East, Central and Southern African
Health Community

MANUAL OF LABORATORY METHODS FOR FORTIFIED FOODS

(Vitamin A, Riboflavin, Iron and Iodine)

PART II

(DETERMINATION OF VITAMIN A IN SUGAR AND OIL)

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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 2 of the Manual for Laboratory Methods for Fortified Foods. It specifies test methods for determination of vitamin A in sugar and oil.

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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Disclaimer

The content of this manual can be adapted to suit country specific contexts. In such a case, the content of the resulting document will be the sole responsibility of the organization adapting the manual and will not represent the views of the authors and that of the ECSA-HC. The Use of the content of this manual should be duly acknowledged.

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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 suchlaboratories 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.

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, which is included as reference, and should be used cautiously. In any case, laboratories using these methods are encouraged to carry out a single-laboratory validation to verify the performance parameters such as precision, recovery, linearity to mention some.

The following sections are included in the manual:

- Definitions and general description of the analytical methods
- Methods for determining vitamin A in sugar and sugar premix
- Methods for determining vitamin A in oil
- HPLC Method for determining Vitamin A in foods

[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.

(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 VITAMIN A IN SUGAR AND OIL

The qualitative and semi-quantitative methods for determining vitamin A in sugar and oil are based on a colorimetric reaction in which a transient blue color is formed when retinol reacts with trichloroacetic acid prepared in dichloromethane, or similar chromogenic reagents. The spectrophotometric quantitative methods for vitamin A are based on the dispersion of the matrix of the fortified food to release vitamin A (retinol and retinyl esters), and then this is followed by extraction of the vitamin A into an appropriate organic solvent. The UV absorbance of the organic extract at 325-326 nm is determined by a UV spectrophotometer. In the case of sugar, the absorbance is solely due to vitamin A, and hence the method is highly selective. For oil, the method is selective but there are other components that may also show absorbance at this wavelength. In order to correct for this effect, variations in the method have been introduced such as running a sample of unfortified oil along with the fortified samples to obtain the blank absorbance. Alternatively, the final extract is irradiated with UV light to destroy any vitamin A present and concentration is based on the difference in absorbance before and after irradiation. It is assumed that the difference in absorbance corresponds only to vitamin A.

In the HPLC method -provided in this manual for reference- the vitamin A is quantified by comparing with standard retinyl palmitate solutions treated in a similar manner. The method can differentiate between free retinol and different retinyl esters, although saponification is usually recommended and hence all vitamin A is transformed into free retinol.

This HPLC method has good selectivity and sensitivity but accuracy and precision of these methods depends of the care given to crucial steps of extraction and saponification. For sugar and oil, recoveries of spiked samples (a measurement of accuracy) are 90% when hexane is used, but it varies depending on the organic solvent used in the extraction. For example, when a mixture of diethyl ether: petroleum ether (1:1) is used, recovery is about 95%, but this mixture is difficult to use especially in warm or highaltitude places. In flours, recoveries are typically 85% or better, and it will depend on the saponification process used.

Repeatability (within-run variation) of the spectrophotometric method for vitamin A in sugar has a variation lower than 5% for extracts from the same weighed sample. Repeatability may be higher in sugar due to the heterogeneity of the sugar samples as a consequence of limitations associated with mixing solid particles of different sizes (micronutrient beadlets and sugar crystals). The repeatability has a 10% or lower variation for extracts from two different replicates of the same sample repeatability and reproducibility of the UV Spectrophotometry method are better when testing vitamin A in oil than in sugar because a liquid sample is more homogeneous than solid samples.

Repeatability when using the HPLC could be lower than 5% and reproducibility could be lower than 10% when performed by an experienced analyst. Precision (repeatability and reproducibility) for the semi-quantitative method is influenced by the stability of the chromogenic reagent among other things.

Table 1 below summarizes the performance parameters of the different methods for vitamin A, compares approximate costs and dependence on equipment.

Table 1. Comparison of performance parameters for methods to determine vitamin A in different matrices

Parameter	Chromogenic (Qualitative)	Chromogenic (Semi-quantitative)	UV Spectrophotometry (sugar and oil)	HPLC (flour)
Cost (US$/assay)	US$0.20	US$ 2.00	US$ 7.50	US$ 50.00
Sophisticated equipment	-	-	++	+++
Selectivity	+++	+++	sugar: +++ oil: ++	+++
Sensitivity	-	+	sugar: +++ oil: ++	+++
Accuracy	(presence)	+ (range is reported)	+++	+++
Precision	+	+	++	++

(B). PROCEDURES FOR DETERMINING VITAMIN A IN FORTIFIED SUGAR AND SUGAR PREMIX

I. FIXED CUT-OFF POINT METHOD TO DETERMINE VITAMIN A IN SUGAR (QUALITATIVE METHOD)

A. References

Calzia R, Martinez C, Dominguez P and Dary O. A cut-off point method to determine vitamin A in sugar and other foods for fast screening in monitoring programs. W35. Institute of Nutrition of Central America and Panama (INCAP/PAHO), Universidad de San Carlos de Guatemala. Presented in the XXI IVACG Meeting, Marrakech, Morocco. 3-5 February, 2003.

Bayfield, R.F. and Cole, E.R. 1980. Colorimetric determination of vitamin A with trichloroacetic acid. In: McCormick, D.B. and Wright, L.D., Eds. *Methods in Enzymology, part F. Vitamins and Coenzymes*. Vol. 67. New York: Academic Press. pp 189-195.

Arroyave, G., Pineda, O. y Funes, C. de. 1974. Enriquecimiento de azúcar con vitamina A. Método rápido para la fácil inspección del proceso. *Arch. Latinoamer. Nutr*. **24**: 155-159.

Principle

This method is qualitative and determines the presence of retinol in sugar at a concentration around the established cutoff points of either 3.5 mg/kg or 5.0 mg/kg. The cut-off point is the minimum concentration that can be detected with relatively good selectivity.

Retinol present in sugar reacts with trichloroacetic acid to form anhydroretinol. During the reaction, a blue color can be observed indicating the presence of retinol in the sample. The blue color is transient, so if the color develops, it must be observed within 10 seconds after adding the reagent.

B. Critical points and cautions

The reagent should be used within 5 days if stored at room temperature and within 14 days if refrigerated. If acetic anhydride is added to the solution, the chromogenic reagent is stable at room temperature for at least 18 days. If refrigerated, it should be removed from the refrigerator 2 to 3 hours prior to use. If necessary, it can be warmed in a water bath between 30-40$_{o}$C. If crystals develop, they can be dissolved by manual agitation of the container.

The chromogenic reagent is corrosive and should be handled with care by trained personnel. Immediately before use, the volume required should be transferred to a beaker, from which it can be drawn into a pipette before being added to the sugar solution. The reagent goes turbid in a humid environment, so it must be kept capped until needed. In addition, the beaker into which it is poured must be dry and at room temperature. After the completion of the analysis, any remaining reagent in the beaker should be discarded appropriately and NOT returned to its original container.

C. Equipment and materials

- Analytical balance
- Hot plate
- Beakers (25, 250 and 600 mL)
- Graduated cylinder (100 and 10 mL)
- Wide-mouth dark glass bottle (to collect used reagent)
- Dark glass bottle with glass stopper
- Disposable rubber gloves
- Test tubes (15mm x100 mm)
- Plastic spoon
- Watch glass
- Polyethylene pasteur pipette
- Glass rod

D. Reagents

- Chromogenic reagent: Trichloroacetic acid/Dichloromethane/acetic anhydride for cut-off point -3.5 mg/kg. Mix 60.0 g trichloroacetic acid (FW: 163.39, 99.5%) with 80.0 g dichloromethane (60.6 mL) (FW: 84.93, 99.5%, d=1.32 g/mL). To dissolve completely, warm up the mixture in a water bath at 50°C stirring constantly. Add 2 mL of acetic anhydride (FW: 102.092) and store in a dark bottle with glass stopper, preferably in a refrigerator.

Table D-1 below shows the amount of reagents needed for both cut-off point 3.5 mg/kg and 5 mg/kg.

TABLE B-1. CHROMOGENIC REAGENT COMPOSITION DEPENDING ON THE CUT-OFF POINT

Reagent	Retinol cut-off point	
	3.5 mg/Kg	5 mg/Kg
Trichloroacetic acid	60 g	30 g
Dichloromethane	80 g (60.6 mL)	80 g (60.6 mL)
Acetic anhydride	2 mL	2 mL

C. Procedure

1. Homogenize the sugar samples within their bags, with gentle rotary movements.
2. In a test tube, place about 1-g sugar measured with a plastic spoon.
3. Add 2 mL distilled water at 35° – 40 °C and dissolve the sugar.
4. Using a polyethylene Pasteur pipette, add 1 mL chromogenic reagent. Write down the result as positive (+) or negative (-) only. If the result is positive, that is retinol is present in levels around the fixed cut-off point, a blue color will be observed.

F. Interpretation of results

Positive results: The result is positive when the color of the solution turns blue or light blue after the addition of the chromogenic agent. The intensity of the color will vary depending on the concentration of vitamin A in the sample. When vitamin A concentration is low, a few sugar crystals will turn light blue and deposit at the bottom of the tube slowly. Register this result as positive.

Negative results: When the light blue color is barely visible or no change in color is observed, the result is negative.

II. SEMI-QUANTITATIVE METHOD FOR DETERMINING VITAMIN A IN FORTIFIED SUGAR

A. References

Arroyave, G., O. Pineda, and C. de Funes, (1974). Enriquecimiento de Azúcar con Vitamina A. Método Rápido parala Fácil Inspección del Proceso. *Arch. Latinoamer. Nutr.* 24: 155-159.

Bayfield, R.F. and E.R. Cole. (1980). Colorimetric Determination of vitamin A with Trichloroacetic Acid. In: *Methods in Enzymology*, vol. 67. Vitamins and Coenzymes, Part F. Eds: McCormick, D.B. and L.D. Wright. Academic Press, New York. pp 189-195.

B. Principle

The method described here is a modification of that proposed by Arroyave, Pineda, and Funes (1974). This method is based on the formation of anhydroretinol when retinol is mixed with a chromogenic reagent prepared by dissolving trichloroacetic acid in dichloromethane. A blue complex is formed and the intensity of the color can be measured semi-quantitatively by visual comparison against a reference scale of copper sulfate solutions. The blue color is transient, so the comparison should be done within 10 seconds of adding the reagent.

C. Critical Points and Cautions

The chromogenic reagent has to be prepared frequently because it is unstable. The reagent should be used within 5 days if stored at room temperature and within 14 days if refrigerated. If acetic anhydride is added to the solution, the chromogenic reagent is stable at room temperature for at least 18 days. If refrigerated, it should be removed from the refrigerator 2 to 3 hours prior to use. If necessary, it can be warmed in a water bath between 30-40$_{o}$C. If crystals develop, they can be dissolved by manual agitation of the container. To verify the quality of the reagent, a control with a known concentration of vitamin A in sugar should be analyzed at the same time, and the intensity of the blue color should match the expected intensity according to the reference scale.

The chromogenic reagent is corrosive and should be handled with care by trained personnel. Immediately before use, the volume required should be transferred to a beaker, from where it can be drawn into a syringe before being added to the sugar solution. A syringe rather than a pipette is used because the addition of the reagent should be vigorous and rapid. The reagent goes turbid in a humid environment, so it must be kept capped until needed. In addition, the beaker into which it is poured must be dry and at room temperature. Any reagent in the beaker that is not used should be discarded appropriately and NOT returned to its original container.

D. Equipment and Materials

- Balance
- Beaker (50-100mL)
- Colorimetric scale of copper sulfate solutions
- Disposable rubber gloves
- Glass test tubes (15mm x 100mm)
- Plastic bottle (50mL)
- Wide mouth glass bottle (to collect used reagent)
- Water bath (50-60°C)
- Bottle (500mL) or thermos (for distilled water)
- Dark glass bottle with glass stopper
- Glass syringe (5-10mL) with 3 cm teflon[5] tip
- Graduated pipettes (10 mL)
- Watch glass

E. Reagents

Chromogenic reagent: Trichloroacetic acid/Dichloromethane/acetic anhydride Mix 120.0 g trichloroacetic acid (FW: 163.39, 99.5%) with 80.0 g dichloromethane (60.6 mL) (FW: 84.93, 99.5%, d=1.32 g/mL). To dissolve completely, warm the mixture in a water bath at 50°C stirring constantly. Add 2 mL of acetic anhydride (FW: 102.092) and store in a dark bottle with glass stopper, preferably in a refrigerator. The chromogenic reagent prepared as stated is sufficient for 25-30 samples.

- Colorimetric scale

[5] The tip material must be resistant to dichloromethane

Prepare the following dilutions from a 300g/L stock solution of copper sulfate ($CuSO_4 \cdot 5H_2O$).

Volume (mL) $CuSO_4 \cdot 5H_2O$-300 g/L to prepare 10 mL	Concentration $CuSO_4 \cdot 5H_2O$ (g/L)	Equivalent Concentration of retinol (mg/kg)
1	30	5
2	60	10
3	90	15
4	120	20

Make up to volume (10 mL) with distilled water.

Measure 5 mL each of the copper sulfate standard solutions into exactly the same type of tubes in which the samples will be analyzed. Close the tubes tightly using a rubber stopper or a screw cap. It is better if the tubes are completely sealed to avoid evaporation of the solution. Identify each tube with its number, indicating the **concentration of retinol in mg/kg** that the color represents. These solutions are stable and can be kept indefinitely at room temperature.

F. Procedure

a. Solubilizing vitamin A from the fortified sugar

1. Mix the sugar sample thoroughly.
2. In a 250-mL beaker, weigh 50-g sugar.
3. Add 50-mL distilled water at 50-60°C. Dissolve the sugar, heating the solution if necessary.
4. Cool solution to room temperature.

b. Preparing for colorimetric reaction

5. Transfer 1-mL sugar solution to a test tube (a tube previously marked at a 1-mL level can be used).
6. Decant enough chromogenic reagent for all the samples to be tested into a clean glass beaker.
7. Wearing disposable rubber gloves, add 3 mL of chromogenic reagent to the sugar sample solution in the test tube using a syringe. Mix immediately and vigorously.
8. Compare the intensity of the blue color of the samples with the copper sulfate standards within 10 seconds of adding the reagent, because the color change is transient.
9. Estimate the approximate concentration of retinol in the sugar sample (mg/kg) by matching the color developed to the closest tube in the reference scale. In most instances, the intensity of the blue color of the sample will fall between two of the reference tubes. The level of retinol in the sugar should be reported as falling within the range corresponding to the reference tubes. For example, if the blue intensity of the sample solution lies somewhere between the levels of 30 and 60 g/L copper sulfate standard solutions, the retinol leve is between 5 and 10 mg/kg. Do not attempt to be more precise.

c. Discharging the used reagents

10. Discard residual chromogenic reagent, including the sugar-reagent mixture, into a glass bottle containing dissolved sodium bicarbonate, slowly adding the reagent to the bottle. The bottle should be clearly labeled as a waste bottle.
11. After the bottle is filled, the content can be discarded appropriately as other organic waste material, burning it in a chemical incinerator equipped with an afterburner and scrubber.

III. QUANTITATIVE SPECTROPHOTOMETRIC METHOD FOR DETERMINING VITAMIN A IN FORTIFIED SUGAR

A. References

Arroyave G. and Funes C. de (1974) Enriquecimiento de Azúcar con Vitamina A. Método para la Determinación Cuantitativa de Retinol en Azúcar Blanca de Mesa. *Arch. Latinoamer. Nutr.* **24**:147-153.

B. Principle

This method is an adaptation of the method developed by Arroyave and Funes (1974). The procedure uses five to ten times less reagent volume than the original method, and its accuracy is similar. In the past, 20 g sugar sample were used and it has been increased to 100 g in order to improve the precision and reproducibility of the analysis.

Sugar sample is dissolved in warm water to dissolve the matrix of vitamin A fortificant compound. The sugar solution is diluted 1:2 with sodium hydroxide and then, the released vitamin A (as retinyl palmitate) is extracted into hexane. Retinyl palmitate concentration is determined by recording the absorbance of this solution at 326 nm. This method does not usually require irradiation with UV light, because the absorbance of the extract at 326 nm is mainly due to the retinyl palmitate in sugar. Fortified sugar is essentially made of pure sugar and the premix constituents which do not interfere with absorbance at 326 nm.

C. Critical Points and Cautions

A spectrophotometer capable of reading 326 nm is essential. This is because the concentration of the retinyl palmitate is measured by spectrophotometric analysis. Given the importance of the spectrophotometer for ensuring the accuracy and reliability of the vitamin A determinations, it should be calibrated frequently following the instructions provided by the manufacturer, especially to confirm the calibration of the monochromator. This confirmation should be carried out frequently and not only when a new lamp is installed.

It is critical that water used to dissolve the sample is 85° C to assure the matrix of the vitamin A compound dissolves completely. Once retinyl palmitate has been extracted in hexane, the analysis should not be interrupted. Based on the experience at the laboratory of the Institute of Nutrition of Central America and Panama (INCAP), if the variability between extracts of the same solution is greater than 5%, the results should be rejected and the extractions repeated.

The recovery of the method is at least 91%.

D. Equipment and Materials

- UV Spectrophotometer (326 nm)
- Beaker (250 mL)
- Vortex mixer
- Test tubes with screw caps (20 and 50 mL)

- Aspiration bulbs for Pasteur pipettes and graduate pipettes
- Black clothing
- Pasteur pipettes
- Spectrophotometer quartz cuvettes (UV)
- Volumetric flasks (200-250 mL)
- Graduated cylinder (100 mL)
- Glass rods
- Spatulas
- Test tube rack
- Volumetric or serologic pipettes (to measure 2, 3 and 8 mL)

E. Reagents

- Absolute ethanol (C_2H_5OH), AR grade, purity=99.8%, FW=46.07, d=0.79 g/mL
- Phenolphthalein solution-1% m/v in ethanol; Phenolphthalein ($C_{20}H_{14}O4$), FW=318.33
- Hexane (C_6H_{14}), AR grade, purity=99%, FW=86.18, d=0.66 g/mL
- Sodium hydroxide solution-0.1 N; Sodium hydroxide (NaOH), purity=97%, FW=40.00

F. Procedure

a. Solubilizing vitamin A from the fortified sugar

1. Homogenize the sugar sample inside the container with gentle rotary movements.

2. Weigh approximately 100 g of sugar, recording the exact weights to two decimal places; place the sugar in a 250-mL beaker and add about 100 mL hot water at 85°C. Use a glass rod to completely dissolve the sample. Cover the beakers with a watch glass or aluminum foil.[6]

3. Cool them to room temperature in a dark place. An ice bath can be used for this purpose.
   Transfer to a 250 mL volumetric flask. Rinse the beaker with small amounts of distilled water and transfer the washings to the volumetric flask. Make up to 250 mL with distilled water and mix.

5. If samples are expected with vitamin A levels above 20 mg/kg, dilute the sugar solution 1:1 with water (same amounts of the sugar solution and water) before proceeding to the following step. This step is going to introduce an additional dilution factor of 2 for the samples with higher content of vitamin A.

b. Extracting vitamin A from the fortified sugar

6. Measure 5 mL of the solution prepared in steps (4 or 5) into a 50 mL test tube. Prepare triplicates for each sample.
7. Add 5 mL of 0.1 N-sodium hydroxide to each tube and mix in a Vortex for 30 seconds.
8. Add 2-3 drops phenolphtalein-1% m/v to the same tubes. Then, add 5 mL absolute ethanol to each tube. Mix in the vortex mixer for 5 seconds.
9. Measure 5 mL of hexane and add it to each tube from step (8). Immediately close with a cap each tube and mix vigorously with the vortex mixer for 30 seconds to ensure complete extraction of the retinyl palmitate. Open the tubes briefly to release the vapor pressure. Allow separation of phases. The aqueous phase has a fuchsia color, and the top organic solvent phase is colorless.

6 This procedure should dissolve sugar and vitamin A premix for most samples. Sometimes samples are difficult to dissolve and they need further heating. In this case place the beakers in a water bath at 55-65°C for 10-15 minutes.

c. Recording absorbance of the extracted vitamin A

10. As soon as possible, transfer the organic phase, using a Pasteur pipette to a 1 cm light path spectrophotometer cuvette and read the absorbance at 326 nm. Adjust the zero of spectrophotometer with hexane before each reading.

G. Calculations

The retinyl palmitate concentration of the sugar sample is calculated using the following equation:

$$\text{retinyl palmitate } (mg/kg) = \frac{Abs_{corrected}}{a} \times \frac{V_{org}}{V_{sugar}} \times \frac{V_i}{w} \times \frac{CF_{spec}}{R} \times D$$

Where:

$$Abs_{corrected} = Abs_{sample} - Abs_{blank}$$

And Abs_{blank} is the average for the three readings, which should be less than 0.050

The equation parameters are:

PARAMETER	EXPLANATION	VALUE
A	Retinyl palmitate absorption coefficient in hexane (mg^{-1} cm^{-1} L)	0.092
V_{org}	Volume of the organic phase (mL)	5.0
V_{super}	Volume of the aliquot analyzed from the sugar solution (mL).....	5.0
V_I	Volume of the initial solution of the sample (mL)	250.0
W	Weight of the sample (g).....	data from weight
R	Recovery	0.906
CF_{spec}	Correction factor of the spectrophotometer. Ideally..	1
D	Dilution Factor: 1 or 2 depending on step (5).....	D

To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinyl palmitate (286.46/524.84 = 0.546), must be taken into consideration. Simplified equations to estimate the unesterified retinol are:

a. For samples without step (5)

$$Retinol\ (mg/kg) = Abs_{corrected}\ x \frac{1639.4}{W} xCF_{spec}$$

b. For samples with step (5): an additional dilution 1:2:

$$Retinol\ (mg/kg) = Abs_{corrected}\ x \frac{3278.8}{W} xCF_{spec}$$

IV. SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN A (RETINOL) IN PREMIX

A. References

Dary, O. and Arroyave, G. (1996*). Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMNI/USAID. pp 9-11.

Henninger, H. Determination of Vitamin A in Dry Vitamin Premixes by Spectrophotometry. In Hofstetter, J*. Analytical Methods for Vitamins in Food/Pharma Premixes*. Vitamins and Fine Chemicals Division, ROCHE. Switzerland. pp 6-7.

B. Principle

This method entails solubilizing water-miscible retinyl palmitate beadlets in hot water, followed by dilution in 2-propanol. The concentration of retinyl palmitate is determined by its spectrophotometric absorbance at 326 nm. This method does not require irradiation with UV light, because the absorbance of the extract at 326 nm is essentially only due to the retinol in the premix.

C. Critical Points and Cautions

Once retinyl palmitate has been solubilized in 2-propanol, the analysis should not be interrupted. Based on the experience at the Institute of Nutrition of Central America and Panama (INCAP) laboratory, if the variability between replicates of the same premix solution is greater than 5 percent, the results should be rejected and the solubilization/extractions repeated. In addition, the results of two independently weighed replicates of the same sample should not differ on average by more than 10 percent. If the variation is greater than 10 percent, the complete procedure should be repeated.

D. Equipment and Materials

- UV Spectrophotometer
- Water bath (50-60°C)
- Beakers (150-200 mL)
- Glass rods
- Spatulas
- Test tube rack
- Volumetric flasks (100 mL)
- Watch glasses
- Vortex mixer
- Aspiration bulbs for Pasteur pipettes and graduate pipettes
- Black clothing
- Pasteur pipettes
- Spectrophotometer UV-cuvettes
- Test tubes with screw caps (20 mL)
- Volumetric or serologic pipettes (to measure 1, 2, 3, 4, 5, 8 mL)

E. Reagents

- 0.1-N HCl solution [Hydrochloric acid (AR). (HCl), purity=37%, FW=36.46 g/mol, d=1.19 g/mL]
- Hexane (AR). (C_6H_{14}), purity=99%, FW=86.18, d=0.66 g/mL
- 2-propanol (AR) (($CH_3CH(OH)CH_3$), purity=99.7%, FW=60.10, d=0.78g/mL

F. Procedure

a. Solubilizing vitamin A from the premix

1. Homogenize the sample mixing it within a medium-size container (providing sufficient space for mixing) with gentle rotary movements.

2. Weigh in duplicate 1.25 g of premix, recording the exact weights to three decimal places. Dissolve the sample with 60-80 mL of distilled hot water (about 80°C) in a 100 mL beaker. Use a glass rod to completely dissolve the sample. Cover each beaker with a watch glass.

3. Incubate in a water bath at 50-60°C for 15 min. Cool to room temperature.

4. Transfer to a 100 mL volumetric flask. Rinse the beaker with small amounts of distilled water; transfer the washings to the volumetric flask, mix well and make up to 100 mL with distilled water and mix. This solution is cloudy.

b. Diluting and extracting vitamin A from the premix solution

5. Measure 2 mL of the solution prepared in step 4 into a 20 mL tube and add 8 mL of 2-propanol (to give a 2:10 dilution). Mix vigorously in a Vortex mixer.

6. Measure 1 mL of the solution prepared in step 5 into a 20 mL tubes and add 9 mL of 2-propanol (to give a 1:10 dilution). Mix using a Vortex mixer for 5 seconds.

7. Transfer in duplicate 3 mL of the solution from step 6 and place in a 20 mL tube. Add 3 mL of 0.1-N hydrochloric acid and 4 mL of hexane. Mix in a Vortex mixer for 30 seconds. After mixing and settling, the organic phase is at the top.

c. Recording absorbance of the extracted vitamin A

8. Adjust the zero of the spectrophotometer with hexane. As soon as possible, transfer the organic phase, using a Pasteur pipette to a 1 cm light path spectrophotometer cuvette and read the absorbance at 326 nm.

G. Calculations

Retinyl palmitate concentration in the premix sample is calculated using the following equation:

$$retinyl\ \ palmitate\ \ (g/kg) = \frac{Abs_{corrected}}{a} \times \frac{V_{osg}}{V_{al}} \times \frac{V_i}{w} \times DF \times CF_{spec}$$

Equation parameters are:

PARAMETER	EXPLANATION	VALUE
$Abs_{Corrected}$	Absorbance of the sample – Absorbance of the blank (hexane) ……. .	?
A	Retinyl palmitate absorption coefficient in hexane (g^{-1} $cm^{-1} \cdot L$) ……. .	92
V_{org}	Volume of the organic phase (mL) …… .	4.0
Val	Volume of the aliquot analyzed from the premix solution (mL)	3.0
V_I	Volume of the initial solution of the sample (mL) ……..	100.0
W	Weight of the sample (g)…….. .	data from weight
DF	Dilution factor (point b.5 and b.6 above) …… .	50
CF_{spec}	Correction factor of the spectrophotometer ……..	1[7]

To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinyl palmitate (286.46/524.84 = 0.546), must be taken into consideration. A simplified equation to estimate the unesterified retinol is:

$$retinol(g/kg) = Abs_{corrected} \; x \frac{39.565}{w} x \, CF_{spec}$$

[7] If the spectrophotomer is in good condition this value should be 1.0.

(C). PROCEDURES FOR DETERMINING VITAMIN A IN FORTIFIED OIL

I. FIXED CUT-OFF POINT METHOD TO DETERMINE VITAMIN A IN OIL (QUALITATIVE METHOD)

A. References

Bayfield, R.F. and Cole, E.R. 1980. Colorimetric determination of vitamin A with trichloroacetic acid. In: McCormick, D.B. and Wright, L.D., Eds. *Methods in Enzymology*, part F. Vitamins and Coenzymes. Vol. 67. New York:

Academic Press. pp 189-195.
Kakuda Y., Firouz J., Varghese A. Comparison of colorimetric assays for vitamin A determination in fortified oil, sugar, salt, flour to HPLC method. No published, 2003.

Makhoka V. and Makhumula P. Qualitative method for detection of vitamin A in fortified oil. Not published, 2007.

B. Principle

This method is qualitative and determines the presence of retinol in oil at a concentration around the cut-off point of 10 mg/L. Retinol present in oil as esters of acetate or palmitate reacts with trifluoroacetic acid (TFA) –or equivalent reagentdissolved in dichloromethane (DCM) to form anhydroretinol. During the reaction of solution of fortified oil and TFA, a blue color is observed indicating the presence of retinol in the sample. The blue color is transient, so if the color develops, it must be observed within 10 seconds after adding the reagent.

C. Critical points and cautions

The chromogenic reagent has to be prepared frequently because it is unstable. The reagent should be used within two weeks if stored at room temperature and within two month if refrigerated. If acetic anhydride is added to the solution, the chromogenic reagent is stable at room temperature for around four months[8]. If refrigerated, it should be removed from the refrigerator 2 to 3 hours prior to use. If necessary, it can be warmed in a water bath between 30-40$_o$C. If crystals develop (in the case of TCA), they can be dissolved by manual agitation of the container. To verify the quality of the reagent, a control with a known concentration of vitamin A in oil should be analyzed at the same time.

After the completion of the analysis, any remaining reagent in the beaker should be discarded appropriately and NOT returned to its original container.

D. Equipment and materials

- Analytical balance
- Graduated cylinder (100 and 25 mL)
- Dark glass bottle with glass stopper
- Test tubes (15mm x100 mm)
- Beakers (50 and 250 mL)
- Wide-mouth dark glass bottle (to collect used reagent)
- Disposable rubber glasses
- Dispensing pipette resistant to dichloromethane

E. Reagents

(a) Chromogenic reagent: Trifluoroacetic acid/Dichloromethane

Mix 15.0 mL trifluoroacetic acid (FW: 114.03, 99.5%) in 120 mL dichloromethane (FW: 84.93, 99.5%, d=1.32 g/mL; DCM is also referred to as methylene dichloride). Store in a brown bottle in a cool environment. When properly stored, the solution has been found to be stable for up to 4 months. The chromogenic reagent prepared as stated is sufficient for 40-45 samples.

[8] This period of time is about a third if TCA is using instead of TFA.

F. Procedure

1. In a test tube, place about 0.5-mL oil.
2. Using a dispensing pipette resistant to dichloromethane, add 3 mL chromogenic reagent. Look for the formation of a blue solution and write down the result as positive (+) or negative (-). If the result is positive, retinol is present in levels around the fixed cut-off point of 10 mg/L.

G. Interpretation of results

Positive results: The result is positive when the color of the solution turns blue or light blue after the addition of the chromogenic agent. The intensity of the color will vary depending on the concentration of vitamin A in the sample.

Register this result as positive.

Negative results: When the light blue color is barely visible or no change in color is observed, the result is negative.

II. SEMI-QUANTITATIVE METHOD FOR DETERMINING VITAMIN A IN FORTIFIED OIL

B. Bayfield, R.F. and E.R. Cole. (1980). Colorimetric Determination of vitamin A with Trichloroacetic Acid. In: *Methods in Enzymology*, Vol 67. Vitamins and Coenzymes, Part F. Eds: McCormick, D.B. and L.D. Wright. Academic Press, New York. pp 189-195. Kakuda Y, Firouz J, Varghese A. Comparison of colorimetric assays for vitamin A determination in fortified oil, sugar, salt, flour to HPLC method. No published, 2003.

C. Principle

The method described is based on the formation of anhydroretinol when retinol or its esters reacts with a chromogenic solution made by dissolving trifluoroacetic acid (TFA) in dichloromethane (DCM). A blue complex is formed and the intensity of the color is proportional to the amount of retinol which can be measured semi-quantitatively by visual comparison against a reference scale of standard copper sulfate solutions. The blue color is transient, so the comparison should be done within 10 seconds of adding the reagent. Other compounds can replace TFA, such as trichloroacetic acid (TCA) and antimony trichloride (Carr Price solution). However, TFA has proved to be easier to handle and does not run cloudy due to moisture absorption as does TCA under humid conditions. DCM is preferred but other solvents such as hexane or chloroform may also be used.

D. Critical Points and Cautions

The chromogenic reagent has to be prepared frequently because it is unstable. The reagent should be used within 2 weeks if stored at room temperature and within 2 months if refrigerated. If acetic anhydride is added to the solution, the chromogenic reagent is stable at room temperature for about four months[9]. If refrigerated, it should be removed from the refrigerator 2 to 3 hours prior to use. If necessary, it can be warmed in a water bath between 30-40°C. If crystals develop (in the case of TCA), they can be dissolved by manual agitation of the container. To verify the quality of the reagent, a control with a known concentration of vitamin A in oil should be analyzed at the same time, and the intensity of the blue color should match the expected intensity according to the reference scale.

[9] This period of time is about a third if TCA is using instead of TFA.

The chromogenic reagent is corrosive and should be handled with care by trained personnel and protective clothing and gloves must be worn. Immediately before use, the volume required should be transferred to a beaker, from where it can be drawn into a syringe before

being added to oil. A syringe rather than a pipette is used because the addition of the reagent should be vigorous and rapid. In addition, the beaker into which it is poured must be dry and at room temperature. Any reagent in the beaker that is not used should be discarded appropriately and NOT returned to its original container.

E. Equipment and Materials

- Balance
- Colorimetric scale of standard copper sulfate solutions
- Disposable rubber gloves
- Glass test tubes (15mm x 100mm)
- Pasteur pipettes and pipettes bulbs
- Wide mouth glass bottle (to collect used reagent)
- Beaker (50-100mL)
- Dark glass bottle with glass stopper
- Glass syringe (5-10mL) with 3 cm Teflon[10] tip
- Graduated pipettes (5-10 mL)
- Volumetric flask, amber (25 mL)

F. Reagents

a. Chromogenic reagent: Trifluoroacetic acid/Dichloromethane

Mix 30.0 mL trifluoroacetic acid (FW: 114.03, 99.5%) in 60 mL dichloromethane (FW: 84.93, 99.5%, d=1.32 g/mL; DCM is also referred to as methylene dichloride). Store in a brown bottle in a cool environment. When properly stored, the solution has been found to be stable for up to 4 months. The chromogenic reagent prepared as stated is sufficient for 25-30 samples.

Colorimetric scale

[10] The tip material must be resistant to dichloromethane

Prepare the following dilutions from a 300 g/L stock solution of copper sulfate ($CuSO_4.5\ H_2O$).

Volume (mL) $CuSO_4.5H_2O$-300 g/L to prepare 10 mL	Concentration of $CuSO_4.\ 5\ H_2O$ (g/L)	Approximate Concentration of Retinol (mg/kg)
1	30	10
2	60	20
3	90	30
4	120	40

Make up to volume (10 mL) with distilled water.

Measure 5 mL of each of the copper sulfate standard solutions into clearly labeled tubes and the type of tubes should be the same as those in which the samples will be analyzed. Close the tubes tightly using a rubber stopper or a screw cap. It is better if the tubes are completely sealed to avoid evaporation of the solution. The color intensity of these copper sulfate solutions are calibrated against the color produced by the reaction of standards retinyl acetate solutions with the chromogenic reagents. It is preferable to express the results in terms of retinol instead of retinyl esters and hence the scale is presented in terms of milligrams of retinol per kilogram. Identify each tube with a number indicating the **concentration of retinol in mg/kg** that the color represents. These standard solutions are stable and can be kept indefinitely at room temperature. Other chromogenic reagents may be used such as trichloroacetic acid (TCA) and antimony trichloride (Carr Price Solution) as described below.

b. **Trichloroacetic acid (TCA):** Dissolve 25 g of TCA (FW: 163.39) in 35 mL of dichloromethane and heat gently to dissolve. Make up the solution to 50 mL with the solvent. Acetic anhydride (15 mL) is also added to increase the stability of the solution normally affected by the presence of moisture. Store in a brown bottle. TCA is readily available and a low cost reagent, but it is corrosive and the complexes formed are less stable than TFA complexes.

c. **Antimony trichloride (Carr Price solution):** The solution is prepared by dissolving 100 g of antimony trichloride ($SbCl_3$, molar mass 228.11) in 300 mL chloroform. Acetic anhydride (15 mL) is also added to increase the stability of the solution normally affected by the presence of moisture. Care should be taken to keep the reagent as dry as possible and away from light.

G. Procedure

a. Diluting the oil sample

1. Place a 25 mL amber volumetric flask on a balance. Amber flasks should be used because vitamin A is light sensitive.
2. Tare the flask and transfer 5.0 g of oil into the flask using a Pasteur pipette. Accurately record the mass to one decimal place.
3. Add DCM to the flask to dissolve the oil and make up to volume and mix thoroughly.

b. Reaction with the chromogenic solution

4. In a tube of similar dimensions to those used for the copper sulfate solutions, pipette 3.0 mL of the TFA solution, and stand next to the tubes containing the copper sulfate solutions.
5. Into this tube containing the TFA, inject (a syringe can be used) rapidly 1.0 mL of the diluted sample solution of oil and mix quickly on a vortex.

c. Interpreting the result

6. Compare the color intensity developed against the set of tubes of copper sulfate within 5 to 10 seconds.
7. Estimate the approximate concentration of retinol in the oil sample by matching the color developed to the closest tube in the reference scale. In most instances, the intensity of the blue color of the sample will fall between two of the reference tubes. The level of retinol in oil should be reported as falling within the range corresponding to the reference tubes. For example, if the intensity of the blue sample solution lies somewhere between the levels of 30 and 60 g/L copper sulfate standard solutions, the retinol level is between 10 and 20 mg/kg. Do not attempt to be more precise.

d. Discharging the used reagents

8. Discard residual chromogenic reagent, including the oil-reagent mixture, into a glass bottle containing dissolved sodium bicarbonate, slowly adding the reagent to the bottle. The bottle should be clearly labeled as a waste bottle.
9. After the bottle is filled, the content can be discarded appropriately in line with local regulations for disposal of hazardous waste. It is recommended to burn it in a chemical incinerator equipped with an after burner and scrubber.

III. QUANTITATIVE SPECTROPHOTOMETRIC METHOD FOR DETERMINING VITAMIN A IN FORTIFIED OIL

A. References

Kambagar, T & Fawzi, A.B. (1978). Spectrophotometric Determination of Vitamin A in Oils and Fats. *J Assoc Off Anal Chem.* 61(3):753-755.

Subramanyam, G.B. & Parrish, D.B. (1976) Colorimetric Reagents for Determining Vitamin A in Feeds and Foods. *J Assoc Off Anal Chem.* 59(5):1125-1136.

B. Principle

The method is applicable to oils fortified with vitamin A in the form of retinyl palmitate or retinyl acetate, and it is based on absorbance of retinol within the UV-VIS region.

Retinol and its esters absorb UV radiation with a maximum of 325 nm. Retinyl esters in the fortified oil are determined by diluting the oil in organic solvents such as dichloromethane, chloroform or hexane, followed by reading the absorbance of the solution at 325 nm. The concentration of retinol is estimated by dividing the absorbance with the extinction coefficient of retinol and its esters in the different solvents. Other substances naturally present in oil such as carotenoids absorb close to 325 nm and so absorbance must be corrected for a blank absorbance of the specific oil using unfortified oil from the same batch. Another option is to read the absorbance of the sample solution before and after exposure to ultraviolet irradiation using a UV Irradiation Chamber described in **Annex 3** of this manual. The difference between the two readings is associated with retinyl esters which are destroyed by the UV-irradiation.

C. Critical Points and Cautions

A spectrophotometer capable of accurately reading absorbance at 325 nm is essential. Given the importance of the spectrophotometer for ensuring the accuracy and reliability of the retinol determinations, it should be calibrated frequently following the instructions provided by the manufacturer, especially to confirm the calibration of the monochromator. This confirmation should be carried out frequently and not only when a new lamp is installed. Low actinic(amber) glassware should be used in the analysis, but if not available, protect samples and glassware containing the samples solutions from light with a piece of black clothing, aluminium foil or install gold-fluorescent light (yellow light) in the room.

D. Equipment and Materials

- UV Spectrophotometer (325 nm)
- Beaker (250 mL)
- Pasteur pipettes
- Volumetric flasks, amber (25 mL)
- Vortex mixer
- Black clothing
- Spectrophotometer quartz cuvettes (UV)
- Graduate pipettes (to measure 2, 3 and 8 mL)

E. Reagents

- Dichloromethane (FW= 84.93, 99.5%, d=1.32 g/mL) or
- Hexane AR. (C_6H_{14}), purity=99%, FW=86.18, d=0.66 g/mL.

F. Procedure

a. the oil sample

1. Place a 25 mL volumetric flask on a balance. Amber flasks should be used because vitamin A is light sensitive.
2. Tare the flask and transfer 2.0 g of oil into the flask using a Pasteur pipette. Accurately record the mass to four decimal places.
3. Add solvent (preferred dichloromethane) to the flask to dissolve the oil and make up to volume and mix thoroughly.
4. Repeat the process above using blank oil (unfortified oil from same batch).

b. Reading the absorbance of samples and unfortified controls

5. Place the solvent used for diluting the samples into 1 cm quartz UV cuvettes and zero the spectrophotometer at 325 nm. Use the solvent as the spectrophotometric blank.
6. Record the absorbance of samples and unfortified controls at 325 nm.

G. Calculations

1. Correct reading of the samples by subtracting the absorbance of the unfortified oil treated in a similar manner. This is the corrected absorbance for the sample to be used for calculations.

2. Estimate the retinyl palmitate concentration of the oil sample using the following equation:

$$\text{retinyl palmitate } (mg/kg) = \frac{Abs_{corrected} \; x \; Vf \; x \; CF_{ppm}}{a \; x \; w}$$

Where $Abs_{corrected} = Abs_{sample} - Abs_{unfortified\ oil}$ and $Abs_{unfortified\ oil}$ is the average absorbance of the unfortified oil treated in similar manner as the samples.

The equation parameters are:

PARAMETER	EXPLANATION	VALUE
A	Retinyl palmitate absorption coefficient in dichloromethane (mg^{-1} cm^{-1} L)	0.094
	or in hexane (mg^{-1} cm^{-1} L)	0.092
Vf	Final volume (mL)	25
W	Weight of the sample (g)	data from weight
CF_{spec}	Correction factor of the spectrophotometer. Ideally	1

To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinyl palmitate (286.46/524.84 = 0.546), must be taken into consideration. A simplified equation to estimate the unesterified retinol, when dichloromethane is used as solvent, is:

$$retinol\ (mg/kg) = Abs_{corrected}\ x \frac{145}{w} x CF_{spec}$$

H. Alternative procedure: Irradiation with UV light in the irradiation chamber[11]

1. Place about 5 mL of the diluted samples into a 10 mm x 75 mm glass test tube transparent to UV light and close it with a cap resistant to dichloromethane or hexane.
2. Irradiate the tubes in the irradiation chamber for 35 minutes (or the time required according to the performance of the irradiation chamber, see **Annex 4**).
3. Adjust the zero of the spectrophotometer with the solvent. Read the absorbance of the irradiated and unirradiated solutions at 325 nm in 1 cm light path quartz cuvettes.
4. Calculate vitamin A concentration with the same equation, but changing the meaning of $Abs_{corrected}$.

$$retinol\ (mg/kg) = Abs_{corrected}\ x \frac{145}{w} x CF_{spec}$$

where $Abs_{corrected}$ is: $Abs_{corrected}$ = (Abs unirradiated sample) – (Abs irradiated sample)

[11] A model of this irradiation chamber is presented in **Annex 3** of this Manual.

IV. DETERMINATION OF VITAMIN A IN FOODS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[12]

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.

[12] 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 apparatus13

E. Reagents

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

13 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 volume0 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 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 path length cell and 2-propanol as blank.

   Calculate purity of retinol palmitate as

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

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):

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

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).

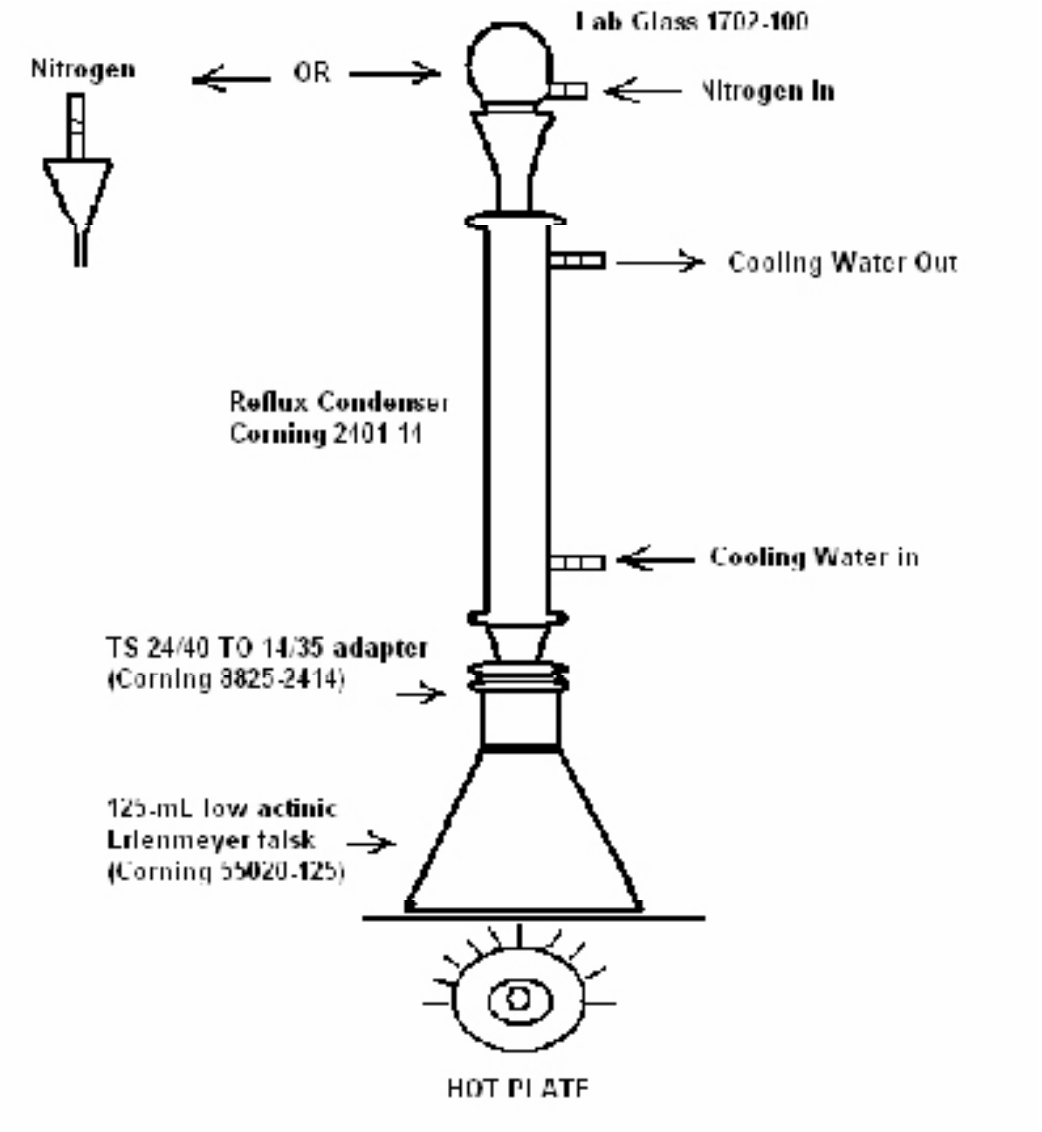
5. Add the corrected peak height or area for *13-cis* isomer to that of the *all-trans* isomer to give total sample peak height or area.

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

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 in 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.

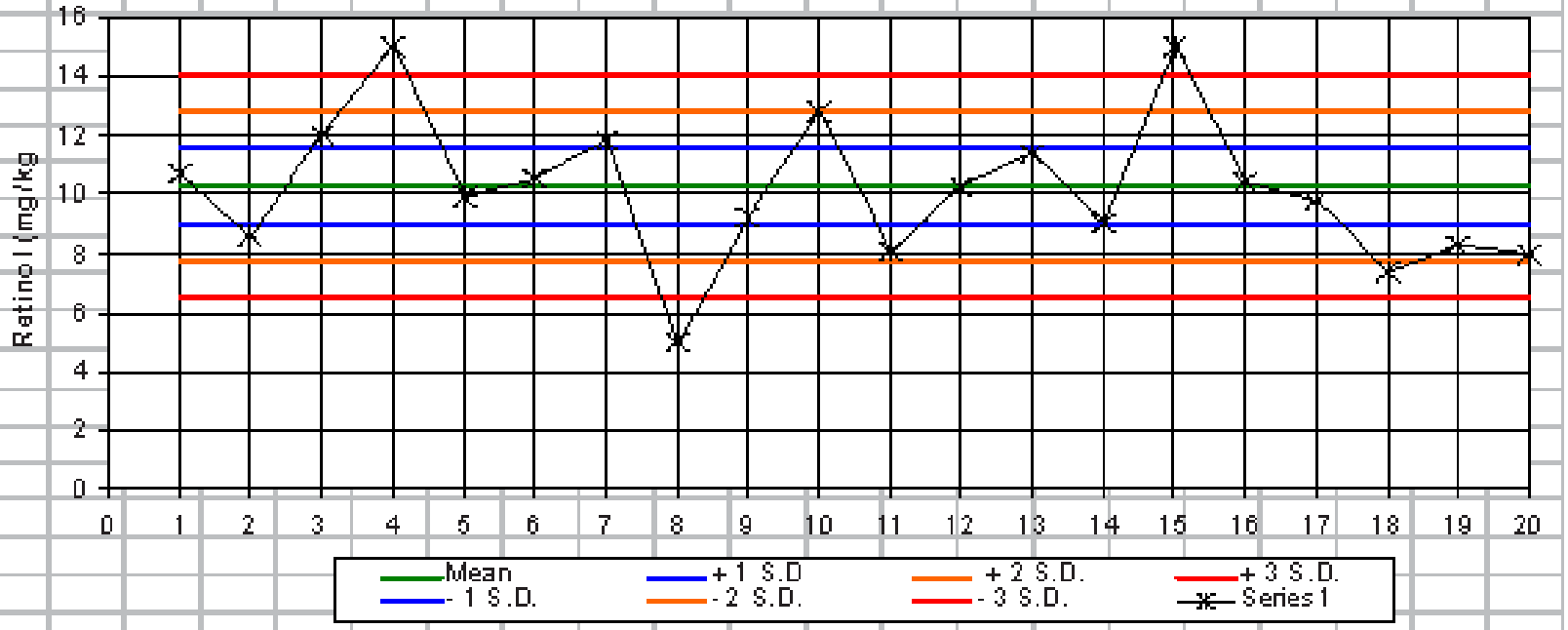
TABLE 1

INTERNAL QUALITY CONTROL OF THE SPECTROPHOTOMETRIC 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.																				

INTERNAL QUALITY CONTROL CHART



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)[14]. 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.

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

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
- Volumetric pipette of (0.5, 25, 50 mL)
- Asbestos plate
- Crucible
- Desiccator
- Oven (100–110 °C)
- Spectrophotometer
- Volumetric flask (100, 500, 1 L)

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. CONSTRUCTION OF AN ULTRAVIOLET IRRADIATION CHAMBER

REFERENCE

Dary, O. and Arroyave, G. (1996*). Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMNI/USAID. pp 71-73.

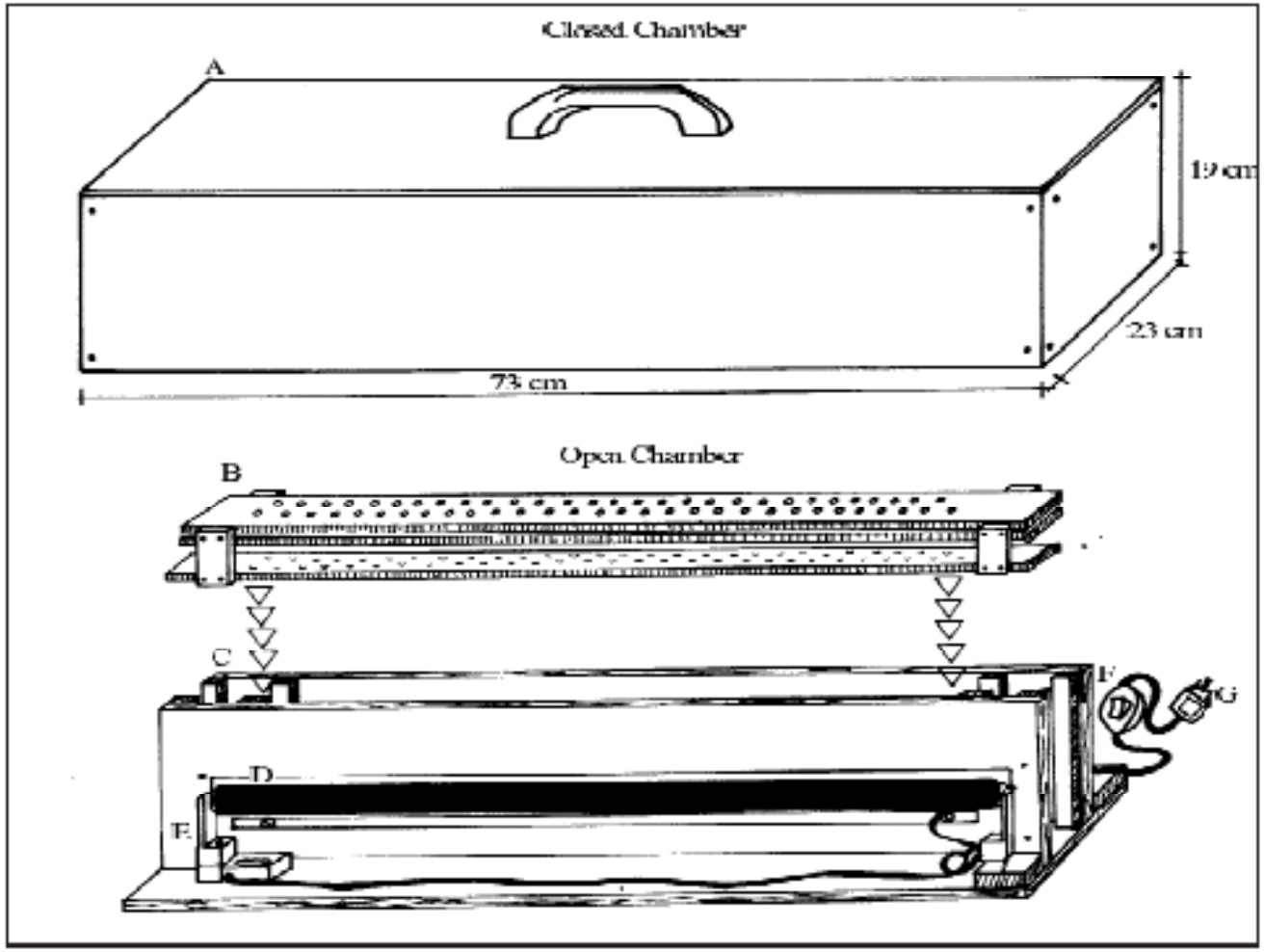
The irradiation unit is a simple, low-cost unit made of wood on which two ultraviolet (UV) lamps (350-390 nm emission) are mounted. The base has two parallel lateral walls (point B, figure 3.1). The lower part of the lateral walls has openings that extend along the length of the walls and expose the test tubes containing the retinol solutions to the UV light (point C, figure 3.1).

The test tube rack slots into the gap between the lateral walls (point D, figure 3.1) where the base contains small depressions to hold the test tubes in place. The holes on one side of the test tube rack alternate with those on the other side. The spacing of the holes on the test tube rack is such that the distance between the centers of any two adjacent tubes is enough to allow the light from both UV lamps to fully reach and expose the extract to the same amount of UV light. In this model, the distance between the holes is two centimeters and the diameter of the test tubes is one centimeter.

The UV lamps and their transformers and starters (point E, figure 3.1) are attached to the base of the unit. Both lamps are operated simultaneously by a switch (point F, figure 3.1) that is outside of the unit. The system is covered by a wooden top (point A, figure 3.1), with a small hole at the side for the electric cord.

Figure 3.2 and 3.3 give details of the construction plan.

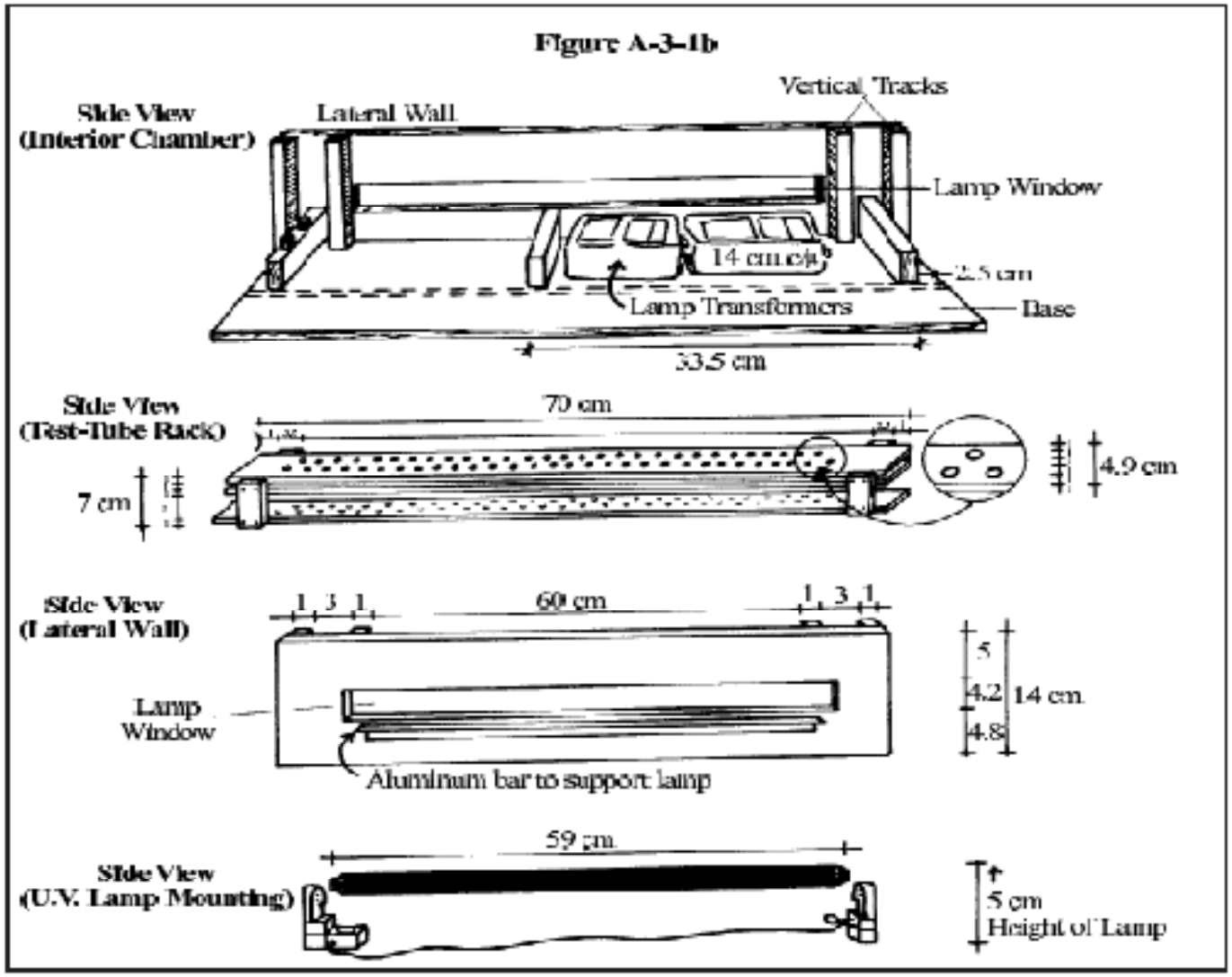
FIGURE 3.1. IRRADIATION CHAMBER



A = wooden cover B = Test Tube Rack C = Lateral Walls D = Lateral Window
E = UV Lampmounting F = Lamp Switch G = Lamp Plug

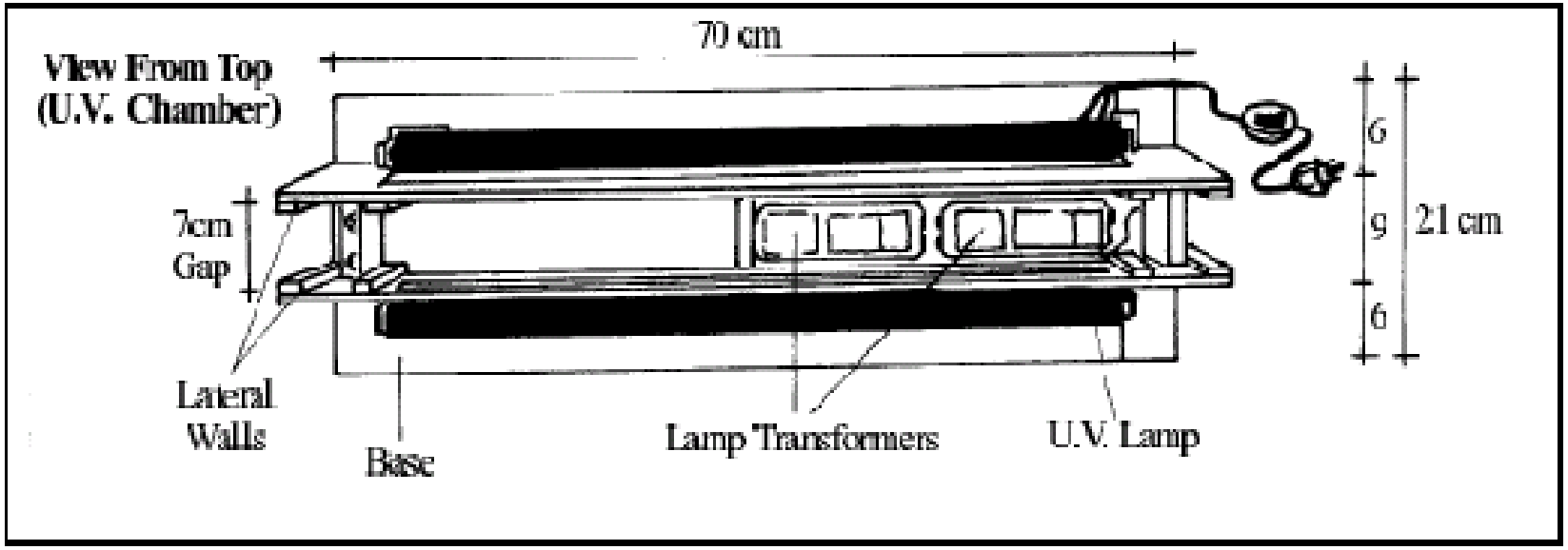
Source: Dary, O. and Arroyave, G. (1996). *Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMNI/USAID.

FIGURE 3.2. DIMENSIONS OF AN IRRADIATION CHAMBER. SIDE VIEW



Source: Dary, O. and Arroyave, G. (1996). *Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMN/USAID.

FIGURE 3.3. DIMENSIONS OF AN IRRADATION CHAMBER. VIEW FROM THE TOP



Source: Dary, O. and Arroyave, G. (1996). *Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMNI/USAID.

ANNEX 4. VERIFICATION OF THE EFFICIENCY OF THE IRRADIATION CHAMBER

A. Reference

Dary, O. and Arroyave, G. (1996*). Sugar Fortification with Vitamin A. Analytical Methods for the Control and Evaluation of Sugar Fortification of Vitamin A. Part 3.* 2nd edition. INCAP/OMNI/USAID. pp 65-68.

B. Principle

The chamber is verified with a standard solution of retinyl palmitate dissolved in the same solvent used for the sample that will be analyzed. Concentration of retinol in the solution should be about 5 µg/mL and its absorbance should be close to 0.9 at 325 nm. The time required to destroy retinol in the standard solution is determined irradiating the retinyl palmitate solution for an hour and measuring the absorbance of the solution every five minutes. The optimal irradiation time is the period of time in which at least 95 percent of the retinol is destroyed.

C. Critical points and precautions

Irradiation is not extended beyond the 95% destruction of retinol because: 1) Destruction of retinol follows an asymptotic logarithmic curve; thus, destruction of the last traces of retinol would take an indefinite period of time that would be impossible to define exactly; 2) the sample extracts may contain substances that are not retinol but that absorb at 325 nm and are destroyed by irradiation after a prolonged period of time; and 3) interfering new complexes that absorb at 325 nm may be formed during prolonged irradiation.

The efficiency of the irradiation unit should be verified periodically. If it is used daily or often, verification should be done at least once a month and every time a new UV lamp is installed. Before the optimal time of irradiation is determined, check that irradiation is uniform along the test tube rack. The retinyl palmitate standard solution should be prepared freshly every time the chamber is verified and protected from direct light at all times. Mark the solvent level in the test tube before placing them in the chamber as evaporation may occur during the irradiation period.

D. Equipment and materials

- Irradiation chamber
- UV/VIS spectrophotometer (325 nm)
- Bulbs for Pasteur pipettes
- Volumetric flask (100 mL)
- Teflon stoppers (for test tubes)
- Stopwatch
- Quartz cuvettes (1 mL, 1 cm light path)
- Test tubes 10mm x 75 mm glass transparent to UV light
- Pasteur pipettes

E. Reagents

- Retinyl palmitate standard solution (aprox. retinol concentration- 5 µg/mL) in organic solvent such as dichloromethane or hexane.

F. Procedure

a. Confirmation of intensity of irradiation for test tubes positions

1. Turn the UV lights in the chamber on and let them warm for about 30 minutes.
2. Fill test tubes with 2 mL retinyl palmitate standard solution and close them with a Teflon cap or any stopper resistant to the solvent being used. Mark with a permanent marker the level of the solvent.
3. Place the test tubes in the chamber and irradiate them for 15 minutes.
4. Check the solvent level in every tube and make up to the mark if necessary.
5. Read the absorbance of the solutions. Do not use the positions that showed the lowest absorbance. This indicates that the UV light does not reach these tubes with the same intensity as the others. These positions are usually the extreme sides of the test tube rack.

b. Verifying the optimal time of irradiation

1. Fill test tubes with 2 mL standard solution. Use three tubes per irradiation time (about 36 tubes)
2. Close the tubes with a Teflon cap or any stopper resistant to the solvent being used. Mark the solvent level in each tube before placing them in the rack.
3. Place the three controls, that is, those not irradiated (0 time) in the dark until read.
4. Every 5 minutes (that is at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 60 minutes) remove randomly three test tubes from the rack and label them with the irradiation time. Check the solvent level and make it to the mark if necessary. Place the tubes in the dark.
5. Continue the process until all the time periods are completed.
6. Adjust the zero of the spectrophotometer using the solvent used to prepare the standard. Read the absorbance of all solutions, including the controls at 325 nm.

G. Calculations

1. Plot a chart the irradiation time (x-axis) against the mean absorbance for the three tubes (y-axis).
2. The optimal irradiation time is the period of time in which at least 95% of the retinol is destroyed.
3. This time should be the one to irradiate the sample extracts. The results have to be corrected by the “efficiency factor”, that is, the percent destruction of the predetermined irradiation time. For example, if it is found that 95% of retinol is destroyed in 35 minutes and this amount of time is used to irradiate the sample extracts, the results are multiplied by 1.053 (100/95).

ANNEX 5. 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

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