Accelerated Shelf-life study of fortified rice: Evaluating micronutrient retention in different packaging options

by

Mehreen Iftikhar

B.S., Karachi University, 2010

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2022

Approved by:

Major Professor
Sajid Alavi

Copyright

Abstract

The number of people in the world affected by hunger continued to increase in 2020 under the shadow of the COVID-19 pandemic. After remaining virtually unchanged from 2014 to 2019, the prevalence of undernourishment (PoU) increased from 8.4 percent to around 9.9 percent between 2019 and 2020. Despite hunger, the biggest problem in developing countries is micronutrient deficiencies whereas in western countries the primary concern is obesity, but micronutrient deficiencies are also present here. In total around 2 billion people suffer from micronutrient deficiencies specially vitamin A,vitamin B, Folic acid, iron, and zinc.

Fortification is considered as an effective strategy to address micronutrient deficiencies. Rice as a staple food used by more than half of the world. It contains mainly starch as other nutrients removed during milling process to produce white rice (Steiger et al 2014), considering wide acceptance worldwide rice has great potential to use effectively as carrier for fortificants for much needed micronutrients. My overall research was to analyze accuracy of measurement methods for micronutrient analysis, conduct accelerated shelf-life study on fortified rice produced using coating technology with different packaging at different temperature conditions and calculate overages of micronutrients needed to produce fortified kernels using extrusion technology.

The first part of this study was to evaluate micronutrient contents in fortified rice produced using two different technologies (coating and extrusion), analyze effect of particle size during sample preparation on accuracy of results and compare different methodologies used by commercial labs for analysis of micronutrients in fortified rice. Different sample grinding methods were evaluated before the micronutrient analyses, and it was found that grinding leading

to 95% of particles through 600 microns was optimal, and further intensity of grinding (example, 95% through 250 microns) did not lead to any improvement in results. Five different methodologies were used for the micronutrient analyses, coded as methods A, B, C, D and E, for the purpose of this study, based on standard protocols employed by various commercial testing labs. The maximum deviation from these standards for vitamin A in coated FRK was observed to be 127.7% (method B) and minimum 8.6% (method D); these maximum and minimum deviations were -63% (method C) and 6.7% (method E), respectively for extruded FRK. In general, the lowest deviations were observed for minerals (iron and zinc; in some cases, less than 1%) as opposed to vitamin concentrations. This study helped to understand the impact of FRK production method, sample preparation and analyses techniques on accuracy of micronutrient concentration measurements and would serve as basis for fortified rice suppliers and food aid organizations to improve quality and efficacy.

The second part of this study focused on conducting accelerated shelf-life study. Fortified coated rice kernels (FRK) were mixed in a ratio of 1:100 with regular rice to produce fortified rice and packaged in woven poly propylene (WPP), laminated woven poly propylene (LWPP) and a new multi-layer hybrid bags (10 kg in size) and placed in 3 different accelerated storage conditions (27°C, 33°C and 43°C at 60%RH) and key attributes micronutrient attributes (Vitamin A, Vitamin B1, Folic acid, Iron and Zinc) and microbial load (yeast and mold) were determined at regular intervals over a period of 6 months. Vitamin A was the most degraded micronutrient. Minerals results were relatively stable throughout the accelerated shelf-life period in all 3 packaging and storage conditions. Sensory results showed significant change in aroma in all 3 packaging and at the extreme storage condition (43°C). Hybrid packaging bags were similar or better than other packaging options for retention of micronutrients and sensory attributes and

minimizing microbial load in fortified rice. Data were fitted to the Arrhenius model to determine the rate constant.

The third and final part of this study was to produce fortified rice kernels using extrusion technology. 4 formulations with different levels of micronutrients were used to produce fortified rice kernels. As per USDA recommendation vitamin A 500IU, vitamin B1 0.5mg, folic acid 0.13mg, Iron 4 mg and zinc 6 mg with 20 ± range should be present in final product. Keeping that standard in mind, our formulation (100% premix) gave best results. Overages more than that would exceed the acceptable level suggested by USDA, so 100% premix formulation is considered as optimum formulation and suggested to be used to produce extruded fortified kernels.

Overall, this research has operational significance for food aid in general. Results will help in understanding gaps in current packaging and transition to new more effective packaging with optimum formulation suggestion to produce fortified rice kernels.

Table of Contents

List of Figures

List of Tables

Acknowledgements

My sincere acknowledgment and gratitude to my major advisor Dr. Sajid Alavi for his guidance, support, and encouragement throughout my program of study. Additionally, I would like to thank all my committee members Dr. Brian Lindshield, Dr. Kaliramesh Siliveru, Dr. Martin Talavera and Dr. John Mathew for sharing their expertise and involvement throughout the progression of this research.

I would also like to thank USDA & USAID for sponsoring this research. Also, several industrial partners including packaging suppliers, fortified rice kernel suppliers, rice supplier and analytical labs for active participation.

My heartfelt gratitude to Eric Maichel and all my lab mates Randall Martin, Delaney Webb, Blake Plattner, Jenna Flory, Tucker Graff and Conrad Kabus for their support throughout this journey.

Dedication

To my parents

without whom none of my success was possible

&

To my husband

for all the support and encouragement.

Chapter 1 - Introduction

Rice is one of the most critically important food crops in the world. It is a food staple of many cultures and is essential to the economy of developing countries. It takes up approximately 11% of the world's cultivated land. Despite being such a popular food, it is not as nutritionally dense as compared to other foods. In a world where there are significant micronutrient deficiencies, it would make sense to enrich a popular food that people gravitate towards naturally. There are several challenges with fortification and generally how the food is prepared. During washing and boiling for example, a popular method of enrichment called dusting, is washed off. In our work, we explore fortification methods and different packaging that can potentially improve and sustain the micronutrients to have them effective delivered to the patient.

Micronutrient are essential nutrients required in small amount to perform different functions of body.Deficiencies of these micronutrients are very common across the world not only in developing countries (western Africa and southeast Asia) but also in western countries. (Migliozzi et al., 2015).As a result, it causes diseases and general suffering. Most common deficiencies include iron which causes anemia. Iron deficiency anemia (IDA) which is most prevalent and widespread nutritional disorder in the world in which body stop producing adequate healthy red blood cells. Zinc deficiency is also very common and leads to several serious health consequences including stunting, low-quality pregnancy, affect birth weight, gastrointestinal, epidermal, reproductive, and skeletal systems (Anand, Rahi, Sharma, & Ingle, 2014) (Shahzad, Rouached, & Rakha, 2014). Vitamin A deficiency is also very common and leads to night blindness and delayed growth. Vitamin B deficiency can cause fatigue, anxiety, neurological and cardiovascular diseases. These are just few examples, the list of problems

associated with these deficiencies are very long, so fortification is a valuable addition to address this effectively.

Fortification is mostly done using 2 technologies coating and extrusion. Coating is the oldest way to add micronutrients into commercial rice using a water-resistant edible coating. There are many different coatings used ex waxes, gums, starches, and cellulosic polymers but as the micronutrients are only layered on top not embedded inside (Steiger et al. 2014). Extrusion is another way to incorporated micronutrients, it uses to make recomposed rice with more uniform distribution of micronutrients, and we show same trend in our results where extruded fortified rice had lower deviations as compared to coated FRK, due to more uniform distribution of micronutrients.

Chapter 2 Objectives

Chapter 2 reviews different ways to evaluate micronutrient contents in fortified rice produced using two different technologies (coating and extrusion), analyze effect of particle size during sample preparation on accuracy of results and compare different methodologies used by commercial labs for analysis of micronutrients in fortified rice. Different sample grinding methods were also evaluated before the micronutrient analyses. Five different methodologies were used for the micronutrient analyses, coded as methods A, B, C, D and E, for the purpose of this study, based on standard protocols employed by various commercial testing labs.

Chapter 3 Objectives

Chapter 3 focused on conducting accelerated shelf-life study on fortified coated rice packaged in existing packaging woven poly propylene (WPP) and improved packaging laminated woven poly propylene (LWPP) and multi-layer hybrid bags (10 kg in size) and placed in 3 different accelerated storage conditions (27°C, 33°C and 43°C at 60%RH) to see the impact

of those condition on micronutrient attributes (Vitamin A, Vitamin B1, Folic acid, Iron and Zinc) and microbial load (yeast and mold) and sensory attributes were determined at regular intervals over a period of 6 months.

Chapter 4 Objectives

The goal for chapter 4 was to produce fortified rice kernels using extrusion technology and analyze different process parameters during extrusion processing. Overages were also determined for production of fortified rice kernels using extrusion technology.

Overall, this research has operational significance for food aid in general. Results helped in understanding gaps in current packaging and transition to new more effective packaging with optimum formulation suggestion to produce fortified rice kernels.

1.2 References

Anand, T., Rahi, M., Sharma, P., & Ingle, G. K. (2014). Issues in prevention of iron deficiency anemia in India. *Nutrition*, *30*(7-8), 764-770.

Migliozzi, M., Thavarajah, D., Thavarajah, P., & Smith, P. (2015). Lentil and kale: Complementary nutrient-rich whole food sources to combat micronutrient and calorie malnutrition. *Nutrients*, *7*(11), 9285-9298

Shahzad, Z., Rouached, H., & Rakha, A. (2014). Combating mineral malnutrition through iron and zinc biofortification of cereals. *Comprehensive Reviews in Food Science and Food Safety*, *13*(3), 329-346

Chapter 2 - Comparison of Methods for Evaluating Micronutrient Concentration in Fortified Rice

Abstract

Micronutrient fortified rice is a cost effective and efficient tool in the fight against global malnutrition, since rice is a staple food and makes up a large percentage of the diet around the world. This study involved evaluation of micronutrient content in fortified rice produced using two technologies – extrusion and coating. The fortified rice kernels (or FRK) in concentrated form are mixed in a ratio of 1:99 with regular rice to produce fortified rice that is ready for distribution. Five different methodologies were used for the micronutrient analyses, coded as methods A, B, C, D and E, for the purpose of this study, based on standard protocols employed by various commercial testing labs. The United States Agency for International Development (USAID) standard concentrations for micronutrient fortified rice (per 100g) are 500 IU, 0.5 mg, 0.13 mg, 4 mg, and 6 mg, respectively, for vitamin A, vitamin B1, folic acid, iron and zinc. The maximum deviation from these standards for vitamin A in coated FRK was observed to be 127.7% (method B) and minimum 8.6% (method D); these maximum and minimum deviations were -63% (method C) and 6.7% (method E), respectively for extruded FRK. All these methods utilized high performance liquid chromatography (HPLC) with the primary differences being sample preparation and solvents used in extraction. Similarly, clear differences were observed between various methods used for other micronutrients resulting in varying deviations from the standard. In general, the lowest deviations were observed for minerals (iron and zinc; in some cases, less than 1%) as opposed to vitamin concentrations. This was expected as the latter are harder to extract and quantify in complex matrices such as rice. It was also observed that results

for extruded FRK had lower deviations from the standard than coated FRK, which might be due to more uniform distribution of micronutrients in the former due to the nature of the fortification technology. Different sample grinding methods were also evaluated before the micronutrient analyses, and it was found that grinding leading to 95% of particles through 600 microns was optimum, and further intensity of grinding (example, 95% through 250 microns) did not lead to any improvement in results. This study helped to understand the impact of FRK production method, sample preparation and analyses techniques on accuracy of micronutrient concentration measurements and would serve as basis for fortified rice suppliers and food aid organizations to improve quality and efficacy.

2.1 Introduction

The number of people in the world affected by hunger continued to increase in 2020 under the shadow of the COVID-19 pandemic. After remaining virtually unchanged from 2014 to 2019, the prevalence of undernourishment (PoU) increased from 8.4 percent to around 9.9 percent between 2019 and 2020. Of the total number of undernourished people in 2020 (768 million), more than half (418 million) live in Asia and more than one-third (282 million) in Africa, while Latin America and the Caribbean accounts for about 8 percent (60 million) (FAO, 2021). To get to milestone of zero hunger across the world, food availability is a big challenge to save lives but the right amount of nutrition at right time is also significantly important to not only save lives but allow people and countries to reach their full potential. There is a significant improvement and progress in fight against hunger over the years, but poor /bad nutrition is still a challenging problem with one in three people in this world affected by some form of malnutrition (WFP, 2018). At times malnutrition is often unnoticeable as people might not get

any clinical symptoms hence why it is also considered as hidden hunger, but if continued, over the time the consequences would become very serious.

Micronutrient is a collective term used for essential vitamins and trace minerals. Inadequate intake or deficiencies of micronutrients are very common worldwide especially in developing countries where there aren't adequate means for healthy diet to everyone. Diets are not balanced and mostly cereal based which barely fulfill the need of essential micronutrients. (Migliozzi et al., 2015). Micronutrient deficiencies make people susceptible to infectious diseases, impairs their physical and mental development, reduce their ability of productivity, and increase the risk of premature death (WFP, 2017). Most common deficiencies include iron which causes anemia. Iron deficiency anemia (IDA) which is most prevalent and widespread nutritional disorder in the world. Zinc deficiency is also very common and leads to several serious health consequences including stunning, low-quality pregnancy, affect birth weight, gastrointestinal, epidermal, reproductive, and skeletal systems (Anand, Rahi, Sharma, & Ingle, 2014) (Shahzad, Rouached, & Rakha, 2014).

2.1.1 Aliments related to Micronutrient Deficiencies

Fat soluble vitamins includes vitamin A, D, E and K. Vitamin A is a fat-soluble vitamin that is stored in liver. It follows the same absorption mechanism as fat. It's typically found in animal products such as meat, fish, poultry, and dairy products in the form of retinyl acetate or retinyl palmitate (Preformed vitamin A) whereas it's also found in plant-based foods such as vegetables and fruits in the form of beta carotene (Pro vitamin A). Vitamin A helps to form and maintain healthy skin, teeth, skeletal and soft tissues. It is also known as retinol because it produces the pigment in the retina of eye (Institute of medicine, food, and nutrition board, 2001). Vitamin A deficiency (VAD) is a common and leading public health problem which leads to

blindness in children, night blindness in pregnant women, severe infections, and risk of maternal mortality (World Health Organization, 2013).

Water soluble vitamins are essential for body cellular functions, growth, and development. It dissolves in water and readily absorbed into tissues for immediate use. Humans cannot synthesize water soluble vitamins (except niacin) so they must obtain from extrinsic sources. (Said, 2015). Vitamin B1 is a member of water-soluble family, also known as thiamin or thiamine. It is found in body via two main sources food which absorbs in small intestine and normal microflora of large intestine which absorbs in colon. Foods that are considered as good sources of thiamine includes edible seeds and nuts, legumes, rice, meat, whole grains cereals. Excessive refining, milling, and polishing of rice and cereals results in losing considerable portions of vitamin B. It performs various function in body such as energy production, breakdown of carbohydrates, immune system activation, communication between brain and nerve cells and signaling or communicating between cells and tissues. Deficiency of vitamin B1 leads to a disease called Beri- Beri which occurs to infants whose mothers are deficient. It could also be found in those individuals who has high intakes of carbohydrates and other anti-thiamin factors such as excessive intake of tea, coffee, raw fish, and shellfish which contains an enzyme thiaminases that destroy thiamine. Wernicke Korsakoff syndrome and optic neuropathy are also caused by vitamin b1 deficiency. It is not only problem of developing countries where there is poor dietary intake food due to limited resources, whereas it is also often found in developed countries due to excessive alcoholism where alcohol causes thiamin deficiency by reducing the rate of absorption (Wiley & Gupta, 2019).

Folic acid and folate are water soluble vitamins and belongs to vitamin B family and categorize as vitamin B9. Folate is a B vitamin that occurs naturally in foods such as green leafy

vegetables, citrus fruits, and beans whereas folic acid is a synthetic form of folate which founds in supplements and added to fortified foods (enriched breads, enriched flours, enriched pasta, enriched rice, enriched corn meals, fortified corn masa flour, fortified breakfast cereals etc). Our body does not store folic acid that means we should regularly consume foods containing folate or add supplements to daily intake. Deficiency of folate causes anemia, growth retardation, problems during pregnancy which could affect child brain (anencephaly) and spine (spina bifida), cardiovascular disease, chronic disease, and increased risk of certain type of cancer (Dary & Hurrell, 2006).

Trace minerals are classified as minerals that are required in the diet in smaller amount per day. These includes copper, zinc, selenium, iodine, chromium, fluoride, manganese, molybdenum, and others. Though these trace minerals required by body in smaller amount, but their deficiency could be detrimental. In this study we focused on two of these minerals which are iron and zinc.Iron is a trace mineral which is naturally present in many foods. It is considered as essential mineral as it is needed to produce an essential component of hemoglobin, an erythrocyte (red blood cell) that is responsible to transfers oxygen from lungs to tissues (Wessling, 2014). Iron also plays important role in physical growth, neurological development, cellular functioning, and some hormones synthesis (Aggett et al, 2012). The bioavailability of iron is highly dependent on its sources. Dietary iron is found in two main forms which is classified as heme and nonheme (Wessling, 2014). Iron deficiency could develop symptoms like fatigue, weakness, and pale skin, shortness of breath, dizziness, swollen / sore tongue and abnormal heart rate. Iron deficiency is one of the most common nutritional deficiencies worldwide affecting primarily children and women. Dietary supplements and fortifying foods are some of the ways to prevent iron deficiency.Zinc is an essential trace mineral and a cofactor for

more than two hundred enzymes in the human body which is needed by our body for structural, regulatory, and catalytic functions. Deficiency of zinc is quite common and lead to hair loss, diarrhea, skin sores, loss of appetite and weight loss.

2.1.2 Micronutrient Fortification of foods

Fortification is the practice of deliberately increasing the content of an essential micronutrient i.e. vitamins and minerals in food, so as to address micronutrient deficiencies and improve the nutritional quality of the food supply. Whereas enrichment refers to adding the original nutrients back into food. Rice is an excellent staple food for over half of the world's population (FAO,2004), As it is one of the most dominant crops consumed worldwide and accounts for over 20 % of global calorie intake, rice when fortified with micronutrients can help aid vulnerable populations at large scale. (Steiger et al., 2014). The United States and other governments and agencies around the world employ fortified rice as a major means of food assistance. To ensure quality and efficacy, it important to analyze and quantify micronutrients in fortified food products

2.1.3 Commonly used Analytical Methods for Micronutrient Analysis

2.1.3.1Advanced Analytical techniques for fat soluble vitamins (FSV)

To perform human body crucial functions, there are several essential micronutrients which are vital including Fat soluble vitamins (FSV). There are several analytical methods developed over the years, but due to considerably low amount and rough natural distribution it is considered as more complex to determine accurate amount of FSVs in matrix. Fat soluble vitamins includes vitamin A, vitamin D, vitamin E and K. Each one of them has a different function and bioavailability. There are several extraction and chromatographic techniques used in the industry but there is lack of standard methods for different foods specially fortified foods

and vitamin premixes. Here is a brief overview of generally used extraction and separation analytical methods for different matrix.

Extraction is one of the most important steps for any vitamin analysis as the results might alter due to chemical instability pertaining (light, oxygen, heat, alkali, and acids), chemical heterogeneity, matrix complexity, low concentrations in representative samples and interaction with other macro components such as polysaccharides, proteins, and lipids. Vitamin A is sensitive to light, oxygen, and acids that's why addition of an antioxidant typically considered during sample extraction. In general, extraction of fat-soluble vitamins is done by saponification. Saponification is defined as process in which a base (such as sodium hydroxide NaOH or potassium hydroxide KOH) is added to hydrolyze an ester resulting in carboxylate salt an alcohol. During this process soaps are formed due alkaline hydrolysis of fats and oils that's why it is referred as saponification (Latin sapon, meaning "soap" and facere, meaning "to make"). Alkaline conditions are provided with addition of antioxidant at ambient or elevated temperatures with inert atmospheric conditions (Md et al, 2020). At times, Hot saponification is used for the extraction of fat-soluble vitamins from food also effective for extraction of FSVs such as vitamin A, D and E, but not considered suitable for vitamin K (Md et al, 2020). Overnight cold saponification is used for vitamin K extraction as vitamin K homologues are sensitive to alkaline conditions under high temperatures. These conventional techniques often result in less recoveries, vitamin degradation, requires excessive solvents, prone to mishandling as its laborious and time consuming. Alterative extraction methods to handle these micronutrients with intense care which are sensitive to light, oxygen, heat, and acids includes Pressurized liquid extraction (PLE) in which elevated temperature and pressure conditions are provided for increase isolation yields. The advantages of this technique include less time, reduce

extracted volumes and the extraction is carried out in stainless steel cell that prevent light and oxygen interference during extraction process which is needed for fat soluble vitamins as they are sensitive to oxygen, light, heat, acid. High cost and variability of extracted volumes are considered as drawback of this extraction method (Fanali et al, 2017). Another alternative extraction technique is dispersive liquid- liquid microextraction (DLLME)which has been developed to overcome shortcomings of conventional extraction methods which allows dispersion of fine droplets of extraction solvent in an aqueous solution rapidly making a cloudy solution. Relatively low solvent (in microliter range) and used for variety of foods (Zgola et al, 2011). It could be used alone or combined with alkaline digestion.

Chromatographic techniques are also very important along with selection of right extraction method. Liquid chromatography (LC) is considered as most suitable for fat soluble vitamins (FSVs) and carotenoids in various foods, which includes high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), nano liquid chromatography (nano-LC), two-dimensional liquid chromatography (2D-LC).These techniques are very versatile in terms of type of chromatographic mode such as normal phase (NP), reverse phase (RP), non-aqueous reverse phase (NARP) and column packings. These LC techniques could also be coupled with different advanced detectors such as ultraviolet/visible (UV/Vis), diode array (DAD), photo diode array (PDA), florescence (FLD), electrochemical (ED), mass chromatography (MS). These aids in technical solution for vitamin characterization in complex food matrices. Among listed detectors MS is considered highly selective and have potential to detect low level of vitamins (Fanali et al, 2017).

2.1.3.2 Analytical techniques for water soluble vitamins (WSVs)

Water soluble vitamins are essential micronutrient including all B group vitamins and vitamin C. There are different techniques used for water soluble vitamins but analysis of WSVs is challenging due low-level concentration and interaction with other compounds like protein, phosphate, presence other biological active forms (vitamers), unstable nature and chemical heterogeneity (Fatima et al, 2019). Recently more reliable methods are developed for sample preparation and analytical techniques. Commonly used methods for vitamin B complex includes protein precipitation acid hydrolysis and/or enzymatic treatment, solid phase extraction (SPE) and dispersive solid phase extraction (DSPE). There are several analytical methods used for B complex vitamin analysis including HPLC which are coupled with different detectors such as ultraviolet UV, florescence FLD, diode array DAD, and electrochemical ED. LC-MS/MS also used for vitamin analysis for the detection of residual chemical compounds, confirmatory identification of small organic molecules, and confirmation and quantitation of contaminants and adulterants in pharmaceutical and food samples (Fatima et al, 2019). Folic acid also belongs to B complex family and there are several methods employed for the determination of folic acid including Spectrophotometry, HPLC, and HPLC couple with mass Spectroscopy, colorimetric, fluorometric, spectrophotometry, electrophoresis, and microbial assay. However microbial assay is time consuming and requires several hours to develop assay (Akbar et al, 2016).

2.1.3.3 Analytical techniques for trace elements/ minerals

Mineral analysis of food samples also requires sample preparation and analytical techniques for quantification of these minerals. There are different ways used for sample preparation which could be either manually or with automated measures. Samples needs to be blended, mixed, grind to be used for mineral analysis, then follow process of digestion to remove

potential interference in the matrices. There are many ways digestion could be performed such as wet acid, dry ash, microwave assisted digestion (MW-AD), and microwave induced combustion (MIC) and using open vessel with atmospheric digestion. After sample is removed from organic matrix, inorganic portion of sample could be analyzed by atomic absorption spectrometer (AAS), Microwave and inductively coupled plasma optical emission spectrometry /Atomic emission spectrometry (ICP-OES/AES), Inductively coupled plasma mass spectrometry (ICP-MS), Electrochemical techniques, techniques using X-rays.

2.1.3.4 Micronutrient Analysis used for rice and rice-based products

There are several methods available for vitamin and mineral analysis for rice and rice-based products including fortified rice. Methods vary based on type of sample being analyzed. Ivarsen et al., 2021 developed methods for quantification of retinyl palmitate, thiamine, niacin, pyridoxine, folic acid, cyancobalamin, zinc and iron using chromatographic methods. Retinyl palmitate were extracted using enzymatic treatment and analyzed with HPLC coupled with UV detector. Thiamine extraction with HCL and analyzed with HPLC coupled with florescence FLD detector. Folic acid extraction with combination of amylase solution and acids analyzed by liquid chromatograph triple quadrupole mass spectrometer (LC-MS/MS). Iron and zinc extraction was done by HCL and analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES). To analyzed stability of Vitamin A, Iron and Zinc in fortified rice Kuong et al., 2016 used reverse phase HPLC for vitamin A using ultraviolet/diode array detector. Iron and zinc were analyzed using inductive coupled plasma-optic emission spectrometry. Solid-liquid extraction (SLE), Saponification and solid phase extraction (SPE) using HPLC with FLD detector preferred for rice matrix for vitamin E isomers. (Fanali et al., 2017) (Huang et al., 2011). ICP-MS (Inductively coupled plasma mass spectroscopy) were used for mineral analysis of

milled rice Jiang et al., 2007. Another study by Losso et al., 2017 used ICP-OES for iron analysis on uncooked and cooked rice samples. Jannasch et al., 2020 also used similar method for iron analyses by using ICP-OES where rice was fortified using parboiling. Lee et al., 2000 studied stability of retinyl palmitate during cooking and storage in rice fortified with ultra-rice fortification technology. Modified method of extraction using direct solvent extraction followed by normal phase HPLC was used. Balakrishna et al., 2020 performed thiamine analysis through thiamine thiochrome fluorescence spectroscopy using AOAC official method of analysis in rice where rice was enriched with natural thiamine using high pressure processing. Shrestha et al., 2003 analyzed folic acid in milled rice using microbiological assay with Lactobacillus casei.

It is clear from the preceding discussion that there are no standardized methods for analyses of micronutrients in fortified rice. Varying analytical methods have been reported in different studies. More sample preparation techniques including sampling size, grinding, etc also differ, and can make a difference to the analyses. This lack of standardization can be a challenge for food agencies and fortified rice manufacturers. The overall focus of this study was to compare accuracy of measurement methods employed by commercial analytical labs for micronutrients in fortified rice. Accuracy comes with both trueness (closeness of the measurement to the expected value), and precision (closeness of the measurements to each other) as shown in Figure 2.1

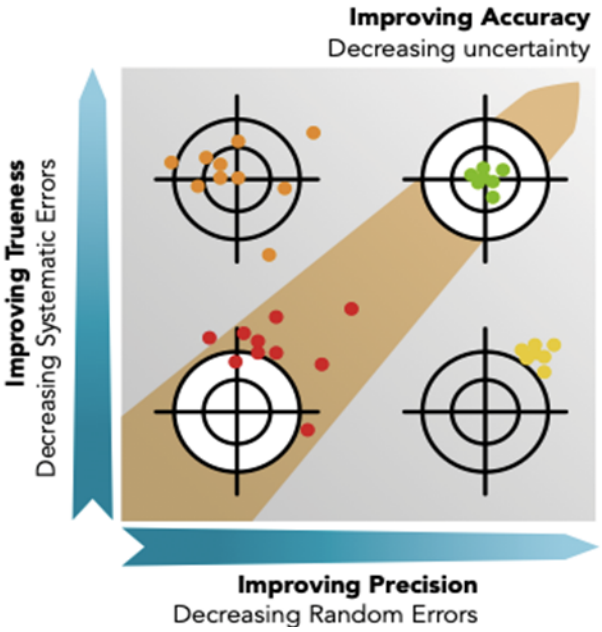


Figure 2.1. Accuracy of measurement (Rimkus,2021)

The specific objectives of this study were to evaluate the accuracy of micronutrient analyses in fortified rice performed by five commercial labs; study the effect of grinding method or particle size during sample preparation for micronutrient analyses; and understand the impact of two different rice fortification technologies (coating and extrusion) on the analyses. The micronutrients studied were vitamin A, vitamin B1 (or thiamine), folic acid, iron and zinc. Z score or standard score was used to compare the results of each lab to a standard normal distribution.

2.2 Materials and Methods

2.2.1 Materials

Fortified rice produced from different technologies (coated and extruded) were donated from two commercial suppliers *Wright Enrichment Inc*. (Crowley, LA) and *Heartland harvest*

Inc. (Kankakee, IL). Long Grain Whole Grain Milled White Rice is used as the commercial rice (non-fortified) and donated from *Supreme Rice, LLC* (Crowley, LA).

2.2.2 Analytical Laboratories

Five different laboratories (coded as A, B, C, D and E) were used to analyze fortified kernels and fortified rice (coated and extruded) which were coded anonymously after grinding using optimized grinding method (as described below) and packed in zip lock bags and then an opaque sealed bag to avoid light related deterioration and stored in freezer. All samples were analyzed for vitamin A, vitamin B1 (thiamin), folic acid, iron, and zinc.

2.2.3 Mixing

Coated and extruded fortified rice kernels (FRK) were mixed 1:99 with the regular rice to produce fortified rice (i.e., 1.5g of fortified kernels mixed with 148.5g of non- fortified rice). All samples were 150g each and mixed homogeneously.

2.2.4 Grinding and Particle Size Determination

Two different methods of grinding were studied to achieve a particle size distribution with 50% through 250 microns and 75% through 420 microns, as per industry standard *Wright Enrichment Inc*. (Crowley, LA). A coffee grinder (Mr. Coffee) and high-speed multifunctional grinder (Moongiantgo Grain Grinder) used at different time intervals for grinding 15, 30, 60, 80 and 100 seconds. After each 20 seconds, grinder was stopped for about 10-15 seconds to avoid sample heating and then further ground at room temperature.

To compare grinding methods used by different commercial analytical labs, fortified rice samples were also sent and requested to grind them as per their practice. Brief details of grind methods are mentioned below.

Lab A: This lab used Hammer Mill with 1.5mm screen size to grind the samples as per their routine practice

Lab B: Lab B ground the samples using Perten Hammer mill with 0.5mm screen for about 5 seconds.

Lab C: Lab C used a Cryogrinder, which is an advanced method to homogenize while avoiding sample to heat up.

Lab D: The samples were ground for 30 – 60 seconds using a Thermomix Vorwerk on the highest speed setting. The heating settings were not used for processing. Samples were processed with minimal light

Lab E**:** This lab used a Grain grinder to grind the samples

Particle size analysis were performed using a Hosokawa alpine jet air sieve *(Hosokawa, Augsburg, Germany*). The series of sieves utilize were 53, 75, 106, 125, 212, 300, 425, 600 and 850 microns)

2.2.5 Micronutrient analysis with different particle size

To evaluate the impact of particle size on micronutrient analysis, samples from 3 different grind duration using grain grinder (15 seconds, 30 seconds, and 100 seconds) sent to Lab. They also performed the analysis based on their own practice of grinding i.e., using a hammer mill.

To verify the results another set of samples sent to another lab for grind 30 seconds and 100 seconds sent to another Lab for micronutrient analysis. All the samples were doubled sealed in opaque bags to block exposure to light, humidity and other external factors till samples are ready for analysis.

2.2.6 Micronutrient analysis methodologies employed across testing labs

2.2.6.1 Vitamin A analysis

Lab A used a modified method AOAC 974.29 in which 10 g of samples were weighed into saponification flasks. Samples were saponified on a steam bath with reagent alcohol, potassium hydroxide, and an antioxidant (BHT). Samples were then cooled, hexane was added and then mixed. Phases were allowed to separate. The organic layer containing the vitamin A is decanted into a separatory funnel. This extraction process was performed a total of three times to ensure complete extraction. The organic collection in the funnels was rinsed with deionized water (DI) and finally filtered through sodium sulfate into volumetric flasks. For retinol, samples were diluted in hexane if necessary. A portion of the sample was transferred into an HPLC vial. Retinol samples were analyzed by HPLC with mobile phase consisting of isorpropaol and hexane. The HPLC was equipped with a silica column and fluorometric detector (Ex 330 nm, Em 480 nm). (AOAC 974.29 Mod)

Lab B used in house industrial standard for vitamin A analysis. 9g and 1g samples of fortified rice and fortified kernels respectively, then were sonicated in an acetic acid solution for 15mins.Isopropyl alcohol was then added, vortexed and the sample shaken for 1 hour. The samples were then centrifuged, syringe filtered and analyzed by normal phase HPLC with UV detection. Note: The laboratory has developed two methods for vitamin A, depending on the matrix and method preference. Neither method involves saponification. They typically measure retinyl palmitate and report what found or can convert it to an equivalent amount of retinol.

Lab C vitamin A analysis method was referred from (Ball, 1988) (Budavari, 1996) (Kirk *et al.,* 1991) (Lambert *et al.,* 1985) (Reynolds *et al.,* 1985) (Segawa*,* 2009). 2.5 g of fortified samples were weighed, homogenized along with 0.25g pyrogallol, 60mL ethanol, and 10mL of

potassium hydroxide solution. These were refluxed for 40minutes under nitrogen to saponify, then to room temperature. 50mL of extraction solvent was then added to a separatory funnel, using extraction solvent and water to rinse the flask. After shaking the funnel, then 5-10mL saturated NaCl solution was added. Layers were allowed to separate and lower aqueous layer was discarded. This process was repeated twice more. The remaining organic layer was drained into a 250-mL flask and evaporated to dryness under nitrogen. It was reconstituted into 25mL methanol, sonicated to mix then filtered through 0.45µm PTFE syringe filter into an auto sampler vial. Samples were then injected and analyze by HPLC at 365 nm.

Lab D used modified AOAC method (2001.13) in which 0.2 - 0.4 g of sample was weighed then pyrogallol and ethanol were added followed by 45% KOH solution. Samples were vortexed for 1 minute then, incubated at 70ºC for 45 minutes, shaking the tube gently by hand every 10 minutes. Samples were then cooled to room temperature and water and hexane added. Samples were shaken for 10 minutes, then centrifuged for 3 minutes at 3000 rpm. The top hexane layer was transferred to another tube, then the hexane extraction procedure was repeated twice more. Sodium sulfate was added before vortexing then centrifuging at 3000 rpm for 3 minutes.0.1 ml of 1-pentanol was added to 0.5 ml of extract before it was dried under nitrogen. This was reconstituted in isopropyl alcohol and sonicated for 5 minutes before being analyzed by HPLC. Mobile phase consisted of methanol: isopropanol (80:20) and water at flow rate of 1.8 ml/min.

Lab E did not share their method details due confidentiality.

2.2.6.2 Vitamin B1 (Thiamin)

Lab A modified AOAC 942.23 method for vitamin B1 analysis.1 g of samples were extracted in 0.1N HCl by autoclaving. Various forms of phosphorylated thiamine were then

converted into free thiamine with an alpha amylase solution. Samples were diluted, centrifuged, and filtered, and then injected in an ultra-performance liquid chromatography (UPLC). After the peak separation on a C18 column, the eluent enters an oxidation loop where thiamine reacts with alkaline ferricyanide and was converted to a fluorescent derivative, thiochrome. Thiochrome was analyzed on a fluorescent emission detector (Ex 363 nm, Em 435 nm).

Lab B used an in-house method by Analytical chemical services of Columbia.1g fortified rice kernels (FRK) and 10g fortified rice were added to Mobile phase. The samples were vortexed and shaken for 1 hour. Samples were then centrifuged, filtered, and analyzed by HPLC.

Lab C method derived from (Fellman *et al.,* 1982) (AOAC (2005) 18th edition, 942.23) (J. Food Comp. and Analysis (1989) Vol. 2(1) 41.). 2–10g sample was weight and homogenized into a 125mL Erlenmeyer flask. 70mL 0.1N HCl were added and autoclaved at 122°C for 30 minutes. It was then cooled to room temperature.3N sodium acetate solution was added until pH 4.0–4.5 reached. Then 5mL of Taka-diastase solution was added and incubated at 52°C for 3 hours or at 35°C overnight then cooled to room temperature. It was then diluted to 100mL with DI water.10mL was pipetted in to test tube along with 5mL potassium ferricyanide and approximately 1mL H2PO4to pH 6.9–7.1. This was then transferred to C18 Sep-Pak, washed with 4mL phosphate buffer, then with 5% methanol buffer solution. It was eluted with 50% methanol water into 5mL volumetric and diluted to volume. It was then analyzed by HPLC-FLD

Lab D used modified method using EN14122:2014 (Determination of vitamin B1 by high performance liquid chromatography) in which acid hydrolysis with enzymatic treatment was performed. No further details were shared.

Lab E did not share their method details due confidentiality.

2.2.6.3Folic acid analysis

Lab A used modified method of AOAC 992.05 (Total Folate (Pteroylglutamic Acid) in Infant Formula - Microbiological Methods).1 g of sample was autoclaved and undergoes enzymatic treatment using creon capsules and chicken pancreas conjugase to release folate from matrix. Sample solution was then mixed with growth media and inoculated with L. Rhamnosus. After overnight incubation, the concentration of total folate in the sample is determined by reading the turbidity of the sample at 600 nm against a series of calibration standards. The amount of growth is directly proportional to the concentration of the analyte in the sample.

Lab B method was derived from (Osseyi *et al.,* 1998) (Gregory *et al.,* 1988) (Pfeiffer *et al.,* 1997).1g fortified rice kernels (FRK) and 9g fortified rice were weighed and extraction solution was added, vortexed and shaken for 1 hour. An alpha-amylase solution was added and mixed. The sample is then heated in a water bath for 1 hour. The sample is then centrifuged, filtered, and analyzed by HPLC.

Lab C used a microbiological method for the quantitative determination of total vitamin (added and natural vitamin) in food, animal feed and pharmaceutical products. The VitaFast® vitamin microtiter plate is read in a micro well plate reader to yield the turbidity measured at 610-630nm, or alternatively, at 540-550nm.The control turbidity readings were used to form the standard curve and the sample turbidity readings were plotted against the curve to calculate the exact concentration of the analyte. Turbidity results yield quantitative results.

Lab D also analyzed samples for folic acid using microbiological assay. Method was derived from EN 14131:2003 (Determination of folate by microbiological assay) / AOAC 944:12 (Folic acid microbiologically with Enteroroccus hirae)

Lab E did not share their method details due confidentiality.

2.2.6.4 Iron and Zinc analysis

Lab A used modified method from (AOAC 984.27, 927.02, 985.01, 965.17) to quantify iron and zinc in a variety of sample matrices.10 g of the sample was weighed into a crucible. It was then ashed in a muffle oven at 5500 °C for greater than 5 hours. The ash then dissolved in mainly hydrochloric acid with a small amount of nitric acid while boiling on a hotplate. This solution was transferred to a volumetric flask and brought to volume with deionized water. An appropriate dilution was performed, and the solution was introduced into the Elemental Analysis by ICP uses Inductively Coupled Plasma Optical Emissions Spectrophotometry (ICP-OES) instrument. The emission signal was measured at 238.2 nm for iron and 206.2 nm for zinc. Calibration standards, drift control standards, and control samples were analyzed with each batch to ensure instrument suitability and acceptable results. The iron and zinc signal were adjusted by gallium internal standard recovery determined using the 294.4 nm wavelength. Measurements were computed by the ICP software (Winlab).

Lab B microwave digested 0.5g of fortified rice kernels and 1g fortified rice was microwave digested with a ramp temperature of 180°C for 15 minutes, hold 10 minutes and cooled to manufactures instructions. The clear digestion was transferred to 100 ml volumetric and filled to volume with DI water as per AACC 40-70.01

Lab C added nitric acid to weighed samples then deionized water was added. Samples were digested with a microwave digester equipped with automatic sampler for sequential digestions (or analogous microwave digester), for a minimum of 10 minutes at ≥200°C.If after digestion the solution is not clear, digestion was continued until the solution was clear or light-straw colored (presence of solid residue insoluble in the applied analytical conditions) and filtered with 0.45µm disposable Teflon filters. The samples were analyzed by ICP-OES and

quantitated using a prepared standard curve for each analyte. (AACC 40-70 & 40-71) (AOAC 985.35 (50.1.14) (Chu, 1995)

Lab D used 0.4730 – 0.4927 g of sample weighed and 50 µL of ~1 ppm Yttirum internal sample added along with 0.5 mL of hydrochloric acid. 1.5 mL of nitric acid allowed to react before capping for microwave digestion. After digestion was completed, samples were transferred to centrifuge tube and digest rinsed using distilled water bring total volume in the centrifuge tube to 50 ml.Tubes were centrifuged at 4000 rpm for 5 min then measured by ICP (AOAC 2011.14)

Lab E did not share their method details due confidentiality.

2.2.7 Data interpretation and Statistical Analysis

Performance characteristics of result are measured with accuracy of data. Accuracy comes with both trueness (closeness of the measurement to the expected value), and precision (closeness of the measurements to each other) which covers both systematic and random errors Rimkus, 2021.Similar approach is used to analyze results as per equations below.

$$\text{Delta \%} = (x - \text{recommended value})/\text{recommended value} * 100$$

$$\text{COV} = (\text{Standard deviation} / \mu) * 100$$

z score or standard score was calculated across all labs

$$z = (x - \mu)/\sigma$$

where x is the mean value of an attribute obtained by each lab, μ mean across lab in a performance measure; σ is standard deviation across the labs for each performance measure. The z score is an absolute quantity that shows if a result is greater than or less than the mean after normalization. Lower z score represents more accurate results, z score is negative when the result

of an attribute is less than the mean and positive when greater. Performance of each lab were analyzed as satisfactory for $|Z| \leq 1$, questionable for $1 < |Z| \leq 2$, and unsatisfactory for $|Z| \geq 2$ Results are shown in Table 2.6 for z scores. (Dias et al, 2015)

For micronutrient analysis data comparing results from the 5 different labs, a four-way analysis of variance (ANOVA) was performed by using the GLM procedure by statistical analysis software (SAS 9.4 Inst. Inc., Cary, NC). The four factors were the different analytical labs (5), micronutrients (5), rice fortification technology (2) and rice dilution (2). Tukey's HSD (Honest Significance Difference) test was applied for the least-squares means separation, with significance considered at a probability $P<0.05$.

2.3 Results

2.3.1 Particle size analysis: In- house trials

There were several iterations of grinding performed using coffee grinder and a high-speed multifunctional Grinder *(Moongiantgo Grain Grinder).*

Wide variation observed in particle size distribution using 2 different grinders (Table 2.1).30 seconds of grind using grain grinder gave 70% while coffee grinder gave only 17% through 300 microns, 85% through 425 microns using grain grinder compared to only 26% using coffee grinder. At 100seconds of grind grinding 97% passed through 300 microns while only 56.3% using the coffee grinder. At 425 microns it was 99% through with grain grinder while it was 77.9% using coffee grinder.

Coffee grinder gave coarser particle size whereas grain grinder gives finer particle upon grinding which might be due size and thickness of blades. After comparing results of different time durations (15, 30, 60, 80 & 100) coffee grinder was excluded from further grinding trials.

Table 2.1. Grinding trials using coffee grinder and grain grinder in % cumulative

Sieve (µ)	CG-15sec	GG-15sec	CG-30sec	GG-30sec	CG-60sec	GG-60sec	CG-100sec	GG-100sec
53	2.0±0.5	7.2±1.3	2.9±0.8	12.3±0.6	7±0.1	23.6±0.3	11.5±0.5	28.2±0.6
75	3.2±0.1	12.0±0.0	4.8±0.7	20.1±1.1	11.3±0.4	35.2±0.1	17.5±1.4	43.1±1.1
106	4.3±3.1	17.8±0.7	6.7±0.1	29.4±0.9	15.6±0.3	48.1±4.7	23.6±0.5	58.5±0.2
125	4.9±0.3	20.8±0.1	7.6±0.2	30.5±0.9	-	54.0±0.1	26.6±0	65.2±0.8
212	7.7±1.2	35.8±3.2	12.5±0.6	56.0±0.7	-	84.6±0.2	42.2±0.1	93.6±1.4
300	10.7±1.5	47.5±2	17.5±0.8	70.9±1.7	40.9±1.1	91.3±0.1	56.3±0.1	97.6±0.1
425	16.3±0.7	62.1±1.1	26.7±0.9	85.6±0.6	60±0.2	98.7±0.3	77.9±0.3	99.9±2.1
600	25.6±0.3	75.1±1.6	41.6±2.1	94.4±2.2	82.3±0.2	99.9±1.1	95.5±1.5	-
850	40.6±0.4	84.7±1	63.1±0.2	98.2±0.1	96±0.2	100.0±1.1	100±1.4	-

CG- Coffee Grinder, GG- Grain Grinder, % Cumulative – showing results of all sieves passed through in sequence.

Figure 2.2 shows the results of particle size distribution at different time of grinding using grain grinder starting with 15seconds, 30 seconds, 100seconds and hammer mill grind. By increasing the grind time finer particle were obtained at initial sieves.

There is no standard range of particle size for micronutrient analysis available but there are some industry practices which suggest 50% through 250 micron and 75% through 420 microns (*Wright Enrichment Inc*.) for micronutrient analysis. 30 seconds of grind using grain grinder gave results close to recommended industrial standards. (Ivarsen et al., 2021) grinded samples 100% through 250 microns for micronutrient analysis in fortified kernels and fortified rice. We also compared KSU grinds using GG with HM (hammer mill) to see any difference in distribution. HM results were close to 100 seconds of KSU grind.

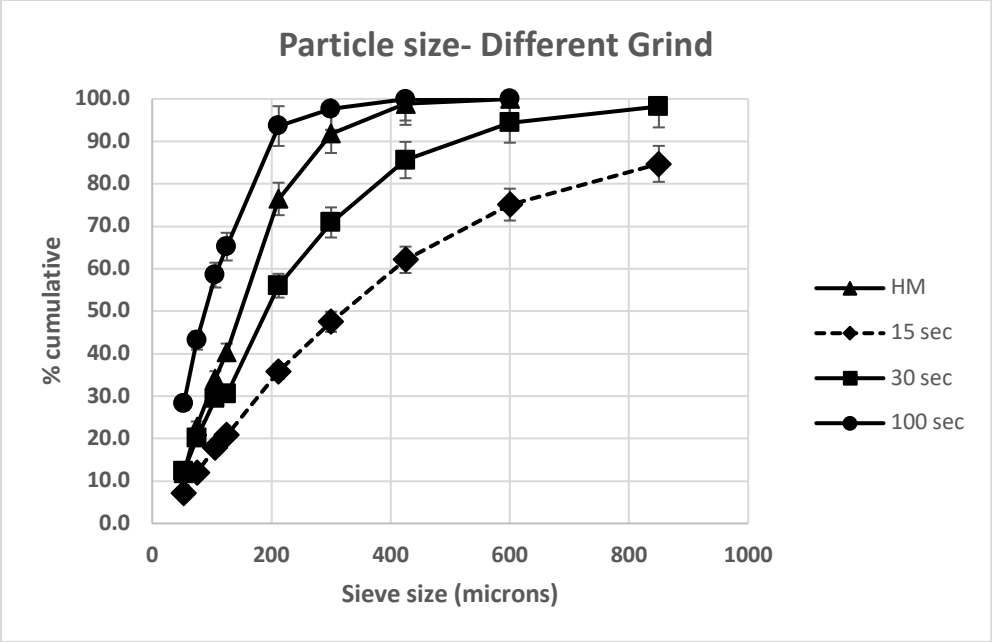


Figure 2.2. Particle size cumulative of different GG duration and HM

2.3.2 Comparison of particle size with different labs

Duplicate samples were sent to different labs for grinding and then particle size analysis was performed in-house using a Hosokawa alpine jet air sieve *(Hosokawa, Augsburg, Germany*). The series of sieves utilize were 53, 75, 106, 125, 212, 300, 425, 600 and 850 microns).

Results showed 69%, 91.9%, 81.5 and 51.8% through 300 microns from Lab A, Lab B, Lab C, and lab D. Our internal lab grinding results (30 seconds of grind using grain grinder) were compared to see the difference in particle size distribution. Variation in the results were observed comparing different labs grinding (Table 2.2).

To analyze the difference quantitatively in grinding iterations, micronutrient analysis was requested from one of the commercial labs.

Table 2.2. Comparison of optimum KSU grind with other labs in % cumulative

Sieve (µ)	KSU	Lab A	Lab B	Lab C	Lab D
53	12.3±0.1	6.2±0.3	11.8±1.2	21.5±2.1	5.5±1.1
75	20.1±1.1	12.3±2.4	22.9±0.1	39.5±0.3	11.6±0.9
106	29.4±.0.7	20.3±3.4	34.2±2.1	49.2±0.1	15.3±0.5
125	30.5±0.7	24.7±0.8	40.3±0.9	53.7±0.1	25.3±0.6
250	56.0±0.7	56.3±0.1	76.4±0.7	74.9±0.7	37.4±1.8
300	70.9±1.9	69.1±0.5	91.8±0.9	81.5±0.3	51.8±0.6
425	85.6±0.4	89.4±0.1	98.8±0.7	93.2±1.9	66.4±2.4
600	94.4±0.6	98.2±0.8	100.0±1.9	99.5±3.3	83.4±2.1
850	98.2±0.8	99.9±0.3	-	100.0±0	96.4±0.5

2.3.3 Micronutrient Results at different particle size

Fortified rice kernels FRK and fortified rice (coated and extruded) from 15,30 and 100 seconds grind and commercial lab grind (hammer mill) results were compared for systematic errors as (delta%) and precision as (coefficient of variance COV) in Table 2.3 and Table 2.4. Higher delta% were observed in different grinding for vitamins (Vitamin A, Vitamin B1 and Folic acid). Vitamin A showed 158.37% and 388% delta for FRK and fortified rice (coated) whereas 179% and 240% delta for FRK and fortified rice (extruded) for hammer mill grind results. Vitamin B1 showed 65% and 168% delta for FRK and fortified rice (coated) whereas 63% and 136% delta for FRK and fortified rice (extruded) for 30 seconds grind results. Higher COV shows less precision of data, it was 39.7 for iron, 38.2 for zinc in fortified coated samples whereas 0.3 and 0 for iron and zinc in coated FRK (hammer mill), which shows that error in lab results increased with sample dilution.

Different grind leads to variation in micronutrient results specially for vitamins which has shown in other studies too. Nakos et al., 2017 showed improper mixing can affect the recovery of sample, sufficient grinding showed better results on vitamin B12 extraction. Kurek et

al., 2017 found that particle size of dietary fiber does affect bio accessibility of vitamin B in fortified wheat bread. Typically, vitamins are less stable, different to extract and involves series of steps which could be the reason for such a high variation in results. Minerals (Iron and Zinc) showed lower deviation as compared to vitamins

In our study, coated samples (FRK and fortified rice) showed more deviation as compared to extruded samples (FRK and fortified rice), because later had micronutrient embedded into the samples and more uniformly distributed. Wieringa et al., 2014 studied stability and retention of different micronutrients (vitamin A, iron, zinc, folic acid, and vitamin B12) in fortified rice using different technologies (hot extrusion, cold extrusion, and coating) and did not find any differences in results for either technology. Kuong et al., 2016 shared over 80% of losses especially for vitamin A at higher temperatures and humidity when coating technique was used for fortifying rice as compared to extrusion technique. Fortification techniques also plays role in the analysis of micronutrients. Extrusion is considered better than traditional fortification techniques as vitamins and minerals premixed with rice flour or broken rice kernels so more homogeneous mixing is achieved, as vitamins and minerals are premixed less exposure to oxidation is expected as compared to traditional fortification methods like coating. On the other hand, due high temperatures in extrusion processing vitamins are more prone to losses so overages are preferred during fortification Atungulu et al., 2014.

Overall, 30 seconds and 100 seconds showed almost similar results and less deviation from expected results as compared to 15 seconds of grind and hammer mill grind. There was not significant improvement observed for 100 seconds of grind.

Table 2.3. Micronutrient results at different grinding- Commercial lab results (Coated)

	FRK-Coated								
Parameters	**USAID Recommended**	**15 sec**		**30 sec**		**100 sec**		**HM**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	69.5±10.4	6.19	127.7±5.7	2.52	131.6±1.7	0.77	158.3±10.6	4.14
Vitamin B1	0.5 mg/1g	378.8±4.3	0.90	65.1±0.2	0.17	73.39±1.3	0.79	31.83±2.7	2.06
Folic Acid	0.13 mg/1g	95.6±4.8	2.50	59.4±0.7	0.48	57.7±0.7	0.49	148.48±3.8	1.53
Iron	4.0 mg/1g	5.6±3.1	2.96	-0.9±5.9	5.99	0.6±0.6	0.62	0.31±0.3	0.31
Zinc	6.0 mg/1g	2.5±4.1	4.07	4.1±2.5	2.40	-4.5±1.2	1.31	-6.67±0	0.00
	Fortified Rice-Coated								
Parameters	**USAID Recommended**	**15 sec**		**30 sec**		**100 sec**		**HM**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	98.2±22.9	11.41	156.1±19.8	7.75	136.2±12	5.13	388.0±118	24.29
Vitamin B1	0.5 mg/100g	257.2±88.1	24.66	168.9±11.6	4.34	177.0±5.2	1.91	78.8±12.3	6.90
Folic Acid	0.13 mg/100g	-99.7±0.01	6.34	-99.6±0	0.53	-99.6±0	0.00	-99.7±0	4.00
Iron	4.0 mg/100g	82.8±1.5	0.85	32.5±6.2	4.71	15.6±6.8	5.95	159.3±103	39.76
Zinc	6.0 mg/100g	113.50±20.8	9.76	60.4±14.5	9.09	37.5±0	0.00	254.1±135	38.26

Delta % is referring to systematic errors whereas COV (coefficient of variance) is showing random errors in data

Table 2.4. Micronutrient results at different grinding- Commercial lab results (Extruded)

	FRK-Extruded								
Parameters	**USAID Recommended**	**15 sec**		**30 sec**		**100 sec**		**HM**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	35.9±19.2	14.13	55.3±2.9	1.88	79.3±.09	0.01	179.3±10.9	3.91
Vitamin B1	0.5 mg/1g	392.8±1.6	0.34	63.2±0.4	0.28	60.7±2.9	1.81	134.4±2.1	0.93
Folic Acid	0.13 mg/1g	108.4±6.1	2.96	64.6±0.7	0.45	65.3±1	0.61	150.0±2.8	1.15
Iron	4.0 mg/1g	-5.6±1.8	1.99	-8.4±2.1	2.39	-7.1±0.3	0.34	-4.6±0.9	0.98
Zinc	6.0 mg/1g	-6.6±0	0.00	-10.8±2.5	2.80	-5.4±6.2	6.61	-3.3±0	0.00
	Fortified Rice-Extruded								
Parameters	**USAID Recommended**	**15 sec**		**30 sec**		**100 sec**		**HM**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	9.3±15.6	14.35	104.0±4.4	2.16	136.1±31.1	13.19	240.9±78	23.14
Vitamin B1	0.5 mg/100g	225.7±50.8	15.62	136.3±1.1	0.47	144.9±2.2	0.90	205.2±63.8	20.92
Folic Acid	0.13 mg/100g	-99.8±0.2	13.37	-99.6±0	0.55	-99.6±0	2.37	-99.7±0	27.94
Iron	4.0 mg/100g	4.6±26.5	25.37	16.8±0.6	0.53	15.0±6.2	5.43	96.8±71.8	36.51
Zinc	6.0 mg/100g	10.6±14.3	12.95	40.5±5.2	3.73	35.3±0	0.00	92.7±46.9	24.34

Delta % is referring to systematic errors whereas COV (coefficient of variance) is showing random errors in data

To further verify the results shown in Table 2.3 and Table 2.4, we sent our samples from 30 and 100 seconds of grind for further analysis to two different commercial labs. Each lab has their own protocol for micronutrient analysis with inhouse modifications. Results were compared in table 2.4 were both labs were coded as Lab A and Lab B.

Significant differences in results were reported when compared (30 and 100 sec) grinds with both labs. Lab A results for vitamin B1was 46.81% with 30 sec of grind whereas it was 48.09% with 100 sec of grind for coated FRKs. Similarly, 29.65% with 30 sec of grind whereas 33.35% with 100 sec of grind on extruded FRKs. Lab B for vitamin B1and other results showed higher deviations. Thiex et al., 1996 comprehensively mentioned potential sources of vitamin A in animal feed and pet food where sample handling and preparation does have a key role.

For vitamin A, Lab A used modified method AOAC 974.29 in which samples were saponified for extraction and analyzed by using HPLC with flourometric detector whereas Lab used modified industrial developed method in which HPLC equipped with UV detector was used. Saez et al., 2019 showed HPLC-FL provide better separation and classification as compared to HPLC-UV while analyzing different nut samples. Also, they concluded less error of prediction of adulteration levels using HPLC-FL. HPLC-FL is more sensitive to HPLC-UV Al-Dirbashi et al., 2001.

Vitamins are organic compounds and harder to extract whereas minerals are inorganic. Minerals have simpler chemical composition compared to vitamins. We found more deviation in our results for vitamins which second this basic difference. Vitamin A is considered as one of the most sensitive and unstable due its chemical structure and composition, therefore its challenging to study the effect of extrusion on retention of vitamin A. There are several factors which aid to

degradation of vitamin A including temperature, light, oxygen, time, and pH (Camire et al.,1990) which make it even more complex to analyze.

Diluted samples fortified rice (coated and extruded) showed high deviations and lower precision as compared to concentrated samples FRK (coated and extruded) which was expected, because the sample size is very small it might not be the true representation of the sample though all samples were mixed homogeneously.

Overall, Lab A at 30 seconds grind showed better results and less deviation as compared to Lab B where both labs followed their own method of analysis for extraction. Though grinding is a key step before sample analysis there is a need for further research to standardize methods for each matrix specially for fortified rice to avoid result variation.

Table 2.5. Comparison of results Lab A vs Lab B at 30 and 100 sec (Coated)

	FRK-Coated								
Parameters	**USAID Recommended**	**30 sec Lab A**		**30 sec Lab B**		**100 sec Lab A**		**100 sec Lab B**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	18.8±1.8	1.52	127.7±5.4	2.52	6.6±3.6	3.38	131.6±1.7	0.77
Vitamin B1	0.5 mg/1g	46.8±3.4	2.34	65.1±0.2	0.17	48.0±0.1	0.09	73.3±1.3	0.79
Folic Acid	0.13 mg/1g	-9.2±10.7	11.86	59.4±0.7	0.48	-18.0±6.5	7.98	57.7±0.7	0.49
Iron	4.0 mg/1g	7.1±0.6	0.58	-0.9±5.9	5.99	3.2±0	0.00	0.6±0.6	0.62
Zinc	6.0 mg/1g	7.2±1.2	1.17	4.1±2.5	2.40	0.8±4.6	4.63	-4.5±1.2	1.31
	Fortified Rice-Coated								
Parameters	**USAID Recommended**	**30 sec Lab A**		**30 sec Lab B**		**100 sec Lab A**		**100 sec Lab B**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	30.0±8.2	6.31	156.1±19.8	7.75	44.6±29	20.06	136.2±12.1	5.13
Vitamin B1	0.5 mg/100g	78.0±0	0.00	168.9±11.6	4.34	128.0±0	0.00	177.0±5.2	1.91
Folic Acid	0.13 mg/100g	10.7±2.3	2.08	-99.6±0	0.53	35.7±17.3	12.75	-99.6±0	0.00
Iron	4.0 mg/100g	48.7±18.7	12.61	32.5±6.2	4.71	60.0±17.5	10.94	15.6±6.8	5.95
Zinc	6.0 mg/100g	67.5±19.1	11.44	60.4±14.5	9.09	82.5±17.5	9.59	37.5±0	0.00

Table 2.6. Comparison of results Lab A vs Lab B at 30 and 100 sec (Extruded)

FRK-Extruded									
Parameters	**USAID Recommended**	**30 sec Lab A**		**30 sec Lab B**		**100 sec Lab A**		**100 sec Lab B**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	11.0±4.8	4.324	55.3±2.9	1.88	2.4±4.2	4.10	79.3±0	0.01
Vitamin B1	0.5 mg/1g	29.6±1.2	0.980	63.2±0.4	0.28	33.3±0.1	0.10	60.7±2.9	1.81
Folic Acid	0.13 mg/1g	-7.6±5.3	5.833	64.6±0.7	0.45	-28.6±0.8	1.19	65.3±1.0	0.61
Iron	4.0 mg/1g	3.2±0	0.000	-8.4±2.1	2.39	3.5±0.2	0.24	-7.1±0.3	0.34
Zinc	6.0 mg/1g	1.4±0.4	0.411	-10.8±2.5	2.80	8.2±5.0	4.68	-5.4±0.3	6.61
Fortified Rice-Extruded									
Parameters	**USAID Recommended**	**30 sec Lab A**		**30 sec Lab B**		**100 sec Lab A**		**100 sec Lab B**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	40.2±1.8	1.28	104.0±4.4	2.16	15.0±2.6	2.26	136.1±31.1	13.19
Vitamin B1	0.5 mg/100g	78.0±0	0.0	136.3±1.1	0.47	65.0±13.0	7.88	144.9±2.2	0.90
Folic Acid	0.13 mg/100g	7.3±5.7	5.37	-99.6±0	0.55	22.6±6.5	5.33	-99.6±0	2.37
Iron	4.0 mg/100g	18.7±3.7	3.15	16.8±0.6	0.53	20.0±2.5	2.08	15.0±6.2	5.43
Zinc	6.0 mg/100g	40.8±0.8	0.59	40.5±5.2	3.73	38.3±1.6	1.20	35.3±0	0.00

2.3.4 Comparison of Micronutrients results with different labs using optimized grinding

Earlier sections showed that results would vary not only based on how samples were ground but also what method of analysis was used. After concluding optimum grind (30 seconds) for samples, we compared results from five different commercial labs to select lab for further study with most accurate results. Vitamin A was 18.8%, 127.7%, 103.6%, 8.6% and 12.17% for FRK (coated) whereas 30%, 156.1%, 161.6%, 64.6% and 12.1% for fortified rice (coated) from Lab A, B, C, D and E respectively. Similarly, vitamin A was 11%, 55.3%, -63.1%, -16.1% and 6.7% for FRK (extruded) 40.2%, 104%, 65.3%, 17.2% and 6.7% for fortified rice (extruded) from Lab A, B, C, D and E respectively (Table 2.5). COV was 1.4 for vitamin A FRK (coated) and 10.3 for fortified rice (coated) from lab C results which shows lab results were not good when diluted samples were analyzed, it decreases the precision in results. Lab D also showed similar trend for vitamin A results.

Results from Lab B and Lab C showed highest deviation from expected results. Extruded FRKs showed better results as compared to coated FRKs as later has micronutrient only on the surface. There are some problems associated with coating technologies which are related to color, taste and loss of micronutrients during washing and cooking Steiger et al., 2014. Coating technology is less acceptable because of odors of waxes and solvents which stays in final product. Weak adhesion layer also facilitates losses after washing and rinsing (Alavi et al., 2008)

Table 2.7. Comparison across all labs using optimized grinding (Coated)

FRK-Coated											
Parameters	**USAID Recommended**	**A**		**B**		**C**		**D**		**E**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	18.8±1.8	1.52	127.7±5.7	2.52	103.6±3.0	1.47	8.6±8.3	7.71	12.1±11.0	10.4
Vitamin B1	0.5 mg/1g	46.8±3.4	2.34	65.1±0.2	0.17	35.4±1.0	0.74	5.2±0.6	0.57	20.0±0	0.1
Folic Acid	0.13 mg/1g	-9.2±10.7	11.86	59.4±0.7	0.48	-79.4±0.9	4.58	20.7±4.6	3.82	1.9±1.9	1.88
Iron	4.0 mg/1g	7.1±0.6	0.583	-0.9±5.9	5.99	18.5±2.2	1.90	9.7±5.9	5.45	-	-
Zinc	6.0 mg/1g	7.2±1.2	1.17	4.1±2.5	2.40	23.3±0.8	0.68	8.9±7.4	6.82	-	-
Fortified Rice-Coated											
Parameters	**USAID Recommended**	**A**		**B**		**C**		**D**		**E**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	30.0±8.2	6.31	156.1±19.8	7.75	161.6±27.0	10.32	64.6±50.9	30.94	12.1±11.7	8.54
Vitamin B1	0.5 mg/100g	78.0±0	0.00	168.9±11.6	4.34	68.0±12.0	7.14	43.0±15.0	10.49	20±0	0.1
Folic Acid	0.13 mg/100g	10.7±2.3	2.08	-99.6±1.6	0.53	-70.6±7.9	27.03	33.8±4.6	3.45	8.0±4.3	1.88
Iron	4.0 mg/100g	48.7±18.7	12.61	32.5±6.2	4.71	21.8±10.6	8.72	166.2±69.7	26.21	-	-
Zinc	6.0 mg/100g	67.5±19.1	11.44	60.4±14.5	9.09	54.1±5.8	3.78	12.4±29.2	25.99	-	-

Table 2.8. Comparison across all labs using optimized grinding (Extruded)

FRK-Extruded											
Parameters	**USAID Recommended**	**A**		**B**		**C**		**D**		**E**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/1g	11.0±4.8	4.32	55.3±2.9	1.88	-63.1±1.2	3.25	-16.1±0.3	0.44	3.2±2.8	2.7
Vitamin B1	0.5 mg/1g	29.6±1.2	0.98	63.2±0.4	0.28	15.2±0.2	0.17	-1.5±1.3	1.32	21.0±1	0.8
Folic Acid	0.13 mg/1g	-7.6±5.3	5.83	64.6±0.7	0.45	-88.2±0.4	3.61	19.2±3.8	3.23	16.5±2.6	2.3
Iron	4.0 mg/1g	3.2±0	0	-8.4±2.1	2.39	-90.1±0	0.13	35.5±28.8	21.26	5.0±0	0
Zinc	6.0 mg/1g	1.4±0.4	0.41	-10.8±2.5	2.80	-90.3±0	0.78	-13.3±15.7	18.14	8.2±3.0	2.8
Fortified Rice-Extruded											
Parameters	**USAID Recommended**	**A**		**B**		**C**		**D**		**E**	
		Delta%	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**	**Delta%**	**COV**
Vitamin A	500IU/100g	40.2±1.8	1.28	104.0±4.4	2.16	65.3±26.6	16.13	17.2±9.2	7.85	3.2±2.8	5.09
Vitamin B1	0.5 mg/100g	78.0±0	0.00	136.3±1.1	0.47	79.0±17.0	9.50	28.0±0	0.00	21.0±1	0.81
Folic Acid	0.13 mg/100g	7.3±5.7	5.38	-99.6±1.6	0.55	-79.6±2.6	13.15	48.4±9.2	6.22	16.5±2.6	2.20
Iron	4.0 mg/100g	18.7±3.7	3.16	16.8±0.6	0.53	-84.1±2.8	18.11	22.7±13.6	11.08	5.0±0	1.41
Zinc	6.0 mg/100g	40.8±0.8	0.59	40.5±5.2	3.73	-88.0±0.3	2.78	30.0±9.4	7.29	8.2±3.0	2.83

Lab selection was based on z score. Table 2.9 shows z score for all five labs where results were considered satisfactory $|Z| \leq 1$, questionable for $1 < |Z| \leq 2$, and unsatisfactory for $|Z| \geq 2$. Lab A showed z score less than 1 for all tested micronutrients whereas Lab B, Lab C, Lab D results did not match our z score acceptance criteria, Lab E did not complete the analysis and excluded from the study. There are couple of different factors which might lead to these variations including sample handling and preparations

standard solution calibration, Extraction, limit of detection equipment **(**Thiex et al., 1996). Extraction is a key step in vitamin analysis because of various challenges which includes chemical instability to light, heat, oxygen, alkali and acids, intra group and inter group chemical heterogeneity, low and different concentrations and matrix complexity (Fanali et al., 2017).

Table 2.9. Z-Score across all labs using optimized grinding method

FRK-Coated						
Parameters	**USAID Recommended**	**A**	**B**	**C**	**D**	**E**
Vitamin A	500IU/1g	-0.70	1.44	0.97	-0.90	-0.83
Vitamin B1	0.5 mg/1g	0.60	1.47	0.06	-1.38	-0.74
Folic Acid	0.13 mg/1g	-0.93	-0.93	-0.55	1.35	1.06
Iron	4.0 mg/1g	-0.21	-1.38	1.43	0.16	-
Zinc	6.0 mg/1g	-0.50	-0.92	1.68	-0.26	-
Fortified Rice-Coated						
Parameters	**USAID Recommended**	**A**	**B**	**C**	**D**	**E**
Vitamin A	500IU/100g	-0.88	1.14	1.22	-0.32	-1.16
Vitamin B1	0.5 mg/100g	0.05	1.83	-0.14	-0.63	-1.11
Folic Acid	0.13 mg/100g	0.67	-1.46	-0.90	1.12	0.57
Iron	4.0 mg/100g	-0.32	-0.60	-0.79	1.71	-
Zinc	6.0 mg/100g	0.88	0.55	0.26	-1.69	-
FRK-Extruded						
Parameters	**USAID Recommended**	**A**	**B**	**C**	**D**	**E**
Vitamin A	500IU/1g	0.32	1.46	-1.60	-0.38	0.21
Vitamin B1	0.5 mg/1g	0.18	1.75	-0.49	-1.27	-0.17
Folic Acid	0.13 mg/1g	0.23	1.38	-1.05	0.66	-1.23
Iron	4.0 mg/1g	0.33	0.05	-1.88	1.10	0.40
Zinc	6.0 mg/1g	0.62	0.28	-1.95	0.21	0.84
Fortified Rice-Extruded						
Parameters	**USAID Recommended**	**A**	**B**	**C**	**D**	**E**
Vitamin A	500IU/100g	-0.19	1.64	0.53	-0.84	-1.14
Vitamin B1	0.5 mg/100g	0.22	1.63	0.25	-0.98	-1.12
Folic Acid	0.13 mg/100g	0.85	-0.90	-0.57	1.52	-0.90
Iron	4.0 mg/100g	0.56	0.51	-1.98	0.66	0.25
Zinc	6.0 mg/100g	0.71	0.70	-1.94	0.48	0.06

Satisfactory $|Z| \leq 1$, Questionable for $1 < |Z| \leq 2$, and Unsatisfactory for $|Z| \geq 2$

Four-way ANOVA results are presented in detail in Appendix A. All the four factors, including the different analytical labs used, micronutrient type, fortification technology type and dilution level, showed significant effect ($p < 0.05$) on the delta or deviation from expected value. Significant interaction ($p < 0.05$) was also found between several pairs of factors including fortification technology and labs, fortification technology and micronutrient type, labs and dilution level, micronutrients and dilution level, and micronutrients and lab.

2.4 Conclusion

Sample preparation prior to analysis plays a very critical role on results specially micronutrient results where uniform distribution of particle lead to better results. Clear differences in particle size distribution were found Table 2.1 and Table 2.2. Excessive grind also does not give better results as it ended up heating the samples and results were not ideal. The method developed at KSU of grinding 30 seconds using grain grinder was considered as optimum grind and will be used for the further study.

In Fortified rice kernels (FRKs) micronutrient premix was in concentration form as compared to fortified rice in which it was 100-fold dilution so greater deviation (delta%) and lower precision (COV) were observed which was expected. Fortification technology used in the study (coated and extruded) also showed that results can improve if uniform distribution of micronutrients achieve during fortification process. In this study, extruded samples showed better results as compared to coated samples.

Minerals has high retention capacity as compared to vitamins thus more stable (Khamila et al, 2020). A similar trend was observed in our results with lower deviation observed for

minerals than vitamins as the latter are hard to extract and quantify in complex matrices. Clear differences in delta%, COV and Z scores were observed among different lab methods.

Micronutrient analysis is a challenging analytical test which is dependent on series of factors, more research is needed to develop standardize methods as per different foods. In our study we standardize grinding so that's not a factor and every lab method leads to different particle size.

2.5 References

AACC (2010) Approved methods of analysis, 11th edn. Method 46-10.01. Method 32-10.01, Method 40-20.01. Method 40-70.01 Cereals and Grains Association, St. Paul, MN, USA

Aggett PJ. Iron. In: Erdman JW, Macdonald IA, Zeisel SH, eds. Present Knowledge in Nutrition. 10th ed. Washington, DC: Wiley-Blackwell; 2012:506-20.

Akbar, S., Anwar, A., & Kanwal, Q. (2016). Electrochemical determination of folic acid: A short review. *Analytical biochemistry*, *510*, 98-105.

Al-Dirbashi, O. Y., Ikeda, K., Takahashi, M., Kuroda, N., Ikeda, S., & Nakashima, K. (2001). Drugs of abuse in a non-conventional sample; detection of methamphetamine and its main metabolite, amphetamine in abusers' clothes by HPLC with UV and fluorescence detection. *Biomedical Chromatography*, *15*(7), 457-463.

Alavi, S., Bugusu, B., Cramer, G., Dary, O., Lee, T. C., Martin, L., ... & Wailes, E. Rice fortification in developing countries: a critical review of the technical and economic feasibility. A2Z Project, Academy for Educational Development. Washington, DC, 2008

Anand, T., Rahi, M., Sharma, P., & Ingle, G. K. (2014). Issues in prevention of iron deficiency anemia in India. *Nutrition*, *30*(7-8), 764-770.

Atungulu, G. G., & Pan, Z. (2014). Rice industrial processing worldwide and impact on macro- and micronutrient content, stability, and retention. *Annals of the New York Academy of Sciences*, *1324*(1), 15-28.

Balakrishna, A. K., & Farid, M. (2020). Enrichment of rice with natural thiamine using high-pressure processing (HPP). *Journal of Food Engineering*, *283*, 110040.

Budavari, S. (1996). The merck Index 12th edition. White house station, NJ: Merck and Co.

Camire, M. E., Camire, A., & Krumhar, K. (1990). Chemical and nutritional changes in foods during extrusion. *Critical Reviews in Food Science & Nutrition*, *29*(1), 35-57.

da Silva Dias, R., Aparecida de Abreu, C., Ferreira de Abreu, M., & Paz González, A. (2015). Statistical methods for evaluating results from soil micronutrient analyses in interlaboratory programs. *Communications in Soil Science and Plant Analysis*, *46*(sup1), 57-71.

Dary, O., & Hurrell, R. (2006). Guidelines on food fortification with micronutrients. *World Health Organization, Food and Agricultural Organization of the United Nations: Geneva, Switzerland*, 3-37.

Fanali, C., D'Orazio, G., Fanali, S., & Gentili, A. (2017). Advanced analytical techniques for fat-soluble vitamin analysis. *TrAC Trends in Analytical Chemistry*, *87*, 82-97.

Fatima, Z., Jin, X., Zou, Y., Kaw, H. Y., Quinto, M., & Li, D. (2019). Recent trends in analytical methods for water-soluble vitamins. *Journal of Chromatography A*, *1606*, 360245.

Fellman, J. K., Artz, W. E., Tassinari, P. D., Cole, C. L., & Augustin, J. (1982). Simultaneous Determination of Thiamin and Riboflavin in Selected Foods by High-Performance Liquid Chromatography. *Journal of Food Science*, *47*(6), 2048-2050. AOAC (2005) 18th edition, 942.23 J. Food Comp. and Analysis (1989) Vol. 2(1) 41.

Gregory III, J. F., & Toth, J. P. (1988). Chemical synthesis of deuterated folate monoglutamate and in vivo assessment of urinary excretion of deuterated folates in man. *Analytical biochemistry*, *170*(1), 94-104.

Huang, S. H., & Ng, L. T. (2011). An improved high-performance liquid chromatographic method for simultaneous determination of tocopherols, tocotrienols and γ-oryzanol in rice. *Journal of Chromatography A*, *1218*(29), 4709-4713.

Ivarsen, E., Andersen, C. P., Jensen, S. M., Pedersen, C. T., & Svaneborg, A. K. (2021). Quantification of retinyl palmitate, thiamine, niacin, pyridoxine, folic acid, cyanocobalamin, zinc, and iron by chromatographic methods in fortified kernels and fortified rice. *Acta Chromatographica*, *33*(2), 145-152.

Jannasch, A., & Wang, Y. J. (2020). Development of a limited-water soaking method on the fortification of rice with calcium and iron by parboiling. *Journal of Cereal Science*, *94*, 103014.

Jiang, S. L., Wu, J. G., Feng, Y., Yang, X. E., & Shi, C. H. (2007). Correlation analysis of mineral element contents and quality traits in milled rice (Oryza stavia L.). *Journal of Agricultural and Food Chemistry*, *55*(23), 9608-9613.

Khamila, S., Sila, D. N., & Makokha, A. (2020). Compliance status and stability of vitamins and minerals in Fortified Maize Flour in Kenya. *Scientific African*, *8*, e00384.

Kuong, K., Laillou, A., Chea, C., Chamnan, C., Berger, J., & Wieringa, F. T. (2016). Stability of Vitamin A, iron and zinc in fortified rice during storage and its impact on future national standards and programs—Case study in Cambodia. *Nutrients*, *8*(1), 51.

Kurek, M. A., Wyrwisz, J., Karp, S., & Wierzbicka, A. (2017). Particle size of dietary fiber preparation affects the bioaccessibility of selected vitamin B in fortified wheat bread. *Journal of cereal science*, *77*, 166-171.

Launay, B., & Lisch, J. M. (1983). Twin-screw extrusion cooking of starches: flow behaviour of starch pastes, expansion and mechanical properties of extrudates. *Journal of Food Engineering*, *2*(4), 259-280.

Lee, J., Hamer, M. L., & Eitenmiller, R. R. (2000). Stability of retinyl palmitate during cooking and storage in rice fortified with Ultra RiceTM fortification technology. *Journal of Food Science*, *65*(5), 915-919.

Losso, J. N., Karki, N., Muyonga, J., Wu, Y., Fusilier, K., Jacob, G., ... & Greenway, F. L. (2017). Iron retention in iron-fortified rice and use of iron-fortified rice to treat women with iron deficiency: A pilot study. *BBA clinical*, *8*, 78-83.

Md Noh, M. F., Gunasegavan, R. D. N., Mustafa Khalid, N., Balasubramaniam, V., Mustar, S., & Abd Rashed, A. (2020). Recent techniques in nutrient analysis for food composition database. *Molecules*, *25*(19), 4567.

Migliozzi, M., Thavarajah, D., Thavarajah, P., & Smith, P. (2015). Lentil and kale: Complementary nutrient-rich whole food sources to combat micronutrient and calorie malnutrition. *Nutrients*, *7*(11), 9285-9298

Nakos, M., Pepelanova, I., Beutel, S., Krings, U., Berger, R. G., & Scheper, T. (2017). Isolation and analysis of vitamin B12 from plant samples. *Food Chemistry*, *216*, 301-308.

Osseyi, E. S., Wehling, R. L., & Albrecht, J. A. (1998). Liquid chromatographic method for determining added folic acid in fortified cereal products. *Journal of Chromatography A*, *826*(2), 235-240.

Pfeiffer, C. M., Rogers, L. M., & Gregory, J. F. (1997). Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. *Journal of Agricultural and Food Chemistry*, *45*(2), 407-413.

Reynolds, S. L., & Judd, H. J. (1984). Rapid procedure for the determination of vitamins A and D in fortified skimmed milk powder using high-performance liquid chromatography. *Analyst*, *109*(4), 489-492.

Rimkus, G. (2021). Verifying laboratory performance and quality control in the context of micronutrient testing of fortified food: a guidance document. *Global Allianc e for Improved Nutrition (GAIN), Geneva, Switzerland*.

Sáez Vigo, R. (2019). Determination of almond adulterations with hazelnut and peanut by HPLC-UV and HPLC-FL fingerprinting, and multivariate calibration methods.

Said, H. M. (2015). Water-soluble vitamins. *Nutrition for the primary care provider*, *111*, 30-37.

Segawa, A. (2009). *Analysis of a Tropical Banana Juice and Effects of Heat Processing Using HPLC*. University of Johannesburg (South Africa).

Shahzad, Z., Rouached, H., & Rakha, A. (2014). Combating mineral malnutrition through iron and zinc biofortification of cereals. *Comprehensive Reviews in Food Science and Food Safety*, *13*(3), 329-346.

Shrestha, A. K., Arcot, J., & Paterson, J. L. (2003). Edible coating materials—their properties and use in the fortification of rice with folic acid. *Food Research International*, *36*(9-10), 921-928.

Steiger, G., Müller-Fischer, N., Cori, H., & Conde-Petit, B. (2014). Fortification of rice: technologies and nutrients. *Annals of the New York Academy of Sciences*, *1324*(1), 29-39.

Thiex, N., Smallidge, R., & Beine, R. (1996). Sources of error in vitamin A analysis. *Journal of AOAC International*, *79*(6), 1269-1276.

Wieringa, F. T., Laillou, A., Guyondet, C., Jallier, V., Moench-Pfanner, R., & Berger, J. (2014). Stability and retention of micronutrients in fortified rice prepared using different cooking methods. *Annals of the New York Academy of Sciences*, *1324*(1), 40-47.

Wiley, K. D., & Gupta, M. (2019). Vitamin B1 thiamine deficiency (beriberi).

Zgoła-Grześkowiak, A., & Grześkowiak, T. (2011). Dispersive liquid-liquid microextraction. *TrAC Trends in Analytical Chemistry*, *30*(9), 1382-1399.

Chapter 3 - Accelerated Shelf-life study of fortified rice: Evaluating micronutrient retention in different packaging options

Abstract

Fortified rice is a primary means for delivering food aid internationally and to address micronutrient-deficiency. In this project, an accelerated shelf-life study was conducted for fortified rice stored in three different types of packaging to evaluate micronutrient retention, using three different controlled storage conditions (temperatures).

Shelf-life testing can be a prolonged exercise especially if the product has a storage life of months or even years, which is often true for low moisture foods. Accelerated shelf-life testing is common for determination of shelf-life of products in a relatively short period of time and was used in this study for fortified rice packaged in different types of bags.

Fortified rice kernels (FRK) in concentrated form produced using coating technology were mixed in a ratio of 1:100 with regular rice to produce fortified rice and packaged in woven poly propylene (WPP), laminated woven poly propylene (LWPP) and a new multi-layer hybrid bags (10 kg in size) and placed in 3 different accelerated storage conditions (27°C, 33°C and 43°C at 60%RH) and key attributes micronutrient attributes (Vitamin A, Vitamin B1, Folic acid, Iron and Zinc) and microbial load (yeast and mold) were determined at regular intervals over a period of 6 months. A descriptive sensory analysis panel was also used to characterize aroma and brittleness of products over time.

The United States Agency for International Development (USAID) standard concentrations for micronutrient fortified rice (per 100g) are 500 IU, 0.5 mg, 0.13 mg, 4 mg and 6 mg, respectively, for vitamin A, vitamin B1, folic acid, iron and zinc. Maximum deviation from standards was observed in Vitamin A over the period of time especially in traditional

packaging (WPP). Minerals results were relatively consistent throughout the accelerated shelf-life period in all 3 packaging and storage conditions. Sensory results showed significant change in aroma in all 3 packaging and at the extreme storage condition (43°C). Hybrid packaging bags were similar or better than other packaging options for retention of micronutrients and sensory attributes and minimizing microbial load in fortified rice. Data were fitted to the Arrhenius model to determine Q10 factor and extrapolated to find shelf-life at target ambient temperature.

This work has significant operational significance for food aid in general. A detailed protocol was developed for shelf-life testing for packaging fortified rice and results will help in understanding gaps in current packaging and transition to new more effective packaging.

3.1 Introduction

Rice (Oryza sativa) is one of the leading staple foods for more than three billion people across the globe due its diverse nutritional properties including fiber, energy, minerals, vitamins and other biochemicals (Burlando & Cornara, 2014) (WFP nutrition division). Rice is enriched with micro and macro nutrients but during milling process most of the micronutrients are removed to produce white rice which is rich in starch. (Steiger et al 2014). Top ranked countries as per World population review 2021 are China, India, USA, Indonesia, and Pakistan for the production of rice. 90% of rice is produced and consumed in Asian countries. In some countries including Bangladesh, Cambodia ad Myanmar, rice contributes as much as 70% of daily energy intake. (WHO program Nutrition division, Italy). Not only Asian countries but also, it's a very important staple food in several other countries including African countries and America. Milled rice is good source of energy but not sufficient for micronutrients. Unpolished rice is considered as good source of vitamin B1, B6, E, and niacin. During polishing of rice, the majority (75-90%) of these vitamins are removed.

Fortification is the practice of deliberately increasing the content of an essential micronutrient i.e., vitamins and minerals (including trace element) in the food, to improve the nutritional quality of the food supply and provides a public health benefit with minimal risk to health. (WHO). As rice is largely consumed across the world, it is potentially an excellent medium for delivering micronutrients to a large population to significantly improve micronutrient deficiencies. Based on available evidence of efficacy, stability and needs, certain micronutrients are recommended for rice fortification including minerals (iron and zinc) and vitamins A, B1 (thiamin), B3 (niacin), B6 (pyridoxine), B9 (folic acid) and B12 (cobalamin). However, these micronutrients deteriorate with time in any fortified food form depending on their type, matrix in which they are embedded, storage conditions including temperature and humidity, and packaging. Micronutrient deterioration is an important factor that determines the shelf life of fortified foods.

3.1.1 Accelerated Shelf-life Testing (ASLT) and the Arrhenius Model

Standard shelf-life studies can be a prolonged exercise especially if the product has a storage life of months or even years, which is often true for low moisture foods. They can also be cumbersome, costly, and even impractical if information on deterioration kinetics is needed over a range of storage conditions.

Accelerated shelf-life testing (ASTL) is common for determination of shelf life of products in a relatively short period of time. ASLT also allows an understanding of changes in rate of deterioration with variation in storage conditions, primarily temperature, thus proving to be a useful and flexible predictive tool that minimizes the need for excessive experimentation (Labuza & Schmidl, 1985).

This and the following sections summarize the basic concepts and theory of ASLT and deterioration kinetics in food products in general and how it has been applied for determining shelf life of a variety of foods in different storage conditions. Also, a survey of studies is provided that are in particular related to shelf life of rice and changes in micronutrient retention with time. The primary aim was to establish accepted practice in scientific literature pertaining to protocols for ASLT testing and shelf-life determination of food products that can be applied to micronutrient retention (and microbial load) kinetics during prolonged storage of fortified rice in varying conditions, which was the overall goal of this study.

The basic process of ASLT involves the following steps (Labuza & Schmidl, 1985; Choi et al., 2017) – i) selection of key kinetically active elements or physico-chemical attributes that determine the shelf-life of a product, ii) conducting a kinetic study or storage experiment under controlled and elevated conditions (usually temperature) that result in a sufficient rate of deterioration over a limited period of time, and iii) extrapolation of results to real or normal storage conditions to be able to calculate the actual shelf life of the product.

In order to successfully extrapolate (step iii) it is essential to use a sound thermodynamic model that can correctly describe the deterioration kinetics and a select set of conditions (temperatures) that give sufficient data points for model fitting.

The classic model that is used by food scientists is based on the Arrhenius equation as described by Labuza (1982; 2000) and has been applied widely for a range of products and conditions. This approach first (Step 1) uses plots with respect to time of the selected kinetic parameter or physico-chemical attribute (say, folate concentration or rancidity score determined by sensory analysis) as obtained experimentally at different temperatures (Ganje et al., 2016; Choi et al., 2017). The reaction order is determined using statistical analysis of the time versus

score/ concentration data and can be verified in a less vigorous fashion by visual inspection of the plots.

As a next step (Step 2), the data at each temperature is fitted to the appropriate kinetic equation based on the reaction order. For example, in many cases changes in physico-chemical attributes of food such as folate content (y) with respect to storage time (t, in days) fits zero-order kinetics the best. In that case the appropriate equation will be:

$$y = y_o - k_T t \qquad (1)$$

where, y_0 and k_T are found by best fit using linear regression and represent the value of the physico-chemical attribute (example, folate content) at the start of storage (t = 0) and the reaction rate or rate constant, respectively. On the other hand, for microbial growth, the best fit equation could very well follow first-order kinetics. So, care should be taken in determination of the reaction order as explained in Step 1.

As Step 3, the reaction rate (K_T) at various temperatures (determined in Step 2) is fitted to the Arrhenius equation, which models the temperature dependence of

$$K_T{:}k_T = A \exp(-E_a RT) \qquad (2)$$

or,

$$\ln k_T = \ln A - E_a RT. \qquad (3)$$

where, E_a, R, T and A are activation energy of the deterioration reaction (J mol^{-1}), universal gas constant (8.314 J $mol^{-1}K^{-1}$), absolute temperature (K) and pre-exponential factor (day^{-1}), respectively. E_a and A for the temperature range in question are thus found by statistical fitting or regression.

Step 4 involves the determination of the Q10 factor between any two temperatures separated by 10°C or 10K within or close to the temperature range of the study, defined as the relationship of the reaction rates at T+10 and T, as follows (Labuza & Schmidl, 1985; Al-Kadamany et al., 2003; Choi et al., 2017):

$$Q10 = k_{T+10}/ k_T \qquad (4)$$

$$Q10 = \exp [10\ E_a/RT(T+10)] \qquad (5)$$

This way, once the activation energy of the deterioration reaction is determined in Step 3, the Q10 factor can be found between any two temperatures separated by 10°C. Typically, the Q10 factor for many physico-chemical attributes of food products during storage lies between 2 and 3 (Choi et al., 2017). In the absence of kinetics data at different temperatures, often a factor of 2 is assumed for the estimation of real time shelf life from ASLT data at a given elevated temperature (Sewald & DeVries, 2003; Chanadang & Chambers, 2019).

As a final step (Step 5), the shelf life (in days, t*) at the actual storage temperature (T*) or the real time shelf life of the product can be found by extrapolation. A cut-off score (y*) related to the physico-chemical attribute or kinetically active element is set to mark the end of shelf life of the product (Cordova et al., 2011; Choi et al., 2017), as the point beyond which the product is considered to be spoiled, contaminated or of sub-standard nutritional quality (minimum folate content, as an example). The reaction rate k_{T*} at the storage temperature is calculated by extrapolation using equation (4) and an estimated Q10 factor (for example, 2) in the absence of temperature kinetics data; or otherwise calculated using equations (3.4) and (3.5) together or directly using equation (3.3). Once k_{T*} is known, the kinetics equation or equation (3.1) is used to calculate the corresponding shelf life t* in days.

3.1.2 ASLT studies for food products

A summary of a few key ASLT studies for food products is provided in this section to illustrate the principles described above. The reviewed articles include products such as grain sorghum-based fortified blended foods used in food aid, hazelnuts, carbonated fruit drink, chili sauce, instant noodles, mango soy fortified yogurt powder, concentrated yogurt, tomato paste, and ready to eat products such as dried apple snacks, semi-dried persimmons and ready-to-eat salad with cereals, tuna and chicken. A range of storage conditions were used in these studies, and different physico-chemical attributes or kinetically active elements were monitored as a determinant of product quality and shelf life as summarized in Table 3.1.

ASLT was conducted for instant noodles made with flour fortified with different iron compounds at three elevated temperatures (30°C, 35°C & 40°C) and predicted shelf life using the Arrhenius model was compared with real time storage at 25°C-28 °C **(**Ong, 2015). Kinetic attributes that were monitored included iron content, peroxide value, free fatty acids, pH, moisture, color and sensory characteristics of appearance, taste and flavor. Due to inadequate separation of temperatures significant change in many of these attributes with respect to time was not found, and differences due to temperature were not observed in many cases. The oxidative stability as peroxide value POV of the raw and roasted hazelnuts was estimated using ASLT at elevated temperatures (55°C, 65°C and 75°C) at water activity (aw) of 0.43. In addition, the samples were maintained for 8 months in real storage or long-term shelf-life testing conditions of 20°C–30°C

for validating the results obtained from short-term ASLT **(**Shafiei et al.,2020). The Arrhenius model was used for prediction of shelf life from ASLT, which resulted in good correspondence with real time studies. However, there can be another pitfall or limitation related to improper

selection of storage conditions when temperature range for ASLT differs substantially from the real time storage temperature. Physicochemical properties, microbiological changes and sensory attributes were studied by (Choi et al.,2017) using acceleration experiments for semi-dried persimmon at -10, 0 and 10^{o}C and comparison with long term or real time storage at -20^{o}C. Arrhenius model was used for prediction of shelf life. The deterioration kinetics at -20^{o}C was found to be dissimilar due to phenomenon of sugar crystallization at the surface of the persimmons, which was not found at the ASLT temperatures. Similar mismatch between ASLT and real time can occur due to other phenomena such as phase changes, glass transition, etc. Therefore, care should be taken to select the temperature range for ASLT such that real time storage temperature is reasonably close to the ASLT range. However, this is difficult to achieve with use of several ASLT temperatures while maintaining a good separation (10^{o}C or more), besides the obvious drawback of associated costs and complexity (Chanadang & Chambers., 2019).

Some studies use just use one ASLT temperature, but the drawback of that approach is that the temperature kinetics is hard to establish, and prediction of shelf life relies on assumption of a value for the Q10 factor rather than its calculation from actual data. (Phan et al.,2014) and (Chanadang & Chambers., 2019) evaluated shelf-life study of fortified blended foods of FBFs based on grain sorghum in real time at 30°C and 65% relative humidity (RH) and compared with shelf life as determined by ASLT at 50°C and 70% RH. For the latter, a Q10 factor of 2 was assumed. Descriptive sensory attributes of aroma and flavor of the FBFs after cooking into porridge were used as kinetic parameters for shelf life. A reasonable correspondence was found between the predicted shelf life using ASLT and the actual shelf life determined in real time, with a few exceptions where deterioration kinetics of the products might have deviated due to

interaction of severe extrusion processing conditions, presence of exceptionally high level of lipids and/or natural and added antioxidants in the FBFs. An ASL study on carbonated beverage drinks was conducted using two elevated storage conditions (14ºC and 40ºC; 90% RH) (Hemanth et al., 2020) to predict shelf life during real-time storage. The Arrhenius model and the Q10 factor was employed for this purpose. In a study to evaluate the potentiality of infrared spectroscopy (FT-IR) in food analysis during storage (Rodiles-López et al., 2020), an accelerated shelf-life study was carried out on three types of chili habanero sauces at only one temperature (50°C) for a period of 24 days. FT-IR chemometrics were used to good effect as a rapid food quality and shelf-life evaluation tool in comparison with traditional measures such as pH, microbial load, and sensory characteristics. A Q10 factor of 2 was assumed to predict the shelf life at a storage temperature of 20ºC, from the ASLT data obtained at 50ºC, with the obvious limitation of such an assumption. The shelf life of mango soy fortified yoghurt powder packed in high density polypropylene and aluminum laminated polyethylene pouches was determined and compared based on free flowness of product under a single accelerated storage condition (38±1ºC, 90%RH) **(**Kumar & Mishra, 2004). Quality parameters free fatty acid, thiobarbituric acid and hydroxymethyl furfural contents, starter count and color change in both packaging were also monitored. The kinetics of quality parameter change was found to be of zero order.

Usually use of two ASLT temperatures, besides the real time temperature is a sound approach that allows a good estimation of shelf life. An example of this is the ASLT study by (Cordova et al., 2011) for dried apple snacks. Two elevated temperatures (25°C and 35°C) were used for ASLT and predicted shelf-life results using Arrhenius model were compared with real time storage at 18ºC. First order deterioration kinetics was observed for all conditions. Differences in shelf life between the temperatures were not found indicating high stability of the

product. Similarly, (Ganje et al., 2016) successfully employed ASLT at two temperatures (40°C and 50°C) and the Arrhenius equation to predict the shelf life of tomato paste containing microencapsulated and encapsulated olive oil extract and compare with real time shelf life at 30°C. In another study, shelf life of concentrated yogurt or labneh was adequately determined using ASLT at 15°C and 25 °C and the Arrhenius equation and compared with real time storage shelf life at 5°C (Al-Kadamany et al., 2003). An ASLT study was conducted on ready to eat salad with cereal, tuna and chicken packed in thermos-sealed trays at 3 different temperature conditions (12°C,18°C and 25°C), it order to predict shelf in real time storage at 4°C **(**Haouet et al., 2018). Samples were assessed for sensory characteristics (texture, color, odor, and aroma), total basic volatile nitrogen or TVB-N as measure of protein degradation or proteolysis to amines and ammonia and pH as a measure of fermentation. The Arrhenius model was used to determine the shelf life.

3.1.3 Shelf-life studies for Rice

There are not many studies reported on shelf life of rice (regular or fortified). A few are summarized in Table 3.1. A real-time shelf-life study (8 months of storage) was conducted for brown rice stabilized for controlling rancidity using three different treatments - dry heat at 60°C, wet heat using steam and microwaving at 800 watts - and different exposure times (Bergonio et al., 2016). In another study focused on improving the shelf life of brown and rough rice (Ding et al., 2015), different drying techniques were compared (infrared, hot air and ambient drying). Infrared treatment was found to be the most effective out of these as it helped to inactivate the lipase enzyme and extend the shelf life of rice. This was also a real-time study conducted at 35°C for a period of 10 months. Other studies were related to rice-based products not rice itself. A real-time study (30°C, 70-85%RH) was conducted (Sirpatrawan., 2009) to study the effect of two

different packaging materials on shelf life of rice cracker with varying fat and initial moisture content. (Kurniadi et al., 2017) conducted a shelf-life analysis of canned-fried rice products sterilized at different conditions (121^{o}C, 15 and 20 min) and stored at elevated temperatures for an ASLT study (35 ^{o}C, 45 ^{o}C and 55°C for 35 days). The Arrhenius model was used to arrive at the shelf life at a storage temperature of 30^{o}C. This effort was also focused on a processed and ready-to-eat rice product, not for rice itself.

Table 3.1. Summary of ASLT studies for a range of foods products in peer-reviewed literature

Product	Temperature/RH (RT)	Temperature/RH (ASL)	Attribute	Reference
Instant noodles	25-28^{o}C; 75-80%RH	30^{o}C, 35^{o}C & 40^{o}C; 80%RH	Iron content, peroxide value, free fatty acids, pH, moisture, color, sensory (appearance, taste & flavor)	Ong, 2015
Hazelnuts	20–30 ^{o} C	55^{o}C, 65^{o}C & 75^{o}C	Peroxide value (PV), para-anisidine value, and total oxidation value	Shafiei et al.,2020
Semi-dried persimmons	-20^{o}C	-10^{o}C, 0^{o}C & 10^{o}C	Color, microbial load, tannin content, total solids and sugar concentrations	Choi et al.,2017
Grain sorghum-based fortified blended foods	30^{o}C; 65% RH	50^{o}C; 70%RH	Aroma & flavor after cooking	Phan et al., 2014
Grain sorghum-based fortified blended foods	30^{o}C; 65% RH	50^{o}C; 70%RH	Aroma & flavor after cooking	Chanadang & Chambers, 2019
Carbonated fruit drink		14^{o}C & 40^{o}C; 90%RH	Total soluble solids, pH, carbonation, color, appearance, taste, flavor, overall acceptability	Hemanth et al., 2020
Chili sauce		50^{o}C	pH, total acidity, total soluble acid & total solids, color, odor, texture, taste	Rodiles-López et al., 2020

Mango soy fortified yoghurt powder in two different packagings		38±1°C; 90%RH	Flowability, free fatty acids, thiobarbituric acid, hydroxymethyl furfural, starter cuture count, color	Kumar & Mishra, 2004
Dried apple snacks	18°C	25°C & 35°C	Water activity, moisture, SO_2 content, instrumental color, sensory (taste, color, aroma & texture)	Cordova et al., 2011
Tomato paste with olive oil extract	30°C	40°C & 50°C	Color, pH	Ganje et al., 2016.
Concenrated yogurt (Labneh)	5°C	15°C & 25°C	Lactic acid bacteria and other microbial, pH, sensory	Al-Kadamany et al., 2003
RTE salad with cereals, tuna & chicken		12° C, 18°C & 25°C	Sensory characteristics (texture, color, odor and aroma),total basic volatile nitrogen, pH	Haouet et al., 2020

Table 3.2. Shelf-life studies for rice and rice products.

Product	Shelf-Life Study Mode	Treatment	Reference
Brown Rice	Real time	Dry heat, wet heat and microwave heat	Bergonio et al., 2016
Brown and rough rice	Real time	Infrared, hot air and ambient drying	Ding et al., 2015
Rice crackers	Real time	Polyethylene and polypropylene packaging	Sirpatrawan, 2009
Canned fried rice	Accelerated or ASLT (35, 45 and 55°C)	Sterilization (121°C, 15 and 20 min)	Kurniadi et al.,2017

3.1.4 Micronutrient Retention Studies for Rice

A few studies have been conducted for micronutrient retention in rice during storage, as summarized in Table 3.3. Multiple fortified quick cooking rice was packed and heat-sealed in 3 types of packaging: (commercial oriented polypropylene/polyethylene, metallized polyethylene terephthalate/polyethylene and laminated aluminum foil/ oriented polypropylene/ polyethylene) and a shelf-life study was conducted in real-time conditions under fluorescent light at 40°C for 3 months. Rancidity was lower in laminated aluminum bags as compared to other packaging (Porasuphatana et al., 2008). Attributes such as sensory quality, lipidoxidation-thiobarbituric acid reactive substances (TBARS), water activity, moisture content, instrumental color, as well as vitamin and mineral content were monitored for determining the shelf life. Another study focused on ASLT for multiple-fortified extruded Ultra Rice^{TM} formulations for developing a shelf stable premix containing iron, zinc, and B vitamins (Li et al., 2008). A single accelerated condition of 40°C and 60% RH was used for storage over a period of 32 weeks to understand the effect of iron sources on micronutrient retention, oxidative stability, and sensory and physical properties. Many other studies on fortified rice are related to micronutrient efficacy and/ or absorption, not storage stability. Although description of these studies is beyond the scope of this

review, one example is Kuong et al. (2019) focusing on improvement of serum zinc and folate concentration in close to 2000 Cambodian school children through feeding multi-micronutrient fortified over a period of 6 months.

Table 3.3. Micronutrient retention studies for rice

Product	Shelf-Life Study Mode	Treatment	Reference
MFQCR (Multiple fortified quick cooking rice) *Sprayed with fortificant solution	Real time	Commercial OPP, metalized polymer and aluminum foil and polymer lamination packaging	Porasuphatana et al., 2008
Multiple fortified Ultra Rice™	ASLT (40°C, 60% RH)	Four iron sources	Li et al., 2008
Fortified Rice (coated and Extruded)	Real time	25±5 °C at 60%RH & 40±5 °Cat 60%RH	Kuong et al., 2016

3.1.5 Types of rice fortification

Shelf life of fortified rice depends on the fortification technology and how the micronutrients are embedded in the rice matrix. There are three different ways/ technologies for the addition of micronutrients added to rice for fortification purpose – dusting, extrusion, and coating. Dusting technology is used for bulk rice directly by rice millers. On the other hand, the extrusion and coating technologies are used to prepare fortified rice kernels (FRK) with concentrated micronutrient levels, which in turn are added to bulk rice in a specified ratio (for example, 1:99) in a separate operation by the rice millers before packaging.

3.1.5.1 Dusting technology

During dusting, micronutrients in the form of fine particles are blended with the bulk rice. This method makes use of the electrostatic forces between the rice surface and the micronutrients (Steiger et al 2014). This technology observed only in the U.S. Consumers are

advised that rice fortified with powdered premixes should not be rinsed before or after cooking nor should the rice be cooked in excessive amounts of water and then drained.

3.1.5.2 Extrusion

Both hot and cold extrusion is used for rice fortification. In the hot extrusion process, rice flour is used which is typically obtain from broken rice kernel or poor-quality rice. The rice flour is mixed with micronutrient fortificant mix, water, binding agents (if needed) and emulsifiers. The extrusion process requires high temperature (70°C-110°C) with low shear, resulting in a product close to natural rice (sheen, transparency, consistency, and flavor). Single screw or twin-screw extruder could be used for hot extrusion, in which the starch gets partly or fully gelatinized due to high pressure, shear and heat during the extrusion. Thermal energy via steam preconditioning and/ or heated barrel jackets can contribute to the cooking, besides the mechanical energy generated by the extrusion screw (Alavi et al 2008). At the end of extruder, rice kernel shaped die orifices are used to give the final product resemblance of rice grain.

Cold extrusion technology is similar, except it utilizes a simple forming extruder also called a pasta press. It is primarily a low temperature (below 70^{o}C) and low shear, forming process resulting in grains that are uncooked, opaque, and easier to differentiate from regular rice kernels. It does not involve any additional thermal energy input before or during the process by preconditioning or heated barrel jackets and relies on the minimal heat generated during the lowe shear process itself (Steiger et al 2014).

3.1.5.3 Coating

In the coating method, ingredients such as waxes and gums are combined with the fortificant mix to create a liquid which is sprayed to the rice in several layers on the surface of grain kernels to form the rice-premix. The rice-premix is then blended with commercial rice for

fortification. The waxes and gums enable the micronutrients to stick to the rice kernel, thus reducing losses when the grains are washed before cooking, which is a common practice in developing countries. The final product is rice covered by a waxy layer; the color depends on the fortificants that are added. *Wright Enrichment Inc*. also uses a coating technology. This proprietary technology involves embedding the enrichment in micro perforations on the rice surface.

The current study focuses on accelerated shelf-life testing for micronutrient fortified rice based on the above-mentioned proprietary coating technology. Three different packaging technologies for rice (woven polypropylene, woven polypropylene with lamination, and multi-layer hybrid packaging) and three controlled storage temperatures were used to understand the kinetics of microbial load (mold and yeast) and micronutrient deterioration (vitamin A, vitamin B1, folic acid, iron and zinc). This information was used to predict the shelf life of packaged fortified rice fortified rice using ASLT theory as described earlier.

3.2. Methodology

3.2.1 Raw Material

The Fortified rice kernels (FRKs) were produced from coating technology and donated by *Wright Enrichment Inc*. (Crowley, LA) FRKs are referred as rice grain fortified with the micronutrient premix. Long Grain Whole Grain Milled White Rice was sourced from *Supreme Rice.* (Crowley, LA). Yellow no.5 color was used to color the FRKs only for study purpose.

3.2.2 Packaging Type

Three different types of packaging were used in this study. WPP (woven polypropylene), LWP (Laminated woven polypropylene) and Hybrid (combination of polyethylene liner and kraft paper. WPP bags were made of 100% polypropylene, white color with 10*10 weave. LWP

bags were made of 100% woven polypropylene with lamination of polypropylene/polyethylene blend with 2.5 mil thickness and clear color. Hybrid bags were PBOM (pinch bottom open mouth) bags consists of different layers including adhesive, coated white kraft, natural kraft, LLDPE (linear low-density polyethylene), HDPE (high density polyethylene). Physical properties include thickness of 17.7 mils, puncture resistance >600 grams. All bags were specially designed to fill 10 kg of fortified rice. WPP & LWPP bags dimensions were 10 x 4 x 21" whereas Hybrid bag dimensions were 11 x 3 x 22" due multilayers. Two-fold sewing was done for WPP & LWPP bags using sewing machine with white thread, whereas hybrid bags were heat sealed (3 min at 250°C) using heat sealer (Sealer sales W series 12" direct heat foot sealer, meshed seal, 15mm seal width). WPP and LWP bags were sourced by *JohnPac LAPAC Manufacturing Inc*. (Crowley, LA), whereas Hybrid bags were sourced by *ProAmpac* (Wapakoneta, OH)

All bags seem were sealed in a way which prevents the product from leaking during handling and storage.

3.2.3 Mixing and Packing

Fortified rice kernels (FRKs) were mixed with milled long grain rice (non-fortified rice) with the ratio of 1:100. A two-step mixing was done for each bag to make sure homogenous mixing in all samples. Each bag consists of 10kgs.

First 100g of FRKs were mixed in 1 kg of milled long grain rice for 30 seconds then it was further mixed with 9 kgs of milled rice using a Hobart mixer.

After homogenous mixing, samples were packed in respective packaging bags and the seam was closed via sewing for WPP & LWPP bags and heat sealing for Hybrid bags.

3.2.4 Storage Condition

The samples were placed in three different temperature conditions including ambient conditions targeting 25 ± 2.5°C and two elevated temperatures including 35± 2.5°C and 45± 2.5 °C with humidity of 55- 60% for all three temperatures. All storage conditions were closely monitored placing 2 onset HOBO (temperature and humidity monitoring device) in each room.

The elevated temperatures were selected to extrapolate results to real or normal storage conditions of warehouses and retailers. Each bag was marked clearly with the details including date stored, date due for analysis, temperature conditions and packaging type to avoid any confusion during sampling and analysis.

3.2.5 Sorting

Typically, in cereal and grains, sorting is considered as a quality assessment to remove / separate undesired seeds (Nitka et al., 2018). In this research the primary object of passing each sample to sorting process was to sort all the FRKs from fortified rice and then incorporate them into rice to individually prepare sample for micronutrient and microbial analysis with a guarantee of similar ratio for all samples.

After taking bags out of each storage conditions at respective time point, passed through a high-speed image-based sorter which was designed to detect and separate different grains based on color and texture. It mimics commercial sorters with a sorting accuracy of > 95 % with a throughput of ~ 25 kg/h of wheat. (Pearson, 2009). The sorting accuracy was > 90% with a throughput of ~ 20kg/h in case of fortified rice with three channels.

The sorted sample from image-based sensor were passed through a multispectral sorting device which has three visible and three near infrared light emitting diodes (LED) (Pearson et al., 2013). This LED sorter has better sorting accuracy as compared to camera sorter (high speed

image-based sorter). The sorting accuracy with fortified rice was >95%. Samples were then hand sorted to achieve 100% of sorting.

3.2.6 Micronutrient Analysis

3.2.6.1 Sample Preparation

To prepare 100g of sample, sorted FRKs were mixed again with milled rice from same bag with mixing ratio of 9:1 (90 g of milled rice with 10g of FRKs). This process was carefully performed for each sample to avoid any sampling errors.

After mixing, each sample was ground as per our internal validated grinding method, which is 30seconds of grind using a high-speed multifunctional Grinder (Moongiantgo Grain Grinder). Samples then carefully transferred to zip lock bags and then further packed with translucent bags to avoid sample exposure with light during storage and transportation.

All micronutrient and microbial analysis were outsourced and performed by a commercial lab. Here are the brief details of micronutrient and microbial method of analysis Vitamin A used a modified method AOAC 974.29 in which 10 g of samples were weighed into saponification flasks. Samples were saponified on a steam bath with reagent alcohol, potassium hydroxide, and an antioxidant (BHT). Samples were then cooled, hexane was added and then mixed. Phases were allowed to separate. The organic layer containing the vitamin A is decanted into a separatory funnel. This extraction process was performed a total of three times to ensure complete extraction. The organic collection in the funnels was rinsed with deionized water (DI) and finally filtered through sodium sulfate into volumetric flasks. For retinol, samples were diluted in hexane if necessary. A portion of the sample was transferred into an HPLC vial. Retinol samples were analyzed by HPLC with mobile phase consisting of isorpropaol and

hexane. The HPLC was equipped with a silica column and fluorometric detector (Ex 330 nm, Em 480 nm). (AOAC 974.29 Mod)

Vitamin B1 (Thiamin) modified AOAC 942.23 method for vitamin B1 analysis.1 g of samples were extracted in 0.1N HCl by autoclaving. Various forms of phosphorylated thiamine were then converted into free thiamine with an alpha amylase solution. Samples were diluted, centrifuged, and filtered, and then injected in a ultra-performance liquid chromatography (UPLC). After the peak separation on a C18 column, the eluent enters an oxidation loop where thiamine reacts with alkaline ferricyanide and was converted to a fluorescent derivative, thiochrome. Thiochrome was analyzed on a fluorescent emission detector (Ex 363 nm, Em 435 nm).

Folic acid analysis used modified method of AOAC 992.05.1 g of sample was autoclaved then cooked to room temperature. Creon capsules and chicken pancreas conjugase were added. Sample solution was then mixed with growth media and inoculated with L. Rhamnosus. After overnight incubation, the concentration of total folate in the sample is determined by reading the turbidity of the sample at 600 nm against that of a series of calibration standards. The amount of growth is directly proportional to the concentration of the analyte in the sample.

Iron and Zinc analysis used modified method from (AOAC 984.27 mod, 927.02 mod, 985.01 mod, 965.17 mod). Elemental Analysis by ICP uses Inductively Coupled Plasma Optical Emissions Spectrophotometry (ICPOES) to quantify iron and zinc in a variety of sample matrices.10 g of the sample was weighed into a crucible. It was then ashed in a muffle oven at 5500°C for greater than 5 hours. The ash then dissolved in mainly hydrochloric acid with a small amount of nitric acid while boiling on a hotplate. This solution was transferred to a volumetric

flask and brought to volume with deionized water. An appropriate dilution was performed, and the solution was introduced into the ICP-OES instrument. The emission signal was measured at 238.2 nm for iron and 206.2 nm for zinc. Calibration standards, drift control standards, and control samples were analyzed with each batch to ensure instrument suitability and acceptable results. The iron and zinc signal were adjusted by gallium internal standard recovery determined using the 294.4 nm wavelength. Measurements were computed by the ICP software (Winlab).

3.2.7 Microbial Analysis

Yeast and mold were analyzed as per FDA BAM Chapter 18 modified method, where samples aseptically pipetted into DRBC (dichloran rose Bengal chloramphenicol) agar. Plates were incubated at 25°C. Results were reported as colony forming units (CFU)/g (Kodaka et al., 2006)

3.2.8 Sensory Analysis

A descriptive sensory analysis was conducted at Kansas State University with 5 trained panelists on appearance, aroma, and texture of samples. Each panelist had more than 120 h of descriptive training, with expertise in testing grain products adapted from (Keane ., 1992)

For sensory evaluation, fortified rice samples were prepared (1:100 ratio) and randomly assigned a 3- digit code. Samples were served in a medium size glass snifter to each panelist in sequential monadic order. The evaluation was divided in three phases. In order to prepare the lexicon to be used, set of samples were reviewed by panelist on orientation day to establish the terminologies used to describe attributes, finalize the attributes with appropriate reference to be used along with samples. The descriptive terms and reference details are shown in Table 3.4

Total 12 attributes were analyzed by panelist which included uniformity of color, color intensity, brightness, translucent, starchy, grain, vitamin, straw, musty/dusty, sweet aromatics, nutty and factorability. All the attributes were scored based on 15-point scale (0= none to 15=

extremely high) with 0.5 increments. (Yoo et al., 2013). This scale has been widely used for a range of product and shown shared in (Yoo et al., 2013). All the samples were analyzed in duplicate. All sensory analysis were performed on uncooked rice only.

Table 3.4. Definition used for sensory analysis of fortified rice

Appearance	
Uniformity of Color	A measurement describing uniformity of color for rice grains (Yes/No).
Color Intensity	The intensity or strength of the white color from light to dark (where lower numbers indicate whiter is present in the sample than higher numbers). Reference: Porter Paint 6902 = 3.0 Porter Paint 6903 = 10.0
Brightness	The chroma (or purity) of the color, ranging from dull, muddied to pure, bright color. Reference: Argo corn starch in water=5.0 Preparation: Mix 1g corn starch in 100 mL water; serve a ¼ cup in a snifter.
Translucent	A measurement of describing translucency of rice grains. Reference: Clear water = 0.0 Argo corn starch in water = ___ Preparation: Serve water in a snifter. Mix 1g corn starch in 100 mL water; serve a ¼ cup in a snifter.
Aroma	
Starchy	The flat aroma note associated with raw or processed starch-based grain products such as wheat, rice, oats, and other grains. Reference: Argo corn starch in water = 3.5 Preparation: Mix 1g corn starch in 100 mL water; serve ¼ cup in a medium snifter.
Grain	The light dusty/musty aromatics associated with grains such as corn, wheat, bran, rice, and oats. Reference: Cereal Mix (dry) = 5.0 Preparation: Mix ½ cup of each General Mills Rice Chex, General Mills Wheaties, and Quaker Quick Oats. Put in a blender and "pulse" blend into small particles. Place 1 Tbsp in a medium snifter.
Vitamin	The aromatics associated with a just opened bottle of vitamin pills (generally thought to be oxidized thiamin) Reference: 1-Nature Made Super B-Complex = 10.0 Preparation: Crush one vitamin pill and place in a medium snifter.
Straw	Somewhat sweet, dry, slightly dusty aromatics with the absence of green; associated with dry grain stems. Reference: Straw = 7.0

	Preparation: Place 5g in a medium snifter
Musty/Dusty	Dry, dirt-like aromatic associated with dry, brown soil. Reference: Kretschmer Wheat Germ = 4.0 Preparation: Place 1tablespoon in a medium snifter.
Sweet Aromatics	An aromatic associated with the impression of a sweet substance. Reference: Nabisco Lorna Done Cookies Preparation: Crush 1 cookie and serve in a medium snifter.
Nutty	A combination of slightly sweet, brown, woody, oily, musty, bitter and astringent aromatics commonly associated with nuts, seeds, beans, and grains. Reference: Kretschmer Wheat Germ = 12.0 Preparation: Serve 1 tablespoon in a medium snifter.
Texture	
Fracturability	The force with which the sample ruptures. Evaluate on the first bite down with the molars. Reference: Corn nuts = ___ Raw almonds = ___ Preparation: Serve in 3.25oz cups.

3.2.9 Statistical Analysis

For sensory results, the descriptive panelists data were recorded using a paper ballot. XLSTAT ANOVA (analysis of variance) was used to determine the statistical significance of the mean (average) ratings. A p-value under 0.05 indicates there is a significant difference between the samples at the 5% level (or 95% confidence level). If a significant difference was identified, the Tukey's pair-wise comparison test was used to determine where the differences lay. Tukey's test results (as indicated by the letter to the right of the mean rating) show that for a sample, the attribute (i.e., color) of the rice is significantly different from the color of the other rice samples at the 5% level.

For ASLT data, a four-way analysis of variance (ANOVA) was performed by using the GLM procedure by statistical analysis software (SAS 9.4 Inst. Inc., Cary, NC). The four factors were the different micronutrients (5), time intervals for sampling (5), packaging type (3) and

storage temperature (3). Tukey's HSD (Honest Significance Difference) test was applied for the least-squares means separation, with significance considered at a probability $P<0.05$

3.2.10 Shelf-life estimation calculation using Arrhenius Model

Arrhenius model is a well-known model used for shelf-life estimation. (Van, 2008) (Syarief et al., 2020) Changes in quality attributes can follow zero order and first order reaction mostly. The rate of change can be described in general equation at concentration c where.

$$r = \frac{dc}{dt} = kc^{n} \qquad (1)$$

Where k is rate constant t is time, n is order of reaction
This equation can be integrated to obtain the concentration with respect to time

$$c^{1-n} = c_o^{1-n} + (n-1)\,kt \qquad \text{for } n \neq 1 \qquad (2)$$

$$c = c_o \exp(-kt) \qquad \text{for } n \neq 1. \qquad (3)$$

Where c_o is initial concentration and t equal to zero. Equation 2 if n=0, it will be

$$-\frac{dc}{dt} = k \qquad (4)$$

By Integration, the equation would be;

$$c = c_o - kt \qquad (5)$$

This equation is used for zero order reactions.
Mostly reactions in foods follow first order reactions where n=1, so the equation will be

$$\frac{dc}{dt} = kc \qquad (6)$$

After integrating the equation will be;

$$c = c_o \exp(-kt) \qquad (7)$$

Converting exponential equation into logarithmic form, it will be;

$$\ln c = \ln c_o - kt. \qquad (8)$$

Whereas c_o is initial concentration, c is concentration content at certain time, k is constant reaction rate (week^{-1}) and t is time (week).Ln c was plotted with time t using equation 8 to obtain k at each temperature and packaging

Now, Introducing Arrhenius equation to see the dependence of k on temperature

$$k = k_0 \exp(-\frac{Ea}{RT}) \qquad (9)$$

In linear form the equation will be

$$lnk = lnk_0 - \frac{Ea}{RT} \qquad (10)$$

Lnk vs 1/T data was fitted to equation 10 for each packaging
Where k_o is the reaction constant at temperature, Ea is activation energy (kJ mol^{-1}), R is gas constant (8,314 J mol^{-1} K^{-1}) and T is absolute temperature.

From equation10, best fit parameters of k reaction constant was determined at any given temperature of interest which was used to estimate shelf life using equation 16

$$ln\frac{c_0}{cl} = kt \qquad (11)$$

Where c_0 is initial concentration, cl is the lowest allowable critical limit to be recommended in the product and kt is reaction rate calculated from Arrhenius equation at each temperature.

3.3 Results and Discussion

3.3.1 Vitamin A

Vitamin A is a fat-soluble vitamin which is stored in liver. It's typically found in animal products such as meat, fish, poultry, and dairy products in the form of retinyl acetate or retinyl palmitate (Preformed vitamin A) whereas it's also found in plant-based foods such as vegetables and fruits in the form of beta carotene (Pro vitamin A). Vitamin A helps to form and maintain healthy skin, teeth, skeletal and soft tissues. It is also known as retinol because it produces the pigment in the retina of eye (Institute of medicine, food, and nutrition board, 2001). Vitamin A deficiency (VAD) is a common and leading public health problem which leads to blindness in

children, night blindness in pregnant women, severe infections, and risk of maternal mortality (World Health Organization, 2013).

Vitamin A as retinyl palmitate was measured at each time point in all packaging. Table 5 showing the retention in fortified rice at Week 0, 6, 13, 19 and 26 in WPP, LWP and Hybrid bags. High temperature drastically decreased the levels in all packaging bags. At 27°C, retention of retinyl palmitate was same in all packaging whereas some variations were observed for other time points like in WPP it was 61%, 78% in LWP and 75% in Hybrid bags at week 19.

At 33°C, in WPP & LWP it was 52% and slightly better for hybrid bags at 56% at week 19. At 43°C it was only 5% for WPP, 6% for LWP and Hybrid bags.

It has been reported earlier that vitamin A stays stable under inert atmosphere and drastic loses are expected at higher temperatures (Lešková et al., 2006). Khov et al., 2015 also mentioned significant losses during shelf-life study of fortified rice produced with different technologies (coated and extrusion). For fortified rice using coating technology the losses ranging from 88% to 93% at 6 months and 1 year respectively when stored at 40ºC & 75% humidity. Overages of vitamin A does not seem to be the solution because there is risk associated with high dose of vitamin A especially for kids. Vitamin A is highly sensitive to oxidation during storage and processing (Ball, 2005). Figure 3.1 showed results at each temperature in WPP, LWP and Hybrid packaging, Higher temperature 43ºC resulted higher degradation in all three packaging.

Table 3.5. Retention of Vitamin A (Retinyl palmitate), as percentage in different packaging and temperature conditions

Temp	Packaging	Week 0	Week 6	Week 13	Week 19	Week 26
27°C	WPP	100	89.0	86.7	61.6	69.6
	LWP	100	89.4	90.1	78.7	69.5
	Hybrid	100	93.9	82.1	75.8	62.9
33°C	WPP	100	86.5	76.8	53.0	39.1
	LWP	100	85.20	76.8	52.3	35.3
	Hybrid	100	86.7	72.6	56.8	47.2
43°C	WPP	100	45.8	14.3	5.5	1.9
	LWP	100	48.2	16.4	6.4	3.1
	Hybrid	100	50.3	20.4	6.5	4.7

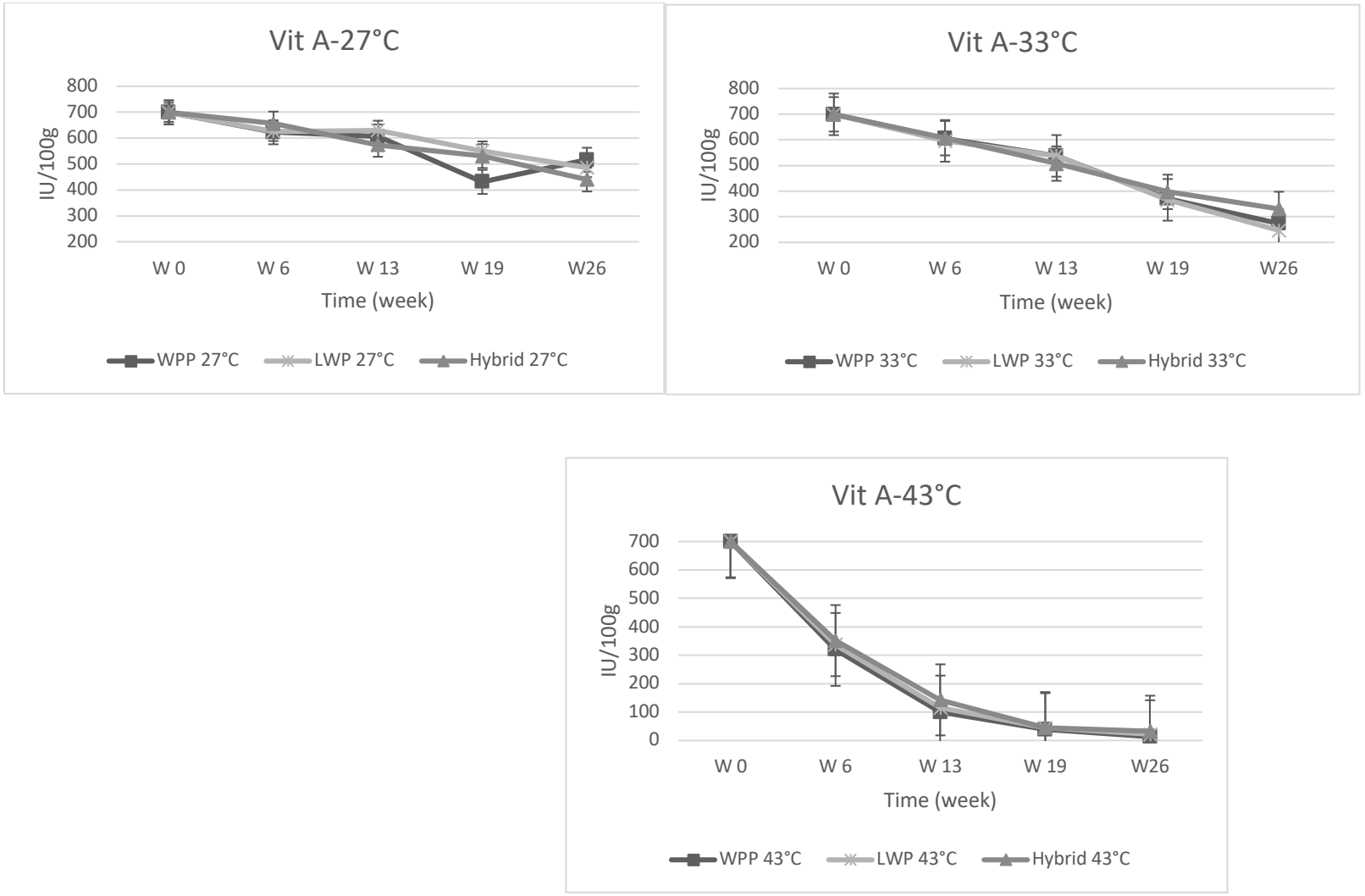


Figure 3.1. Results of vitamin A retention at 27°C, 33°C and 43°

3.3.2 Shelf-life estimation calculation using Arrhenius Model

Vitamin A was considered as one of the key indicators for the shelf-life study as significant reduction was observed over the time. First order kinetic was used in which (ln K) was plotted against storage time (measured in weeks) Figure3. 2, which gave rate constant at each temperature and packaging. In Table 3.6 rate constant increased as the temperature increase showing the impact of temperature, as vitamin A is very sensitive to temperature. After getting the rate constants, log of rate constant (ln K) was plotted against inverse temperature (1/T in Kelvin degree). Equation 10 (Arrhenius equation) was used to determine activation energy, this equation show the relationship between reaction rate and temperature, which shows dependency of k (reaction rate) on temperature (Anwar et al., 2019). Activation energy is listed in Table 3.7 WPP gave highest activation energy making it more susceptible to environmental conditions.

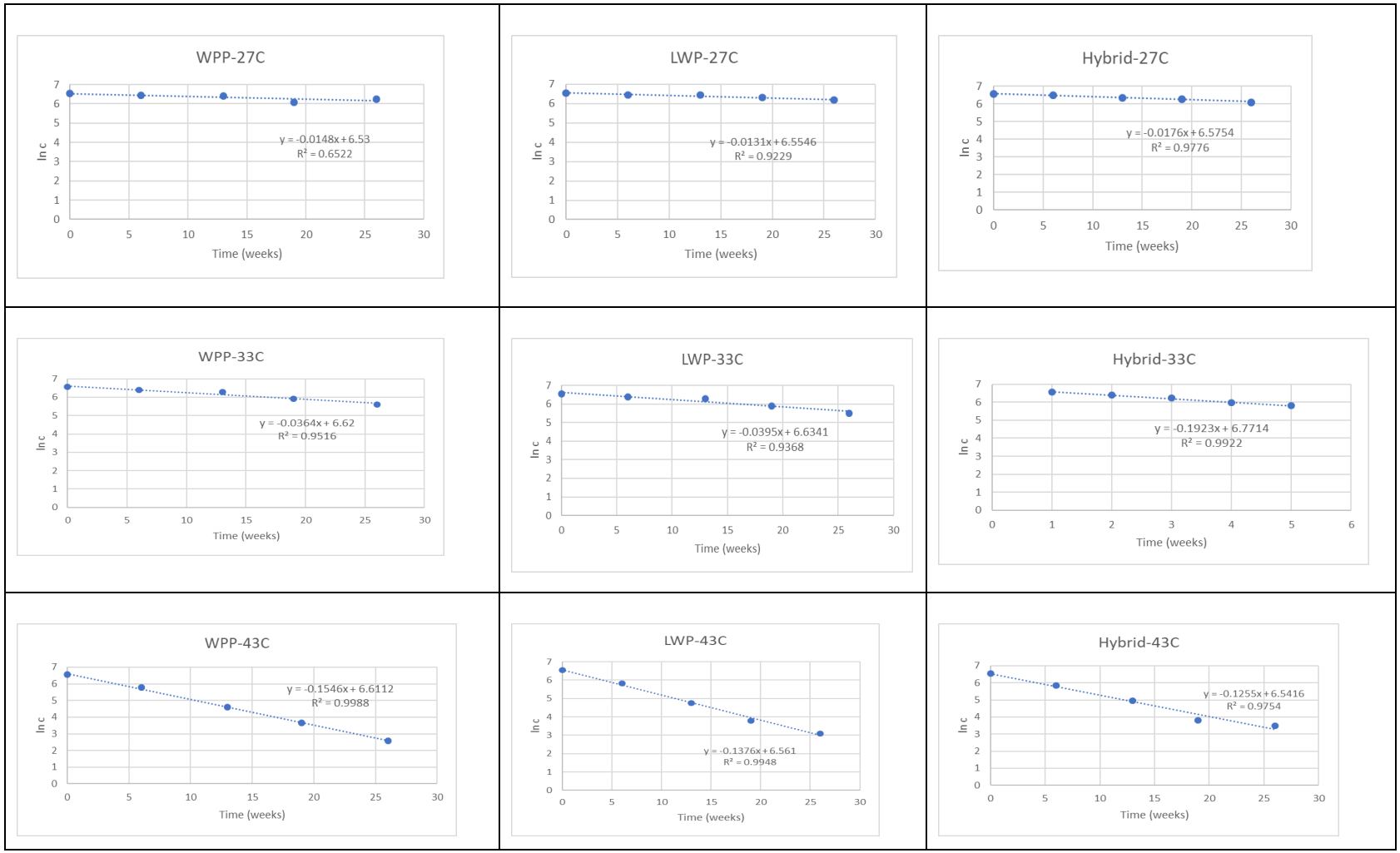


Figure 3.2. Retention of vitamin A during storage

Table 3.6. Rate constants of first order for Vitamin A under different storage temperature and packaging

	27°C			33°C			43°C		
	K (1/week*10^{-4})	c_0 (IU)	R2	K (1/week*10^{-4})	c_0 (IU)	R2	K (1/week*10^{-4})	c_0	R2
WPP	166	685.40	0.7511	364	749.95	0.9516	1,546	743.37	0.9988
LWP	131	702.47	0.9229	395	760.59	0.9368	1,376	706.98	0.9948
Hybrid	176	717.23	0.9776	296	715.44	0.9917	1,255	693.40	0.9754

Table 3.7. Constants and fitting rates of regression lines using Arrhenius equation

Packaging	Ea (kJ mol^{-1}	Ln K	R2
WPP	110601.142	40.192	0.9984
LWP	114550.292	41.648	0.9912
Hybrid	98620.668	35.382	0.9816

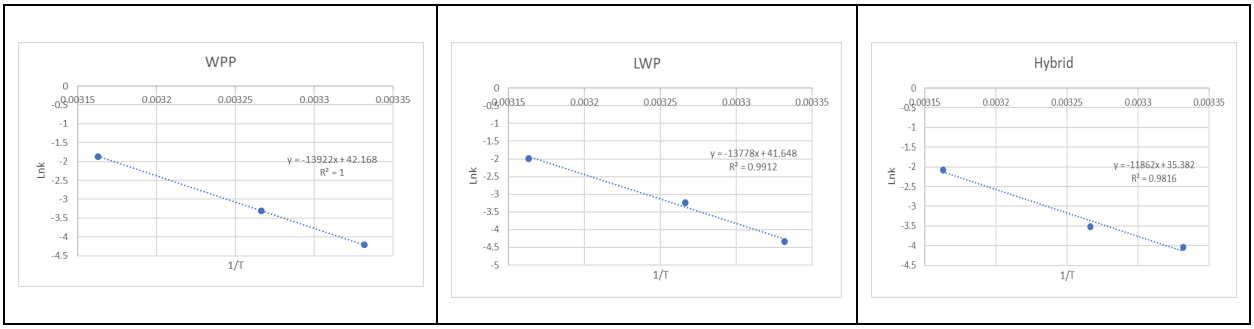


Figure 3.3. Arrhenius plot for vitamin A in different packaging using first order kinetics

Figure 3.3 shows Arrhenius plot for vitamin A. The slope of regression line gives values for Ea and Ln K. Higher Ea confirmed the vitamin A is highly affected in extreme temperature conditions. After getting the rate constant, shelf life for vitamin A was estimated for our targeted temperatures i.e 27°C, 33°C and 43°C as shown in Table 3.8 considering all packaging and temperatures. Great losses were seen at higher temperature irrespective of packaging.

Table 3.8. Shelf-life estimation for vitamin A in weeks at different temperature and packaging

Packaging	27°C	33°C	43°C
	Shelf-life estimation in weeks		
WPP	13.86	5.82	1.47
LWP	15.73	6.40	1.54
Hybrid	13.99	6.45	1.89

3.3.3 Vitamin B1

Vitamin B1 is a member of water-soluble family, also known as thiamin or thiamine. It is found in body via two main sources food which absorbs in small intestine and normal microflora of large intestine which absorbs in colon. It performs various function in body such as energy production, breakdown of carbohydrates, immune system activation, communication between brain and nerve cells and signaling or communicating between cells and tissues.

Retention of vitamin B1 was measured in all packaging and temperature conditions. At 27°C the retention was more than 88% in all packaging. At 33°C the retention was more than 85% in all packaging conditions whereas at 43°C the retention went 69.13% at week 19 with hybrid bags Table 3.9. Vitamin B1 and B2 stayed stable during storage study on freeze dried meal where samples were stored at 1°C, 30°C and 40°C for 6-, 12- and 24-months Coad et al.,2020. Another study on pediatric formulations containing high amounts of calcium were conducted to study vitamins (B1, B2, B6 and C). Vitamin B1 variation were not statistically significant under studied temperature (4°C & 25°C) (Riberio et al., 2011). Two salt forms of thiamine (Thiamine mononitrate and thiamine chloride hydrochloride) were studied under different PH and concentrations at 25°C, 40°C, 60°C, 70°C and 80°C to represent storage temperatures where reduction was observed at higher temperatures (Voelker et al., 2021).

Vitamin B1 as thiamine mononitrate showed 3- 13% of reduction after extrusion process. Water soluble vitamins like vitamin B is also considered sensitive to high temperatures and increase temperature during extrusion process can result in decrease of thiamin retention (Riaz et al., 2009).

Over the time, vitamin B1 did showed reduction but there was not any significant effect of packaging observed. Table 3.9 shows % retention at each temperature and packaging. Highest reduction was 33% at week 26 when stored in LWP at 43°C. When plotted using first order kinetic figure 3.4 showed a slight decreasing trend at 43°C. Table 10 showed rate of reaction was much smaller as compared to vitamin A (Table 3.6). At WPP at 27°C and LWP at 33°C, rate of reaction was positive so did not included in table.

Table 3.9. Retention of Vitamin B1 as percentage in different packaging and temperature conditions

Temp	Packaging	Week 0	Week 6	Week 13	Week 19	Week 26
27°C	WPP	100	88.4	100.5	93.9	92.1
	LWP	100	95.5	91.3	93	93.2
	Hybrid	100	100.1	99.0	91.6	87.0
33°C	WPP	100	95.7	102.6	85.7	82.7
	LWP	100	96.6	101.5	85.1	80.5
	Hybrid	100	93.9	104.5	90.6	81.1
43°C	WPP	100	83.9	89.0	73.2	70.5
	LWP	100	94.0	92.2	75.8	67.0
	Hybrid	100	90.4	90.6	69.1	70.3

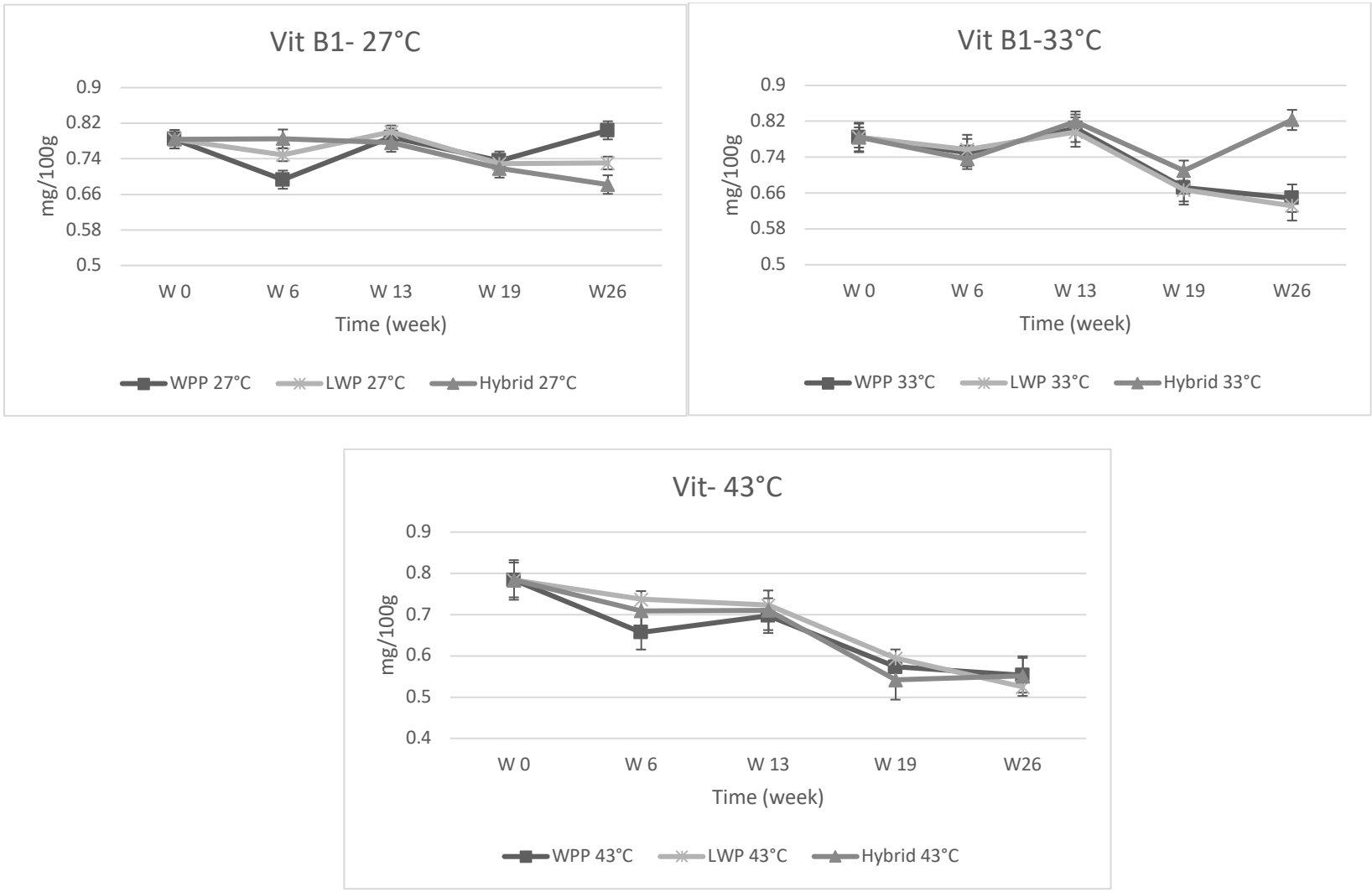


Figure 3.4. Retention of Vitamin B1 in different packaging and temperature conditions

Table 3.10. Rate constant for vitamin B1

Packaging	K (27°C) (1/week*10^{-4})	K (33°C) (1/week*10^{-4})	K (43°C) (1/week*10^{-4})
WPP	15	74	13
LWP	26	86	155
Hybrid	58	69	15

3.3.4 Folic Acid

Folic acid and folate are water soluble vitamins and belongs to vitamin B family and categorize as vitamin B9. Folate is a B vitamin that occurs naturally in foods such as green leafy vegetables, citrus fruits, and beans whereas folic acid is a synthetic form of folate which founds in supplements and added to fortified foods (enriched breads, enriched flours, enriched pasta, enriched rice, enriched corn meals, fortified corn masa flour, fortified breakfast cereals etc). Our body does not store folic acid that means we should regularly consume foods containing folate or add supplements to daily intake. Deficiency of folate causes anemia, growth retardation, problems during pregnancy which could affect child brain (anencephaly) and spine (spina bifida), cardiovascular disease, chronic disease, and increased risk of certain type of cancer (Dary & Hurrell, 2006) (Vitamins and minerals- USDA). Li et al., 2011 studied extruded fortified ultra-rice with vitamin A, Iron , vitamin B1 and folic acid, where folic acid was stable under high temperature and humidity (40°C, 60RH%). Fortified flour with folic acid were studied in different packaging bags at different temperatures where not significant losses of folic acid were not observed (Hemery et al., 2020). No significant losses in folic acid were noted while studying folic acid enriched corn masa flour, tortillas and chips during six months shelf-life study Phillips et al., 2017. Degradation of folic acid were found significant in fortified

vitamin juice (Frommherz et al., 2014). Further studies showed little or no loss for retention of folic acid in fortified breakfast cereals and vitamin- mineral premixes (Berry et al., 2010)

In our study no significant reduction noted during storage was found.

Table 3.11. Retention of Folic acid as percentage in different packaging and temperature conditions

Temp	Packaging	Week 0	Week 6	Week 13	Week 19	Week 26
27°C	WPP	100	77.5	76.9	87.3	92.5
	LWP	100	90.8	89.1	91.7	89.4
	Hybrid	100	90.0	86.7	87.8	81.3
33°C	WPP	100	95.8	93.8	86.3	91.3
	LWP	100	85.8	82.3	80.3	94.2
	Hybrid	100	73.3	76.2	92.1	111.3
43°C	WPP	100	61.6	81.2	82.1	93.8
	LWP	100	78.3	77.5	77.5	75.0
	Hybrid	100	77.5	79.8	88.5	99.6

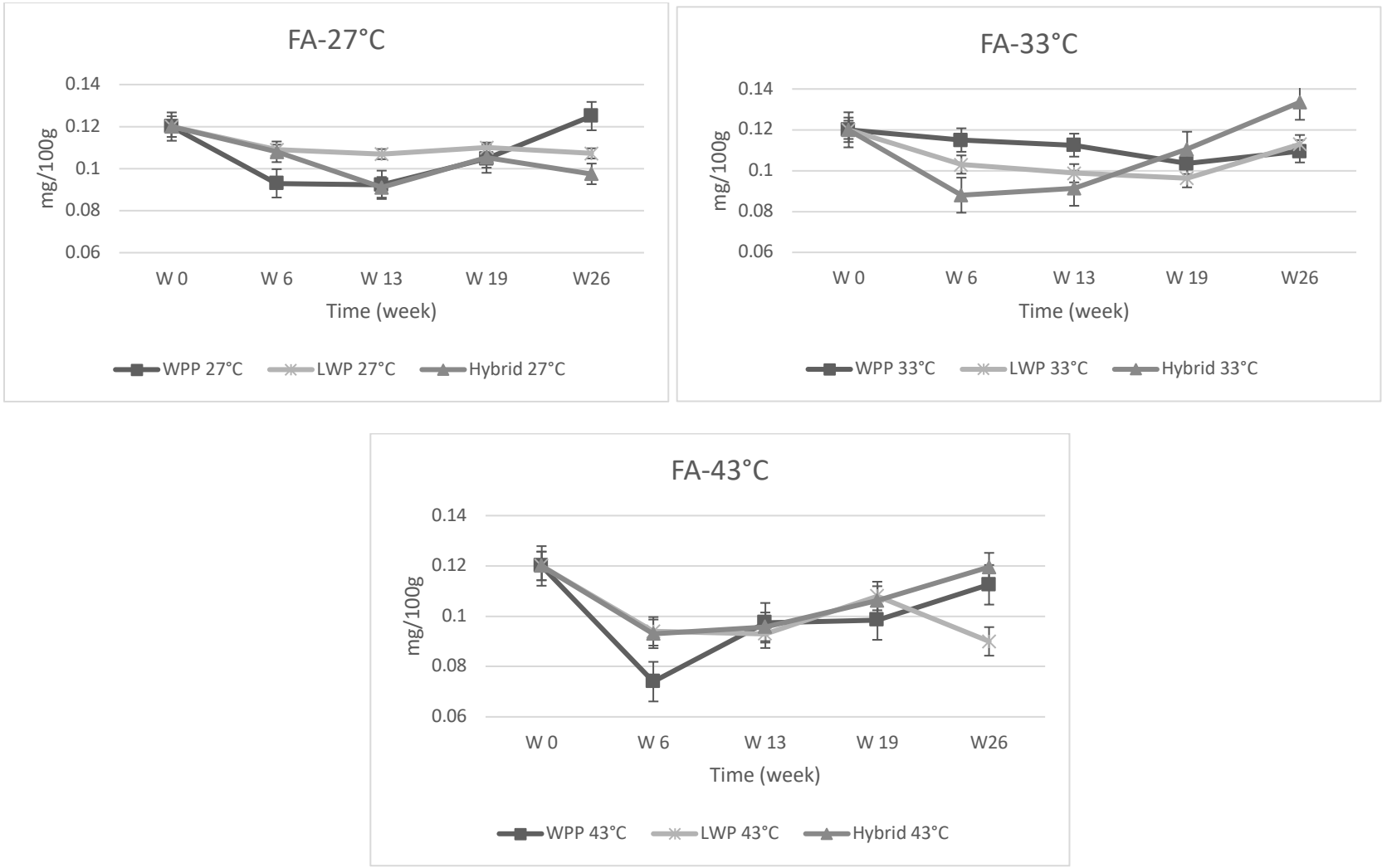


Figure 3.5. Retention of Folic acid in different packaging and temperature conditions

Table 3.12. Rate constant for Folic acid

Packaging	K (27°C) (1/week*10^{-4})	K (33°C) (1/week*10^{-4})	K (43°C) (1/week*10^{-4})
WPP	5	44	-
LWP	33	27	89
Hybrid	68	-	101

3.3.5 Iron and Zinc

Minerals are naturally occurring inorganic element that are hard to decompose with simple chemical reaction. Li et al., 2008 found that iron and zinc retention stayed same in different temperatures condition while studying effect of iron compounds on the storage stability of multiple fortified ultra-rice. Kuang et al., 2016 studied fortified rice a case study in Cambodia focusing on Iron and zinc retention over the time in different storage conditions and different time points also showed no significant differences with average retention of 90%-100%. Hemery et al., 2018 validated similar results while testing fortified flour in different storage time, temperature, relative humidity, and packaging type. Minerals are generally considered as heat stable and expected to have no impact during extrusion processing. (Singh et al., 2007). Minerals are considered as stable as they don't get degraded if exposed to light, heat, oxidizing agents or other extreme factors that can affect vitamins (de Silva et al., 2016)

Table 11 showed similar results in our study in which iron and zinc did not have significant effect on temperature and packaging. In all conditions the retention was over 85% for both iron and zinc.

Table 3.13. Retention of Iron as percentage in different packaging and temperature conditions

Temp	Packaging	Week 0	Week 6	Week 13	Week 19	Week 26
27°C	WPP	100	100.7	101.7	98.6	104.7
	LWP	100	102.2	105.3	100.8	102.4
	Hybrid	100	102.9	98.9	100.1	94.7
33°C	WPP	100	103.0	106.7	95.7	95.8
	LWP	100	99.1	111.6	95.7	91.3
	Hybrid	100	101.7	108.7	100.0	98.0
43°C	WPP	100	95.2	107.2	97.2	95.8
	LWP	100	103.3	108.6	96.3	94.7
	Hybrid	100	105.2	109.2	90.3	93.5

Table 3.14. Rate constant for Iron

Packaging	K (27°C) (1/week*10^{-4})	K (33°C) (1/week*10^{-4})	K (43°C) (1/week*10^{-4})
WPP	-	24	9
LWP	-	33	28
Hybrid	22	9	44

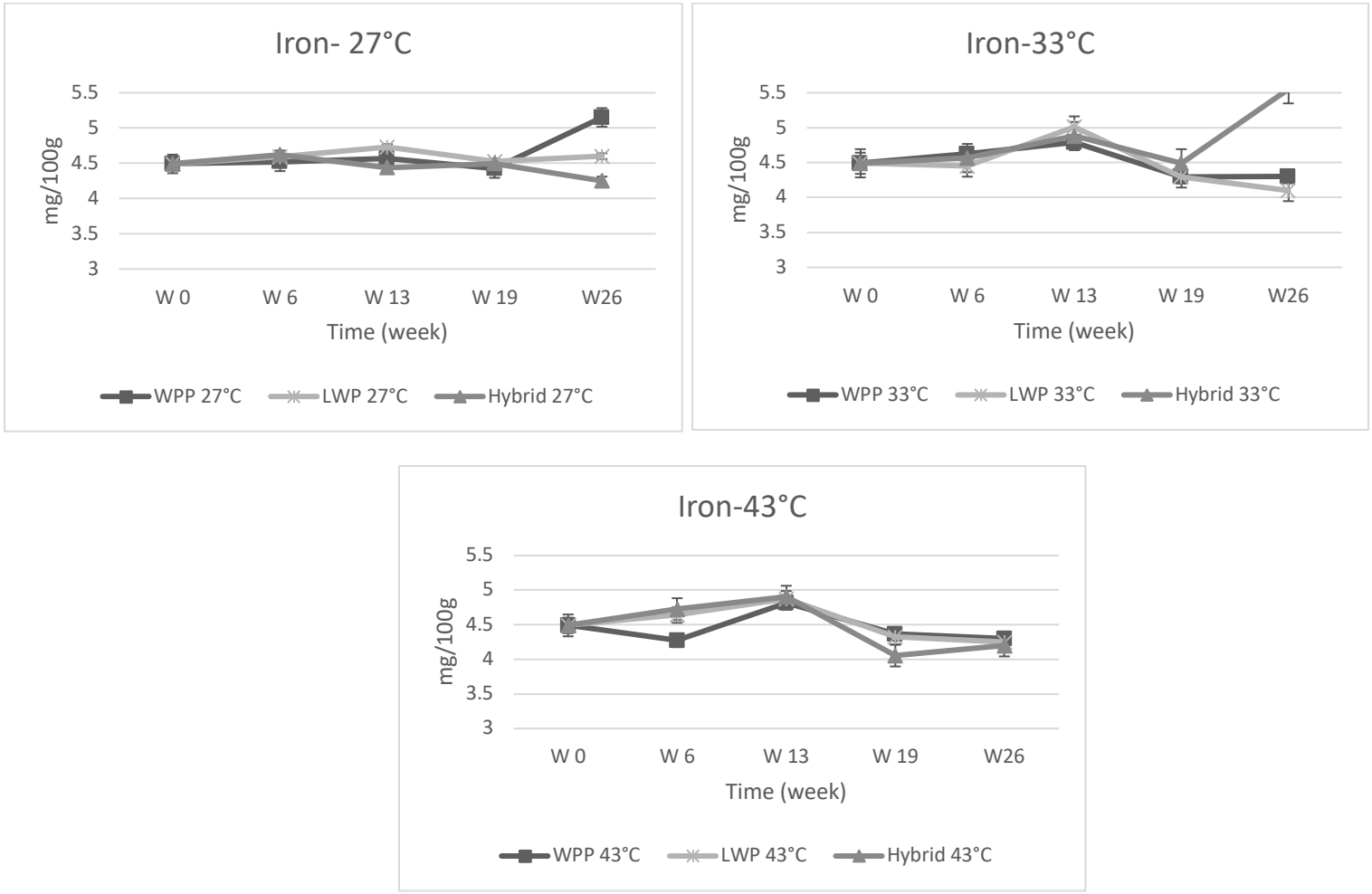


Figure 3.6. Retention of Iron in different packaging and temperature conditions

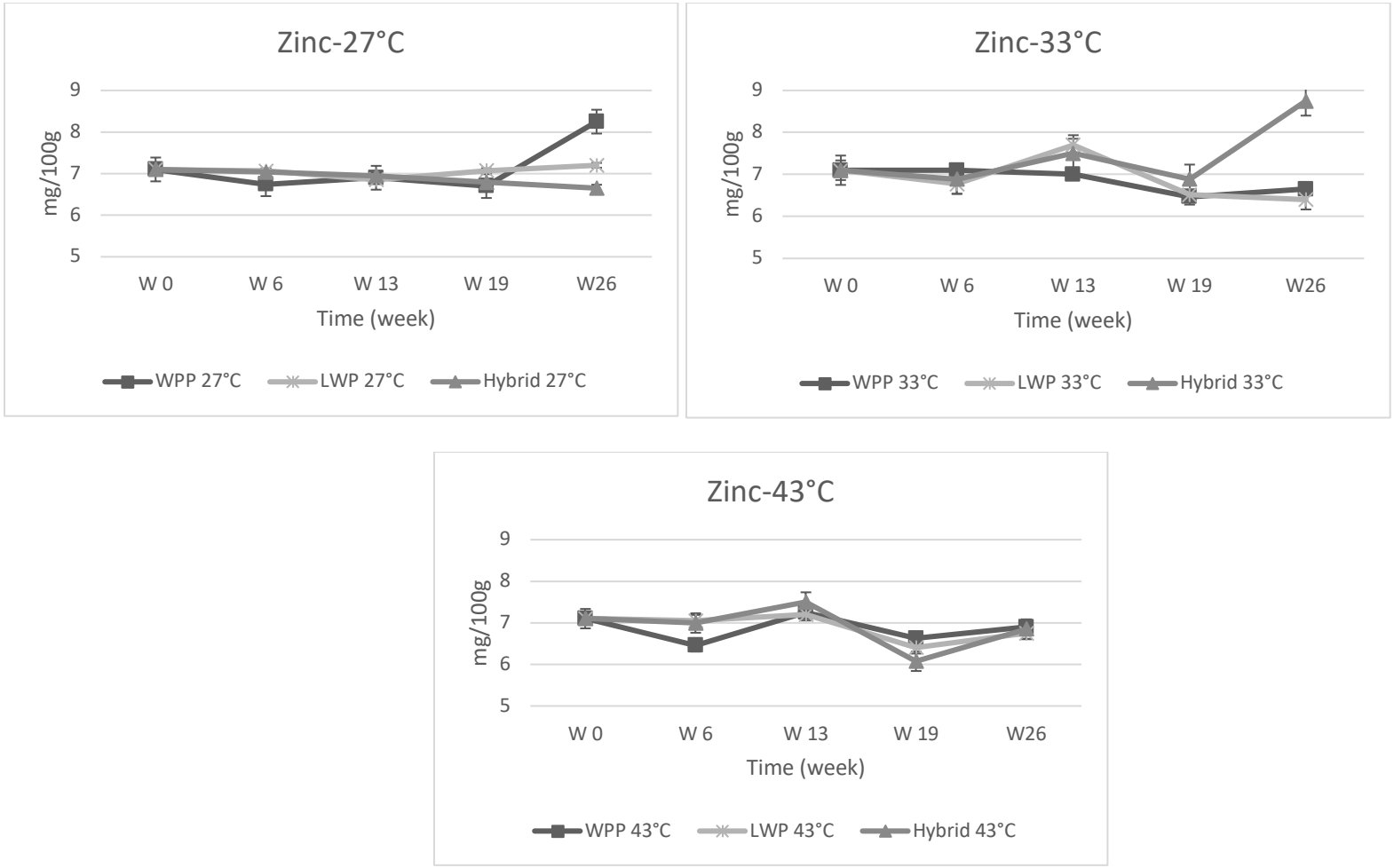


Figure 3.7. Retention of Zinc in different packaging and temperature conditions

Table 3.15. Rate constant for Zinc

Packaging	K (27°C) (1/week*10^{-4})	K (33°C) (1/week*10^{-4})	K (43°C) (1/week*10^{-4})
WPP	-	34	4
LWP	-	37	3
Hybrid	26	63	31

Table 3.16. Retention of Zinc as percentage in different packaging and temperature conditions

Temp	Packaging	Week 0	Week 6	Week 13	Week 19	Week 26
27°C	WPP	100	95.0	97.2	94.4	107.0
	LWP	100	99.5	96.5	99.6	101.4
	Hybrid	100	99.2	97.9	95.6	93.7
33°C	WPP	100	100.0	98.6	91.1	93.7
	LWP	100	95.4	108.5	91.8	90.1
	Hybrid	100	97.0	105.6	97.0	81.7
43°C	WPP	100	90.9	102.1	93.3	97.2
	LWP	100	99.2	101.4	90.2	95.1
	Hybrid	100	98.5	105.6	85.6	96.5

Four-way ANOVA results are presented in detail in Appendix B. All three factors, including the micronutrient type, storage temperature and time of sampling, showed significant effect ($p < 0.05$) on the micronutrient level detected. The packaging type did not have a significant effect on the micronutrient level. Significant interaction ($p < 0.05$) were also found between several pairs of factors.

3.3.6 Microbial Results

Microbial count was analyzed by yeast and mold count. At all-time points, yeast and mold were analyzed. Yeast and Mold count was low till the end of study. There was a slight

increasing trend during storage but all within acceptable range (USDA recommends microbial count to be less than 1000cfu) Table 3.17. Estimated shelf life based on

Table 3.17. Microbial Results during study (Mold 35 cfu/g, yeast <10 cfu/g at the start of study)

		Week 6		Week 13		Week 19		Week 26	
Packaging	**Storage Condition**	**Mold** (cfu/g)	**Yeast** (cfu/g)	**Mold** (cfu/g)	**Yeast** (cfu/g)	**Mold** (cfu/g)	**Yeast** (cfu/g)	**Mold** (cfu/g)	**Yeast** (cfu/g)
WPP	27°C	40	<10	85	40 (est)	165	180	180	120
	33°C	20	<10	25 (est)	<10	130	100	150	50
	43°C	15	<10	60 (est)	<10	480	85	100	10
LWP	27°C	15	<10	105	<10	245	130	60	50
	33°C	60	<10	15 (est)	<10	130	25	590	90
	43°C	<10	<10	15 (est)	55(est)	220	65	410	120
Hybrid	27°C	<10	<10	55	<10	225	65	580	430
	33°C	<10	<10	15 (est)	20 (est)	230	50	30	20
	43°C	<10	<10	65 (est)	65 (est)	285	40	510	150

3.3.7 Sensory Analysis

Twelve attributes were analyzed on different time interval samples (Week 0, week 6, week 19 & week 26) in all packaging (WPP, LWP & Hybrid) at 27°C and 43°C. Principal component analysis (PCA) helped to visualize and summarize the information Figure 3.8. The graphs show a 67.5% of variability, which means this accounts for 67.5% of the variability among products. Shift of the products towards the right over the time means a change in the overall characteristics towards more intense aromas in grain, starchy, nutty etc. Staleness does not seem to be a feature of the aged samples, but musty/dusty does. There is not a big difference between products at week 19 or week 26 but there is a difference with the week 6 products that

seems to be mostly closer to the control (left side). PCA could tend to exaggerate some relationships, so individual results were confirmed with the numeric data. (Table 3.18).

Table 3.18. Rice Descriptive Results -Mean and p-value

Sample Details	Color intensity	Brightness	Translucent	Starchy (a)	Grain (a)	Musty/Dusty (a)	Straw (a)	Vitamin (a)	Sweet Aromatics (a)	Nutty (a)	Stale (a)	Fracturability (t)
Control	6.5^{cde}	4.2 ^{b}	11.8	3.4^{f}	3.3^{f}	3.3^{e}	3.1^{d}	3.6^{ab}	1.0^{d}	1.3 ^{b}	3.0 ^{a}	10.3
Hybrid 27°C Week 6	3.1^{h}	4.4^{ab}	11.8	4.4^{bcde}	4.4^{abcd}	3.8^{bcde}	3.9^{abc}	3.8^{ab}	2.1^{bc}	3.0 ^{a}	0.0 ^{e}	10.8
Hybrid 27°C Week 19	7.6^{abc}	4.6^{ab}	11.8	4.6^{abcde}	4^{cdef}	3.9^{abcde}	3.9^{abc}	3.8^{ab}	2.2^{bc}	2.8 ^{a}	1.6 ^{b}	10.7
Hybrid 27°C Week 26	7.9 ^{ab}	4.9^{ab}	11.8	4.6^{abcde}	4^{cdef}	3.5^{cde}	3.8^{abcd}	3.7^{ab}	2.7^{a}	2.6 ^{a}	0.0 ^{e}	10.4
Hybrid 43°C Week 6	6.5^{cde}	4.8^{ab}	11.8	4.0^{ef}	3.5 ^{ef}	4.0^{abcd}	3.3^{cd}	3.4^{ab}	1.8^{c}	2.6 ^{a}	0.0 ^{e}	9.9
Hybrid 43°C Week 19	6.4^{de}	4.4^{b}	11.8	5^{abc}	4.8^{a}	4.2^{abc}	4.0^{abc}	3.3^{ab}	2.3^{ab}	2.9^{a}	0.0^{e}	11.0
Hybrid 43°C Week 26	6.9^{bcd}	4.3^{b}	11.8	4.6^{abcde}	4.5^{abcd}	3.9^{abcde}	3.7^{abcd}	3.0^{b}	2.2^{bc}	2.6^{a}	1.0^{bc}	11.0
WPP 27°C Week 6	4.2^{gh}	5.2^{a}	11.8	4.5^{bcde}	3.9^{def}	3.7^{bcde}	3.6^{bcd}	4.1^{a}	2.1^{bc}	2.8^{a}	0.0^{e}	10.2
WPP 27°C Week 19	4.0^{gh}	4.4^{ab}	11.8	4.4^{bcde}	4.1^{bcde}	4.0^{abcd}	3.3^{cd}	3.2^{ab}	2.2^{bc}	2.6^{a}	1.0^{bc}	10.5
WPP 27°C Week 26	6.1^{def}	4.6^{ab}	11.8	4.6^{abcde}	4.6^{abcd}	4.1^{abcd}	4.4^{a}	3.3^{ab}	2.3^{abc}	2.9^{a}	0.9^{cd}	10.6
WPP 43°C Week 6	6.9^{bcd}	4.5^{ab}	11.8	4.7^{abcd}	4.4^{abcd}	3.9^{abcde}	3.9^{abc}	4.1^{a}	2.2^{bc}	2.9^{a}	1.4^{bc}	10.8
WPP 43°C Week 19	8.4^{a}	4.2^{b}	11.8	4.7^{abcde}	4.2^{abcde}	4.3^{ab}	3.6^{bcd}	3.6^{ab}	2.3^{ab}	2.8^{a}	0.0^{e}	11.5
WPP 43°C Week 26	8.4^{a}	4.3^{b}	11.8	4.6^{abcde}	4.3^{abcd}	4.1^{abcd}	3.6^{bcd}	3.1^{ab}	2.3^{ab}	2.8^{a}	0.0^{e}	10.4
LWP 27°C Week 6	5.0^{fg}	4.4^{b}	11.8	4.1^{def}	3.9^{cdef}	3.4^{de}	3.3^{cd}	3.6^{ab}	2.1^{bc}	2.4^{a}	1.0^{bc}	10.5
LWP 27°C Week 19	7.6^{abc}	4.7^{ab}	11.6	4.8^{abcd}	4.6^{abc}	4.2^{abc}	3.8 ^{abcd}	3.6^{ab}	2.4^{ab}	2.9^{a}	0.0^{e}	11.0

LWP 27°C Week 26	4.1^{gh}	4.5^{ab}	11.8	4.4^{bcde}	4.4^{abcd}	4.1^{abcd}	3.8 ^{abcd}	3.3^{ab}	2.2^{bc}	2.9^{a}	0.2^{de}	11.5
LWP 43°C Week 6	7.9^{ab}	4.4^{ab}	11.8	5.1^{ab}	4.8^{ab}	4.1^{abcd}	3.6 ^{bcd}	3.4^{ab}	2.4 ab	3.0^{a}	0.0^{e}	10.2
LWP 43°C Week 19	7.6^{abc}	4.7^{ab}	11.6	4.8^{abcd}	4.6^{abc}	4.2^{abc}	3.8 ^{abcd}	3.6^{ab}	2.4 ab	2.9^{a}	0.0^{e}	11.0
LWP 43°C Week 26	7.8^{ab}	4.5^{ab}	11.8	5.3^{a}	4.6^{abc}	4.6^{a}	4.1 ab	3.6^{ab}	2.3 ab	2.9 a	0.0^{e}	10.8
Pr > F(Model)	<0.0001	0.699	1.000	0.001	0.001	0.064	0.176	0.854	<0.0001	0.002	<0.0001	0.907
Significant	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

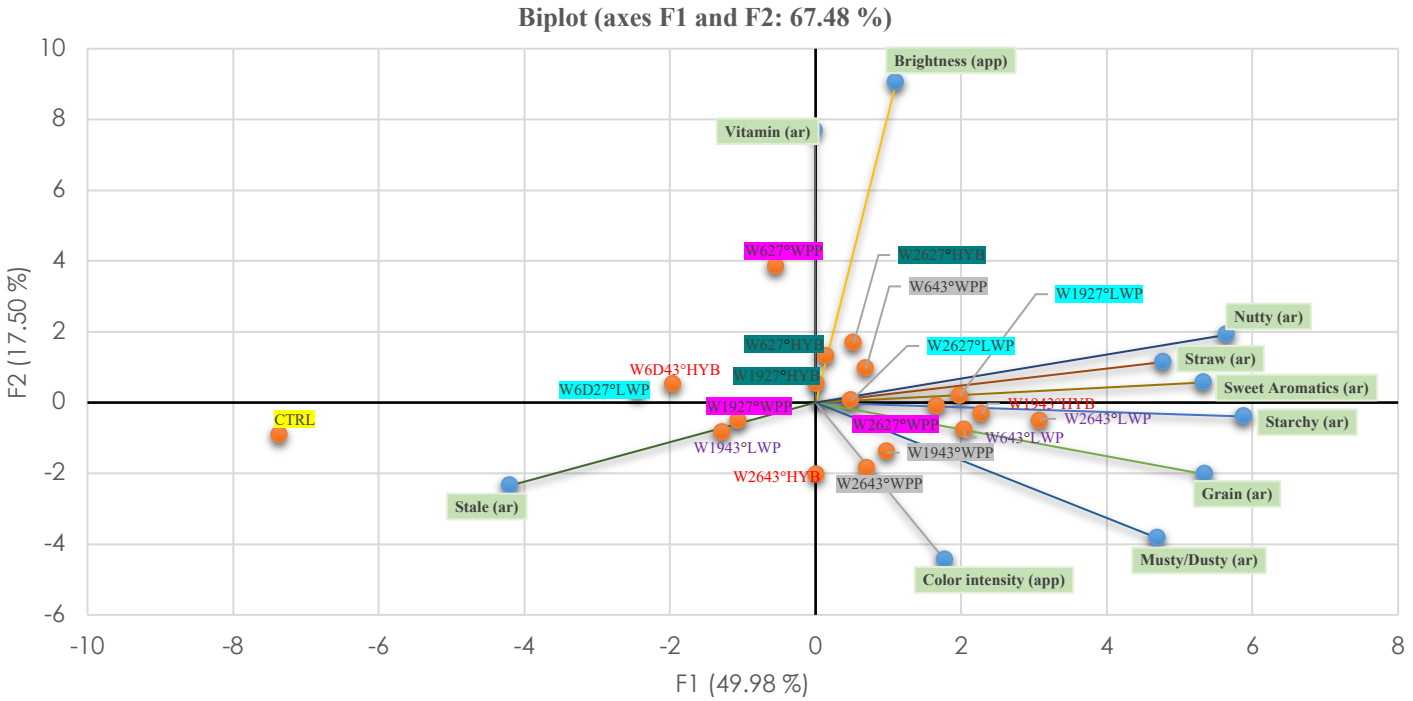


Figure 3.8. Principle Component Analysis (PCA)

Statistically significant difference was found between the samples for the attributes color intensity(appearance), starchy(aroma), grain(aroma), musty/dusty(aroma), sweet aromatics(aroma), nutty(aroma), stale(aroma).

For color intensity(appearance) attribute, samples at week 6 in WPP (27°C), LWP (27 & 43°C) and Hybrid (27°C), week 19 in WPP (27°C & 43°C), week 26 WPP (43°C), Hybrid (27°C), LWP (27°C) are significantly different from control.

For aroma attribute starchy, except for samples at week 6 in LWP and Hybrid packaging, all others are significantly different from the control.

For grain(aroma)attribute, samples at week 6 in WPP (27°C), LWP (27°C), Hybrid (43°C) week 19 Hybrid (27°C), LWP (43°C) and week 26 in Hybrid doesn't have a significant difference from the control.

No statistical difference was found between the samples at week 6 in WPP (27°C & 43°C), LWP (27°C), Hybrid (27°C), week 19 in Hybrid (27°C), week 26 in hybrid (27°C and 43°C) and control, for Musty/dusty aroma.

For Nutty and sweet aromatics, all the samples are statistically different from the control.

Statistical difference was found between the samples and control, except for sample at week 19 in LWP at 43°C for the stale attribute.

3.4 Conclusion

This study showed that shelf life of fortified rice could be estimated by using Arrhenius model based on accelerated shelf-life study. Vitamin A was considered as key indicator for estimating the shelf life as great losses were observed in all packaging which question if rice is a suitable carrier for vitamin A, especially with coating technology. LWP and Hybrid packaging shows better results for vitamins as compared to WPP as both have better barrier properties and

could be a good alternative for effective packaging. Other vitamins (vitamin B1 and Folic acid) results did not show noticeable deterioration over the time.

Similarly, iron and zinc losses during storage with high temperature and packaging variations were negligible, which affirm that fortified rice is an effective carrier to improve iron and zinc deficiencies.

3.5 References

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. 2003. Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. LWT-Food Sci Technol. 36: 407–414.

Alavi, S., Bugusu, B., Cramer, G., Dary, O., Lee, T. C., Martin, L., ... & Wailes, E. Rice fortification in developing countries: a critical review of the technical and economic feasibility. A2Z Project, Academy for Educational Development. Washington, DC, 2008.

Anwar, S. H., Ayun, S. Q., & Nasution, I. S. (2019, October). Shelf-life estimation of red dragon fruit jam using accelerated shelf-life testing (ASLT) method. In *IOP Conference Series: Earth and Environmental Science* (Vol. 365, No. 1, p. 012029). IOP Publishing.

Ball, G. F. (2005). *Vitamins in foods: analysis, bioavailability, and stability*. CRC press.

Bergonio,K., Lucatin,L., Corpuz,G., Ramos,N., & Duldulao, J.2016. Improved Shelf Life Of Brown Rice By Heat and Microwave Treatment. J. Microbiology, Biotechnology and Food Science:5(4):378-385.

Berry, R. J., Bailey, L., Mulinare, J., Bower, C., & Dary, O. (2010). Fortification of flour with folic acid. *Food and nutrition bulletin*, *31*(1_suppl1), S22-S35.

Burlando, B., & Cornara, L. (2014). Therapeutic properties of rice constituents and derivatives (Oryza sativa L.): A review update. *Trends in food science & technology*, *40*(1), 82-98.

Chanadang, S., & Chambers, E. 2019. Sensory Shelf Life Estimation of Novel Fortified Blended Foods under Accelerated and Real-Time Storage Conditions. J. Food Science:84(9):2638-2645.

Coad, R., & Bui, L. (2020). Stability of vitamins B1, B2, B6 and E in a fortified military freeze-dried meal during extended storage. *Foods*, *9*(1), 39.

Cordova, A., Quezada, C., & Saavedra. J. 2011. A MALST method comparison over univariate kinetic modeling for determination of shelf-life in cereal snack of dried apples. Procedia Food Sci. 1: 1045–1050.

Choi, J. Y., Lee, H. J., Cho, J. S., Lee, Y. M., Woo, J. H., & Moon, K. D. (2017). Prediction of shelf-life and changes in the quality characteristics of semidried persimmons stored at different temperatures. *Food Science and Biotechnology*, *26*(5), 1255-1262.

Dary, O., & Hurrell, R. (2006). Guidelines on food fortification with micronutrients. *World Health Organization, Food and Agricultural Organization of the United Nations: Geneva, Switzerland*, 1-376.

Ding, C., . Khir, R., Pan,Z., Zhao, L.,Tu, K., El-Mashad, H., & McHugh, T.2015.Improvement in Shelf life of rough and brown rice Using infrared Radiation heating. J. Food and Bioprocess Technology:8(5):1149-1159.

Frommherz, L., Martiniak, Y., Heuer, T., Roth, A., Kulling, S. E., & Hoffmann, I. (2014). Degradation of folic acid in fortified vitamin juices during long term storage. *Food chemistry*, *159*, 122-127.

Ganje, M., Jafari, S.M., Dusti, A., Dehnad, D., Amanjani, M., & Ghanbari, V. 2016. Modeling quality changes in tomato paste containing microencapsulated olive leaf extract by accelerated shelf life testing. Food Bioprod Process. 97: 12–19.

Haouet, M. N., Tommasino, M., Mercuri, M. L., Benedetti, F., Di Bella, S., Framboas, M., ... & Altissimi, M. S. (2018). Experimental accelerated shelf life determination of a ready-to-eat processed food. *Italian journal of food safety*, *7*(4).

Hemanth, K. J., Hema, M. S., Sinija, V. R., & Hema, V. (2020). Accelerated shelf-life study on protein-enriched carbonated fruit drink. *Journal of Food Process Engineering*, *43*(3), e13311.

Hemery, Y. M., Fontan, L., Laillou, A., Jallier, V., Moench-Pfanner, R., Avallone, S., & Berger, J. (2020). Influence of storage conditions and packaging of fortified wheat flour on microbial load and stability of folate and vitamin B12. *Food chemistry: X*, *5*, 100076.

Hemery, Y. M., Laillou, A., Fontan, L., Jallier, V., Moench-Pfanner, R., Berger, J., & Avallone, S. (2018). Storage conditions and packaging greatly affects the stability of fortified wheat flour: Influence on vitamin A, iron, zinc, and oxidation. *Food chemistry*, *240*, 43-50.

Jafari, S. M., Ganje, M., Dehnad, D., Ghanbari, V., & Hajitabar, J. (2017). Arrhenius equation modeling for the shelf life prediction of tomato paste containing a natural preservative. *Journal of the Science of Food and Agriculture*, *97*(15), 5216-5222.

Keane, P. (1992). *The flavor profile*. ASTM International.

Kodaka, H., Mizuochi, S., Teramura, H., Nirazuka, T., Oda, S., Tanaka, H., ... & Betts, R. (2006). Comparison of the Compact Dry YMwith the FDA BAM Method for Enumeration of Yeasts andMolds in Fruit-Based Products: Performance-Tested MethodSM 100401. *Journal of AOAC International*, *89*(1), 127-138.

Kumar, P., & Mishra, H. N. (2004). Storage stability of mango soy fortified yoghurt powder in two different packaging materials: HDPP and ALP. *Journal of Food Engineering*, *65*(4), 569-576.

Kuong, K., Laillou,A., Chea,C., Chamnan,C., Berger,J., & Wieringa, F. 2016. Stability of Vitamin A, Iron and Zinc in Fortified Rice during Storage and Its Impact on Future National Standards and Programs—Case Study in Cambodia. Nutrients :8(1). Open Access.

Kuong, K.,Tor,P., Perignon, M., Fiorentino,M., Chamnan, C.,Berger, J., Burja, K., Dijkhuizen, M., Parker, M., Roos, N., & Wieringa, F. 2019.Multi-Micronutrient Fortified Rice Improved Serum Zinc and Folate Concentrations of Cambodian School Children. A Double-Blinded Cluster-Randomized Controlled Trial. Nutirent: 11(12).

Kurniadi, M., Bintang, R., Kusumaningrum, A., Nursiwi, A., Nurhikmat, A., Susanto, A., ... & Frediansyah, A. (2017, December). Shelf life prediction of canned fried-rice using accelerated shelf life testing (ASLT) arrhenius method. In *IOP Conference Series: Earth and Environmental Science* (Vol. 101, No. 1, p. 012029). IOP Publishing.

Labuza, T. 1982. Shelf Life Dating of Foods. Food and Nutrition Press, Westport, New Zealand. pp. 41–88.

Labuza, T.P. 2000. The search for shelf life. Food Testing and Analysis.6: 26–36.

Labuza, T.P., & Schmidl, M.K. 1985. Accelerated shelf-life testing of food. J. Food Technol. 39: 57–62.

Lacerda,D., Soares Junior,M., Bassinello,P., Caliari, & Castro, M. 2013.The kinetics of lipase activity and hydrolytic rancidity of raw, parboiled, and extruded rice bran during storage. J. Food Science and Technology :33(2):376-381.

Lešková, E., Kubíková, J., Kováčiková, E., Košická, M., Porubská, J., & Holčíková, K. (2006). Vitamin losses: Retention during heat treatment and continual changes expressed by mathematical models. Journal of Food Composition and analysis, 19(4), 252-276.

Li, Y. O., Diosady, L. L., & Jankowski, S. (2011). Folic acid stability in the presence of various formulation components including iron compounds in fortified extruded Ultra Rice® over prolonged storage at 40 C and 60% relative humidity (RH). *International Journal of Food Science & Technology*, *46*(2), 379-385.

Li, Y., Diosady, L. L., & Jankowski, S. (2008). Effect of iron compounds on the storage stability of multiple-fortified Ultra Rice®. *International journal of food science & technology*, *43*(3), 423-429.

Nitka, A., & Sioma, A. (2018, October). Design of an automated rice grain sorting system using a vision system. In *Photonics Applications in Astronomy, Communications, Industry, and High-Energy Physics Experiments 2018* (Vol. 10808, p. 1080816). International Society for Optics and Photonics.

Ong, E. 2015. Accelerated shelf life evaluation of instant noodles made with fortified flour. Food Innovation and Resource Centre of the Singapore Polytechnic and Flour Fortification Initiative, Atlanta, Georgia, USA. Accessed at: http://www.ffinetwork.org/about/stay_informed/ publications/documents/InstantNoodleShelfLife.pdf

Pearson, T. (2010). High-speed sorting of grains by color and surface texture. *Applied engineering in agriculture*, *26*(3), 499-505.

Pearson, T., Maghirang, E., & Dowell, F. (2013). A multispectral sorting device for wheat kernels.

Phan, U. T. X., Chambers IV, E., Padmanabhan, N., & Alavi, S. (2014). Accelerated vs. real time modeling for shelf life: an example with fortified blended foods. *Science and Technology Development Journal*, *17*(3), 83-91.

Phillips, R., Pike, O. A., Eggett, D. L., & Dunn, M. L. (2017). Folate stability in folic acid enriched corn masa flour, tortillas, and tortilla chips over the expected shelf life. *Cereal Chemistry*, *94*(6), 917-921.

Porasuphatana, S., Chavasit, V., Vasinrapee, S., Suthutvoravut,U., & Hurrell, R. 2008. Production and Shelf Stability of Multiple-Fortified Quick-Cooking Rice as a Complementary Food. Journal of Food Science 73(7).

Ribeiro, D. O., Pinto, D. C., Lima, L. M. T., Volpato, N. M., Cabral, L. M., & de Sousa, V. P. (2011). Chemical stability study of vitamins thiamine, riboflavin, pyridoxine and ascorbic acid in parenteral nutrition for neonatal use. *Nutrition journal*, *10*(1), 1-9.

Riaz, M. N., Asif, M., & Ali, R. (2009). Stability of vitamins during extrusion. *Critical Reviews in Food Science and Nutrition*, *49*(4), 361-368.

Rodiles-López, J. O., García-Rodríguez, D. A., Gómez-Orozco, S. Y., Tiwari, D. K., & Coria-Téllez, A. V. (2020). Food quality evaluation of accelerated shelf life of chili sauce using Fourier transform infrared spectroscopy and chemometrics. *Journal of Food Processing and Preservation*, *44*(3), e14350.

Saha, S., Jha, S., Tiwari, A., Jayapalan, S., & Roy, A. (2021). Considerations for improvising fortified extruded rice products. *Journal of Food Science*, *86*(4), 1180-1200.

Sewald, M., & DeVries, J. 2003. Food product shelf life (pp. 1–10). Minneapolis, MN: Medallion Laboratories Analytical Progress Report.

Shafiei, G., Ghorbani, M., Hosseini, H., Sadeghi Mahoonak, A., Maghsoudlou, Y., & Jafari, S. M. (2020). Estimation of oxidative indices in the raw and roasted hazelnuts by accelerated shelf-life testing. *Journal of Food Science and Technology*, *57*(7), 2433-2442.

Steiger, G., Müller-Fischer, N., Cori, H., & Conde-Petit, B. (2014). Fortification of rice: technologies and nutrients. *Annals of the New York Academy of Sciences*, *1324*(1), 29-39.

Sirpatrawan, U.2009.Shelf-Life Simulation Of Packaged Rice Crackers. J. Food Quality :32(2): 224-239.

Syarief, R., Hermanianto, J., & Nurtama, B. (2020, September). Kinetics of quality changes and shelf life estimation of frozen coated Tumpi-Tumpi using accelerated shelf-life testing (ASLT) method with Arrhenius approach. In *IOP Conference Series: Earth and Environmental Science* (Vol. 564, No. 1, p. 012048). IOP Publishing.

Van Boekel, M. A. (2008). Kinetic modeling of food quality: a critical review. *Comprehensive Reviews in Food Science and Food Safety*, *7*(1), 144-158.

Voelker, A. L., Taylor, L. S., & Mauer, L. J. (2021). Effect of pH and concentration on the chemical stability and reaction kinetics of thiamine mononitrate and thiamine chloride hydrochloride in solution. *BMC chemistry*, *15*(1), 1-14.

Yoo, J., Alavi, S., Adhikari, K., Haub, M. D., Aberle, R. A., & Huber, G. (2013). Rice-shaped extruded kernels: physical, sensory, and nutritional properties. *International Journal of Food Properties*, *16*(2), 301-321.

Chapter 4 - Extrusion Processing of Fortified Rice with different levels of Micronutrient Premix

Abstract

Rice (Oryza sativa) is one of the leading staple foods for more than three billion people across the globe. To effectively use rice by adding extra nutrients in it would make it a very valuable food. Fortification is a well-known, cost effective and sustainable approach to address micronutrient deficiencies which benefits large population. There are different ways for fortification including coating, dusting, and extrusion.

Extrusion is a well-known and cost-effective technique used for the production and fortification of food. Rice shaped kernels with different levels of micronutrients were produced using rice flour to investigate losses of micronutrient during the process of hot extrusion. Four formulations were used including control with no micronutrients, recommended levels as per daily intake, 25% over recommended and 50% over recommended levels were extruded. Micronutrient analysis includes vitamin A, Vitamin B, Folic acid, Iron and Zinc.

RVA results showed decrease in peak viscosity as rice flour levels lowers and premix was added. Similar trends were observed with SME for 91% control to 71% in 100% premix formulation recipe. Micronutrient results showed around 34%- 37% of losses for vitamin A, 3.55-13% for vitamin B1, 32% - 36% for folic acid, 4%-15% for iron, 1%-7% for zinc during extrusion and drying process.

This research helped to understand degree of micronutrient losses during hot extrusion-based production of fortified rice kernels and effect of formulation change in process variation and could be used as useful guide to produce fortified extruded kernels to deliver recommended levels of micronutrients as per daily intake.

4.1 Introduction

Rice is a staple food used by more than half of the world. Naturally rice has high starch and low protein and micronutrients, which mostly lost during milling process.

Food fortification is a well-known, cost effective and sustainable approach to address micronutrient deficiencies which benefits large population. Adding extra nutrient to rice and elevate nutritional deficient people around the world has been used for years.

There are different technologies that could be used to add nutrients in rice including coating technology in which coating agents like waxes and/or gums are mixed with fortificant premix to make a slurry which serves as coating and sprayed to rice in several layers for uniform distribution on the surface of rice. As the fortificant premix is only on the surface of rice, high losses are expected especially for vitamin A.

Dusting is another way of fortifying rice in which micronutrients as fine particles are blended with rice, typically it uses electrostatic forces to mix rice with micronutrient (Steiger et al 2014). It advised to not rinse or wash before or after cooking to retain all added micronutrients.

Extrusion is defined as process to push raw material mix through a small opening which is called die, to form and shape the material as desired (Launay and Lisch .,1983). The product which came out of die is referred as extrudates, it is a well-developed and versatile process used to fortify food including rice. (Li et al., 2009, 2011; Ebuehi & Oduwole, 2010). This processing system mostly utilize single screw or a set of screws. Typical process involves mixing, conveying, kneading, heating, melting, shearing, shaping, cooking and forming final product (Dalbhagat et al 2019.,) (Xu et el., 2016). Pressure, shear, and temperatures created by screws

plays very important role in forming extruded product. Hot extrusion process required typically high temperatures (70-110C) with low shear and results in a product with is close to natural rice.

4.1.1 Dusting technology

During dusting, micronutrients in the form of fine particles are blended with the bulk rice. This method makes use of the electrostatic forces between the rice surface and the micronutrients (Steiger et al 2014). This technology observed only in the U.S. Consumers are advised that rice fortified with powdered premixes should not be rinsed before or after cooking nor should the rice be cooked in excessive amounts of water and then drained.

4.1.2 Coating

In the coating method, ingredients such as waxes and gums are combined with the fortificant mix to create a liquid which is sprayed to the rice in several layers on the surface of grain kernels to form the rice-premix. The rice-premix is then blended with commercial rice for fortification. The waxes and gums enable the micronutrients to stick to the rice kernel, thus reducing losses when the grains are washed before cooking, which is a common practice in developing countries. The final product is rice covered by a waxy layer; the color depends on the fortificants that are added. *Wright Enrichment Inc*. also uses a coating technology. This proprietary technology involves embedding the enrichment in micro perforations on the rice surface.

4.1.3 Extrusion

Both hot and cold extrusion is used for rice fortification. In the hot extrusion process, rice flour is used which is typically obtain from broken rice kernel or poor-quality rice. The rice flour is mixed with micronutrient fortificant mix, water, binding agents (if needed) and emulsifiers. The extrusion process requires high temperature (70-110^{o}C) with low shear, resulting in a

product close to natural rice (sheen, transparency, consistency, and flavor). Single screw or twin-screw extruder could be used for hot extrusion, in which the starch gets partly or fully gelatinized due to high pressure, shear and heat during the extrusion. Thermal energy via steam preconditioning and/ or heated barrel jackets can contribute to the cooking, besides the mechanical energy generated by the extrusion screw (Alavi et al 2008). At the end of extruder, rice kernel shaped die orifices are used to give the final product resemblance of rice grain.

Cold extrusion technology is similar, except it utilizes a simple forming extruder also called a pasta press. It is primarily a low temperature (below 70^{o}C) and low shear, forming process resulting in grains that are uncooked, opaque, and easier to differentiate from regular rice kernels. It does not involve any additional thermal energy input before or during the process by preconditioning or heated barrel jackets and relies on the minimal heat generated during the lower shear process itself (Steiger et al 2014).

This section focuses on investigating the degree of micronutrient losses during hot extrusion-based production of fortified rice kernels using rice flour and micronutrient premix where final product has similar appearance as regular rice

4.2 Materials and Methods

4.2.1 Raw Materials and Experimental Design

Rice flour was commercially sourced from St. Charles Trading Inc. whereas vitamin/ Mineral premix was donated by *Wright Enrichment Inc*. (Crowley, LA). Minor ingredients Salt, Monoglyceride and yellow color No.5 were also used. Ingredients were mixed in ratio shown in Table 4.1, which represents a simple experimental design with addition of 4 levels of micronutrient premix. The control formulation had no micronutrients added. The formulation labeled 100% had the recommended level of micronutrient premix added to target the USDA

mandated levels of vitamin A, vitamin B1, folic acid, iron and zinc in the final fortified rice provided the dilution level of the fortified rice kernel was 1:99. The formulations labeled 125% and 150% had 25% and 50% overages of the micronutrient premix added, leading to higher than targeted levels.

Table 4.1. Treatment Formulation %

Treatment	Control	100%	125%	150%
Rice Flour	97.65	88.85	86.65	84.5
Vitamin/Mineral-Premix	-	8.8	11	13.2
Salt	1	1	1	1
Monoglyceride	0.75	0.75	0.75	0.75
Yellow # 5	0.6	0.6	0.6	0.6

4.2.2 Raw Material Analysis

All raw material mixes were analyzed with Rapid visco analyzer to see the rheological changes with different formulations as shown in Table 4.1. For comparison commercial rice were also analyzed on RVA.

A rapid visco analyzer (RVA) (RVA 4500, Perten Instruments, and Waltham MA) was used to measure the viscosity of each treatment using AACC Method 76-21.02 STD1. All treatment slurries at 14% solid concentration (w/v) were mixed and placed in the RVA. After putting slurries in the chamber, it was heated to 50 °C, stirred at 960 rpm for 10 seconds. After 1 min at 50 °C, slurries were heated to 95 °C at 12 C/min, then held at that temperature for 2.5 min. Slurries were then cooled to 50 °C. Peak and final viscosities were recorded, as well as the temperature and time the peak viscosity was reached. (Ramirez et al., 2021)

4.2.3 Pilot Scale Extrusion Parameters and Calculations

All treatments were mixed for 5 minutes using a batch ribbon blender (Wenger Manufacturing, Sabetha, KS, USA). Extrusion was done on a pilot- scale, co-rotating twin screw

extruder (TX-52, Wenger Manufacturing, with a screw diameter of 52mm and L/D ratio of 19.5. The dry material feed rate was 55 kg/hr with the speed of 9rpm/Hz. In pre-conditioner the cylinder speed was 361 RPM, where steam and water were added as per Table 4.2. Three temperature zones were used at 30, 90 & 105 °C from inlet of extruder barrel to the outlet, with die temperature ranging from 100- 104 °C.

The screw profile consists of double flighted elements including six full pitch forward double flights with two ½ pitch double flight forward elements followed by conical cut element of ¾ pitch at the end (Figure 4.1)

A 7 opening rice die was used in which each opening was 0.047" x 0.204". Only 4 openings were used keeping 3 openings blocked. A Flex knife with 4 hard blades were used with a speed of 3590 RPM. Fortified rice kernels were dried at 155 F for 48 minutes and cooled for 11 minutes in a dual pass drier (4800, Wenger Manufacturing). Dried FRKs were collected and stored in the freezer at -5°C.

Table 4.2. Extrusion Process Parameters

Parameters	Control	100%	125%	150%
Feed Rate(kg/hr)	55	55	55	55
IBM (%)	43.36	35.22	36.85	36.85
Screw Speed (RPM)	174	196	264	307
Die Temp (°C)	100-104	102-104	100-103	100

Specific mechanical energy (SME) was calculated using equation

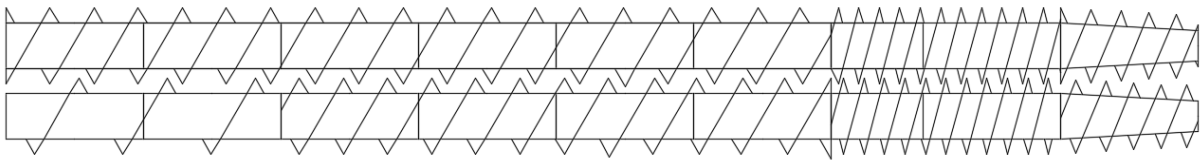
$$\textbf{SME}\ (\mathrm{kJ/kg}) = \frac{\left(\frac{\tau - \tau_0}{100}\right) \times \frac{N}{N_r} \times P_r}{\dot{m}}$$

where τ is the % torque, τo is the no-load torque, N is the measured screw speed in RPM, N_r is the rated screw speed (336 rpm), P_r is the rated motor power (22.4 kW) and m is mass flow rate in kg/s.

For In- barrel moisture (IBM), below equation was used

$$\textbf{IBM}\ (\%\ \text{wb}) = \frac{m_f \times X_{fw} + m_{ps} + m_{pw} + m_{ew}}{m_f + m_{ps} + m_{pw} + m_{ew}} * 100$$

Where m_f is the dry feed rate in kg/h, X_{fw} is the moisture content of the dry feed material



1 Full pitch, double flight, 9U
2 Full pitch, double flight, 9U
3 Full pitch, double flight, 9U
4 Full pitch, double flight, 9U
5 Full pitch, double flight, 9U
6 Full pitch, double flight, 9U
7 ½ pitch, double flight, 6U
8 ½ pitch, double flight, 6U
9 ¾ pitch, double flight, cone

Figure 4.1. Extruder Screw Configuration

4.2.4 Fortified Rice Kernels (FRK) Analysis

FRKs from all treatments were ground as per our internal validated grinding method, which is 30seconds of grind using a high-speed multifunctional Grinder (Moongiantgo Grain Grinder). All the samples were doubled sealed in opaque bags to block exposure to light, humidity and other external factors till samples are ready for analysis.

All micronutrient analysis were outsourced and performed by a commercial lab. 100g of sample from each treatment were sent in duplicate for analysis.

Vitamin A used a modified method AOAC 974.29 in which 10 g of samples were weighed into saponification flasks. Samples were saponified on a steam bath with reagent alcohol, potassium hydroxide, and an antioxidant (BHT). Samples were then cooled, hexane was added and then mixed. Phases were allowed to separate. The organic layer containing the vitamin A is decanted into a separatory funnel. This extraction process was performed a total of three times to ensure complete extraction. The organic collection in the funnels was rinsed with deionized water (DI) and finally filtered through sodium sulfate into volumetric flasks. For retinol, samples were diluted in hexane if necessary. A portion of the sample was transferred into an HPLC vial. Retinol samples were analyzed by HPLC with mobile phase consisting of isorpropaol and hexane. The HPLC was equipped with a silica column and fluorometric detector (Ex 330 nm, Em 480 nm). (AOAC 974.29 Mod)

Vitamin B1 (Thiamin) modified AOAC 942.23 method for vitamin B1 analysis.1 g of samples were extracted in 0.1N HCl by autoclaving. Various forms of phosphorylated thiamine were then converted into free thiamine with an alpha amylase solution. Samples were diluted, centrifuged, and filtered, and then injected in an ultra-performance liquid chromatography (UPLC). After the peak separation on a C18 column, the eluent enters an oxidation loop where

thiamine reacts with alkaline ferricyanide and was converted to a fluorescent derivative, thiochrome. Thiochrome was analyzed on a fluorescent emission detector (Ex 363 nm, Em 435 nm).

Folic acid analysis used modified method of AOAC 992.05.1 g of sample was autoclaved then cooked to room temperature. Creon capsules and chicken pancreas conjugase were added. Sample solution was then mixed with growth media and inoculated with L. Rhamnosus. After overnight incubation, the concentration of total folate in the sample is determined by reading the turbidity of the sample at 600 nm against that of a series of calibration standards. The amount of growth is directly proportional to the concentration of the analyte in the sample.

Iron and Zinc analysis used modified method from (AOAC 984.27 mod, 927.02 mod, 985.01 mod, 965.17 mod). Elemental Analysis by ICP uses Inductively Coupled Plasma Optical Emissions Spectrophotometry (ICPOES) to quantify iron and zinc in a variety of sample matrices.10 g of the sample was weighed into a crucible. It was then ashed in a muffle oven at 5500 °C for greater than 5 hours. The ash then dissolved in mainly hydrochloric acid with a small amount of nitric acid while boiling on a hotplate. This solution was transferred to a volumetric flask and brought to volume with deionized water. An appropriate dilution was performed, and the solution was introduced into the ICP-OES instrument. The emission signal was measured at 238.2 nm for iron and 206.2 nm for zinc. Calibration standards, drift control standards, and control samples were analyzed with each batch to ensure instrument suitability and acceptable results. The iron and zinc signal were adjusted by gallium internal standard recovery determined using the 294.4 nm wavelength. Measurements were computed by the ICP software (Winlab).

4.2.5 Statistical Analysis

Samples were analyzed in duplicate. ANOVA was conducted to compare the results using SAS software (SAS, Cary, NC). Significance of differences was determined by Tukey's test ($p < 0.05$).

4.3. Results and Discussion

4.3.1 Pasting properties using Rapid Visco Analyzer Viscosity

All raw material mixes were analyzed on RVA to see the potential changes in viscosity with changes in formulation. Pasting profile decreased as starch level decreases due addition of micronutrient premix in the formulation as shown in Table 4.3. Viscosities of extruded rice flour decreased when compared with raw rice as extruded samples were already gelatinized during extrusion process (Guha et al.,1998). Extrusion parameters like barrel temperature and screw speed also affect the gelatinization properties of extruded rice. Higher screw speed resulted in lowest peak viscosity in formulation 4 where highest premix was added which also contributed to reducing the starch content. Starch granules undergoes gelatinization and degradation by heat and moisture on hydrogen bonding which is present in polysaccharide chains (Camire et al., 1990). Functional properties of starch-based products are mostly analyzed with pasting properties using RVA, which shows relative measure of degradation of starch, disintegration, swelling, and gelling properties occur during extrusion process (Ryu et al., 1993). Peak viscosity measures the ability to make a paste during heating phase when starch granule starts to swell.

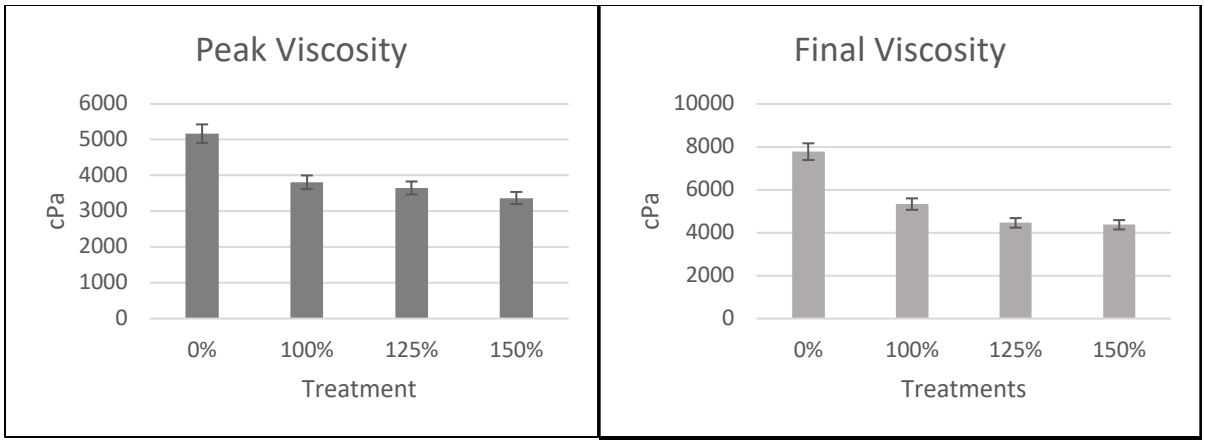


Figure 4.2. Peak and final Viscosity for all formulations

Table 4.3. Pasting properties of all formulations

Recipe	Peak viscosity (cP)	Final viscosity (cP)	Pasting Time (s)	Pasting Temp (°C)
Control	5169± 113[a]	7774.5 ± 87[a]	358 ± 5 [d]	83.12 ± 0[a]
100%	381± 136 [b]	5332.5 ± 48.5[b]	403 ± 2 [b]	84.37 ± 0[ab]
125%	3650± 94 [b]	4458 ± 84[c]	420 ± 1 [a]	84.8 ± 0[c]
150%	3371± 37 [b]	4271.5 ± 97.5[c]	417 ± 2 [a]	85.15 ± 0[d]

4.3.2 Specific Mechanical Energy

In our study, we found that higher starch formulation (control) showed highest peak viscosity and required more mechanical energy. There are several factors involve in the operation of extrusion including barrel temperatures, die temperatures, pressure, screw speed, moisture, flow rate of material, die configuration, extruder design (single vs twin screw extruder), which control mechanical energy and residence time in the extruder (Owusu-Ansah et al., 1983). Low in barrel moisture increases expansion and increased barrel temperature, screw speed, throughput and specific energy input increase reduction of vitamins during extrusion (Killeit 1994).

It is also linked linearly to in barrel moisture and screw speed, whereas barrel temperature and feed rate also significantly impact SME (Onwulata et al., 1994). Figure 4.4 showed as we decrease starch in formulation, less mechanical energy was required during extrusion. As we increase screw speed, SME was also expected to increase which was observed in our formulation with 150% premix. Screw speed of 307RPM showed 80.38 (KJ/kg) in 150% formulation.

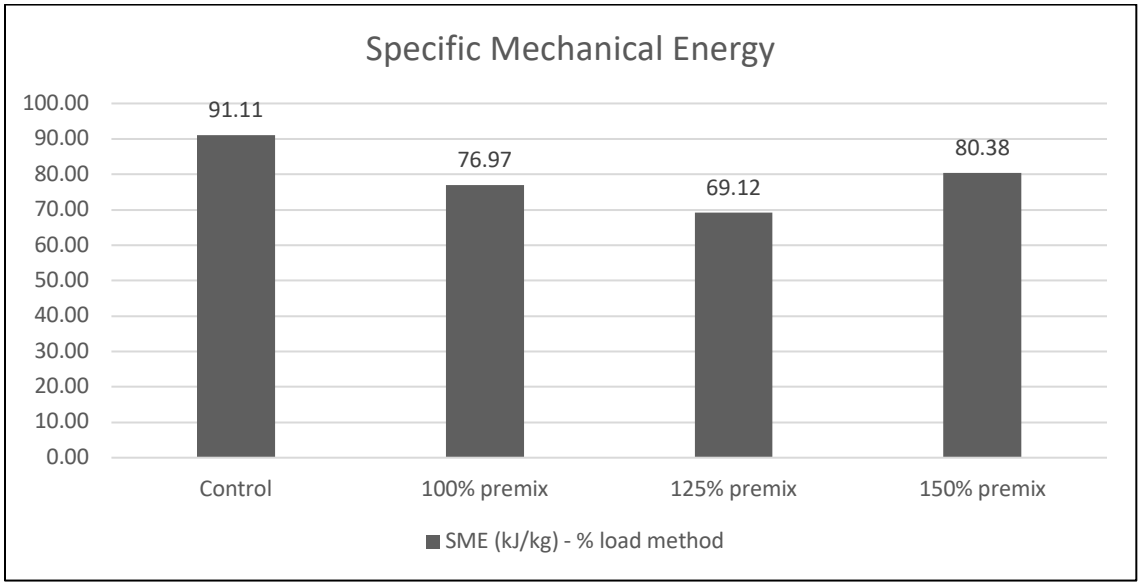


Figure 4.3. SME Calculations (% load) for all formulations

4.3.3 Fortified Rice kernels (FRKs)- Visual analysis

Significant color changes were observed in the final product when micronutrient premix was added. Highest premix level showed darker FRKs. Moretti et al., 2005 in the study for the development of iron fortified extruded kernels showed difference in the color due color masking properties of ferric pyrophosphate. In our study, no instrument was used to differentiate the color changes in different formulations.

4.3.4 Micronutrient Analysis

All products micronutrient analysis were performed as shown in Table 4.4. Using supplier specifications for micronutrient premix, levels of micronutrients were estimated as we increase premixes in the formulation to relate the losses during extrusion.

Table 4.4. Micronutrient Results and Raw material estimations

Parameters	Control		100 % Premix			125% Premix			150% Premix		
	RM Estimation	Analytical Results	RM Estimation	Analytical Results	% Reduction	RM Estimation	Analytical Results	% Reduction	RM Estimation	Analytical Results	% Reduction
Vitamin A	No Premix	1.97	780.75	512.00	-34.42	975.93	649.00	-33.50	1171.12	736.50	-37.11
Vitamin B1		0.00	0.74	0.71	-3.55%	0.92	0.86	-6.62	1.11	0.97	-13.02
Folic Acid		0.00	0.163	0.10	-36.17	0.20	0.13	-37.42	0.24	0.16	-32.92
Iron		0.02	4.4	3.71	-15.68	5.5	4.88	-11.27	6.6	6.33	-4.09
Zinc		0.04	6.17	5.71	-7.54	7.71	7.48	-3.01	9.25	9.12	-1.46

RM= raw material micronutrient estimation based on supplier COA, Analytical results = Final product results from lab

4.3.5 Effect of Extrusion on micronutrient reduction

Minerals are considered as essential element, found as minor portion of food composition while playing vital role for humans from nutritional aspect. Minerals are inorganic elements that are hard to decompose with simple chemical reactions. Calcium, sodium, phosphorus, potassium, magnesium, sulfur and chloride falls under macro-minerals category whereas iron, zinc, copper, iodine, chromium, manganese, selenium and molybdenum are considered as micro-minerals as required in small amounts. Though required in small amount but they perform key functions. For example, Iron helps to prevent anemia (condition in which body stop producing adequate healthy red blood cells RBCs), zinc boosts immune system, copper needed for iron metabolism, iodine helps in growth and metabolism, chromium works with insulin to regulate blood sugar levels, manganese and molybdenum are part of many enzymes, selenium works as antioxidant (Hänsch & Mendel., 2009).

To prevent mineral deficiencies and improve nutritional value mostly they are fortified in food in different chemical forms. Extrusion processing helps to reduce the inhibiting factors like phytate and improve absorption of minerals (Alonso et al., 2001). Phytates are compounds naturally present in cereals and grains, despite beneficial aspect they reduce mineral absorption and plays role in mineral deficiency in human and animals (Nikmaram et al., 2017). Extrusion processing generally affects macromolecules like starch, minerals typically not affected by extrusion processing (Camire et al., 1990).

Minerals are generally considered as heat stable and expected to have no impact during extrusion processing. (Singh et al., 2007). Minerals stability has not been extensively studied (Camire et al., 1990) but there are some recent studies on mineral stability where extrusion enhanced absorption of most minerals in bean-based diets (Signh et al., 2007)

Iron act as a catalyst especially when present in the form of ferrous state and catalyze lipid oxidation and affect shelf life of food (Camire et al., 1990) Fortification of rice with iron and zinc were done using hot and cold extrusion techniques to study the retention of these minerals at different storage conditions (25 °C/ 60% RH & 40 °C/75%RH), no significant changes were observed at different conditions except one point, where zinc reduction were recorded at 40 °C/75%RH (Kuong et al., 2016). Singh et al., 2000 results showed increase in mineral levels with addition of wheat bran and no reduction in the mineral content after extrusion. Minerals are considered as stable as they don't get degraded if exposed to light, heat, oxidizing agents or other extreme factors that can affect vitamins (de Silva et al., 2016)

In general, extrusion helps improves the absorption of many minerals and showed positive aspect to reduce anti nutritional factors including phytate and tannins

In our experiment Iron is in the form of ferric pyrophosphate and zinc as zinc oxide were incorporated in different levels to produce fortified rice kernels as shown in Table 4. We found reduction from 4.09% – 15.68% for iron and 1.46% - 7.54% for zinc. Extrusion typically does not have any significant impact on minerals as proven from other studies, but this deviation might be due to sampling and other limitation due analytical methods.

Fat soluble vitamins includes vitamin A, D, E and K. Vitamin A is a fat-soluble vitamin that is stored in liver. It follows the same absorption mechanism as fat. It's typically found in animal products such as meat, fish, poultry, and dairy products in the form of retinyl acetate or retinyl palmitate (Preformed vitamin A) whereas it's also found in plant-based foods such as vegetables and fruits in the form of beta carotene (Pro vitamin A). Vitamin A helps to form and maintain healthy skin, teeth, skeletal and soft tissues. It is also known as retinol because it produces the pigment in the retina of eye (Institute of medicine, food, and nutrition board, 2001).

Vitamin A deficiency (VAD) is a common and leading public health problem which leads to blindness in children, night blindness in pregnant women, severe infections, and risk of maternal mortality (World Health Organization, 2013).

Fortification of vitamin A is one of the ways to address VAD. Retention of vitamin A is challenging as it is sensitive to heat, light, oxygen, humidity, and acid. Vitamin A is considered as one of the most sensitive and unstable due its chemical structure and composition, therefore its challenging to study the effect of extrusion on retention of vitamin A. There are several factors which aid to degradation of vitamin A including temperature, light, oxygen, time and pH (Camire et al .,1990). Fortified rice kernels using hot extrusion were studied for retention of retinyl palmitate and its stability at different conditions, showed around 40% of degradation (including extrusion, drying, storage and cooking) were recorded during storage of 18 weeks. Hot extrusion of FRKs with retinyl palmitate, iron and zinc were only 5.3% during extrusion processing, 28.5% during storage at 30C in plastic packaging and 9.8% during cooking. Study showed good retention of retinyl palmitate during hot extrusion in the presence of iron and zinc (Pinkaew et al., 2012)

Morin et al., 2021 reviewed vitamin retention in different food including pet food and showed high temperature >100C during extrusion, can degrade vitamins specially vitamin A. 26%-93% retention of vitamin A after extrusion were reported due sensitivity to heat, light, oxygen and acid. Similar results were found for thiamine where 10-100% of retention after extrusion were reported. Vitamin A degrade in corn/soybean/groundnut mixture around 52.5% during extrusion (de Muelenaere & Buzzard, 1969). Several other studies showed range of % retention after extrusion which showed extrusion could play role in degradation of vitamins (Riaz et al., 2009)

Vitamin A palmitate was used in our study, around 37% of reduction was found in all three formulations, correlating results with previous studies.

Vitamin B1 as thiamine mononitrate showed 3- 13% of reduction after extrusion process. Water soluble vitamins like vitamin B is also considered sensitive to high temperatures and increase temperature during extrusion process can result in decrease of thiamin retention (Riaz et al., 2009)

Li et al., 2011 studied extruded fortified ultra-rice with vitamin A, Iron, vitamin B1 and folic acid, where folic acid was stable under high temperature and humidity (40C, 60RH%). Fortified flour with folic acid were studied in different packaging bags at different temperatures where not significant losses of folic acid were not observed (Hemery et al., 2020). No significant losses in folic acid were noted while studying folic acid enriched corn masa flour, tortillas, and chips during six months shelf-life study Phillips et al., 2017. Degradation of folic acid were found significant in fortified vitamin juice (Frommherz et al., 2014). Further studies showed little or no loss for retention of folic acid in fortified breakfast cereals and vitamin- mineral premixes (Berry et al., 2010).

Our study showed 32% of reduction for folic acid as a result of extrusion process which is comparable to Riaz et al., 2009 where 27% of reduction during extrusion was reported.

4.3.6 Recommendation for Optimum formulation for extrusion

In our study, we tried to see the impact of extrusion on different micronutrients and recommendation for optimum formulation to be used for rice fortification using extrusion. USDA and USAID have recommended levels to be present in the final product. For vitamin A 500IU, vitamin B1 0.5mg, folic acid 0.13mg, Iron 4 mg and zinc 6 mg (USDA MR26 Table1), with 20 ± range for all micronutrients. Keeping that standard in mind, our formulation (100%

premix) gave best results. Overages more than that would exceed the acceptable level suggested by USDA, so 100% premix formulation is considered as optimum formulation at production level and suggested to be used to produce extruded fortified kernels.

4.4 Conclusion

This research could be used as guideline to estimate the levels of micronutrients that needs to be added extra to obtain desired levels of micronutrient in final products. Micronutrient results showed around 34%- 37% of losses for vitamin A, 3.55-13% for vitamin B1, 32% - 36% for folic acid, 4%-15% for iron, 1%-7% for zinc during extrusion and drying process, so formations could be done accordingly to get right levels of micronutrients in the final product.

4.5 References

Alavi, S., Bugusu, B., Cramer, G., Dary, O., Lee, T. C., Martin, L., ... & Wailes, E. (2008). Rice fortification in developing countries: a critical review of the technical and economic feasibility. *Institute of Food Technologists, Washington DC*.

Alonso, R., Rubio, L. A., Muzquiz, M., & Marzo, F. (2001). The effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. *Animal Feed Science and Technology*, *94*(1-2), 1-13.

Berry, R. J., Bailey, L., Mulinare, J., Bower, C., & Dary, O. (2010). Fortification of flour with folic acid. *Food and nutrition bulletin*, *31*(1_suppl1), S22-S35.

Camire, M. E., Camire, A., & Krumhar, K. (1990). Chemical and nutritional changes in foods during extrusion. *Critical Reviews in Food Science & Nutrition*, *29*(1), 35-57.

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food chemistry*, *196*, 309-323.

Dalbhagat, C. G., Mahato, D. K., & Mishra, H. N. (2019). Effect of extrusion processing on physicochemical, functional and nutritional characteristics of rice and rice-based products: A review. *Trends in Food Science & Technology*, *85*, 226-240.

De Muelenaere, H. J. H., & Buzzard, J. L. (1969). Cooker extruders in service of world feeding. *Food technology*.

Espinosa-Ramírez, J., Rodríguez, A., De la Rosa-Millán, J., Heredia-Olea, E., Pérez-Carrillo, E., & Serna-Saldívar, S. O. (2021). Shear-induced enhancement of technofunctional properties of whole grain flours through extrusion. *Food Hydrocolloids*, *111*, 106400.

Espinosa-Ramírez, J., Rodríguez, A., De la Rosa-Millán, J., Heredia-Olea, E., Pérez-Carrillo, E., & Serna-Saldívar, S. O. (2021). Shear-induced enhancement of technofunctional properties of whole grain flours through extrusion. *Food Hydrocolloids*, *111*, 106400.

Frommherz, L., Martiniak, Y., Heuer, T., Roth, A., Kulling, S. E., & Hoffmann, I. (2014). Degradation of folic acid in fortified vitamin juices during long term storage. *Food chemistry*, *159*, 122-127.

Guha, M., Ali, S. Z., & Bhattacharya, S. (1998). Effect of barrel temperature and screw speed on rapid viscoanalyser pasting behaviour of rice extrudate. *International journal of food science & technology*, *33*(3), 259-266.

Hänsch, R., & Mendel, R. R. (2009). Physiological functions of mineral micronutrients (cu, Zn, Mn, Fe, Ni, Mo, B, cl). *Current opinion in plant biology*, *12*(3), 259-266.

Hof, J. (2006). Vitamin and mineral retention and sensory evaluation of extruded fortified rice.

Hussain, S. Z., Singh, B., & Naik, H. R. (2014). Viscous and thermal behaviour of vitamin A and iron-fortified reconstituted rice. *International Journal of food science & Technology*, *49*(5), 1324-1329.

Hemery, Y. M., Fontan, L., Laillou, A., Jallier, V., Moench-Pfanner, R., Avallone, S., & Berger, J. (2020). Influence of storage conditions and packaging of fortified wheat flour on microbial load and stability of folate and vitamin B12. *Food chemistry: X*, *5*, 100076.

Killeit, U. (1994). Vitamin retention in extrusion cooking. *Food Chemistry*, *49*(2), 149-155.

Kuong, K., Laillou, A., Chea, C., Chamnan, C., Berger, J., & Wieringa, F. T. (2016). Stability of Vitamin A, iron and zinc in fortified rice during storage and its impact on future national standards and programs—Case study in Cambodia. *Nutrients*, *8*(1), 51.

Launay, B., & Lisch, J. M. (1983). Twin-screw extrusion cooking of starches: flow behaviour of starch pastes, expansion and mechanical properties of extrudates. *Journal of Food Engineering*, *2*(4), 259-280.

Li, Y. O., Diosady, L. L., & Jankowski, S. (2011). Folic acid stability in the presence of various formulation components including iron compounds in fortified extruded Ultra Rice® over prolonged storage at 40° C and 60% relative humidity (RH). *International Journal of Food Science & Technology*, *46*(2), 379-385.

Moretti, D., Lee, T. C., Zimmermann, M. B., Nuessli, J., & Hurrell, R. F. (2005). Development and evaluation of iron-fortified extruded rice grains. *Journal of food science*, *70*(5), S330-S336.

Morin, P., Gorman, A., & Lambrakis, L. (2021). A literature review on vitamin retention during the extrusion of dry pet food. *Animal Feed Science and Technology*, *277*, 114975.

Nikmaram, N., Leong, S. Y., Koubaa, M., Zhu, Z., Barba, F. J., Greiner, R., ... & Roohinejad, S. (2017). Effect of extrusion on the anti-nutritional factors of food products: An overview. *Food control*, *79*, 62-73.

Onwulata, C. I., Mulvaney, S. J., & Hsieh, F. (1994). System analysis as the basis for control of density of extruded cornmeal. *Food Control*, *5*(1), 39-48.

Owusu-Ansah, J., Van de Voort, F. R., & Stanley, D. W. (1983). Physicochemical changes in cornstarch as a function of extrusion variables. *Cereal Chemistry*, *60*(4), 319-324.

Pinkaew, S., Wegmuller, R., & Hurrell, R. (2012). Vitamin A stability in triple fortified extruded, artificial rice grains containing iron, zinc and vitamin A. *International journal of food science & technology*, *47*(10), 2212-2220.

Riaz, M. N., Asif, M., & Ali, R. (2009). Stability of vitamins during extrusion. *Critical Reviews in Food Science and Nutrition*, *49*(4), 361-368.

Ryu, G. H., Neumann, P. E., & Walker, C. E. (1993). Pasting of wheat flour extrudates containing conventional baking ingredients. *Journal of food science*, *58*(3), 567-573.

Singh, D., Chauhan, G. S., Suresh, I., & Tyagi, S. M. (2000). Nutritional quality of extruded snacks developed from composite of rice brokens and wheat bran. *International Journal of Food Properties*, *3*(3), 421-431.

Singh, S., Gamlath, S., & Wakeling, L. (2007). Nutritional aspects of food extrusion: a review. *International Journal of Food Science & Technology*, *42*(8), 916-929.

Steiger, G., Müller-Fischer, N., Cori, H., & Conde-Petit, B. (2014). Fortification of rice: technologies and nutrients. *Annals of the New York Academy of Sciences*, *1324*(1), 29-39.

Xu, E., Wu, Z., Pan, X., Long, J., Wang, F., Xu, X., ... & Jiao, A. (2016). Effect of enzymatic (thermostable α-amylase) treatment on the physicochemical and antioxidant properties of extruded rice incorporated with soybean flour. *Food chemistry*, *197*, 114-123.

Chapter 5 - Conclusion and Future Work

Overall, this research has significant impact specially for food aid purposes. This research highlighted the importance of sample preparation and grinding on micronutrient results. Optimum grinding is required to achieve better results. Coarser grind (15 sec) or excessive grind (100 sec) are not ideal where less grinding would not allow proper sample extraction and extra grind also does not improve results with high chances of micronutrient losses during sample preparation. Significant differences in analytical techniques leads to results deviation as highlighted in chapter 2. Fortified rice kernels (FRKs) showed better results where micronutrient premix was in concentration form as compared to fortified rice in which it was 100-fold dilution. Greater deviation (delta%) and lower precision (COV) were observed for fortified rice, which was expected, because the sample size is very small it might not be the true representation of the sample though all samples were mixed homogeneously. Minerals are more stable and easier to extract as compared to vitamins which needs series of steps for extraction and sensitive to environmental factors.

Accelerated shelf-life study showed that vitamin A as retinyl palmitate is very susceptible to heat and huge losses were noted irrespective of packaging which questions If rice is a suitable carrier to address vitamin A deficiencies. Minerals retention was stable as compared to vitamin A which further affirms that minerals does not affect by heat. Descriptive sensory analysis also gives valuable results to analyze changes in fortified rice. Color and aroma significantly changed over the time whereas factorability was not statistically significant for analyzed samples in all packaging and all three temperature conditions.

Production of fortified rice kernels (FRKs) investigated the degree of micronutrient losses during hot extrusion-based production of fortified rice kernels using rice flour and

micronutrient premix. This study showed over 37% of losses for vitamin A during extrusion and drying process and could be used as guideline for to attain recommended levels as per daily intake.

It is clear from this study that different analytical labs and methods lead to vastly different results for micronutrient analyses, and it is important to standardize analytical methods for fortified rice to ensure reliable results. The three different forms of packaging used in the study did not differ statistically in terms of preventing micronutrient deterioration. The extremely high deterioration rates of vitamin A irrespective of packaging type, leads to the conclusion that either a different form of fortification (example, cooking oil) or a more impervious packaging should be explored for increasing the shelf life of fortified rice. Also, this study needs to be replicated for extruded fortified rice kernels, as the data described here are based on coated fortified rice kernels.

This study has great operational significance for food aid and would serve as basis for fortified rice suppliers and food aid organizations to improve quality and efficacy. These results will help in understanding gaps in current packaging and transition to new more effective packaging with optimum micronutrients forms for fortified rice.

Appendix A-Chapter 2, 4- way ANOVA Results

Use this as only significant interactions are included

The GLM Procedure

Dependent Variable: delta

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	99	661931.0878	6686.1726	22.68	<.0001
Error	100	29486.9265	294.8693		
Corrected Total	199	691418.0143			

R-Square	Coeff Var	Root MSE	delta Mean
0.957353	62.16719	17.17176	27.62190

Source	DF	Type I SS	Mean Square	F Value	Pr > F
rice	1	32005.5120	32005.5120	108.54	<.0001
dil	2	62759.8355	31379.9178	106.42	<.0001
lab	4	154364.3320	38591.0830	130.88	<.0001
micro	4	60943.1246	15235.7812	51.67	<.0001
rice*lab	4	35453.6822	8863.4206	30.06	<.0001
rice*micro	4	17194.4188	4298.6047	14.58	<.0001
dil*lab	8	29007.8495	3625.9812	12.30	<.0001
dil*micro	8	6463.7284	807.9660	2.74	0.0089
lab*micro	16	190247.0358	11890.4397	40.32	<.0001
rice*lab*micro	16	26573.5628	1660.8477	5.63	<.0001
dil*lab*micro	32	46918.0062	1466.1877	4.97	<.0001

Appendix B- Chapter 3, 4-Way ANOVA Results

The GLM Procedure

Class Level Information

Class	Levels	Values
temp	3	27 33 43
pack	3	HYBRID LWP WPP
time	5	0 6 13 19 26
micro	5	FA Iron VitA VitB Zinc

Number of Observations Read	450
Number of Observations Used	450

The GLM Procedure

Dependent Variable: conc

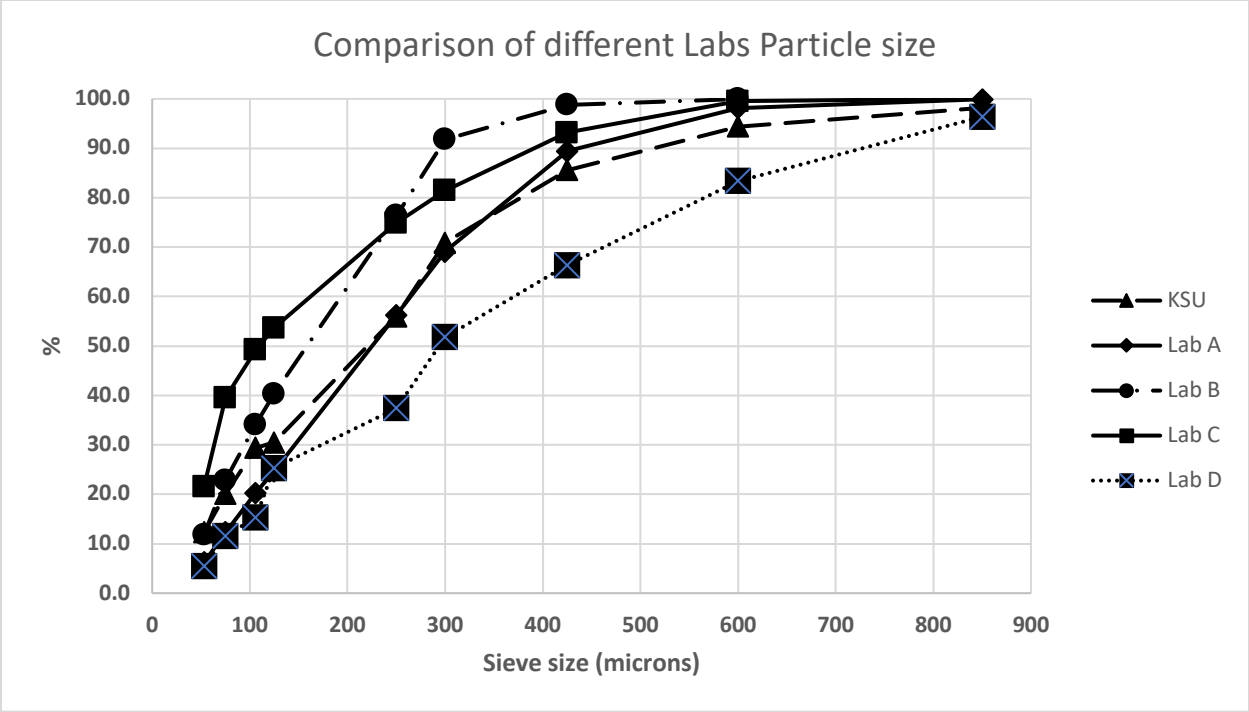
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	144	18591249.68	129105.90	858.07	<.0001
Error	305	45890.30	150.46		
Corrected Total	449	18637139.98			

R-Square	Coeff Var	Root MSE	conc Mean
0.997538	13.48979	12.26621	90.92962

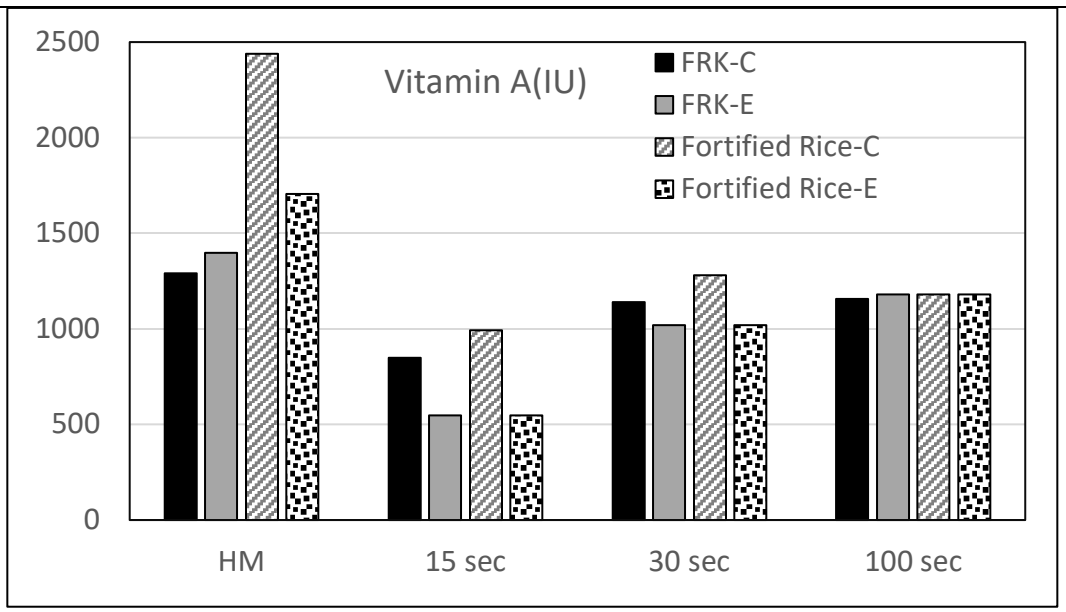
Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	376649.62	188324.81	1251.66	<.0001
pack	2	460.97	230.48	1.53	0.2178
time	4	446724.05	111681.01	742.26	<.0001
micro	4	13891724.34	3472931.09	23082.1	<.0001
temp*pack	4	944.58	236.15	1.57	0.1823
temp*time	8	114692.69	14336.59	95.29	<.0001
temp*micro	8	1502628.31	187828.54	1248.36	<.0001
pack*time	8	1810.18	226.27	1.50	0.1550
pack*micro	8	1785.52	223.19	1.48	0.1624
time*micro	16	1783928.57	111495.54	741.03	<.0001
temp*pack*time	16	5068.40	316.78	2.11	0.0082
temp*time*micro	32	457727.74	14303.99	95.07	<.0001
pack*time*micro	32	7104.70	222.02	1.48	0.0518

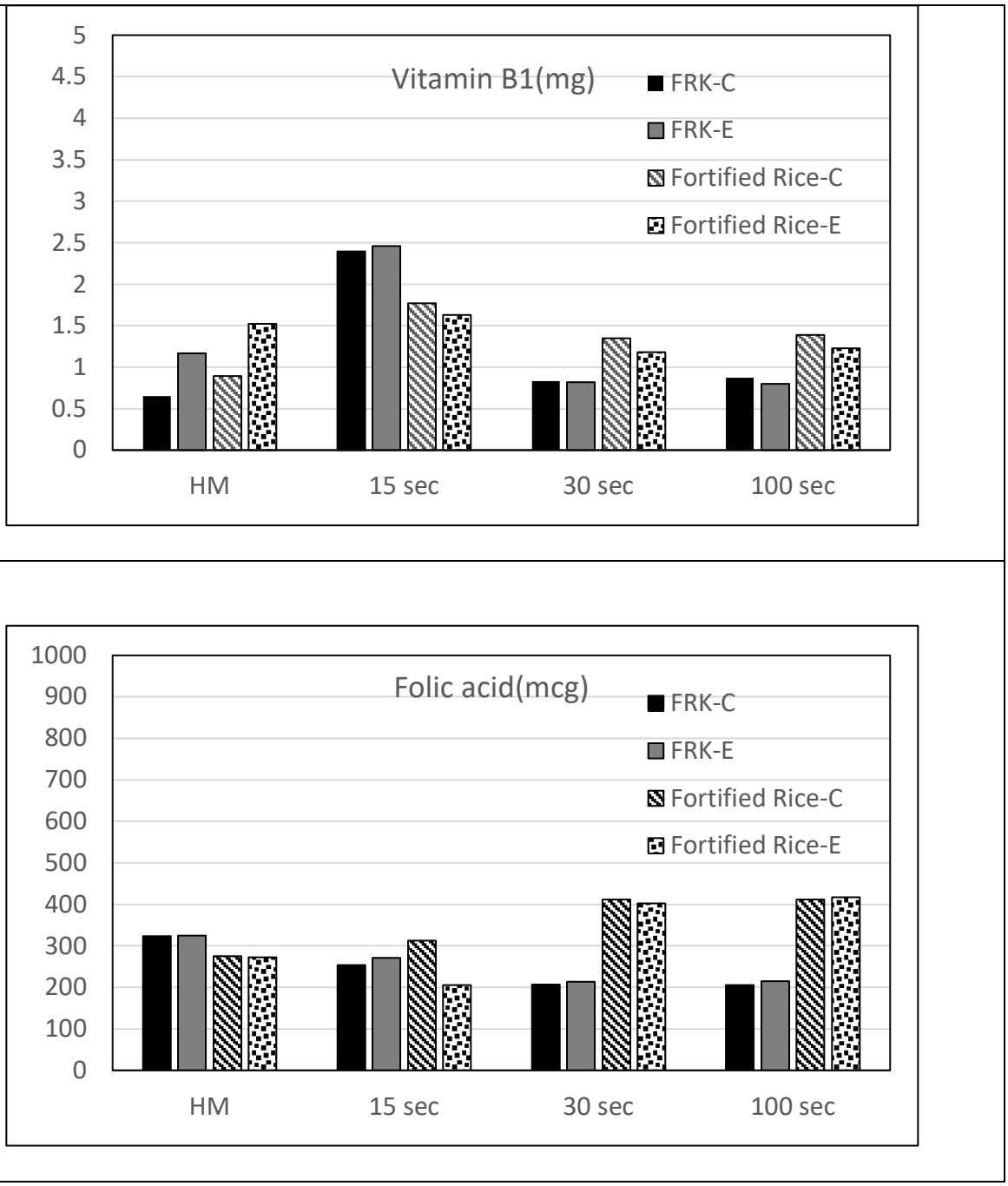
Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	376649.62	188324.81	1251.66	<.0001
pack	2	460.97	230.48	1.53	0.2178
time	4	446724.05	111681.01	742.26	<.0001
micro	4	13891724.34	3472931.09	23082.1	<.0001
temp*pack	4	944.58	236.15	1.57	0.1823
temp*time	8	114692.69	14336.59	95.29	<.0001
temp*micro	8	1502628.31	187828.54	1248.36	<.0001

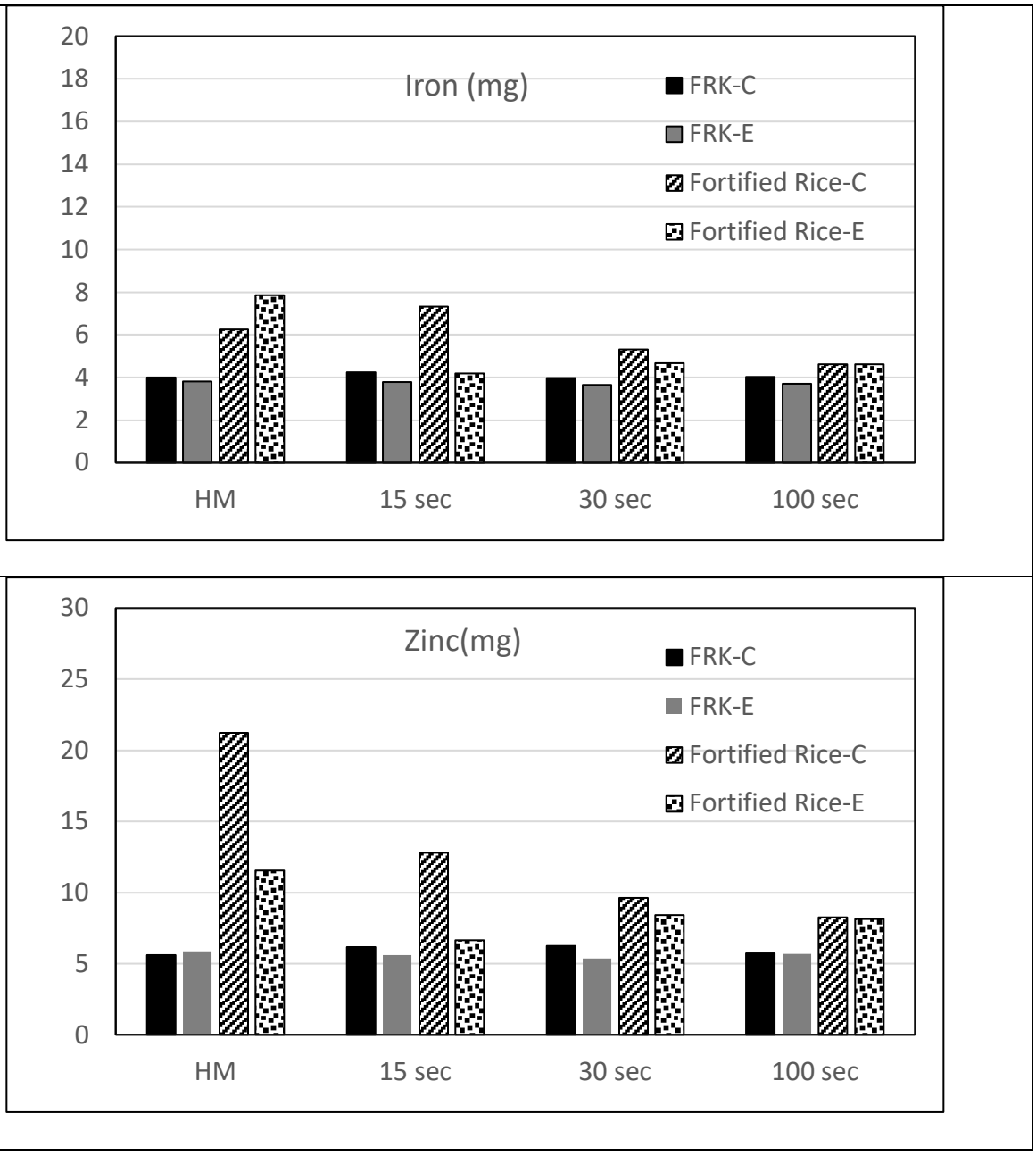
Source	DF	Type III SS	Mean Square	F Value	Pr > F
pack*time	8	1810.18	226.27	1.50	0.1550
pack*micro	8	1785.52	223.19	1.48	0.1624
time*micro	16	1783928.57	111495.54	741.03	<.0001
temp*pack*time	16	5068.40	316.78	2.11	0.0082
temp*time*micro	32	457727.74	14303.99	95.07	<.0001
pack*time*micro	32	7104.70	222.02	1.48	0.0518



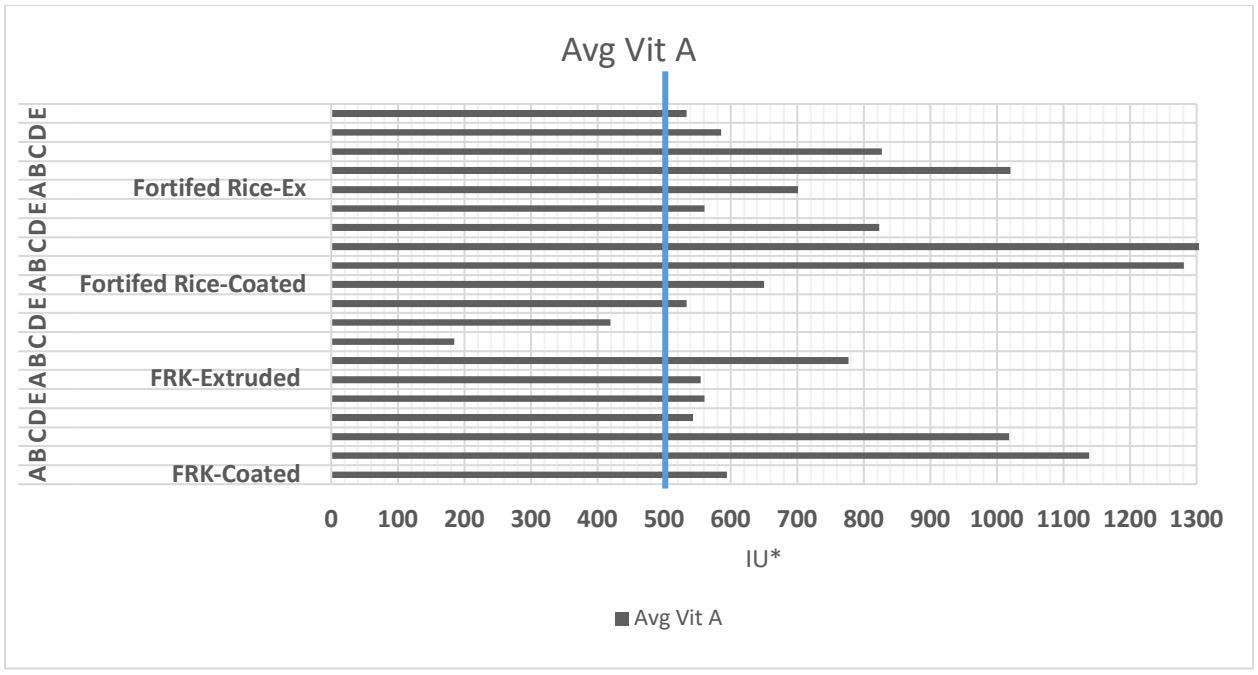
Particle size comparison across all labs

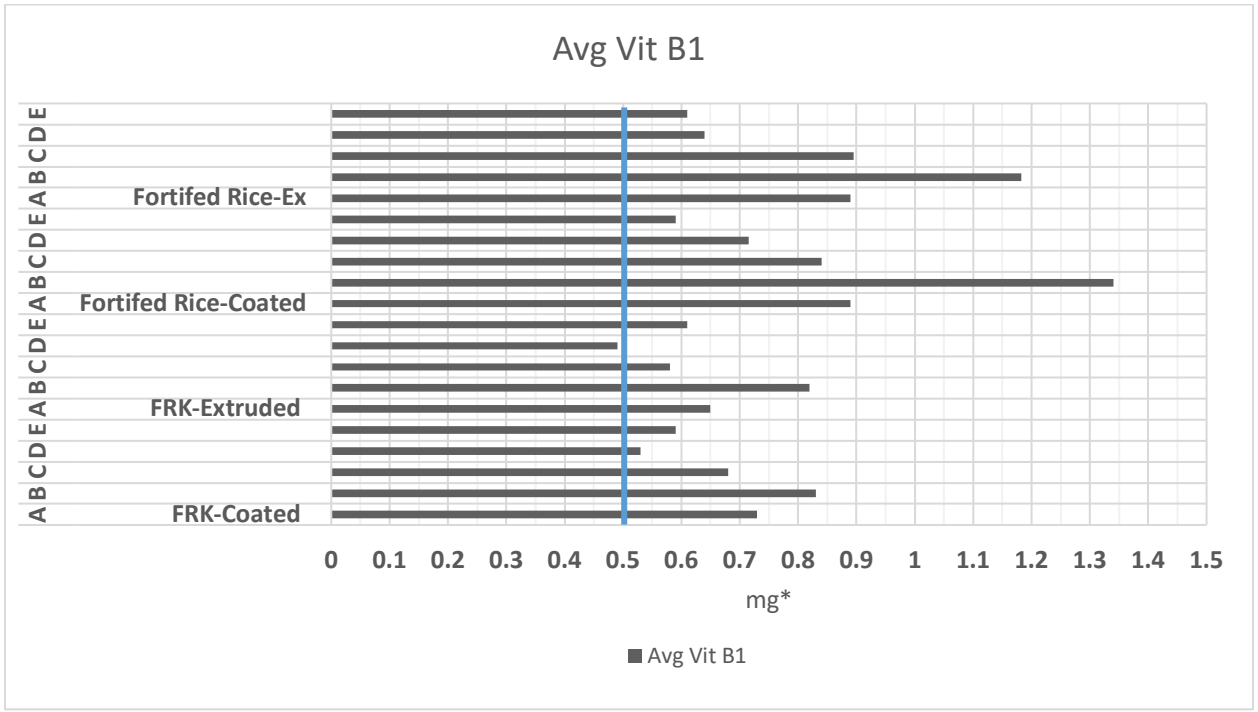


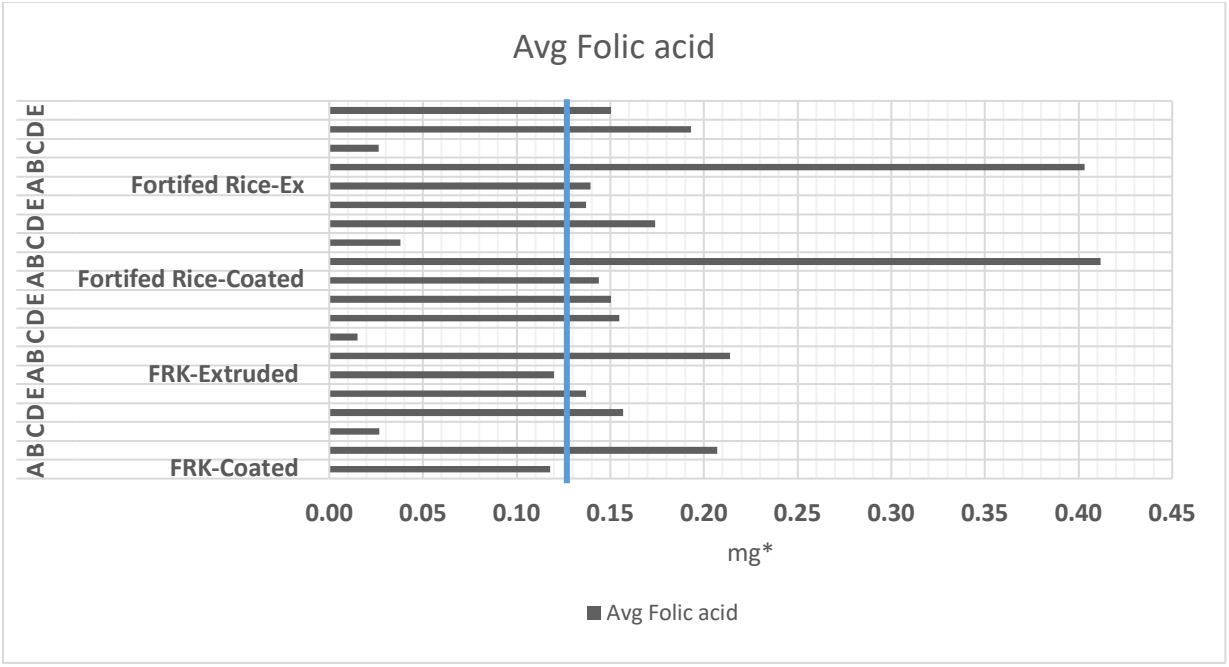

Vitamin B1(mg)
FRK-C
FRK-E
Fortified Rice-C
Fortified Rice-E
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
HM
15 sec
30 sec
100 sec
Folic acid(mcg)
FRK-C
FRK-E
Fortified Rice-C
Fortified Rice-E
1000
900
800
700
600
500
400
300
200
100
0
HM
15 sec
30 sec
100 sec

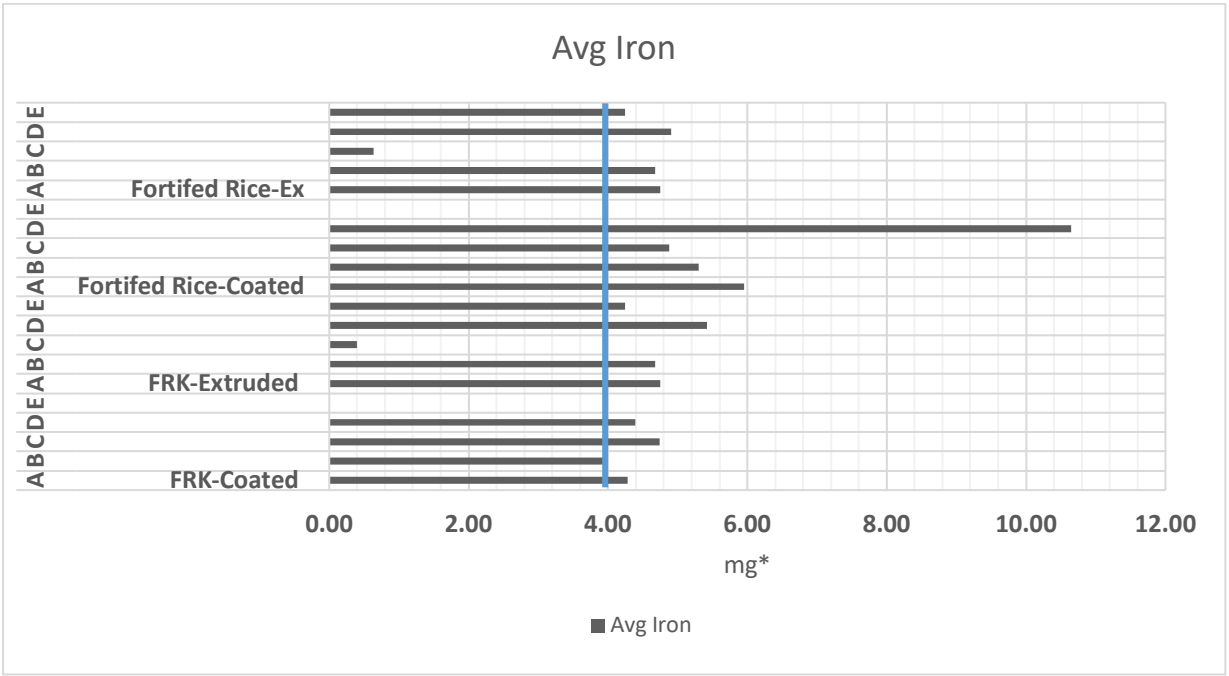

Iron (mg)
FRK-C
FRK-E
Fortified Rice-C
Fortified Rice-E
20
18
16
14
12
10
8
6
4
2
0
HM
15 sec
30 sec
100 sec
Zinc(mg)
FRK-C
FRK-E
Fortified Rice-C
Fortified Rice-E
30
25
20
15
10
5
0
HM
15 sec
30 sec
100 sec

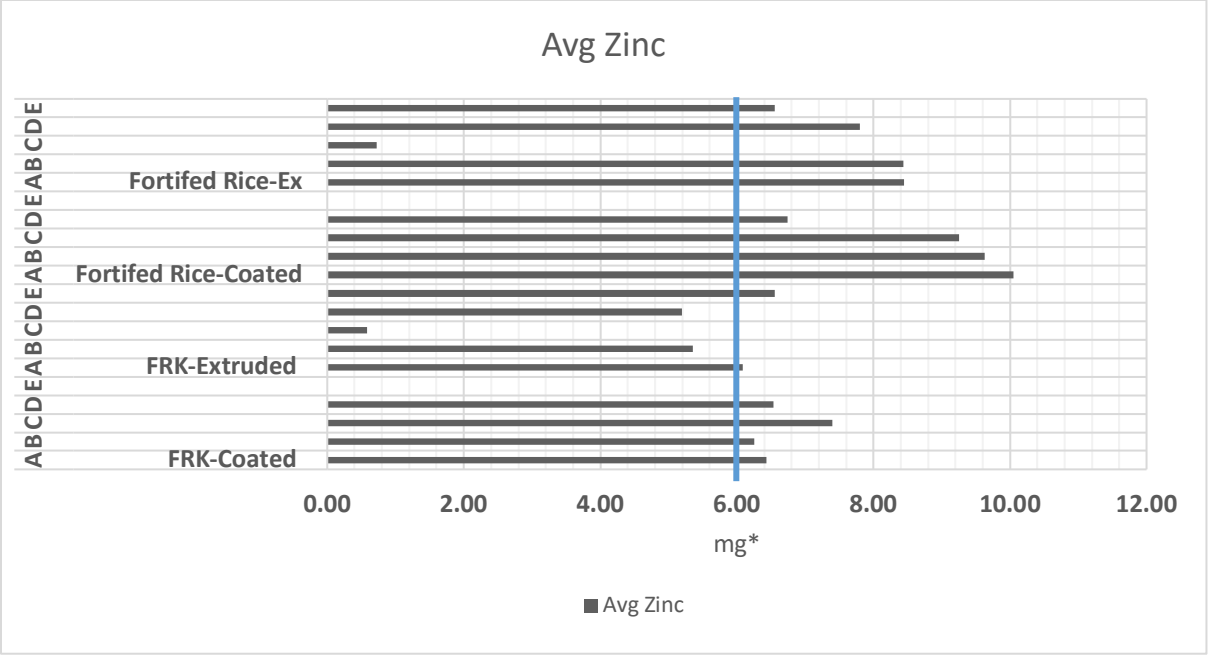
Comparison of different grind with each micronutrient






Avg Folic acid
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
Fortifed Rice-Ex
Fortifed Rice-Coated
FRK-Extruded
FRK-Coated
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
mg*
Avg Folic acid


Avg Iron
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
A
B
C
D
E
Fortifed Rice-Ex
Fortifed Rice-Coated
FRK-Extruded
FRK-Coated
0.00
2.00
4.00
6.00
8.00
10.00
12.00
mg*
Avg Iron



Comparison of micronutrients with different techniques, dilution, and methods