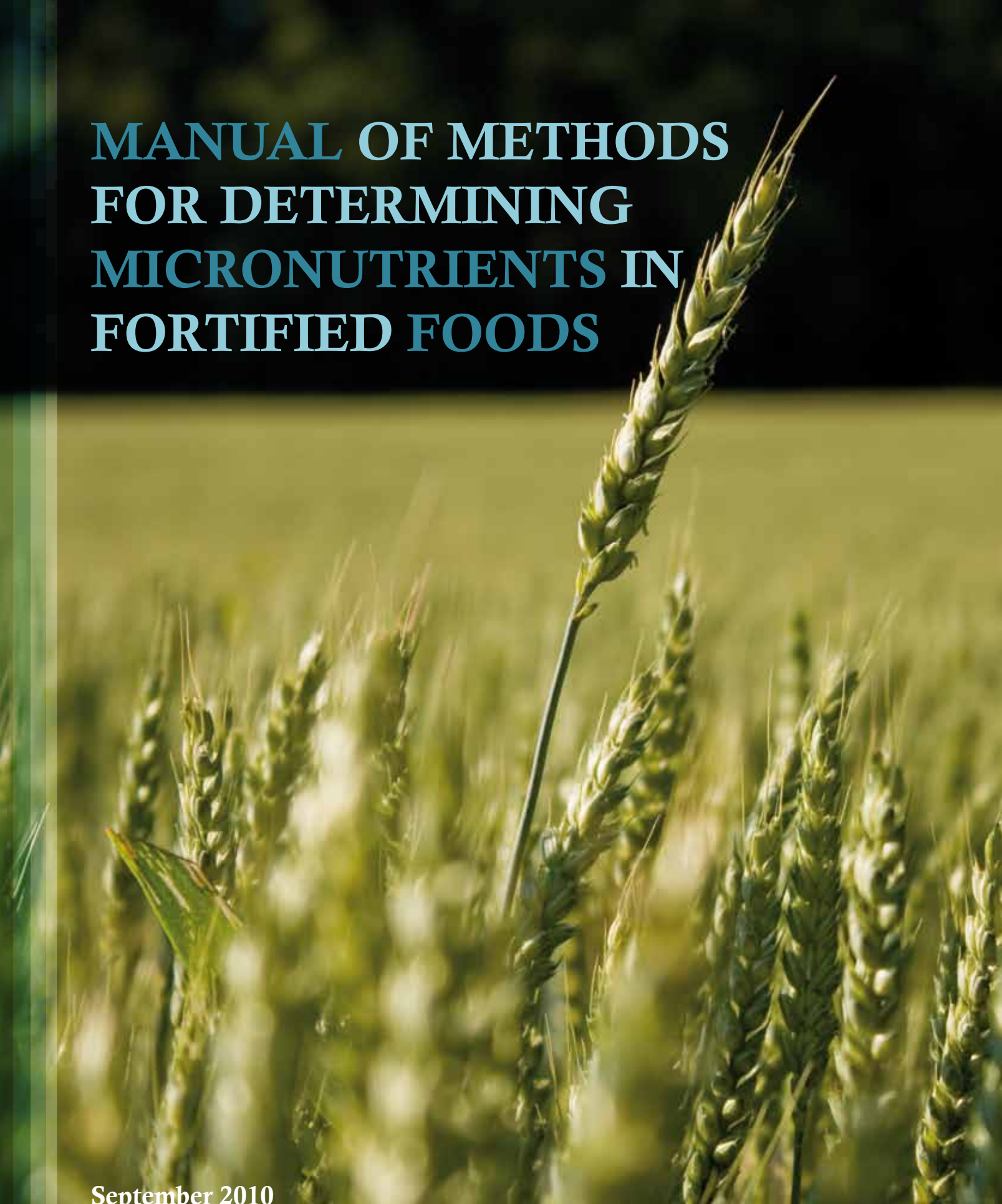
MANUAL OF METHODS FOR DETERMINING MICRONUTRIENTS IN FORTIFIED FOODS



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Copies of the manual can be obtained from the Ministry of Health- Central Public Health Laboratory.

Foreword

A2Z is proud to announce the completion of the "Manual of Methods for Determining Micronutrients in Fortified Foods" mainly wheat flour, which can also be applied to bread and other fortified foods such as cereal-based products, milk and edible oil. This manual will be used by the Palestinian Ministry of Health- Central Public Health Laboratory to strengthen the supervisory monitoring system of the national fortification program.

With sincere thanks and appreciation of the project to the A2Z food fortification consultant Mrs. Monica Guamuch for her very active participation and effort in developing and formulating the manual in the West Bank in coordination with the Palestinian Ministry of Health.

The project would also like to pass special thanks for the high level of support of Dr. Asad Ramlawi, the Director General of the Primary Health Care Department and Mr. Ibrahim Salem, the Director of the Central Public Health Lab.

A2Z acknowledges also the efforts of the Central Public Health Laboratory staff for their high level of dedication, commitment and contribution to the development of the Manual, namely:
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Table of contents

I. Introduction

Food fortification is one of the nutritional interventions used to improve the dietetic intake of micronutrients by the population. Typical foods fortified around the world are cereal flours, mainly wheat and maize flours, pasta and noodles, milk, oil and margarines, among others.

Wheat flour fortification is carried out in many countries around the world to provide vitamins and minerals through bread, pasta and other baking goods. The Palestinian Wheat Flour Fortification Standard issued in 2005 establishes that wheat flour must be fortified with iron, zinc, vitamin A, vitamin D, thiamin (B_1), riboflavin (B_2), niacin (B_3), pyridoxine (B_6), folic acid (B_9) and vitamin B_{12}. The micronutrient formulation for wheat flour in other countries usually includes only iron, thiamin, riboflavin, niacin and folic acid.

Food industry plays an essential role in food fortification, since the food plants and, in this case, wheat mills are responsible to add the minerals and vitamins premix to the flour in the adequate amounts to comply with the requirements indicated in the Standard. On the other hand, the Ministry of Health verifies that the Standard requirements are being complied, through inspection and sampling. Samples are analyzed in the Central Public Health Laboratory (CPHL) and reports indicate whether the samples complied with the Standard.

Vitamins analysis is expensive, because the procedures are long, require sophisticated equipment, reagents and materials, and skilled and trained personnel. Iron analysis is cheaper compared to vitamin analysis and this micronutrient is usually used as "Indicator" of compliance for fortification taking into account that the same premix contains all the vitamins and minerals. However, analyzing vitamins is important to confirm compliance with the standard and not only with iron. This is especially important for flour imported from Israel and other countries, where wheat flour fortification is not mandatory or the micronutrient formulation does not include all the micronutrients specified in the Palestinian Standard. Furthermore, it is important to test the flour and not only the micronutrient premix with the purpose of verifying that indeed the right premix is being used for the fortification of the flour.

This Manual presents the methods applied in the Central Public Health Laboratory for the analysis of wheat flour and fortified foods. Although vitamin analysis is not applied routinely due to the cost, it may be applied periodically to random samples. Moreover, these methods are important for completing and updating food composition tables based on unique Palestinian dishes.

II. Methods of analysis for iron

Iron is the fourth most abundant element on earth, but iron deficiency in humans is one of the most widespread nutritional problems in the world, because the human intestine has reduced absorption to most iron compounds. In solid form, metal exists as a malleable metal, which readily oxidizes in moist air.

General Properties

- Symbol — Fe
- Mol wt. — 55.845 g/mol
- Oxidation states — -2 to +6, but +2 and +3 are the most common states.
  - Ferrous ion — Fe^{+2}
  - Ferric ion — Fe^{+3}
- Soluble in mineral acids — Hydrochloric acid, sulfuric acid and nitric acid.
- Ferrous salts oxidize to the ferric form in the presence of moist air.
- Reacts with chromogenic agents to form complexed colored compounds. The color of the complex will vary depending on the oxidation state of iron. This is helpful when determining the presence of either ferrous or ferric iron.

A general scheme of the steps involved in the different techniques for measuring iron in food is presented in **Figure 1**. Sample is digested to destroy organic matter and reduce complex molecules to their elements, using either wet or dry digestion. A solution from the digested product is prepared with diluted acid and then the iron content is measured using Atomic Absorption (AA), Induced-Coupled Plasma (ICP) or visible spectrophotometry. The visible spectrophotometry technique needs reduction of ferric ion to the ferrous form in order to use chromogenic methods that have larger analytical sensitivity than those specific for ferric iron.

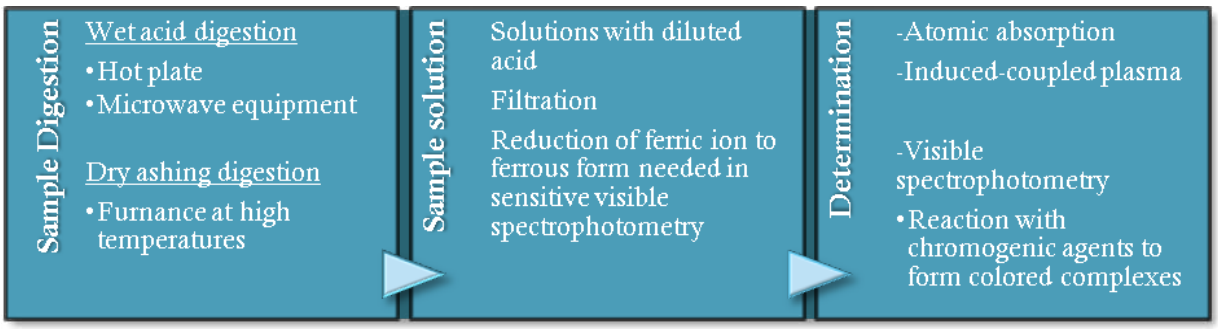


Figure 1. General scheme for determining iron in foods.

Several iron compounds are used for wheat flour fortification, which have different price, bioavailability and oxidation state. Fortification regulations specify the source of iron to be used in wheat flour. However, methods for determining total iron neither distinguish between natural iron and added iron nor the type of iron added (e.g. ferrous, ferric or electrolytic). A method to determine soluble iron from ferrous sulfate was developed at Birzeit University and it has been applied routinely in the Central Public Health Laboratory to determine whether the source of iron used is ferrous sulfate or other. This manual presents three methods for analyzing iron in wheat flour:

- Iron spot test: a qualitative method to determine the presence of iron from fortification, regardless of its type, in flour.
- Quantitative method for determining soluble iron from ferrous sulfate.
- Quantitative method for determining total iron.

A. Qualitative method to determine iron in wheat flour (Spot test for determining added iron)

I. References

- AACC Method 40-40. Iron-Qualitative Method. First approval 5-5-60; reviewed 10-27-82.

II. Principle

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.

Here, it is important to state that the presence of electrolytic or reduced iron may be determined visually when a magnet is inserted into flour slurry. After stirring the slurry for ten minutes, iron particles stick to the magnet.

III. Materials

- Filter paper Whatman # 1 or other type of filter paper
- Manual sieve
- Watch glass

IV. Reagents

- Hydrochloric acid (HCl), p.a. 37%, d= 1.19 g/mL, mol wt. 36.
- Hydrogen peroxide (H_2O_2), p.a., 30% v/v, mol wt. 34.0147 g/mol
- Potassium thiocyanate (KSCN), p.a., mol wt. 97.181 gm/mol

V. Solutions

- Hydrochloric acid solution–2N (HCl). To a 500 ml beaker, add 100 ml distilled water. Then pour slowly 17 ml of concentrated HCl[1] (37%), and finally add 83 mL more of water.

- Potassium Thiocyanate-10%. Dissolve 10 g of KSCN in 100 ml water. Prior to use, prepare a 5% solution by mixing 10 mL of this solution with 10 mL of the 2-N HCl.

- Hydrogen peroxide (H_2O_2) - 3% (required when iron is elemental iron or a ferrous salt). Add 5 ml concentrated 30% H_2O_2 to 45 mL distilled water. Prepare daily. Discard after completing the analysis.

[1] If hydrochloric acid has a concentration other than 37%, calculate the volume of HCl to get a HCl-2N solution.

VI. Procedure

1. Place the filter paper over the watch glass

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

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

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

5. Add small amounts of the H_2O_2-solution. Let it stand for a few minutes for the reaction to occur (oxidation of any form of iron to iron(III)). Red spots indicate the presence of added iron from any source.

VII. Interpretation

- Unfortified samples of wheat flour might show a reddish coloration, but not well defined red spots as shown on the picture below.

- Number and density of spots might be associated to the iron level in the sample. The more red spots appear, the higher the concentration of iron in the sample. The picture below compares the number of spots in two samples with different concentrations of iron. The first sample (left) shows only a few spots indicating the iron content is low, whereas the second sample (right) shows more spots indicating the iron content is higher.

- Although there is no rule for the size of the spots, the appearance of them might vary from small, well defined, to large spots tending to diffuse as iron solubilizes (see picture below). This might be due to the source and quality of iron used to fortify flour and although no conclusion can be drawn based on this, keep in mind that you might find different shapes when analyzing samples from different mills or countries.

B. Quantitative spectrophotometric method for determiningsoluble iron from ferrous sulfate in flours

I. References

Method for determination of iron from $FeSO_4$ was designed by Hana Ali from the Palestinian University of Birzeit, and Omar Dary from A2Z/the USAID Micronutrient and Child Blindness Project.

II. Principle

The ferrous ion (Fe^{2+}) can be determined spectrophotometrically by forming red colored complexes using several chromogens that interact with iron (Fe^{2+}) such as 1,10-phenanthroline.H_2O; bathophenanthroline, (a disulphonic salt of 4,7- diphenyl – 1,10 phenanthrolyne); α,α - dipyridile (2,2' bipyridine). The color reaction has to be performed under pH-controlled conditions suitable for the chromogen. In order to reduce the competition by hydronium ions (H_3O^+) for the ligand, a solution of 2-M sodium acetate is added.

Phenantroline also reacts with ferric iron, and it forms a light blue complex.

The determination of iron in flours fortified with ferrous sulfate neither requires digestion of the sample nor the reduction step with hydroxylamine. In this case, the iron from ferrous sulfate is extracted into a water/acetone mixture and in the presence of trichloroacetic acid. The latter reagent is needed to precipitate proteins and avoid the formation of the dough when the flour comes in contact with water.

The experience of the laboratory has showed that the recovery of the method extracting soluble iron from flour fortified with ferrous sulfate is 99%. However, the method also extracts some intrinsic iron and elemental iron from fortified flour. Nevertheless, the amount is small since the solubility of these types of iron is not as good as the one from ferrous sulfate. The laboratory has determined that the limit of quantitation for soluble iron from ferrous sulfate is 10 mg/kg. Results below this value will indicate that soluble iron extracted might come from any iron source, either intrinsic iron or an iron salt added to flour.

III. Critical points

- Clean and wash all glassware following appropriate cleaning procedures for analysis of minerals.
- All reagents must be analytical grade with the minimum possible content of iron.
- Use distilled and deionized water.
- Maintain the pH of solutions between 5-6. If necessary, more sodium acetate can be added to increase the pH.

IV. Equipment and materials

- Analytical balance
- Centrifuge
- Centrifuge tubes (50mL)
- Cuvettes (1 or 3 mL capacity, 1 cm pathlight and suitable for reading in visible light)
- Graduated cylinders
- Spectrophotometer VIS (λ=510 nm)

- Refrigerator
- Volumetric flasks (25, 100, 250 mL)
- 250 mL Erlenmeyer flasks
- Volumetric and graduate pipettes
- Parafilm
- Vortex mixer

V. Reagents

- Hydrochloric acid (HCl). p. a. 37%, d=1.19 g/mL, mol wt 36.46.
- Sodium acetate trihydrated, ($CH_3COONa.3H_2O$), p.a., 99% Fe < 200μg/kg, mol wt 136.08.
- Trichloroacetic acid (CCl_3CO_2H), 99+%, p.a., mol wt 163.39
- Acetone (CH_3COCH_3), p.a. mol wt 58.08
- 1,10-phenanthroline-monohydrate, p.a., mol wt= 198.23.
- Iron Standard Ammoniacal Ferrous Sulfate[2], Fe $(NH_4)_2(SO_4)_2 .6H_2O$, mol wt 392.14

VI. Solutions

a. Water: Acetone 80:20 Solution

Note: Prepare freshly before using it.

In 100 mL graduated cylinder, add deionized water to the 80 mL mark and then continue to the 100 mL mark using acetone. Mix well and close.

b. Chromogen B-1: 1,10-phenanthroline.H_2O

Dissolve 0.1 g 1,10-phenanthroline.H_2O in ca 80 mL H_2O at 80° C, let it cool down, and dilute to 100 mL. Store in a dark bottle in the refrigerator. The solution is stable for several weeks. Discard if the solution turns lightly pink, indicating that it has been contaminated with iron.

c. 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.

VII. Standard solutions

a. Primary Standard Solution of Iron – 1000 mg/L

Dissolve 3.512 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ 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.

b. 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

2 Ammonium ferrous sulfate is more stable to oxidation than ferric chloride, which is also used as standard, and there is no need for reduction to use it to measure ferrous iron.

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.

c. Standard Solutions for the Calibration Curve

Solutions for the calibration curve will have iron levels from 0.0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 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, and then make up to volume with deionized water.

Iron (mg/L, ppm)	Volume of the Secondary Solution (10 mg/L) to be added (mL)
0.0	0.0
0.2	2.0
0.5	5.0
1.0	10.0
1.5	15.0
2.0	20.0
3.0	30.0
4.0	40.0
5.0	50.0

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.

VIII. Procedure

1. Mix thoroughly 100 g of flour.

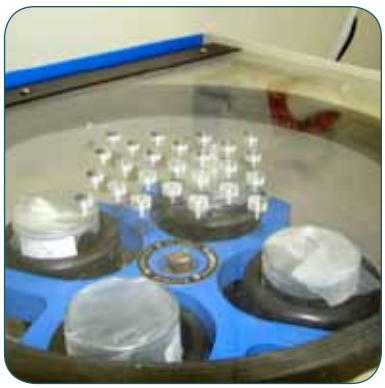
2. Weigh10g of flour with significance in milligram (0.001 g) and pour slowly into 250 mL Erlenmeyer flask, already containing 1.0 g TCA dissolved in about 100 mL of water: acetone (80:20).

3. Stir with a magnetic stirrer for 10 minutes.

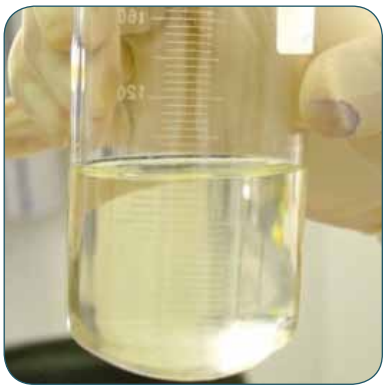
4. Seal the flask with parafilm and leave it in the refrigerator for at least 1 -1.5 hr.

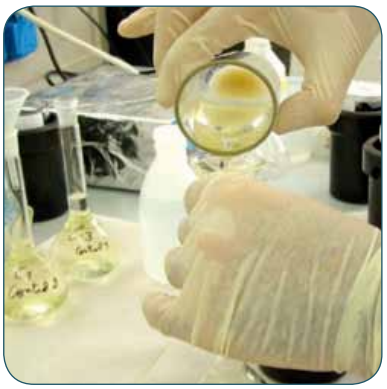
5. Decant the supernatant in equal amounts into two centrifuge tubes.

6. Centrifuge (~ 3500 rpm) for at least 15 min.



7. Transfer both supernatants to a 100 mL-volumetric flask. The supernatant must be clear. Make up to volume (100 mL) with deionized water.





8. Pipet 10.0 mL aliquots of sample solutions and standard solutions into different 25 mL volumetric flasks.

9. Add 5.0mL acetate buffer and 4.0mL of 1,10-phenanthroline to each flask. Mixwell and color will start developing.

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

11. Turn on the spectrophotometer and warm it up for 15-20 minutes prior reading the absorbance.

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

13. Set the instrument to zero absorbance using deionized water.

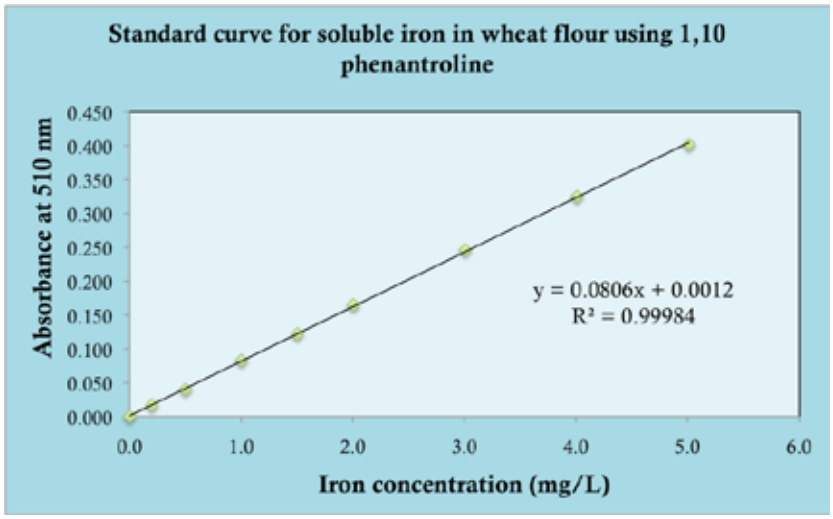
14. Read the absorbance of the 0 mg/L standard solution (blank) and record the absorbance.

15. Read the absorbance for the standard solutions and flour sample solutions. The solutions will get a red-orange color. The more intense of the color, the higher the concentration of iron in the sample.

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

IX. Calculations

1. Plot a graph of the absorbance values of the standard solutions (y-axis) against concentration (x-axis) and obtain the equation of the standard curve (a typical equation is shown below the standard curve.



2. Calculate the concentration of soluble iron in the sample solution solving the standard curve equation for x. For example:

$$x = \frac{(y - 0.0012)}{0.0806}$$

3. Calculate the concentration of soluble iron in the flour sample using the equation below.

$$Soluble\ iron\ (mg/kg) = \frac{[Fe]\ x\ 100}{w}$$

Where *w* is around 10.0 g.

C. Quantitative spectrophotometric method for determination of total iron in wheat flour

I. References

Cunnif, D (Ed). Official Methods of Analysis of AOAC International. 1997. 16a ed. AOAC International, Gaithersburg. No.944.02.

AOAC. Official Methods944.02

II. Principle

The determination of total iron in foods usually includes the total combustion of organic materials leaving only the ash, which contains the mineral part of foods. This process transforms all iron present to the oxidized ferric form (Fe^{3+}). A solution of the ash is prepared using hydrochloric acid and the iron (III) is reduced to iron(II) using hydroxylamine hydrochloride. The ferrous ion (Fe^{2+})can be determined spectrophotometrically by forming colored complexes using several chromogens that interact with iron (Fe^{2+}) such as 1,10-phenanthroline.H_2O; bathophenanthroline, (a disulphonic salt of 4,7- diphenyl – 1,10 phenanthrolyne); α,α - dipyridile (2,2' bipyridine); or ferrozyne (acid[3–(2-pyridyle)- 5,6 –bis-(4- phenylsulphonic) –1,2,4- triazine). The color reaction has to be performed under pH-controlled conditions suitable for the chromogen. In order to reduce the competition by hydronium ions (H_3O^+) for the ligand, a solution of 2 M sodium acetate is added.

III. Critical points

- Clean and wash all glassware following appropriate cleaning procedures for analysis of minerals.
- All reagents have to be analytical grade with the minimum possible content of iron. The water used has to be distilled and deionized, with less than 2μ Si/cm conductivity, or 10^{-6} $(ohm.\ cm)^{-1}$.
- It is critical to maintain the pH of solutions between 5-6. If necessary, more sodium acetate can be added to increase the pH.

IV. Equipment and materials

– Analytical balance
– Cuvettes(1 or 3 mL capacity, 1 cm pathlight and suitable for reading in visible light)
– Furnace (Temperature > 500 °C)
– Funnels
– Graduated cylinders
– Porcelain crucibles
– Spectrophotometer UV/VIS
– Volumetric flasks (25, 100, 250 mL)
– Volumetric and graduated pipettes
– Vortex mixer

V. Reagents

– Hydrochloric acid (HCI), 37%, p.a., d =1.19 g/mL, Fe < 28 μg/mL, mol wt 36.46.
– Nitric acid (HNO_3), p.a., 65 %, d = 1.39 g/mL, Fe < 1 μg/mL, mol wt 63.01.
– Sodium acetate trihydrated, ($CH_3COONa.3H_2O$), p.a., 99% Fe < 200μg/kg, mol wt 136. 08.

- 1,10-phenanthroline-monohydrate, p.a., mol wt.= 198.23.
- Hydroxylamine hydrochloride ($NH_2OH.HCl$), p.a., mol wt = 69.49.
- Glacial acetic acid (CH_3COOH), p.a., mol wt. 60.05.
- Standards Solution for iron Ammoniacal Ferrous Sulfate, Fe $(NH_4)_2(SO_4)_2 .6H_2O$, mol wt 392.14

VI. Solutions

a. 1,10-phenanthroline.H_2O

Dissolve 0.1 g 1,10-phenanthroline.H_2O in ca 80 mL H_2O 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.

b. 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.

c. Hydroxylamine Hydrochloride –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.

VII. Standard solutions

a. Primary Standard Solution of Iron – 1000 mg/L

Dissolve 3.512 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ 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.

b. 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.

c. Standard Solutions for the Calibration Curve

Solutions for the calibration curve will have iron levels from 0.0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0,4.0 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, and then make up to volume with deionized water.

Iron (mg/L, ppm)	Volume of the Secondary Solution (10 mg/L) to be added (mL)
0.0	0.0
0.2	2.0
0.5	5.0
1.0	10.0
1.5	15.0
2.0	20.0
3.0	30.0
4.0	40.0
5.0	50.0

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.

VIII. Procedure

a. Dry digestion (ashing)

1. Clean the porcelain crucibles, and label using a high-temperature proof marker.
2. Dry crucibles in the oven at 110 ˚C and cool in a dessicator. Repeat until constant weight is attained.
3. Take about 100 g of the flour and grind in a mortar and pestle and mix well.
4. Weigh 1 g of the previously homogenized sample in duplicate. Weigh by difference directly into the crucibles using an analytical balance and record the weights accurately to 3 decimals (0.001 g).
5. Place the crucibles into the muffle furnace at 550 ˚C and heat for 6 hours.
6. Turn the oven off and wait until the temperature has decreased.
7. The ashing is complete when a white or grayish ash is obtained. If this is not the case, continue the ashing until white/grayish ash is obtained.
8. Let the crucibles cool down for 5 minutes and place in a dessicator for 1 hour until they reach room temperature.

b. Preparation of the ash solution

1. Add 5 mL of concentrated HNO_3 to the crucible, pouring the acid onto the inside walls of the crucible.

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

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

4. Let the crucible cool down and transfer the solution quantitatively into a 25.0 mL volumetric flask. Wash crucible with distilled water and bring to volume with deionized water.

c. Determination of iron

1. Pipet 10.0 mL of the sample solution into 25.0 mL volumetric flask, then add 1.0 mL of hydroxylamine hydrochloride solution, mix well and let it stand for 5 minutes.

2. Pipet 10.0 mL of the standard solutions prepared in VII.c, into 25.0 mL volumetric flasks, and follow the same procedure as for the samples.

3. Add 5.0 mL acetate buffer and 4.0 mL of 1,10-phenanthroline to each flask. Mix well and color will start developing.

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

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

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

7. Set the instrument to zero Absorbance using deionized water.

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

9. Read the absorbance for the standard solutions and flour sample solutions.

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

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3 This reducing step is very important because iron oxidizes to Fe (+3) during ashing and reaction with concentrated acids.

4 In the case of the other chromogenic agents (bathophenantrholine, a-dipyridyle, or ferrozine) use 2 mL of the solutions instead of 4 mL.

5 For the other chromogenic agents, the corresponding wavelengths are: bathophenantroline: 535 nm; a-dipyridyle: 521 nm; and ferrozine: 562 nm.

IX. Calculation

1. Plot a graph of the absorbance values of the standard solutions (y-axis) against concentration (x-axis) and obtain the equation of the standard curve. The equation will be similar to the one obtained for soluble iron.

2. Calculate the concentration of soluble iron in the sample solution solving the standard curve equation for x.

3. Calculate the concentration of soluble iron in the flour sample using the equation below.

$$Iron\ (mg/kg) = \frac{[Fe]\ x\ 25}{w}$$

Where *w* is around 1.0 g or the weight used in the ashing steps.

III. Methods to determine vitamin A in wheat flour

Vitamin A is the generic name applied to a group of fat soluble compounds that have the biological activity of all-*trans*-retinol and includes: retinol (alcohol), retinal (aldehyde), retinoic acid (carboxylic acid), and pro-vitamin A carotenoids such as ß-carotene. Retinol and its related compounds consist of four isoprenoid units joined head to tail, and contain five conjugated double bonds.

Pro-vitamin A carotenoids are found in fruits and vegetables, as well as in egg yolk, and they are transformed in the organism to all-*trans*-retinol. Retinol is referred to as preformed vitamin A and it is found in liver, milk, butter, cheese, eggs and fish liver oils, mainly esterified with fatty acids. Retinyl palmitate and retinyl acetate are the two main retinyl esters used in food fortification. The vit. A compound for flour fortification is usually embedded into a protecting matrix that contains starches and antioxidants to provide stability and dispersibility in water.

General properties

Retinol

- Formula: $C_{20}H_{30}O$
- Mol wt: mol wt. 286.45 g/mol
- UV max.: Ethanol: 324-325 nm. $E^{1\%}_{1cm}1835$; 2-propanol: $E^{1\%}_{1cm}1824$
- Solubility: Soluble in absolute ethanol, methanol, chloroform, ether, fats and oils.
- Exhibits a yellow-green fluorescence (emission: 470 nm) when irradiated with UV light at 325 nm (excitation wavelength)

Retinyl acetate

- Formula: $C_{22}H_{32}O_2$.
- Mol wt: 328.49 g/mol
- Pale yellow prismatic crystals. Melting point: 57-58 ºC
- UV max: Ethanol 325 nm. $E^{1\%}_{1cm}1550$; 2-propanol: $E^{1\%}_{1cm}1523$

Retinyl palmitate

- Formula: $C_{36}H_{60}O_2$.
- Mol wt: 524.86 g/mol.
- Morphous or crystaline. Melting point: 28-29 ºC.
- UV max: Ethanol, 325-328 nm, $E^{1\%}_{1cm}975$; 2-propanol: $E^{1\%}_{1cm}953$
- All retinoids are susceptible to isomerization and oxidation when exposed to light, oxygen, reactive metals and elevated temperatures

1 IU Vitamin A = 0.3 μg retinol

Retinol and its esters react with trifluoroacetic and trichloroacetic acids to form a blue color in anhydrous solvents such as chloroform, dichloromethane, or heptane. This reaction is the principle of the qualitative method to determine vitamin A in wheat flour. Quantitative determination of retinol is carried out injecting the sample extract in a high-performance liquid chromatographer (HPLC) after the sample has been saponified, and retinol has been extracted from the matrix. The manual presents two methods:

- Qualitative method to determine vitamin A in wheat flour
- Quantitative method to determine vitamin A in foods by high-performance liquid chromatography (HPLC)

A. Qualitative method for determining vitamin A in fortified wheat flour

I. References

[Developed by Phillip Makhumula and AsumaniRatibu as part of a regional A2Z/ECSA fortification project to support national laboratories in building capacity for determining levels of micronutrients in fortified foods].

- BASF Method for vitamin A determination in flour, Analytical method, QM 02099QA000
- Manual for Sugar Fortification with Vitamin A Part 3", Omar Dary, Ph.D.; Guillermo Arroyave, Ph.D.
- "Colorimetric Determination of Vitamin A with trichloroacetic acid", D. B. McCormick and L. D. Wright, Eds. Methods in Enzymology; Part F, Vitamins and Coenzymes 67: 189-95, New York: Academic Press.

II. Principle

Vitamin A (retinyl palmitate) used for fortifying flours is extracted into organic solvents after mixing the flour with water and 2-propanol. The organic solution containing vitamin A is then reacted with chromogenic solutions to produce a blue solution. The procedure described here improves on prior methods that did not provide results that were reproducible and easy to interpret when used for fortified flours. The limitation of the traditional methods is attributed to two main reasons:(1) the amount of vitamin A added to flour is low and hence the blue color is pale, and (2) the color produced is transient and all decisions should be done swiftly within 10-15 seconds of mixing the vitamin A extract with the chromogenic solution. A new proposed modification to extend the life of the blue color involves the addition of florisil to the vitamin A extract before addition of the chromogenic solution. This adsorbent (florisil) adsorbs the vitamin A, and the blue complex developed by the reaction with the chromogenic reagent takes place in this solid matrix. When the concentration of vitamin A is high enough (above 1 mg/kg), the blue color lasts for a few minutes before changing to a light brown redish color. Because the results are not reproducible at low concentrations of vitamin A, the use of florisil is not recommended, unless the concentration of vitamin A is above 1mg/kg.

Based on results obtained in the laboratory, solutions giving a blue or light blue color will be reported as positive, with a concentration above 0.5 mg/kg.

III. Critical points

- Carry out the test under the fume hood and cover the solutions when they are transported for centrifugation to avoid contact with the fumes from the solvents used in the test.
- The disposal of reaction solutions needs to be done appropriately as any other organic waste solutions.
- Due to the low levels of the vitamin A in flour the amount of flour is significantly high and slurry (paste) is usually formed with the wheat flour when water is added. The addition of the 2-propanol however produces a suspension of flour in the solvent.
- Separation between solid and liquid phases is achieved using centrifugation.
- Thorough agitation of the wheat flour after addition of the different reagents is essential in order to solubilize vitamin A and then extract it into the n-heptane layer.
- Measure the shaking times indicated in the method with a stopwatch to ensure reproducibility of results.

6 A2Z/ECSA Consultant in Food Fortification, Analytical Chemist
7 Senior Laboratory Analyst from Uganda Industrial Research Institute (UIRI), Kampala, Uganda. Laboratory work conducted in UIRI Analytical Chemistry Laboratory (April 2008)

IV. Equipment and materials

– 50 mL centrifuge tubes
– 50 mL Volumetric flask
– Centrifuge, 3500 rpm, capacity to hold 50 mL tubes
– Vortex mixer
– Automatic pipette to discharge 5 mL or glass syringe
– Measuring cylinders 20 mL, 10 mL
– Balance to weigh 10g, 20g
– Pasteur Pipettes
– Test tubes (15mm x 100mm)

V. Reagents

All reagents are of analytical grade unless otherwise stated.
– Distilled water
– 2-propanol
– N-heptane
– Dichloromethane
– Trifluoroacetic acid (TFA). F_3CCOOH, mol wt 163.39.

VI. Solutions

– **Trifluoroacetic acid (TFA)** -20% v/v.

Prepare a reagent solutions as follows: In a volumetric flask, place 10 mL of TFA and add dichloromethane and make up to 50 mL. Mix solution thoroughly.

VII. Procedure

1. Weigh 10 g of wheat flour into a 50 mL centrifuge tube.

2. Add 20 mL of distilled water using a measuring cylinder and shake well manually for 1 minute. Measure the time with a stopwatch.

3. Add 10 mL of 2-propanol and shake thoroughly manually for 1 minute. If a vortex mixer is used instead, ensure that complete mixing takes place to solubilize the vitamin A.

4. Add 10 mL of n-heptane and a small volume of saturated saline solution to improve separation. Shake in a vortex mixer for 1 minute using a stopwatch.


5. Centrifuge the tube for 10 minutes at 3000 rpm. A clear separation between the solids-aqueous layer and the organic layer must be obtained. The organic layer will be yellow (see picture below).

6. Using a Pasteur pipette, transfer the organic phase (~10mL) to a clean test tube (See test tubes with the yellow solution in the pictures shown below in step 8).

7. Using a pipette, transfer 2 mL of the organic extract to another test tube.

8. Pipette 3 mL of the TFA solution and add them fast and vigorously into the tube containing the extract, so the addition will mix the solution. **The development of a blue color indicates the presence of vitamin A in the flour and the intensity of the color is directly proportional to the concentration of vitamin A in the sample.** The pictures below show different shades of blue in two samples. The concentration of vitamin A in the picture to the left is higher than the concentration in the sample from picture to the right.


9. If a blue or light blue color is observed, report the results as positive and above 0.5 mg/kg.

VIII. Qualitycontrol

Run the flour control with a known concentration of vitamin A every time wheat flour samples are analyzed. If the reaction is negative with the control, check the procedure and the stability of the TFA solution.

B. Determination of vitamin A in foods by high-performance liquid chromatography

I. References

Horwitz W and GW Latimer (Eds.). AOAC Official Method 2001.13.Vitamin A (Retinol) in Foods.Vitamins and Other Nutrients.Chapter 45, p.53-56.Official Methods of Analysis of AOAC International. 18thed, Revision 2, 2007. Maryland.

II. Principle

Standards and samples are saponified in basic ethanol-water solution, neutralized, and diluted. This process converts fats to fatty acids, and retinyl esters to retinol and the corresponding fatty acids. Extract clean-up is carried out with a C18 cartridge and vitamin A is concentrated eluting with a smaller volume of isopropanol than the aliquot taken to clean. Retinol is quantified in an LC system, using UV detection at 326 nm. Concentration is calculated by comparison of peak heights or peak areas of retinol in test samples with those of standards.

Recovery of vitamin A in Infant Formula 1849 (NIST) was 99% as measured in the CPHL Laboratory.

III. Critical points and cautions

Due to the labile nature of retinol, it is important to saponify the samples under a nitrogen atmosphere and in the presence of pyrogallic acid.

Potassium hydroxide is extremely caustic and it can cause severe burns. Protect skin and eyes while performing this method. This method involves the use of flammable liquids. Perform behind a barrier when using hot water, steam or an electric heating mantle. Use an effective fume removal device to remove flammable vapors produced. Leave ample headroom in flask.

Protect samples from light by covering the glassware containing the sample extracts with aluminum foil or a piece of black cloth, and work under subdue light.

IV. Equipment and materials

- HPLC system
  - Pump operating continuously at 1.0-2.0 mL/min with a flow precision of ± 1% or better
  - Injector. A manual injector or autosampling injector with a 100 µL fixed loop having a typical sampling precision of ±0.25% or better
  - Reverse-phase C18 column, 5 µm (4.6x250 mm) capable of separating cis and trans isomers of retinol with a resolution of 1.0 or greater.
  - Photometric detector monitoring absorbance at 326 nm.
  - Data collection system or integrator
- Sep-pak Cartridges C18 Vac 3cc (500 mg). Waters.or equivalent.
- Erlenmeyer flasks (125 mL) with neck adapted for connecting reflux condenser
- Hot plate
- Reflux condensers

- Volumetric flasks (100 and 10 mL)
- Nitrogen blanket apparatus[8]

V. Reagents

- Certified vitamin A acetate concentrate (USP) or
- Retinyl palmitate, all-trans.
- Acetic acid glacial, AR
- Acetonitrile, AR
- Isopropanol, AR
- Methanol, HPLC grade
- Absolute ethanol AR
- Tetrahydrofuran (THF), AR grade
- Hexane (n-Hexane 95% for HPLC)
- Pyrogallic acid, crystal, AR grade

VI. Solutions

a. Mobile phase: Combine 890 mL methanol and 110 mL distilled water. Mix well. Stir overnight to degas or prior to use.

b. THF-methanol [50+50]: Combine 500 mL tetrahydrofuran and 500 mL 95% ethanol. Mix well.

c. Potassium hydroxide solution-50%: Slowly add 500 g of KOH pellets to 500 mL water contained in a 2L thick walled Erlenmeyer flask. The solution gives off substantial heat while KOH is dissolving. Add the KOH in 100 g portions while the flask is being cooled with cold water. Swirl the flask gently to aid in dissolution of the KOH. Store in glass container with cork stopper.

d. Washing solution-acetonitrile-20% in water: Combine 80 mL water and 20 mLacetonitrile. Mix well.

e. Vitamin A working standard (ca 5 μg retinol/mL)

1. Using USP standard: Weigh 50 mg retinyl acetate concentrate into a 100-mL volumetric flask. Record weight to nearest 0.1 mg. Record concentration in mg/g per USP certification. Add a small amount of acetone (less than 3 mL) to aid dissolution. Dilute to volume with absolute ethanol. Store at 4°C in dark. Solution is stable for two weeks.

2. Using retinyl palmitate:

Stock solution: Weigh 55 mg retinyl palmitate into 100-mL volumetric flask. Record weight to nearest 0.1 mg. Record purity per supplier certification or purity test. Add pea-sized piece of pyrogallic acid. Dissolve and dilute to volume with hexane.

Working solution: Pipet 2 mL solution to second 100-mL flask and dilute to volume with absolute ethanol. Store at 4°C in dark. Solution is stable for two weeks.

8 A supply of nitrogen gas with appropriate tubing and connectors to provide a constant nitrogen atmosphere blanket in the reflux apparatus during saponification.

Check the concentration of retinyl palmitate stock solution every time is used. Pipette 2 mL stock retinyl palmitate solution into a 100-mL volumetric flask and dilute to volume with hexane. Read the absorbance at the maximum wavelength (325-328 nm) using a 1-cm pathlength cell and hexane as blank. Calculate the purity of retinol palmitate for the working day as:

$$Purity\ (\%) = \frac{A_{max}\ x\ (5x10^6)}{920\ x\ w}$$

Where *w* = weight to prepare the stock retinyl palmitate in hexane in mg.

Purity of stock solution when a new standard is opened:

Check purity as follows: Dissolve 50 mg (record to nearest 0.1 mg) of retinyl palmitate standard in 2-propanol (UV-spectroscopy grade) in a 500-mL flask and dilute to volume. Dilute 10 mL of this solution to 100 mL with 2-propanol (final concentration is approximately 10 mg per liter). Measure maximum absorbance obtained at 325-328 nm using a 1-cm pathlength cell and 2-propanol as blank. Calculate purity of retinol palmitate as

$$Purity\ (\%) = \frac{A_{max}\ x\ (5x10^6)}{960\ x\ w}$$

where A_{max} = absorbance maximum; ($5x10^6$) = combined dilution factors, conversion to 1% equivalent solution, and conversion to percent; 960= absorbance of pure retinyl palmitate (1% solution in 1-cm cell), and *w*=weight of retinyl palmitate standard in mg.

VII. Procedure

a. **Preparation of sample**

1. Solid samples should be ground to pass a 40-mesh sieve. Liquid or wet samples should be blended to homogeneity and stored at or below 4°. All samples should be stored in the dark.

b. **Saponification and extraction of sample**

2. Turn on hot plate to preheat. Start and adjust cooling water flow to precool reflux condensers. Reflux system should be arranged as shown in the picture below.

3. Standards

- High standard: Pipet 4 mL vitamin A working standard into 125-mL Erlenmeyer flask. Add 25 mL 95% ethanol. Proceed to step 5.
- Intermediate standard: Pipet 3 mL vitamin A working standard into a second 125-mL Erlenmeyer flask. Add 33 mL 95% ethanol. Proceed to step 5.
- Low standard 1: Pipet 2.0 mL vitamin A working standard into a third 125-mL Erlenmeyer flask. Add 35 mL 95% ethanol. Proceed to step 5.
- Low standard 2 (prepare this standard when running samples with vitamin A concentrations below 1.5 mg/kg): Pipet 1 mL vitamin A working standard into a 125-mL Erlenmeyer flask. Add 37.5 mL 95% ethanol. Proceed to step 5.

IMPORTANT: For samples with vitamin A concentrations below 1.5 mg/kg, such as wheat flour, run Intermediate standard, Low standard 1 and Low standard 2, only.

4. Samples

- Low fat (less than 40% fat). Weigh sample (not more than 5 g) to give approximately 50 µg vitamin A into 125-mL Erlenmeyer flask. For samples high in sugar, add 3 mL water and disperse sample as slurry. Add 40 mL 95% ethanol.
- High-fat. Weigh sample (not more than 2 g) to give approximately 50 µg vitamin A into 125-mL Erlenmeyer flask. Add 40 mL 95% ethanol.
- **Wheat flour**: Weigh 10 g sample, considering the vitamin A concentration is lower than 1.5 mg/kg. Add 40 mL 95% ethanol.

5. Add a pea-sized piece (approximately 50 mg) of pyrogallic acid (antioxidant) to each standard and sample flask. Add a glass bead to promote even boiling.

6. Swirl all flasks to ensure that all samples are thoroughly dispersed in the solution.

7. Turn on nitrogen flow and ensure a nitrogen atmosphere for all flasks while refluxing.

8. Pipet 10 mL 50% KOH solution into each flask and immediately place flask on hot plate under reflux condenser. Swirl.

9. Reflux 45 min. Swirl flasks every 10 min.

10. Remove reflux flasks from hot plate, stopper with corks, and quickly cool flasks to room temperature, using cold water or ice water.

11. Pipet 10 mL glacial acetic acid solution into each flask to neutralize the KOH. Mix well and let flasks cold again to room temperature.

12. Quantitatively transfer solution in each flask to 100 mL volumetric flaks, using 50:50 THF:ethanol. Dilute to volume with same.

13. Stopper and invert volumetric flasks 10 times.

14. Allow samples to set for at least 1 hour at room temperature and preferably overnight in refrigerator to allow fatty acid salts formed during saponification to precipitate. In some cases, centrifugation may be helpful to reduce settling time. The color of solution will be dark (see picture below) and a whitish precipitate will form in samples at the bottom of the flask along with the solids. The higher the fat content in the sample, the more precipitate will form.

c. **Clean-up and concentration procedure**

Note: The assembly shown in the picture below is helpful for processing the samples.

15. Condition the C18 cartridge with 10 mL ethanol, then pass 10 mL water.

16. Pass 10 mL of the sample extract through the C18 cartridge, controlling the flow in order to allow interaction between the extract and the resin.

17. Wash the cartridge with 5 mL washing solution (acetonitrile-20% in water).

18. Wash again the cartridge with 5 mL washing solution (acetonitrile-20% in water). The picture below shows the difference between the cartridge after the sample has been loaded (right) and after the cartridge has been washed to eliminate sample impurities other than vitamin A (left).

19. Elute vitamin A with 1.5 mL isopropanol.

d. **Determination**

20. The optimized conditions for the analysis used in the CPHL Laboratory are:

    - Mobile phase: Methanol:water (89:11)
    - Flow rate: 1.3 mL/min
    - Wavelength: 323 nm
    - Retention time: Around 11-12 min.

21. Start HPLC system and allow to warm up and equilibrate for minimum of 30 min with mobile phase flowing. Flow rate should be 1.3 mL/min.

22. Inject vitamin A standard onto vitamin A HPLC system. Adjust mobile phase to achieve a resolution of 1.5 or better for cis and trans forms. All trans retinol should elute in approximately 6 min or longer.

23. Inject the standards. Repeat injection of standards until peak height(s) or areas are reproducible.

24. Inject sample solutions. Intersperse with standard solution injections after every nine samples to assure accurate quantitation. (If retinol peak height or area exceeds the one for the high standard by more than 25%, dilute sample solutions using a solution of 10 mL 50% KOH solution, 40 mL 95% ethanol, 10 mL glacial acetic acid, and 40 mL 50:50 THF: ethanol solution).

VIII. Calculations

Results can be calculated using either a standard curve or a response factor. Choose one procedure and use it for the calculations in the lab. Both procedures are based on the premise that the detector response is linear in the concentration range use in the method.

a. **Procedure using a standard curve**

1. Calculate the equation of the standard curve using Area (y) vs. concentration-mg/L (x) for the three standard concentrations injected. The equation will of the type:

$$y = mx + b$$

2. Calculate the retinol concentration (mg/L) in the injected sample solution using the equation.

3. Using the concentration of sample obtained in step 2, calculate retinol concentration in the sample using the following formula:

$$Vitamin\ A(mg/kg) = \frac{C_s x V_i}{W_s}$$

Where:

Parameter	Explanation	Value
C_s	Concentration of retinol in the sample (mg/L)	?
V_i	Initial volume (mL)	100
W_s	Sample weight (g)	Around 10

b. **Procedure using a response factor**

Calculate µg/g vitamin A (as retinol) as follows:

1. Measure peak heights or areas of standards.

   a. Using USP standard

   Response factor for vitamin A (RFA):

$$RF_A = \frac{mg_{std}\ x\ ml_{std}\ x\ C_{std}}{PH_{std}\ x10,000}$$

Where

PARAMETER	EXPLANATION	VALUE
$mg_{std=}$	mg of USP standard weighed in reagents (6.e.1)	?
$mL_{std}=$	mL of standard used in procedure step 7.b.3	?
$C_{std}=$	concentration of USP vitamin A (as retinol) per USP certification (mg/g)	?
$PH_{std}=$	peak height or area of standard from chromatogram	?
10,000 =	combined dilution factors for vitamin A standard	10,000

b. Using retinylpalmitate

Response factor for vitamin A (RF_A):

$$RF_A = \frac{mg_{std} \times mL_{std} \times P_{std} \times 0.5458}{PH_{std} \times 500}$$

Where

Parameter	Explanation	Value
$mg_{std=}$	mg retinyl palmitate weighed in reagent step (6.e.2)	?
$mL_{std}=$	mL of standard used in procedure step 7.b.3	?
$P_{std}=$	Percent purity certified by supplier (or determined), divided by 100	?
$PH_{std}=$	peak height or area of standard from chromatogram	?
0.5458 =	ratio of retinol to retinyl palmitate molecular weights	0.5458
500 =	combined dilution factors and conversion from mg to µg	500

2. RF_A values of low, medium and high standards should agree with each other within 3% relative since detector response should be linear across the concentration range used here. Average of RF_A values calculated from high, medium, and low standards should be used for sample quantitation.

3. Measure peak heights or areas corresponding to retinol in sample extracts. The *13-cis* isomer of retinol (eluting immediately before the *all-trans* isomer) might be present in some samples.

4. Calculate vitamin A content using the following formula:

$$Vitamin\ A, \mu g/g\ (as\ retinol) = \frac{RF_A \times PH_{sam} \times 100}{w}$$

Where:

PH_{sam} = total sample peak height or area of *all-trans*
100 = dilution volume of sample
w = weight of sample in g

IV. Methods to determine water-soluble vitamins in foods

Water soluble vitamins included in here are thiamin (vitamin B_1), riboflavin (vitamin B_2), niacin (B_3) and folic acid (B_9). As other vitamins, these vitamins are susceptible to exposure to different conditions that may destroy them during the analysis. The general properties of the vitamins are described below.

Thiamin (Vitamin B1)

Thiamin is an essential nutrient required for the carbohydrate metabolism and the nerve function. It is found in whole grains, meat products, vegetables, milk, legumes and fruit, and in fortified foods. There are two forms of thiamin commercially available: thiamin mononitrate and thiamin hydrochloride. Thiamin mononitrate is more stable than the hydrochloride, but the latter is more soluble in water than the mononitrate form. Foods are usually fortified with thiamin mononitrate, but both forms are used as standard in the analysis of thiamin.

General Information

- Formula: Hydrochloride. $C_{12}H_{17}ClN_4OS$.
- Mol wt: 300.81. Hydrochloride: 337.27 g/mol.
- Sensitive to heat, oxygen, alkali, radiation and sulfites.
- Dry, crystalline vitamin is very stable.
- Solutions in dilute mineral acids are very stable if protected from UV light.
- Maximum UV absorption in aqueous solutions: 245 nm.
- Reacts with ferricyanide in alkali solution to form thiochrome, a fluorescent compound.

Riboflavin (Vitamin B2)

Riboflavin is present in milk, eggs, malted barley, liver, kidney, leafy vegetables, yeast and fortified foods.

General Information

- Formula: $C_{17}H_{20}N_4O_6$
- Mol wt: 376.36
- Yellow-orange crystals
- Absorption max: 220-225, 266, 371, 444, 475 nm
- Shows natural green fluorescence with max. at 565 nm
- Relatively insoluble in water, very soluble in diluted alkalis (with decomposition)
- Destroyed by light, either in alkaline or acid solutions
- Sensitive toalkalies and stable to mineral acids in the dark heat, oxygen, alkali, radiation and sulfites.
- Dry, crystalline vitamin is very stable.
- Solutions in dilute mineral acids are very stable if protected from UV light.
- Maximum UV absorption in aqueous solutions: 245 nm.
- Reacts with ferricyanide in alkali solution to form thiochrome, a fluorescent compound.

Niacin

The term niacin is applied to nicotinic acid and its derivative niacinamide. Dietary sources include liver, fish, yeast and cereal grains, including fortified wheat flour.

General Information

- Nicotinic acid: $C_6H_5NO_2$. Mol wt. 123.11
- Niacinamide: $C_6H_6N_2O$. Mol wt. 122.12
- Absorption max: Nicotinic acid: 263 nm.
- Niacinamide: 261 nm ($A^{1\%}_{1cm} = 451$)
- Both nicotinic acid and nicotinamide are stable when exposed to heat, light, air and alkali.

Folic acid (pteroylglutamic acid)

Folate is a generic name applied to a group of compounds that have similar activity in the living organisms. Naturally occurring folates are pteroylpolyglutamic acids with two to eight glutamic acid groups. Folic acid is a synthetic folate known as pteroylglutamicacid which gives the basic structure for folates. This is used for vitamin supplements and food fortifications and is more stable than naturally occurring folates.

General Information

- Formula: $C_{19}H_{19}N_7O_6$
- Mol wt.: 441.40
- Appearance: Yellowish-orange crystals.
- UV max (pH 13) 256, 283, 368 nm
- Very slightly soluble in cold water
- Relatively soluble in acetic acid, solutions of alkaly hydroxides and carbonates.
- Soluble in hot dilute hydrochloric and sulfuric acids.
- Stable in alkaline, but unstable in acid solutions.
- Affected byair, heat, sunlight and UV rays, oxidizing and reducing agents.

A. Method to determine riboflavin in fortified foods

I. References

Schüep, W. y Steiner, K. Determinationof Vitamin B_2 in Complete Feeds and Premixes with HPLC. En: Keller, H.E. *Analytical Methods for Vitamins and Carotenoids in Feeds*. Animal Nutrition and Health Vitamins and Fine Chemicals Division, Roche. Switzerland. pp. 30-32.

II. Principle

Riboflavin added in fortification is extracted from the sample in an autoclave with dilute sulfuric acid. An amylase suspension is used to destroy the starch in the flour and improve filtration. After filtration of the flour suspension, a portion of the filtrate is diluted with methanol and any precipitate is removed by centrifuge. The riboflavin content is determined by HPLC on a reversed phase column (C18) with fluorimetric detection. This method is useful for riboflavin content above 0.5 mg/kg. Wheat flour is fortified to a minimum level 2.5 mg/kg.

III. Critical points and precautions

Riboflavin is labile to light; therefore, samples and sample solutions must be protected from light at all times. Prepare the stock standard solution in a separate room than the samples to avoid contamination. Sample extracts loose 9% riboflavin from one day to the other. Therefore, sample extracts must be injected in the HPLC on the same day of extraction, unless it is impossible to do so. In that case, correct for losses when calculating the final riboflavin concentration.

Riboflavin stock solution is stable up to 1 month, but its concentration decays gradually and actual concentration must be determined every time it is used using the extinction coefficient. It is preferable to prepare a fresh standard every time samples are analyzed if the method is not applied routinely.

Riboflavin concentration in flour samples is calculated using the peak height. Calculations made in the laboratory showed that riboflavin concentration is overestimated when using area.

Prior to injecting standards and samples in the injection valve, the column must be stabilized with the mobile phase. At the end of the run, rinse the column thoroughly with HPLC-water to eliminate all salt residues from the mobile phase. Then, wash it with methanol. NEVER leave the mobile phase in the column.

IV. Equipment and materials

- Autoclave (121-123°C)
- Agitator Vortex type
- Analytical balance (± 0.0001 g)
- Water bath (40°C)
- Centrifuge (3000 rpm)
- HPLC system
  - Pump operating continuously at 1.0-2.0 mL/min with a flow precision of ± 1% or better
  - Injector. A manual injector or auto sampling injector with a 50 µL fixed loop having a typical sampling precision of ±0.25% or better
  - Data collection system or integrator
- Volumetric flasks (25, 100 and 1000 mL)

- Beakers (25, 100 and 1000 mL)
- Glass funnels
- Amber glass vessels
- Volumetric pipettes
- Graduated cylinders
- Centrifuge tubes (50 and 10 mL)
- Test tubes (10 mL)
- Glass rods
- Filter paper Whatman No. 41

V. Reagents

1. **Acetic acid - 0.02M:** ((CH_3COOH), 99.8%, FW: 60.05, d=1.05 g/mL). In a 1L volumetric flask containing around 500 mL of distilled water add 1.2 mL of glacial acetic acid. Agitate and make up to volume with distilled water.

2. **Amylase-5% w/v:** Weigh 1.25 g amylase and add around 5 mL distilled water. Let it stand until is completely hydrated. Make up to volume 25 mL with distilled water and agitate thoroughly. Prepare only the amount to be used.

3. **Sodium acetate-2M:** (CH_3COONa), 99.5%. Dissolve 164 g of sodium acetate anhydrous in distilled water and dilute to 1 L.

4. **Sulfuric acid-0.1M:** (H_2SO_4, 95-97%, 1.84 g/mL, FW 98.08). In a beaker containing around 600 mL distilled water, add 10 mL concentrated sulfuric acid. Agitate and make up to 1 L with distilled water.

5. **Mobile phase (HTAA:Methanol, 83:17)**[9]

   Solution A, HTAA (Sodium hexanosulfonate-5mM, triethylamine-0.13 %, acetic acid-1%)

   In a 25-mL beaker weigh 0.9602 g sodium hexanosulfonate ($C_6H_{13}O_3SNa$, Sigma Ultra 98%, FW: 188.2 g/mol, Sigma H-9026). Dissolve in HPLC grade water and transfer quantitatively to a 1-L volumetric flask. Add 1.3 mL triethylamine (($C_6H_{15}N$), > 99%, FW 101.19, d=0.73 g/mL) and 10 mL glacial acetic acid ((CH_3COOH), 99.8%, FW=60.05, d=1.05 g/mL). Make up to volume with HPLC grade water.

   In a 1-L Erlenmeyer flask add 830 mL solution A and 170 mL methanol. Mix well and filter the solution through a 0.45 μm filter. Degas the solution prior to use. Prepare only the volume of mobile phase to be used.

9 Dong, M., Lepore, J. y Tarumoto, T. 1988. Factors Affecting the Ion-Pair Chromatography of Water Soluble Vitamins. *J. Chromatogr.* **442**: 81-95.

VI. Standard solutions

1. **Riboflavin stock standard solution-100 mg/L:** Dry riboflavin USP reference standard for 1 to 2 hours at 60-70°C in a vacuum oven. Keep the dried standard in a tightly closed container in the dessicator. Weigh accurately 50 mg of riboflavin into a 500 mL-volumetric flask and dissolve in acetic acid-0.02 M. Place the solution in a ultrasonic bath for 30 minutes or until riboflavin is completely dissolved. Make up to volume with acetic acid-0.2M.

   Calculate the actual concentration of the solution from the absorbance read in the standard solution-1 mg/L.

   Store the solution in an amber flask in the refrigerator and it can be used up to one month.

2. **Riboflavin standard solution – 1 mg/L:** Place 1 mL stock standard solution-100 mg/L in a 100-mL volumetric flask. Make up to volume with distilled water. Prepare this solution every time samples are run and discard it after finishing work.

   Determine the actual concentration of the solution by reading the absorbance at 266 nm in UV light. Calculate the actual concentration using the extinction coefficient = 870 for riboflavin. Read the absorbance and calculate the actual concentration every time samples are run.

3. **Riboflavin working standard solutions:** Prepare three working standard solutions with concentrations 0.04 mg/L, 0.08 mg/L and 0.12 mg/L. In 25 mL volumetric flasks, place 1, 2 and 3 mL of riboflavin standard solution-1 mg/L. Make up to volume with water. Prepare these solutions every time samples are run and discard them after finishing work.

VII. Procedure

A. Extraction

1. In a 100 mL beaker, weigh accurately 10 g flour in duplicate.

2. Add 10-20 mL sulfuric acid-0.1M. Agitate the sample with a glass rod and add more 0.1 M-sulfuric acid to around 50 mL.A slurry must be obtained, without any lumps.

3. Cover the beaker with aluminum foil or a watch glass and sterilize in an autoclave for 15 minutes at 121-123°C.

4. Transfer the hot solution to a 100-mL volumetric flask containing 8 mLof 2 M-sodium acetate.

5. Let the solution cool down and add 5 mL of the 10% amylase suspension.

6. Incubate at 40°C for 20 minutes. Cool down the solution and make up to volume with distilled water.

7. Filter the solution through a glass funnel with filter paper Whatman No. 41. Discard the first 5-10 mL of the filtrates.

8. Pipette exactly 4.0 mL of the filtrate obtained into a centrifuge tube which contains 4.0 mL methanol. Mix and use the centrifuge to separate the precipitate from the supernatant liquid.

9. Pipette 4.0 mL of the clear supernatant into a test tube, dilute with 2.0 mL water and mix on a Vortex mixer. This is the final extract of the sample for HPLC. Filter this solution through a 0.45 µm membrane.

B. Chromatography

10. Start HPLC system and allow to warm up and equilibrate the column for at least 1 hour with mobile phase flowing. Flow rate should be 1.0 mL/min. Use the chromatographic conditions indicated in Table 1.

11. Adjust flow to 1.5 mL/min and inject the working standard solution in duplicate, in the following order 0.04 mg/L, 0.08 mg/L and 0.12 mg/L.

12. Inject the samples under the same conditions as the riboflavin standard and intersperse with standard solution injections after every nine samples to ensure accurate quantification.

Table 1. Chromatographic conditions to determine riboflavin

Parameter	Condition
Column	C18. Waters. 150 mm x 4.1mm ID
Flow	1.5 mL/min
Detector	Fluorescence: Excitation wavelength: 423 nm Emmision wavelength: 525 nm
Injection volume	50 µL

VIII. Calculations

1. Calculate the equation of the standard curve using height (y) vs. concentration-mg/L (x) for the three standard concentrations injected. An equation similar to the following will be obtained:

$$y = 1E08\,x - 144138$$

The correlation coefficient (r) should be 0.999 or above.

2. Calculate the riboflavin concentration (mg/L) in the injected solution using the regression equation.

3. Using the concentration of sample in mg/L, calculate riboflavin concentration in the sample using the following equation.

$$Riboflavin\,(mg/kg) = C_s\,x\,\frac{Vi}{W_s}\,x\,\frac{D}{10}\,x\,1000$$

Where:

Parameter	Explanation	Value
C_s	Concentration of riboflavin in the sample (mg/L) obtained from the regression equation.	?
D	Sample dilution	3
V_i	Initial volume (mL)	100
W_s	Sample weight (g). Around 10 g.	?

B. Determination of thiamin in flours by high-performance liquid chromatography

I. References

Rettenmaier R, Vuilleumier JP and Muller-Mullot W. Determination of Vitamin B1 in Complete Feeds. In: Keller, H.E. Analytical Methods for Vitamins and Carotenoids in Feeds. Animal Nutrition and Health Vitamins and Fine Chemicals Division, Roche. Switzerland. pp 23-26

II. Principle

Thiamin is extracted in an autoclave with diluted sulfuric acid. After enzyme hydrolysis, thiamin is oxidized with potassium ferricyanide in sodium hydroxide to form the thiochrome, which is fluorescent. The extract is injected into a HPLC onto a reverse phase column (C18) with fluorescence detection: excitation at 370 nm and emmision at 430 nm.

Recovery of Thiamin in Infant Formula 1849 (NIST) was 95.8 % as measured in the CPHL Laboratory.

III. Critical points and precautions

Thiamin is labile to light and heat. In acid medium, below pH 5.5, thiamin is more heat-resistant, whereas is unstable in alkaline and neutral medium. Samples and standard solutions must be protected from light and a thin layer of liquid paraffin is added during autoclaving to protect from heat.

The oxidizing agent must be prepared freshly, right before the reaction is carried out. The reaction to form the thiochrome must be done a few minutes before injecting in the HPLC to avoid underestimating thiamine content due to losses caused by the basic pH of the oxidizing agent.

Prior to injecting standards and samples in the injection valve, the column must be stabilized with the mobile phase. At the end of the run, rinse the column thoroughly with HPLC-water to eliminate all salt residues from the mobile phase. Then, wash it with methanol. NEVER leave the mobile phase in the column. Follow the washing protocol recommended for the column used.

IV. Equipment and materials

- Autoclave 121-123°C
- Agitator Vortex type
- Analytical balance (± 0.0001 g)
- Water bath (40°C)
- HPLC system
  - Pump operating continuously at 1.0-2.0 mL/min with a flow precision of ± 1% or better
  - Injector. A manual injector or auto sampling injector with a 100 µL fixed loop having a typical sampling precision of ±0.25% or better
  - Reverse-phase C18 column, 5μm (4.6x150 mm)
  - Fluorescent detector: Excitation wavelength: 370 nm; emission wavelength: 430 nm.
- Volumetric flasks (25, 100 and 1000 mL)
- Beakers (25, 100 and 1000 mL)

- Glass funnels
- Amber glass vessels
- Volumetric pipettes
- Graduated cylinders
- Test tubes (10 mL)
- Glass rods
- Filter paper Whatman No. 41

V. Reagents

- Glacial acetic acid. (CH_3COOH), p.a. 99.8%, mol wt. 60.05, d=1.05 g/mL
- Liquid paraffin (mineral oil). d~ 0.84 g/mL.
- Methanol (CH_3OH), HPLC.
- Potassium ferricyanide($K_3Fe(CN)_6$), p.a.
- Sodium acetate (CH_3COONa), p.a. 99.5%.
- Sodium hexanosulfonate ($C_6H_{13}O_3SNa$), Sigma Ultra 98%, mol wt.188.2 Sigma H-9026.
- Sodium hydroxide (NaOH)
- Sulfuric acid (H_2SO4), p.a., 95-97%, 1.84 g/mL, mol wt. 98.08.
- Thiamin mononitrate or hydrochloride, Standard.
- Triethylamine ($C_6H_{15}N$), > 99%, mol wt. 101.19, d=0.73 g/mL
- αAmylase.

VI. Solutions

1. **Amylase-5% w/v:** Weigh 2.5 g amylase and add around 5 mL distilled water. Let it stand until is completely hydrated. Make up to volume 50mL with distilled water and agitate thoroughly.

2. **Potassium ferricyanide-1%**: Dissolve 1 g potassium ferricyanide in water and dilute to 100 mL. Prepare fresh daily.

3. **Oxidizing reagent:** Mix 4.0 mL 1% potassium ferricyanide solution with sufficient 15% sodium hydroxide (w/v) solution to make 100 mL. Use this reagent within 4 h after preparation.

4. **Sodium acetate-2.5M:**Dissolve 205 g of sodium acetate anhydrous in distilled water and dilute to 1 L.

5. **Sodium hydroxide-15% (w/v):** Weigh 15 g sodium hydroxide and dissolve in distilled water. Cool the solution down and bring to 100 mL.

6. **Sulfuric acid-0.1M:** (H_2SO_4, 95-97%, 1.84 g/mL, FW 98.08). In a beaker containing around 600 mL distilled water, add 10 mL concentrated sulfuric acid. Agitate and make up to 1 L with distilled water.

7. **Mobilephase (HTAA:Methanol, 83:17)**[10]

   Prepare the mobile phase as follows:

   Solution A, HTAA (Sodium hexanosulfonate-5mM, triethylamine-0.13 %, acetic acid-1%)

10 Dong, M., Lepore, J. y Tarumoto, T. 1988. Factors Affecting the Ion-Pair Chromatography of Water Soluble Vitamins. *J. Chromatogr.* **442**: 81-95.

In a 25-mL beaker weigh 0.9602 g sodiumhexanosulfonate. Dissolve in HPLC grade water and transfer quantitatively to a 1-L volumetric flask. Add 1.3 mLtriethylamine and 10 mL glacial acetic acid. Make up to volume with HPLC grade water.

In an Erlenmeyer flask add 83 mL solution A and 17 mL methanol. Mix well and filter the solution through a 0.45 μm filter. Degas the solution prior to use. Prepare only the quantity of mobile phase to be used.

8. **Thiamine stock standard solution-100 mg/L:** Dry Thiamine Mononitrate USP Reference Standard for 1 to 2 hours at 60-70 °C in a vacuum oven until it reaches constant weight. Keep the dried standard in a tightly closed container in the dessicator. Weigh accurately Thiamine mononitrate equivalent to 50 mg Thiamine into a 500 mL-volumetric flask and dissolve in sulfuric acid-0.1 M. Make up to volume with sulfuric acid-0.1 M. Store the solution in an amber flask in the refrigerator and it can be used up to one month.

9. **Thiamine standard solution – 10 mg/L:** Place 10 mL stock standard solution-100 mg/L in a 100-mL volumetric flask. Make up to volume with sulfuric acid-0.1 M. Prepare this solution every time samples are run and discard it after finishing work.

10. **Thiamine working standard solutions:** Prepare three working standard solutions with concentrations **0.05 mg/L, 0.1 mg/L and 0.2 mg/L.** In 100 mL volumetric flasks, place 0.5, 1.0 and 2 mL of thiamine standard solution-10mg/L. Make up to volume with sulfuric acid-0.1 M. Prepare these solutions every time samples are run and discard them after finishing work.

VII. Procedure

A. Extraction

1. In a 100 mL beaker, weigh accurately 10 g flour.

2. Add 10-20 mL sulfuric acid-0.1M. Agitate the sample with a glass rod and add more 0.1 M-sulfuric acid to around 50 mL, and add 1-2 mL paraffin oil.

3. Cover the beaker with aluminum foil or a watch glass and sterilize in an autoclave for 15 minutes at 121-123°C.

4. Let the solution cool down and transfer it to a 100-mL volumetric flask containing 8 mLof 2.5 M-sodium acetate to adjust pH to 4.5.

5. Add 5 mL of the 5% amylase suspension.

6. Incubate at 40°C for 20 minutes. Cool down the solution and make up to volume with distilled water without taking into account the paraffin layer.

7. Filter the solution through a glass funnel with filter paper Whatman No. 41. Discard the first 5-10 mL of the filtrates.

B. Thiochrome reaction

8. Take 10 mL of the filtrate and add 5 mL of the oxidizing solution. Agitate and immediately, neutralize the solution with Glacial acetic acid (about 1.5 mL) to bring to pH 4-5.
9. Filtrate the solutions with a 0.45 um filter and put into an autosampler vial.

10 Dong, M., Lepore, J. y Tarumoto, T. 1988. Factors Affecting the Ion-Pair Chromatography of Water Soluble Vitamins. J. Chromatogr. 442: 8195-.

C. Chromatography

10. Start HPLC system and allowto warm up and equilibrate for minimum of 1 hour with mobile phase flowing. Flow rate should be 1.0 mL/min.Use the chromatographic conditions indicated in the table below.

Parameter	Condition
Column	XBridge C18 Column, 5 µm, 4.6x 150 mm
Flow	1.0 mL/min
Fluorescence Detector	Excitation wavelength: 370 nm; Emission wavelength: 430 nm
Injection volume	100 µL

11. Adjust flow to 1 mL/min and inject the working standard solution in duplicate, in the following order 0.05 mg/L, 0.1 mg/L and 0.2 mg/L. if necessary, increase the flow to 1.5 mL/min.

12. Inject the samples under the same conditions as the thiamine standard and intersperse with standard solution injections after every nine samples to ensure accurate quantification.

VIII. Calculations

1. Calculate the equation of the standard curve using Area (y) vs. concentration-mg/L (x) for the three standard concentrations injected. An equation similar to the following will be obtained:

$$y = 1E08x - 144138$$

2. The correlation coefficient (r) should be 0.999 or above.

3. Calculate the Thiamine concentration (mg/L) in the injected solution using the regression equation.

4. Using the concentration of sample in mg/L, calculate Thiamine concentration in the sample using the following equation.

$$Thiam-ine(mg/kg) = C_s x \frac{V}{W_s}$$

Where:

Parameter	Explanation	Value
C_s	Concentration of Thiamine in the sample (mg/L) obtained from the regression equation.	?
V_i	Initial volume (mL)	100
W_s	Sample weight (g).	Around 10 g

C. Determination of niacin in flours by high performance liquid chromatography

I. Reference

Tyler TA and Genzale JA. 1990.Liquid Chromatographic Determination of Total Niacin in Beef, Semolina, and Cottage Cheese. J Assoc Off Anal Chem 73 (3):467-469.

II. Principle

Niacin is extracted with an alkaline digestion with calcium hydroxide. Excess calcium is precipitated and pH is adjusted prior to solid-phase cleanup. Sample extract is cleaned up using a C18 cartridge. Niacin is collected after impurities have been separated. The pH in the sample extract is acidified and the extract is injected in a C18 column using sodium dodecyl sulfate and acetonitrile in the mobile phase.

III. Critical points

Adjusting pH is critical in the different steps. First, calcium hydroxide is eliminated by precipitation with oxalic acid, decreasing pH to 6.5-7.0. Then, pH must be neutral since niacin is not retained on the C18 column at this pH, whereas a large number of colored compounds are retained, obtaining a cleaned extract. Finally, the pH is acidified to reach the same pH as the mobile phase.

The filtration process is slow and time consuming. It is recommended to centrifuge the extracts prior to filtration to accelerate the process.

A good separation is obtained in the chromatogram when using the combination of SDS and acetonitrile in the mobile phase, but both reagents must be very pure to obtain a stable baseline.

IV. Materials and equipment

- Autoclave 121-123°C
- Agitator Vortex type
- Analytical balance (± 0.0001 g)
- HPLC system
  - Pump operating continuously at 1.0-2.0 mL/min with a flow precision of ± 1% or better
  - Injector. A manual injector or auto sampling injector with a 100 µL fixed loop having a typical sampling precision of ±0.25% or better
  - Reverse-phase C18 column, 5 μm (4.1x250 mm)
  - UV detector

- C_{18} cartridges (Sep-pak). Waters.Sep-pak Cartridges C_{18} Vac 3cc (500 mg).
- Erlenmeyer flasks (250, 500 mL)
- Volumetric flasks (25, 100 and 1000 mL)
- Beakers (25, 100 and 1000 mL)
- Glass funnels
- Volumetric pipettes
- Graduated cylinders
- Glass rods
- Filter paper Whatman No. 42 and 2.

V. Reagents

- Acetonitrile (CH_3CN), mol wt. 41.05, HPLC grade.
- Calcium hydroxide. ($Ca(OH)_2$), mol wt. 74.09, p.a
- Ethanol.
- o-Phosphoric acid (H_3PO_4), 85% purity, mol wt. 98.00, p.a.
- Oxalic acid ($C_2H_2O_4.2H_2O$), mol wt. 126.07, p.a.
- Sodium dodecylsulfate ($CH_3(CH_2)_{11}OSO_3Na$), ≥ 99% purity for ion-pair chromatography, mol wt. 288.38. Fluka 71726.

- **Mobile phase:**

  Solution A (acidified water): Dilute 1.00 mL o-phosphoric acid in 600 mL water and filter through 0.45 um.

  Solution B: In a 500 mL beaker, mix 120 mL water and 230 mL acetonitrile. Dissolve 1.00 g sodium dodecyl sulfate in this solution. Bring total volume to 400 mL with water. Filter through 0.45 um.

  Mix solution A and B to make 1L mobile phase. Mix well.

- **Stock niacin standard solution (2000 mg/L):**
  Weigh 200 mg niacin and dissolve in ethanol:water (1+1) in a 100 mL volumetric flask. Bring to volume with the mixture ethanol:water (1+1) and mix well. This solution is stable at least 2 weeks at room temperature.

- **Working Standard solution-2 mg/L:**
  Take 5 mL of stock standard solution and dilute to 100 mL with water. Then take 2 mL of the dilution and dilute to 100 mL with water.

VI. Procedure

1. Weigh 2 g flour in a 500 mL blender jar. The sample weight should contain about 0.04 mg niacin.

2. Add 198 mL water and add 10 g calcium hydroxide.

3. **Standard treatment:** Prepare three standard solutions with different concentrations.

   - Low standard: Pipet 10 mL of the working standard solution (2 mg/L) into 500 mL Erlenmeyer flask containing 190 mL water.
   - Intermediate standard: Pipet 20 mL of the working standard solution into 500 mL Erlenmeyer flask containing 180 mL water.
   - High standard: Pipet 40 mL of the working standard solution into 500 mL Erlenmeyer flask containing 160 mL water.

   Add 10 g calcium hydroxide and treat the standard as the sample starting from step 4.

4. Blend aprox. 30 seconds at high speed using a vertical stirrer or blender.

5. Autoclave 15 min at 121°C.

6. Cool in ice bath at least 30 min.

7. Transfer the extract to a 250 mL volumetric flask and bring to volume with water.

8. Filter the cold solution through Whatman paper No. 2V, try to filter only the supernatant. Centrifuging the solution will help to obtain a clear filtrate.

9. Transfer 100 mL of the filtrate, measured with a volumetric pipette, to 250 mL Erlenmeyer flask containing 300 mg oxalic acid.

10. Mix well and adjust final pH to 6.5 to 7.0 by dropwise addition of filtrate or by addition of a few crystals of oxalic acid.

11. Centrifuge the filtrate at 5,000 rpm for 10 minutes. Filter slowly through 1 or 2 pieces of Whatman No. 42 paper so that clear filtrate is obtained.

12. Condition C18 cleanup cartridge with 10 mL ethanol and then pass 10 mL water.

13. Slowly pass 10 mL clear sample filtrate through cartridge.

14. Discard first 6 mL and collect next 3.5 mL in sample vial.

15. Add 1 drop 85% fosforic acid (H_3PO_4) and mix well.

16. Inject 100 μL standard and sample solutions using the chromatographic conditions described below.

Parameter	Condition
Column	C18. Waters. 250 mm x 4.1mm ID
Flow	1.5 mL/min
Detector	Ultraviolet Wavelength: 254 nm
Injection volume	100 μL

Note: The peak shape and retention time may be modified by adding acetonitrile (95% mobile phase: 5% acetonitrile).

VII. Calculations

1. Calculate the equation of the standard curve using height (y) vs. concentration-mg/L (x) for the three standard concentrations injected.

2. The correlation coefficient (r) should be 0.999 or above.

3. Calculate the niacin concentration (mg/L) in the injected solution using the regression equation.

4. Using the concentration of sample in mg/L, calculate niacin concentration in the sample using the following equation.

$$Niacin\ (mg/kg) = C_s x \frac{Vi}{W_s}$$

Where:

Parameter	Explanation	Value
C_s	Concentration of niacin in the sample (mg/L) obtained from the regression equation.	?
V_i	Initial volume (mL)	250
W_s	Sample weight (g). Around 2 g.	?

D. Determination of folic acid (pteroylglutamic acid) in fortified foods by microbiology

I. References

Horwitz W and GW Latimer (Eds.). AOAC Official Method 944.12.Folic Acid (Pteroylglutamic Acid) in Vitamin Preparations. 45.2.03. Official Methods of Analysis of AOAC International. 18thed, Revision 2, 2007. Maryland.

II. Principle

The microbiological assay to determine folic acid in fortified foods is based on the requirement of *Enterococcushirae* (ATCC 8043) for free folic acid, which must be taken from the culture environment. The concentration of folic acid in the samples is measured indirectly by the growth of the microorganism, considering that the content of folic acid in the standards or samples is the limiting factor for the microorganism growth. This growth is compared with that in a folic acid standard solution. Turbidity is measured in the solutions and based on the response obtained for the standard solutions the folic acid concentration in the samples is calculated.

The implementation of the folic acid assay requires several steps prior to testing samples. The bacteria (*Enterococcushirae*) must be propagated and maintained for the analysis. The optimum growth time must be determined in order to get an adequate response and spectrophotometer should be calibrated for the turbidity assay.

III. Critical Points

- The method is not applicable when extraneous turbidity or color interferes with the turbidimetric measurements.
- Protect solutions from undue exposure to light throughout all stages.
- Media is highly hygroscopic, therefore they must be stored in cool dry places or refrigeration.
- When preparing media, cool down rapidly after sterilization to avoid pH changes, unwanted precipitation, loss of nutrients and darkening of the medium due to Maillard reaction.
- Media from Hi Media have been used in the implementation of the method. Other brands can be used provided that they meet the nutrient requirements described in the AOAC method for the bacteria propagation, maintenance, inoculum and assay, but the bacteria growth may vary and the method might need to be standardized again with the new media brand.
- Glassware used in the analysis must be washed with a neutral soap that do not stimulate the bacterial growth. After soaking the glassware in soap, it must be rinsed thoroughly with ultrapure water and heated in the oven to eliminate any trace of contamination.
- Water used in the analysis must be ultrapure and treated with activated charcoal to eliminate any color impurities that might affect the analysis.
- Use of inoculated and non-inoculated blanks is essential as controls to check contamination.
- Periodically, check the purity of the inoculum by culturing some cells in Blood Agar and verify that only *Enterococcushirae* has grown. A Gram staining is recommended.

IV. Equipment

- Analytical balance (± 0.0001 g)
- Autoclave
- Automatic pipette
- Automatic pipette 1 mL
- Automatic pipette 50 μL
- Blender
- Bunsen burner
- Centrifuge (5000 rpm)
- Dessicator
- Freezer (-10 to -20 °C)
- Glass or disposiblecuvettes for readings in visible light
- Hot plate with agitator
- Incubator (35-37 °C)
- pH meter (pH 1-14)
- Quart cuvettes, 1 mL for readings in UV light
- Refrigerator (4-8 °C)
- UV/Visible Spectrophotometer
- Thermometer (0-100 °C)
- Vortex mixer
- Water bath for incubation (35-40 °C)

V. Materials

- Beakers (50, 250, 600 mL)
- Erlenmeyers (250, 500, 1000 mL)
- Glass sterile vials of 5 mL
- Membranes of 0.45 μm
- Parafilm
- Plastic sterile tubes for centrifuge (10, 25 and 50 mL)
- Quantitative filter paper Whatman No. 1.
- Quantitative filter paper Whatman No. 5.
- Sterile pipette tips 2-200 μL
- Sterile pipette tips for 50-1000 μL
- Test tube rack
- Test tubes 20mm x 150 mm with caps
- Volumetric flasks (100, 200, 250, 500 mL)
- Volumetric pipettes (1, 2, 3, 4 and 5 mL)

VI. Reagents

- Bacteria*Enterococcushirae* ATCC 8043
- α-Amylase from Bacillus sp.
- Hydrochloric acid (HCl) p.a., 37%, mol wt. 36.46, d=1.19 g/mL,
- Folic acid, grade USP. ($C_{19}H_{19}N_7O_6$), mol wt. 441.4
- Broth for inoculum preparation for microbiological assays of vitamins (Micro vitamin test inoculum broth, Hi Media M133)

- Powdered activated carbon, p.a.
- Potassium chloride (KCl) p.a., mol wt. 74.56
- Sodium chloride (NaCl) p.a., mol wt. 58.44
- Sodium lauryl sulfate or Neutral Extran
- Potassium dihydrogen phosphate (KH_2PO_4), p.a., mol wt. 136.09 g/mol.
- Sodium dihydrogen phosphate (Na_2HPO_4) p.a., mol wt. 141.96
- Sodium hydroxide (NaOH) p.a., mol wt. 40.0
- Medium for folic acid analysis (Folic acid medium AOAC, HiMediaM126)
- Medium for strains maintenance in microbiological assays of vitamins (Micro vitamin test cultura agar, HiMedia M132D)
- Medium for strain propagation Brain Heart Infusion or Trypticase Soy Broth
- Pancreatin
- Toluene (C_7H_8) p.a., mol wt. 92.14, d=0.87 g/mL

VII. Solutions

A. Reagents

1. **Hydrochloric acid-0.1N:** In a 1-L volumetric flask containing around 800 mL distilled water, add 8.3 mL concentrated hydrochloric acid. Agitate and bring to volume with distilled water. Work in a fume hood. Store in a glass container in a cool dark place, separated from bases.

2. **Distilled water treated with activated carbon:** Add 10 mg activated carbon to 1 L distilled water (picture to the left). Agitate and let settle for one day. Filter the solution through filter paper Whatman No. 1 (picture to the right). Prepare solution every time it will be used.

3. **Phosphates buffer-0.1M, pH=7.** Weigh 9 sodium chloride, 0.2 g potassium chloride, 1.44 g Na2HPO4, 0.24 g KH2PO4 and dissolve in 500 mL distilled water treated with activated carbon. Adjust pH to 7 with KOH 4N. Transfer the solution to a 1-L volumetric flask and bring to volume with distilled water treated with activated carbon. Prepare a fresh buffer every time. If stored, sterilize in autoclave at 121-124°C for 30 minutes and keep in the refrigerator.

4. **Sterile glycerine-15% v/v:** Add 17 mL glicerine 87% to a glass bottle with screw cap containing 83 mL isotonic saline solution. Sterilize in autoclave at 121-123°C for 30 minutes and cool down to room temperature. Prepare a fresh solution every working day.

5. **Ammonium hydroxide-(2+3) around 40% v/v:** In a 100-mL volumetric flask containing around 30 mL distilled water, add 40 mL concentrated ammonia (NH3) and bring to volume with distilled water. Store in a plastic container, separated from acid solutions. Solution is stable indefinitely.

6. **Ammonium hydroxide-0.1M:** In a 100-mL volumetric flask with 50 mL distilled water, add 1.4 mL concentrated ammonia and bring to volume with distilled water.

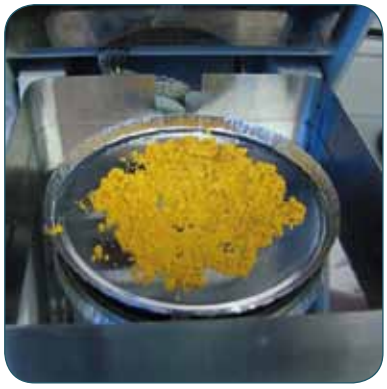
7. **Sodium hydroxide-0.1N:** Weigh 4 g sodium hydroxide. Dissolve in a 500-mL beaker with 300 mL distilled water. Cool the solution down in a cold water bath. Transfer the solution to a 1-L volumetric flask. Wash the beaker with distilled water and transfer the washings to the flask. Bring to volume with distilled water. Store the solution at room temperature in a polyethylene container. Solution is stable indefinitely. Do not use for titrations.

8. **Sodium hydroxide-0.01N:** In a 1-L volumetric flask containing distilled water, add 100 mL sodium hydroxide solution-0.1N and bring to volume with water. Store at room temperature in a polyethylene container. The solution is stable indefinitely.

9. **Sterile isotonic saline solution-0.9% p/v:** Weigh 9 g sodium chloride in a 250-mL, dissolve with 100 mL distilled water. Transfer the solution to a 1-L volumetric flask. Wash the beaker and transfer the washings to the flask. Bring to volume with distilled water. Transfer the solution to a bottle and sterilize in an autoclave at 121-124°C for 30 minutes.

B. Standard solutions of folic acid

1. **Stock solution-100mg/L:** Dry around 1 g standard folic acid (USP) in a porcelain crucible in an oven at 100-110°C to constant weight. As an alternative, it can be dried in a moisture analyzer (see picture below). Moisture content is compared to the maximum moisture content in the certificate and correction for purity and moisture is calculated when calculating the concentration.



2. Weigh 50 mg standard and dissolve with phosphates buffer-0.1M pH=7 and bring to volume to 500 mL. Store the solution in the refrigerator in a dark container with a 2-mm toluene layer. Solution is stable for two months.

Concentration:

- Transfer 10 mL stock standard solution-100 mg/L to a 100-mL volumetric flask and bring to volume with phosphates buffer-0.1M, pH=7.
- Read the absorbance of the solution at 282nm and 346 nm in a UV-spectrophotometer with 1-cm light path cuvettes. Zero the instrument with phosphates buffer 0.1M, pH=7. Get three reading at each wavelength.
- Calculate the average of the absorbances and multiply the results by 160 for the absorbances at 282 nm and by 613.33 for the readings at 346 nm.

3. **Intermediate standard (1)-1mg/L:** Transfer 1 mL stock solution to a 100-mL volumetric flask with around 50 mL distilled water. Adjust pH between 7-8 with HCl or NaOH-0.1 N drop by drop, and using a potenciometer. Bring to volume with distilled water. Store in a dark container with a 2-mm toluene layer. Solution is stable for two weeks.

4. **Intermediate standard (2)-100 ng/mL:** Transfer 10 mL intermediate standard solution-1 to a 100 mL volumetric flask and bring to volume with distilled water. Prepare a fresh soluton every time is needed.

5. **Working solution-1 ng/mL:** Transfer 1mL intermediate solution-2 to a 100-mL volumetric flask and bring to volume with distilled water treated with activated carbon.

C. Culture medium for HiMedia brand

According to the experience in the laboratory, only the amount of medium needed is prepared every time. Follow the instruction in the container to adjust the amount to be weighed according to the volume to be used.

1. **Trypticase soy broth**: Weigh 30 g broth and suspend in 1L distilled water. Heat until all the solids have dissolved. Adjust pH to 7.3±0.2 (25°C) with HCl 0.1N or NaOH 0.1 N. Sterilize in an autoclave at 121-124°C for 15 minutes. Dispense in tubes. Incubate 24 hours at 35-37°C. If no microbial growth is observed, use it or store it. For storage, seal the tubes with parafilm, store at 2-8°C. The medium expires after two months of preparation.

2. **Medium for maintenance of strains used in vitamin microbiological assays (M132D):** Weigh 11.1 g agar, suspend in 1L distilled water and boil 2-3 minutes. Cool down rapidly in a cold water bath. When the medium is at room temperature, the final pH should be 6.7±0.2 (25°C). If not, adjust it, but it should not be necessary. Dispense 10 mL medium in tubes 16-20 mm diameter and sterilize in autoclave at 121-124°C for 15 minutes. Cool down at room temperature on top of an inclined surface to get a slant. Incubate 24 hours at 35-37°C. It can be used if no microbial growth is observed. Seal the tubes with parafilm, store at 2-8°C. The medium expires after two months of preparation.

3. **Inoculum broth for vitamin microbial assays (HiMedia 133):** Weigh 52.1 g medium, suspend in 1 L distilled water. When the medium is at room temperature, the final pH should be 6.7±0.2 (25°C). If not, adjust it, but it should not be necessary. Dispense 10 mL in tubes 16-20mm diameter and sterilize in an autoclave at 121-124°C for 15 minutes. Cool down rapidly in a cold water bath. Incubate 24 hours at 35-37°C. Use it provided that no microbial growth was observed. Seal the tubes with parafilm and store at 2-8°C and discard after two months of preparation.

4. **Medium for folic acid analysis (HiMedia M126):** Weigh 11.1 g medium and suspend in 100 mL distilled water. Let boil 2-3 minutes. Cool down rapidly in a water bath. When the medium is at room temperature, the final pH should be 6.7±0.2 (25°C). If not, adjust it, but it should not be necessary. Prepare the amount needed for each run only.

VIII. Procedure

A. Strain propagation

1. In aseptic conditions, take a minimum quantity of the freeze-dried strain and inoculate in Trypticase soy broth. Incubate 24 hours at 37ºC.*Enterococcushirae* is a facultative anaerobic bacteria. The growth will look as a precipitate at the bottom of the tube (see picture below).

2. After the bacteria have grown in agar for maintenance (Part VIII.B), discard the vial.

B. Strain maintenance (Stock culture)

Once the microorganism has been reconstituted and is growing adequately, prepare fresh stab culture every week. Bacteria growing in this culture are the source of inoculum for the analysis. Do not use for preparing inoculum if it more than one week old.

1. Take a portion of the reconstituted strain and cultivate it in a tube with agar for strain maintenance used in the microbiological assay for vitamins (HiMedia 132D) and incubate 24 h at 37°C. The bacteria will look as shown in the picture below.

2. Store at 4-8°C. Repeat this procedure weekly. Before using new culture in assay, make several successive transfers of culture in 1-2 week period. From the maintenance tube, prepare the inoculum for the analysis of folic acid in food.

C. Preserving the strain for a long time

It is recommended to have a stock culture, to get the bacteria at any time. The strain is preserved in the stock culture for over three months.

1. Inoculate *Enterococcushirae* ATCC 8043 in 1 mLTrypticase soy broth. Incubate 24 hours at 35-37°C.

2. Add 1 mL glycerine-15% and store at -70°C.

3. Repeat this procedure during preservation of the culture in Trypticase soy broth with glycerol-15% from the transference of the strain.

D. Inoculum preparation

1. Transfer cells from the stock culture of *Enterococcushirae* ATCC 8043 to sterile tube containing 10 mL broth for inoculum (HiMedia 133).

2. Incubate 16-18 h at 35-37 °C.

3. Under aseptic conditions, centrifuge culture at 3,000 rpm for 5 minutes and decant the supernatant. Wash cells with 10 mL sterile 0.9% saline solution, resuspending the cells in the pellet by agitation. Repeat this procedure two times more and resuspend the pellet in 10 mL sterile 0.9 % saline solution. This suspension is the stock inoculum.

a) Appearance of the inoculum after centrifugation for the first time. Bacteria cells form a pellet at the bottom of the tube. b) After eliminating the media and resuspending the pellet in isotonic saline solution, the liquid in the tube looks turbid. c) The washed cells form a pellet at the bottom of the tube after the final centrifugation.

4. To obtain the work inoculum, take 10 μL of the stock inoculum and add it to 10 mL sterile isotonic saline solution.

5. A larger quantity of inoculum might be prepared following the proportion of stock inoculum/isotonic saline solution. Store it in 5-mL sterile vials.

6. Label the vials with the medium name, lot number and expiration date, and store in the refrigerator.

7. Use the amount of inoculum needed at room temperature the day of the analysis.

Note: Prior to start analyzing samples, determine the optimum growth time and calibrate the spectrophotometer following the steps described in Steps X and XI.

E. Sample preparation

1. Grind solid samples and homogenize them.
2. In a 250-mL Erlenmeyer flask, weigh 2 g wheat flour.
3. Add 50 mL distilled water treated with activated carbon and mix well.
4. Add 5 mL amylase-10% to the cereal based samples or 1 mL pancreatine-4% to the dairy samples. Cover with a 50-mL beaker or with aluminum paper and incubate at 37°C overnight.
5. Sterilize the solution in an autoclave at 121-123°C for 15 minutes.
6. Mix well and cool the flasks down in a water bath with ice until they reach room temperature.
7. Transfer to a 200 mL volumetric flask and bring to volume with distilled water treated with activated charcoal and agitate the solution.
8. Centrifuge 25 mLsolution at 3,000 rpm for 5 minutes.
9. Take 5 mL supernatant and transfer to a 100 mL volumetric flask with distilled water treated with activated charcoal and agitate.
10. If the solution is turbid, filter through filter paper Whatman No. 5 and if turbidity persists, filter through a 0.45 μm membrane.

F. Microbiological assay

1. Prepare the following tubes with screw cap:

- 3 empty tubes for uninoculated blanks which will be used as sterilization controls.
- 3 empty tubes for inoculated blanks. Controls to check chemical contamination of reagents, media and water used in the analysis, as well as the inoculum purity.
- 10 tubes containing 0.5, 1, 2, 3, 4 and 5 mL folic acid working standard solution-1 ng/mL, respectively. Prepare each volume in duplicate.
- Per sample: 10 tubes containing 0.5, 1, 2, 3 and 4 mL sample final extract, in duplicate.

2. Add distilled water treated with activated charcoal to the tubes to get a final volume of 5 mL, as indicated in the following table:

mL standard or sample/tube	mL distilled water to be added
0 (blanks)	5
0.5	4.5
1	4
2	3
3	2
4	1
5	0

3. Add 5 mL agar for folic acid assays (HiMedia 126) to all the tubes and close them.

4. Sterilize in autoclave for 5 minutes at 121-124°C.

5. When the tubes are out of the autoclave, cool them down as rapidly as practicable to keep color formation at minimum. An ice water bath is useful. Take precautions to keep sterilizing and cooling conditions uniform throughout assay.

6. Aseptically inoculate each tube adding 25 μL inoculum with an automatic pipette and sterile tips, except to the blanks without inoculation.

7. Incubate 16 hours at 35-37°C in a water bath.

8. After incubation, check for growth in the blanks. If any growth is obtained, contamination might have occurred and this invalidates the assay.

The tube to the left shows growth (samples or standards), whereas the solution to the right is clear showing no growth (blanks).

9. Before reading each tube, agitate for 10 seconds in a vortex mixer.

10. Warm the spectrophotometer up and set the wavelength at 550 nm.

11. After 15-30 minutes, adjust the instrument to 100% transmittance with the blanks without inoculation.

12. Read the % transmittance of the inoculated blanks.

13. Set 100% transmittance with the inoculated blanks and read the % transmittance of the rest of the tubes.

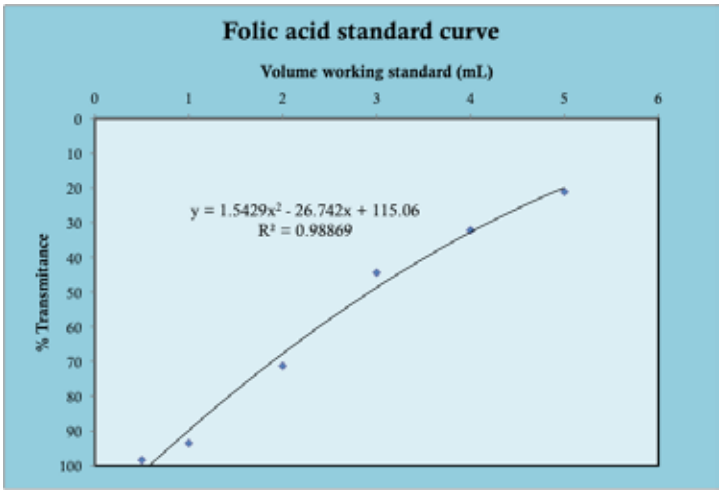
14. Discard the tubes and wash the glassware as described in the glassware cleaning procedure.

IX. Calculations

1. Calculate the average of the %T for each standard concentration.

2. Plot "y" (the average %T)vrs. "x" (volume of standard solution-1 ng/mL in each tube (mL)). A curve similar to the one below will be obtained, which is practically a parabolic curve. Calculate the second-degree equation for the curve which will be:

$$y = ax^2 + bx + c$$



The response curves from different assays will not be necessarily coincident.

3. Determine the "equivalent volume of folic acid standard in each tube" of sample assay solution by interpolating from standard curve or solve the second degree equation for x, using the following formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4a(c - \%T)}}{2a}$$

Do not use the values for 0.5 mL and 5 mL.

4. Divide each value obtained by the quantity in mL sample extract added to each tube. That is 1, 2, 3 or 4 mL. The value obtained will be "the equivalent mL standard/mL".

5. Sum all the values obtained for one sample and then divide by the total number of tubes used (8). This will be the first average.

6. Calculate the range: average (obtained in the previous step) ± 10 %.

7. Calculate a new average after discarding the %T values that are not within the calculated range. Do not discard more than 2/3 values, that is more than 3 values. If more than 3 values are discarded, then the assay is invalid and it must be repeated.

8. Calculate the concentration of folic acid with the following equation:

$$Folic\ acid\,(mg/kg) = \frac{\overline{X}}{1000} x \frac{V_3}{V_2} x \frac{V_1}{w_s}\ x\ C_{std}$$

Where the parameter of the equations are:

Parameter	Explanation	Value
$\overline{X}$	New average after discarding outlier results	?
w_s	Sample weight (g)	?
V1	Initial volume (volume 1)	200 mL
V2	Aliquot volume (volume 2)	5 mL
V3	Volume 2nd dilution (volume 3)	100 mL
C_{std}	Actual concentration stock solution of folic acid (~1 ng/mL)	?

X. Optimum incubation time

1. Prepare two tubes with 0 mL folic acid standard and 5 mL distilled water.
2. Prepare two tubes with 5 mL folic acid standard solution-1 ng/mL.
3. This makes 4 tubes per incubation time. It is recommended to determine the % transmittance of tubes at 16, 18, 20, 22 and 24 hours after inoculating the tubes. Prepare 20 tubes in total.
4. Add distilled water to complete the final volume to 5 mL in the blank tubes.
5. Add 5 mL medium for folic acid analysis (HiMedia 126). Sterilize, cool down and add 25μl inoculum and incubate at 37 °C.
6. Mix and set the wavelenght at 550 nm. Set the 100% transmittance with the tube with 0 mL standard solution. Read the % transmittance of the tubes containing 5 mL standard solution.
7. Repeat this procedure to complete 24 hours. When the change in % transmittance between two different incubation times is lower than 3 units, that is the optimum incubation time.

XI. Spectrophotometer verification

A. Preparation

1. Wash a porcelain crucible with soap, tap water and rinse it with distilled water.
2. Dry the crucible in an oven at 110°C until constant weight.
3. Cool the crucible down in a dessicator and weigh it before using it.
4. Prepare the inoculum as described in the procedure.

B. Verification

1. In an Erlenmeyer flask containing 300 mL medium for folic acid assay (HiMedia 126) aseptically add 1 mL standard folic acid solution-100 μg/mL.

2. Sterilize in autoclave for 5 minutes at 121°C and cool down.

3. Add 1 mL working inoculum suspension.

4. Incubate for 16 hours at 37 °C.

5. Aseptically, centrifuge at 3000 rpm for 5 minutes and wash with 50 mL0.9% sterile saline solution. Repeat this step three times and resuspend the cells in 25 mL isotonic sterile solution.

6. In aseptic conditions and using a sterile volumetric pipette, take 10 mL suspension prepared in step 5 and dispense it in a porcelain crucible.

7. Evaporate this aliquot in a water bath at 100°C.

8. Dry to constant weight in a vacuum oven at 110 ºC.

9. Considering that 10 mL saline solution have 0.09 g sodium chloride, calculate the weight of bacteria (W_B) (g) using the following formula:

$$W_B\ dry(g) = (Crucible\ weight + dry\ solids) - Crucible\ weight - 0.09$$

10. Prepare 50 mL bacteria solution where every mL is equivalent to 0.5 mg dry cells. For this, take an aliquot of solution prepared in step 4. Calculate the volume of this aliquot with the following equation:

$$Aliquot\ volume\ (mL) = 50\,mL\,x\,\frac{10\ mL}{W_B\ dry\ (g)}\ x\,\frac{1\ g}{1000\ mg} \times \frac{0.5\ mg}{1\ mL}$$

11. In different tubes, add 0 (Blank), 1, 1.5, 2, 2.5, 3, 4 and 5 mL diluted aliquot in step 10. Carry out this step in duplicate. Prepare two tubes for blanks without inoculum.

12. Add 0.9%-sterile saline solution to the tubes as necessary to complete the volume to 5 mL.

13. Add 5 mL medium for folic acid assays (HiMedia 126).

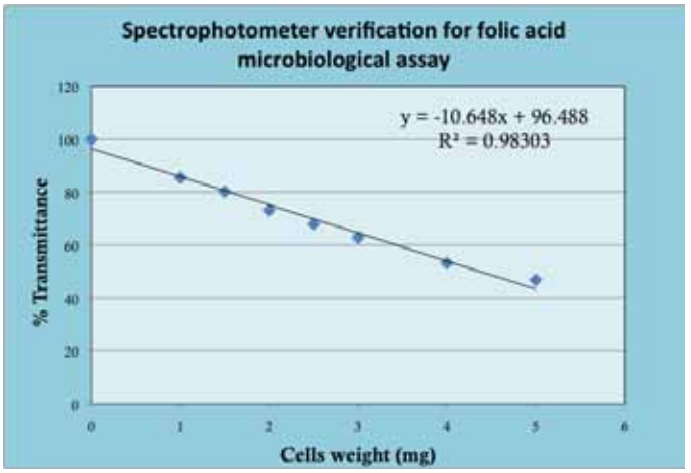
14. Agitate and read at 550 nm, setting 100% transmittance with the tubes with 0 mL or blanks.

C. Calculations

15. Calculate the average of the % transmittance for each tube with the same volume of solution.

16. Calculate the weight of cells per tube (mg cells in dry weight).

17. Plot a chart with the content of cells (mg cells in dry weight, X axis) vrs. % transmittance in each solution, y axis. Calculate the equation and calculate the correlation coefficient.



18. Correlation coefficient should be higher than 0.99 to confirm that there is a relation between the instrument response and the concentration of bacteria in suspension.

Repeat appropriate verification step at least twice more for the spectrophotometer to be used in the assay. Draw composite curve, best representing 3 or more individual curves, relating % T to mg dried cell weight under conditions for the assay.Once appropriate curve is established, all subsequent relationships between % T and cell weight are determined directly from this curve. Respective assay limits expressed as mg dried cells weight/tube are so determined.

XII. Cleaning glassware procedure

1. Autoclave the glassware which was in contact with the bacteria for 30 minutes at 121°C.

2. Rinse the glassware three times with tap water.

3. Soak the glassware in Sodium Lauryl Sulfate (5% v/v) for 24 hours.

4. Clean the test tubes with a brush.

5. Rinse the glassware eight times with tap water.

6. Rinse two times with distilled water.

7. Dry the glassware in the oven at 250 °C.

V. References

- Bowman BA and Russell RM (Eds). **Present Knowledge in Nutrition**. 8th ed. ILSI Press. Washington, DC. 2001. 805 p.

- O'Neil MJ (Ed), Heckelman PE, Koch CB, Roman KJ, Kenny CM, D'Arecca MR.**The Merck Index.** 14th ed. Merck & Co, INC. NJ. USA. 2006.

- Spitzer V (Ed.). **Vitamin Basics The Facts about Vitamins in Nutrition.** 3rd. ed. DSM Nutritional Products. Germany. 2007. 97 p.