Manual for Sugar Fortification with Vitamin A

Part 3

Analytical Methods for the Control and Evaluation of Sugar Fortification with Vitamin A

Omar Dary, Ph.D.
Guillermo Arroyave, Ph.D.

with

Hernando Flores, Ph.D.,
Florisbela A. C. S. Campos, and
Maria Helena C. B. Lins

Dr. Omar Dary is a research biochemist at the Institute of Nutrition of Central America and Panama (INCAP), Guatemala.

Dr. Guillermo Arroyave is an international consultant in micronutrients residing in San Diego, California.

Dr. Hernando Flores, Ms. Campos, and Ms. Lins are biochemists at the Universidad de Pernambuco, Brazil.

MANUAL FOR SUGAR FORTIFICATION

PART 3

TABLE OF CONTENTS

TABLES

FIGURES

ACKNOWLEDGMENTS

This manual is based on experiences in sugar fortification at the Institute of Nutrition of Central America and Panama (INCAP). Many people have been involved in this program over the last 25 years. This publication represents the work of all those involved, who are too numerous to mention.

The presentation and content of parts 1, 2, and 3 of the manual benefited greatly from the comments and suggestions of the reviewers, whom the authors would like to thank. These include Drs. Frances R. Davidson and Timothy Quick, United States Agency for International Development (USAID); Dr. Juan R. Aguilar, United Nations Children's Fund (UNICEF), Peru; Dr. Jose O. Mora, International Sciences and Technology Institute (ISTI); Dr. Luis Mejia, Kellogg Latin America; Mr. Alberto Nilson, Hoffman-La Roche, Brazil; Dr. Kenneth Brown, University of California at Davis; Mr. Leonardo de Leon, INCAP; Drs. Jean Humphrey and Esse Yamini, Johns Hopkins University; and especially Dr. Penelope Nestel, Opportunities for Micronutrient Interventions (OMNI) Project.

Part 2 is based on the collective experience of the following individuals who have been instrumental in improving the sugar fortification process in Central America over the last 10 years: Mr. Leonel Anleu, Sugar Producers' Association, Guatemala; Dr. Oscar Pineda, formerly of INCAP, Guatemala; Dr. Vilma Estrada, Dr. Doris Chinchilla, Mr. Plutarco Murillo, and Ms. Victoria Sarai Bejarano, Honduras; and Dr. Manuel de Gracia and Dr. Enrique Murillo, Panama.

Part 3 presents the methods used at the Chemistry and Biochemistry Laboratory, INCAP, and is based on work carried out by Carolina Martinez, Dora Ines Mazariegos, Maria Elena Estrada de Sandoval, Esmeralda Morales, Monica Guamuch, and Gerardo Pirir.

The manual was translated into English by Maritza Mendez de Oliva and Carlos Cipriani. Odette Sanabria de Osorio drew the illustrations. Ingrid Cabrera, Hazel de Orellana, and Ana Ruth de Burckard typed the Spanish edition, and Lisa Sherburne was instrumental in compiling and formatting and Pamela Cubberly in copyediting and formatting the English edition; their assistance is greatly appreciated.

Support activities carried out from 1992 to 1994, which have served in part as the basis for this publication, were financed by the Regional Office for Central America and Panama (ROCAP)/USAID Project 596-0169.

FOREWORD

In many countries, vitamin A deficiency is a widespread problem that is not necessarily limited to specific groups of people or isolated communities. Among the interventions available, food fortification is an accepted method of delivering micronutrients to the population at large and is widely practiced in developed countries. In these countries, foods such as milk powder, butter and margarine, complementary infant foods, and breakfast cereals are routinely fortified with micronutrients, including vitamin A. The above foods, however, are not regularly consumed by the vast majority of the population in developing countries, especially among those at greatest risk of vitamin A deficiency. One food that is consumed by nearly the entire population in developing countries is sugar, which can be fortified with vitamin A. Sugar fortification is practical because target populations do not need to alter or adapt a new or costly distribution system. Indeed, sugar fortification only requires the existence of a well-established sugar production and marketing system. This allows for the uniform addition of vitamin A as well as the monitoring of its content. Fortification of sugar with vitamin A is one of the safest, most efficacious, and most cost-effective interventions to prevent and control vitamin A deficiency.

This manual in which technical guidelines are presented to systematize and facilitate the establishment and execution of a vitamin A sugar fortification program is divided into three parts. Part 1, *Guidelines for the Development, Implementation, Monitoring, and Evaluation for Vitamin A Sugar Fortification Program,* describes why it is important to prevent and reduce vitamin A deficiency and how to go about establishing such a program. Existing strategies are discussed and the basic elements to be considered in establishing an appropriate program for vitamin A sugar fortification are described in detail. In addition, part 1 offers an overview of the entire program so that public and private sector officials who manage and coordinate sugar-processing activities have information on the essential components to ensure an adequate operation. Technical areas presented in this document will also be useful to specialists involved in specific components of the fortification process. These include the operations involved in sugar fortification, determinates for both the efficiency and efficacy of intervention, and guidelines for determining program costs.

Part 2, *Technical and Operational Guidelines for Preparing Vitamin A Premix and Fortified Sugar*, is written specifically for technical personnel responsible for implementing sugar fortification. Chapter I covers general aspects if the fortification process, Chapter II describes how to manufacture the premix, and Chapter III describes procedures for adding the vitamin A premix to sugar. It also contains a detailed description of quality control procedures.

Part 3, *Analytical Methods for the Control and Evaluation for Sugar Fortification with Vitamin A*, presents field and laboratory methods to estimate the content of vitamin A in the premix and in fortified sugar. It also gives details on how to determine retinol levels in biological samples critical in evaluating the impact of the fortification program. Part 3 is written primarily for laboratory personnel who will be responsible for laboratory analyses.

Each part of the manual is relatively self-sufficient in the essential areas of program design and implementation. Ideally, however, it is recommended that the three parts be considered as theoretical and practical units to be used together.

Research on sugar fortification with vitamin A first began at the Institute of Nutrition of Central America and Panama (INCAP) in the 1960s under the leadership of Guillermo Arroyave with the support of Dr. M. Forman of USAID. The technology was developed over a 10-year period. Starting in 1974 Costa Rica, Guatemala, Honduras, and Panama legislated that all sugar must be fortified with vitamin A. With USAID support, the INCAP team was able to show conclusively that sugar fortification with vitamin A is both efficacious and cost-effective. USAID support, which has continued over the years, most recently through the OMNI project, has been important in ensuring that sugar fortification programs have continually improved in Central America countries. It has also stimulated the successful development of sugar fortification programs in other Latin American countries.

A sustainable sugar fortification program reflects the collaborative efforts between sugar producers, the public sector, researchers, and donors. The purpose of this document is to share the experiences of those involved in sugar fortification in Central America, so that other countries can plan and implement this important intervention to eliminate and prevent vitamin A deficiency.

Frances Davidson
Office of Health & Nutrition, USAID
Washington, D.C.

Hernán Delgado
INCAP/PAHO
Guatemala

I. INTRODUCTION

Access to a laboratory is essential to having an effective vitamin A sugar fortification program. This is because chemical analyses are required to verify the retinol levels in both premix and fortified sugar. Furthermore, the biological impact of sugar fortification is determined from the retinol levels in serum and breast milk.

It is important for the reader of this manual to note that any laboratory working on retinol determinations should have two basic facilities: first, to ensure the quality of results, an area away from direct sunlight with indirect incandescent yellow light and, second, to ensure the safety of laboratory personnel, a laboratory hood to reduce the risks associated with using volatile flammable solvents. If the appropriate lighting is not available, all test tubes and flasks with retinol-containing solutions can be covered with black fabric to reduce the risk of retinol losses.

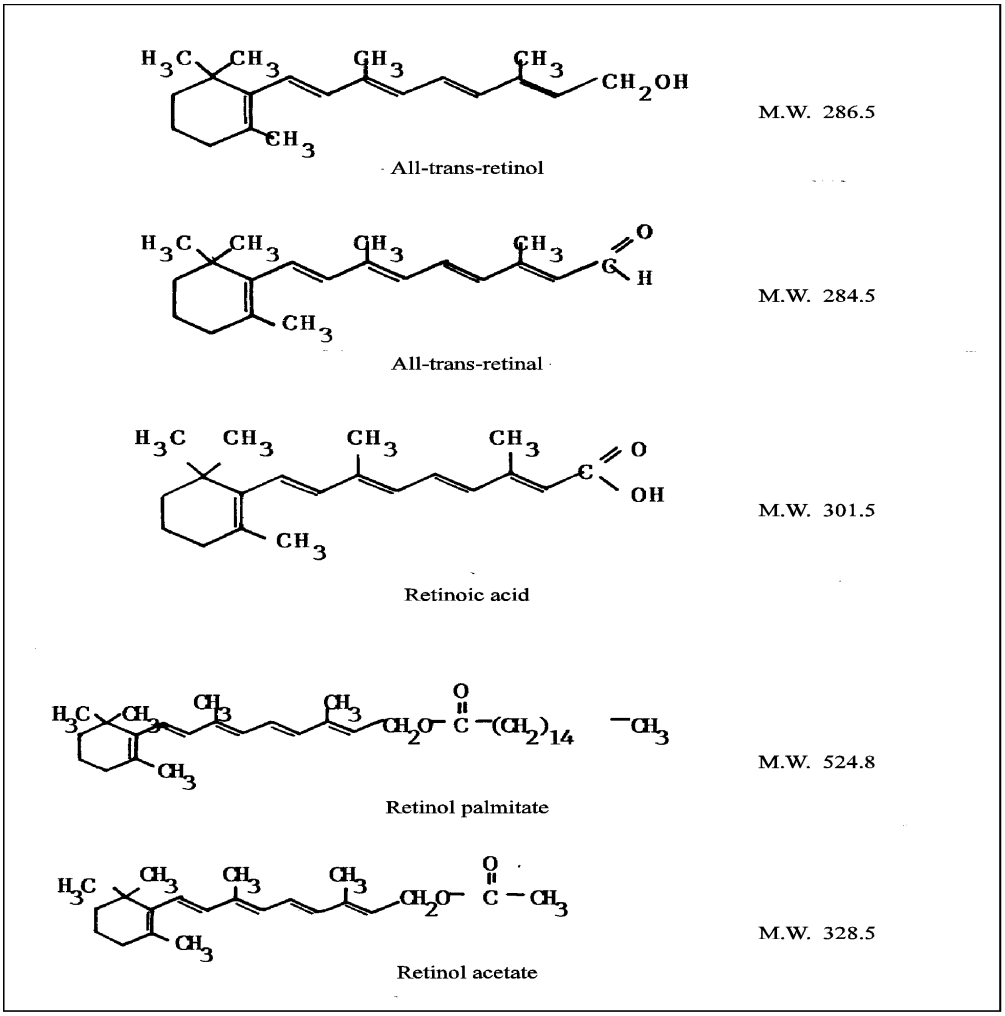
This document starts by describing the general properties of retinol and the retinol compound used in sugar fortification as the basis for understanding the analytical methods presented. It is followed by sections that describe common principles behind and details of laboratory methods[1] to determine the retinol content in *(a)* premix and fortified sugar (including measuring peroxide levels in vegetable oil, which is used in the premix), *(b)* blood, and *(c)* breast milk. Included for each substance are guidelines for collecting samples and a comparison of the advantages and limitations of principles and procedures. A concluding section then describes procedures for quality control of the analytical methods. Appendixes include information on how to construct an ultraviolet irradiation chamber and properly clean glassware and spectrophotometric cuvettes and a list of addresses for suppliers of the equipment and reagents needed for the assays described here.

1. Unless stated, the methods described in this manual are currently those being used in the Chemistry and Biochemistry Laboratory at the Institute of Nutrition of Central America and Panama (INCAP).

II. PROPERTIES OF RETINOL AND RETINOL COMPOUNDS USED IN SUGAR FORTIFICATION

Retinol is the active form of vitamin A and is found in animal tissues and fluids but not plants.[2] It is most commonly found as a fatty acid ester, predominantly as retinyl palmitate. A number of retinol derivatives with specific biochemical functions also exist. These include retinal and retinoic acid in which the -CHO and -COOH radicals, respectively, replace the -OH radical. The chemical structures of the retinol and derived compounds are shown below in figure 3.1.

Figure 3.1:
CHEMICAL STRUCTURE OF RETINOL AND DERIVATIVES



2. Nevertheless, about 600 compounds in plant tissues whose structures are related to retinol are known as carotenoids. Only fifty of these natural compounds can be converted to retinol in animals and are known as active carotenoids or precursors of vitamin A. The most active compound is beta-carotene, making it the most important carotenoid from a nutritional perspective.

Retinol in blood is bound to its carrier protein, known as retinol binding protein, but, in breast milk, it is esterified with several fatty acids. To determine blood retinol levels, the plasma or serum samples require treatment with alkaline ethanol to destroy the protein complex, which precipitates the protein and liberates the retinol. In breast milk, retinol should be separated from its esters by saponification.

All the retinol compounds commercially available for industrial or pharmaceutical use are synthetic. Most are in the form of esters, often palmitate or acetate, which are more stable and easier to handle. Retinol and its esters are highly soluble in organic solvents and fats but not water. For this reason, retinol is not suitable for fortifying water-based foods; however, the advent of water-miscible, dry, vitamin A compounds, including retinyl palmitate beadlets, has facilitated the fortification of water-based foods, including sugar.

The prototype retinyl palmitate used in sugar fortification is 250-CWS,[3] in which retinyl palmitate is embedded in a gelatin matrix containing antioxidants as stabilizers, as well as other compounds that make it water-miscible. The 250-CWS beadlets are spherical in shape, light yellow in color, and have a slight but distinct odor. Its stability and other characteristics makes 250-CWS a suitable fortificant for white sugar. Because retinol is fat soluble, it is easily extracted with organic solvents; however, the water-miscible beadlets must first be dispersed in water to release the retinyl palmitate from the other beadlet components.

Once retinol and its esters have been extracted, their distinct characteristics become evident. These characteristics, often the basis for determining retinol levels or important considerations in assays, include the following:

- *Destruction by ultraviolet (UV) light*. Retinol is destroyed when irradiated with UV light; thus, specific determinations can be made using retinol absorbance before and after its selective destruction with UV light. At the same time, this property requires that samples and specific solutions containing retinol be protected from direct light during preparation and handling.

- *Absorption of electromagnetic energy in the UV and shortest wavelengths of the visible spectrum*. Due to the conjugated double bond in retinol, this molecule absorbs radiant energy around 325 nm. This property is generally used to determine quantitatively the level of retinol and is done by measuring its absorbance at maximum absorption wavelength, which could vary slightly depending on the retinol derivative and solvent.

- *Oxidation*. Retinol readily takes up oxygen to form peroxide bonds, a reaction directly related to temperature; thus, solutions containing retinol should be protected from prolonged exposure to air and kept at a maximum temperature of 10°C.

3. Produced by Hoffman-La Roche and BASF, Germany (see appendix 3.3).

III. PRINCIPLES FOR DETERMINING RETINOL IN VITAMIN A PREMIX AND FORTIFIED SUGAR

The retinol concentration in premix or fortified sugar can be determined quantitatively using a spectrophotometric method or semi-quantitatively using a colorimetric method that is based on a chromogenic reaction.

A. Spectrophotometric method

1. *Collection and handling of samples*. A minimum of 5 g of premix and 50 g of fortified sugar is needed. Samples should be stored in opaque glass or plastic bottles that are tightly capped and protected from direct sunlight, heat, and humidity. Once at the laboratory, samples should be stored in a dry, cool place. Before weighing the amount to be analyzed, the premix or fortified sugar should be thoroughly mixed.

2. *Dispersion of the retinyl palmitate beadlets*. To ensure complete dispersion of the 250-CWS in water, the beadlets should be dissolved and preferably incubated in water at 50°–60°C for 15 minutes.

3. *Extraction*. Retinyl palmitate is extracted from the aqueous layer using an organic solvent. The most appropriate is hexane. Because of the high concentration of retinyl palmitate in the premix, extraction is unnecessary. Instead, dilution of the aqueous phase with 2-propanol is sufficient.

4. *Determining retinol concentration*. Retinol determination is based on its absorbance at 325 nm using a spectrophotometer. The high retinyl content in premix and fortified sugar allows for a higher wavelength to be used if necessary but always below 350 nm; however, the accuracy and sensitivity decrease when wavelengths over 325 nm are used, which means that a correction factor must be used. This can be easily obtained by dividing the absorbance of a retinol solution at 325 nm in a reference spectrophotometer by the absorbance of the same solution at the other wavelength used. Table 3.1 on the next page shows the correction factors for retinol at different wavelengths as determined at INCAP.

Table 3.1: Correction Factors for Retinol Absorbance at Different Wavelengths Using a Source of Visible[4] Light

Wavelength (nm)	Correction factor
325	1.007
330	1.066
335	1.117
340	1.360
345	1.622
350	2.030

5. *Increasing the analytical specificity*. This is necessary when the samples have a significant amount of interfering substances and can be achieved in two ways: first, by separating the retinol from other substances that absorb radiant energy at equal or similar wavelengths to retinol using high performance liquid chromatography (HPLC) or, second, by measuring the absorbance of the extracts before and after selectively destroying the retinol through exposure to UV light. The difference in absorbance between the unirradiated and irradiated extracts is specifically due to retinol and is used to calculate the concentration of retinol in the sample. This procedure is used to assay premix samples. Fortified sugar essentially contains no interfering substances; thus, the retinol can be specifically determined simply by subtracting the absorbance of the reagent's blank from the absorbance of the sample.

B. Colorimetric method

The other method that can be used to determine retinol levels in fortified sugar[5] is colorimetric, which is faster and does not require a spectrophotometer, making it adaptable to field conditions. The method is based on the Carr-Price reaction, in which retinol is converted to anhydroretinol when combined with a chromogenic reagent containing trichloracetic acid and dichloromethane. A blue color develops from the transient protonation of anhydroretinol, the intensity of which is proportional to the concentration of retinol in the sample. This method is less accurate and precise than the spectrophotometric one; thus, the validity of the results should be verified against those using the spectrophotometric method.

4. Reference value for ultraviolet radiation source at 325 nm = 1.000.
5. This method is not sufficiently accurate to be used as a quality control parameter for premix.

IV. SPECTROPHOTOMETRIC DETERMINATION OF RETINOL IN PREMIX

A. References[6]

None are available.

B. Principle

This method entails solubilizing water-miscible retinyl palmitate beadlets in hot water, followed by dilution in 2-propanol. The concentration of retinol in retinyl palmitate is determined by its spectrophotometric absorbance at 325 nm.

C. Critical points and cautions

A spectrophotometer capable of reading 325 nm is essential. This is because the concentration of the retinol standards has to be verified by spectrophotometric analysis. 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.[7] This confirmation should be carried out frequently and not only when a new lamp is installed.

A UV light irradiation system, in which the retinol is destroyed, is also required. This system can be as simple as a UV lamp and curtain to protect technicians from exposure to this light. The critical factor is to establish exactly both the optimal irradiation time and appropriate distance between the UV light and the solutions. The efficiency of the irradiation system should be checked periodically (every 3 months) and each time a new lamp is installed. Appendix 3.1 gives details of an irradiation system.

Once the sample has been solubilized in 2-propanol, the analysis should not be interrupted.

6. Based on an unpublished procedure described by O. Pineda in *Fortificación de Azúcar con Vitamina A, Manual de Operaciones*, Guatemala City: INCAP.
7. Section XIV.C.1 includes a description of a simple procedure to calibrate spectrophotometers routinely used in clinical laboratories.

Based on experience at the INCAP laboratory, if the variability between duplicates of the same solution is greater than 3 percent, the results should be rejected and the readings repeated. In addition, the results of two independently weighed subsamples of the same sample should not differ on average by more than 8 percent. If the variation is greater than 8 percent, the assay should be repeated.

D. Equipment and materials

Vortex mixer
Water bath (50°–60°C)
Irradiation chamber with ultraviolet light (described in appendix 3.1)
UV/VIS spectrophotometer
100 mL volumetric flasks
Spectrophotometer cuvettes (preferably quartz)
Graduated serological pipettes
Volumetric pipettes
20 mL test tubes
10 mm x 75 mm glass tubes transparent to UV light
Glass rods
200–250 mL beaker
Spatulas
Aluminum foil

E. Reagents

1. Analytical grade 2-propanol ($(CH_3CH(OH)CH_3)$, purity = 99.7%, MW = 60.10, d = 0.78 g/mL).

F. Procedure

1. Mix the premix sample thoroughly.

2. Weigh duplicate 1 g samples, recording the exact weights to three decimal places, and dissolve each sample in 80 mL of distilled water at 50°–60°C in a beaker. Use a glass rod to completely dissolve the sample.

3. Incubate in a water bath at 50°–60°C for 15 minutes. Cool at room temperature. Transfer to a 100-mL volumetric flask. Rinse the beaker with small amounts of distilled water, transfer the washings to the volumetric flask, and make up to volume with distilled water, and mix. This solution is cloudy.

4. Measure 2 mL of the solution in step 3 into a 20 mL test tube and add 8 mL of 2-propanol (to give a 2:10 dilution). Mix vigorously in a Vortex mixer.

5. Measure 1 mL of solution from step 4 into a 20-mL test tube and add 9 mL of 2-propanol (to give a 1:10 dilution). Mix using a Vortex mixer.

6. Separate off 1 mL of the solution from step 5 and place in a 10 mm x 75 mm glass test tube transparent to UV light. Irradiate this solution in the irradiation chamber for 35 minutes (or the time required according to the performance of the irradiation chamber).

7. Adjust the zero of the spectrophotometer with 2-propanol. Read the absorbance of the irradiated and unirradiated solutions at 325 nm[8] in 1 cm light path quartz cuvettes.

G. Calculations

The concentration of retinyl palmitate in the premix samples is calculated using the following equation:

$$Retinyl\ palmitate\ (mg/g) = \frac{(Abs_o - Abs_{irrad})}{a} \times \frac{Vl}{p} \times FD \times FC_{spe}$$

where:

Abs_o = reading at time zero

Abs_{irrad} = reading after irradiation.

8. If a UV-light spectrophotometer is not available, a visible light spectrophotometer may be used but at a wavelength below 350 nm (see table 3.1).

The parameters for the equation are:

Parameter	Explanation	Value
å	Retinyl palmitate absorption coefficient *(mg^{-1}cm^{-1}mL)*	94.0
Vl	Volume of the initial solution of the sample (mL)	100.0
p	Weight of the sample *(g)*	weight at step IV.F.2
FD	Dilution factor	50.0
Fc_{spe}	Correction factor of the spectrophotometer,[9] ideally	1.000

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 the following:

$$Retinol\ (mg/g)\ =\ (Abs_{o}\ -\ Abs_{irrad}) \times \frac{29.04}{p} \times FC_{spe}$$

9. See section XIV.C.1.

V. SPECTROPHOTOMETRIC DETERMINATION OF RETINOL IN FORTIFIED SUGAR

A. References

Arroyave, G. and C. de Funes. 1974. Enriquecimiento de azúcar con vitamina A. Método para la determinación cuantitativa de retinol en azúcar blanca de mesa." *Arch. Latinoamer. Nutr.* 24: 147–53.

B. Principle

This method is an adaptation of the method developed by Arroyave and Funes (1974). The procedure uses five to ten times less reagents than the original method, and its accuracy is similar. The precision, however, is somewhat lower, although highly satisfactory. The method requires the extraction of retinyl palmitate in hexane. Retinol concentration is determined by its absorbance at 325 nm. This method does not usually require irradiation with UV light, because the absorbance of the extract at 325 nm is essentially only due to the retinol in sugar.

C. Critical points and cautions

A spectrophotometer capable of reading 325 nm is essential. This is because the concentration of the retinol standards has to be verified by spectrophotometric analysis. 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.[10] This confirmation should be carried out frequently and not only when a new lamp is installed.

A UV-light irradiation system in which the retinol is destroyed is also required. This system can be as simple as a UV lamp and curtain to protect technicians from exposure to this light. The critical factor is to establish exactly both the optimal irradiation time and appropriate distance between the UV light and the solutions. The efficiency of the irradiation system should be checked periodically (every 3 months) and each time a new lamp is installed. Appendix 3.1 gives details of an irradiation system.

10. Section XIV.C.1 includes a description of a simple procedure to calibrate spectrophotometers routinely used in clinical laboratories.

Once the sugar has been extracted in hexane, the analysis should not be interrupted.

Based on experience at the INCAP laboratory, if the variability between replicates of the same solution is greater than 5 percent, the results should be rejected and the extractions repeated. The recovery of the method is at least 91 percent.

D. Equipment and materials

Vortex mixer
Water bath (50°–60°C)
UV/VIS spectrophotometer
100 mL volumetric flasks
Spectrophotometer cuvettes (preferably quartz)
Graduated serological pipettes
Volumetric pipettes
Pasteur pipettes
20 mL test tubes with screw caps
Glass rods
200–250 mL beaker
Aspiration bulbs for Pasteur and serological pipettes
Spatulas

E. Reagents

1. Analytical grade absolute ethanol ((C_2H_5OH), purity = 99.8%, MW = 46.07, d = 0.79 g/mL)

2. Analytical grade hexane ((C_6H_{14}), purity = 99%, MW = 86.18, d = 0.66 g/mL)

3. 0.1N sodium hydroxide ((NaOH), purity = 97%, MW = 40.00). Dissolve 4 g NaOH in 1 liter of distilled water. Store in polyethylene or polypropylene bottle.

F. Procedure

1. Mix the sugar sample thoroughly.

2. Weigh approximately 20 g of sugar, recording the exact weights to three decimal places and dissolve in 60–80 mL 0.1N NaOH in a 200–250 mL beaker. Use a glass rod to completely dissolve the sample.

3. Incubate in water bath at 50°C for 15 minutes. Cool at room temperature. Transfer to a 100 mL volumetric flask. Rinse the beaker with small amounts of 0.1N NaOH, transfer the washings to the volumetric flask, and make up to 100 mL with 0.1N NaOH and mix.

4. Measure 4 mL of the solution in step 3 into three 20 mL test tubes. Prepare in triplicate a reagent blank with 0.1N NaOH following the same procedure as for the samples.

5. Add 4 mL of absolute ethanol to each tube. Mix in the Vortex mixer for 5 seconds.

6. Measure 5 mL of hexane and add it to each tube from step 5. Immediately stopper 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.

7. Allow separation of the top organic solvent phase.

8. 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 325 nm.[11] Adjust the zero of the spectrophotometer with hexane before each reading.

11. If a UV-light spectrophotometer is not available, a visible light spectrophotometer may be used but at a wavelength below 350 nm.

G. Calculations

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

$$Retinyl\ palmitate\ (\mu g/g) = \frac{Abs_{corrected}}{å} \times \frac{Vh}{Vaz} \times \frac{Vi}{p} \times FC_{spe}$$

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 parameters for the equation are:

Parameter	Explanation	Value
å	Retinyl palmitate absorption coefficient in hexane ($\mu g^{-1} cm^{-1} mL$)	0.092
Vh	Volume of the organic phase (mL)	5.0
Vaz	Volume of the aliquot analyzed from the sugar solution (mL)	4.0
VI	Volume of the initial solution of the sample (mL)	100.0
p	Weight of the sample (g)	Weight at step V.F.2
FC_{spe}	Correction factor of the spectrophotometer,[12] ideally	1.000

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:

$$Retinol\ (\mu g/g) = Abs_{corrected} \times \frac{741.85}{p} \times FC_{spe}$$

12. See section XIV.C.1.

H. Verification of the efficiency of the extraction

To verify the efficiency of the extraction, a recovery assay should be done using the following suggested procedures:[13]

1. Using a sample of unfortified sugar, follow steps V.F.1–4 of the analytical procedure. At this point, add to two of three test tubes 3 mL of absolute ethanol and then 1 mL of an ethanol solution of retinyl palmitate of a known concentration (approximately 20 µg/mL[14]), that is, the control. To the third tube, that is, the blank, add 4 mL of absolute ethanol. Continue the analytical procedure from step V.F.6. Read the absorbance of the retinyl palmitate controls and the blank. Subtract the absorbance of the blank from the mean absorbance of the controls. This is the absorbance due to the retinyl palmitate added (*s*).

2. Independently prepare a retinyl palmitate solution as follows. Measure 1 mL of the same retinyl palmitate solution that was used above into a 5 mL volumetric flask. Make up to volume with ethanol. Read the absorbance of this solution and multiply by 0.98 to compensate for the higher absorbance of retinyl palmitate in ethanol than in hexane. This figure (*t*) is the theoretical absorbance that would have been found if recovery efficiency was 100 percent.

3. Calculate recovery (*R*) as follows:

$$R = \frac{s}{t} \times 100$$

13. This assay uses pure retinyl palmitate rather than retinyl palmitate beadlets; however, in the experience of INCAP, the results are practically the same.
14. Prepare from a primary solution of 100 µg/mL of retinyl palmitate in ethanol; keep at !20°C in a nitrogen atmosphere and a dark container. The 20 µg/mL solution should have an absorbance near 2.0 at 325 nm against absolute ethanol.

VI. SEMIQUANTITATIVE COLORIMETRIC DETERMINATION OF RETINOL IN FORTIFIED SUGAR

A. References

Arroyave, G., O. Pineda, and C. de Funes, 1974. "Enriquecimiento de azúcar con vitamina A. Método rápido para la fácil inspección del proceso." *Arch. Latinoamer. Nutr*. 24: 155–59.

Bayfield, R. F. and E. R. Cole. 1980. "Colorimetric Determination of Vitamin A with Trichloroacetic Acid." In D. B. McCormick and L. D. Wright, eds. *Methods in Enzymology*; *Part F, Vitamins and Coenzymes*. 67: 189–95. New York: Academic Press.

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 containing trichloroacetic acid and dichloromethane. A blue complex is formed and the intensity of the color can be measured semiquantitatively 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 (25°C) and within 14 days if refrigerated. If refrigerated, it should be removed from the refrigerator 2 to 3 hours prior to use. 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 retinol in sugar should be analyzed and the intensity of the blue color should match the expected intensity according to the reference scale.

The chromogenic reagent is highly 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 syringe before being added to the sugar. 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. Materials

50 mL plastic bottle

Wide mouth glass bottle (to collect used reagent)

500 mL bottle or thermos (for distilled water)

Dark glass bottle with glass stopper

5–10 mL glass syringe with 3 cm polyethylene tip

10 mL graduated pipettes

15 mm x 100 mm glass test tubes with meniscus level marked to show 1 mL and another to show the volume occupied by 10 g of sugar

50–100 mL beaker

Copper sulfate solutions (colorimetric scale), which are described below

Disposable rubber gloves

E. Reagents

1. Chromogenic reagent: Trichloroacetic acid/dichloromethane

   Mix 120.0 g trichloroacetic acid with 80.0 g of dichloromethane (60.6 mL). To dissolve completely, warm the mixture in a water bath at 50°–60°C stirring constantly. Store in a dark bottle with glass stopper, preferably in a refrigerator. The chromogenic reagent prepared as stated is sufficient for 25–30 samples.

2. Colorimetric scale

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

$CuSO_4.5\ H_2O$ *(g/L)*	**Approximate equivalent concentration** *(µg/g retinol in sugar)*
30	5
60	10
90	15
120	20

   Measure 4 mL of each of the standard copper sulphate solutions in the same type of tubes in which the samples will be analyzed. Close the tubes very tightly using a rubber stopper. Identify each tube with its number, indicating the concentration of retinol in

µg/g that the color represents. These solutions are stable and can be kept indefinitely at room temperature.

F. Procedure

1. Mix the sugar sample thoroughly.

2. In a 50 mL wide mouth flask, weigh 10 g of sugar (or use the test tube marked to show the equivalent volume).

3. Add 10 mL of water at 50°C, preferably distilled. Dissolve the sugar, heating the solution, if necessary.

4. Cool solution at room temperature.

5. Transfer the sugar solution to a test tube up to the previously marked 1 mL level.

6. Pour enough chromogenic reagent for all the samples into a clean glass beaker

7. Wearing disposable gloves, add 3 mL of chromogenic reagent to each 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 (µg/g) 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 intensity of the blue lies somewhere between the levels of 30 g/L and 60 g/L copper sulfate, the retinol level is between 5 µg/g and 10 µg/g sugar.

10. Discard residual chromogenic reagent, including the sugar-reagent mixture, in a glass bottle and take to the laboratory for proper disposal.

VII. VOLUMETRIC METHOD TO DETERMINE PEROXIDE LEVELS IN OILS

A. References

Association of Official Analytical Chemists. 1984. *Official Methods of Analysis of the Association of Official Analytical Chemists*. 14th. ed., Arlington, Virginia.

B. Principle

This method is recommended for determining the peroxide level in the vegetable oil used in manufacturing the vitamin A sugar premix. Peroxides and similar substances arise from fat and oil oxidation. These substances oxidize iodide to iodate, which can be quantitatively measured by their redox reaction with sodium thiosulfate. The reaction takes place in a slightly acid medium and in the presence of an excess of iodide ions. Due to the hydrophobic nature of the oil, titration is carried out in a mixture of acetic acid and chloroform.

C. Critical points and cautions

Reagents should be kept free of oxygen using an inert gas (CO_2 or N_2).

D. Equipment and materials

Magnetic mixer
500 mL and 1,000 mL volumetric flasks
Burette
250 mL Erlenmeyer flask preferably with glass stoppers
Dropper bottle
1,000 mL graduated cylinder
Glass rods
100 mL and 400 mL beaker
Spatula

E. Reagents

1. Acetic acid/chloroform

   Mix 3 volumes glacial acetic acid ((CH_3COOH), purity = 99.7%, MW = 60.05, d = 1.05 g/mL) with 2 volumes of chloroform USP (CH_3Cl_3)). Prepare only the quantity needed and place in a glass bottle.

2. 1 percent indicator starch solution

   Put 0.5 g of analytical grade starch in a 100 mL beaker and add 5 mL of hot, distilled water. Mix by constantly stirring in 45 mL of distilled water. Boil for a few minutes, let cool, and filter. Store in a dropper bottle for no more than 1 week.

3. 0.1N sodium thiosulfate

   Dissolve approximately 25 g of analytical grade sodium thiosulfate (($Na_2S_2O_3.5H_2O$), purity = 99.5%, MW = 248.18) in 1 liter of distilled water. Bring to a boil and gently boil for 5 minutes. Allow to cool. Transfer to a dark bottle, previously washed with a chromic cleaning mixture and rinsed with boiled water, and store. The solution remains stable for about 6 months. Discard the solution if it becomes turbid. The solution should be standardized before use (described below). Diluted solutions are prepared using boiled water just before use.

4. Potassium iodide free of iodate

5. 1N hydrochloric acid

   Slowly add 82.8 mL of concentrated hydrochloric acid ((HCl), 37%, MW = 36.46, d = 1.2g/mL) to 300 mL distilled water in a 1,000 mL volumetric flask. Cool at room temperature and make up to 1,000 mL with distilled water. Store in a tightly capped glass bottle to avoid contact with vapors and alkaline solutions.

6. Potassium dichromate (($K_2Cr_2O_7$), purity = 99.0, MW = 294.19)

F. Procedure

1. Standardizing the sodium thiosulfate solution

Every time a batch of samples is processed, the following procedure should be carried out in triplicate:

a. Weigh between 0.2000 g and 0.2300 g of potassium dichromate, previously dried for 2 hours at 105°C and stored in a desiccator, and transfer to a 250 mL Erlenmeyer flask.

b. Add about 80 mL of distilled water, 2 g of potassium iodide, and 20 mL of 1N HCl, and mix with a magnetic stirrer. Let stand for 10 minutes in the dark.

c. With continuous stirring, titrate the potassium dichromate solution with 0.1N sodium thiosulfate, until the yellow color almost disappears. At this point, add ten drops of the 1 percent starch solution. Continue titrating drop by drop. The titration is complete when the blue color disappears. Record the final volume of thiosulfate solution required to complete the titration.

The normality of the thiosulfate solution is calculated as follows:

$$Na_2S_2O_3(eq/mL) \text{ ' } \frac{gK_2Cr_2O_7}{mLNa_2S_2O_3} \times \frac{1eqCr_2O_7}{294.20gCr_2O_7} \times \frac{6eqS_2O_3}{1eqCr_2O_7}$$

which can be simplified to:

$$Normality\ \ Na_2S_2O_3(eq/L) \text{ ' } \frac{gK_2Cr_2O_7}{mLNa_2S_2O_3} \times \frac{1{,}000(mL/L)}{49.032}$$

2. Titration of samples

Each sample should be run in duplicate.

a. Weigh 5 (±0.05) g of each sample in a 250 mL Erlenmeyer flask with glass stopper.

b. Add 30 mL of acetic acid/chloroform solution and gently mix until dissolved.

c. Add approximately 1 g of potassium iodide and mix. Let stand for 1 minute.

d. Add 30 mL of distilled water and ten drops of the 1 percent starch solution. Stirring continuously, slowly titrate with 0.1N sodium thiosulfate solution until the blue color suddenly disappears. If the titration uses less than 0.5 mL 0.1N sodium thiosulfate, reduce the normality of the solution to 0.01N (1:10 dilution) with boiled distilled water and repeat the titration.

   The intermediate iodine formed is soluble in the organic phase; therefore, the disappearance of the color (first yellow and then blue once the starch has been added) should be observed in this phase.

3. Blank titration

In each run, repeat the procedure for a blank, in which no oil is added (less than 0.1 mL of sodium thiosulfate solution should be needed). Subtract the titration volume for the blank from the titration volume for each of the samples.

G. Calculations

The milliequivalent of peroxides per kilogram of sample are calculated as follows:

$$Perox.\ (meq/kg) = \frac{mL\ S_2O_3}{g\ sample} \times S_2O_3\ (eq/L) \times 1{,}000(meq/eq)$$

The maximum permitted level for peroxides in vegetable oil is 5 meq/kg.

VIII. PRINCIPALS OF METHODS TO DETERMINE RETINOL IN BLOOD

A. Introduction

Evaluating the nutritional impact of a sugar fortification program on vitamin A status can be done by measuring retinol levels in blood both before and after the intervention.

Two methods for determining blood retinol levels are presented here. The first is an adaptation of the classic spectrophotometric method of Bessey et al. (1946) (see section IX). The specificity of this method is based on the selective destruction of free or esterified retinol present in blood by irradiation with UV light. The second method requires an apparatus that does high performance liquid chromatography (HPLC) to separate the free retinol from other substances (see section X). This method is more specific and sensitive than the spectrophotometric method; however, the spectrophotometric method has the advantage of being lower in cost and allowing for two or three times as many samples to be analyzed during the same period of time. A limitation of the spectrophotometric method is that it does not differentiate between free retinol and its esters. This would be important only if used in populations with very high intakes of retinol in postprandial conditions. This limitation does not apply in countries in which vitamin A deficiency is a public health problem.

The choice of the method to use will depend on the purpose of the study and the resources available. While the HPLC is undoubtably more accurate and precise, both methods can provide the necessary information for evaluation purposes.

The determination of blood retinol levels involves the following laboratory steps:

1. *Treatment with alkaly*. Theoretically, this treatment hydrolyzes the retinyl esters in the sample. For the spectrophotometric method, this hydrolysis is incomplete, but this does not affect the usefulness of the method. In serum or plasma, this treatment is necessary to destroy the retinol-retinol binding protein complex and precipitate proteins and other interfering substances.

2. *Extraction*. Retinol and nonhydrolyzed esters are extracted with an organic solvent. A xylene-kerosene mixture is preferred in the spectrophotometric determination, while hexane or another low-boiling-point solvent is used in the HPLC determination.

3. *Determining absorbance*. In both the spectrophotometric and HPLC methods, the absorbance of retinol at 325 nm is used for its quantitative determination.

4. *Selective destruction of retinol*. To ensure that the absorbance is from retinol only, absorbance is determined in the sample extracts both before and after selective destruction of the retinol by irradiation with UV light. The HPLC method does not require this step because, before measuring the absorbance of retinol, it is chromatographically separated from other interfering substances including its esters.

B. Collection and management of blood samples

Blood retinol can be measured in both serum or plasma. Serum is preferable when blood is processed within 6 hours at the site of sample collection. If these conditions cannot be met, as is often the situation in many surveys, plasma is recommended because there is less risk of hemolysis. Centrifugation of plasma can be delayed for up to 24 hours, provided the samples are refrigerated.

Blood can be obtained from venipuncture or, if the laboratory uses microadaptations of the analytical methods, from finger pricks. When venipuncture is used, between 3 and 5 mL should be drawn from an antecubital vein using standard safety procedures. A 21- or 22-gauge needle can be used for preschool children, while a 20-gauge needle can be used for adults. When a tourniquet is applied around the forearm, it should be removed as soon as blood begins to flow into the tube so as to minimize the risk of hemolysis. It is preferable to draw blood directly into a vacutainer tube, covered with aluminum foil. The tubes and their aluminum foil should be labeled with indelible ink. If collecting blood directly into a vacutainer is not possible or is difficult, it can be drawn into a syringe and injected into the vacutainer tube. To avoid this double step, an alternative is to use a Sarstedt Monovette test tube syringe, but they are more expensive than syringes and vacutainer tubes. Vacutainer tubes containing serum or plasma gel separators will help to prevent hemolysis, but only if the blood is centrifuged before it is transported.

Blood samples should not be exposed to air or direct sunlight, nor should they come into direct contact with ice. The blood samples should be packed and transported carefully to ensure that they do not become hemolyzed from too much shaking while in transit. If the blood is exposed to air, it is suggested that the atmosphere above the sample be filled with nitrogen by blowing the gas into the tube for 30 to 60 seconds without disturbing the sample, after which the tube should be tightly capped.

Serum is separated from coagulated blood by centrifugation at 2,500 to 3,000 rpm for 15 minutes. Unclear serum should be transferred with a Pasteur pipette to another centrifuge tube and recentrifuged. The serum should be stored at !20°C in adequately sized vials so that the dead space between the surface of the sample and cap is minimal, leaving enough space for expansion during freezing.

Plasma is separated from the blood cells using the same process as for serum, but the centrifugation need only be done for 10 minutes. Plasma samples should be handled exactly the same as

serum samples and should also remain frozen until analyzed. Under this condition, both kinds of samples will remain viable for up to one year.

When centrifugation is done in the field, serum and plasma can be temporarily stored and transported frozen, for which liquid nitrogen or dry ice can be used. It is essential that, once frozen, samples remain frozen at all times even when being transferred between laboratories; thus, sufficient amounts of liquid nitrogen or dry ice must be available to ensure this.

Where finger-prick blood samples are collected, an automatic lancet that pricks to a given depth and minimizes bruising is preferable to an ordinary lancet, although they cost slightly more. It is important when collecting finger-prick blood samples that blood is not squeezed out of the finger. Blood flow can be made easier if the subjects are asked to shake their hand vigorously up and down before their finger is pricked. The blood can be easily collected in a serum or plasma microvacutainer,[15] which can be centrifuged. After centrifugation, the serum or plasma should be transferred to a vial and stored as for venipuncture blood.

To verify that the samples have not deteriorated during storage, aliquots of a serum or plasma control whose retinol value is known should be stored under the same conditions and analyzed at the same time.

15. Microvacutainers with a lip will facilitate directing the flow of blood into it.

IX. SPECTROPHOTOMETRIC DETERMINATION OF BLOOD RETINOL BY ULTRAVIOLET DESTRUCTION OF RETINOL

A. References

Bessey, O. A., O. H. Lowry, M. J. Brock, and J. A. López . 1946. The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.* 166: 177–88.

Araujo, C. R. C. and H. Flores. 1978. Improved spectrophotometric vitamin A assay. *Clin. Chem.* 24: 386.

B. Principle

The procedure described below is an adaptation of the method proposed by Bessey et al. (1946). This method consists of precipitating the proteins with an alcoholic potassium hydroxide solution and subsequent extraction of retinol (and any esters) in a kerosene-xylene mixture. Saponification, although incomplete, facilitates retinol extraction and eliminates interfering substances. The retinol in the extract is selectively destroyed by irradiation of ultraviolet light. The difference between absorbance at 328 nm[16] before and after irradiation is attributable and proportional to total retinol.

C. Critical points and cautions

A spectrophotometer capable of reading 325 nm is essential. This is because the concentration of the retinol standards have to be verified by spectrophotometric analysis. 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.[17] This confirmation should be carried out frequently and not only when a new lamp is installed.

A UV-light irradiation system in which the retinol is destroyed is also required. This system can be as simple as a UV lamp and curtain to protect technicians from exposure to this light. The critical factor is to establish exactly both the optimal irradiation time and appropriate distance between the UV light and the solutions. The efficiency of the irradiation system should be checked periodically (every 3 months) and each time a new lamp is installed. Appendix 3.1 gives details of an irradiation system.

16. Maximum absorbance wavelength of retinol in a kerosene-xylene mixture.
17. Section XIV.C.1 includes a description of a simple procedure to calibrate spectrophotometers routinely used in clinical laboratories.

Once the retinol has been extracted into the organic solvent, the analysis should not be interrupted.

Based on the experience at the INCAP laboratory, two independently analyzed subsamples of the same sample should not differ on average by more than 10 percent if the concentration of retinol is equal to or greater than 30 µg/dL or by more than 15 percent if the concentration of retinol falls between 15 and 30 µg/dL. If these criteria are not met, the assay should be repeated. Samples having a retinol concentration below 15 µg/dL should be reported as 15 µg/dL. The recovery of retinol using this method is 93 percent or higher.

D. Equipment and materials

Vortex mixer
Water bath (56°C)
UV-light irradiation chamber with ultraviolet light (example in appendix 3.1)
Refrigerated centrifuge
Stopwatch
UV/VIS spectrophotometer
Black walled quartz cuvettes (maximum 1 mL capacity)
Dark glass bottle
Graduated serological pipettes
Automatic pipettes (Eppendorf type)
Pasteur pipettes
Graduated cylinder
10 mm x 75 mm glass test tubes transparent to UV light
12 mm x 75 mm glass test tubes
Glass rods
Aspiration bulbs for Pasteur and serological pipettes

E. Reagents

1. Alcoholic potassium hydroxide

   Combine 10 volumes of analytical grade absolute ethanol ((C_2H_5OH), purity = 99.8%, MW = 46.07, d = 0.79 g/mL) with 1 volume of 11.0N potassium hydroxide (KOH, MW = 56.11). Mix fifty times using a stirring rod. Centrifuge at 3,000 rpm for 15 minutes. After centrifugation, a small white precipitate or a separate phase may be observed at the bottom of the tube; this is caused by potassium carbonate that is present in some KOH preparations. The clear supernatant solution should be used within 60 minutes of preparation.

2. Kerosene/xylene 1:1 volume

   Mix, just before using, the necessary amounts of kerosene (fraction distilled in glass at 206°–216°C [18]) and xylene (fraction distilled in glass at 138°–143°C[19]) until the solution turns clear. Both the individual solvents and the mixture should be stored in dark bottles.

F. Procedure

1. If the samples have been in the freezer, thaw at room temperature (20°–25°C) and mix well in a Vortex mixer. Centrifuge at 2,500 rpm for 10 minutes preferably in a refrigerated centrifuge to eliminate any interference due to fibrin formation.

2. Measure in duplicate 0.5 mL serum or plasma into 12 mm x 75 mm glass tubes.

3. Prepare in duplicate a reagent blank with 0.5 mL of distilled water and a control serum with 0.5 mL of serum of known retinol concentration.

18. These temperatures must be adjusted to the specific barometric pressure of each place. Thus:

$$t_{corr} = t_{ref} - \frac{(273 - t_{ref}) \times (760 - mmHg)}{10{,}000}$$

19. See footnote 18.

4. Add 0.5 mL of alcoholic KOH to all tubes. Mix in a Vortex mixer for 10 to 20 seconds.

5. Close the tubes with rubber stoppers and incubate in a water bath at 56°C for 20 minutes.

6. Cool at room temperature. Add 1.0 mL of 1:1 kerosene/xylene mixture. Mix in the Vortex mixer for 30 seconds measured with a stopwatch. Agitation should be vigorous to ensure a complete extraction.[20]

7. Centrifuge at 3,000 rpm for 10 minutes preferably in a refrigerated centrifuge.

8. Remove the organic phase using a Pasteur pipette and transfer to a spectrophotometric cuvette. Be careful not to transpose any of the aqueous phase. Read the absorbance of the organic phase at 328 nm against xylene/kerosene.

9. Using a Pasteur pipette, transfer the organic extract from the spectrophotometric cuvette to a 10 mm x 75 mm tube transparent to ultraviolet light. Irradiate the tubes with ultraviolet light for 35 minutes or the time determined necessary to destroy retinol (see section XIV.C.2).

10. Between samples, aspirate the residue in the spectrophotometric cuvette with a Pasteur pipette connected to a vacuum system. Wash the cuvettes with the kerosene/xylene mixture every 15 readings.

11. Read the absorbance of every irradiated extract against the mixture of xylene/kerosene at 328 nm.

20. The volume ratio for serum:alcoholic KOH:xylene/kerosene described in the procedure is 1:1:2. An increase in sensitivity may be achieved by either *(a)* increasing proportionally the serum (or plasma) volume and alcoholic KOH but keeping the 1:1 ratio without changing the kerosene/xylene volume. In this case, the test tube size should be increased to hold the larger volumes or *(b)* decreasing the kerosene/xylene volume while keeping the serum or plasma and alcoholic KOH volumes unchanged. In these cases, the irradiation chamber may have to be slightly modified to ensure complete irradiation of the samples by UV light. Keep in mind that any variation in serum or plasma and kerosene/xylene volumes implies a different dilution factor in the equation for the calculations.

G. Calculations

1. Correct the absorbance of each sample before irradiation by subtracting the absorbance of the unirradiated blank. Subtract the absorbance of the irradiated blank from the absorbance of the irradiated sample. Sometimes the absorbance of the reagent blank is negative, therefore, the value should be added instead of subtracted.

2. Calculate the specific absorbance due to retinol by subtracting the corrected readings of the irradiated tubes from the readings of the unirradiated ones and correct by the "efficiency factor" of the irradiation chamber (see section XIV.C.2)

3. Calculate the retinol concentration using the following equation:

$$Retinol\ (\mu g/dL) = \frac{Abs_{Retinol} \times Vs \times 10^6}{å \times Vm}$$

where:

$Abs_{retinol}$ is the absorbance of each sample obtained in step IX.G.2.

The parameters for the equation are:

Parameter	Explanation	Value
å	Retinol absorption coefficient ($g^{-1}cm^{-1}dL$) in kerosene:xylene at 328 nm	1,570
Vs	Volume of organic phase (mL)	1.0
Vm	Volume of the sample (mL)	0.5

The equation is simplified to:

$$Retinol\ (\mu g/dL) = Abs_{retinol} \times 1{,}273.9$$

H. Verification of the method's reproducibility

To verify the method's reproducibility through independent runs, a pool of serum should be prepared and its exact retinol concentration determined. To do this, prepare a series of vials with 1.5 mL of control serum, leaving as little space as possible between the surface of the sample and the vial cap. Blow inert gas (nitrogen) in the tubes and close tightly. Store the control vials in the freezer at !20°C or lower. Include a duplicate serum control in every analytical run. After ten or more runs of the control, calculate the method's between-run coefficient of variation.

I. Verification of the method's recovery

The recovery of the retinol extraction should be verified periodically. The following procedure is suggested:

1. Irradiate a plasma or serum sample with UV light for 1 hour. Then, divide the sample into two portions. To one portion, add 10 µL of a 50 mg/mL retinol solution in absolute ethanol[21] for each milliliter of the serum or plasma (control). To the second portion (the blank), add 10 µL of ethanol for every milliliter of serum or plasma. Treat both portions as independent samples following the analytical procedure from step IX.F.2.

2. Calculate the absorbance due to the added retinol as follows:

$$Abs_{retinol} = (Abs_{cont} - Abs_{blank} - Abs_{contirr} + Abs_{blankirr})$$

where Abs_{cont} and $Abs_{contirr}$ are the absorbances before and after irradiating the retinol-containing controls and Abs_{blank} and $Abs_{blankirr}$ are the absorbances for the blanks before and after irradiation.

The value from this calculation (*s*) is the absorbance due to the added retinol.

3. Independently, place 50 mL of the same 50 µg/mL retinol solution in a 10-mL volumetric flask and make up to volume with the kerosene/xylene mixture. Read the absorbance of this solution at 328 nm against the kerosene/xylene mixture and multiply by 0.99, which is the ratio between this dilution and the dilution occurring during the recovery assay (200/202). This value (*t*) is the theoretical absorbance that would have been found if recovery of retinol was 100 percent efficient.

21. A dilution of this solution should have an absorbance near to 0.9 when read at 325 nm against ethanol.

4. Calculate recovery (*R*) as follows:

$$R = \frac{s}{t} \times 100$$

J. Variations in assay

If a spectrophotometer capable of reading absorption accurately using smaller cuvettes is available, samples and reagent volumes can be proportionally reduced; however, the size of the test and irradiation tubes, as well as the irradiation chamber will also have to be adjusted.

When a micromethod is used, the spectrophotometer's capability of reading small cuvettes should be verified. To do this, prepare dilutions of a 50 µg/mL retinol in ethanol solution at 1:100, 2:100, 3:100, 4:100, and 5:100. Read the absorbance of these solutions at 325 nm using normal quartz cuvettes (3 mL) and compare the absorbances with those from semimicro (1 mL) or micro (less than 1 mL) quartz cuvettes. The reading for the latter two cuvettes should coincide with those for the normal cuvettes. The above retinol in ethanol solutions should have absorbances ranging from 0.100 to 0.500.

X. DETERMINATION OF BLOOD RETINOL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A. References

Bankson, D. D., R. M. Russell, and J. A. Sadowski. 1986. Determination of retinyl esters and retinol in serum or plasma by normal-phase liquid chromatography: method and applications. *Clin. Chem.* 32: 35–40.

Bieri, J. G., T. J. Tolliver, and G. L. Catignani. 1979. Simultaneous determination of á-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.* 32: 2143.

DeRuyter, M. G. M. and A. P. Leenheer. 1976. Determination of Serum retinol (vitamin A) by high-speed liquid chromatography. *Clin. Chem.* 22: 1593–95.

Packer L. 1990. *Methods in Enzymology, Retinoids. Part A, Molecular and Metabolic Aspects.* 189: 75–76, 155–67, 170–75. New York: Academic Press.

B. Principle

This is a highly selective and accurate method that requires neither blanks nor irradiation of the retinol-containing extracts. The sample does not require saponification, because the method is designed to measure specifically the nonesterified retinol in blood. The method can be modified if retinyl ester determinations are needed.

Serum or plasma is diluted 1 to 2 with a retinyl acetate solution in ethanol. The retinyl acetate acts as the internal standard, and the ethanol precipitates the proteins, which releases the retinol that is then extracted with hexane. The extract is evaporated in a nitrogen atmosphere and the residue resuspended in methanol. Retinol is separated by high performance liquid chromatography using a fixed apolar phase (C_{18}) and 95 percent methanol as the mobile phase. Retinol is detected with an ultraviolet detector at 325 nm, and its concentration is determined from the ratio of its peak area to that of the retinyl acetate.

C. Critical points and cautions

A spectrophotometer capable of reading 325 nm, even when high pressure liquid chromatography (HPLC) is used, is essential. This is because the concentration of the retinol standards has to be verified by spectrophotometric analysis. 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.[22] This confirmation should be carried out frequently and not only when a new lamp is installed.

Once retinol has been extracted in the organic phase, the analysis should not be interrupted.

Because the retinol standard and retinol acetate internal control are not completely pure, their purity must be estimated. This can be done by calculating the percent of the peak area for each analyte over the total area of all the peaks detected in the chromatogram.

In situations in which no HPLC-grade water is commercially available, it can be prepared in the laboratory by demineralizing, filtering, and boiling water that has been doubly distilled.

Based on the experience at the INCAP laboratory, two independently analyzed subsamples of the same sample should not differ on average by more than 10 percent. If the variation is greater than 10 percent, the assay should be repeated. The recovery of retinol using this method is 95 percent or more.

D. Equipment and materials

Vortex mixer
Refrigerated centrifuge
Liquid chromatograph with UV detector
Micropak column SP-18-5 of 150 x 4 (DI) mm
Stopwatch
UV/VIS spectrophotometer
Spectrophotometer quartz cuvettes
10 mL and 100 mL volumetric flasks
Dark glass bottles

22. Section XIV.C.1 includes a description of a simple procedure to calibrate spectrophotometers routinely used in clinical laboratories.

Graduated serological pipettes
Automatic pipettes (Eppendorf type)
Pasteur pipettes
Conical centrifuge tubes (9 mm x 76 mm)
500 µL amber micro centrifuge tubes
10 mL beaker
Aspiration bulbs for Pasteur and serological pipettes
45 mm millipore membrane

E. Reagents

1. Analytical grade absolute ethanol ((C_2H_5OH), purity = 99.8%, MW = 46.07, d = 0.79 g/mL)

2. HPLC grade hexane ((C_6H_{14}), purity = 95%, MW = 86.18, d = 0.66 g/mL)

3. HPLC grade methanol ((CH_3OH), purity = 100 %, MW = 32.04, d = 0.79 g/mL)

4. Nitrogen gas (99.5 percent purity)

5. Internal standard (retinyl acetate)

   a. Stock solution (about 500 mg/mL)

      Weigh 0.056 g of retinyl acetate of the highest purity (MW = 328.5, 90% purity) in a 10 mL beaker and dissolve with absolute ethanol. Transfer to a 100 mL volumetric flask. Rinse the beaker several times with absolute ethanol and transfer the washings to the volumetric flask. Add approximately 0.002 g of BHT (3,5-bis-(t-butyl)-4-hydroxy-toluene). Make up to 100 mL with absolute ethanol. Store the solution in the freezer (!20°C) in a dark glass bottle with glass stopper. The concentration of this solution is calculated from the concentration of the secondary solution.

   b. Primary solution (about 50 µg/mL)

      Dilute 1 mL of stock solution to 10 mL with absolute ethanol in a 10 mL volumetric flask. Store as indicated for the stock solution and calculate its

retinol concentration from the concentration of the secondary solution.

c. Secondary solution (about 0.5 µg/mL)

Dilute 0.5 mL of primary solution to 50 mL with absolute ethanol in a 50 mL volumetric flask. Store the solution in a refrigerator. Discard the remainder after using it.

The concentration of retinyl acetate in the secondary solution is determined in a spectrophotometer as follows:

i. Zero the spectrophotometer using absolute ethanol. Read in triplicate the absorbance of the secondary solution of retinyl acetate at 325 nm.

ii. Divide the average value of the absorbance by 0.156[23] and multiply by the degree of purity of the reagent as determined in HPLC (peak area of retinyl acetate/total area of all peaks.) The result is the actual concentration of retinyl acetate in the secondary solution in µg/mL.

The µg/mL retinyl acetate concentration in the primary and stock solutions is obtained by multiplying this value by 100 and 1,000, respectively.

6. Standard retinol solutions

a. Stock solution (about 500 µg/mL)

Weigh 0.071 g retinol of the highest purity (MW = 286.5, 70% purity) and dissolve with absolute ethanol in a 10 mL beaker. Transfer to a 100 mL volumetric flask. Rinse the beaker several times with absolute ethanol, and transfer the washings to the volumetric flask. Add approximately 0.002 g of BHT. Make up to 100 mL with absolute ethanol. Store the solution in the freezer (!20°C) in a dark bottle with glass stopper. The concentration of this solution is calculated from that of the secondary solution.

23. This value is the absorption coefficient ($mg^{!1}cm^{!1}mL$) for retinyl acetate in ethanol at 325 nm.

b. Primary solution (about 50 µg/mL)

Dilute 1 mL stock solution to 10 mL absolute ethanol in a 10 mL volumetric flask. Store as indicated for the stock solution and calculate its concentration from the concentration of the secondary solution.

c. Secondary solutions (about 1–10 µg/mL)

Dilute 5 mL primary solution to 25 mL of absolute ethanol in a 25 mL volumetric flask. Dilute the secondary retinol solution with absolute ethanol to obtain at least 10 mL of five solutions with different concentrations of retinol ranging from 1 to 10 µg/mL. Store the secondary solutions in the refrigerator. Discard the solutions after using them.

The concentration of retinol in the most concentrated secondary solutions is determined in a spectrophotometer as follows:

i. Zero the spectrophotometer using absolute ethanol. Read in triplicate the absorbance of the 10 µg/mL secondary retinol solution at 325 nm.

ii. Divide the average value of the absorbance by 0.1845[24] and multiply by the degreee of purity of the reagent as determined in HPLC (peak area of retinol/total area of all peaks.) The result is the actual concentration of retinol in the secondary solution in µg/mL. The concentration of retinol in the other solutions is calculated by dividing each one's average value for absorbance by its dilution factor.

The retinol concentration in the primary and stock solutions is obtained by multiplying the concentration of the 10 µg/mL and secondary solutions by 5 and 50, respectively.

24. This value is the absorption coefficient ($mg^{-1}cm^{-1}mL$) for retinol in ethanol at 325 nm.

F. Procedure

1. Calibration curve

a. Measure 0.1 mL of primary retinyl acetate solution (50 µg/mL) and 1.0 mL of each secondary retinol solution into individual 10 mL volumetric flasks. Make up to volume with HPLC-grade absolute methanol.

b. Filter each solution through the 45 µ millipore membrane. Run the solutions in the HPLC.

c. Regress the ratio of the area of the peaks of retinol to the area of the peak of retinyl acetate multiplied by the concentration of the internal standard of retinyl acetate (y-axis) on the retinol concentration of the secondary retinol solutions in µg/mL (x-axis). The regression equation used to calculate the retinol levels of the samples is:

$$Y = mX + b$$

where:

Y = retinyl acetate (µg/mL) x (area peak retinol/area peak retinyl acetate)
X = retinol (µg/mL)
m = slope (Y/X)
b = intercept on y-axis

The regression equation should be calculated every fifteen days to confirm the consistency of the analytical conditions. However, for each run, at least one retinol standard must be analyzed to confirm the validity of the curve.

2. Extraction

One extraction is sufficient for plasma or serum and is recommended because more samples can be processed and few sources of error will be introduced.

a. If samples are frozen, thaw at room temperature (20°–25°C) and mix thoroughly using a Vortex mixer. Centrifuge at 2,500 rpm for 10 minutes to eliminate interference due to fibrin formation if plasma is being used.

b. Measure 0.25 mL of serum or plasma into duplicate 9 mm x 76 mm conical centrifuge tubes.

c. Add 0.25 mL of secondary solution (0.5 µg/mL) of the internal standard (retinyl acetate). Mix in the Vortex mixer for 1 minute.

d. Add 0.5 mL hexane and mix vigorously in the Vortex mixer for 1 minute to ensure complete extraction.

e. Centrifuge at 2,500 rpm for 2 minutes. A refrigerated centrifuge should be used where one is available.

f. Using a Pasteur pipette, transfer the organic phase to an amber micro centrifuge tube.

g. Evaporate the organic phase by blowing nitrogen into the micro centrifuge tube inside a laboratory hood for organic solvents.

h. Resuspend the extract with 0.25 mL HPLC-grade methanol. Mix in the Vortex mixer for 1 minute.

i. Centrifuge at 2,000 rpm for 5 minutes.

3. Chromatography

a. Using the HPLC syringe, take an appropriate amount of sample solution and inject into the machine following the optimal conditions for the apparatus, as determined by the laboratory. For example:

Mobile phase	95% methanol/5% water
Flow	1.6 mL/min
Detector	UV at 325 nm with 0.002 UA/mV sensitivity
Integrator attenuation	4
Paper speed	1 cm/min
Injection volume	100 µL
Elution time	Retinol: 2.5 min Retinyl acetate: 4.5 min

b. At the end of every day, wash the HPLC system for at least 30 minutes at a flow of 0.5 mL/min with a mixture of 95 percent methanol/5 percent water, followed by 100 percent methanol for 15 minutes.

G. Calculations

1. Calculate the ratio of the peak of retinol over the peak of retinyl acetate for each sample and multiply these ratios by the concentration of the secondary retinyl acetate internal standard.

2. The retinol concentration in the samples is calculated directly using the calibration curve.

3. To report retinol levels in the samples in µg/dL, *multiply* the values calculated in the preceding step by 100.

H. Verification of the recovery of the method

Even though it is not essential for the calculations, it is recommended that the recovery of the method be verified every 3 months. For this purpose the following procedure is suggested:

1. Irradiate a plasma or serum sample with ultraviolet light for 1 hour and separate into two portions. To one portion, add 10 µL of the primary 50 µg/mL retinol solution for each milliliter of the sample (control).[25] To the second portion, add 10 µL of ethanol for every milliliter of sample (blank). Treat both portions as independent samples and follow the chromatographic process.

2. Using the calibration curve, calculate the retinol level for both portions. Subtract the retinol value of the blank from that of the control. The result of this calculation is the amount of recovered retinol (*s*).

3. Independently, place 0.1 mL of primary retinol solution (50 mg/mL), 0.1 mL of primary acetate solution (50 µg/mL) and make to volume with HPLC grade methanol in a 10 mL volumetric flask. Run this solution in the HPLC system.

4. Determine the retinol level using the calibration curve. Divide this value by 1.01 to adjust for the dilution of the primary retinol solution in the control sample. This value (*t*) is the theoretical amount of retinol that would have been found if the recovery was 100 percent efficient.

5. Calculate recovery (*R*) as follows:

$$R = \frac{s}{t} \times 100$$

25. A 1:10 dilution of the primary retinol solution should have an absorbance around 0.9 at 325 nm against ethanol.

XI. PRINCIPALS OF METHODS TO DETERMINE RETINOL IN BREAST MILK

A. Introduction

Evaluating the nutritional impact of a sugar fortification program on vitamin A status can be done by measuring retinol levels in breast milk both before and after the intervention.

Two methods for determining breast milk retinol levels are presented here. The first is an adaptation of the classic spectrophotometric method of Bessey et al. (1946) (see section XII). The specificity of this method is based on the selective destruction of free or esterified retinol present in breast milk by irradiation with UV light. The second method requires an apparatus that does high performance liquid chromatography (HPLC) after saponification and extraction of the sample (see section XIII). This method is more specific than the spectrophotometric method; however, the spectrophotometric method has the advantage of being lower in cost and allowing for two or three times as many samples to be analyzed during the same period of time.

The choice of the method to use will depend on the purpose of the study and the resources available. While the HPLC is undoubtably more accurate and precise, both methods can provide the necessary information for evaluation purposes.

The determination of breast milk retinol levels involves the following laboratory steps:

1. *Saponification*. Samples are treated with alcoholic potassium hydroxide to hydrolyze the retinyl esters in the sample. Most of the vitamin A in breast milk is in the esterified form and therefore saponification is necessary to hydrolyze the esters and obtain a total retinol value. In the spectrophotometric method, saponification also improves the accuracy of the determination by avoiding errors due to differences in extraction rates and thus spectrophotometric characteristics between free retinol and its esters.

2. *Extraction*. Retinol and nonhydrolyzed esters are extracted with an organic solvent. A xylene-kerosene mixture is preferred in the spectrophotometric determination, while hexane or another low-boiling-point solvent is used in the HPLC determination.

3. *Determining absorbance*. In both the spectrophotometric and HPLC methods, the absorbance of retinol at 325 nm is used for its quantitative determination.

4. *Selective destruction of retinol*. To ensure that the absorbance is from retinol only, absorbance is determined in the sample extracts both before and after selective destruction of the retinol by irradiation with UV light. The HPLC method does not require this step because, before measuring the absorbance of retinol, it is chromatographically separated from other interfering substances including its esters.

B. Collection and management of breast milk samples

Ideally, the milk sample should be the total milk from one breast that has not been emptied for at least 2 hours, because the concentration of certain milk components, in particular fats and fat-soluble substances such as vitamin A, increase over time during suckling. This is impractical in field work, however, and it is recommended that the first 10 mL from a breast that has not been suckled for at least 1 hour be taken. It is essential that the procedure followed in collecting breast milk samples be clearly documented and consistently followed.

Breast milk can be expressed manually or with a hand pump into a 20 to 30 mL wide mouth, screw top tube/bottle made of glass or polypropylene (for example, Nalgene). Samples should be kept cool and away from air, sunlight, bright light, and high temperatures. Ideally, precise alliquots to be used in the analysis should be removed and placed in separate vials for long-term storage as soon after collection as possible. If the samples are likely to remain frozen for a long period of time, the atmosphere above the sample surface should be filled with nitrogen by blowing the gas without disturbing the sample for 30 seconds, after which they should be tightly closed. Samples should be frozen as soon as possible after collection and not thawed until ready for analysis. Prior to analysis, breast milk samples should be completely homogenized, by sonification wherever possible.

XII. APPLICATION OF THE SPECTROPHOTOMETRIC METHOD BY ULTRAVIOLET DESTRUCTION FOR THE DETERMINATION OF TOTAL RETINOL IN BREAST MILK

Even though the method described in section IX was originally proposed for the spectrophotometric determination of retinol in serum or plasma, it can be easily applied to breast milk. The principle of the method, as well as the equipment, materials, and reagents are the same for both blood and breast milk.

Milk samples should be carefully homogenized, treated, and analyzed in exactly the same way as for serum or plasma. The size of the aliquot remains the same, because the retinol concentration ranges are similar for both fluids. The results are also calculated using the same equations.

It should be noted that this method measures total preformed vitamin A and does not differentiate between free and esterified retinol. Because breast milk contains a sizable proportion of retinyl esters, the accuracy of the results, in terms of free retinol, is inferior to that of the method for blood. This is because saponification is incomplete and residues of nonhydrolyzed retinyl esters may behave slightly different during extraction. This limitation is amply compensated for by the practicability and low cost of this method, which are characteristics that make it suitable for surveys to evaluate the impact of vitamin A interventions. In these situations, the total content of retinol in breast milk (free and esters), that is, its activity as preformed vitamin A, is the main concern, because evidence exists that this parameter provides a good picture of the vitamin A status of the lactating women.

XIII. DETERMINATION OF BREAST MILK RETINOL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)[26]

A. References[27]

B. Principle

Retinol in breast milk is found mostly esterified to fatty acids. The ester linkage must be saponified to release the retinol. 3,4-Didehydroretinyl acetate, added to the milk before saponification, is used as an internal standard to determine extraction efficiencies. After saponification, the retinol and the didehydroretinol are extracted and analyzed by HPLC using a C_{18} reversed-phase column and a methanol/water mobile phase.

C. Critical points and cautions

A spectrophometer capable of reading 325 nm, even when high pressure liquid chromatography (HPLC) is used, is essential. This is because the concentration of the retinol standards has to be verified by spectrophotometric analysis. 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[28]. This confirmation should be carried out frequently and not only when a new lamp is installed.

Once retinol has been extracted in the organic phase, the analysis should not be interrupted.

In situations in which no HPLC-grade water is commercially available, it can be prepared in the laboratory by demineralizing, filtering, and boiling water that has been doubly distilled.

Based on the experience of the Department of Biochemistry and Biophysics, Iowa State University, two independently analyzed subsamples of the same sample should not differ on average by more than 10 percent. If the variation is greater than 10 percent, the assay should be repeated.

---

26. This section was written by Sherry Tanumihardjo, Department of Biochemistry and Biophysics, Iowa State University.
27. This method is based on the procedure developed by S. Tanumihardjo and J.A. Olson from the Department of Biochemistry and Biophysics, Iowa State University.
28. Section XIV.C.1 includes a description of a simple procedure to calibrate spectrophotometers routinely used in clinical laboratories.

D. Equipment and materials

Vortex mixer
Clinical centrifuge
Liquid chromatograph with UV detector
Water "Resolve" 5 µm C_{18} reverse-phase column
Stopwatch
UV/VIS spectrophotometer
Spectrophotometer quartz cuvettes
25 mL volumetric flask
Graduated serological pipettes
Automatic pipettes (Eppendorf type)
Pasteur pipettes
Aspiration bulbs for Pasteur and serological pipettes
10 mL screw top centrifuge tubes
7 mL test tubes with caps

E. Reagents

1. Analytical grade absolute ethanol ((C_2H_5OH), purity=99.8%, MW=46.07, d=0.79 g/mL)

2. Analytical grade 2-propanol (($CH_3CH(OH)CH_3$), purity=99.7%, MW=60.10, d=0.78 g/mL)

3. 50:50 w/v potassium hydroxide in water (KOH/H_2O)

4. Purified water

5. HPLC-grade hexane ((C_6H_{14}), purity=95%, MW=86.18, d=0.66 g/mL)

6. HPLC-grade methanol ((CH_3OH), purity=100%, MW=32.04, d=0.79 g/mL)

7. HPLC-grade ethylene dichloride (($ClCH_2CH_2Cl$), purity=99.8%, MW=98.96, d=1.25 g/mL) or HPLC-grade methylene dichloride ((CH_2Cl_2), purity=99.9%, MW=84.94, d=1.32 g/mL).

8. Nitrogen or argon gas (99.5 percent purity)

9. Internal standard solution of 3,4-didehydroretinyl acetate (DRA)[29] ($C_{22}H_{30}O_2$, MW=326)

   DRA is an excellent choice for an internal standard because:

   a. DRA can be added to the breast milk before the analysis is started and can be carried through until the HPLC determination.

   b. DRA is in the esterified form and will therefore undergo hydrolysis as do the retinyl esters in the breast milk.

   c. By monitoring a few HPLC runs longer than usual, the completeness of the saponification step can be determined in each laboratory.

   d. Didehydroretinol is easily separated from retinol during the HPLC analysis.

   DRA is prepared as follows:

   From the DRA stock solution (35 mM in corn oil), take 10 µL and mix with 25 mL isopropanol in a volumetric flask. This is the primary DRA solution.

29. Small quantities of this reagent dissolved in corn oil for use as an internal standard for breast milk analysis can be obtained from Iowa State University for a shipping and handling charge.

10. Standard retinol solutions

a. Stock solution (about 500 µg/mL)

Weigh 0.050 g retinol of the highest purity (MW=286.5 70% purity) and dissolve with absolute ethanol in a 10 mL beaker[30]. Transfer to a 100 mL volumetric flask. Rinse the beaker several times with absolute ethanol, and transfer the washing to the volumetric flask. Add approximately 0.002 g of BHT (3,5-bis-(t-butyl)-4-hydroxytoluene). Make up to 100 mL with absolute ethanol. Store the solution in the freezer (-20°C) in a dark bottle with glass stopper. The concentration of this solution is calculated from that of the secondary solution.

b. Primary solution (about 50 µg/mL)

Dilute 1 mL stock solution to 10 mL absolute ethanol in a 10 mL volumetric flask. Store as indicated for the stock solution and calculate its concentration from the concentration of the secondary solution.

c. Secondary solutions (about 1-10 µg/mL)

Dilute 5 mL primary solution to 25 mL of absolute ethanol in a 25 mL volumetric flask. Dilute this secondary retinol solution with absolute ethanol to obtain at least 10 mL of five solutions with different concentrations of retinol ranging from 1 to 10 µg/mL. Store the secondary solutions in the refrigerator. Discard the solutions after using them.

The concentration of retinol in the most concentrated secondary solutions is determined in a spectrophotometer as follows:

i. Zero the spectrophotometer using absolute ethanol. Read in triplicate the absorbance of the 10 µg/mL secondary retinol solution at 325 nm.

30. If retinol of high purity is not available in the market, it can be prepared by the following methods: (a) reduction of retinaldehyde (which is chemically more stable) with sodium borohydride ($NaBH_4$); (b) saponification of retinyl acetate with alcoholic potassium hydroxide (KOH); or (c) HPLC purification of commercial retinol by injecting a concentrated solution onto the HPLC and collecting the retinol peak as it elutes from the column.

ii. Divide the average value of the absorbance by 0.1845[31], and multiply by the degree of purity of the reagent as determined in HPLC (peak area of retinol/total area of all peaks).

The retinol concentration in the primary and stock solutions is obtained by multiplying the concentration of the 10µ/mL secondary solution by 5 and 50, respectively.

F. Procedure

1. Calibration curve

a. Measure 1.0 mL of each secondary retinol solution into individual 10 mL volumetric flasks. Make up to volume with isopropanol.

b. Filter each solution through the 45 µm millipore membrane. Run the solutions in the HPLC.

c. Construct a standard curve for retinol by plotting integrator peak areas versus the amount of retinol injected (ng). Determine the best fit straight line:

$$Y = mX + b$$

Where:

Y = peak area of retinol
X = mass of retinol injected (ng)
m = slope (Y/X)
b = intercept on y-axis

Determine the mass of retinol in the samples from their peak area either directly from the graph or from the relationship:

$$x = \frac{(y-b)}{m}$$

31. This value is the absorption coefficient ($mg^{-1}cm^{-1}mL$) for retinol in ethanol at 325 nm.

The regression equation should be calculated every fifteen days to confirm the consistency of the analytical conditions. However, for each run, at least one retinol standard must be analyzed to confirm the validity of the curve.

2. Saponification

a. Pipet an aliquot of breast milk (0.5 mL) into a 10 mL screw top centrifuge tube.

b. Add 40 µL of the DRA primary solution (internal standard).

c. Suspend in 0.75 mL ethanol by mixing in a Vortex mixer for 15 seconds.

d. Add 0.4 mL 50:50 (w/v) KOH/H_2O. Screw on top. Mix in a Vortex mixer for 15 seconds.

e. Place in a water bath at 45°C for 1 hour. Mix in the Vortex mixer every 15 minutes for about 15 seconds.

3. Extraction

a. After saponification extract by adding 1 mL hexane. Mix in the Vortex mixer for 30 seconds and centrifuge at 2,000 rpm for 30 seconds. Using a Pasteur pipette, transfer the top organic layer to a clean test tube. Repeat the extraction with hexane two more times, that is, mixing in the Vortex mixer and centrifuging.

b. Pool the organic layers and evaporate by blowing argon or nitrogen into the test tube, inside a laboratory hood for organic solvents.

c. Resuspend the extract with 100 µL of the appropriate solvent (i.e., 50:50 methanol:ethylene [or methylene] dichloride). Mix in the Vortex mixer for 15 seconds, centrifuge, and keep on ice packs until injection into the HPLC system.

4. Chromatography

a. Using the HPLC syringe, take 25 µL of sample solution and inject into the machine following the optimal conditions for the apparatus, as determined by the laboratory. For example:

Mobile phase	85% methanol/15% water **OR** 90% methanol/10% water
Flow	1 mL/min
Detector	UV at 335 nm[32]
Integrator attenuation	6
Paper speed	0.5 cm/min
Elution time	Retinol: 5.6 min Didehydroretinol: 4.6 min

b. At the end of every day, wash the HPLC system at least for 15 minutes at a flow of 1.0 mL/min with a mixture of 50% methanol/50% methylene dichloride, followed by 100% methanol for 15 minutes.

G. Calculations

1. Determine the amount of retinol in ng in the injected aliquot from the calibration curve.

2. Divide by the equivalent amount of breast milk volume used (i.e. 125 µL).

3. Correct the value of each sample by dividing it by the recovery proportion (see section XIII.H below).

4. To report retinol levels in the samples in µg/dL, multiply the values calculated in the preceding step by 100.

32. This is an intermediate point between the wavelengths of maximum absorbance for retinol, 325 nm, and for didehydroretinol, 350 nm.

H. Calculation of the recovery proportion

The following procedure is suggested:

1. Dilute 0.4 mL of the primary DRA solution to 1 mL with isopropanol, and inject 25 µL in the HPLC system.

2. Wait for the DRA to elute (about 10 minutes on a 15 cm C_{18} column). Depending on the column, the *cis-trans* isomers may be separated. If so, add the integrator areas together; this is the area if 100 percent recovery of the didehydroretinol has been acheived after saponification and extraction *(t)*. On reverse-phase columns, the dehydroretinol isomers most likely will not be separated. Each laboratory may want to verify this on their HPLC system.

3. Independently, add 40 µL of the primary DRA solution into 0.5 mL water. Then, follow the procedure from step F.1.c. to F.4.a. The integrator area of didehydroretinol is the actual amount extracted in the system*(s)*.

4. Calculate the recovery proportion of didehydroretinol *(R)* as follows:

$$R = \frac{s}{t}$$

   Each sample will have a unique value for *(s)* and should be corrected by dividing by *(R)*.

I. Variations

When expressing the vitamin A content by volume, the collection period for breast milk samples needs to be randomly distributed throughout the day to account for the wide variation in the fat content of the milk. If this is not possible, the fat content of the fresh milk should be accounted for by using a "creamatocrit" method. If fresh samples cannot be analyzed, the fat content must be determined using a chemical method[33].

The creamatocrit method is as follows:

1. Draw about 75 µL of well-mixed fresh breast milk into capillary tubes. Seal at one end and spin in a hematocrit centrifuge.

2. Measure the length of the cream layer and determine the amount of fat in reference to a standard curve of fat concentrations established by a chemical method.

3. Express the retinol amount on a per gram milk fat basis.

33. Various chemical methods can be found in: Ferris, A.M. and R.G. Jensen. 1984. Lipids in human milk: a review. 1:Sampling, determination, and content. *J. Pediatr. Gastroenterol. Nutr.* 3: 108-122.

XIV. LABORATORY QUALITY CONTROL PROCEDURES

A. Criteria of quality control[34]

All analytical methods have their own intrinsic characteristics, which determine their performance under optimal working conditions. This section focuses on the systematic quality control of laboratory operations rather than the intrinsic characteristics of each particular method. Analytical procedures are subject to errors, depending on the conditions under which they are carried out. These include the "human factor"; environmental factors such as temperature, humidity, and exposure to light; and the characteristics of the equipment used.

The two qualities of an analytical method that are subject to variation are *accuracy*, which is the degree to which the results represent the true or real value, and *precision*, which is the reproducibility of the results obtained under normal day-to-day conditions.

1. Determining accuracy

When setting up an analytical method, every laboratory should confirm its accuracy. Ideally, the accuracy of the method should be close to that of the original method, run under optimal conditions, as described in the literature. The approach to determining the accuracy of a method under routine normal conditions may vary from method to method. The most common approach involves the recovery of a known amount of the compound being analyzed. In this case, known amounts of retinol or retinyl palmitate are added to a portion of the sample. The samples with and without the added analyte are treated and analyzed in exactly the same way. The quantity of the analyte recovered, expressed as the percent of the quantity added, is the mathematical expression for the accuracy of the method. The validity of this assessment is improved if three concentrations of the analyte are used. These concentrations should correspond to levels in the "insufficient," "adequate," and "high" ranges of retinol as found under actual conditions in the samples. For example, a high level of retinol in fortified sugar could be 25 µg/g, an adequate level, 15 µg/g, and an insufficient level, 5 µg/g.

Another method for determining accuracy is to analyze *certified standard controls,* prepared by centers that specialize in producing controls such as the National Institute of Standards and Technology (NIST) (see appendix 3.3). The values obtained should be the same as those indicated by the issuing laboratory.

34. This section was prepared with the assistance of D. I. Mazariegos, INCAP.

An alternative way to determine accuracy is to compare the results obtained in the laboratory doing the testing with those obtained from the same samples in a reference laboratory. This is known as external validation. It is suggested that a minimum of twenty samples containing different levels of retinol be analyzed in both laboratories. The results should be plotted on a graph with the results of the reference laboratory plotted on the x-axis and those of the laboratory being tested on the y-axis. The correlation coefficient should also be calculated. Ideally, the slope of the regression line should be one and its intercept zero.

2. Determining precision

Precision is the expression of the method's reproducibility and has two components: the *within-run* variation, which is the random variation between replicates in the same run, and the *between-run* variation, which is the variation between independent runs. The way to determine the within- and between-run variations is described below.

Within-run variation. Select three retinol-containing samples with different concentrations corresponding to the "insufficient," "adequate," and "high" ranges that would be expected for the samples to be analyzed. Analyze each of the three samples at least ten times. The precision of the method is expressed as the coefficient of variation (CV), which is calculated as follows:

$$CV\ (\%)\ =\ \frac{standard\ \ deviation}{mean}\ \times\ 100$$

Between-run variation. Take the same three samples used in the within-run variation and analyze them in duplicate in ten different independent runs. The precision of the method is expressed as the coefficient of variation, using the same equation shown above. Because this test is done over time, it is important that the samples are preserved so that their concentration of retinol does not vary during the period of time required to complete the assays.

Total analytical error. The total analytical error (E_a) is the variation that results from the added effect of the within- and between-run variations, and is calculated as follows:

$$E_a\ =\ \frac{1}{2}\sqrt{CV^2_{within\ run}\ +\ CV^2_{betwn.\ run}}$$

As a general rule, a method that has an E_a of 5 percent is considered a good method. A method having an E_a of 10 percent may be considered acceptable when the analytical method is very complex. Each laboratory will have to decide on the lower limit of accuracy and precision acceptable for their

purposes.

B. Routine quality control

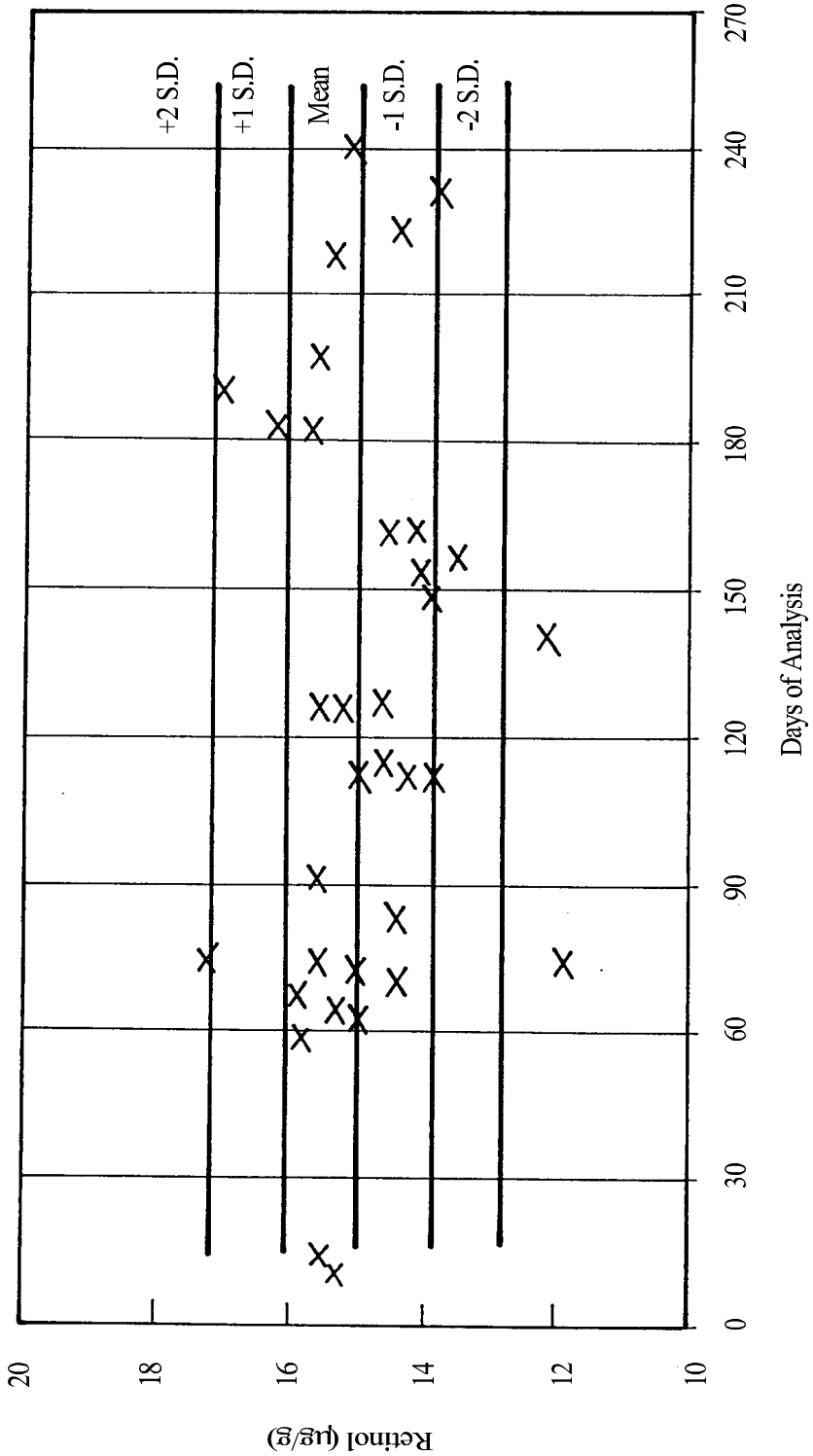
Once the method has been initially validated, a routine systematic approach must be put in place to ensure that the accuracy and precision of the method is maintained over time. All analytical methods should be run, at least in duplicate, as a standard procedure in any laboratory. Results should only be accepted if the value of each duplicate does not deviate from the average by more than twice the within-run precision of the method. When it is not possible to analyze all samples in duplicate, the reproducibility of the assay should be confirmed by analyzing duplicates of some of the samples (for example, 10 percent) selected randomly. The above situation should only be used when the number of samples surpasses the capacity of the laboratory, when the amount of each sample is limiting, or when the assay is too expensive.

In addition to the initial determinations of accuracy and precision, possible changes in these characteristics with time should be checked using reference control samples at three concentrations of retinol ("insufficient," "adequate," and "high") every time the assay is done. The complete run should be repeated if the results for two of the three reference controls is outside the limits of two standard deviations around the previously estimated mean for those reference controls. If only one reference control can be included in each run, the "insufficient" level should be used, because retinol assays are generally concerned with identifying samples below the low critical level.

The results of the accuracy and precision assays through time should be plotted on a graph of quality control, when the x-axis is the ordinal number or date of each run and the y-axis is the retinol value found for the reference control sample (see figure 3.2 on the next page). In this graph, lines parallel to the x-axis correspond to the values of the average plus or minus one and two standard deviations and are based on calculations reflecting between-run variations in the method.

If the method uses a calibration curve calculated with standards, this curve should be obtained ideally for each run by analyzing at least five different concentrations of retinol covering the range of retinol that the laboratory is interested in. The correlation coefficient (r^2) for each calibration curve should be equal or greater than 0.98. Samples falling outside the upper limit on the calibration curve must be diluted and reanalyzed. Those that have results that fall below the standard having the lowest concentration should be reported as having a "concentration lower than" the value of the lowest standard. It is incorrect to extrapolate the calibration curve beyond the experimentally determined range. Finally, it is important to stress that the approval of a method by the laboratory manager depends on whether the expected quality of the method is maintained through time; therefore, when new factors are introduced (for example, modification of the technique or changes in procedures, new personnel, or new equipment), the method should be repeated until acceptable values for accuracy and precision are again achieved.

Figure 3.2:
FORTIFIED SUGAR QUALITY



C. Equipment calibration

1. Spectrophotometer calibration

A simple spectrophotometer can be used in retinol assays if previously calibrated against a reference spectrophotometer. To do this, the absorbance range of a retinol solution in ethanol or hexane should be obtained. The spectrum obtained for both spectrophotometers must match. After this, the absorbance at the peak of maximum absorption should be obtained in both spectrophotometers using a standard retinol solution. The correction factor (CF) for the calibrating spectrophotometer is:

$$CF = \frac{Absorbance_{reference}}{Absorbance_{calibrated}}$$

The range of linearity of the spectrophotometer should also be tested using retinol solutions in ethanol with concentrations ranging from 0.1 to 5.0 µg/mL (absorbances should be between 0.020 and 1.000), which can be obtained by appropriate dilution of a 50 µg/mL retinol solution, for example, 0.2:100, 0.5:100, 1:100, 2:100, 5:100, and 10:100. Readings should be made using standard-sized quartz cuvettes (3 mL).

2. Verification of the efficiency of the irradiation chamber

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.

A standard solution of retinol or retinyl palmitate is prepared (using the same solvent used for the sample) with a retinol concentration of 5 µg/mL. The absorbance of this standard solution should be close to 0.9 at 325 nm. The standard should be prepared freshly every time and protected from direct light at all times.

The unit is calibrated by determining the time required to destruct the retinol in the standard solution. To do this, two procedures are needed. First, confirm that all the positions in the irradiation chamber's test tube rack receive the same intensity of irradiation. Second, determine the optimal time of irradiation.

To check that irradiation is uniform along the test tube rack, all the test tube positions must be occupied by a tube containing the same retinol solution. After a fixed period of irradiation, for example, 15 minutes, the absorbance of each solution is determined. The test tube positions that destroy retinol at a lower magnitude than the norm should be labeled and not used; these are usually the positions at either end of the rack.

To determine the optimal time of irradiation, the same standard solution of retinol is irradiated in triplicate tubes for specific periods of time, for example, 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 minutes.

The procedures for calibrating the unit are as follows:

a. Turn on the UV lamps at least 30 minutes before use.

b. Place 1 mL of standard solution in all the tubes labeled to indicate the irradiation time. It is essential that the label is positioned so that it does not obstruct the path of the UV light.

c. Close the tubes using a stopper made of Teflon or high quality rubber, for example, vacutainer type. Mark the meniscus level in each tube before placing the tube in the rack.

d. Place the three controls, that is, those not irradiated (0-time) in the dark until read.

e. At the end of each irradiation time period, remove the tubes corresponding to that time period and place them in the dark together with the 0-time controls.

f. Continue the process until all the time periods are completed.

g. Check the level of liquid in each tube to ensure that there was no loss of solvent by evaporation. If it is lower than the mark (see step c), add solvent up to the level of the mark and mix the contents. (If the tubes are properly closed, there will be no evaporation.)

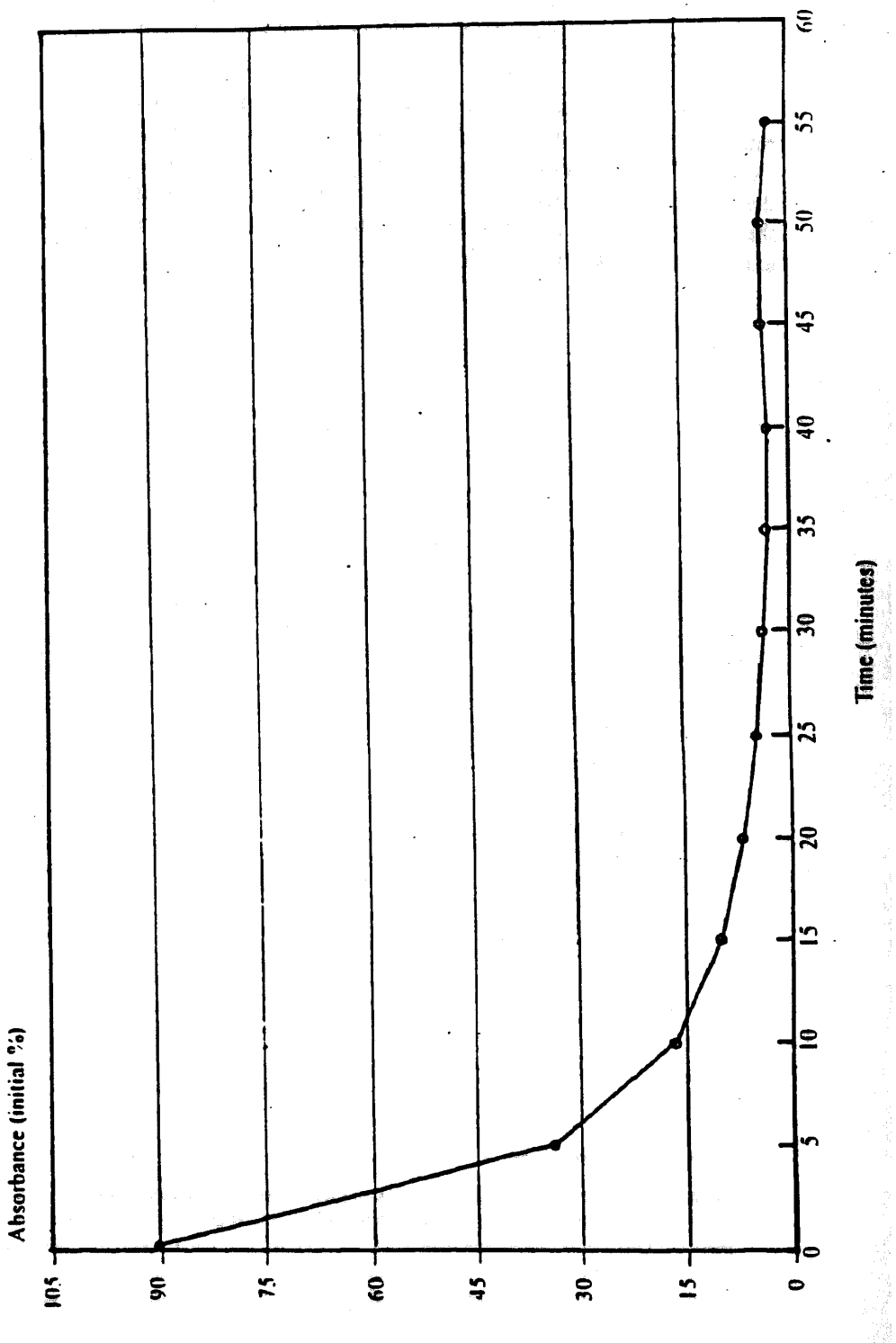
h. Adjust the zero of the spectrophotometer, using the solvent used to prepare the standards. Measure the absorbance of all solutions, including the controls, at 325 nm, using the same spectrophotometric cuvettes used for the sample extracts and under the same conditions.

i. Plot a graph of irradiation time (x-axis) against the mean absorbance for each point (y-axis) (see figure 3.3 on the next page).

The optimal irradiation time is the period of time in which at least 95 percent of the retinol is destroyed.[35] Once determined, this time should be the one used 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 percent of the 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).

35. Irradiation is not prolonged until retinol is completely destroyed for three reasons. First, 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. Second, 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. Third, interfering new complexes that absorb at 325 nm may be formed during prolonged irradiation.

Figure 3.3:
RETINOL ABSORBANCE BY IRRADIATION TIME



XVI. SUGGESTED READING

Arroyave, G., C. O. Chichester, H. Flores, J. Glover, L. Mejía, J. A. Olson, K. L. Simpson, and B. A. Underwood. 1982. *Biochemical Methodology for the Assessment of Vitamin A Status*. Washington, D.C.: IVACG.

Carey, N. and C. Garber. 1989. "Evaluation of Methods." In L. Kaplan and A. Pesce, eds. *Clinical Chemistry: Theory, Correlation, and Analysis*. 2nd edition. Chapter 19. pp. 290–310. Saint Louis: Mosby Company.

Conacher, H. 1990. Validation of methods used in crisis situations: task force report. *J. Assoc. Off. Anal. Chem.* 73(2): 332–34.

Cornbleet, J. and N. Gochman. 1979. Incorrect least squares regression coefficients in method-comparison analysis. *Clin. Chem.* 25(3): 432–38.

Association of Official Analytical Chemists. 1988. Guidelines for collaborative study procedure to validate characteristics of a method of analysis. *J. Assoc. Off. Anal. Chem.* 71(1): 161–73.

Ruig, W., R. Stephany, and J. Dijkstra. 1989. Criteria for the detection of analytes in test samples. *J. Assoc. Off. Anal. Chem.* 72(3): 487–90.

Appendix 3.1
CONSTRUCTION OF AN ULTRAVIOLET IRRADIATION CHAMBER

The irradiation unit (see appendix figure 3.1a on the next page) is a simple, low-cost unit made of wood on which two ultraviolet (UV) lamps (350–390 nm emission)[36] are mounted.

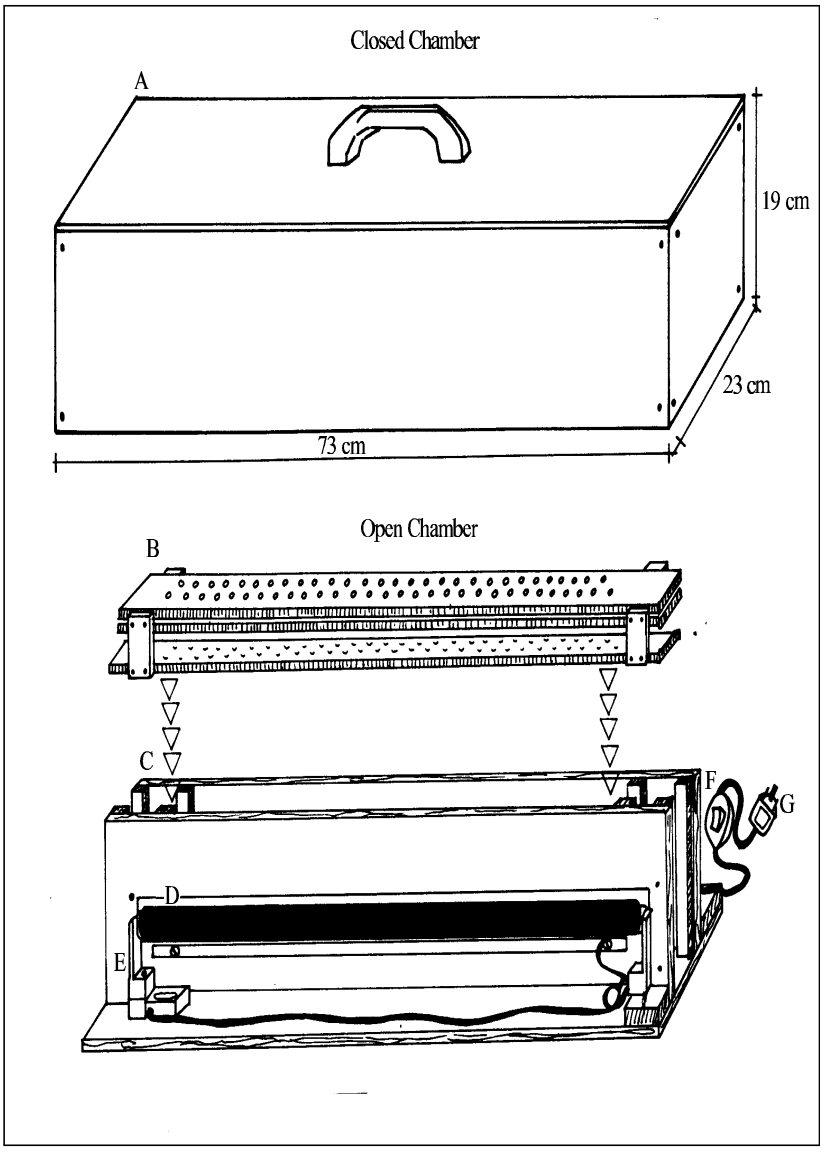
The base has two parallel lateral walls (point B, appendix figure 3.1a). The lower part of the lateral walls has openings that extend along the length of the walls and expose the test tubes containing the retinol solutions to the UV light (point C, appendix figure 3.1a). The test tube rack slots into the gap between the lateral walls (point D, appendix figure 3.1a), when 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 center of any two adjacent tubes is enough to allow the light from both UV lamps to fully reach and expose the extracts 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, appendix figure 3.1a) are attached to the base of the unit. Both lamps are operated simultaneously by a switch (point F, appendix figure 3.1a) that is outside of the unit. The system is covered by a wooden top (point A, appendix figure 3.1a), with a small hole at the side for the electric cord.

Appendix figure 3.1b gives details of the construction plan.

36. General Electric F20 T 12/B<13 20-watt lamps have proved good.

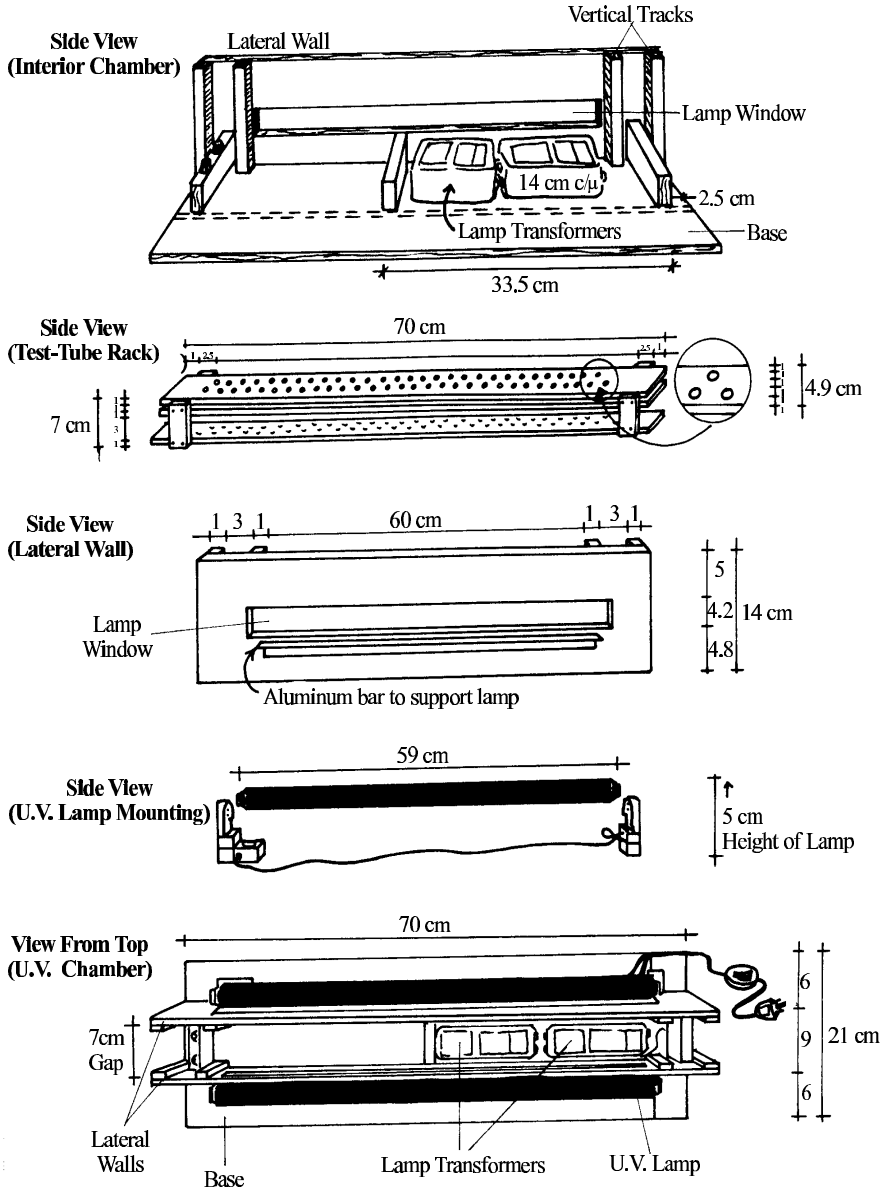
Appendix Figure 3.1a
IRRADIATION CHAMBER



A = Wooden Cover
B = Test Tube Rack
C = Lateral Walls
D = Lateral Window
E = U.V. Lamp-Mounting
F = Lamp Switch
G = Lamp Plug

Appendix Figure 3.1b
DIMENSIONS OF AN IRRADIATION CHAMBER

Figure A-3-1b



Appendix 3.2
SUGGESTIONS FOR CLEANING GLASSWARE AND SPECTROPHOTOMETRIC CUVETTES

The cleanliness of glassware and spectrophotometric cuvettes is essential for obtaining reliable results. The spectrophotometric cuvettes should be carefully washed and handled to avoid scratching and staining. The following steps should be followed to ensure that the condition of the glassware and spectrophotometric cuvettes do not affect the results.

I. Glassware

A. Discard any sample residues and reagents in appropriate containers. Organic solvents should be incinerated or, in some instances, recovered by distillation. Water-soluble or innocuous substances can be poured down the drain followed by abundant water. Before discarding biological materials, they should be decontaminated by treatment with a sodium hypochlorite solution for 24 hours or autoclaved for 30 minutes at 121°C.

B. Submerge the glassware in tap water. Large pieces of glassware should be brushed with a 2–3 percent solution of an alkaline detergent (for example, Extran or Teepol). Small glassware, vials, and pipettes should be boiled for 15 minutes in a 5 percent alkaline detergent solution.

C. Rinse with tap water.

D. Soak overnight in a 10 percent nitric acid solution. Stained glassware can be treated with a 50 percent solution of nitric acid.

E. Rinse twenty times with tap water and then ten times with distilled water.

F. Dry in an oven at 60°C.

G. Store in a closed cabinet.

II. Spectrophotometric cuvettes

A. After each use, rinse the cuvettes with the solvent used to dilute the samples. Then, rinse in a 1:2:1 solution of water/95 percent ethanol/concentrated hydrochloric acid.

B. If the cuvettes are frequently used, store submerged in the above 1:2:1 solution.

C. If the cuvettes are infrequently used, remove from the solution, rinse five times with acetone, and blow the inside dry with air filtered through a dry cotton filter. *Never dry the cuvettes using heat or in an oven.*

D. Store cuvettes in a closed container.

E. Prior to using the cuvettes, rinse the inside with the solvent used to dissolve the samples and suck out the remaining solvent using a vacuum.

As a routine practice, especially to detect any problems with a cuvette (dirt, stains, and scratches), it is important to verify that the absorbance reading of all the cuvettes in any position, including that for the reference cuvette, is essentially the same when filled with the same solution. Sometimes a small, but constant, difference may be found among cuvettes. In this case, this difference should be corrected for.

Appendix 3.3
SUPPLIERS FOR LABORATORY EQUIPMENT AND REAGENTS

I. Suppliers

A. ALDRICH
P.O. Box 355
Milwaukee, WI 53201-9358
Tel: (414) 273-3850
Fax: (414) 273-4979

B. BASF
6700 Ludwigfhafen-Rhein, Ludwingfhafen, Germany
Tel: (049) 621-600
Fax: (049) 622-525

C. Beckman Instruments Inc.
2500 Harbor Boulevard, Fullerton, CA 92634
Tel: (714) 521-3700
Fax: (714) (213) 691 -0841

D. Fisher Scientific
Headquarters
711 Forbes Avenue
Pittsburgh, PA 15219-4785
USA
Tel: (412) 562-8300

International Headquarters, Latin America, Mexico, and Caribbean
50 Fadem Road
Springfield, NJ 07081-3193
USA
Tel: (201) 467-6400

Fax: (201) 379-7415

Europe-Mid East/Africa
New Enterprise House
St. Helen's Street
Derby, DE 1 3GY
United Kingdom
Tel: (44) 332-200755
Fax: (44) 332-296546

Asia and Pacific
No. 1 101 Thomson Road
16-05 United Square
Singapore 1130
Tel: (65) 250-9766
Fax: (65) 253-2286

E. Hoffman-La Roche
CH-4002, Basel, Switzerland
Tel: (061) 688-1111
Fax: (061) 691-9600

F. J. T. Baker
222 Red School Lane
Phillipsburg, NJ 08865
USA
Tel (908) 859-2151
Fax: (908) 857-4318

G. Millipore Intertech
P.O. Box 10
Marlborough, MA 01752
USA
Tel: (508) 624-8400
Fax: (508) 624-8630

H. National Institute of Standards and Technology (NIST)
Bldg. 202, Room 204
Gaithersburg, MD 20899
Tel: (301) 975-6776
Fax: (301) 948-3730

I. Perkin Elmer Corporation
761 Main Ave., Norwalk, CT 06859-0012
USA
Tel: (203) 762-1000
Fax: (203) 762-6000

Bodenseewerk Perkin-Elmer GmbH
Postfach 10 11 64, D-7770 Uberlingen, Germany
Tel: (075) 51-81-0
Fax: (075) 51 -1612

Perkin-Elmer Ltd.
Post Office Lane, Beaconsfield, Bucks HP9 1 QA
England
Tel: (0494) 676161
Fax: (0494) 679333

J. Sarstedt
P.O. Box 468, Newton, NC 28658-0468
USA
Tel: (704) 465-4000
Fax: (704) 465-4003

K. Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178-9916
USA
Tel: (314) 771-5750
Fax: (314) 771-5757

L. VARIAN
Varian Associates
Parts and supplies center
220 Humboldt Court
Sunnyvale, CA 94089
USA
Tel: (408) 734-5370
Fax: (408) 744-0261

II. Equipment (examples)

A. Spectrophotometer UV/VIS, VARIAN DMS- 100S or Perkin Elmer Lamda 3B HPLC

B. Varian Chromatography 5500 with UV detector. Includes Autosampler Vista 9090, interphase Varian IIM-A, DS-650 computer, and HP ThinkJet printer

C. Micropak Column Sp-18-5 of 150 x 4 (Dl) mm (Varian 03-912042.42)

D. Test tubes 10 x 75 mm. Kimble KG-33

E. Masked semi-microcell spectrophotometer cuvettes. Beckman, Product 533043

III. Reagents (examples of supliers with catalogue number)

A. *Spectrophotometric determination of retinol in premix*
2-propanol. Merck Art. 9634

B. *Spectrophotometric determination of retinol in fortified sugar*
Absolute ethanol. Merck Art. 983.25
Hexane. Sigma Art. H-9379
Sodium hydroxide. Sigma S-0899

C. *Colorimetric determination of retinol in fortified sugar*
Trichloroacetic acid. Merck No. 1839
Dichloromethane. Merck No. 1593
Cupric sulfate. Fisher Scientific Company Art. No. 493

D. *Peroxides in oil*

Glacial acetic acid. Sigma Art. A-0808
Chloroform, USP. Aldrick 31,998-8
Starch. Merck Art. 1252
Sodium thiosulfate. Merck Art. 6516
Potassium iodide. Merck Art. 5043 Kl
Concentrated hydrochloric acid. Sigma Art. H-7020
Potassium dichromate. Sigma Art. P-6435

E. *Spectrophotometric determination of blood and milk retinol by UV destruction*

Absolute ethanol. Merck Art. 983.25
Potassium hydroxide. Aldrick Art. 30,656-8
Deorodized kerosene. J.T. Baker Art. P339-00
Xylene (analytical grade). J.T. Baker Art 9490-03

F. *HPLC determination of blood and milk retinol*

Absolute ethanol. Merck Art. 983.25
2-propanol. Merck Art. 9634
Hexane (HPLC grade). J.T. Baker Art. 9303-3
Methanol (HPLC grade). J.T. Baker Art. 9093
Ethylene dichloride (HPLC grade). Sigma-Aldrich Art. 27,056-3
Methylene dichloride (HPLC grade). Sigma-Aldrich Art. 27,057-1
Retinyl acetate. Sigma Art. R-3513
BHT. Sigma Art. B-1378
Retinol. Sigma Art. R-7632

G. *Irradiation chamber*

Irradiation lamps. General Electric F20 T 12 BLB