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Department of Applied Human Nutrition

Formulating a salt premix solution co-fortified with thiamine and iodine

by
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Abstract

Introduction: Salt fortification is a large-scale, cost-effective method for preventing population-wide nutrient deficiencies. Thiamine (vitamin B_1) deficiency is a national public health concern in Cambodia. Salt iodization is already mandated in Cambodia, so co-fortifying salt with both iodine and thiamine could be an efficient solution, especially if existing spray fortification equipment is employed. Iodine and thiamine react unfavourably in aqueous solution: Thiamine chloride hydrochloride (TClHCl) lowers pH, catalyzing reduction-oxidation reactions leading to discolouration, precipitation, and rapid losses of iodine. However, both thiamine and iodine may be stabilized at $5.00<pH<6.00$, potentially minimizing losses of either nutrient.

Objective: To explore the viability of various thiamine-iodine solutions designed for the spray fortification of salt in Cambodia.

Methods: Twenty-four solutions were formulated to achieve a target of $5.00<pH<6.00$ using potassium iodate (KIO_3) or potassium iodide (KI), TClHCl, sodium acetate, sodium carbonate, sodium citrate tribasic dihydrate, dextrose, sodium hydroxide (NaOH), carbonate buffer, and/or citrate buffer. All solutions were formulated with a minimum of 3% w/v iodine and 7.8% w/v thiamine, and were stored under typical Cambodian conditions (30°C, 65% humidity) for up to four weeks. Data were collected at 0, 6, 12, 24, and 48 hours, and at 1, 2, 3, and 4 weeks, where viable. pH was measured using an electronic pH meter, and observations pertaining to colour, odour, and other physical characteristics were recorded. Photographs of each solution were also taken. Thiamine and iodine concentrations were analyzed using high performance liquid chromatography and high-pressure ion chromatography, respectively, among a subset of viable solutions.

Results: All solutions formulated with KIO_3 developed sharp odours, pigments, and precipitate. Those with KI were less odourous, clear or white in appearance, and exhibited sedimentation during storage. No experimental solution maintained pH within the desired range of $5.00<pH<6.00$, regardless of thiamine concentration (7.8–21% w/v), source of iodine (KIO_3 or KI), alkalizing agent or stabilizer used. Still, solutions analyzed for micronutrient content retained ≥80% of both iodine and thiamine after 2 weeks of storage, and >60% after 4 weeks. Three solutions formulated with KI and TClHCl, along with either NaOH, 0.2 M carbonate buffer, or no alkalizing agent, maintained acceptable concentrations of both thiamine and iodine through the duration of the experiment.

Conclusion: Findings suggest that the premix solution formulated with KI and TClHCl without additional reagents is the most viable for the spray fortification of salt with iodine and thiamine. Future work should explore the viability of this solution when sprayed onto locally produced salt and stored in typical Cambodian conditions. Sensory evaluation and efficacy trials will be needed to determine the acceptability and efficacy of any prospective co-fortified salt, along with cost-benefit and quality assurance analyses, prior to implementation of a national thiamine-iodine co-fortified salt program in Cambodia.

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List of Acronyms and Abbreviations

aq	Aqueous state
CDHS	Cambodia Demographic and Health Survey
Cl^-	Chloride
DFS	Double fortified salt
EAR	Estimated average requirement
H^+	Hydrogen
H_2SO_4	Sulfuric acid
HPLC	High performance liquid chromatography
I^-	Iodide
I_2	Elemental iodine
I_3^-	Triiodide
IO_3^-	Iodate
KI	Potassium iodide
KIO_3	Potassium iodate
Lao PDR	Lao People's Democratic Republic
LMIC	Lower- and middle-income country
MDD	Minimum dietary diversity
MDD-W	Minimum dietary diversity for women
NaI	Sodium iodide
$NaIO_3$	Sodium iodate
NaOH	Sodium hydroxide
NO_3^-	Nitrate
ppm	Parts per million
RDA	Recommended dietary allowance
s	Solid state
SRMA	Systematic review and meta-analysis
TClHCl	Thiamine chloride hydrochloride
TDD	Thiamine deficiency disorder
TFS	Triple fortified salt
ThDP	Thiamine diphosphate

ThMP	Thiamine monophosphate
ThTP	Thiamine triphosphate
TMN	Thiamine mononitrate
UL	Tolerable upper-level intake
UNICEF	United Nations Children's Fund
w/v	Weight per volume
WRA	Women of reproductive age

1.0 Introduction

Thiamine, or vitamin B_1, is a water-soluble micronutrient essential to carbohydrate metabolism and infant neurological development. Insufficient daily thiamine intake can lead to deficiency and can manifest as a cluster of syndromes affecting the nervous and cardiovascular systems, collectively termed Thiamine Deficiency Disorders (TDDs). TDDs are most prevalent in low- and middle-income countries, specifically in South and Southeast Asia, where staple foods are low in thiamine and dietary diversity is often poor. In Cambodia, the diet is comprised primarily of thiamine-poor white, polished rice. As many as four in five women of reproductive age (WRA) may be thiamine deficient, and more than two-thirds of infants aged 6–12 months may be at risk of potentially fatal TDD. Thiamine deficiency among WRA in Cambodia is a national public health concern that must be addressed promptly. Food fortification is a reliable, safe, and cost-effective method for preventing population-wide nutrient deficiencies. The fortification of table salt with iodine is a widely celebrated public health strategy for preventing iodine deficiency in most countries globally. Previous work has demonstrated that salt is a suitable vehicle for thiamine fortification in Cambodia, where no thiamine fortification programs exist to date. Given the existing public health messaging and spray-fortification infrastructure in Cambodia, co-fortifying salt with thiamine and iodine holds promise as an inexpensive and sustainable means of preventing thiamine deficiency in Cambodia. Prior to implementing a novel thiamine fortification program, the stability of a co-fortification premix solution which will be sprayed onto the salt must be assessed. Preliminary investigations have demonstrated that thiamine and iodine do not co-exist favourably in solution. As per Cambodian regulations, salt is routinely fortified with potassium iodate. Iodate is susceptible to reduction to elemental iodine in the presence of acid, moisture, and hygroscopic impurities common to crude salt, such as magnesium chloride. The reduction of iodate causes rapid losses of iodine due to sublimation of elemental iodine out of solution. In addition, thiamine is a highly unstable vitamin. Thiamine is most stable in acidic solutions and exhibits rapid degradation in neutral or alkaline conditions. Given these technical challenges, it is essential to test potential co-fortification premix formulations for stability in advance of human efficacy trials. Thus, this work aims to assess various strategies to stabilize and maintain concentrations of both thiamine and iodine in a fortification premix for the prospective co-fortification of commercially available salt in Cambodia.

2.0 Literature Review

2.1 Cambodia

Cambodia is a lower-middle income country in Southeast Asia bordered by Thailand to the northwest, Lao People's Democratic Republic (Lao PDR) to the northeast, Vietnam to the east and southeast, and the Gulf of Thailand to the southwest (1). Cambodia is the smallest country by landmass on the Indochina peninsula (2) and has been topographically described as "saucer-shaped" (3), such that low-lying wetlands and floodplains are fringed by highlands and mountainous regions at the country's perimeter (1,3). Central to the wetlands are the Mekong and Bassac rivers and the Tonlé Sap lake (1,3). The climate in Cambodia is dictated by monsoonal winds and is dichotomized into two distinct seasons: a "wet" season, with tropical winds, heavy rainfall, and high humidity from mid-April to October; followed by a "dry" season running November to mid-March (1,3). Flooding of the Mekong river during the wet season renders the surrounding wetland highly fertile (3); however, much of the land in Cambodia is void of nutrients and is not arable (1–3). Indeed, the topography and climate heavily influence settlement, occupation, agriculture, food availability, and dietary patterns nation-wide (4,5).

The population of Cambodia is approximately 17 million (51.1% female), growing at an average rate of 1.5% per year (6). Though the number of urban households is increasing at an annual rate that is 50% faster than that of rural households (7), the 2021 *Cambodia Socio-Economic Survey* reports that 61% of the population still reside in rural villages (7,8) where agriculture is the primary source of income (1,7,8). Agriculture is a prominent economic sector in Cambodia (1,2,9), representing 22% of the GDP in 2022 (10). Rice is the most abundant and important cultivar (7,9,11), followed by fruits, then starchy crops; reflecting an estimated 61–70%, 20%, and 9% of agricultural outputs, respectively (7). Aquaculture and the raising of livestock are also important sources of income for Cambodians, particularly in rural areas (9,11,12). Given their economic importance, agricultural and other rearing activities largely shape the dietary patterns of people living in rural Cambodia (9,10).

2.1.1 Frequently consumed foods

Rice is an abundant crop and ubiquitous staple food in Cambodia. In *et al.* report that all participants (*n*=941) in a nationally-representative survey consumed rice at a minimum of twice

per day (13). Adults consumed an average of 823 (± 334) g of cooked rice per day (13), contributing to nearly 70% of total energy intake (14). Other starchy foods such as cassava, taro, and maize are also consumed in Cambodia, but in smaller volumes (13). Common vegetables in the Cambodian diet are morning glory, bitter melon, and lotus root (15). While In *et al.* report that 98.5% of participants consumed some amount of vegetables daily, the quantity of fruit and vegetables consumed remains low (13), with a combined average consumption of just over 80 g/day (14).

Fish is another important and ubiquitous food source in the traditional Cambodian diet (13,16). Fermented fish paste, *prohok*, is of particular importance, as it contributes meaningfully to intakes of protein, iron, and essential fatty acids (16). Quantities of fish consumed in Cambodia are reported to increase during the dry season compared to the wet season, while those of rice, other starchy foods, vegetables, and flesh foods decrease (13). Flesh foods, such as chicken, beef, and pork, are consumed in Cambodia, but only in small amounts, especially amongst women (13,17). The consumption of flesh foods is also generally higher amongst urban populations than rural (13,18). Plant-based proteins, particularly beans, legumes, and pulses, are not abundant in the Cambodian diet (14,19). Alcohol is not commonly consumed in Cambodia; however, both the proportion of consumers and the quantity of alcohol consumed nearly double in the dry season compared to the wet (3.4% of alcohol consumers in the dry season compared to 7.6% the wet; average consumption of 240.2 mL alcohol/person/day in the dry season compared to 355.8 mL alcohol/person/day in the wet) (13).

2.1.2 Malnutrition and food security

Dietary patterns in Cambodia and the types of foods available vary markedly by province and by urban/rural setting (13), as do rates of poverty and malnutrition (20). Though its prevalence has declined over the past two decades (21), undernutrition is still prevalent amongst women of reproductive age (WRA) and children in Cambodia, with notable inequities between those living in urban areas compared to rural (19). Poor nutritional quality and limited diversity of foods consumed contribute to undernutrition (14,22). As reported in the *Cambodia Demographic and Health Survey 2021-2022* (CDHS), less than half (49.8%) of Cambodian women aged 15–49 years living in rural areas achieved adequate dietary diversity (19). From the same report, 50% of

infants aged 0–5 months were exclusively breastfed, 40% of children aged 6–23 months were still breastfed, and 82% of children 0–24 months were ever breastfed (19). Limited maternal dietary diversity increases the risk for micronutrient deficiencies amongst infants and young children in Cambodia whose diets are comprised predominantly or exclusively of human milk—especially for thiamine, given that the thiamine content of human milk is directly related to maternal thiamine intake (23).

2.2 Thiamine

Thiamine, also called vitamin B_1, is a water-soluble micronutrient essential to carbohydrate metabolism and infant neurological development (24–27). It is important to the functioning of several systems in the human body (28), but is of most importance for the neurological and cardiovascular systems (29). Thiamine can exist in the human body as free thiamine, or may be phosphorylated into its mono- (ThMP), di- (ThDP), or triphosphate (ThTP) derivatives (29). It is most abundant as the biologically active form, ThDP (24), which accounts for roughly 80% of total circulating thiamine (29). ThDP serves as a cofactor for enzymatic processes critical to the metabolism of carbohydrates and amino acids (29–31), and to the synthesis of neurotransmitters (25,32). Thiamine occurs naturally in many foods, with pork, whole grains, and legumes amongst the richest sources (30,31). Thiamine is also regularly added to refined cereal and grain products in high-income settings (30,31). In low-resource settings where the diet is repetitive and comprised of thiamine-poor staples, such as polished white rice, adequate daily intakes of thiamine are difficult, if not impossible, to achieve (22).

Thiamine deficiency may affect a large proportion of the Cambodian population (29). Nearly 2,500 kcal/day per capita are available in the Cambodian national food supply (14); yet the estimated mean thiamine availability per capita is less than the recommended dietary allowance (RDA) for adult men (1.2 mg/day; see **Table 2-1** for the dietary reference intakes for thiamine) (29). A majority of the dietary energy consumed in Cambodia is derived from polished white rice, which has had much of its micronutrient content discarded following removal of the bran and germ (24,29). While thiamine-rich foods—namely pork and some varieties of fish (13,17)—may be consumed as part of the typical Cambodian diet, the frequency and/or the quantity in which these are consumed is not sufficient to achieve adequate thiamine intake. This is

especially true amongst perinatal women and infants (22), who have the most demanding thiamine requirements, but often the lowest intake (33).

Table 2-1: Dietary reference intakes for thiamine, adapted from (24)

	Dietary reference intake[1] (mg/d)			
Life-stage group	EAR	RDA	AI	UL[2]
Infants				
0–6 mo	-	-	0.2	-
7–12 mo	-	-	0.3	-
Children				
1–3 y	0.4	0.5	-	-
4–8 y	0.5	0.6	-	-
9–13 y	0.7	0.9	-	-
Female				
14–18 y	0.9	1.0	-	-
19+	0.9	1.1	-	-
Pregnancy	1.2	1.4	-	-
Lactation	1.2	1.4	-	-
Male				
14–18 y	1.0	1.2	-	-
19+	1.0	1.2	-	-

[1]Abbreviations: EAR, estimated average requirement; RDA, recommended dietary allowance; AI, adequate intake; UL, tolerable upper-level intake.
[2] No UL for thiamine has been established, as no adverse events related to excessive intake have been reported (41).

During pregnancy and lactation, the RDA for thiamine increases to 1.4 mg/day, up from 1.1 mg/day for non-pregnant WRA (24). No tolerable upper intake level (UL) for thiamine has been established, as no adverse events related to excessive intake have been reported (31). Insufficient maternal thiamine intake can be detrimental to exclusively-breastfed infants, who rely solely on human milk for the thiamine needed for optimal growth and cognitive development (23,29). Relative to thiamine requirements (0.2 mg/day for infants aged 0–6 months (24)), growth and development are considerable during the first months of life, and clinical manifestations of deficiency advance more quickly in infants than in any other life-stage group (29). Accordingly, infants are most vulnerable to the effects of thiamine deficiency (24,29).

2.2.1 Thiamine deficiency disorders

Clinical and subclinical manifestations of thiamine deficiency are collectively referred to as Thiamine Deficiency Disorders (TDDs). TDDs occur most commonly in low- or middle-income

countries (LMIC) due to poor dietary thiamine intake (29). Exacerbating poor thiamine intake in Cambodia are the increased thiamine requirements caused by higher physiological demands for thiamine necessitated by elevated carbohydrate intake (*i.e.*, >65% of total energy intake) related to the high volume of rice consumed (24). Low levels of circulating thiamine compromise energy metabolism and increase intracellular oxidative stress (28,31), which can significantly impair cognitive development and may lead to fatal heart failure in infants and young children (29).

Historically, TDDs have been categorized as Wernicke's encephalopathy (or Wernicke-Korsakoff syndrome), which is characterized by neurological symptoms such as tingling or loss of function in the extremities and face; or as beriberi, which can be further categorized into "wet" and "dry" presentations that predominantly affect the cardiovascular system or nervous system, respectively (29). Given that Wernicke's encephalopathy is often less fatal than pediatric presentations of thiamine deficiency (29), this form of TDD will not be discussed further. Of particular concern is infantile beriberi, such that the risk of TDD is highest in the first year of life (29). Manifestations of infantile beriberi are multitudinous and non-specific (25,29,34). Generally, symptoms of infantile beriberi can fall along a spectrum, including the acute cardiologic form, characterized by colic, refusal of bottle or breast, vomiting, and sudden cardiac shock; the aphonic form, characterized by a piercing or silent (aphonic) cry, restlessness, and shortness of breath; or the pseudo-meningitic form, characterized by involuntary eye movement, muscle twitching, and unconsciousness (29). The onset of symptoms is often rapid and may lead to fatality within a few days following the initial presentation of symptoms (35).

There is a lack of consensus on a biomarker cut-off value to define thiamine deficiency and/or insufficiency, making it difficult to ascertain the true pervasiveness of thiamine deficiency amongst WRA and infants in Cambodia. In 2017, Whitfield *et al.* estimated that anywhere from 27–78% of mothers and 38–70% of children aged 6–12 months in Cambodia were thiamine deficient, depending on the cut-off value used (36). Despite the true prevalence of thiamine deficiency remaining unknown, given the poor maternal intakes and serious adverse outcomes of infantile deficiency, coupled with high breastfeeding rates, it is evident that thiamine deficiency

among WRA in Cambodia is a national public health concern that must be addressed promptly (36). As such, the remainder of this review will focus on thiamine.

2.3 Interventions

There exist several public health interventions to improve the thiamine status of WRA in Cambodia. Specifically regarding thiamine, Whitfield *et al.* have described nutrition education, supplementation, and food fortification as promising strategies to increase thiamine intakes in countries where WRA and infants are vulnerable to thiamine deficiency and TDDs (29).

2.3.1 Nutrition education

The evidence supporting the use of nutrition education to improve the nutritional status of WRA in LMIC is situational and context-dependent (37). Increasing the diversity of foods consumed is the preferred method of improving micronutrient status, such that it is the most sustainable means to increase micronutrient intake (38,39). Unfortunately, this is not feasible in many regions globally due to poverty and limited access to—or availability of—diverse nutritious foods (38,39). In Cambodia, nearly four million individuals lack year-round access to adequate quantities of nutritious food (5,40). Given that underlying causes of thiamine deficiency extend beyond those that can be governed by the individual, such as natural disasters leading to food insecurity and poverty (29), nutrition education is not an ideal public health intervention to increase thiamine intakes of WRA in Cambodia.

2.3.2 Supplementation

A number of the most efficacious interventions for improving both maternal and child health outcomes worldwide involve the provision of one or more micronutrients through supplementation (41). Importantly, many of the most effective supplementation programs, such as the provision of vitamin A supplements, demand supplementation only biannually, given the storage physiology of this fat-soluble vitamin (42). The metabolism of thiamine is very different, and thus necessitates adequate near-daily intake through food or supplementation to maintain sufficient thiamine status (29). Gallant *et al.* recently assessed the effect of daily thiamine supplementation provided to lactating women (n=335) in Kampong Thom province, Cambodia, from 2 through 22 weeks postnatal, on total thiamine concentrations of expressed human milk

(43). Three dosages were trialed (low [1.2 mg thiamine/day], moderate [2.4 mg/day], and high [10 mg/day]), alongside a placebo (0 mg/day). All three experimental dosages significantly improved total thiamine concentrations of human milk at all collection points (2, 4, 12, and 24 weeks) compared to the placebo, and there was no statistically significant difference in total thiamine concentration of human milk found between the three experimental groups after 22 weeks of daily oral thiamine administration (43). Given this, lactating women require only 1.2 mg thiamine daily to maximize human milk thiamine concentrations. However, using data from the same trial, Measelle *et al*. reported that benefits to infants' language skill development were most prominent in infants whose mothers were receiving the highest dose of thiamine (10 mg/day) (44). While these data demonstrate the efficacy of relatively low-dose thiamine supplementation for lactating women in Kampong Thom province, there exist potential barriers to any prospective thiamine supplementation program targeting WRA nation-wide. Namely, difficulty accessing necessary supplements in rural areas, a lack of federal support and/or external aide to manufacture or procure supplements, and potential disruptions in the supply chain (45).

2.3.3 Fortification

Food fortification is the practice of adding one or more nutrients to commonly consumed foods during processing to increase their nutritional value (46,47). It is a reliable, safe, and cost-effective method for preventing population-wide nutrient deficiencies long-term (38,46–48). Food fortification does not require behaviour change at the individual level; instead, if an ideal food vehicle is selected, it is passively consumed as part of the habitual diet (47). As such, fortification is a very attractive public health intervention to increase the consumption of key nutrients otherwise lacking in the diet. There exist three primary domains of food fortification: large-scale or mass fortification, biofortification, and point-of-use ("at home") fortification (46–49). The latter two approaches are explained in greater detail elsewhere (46,50,51).

Successful large-scale fortification programs rely on the selection of an appropriate food vehicle to carry the fortificant. Candidate foods must be centrally processed; inexpensive, and thus, easily accessible in LMIC; and consumed regularly in fairly consistent quantities (47). Mannar and Khan describe three tiers of potential food vehicles suitable for fortification, including staple foods (rice, oil, salt), basic foods (packaged cereals, dairy products), and value-added foods

(condiments, beverages) (47). Staple foods are the most economical vehicles for large-scale fortification, and are the most appropriate for the Cambodian context. Staple foods also offer the greatest potential for micronutrient delivery across the population—especially to those most vulnerable to deficiency (47). In Cambodia, the most abundant staple food is polished white rice (24), which is consumed daily by nearly every member of the population (13).

Naturally, rice appears to be the ideal fortification vehicle. In fact, fortification of rice with at least one micronutrient is mandatory in many other countries (52). Nevertheless, there exist several limitations to the large-scale fortification of polished rice in Cambodia. The coating and dusting technologies for rice fortification (described elsewhere (24,53)) utilized in other countries are not appropriate for use in Cambodia, where the kernels are repeatedly rinsed and soaked before cooking (24), and thus, stripped of the added nutrients (24,29). Moreover, rice milling is largely decentralized in Cambodia, which would greatly complicate distribution of the fortification premix, would impede quality control, and would necessitate the procurement of specialized machinery by many independent, small-scale producers (24).

Considering the drawbacks of fortifying intact kernels, fortifying grain as a milled flour is much more practical (52). Still, wheat flour fortification is not suitable for the provision of micronutrients in Cambodia (54), where consumption is insignificant, and disparities in consumption exist between wealth quintiles and between rural and urban dwellers (19,55). Other commodities such as vegetable oil, soy sauce, and fish sauce have been identified as more suitable fortification vehicles in this setting (56); however, Whitfield *et al.* note that the decentralized production of fish sauce complicates enforcement of fortification regulations (29). Many rural households make their own fish sauce, rendering large-scale fortification of this condiment ineffective for those who would otherwise be the largest beneficiaries of such a program. To date, the only mandatory fortification program in Cambodia is salt iodization (55).

2.3.4 Salt iodization

One of the most widely celebrated fortification programs globally is the addition of iodine to table salt, otherwise known as salt iodization. First introduced in Switzerland in the 1920s, salt iodization is now mandatory in over 124 countries worldwide (57), including Vietnam, Thailand,

Lao PDR, and Cambodia (55). As of 2018, nearly 90% of the global population used iodized salt (57). Iodine is a micronutrient required in trace amounts; yet, it is an essential component of thyroid hormones, and is vital to fetal and infantile development and to lifelong cognition (58,59). The fortification of table salt with iodine is the most cost-effective public health intervention to reduce the burden of iodine deficiency disorders within a population (57,60–62). Much like thiamine, daily requirements for iodine increase during pregnancy and lactation, making perinatal women and infants most at risk for deficiency (60).

Across Cambodia, salt is an affordable and universally used condiment. Salt is consumed in relatively consistent quantities throughout the year by all life-stage groups regardless of economic class or geographic region (13,24), making it an ideal fortification vehicle. The national average salt intake was estimated to be 8.5 g/day per person over 18 years of age in 2016 (14). More recently, Chan *et al.* estimated salt intake specifically amongst lactating women living in rural Cambodia—the target population of any prospective national thiamine fortification program—to be 7.7 g/day/person (an adjusted value reflecting intake from all food sources) (63). Importantly, women's salt consumption does not appear to be affected by cultural peripartum food avoidances (64), and is thus expected to remain consistent irrespective of life-stage or parity.

2.4 Salt as a fortification vehicle in Cambodia

Most (~85%) of the salt consumed in Cambodia is produced domestically in Kep and Kampot provinces (65). Both coarse and refined salt are produced by distilling sea water through a series of evaporation pools (66), the latter requiring further refining via boiling to yield a smaller crystal (65,67). The Salt Producers Community of Kampot and Kep are responsible for the marketing, sales, and iodization of all domestically produced salt (65). Coarse salt undergoes spray fortification, in which a potassium iodate (KIO_3) premix solution is sprayed onto the salt and is let to dry; whereas refined salt can be either spray fortified, or fortified by mixing the salt with powdered solid KIO_3 (67). While the iodization of salt became mandatory in Cambodia in 2004 (65,68), the presence of iodized salt in the household varies markedly year-to-year (69) and between rural and urban populations (19). According to 2021–22 CDHS data, iodized salt was present in less than half (49%) of all households where salt was tested, down considerably from

previous years (19,69). Moreover, Laillou *et al.* tested the iodine content of 1862 salt samples collected from various markets across 24 Cambodian provinces in 2014, and found that only 8% of tested samples were adequately iodized (67). This is likely explained by UNICEF ceasing procurement of iodine premix for the country, coupled with substandard quality control measures in place at the time (29,67,69). In spite of the inconsistent compliance to national salt fortification legislature within the past decade, salt remains a promising vehicle for micronutrient delivery in Cambodia due to its universal consumption and recent government efforts to control domestic salt iodization practices (69).

In 2018, the Government of Cambodia released *Prakas* 85, which introduced more stringent regulations for salt iodization, along with a new logo to indicate certified iodized salt (70). Following the new regulations, hand-spraying for spray fortification was no longer permitted (69). As such, most producers shifted to using a manually operated spray fortification machine—whereby a small amount of a standard iodine premix solution is thoroughly incorporated into the salt, which is then air-dried on a conveyor belt—with purchasing assistance from UNICEF (69,70). These machines are low-cost and do not require electricity, making them ideal for salt fortification in rural settings. Considering the iodization procedures and public health messaging surrounding the importance of using fortified salt are already in place (29), leveraging the existing machinery to fortify salt with both iodine and thiamine holds promise as an inexpensive and practical means to increase the thiamine intake of perinatal women, and hence, to prevent thiamine deficiency and subsequent TDDs amongst infants and young children in Cambodia.

2.5 Co-fortification of salt

The co-fortification of salt with two or more micronutrients is not a novel concept. The first attempts to doubly fortify salt with iodine and iron were made in India in the 1990s (71), using a rudimentary formulation that ultimately rendered the product undesirable and impractical (72). Since, several techniques have been developed to improve the overall desirability and potentiality of a double-fortified salt (DFS). In relevant literature, "DFS" refers specifically to that containing both iron and iodine. Given that deficiencies in iron and iodine are amongst the most prevalent worldwide (73), a large majority of the current research investigating the co-fortification of salt focuses specifically on iron-iodine DFS. A 2021 systematic review and meta-

analysis (SRMA) of 22 studies examining the effects of DFS on several indicators across various sex and life-stage groups found significant improvements in hemoglobin concentration (standardized mean difference [95% CI]: 0.33 [0.18, 0.48]), ferritin (0.46 [0.21, 0.71]), anemia (RR [95% CI]: 0.80 [0.70, 0.92]), and iron deficiency anemia (RR 0.36 [0.24, 0.55]) compared to standard iodized salt (74). These trials included several formulations of DFS, differing in the form of iron used, the method of incorporation, and in the concentrations of iron and iodine of the final salt product. Select methods will be reviewed in greater detail to inform plausible means to co-fortify salt with iodine and thiamine.

The development of an economical co-fortified salt is technically challenging given that the physical and chemical properties of salt do not favour the coexistence of fortificants (75). Fortificants may react unfavourably during periods of storage or when exposed to high temperatures (*i.e.*, when cooking), resulting in changes to organoleptic properties (flavour, odour, colour) and in potential losses of one or both nutrients (72,75,76). Also important to consider are the rate of degradation of the selected fortificants (77), and the amount of the fortificants required to achieve the desired nutrient content per serving. Accordingly, any successful method employed to co-fortify salt must either place a physical barrier between the selected fortificants, or must utilize stabilizing compounds to limit their reactivity and to retain nutrient content and optimal sensory characteristics. Oftentimes, this is achieved through microencapsulation of the fortificant (75).

The most successful method to fortify salt with multiple micronutrients described to date was pioneered at the University of Toronto, and involves the dry-mixing of an extruded, microencapsulated ferrous fumarate (iron) premix with ordinary iodized salt in a ratio of 1:200 by weight to create DFS (77,78). This technology prevents the chemical interactions between iron and iodine that would otherwise cause unfavourable losses of iodine (82) and adverse changes to the colour and odour of the salt (79). Beyond retaining nutrient content throughout cooking and periods of storage, this formulation of DFS has been demonstrated effective at delivering iron and iodine in large-scale operations in India (77). Still, the development of a microencapsulated thiamine premix would not be practical for use in Cambodia, given the high

costs of specialized equipment and the large number of independent producers that would need to adopt this technology (67).

Encouragingly, alternative methods to fortify salt with iodine and various B vitamins have been documented that are more appropriate for low-resource settings. Modupe *et al.* assessed the stability of folic acid and iodine in various aqueous premix solutions intended for the spray fortification of salt, after two months of storage at three different temperatures (80). Premix solutions were prepared using purified water, 0.5–1% weight/volume[1] folic acid, and 2% w/v iodine; and the pH was corrected to 9 or 11.2 using a 0.1 M sodium carbonate solution. After two months of storage at 25, 35, or 45 ºC, >70% of both the folic acid and iodine were retained in all premix solutions. Iodine retention was not impacted by initial folic acid concentration, pH, or temperature, while folic acid retention was slightly greater at pH 11.2 compared to at pH 9 (80).

The same authors (80) also tested the stability of folic acid and iodine in a fortified salt. Premix solutions were sprayed onto food-grade refined salt, which was then air-dried and mixed with microencapsulated ferrous fumarate to create triple fortified salt (TFS). Experimental concentrations of folic acid, iodine, and iron in the final TFS samples were 12.5 or 25 ppm, 50 ppm, and 1000 ppm, respectively, equal to at least 50% of the RDA for each nutrient per 10-gram serving (80). Samples were packaged in clear polyethylene freezer bags prior to storage. Importantly, the initial moisture content within the packaged TFS samples was kept low (<0.06%), given that high moisture content accelerates iodine losses (38,81). Following six months of storage at 25, 35, or 45 ºC and 60–70% relative humidity (RH), 70–85% of folic acid and 85–95% of iodine was retained in all TFS samples. Initial folic acid concentrations did not greatly affect iodine retention in any of the stored TFS samples. In fact, authors speculate that the higher concentrations of folic acid may have stabilized the iodine (80).

In a similar analysis by Modupe and Diosady, 0.01% w/v vitamin B_{12} was also added into experimental premix solutions containing 2% w/v iodine and 1% w/v folic acid, adjusted to pH

[1] In relevant literature, "% w/v" is generally used to indicate concentrations of compounds in aqueous premix solutions, while "ppm" is used to indicate concentrations of compounds in the finished salt product. The same conventions will be followed herein.

8–9 (82). After only two months of storage, B_{12} losses were up to 90%, even with the addition of stabilizing antioxidizing agents. Interestingly, premix solutions containing only vitamin B_{12} and iodine (*i.e.*, no folic acid), at 0.01% w/v and 2% w/v, respectively, adjusted to pH 6.9, were much more stable, exhibiting >75% retention after two months of storage across all three experimental temperatures (25, 35, or 45 ºC). From these trials, it appears that the co-existence of iodine and thiamine in a single fortification premix would be viable, so long as the concentrations of the fortificants are appropriate to provide sufficient iodine and thiamine to meet the target population's intake, and that various factors affecting the stability of both nutrients, as described below, are considered and accounted for in the premix formulation.

2.6 Iodine as a fortification ingredient

Iodine is a trace element required in only small amounts (30); yet, inadequate intake can cause severe deficiency in adults and mortality in infants and young children (61,83). While present naturally in considerable amounts in foods of marine origin, including seaweed, cod, and oysters (58,83), many people globally rely on iodine-fortified foods to maintain adequate intake (58,83). Iodine may be added to several foods, but is most widely consumed through iodized salt (57,83). Salt may be iodized with various iodides (e.g., sodium iodide (NaI), cuprous iodide (CuI), potassium iodide (KI)) or iodates (e.g., sodium iodate ($NaIO_3$), KIO_3) (58,68,84). Most commonly, iodine is added to salt as KI or KIO_3 (83,84). In Cambodia, government regulations require the addition of 50–60 mg iodine/kg salt (68), which is equivalent to approximately 84–101 mg KIO_3. KIO_3 is the iodine fortificant recommended by the World Health Organization for salt iodization (85), given that it is less soluble and more stable than KI when exposed to heat, and is therefore more appropriate for the fortification of salt in humid, tropical climates such as Cambodia (61,75,86,87).

2.6.1 Redox reactions

Unbuffered solutions of KIO_3 (0.35–2% w/v) formulated with purified water remain stable when stored at temperatures ranging from 25–45ºC, retaining >95% of iodine after up to four months of storage (80,88). This suggests that KIO_3 is relatively stable as a single solute in solution. That said, iodine is a strong oxidizing agent (89). Iodate (IO_3^-) may be reduced to iodide (I^-) or elemental iodine (I_2) by antioxidants or other reducing agents in reduction-oxidation (redox)

reactions. Reduced I_2 readily sublimes and diffuses out of solution, causing rapid losses of iodine (86,90,91). Such reactions are accelerated in the aqueous phase, and in the presence of heat, acid, humidity/moisture, or other impurities (90,92). A notable impurity common to the "crude" salt produced in LMIC is magnesium chloride, a hygroscopic compound that draws ambient moisture onto the salt, thereby accelerating the degradation of iodine (90,93). Other impurities, such as organic or insoluble matter, or salts of calcium, magnesium, carbonates, and sulphates, may destabilize iodine (90). In both the solid and aqueous states, the stability of iodine is influenced by the presence of reducing agents (92,94); however, the reducing power of these agents is mediated largely by moisture and heat (94), and to a lesser extent, by pH (91). That is, should a neutral iodine fortification premix solution or a bag of iodized salt be stored in a cool, dry environment—or alternatively, in well-sealed impervious packaging—the iodine should remain stable despite the potential presence of reducing agents within these media (90,91,93). Likewise, salt of high purity iodized with KIO_3 will retain considerable amounts of iodine even when stored in open containers under conditions of elevated temperature and high RH (90,91).

2.6.2 pH

Sultan *et al.* (75) suggest that iodine is most stable under alkaline conditions; however, there appears to be a lack of consensus on the extent to which pH affects the stability of iodine. Shi has put forth that the oxidation potential of IO_3^- in solution is a function of the concentration of H^+ ions, among other factors (92); whereby the lower the pH, the higher $[H^+]$, and the more rapid the rate of reduction (94):

$$IO_3^- + 6\ H^+ + [\text{reducing material}] \rightarrow \tfrac{1}{2}\ I_2 + 6\ H_2O + [\text{oxidized material}] \qquad (92)$$

Other authors suggest the effect of pH is dependent on the form in which the iodine is present. Indeed, Kelly reports that samples of KI-iodized salt treated with alkali exhibit greater iodine retention (98%) when heated at 80ºC for 77 hours, compared to those treated with acid or kept neutral, exhibiting 74% and 71% iodine retention, respectively (93). When samples of KIO_3-fortified salt were exposed to the same conditions at neutral pH, 100% of the iodine was retained. Unfortunately, the author did not assess the stability of KIO_3-fortified salt in acid or alkali, so no accurate conclusions regarding the effect of pH on the different forms of iodine can be made. In

another analysis of several salt samples with various geographic origins, Diosady *et al.* note that the effect of pH on iodine stability, specifically as IO_3^-, was "not clear-cut" (91). All samples included (n=23) were of alkaline pH (7<pH<10), other than one sample from Canada, with a pH of 6.25. Here, pH was influenced by the type and quantity of impurities drawing moisture onto the salt samples. No statistically significant trend was observed between iodine losses over the 12-month storage period and the initial pH of the samples, indicating that iodine losses are mediated through elevated moisture and the presence of impurities more so than through pH (91). Still, referring back to the equation above, it would seem that iodine stability is a factor of $[H^+]$ when reducing materials are present.

2.7 Methods to stabilize iodine in fortification premixes and fortified salt

Various methods promoting the stability of iodized salt have been explored in relevant literature. Generally, these methods can be described as the addition of stabilizers, removing hygroscopic impurities, the selection of optimal packaging, and controlling pH. Such methods may be leveraged to formulate a stable thiamine-iodine co-fortification premix solution or co-fortified refined salt for use in Cambodia.

2.7.1 Stabilizers

There exist several compounds that may be added to salt to promote the stability of the added iodine, at both the experimental and the commercial level. These include calcium carbonate, calcium silicate, and various sodium salts (sodium hexametaphosphate, sodium citrate, sodium ascorbate, sodium erythorbate) (81,82,86,90). Many of these compounds are anti-caking agents, desiccants, pH regulators, and/or emulsifiers. Interestingly, when added as a calcium salt, carbonates effectively promote the retention of iodine in fortified salt; whereas carbonates occurring organically in crude salt, such as that produced in Cambodia, do not appear to have a stabilizing effect (91). Another compound of note is calcium silicate, a desiccant used as a drying and an anti-caking agent in several formulations of iodized salt intended for use in tropical climates (86). Considering the high humidity and average temperature in Cambodia (65% RH and 30ºC), utilizing a small amount (5,000 ppm (86)) of calcium silicate in a co-fortified salt for use in this setting would be a viable means to control moisture and increase the stability of iodine in the co-fortified salt during storage.

Sugars and small polysaccharides are also used to stabilize concentrations of iodine, both in experimental solutions and commercial fortified salt. When evaluating the stability of solutions of KI, iodine, thiamine, and either glucose or sucrose, Murphy and Goodyear hypothesized that the stability of iodine-glucose preparations was related to interactions between the iodine and glucose that prevented the formation of a precipitate (95). Similarly, Diosady *et al.* note that samples of high-purity salt fortified with KI only or KI and ferrous sulfate (iron), mixed with 500 ppm dextrose and 5,000 ppm calcium silicate, exhibited markedly higher stability after six months of storage at 40ºC and 60% RH compared to those without dextrose (86). In fact, a brand of commercially available iodized salt in Canada utilizes invert sugar (300 ppm) to improve iodine stability (96). Dextrose is traditionally used to stabilize iodine in salt fortified with KI, as it helps to prevent the oxidation of I^- to I_2 (86,91). Though the use of dextrose for the stability of iodine in KIO_3-fortified salt is not reported in the literature, it is possible that the addition of a small amount of dextrose to the premix solution may prevent the oxidation of intermediate I^- anions formed in redox reactions between IO_3^- and other solutes. Thus, dextrose remains a viable stabilizing agent for the prospective thiamine-iodine co-fortification premix solution.

2.7.2 Removing hygroscopic impurities

Considering that iodine losses are accelerated by humidity and moisture, removing the hygroscopic impurities common to crude salt (notably, sea salt (94)) is sensible. Indeed, Cambodian regulations stipulate that salt intended for human consumption may contain no more than 0.5% magnesium chloride (68), which is equal to 5,000 ppm. One method described by Shi is the purification of the seawater brine from which salt is extracted, wherein the brine is treated to remove calcium, magnesium, and sulfate ions prior to evaporation (92). While possible, this technique is costly and not technically feasible in many LMIC due to lack of proper heating equipment (91). An alternative, yet similar method proposed by Shi is the purification of the salt slurry that is produced after the serial evaporation of seawater, rather than purifying the seawater brine (92). This method involves the addition of a sodium hypochlorite-sodium hydroxide solution to "wet" salt (*i.e.*, a supersaturated slurry of salt and seawater brine), which is then dried in a fluidized bed drier set at 120ºC for six minutes prior to fortification with KIO_3. The author argues that this method is more economical than purifying the initial brine (92), yet critics have

noted that the heating and drying process required to sufficiently remove traces of the purifying solution from the final salt is costly (94), and thus, is equally impractical for use in LMIC.

2.7.3 Packaging

The micronutrient content of a fortification premix solution and/or a fortified salt must be retained throughout production, transport, distribution, and various stages of storage (86). In fact, Diosady *et al.* note that the selection of appropriate packaging is "critical to the stability of the added iodine" (91). The optimal packaging for a fortification premix and for a fortified salt should be well-sealed and impervious, limiting exposure to ambient moisture, heat, and contaminants. Though many materials are utilized as packaging for salt in LMIC, the best packaging material to promote the stability of iodine is solid, low-density polyethylene, which is impenetrable to moisture (91). If kept intact and adequately sealed after each use, packaging of this type should curtail the adsorption of ambient moisture onto the salt throughout distribution and storage at the household level (91), thereby minimizing redox reactions that result in rapid iodine losses (86). In Cambodia, salt may be packaged in clear polyethylene bags, or may be sold loosely in bulk (67), rendering the selection of packaging materials to be largely at the discretion of the consumer. While household salt is commonly stored in clear plastic containers with plastic screw-top lids (Hou Kroeun, personal communication, December 2023) limiting moisture drawn onto the salt, packaging should not be a primary method of micronutrient stabilization, given the wide variation in market practices in Cambodia.

2.7.4 Controlling pH

Pure sodium chloride (NaCl) is neutral in the aqueous state. Of course, common salt is not purely NaCl; present are also minerals or other environmental contaminants that can affect pH (91). Considering IO_3^- may be vulnerable to reduction at pH <7, adding an alkaline compound, such as sodium carbonate, to iodized salt is proposed as a means to improve the stability of iodine (92). That said, this method is not favourable, as it may yield undesirable organoleptic alterations and may increase moisture of the salt as a result of neutralization reactions (92). Instead, aqueous sodium carbonate may be added to the micronutrient premix solution that is sprayed onto the salt and then dried, as done routinely by Modupe and colleagues (80–82).

pH may also be controlled using a buffering system. While buffers are not often used to control the pH of salt fortified singly with iodine, several successful doubly-, triply-, and even quadruply-fortified salt formulations utilize buffers in their micronutrient premix solutions to improve the stability of the aqueous fortificants (80,82,88). To stabilize iodine concentrations in fortification premixes containing 1–3% w/v iodine (added as KIO_3) and 1–3% w/v folic acid, McGee *et al.* utilized a carbonate buffer (0.1–0.2 M) to maintain pH 9 throughout storage (88). After two months of storage at 45ºC, the average pH of all premix solutions was maintained within 0.1 of their initial pH. Following five months of storage at the same temperature, the retention of both iodine and folic acid in all solutions was >85% (88).

The same authors (88) tested the stability of folic acid and iodine in refined salt samples after micronutrient premixes containing 1–2% w/v folic acid, 1–3% w/v iodine (added as KIO_3), and 0.1–0.2 M carbonate buffer to adjust to pH 9, were sprayed onto Canadian salt and stored. Following 20 months of storage at 45ºC and RH cycling between 20% and 60%, >85% of the iodine and >50% of the folic acid was retained in all salt samples. Authors did not comment on the micronutrient stability in samples sprayed with unbuffered premix solutions of this type. That said, it has been previously reported (97) that the addition of folic acid to already-iodized salt via spraying or dry-mixing in amounts equivalent to 20 µg or 2 mg folic acid/g salt, respectively, resulted in up to 30% greater iodine losses following nine months of storage at 40ºC and 60% RH, compared to the iodine-only controls. This suggests that controlling pH through the addition of a buffer, as done by McGee *et al.* (88), does improve iodine stability in co-fortification premix solutions and co-fortified salt stored for prolonged periods in adverse conditions.

Nevertheless, the importance of controlling pH to favour the stability of iodine in a co-fortification premix solution may be contingent on the specific fortificants or other compounds also present in solution and/or in the salt. As noted above, Diosady *et al.* report no discernable effect of initial pH of the salt samples on iodine retention over the 12-month storage period (91). Samples exhibiting the greatest iodine retention contained various concentrations of other compounds, including calcium (ranging from 290–3,800 ppm), magnesium (210–4,100 ppm), and sulfur (160–4,700 ppm), and ranged from pH 6.25–9.35. This suggests that the pH required to support the stability of iodine in any prospective thiamine-iodine fortification premix solution

may be somewhat variable (91). Conceivably, so long as the organic impurities present in Cambodian salt are considered and accounted for by other means, pH may be adjusted to accommodate the conditions optimal for the stability of thiamine. Indeed, controlling pH through the addition of a buffer is the most feasible means to stabilize iodine concentrations in a co-fortification premix solution in Cambodia.

2.8 Thiamine as a fortification ingredient

Thiamine can be added to many foods or beverages to increase thiamine content. As a free molecule, thiamine is composed of a thiazole ring bound by a methylene group to a pyrimidine ring (98), and has a molecular formula of $C_{12}H_{17}N_4OS^+$ (99). As a fortification ingredient, thiamine is present as a thiamine salt, most commonly as thiamine mononitrate (TMN) or as thiamine chloride hydrochloride (TClHCl; referred to frequently simply as thiamine hydrochloride (100)) (101); though ThDP has also been used for fortification purposes[2] (103). Both TMN and TClHCl are soluble in water, with respective solubilities of approximately 30 g/L, and greater than 500 g/L (100). The higher solubility of TClHCl renders it more appropriate for aqueous fortification than TMN, which is better suited for dry applications (24). While the addition of thiamine to foods presents few technical challenges regarding safety, cost, and organoleptic alterations (24), thiamine is considered amongst the most unstable vitamins (104), given its sensitivity to light, humidity, and radiation (24,104), and its susceptibility to hydrolytic, thermal, alkaline, and oxidative degradation (101,104). In solution, thiamine stability is also influenced by the water activity and polarity of the solvent (105).

2.8.1 pH and its effects on thiamine degradation

At physiologic pH, thiamine has a single positive charge on the nitrogen at position three (N3) of the thiazole moiety (106) (**Fig. 2-1**). In solutions of pH <6, thiamine exists most abundantly as a doubly protonated species (100,106). There is heterogeneity in the literature regarding the exact position of the additional positive charge; Windheuser and Higuchi propose that the primary amino group of the pyrimidine ring is the site of the protonation (107), whereas Voelker *et al.* (100) and Edwards *et al.* (106) propose it is the pyrimidine N1 that holds this charge. In any

[2] When used for fortification, ThDP is in the form of a chloride salt with chemical formula $C_{12}H_{19}ClN_4O_7P_2S$ and molecular weight of 460.77 g (102).

case, it is unanimously recognized that the non-oxidative degradation of thiamine in strongly acidic conditions results in the formation of the thiamine antagonist, oxythiamine, via substitution of the primary amino group of the pyrimidine ring with a hydroxyl group (107–109). In solutions with pH ≥6, the pyrimidine N1 loses its positive charge (100,106), yielding a notably less stable molecule than the protonated counterpart, and thus eliciting rapid hydrolytic degradation via cleavage of the methylene bridge at high pH (100,107). Particularly in solutions of pH >7, the free thiazole moiety undergoes further degradation into numerous sulfur-containing compounds (110), leading to discoloration of the solvent (100) and the production of pungent odours akin to those of rotten egg (111).

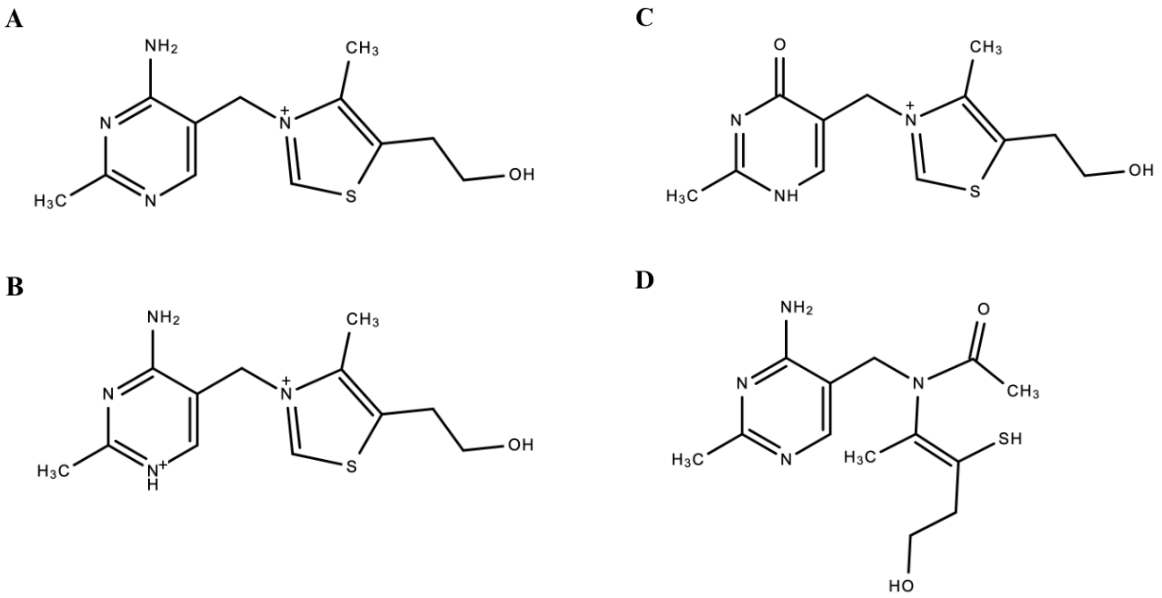

Figure 2-1. Chemical structures of thiamine species at different pH. A. Thiamine at physiologic pH **B.** Thiamine at pH <6 **C.** Oxythiamine; present at pH <3 **D.** Thiamine at pH >7. Figure created using ChemSpider.

Degradation of thiamine has been reported by many to follow first-order or pseudo-first-order kinetics (100,101,104,105,108,112). Voelker *et al.* have determined that the degradation reactions of aqueous TMN and TClHCl follow pseudo-first-order kinetics in mildly acidic solutions (pH ~6), where the rate of degradation is dependent on the initial thiamine concentration; in mildly acidic solutions of higher initial concentration, thiamine degradation proceeds more rapidly (101). In more acidic solutions (pH ~3), thiamine degradation is independent of initial thiamine concentration, and follows first-order kinetics more faithfully

(101). The reaction kinetics and resulting products of thiamine degradation are dependent on several factors beyond those described above, including the type and the concentration of other thiamine species in solution, the presence and concentration of other solutes, the ionic strength of the solution (101), and, if used, the properties (concentration, type) of a buffer (108). Should no buffer be added, it is the anions present in the aqueous thiamine salts that affect pH of the resulting solution; solutions of TMN are near neutral, whereas solutions of TClHCl are acidic. The exact pH of these solutions ranges from 5.4–7.0 and 1.1–3.6, respectively, depending on the initial concentrations of the thiamine salt in solution (100).

2.8.2 Buffers

It is well established that thiamine is most stable in acidic solutions (24,100,101,108), irrespective of the presence of a buffer (100,101). Voelker *et al.* write, "regardless of the counterion(s) present in solution, the thiamine degradation reaction proceeded in the same manner" (101). Here, authors examined the effect of exogenous nitrate (NO_3^-) and chloride (Cl^-) counterions in buffered solutions of 0.1% w/v and 2% w/v TClHCl or TMN to elucidate the effect of the counterion (either NO_3^- or Cl^-) present in aqueous thiamine salts on the degradation of thiamine at pH 3 and 6. It was concluded that counterions—and therefore, buffer type—had no effect on the rate or pathway of thiamine degradation (101). Indeed, NO_3^- and Cl^- are already present in TMN and TClHCl, respectively; thus, it is unsurprising that these anions are inert in the kinetics of thiamine degradation.

By contrast, Pachapurkar and Bell note that both the concentration and the type of buffer used may impact thiamine stability (108). Authors analyzed the stability of eight solutions of 0.02% w/v TClHCl, differing in the type (phosphate or citrate) and concentration (0.02 M or 0.1 M) of buffer used, corrected to one of four pH values (pH 4–7). Triplicate samples of the solutions were stored at 25ºC for 42 weeks. Solutions corrected to pH 4 using 0.02 M phosphate buffer exhibited the greatest thiamine retention over the storage period, though a few other buffered solutions retained >75% of the initial thiamine content, even beyond the 42-week examination period. While all solutions exhibited the greatest stability at pH 4 and 5, solutions with citrate buffer exhibited greater thiamine retention than those with phosphate buffer at pH 6 and 7, regardless of buffer concentration (0.02 or 0.1 M). This suggests that thiamine stability is more

vulnerable to differences in pH in solutions with phosphate buffer than in those with citrate buffer. Authors hypothesize this increased sensitivity to changes in pH is likely mediated through interactions between thiamine and the counterions present in the phosphate buffer (*i.e.*, hydrogen phosphate, HPO_4^{2-}) that were not present in solutions buffered with citrate (108). As reported, it is likely that certain buffer salts may catalyze thiamine degradation. Therefore, if a buffer is included in the formulation of co-fortification premix solution to stabilize thiamine, careful consideration must be made when selecting the type of buffer to minimize counterion interaction and maximize thiamine stability.

2.9 Thiamine-iodine interactions

Given that iodine has been added to salt for several decades, an abundance of research exists detailing the stability and/or reaction kinetics of KI and KIO_3 when added to salt in various conditions or alongside additional nutrients. The literature examining thiamine as fortificant is much more sparse, notably regarding its interactions with other nutrients. In 1948, Murphy and Goodyear examined the stability of TClHCl in solution with 0.2% w/v KI, 0.1% w/v iodine, and either 25% w/v sucrose or 37.5% w/v glucose (95). Solutions ranged from pH 2.2–3.0. After four years of storage at 23–33 ºC, ≥85% of the thiamine was retained in all solutions. Authors concluded that the experimental concentrations of KI and iodine in aqueous solutions with TClHCl did not exert a destructive effect on *thiamine* in the presence of glucose or sucrose (95). Unfortunately, authors did not investigate the stability of iodine in these solutions, nor did they report on changes to odour or colour of the solutions during and following the extended period of storage.

More recently, McSweeney *et al.* attempted to co-fortify salt with thiamine and iodine via spray fortification using a premix solution composed of TClHCl, KIO_3, and water, in amounts equivalent to 500 ppm thiamine and 50–60 ppm iodine in fortified salt (113). Though the salt was found to have acceptable sensory characteristics, authors found that nearly 80% of the initial iodine content was lost after four months of storage in typical Cambodian conditions (approximately 30°C, 65% RH, dark location), whereas >90% of the thiamine was preserved (113). While iodine may not exert a destructive effect on thiamine, as suggested by Murphy and

Goodyear (95), it is evident from the trial by McSweeney *et al*. that thiamine and iodine do not co-exist favourably, explored in greater detail below.

2.10 Rationale and research gap

In Cambodia, as many as 78% of WRA may be thiamine deficient, and more than two-thirds of infants aged 6–12 months may be at risk of potentially fatal TDDs (36). Previous work has demonstrated that salt is a suitable vehicle for thiamine fortification in Cambodia, where no thiamine fortification programs exist to date. Given the existing public health messaging and spray fortification infrastructure, co-fortifying salt with thiamine and iodine holds promise as an inexpensive and sustainable means of preventing thiamine deficiency among WRA and their exclusively-breastfed infants in Cambodia. However, pilot data from our lab suggest that thiamine may react unfavourably with iodine in a solution of TClHCl and KIO_3, producing unpleasant odours, and more concerningly, catalyzing losses of iodine via sublimation. Thiamine, particularly in the thiol form, has been described as an antioxidant (114,115), which is a form of reducing agent (116). Regrettably, IO_3^- is susceptible to reduction in the presence of reducing agents, as described above. Indeed, Dwivedi and Arnold (110) write in their review, "thiamine can be oxidized by iodine to form the corresponding disulfide derivative without loss of thiamine activity". In other words, thiamine may reduce iodine (IO_3^-) without losing vitamin activity, while iodine (I_2) is lost via sublimation. The reduction of IO_3^- is catalyzed by elevated temperature, humidity, and acid—the first two being conditions characteristic of the climate in Cambodia, the latter being the condition necessary for thiamine stability. Consequently, it may be challenging to formulate a fortification premix solution or co-fortified refined salt with stable concentrations of both thiamine and iodine that can take advantage of the existing fortification procedures and machinery utilized in Cambodia at present. With that, the goal of this research was to assess various strategies to stabilize and maintain concentrations of both thiamine and iodine in a fortification premix solution for the prospective co-fortification of commercially available salt in Cambodia.

3.0 Methods

3.1 Objectives

The objectives of this research were threefold:

1. To monitor odour, colour, precipitation, and other physical changes to experimental premix solutions for up to four weeks of storage.
2. To assess pH of experimental premix solutions for up to four weeks of storage.
3. To determine which, if any, of the experimental premix solutions maintain satisfactory concentrations of both iodine and thiamine over four weeks of storage in typical Cambodian conditions (30ºC, 65% RH).

These objectives were met through a series of three experiments, outlined below.

3.2 Materials

Table 3-1 details all reagents used for premix preparation by experiment, including brand and grade. KIO_3, KI, sodium citrate tribasic dihydrate, sodium carbonate, and sodium acetate were purchased from MilliporeSigma Canada (Oakville, Ontario, Canada). TClHCl and sodium hydroxide (NaOH) were purchased from Thermo Fischer Scientific Chemicals Inc (Ottawa, Ontario, Canada). Citric acid was purchased from VWR International (Mississauga, Ontario, Canada), and dextrose from Elo's Premium (Toronto, Ontario, Canada). Sodium bicarbonate (Arm and Hammer™) and distilled water (President's Choice®) were purchased from Church & Dwight Canada Corp (Mississauga, Ontario, Canada) and Loblaw Companies Ltd (Brampton, Ontario, Canada), respectively.

Table 3-1: Materials used for solution preparation by experiment

Material	Brand	Grade[1]
Experiment 1		
Potassium iodate	Supelco® Emsure®	Reagent grade (ACS)
Thiamine chloride hydrochloride	Thermo Scientific	Reagent grade (HPLC)
Dextrose	Elo's Premium	Food grade
Sodium citrate tribasic dihydrate	Sigma-Aldrich®	Reagent grade (ACS)
Sodium acetate	Sigma-Aldrich®	Reagent grade (ReagentPlus®)[2]
Sodium carbonate	Sigma-Aldrich®	Reagent grade (ACS)
Distilled water	President's Choice®	Food grade
Experiment 2		
Potassium iodide	Sigma-Aldrich®	Reagent grade (ACS)
Potassium iodate	Supelco® Emsure®	Reagent grade (ACS)
Thiamine chloride hydrochloride	Thermo Scientific	Reagent grade (HPLC)
Dextrose	Elo's Premium	Food grade
Sodium carbonate	Sigma-Aldrich®	Reagent grade (ACS)
Sodium bicarbonate	Arm and Hammer™	Food grade
Distilled water	President's Choice®	Food grade
Experiment 3		
Potassium iodide	Sigma-Aldrich®	Reagent grade (ACS)
Thiamine chloride hydrochloride	Thermo Scientific	Reagent grade (HPLC)
Sodium hydroxide	Elo's Premium	Reagent grade (ACS)
Sodium citrate tribasic dihydrate	Sigma-Aldrich®	Reagent grade (ACS)
Citric acid	VWR International	Reagent grade (ACS)
Sodium carbonate	Sigma-Aldrich®	Reagent grade (ACS)
Sodium bicarbonate	Arm and Hammer™	Food grade
Distilled water	President's Choice®	Food grade

[1]Abbreviations: ACS, American Chemical Society; HPLC, high-performance liquid chromatography.
[2]ReagentPlus® is a grading category registered by Sigma Aldrich indicating ≥98.5% purity (117).

3.3 Solution preparation, sampling, and storage

A total of twenty-four premix solutions (*a*–*x*) were prepared over three experiments. All solutions were prepared and stored at Mount Saint Vincent University (MSVU) in Halifax, Nova

Scotia, Canada. Though TClHCl and KIO_3 or KI were used as the sources of thiamine and iodine, respectively, concentration (% w/v) of these fortificants will be reported on a nutrient basis (*i.e.*, % w/v thiamine and % w/v iodine), as done routinely in similar works (80,82,88), unless explicitly indicated otherwise. Concentrations of all other reagents will be reported as % w/v of the total compound added to solution. Also of note, despite using the term *solution* throughout the following sections, several of the premix "solutions" described herein would be more appropriately referred to as *suspensions* due to the settling of particulates upon standing (118). However, for uniformity, the term *solutions* will be used to refer to all aqueous premixes formulated during the experiments presented below.

3.3.1 Experiment 1

Experiment 1 had two phases: Experiment 1A and 1B. For Experiment 1A, solutions were formulated to achieve an initial concentration of 60 ppm iodine in fortified salt as per Cambodian regulations (68), equivalent to approximately 3% w/v iodine in solution. KIO_3 is the fortificant used by Cambodian salt manufacturers at present (69); thus, all solutions in Experiment 1 were formulated with KIO_3. Chan *et al.* modelled a thiamine fortification scenario and determined an acceptable dose of thiamine to be 275 mg/kg salt (275 ppm), based on an average daily salt intake of 7.7 g/day among lactating Cambodian women (63). This model assumed no stability losses. Given that the rate of thiamine degradation increases with increasing pH, solutions here were formulated to reflect 50% overages to achieve an initial concentration of 415 ppm thiamine in fortified salt, equivalent to 21% w/v thiamine in the premix solutions. If used to fortify salt, these solutions would yield a mean daily intake of approximately 460 µg iodine and 3.2 mg thiamine from fortified salt (assuming daily consumption of 7.7 g/day and zero nutrient losses through storage), which satisfies the dietary intake requirements for both nutrients for all sex and life-stage groups (119,120).

Three control solutions (*a*–*c*) and four experimental solutions (*d*–*g*) were formulated in Experiment 1A, for a total of seven solutions:

a. KIO_3 only
b. TClHCl only
c. KIO_3 and TClHCl

d. KIO_3 and TClHCl with dextrose

e. KIO_3 and TClHCl with sodium citrate tribasic dihydrate

f. KIO_3 and TClHCl with sodium acetate

g. KIO_3 and TClHCl with sodium carbonate

A research assistant assisted with solution preparation during Experiment 1A. All solutions were prepared in the order listed above, directly in BRAND® 1000-mL, brown-coloured, low-density polyethylene wide-mouth bottles (MilliporeSigma Canada, Oakville, Ontario, Canada). Bottles were labelled according to solution type. TClHCl was added to all bottles except that of solution *a*, followed by KIO_3 to all but that of solution *b*. Dextrose was added to bottle *d*. Distilled water was added to dissolve the solute(s) in each bottle, and all solutions were mixed individually using the Corning Stirrer PC-353 magnetic mixer (Corning Incorporated, Corning, New York, USA) on moderate to high speed for 90 seconds. Additional water was added until each bottle was filled to the base of the neck to achieve a final volume of approximately 1000 mL. Crystalline sodium citrate tribasic dihydrate, sodium acetate, and sodium carbonate were added to solutions *e*–*g*, respectively, in increments of 5–10 g at a time, in attempt to attain pH 5.50. Solutions were mixed on moderate speed throughout, and pH was measured after each addition (see section 3.4.3 for details on pH measurement). None of solutions *e*–*g* achieved pH 5.50, despite considerable volumes of each basic salt being added to their respective solutions; therefore, addition of basic salts to solutions *e*–*g* was terminated before reaching target pH based on empirical judgement (*i.e.*, evident saturation, strong pigmentation, precipitation). Total mass of each basic salt added to solutions *e*–*g* was recorded. All premix solutions prepared in Experiment 1A, the reagents, and their respective concentrations (% w/v) are summarized in **Table 3-2**.

Table 3-2: Formulations of premix solutions prepared in Experiment 1A

Solution	Reagent (% w/v)					
	Iodine	Thiamine	Dextrose	Sodium citrate tribasic dihydrate	Sodium acetate	Sodium carbonate
a	3	0	0	0	0	0
b	0	21	0	0	0	0
c	3	21	0	0	0	0
d	3	21	0.05	0	0	0
e	3	21	0	20	0	0
f	3	21	0	0	7.5	0
g	3	21	0	0	0	3

Total volume of each solution was approximately 1000 mL. All values are approximate.

Samples from each solution were collected at 0, 6, 12, 24, and 48 hours. Immediately following preparation (*t*=0), four 8-mL aliquots were taken from each solution and were stored in amber cryovials at –80°C. An additional 30–40 mL of each solution was withdrawn and dispensed into labelled 50-mL clear conical tubes for visual inspection, photographing, and pH measurement. After initial sampling, solutions were transferred to a Fisher Scientific 650D Isotemp® incubator (Fisher Scientific, Ottawa, Ontario, Canada) for storage. The incubator was set to 30°C and outfitted with three water baths (totaling approximately 2100 cm^2) to maintain 65% RH. At each subsequent collection point, solution storage bottles were individually removed from the incubator and vigorously shaken by hand[3] for 3–5 seconds to resuspend the solutions prior to sampling, following the same procedures as at *t*=0. Experiment 1A was terminated after 48 hours due to evident futility.

3.3.1.1 Experiment 1A troubleshooting

Additional experimental solutions of lesser volume were formulated extemporaneously during Experiment 1A due to unsatisfactory pH and changes to physical properties observed upon preparation of solutions *e–g.* Two sets of modified solutions (*h–j* and *k–m*) similar in formulation to solutions *e–g* were attempted in Experiment 1B, and were prepared in a 100-mL beaker to a final volume of 50 mL each. The concentrations of iodine and thiamine in solutions *h–j* were

[3] Initially the stir plate was employed; however, this was ineffective for various solutions due to the formation of a very thick, sticky precipitate. Shaking each solution by hand allowed for greater consistency across samples.

consistent with solutions previously prepared in Experiment 1A (3% w/v and 21% w/v, respectively). Rather than adjusting pH after both fortificants were in solution, pH of just TClHCl in solution was adjusted prior to the addition of KIO_3, using one of sodium citrate tribasic dihydrate, sodium acetate, or sodium carbonate. KIO_3 was then added, in addition to distilled water to total volume of 50 mL. For solutions *k–m*, the concentration of iodine remained consistent at 3% w/v, whereas that of thiamine was decreased to 7.8% w/v to lower the concentration of both hydrogen ions (H^+) and of total solutes, in an attempt to limit precipitation as experienced in Experiment 1A. This concentration of thiamine corresponds to the estimated average requirement (EAR) for thiamine for pregnant and lactating women (1.2 mg/day) and is equivalent to 57% of the dose recommended by Chan *et al.* (63,120). The second set of modified solutions was prepared in the same manner as the first, where pH was adjusted prior to the addition of KIO_3. All solutions prepared in Experiment 1B were to assess physical properties only (objective 1). Experiment 1B solutions, the reagents, and their respective concentrations (% w/v) are summarized in **Table 3-3**.

Table 3-3: Formulations of premix solutions prepared in Experiment 1B

	Reagent (% w/v)					
Solution	Iodine	Thiamine	Dextrose	Sodium citrate tribasic dihydrate	Sodium acetate	Sodium carbonate
h	3	21	0	10	0	0
i	3	21	0	0	5	0
j	3	21	0	0	0	1.6
k	3	7.8	0	5.6	0	0
l	3	7.8	0	0	2	0
m	3	7.8	0	0	0	1.1

Total volume of each solution was approximately 50 mL. All values are approximate.

Sampling procedures described for Experiment 1A were not reproduced during Experiment 1B, as solutions *h–m* were prepared in small volumes to assess initial physical properties only. No aliquots were taken. Solutions were poured into 50-mL clear conical tubes and were stored upright at room temperature (~21°C) for up to 20 hours. pH of each solution was measured upon preparation only. Photographs were taken at "*t*=0" and at irregular intervals over the brief observation period (see section 3.4 for detailed data collection procedures). Though dissimilar in

experimental design and data reporting, data collected during Experiment 1B are retained in the present thesis to demonstrate the effect of adjusting pH of TClHCl solutions prior to the addition of KIO_3 on initial pH, colour, and precipitation.

3.3.2 Experiment 2

A second experiment was conducted to address objectives 1 (monitor physical changes) and 2 (assess pH) only. As with the previous experiment, these solutions were formulated to achieve an initial concentration of approximately 3% w/v iodine; however, KI was used as the iodine fortificant instead of KIO_3 in all but one solution, as iodine in its reduced form (I^-) may act more favourably than in its oxidized form (IO_3^-) when in solution with thiamine, which acts as a reducing agent (116). In addition, the concentration of thiamine in each solution was decreased in this experiment. Given the very high volume of TClHCl required to achieve the 21% w/v thiamine attempted in Experiment 1 (the equivalent of 415 ppm thiamine in fortified salt), and that thiamine losses increase as the concentration of the thiamine salt increases in solutions of pH ≥6 (101), solutions in Experiment 2 were formulated to contain approximately 14% w/v thiamine, equivalent to the fortification dose proposed by Chan *et al.* without overages (275 ppm). If used to fortify salt, these solutions would yield a mean daily intake of approximately 460 µg iodine and 2.1 mg thiamine from fortified salt, which again, assuming daily consumption of 7.7 g/day and zero nutrient losses through storage, continues to satisfy the dietary intake requirements for both nutrients for all sex and life-stage groups (119,120).

Four experimental (*n*–*q*) solutions were formulated in Experiment 2, for a total of four solutions:

n. KI and TClHCl only
o. KI and TClHCl with dextrose
p. KI and TClHCl with carbonate buffer
q. KIO_3 and TClHCl with carbonate buffer

Unlike in Experiment 1, these solutions were prepared in a 2000-mL glass beaker prior to being poured into the brown plastic bottles for storage. Each solution was prepared to a total volume of 480 mL instead of 1000 mL, as this experiment was planned to assess viability over only one week of storage at typical Cambodian conditions.

To prepare solution *n*, a small volume of distilled water was poured into the beaker prior to the addition of TClHCl to facilitate dissolution. The mixture was stirred on moderate to high speed for 90 seconds. KI was added to the solution with distilled water to 480 mL, and mixed. The solution was mixed for an additional 4.5 minutes; however, solution *n* would not solvate completely, with sediment continuing to settle at the bottom of the beaker each time mixing was paused. Solution *o* was prepared by stirring TClHCl, dextrose, and a volume of distilled water on moderate to high speed for 90 seconds. KI was added to the beaker with distilled water to 480 mL, and was stirred for an additional 90 seconds.

Solutions *p* and *q* were prepared with 0.2 M carbonate buffer as the solvent, as per expert recommendation (Dr. Levente Diosady, personal communication, July 2024). Stock solutions of 0.2 M carbonate and 0.2 M bicarbonate were prepared with distilled water and sodium carbonate or sodium bicarbonate, respectively. The buffer was prepared by mixing 600 mL of the carbonate solution and 400 mL of the bicarbonate solution. Solutions *p* and *q* were prepared with KI and KIO_3, respectively. All premix solutions prepared in Experiment 2, the reagents, and their respective concentrations (% w/v) are summarized in **Table 3-4**.

Table 3-4: Formulations of premix solutions prepared in Experiment 2

	Reagent (% w/v)				
Solution	Iodine	Thiamine	Dextrose	Sodium carbonate	Sodium bicarbonate
n	3	14	0	0	0
o	3	14	0.5	0	0
p	3	14	0	1	1
q	3	14	0	0.6	0.6

Total volume of each solution was 480 mL. All values are approximate.

Samples were collected at 0, 6, 12, 24, and 48 hours, and at 1 week. During this experiment, two 8-mL aliquots were taken from each solution at each timepoint (as opposed to four as done in Experiment 1A) and were stored in amber cryovials at –80°C. As with Experiment 1A, an additional 30–40 mL of each solution was withdrawn and dispensed into labelled 50-mL clear conical tubes for visual inspection, photographing, and pH measurement. All solutions were stored as per Experiment 1A (30°C, 65% RH), and all subsequent sampling done likewise.

3.3.3 Experiment 3

A third and final experiment was conducted using insights gained from Experiments 1 and 2: Solutions formulated with KI appeared more viable than those with KIO_3; therefore, KI was used as the iodine fortificant in Experiment 3. Solution *p* (TClHCl and KI with carbonate buffer) prepared during Experiment 2 was tested again in Experiment 3. In addition, a novel solution formulated with a carbonate *solution* (100% sodium carbonate) instead of a carbonate *buffer* (60% sodium carbonate, 40% sodium bicarbonate) was also attempted to achieve target pH. Additional solutions were formulated with NaOH to adjust pH, as per expert opinion (Dr. Kelly Resmer, personal communication, October 2024), both with and without citrate buffer. Dextrose was not used in Experiment 3.

All solutions were formulated to achieve an initial concentration of approximately 3% w/v iodine, equivalent to 60 ppm in fortified salt. The concentration of thiamine in each solution was reduced to 7.8% w/v, equivalent to 156 ppm thiamine in fortified salt, in effort to decrease both pH and total solute concentration of the solutions to prevent saturation. Assuming a mean daily salt consumption of 7.7 g, this dose of thiamine would provide 1.2 mg thiamine/day through fortified salt, equal to the EAR for pregnant and lactating women (120). Assuming zero nutrient losses through storage, this level of fortification satisfies the EAR for both iodine and thiamine for all sex and life-stage groups (119,120).

Two control solutions (*r* and *s*) and five experimental solutions (*t*–*x*) were formulated in Experiment 3, for a total of seven solutions:

r. TClHCl only
s. KI only
t. KI and TClHCl
u. KI and TClHCl with NaOH
v. KI and TClHCl with citrate buffer
w. KI and TClHCl with carbonate buffer
x. KI and TClHCl with carbonate solution

Each solution was prepared in a 2000-mL beaker as done in Experiment 2, to a total volume of 1000 mL. To prepare solutions *r* and *s*, TClHCl and KI, respectively, were individually mixed with distilled water on the stir plate for 90 seconds at moderate speed. Solution *t* was prepared by mixing TClHCl with a small volume of distilled water prior to the addition of KI and more water to the desired volume. As observed with solution *n* in Experiment 2, the solution would not solvate completely, despite a considerable amount of time on the stir plate. The sediment visible in solution *t* was thought to be precipitation due to overmixing; therefore, when preparing solution *u*, TClHCl and KI were stirred on moderate speed for only 30 seconds. The decreased stir time effectively dissolved reagents such that there was no perceptible precipitation. A 1.0 M NaOH solution was added dropwise to solution *u* to adjust pH to ~5.50. pH was measured after each addition (see section 3.4.3 for details on pH measurement). The solution became resistant to increases in alkalinity around pH 4.60, despite considerable volumes of NaOH being added after this pH was attained (total of 75 mL 1.0 M NaOH).

Solution *v* was prepared with 0.2 M citrate buffer, prepared according to volumes presented on AAT Bioquest (121), modified to produce a 0.2 M solution with initial pH of 5.50. TClHCl and KI were dissolved in separate volumes of citrate buffer before being combined to ensure each solute dissolved fully. pH of solution *v* was adjusted with 1.0 M NaOH. As with solution *u*, solution *v* became resistant to further changes in pH around pH 4.70 (total of 125 mL 1.0 M NaOH). Solution *w* was prepared with carbonate buffer, as described in section 3.3.2, to a final volume of 1000 mL. Last, solution *x* was prepared using 0.2 M sodium carbonate solution as the solvent. TClHCl and KI were dissolved in separate volumes of carbonate solution before being combined to ensure each solute was fully dissolved prior to combining. Once prepared, each of solutions *r*–*x* was poured into a narrow-mouth, amber glass bottle (VWR International, Radnor PA, USA). Bottles were labelled according to solution type. All solutions prepared in Experiment 3, the reagents, and their respective concentrations (% w/v) are summarized in Table **3-5**.

Table 3-5: Formulations of premix solutions prepared in Experiment 3

Sample	Reagent (% w/v)						
	Iodine	Thiamine	NaOH	Sodium citrate tribasic dihydrate	Citric acid	Sodium carbonate	Sodium bicarbonate
r	3	0	0	0	0	0	0
s	0	7.8	0	0	0	0	0
t	3	7.8	0	0	0	0	0
u	3	7.8	0.3	0	0	0	0
v	3	7.8	0.5	3.5	1	0	0
w	3	7.8	0	0	0	1.3	0
x	3	7.8	0	0	0	0.8	0.4

Total volume of each solution was approximately 1000 mL. All values are approximate.

In Experiment 3, samples were collected at 0, 6, 12, 24, and 48 hours, and at 1, 2, 3, and 4 weeks. Sampling and storage procedures followed during Experiment 3 mimicked those of Experiment 1A: Immediately following preparation (t=0), four 8-mL aliquots were taken from each solution and were stored in amber cryovials at –80°C. An additional 30–40 mL of each solution was withdrawn and dispensed into labelled 50-mL clear conical tubes for visual inspection, photographing, and pH measurement; after which, solutions were transferred to an incubator set to 30°C and 65% RH for storage. At each timepoint thereafter, solution storage bottles were individually removed from the incubator and vigorously shaken by hand for 3–5 seconds to resuspend the solutions prior to sampling.

3.4 Data collection

Samples obtained during Experiments 1–3 were used to collect various data, detailed below.

3.4.1 Observations

Remarks pertaining to solution colour, clarity, texture, odour, and precipitation were recorded in a bench-top lab book at each timepoint. Comments were transcribed into a Microsoft Word document immediately following each collection point and were subject to elaboration as needed.

3.4.2 Photographing

Photographs of each clear conical tube were captured at each timepoint with an iPhone 14 (Apple Inc, Cupertino, California, United States) prior to pH measurement to document colour, presence of precipitate (if any), and other visual characteristics. Conical tubes were held up against a white wall with strong overhead lighting for each photograph. A minimum of two photographs were captured for each solution: one with the label clearly visible, one with the label offset to better capture visual characteristics of the solution. Solutions with visible separation or distinct layering were captured a minimum of four times: twice as just described, then twice after the solution was vigorously shaken to resuspend the solutes and homogenize the layers. Additional photographs were taken to capture details that were not sufficiently represented in the "standard" photographs.

3.4.3 pH measurement

The pH of all samples was measured in triplicate (at a minimum) at each indicated timepoint. pH was analyzed using the edge® Multiparameter pH Meter model HI2020 (Hanna Instruments, Woonsocket, Rhode Island, USA) and the Digital Glass Body pH Electrode for General Purpose model HI11310 (Hanna Instruments, Woonsocket, Rhode Island, USA), calibrated using Hanna Instruments pH 1.68 (HI7001L), 4.01 (HI7004L), 7.01 (HI7007L), and 10.01 (HI7010L) calibration solutions. The electrode was rinsed thoroughly with distilled water and dried using Kimberly-Clark Professional™ Kimtech Science™ Kimwipes™ Delicate Task Wipers between each replicate measurement. If measurements of the same sample at the same timepoint differed by ≥0.15, another three measurements were taken. All measurements were recorded in a bench-top lab book and were input into a Microsoft Excel spreadsheet after collection. Mean pH for each solution at each timepoint was calculated and recorded.

3.4.4 Thiamine assessment

Thiamine content of each sample was analyzed at the Acadia Laboratory for Agri-Food and Beverage (Acadia University, Wolfville, Nova Scotia, Canada) using a method adapted from Lu and Frank (122). Briefly, samples were diluted 2000-fold and analyzed by high performance liquid chromatography (HPLC) using an Agilent 1290 Infinity Series HPLC on an Agilent Poroshell 120 Aq-C18 column (100 x 4.6 mm, 2.7 µm), with a gradient of 10 mM monosodium

phosphate, pH 2.5, and acetonitrile at 1 mL/min. Standard curves were prepared with TClHCl (MilliporeSigma Canada, Oakville, Ontario, Canada). Thiamine content is reported on a thiamine basis.

3.4.5 Iodine assessment

Iodine was analyzed on an iodide (I^-) basis, but will be referred to as "iodine" for simplicity. Iodine content of each sample was also analyzed at the Acadia Laboratory for Agri-Food and Beverage (Acadia University, Wolfville, Nova Scotia, Canada) using a method adapted from Alahmad *et al.* (123). Samples were diluted 10,000-fold and analyzed by high pressure ion chromatography (HPIC) using a Thermo Scientific Dionex™ Integrion™ HPIC™ system on a Dionex IonPac AS18 (4 × 250 mm) Anion column with a 100 mM potassium hydroxide eluent generator at a flow rate of 1 mL/min. Standard curves were prepared with an iodide reference solution (MilliporeSigma Canada, Oakville, Ontario, Canada).

3.5 Data analysis

Analyses are reported by objective, as detailed below.

3.5.1 Objective 1: Monitoring sensory properties and physical characteristics

Sensory properties and physical characteristics of the solutions, such as colour, clarity, odour, precipitation, viscosity, and homogeneity, are described qualitatively, with an emphasis on observed changes to any of these characteristics over time.

3.5.2 Objective 2: pH assessment

Mean pH measurements are reported in a table, and are plotted graphically to visualize changes in pH over time.

3.5.3 Objective 3: Micronutrient content

Micronutrient content of solutions was evaluated in terms of both stability and absolute concentration. The stability of iodine and thiamine in all solutions is represented as a percentage of their initial concentrations (% retention) over time, such that the concentration of either nutrient at t=0 reflects 100% retention, consistent with similar works (80–82,88,90,91,113). The

calculated % retention of iodine and thiamine in each solution is plotted graphically by timepoint. Absolute concentrations (% w/v) of thiamine and iodine in each solution at each timepoint were used to determine the acceptability of the solutions' nutrient content over time. These data are reported in a colour-coded figure, where green indicates acceptable, red indicates unacceptable, and grey indicates controls. Solutions were deemed acceptable if they maintained satisfactory concentrations of both iodine *and* thiamine over four weeks of storage. Solutions that satisfied the minimum concentration for only one nutrient were not deemed acceptable. Acceptable concentrations of both nutrients are defined below.

3.5.3.1 Acceptable iodine content

The minimum acceptable concentration of iodine in the premix solutions at any point during storage was defined as ≥2.5% w/v. This is equivalent to 50 ppm in fortified salt, which is the minimum iodine content permissible in salt at the point of production per Cambodian regulations (68). Conversely, outlined by the same regulations, the maximum iodine content permissible in salt at any point during production or distribution is 60 ppm, equal to 3% w/v in the premix solution. That said, no true "maximum" for iodine concentration was established for the solutions prepared herein, given that all solutions were formulated with 3% w/v iodine in consideration of the aforementioned regulations. It was assumed that no solution would exhibit an iodine concentration >3% w/v; therefore, the "maximum" iodine content of solutions is defined as ≥3% w/v.

3.5.3.2 Acceptable thiamine content

Coats *et al.* have previously estimated the mean daily thiamine intake of WRA in Cambodia to be 0.75 mg/day from food (124). To achieve the RDA for pregnant and lactating women of 1.4 mg thiamine/day (120), a daily minimum of 0.65 mg thiamine would need to be consumed through fortified salt to make up the difference. Assuming a mean daily salt intake of 7.7 g among this population (63), and estimating ~50% thiamine losses over storage, the minimum acceptable concentration of thiamine in the premix solutions at any point during storage was determined to be ≥6.3% w/v, equivalent to 126 ppm in fortified salt. Again, no true "maximum" concentration was established for the thiamine content of solutions, given that there is no established UL for this vitamin (120). As with iodine, it was assumed that no solution would

exhibit a thiamine concentration >7.8% w/v, given that solutions analyzed for micronutrient content (those prepared in Experiment 3) were only formulated to contain 7.8% w/v thiamine. Accordingly, the “maximum” thiamine content of solutions is defined as ≥7.8% w/v.

3.6 Dissemination of findings

Preliminary findings from this project were summarized in a final report for Research Nova Scotia (required by all their funding recipients (125)) that was submitted in August 2024. This research was shared at the 2025 Science Atlantic Foods and Nutrition Conference via virtual oral presentation, and will also be shared at the 2025 Canadian Nutrition Society Annual Conference in a poster presentation. Additionally, an abstract will be published in the journal *Applied Physiology, Nutrition, and Metabolism*, and we will prepare a manuscript for submission to a peer-reviewed journal such as the *Journal of Food Engineering*. Importantly, findings from this project will be used to inform further developments of a thiamine-iodine co-fortified salt in Cambodia. Insights from this project will be shared with relevant personnel of the Royal Government of Cambodia Ministry of Planning and with the Cambodian National Nutrition Working Group to provide an update regarding the development of a technology for the co-fortification of commercially available salt for the prevention of thiamine and iodine deficiencies nationwide.

4.0 Results

4.1 Sensory and physical properties

The photographs captured of each solution at each timepoint are displayed in **Figures 4-1** (Experiment 1A), **4-2** (Experiment 1B), **4-3** (Experiment 2), and **4-4** (Experiment 3). Control solutions *a* (KIO_3 only), *b* (TClHCl only [21% w/v]), *r* (KI only), and *s* (TClHCl only [7.8% w/v]) remained transparent and colourless or nearly colourless throughout storage, and exhibited no precipitation. Experimental solution *t* (KI and TClHCl) remained transparent and nearly colourless throughout storage, with some precipitation at t=0 that was not observed at any other timepoint. No other experimental solution (*d–q, u–x*) yielded a completely favourable sensory profile. All co-fortified solutions formulated with KIO_3 (solutions *c–m*, *q*) developed deep red, brown, and/or amber pigments; pungent odours that burned the eyes and nose; and a flaky red or black precipitate with a metallic luster. Conversely, solutions *p* and *u–x*, all of which were formulated with KI, were a homogenous, opaque white with a noticeable luster at t=0, and later separated into distinct layers with sedimentation during storage. Odours characteristic of thiamine were emitted from these solutions that grew more pronounced over the observation period. Notably, a very thick, heavy foam developed on the surface of solutions *g* (KIO_3 and TClHCl with carbonate), *u* (KI and TClHCl with NaOH), and *v* (KI and TClHCl with citrate buffer) during preparation that later settled as precipitate. Solutions *p* (KI and TClHCl [14% w/v thiamine] with carbonate buffer), *w* (KI and TClHCl [7.8% w/v thiamine] with carbonate buffer), and *x* (KI and TClHCl with carbonate solution) were nearly entirely aerated at t=0, such that they were voluminous and stiff; similar in appearance and texture to steamed cow's milk or egg whites whipped to stiff peaks. The foam persisted atop solutions *p*, *w*, and *x* through the duration of the experiment, and became increasingly more difficult to fully reincorporate into solution.

The observed changes to sensory and physical properties over time suggest that only select solutions formulated with KI maintain a sensory profile reasonable for the fortification of salt in Cambodia. See **Table 4-1** for a summary of key observations pertaining to the colour, clarity, odour, precipitation, and other characteristics of the 24 solutions prepared in Experiments 1–3.

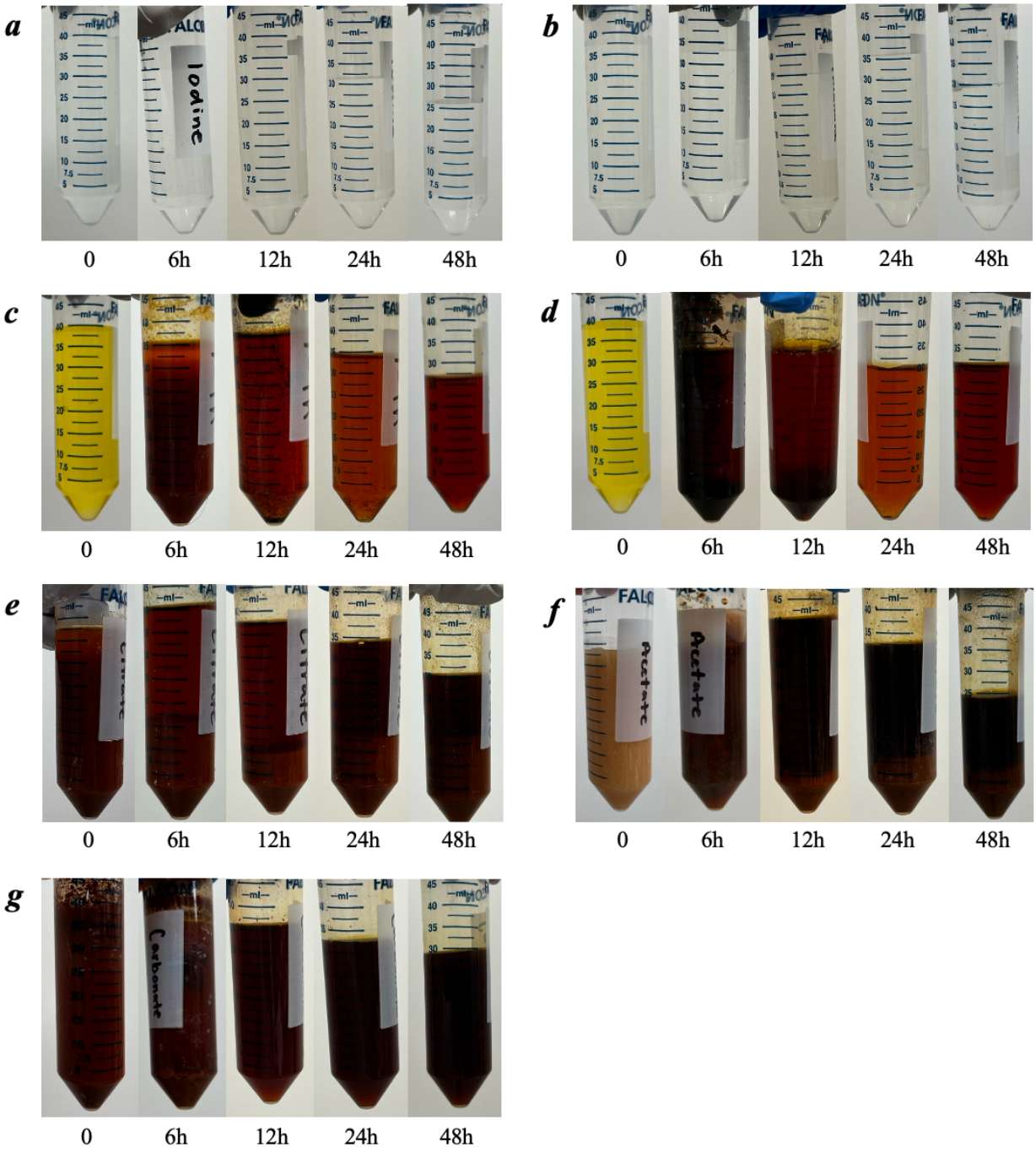

Figure 4-1. Photographs of solutions prepared during Experiment 1A at various timepoints.
a, KIO_3 only; ***b***, TClHCl only; ***c***, KIO_3 and TClHCl; ***d***, KIO_3 and TClHCl with dextrose; ***e***, KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***f***, KIO_3 and TClHCl with sodium acetate; ***g***, KIO_3 and TClHCl with sodium carbonate.

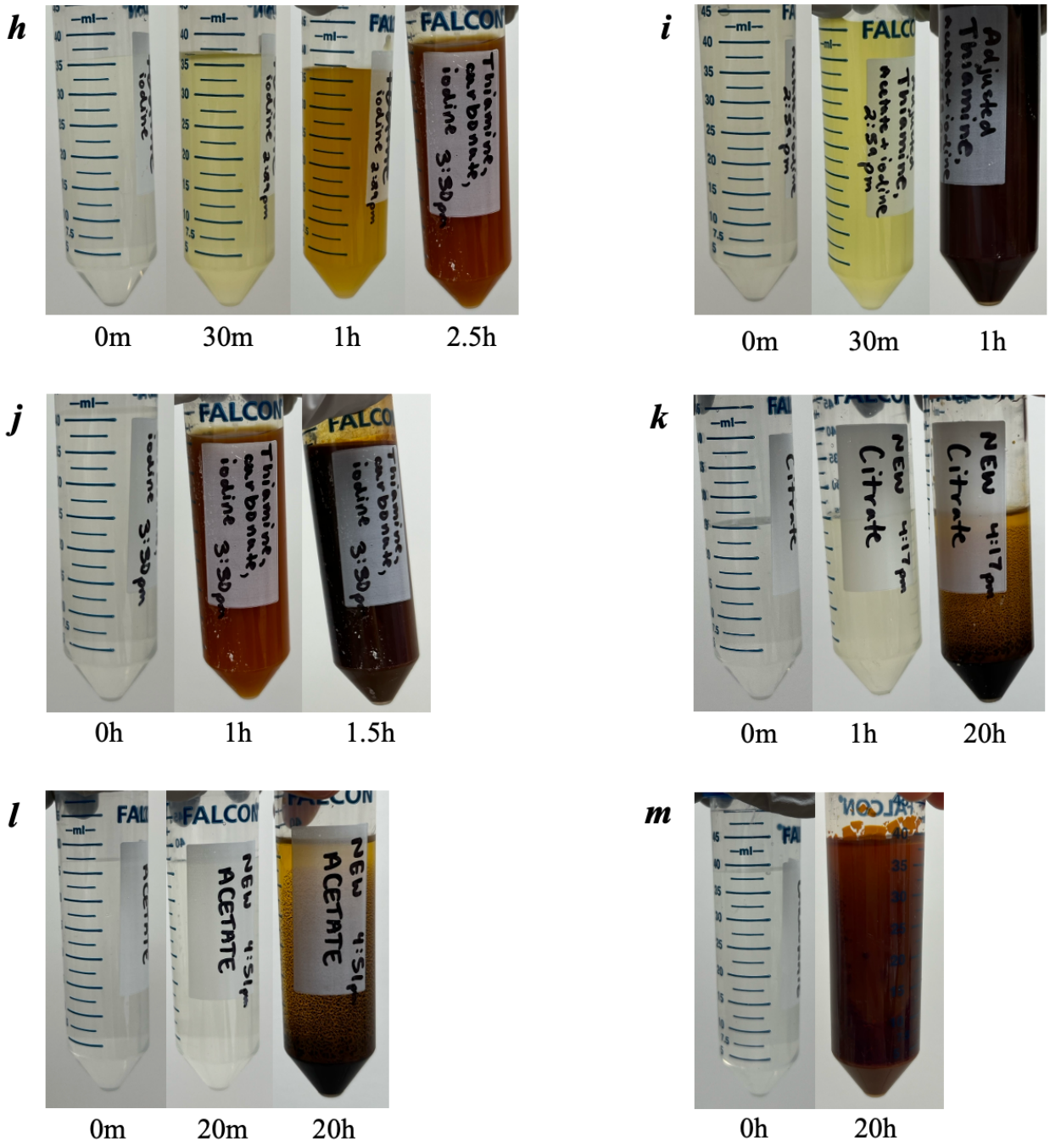

Figure 4-2. Photographs of solutions prepared during Experiment 1B at various timepoints.
h, first modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***i***, first modified KIO_3 and TClHCl with sodium acetate; ***j***, first modified KIO_3 and TClHCl with sodium carbonate; ***k***, second modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***l***, second modified KIO_3 and TClHCl with sodium acetate; ***m***, second modified KIO_3 and TClHCl with sodium carbonate.

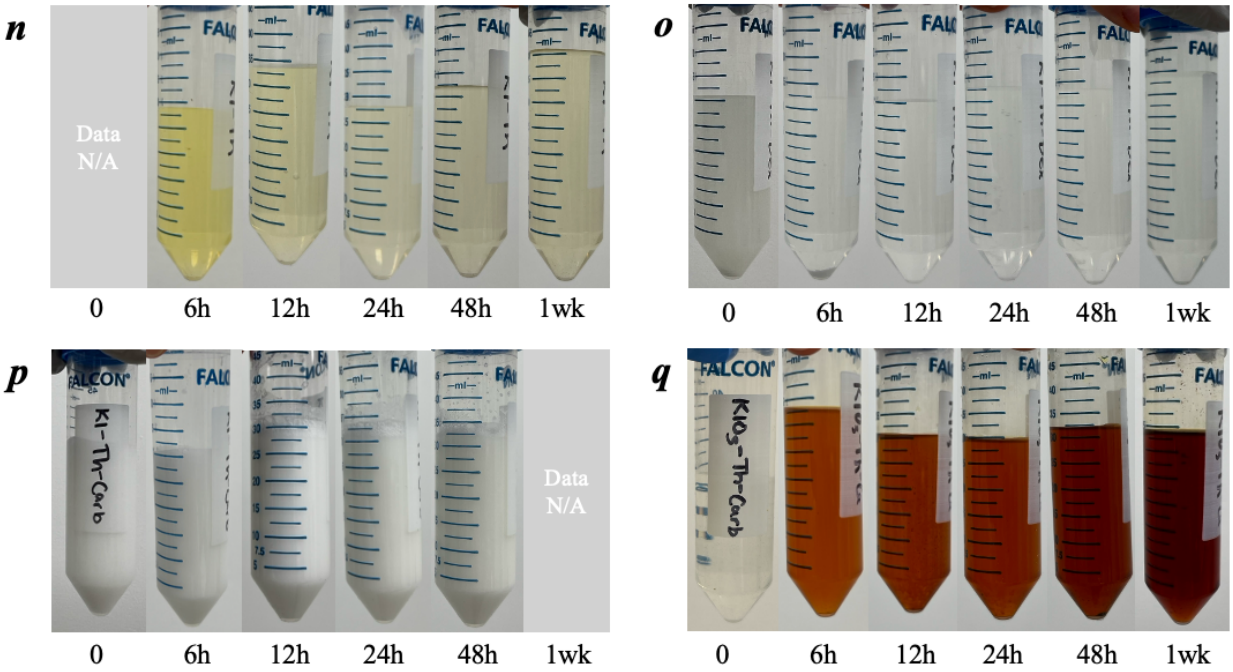

Figure 4-3. Photographs of solutions prepared during Experiment 2 at various timepoints.
n, KI and TClHCl; ***o***, KI and TClHCl with dextrose; ***p***, KI and TClHCl with carbonate buffer; ***q***, KIO_3 and TClHCl with carbonate buffer.

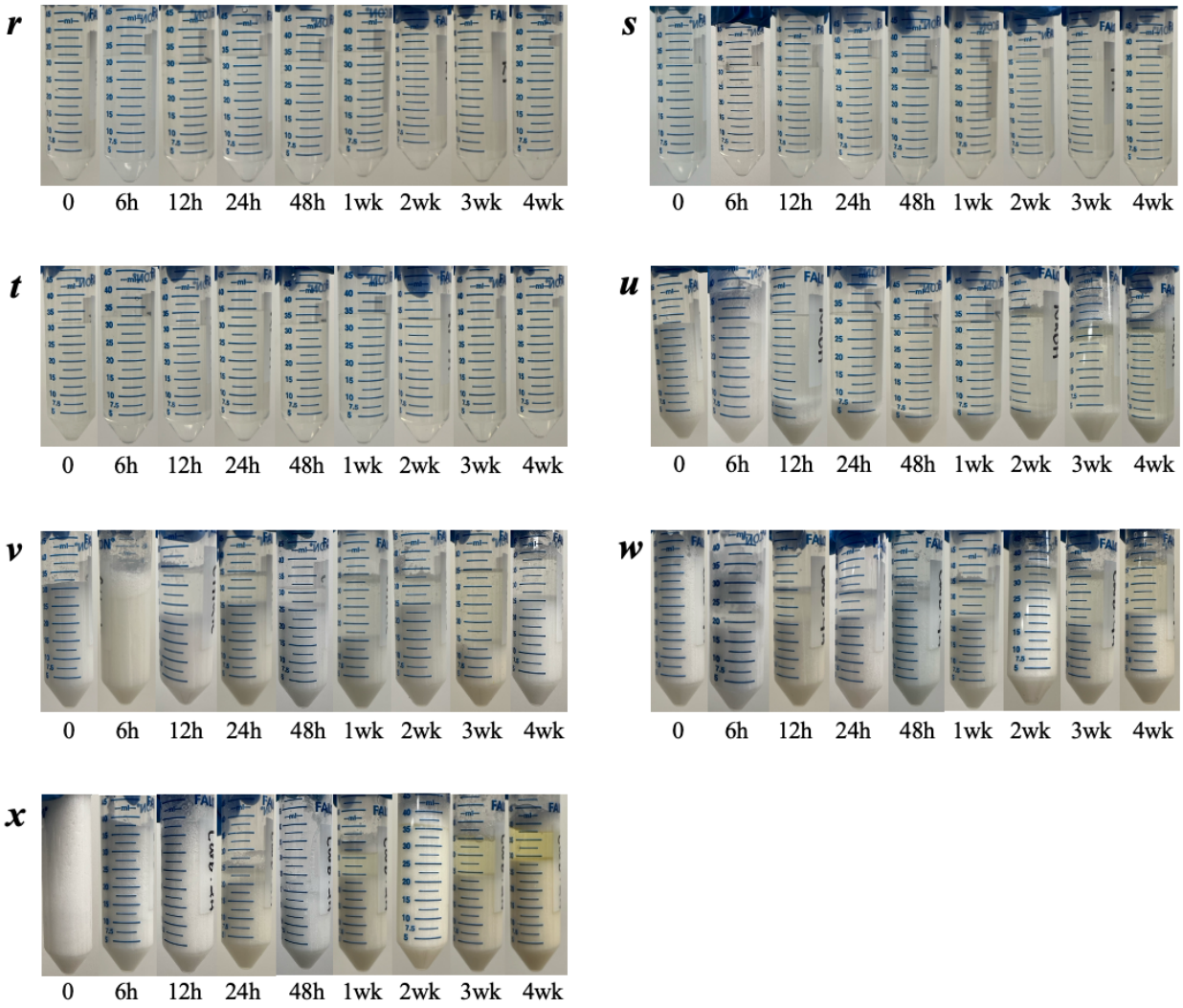

Figure 4-4. Photographs of solutions prepared during Experiment 3 at various timepoints.
r, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x,*** KI and TClHCl with carbonate solution.

Table 4-1: Physical characteristics of all solutions prepared in Experiments 1–3

Sample	Colour	Clarity	Odour	Precipitate	Other/Comments
Experiment 1A					
a	Colourless	Transparent	None	None	Remained constant throughout storage.
b	Extremely faint yellow; nearly colourless.	Transparent	Umami, sulfurous	None	Odour first recorded at *t*=12h and grew stronger during storage.
c	Bright yellow at *t*=0; rust-red at *t*=6h; deep red-brown at *t*=12h and beyond.	Translucent	Pungent, acrid; burned eyes and nose	Red flecks at *t*=6h; black and very sticky at *t*=12h and beyond	Odour first recorded at *t*=12h and grew stronger during storage. Precipitate was settled at bottom of container, could not be agitated or reincorporated into solution.
d	Bright yellow at *t*=0; deep brown at *t*=6h and beyond.	Translucent	Pungent, acrid; burned eyes and nose	Red flecks at *t*=6h; black and very sticky at *t*=12h and beyond	Odour first recorded at *t*=12h and grew stronger during storage. Precipitate was settled at bottom of container; difficult to agitate; could not be reincorporated into solution.
e	Deep rust-red	Opaque	Pungent, acrid; burned eyes and nose	Red flecks at *t*=0; black and very sticky at *t*=6h and beyond	Odour first recorded at *t*=12h and grew stronger during storage. Precipitate was settled at bottom of container; difficult to agitate; could not be reincorporated into solution.
f	Light orange at *t*=0, deep rust-red at *t*=6h and beyond.	Opaque	Pungent, acrid; burned eyes and nose	White flecks at *t*=0; red flecks at *t*=6h and beyond	Odours first recorded at *t*=12h and grew stronger during storage. Precipitate was settled at bottom of container.
g	Deep rust-red	Opaque	Pungent, acrid; burned eyes and nose	Brick-red emulsion at *t*=0; black and very sticky, settled at bottom, at *t*=6h and beyond	Formation of a thick, heavy brick-red emulsion at *t*=0. Precipitate was settled at bottom of container at *t*=6h; difficult to agitate; could not be reincorporated into solution.

Experiment 1B					
h	Faint yellow at *t*=0; rust-red within 3 hours.	Transparent at *t*=0, opaque within 3 hours	Not reported	Dark red flecks	None
i	Faint yellow at *t*=0; rust-red within 3 hours.	Transparent at *t*=0, opaque within 3 hours	Not reported	Dark red flecks	None
j	Very faint yellow at *t*=0; rust-red within 3 hours.	Transparent at *t*=0, opaque within 3 hours	Not reported	Dark red flecks	None
k	Colorless at *t*=0; deep amber within 24 hours.	Transparent	Not reported	Black crystals along sides of conical tube	None
l	Colorless at *t*=0; deep amber within 24 hours.	Transparent	Not reported	Black crystals along sides of conical tube	None
m	Colorless at *t*=0; deep amber within 24 hours.	Transparent	Not reported	Black crystals along sides of conical tube	None
Experiment 2					
n	Yellow	Translucent at *t*=0; transparent at *t*=6h and beyond	Odourless	White flecks at *t*=0	Yellow hue was most pronounced at *t*=6h.
o	Very faint yellow	Translucent at *t*=0; transparent at *t*=6h and beyond	Umami, sulfurous; acrid undertone	White flecks at *t*=0	Nearly clear at *t*=1 week. Irritating odour.
p	White/colourless at *t*=0. Distinct luster.	Opaque	Umami, sulfurous	White, lustrous emulsion that settled during storage	Foam at *t*=0 similar in appearance to steamed whole milk or egg whites whipped to stiff peaks. Solution separated into two distinct phases during storage (clear top layer, opaque white bottom layer) that homogenized temporarily after being shaken.
q	Faint yellow at *t*=0; deep rust-red at *t*=6h.	Transparent at *t*=0; translucent at *t*=1 week	Pungent, acrid; burned eyes and nose	Red flecks at *t*=12h that were black at *t*=1 week	Odours first recorded at *t*=6h and grew stronger over storage.

Experiment 3					
r	Colourless	Transparent	Odourless	None	Remained constant throughout storage.
s	Extremely faint yellow; nearly colourless.	Transparent	Umami, sulfurous	None	Odour first recorded at *t*=6h and grew stronger during storage.
t	Extremely faint yellow; nearly colourless.	Transparent	Umami, sulfurous	White flecks at *t*=0; not observed at any other timepoint	Odour first recorded at *t*=6h and grew stronger during storage.
u	White at *t*=0. Distinct luster. Became slightly more yellow through storage.	Opaque	Umami, sulfurous; very faint.	White, lustrous foam that settled during storage	Odour first recorded at *t*=24h. Stiff foam. Solution separated into three distinct layers (white, frothy top layer; clear middle layer with suspended particulates; opaque white bottom layer) that homogenized temporarily after being shaken. Top layer became increasingly more difficult to reincorporate into solution through the duration of experiment, and layers would separate more quickly after being shaken at each subsequent collection point.
v	Bright white, distinct luster.	Opaque	Umami, sulfurous; very faint.	White, lustrous foam that settled during storage	Odour first recorded at *t*=24h and grew stronger during storage. Stiff foam at *t*=0. Solution separated into three distinct layers (white, frothy top layer; clear middle layer with suspended particulates; opaque white bottom layer) that homogenized temporarily after being shaken. Top layer became increasingly more difficult to reincorporate into solution through the duration of experiment, and layers would separate more quickly after being shaken at each subsequent collection point.

					Odour first recorded at *t*=1 week.
w	Bright white with faint luster at *t*=0. Became slightly more yellow through storage.	Opaque	Nondescript; unlike that of other solutions. Faint.	White, lustrous foam that remained suspended during storage	Voluminous, aerated, and very dense at *t*=0, similar in appearance to steamed whole milk or egg whites whipped to stiff peaks. Solution separated into two distinct layers (a foamy white top layer and near-clear bottom layer) that would not integrate completely when the storage bottles were shaken by hand.
x	Bright white with faint luster at *t*=0. Became more yellow through storage.	Opaque	Nondescript; unlike that of other solutions. Faint.	White, lustrous foam that remained suspended during storage	Odour and yellow hue first recorded at *t*=1 week. Voluminous, aerated, and very dense at *t*=0, similar in appearance to steamed whole milk or egg whites whipped to stiff peaks. Solution separated into two distinct layers (a foamy white top layer and near-clear bottom layer) that would not integrate completely when the storage bottles were shaken by hand.

a, KIO_3 only; ***b***, TClHCl only; ***c***, KIO_3 and TClHCl; ***d***, KIO_3 and TClHCl with dextrose; ***e***, KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***f***, KIO_3 and TClHCl with sodium acetate; ***g***, KIO_3 and TClHCl with sodium carbonate; ***h***, first modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***i***, first modified KIO_3 and TClHCl with sodium acetate; ***j***, first modified KIO_3 and TClHCl with sodium carbonate; ***k***, second modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***l***, second modified KIO_3 and TClHCl with sodium acetate; ***m***, second modified KIO_3 and TClHCl with sodium carbonate; ***n***, KI and TClHCl; ***o***, KI and TClHCl with dextrose; ***p***, KI and TClHCl with carbonate buffer; ***q***, KIO_3 and TClHCl with carbonate buffer; ***r***, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x,*** KI and TClHCl with carbonate solution.

4.2 pH

The pH of solutions at each timepoint is reported in **Table 4-2**. The largest changes in pH were recorded within the first 48 hours; therefore, **Figure 4-5** portrays changes in pH within the first 48 hours for solutions *a*–*g* (**A**), *n*–*q* (**B**), and *r*–*x* (**C**). pH of solutions *h*–*m* was not recorded beyond *t*=0, therefore solutions *h*–*m* are not included in the figure. Only control solution *a* (KIO_3 only) maintained pH within the desired range (5.00<pH<6.00). Most solutions (*c*, *d*, *f*, *n*–*q*, *s*–*u*) grew increasingly more acidic (*i.e.*, mean pH decreased) over time, while solutions *r*, *w*, and *x* became more basic (*i.e.*, mean pH increased). Grouping solutions by chemical source of iodine (KIO_3 or KI) or stabilizing agent did not reveal obvious trends in pH according to solution type. However, when grouped by concentration of TClHCl (*i.e.*, by experiment), it appears that solutions formulated with the lowest concentration of TClHCl (7.8% w/v thiamine) generally achieved higher pH than those with higher concentrations (14% w/v and 21% w/v; see **Appendix A** for visuals). Nonetheless, no experimental solution maintained a pH within the desired range of 5.00<pH<6.00, regardless of the source of iodine, concentration of thiamine, or the presence of an alkalizing agent or buffer.

Table 4-2: Mean pH of all solutions prepared during Experiments 1–3

	Timepoint								
Solution	*t*=0	*t*=6h	*t*=12h	*t*=24h	*t*=48h	*t*=1wk	*t*=2wk	*t*=3wk	*t*=4wk
a	5.59	5.89	5.78	5.90	5.86	-	-	-	-
b	2.36	2.29	2.35	2.43	2.52	-	-	-	-
c	2.58	2.28	0.93	0.78	0.81	-	-	-	-
d	2.75	1.31	0.96	0.77	1.07	-	-	-	-
e	4.50	4.50	4.42	4.35	4.50	-	-	-	-
f	4.87	4.23	4.23	4.21	4.29	-	-	-	-
g	4.38	4.20	4.20	4.17	4.20	-	-	-	-
h	5.20	-	-	-	-	-	-	-	-
i	4.80	-	-	-	-	-	-	-	-
j	5.50	-	-	-	-	-	-	-	-
k	5.55	-	-	-	-	-	-	-	-
l	5.20	-	-	-	-	-	-	-	-
m	5.45	-	-	-	-	-	-	-	-
n	3.23	2.78	2.77	2.78	2.74	2.24	-	-	-
o	3.13	2.80	2.88	2.86	2.85	2.36	-	-	-
p	4.54	4.38	4.37	4.43	4.42	3.94	-	-	-
q	5.48	2.80	2.81	2.73	2.64	2.30	-	-	-
r	5.92	6.36	6.23	6.17	6.52	6.50	6.93	6.47	6.52
s	3.67	3.06	2.92	2.99	3.37	2.85	3.18	2.50	2.70
t	3.23	3.16	3.09	3.16	3.37	2.97	3.35	2.67	2.81
u	4.49	4.23	4.28	4.45	4.55	4.21	4.63	3.82	3.97
v	4.66	4.68	4.93	5.02	4.84	4.93	5.13	4.28	4.54
w	5.11	6.66	6.63	6.96	6.84	7.00	6.95	6.71	6.55
x	6.36	6.82	6.98	7.43	7.45	7.61	7.53	7.40	7.17

a, KIO_3 only; ***b***, TClHCl only; ***c***, KIO_3 and TClHCl; ***d***, KIO_3 and TClHCl with dextrose; ***e***, KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***f***, KIO_3 and TClHCl with sodium acetate; ***g***, KIO_3 and TClHCl with sodium carbonate; ***h***, first modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***i***, first modified KIO_3 and TClHCl with sodium acetate; ***j***, first modified KIO_3 and TClHCl with sodium carbonate; ***k***, second modified KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***l***, second modified KIO_3 and TClHCl with sodium acetate; ***m***, second modified KIO_3 and TClHCl with sodium carbonate; ***n***, KI and TClHCl; ***o***, KI and TClHCl with dextrose; ***p***, KI and TClHCl with carbonate buffer; ***q***, KIO_3 and TClHCl with carbonate buffer; ***r***, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x,*** KI and TClHCl with carbonate solution.

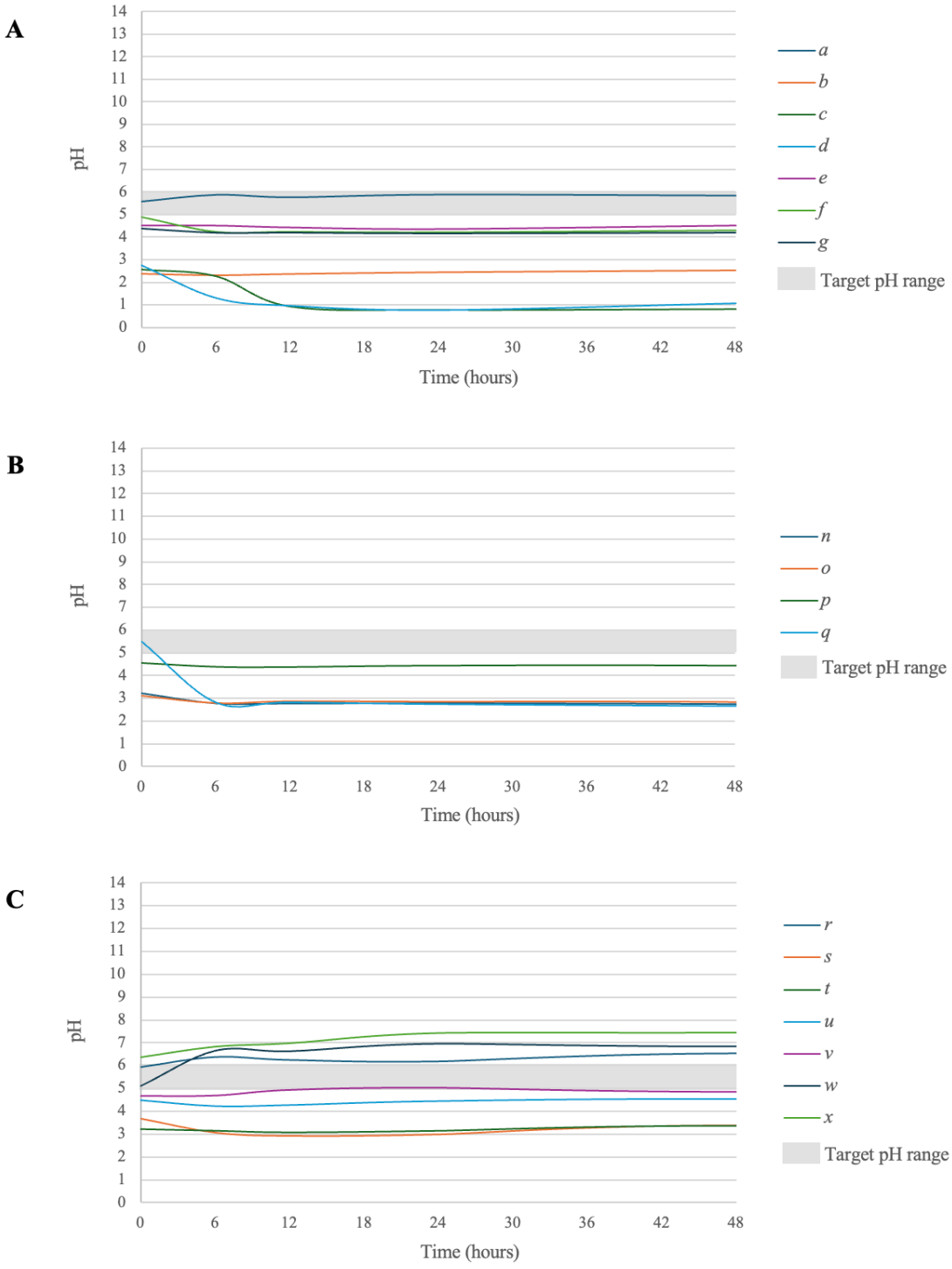

Figure 4-5. pH of solutions over 48 hours of storage. **A.** solutions *a–g* **B.** solutions *n–q* **C.** solutions *r–x*. ***a***, KIO_3 only; ***b***, TClHCl only; ***c***, KIO_3 and TClHCl; ***d***, KIO_3 and TClHCl with dextrose; ***e***, KIO_3 and TClHCl with sodium citrate tribasic dihydrate; ***f***, KIO_3 and TClHCl with sodium acetate; ***g***, KIO_3 and TClHCl with sodium carbonate; ***n***, KI and TClHCl; ***o***, KI and TClHCl with dextrose; ***p***, KI and TClHCl with carbonate buffer; ***q***, KIO_3 and TClHCl with carbonate buffer; ***r***, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x***, KI and TClHCl with carbonate solution.

4.3 Micronutrient content

Thiamine and iodine analyses were not completed for solutions *a–q* because of undesirable physical and sensory characteristics. All solutions for which micronutrient analyses were conducted (*i.e.*, solutions *r–x*) exhibited adequate nutrient stability for the entirety of the four-week experimental period. **Figure 4-6** displays the retention of iodine (**A**) and thiamine (**B**) in solutions *r–x* at each timepoint, calculated as a percentage of their respective concentrations in each solution at *t*=0. After four weeks of storage, both single-nutrient control solutions had minimal to no losses: *r* (KI only) retained >100% of its initial iodine content, while *s* (TClHCl only) retained nearly 90% of its initial thiamine content. Nutrient retention of co-fortified solutions *t–x* was similar to that of the controls. After two weeks of storage at 30°C and 65% RH, all experimental solutions retained ≥80% of their initial concentrations of both thiamine and iodine. Even after four weeks of storage, double the duration in industry settings, retention of both nutrients was ≥70% in all solutions but solution *w* (KI and TClHCl with carbonate buffer), which exhibited a retention of 63% and 79% of initial thiamine and iodine, respectively.

Figure 4-7 displays the absolute concentrations of thiamine and iodine in each solution at each timepoint, colour-coded according to level of concentration, with grey indicating controls, red indicating unacceptably low concentrations, and green indicating acceptable concentrations. Of note, despite solutions being formulated to contain 7.8% w/v thiamine, all solutions exceeded this expected maximum: concentrations of thiamine in the premix solutions at *t*=0 ranged from 8.79–10.59% w/v. Iodine concentrations at *t*=0 were as expected (~3% w/v). Experimental solutions *t–w* contained equal to or greater than the minimum acceptable concentrations of both iodine (≥2.5% w/v) and thiamine (≥6.3% w/v) at *t*=2 weeks, with concentrations of iodine and thiamine ranging from 2.78–3.36% w/v and 8.58–9.18% w/v, respectively. At *t*=4 weeks, all experimental solutions exceeded the minimum acceptable thiamine concentration (6.32–8.95% w/v), whereas only experimental solutions *t* (KI and TClHCl), *u* (KI and TClHCl with NaOH), and *x* (KI and TClHCl with carbonate solution) met or exceeded the minimum acceptable concentration of iodine at this timepoint (2.72%, 2.81%, and 3.08% w/v, respectively). Solutions *t*, *u*, and *x* maintained acceptable concentrations of both thiamine and iodine throughout the experimental period; hence, these solutions are deemed the most viable by way of micronutrient content.

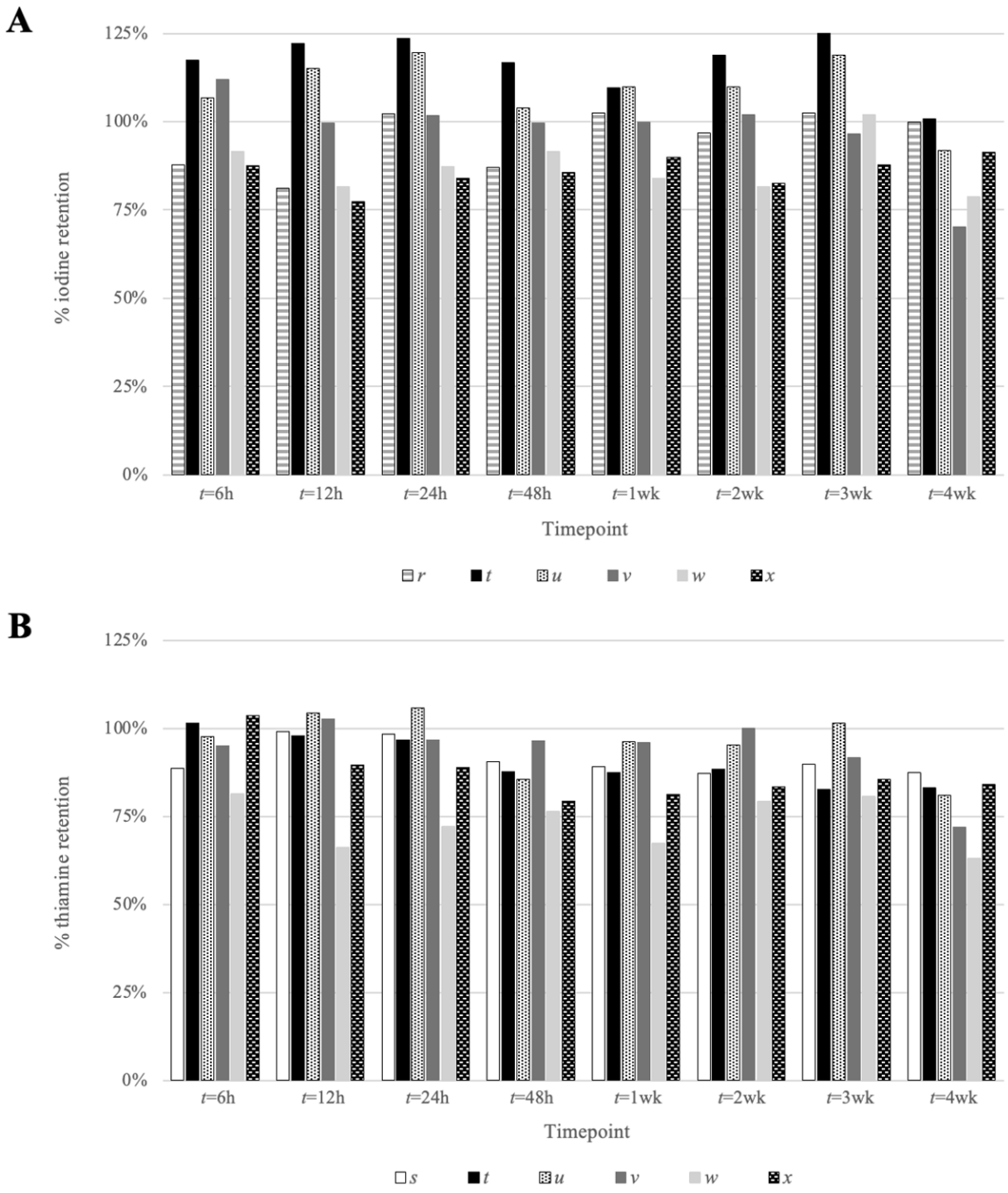

Figure 4-6. Retention of iodine (A) and thiamine (B) in solutions as a percentage of initial iodine or thiamine concentration over time. ***r***, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x,*** KI and TClHCl with carbonate solution.

Solution		Timepoint								
		t=0	*t*=6h	*t*=12h	*t*=24h	*t*=48h	*t*=1wk	*t*=2wk	*t*=3wk	*t*=4wk
r	*I*	3.12	2.74	2.53	3.19	2.72	3.20	3.02	3.20	3.12
	Th	0	0	0	0	0	0	0	0	0
s	*I*	0	0	0	0	0	0	0	0	0
	Th	10.22	9.05	10.12	10.06	9.25	9.11	8.91	9.18	8.95
t	*I*	2.70	3.17	3.30	3.34	3.15	2.96	3.21	3.39	2.72
	Th	10.26	10.42	10.06	9.93	8.99	8.97	9.06	8.49	8.53
u	*I*	3.06	3.27	3.52	3.66	3.18	3.36	3.36	3.64	2.81
	Th	9.63	9.41	10.06	10.19	8.24	9.26	9.18	9.77	7.80
v	*I*	2.95	3.30	2.94	3.00	2.94	2.95	3.01	2.85	2.07
	Th	8.79	8.35	9.02	8.51	8.48	8.44	8.80	8.06	6.32
w	*I*	3.01	2.76	2.46	2.63	2.76	2.53	2.46	3.07	2.37
	Th	10.59	8.62	7.00	7.63	8.10	7.13	8.39	8.54	6.68
x	*I*	3.37	2.95	2.61	2.83	2.89	3.03	2.78	2.96	3.08
	Th	10.30	10.67	9.22	9.15	8.18	8.37	8.58	8.82	8.66

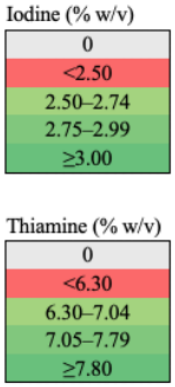

Figure 4-7. Acceptability of thiamine and iodine concentrations in each solution over time.
r, KI only; ***s***, TClHCl only; ***t***, KI and TClHCl; ***u***, KI and TClHCl with NaOH; ***v***, KI and TClHCl with citrate buffer; ***w***, KI and TClHCl with carbonate buffer; ***x***, KI and TClHCl with carbonate solution. *I*, iodine; *Th*, thiamine. Red indicates unacceptably low concentrations of iodine (<2.50% w/v) or thiamine (<6.30% w/v). Green indicates acceptable concentrations of iodine (≥2.50% w/v) or thiamine (≥7.80% w/v), whereby the darker the shade of green, the higher the concentration. Grey indicates expected zeros (control solutions with no added thiamine *or* no added iodine).

5.0 Discussion

This research describes temporal changes in sensory and physical properties, pH, and micronutrient concentration of 24 salt fortification premix solutions containing thiamine and iodine when stored at average 30°C and 65% RH for up to four weeks. The findings demonstrate that solutions formulated with TClHCl and KI are much more viable for the spray fortification of salt than those with TClHCl and KIO_3. Solutions of TClHCl and KIO_3 produced obvious adverse properties such as off-putting colours, odours, and precipitate that are highly inappropriate for the spray fortification of salt. By contrast, nearly all solutions with TClHCl and KI remained clear or white, were less odourous, and exhibited minimal precipitation. Though none of the experimental premix solutions maintained $5.00<pH<6.00$, it appears that pH did not affect micronutrient stability as much as initially hypothesized. Encouragingly, three solutions analyzed for micronutrient content maintained acceptable concentrations of both thiamine and iodine after four weeks of storage, with one solution considered highly viable for the spray co-fortification of salt in Cambodia.

5.1 Selection of fortificants

5.1.1 Thiamine fortificants

Three forms of thiamine have been used in the literature as thiamine fortificants, and thus, were initially considered in this research for the formulation of the premix solutions. These are TClHCl, TMN, and ThDP. Despite being the biologically active form of this vitamin in humans, ThDP has also been used as a source of thiamine for food fortification; albeit, much more seldomly than TClHCl or TMN. This is likely because ThDP is not listed as a permitted food additive in the United States by the Food and Drug Administration (126) or in the Codex Alimentarius General Standard for Food Additives (127); the latter of which guides food standards in Cambodia (128). That said, The European Union does permit the addition of ThDP to foods in the form of thiamine pyrophosphate chloride (129). Only one study using ThDP as a thiamine fortificant was identified (103). In this report, authors used ThDP or TClHCl to fortify potato dumplings with various freeze-dried vegetable-thiamine matrices, and found that thiamine losses were significantly greater in samples prepared with ThDP compared to with TClHCl ($p<0.05$) (103). Accordingly, not only would ThDP not be permitted for use in Cambodia, but it

is also unlikely that its stability would be more favourable in aqueous premix preparations than other readily available thiamine fortificants.

An important factor to consider when selecting a thiamine fortificant for an aqueous premix solution designed for the spray fortification of salt is solubility. The selected fortificant must be adequately soluble to achieve even distribution of the nutrient throughout the solution in appropriate concentrations for the desired level of fortification (88). To achieve a thiamine dosage of 275 ppm in fortified salt as recommended in Chan *et al.* (63), the premix solution must contain a minimum of 13.8% w/v (138 g/L) thiamine, assuming the industry standard of 2 mL of premix are used per kg salt (Hou Kroeun, personal communication, December 2023). This concentration of thiamine is equivalent to 175 g/L TClHCl, 170 g/L TMN, or 240 g/L ThDP. However, the solubilities of these compounds are approximately 1000 g/L, 27 g/L, and 50 g/L, respectively (100,102,130), indicating that only TClHCl is adequately soluble in water to achieve 275 ppm of thiamine in fortified salt. If a lesser thiamine dosage was desired, such as that attempted in Experiments 1B and 3 (156 ppm), this would require 7.8% w/v (78 g/L) thiamine, equivalent to 100 g/L TClHCl, 96 g/L TMN, or 135 g/L ThDP. Again, only TClHCl is adequately soluble in water to achieve this level of fortification. Accordingly, the only thiamine salt suitable for use in a high-concentration aqueous premix solution designed for spray fortification is TClHCl, and thus, was the fortificant selected as the source of thiamine for all solutions prepared in this series of experiments.

5.1.2 Iodine fortificants

KIO_3 was selected as the iodine fortificant for solutions prepared during Experiment 1 in keeping with current salt iodization practices employed by the Salt Producers Community of Kampot and Kep. KIO_3 has long been the preferred fortificant for salt iodization in hot, humid climates due to its greater stability as a single fortification ingredient in these conditions compared to KI (75,85,86). Even so, co-fortification premix solutions formulated with KIO_3 were evidently not viable for the spray fortification of salt with thiamine and iodine due to their rapid discolouration, precipitation, and malodour, necessitating the selection of an alternative source of iodine for this purpose. The Government of Cambodia has approved KIO_3, KI, $NaIO_3$, and NaI for the iodization of salt (68). Potassium salts of iodate and iodide are preferred over the sodium

salts for salt iodization given the increased stability of iodine in fortified salt when potassium salts are used (131). Despite being an approved fortificant in Cambodia, KI is not traditionally used to iodize salt in hot and humid climates, or with high levels of impurities common to salt in LMIC, due to its susceptibility to oxidation in these conditions (90,91). Such oxidation causes rapid losses of iodine, necessitating the addition of stabilizers; particularly dextrose, a reducing sugar, and calcium silicate, an anti-caking agent (86,90,91). At present, the use of KI as a fortificant for salt is largely limited to economically developed nations with robust salt refining techniques, mild climates, access to impervious packaging materials, and/or the ability to consistently add stabilizers to the fortified salt at the point of production (86). Nonetheless, KI was selected as an alternative iodine fortificant in Experiments 2 and 3, and promisingly, produced more favourable physical and sensory outcomes in the co-fortified solutions than did KIO_3.

There are several benefits to using KI in place of KIO_3 for the co-fortification of salt with thiamine and iodine in Cambodia. KI is more economical than KIO_3, due to both its lower price per gram compared to KIO_3 and its higher proportion of iodine (by weight) per mole (KI is 76% iodine by weight, whereas KIO_3 is only 59% iodine by weight) (131), thereby requiring less mass of KI per L of premix solution than would be required of KIO_3 to attain the same iodine concentration. KI is also more soluble in water than KIO_3, rendering it more appropriate for application in high-concentration solutions such as those described here. Most encouragingly, given that thiamine itself is a reducing agent (114–116) and is necessarily present in high concentrations in the co-fortified premix solutions, it is likely that no additional reducing agents or stabilizers would be required in the premix, keeping the formulation simple and practical for low-resource settings.

5.2 pH

pH data were collected as part of this research to assess the impact of pH on various outcomes related to solution viability, including sensory properties and micronutrient retention. Despite none of the co-fortified solutions maintaining $5.00<pH<6.00$ as initially desired to stabilize both nutrients (91,100), the pH of solutions did not appear to influence sensory properties or micronutrient retention to any great degree. What appeared to have the greatest effect on sensory

and physical properties of the solutions was the source of iodine, which was switched from KIO_3 to KI in light of these findings, explored below.

5.3 Organoleptic and physical properties

To co-fortify salt with thiamine and iodine using spray fortification, the ideal spray solution would be clear and colourless (or nearly so), with evenly dispersed and fully dissolved solutes, no precipitate, and no malodour (131). This would favour end-product safety and desirability, while protecting manufacturers from hazardous fumes and maintaining optimal functionality of equipment. Here, many solutions formulated with KIO_3 developed colours, odours and precipitate that are highly undesirable for the spray fortification of salt.

5.3.1 Colour

5.3.1.1 Iodine and its derivatives

Solutions formulated with TClHCl and KIO_3 were not viable due to the rapid development of unfavourable pigments. All of these solutions were a deep red-brown colour within six hours of storage. This may be explained by the formation of aqueous I_2 and triiodide (I_3^-) in a series of redox reactions as follows[4,5]:

(1) $IO_3^- + 3$ [reducing material] $\rightarrow I^- + 3$ [oxidized material]

(2) $IO_3^- + 5\ I^- + 6\ H^+ \rightarrow 3\ I_2 + 3\ H_2O$

(3) $I_2 +$ [reducing material] $\rightarrow 2\ I^- +$ [oxidized material]

(4) $I_2 + I^- \rightleftharpoons I_3^-$.

Though simple aqueous solutions containing I^- are colourless, those containing I_2 or I_3^- appear red to deep brown in colour (136–138). Accordingly, formation of I_2 and/or I_3^- likely contributed to the pigmentation observed in all solutions formulated with KIO_3 and TClHCl. By contrast, solutions formulated with KI and TClHCl did not develop red-brown pigments at any point during storage, suggesting that I_2 and/or I_3^- were not formed in these solutions. Considering the

[4] This series of reactions has not been published explicitly, but was extrapolated using the equations and reasoning presented in (92,132–135).

[5] $3\ O^{2-}$ have been omitted from the reaction products of reaction (1) due to ambiguity of the reducing material employed.

reaction illustrated above, this is unsurprising, given that I^- in these solutions cannot be further reduced by reducing materials such as thiamine (114–116), and thus would not react to form the pigmented iodine compounds I_2 and I_3^-.

5.3.1.2 Thiamine and its derivatives

The degradation of thiamine may have also contributed to the unfavourable colours observed from solutions with KIO_3. The bright yellow colour of solutions *b* (KIO_3 and TClHCl) and *d* (KIO_3 and TClHCl with dextrose) observed at t=0 was likely due to the formation of a thiamine derivative, thiochrome, that is produced when thiamine is in solution with a strong oxidizing agent (110). IO_3^- is a relatively strong oxidizing agent when in acidic conditions ($E°$ +1.195 V (139)) such as those of solutions here, so it is possible that thiamine was oxidized to thiochrome nearly instantaneously upon dissolution with KIO_3 to produce a fluorescent yellow pigment. The same fluorescent hue was not observed in solution *q* (KIO_3 and TClHCl with carbonate buffer) at t=0, likely due to the higher alkalinity of the solution (pH 5.48 at t=0) where the reduction potential of IO_3^- is much lower ($E°$ +0.257 V at pH 7 (139)), and is thus not strong enough to oxidize thiamine to its thiochrome form.

Solutions prepared in Experiments 2 and 3 (*o* [KI and TClHCl with dextrose], *s* [TClHCl only], *w* [KI and TClHCl with carbonate buffer], and *x* [KI and TClHCl with carbonate solution]) developed only a faint yellow pigment over the storage period, different to that of thiochrome, that would be much more acceptable if sprayed onto salt. Indeed, solutions of thiamine salts are known to have a slight yellow hue that deepens over time and with increasing temperatures. For instance, Voelker *et al.* found that solutions of TClHCl (27, 100, and 500 g/L; equal to 2.1, 7.8, and 39 % w/v thiamine in solution, respectively) were clear and faintly yellow at t=0, but developed deep red-brown pigments after 31 days of storage at 80°C (100). However, solutions of only TClHCl formulated for this thesis did not develop pigments like those described by Voelker and colleagues, likely due to the different storage temperatures used between the two studies (30°C here versus 80°C used by Voelker *et al.*; the latter causing more rapid changes in colour than the former (100)). Given that premix solutions will not be stored at 80°C in practice, it is unlikely that solutions of KI and TClHCl will develop unfavourable red pigments that were

not observed in the current experiments if used in industry settings, thereby maintaining acceptability by way of colouring.

5.3.2 Odour

5.3.2.1 Iodine

In addition to the development of pigments and precipitate, many solutions prepared in Experiments 1–3 became increasingly more odourous throughout storage. Odours produced by solutions prepared with TClHCl and KIO_3 during Experiment 1 were particularly offensive, burning the eyes, nose, and throat after just brief exposure while wearing appropriate lab attire and personal protective equipment. Such irritation is indicative of the production of I_2 gas, which is toxic, corrosive, and cautioned as an irritant of the skin, eyes, and respiratory tract (140–142). Moreover, I_2 gas is known to have a distinctive sharp odour (140), which was detected in solutions *c*–*g*. The release of I_2 gas from various solutions formulated with TClHCl and KIO_3 is in keeping with known behaviours of elemental iodine, which volatilizes readily in standard conditions. Such volatilization is not ideal for salt fortification as it causes rapid losses of iodine (86,90,91,140,141). Importantly, these irritating vapours and distinctive odours were not noted in solutions formulated with KI and TClHCl, suggesting greater iodine retention in solution and a more favourable sensory profile.

5.3.2.2 Thiamine

The distinctive, meaty odour characteristic of thiamine was detected in several solutions ranging in intensity from faint to noxious. A number of compounds contributing to its aroma have been identified, including furans, pyrimidines, thiols, thiazoles, sulfides, disulfides, and elemental sulfur (143–145). These compounds form readily under conditions of high moisture and low pH (100,143,146); both of which are the conditions of the solutions prepared here.

Aromatic compounds derived from thiamine are known to contribute favourably to the aroma and flavour of cooked meats and other savoury dishes (144,147), and are not necessarily linked to loss of vitamin activity (100). Should any of the premix solutions prepared here be used to fortify salt, thiamine-derived aromatics imparted onto the salt by the premix solution may actually be advantageous to consumer acceptability of the salt, given that umami is an important

flavour component of traditional Cambodian cuisine (148). For instance, in a sensory analysis of thiamine-fortified salt by McSweeney *et al.* among Cambodian consumers, thiamine-fortified salt was highly rated by participants across all five sensory categories evaluated (appearance, aroma, taste, texture, overall liking) when used both in soup- and porridge-style meals (n=499) and in stir-fried and grilled meals (n=430). In either instance, the liking of thiamine-fortified salt was scored at an average of 4.5/5 across the evaluated categories, where a score of 1 indicated "dislike it very much", and 5 indicated "like it very much" (113). As such, the thiamine-derived aromatic compounds produced by premix solutions are not of concern, and may actually enhance sensory acceptability of co-fortified salt in Cambodia. That said, the transferability of such fortified salt to populations who use salt for non-savoury cooking and baking would need to be explored before scaling this intervention in other countries or cultural settings.

5.3.3 Precipitation

Precipitation was observed in several solutions across the three experiments. Indeed, many of the "solutions" prepared here are more appropriately referred to as suspensions, given the settling of undissolved particulates at the bottom of the storage bottles upon standing, that would resuspend with agitation. Other solutions developed large precipitate that would not resuspend. Precipitation of premix solutions designed for spray fortification is not desirable, as the particulates may accumulate on the interior of the fortification equipment, causing damage or limiting functionality. Large precipitate may obstruct the tubing supplying the machine with premix or may not be siphoned onto the salt due to its mass, resulting in poor fortification in either case. Solutions with precipitated matter would also be challenging to evenly distribute throughout the salt, potentiating unequal nutrient distribution within each batch and leading to inconsistent levels of fortification across packages (90).

Many of the solutions were not viable because of unfavourable colours and odours, and are similarly inviable due to precipitation. Of interest here are the three solutions with promise, namely solutions *t* (KI and TClHCl only), *u* (KI and TClHCl with NaOH), and *x* (KI and TClHCl with carbonate solution), which exhibited reasonable physical and sensory properties and adequate nutrient stability. That said, two of these solutions, namely, *u* and *x*, became foamy during preparation with the addition of NaOH or carbonate solution, respectively, later resulting

in sedimentation that may not be optimal for the spray fortification of salt (90). By contrast, solution *t* exhibited little to no precipitation through the four-week observation period, which is most ideal for a co-fortification premix solution for reasons described above (88).

5.4 Micronutrient retention and absolute concentration

Micronutrient retention and absolute concentration of the solutions for which such analyses were conducted (*r*–*x*) were very encouraging. Micronutrient retention was calculated to better conceptualize the stability of nutrients in the solutions, while absolute concentration of each nutrient was used to dichotomize the solutions as acceptable or not acceptable for the spray co-fortification of salt. Minimum acceptable concentrations were defined as ≥2.5% w/v iodine and ≥6.3% w/v thiamine at any given timepoint; the former derived from Cambodian regulations (68), the latter calculated using the RDA for thiamine for WRA (1.4 mg/d) and mean thiamine intakes among this population in Cambodia previously estimated by Coats *et al.* (0.75 mg/d) (124). Solutions with satisfactory concentrations of only one nutrient were not considered appropriate for spray fortification.

Overall nutrient retention was excellent. Each solution maintained ≳80% of both thiamine and iodine over two weeks of storage, which is the typical storage duration of premix solutions used by Cambodian salt manufacturers at present (Hou Kroeun, personal communication, December 2023). Ultimately, solution *t* is likely the most viable solution for spray fortification, given its more favourable sensory properties compared to solutions *u* and *x* (*t* was transparent, with minimal precipitation observed at *t*=0 only, nearly colourless, and had umami aromas that may be favourable in Cambodian cuisine; *u* and *x* both developed thick foams during preparation and exhibited sedimentation during storage, the latter also developing a bright yellow hue near the end of the storage period) and its simple formulation of just KI, TClHCl, and water.

While promising, there are a few factors to consider when interpreting the nutrient retention data. For instance, several solutions, namely *r* (KI only), *t* (KI and TClHCl only), *u* (KI and TClHCl with NaOH), and *v* (KI and TClHCl with citrate buffer), exhibited nutrient retention that seemed to increase considerably over time (e.g. solution *t* exhibited a reported iodine retention as high as 126% of its initial content at *t*=3 weeks). These detected increases are likely not due to bona fide

increases in absolute iodine content over time, but are instead likely a result of sampling errors owing to the colloidal nature of the "solutions", again more appropriately referred to as suspensions. It is most likely that I^- was not evenly distributed throughout the solution, resulting in total moles of I^- withdrawn from the aqueous premixes at each collection point to be different than what would be expected with uniform distribution, thereby skewing the reported molarity of the solution at points of collection. The sedimentation and other precipitation-type (foaming) reactions observed in solutions at t=0 would have also impacted the iodine concentration in the aliquots for much the same reason. In either instance, sampling errors due to the uneven distribution of fortificant in the premix solutions may have resulted in reported values that do not accurately reflect the true iodine content of the aqueous premixes at any given timepoint. Nonetheless, the iodine content of all solutions remained impressively high relative to the initial concentration (nearly all retained ≳80% of iodine at each timepoint), particularly in view of the rapid volatilization that is typical of iodine in conditions of high humidity and heat, often resulting in poor iodine retention (86,91,149).

To prevent inconsistent fortification in practice, it may be advantageous to prepare only one to two days' worth of premix solution at a time, as opposed to two weeks' worth, to prevent the sedimentation of particulates that may occur over prolonged storage. The solution may also be stirred or agitated prior to being sprayed on the salt in effort to resuspend any settled matter. However, it is not certain how impactful these measures would be in practice. The likely most practical means to uniformly fortify salt would be to use a premix formulation with little to no precipitation, such as solution *t* prepared here. If used to fortify salt, further work will be needed to assess the uniformity of fortification within and across batches of the co-fortified *salt* to determine if any of the measures just described to prevent sedimentation are indeed warranted at the point of production.

5.4.1 Impact of pH on nutrient stability

It was initially hypothesized that maintaining solution pH of 5.00–6.00 would favour the stability of both thiamine and iodine (91,100), preventing significant losses of either micronutrient. It appears this was not the case: pH did not seem to impact the stability of thiamine nor iodine, given that the three solutions maintaining adequate nutrient concentration through storage also

maintained considerably different mean pH over the four-week storage period. These solutions, *t* (KI and TClHCl), *u* (KI and TClHCl with NaOH), and *x* (KI and TClHCl with carbonate solution), maintained mean pH of 2.98, 4.29, and 6.86, respectively, while each retained 91–100% of initial iodine content and 81–84% of initial thiamine after four weeks' storage.

Disoady *et al.* previously found that pH is not the sole mediator of iodine stability in fortified salt through analyses of iodized salt samples ranging in pH from 6.25–9.77 (91). Iodine stability is more likely influenced by other compounds present, such as magnesium chloride (90), that draw moisture onto the salt. While all premix solutions prepared here demonstrated adequate nutrient stability, data pertaining only to the *solutions* were collected, and were extrapolated to interpret their suitability for the spray fortification of *salt*. Future experiments are needed to determine how nutrient stability may be influenced by the presence of impurities within the salt when the co-fortified premix solutions are used for its fortification, explored in greater detail below.

5.5 Future directions and opportunities

This project is an exciting first step in the development of a co-fortified salt to prevent thiamine and iodine deficiencies in Cambodia; however, more work is needed to determine the viability of a thiamine-iodine co-fortified salt for use in this setting.

5.5.1 Using acceptable solutions to fortify salt

This project explored the viability of thiamine-iodine premix solutions specifically designed for the spray fortification of salt. Given that spraying technologies are currently utilized to fortify salt in Cambodia, it would be ideal to create a premix solution compatible with the existing equipment and procedures to facilitate uptake among salt producers without drastically increasing costs incurred. Here, three different experimental premix solutions designed for the spray fortification of salt maintained acceptable concentrations of both thiamine and iodine after four weeks of storage in simulated Cambodian conditions (30°C, 65% RH). These were prepared with KI and TClHCl, along with either NaOH, citrate buffer and NaOH, or no additional reagents (*i.e.*, KI and TClHCl only). While all were considered "viable" by way of nutrient content, the most practical and technically feasible solution for spray fortification among these three solutions was that with KI and TClHCl only, given its simple formulation and only minor

alterations to physical and sensory properties that would likely not influence consumer acceptability of the co-fortified salt (113).

Future experiments are necessary to ascertain the viability of the KI and TClHCl premix solution once sprayed onto salt, particularly in relation to the stability of thiamine and iodine in the *salt* over time when stored under typical Cambodian conditions. In these experiments, it will be important to evaluate how duration of storage, storage temperature, % RH, and different types of packaging materials (*i.e.*, low-density polyethylene, plastic containers with screw-top lids, or other packaging materials commonly employed at the household level) may affect nutrient stability. It may also be beneficial to evaluate nutrient stability in fine salt compared to coarse, given that the crystal size and the presence of impurities common to coarse salt are known to impact iodine stability (67,86,150). Hygroscopic impurities common to coarse or "crude" sea salt draw moisture onto the salt from the surrounding environment, causing rapid reduction of IO_3^- to I_2, particularly in the presence of reducing agents or minerals (90,91). Given that we are suggesting the use of KI instead of KIO_3 in this premix formulation, which is seldomly done in LMIC (151), it is unknown how hygroscopic impurities in the salt may affect iodine stability, particularly in the presence of thiamine. Notably, considering the antioxidation properties of thiamine (114–116), it may be that iodine stability in the co-fortified salt will not be impacted by moisture or impurities present that would otherwise cause iodine volatilization due to the rapid oxidation of I^- in these conditions (90,91). In fact, thiamine and iodine may have a mutually stabilizing effect in co-fortified salt, as was similarly observed by Modupe and Disoady between folic acid and iodine in a quadruple fortified salt with iodine, folic acid, iron, and vitamin B_{12} (82). Still, future experiments will need to assess the stability of thiamine and iodine in a co-fortified salt before any conclusions regarding their reciprocal stability can be drawn.

5.5.2 Formulating additional premix solutions

Solutions were initially formulated with 21% w/v thiamine, equivalent to 264 g/L TClHCl in solution and 415 ppm thiamine in fortified salt, in attempt to achieve the thiamine fortification dose for salt recommended by Chan *et al.*, with 50% overages (63). This high concentration of TClHCl affected pH of the solutions and likely contributed to the unfavourable physical and sensory outcomes observed in the first set of experiments. Accordingly, thiamine dosing was

titrated down to 14% w/v and 7.8% w/v in subsequent experiments to decrease the concentration of both H^+ and total solutes in solution. Solutions with lower concentrations of thiamine exhibited much more favourable outcomes, particularly in terms of physical and sensory properties. That said, the source of iodine was also switched from KIO_3 to KI in these experiments, and nutrient analyses were only carried out for solutions with 7.8% w/v thiamine; therefore, it is unknown how KI may react in solutions with thiamine concentrations >7.8% w/v. Furthermore, while the solutions containing 7.8% w/v thiamine would yield a fortification dosage that would satisfy the RDA for thiamine among WRA in the context of an existing baseline intake of thiamine from non-salt dietary sources (124), it would be valuable for future experiments to reformulate a premix solution with KI and greater concentrations of TClHCl that more closely align with the fortification dose recommended by Chan *et al.* (63) to evaluate how greater concentrations of thiamine may influence solution viability.

5.5.3 Microencapsulation

Despite the ease of leveraging existing spray fortification machinery in Cambodia to co-fortify salt with thiamine and iodine, an alternative, very intriguing alternative method is microencapsulation. Encapsulating one or both fortificants with food-safe coating materials creates a physical barrier to inhibit their interaction, as well as interaction with impurities within the salt or with the environment, that may otherwise cause a loss of nutrient content and unfavourable changes to organoleptic properties (77). Though technically feasible (152), investment in the technology to microencapsulate iodine would be redundant given the spray fortification infrastructure already in place in Cambodia (77). Therefore, should microencapsulation be of interest, the most practical means to co-fortify salt with thiamine and iodine using this technology would be to encapsulate pellets of a solid thiamine premix.

Briefly, an edible dough comprised primarily of a milled flour, vegetable oil, and water, would be fortified with TMN rather than TClHCl, as TMN is the preferred thiamine fortificant for dry applications (104). While durum semolina is traditionally used as the binding starch for pellet-type micronutrient premixes (77), rice flour also yields an acceptable extrudate when food-grade dextrin is incorporated as a secondary binder (153). Rice flour may be a more cost-effective and culturally relevant starch for the production of a solid thiamine premix, given the abundance and

cultural significance of rice in Cambodia (11). In either case, the thiamine-fortified dough would be extruded in thin strands, cut into pieces of ~0.5 mm in diameter to mimic the size of salt crystals, and encapsulated with a food-safe, water-resistant coating, as described by various authors elsewhere (77,131,153). The encapsulated thiamine pellets would then be mixed with salt spray-fortified with KIO_3 or KI in an appropriate ratio to attain the target dose of 275 ppm thiamine (63) and 50–60 ppm iodine (68) in the co-fortified salt. Of course, future experiments would have to assess the stability of both KIO_3-fortified and KI-fortified salts alongside the thiamine pellet to ensure adequate retention of both thiamine and iodine in the co-fortified salt when stored or used for cooking.

A secondary advantage of encapsulating a thiamine extrudate is that the formulation of the solid thiamine premix may be modified to include additional nutrients, either within the extrudate or one of the additional coating layers, as described elsewhere (80,82,131). The pellet may be easily reformulated to reflect the evolving micronutrient needs of the Cambodian population, or to reflect changes to daily salt intake in light of the World Health Organization's sodium reduction initiatives (154). The formulation may also be modified to accommodate the salt intake patterns and varying micronutrient needs of other populations, such as the addition of retinyl palmitate and ferrous fumarate to a thiamine pellet for salt fortification in Lao PDR, where vitamin A and iron deficiencies remain prevalent (155), or zinc oxide to that for Vietnam, where zinc deficiency remains a concern (156).

5.5.4 Sensory evaluations and human efficacy trials

Regardless of the method used to co-fortify salt with thiamine and iodine in a manner that preserves both nutrients over an extended period of storage in typical Cambodian conditions, it is imperative that sensory evaluation and human efficacy trials are conducted to ensure acceptance and efficacy of the co-fortified salt prior to national programmatic implementation. Sensory trials and colour analyses should also be conducted to assess acceptability of the product among Cambodian consumers compared to traditional iodized salt. Importantly, human efficacy trials would be required to evaluate the impact of the thiamine-iodine salt on outcomes of interest, such as erythrocyte ThDP concentrations and urinary iodine excretion (36,157), with a particular

focus on outcomes among a nationally-representative sample of WRA and their infants, given that these populations are the intended beneficiaries of any such intervention (36).

In addition to assessing the efficacy of the thiamine-iodine salt, future work will need to evaluate the economic and programmatic feasibility of implementing a co-fortified salt program in Cambodia. This may be done using evidence from previous or existing multiple-micronutrient salt fortification programs worldwide, or ex-ante modelling of prospective programs (158–160). Indeed, the success of a nationwide co-fortified salt program relies on the tremendous buy-in from multiple stakeholders across multiple sectors; robust, ongoing consumer education to—among other objectives—illuminate the merits of co-fortified salt and to rationalize the modest surcharge when compared to traditional iodized salt; financial assistance and/or technical assistance for procurement of necessary equipment and consumables; strict enforcement of quality standards; and regular quality assurance during production and distribution of the co-fortified salt (161–164). It is advised to engage all relevant stakeholders in each step of the planning and piloting phases of any national co-fortified salt intervention to ensure there is sufficient support and clear comprehension of the production and distribution channels, in addition to sophisticated consumer education and quality control, to ensure long-term prosperity of the prospective thiamine-iodine co-fortified salt program in Cambodia (161,162).

5.5.5 Quality control

A crucial component to the success of any prospective national co-fortified salt program is regular monitoring and ongoing quality control at multiple points throughout the supply chain (161). Cambodia has previously experienced complications with national salt iodization due to inconsistent funding, insufficient manufacturing standards, and gaps in quality assurance (69). However, since 2018, government enforcement of strict quality control measures at the point of production, distribution, and purchasing have improved greatly (69).

Generally, assessments for the presence of iodine in salt can be categorized as quantitative and qualitative (165). Quantitative assessments determine the amount (ppm) of iodine in a salt sample, often using laboratory methods such as titration, while qualitative assessments simply indicate if iodine is present in the salt or not, often using rapid field test kits (165,166). Rapid

field test kits are inexpensive, easy to use and interpret, and do not require electricity or running water to operate; therefore, these kits are imperative for assessing the quality of iodized salt in low-resource settings where literacy rates may be poor (151,166,167).

Rapid field test kits for iodine in salt rely on the reduction or oxidation of iodine species to I_2, and the subsequent binding of I_2 to starch to form a dark blue colour in the presence of iodine in the salt being tested. There exist distinct test kits for salt iodized with KIO_3 and salt iodized with KI, given that IO_3^- must be reduced to I_2, while I^- must be oxidized, to produce the blue colour indicative of iodine's presence (151,168). Currently, KIO_3 is used for salt iodization in Cambodia, meaning that all test kits in circulation are designed specifically to identify the presence of IO_3^- in salt. Should a salt co-fortified with thiamine and KI be implemented at any scale, the test kits provided to consumers or authorized quality control agents must be those specifically for *iodide* (I^-) assessment, as the *iodate* (IO_3^-) -specific kits currently in use will not indicate the presence of iodine in salt fortified with KI. UNICEF has recently updated the packaging of their rapid test kits for iodized salt, such that test kits for salt fortified with KIO_3 are purple, and those for salt fortified with KI are in blue, enabling easy distinction between the two kit types regardless of literacy or language (165). Nonetheless, adequate education will be imperative to ensure any consumer or personnel using a rapid test kit to determine the quality of fortified salt understands which type of salt they are testing (traditionally iodized salt with KIO_3, or co-fortified salt with KI) and which test kit to use in either instance, to ensure rapid test kits are accurately reflecting the nutrient quality of the salt being measured.

5.6 Strengths and limitations

This research has many strengths. It is the first study to assess the viability of premix solutions containing both thiamine and iodine for the spray co-fortification of salt, as well as the first study to report on the interactions of KIO_3 or KI in solution with TClHCl at high concentrations. Here, a wide range of formulations were attempted, including various concentrations and types of fortificants, several alkalizing agents, and dextrose, a customary stabilizer for iodized salt. Solutions were stored at 30°C and 65% RH to mimic the average premix storage conditions in Cambodia. Where viable, solutions were also stored for twice the typical storage period (*i.e.*, up to four weeks) to monitor changes to pH, colour, and odour over time as they would occur in the

field. This thesis reports on organoleptic properties, pH, and micronutrient retention of experimental solutions to present a complete depiction of the viability of each.

Despite these strengths, this research is not without its limitations. The considerable discrepancy between the intended thiamine concentration of solutions prepared in Experiment 3 (~7.8% w/v) compared to the concentration of these solutions determined by the nutrient analyses (~10% w/v) is perplexing. Despite calibrating the scale used for solution preparation and taring it appropriately during premix formulation, using standard curves of known thiamine concentration in analysis, and cross-referencing the Certificate of Analysis for the specific lot number of the TClHCl used, the source of the discrepancy was not identified. That said, the unknown error is thought to be systematic; though not accurate, the mass of thiamine added to each solution was precise, as evidenced by the close alignment in reported values across solutions (most within ±0.4% w/v of one another), and notably, with the thiamine-only control sample (solution *s*). Importantly, this systematic error does not detract from the tremendous stability of thiamine exhibited by the analyzed solutions, given that % retention was calculated based on the reported concentrations at t=0 rather than the amount of fortificant added to solutions.

Another notable limitation to this research is the colloidal nature of the premix "solutions" which complicated pH assessment and nutrient analyses. For instance, suspensions and other heterogenous mixtures may impede the functioning of the pH meter, resulting in issues such as delayed electrode response, which could introduce measurement errors (169). Similarly, unequal distribution of solutes and/or suspended particulates within the solutions likely influenced the micronutrient analyses, such that the reported concentrations of thiamine and iodine in the aliquots may not have accurately reflected the true concentration of the solution. Such discrepancies in reported concentration could result in unwitting harmful under-fortification if used to fortify salt, leading to potential deficiencies in iodine and perpetuating those in thiamine. Further, given that there were considerable discrepancies observed in iodine concentration from one timepoint to the next, wherein concentration reportedly *increased* over time in solutions *r*, *t*, *u*, and *v*, it is likely that the suspended matter was not homogenously distributed in the continuous phase, resulting in inconsistent measurements and skewed retention data. Nonetheless, as concluded similarly in regards to thiamine stability, this limitation does not

detract from the remarkable stability of iodine observed in the analyzed solutions, seeing as iodine retention remained ≥70% in all samples at each timepoint. With this, the most promising solution is that of just KI and TClHCl, which exhibited tremendous retention of both thiamine and iodine while remaining nearly colourless, clear, and without foam or other precipitate.

6.0 Conclusion

The implementation of a national thiamine-iodine co-fortified salt program has the potential to reduce thiamine deficiency and its related disorders among the most vulnerable populations in Cambodia, while conserving the national salt iodization program currently in place. This research assessed the viability of many formulations of fortification premix solutions containing thiamine and iodine intended for the spray fortification of commercially available salt in Cambodia. Of the 24 formulations tested, three premix solutions maintained acceptable concentrations of thiamine and iodine over the four-week study period. The solution formulated with 3% w/v iodide and 7.8% w/v thiamine without stabilizing reagents was deemed most suitable for spray fortification given its remarkable stability despite no additional stabilizers, with minimal alterations to sensory and physical characteristics over time. As the current experiments evaluated the viability of premix solutions only, future experiments should explore the stability of thiamine and iodine in co-fortified *salt* over a period of storage in typical Cambodian conditions using insights gained from the experiments presented herein. All future experiments exploring the viability of salt co-fortified with thiamine and iodine should be succeeded by sensory evaluation and efficacy trials to determine the acceptability and potential efficacy of any prospective co-fortified salt, and should be accompanied by quality assurance and cost-benefit analysis research, prior to programmatic implementation in Cambodia to ensure the intended benefits of any such program are realized specifically by WRA, and most importantly, their exclusively breastfed infants.

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8.0 Appendices

Appendix A: Additional visualization of pH data

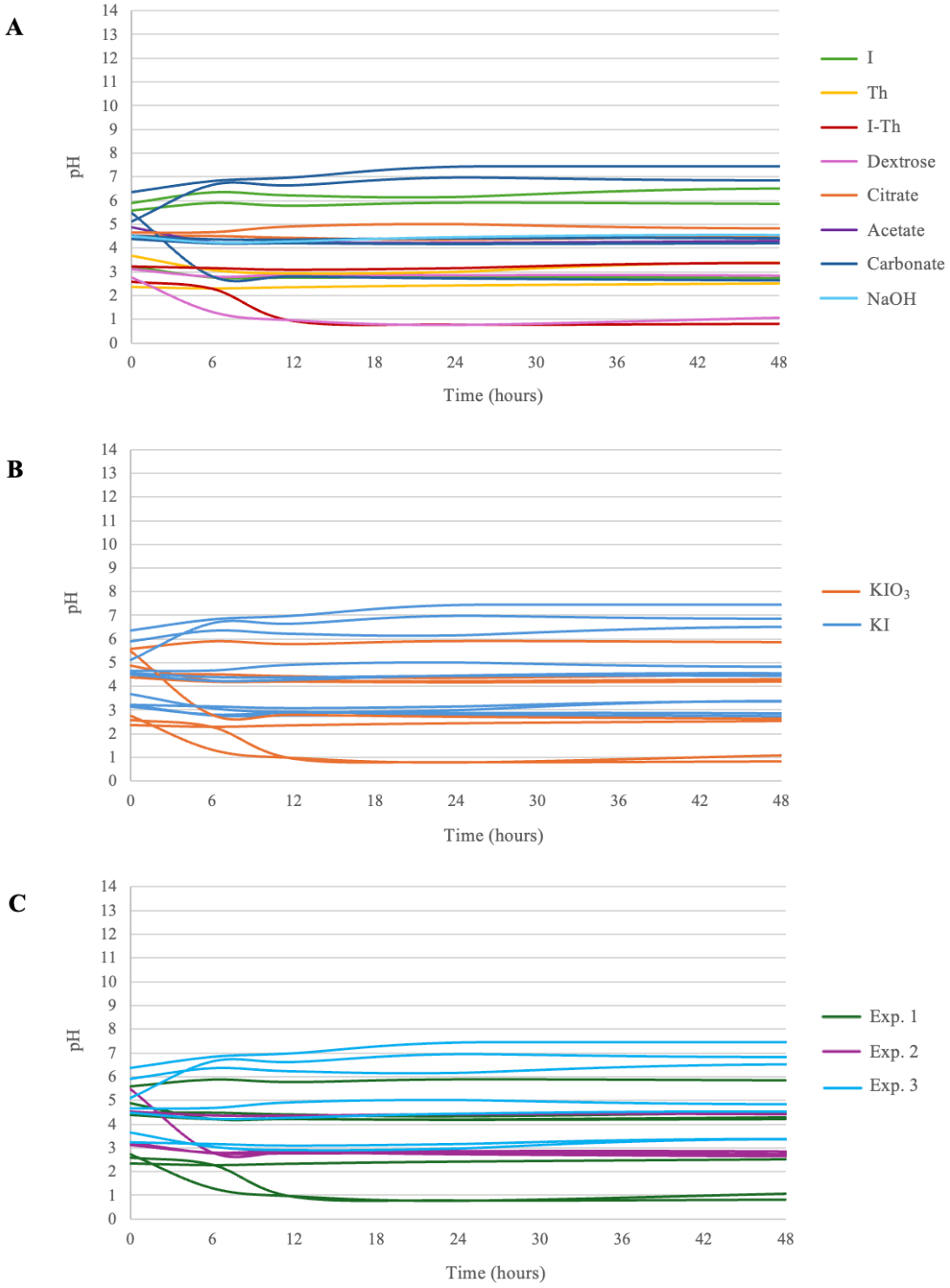

Figure 8-1. pH of solutions by stabilizing compound (A), source of iodine (B), and experiment (C). Abbreviations: I, Iodine only; Th, Thiamine only; NaOH, sodium hydroxide; KIO_3, potassium iodate; KI, potassium iodide; Exp. 1, Experiment 1; Exp. 2, Experiment 2; Exp. 3, Experiment 3.