East, Central and Southern Africa Health Community

Fostering Regional Cooperation for Better Health

# Laboratory methods for fortified foods
# Part II
# Determination of Vitamin A as retinol in Sugar, Sugar premix and Edible Oils & Fats using Semi-quantitative and Quantitative Spectrophotometric method

Second Edition 2017

## Foreword

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

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

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

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

ii) Inspection and Enforcement; and

iii) Consumption Monitoring and Program Impact. The groups were tasked with identifying capacity and resource gaps and propose ways of filling these gaps in each of the technical areas. Subsequently, they identified priority activities, targets, and developed road maps on how the activities would be

implemented to achieve the set targets. Target 2 of the Inspection and Enforcement Working Group was to review the Regulatory Monitoring Frameworks used by countries in the Region. To inform this review, a workshop was organized for this group at the Imperial Resort Beach Hotel in Entebbe, Uganda from the 7th to 10th November 2016. Its aim was to review the existing guidelines (that countries are using) for gaps and weaknesses and use recommendations from this review to develop harmonized and practical guidelines that all countries can adopt and apply in inspection of fortified foods and specialized nutritional products. A key recommendation of the Entebbe workshop was that the inspection manuals be merged and be developed into two guidelines namely internal and external monitoring of fortified foods and that of commercial and points of entry inspection guidelines. The same meeting recommended that the test methods be reviewed by the laboratory working group which was previously a sub-working group of the inspection and enforcement working group and had become the fourth working group.

ECSA-HC with technical support from GAIN and financial support of USAID hosted a regional food fortification workshop for laboratory analysts between 13 – 16th December 2016 in Nairobi, Kenya that recommended review of the manuals to update of any new updates in the reference methods, format them as guided by ISO/IEC directive 2 and presenting them as a simple test method format for use by the laboratories.

The first editions of ECSA manuals of laboratory methods are recognized as primary reference materials and have guided the development of this edition. In addition, reference has also been made to the latest editions of ISO, AOAC and Codex standards and duly recognized under the bibliography.

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

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

**DIRECTOR GENERAL.**

**ECSA-HC**

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***Disclaimer***

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

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## Acronyms and Abbreviation

| | |
|---|---|
| AOAC | Association of Official Analytical Chemists |
| ECSA – HC | East, Central and Southern Africa – Health Community |
| GAIN | Global Alliance for Improved Nutrition |
| ISO | International Organization for Standardization |
| ISO/IEC | International Organization for Standardization/International Electrotechnical Commission |
| USAID | United States Agency for International Development |
| SWOT | Strength, Weakness, Opportunities and Threats |

## Introduction

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

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

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

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[1] **Accuracy** Is the capacity of the analytical method to determine the amount of the analyte as close as possible to the true value.
[2] **Precision** Is a general term for the variability among repeated tests under specified conditions. Two types can be identified: **repeatability**, or the variation within runs; and **reproducibility** of variations between runs.
[3] **Specificity** is the ability of the method to respond exclusively to the substance that is being measured and not to any degrading impurities or other components present in the food matrix.
[4] **Sensitivity** Is the degree of certainty that an analytical method can differentiate between two very similar amounts of the analyte. The smallest amount of substances in a sample that can accurately be measured by a method is called **limit of detection**.
[5] **Ruggedness** is defined as the degree to which the same method produces the same results in different laboratories and used by different technicians.

This section of the manual is dedicated to method for determination of vitamin A (in the form of retinyl esters) in both sugar and vegetable oil, by an UV/Visible spectrophotometric method. An annex includes a semi-quantitative UV/Vis method based on a chromogenic reaction.

# Determination of vitamin a in sugar, sugar premix, edible oils and Fats

## 1.0 Scope of the method

This part of the ECSA-HC manual of methods for the determination of micronutrients added to fortified foods provides guidance on the use of spectrophotometric method to determine vitamin A in sugar, sugar premix, edible oils and fats as alternative to the use of High-Performance Liquid Chromatography (HPLC). Annexes A and B describe the methods for semi-quantitative determination of retinol in the same food matrixes.

The method is applicable to products fortified with vitamin A in the form of retinyl palmitate or retinyl acetate.

## 2.0 Normative references

ECSA – HC (2007) guidelines on food fortification, 1st Edition.

ECSA –HC (2007) Guidelines on internal and external monitoring of fortified foods 1st Edition.

ECSA – HC (2007) Guidelines on commercial and points of entry inspection 1st Edition.

## 3.0 Terms and Definition

### 3.1 Accuracy

is the capacity of the analytical method to determine the amount of the analyte as close as possible to the true value

### 3.2 Laboratory sample

is a sample as prepared (from the lot) for sending to the laboratory and intended for inspection or testing

### 3.3 Lot

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

### 3.4 Precision

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.

### 3.5 Qualitative analysis

Is a method used to determine the presence or absence of a nutrient and is ideal for screening samples to determine if the samples contain the targeted nutrient.

### 3.6 Semi-quantitative analysis

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

### 3.7 Quantitative analysis

Is a method which accurately determine the content (amount) of micronutrients in the food.

### 3.8 Sensitivity

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

### 3.9 Test portion

Quantity of material drawn from the test sample (or from the laboratory sample if both are the same)

### 3.10 Test sample

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

## 4.0 Principle

### 4.1 Vitamin A in Sugar and sugar premix

Sugar sample is dissolved in warm water to dissolve the matrix and the microencapsulated that contains the palmitate ester of retinol (vitamin A), as this resist dissolution in cold water. Then, the sugar solution is diluted in the ratio 1:2 with a solution of sodium hydroxide. The released vitamin A 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 and not to other compounds present in sugar or the microcapsule. Fortified sugar is essentially made of pure sugar and the premix constituents which do not interfere with absorbance at 326 nm.

### 4.2 Edible fats and oils

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. The difference between the two readings is associated with retinyl esters which are destroyed by the UV-irradiation.

## 5.0 Reagents

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

**5.1 Absolute ethanol ($C_2H_5OH$),** AR grade, purity=99.8%, FW=46.07, d=0.79 g/mL

**5.2 Phenolphthalein solution**-1% m/v in ethanol; Phenolphthalein ($C_{20}H_{14}O4$), FW=318.33

**Note:** weigh 1g of phenolphthalein and dissolve in 100 mL ethanol

**5.3 Sodium hydroxide solution**-0.1 N; Sodium hydroxide (NaOH), purity=97%, FW=40.00

**Note**: weigh 4g of NaOH and dissolve in 1000 mL of distilled water

**5.4 Dichloromethane** (FW= 84.93, 99.5%, d=1.32 g/mL)

**5.5 Hexane AR**. ($C_6H_{14}$), purity=99%, FW=86.18, d=0.66 g/mL.

**5.6 2- Propanol AR $(CH_3)_2CHOH$, FW=60.10**

## 6.0 Apparatus

**Caution**: Amber flasks should be used because vitamin A is light sensitive. Usual laboratory apparatus and, in particular, the following:

**6.1 UV/VIS Spectrophotometer**

**6.2 Vortex mixer**

**6.3 Beaker**, 250 mL

**6.4 Test tubes with screw caps**, 20 mL and 50 mL

**6.5 Aspiration bulbs** for Pasteur pipettes and graduate pipettes

**6.6 Graduated measuring cylinder**,100 mL

**6.7 Graduated pipettes**, 2 mL, 5 mL, 10 mL

**6.8 Black clothing or aluminum foil**

**6.9 Glass rods**

**6.10 Pasteur pipettes**

**6.11 Spatulas**

**6.12 Spectrophotometer quartz cuvettes,** 1cm light path

**6.13 Test tube rack**

**6.14 Volumetric flasks**, 25 mL, 100 mL, 200 mL - 250 mL

**6.15 Analytical balance**

## 7.0 Sampling

A representative laboratory sample from the lot and whose integrity has been maintained during transportation and storage should be sent to the laboratory; at least 50 grams of sugar premix, 500 grams of sugar, and 500 mL of oil.

# 8.0 Procedure

### 8.1 Sugar and sugar premix

### 8.1.1 Solubilizing vitamin A from fortified sugar and sugar premix

a) Homogenize the sugar sample or the sugar premix inside the container with gentle rotary movements.

b) 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 of hot water at 85°C. Use a glass rod to completely dissolve the sample or a magnetic stirrer. Cover the beakers with a watch glass or aluminum foil.

**Note 1:** For premix use approximately 1.25 g

c) Cool them to room temperature in a dark place. An ice bath can be used for this purpose. Transfer quantitatively 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.

**Note3:** For premix bring to 100 mL final volume with distilled water and mix. This solution is cloudy.

### 8.1.2 Extracting Vitamin A from the fortified sugar and sugar premix

a) Measure 5 mL of the solution prepared in steps 8.1.1 c and d into a 20 mL test tube. Prepare triplicates for each sample.

b) Add 5 mL of 0.1 N-sodium hydroxide to each tube and mix on a Vortex for 30 seconds. This is a 1:2 dilution of the original sugar solution.

c) Measure 5 mL of the solution prepared in steps 8.1.2 b into a 20 mL test tube.

d) Add 2-3 drops phenolphtalein-1% m/v to the same tubes. Add 3 mL absolute ethanol to each tube. Mix on the vortex mixer for 5 seconds.

e) Measure 3 mL of hexane and add it to each tube. Immediately close with a cap each tube and mix vigorously on 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 fucsia color, and the top organic solvent phase is colorless.

**Note 4**: The premix requires a different extraction procedure. Thus:

f) Measure 2 mL of the premix solution prepared in section 8.1.1 into a 20 mL tube and add 8 mL of 2-propanol (to give a 2:10 dilution). Mix vigorously in a Vortex mixer.

g) Measure 1 mL of the solution prepared above into a 20 mL tubes and add 9 mL of 2-propanol (to give a 1:10 dilution). Mix vigorously in a Vortex mixer.

h) Transfer in duplicate 3 mL of the last solution in a 20 mL tube. Add 3 mL of 0.1 N-hydrochloric acid and 4 mL of hexane. Mix in a Vortex for 30 seconds. After mixing and settling, the organic phase is at the top.

### 8.1.3 Measure and record absorbance reading of the extracted vitamin A

Adjust the spectrophotometer to zero with hexane as a blank before each reading. 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.

## 8.2 Edible fats and oils

### 8.2.1 Preparation of test portion

a) Weigh 2.0 g of the sample in a 25 mL volumetric flask and accurately record the mass to four decimal places.

b) Add solvent (preferred dichloromethane) to the flask to dissolve the oil and make up to 25 mL volume and mix thoroughly.

c) Repeat the process using blank oil (unfortified oil from a similar batch)

### 8.2.2 Reading absorbance of test portions with spectrophotometer

a) Place the solvent used for diluting the test portion into 1 cm quartz UV cuvettes and zero the spectrophotometer at 325 nm. Use the solvent as the spectrophotometric blank.

b) Record the absorbance of test portion and unfortified blanks at 325 nm.

**Note:** Where a blank sample is not available, irradiation in UV should be done as described in the alternate method below.

### 8.2.3 Alternate method using UV irradiation

**Note: (see annex D for design of Irradiation Chamber. Extract from the first edition (annex 3))**

a) Place about 5 mL of the extracted 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.

Irradiate the tubes in the irradiation chamber for 35 minutes (or the time required according to the performance of the irradiation.

b) 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.

# 9 Calculations

## 9.1 Vitamin A in sugar

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

$$Retinyl\ palmitate\ mg.kg^{-1} = \frac{Abs_{Corrected}}{Q} \times \frac{V_{org}}{V_{sugar}} \times \frac{V_i}{w} \times \frac{CF_{Spec}}{R} \times D$$

Where:

Abs is absorption reading

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

*Note: $Abs_{blank}$ should be average of 3 readings*

$Q$ is retinyl palmitate absorption coefficient in hexane (0.092 $mg^{-1}$ $cm^{-1}$ L)

$V_{org}$ is volume of the organic phase (3 mL)

$V_i$ is volume of the initial solution of the sample (250 mL)

$V_{sugar}$ is the volume of the aliquot analyzed from the sugar solution ( 5 mL)

w is weight of the sample (100 g)

R is recovery (0.905, for example[6])

[6] The recovery factor should be calculated by each laboratory, according to Thompson *et al.*, *Pure & Appl. Chem.*; 1999; **71** (2): 337–348, Harmonized Guidelines for the Use of Recovery Information in Analytical Measurement. If a recovery factor is used, it should be declared in the results report.

$CF_{spec}$ is correction factor of the spectrophotometer (1 if the spectrophotometer is in good condition, has received periodic maintenance and has been verified)

D is the dilution factor x 2

**IMPORTANT:** To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinol palmitate (1866.45/524.84 = 0.546) should be taken in consideration.

The above equation is simplified to:

$$Retinol \frac{mg}{kg} = Abs_{corrected} \times \frac{1967.3}{w} \times CF_{Spec}$$

**9.2 Vitamin A in sugar premix**

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

$$Retinyl\ palmitate\ g.kg^{-1} = \frac{Abs_{Corrected}}{Q} \times \frac{V_{org}}{V_{premix}} \times \frac{V_i}{w} \times \frac{CF_{Spec}}{} \times D$$

Where:

Abs is absorption reading

$Abs_{Corrected} = Abs_{sample} - Abs_{blank\ (hexane)}$

$Q$ is retinyl palmitate absorption coefficient in hexane (92 $g^{-1}$ $cm^{-1}$ L)

$V_{org}$ is volume of the organic phase (4 mL)

$V_i$ is volume of the initial solution of the sample (100 mL)

$V_{premix}$ is the volume of the aliquot analyzed from the sugar premix solution (3 mL)

w is weight of the sample (1.25 g)

$CF_{spec}$ is correction factor of the spectrophotometer (1 if the spectrophotometer is in good condition)

D is dilution factor: 50

**IMPORTANT:** To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinol palmitate (1866.45/524.84 = 0.546) should be taken in consideration. The above equation is simplified to:

$$Retinol\frac{mg}{kg} = Abs_{corrected} \times \frac{39.565}{w} \times CF_{Spec}$$

### 9.3 Vitamin A in fats and oils

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

$$Retinyl\ palmitate \frac{mg}{kg} = \frac{Abs_{corrected} \times V_f \times CF_{spec}}{Q \times w}$$

where

$Abs_{Corrected} = Abs_{sample} - Abs_{blank}$ *or Abs before irradiation – Abs after irradiation*

Q is retinyl absorption coefficient in dichloromethane (0.094 $mg^{-1}cm^{-1}$ L)

$V_f$ is final volume (25 mL)

w is weight of the sample (2.0 g)

$CF_{spec}$ Correction factor of the spectrophotometer (1 if the spectrophotometer is in good condition)

**IMPORTANT:** To express the results as unesterified retinol, the ratio of the molecular weights of retinol/retinol palmitate (1866.45/524.84 = 0.546) should be taken in consideration.

This equation when dichloromethane is used is simplified to:

$$Retinol\ \left(\frac{mg}{kg}\right) = Abs_{Corrected} \times \frac{145}{w} \times CF_{Spec}$$

## Annex A (Informative): Semi-quantitative method for Vit. A in Sugar

**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.

**Caution**

The chromogenic reagent has to be prepared and used within 5 days if stored at room temperature and within 14 days if refrigerated because it is unstable. 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◦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 is unstable to water 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.

**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.

**Apparatus**

a) Balance
b) Water bath (50-60°C)
c) Beaker (50-100 mL)
d) Amber bottle (500 mL) or thermos (for distilled water)
e) Colorimetric scale of copper sulfate solutions
f) Dark glass bottle with glass stopper
g) Disposable rubber gloves
h) Glass syringe (5-10 mL) with 3 cm teflon[5] tip
i) Glass test tubes (15 mm x 100 mm)
j) Graduated pipettes (10 mL)
k) Plastic bottle (50 mL)
l) Watch glass
m) Wide mouth glass bottle (to collect used reagent)

**Reference scale for comparing the intensity of the blue color**

Prepare dilutions as indicated below from a stock of 300 g/L stock solution of copper sulfate ($CuSO_4 \cdot 5H_20$)

| Volume (mL) $CuSO_4 \cdot 5H_20$ – 300 g/L to prepare 10 mL | Concentration $CuSO_4 \cdot 5H_20$ (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.<br>Measure 5 mL each of the copper sulfate standard solutions into exactly the same type of tubes in which the samples will be analyzed.<br>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. | | |

**Procedure**:

**Solubilizing vitamin A from sugar**

a) Mix the sugar sample thoroughly.

b) In a 250 mL beaker, weigh 50 g sugar.

Add 50 mL distilled water and dissolve sugar with a glass rod or magnetic stirred. **Preparing for colorimetric reaction**

a) Transfer 1 mL sugar solution to a test tube (a tube previously marked at a 1 mL level can be used).
b) Transfer enough chromogenic reagent for all the samples to be tested into a clean glass beaker
c) Wearing disposable rubber gloves, add rapidly 3 mL of chromogenic reagent to the sugar sample solution in the test tube using a syringe. The speed of the addition works as an aid to mix the reagents since the color develops immediately.
d) 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 is transient.
e) 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 level is between 5 and 10 mg/kg. Do not attempt to be more precise.

## Annex B (Informative): Semi-quantitative method for Vit. A in Fats and Oils

### 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.

### Caution

Same as in annex A

### Reagents

**Chromogenic reagent**, Trifluoroacetic acid/Dichloromethane (DCM). Mix 30 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 an amber 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.

Note: 1 mL of TFA in 25 mL DCM can be sufficient to run 20-30 samples. Use florisil to retain the blue colour, will be stable for more than 10 minutes.

**Apparatus**

Same as annex A

**Reference scale for comparing the intensity of the blue color**

Prepare dilutions as indicated below from a stock of 300 g/L stock solution of copper sulfate ($CuSO_4 \cdot 5H_20$)

| **Volume (mL) $CuSO_4 \cdot 5H_20$ – 300 g/L to prepare 10 mL** | **Concentration $CuSO_4 \cdot 5H_20$ (g/L)** | **Equivalent 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.<br>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 solutions**

**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 an amber bottle. TCA is readily available and a low cost reagent, but it is corrosive and the complexes formed are less stable than TFA complexes.

**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.

**Procedure**

**Diluting oil test portion**

a) Place a 25 mL amber volumetric flask on a balance. Amber flasks should be used because vitamin A is light sensitive.
b) 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.
c) Add DCM to the flask to dissolve the oil and make up to volume with DCM and mix thoroughly.

**Reaction with the chromogenic solution**

a) 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.
b) Into this tube containing the TFA, inject (a syringe can be used) rapidly 1.0 mL of the diluted sample solution of oil, ensuring that the speed of addition works as a mixing aid

**Interpreting results**

a) Compare the color intensity developed against the set of tubes of copper sulfate within 5 to 10 seconds.
b) 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.

**Annex C (Informative): Qualitative method for Vit. A in sugar**

**Principle**

**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.

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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 30-40oC. 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**

**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.

## Annex D (Informative): Ultraviolet Irradiation Chamber (Construction and verification)

### 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.

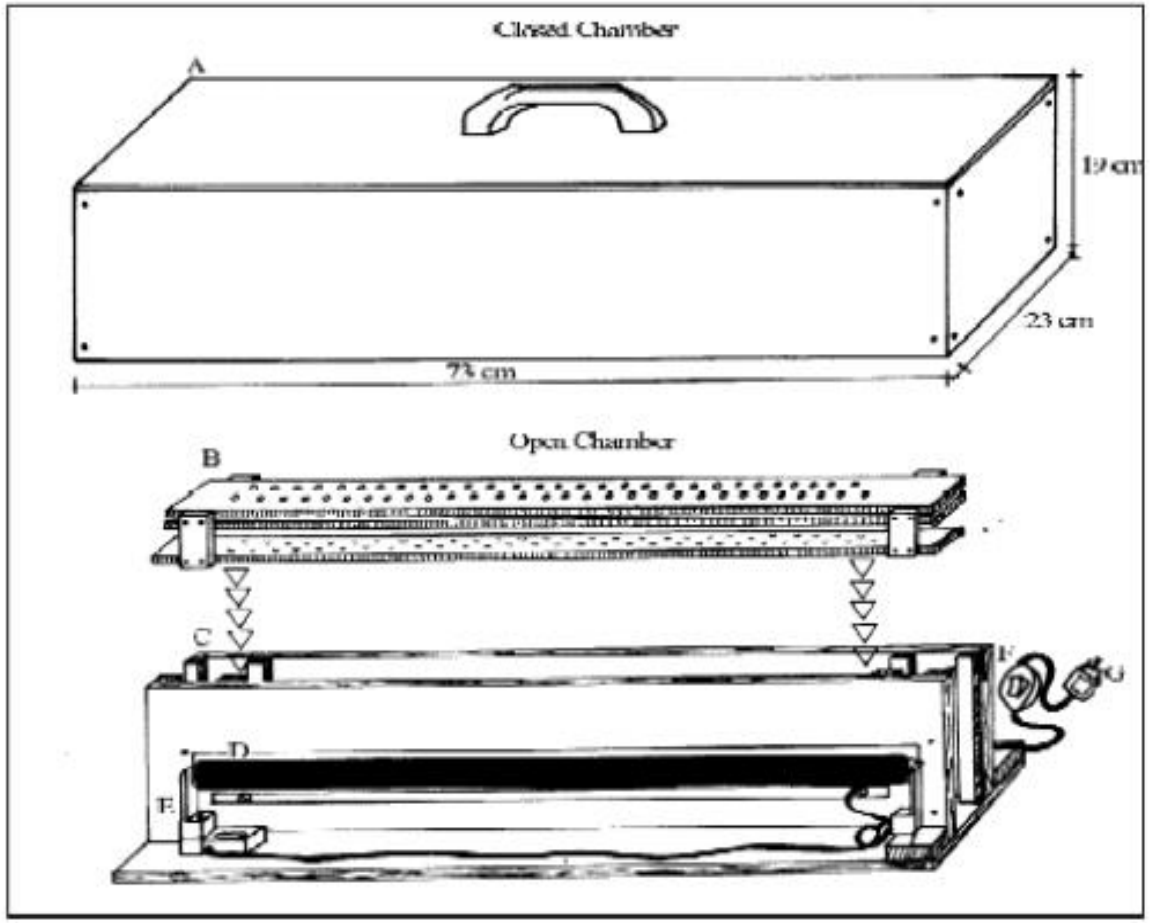
The irradiation unit is a simple, low-cost unit made of wood on which two ultraviolet (UV) lamps (350-390 nm emission) are mounted. The base has two parallel lateral walls (point B, figure 3.1). The lower part of the lateral walls has openings that extend along the length of the walls and expose the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

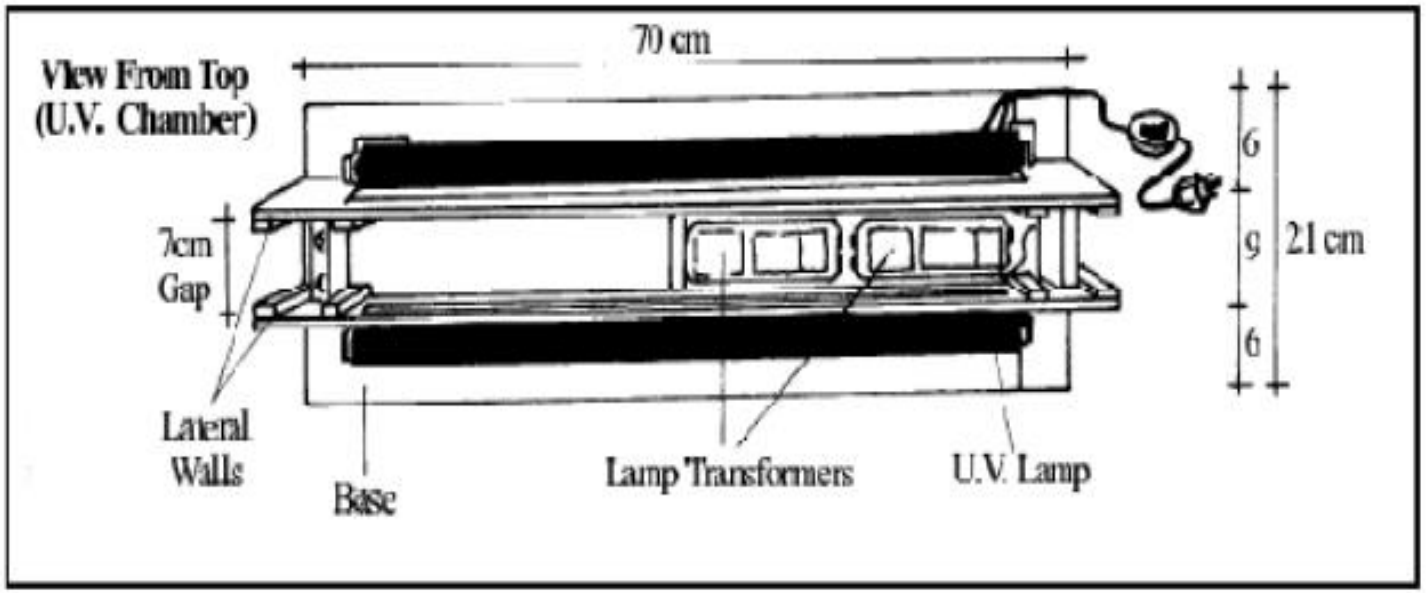
**FIGURE 3.1. IRRADIATION CHAMBER**




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 3.3. 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.

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