Laboratory methods for fortified foods

Part III

Quantitative and Spot-test Determinations of Iron in Fortified Flours

Second Edition 2017
Foreword

ECSA-HC has been working with partners in direct response to resolutions of the Conference of Health Ministers to scale up Food Fortification initiatives as a critical strategy in managing micronutrient malnutrition among populations of the member states.

Part of the outcome of the intensified collaborative initiatives, was publication of three parts of the first edition ECSA-HC manuals on laboratory methods of fortified foods namely part 1 on determination of iodine in salt, part II on determination of vitamin A in sugar and oils and Part III on determination of iron, vitamin A and riboflavin in fortified flours. The manuals have been implemented in the last 10 years.

During the food fortification workshop held in Arusha-Tanzania in September 2015, three working groups through which the ECSA-HC capacity building Initiative co-implemented by ECSA-HC and GAIN and supported by USAID were formed:

  i) Production, Food Safety and Quality Assurance/Quality Control;

  ii) Inspection and Enforcement; and

  iii) Consumption Monitoring and Program Impact. The groups were tasked with identifying capacity and resource gaps and propose ways of filling these gaps in each of the technical areas. Subsequently, they identified priority activities, targets, and developed road maps on how the activities would be
implemented to achieve the set targets. Target 2 of the Inspection and Enforcement Working Group was to review the Regulatory Monitoring Frameworks used by countries in the Region. To inform this review, a workshop was organized for this group at the Imperial Resort Beach Hotel in Entebbe, Uganda from the 7th to 10th November 2016. Its aim was to review the existing guidelines (that countries are using) for gaps and weaknesses and use recommendations from this review to develop harmonized and practical guidelines that all countries can adopt and apply in inspection of fortified foods and specialized nutritional products. A key recommendation of the Entebbe workshop was that the inspection manuals be merged and be developed into two guidelines namely internal and external monitoring of fortified foods and that of commercial and points of entry inspection guidelines. The same meeting recommended that the test methods be reviewed by the laboratory working group which was previously a sub-working group of the inspection and enforcement working group and had become the fourth working group.

ECSA-HC with technical support from GAIN and financial support of USAID hosted a regional food fortification workshop for laboratory analysts between 13 – 16th December 2016 in Nairobi, Kenya that recommended review of the manuals to update of any new updates in the reference methods, format them as guided by ISO/IEC directive 2 and presenting them as a simple test method format for use by the laboratories.
The first editions of ECSA manuals of laboratory methods are recognized as primary reference materials and have guided the development of this edition. In addition, reference has also been made to the latest editions of ISO, AOAC and Codex standards and duly recognized under the bibliography.

This part of revised test method is meant to directly contribute to the overall effort to strengthen food fortification in the region.

It is our hope that the use of this test method will help strengthen food control activities in our countries in order to deliver safe and quality fortified foods to the ECSA-HC population.

DIRECTOR GENERAL.

ECSA-HC
Acknowledgement

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ECSA-HC is sincerely grateful to all officials from various government institutions in particular the food control departments and National Standards Bodies of the following ECSA countries: Burundi, Ethiopia, Kenya, Lesotho, Malawi, Mozambique, Rwanda, Swaziland, South Africa, Tanzania, Uganda, Zambia and Zimbabwe. Your contributions and dedication towards development, review and finalization is highly acknowledged.

ECSA-HC also convey profound thanks to the authors of the first edition; Dr Omary Dary, Mr Philip Makhumula and Monica Guamuch and other regional and international food fortification experts who provided technical support to review and input to the current edition.

ECSA-HC appreciates the Dormus Food Safety System Consultants who contributed to the development of this manual.
Disclaimer

The content of these guidelines can be adapted to suit country specific contexts. In such a case, the content of the resulting document will be the sole responsibility of the organization adapting the guideline and will not represent the views of ECSA-HC. The Use of the content of these guidelines should be duly acknowledged.
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Annex A: Spot Test for determining presence of iron in fortified flours

Annex B (Informative): Glassware cleaning procedure

Bibliography
# Acronyms and Abbreviation

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>BMGF</td>
<td>Bill and Melinda Gates Foundation</td>
</tr>
<tr>
<td>ECSA – HC</td>
<td>East, Central and Southern Africa – Health Community</td>
</tr>
<tr>
<td>GAIN</td>
<td>Global Alliance for Improved Nutrition</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>ISO/IEC</td>
<td>International Organization for Standardization/International Electrotechnical Commission</td>
</tr>
<tr>
<td>USAID</td>
<td>United States Agency for International Development</td>
</tr>
<tr>
<td>SWOT</td>
<td>Strength, Weakness, Opportunities and Threats</td>
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Introduction

Analytical methods of fortified food products are critical components of successful implementation of set standards and/or regulations by both the industry as well as the food control regulatory authorities.

The success of internal, external, commercial and points of entry monitoring, inspection and audit heavily relies on the accuracy\(^1\), precision\(^2\), specificity\(^3\), sensitivity\(^4\), ruggedness\(^5\), and easy applicability of the methods to release reliable results of collected samples for action.

It is recommended that both industry and regulatory agencies use similar procedures and method in determining compliance to avoid possible disputes of the results. However, although these methods have been shown to be reliable and useful, other validated methods as listed in the annex may also be used. It is also important to note that for routine internal monitoring, industry may choose to use either qualitative or semi quantitative methods in checking for the presence and appropriate addition of micronutrients to the fortified foods but they should have access to quantitative analysis at determined intervals to confirm the performance of the fortification process.

This section of the manual is dedicated to method for determination of total iron in flours, through either atomic absorption spectrophotometry (AAS), or UV/Visible spectrophotometry. An annex describes the semi-quantitative chromogenic method (spot test) for the iron added to fortified flours.

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1 **Accuracy** Is the capacity of the analytical method to determine the amount of the analyte as close as possible to the true value.

2 **Precision** Is a general term for the variability among repeated tests under specified conditions. Two types can be identified: **repeatability**, or the variation within runs; and **reproducibility** of variations between runs.

3 **Specificity** is the ability of the method to respond exclusively to the substance that is being measured and not to any degrading impurities or other components present in the food matrix.

4 **Sensitivity** Is the degree of certainty that an analytical method can differentiate between two very similar amounts of the analyte. The smallest amount of substances in a sample that can accurately be measured by a method is called **limit of detection**.

5 **Ruggedness** is defined as the degree to which the same method produces the same results in different laboratories and used by different technicians.
DETERMINATION OF IRON IN FLOUR

1.0 Scope of the method

This part of the ECSA-HC manual of methods for the determination of micronutrients added to fortified foods provides guidance on the determination of total iron in flours using either by Atomic Absorption Spectrometry (AAS) or by UV/Visible spectrophotometry. Annex A describes the method for semi-quantitative determination of iron on fortified flour by the spot-test.

2.0 Normative references


3.0 Terms and Definitions

3.1 Accuracy

Is the capacity of the analytical method to determine the amount of the analyte as close as possible to the true value
3.2 Laboratory sample
Is a sample as prepared (from the lot) for sending to the laboratory and intended for inspection or testing

3.3 Lot
Is the quantity of product that is assumed to be of the same production process and represented by specified sampling rules

3.4 Precision Two types of precision have been found necessary for describing the variability of a test method: 1) within-run variation also known as **repeatability**, and 2) between-run variation also named as **reproducibility**.

Is a general term for the variability among repeated tests under specified conditions.

3.5 Qualitative analysis
Is a method used to determine the presence or absence of a nutrient and is ideal for screening samples to determine if the samples are fortified with targeted nutrient.

3.6 Semi-quantitative analysis
Is a method mainly used to determine the micronutrient content in the finished product during the fortification process at the factory. These methods are based on their respective qualitative methods, but are adapted to introduce comparative assessment based on intensity of color development or spot density

3.7 Quantitative analysis
Is a method which accurately determine the micronutrient content in the food
3.8 Sensitivity

Is the degree of certainty that an analytical method can differentiate between two very similar amounts of the analyte. The smallest amount of substances in a sample that can accurately be measured by a method is called limit of detection.

3.9 Test portion

The actual material weighed or measured for the analysis. Reference: AOAC
http://www.aoac.org/aoac_prod_imis/aoac/AOAC_Member/PUBSCF/GARCF/TERMS_A.aspx

3.10 Test sample

Subsample or sample prepared from the laboratory sample and from which test portions will be taken.
4.0 Principle

The determination of total iron in foods (i.e. without differentiating intrinsic iron and iron added in fortification) usually includes the total combustion of organic materials leaving only the ash, which contains the mineral part of foods. The sample is ashed at 550°C ± 10 in the muffle furnace for 5 hours. Add HNO₃ and evaporate to dryness. This process transforms all iron present to the oxidized ferric form (Fe³⁺). A solution of the ash is prepared using hydrochloric acid, and the total is determined using an iron-specific lamp on the AAS or ICP or making the iron to react with a chromogenic substance to read the colored complex in UV/Vis spectrophotometry. The color reaction has to be performed under pH-controlled conditions suitable for the chromogen.
5.0 Reagents

**WARNING** — Analysts should take into account the relevant national laws/ regulations on handling hazardous substances as appropriate, as well as ensuring that technical, organizational and personal safety measures are adhered to. Analysts must read the material Safety Data sheets (MSDS) of all the reagents and must have a procedure in their laboratory to handle the chemical waste correctly.

Unless otherwise specified, use only reagents of analytical grade. Only distilled or deionized water with less than 2 mS/cm conductivity, or $10^{-6}$ (ohm. cm)$^{-1}$ shall be used. All glass ware shall be cleaned using the procedure annexed to this method as **Annex B**

5.1 Reagents

5.1.1 Nitric acid (HNO$_3$), AR grade, 65 %, $D = 1.39$ g/mL, Fe < 1 µg/mL.

5.1.2 Hydrochloric acid (HCl), 37%, AR grade, $D = 1.19$ g/mL, Fe < 28 µg/mL, MW=36.46.

5.1.3 Standards Solution for iron, recommended: Ammoniacal Ferrous Sulfate, Fe (NH$_4$)$_3$(SO$_4$)$_2$.6H$_2$O, MW=392.14
5.1.4 Distilled or deionized water

Only for the chromogenic UV/Vis method:

5.1.4 Sodium acetate trihydrated, (CH$_3$COONa.3H$_2$O), AR grade, 99% Fe < 200μg/kg, MW= 136.08.

5.1.5 1,10-phenanthroline-monohydrate, AR grade, MW= 198.23

5.1.6 Hydroxylamine hydrochloride (NH$_2$OH.HCl), AR grade, MW= 69.49.

5.1.7 Acetic acid (CH$_3$COOH), AR grade

5.2 Solutions for the chromogenic UV/Vis method

5.2.1 Hydroxylamine Hydrochloride (8 –10 %): Add 10 g of hydroxylamine hydrochloride into a beaker, and dissolve with 100 mL of deionized water with the aid of a glass rod. Transfer the solution into a glass flask with hermetical cover. The solution is stable for indefinite time.

5.2.2 Acetate Buffer-2 M: In a 500 mL beaker add 68 g sodium acetate trihydrate, and dissolve in approximately 100 mL of deionized water. Add 60 mL of glacial acetic acid and dilute to 500 mL. Transfer the solution into a glass flask with hermetical cover. The solution is stable for indefinite time.

5.2.3 Chromogen: Phenanthroline: Dissolve 0.1 g 1, 10-phenanthroline.H$_2$O in ca 80 mL H$_2$O at 80°C, let it cool down, and dilute to 100 mL. Store in a dark bottle under refrigeration. The solution is stable for several weeks. Discard if the solution turns lightly pink, indicating that it has been contaminated with iron.
5.3 Standards solutions

5.3.1 Primary standard Solution of Iron – 1000 mg/L, dissolve 3.512 g of Fe(NH$_4$)$_2$(SO$_4$)$_2$.6H$_2$O in distilled water, and add a few drops of concentrated HCl. Dilute to 500 mL in a volumetric flask. Transfer the solution to a plastic bottle. This solution is stable for indefinite time, unless a light pink color is observed indicating contamination.

5.3.2 Secondary Standard Solution of Iron-10 mg/L, into a 500 mL volumetric flask pipette 5 mL of the Primary Standard Solution (1000 mg/L). Add 2 mL concentrated HCl. Fill with distilled water up to the 500 mL mark. Transfer the solution to a plastic bottle and store it in a cool dry place. This solution is stable for about 6 months.

5.3.3 Standard Solutions for the Calibration Curve, solutions for the calibration curve will have iron content between 0.0 and 5.0 mg/L (ppm). Into 100 mL volumetric flasks, pipet the amounts of the Secondary Standards Solution (10 mg/L) that are specified in the table 1, and then make up to volume with distilled or deionized water. Mix thoroughly by inverting the flask several times. Transfer the solutions into properly labeled plastic bottles. These standard solutions are stable for approximately six months.
<table>
<thead>
<tr>
<th>Iron (mg/L, ppm)</th>
<th>Volume of secondary solution (10 mg/L) to be added (mL)</th>
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<tbody>
<tr>
<td>0.0</td>
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<tr>
<td>0.2</td>
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<td>40.0</td>
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<td>5.0</td>
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</table>
6.0 Apparatus

6.1 AAS of wavelength set a 248.3 nm with Air – acetylene gas, UV/Vis spectrophotometer and Other equipment like ICP can be used

6.2 Beakers

6.3 Pipette fillers

6.4 Muffle Furnace

6.5 Volumetric flask 50 mL, 100 mL, 500 mL and 1000 mL

6.6 Volumetric and graduated pipettes to discharge volumes in Table 1

6.7 Erlenmeyer flasks, 250 mL

6.8 Porcelain crucibles, cups

6.9 Graduated measuring cylinders

6.10 Analytical balance

6.11 Hot plate

6.12 Hot air Oven

6.13 Desiccator
7.0 Sampling

A representative laboratory sample from the lot and whose integrity has been maintained during transportation and storage should be sent to the laboratory. At least 500 grams of flour per sample should be sent to the laboratory.

Sampling is not part of the method specified here. A recommended sampling method is given in the ECSA–HC guidelines for internal and external monitoring of fortified foods.

8.0 Preparation of the test sample

A test sample should be a representative sample of the samples received by the laboratory. For preparing it, mix well each one of the individual samples. Then, take equal portions of each one to add to a minimum of 100 grams, and mix well. This is a composite sample that should be thoroughly mixed to ensure that a good homogenization is achieved. It is from this compositied test sample where test portion should be drawn.
9.0 Procedure

9.1 Test portion

9.1.1 Clean and label the crucibles with a graphite pencil. Dry them in the oven at 110 °C and cool them in a desiccator

9.1.2 Using an analytical balance weigh directly into crucibles 4g to the nearest 1 mg (or to 3 decimals e.g. 0.001g) of the test portion in duplicate and record the weight

9.2 Dry ashing the sample

9.2.1 Transfer the crucibles containing the test portion to the muffle furnace and increase the temperature to 250 °C and heat for 1 hour. The temperature is then increased to 550 °C and ash for 5 hours.

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**Note 1:** Ashing is complete when a white or greyish ash is obtained. If this is not obtained extend the ashing time until it is achieved. If carbon is still present following the initial incineration, several drops of water, 1M magnesium nitrate or nitric acid should be added; then the sample should be re-ashed.

**Note 2:** Once the ashing is achieved, turn off the furnace and wait for 30 minutes before opening the door. Turn off furnace and wait to open it until the temperature has dropped to at least 250 °C, preferably lower. Open door carefully to avoid losing ash.
9.2.2 Let the crucibles cool down for 5 minutes and place in a desiccator for 1 hour until they attain room temperature.

9.3 Dissolving of ash

9.3.1 Add 5 mL of concentrated HNO₃ to the crucible, pouring the acid onto the inside walls of the crucible.

9.3.2 Evaporate the acid by heating the crucibles on top of a hot plate at low temperature, solution should not boil.

9.3.3 Dissolve the remaining residue by adding 2 mL of conc. HCl, and heat for few minutes, taking care that the solution does not spill out the crucible.

9.3.4 Let the crucible cool down and transfer the solution quantitatively into a 25.0 mL volumetric flask. Wash crucible with distilled/deionized water and bring to volume with distilled / deionized water
10.0 Determination of total Iron content using AAS (for other equipment like ICP-equipment operation procedure should also be followed accordingly)

10.1 Turn on Atomic Absorption Spectrometer 15-20 minutes before using it to warm up. Adjust the Atomic Absorption Spectrometer in accordance with the manufacturer's instructions. Optimize the response of the instrument for measurement with the air-acetylene flame.

10.2 Adjust the wavelength to 248.3 nm and slit of 0.2 nm.

10.3 Set the instrument to zero Absorbance using distilled/deionized water.

10.4 Read the absorbance of the 0.0-mg/L standard solution (blank) and record the absorbance.

10.5 Read the absorbance for each standard solution and each flour sample solution.

10.6 Draw (plot) a calibration curve by plotting the absorbance of standard on y – axis against the content (concentration) of Fe on x – axis (minimum r=0.999) and determine the concentration of iron in the flour sample as described in clause 12.
11.0 **Determination of total Iron content in UV/Vis spectrophotometry**

11.1 Pipet 10.0 mL of the sample solution into 25.0 mL volumetric flask, then add 1.0 mL of hydroxylamine hydrochloride solution (5.2.1), mix well and let it stands for 5 minutes. With the standards, pipet 10.0 mL of each standard solutions (prepared in 5.3 above) into 25.0 mL volumetric flasks, and proceed as the samples.

11.2 **Add 5.0 mL acetate buffer (5.2.2) and 4.0 mL of phenanthroline solution (5.2.3) to each flask. Mix well and color will start developing.**

11.3 Let stand it for 30 min and then make up to volume (25 mL) using deionized water.

11.4 Turn on the spectrophotometer 15-20 minutes before using it to warm up.

11.5 Adjust the wavelength to 510 nm. Set the mode to Absorbance.

11.6 Set the instrument to zero Absorbance using deionized water.

11.7 Read the absorbance of the 0.0 mg/L standard solution (blank) and record the absorbance.

11.8 Read the absorbance for each standard solution and each sample solution.

11.9 If color intensity of the samples is too high, make appropriate dilution of the sample solutions and record the absorbance again.

11.10 Draw (plot) a calibration curve by plotting the absorbance of standard on y – axis against the content (concentration) of Fe on x – axis and determine the concentration of iron in the flour sample as described in clause 12.
12 Calculation

12.1 Using data in 10.5 (or 11.8) for the standard solutions, calculate the regression line of absorbance \( y \) versus iron concentration \( \text{Fe} \) in mg/L \( x \), as presented in the following equation

\[
y = mx + c
\]

Where:

- \( y \) is the absorbance reading
- \( m \) is the slope of the line
- \( c \) is the \( y \) intercept of this regression plot

Therefore, \( x \) (concentration of iron) = \( \{y \text{ (absorbance reading of sample)} - c \text{ (intercept)}\} \div m \)

12.2 In order to report the result as mg/kg, the equation below applies

\[
\text{Iron (mg.kg}^{-1}) = \frac{F_{\text{e} (x)} \times V_i}{W} \times DF
\]

Where,

- \( F_{\text{e} (x)} \) is Iron concentration based on the calibration curve in mg/L
- \( V_i \) is Volume of the initial solution in mL (Clause 9.3.4, i.e. 25)
- \( W \) is Weight of the sample in grams (Clause 9.1.2)
DF is the dilution factor for those samples that required dilution for the absorbance to be within the linear range of standard curve.

If available, the uncertainty values should be included when reporting the results.
Annex A: Spot Test for determining presence of iron in fortified flours

1.0 Principle

This method only detects added iron through fortification, and which is detected by means of colored spots on the surface of a layer of the flour. Ferric iron, in an acidic medium, reacts with a solution of potassium thiocyanate (KSCN) to form an insoluble red pigment. Other types of iron, such as ferrous iron and elemental iron can also react in a similar manner once they are oxidized to the ferric form using hydrogen peroxide. Ferrous iron, mostly from ferrous sulfate, can be specifically identified by formation of an insoluble bright blue pigment called Turnbull’s blue by reaction with ferricyanide. The reaction may also occur with ferrous fumarate but it may be slow or even because the low solubility of this salt in water.

2.0 Materials

2.1 Filter paper, Whatman No.1

2.2 Manual sieve

2.3 Watch glass

3.0 Reagents

3.1 Hydrochloric acid solution, (2N HCl). To a 500 mL beaker, add 100 mL distilled water. Then pour slowly 17 mL of concentrated HCl, and finally 83 mL more of water.

3.2 Hydrochloric acid solution, (0.003N HCl). Prepare 1 L of solution by adding 600 mL distilled water to a 1 litre volumetric flask. Then pour 1.5 mL of the 2N HCl- and make up to volume with distilled water.
3.3 **Potassium Thiocyanate** (10%). Dissolve 10 g of KSCN in 100 mL water. Prior to use, mix 10 mL of this solution with 10 mL of the 2N HCl.

3.4 **Hydrogen peroxide** (H₂O₂) 3%, (required when iron is elemental iron or a ferrous salt). Add 5 mL concentrated 30% H₂O₂ to 45 mL distilled water. Prepare daily. Discard after completing the analysis.

3.5 **Potassium Ferricyanide**, 10%. Dissolve 10 g of K₃Fe(CN)₆ in 100 mL water. Prior to use, mix 10 mL of this solution with 10 mL of 0.003N HCl.
4.0 Procedure

4.2 Determining iron of any type (Thiocyanate Method)

4.2.1 Place a filter paper over a watch glass.

4.2.2 Wet the surface of the filter paper with the solution of potassium thiocyanate. Let the liquid penetrate the paper fibers.

4.2.3 Using a hand sieve, sift portion of the flour test sample in order to load a thin layer over the entire wet area. Shake off or scrape off any excess flour.

4.2.4 Add a little more of the acidic solution of potassium thiocyanate over the flour layer.

4.2.5 Add small amounts of the H$_2$O$_2$-solution. Let it stand for a few minutes for the reaction to occur.

4.2.6 Red color spots indicate the presence of iron of any type (NaFeEDTA, ferrous sulfate, ferrous fumarate, electrolytic iron, reduced iron) added by fortification.

4.3 Determining presence of ferrous sulfate (Ferricyanide Method)

4.3.1 Place the filter paper over the watch glass

4.3.2 Wet the surface of the filter paper with the solution of potassium ferricyanide-10% and 0.003N HCl. Let the liquid penetrate the paper fibers.

4.3.3 Using a hand sieve, sift portion of the flour test sample in order to load a thin layer over the entire wet area. Shake off or scrape off any excess flour.

4.3.4 Add a little more of the acidic solution of potassium ferricyanide over the flour layer.

4.3.5 Let it stand for a few minutes for the reaction to occur.
4.3.6 A fast reaction with clearly distinct blue spots (within 2 minutes of adding the ferricyanide) is indicative of the presence of ferrous sulfate. Ferrous fumarate and some types of elemental iron types may also react but reaction is slow (6-7 minutes or longer).

5.0 Interpretation

Number and intensity of spots as described in the results is indicative of the iron content in the test sample. Controls of known amounts of the same type of iron may be used to semi quantify the amount of iron in the flour.
Annex B (Informative): Glassware cleaning procedure

a. Remove all labels and any writing on the glasses with ethanol.
b. Wash the glassware using the normal procedure for laboratories, (washing with glassware detergent, then clean with normal water and finally rinse with distilled water).
c. After that, rinse glassware 5 times with distilled/deionized water.
d. Submerge the glassware into a container/bath filled with diluted nitric acid (20% HNO₃, 80% H₂O distilled /deionized water).
e. It has to be totally submerged without air bubbles. Soak the glassware overnight.
f. Put a label outside the bath indicating date, time and quantity of glassware.
g. Take the glassware out carefully using gloves, and leaving the acid inside the container.
h. Rinse the glassware 5 times with distilled/deionized water.
i. Let the glassware dry away from dust and other contaminants and keep well covered (with saran wrap).
j. Keep the glassware in new plastic bags, hermetically closed if possible.
Bibliography


AOAC Methods. 999.11 Iron in foods .

AOAC Methods. 985.35 minerals in infant formula


ISO 6498 Animal feeding stuffs — Guidelines for sample preparation
