Food Fortification Initiative
Enhancing Grains for Healthier Lives

Documenting a field-use rapid qualitative assay for fortified rice
22 September 2015

BACKGROUND

Rapid, qualitative fortified rice assays are currently used by fortified kernel producers and pilots/programs that distribute fortified rice. However, many of these Standard Operating Procedures (SOPs) are not documented for non-laboratory use, and require adaptation for use in the field, as may be the case in regulatory monitoring settings.

OBJECTIVE

Among assays currently used to identify fortified rice, select and adapt an SOP for a rapid, qualitative fortified rice assay for programmatic and regulatory monitoring use, prioritizing low-cost and ease of use in field settings.

METHODS

The Food Fortification Initiative (FFI) collected details on current rapid fortified rice assays from partners active in rice fortification. Five qualitative assays were identified for fortified rice (Table 1). Although assays exist for B vitamins and vitamin A, a focus was placed on qualitative assays for iron, given anticipated widespread use of iron in rice fortification programs.

A grading matrix was developed to score each of the rice assays on eight characteristics, considering assay costs, chemical safety, and applicability across rice fortification technologies (Table 3). Prices for the reagents and lab equipment, and chemical safety details were estimated using quotes and material safety data sheets (MSDS) from suppliers in the United States (Sciencelab.com, Fisher Scientific, JOST Chemical, Sigma Aldrich, Flinn Scientific, Mallinckrodt Chemicals, Alfa Aesar, Acros Organic).

FFI requested fortified kernels or fortified rice using three technologies (hot extruded, warm extruded, coated) from five fortified rice producers to test applicability of the assay across technologies. Six fortified rice samples were collected from fortified kernel producers (Table 2). Cold extruded kernels were not sought as no company currently produces fortified kernels via cold extrusion. Fortified kernels were blended with non-fortified rice (produced in the USA) to create fortified rice. All samples were anonymized during assay testing.

FFI contracted a grain fortification consultant (Jeff Gwirtz, JAG Services Inc.) to document the assays with assistance by local laboratory personnel at two locations, Research Products (Salina, Kansas, USA) and Wright Group (Crowley, Louisiana, USA).

RESULTS

Assay 1, Iodine (tests for starch):
Because this assay identifies gelatinized starch, rather than a nutrient, it was anticipated that the assay would identify extruded, but not coated kernels. This test produced colored kernels, but the color quickly faded upon exposure to air so results had to be read quickly. The parboiled fortified rice and

coated rice also took longer to stain. The assay will also give a false positive to non-fortified reconstituted[1] kernels, as it only tests for starch, and not iron presence.

Assay 2, Citric acid, potassium hexacyanoferrate:
This assay produced discrete, blue-colored kernels. However, this assay requires a heating step, which none of the other assays require.

Assay 3, Hydrochloric acid, potassium ferricyanide/ferrocyanide:
This assay produced discrete, blue-colored kernels.

Assay 4, Hydrochloric acid, Potassium thiocyanate:
This assay uses the same chemicals as the Method 40-40.01: Iron Qualitative Method as approved by the American Association of Cereal Chemists for wheat flour fortification. Commonly called the iron spot test, this includes hydrogen peroxide as a reagent. This reagent is unnecessary in fortified rice because the iron compound has already been reduced to the ferric form. This assay produced discrete, dark red colored kernels.

Assay 5, Nitric acid, silver nitrate:
This test was dropped as a testing option due to the advised high cost of silver nitrate as a reagent.

Assay 6, Sulfuric acid, potassium persulfuric acid, Potassium thiocyanate:
Dissolving potassium persulfuric acid and achieving uniform coating with a spray bottle to disperse the reagents was difficult. This assay produced discrete, dark red colored kernels, but compared to the other assays, it requires more reagents (three versus two) and the use of strong salt and strong acid reagents.

Scoring
Assays were scored on eight characteristics (Table 3). Assays four, three, and one were the easiest to perform. They also had the lowest scores for set-up, time, and expertise required.

Selecting an assay for documentation
Given that assays four and thee can be conducted on unheated, coated and extruded kernels, SOPs were documented for these two assays (Appendix A). The SOPs were written for field use and to avoid precise requirements for measurements where possible.

CONCLUSION
The SOPs documented as part of this exercise provide a simple, field-suitable assay that does not require the end user to know what kind of rice fortification technology has been used to fortify the rice. Neither assay requires complicated equipment or precise measurements, and the chemicals are adequately safe in settings without hazardous waste disposal systems available. The chemicals are relatively common reagents that should be easily procured at most chemical suppliers. Currently ferric pyrophosphate is the preferred iron compound for rice fortification due to its white color; however if in the future other (ferric) iron compounds are used, Assay 4 can be modified to include hydrogen peroxide (as is now used for fortified wheat flour).

[1] Reconstituted kernels are a “rice-based, rice kernel-like food product” that are the base for adding micronutrients to create fortified kernels: https://www.google.com/patents/EP2317875A1?cl=en

Although both assay four and three have the same ease and cost of use, the widespread use of the iron spot test for fortified wheat flour gives assay four 4 an advantage in countries or programs monitoring both fortified wheat flour and fortified rice.

ACKNOWLEDGEMENTS
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Tables 1-3

Table 1: Qualitative assays by chemicals and identifying source

Assay #	Tests for	Chemicals
1	Starch	Iodine
2	Iron	Citric acid Potassium hexacyanoferrate
3	Iron	Hydrochloric acid Potassium ferriccyanide/ferrocyanide
4	Iron	Hydrochloric acid Potassium thiocyanate
5	Iron	Nitric acid Silver nitrate*
6	Iron	Sulfuric acid Potassium persulfuric acid Potassium thiocyanate

*This assay was later dropped and not tested due to the advised high cost of silver nitrate as a reagent.

Table 2: Fortified kernel samples used in the exercise

Technology	Details	Iron compound and accompanying nutrients
Hot Extruded	Fortified kernels, target blend of 1%	Iron (micronized ferric pyrophosphate), niacin, thiamin
Hot Extruded	Fortified kernels, target blend of 1%	Iron (micronized ferric pyrophosphate)
Hot Extruded	Parboiled fortified rice, blended at 1%*	Iron (micronized ferric pyrophosphate), cyanocobalamin, folic acid, thiamin, vitamin A, zinc
Warm Extruded	Fortified kernels, target blend of 5%	Iron (micronized ferric pyrophosphate), folic acid, thiamin, zinc
Coated	Fortified kernels, target blend of 1%	Iron (ferric pyrophosphate), cyanocobalamin, folic acid, niacin, pyridoxine, thiamin, vitamin A, zinc
Coated	Fortified kernels, target blend of 1%	Iron (ferric pyrophosphate), cyanocobalamin, folic acid, niacin, thiamin, pyridoxine, vitamin A, zinc

*The parboiled rice sample was sent to FFI as a final product; i.e. already blended with non-fortified rice at 1%.

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Table 3: Assay Matrix

Assays were scored by JAG Services in the matrix below as:

1. Safety of chemicals used…..1 is safe, 5 is very dangerous
2. Difficulty/ease of sourcing chemicals……scale 1 is easy, 5 is very difficult
3. Expertise needed to conduct each test, expertise……1 is easy, 5 is significant training required
4. Time/labor needed to conduct each test, Elapsed time in minutes
5. Materials (reagents) needed to conduct each test, Expenses in USD
6. Estimated cost per test, USD, Manhattan, Kansas, USA
7. Iron test applicability for iron tests including micronized/non-micronized ferric pyrophosphate
8. Starch test applicability to Fortified Kernel Types, yes, no, maybe

	Safety, Training and Availability			Labor, Capital and Materials			Iron Forms		Fortified Kernel Types		
Test Method	Safety of Chemical	Chemical Sourcing	Expertise Required	Time for Test	Set Up USD*	USD/Test	Micronized FePP	Non-micronized FePP	Hot Extruded	Warm Extruded	Coated
Assay 1: Iodine Starch Assay	1	1	1	5	10.20	>0.15	Further testing required				No
Assay 2: Citric acid, potassium Hexacyanoferrate	2	1	4	30	462.01	1.06	Yes	Yes	Yes	Yes	Yes
Assay 3: Hydrochloric acid, potassium ferric/ferrocyanide	2	1	2	10	433.24	0.02	Yes	Yes	Yes	Yes	Yes
Assay 4: Hydrochloric acid, potassium thiocyanate	2	1	2	10	433.24	0.02	Yes	Yes	Yes	Yes	Yes
Assay 6: Sulfuric acid, potassium persulfuric acid, potassium thiocyanate	5	1	5	10	510.87	1.03	Yes	Yes	Yes	Yes	Yes

*Set Up USD shown is an approximation of the USD value of materials (reagents) needed for the test.

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Appendix A

Standard Operating Procedure:
Qualitative Spot Test to Detect Fortificant Iron in Fortified Rice

Reagents: Hydrochloric acid, Potassium thiocyanate

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Purpose:

The purpose of this test is to quickly and inexpensively assess the presence of fortified kernels (containing iron) in fortified rice. The dark brown-red pigment is generated by the reaction of the reagents with ferrous (iron) ions in the fortified kernels. Thus, this test will not work on fortified rice using fortified kernels that do not contain iron or iron in the non-ferric form. This is a qualitative test only (i.e. detects if iron is present/not present); quantitative details, including iron level and mixture homogeneity, are beyond the scope of this procedure.

Time required:

Less than five minutes

Equipment:

- Scale to weigh the potassium thiocyanate (KSCN)[2] (if Reagent 2 is not already prepared)
- Non-metal, non-porous container to hold fortified rice sample (for example, plastic cup or tray)
- Reagent 1: Hydrochloric acid in dilute 2N form (2N HCl)[3], in a non-metal, labeled bottle
- Reagent 2: Potassium thiocyanate (KSCN) 10% solution, in a non-metal, labeled bottle

Both HCl and KSCN 10% are clear, so labeling the solutions is necessary! Solutions will last for approximately 1 month. Store bottles in a cool and dark room or in a refrigerator used for non-food storage purposes.

To prepare Reagent 2:

1. Mix 10 grams of KSCN with 100 mL of water (distilled or bottled), by lightly swirling the water with the KSCN or stirring to mix.

Procedure for testing fortified rice for the presence of iron:

The test is to be conducted in a well-ventilated area. Wearing of goggles and disposable gloves is recommended.

1. Place at least 50 grams of fortified rice in a plastic cup, tray, or similar container.
2. Pour Reagent 1 (2N HCl) on the rice until all of the rice is wet.
3. Pour a similar amount of Reagent 2 (10% KSCN) on the wet rice sample.
4. Immediately, fortified kernels will turn red to dark red (black upon drying) indicating the presence of iron fortified kernels (Figure 1).

[2] KSCN is a dry, crystalline reagent.

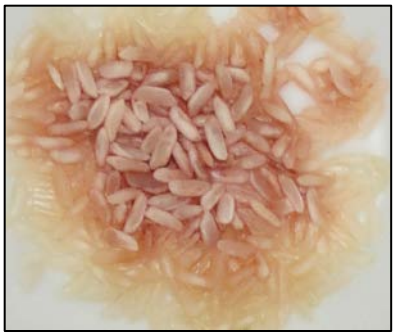
[3] HCl can be purchased in the concentrated 37% solution but it is a dangerous reagent. If conducting this assay in a non-laboratory setting, purchase the diluted 2N solution. N indicates the unit of normality, or concentration per liter of solution.

Figure 1: Fortified kernels in fortified rice will turn dark red/black, indicating the presence of iron.

Log the results and dispose of sample:
Record the results (are there fortified kernels, Yes or No?). Throw rice sample in the trash. If disposing of the reagents, HCl 2N needs to be neutralized with baking soda (sodium bicarbonate) by adding HCl to baking soda (do not add baking soda to HCl) until the bubbles stop rising. The HCl and baking soda mixture can be then poured down the sink with water running. Wash hands.

Notes:
Although the reagents are in diluted form, if any of the reagents come in contact with skin, wash the skin immediately.

Fortified rice using coated or extruded technology is usually fortified in a 0.5%-2% blending ratio, which will result in a discrete, iron-fortified kernel result as shown above. However, in the United States, dusting is the most commonly used fortification technology, and this is applied to all of the rice. Dusting technology is not suitable for populations where the cooking practices include rinsing rice prior to cooking or disposing of excessive cooking water. For identification purposes, below is the iron spot test used on dusted rice and non-fortified rice:



Rice fortified with iron using dusting technology

Non-fortified rice

Reference:
AACC International Approved Methods, Inorganic Constituents. AACCI Method 40-40.01, Iron -- Qualitative Method (This method qualitatively determines iron added to flour and has been adapted for use with fortified rice.)

Disclaimer:
Reference herein to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement to the exclusion of other commercial products, process, or service that may be suitably applicable.

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Standard Operating Procedure:
Qualitative Spot Test to Detect Fortificant Iron in Fortified Rice
Reagents: Hydrochloric acid, Potassium ferricyanide/ferrocyanide
22 September, 2015

Purpose:
The purpose of this test is to quickly and inexpensively assess the presence of fortified kernels (containing iron) in fortified rice. Prussian blue, the deep blue pigment in blue printing, is generated by the reaction of the reagents with ferrous (iron) ions in this test. Thus, this test will not work on fortified rice using fortified kernels that do not contain iron. This is a qualitative test only (i.e. detects if iron is present/not present); quantitative details, including iron level and mixture homogeneity, are beyond the scope of this procedure.

Time required:
Less than five minutes

Equipment:
- Scale to weigh the potassium ferricyanide/ferrocyanide reagent[4] (if Reagent 2 is not already prepared)
- Non-metal, non-porous container to hold fortified rice sample (for example, plastic cup or tray)
- Reagent 1: Hydrochloric acid (HCl), in dilute 2N form[5], in a non-metal, labeled bottle
- Reagent 2: Potassium Ferricyanide ($C_6N_6FeK_3$) or Potassium Ferrocyanide ($K_4[Fe(CN)]_6 \cdot 3H_2O$), 10% solution, in a non-metal, labeled bottle

Both Reagents 1 and 2 are clear, so labeling the solutions is necessary! Prepare the Potassium ferricyanide/ferrocyanide solution fresh for each test. The HCl solution will last for approximately 1 month. Store bottles in a cool and dark room or in a refrigerator used for non-food storage purposes.

To prepare the 10% potassium ferricyanide/ferrocyanide solution:
1. Mix 10 grams of potassium ferricyanice/ferricyanide with 100 mL of water (distilled or bottled).

Procedure for testing fortified rice for the presence of iron:
The test is to be conducted in a well-ventilated area. Wearing of goggles and disposable gloves is recommended.

1. Place at least 50 grams of fortified rice sample in a plastic cup, tray, or similar container.
2. Pour Reagent 1 (2N HCL) on the rice sample until all of the rice is wet.
3. Pour a similar amount of Reagent 2 (10% potassium ferricyanide/ferrocyanide solution) on the wet rice sample.
4. After 1-2 minutes, fortified kernels will turn dark blue, indicating the presence of iron-fortified kernels (Figure 1).

[4] Potassium ferricyanide/ferrocyanide is a dry, crystalline reagent.
[5] HCl can be purchased in the concentrated 37% solution but it is a dangerous reagent. If conducting this assay in a non-laboratory setting, it is recommended that the diluted 2N solution is purchased.

Figure 1: Fortified kernels in fortified rice will turn dark blue, indicating the presence of iron.

Log the results and dispose of sample:
Record the results (are there fortified kernels, Yes or No?). Throw the sample in the trash. If disposing of the reagents, HCl 2N needs to be neutralized with baking soda (sodium bicarbonate) by adding HCl to baking soda (do not add baking soda to HCl) until the bubbles stop rising. The HCl and baking soda mixture can be then poured down the sink with water running. Wash hands.

Notes:
Although the reagents are in diluted form, if any of the reagents come in contact with skin, wash the skin immediately.

Reference:
United States Department of Agriculture, Grain Inspection, Packers and Stockyards Administration. Field Management Division: Policies, Procedures, and Market Analysis Branch. Reference #247: Milled Rice Iron Enrichment Testing

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