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Foreword

Over the last five years, the East, Central and Southern African Health Community (ECSA-HC) has continued to undertake advocacy and technical assistance to assist member countries to embrace and scale up food fortification initiatives as a key strategy to reduce micronutrient malnutrition in the region.

ECSA has been working with partners in direct response to resolutions of the Conference of Health Ministers to scale up food fortification initiatives as a critical strategy in fighting the devastating effects of micronutrient malnutrition among populations of member states. ECSA partners in the Regional Food Fortification Initiative include the A2Z Project, USAID, UNICEF, Micronutrient Initiative (MI), and ICCIDD, among others.

Part of the outcome of the intensified collaborative initiative, is a series of fortification guidelines developed to guide the industry during the fortification process of staple foods and provide government food inspectors a reference point in enforcing the standards.

In order to ensure compliance with the set standards, a manual on laboratory tests for fortified foods was developed. This manual provides agreed upon test methods that have been tested over the years and provided reliable results in the analysis of micronutrients in fortified foods, within the region.

This manual is Part 3 of the Manual for Laboratory Methods for Fortified Foods. It specifies test methods for determination of iron, vitamin A and riboflavin in fortified flours.

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

Steven Shongwe
Executive Secretary
ECSA Health Community

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The manual is as a result of joint work by distinguished food fortification experts in developing countries. During the drafting of this manual, consultations with senior officers from food control departments of the ECSA member states were made and input incorporated.

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ECSA is deeply thankful to the above authors for preparing this manual.

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INTRODUCTION

MANUAL OF LABORATORY METHODS FOR FORTIFIED FOODS

The ability to rapidly test for added micronutrients in fortified foods allows program managers to readily determine if the fortified food complies with the technical specifications, and it is an objective measurement of the program performance.

It is therefore necessary to have easy access to laboratories which can determine the presence and content of common indicator micronutrients such as vitamin A, iron, riboflavin and iodine. The results that are generated from such laboratories provide vital information for establishing if:

(i) the fortification process at the factory level is working properly and micronutrient levels are within specified requirements, based on sampling and testing by the Quality Assurance and Quality Control Department, and on results from inspection and enforcement activities of the Food Control Unit of the government;

(ii) the fortified foods are reaching the retail stores with the expected conditions of fortification; and

(iii) imported fortified foods contain the micronutrient levels required in the national regulations and standards.

Ideally, all methods used for testing micronutrients should be selective, sensitive, accurate, precise, fast, and simple and have a low cost. In practice, the performance, complexity and cost of the methods will depend on several factors such as:

the nature of the matrix (i.e. sugar or salt vs. wheat flour), the fortification compound used to fortify (i.e. reduced iron vs. ferrous salts), the available methods for detecting the analyte or micronutrient and the type of parameter recorded for quantification (i.e. titration volume based on change in color vs. absorbance readings for UV/Vis spectrophotometry). It is difficult to have single methods that fulfill all the characteristics mentioned above and so several methods have been developed for use at different levels of food enforcement, accordingly to the specific conditions and needs. The methods provided in this manual have proved to be cost-effective and adequate for the purposes outlined above. The methods are present in three categories namely qualitative, semi-quantitative and quantitative methods.

Qualitative methods: These are used to determine the presence of a nutrient and are ideal for screening samples to determine if the samples are fortified with the indicator micronutrient. Qualitative methods are usually simple, fast and cheap. These methods may also be used to determine samples which contain the indicator nutrients around a cut-off point in order to estimate percent of fortified food. Where possible, the initial screening of samples earmarked for quantitative tests using qualitative methods is beneficial. It helps in reducing the time and resources wasted in performing complicated and expensive quantitative tests on samples that are presumed fortified but do not contain the nutrient of interest.

2***Semi-quantitative methods:*** These methods are mainly used to monitor the micronutrient levels in the finished product during the fortification process at the factory. These methods are based on their respective qualitative methods, but are adapted to introduce comparative assessment based on intensity of color development or spot density. Quantification is based on comparing color intensity or spot density of fortified samples against standard samples with known amounts of micronutrients. The results are reported as a range of values because of the uncertainties related to the determination of color intensity and spot density by analysts. Despite of this limitation, semi-quantitative methods help to determine

whether the micronutrients added are within a specific acceptable range as determined by the local standards. The advantage of these methods is that they are fast, simple, cheap, and provide concentrations which, together with other factory parameters as amount of premix used per quantity of food produced, can be used for making timely decisions if problems are found. Results obtained using these methods have to be confirmed periodically using quantitative methods, either in the factory laboratory or by sending samples to external laboratories.

Quantitative methods: These methods are meant to accurately determine the concentration of micronutrients in the food. Results from quantitative testing of samples taken by the Food Control Units during inspection activities are crucial for determining whether a factory, distributor or brand is complying with the standards and regulations. Because the concentration levels determined during inspection have legal implications, quantitative, accurate and precise results are a must. These methods are also applicable to the quality control section in factories for verification purposes, and quantitative results are used to justify decisions regarding inspection activities at production centers, importation sites and retail stores. Most quantitative methods for micronutrients are time consuming, need special equipment and skillful and trained technicians, and as a result they are expensive. The only exception is the quantitative test for iodate in salt which is relatively less involving and low cost.

This manual presents analytical methods that have been used in the food fortification programs for several years. The spot test for iron in wheat flour, the methods for determining vitamin A in sugar and oil, and the method for determining iodate/iodine in salt are applied worldwide in the fortification programs. This manual also includes the determination of riboflavin in flours, as an indicator for the vitamins[1]. Other methods have been applied only in some countries, and thorough validation is still needed. This is the case of the method to determine iron from ferrous sulfate in wheat flour.

[1] Although iron is an effective indicator of choice for flour fortification, it is important to confirm compliance of the fortification formula in terms of vitamin content. Vitamin A is usually a vitamin of choice to complement iron determination. However, in cases where vitamin A is not added to the flours, riboflavin is a good alternative.

The following sections are included in the manual:

Definitions and general description of the analytical methods

Methods for determining iron, vitamin A and riboflavin in flours (wheat/maize flour) HPLC determination of Vitamin A in food

(A). DEFINITIONS OF ASSAY PARAMETERS AND GENERAL DESCRIPTION OF THE ANALYTICAL METHODS

I. ASSAY PARAMETERS

In this manual, the following definitions are used to characterize the performance of the analytical assays:

Specificity[2] is the ability of a method to respond exclusively to the target analyte and not to any degrading impurity, or other component of the matrix. Since very few methods are absolutely specific, so the term **selectivity** is often used for this property and is defined as the degree to which a method can quantify the **analyte** (i.e. the micronutrient of interest) accurately in the presence of interferents[3]. The smallest quantity of the analyte that can be distinguished from the background response or analytical noise by the method is known as the **limit of detection**.

Sensitivity[4] is defined for the purpose of this manual as the degree of certainty that an analytical assay can differentiate between two very similar amounts of the analyte. The minimum amount of the analyte that can be quantitatively determined with suitable precision and accuracy is known as **limit of quantification**.

Accuracy is the capacity of the analytical method to determine the amount of the analyte as close as possible to the reality. Frequently, this property is checked by means of spiking the unfortified foods with known amounts of the nutrient (analyte) or analyzing Certified Reference Material (CRM). However, for fortified foods, CRMs are not readily available.

Precision[2] is a general term for the variability among repeated tests under specified conditions. Two types of precision have been found necessary for describing the variability of a test method: 1) within-run variation also known as **repeatability**, and 2) between-run variation also named as **reproducibility**.

Ruggedness defines the degree to which the same method produces the same results in different laboratories and with different laboratory technicians. This is an important parameter to consider when an analytical method has sufficient reliability. Laboratory proficiency testing that compares results from different laboratory for the same samples is a practical way to confirm the ruggedness of a method.

[2] Horwitz W. (Ed.). 2000. AOAC Official Methods of Analysis. 17th edition. AOAC Int., New York. Laboratory Quality Assurance. Appendix E. p3.
[3] Thompson M, Ellison SLR and Wood R. 2002. Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis. (IUPAC Technical Report). ***Pure Appl. Chem.*** 74(5):835–855.

[4] Sensitivity in epidemiology has a different meaning, and it refers to the property of methods to respond to the parameter of interest.

(II). METHODS FOR DETERMINING IRON IN FLOURS

Selectivity of the methods to determine iron will depend whether total iron or iron from a specific source of iron is measured.Spot test using potassium thiocyanate in an acidic solution can detect the presence of iron from NaFeEDTA, which contains iron in the ferric form. Other types of iron (ferrous form and elemental iron) can also be determined using potassium thiocyanate, but in this case the iron forms mustfirst be oxidized to the ferric form using hydrogen peroxide.

Ferrous salts (ferrous sulfate or ferrous fumarate) can specifically be identified by means of their reaction with potassium ferricyanide in hydrochloric acid. Ferrous sulfate reacts fast forming a blue compound, but ferrous fumarate may require a long time or even fail to produce any observable color. Magnetic properties of elemental iron (i.e. reduced or electrolytic iron or other types) are used to identify its presence in flours, making it easier to distinguish it from the other ferrous salts which do not have this property.

Quantitative methods to determine iron are highly selective; visible spectrophotometry or atomic absorption spectrophotometry (AAS) are used for quantifying. This manual contains visible spectrophotometric methods, but once the sample solution is prepared after ashing, the iron content can be measured by using iron-chromogenic agents in visible spectrophotometry, or directly measured specifically for iron by AAS. The choice of method will depend on the availability of equipment in the laboratory. The AAS method may be faster than the spectrophotometric alternative, but the general disadvantage of AAS is that it will determine total iron, regardless of its salt of origin if the sample is ashed.

Special extraction procedures allow extracting ferrous iron from the sample and determining it by visible spectrophotometry. In terms of accuracy and precision, recoveries obtained with visible-spectrophotometric and AAS methods are higher than 90% and reproducibility and repeatability have variations lower than 10%.

Table 1 below summarizes the information for the assays.

Table 1. Comparison of performance parametes for methods to determine iron in flours

Parameter	Spot test (Qualitative and Semi-quantitative)	Visible Spectrophotometry	Atomic Absorption Spectrophotometry
Cost (US$/assay)	US$ 0.10	US$ 5.00	US$ 10.00
Sophisticated equipment	-	++	+++
Selectivity	+++	++	+
Sensitivity	+	+++	+++
Accuracy	+	+++	+++
Precision	+	+++	+++

(III) METHODS FOR DETERMINING VITAMIN A IN FLOURS

The qualitative and semi-quantitative methods for determining vitamin A in flours are based on a colorimetric reaction in which a transient blue color is formed when retinol reacts with trifluoroacetic acid prepared in dichloromethane, or similar chromogenic reagents. Precision (repeatability and reproducibility) for the semi-quantitative method is influenced by the stability of the chromogenic reagent among other things, and the level of vitamin A in flours. In general, semi-quantitative interpretation is difficult, and hence the chromogenic assays are preferable used to identify if the flour contains or not vitamin A.

Measuring vitamin A of fortified wheat flour by spectrophotometry is very difficult, because the complexity of the matrix where some absorbing species are present in the extract, and the low content of vitamin A. Therefore, it is recommended to use a HPLC method. However, with the purpose of simplifying the procedure and reducing costs, samples might be screened first for the presence of vitamin A using a qualitative chromogenic assay.

The HPLC method allows for separation of vitamin A from interferents in the flour matrices. The vitamin A is quantified by comparison with standard retinyl palmitate solution treated in a similar manner. The HPLC methods can differentiate between free retinol and different

retinyl esters although, usually, saponification is necessary to convert all vitamin A into free retinol. The HPLC method has good selectivity and sensitivity, but accuracy and precision depends of the care given to the two crucial steps of extraction and saponification. Variation because repeatability (within-run variation), when using the HPLC, could be lower than 5%, and that of reproducibility could be lower than 10% when performed by an experienced analyst.

Table 2 below summarizes the performance parameters of the different methods for vitamin A, compares approximate costs and dependence on equipment.

Table 2. Comparison of performance parameters formethods to determine vitamin A in different matrices

Parameter	Chromogenic (Qualitative for flour)	UV Spectrophotometry (sugar and oil)	HPLC (flour)
Cost (US$/assay)	US$ 2.00	US$ 7.50	US$ 50.00
Sophisticated equipment	-	++	+++
Selectivity	+++	sugar: +++ oil: ++	+++
Sensitivity	+	sugar: +++ oil: ++	+++
Accuracy	(presence)	+++	+++
Precision	+	++	++

(B). PROCEDURES FOR DETERMININING IRON IN FLOURS

I. SPOT TESTS FOR DETERMINING ADDED IRON IN FORTIFIED WHEAT FLOUR

A. References

AOAC Methods. 12 ed. Ferrous salts. Official Final Action (7.74).

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

Modifications for sequential identification of different types of iron were done by Hana Ali from the Palestinian University of Birzeit, and Omar Dary from A2Z/the USAID Micronutrient and Child Blindness Project.

B. 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. The presence of electrolytic or reduced iron may be determined visually when a magnet is inserted into a flour sample and iron particles stick to it. Ferrous iron can be identified by formation of an insoluble bright blue pigment called Turnbull's blue by reaction with ferricyanide. The reaction is fast with ferrous sulfate, but can be slow or even negative with ferrous fumarate because the low solubility of this salt in water. Reaction may also occur with electrolytic iron after the oxidation of the iron to Fe2+., but the reaction rate is slow.

C. Materials

- Filter paper Whatman # 1
- Manual sieve.
- Watch glass.

D. Reagents

- Hydrochloric acid solution–2N HCl). To a 500 ml beaker, add 100 ml distilled water. Then pour slowly 17 ml of concentrated HCl, and finally 83 mL more of water.
- Hydrochloric acid solution- 0.003N (HCl). Prepare 1 L of solution by adding 600 mL distilled water to a 1 litre volumetric flask. Then pour 1.5 mL of the 2N HCl- and make up to volume with distilled water.
- Potassium Thiocyanate-10%. Dissolve 10 g of KSCN in 100 ml water. Prior to use, mix 10 mL of this solution with 10 mL of the 2N HCl.
- 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.
- Potassium Ferricyanide-10%. Dissolve 10 g of $K_3Fe(CN)_6$ in 100 mL water. Prior to use, mix 10 mL of this solution with 10 mL of 0.003N HCl.

E. Procedure

a. Determining iron from samples containing elemental iron (electrolytic, reduced iron and others)5

1. Take a magnet and insert it into a 1-kg sample of flour.
2. Move it thoroughly inside the sample and then take it out.
3. The presence of electrolytic or reduced iron is shown by the presence of small iron particles on the magnet.

5 The method works better with premixes used for fortifying the flour.

b. Determining iron from samples containing NaFeEDTA

1. Place a filter paper over a 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. Let it stand for a few minutes for the reaction to occur.
5. Red color spots indicate the presence of a ferric salt, such as NaFeEDTA.

c. Determining iron from other sources (including elemental iron)

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 H2O2-solution. Let it stand for a few minutes for the reaction to occur (formation of iron(III)).
6. Red color spots indicate the presence of added iron from any source. [Note: If the result for electrolytic or reduced iron was negative with the magnet test, and the confirming test for ferrous sulfate fails (see next section), then it is likely that iron comes from a ferrous salt].

d. Confirming the presence of a ferrous salt (mainly ferrous sulfate)

1. Place the filter paper over the watch glass
2. Wet the surface of the filter paper with the solution of potassium ferricyanide-10% and 0.003N HCl. Let the liquid penetrate the paper fibers.
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 ferricyanide over the flour layer.
5. Let it stand for a few minutes for the reaction to occur. A fast reaction with clearly distinct blue spots (within 2 minutes of adding the ferricyanide) is indicative of the presence of ferrous sulfate. Ferrous fumarate and some types of elemental iron types may also react but reaction is slow (6-7 minutes or longer). The elemental forms can be identified by the magnetic reaction as described above. If weak brown-greenish spots appear, the reaction is positive for NaFeEDTA.

F. Interpretation

Number and density of spots might be associated to the iron level in the sample. Use flour controls with known amounts of the same type of iron that is added to the flour to make a comparative coarse assessment of the range of the iron content in the samples.

II. QUANTITATIVE SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF TOTAL IRON AND IRON FROM FERROUS SULFATE IN FLOURS AND PREMIXES

A. References

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

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.

B. 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) iron 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); ±,± - 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.

The determination of iron in flours fortified with ferrous sulfate does not require 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 in order to precipitate proteins and avoid the formation of the dough when the flour comes in contact with water. This method is rarely used and its ruggedness still needs to be determined.

Likewise, iron from NaFeEDTA may only need the extraction in water/acetone, without the ashing step, although it still requires the use of hydroxylamine to form the colored complexes with the chromogens mentioned above. Another alternative for NaFeEDTA is to use thiocyanate as a specific chromogen for soluble forms of Fe^{+3}.

[6] A specific method for iron from NaFeEDTA in flours is being developed by AkzoNobel and colleagues carrying out the GAIN-sponsored project in Pakistan. Once this assay has been characterized, authorization will be requested for adding this method in reviewed future versions of this manual.

C. Critical points and precautions

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)$^{-1}$.

For some of the chromogenic agents, it is critical to maintain the pH of solutions between 5-6. If necessary, more sodium acetate can be added to increase the pH.
In the case of ferrozine some interference between iron and other bivalent cations have been reported. However, under the conditions used in the micronutrient premix and fortified wheat flour, the procedure is specific for iron.

D. Equipment and materials

- Agitator vortex
- Analytical balance
- Centrifuge
- Spectrophotometer UV/VIS
- Furnace
- Freezer (or refrigerator)
- Volumetric flasks (25, 100, 250 mL)
- 250 mL Erlenmeyer flasks
- Volumetric and graduate pipettes
- Porcelain crucibles (cups).
- Centrifuge tubes (50mL)
- Graduated cylinders
- Parafilm

E. Reagents

- Sodium acetate trihydrated, ($CH_3COONa.3H_2O$), AR grade, 99% Fe < 200µg/kg, MW= 136. 08.
- Nitric acid (HNO_3), AR grade, 65 %, D = 1.39 g/mL, Fe < 1 µg/mL.
- Hydrochloric acid (HCl), 37%, AR grade, D =1.19 g/mL, Fe < 28 µg/mL, MW=36.46.
- *[7]Trichloroacetic acid (CCl_3CO_2H), 99+%, ACS grade, MW = 163.39
- * Acetone (CH_3COCH_3), AR grade.
- 1,10-phenanthroline-monohydrate, AR grade, MW= 198.23.
- Hydroxylamine hydrochloride ($NH_2OH.HCl$), AR grade, MW= 69.49. (not necessary for determination of iron from $FeSO_4$)
- Acetic a cid (CH_3COOH), AR grade.
- Standards Solution for iron, recommended: Ammoniacal Ferrous Sulfate, $Fe(NH_4)_2(SO_4)_2.6H_2O$, MW=392.14
  Alternative chromogenic agents:
- Bathophenanthroline, disulphonic salt of 4,7- dyphenyl –1, 10 phenanthroline ($C_{24}H_{16}N_2O_6S_2$), MW= 492.5 (anhydrous free acid); or
- ±, ±- dipyridyle (2,2` bipyridyne) ($C_{16}H_8N_2$), MW= 156.19; or Ferrozine, acid [3- (2- pyridyle)-5,6- bis-(4- phenylsulphonic) – 1,2,4 – triazyne dysodic salt, $C_{24}H_{16}N_2O_6S_2$, MW=514.36.

F. Solutions

a. Water: Acetone 80:20 Solution (only for the determination of iron from $FeSO_4$**)**

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.

[7] * Only for determination of iron from $FeSO_4$.

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 under refrigeration. 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.

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

G. 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.

[8] only required for standards made with ferric iron, such as NaFeEDTA, or for digested samples

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.

H. Procedure for Total Iron in Flours

a. Ashing the sample

1. Clean the porcelain crucibles, and label using a marker that withstands high temperatures.
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 for 30 minutes to open the door gradually.
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

9. Add 5 mL of concentrated HNO_3 to the crucible, pouring the acid onto the inside walls of the crucible.
10. Evaporate the acid by heating the crucibles on top of a hot plate at low temperature, solution should not boil.
11. Dissolve the remaining residue by adding 2 mL of conc. HCl, and heat for few minutes, taking care that the solution does not spill out the crucible.
12. 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

13. 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[9]. With the standards, pipet 10.0 mL of the standard solutions prepared in (c) above into 25.0 mL volumetric flasks, and proceed as the samples.
14. Add 5.0 mL acetate buffer and 4.0 mL of 1,10-phenanthroline[10] to each flask. Mix well and color will start developing.
15. Let stand it for 30 min and then make up to volume (25 mL) using deionized water.
16. Turn on the spectrophotometer 15-20 minutes before using it to warm up.
17. Adjust the wavelength to 510 nm[11]. Set the mode to Absorbance.
18. Set the instrument to zero Absorbance using deionized water.
19. Read the absorbance of the 0.0-mg/L standard solution (blank) and record the absorbance.
20. Read the absorbance for the standard solutions and flour sample solutions.
21. If color intensity of the samples is too high, make appropriate dilution of the sample solutions and record the absorbance again.
22. Plot a graph of the absorbance values of the standard solutions (y-axis) against concentration (x-axis) and determine the concentration of iron in the flour sample as described in the section on **calculations**.

9 This reducing step is very important because iron oxidizes to Fe (+3) during ashing and reaction with concentrated acids.
10 In the case of the other chromogenic agents (bathophenantrholine, ±-dipyridyle, or ferrozine) use 2 mL of the solutions instead of 4 mL.
11 For the other chromogenic agents, the corresponding wavelengths are: bathophenantroline: 535 nm; ±-dipyridyle: 521 nm; and ferrozine: 562 nm.

d. Calculation

1. Calculate the regression line of absorbance (y) versus iron concentration (Fe) in mg/L (x) , as presented in the following equation:

   (y) = m **x** (x) **+** cr should be 0.99 or better.

2. Calculate the iron concentration of the sample (mg/L) (x) directly from the regression equation, substituting the value of (y) with the absorbance of the sample. (x) = [(y) **–** c] ÷ m

3. In order to report the content of iron in mg of iron per kilogram of food, multiply the previous result as follows:

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

Where:

PARAMETER	EXPLANATION	VALUE
[Fe]	Iron concentration calculated based on the calibration curve (mg/L) …	?
Vi	Volume of the initial solution (mL) …….	25
w	Weight of the sample(g) about …….	1

The equation can be simplified as:

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

I. Procedure for Iron from $FeSO_4$ in Flours

1. Mix thoroughly 100 g of flour. Weigh 10 g 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). Stir with a magnetic stirrer for 10 minutes.
2. Seal the flask with parafilm and leave it in the freezer for at least 1 -1.5 hr.
3. Decant the supernatant in equal amounts into two centrifuge tubes.
4. Centrifuge (~ 3500 rpm) for at least 15 min.
5. Transfer both supernatants to a 100 mL-volumetric flask. Make up to volume (100 mL) with deionized water.
6. Pipet 10.0 mL aliquots of sample solutions and standard solutions into different 25 mL volumetric flasks.
7. Then, proceed following instructions from step 14 of section H.c above (***Determination of Iron***; i.e. do not add hydroxylamine to the samples).
8. Calculate the concentration of iron in the flour sample using the standard curve as described in the section d above (***Calculations***).

The equation in this case simplifies to:

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

Where ***w*** is around 10.0 g.

J. Procedure for Total Iron Content in Premixes

1. Mix thoroughly the premix sample and weigh accurately 0.25 g (to 3 decimal places) into a clean crucible.
2. Add 80 mL deionized water and 5 mL of concentrated hydrochloric acid (HCl, 37%) or concentrated nitric acid (HNO_3) and heat over a hot plate until the premix is dissolved.
3. Transfer the solution quantitatively to a 250 mL volumetric flask and make up to volume with deionized water.
4. Pipet 5.0 mL of this solution and transfer to a 100 mL volumetric flask. Make up to volume with deionized water and mix well.
5. ***Total iron:*** Follow procedure from step 13 in section H.c above for Total Iron in Flours.
6. Calculate the concentration of iron in the flour sample using the standard curve as described in the section d above (***Calculations***). .

Equation in this case simplifies to:[12]

$$Iron\ (g/kg) = \frac{[Fe]\ x\ 5}{w}$$

Where ***w*** is around 0.250 g.

[12] This value has taken in consideration a dilution 5:100 of the specific step (4) for premixes, as well as division by 1,000 to transform milligrams of iron into grams. The equation also includes the fact that the initial solution was 250 mL instead of 25 mL.

K. Use of other chromogenic agents

a. B2: Bathophenanthroline – 0.05 % in 2M Sodium Acetate.

Into a 150 mL beaker, add 21.76 g of sodium acetate trihydrate (or 13.12 g of anhydrous sodium acetate) and 0.05 g of bathophenanthroline, as disulfonic salt. Add 100 mL of deionized water. Dissolve using a glass rod and apply low heat if necessary. Make sure that the bathophenanthroline is dissolved, because sometimes it does not dissolve well at room temperature. Let solution cool to room temperature and store in a transparent plastic bottle. Discard the solution if it turns pink (due to iron contamination). The solution is stable for 3-4 months.

b. ±. ±- dipyridyle – 0.1% in sodium acetate 2-M.

Into a 150 mL beaker, add 21.76 g of sodium acetate trihydrate (or 13.12 g of anhydrous sodium acetate) and 0.10g of dipyridyle. Add 100 mL of deionized water. Dissolve using a glass rod and low heat if necessary. Make sure that the reagent is dissolved as it dissolves slowly at room temperature. Let solution cool to room temperature and store in a transparent plastic bottle. Discard solution if it becomes pink (due to iron contamination). Solution is stable for 3-4 months.

c. Ferrozine- 0.05 % in sodium acetate 2M

Into a 150 mL beaker, add 21.76 g of trihydrated sodium acetate, (or 13.12 g of anhydrous sodium acetate) and 0.05g of ferrozine, add 100 mL of deionized water. Dissolve using a glass rod and low heat if necessary. Make sure that the reagent dissolves completely as it dissolves slowly at room temperature. Let solution cool and store in a transparent plastic bottle. Discard the solution if it turns pink (due to iron contamination). The solution is stable for 3-4 months

L. Recommended glassware cleaning procedure for mineral analysis

1. Remove all labels and any writing on the glasses with ethanol.
2. Wash the glassware using the normal procedure for laboratories, (washing with glassware detergent, then clean with normal water and finally rinse with distilled water).
3. After that, rinse glassware 5 times with deionized water.
4. Submerge the glassware into a container/bath filled with diluted nitric acid (20% HNO_3, 80% H_2O distilled and deionized water). It has to be totally submerged without air bubbles. Soak the glassware overnight.
5. Put a label outside the bath indicating date, time and quantity of glassware.
6. Take the glassware out carefully using gloves, and leaving the acid inside the container.
7. Rinse the glassware 5 times with deionized water.
8. Let the glassware dry away from any contaminants and keep well covered (with saran wrap).
9. Keep the glassware in new plastic bags, hermetically closed if possible.

C. PROCEDURES FOR DETERMININING VITAMINS IN FLOURS

I. QUALITATIVE METHOD FOR DETERMINING VITAMIN A IN FLOURS

[Developed by Phillip Makhumula[13] and Asumani Ratibu[14] as part of a regional A2Z/ECSA fortification project to support national laboratories in building capacity for determining levels of micronutrients in fortified foods].

A. REFERENCES

- 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.
- The introduction of florisil as an adsorbent for the blue complex resulting from the reaction of vitamin A with the chromogenic reagent was a result of experiments carried out by Dr. Aloysius U. Baes and Dr. Norriel Nipales, from the Philippines, under a contract sponsored by MOST/USAID in 2004-2005.

B. 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. The method described here is based on the principle of the methods listed in the references but displays improved performance in that the vitamin A is more concentrated in an organic extract whose blue color is deeper and sustained over a longer period (at least 7 minutes) as opposed to previous 10-15 seconds15. Another 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 matrix. The blue color lasts for more than one hour before fading to a light brown color.

[13] A2Z/ECSA Consultant in Food Fortification, Analytical Chemist

[14] Senior Laboratory Analyst from Uganda Industrial Research Institute (UIRI), Kampala, Uganda. Laboratory work conducted in UIRI Analytical Chemistry Laboratory (April 2008)

C. CRITICAL POINTS

The chromogenic solutions used produce fumes which makes it important to conduct the tests under controlled laboratory conditions. The disposal of reaction solutions need to 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 a 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 is done using a centrifuge since mixture does not easily separate especially for the extraction of vitamin A from wheat flour.

D. EQUIPMENT AND MATERIALS

- 50mL centrifuge tubes
- Centrifuge to spin 1000 rpm and hold 50 mL tubes
- Eppendorf pipette , to discharge 5 mL
- Balance to weigh 10g, 20g
- Test tubes (15mm x 100mm)
- 50mL Volumetric flask
- Vortex mixer
- Measuring cylinders, 20 mL, 10 mL
- Pasteur Pipettes

[15] Work is still ongoing to improve further on the method and render it useful for semi-quantitative determination of vitamin A in flours. In its present form, the method is ideal as a qualitative method. The performance of other chromogenic solutions will also be investigated.

E. REAGENTS

All reagents are of analytical grade unless otherwise stated.

- 2-propanol
- N-heptane
- Florisil
- Dichloromethane
- Trifluoroacetic acid (TFA) - (F3CCOOH, molar mass – 163.39). Prepare a reagent solutions as follows: to 5 mL of TFA, add dichloromethane and make up to 50 mL. Mix solution thoroughly.
- Distilled water

F. PROCEDURE

1. Weigh 20 g of maize flour/meal (**Use 10 g when testing wheat flour**16) into a 50 mL centrifuge tube
2. Add 20 mL of distilled water using a measuring cylinder and vortex for 1 minute
3. Add 10 mL of 2-propanol and vortex for 1 minute
4. Add 10 mL of n-heptane and vortex for 1 minute
5. Centrifuge the tube for 10 minutes at 1000 rpm.
6. Using a Pasteur pipette, transfer the organic phase (~10mL) to a clean test tube.
7. Using a pipette, transfer 2 mL of the vitamin A extract to another test tube. (If a delay in the disappearance of the blue color beyond 10 minutes is desirable, a spatula full of florisil is added to the test tube at this point).
8. Pipette 3 mL of the TFA reagent solution into the tube containing the 2 mL water/2-propanol extract and mix quickly. **The development of a blue color indicates the presence of vitamin A in the flour.** The color remains visible for 7-10 minutes in solution, and for about one hour when florisil is added.

[16] When testing wheat flour, a paste is formed in step (2) but a suspension is realized on addition of 2-propanol in step (3)

II. DETERMINATION OF VITAMIN A IN FOODS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[17]

A. References

AACC 86-06. Analysis of Vitamin A and E by High Performance Liquid Chromatography.
De Vries J and Silvera K. (2002) Determination of Vitamins (Retinol) and E (alpha-Tocopherol) in Foods by Liquid Chromatography: Collaborative Study. ***J AOAC Int.*** 85(2):424-434.

B. 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. Retinol is quantified in a High Performance Liquid Chromatography (HPLC) system, using UV detection at 328 nm. Concentration is calculated by comparison of peak heights or peak areas of retinol in test samples with those of standards solutions.

C. 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 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 and add boiling chips before heating is begun.

[17] Based on the American Association of Cereal Chemistry **Method 86-06** and reproduced here in part with expressed permission from the Director of Publication and Production, AACC – Minnesota, United States of America. We thank this generous contribution of the AACC to this ECSA project.

D. 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 auto sampling injector with a 20 µL fixed loop having a typical sampling precision of ±0.25% or better Reverse-phase C18 column, 10 µ (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 328 nm.
- Data collection system or integrator
- Erlenmeyer flasks (125 mL) with neck adapted for connecting reflux condenser
- Hot plate
- Reflux condensers
- Volumetric flasks (10,100 and 500 mL)
- Nitrogen blanket apparatus18

E. Reagents

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

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

F. Solutions

a. **Mobile phase:** Combine 860 mL methanol and 140 mL distilled water. Mix well. Stir overnight and degas 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 2-L thick walled Erlenmeyer flask. The solution gives off substantial heat while KOH is dissolving. Add the KOH in 100g 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. **Vitamin A working standard (ca 15 µg/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 95% ethanol. Store at 4°C in dark. Solution is stable for two weeks.

2. **Using retinyl palmitate**: 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 (see below). Add pea-sized piece of pyrogallic acid. Dissolve and dilute to volume with hexane. Pipet 5 mL solution to second 100-mL flask and dilute to volume with 95% ethanol. Store at 4°C in the dark. This working solution is stable for two weeks.

3. **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 100mL with 2-propanol (final concentration is approximately 10 mg per liter). Measure maximum absorbance obtained at 325-328 nm using a 1-cm path length 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 in 2-propanol (1% solution in 1-cm cell), and ***w***=weight of sample in mg.

G. 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 the hot plate to preheat. Start and adjust cooling water flow to precool reflux condensers. Reflux system should be arranged as shown in Figure 1 at the end of this section.
3. Standards
   - High standard: Pipet 5 mL vitamin A working standard into 125-mL Erlenmeyer flask. Add 25 mL 95% ethanol. Proceed to step 5.
   - Intermediate standard: Pipet 2 mL vitamin A working standard into a second 125-mL Erlenmeyer flask. Add 33 mL 95% ethanol. Proceed to step 5.
   - Low standard: Pipet 0.5 mL vitamin A working standard into a third 125-mL Erlenmeyer flask. Add 37.5 mL 95% ethanol. Proceed to step 5.
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 a 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.
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 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 of the 50% KOH solution into each flask and immediately place flask on hot plate under reflux condenser.
9. Reflux for 45 minutes. Swirl flaks 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 cool again to room temperature.
12. Quantitatively transfer solution in each flask to 100 mL volumetric flasks, using a 50:50 THF:ethanol solution. Dilute to volume with same solution.
13. Stopper and invert volumetric flasks 10 times to mix thoroughly.
14. Allow samples to set for at least 1 hour at room temperature and preferably overnight in a refrigerator so as to allow fatty acid salts formed during saponification to precipitate. In some cases, centrifugation may be helpful to reduce settling time.

c. Determination

15. Start HPLC system and allow to warm up and equilibrate for a minimum of 30 min with mobile phase flowing. Flow rate should be 1.0 mL/min.
16. Inject vitamin A standard into the 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.
17. Inject the high, medium and low standards. Adjust detector sensitivity to give peak heights of 50-90% of full scale for the high standard. Repeat injection of standards until peak height(s) are reproducible.
18. Inject sample solutions. In order to ensure consistent performance of the HPLC, inject known standard solution after every nine samples and verify the peak height. (If retinol peak height **exceeds that of 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).

H. Calculations

Calculate concentration of vitamin A as retinol (in mg/kg) as follows:

1. Measure the peak heights or areas of standard solutions and calculate the response factor as follows.

a. Using USP standard

Response factor for vitamin A (RF_A):

$$RF_A = \frac{mg_{std} \times mL_{std} \times C_{std}}{PH_{std} \times 10{,}000}$$

Where

PARAMETER	EXPLANATION	VALUE
mg_{std}=	Mass (in mg) of USP standard reagent used in F.d1	?
mL_{std}=	mL of standard used in procedure step G.b3	?
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 retinyl palmitate

Response factor for vitamin A (RF_A):

$$RF_A = \frac{mg_{std} \times mL_{std} \times P_{std} \times 0.5458}{PH_{std} \times 200}$$

Where

PARAMETER	EXPLANATION	VALUE
mg_{std}=	mg retinyl palmitate weighed in reagent step F.d2	?
mL_{std}=	mL of standard used in procedure step G.b3	?
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
200 =	combined dilution factors and conversion from mg to µg	200

2. RF_A values of low, medium and high standards (from G.b3) should agree with each other within 3% since detector response should be linear across the concentration range used here. The 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) may be present in some samples. Measure the ***13-cis*** peak also.

4. Multiply the height or area of the ***13-cis*** retinol peak by 1.08 (to compensate for difference in absorbance compared to that of the ***trans*** isomer).

5. Add the corrected peak height or area for ***13-cis*** isomer to that of the ***all-trans*** isomer to give total sample peak height or area.

$$Vita\min A, mg/kg\ (as\ retinol) = \frac{RF_A\ x\ PH_{sam}\ x\ 100}{w}$$

Where: PH_{sam} = total sample peak height or area of ***all-trans*** and ***13-cis*** retinol 100 = dilution volume of sample w = weight of sample i

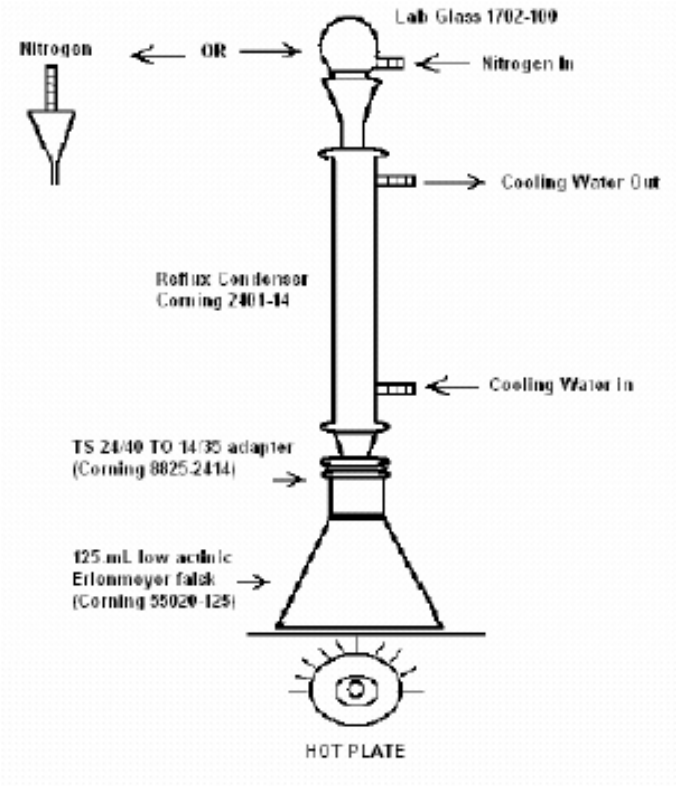


Figure 1. Reflux apparatus.

III. DETERMINATION OF RIBOFLAVIN FROM FORTIFICATION IN FLOURS

A. References

Schüep, W. y Steiner, K. Determination of 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.

B. Principle

Riboflavin added in fortification is extracted from the sample in an autoclave with dilute sulfuric acid. The extract 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.

C. Critical points and precautions

Riboflavin is labile to light; therefore, samples and sample solutions must be protected from light at all times. 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 residue from the mobile phase. Then, wash it with methanol. NEVER leave the mobile phase in the column.

D. Equipment and materials

- Autoclave
- Analytical balance (± 0.0001 g)
- Centrifuge (3000 rpm)
- Agitator Vortex type
- 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 50 µL fixed loop having a typical sampling precision of ±0.25% or better Reverse-phase C18 column, 10 µ 4.6x250 mm)
- Fluorescent detector. Excitation wavelength: 423 nm, emission wavelength: 521 nm.
- 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

E. Reagents

- **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.
- **Amylase-10% w/v:** Weigh 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.
- **Sodium acetate-2M:** (CH_3COONa), 99.5%. Dissolve 164 g of sodium acetate anhydrous in distilled water and dilute to 1 L.
- **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.
- **Citric acid solution-200 mg/L**: Dissolve 200 mg citric acid in 1 L distilled water.
- **Mobile phase**: Combine 300 mL methanol and 700 mL of the 200 mg/L citric acid solution.. Mix well. Degas this solution prior to use.
- **Alternative mobile phase[19]: Mobile phase (HTAA:Methanol, 83:17)**

Prepare the mobile phase as follows

1. **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: 188.2g/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.
2. 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
   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. Weigh accurately 100 mg of riboflavin into a 1L-volumetric flask and dissolve in acetic acid-0.2M. Make up to volume with acetic acid-0.2M.

- **Riboflavin working standard solution-0.12 mg/L:** Dilute 1.0 mL of the stock standard solution in water and make up to100 mL with distilled water. Transfer 3 mL of this solution to a 25 mL volumetric flask and make up to volume with water. Prepare this solution every time it is needed and discard any remaining solution.

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

F. 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.
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 mL of 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 for minimum of 1 hour with mobile phase flowing.
    Flow rate should be 1.0 mL/min.

11. Adjust flow to 1.5 mL/min and inject the 0.12 mg/L riboflavin solution-into the HPLC system.

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.

G. Calculations

Riboflavin concentration is calculated using the following equation:

$$Riboflavin(mg/100g) = \frac{H_s}{H_{std}} x \frac{C_{std}}{R} x \frac{Vi}{W_m} x \frac{D}{10}$$

Where:

Parameter	Explanation	Value
H_s	peak height of Riboflavin in the sample	?
H_{std}	peak height of Riboflavin in the standard solution	?
C_{std}	Concentration of the riboflavin standard solution (µg/mL)	0.12
D	Sample dilution	3
V_i	Initial volume (mL)	100
R	Recovery (%)	98
W_m	Sample weight (g)	?

ANNEX 1. INTERNAL QUALITY CONTROL OF METHODS

A. References

Thompson M and Wood R. 1995. “Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories”. ***Pure & Appl. Chem***. **67**(4):649-666.
Westward JO, Barry PL and Hunt MR. 1981. “A Multi-Rule Shewhart Chart for Quality Control in Clinical Chemistry”. ***Clin. Chem.*** **27**(3):493-501.

B. Principle

Guaranteeing reliability of results reported by a laboratory is one of the major challenges laboratories face everyday. The implementation of an internal quality control program to monitor the performance of methods and results of measurements to decide whether they are reliable enough to be informed is fundamental in the analytical laboratory.

This chapter has been included as a guide to implement an internal quality control program in the laboratory for the analytical methods. The steps here refer to sugar, but they can be also adapted to sugar premix, oil, flours and salt. The program comprises preparing a control sample of the fortified food (in-house reference material) that is run along the samples received in the laboratory. The average micronutrient concentration and its standard deviation are determined for the control sample. A Shewhart chart is built based on these parameters and control limits are plotted.

These control limits are numeric limits within which the control sample values should fall in every run. The control sample values are assessed every time samples are analyzed to verify that the analytical process is within control. Inhouse control material is used here for two reasons: 1) Certified Reference Materials (CRM’s) for fortified foods are not available, except for some flours; and 2) analyzing the CRM in every run would be too expensive.

C. Procedure

1. Take about 15 kg unfortified sugar and fortify it with 10-15 g vitamin A premix to obtain a retinol level between 10-15 mg/kg. Homogenize the sugar. This amount of control sample should last 3-4 months.
2. Determine the retinol content in the control sample until 20 values are obtained. The analysis should be performed by different analysts preferably.
3. ***Control parameters:*** Calculate the arithmetic mean, the standard deviation and the coefficient of variation.
4. Build a Shewhart chart (Levy-Jennings) setting the days or runs (1 to 20) in which the control will be used along the x- axis. The y-axis corresponds to the retinol concentration expressed in mg/kg.

5. Plot the values for the mean, mean ± SD (normal limits), mean ± 2 SD (caution limits) and the mean ± 3 SD (action or rejection limits)[20]. It is recommended to use colors to differentiate the limits to facilitate the visual interpretation of results. Then, green is assigned to the retinol mean, blue to the normal limits, orange to the caution limits and red to the action or rejection limits. Table F-1 presents an example of the table to record results and the chart obtained.

6. Every time sugar samples are analyzed, take a portion of the control and determine its retinol content using the same method applied to the samples.

7. Calculate the retinol concentration in the control and plot the results in the chart.

D. Interpretation

The analytical system is out of control when:

1. The control value falls outside the action limits (mean ± 3 SD). The run is rejected and samples should be analyzed again.

2. The control values from the current and previous runs fall outside the caution limits (mean ± 2 SD), but within the action limits.

3. Nine consecutive control values fall on the same side of the mean. In the case of retinol this can happen because of the decrease of retinol concentration in the control. Therefore, determine again the control limits or change the control.

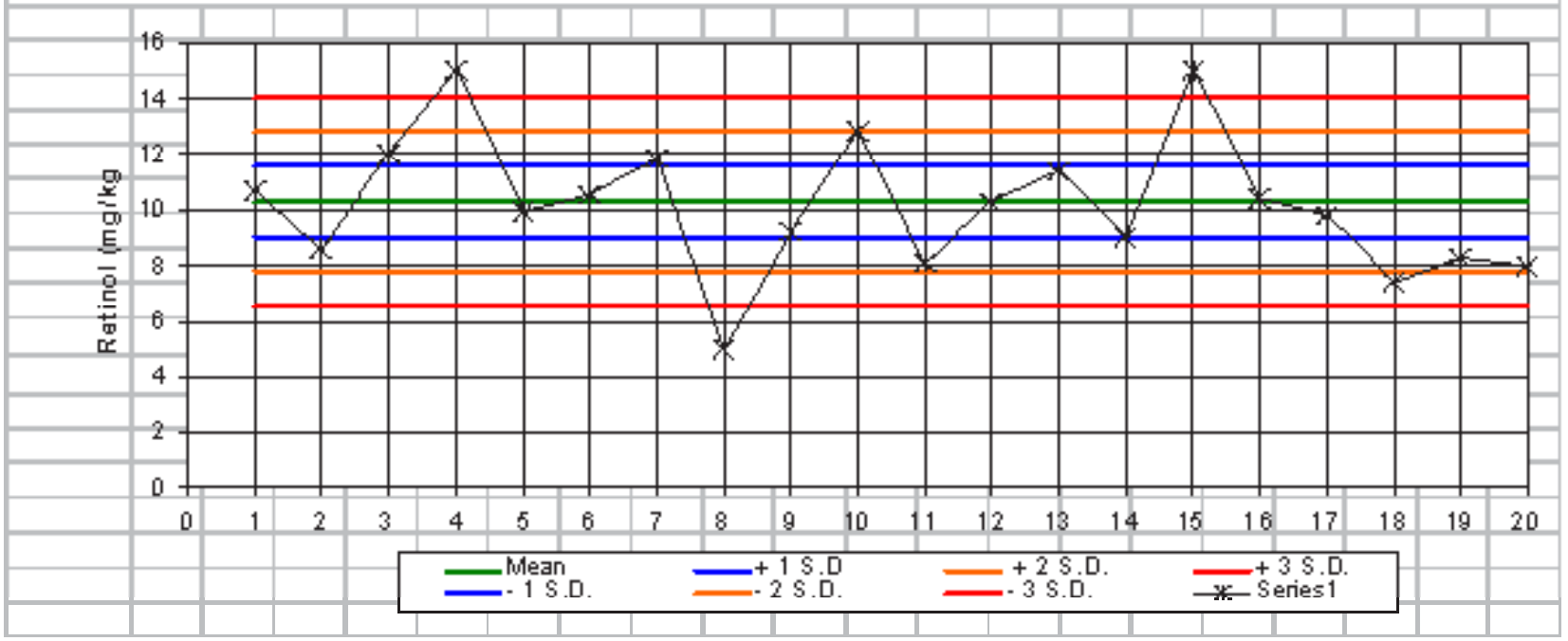
4. If none of the conditions mentioned above are found, the run is accepted.

[20] The caution and control limits could be modified based on the reproducibility and expected variation of the method.

TABLE 1

INTERNAL QUALITY CONTROL OF THE SPECTROPHOTO METRIC METHOD FOR DETERMINING RETINOL IN SUGAR FORTIFIED WITH VITAMIN A

Control:	Sugar									Method:	Spectrophotometric determination of retinol in sugar										
Analyte:	Retinol									Expressed in:	mg/kg										
Year:	2007																				
Date																					
R-1																					
R-2																					
Sum																					
Mean																					
S.D.																					
C.V.																					



ANNEX 2. PROCEDURES FOR PERIODICAL VERIFICATION OF THE SPECTROPHOTOMETER

A. References

Martin, P.G. 1986. ***Manual of food quality control. 1. The food control laboratory.*** Food and Agriculture Organization of the United Nations. Rome. 67 pp.

B. Principle

The periodic checkup of the spectrophotometer is carried out in order to confirm its adequate operation, utilizing potassium dichromate standard solutions in an acid medium. A shift in the wavelength maximum is determined obtaining an absorption spectrum potassium dichromate solution (maximum at 351 nm). The photometric accuracy and linearity of the spectrophotometer are determined by means of a calibration curve of different concentrations of potassium dichromate at the indicated wavelength.

C. Equipment and materials

- Analytical balance (± 0.0001 g)
- Beaker 50 mL
- Cuvettes for spectrophotometer (1 cm)
- Glass rod
- Spatula
- Tongs for crucible
- Asbestos plate
- Crucible
- Desiccator
- Oven (100–110 °C)
- Spectrophotometer
- Volumetric flask (100, 500, 1 L)
- Volumetric pipette of (0.5, 25, 50 mL)

D. Reagents

- Concentrated Sulfuric acid analar. (H_2SO_4), 95-97%, 98.08 g/mol, 1.84 g/mL 0.01N Sulfuric acid
- Potassium dichromate ($K_2Cr_2Or_7$), primary standard, (M.W. 294.19 g/mol)
- Potassium dichromate stock solution-1mM
- Potassium dichromate 0.0625 mM, 0.125-mM, 0.200-mM, 0.250-mM

E. Procedure

a. Wavelength and photometric accuracy

1. Turn on the visible and UV lamps of the instrument 10 minutes before you start reading.
2. Set the zero of the instrument with the solution of sulfuric acid -0.01 N at each one of the wavelengths you will read. If the instrument has double beam, place also the solution of sulfuric acid in the reference cuvette.
3. Scan the potassium dichromate-0.200 mM solution, between 210 and 400 nm. Read the absorbance of the solution at the following wavelengths: 235, 257, 313 and 350 nm.

b. Calibration curve

4. Set the wavelength of the apparatus to 351 nm (theoretical maximum of absorption of potassium dichromate in the visible range).
5. Set the zero of the instrument with the 0.01 N-sulfuric acid solution.
6. Read the absorbance of the potassium dichromate of 0.0625, 0.125, 0.200, and 0.250 mM, in triplicate.

F. Calculations

a. Absorption spectrum

1. Plot the wavelength (x) vs. absorbance (y) to obtain the absorption spectrum.
2. Determine the wavelength of maximum absorption of the potassium dichromate solution.

b. Calibration curve and extinction coefficient

3. Calculate the average of the absorbance of each solution.
4. Calculate the equation for the set of dichromate solutions-mM (x) vs. the average absorbance.

c. Experimental extinction coefficient:

5. Multiply the slope by 3.399. The calculated value is the extinction coefficient of potassium dichromate ($mg^{-1}cm^{-1}mL$) in acid medium. The theoretical value is 10.741 $mg^{-1}cm^{-1}mL$.

d. Correction factor:

6. Divide the experimental extinction coefficient by 10.741 and the result is the correction factor for the apparatus.

e. Photometric accuracy

7. The following absorbances (± 1% full scale) using the solution of 0.2 mM-potassium dichromate should occur at the indicated wavelengths if the spectrophotometer is working adequately:

Wavelength (nm) Absorbance
235 0.747
257 0.869
313 0.293
350 0.644

ANNEX 3. SUPPLIERS FOR LABORATORY EQUIPMENT AND REAGENTS

1. **Sigma-Aldrich**
   3050 Spruce St.
   St. Louis, MO 63103
   Tel: (314) 771-5765; Fax: (314) 286-7817
   www.sigmaaldrich.com

2. **BASF-Denmark**
   Health & Nutrition
   Malmparken 5
   2750 Ballenip
   Tel: (45) 447-30100; Fax: (45) 447-30101
   www.corporate.basf.com

3. **Taylor Scientific,**
   950 Hanley Industrial Court
   St Louis, Missouri, MO 63144
   USA
   Tel: +1-314-962-5555
   Fax: +1-314-962-9382; Toll Free: 800-727-0467
   www.taylorscientific.com

4. **Beckman Coulter, Inc.**
   4300 N. Harbor Boulevard
   Fullerton, CA 92634-3100
   PO Box 3100
   Tel: (800) 742-2345; Fax: (800) 643-4366
   www.beckmancoulter.com

5. **Fisher Scientific**
   2000 Park Lane
   Pittsburgh, PA 15275
   USA
   Tel: (800) 766-7000; Fax: (800) 926-1166
   www.fishersci.com

6. **DSM Human Nutrition & Health**
   PO Box 3255
   Building 241
   CH-4002, Basel
   Switzerland
   Tel: 41(61)688-3333; Fax: 41(61) 688-3330
   www.dsm.com

7. **Mallinckrodt Baker, Inc.**
222 Red School Lane
Phillipsburg, NJ 08865
USA
Tel: (908)859-2151; Fax: (908) 859-9318
www.mallbaker.com

8. **Millipore**
290 Concord Rd
Billerica, MA 01821
USA
Tel: (978) 715-4321
www.millipore.com

9. **National Institute of Standards and Technology (NIST)**
Standard Reference Materials Program
100 Bureau Drive Stop 2300
Gaithersburg, MD 20899-2330
Tel: (301) 975-6776; Fax: (301) 948-3730
www.nist.gov

10. **Perkin Elmer, Life and Analytical Sciences, Inc.**
710 Bridgeport Ave.
Shelton, CT 06484-4794, USA
Tel: (203) 925-4600; Fax: (203) 944-4904
www.perkinelmer.com

11. **Merck KGaA**
Frankfurter St. 250
6293 Darmstadt, Germany
Tel: (49) 6151-72-0; Fax: (49) 6151-72-2000
www.merck.de

12. **EMD Chemicals Inc.**
Analytics & Reagents Division, Life Science Prod. Division
480 South Democrat Rd
Gibbstown, NJ 08027. USA
Tel: (856) 423-6300; Fax: (856) 423-4389
www.emdchemicals.com

13. **Cole Palmer Instruments Co**
635 East Bunker Court
Vernon Hills, IL 60061-1844, USA
Tel: (847) 323-4340
www.coleparmer.com