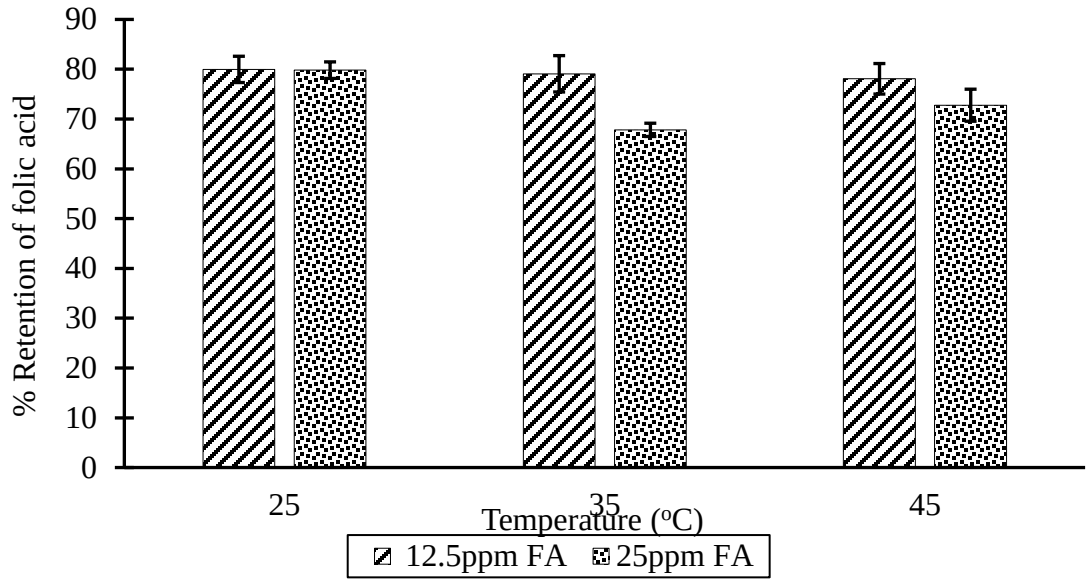


Figure 57:Effect of folic acid concentration on folic acid stability in TFS after 6 months at different storage temperatures

Table 18: L*a*b* Colour Properties of Double and Triple Fortified Salt Samples

Salt Sample	L*	a*	b*
Iron + I	98.7	0.0	1.2
Iron + I +12.5 ppm Folic Acid	95.4	-1.5	8.4
Iron + I +25 ppm Folic Acid	94.3	-2.9	13.5

The concentrations of iron and iodine are 1000 ppm and 50 ppm respectively in all the salt samples. L* (+ = lighter; - = darker); a* (+ = red; - = green); b* (+ = yellow; - = blue)

5.5.4 Optimizing the Process of Triple Fortification of Salt

Given the colour problem observed with spraying folic acid solution on salt, folic acid was removed from the spray solution and was added to the iron premix to form a Fe-FA premix. There were two designs for Fe-FA premix- either iron and folic acid were in the core of the premix (Fe +FA) or folic acid was separated from the iron in the core by a thin layer of TiO_2 ($Fe_{extrudate}$ + FA) as illustrated in Figure 58. Iron and folic were coextruded to have both micronutrients in the core of the premix. Coextruding iron and folic acid was a straight forward process; it only involves adding folic acid to ferrous fumarate while making the premix. Separating folic acid from the core was achieved by two different routes:

a) adding folic acid as a uniform suspension in water to colour masked iron extrudate, or
b) adding a suspension of folic acid in 2.5% HPMC (in a 1:1 ethanol and dichloromethane solvent system).

The folic acid suspension in water was added directly to TiO_2 colour masked extrudate in a beaker and was thoroughly mixed. The suspension of folic acid in 2.5% HPMC was sprayed on colour masked extrudate tumbled in a pan coater. The ratio of the solvents that made up the solvent system is vital to having a uniform suspension of folic acid that is volatile enough for pan coating. A 50:50 dichloromethane:ethanol solvent system was used. The use of more dichloromethane caused folic acid to settle at the bottom of the spray flask, while more ethanol caused the colour masked iron extrudate to clump inside the pan coater. After either of the route, the premix was colour masked and coated. Spraying the suspension of folic acid in 2.5% HPMC resulted in a more uniform distribution of the folic acid when compared with the other method (Figure 59).

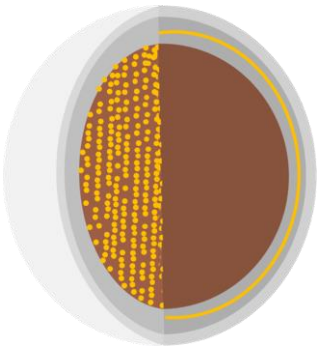


Figure 58:Schematics of Iron-Folic Acid Premix- (Fe + FA) has iron and folic acid in the core of the premix; ($Fe_{extrudate}$ + FA) has iron in the core and folic acid separated by a tiny layer of TiO_2

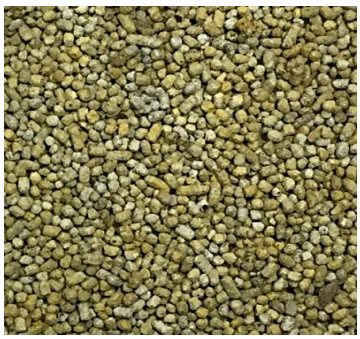
a  b 

Figure 59: Distribution of folic acid in Iron-folic acid premix ($Fe_{extrudate}$ + FA).
a: was made by spraying a suspension of folic acid in 2.5% HPMC (in ethanol and dichloromethane solvent system) on colour masked iron extrudate; b: was made by mixing a suspension of folic acid in water with colour masked iron extrudate.

The iron-folic acid premix ($Fe_{extrudate}$ + FA) with uniform distribution of folic acid and the coextruded iron and folic acid premix (Fe-FA) were subsequently used to formulate TFS. In the optimized process, folic acid will no longer impact the colour of the TFS, as folic acid was hidden with iron by the colour masking and coating agents of the premix. Over 75% of the added folic acid and iodine were retained after 6-month storage. With regards to the two premix samples used, the loss of iodine in the salt did not follow any particular trend (Figure 60). Folic acid was more stable in the (Fe+FA) premix than the ($Fe_{extrudate}$ + FA) premix. TiO_2 being in contact with folic acid may have initiated photocatalytic degradation, which led to the significant loss of folic acid in ($Fe_{extrudate}$ + FA) premix. Putting folic acid in the dark core of the ferrous fumarate (Fe + FA) prevented such photocatalytic degradation. Folic acid was more stable in the TFS formulated with Fe-FA premix than in TFS formulated by spraying folic acid and iodine solution on salt. Iron seems to have enhanced the stability of folic acid in the salt. The same pattern was shown by Li et al. (2011) in fortified rice that contained folic acid and iron. McGee et al. (2017) suggested that the loss of folic acid in a salt fortified with iodine and folic acid is due to oxidative stress. These studies and stability of folic acid in the TFS may shine more light into the oxidative degradative pathway of folic acid in salt reported by Modupe et al. (2019). The reductive potential of ferrous iron may have prevented the oxidative degradation of folic acid in the salt. Aside from the enhanced stability of folic acid in the (Fe+FA) premix, it is easier to make when compared with the ($Fe_{extrudate}$ + FA) premix. Going forward, TFS should be formulated by adding folic acid and iron as microencapsulated coextrudate, and iodine added by the traditional method of spraying iodine solution. The TFS will deliver 200% iodine's RDA, 56% iron RDA and 100% folic acid RDA based on the consumption of 10g of salt per day (Vinodkumar and Rajagopalan, 2009).

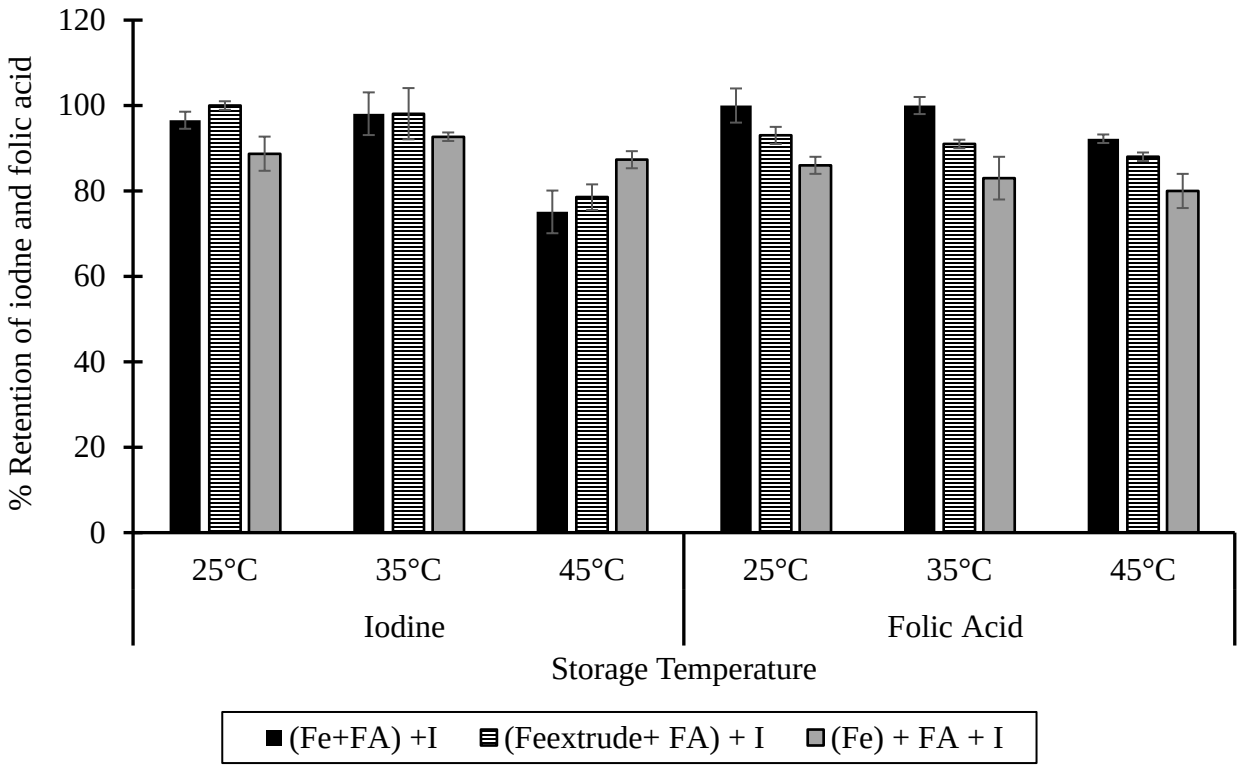


Figure 60: Stability of iodine and folic acid in TFS formulated with Fe-FA premix after 6 months at different storage temperatures

(Fe+FA) has both iron and folic acid in the core of the premix; ($Fe_{extrudate}$ + FA) has folic acid separated by a tiny layer of TiO_2 in the premix; (Fe) has only iron in the premix, folic acid and iodine were added as a solution

5.6 Quadruple Fortification of Salt

The metabolisms of folic acid and vitamin B_{12} are intertwined (Fenech, 2001). There is evidence that folic acid masks the early symptoms of vitamin B_{12} deficiency (Reynolds, 2006; Dwarkanath *et al.*, 2013). Also, vitamin B_{12} deficiency can cause anaemia and prenatal and postnatal complications (Townsley, 2013). Hence, it is imperative to add vitamin B_{12} to the TFS if the impact of adding folic acid to salt will be felt. Three possible routes for adding B_{12} were explored:

a) Addition through a spray solution that contained iodine, folic acid, and vitamin B_{12},
b) Addition through Fe-B_{12} premix, and
c) Addition through Fe-B_{12}-FA premix.

The targeted concentrations of iron, iodine, folic acid, and vitamin B_{12} in the Quadruple Fortified Salt (QFS) were 1000 ppm, 50ppm, 25ppm, and 0.25ppm, respectively.

5.6.1 Formulation of Spray Solution for Quadruple Fortification of Salt

The addition of vitamin B_{12} through spray solution was the simplest of the three possible routes. Hence, it was first explored. Initially, spray solutions containing a sodium carbonate buffer and 0.015% B_{12}, 2% folic acid, and 3% iodine, reflecting the target amounts of the micronutrients in the salt, were prepared. The impact of citrate, ascorbate, and erythorbate on the stability of the vitamin B_{12} in the solution was studied. All the spray solutions became cloudy, except for the vitamin B_{12} solution and folic acid + iodine + vitamin B_{12} + citrate solution (*Figure 61*). The folic acid + iodine + vitamin B_{12} + ascorbate solution did not become cloudy at 45 ^{o}C.

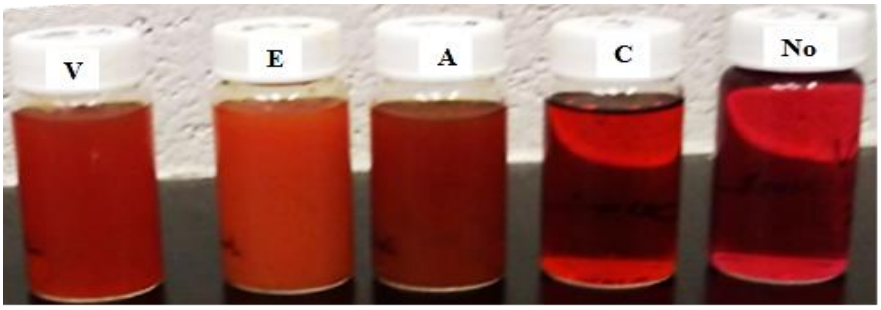


Figure 61:The effect of the constituents of spray solutions on their solubility
(V) 0.015% vitamin B_{12} +3% iodine + 2% folic acid; (E) 1% erythorbate +0.015% vitamin B_{12}+3% iodine +2% folic acid (A) 1% ascorbate+0.015% vitamin B_{12}+3% iodine +2% folic acid; (C) 1% citrate,+0.015% vitamin B_{12}+3% iodine +2% folic acid; and (No) 0.015% vitamin B_{12}

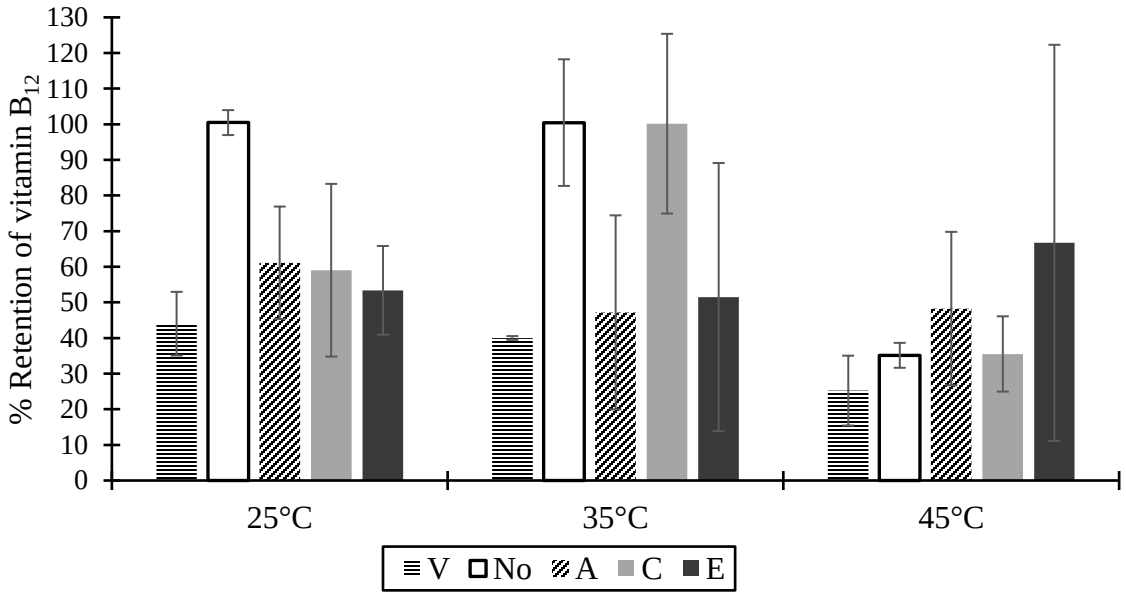


Figure 62: Vitamin B_{12} retention in spray solution after 1 month at different storage temperatures.
V contained 0.015% ; No contained 0.015% + 2% folic acid + 3% iodine; A contained 0.015% + 2% folic acid + 3% iodine + 1% ascorbate; C contained 0.015% + 2% folic acid + 3% iodine + 1% citrate; E contained 0.015% + 2% folic acid + 3% iodine + 1% erythorbate. The pH of all the solutions were adjusted to 9.

Vitamin B_{12} was not stable in the solution. About 40-75% of the B_{12} was lost at 45 ^{o}C after one-month storage (Figure 62). The stability of vitamin B_{12} in the solution is not acceptable, as at least 75% retention of vitamin B_{12} is the target for 2-month storage. None of the antioxidants significantly enhanced the stability of vitamin B in the solution. The observed instability of vitamin B_{12} in the solution may be due to the pH of the solution. The pH spray solutions were adjusted to 9 for folic acid solubility, but vitamin B_{12} is only stable in slightly acidic solutions.

Given that vitamin B_{12} was not stable at pH 9, the impact of allowable pH (due to folic acid) on the stability of B_{12} was investigated in a new set of spray solutions. The solubility of folic acid is dependent on the concentration of the sodium carbonate in the solution, which determines the pH. For the targeted concentrations of the micronutrients in the solution, pH 8 is the lowest possible pH for folic acid, iodine, and vitamin B_{12} solution that can be obtained by adjusting with 0.1M sodium carbonate. Hence, the impact of pH 8-11 (11 being the highest possible pH) on the stability of vitamin B_{12} was evaluated. The stability of vitamin B_{12} was dependent on the pH of the solution (*Figure 63*). At 45 ^{o}C, all the B_{12} in the solutions were lost at pH values above 8. Even at pH 8, only 50% of B_{12} was retained.

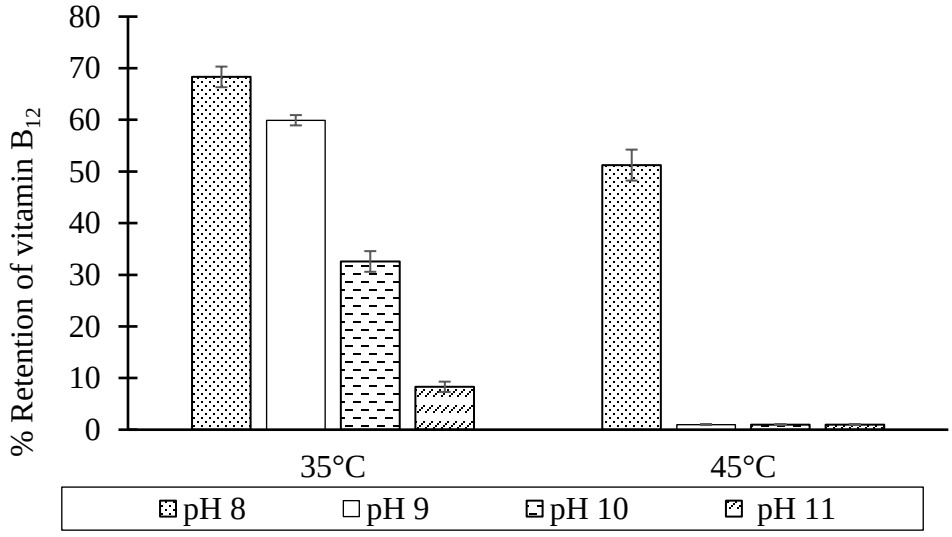


Figure 63:Effect of pH on the stability of vitamin B_{12} after 1 month at different storage temperatures

The impact of citrate and ascorbate (as antioxidants), and the exposure to natural light on the stability of vitamin B_{12} was evaluated in the last set of spray solutions. Iodine and folic acid were very stable in all the solutions: less than 11% of the added iodine and folic acid was lost after 2-

month storage, even at 45 ^{o}C. The stability of folic acid (Table 19) and iodine were not impacted by the exposure to light Exposure to light significantly affected the stability of B_{12} in solutions that contained ascorbate. For the solution that contained ascorbate, the colour of the solution changed from pink to yellow on exposure to light (Figure 64). In other solutions, exposure to light either marginally affected or did not significantly impact the stability of vitamin B_{12} (Table 19). This result is unexpected, as vitamin B_{12} is known to be photosensitive. The pH of the solution may be responsible for the observation. The photodegradation of vitamin B_{12} is pH-dependent (Ahmad, Hussain and Fareedi, 1992).

Table 19:Impact of Light Exposure to Folic Acid and Vitamin B_{12} Stability (2-Month Storage)

Solution Description	Stability of Folic Acid (%)		Stability of Vitamin B_{12} (%)	
	Light	Dark	Light	Dark
B_{12}+FA+I adjusted to pH 9 by Na_2CO_3	94.4±0.9	95.7±0.9	77.7±4.0	72.6±1.6
B_{12}+FA+I adjusted to pH 8 by Na_2CO_3	93.4±1.6	94.1±0.9	68.5±1.1	74.9±11.7
B_{12} unadjusted pH 6.5			77.2±2.1	82.8±3.6
B_{12} + I unadjusted pH 6.8			87.5±5.1	70.9±0.8
B_{12} adjusted to pH 2.8 by ascorbate			4.0±0.2	21.5±0.7

Figure 64: Discolouration of a solution of vitamin B_{12} and ascorbate due to light exposure
The covered scintillation vial was kept in the dark (pink, the standard colour of vitamin B12); uncovered vial was exposed to light

Ascorbate (AH_2) and pH were the significant factors that negatively affected the stability of B_{12} in the solutions. Ascorbate promoted the degradation of B_{12} in the solution. Ahmad et al. (2017) and (2014) described the product of degradation as hydroxocobalamin, which is further rapidly degraded to products that do not elicit the vitamin functions. The degradation of cyanocobalamin

to hydroxocobalamin involves the loss of the CN-group. The degradation is a reductive decyanation reaction that occurs slowly in the presence of light; it was catalyzed in this case by ascorbate(Ahmad *et al.*, 2014). This involves the reductive potential of the ascorbate.

Given that the pKa of ascorbate is 4.2 (Du, Cullen and Buettner, 2012), it is predominantly in its conjugate acid form in the solution (pH 2.8), which has high reductive potential to drive the decyanation. The spray solution that contained ascorbates was discoloured when exposed to light for one month. The change in colour implies the reduction of the central cobalt from Co^{3+} to Co^{2+} and the oxidative cleavage of the corrin ring (Frost, Lapidus and Armstrong, 1952). Since Co^{2+} moiety in vitamin B_{12} can be easily oxidized to Co^{3+} (Fedosov *et al.*, 2011) and that the loss of vitamin function is through oxidation, the oxidative cleavage poses more significant damage to the stability of vitamin B_{12} than reductive decyanation.

$$[Co^{3+}CN] \xrightarrow{hv/AH_2} [Co^{3+}OH] + CN^- \xrightarrow{hv/AH^-} [Co^{2+}OH] \overset{O_2}{\rightarrow} \textit{Corrin ring cleavage products}$$

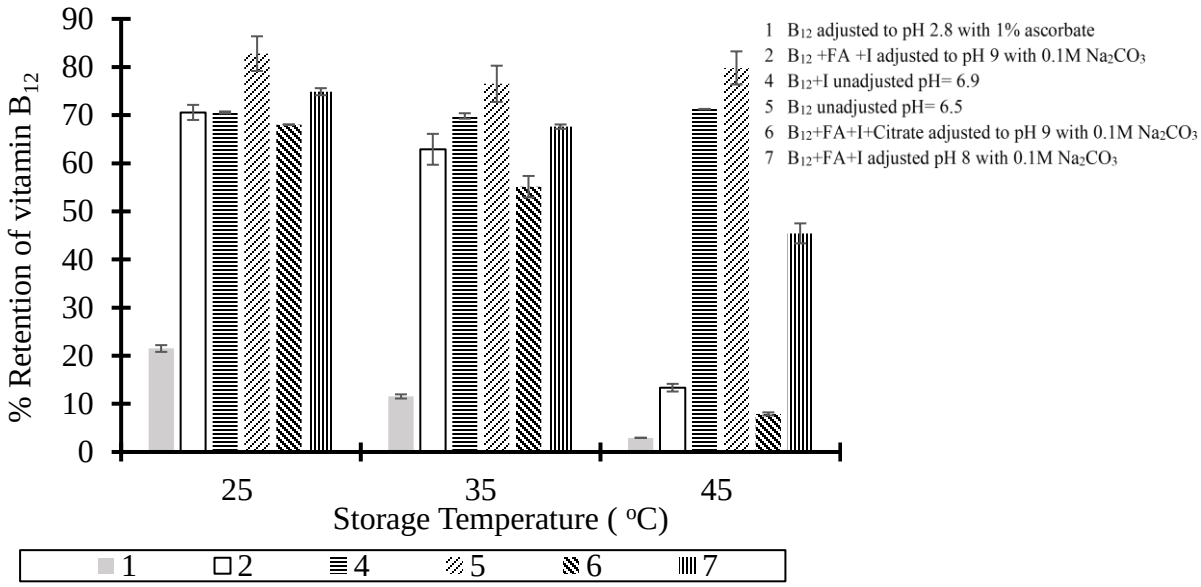


Figure 65: Stability of B_{12} in the new set of solution for the quadruple fortification of salt after 2 months at different storage temperatures

In the absence of ascorbic acid, pH significantly impacted the stability of B_{12}. pH 9 is essential for the stability and solubility of folic acid. At this pH, B_{12} is not stable. By adjusting the solution to pH 8, the stability of B_{12} was significantly improved, but the stability was not adequate (Figure 65). Frost et al. (1952) showed that the optimal pH for B_{12} is 4-6.5 and that at pH 7.5 and above,

there is a rapid loss of vitamin B_{12}. Salnikov and Makarov (2019) suggest that alkaline solution enhances the reduction of Co^{3+} to Co^{2+} through the deprotonation of the corrin ring (acting as a reducing agent). Also, the C3, C8, and C13 of vitamin B_{12} are susceptible to this deprotonation under this condition.

Comparing Solutions 4 (B_{12} unadjusted pH=6.5) and 5 (B_{12} + I unadjusted pH=6.9), iodate or increase in pH seems to have protected B_{12} from photolysis. Ahmad et al. (1992) showed that, in the pH range of 1to 8, increasing the pH of B_{12} solutions protects B_{12} from photolysis. So, the protective effect is probably due to pH rather than the iodate. Within the tested parameters, neither pH nor antioxidants had a significant effect on the stability of iodine and folic acid. B_{12} was very stable (70% and 76%, even at 45 ^{o}C) in solutions 4 and 5, respectively (Figure 65).

From the results, it became clear that formulating a solution that contained folic acid and B_{12} is not feasible for making QFS. Therefore, three further options for making QFS were proposed.

1) Adding iodine and B_{12} as solution and iron and folic acid as encapsulated solids
2) Adding iodine and folic acid as solution and iron and B_{12} as encapsulated solids
3) Adding iodine as solution and iron, folic acid and B_{12} as encapsulated solids

The first option was not considered given that the stability of B_{12} (70%) was lower in the solution in option (1) than the stability of folic acid (91%) in the solution in option (2). The remaining two options will require that the technology for making iron premix be modified to accommodate additional micronutrient(s). Option 2 being simpler than Option 3 was first considered. Option 2 involves making an iron-B_{12} premix.

5.6.2 Formulation of Quadruple Fortified Salt (QFS)

The technology for making iron premix consists of extrusion, cutting, size matching, colour masking, and coating. Four of these steps were considered as the point of B_{12} addition:

a) Coextrusion of iron (ferrous fumarate) and B_{12} (A),
b) Spraying a solution of B_{12} on size screened iron extrudate before colour masking (B),
c) Using a mixture of B_{12} and TiO_2 for colour masking (C), and
d) Using a mixture of B_{12} and HPMC for coating (D).

The impact of these four options on the stability and feasibility of the process was evaluated. Co-extruding iron and vitamin B_{12} (A) was the simplest of these four options. Option C was not

technically feasible. At the time of formulating this premix, colour masking agent (TiO_2) was held on the surface of the extrudate by a weak electrostatic force before it was glued to the surface by the coating material. The process of applying the coating material required the use of compressed air, which blew some TiO_2 away. With this method, the B_{12} was also blown away with the TiO_2. Also, Option B required a solution of B_{12} to be sprayed on iron extrudates. The addition of the solution will increase the moisture content and water activity of the premix. The moisture may aid the degradation of micronutrients and microbial growth. Finally, Option D was not feasible due to a colour problem. Soy stearin being translucent cannot sufficiently mask the pink colour of the mixture of B_{12} and HPMC used for holding TiO_2 in place. Hence, the premix made with Option D was pink in colour. There were noticeable pink spots in the QFS formulated with premix (D). Given all these technical issues, the co-extrusion of iron and B_{12} was preferred among the four routes proposed.

Only Premix A, B, and D were used to formulate QFS and TFS* (salts that conatined three micronutrients but not a combination of iron, iodine and folic acid): they were mixed with salt fortified with iodine and folic acid. The premix samples were also added to iodized salt and salt fortified with folic acid. The concentrations of micronutrients in the fortified salt were 1000pmm, 50ppm, 25ppm, and 0.25ppm for iron, iodine, folic acid, and vitamin B_{12}, respectively. In all the salt samples, less than 20% of iodine and 30% of the added folic acid were lost after 6-month storage (Table 20).

Table 20:Stability of folic acid and iodine in QFS and TFS* formulated with Fe-B_{12} Premix

Type of		**Retention of Iodine (%)**			Retention of Folic Acid (%)		
Solid premix	Spray solution	25 ^oC	35 ^oC	45 ^oC	25 ^oC	35 ^oC	45 ^oC
A	I +FA	100.4±2.0	101.5±4.1	99.7±4.5	97.2±2.9	94.4±0.9	93.2±0.4
B	I +FA	98.4±1.8	93.8±1.2	85.8±2.0	86.8±1.8	85.6±1.0	78.4±2.8
D	I +FA	100.7±2.6	98.1±3.3	93.8±3.0	86.9±4.5	83.7±1.7	77.0±1.9
A	I	92.5±2.2	94.3±1.2	89.4±0.7			
D	I	93.1±1.1	94.1±0.3	92.0±1.4			
A	FA				83.9±2.9	84.6±0.9	73.5±1.6
D	FA				85.7±2.5	80.9±1.8	72.1±3.1
Iron premix	I + FA+ B_{12}	97.0±2.4	89.9±2.3	86.2±4.7	87.7±3.0	82.4±6.5	79.4±0.3

I= 2% Iodine; FA=1% folic acid; A=iron and B_{12} were coextruded; B=B_{12} sprayed on iron extrudate before colour masking; D= a mixture of B_{12} and HPMC was used for coating iron premix

5.6.2.1 Effect of the Position of Vitamin B_{12} in the Premix on the Stability of Iodine and Folic Acid in QFS

The impact of the position of B_{12} in the premix on the stability of folic acid and iodine was evaluated. In the salt, the emphasis was on the stability of folic acid and or iodine because B_{12} was very stable in the three premix samples (A, B & D); more than 95% of the added vitamin B_{12} was retained in the premix after 6-month storage. In terms of iodine and folic acid stability, Premix A (coextruded iron and B_{12}) was better than the other premix samples. The QFS formulated with Premix A had more than 90% of the added folic acid and iodine retained in the salt for 6 months, even at 45 °C/60-70% RH (Figure 66).

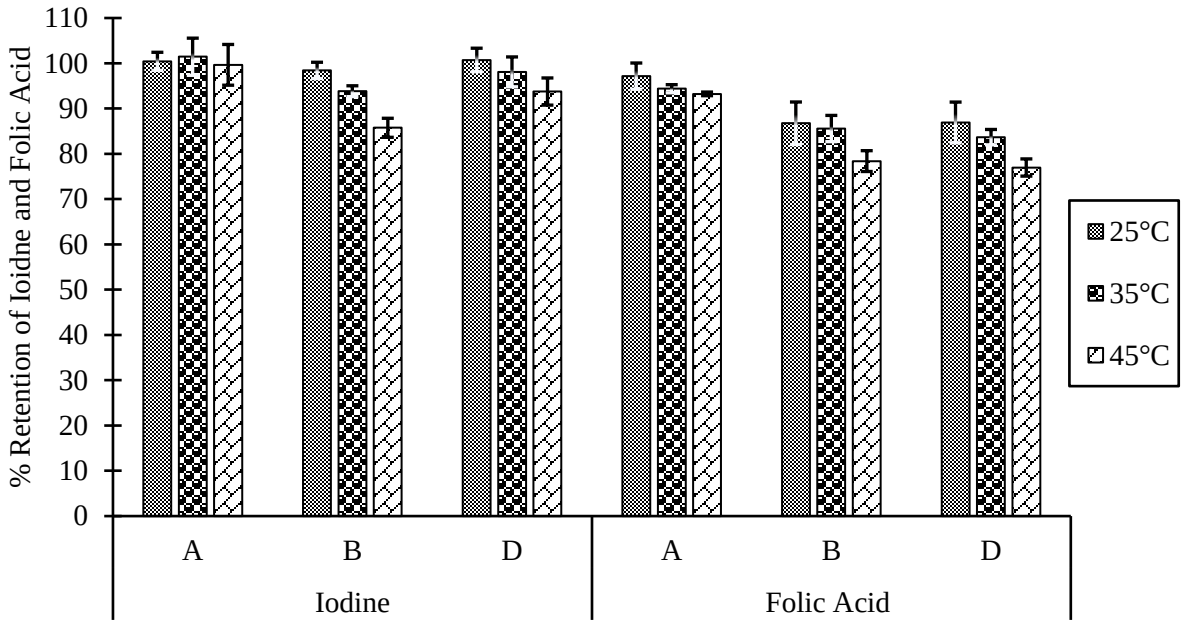


Figure 66:Effect of the position of B_{12} in premix on the stability of iodine and folic acid in the QFS after 6 months at different storage temperatures

A=iron and B_{12} were coextruded; B=B_{12} sprayed on iron extrudate before colour masking; D= a mixture of B_{12} and HPMC was used for coating iron premix

5.6.2.2 Effect of Vitamin B_{12} on the Stability of Iodine and Folic Acid in QFS

In this batch of QFS, there was a sample formulated with a freshly prepared solution of iodine, folic acid, and vitamin B_{12} and iron premix. The stability of folic and iodine in this QFS sample was compared with the QFS formulated with a solution of folic acid and iodine, and premix A. In the latter QFS, B_{12} was separated from the iodine and folic acid; in the former, the three micronutrients were not separated. In both QFS samples, more than 70% folic acid and 85% iodine were retained after 6-month storage. Separating B_{12} from folic acid and iodine in the QFS

significantly improved the stability of folic acid and iodine in the salt. This further supports the idea of not adding vitamin B_{12} to the salt through the spray solution but through the premix.

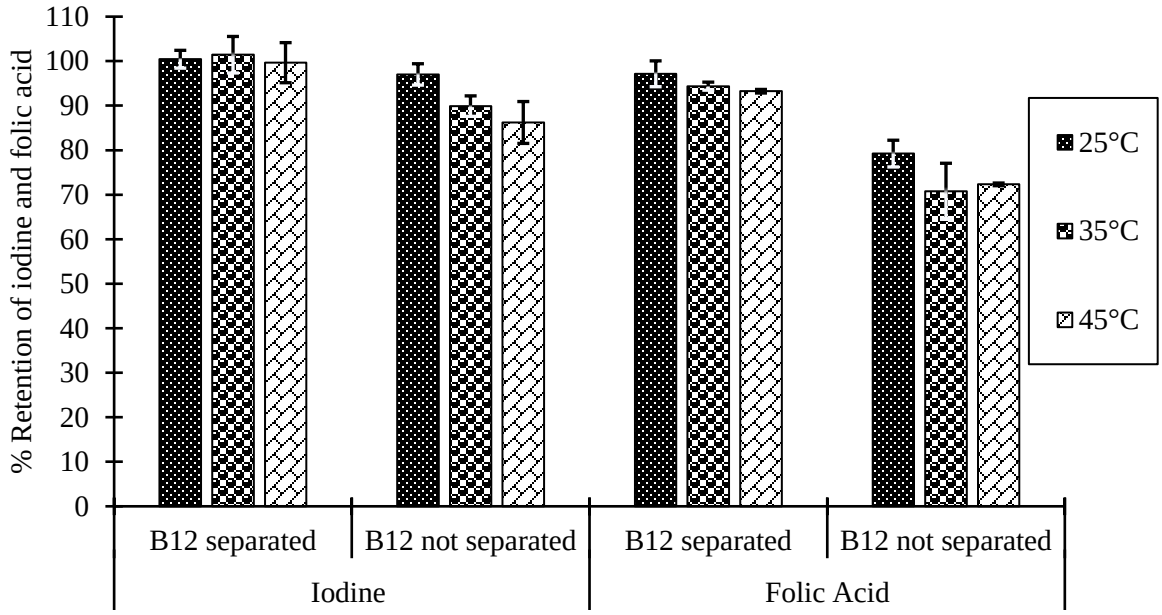


Figure 67:Effect of B_{12} on the stability of iodine and folic acid in the QFS after 6 months at different storage temperatures

The coextrusion of B_{12} and iron (ferrous fumarate) has several advantages:

It is the most straightforward of the four techniques for making Fefum-B_{12} premix. The technique physically separates the incompatible fortificants in the spray solution. This will maintain their potency and enhance their stability in salt. The encapsulation of the extrudate may facilitate the acceptability of the QFS; the pink colour of the vitamin B_{12} is eliminated by the colour masking and coating excipients of the premix. The coats protect B_{12} from photodegradation. The existing infrastructure for DFS production can handle this technology. Finally, the micronutrients are very stable in the QFS; more than 90% of all the added micronutrients were retained after 6-month storage in the coextruded sample. So, the technology has the potential of delivering the four micronutrients to vulnerable populations. However, just as with the TFS made with folic acid and iodine solution and iron premix, the colour of the salt was yellow. The colour may affect the acceptance of the salt by consumers. Hence, the process was futher optimized.

5.6.3 Optimizing the Process of Quadruple Fortification of Salt

The process for adding iron, iodine, folic acid, and vitamin B_{12} to salt, initially described, was optimized to minimise the yellow colouration of the salt by folic acid. In the optimized process, FeFum-B_{12}-FA premix was made instead of the FeFum-B_{12} premix used in the initial process.

Two designs were developed for the FeFum-B_{12}-FA premix.

a) All the micronutrients were in the core of the premix (FeFum+B_{12}+FA).
b) The FeFum-B_{12} core was separated from the folic acid by a thin layer of TiO_2 ((FeFum+B_{12})extrudate +FA).

The choice of FeFum-B_{12} over a FeFum-FA core was due to higher stability of B_{12} in the FeFum-B_{12} premix relative to folic acid stability in the FeFum-FA (as shown in previous sections '5.3.4' and '5.4.2.1'). The concentrations of iron, iodine, folic acid, and vitamin B_{12} in the QFS were maintained as in the QFS discussed in Section 5.3.4

The two designs had no significant impact on the stability of iodine in the salt (Figure 68). The result was expected because irrespective of the premix designs, they were colour masked and coated by the same materials. The high retention of iodine in the QFS samples confirms that the coating formed an adequate physical barrier, keeping the iron in the premix from being in contact with iodine in the salt. At 45 ^{o}C, folic acid improved the stability of iodine in the salt (FeFum+B_{12} vs. FeFum+B_{12}+FA). This observation is consistent with previous reports (McGee, Sangakkara and Diosady, 2017; Modupe, Krishnaswamy and Diosady, 2019).

Folic acid was very stable in the QFS except in the salt formulated with the (FeFum+B12)extrudate +FA Premix (in this premix folic acid was sandwiched between the thin layers of TiO_2). The coextrusion of the iron, folic acid, and B_{12} significantly improved the stability of folic acid when compared with the sample that has its folic acid separated from iron and B_{12} by a thin layer of TiO_2. When the three QFS samples were compared, the stability of folic acid in the salt was impacted by TiO_2 and iron. Sandwiching the folic acid between two layers of TiO_2 may have negatively impacted the stability of folic acid in (FeFum+B12)extrudate +FA sample. Folic acid is photolabile. TiO_2 might have induced photocatalytic degradation of folic acid in the sample (Jonidi-Jafari *et al.*, 2015). At 25 and 35 ^{o}C, contact with iron in the premix significantly improved the stability of folic acid in the QFS samples. McGee et al. (2017) and Modupe et al. (2019) proposed oxidative degradation as the possible path for the loss of folic acid in fortified salt. The ferrous iron used may have inhibited the oxidative degradation of folic acid. This observation is

consistent with that of Modupe et al. (2019) and Li et al. (2008). Both studies observed that ferrous iron enhanced the stability of folic acid.

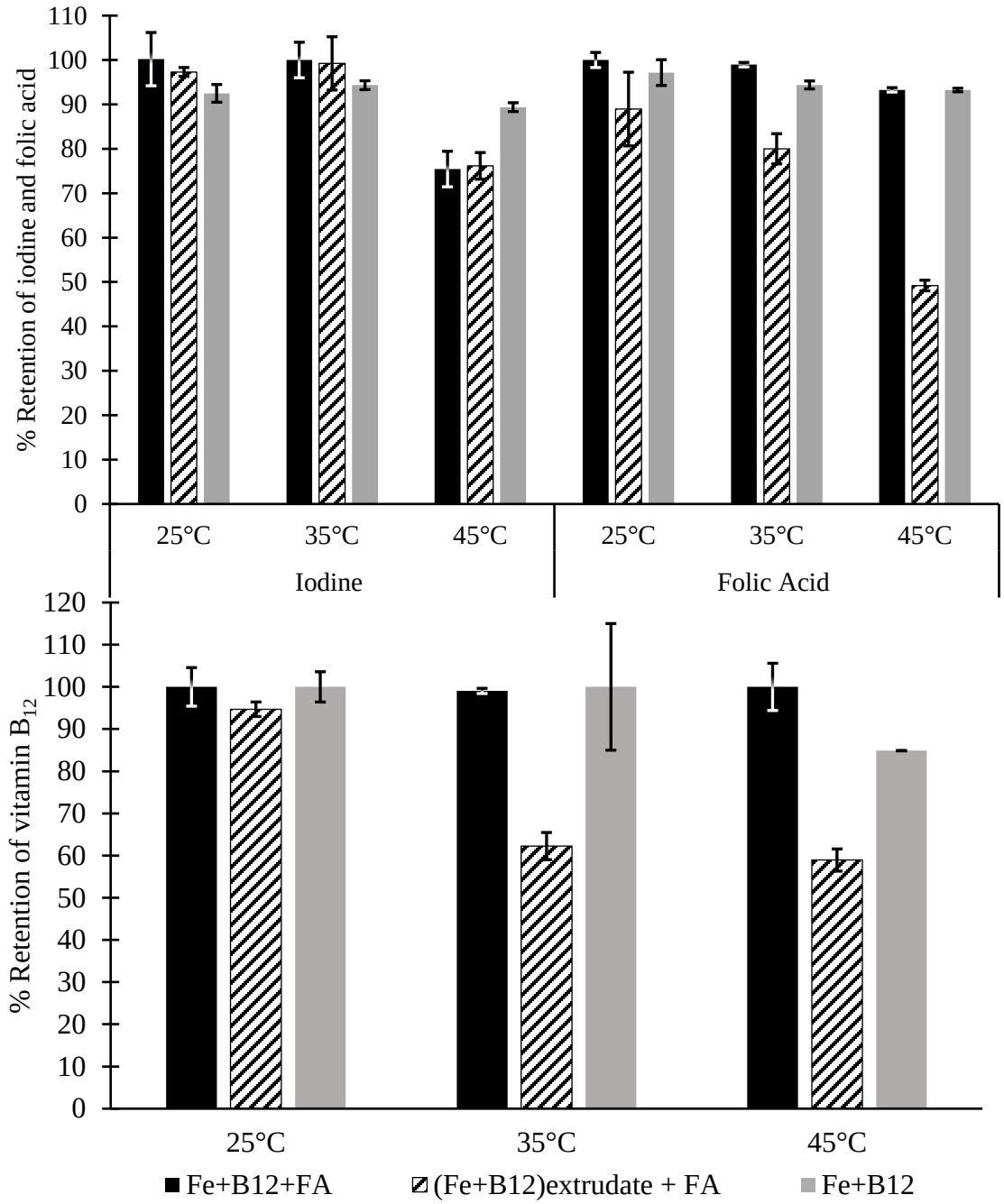


Figure 68:Stability of iodine, folic acid and vitamin B_{12} in the optimized QFS after 6 months at different storage temperatures

Fe+B12+FA: iron, vitamin B_{12} and folic acid were in the core of the premix that was added to iodized salt; (Fe+B12)extrudate +FA: iron and vitamin B_{12} were in the core while folic acid was sandwiched between two titanium dioxide layers of the premix that was added to iodized salt; Fe+B12: iron and vitamin B_{12} were in the core of the premix that was added to the salt sprayed with a solution of folic acid and iodine.

Unlike folic acid, the presence of iron significantly improved the stability of vitamin B_{12} in the salt only at 45 ^{o}C. The reason for this is unknown. The impact of ascorbic acid on the stability of vitamin B_{12} (in Section 5.4.1) suggested that reducing agents negatively affect the stability of vitamin B_{12}. This result did not support this suggestion. Several studies showed that iron irrespective of its oxidation state helps to stabilize vitamin B_{12} (Zuck and Conine, 1963; Newmark, 1958; Mukherjee and Sen, 1959; Mukherjee and Sen, 1957). An insight into the mechanism of reduction by ferrous iron and ascorbate may help to reconcile this contradiction. While ferrous iron is just an electron donor, ascorbate is both an electron and a proton donor. This difference may further shed light on the mechanism of degradation of vitamin B_{12} in the presence of ascorbate. Ahmad et al. (2014) showed that the loss of vitamin B_{12} due to ascorbate was higher at the pH that supports the production of H^+ ion from ascorbate. Hence, the protonation of vitamin B_{12} by H^+ ion from ascorbate may be responsible for the observed loss of vitamin B_{12}. The studies by Argüello et al. (2005) and Kim et al. (2008) suggest that protonation is the likely mechanism for the homolysis of alkyl and cyanide group in vitamin B_{12}.

Just as with other micronutrients, coextrusion of iron, folic acid, and vitamin B_{12} significantly improved the stability of vitamin B_{12} when compared with the other design. Hence, the optimal formulation of QFS involves adding iron, folic acid, and vitamin B_{12} as a coextruded and encapsulated solid to iodized salt. The route did not only solve the colour problem associated with spraying folic acid directly on salt but also shielded folic acid and vitamin B_{12} from the potential adverse effect of their exposure to light. The formulated QFS will deliver 200% iodine RDA, 56% iron RDA, 100% folic acid RDA and 100% vitamin B_{12} RDA based on the consumption of 10g of fortified salt per day (Vinodkumar and Rajagopalan, 2009).

5.7 Kinetic Tools for Predicting Stability of Iodine and Folic Acid in Fortified Salt

Kinetic tools, such as degradation constant and Gibb free energy, are vital to predicting the stability of micronutrients in the salt samples. With these tools, the stability of micronutrients in the distribution channels can be predicted.

5.7.1 Calculation and Validation of the Kinetic Parameters for the Stability of Iodine and Folic Acid in TFS

The data obtained from the 6-month stability study were used to calculate the kinetic parameters for micronutrient stability in salt (Figure 69). The linear regression (R^2) of the different rate laws of degradation of the micronutrients was used to predict the order of degradation. For Triple Fortified Salt, the correlation regression (R^2) of first-order was slightly higher than that of the zero- and second-order. Since the degradation of iodine and folic acid were individually accounted for, the degradation of iodine and folic acid in the Triple Foritfed Salt may be first-order or pseudo-first-order kinetics. Given the stability of micronutrients in the salt, folic acid may be a degradation rate-limiting fortificant because it is less stable than iodine. Folic acid in the salt is degraded through oxidative interaction with iodate, the source of iodine. Diffusion may play a role in their interaction. The diffusion is a zero-order rate reaction, while oxidation is more likely the first-order rate reaction; hence, the closeness in the value of R^2 for the zero and first-order degradation rate. However, I assume the first-order degradation for folic acid because oxidation caused its degradation and the slightly higher value of R^2. The slope of Figure 69 (the degradation constant) was used to plot the Arrhenius graph for the zero- and first-order degradation of iodine and folic acid in the salt (Figure 70). The activation energy for the degradation of the micronutrients in the fortified salt was calculated using the slope of the Arrhenius plot and Equation 16. Although the activation energy for the first-order degradation was higher than that of the zero-order, they showed similar trends (Table 21).

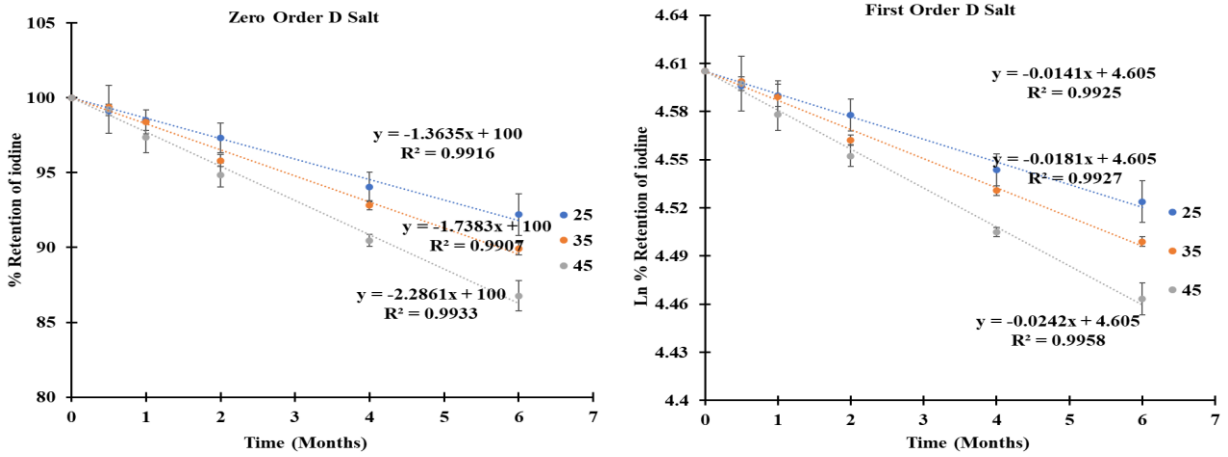


Figure 69: Sample of the zero- and first-order degradation kinetics of iodine in a fortified salt obtained from 6 month stability study

$$E_a = -(slope\ of\ Arrhenius\ plot\ \times R\ ) \qquad Equation\ 16$$

Where E_a = activation energy (J/mole)

R= gas constant (8.314 J/K·mole)

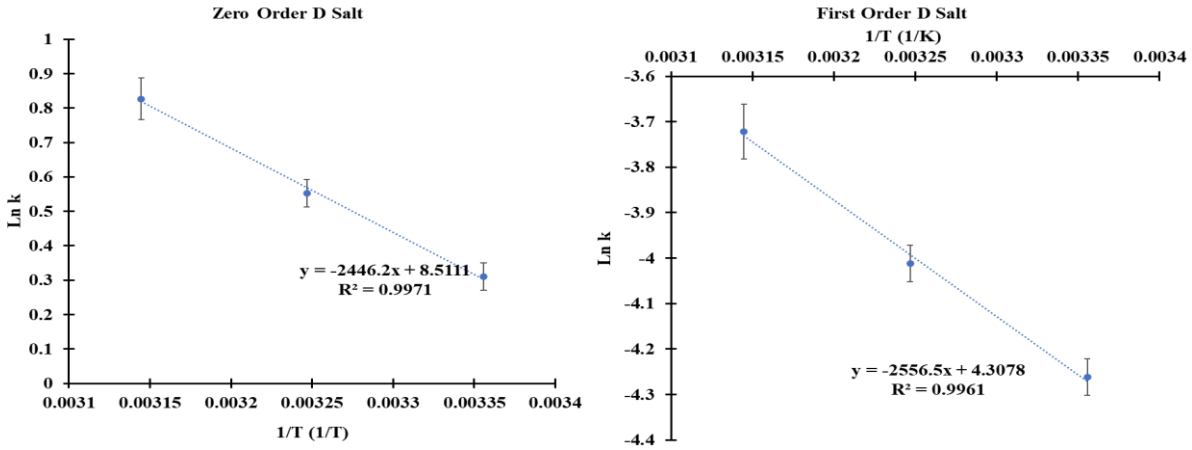


Figure 70: Sample of the Arrhenius plot for the zero- and first-order degradation kinetics of iodine in a fortified salt obtained from 6 month stability study

Table 21:Kinetic parameters of the degradation of folic acid and iodine in DFS* and TFS

Salt Samples	**Zero Order**				**First Order**			
	k (g/ml. month^{-})			**Activation Energy (kJ/mole)**	**k (10^{-2} month^{-})**			**Activation Energy (kJ/mole)**
	25 ºC	**35 ºC**	**45 ºC**		**25 ºC**	**35 ºC**	**45 ºC**	
Folic Acid								
C	1.8±0.1	2.1±0.1	2.6±0.1	14.1±0.7	2.0±0.1	2.3±0.1	3.2±0.1	14.1±0.7
D	2.7±0.2	3.1±0.1	3.7±0.2	12.8±0.6	3.2±0.2	3.9±0.1	4.6±0.2	15.7±0.7
CA	2.0±0.1	3.0±0.2	3.7±0.1	27.6±1.1	1.9±0.1	3.2±0.2	4.1±0.1	30.1±1.2
DA	2.3±0.1	2.8±0.2	3.4±0.2	16.2±0.9	2.8±0.1	3.6±0.3	4.6±0.2	19.7±1.1
Iodine								
C	0.8±0	1.2±0	1.6±0	28.5±0.8	0.8±0	1.2±0	1.9±0.1	33.5±0.8
D	1.4±0	1.7±0	2.3±0.1	20.3±0.5	1.4±0	1.8±0	2.4±0.1	21.3±0.5
CA	1.0±0	1.5±0.1	2.0±0.1	26.4±1.0	1.1±0	1.8±0.1	2.2±0.1	26.3±1.0
DA	1.0±0	1.2±0	1.5±0	15.4±0.4	1.1±0	1.3±0	1.9±0.1	16.4±0.4

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm.

The activation energies of the C salts were higher than those of the D salts. The presence of iron premix increased the activation energies for folic acid in the salt but reduced the activation energies of iodine in the salt. These observations imply that folic acid and iodine were more stable in the C salts than in D salts and that iron seems to improve the stability of folic acid while it decreases that stability of iodine in the salts. As stated in earlier sections, the reducing power of the ferrous fumarate may have played a role in this. While it reduces the iodate to iodine, which is then lost by sublimation, the iron may have reduced the oxidative degradation of folic acid. These deductions are consistent with the trend of the stability of folic acid and iodine in TFS samples.

Table 22:Validation of the Kinetic Parameters Estimated from 6-Month Storage with 12-Month Storage Data (TFS)

Salt Sample	**Folic Acid (Months)**	**Iodine (Months)**
C	11.6 ± 1.0	11.4 ± 1.4
D	11.7 ± 0.1	11.4 ± 1.0
CA	10.4 ± 2.3	11.4 ± 1.0
DA	11.8 ± 1.8	11.9.± 0.5

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm.

The kinetic parameters of the degradation of micronutrients in the TFS were derived based on a 6-month stability study. The stability of micronutrients in salt in a 12-month study was used to validate the calculated kinetic parameters in Table 21. By using Equation 17 (derived from the first-order rate integral equation), corresponding stability times were calculated; their closeness to 12 months validates the degradation constant for TFS (Table 22). Using Equation 17, the time it will take to lose 25% of the iodine, and folic acid ($R_{(T)}$ = 75%) in TFS samples was calculated. From the calculations and for TFS with the best stability outcome, it will take 15, 9, and 7 months to lose 25% folic acid in TFS at 25, 35, and 45 ^{o}C, respectively. For iodine, it will take 26, 16, and 13 months, respectively (Table 23). In all samples, the micronutrients can be projected to retain at least 75% of the added micronutrients for more than 6 months.

$$t_{(R,T)} = \frac{ln\left(\frac{1}{R_{(T)}}\right)}{k_{(T)}} \qquad Equation\ 17$$

Where;

$t_{(R,T)}$= time (in months) required to retain micronutrient (%) in fortified salt at a given temperature

$R_{(T)}$= Retention of micronutrients in fortified salts (%) for a given temperature; values were obtained from the 12-month stability study

$k_{(T)}$= degradation constant obtained from the 6-month stability study

Table 23:Estimated Time (Months) for the Loss of 25% and 50% of Folic Acid and Iodine in TFS

Micronutrients	Salt Samples	25 ºC		35 ºC		45 ºC	
		25%	50%	25%	50%	25%	50%
Folic Acid	C	14.7±0.7	35.4±1.7	12.3±0.7	29.6±1.7	9.5±0.4	23.0±1.0
	D	9.3±0.5	22.5±1.2	7.3±0.3	17.6±0.7	6.3±0.3	15.1±0.7
	CA	14.9±0.6	35.9±1.4	9.0±0.5	21.7±1.2	7.0±0.2	16.8±0.5
	DA	11.1±0.4	24.9±1.0	8.4±0.6	19.4±1.5	6.4±0.3	15.1±0.7
Iodine	C	36.4±0.8	87.7±1.9	23.6±0.6	56.8±1.4	15.6±0.5	37.5±1.2
	D	20.4±0.6	49.2±1.4	15.9±0.2	38.3±0.5	11.9±0.3	28.6±0.7
	CA	25.9±0.9	62.5±2.1	16.3±0.6	39.2±1.4	13.3±0.4	32.1±1.0
	DA	27.1±0.7	65.4±1.7	22.8±0.3	55.0±0.7	17.9±0.6	43.1±1.4

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm.

5.7.2 Calculation and Validation of the Kinetic Parameters for the Stability of Iodine and Iron in QFS

The kinetic data for folic acid and iodine in QFS were calculated just as it was done for TFS. The calculations were based on the stability of folic acid and iodine in the Quadruple Fortified Salts monitored for 6 months (Figure 71). There are two QFS samples for which the kinetic parameters were estimated. In the first sample (QFS*), a solution of iodine, folic acid, and vitamin B_{12} was sprayed on salt, while the iron was added as an extruded and encapsulated iron. In the second sample (QFS), a solution of folic acid and iodine was sprayed on salt while iron and B_{12} were added as an extruded and encapsulated particle. As observed for TFS, the R^2 of the first order of degradation of the micronutrients was higher than the zero- and second-orders. The degradation of folic acid and iodine in the QFS sample may have obeyed the first-order rate or pseudo-first-order rate because the degradation of iodine and folic acid were accounted for individually while vitamin B_{12} was not considered (it was very stable). Given the same reasons as that of TFS, I assumed a first-order degradation rate. The activation energy was extrapolated from the Arrhenius plot

(Figure 72) and Equation 16. The activation energy for the degradation of iodine was higher than that of folic acid (Table 24). The activation energy for the degradation of folic acid and iodine in QFS was higher than in QFS*. This is consistent with the stability study- aside from the instability of B_{12} in the spray solution, micronutrients were more stable in QFS if folic acid and B_{12} were not added together as a solution sprayed on salt.

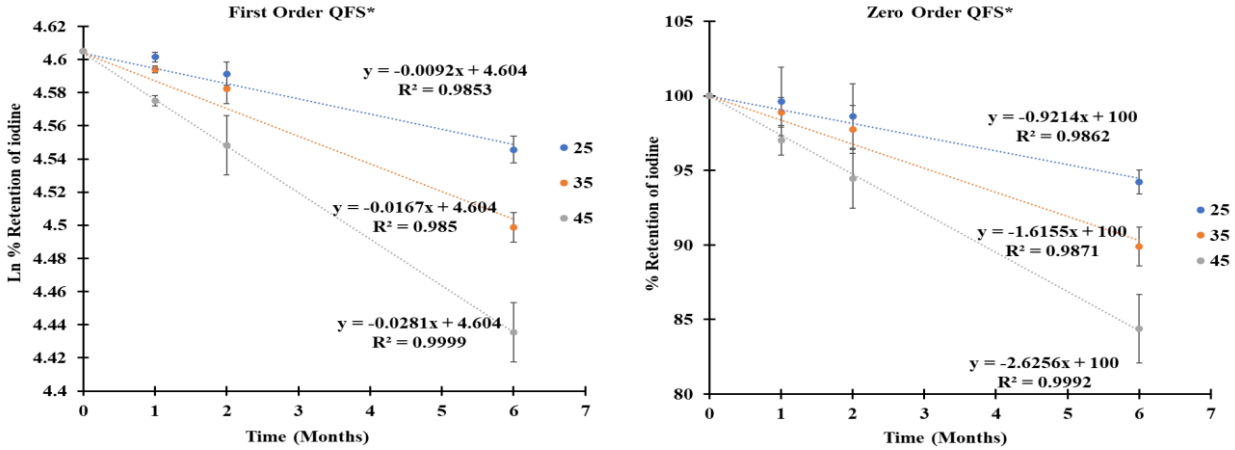


Figure 71: Sample of the zero- and first-order degradation kinetics of iodine in a QFS obtained from 6-month stability study

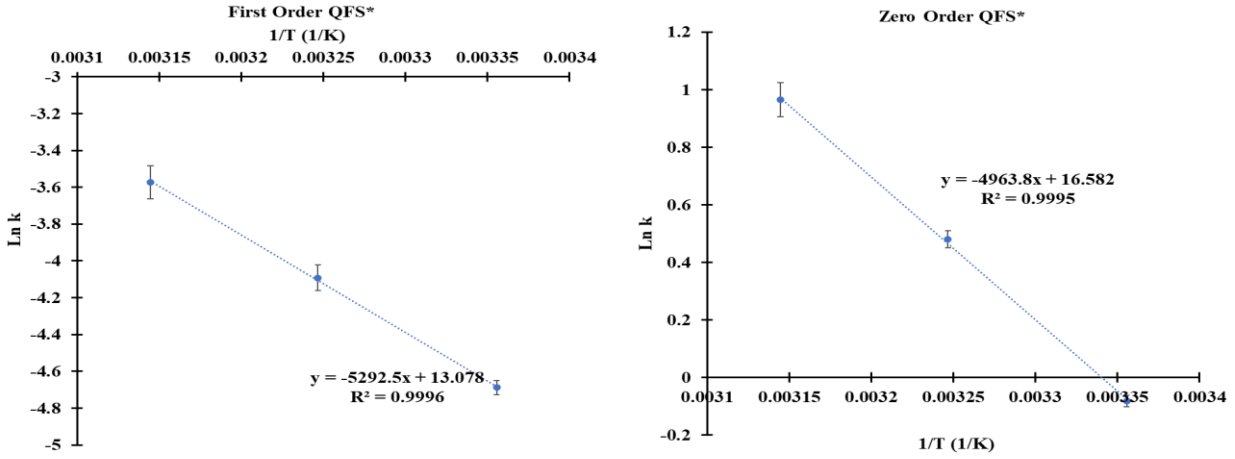


Figure 72: Sample of the Arrhenius plot for the zero- and first-order degradation kinetics of iodine in a QFS obtained from 6 month stability study

Table 24:Kinetic parameters of the degradation of folic acid and iodine in QFS

Salt Samples	Zero Order				First Order			
	k (g/ml. month^{-})			Activation Energy (kJ/mole)	k (10^{-2}. month^{-})			Activation Energy (kJ/mole)
	25 ^{o}C	35 ^{o}C	45 ^{o}C		25 ^{o}C	35 ^{o}C	45 ^{o}C	
Folic Acid								
QFS*	1.3±0	1.7±0.1	2.4±0.2	24.0±1.2	1.4±0	1.8±0.1	2.7±0.3	29.4±1.5
QFS	0.9±0	1.3±0	1.8±0.1	29.0±1.2	0.9±0	1.4±0	2.0±0.1	35.3±1.4
Iodine								
QFS*	0.9±0	1.6±0	2.6±0.1	41.3±1.2	0.9±0	1.7±0.1	2.8±0.1	44.0±1.2
QFS	0.3±0	0.5±0	0.9±0	49.2±1.8	0.2±0	0.5±0	0.9±0	53.0±1.9

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.

The kinetic parameters calculated for folic acid and iodine in the Quadruple Fortified Salt were validated with data obtained from the 12- month stability study of Quadruple Fortified Salt. Just as carried out for TFS (using Equation 17), the kinetic parameters and the percentage stability of folic acid and iodine in the salt were used to predict the time of retention of the corresponding percentage stability. The predicted time was about 12 months, which validated the kinetic parameters from Table 24 (Table 25). The time it will take for 25% of the micronutrient to be lost ($R_{(T)}$ = 75%) was estimated. For the Quadruple Fortified Salt with a better outcome (QFS), it will take 32, 20, and 15 months to lose 25% folic acid in the QFS at 25, 35, and 45 ^{o}C, respectively. For iodine, it will take 122, 62, and 32 months, respectively (Table 26). This kinetic tool can be used to predict the loss of micronutrients in salt in the field by using the data obtained from laboratory storage studies, provided that all the other factors that may affect the stability of the micronutrients in the field can be simulated in the laboratory.

Table 25: Validation of the Kinetic Parameters Estimated from 6-Month Storage with 12-Month Storage Data (QFS)

Salt Samples	Folic Acid	Iodine
QFS*	12.2 ± 0.6	11.5 ± 1.4
QFS	12.1 ± 1.5	13.0 ± 1.0

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.

Table 26:Estimated Time (Months) for the Loss of 25% and 50% of Folic Acid and Iodine in QFS

Micronutrients	**Salt Samples**	**25 ºC**		**35 ºC**		**45 ºC**	
		25%	**50%**	**25%**	**50%**	**25%**	**50%**
Folic Acid	QFS*	21.0±0.3	50.6±0.7	15.9±0.7	38.3±1.7	10.7±1.1	25.8±2.7
	QFS	32.0±0.9	77.0±2.1	20.1±0.6	48.5±1.4	14.6±1.0	35.2±2.4
Iodine	QFS*	31.3±0.6	75.3±1.4	17.2±0.4	41.5±1.0	10.2±0.4	24.7±1.0
	QFS	122.4±2.5	295.0±6.0	61.7±2.3	148.7±5.5	31.9±1.9	76.8±4.6

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.

5.8 The Effect of Boiling on the Degradation of Folic Acid and Vitamin B_{12}

For this fortification technology to indeed have an impact on a population, the micronutrients added to food must be able to survive the cooking techniques used for making food. Rice, the most commonly consumed food, is cooked by boiling (Muthayya *et al.*, 2014). Hence, in a preliminary study, the effect of boiling on the stability of folic acid and vitamin B_{12} was evaluated. About 93% of the folic acid and 75% of B_{12} added was retained after boiling for 30 minutes - the average cooking time. The result showed that the micronutrients were sufficiently stable, and folic acid was comparatively more stable than vitamin B_{12}. The kinetics of degradation of folic acid and B_{12} fit the pseudo-second-order kinetics, as shown in Figure 73 (Robati, 2013).

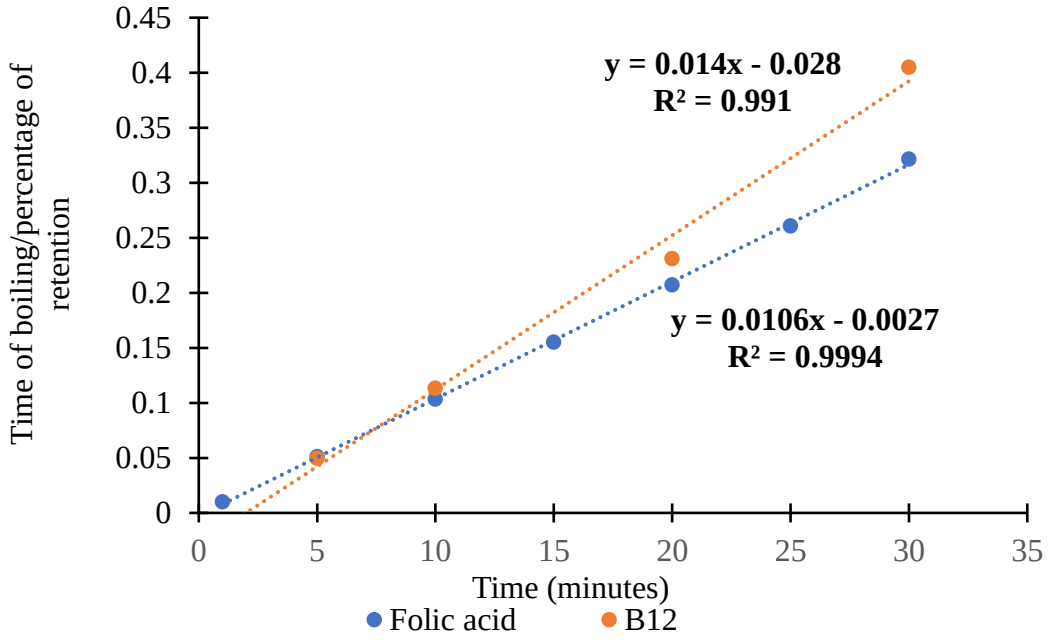


Figure 73: Pseudo second order degradation kinetics of folic acid and B_{12} in boiling water

After the preliminary study, the stability of the folic acid and B_{12} in rice cooked with QFS was studied. Several attempts failed because of the difficulty in extracting the micronutrients from the cooked food. The extraction involves sieving, and the high content of amylopectin in rice made sieving impossible. The use of sodium carbonate as a flocculate did not solve the problem. After several failed attempts, cooking rice with excess water (1:9, rice: water) helped to resolve this problem. Even then, the filtration was nearly impossible. Also, the impact of microwave heating on the stability of the micronutrients was evaluated. The results showed that the rice was already enriched with folic acid and vitamin B_{12}. The salt contributed to the level folic level in the rice but did not for B_{12}. Given the amount of salt added to the rice and concentration of folic acid in the unfortified and fortified cooked rice, over 70% of the folic acid due to added QFS was retained in the cooked rice. Cooking alone did not significantly impact the stability of B_{12} in the cooked rice. However, microwave heating significantly degraded B_{12} but not folic acid (Figure 74). There was no observed sensory difference between the rice cooked with or without the QFS.

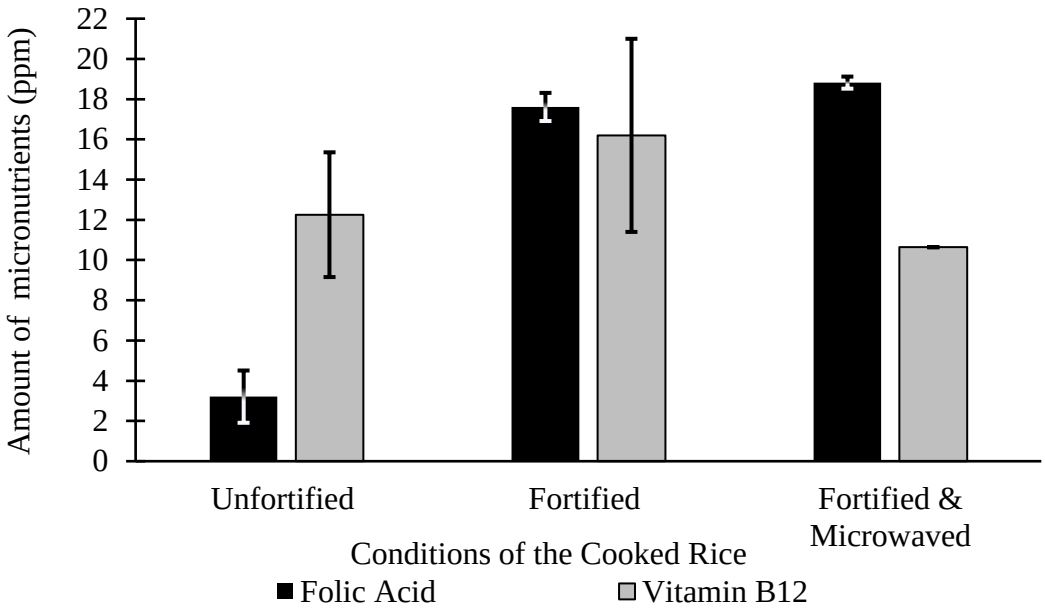


Figure 74:Stability of folic acid and vitamin B_{12} in cooked and microwaved rice

This study showed that the micronutrients added to food should be able to survive the cooking conditions and that the salt did not cause any change in the sensory properties of the cooked rice. Moreover, the micronutrients were very stable for more than six months of storage in salt, even at 45 ^{o}C/ 60-70% RH. Given that the projected additional price for adding these micronutrients to

salt is about $0.31 per person in a year, QFS can effectively deliver four micronutrients to a vulnerable population cost-effectively. In the optimized QFS, the premix is indistinguishable in the salt. Hence, the addition of the micronutrients to salt will not affect consumers' acceptance of QFS.

5.9 Small Scale -Sensory Survey for Food Cooked with Quadruple Fortified Salt

The acceptance of a fortified product is essential to achieving its intended impact. The World Health Organisation advocated for the use of education to improve the acceptance of fortified food, especially in populations with a high level of illiteracy (Mannar, 2006). A fortified food with sensory properties (appearance, taste, and aroma) which a population is familiar with tends to be accepted (Tuorila, 2007).

Hence, a study was carried out to get feedback on the appearance, taste, and aroma of rice prepared using some samples of the Quadruple Fortified Salt. At the time of carrying out this study, the available Quadruple Fortified Salt samples were yellow due to folic acid. Rice samples were prepared with QFS and iodized salt (control). The impact of the yellow colour of the salt on the acceptability of the rice cooked was evaluated. The results are presented based on the responses of respondents. Ten respondents participated in the survey; they identified themselves as six females and four males. The food samples were number coded to aid random independent responses. Also, of the three food samples represented to the respondents, two of them (523 and 596) were prepared with QFS while the third (535) was prepared with iodized salt.

Table 27: Response on taste and aroma of the rice prepared with different salt samples

Samples	Taste			Aroma		
	Unacceptable (%)	Indifferent (%)	Acceptable (%)	Unacceptable (%)	Indifferent (%)	Acceptable (%)
523	0	30	70	0	20	80
535	10	50	40	0	30	70
596	10	10	80	0	40	60

Table 27 shows the respondents' responses to the taste and aroma of rice samples prepared with the different salts. The survey shows that majority of the respondents accepted the taste of the rice prepared with QFS. The same trend was observed for the aroma of the cooked rice. In terms of these sensory properties, the rice was well accepted. Only one of the respondents marked the rice

prepared with QFS as ‘unacceptable’. The same respondent marked the rice prepared with the iodized salt as ‘unacceptable’.

Table 28 shows the respondents’ responses to the colour of the rice prepared for the salt samples. The survey showed that the rice sample prepared with QFS has high acceptability in terms of the colour of the rice samples. The response of the respondent to the colour of the rice sample is surprising as few of the respondents preferred the rice prepared with QFS to that prepared with iodized salt.

Table 28: Response on Colour of the Rice Prepared with Different Salt Samples

Rice Samples	Salt Samples	Unacceptable (%)	Indifferent (%)	Acceptable (%)
523	QFS	0	10	90
535	Iodized Salt	20	30	50
596	QFS	0	20	80

Table 29 shows the respondents’ responses to the overall acceptability of the rice samples prepared with the salt sample. Just as the previous survey data has shown, all the rice samples were acceptable. The rice samples prepared with QFS were more acceptable than rice prepared with iodized salt. From the result obtained, the salt did not have any effect on the acceptability of the cooked rice.

Table 29: Response on Overall Acceptability of the Rice Prepared with Different Salt Samples

Samples	Salt Samples	Unacceptable (%)	Indifferent (%)	Acceptable (%)
523	QFS	0	10	90
535	Iodized Salt	20	30	50
596	QFS	0	10	90

Although the colour of the QFS did not affect the sensory acceptance of the cooked food, there is no guarantee that consumers (especially in an illiterate population) will buy a salt that has a different colour (in this case, yellow) from the traditional white salt. Also, the process of making this salt is not feasible for a small-scale industrial practice, as spray solution is typically stored for weeks. Almost all the Vitamin B_{12} would have been lost before the solution is used up. Hence, as discussed in earlier Section 5.6.3, the process has been optimized, so that iron, folic acid (which

caused the yellow colour and vitamin B_{12} (that is not stable in solution) are added as an extruded and microencapsulated particles.

5.10 Cost Analysis of the Technology

Although everybody can use the fortified salt, the technology is targeted at the poor populations. Hence, the cost implication for adding the four micronutrients through this technology was examined. The technology is compatible with the technology of making iodized salt. The technology for making and admixing the micronutrient premix is the only addition to the current practice. The described technology can be carried out by already employed personnel of traditional salt companies so that additional labour costs can be as little as $3/ kg premix. Additional utility costs should be negligible. The analysis assumed that grants and philanthropic donations would cover the fixed cost. This has been the model used so far in India, where similar technology has been deployed (Diosady, Mannar and Krishnaswamy, 2019).

Table 30:Cost analysis for the quadruple fortification of salt

Constituents of QFS	USD/kg	Amount needed/ kg of salt	Cents/kg salt
Ferrous fumarate	12.00	2.91 g	3.49
Semolina	1.80	0.73 g	0.13
Fat	1.57	0.09 g	0.01
TiO_2	22.00	0.91 g	2.00
Soy Stearin	2.00	0.18 g	0.04
HPMC	52.00	0.18 g	0.95
Sodium Carbonate	9.70	0.02 g	0.02
Potassium Iodate	6.00	0.08 g	0.05
Folic Acid	90.00	0.03 g	0.23
Cyanocobalamin	5000.00	250 mg	0.13
Personnel cost for Premix	3.00	5g premix	1.50
Total cost/kg of salt			8.54
Cost per person/ year			31.12

Most of the prices were obtained from the Alibaba website

The additional material cost for adding the four micronutrients is 8.5 cents/kg salt. Based on the assumption that 10g of salt is consumed per person per day in the target populations, 3.65 kg is consumed per person annually. The additional cost of adding iron, iodine, folic acid, and vitamin B_{12} to salt is about 31 cents per person per year (Table 30). This cost analysis is very close to that projected at the Pilot Plant. The described technology is a cost-effective means of reducing the prevalence of micronutrient deficiencies.

6.0 Conclusions

6.1 Summary

This research forms an integral contribution to the development of a multiple fortification platform for salt and granular foods for which a patent has been filed. The fortification of staple foods with micronutrients serves as an easy, rapid, and cost-effective means of delivering essential micronutrients to vulnerable populations. The adverse interactions among the micronutrients and adverse effects of micronutrients on sensory properties of staple food pose a great challenge to the effectiveness of fortification programs. **A novel technology was developed for adding iodine, iron, folic acid, and vitamin B_{12} to salt. The extrusion-based microencapsulation technology was employed to match the size of some micronutrients with salt particles and to prevent interactions among the micronutrients and the potential effect of the micronutrients on the sensory properties of salt.**

The novel contributions of this research will be categorized into both scientific and engineering contributions

Scientific Contributions

1. The hydrophobic nature of soy stearin, the outer coat of the extruded and microencapsulated ferrous fumarate, prevented the adverse moisture aided interactions among the micronutrients.

2. The analytical methods for quantifying folic acid and determining the products of degradation of folic acid were developed
    a. The products of degradation of folic acid were determined by carrying out differential analysis on the total ion current chromatographs obtained for TFS (freshly prepared and stored) using uHPLC-MS.

3. Glutamic acid, the chloride additive of pteroic acid, and decarboxylated folic acid were identified as the products of folic acid degradation in the salt, which indicated oxidative degradation of folic acid.

4. The iron source, ferrous fumarate, significantly reduces the oxidative degradation of folic acid and vitamin B_{12} when the iron and folic acid were coextruded

5. The pH of the spray solution must be higher than pH 8 for folic acid to be fully dissociated, which makes it soluble and stable by the formation of sodium salts of folic acid. Vitamin B_{12} is not stable at this pH.

6. A kinetic model developed and validated based on the data from the degradation of folic acid and iodine in salt showed that the folic acid and iodine degradation kinetics fit the first-order rate law
    a. The kinetic model was used to predict the stability of folic acid and iodine in fortified salt and confirms that more than 75% of the micronutrients will be retained in the salt for more than 6 months, even at 45 ^{o}C.

Engineering Contributions

1. The amount of TiO_2, type of coating material, and technique used for microencapsulation critical to the effectiveness of the fortification were determined.
    a. Coating with soy stearin (10% $^{w}/_{w}$) caused the floating of the micronutrient premix, while the use of an excessive amount of TiO_2 (more than 30% $^{w}/_{w}$) resulted in an ineffective coating and led to the formation of dark spots on the micronutrient premix.
    b. Double coating first with 5% $^{w}/_{w}$ HPMC and 5% $^{w}/_{w}$ soy stearin solved the floating problem.
    c. Ultimately, coating with a suspension of 15% $^{w}/_{w}$ TiO_2 in a solution of 10% $^{w}/_{w}$ soy stearin solved the problems of floating and dark spots on the micronutrient premix.

2. The process developed for triple fortification that can deliver 50-200% iodine, iron, and folic acid RDAs was developed (the TFS has 1000ppm iron, 50ppm iron, and 25ppm folic acid)
    a. The process involved mixing a solution of iodine or iodine and folic acid and a premix of iron-folic acid or iron with salt.
    b. More than 80% of the micronutrients were retained in a solution of 1% folic acid and 2% iodine made with 70 mM sodium carbonate (pH 9); antioxidants did not significantly impact the stability of the micronutrients; hence, they are not needed in the formulation of the spray solution.

c. More than 70% of the micronutrients were retained in the TFS made with the solution of folic acid and iodine and iron premix.
d. The iron-folic acid premix prevented the discolouration of the salt that resulted from the addition of folic acid as a solution; iron inhibited the oxidative loss of folic acid, such that over 90% of folic acid was retained in the TFS made with the iron-folic acid premix

3. The process developed for quadruple fortification that can deliver 50-200% iodine, iron, folic acid, and vitamin B_{12} was developed (the QFS has 1000ppm iron, 50ppm iron, 25ppm folic acid, and 0.25ppm vitamin B_{12})
    a. The process involved mixing a solution of iodine or iodine and folic acid and a premix of iron-folic acid-vitamin B_{12} or iron-vitamin B_{12} with salt.
    b. A solution of iodine, folic acid, and vitamin B_{12} was not used because folic acid and vitamin B_{12} were not compatible in solution
    c. The understanding of the chemistry of folic acid and vitamin B_{12} and colour of folic acid define the optimal process- mixing iron-folic acid-vitamin B_{12} premix with iodized salt.
    d. In the optimized QFS, more than 90% folic acid and vitamin B_{12} were retained after 6 months (25-45 ^{o}C/60-70% RH); the micronutrients did not affect the sensory properties of the salt.

4. The micronutrients in the salt were stable in cooking (more than 70% was retained) and did not affect the sensory properties of cooked food.

6.2 Recommendations

This work focused on developing a process for adding iron, iodine, folic acid, and vitamin B_{12} to salt. JVS Food Pvt., India has scaled up the process while Cornell University (funded by the Center for Disease Control) will be testing the efficacy of QFS in India later this year. The following recommendations are made:

1) Although coating premix with a mixture of TiO_2 and soy stearin with pan or drum coater solved the two challenges (floating and dark spots on premix) that were encountered during the field test of Double Fortified Salt, the impact of the coat to prevent interaction between iron and iodine in the salt matrix is yet to be evaluated. It will be essential to study the

effectiveness of the coating to prevent iodine loss in a Double Fortified Salt at the laboratory scale before the optimized encapsulation process is scaled-up.

2) Although the multi-layer design for the premix did not add any significant advantage to the triple and quadruple fortification of salt, it may be useful for the addition of other micronutrients. It may be useful to evaluate the possibility of adding other micronutrients, such as vitamins A and B_1, zinc, to extra layer created by the design of premix.

3) The product of degradation of folic acid and the pathway for degradation was elucidated in this work. It will be useful to do the same for vitamin B_{12}. More importantly, it will be useful to develop analytical methods for the products of degradation of folic acid and vitamin B_{12} in the salt system. It will significantly lead to having a robust quality control for the fortification process.

4) Although rice is one of the most consumed foods and boiling is one of the most used techniques for cooking, it will be useful to evaluate the impact of other cooking techniques on the stability of the micronutrients in the salt and impact of the salt on the sensory characteristics on other food matrices.

5) The use of the co-axial airflow encapsulation nozzle to extrude a mixture of compatible micronutrients and sodium alginate into a solution of calcium carbonate may be a more viable and cost-effective alternative to the developed extrusion-based microencapsulation. Less sophisticated equipment than the extruder used in the described process will be required.

6) An accelerated storage study using high temperatures (lower than 69 ^{o}C, the melting point of soy stearin) and a short time may provide valuable kinetic data that can be used to develop a kinetic tool for predicting the stability of micronutrients in the salt samples during the field testing.

7) There may be a need to develop a colour-based qualitative analytical method for detecting the presence of the added micronutrients in the salt. This qualitative method will aid a rough and fast determination of the quality of the salt during field testing.

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8.0 Appendix

8.1 Supplementary Methodology used for Analytical Method Developed for Folic Acid

8.1.1 Determination of Limit of Detection and Quantification

The limits of detection (LOD) and limit of quantification (LOQ) of folic acid in salt were calculated using the expressions as described by ICH Guideline, 2005 as expressed in Equation 12&13.

$$LOD = \frac{3.3\,\sigma}{S} \qquad Equation\ 12$$

$$LOQ = \frac{10\sigma}{S} \qquad Equation\ 13$$

Where σ = standard deviation of the response of blank at the chosen wavelength, and
S = slope of the calibration curve

8.1.2 Percentage Recovery

The percentage recovery from spray solution and fortified salt was evaluated as expressed in Equation 14. Based on the linear regression equation of the calibration curve, the absorbance of folic acid extracted from salt and spray solution was measured on different days and in 2 different laboratories.

$$\%\ Recovery = \frac{Amount\ of\ folic\ acid\ analyzed\ in\ salt\ or\ solution}{Amount\ of\ folic\ acid\ added\ to\ salt\ or\ solution} \times 100 \qquad Equation\ 14$$

8.2 Detailed Experimental Designs for Formulating Iron Premix

Table 31 shows the composition of the premix formulated at JVS Food Pvt., India

Table 31:Experimental design for the formulation of iron premix in JVS Foods Pvt. India

Batches	**% Iron fumarate**	**% Binder**	**% TiO_2**	**Coating Material (%)**
1	75	25	25	HPMC (10%)
2	80	20	30	Sepifilm (10%)
3	80	20	30	Soy Stearin (5%)
4	80	20	35	HPMC (5%)
5	80	20	35	HPMC (7.5%)
6	80	20	35	HPMC (10%)
7	80	20	30	HPMC (5%)
8	80	20	30	HPMC (7.5%)
9	80	20	30	HPMC (10%)

*Baker percentage is used to express the percentage of material used in dry basis in extrusion formulation

Table 32 shows the combination of material used for coating iron premix in a preliminary study to elucidate what was responsible for the floating of iron premix in water.

Table 32: Experimental design for the preliminary coating of iron premix in the laboratory

Premix	**% HPMC (H) (dry basis in extrusion formulation)**	**% Soy Stearin (SS) (dry basis in extrusion formulation)**	**% Maltodextrin (MD) (dry basis in extrusion formulation)**	**% Lecithin (L) (dry basis in extrusion formulation)**
SS		10		
S		5		
H,SS[a]	5	5		
H,SS	5	5		
H,SS[b]	5[b]	5		
H,SS[c]	5[c]	5		
MD, SS		5	5[b]	
MD			10[b]	
(MD+SS)		5[d]	5[d]	
(L+SS)		5[d]		5[d]
(H+SS)	5[d]	5[d]		
SS, H	5[e]	5[e]		
H	10			
CTRL				

[a] Premix was obtained from PVS Foods Pvt., India

[b] The premix was first colour masked and coated with 50% of the required TiO_2 and coating material, the operation was stopped, and then the other 50% was applied;

[c] The premix was sprayed with little water before colour masking with TiO_2;

[d] The two coating materials were mixed, then applied using a pan coater;

[e] Unlike the usual protocol, premix was coated with soy stearin first before HPMC

Table 33 shows the final combination of material used to solve the floating problem of iron premix

Table 33:Experimental design for determining the optimal coating that will prevent the floating of premix

Samples	**%HPMC**	**% Soy stearin (SS)**	**% Lecithin (L)**
SS	0	10	0
H	10	0	0
(L+SS)[a]	0	10	1
H,SS[b]	5	5	0
H,SS	5	5	0

[a] Coat composition is different from (L+SS) described in Table 32;

[b] Premix was obtained from PVS Foods Pvt., India

Table 34 shows the experimental design for determining the optimal amount of TiO_2 for colour masking

Table 34:Experimental design for determining the optimal amount of TiO_2 required for colour masking

Samples	**TiO_2 (% $^{w}/_{w}$)**	**Method**
FD 25	25	Fluidized Bed
FD 12.5	12.5	Fluidized Bed
PC 5	5	Pan Coater
PC 10	10	Pan Coater
PC 15	15	Pan Coater
PC 20	20	Pan Coater

Table 35 shows blends of different combinations of coating material and titanium dioxide used for coating iron premix

Table 35:Experimental design for coating with a mixture of TiO_2 and soy stearin or HPMC

Type of particle coated	Amount of coating material used (% $^{w}/_{w}$)		
	TiO_2	HPMC	Soy stearin
Brown extrudate			
Brown extrudate			10
Brown extrudate		10	
Brown extrudate	5		10
Brown extrudate	10	10	
Brown extrudate	10		10
Brown extrudate	15	10	
Brown extrudate	15		10
Brown extrudate	20		10
Colour masked extrudate	10	10	
Colour masked extrudate	10		10
Colour masked extrudate*	15	5	5
Colour masked extrudate	15	10	
Colour masked extrudate	15		10

* has multiple coating layers of HPMC and soy stearin. For the colour masked extrudate, the iron extrudate was dusted with TiO_2 before the coating was applied; in the other type, a mixture of TiO_2 and coating material was applied.

8.3 Supplementary Results for Premix coating

Figure 1 shows the microscopic images of the iron premix obtained from JVS Foods Pvt. India

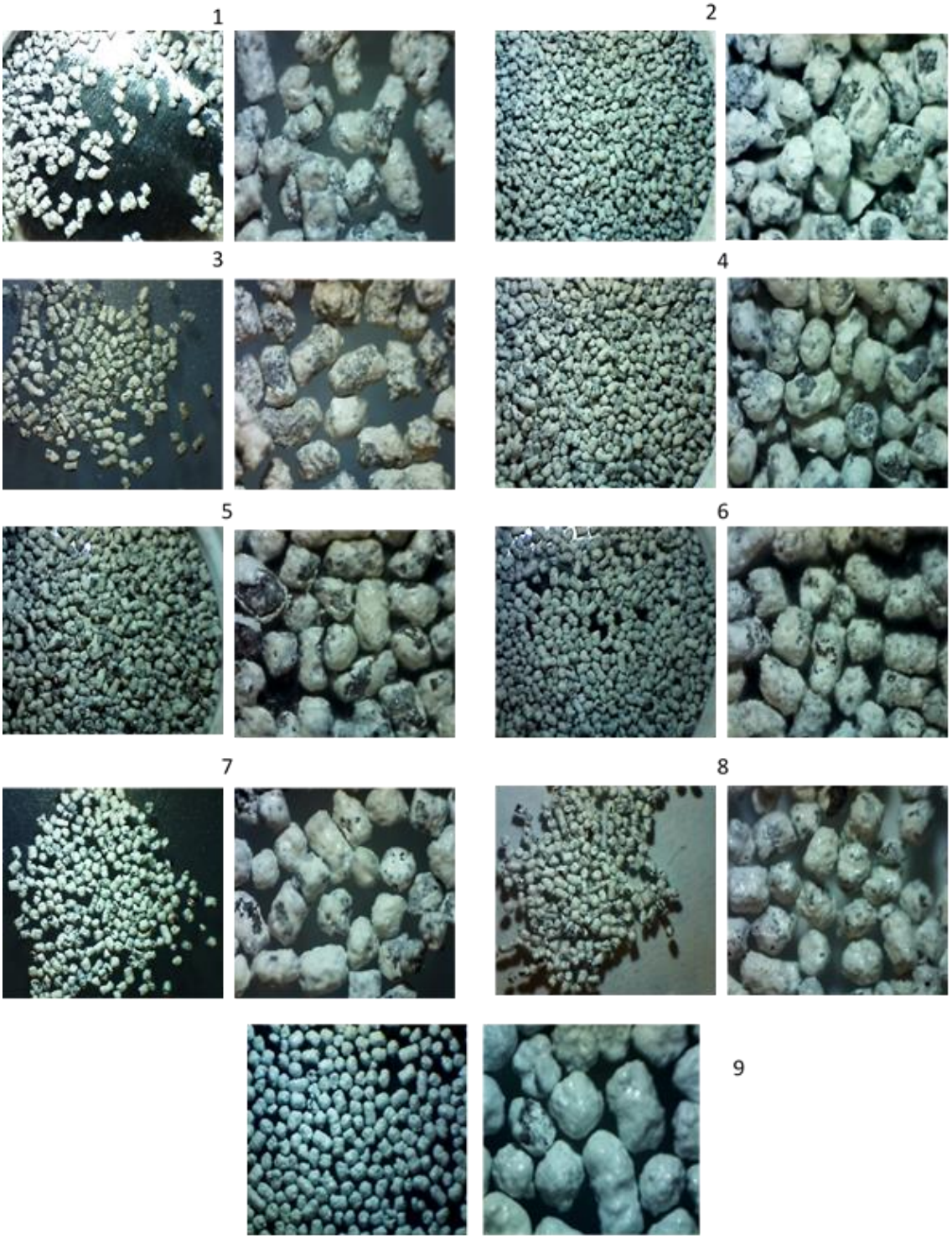


Figure 75:Image of Iron Premix Obtained from Pilot Plant, India

Aside from coating with 10% soy stearin, 10% HPMC, blend of 10% soy stearin and 1% lecithin and (5% HPMC and 5% soy stearin) reported in the thesis, the premix was also coated with 10% maltodextrin, (5% maltodextrin and 5% soy stearin), the blend of 5% maltodextrin and soy stearin, blend of 5% HPMC and 5% soy stearin. Figure 76 showed the effect of the material used to coat on the amount of iron on the surface of the premix. The results showed why we choose a combination of HPMC and soy stearin over of the coating material.

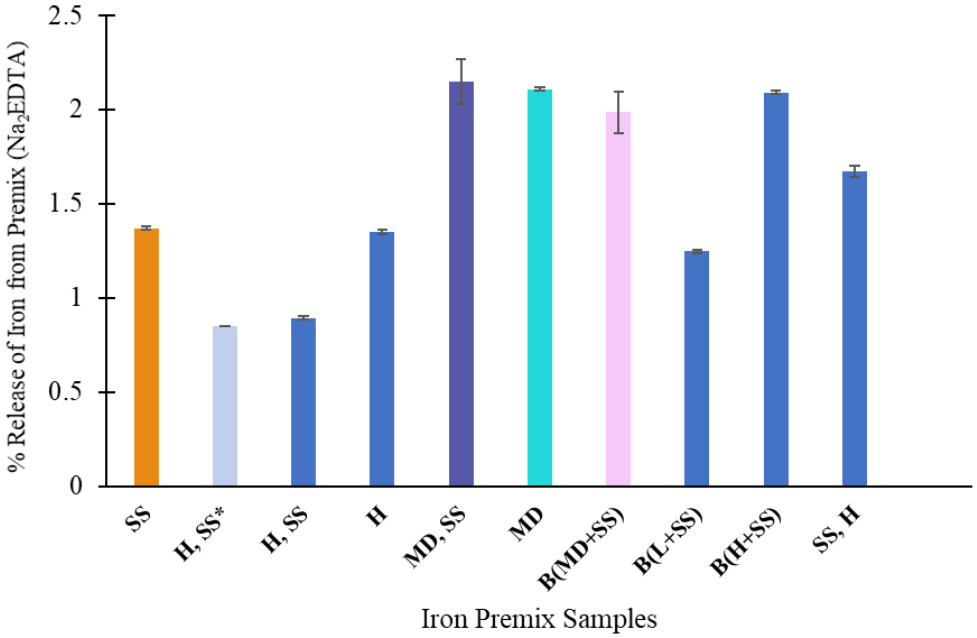


Figure 76:The effect of coating material on the amount of iron exposed on the surface of the premix
SS: soy stearin, H: hydroxypropyl methylcellulose (HPMC), MD: maltodextrin, H, SS: HPMC was applied first before soy stearin, MD, SS: maltodextrin was applied first before soy stearin, B: blend of the material was applied, SS, H: soy stearin is applied first before HPMC. * the premix was obtained from India.

8.4 Preparation of suitable coating solution for pan coating

A solution of HPMC in ethanol was previously used for coating iron premix using a fluidized bed. The solution is not suitable for pan coating as it is not volatile enough for the process. A more volatile solvent system is required. Unfortunately, HPMC is not soluble in most of the 'useable' solvent. Hence, a mixture of ethanol that dissolved HPMC and very volatile solvents (in this case, diethyl ether and dichloromethane) was evaluated for their suitability for pan coating.

First, the ratio of the solvents that are completely miscible was determined. The mixture becomes more miscible as the proportion of ethanol in the solvent system increases (*Figure 77*). This observation was consistent with dichloromethane or diethyl ether mixture with ethanol. However,

a higher proportion of ethanol signifies a less volatile solvent system. Hence, a 1:1 ratio of ethanol with the solvent was explored.

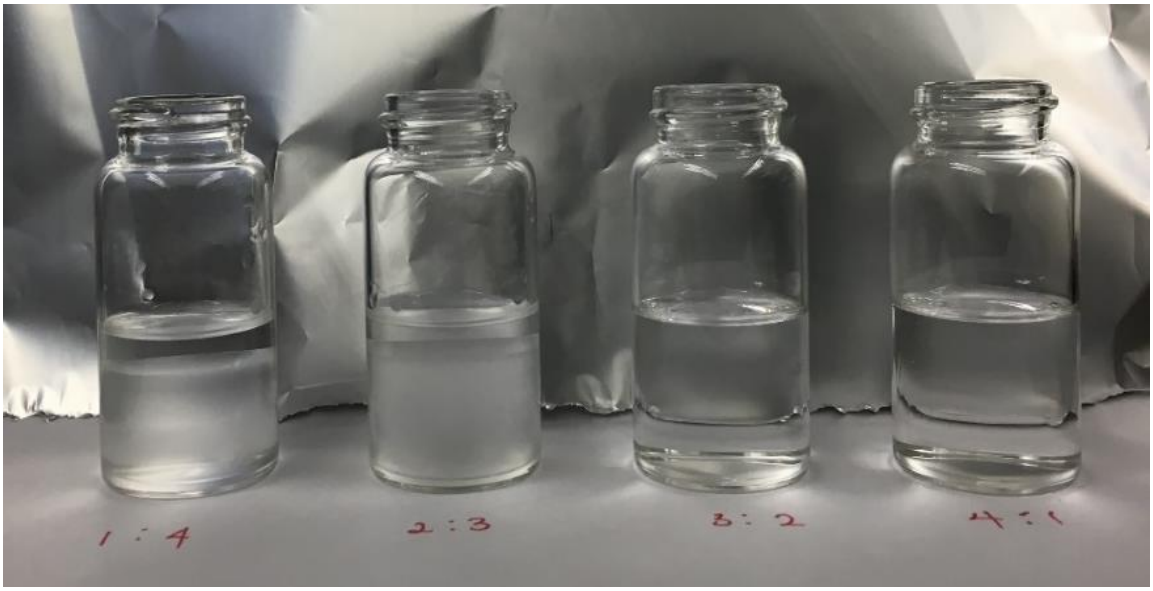
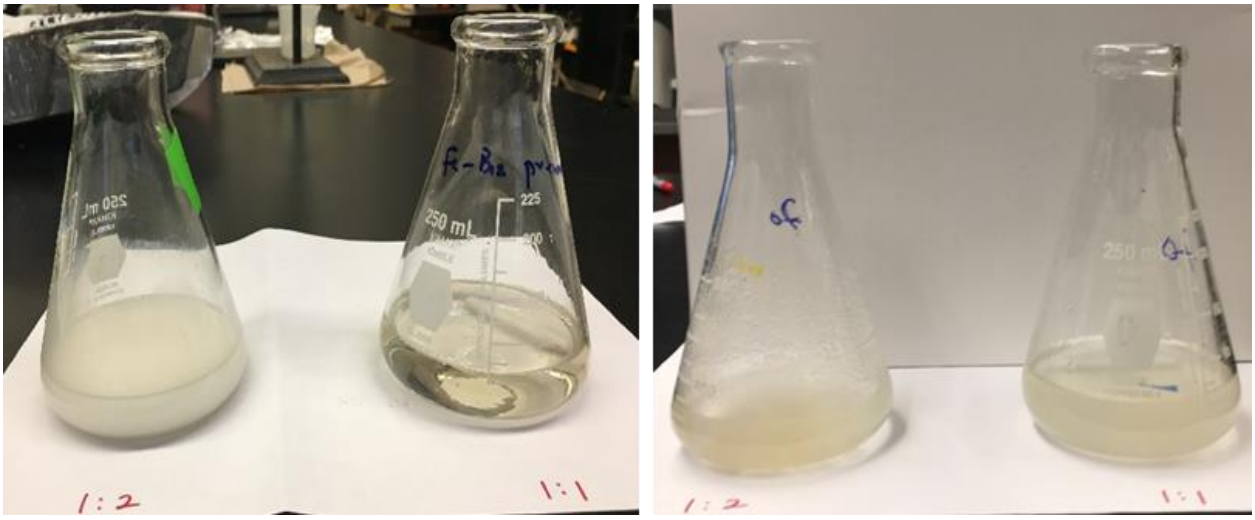


Figure 77:Miscibility of diethyl ether/dichloromethane: ethanol

The ratio 1:1 dissolved HPMC, higher proportion of dichloromethane or diethyl ether did not dissolve HPMC (*Figure 78*). HPMC was more soluble in the solvent that contained diethyl ether, but for its being flammable, dichloromethane was used. The possibility of using the solvent system to spray folic acid on the surface of the iron premix was explored. Folic acid was only able to form a uniform suspension with the ratio 1:1(*Table 36*).



Ethanol : diethyl ether

Ethanol : dichloromethane

Figure 78:Solubility of HPMC in different of ethanol: diethyl ether or dichloromethane ratios

Table 36:Overall Remarks on the Ratio of Solvents used

Diethyl ether / dichloromethane (%)	Ethanol (%)	Remarks
20	80	• Dissolved HPMC • Wetted the premix • Premix clumped
40	60	• Dissolved HPMC • Wets the premix • Premix clumped
50	50	• Dissolved HPMC • Did not wet the premix • Premix did not clump • Formed a uniform suspension of folic acid
60	40	• Slightly dissolved HPMC • Folic acid precipitated
80	20	• Did not dissolved HPMC

8.5 Impact of coating with lecithin on the stability of iodine in DFS

The use of a blend of soy stearin and lecithin was one of the solutions for preventing iron premix from floating in the water. Lecithin being an emulsifier, allows water to wet the surface of the premix, causing it to sink. The physical barrier between iron and iodine due to the coating was compromised by lecithin, as it allowed moisture to penetrate the premix coat, enhancing interaction between iron and iodine. This led to a significant loss of iodine in DFS formulated with iron premix coated with a mixture of lecithin and soy stearin after 2-month storage. Hence, the premix coated with a mixture of lecithin and soy stearin was dropped from further use.

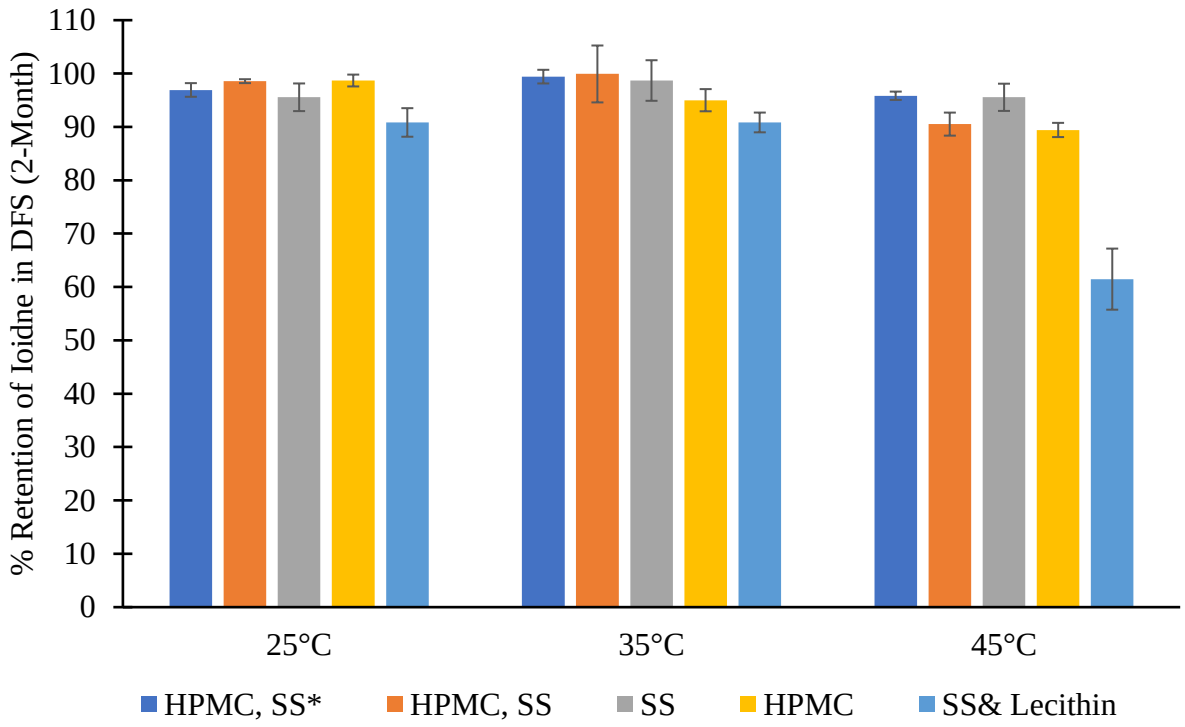


Figure 79:Impact of a mixture of lecithin and soy stearin on the stability of iodine in DFS

8.6: Formulation of Spray Solutions for Triple Fortification of Salt

Table 37 shows the first set of spray solution that was formulated for the triple fortification of salt. It was made with sodium carbonate buffers of varying concentrations.

Table 37:First set of spray solution for the triple fortification of salt

Amount of iodine (% $^{w}/_{v}$)	Amount of Folic Acid (% $^{w}/_{v}$)	Conc. of sodium carbonate buffer (M)
2	1	0.3
3	2	0.3
3	3	0.1
3	3	0.2
3	3	0.3

Table 38 shows the second set of spray solutions that was formulated for the triple fortification of salt. The impact of pH on the stability of iodine and folic acid in the spray solution was studied. The solution with pH 8-10 was formulated. 0.1M sodium carbonate buffer was used to dissolve folic acid and to adjust the pH.

Table 38:Second Set of Spray Solution for Triple Fortification of Salt

Amount of iodine (% $^{w}/_{v}$)	Amount of Folic Acid (% $^{w}/_{v}$)	Adjusted pH
2	1	7
2	1	8
2	1	9
2	1	10

There was a switch from the use of sodium carbonate buffer to just sodium carbonate solution. The switch was to make the process of formulating the spray solution more straightforward. *Table 39* compares the steps that were involved in the formulation of the spray solution, using sodium carbonate buffer or just the sodium carbonate solution.

Table 39:Comparison between the existing and new approach of making spray solution for the triple fortification of salt

Existing Approach	New Approach
• Prepare 0.1 M Na_2CO_3 • Prepare 0.1 M $NaHCO_3$ • Mix 0.1 M Na_2CO_3 with 0.1 M $NaHCO_3$ • Adjust to pH 9 • Mix FA and I with the buffer solution • Adjust to pH 9 with 0.1 M Na_2CO_3 • Measure the pH	• Weigh 0.742 g of Na_2CO_3 • Add FA and I and water • Mix • Measure the pH

Table 40 shows the third set of spray solution formulated for the triple fortification of salt. The solution was made or adjusted to pH 9 with a 0.1M sodium carbonate solution. The impact of the reduction of folic acid concentration and the addition of citrate on the stability of iodine and folic acid in the solution was investigated with the solution.

Table 40:Third Set of Spray Solution for Triple Fortification of Salt

Folic acid (%)	**Iodine (%)**	**Citrate (%)**
pH adjusted to 9		
0.5	0	0
1.0	0	0
0.5	2	0
1.0	2	0
0.5	0	1
1.0	0	1
0.5	2	1
1	2	1
2	0	0
2	0	1
pH not Adjusted (~ 11.2)		
0.5	0	0
1.0	0	0
0.5	2	0
1.0	2	0

8.7: Formulation of Triple Fortified Salt

Some of the iron premix and spray solution adjusted to pH 9 with 0.1M sodium carbonate solution were used to formulate Triple Fortified Salt (Table 41). The impact of folic acid concentration on the stability of iodine and folic acid in the salt samples and the impact of citrate on the stability of folic acid and iodine in the salt samples were investigated.

Table 41:Formulation of Triple Fortified Salt

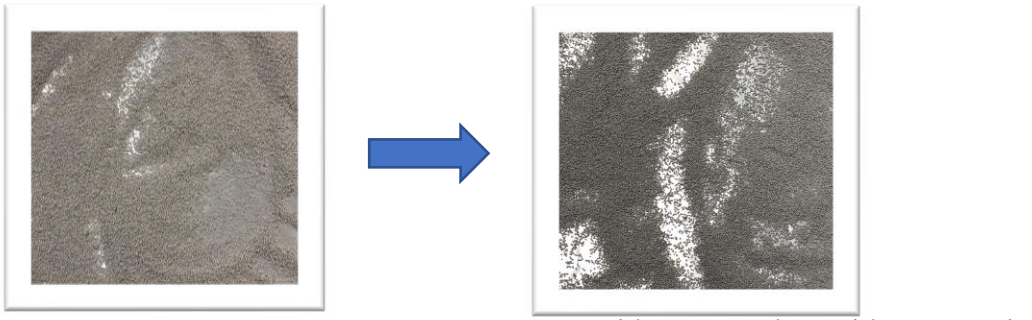
Type of premix	Type of solution	Conc. of Iron (ppm)	Conc. of iodine (ppm)	Conc. of folic acid (ppm)	Conc. of citrate
-	C	-	50	12.5	-
	D	-	50	25	-
	L	-	50	25	25 ppm
10% Soy stearin	C	1000	50	12.5	-
	D	1000	50	25	-
	L	1000	50	25	25 ppm
10% HPMC	C	1000	50	12.5	-
	D	1000	50	25	-
	L	1000	50	25	25 ppm
5% HPMC & 5% soy stearin	C	1000	50	12.5	-
	D	1000	50	25	-
	L	1000	50	25	25 ppm
* 5%HPMC & 5% soy stearin	C	1000	50	12.5	-
	D	1000	50	25	-
	L	1000	50	25	25 pm

*Premix sample was obtained from JVS Foods Pvt., India; C: solution contained 0.5% folic acid and 1% iodine; D: solution contained 1% folic acid and 1% iodine; L: solution contained 1% folic acid, 1% iodine and 1% citrate

Two Designs used for Formulating of Iron-Folic Acid Premix

Design A

Coat (Fe+ B12 + FA) extrudate with TiO_2



Coat with HPMC, then with soy stearin

Design B

Coat with TiO_2 Coat with TiO_2

Spray HPMC & folic acid

Coat with HPMC, then with soy stearin

8.8 Supplementary Result for Triple Fortification of salt

Figure 80 shows the effect of the concentration of sodium carbonate buffer on the solubility of folic acid in the spray solution. The concentration of sodium carbonate solution has a positive correlation with the solubility of folic acid in the spray solution.

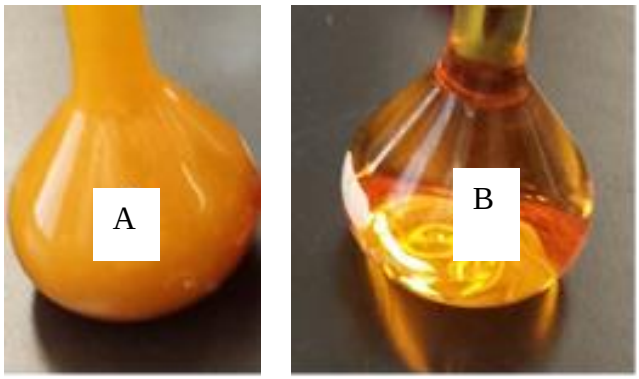


Figure 80:Effect of the concentration of carbonate buffer on the solubility of folic acid

(A) 3% FA + 3% I + 0.1M carbonate buffer (B) 3% FA + 3% I + 0.3M carbonate buffer

The impact of the composition of sodium carbonate buffer on the pH of the spray solution was investigated. The pH of the solution increases as the proportion of sodium carbonate in the buffer increases. About 2:3 ratio of 0.2M sodium carbonate to 0.2M sodium hydrogen carbonate is required for the solution of 1% folic acid and 2% iodine to be pH 9 (*Figure 81*).

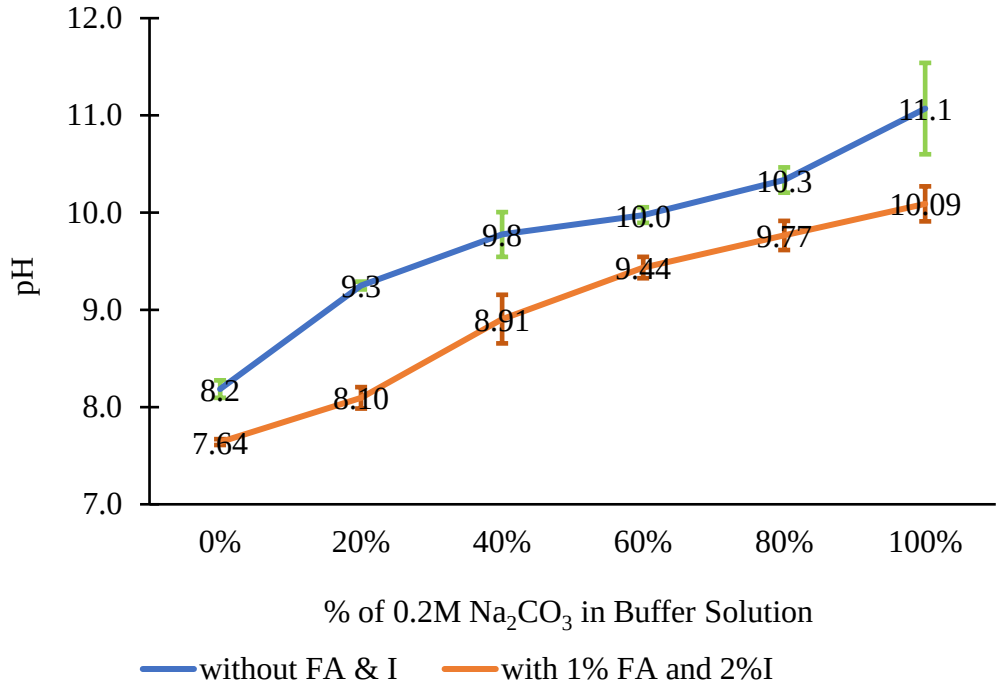


Figure 81:Effect of the composition of the sodium carbonate buffer on the pH of the spray solution formulated for the triple fortification of salt

Since pH has significant effects on the solubility and stability of folic acid in the spray solution, the impact of pH on the stability of iodine in the spay solution was investigated.

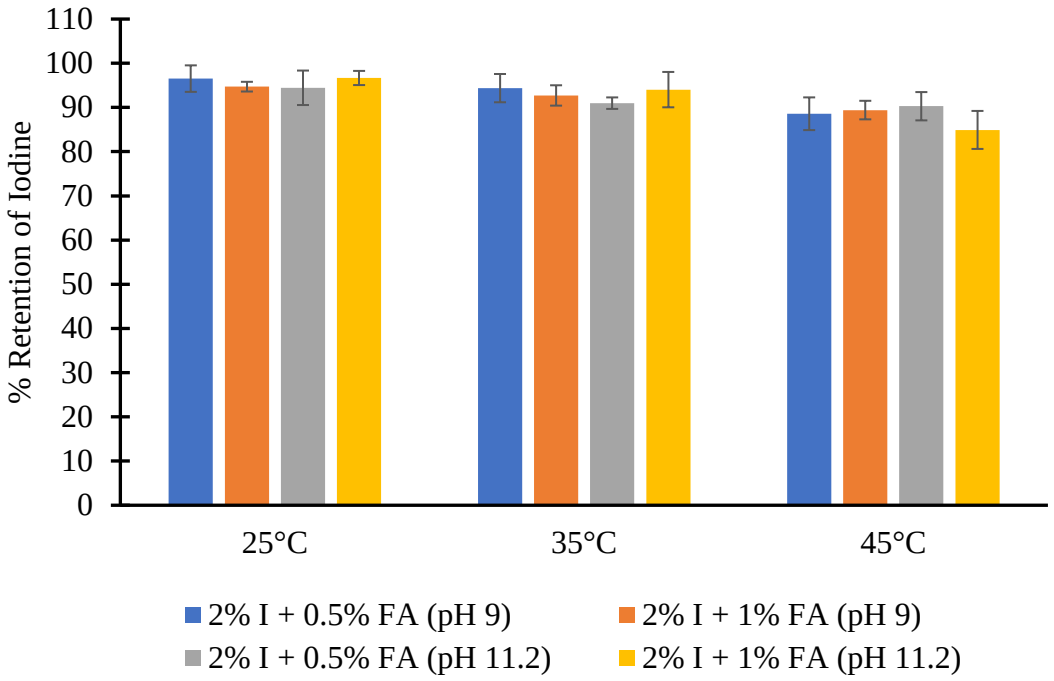


Figure 82 shows that the pH of the solution did not have any significant effect on the stability of iodine.

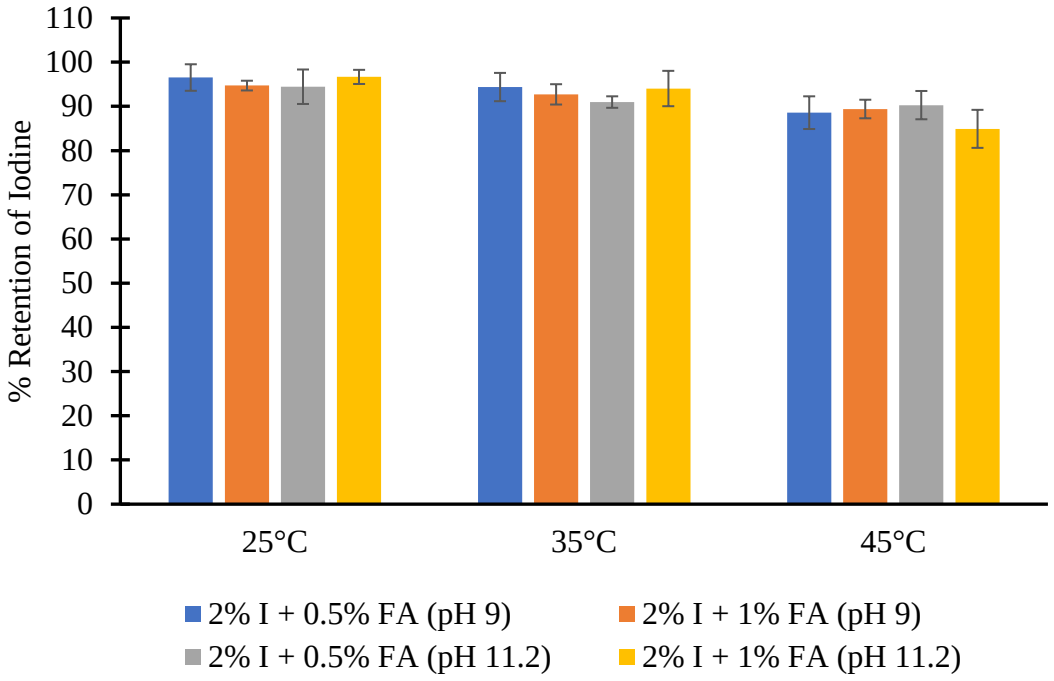


Figure 82:Effect of pH on the stability of iodine in the spray solution

Figure 83 shows that the concentration of folic acid in the spray solution had no significant effect on the stability of iodine in the spray solution

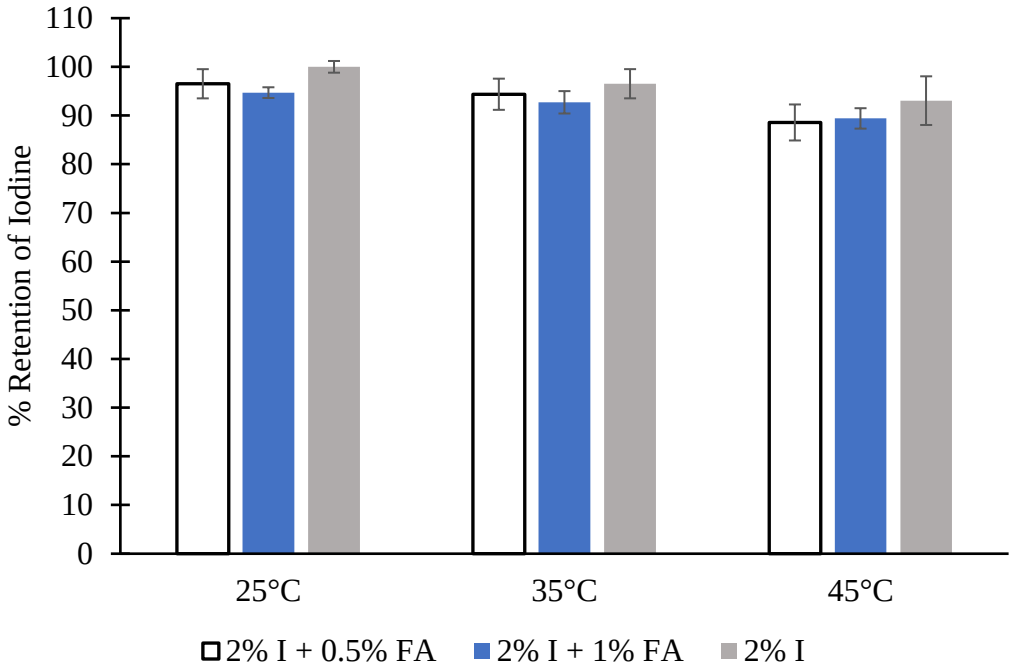


Figure 83:Effect of folic acid concentration on the stability of iodine in the spray solution

Figure 84 shows that, like folic acid, citrate had no significant effect on the stability of iodine in the spray solution.

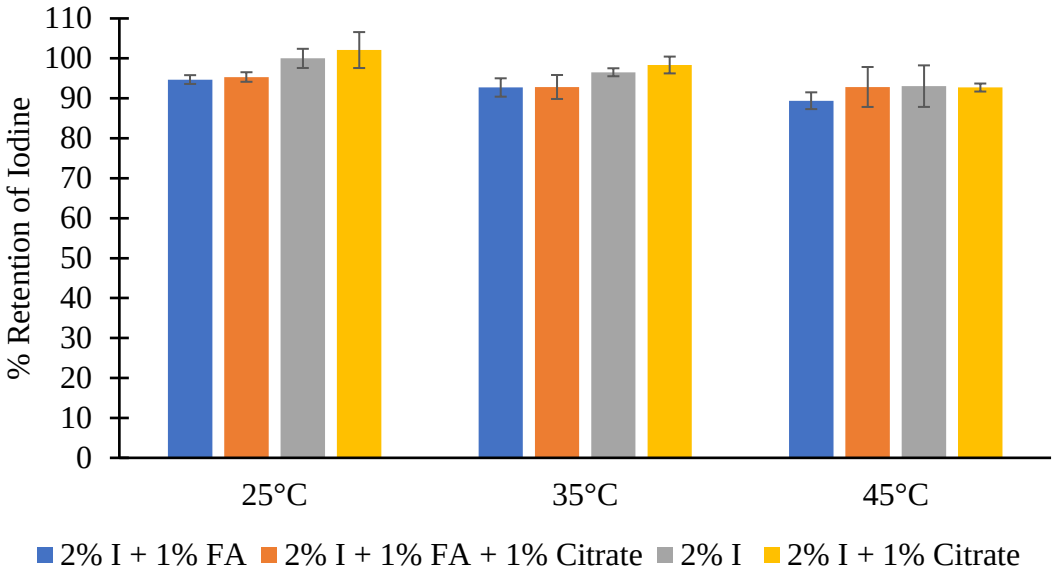


Figure 84:Effect of citrate on the stability of iodine on spray solution

Figure 85 shows that iodine affected the stability of folic acid in the salt samples (significantly at 35 ^{o}C). I have earlier postulated that potassium iodate when in the spray solution enhanced the formation of sodium salts of folic acid, which makes folic acid more stable.

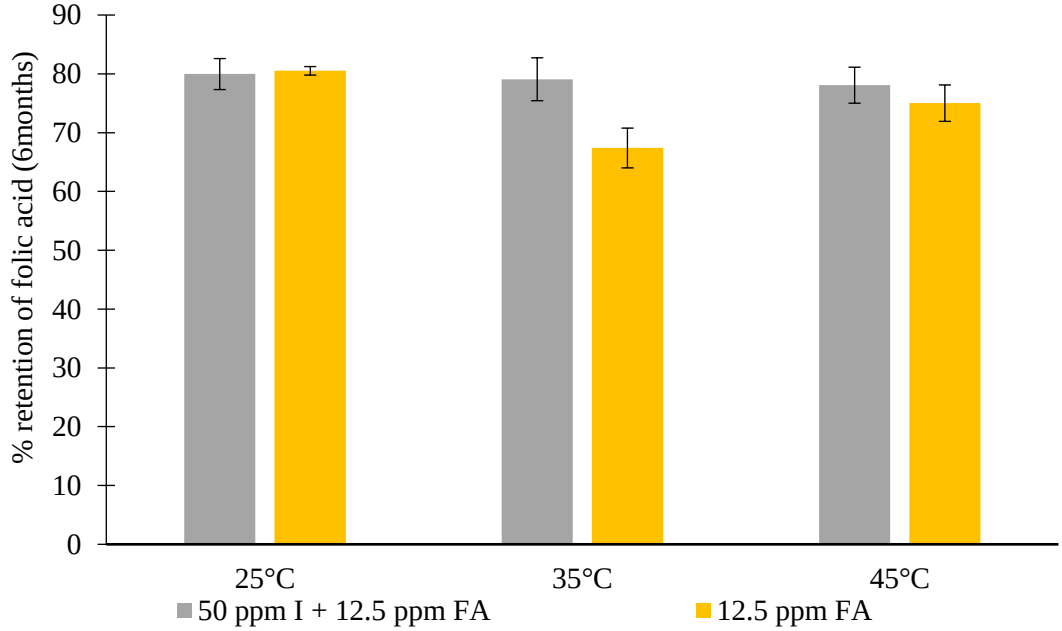


Figure 85:Effect of iodine on the stability of folic acid

The presence of folic acid and its concentration did not seem to impact the stability of iodine in the salt after six months of storage (*Figure 86*)

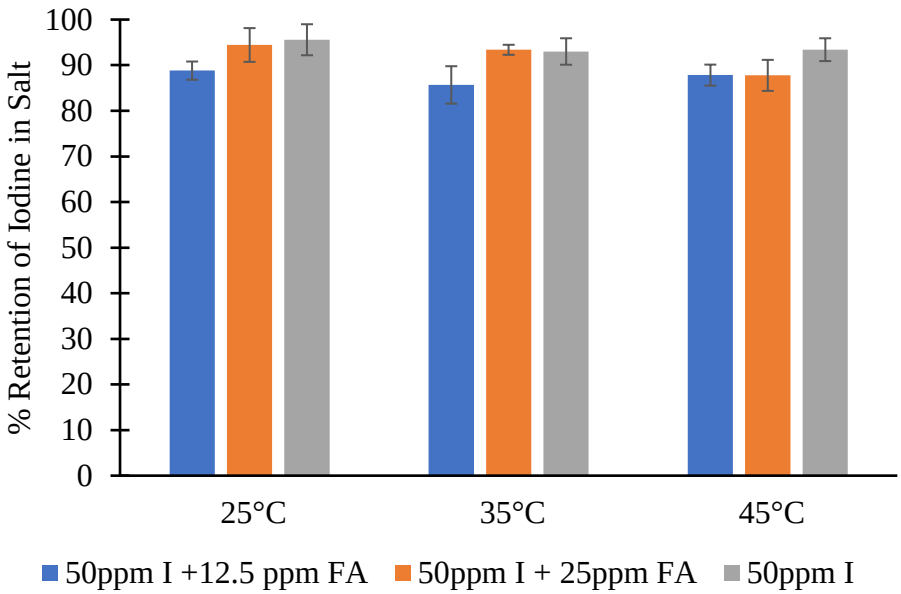


Figure 86:Effect of folic acid on the stability of iodine in Fortified Salt

8.9 Formulation of Spray Solution for Quadruple Fortified Salt

Table 42:First set of spray solution for quadruple fortification of salt

Conc. of Iodine ($^{w}/_{v}$ %)	Conc. of Folic Acid ($^{w}/_{v}$ %)	Conc. of Vit. B_{12} ($^{w}/_{v}$ %)	Conc. of Citrate ($^{w}/_{v}$ %)	Conc. of Ascorbate ($^{w}/_{v}$ %)	Conc. of erythorbate ($^{w}/_{v}$ %)
0	0	0.015	0	0	0
3	2	0	0	0	0
3	2	0.015	0	0	0
3	2	0.015	1	0	0
3	2	0.015	0	1	0
3	2	0.015	0	0	1

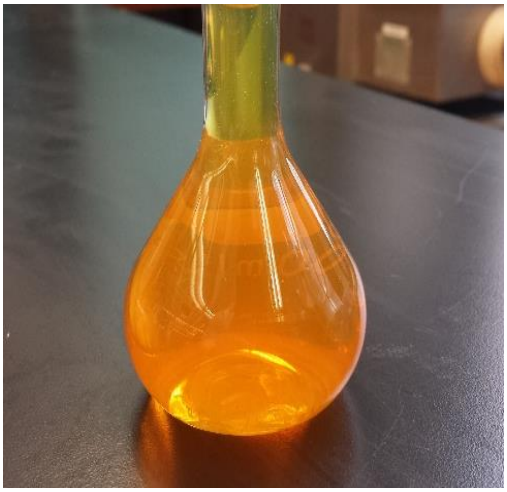
Table 43: Second set of spray solution for quadruple fortification of salt

Conc. of Iodine ($^{w}/_{v}$ %)	Conc. of Folic Acid ($^{w}/_{v}$ %)	Conc. of Vit. B_{12} ($^{w}/_{v}$ %)	pH
0	0	0.01	6.5 unadjusted
0	0	0.01	2.8 adjusted by ascorbic acid
2	1	0	9 adjusted by sodium carbonate

2	0	0.01	6.9 unadjusted
2	1	0.01	8 adjusted by sodium carbonate
2	1	0.01	9 adjusted by sodium carbonate
2	0.5	0.01	9 adjusted by sodium carbonate
3	2	0.015	8 adjusted by sodium carbonate
3	2	0.015	9 adjusted by sodium carbonate
3	2	0.015	9 adjusted by sodium carbonate and citric acid

8.10 Supplementary Result for Quadruple Fortification of salt

Folic acid and vitamin B_{12} affected the colour of the spray solution (*Figure 87*) and salt when sprayed directly unto it. Folic acid alone or when combined with vitamin B_{12} turned the colour of the salt from white to yellow. Vitamin B12 alone only brightened the white colour of the salt.

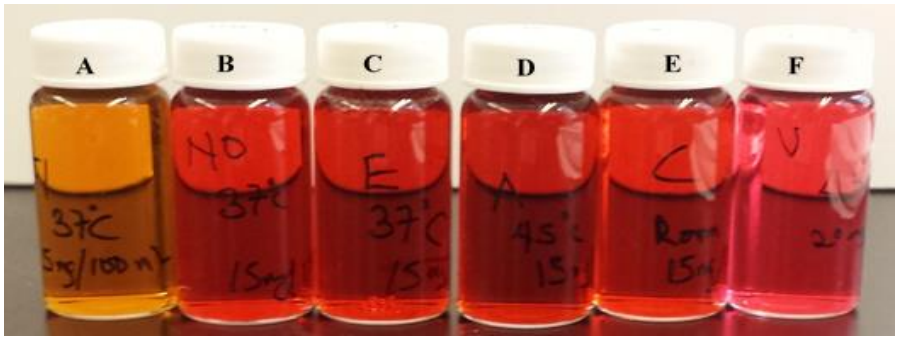


3% I + 2% FA

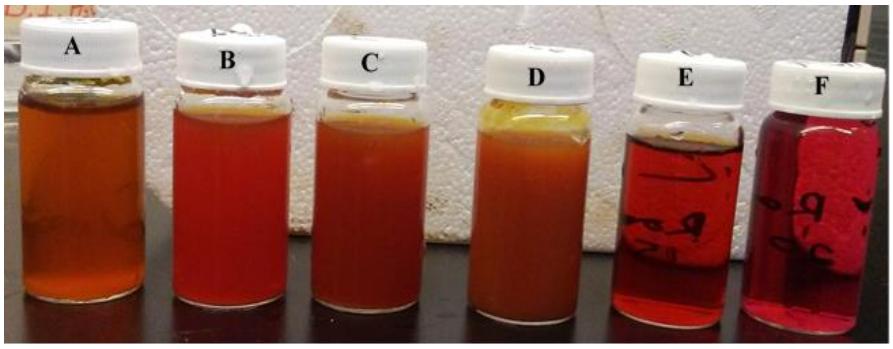
3% I + 2% FA + 0.015% B_{12}

Figure 87:Impact of folic acid and vitamin B12 on the colour of the spray solution

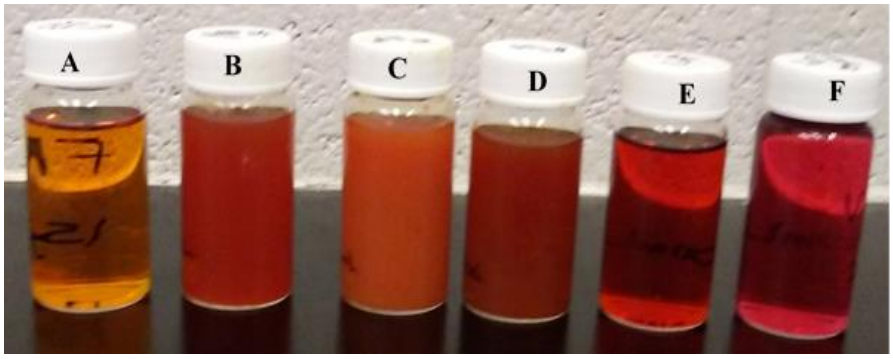
Figure 88 shows the the precipitation of micronutrients in spray solution that contained folic acid and vitamin B_{12}, especially at 25 ^{o}C. Citrate was able to maintain the solubilty of the micronutrients in solution even at 25 ^{o}C. Ascorbate improved the solubility of the micronutrients at 35 and 45 ^{o}C



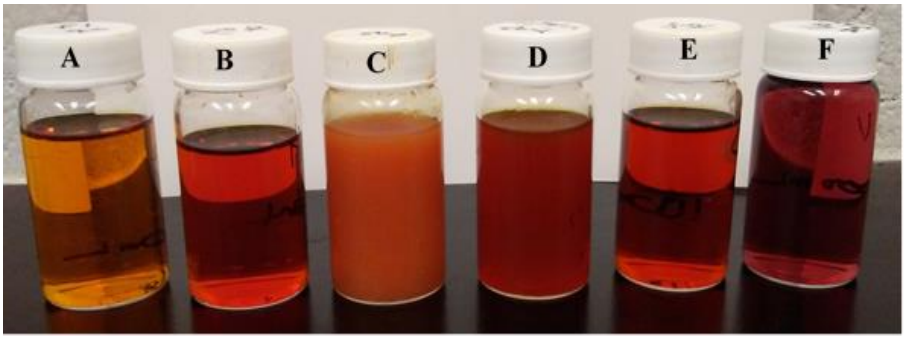
Freshly prepared solutions



Solutions stored for one month at 25 ^{o}C



Solutions stored for one month at 35 ^{o}C



Solutions stored for one month at 45 ^{o}C

Figure 88: Precipitation observed in solution formulated for the quadruple fortification of salt (Preliminary Study)

(a) 3% I + 2% folic acid; (b) 3% I + 2% FA+ 0.015% B_{12}; (c) 3% I + 2% FA + 0.015% B_{12} erythorbate; (d) 3% I + 2% FA+ 1% Ascorbate; (e) 3% I + 2% FA+ 0.015% B_{12} + 1% citrate; and (f) 0.015% vitamin B_{12}

The impact of the composition of sodium carbonate buffer on the pH of the spray solution was investigated. The pH of the solution increases as the proportion of sodium carbonate in the buffer increases. About 2:3 ratio of 0.2M sodium carbonate to 0.2M sodium hydrogen carbonate is required for the solution of 1% folic acid, 2% iodine, and vitamin B_{12} to be pH 9 (*Figure 89*).

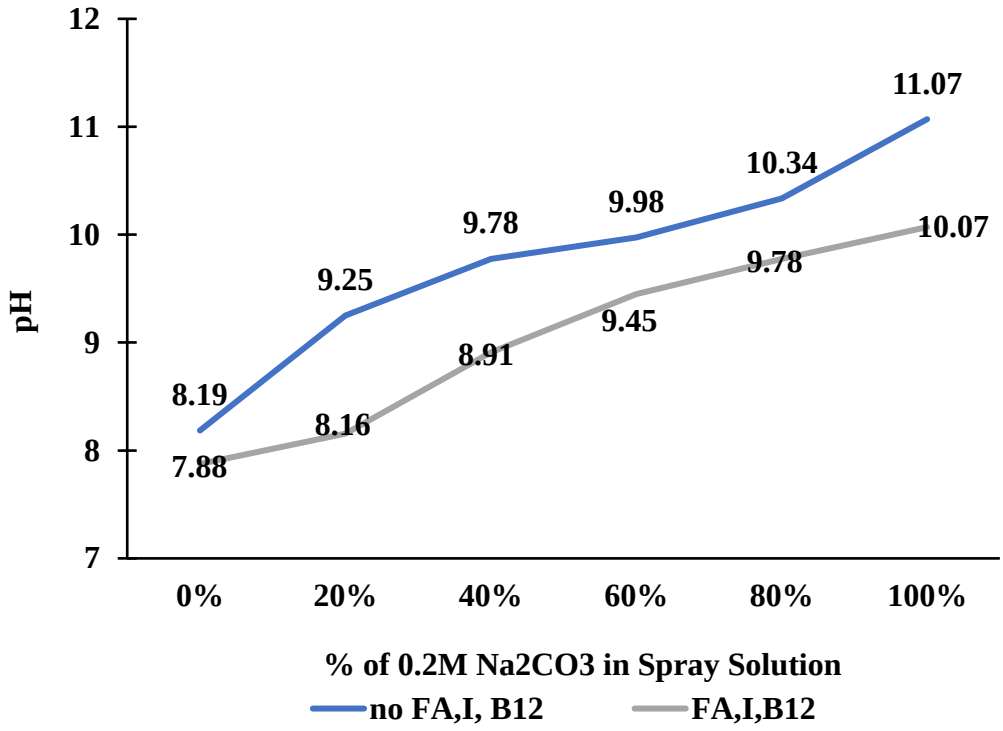


Figure 89:Effect of the composition of the sodium carbonate buffer on the pH of the spray solution formulated for the triple fortification of salt

Figure 90 shows that the addition of vitamin B_{12} to the spray solution did not significantly affect the stability of folic acid in the spray solution, just as *Figure 91* shows that pH has no impact on the stability of folic acid. The same trend was observed for iodine.

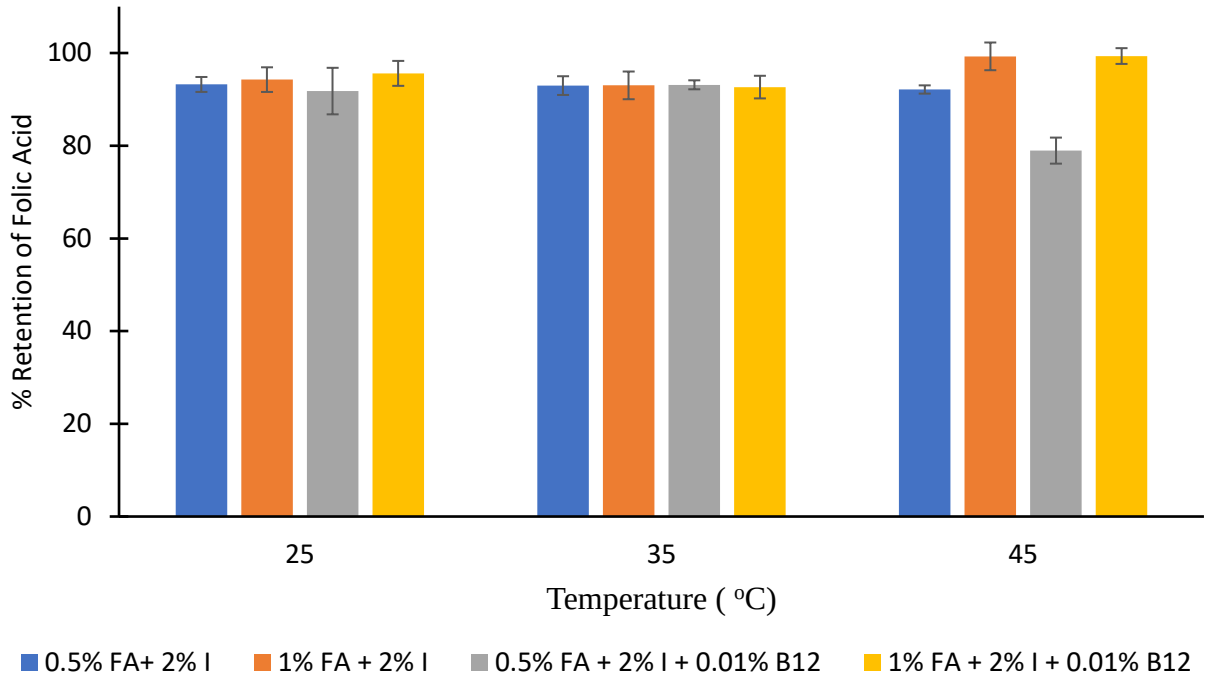


Figure 90:Effect of vitamin B_{12} on the stability of folic acid in the spray solution formulated for the quadruple fortification of salt

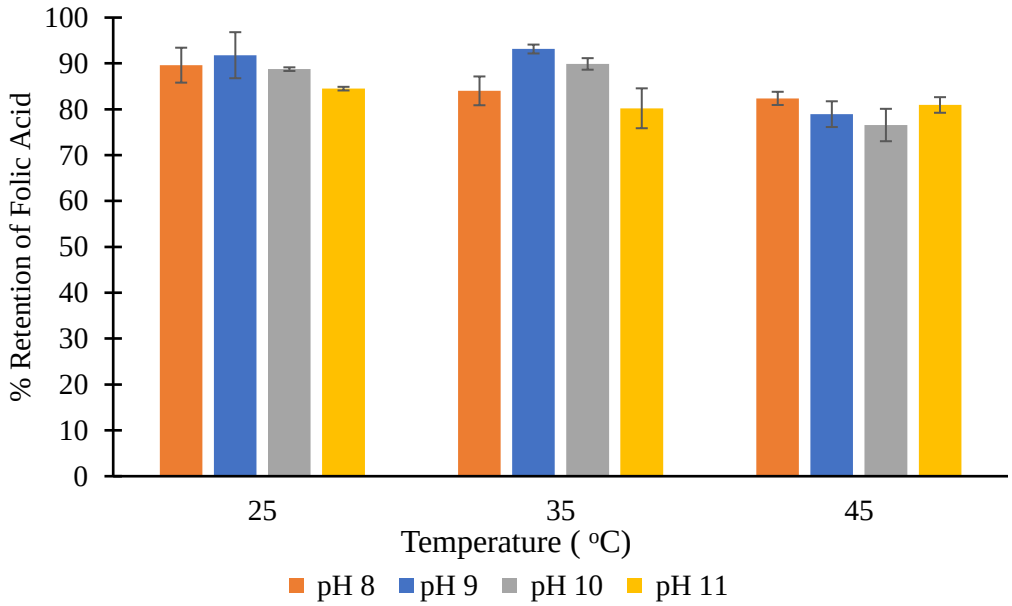


Figure 91:Effect of pH on the stability of folic acid in the spray solution formulated for the quadruple fortification of salt

Figure 92Figure 93 shows that citrate had no significant impact on the stability of folic acid and iodine in the spray solution

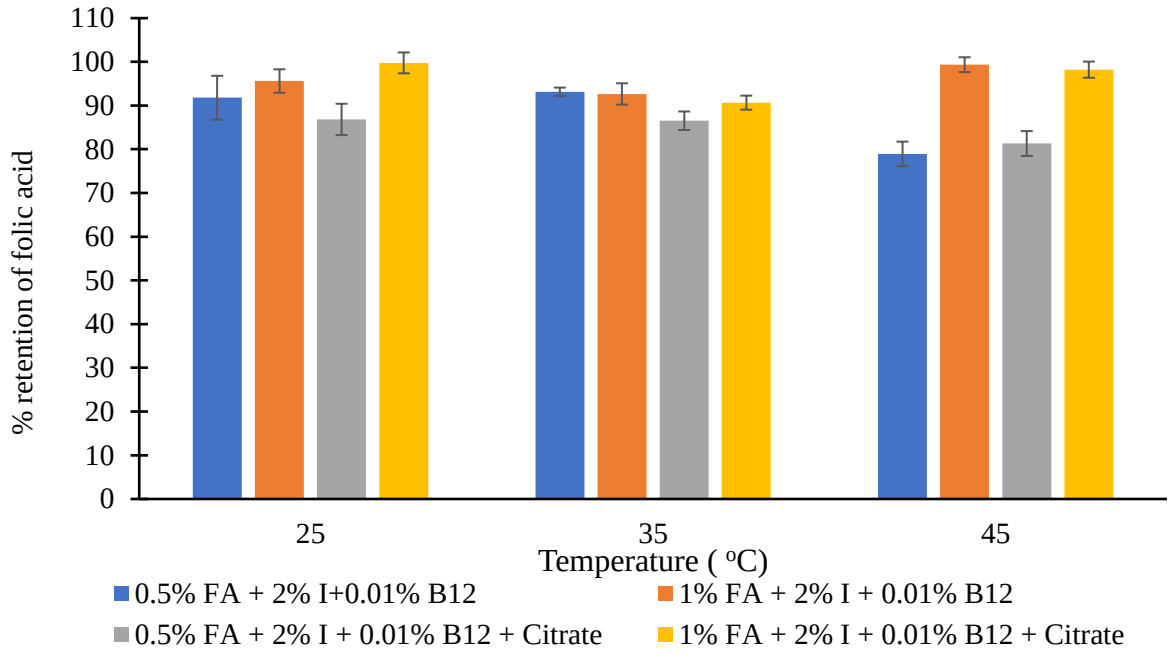


Figure 92:Effect of citrate on the stability of folic acid in the spray solution formulated for the quadruple fortification of salt

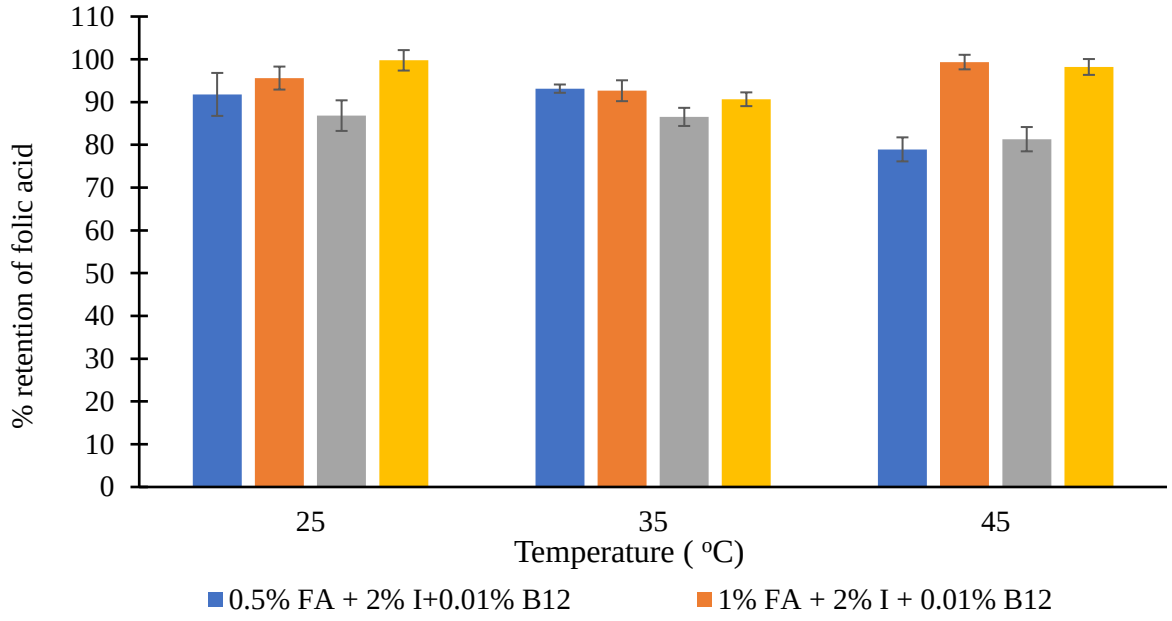


Figure 93:Effect of citrate on the stability of iodine in the spray solution formulated for the quadruple fortification of salt

Figure 94 shows that just as with the spray solution, the addition of vitamin B_{12} directly to salt as a solution that contained folic acid and iodine did not have any significant impact on the stability of folic acid in the fortified salt. The same trend was observed for iodine (*Figure 95*). *Figure 95* also shows that the concentration of folic acid in the salt had no significant effect on the stability of iodine.

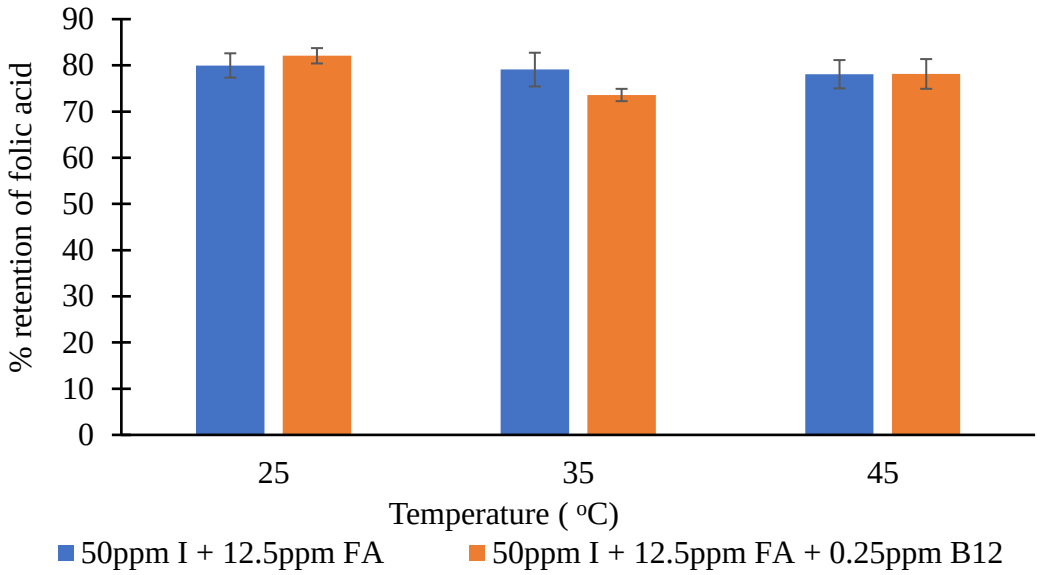


Figure 94:Effect of vitamin B12 on the stability of folic acid in QFS

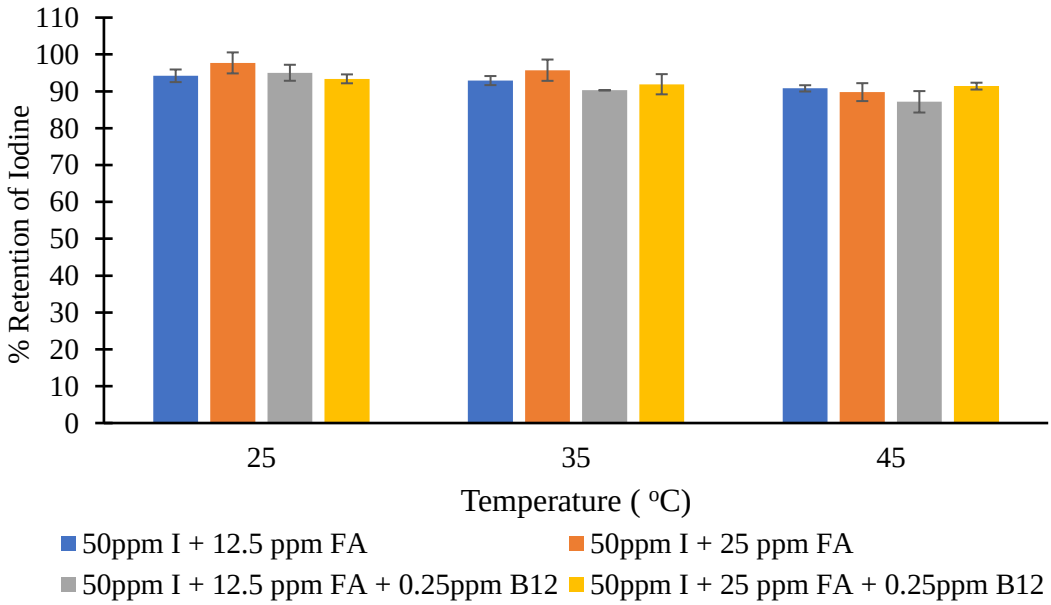


Figure 95:Effect of vitamin B_{12} and concentration of folic acid on the stability of iodine in QFS

Using a solution that contained iodine, folic acid, and vitamin B_{12} of different pH to formulate QFS had a significant effect at 45 ^{o}C and marginally at 35 ^{o}C.

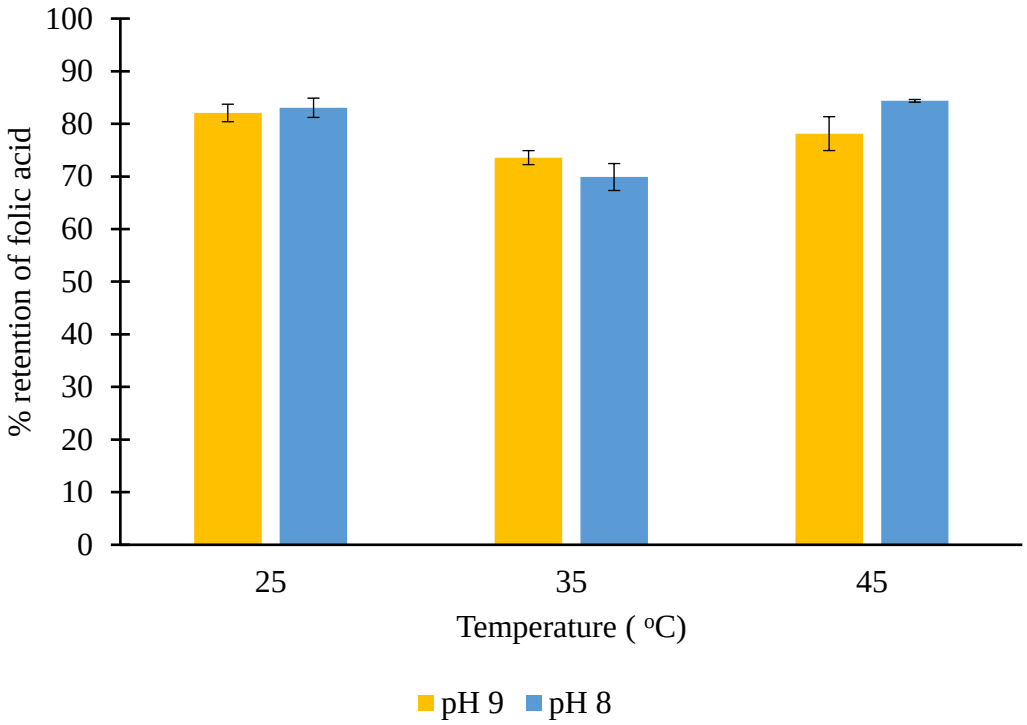


Figure 96:Effect of pH on the stability of folic acid in QFS

Figure 97 shows that vitamin B12 was very stable in the iron-B12 premix. More than 95% of the vitamin was retained at 25 ^{o}C irrespective of how it was made. The premix A was used further in the formulation of QFS because the process of formulating it was straightforward and more compatible with the established process of making iron premix.

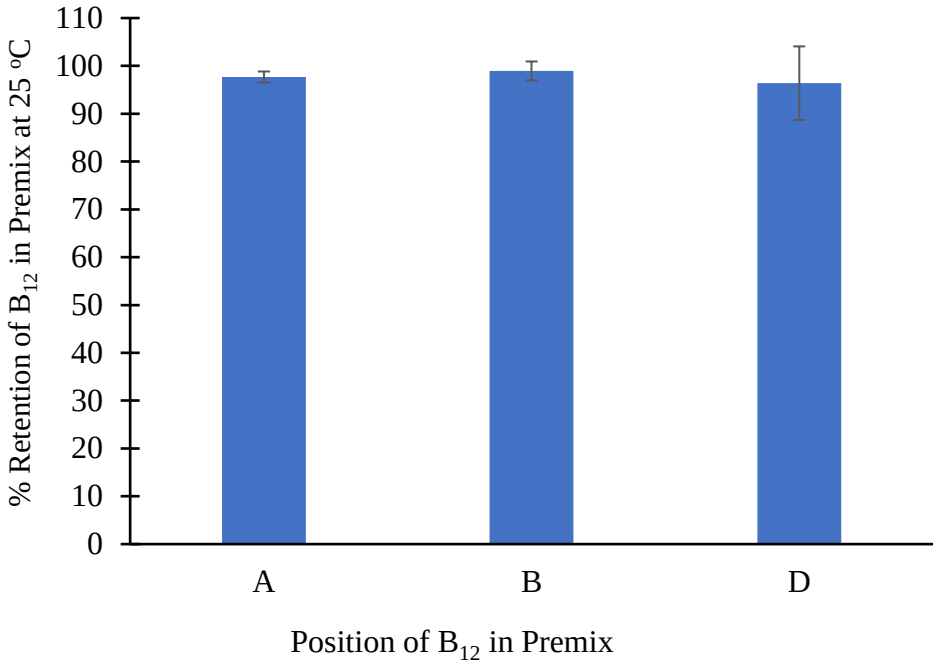


Figure 97: Effect of the position of B_{12} on the stability of vitamin B_{12} in Fe-B_{12} Premix
A=iron and B_{12} were coextruded; B=B_{12} sprayed on iron extrudate before colour masking; D= a mixture of B_{12} and HPMC was used for coating iron premix

8.11 Pilot Scale Production of Quadruple Fortified Salt

The process that I developed for the quadruple fortification of salt has been scaled up by JVS Foods Pvt., India. The technology involved adding iron- B_{12} premix to a salt priorly fortified with folic acid and iodine. A sample of the QFS made by the company was collected and stored in the laboratory. The stability of micronutrients in the salt was compared with the QFS made in the laboratory.

There was no significant difference in the stability of folic acid in the QFS obtained from India and the QFS made in the laboratory. More than 75% of the folic acid was retained in the QFS samples, even at 45 ^{o}C (Figure 98).

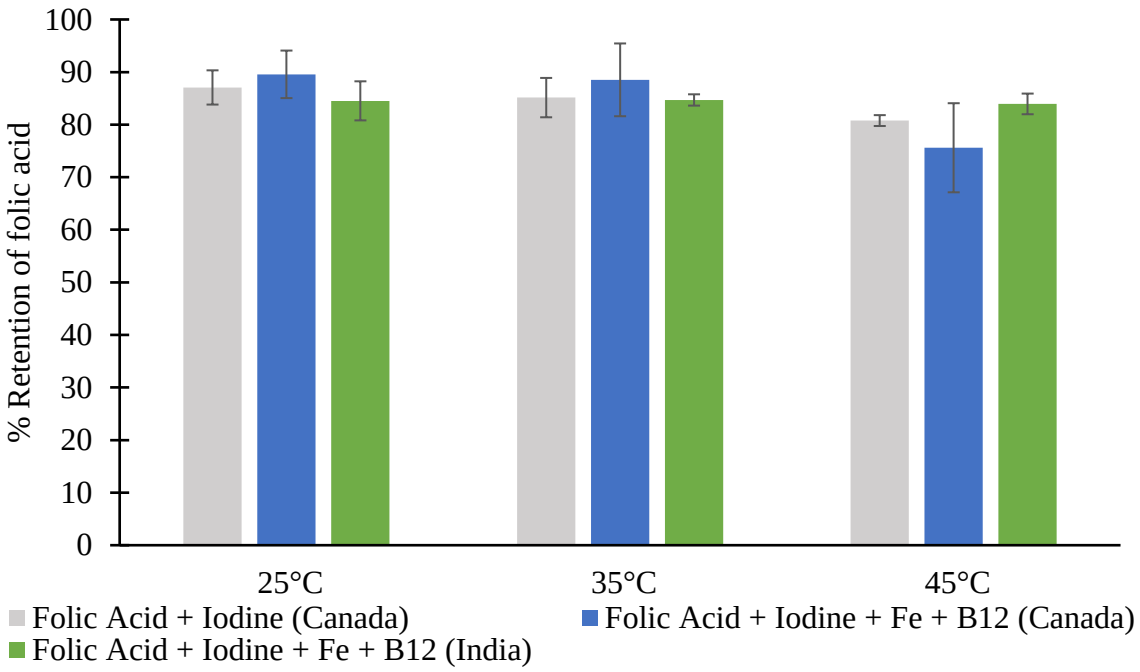


Figure 98: Comparing the stability of folic acid in QFS made in the laboratory and JVS Foods Pvt.

Unlike folic acid, there were significant differences between the stability of iodine and vitamin B_{12} in the QFS made in the laboratory, and the one made by JVS Foods Pvt. Iodine and vitamin B_{12} were more stable in the QFS made in the laboratory than QFS made by JVS Foods. For iodine, over 80% iodine was retained in the salt, even in the QFS made by JVS Food Pvt (Figure 99). The stability of vitamin B_{12} in the QFS made by JVS only fell below the set target at 45 ^{o}C. At 25 and 35 ^{o}C, over 70% of the added vitamin B_{12} was retained (Figure 100). The seemly instability of vitamin B_{12} may be linked to the purity of the cyanocobalamin used as the fortificant.

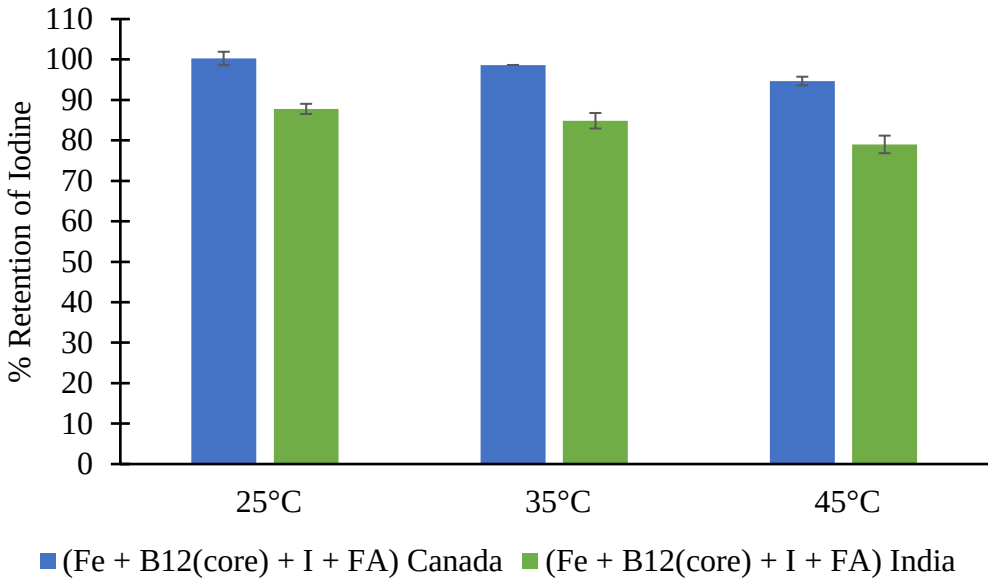


Figure 99: Comparing the stability of iodine in QFS made in the laboratory and JVS Foods Pvt.

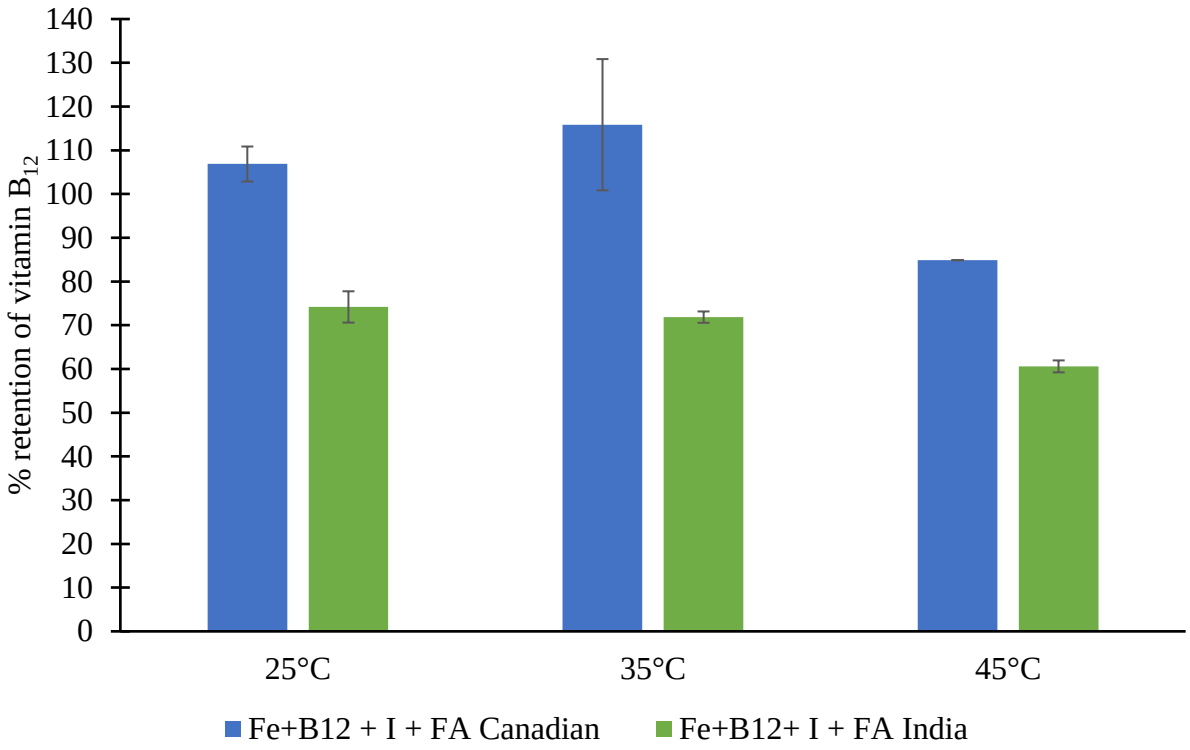


Figure 100: Comparing the stability of vitamin B_{12} in QFS made in the laboratory and JVS Foods Pvt.

8.12 Supplementary Results on the Kinetic Analysis of the Degradation of Folic Acid and Iodine in TFS and QFS

Table 28:Regresion analysis of the degradation of folic acid and iodine in TFS

Samples	Zero Order				First Order				Second Order			
	R^2			Activation Energy (kJ/mole)	R^2			Activation Energy (kJ/mole)	R^2			Activation Energy (kJ/mole)
	25 °C	35 °C	45 °C		25 °C	35 °C	45 °C		25 °C	35 °C	45 °C	
Folic Acid												
C	0.953	0.984	0.986	14.1	0.976	0.993	0.992	14.1	0.974	0.992	0.992	18.4
D	0.999	0.992	0.989	12.8	0.993	0.996	0.989	15.7	0.981	0.969	0.977	17.9
C A	0.996	0.990	0.999	27.6	0.998	0.996	1.000	30.1	0.995	0.998	1.000	32.1
D A	0.996	0.992	0.994	16.2	1.000	0.998	0.993	19.7	0.999	1.000	0.990	21.7
Iodine												
C	0.974	0.971	0.988	28.5	0.983	0.977	0.986	33.5	0.978	0.976	0.987	33.7
D	0.993	0.991	0.990	20.3	0.996	0.993	0.992	21.3	0.997	0.994	0.993	22.4
C A	0.969	0.981	0.994	26.4	0.977	0.983	0.991	26.3	0.975	0.982	0.990	27.0
D A	0.995	0.964	0.916	15.4	0.994	0.971	0.938	16.4	0.994	0.971	0.938	17.2

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

Table 28:Regresion analysis of the degradation of folic acid and iodine in QFS

Samples	Zero Order				First Order				Second Order			
	R^2			Activation Energy (kJ/mole)	R^2			Activation Energy (kJ/mole)	R^2			Activation Energy (kJ/mole)
	25 °C	35 °C	45 °C		25 °C	35 °C	45 °C		25 °C	35 °C	45 °C	
Iodine												
QFS*	0.972	0.978	0.999	41.3	0.979	1.000	0.990	44.0	0.968	1.000	0.989	45.2
QFS	0.969	1.000	0.989	49.2	0.969	1.000	0.990	53.0	0.968	1.000	0.989	50.7
Folic Acid												
QFS*	0.979	0.957	0.966	24.0	0.986	0.971	0.983	29.4	0.991	0.979	0.988	26.5
QFS	0.990	0.839	0.842	29.0	0.992	0.874	0.874	35.3	0.992	0.863	0.872	30.9

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.

Degradation kinetics of Iodine in the Triple Fortified Salt

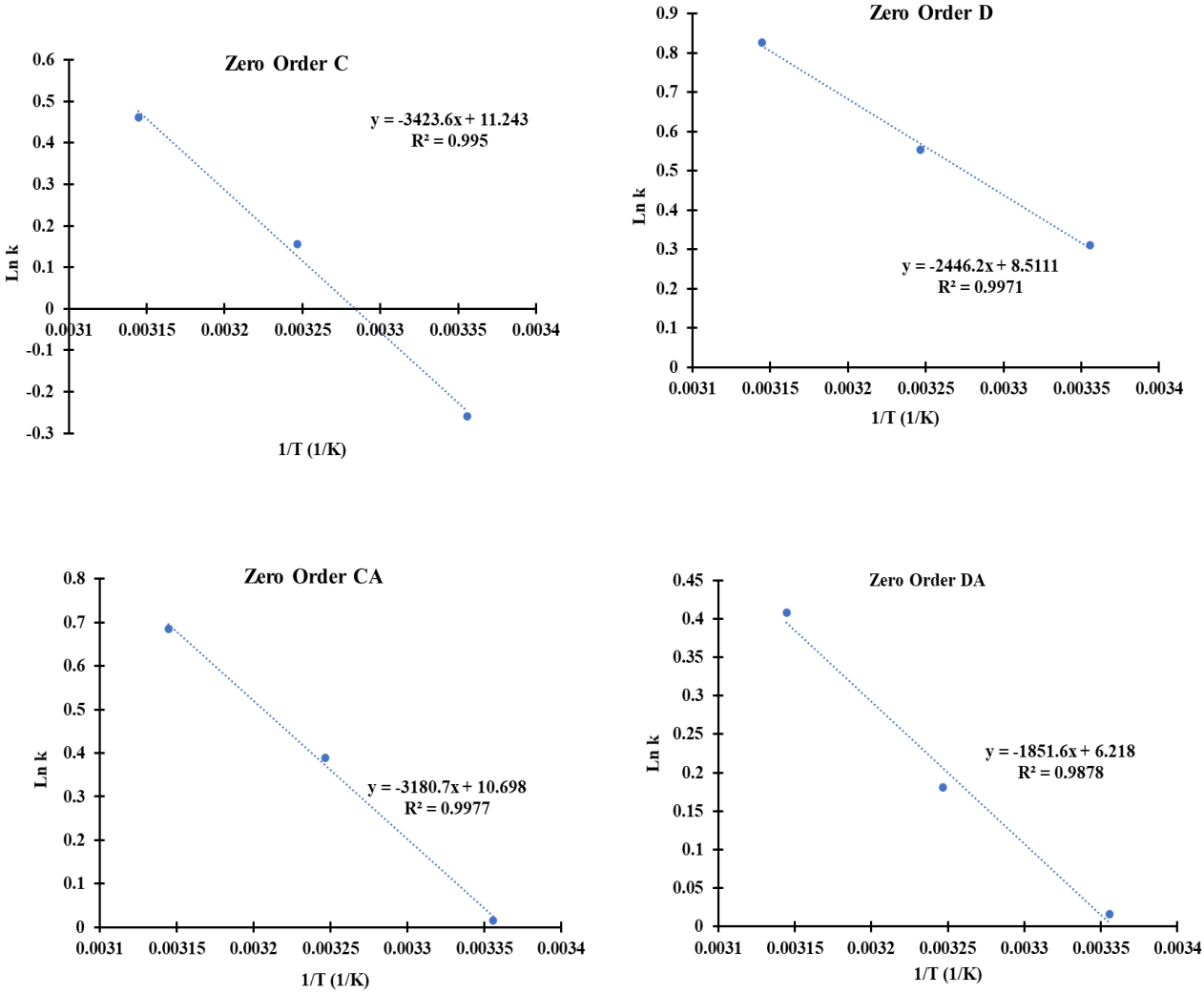


Figure 101:Arrhenius plot for the zero order degradation rate for iodine in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

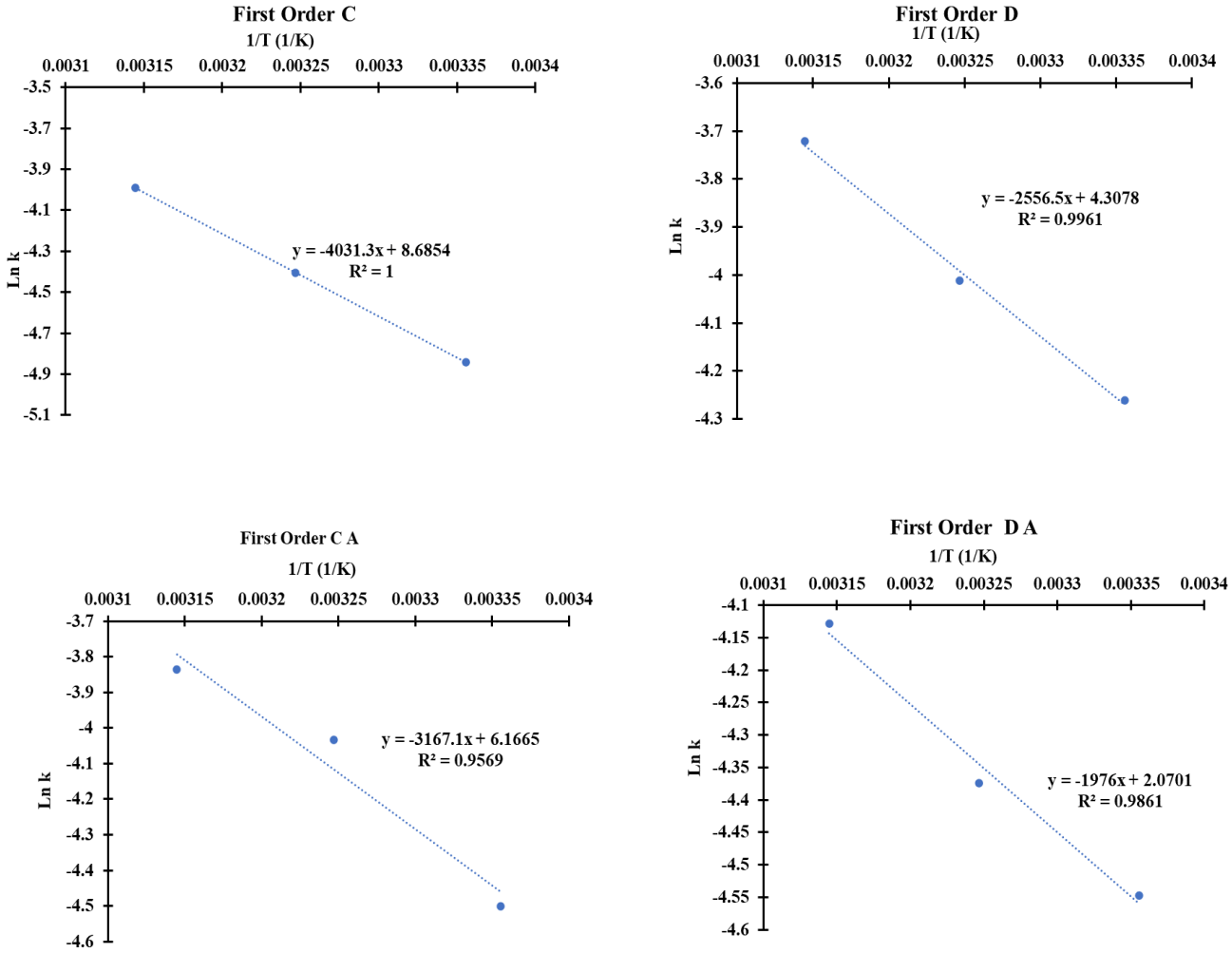


Figure 102:Arrhenius plot for the first order degradation rate for iodine in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

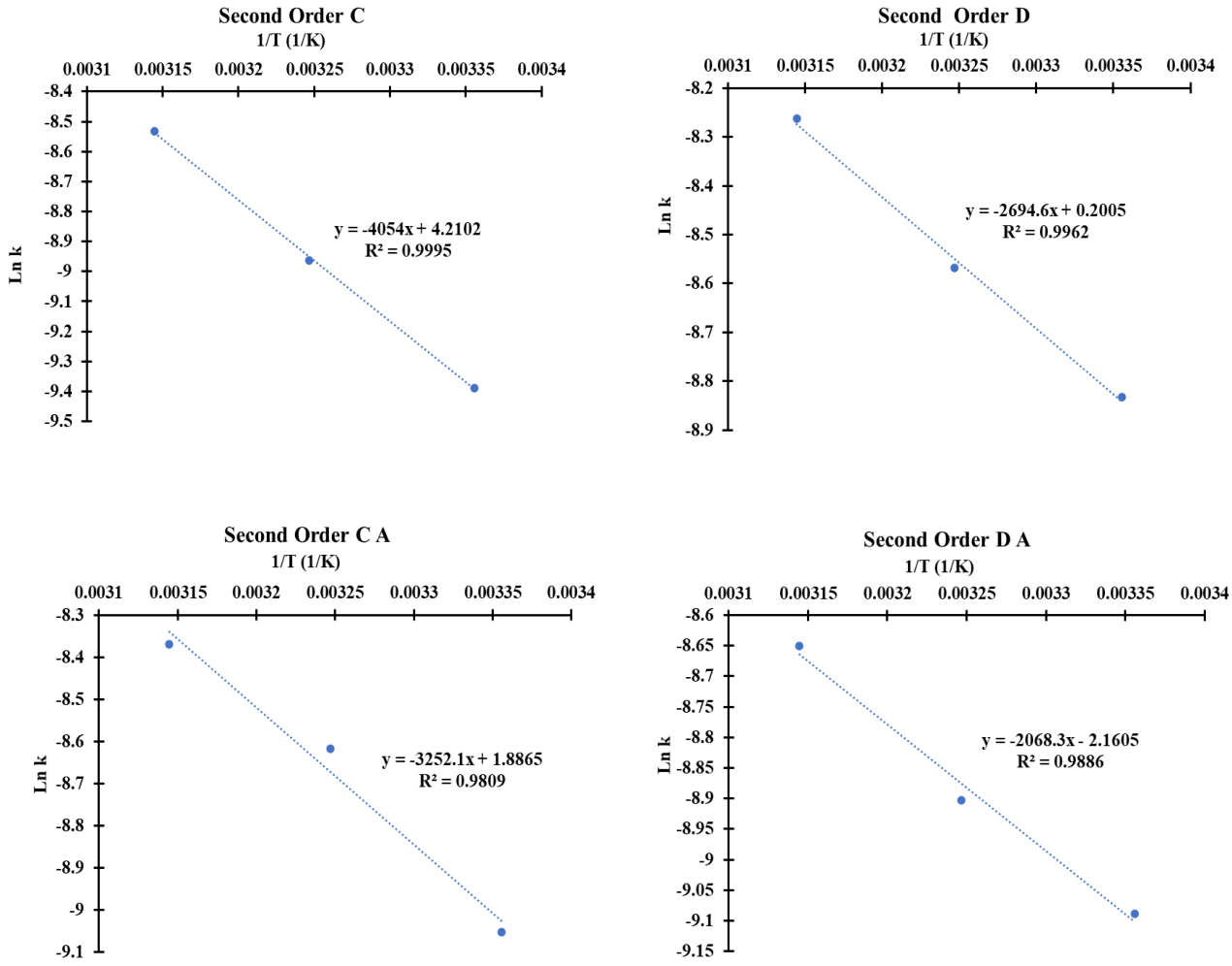


Figure 103:Arrhenius plot for the second order degradation rate for iodine in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

Degradation kinetics of Folic Acid in the Triple Fortified Salt

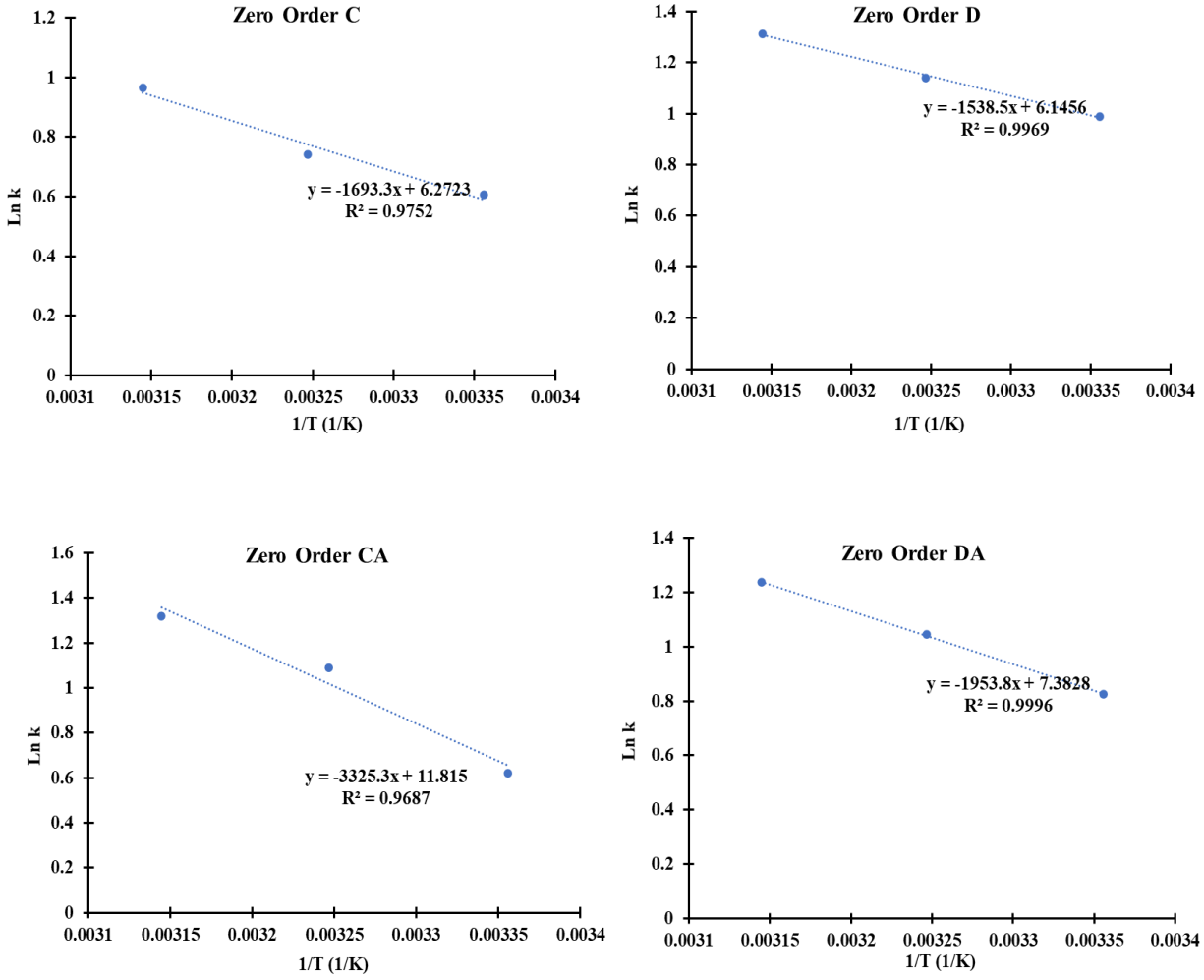


Figure 104:Arrhenius plot for the zero order degradation rate for folic acid in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

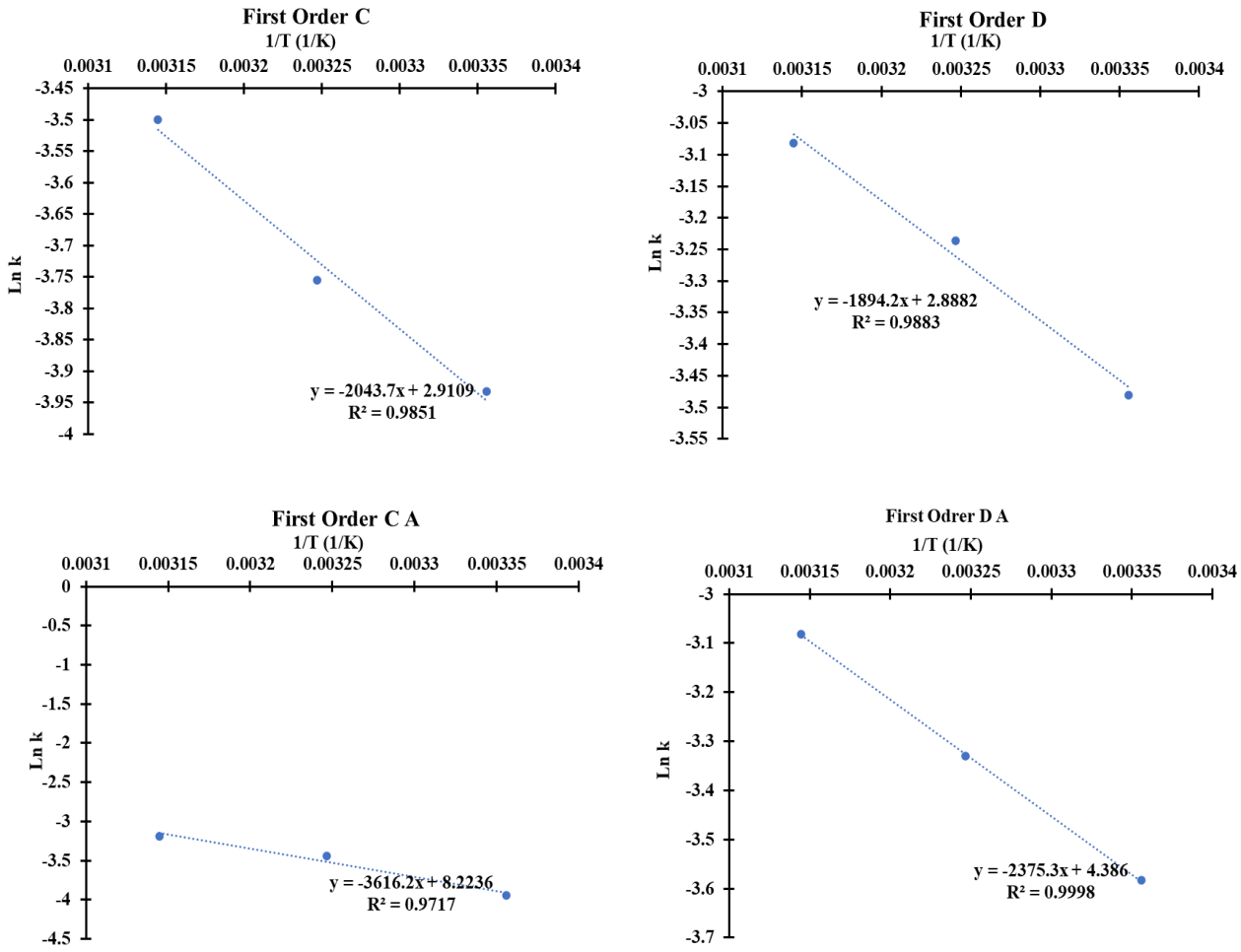


Figure 105:Arrhenius plot for the first order degradation rate for folic acid in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

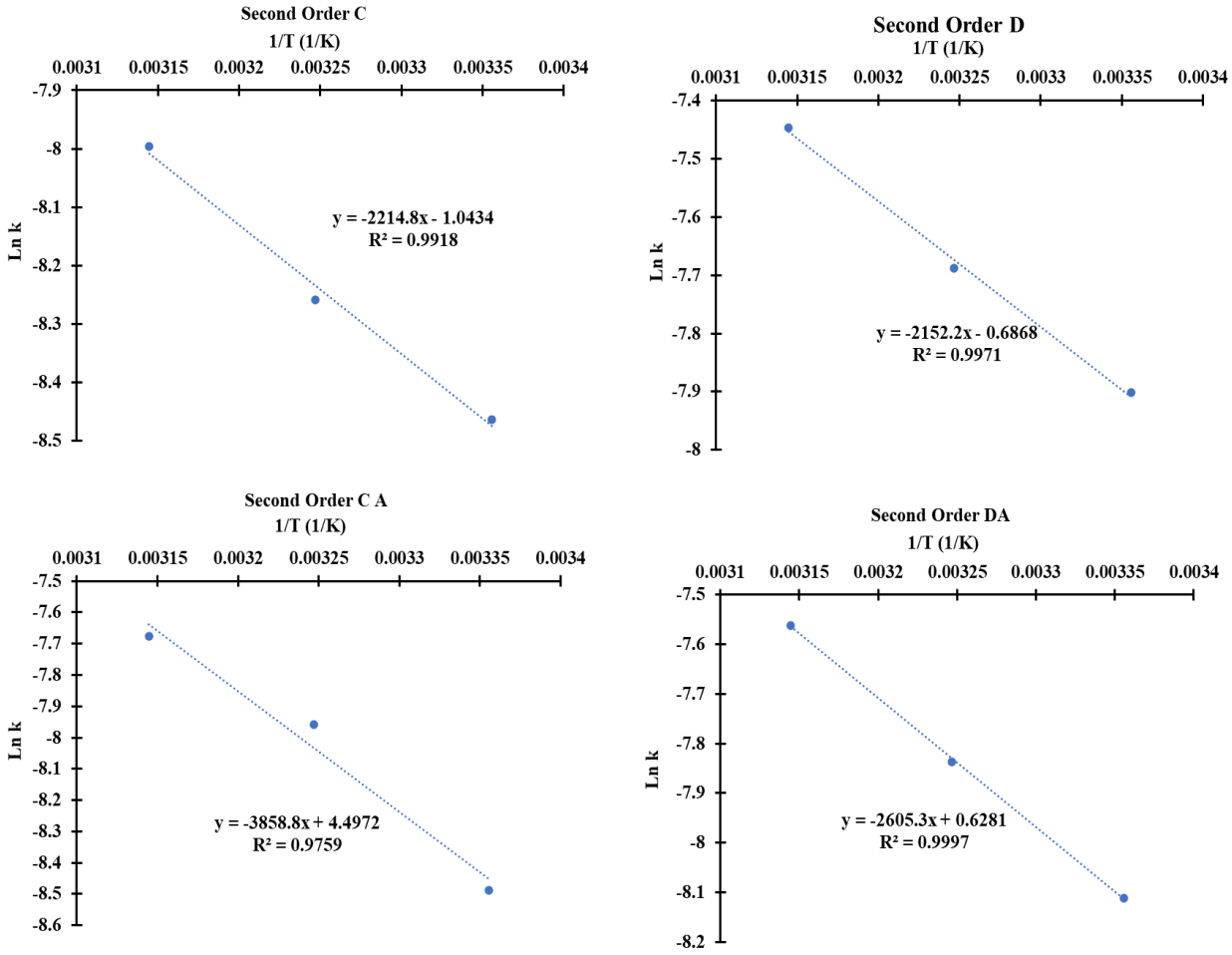


Figure 106:Arrhenius plot for the second order degradation rate for folic acid in TFS

The C and D salt (DFS*) are fortified by spraying a solution of folic acid and iodine on salt. They differ in the concentration of folic acid; the C salt had 12.5ppm of folic acid, while the D salt had 25ppm folic acid. The CA and DA salt samples had iron at 1000ppm

Degradation kinetics of Folic Acid in the Quadruple Fortified Salt

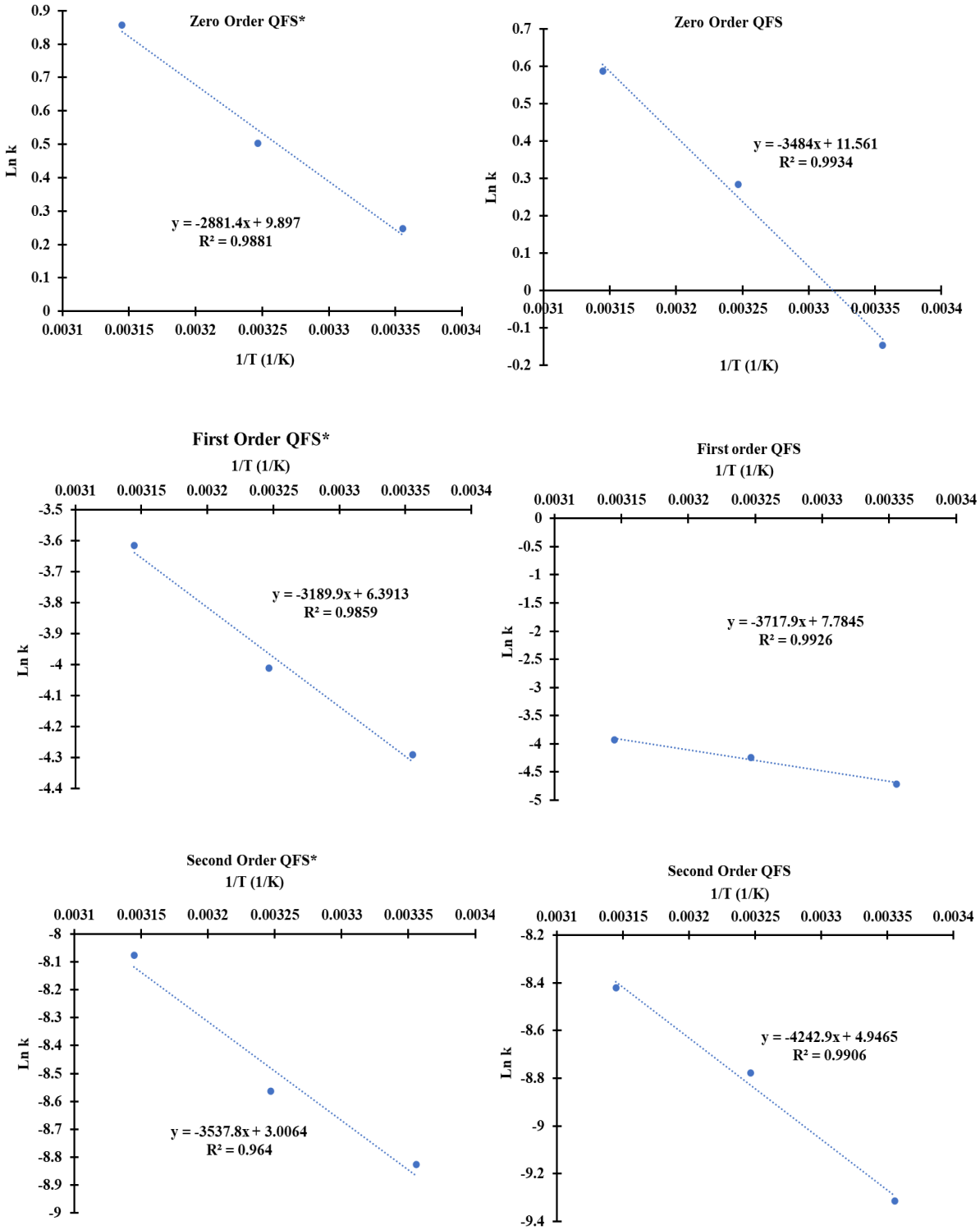


Figure 107:Arrhenius plot for degradation rate for folic acid in QFS

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.

Degradation kinetics of Iodine in the Quadruple Fortified Salt

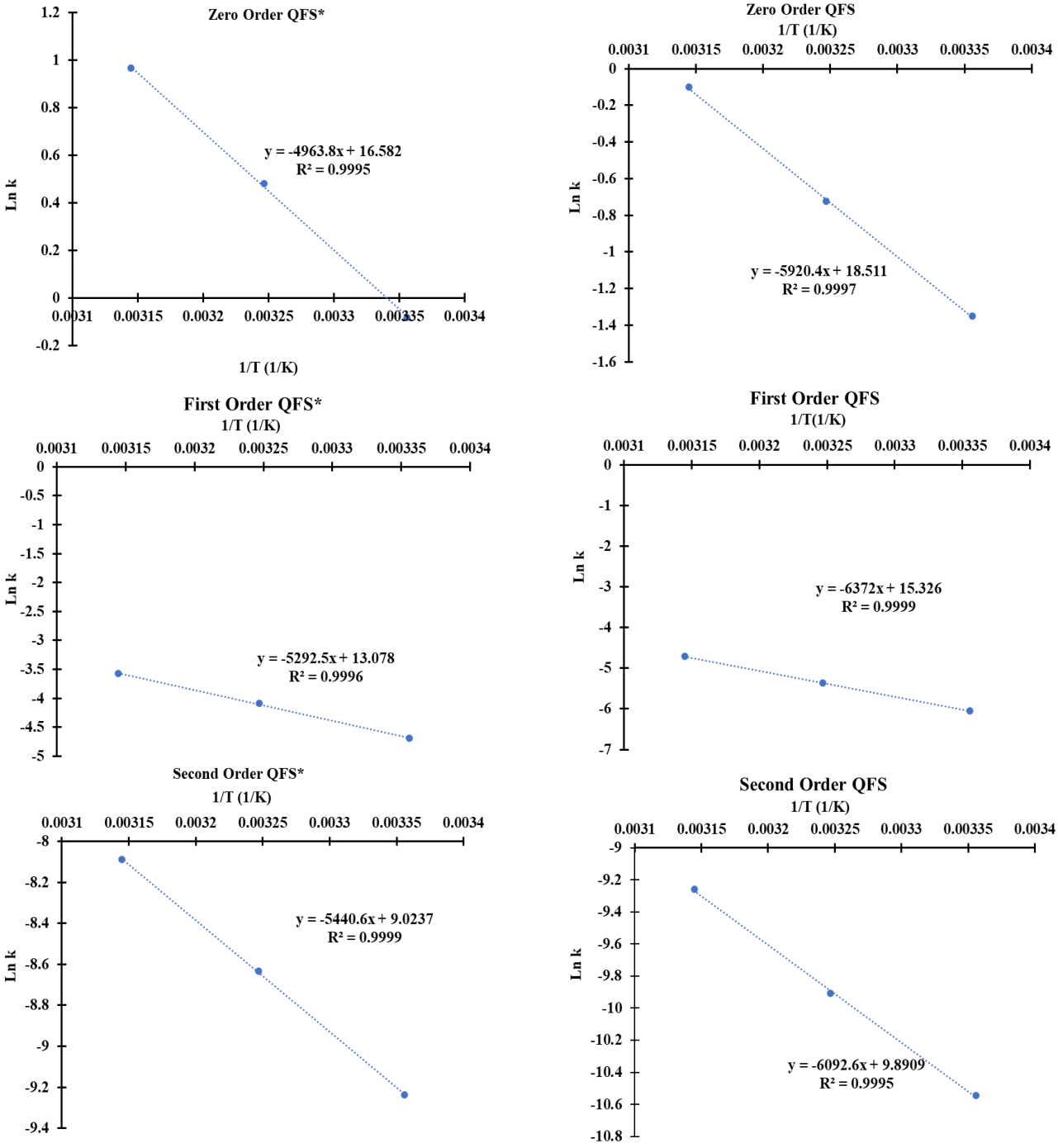


Figure 108:Arrhenius plot for the degradation rate for Iodine in QFS

QFS* was formulated with a solution of iodine, folic acid, and vitamin B_{12} and iron premix; QFS was formulated with a solution of iodine and folic acid and iron-B_{12} premix.