



MANUAL FOR WHEAT FLOUR FORTIFICATION WITH IRON

ANALYTICAL METHODS FOR MONITORING
WHEAT FLOUR FORTIFICATION WITH IRON

MANUAL FOR

WHEAT FLOUR FORTIFICATION WITH IRON

Part 3

**Analytical methods for monitoring wheat flour
fortification with iron**

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Foreword

Anemia is the most widespread micronutrient deficiency in the world; it affects all age groups in both developed and developing countries. The lower levels of anemia found in developed countries are attributed to higher levels of heme-iron intake and the fortification of staple cereals and other foods such as breakfast cereals. In developing countries, the regular consumption of high phytate meals precipitates anemia because much of the iron ingested is unavailable for absorption. Other micronutrient deficiencies, such as inadequate intake of folic acid and vitamin A, and parasites also cause anemia. Much of the anemia found in developing countries is not due solely to iron deficiency; thus, multiple interventions are needed.

Iron deficiency anemia can be controlled through iron supplementation and food fortification. Wheat-based foods are consumed in many countries, and their consumption is increasing. Fortification of wheat flour with iron (and other micronutrients) is a practical intervention because target populations do not need to alter their eating habits and programmers do not need to adapt a new or costly distribution system; wheat fortification only requires the existence of a well-established milling and marketing system that allows for the uniform addition of iron and monitoring the iron content in flour. Fortification of wheat flour with iron is safe and can be used to prevent, but not cure, iron deficiency anemia.

In this three-part manual, technical guidelines are presented to systematize and facilitate the establishment and implementation of a program for iron fortification of wheat flour. Part 1 describes why it is important to prevent and reduce both iron deficiency and iron deficiency anemia and how to go about establishing such a program. Existing strategies are discussed and the basic elements to be considered in establishing an appropriate program for iron fortification of wheat flour are described in detail. In addition, Part 1 offers an overview of the entire program so that public and private sector officials who manage and coordinate flour milling activities have information on the essential components to ensure adequate operation. Technical areas described in this document will also be helpful to specialists for specific components of the fortification process. These include the operations involved in wheat flour fortification, determinants of both the efficiency and efficacy of intervention, and guidelines for determining program costs.

Part 2, Technical and Operational Guidelines, is written specifically for technical personnel responsible for implementing wheat flour fortification with iron. It covers the different forms of iron compounds, composition and preparation of premixes, procedures for adding iron to wheat flour, and a description of quality control procedures.

Part 3, Analytical Methods for Monitoring Wheat Flour Fortification with Iron, presents laboratory methods to determine the content of iron in the premix and in fortified wheat flour. Part 3 is written primarily for laboratory personnel who will be responsible for laboratory analyses.

Each part of the manual is relatively self-sufficient in the essential areas of program design and implementation. Ideally, however, it is recommended that the three parts be considered as theoretical and practical units to be used together.

A sustainable program for iron fortification of wheat flour reflects collaborative efforts of millers, producers, the public sector, researchers, and donors. The purpose of this document is to share the experiences of those involved in wheat flour fortification so that other countries can plan and implement this important intervention to eliminate and prevent iron deficiency and iron deficiency anemia.

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Abbreviations

AACC	American Association of Cereal Chemists
AAS	atomic absorption spectrophotometer
AOAC	Association of Official Analytical Chemists
INCAP	Institute of Nutrition of Central America and Panama
NIST	National Institute of Standards and Technology
ppm	parts per million; a unit of measurement; it is equivalent to micrograms per gram or milligrams per kilogram.
ppb	parts per billion; a unit of measurement; it is equivalent to 10^{-3} micrograms per gram.

Glossary

Ashing: the process of disintegrating organic constituents of food to produce oxides of inorganic elements (minerals). Dry ashing at high temperatures in a muffle furnace or wet ashing with concentrated acids and oxidizing agents are commonly used in mineral analyses.

Chromogenic reagent: a reagent that specifically reacts with the compound of interest and produces a color. For example, bathophenanthroline (for ferrous iron).

Qualitative method: provides information on the presence or absence of a certain compound without reference to the amount of the compound in foods. For example, the qualitative spot test for iron in flour.

Quantitative method: determines the exact amount of a particular compound in foods. For example, the spectrophotometric method for iron in flour.

Semi-quantitative method: provides an approximate estimate within a predefined range (for iron, 10 to 20 $\mu\text{g/g}$) of the amount of a certain compound in foods. For example, the semi-quantitative spot test for iron in flour.

I. Introduction

Analyzing the iron content of flour determines whether the required level of iron is present in fortified flour, which is critical to the success of a flour fortification program. Several analytical methods are available for determining the level of iron in flour ranging from simple rapid tests to ones that require sophisticated instruments. The official methods approved by reputed analytical associations, such as the Association of Official Analytical Chemists (AOAC) and the American Association of Cereal Chemists (AACC), are generally followed by the flour industry in developed countries, and in an increasing number of developing countries, in order to meet regulatory and purchasing quality standards.

This part of the manual provides a comprehensive collection of the analytical techniques available for determining iron in flour products, including the official methods approved by the AOAC¹ and AACC² and simple assays developed by established research institutions such as INCAP³. The methods are applicable to all iron fortificants and can be used by flour mills, government regulatory agencies, premix suppliers, or other laboratories involved in food iron analysis. In the following sections, the principle, advantages, and limitations of qualitative, semi-quantitative, and quantitative methods are briefly discussed. This information can help to identify and select the most appropriate assay method for the resources available. This next portion is followed by various appendices, which include a list that summarizes the main analytical instruments and equipment, their costs, and supplies (Appendix 3.1) and a list of the major commercial suppliers for laboratory equipment and chemicals (Appendix 3.2). Appendices 3.3 to 3.10 contain the different iron assay procedures, listed below along with their applications:

- **Qualitative:**
 - Spot test method (AACC 40-40, Appendix 3.3) — Fortified flours
- **Semi-quantitative:**
 - Spot test method (INCAP method IV, Appendix 3.4) — Flour and premixes
 - Colorimetric method (INCAP method V, Appendix 3.5) — Flour, premixes, and other products
- **Quantitative:**
 - Spectrophotometric method (AACC 40-41B, Appendix 3.6; INCAP method VI, Appendix 3.7) — Cereals, cereal products, premixes, various other food products and ingredients
 - Atomic absorption method (AACC 40-70, Appendix 3.8; AOAC 975.03, Appendix 3.9; Wet ashing, Appendix 3.10) — Cereals, cereal products, premixes, and other plant foods

All methods have been cited and printed with permission from the respective organizations.

A major concern when conducting iron assays in a laboratory is contamination of non-fortificant iron from rusted metal fixtures, equipment, and furniture. All analyses must, therefore, be conducted with utmost care to avoid contamination. This means that all glassware must be soaked in dilute nitric or hydrochloric acid, thoroughly rinsed with deionized distilled water, and dried in an oven. Moreover, all reagents must be of analytical grade suited for mineral analyses.

¹ AOAC Official Methods of Analysis (2000). 17th edition. Association of Official Analytical Chemists International, Arlington, Virginia, USA.

² Approved methods of the American Association of Cereal Chemists (2000). 10th edition. American Association of Cereal Chemists, Inc., Minnesota, USA.

³ Methods for iron determination were obtained through personal communication with Dr. Omar Dary at the Institute of Nutrition of Central America and Panama, Guatemala City, Guatemala.

Care should always be taken when handling acids because they are corrosive and cause burns. Technicians must wear protective clothing, including gloves, and work under a hood when handling concentrated acids and other corrosive agents. (See Appendix 3.11 for laboratory safety.)

II. 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 a 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 pretreatment.
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

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

Notes

1. 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.

III. 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. (See Appendix 3.4.)

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.

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 the α,α -dipyridyl chromogen. However, slight differences in color become more difficult to distinguish with the dipyriddy reagent.

IV. 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 reagent, orthophenanthroline, in the presence of a reducing agent (such as 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 µg/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 ortho-phenanthroline, such as α,α -dipyridyl, bathophenanthroline, and ferrozine, also react with ferrous iron and are widely used for iron determinations in various food 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.

B. Atomic absorption spectrophotometric (AAS) method

This method is approved by AACC and AOAC for iron determinations in various products, including cereals, cereal products, milk, and milk products. (See Appendices 3.8 and 3.9.)

Principle

Inorganic elements, including iron, are solubilized after dry or wet ashing the food sample. The iron-containing solution is then atomized in an air-acetylene flame, and the absorption is measured at a specific wavelength (248 nm) to determine the iron concentration.

Advantages

1. It is applicable to various types of food products and ingredients.

2. It is a sensitive technique with a detection limit of 10 to 20 ppb and a linear range between 1 and 20 ppm of iron.
3. It is highly reliable, accurate, and precise.
4. Once the sample is ashed, the solution can be used for determining other elements.
5. Although the AAS instrument is expensive, it has a unique advantage, i.e. the ability for simultaneous determinations of calcium, iron, copper, magnesium, zinc, and manganese.
6. It is ideal for analyzing a large number of samples.

Limitations

1. It requires skilled personnel who are trained to handle corrosive chemicals. Also, personnel must be well trained in the use of the AAS instrument to optimize the instrument settings and air-acetylene flow parameters.
2. The AAS method is a time-consuming procedure involving overnight dry ashing. Samples with high protein content may take longer to be ashed.
3. The AAS instrument has high capital and maintenance costs. (See Appendix 3.1.)

Notes

1. Careful calibration of the AAS instrument with an iron standard solution is necessary. An internal standard may also be needed to minimize matrix interference.
2. Both dry and wet ashing procedures are approved by AOAC for the preparation of plant-based food samples for iron determination by AAS (AOAC 975.03, Appendix 3.9).
3. Wet ashing can also be performed using sulfuric and nitric acids. (See University of Chile method, Appendix 3.10.)

Appendix 3.1

Cost of selected analytical instruments/ equipment from different suppliers

Equipment	Product name	Cost (US\$)	Supplier
AAS	AAS-Analyst 100	16,500 - 20,000	Perkin-Elmer
	Flame AAS	17,000	Thermo Jarrell-Ash
Ashing/fume Hood	SafeAire® Basic	3,400 – 7,600	Fisher Scientific
	Labconco® Acid digestion	13,120	Fisher Scientific
	Labconco® Acid digestion	12,495 – 16,625	VWR
Muffle Furnace	Isotemp® Basic	1,600 – 2,230	Fisher Scientific
	Thermolyne Benchtop	1,150 – 3,200	Fisher Scientific
	Thermolyne Heavy-duty	1,670 – 3,170	VWR
Spectrophotometer	Lambda UV/Vis Series	8,510 - 15,750	Perkin-Elmer
	DU600 Series	10,100 - 11,700	Beckman Instruments

Note: Equipment may be available from local suppliers at a lower cost. Readers are advised to explore such possibilities.

Appendix 3.2

List of suppliers for laboratory glassware, equipment, and chemicals

Aldrich P.O. Box 355 Milwaukee, WI 53201-9358	Tel: (414) 273 3852 Fax: (414) 273 4979	Chemicals, glassware, lab equipment
Baxter Diagnostics Inc. Scientific Products Division 1430 Waukegan Road McGaw Park, IL 60085	Tel: (847) 229 0180 Tel: (800) 873 8971 Fax: (847) 229 0668	Glassware, lab equipment, & supplies
Beckman Instruments, Inc. 2500 Harbor Blvd. Fullerton, CA 92634	Tel: (714) 871 4848 Tel: (800) 854 8067 Fax: (714) 521 3700	Spectrophotometer
Fisher Scientific 711 Forbes Ave. Pittsburgh, PA 15219-4785	Tel: (412) 490 8300 Fax: (800) 926 1166 Fax: (201) 379 7638	Glassware, lab equipment, & supplies
NIST^a Bldg. 202, Room 204 Gaithersburg, MD 20899	Tel: (301) 975 6776 Fax: (301) 948 3730	Standards, Reference materials
Perkin Elmer 761 Main Ave. Norwalk, CT 06859-0012	Tel: (203) 762 1000 Fax: (203) 762 6000	AAS instrument, Spectrophotometer
Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178-9916	Tel: (314) 771 5750 Fax: (314) 771 5757	Chemicals, test kits
Thermo Jarrell-Ash Co. 27 Forge Parkway Franklin, MA 02038	Tel: (508) 520 1880	AAS instrument
VARIAN 220 Humboldt Court Sunnyvale, CA 94089	Tel: (408) 734 5370 Fax: (408) 744 0261	Spectrophotometer
VWR International P.O. Box 1002 S. Plainfield, NJ 07080	Tel: (908) 757 4045 Fax: (908) 757 0313	Glassware, lab equipment, & supplies

^a National Institute of Standards and Technology

Revision of the original table by Dary, O., G. Arroyave, H. Flores, F.A.C.S. Campos, M.H.C.B. Lins. 1996. Sugar Fortification with Vitamin A. Part 3. Analytical methods for the control and evaluation of sugar fortification with vitamin A. USAID/INCAP.

Note: The above table only lists suppliers based in the United States. However, equipment and materials may be obtained through local suppliers in most countries.

Appendix 3.3

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 rigid galvanized iron plate, about 12 x 8 cm
Flour trier

II. Equipment

None

III. Reagent

Thiocyanate reagent - Dissolve 10 g KSCN in 100 ml 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 the two flours, in amount sufficient to wet area approximately 1 inch in diameter.
3. Let stand at least 10 min. 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 min, indicating location of individual particles of iron compound. (This affords some estimation of uniformity of mixing.)

Determination of ferrous iron

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

V. Reference

Schlesinger, H.I., and Van Valkenburgh, H.B. 1931. The structure of ferric thiocyanate and the thiocyanate test for iron. J. Am. Chem. Soc. 53:1212.

METHOD 14-10—Pekar Color Test (Slick Test)

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

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.

Apparatus

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

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 in 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 the two flours should be distinct. Note difference in color.
3. To 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° 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.

Reference

Alcock, A.W., and Ediger, N.J. 1929. Influence of flour moisture on the Pekar test. *Cereal Chem.* 6:410.

Appendix 3.4

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 100 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.

VI. Notes

None

Appendix 3.5

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 1g, 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): To a 500 ml beaker add 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 Fe(NH₄)₂(SO₄)₂·6H₂O in distilled water, add 2 drops of concentrated HCl and dilute to 500 ml.

Standard, 10 ppm: To a 100 ml volumetric flask, add 1.0 mL (measured with a volumetric pipette) of 1000 ppm iron standard. Then add 16 mL of 6M 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 are equivalent to 0, 20, 40, 60, and 80 ppm (mg/kg) of iron in flour, add 2 ml 6M 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 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 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 of the crucible.
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 solutions 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. References

AOAC Official Methods of Analysis (1984), No. 14.011. JAOAC 27: 86, 396, 1944; 28: 77, 1945.

Appendix 3.6

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 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 °
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 greater diameter 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 *o*-phenanthroline in about 80 ml water at 80°, 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 0.1g 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 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°) 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
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 $\leq 550^\circ$. See Notes.
4. Remove crucible from furnace and cool to room temperature.
5. Carefully add 5 ml 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 min 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 min, add 5 ml buffer solution and 1 ml *o*-phenanthroline; dilute to volume. Mix thoroughly.
10. Let stand 30 min, then measure absorbance of samples, standards, and blank solutions in spectrophotometer at 510 nm. Note:
 - a. If color intensity is 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 vs 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) = $(C \times DF \times 10) \div W$

where C = concentration of sample solution (in ppm), DF = dilution factor (if any) from step 10, note a, W = sample weight in grams.

VI. 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 on dish.

VII. References

1. Andrews, J.S. and Felt, C. The iron content of cereals. Cereal Chem 18:8 19.
2. AOAC International. 1998. Official Methods of Analysis of AOAC International, 16th ed., 4th rev, Method 944.02. The Association, Gaithersburg, MD.
3. Howe, M. 1944. Report of the 1943-44 methods of analysis subcommittee on the determination of iron in cereal products. Cereal Chem. 21:412.

Appendix 3.7

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-diphenyl-1,10-phenanthroline-disulfonic 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 the color is sensitive to pH, thus the pH of the solution is maintained through the addition of 2M sodium acetate buffer.

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

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

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
 α,α -dipyridyl (2,2' bipyridine) ($\text{C}_{10}\text{H}_8\text{N}_2$), PM 156.19 Fisher D-95
or
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 standards, choose from the following:

- Electrolytic iron, Merck 3810 or Baker 2234
- Ferrous ammonium sulfate, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ Merck 3792
- Iron standard, Merck 19781

IV. Procedure

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 cook and 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.

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

Hydroxylamine hydrochloride, 10%: To a 500 ml beaker add 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: To a 500 ml beaker add 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 contamination with iron. The solution is stable for 3 to 4 months.

Bathophenanthroline, 0.025% in sodium acetate, 2M (Bathophenanthroline-0.025%/CH₃COONa-2M): To a 500 ml beaker add 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 it in a glass or plastic flask. Discard solution if pink color develops (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 $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ in distilled water, add 2 drops of concentrated HCl and dilute to 500 ml.

Standard, 10 ppm: To a 100 ml volumetric flask, add 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 flasks. 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, 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 stoppers. 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
0.6	6
1.2	12
2.4	24
4.0	40

C. Ashing the sample

1. Accurately weigh out about 2 g 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.

D. 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 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. To each corresponding tube, add 5 ml of standard, control, or sample.
3. Add 0.5 mL 10% hydroxylamine solution. Vortex.
4. Add 4 mL of dipyridyl-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 dipyridyl or 535 nm for bathophenanthroline. Adjust to zero using distilled water.

V. 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:

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

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

VI. Notes

1. Make sure that all glassware are appropriate for mineral analysis. Reagents should be 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 $10^{-6}(\text{ohm.cm})^{-1}$.
3. When using the dipyridyl 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 10 ppm), control, and samples.
2. To each corresponding tube, add 1 mL of standard, control or sample.
3. Add 0.1 mL 10% hydroxylamine solution and vortex.
4. Add 0.75 mL of dipyridyl-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 dipyridyl or 535 nm for bathophenanthroline. Adjust to zero using distilled water.

VII. References

AOAC Official Methods of Analysis (1984), No. 14.011. JAOAC 27: 86, 396, 1944; 28: 77, 1945.
Measurement of iron status. 1985. Report of the International Nutritional Anemia Consultative Group (INACG).

Appendix 3.8

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 I** (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 I. Operating Parameters

Element	Wavelength (Å)	Flame ^a	Range (µg/ml)	Remarks
Ca	4227	Rich Air-C ₂ H ₂	2-20	1% La, 1% HCl
	4227	Rich N ₂ O-C ₂ H ₂	2-20	Requires special burner
Cu	3427	Air-C ₂ H ₂	2-20	
Fe	2483	Rich Air-C ₂ H ₂	2-20	
Mg	2852	Rich Air-C ₂ H ₂	0.2-2	May need La
Mn	2795	Air-C ₂ H ₂	2-20	
Zn	2138	Air-C ₂ H ₂	0.5-5	

^a C₂H₂ = acetylene

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. Calcium, 25 μg Ca/ml. Dissolve 1.249 g CaCO_3 in minimum amount 3N HCl. Dilute to 1 liter. Dilute 50 ml to 1 liter.
 - b. Copper, 1000 μg Cu/ml. Dissolve 1.000 g pure Cu metal in minimum amount HNO_3 and add 5 ml HCl. Evaporate almost to dryness and dilute to 1 liter with 0.1N HCl.
 - c. Iron, 1000 μg Fe/ml. Dissolve 1.000 g pure Fe wire in about 30 ml 6N HCl with boiling. Dilute to 1 liter.
 - d. Magnesium, 1000 μg Mg/ml. Place 1.000 g pure Mg metal in 50 ml water and slowly add 10 ml concentrated HCl. Dilute to 1 liter.
 - e. Manganese, 1000 μg Mn/ml. Dissolve 1.582 g MnO_2 in about 30 ml 6N HCl. Boil to remove Cl and dilute to 1 liter.
 - f. 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 La_2O_3 (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

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 HNO_3 , 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, take this into account 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 a re-ash blank, using the same amount of concentration HCl or HNO_3 as for the samples.
4. Phosphorus interferes in calcium and may interfere in magnesium determination with air- C_2H_2 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 $\text{N}_2\text{O-C}_2\text{H}_2$ 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

1. AOAC International. 1995. Official Methods of Analysis of AOAC International. 16th ed. Method 965.09. The Association, Arlington, VA.
2. Gatehouse, B.M., and Willis, J.B. 1961. Performance of a simple atomic absorption spectrophotometer. *Spectrochim. Acta* 17:710.
3. Zook, E.G., Greene, F.E., and Morris, E.R. 1970. Nutrient composition of selected wheats and wheat products. VI. Distribution of manganese, copper, nickel, zinc, magnesium, lead, tin, cadmium, chromium, and selenium as determined by atomic absorption spectroscopy and colorimetry *Cereal Chem.* 47:720.

Appendix 3.9

AOAC official method 975.03

Metals in plants and pet foods

Atomic absorption spectrophotometric method

First Action 1975; Final Action 1988
(Applicable to calcium, copper, iron, magnesium, manganese, potassium, and zinc)

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/Apparatus

Vortex mixer
 Analytical balance
 Hot plate
 Muffle furnace with pyrometer and capable of operating up to 525°C or a Wet ashing fume hood
 Eppendorf pipette (100 and 500 mL)
 Atomic absorption 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 general outline of operating parameters is given in Table 965.09 (below). Operators must become familiar with settings and procedures adapted to their own apparatus and use table only as guide to concentration ranges and flame conditions. (Caution: See **Appendix 3.11** for safety notes on atomic absorption spectrophotometer)

Table 965.09 Operating parameters

Element	Wavelength (Å)	Flame	Range (µg/ml)	Remarks
Ca	4227	Rich Air-C ₂ H ₂	2-20	1% La, 1% HCl
	4227	Rich N ₂ O-C ₂ H ₂	2-20	Requires special burner
Cu	3427	Air-C ₂ H ₂	2-20	
Fe	2483	Rich Air-C ₂ H ₂	2-20	
Mg	2852	Rich Air-C ₂ H ₂	0.2-2	May need La
Mn	2795	Air-C ₂ H ₂	2-20	
Zn	2138	Air-C ₂ H ₂	0.5-5	

III. Reagents

Deionized H₂O may be used.

(a) *Potassium stock solution.* - 1000 µg K/mL. Dissolve 1.9068 g dried (2 h at 105°C) KCl in H₂O and dilute to 1 L. Use following parameters for Table 965.09: 7665 Å (766.5nm), air-C₂H₂ flame, and 0.04-2 µg/mL range.

(b) *Calcium stock solutions.* - Prepare Ca stock solution and working standards as in 965.09B (below).

(c) *Cu, Fe, Mg, Mn, and Zn stock solutions.* - Prepare as in 965.09B(b), (c), and (e)-(g) (below).

(d) *Working standard solutions.* - Dilute aliquots of solutions (c) with 10% HCl to make ≥ 4 standard solutions of each element within range of determination.

965.09 B. Standard solutions

(Do not use <2 mL pipettes or <25 mL volumetric flasks. Automatic dilution apparatus may be used. Prepare standard solutions in 0-20 µg range fresh daily.)

a. Calcium solutions – 1) Stock solution – 25 µg Ca/mL. Dissolve 1.249 g CaCO₃ in minimum amount 3N HCl. Dilute to 1 L. Dilute 50 mL to 1 L. 2) Working standard solutions – 0, 5, 10, 15, and 20 µg Ca/mL containing 1% La. To 25 mL volumetric flasks add 0, 5, 10, 15, and 20 mL Ca stock solution. Add 5 mL La stock solution and dilute to 25 mL.

b. Copper stock solution – 1000 µg Cu/mL. Dissolve 1.000 g pure Cu metal in minimum amount HNO₃ and add 5 mL HCl. Evaporate almost to dryness and dilute to 1 L with 0.1N HCl.

c. Iron stock solution – 1000 µg Fe/mL. Dissolve 1.000 g pure Fe wire in ca 30 mL 6N HCl with boiling. Dilute to 1 L.

d. Lanthanum stock solution – 50 g La/L. Dissolve 58.65 g La₂O₃ in 250 mL HCl, adding acid slowly. Dilute to 1 L.

e. Magnesium stock solution – 1000 µg Mg/mL. Place 1.000 g pure Mg metal in 50 mL H₂O and slowly add 10 mL HCl. Dilute to 1 L.

f. Manganese stock solution – 1000 µg Mn/mL. Dissolve 1.582 g MnO₂ in ca 30 mL 6N HCl. Boil to remove Cl and dilute to 1 L.

g. Zinc stock solution – 1000 µg Zn/mL. Dissolve 1.000 g pure Zn metal in ca 10 mL 6N HCl. Dilute to 1 L.

h. Other standard solutions – Dilute aliquots of solutions b, c, e, f, and g with 0.5N HCl to make ≥ 4 standard solutions of each element within range of determination.

IV. Procedure

A. Preparation of Sample

1. *Dry ashing.* Accurately weigh 1 g test portion, dried and ground as in 922.02(a) (below), into glazed, high-form porcelain crucible. Ash 2 h at 500°C, and let cool. Wet ash with 10 drops H₂O, and carefully add 3-4 mL HNO₃ (1 + 1). Evaporate excess HNO₃ on hot plate set at 100-120°C. Return crucible to furnace and ash additional 1 h at 500°C. Cool crucible, dissolve ash in 10 mL HCl (1 + 1), and transfer quantitatively to 50 mL volumetric flask.

922.02(a) Preparation of Sample

(a) *For mineral constituents.* Thoroughly remove all foreign matter from material, especially adhering soil or sand, but to prevent leaching, avoid excessive washing. Air or oven-dry as rapidly as possible to prevent decomposition or weight loss by respiration, grind, and store in tightly stoppered bottles. If results are to be expressed on fresh weight basis, record sample weights before and after drying. When Cu, Mn, Zn, Fe, Al, etc. are to be determined, avoid contaminating sample by dust during drying and from grinding and sieving machinery.

2. *Wet ashing.* Accurately weigh 1 g test portion, dried and ground as in 922.02(a), into 150 mL Pyrex beaker. Add 10 mL HNO₃ and let soak thoroughly. Add 3 mL 60% HClO₄ and heat on hot plate, slowly at first, until frothing ceases. Heat until HNO₃ is almost evaporated. If charring occurs, cool, add 10 mL HNO₃, and continue heating. Heat to white fumes of HClO₄. Cool, add 10 mL HCl

(1 + 1), and transfer quantitatively to 50 mL volumetric flask. (Caution: See **Appendix 3.11** for safety notes on wet ashing.)

B. Determination

To solution in 50 mL volumetric flask, add 10 mL 5 % La solution, and dilute to volume. Let silica settle, decant supernate, and proceed as in 965.09D (below).

Make necessary dilutions with 10% HCl to obtain solutions within range of instrument.

965.09D Determination

(P interferes in Ca and may interfere in Mg determination with air-C₂H₂ burners. Eliminate interference by adding La stock solution to standard and sample solutions so that final dilutions contain 1% La. P does not interfere with Ca determination with N₂O-C₂H₂ flame is used.)

Set up instrument as in Table 965.09, or previously established optimum settings for apparatus to be used. Less sensitive secondary lines (Gatehouse and Willis, Spectrochim. Acta 17:710, 1961) may be used to reduce necessary dilution, if desired. Read ≥ 4 standard solutions within analysis range before and after each group of 6-12 samples. Flush burner with H₂O between samples, and re-establish 0 absorption point each time. Prepare calibration curve from average of each standard before and after sample group. Read concentration of samples from plot of absorption against $\mu\text{g/mL}$.

V. Interpretation/Calculations

Element, ppm ($\mu\text{g/g}$) = ($\mu\text{g/mL}$) x $F/\text{g sample}$

Element, % = ppm ($\mu\text{g/g}$) x 10^{-4}

where $F = (\text{mL original dilution} \times \text{mL final dilution}) / \text{mL aliquot if original 50 mL is diluted}$.

VI. Notes

None

VII. Reference

JAOAC 58:436 (1975).

Revised: March 1996

Appendix 3.10

Wet digestion using sulfuric and nitric acids

Used by the Institute of Nutrition and Food Technology, University of Chile

I. Materials

Volumetric flask, 1 L
Volumetric flasks, 250 mL
Volumetric flasks, 100 mL
Volumetric flasks, 25 mL
Beakers, 250 mL
Volumetric pipettes, 100 mL, 500 mL, 200-1000 mL
Graduated tubes
Test tubes, 10 mL

II. Equipment

Analytical balance
Hot-block digestion unit
Wet ashing fume hood
Kjeldahl flasks

III. Reagents

A. Iron Standard Solutions

1. Stock standard solution. Fe 1000 mg/L. Dissolve 1.0 g Fe wire metal in 10 mL (65%) HNO₃ ultrapure. Dilute to 1000 mL with deionized water.
2. Intermediate standard solution. Fe 10 mg/L. Dilute 10 mL stock standard solution to 1000 mL with deionized water.
3. Working standard solution. Fe 1 µg/mL. Dilute 10 mL intermediate standard solution to 100 mL with deionized water.

IV. Procedure

Rinse all glassware with concentrated HCl diluted 1:1 with deionized water.

1. Weigh 1 g homogenized sample (dry weight) into a 100 mL Kjeldahl flask.
2. Add 6 mL of concentrated (97%) H₂SO₄ ultrapure and then 4 mL of (65%) HNO₃ ultrapure to the sample, and predigest at room temperature for 12 hours.
3. Place the flasks in a hot-block digestion apparatus and heat at 150°C for 4 hours.
4. Remove the flask from the hot-block, and allow to cool at room temperature.
5. After adequate cooling add 10 mL of 1 N HCl ultrapure, return the flasks to digestion rack and heat at 90°C until the samples are clear.
6. Subsequently, remove the Kjeldahl flasks and transfer the sample digest to 50 mL volumetric flask (rinse the Kjeldahl flask five times with 5 mL of deionized water), and make up to volume with deionized water.

V. Interpretation

Iron is measured by atomic absorption spectrophotometric method using the following conditions: wavelength 248 nm, slit 0.2 nm, and flame air-C₂H₂ (lean-blue).

VI. Notes

None

VII. Reference

Manuel Olivares, MD, Fernando Pizarro MT, Angelica Letelier MT, Eva Hertrampf, MD, and Tomas Walter, MD. Instituto de Nutricion y Tecnologia de los Alimentos, Universidad de Chile.

Appendix 3.11

Laboratory safety

Selected notes taken from: Cole, EC. Laboratory Safety. In: Methods of Analysis for Nutrition Labeling, DM Sullivan and DE Carpenter (Eds.), AOAC International, 1993

These precautionary notes and safe handling techniques serve only as a reminder of possible hazards involved in the use of particular operations or substances. Readers must follow safety requirements of their organization and state, provincial, or federal government.

Glassware

Dispose of chipped or broken glassware in special containers; minor chips may be fire-polished and glassware retained. If glassware is to be repaired, mark defective area plainly and store in special location until repairs are completed. Use heat-resistant glassware for preparation of solutions that generate heat (e.g., not bottles or graduate flasks).

Atomic absorption spectrophotometer

Follow all manufacturer's instructions for installation, operation, safety, and maintenance. Use only hose/tubing to conduct gases approved by manufacturer and supplier. Use effective fume removal device to remove gaseous effluents from burner. Use only C_2H_2 which is dissolved in solvent recommended by manufacturer. Open C_2H_2 tank stem valve only one-quarter turn. Change tank when C_2H_2 pressure shows 75-100 lb. If instrument has a drain trap, ensure that it is filled with H_2O before igniting burner. Following repair to C_2H_2 supply line, check for gas tightness at all connections with soap solution or combustible gas detection system. Whenever solutions are aspirated which contain high concentrations of Cu, Ag, or Hg, spray chamber should be rinsed with 50-100 mL H_2O before shutting down to clean these metals from chamber. See safety notes on compressed gas cylinders.

Compressed gas cylinders

Identify by name(s) of gas(es) contents of compressed gas cylinders on attached decal, stencil, or tag, instead of by color codes. Move cylinders (with protective cap) upright secured to cart. Secure cylinders in upright position by means of strap, chain, or non-tip base. Let contents of C_2H_2 cylinders settle and let all cylinders come to room temperature prior to opening. Use only correct pressure gauges, pressure regulator, flow regulator, and hose/tubing for each size of gas cylinder and type of gas as specified by supplier. Use soap solution or combustible gas detection system to check all connections, especially which system is pressurized and gas is not flowing, to check for slow leak. Use special heater on N_2O gas line. Close gas tank valve and diaphragm on regulator (turn counter-clockwise) when gas is not in use. Service regulator at least yearly. Use toxic gases only in effective fume removal device. When burning gas, use flashback prevention device in gas line on output side of regulator to prevent flame being sucked into cylinder.

Hazardous radiations

UV radiation is encountered in AA spectrophotometry, fluorometry, UV spectrophotometry, and both long- and shortwave UV lamps. Never expose unprotected eyes to UV light from any source either direct or reflected (e.g., flames in flame photometer, lamps, electric arcs, etc.). Always wear appropriate eye protection such as goggles having uranium oxide lenses, welder's goggles, etc., when such radiations are present and unshielded. Keep skin exposure to UV radiation to minimum.

Pipettes

Do not pipet hazardous liquids by using mouth suction to fill pipette. Use pipette fillers or rubber tubing connected through trap to vacuum line for this purpose.

Wet ashing

This technique is among the most hazardous uses of acids but can be performed safely. Observe precautions for particular acids used and rigorously follow directions given in the specific method being used.

Acids

Use effective acid-resistant fume removal device whenever heating acids or performing reactions which liberate acid fumes. In diluting, always add acid to water unless otherwise directed in method. Keep acids off skin and protect eyes from spattering. If acids are spilled on skin, wash immediately with large amounts of water.

Copious fumes are evolved when concentrated nitric acid and concentrated hydrochloric acid are mixed. Avoid premixing. Use effective fume removal device when fumes are generated. Handle with disposable polyvinyl chloride, not rubber, gloves.

Hydrogen peroxide

Hydrogen peroxide of 30% strength is hazardous and can cause severe burns. Drying H_2O_2 on organic material such as paper or cloth can lead to spontaneous combustion. Copper, iron, chromium, other metals, and their salts cause rapid catalytic decomposition of H_2O_2 . Hazardous with flammable liquids, aniline, and nitrobenzene. Since it slowly decomposes with evolution of O_2 , provide stored H_2O_2 with vent caps. Wear gloves and eye protection when handling.