

Chromogenic Spot and Test Tube Techniques for Qualitative Zinc Detection in Fortified Wheat Flour: Method Development and ICP-MS Validation

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Abstract

Zinc fortification of wheat flour is widely implemented in Ethiopia, yet simple, field-deployable tools to verify the presence of zinc remain scarce. We developed and validated rapid qualitative chromogenic assay, as a spot test format and a complementary test-tube method format for detecting zinc in fortified wheat flour, using inductively coupled plasma mass spectrometry (ICP-MS) as a reference method. Experimental methods were evaluated with 31 wheat flour samples from mills that had initiated fortification and 20 unfortified wheat flour samples. The finding revealed that as the ICP-MS as reference method, the spot assay achieved 94% overall accuracy, with 96.8% sensitivity (30/31 fortified samples correctly identified) and 90.0% selectivity (18/20 unfortified samples correctly classified); two unfortified samples yielded false positive. Cohen's kappa coefficient was 0.88, indicating "almost perfect" agreement beyond chance. Both formats delivered results in approximately 5 minutes, required minimal equipment, and showed no substantial interference from common matrix components. We propose the spot assay as a primary screening tool for routine monitoring in flour mills, regulatory inspections, and resource-limited settings, with the test-tube format available as a confirmatory or alternative procedure.

1. Introduction

Zinc is an essential trace element for human growth, immune function, and development, and inadequate intake is widespread. At least 25% of the global population is at risk of zinc deficiency due to low dietary intake, with particularly high prevalence among pregnant women; deficiency is associated with impaired growth, immune dysfunction, and adverse pregnancy outcomes, while excessive intake can cause gastrointestinal and systemic symptoms (Prasad 1996; Wang et al. 2012). Maintaining adequate zinc status therefore requires sustained public-health strategies, including food-based approaches such as fortification of staple foods.

Wheat is a major staple worldwide, contributing substantially to daily energy and protein intake. Fortifying wheat flour with zinc offers a practical pathway to increase population zinc intake and reduce deficiency. In Ethiopia, mandatory wheat flour fortification has been adopted under the National Food and Nutrition Policy and the 2022 mandatory fortification standard to address micronutrient deficiencies (Federal Democratic Republic of Ethiopia, 2018). Effective implementation depends on routine quality control to verify that zinc is present at intended levels at mills, distribution points, and regulatory checkpoints.

Reference methods for zinc quantification such as flame atomic absorption spectrophotometry, ion chromatography, and inductively coupled plasma mass spectrometry are accurate and suitable for regulatory or laboratory testing (Jorhem et al. 2000; Vrček & Vinković Vrček 2012; USP 2023). However, they require costly instrumentation, specialized personnel, and controlled laboratory conditions, limiting their utility for rapid, on-site verification in resource constrained settings. By contrast, simple qualitative spot assays have been successfully deployed for iron in fortification programs and are used routinely for field screening (ECSA-HC 2007). A comparable, validated qualitative tool for zinc is needed to support

Ethiopia's zinc-fortified wheat flour program, especially because iron is not included in the current formulation and iron spot tests cannot be used as a proxy.

Dithizone (diphenylthiocarbazone, DTZ) is a sulfur-containing organic reagent that forms colored complexes with multiple metal ions, including zinc. Since its early use in histochemical detection of zinc, DTZ has been applied to localize and detect zinc in diverse biological matrices and cereals, including wheat (Ozturk et al. 2006). Its rapid color response and minimal equipment requirements make it a promising basis for a field-deployable screening assay, provided that selectivity and matrix effects are characterized in fortified flour.

The Ethiopian government has endorsed the National Food and Nutrition Policy, and its implementation strategy to reduce any form of malnutrition (Federal Democratic Republic of Ethiopia 2018). As part of the strategy Ethiopia have turned to mandatory food fortification programs to address micronutrient deficiencies in their populations. In 2022, the Government has endorsed the mandatory food fortification standard for wheat flour fortification. Unlike many countries, the wheat flour fortification program in Ethiopia lacks iron and this makes unable to use iron spot test to support wheat fortification if the appropriate amount of nutrients (zinc, folic acid, and other B Vitamins) is consistently present in the product at the factory level before the product release to the market. Establishing quality control for zinc spot qualitative detection methods at factory, port and regulatory bodies is very crucial to the smooth roll out of the wheat flour fortification program in the country.

To address this gap, there is a need for simple, rapid, and low-cost qualitative methods that can be deployed in flour mills, regulatory inspections, and resource-limited laboratories to screen for the presence of zinc in fortified wheat flour. While quantitative methods remain essential for compliance testing and detailed surveillance, a robust qualitative screening tool can triage samples, flag non-compliant products, and guide targeted confirmatory analysis.

In this study, we developed a qualitative chromogenic spot assay, with a complementary test-tube format, for the detection of zinc in fortified wheat flour. The methods were validated against ICP-MS as a reference technique using 31 wheat flour samples collected from milling factories that had initiated fortification and 20 unfortified wheat flour samples. Performance characteristics-including accuracy, sensitivity, selectivity, and agreement (Cohen's kappa)-were evaluated in line with principles for validation of non-standard, laboratory-developed methods under ISO/IEC 17025:2017 and relevant screening-method guidance.

2. Materials and Methods

2.1. Reagents and materials

All reagents were of analytical grade and used without further purification. Absolute ethanol, sodium acetate (GR, $\geq 99.5\%$), and glacial acetic acid were employed for the preparation of the chromogenic reagent (Dithizone (98%)) and buffer solutions, respectively. Concentrated nitric acid (HNO_3) and

hydrogen peroxide (H_2O_2) were used for sample digestion prior to ICP-MS analysis. Ultra-pure water was used for all dilutions and solution preparations.

2.2. Sample collection and description

A total of 51 wheat flour samples were analyzed. Thirty-one (31) samples were collected from mills that had initiated zinc fortification of wheat flour in and around Addis Ababa, under monitoring by the Ethiopian Food and Drug Authority (EFDA). These samples were intended to represent commercially produced fortified flour under routine operating conditions. Twenty (20) wheat flour samples known to be unfortified (no added zinc containing premix) were included as negative controls to assess the selectivity of the qualitative test.

All samples were collected in clean, dry, food-grade containers and labeled with a unique code, factory identifier, and date of collection. Samples were stored in a cool, and dry place prior to analysis and analyzed within weeks of collection.

2.3. Reagent preparation

1. **Chromogenic reagent solution:** Prepare a stock solution of dithizone (0.05% w/v) by dissolving 0.05 g of analytical-grade dithizone in absolute ethanol and diluting to 100.0 mL. Store the solution in an amber glass bottle at 4°C, protected from light. The reagent was freshly prepared every 2 weeks and used within its stability period.
2. **Aqueous extraction:** Dissolve 0.82 g of anhydrous sodium acetate in 80 mL of deionized water. Adjust the pH to 5.5–6.0 dropwise using 1 M acetic acid and a pH meter. Finally, dilute the solution to the mark in a 100 mL volumetric flask with deionized water.
3. **Reference Standard Enriched in the Laboratory:** The laboratory-fortified reference standard sample is prepared by homogenously blending a baseline matrix of unfortified wheat flour with an analytical-grade zinc salt, such as zinc sulfate heptahydrate, to achieve a predetermined target concentration corresponding to a mandatory regulatory threshold (e.g., 80 mg/kg zinc).

2.4. Optimization of the qualitative assay

A stock chromogenic reagent solution was prepared at a concentration of 0.5 mg mL^{-1} in absolute ethanol. Based on preliminary observations with this solution, two additional working concentrations (0.34 mg mL^{-1} and 0.17 mg mL^{-1}) were prepared by appropriate dilution with ethanol. The reagent exhibited a characteristic deep blue color in solution.

To optimize the spot assay, aliquots of wheat flour (250, 500, 750, and 1000 mg) were weighed and transferred onto a clean, non-reflective white sampling trays. Volumes of 250–1000 μL of each chromogenic reagent concentration (0.5 , 0.34 , and 0.17 mg mL^{-1}) were added to the flour samples. The development of color was monitored visually, and the time required for a distinct color change was recorded for each combination of sample mass, reagent volume, and reagent concentration.

For the test-tube format, 250, 500, and 1000 mg of both fortified and unfortified wheat flour were weighed into separate tubes and suspended in 5 mL of sodium acetate buffer. Volumes of 500, 750, and 1000 μL of each chromogenic reagent concentration (0.5, 0.34, and 0.17 mg mL^{-1}) were added. As for the spot assay, the color change was observed by the naked eye and the time to color development was recorded for each mixture.

Based on these experiments, the combination of sample mass, reagent concentration, and reagent volume that provided the most distinct and rapid color response with minimal reagent consumption was selected as the optimized condition for subsequent validation. The final optimized procedures for the spot and test-tube assays are described in the following subsections.

2.5. Qualitative spot assay (primary method)

Procedure:

1. A precise mass of 1.0 gram of the unknown wheat flour sample and a separate 1.0-gram mass of the 80 mg kg^{-1} laboratory-fortified reference standard sample are measured and placed side-by-side on non-reflective white sampling trays.
2. The surfaces of both 1.0-gram piles are firmly compressed and flattened using a clean plastic blade to establish uniform, smooth testing planes.
3. A volumetric dosage of approximately 20 drops (equivalent to 1.0 mL) of the ethanol-based dithizone reagent solution is applied directly onto both flattened sample surfaces.
4. Allow the reaction to proceed for 5 minutes. Observe the color change directly on the flour surface under white light.

2.6. Complementary test-tube format

Procedure:

1. Weigh around 0.1 grams of the unknown flour sample and place it in a clear, cylindrical glass test tube marked "Unknown". Take 0.1 gram of the standard that has been fortified with zinc sulphate in the laboratory and place it in another glass test tube, marked "Standard".
2. Place exactly 5.0 ml of the aqueous buffer solution in both test tubes. Seal the test tubes tightly and shake them for 1 minute to release the zinc ions in their liquid form.
3. Use the pipette and add 1.0 ml (around 20 drops) of the blue chromogenic reagent solution to both test tubes.
4. Gentle swirling and waiting for 5 minutes to observe color development.

2.7. Reference method: ICP-MS

Zinc concentrations in wheat flour samples were determined using inductively coupled plasma mass spectrometry (ICP-MS) as the reference method. Approximately 0.5 g of homogenized wheat flour was accurately weighed into a digestion vessel. To each sample, 10 mL of concentrated nitric acid (HNO_3)

and 2 mL of hydrogen peroxide (H₂O₂) were added. The mixtures were heated on a hot plate (AS ONE Corporation, Model HPRB-6040) until clear, colorless solutions were obtained, indicating complete digestion. After cooling, the digests were filtered through qualitative filter paper (Whatman No.1) and quantitatively transferred to 50 mL volumetric flasks, then diluted to volume with ultrapure water.

Zinc was quantified using an Agilent 7900 ICP-MS instrument. Calibration was performed using multi-element standards covering a range of 5–40 ppm, with a correlation coefficient (R²) of 0.999. A suitable certified reference material (CRM), NIST Standard Reference Material® Wheat flour (SRM 1576b), was analyzed alongside the samples to verify method accuracy and instrument performance. All reagents were of analytical grade or higher, and ultrapure water was used throughout to minimize contamination.

2.8. Method validation design

The qualitative chromogenic spot assay (primary) and complementary test-tube format were evaluated against ICP-MS as the reference method for zinc. Each flour sample was analyzed by both the qualitative test and ICP-MS. Based on the ICP-MS results and known fortification status, samples were classified as fortified (positive) or unfortified (negative) for the purpose of calculating diagnostic performance metrics.

Performance characteristics assessed included:

- **Accuracy (percent agreement)** between the qualitative test and ICP-MS.
- **Sensitivity** (proportion of fortified samples correctly identified as positive).
- **Selectivity (specificity)** (proportion of unfortified samples correctly identified as negative).
- **Cohen's kappa coefficient** to assess agreement beyond chance.

Validation was conducted in accordance with principles for laboratory-developed, non-standard methods under ISO/IEC 17025:2017 and relevant screening-method guidance (e.g., FDA ORA-LAB.5.4.5).

3. Results

Optimization of reagent concentration and sample amount

Optimization experiments evaluated sample masses of 250–1000 mg for both the spot and test-tube assays, chromogenic reagent concentrations of 0.50, 0.34, and 0.17 mg mL⁻¹, and reagent volumes of 250–1000 µL. The clearest color discrimination between fortified and unfortified wheat flour was achieved with 1.0 g of sample with 1.0 mL of 0.34 mg mL⁻¹ chromogenic reagent for the spot assay, and with 0.1 g of sample with 0.34 mg mL⁻¹ chromogenic reagent for the test-tube assay. These conditions produced a distinct color change within a practical time frame and were adopted for subsequent validation of the qualitative method. Representative images of the optimized spot and test-tube assays under these conditions are shown in Figs. 1 and 2, respectively.

Diagnostic performance of the qualitative test

Zinc concentrations in the 51 wheat flour samples were determined by ICP-MS and ranged from 6.90 to 91.34 mg kg⁻¹. Among the 31 samples collected from mills practicing fortification, the mean zinc concentration was 85.04 ± 29.1 mg kg⁻¹, whereas the 20 unfortified flour samples had a mean concentration of 8.70 ± 1.01, mg kg⁻¹. A decision threshold of 80 ± 10 mg kg⁻¹ was applied to classify samples as fortified (positive) or unfortified (negative) for evaluation of the qualitative assay.

The qualitative test method was evaluated using 31 fortified wheat flour samples and 20 unfortified wheat flour samples. Using ICP-MS as the reference method, the qualitative method achieved an overall accuracy of 94.1% (48/51), corresponding to a misclassification rate of 5.9%.

The method demonstrated high diagnostic sensitivity (96.8%), correctly identifying zinc in 30 of 31 fortified samples. Selectivity (specificity) was 90.0%, with 18 of 20 unfortified samples correctly classified as negative and 2 samples yielding false positive results. The distribution of true positives, false negatives, true negatives, and false positives is summarized in Table 1.

Table 1
Validation contingency table

Samples	Replicates	TP	TN	FP	FN
Negative (non-fortified)	20	0	18	2	0
Positive (fortified)	31	30	0	0	1
Overall	51	30	18	2	1

The Cohen’s kappa coefficient was 0.88, indicating “almost perfect” agreement between the qualitative test and ICP-MS according to the Landis and Koch interpretation scale. ICP-MS measurements showed that zinc concentrations were generally higher in fortified flour samples, which had a median concentration of 78.95 mg kg⁻¹, compared with 8.41 mg kg⁻¹ in unfortified samples. Based on these performance characteristics and the intended use as a screening assay, the method is considered suitable as a primary qualitative screening tool in accordance with ISO/IEC 17025:2017 and FDA ORA-LAB.5.4.5 guidelines for method validation.

Visual representation of the spot and test-tube assays

Representative results for the spot assay are shown in Fig. 1, displaying the color response for an unfortified control and a flour sample fortified at the standard level. A corresponding set of results for the test-tube format is presented in Fig. 2, illustrating color development under identical conditions. In both formats, fortified samples exhibited a distinct color change compared to unfortified controls, aligning with the qualitative classification used during validation.

Analytical sensitivity and dependence on concentration for color generation by direct contact with surface spots were evaluated for zinc fortification ranging from 10 ppm to 80 ppm, alongside an unfortified control sample (Fig. 3). As demonstrated in Fig. 3, a clear visible gradient of colorimetric

changes is observed. The control sample appears yellow-green, representing the non-reactive matrix of the dithizone reagent. Upon addition of zinc ions, an immediate bathochromic shift occurs. At 10 ppm, a faint pink-brown ring is visible along the outer edge of the compressed flour. Increasing the concentration to 20 ppm and 40 ppm results in more intense coloration that extends inward. At higher concentrations of 60 ppm and 80 ppm, a highly saturated red-to-pink complex develops.

This concentration-dependent visual change confirms that the technique can effectively detect zinc presence in unfortified, under-fortified, and well-fortified wheat flour samples in situ.

4. Discussion

Interpretation of analytical performance

The qualitative chromogenic spot assay, along with its complementary test-tube format, showed a high overall agreement of 94% with ICP-MS for the detecting zinc in wheat flour. A sensitivity of 96.8% demonstrates the method's strong ability to correctly identify truly fortified samples, which is critical for a screening tool designed to minimize false negatives in fortification monitoring. The observed selectivity of 90.0% indicates a modest false-positive rate (2 of 20 unfortified samples), acceptable for a primary screening assay where the goal is to flag potentially non-compliant samples for confirmatory quantitative analysis rather than to make final compliance decisions based solely on the qualitative result.

The Cohen's kappa coefficient of 0.88 confirms "almost perfect agreement" beyond chance between the qualitative test and the reference ICP-MS method, underscoring the statistical robustness of the assay despite the slight deviation from an idealized 95% threshold for some performance metrics. This level of agreement aligns well with the method's intended use as a triage tool within a broader monitoring framework that includes periodic instrumental verification.

Practical implications in the local fortification context

In this study, all fortified samples were obtained from mills that had implemented wheat flour fortification, while unfortified samples represented wheat flour produced without added zinc containing premix. In regions where mandatory fortification with iron is not enforced, the risk of interference from iron in the chromogenic reaction is minimized, simplifying both method deployment and interpretation. The spot assay requires minimal equipment, short reaction times, and demand only operator training, making it suitable for routine use in flour mills, point-of-sale inspections, and resource-limited laboratories. The complementary test-tube format provides an alternative under slightly more controlled environment, such as central quality-control laboratories or when a second independent qualitative assessment is needed.

Positioning this assay as a primary screening tool aligns with ISO/IEC 17025:2017 guidelines for the validation of laboratory-developed, non-standard methods, where performance characteristics must be

relevant to the intended use and customer needs. Its high sensitivity and strong agreement with ICP-MS support its use to rapidly distinguishing likely fortified from likely non-fortified flours, reserving ICP-MS or other quantitative methods for confirmatory testing of borderline or disputed samples.

Limitations and future work

Limitations of this study include the qualitative nature of the assay, which prevents direct assessment of compliance with specific fortification levels, and the potential for matrix effects or reagent variability when applied to untested flour types or under extreme environmental conditions. Although the sample set reflects local milling practices, it was limited to Ethiopia and may not represent the full diversity of flour matrices encountered worldwide. Future research could focus on developing semi-quantitative approaches (such as smartphone-based colorimetry), conducting broader field validations across diverse geographic regions and supply chains, and pursuing formal standardization of the procedure as a recommended screening tool for fortification programs.

Conclusions

The qualitative chromogenic spot assay and its complementary test tube format offer a rapid, cost-effective, and user-friendly screening method for detecting zinc fortification in wheat flour. With high sensitivity and strong agreement with ICP MS, these assays provide a reliable tool for routine monitoring in various settings, including flour mills and resource-limited laboratories. While the method's qualitative nature limits precise quantification, its effectiveness in distinguishing fortified from unfortified samples supports its role as a primary screening approach within broader fortification monitoring frameworks. Further validation and standardization will enhance its applicability across diverse regions and flour types, contributing to improved public health outcomes through better fortification compliance.

Declarations

Author Contribution

Tadesse G/giyorgis developed the colorimetric method, performed the experimental work, and wrote the main manuscript. Dr. Adamu Tessema identified the research problem (the absence of a validated zinc testing method in Ethiopia) and critically reviewed the manuscript. Dr. Masresha Tessema served as the overall coordinator of the project, provided supervision, and facilitated budgetary and administrative support. Teshome Assefa carried out the ICP-MS instrument analysis.

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Figures



Figure 1

Representative results of the qualitative chromogenic spot assay for zinc in wheat flour. Left panel: negative control (green-to-muddy color)); right panel: positive control (pink-to-red color)

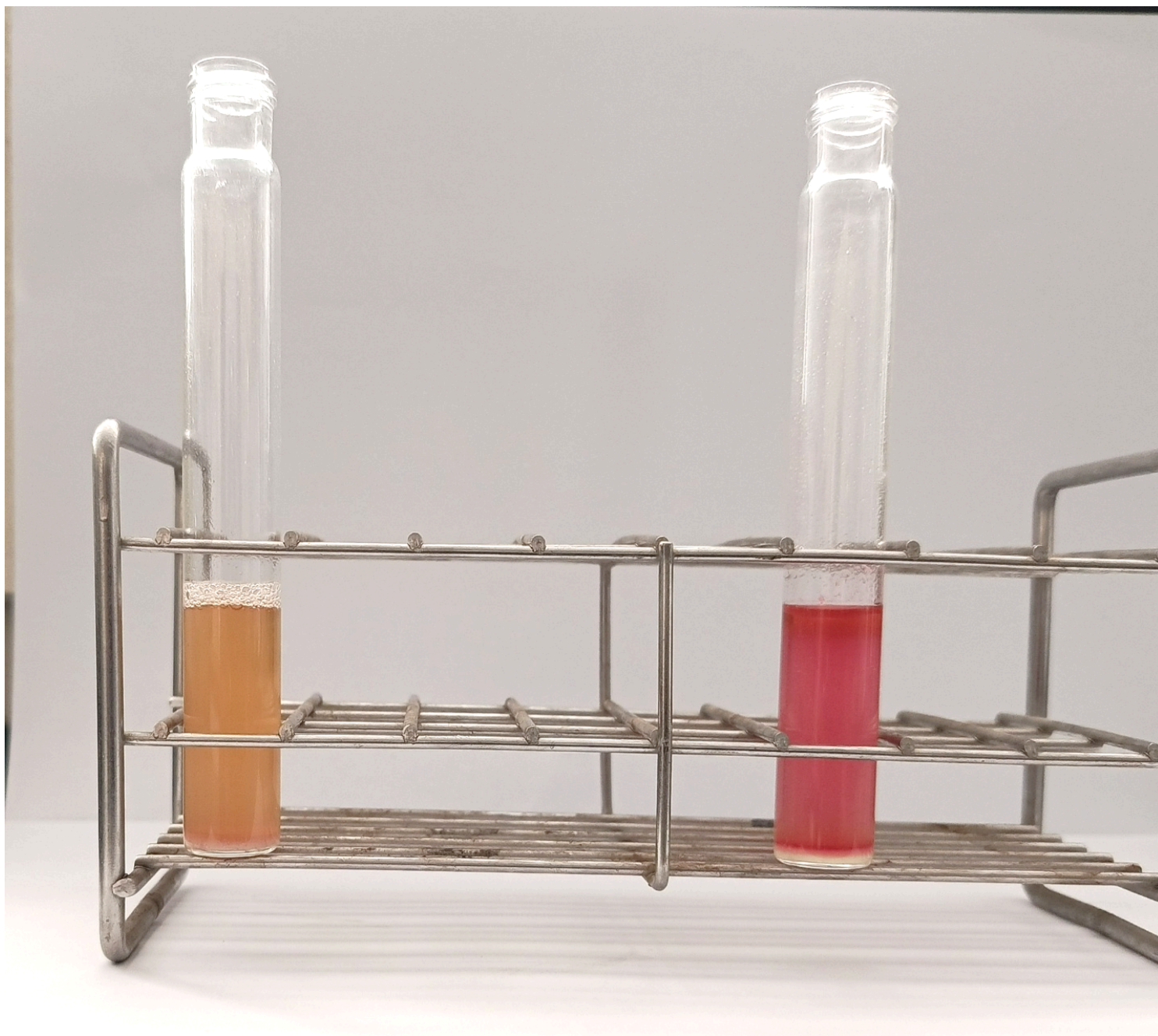


Figure 2

Representative results of the complementary qualitative chromogenic test-tube assay for zinc in wheat flour. Left panel: negative control (muddy amber-orange color); right panel: positive control (pink red color)

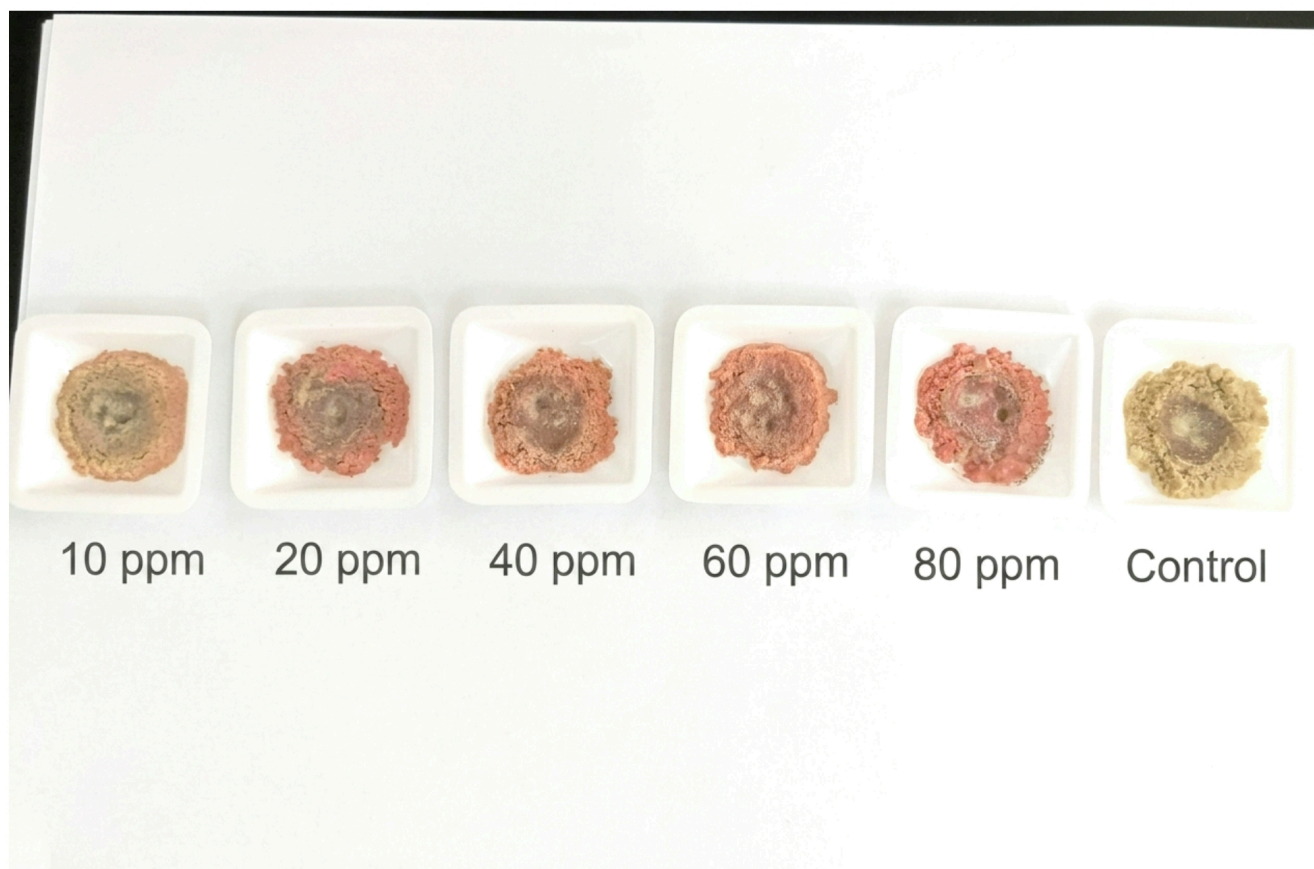


Figure 3

Visual colorimetric gradient of the direct surface-contact spot test on compressed wheat flour matrices

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