FORMULATION OF FORTIFIED DRIED BROKEN RICE AS COMPLEMENTARY FOOD

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Thesis
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ABSTRACT

Complementary food plays an important role in contributing adequate nutrients to infants after the first four to six months. Calcium and iron were found to be inadequate in complementary food of breast-fed infants in Thailand, while thiamin, and folate were potentially the problem nutrients. At least 90% of the Thai mothers prepared complementary food themselves by using rice as a basic ingredient. Dried broken rice was a convenient product that was widely used for preparing complementary food. In this study, the feasibility of fortifying dried broken rice with calcium, iron, thiamin and folate at the levels recommended in the WHO guideline (2001) was conducted. Four fortification processes were studied. The most feasible process was pre-drying broken rice at 90^{o}C for 1 h, soaking in vitamin and mineral solution (at ratio 2:1, rice:solution), and finally drying at 70^{o}C for 1 h 50 min. The product needed 8-9 min. for cooking. Calcium lactate or calcium lactate gluconate was the calcium source while ferrous sulfate, ferrous lactate, or sodium iron EDTA was the iron source. Vitamin sources included thiamin hydrochloride and folic acid. Per serving (20 g), the product consisted of 40 mg Ca, 5.3 mg Fe, 0.08 mg thiamin, and 11 μg folate. The result from a home use test in 52 infants aged 4-24 mo. indicated that the complementary foods prepared by using the product as a basic ingredient was accepted by mothers, caretakers, and infants in terms of sensory quality and convenience. Approximately 5 and 12% of Ca and Fe, respectively was lost during processing, while thiamin loss was approximately 20% and folate loss ranged from 15-41%. Losses of thiamin during accelerated storage condition (42^{o}C for 3 mo.) were not significant ($p>0.05$). Both metallized and commercial plastic bags could not prevent rancidity in most products except the one fortified with NaFeEDTA during storage. An *In vitro* bioavailability study indicated that NaFeEDTA was the most effective as compared to ferrous sulfate and ferrous lactate.

KEY WORDS: COMPLEMENTARY FOOD/FORTIFICATION/MINERALS/ VITAMINS/ IRON BIOAVAILABILITY

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(FORMULATION OF FORTIFIED DRIED BROKEN RICE AS COMPLEMENTARY FOOD)

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บทคัดย่อ

ในวัยทารกที่มีอายุตั้งแต่ 4หรือ 6 เดือนขึ้นไป แหล่งสำคัญของสารอาหารคืออาหารทารก. แคลเซียมและเหล็กในอาหารทารกไทยยังมีปริมาณไม่เพียงพอ นอกจากนี้วิตามินบี1 และโฟเลทยังเป็นสารอาหารสำคัญที่มีปัญหา อย่างน้อย 90%ของแม่เตรียมอาหารทารกเองโดยใช้ข้าวเป็นส่วนประกอบหลัก ข้าวหักอบแห้งมีการใช้เป็นอาหารทารกอย่างแพร่หลายเนื่องจากมีความสะดวกในการปรุง การศึกษานี้เป็นการศึกษาถึงความเป็นไปได้ในการเสริมสารอาหารซึ่งประกอบไปด้วยแคลเซียม เหล็ก วิตามินบี1และโฟเลทตามข้อแนะนำความต้องการสารอาหารจากอาหารทารกของ FAO ในข้าวหักอบแห้ง วิธีผลิต4วิธีถูกพัฒนาขึ้น อย่างไรก็ตามวิธีที่มีความเป็นไปได้และเหมาะสมในด้านการผลิตคือการอบแห้งข้าวหักที่อุณหภูมิ90°ซ เป็นเวลา 1ชม.และแช่ในสารละลายวิตามินและแร่ธาตุ และอบที่70 ° ซ เป็นเวลา 1 ชม.50นาที วิธีการปรุงผลิตภัณฑ์คือนำไปต้มในน้ำเดือดเป็นเวลา 8-9 นาที สารอาหารที่ใช้เสริมประกอบไปด้วย calciumlactate, calciumlactate gluconate, ferrous sulfate, ferrous lactate, sodiumironEDTA, thiaminhydrochloride,และfolic acid โดยระดับในการเสริมกำหนดไว้ที่แคลเซียมและเหล็ก 40 และ 5.3 มิลลิกรัมตามลำดับ วิตามินบี1และโฟเลท 0.08มิลลิกรัมและ11ไมโครกรัมตามลำดับในหนึ่งหน่วยบริโภคของข้าวอบแห้งเสริมสารอาหาร20กรัม ผลการศึกษาโดยใช้การทดสอบแบบ home use test ในทารก52คนช่วงอายุ 4-24 เดือนพบว่าการปรุงอาหารสำหรับทารกโดยใช้ผลิตภัณฑ์นี้เป็นส่วนประกอบหลักได้รับการยอมรับจากกลุ่มผู้ทดสอบทั้งในด้านคุณภาพและความสะดวกในการเตรียม การสูญเสียแคลเซียมและเหล็กจากกระบวนการผลิตเกิดขึ้นประมาณ5และ12% ตามลำดับ ในขณะที่วิตามินบี1และโฟเลทสูญเสีย20% และ15-41%ตามลำดับ การศึกษาอายุการเก็บพบว่าวิตามินซึ่งใช้เป็นอินดิเคเตอร์ในการทดสอบไม่มเกิดการสูญเสีย นอกจากนี้พบว่า ถุงพลาสติกที่ใช้ในเชิงพานิชย์และเมททอลไลซ์ไม่สามารถป้องกันการเกิดกลิ่นเหม็นหืนจากปฏิกิริยาออกซิเดชั่นได้ยกเว้นผลิตภัณฑ์ที่เสริมNaFeEDTA ในการศึกษาความสามารถในการนำธาตุเหล็กไปใช้ประโยชน์ได้โดยวิธีเลียนแบบการย่อยและการดูดซึมในหลอดทดลอง พบว่า NaFeEDTA ที่เสริมในผลิตภัณฑ์นี้มีปริมาณธาตุเหล็กที่ใช้ประโยชน์ได้มีค่ามากกว่า ferrous sulfate และ ferrous lactatae

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CHAPTER I
INTRODUCTION

Complementary food gradually plays more important role in contributing adequate nutrients to infant after 6 months of age. In developing countries, the first year of life is vulnerable period for developing malnutrition that usually coincides with the introduction of complementary food (1,2,3). Inappropriate complementary food can have impact on growth pattern due to insufficient or inappropriate dietary intakes and frequent infection, which in turn impact negatively a social and economic development. Since the 8th National Economic and Social development Plan (1997-2001), Thailand had planned to eliminate, control and reduce nutrition problem including protein energy malnutrition, iron deficiency anemia, iodine and vitamin A deficiencies of preschool children (2). As being reported by Souvaphapsopha (2001), up to 90% of mothers in Thailand prepared complementary foods by themselves and most of them used rice as the basic ingredient. The preparation was normally based on the guideline of Ministry of Public Health for complementary feeding (4). Studies by Souvaphapsopha (2), and Porniammonkhon (5) indicated that most home prepared complementary food could not provide adequate nutrients if infants were breast-fed especially, in the age group 3-24 months. The problem nutrients found inadequate were calcium and iron. In addition, Ministry of Public Health also had concerns on deficiencies in vitamin B1 and folate in infants (personal communication, Sinawat). Low calcium intake in children may induce rickets, growth retardation and low iron intake can cause iron deficiency anemia leading to reduce psychomotor and mental development in infants (6,7). Only 10% of infants in Thailand are fed with these commercial complementary foods, while the rest still prepare by themselves based on the guideline developed by Ministry of Public Health (2,8). Even, the powdered instant form normally contains adequate nutrients, however the product cannot be used to condition (educate and acculturate) infants and young children to wider diet at weaning by providing experience with transitional liquid to-solid variations in

consistency, texture and taste (9). While the ready-to-eat type that is normally packed in glass jar is not well accepted by the Thai consumers due to western style and high cost. Besides the ready-to-eat type mainly aims to educate infants instead of providing enough nutrients within one serving. Ideal complementary foods for agricultural developing country like Thailand should be based on locally available raw material at affordable price. In addition, it should be an educational tool for developing infant to learn different characteristics and varieties of food that are seasonally available in the country.

The complementary foods used in Thailand are rice-based and mostly home-prepared (8). It traditionally is given to Thai infants aged 6-12 months with certain kinds of fruits and animals such as ripe banana, egg yolk, pork, liver, fishes, broth for 2-3 times a day (5). Rice is therefore the suitable choice for fortification with the problem nutrients since it is widely consumed by the children from all socio-economic status. Rice is also the base for complementary food preparation based on the guidelines of Ministry of Public Health, which is the good strategy for nutrition education and utilization of locally available raw materials. While fortification of rice can guarantee the adequacy of the problem micronutrients.

CHAPTER II
OBJECTIVES

General Objective

To study on the feasibility in production of dried broken rice fortified with problem vitamins and mineral to be used for preparing complementary food for Thai infants aged 6-12 months.

Specific objectives

1. To formulate dried broken rice fortified with thiamin, folic acid, vitamin A calcium and iron.
2. To analyze the retention of the fortified vitamins and minerals in the finished product.
3. To evaluate the shelf life of the fortified products.
4. To test *in vitro* iron bioavailability of the fortified product.

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CHAPTER III
LITERATURE REVIEW

3.1 Nutrient requirement for normal infants

3.1.1 Energy requirement

Per unit of body weight, the daily energy requirements of normal infants are three to four times greater than those of the adult (10). Current knowledge (WHO, 1998) about energy requirements of children less than 2 years of age was based don TEE (Total Energy Expenditure) plus the energy cost of growth which was considerably less than the earlier FAO/WHO/UNU recommendations that based on dietary intake (6). Energy expenditure is generally considered to consist of a basal energy component and a component of expenditure of above the basal level (11). Energy expenditure of crying infant, for example, is much above the basal level. **Table 1** shows the energy requirement of infants of different age groups.

3.1.2 Protein, vitamin, and mineral requirement

Over 40% of total protein intake should come from essential amino acid. The goal is satisfied by either breast milk or infant formula. Total protein intake should not exceed 20% of energy needs because the excess nitrogen and minerals supplied by high protein diets would exceed the ability of infant's kidneys to excrete the resulting metabolic waste products (12). The iron stores present in newborns generally are depleted by the time birth weight doubles, at 4 to 6 mo of age. Breast-fed infants need solid food, such as iron- fortified infant cereal or meat to supply extra iron at 6 mo of age. The nutrient requirement during the first year of life, the

recommended nutrient intake per day for different age group of infants, and Dietary Reference Intake for Thai infants are presented in **Table 2, 3, and 4**.

Table 1 Recommended energy intake during the first year of life

Age group (mo)	From Butte, 1995 [1]	
	Kcal / kg/d	Kcal/d
0-2	88	404
3-5	82	550
6-8	83	682
9-11	89	830

[1] Based on energy requirement for total energy expenditure plus growth of breast-fed infants

Source: Brown KH, Dewey KG, Allen LH, 1998

Table 2 Nutrient daily requirement during the first year of life [1]

Nutrient	Recommended nutrient intake[2]				SNI[3]
	Age group (mo)				
	0-2	3-5	6-8	9-11	
Protein(g)	9.6	8.5	9.1	9.6	
Vitamin A (μgRE)	350	350	350	350	
Biotin (μg)					10-200
Folate (μg)	16	24	32	32	
Niacin (mg)	3	3	4	5	
Pantothenic acid (mg)					1.7
Riboflavin (mg)	0.4	0.4	0.4	0.4	
Thiamin (mg)	0.2	0.2	0.2	0.3	
VitaminB6 (mg)	0.2	0.2	0.3	0.4	
Vitamim B12 (μg)	0.3	0.3	0.4	0.4	
Vitamin C (mg)	25	25	25	25	
Vitamin D (μg)	8.5	8.5	7	7	

Table 2 Nutrient requirement during the first year of life [1](cont).

Nutrient	Recommended nutrient intake [2]				SNI [3]
	Age group (mo)				
	0-2	3-5	6-8	9-11	
Vitamin E (mg/g PUFA)					0.4
Vitamin K(μg)					10
Calcium (mg)	525	525	525	525	
Chlorid (mg)	320	400	500	500	
Copper (mg)	0.2	0.3	0.3	0.3	
Fliorid (mg)					0.5
Iodine(μg)	50	60	60	60	
Iron (mg)		11	11	11	
Magnesium (mg)	55	60	75	80	
Maganese (μg)					16
Phosphorus (mg)	400	400	400	400	
Potassium (mg)	800	850	700	700	
Selenium (μg)	10	13	10	10	
Sodium (mg)	210	280	320	350	
Zinc (mg)	4.0	4.0	5.0	5.0	

[1] Dietary Reference Values for energy and nutrients for the United Kingdom (Dept of Health, 1991), unless otherwise indicated

[2]Recommended nutrient intake (RNI) is the estimated average requirement plus a safety factor of 2 SD

[3]Safe nutrient intake (SNI) from British Dietary Reference Values

Source: Brown KH, Dewey KG, Allen LH, 1998

Table 3 Recommended nutrient intake per day for different age group of infant WHO 2002

Nutrient	Recommended nutrient intake		
	6-8 mo	9-11 mo	12-23 mo
Protein (g)	NA	NA	NA
Vitamin A (μg RE)	400	400	400
Folate (μg)	80	80	160
Niacin (mg)	4	4	6
Pantothenic acid (mg)	1.8	1.8	2.0
Riboflavin (mg)	0.4	0.4	0.5
Thiamin (mg)	0.3	0.3	0.5
Vitamin B_6 (mg)	0.3	0.3	0.5
Vitamin B_{12} (ng)	0.5	0.5	0.9
Vitamin C (mg)	30	30	30
Vitamin D (μg)	5	5	5
Vitamin K (μg)	10	10	15
Calcium (mg)	400	400	500
Chloride (mg)	NA	NA	NA
Copper (mg)	NA	NA	NA
Fluoride (μg)	NA	NA	NA
Iodine (μg)	90	90	90
Iron (mg)	9.3	9.3	5.8
Magnesium (mg)	54	54	60
Manganese (mg)	NA	NA	NA
Phosphorus (mg)	NA	NA	NA
Potassium (mg)	NA	NA	NA
Selenium (μg)	10	10	17
Sodium (mg)	NA	NA	NA
Zinc (mg)	4.1	4.1	4.1

NA= Not yet available

Source: Brown KH, Dewey KG, 2003

Table 4 Dietary Reference Intake Tables for Thai infants 2003

Nutrient	Nutrient recommended per day	
	6-11 m0	12-36 mo
Energy (kcal)[1]	800	1000
Protein (g) [1]	16	19
Vitamin A (μg RE)	400	400
Vitamin C (mg)	35	40
Vitamin D (μg)	5	5
Vitamin E (mg)	5	6
Vitamin K (μg)	2.5	30
Thiamin (mg)	0.3	0.5
Rioflavin (mg)	0.4	0.5
Niacin (mg)	4	6
Viamin B6 (mg)	0.3	0.5
Folate (μg)	80	150
Vitamin B12 (μg)	0.5	0.9
Panthotenic acid (mg)	1.8	2
Biotin (μg)	6	8
Choline (mg)	150	200
Calcium (mg)	270	500
Phosphorus (mg)	275	460
Magnesium (mg)	30	60
Fluoride (mg)	0.4	0.6
Iodine (μg)	90	90
Iron (mg)	9.3	5.8
Copper (μg)	2200	3400
Zinc (mg)	3	2
Selenium (μg)	20	20
Manganese (mg)	0.6	1.2

[1]Based on body weight 8, and 13 kg for 6-11 mo, and 12-36 mo

3.2 Complementary feeding

The complementary feeding is defined as the process starting when breast milk alone is no longer sufficient to meet the nutritional requirements of infants, and therefore other foods and liquids are needed, along with breast milk (**Figure 1**). The target age range for complementary feeding is generally taken to be 6 to 24 mo of age, even though breastfeeding may continue beyond two years (13).

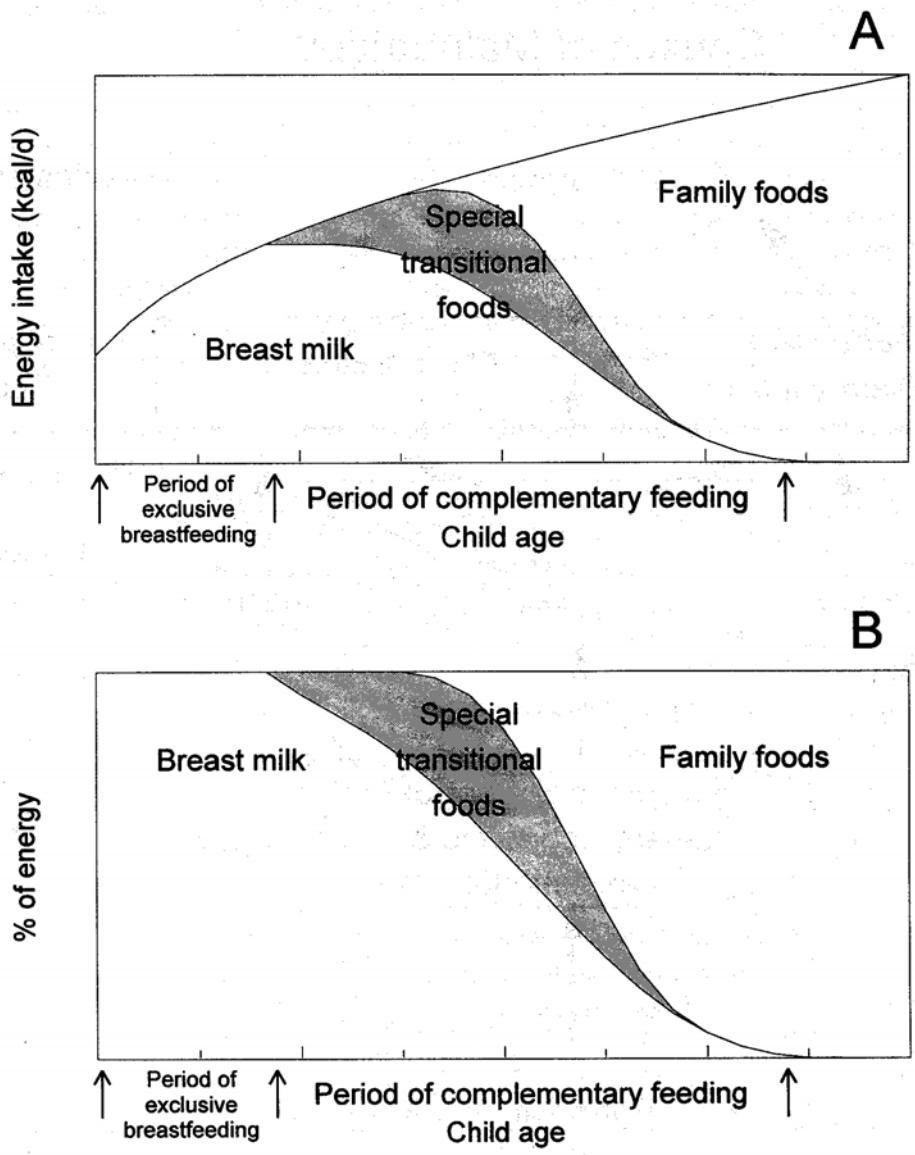

Figure 1 Contribution of different food sources to young children's energy intake

3.2.1 Infant feeding guidelines

There are many suggestions on infant feeding guidelines. The resent guidelines are developed from discussions at several technical consultations and documents on complementary feeding (WHO/UNICEF, 1998; WHO/UNICEF Technical Consultation on Infant and Young Child Feeding, 2002; WHO Global Consultation on Complementary Feeding, 2001), as follows. (13).

1.Waiting until six months to introduce other foods outweigh any potential risks

2 Breastfeeding continues to make an important nutritional contribution well beyond the first year of life

3.Optimal complementary feeding depends not only on what is fed, but also on how, when, where, and by whom the child fed.

4.The peak incidence of diarrhea disease is during the second half year of infancy as the intake of complementary foods increases. Because they are difficult to keep clean, feeding bottles are a particularly important route of transmission of pathogens

5. Starting with small amount of foods and increase the quantity as the child gets older, while maintaining frequent breastfeeding.

6. Gradually increase food consistency and variety as the infant gets older, adapting to the infant's requirement and abilities

7. Increase the number of times that the child is fed complementary foods as he gets older. For the average healthy breastfed infant, meals of complementary foods should be provided 2-3 times per day at 6-8 mo of age and 3-4 times per day at 9-11 and 12-24 mo of age

8. Use fortified complementary foods or vitamin-mineral supplements for the infant, as needed

9. Increase fluid intake during illness, including more frequent breastfeeding, and encourage the child to eat soft, varied, appetizing, favorite foods

In 2000, the Guideline of Ministry of Public Health developed a guideline for complementary food of infants aged 0-12 mo was developed (4). It includes different food and commodities that should be given to infants, as follows

4 months: ground rice and soup, egg yolk, liver and breast milk

5 months: add fish, green-leaf vegetable, pumpkin, tomato, carrot, and breast milk

6 months: begin one meal, fruit and breast milk

7 months: add ground meat and whole egg, ripe fruit and breast milk

8-9 months: begin 2 meals and breast milk

10-12 months: begin 3 meals and breast milk

3.2.2 Introduction of complementary food

Professional recommendations (by WHO Expert Committee) to introduce non-milk feeding between 4th and 6th months have been around since the 1960s. Concept of the appropriate timing for introduction of complementary foods was based on a comparison of the theoretical energy requirements of young infants and their energy intake from breast milk at different ages. Thus, it is assumed that when the average energy intake from breast milk falls below these theoretical requirements, additional energy source need to be offered (5).

The age of introduction of complementary food for individual infants cannot be set rigidly; rather it depends on rate of growth, stage of development, and level of activity (2).

The goal of feeding during the first two years of life is to provide developmentally appropriate, nutritious foods in positive mealtime experiences so that an infant can

(i) Achieve normal growth and development,

(ii) Learn to accept and enjoy a variety of nutritious foods

(iii) Make a smooth transition from dependent (being fed to competent self-feeding (15).

The non-nutritional role of complementary foods introduced to older infants should also be recognized. These foods help to condition (educate or acculturate)

infants and young children to a wider diet at weaning by providing experience with transitional liquid to – solid variations in consistency, texture, and taste (9).

To introduce complementary food, it is customarily to begin with a single food (most commonly cereal or fruit) rather than with a mixture of foods, and to allow at least several days to elapse before introducing the next new food. Moreover, it should be start with small amounts of food and increase the quantity as the child gets older, while maintaining frequent breastfeeding (9,13).

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3.2.3 Micronutrient required from complementary food

In the WHO/ UNICEF, 1998 reported the amount of protein and micronutrients needed from complementary foods were estimated by subtracting the amounts provided by human milk from the recommended nutrient intakes (RNI) for each of age intervals (6-8, 9-11, and 12-23 mo). These were then converted into desired nutrient densities (per 100 Kcal of complementary food) by dividing by the amount of energy needed from complementary foods at each age (3). **Table 5** showed the energy requirement from the complementary food. There were a decrease in energy of 10-18% kcal per day and 25-32% decrease in energy from CF per day in the revised new recommendation by FAO, 2001 compared to the recommendation by WHO, 1998 (3, 16). **Table 6** shows the nutrient densities (mg/100 kcal) in complementary foods (CF) by age desired nutrient densities (i.e., assuming an average breastmilk intake) of based on the current (WHO/UNICEF, 1998) and revised recommendations (FAO, 2001).

Table 5. Energy requirements from complementary foods according to age group

Age group (mo)	Total energy requirements		Milk energy intake	Energy requirement from CF [1]	
	WHO/ UNICEF 1998	US longitudinal data		WHO UNICEF1998	US longitudinal data
	Kcal / day				
6-8	682	615	413	269	202
9-11	83o	686	379	451	307
12-23	1092	894	346	746	548

[1] Based on total energy requirements proposed by IDECG (as presented in the WHO/UNICEF 1998-publication

Source: Brown KH, Dewey KG, 2003

Table 6 Current (WHO/UNICEF, 1998) and possible revisions (FAO, 2001) in nutrient densities per 100 kcal of complementary foods using new DRI (daily recommended intake), by age

.

Nutrient	**6-8 mo**		**9-11 mo**		**12-24 mo**	
	Current	**Revision**	**Current**	**Revision**	**Current**	**Revision**
Protein (g)	0.70	1.00	0.70	1.00	0.70	0.90
Calcium (mg)	125.00	40.00	78.00	32.00	26.00	63.00
Iron (mg)	4.00	5.30	2.40	3.50	0.80	1.20
Thiamin (mg)	0.04	0.08	0.04	0.06	0.05	0.07
Folate (μg)	0.00	11.00	0.00	9.00	0.00	19.00

Source: Sanghvi TG, 2002

3.3 Complementary food in Thailand

The complementary foods used in Thailand are rice-based and mostly home prepared. Therefore, infants in Thailand have a high prevalence of thiamin deficiency since polished white lost several essential nutrients, particularly thiamin during the milling process. Moreover, maternal deficiency of thiamin rapidly and severely depletes the amount of the vitamin secreted in milk. As a result of this, and low infant stores, infantile beriberi can appear within a few weeks after birth. Most of the cases occur within the first 6 months of life (personal communication, Dr Sinawat S, 2003)

Porniammongkhon (2001) found that iron and calcium intake is very low in breast-fed infants in rural as well as urban areas. In the rural area, the iron adequacy is 11% and 19.5% in the 3-5 mo, and 12-23 mo old infants respectively (5). The iron adequacy in the urban area varies between 6.8% and 21.3% for the age 3-5 mo and 9-11 mo respectively (5).

Porniammongkhon also found that Infants in the rural area only reach 34% (9-11 mo old) and 59% (12-23 mo old) of the required daily calcium intake. The situation looks similar in the urban areas. The intake varies between 37% and 56% for the infants aged 9-11 mo and 12-23 mo respectively (5). Even if the complementary food were cooked based on the Thai guideline for infant feeding, the intake of calcium would be very low. The situation is much better in infants fed with infant formula.

One problem is that milk and dairy products, excellent sources of calcium, are expensive and therefore cannot be afforded by low-income families.

Both adults and children need folic acid to make normal red blood cells and prevent anemia. In infants and children, folic acid deficiency can slow growth rate (10).

Although, only a few studies have been performed on the problem of folic acid deficiency in Thailand, the WHO suggests fortifying complementary food for preventative folic acid deficiency in the developing country (16). Low serum folacin levels and megaloblastic anemia have been reported in association with conditions of nutritional stress such as pregnancy, infancy, alcoholism and disease state (17).

3.4 Food fortification

3.4.1 Fortificants

3.4.1.1 Iron fortificant

Iron is the most difficult mineral to add to foods and ensure adequate absorption. The main problem is that the most soluble and absorbable iron compound often cause unacceptable color and flavor changes in the food vehicle. On the other hand, insoluble compound such as elemental iron powder not cause sensory changes but may be so poorly absorbed as to be of little or nutritional benefit (18,19).

Iron fortificants list as GRAS (generally recognized as safe) by the US Food and Drug Administration (FDA) including reduced iron, ferric phosphate, ferric pyrophosphate, ferric-sodium-pyrophosphate, ferrous gluconate, ferrous lactate, and ferrous sulfate, however, some non-GRAS iron fortification are needed to corroborate their safety, such as iron amino acid chelate, sodium-iron EDTA (20, 21).

The iron compound selected for food fortification should be the one with the highest RBV (relative bioavailability) that cause no sensory change when added to the food vehicle. However, the processing of the fortified dried rice was the liquid system so that the freely water-soluble iron compounds would be the first choice. Three selected soluble iron compound are present as follow.

(i) Ferrous sulfate ($FeSO_4$)

Ferrous sulfate is used to fortify infant cereal formula, bread, and pasta (18). Moreover, it can also be added to wheat flour when stored for short period (22), but possibly trigger fat oxidation and off flavors in wheat and other cereal flours stored for longer periods (23, 24). Ferrous sulfate and other soluble iron compounds have been reported to cause unacceptable color changes in infant cereals (24), cocoa product (25), salt (26), and tortillas. Soluble iron compounds may cause a metallic taste in bouillon cubes and fruit drinks, and may cause precipitates in soy sauce, fish sauce, and tea infusions

Cook et al (1983) demonstrated that ferrous sulfate was useful for fortifying wheat flour that storaged for the short times (22). But for longer storage times, Hurrell et al. (1984, 1989) reported that ferrous sulfate can provoke fat oxidation and off flavors in wheat flour and also cause unacceptable color changes in infant cereals (23, 24).

(ii) Ferric sodium ethylenediaminetetraacetic acid (NaFeEDTA)

The attractive advantage of NaFeEDTA over other fortification compounds is that it prevents iron binding to inhibitors of iron fortification, especially for phytic acid. Hence iron absorption from NaFeEDTA, which is added to cereal foods or to meals consisting high amount of phytic acid, is two to three times higher than from ferrous sulfate (27). In cast of the lower level of dietary inhibitors, Fidler et al. (2001) reported that the absorption of NaFeEDTA might be similar to the absorption of ferrous sulfate (28). NaFeEDTA is slowly water-soluble and thus may cause unacceptable color changes in some food vehicle, although it does not induce fat oxidation in stored wheat flour (29). Garby et al. (1974) and Thuy et al. (2001) demonstrated the improved iron status in human consuming NaFeEDTA fortified fish sauce (30). The same results in human absorption are observed by Viteri et al. (1995)'s study in fortified sugar and Ballot et al. (1989)'s study in curry powder (31,32). It is about six times more expensive than ferrous sulfate. It is a useful compound fortification of cereal foods, fish sauce, and soy sauce.

(iii) Ferrous lactate(CH_3-CHOH-COO)$_2$.3H_2O)

Ferrous lactate is a yellow-green powder with a slight odor and sweet taste. It contains a minimum of 19% iron and is soluble in water. In studies with pharmaceutical dose, it has shown a similar bioavailability to ferrous sulfate. Anemic rat fed liquid soya-based or milk-based infant formulas likewise absorbed iron from ferrous lactate and ferrous sulfate equally well. It presumably has the same disadvantages as the soluble ferrous salts, and has been reported to give slight off-flavors in liquid whole milk and skim milk and to cause a darkening in the color of chocolate milk (18).

3.4.1.2 Calcium fortificant

Many factors must be considered such as high nutritional value of calcium salt, cost effectiveness, solubility, interaction with dietary component, acceptability taste and appearance, and bioavailability of finished product. Several commercial calcium salts are available to the food manufacturers for calcium fortification, including calcium carbonate, phosphate, citrate, lactate, and gluconate (33). Typically, calcium doesn't have an overwhelming effects on a fortified's flavor, but in a bland system, certain flavor arise. Calcium lactate and calcium gluconate are considered to be the blandest of the calcium salt. Calcium carbonate comes across as soapy or lemon. Calcium citrate tastes acidic and the chloride salt may promote bitterness. Tricalcium phosphate has a bland flavor but give a gritty mouthfeel. Moreover high level of calcium, particularly insoluble forms can produce a chalky mouthfeel often picked up as part of the flavor. They also promote astringency in the product. Large particle sizes of insoluble calcium salts might feel gritty in the mouth (34,35). However, solubility is of prime importance for fortified dried rice because the processing of it exist as a liquid system. Three selected calcium salts and their characteristics are presented as follow

(i) Calcium lactogluconate

Calcium lactogluconate is a molar mixture of calcium lactate and calcium gluconate. It is highly soluble in water and has neutral taste but contains about 10% of calcium (36).

(ii) Calcium lactate

Calcium lactate has a very good solubility (9%w/w) compared to other calcium sources used in food products and its taste is neutral. Stiudies have shown that calcium lactate is very well absorbed by human (37).

3.4.2 Stability of minerals and vitamins

3.4.2.1 Stability of Thiamin

Thiamin salts have a characteristic odor and slightly bitter taste (38). They are relatively stable to atmospheric oxygen in absence of light and moisture and both are normally considered to be very stable when used in dry products with light and moisture-proof packaging (38, 39). At a pH of 5 or higher, thiamin is destroyed by autoclaving and at pH of 7 and it is more easily destroyed by boiling or merely storing at room temperature (38). Of all the B vitamin, the most studied and probably most sensitive to temperature is thiamin (40).

Schultz et al. (1942) studied the loss of thiamin in enriched bread food and found that thiamin lost 21-22% (41). Rice et al. (1944) report data that at 63^{o}C, the highest temperature reported, 20hours are required for a 50% loss of thiamin in dried pork (40). Coppock et al. (1956) studied again the loss of thiamin in enriched bread and found that thiamin lost 17-23%(41). Abdel-Rahman (1982) found that thiamin was reduced by 42 and 47.5% after cooking spaghetti for 10 and 15 min, respectively, and the loss after 20 min was equal to the loss at 15 min of cooking (42). Ranhotra et al (1983) found that thiamin loss in cooking of spaghetti, noodle and macaroni ranged in 41.6-54.4% (43,46). Ranhotra et al. (1986) studied the stability of added vitamins of cookies and bread and found that thiamin lost 11% and 83% for baking at 425^{o}F 22min and 360^{o}F15min respectively (44). Schlude (1987) found that thiamin loss during extrusion cooking, high-temperature short time, was 6-62% and Roche who compare vitamin stability of pre and post extrusion and found that thiamin lost 15.6 and 9.4%, respetively (43). Vandrasek H.T. et al. (1987) studied thiamin partitioning and retention in cooked rice and pasta products and found that thiamin loss from cooking was only through leaching making the retention of cooked-enriched pasta product ranged between 66-47% (45). They mentioned that leaching could play an important role in thiamin loss where part of the cooking liquid is not consumed. Buhler Brothers et al (1989) found that thiamin retention in spaghetti after drying at 50 ^{o}C 9-12 hours and after cooking were 98% and 67%, respectively (43).

3.4.2.2 Stability of folic acid

Folic acid is the most sensitive vitamin of B complex, considerable changes may occur particularly during thermal processing. Oxidizing and reducing agents decompose it. Sunlight and particularly ultraviolet radiation, has serious effect on the stability of folic acid (46, 39). Losses of around 10% are found in boiled eggs, while other forms of cooking (fried, poached, scrambled) give between 30 and 35% loss and total folic acid losses from vegetable as a result of heating and cooking process can be as high as 50% (39).

Keagy P.M. et al (1975) found that folacin loss from baking enriched bread at 425^{o}F 25 min was 8- 14% (17). Steele (1976) found that average processing loss of folate in enriched breakfast cereal (RTE) was 25% and Cort found the enriched rice in condition of cooking in double boiler, not drained lost folate 0% (41). Connor and Keagy (1981) found that enriched cookies made with enriched flour lost folate 15% (39).

3.4.2.3 Stability of calcium and iron

Compared with vitamins, minerals are very stable under extreme processing condition. The primary mechanism of loss of minerals is through leaching of water-soluble materials (47). Some may be oxidized to higher valences by exposure to oxygen, but there is no convincing evidence that nutritional value is affected (48).

Futer et al. (1947) found that no loss of iron fortified in enriched corn grits if cooking water was not discarded (41). Abdel-RahmanAHY (1982) studied effect of cooking on the loss of minerals in spaghetti and found that trace element (Fe, Mn, Cu, Mo, Ca) became stable after 15 and 20 min of cooking and the loss of different minerals in water are relatively small for all cooking times, major losses found in discarded water (40). Ranhotra et al. (1985) found that cooking led to lose Fe and Calcium in enriched pasta 0-20% (49). Calcium addition is difficult because it is bulky, affects the product pH and can create a “ chalky’ texture and mouthfeel in certain foods. The biggest challenge was to add the calcium without changing the flavor, texture or color of the reformulated cereal (50).

One important factor that should be carefully assessed in the preparation of mineral premixes (as ingredients for food fortification) is type of salt to be fortified (47). Ionization, dehydration, or a change in valence, where possible, might also occur. Any or all of these reactions could result in undesirable quality changes and consideration must be given to such an occurrence. Mineral fortification always causes change in color, flavors in lipid-containing foods (51). Abrams & Alkinsom recommend the level of calcium fortification to be 100-200 mg per daily ration. This amount is safe and could be incorporated into the food with no undesirable organoleptic change (52).

3.4.3 Food vehicles

Criteria for choosing a food vehicle are illustrated as follow (53):

a. Consumption

- High proportion of population covered,
- Regular consumption is relatively constant amounts,
- Minimal variation in consumption pattern between individual,
- Minimal regional variation in consumption pattern,
- Appropriate serving size to meet a significant part of daily dietary requirement of nutrient added
- Consumption not related to socioeconomic status,
- Low potential for excessive intake (to avoid possible toxicity),
- No change in consumer acceptability after fortification, and
- No change in quality as a result of micronutrient addition.

b. Processing and storage

- Centralized processing,
- Simple, low cost technology,
- Good masking qualities (dark color and strong odor of the vehicle to mask slight changes to original color and odor),
- High stability and bioavailability of added micronutrient in final product,

- Minimal segregation of the fortificant and vehicle,
- Good stability during storage, and
- No micronutrient interaction.

c. Marketing

- Appropriate packaging that will ensure stability,
- Labeling according to prescribed standards, and
- Adequate turnover rate.

3.5. Bioavailability

Bioavailability is the amount of nutrient that is absorbed when entered the body and available for metabolic use. Bioavailability is significant because all nutritional intake must be available to various body systems for maintaining biological functions. No matter how high the nutrient levels or how well formulated the product, if it is not available, then money and effort have been worthless (54).

Abraham et al. (1986) studied the absorption of fortificaton iron from formulus in infants and found that iron absorption of ferrous sulfate between 10 and 19 per litter mg added varied from 2.9-5.1% (55).

Romera et al. (2000) examined the bioavailability of iron from fortified rice and the inhihitor effect of bran phytate. Rice fortified with electrolytic iron was simulated to brown rice by adding 4.76 and 9.09% rice bran. The result showed that reduced the iron bioavailability of fortified rice with or without bran. After phytic acid hydrolysis, iron bioavailability was increased. Multiple phytic acid would be primarily responsible for iron bioavailability (56).

Trinidad et al. (2002) studied the bioavailability of fortified wheat flour using *in vitro* method. The result indicated that the percentage of dialyzable iron from NaFeEDTA (15.7%) and ferrous sulfate (13.2%) was greater than other iron forms. In addition, by using *in vivo* method to evaluate iron bioavailability of fortified rice, the result showed that iron absorption from the rice fortified with NaFeEDTA (0.44mg) was greater than ferrous sulfate (0.22mg) (57, 58).

3.5.1 Method for measuring iron bioavailability

Bioavailability of iron has become the important issue, as many foods that are potentially good sources of iron are limited by the bioavailability of iron, resulting in the high prevalence of iron deficiency. Therefore, a rapid assessment of iron bioavailability is required to establish the knowledge for controlling and preventing iron deficiency.

3.5.1.1 *In vivo* methods

a. Non-isotopic method

Chemical balance

This original technique is based on measuring the difference between the iron intake and excretion. The tested diet is fed for two weeks with the fecal marker to ensure that previous meals have been excreted from the lumen, then intake and excretion are measured for at least six days. This method was employed prior to the ready availability of radioisotopes. Prolonged stool collections make this a cumbersome method and the small amounts of iron absorbed each day result in this approach being highly inaccurate (59, 60).

Change in hemoglobin and ferritin concentrations

Measurements of increase in plasma iron concentration with iron intake provide an indirect estimation of the amount of iron absorbed. Ferritin concentrations are more responsive to the changes of iron status because an increase in iron storage would be expected in more subjects. Similarly to balance method, this approach provides variable absorption. Changes in plasma iron level following pharmacological doses of iron; however, are useful as a crude screening test for gastrointestinal malabsorption of iron (59, 60).

b. Radioisotope method

Progress in identifying iron bioavailability and absorption was facilitated in the past decades by the application of radioisotopes in humans. Absorption were evaluated from the amount of radioactive iron (usually ^{59}Fe of ^{55}Fe) incorporated into

the circulating erythrocytes 2 to 3 weeks after ingestion of the radioactive label. Alternatively, whole body counters were used to assess entire body retention of radioactive iron. Food under this study could be labeled either intrinsically or extrinsically tagging. Intrinsic labeling relies on either hydroponic cultivation of plant or biosynthetic labeling of animal. Extrinsic tagging is an easier and valid indicator of bioavailability and absorption because the extrinsic factor accesses the common pool with intrinsic iron; enhancing and inhibitory affect the common pool in aggregation, and neither affect the intrinsic or extrinsic pool selectively. The main disadvantages of this method are that additional isotope must be ingested. In addition, day to day variation in individual absorption may affect the experiment outcomes. Furthermore, radio labeled foods cannot be given to children, pregnant, lactating mother, or women at risk of becoming pregnant (59, 60).

c. Stable isotope method

Because of general concern about radioisotopes in human subjects, particularly in children and pregnant women, stable isotopes of iron including ^{54}Fe, ^{56}Fe, ^{57}Fe, and ^{58}Fe have been evaluated as alternative tracers. These techniques are safe to use even in vulnerable groups such as infant, children, pregnant and lactating women. The utilization of staple radioisotope is limited because these approaches are extremely costly and require highly sophisticated laboratory facilities (59, 60).

d. Animal models

Ideally, determination of iron bioavailability should be quantified only the total amount of iron metabolite presenting in the source. Because the amount absorbed by human and animal varies directly with their iron status, bioavailability values are confounded to an unknown extent by the characteristic of food and the physiological characteristics of model subjects. In animal model, this complexity can be minimized by using an iron deficient animal given the quantities of iron lower its known capacity for absorption. The amount of iron absorption is thus considered to reflect which metabolite in the source is consumed.

Rats have been used to study the bioavailability of iron supplements and the effect of processing on bioavailability. Most of these researches have been directed

towards evaluating the bioavailability of unknown sources relative to that of a reference iron source. Mahoney and Hendrick (1984) indicated that rats and humans response similarly to many dietary and physiological factors known to affect iron utilization. It was found that iron absorption by rats was highly correlated (r = 0.94) with that of man (61).

3.5.1.2.*In vitro* methods

In vitro methods generally consist of simulation of *in vivo* digestive process, including acid hydrolysis, proteolytic digestion and addition of bile acids, followed by determination of how much of the element is soluble or dialyzes through a membrane of certain pore size. Such methods provide a crude prediction of bioavailability of food iron. Although superior to certain animal models, these *in vitro* techniques are the best indicator of trends in bioavailability rather than an indicator of absolute levels of iron absorption. Schricker et al. (1981) compared the *in vitro* and *in vivo* study of bioavailability in rats and man by measuring iron bioavailability according to Miller's method. The significant correlation between the *in vitro* and *in vivo* methods was observed in this study (62) and also confirmed by other experiments (62-66). These methods reject the need to understand and regulate all physiological factors that affect the efficiency of which iron form is absorbed. Theoretically, this method should provide a consistent and reproducible means of assessing the effect of dietary varies on absorbable iron.

3.5.2 Effect of calcium on the iron absorption

Iron and calcium are essential nutrient and the requirement for both nutrients are especially high in the same groups of subjects: infants, children, teenagers, and pregnant women, however; there is a potential risk that fortification with mineral salts will reduce the bioavailability of other minerals in food by either changing their intestinal solubility or competing for uptake at absorption size (67). Calcium phosphate reduced the absorption of nonheme iron from semi-synthetic meal by 50%, whereas calcium alone did not (68).

James D Cock (1991) found that calcium carbonate did not inhibit the absorption of ferrous sulfate with doses of either 300 mg Ca or 37 mg Fe or 600 mg Ca and 18 mg Fe, when taken without food but, at the latter levels, calcium citrate and calcium phosphate reduced iron absorption by 49% and 62% (69).

Hallberg L. et al (1992) found that no inhibiting effect on iron absorption when adding 3 mg calcium to 0.01 mg iron (molar ratio Ca/Fe=420)(70).

Geerup et al. (1995) demonstrated that the inhibiting effect of calcium was dose-dependent at maximal 300 mg calcium. However, when the amount of calcium exceeded 300 mg, the inhibitory effect was negligible. In addition, the simultaneous consumption of calcium rich foods with iron rich foods in a meal also inhibited iron absorption. Approximately 30 to 50% more iron was absorbed when no milk or cheese was served with lunch or dinner to Swedish subjects fed a common Scandinavian diet containing 1.4 mg more iron per day were absorbed when milk or cheese (providing 937 mg calcium per day) were consumed separately from the main meals (lunch and dinner) (71).

Ranhotra et al. (1997) studied bioavailability of calcium in breads fortified with different calcium sources and suggested that food fortified with Ca carbonate or Ca sulfate, which are commonly used for this purpose, may be as good a source of utilizable Ca as more expensive Ca sources. However, some data show that addition of a moderate amount of calcium, 39-156 mg, was beneficial to calcium absorption and did not interfere with iron absorption (49).

Wauben (1999) determined whether dietary calcium: iron ratio similar to that often consumed by premature human infants inhibited iron absorption in infant piglets. The result showed that in vivo iron absorption from the normal calcium (2.0g/L) diet did not differ from the high calcium diet (4.67 g/L) (72).

CHAPTER IV
MATERIALS AND METHODS

A schematic diagram indicating process for developing and evaluating product quality of dried-rice based complementary food is demonstrated in **Figure 2**.

4.1 Development of method for fortification

Four processes potentially used for production and fortification were developed by considering their practicality at industrial scale and loss of fortified nutrients (**Table7)**. Details of each process were described as follows.

1st process: Broken rice was cleaned by first washing in tap water and then deionized water. The cleaned rice was cooked in mineral and vitamin solution at ratio rice to solution 1:2 by using electric rice cooker. The fortified cooked rice was spread in an aluminum tray and dried in a hot air oven at 70°C for 12 h. The dried rice was finally disintegrated in a hammer mill.

2nd process: This process was slightly different from the 1st process. Only mineral solution was used for rice cooking, while vitamin solution was used for spraying after the rice had been cooked and dried. After spraying, the rice was again dried at the same temperature for 40 min.

3rd method: Broken rice was cooked, dried and then sprayed with vitamin and mineral solutions.

4th process: Broken rice was cleaned by washing with tap water and then deionized water. The cleaned rice was dried in a hot air oven at 90°C for 1 h. The dried rice was soaked in vitamin and mineral solutions for 10 min at the ratio 2:1 (dried rice:solution). The fortified rice was then dried in a hot air oven at 70°C for 1:50 h.

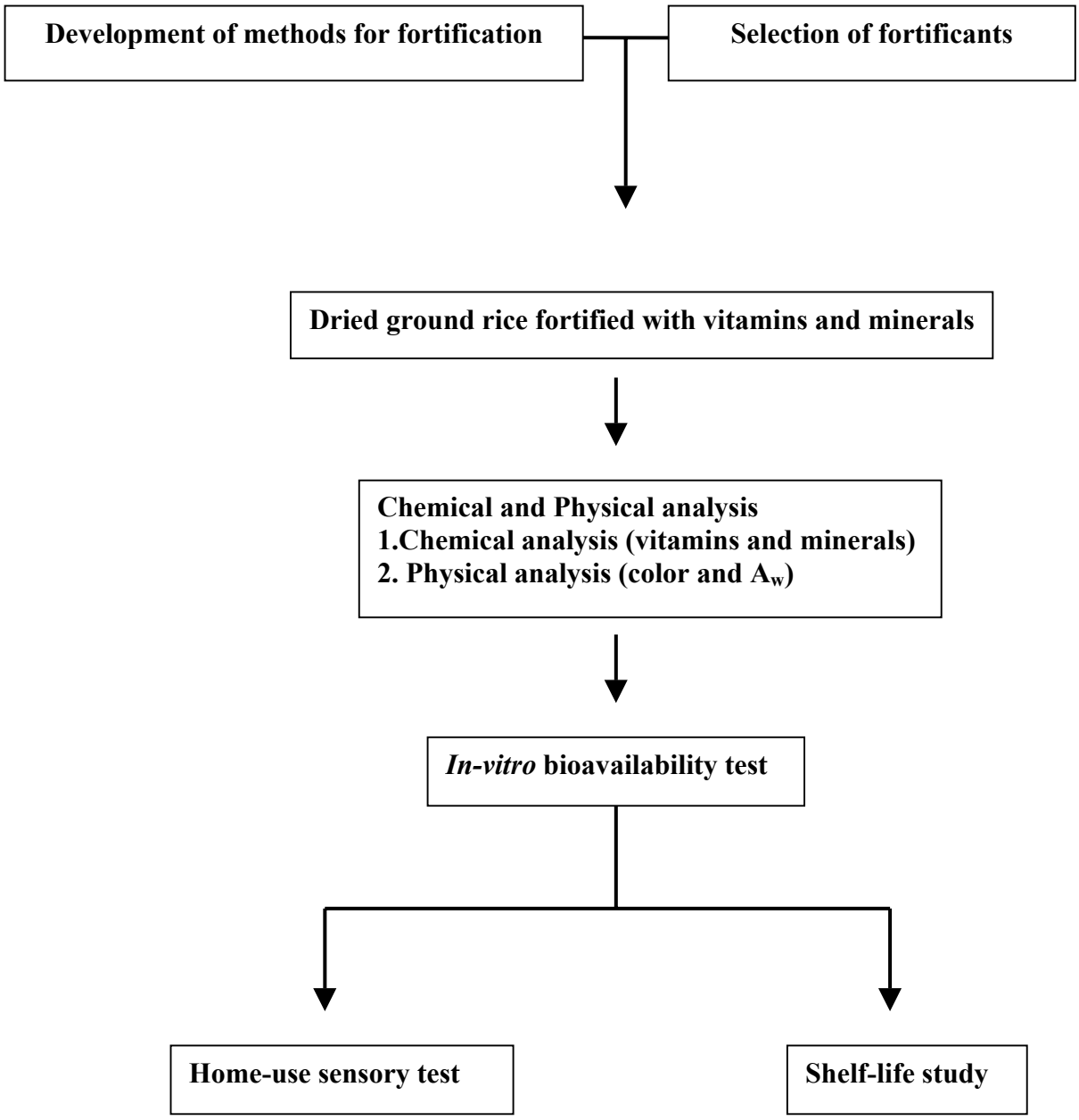

Figure 2. Schematic diagram of the experiment design.

Table 7 Processes of fortification

Process	Description
1	Broken rice → clean → cook in fortificant solution → dry → disintegrate.
2	Broken rice→clean → cook in mineral solution → dry→ spray with vitamin solution → dry → disintegrate
3	Broken rice→clean → cook in fortificat solution → dry→ spray with vitamin and mineral solution → dry →disintegrate
4	Broken rice → clean → dry → soak in fortificant solution → dry

4.2 Type and level of fortificants

The criterion for selecting fortificants was solubility in water. Vitamins obtained from Roche Co., Bangkok, Thailand included dry vitamin A acetate type 325 CWS/F with minimum 325,000 IU per g, thiamin hydrochloride, and folic acid. Calcium sources obtained from Nutrition Ltd. Partnership, Bangkok, Thailand included calcium lactate (13% Ca) and calcium lactogluconate (10% Ca). Three sources of iron were selected based on iron content , solubility, bioavailability, and cost. The iron sources used included dried ferrous sulfate (33% Fe) from Ajax Co. (Aubern, Australia), ferrous lactate (19% Fe) from Vicky Consolidate Co., Ltd. (Bangkok, Thailand), and ferric sodium ethylenediaminetetraacetic acid , NaFeEDTA (13% Fe) from Akzo Nobel Functional Chemicals Co. (Arnhem, Netherlands).

Since iron could cause change in product appearance fortification with iron content of 3, 5, and 7 mg per serving would be pre-tested for their sensory acceptability in 35mothers who were faculties staff of Institute of Nutrition, Mahidol University. Cooked products fortified with calcium lactate gluconate and two forms of iron i.e. ferrous sulfate or NaFeEDTA at different levels, which were 3,5, and 7 mg were tested in air condition individual booth under daylight fluorescent bulb of the sensory science laboratory at the Institute of Nutrition, Mahidol University. The products that were fortified with different forms of iron were tested on the different days. The products was served in small white melanine bowl (dia 3.5 in) and randomly served with cooked chicken. Each sample was coded with three random digit numbers. Panelists were asked to rinse their mouths with water between samples.

Appropriateness for using the product as complementary food in terms of general and overall appearances, color, taste, and aroma were tested on 9 point hedonic scales, which rated 9 as “ the most appropriate”, 5 as “ neither appropriate nor non appropriate” and 1 as “the least appropriate”. The questionnaires used in this study were demonstrated in **Appendix A.**

In final, level of fortification used followed the FAO revised recommendation, 2000 (3). Per serving size (20 g dried or 180g cooked), the fortified broken rice contained 40 mg calcium, 5.30 mg iron, 0.08 mg thiamin, and 11.00 μg folate.

4.3 Production at laboratory scale

The fortified dried broken rice of each process was prepared in triplicate for quality evaluation, shelf-life study, and bioavailability test. The fortified dried broken rice was packed in plastic laminated aluminum foil and kept in freezer for quality analysis and bioavailability test. For shelf-life study, the products were packed in 2 kinds of packaging including polyethylene plastic bag that normally used in commercial and polypopyrene coated with aluminium bag.

4.4 Quality evaluation of products

Both dried and cooked fortified products were determined for their physical and chemical qualities. Three batches of the products were prepared for analyses. In order to determine degree of homogeneity of the fortified nutrients, a batch of dried fortified rice was sampled for 10 spots, and sample from each spot was analyzed for iron content.

Cooked fortified rice was prepared by boiling 20 g of the dried fortified rice 280 g (1:14) water for 8 min. The products were blended in electric blender and stored in white plastic bottles at –40^{o}C until analysis.

4.4.1 Physical property

Both fortified dried broken and cooked rice products were tested for the physical properties including (i) color as Hunter Lab value (L = white-dark, a = red-

green, b = yellow-blue) by using Spectro Colorimeter Model JS 555 (Japan), (ii) water activity by using water activity instrument, NOVASINA MIK 3000 (Switzerland), (iii) pH by using pH meter model DH340, only cooked products were measured pH (Germany).

4.4.2 Analysis of nutritive value of the product

Nutritive value of each sample was analyzed in duplicate. Moisture content was analyzed by drying the sample in a hot air oven at 110 ± 5^{o}C until obtaining constant weight. Percent dry matter was determined by subtraction % moisture content from 100. Calcium content was determined in Atomic Absorption Spectrophotometer after being ashed in muffle at 550^{o}C. Total iron was analyzed in Atomic Absorption Spectrometry after wet digestion. Thiamin residue was determined by using HPLC after acid and enzyme digestion. Folic acid was analyzed by microbial assay, which based on the observation on the growth of *Lactobacillus casei* ATCC 7469. Details for the analytical methods were shown in **Appendix B, C, D, E, F, and G** respectively.

4.5 Shelf-life study

Three batches of samples used for shelf life study were prepared from Chiensiri Co., Ltd., Bangkok, Thailand. Approximately 60 g of sample was packed in 2 kinds of packaging i.e. nylon laminated PE plastic bag that was normally used for commercial, and metallized plastic bag (OPP/MCPP). After the bags were sealed, they were stored in an incubator at 42^{o}C for 3 mo. The products were periodically sampled at 2 wk, 1 mo, 2 mo and 3 mo to determine for moisture content and water activity, while thiamin content was determined as indicator for nutrient loss.

4.6 Evaluation of iron bioavailability

In Vitro Gastrointestinal Digestion method was used for determining iron bioavailability of the cooked products. The determination was based on analysis of iron dialyzability under simulated physiological conditions according to the *in vitro* method of Miller et al., 1981 (61) (**Appendix H**). The method involved 2 digestion stages by using pepsin and pancreatin enzymes. Iron bioavailability was determined as the amount of iron that permeated through dialysis bag after digestion in the pancreatin enzyme.

4.6.1 Enzymes and chemical for *in vitro* digestion

The enzymes and chemical used for *in vitro* digestion included

1. Bile extract porcine (B8631) from Sigma Chemical Co. St. Louis. Mo 63178 USA
2. Panceatin (P1750) from Sigma Chemical Co. St. Louis. Mo 63178 USA
3. Pepsin from porcine stomach (P7125) from Sigma Chemical Co. St. Louis. Mo 63178 USA
4 $NaHCO_3$. From Merck Co. (Frankfurt, Germany)

4.6.2 Sample preparation

After the fortified rice had been cooked in deionized water then it was freeze- dried, blended, and stored in acid washed plastic bottle. Total iron content of each cooked sample was analyzed in duplicate.

4.6.3 In vitro digestion

The digestion was divided into 2 stages including

Stage I: Peptic digestion

The frozen cooked product was thawed at room temperature and blended in electric blender (National super blender). Approximately 10 g of the blended product

(containing 1-2 mg iron) was first homogenized with in approximately 80 ml deionized water in homogenizer (Nissei AM-1 Homogenizer), then adjusted to pH 2.0±0.05 by using 6 N HCl. Volume of the slurry was adjusted to be 100 ml and added with 3.2 ml of freshly prepared pepsin solution. The slurry was then incubated at 37°C for 2 h in a shaking water bath.

Stage II: Pancreatin digestion

After 2h of the peptic digestion, the content of each flask were divided into two 20 g aliquots. One of the two was frozen, while the other was mixed with 5 ml of pancreatin-bile suspension and titrated to pH 7.5 with 0.5 N KOH. Dialysis sac was filled with 0.5 N $NaHCO_3$ at the same volume of KOH used for titration, and then with DI water to bring the total volume to 25 ml. The filled dialysis sac was then soaked in another aliquot that had been thawed, and incubated in 37°C shaking water bath for 30 min. Then 5 ml of pancreatin-bile suspension was added, and incubation was continued for 2h. After incubation, the dialysate was weighed and analyzed for iron.

4.6.4 Calculation of iron dializability

Iron dialyzability (%) was calculated as follow:

$$\text{Dialyzability (\%)} = [D / (W \times A)] \times 100$$

Where D is the total amount of iron in dialysate; W is weight of sample used (g); and A is concentration of iron in sample (mg/g).

4.7 Statistical analysis

Statistical analysis was performed using the Statistic Package for the Social Science for Windows version 9.0 (SPSS Inc). Means of the results from chemical analyses were tested for significant difference at $p = 0.05$ by using One-Way Analysis of Variance (ANOVA) and Schefee's multiple comparison test. Significant differences

of the sensory qualities were analyzed using Friedman and Wilcoxon tests. The effect of packaging materials on the product's shelf life was tested by using Paired Samples T-Test.

4.8 Home-use test

Sensory acceptability of the fortified products was studied by using Home-use test. The study was performed in 52 mothers of infants aged 4-24 mo, at Nongpho subdistrict, Potharam, Ratchaburi province, Thailand. Seventy grams of the product was packed in aluminum foil laminated polyethylene bag, and given to each mother to cook for infant by using her own recipe. On the next day, mother was interviewed for her opinion on product acceptabilities in infant and mother on 5 point smiley scales. Other questions i.e. suitable price, convenience, affordability, willingness to buy and foods used in combination with the product were also included in the interview.

CHAPTER V
RESULTS

5.1. Development of method for fortification

5.1.1 Selection of fortification method

From our primary trials on the practicality of the 4 methods for fortification, it was found that the 3rd method was not practical even at laboratory level since the required amount of calcium could not be totally soluble in the water used for spraying **(Table 8)**. While the 1st and 2nd methods could be produced at the laboratory level, however they were commented by food industry that the drying period of 12 h was too long and unpractical. Besides, the cooked rice was not easily spreadable on the drying tray. Therefore the 4th method was selected for further study.

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5.1.2 Fortification dosage

Our preliminary study indicated that vitamin A loss due to processing with the 4th method was as high as 80%. Therefore, vitamin A was not included in the fortification. Based on the assumption that one serving of complementary food was 180 g (2), the amount of fortificants was calculated as shown in **Table 9**.

Table 8 Amounts of fortificants based on 1 kg of raw materials.

Fortificants	Amount of fortificant	Amount of Nutrients Per Serving (20g)
Ferrous sulfate	0.083 g	5.3 mg Fe
NaFeEDTA	0.189 g	5.3 mg Fe
Ferrous lactate	0.135 g	5.3 mg Fe
Calcium lactate gluconate	2.000 g	40 mg Ca
Calcium lactate	1.538 g	40 mg Ca
Thiamin	0.0004 g	0.08 mg Thiamin
Folic acid	0.55 mg	11 μg Folic acid

Table 9 Sensory test in mothers on appropriateness of the prototype products for using as a complementary food

Iron forms	**Fe content (mg / serving)**	**Before tasting** [1,2,3]		**After tasting** [1, 2,3]		
		General appearance	**Color**	**Overall acceptability**	**Taste**	**Aroma**
NaFe EDTA	3	7.60±0.77^{ac}	7.60±0.77^{ab}	7.69±1.08 ^{ab}	7.49±0.95 ^{a}	7.71±0.83 ^{a}
	5	7.83±0.66^{ab}	7.86±0.65^{ad}	7.60±0.85^{ab}	7.69±0.94 ^{a}	7.77±0.69 ^{a}
	7	7.66±0.87^{abc}	7.77±0.84 ^{ab}	7.54±0.98 ^{acb}	7.80±0.73 ^{a}	7.74±0.74 ^{a}
Fe sulfate	3	7.60±0.69^{a}	7.60±0.69 ^{ab}	7.69±0.76 ^{b}	7.66±0.73 ^{a}	7.54±0.74^{ab}
	5	7.43±0.95^{c}	7.29±0.89 ^{bc}	7.29±0.89^{ac}	7.37±0.94 ^{a}	7.23±1.06 ^{bc}
	7	7.23±1.00 ^{c}	7.14±0.73 ^{c}	7.17±0.82 ^{c}	6.91±0.95 ^{b}	6.86±1.17 ^{c}

[1]Result indicates as means ± SD from 35 subjects.

[2]Rate on 9-point hedonic scales (1, the least appropriate; 5, neither appropriate nor non-appropriate, 9, the most appropriate)

[3]Means within the same column with the same alphabet are not significantly different ($p < 0.05$).

Scores for appropriateness of the products for using as a complementary food were shown in **Table 9**. The difference in NaFeEDTA content did not affect qualities of the cooked products. In case of ferrous sulfate, the scores of general appearance and overall acceptability became lower as the iron content was higher, due to the lower acceptabilities in color, aroma and tastes. However, the iron fortification level of 5.3 mg content would be appropriate for using as complementary food in mother opinion.

5.2 Quality evaluation

5.2.1 Degree of homogeneity

Degree of homogeneity was evaluated by considering iron that was distributed within the product. Iron contents in the products that had been sampled from 10 spots of the same production batch were shown in **Table 10**. The 4th fortification method resulted in a homogeneous product with % CV of only 3.21%.

Table 10 Homogeneity of fortified dried broken

Sample position	**Iron content (mg%)**
1	20.64
2	20.32
3	21.58
4	19.96
5	20.96
6	20.72
7	21.02
8	20.90
9	19.17
10	20.44
Mean	20.57
% CV	3.209

5.2.2 Physical property

5.2.2.1 Color value

Colors of the fortified products were different from the unfortified ones (**Table 11**). The L value indicated that color of fortified product was duller. In addition, different kinds of fortificants also resulted in products of different color tones. The product fortified with calcium lactate gluconate tended to be more yellow (higher b value) and whiter (higher L value) than the one fortified with calcium lactate. Most of the dried fortified rice had the color in the tone of greenish yellow (-a and +b values). General appearances of the products were shown in the **Figure 3**.

Figure 3 Fortified dried broken rice; CLG = Calcium lactate gluconate, CL = Calcium lactate

After being cooked, the products fortified with ferrous sulfate or ferrous lactate, and calcium lactate had higher brown color tone than the ones containing calcium lactate gluconate. The cooked products containing ferrous sulfate or ferrous lactate with calcium lactate gluconate had similar color, which had slightly less yellow and greener tone. The cooked products containing NaFeEDTA had the color quality similar to the unfortified cooked product. As considering from **Figure 4**, the colors of the cooked products containing NaFeEDTA were much whiter than the products fortified with other iron fortificants (**Table12**).

Figure 4 Cooked fortified dried broken rice; CLG = Calcium lactate gluconate, CL = Calcium lactate

Table 11 Colors of the dried broken rice fortified with different calcium and iron fortificants.

Sample [1]	**L***	**a***	**b***
Control	92.75±0.23	-2.11±0.13	10.88±0.21
CLG + $FeSO_4$	91.16±0.27	-2.91±0.08.0	13.68±0.27
CLG + NaFeEDTA	88.29±0.61	-0.32±0.12	11.44±0.22
CLG + Fe lactate	90.73±0.60	-2.98±0.03	13.85±0.50
CL+ $FeSO_4$	87.77±0.51	-1.53±0.12	14.86±0.08
CL+ NaFeEDTA	87.82±0.41	-0.22±0.29	10.56±0.46
CL+ Fe lactate	88.40±0.20	-1.45±0.11	14.03±0.06

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

Table 12 Colors of cooked dried broken rice that had been fortified with different calcium and iron fortificants.

Sample [1]	L*	a	b
Control	68.33±0.16	-3.23±0.08	6.34±0.06
CLG + $FeSO_4$	70.41±0.10	-5.18±0.14	5.00±0.03
CLG + NaFeEDTA	71.00±0.62	-4.3±1.33	3.17±3.23
CLG + Fe lactate	72.93±0.57	-3.56±0.57	2.21±3.54
CL+ $FeSO_4$	72.65±0.86	-4.45±0.79	6.72±0.82
CL+ NaFeEDTA	71.20±0.37	-3.11±0.49	7.47±0.10
CL+ Fe lactate	70.83±1.13	-2.91±0.37	6.99±0.67

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

5.2.2.2 pH value of cooked product and water activity of fortified dried broken rice

The pH values of cooked fortified products were slightly lower than the unfortified one. Moreover, the cooked products fortified with NaFeEDTA had slightly higher pH than the other fortified cooked products (**Table 13**). All the products had water activities of approximately 0.2.

Table 13 pH value of cooked product and water activity of fortified dried broken rice broken rice

Sample1 [1]	pH	Aw (water activity)
Control	6.27 ± 0.08	0.21±0.03
CLG + $FeSO_4$	5.25 ±0.02	0.22± 0.03
CLG + NaFeEDTA	5.69 ±0.02	0.26 ±0.13
CLG + Fe lactate	5.33 ±0.03	0.18 ±0.05
CL+ $FeSO_4$	5.24 ±0.01	0.20 ±0.05
CL+ NaFeEDTA	5.72 ±0.03	0.17± 0.02
CL+ Fe lactate	5.22 ±0.03	0.21 ±0.05

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

5.2.3 Nutrient composition and loss due to processing and cooking

5.2.3.1 Calcium

After fortification, the calcium contents were approximately 199 mg%, which was slightly lower than the expected content (inherited + fortified = 8 + 200 mg%). There was no significant difference in calcium content and % loss in the products fortified with different kinds of fortificants ($p > 0.05$). Only up to 6% of calcium was lost during processing and approximately 3% was lost during cooking (**Table 14**).

Table 14 Calcium content and losses due to processing and cooking

Sample[1]	Calcium content [2,3] mg %		% Processing loss [4]	% Cooking loss [5]
	Raw products	Cooked products		
Control	8.00	-	-	-
CLG+ $FeSO_4$	196.44±1.56	191.65±9.97	5.5 ± 0.8	2.6±2.1
CLG+ NaFeEDTA	193.59±5.09	203.25±9.71	5.9 ± 2.4	0.0±0.0
CLG+ Fe lactate	194.41±5.97	198.98±17.08	6.5 ± 2.8	2.4±2.1
CL+ $FeSO_4$	200.00±1.29	200.67±4.98	3.7 ± 0.6	0.6±1.0
CL+ NaFeEDTA	202.07±6.31	201.38±14.4	2.6 ± 2.2	3.3±5.7
CL+ Fe lactate	203.45±6.21	200.54±12.90	4.7 ± 2.5	3.2±5.6

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

[2]Samples were obtained from three production batches

[3]The values of the fortified products in each column were not significant difference ($p > 0.05$)

[4]Calculated based on initial value of 208 mg%

[5]Calculated based on difference in dry basis between dried and cooked products

5.2.3.2 Iron contents

Trace amount of iron was found in the unfortified product. As compared to the fortified dosage (26.5 mg%), approximately 5-16% of iron was found lost in the processed products. After cooking, the iron contents were higher than uncooked products. There were no significant differences among iron contents in the products containing different fortificants (**Table 15**).

Table 15 Iron contents and loss due to processing and cooking

Sample[1]	Iron content [2,3] mg %		% Processing loss [4]	% Cooking loss [5]
	Raw products	Cooked products		
Control	0.034	-	-	-
CLG+ $FeSO_4$	23.29±0.77	25.49±1.21	9.0±3.0	0
CLG+ NaFeEDTA	22.50±0.46	25.48±0.51	16.0±1.8	0
CLG+ Fe lactate	24.29±0.68	25.99±1.39	5.1±2.6	0
CL+ $FeSO_4$	22.29±0.33	26.76±0.15	12.9±1.3	0
CL+ NaFeEDTA	23.44±0.93	24.85±0.34	8.4±3.6	0
CL+ Fe lactate	24.13±0.57	26.33±0.45	5.8±2.2	0

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

[2]Samples were obtained from three production batches

[3]The values of the fortified products in each column were not significant difference ($p > 0.05$)

[4]Calculated based on initial value of 26.5 mg%

[5]Calculated based on difference in dry basis between dried and cooked products

5.2.3.3 Thiamin content

Thiamin loss due to processing was up to 20% (based the fortified dosage of 0.4 mg%). The loss was not found during cooking. There was no significant difference ($p > 0.05$) in thiamin contents in all fortified products (**Table 16**).

Table 16 Thiamin content and loss due to processing and cooking

Sample[1]	Thiamin content [2,3] mg %		% Processing loss [4]	% Cooking loss [5]
	Raw products	Cooked products		
Control		-	-	-
CLG+ $FeSO_4$	0.354±0.012	0.343±0.001	11.5±2.9	3.0±2.6
CLG+ NaFeEDTA	0.358±0.012	0.367±0.008	10.5±2.6	0.1±0.2
CLG+ Fe lactate	0.316±0.006	0.361±0.010	20.9±1.5	0.0±0.0
CL+ $FeSO_4$	0.361±0.012	0.350±0.020	9.9±5.2	3.4±5.9
CL+ NaFeEDTA	0.356±0.020	0.355±0.010	16.6±5.0	0.1±0.2
CL+ Fe lactate	0.354±0.027	0.357±0.007	11.5±6.8	1.7±1.5

[1]

CLG = Calcium lactate gluconate, CL = Calcium lactate

[2]Samples were obtained from three production batches

[3]The values of the fortified products in each column were not significant difference ($p > 0.05$)

[4]Calculated based on initial value of 0.4 mg%

[5]Calculated based on difference in dry basis between dried and cooked products

5.2.3.4 Folic acid

Folic acid contents determined by microbiological assay are shown in **Table 17.** Folic acid content of unfortified products was 56μg%, which was as high as amount of adding (55 μg%). Folic acid contents ranked from 64.76 to 94.25 μg%. Processing loss ranked from 15 to 42 percent.

Table 17 Folic acid content and loss due to processing of fortified dried broken rice

Sample[1]	Folic acid content [2,3] μg %		% Processing loss [4]	% Cooking loss [5]
	Raw products	Cooked products		
Control	59.14	-	-	-
CLG+ $FeSO_4$	94.25±12.823 [b]	68.973	15.1±11.6 [b]	40.3
CLG+ NaFeEDTA	83.31±3.159 [ab]	89.432	24.9±2.8 [ab]	22.4
CLG+ Fe lactate	67.52±5.661 [a]	88.714	39.2±5.1 [a]	22.5
CL+ $FeSO_4$	74.90±6.367 [ab]	70.801	32.5±5.7 [ab]	40.6
CL+ NaFeEDTA	64.76±4.118 [a]	72.171	41.7±3.7 [a]	37.2
CL+ Fe lactate	69.70±3.923 [a]	84.112	37.2±3.5 [a]	26.7

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

[2]Samples were obtained from three production batches

[3]The values of the fortified products in each column were not significant difference ($p > 0.05$)

[4]Calculated based on initial value of 111 μg%

[5]Calculated based on difference in dry basis between dried and cooked products

5.2.3.5 Nutritive value

Table 18 showed the nutritive value of the dried broken rice, which had been fortified based on the possible revised guidelines for nutritive value per 100 kcal compared to the current (WHO/UNICEF 1998) and revised (FAO, 2001) recommendations (3). The energy density of fortified products was 0.7 kcal/ g of cooked product. The fortified nutrients per 100 kcal were in the amounts at higher than 100% and most of the fortified nutrients were approximately 50% of the standards. **Table 19** showed % adequacy of nutrient based on the DRI, 2003 and WHO, 2002. Most of fortified nutrients were approximately 20-40% of both recommendations.

Table 18 Nutrient contents of the fortified dried broken rice and their adequacies based on current (WHO/UNICEF, 1998) and possible revised recommendations (FAO, 2001) of nutrients required from **complementary food per day** for children of different age groups.

Nutrient	Content		Adequacy based on the recommendation for complementary food[1] (%)					
	/100 kcal	/serving (20g)	6-8 mo[2]		9-11 mo[2]		12-23 mo[2]	
			Current[3]	Revision[4]	Current[3]	Revision[4]	Current[3]	Revision[4]
Energy	100	76.6	28	38	34	51	30	42
CHO (g)	22.7	17.40	NA	NA	NA	NA	NA	NA
Fat (g)	0.18	0.14	NA	NA	NA	NA	NA	NA
Protein (g)	1.9	1.44	77	72	91	96	84	87
Calcium (mg)	52	39.88	37	50	23	83	63	36
Iron (mg)	6.73	5.20	48	49	96	99	258	235
Thiamin (mg)	0.09	0.07	66	45	79	79	57	55
Folate (μg)	20.6	15.80	NA	72	NA	95	NA	45
Energy density = 0.7 (Kcal/g cooked product)								

[1]NA= Not applicable

[2]1 meals for 6-8 mo ; 2 meals for 9-11; and 3 meals for 12-23 mo.

[3]Energy required from complementary food=269 kcal, 451 kcal, 746 kcal for 6-8, 9-11, and 12-23 respectively (WHO/UNICEF, 1998)

[4]Energy required from complementary food=200 kcal, 300 kcal, 550 kcal for 6-8, 9-11, and 12-23 respectively (FAO, 2001)

Table 19 Nutrient contents of the fortified dried broken rice and their adequacies based on **Dietary Reference Intake (DRI) for Thais, 2003 and WHO Recommendations, 2002** of nutrients required per day for children of different age groups

Nutrient	**Content / serving (20g)**	**% Adequacy[1]**					
		6-8 mo[2]		**9-11 mo[2]**		**12-23 mo[2]**	
		Thai DRI 2003	WHO 2002	Thai DRI 2003	WHO 2002	Thai DRI 2003	WHO 2002
Energy (kcal)	76.60	10	NA	19	NA	45	NA
CHO (g)	17.40	NA	NA	NA	NA	NA	NA
Fat (g)	0.14	NA	NA	NA	NA	NA	NA
Protein (g)	1.44	9	NA	18	NA	45	NA
Calcium (mg)	39.88	15	10	29	20	24	24
Iron (mg)	5.20	55	55	111	111	267	267
Thiamin (mg)	0.07	24	24	47	47	42	42
Folate (μg)	15.8	20	20	39	39	33	30

[1]NA= Not applicable

[2]1 meal for 6-8 mo; 2 meals for 9-11 mo; and 3 meals for 12-23 mo.

5.3 Shelf-life study

During the accelerating storage condition (42^{o}C for 3 mo), changes in qualities of the fortified dried rice products that were packed in commercial polypropylene plastic bag and metallized bag were reported in **Table 20** and **Table 21**.

5.3.1 Moisture content and water activity

Moisture contents of the products packed both in plastic and metallized bags increased gradually. However, higher increase was found in the products packed in the plastic bag. As a consequence, water activity was also found to slightly increase (**Table 20**).

5.3.2 Thiamin

Loss of thiamin during storage was shown in **Table 21**. No losses were found in all fortified products. There were also no differences in thiamin contents of the products packed in polyethylene plastic and metallized bags. After 2 weeks, the rancidity smell of products both packed in plastic and metallized occurred, except the product fortified with NaFeEDTA packed in metallized bag.

Table 20 Moisture content and water activity of product kept for shelf life study

Sample [1]	Storage time	%Moisture		Water activity (Aw)	
		Metallized	Plastic	Metallized	Plastic
CLG +$FeSO_4$	0 thday	5.15±0.35	5.15±0.35	0.22±0.03	0.22±0.03
	2 wk	5.16±0.47	5.31±0.60	0.24±0.04	0.24±0.04
	1 mo	5.43±0.25	6.04±0.18	0.24±0.04	0.26±0.03
	2 mo	5.67±0.44	5.96±0.28	0.25±0.03	0.26±0.02
	3 mo	5.70±0.56	5.9±0.29	0.25±0.03	0.28±0.03
CLG +NaFeEDTA	0 th day	5.44±2.03	5.44±2.03	0.26±0.13	0.26±0.13
	2 wk	6.4±1.49	6.57±1.35	0.27±0.13	0.28.±0.12
	1 mo	6.53±1.44	6.89±1.47	0.28±0.13	0.29±0.10
	2 mo	6.17±1.64	6.66±1.15	0.26±0.01	0.26±0.02
	3 mo	6.15±1.60	6.52±1.08	0.27±0.09	0.28±0.04
CLG +Fe lactate	0 th day	4.32±0.30	4.32±0.30	0.18±0.05	0.18±0.05
	2 wk	5.18±0.58	5.4±0.31	0.20±0.05	0.20±0.05
	1 mo	5.41±0.53	5.66±0.53	0.20±0.05	0.20±0.02
	2 mo	5.99±0.15	5.69±0.01	0.23±0.04	0.21±0.03
	3 mo	5.58±0.32	5.96±0.2	0.22±0.04	0.27±0.04
CL + $FeSO_4$	0 th day	4.77±0.33	4.77±0.33	0.20±0.05	0.20±0.05
	2 wk	5.23±0.51	5.38±0.22	0.20±0.03	0.19±0.03
	1 mo	5.92±0.13	5.88±0.15	0.22±0.04	0.26±0.04
	2 mo	5.83±0.54	5.76±0.32	0.24±0.03	0.21±0.02
	3 mo	5.74±0.42	5.71±0.50	0.23±0.01	0.27±0.02
CL +NaFeEDTA	0 th day	4.16±0.17	4.16±0.17	0.17±0.02	0.17±0.02
	2 wk	4.53±0.60	5.14±0.60	0.20±0.02	0.19±0.02
	1 mo	5.23±0.54	5.45±0.53	0.18±0.03	0.22±0.03
	2 mo	5.39±0.14	5.67±0.04	0.21±0.01	0.21±0.03
	3 mo	5.13±0.23	5.69±0.10	0.21±0.01	0.26±0.02
CL +Fe lactate	0 th day	4.32±0.26	4.32±0.26	0.21±0.05	0.21±0.05
	2 wk	4.87±0.29	5.65±0.57	0.20±0.07	0.23±0.02
	1 mo	5.50±0.61	5.96±0.42	0.22±0.01	0.25±0.03
	2 mo	5.55±0.45	5.66±0.13	0.23±0.02	0.20±0.01
	3 mo	5.63±0.57	5.81±0.12	0.24±0.02	0.27±0.01

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

Table21 Thiamin contents in fortified dried broken rice packed in different packaging materials during shelf-life study

Sample [1]	Storage time	Thiamin	
		Metallized	Plastic
CLG +$FeSO_4$	0^{th}day	0.354 ± 0.012	0.354 ± 0.012
	2 wk	0.344 ± 0.005	0.330 ± 0.028
	1 mo	0.329 ± 0.028	0.324 ± 0.012
	2 mo	0.347 ± 0.011	0.350 ± 0.009
	3 mo	0.345 ± 0.016	0.323 ± 0.028
CLG +NaFeEDTA	0^{th} day	0.358 ± 0.010	0.358 ± 0.010
	2 wk	0.354 ± 0.010	0.332 ± 0.020
	1 mo	0.365 ± 0.023	0.350 ± 0.012
	2 mo	0.356 ± 0.015	0.351 ± 0.017
	3 mo	0.352 ± 0.016	0.358 ± 0.017
CLG +Fe lactate	0^{th} day	0.364 ± 0.007	0.364 ± 0.007
	2 wk	0.331 ± 0.016	0.332 ± 0.027
	1 mo	0.331 ± 0.027	0.326 ± 0.038
	2 mo	0.356 ± 0.005	0.321 ± 0.032
	3 mo	0.359 ± 0.002	0.330 ± 0.021
CL + $FeSO_4$	0^{th} day	0.360 ± 0.020	0.360 ± 0.020
	2 wk	0.338 ± 0.020	0.303 ± 0.018
	1 mo	0.337 ± 0.009	0.329 ± 0.002
	2 mo	0.351 ± 0.025	0.318 ± 0.013
	3 mo	0.348 ± 0.020	0.307 ± 0.005
CL +NaFeEDTA	0^{th} day	0.356 ± 0.014	0.356 ± 0.014
	2 wk	0.326 ± 0.028	0.324 ± 0.027
	1 mo	0.333 ± 0.020	0.326 ± 0.017
	2 mo	0.339 ± 0.010	0.331 ± 0.012
	3 mo	0.338 ± 0.012	0.319 ± 0.007
CL +Fe lactate	0^{th} day	0.354 ±0.005	0.354 ±0.021
	2 wk	0.333 ± 0.020	0.293 ± 0.016
	1 mo	0.330 ± 0.016	0.323 ± 0.012
	2 mo	0.326 ± 0.007	0.317 ± 0.017
	3 mo	0.329 ± 0.012	0.303 ± 0.028

[1]CLG = Calcium lactate gluconate, CL = Calcium lactate

5.4 Estimation of bioavailability of various iron sources fortified in dried broken rice model by *in vitro* digestion method

Table 22 Percentage of dialyzable iron from dried rice based complementary food[1]

Samples	Dialyzable iron (%)
Control	ND
CLG + Ferrous sulfate	2.5±0.4 [c]
CLG + NaFeEDTA	61.1±2.8 [a]
CLG + Ferrous lactate	5.2±0.7[c]
CL+ Ferrous sulfate	1.6±0.2[c]
CL + NaFeEDTA	56.4±4.0[b]
CL+ Ferrous lactate	2.0±0.1[c]

[1] Results are the means of 6 determinations ± SD. Means within the same column followed by same letter are not statistically significant different using one-way ANOVA test ($p < 0.05$): ND = Not detectable, CLG=calcium lactate gluconate, CL=calcium lactate,

In this study, the bioavailability of iron was expressed as percentage of dialyzable iron obtained from *in vitro* digestion technique. *In vitro* percentage of dialyzable iron from fortified dried broken rice was compared (**Table 22**). The bioavailability of native iron in control dried broken rice was very poor. Fortification of cooked dried broken rice with iron compounds significantly increased both the iron content and iron bioavailability. The percentage of dialyzable iron from NaFeEDTA fortified dried broken rice, which was approximately twenty times the amount of dialyzable iron compared to other iron forms was significantly highest when compared with the other iron form fortified dried broken rice ($p < 0.05$). However, the different kinds of calcium salts caused different in percent dialyzable iron. The percentage of dialyzable iron of products added calcium lactate gluconate was higher than one added calcium

lactate with the same types of iron sources. No significant different were observed on the percentages of dialyzable iron between ferrous sulfate and ferrous lactate fortified dried broken rice with any calcium salts. It should be noted that the results of this study only reply the trend of iron bioavailability rather than the absolute absorption.

5.5 Home use test

The overall acceptance by the mothers/caretakers was very good. None of them strongly disliked the product. Most of them liked the product as “slightly” to “very much”. As compared to normal rice, the cooked fortified dried broken rice was told to be “more convenient” to “very convenient” and the majority mentioned that the price 10 baht was affordable.

CHAPTER VI
DISCUSSION

6.1 Development of method for fortification

6.1.1 Selection of fortification method

The fortification methods that used cooked rice i.e. 1st and 2nd methods needed drying period of at least 12 h. Such methods were commented by food industry that the drying period was not practical since it was too long. Besides, the cooked rice was too stick and not easily spreadable on drying tray. While the 3rd method was totally unpractical since high amount of calcium needed could not be dissolved in limited volume of water. The 4th method was modified from the method currently practiced at the factory, it was therefore the most practical and acceptable since it needed less than 3 h for drying and resulted in homogeneous fortified nutrients (**Table 10**). The products produced from all methods mentioned above were convenient since they required only 8-9 min for cooking as comparing to the normal rice that normally took at least 20 min.

6.1.2 Fortificant dosage

Our preliminary study indicated that vitamin A was lost up to 80% during processing of all methods, even the 4th method. Vitamin A was sensitive to atmospheric oxygen, ultraviolet light, high temperature and the presence of metal, since these factors could accelerate the oxidation reaction (39,73). Vitamin A was therefore not included in the fortification. The fortification level was calculated based on the assumption in Souvaphapsopha's study (2), that the appropriate serving size of the cooked complementary food was 180 g. Since mother was not real consumer of

the product, she was therefore asked only for her opinion on appropriateness instead of acceptability. At the fortification level of iron that met the recent FAO standard, (5.3 mg/100kcal) the product was still rated to be appropriate for using as complementary food. Calcium lactate gluconate was selected for this study since it was the form that was more practical for being used at the industry scale. Calcium lactate gluconate had better solubility and also resulted in a better appearance of the cooked products. Even calcium lactate gluconate costs more, it still could satisfy the manufacturer. For the iron sources, ferrous sulfate and NaFeEDTA were selected in the study due to their wide uses in many food products. As compared to NaFeEDTA, ferrous sulfate provided products that had an unacceptable duller color tone, however it was still more preferred by manufacturer since it had a lower cost, better solubility, and did not affect the quality of cooked product.

6.2 Quality evaluation

6.2.1 Degree of homogeneity

Since the fortificants used were homogeneously mixed into solution before being used, only iron that was more stable and easily analyzed was therefore used as indicator of nutrient homogeneity due to the fortification process. The coefficient of variation of iron in the product was only 3.2%, which indicated that the fortification method used resulted in a homogeneous product. For industrial implementation, the fortificants however would be prepared as a homogeneous premix powder from the supplier. Therefore, the 4^{th} fortification method to be used in industry could be used regardless of the amount used.

6.2.2 Physical property

6.2.2.1 Color

Iron and calcium fortificants of different sources were found to affect on sensory quality of the products differently, especially on color. Iron fortification normally was quite complicated since it always affected on color changes in food vehicles. Iron fortifications with ferrous sulfate, ferrous lactate and NaFeEDTA resulted in yellowish to light-brownish colored products. However, such effect was not consequently found in the cooked product that was fortified with NaFeEDTA. The cooked product fortified with NaFeEDTA had a normal white color, which was different from the cooked products fortified with the other 2 fortificants that had slightly yellow color. Calcium fortification with calcium lactate resulted in product with more dull white color than the fortification with calcium lactate gluconate. However, the products fortified with both calcium sources became normal and no difference after being cooked. Combination of NaFeEDTA either calcium source resulted in product with the most normal sensory characteristics. The use of calcium lactate in combinations with other iron sources i.e. ferrous sulfate and ferrous lactate resulted in cooked dull yellow color, while the use of calcium lactate gluconate with these iron sources resulted in brighter yellow color.

6.2.2.2 Water activity

Due to the very low water activity (0.2), the dried fortified product was very stable in term of microbial quality. However, lipid oxidation was the problem that needed to be aware since oxidative rancidity could be accelerated under low water activity condition (lower than 0.3) especially with iron as a catalyst (73).

6.2.2.3 pH

The pH values of cooked fortified products were slightly lower than the unfortified one (**Table 13**). Regarding to our previous study (unreported data), pH of the water was affected by iron not calcium fortificants. Iron of different sources could affect pH of water differently. With the same amount of iron obtained, the pH of water became 3.58, 4.89, and 5.49 for ferrous sulfate, NaFeEDTA, and ferrous lactate,

respectively. While pH value of calcium lactate and calcium lactate gluconate solution were 6.56 and 7, respectively. Therefore, pH values of the cooked were mainly affected by the iron fortificant used.

6.2.3 Nutrient composition and loss due to processing and cooking

6.2.3.1 Calcium and iron

Calcium content in the product was only approximately 6 % lower than what expected (208mg/100 g). Calcium was a stable nutrient that normally lost only due to leaching. In this fortification process, most of the fortificant solution was absorb into rice, therefore only small amount of calcium was in the unabsorbed solution. Loss of calcium during drying and cooking would unlikely occur since calcium would not be destroyed by oxidation and heat at cooking temperature (34). Besides, the water used for cooking was not drained out.

As compared to calcium, iron loss was found to be higher which was 8-19 % in the dried product. The cause of loss should be the same as calcium, however more iron might be adsorbed on the surface of plastic basin that was used for soaking rice. Regarding to our previous study (unreported data), approximately 2 mg of iron was found adsorbing on surface of the plastic basin. Iron content was however found increase up to 1.7 mg as the products was cooked in a stainless steel pot. The increase was due to dissolved iron from the pot during cooking for 8-9 min. Such evidence was not found as the water (pH 6.9) was cooked in the same pot. The acidic condition (pH 4.6) occurred after iron compound had been added could be the main reason for iron dissolution.

6.2.3.2 Thiamin and folic acid

As a water-soluble vitamin, thiamin lost up to 20%. High temperature was the main reason for thiamin loss, since it was relatively stable to atmospheric oxygen in absence of light and moisture. Cooking slightly affected thiamin loss due to the short period of only 8 min. Vandrasek H.T. et al (1987) studied thiamin partitioning and retention in cooked rice and pasta products and found that thiamin loss from cooking was only through leaching making the retention of cooked-enriched pasta

product ranged between 66-47%. They mentioned that leaching could play an important role in thiamin loss where part of the cooking liquid is not consumed (45). The fortification dosage of thiamin should be 20% more the requirement in order to compromise for the loss during processing

Folic acid contents of the dried products ranged from 64-93 μg%, which was higher than the fortified dosage of 55 μg%. It was found that the unfortified product contained up to 56 μg%. Therefore high loss of folic acid in fact occurred during drying. During cooking, loss in folic acid was still found up to 40%. The cooked product however still contained folic acid in the amount that could meet the requirement (> 55 μg%). Besides vitamin C, thiamin and folic acid were the most heat sensitive (74).

Sunlight and particularly ultraviolet radiation, has serious effect on the stability of folic acid (46, 39). Losses of around 10% are found in boiled eggs, while other forms of cooking (fried, poached, scrambled) give between 30 and 35% loss and total folic acid losses from vegetable as a result of heating and cooking process can be as high as 50% (39).

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6.2.3.3 Nutritive value of fortified dried broken rice

Only fortified broken rice could not fulfill the needs for certain nutrients, especially energy. Energy density of the cooked fortified broken rice was too low. Such weakness was not the problem since the product was normally treated as rice that was used as a base for mixing with other foods such as egg yolk, meat, liver, vegetable oil, as recommended in the Guideline of Ministry of Public Health, 2000 (4). Most of the fortified nutrients were approximately 50% higher than the standards, which would compensate for the additional energy after other foods had been added. Since the formulation was based on complementary food standard of infant aged 6-8 mo, most nutrients were therefore approximately 100% of the Revised FAO, 2001 standard for complementary food (40 mg calcium, 5.3 mg iron, 0.08 mg thiamin, and 11 μg folate per 100 kcal, **Table 18**).

In case of infants of older ages, this product if fed for 3 meals provided too high iron, that was up to 250% of the standard for complementary food (**Table 18**),

which consequently affected its adequacy based on Thai DRI, 2003 and WHO recommendation 2002 to be too high (**Table 19**). However, older infants were normally not solely relied on this complementary food for 3 meals but liked to enjoy varieties of food, especially from the family pot, which will be benefit for feeding practice.

6.3 Shelf-life study

During the accelerating storage condition, both commercial polyethylene plastic bag and metallized bag could not absolutely protect moisture; the moisture contents of products packed the former kind of bag tended to increase at a higher rate. However, the final water activities were still only about 0.2-0.3, which was quite stable in term of microbial growth. Polyethylene normally is good barrier for moisture but not oxygen. During the test, all irons except NaFeEDTA catalyzed oxidation reaction and caused product rancidity within two weeks. NaFeEDTA was the best iron fortificant in this study. Thiamin which was used as the indicator for nutrient loss during storage did not lose in both commercial polyethylene plastic bag and metallized bag, since it is not sensitive to oxygen and light.

6.4 Estimation of bioavailability of various iron sources fortified in dried broken rice model by *in vitro* digestion method

Bioavailabilities of the cooked dried broken rice fortified with different iron sources were different, which NaFeEDTA was the most effective as compared to ferrous sulfate and ferrous lactate. NaFeEDTA was relatively soluble in different food matrices and non-reactive, which could prevent iron binding with dietary inhibitors such as phytates and polyphenols. In cereal that contained high phytates, the iron bioavailability from NaFeEDTA is 2-3 times higher than ferrous sulfate (27). Trinidad (2002) studied the bioavailability of ferrous sulfate and NaFeEDTA in fortified rice by

using both *in vitro* and *in vivo* method. *In vivo* study showed that the percentage of iron absorption from NaFeEDTA was higher than ferrous sulfate, while no difference was found *in vitro* method (57). Different sources of calcium also affected iron bioavailability. Gluconate on calcium lactate gluconate has been reported as an enhancer for the iron absorption. Pirapatdit (2003) studied on bioavailability of various forms of iron fortified in fresh wheat noodles model by using in vitro method and found that the percentage of iron dialyzability of product fortified with NaFeEDTA was the highest when compared with ferrous sulfate, ferrous fumarate, and encapsulated reduced elemental iron powder (75).

6.5 Home use test

Most infants accepted the cooked product with the foods of their caretakers' choices. And they could consume at the same amount as usual. The acceptability of mother was another important and significant factor. The attractive factors for mothers were convenience and nutritive value of the product. The product needed shorter cooking period, therefore it did not stick to the pot. While, mothers would like their infant to be healthy by consuming the fortified products. By using the product as rice for mixing with other foods, it was found that the prepared complementary foods had adequate nutrient values based on WHO/UNICEF (1998) and FAO (2001) standards.

The price of 10 baht was mentioned to be reasonable by most mothers and producers. This price could be affordable by consumers however was high enough to provide marketing opportunity to the producer.

CHAPTER VII
CONCLUSION

It was feasible to produce dried broken rice product fortified with calcium, iron, thiamin and folate at the levels recommended in FAO guideline (2001). During processing, folate loss was found to be highest, while losses of minerals were also found mainly due to absorption on the surface of soaking container. The products fortified with iron sources except NaFeEDTA could not be stored in commercial economical packagings i.e. plastic and metallized bags due to oxidative rancidity. As the product was used as a basic ingredient, the complementary foods prepared based on the Thailand Ministry of Public Health guideline could fulfill the recommended daily allowance (RDA) of most nutrients for infants aged 6-24 mo. NaFeEDTA was the most appropriate iron fortificant due to product stability and high *in vitro* bioavailability.

For further studies, bioavailabilities of nutrients under the real consumption condition (with other foods) must be performed, especially *in vivo*. Studies on appropriate antioxidants or packagings should also be conducted in order to evaluate the feasibility in extending the shelf stabilities of the products fortified with lower cost iron sources.

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APPENDIX A

Questionnaire

APPENDIX A

แบบประเมินผลทางประสาทสัมผัส
ผลิตภัณฑ์ข้าวต้มเสริมวิตามินและแร่ธาตุสำหรับทารก 6-12 เดือน

สูตรหมายเลข.....

ผู้ประเมินอายุ..... **วันที่**.....

ผลิตภัณฑ์นี้เป็นข้าวต้มกึ่งสำเร็จรูปเสริมวิตามินและแร่ธาตุที่เป็นประโยชน์ต่อทารก ในความเห็นของท่านซึ่งมีความคุ้นเคยในการเลี้ยงทารก ***ท่านคิดว่าผลิตภัณฑ์นี้เหมาะสมในการใช้เลี้ยงทารกเพียงไร***

ตอนที่1 ก่อนชิมเมื่อท่านได้รับผลิตภัณฑ์อย่าเพิ่งชิม กรุณาให้คะแนนความเหมาะสมของผลิตภัณฑ์โดยการมองแล้ว ขีดเครื่องหมาย√ ลงในช่องที่ตรงกับความคิดเห็นของท่านมากที่สุด

1 ความเหมาะสมต่อลักษณะโดยทั่วไป หมายถึง ความเหมาะสมในการใช้เลี้ยงทารก

..... เหมาะสมมากที่สุด
..... เหมาะสมมาก
..... เหมาะสมปานกลาง
.....เหมาะสมเล็กน้อย
.....เฉยๆ
..... ไม่เหมาะสมเล็กน้อย
.....ไม่เหมาะสมปานกลาง
.....ไม่เหมาะสมมาก
..... ไม่เหมาะสมมากที่สุด

ข้อเสนอแนะ.....
.....
.....

2. ความเหมาะสมของสี

.....เหมาะสมมากที่สุด
.....เหมาะสมมาก
.....เหมาะสมปานกลาง
..... เหมาะสมเล็กน้อย
..... เฉยๆ
.....ไม่เหมาะสมเล็กน้อย
.....ไม่เหมาะสมปานกลาง
.....ไม่เหมาะสมมาก
.....ไม่เหมาะสมมากที่สุด

ข้อเสนอแนะ.....
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เฉย ๆ คือไม่สามารถบอกได้ว่าเหมาะสมหรือไม่เหมาะสม

ตอนที่ 2 กรุณาชิมตัวอย่าง กับไก่คั่วเค็มแล้วให้คะแนนความเหมาะสมเฉพาะข้าวต้มเพียงอย่างเดียวในการใช้เลี้ยงทารก โดยขีด √ ลงในช่องที่ตรงกับความเห็นของท่านมากที่สุด

1.ความเหมาะสมของผลิตภัณฑ์โดยรวม หมายถึง ความเหมาะตามความเห็นของท่านหลังจากได้ชิมผลิตภัณฑ์แล้ว

..... เหมาะสมมากที่สุด
..... เหมาะสมมาก
..... เหมาะสมปานกลาง
.....เหมาะสมเล็กน้อย
.....เฉยๆ
..... ไม่เหมาะสมเล็กน้อย
.....ไม่เหมาะสมปานกลาง
.....ไม่เหมาะสมมาก
..... ไม่เหมาะสมมากที่สุด

ข้อเสนอแนะ……
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2. ความเหมาะสมในกลิ่นของข้าวต้ม

.....เหมาะสมมากที่สุด
.....เหมาะสมมาก
.....เหมาะสมปานกลาง
..... เหมาะสมเล็กน้อย
..... เฉยๆ
.....ไม่เหมาะสมเล็กน้อย
..... ไม่เหมาะสมปานกลาง
.....ไม่เหมาะสมมาก
.....ไม่เหมาะสมมากที่สุด

ข้อเสนอแนะ……..
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3 ความเหมาะสมในรสชาติของข้าวต้ม

.....เหมาะสมมากที่สุด
.....เหมาะสมมาก
.....เหมาะสมปานกลาง
.....เหมาะสมเล็กน้อย
.....เฉยๆ
.....ไม่เหมาะสมเล็กน้อย
.....ไม่เหมาะสมปานกลาง
.....ไม่เหมาะสมมาก
.....ไม่เหมาะสมมากที่สุด

ข้อเสนอแนะ……..
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เฉยๆ คือไม่สามารถบอกได้ว่าเหมาะสมหรือไม่เหมาะสม

APPENDIX B

Determination of moisture

Hot-air-oven method; AOAC 1990, 952.45

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Determination of moisture

Hot-air-oven method; AOAC 1990, 952.45

Principle:

A well homogeneous sample is dried in an oven (usually at 100 + 5 ^{o}C) until constant weight is obtained. The loss of weight is taken as a measure of moisture content in the sample. Acid washed sand is used to mix with the wet sample prior to dry in order to increase area for rapid and complete evaporation of water from the wet sample

Procedure:

1. Weigh approximately 20 g of acid washed sand into a porcelain dish containing a small glass stirring rod and dry in hot air oven at 100 + 5^{o}C for 30 min.
2. Remove the sand dish and cool in the desicator.
3. Weigh sand dish (=a) and then approximately 5 g sample. Reweigh(=b g)
4. Add small amount of distilled water to disperse the sample evenly and evaporate the water as mush possible on the boiling water bath. The sample dish should be frequently mix until dries.
5. Transfer the sample dish to hot air oven and dry the sample at 100 + ^{o}C for 2 hr.
6. Remove the sample dish and cool in a desicator and weigh (=c g)
7. Return the sample dish to the hot air oven and dry until a constant weight is obtained. Reweigh every 30-min.
8. The different weight between each interval time should not be more than 1-3 mg

Calculation:

% Moisture = (b-c)/(b-a)x 100 (w/w)

APPENDIX C

Determination of Ash (Dry Ashing)

APPENDIX C

Determination of Ash (Dry ashing)

Principle:

The ash content is determined by incinerating a known quality of foodstuff, previously dried, in a muffle furnance at 550^{o}C until constant weight. The residue (the ash) is dissolved in acid solution, then subjected to analysis of minerals (Calcium, phosphorus, sodium and potassium was determined from the ash obtained).

Reagent and Instrument:

- 50% nitric acid
- Porcelain crucible
- Muffle furnace
- Water bath
- Bunsen burner

Procedure:

1. Dried acid washed porcelain crucibles in the furnace at 550 ^{o}C for 30 min.
2. Cool the crucible in a desicator for 20 min. Weighed, nearest to 1 mg (=a g)
3. Weighed exactly 2-10 g of homogeneous sample into the weighed crucible.
4. Heat the dried sample over a low flame of bunsen burner or on an electric hot plate until clear. This is to burn away some of organic matter.
5. Incinerated the charred sample in a furnace at 550^{o}C until complete ashing was obtained (white or light gray ash). Removed the crucible and cooled in a desicator.
6. Weighed nearest to 1 mg (=b g).
7. If the sample is not completely ashed, 2ml 50% nitric acid is carefully added and mixed. Evaporate off the acid and dry the sample over the flame or heater and reincinerate until complete ashing is obtained.

Calculation

$$\text{Ash} = \frac{(b-a) \times 100}{\text{Weight or volume of sample}} \text{ \% w/w or \% w/v}$$

APPENDIX D

Determination of Calcium

APPENDIX D

Determination of Calcium

Principle:

Sample was prepared by dry ashing. Ash was dissolved in 4N HNO_3 and dilute in deionized water.

Reagents

- Standard calcium
- 5% stock lanthanum solution (for calcium):58.659 of lanthanum oxide was dissolve in 250 ml of concentrated hydrochloric acid. The solution was stirred until clear and then dilute to 1000 ml with deionized distilled water.
- 4 N nitric acid

Procedure:

The sample filtrate (from ash) obtained was diluted appropriately with 10% 4N nitric acid and 20%(v/v) of 5% lanthanum solution in a volumetric flask and finally the calcium concentration in filtrate was adjusted with deionized distilled water. Calcium determination was conducted using flame atomic absorption spectrophotometer (Varian; spectr AA-20). The normal working condition of atomic absorption spectrophotometer (AAS) for analyzing calcium as follow:

Flame:	air/acetylene
Lamp current	4 mA
Slit width	0.5 nm
Wavelength	422.7 nm

Calculation:

$$\text{Ca (mg/100g)} = \frac{\text{Y x (b-a) x 0.015287 x 100 x100}}{\text{Weight of sample}}$$

Note:

1. Y is dilution of sample (used for ash determination)
2. If b-a is more than 1.5 g, dilute the sample and reanalysed
3. Clean glassfilter drucible with deionized distill water, then 4 N NaOH, distilled water, 4 N nitric acid, 70% ethanol, and ether, respectively.

APPENDIX E

Determination of total iron

(Wet digestion)

APPENDIX E
Determination of total iron
(Wet digestion)

Principle:

Wet ashing technique was used to prepare the sample for the determination total iron. The samples were digested by nitric acid and perchloric acid at ratio 5:1 and then determined the iron content using an atomic absorption spectrophotometer at a wavelength of 248.3 nm.

Reagent:

1. Conc. Nitric acid (Merk # 1.00456.2500)
2. Conc. Perchloric acid (JT Baker 3 9625-04)
3. Ferric standard solution (Merk # 1.09972): Stock standard solution (1000 ppm)
4. Intermediate standard: Dilute 10 ml of stock standard to 100 ml with deionized water to make 0.1 mg Fe/ml standard solution.
5. Working standard: 0.5-2.0 mg Fe/100 ml. Prepared by an appropriate dilution of the intermediate standard with deionized water, in the presence of 10% 4N nitric acid.

Instrument:

1. Teflon
2. Volumetric flask
3. Filter paper # 42 and funnels
4. Atomic absorption spectrophotometer (Varian; Spectr AA-20)

Procedure:

1. Weigh 1-5 g of the homogeneous sample (depending on expected iron content) into teflon.
2. Add 5 ml conc. Nitric acid and 1 ml perchloric acid to eaach of teflon sample and tightly covered with lids.

3. Keep the teflon sample under fume hood at room temperature for predigestion overnight.
4. Place the teflon sample in hot air oven for 16-20 hr or until the solution clear.
5. Transfer the digested sample to an appropriate volume of volumetric flask and dilute with deionized distilled water.
6. Measure the diluted sample, working standard iron and reagent blank by atomic absorption spectrophotometer.

The insttrument setting:

Flame	air/ acetylene
Lamp	5 mA
Spectral band pass	0.5 nm
Wavelength	248.3 nm
Flame stoichiometry oxidizing	

Calculation:

$$\text{Total iron (mg/100 g)} = \frac{\text{absorbance x standard curve x 100}}{\text{Weight of sample (g)}}$$

APPENDIX F

Determination of Thiamin

APPENDIX F

Determination of Thiamin

Principle:

The vitamins are extracted from the food by acid and enzyme digestion. The aqueous extract is injected on to a reverse phase HPLC column. The fluorescence of ribloflavin is measured and thiamin is determined after post column derivatisation to convert the thiamin to thiochrome.

Reagent:

1. Acetic acid, glacial
2. 1-Hexanesulphonate sodium salt
3. 5% Sodium hydrogen carbonate: Dissolve 5g of sodium hydrogen carbonate in 100 ml of deionized water
4. Hydrochloric acid 0.1 N: Add 8.3 ml concentrated HCl to 100 ml of deionized water
5. Sodium acetate, 2.5M: Dissolve 205g anhydrous sodium acetate or 340g sodium acetate-trihydrate in deionized water and dilute to 1000 ml
6. Takadiastase 10%: Dissolve 10g of the enzyme (Fluka Cat. No. 86250) in 100ml of queous sodium acetate
7. Potassium ferriccyanide
8. Sodium hydroxide
9. Acidified 20% ethanaol: 20%ethanol adjusted to pH 3.5-4.3 with 0.1N HCl
10. 0.02N CH_3COOH: Add 1.14ml glacial acetic acid to 500ml of deionized water and dilute to 1L with deionized water.

Thiamin standard

(a) Stock thiamin, 100 μg/ml: accurately weigh 0.0224g thiamin-HCl (Sigma Cat. No T-4625) into a 200 ml volumetric flask. Dissolve and dilute to the volume with the acidified 20% ethanol.(Stock standards kept at 4^{o}C and

protected from light in amber glass bottles. Prepare fresh stock standard every two months)

(b) Mixture of intermediate standard I, 20 μg/ml (for %recovery test): pipette 10ml stock thiamin into a 100ml volumetric flask. Dilute to volume with deionized water.

(c) Mixture of intermediate standard II, 20 μg/ml. (for standard curves): pipette 10ml of stock thiamin into 100ml volumetric flask. Dilute to the volume with deionized water.

Standard curve: Pipette 10 ml of (c) into an erlenmeyer flask. Treat the working standard in the same way as samples or three times (begin, middle and end) through out the determination. Prepare 3 concentration of calibrating standards from extracted standard follows.

Calibrating standard	Concentration (ng/ml)	Volume of extracted standard	Final volume
A	200	10	50
B	400	10	25
C	500	10	10

Procedure:

Weigh accurately about 1-6g dry sample or pipette 10ml of liquid sample into a 125ml erlenmeyer flask. For %recovery study, spike the recovery sample with 2ml of 10 μg/ml standard solution (c). Add about 60ml 0.1M hydrochloric acid or ≥ 10 times dry weight sample in gram to all flasks then cap with aluminium foil and mix. Place the flask in the boiling water bath for 30 min, with further mixing at 10 min intervals or autoclave mixture 30 min at 121^{o}C.

Warning: Hot acid solutions in bottles under pressure. Wear rubble groves when handling.

Remove flasks from water bath, then cool to room temperature. Add 5ml of 10% Takadiastase solution, cap flask, mix and incubate in a 37^{o}C incubator for overnight or 45-50 ^{o}C water bath for 3 hr or 55^{o}C water bath for1 hr with intermittent mixing. Cool to room temperature and quantitatively transfer into 100ml volumetric flask. Dilute to the volume with deionized water. Then filter through filter paper whatman 42 and

collect the filtrate in a 125ml erlenmeyer flask. Pass 10ml filtrate through a 0.45μm membrane fliter discs (Gelman; type Nylaflo) and collecting an aliquot into 5ml amber glass vial which is ready for HPLC (CONSIAMETRIC 3200) analysis. Set up the HPLC system using the following conditions:

Column: As above

Mobile Phase: 0.005M hexanesulphonic acid (sodium salt) in 85:15 methanol: water, pH 6.0. Dissolve 0.5156 g of 1-hexanesulphonic acid sodium salt monohydrate in 500 ml of 85% methanol in water. Add 1ml of glacial acetic acid, and adjust pH of solution to 6.0 by addition of 5% aqueous $NaHCO_3$. Filter and degas before use.

Oxidant: 1 mM potassium ferricyanide in 0.375 M Sodium hydoxide. Dissolve 0.33g potassium ferricyanide in 100ml of water, mix with 200 ml water containg 15g NaOH and dilute the mixture to 1L. Store in an amber glass container. Prepare freshly before use.

Detector: Fluorescence, excitation 360nm, emission 435 nm, gain 100, atten 8.

Injection: 10μl

Flow Rates: 1.3 ml/min (mobile phase)
1.0 ml/min (oxidant)

Retention Time: 3-4 min.

Oxidant is introduced at the T-piece, what is placed between the column and the detector.

Note: It is necessary to use an inert plastic filter in the oxidant pump intake line

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APPENDIX G

Determination of Folic acid

APPENDIX G

Determination of Folic acid

Principle:

The method is based on the observation that certain organisms require specific vitamins for growth. Using the basal medium containing all nutrients except that to be assayed, growth responses of the organism are then compared quantitatively with standard of known concentration.

Reagent:

- 0.2 M Stock phosphate buffer*.

(A) 31.20 g NaH2PO4 (MW156.01) dilute to 1 L with deionized water

(B) 28.39 g NHPO (MW 141.96) dilute to 1 L with deionized water

- Form of available salts must be checked carefully. Weight of reagent ussed varies to the form of salts.

Working buffer: prepare freshly before use.

212.5 ml (A)+35.5 ml (B) dilute to nearly 1000ml with deionized water, add ascorbic acid to the buffer in the concentration of 0.1 % (W/V), adjust pH to pH 6.1 with (B) or (a) and dilute to 1000 ml with deionized water.

- Microorganism: *Lactobacillus casei* ATCC 7469
- Stock medium: Bacto-Micro Assay Culture Agar (MACA).
- Stock culture: Stab culture (3 tubes) in MACA. Incubate 35-37 ^{o}C, 24-48 h. Store 3 tubes of the stock culture in a refrigerator. Subculture monthly in triplicate.
- Culture medium: Micro-inoculum broth
- Inoculum: Subculture L. casei from a stock culture to Micro-inoculum broth. Incubated at 35-37 ^{o}C,18-24 h . Under aseptic condition, wash cells with 3 x 5 ml portions of steriled 0.9% NaCl solution(NSS). Decanted the last supernate. Diluted the inoculum to an appropriate concentration with steriled NSS (McFaland No.0.5)
- Assay medium: Bacto-Folic acid assay Medium.

- Chicken pancreas powder (Difco)
- Folic acid standard: Folic acid

Stock folic acid standard (100 μg/ml): dissolve 25 mg dried folic acid in 0.1 N NaOH by adding NaOH little by little until the solution is clear. Then adjust the pH of solution to 7 with 0.05N HCl. Made up to 250 ml with 20% ethyalcohol.

Intermediate standard I: 2 ml (100μg) 200 ml (1000ng/ml)

Intermediate standard II: 2 ml (1000 μg) 200 ml (10ng/ml)

Working standard: 20 ml (10ng/ml) 250 ml(0.8 ng/ml)

Standard curve preparation: pipette in triplicate

Folic acid concentration (ng/tube)	0	0.2	0.4	0.8	1.2	1.6
Working standard (ml)	0	0.25	0.5	1.0	1.5	2.0
Deionized water (ml)	2	1.75	1.5	1.0	0.5	0
Folic acid assay medium (ml)	2	2	2	2	2	2

Blank: 2 sets of uninoculated blank for zero setting

Procudure:

Weigh x grams of sample + 100 ml buffer, autoclave 120 ^{o}C/10 min. After cooling, added 20 mg chicken pancreas powder (2 ml of 1% buffered enzyme solution) per g of sample. Incubated 45 ^{o}C for 1 h (or adding 0.5-ml toluol and incubated 37^{o}C 16 h). Autoclave 120 ^{o}C/5 min (or boiling in a water bath at 100 ^{o}C/15 min). After cooling, diluted to 200 ml and filter. Adjust pH of a portion of the clear filtrate to pH 6.2 and diluted to appropriate concentration of about 0.25-0.3 ng folic acid /ml.

Sample test :pipette in duplicate

ml extract	0.5	1	2
ml deionized water	1.5	1	0
ml medium	2	2	2

After mixing, the standard and sample tubes were steriled by boiling at 100 ^{o}C for 15 min (or autoclaving at 120 ^{o}C for 5 min). Aseptically inoculate each tube with 1 drop

of appropriate inoculum, using a steriled pasteur pipette. Mix thoroughly and incubated the set at 35-37 ^{o}C for 18-24 h. Stop growth by boiling at 100 ^{o}C for 5 min. Cool and measure growth of the test organism by the turbidimetric method at the wavelength of 620 nm.

APPENDIX H

Determination of dialyzable iron

(*In vitro* digestion technique)

APPENDIX H

Determination of dialyzable iron

(*In vitro* digestion technique)

Principle:

In vitro digestion method involves a simulated digestion with pepsin in the peptic digestion stage followed with the pancreatin-bile extract in the intestinal digestion stage. Dialyzability of the element is used as an estimated trend of how well the element is absorbed.

Reagent:

1. A pepsin solution is prepared by dissolving 16 g pepsin (Sigma P-7125, from porcine stomach mucosa) in 100 ml of 0.1 M HCl
2. Pancreatin-bile extract mixture contains 4 g pancreatin (Sigma P-1500, from porcine pancreas) and 25 g bile extract (Sigma B-8631, porcine)in 1 ml of 0.1 M $NaHCO_3$
3. 0.5 M $NaHCO_3$: weigh 4.2 g NaHCO3 (Merk 1.06329.1000) and dissolve in 100 ml of deionized distilled water.
4. 0.5 M KOH: weigh 2.8 g KOH (Merk 1.05033.1000) and dissolve in 100 ml of deionized water.
5. 6 N HCl: measure 50 ml of 12 M HCl (Merk 1.00317.2500) and dilute to 100 ml deionized distilled water.

Instruments:

1. Erlenmeyer flask 250 ml and 125 ml.
2. Homogenizer NiSSei AM-1.
3. PH meter ORION Model SA 720
4. Thermometer
5. Shaker-water bath (Precision)
6. Atomic absorption spectrometer (Varian;Spectr AA-20)

7. Dialysis membrane (Spectr/Por Membrane, molecular mass cut-off of 6000-800 Da and diameter 14.6).

Procedure:

In vitro method with equilibrium dialysis:

Prior to the in vitro digestion method, iron content in sample and dialysate fractions are determined by atomic absorption spectrometry (as described in Appendix E)

1. Weight the sample approximately 10 g
2. Add deionized distilled water (80 ml) to sample and then homogenize with electrical homogenizer
3. Trasfer the homogenized sample to 250 ml Erlemeyer flask.
4. Adjust pH sample to 2.0 with 6 M Hl. Sample is made up to 100 g with deionized water.
5. Add 3.2 ml of freshly prepared pepsin solution to sample.
6. Incubate the mixture in a shaking water bath for 2 h at 37^{o}C
7. At the end of 2 h interval, duplicate 20 g peptic digestion is tranfered to 125 ml Erlemeyer flask
8. Place the dialysis tube comtaining 25 ml water and an amount of $NaHCO_3$ equivalent to the titrable acidity previously determined in the sample flask
9. Incubate sample flask containing dialysis bag in a shaking water bath for 30 min at 37^{o}C
10. After 30 min, 5 ml of pancreatin-bile extract is added to sample flask.
11. Continues the incubation of sample flask for an addition 2 h
12. At the termination of 2 h incubation, dialysis tube is removed, rinsed with deionized distilled water.
13. Dialysate is analyzed for the total iron content by Atomic Absorption Spectrometry

Tritratable acidity:

Titrable acidity is determined on 20 g of the pepsin digest incubated 5 ml pancreatin-bile extract. Titratable acidity is defined as the number of equivalents of

KOH required to amount of the mixture of peptic digest and pancreatin-bile extract at pH 7.5.

Calculation of iron bioavailability (dialyzability):

The iron dialyzability is expressed as a percentage.

Dialyzability (%)= [D/ (W x A)] x 100

Where; D is the total amount of iron in dialysate solution; W is the weight of sample used for digestion (g); and A is the concentration of iron in the sample (mg/g)

BIOGRAPHY

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