

Draft

**Ministry of Health and Social Development of the Republic of Kazakhstan
Committee of Consumer Rights Protection of the Ministry of National Economy of the
Republic of Kazakhstan
Kazakh Academy of Nutrition**

**EXTERNAL AND INTERNAL QUALITY CONTROL OF IRON FORTIFICATION OF
FLOUR
(including iron in vitamin-mineral mixtures)**

Guidelines

Almaty, 2016

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These Guidelines are based on the requirements of the Code of the Republic of Kazakhstan, dated of 18 September, 2009, “On Health of Population and Health Care System”, the Law of the Republic of Kazakhstan “On Technical Regulation”, dated 9 November, 2004, and the “Rules of Fortification of Foods Requiring Sanitary and Epidemiology Surveillance”, by the Order of the interim Minister of National Economy of the Republic of Kazakhstan, dated of 24 February, 2015, No. 123 (registered in the Registry of Public Registration of Legal Enactments under the No. 10564.

1. Annotation

These Guidelines “External and Internal Quality Control of Iron Fortification of Flour (including iron in vitamin-mineral mixtures)” describe the organization and management of external and internal quality control of iron fortification of flour. They are intended for the public services and organizations of sanitary-epidemiology surveillance and managers of quality control of flour fortification at flour mills. These Guidelines are based on appropriate international experience and approaches for ensuring flour fortification and quality and safety of end products.

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2. List of Abbreviations

In these Guidelines the terms and definitions derived from the “Rules of Fortification of Foods Requiring Sanitary and Epidemiology Surveillance” (Rules No.123) , by the Order of the interim Minister of National Economy of the Republic of Kazakhstan, dated of 24 February, 2015, No. 123 (registered in the Registry of Public Registration of Legal Enactments under the No. 10564) have been used.

ExtC	external control
VMM	vitamin-mineral mix
IntC	internal control
QC	quality control
QA	quality assurance
PS	production standards

3. Explanatory Note

Flour fortification and its quality control in Kazakhstan are regulated by the law on mandatory fortification of high extraction flour, dated of 2009. At the same time, there is a need for ensuring a uniform observance of norms and standards, in order to assure universal algorithm of fortification quality control irrelevant of the size, equipment fleet of the mill and other parameters, which might affect the quality of the end product. In this regard, these guidelines focus on uniform understanding and observance of quality control activities and realization of a collective responsibility for following the production standards.

The goal of the Guidelines is to render a methodological aid to public bodies, individuals and legal entities regardless of their type of property both on independent and institutional levels, which are in charge of quality control of products, for uniform methods and interpretation of results. The guidelines will promote interaction between the external and internal quality control of fortified products and increase consumer satisfaction, as well as motivation of mills in terms of compliance with appropriate standards.

4. Introduction

Among the CIS countries, laws on mandatory fortification of baker's flour with vitamin-mineral premix have been endorsed in Kazakhstan and Kyrgyzstan (in 2009). In Turkmenistan, the mandatory flour fortification is regulated by the Presidential Decree of 1994. Among other CIS countries, the flour fortification programs have been adopted in Uzbekistan (by Presidential Decree), Tajikistan, and by Georgia. At the same time, there is no document justifying the uniform approach to the external and internal quality control. These Guidelines are based on the assessment of quality assurance and quality control of flour fortification with iron in the composition of fortification mixes, according to the Rules No. 123.

Quality assurance (QA) and Quality control (QC) are the procedures to be established at each mill, with a purpose to ensure both systematic and periodical checks of compliance of the mill with the existing standards of wheat flour fortification with vitamin-mineral mix (VMM), or any other fortificant(s) containing iron, with simultaneous observance of established QA and QC procedures and good manufacturing practices, and eventual goal to maintain processes of quality improvement and standard product quality.

Table – Summary of Characteristics of QA and QC and Their Interdependence in the Whole Process of Good Manufacturing

Issue	Quality assurance	Quality control
Definition	QA is a set of activities for ensuring quality in the processes by which products are manufactured.	QC is a set of activities for monitoring quality in products. The activities focus on identifying defects in the products produced.
Focus on	QA aims to prevent defects with a focus on the process used to make the product. It is a proactive quality process.	QC aims to identify (and correct) defects in the finished product. QC, therefore, is a reactive process.
Goal	The goal of QA is to improve manufacturing processes so that defects do not arise.	The goal of QC is to identify defects during, after a product is manufactured, and before it is released.
How	Establish a quality management system, which defines the procedures for manufacture.	Finding and eliminating quality problems using tools and equipment, so that customer's requirements are continually.
What	Prevention of quality problems through planned and systematic activities, including documentation.	The activities or techniques used to achieve and maintain the product quality, process and service.
Responsibility	Everyone on the team involved in manufacturing the product is responsible for quality assurance.	Quality control is usually the <u>responsibility</u> of a specific team that tests the product for defects.
Example	Establishing an approved supplier list is an example of QA.	Laboratory testing the moisture content of flour is an example of QC.
As a tool	QA is a managerial tool.	QC is monitoring tool.

To ensure the quality of fortified flour, there exist independent systems of external control and quality surveillance responsible for:

- I) Assessment of producers' capacity to conform with production standards;
- II) Periodical inspections and audits of equipment, premises, methods and knowledge of staff of a mill;
- III) benchmark testing of final products at established time intervals;
- IV) control of corrective measures and recommendations on compliance with production standards and consumer expectations;

5. Organization of external and internal quality control

Overall goal of the External Control (ExtC) is to check the compliance with the established standards of Good Manufacturing Practices (GMP), with a purpose to protect population and consumers from negative effects of inferior quality products. ExtC must ensure public security, protection of rights and freedoms of consumers.

ExtC is executed by authorized public bodies and specialized, accredited private organizations, and controlling bodies according to the established schedule, as well as by the requests of mills, public organizations and within the boundaries of established periodicity of checks. In case of flour fortification, public sanitary and epidemiology services are in charge of producer checks. Such checks provide information and identify potential sources of violation of Internal Control (IntC) at the mills, and allow to give specific recommendations on corrective measures to comply with existing Good Manufacturing Practices (GMP) and requirements of normative documents.

ExtC and IntC have a common goal to ensure the required quality level of the final product offered to consumers, and they should collaborate in this direction and ensure mutually additive positive effect.

The table below describes main characteristics and differences between ExtC and IntC

Issue	External control	Internal control
Definition	To be defined in normative requirements (standards)	To be defined by mill leadership based on standard recommendations, normative standards and customer requirements.
Focus	Focus on compliance with normative requirements	Focus on conformity to internal Quality Management System
Goal	Goal of the external control is to ensure conformity of existing standards and normative documents to the requirements of GMP	Goal of internal control is to ensure conformity to internal Quality Management System
How	Periodical checks of documentations and assessment of staff competence	Regular monitoring with corrective and preventative action
What	Standards of external control, including proper documentation	Regular and scheduled activities or technologies chosen by a mill to achieve and maintain proper quality of products, processes and services
Responsibility	External organization authorized by competent body	Internal responsibility of a mill
Example	Public Health inspection is an example of ExtC. Audit is an example of ExtC	Regular testing of product samples are an example of IntC
Tool	ExtC is a tool of assessment of compliance of production processes to rules and recommendations on corrective activities	IntC is a response (reacting to production defect) and corrective tool to ensure conformity with GMP and standards in regard of final quality of the product

Mill auditing bodies should analyze the findings of internal control, in order to spare time and resources and avoid duplication of efforts, and focus on ExtC to identify potentially critical elements. Interaction of ExtC and IntC ensures better effect on end result subject to coordination of checks, exchange of reports, access to operational documentation, joint reports to proper leadership and common documentation patterns.

Each of listed responsibilities of ExtC can be specified also as follows:

- 1) Assessment of producer's capacity to follow the normative requirements and standards.
- 2) Availability and knowledge of rules related to flour fortification, in accordance with the following checklist:
 - Appropriate standard of Republic of Kazakhstan, and technological instructions;
 - Copies of effective standards or guides on flour fortification and appropriate requirements of the competent body in charge of sanitary and epidemiological security of population;
 - Specifications of the equipment required for flour fortification;
 - A log of preliminary and operative instructing of staff on safety measures at operating with flour fortification;
 - A log of regular technical checks of fortification equipment;
 - *A log for registration of laboratory samples and tests of fortified flour with established time interval and analyses of external control (it is a recommended log, according to effective standards currently flour fortification at a mill is monitored by weight method only. No analyses are made).*
 - Logs to every day records of quality control at a mill (see, annex 3).
 - Warehouse logs recoding route of premix within the mill and to technological lines.
- 3) Regular checks and audits
 - Assessment of regularity of order and delivery of fortificants, suppliers, quality documents certificate of origin, certificate of public registration of premix, etc.);
 - Assessment of premises and conditions of storage, release, record of use of fortificants;
 - Visual assessment of mixing equipment and production process;
 - Sampling, sample control, recording and registration of samples and proper documentation.

A. External control by governmental bodies:

State audit: there exist two types of audit.

One is called **technical audit**, or the extra-enterprise audit, when the competent body in charge of sanitary and epidemiological security of population examines information provided by a mill or association of mills, if the latter is authorized to collect such information, with a purpose to determine documentary quality of flour fortification. This type of audit can be made once a quarter (see, the example of mill's report).

Second type of auditing is **the inspection or field examination** when the competent body in charge of sanitary and epidemiological security of population inspect a mill with a purpose to assess the conformity of enterprise to flour fortification requirements and double checking of data provided for technical audit.

The competent body in charge of sanitary and epidemiological security of population determine frequency of field inspections based on the findings of previous operation of a mill and number of claims or violations identified in regard of the specific enterprise.

One of important stages is the check of observance of the procedure of acceptance of a premix, including registration of the following information:

- 1) Check of package for any damage and recording of such damages in the documentation. It is acceptable to have small damages, but it is unacceptable to have serious damaging by water and ruptures of internal bags;
- 2) Record of date of acceptance and name of controller;
- 3) Data on type or name of commodity and number of boxes/bags or total mass, comparison with ordered items;
- 4) Record of batch numbers;
- 5) Check the availability and recording in the documentation of a test certificate of the premix and certificate of public registration. It may be put in one of boxes or sent separately by fax or e-mail. All this information should be recorded in the "Report on Premix Acceptance".
- 6) Each box must be labeled. The label shall bear: a) name of commodity; b) intended use of commodity; c) name of producer and contact information; d) safety measures; d) date of production and shelf life, sometimes presented in batch number; e) batch number; f) recommended use ration; g) net weight; h) composition.

B. Premix producer must provide a Certificate of public registration of premix (or excerpt from the Registry of certificates of public registration), test certificate or test protocol (in case of the Kazakh regulations). For each premix batch and for all micronutrients in the composition of premix, there should be provided a proper document/certificate proving quality of a product. Test certificate (sometimes called "Quality certificate") is a formal document confirming the quality of premix. Test certificates for each batch of premix must be stored and presented in case of control checks. The test certificate shall bear the following information:

Results of chemical analysis of the premix batch in regard of each nutrient in its composition. The certificate may provide reference data on minimum and maximum levels of elements in the premix, batch or lot number, date of production and shelf life, if these data are not included in the batch number.

Premix suppliers shall provide: information on commodity or a data table. Such document should be stored at a mill and provided upon request to ExtC representatives, production staff and department of quality control. This document shall contain the following information:

- 1) Premix name;
- 2) Name and contact information of producer;
- 3) Intended use of premix;
- 4) Premix composition – as a rule, in descending order;
- 5) Nutritive status of ingredients (that is, a class according to classification of chemical substances allowed for using in food industry);
- 6) Recommended proportion of addition of premix to flour and levels of content of micronutrients at such proportion;
- 7) Standards of minimum composition of a premix, as well as standards of maximum composition (if available);
- 8) Storage and transportation instructions;
- 9) Shelf life of a premix;
- 10) Content of pathogens and other microorganisms of concern

6. Control methods

The control is accomplished by using the following assessment methods:

- A. Assessment of knowledge and skills of laboratory staff;
- B. Assessment of availability of equipment and reagents for qualitative and quantitative tests;
- B. Control sampling and exchange of findings with a mill;
- Г. Provision of an option of qualification tests.

External quantitative analysis. Getting a mixed sample of fortified flour:

- 1) Mixed samples are made by mixing samples of fortified flour collected at established time intervals during the production shift;
- 2) Mixed samples consist of 5-10 samples collected from production line, for example, during a 8-hour shift;
- 3) Samples used for preparation of a mixed sample shall of the same volume and be collected at even intervals during the production shift to be representative for the whole product manufactured during the certain period;
- 4) The goal of the mixed sample is to assess a mean content of nutrients in a certain batch. Use of mixed samples allows decreasing the cost of analyses of fortification via reducing the number of samples required for getting average value.
- 5) Use of quantitative analysis for checking one or several cases of too high or too low content of nutrients is senseless. Its main goal is to monitor the evolving trends, and as such, the quantitative analysis is a valuable tool.

7. Ensuring correcting activities and recommendations on observance of normatives and consumer demands

First, it is necessary to organize a regular reporting on the outcomes of external control activities;

Second, it is necessary to hold discussions with a mill on corrective activities and deliver methodological support in the development of recommendations to ensure compliance with Good Manufacturing Practices and to organize, if necessary, (re)training of specialists, taking into consideration employee turnover, replacement of equipment, etc.;

Third, to establish submissions of follow-up reports on operation of a mill on ensuring the established level of quality before the controlling bodies.

8. Role of laboratory control

As the laboratory methods constitute an important part of both external and internal control of fortification quality, the Annex 1 and Annex 4 provide detailed description of methods commonly used around the world for measuring iron, which are recommended for official use by sanitary, and epidemiology services and mills in Kazakhstan.

With evolution of laboratory techniques, currently many started shifting to inductively coupled plasma mass spectrometry (ICP-MS) for determination of iron and other inorganic substances. ICP-MAS is a type of mass spectrometry which is capable of detecting metals and several non-metals at concentrations as low as one part in 10^{15} (part per quadrillion, ppq) on non-interfered low-background isotopes. This is achieved by ionizing the sample with inductively coupled plasma and use of a mass spectrometry to separate and quantify ions. Compared to atomic absorption spectroscopy, ICP-MS has greater speed, precision, and sensitivity.

Annex 1

Recommended (reference) methods of iron determination in flour in the frame of ExtC (Spectrophotometric methods are given in the Annex 4)

HPLC method for determination of iron

Iron determination with use of Dionex-500 chromatograph

Reagents

- 1) De-ionized water, nano-purification 18.0 mOhm
- 2) Concentrated HCl 36%
- 3) Iron standard 100 µg/ml in HNO₃, commercial standard
- 4) Post column solvent MetPacDionex Corp. P/N46094
- 5) Concentrated dilutor MetPacDionex Corp. P/N46088
- 6) 4-(2-pyridazo) resorsinol, analytical grade
- 7) Monosodium monohydrate

Equipment

- 1) Analytical balance capable to weigh 0.01 mg
- 2) Measuring glass, 400 ml and 1,000 ml
- 3) Column and breaker Dionex IonPac CS5 A and IonPac CG5A
- 4) Alumina crucible, 50 ml
- 5) Flasks, 100 ml
- 6) Graduated cylinders, 500 ml and 1,000 ml
- 7) Gas stove 12"x24", 220 V, with remote control
- 8) HPLC system (Dionex-500, with AD20 absorption detector, pump GP50, sampler AS40)
- 9) Magnetic rods for mixer, octagonal 15"x3/8", teflon-coated
- 10) Muffle furnace, 675 °C
- 11) Sonic bath
- 12) Mixer
- 13) Disposable pipette, 5 ml
- 14) Watch glass, 65 mm
- 15) Volumetric flask, 250 ml
- 16) Volumetric pipette, class A: 1, 3, 7, 10 and 15 ml

Preparation of solution

Iron standards

Using 1, 3, 7, 10 and 15 pipettes, transfer 100 µg/ml iron standards in labelled volumetric (250 ml) flasks, dilute to volume with deionized water, cap and mix. These solutions are equivalent to 3.629 mg/pound, 10.866 mg/pound, 25.402 mg/pound, 36.288 mg/pound and 54.432 mg/pound of iron, respectively. Label the day of preparation.

One pound = 453.6 g

Mobile phase

Add 800 ml deionized water to 1,000 ml graduated cylinder. Fill to volume with dilutor PDCA. Transfer to the reservoir with mobile phase to deliver to HPLC system.

Post-column solvent

Weigh 0.0600±0.0010 g of the solvent, transfer in 1,000 ml flask with screwcap. Place the flask in sonic bath for five minutes, connect the flask to post-column pump.

Preparation of samples

- 1) Sample 5.00 ± 0.01 g flour and place in alumina crucible. Put the crucible in muffle oven. Record the sample number and its position in oven. Repeat the same with all samples and prepare flour.
- 2) Put muffle temperature for 675°C and leave crucibles overnight.
- 3) Switch off the oven next morning and carefully open the door to speed up the cooling of crucibles. Take cool crucible from oven and place on gas stove located in fume hood.
- 4) Turn on the fume hood, and add 2 ml of concentrated HCl in each crucible, around evaporation plate. Close crucible with watch glass. Heat crucible until about 1 ml solution remains. Take crucible off the gas stove and let it cool.

Notes

Avoid full evaporation of hydrochloric acid! If occurs, add 1 ml of concentrated hydrochloric acid and heat it again.

Wash off condensate on watch glass with deionized water in labeled reaction tubes. Move the content of crucible in a flask and collect in it deionized water used for washing the crucible several times. Add deionized water to 100 ml volume, cap the flask and mix by tilting.

Fill labelled tube with sample and place in sample conveyor. After charging put the container in automatic sampler AS40 and make sure the black point is on the right side of conveyor at looking on the front side of sampler.

Analysis

- 1) Open the start window PeakNet. To load schedule, click on second icon on the left. Close the schedule developed in step 3.3.2. after the click, press the last icon (OK), pump starts operation.
- 2) Switch on post-column pump and press Start. Let the system work for 30 minutes.
- 3) Load samples in automatic sampler AS40 as appropriate and press button Start.
- 4) "Dionex" software automatically calculates iron in mg/pound and prints out results of each standard and samples.

Elements by atomic absorption spectrophotometry AACC method 40-70

Final approval October 16, 1991; Reapproval November 3, 1999

This method determines calcium, copper, iron, magnesium, manganese, and zinc in grains and cereal products.

I. Materials

- Volumetric flask, 1 L
- Volumetric flasks, 250 mL
- Volumetric flasks, 100 mL
- Volumetric flasks, 25 mL
- Beakers, 250 mL
- Manual volumetric pipettes (200-1000 mL)
- Porcelain crucibles
- Watch glasses
- Pipette tips
- Graduated tubes
- Tips for 'blue' pipettes
- Test tubes, 10 mL

II. Equipment

Atomic absorption cereal products spectrophotometer. Several commercial models are available.

Since each design is somewhat different, with varying requirements of light source, burner flow rate, and detector sensitivity, only the general outline of operating parameters is given in Table 1 (below). Operator must become familiar with settings and procedures adapted to own apparatus and use table only as guide to concentration ranges and flame conditions. Single-slot burner may require that lanthanum be added to standard and sample solutions for all elements.

Ashing vessels, 150 ml beaker (Pyrex or Vycor) or 30 ml Vycor crucible.

Muffle furnace capable of operating at temperatures up to 525°.

Vortex mixer

Analytical balance

Hot plate

Eppendorf pipette (100 and 500 mL)

Table 1. Operating Parameters

Element	Wavelength	Flame	Range (µg/ml)
Fe	2483	Rich air-acetylene	2-20
Zn	2138		0.5-5

III. Reagents

- 1) Water, distilled-deionized (greater than 10 megohm resistance). Use throughout procedure in all preparation and dilution of solutions.
- 2) Stock solutions. See Note 1.
 - a. Iron, 1000 µg Fe/ml. Dissolve 1.000 g pure Fe wire in about 30 ml 6N HCl with boiling. Dilute to 1 liter.
 - b. Zinc, 1000 µg Zn/ml. Dissolve 1.000 g pure Zn metal in about 10 ml 6N HCl. Dilute to 1 liter.
- 3) Lanthanum stock solution, 50 g La/liter ~5% HCl. Dissolve 58.65 g La₂O₃ (99.99%, low calcium content) in 250 ml concentrated HCl, adding acid slowly. Dilute to 1 liter.
- 4) Working standard solutions.
 - a. Calcium, 0, 5, 10, 15, and 20 µg Ca/ml containing 1% La and ~1% HCl. To 25 ml volumetric flasks, add 0, 5, 10, 15, and 20 ml Ca stock solution (reagent 2a). Add 5 ml La solution and dilute to volume.
 - b. Other standard solutions. Dilute aliquots of solutions 2b, 2c, 2d, 2e, and 2f with 0.5N HCl, to make at least four standard solutions of each element within range of determination.

IV. Procedure

A. Preparation of sample

B.

- 1) Accurately weigh 1-10 g ground sample (depending on anticipated concentration of element) into ashing vessel. See Note 2. Begin preparation of reagent blank at this point.
- 2) Char on hot plate or in muffle, then ash at 500° overnight. If not completely ashed, cool sample and wet with a few drops of concentrated HCl or HNO₃, dry at low heat, and re-ash. See Note 3.

- 3) Break up cake with stirring rod and dissolve in 10 ml concentrated HCl. Boil and evaporate solution nearly to dryness on hot plate. Do not bake residue.
- 4) Redissolve residue in 20 ml 2N HCl, boiling gently if necessary.
- 5) Filter through fast paper into 100 ml volumetric flask, washing paper and residue thoroughly with water. Dilute to 100 ml and mix.
- 6) Measure absorption of solution directly, or dilute with 0.5 N HCl to obtain solutions within ranges of instrument. If Ca is to be determined, add enough La stock solution to make final dilution 1% La (i.e., 5 ml La solution to 25 ml flask, 20 ml to 100 ml flask, etc.). See Note 4.

B. Method

- 1) Set up instrument as in Table I or by previously established optimum settings for apparatus to be used. Secondary or less sensitive lines may be used to reduce necessary dilution if desired. See Ref.2.
- 2) Read at least 4 standard solutions within analytical range before and after each group of 6 to 12 samples. Flush burner with water between samples, and reestablish zero absorption point each time.
- 3) Prepare calibration curve from average of each standard before and after sample group. See Note 5.
- 4) Read concentration of samples from plot of absorption against $\mu\text{g/ml}$.

V. Interpretation/Calculations

$$\text{Element (ppm)} = (\mu\text{g/ml} \times 100) \div \text{sample weight (g)}$$

If original 100 ml volume is diluted, consider this in final calculation.

VI. Notes

- 1) Do not use <2 ml pipettes or <25 ml volumetric flasks in making standards. Prepare working solutions in 0-20 $\mu\text{g/ml}$ range fresh daily. Automatic dilution apparatus may be used. Alternatively, purchased standards may be substituted for stock solutions.
- 2) Some grains and unfortified products may require ashing a larger sample to increase the concentration of elements to detectable ranges. See Ref. 3.
- 3) Contamination from labware and reagents can contribute significant amounts of analytical error. Blanks should be run to monitor for contamination. Cleaning protocols including rinses in mineral acids (e.g., nitric and hydrochloric acids) and multiple rinses in distilled/deionized water have proven effective in minimizing metal contamination in labware. If re-ashing is necessary, prepare re-ash blank, using the same amount of concentration HCl or HNO₃ as for the samples.
- 4) Phosphorus interferes in calcium and may interfere in magnesium determination with air-C₂H₂ burners. Eliminate interferences by adding La stock solution to standard and sample solutions so that final dilutions contain 1% La. Phosphorus does not interfere with calcium determination when an N₂O-C₂H₂ burner is used.
- 5) It is recommended that a standard reference material (such as those available from the National Institute of Standards and Technology) be analyzed frequently to ensure accuracy of the determination. Choose reference material that matches as closely as possible the matrix of the sample being analyzed.

VII. References

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Annex 2 - FLOUR FORTIFICATION METHODOLOGY

Ensuring standardized monitoring of fortification procedures and records:

- 1) Store fortification well protected from exposure to light or under the conditions laid down by the manufacturer. It is ideal to keep fortification mixes in their original containers. Once opened, exposure to the light and air should be minimized to prevent product degradation.
- 2) Obtain and keep on record on record a certificate of compliance for every batch of fortification mix.
- 3) Employ, and adhere to, strict stock rotation procedures to prevent old stock losing potency and to comply with the shelf life expiry date. It is recommended to employ “the first in, first out” (FIFO) system for this purpose.
- 4) Keep records of grain procurement;
- 5) Keep records of fortification mix inventory and usage;
- 6) Keep production records of the amount of fortified bread flour produced;
- 7) Keep monthly records of the amount of fortification mixes used every month. These records should correspond with the monthly production records;
- 8) Ensure that all critical stages of the manufacturing process are monitored to ensure the correct dosage levels are maintained through the following measures:
 - 9) Checking of fortification mix feeders to ensure they are delivering the correct dosage levels. This can be done by measuring the weight of fortification mix discharged over a specific time (1 or 2 minutes) and comparing the measurements with the target weight of fortification mix.
 - 10) Performing frequent visual checks to ensure fortification mixes are being used and that no blockages have occurred, and keeping a record of this.
 - 11) Performing regular and random iron spot tests on the bread flour using composite samples of fortified flour.
 - 12) Collect individual samples of fortified flour every 2 hours to form average per shift sample to determine the fortifying iron.

Analytical error

To minimize analytical and sampling errors, laboratories shall thoroughly mix the samples received from the inspector to form an average shift sample.

Then the laboratories of mills or authorized body (in case of external control) shall analyze the sample – possibly in duplicate and, more than likely, twice on the same extract rather than twice from the same sample.

Total error

Laboratory error can be high – at 95% confidence level for vitamin analysis in fortified product the result is $\pm 15-20\%$.

Distributing 200 g of pre-mix in 1,000 kg of flour is not easy even with a very good mixer, so we could have a variation $>30\%$.

Quality control of the iron fortification at the mill

Taking the following actions during the receipt and storage of the iron fortificant will help the mill to assure the quality of the iron in the final product:

- 1) Request that suppliers include a certificate of compliance with each shipment. A copy of the certificate of compliance should be kept with the permanent records.
- 2) Maintain a “first in, first out” policy. Number lots consecutively in order that they are received. For each lot, write the lot number and the date of receipt on the sticker.
- 3) Arrange storage areas to facilitate the “first in, first out” policy. Store bags or boxes of the iron fortificant in consecutive order, so that the oldest can be withdrawn first.

- 4) Store the iron fortificant in conditions that maintain its quality and prevent contamination or deterioration.
- 5) Record and monitor the movement of the iron fortificant in and out of the warehouse, and control the amounts used in manufacturing.
- 6) Maintain records of returned materials and their use and control.
- 7) Maintain sufficient stocks of iron fortificant. Ask the purchasing department to order a new shipment in sufficient time to avoid running out of iron fortificant.
- 8) To confirm that the composition of the iron fortificant meets specifications, send samples for laboratory analysis within three months of arrival.

Packaging, storage, and handling the premix

Premixes are concentrated sources of iron or other micronutrients. Excessive intake of iron in a single dose is poisonous and can be fatal; lower concentrated doses over a prolonged period can also be harmful. For this reason, the boxes must be carefully labelled “Not suitable for direct consumption”, and workers at the mill must understand and respect this warning. Operators at mill should take precautions such as wearing a dust mask, gloves, etc., to prevent inhalation of and exposure of skin to the premix or iron fortificant.

The premix and iron fortificant must be properly handled to minimize degradation of the compound. They must be stored in well-ventilated rooms at low or mild temperatures (preferably not higher than 25°C), and exposure to humidity must be avoided. The amount of commercial premix or iron fortificant needed should be estimated and obtained in quantities small enough so that it does not need to be stored for long periods of time. The production lot number(s) should be properly recorded and the premix used on a first-in/first-out basis. Once a commercial premix or iron fortificant box has been opened, it should be used within the period specified for its shelf life.

Fortification of flour in continuous and batch systems

Volume or weight feeders are used to introduce a premix or mixture in the flow of flour.

In a continuous milling system, the iron fortificant or premix is continuously and gradually added as a free-flowing dry powder at a rate dependent on and compatible with the flow of flour along the production conveyor (about 150 g per 1 ton of higher grade flour and 120 g per 1 ton of first grade flour). The homogeneity of micronutrients in the fortified flour is largely dependent on the location of the feeder, and it is very important that good mixing of the micronutrients in flour occurs. The two most common sites for adding micronutrients are 1) before packaging, which facilitates good mixing and 2) where flours from different streams converge, which facilitates excellent mixing.

Small- and medium-sized mills may choose to fortify flour using a batch system, where flour is fortified in individual batches rather than in a continuous process. In batch fortification, iron is measured out by weight or volume and added to a batch mixer that is partially or totally filled with flour. The well-mixed, fortified flour is then transferred to pack-out bins and another batch of flour is fortified. The time of satisfactory mixing up is determined empirically.

Quality control of the iron fortificant or commercial premix

A recording and storage system must be in place to log the date the iron is received. The iron or premix must be stored under conditions specified by the manufacturer. Physical characteristics such as color, texture, and odor can be visually examined. The iron or premix should be free-flowing with no lumps or off-odor. The Certificate of Analysis provided by the manufacturer should be reviewed for the grade, particle size, and concentration of iron. The iron should be analyzed using semi-quantitative spot tests to ensure the iron level; the analytical methods are presented in Part 3 of this manual. All these observations along with any corrective actions taken when the required specifications were not met must be properly recorded and filed for easy access. Annex 3 gives an example of the type of recording form that can be used.

Quality control of iron-flour premix made at the mill

The iron-flour premix made at the mill should be analyzed using quantitative methods to verify the concentration of iron in the iron-flour premix. The amount of iron in the premix must be within 10% on either side of the specified mean. The analytical methods to determine the amount of iron in the iron-flour premix and fortified iron are presented in Part 3 of this manual.

The mill manager should be responsible for monitoring the quality of iron-flour premix, the date of manufacture, and the addition to bulk flour. The dates and results of all chemical analyses must be properly recorded. The mill manager also is responsible for any corrective actions. All records should be filed for easy access. Annex 3 gives an example of the type of recording form that can be used.

Quality control of the fortified flour

Four methods can be used to supervise and control the addition of iron to flour. Proper recording of all the results of the quality control procedures is essential. An example for recording the quality control results of fortified flour is given in Annex 3.

1. Premix inventory control

Comparing the amount of premix used against the flour production records provides a simple way of determining if the correct amount of premix is being used. Another way is to obtain the ratio of the amount of fortified flour produced to the amount of premix used in a predetermined period of time. These methods provide general information on whether the required amount of premix is being added. The mill manager may add this task to his routine quality control activities.

2. Regular weight checks

In this case, a plate is put under the feeder to collect the fortificant for usually one to three minutes and the fortificant is weighed to determine if the feeder is dispensing the correct amount of fortificant at that setting; longer collection times are used for lower addition rates. Weight checks should be done every time flour samples are taken for routine quality analysis – approximately every two hours. The operator should record the time, weight, and feeder setting. Automated, loss-of-weight feeders are also available, wherein the disappearance of iron fortificant should correspond with calculated usage and any adjustments to the feeder can be made based on these results. The mill manager may add this task to his routine quality control activities.

3. Spot tests

Mills can use a simple qualitative spot test for iron to determine if the flour has been fortified or a semi-quantitative spot test that allows the determination of whether the flour is grossly under- or over-fortified. Spot tests are quick and simple, and can be used at various points in the production line – using them at the point of pack-out or packed bags will ensure that the flour fortified within a specific range of iron content. The results of qualitative or semi-quantitative tests, however, are not precise enough to make feeder adjustments. It is recommended that semi-quantitative spot tests be conducted every time the mill conducts routine quality control procedures (for example, for moisture or color), approximately once per shift in a continuous fortification process using average shift sample for 12 hours or for every batch of flour produced using the batch fortification process. The laboratory manager or a laboratory technician should be responsible for conducting these tests.

4. Quantitative tests

Quantitative tests provide accurate data on the amount of iron in the flour and reflect the efficiency of the entire production process. Ideally, the same flour samples collected for semi-quantitative spot tests should be pooled for each production shift, mixed well, and analyzed. However, it is recommended that quantitative tests be conducted as often as routine tests for

protein in flour. The tests may need to be performed more frequently when troubleshooting problems in the fortification process. The laboratory manager or a laboratory technician should be responsible for conducting these tests.

Sampling the flour for chemical analysis

The point at which the flour is sampled, the sampling procedure, and how the sample collected and handled are critical in obtaining reliable and useful results. Samples should be collected at a point in the production line after the addition of the fortificant as well as the blending of fortified flour. Samples must be kept protected from high temperatures and humidity until analyzed. A good sample represents the entire production run averaging out any momentary variation in the fortification process. A composite sample can be obtained by taking a “grab” of flour at a specified point in the production line every two hours over a shift and mixing them together. This composite sample of fortified flour can then be analyzed for iron content.

STORAGE OF FLOUR

It is important to organize the zone of flour storage operating by the order “first in – first out”, that is to store the bags or package in consecutive order to release first the batches which came to storage are earlier.

Annex 3 – Examples of logs for registration of flour fortification with iron

An example of a quality control log for iron fortificant or commercial iron premix

Date received	Batch #	Results of visual examination			Certificate of analysis check	Iron (ppm) (semi-quantitative test)	Observations / corrective actions
		Color	Free-flowing	Odor			

Mill manager _____

Date _____

An example of a quality control log for iron-flour premix made at the mill

Date manufactured	Batch number	Date tested for iron	Iron (ppm) (quantitative test)	Date used in bulk flour	Observations / corrective actions

Mill manager _____

Date _____

An example of a quality control log for fortified flour

Date _____

Shift and time	Premix inventory control				Regular weight checks			Semi-quantitative spot test (ppm) ¹	Quantitative test (ppm)	Observations / corrective actions
	Time (hours)	No. flour sacks produced (A)	No. fortificant bags used (B)	A † B	Collection time (min & sec)	Weight (mg)	Feeder setting			

Mill manager _____

Laboratory manager _____

Annex 4 – Methods of assessment of iron fortification recommended for mills (Internal control)

QUALITATIVE METHOD

A. Spot test method

This method, approved by the AACC, is applicable for qualitative determinations of iron in enriched flour (See Appendix 3.3).

Principle

Ferric iron added to flour reacts with a thiocyanate (KSCN) reagent to form a red colored complex. A higher number of red spots and deeper red color appear with enriched and fortified flour compared with untreated flour.

Advantages

- 1) It is a simple, fast and easy technique requiring no sample treatment.
- 2) It is inexpensive: only two reagents, KSCN and HCL, are needed.
- 3) Personnel with minimal training can conduct this assay.
- 4) It does not require a laboratory; it can be conducted in the flour mill.

Limitations

It is not quantitative, i.e., it does not determine the amount of iron present in the sample.

Notes

This method shows only ferric iron. If iron is added in the ferrous form, the sample needs to be oxidized with hydrogen peroxide to convert the ferrous to ferric iron before analysis.

Description of the method 1:

Iron qualitative method

AACC Method 40-40

First approval May 5, 1960; reapproval November 3, 1999

This method qualitatively determines iron added to flour

I. Materials

Rectangular glass or rich galvanized iron plate, about 12 x 8 cm
Flour trier

II. Equipment

None

III. Reagent

Thiocyanate reagent – Dissolve 10 g KSCN in 100 ml of water. Mix with equal volume 2N HCL just prior to use.

Hydrogen peroxide, 3%

IV. Procedure

Determination of ferric iron

- 1) Slick untreated and enriched flour side by side in usual manner (See Method 14-10 below).
- 2) Drop approximately 1 ml thiocyanate reagent at junction of two flours, in amount sufficient to wet area approximately 1 inch in diameter.

3) Let stand at least 10 minutes. If added ferric compounds are present, deeper red color will be formed than in untreated flour. Small local areas of intense red show up after 20 minutes, indicating location of individual particles of iron compound. (This affords some estimation of uniformity of mixing.

V. Determination of ferrous iron

Follow steps 1 and 2 above.

- 1) Drop approximately 1 ml of 3% hydrogen peroxide over same area wet by thiocyanate reagent.
- 2) Repeat step 3 above. The ferrous iron will have been oxidized to ferric state by the hydrogen peroxide.

VI. Sampling frequency:

Twice per shift

Description of the method 2:

Method 14-10 – Pekar Color Test (Slick test)

Final approval April 13, 1961; Reapproval November 3, 1999

I. Objective

Flour color, like ash content, is an indication of milling efficiency. This method is a qualitative test for flour color. The color of flour is visually compared to that of a standard flour.

II. Apparatus

- 1) Rectangular glass or rigid galvanized iron plate, about 12 cm long and 8 cm wide.
- 2) Flour slick.

III. Procedure

- 1) Place approximately 10-15 g flour on glass or iron plate. Pack one side in straight line by means of flour slick. Treat same quantity of standard flour used by comparison in same manner, so that straight edges of two flours are adjacent.
- 2) Carefully move one of the portions so that it will be on contact with the other and “slick” both with one stroke of flour slick in such a manner that thickness of layer diminishes from about 0.5 cm in middle of plate to thin film at edge. Line of demarcation between two flours should be distinct. Note difference in color.
- 3) Two emphasize differences in color, cut off edges of layer with flour slick to form rectangle and carefully immerse plate with flour in cold water for 1 minute.
- 4) Dry at 100°C and note color difference when still moist and when completely dried.

Note

Since original moisture content of flour has a marked influence on the results of the Pekar test, standard flour and flour tested should be of approximately the same moisture content.

IV. Sampling frequency: Twice per shift

II. Semi-Quantitative Methods

A. Spot test method

This method is an adaptation of the AACC's qualitative spot test method. It was developed and is routinely used at INCAP for semi-quantitative determinations of iron in flour.

Principle

Ferric or ferrous iron added to flour reacts with a thiocyanate (KSCN) reagent, in the presence of hydrogen peroxide, to form a red-colored complex. The number of spots reflects roughly the amount and homogeneity of the iron in the sample.

Advantages

- 1) It is a simple, fast, and easy technique requiring no sample pretreatment.
- 2) It is inexpensive.
- 3) Personnel with minimal training can conduct this assay.

Limitations

- 1) It is semi-quantitative and provides only a rough estimate of the amount of iron in the sample.

Notes

- 1) Iron added to flour in either ferric or ferrous iron can be detected.
- 2) Flour samples with known concentrations of iron can be tested and the results can be used as a reference for more accurate estimations.

Description of the method 1:

Semi-quantitative spot test for iron INCAP method IV

Adaptation of the AACC 40-40 qualitative method for iron

I. Materials

Watch glass
Droppers

II. Equipment

None

III. Reagents and Solutions

Hydrochloric acid, HCl, 37% Merck 317

Hydrogen peroxide, H₂O₂, 30% Merck 7209 (it is possible to replace this reagent with commercial oxygenated water).

Potassium thiocyanate, KSCN, Merck 5124 or 5125

KSCN – 10%: Dissolve 10 g of KSCN in 100 ml distilled water.

HCl – 2M: To a 500 ml beaker add 100 ml distilled water, then, 17 ml concentrated HCl, and finally 83 ml distilled water.

H₂O₂ – 3%: Add 9 ml concentrated H₂O₂ (30%) to 81 ml distilled water.

Reagent 1

Immediately before using, mix equal amounts of 10% KSCN and 2M HCl. Mark the levels of 20 and 40 ml on a flask using a pipette. Add 2M HCl up to the first mark and then add 10% KSCN up to the second mark. This is reagent 1. Use within 1 day. Discard the remainder.

Reagent 2

3% H₂O₂. Discard remaining solution at the end of the day.

IV. Procedure

- 1) Take a sample of 10.0 g of flour and place it on the watch glass. With the lower part of another watch glass, press on the flour sample so that it forms a flat surface.
- 2) Add 5 drops of reagent 1 with the dropper so that it covers an area of 4x4 cm (1.5x1.5 inches). Let stand 15 to 30 seconds
- 3) Add 5 drops of reagent 2 on the surface covered by reagent 1. Let stand 1 to 2 minutes.

V. Interpretation

The appearance of red-colored spots indicates the presence of iron. The number of spots is a rough estimate of the amount and homogeneity of iron in the sample. If a more accurate estimation is required, test with known concentrations of iron (30, 60 and 90 ppm) and compare the results with those of the test samples.

B. Colorimetric method

This method was developed by INCAP and is routinely used in its laboratory for the semi-quantitative determination of iron in flour (See Appendix 3.5).

Principle

After total combustion of the organic material, by ashing the sample, iron is dissolved in a mildly acidic solution and reduced through the addition of hydroxylamine hydrochloride. Ferrous iron reacts with the chromogen, bathophenanthroline, to form a pink-colored complex. The intensity of the color provides an approximate estimation of the amount of iron in the sample.

Advantages

- 1) It is applicable to various types of food products and ingredients.
- 2) It is relatively inexpensive.
- 3) Personnel with minimum training can perform the analyses.

Limitations

- 1) It is semi-quantitative but provides a more accurate estimate than the semi-quantitative spot test.
- 2) It is a time-consuming procedure involving overnight dry ashing. Samples with a high protein content may take longer to be ashed.
- 3) It involves handling hot acid solutions; thus, appropriate safety precautions must be taken.

Notes

- 1) The test is based on visual differentiation or categorization and does not require a spectrophotometer.
- 2) Hydroxylamine hydrochloride reagent must be stored in the refrigerator.
- 3) It is possible to replace bathophenanthroline reagent with α,α -dipyridyl chromogen. However, slight differences in color become more difficult to distinguish with the dipyriddy reagent.

Description of the method 2:

Semi-quantitative colorimetric determination of iron in flour

INCAP method V

INCAP, Chemistry and Biochemistry Laboratory

Revision No.2; April, 1997

This method entails total combustion of the organic material by ashing the sample. Then the ash is dissolved in acid and the iron present is reduced through the addition of hydroxylamine hydrochloride reagent. Ferrous iron is determined through the formation of a pink color in the presence of the chromogen bathophenanthroline (4,7-diphenyl-1,10-phenanthroline-disulfonic acid).

I. Materials

Volumetric flasks – 25, 100, and 1000 ml
Beakers – 500 ml
Burette – 25 ml
Porcelain crucibles
Graduated tubes
Serological pipettes – 5 and 10 ml
Volumetric pipettes – 1, 2, and 5 ml
Test tubes – 10 ml

II. Equipment

Analytical balance (1 mg)
Hot plate
Muffle furnace

III. Reagents

Sodium acetate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$), 99%, Fe<200 mg/kg, PM 136.08, Merck Art. 6267
Hydrochloric acid (HCl), 37%, 1.19 g/ml, Fe<28 mg/ml, PM 36.46, Merck Art. 317
Bathophenanthroline, 4,7-diphenyl-1,10-phenanthroline-disulfonic acid ($\text{C}_{24}\text{H}_{16}\text{N}_2\text{O}_6\text{S}_2$), PM (free acid, anhydrate) 492.5, Sigma B-1375
Hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$), PM 69.49, Baker 2196
Iron standard 1 g, Baker DILUT-IT 4777, or any other iron standards, including ferrous ammonium sulfate
Deionized water, Fe<1mg/dl

A. Preparation of solutions

Hydrochloric acid (HCl), 6M: Add 200 ml deionized water to a 500 ml beaker. Slowly add 250 ml concentrated HCl. Let it cool and transfer to a 500 ml volumetric flask and make to volume with deionized water. Transfer to a glass flask and close with a glass stopper. This solution is stable indefinitely.

HCl, 0.96M: Add 168 ml deionized water to a 500 ml beaker. Slowly add 32 ml 6M HCl. Transfer to a glass flask and close with a glass stopper. This solution is stable indefinitely.

Hydroxylamine hydrochloride, 10%: To a 500 ml beaker add 50 g hydroxylamine hydrochloride and then 400 ml deionized water. Stir with a glass rod. After it is completely dissolved, transfer to a 500 ml volumetric flask and make to volume with deionized water. Transfer to a glass flask and close with a glass stopper. This solution is stable indefinitely.

Bathophenanthroline, 0.025% in sodium acetate, 2M (Bathophenanthroline-0.025%/CH₃COONa-2M): Add to a 500 ml beaker 108.8 g sodium acetate trihydrate and 0.10 g bathophenanthroline. Add 400 ml deionized water. Dissolve completely with a glass rod using gentle heat if necessary. Make sure that bathophenanthroline is fully dissolved because it is poorly soluble at room temperature. Store in a glass or plastic flask. Discard solution if pink color develops, because this indicates contamination with iron. The solution is stable for 3 to 4 months.

B. Preparation of iron standards

Standard, 1000 ppm: Dilute reagent DILUT-IT, according to manufacturer's instructions, in a 1 L flask using deionized water. Alternatively, dissolve 3.512 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4) \cdot 6\text{H}_2\text{O}$ in distilled water, add 2 drops of concentrated HCl, and dilute to 500 ml.

Standard, 10 ppm: Add to a 100 ml volumetric flask 1.0 ml (measured with a volumetric pipette) of 1000 ppm iron standard. Then add 16 ml of 6N HCl and adjust to volume with deionized water.

Standards for visual comparison: Use 25 ml volumetric flasks. To make concentrations of 0.0, 0.8, 1.6, 2.4, and 3.2 ppm, which equivalent to 0, 20, 40, 60, and 80 ppm (mg/kg) of iron in flour, add 2 ml 6N HCl to each flask, then add 2.5 ml 10% hydroxylamine solution, and a corresponding quantity of the 10 ppm iron standard (see table below). Adjust to volume with deionized water. Cover with glass stoppers. These standards are stable for 2 to 4 weeks.

Equivalent concentration in mg iron/kg flour	Real concentration in mg iron/l (ppm)	Volume of 10 ppm iron standard to be added (ml)
0	0.0	0.0
20	0.8	2.0
40	1.6	4.0
60	2.4	6.0
80	3.2	8.0

IV. Procedure

A. Ashing the sample

- 1) Accurately weigh 2 g of previously homogenized sample. Weigh samples in duplicates. Transfer sample to a porcelain crucible.
- 2) Ash sample in a muffle furnace at 500°C for 4 hours. The sample is adequately ashed when a white or grey ash is obtained. Cool to room temperature.

B. Solubilizing the ash.

- 1) Add 5 ml of 6M HCl to the porcelain crucible allowing the acid to wash the walls of the crucible and evaporate until dry on the hot plate, taking care that the sample does not splash outside the crucible at any time.
- 2) Dissolve the residue in exactly 5 ml of 6M HCl and leave for 5 minutes on the hot plate.
- 3) Filter into a 50 ml flask using a Pasteur pipette. Wash the crucible using several portions of distilled water and quantitatively transfer the contents to the flask.
- 4) Add 5 ml of 10% hydroxylamine solution to the flask and mix by gently rotating the flask. Adjust to volume with deionized water.

C. Iron determination

- 1) Label five 10 ml test tubes for 0, 20, 40, 60, and 80 ppm of iron standards and two test tubes for ashed flour samples.
- 2) Add 2 ml each of iron standard and ashed flour sample solutions using a volumetric pipette.
- 3) Add 6 ml of bathophenanthroline-0.025%/sodium acetate-2M solution using a burette. Leave for 20 minutes.
- 4) Compare the color of the sample to that of the standards.

V. Interpretation

Report results in intervals of 0 to 20, 20 to 40, 40 to 60, or 60 to 80 ppm. If the intensity of the color exceeds that of the 80 ppm standard, dilute 5 ml of the sample solution with 5 ml of

0.96M HCl and mix. Repeat analysis with the diluted sample. If the color of the diluted sample falls in the 20 to 40 ppm interval, the actual concentration is 40 to 60 ppm of iron.

VI. Notes

The bathophenanthroline reagent can be replaced with α,α -dipyridyl (2,2'-bipyridine) [C₁₀H₈N₂, PM 156.19, Fisher D-95] reagent. In this case, add 4 ml of the chromogen solution instead of 6 ml. the disadvantage to using the dipyridyl reagent is that slight differences in color are more difficult to distinguish.

VII. Sampling frequency: once a day

III. QUANTITATIVE METHODS

A. Spectrophotometric method

This method is approved by AACC for quantitative determinations of iron in cereals and cereal-based food products (See Appendix 3.6).

Principle

Organic constituents in a food sample are broken down by dry or wet ashing at a high temperature and the inorganic constituents are dissolved in a mildly acidic solution. Solubilized ferrous iron is then reacted with a chromogenic agent, orthophenanthroline, in the presence of a reducing agent (such as a hydroxylamine hydrochloride), resulting in a pink-colored complex. The concentration of iron is determined by its Spectrophotometric absorbance at 510 nm.

Advantages

- 1) It is applicable to various types of food products and ingredients.
- 2) It is a sensitive technique with a detection limit of less than 1 ppm (1 mcg/g or 1 mg/kg) of iron in sample.
- 3) It is relatively inexpensive compared with atomic absorption spectroscopy (AAS) or emission spectroscopy methods.
- 4) The ashed solution can be used for the determination of other inorganic elements.

Limitations

- 1) It requires personnel trained to handle corrosive chemicals and to operate the spectrophotometer or colorimeter.
- 2) It is a time-consuming procedure involving overnight dry ashing. Samples with high protein content may take longer to be ashed.
- 3) It is relatively expensive involving costs of reagents, muffle furnace (approximately US\$1.500), and spectrophotometer (between US\$8.500 and 12.000; Appendix 3.1). A fume hood (between US\$3.500 and 13.000) is needed for wet ashing of samples.

Notes

- 1) It requires the preparation of an iron standard curve.
- 2) The reagents must be stored in the refrigerator.
- 3) Chromogens other than orthophenanthroline, such as α,α -dipyridyl, bathophenanthroline, and ferrozine, also react with ferrous iron and are widely used for iron determinations in various laboratories (See INCAP method VI, Appendix 3.7).
- 4) Although dry ashing is recommended, wet ashing may also be used for Spectrophotometric iron determination. Wet ashing procedures are described in Appendices 3.9 (sample preparation) and 3.10.

Description of the method 1:

Iron spectrophotometric method

AACC method 40-41B

Final approval May 5, 1960; reapproval November 3, 1999

This method determines iron content by reaction with orthophenanthroline and spectrophotometric measurement. It is applicable to cereals and cereal-based products.

I. Materials

Volumetric flask, 1 L
Volumetric flasks, 250 ml
Volumetric flasks, 100 ml
Volumetric flasks, 25 ml
Beakers, 250 250 ml
Manual volumetric pipettes, 1000 ml
Watch glasses
Pipette tips
Graduated tubes
Tips for 'blue' pipettes
Test tubes, 10 ml

II. Equipment

Muffle furnace capable of maintaining 550°C
Platinum, silica, or porcelain crucible, approximately 60 mm diameter, 35 ml capacity.
Porcelain evaporating dishes of about 25 ml capacity are satisfactory. Do not use flat-bottomed dishes of diameter greater than 60 mm.
Spectrophotometer or colorimeter
Eppendorf pipettes, 100 ml and 500 ml
Analytical balance
Vortex mixer
Hot plate

III. Reagents

- 1) Orthophenanthroline solution. Dissolve 0.1 g orthophenanthroline in about 80 ml of water at 80°C, cool, and dilute to 100 ml. store in amber bottle in refrigerator. (Stable for up to several weeks).
- 2) Iron standard solution, 10 µg Fe/ml. a) Dissolve 0.1 g analytical grade Fe wire in 20 ml HCl and 50 ml water, and dilute to 1 liter. Dilute 100 ml of this solution to 1 liter; or b) Dissolve 3.512 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ in water, add 2 drops of HCl, and dilute to 500 ml. dilute 10 ml of this solution to 1 liter.
- 3) Hydroxylamine hydrochloride solution. Dissolve 10 g $\text{NH}_2\text{OH} \cdot \text{HCl}$ in water and dilute to 100 ml. store in amber bottle in refrigerator. (This solution is stable for several weeks).
- 4) Acetate buffer solution. Dissolve 8.3 g anhydrous sodium acetate (previously dried at 100°C) in water, add 12 ml acetic acid, and dilute to 100 ml. (It may be necessary to redistill the acetic acid and purify sodium acetate by recrystallization from water, depending on amount of Fe present).
- 5) Prepare working standards as follows: Place aliquots of the 10 µg/ml standard solution according to table below into 100 ml volumetric flasks, add 2 ml concentrated HCl to each, and dilute to volume.

Aliquot of 10 µg/ml solution taken (ml)	Final Fe concentration (ppm)
0	0

Aliquot of 10 µg/ml solution taken (ml)	Final Fe concentration (ppm)
2	0.2
5	0.5
10	1.0
15	1.5
20	2.0
25	2.5
30	3.0
35	3.5
40	4.0
45	4.5

Mix thoroughly by inverting flask 10-20 times. Using 10 ml of each of these standard solutions, continue under procedure beginning with step 8.

- 6) Ashing aid
 - a. Magnesium nitrate solution. Dissolve 50 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in water and dilute to 100 ml
 - or*
 - b. Redistilled HNO_3

IV. Procedure

- 1) Accurately weigh 2-10 g of sample (depending on concentration of iron expected) into clean crucible. (Begin to prepare blank solution at this point in same manner as sample).
- 2) Char on hot plate or under infrared lamp (optional).
- 3) Ash overnight in muffle furnace at $\leq 550^\circ\text{C}$. See Notes.
- 4) Remove crucible from furnace and cool to room temperature.
- 5) Carefully add 5 ml of concentrated HCl, letting acid rinse upper portion of crucible; evaporate to dryness on steam bath.
- 6) Dissolve residue by adding 2 ml concentrated HCl, accurately measured; cover with watch glass and heat 5 minutes on steam bath.
- 7) Rinse watch glass with water, filter quantitatively into 100 ml volumetric flask, dilute to volume, and mix thoroughly.
- 8) Pipet 10 ml aliquot into 25 ml volumetric flask, and add 1 ml hydroxylamine HCl solution. Mix thoroughly.
- 9) After 5 minutes, add 5 ml buffer solution and 1 ml orthophenanthroline; dilute to volume. Mix thoroughly.
- 10) Let stand 30 minutes, then measure absorbance of samples, standards, and blank solutions in spectrophotometer at 510 nm.

Note:

- a. If color intensity too great, make appropriate dilution of ash solution and continue beginning at step 8.
- b. Color produced is permanent for several hours. Keep out of direct sunlight.

V. Interpretation / Calculations

- 1) Plot absorbance versus concentration (in ppm) for standard solutions.
- 2) Obtain concentration of sample solutions from standard curve, subtracting blank value from each.
- 3) Iron content (mg/100 g) = $(C \times \text{DF} \times 10) \div W$
 where, C = concentration of sample solution (in ppm), DF = dilution factor (if any) from step 10, Note a, W = sample weight in grams.

Notes

To diminish ashing time or for samples that do not burn practically carbon-free, use one of the following ashing aids: Moisten ash with a) 0.5-1.0 ml magnesium nitrate solution *or* b) redistilled HNO₃. Dry contents and carefully ignite in muffle to prevent spattering. (A white ash with no carbon results in most cases). Do not add these ashing aids to self-rising flour (products containing NaCl) in platinum dish, because of vigorous action.

VI. Sampling frequency: once a day

Description of the method 2:

Spectrophotometric analysis for quantitative determination of iron in foods INCAP method VI

INCAP, Chemistry and Biochemistry Laboratory
Revision No.3; April, 1997

To analyze inorganic iron in foods, the organic material is first combusted by ashing. The resultant ash is then solubilized and iron reduced to the ferrous form by adding hydroxylamine. The ferrous iron is determined spectrophotometrically after forming a colored complex with any of the following chromogens: α,α -dipyridyl (2,2'-bipyridine), bathophenanthroline (4,7-1,10-phenanthroline-diphenylsulfonic acid), or ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine). The absorption maxima of the colored solution is 521 nm for dipyridyl, 535 nm for bathophenanthroline, and 562 nm for ferrozine. The reaction leading to the formation of color is sensitive to pH, thus the pH of the solution is maintained through the addition of 2M sodium acetate buffer.

A. Materials

Volumetric flask, 1 L
Volumetric flasks, 250 ml
Volumetric flasks, 100 ml
Volumetric flasks, 25 ml
Beakers, 250 ml
Manual volumetric pipettes (200-1000 ml)
Porcelain crucibles
Watch glasses
Pipette tips
Graduated tubes
Tips for 'blue' pipettes
Test tubes, 10 ml

B. Equipment

Vortex mixer
Analytical balance
Spectrophotometer (521, 535, or 562 nm)
Hot plate
Muffle furnace
Eppendorf pipette (100 and 500 ml)

C. Reagents

Sodium acetate (CH₃COONa·3H₂O), 99%, Fe<200 mg/kg, PM 136.08, Merck Art. 6267
Hydrochloric acid (HCl), 37%, 1.19 g/ml, Fe<28 mg/ml, PM 36.46, Merck Art. 317
 α,α -dipyridyl (2,2'-bipyridine) (C₁₀H₈N₂), PM 156.19 Fisher D-95

Or

Bathophenanthroline, 4,7-diphenyl-1,10-phenanthroline-disulfonic acid ($C_{24}H_{16}N_2O_6S_2$), PM (free acid, anhydrate) 492.5, Sigma B-1375

Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$), PM 69.49, Baker 2196

Iron standards, choose from the following:

- Electrolytic iron, Merck 3810 or Baker 2234
- Ferrous ammonium sulfate, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, Merck 3792
- Iron standard, Merck 19781

D. Procedure

A. Preparation of solutions

Hydrochloric acid (HCl), 6M: Add 200 ml deionized water to a 500 ml beaker. Slowly add 250 ml of concentrated HCl. Let it cool and transfer to a 500 ml volumetric flask and make the to volume with deionized water. Transfer to a glass flask and seal. This solution is stable indefinitely.

HCl, 0.96M: Add 168 ml deionized water to a 500 ml beaker. Then slowly add 32 ml 6V HCl. Transfer to a glass flask and seal. This solution is stable indefinitely.

Hydroxylamine hydrochloride, 10%: Add to a 500 ml beaker 50 g hydroxylamine hydrochloride, then add 400 ml deionized water. Stir with a glass rod. After it is completely dissolved, transfer to a 500 ml volumetric flask and make to volume with deionized water. Transfer to a glass flask and seal. This solution is stable indefinitely.

Dipyridyl, 0.025% in sodium acetate, 2M: Add to a 500 ml beaker 108.8 g sodium acetate trihydrate and 0.10 g dipyridyl. Add 400 ml deionized water. Dissolve completely using gentle heat if necessary. Make sure that bathophenanthroline is fully dissolved because it is poorly soluble at room temperature. Store in a glass or clear plastic flask. Discard solution if pink color develops as this indicates to contamination with iron. The solution is stable for 3 to 4 months.

Bathophenanthroline, 0.025% in sodium acetate, 2M (Bathophenanthroline-0.025%/CH₃COONa-2M): Add to a 500 ml beaker 108.8 g sodium acetate trihydrate, and 0.10 g bathophenanthroline. Add 400 ml deionized water. Dissolve completely using gentle heat if necessary. Make sure that bathophenanthroline is fully dissolved because it is poorly soluble at room temperature. Store in a glass or clear plastic flask. Discard solution if pink color develops (indicates to contamination with iron). The solution is stable for 3 to 4 months.

B. Preparation of iron standards

Standard, 1000 ppm: Dilute reagent DILUT-IT according to manufacturer's instructions, in a 1 L flask, using deionized water. Alternatively, dissolve 3.512 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water, add 2 drops of concentrated HCl and dilute to 500 ml.

Standard, 10 ppm: Add to a 100 ml volumetric flask 1.0 ml (measured with a volumetric pipette) of 1000 ppm iron standard. Then add 16 ml of 6M HCl and make to volume with deionized water.

Preparation of standards: Prepare standards in 100 ml volumetric flask. To make concentrations of 0.0, 0.3, 0.6, 1.2, 2.4, and 4.0 ppm, which are equivalent to 0, 7.5, 15, 30, 60, and 100 ppm (mg/kg) of iron in flour, add 8 ml 6M HCl to each flask, and then add a corresponding quantity of 10 ppm iron standard (see table below). Adjust to volume with deionized water. Store in dark glass flasks and cover with glass stopper. These standards are stable for 2 to 4 weeks.

Real concentration in mg iron/L (ppm)	Volume of 10 ppm iron standard to be added (ml)
0.0	0
0.3	3

Real concentration in mg iron/L (ppm)	Volume of 10 ppm iron standard to be added (ml)
0.6	6
1.2	12
2.4	24
4.0	40

C. Ashing the sample

- 1) Accurately weigh out about 2 g of previously homogenized sample. Weigh samples in duplicates. Transfer sample to a porcelain crucible.
- 2) Ass sample in s muffle furnace at 500°C for 4 hours. The sample is adequately ashen when a white or grey ash obtained. Cool to room temperature.

D. Solubilizing the ash

- 1) Add 5 ml of 6M HCl to the porcelain crucible allowing the acid to wash walls of the crucible and evaporate until dry on the hot plate, taking care that the sample does not splash outside the crucible at any time.
- 2) Dissolve the residue in exactly 5 ml of 6M HCl and leave for 5 minutes on the hot plate.
- 3) Filter into a 50 ml flask. Wash the crucible with several portions of distilled water, and quantitatively transfer the contents of the crucible.

E. Iron Determination

- 1) Label, in duplicate, 10 ml test tubes for standards (0.0, 0.3, 0.6, 1.2, 2.4, 4.0, and up to 10 ppm), control, and samples.
- 2) Add to each corresponding tube 5 ml of standard, control, or sample.
- 3) Add 0.5 ml of 10% hydroxylamine solution. Vortex.
- 4) Add 4 ml of dipyrldyl-0.025%/sodium acetate-2M or bathophenanthroline-0.025%/sodium acetate-2M solution. Vortex and leave for 20 minutes.
- 5) Read absorbance of the solution in each tube in a spectrophotometer at 521 nm for dipyrldyl or 535 nm for bathophenanthroline. Adjust to zero using distilled water.

F. Interpretation / Calculations

- 1) Plot concentration of iron in ppm (y) versus absorbance (x).
- 2) The concentration of iron can be calculated directly using a regression equation. To report the concentration of iron in mg of iron per kg of food, multiply the results obtained in ppm (mg/l) as:

$$\text{Iron (mg/kg)} = \text{conc.of iron (mg/l)} \times [(50 \times 10^{-3} \text{ L}) / (\text{sample in kg})]$$

$$\text{Iron (mg/kg)} = \text{conc. (ppm)} / \text{weight (g)} \times 50$$

G. Notes

- 1) Make sure that all glassware are appropriate for mineral analysis. Reagents should of analytical grade with as low concentration of iron as possible.
- 2) Use only distilled and deionized water with a conductivity less than 2 mSi/cm or $10^{-6}(\text{ohm}\cdot\text{cm})^{-1}$.
- 3) When using the dipyrldyl chromogen, it is critical to maintain the pH of the solution between 5 and 6. If necessary, add sodium acetate buffer solution.

Micro assay: If manual and Eppendorf pipettes are available, as well as a spectrophotometer capable of reading small cells, this method can be performed in the “micro” version. The calculations are similar to the “macro” method.

- 1) Identify, in duplicate, 10 ml tubes for standards (0.0, 0.3, 0.6, 1.2, 2.4, 4.0, and up to 100 ppm), control, and samples.
- 2) Add to each corresponding tube 1 ml of standard, control, or sample.
- 3) Add 0.1 ml 10% hydroxylamine solution and vortex.
- 4) Add 0.75 ml of dipyritydyl-0.025%/sodium acetate-2M or bathophenanthroline-0.025%/sodium acetate-2M solution. Vortex and leave for 20 minutes.
- 5) Read the absorbance of the solution in each tube in a spectrophotometer at 521 nm for dipyritydyl or 535 nm for bathophenanthroline. Adjust to zero using distilled water.

H. Sampling frequency: Once a day

NOTES: Management of a mill should keep in mind, that if one cannot measure it, one cannot control it.

- 1) Just because you can measure, it does not mean you have to.
- 2) Make all of such records available for inspection when required by the authorities who are responsible for monitoring the fortification program and in implementing inspection or monitoring for all fortified food products.
- 3) Codex CAC GL 50 recommends that the inspector samples from the square root of the number of packages, i.e., if a warehouse has 60,000 bags, then the inspector needs to take samples from 245 bags, combine them, mix thoroughly and sub-sample.
- 4) Taking a package from the packing line is not sampling.
- 5) Mill will have kept a small sample from each hours production and combined them – inspector has the mandate to take a sample from there.

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