

Qualitative Spot Test for Zinc in Fortified Wheat Flour and Rice using Xylenol Orange

Standard Operating Procedure

Purpose

The purpose of this test is to quickly and inexpensively assess the presence of added zinc in fortified wheat flour and rice.

Scope

Use this method to test for the presence of added zinc in fortified foods, specifically refined wheat flour and white rice¹ using extruded or coated kernels.²

Synopsis

Zinc in fortified refined wheat flour and fortified white rice (using extruded or coated kernels) reacts with an aqueous solution of xylenol orange at pH 6.5 (phosphate buffer) to form dark pink/purple-colored spots.

Time Required³

10–15 minutes

WARNING

Refer to pages 5–6 for reagent safety information prior to preparing reagents and conducting the assay.

Equipment

Prepare the reagent in a laboratory setting so you can take appropriate safety precautions if needed. The completed xylenol orange reagent may be used in both laboratory and field environments.

Table 1. Equipment for Reagent Preparation

Laboratory Equipment	Qty	Non-Laboratory Alternative	Qty
Analytical balance capable of weighing to at least 0.1 milligram (mg)—Ohaus Explorer Precision or equivalent	1	not applicable (N/A)	

¹ This assay was also tested in brown rice fortified with extruded and coated fortified kernels; Annex 2 shows the results.

² The assay was tested on fortified maize meal, but this use is not recommended; Annex 3 shows the results.

³ This is the amount of time needed from sample preparation to time of result interpretation and recording; it does not include time required for reagent solution preparation.

Laboratory Equipment	Qty	Non-Laboratory Alternative	Qty
A balance capable of weighing ~25 grams (g)	1	N/A	
Weigh boats—250 milliliter (mL), VWR 10803-170 or equivalent	1	~20 centimeter ² sheet parchment paper	
General purpose disposable polyethylene ⁴ transfer pipettes—VWR 16001-174 or equivalent	1	N/A	
Amber volumetric flask—100 mL, 1 liter (L)	1	N/A	
Beakers—50 mL, 250 mL.	1	N/A	
Graduated cylinder—500 mL, 1.0 L	1	N/A	
Polyethylene reagent bottles ⁴ —100 mL, 500 mL, 1.0 L	2	Any plastic or polyethylene capable of containing 100 mL, 500 mL, 1.0 L	2
Vortex mixer—VWR 58816-121 or equivalent	1	Shake solution (if bottle lid is tight) or mix manually (e.g., with a plastic spoon) ⁵	
pH meter—Thermo Scientific™ Elite pH pocket tester (CAS 13-643-120) or equivalent	1	N/A	
Disposable gloves (as required)	-	N/A	
Spray bottle ⁶ —VWR 10216-894 or equivalent	1	N/A	

Reagents

- 2.23×10^{-5} molarity (M) xylene orange solution in pH 6.5 0.1 M phosphate buffer⁷

Procedure

The use of disposable gloves is recommended. The test does not require a fume hood. Use clean materials to avoid contaminating the sample.

Fortified Wheat Flour Samples

1. Weigh approximately 25 g (~55 mL) sample into a weigh boat or, by volume, using a graduated 100-250 mL beaker and transfer it to a flat surface, a petri dish, or a white tile.
2. Spread the top of the flour pile to create a well in the sample, using the bottom of a small, clean 50 mL beaker or spatula.
3. Using a polyethylene transfer pipette, carefully add 10–15 drops of the xylene orange solution to wet the flattened surface area.

⁴ Should not be glass

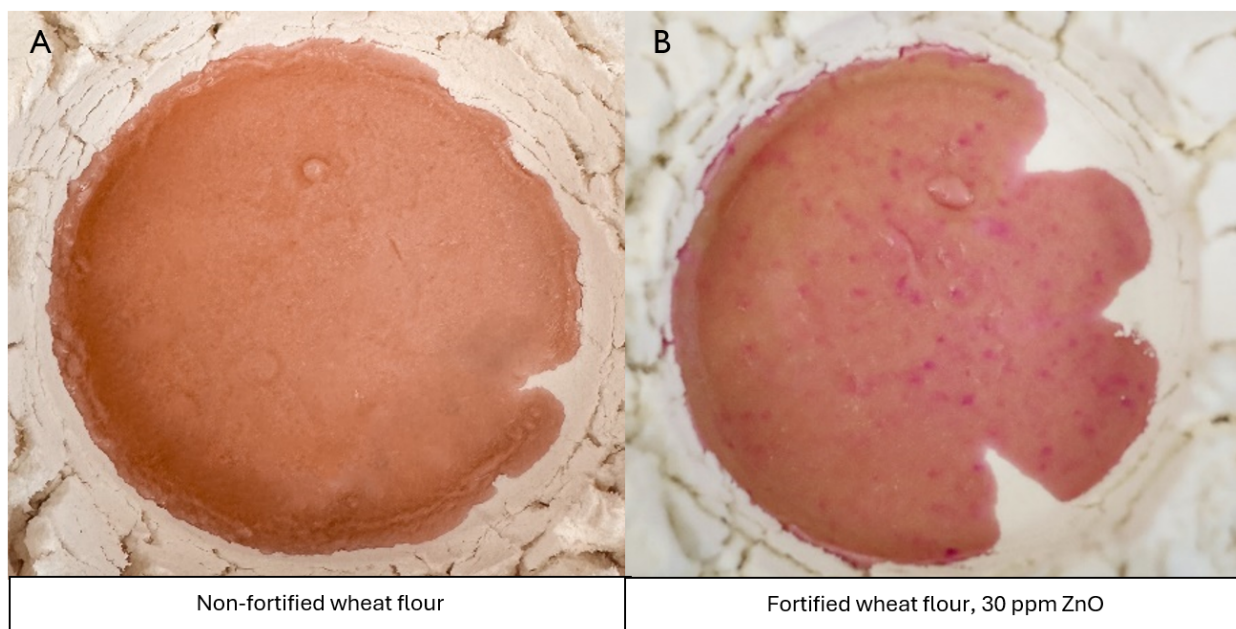
⁵ Plastic is ideal for minimizing metal contaminants.

⁶ Required only for rice applications. See Annex 4.

⁷ See Annex 1 for reagent preparation instructions.

4. Set aside for 10 minutes; if pink/purple-colored spots appear, added zinc is present.

Figure 1. Zinc Spot Test in Wheat Flour Samples



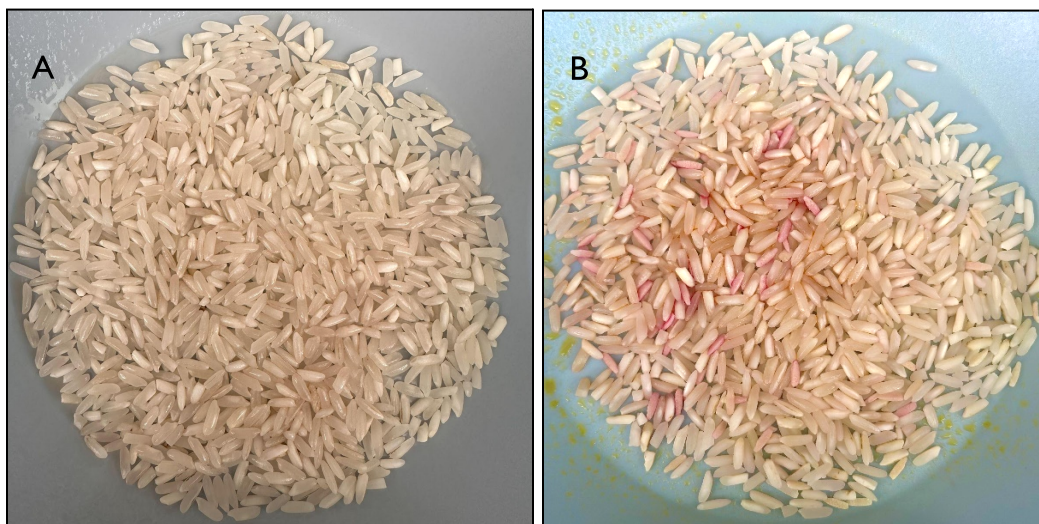
Dark pink/purple spots indicate the presence of added zinc in wheat flour (B, on the right) compared to a non-fortified control sample (A, on the left).

Fortified Rice Samples

1. Weigh approximately ~50 g sample into a weigh boat or, by volume, using a 100-250 mL graduated beaker and place on a flat surface, a petri dish, or a white tile.
2. Spread the rice to make a flat thin layer of the sample.
3. Using a spray bottle,⁸ spray the xylenol orange/buffer solution over the sample surface to moderately wet the surface.
4. Look for a reaction within 10 minutes (results may be seen 1–2 minutes after application). The appearance of pink/purple-colored spots indicates the presence of added zinc. Reading after 10 minutes may complicate test interpretation.

⁸ Spray application of reagent is preferred to dripping/pouring in assays for fortified rice. See Annex 5.

Figure 2. Zinc Spot Test in White Rice Samples



Dark pink/purple spots indicate the presence of added zinc in fortified white rice kernels (B, on the right), compared against a negative control (A, on the left).

Safety of Reagents

Xylenol Orange

According to the Materials Safety Data Sheet (MSDS), this reagent is not a hazardous compound. However, as with any laboratory agents, take care when handling.

First aid measures:

- If inhaled, move to an area with fresh air.
- In the event of skin contact, take all contaminated clothing off immediately. Rinse skin with water/in a shower.
- In the event of eye contact, rinse out with plenty of water. Remove contact lenses.
- If swallowed, drink up to 16 ounces of water.
- Consult a doctor if you are experiencing symptoms of gastrointestinal distress (e.g., nausea, abdominal pain, diarrhea).

0.1 M Phosphate Buffer, pH 6.5

The buffer solution may cause mild skin and eye irritation when in contact. Use gloves when handling and avoid contact with skin or eyes. Upon contact with skin, wash with water thoroughly. If in the eye, rinse with water for several minutes.

Disposal of Samples After Testing

- Containment and labeling: place tested samples in a sealable, clearly labeled container, such as a sealable plastic bag. The label should include the contents and any relevant hazard information.

- Check your local, state, or national regulations regarding the disposal of chemically contaminated materials.
- If in a laboratory or institutional setting, consult with your environmental health and safety officer or waste management team for specific instructions.

Acknowledgments

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Annex I. Reagents and Preparation of Solutions

Reagents

- Xylenol orange tetrasodium, water-soluble—Spectrum CAS 3618-43-7 (760.59 g/mole [mol]), analytical grade
- Potassium phosphate monobasic or potassium dihydrogen phosphate—VWR 7778-77-0 (136.09 g/mol), analytical grade
- Potassium phosphate dibasic—VWR 7758-11-4 (174.18 g/mol), analytical grade
- Deionized water

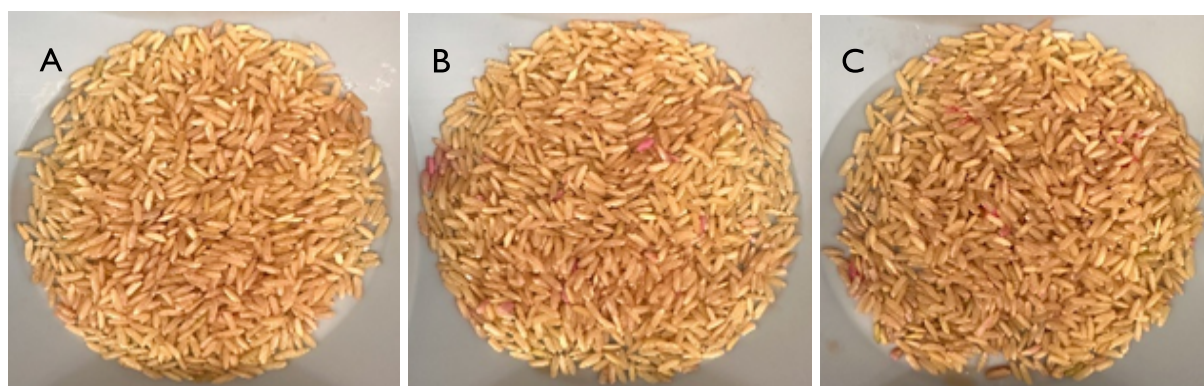
Preparation of Solutions

- 1 M potassium phosphate monobasic:
 - Add 87.09 g potassium phosphate monobasic to ~300 mL deionized water in a 500 mL graduated cylinder or beaker and stir to dissolve. Bring to 500 mL with deionized water.
 - Use the solution within six months of preparation and store it at ambient temperature.
- 1 M potassium phosphate dibasic:
 - Add 68.05 g potassium phosphate dibasic to ~300 mL deionized water in a 500 mL graduated cylinder or beaker and stir to dissolve. Bring to 500 mL with deionized water.
 - Use the solution within six months of preparation and store it at ambient temperature.
- pH 6.5, 0.1 M phosphate buffer:
 - Combine 70 mL 1 M potassium phosphate monobasic and 30 mL 1 M potassium phosphate dibasic in a 1 L volumetric flask. Dilute to 1 L with deionized water.
 - Check that the solution reads pH 6.5 (± 0.05) with a handheld pH meter or pH test strips.
 - Use the solution within three months of preparation and store it at ambient temperature.
 - 2.23×10^{-5} M xylenol orange solution in pH 6.5 phosphate buffer: Weigh 17 mg of xylenol orange tetrasodium salt into a 100 mL beaker and add 50 mL of the pH 6.5 buffer. Dissolve completely and transfer to a 100 mL volumetric flask. Bring to 100 mL with deionized water.
 - Use the solution within three months of preparation and store it at ambient temperature.

Annex 2. Zinc Spot Assay Applied to Fortified Brown Rice Samples

The zinc spot assay using xylenol orange was opportunistically applied to brown rice samples fortified with both extruded and coated fortified kernels. Results below show that the reagent successfully reacts with present zinc to form purple/pink-colored spotting; however, visibility is not as clear as with white rice.

Figure 3. Zinc Spot Test in Brown Rice Samples

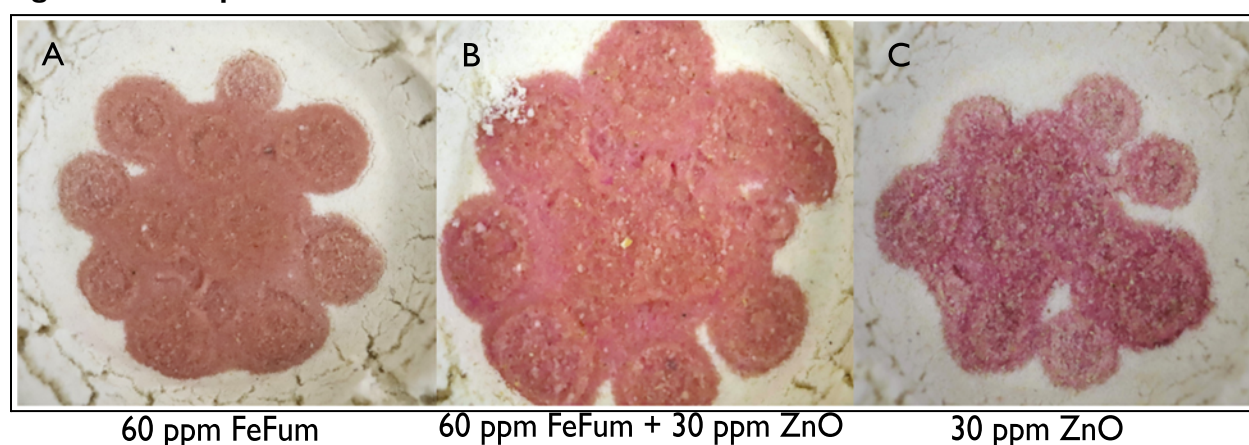


Purple/pink spots indicate the presence of added zinc in fortified brown rice samples (B, C, on the right), compared against a negative control (A, on the left).

Annex 3. Zinc Spot Test Applied to Fortified White Maize Meal

As seen below, there is a visual color change in maize meal fortified with zinc, however, identification of individual spots is difficult due to the maize meal texture. As compared against a sample fortified with only iron (A on left), samples fortified with zinc show intensified pink/purple coloring (B in middle; C on right). Due to the difficult visual distinction between the control and fortified samples (Figure 4), this SOP is not recommended for application to fortified maize flour.⁹

Figure 4. Zinc Spot Test in Maize Meal



Maize meal samples fortified with 60 ppm ferrous fumarate (FeFum), 60 ppm FeFum and 30 ppm zinc oxide (ZnO), and 30 ppm ZnO were treated with 2.23×10^{-5} M xylenol orange in pH 6.5 phosphate buffer following the application method for wheat flour samples.

⁹ Alternatively, a zinc spot assay by dithizone may be considered. (See: USAID Advancing Food Fortification Opportunities to Reinforce Diets. 2024. *Standard Operating Procedure: Qualitative Spot Test for Zinc in Fortified Maize Flour and Rice using Dithizone*. Arlington, VA: USAID AFFORD.)

Annex 4. Zinc Spot Test Applied by Dripping onto Fortified Rice

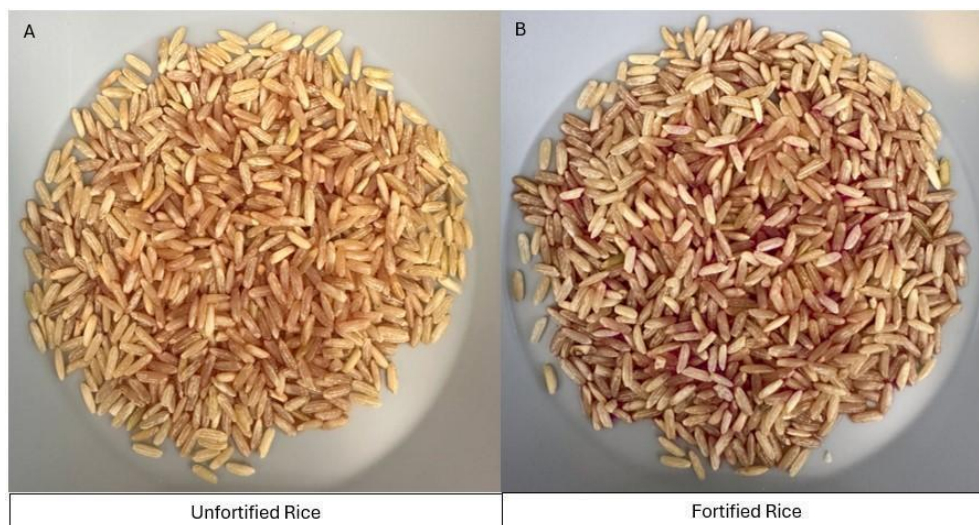
Spraying is recommended because dripping or pouring the xylenol orange reagent onto fortified rice samples is less effective at identifying fortified kernels compared to a spray application of the reagent, although dark purple/pink spotting of fortified kernels may still be observed.

Figure 5. Zinc Spot Test Drip Application in White Rice



Dark pink/purple spots indicate the presence of added zinc in fortified kernels in white rice (B, on the right), compared against a negative control (A, on the left), after dripped application.

Figure 6. Zinc Spot Test Drip Application in Brown Rice



Dark pink/purple spots indicate the presence of added zinc in fortified kernels in brown rice (B, on the right), compared against a negative control (A, on the left), after dripped application.



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