Vitamin A and zinc efficacy in triple fortified extruded rice

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VITAMIN A AND ZINC EFFICACY IN TRIPLE FORTIFIED EXTRUDED RICE

A dissertation submitted to

ETH ZURICH

for the degree of
Doctor of Sciences

presented by

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2012
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AGP</td>
<td>α1-acid-glycoprotein</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CIC</td>
<td>Conjunctival impression cytology</td>
</tr>
<tr>
<td>CRABP</td>
<td>Cellular retinoic acid-binding protein</td>
</tr>
<tr>
<td>CRBP</td>
<td>Cellular retinol-binding protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DALYs</td>
<td>Disability adjusted life years</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal transporter-1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>DRD</td>
<td>Deuterated-retinol-dilution</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated average requirement</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FAZ</td>
<td>Fractional zinc absorption</td>
</tr>
<tr>
<td>FBS</td>
<td>Food balance sheet</td>
</tr>
<tr>
<td>GCCIRMS</td>
<td>Gas chromatography-combustion-isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross domestic product</td>
</tr>
<tr>
<td>HAZ</td>
<td>Height for age Z-score</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
</tr>
<tr>
<td>IDD</td>
<td>Iodine deficiency disorders</td>
</tr>
<tr>
<td>IVACG</td>
<td>International Vitamin A Consultative Group</td>
</tr>
<tr>
<td>LIFDs</td>
<td>Low income food deficit countries</td>
</tr>
<tr>
<td>LRAT</td>
<td>Lecithin:retinol acyltransferase</td>
</tr>
<tr>
<td>MGFP</td>
<td>Micronized ground ferric pyrophosphate</td>
</tr>
<tr>
<td>MSG</td>
<td>Monosodium glutamate</td>
</tr>
<tr>
<td>NE</td>
<td>Northeastern</td>
</tr>
<tr>
<td>PT</td>
<td>Pupillary threshold score</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>RDR</td>
<td>Relative Dose Response Test</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>SZn</td>
<td>Serum zinc</td>
</tr>
<tr>
<td>TAZ</td>
<td>Total quantity of absorbed zinc</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>UI</td>
<td>Urinary iodine</td>
</tr>
<tr>
<td>USI</td>
<td>Universal salt iodization</td>
</tr>
<tr>
<td>VAD</td>
<td>Vitamin A deficiency</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>EZP</td>
<td>Exchangeable zinc pool</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>WHZ</td>
<td>Weight for height Z score</td>
</tr>
<tr>
<td>PEM</td>
<td>Protein energy malnutrition</td>
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<tr>
<td>PATH</td>
<td>Program for Appropriate Technology in Health</td>
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SUMMARY

Background: Iron, vitamin A and zinc deficiencies continue to be a major public health problem in Asia. These can result in a lowered immune defense, physical and mental retardation and impaired reproductive function especially affecting the high risk groups of people such as women of reproductive age and children. Multi-micronutrient-fortification is recommended as a more cost-effective strategy than single fortification as micronutrient deficiencies often coexist in the same individual. Rice is widely consumed especially in South and Southeast Asia and contains only low amounts of phytate which makes it a promising food vehicle for fortification. Thus, triple fortified rice with iron, zinc and vitamin A could be an effective and sustainable approach to combat micronutrient deficiencies in a rice-eating population. However, due to the high susceptibility of vitamin A to degradation, producing triple fortified rice grains is technically challenging. The impact of triple fortified extruded rice grains on improving vitamin A and zinc status have not been assessed so far.

Aim: The aims of this thesis were

I. To develop triple fortified artificial rice grains containing iron, zinc and vitamin A by using the hot extrusion technology with a good vitamin A stability for use in tropical countries.

II. To demonstrate an improvement of vitamin A status in school children in Southern Thailand who consumed triple fortified rice grains by comparing 2 different indicators: 1) estimated change of total body vitamin A pool size or total body reserves of vitamin A (TBR of vitamin A) using a paired stable isotope dilution technique of labeled $^{13}$C retinyl acetate and 2) serum retinol (SR) concentration.

III. To determine the impact of triple fortified extruded rice on zinc status in school children in Southern Thailand with additionally monitoring iron and vitamin A status.
**Study I.** The artificial rice grains fortified with iron (micronized ground ferric pyrophosphate: MGFP), zinc (either ZnO, ZnSO$_4$, ZnCO$_3$) and vitamin A (retinyl palmitate: RP) were produced by hot extrusion. Vitamin A losses were measured during the whole processing steps as well as during cooking. The stability of vitamin A was also evaluated when stored in different types of packaging for 18 weeks under simulated tropical conditions and simulated day light in a climate chamber. Color changes of triple fortified rice grains were quantified with colorimetric measurements. The highest loss of RP occurred during storage (28.5%), followed by cooking (9.8%), and the extrusion process (5.3%). After 18 weeks of storage, aluminum foil bags (no light) were better in protecting vitamin A degradation with an 18% higher RP retention than when stored in transparent plastic bags. In transparent bags iron and zinc had no influence on vitamin A losses, while in the aluminum foil bags, iron containing rice grains showed significantly higher vitamin A losses and zinc alone had no effect on retention of RP. The rice grains fortified with ZnSO$_4$, MGFP and vitamin A showed the highest RP losses during storage while losses in grains single fortified with vitamin A were lowest. Zinc compounds helped in decreasing the yellowish color of grains containing RP and MGFP.

**Study II.** The efficacy of vitamin A of triple fortified extruded rice with vitamin A (RP), zinc (ZnSO$_4$) and iron (MGFP) was evaluated in 8-12 y old school children (n=50). Children were randomly assigned to receive either triple fortified rice (n=25) or normal rice (n=25) as a part of the lunch meal for 2 months. The fortified rice grains were mixed into normal rice at the ratio 1:50 before cooking and this provided an extra 10 mg iron, 9 mg zinc and ~890 µg vitamin A per feeding day. A paired stable isotope dilution technique with labeled $^{13}$C retinyl acetate was used to quantify VA pool size at the beginning and end of the feeding period compared to SR concentration. Anthropometric measures and C-reactive protein (CRP) were also measured at
both time points. The baseline results showed that liver vitamin A concentration was only slightly higher (0.113 µmol/g) than the upper cut-off for marginal vitamin A status (0.07-0.1 µmol/g) with 24% showing vitamin A deficiency (VAD) based on liver vitamin A concentration while using SR none of the children were found with VAD and SR was well above the cut-off for VAD. At the end of the study, triple fortified rice showed its efficacy in improving vitamin A status by significantly increasing TBR of vitamin A (from 0.105 mmol retinol to 0.189 mmol retinol) and liver vitamin A concentration (from 0.122 µmol/g to 0.22 µmol/g) in children who consumed the triple fortified rice and these parameters were significantly higher than in the control group which remained unchanged compared to baseline. On the other hand, SR concentration did not change in any of the 2 groups over the course of the intervention. Triple fortified extruded rice with vitamin A, zinc and iron is an efficacious food vehicle in improving vitamin A status which was evaluated by the $^{13}$C stable isotope measurement of TBR of VA but not by changes in SR.

**Study III.** The efficacy of triple fortified extrude rice grains (with MGFP, ZnSO$_4$, RP) was tested in a randomized, controlled double-blind study in school children aged between 7 and 12 y (n=203) with low serum zinc (SZn) concentration at baseline. Children were randomized to receive either triple fortified extruded rice (n=101) or normal rice (n=102) as a component of the school lunch meal every day 5 times per week for 5 months. The fortified rice provided an extra 10 mg iron, 9 mg zinc and ~890 µg vitamin A per school feeding day. SZn, hemoglobin, serum ferritin (SF), SR, CRP concentrations and anthropometric measures were assessed at baseline and at the end of the study. Pilot study and baseline result showed that VAD, anemia and ID were not prevalent in this population group, but Zn deficiency with a prevalence of 44%. Rice is the main Zn source and contributed with 27% to the total dietary zinc intake. At post
intervention, SZn significantly increased in both groups, to 72.8 µg/dL in the intervention group and to 69.6 µg/dL in the control group, compared to baseline (61.3 µg/dL). This is mainly due to the proper foods provided the school lunch program and school milk which were given to every child at school. However, triple fortified rice demonstrated its efficacy with a significantly higher increase in SZn in the intervention group than in the control group at the end of the study. The prevalence of zinc deficiency significantly decreased from 100% to 29% and 39% in the intervention and the control group, respectively. In addition to this, iron and vitamin A status also improved in the fortified group with a significant increase in SR (from 1.01 µmol/L to 1.09 µmol/L) over time (not seen for the control group) and a significantly lower prevalence of ID compared to the control group (2.2% in the intervention, 9.5% in the control group) at the end of the study.

**Conclusion:** Triple fortified rice grains containing iron, zinc and vitamin A with good vitamin A stability and acceptable color can be produced by using the hot extrusion technology. Based on RP stability, color and costs, ZnO is the most suitable Zn compound to be added together with MGFP and RP to triple fortified rice. Triple fortified extruded rice grains were efficacious in improving vitamin A and zinc status of school children in Southern Thailand. A stable isotope dilution technique of labeled \(^{13}\)C retinyl acetate can be used for evaluating the impact of an intervention of a vitamin A fortified food with the advantages of using less participants and feeding time compared to other methods. However the higher costs need to be considered. We demonstrated an improvement of zinc status by using SZn as indicator. Therefore SZn could be used to monitor an intervention program using a low phytate food, subjects with low zinc status and low level of infection, and a relatively long intervention period (5 months in our case).
ZUSAMMENFASSUNG


Reis ist ein weitverbreitetes Grundnahrungsmittel, besonders in Süd- und Südostasien und enthält nur geringe Mengen an Phytat, weshalb es ein vielversprechendes Lebensmittel für die Anreicherung ist. Aus diesem Grund könnte mit Eisen, Zink und Vitamin A, also dreifach angereicherter Reis, eine wirksame und nachhaltige Methode sein, zur Bekämpfung von Mikronährstoffmängeln in einer Bevölkerung die hauptsächlich Reis verzehrt. Anreicherungen mit Vitamin A sind allerdings, auf Grund der hohen Instabilität, eine technische Herausforderung. Die Wirkung von solch dreifach angereicherten, extrudierten Reiskörnern zur Verbesserung des Vitamin A- und Zinkstatus, wurde bisher noch nicht bewertet.

Ziel: Die Ziele von dieser Doktorarbeit waren

Mittels Heissextrusion, künstliche dreifach angereicherte Reiskörner herzustellen, die zugleich Eisen, Zink und Vitamin A enthalten und dabei eine gute Vitamin A Stabilität aufweisen und für den Gebrauch in tropischen Ländern geeignet sind.
Eine Verbesserung des Vitamin A Status in Schulkindern in Südthailand nachzuweisen, welche die dreifach angereicherten Reiskörner verzehrt haben, durch den Vergleich von 2 verschiedenen Indikatoren: 1) abgeschätzte Veränderung der Grösse des Gesamt-Körper-Vitamin A-Pools oder Gesamt-Körper-Vitamin A-Reserven (TBR oder Vitamin A) mittels Anwenden der Isotopenverdünnungstechnik mit $^{13}$C-markiertem Retinylacetat und 2) Konzentration von Serumretinol (SR)

Die Wirkung des dreifach angereicherten Reises auf den Zinkstatus von Schulkindern in Südthailand zu bestimmen, unter zusätzlicher Beobachtung des Eisen- und Vitamin A-Status.

**Studie I.** Die künstlichen Reiskörner angereichert mit Eisen (mikronisiertem Eisenpyrophosphat, MGFP), Zink (entweder ZnO oder ZnSO$_4$ oder ZnCO$_3$) und Vitamin A (Retinylpalmitat: RP) wurden mittels Heissextrusion hergestellt. Verluste von Vitamin A wurden während allen Fabrikationsschritten und des Kochens gemessen. Die Stabilität von Vitamin A wurde auch nach 18 wöchigem Lagern in verschiedenen Verpackungen und unter simulierten tropischen Bedingungen und simuliertem Tageslicht in einer Klimakammer evaluiert. Farbveränderungen der dreifach angereicherten Reiskörner wurden mittels kolorimetrischen Messungen quantifiziert. Der grösste Verlust von RP fand während der Lagerung statt (28.5%), gefolgt vom Kochen (9.8%), und dem Extrusionsverfahren (5.3%). Beutel aus Aluminiumfolie (Licht undurchlässigkeit) schützten das Vitamin A besser gegen Abbauen nach 18 wöchingerLagerung, mit einer 18% höheren RP Retention als wenn gelagert in transparenten Beuteln aus Plastik. In transparenten Beuteln hatten Eisen und Zink keinen Einfluss auf Vitamin A Verluste, hingegen in Beuteln aus Aluminiumfolie zeigten Reiskörner, welche Eisen enthalten höhere Vitamin A Verluste und Zink alleine hatte keinen Effekt auf die Retention von RP. Reiskörner, welche mit ZnSO$_4$ fortifiziert waren, MGFP und Vitamin A zeigten die
höchsten Verluste an RP während der Lagerung während die Verluste in Reiskörnern, welche nur mit Vitamin A angereichert wurden am geringsten waren. Zink Verbindungen halfen die gelbbliche Verfärbung der Körner, welche RP und MGFP (enthalten), zu reduzieren.

**Studie II.** Die Wirksamkeit von dreifach angereichertem Reis mit Vitamin A (in der Form von Retinylpalmitat, RP), Zink (ZnSO₄) und mikronisiertem Eisenpyrophosphat (MGFP) wurde in 8-12-jährigen Schulkindern (n=50) evaluiert. Die Kinder wurden in zwei Gruppen randomisiert und erhielten entweder den dreifach angereicherten Reis (n=25) oder normalen Reis (n=25) als Teil des Mittagessens während zweier Monate. Die angereicherten extrudierten Reiskörner wurden vor dem Kochen im Verhältnis 1:50 mit normalen Reiskörnern gemischt. Die Anreicherung führte zu einer zusätzlichen Dosis von 10 mg Eisen, 9 mg Zink und ~890 µg Vitamin A pro Tag. Eine Isotopenverdünnungstechnik mit ¹³C-markiertem Retinylacetat wurde zur Quantifizierung der Vitamin A-Reserven zu Beginn und nach Abschluss der Interventionsphase verwendet. Anthropometrische Parameter und C-reaktives Protein (CRP) wurden ebenfalls zu beiden Zeitpunkten gemessen. Die Baseline-Messungen vor der Intervention ergaben, dass die durchschnittlichen Leber-Vitamin-A-Konzentrationen (0.113 µmol/g) nur wenig über dem oberen Grenzwert für marginalen Vitamin-A-Status lagen (0.07-0.1 µmol/g). Basierend auf den Leber-Vitamin-A-Grenzwerten litten demnach 24% der Kinder an Vitamin A-Mangel (VAD), während unter Verwendung der Serum-Retinol (SR)-Werte keines der Kinder mit VAD diagnostiziert wurde. Am Ende der Studie konnte die Wirksamkeit von dreifach angereichertem Reis im Bezug auf eine Verbesserung des Vitamin A-Status anhand signifikant erhöhter Gesamt-Vitamin-A-Reserven (von 0.105 mmol zu 0.189 mmol Retinol) und signifikant höherer Leber-Vitamin-A-Konzentrationen (von 0.122 µmol/g zu 0.220 µmol/g) in der angereicherten Reisgruppe gegenüber der Kontrollgruppe nachgewiesen werden. In
letzterer wurden keine Veränderungen gegenüber der Baseline festgestellt. Andererseits blieb die SR-Konzentration in beiden Gruppen während der Intervention unverändert. Unter Berücksichtigung dieser Ergebnisse lässt sich zusammenfassen, dass der untersuchte dreifach angereicherte Reis (mit Vitamin A, Zink und Eisen) eine wirksame Strategie zur Verbesserung der Vitamin-A-Versorgung darstellt, was durch die $^{13}$C-Methodik zur Bestimmung der Gesamt-Körper-Reserven an Vitamin A, jedoch nicht über eine Verbesserung in den Serum-Retinol-Werten belegt werden konnte.

**Studie III.** Die Wirksamkeit von dreifach angereicherten, extrudierten Reiskörnern (MGFP, ZnSO$_4$ und RP) wurde in einer randomisierten Doppelblindstudie in Schulkindern, im Alter von 7 bis 12 Jahren (n=203) mit geringer Serumzinkkonzentration (SZn) zum Studienstart, untersucht. Die Kinder wurden in zwei Gruppen randomisiert. Eine Gruppe (n=101) erhielt dreifach angereicherten Reis als Teil des Mittagessens in der Schule, fünf Mal die Woche über 5 Monate, während die andere Gruppe (n=102) normalen Reis serviert bekam. Durch den angereicherten Reis wurden pro Schulmahlzeit zusätzlich 10mg Eisen, 9 mg Zink und ungefähr 890 µg Vitamin A zur Verfügung gestellt. SZn-, Hämoglobin-, Serumferritin (SF)-, SR-, CRP- Konzentrationen und anthropometrische Daten wurden zum Beginn und am Ende der Studie bestimmt. Die Pilotstudie sowie die Daten vom Beginn der Studie zeigten, dass VAD, Anämie und ID in dieser Bevölkerungsgruppe nicht verbreitet sind. Die grösste Zinkquelle stellt Reis dar, der allein 27% zur gesamten Zinkaufnahme aus der Nahrung beiträgt. Nach der Intervention war die SZn-Konzentration in beiden Gruppen signifikant angestiegen, von 61.3 µg/dL zu Beginn auf 72.8 µg/dL in der Interventionsgruppe und 69.6 µg/dL in der Kontrollgruppe. Dieser Effekt resultiert vor allem aus dem Mittagessenprogramm der Schulen und der Schulmilch, die an jedes Kind ausgegeben wurde. Dennoch konnte die
Wirksamkeit von dreifach angereichertem Reis in dieser Studie, durch den signifikant höheren Anstieg in der Interventionsgruppe im Vergleich zur Kontrollgruppe, gezeigt werden. Die Häufigkeit von Zinkmangel wurde signifikant reduziert, von 100% auf 29% in der Interventionsgruppe beziehungsweise auf 39% in der Kontrollgruppe. Zusätzlich verbesserte sich der Eisen- und Vitamin A-Status in der Gruppe, die den angereicherten Reis erhielt. SR ist signifikant angestiegen während der Studie (von 1.01 µmol/L auf 1.09 µmol/L) aber nicht in der Kontrollgruppe. Das Vorkommen von ID ging im Vergleich zur Kontrollgruppe auch deutlich zurück (2.2% in der Interventions- bzw. 9.5% in der Kontrollgruppe am Ende der Studie).


Die Verwendung von Isotopenverdünnungstechnik mit $^{13}$C-markiertem Retinylacetat kann zur Abschätzung des Einflusses einer Intervention von Vitamin A angereicherter Lebensmittel herangezogen werden. Weniger Studienteilnehmer und kürzere Fütterungszeiten sind die Vorteile dieser Methode, was aber gegen und muss aber gegen die erhöhten Kosten abgewogen werden muss.

Da eine Verbesserung des Zinkstatus anhand SZN als Indikator gezeigt werden konnte, könnte SZN zum Monitorieren in Interventionsprogrammen eingesetzt werden mit Mahlzeiten mit
niedrigem Phytinsäuregehalt, Studienpopulationen wenn niedrigem Zinkstatus zu Beginn und niedriger Infektionsrate und relativ langen Interventionsperioden (5 Monate in unserer Studie) verwendet werden.
INTRODUCTION

Over the past few decades, developing countries have gone through rapid changes in their economic and human development; nevertheless, micronutrient deficiencies still remain a major public health problem especially in Asia [1, 2]. Among all micronutrient deficiencies, vitamin A and zinc deficiencies have been reported to be the largest disease burden and to be responsible for \( \sim 1 \) million deaths in children < 5 y of age while iron deficiency (ID) is a risk factor for maternal mortality accounting for 115,000 deaths annually [3].

WHO reports that vitamin A deficiency (VAD) affects 119 million preschool aged children and 9 million pregnant women worldwide [4]. The adverse health consequences of VAD are present in many forms in different tissues such as the clinical ocular symptoms of xerophthalmia which may lead to blindness [5], weakened immune defense [6], and may exacerbate anemia [7]. It is estimated that almost 2 billion people worldwide have a low intake of zinc and are at risk of zinc deficiency based on calculations from food balance sheet (FBS) while data on prevalence of zinc deficiency at country level are still lacking [8]. The clinical signs of zinc deficiency in humans include growth retardation, delayed sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired appetite and increased susceptibility to infections [9]. The consequences of ID have an impact in both developed and developing countries which cause adverse health effects on pregnancy outcome, infant growth, cognitive performance, immune status and work capacity [10].

Coexistence of micronutrient deficiencies are common among high risk groups including pregnant women [11], women of reproductive age [12] and children [13] especially in those who live in the developing world where people cannot afford a healthy and well-balanced diet. There is evidence that these micronutrients interact in the metabolism [14-17], thus giving
single nutrient in supplementation or fortification programs may fail to show a beneficial impact on nutritional status [18, 19].

Multiple micronutrient fortification is a potential solution of the above mentioned problem since food fortification is considered as the most cost-effective, long-term strategy to combat micronutrient deficiencies [20]. Rice is one of the leading staple foods as it is widely consumed in poorer populations, especially in Asia. Because rice contains low amounts of iron and zinc and no vitamin A, people who rely mainly on a rice based diet are at high risk of these deficiencies [21]. Therefore, triple fortified extruded rice containing iron, zinc and vitamin A could be a sustainable and promising strategy for helping people suffering from those deficiencies and living in rice-consuming countries.

However, the challenge in the production of triple fortified extruded rice containing iron, zinc and vitamin A is the stability of vitamin A in combination with the other two micronutrients as well as the color of such triple fortified grains. Further, the efficacy of improving vitamin A and zinc status in high risk populations needs to be proven.

The present thesis consists of a literature review and three manuscripts. The literature part is divided into 5 chapters starting with micronutrient deficiencies in Asia followed by the details with respect to VAD and zinc deficiency covering characteristic, functionality, metabolism, etiology, health consequences, prevalence, assessment indicators and prevention and combating strategies. In the next chapters the impact of coexisting micronutrient deficiencies and their interactions are discussed and techniques for rice fortification and problems of rice-consuming populations are described. The first manuscript of this thesis investigates the stability of vitamin A in triple fortified extruded rice grains with iron, zinc and vitamin A during the whole processing steps and while stored, as well as color changes during storage. In the
second and third manuscript, the efficacy of vitamin A and zinc in triple fortified rice grains was tested in school children in Southern Thailand.

References

LITERATURE REVIEW
1. Micronutrient deficiencies in Asia

Despite rapid economic and human development in the recent decade, micronutrient deficiencies continue to be a major public health problem in Asia, especially in South Asia with a large proportion of the world’s malnourished children living in India. Thus, improving nutritional status in that country will markedly influence the global prevalence [1, 2]. Across the world, micronutrient deficiencies are one of the most important risk factors for illness and death, with hundreds of millions of pregnant women and young children particularly affected [3]. The degree and distribution of micronutrient deficiencies in those population groups depends on many factors including the political and economic situation, the level of education and sanitation, the season and climate conditions, food production, cultural and religious food customs, breast feeding habits, prevalence of infectious diseases, the existence and effectiveness of nutrition programs and the availability and quality of health services [4, 5]. Among those factors, poor dietary quality is often the major cause of micronutrient deficiencies mostly due to the consumption of mainly lower cost plant source foods with very small amounts of animal source foods due to their higher price [6]. Environmental factors such as the content of trace elements in soil can also contribute to an inadequate intake of iodine, selenium and zinc. For example Northeast (NE) Thailand, where a high prevalence of zinc and iodine deficiencies in children has been reported [6, 7], is one of the regions with low soil iodine and low soil zinc concentration. On occasions, this low level of mineral may be further aggravated by leaching due to flooding [8, 9]. There is a report focusing on the outcome of climate changes in Southeast Asia which shows that many parts of this region such as Bangkok, Jakarta, Manila, and Ho Chi Minh City, have been affected by the high risk of the rising of sea level together with long heat waves and urban smog. These climatic conditions could bring
more limited access to food and increase food insecurity to people in these affected areas. As a result of climatic and other adverse forms of global environmental changes, good health of the Southeast Asian population is far from assured [2].

Undernutrition is reported to be higher among children from indigenous groups. This is partly because most of them live in rural regions and belong to poorer families with limited access to health services. For example, in Cambodia, micronutrient deficiencies are more prevalent in ethnic groups living predominantly in highland area. This is comparable to Thailand, where malnutrition remains most prevalent in the hill tribe ethnic groups living in the north and NE [1], while people living in the cities of NE Thailand have shown normal ranges of serum retinol (SR) and serum zinc (SZn) concentration [10].

Micronutrient deficiencies are reported to contribute almost 11% to the causes of death in children under 5 years old worldwide [11]. Among the deficiencies of vitamins and minerals, the largest disease burdens have been attributed to vitamin A and zinc deficiencies. Vitamin A deficiency (VAD) in newborn babies, infants and children resulted in about 6% of under-5 deaths, 5% of under-5 disability-adjusted life-years (DALYs), and 1.7% of total DALYs. Zinc deficiency resulted in about 4% of under-5 deaths and DALYs and 1% of total DALYs. The regional pattern for disease burden attributed to vitamin A and zinc deficiencies are similar. The highest burden for each is in South-central Asia followed by several sub regions of Africa [12].

Among micronutrient deficiencies vitamin A, iron, iodine and zinc are the most prevalent in the populations at risk and they continue to be a major public health problem. In children, such deficiencies can lead to the impairment in growth, immune competence and mental and physical development [13].
1.1 Vitamin A deficiency (VAD)

Low consumption of vitamin A rich foods especially in the form of preformed vitamin A is the primary cause of VAD [14]. Thus, a higher consumption of vitamin A in the form of provitamin A carotenoids (70-75% of total consumption of vitamin A) which mainly comes from dark green leafy vegetables and fruits in Southeast Asia results in a higher prevalence of VAD than it is found in industrialized countries where only 30-35% of total consumption of vitamin A comes from provitamin A and 65-70% from preformed retinol [15-17]. Similar, data from Indonesia found that when people spend more money on animal-based food including eggs and dairy products, the prevalence of night blindness among nonpregnant women decreased significantly [18].

The latest updated report of the World Health Organization (WHO;1995-2005) looking at pre-school aged children and pregnant women excluded countries where the GDP was > 15,000 US$ which were classified as high income countries and assumed not to be at risk of VAD as a public health problem. They estimated that Asia by WHO region and especially Southeast Asia contained the highest proportion of the population, as well as the largest number of pre-school aged children (49.9%, 91.5 million) and pregnant women (17.3%, 6.69 million), that were affected by biochemical VAD as indicated by SR < 0.7 µmol/L [19].

Although, school aged children are reported to be of lower public health concern as compared to pre-school aged children and pregnant and lactating women, Sigh and West (2004) estimated the prevalence of VAD in school-aged children as 23.4% in WHO region of Southeast Asia. This corresponded to around 83 million vitamin A deficient children in the region, of whom 10.9% (9 million, at an overall prevalence of 2.6%) had mild xerophthalmia (night blindness or Bitot’s spot). The prevalence values of VAD in this region range from 5.2%
in Thailand to a high prevalence of 34.2% in Indonesia [20]. The largest number of vitamin A deficient school children in this region with around 56.4 million can be found in India. When using 15% as the cut off for VAD prevalence to determine the public health significance; Bangladesh, Bhutan, India, Indonesia and Myanmar show a VAD problem of public health importance among school aged children [21].

Women of childbearing age are also at a high risk of night blindness. In a study conducted in rural Nepal, the consequences of VAD were compared between two groups (with or without night blindness) in a vitamin A supplementation trial in pregnant women. Women who were night blind during pregnancy had a 4-fold greater risk of mortality during the first 2 years postpartum and there was a greater mortality among their infants during the first 6 months of life [22]. Another study has shown that VAD can also lower the immune system resulting in an estimated 30–40% of preschool children in South Asia being at increased risk of illness and death [23].

Among the three major strategies to combat VAD which are food fortification, supplementation and food diversification, food fortification is well recognized as the most cost-effective and sustainable approach[24]. There are many countries in Asia such as India, Pakistan, Philippine and Thailand who have adopted this strategy and products such as cow milk, cooking fat, margarine and condensed milk fortified with vitamin A can be found on the market [23].

1.2 Iron deficiency anemia (IDA)

Iron deficiency anemia (IDA) is considered as one of the most important contributing factors to the global burden of disease [25]. Its consequences can affect all population and age groups and as a result the development of a nation through increased maternal and newborn mortality, impaired health and mental development of infants and children, limited learning
capability, impaired immune function and reduced working and productive capacity. Predominantly women of reproductive age, pregnant women and young children are at risk of suffering from IDA [26, 27]. The results from large multi-country studies in Southeast Asia (Thailand, Vietnam and Indonesia) show that infant boys had a higher risk of anemia and iron deficiency (ID) than infant girls. The authors suggest that infant boys experienced a stronger decline in iron store during the second half of infancy period and have a higher iron requirement due to the higher growth rate of boys than girls at that age [28]. In addition to inadequate intake of iron, especially heme iron from meat products, the other important contributors to IDA in Southeast Asia are consumption of low bioavailable iron from cereal-based diets and hookworm (*Ancylostoma duodenale* and *Necator americanus*) infection, which causes chronic gastrointestinal blood loss [26, 29]. Support for this reasoning comes from a study with healthy women in Nepal which showed that 54% of the participants consumed less than the recommended average intake of iron and iron intake mainly came from rice, wheat flour and green, dry vegetables [30]. Another study from Vietnam reported that a non-anemic population was highly associated with consuming higher quantities of meat (more than 3 times per week) compared to an anemic population of women of reproductive age [27]. In Bangladesh, one of the major sources of iron intake is from drinking groundwater which is also low in bioavailability [31]. Widespread prevalence of intestinal worm infections can be found in all the countries in Southeast Asia with prevalence between 46-90% [26]. One study in women of reproductive age in Vietnam found that hookworm infection was a strong risk factor for IDA with 78% of women in the study infected [27].

The major cause of anemia in the region however, is reported to be thalassemia and hemoglobinopathies with a prevalence varying between 5% and 60%. Several studies conducted in NE Thailand indicated that thalassemia and hemoglobinopathies rather than IDA...
were the major causes of anemia [32, 33]. Vitamin A deficiency which is also prevalent in this area, as mentioned previously, may also contribute to anemia as vitamin A has been shown to have an important role in iron utilization [34].

However, as many as 600 million people in Southeast Asia are suffering from IDA. The prevalence of IDA in pregnant women in this region is 74% with a wide range of 13.4% in Thailand to 87% in India [26]. Two-thirds of pregnant women in south Asia suffer from IDA, which is the highest prevalence rate in the world [35-37]. For women of childbearing age, ID was reported to affect around 22-23% in Vietnam [27, 38]. For infancy, the rates are somewhat lower. Studies conducted in Thailand and Vietnam reported the prevalence of IDA was 0.4 and 12.5%, respectively [39]. This is comparable to a prevalence of 15% from another study in Cambodian infants and toddlers [40]. In rural Bangladesh, the prevalence of anemia, ID and IDA in infants was reported to be about 46%, 21% and 13%, respectively [41]. A recent publication looking at school children in Southern India observed that 58% and 10% of these children were ID and IDA, respectively [42].

National programs to prevent and control IDA such as iron and folate supplementation of pregnant women are implemented only in some countries and have failed to have the foreseen impact [26]. In order to more effectively control IDA, several countries have now initiated an integrated and more comprehensive approach including provision of deworming as well as iron and folate supplements to pregnant women, nutrition education with respect to the consumption of food rich in iron, and health education on personal hygiene and sanitation [26, 27]. Triple fortification of instant noodle with iron, vitamin A and iodine in Thailand has been launched in 1996. This has been accepted by the customers in the market [43]. In China, fortified wheat flour with NaFeEDTA was introduced in several provinces without any problem reported so far [44].
1.3 Iodine deficiency disorders (IDD)

Iodine is an important trace element used for the production of thyroid hormones by the thyroid gland. Thus, when iodine intake is lower than recommended iodine deficiency can develop. Because this results in various adverse impacts on growth and development throughout the life cycle, iodine deficiency disorders (IDD) is the collective term for those symptoms [45, 46]. The visible manifestation of IDD is goiter (hypothyroidism). IDD can happen at any stage of life and may lead to stillbirth, miscarriage, poor growth, cognitive impairment as well as poor fetal development. The most damaging causes of IDD are cretinism and irreversible mental retardation which leads to poor school performance, reduced intellectual ability, and impaired work capacity [46, 47]. Therefore, iodine deficiency is the world’s greatest single cause of preventable brain damage, and this fact is the primary motivation behind the current worldwide drive to eliminate iodine deficiency [48].

Iodide is most abundant in the ocean; thus seafood and other marine plants like seaweed are good sources of iodine. IDD also occurs in populations living in areas with low soil iodine content. Such regions are highlands like the Alps, Andes, Atlas, and Himalayan, areas of past glaciations, areas with repeated leaching effects of snow or frequent flooding especially in South and Southeast Asia including many places of inland areas such as central Asia, Africa and central and Eastern Europe. Crops grown in these areas have low iodine content [45, 47]. People living in many parts of Asia are at a high risk of suffering from IDD. Data of school-aged children are most commonly used to investigate iodine status in a population because this is the most efficient and practical group to survey and usually reflects the status of the general population.

Iodine deficiency is considered to be a public health problem in populations when school-aged children have a median urinary iodine (UI) <100 µg/L or goiter rate is above 5% [48]. However,
a study performed in central Thailand measuring UI from pairs of pregnant women and school-aged children from the same family showed that the median UI in school-aged children may not be an adequate surrogate for monitoring iodine nutrition in pregnant women since the results indicate optimal iodine status in the children but mild-to-moderate iodine deficiency in their pregnant mothers [49]. The most recent worldwide data of IDD of 2007 revealed that 2 million people (~31% of global population) have insufficient iodine intake. The most effected region is Southeast Asia (WHO region) where ~580 million of people have low iodine consumption while the highest of the prevalence is found in Europe (~52%). When comparing data of 2003 and 2006, the largest improvement of IDD status in school aged children was achieved in Southeast Asia where the prevalence of low iodine intake decreased by 9.6% while the global prevalence has decreased by 5% [46].

The most widely used strategy to control IDD is universal salt iodization (USI) and its effectiveness has been shown in Nepal and China [50, 51]. Nevertheless, IDD remains a major contributor to impaired health and development of people across the world especially among preschool children and pregnant woman. For example, in south Asia, household coverage of iodized salt is only 49% and more than 17 million infants could suffer from brain damage annually [52]. Therefore, the best strategy to control iodine deficiency is a carefully monitored universal salt iodization program which is one of the most cost-effective ways to contribute to economic and social development [45]. Besides table salt, fish sauce is one of the potential food vehicles which is widely consumed in Southeast Asia and drinking water is also often fortified with iodine in area of high risk of IDD such as in Northern Thailand [44].
1.4 Zinc deficiency

Zinc deficiency is one of the major contributors to high morbidity and mortality in developing countries, particularly among young children. There is, however, only limited information on the global prevalence of zinc deficiency [53]. WHO [54] estimated that zinc deficiency may be responsible for approximately 800,000 deaths of children under 5 years of age worldwide annually. A recent publication revealed that zinc deficiency may have contributed to 182,546 deaths of pre-school children in Asia with the largest number found in India [55]. Like the other micronutrient deficiencies, zinc deficiency is mainly caused by inadequate intake or consumption of poorly bioavailable zinc (due to absorption inhibitors such as phytate) but excess losses of zinc during diarrhea can also play a role [54]. Several studies from south Asia show a high prevalence of zinc deficiency and that it is related to the source of dietary zinc [56-58]. In rural Bangladesh, it has been reported that about half (55%) of pregnant women suffer from zinc deficiency, which was found to be related to seasonal variations. The authors found that zinc deficiency was most prevalent in seasons with mild temperature, while the lowest prevalence was found during the hot and dry season. The authors suggested that the difference in food availability and the price of food varying throughout the year may cause the changes in dietary habits and micronutrient intake [56]. A study in Nepal found that zinc deficiency in both pregnant and non-pregnant women was related to low intake of meat, with the main source of zinc being rice [57]. Another study in women of reproductive age in Nepal reported that rice contributed 50% to the total estimated daily zinc intake while wheat and meat each contributed with 15% [58]. A report from NE Thailand indicated that school children mainly consume glutinous rice (Oryza glutinosa) which is low in both phytate and zinc content therefore, zinc deficiency in this area was not related to the inhibitory effect of phytate but may
partly result from the rice grown on the low-zinc soils of NE Thailand as well as from a lower intake of animal protein [59].
2. Vitamin A

2.1 Characteristic of vitamin A (physical, chemical and functions)

Vitamin A is a nutritional term used for describing fat soluble substances that are structurally related to the lipid alcohol retinol and share its biological activity. This includes provitamin A carotenoids, the precursors of retinol. Retinol is a pale yellow crystalline solid with a molecular mass of 286.46. All forms of vitamin A have a cyclohexenyl (β-ionone) ring to which an isoprenoid chain is attached, called a retinyl group [60]. Retinol can also be called preformed vitamin A and it can be converted to two other biological forms: retinal and retinoic acid (RA) [23].

![Chemical structures of important functional forms of vitamin A. Retinol and retinyl esters (palmitate shown) are the dietary forms of preformed vitamin A. Retinal is essential in vision, and retinoic acid is involved in growth and cellular differentiation [61]](image)

In natural products vitamin A can be found in 2 principal forms; preformed retinol and provitamin A carotenoids. The preformed retinol is found in animal products which in nature is esterified with long-chain fatty acids (mainly palmitate and stearate) predominantly retinyl
LITERATURE REVIEW

Palmitate. These forms are more stable than the retinol. Carotenoids are found in green, orange, and yellow plants and are synthesized by the plant itself. It can also be found in animal products resulting from their feed [60, 62]. Carotenoids are the most abundant of the naturally occurring pigments. Among these, all-trans-β-carotene is the one with the highest vitamin A activity while lutein, lycopene and zeaxanthin are the three main carotenoids which cannot be converted to vitamin A. The highest content of vitamin A in nature is found in liver of some fish including halibut, cod and shark [23, 63].

Because of the double bond, vitamin A/retinoids are highly susceptible to isomerization, oxidation, polymerization as well as to the presence of trace metals (especially iron) and to acid solutions (pH < 5) [64, 65]. Therefore, they must be protected from light, oxygen and high temperature. Retinoids are more stable when stored at low temperature in the crystalline form, oil, or some organic solvents and in the absence of light and oxygen. In tissue specimens that have been kept sealed and deep frozen (preferably at -70°C), retinol and its ester have been stable for several years [63].

Vitamin A plays a role in a variety of functions throughout the body. The most well known function of vitamin A is its important role for vision. It is important for the function of the eye in two distinct forms for two distinct processes: a) as 11-cis-retinal, vitamin A functions in the retina in the transduction of light into the neutral signals necessary for vision and b) as RA, vitamin A maintains normal differentiation of the cells of the conjunctival membranes, cornea, and other ocular structures preventing xerophthalmia. Vitamin A also appears to maintain normal skin health and RA plays a key, hormone-like role in cell differentiation of tissues and organs throughout the body. Moreover, vitamin A has been shown to control the enzymes involved in the synthesis of glycoprotein and glycosaminoglycan which are compounds present
in the cell surface molecules. Impairment of this function by VAD may cause the lack of mucin secretion and liquefaction of the cornea seen in xerophthalmia [60]. In the immune system, vitamin A has a function in maintaining the lymphocyte pool and T-cell-mediated responses as well as immunoglobulin production [66]. When the mucous secretion declines, this results in the loss of cellular integrity in those who have low vitamin A status which also impairs the body’s ability to resist invasion from pathogenic organism which then compromises the immune system [67]. For the reproduction system, RA is important for the testosterone production [68]. Vitamin A is required for a full haematologic response; the mechanism however remains unclear. It is most likely that vitamin A has an effect on the absorption and/or metabolism of iron or might act directly on hematopoiesis [34]. RA is known to have a hormone like function in the control of growth and development of tissue in the musculo-skeletal system and human growth hormone secretion [69]. Moreover, vitamin A also has a function in gene transcription and some of the carotenoids have been reported to have antioxidant properties, which protect cells from free radicals [23, 63].

2.2 Metabolism of vitamin A

There are numerous cycles of hydrolysis and re-esterification which are the characteristics of vitamin A’s metabolism in the intestine, liver and other tissues [60]. After ingestion, retinyl esters are hydrolyzed in the intestinal mucosa by the pancreatic enzyme, pancreatic triglyceride lipase and intestinal brush border enzymes releasing retinol and free fatty acids [70] while carotenoids are converted via two enzymatic steps to retinol [71]. The absorption of vitamin A in the intestinal tract depends on bile salt and dietary fat and the uptake of retinol to the enterocytes seems to occur by facilitated diffusion [60, 72, 73]. In the enterocytes, free retinol is bound to the cellular retinol binding protein (CRBP) II before it undergoes the
esterification which is carried out by two enzymes; lecithin: retinol acyltransferase (LRAT) and acyl CoA: retinol acyltransferase. The retinyl esters are then combined with other triglycerides or cholesteryl esters prior to incorporation into chylomicrons and absorbed via the lymphatic pathway to the liver. During transportation to the liver, chylomicrons are partially degraded by lipoprotein lipase resulting in the products of "chylomicron remnant" with the major part of retinyl esters remaining in it [72, 73]. The chylomicron remnants are then taken up by and stored in the liver. Under normal conditions of adequate vitamin A status, liver is the main storage organ of vitamin A in the human body with around 95% found in the form of retinyl ester as palmitate and stearate [70]. Retinyl esters are hydrolyzed to retinol when taken up by the hepatocytes in the liver before being transferred for storage to the stellate cells where retinol is again esterified to retinyl acetate. Stellate cells contain more than 80% of the vitamin A in the liver [72-74].

When needed, the retinyl esters are hydrolyzed in the stellate cells and then the free retinol is transferred and binds in the parenchymal cells with the retinol-binding protein (RBP). The mobilization of vitamin A from the liver is a very efficient process and more than 90% of the plasma retinol in the circulation is bound to RBP (Holo-RBP, 1:1 mol/mol). Before secretion from the liver, holo-RBP is combined with another transport protein, namely, prealbumin or transthyretin (TTR). This larger size helps it to prevent vitamin A losses through kidney filtration [23, 72, 75]. At the cell where vitamin A uptake occurs, retinol most possibly enters into the cell by 2 pathways: by passive diffusion and by the way of RBP receptor which may vary from one tissue to another [76]. Depending on the tissue, retinol is either stored in the adipose tissue as lipid droplets in the form of retinyl esters, or as the activated form of RA acid, or retinal in eyes.
or in the lung. RA is bound by the cellular RA-binding protein (CRABP) type I or II to facilitate its distribution into the target cell [77].

Before the catabolism, retinol undergoes extensive recycling between liver, blood circulation and peripheral tissues while RBP is not reused after retinol dissociation. Because the capacity of vitamin A storage in the body is high and the rate of catabolism is low the risk of excess vitamin A in tissues is high [60]. The catabolism of retinal involves the transformation to RA as an intermediate and which cannot be converted to retinol or retinal [78]. RA is then metabolized by several reactions including isomerization, decarboxylation and glucuronidation which produces the metabolite all-trans-RA including 13-cis-RA, 9-cis-RA, retinoyl b-glucuronide, 5,6-epoxy RA, 4-hydroxy RA, 4-oxo RA, 3,4-didehydro RA and 18-OH RA. Some of these metabolites are biologically active products while others are just catabolic products [79, 80]. Normally vitamin A is excreted from the body in the urine only as inactive metabolites resulting from tissue utilization and in bile as potentially recyclable active glucuronide conjugates of retinol [67].

2.3 Vitamin A deficiency

2.3.1 Etiology

WHO defines people at risk of biochemical VAD if SR concentration is < 0.7 µmol/L [19]. VAD can be found at any age. However, population groups potentially at risk are children under 6 years of age as well as women of reproductive age during pregnancy and lactation. The former group has high requirements of vitamin A to support their rapid growth together with the transition from breastfeeding to the dependence on other dietary food sources of vitamin A [67] while the higher requirement in pregnant and lactating women is due to the increased demand
for the development of the fetus and breast milk production [19]. VAD is commonly found in areas where populations consume mainly plant-based diets containing provitamin A carotenoids and little fat [81]. Approximately 50-90% of ingested retinol is absorbed [82]. For provitamin A carotenoids, the absorption depends on the type of plant sources and the amount of fat in the meal as well as on the individual vitamin A status. Increased fat intake is likely to improve the absorption of vitamin A in the body [67, 83]. Fat helps absorption by stimulating bile flow, forming of lipid micelles in the small intestine and facilitating the uptake of vitamin A into mucosal cells and the transport of vitamin A to the lymphatic or portal circulation [84, 85].

Retinyl esters, the highly bioavailable sources of vitamin A, are found in liver, egg and milk with the highest concentrations of preformed vitamin A found in liver and fish liver oils [23, 60]. Plants and some lower organisms (e.g. algae) cannot synthesize retinoid directly. However, they do produce carotenoids that serve as the precursors of vitamin A which humans and animals then convert to retinol after digestion [60]. Dark green leafy vegetables, yellow fruits, orange roots (mainly carrots) and palm oil are the main sources of carotenoids [63]. More than 700 carotenoids have been identified in nature with about 50 which can be cleaved to vitamin A. Among these, only three forms, namely β-carotene, α-carotene and β-cryptoxanthin, represent major sources in the human diet [86]. Bioavailability of provitamin A carotenoids from plant food sources is uncertain and can be affected by several factors [83, 87] including species of carotenoids, host-related factors and factors influencing absorption i.e. fat content in food, the relative amount of carotenoids and resistant starch which may interfere with the release of carotenoids form the food matrix during digestion [86]. The term of the bioconversion factor is defined as the proportion of absorbed provitamin A carotenoids which is converted to retinol (1µg) and which varies due to the above mentioned factors [86]. The bioconversion factors were reported to be of 2 µg for β-carotene dissolved in oil [88], 9.5 µg for
Indian spinach [89], 12 µg for orange and yellow fruits and vegetables [88], 13 µg for sweet potato [89] and ≈ 26 µg for green leafy vegetables [88].

The difference of the bioconversion factors between the Indian spinach and green leafy vegetables can be explained by the methods that were used for assessing the change in vitamin A status, food preparation techniques and the treatment of intestinal helminthes in different studies [89].

The traditional retinol conversion factors from the National Research Council [90] of 6 µg for β-carotene and 12 µg for other provitamin A carotenoids to 1 µg retinol were revised to 12 µg for β-carotene and 24 µg for other provitamin A carotenoids by the Institute of Medicine [91] as a result of new research findings.

In some places, food habits and taboos as well as the culture-specific factors for feeding children, adolescents, pregnant and lactating women may restrict the consumption of potentially good food sources of vitamin A [92, 93]. VAD often coexists with severe infections such as measles and the frequent infections causing diarrhea and respiratory diseases. This synergistic effect can cause lower intake through depressed appetite and absorption and may also deplete body stores of vitamin A through excessive metabolism and also lower SR concentration [19]. Vitamin A status depends on many factors including the adequate intake of fat, protein, vitamin E, iron, zinc as well as the season which affects the availability of carotenoid rich fruits and vegetables [23]. Diarrhea, parasitic infections and other intestinal disorders can reduce the absorption of vitamin A [94].

### 2.3.2 Consequences

The consequences of VAD appear differently by a number of symptoms in different tissues. The eye’s symptoms, resulting from a gradual depletion of vitamin A stores lead to
xerophthalmia. Xerophthalmia is the term for the clinical symptoms of VAD which with increasing severity, manifests as night blindness, conjunctival xerosis and Bitot's spot, corneal xerosis and corneal ulceration/keratomalacia. The latter symptom usually leads to blindness [82].

VAD also causes keratinization of the tracheal epithelium and thinning of the intestinal epithelium. Xerophthalmia was reported to be accompanied by upper respiratory infections and diarrhea which is exacerbated by protein-energy malnutrition. Vitamin A deficiency is also related to an increase in mortality in young children as a result of lower resistance to infection particularly diarrhea and measles [95]. Meta-analyses provide convincing evidence that improvement of vitamin A status of deficient children aged 6 months to 6 years reduces their risk of dying by 20-30% [96, 97]. Moreover, VAD exacerbates anemia [34] and other conditions not yet identified or clarified such as impaired growth and development [23].

2.3.3 Prevalence

VAD is a concern as a major public health problem especially in poor societies of lower income countries. In a previous section of chapter 1, the impact of VAD in Asia is already described. Therefore, this part deals with the global prevalence of VAD. The latest report of WHO estimates around 190 million preschool aged children and 19.1 million pregnant women worldwide to have a SR concentration < 0.7µmol/L. From these data, there are 122 and 88 countries, which are classified as having a moderate to severe public health problem in preschool aged children and pregnant women, respectively. This corresponds to 33.3% of preschool aged children and 15.3 % of pregnant women being at risk of VAD globally. The highest proportion of preschool aged children with low SR concentration can be found in Africa and Southeast Asia with the biggest number of affected children and pregnant women in Southeast Asia as shown in table 1 [19].
Table 1  Prevalence of serum retinol concentration < 0.7 µmol/L and number of individuals affected among preschool aged children and pregnant women in populations of countries at risk of vitamin A deficiency 1995-2005, globally and by WHO region[19].

<table>
<thead>
<tr>
<th>WHO region</th>
<th>Preschool-age children</th>
<th>Pregnant women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence (%)</td>
<td># affected (millions)</td>
</tr>
<tr>
<td>Africa</td>
<td>44.4 (41.3-47.5)</td>
<td>56.4 (52.4-60.3)</td>
</tr>
<tr>
<td>Americas</td>
<td>15.6 (6.6-24.5)</td>
<td>8.68 (3.70-13.7)</td>
</tr>
<tr>
<td>South-East Asia</td>
<td>49.9 (45.1-54.8)</td>
<td>91.5 (82.6-100)</td>
</tr>
<tr>
<td>Europe</td>
<td>19.7 (9.7-29.6)</td>
<td>5.81 (2.87-8.75)</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>20.4 (13.2-27.6)</td>
<td>13.2 (8.54-17.9)</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>12.9 (12.3-13.5)</td>
<td>14.3 (13.6-14.9)</td>
</tr>
<tr>
<td>Global</td>
<td>33.3 (31.1-35.4)</td>
<td>190 (178-202)</td>
</tr>
</tbody>
</table>

a population subgroups: Preschool-age children (< 5 years); Pregnant women.

b Numerator and denominator excludes countries with a 2005 GDP > US $ 15 000.

c 95% Confidence intervals.

2.4 Assessment of vitamin A status and limitations

The first step in assessing vitamin A status took place in 1974 when WHO set up an expert group with knowledge of VAD and xerophthalmia, including the classification of the eye lesions. Because of the limited knowledge at that time, the magnitude of VAD as a public health problem was based on the prevalence of negative eyes signs combined with low SR concentration (<0.35 µmol/L) [63]. Nowadays, clinical signs of VAD are rare as a result of intervention programs and economic development. Biochemical or marginal vitamin A status,
in which clinical signs or symptoms are absent, is more prevalent and can also lead to adverse health consequences. A population with marginal vitamin A status is more susceptible to infection, and even a single bout of infection can rapidly deplete VA stores in the body [98].

Multiple indicators have been developed to assess the different degrees of vitamin A status [61]. Several assessment techniques to determine the vitamin A status of populations across a continuum of liver reserves are shown in figure 2 which has recently been proposed by Tanumihardjo [61]. Among these methods, only the isotope dilution technique and the dose response tests have been compared with direct measurement of liver reserve, "the gold standard". Obviously, these two methods are more practical than the liver biopsy, which is not widely used in the real world due to the invasiveness [61, 99].

<table>
<thead>
<tr>
<th>INDICATOR</th>
<th>VA STATUS CONTINUUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deficient (&lt; 0.07)</td>
</tr>
<tr>
<td></td>
<td>Marginal (0.07 - 0.1)</td>
</tr>
<tr>
<td></td>
<td>Adequate (0.1 - 1.0)</td>
</tr>
<tr>
<td></td>
<td>Sub-toxic (&gt;1.0)</td>
</tr>
<tr>
<td></td>
<td>Toxic (10 µmol/g)</td>
</tr>
<tr>
<td>Clinical signs and tests</td>
<td>□</td>
</tr>
<tr>
<td>Serum retinol</td>
<td>□</td>
</tr>
<tr>
<td>Breast milk retinol</td>
<td>□</td>
</tr>
<tr>
<td>Dose response tests</td>
<td>□</td>
</tr>
<tr>
<td>Isotope dilution</td>
<td>□</td>
</tr>
<tr>
<td>Liver sample</td>
<td>□</td>
</tr>
</tbody>
</table>

**Figure 2** Recommendation of biomarkers of vitamin A status in relation to liver-reserve concentrations (µmol vitamin A/ g liver) [61]

Vitamin A indicators can be divided into two main categories as follows: 1) clinical, functional, and histologic indicators and 2) qualitative and quantitative biochemical indicators [61]. The two categories will be discussed in the following two sections.
2.4.1 Clinical, functional and histological indicators

Xerophthalmia

In the past, since the recognition that xerophthalmia was the most common cause of blindness in young children around the world, traditionally clinical eye signs and symptoms of xerophthalmia were used to identify populations with VAD as recommended by The International Vitamin A Consultative Group (IVACG) [63, 99]. As describes before, the term xerophthalmia refers to the range of ocular manifestation of VAD, from the milder stage of night blindness and Bitot’s spots which are reversible when treated with vitamin A supplements, to irreversible blindness resulting from corneal xerosis, ulceration and necrosis (keratomalacia) [61, 82, 98] as listed in Table 2. Since the corneal disease is rare, the most commonly assessed stages are night blindness and Bitot’s spot which represent moderate-to-severe VAD [19]. Details of the standard procedure to classify xerophthalmia were published by Sommer [82].

Table 2 Classification of xerophthalmia [100] range by the severity of condition from milder stage of night blindness (XN) to most severe of keratomalacia (X3B)

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>XN</td>
<td>Night blindness</td>
</tr>
<tr>
<td>X1A</td>
<td>Conjunctival xerosis</td>
</tr>
<tr>
<td>X1B</td>
<td>Bitot’s spot</td>
</tr>
<tr>
<td>X2</td>
<td>Corneal xerosis</td>
</tr>
<tr>
<td>X3A</td>
<td>Corneal ulceration/keratomalacia (&lt; 1/3 corneal surface)</td>
</tr>
<tr>
<td>X3B</td>
<td>Corneal ulceration/keratomalacia (≥ 1/3 corneal surface)</td>
</tr>
<tr>
<td>XS</td>
<td>Corneal scar</td>
</tr>
<tr>
<td>XF</td>
<td>Xerophthalmic fundus</td>
</tr>
</tbody>
</table>
Conjunctival impression cytology (CIC)

Evaluation of Conjunctival impression cytology (CIC) has been proposed as a field-operative test [101]. During VAD, mucin-containing goblet cells decrease. CIC is based on the lack of normal goblet cells and the presence of enlarged epithelial cells in the conjunctiva of vitamin A-deficient people. For measurement, cells are transferred from the conjunctiva to filter paper by quickly touching the eye surface using manual pumping. Then, the filter paper is stained to differentiate the goblet cells from the endothelial cells. The goblet cells are counted and scored under the microscope for classification of eye abnormality [61, 101]. Nevertheless, this method has not been widely used because of its limitation when used with groups with infectious trachoma [102] and with subclinical deficiency in young children [103].

2.4.2 Qualitative and quantitative biochemical indicators

Serum retinol

SR is the most widely used indicator for assessing vitamin A status and can be measured with a simple HPLC method [104]. However it can only be used at the population level because SR is homeostatically controlled over a wide range of body stores and thus concentration will only change when the stores are either very high or very low [63, 98]. Moreover, SR is decreased with infection and inflammation presumably because retinol is bound to the negative acute phase protein (RBP) which is depressed with protein malnutrition and an acute phase response [61, 105]. It is recommended to include the measurement of C-reactive protein (CRP) and/or α1-acid-glycoprotein (AGP) concentration (two acute-phase proteins) for the interpretation of the prevalence of VAD in areas where infection and inflammation are common[106]. SR is not really suitable for assessing the change in vitamin A status in response to an intervention because SR would only be expected to increase if initial values are low and subclinical infection is not present [98]. In addition, deficiencies of other micronutrients,
such as iron, might interfere and negatively affect SR levels[107]. According to WHO guideline [19], SR values below a cut-off of 0.35 and 0.70 µmol/L represent severe VAD and moderate VAD, respectively, while a cut-off of 1.05 µmol/L has been proposed for low vitamin A status in pregnant and lactating women.

**Breast-milk retinol concentration**

Breast-milk retinol concentrations are a useful tool and a unique indicator in lactating women. The status of the mother can usually be predictive of the nursing infant [108]. The advantage of this method is that it is less invasive and there is no need for further processing at the field station which also shortens sample preparation [99]. The limitation of this biomarker is that breast-milk retinol concentrations may rather reflect recent dietary intakes than true vitamin A status [61] and the response to supplementation was shown to only be modest in lactating women in Kenya [109].

**Relative dose response (RDR) and Modified relative dose response (MRDR)**

The dose response test is based on the metabolism of RBP which is accumulated in liver as apo-RBP when vitamin A in liver becomes low, which occurs before SR levels decrease [110]. After the challenge dose of retinyl ester or 3,4 didehydroretinyl ester is administered, the accumulated RBP binds to retinyl ester or 3,4 didehydroretinyl ester as a holo-RBP-retinol complex and is then rapidly released from the liver to the serum [61]. A blood sample is taken at time 0 (before dosing) and 5 h (after dosing) [99]. The RDR value is presented as the percentage using the following calculation where A0 and A5 is the SR at baseline and 5 h post dosing with retinyl ester respectively [61, 111].

\[
RDR = \left( \frac{A5 - A0}{A5} \right) \times 100
\]

If the RDR is > 20% then that person is considered having inadequate liver reserves (< 0.07 µmol/g). The drawback of this method is that it requires 2 blood drawings within 5 h [61].
Therefore, the MRDR was developed by using 3,4 didehydroretinyl acetate and only 1 blood sample drawn 4-6 h after the dose [112]. This is possible because the circulating concentration of 3, 4 didehydroretinol is low in serum. The calculation of MRDR is the ratio of 3, 4 didehydroretinol to retinol. MRDR values ≥ 0.06 indicate low liver reserves. The MRDR technique has been used extensively in several countries to evaluate interventions as it is more responsive than SR and it can be used to diagnose subclinical vitamin A status [113-115]. Although the MRDR test can be applied to people with a wide range of vitamin A status, from deficient to normal vitamin A status, it still has limitations in defining the sub-toxic and toxic stages of liver reserves [61].

**Isotope dilution technique**

This is the most sensitive indicator of vitamin A status and measures liver reserves of vitamin A [98, 104]. The method allows the evaluation of vitamin A status over a wide range from deficiency to toxic states, by using stable isotopes as tracer to calculate an estimate of total body reserves (TBR) of vitamin A (or total body vitamin A pool size) [61]. The isotope dilution technique (illustrated in figure 3) consists of: 1) giving an oral dose of an appropriate stable isotope (tracer) to subjects, 2) obtaining a blood sample after the tracer has mixed with the endogenous vitamin A, 3) measuring of the plasma or serum isotopic ratio of labeled (tracer) to non-labeled retinol (tracee) and 4) calculating an estimate of TBR of vitamin A in the body [98, 104] by using the fundamental mass balance equation [116].

\[
(Fa \times a) + (Fb \times b) = (Fc \times C)
\]

\[
Fa = 0.1
\]

(fraction of dose labeled (in this example, \(^{13}\)C\(_2\) retinol has 2 \(^{13}\)C out of 20 carbon)= 2/20)
Fb, Fc = the decimal form of At% $^{13}$C of serum retinol at baseline and 14 days after administered respectively

(AT% is an expression of the percent of 13C atoms to 12C atoms in the retinol extracted = $\frac{100}{1 + (1/R)}$ where R is the ratio $^{13}$C/$^{12}$C)

- $a = \mu$mol VA absorbed from labeled dose and stored = $\mu$mol VA administered x 0.5 [117]
- $b = \text{uncorrected baseline TBR (unknown)}$ in $\mu$mol
- $c = \text{TBR after dosing} = a + b$ in $\mu$mol

uncorrected baseline TBR (unknown) = $b = [(F_c \times a) - (F_a \times a)] / (F_b - F_c)$, with $c = a + b$

corrected TBR ($\mu$mol) = $b \times e^{(-kt)}$

where $k = \ln(2)/140$ and $t =$ time in days (either 3, 7 or 14 day after dosing) [104].

**Estimation of liver vitamin A concentration (µmol/L)**

The calculation assumes that, for example in children, liver weight is 3% of body weight [118] and 90% of total body VA (TBR) is stored in the liver [119]. Subjects are considered as VAD when liver vitamin A concentration $< 0.07 \mu$mol/g [98].

There are 2 stable isotopes that are routinely used for this technique: deuterated retinyl acetate is the most widely used and $^{13}$C-retinyl acetate which has been developed in recent years [116, 120]. The difference among these two types of stable isotopes is that different types of equipment is used for analysis: the deuterated retinyl acetate is measured by using conventional gas chromatography-mass spectrometry (GC-MS) while the $^{13}$C-retinyl acetate is measured by gas chromatography-combustion-isotope ratio mass spectrometry (GCCIRMS) [98, 104]. The latter machine requires a lower dose of tracer, which does therefore not perturb vitamin A trafficking, but sample preparation is more demanding when compared to GC-MS [61, 121].
The isotope dilution technique has several uses. It can be used to assess vitamin A status, to evaluate the efficacy or effectiveness of vitamin A intervention programs [122-124], to study the conversion of provitamin A carotenoids to vitamin A and estimate their bioequivalence [89, 125, 126], and to obtain information on dietary requirements of vitamin A [127, 128]. Although this tracer method has a number of advantages, it is more expensive and technically demanding than other methods and requires the acceptance of ethical committees to infuse stable isotopes and take blood samples which can be a problem in children. This higher cost can however be compensated by the lower number of subjects and the shorter intervention period thus, studies should be well designed. Isotope dilution technique is thus the method of choice when an estimate of vitamin A pool size (or TBR of vitamin A) is required and when critical decision-making is needed; for example to estimate quantitatively the amount of

Figure 3  The principles of the tracer methodology [98]
vitamin A retained in the body in response to an intervention with different source of vitamin A [98].

**Dietary assessment**

The advantage of these techniques to estimate vitamin A status is that they are neither invasive, expensive nor complicated. The dietary pattern and food habits of populations at risk of VAD can be easily assessed. However, the drawback of this method it that there is still an uncertainty with respect to the bioconversion factors of provitamin A carotenoids to retinol and vitamin A is well preserved in the body. Short-term dietary recall such as 24 h dietary recall to measure intake may have little to do with long term status of an individual, but it may be useful in population studies [63, 101].

**2.5 Strategies used for prevention and combat of VAD**

**2.5.1 Supplementation**

Prophylactic and therapeutic vitamin A supplements have been successfully used in the prevention and control of VAD for more than 20 years [129]. It is for example recommended that vitamin A capsules are regularly given to all children between the age of 6 months and 5 years in countries where over 70 of 1,000 children die before reaching the age of 5 years. Supplementation with vitamin A therefore has not been based on VAD but on child mortality. A high-dose of vitamin A (100,000 IU for 6-12 months old babies and 200,000 IU for children between 1 and 5 years) should be given twice yearly [130].

This supplement maximizes liver stores of the vitamin [131] and results in a cyclic pattern of changing in liver reserves [132]. Several studies have reported the efficacy of vitamin A supplementation in improving vitamin A status in vulnerable groups such as infants, preschool
aged children, pregnant and lactating women as well as the reduction in mortality and morbidity rates [129, 131]. While vitamin A supplements have reduced morbidity and mortality in some populations, particularly where measles are common, other studies [133, 134] as well as a meta-analysis [135] reported that vitamin A supplements have no consistent impact on overall protection from mortality and morbidity. Moreover, an adverse effect of high doses of vitamin A on respiratory infection has been reported, particularly in children with adequate nutritional status [136, 137]. The underlying cause of this was explained by the high dose of vitamin A supplements which as a non-physiological dose may cause immune dysregulation, especially in children with good vitamin A status [137].

The drawbacks of this strategy are that it not only requires recurrent funding each year for the vitamin A supplements but also the cost for the staff working for the distribution and the monitoring of the programs [138].

2.5.2 Fortification

The advantages of food fortification as a cost-effective and safe intervention are presented through examples from various parts of the world with different staple foods and food products. Food fortification has many advantages: it is generally socially acceptable, it requires minimal changes in food habits, it usually adds < 2% to the cost of the unfortified food, the delivery system is already set up and it can become sustainable [24].

Oil, margarine and other hydrogenated oil products are the most suitable vehicles for vitamin A fortification because they facilitate the absorption of vitamin A. Moreover, oil stabilizes retinol and minimizes oxidation of vitamin A. Vitamin A fortified margarine was first produced in Denmark in the 1920s [139] and has since been introduced to many countries across the world with the same purpose of imitating the nutrition value of butter and preventing VAD. Vitamin A fortified margarine was tested for its efficacy in the Philippines for 6 months and results
showed an increase in SR in the intervention group with the control group decreasing over the same time period [140]. Fortification of cereal flours with vitamin A has also been widely implemented in many parts of the world; for example, in 1993, Venezuela launched a national program for fortifying precooked corn flour [141] and the Philippines for fortifying wheat flour [142]. Fortification of cereal flours with vitamin A is feasible because stability is good during cereal flour production since antioxidants and stabilizing agents are added [24].

Sugar is another useful vehicle for vitamin A fortification when consumption is high and production is centralized. Sugar fortification was a success when implemented in Central America in the 1970s. Evaluation of the effectiveness after 1 year of the implementation of a national program of vitamin A fortified sugar in Nicaragua was done by using the deuterated-retinol-dilution (DRD) technique. The liver retinol reserves of Nicaraguan children increased from 0.57 µmol/g liver to 1.2 µmol/g liver [124]. Vitamin A fortified monosodium glutamate (MSG), another potential vehicle for vitamin A fortification, was successfully developed and the efficacy of MSG was demonstrated in the Philippines [143] and Indonesia [144].

Several other food vehicles have been used for vitamin A fortification including whole grain wheat, rice, tea, instant noodles, yogurt and salt [24, 145, 146]. Among these food vehicles, rice is the most attractive because of its wide consumption in developing countries where VAD is highly prevalent. Several techniques have been used to add vitamin A to the rice grains including coating [147] and making artificial rice grains by using the Ultra Rice technology [148] which had been proved to be efficacious [149].

Normal losses of vitamin A in dry fortified foods have been estimated to be around 30-50% occurring during transportation, storage as well as food preparation [24]. These losses need to be taken into account when defining the fortification level. Apart from monitoring the stability of
vitamin A in the fortified products, monitoring of vitamin A status to ensure adequate and not excessive levels of total body vitamin A is also necessary [98].

2.5.3 Dietary diversification

Compared to supplementation and fortification, dietary diversification has been less emphasized even though it is considered as potentially the most sustainable approach [132, 137]. It is difficult to get populations to change dietary habits particularly if the modified diets are more expensive as with vegetables and some fruits. Also it is perceived that provitamin A carotenoids from plant sources are poorly bioavailable [150] and that many factors affect the bioavailability of provitamin A carotenoids [83]. Using the stable isotope dilution technique in humans, studies have shown improved or maintained liver vitamin A stores with provitamin A carotenoids [85]. Nowadays, since foods fortified with vitamin A are becoming common and are being introduced in many parts of the world, there is a danger of hypervitaminosis A when preformed retinol is used [61, 132]. Provitamin A however would offer a natural protection against hypervitaminosis A as the bioconversion of provitamin A carotenoids to retinol decreases when liver reserves of vitamin A are high [132].

2.5.4 Biofortification

Biofortification is an agricultural strategy for increasing the micronutrient concentration in cereal seeds by using conventional breeding or targeted genetic engineering [151], or by use of agronomic practices such as applying fertilizers. The advantage of biofortification over fortification is that there is no need of a food vehicle that is centrally processed and biofortified food is accessible by the local people or farmers who do not purchase manufactured foods [152]. Biofortification could be considered either as a form of dietary diversification [138] or fortification [131]. This is a rather new technology which is a potential long-term and sustainable approach to improve vitamin A status of people especially those living in remote
areas where it is difficult to reach the population with fortified foods and supplementation [138]. There are several crops which have been used for biofortification with provitamin A carotenoids including sweet potato [153], maize [154, 155], cassava [156] and rice [157]. Traditional breeding methods have been applied for sweet potato, maize and cassava. For the rice grain, since provitamin A carotenoids do not exist naturally in rice, genetic engineering has been applied [158]. To date the highest carotenoid concentration of Golden Rice is 37 μg/g dry weight [159] with a conversion factor to retinol comparable to the conversion factor of pure β-carotene (2.1 μg for Golden Rice and 2 μg for pure β-carotene to 1 μg retinol) [86]. Efficacy [160] and effectiveness [161] studies have reported improved vitamin A status when assessed with the MRDR technique and by SR concentration, in African children who consumed orange-fleshed sweet potatoes which contain a high concentration of β-carotene (100-1600 μg retinol/100g).
3. Zinc (Zn)

3.1 Characteristics of zinc (physical, chemical and functional)

Zinc is a metallic chemical element with a bluish-white and shiny surface. It ranges in the 24th most abundant element with the atomic number of 30. Since it's the first member in group 12 of the periodic table it has a fairly active and strong reducing property with the oxidation state of +2. It can therefore dissolve in both acids and alkalis. In soil, zinc is contained at levels between 5 and 770 ppm with an average of 64 ppm [162, 163]. People living in countries where soil contains low amounts of zinc have a higher risk of zinc deficiency. Countries such as Afghanistan, Bangladesh, Brazil, China, India, Iran, Iraq, Pakistan, Sudan, Syria, Turkey, Australia, Philippines, many states in the USA and parts of Europe belong to these regions [8].

In human adults, zinc content in the whole body is approximately 2 g with higher average content in men (2.5 g) than in women (1.5 g). Zinc is found in all organs, tissues, fluids and secretions with 83% of total zinc in the body located in the skeletal muscle and bone mass [164]. Only 0.2% of total zinc is found in plasma which has a rapid turnover rate [165]. When total body zinc is depleted due to low intake, proportionately more zinc is lost from bone, liver, testes and plasma while zinc content is better preserved in the skeletal muscle, skin and heart [166]. Zinc is the most abundant intracellular trace element and is involved in many parts of catalytic, structural and regulatory functions in the human body. Zinc is an essential component of enzymes with over 200 zinc enzymes which can be found in the biologic system. They participate in the synthesis and degradation of carbohydrates, lipids, proteins and nucleic acids as well as in the metabolism of other micronutrients. Zinc has a crucial role as it stabilizes the molecular structure of cellular components and biomembranes. This function contributes to the maintenance of cell and organ integrity, for example through the zinc-fingers which enable
polypeptides that are too small to fold by themselves to fold in a stable fashion when stabilized by bound zinc. Zinc is necessary for ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and ribosome stabilization as well as for polynucleotide transcription. Moreover, zinc has an essential role in the immune system. Its involvement in numerous fundamental functions probably accounts for the essentiality of zinc for all life stages [67, 167].

As a consequence of homeostasis controlling zinc levels in the body over a broad range of exposure, zinc has a relatively low toxicity. Therefore, adverse health effects due to zinc intoxication are quite rare [168].

3.2 Metabolism of zinc

Although the homeostatic regulation of zinc metabolism in humans is not well defined, it appears that both absorption and excretion are involved [169]. As opposed to zinc absorption, endogenous excretion rapidly changes when zinc intakes are just above or below the optimal levels. Changes in zinc absorption are rather slow in the response to changes in zinc intake but are capable of managing large fluctuations in dietary zinc. In the situation of low zinc intake or with prolonged marginal intakes, there are secondary homeostatic adjustments which include changes in urinary excretion, changes in plasma zinc turnover rates, and zinc released from selected tissues such as bone, to maintain its function [170].

Absorption

Zinc absorption can be defined in two ways: 1) the fractional zinc absorption or FAZ is the fraction or percent of consumed zinc that is absorbed and 2) the total quantity of absorbed zinc (TAZ) over the whole day [171]. Several studies have proven that zinc absorption is influenced by dietary zinc intake rather than zinc status and it affects both FAZ and TAZ differently. When zinc intake increases, FAZ declines whereas TAZ increases. Studies in animals and humans
show that short and long term past intakes have no influence on FAZ and TAZ but efficiency and the amount of zinc absorbed are determined by current zinc intakes [172-175].

There are two mechanisms for the intestinal transport of zinc from the lumen of the intestine and on to the portal circulation: transcellular (saturable process or active transport) and paracellular transport (non-saturable process, passive diffusion). In the transcellular transport, zinc moves across the apical membrane through the cell exiting at the basolateral membrane, which is a carrier-mediated process. Paracellular transport is a simple diffusion by which zinc diffuses through the tight junctions between intestinal cells and it occurs when the concentration of zinc in the intestinal lumen is higher than the ability of the transcellular mechanism to transport zinc. As the transcellular mechanism becomes saturated with increasing zinc concentrations in the intestinal lumen, absorption by simple diffusion (paracellular transport) predominates increasingly [176].

In the transcellular transport, the zinc absorption process is facilitated by not only the saturation kinetics but also by the regulation of zinc transporters that help zinc migrate into and across the enterocyte. These are the principal mechanism by which whole-body zinc homeostasis is maintained [177]. There are 2 gene families of zinc transporters: the ZnT protein family functions in lowering the cellular zinc concentration (until now 10 members have been discovered) while ZIP proteins with 14 members act to increase cellular zinc [178, 179].

The discoveries of zinc transporters have clarified the mechanism of zinc absorption. When zinc intake is below 9 mg/day, Zip4 is the major importer responsible for zinc uptake into the enterocyte. At the basolateral membrane ZnT1 transports zinc from the enterocytes into the circulation [180]. The mechanism of the intracellular transport of zinc from apical to basolateral intracellular surface of enterocytes is still not clear at this time [176]. It appears that zinc may
be sequestered in the cell by incorporation into the Golgi apparatus via ZnT7 or binding to metallothionein [179]. Zinc is distributed through the whole body via the serum/plasma by binding to several proteins such as albumin, α-microglobulin and transferrin [181].

![Figure 4](image.png)

**Figure 4** Overview of major zinc transporters expressed in intestinal epithelial cells. ZIP4 is a major importer and is regulated by zinc, ZIP14 is responsive to proinflammatory conditions and is postulated to be at both the apical and basolateral surface of enterocytes. ZnT1 and ZIP5 influence zinc trafficking at the basolateral membrane. ZnT7 influences the apparent transcellular movement of zinc [179]

**Excretion**

In general, most of the zinc losses are through endogenous fecal losses which are the losses through feces (90%) while only small amounts of zinc is lost through urine (<10%) [176]. Endogenous fecal losses can vary from less than 1 mg zinc/day to more than 5 mg zinc/day [182]. This plays an important role in the maintenance of zinc homeostasis and depends on recent absorption and zinc status [177]. Fecal zinc losses are mainly from unabsorbed dietary zinc and endogenous zinc secretions. Pancreatic secretions are a major source of endogenous
zinc [167]. A daily amount of 0.5-0.7 mg zinc is lost from urine and integument which depend less on normal variations in zinc intake [67]. Under basal conditions, up to 95% of filtered zinc is reabsorbed in the distal parts of renal tubule [183]. Muscle catabolism during severe burns, major surgery and other trauma or starvation also cause clinically significant increases in urinary zinc losses. Chelating agents, such as ethylene-diamine-tetra-acetic acid (EDTA), also elevates urinary zinc level. Other sources of loss include semen and menstrual secretions [167] as well as a condition of strenuous exercise and high temperature through perspiration [67].

Storage

When animals are fed with low zinc diets their zinc status rapidly declines. This suggests that the body has no specific zinc storage [67, 176]. Increasing zinc intake elevates content of zinc in bone, liver and intestine. Release of zinc from those tissues during depletion may delay the onset of zinc deficiency symptoms [184]. It appears that a small cellular zinc reserve exists in all tissues, which is readily available to maintain function during short-term, insufficient intakes. With a subsequent increase in intake, positive zinc balance occurs and the reserve is replenished [180].

3.3 Zinc deficiency

3.3.1 Etiology

The major causes of zinc deficiency are inadequate dietary intake and consumption of a diet with low bioavailable zinc [54]. Excess losses and impaired utilization during diarrhea or parasitic infections, increased sweat through hot and humid climates, as well as increased requirements during pregnancy, lactation, young childhood, and adolescence also contribute to
zinc deficiency. In addition, food taboos in some Asian countries prevent pregnant and lactating women as well as young children from consuming adequate zinc and protein-rich foods [185]. People who live in environments of low zinc content in soil as a result of erosion or leaching by heavy rainfall may have a high risk of zinc deficiency because plants grown in these areas may be zinc deficient [186]. Good sources of zinc are shell fish, red meat, liver and egg. Cereals with low extraction rates, chicken, pork or meat with a high fat content have a moderate zinc content while roots and tubers, green leafy vegetables and fruits are only modest sources of zinc. Saturated fats and oils, sugar and alcohol have very low zinc contents [67, 167]. People who rely on plant based diets, and also have a low meat consumption, may have difficulties in meeting their daily zinc requirements, especially those consuming staple foods with a high phytate content which reduces zinc bioavailability [186]. Micronutrient malnutrition can become a serious problem in children and infants in developing countries who consume high phytate cereal based complementary foods with phytate:mineral molar ratios at levels likely to inhibit absorption of iron, zinc, and calcium [187]. The suggested desirable levels for adequate zinc absorption is a phytate:zinc molar ratio < 18, which corresponds to a mixed or refined vegetarian diet, whereas a phytate:zinc molar ratio of > 18 corresponds to an unrefined cereal-based diet resulting in a reduced bioavailability [188]. In the presence of calcium, the phytate-zinc complex is stabilized further and therefore WHO recommends that diets that have a phytate:zinc molar ratio > 15 together with more than 1 g/day of calcium, are categorized as low zinc bioavailability [67]. There are some studies suggesting that Na$_2$EDTA [189] and components in milk and infant formulas such as lactose, glucose polymers and caseinphosphopeptides may improve zinc absorption [190].

It has also been reported that zinc deficiency is associated with malabsorption syndromes, alcoholism, chronic renal diseases and chronic debilitating diseases [191, 192]. Acrodermatitis
enteropathica is a rare autosomal recessive genetic disorder which causes zinc malabsorption [193].

3.3.2 Consequences

Zinc deficiency is one of the ten biggest factors contributing to the burden of disease in the developing world and leading to a high mortality in such countries [25]. Because of the important role of zinc in numerous enzymes, as well as other functions such as in gene expression, zinc deficiency could jeopardize many essential metabolic functions. Cell division and protein synthesis for growth are the principal roles of zinc and are especially important for infants, children, adolescents, and pregnant women. Therefore, these groups of population are suffering most from inadequate zinc intake [67].

Zinc deficiency develops differently as compared to most of the other nutrient deficiencies where growth reduction is a late manifestation of the deficiency. When zinc intake is inadequate, the first responses are a reduction in growth (in a growing organism) and a decrease in endogenous losses of zinc to conserve tissue zinc. This is because it is crucial to preserve tissue zinc for maintaining zinc's role for transcription factors and regulated gene expression [167]. The very first cases of zinc deficiency were reported in humans consuming a diet high in phytate and low in meat (the classic studies of zinc deficiency in Iranian adolescent males) in 1958 with the clinical symptoms of dwarfism, hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy, geophagia and iron deficiency anemia [194]. Since then, zinc deficiency has been studied extensively [176]. The clinical manifestations of severe zinc deficiency in human include growth retardation, delayed sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired appetite, increased susceptibility to infections mediated
via defects in the immune system, and the appearance of behavioral changes [195]. Moreover, zinc deficiency may contribute to many diseases including malabsorption syndrome, chronic liver disease, chronic renal disease, sickle cell disease, diabetes, malignancy and other chronic illnesses. It is probable that other trace elements and vitamins are also involved in all the above mentioned conditions, since zinc deficiency does not occur alone in developing countries [196].

3.3.3 Prevalence
A recent report in the Lancet estimated that zinc deficiency is responsible for 500,000 deaths per year [12] while data on prevalence of zinc deficiency at country level are still lacking. The use of zinc intake estimations from national food balance sheets (FBS) or the prevalence of other relevant clinical manifestations such as stunting and diarrhea in children have been used as a surrogate of prevalence of zinc deficiency although these data have been claimed as the less precise estimates [58, 197, 198]. About almost 2 billion people worldwide are at risk of zinc deficiency based on calculations from indirect indicators of FBS. When combining two sets of data, FBS (percent of individuals at risk of inadequate zinc intake) and stunting rate (percent of preschool children who are stunted), more reliable data of the risk of zinc deficiency are obtained. Results from the above estimations indicate that countries in Southern and Southeast Asia, Africa, Central America and Andean region are classified to either high or moderate risk of zinc deficiency [197].

3.4 Assessment of Zn status and limitations
Data on the prevalence of zinc deficiency is not well recorded. The main reason is a lack of reliable and widely accepted indicators of zinc status, especially for the assessment of mild or
marginal deficiency, which is most prevalent and often underestimated [199, 200]. It is essential to know the prevalence and the severity of zinc deficiency in a population when setting up an appropriate zinc intervention program and when monitoring its effectiveness in improving the health and well-being of high risk populations [201]. Serum and plasma zinc levels are the most widely used biochemical indicators of zinc status. Zinc content in other tissues have also been investigated as possible indicators for zinc status, including various blood cell types, hair and nails, in a number of zinc binding proteins such as metallothionein, as well as in zinc metalloenzymes. But these indicators do not have any advantage in sensitivity or analytic convenience when compared to SZn concentration [197, 201, 202].

There are 3 indicators which have been recommended by WHO/UNICEF/IAEA/IZiNCG for assessing the prevalence of zinc deficiency at the population level: 1) percentage of the population with low SZn concentration, 2) prevalence of inadequate zinc intake, and 3) percentage of stunting in children < 5 years of age [203].

### 3.4.1 Serum Zinc

Less than 0.2% of the total zinc in the human body is found in plasma [165]. However, serum or plasma zinc is claimed to be the best available biomarker for assessing the risk of zinc deficiency in populations because it had been proven under the following criteria: a) response of SZn concentrations to zinc intake, b) use of SZn levels to predict functional responses to zinc interventions, and c) relationship between initial SZn and change in SZn in response to the intervention [198, 201].

For the population assessments, the percentage of people who have low SZn concentration with respect to age/sex/time of day specific appropriate lower cut-offs is used as an indicator. Zinc deficiency is recognized as a public health concern and the risk is considered to be
elevated when the prevalence of low SZn in the population is more than 20%. In this circumstance, an intervention to improve zinc status of the population is recommended [197]. The prevalence of low SZn levels can be used to evaluate the success and impact of an intervention program. In general, SZn levels of an individual vary in a fairly narrow range due to the control of the homeostasis mechanism. Therefore, this indicator can only be used to assess individual zinc status under extreme dietary conditions [201]. Several factors can affect SZn concentration. These include factors involved in the measurement technique [204] as well as biological factors like certain genotypes, mutations, age, gender, nutritional status, interaction with drugs and nutrients (iron, calcium) and infection/inflammation which can cause an increase or decrease of SZn levels [200]. Thus, when interpreting such data these possible confounders must be carefully considered [205]. Recent studies in men and young children [206, 207] found that SZn responded within 2 weeks in the group receiving supplements but not in the fortification group. Serum zinc concentration may therefore not be a sensitive parameter for the evaluation of a short-term fortification program [206]. However, compiling data [201] from several studies show the relationship between dietary zinc intake and SZn concentration (Figure 5). This review indicated that SZn decreases sharply when zinc intake is less than ~2 to 3 mg/day, and that with an increasing zinc intake, SZn increases continuously until reaching a plateau when the intake is as high as ~25 to 30 mg/day.
3.4.2 Dietary indicators for assessing the adequacy of population zinc intake

The prevalence of inadequate zinc intake can provide the relative magnitude of the risk of zinc deficiency in a population and identified subpopulations, because inadequate zinc intake is the main cause of zinc deficiency. These data can also provide information on the dietary pattern, and how this contributes to inadequate zinc intake, and they can be used for the development of an appropriate food-based intervention for improving zinc status in a distinct community [208]. A prevalence of inadequate zinc intake of higher than 25% is considered to be an elevated risk for zinc deficiency in a population and a public health concern [209]. The possible confounding factors that might lead to inconsistent findings include setting of the estimated average requirement (EAR) values for toddlers, children, women, including pregnant and lactating women, and the elderly [200] as well as the small and non-representative nature of
the study groups investigated [208]. Additionally there is also a need to estimate bioavailability as recommended intakes increase for populations consuming lower bioavailability diets.

3.4.3 Functional indicators of zinc status: prevalence of stunting in children < 5 years of age

Positive results of zinc supplementation trials confirmed that an effect of zinc supplementation on linear growth can be expected when children with initially low height for age Z-scores (HAZ) are recruited, irrespective of age of children or whether they are from a developed or less-developed country [210, 211]. Thus, height- or length- for age was chosen as indirect indicator for assessing zinc status of population’s risk of zinc deficiency [211]. The risk of zinc deficiency is considered to be elevated and of public health concern when the prevalence of stunting in children < 5 years of age is more than 20% [197, 212]. The advantage of this method is that it is less invasive, easier to perform and more directly related to disease mechanisms or health status than measurement of biochemical indicators. However, the functional indicators should be evaluated by their response to zinc supplementation trials in randomized controlled trials (RCT) which is an expensive approach because it needs a large number of participants and long periods of time to detect such effects [208].

3.4.4 Other biomarkers of zinc status

Hair zinc

The use of zinc content in hair as an indicator of zinc status has been controversial since results from several studies have shown both positive and negative correlation of hair zinc and either functional or clinical signs of zinc deficiency or dietary index [197]. The limitation of using hair zinc concentration as zinc indicator include lacking of established cut-offs for most age
groups as well as variation with age, sex, season, hair growth rate, severity of malnutrition, and possibly hair color and other hair cosmetic products. The advantage of this indicator is that it is less invasive than SZn and it can therefore be used as an alternative option when blood taking in children is not allowed. Moreover, unlike SZn, hair zinc is more stable and not affected by diurnal variation, prolonged fasting, meal consumption and inflammation [197, 208].

**Urinary zinc excretion**

Urinary zinc concentrations are fairly stable when zinc consumption is adequate. However, diurnal variations have been reported. Urinary excretion rates are diminished in severe deficiency states. It is also affected by a range of clinical conditions that increase zinc excretion including liver and kidney diseases, infections, burns, alcoholism and sickle cell disease. Moreover, catabolism and anabolism of the muscle can increase and decrease excretion, respectively [213].

**Other potential biomarkers**

A systematic review [205] reported on other potential useful indicators for zinc status and emphasized that there were insufficient data to confirm their usefulness. The potential biomarkers included aminolevulinic acid dehydratase, erythrocyte metallothionein, monocyte metallothionein DNA, saliva zinc, saliva-sediment zinc, mixed saliva zinc, plasma extracellular superoxide dismutase, lymphocyte zinc, lymphocyte ecto-5’-nucleotidase, nail zinc, plasma angiotensin-converting enzyme, neutrophil zinc, T lymphocyte metallothionein-2A mRNA, plasma 5’-nucleotidase, endogenous zinc excretion, plasma zinc flux, exchangeable zinc pool, carbonic anhydrase, fecal zinc, neutrophil a-D-mannosidase, neutrophil alkaline phosphatase, erythrocyte membrane zinc, erythrocyte membrane alkaline phosphatase, and erythrocyte membrane NP.
In the future, molecular techniques like polymerase chain reaction (PCR) assays and kinetic markers may be used. For PCR, it is being used increasingly to measure messenger ribonucleic acid (mRNA), e.g. metallothionein mRNA in various tissues and zinc transporter proteins. Isotope techniques are used for measuring kinetic markers such as exchangeable pool of zinc (EZP) and plasma zinc turnover rates. However, at present more research is needed to establish the specificity, sensitivity, validity and feasibility of the above mentioned techniques [208].

3.5 Strategies used for prevention and combat of zinc deficiency

There are several strategies for improving zinc status in populations at risk of deficiency [214]. These are food fortification, supplementation, biodiversification/modification and biofortification. Among these strategies, zinc supplementation and fortification are the most promising with respect to its rapid implementation [215]. However, in order to select the most suitable strategy, it is essential to know the magnitude/severity of deficiency in different population subgroups as well as financial and technological resources that are available so that the sustainability of the intervention can be evaluated [197, 216].

3.5.1 Supplementation

A recent meta-analysis [217] examined the impact of zinc supplementation on morbidity, mortality, physical growth and SZn concentration. It found that zinc supplementation can reduce the incidence of diarrhea and acute lower respiratory tract infection by ~20% and ~15%, respectively. Most of the studies found a positive effect on growth (both weight and height but not for weight for height Z score (WHZ)) [210, 217, 218]. This is on the contrary to a recent study from Ramakrishnan et al [219] which did not find a beneficial effect on weight and
height but a small effect on WHZ. The latter meta-analysis concluded that this might be due to the different age groups evaluated and because subjects in this recent study may have better nutritional status which might have attenuated the concurrent benefit of zinc supplementation on growth. Zinc supplementation has been demonstrated to reduce child mortality rate in developing countries by 6-9% [217, 218]. This effect however may be restricted to children > 12 months old showing an 18% reduction in mortality rate [217].

3.5.2 Fortification

Food fortification is recognized as the most economical approach to combat nutritional deficiencies because of its relatively low cost and long term sustainability but the data on efficacy trials of zinc fortification is very limited [215]. Studies have reported that zinc fortification can increase both dietary zinc intake and total daily zinc absorption [220-223] and that zinc oxide seems to be absorbed as well as zinc sulfate when fortifying cereal staples [224]. As opposed to supplementation, zinc added to food appears to have no adverse effect on the absorption of other minerals such as iron [189, 225]. Only a few intervention trials of zinc fortification have found a positive effect on biochemical and functional indicators such as SZn concentration and growth. This may be related to several factors such as food vehicles, age group and initial zinc status of participants or differences in the study protocol [216]. Protocol differences include the intervention period not being long enough to show an effect of fortified food on SZn concentration or an effect on growth [189, 220]. In efficacy studies of fortified infant formula, the results are inconsistent with only about half of all studies finding a positive effect on SZn concentration [226-229]. An improvement in physical growth indicators has been reported mainly when premature or malnourished (protein energy malnutrition:PEM) infants have been recruited [229, 230] while these effects were not shown in healthy term
infants [226, 227]. Cereal products such as breakfast cereals [231], rice flour [189] wheat products [220] and cereal porridges [215] have also been investigated as food vehicles for zinc fortification. From these studies, only the study of Hambidge et al. [231] reported a positive effect on SZn concentration in the intervention group, whereas another study using a cereal-based porridge detected a positive impact on reducing the prevalence of underweight [232]. In Thailand, a study using a multiple micronutrients fortified seasoning powder added to school lunch meals has shown an increased SZn concentration in the group receiving the fortified powder [233]. This positive finding may be related to the age of the subjects (school aged children) or to the nature of the fortification vehicle which had low phytate [216] and was added to a low phytate meal. There are also reports that zinc fortification improves morbidity outcomes by reducing the diarrhea rate [234] and the incidence of respiratory-related diseases [235].

3.5.3 Dietary diversification

Dietary diversification can improve zinc nutritional status as well as other coexisting micronutrient deficiencies at the same time and has the advantage of preventing antagonistic interactions. Strategies to increase intake and/or bioavailability of zinc include interventions based on agriculture such as production or promotion of animal-source foods through animal husbandry or aquaculture and commercial and household processing strategies [236]. Although these are potentially promising strategies to combat zinc deficiency, they have only been implemented at small scale and have not been proven to affect nutritional status or micronutrient indicators on a large scale [218]. The latest study in New Zealand toddlers didn’t find any improvement in serum and hair zinc concentration when subjects were fed with either red meat or a fortified manufactured toddler milk drink although the diets increased zinc intake
in both groups. The authors suggested to reevaluate both the dietary and biochemical criteria used to define low zinc status in toddlers [237].

The promotion of breastfeeding at least until the first 6 months of age is very important since this can ensure adequate zinc status of young children [238]. This was confirmed by the studies in term infants living in industrialized countries [239-241] which didn’t find a positive impact of supplemental zinc on growth of breastfed infants younger than 6 months. This indicated that breast milk alone provides enough zinc for term infants during the first 6 months of life.

3.5.4 Biofortification

Crops that have been targeted for zinc biofortification include rice, wheat, maize, and pearl millet [152]. Conventional breeding identifies the traits of high zinc concentration in seeds and then introduce them into locally adopted cultivars [242]. At present, the minimum target zinc level in various food crops have been set to provide ~ 40% of the physiological requirement for absorbed zinc for non-pregnant women and children 4-6 years old [152]. The application of zinc containing fertilizers can improve zinc content of grains in some areas with low soil zinc concentration [152, 243]. Such studies with rice and wheat grains have reported an increase in yield and concentration of zinc when using ZnSO$_4$ or zinc enriched/coated urea to fertilize the soil [244, 245]. Genetic modification is a novel technique and a potential alternative method when the desired zinc levels cannot be achieved by the other two methods. The inhibitory effect of phytate on zinc can be overcome by mutagenesis to reduce the synthesis of phytate or increase the level of phytase by over-expressing the phytase gene [242]. Results from an absorption study in women who consumed either 300 g of biofortified wheat (41 µg zinc /g) or control (24 µg zinc /g) showed that women absorbed 0.5 mg/d more zinc from biofortified
wheat than from the control ($p < 0.05$) [246]. It remains to be seen whether an increase in zinc intake can produce a measurable increase in zinc status.
4. Coexistence of micronutrient deficiencies

4.1 Overview and magnitude of the problem

Multiple micronutrient deficiencies are commonly found among high risk groups including pregnant and lactating women, women of reproductive age and children especially those who live in the developing world. Coexistence of micronutrient malnutrition in children has frequently been found in both developing and developed countries where people cannot afford a healthy and well-balanced diet including meat, fish, dairy products, fruits and vegetables. The other risk factors of multiple micronutrient deficiencies are relatively similar to those for single micronutrient deficiencies and include parasitic infections or diarrhea and increased requirements for physical growth and mental development in children. In addition, school-aged children tend to develop a more independent eating pattern, which without supervision can lead to an intake of foods of low nutritional value, such as soft drinks and salty snacks in place of micronutrient-rich foods [247].

Micronutrient deficiencies coexist and overlap because of the common etiology and underlying mechanisms. For instance, in developing countries consumption of diets low in animal protein and high in phytate is common and leads to a lower intake and absorption of iron and zinc [248]. Coexisting nutritional deficiencies can reduce the beneficial impact of a single nutrient supplement or fortification in improving nutrition status and morbidity [249, 250]. Therefore, the concern of single micronutrient interventions have been raised and more evidence on the efficacy and/or effectiveness of food-based interventions is required in order to establish policy and program options to deal with coexistence of multiple micronutrients deficiencies [250].

A study in Indonesia showed that the coexistence of iron, vitamin A and zinc deficiency was high in lactating women and their infants. Furthermore, the authors found that infants with VAD
had a 3 times greater risk of zinc deficiency than those without VAD [251]. A high prevalence of vitamin B2 (89%) and folic acid (25%) deficiencies but a low prevalence of vitamin A and C deficiency was found in anemic adolescent girls in Bangladesh [252]. Another study in adolescent girls conducted in the Sudan found that 14% had ≥ 2 micronutrient deficiencies. A positive correlation between low Hb and zinc deficiency and low Hb and copper deficiency has been reported, suggesting potential metabolic interactions possibly derived in part from a shared deficit in the food sources [253]. Jiang et al reported that multiple micronutrient deficiencies were common among pregnant women during the first trimester in Nepal with more than 80% of women having ≥2 micronutrient deficiencies with zinc deficiency being the most commonly found in this population (60%) [57]. A study conducted among school children in NE Thailand [6] reported that 60% of children were at risk of ≥ 2 coexisting micronutrient deficiencies with the highest frequency being iodine and zinc deficiency. A recent study in India demonstrated that multiple micronutrient deficiencies are widespread among young, rural and tribal women of reproductive age. The prevalence of two, three, or more micronutrient deficiencies in this population was 40%, 40%, and 10%, respectively, with the concurrent prevalence of zinc deficiency and anemia being the highest (34%) [254].

In order to combat coexisting micronutrient deficiencies, multiple micronutrient fortification of staple foods and condiments has been developed as well as the various forms of micronutrient administration for infants and young children. These include sprinkles which are used for in home fortification, and foodlets and microcapsules which are usually consumed without food and used instead of a medical tablet. However, there are potential interactions between micronutrients within the formulations for both fortification and supplementation and this remains a technological challenge [250]. There are also interactions between the deficiencies at the physiological level and these are described below.
4.2 Physiological consequences of micronutrient (iron, zinc and vitamin A) interactions

4.2.1 Iron and vitamin A

The relationship between VAD and anemia has been recognized when pooled data from surveys in 8 developing countries (Vietnam, Chile, Brazil, Uruguay, Ecuador, Venezuela, Guatemala and Ethiopia) demonstrated a positive correlation between SR and Hb concentrations \( r = 0.77, p < 0.0001 \) [255]. In a cross-sectional survey of Chinese children, a significant correlation between SR and Hb \( r = 0.22, p < 0.01 \) but not between SR and SF was found, and VAD was associated with an increased prevalence of anemia [256]. These associations are in agreement with other studies which were conducted in children in developing countries [257, 258]. There are a number of reports indicating the positive effects of vitamin A supplementation/fortification on improving iron status in anemic people by increasing Hb or reducing anemia, although not all studies agree [259].

Several mechanisms on how VAD could have an influence on anemia have been suggested and will be discussed in detail below: these include 1) decreased resistance to infection in individuals with VAD which can increase the anemia caused by infection; 2) effects on iron absorption and/or metabolism; and 3) modulation of erythropoesis [259].

**Vitamin A and the anemia of infection/inflammation**

The anemia of infection means the anemia observed in individuals in areas with chronic infection, and is considered as a syndrome within the broader category of the “anemia of chronic disease” [260]. Since vitamin A has an important role in immune function [66] VAD results in increased morbidity and mortality from some infectious diseases such as diarrhea, measles, tuberculosis and malaria [261] which may be related to the anemia of infection.
However, to date there is little evidence that supports this hypothesis [259]. Inflammatory cytokines have been involved in the anemia of infection, as they appear to interfere with erythropoiesis by impaired erythropoietin (EPO) production, impaired ability of the erythroid progenitors to response to EPO, and impaired mobilization of reticuloendothelial system iron stores. The other biological mechanism which could be involved in anemia of infection is the shortened red cell survival [260].

More recently, hepcidin has been shown to be the key regulator of iron metabolism and a mediator of anemia of inflammation [262] since an overproduction of hepcidin is found during infection and inflammation [263]. Hepcidin negatively regulates iron uptake by the small intestine, as well as iron release from macrophages [264] thus restricting iron entry into the plasma and iron supply for erythropoiesis. In patients with anemia of inflammation, hepcidin production is increased up to 100 fold [265].

Effect of VAD on iron absorption and/or metabolism

Vitamin A might play a role in enhancing iron absorption by its physicochemical property. A series of studies done by a group of researchers in Venezuela revealed that the addition of vitamin A, beta-carotene [266] and non-provitamin A carotenoids (lycopene, lutein, and zeaxanthin) [267] can improve the absorption of iron from meals with high phytate and polyphenol content. These studies suggest that vitamin A, beta-carotene and non-provitamin A carotenoids potentially diminishes the inhibitory effect of non-heme iron absorption inhibitors by forming complexes with iron which are soluble in the intestinal lumen. However, these findings were not confirmed by studies conducted in Europe and these conflicting results may be related to a possible lower overall nutritional status in the Venezuelan subjects who were from a lower socioeconomic population, compared to the subjects from Switzerland and Sweden.
Davidsson and coworkers [269] investigated the influence of retinyl palmitate (3.5 µmol) added to a test meal consisting of a Fe-fortified maize porridge on iron absorption in children with VAD, before and after a single dose vitamin A supplementation (210 µmol retinyl palmitate). Added vitamin A to test meals significantly decreased iron absorption (before giving a single dose of vitamin A), but this influence was not detected after 3 week of provision of high dose vitamin A. This was most probably because the children in this study were also iron deficient, which might have influenced vitamin A metabolism.

According to several animal studies, VAD increases iron concentration in the liver [270], spleen [271] and femur [272]. These results further suggest the accumulation of iron in those specific organs is due to impaired iron mobilization from the ferritin stores to the bone marrow for erythropoiesis. In line with the animal studies, a study in school children, who were given 200,000 IU of vitamin A as a supplement, resulted in an increase of mean Hb and reduced prevalence of anemia [257]. Moreover, EPO, a stimulant of erythropoiesis also increased. These results suggest that during vitamin A repletion, an increase in EPO circulation may mediate improvements in Hb. Another possible mechanism is that VAD decreases transferrin synthesis and thus reduces iron transport to the bone marrow [273]. As of to date, the details of the iron and vitamin A metabolic interaction are complex and remain unresolved. Several factors including subject characteristics and analytical methods could explain the contradictory reports between the studies [274].

**Vitamin A and erythropoiesis**

Vitamin A may play a role in erythropoiesis via retinoic acid, which is a key signaling molecule, and is possibly directly involved in the late stage of the red cell formation process [275, 276]. In addition, vitamin A can enhance EPO production. The 3’- enhancer region of the EPO gene
contains a sequence homologous to DR-2, a steroid-responsive element that appears to be regulated by retinoic acid [277, 278].

On the other hand, there is evidence from both animal [279-281] and human [282, 283] studies indicating that ID seems to alter vitamin A metabolism by a reduction in SR and an increase in hepatic retinol and retinyl ester. The mechanism may involve an increase in retinol sequestration in the liver [284] and/or impairment in the activity of one or more retinyl ester hydrolases [279].

4.2.2 Zinc and iron

The coexistence of zinc and iron deficiencies in humans has been known since the discovery of human zinc deficiency in Iran and Egypt in late 1950 [194]. Several surveys have found a correlation between iron and zinc status in low-income African, American and Hispanic children in the US where anemic children had a significantly lower zinc status than non-anemic children [285]. These results are consistent with those of a study in African pregnant women who consumed a diet low in animal protein [286]. A high association between zinc status (SZn and zinc pool size) and iron status (serum iron and SF) was observed in premenopausal non-anemic women with normal iron status [287]. Moreover, the co-occurrence of zinc and iron deficiency was found in studies from Thailand [288], India [289] and Sri Lanka [290].

The underlying causes of the association between zinc and iron status can be explained by: 1) Good sources of both iron and zinc share the same foods like red meat. Moreover, phytate is an important inhibitor of iron and zinc absorption, which is found in plant foods especially whole-grain products such as the staple foods wheat and maize. [286, 287, 291] 2) zinc has an important role in Hb synthesis through the activity of several zinc dependent enzyme systems,
including aminolevulinic acid dehydrase that mediates a step in the synthesis of heme and thymidine kinase and DNA polymerase, which are involved in DNA synthesis [292]. More recently, the zinc-finger transcription factor, GATA-1, has also been confirmed as essential for normal erythropoiesis [293]. Other potential mechanisms may involve the stimulation of hematopoiesis by zinc-induced increases in plasma insulin-like growth factor-1 levels [294] and the role of zinc in stabilizing cell membranes [295].

Results from several intervention trials have raised a concern of an iron and zinc interaction when they are given together as supplements in which one inhibits the absorption of the other [107, 296-298]. It was observed that high iron intake reduced zinc absorption when iron and zinc were given in a water solution [299] which was not observed when iron and zinc were given with a meal. There was no effect of zinc fortification on iron absorption in school children when adding 1.5 mg of zinc as zinc oxide to fortified rice products [300] and wheat dumplings [301]. Davidsson et al. also reported no negative effect of bread fortified with NaFeEDTA on zinc absorption in women [302]. Moreover, this study observed an increase in zinc absorption in a low-bioavailability diet when using NaFeEDTA. However, the beneficial role in improving zinc absorption was not found when cereals fortified with NaFeEDTA were used in an infant study [303]. Previously, it was reported that the interaction between zinc and iron might be due to the competition for the transportation by the divalent metal transporter-1 (DMT1) in the small intestine. However, this hypothesis was dismissed because it was later found that iron and zinc are transported by different transporters [304]. Recently, the results from Caco-2 cells (a model of absorptive enterocytes) confirmed that zinc is a noncompetitive inhibitor of iron uptake and it was suggested that dietary ligands can modulate iron-zinc interactions [305]. It has been proposed that a Fe:Zn molar ratio ≥ 2:1 and a total amount of > 25 mg iron may contribute to
the detrimental effect of iron on zinc absorption [306, 307]. Lönnerdal suggested a ratio of iron to zinc of ~ 1:1, is likely to exclude the possibility of an interaction between iron and zinc [308]. Another possible mechanism was postulated by David in 1980 [309]. He proposed that high levels of zinc may affect iron metabolism by impairing the incorporation of iron into or release from ferritin. Moreover, he suggests that high levels of zinc lead to a faster turnover of iron due to shortened red blood cell life spans [309].

4.2.3 Zinc and vitamin A

Zinc participates in several aspects of vitamin A metabolism including absorption, transportation and utilization most probably through the involvement of zinc in protein synthesis and cellular enzyme functions. On the other hand, there is also evidence that vitamin A affects zinc metabolism. Therefore, deficiency of one or both may alter the metabolism of the other [310].

In both cross-sectional and intervention studies, the effect of supplementation and the relation between zinc and vitamin A status are inconsistent. However, a positive correlation is often seen in malnourished populations with coexisting vitamin A and zinc deficiency [310]. It has been observed that increased zinc intake may improve vitamin A status when subjects are moderately to severely protein-energy deficient [311]. Results from a study in children in NE Thailand showed that low SR concentration (< 1.05 µmol/L) (odds ratio of 1.65; \( p = 0.028 \)) was a predictor of low SZn [6]. However this was in contrast to a survey in Cambodian children, which reported a high prevalence of coexisting micronutrient deficiencies, but no correlation between SR and SZn concentration [312]. A synergistic effect of combined zinc and vitamin A supplementation was detected in children living in urban slums in Bangladesh. Among children with VAD, the prevalence of VAD was significantly lower in the combined zinc and
vitamin A supplementation group (13.3%) when compared to the placebo group (47%; \( p = 0.05 \)) and compared to the group given vitamin A alone (37.5%) or zinc alone (40.6%) alone [313]. Moreover, plasma vitamin A concentrations were significantly increased proportionally to the zinc dose levels given to middle-aged and older European people [314].

The mechanism which may explain a potential dependency of vitamin A on zinc is that zinc deficiency can lower the hepatic synthesis of RBP which leads to a decrease in RBP in plasma, which is required for mobilization of retinol from the liver [315, 316]. Moreover, zinc may play a specific role in the conversion of \( \beta \)-carotene to retinol via the enzyme 15-15 dioxygenase [317]. Conversely, severe VAD may manipulate zinc metabolism by reducing absorption and lymphatic transport of zinc by altering the synthesis of zinc dependent binding proteins [310].

![Functions of zinc in the retina and retinal pigment epithelium](image)

**Figure 6** Functions of zinc in the retina and retinal pigment epithelium [318]
Zinc deficiency is related to ocular function since it is present in a relatively high amount in the eye especially in the retina, retinal pigment epithelium and choroid. Zinc deficiency may cause altered vision, electroretinograms, and oscillatory potentials. In severe cases, ultrastructure changes have been detected in the retina and retinal pigment epithelium. The known functions of zinc in the retina and retinal pigment epithelium include modulation of synaptic transmission, regulation of the light-rhodopsin reaction, interaction with taurine and vitamin A in the photoreceptor, modifier of photoreceptor plasma membranes and antioxidants [318].

The interaction between zinc and vitamin A has also been observed in patients suffering from various pathological conditions that severely compromise hepatic function, such as alcoholic cirrhosis, cystic fibrosis (CF) and idiopathic haemochromatosis [310]. Recently, it has been reported that giving zinc therapy alone to CF patients with VAD can improve the symptom of night blindness [319].
5. Rice fortification

5.1 Rice production and consumption

Rice is one of the world’s most important cereals for human consumption and is particularly important for food security, as it is mainly produced in the developing countries especially in low income food deficit countries (LIFDs). In the world, there are only two major species of cultivated rice: *Oryza sativa*, or Asian rice, and *Oryza glaberrima*, or African rice. The dominant rice varieties grown across the world belong to the *O. sativa* species [320].

Rice production

Rice can be cultivated in a wide range of locations and climates but geographically, Asia is the biggest rice producer with 90% of the world rice’s production, of which China and India together have a share of about 50% of the global production followed by Indonesia, Bangladesh, Vietnam and Thailand respectively [320]. Among these countries, Thailand and Vietnam are the two leading rice exporters. The global rice production in 2010 was 466 million tons (milled rice) which corresponds to an increase of 2.3% from 2009 [321]. Data from 1980-2005 show a trend of increased rice production which is mostly due to an increase in productivity rather than in area [320].

Rice consumption

About 90% of the rice production is used for human consumption, while the remainder is used for other purposes [322]. Forecasts for 2011 estimate a global rice utilization, including food, feed and other uses, of around 460 million tons, 1.6 percent, or 7 million tons more than the current estimate for 2010 [323]. Rice is the staple food in East, Southeast, and South Asia, where 90% of the world’s rice crop is produced and consumed [324] with an average of 56.9 kg per capita consumption [323]. Since the mid 1990s, global rice consumption as food has risen at an annual rate of 1.1% [322]. Rice is the single most important food item in terms of
providing the calorie intake. On average more than 20% of the human dietary energy intake comes from rice, although this value can approach 70% in Bangladesh, Cambodia and Myanmar, and is higher than 20% in 34 countries. They are mostly in Asia, but also in Africa (e.g. Madagascar, Sierra Leone and Guinea Bissau) and LIFDs (e.g. Guyana, Suriname and Cuba) [320].

5.2 Nutritional problems in rice eating populations

The nutritional situation in rice-consuming countries depends on many factors such as socioeconomic, developmental, cultural, environmental and dietary factors. Among the major nutritional problems prevalent in rice-consuming countries, inadequate and unbalanced dietary intake is the most important one [325]. Rice is the major source of energy and protein in this group of people: 100 grams of raw white rice provide 361 kcal and 6 grams of protein. Brown rice is a good source of B vitamins such as thiamin, riboflavin and niacin but contains little or no calcium, iron, zinc, vitamin C, vitamin D and vitamin A. The amino acid profile of rice is high in glutamic and aspartic acids but low in lysine. The nutrients composition of rice grains however, varies depending on the rice variety and degree of polishing; and using the highest degree of polishing provides the lowest level of proteins, vitamins and minerals in the final product [320, 322]. Daily energy intakes from rice in Asia (783 kcal/capita/day), especially in South (715 kcal/capita/day) and Southeast Asia (1270 kcal/capita/day), are much higher than in other continents of the world. These contribute 30% and 49% to total energy intake per day for people who live in South and Southeast Asia, respectively [326]. The contribution of protein from rice to total protein requirements was 69% in South Asia and 51% in Southeast Asia, based on FAO Food balance sheets for 1979-81. These percentages are higher than the contribution of any other cereal protein in any region of the world. However rice contains lower
protein levels than other cereals and of importance is the incompleteness of essential amino acids important for child growth [322, 325]. Therefore, prevalence of children under 5 who are suffering from stunting and protein energy malnutrition is generally higher in the major rice-consuming countries. This suggests the possibility of protein calorie malnutrition in association with heavy rice consumption [322, 325]. People in rice eating countries however are also at risk of micronutrient deficiencies as micronutrients which are present in the whole grain such as B vitamins, iron, zinc and calcium are removed during milling [320, 327]. For example, up to 80% of vitamin B1 is removed during the process of milling and the disease (beriberi) is found predominantly in those regions where people rely on diets containing large amounts of polished rice. Beside beriberi, other potential micronutrient deficiencies found in rice eating populations, are IDA, pellagra and ariboflavinosis which are caused by low intake of iron, niacin and vitamin B2 (riboflavin), respectively [328]. Additionally, deficiencies of iodine, zinc, calcium and vitamin A are common in this area because rice contains only small amounts of these micronutrients especially when iodine and zinc content in the soil is low [325]. For example, in rural Bangladesh, where rice is the predominant diet with a low consumption of animal-food sources, zinc deficiency is a potential health problem based on the high prevalence of low zinc intake in children (22%) and women (73%) [329]. A recent study in Indonesia shows that, women of childbearing age belonging to families consuming a lot of rice which contains no vitamin A and little of preformed vitamin A from animal and dairy products as well as low levels of provitamin A carotenoids from plant-based foods are at risk of VAD [18].

5.3 Methods used for rice fortification

As mentioned previously rice is the main staple food in many parts of the world, but mostly in developing countries where micronutrient deficiencies are most common. Therefore, rice is an
attractive food vehicle for micronutrient fortification. However, rice fortification faces a different challenge, as unlike the other food vehicles such as sugar, wheat or maize, rice is preferably consumed as whole grains and not milled into flour before consumption. In addition, in some countries rice is traditionally washed before cooking and usually cooked in excess water and drained. The small quantities of rice that are milled before consumption are produced by small-scale millers and are not centrally processed, which makes fortification even more difficult. Early rice fortification technologies such as parboiling focused on restoration of micronutrients which are lost during milling and only recently other technologies have been developed for rice fortification [327]. When compared to other food fortification vehicles, rice fortification is relatively expensive due to the use of sophisticated technologies or because special machines are needed to produce artificial fortified kernels or to coat natural kernels [330].

5.3.1 Parboiled rice

Parboiled rice has been known as the method of rice fortification for many years and has been consumed by more than 50% of rice consumers globally. Originally, this method consisted of heating paddy grains in a jar of water before the grains are sun dried and polished. Today, several methods have been developed to improve the original technique. One of the more recent techniques has been called “converted rice”, and in this method the air is removed from the rice before soaking and pressure is used in order to facilitate the transfer of nutrients into the grain. The process results in retaining of vitamins such as thiamine which can be transferred into the endosperm by 50-90%. Moreover it can reduce the damage due to breaking of rice grain and protect from vermin. On the other hand, it also results in lowered consumer acceptance by giving a golden color, an earthy woody flavor and grains with a harder texture [327, 331, 332]. Recently, studies have been reported on the improvement of
the iron [333], zinc [334] and iodine [335] content of parboiled rice by soaking paddy grains in the respective micronutrient solutions. For example, iron (FeSO$_4$) fortification of parboiled rice significantly increased the total iron concentration at a level of 140 mg/kg dry weight (compare to 7 mg/kg of polished normal rice) with a good retention after rinsing and soaking [333].

5.3.2 Coating

This method was developed by R.R. Williams and coworkers [327]. After that in 1946, Hoffmann-La Roche used this method in a highly successful study to prevent beriberi in the Bantaan province of the Philippines in 1948-1950 [336]. A mixture of fortificants and other ingredients such as waxes or gums are sprayed on to the surface of polished rice in a rotary cylinder and then dried by hot air. The waxes and gums enable the micronutrients to stick to the rice kernel, thus reducing losses when the grains are washed before cooking. In some case, after drying, talc is applied to prevent the grains from sticking together. The disadvantage of this method is that the rice grains often have a distinctive color, smell and taste that are objectionable to some consumers [327, 337]. The coated grains need to be mixed with normal rice e.g. at a ratio of 1:200 [338]. Recently, flour gel was used as the coating material for iodine and iron fortified rice with excellent retention after washing and cooking. For example washing or cooking of the iodine-enriched rice resulted in about 99% and 94% retention of original iodine content, respectively [339, 340].

5.3.3 Dusting/powder enrichment

The process of dusting or powder enrichment is similar to fortification of flours in which the mixture of fortificants and other ingredients are applied on polished rice. The fortificants stick to the rice grain because of electrostatic forces. However, the added micronutrients can easily be lost by washing and rinsing and therefore this type of fortification is not suitable for developing
countries where rice grains are often washed and rinsed before cooking [337]. The other disadvantages of this method are that micronutrients are less stable because they easily react with other ingredients in the product. However, this method was claimed to be the least expensive when compared with other forms of enrichment [338].

5.3.4 Cold extrusion

Ultra Rice® was developed by Bon Dente and the Program for Appropriate Technology in Health (PATH) using the cold extrusion process. This technology is suggested to hold great promise for alleviating micronutrients deficiencies in rice eating populations [341]. The process uses a pasta-like machine that cuts the dough into rice-shaped grains. The dough consists of a rice flour mix containing fortificants, binding agents, water and antioxidants [342]. Rice flour is usually made from broken rice grains which normally have a low price and are used for animal feed; thus this represents a secondary benefit as a way to increase overall rice yields [327]. As the process uses low temperatures (only the heat generated by low shear forces), the grains are uncooked, opaque, and easier to differentiate from regular rice kernels [330, 342]. To prevent the loss of vitamin A, PATH has developed two separate fortified rice premixes; one fortified with vitamin A, the others fortified with iron, zinc, thiamin and folic acid. The fortified rice grains are blended with normal rice with a ratio that can vary depending on the requirement of the target population in different areas, but usually is around 1:100 [148]. Several studies have proven good shelf life and micronutrient stability during storage and cooking under various storage conditions [341, 343, 344].

5.3.5 Hot extrusion

Unlike cold extrusion, hot extrusion uses relatively high temperatures (70-110°C) [337]. Rice flour is mixed with fortificant(s), water, binding agents and stabilizers then passed through a
single/twin screw extruder in which the paste is subjected to high pressure, shear and heat during the process and then cut into grain-like kernels at the end of the extruder by passing through a special die and knife. The high temperatures result in partially or fully cooked fortified rice grains that have similar appearance (sheen, transparency, consistency and flavor) as natural rice kernels. This method is suitable for population groups who prefer homogeneous grains in form, size, consistency, flavor and color [337]. Kapanidis and Lee [345] developed a hot extrusion process for iron fortified rice. Artificial rice grains were fortified with ferrous sulfate under acid conditions in order to diminish/eliminate unacceptable color and resulted in good sensory and color acceptance when mixed with jasmine rice at a ratio of 1:100 and 1:200. However, extended storage tests showed discoloration. In a further trial, iron fortified rice grains were produced by using micronized ground ferric pyrophosphate (MGFP) [346]. These grains had comparable sensory properties to normal rice and showed low losses during rinsing. Hot extrusion is best suited for the mass large scale production since the cost of production is relatively higher than the other methods [337].

5.4 Nutritional studies with fortified rice

During 1948-50, one of the most successful rice fortification studies was conducted in the Bantaan province of the Philippines demonstrating the value of rice fortification. Previously, the intake of thiamin in this area was very low (0.7 mg/day) resulting in the high incidence of beriberi, which affected about 13% of the population and caused 164 deaths among 98,000 people over one year. After consuming coated rice fortified with thiamin, nicotinamide and iron for 3 months, there was a decrease in mortality. After 21 months of the intervention there were no deaths from beriberi. As a result of this study, fortification of rice became an accepted practice in many countries but was legally enforced in only a few [327, 328].
ID is a major public health problem and several studies have been performed on iron fortified rice. Iron (using MGFP as iron fortificant) fortified rice produced by hot extrusion was fed in an efficacy trial to children during a school lunch meal in India. The rice provided ≈ 20 mg iron/day for 7 months to the group receiving the iron fortified rice. Results showed that there was an increase in body iron stores in both, the fortified and control groups but the prevalence of ID was significantly lower in the intervention group compared to the control group at the end of the study. Despite of an improvement in iron status, hemoglobin (Hb) concentration in the intervention group did not improve. The authors suggested that children might also have coexistence of other micronutrient deficiencies (riboflavin, vitamin A) and infection/inflammation [347]. Recently, a study in 6-24 mo old children has demonstrated comparable results with an improvement in both serum ferritin (SF) and Hb in young children who received either iron fortified rice with MGFP using the Ultra Rice technology or from supplemental iron drops as FeSO₄ with a significantly larger increase in SF and Hb in children who had fortified rice ($p < 0.01$) [348]. In an earlier efficacy trial using Ultra Rice fortified with iron in nonpregnant and nonlactating women in Mexico, the rice was fortified with microencapsulated micronized ferric pyrophosphate with a much smaller particle size of ca. 0.3 µm compared to ~2.5 µm of MGFP. The subjects in the intervention group consumed on average 13 mg iron/day, 5 days per week for a 6 months period. A significant improvement in iron status was found in the intervention group with a significant increase in SF and estimated body iron stores and a significant decrease in transferrin receptor. For Hb, a significant increase was only found when subjects had a baseline Hb < 12.8 g/dL [349].

Rice fortified with iodine (KIO₃) was tested in 10 adult Thai subjects by measuring iodine concentration of pooled 24 h urine sample. Fortified rice (containing 50 µg iodine per 100 g
rice) was given to all subjects on the third day (of a total of 5 study days) while they were given normal rice for the rest of the feeding period. Although, the study was rather short with only 5 days and was focusing more on sensory and stability properties than on the impact of nutritional status, urinary iodine was highest with around 100-300 µg/L in 8 from 10 subjects on study day 3 on which all subjects were fed with iodine fortified rice [339]. A recent efficacy study with Ultra Rice fortified with vitamin A was made in night blind pregnant Nepali women and compared Ultra rice with other modes of delivery, namely; vitamin A supplements at two levels (850 and 2000 µg/day), goat liver, amaranth leaves, or carrots. Except for subjects receiving the higher vitamin A supplementation doses, all subjects received 850 µg retinol equivalents per day for 6 days per week. After 6 weeks of the study, the improvement in dark adaptation by measuring pupillary threshold (PT) was significantly lower in the liver group compared to the vitamin A fortified rice group while there were no other differences by treatment group. SR was significantly greater in the goat liver group than in the fortified rice, amaranth leaves, carrots and low dose vitamin A supplement groups [350]. Since iron and riboflavin affect vitamin A utilization and photoreceptor function, in a subsequent study iron and riboflavin supplements were also given to the same population together with vitamin A fortified rice; with the same amount of vitamin A and for the same period of time as in the former study and compared to the group receiving vitamin A fortified rice only (control group). In the supplement group, women who were iron deficient at the beginning had a significantly greater improvement in PT score than those iron deficient in the control group. The author concluded that ID may limit the efficacy of vitamin A to normalize dark adaptation in pregnant Nepali women [351]
The RDR has been used to assess the bioavailability of vitamin A in Ultra Rice. Eighty-three children aged 11-77 months were selected for the study. Ultra Rice fortified with vitamin A (1500 IU of vitamin A) cooked with sugar and milk was fed to deprived subjects as the challenge dose for the RDR test. There was a positive test (response > 20%) in deficient subjects which indicating that vitamin A in the test meal was adequately absorbed and transported [149].
References


LITERATURE REVIEW


Manuscript 1: Vitamin A stability in triple fortified extruded, artificial rice grains containing iron, zinc and vitamin A.

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Summary

Multi-micronutrient fortified rice could be an effective and sustainable approach to combat micronutrient deficiencies. We produced hot-extruded artificial rice grains fortified with 10 mg iron (as micronized ground ferric pyrophosphate), 5 mg zinc (as oxide, sulfate or carbonate) and 750 µg vitamin A/g (as retinyl palmitate (RP)) and measured RP stability. The rice was designed to be mixed 1:200 with natural rice. Mean RP losses were 5.3% during extrusion, 28.5% during storage and 9.8% during cooking. Storage losses after 18 weeks at 30°C in plastic packages exposed to light were ca 40% with iron and zinc causing no further losses. In aluminum packages (no light), mean RP losses were ca 20%. Iron, but not zinc, increased RP degradation. Zinc sulfate increased the negative effect of iron. The relatively good stability of RP during hot extrusion can be explained by the closed surface and dense nature of the artificial grain protecting RP from oxidation.
Introduction

Vitamin A (VA), iron (Fe) and zinc (Zn) deficiencies are common among children and young women in the developing countries of South and Southeast Asia and these deficiencies often coexist in the same individual (Brown et al., 2002). Due to a low intake of preformed retinol from animal source foods and a low supply of pro-vitamin carotenoids from plant foods, more than 5 million preschool children worldwide and nearly 10 million pregnant women are reported to be affected by night blindness, and 200 million individuals in these population groups have subclinical VA deficiency (WHO, 2009). VA deficiency in young children is a major health concern and due to a weakened immune defense may increase mortality 3-4 fold by contributing to reduced survivability in response to respiratory and diarrheal diseases (Murphy, 1996).

Pre-school aged children, women of child bearing age and pregnant women in poor socioeconomic environments are also at highest risk of Fe deficiency and Fe deficiency anemia (IDA). Iron deficiency (ID) is a major cause of anemia, which affects some 315 million women and children in Southeast Asia (WHO, 2008). IDA has adverse health effects on pregnancy outcome, infant growth, cognitive performance, immune status and work capacity (WHO/UNICEF/UNU, 2001).

Most countries in South and Southeast Asia are additionally expected to be at high risk for Zn deficiency especially when low Zn rice based diets include high phytate legume seeds and few animal source foods. An estimated 30% of the population is considered at risk for inadequate Zn intake (IZiNCG, 2004). Zn deficiency can result in impaired physical growth, decreased immune defense, and poor reproductive function and neural development (IZiNCG, 2004).

The most cost effective, long-term strategy to combat micronutrient deficiencies is food fortification (WHO/FAO, 2006) and, due to its wide consumption by the poorer populations, rice
would be an ideal vehicle for South East Asia. However, as rice is mainly consumed as a grain without milling, micronutrient fortification is technically more difficult and not widespread. Several techniques have been proposed including hot extrusion (Kapanidis et al., 1996) and cold extrusion (Li et al., 2009) processes to manufacture artificial fortified grains. Other techniques include coating (Tulyathan & Prunglumpu, 2009) or dusting (Alavi et al., 2008) the native grains with micronutrient powders. In the extrusion and coating procedures, a rice-premix is first manufactured and subsequently blended with native rice. The dusting technique applies a micronutrients premix directly on to the surface of native rice (Alavi et al., 2008). We have previously fortified rice with Fe using hot extrusion with micronized ground ferric pyrophosphate (MGFP) as the Fe compound (Moretti et al., 2005). When these artificial, extruded Fe fortified rice grains were mixed into native rice and fed to Indian children within a school lunch program, Fe status was significantly improved (Moretti et al., 2006).

The ultimate goal of our program is to produce hot extruded, triple fortified artificial rice grains containing Fe, Zn and VA. Adding VA is technically more challenging due its sensitivity to processing and storage losses especially in the presence of Fe. The many double bonds in the structure of retinol are sensitive to degradation in the presence of oxygen, ultra violet light, heat and low pH (Wirakartakusumah & Hariyadi, 1998) and the degradation is accelerated in the presence of Fe (Manan et al., 1991). Adding VA however would be expected to improve both Fe and VA status. This is because VA is needed for optimum Fe metabolism as it is essential for the efficient production of erythropoietin, which controls red blood cell production, and the mobilization of Fe from existing stores (Semba & Bloem, 2002; Zimmermann et al., 2006). In this study, we report the losses of VA (in the form of retinyl palmitate (RP)) during the hot extrusion of artificial rice grains containing Fe, Zn and VA. We also investigated the influence
of cooking, packaging and storage of the extruded rice grains containing different combination of VA, Fe and Zn, and color changes were quantified instrumentally.

Materials and Methods

Fortification compounds

For the production of the fortified extruded rice grains, the following Fe, Zn and VA compounds were used: micronized ground ferric pyrophosphate (MGFP) with a mean particle size ≈ 2.5 µm; zinc oxide (ZnO) with a mean particle size of <0.05 mm; zinc sulfate (ZnSO₄) with a mean particle size of <0.25 mm; and zinc carbonate (ZnCO₃) with a mean particle size <0.044 mm. All above compounds were provided by Dr. Paul Lohmann GmbH KG (Emmerthal, Germany). The VA compound used was retinyl palmitate (RP) dispersed in a matrix of modified starch, sucrose, butylated hydroxytoluene (BHT) and fractionated coconut oil (Dry vitamin A palmitate 250 S/N, DSM Nutritional Products Ltd., Basel Switzerland)

Production of Fortified Extruded Rice Grains

Two batches of rice grains, each containing one of 8 different combinations of micronutrients, were produced using a hot extrusion process (Table 1). For the production of the fortified extruded rice grains, rice flour (Haefliger AG – melior gourmet, Herzogenbuchsee, Switzerland) was first mixed for 20 minutes in a drum hoop mixer (J.Engelmann AG, Ludwigshafen, Germany) with the micronutrient fortificants (Table 1) and with distilled monoglycerides (DIMODAN HP® 75/B KOSHER, Danisco A/S, Copenhagen, Denmark) which were added as an emulsifier. The premixes consisted of approximately 94.5% rice flour, 0.5% emulsifier, 3.75% MGFP, 1% of Zn as ZnO, ZnSO₄ or ZnCO₃ and 0.25% RP. The desired final fortification levels, set to provide young children with 85%, 70% and 80 % of their daily requirement for Fe, Zn and VA, respectively (WHO/FAO, 2004), were 10 mg Fe, 5 mg Zn and 750 µg VA (retinol)
per gram of fortified extruded rice grains. These levels were based on the recommended nutrient intake (RNI) for school aged children (with pre menarche girls) (WHO/FAO, 2004) of 8.9 - 14.6 mg/day for Fe, 5.6 - 8.6 mg for Zn and 500 - 600 µg for VA, mixing of the fortified extruded rice grains with non-fortified grains at a ratio of 1:200, an estimated rice consumption in Thai school-aged children of 195 g (Sirichakwal & Chittchang, 2005), a relative bioavailability of MGFP of approximately 70% compared to FeSO₄ in rats (Wegmueller et al., 2004), a moderate bioavailability for Zn (30% absorption) (WHO/FAO, 2004), estimated losses of vitamin A of approximately 40% during production, cooking and storage.

The extrusion experiments were conducted with a co-rotating, twin-screw extruder (Model OEVB-D-30.5, Bühler AG, Utzwil, Switzerland) consisting of 5 barrel segments. After a preliminary mixing, the dry premix was transferred into the feeder (K-TronSchweiz AG, Niederlenz, Switzerland) of the extruder and was discharged gravimetrically at a dosing rate of approximately 8 – 9 kg/h into the first barrel segment of the extruder. For all premixes, the extrusion was done at two different temperatures (80°C and 95°C in barrel segments 2, 3, and 4). Water was pumped by a dosing pump (Grundfos, Fällanden, Switzerland) into the second barrel at a rate of approximately 2 L/h. The screw speed was fixed at 150 rpm. A specially manufactured four-oval hole die together with a cutter (Bühler AG, Utzwil, Switzerland) attached at the end of the die, were used to form correctly shaped rice grains. After extrusion, the rice grains were air dried for 2 nights in a dark room prior to storage in a climate chamber. RP losses during extrusion, drying, cooking and storage were determined. The sampling procedure is illustrated in Table 2.
Table 1 Micronutrient combinations used for the production of single, dual and triple fortified extruded rice grains

<table>
<thead>
<tr>
<th>Single fortified</th>
<th>Dual fortified</th>
<th>Triple fortified</th>
</tr>
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<tbody>
<tr>
<td>RP*</td>
<td>RP+MGFP**</td>
<td>RP+MGFP+ZnO</td>
</tr>
<tr>
<td>RP+ZnO</td>
<td>RP+MGFP+ZnSO₄</td>
<td></td>
</tr>
<tr>
<td>RP+ZnSO₄</td>
<td>RP+MGFP+ZnCO₃</td>
<td></td>
</tr>
<tr>
<td>RP+ZnCO₃</td>
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</tbody>
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* RP is retinyl palmitate** MGFP is Micronized ground ferric pyrophosphate

Table 2 Sampling plan and measurements performed

<table>
<thead>
<tr>
<th>Sample detail</th>
<th>Measurements performed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RP**</td>
</tr>
<tr>
<td>1. Dry premix before extrusion</td>
<td>x</td>
</tr>
<tr>
<td>2. Extruded rice grains: right after extrusion</td>
<td>x</td>
</tr>
<tr>
<td>3. Extruded rice grains: after drying (baseline)</td>
<td>x</td>
</tr>
<tr>
<td>Storage test*</td>
<td></td>
</tr>
<tr>
<td>4. Extruded rice grains: 6 wk</td>
<td>x</td>
</tr>
<tr>
<td>5. Extruded rice grains: 12 wk</td>
<td>x</td>
</tr>
<tr>
<td>6. Extruded rice grains: 18 wk</td>
<td>x</td>
</tr>
<tr>
<td>Cooking test*</td>
<td></td>
</tr>
<tr>
<td>7. Extruded rice grains: after drying (baseline) and after 6 wk of storage</td>
<td>x</td>
</tr>
</tbody>
</table>
*Measurements performed in samples stored in both plastic and aluminum foil bags

**RP is retinyl palmitate

**Preparation of cooked rice samples**

For the cooked rice samples, 100 g of extruded rice grains were cooked with 180 g of water in a rice cooker for 15 minutes (Type RK 2422, Severin Elektrogeräte GmbH, Sundern, Germany) and then chopped and mashed with a knife until the rice grains were transformed into a paste.

**Stability testing**

From each premix, two batches of extruded rice grains were randomly produced at different times, both at two different extrusion temperatures (80°C and 95°C) and stored in 1.5 kg portions in transparent polyethylene (PE) bags and light protected aluminum foil (AF) bags in a climate chamber at 30°C and 76% RH for 18 weeks. Artificial light was present throughout the whole storage period. The stability of RP and color during storage were measured at baseline and every 6 weeks up to 18 weeks. Fe and Zn content were determined in the dry premixes as well as in the rice grains at the end of storage.

**Laboratory Analysis**

**Vitamin A as retinyl palmitate**

Approximately 100 g of dry premix, of extruded rice samples both directly after extrusion and after drying, as well as 200 g of each stored sample from both the PE and the AF bags at each time point (baseline, after 6, 12 and 18 weeks) were analyzed for RP concentration. Prior to analysis, the uncooked extruded rice grains were finely milled in a centrifuge mill (Type ZM1, Retch, Haan, Germany) with a 0.25 mm titan sieve.

About 2 g of the sample (cooked or uncooked) was weighed and the starch matrix of the RP was digested by the enzyme Taka-Diastase (Taka-Amylase A, 96.2 U/mg, Fluka, Buchs,
Switzerland). The RP was then extracted by a solvent mixture of 1:1 acetone (Fluka, Buchs, Switzerland) and tetrahydrofurane (Fluka, Buchs, Switzerland). The extract was filtered and RP content measured by reverse phase HPLC (Merck, Zug, Switzerland) with the mobile phase of 100% methanol (HPLC Grade, Fluka, Buchs, Switzerland). RP content of each sample was calculated by comparing the peak area of the internal retinyl acetate standard and that of the sample. The analyses were performed in duplicates and with two injections into the HPLC per replicate.

**Moisture content**
Moisture content of the premix, the milled rice samples and the cooked rice samples was determined by a Halogen Moisture Analyzer Type HR73 (Mettler Toledo, Greifensee, Switzerland).

**Fe and Zn content**
Aliquots (0.5 g) of the premix and of the milled extruded rice samples at 18 weeks of storage were digested with 7 ml 65% HNO$_3$ (Merck, Zug, Switzerland) and 3 ml 30% H$_2$O$_2$ (trace selected Fluka, Buchs, Switzerland) in a microwave digestion system (MLS Ethos Plus, MLS Laboratory System, Leutkirch, Germany) and analyzed by flame atomic absorption spectroscopy (SpectraAA 400, Varian, Mulgrave, Australia). For the determination of Fe, the standard addition technique was used in order to minimize matrix effects. Zn content was measured using the external calibration technique. Each sample was measured in duplicate.

**Color stability**
Color of the fortified rice grains was measured at baseline (after drying for 2 nights), and after storage for 6, 12, and 18 weeks in the climate chamber and the values were compared to
natural jasmine rice imported from Thailand (Migros, Switzerland). Approximately 35 g of rice grains were filled into a round container with a glass bottom for color measurement using the Hunter scale by a Spectral Photometer (Chroma-Meter CR-310, Minolta, Dietikon, Switzerland). The illuminant D_{65} (average daylight, including ultraviolet spectra) and a 0° observer angle with a large reflectance spectrum were used (Wegmüller et al., 2003). The color difference was expressed by a single value of ΔE_{ab}, which contained the absolute value of the color difference but not the direction of the difference. ΔE_{ab} was calculated using the following equation:

\[ ΔE_{ab} = \sqrt{(ΔL)^2 + (Δa)^2 + (Δb)^2} \]

Where ΔL, Δa and Δb describe the difference between the color of the fortified extruded rice grains and the reference color of natural jasmine rice.

**Statistical Analysis**

Color measurements and iron, zinc and RP data were expressed as mean ± SD. RP content in differently fortified rice grains during storage at 0, 6, 12 and 18 weeks were compared using one way-ANOVA, with LSD as the post hoc test (version 18.0; SPSS Inc, Chicago, IL). P values <0.05 were considered significant.

**Results and Discussion**

**Losses of RP during extrusion, cooking and storage**

The mean losses of RP during the different production steps of extruded fortified rice grains (extrusion, drying, cooking) as well as during storage of the differently fortified grains are shown in figure 1. Overall around 40% of the added RP was degraded. Losses were highest during storage (18 weeks at 30°C, 76% RH) with 28.5±16.7% degradation of RP, and lowest
during drying (0.1±4.5%). The negligible losses during drying in our study can be explained by the mild conditions used (room temperature and darkness) while greater losses can be expected using hot air drying (Li et al., 2008a). RP losses were around 5% during extrusion and 10% during cooking.

**Figure 1** Mean losses and standard deviations (SD) of retinyl palmitate in extruded rice grains (single (RP), dual (RP+MGFP or RP+ZnSO₄/ZnO/ZnCO₃) and triple (RP+MGFP+ZnSO₄/ZnO/ZnCO₃) fortified) at different processing steps during hot extrusion and after storage at 30°C and 76% RH.

**Stability of RP during extrusion.** RP displayed a high retention during the hot extrusion process in the presence of Fe and Zn. Only 5.3±3.0% degradation was observed in our studies which is comparable to VA stability in mixed feed products produced by hot extrusion under similar conditions (barrel temperatures 100-130°C, and moisture 12-22%)
Extrusion cooking is a high temperature short time (HTST) processing technology in which degradation of vitamins depends not only on temperature but also on other factors including oxygen, light, moisture, pH, time as well as the technical processing variables (Killeit, 1994). There was no significant difference of RP retention in extruded rice grains when using a barrel temperature of 80°C or 95°C (p>0.05) (results not shown). The relatively low losses of RP during our extrusion process could be due to the relatively mild extrusion conditions employed with a high moisture content (18-20%) and low temperature (80-95°C). Higher extrusion temperatures (≥200°C) and lower moisture contents (<15%) have been shown to adversely impair the nutritional quality of foods (Singh et al., 2007).

Influence of packaging material on RP stability during storage. In general, VA is degraded by two main mechanisms. The first is by photo isomerization, which has been investigated in our study by using different packaging materials and is reported below. The second is by oxidative degradation caused by free-radical attack on the VA molecule (Li et al., 2009). This was investigated in our studies with RP in the presence of Fe and Zn but in the absence of light. Results from these investigations are reported in the subsequent section. Our results from the RP stability studies with rice grains stored in different packages showed that the AF bag; by protecting the grains from light, decreased losses compared to the transparent PE bag (Figure 2). RP retention was 18% higher (p<0.05) after storage for 18 weeks in AF bags as compared to storage in PE bags which resulted in an about 40% degradation. These findings are in agreement with results published by Murphy et al (1988) who investigated the extent of isomerization and degradation of all trans retinyl palmitate fortified skim milk in 3 types of containers (paperboard, polyethylene and glass) under
fluorescent lighting. They found no significant loss of RP in paperboard containers during 3 days of storage, but significant losses of RP occurred in plastic and glass containers even after the relatively short storage period. Photoisomerization of RP from its all trans form to the either 9-cis or 13-cis forms has been found to be the cause of this photo degradation (Kim et al., 2000; Zahar et al., 1987; Murphy et al., 1988). Figures 3 and 4 show the percentage of retention of RP in PE and AF bags respectively when the rice grains were fortified in 4 different ways: (1) RP alone (2) RP+MGFP (3) RP+Zn and (4) RP+MGFP+Zn. Overall, retention of RP in rice grains stored in AF bags was > 70% at 18 wk of storage for all groups (72.4-92.8%, Figure 4) whereas retention dropped when stored in PE bags (55.5-68.4%, Figure 3). In the PE packaging, RP retention was not significantly different with and without the addition of iron and zinc compounds at all time points, while in the AF bags, after 18 weeks of storage, iron containing rice grains showed significantly higher losses. These studies showed that light is a major risk factor in the degradation of RP but in the absence of light, iron will increase the degradation. Zn on the contrary does not appear to increase RP degradation.
Figure 2 Mean±SD retention of retinyl palmitate in extruded rice grains (single (RP), dual (RP+MGFP or RP+ZnSO₄/ZnO/ZnCO₃) and triple(RP+MGFP+ZnSO₄/ZnO/ZnCO₃) fortified) during storage in transparent polyethylene (PE) and aluminum foil bags (AF).

Figure 3 Retention of RP (%) in different groups of fortified extruded rice grains using transparent plastic bags during storage.
Figure 4 Retention of RP (%) in different groups of fortified extruded rice grains using aluminum foil bags during storage

Factors influencing RP retention in the absence of light. Retention of RP in differently fortified rice grains stored in AF packaging during 6, 12 and 18 weeks was compared to baseline (grains after drying) values (Table 3).

Stability of RP in the absence of Fe and Zn. In the fortified rice grains containing RP alone, RP was very stable and only 7% of the RP was destroyed during storage for 18 weeks at 30°C in the AF packaging (Table 3). When comparing our results with artificially fortified rice grains produced by cold extrusion using the Ultra Rice® technology of the Program for Appropriate Technology in Health (PATH) (PATH, 2005) losses of RP were in general greater after cold extrusion and storage than the losses we found in the present study. For example, a 25% loss of RP was reported when Ultra Rice® was stored up to 180 days protected from direct light at ca. 26°C (aw ca. 0.7) (Flores et al., 1994) compared to 7% loss after storage for 18 weeks (126 days) at 30°C and 76% RH in our study. Only when Ultra Rice® samples were stored at 0°C
and under N\textsubscript{2} for 24 weeks, the RP losses were low and comparable with our study (Lee et al., 2000). The latter study reported that the stability of RP was more affected by storage temperature than by relative humidity.

Several factors could explain the higher retention of RP in our study when the hot extruded rice grains were stored in AF bag as compared to those studies with the cold extruded Ultra Rice®. These include the type of VA compound used, the addition of antioxidants and the processing technology. Several types of VA have been used in the published studies. These include all trans retinyl palmitate beads ® 500 (Lee et al., 2000), retinyl palmitate 250 SD (RP 250 SD) (Murphy et al., 1992) and all – trans retinyl palmitate type VI (Flores et al., 1994). These VA compounds are either coated with different materials or in the oily form, as trans retinyl palmitate type VI, and contain other ingredients, which are added to different food products for specific purposes. All of the above compounds contain either or both BHT and BHA as an antioxidant. PATH (2005) reported that using RP in the oily form containing minimum quantities of BHT and BHA resulted in high VA losses.

In 1996, RP 250 SD was claimed as the most stable form of VA for fortified rice grains. In this product, RP is dispersed in acacia with lactose as a coating material and BHT and BHA are added as antioxidants (Murphy, 1996). The authors reported the estimated half-life of RP in fortified rice grains with RP 250 SD at 25°C, aw = 0.75 was improved from only 20 days when BHT and BHA were added to the rice flour before extrusion to more than 1 year when tocopherol, ascorbic acid and saturated fat were added to the rice flour (Murphy et al., 1992).

In contrast to this, the hot extrusion process used in our study without the addition of antioxidants still showed excellent RP stability with 97% retention after storage for 18 weeks at 30°C and 76% RH. The better retention may also be due to the retinyl palmitate 250 S/N containing modified starch and sucrose as a coating material instead of acacia and lactose.
Johnson et al (1988) have reported that sucrose is the simplest and most often used oxygen barrier compound when vitamins are sprayed onto fortified breakfast cereals. Dry retinyl palmitate 250 S/N is a new product specifically developed for the enrichment of staple foods, especially flour (DSM, 2009).

In addition to antioxidants, the amount and type of fat could also influence RP stability. Using saturated fats and antioxidants in the formulations for extruded grains greatly improved the stability of VA in Ultra Rice® (Li et al., 2009; Murphy et al., 1992). Our extruded rice grains contained only 0.5% fat in the form of distilled monoglycerides, while Ultra Rice® contains 2-3% of shortening as a necessary lubricant for extrusion which could lead to lipid oxidation (Li et al., 2008a). The most recent study on stability of RP investigated the effect of different antioxidant systems on RP stability in Ultra Rice®. The greatest stability was obtained either using the combination of BHA and BHT or using tertbutylhydroquinone (TBHQ) together with ascorbic acid, citric acid and sodium tripolyphosphate (STPP) which resulted in retentions of >85% and 70% at 25°C and 45°C (Li et al., 2009).

The hot extrusion process in our study used temperatures of 80 and 95°C compared to the Ultra Rice® process using the cold extrusion technology (no heat applied in the process). A higher RP retention with hot extrusion has been explained by the formation of amylose-lipid complexes (Tran et al., 2008; Singh et al., 2007; Bjorck & ASP, 1983) retarding lipid oxidation due to the lipids trapped in a dense carbohydrate matrix (Kim et al., 2000). Scanning electronic micrograph (SEM) pictures from the fortified extruded rice grains produced by hot extrusion in our laboratory showed a regular, smooth and close structure. Such a structure may hinder oxygen diffusion and thereby prevent RP degradation.
Table 3  Retention of RP (%) compared to baseline (mean±SD) in differently fortified rice grains stored in aluminum foil bags for 6, 12 and 18 weeks at 30°C and 76% RH

<table>
<thead>
<tr>
<th>Premix</th>
<th>6 week</th>
<th>12 week</th>
<th>18 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>cd 95.6±10.7</td>
<td>d 99.0±13.5</td>
<td>d 92.8±10.1</td>
</tr>
<tr>
<td>RP+MGFP</td>
<td>cd 94.6±4</td>
<td>bc 78.5±10.3</td>
<td>b 72.4±9.5</td>
</tr>
<tr>
<td>RP+ZnO</td>
<td>d 102.8±9.2</td>
<td>bcd 87.3±18.4</td>
<td>cd 89.0±15.4</td>
</tr>
<tr>
<td>RP+ZnSO₄</td>
<td>bcd 93.5±9</td>
<td>bcd 84.4±15.4</td>
<td>cd 88.9±2.4</td>
</tr>
<tr>
<td>RP+ZnCO₃</td>
<td>bc 88.3±4.6</td>
<td>cd 95.3±7.2</td>
<td>bcd 82.2±6</td>
</tr>
<tr>
<td>RP+MGFP+ZnO</td>
<td>bcd 90.3±12.9</td>
<td>ab 75.1±3.9</td>
<td>cd 86.5±8.4</td>
</tr>
<tr>
<td>RP+MGFP+ZnSO₄</td>
<td>a 75.0±12.2</td>
<td>a 59.8±15.9</td>
<td>a 53.8±9.9</td>
</tr>
<tr>
<td>RP+MGFP+ZnCO₃</td>
<td>ab 80.6±5.1</td>
<td>bcd 81.7±7</td>
<td>bc 77.9±6.3</td>
</tr>
</tbody>
</table>

*Values in the same column that are followed by the same letter are not significantly different (p > 0.05)

** RP is retinyl palmitate

***MGFP is micronized ground ferric pyrophosphate

Stability of RP in the presence of Fe and Zn. In AF packaging, only the addition of Fe decreased RP stability. RP retention after 18 weeks storage in the absence of Fe and Zn was 92.8% and this was not significantly decreased (p>0.05) in the dual fortified samples with Zn (ZnO, ZnCO₃ and ZnSO₄) and in the triple fortified sample with ZnO and MGFP. However,
after 18 weeks storage, the RP retention in the grains containing RP+MGFP+ZnSO$_4$ and in those containing RP+MGFP was significantly lower (p<0.005) at 53.8 and 72.4% respectively (Table 3). Manan et al. (1991) studied the influence of mineral fortification on the stability of all-trans retinol in a microcrystalline cellulose model system stored at an a$_w$ of 0.42 and a temperature of 30°C. The study demonstrated that Fe (FeSO$_4$) and copper (CuSO$_4$) supplements result in a decrease in the retinol half-life of 50% and 25%, respectively, when compared to the system containing retinol alone, while Zn (ZnO) and calcium (CaCO$_3$) had smaller effects. The investigators suggested that these results could be due to the greater ionic mobility of Fe and copper in addition to lower solubility characteristic of the Zn (ZnO) and calcium (CaCO$_3$) compounds, which had been used. Our findings with respect to the good RP stability in the rice grains dual fortified with RP+Zn (ZnSO$_4$, ZnO, ZnCO$_3$) as well as the significant degradation with MGFP are in line with the results of Manan et al. (1991).

In order to protect the VA from losses due to the interaction with other micronutrients in the Ultra Rice®, PATH has developed two separate fortified rice premixes. One premix is fortified with VA alone, and the other premix is fortified with Fe, Zn, thiamin and folic acid (PATH, 2005). In our study, we have however found good stability of RP in the presence of Fe when using poorly soluble Zn compounds such as ZnO and ZnCO$_3$. In triple fortified rice grains with ZnO, RP retention was comparable to retention with RP alone after 18 weeks of storage. Using ZnSO$_4$, a soluble Zn compound, however significantly decreased the RP stability. ZnSO$_4$ has a pH around 4-5 and the decreased RP stability might be explained by the sensitivity of VA to acid conditions (Wirakartakusumah & Hariyadi, 1998) and the acidity of ZnSO$_4$ together with the catalytic effect of Fe (MGFP) may thus have promoted the degradation of RP in the grains triple fortified with RP+MGFP+ZnSO$_4$, while the higher retention of RP in triple fortified rice grains using ZnO or ZnCO$_3$ as Zn fortificant may be due to their lower acidity characteristics. In
addition, these two compounds may reduce the catalytic effect of Fe (MGFP). Our results are similar to the findings of Kim et al (2000). They investigated the loss of RP and isomers formed in corn flakes fortified with RP alone or with a multiple vitamin mixture (vitamins A, B₁, B₆, B₁₂, C and D) stored at ambient (23°C) and elevated temperature (45°C). They found that the presence of other vitamins retarded the loss of RP and suggested 2 possible mechanisms. The first was the antioxidant activity of vitamin C and the second was by a dilution effect from other micronutrients, which would decrease the contact of RP with free radicals. This second mechanism may be the reason for good RP stability in our triple fortified rice grains with ZnO and ZnCO₃. However, the losses in either double fortified grains containing Fe would be expected to be higher if we had used a high solubility iron compound such as FeSO₄ instead of MGFP (Manan et al., 1991).

**Stability of RP during cooking.** Mean losses during cooking were 9.8±6.3%, which is also low compared to other studies, most of which have reported losses of more than 20% (Murphy et al., 1992; Flores et al., 1994; Lee et al., 2000). However, in these published studies, the extruded fortified rice grains were produced by the Ultra Rice® technology using cold extrusion. Lee et al (2000) compared three different cooking methods (rice cooker, boiling with little of water and boiling with excess water) without showing any difference (p>0.05) in RP losses, which were 13-25%. By adding a combination of BHT and butylate hydroxyanisole (BHA) as antioxidants, these losses could be reduced to 4% (Murphy et al., 1992).

**Stability of Fe and Zn**

The concentration of Fe and Zn were stable in all samples over the course of processing and storage for 18 weeks. Since Fe and Zn belong to the minerals which cannot decompose or be synthesized by ordinary chemical reactions (Singh et al., 2007) this is expected and has previously been reported (Li et al., 2008b).
Color stability

Color differences of the fortified extruded rice grains presented as $\Delta E$ values compared to jasmine rice are shown in Figure 5. The lowest $\Delta E$ values were found in the grains dual fortified with RP and Zn with the lowest $\Delta E$ values of $\approx 2-4$ for grains containing RP+ZnO and RP+ZnSO$_4$. The most probable reason is the whiteness of the Zn compounds, which decreased the $\Delta E$ value compared to the grains fortified with RP alone which has a yellowish color. The addition of the yellowish colored MGFP increased the $\Delta E$ values in all the grains. The highest $\Delta E$ value and the biggest difference when compared to natural jasmine rice were found in the grains containing RP+MGFP+ZnCO$_3$ ($\Delta E=14$). The addition of ZnSO$_4$ improved color stability ($\Delta E = 9$) when compared to grains containing RP+MGFP ($\Delta E = 12$) and would therefore be the Zn compound of choice with respect to color. In a previous study (Moretti et al., 2005) much lower $\Delta E$ values of $\approx 4-5$ and 5-6 were reported when comparing rice grains fortified with MGFP alone to jasmine rice and Indian basmati rice, respectively. Possible reasons for higher color difference in our study may be the use of higher extrusion temperatures with the twin screw extruder (80°C and 95°C) compared to 70°C with the single screw extruder in the study of Moretti et al (2005) and the addition of the yellowish colored RP compound in our study which was not added by Moretti.
Figure 5 The ΔE values of extruded fortified rice grains containing different fortification compounds compared to natural jasmine rice during storage

Conclusions

Our results demonstrate that it is possible to produce extruded rice grains triple fortified with vitamin A (RP), iron (MGFP) and zinc (ZnSO₄, ZnO, ZnCO₃) with good RP stability. The hot extrusion technology has the advantage of producing grains with a closed surface and a dense carbohydrate matrix, which protects losses of RP through oxidation. Mean RP losses were 5% during extrusion, 28.5% during storage for 18 weeks at 30°C and 9.8% during cooking. RP storage losses increased up to 40% in plastic packaging but could be decreased by half with aluminum packages. Our findings show that light has a stronger effect on RP stability than addition of iron or zinc, which had no influence on RP stability in plastic packaging. In the absence of light, Zn compounds alone also had no influence on RP stability, but iron added as
MGFP decreased RP stability when added alone or when added with zinc sulphate or zinc carbonate. Zinc sulphate appeared to further increase RP losses caused by iron. All Zn compounds greatly decreased the yellowing of the grains caused by the addition of RP alone. Based on RP stability, color and cost we propose ZnO as the most suitable Zn compound to add with MGFP and RP in triple fortified, hot extruded artificial rice grains. These studies have provided evidence to minimize RP losses during storage of the triple fortified grains containing Fe, Zn and RP, nevertheless total losses during extrusion, storage and cooking can approach 40% and must be taken into account when formulating the composition of the rice grains to meet the requirements of the target population.
References


**Acknowledgements**

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Manuscript 2: Triple fortified rice grains containing vitamin A overcome vitamin A deficiency and increase vitamin A liver stores in school-aged Thai children

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Abstract

Vitamin A (VA) fortified rice is a potential intervention strategy to prevent VA deficiency in at-risk populations. Triple fortified artificial rice grains with added VA, zinc and iron were produced by hot extrusion technology and their ability to improve VA status was tested in Thai school children. A paired stable isotope dilution technique with labeled $^{13}$C retinyl acetate was used to quantify VA pool size at the beginning and end of the feeding period. Fifty healthy school children (SR > 0.7 µmol/L) were randomized into 2 groups to receive either triple fortified rice (n=25) or natural rice (n=25) for 2 months as part of the daily school meal. The fortified grains, mixed 1:50 with regular rice, were estimated to provide an extra 890 µg VA/d, 5d/week. Labeled $^{13}$C retinyl acetate was administered orally to each child before and at the end of the feeding period to estimate total body reserve of vitamin A (TBR of VA) which increased significantly (p<0.05) in the intervention group from 0.105 µmol retinol at baseline to 0.184 µmol retinol after 2 months feeding. There was no change in the TBR of VA in the control group (0.084 vs 0.087 µmol retinol, p= 0.22). Serum retinol (SR) remained unchanged in both groups. We conclude that VA fortified, hot extruded rice is an efficacious vehicle to provide additional VA to at-risk populations, and that the efficacy of VA fortified foods can be usefully monitored by the $^{13}$C stable isotope measurement of TBR of VA but not by changes in SR.
Introduction

Vitamin A deficiency (VAD) is an important cause of blindness in children and contributes to mortality and morbidity from infection, especially in children and pregnant women (1). The latest report of WHO estimated that around 190 million preschool aged children and 19.1 million pregnant women worldwide suffer from VAD based on biochemical measurements (1). Although clinical signs of VAD are globally less common, marginal deficiency is still considered to be highly prevalent and difficult to diagnose (2). Although vitamin A (VA) status can be assessed using a variety of biological, functional, histological and biochemical methods (2, 3), few are suitable to monitor changes in status following interventions. Serum retinol concentration (SR) has been most widely used to monitor VA status. This measurement however is a weak biomarker because of its homeostatic control over a wide range of body VA stores, resulting in changes only when the stores are either very high or very low (3, 4). Additionally, infection and inflammation significantly influence SR concentrations because retinol binding protein (RBP), its major transporter in the blood, is a negative acute phase protein, and both SR and RBP decline with infection (5).

A more robust method to measure vitamin A status and to monitor the efficacy of vitamin A interventions is the isotope dilution technique. This method, which uses VA labeled with a stable isotope ($^2$H or $^{13}$C) to quantitatively estimate total VA body stores, has generated estimates in close agreement with the direct measurement of liver VA reserves (6, 7). Moreover, this method can be used to assess a wide range of VA status from deficiency to toxic status (4). The principle of isotope dilution is used to estimate the total body VA pool or total body reserve (TBR) of VA (8). The stable isotope dilution technique with deuterated retinyl acetate has been successfully used to demonstrate an increase in the VA pool size in
response to both supplementation and fortification with VA (9, 10) or with provitamin A carotenoids (11, 12). More recently an isotope dilution method using $^{13}$C retinyl acetate has been developed with the advantage of requiring lower doses of the tracer, but with a more demanding sample preparation procedure (2, 13). The $^{13}$C isotope dilution technique has so far only been used in rats (14) which demonstrated a marked increase in TBR of VA with a 2 to 10 fold increase in VA intake, where SR concentrations remained unchanged, and in Rhesus monkeys with hypervitaminosis A (15).

The main etiology for VAD is a low VA intake, either as preformed retinol from dairy products and eggs, or as pro vitamin A carotenoids from fruits and vegetables. The population groups potentially most at risk are children under 6 years of age as well as women of reproductive age during pregnancy and lactation (1). The former group has high requirements of VA to support their rapid growth together with the transition from breastfeeding to the dependence on other dietary food sources (16) while the higher requirement in pregnant and lactating women is due to their increased demand for the transfer to the fetus and breast milk production (1).

The two main strategies to increase VA intake and combat and control VAD are periodic high dose supplementation (17) and food fortification (18). The need for high dose supplements in children has been based on childhood mortality rates (19) rather than VAD and has successfully decreased morbidity and mortality (20), although supplements to children without VAD have recently been criticized (21). Over the years, vitamin A in the form of retinol has been added to a variety of foods including margarine (22), corn flour (23), wheat flour (24), sugar (9) and rice (25). One year after the introduction of vitamin A fortified sugar in Nicaragua, VA liver stores, as measured by the deuterium isotope dilution technique, doubled (9).
Rice can be fortified with micronutrients by adding artificial extruded rice grains containing the micronutrients to regular rice at a ratio of 1:50 or 1:100 (25). Rice grains can be produced using a cold extrusion similar to pasta making (26) or by hot extrusion (27). The cold extrusion, or Ultra Rice technology, has been used to produce Vitamin A fortified grains which increased serum retinol when fed to a target population (25). The advantage of the hot extrusion is providing better vitamin A stability during storage (Pinkaew et al, in press). We have used the hot extrusion technique to manufacture artificial triple fortified extruded rice grains containing vitamin A, iron (Fe) and zinc (Zn). The product showed good vitamin A stability (Pinkaew et al, in press) and feeding the grains to school children improved both Fe (28) and Zn (Pinkaew et al, submitted) status.

The aim of the present study was to evaluate the impact of extruded rice grains triple fortified with VA, Fe and Zn on VA status of school children in Southern Thailand. The change in VA status was quantified by measuring total body VA pool size at baseline and 2 months after consumption of the triple fortified rice grains within a school meal program. VA pool size was measured using a paired stable isotope dilution technique of labeled $^{13}$C retinyl acetate. The change in VA pool size was compared to the change in SR concentration.

**Subjects and Methods**

**Study site**

The study was carried out in a peri-urban area of Muang district, Satun province located on the west coast of Southern Thailand. The majority of the population was Muslim and the subjects were primarily from low income families. One primary school in the Muang district, with children from 4-12 years old, was selected for the study. The school provided a free lunch meal (5
days/week) which was partly subsidized by the government. The lunch meals were prepared in a rotating order and always contained rice together with another dish prepared from chicken, fish, vegetables or beef (rarely). Free milk was also provided by the government to all children at the school.

**Subjects**

School children aged between 8 and 12 years were recruited for the study. Only children with general good health and no major chronic diseases were eligible to participate in the study. At the time of VA status assessments children were free of acute illness, febrile conditions and gastrointestinal problems. Children who had consumed the triple fortified rice in a previous study (Pinkaew et al, submitted) or showing clinical symptoms of VAD (Bitot’s spot or ocular sign of xerophthalmia), or SR values below 0.7µmol/L, were excluded. Informed consent was obtained from each subject as well as from their parents or guardians. The study protocol was approved by the ethics committees of the ETH Zurich, Switzerland and Mahidol University, Thailand.

**Extruded rice production**

Triple fortified extruded rice grains containing VA (retinyl palmitate; RP, Dry vitamin A palmitate 250 S/N, DSM Nutritional Products Ltd., Basel Switzerland), Fe (micronized ground ferric pyrophosphate; MGFP, Dr. Paul Lohmann GmbH KG (Emmerthal, Germany)) and Zn (zinc sulfate; ZnSO₄, Dr. Paul Lohmann GmbH KG (Emmerthal, Germany)) were produced by the hot extrusion technology with a co-rotating twin-screw extruder (Model OEVB-D-30.5, Bühler AG, Uzwil, Switzerland) at ETH Zurich. The extrusion process has been described previously (Pinkaew et al, in press). After extrusion, the grains were air dried for 2 nights in a dark room.
before packing into aluminum foiled bags under vacuum. After transportation to Thailand, the grains were stored in the fridge until mixing with normal unfortified rice. The fortification levels of extruded rice grains were 10 mg Fe, 9 mg Zn and 1050 µg VA per g extruded rice. These levels were calculated based on the rice consumption of school lunch meals (≈140 g cooked rice which corresponds to ≈50 g of dry uncooked rice) as well as on the estimation of nutrient intake data from 3-day weighed food records performed during a pre-survey in March 2008. The fortified rice grains were mixed with the normal rice at a ratio of 1:50. These fortification levels were estimated to bring 97.5% of the population above the EAR for VA, Fe and Zn (16, 29) when EARs for Fe and Zn are based on a 10% bioavailability and a moderate bioavailability, respectively. Based on the results of previous VA stability tests (Pinkaew et al, in press), vitamin A was added at a 40% excess to allow for the maximum losses of VA during production, storage under tropical conditions and cooking. As mentioned earlier, vacuum packaging in light proof packaging and cold temperature were used to store the fortified rice grains so as to minimize storage losses. The children were estimated to consume an additional 890 µg VA, 10 mg Fe, and 9 mg Zn per day, from the triple fortified rice after cooking. These levels are well below the tolerable upper intake level (UL) for all micronutrients (29).

**Preparation of the rice component of the lunch meal**

One batch of regular rice, the type frequently consumed in the study area, and one batch of the fortified rice were cooked every day. The rice was prepared at a central kitchen in the city of Satun by a trained cook. For the fortified rice, normal rice was mixed before cooking with triple fortified extruded rice grains at a ratio of 50:1 according to a previous study by Moretti et al (27). The portions of 140 g of cooked rice (triple fortified rice or unfortified rice) were packed
into individual containers, which were labeled with the child’s name. The quality and quantity of the rice portions were randomly checked by research assistants every day.

**Intervention trial**

The VA efficacy study was a double-blind, randomized, controlled trial. Forty school children (20 per intervention group) were considered necessary for the study based on an expert group opinion (6). In order to allow for dropouts, we recruited 50 children (25 per intervention group) (4). Eligible subjects were randomized in 2 groups. One group was given the triple-fortified rice containing Fe, Zn and VA (fortified group) and the other group was given non-fortified rice (non-fortified group/control) as a component of the school lunch meal.

The individually packed rice portions were transported by research assistants from the central kitchen to the school each day. During lunch time, the rice was consumed along with other foods given as part of the school lunch including soup, curry or stir fried dishes. The rice consumption was supervised by teachers and research assistants daily. The leftovers from meals were estimated for each child and recorded every second day. The rice meal was fed 5 days a week for a total duration of 2 months. The study was conducted from August 2010 to November 2010.

**Estimation of changes in TBR of VA by paired stable isotope dilution technique**

The $^{13}$C-retinol isotope dilution technique was used to measure total body VA stores at baseline and again after 2 months of consumption of the vitamin A fortified rice. As an alternative status measure, SR was also measured at baseline and after 2 months. Figure 1 shows the procedures study participants followed:
On study day 0, children received the first oral dose of $^{13}$C-labeled VA (30) together with 5 g of potato chips. Labeled VA, $[^{13}$C$_2$]-retinyl acetate (Department of Nutritional Sciences, University of Wisconsin-Madison, USA) was used for the study. One $\mu$mol (168.2 $\mu$L) retinol equivalents (RE) of labeled retinyl acetate dissolved in corn oil was given to each child by a positive displacement pipette. Weight and height of all participants were measured on the same day. Subjects then consumed their normal diet for 14 days. The first blood sample (5 ml) was collected 14 days after administration of the labeled compound (day 14) for quantitative estimation of initial VA pool size (15) as well as for the determination of SR and C-reactive protein (CRP) concentration. CRP was measured to detect children with infection or inflammation. On study day 15, children started the intervention, either consuming the triple fortified rice meal or the non-fortified rice meal according to the random allocation. This
intervention ended on study day 75 (a total of 60 days of feeding). Thereafter, the children consumed their usual diet for 7 days (study day 76 to 82). The second blood sample (5 ml) was drawn on study day 83 in order to measure the quantity of isotopes remaining from the 1st dose, as well as SR and CRP. On the same day, after the blood draw, a second dose of $^{13}$C-labeled VA was administered together with 5 g of potato chips. The 3rd and final (5 ml) blood sample was taken 14 days later (day 97) for the determination of the final VA pool size. To assess the natural enrichment of $^{13}$C at baseline, 5 ml blood samples from 5 children not participating in the study were collected (31).

**Laboratory analyses**

Venous blood samples were collected into evacuated blood collection tubes which were handled so as to protect the blood from heat and light. Blood was centrifuged at 3000 rpm for 15 minutes, serum was separated and pipetted into dark cryovials and stored at -20°C at the hospital in Satun. Frozen serum samples were then transported to Mahidol University on dry ice. Samples for measuring $^{13}$C-retinol were kept at -20°C until shipment on dry ice to the University of Wisconsin-Madison where they were kept frozen until analysis.

The serum samples were analyzed for $^{13}$C-retinol enrichment by separating retinol from other constituents of serum using High Performance Liquid Chromatography (HPLC), collecting the retinol fraction, and using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to analyze the $^{13}$C/$^{12}$C in the purified retinol (31).

SR was measured at the Institute of Nutrition, Mahidol University, using HPLC according to the method of The International Vitamin A Consultative Group (32) with reference material from the National Institute of Standard and Technology (Gaithersburg, MD, USA). CRP concentration was determined in serum samples using an automated solid-phase, two-site chemiluminescent
immunometric assay (Immuliite One, Diagnostic Products Corporation (DPC), Los Angeles CA, USA) at ETH Zurich. A three levels serum control (DPC, Los Angeles CA, USA) was analyzed with each set of measurements. A cutoff of ≥ 5 mg/L is used to indicate the presence of inflammation or infection (33). SR values of samples with elevated CRP levels were excluded from analysis.

**Estimation of TBR of VA (or total body VA pool size)**

The TBR of VA (µmol) was calculated with the following mass balance equation (15)

\[(Fa \times a) + (Fb \times b) = (Fc \times C)\]

\[Fa = 0.1 \text{ (fraction of dose labeled (in this study, } ^{13}\text{C}\text{2 retinol has } 2 \text{ of } ^{13}\text{C out of } 20 \text{ carbon)= 2/20)}\]

\[Fb, Fc = \text{ the decimal form of } \text{At\% } ^{13}\text{C of SR at baseline and 14 days after administered respectively}\]

(AT\% is an expression of the percent of 13C atoms to 12C atoms in the retinol extracted = 100/1+(1/R) where R is the ratio 13C/12C )

\[a = \mu \text{mol VA absorbed and store from labeled dose = } \mu \text{mol VA administered } \times 0.5 \text{ (34)}\]

\[b = \text{ uncorrected base line TBR (unknown) in } \mu \text{mol}\]

\[c = \text{TBR after dosing = } a + b \text{ in } \mu \text{mol}\]

uncorrected base line TBR (unknown) = \((Fc \times a) - (Fa \times a)) / (Fb -Fc)\), with \(c = a + b\)

corrected TBR (µmol) = \(b \times e^{(-kt)}\) where \(k = \ln(2)/140 \text{ and } t = \text{ time in days (14 day after dosing)}\)

(this is the results after correct for the decay of retinol or half-life correction) (8)

**Estimation of liver vitamin A concentration (µmol/g)**

The calculation assumed that, in this age group, liver weight is 3% of body weight (35) and that 90% of total body VA (TBR) is stored in the liver (36).
Statistical analyses

SPSS (Version 18, Microsoft, Seattle) software was used to perform the statistical analyses. Normality of data was checked before analysis with the Kolmogoroff-Smirnoff test and not normally distributed data were log transformed. A repeated-measures analysis of variance was done to compare effects of time x group for SR, TBR of VA and liver VA concentration. Post hoc comparisons were done by using unpaired t-tests between groups and paired t-tests within groups. Group effect for the binary variables of low VA status (SR < 1.05 μmol/L) was tested by using Pearson’s chi-square test, and the time effect was tested by using McNemar’s test. Significance was set at $P < 0.05$. Two outliers (mean ± 3 SD) were excluded from statistical analysis.

Results

Subject characteristics

Of the 50 children who started the intervention (25 children in each group), 45 children completed it. Two children dropped out because they moved out of the study area (1 child in each group) and 3 children (1 in the intervention group and 2 in the control group) were excluded due to low compliance (< 80% during the feeding period). Furthermore, the isotopic ratio could not be measured in 4 subjects (3 in the intervention group and 1 in the control group). Thus at the end point, data from 41 children were eligible for data analysis. The baseline characteristics of the participating children are shown in Table 1. There was no significant difference in any of the characteristics between the two groups except for BMI-for-age Z scores. The prevalence of stunting (20%) and underweight (19%) was relatively high in this population group (37) while 6% of children had a BMI-for-age Z score < -2 SD.
Table 1 Baseline characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Intervention (n=25)</th>
<th>Control (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:Female</td>
<td>12:13</td>
<td>12:13</td>
</tr>
<tr>
<td>Age (y)</td>
<td>9.2 ± 1.1</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>26.1 (19-66.3)</td>
<td>23.1 (16.6-33.2)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>123.3 (114.5-144.7)</td>
<td>122.2 (115.3-144.2)</td>
</tr>
<tr>
<td>Weight-for-age Z score (WAZ)*</td>
<td>-1.0 (-3.2-4.9)</td>
<td>-1.1 (-3.9-(-0.01))</td>
</tr>
<tr>
<td>Prevalence of underweight* (%)</td>
<td>19</td>
<td>18.8</td>
</tr>
<tr>
<td>Height-for-age Z score (HAZ)</td>
<td>-1.4 (-2.8-2.5)</td>
<td>-1.3 (-2.5-0.3)</td>
</tr>
<tr>
<td>Prevalence of stunting (%)</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>BMI-for-age Z score</td>
<td>-0.1 (-2.5-4.3)**</td>
<td>-0.9 (-3.5-0.7)</td>
</tr>
<tr>
<td>Prevalence of BMI &lt; -2 SD (%)</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

*Value based on WHO references 2007 for which weight for age reference data are not available for children older than 10 years

** Significantly different from control group

Compliance with feeding

The total feeding period was 58 days, of which 30 days were on school days and 28 days were during the semester break when the children voluntarily came back to school for the free meal. Taking compliance into account, the children consumed the rice meals on average for 53.7 days, with similar consumption in the intervention (53.5 days) and the control group (53.9 days). Mean rice meal intake was 123 g/day with a slightly lower (but not statistically significant, p= 0.17) consumption in the control group (119.2 ± 19.9 g/day) than in the
intervention group (126.7 ± 14.2 g/day). The fortified rice provided an additional ~43'000 µg of VA during the whole study period (or ~800 µg of VA per day) to the intervention group.

**Serum retinol concentration and C-reactive protein**

The mean initial SR concentrations in the intervention and the control group (Table 2) were not significantly different. SR concentration did not change over the course of the study in both groups. However, at the end of the study, the prevalence of low VA status as defined by SR < 1.05 µmol/L (1) was significantly higher in the control group (44%) than in the intervention group (10%).

The overall prevalence of elevated CRP was 12.5% and 7.3% at baseline and at the end of the study, respectively, with a higher proportion in the intervention group compared to the control group at both time points (Table 2).
Table 2 Serum retinol concentration (SR), prevalence of SR < 1.05 µmol/L and prevalence of elevated CRP

<table>
<thead>
<tr>
<th></th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SR (µmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.21 ± 0.19</td>
<td>1.18 ± 0.26</td>
</tr>
<tr>
<td>n*=20</td>
<td></td>
<td>n=22</td>
</tr>
<tr>
<td>End point</td>
<td>1.28 ± 0.27</td>
<td>1.15 ± 0.23</td>
</tr>
<tr>
<td>n=20</td>
<td></td>
<td>n=18</td>
</tr>
<tr>
<td><strong>Prevalence of SR &lt;1.05 µmol/L (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>20</td>
<td>36.4</td>
</tr>
<tr>
<td>End point</td>
<td>10(^a)</td>
<td>44.4</td>
</tr>
<tr>
<td><strong>Prevalence of elevated CRP value (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.7</td>
<td>8.3</td>
</tr>
<tr>
<td>End point</td>
<td>13</td>
<td>0(^b)</td>
</tr>
</tbody>
</table>

*Number of analyzed samples for SR concentration at baseline and end point after excluding samples of children with high CRP, hemolysis, not enough volume for analysis, and samples of children who have moved out of the study region during the study and of children with low compliance

\(^a\)Significantly different from control group at the same time point (p<0.05)

\(^b\)Significantly different from baseline (p < 0.05)
Total body reserve of vitamin A (TBR of VA) and liver vitamin A concentration

Table 3 shows results of TBR of VA and liver VA concentration at baseline and after the 2 month intervention. We found a significant time x treatment interaction in both TBR of VA and hepatic VA concentration. At baseline both parameters were comparable between the intervention and the control group. After the 2 months feeding period, TBR of VA and liver VA concentration significantly increased and were nearly doubled in the intervention group when compared to baseline. Moreover, these values were significantly higher at endpoint when compared to the control group in which no change from baseline was observed. The estimated increase in TBR of VA in the intervention group during the whole study using the $^{13}$C stable isotope dilution technique was $\sim$24,066 µg for a total additional VA intake of 43,000 µg, indicating that about half of the additional VA intake from the fortified rice had been laid down in the liver stores.

At baseline, the mean liver VA concentration in both groups were above the cut off for VAD (<0.07 µmol/g) (4) although 4/25 subjects in the intervention group (16%) and 8/25 subjects in the control group (32%) were classed as VAD. At the end of the study, no subject in the intervention group was deficient, whereas the same 6 out of 8 subjects in the control group still had liver vitamin A concentrations <0.07 µmol/g and were classed as VAD. None of study participants showed a sub-toxic (>1 µmol/g) or toxic (10 µmol/g) VA status based on liver stores (2).
**Table 3** Total body reserve (TBR) of vitamin A, liver vitamin A concentration and prevalence of low liver vitamin A concentration

<table>
<thead>
<tr>
<th></th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TBR of vitamin A</strong> (mmol retinol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>0.105 ± 0.044</td>
<td>0.084 ± 0.051</td>
</tr>
<tr>
<td></td>
<td>n = 25</td>
<td>n = 25</td>
</tr>
<tr>
<td><strong>After intervention</strong></td>
<td>0.189 ± 0.104&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.087 ± 0.062</td>
</tr>
<tr>
<td></td>
<td>n* = 19</td>
<td>n* = 20</td>
</tr>
<tr>
<td><strong>Liver vitamin A</strong> (µmol/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>0.122 ± 0.054</td>
<td>0.105 ± 0.06</td>
</tr>
<tr>
<td><strong>After intervention</strong></td>
<td>0.220 ± 0.141&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.108 ± 0.078</td>
</tr>
<tr>
<td><strong>Prevalence of vitamin A deficiency</strong> (%&lt;sub&gt;1&lt;/sub&gt;, liver VA &lt;0.07 µmol/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td><strong>After intervention</strong></td>
<td>0&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>35</td>
</tr>
</tbody>
</table>

*Number of analyzed samples for TBR of vitamin A and liver vitamin A at end point after excluding samples of children with low compliance, samples of children who have moved out of the study region, samples who couldn’t be measured and outliers (mean ± 3 SD)

<sup>a</sup> Significant time x treatment interaction (p < 0.05)

<sup>b</sup> Significantly different from baseline (p<0.05)

<sup>c</sup> Significantly different from control group at the same time point (p<0.05)
Discussion
The extruded triple fortified rice grains containing VA were highly efficacious at improving VA status in Thai school children when status was measured using the $^{13}$C - retinyl acetate isotope dilution technique. Using this technique, the mean TBR of VA in the children increased from 0.105 mmol retinol at baseline to 0.189 mmol after the 2 month intervention ($p<0.05$). Over the same period, the liver VA concentration increased from 0.122 to 0.220 µmol/g ($p<0.05$), indicating that much of the retinol added to the extruded grains had withstood the processing, cooking and storage procedures and had been absorbed from the school meals and, as it was in excess of immediate requirements, was stored in the liver. Some 50% of the additional vitamin A intake from the fortified rice grains was stored in the liver.

In contrast, consumption of the VA fortified rice resulted in no change in VA status when this was monitored using SR. This could perhaps be expected since the number of subjects selected for the stable isotope dilution technique would be too low to detect differences in SR. Nevertheless, SR has been shown in earlier studies not to always change in response to a dietary intervention (2, 10, 12, 38). This is presumably due to the homeostatic control of SR concentration over a wide range of liver reserves in addition to the many other factors that can affect SR concentration (2, 4).

The baseline mean TBR of VA in the present study (0.096 mmol combining both groups) is comparable to the TBR of VA reported in Chinese children (0.09-0.13 mmol) (11) and in Filipino school children (0.078-0.089 mmol) (12), but much lower than that reported in US school children (1.02 mmol) (39), and also lower than in Nicaraguan children (0.39 mmol) (9) and in a second study with Chinese children (0.27 mmol) (40). The lower liver VA concentrations in our study children can perhaps be expected since they came from a low socioeconomic population with some 20% underweight and 20% stunting (Table1). The mean
baseline liver VA concentrations in our study children (0.113 µmol/g averaged over both groups) were only about half the liver VA concentrations reported on autopsy of Thai children who died from accidents (0.22 µmol/g) (41). The initial mean liver VA stores of 0.113 µmol/g (0.122 µmol/g in the intervention group and 0.105 µmol/g in the control group) are only slightly higher than the upper cut-off for marginal VA status (0.07-0.1 µmol/g) (4) and indicate that VA status of school children in this area should be regularly monitored. Interestingly, none of the children were classed as VAD based on SR < 0.7 µmol/L (according to the exclusion criteria) (1) whereas based on liver VA concentration at baseline, 24% of children (16% in the intervention group and 32% in the control group) were considered to be VA deficient. Unfortunately the cost and technical difficulties in using stable isotopes rule out its use to measure VA status. Other methods such as the modified relative dose response test (2) may be better alternatives to SR. In the present study, the increase in TBR of VA of 84 µmol retinol in the intervention group (189 (end point) -105 (baseline) µmol retinol) consuming 890 µg retinol/d over the 2 month study was comparable to that reported in school children in the Philippines with an average increase of 89 µmol retinol after a 9 week period of daily consumption of 4.2 mg provitamin A carotenoids (12). TBR of VA however increased to a somewhat greater extent in our study children than in Bangladeshi men who were fed for 60 days with 750 µg retinol equivalent per day of either sweet potato (increase of 29 µmol retinol), Indian spinach (increase of 41 µmol retinol), retinyl palmitate (increase of 65 µmol retinol) and β-carotene (increase of 62 µmol retinol) (10).

All the above cited studies used the deuterated-retinol-dilution (DRD) technique and the present study is the first study using the $^{13}$C technique in human to be published. In order to estimate the TBR of VA both stable isotope dilution techniques assume that 50% of the daily dose of VA is absorbed from the meal and stored (34). In our study, this would be ~21,500 µg
(43,000 x 0.5) which is close to 24,066 µg estimated using the $^{13}$C stable isotope dilution technique, and demonstrates the reliability of the labeled $^{13}$C retinyl acetate method. Several studies have similarly demonstrated the reliability of the DRD technique (9, 10, 42).

In conclusion, this study shows the usefulness of the stable isotope dilution technique with labeled $^{13}$C retinyl acetate for assessing VA status and measuring the impact of an intervention with a VA fortified food. While the method is too expensive and complicated for the measurement of VA status, the stable isotope dilution methods would appear to be the methods of choice for monitoring VA interventions. The triple fortified extruded rice grains tested in this study were shown to be highly efficacious in improving VA status of school children in Thailand. Earlier studies with the triple fortified rice had similarly demonstrated improved Fe and Zn status in children consuming the rice. Such rice would therefore be an effective strategy to prevent VA, Fe and Zn deficiencies in at risk populations both in Thailand as well as in other rice eating populations.
References

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Manuscript 3: Extruded rice grains fortified with zinc, iron and vitamin A improve zinc status of Thai school children when incorporated into a school lunch program

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Iron (Fe), zinc (Zn) and vitamin A deficiencies are common among children in developing countries and often occur in the same individual. Rice is widely consumed in the developing countries of Asia and the low phytate in polished rice makes it ideal for Zn and Fe fortification. Triple fortified rice grains with Zn, Fe and vitamin A were produced using the hot extrusion technology. The main objective of the present study was to determine the impact of triple fortified extruded rice on Zn status in school children in Southern Thailand. Fe and vitamin A status were also followed. School children with low serum Zn (SZn) (n=203) were randomized into 2 groups to receive either triple fortified rice (n=101) or natural rice (n=102) as a component of school lunch meals for 5 months. SZn, hemoglobin, serum ferritin, serum retinol and C-reactive protein concentration were measured at baseline and at the end of the study. After the intervention, SZn significantly increased in both groups (p< 0.05), most likely due to the proper implementation of the school lunch and school milk programs, however, SZn increased to a significantly greater extent (p<0.05) in the group receiving the triple fortified rice. We conclude that the Zn fortification of extruded rice grains is efficacious and can be used to improve Zn status in school children.
**Introduction**

Deficiencies in iron (Fe), zinc (Zn) and vitamin A still remain major public health problems in developing countries [1]. These deficiencies have adverse health consequences on growth and cognitive development, pregnancy outcome, immune and reproductive function [2, 3] and additionally increase mortality and morbidity from infections, including measles, diarrhea and malaria [4]. Moreover, the coexistence of micronutrient (MN) deficiencies is common [5] and is often found in Thailand. In North East (NE) Thailand, 60% of school children are at risk of two or more coexisting MN deficiencies [6].

Although the national prevalence of Zn deficiency in Thailand is currently unknown, the most recent study in NE Thailand, based on serum zinc (SZn) levels reported that 57% of school children were Zn deficient [6], while in the same region, 20% of the same children had marginal vitamin A deficiency (VAD) [7]. Anemia however still remains the major public health problem and the latest Thai national nutrition survey in 2003 [8] reported 56% and 26% respectively of 6-11 mo old and 1-5 yr old children were anemic, a higher prevalence than in the previous survey. Several studies conducted in NE Thailand indicated that thalassemia and hemoglobinopathies rather than ID are the major causes of anemia [7, 9, 10]. The latest Thai national nutrition survey also found the highest prevalence of stunting, a potential sign of Zn deficiency, in children in the southern region of Thailand [8].

Due to its wide consumption by poor population groups, rice is a promising vehicle for food fortification. Rice provides ca. 41% of total energy in Thailand [11]. In addition, polished rice is an advantageous vehicle for Zn and Fe fortification because of its relatively low phytate content. In previous studies, we have successfully fortified rice with Fe using the hot extrusion technology and micronized ferric pyrophosphate as the iron source [12]. These artificial, iron
fortified rice grains, when mixed into natural rice, improved the iron status of Indian children when fed as part of a school lunch program [13].

Simultaneous fortification of rice with Fe, Zn and vitamin A could be a novel approach to control these deficiencies due to beneficial interactions. There are several potential interactions of vitamin A in Fe metabolism [14] such as within the erythropoietin pathway [15] and during the recovery of Fe from the ferritin stores [16]. On the other hand, iron is also needed to recover VA from its liver stores [17]. Zinc, vitamin A and Fe [18] substantially contribute to improved immune competence. We have recently produced artificial rice grains triple fortified with Fe, Zn and vitamin A by the hot extrusion technology (Pinkaew et al submitted for publication). Storage tests demonstrated relatively low and acceptable vitamin A losses on storage in tropical conditions.

Previous studies of Zn supplementation in pharmacological Zn doses have reported increased SZn [19], increased growth [20, 21] and a decrease in the incidence of diarrhea [21]. Previous reports on the efficacy of Zn fortified foods however are inconsistent [22], and several studies have failed to show an improvement in SZn with regular consumption of Zn fortified foods [19, 23, 24], although one study in NE Thailand reported a significant increase in SZn of children with low serum zinc at baseline with regular consumption of a Zn containing multinutrient fortified spice mix [25].

The aim of the present study was to demonstrate an improvement in SZn in school children in Southern Thailand when adding the triple fortified rice grains to the school lunch program. Iron and vitamin A status were also monitored.
Subjects and Methods

Study site

The study was conducted in Satun province which is located on the west coast of Southern Thailand where the majority of the population is Muslim. The study was performed in 8 primary schools in the Muang district which included mainly children from low-income families. The schools included 4-12 years old children (kindergarten – grade 6) who were offered a school lunch program (5 days/week) partly subsidized by the government. Lunch menus were prepared in rotating order and usually consisted of rice together with chicken or fish, and occasionally with vegetables. The schools also provided free milk to all children. Fe supplement tablets, which had been given to the children by health officers/village health volunteers before the intervention, were not provided during the intervention. The study protocol was approved by the ethics committees of the ETH Zurich, Switzerland and Mahidol University, Thailand.

Pilot study to measure Zn, Fe and vitamin A intake and status

One year prior to the efficacy study (March 2008), a small 3 day food intake survey and status study was conducted in 2 schools of the same area during the school vacation so as to measure current Zn, Fe and vitamin A intake and status, the main dietary components, and the suitability of the schools for a Zn intervention study.

Dietary assessment. 3-day weighed food records were conducted in all members of 20 families. The households were randomly selected from families with children aged between 7 and 12 y. The assessment was done on 3 consecutive days (including 2 week days and 1 weekend day) and during that time, the participants were asked to maintain their usual food
habits. Edible portions of all food and beverages were weighed during preparation and consumption using food-scales with a precision of ±1 g. After the meal, the food that was not eaten was weighed. Food consumed outside home was weighed by asking the participant to buy the same amount of food they have eaten (money given by the staff) or by recalling the food consumed.

Nutrient intake was calculated using the software program INMUCAL (Mahidol University, Thailand, version WD 2.1). Zinc bioavailability was estimated based on the phytic acid:Zn molar ratio [26].

**Micronutrient status.** Children aged between 7 and 12 y, with no visible signs of infectious disease and not taking micronutrient supplements (n=92) were randomly selected from both schools. Informed consent was obtained from all participants as well as their parents/guardians. The anthropometric measurements were collected before ≈ 5 ml blood was drawn from all participants by venipuncture. The blood was equally divided into 2 tubes; 1 trace element free tube and 1 EDTA containing tube. The whole blood/serum samples were used to measure the following biochemical indicators: hemoglobin (Hb), SZn, serum ferritin (SF), serum retinol (SR) and C-reactive protein (CRP).

**Efficacy study: preliminary screening and intervention.**

A total of 744 children participated in the baseline screening. Informed consent was obtained from participating children and parents/guardians. Weight and height were measured and ≈ 5 ml of whole blood (divided in 2 tubes; 1 trace element free tube and 1 EDTA containing tube) was collected by venipuncture for determination of Hb, SZn, and CRP.

The efficacy study was a double-blind, randomized, controlled trial. All children with Zn deficiency (low SZn concentration) were invited to join the intervention study. Zinc deficiency
was defined as \( \text{SZn} < 65 \, \mu\text{g/dL} \) for children \(< 10 \, \text{y} \), \(< 66 \, \mu\text{g/dL} \) for female subjects \( \geq 10 \, \text{y} \) and \(< 70 \, \mu\text{g/dL} \) for male subjects \( \geq 10 \, \text{y} \), respectively, from a morning non-fasting blood sample [2]. Children showing severe anemia (Hb \(< 8.0 \, \text{g/dL} \)) or VAD (Bitot’s spot or ocular signs of xerophthalmia) or \( \text{SZn} \) concentration \(< 54 \, \mu\text{g/dL} \) were excluded and treated according to local policy. From the children included in the study, SF and SR were additionally measured in the Zn deficient children at baseline. All parameters (SZn, CRP, Hb, SF and SR) were measured again at the end of the intervention. Eligible subjects (n=203) were randomized in 2 groups. One group was given the triple-fortified rice containing Fe, Zn and vitamin A and the other group was given non-fortified rice. Sample size calculations indicated that 76 children were needed in each group, based on 90% power to detect a difference of 5 \( \mu\text{g/dL} \) in mean \( \text{SZn} \) concentration with a significance level of 0.05 (2-tailed) calculated from the standard deviation (SD) of \( \text{SZn} \) concentration of the prescreening survey. Anticipating a drop-out rate of 10-15%, these estimates indicated that at least 88 subjects were required in each group. The study was conducted from July 2009 to May 2010. Figure 1 shows an outline of the Zn efficacy study.
Figure 1 Efficacy study outline
Rice fortification

Triple fortified extruded rice grains were produced using the hot extrusion technology with a co-rotating twin-screw extruder (Model OEVB-D-30.5, Buhler AG, Utzwil, Switzerland) consisting of 5 barrel segments. Rice flour (Haefliger AG – melior gourmet, Herzogenbuchsee, Switzerland) was first dry mixed with zinc sulfate, micronized ground ferric pyrophosphate (Dr. Paul Lohmann GmbH KG, Emmerthal, Germany), retinyl palmitate (Dry vitamin A palmitate 250 S/N, DSM Nutritional Products Ltd., Basel Switzerland) and distilled monoglycerides (DIMODAN HP® 75/B KOSHER, Danisco A/S, Copenhagen, Denmark) as an emulsifier in a drum hoop mixer (J.Engelmann AG, Ludwigshafen, Germany).

After mixing of all the ingredients, the dry premix was filled into the feeder (K-Tron Schweiz AG, Niederlenz, Switzerland) of the extruder and was discharged gravimetrically at a dosing rate of approximately 8 – 9 kg/h into the first barrel segment of the extruder. The extrusion process was done at 50°C and 80°C at barrel numbers 1, 5 and numbers 2, 3, and 4 respectively. Water was pumped by a dosing pump (Grundfos, Fällanden, Switzerland) into the second barrel at a rate of approximately 2 L/h. The screw speed was fixed at 150 rpm. A special manufactured four-oval hole die together with a cutter (Bühler AG, Utzwil, Switzerland) attached at the end of the die, were used to form the shape of the rice grains. After extrusion, the rice grains were air dried for 2 nights in a dark room before packing into aluminum foil bags under vacuum.

The fortification level of the extruded rice grains was 10 mg Fe, 9 mg Zn and 1050 µg vitamin A per g extruded rice. These levels were based on a consumption of ≈140 g cooked rice per school lunch meal per child which corresponds to ≈ 50 g of dry uncooked rice, diluting the fortified rice 1:50 with natural rice, and the current intakes of Zn, Fe and vitamin A as
measured in the pilot study. They were estimated to increase the intake of these micronutrients in 97.5% of the school children to above their EAR values [26, 27] when the EARs for Fe and Zn are based on a 10% bioavailability and a moderate bioavailability, respectively. For vitamin A, a 40% loss was assumed to have taken place during extrusion, drying, cooking and storage under tropical conditions (Pinkaew et al in press), although during the intervention the rice was refrigerated and stored in light protected vacuum packaging. We estimated that the children received an extra 10 mg Fe, 9 mg Zn and ~890 µg vitamin A per school feeding day from the triple fortified rice during the intervention. These levels remain under the tolerable upper intake level (UL) [27].

**Preparation and feeding of the lunch meal**

Natural rice, as consumed in the study region, was mixed with the triple fortified extruded rice grains at a ratio of 50:1 as described by Moretti et al [12]. For monitoring, at the beginning of each month, Zn was measured in 100 g of cooked rice.

The rice was prepared by local cooks at a central kitchen in Satun town which had been specifically set up for the study. The cooked rice was weighed into individual portions of 140 g into a color-coded container which was labeled with the child’s name. The weight was regularly controlled by research assistants. The rice was transported to the 8 schools by the research assistants and the 140 g of cooked rice (triple fortified rice or unfortified rice) was given to each child. The rice was consumed with foods such as soup or curry which was provided by the school lunch program. The lunch meals contained mainly chicken (2-4 time/week), whereas egg and fish were served with less frequency (1-2 time/week). Vegetables such as cabbage, cucumber and yard long bean were used in some menus. School milk was given every day to all children. The feeding was constantly monitored by research assistants and teachers. The
leftover rice from every child was estimated and recorded every second day. The study duration was 6 months with a break of 1 month due to school holidays (feeding time of 5 months). The rice meal was fed 5 days a week. After completion of the study, all children who remained deficient in any of the micronutrients in either group received supervised treatment of the respective micronutrient(s) according to local policies.

**Laboratory analysis**

Blood samples were stored in a cold box immediately after the blood was taken. The separation of serum from the trace element free tubes for determination of SZn and CRP was done in the field within 30-40 minutes according to the instructions recommended by IZiNCG [2]. Separation of serum from EDTA containing tubes for the analysis of SF and SR was done at the hospital laboratory after analysis of Hb on the same day. Serum samples were stored at -20°C prior to analysis of SF, SZn, SR and CRP.

Hemoglobin was measured using electronic complete blood count (Cell Dyn 3700, Abbott Diagnostic, Santa Clara CA, USA) and 3-level controls provided by the manufacturer (Cell Dyn calibrator and control, Abbott Diagnostic, Santa Clara CA, USA). Anemia was defined as < 120 g/L in children ≥ 12 years old and Hb < 115 g/L in children 5-11 years old [27]. Serum ferritin and CRP were measured using a chemiluminescent immunometric assay (IMMULITE®, Diagnostic Products Corporation (DPC), Los Angeles CA, USA) together with a 3-level serum control (DPC, Los Angeles CA, USA) with every set of measurement. Iron deficiency was defined as SF < 15 µg/L [27] and a cut-off of > 5 mg/L for CRP was used to indicate the presence of inflammation or infection [28]. Serum Zn was measured by flame atomic absorption spectrophotometry (SpectraAA-400, Varian, Mulgrave, Australia). Controls
(Seronorm Trace Elements Serum level 2, IG Instrument-Gesellschaft AG, Zurich, Switzerland) were used to check the precision and accuracy of the SZn analysis. Zinc deficiency was defined as SZn < 65 µg/dL for children < 10 y, < 66 µg/dL for female subjects ≥ 10 y and < 70 µg/dL for male subjects ≥ 10 y, respectively, from a morning non-fasting blood sample [2]. Serum retinol was measured using High Performance Liquid Chromatography (HPLC) according to the method of The International Vitamin A Consultative Group [29] with reference material from the National Institute of Standard and Technology (Gaithersburg, MD, USA). Vitamin A deficiency was defined as SR < 0.07 µmol/L [4]

Statistical analysis

SPSS (Version 18, Microsoft, Seattle) software was used. Normality of data was checked before analysis with the Kolmogoroff-Smirnoff test and not normally distributed data were log transformed. A repeated measures analysis of variance was done to compare effects of time x group for Hb, SF, SZn, SR. Post hoc comparisons were done by using unpaired t-tests between groups and paired t-tests within groups and adjusted for multiple comparisons (Bonferroni correction). Group effect for the binary variables of anemia, ID, IDA, Zn and vitamin A deficiency was tested by using Pearson’s chi-square test, and the time effect was tested by using McNemar’s test. Significance was set at $P < 0.05$.

Results

Pilot study for Zn, Fe and vitamin A intake and status

Table 1 shows Zn, Fe and vitamin A intake and status of school children from the study area. The intakes are also given as % of EAR. The phytic acid to Zn molar ratio was 1.7 indicating a high bioavailability diet. The mean Zn intake at 2.2 mg per day was relatively low.
corresponding to 71% of the EAR values for a high bioavailability Zn diet or only 41% of the EAR for a moderate bioavailability Zn diet. With this low Zn intake 90 % of the children failed to reach their EAR for a high bioavailability Zn diet. This would increase to 100% with a diet of moderate Zn bioavailability [26, 27]. Iron (estimated 10% bioavailability) and vitamin A intakes were also low, corresponding to 81% and 52% of the EAR values and indicated that 85% and 90% of the children, respectively, failed to reach their EAR.

Rice and rice products, such as rice noodles and fermented rice noodles were the main staple foods. The diet contained 63% of total energy as carbohydrate, 26% as fat and 11% as protein. Since the majority of people was Muslim, chicken was the most widely consumed animal protein source followed by mackerel, beef and egg, while rice and rice products provided the highest contribution of plant proteins. Traditional curry, soup and stir fry were commonly prepared with vegetables such as cabbage, cucumber and Chinese cabbage. Protein foods such as chicken, beef, egg, fish and sea food were major sources of Zn contributing with 53% of total zinc intake. The main individual Zn sources however were rice and chicken, which contributed 27% and 17%, respectively, to the total Zn intake. Rice and egg were the main sources of Fe and vitamin A respectively. Milk and milk products were little consumed at the household level.

Biochemical indices of status were more or less in line with the measured intakes for Zn and 44.3% of school aged children had a SZn concentration below the respective age and sex specific cut-offs and were classed as Zn deficient. On the other hand, only 1 % of the children were found to be VAD, 5% had ID and 8% were anemic.
Table 1  Pilot study to estimate approximate intake of Fe, Zn, vitamin A, phytic acid and the micronutrient status of school children in Satun province

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dietary intake (n=20, male=10, female=10)</th>
<th>% of EAR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (mg/d)</td>
<td>7.3±2.5</td>
<td>81</td>
</tr>
<tr>
<td>Zn (mg/d)</td>
<td>2.2±1.1</td>
<td>71(42)</td>
</tr>
<tr>
<td>Vitamin A (µg/d)</td>
<td>202.9±143.9</td>
<td>52</td>
</tr>
<tr>
<td>Phytic acid (mg/d)</td>
<td>37.6±18.3</td>
<td></td>
</tr>
<tr>
<td>Phytic:Fe (molar ratio)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Phytic:Zn (molar ratio)</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemical indicators</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/L)</td>
<td>127.1±8</td>
<td>n=92</td>
</tr>
<tr>
<td>SF (µg/L)</td>
<td>44±22.3</td>
<td>n=88</td>
</tr>
<tr>
<td>SZn (µg/dL)</td>
<td>69.4±10.5</td>
<td>n=88</td>
</tr>
<tr>
<td>SR (µmol/L)</td>
<td>1.1±0.3</td>
<td>n=89</td>
</tr>
<tr>
<td>Prevalence of anemia (%)</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Prevalence of ID (%)</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Prevalence of Zn deficiency (%)</td>
<td>44.3</td>
<td></td>
</tr>
<tr>
<td>Prevalence of VAD (%)</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

*EAR (Estimated Average Requirements) [26, 27] of children 7-12 y, based on 10% bioavailability for Fe and high (or moderate) bioavailability for Zn.

EAR of Fe (base on 10% bioavailability) is 8.9 mg/day, 8.8 mg/day and 10.4 mg/day for children 7-10 y, female 11-14 y (pre-menarche) and male 11-14 y respectively.

EAR of Zn (high bioavailability) is 2.7 mg/d, 3.6 mg/d and 4.3 mg/d for children 7-9 y, female 10-18 y and male 10-18 y respectively.

EAR of Zn (moderate bioavailability) is 4.7 mg/d, 6 mg/d and 7.2 mg/d for children 7-9 y, female 10-18 y and male 10-18 y respectively.

EAR of vitamin A is 357 µg/day, 429 µg/day for children 7-9 y and children 10-18 y respectively.
Efficacy study: Characteristics of subjects

Of the 744 children who were recruited in the prescreening phase, 203 eligible children were invited and agreed to participate in the efficacy trial (Figure 1). One hundred and one subjects and 102 subjects were randomly assigned to the triple fortified rice or the unfortified rice group (control), respectively. At baseline, subject characteristics including gender ratio, age, and anthropometric measurements (table 2) as well as the biochemical indicators of status (table 3) were not significantly different between the intervention and the control group. Of all participating children, 14.5% were stunted (HAZ < -2.0) and 7% underweight (WAZ < -2.0) [30].

The dropout rate was very low (total ~3%, triple fortified rice ~2%, control ~4%) and was mainly due to loss of interest. Fourteen children (5 in the triple fortified group, 9 in the control group) were excluded from data analysis because of low school attendance (< 80% during the feeding period) as was planned in the protocol. One child in the control group was sick on the day of final blood taking. In total, 21 subjects (~10%) were lost for final analysis and 182 completed the study according to the protocol. There was a low infection rate in this area with only 4% of children showing elevated CRP values.
Table 2 Baseline characteristics of the subjects in the intervention and control group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Intervention (n=101)</th>
<th>Control (n=102)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:Female (n)</td>
<td>49:52</td>
<td>49:53</td>
</tr>
<tr>
<td>Age (y)</td>
<td>9.5 ± 1.8</td>
<td>9.5 ± 1.8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>28.4 ± 8.2</td>
<td>27.7 ± 8.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>130.4 ± 12.3</td>
<td>129.8 ± 10.9</td>
</tr>
<tr>
<td>Weight- for-age Z score (WAZ)</td>
<td>-0.63 ± 1.13</td>
<td>-0.67 ± 1.29</td>
</tr>
<tr>
<td>Height- for-age Z score (HAZ)</td>
<td>-0.84 ± 0.95</td>
<td>-0.84 ± 1.13</td>
</tr>
<tr>
<td>BMI- for-age Z score</td>
<td>-0.23 ± 1.19</td>
<td>-0.38 ± 1.29</td>
</tr>
</tbody>
</table>

1There were no significant differences between groups

Compliance with feeding

On average, subjects received the test meal for 129.7 ± 14.9 days and this was comparable between the intervention (128.9 ± 14.9 days) and the control group (130.6 ± 14.7 days). The mean serving size of cooked rice (both triple fortified and unfortified rice) was 141.8 ± 0.4 g per day. Taking into account the leftovers of rice which were monitored and estimated by research assistants, estimated average amounts of rice consumed by subjects was 122.7 ± 18.0 g/d (123.7 ± 17.6 g/d for the intervention group, 121.6 ± 18.3 g/d for the control group). This corresponds to the provision of an additional amount of 8 mg of Zn, 8.8 mg of Fe and ~780 µg of vitamin A per day for children in the intervention group.
Biochemical indicators of nutritional status

Results of biochemical indicators before and after the intervention are shown in table 3. We found a significant time x treatment interaction for SZn concentration only. SZn was significantly increased in both groups (p < 0.05) at the end of the 5 months feeding period. In addition, at the end of the study SZn concentration was significantly higher in the intervention group than in the control group (p = 0.018). Results of SR, Hb and SF indicated that VAD, anemia and ID were not widespread in this population group. However, in the triple fortified group, SR concentration significantly improved over time (p=0.006) while there was no change in the control group. Hb concentration decreased in both groups at the end of the study but the decrease was only significant in the control group (p=0.034). However, mean Hb concentration after the intervention remained higher than the cut-off for anemia (> 120 g/L). SF values were high at the beginning of the study and did not change over time in both the intervention and the control group.

At baseline the prevalence of all micronutrient deficiencies were not significantly different between the two groups except for the prevalence of anemia (figure 2). Compared to baseline, the prevalence of zinc deficiency significantly decreased from 100% to 29% and 39% in the intervention and the control group, respectively. As mentioned above, the prevalence of VAD, anemia and ID were low at the beginning and remained unchanged at the end of the study in both groups except for the prevalence of ID which was significantly higher in the control group (9.5%) than in the intervention group (2.2%) at the end of the study (p=0.039).
**Table 3** Biochemical indicators of children in the intervention and control group during the efficacy trial.

<table>
<thead>
<tr>
<th>Biochemical indicator</th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SZn (µg/dL)</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Endpoint</strong></td>
</tr>
<tr>
<td></td>
<td>61.3 ± 4.2</td>
<td>61.3 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>n=101</td>
<td>n=102</td>
</tr>
<tr>
<td></td>
<td>72.8 ± 8.8</td>
<td>69.6 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>n=90</td>
<td>n=82</td>
</tr>
<tr>
<td><strong>SR (µmol/L)</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Endpoint</strong></td>
</tr>
<tr>
<td></td>
<td>1.01 (0.61 - 2.52)</td>
<td>1.07 (0.57 - 2.32)</td>
</tr>
<tr>
<td></td>
<td>n=101</td>
<td>n=102</td>
</tr>
<tr>
<td></td>
<td>1.09 (0.67 - 1.83)</td>
<td>1.07 (0.55 - 2.53)</td>
</tr>
<tr>
<td></td>
<td>n=91</td>
<td>n=84</td>
</tr>
<tr>
<td><strong>Hb (g/L)</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Endpoint</strong></td>
</tr>
<tr>
<td></td>
<td>127 (80 - 141)</td>
<td>126 (99 - 152)</td>
</tr>
<tr>
<td></td>
<td>n=101</td>
<td>n=102</td>
</tr>
<tr>
<td></td>
<td>125 (102.0 - 147.0)</td>
<td>124 (103 - 149)</td>
</tr>
<tr>
<td></td>
<td>n=94</td>
<td>n=88</td>
</tr>
<tr>
<td><strong>SF(µg/L)</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Endpoint</strong></td>
</tr>
<tr>
<td></td>
<td>36.6 ± 25.7</td>
<td>34.9 ± 25.5</td>
</tr>
<tr>
<td></td>
<td>n=91</td>
<td>n=98</td>
</tr>
<tr>
<td></td>
<td>37.1 ± 17.9</td>
<td>34.8 ± 28.4</td>
</tr>
<tr>
<td></td>
<td>n=91</td>
<td>n=84</td>
</tr>
</tbody>
</table>

*values are mean ± SD

** values are median (range)

***values are geometric mean ± SD

\(^1\) significant time X treatment interaction (p<0.05)

\(^a\) significantly different from base line (p<0.05)

\(^b\) significantly different from the control group at the same time point (p<0.05)
Figure 2 The prevalence of zinc deficiency (Zn def), vitamin A deficiency (VAD), anemia and iron deficiency (ID) the in intervention (Int.) and control (Cont.) group.

* significantly different from baseline (p<0.05)

** significantly different from control group at the same time point (p<0.05)

Discussion

The SZn levels of the Zn deficient Thai school children consuming the triple fortified rice increased markedly after the 5 month intervention with 71% of the children no longer being zinc deficient at the end of the study (Figure 2). Rather surprisingly, the control group fed the unfortified rice also markedly increased their SZn levels and 61% of the Zn deficient children were no longer Zn deficient at the end of the study. The improvement of the children’s Zn status with the Zn fortified rice was significantly better than with the non-fortified rice, demonstrating efficacy of the Zn fortified product. However, the major improvement in Zn status was due to the school lunch program itself. The Thai government subsidizes both school
lunch and free milk but the programs are not always constantly provided. During our intervention study however, the children regularly received white meat and fish with their rice for school lunch and they consumed free milk each school day. Other studies in Thailand where Zn was added to school lunch programs have also reported significant improvements in the SZn concentrations in the control groups [25, 31] and these findings were thought to be related to better dietary quality of food given at school and to an increased awareness of teachers and parents to the child’s nutrition.

We believe the improvement in Zn status of the control children in our study can be explained by the extra Zn provided by the school meal and free school milk. In our pilot study, the estimated Zn intake of children not receiving the school meals was 2.2 mg/day (0.73 mg/meal) which is lower than the EAR. The EAR of Zn for a high bioavailability diet is 2.7 mg/d, 3.6 mg/d and 4.3 mg/d for children 7-9 y, female 10-18 y and male 10-18 y respectively (EAR = Recommended Nutrient Intakes (RNI)/1.2). [26]. We estimate that the school lunch and free milk (200 ml/d) provided an extra 1.65 mg/d per day (0.97 mg from school lunch and 0.68 mg from milk). The calculation was based on portion sizes and food items described for the standard Thai school lunch [32] together with the menus recorded during the study period. The Zn content was calculated using a nutrient database of Thai foods (INMUCAL, Institute of Nutrition, Mahidol University, version WD 2.1). The additional Zn contained in the school meals and the free milk was consumed by both, the children receiving the fortified rice and those receiving the non-fortified rice. This extra 1.65 mg Zn would increase Zn intake of the control group to \( \approx 3.1 \) mg/d which is within the range of their recommended Zn intakes of 2.7 – 4.3 mg/d based on the EAR and could thus explain the increase in SZn. The fortified group received an additional 8 mg Zn per day which exceeds their requirement and could explain the
further improvement in SZN and Zn status. In support of our suggestion, Hess et al [33] reported that SZN decreased markedly at Zn intakes of 2-3 mg per day but rose sharply with additional Zn, reaching a plateau at the intake around 25-30 mg/day. In our study, it is likely that in the absence of the school lunch and free milk, the fortified rice alone would have resulted in a much stronger increase in SZN. Most previous Zn supplementation studies in young children have reported an increase in SZN concentration [19] whereas there has been a lack of response of SZN to Zn-fortified foods, even though they contained the same or slightly higher amounts of total Zn. This suggests that Zn may be less well absorbed in the presence of foods [19, 24, 34]. Serum zinc however is not a robust measure of Zn status. Recent studies in men and young children [35, 36] reported that SZN responded within 2 weeks in subjects receiving supplements but not in subjects consuming Zn fortified foods, indicating that SZN may not be as useful for monitoring short-term fortification programs [35]. Other reasons for the failure to show efficacy of Zn fortified foods include increased growth and infections in the study subjects, relatively high SZN concentrations at baseline, and a food vehicle inhibitory to Zn absorption [22]. Our success in demonstrating an increase in SZN by incorporating the triple fortified rice into the school lunch program could be due to the low phytate content of the fortification vehicle and the meals, the low Zn status of the study children at baseline, a relatively long 5 month intervention, and a low level of infections. A previous study in NE Thailand, in which a Zn containing micronutrient enriched seasoning powder was added to low phytate rice/noodle lunch meals, also reported a significant increase in SZN [25]. On the other hand, Zn added to high phytate wheat and maize products failed to improve Zn status [24, 37, 38]. Hess et al [22] have suggested that Zn fortified foods only increase SZN when not co-fortified with Fe, however our results do not support this suggestion as we found a positive impact on SZN concentration with a rice that was co-fortified with Zn, Fe and vitamin A. Since
our subjects were not growth restricted at baseline (mean HAZ, -0.8 and mean WAZ, -0.6; Table 2), we did not measure the impact of the triple fortified rice on growth. Previous Zn supplementation studies have shown a growth response only when children have an initial mean HAZ of < about -1.5 at baseline [20].

We have previously reported that similar extruded rice grains fortified with MGFP increased iron stores and decreased the prevalence of ID in Indian school children with low iron stores [13]. The current study was not designed to show an improvement in Fe status as most of the Zn deficient children in the study had adequate iron stores at baseline. Nevertheless, at the end of the study, there was a small but significantly lower prevalence of ID in the intervention children than the control group (Figure 2) and blood hemoglobin level had fallen slightly but significantly in the control children but not the intervention children (Table 3). Similarly the study was not designed to demonstrate the efficacy of the vitamin A fortification as the prevalence of VAD was <5% in all children at baseline. However, there was still a small but significant increase in SR in the intervention group but not the control group which confirms a good stability of vitamin A in the triple fortified rice grains.

In conclusion, this study demonstrated the efficacy of zinc sulfate in extruded rice grains triple fortified with Zn, Fe and vitamin A. It suggests that SZn can be used to monitor Zn efficacy studies with fortified foods provided the subjects have low SZn and a low infection rate at baseline, that the meals are low in phytate, and that the study is of sufficiently long duration (in our case 5 months). It also suggests that a more constant implementation of the current Thai school lunch programs containing meat and fish, and the regular consumption of free school milk, will by themselves substantially improve Zn status of school children in Southern Thailand.
Acknowledgement

We would like to thank the school teachers, children and families who participated in the study, staffs of Satun Provincial Health Office, Satun Hospital, and faculty of Agro-Industry Prince of Songkla University, Thailand. S.P., P.W., R.W. and R.H. designed research; S.P., P.W. and R.W. conducted research; S.P. and R.W. performed the statistical analyses; S.P. wrote the first draft of manuscript; S.P. and R.H. had primary responsibility for final content. All authors read and approved the final manuscript.
References

CONCLUSIONS AND PERSPECTIVES

The major goal of this PhD thesis was to develop triple fortified extruded rice grains containing iron, zinc and vitamin A with good stability of vitamin A and to demonstrate an improved vitamin A and zinc status in the target population of school children in Thailand.

Although fortified rice with vitamin A alone has already been successfully developed by PATH using cold extrusion [1], and its ability to improve vitamin A status in a target population demonstrated [2], the challenge is to add other micronutrients, especially iron, without a substantial degradation of vitamin A. This has not been achieved either by cold or hot extrusion.

In the first study, we successfully produced triple fortified rice grains containing iron, zinc and vitamin A using the hot extrusion technology. Grains containing ZnO+MGFP+vitamin A showed that vitamin A retention was comparable to that in grains fortified with vitamin A alone, even after storage under tropical conditions for 18 weeks. Although other zinc fortification compounds are possible with no sensory changes or loss of vitamin A, ZnO is the least expensive [3] and its absorption from fortified wheat based foods has been reported to be similar to ZnSO$_4$ [4]. However, it would be prudent to ascertain the absorption of different zinc compounds from multi-micronutrient fortified extruded rice grains to confirm the use of ZnO.

As expected, we found that light had a strong effect on stability of vitamin A. In the absence of light, iron caused vitamin A losses. Using ZnSO$_4$ caused higher losses of vitamin A when added together with iron (MGFP), compared to using poorly soluble zinc compounds with lower acidity such as ZnO and ZnCO$_3$. The latter two compounds reduced the catalytic effect of iron (MGFP) and losses of vitamin A in triple fortified extruded rice. The same combination of
micronutrient premix could be used for adding iron, zinc and vitamin A to other food vehicles, however the physical nature of food needs to be considered. Vitamin A fortification to food vehicles in powder form (e.g. wheat flour, maize flour) resulted in a higher loss than food vehicles in solid form (e.g. rice grain) due to oxidative degradation. In terms of sensory, although, the color differences ($\Delta E_{ab}$) between triple fortified rice and jasmine rice were relatively high ($\Delta E_{ab} \approx 9-14$), once the triple fortified extruded rice grains were mixed with normal rice (normally consumed in study area) at the ratio of 1:50, the taste and color were not distinguishable from the normal rice alone. Therefore, the possibility to reject the triple fortified rice is unlikely.

The key success factors for developing triple fortified extruded rice with good vitamin A stability are production methods, packaging and the characteristics of the fortificants. To formulate a product with appropriate fortification levels, vitamin A losses during the entire production process, losses during storage need to be carefully assessed. Additional measures, such as, cold storage conditions and vacuum packaging can be applied, however, cost of the fortified rice could become prohibitive.

Triple fortified extruded rice is efficacious in improving vitamin A (study 2) and zinc (study 3) status in school aged children, by significantly increased TBR of vitamin A, liver vitamin A concentration, and $\text{SZn}$ in the intervention group compared to the control group (receiving normal rice).

In study two, the stable isotope dilution technique using $^{13}$C retinyl acetate demonstrated a significant increase in TBR of vitamin A and liver vitamin A ($p < 0.05$) in children who received triple fortified rice for ~ 2 months, while this improvement could not be detected when using SR concentration as indicator. On the other hand, an increase in TBR of vitamin A and liver
vitamin A (from 0.122 to 0.220 µmol/g) which were almost double from baseline values warrants a concern for hypervitaminosis A if the intervention prolonged. This is similar to the case of vitamin A fortified sugar in Nicaraguan children, where 1 y after the intervention, 9 of 21 children had liver vitamin A concentration > 1.05 µmol/g [5] which is considered above the adequate level (0.1-1.0 µmol/g) [6]. Therefore, vitamin A fortification program using preformed vitamin A needs to be carefully monitored for both the level of vitamin A in the fortified food and vitamin A status of the target population to ensure that there is no risk of adverse health effects [7, 8]. With the improved processing method, loss of vitamin A as observed in this study was much less than that using other process. The level of vitamin A fortification could be reduced from that allowed for loss of 40% as used in the current study.

Regarding method for assessing vitamin A status, the isotope dilution method classified the children in our study as having only slightly above adequate status and almost have marginal vitamin A deficiency (mean liver vitamin A 0.133 µmol/g) while the SR method was less sensitive. Thus, the stable isotope dilution technique with labeled $^{13}$C retinyl acetate may be used as an alternative method for monitoring vitamin A status and impact of intervention programs. Its advantage includes requiring a small number of subjects and a shorter intervention duration to detect change and flag the potential risk of too high vitamin A. This method would be method of choice if the aim of the study is to determine the changes in vitamin A status in response to vitamin A intervention program, and to prevent from hypervitaminosis A, which cannot be assessed early enough by other methods [8]. It could be an alternative evaluation model, using a small number of children participated in the program without a need to conduct a large scale effectiveness evaluation. Moreover, the fortification level of vitamin A could be timely adjusted to optimize the vitamin A status of target population.
The third study showed that the prevalence of zinc deficiency was relatively high, with 44% of children in the province of Satun, Southern Thailand, showing low SZn concentration. This magnitude is considered a public health concern and intervention programs to improve zinc status of the population are recommended [3]. Based on these results, zinc status of other at-risk population group in the same area e.g. women of childbearing age, small children also need to be evaluated. Only a few of the previously reported zinc fortification efficacy found a positive impact of fortification Zn on SZn concentration [9]. In our study, we successfully showed an improvement in SZn (p < 0.05), as well as a lower rate of zinc deficiency in children in the intervention group after 5 months of intervention. These results indicate that SZn concentration can be used to evaluate the impact of a fortification program for children having low SZn concentration. Low infection rate, consumption of foods which are low in phytate and a relatively long study duration (5 months) may be key factors for the observed impacts.

Although, the change in SZn concentration from the triple fortified extruded rice group was higher than that in the control group, SZn of the control group also increased significantly over the course of the study. We speculated that this was due to a better adherence of the schools to providing free school lunch and free milk during the school semester, which provided additional sources of zinc to the habitual diet. Nutritional status of children in this area are likely lower during semester break when children rely only on food from home which normally contain lower in quality and quantity compare to school lunch meal. Thus, another important finding of this thesis is that every attempt should be made to ensure that the schools follow the government directives regarding subsidized school meals and milk. In addition, the awareness of teachers and parents on benefit of micronutrients for their children’s health may contribute to the good adherence to the intervention.
CONCLUSIONS

For nutrients which there are no storage in the body, such as zinc, intervention program is necessary and triple fortified extruded rice could be used. Triple fortified extruded rice may be introduced into the school lunch program by using the same delivery channel.

Extruded fortified rice, especially hot extrusion, is superior to other fortification methods in term of the appearance, taste and stability of micronutrients, the higher cost may be offset if this method is used in mass production [10]. The fortified extruded rice could be centrally produced with different micronutrient combinations to fit with the requirements of different subpopulations, e.g., geographical regions of Thailand. Using such triple fortified extruded rice is a potential strategy to combat micronutrient deficiencies in rice-consuming countries. Combining this approach with the continuous support and monitoring of school lunch programs by the government are important ways of maintaining/improving health status of school aged children.

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