Manual for the determination of retinol and carotenoids in blood and human milk

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January 2003

This work was carried out with the aid of a grant from the Micronutrient Initiative, Ottawa, Canada.

Acknowledgements

This manual was made possible by the contribution of many people to whom we are grateful.

The manual was reviewed by:

- *Neal E. Craft, Craft Technologies, Inc., Wilson, NC 27893 USA*
- *Omar Dary, Institute of Nutrition of Central America and Panama, Guatemala City, Guatemala (present address, MOST Project/USAID Micronutrient Program, Arlington VA 22209 USA)*
- *Juergen Erhardt, University of Hohenheim, Hohenheim, Germany*
- *Laila Hussein, National Research Centre, Cairo, Egypt*
- *Edouard Ngou-Milama, Faculty of Medicine, BP 4009, Libreville, Gabon*
- *Delia B. Rodriguez-Amaya, Universidade Estadual de Campinas, Campinas, Brazil*
- *Liz van Stuijvenberg, Medical Research Council, Tijgersberg, South Africa*
- *David I. Thurnham, University of Ulster, Coleraine, Nothern Ireland, UK*

The late Peter van de Bovenkamp gave advice on laboratory techniques. Joke Barendse-van Leeuwen adviced on specimen collection.

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CHAPTER 1: GENERAL INTRODUCTION

A large proportion of the population in most developing countries continues to suffer from a deficiency of micronutrients. Such nutrients are essential and need to be consumed regularly in small amounts of the order of milligrams per day. The micronutrients that are most commonly deficient are vitamin A, iron, iodine, and probably zinc. It is not exceptional for more than 50% of women and children in a country to suffer from a deficiency of one or more of these nutrients. As a consequence, maternal and childhood mortality can be increased by about 30%, mental and motor development of young children can be reduced by 10-40%, and the work productivity of adults can be reduced by about 10%.

Population-based surveys suggest that as many as 175 million children of pre-school age are vitamin A deficient (UNICEF 1998). Especially in sub-Saharan Africa, clinical deficiency and deficiency as measured by biochemical markers ($\geq$ 20% prevalence of retinol value $\leq$ 0.70 μmol/L) frequently occur. This deficiency increases child morbidity and mortality and is the single most important cause of blindness among children in developing countries (Sommer 1996). Supplementation with vitamin A is estimated to lower child mortality by on average 23%, especially for mortality associated with diarrhoea and measles (Beaton et al. 1993). Governments working with UN agencies and non-governmental organisations aim to improve the vitamin A status of young children through interventions. National surveys, such as the UNICEF Multiple Indicator Cluster Survey (MICS), are implemented to evaluate the impact of intervention programs (UNICEF 1999). In countries where vitamin A deficiency is endemic, laboratories should be able to measure biochemical indicators of nutritional status to acceptable standards.

In general, the quality of data produced in a laboratory is an integrated result of 5 Ms: methodology (status of analytical methods, validation procedures, analytical quality control), machine (maintenance of equipment, support, state of art, calibration), men (skills, training and experience of staff and technicians), manipulation of data (calculation, modes of expression, documentation) and materials used (purity of standards and reagents, sample collection, treatment and storage). There is a continuing need for laboratories to demonstrate that their results are valid and fit for purpose. The results generated in inter-laboratory test comparisons, which provide a continuing assessment of the competence of participating laboratories, are indispensable for ensuring the quality of data produced in a laboratory.

In a recent study (Hulshof et al. 2002), the proficiency of 16 selected laboratories including 12 from Africa to quantify retinol in serum was investigated. The repeatability and the accuracy of the measurements from 9 laboratories were not satisfactory. Thus it was suggested to improve:

- Standardisation of the pre-analytical stage (sampling, sample preparation, transport and storage) in the assessment of retinol.

- Repeatability through monitoring and controlling within-laboratory variation by using in-house control material and standard quality control techniques.
- Accuracy through participation in inter-laboratory trials and by analysing Certified Reference Materials.

This manual has been prepared in order to provide laboratories with a useful tool for the measurement of retinol and carotenoids in blood and human milk to acceptable levels of repeatability (referred to as precision) and accuracy. Assuming that the total variability in a test result is the sum of the biological variation, the pre-analytical variation and the analytical variation, the manual focuses on improving both the pre-analytical and analytical stages in the assessment. In Annex 1, the importance of proper and standardised collection and handling of samples is illustrated with an example. The manual is divided in two sections: Section One presents guidelines for the collection, transport and storage of blood and human milk and also standard operating procedures for the analytical determination of retinol and carotenoids in blood and in human milk. Section Two presents information on good laboratory operations, guidelines for establishing in-house quality control procedures, and some performance characteristics in relation to method validation. Section Two is not specific for the assessment of the concentration of retinol and carotenoids but is in principle applicable to any method applied in a laboratory.

References

- *Beaton GH, Martorell R, Aronson KJ, Edmonston B, McCabe G, Ross AC, Harvey B. Effectiveness of vitamin A supplementation in the control of young child morbidity and mortality in developing countries. Toronto: University of Toronto, 1993.*
- *Hulshof PJM, Brouwer JT, Burema J, West CE. Bias and random error in retinol measurements of laboratories in countries with populations with mild to severe vitamin A deficiency. Clin Chem 2002; 48: 2061-2063.*
- *Sommer A, West KP. Vitamin A deficiency: health, survival and vision. New york: Oxford University Press, 1996.*
- *UNICEF. Multiple Indicator Cluster Survey. A survey tool for social change. New York: UNICEF, 1999.*
- *UNICEF. The state of the world's children 1998; focus on nutrition. Oxford: Oxford University Press, 1998.*

SECTION ONE: ANALYTICAL METHODS FOR THE DETERMINATION OF RETINOL AND CAROTENOIDS IN BLOOD AND HUMAN MILK

CHAPTER 2 : COLLECTION, HANDLING, TRANSPORT AND STORAGE OF SAMPLES

2.1 Collection and handling of blood

Before starting with the collection of samples, readers are advised to consult Chapter 4.4 on health and safety aspects of blood collection.

Reference made to specific brands does not imply that other brands cannot be used in the procedures given. The validity of any procedure should be demonstrated in the environment of the laboratory.

2.1.1 Standardisation of collection

2.1.1.1 Time of collection

For determination of retinol and carotenoids in plasma or serum, it does not seem important whether blood is drawn either in fasting or non-fasting state, as meals do not increase blood level of unesterified retinol or carotenoids (Mejia 1983). The following is recommended:

- Take blood samples in the morning to enable preparation for transport and storage.
- In surveys, blood sampling will take place over a scheduled period during the day. However, for intervention studies, blood should be collected from each individual at the same time to reduce possible diurnal effects.
- Document the time of sampling.

2.1.1.2 Collection tubes

Both serum and plasma can be used for the assessment of retinol and carotenoids. There are advantages of using plasma over serum:

- It saves time as clotting is eliminated from the preparation procedure.
- A little more plasma than serum can be obtained from the same amount of blood.
- There is a lower risk of haemolysis when plasma rather than serum is prepared.

Collection tubes containing EDTA should not be used for the preparation of plasma, as EDTA removes water rapidly from retinol to produce anhydroretinol (Furr et al. 1992, Guder et al. 1996). Tubes containing lithium-heparin as an anti-coagulant should be used instead. For the preparation of serum, blood is collected in a tube without anticoagulant. When serum separation tubes (tubes containing an inert gel barrier) are used, serum can be separated effectively from coagulum after clotting and centrifugation (Walters et al. 1995). Once the choice of a specific collection tube has been made, this type of collection tube should be used throughout the whole study.

The volume of the blood to be collected should be twice the volume of serum or plasma required for the laboratory test (to allow duplicate dterminations) plus the dead volume of the sample cup.

2.1.1.3 Position and preparation of the subject

The identity of the subject should always be checked before sampling. This is done by asking the subjects' name and checking the name with the laboratory request form and the label on the collection tubes. The blood collection procedure should be explained to the subject and the subject should be made aware of possible impending pain. Those taking blood samples should always be prepared to support a subject who may faint and be able to administer first aid if necessary. In addition, if blood has to be collected from a child, extra assistance should be requested during the collection procedure. The caretaker should not be expected to assist when extra help is needed. The subjects should be informed in advance that they should avoid strenuous exercise before blood collection. Otherwise, differences in concentration of blood constituents arising from variations in blood volume will occur (Burtis 1994).

The posture of a subject during the blood collection affects the concentration of the analyte measured in serum or plasma. When the position of the subject is changed from supine to upright, the filtration pressure in the lower extremities will increase. Intravascular water moves into the interstitial tissue, leaving blood particles as proteins behind. The concentration of protein in serum/plasma can increase up to 10% and so can that of retinol because it is protein-bound. The preference of the subject should be checked in advance and the posture of the subject at the initial blood collection should be used during subsequent blood collections throughout the study (Burtis 1994, Guder et al.1996).

A tourniquet is applied to facilitate finding an appropriate vein for venipuncture (Paragraph 2.1.2.3). The tourniquet slows the blood flow and makes veins more prominent. However, the tourniquet should not be so tight that the pulse of the subject is no longer palpable. As soon as blood flows into the collection system, the tourniquet should be released. If the constriction time is too long during venipuncture, stasis or haemoconcentration may occur in the vein. In addition, fluid and low molecular compounds move from the intravascular space into the interstitial tissue. Macromolecules, compounds bound to macromolecules including retinol, and blood cells do not penetrate the capillary wall, thus their concentration increases. A constriction time of one minute with subsequent release of the tourniquet has no consequences for the concentration of analytes in blood/plasma/serum.

The arm should be supported and extended in a straight line from shoulder to wrist, in order to help the phlebotomist to identify the best vein for the venipuncture. (Guder et al. 1996, Walters et al. 1995).

2.1.1.4 Avoiding of haemolysis

The degree of haemolysis in blood samples can be regarded as a quality marker of blood collection. When haemolysis takes place, damaged red blood cells release substances such as haemoglobin, lactate dehydrogenase, and potassium into serum. This results in changes in the composition of the sample. The following precautions should be taken to avoid haemolysis:

- Avoid squeezing or milking of the puncture site.
- Transfer blood from syringe to tube as gently as possible (if a non-vacuum blood collection system is used).
- Allow clotting for a minimum of 30 minutes at room temperature (20-22°C) when preparing serum.
- Centrifuge the blood and separate the cells from plasma or serum as soon as possible thereafter.
- Separate serum or plasma from blood cells before freezing.
- If it is necessary to transport blood from the collection site to the laboratory for serum/plasma preparation, avoid shocks and turbulence, as this will increase the risk of haemolysis.

2.1.2 Venipuncture

2.1.2.1 General

The venipuncture procedure is a commonly used method for blood collection. It involves the collection of blood from a vein in the forearm (Figure 2.1). A skin puncture can be an alternative method for blood collection when only small quantities of blood are required or when subjects are young (Paragraph 2.1.4).

2.1.2.2 Materials for venipuncture

The following materials are required:

- Gloves (Vinyl type: Tru-Touch or Nitryl type: Sempermed)
- Blood collection system:
  a) Sterile needle, sterile syringe (5-10 mL) and sterile blood collection tube (5-10 mL) or
  b) Sterile needle, holder and vacuum tube (5-10 mL with or without gel), or
  c) Sterile butterfly needle with a syringe and blood collection tube (5-10mL)
- Disinfectant pads (BonSwab) or gauze/cotton wool impregnated with disinfectant or with 70% (v/v) alcohol
- Tourniquet
- Gauze/cotton wool for covering the arm lesion
- Plaster for adhering gauze/cotton wool to the arm
- Biohazard sharps container

2.1.2.3 Method for venipuncture

The following steps should be carried out:

- Verify the identity of the subject with the laboratory request form and collection tubes.
- The subject should be seated or lying down if seating is not feasible.
- Put on gloves
- Check the needle, and syringe for burrs and defects.
- Support the subjects arm on a cushion.
- Ask the subject to stretch the arm in a straight line from shoulder to wrist and to clench their fist in order to help the phlebotomist to identify the best vein (Figure 2.1).
- Place the tourniquet around the arm above the elbow
- Clean the area of the puncture with disinfectant.

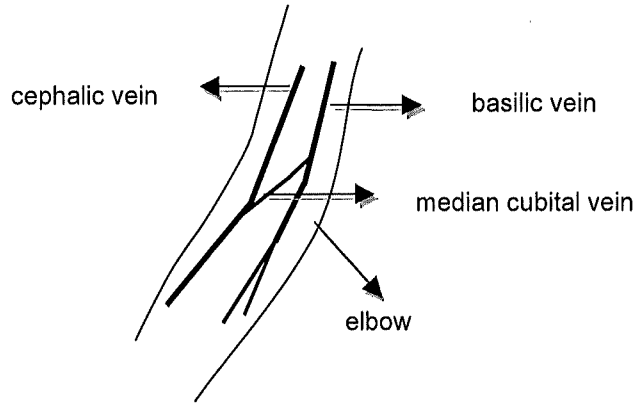


Figure 2.1: Sites for venipuncture

- Dry the site well with sterile gauze/cotton wool as a residue of disinfectants may dilute the specimen.
- Do not touch the cleaned site again, except with the gloved hand for entering the vein with the sterile needle (within one minute after applying the tourniquet).
- Release the tourniquet, as soon as blood flows into the collection tube.
- When sufficient blood has been collected: place gauze over the puncture site, withdraw the needle and apply pressure. Instruct the subject to press the gauze for 2-5 minutes with the arm stretched to ensure that no bleeding and no swelling will occur. Check the venipuncture site before the subject leaves and apply plaster to hold gauze on arm if necessary.
- When blood is drawn with a syringe, the blood should be transferred quickly by gentle ejection into the appropriate tubes. The tubes should then be capped to avoid evaporation. If the collection tube contains an additive or anticoagulant, the tube should be gently mixed by slowly inverting five times.
- Discard used needle into a biohazard sharps container; never recap used needles.
- Check again that tubes are correctly labelled with the subjects' identification data.
- The blood sample is now ready for serum/plasma preparation.

- If serum/plasma can not be prepared at the collection site, see Paragraph 2.1.5 for additional information on conditions during transportation of the blood.

2.1.3 Preparation of serum/plasma

2.1.3.1 Materials for preparation of serum/plasma

The following materials are required:

- Centrifuge capable of operating at 500-1500 g
- Pipette, graduated 1-5 mL, plastic or glass
- Freezer-proof polypropylene tubes (2-5 mL)
- Freezer-proof labels
- Low temperature resistant ink (or a pencil)
- Aluminium foil

2.1.3.2 Method for preparation of serum/plasma

The following steps should be carried out, preferably within two hours after the blood collection:

- For plasma preparation: centrifuge the blood sample as soon as possible after collection at 1200 g for 10-15 minutes.
- For serum preparation: let the blood sample clot for 30 minutes at room temperature (20-22°C) in a dark place or cover the tube completely with aluminium foil. Centrifuge the clotted blood sample at 1200 g for 10-15 minutes.
- Transfer serum or plasma to a freezer-proof labelled tube and close firmly.
- Samples are now ready for transport or storage (serum/plasma samples should never be stored in oversized tubes. If the size of a storage tube is too large, the dead volume increases and evaporation becomes a significant factor (see also Paragraph 2.1.5).

2.1.4 Skin puncture/dried blood spot

2.1.4.1 General

A skin puncture can be an alternative for the venipuncture when:

- A small quantity of blood is sufficient, e.g. for dried blood spot analysis.
- The subject has veins which hamper venipuncture.
- Field settings do not support venipuncture.
- The subject is a child ≤ 2 years.

In adults, the site for skin puncture is the third or fourth finger. The finger that is less hardened should be chosen. The blood should be taken from the palmar surface of the distal phalanx of the finger (Figure 2.2). The sides or tips of the finger should be avoided. To avoid injuring the bone in

the finger, the preferred puncture site in children ≤ 2 years, is the heel (Figure 2.3) (Meites 1988). Do not make a puncture in oedematous tissue and do not lance on a previous puncture site (PAMM 1993).

Only a special type of specimen collection paper should be used for the dried blood spot preparation. Do not use regular filter paper. Cryogenic labels and low temperature resistant ink or a pencil should be used to ensure proper labelling of specimen collection paper during storage (Paragraph 4.3.1).

2.1.4.2 Materials for skin puncture/dried blood spot

The following materials are required:

- Gloves (Vinyl type: Tru-Touch or Nitryl type: Sempermed)
- Disinfectant pads (BonSwab) or gauze/cotton wool impregnated with disinfectant or with 70% (v/v) alcohol
- Sterile gauze/cotton wool for drying finger and wiping away first drop of blood
- Disposable Lancets (BD Microtainer Safety Flow Lancet: for children ≤ 2 years, <2.0 mm; for children ≥ 2 years and adults, < 2.5 mm)
- Gauze/cotton wool for covering finger lesion
- Plaster for adhering the gauze/cotton wool to finger
- Biohazard sharps container
- Specimen collection paper for body fluid collection (Schleicher & Schuell, no 903: (http://www.schleicher-schuell.com/icm11be.nsf/(html)/FramesetBioScience)
- Drying rack for specimen collection cards
- Weighing sheets (glassine weighing paper)
- Freezer proof plastic bags for storage of specimen collection cards (Ziploc bags)
- Low temperature resistant ink or a pencil
- Desiccant (silica gel desiccant packs 2 gram, e.g. available from http://preservesmart.com/)

2.1.4.3 Method for skin puncture/dried blood spot

The following steps should be carried out:

- Verify the identity of the subject with the laboratory request form and the specimen collection card.
- The subject should be seated or lying down if seating is not feasible.
- Put on gloves.
- Chose the appropriate lancet and check for burrs and defects
- Chose the appropriate collection site:
  For children > 2 years and adults, blood can be taken from the palmar surface of the finger (Figure 2.2). For children ≤ 2 years, blood can be taken from the lateral or medial plantar surface of the heel pad (Figure 2 3).

- Clean the area of the puncture with disinfectant.
- Dry the site well with sterile gauze/cotton wool as a residue of disinfectants may dilute the specimen.

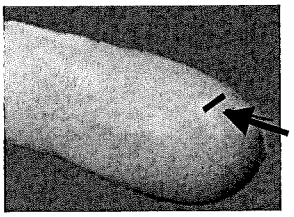


Figure 2.2 Puncture site on the palmar surface the third and fourth fingers.

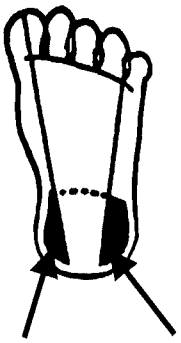


Figure 2.3: Recommended sites, shown in black, for obtaining blood from the heel of infants and young children.

- To perform the puncture, place lancet on the puncture site and depress the plunger and release. Then remove lancet and deposit in the sharp container.
- Wipe away first drop of blood with dry sterile gauze/cotton wool to avoid dilution of the blood drop with tissue fluid (Meites 1988).
- Allow one full size drop of blood to form by gravity and allow the drop to touch the centre of a circle on the specimen collection card (Figure 2.4). Do not press the skin on the surface of the card. Do not layer successive drops of blood on the circle spot.
- Make one application per circle. Fill at least two circles with blood.
- Apply a plaster on the adult puncture site. It is advised not to put a plaster over the puncture site of a child ≤ 3 years, because of skin sensitivity and because the plaster could loosen and be swallowed by the child.
- Check again that the specimen collection card is correctly labelled with the subjects' identification data.
- Let the paper dry thoroughly by air in the dark for a period of two to three hours at room temperature (20-25°C) in a simple drying rack. In humid environments the samples should dry for at least 3 hours or longer. Keep cards horizontal and avoid touching the collection areas with your hand.
- Samples are now ready for transport or storage (Paragraph 2.1.5).

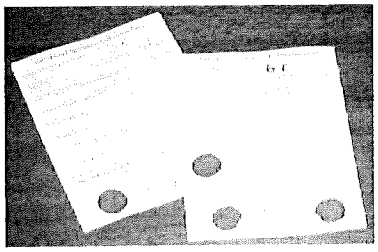


Figure 2.4: Specimen collection paper

2.1.5 Transport and storage of blood

A list with code numbers corresponding with the code numbers on the tubes or blood collection cards should be included in the transport unit.

2.1.5.1 Transport of blood samples

When blood samples cannot be processed at the collection site, the blood samples need to be transported to another central facility to be processed.

- Polystyrene boxes or Styrofoam mailers should be used for the transport of samples.
- Keep samples at about 4°C in the dark during transportation, by placing tubes in a separate container in a box/mailer with cooling elements or on ice (avoid direct contact to prevent freezing of samples). No important changes in retinol and carotenoids concentration are to be expected when the transportation of the blood samples (at 4°C) is within 24 hours after the collection of the blood (Key et al. 1996, Hankinson et al. 1989).
- During transport, the temperature of the samples need to be monitored and controlled (Chapter 5).
- Upon arrival at the processing site, the blood sample should be processed to prepare serum/plasma (Paragraph 2.1.3).

2.1.5.2 Transport and storage of serum/plasma samples

When serum/plasma samples cannot be stored at the collection site at ≤ -20°C, the samples need to be transported to another central facility to be stored and analysed:

- Polystyrene boxes or Styrofoam mailers should be used for the transport of samples.
- Serum/plasma samples should be kept at room temperature (20-22°C) in the dark during transportation, by placing tubes in a separate container in a box/mailer. No important changes in retinol and carotenoids concentration are expected when the samples are kept at room temperature for up to 24 hours after preparation of serum/plasma (Craft et al. 1988).
- During transport, the temperature of the samples should be monitored and kept below 22°C (Chapter 5).

At arrival the samples should be stored at a temperature according to the time that the samples will be stored:

- Serum/plasma samples can be frozen at ≤ -20°C if analysis of the samples will take place within 5 months after the initial collection of the blood sample.
- Serum/plasma samples should be stored at ≤ -70°C if analysis of the samples will take place after 5 months after the initial collection of the blood sample.

Retinol and β-carotene in serum are stable up to 6 months at –20°C. Serum retinol is stable for at least 5 year at –80°C, and serum β-carotene is stable up to 28 months at –70°C (Craft et al.1988, Brown Thomas et al. 1998).

- If a power cut occurs, samples are likely to thaw. Therefore an emergency generator should be sought as soon as possible to restore power. Otherwise, dry-ice (solid carbon dioxide) could be used to maintain the samples frozen for some days.

2.1.5.3 Transport and storage of dried blood spots samples

When dried blood spots collection paper samples cannot be stored at the collection site the samples need to be transported to another central facility to be stored and analysed. The dried blood spots collection paper card should be kept at room temperature (20-22°C) for a week. This is necessary to get a stable retinol value, which is approximately 20% lower for every spot in comparison to a fresh sample (Erhardt, 2002). This period of one week can then be used to transport the samples to the final place for storage and analysis.

- Polystyrene boxes or Styrofoam mailers should be used for the transport of samples.
- If cards are to be placed together in a plastic bag, separate cards from each other by weighing sheets.
- Place the collection paper card with the dried blood spots with desiccants in a sealed freezer proof plastic bag in the dark.
- Keep the samples during transportation at room temperature (20-25°C) before storage at < -20°C after one week. Assure that the samples are kept in the dark. This can be done by covering the plastic bag with foil.
- During transport, the temperature of the samples should be monitored and kept below 25°C (Chapter 5).
- At arrival, the samples should be stored before samples are to be analysed:
  att room temperature, if the samples have not been stored for a week, thereafter
  at a temperature < -20°C.

2.2 Collection and handling of human milk

After collection, human milk samples should be frozen and stored at ≤ –20°C for subsequent analysis.

Reference made to specific brands does not imply that other brands cannot be used in the procedures given. The validity of any procedure should be demonstrated in the environment of the laboratory.

2.2.1 Standardisation of collection

2.2.1.1 Time of collection

The best way of obtaining milk samples would be the complete emptying of one of the breasts that has not been used to feed the child. Under field conditions, this is often impractical. A smaller sample size of 5 -10 mL seems more feasible. The composition of human milk is influenced by several factors, which need standardisation to be able to compare results at the population or individual level:

- The first milk after birth (colostrum, 4-6 days postpartum) contains the highest retinol and β-carotene concentrations. These concentrations decrease gradually during the transitional period (7-21 days postpartum) and stabilise around 21 days after birth in the so-called 'mature' milk. To estimate vitamin A status, milk samples should be collected from one month after delivery until eight months-postpartum (Neville 1995). Always record the age of the breast-fed child.
- The retinol concentration of human milk is related to the fat concentration, which changes throughout the day: it rises in the morning from 05.00 to 10.00 and declines thereafter. To determine the retinol concentration in milk it is necessary to randomise milk sampling at various times of the day and various feeding intervals. If randomised sampling is not possible, milk retinol values should be expressed relative to the fat concentration in milk (Paragraph 3.7) to adjust for diurnal variation and this facilitates the comparisons with other studies (Stoltzfus et al. 1995, WHO 1996, WHO 1985).
- For vitamin A assessment at an individual level, the change of vitamin A concentration during one feed needs to be taken into account. Equal volumes of fore and hind milk should be taken, poolled and mixed (Kim et al.1990, WHO 1996).

2.2.1.2 Conditions at the collection area

The person involved in the sample collection should assure the mother that giving a milk sample will not reduce the amount of milk available for her baby. Rather the opposite is true: the breast

will be stimulated to produce even more milk. At all times, the privacy of the mother should be respected.

The following precautions should be taken when collection of breast milk is done in the open air:

- Search for a shaded place to avoid light exposure as much as possible.
- Protect the container directly after collection of the milk from light by covering the container with foil and keep cool. Assure the container is covered totally when using aluminium foil.
- Aliquot the milk sample into smaller quantities immediate after collection to assure homogeneous samples.
- Document where and how the milk was collected and how it was protected against light and/or heat.

2.2.1.3 Sampling for analysis

If aliquoting is not possible directly after collection of milk, care should be taken to assure that the milk sample is homogenised after arrival in the laboratory (Paragraph 2.2.3). If available, a sonicator should be used to assure homogenisation before aliquoting. The aliquoted milk sample should be frozen as soon as possible after milk collection. Short term storage up to one month should be at –20°C. For longer-term storage, a temperature of ≤–70 °C is required (Neville 1995, Vidal-Viverda et al. 1992).

Thawed samples should be well mixed before analysis to assure appropriate dispersion of the fat in the milk. If available, a sonicator should be used.

2.2.2 Milk collection

2.2.2.1 General

Human milk can be obtained by manual expression of the breast or by using either a manual or an electric breast pump. The choice will depend on the availability of equipment and on the preference of the mother and staff in the field. If it is difficult to express the milk, the mother can allow her infant to suckle from one breast to facilitate the milk 'let down' reflex and thereby aiding expressing from the other breast (WHO 1996).

All materials need to be cleaned very well before the milk collection. Wash materials with soap and hot water; rinse with distilled water and dry well. If a milk pump (manual or electric) is used it should be checked and cleaned every time a milk sample is collected. All parts of the materials, which can be sterilised, need to be sterilised according to the specifications of the manufacturer. Replace cracked and scratched pieces to avoid entry and growth of micro-organisms.

Cryogenic labels and low temperature resistant ink or a pencil should be used to ensure proper labelling of specimen collection paper during storage (Paragraph 4.3.1).

2.2.2.2 Materials for milk collection

The following materials are required:

- Cleaning material: detergent e.g. baby shampoo
- Clean tissue paper
- Breast pump (Kaneson, a type we found satisfactory)
- Collection container, preferably dark coloured (e.g. 25 - 50 mL)
- Aluminium foil

2.2.2.3 Method for milk collection

The following steps should be carried out:

- Clean the nipple and area around with detergent and clean water and pat dry with clean tissue paper.
- Collect approximately 5-10 mL milk from one of the breasts by manual expression or with the help of a pump into a collection container. In case the milk is collected with a pump, see the instructions of the manufacturer.
- Protect the milk in the collection container directly from light by covering with aluminium foil.
- The sample is now ready for sampling for analysis.

2.2.3 Sampling for analysis

2.2.3.1 Materials for sampling for analysis

The following materials are required:

- Freezerproof polypropylene storage tubes (3-6 mL) with air tight caps.
- Air tight caps.
- Pipette, graduated 1 - 5 mL.
- Ultrasonic bath or sonicator (Eurosonic 22, Wilten Woltil, de Meern, the Netherlands)

2.2.3.2 Method for sampling for analysis

The following steps should be carried out:

- Mix the milk gently to disperse the cream well throughout the milk, use a sonicator if available.
- Transfer approximately 2 - 5 mL of well-mixed milk in freezer-proof storage tubes (3-6 mL) and close the tube firmly with cap (Figure 2.5).
- The sample is now ready for storage at low temperature (see Paragraph 2.2.5).

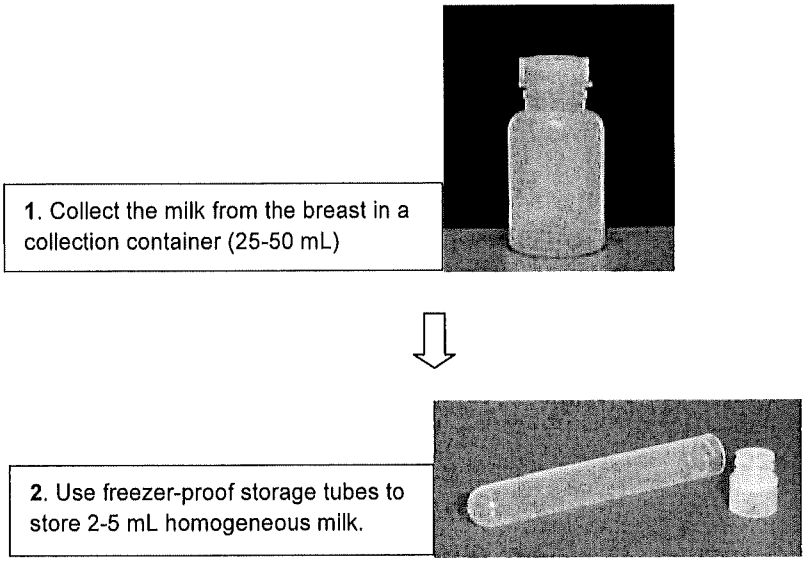


Figure 2.5 Milk aliquoting

2.2.4 Transport and storage of milk

When sampling for analysis or storage of milk samples cannot be done at the collection site the samples should be transported to another facility for further processing. A list with code numbers corresponding with the code numbers on the tubes should be included in the transport unit.

- Polystyrene boxes or Styrofoam mailers should be used for the transport of samples.
- Keep samples at about 4°C in the dark during transportation, by placing tubes in a separate container in a box/mailer with cooling elements or on ice (avoid direct contact to prevent freezing of samples) within 24 hours after the collection of the milk.
- During transport, the temperature of samples should be monitored and controlled (Chapter 5).

At arrival, the samples are ready for sampling for analysis (Paragraph 2.2.3) or storage, the conditions of which will depend on the time envisaged before analysis:

- Milk samples should be stored at $\leq$ -20°C if analysis of the samples will take place in one month after the initial collection of the milk.
- Milk samples should be stored at $\leq$ -70°C if analysis of the samples will take place thereafter.

The retinol concentration does not change when milk samples are stored for one month at -20°C. If storage for a longer period is foreseen, the samples should be stored at $\leq$ -70°C (Vidal-Viverda et al. 1992).

- Milk samples should never be stored in oversized tubes. If the size of a storage tube is too large, the dead volume increases and evaporation becomes a significant factor.

- If a power cut occurs, samples are likely to thaw. Therefore an emergency generator should be sought as soon as possible to restore power. Otherwise, dry-ice (solid carbon dioxide) could be used to maintain milk frozen for some days.

References

- *Brown Thomas J, Duewer DL, Kline MC, Sharpless KE. The stability of retinol, alpha-tocopherol, trans-lycone and trans-beta-carotene in liquid-frozen and lyophilised serum. Clin Chim Acta 1998; 276:75-87.*
- *Burtis CA, Ashwood ER (Eds). Tietz textbook of clinical chemistry. Philadelphia: WB Saunders Company, 1994.*
- *Craft NE, Brown ED, Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988; 34:44-48.*
- *Erhardt JG, Craft NE, Heinrich F, Biesalski HK. Rapid and simple measurement of retinol in human dried whole blood spot. J Nutr 2002; 132:318-21.*
- *Furr HC, Barua AB, Olson JA. Retinoids and carotenoids. In: De Leenheer AP, Lambert WE, Nelis HJ (Eds). Modern chromatographic analysis of the vitamins. New York: Marcel Dekker, 1992.*
- *Guder WG, Narayanan S, Wisser H, Zawta B. Samples: From the subject to the laboratory. The impact of preanalytical variables on the quality of laboratory results. Franfurt: GIT Verlag, 1996.*
- *Hankinson SE, London SJ, Chute CG et al. Effect of transport conditions on the stability of biochemical markers in blood. Clin Chem 1989; 35:2313-2316.*
- Key T, Oakes S, Davey G, et al. Cancer Epidemiol Biomarkers Prev 1996; 5:811-814.
- *Kim Y, English C, Reich P, Gerber P, Simpson KL. Vitamin A and carotenoids in human milk. J Agric Food Chem 1990; 38:1930-1933.*
- *Mejia AL, Arroyave G. Determination of vitamin A in blood. Some practical considerations on the time of collection of the specimens and the stability of the vitamin. Am J Clin Nutr 1983; 37:147-151.*
- *Meites S. Skin puncture and blood collection technique for infants: Update and problems. Clin Chem 1988; 34:1890-1894.*
- *Neville MC. Sampling and storage of human milk. In: RG Jensen (Eds), Handbook of milk* composition. *p 63-79. London: Academic Press, 1995.*
- *PAMM. Laboratory quality assurance, DBS analysis, Laboratory methods manual (draft), 1993.*
- *Stoltzfus RJ, Underwood BA. Breast-milk vitamin A as an indicator of the vitamin status of women and infants. Bull World Health Organisation 1995; 73:703-711.*
- *Vidal-Valverde C, Ruiz R, Medrano A. Stability of retinol in milk during frozen and other storage conditions. Z-Lebens Unters Forsch 1992; 195:562-565.*
- *Walters NJ, Estridge BH, Reynolds AP. Basic medical laboratory techniques. Delmar Publishers. Third edition 1995.*
- *WHO. Indicators for assessing vitamin A deficiency and their application in monitoring and evaluating intervention programmes. Geneva: World Health Organization, 1996.*
- *WHO. The quantity and quality of breast milk. Report of the WHO collaborative study on breast-feeding. Geneva: World Health Organization, 1985.*

CHAPTER 3: STANDARD OPERATING PROCEDURES FOR THE DETERMINATION OF RETINOL AND CAROTENOIDS

3.1 Introduction

Several methods for the determination of retinol and carotenoids in serum/plasma and milk have been described (AOAC 1995, Arroyave et al. 1982a, Frolik and Olson 1994, Furr et al. 1992, Furr et al.1994, ISO-12080-2 2000, Sowell et al. 1994, Nomura et al., 1997). The methods presented in this manual allow determination of retinol and carotenoids in one analytical run or determination of retinol on its own. Other methods of course may give valid results and may be preferred, depending on the inherent and specific laboratory conditions. But it should be emphasised that:

- All analytical procedures should be described in an unequivocal way.
- Good laboratory operations can only be done using calibrated equipment and with documentation of samples and methods (Chapter 4).
- Skilled personnel, using appropriate quality control procedures (Chapter 5) should carry out the method.
- Appropriate internal and external validation of procedures should be carried out (Chapter 6).

Notes:

1. The worksheets in Annexes 2, 3 and 4 have been prepared to assist in ensuring that standard operation procedures are followed correctly.
2. For practical reasons, conventional units are sometimes preferred above S.I units. Conversion factors are supplied in the remarks paragraphs to be able to convert to S.I units if desired.

Reference made to specific brands does not imply that other cannot be used in the procedures given. The validity of any procedure should be demonstrated in the environment of the laboratory.

3.2.1 Retinol and carotenoids in serum/plasma (gradient elution)

1 Title

Standard operating procedure for the determination of retinol and carotenoids in serum/plasma, using reversed-phase high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure

3 Scope

The method described here is suitable for the measurement of retinol and carotenoids in serum or plasma.

4 Principle

Serum/plasma is denatured with ethanol containing an internal standard. The sample is extracted with hexane, and retinol and carotenoids in the extract are determined by reversed-phase high-performance liquid chromatography (HPLC) based on Craft et al. (1992).

5 Precautions

The presence of highly conjugated double bonds renders retinol and carotenoids generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep samples capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes, 10 mL, with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL

6.3 Pipettes, Pasteur: 150 mm

6.4 Flasks, glass stoppered volumetric: 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL and 2000 mL

6.5 Flasks, conical: 100 mL

6.5 Cylinders, measuring: 100 mL and 250 mL

6.6 Crimpvials, 2 mL amber coloured (Phasesep 403682)

6.8 Inserts, 200 µL, for crimpvials (Phasesep 403814)

6.9 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Dispenser for 0-10 mL

7.4 Laboratory shaking machine, to-and-fro and orbital motions (Edmund Buehler SM 25)

7.5 Centrifuge, with adjustable speed up to 3000 g (Sigma 4-10)

7.6 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.7 Cuvettes, quartz glass, 3 cm path length for measurements between 200 and 500 nm

7.8 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl see **14.1**

7.9 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.10 Multiped, 25 mL (Eppendorf)

7.11 Combitips for multiped (Eppendorf 0030-048.440)

7.12 Dry-block heater (Techne) connected with an evaporating unit and nitrogen connection (Pierce Reacti-VAP evaporator 18785)

7.13 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.14 Rotary evaporator with nitrogen connection (with one way valve) and temperature adjustment (35°C)

7.15 HPLC – system (Thermoquest) with:

- P4000 Gradient pump
- SCM 400 Solvent de-gasser
- AS 3000 autosampler with cooling option at 4°C
- UV 2000 detector
- Data recording system
- Vydac 218TP53 reversed phase column (length: 25 cm, diameter: 3.2 mm, particle size: 5µm, metal free frits) with Vydac replaceable guard-column 218GD54

8 Chemicals

8.1 Sodium chloride (Merck 1.06400)

8.2 Distilled water

8.3 Methanol, HPLC grade (Labscan C2517)

8.4 Butylhydroxytoluene (BHT, Sigma B-1378)

8.5 Tetrahydrofuran, HPLC grade (THF, Labscan C2520)

8.7 Ethanol p.a. (Merck 1.00983)

8.8 Hexane, HPLC grade (Rathburn RH 1002)

8.6 Triethylamine (Sigma T0886)

8.9 Dichloromethane, HPLC grade (Labscan C2510)

8.10 Nitrogen (highest purity and oxygen-free)

8.11 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Retinyl acetate in oil (Fluka 95138 – 5 g)
- Lutein (Fluka 95507 – 1 mg)
- Zeaxanthin (Roth 5672.1 – 1 mg)
- β-Cryptoxanthin (Roth 5675.1 – 1 mg)
- α-Carotene (CaroteNature 0007)
- β-Carotene (Sigma C4582 – 5 mg)
- Lycopene (Sigma L9879 – 1 mg)

9 Preparation of reagents and standards

9.1 Sodium chloride (0.9% w/v)

- Dissolve 0.9 gram NaCl (**8.1**) in distilled water (**8.2**) in a volumetric flask of 100 mL.
- Fill to the graduation mark with distilled water (**8.2**) and mix.

9.2 Methanol – tetrahydrofuran (3:1 v/v) containing BHT (0.01% w/v)

- Transfer 375 mL methanol (**8.3**) into a volumetric flask of 500 mL with a measuring cylinder.
- Dissolve 50 mg butylhydroxytoluene (**8.4**).
- Fill to the graduation mark with tetrahydrofuran (**8.5**) and mix.

9.3 Eluent A for HPLC: methanol - tetrahydrofuran - water – triethylamine (87.9:2:10:0.1 v/v/v/v)

- Transfer 100 mL distilled water (**8.2**) into a volumetric flask of 1000 mL with a measuring cylinder.
- Add 20 mL tetrahydrofuran (**8.5**) with a measuring cylinder.
- Add 1 ml triethylamine (**8.6**) with an adjustable pipette.
- Fill to the graduation mark with methanol (**8.3**) and mix.

9.4 Eluent B for HPLC : methanol – tetrahydrofuran – triethylamine (92.4:7.5:0.1 v/v/v)

- Transfer 150 mL tetrahydrofuran (**8.5**) into a volumetric flask of 2000 mL with a measuring cylinder.
- Add 2 mL triethylamine (**8.6**) with an adjustable pipette.
- Fill to the graduation mark with methanol (**8.3**) and mix.

9.5 Sampler eluent for HPLC: methanol – tetrahydrofuran (50:50 v/v)

- Transfer 250 mL tetrahydrofuran (**8.5**) into a volumetric flask of 500 mL with a measuring cylinder.
- Fill to the graduation mark with methanol (**8.3**) and mix.

9.6 Preparation of internal standard solution

9.6.1 Stock solution

- Dissolve 150 mg retinyl acetate (**8.11**) in ethanol (**8.7**) in a 100 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

9.6.2 Diluted stock solution

- Transfer 1.0 mL stock solution of retinyl acetate (**9.6.1**) to a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

9.6.3 Internal standard solution

- Transfer 2.5 mL diluted stock solution of retinyl acetate (**9.6.2**) to a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

Prepare the internal standard of retinyl acetate daily from the diluted stock solution (**9.6.2**).

9.7 Preparation of standard solutions

9.7.1 Stock solutions

- Prepare the stock solutions by dissolving the specified quantity (Table 1) of the standards (**8.11**) with the appropriate solvent in the requested volumetric flasks.
- Fill to the graduation mark with solvent and mix.

Table 1 Stock solutions

Standard	Weight (mg)	Volumetric flask (mL)	Solvent
Retinol	40	100	Ethanol
Retinyl acetate	150	100	Ethanol
Lutein	1	50	Tetrahydrofuran*
Zeaxanthin	1	50	Tetrahydrofuran*
β-Cryptoxanthin	1	25	Tetrahydrofuran*
α-Carotene	1	50	Tetrahydrofuran*
β-Carotene	5	50	Tetrahydrofuran*
Lycopene	1	25	Tetrahydrofuran*

*Tetrahydrofuran containing 0.01% BHT

9.7.2 Diluted stock solutions

- Prepare the diluted stock solutions by bringing the specified quantities (Table 2) of the stock solutions (**9.7.1**) in the requested volumetric flasks (Table 2).
- The solvent of the stock solution needs to be evaporated under a nitrogen atmosphere before the volumetric flask is filled with the desired solvent (except for retinol and retinyl acetate).
- Fill to the graduation mark with the appropriate solvent (Table 2) and mix.

Table 2 Diluted stock solutions

Standard	Stock solution (mL)	Volumetric flask (mL)	Solvent
Retinol	1	50	ethanol
Retinyl acetate	1	100	ethanol
Lutein	2	25	ethanol
Zeaxanthin	2	25	ethanol
β-Cryptoxanthin	2	25	hexane
α-Carotene	2	25	hexane
β-Carotene	1	25	hexane
Lycopene	3	25	hexane

9.8 Determination of the concentration of diluted stock solutions

By measuring the absorbance of diluted stock solutions (**9.7.2**) in the spectrophotometer, the concentration can be calculated with the *Lambert-Beer Law.*

- Assure the spectrophotometer is calibrated before analysis (Chapter 5).
- For greatest accuracy, use a cuvette with a 3 cm light path.
- Measure the absorbance at the specified wavelength against the solvent blank (Table 3).

Table 3: Wavelength and $E^{1\%}_{1cm}$ †

Standard	Wavelength (nm)	$E^{1\%}_{1cm}$
Retinol	325	1850
Retinyl acetate	325	1550
Lutein	445	2550
Zeaxanthin	450	2540
β-Cryptoxanthin	452	2386
α-Carotene	444	2800
β-Carotene	453	2592
Lycopene	472	3450

† $E^{1\%}_{1cm}$: Absorption coefficient in ethanol

Concentration: $c = (\text{absorbance} / \text{absorption coefficient}) \times 10^6$ (µg/dL)

For example: Measured absorbance of β-carotene: 0.706 ⇒

$c = 0.706 / 2592 * 10^6 = 272.4$ µg/dL

N.B. *To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see* ***14.2.***

9.9 Preparation of working standards

- Prepare the working standards by transferring the desired volumes (Table 4) of the diluted stock solutions (**9.7.2**) with a volumetric pipette to a 100 mL conical flask.
- Evaporate the solvent with a rotary evaporator at 35°C under a nitrogen atmosphere.
- Dissolve the residue in methanol : tetrahydrofuran (**9.2**) and transfer quantitatively into a 25 mL volumetric flask.
- Fill to the graduation mark with methanol : tetrahydrofuran (**9.2**), mix and close with stopper.

Table 4 Working standards

Diluted stock solution	Standard 1 (mL)	Standard 2 (mL)	Standard 3 (mL)	Standard 4 (mL)
Retinol	0	1	2	4
Retinyl acetate	0	2,5	2,5	2,5
Lutein	0	1.25	2.5	5
Zeaxanthin	0	0.5	1	2
β-Cryptoxanthin	0	0.5	1	2
α-Carotene	0	1.25	2.5	5
β-Carotene	0	2	4	8
Lycopene	0	1.25	2.5	5

The approximate concentrations of the different components in the working standards are shown in Table 5.

Table 5 Concentration of components in working standards

Component	Standard 1 (µg/dL)	Standard 2 (µg/dL)	Standard 3 (µg/dL)	Standard 4 (µg/dL)
Retinol	0	30	60	120
Retinyl acetate	0	80	80	80
Lutein	0	6	12	24
Zeaxanthin	0	2.5	5	10
β-Cryptoxanthin	0	5	10	20
α-Carotene	0	6	12	24
β-Carotene	0	25	50	100
Lycopene	0	25	50	100

In order to determine the exact concentration of the different components in the working standards, it is necessary to determine the purity of the different components with HPLC:

- Ensure that the HPLC-system is calibrated before analysis (Chapter 5).
- Transfer 1 mL of the diluted stock solutions (**9.7.2**) in crimpvials and inject the samples into the HPLC system according the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100%

With this correction for purity, the exact concentration of the different components in the standard solution can be calculated.

10 Procedure

10.1 Allow frozen serum or plasma samples thaw gently at room temperature (20-25°C) prior to analysis and mix well.

10.2 Transfer 0.5 mL of serum, plasma, or control sample into a Kimax tube using an adjustable pipette.

10.3 Add 0.5 mL NaCl solution (**9.1**) with a multiped and combitip.

10.4 Add 1.0 mL internal standard solution (**9.6.3**) with an adjustable pipette.

10.5 Mix for 30 seconds on a vortex mixer.

10.6 Add 2 mL hexane (**8.8**) with a dispenser and close firmly with screw caps.

10.7 Shake the tube horizontally in a laboratory shaking machine for 5 minutes at 250 reciprocations per minute.

10.8 Centrifuge for 2 minutes at 3000 g.

10.9 Transfer as much as possible of the hexane-phase with a Pasteur pipette into a clean Kimax tube.

10.10 Repeat the extraction steps **10.5 – 10.7**.

10.11 Transfer as much as possible of the hexane-phase with a Pasteur pipette into the tube containing the first hexane fraction.

10.12 Evaporate the hexane in a dry-block heater at 35°C under a nitrogen atmosphere (**8.10**).

10.13 Add, as quickly as possible after drying, 250 µL methanol-tetrahydrofuran (**9.2**) with an adjustable pipette.

10.14 Close the tube immediately with screw cap and mix well on a vortex mixer.

10.15 Transfer the sample with a Pasteur pipette into a crimpvial with insert and close well. The sample is ready for HPLC analysis.

11 HPLC Analysis

11.1 Pump

Eluent A (**9.3**) and eluent B (**9.4**) are used at a constant flow of 0.7 mL/min according to the following schedule.

Table 6 Time schedule for mobile phases

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
0.25-0.75	100 → 0	0 → 100
0.75-20	0	100
20-25*	100	0

* Preparing for next run

11.2 Detector

The UV 2000 detector is used for the measurement of carotenoids (at 450 nm), and of retinol and retinyl acetate (at 325 nm) with the following time schedule:

Table 7 Time schedule wavelength

Time (min)	Wavelength (nm)
0 – 5.75	325
5.75 - 20	450
20 –25*	325

* Preparing for next run

11.3 Sampler

- If an automatic sampler is available, keep the temperature of the sampler tray at 4°C.
- Use amber vials.
- Set the injection volume at 25 µL.
- The tip of the sampler is rinsed after each injection with methanol:tetrahyrofuran (**9.5**).
- Add a solvent blank with each run.
- For analysis that are run overnight, the extracts can be kept for 18 hours in HPLC solvents without experiencing any change in the concentration of retinol or carotenoids (Craft et al. 1988).

11.4 Integration

An internal standard method is used to calibrate the three standards. A computerised programme is incorporated to facilitate automatic integration. The different components have approximately the following retention times under the conditions described:

Table 8 Retention times of retinol and carotenoids

Component	Retention time (min)
Retinol	4.2
Retinyl acetate	5.0
Lutein	6.3
Zeaxanthin	6.6
β-Cryptoxanthin	8.7
α-Carotene	11.5
β-Carotene	12.4
Lycopene	14.7

The concentration of the different components in the samples is reported in µg/dL. When 0.5 mL of serum or plasma is dissolved in 250 µL methanol:tetrahydrofuran (**9.2**) the scale factor of 0.5 needs to be incorporated. In the calculation model the 'bracketing' of standards is used.

11.5 Sequence

In a run of 48 samples, 2 control samples (**14.1**) and three series of 4 standards are placed as follows:

Table 9 Sequence of samples and standards

4 standards
2 control samples
22 subject samples
4 standards
24 subject samples
4 standards

12 Data processing and reporting

For every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control:

For the purpose of quality control (see Chapter 6), a control sample of serum/plasma (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 When the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value by the conversion factor (table 10).

Table 10 Conversion factors for conventional and SI units

Analyte	Conventional units	Conversion factor: conventional→ S.I.	S.I. units
Retinol	µg/dL	0.0349	µmol/L
Retinyl acetate	µg/dL	0.0304	µmol/L
Lutein	µg/dL	0.0176	µmol/L
Zeaxanthin	µg/dL	0.0176	µmol/L
β-Cryptoxanthin	µg/dL	0.0181	µmol/L
α-Carotene	µg/dL	0.0186	µmol/L
β-Carotene	µg/dL	0.0186	µmol/L
Lycopene	µg/dL	0.0186	µmol/L

14.3 Ten percent of the samples are analysed in duplicate.

14.4 The pre-column needs to be replaced regularly, generally after 150-200 samples, depending on the extent of contamination of the column. Less efficient separation can be recognised through an increase in column pressure, and/or decrease in peak symmetry, and/or a decrease in the separation, which is characterised, especially for vitamin A, by a 'shoulder peak'. Contaminated pre-columns can be rinsed for re-use. This can be done by reversing the pre-column and rinsing with 10 mL dichloromethane (**8.9**), followed by 10 mL methanol (**8.3**), both at flow rate of 1.0 mL/minute.

14.5 The working standards can be stored for about 6 weeks at –20°C without change in concentration.

15 References

- *Arroyave G et al. Reprints of selected methods for the analysis of vitamin A and carotenoids in nutrition surveys. Washington DC: IVACG 1982.*
- *Craft NE, Brown ED, Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988; 34: 44-48.*
- *Craft NE, Wise SA, Soares JH. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chrom 1992; 589: 171-176.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids. In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Retinoids and carotenoids. In: De Leenheer AP, Lambert WE, Nelis HJ (Eds). Modern chromatographic analysis of the vitamins. New York: Marcel Dekker, second edition, pp 1-72, 1992.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds.). The retinoids, biology, chemistry and medicine. New York: Raven Press, second edition, pp179-210, 1994.*
- *Olson JA. Vitamin A, retinoids and carotenoids. In: Shils ME (Eds.). Modern Nutrition in health and disease. Baltimore: Williams & Wilkins, eight edition, 1994.*

- *Sowell AL, DL Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multi wavelength detection. Clin Chem 1994; 40:411-416.*

16 Annexes

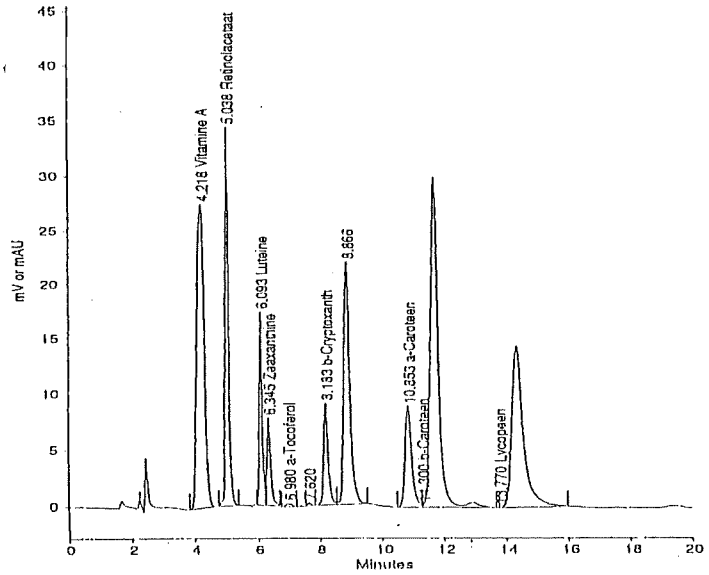


Figure 1 Chromatogram of a working standard solution at 325 nm (0-5.75 minutes) and 450 nm 5.75-20 minutes).

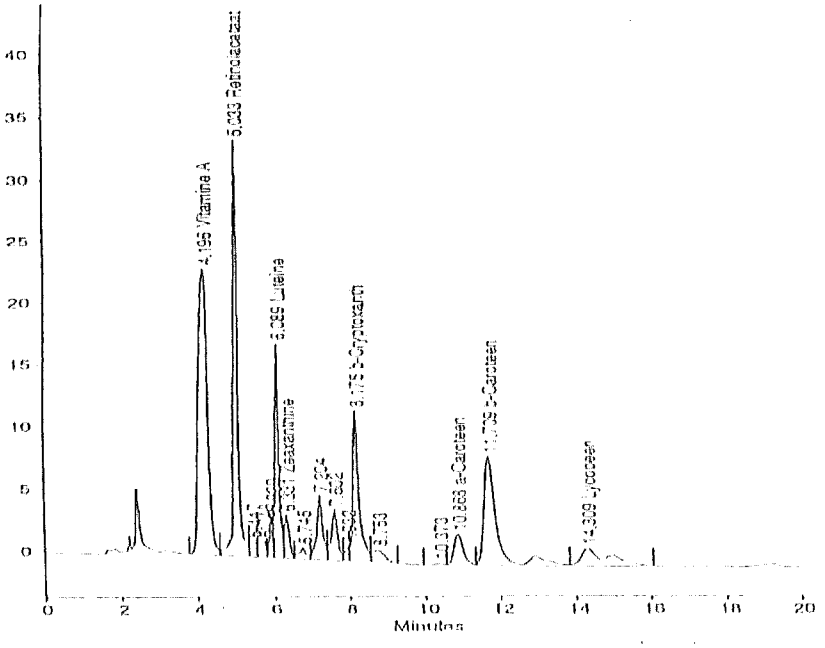


Figure 2 Chromatogram of a control sample of serum at 325 nm (0-5.75 minutes) and 450 nm (5.75-20 minutes).

3.2.2 Retinol and carotenoids in serum/plasma (isocratic elution)

Note: *During the preparation of this manual, we were asked to include a Standard Operating Procedure based on an isocratic elution procedure. Therefore we have modified our previously described SOP* ***(3.2.1)****. The main difference is the choice of the column, mobile phase and elution technique. The description is based on the literature and on a personal communication from Neal E Craft (Craft Technologies, Inc., Wilson USA) but has NOT been tested in our laboratory.*

1 Title

Standard operating procedure for the determination of retinol and carotenoids in serum/plasma, using reversed-phase high performance liquid chromatography based on isocratic elution.

2 Update and review summary

Not applicable in this standard operating procedure

3 Scope

The method described here is suitable for the measurement of retinol and carotenoids in serum or plasma.

4 Principle

Serum/plasma is denatured with ethanol containing an internal standard. The sample is extracted with hexane, and retinol and carotenoids in the extract are determined by reversed-phase high-performance liquid chromatography (HPLC).

5 Precautions

The presence of highly conjugated double bonds renders retinol and carotenoids generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep sample capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes, 10 mL, with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL

6.3 Pipettes, Pasteur: 150 mm

6.4 Flasks, glass stoppered volumetric: 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL and 2000 mL

6.5 Flasks, conical: 100 mL

6.5 Cylinders, measuring: 100 mL and 250 mL

6.6 Crimpvials, 2 mL amber coloured (Phasesep 403682)

6.8 Inserts, 200 µL, for crimpvials (Phasesep 403814)

6.9 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Dispenser for 0-10 mL

7.4 Laboratory shaking machine, to-and-fro and orbital motions (Edmund Buehler SM 25)

7.5 Centrifuge, with adjustable speed up to 3000 g (Sigma 4-10)

7.6 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.7 Cuvettes, quartz glass, 3 cm path length for measurements between 200 and 500 nm

7.8 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl see **14.1**

7.9 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.10 Multiped, 25 mL (Eppendorf)

7.11 Combitips for multiped (Eppendorf 0030-048.440)

7.12 Dry-block heater (Techne) connected with an evaporating unit and nitrogen connection (Pierce Reacti-VAP evaporator 18785)

7.13 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.14 Rotary evaporator with nitrogen connection (with one way valve) and temperature adjustment (35°C)

7.15 HPLC – system (Thermoquest) with:

- SCM 400 Solvent de-gasser
- AS 3000 autosampler with cooling option at 15°C
- UV 2000 detector
- Data recording system
- Spherisorb ODS2 column (250 x 4.0 mm; 3 µm particle size; Ti frits) with Vydac replaceable guard-column 218GD54

8 Chemicals

8.1 Sodium chloride (Merck 1.06400)

8.2 Distilled water

8.3 Methanol, HPLC grade (Labscan C2517)

8.4 Butylhydroxytoluene (BHT, Sigma B-1378)

8.5 Tetrahydrofuran, HPLC grade (THF, Labscan C2520)

8.7 Ethanol, p.a. (Merck 1.00983)

8.8 Hexane, HPLC grade (Rathburn RH 1002)

8.6 Triethylamine (Sigma T0886)

8.9 Dichloromethane, HPLC grade (Labscan C2510)

8.10 Acetonitile, HPLC grade

8.11 p-Dioxane, HPLC grade

8.12 Isopropanol, HPLC grade

8.13 Ammonium acetate

8.14 Ethyl acetate, HPLC grade

8.15 Nitrogen (highest purity and oxygen-free)

8.16 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Retinyl acetate in oil (Fluka 95138 – 5 g)
- Lutein (Fluka 95507 – 1 mg)
- Zeaxanthin (Roth 5672.1 – 1 mg)
- β-Cryptoxanthin (Roth 5675.1 – 1 mg)
- α-Carotene (CaroteNature 0007)
- β-Carotene (Sigma C4582 – 5 mg)
- Lycopene (Sigma L9879 – 1 mg)

9 Preparation of reagents and standards

9.1 Sodium chloride (0.9% w/v)

- Dissolve 0.9 gram NaCl (**8.1**) in distilled water (**8.2**) in a volumetric flask of 100 mL.
- Fill to the graduation mark with distilled water (**8.2**) and mix.

9.2 Eluent for HPLC: acetonitrile - p-dioxane - methanol/isopropanol (50:50 v/v containing 150 mM ammoniumacetate) – triethylamine (79.9:15:5:0.1 v/v/v/v)

- Transfer 750 mL p-dioxane (**8.11**) and 250 mL methanol/isopropanol (50:50 v/v%) (**8.3** and **8.12**) containing 2.5 g ammonium acetate (**8.13**) into a volumetric flask of 5000 mL.
- Add 5 mL triethylamine (**8.6**) with measuring cylinder.
- Fill to the graduation mark with acetonitrile (**8.10**) and mix.

9.3 Sampler eluent for HPLC: acetonitrile – isopropanol (50:50 v/v)

- Transfer 250 mL acetonitrile (**8.10**) into a 500 mL volumetric flask with a measuring cylinder.
- Fill to the graduation mark with isopropanol (**8.12**) and mix.

9.4 Preparation of internal standard solution

9.4.1 Stock solution

- Dissolve 150 mg retinyl acetate (**8.11**) in ethanol (**8.7**) in a 100 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

9.4.2 Diluted stock solution

- Transfer 1.0 mL stock solution of retinyl acetate (**9.4.1**) to a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

9.4.3 Internal standard solution

- Transfer 2.5 mL diluted stock solution of retinyl acetate (**9.4.2**) to a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.7**) and mix.

Prepare the internal standard of retinyl acetate daily from the diluted stock solution (**9.4.2**).

9.5 Preparation of standard solutions

9.5.1 Stock solutions

- Prepare the stock solutions by dissolving the specified quantity (Table 1) of the standards (**8.16**) with the appropriate solvent in the requested volumetric flasks.
- Fill to the graduation mark with solvent and mix.

Table 1 Stock solutions

Standard	Weight (mg)	Volumetric flask (mL)	Solvent
Retinol	40	100	Ethanol
Retinyl acetate	150	100	Ethanol
Lutein	1	50	Tetrahydrofuran*
Zeaxanthin	1	50	Tetrahydrofuran*
β-Cryptoxanthin	1	25	Tetrahydrofuran*
α-Carotene	1	50	Tetrahydrofuran*
β-Carotene	5	50	Tetrahydrofuran*
Lycopene	1	25	Tetrahydrofuran*

*Tetrahydrofuran containing 0.01% BHT

9.5.2 Diluted stock solutions

- Prepare the diluted stock solutions by bringing the specified quantities (Table 2) of the stock solutions (**9.5.1**) into the requested volumetric flasks (Table 2).
- The solvent of the stock solution needs to be evaporated under a nitrogen atmosphere before the volumetric flask is filled with the desired solvent (except for retinol and retinyl acetate).
- Fill to the graduation mark with the appropriate solvent (Table 2) and mix.

Table 2 Diluted stock solutions

Standard	Stock solution (mL)	Volumetric flask (mL)	Solvent
Retinol	1	50	ethanol
Retinyl acetate	1	100	ethanol
Lutein	2	25	ethanol
Zeaxanthin	2	25	ethanol
β-Cryptoxanthin	2	25	hexane
α-Carotene	2	25	hexane
β-Carotene	1	25	hexane
Lycopene	3	25	hexane

9.6 Determination of the concentration of diluted stock solutions

By measuring the absorbance of diluted stock solutions (**9.5.2**) in the spectrophotometer, the concentration can be calculated with the *Lambert-Beer Law*.

- Ensure the spectrophotometer is calibrated before analysis (Chapter 5).
- For greatest accuracy, use a cuvette with a 3 cm light path.
- Measure the absorbance at the specified wavelength against the solvent blank (Table 3.).

Table 3: Wavelength and $E^{1\%}_{1cm}$ †

Standard	Wavelength (nm)	$E^{1\%}_{1cm}$
Retinol	325	1850
Retinyl acetate	325	1550
Lutein	445	2550
Zeaxanthin	450	2540
β-Cryptoxanthin	452	2386
α-Carotene	444	2800
β-Carotene	453	2592
Lycopene	472	3450

† $E^{1\%}_{1cm}$: Absorption coefficient in ethanol

Concentration: c = (absorbance / absorption coefficient) x 10^6 (µg/dL)

For example: Measured absorbance of β-carotene: 0.706 ⇒

c = 0.706 / 2592 * 10^6 = 272.4 µg/dL

N.B. *To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see* ***14.2****.*

9.7 Preparation of working standards

- Prepare the working standards by transferring the desired volumes (Table 4) of the diluted stock solutions (**9.5.2**) with a volumetric pipette to a 100 mL conical flask.
- Evaporate the solvent with a rotary evaporator at 35°C under a nitrogen atmosphere.
- Dissolve the residue in mobile phase (**9.2**) and transfer quantitatively into a 25 mL volumetric flask.
- Fill to the graduation mark with mobile phase (**9.2**), mix and close with stopper.

Table 4 Working standards

Diluted stock solution	Standard 1 (mL)	Standard 2 (mL)	Standard 3 (mL)	Standard 4 (mL)
Retinol	0	1	2	4
Retinyl acetate	0	2,5	2,5	2,5
Lutein	0	1.0	2.0	4
Zeaxanthin	0	0.5	1	2
β-Cryptoxanthin	0	0.5	1	2
α-Carotene	0	1.0	2.0	4
β-Carotene	0	2	4	8
Lycopene	0	1.0	2.0	4

The approximate concentrations of the different components in the working standards are shown in Table 5.

Table 5 Approximate concentration of components in working standards

Component	Standard 1 (µg/dL)	Standard 2 (µg/dL)	Standard 3 (µg/dL)	Standard 4 (µg/dL)
Retinol	0	30	60	120
Retinyl acetate	0	80	80	80
Lutein	0	6	12	24
Zeaxanthin	0	3	6	12
β-Cryptoxanthin	0	6	12	24
α-Carotene	0	6	12	24
β-Carotene	0	30	60	120
Lycopene	0	20	40	80

In order to determine the exact concentration of the different components in the working standards, it is necessary to determine the purity of the different components with HPLC:

- Ensure that the HPLC-system is calibrated before analysis (Chapter 5).
- Transfer 1 mL of the diluted stock solutions (**9.5.2**) in crimpvials and inject the samples into the HPLC system according to the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100%

With this correction for purity, the exact concentration of the different components in the standard solution can be calculated.

10 Procedure

10.1 Allow frozen serum or plasma samples thaw gently at room temperature (20-25°C) prior to analysis and mix well. Transfer 0.5 mL of serum, plasma, or control sample into a Kimax tube using an adjustable pipette.

10.2 Add 0.5 mL NaCl solution (**9.1**) with a multiped and combitip.

10.3 Add 1.0 mL internal standard solution (**9.4.3**) with an adjustable pipette.

10.4 Mix for 30 seconds on a vortex mixer.

10.5 Add 2 mL hexane (**8.8**) with a dispenser and close firmly with screw caps.

10.6 Shake the tube horizontally in a laboratory shaking machine for 5 minutes at 250 reciprocations per minute.

10.7 Centrifuge for 2 minutes at 3000 g.

10.8 Transfer as much as possible of the hexane-phase with a Pasteur pipette into a clean Kimax tube.

10.9 Repeat the extraction steps **10.5 – 10.7**.

10.10 Transfer as much as possible of the hexane-phase with a Pasteur pipette into the tube containing the first hexane fraction.

10.11 Evaporate the hexane in a dry-block heater at 35°C under a nitrogen atmosphere (**8.10**).

10.12 Add, as quickly as possible after drying, 50 µL ethyl acetate (**8.14**) to dissolve the sample and add 200 µL mobile phase (**9.2**) with an adjustable pipette.

10.13 Close the tube immediately with screw cap and mix well on a whirl mixer.

10.14 Transfer the sample with a Pasteur pipette into a crimpvial with insert and close well. The sample is ready for HPLC analysis.

11 HPLC Analysis

11.1 Pump

The eluent (**9.2**) is pumped with a constant flow of 1.0 mL/min.

11.2 Detector

The UV2000 detector is used for measurement of retinol and retinyl acetate at 325 nm, and than switched to 450 nm for the measurement of carotenoids. Total run time: approximately 20 min.

11.3 Sampler

- If an automatic sampler is available keep the temperature of the sampler tray at 15°C.
- Use amber coloured vials.
- Set the injection volume at 20 µL.
- The tip of the sampler is rinsed after each injection with acetonitrile:isopropanol (**9.3**).
- Add a solvent blank with each run.
- For analysis that are run overnight, the extracts can be kept for 18 hours in the HPLC solvent without experiencing any change in the concentration of retinol or carotenoids (Craft et al. 1988).

11.4 Integration

An internal standard method is used to calibrate the three standards. A computerised programme is incorporated to facilitate automatic integration.

The concentration of the different components in the samples is reported in µg/dL. When 0.5 mL of serum or plasma is dissolved in 250 µL mobile phase (**9.2**) the scale factor of 0.5 needs to be incorporated. In the calculation model the 'bracketing' of standards is used.

11.5 Sequence

In a run of 48 samples 2 control samples and three series of 4 standards are placed as follows:

Table 6 Sequence of standards and samples

4 standards
2 control samples
22 subject samples
4 standards
24 subject samples
4 standards

12 Data processing and reporting

For every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control:

For the purpose of quality control (see Chapter 6), a control sample of serum/plasma (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value with the conversion factor:

Table 7 Conversion factors for conventional and SI units

Analyte	Conventional units	Conversion factor: conventional→ S.I.	S.I. units
Retinol	µg/dL	0.0349	µmol/L
Retinyl acetate	µg/dL	0.0304	µmol/L
Lutein	µg/dL	0.0176	µmol/L
Zeaxanthin	µg/dL	0.0176	µmol/L
β-Cryptoxanthin	µg/dL	0.0181	µmol/L
α-Carotene	µg/dL	0.0186	µmol/L
β-Carotene	µg/dL	0.0186	µmol/L
Lycopene	µg/dL	0.0186	µmol/L

14.3 Ten percent of the samples are analysed in duplicate.

14.4 The pre-column needs to be replaced regularly, generally after 150-200 samples, depending on the extent of contamination of the column. Less efficient separation can be recognised through an increase in column pressure, and/or decrease in peak symmetry, and/or a decrease in the separation, which is characterised, especially for vitamin A, by a 'shoulder peak'. The contaminated pre-column can be rinsed for re-use. This can be done by reversing the pre-column and rinse with 10 mL dichloromethane (**8.9**), followed by 10 mL methanol (**8.3**), both at with a flow rate of 1.0 mL/min.

14.5 The working standards can be stored for about 6 weeks at –20°C without change in their concentration.

15 References

- *Arroyave G et al. Reprints of selected methods for the analysis of vitamin A and carotenoids in nutrition surveys. Washington DC: IVACG, 1982.*
- *Craft NE, Brown ED, Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988; 34:44-48.*
- *Craft NE, Wise SA, Soares JH. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chrom 1992; 589:171-176.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids. In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Retinoids and carotenoids. In: De Leenheer AP, Lambert WE, Nelis HJ (Eds). Modern chromatographic analysis of the vitamins. New York: Marcel Dekker, , second edition, pp 1-72, 1992.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds.). The retinoids, biology, chemistry and medicine. New York: Raven Press, second edition, pp 179-210, 1994.*
- *Olson JA. Vitamin A, retinoids and carotenoids. In: Shils ME (Eds.). Modern Nutrition in health and disease. Baltimore: Williams & Wilkins, eight edition, 1994.*
- *Sowell AL, DL Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multi wavelength detection. Clin Chem 1994; 40:411-416.*

3.3.1 Retinol and carotenoids in human milk (gradient elution)

1 Title

Standard operating procedures for the determination of retinol and carotenoids in human milk using reversed-phase high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure.

3 Scope

The method described here is suitable for the measurement of retinol and carotenoids in human milk.

4 Principle

The fat in human milk is extracted with diethyl ether and petroleum ether, and saponified with ethanolic potassium hydroxide (based on the Roese-Gottlieb method, AOAC 1999). Retinol and carotenoids are extracted with hexane, and determined by reversed-phase high performance liquid chromatography (HPLC).

5 Precautions

The presence of highly conjugated double bonds renders retinol and carotenoids generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep samples capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes, 10 mL, with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL

6.3 Pipettes, Pasteur: 150 mm

6.4 Flasks, glass stoppered, volumetric: 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL and 2000 mL

6.5 Flasks, conical: 100 mL and 250 mL

6.6 Cylinders, measuring: 100 mL and 250 mL

6.7 Crimpvials, 2 mL amber coloured (Phasesep 403682)

6.8 Inserts, 200 µL, for crimpvials (Phasesep 403814)

6.9 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Shaker for tubes (IKA Vibrax VXR with VX2-V)

7.4 Dispenser for 0-10 mL

7.5 Laboratory shaking machine, to-and-fro with orbital motions (Edmund Buehler SM 25)

7.6 Centrifuge with adjustable speed up to 3000 g (Sigma 4-10)

7.7 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.8 Cuvettes, quartz glass, 3 cm path length for measurements between 200 and 500 nm

7.9 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl see Remarks **14.1**.

7.10 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.11 Multiped, 25 mL (Eppendorf)

7.12 Combitips for multiped (Eppendorf 0030-048.440)

7.13 Dry-block heater (Techne) connected with an evaporating unit and nitrogen connection (Pierce Reacti-VAP evaporator 18785)

7.14 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.15 Rotary evaporator with nitrogen connection (with one way valve) and temperature adjustment (35°C)

7.16 Sonicator (Eurosonic 22, Wilten Woltil, NL)

7.17 HPLC – system (Thermoquest) with:

- P4000 Gradient pump
- SCM 400 Solvent degasser
- AS 3000 autosampler with cooling option at 4°C
- UV 2000 detector
- Data recording system
- Vydac 218TP53 reversed phase column (length: 25 cm, diameter: 3.2 mm, particle size: 5µm, metal free frits) with Vydac replaceable guard-column 218GD54

8 Chemicals

8.1 Butylhydroxytoluene (BHT, Sigma B-1378)

8.2 Diethylether (Labscan A35095)

8.3 Potassium hydroxide (Merck 1.5033)

8.4 Ethanol 96%

8.5 Pyrogallol (Merck 100612)

8.6 Methanol, HPLC grade (Labscan C2517)

8.7 Tetrahydrofuran, HPLC grade (THF, Labscan C2520)

8.8 Distilled water

8.9 Triethylamine (Sigma T0886)

8.10 Ammonia solution 25% w/v (Merck 5432)

8.11 Petroleum ether (40/60, Merck 1.01775)

8.12 Nitrogen (highest purity and oxygen-free)

8.13 Hexane (Rathburn RH 1002)

8.14 Ethanol p.a. (Merck 1.00983)

8.15 Dichloromethane, HPLC grade (Labscan C2510)

8.16 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Lutein (Fluka 95507 – 1 mg)
- Zeaxanthin (Roth 5672.1 – 1 mg)
- β-Cryptoxanthin (Roth 5675.1 – 1 mg)
- α-Carotene (CaroteNature 0007)
- β-Carotene (Sigma C4582 – 5 mg)
- Lycopene (Sigma L9879 – 1 mg)

9 Preparation of reagents and standards

9.1 Diethylether containing BHT (0.0025% w/v)

- Dissolve 25 mg butylhydroxytoluene (**8.1**) in diethylether (**8.2**) in a volumetric flask of 1000 mL.
- Fill to the graduation mark with diethylether (**8.2**) and mix.

9.2 Potassium hydroxide (5% w/v) in ethanol containing pyrogallol (0.2% w/v)

- Dissolve 5 gram potassium hydroxide (**8.3**) in 100 mL ethanol (**8.4**) on a water bath of (60°C).
- Dissolve 200 mg pyrogallol (**8.5**).

The solution has a brown colour and has to be prepared daily.

9.3 Methanol – tetrahydrofuran (3:1 v/v) containing BHT (0.1% w/v)

- Transfer 375 mL methanol (**8.6**) into a 500 mL volumetric flask with a measuring cylinder.
- Dissolve 50 mg butylhydroxytoluene (**8.1**).
- Fill to the graduation mark with tetrahydrofuran (**8.7**) and mix.

9.4 Eluent A for HPLC: methanol - tetrahydrofuran - water – triethylamine (87.8:2:10:0.1 v/v/v/v)

- Transfer 100 mL distilled water (**8.8**) into a 1000 mL volumetric flask with a measuring cylinder.
- Add 20 mL tetrahydrofuran (**8.7**) with a measuring cylinder.
- Add 1 mL triethylamine (**8.9**) with an adjustable pipette.
- Fill to the graduation mark with methanol (**8.6**) and mix.

9.5 Eluent B for HPLC: methanol – tetrahydrofuran – triethylamine (92.4:7.5:0.1 v/v/v)

- Transfer 150 mL tetrahydrofuran (**8.7**) into a 2000 mL volumetric flask with a measuring cylinder.
- Add 2 mL triethylamine (**8.9**) with an adjustable pipette.
- Fill to the graduation mark with methanol (**8.6**) and mix.

9.6 Sampler eluent for HPLC: methanol – tetrahydrofuran (50:50 v/v)

- Transfer 250 mL tetrahydrofuran (**8.7**) into a 500 mL volumetric flask with a measuring cylinder.
- Fill to the graduation mark with methanol (**8.6**).

9.7 Preparation of standard solutions

9.7.1 Stock solutions

- Prepare the stock solutions by dissolving the specified quantity (Table 1) of the standards (**8.16**) with the appropriate solvent in the requested volumetric flasks.
- Fill to the graduation mark with solvent and mix.

Table 1 Stock solutions

Standard	Weight (in mg)	Volumetric flask (mL)	Solvent
Retinol	40	100	Ethanol
Lutein	1	50	Tetrahydrofuran*
Zeaxanthin	1	50	Tetrahydrofuran*
β-Cryptoxanthin	1	25	Tetrahydrofuran*
α-Carotene	1	50	Tetrahydrofuran*
β-Carotene	5	50	Tetrahydrofuran*
Lycopene	1	25	Tetrahydrofuran*

* Tetrahydrofuran containing 0.01% BHT

9.7.2 Diluted stock solutions

- Prepare the diluted stock solutions by bringing the specified quantities (Table 2) of the stock solutions (**9.7.1**) in the requested volumetric flasks (Table 2).
- The solvent of the stock solution needs to be evaporated under a nitrogen atmosphere before the volumetric flask is filled with the desired solvent (except for retinol).
- Fill to the graduation mark with the appropriate solvent (Table 2)

Table 2 Diluted stock solutions

Standard	Stock-solution (mL)	Volumetric flask (mL)	Solvent
Retinol	1	50	Ethanol
Lutein	2	25	Ethanol
Zeaxanthin	2	25	Ethanol
β-Cryptoxanthin	2	25	Hexane
α-Carotene	2	25	Hexane
β-Carotene	1	25	Hexane
Lycopene	3	25	Hexane

9.8 Determination of the concentration of diluted stock solutions

By measuring the absorbance of the diluted stock solutions (**9.7.2**) in the spectrophotometer, the concentration can be calculated with the *Lambert-Beer Law*.

- Ensure that the spectrophotometer is calibrated before analysis (Chapter 5).
- For greatest accuracy, use a cuvette with a 3 cm light path.
- Measure the absorbance at the specified wavelength against the solvent blank (Table 3).

Table 3 Wavelength and $E^{1\%}_{1cm}$ †

Standard	Wavelength (nm)	$E^{1\%}_{1cm}$
Retinol	325	1850
Lutein	445	2550
Zeaxanthin	450	2540
β-Cryptoxanthin	452	2386
α-Carotene	444	2800
β-Carotene	453	2592
Lycopene	472	3450

† $E^{1\%}_{1cm}$: Absorption coefficient in ethanol

Concentration: c = (absorbance / absorption coefficient) x 10^6 (µg/dL)

For example: Measured absorbance of β-carotene: 0.706 ⇒

$c = 0.706 / 2592 * 10^6 = 272.4$ µg/dL

N.B. *To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see* ***14.2****.*

9.9 Preparation of working standards

- Prepare the working standards by transferring the desired volumes (Table 4) of the diluted stock solutions (**9.7.2**) with a volumetric pipette into a 100 mL conical flask.
- Evaporate the solvent with a rotary evaporator at 35°C under a nitrogen atmosphere.
- Dissolve the residue in methanol:tetrahydrofuran (**9.3**) and transfer quantitatively into a 25 mL volumetric flask.
- Fill to the graduation mark with methanol:tetrahydrofuran (**9.3**), mix and close with stopper.

Table 4 Working standards

Diluted stock solution	Standard 1 (mL)	Standard 2 (mL)	Standard 3 (mL)	Standard 4 (mL)
Retinol	0	1	2	4
Lutein	0	1.0	2.0	4
Zeaxanthin	0	0.5	1	2
β-Cryptoxanthin	0	0.5	1	2
α-Carotene	0	1.0	2.0	4
β-Carotene	0	2	4	8
Lycopene	0	1.0	2.0	4

The approximate concentrations of the different components in the working standards are shown in Table 5.

Table 5 Concentration of components in working standards

Component	Standard 1 (µg/dL)	Standard 2 (µg/dL)	Standard 3 (µg/dL)	Standard 4 (µg/dL)
Retinol	0	30	60	120
Lutein	0	6	12	24
Zeaxanthin	0	2.5	5	10
β-Cryptoxanthin	0	5	10	20
α-Carotene	0	6	12	24
β-Carotene	0	25	50	100
Lycopene	0	20	40	80

In order to determine the exact concentration of the different components in the working standards, it is necessary to determine the purity of the different components with HPLC:

- Ensure that the HPLC is calibrated before analysis (Chapter 5).
- Bring 1 mL of the diluted stock solutions (**9.7.2**) in crimpvials and inject the samples into the HPLC system according to the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100 %.

With this correction for purity, the exact concentration of the different components in the standard solution can be calculated.

10 Procedure

10.1 Allow frozen milk samples thaw gently at room temperature (20-25°C) and mix well to disperse the cream in the milk prior to analysis. The sample can also be placed in an ultrasonic bath for five minutes to homogenise before starting the analysis.

10.2 Transfer 1.0 mL of homogenised milk or control sample into a Kimax tube using an adjustable pipette.

10.3 Add 0.25 mL ammonia solution (**8.10**) with multiped and mix for 30 seconds on a vortex mixer.

10.4 Add 1 mL ethanol (**8.4**) with a multiped with combitip.

10.5 Mix for 30 seconds on a whirl mixer.

10.6 Add 2 mL diethylether with BHT (**9.1**) with a dispenser.

10.7 Add 2 mL petroleum ether (**8.11**) with a dispenser and close firmly with screw cap.

10.8 Shake the tube horizontally in a laboratory-shaking machine for 5 minutes at 250 reciprocations per minute.

10.9 Centrifuge for 2 minutes at 3000 g.

10.10 Transfer as much as possible of the ether-phase with a Pasteur pipette into a clean Kimax tube.

10.11 Repeat the extraction steps **10.6 – 10.9**.

10.12 Transfer as much as possible of the ether-phase with a Pasteur pipette in the tube containing the first ether-fraction.

10.13 Evaporate the ether in a dry block heater at 35°C under nitrogen atmosphere (**8.12**).

10.14 Add 1.5 mL KOH/pyrogallol solution (**9.2**) with a multiped and combitip.

10.15 Purge several seconds nitrogen (**8.12**) through the tube, but not in the solution and close firmly with screw cap.

10.16 Place the tube in a tube shaker in the dark, and swing for three hours at 200 reciprocations per minute.

10.17 Add 1.5 mL distilled water (**8.8**) with a dispenser.

10.18 Add 3 mL hexane (**8.13**) with a dispenser and close tube firmly with screw cap.

10.19 Shake the tube horizontally in a laboratory-shaking machine for 5 minutes at 250 reciprocations per minute.

10.20 Centrifuge for 2 minutes at 3000 g.

10.21 Transfer as much as possible of the hexane-phase with a Pasteur pipette into a clean Kimax tube.

10.22 Repeat the extraction steps **10.18-10.20**.

10.23 Transfer as much as possible of the hexane-phase with a Pasteur pipette into the tube containing the first hexane-fraction.

10.24 Evaporate the hexane in a dry-block heater at 35°C under a nitrogen atmosphere (**8.12**).

10.25 Add, as quickly as possible after drying, 250 µL methanol-tetrahydrofuran (**9.3**) with an adjustable pipette.

10.26 Close the tube immediately with screw cap and mix well with the whirl mixer.

10.27 Transfer the sample with a Pasteur pipette into a crimpvial with insert and close well. The sample is ready for HPLC analysis.

11 HPLC Analysis

11.1 Pump

The eluent A (**9.4**) and eluent B (**9.5**) are used with a constant flow of 0.7mL/min according the following schedule (table 6)

Table 6 Time schedule for mobile phases

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
0.25-0.75	100 → 0	0 → 100
0.75-20	0	100
20-25*	100	0

* Preparing for next run

11.2 Detector

The UV2000 detector is used for the measurement of carotenoids (at 450 nm) and of retinol (at 325 nm) with the following time schedule:

Table 7 Time schedule wavelength

Time (min)	Wavelength (nm)
0-5.75	325
5.75-20	450
20-25*	325

* preparation for next run

11.3 Sampler

- If an automatic sampler is available keep the temperature of the sampler tray at 4°C.
- Use amber vials.
- Set the injection volume at 25 µL.
- The tip of the sampler is rinsed after each injection with methanol:tetrahydrofuran (**9.6**)
- Add a solvent blank with each run.
- For analysis that are run overnight, the extracts can be kept for 18 hours in the HPLC solvent without experiencing any change in the concentration of retinol or carotenoids (Craft et al. 1988).

11.4 Integration

An internal standard method is used to calibrate the four standards. A computerised programme is incorporated to facilitate automatic integration. The different components have approximately the following retention times under the conditions descibed.

Table 8 Retention times of retinol and carotenoids

Component	Retention time (min)
Retinol	4.2
Lutein	6.3
Zeaxanthin	6.6
β-Cryptoxanthin	8.7
α-Carotene	11.5
β-Carotene	12.4
Lycopene	14.7

Retinol is used as a reference peak. The peaks of isomers of β-carotene and lycopene can be accumulated with the integration programme to report total β-carotene and total lycopene. In the calibration table the concentration of the different components of the 4 different standards are entered as µg/dL. The concentration of the different components in the samples is reported in µg/dL. When 1 mL of milk is dissolved in 250 µL methanol-tetrahydrofuran (**9.3**), the scale factor of 0.1 needs to be incorporated. In the calculation model the 'bracketing' of standards is used.

11.5 Sequence

In a run of 48 samples two control samples (**14.1**) and three series of 4 standards are placed as follows:

Table 9 Sequence of samples and standards

4 standards
2 control samples
22 subject samples
4 standards
24 subject samples
4 standards

12 Data processing and reporting

For every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control

For the purpose of quality control (see Chapter 6), a control sample of human milk (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value by the conversion factor:

Table 10 Conversion factors for conventional and SI units

Analyte	Conventional units	Conversion factor: conventional→ S.I.	S.I. units
Retinol	µg/dL	0.0349	µmol/L
Retinyl acetate	µg/dL	0.0304	µmol/L
Lutein	µg/dL	0.0176	µmol/L
Zeaxanthin	µg/dL	0.0176	µmol/L
β-Cryptoxanthin	µg/dL	0.0181	µmol/L
α-Carotene	µg/dL	0.0186	µmol/L
β-Carotene	µg/dL	0.0186	µmol/L
Lycopene	µg/dL	0.0186	µmol/L

14.3 Ten percent of the samples are analysed in duplicate.

14.4 The retinol and carotenoid content can also be expressed per gram fat in human milk (Paragraph 2.2.1.1). Paragraph 3.7 provides additional information for the fat determination in human milk.

14.5 The pre-column needs to be replaced regularly, generally after 150-200 samples, depending on the extent of contamination of the column. Less efficient separation can be recognised through an increase in pressure, and/or a decrease in peak symmetry, and/or a decrease in the separation, which is characterised, especially for vitamin A, by a 'shoulder peak'. Contaminated pre-columns can be rinsed for re-use. This can be done by reversing the pre-column and rinsing with 10 mL dichloromethane (**8.15**), followed by 10 mL methanol (**8.6**), both at a flow rate of 1.0 mL/minute.

14.6 The working standards can be are stored for about 6 weeks at –20°C without change in their concentration.

15 References

- *AOAC. Official methods of analysis. Vitamin A in milk, milk based infant formula, 992.04 50.1.02 – 50.1.03. Gaithersburg, Maryland: AOAC International, 1995.*
- *AOAC. Official methods of analysis of AOAC International. Fat in milk, Roese-Gottlieb method, 905.02. Gaithersburg, Maryland: AOAC International 1999.*
- *Arroyave G et al. Reprints of selected methods for the analysis of vitamin A and carotenoids in nutrition surveys. Washington DC: IVACG 1982.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids. In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Retinoids and carotenoids. In: De Leenheer AP, Lambert WE, Nelis HJ (Eds). Modern chromatographic analysis of the vitamins. New York, Marcel Dekker, second edition, pp 1-72, 1992.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds.). The retinoids, Biology, Chemistry and Medicine. New York: Raven Press, second edition, pp 179-210, 1994.*

- *ISO 12080-2, Dried skimmed milk – Determination of vitamin A content. Geneva: International Organization for Standardization, 2000.*
- *Lucas A, Gibbs JAH, Lyster RLJ, Baum JD. Creamatocrit: simple clinical technique for estimating fat concentration and energy value of human milk. BMJ 1978; 1:1018-1020.*
- *Olson JA 1994, Vitamin A, retinoids and carotenoids. In: Shils ME (Eds.) Modern Nutrition in health and disease. Baltimore; Williams & Wilkins, eight edition, 1994.*
- *Sowell AL, Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multi wavelength detection. Clin Chem 1994; 40:411-416.*

16 Annexes

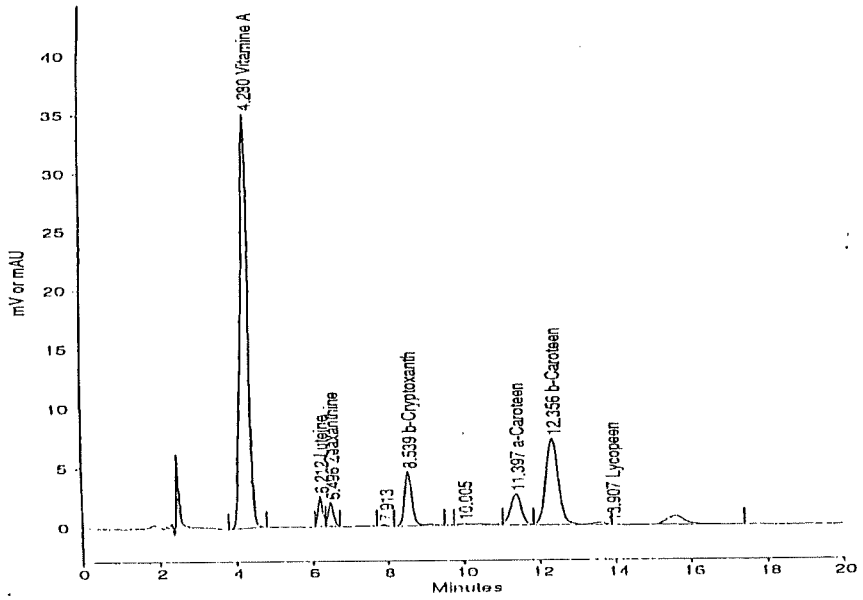


Figure 1 Chromatogram of a working standard solution at 325 nm (0-5.75 min) and 450 nm (5.75-20 min).

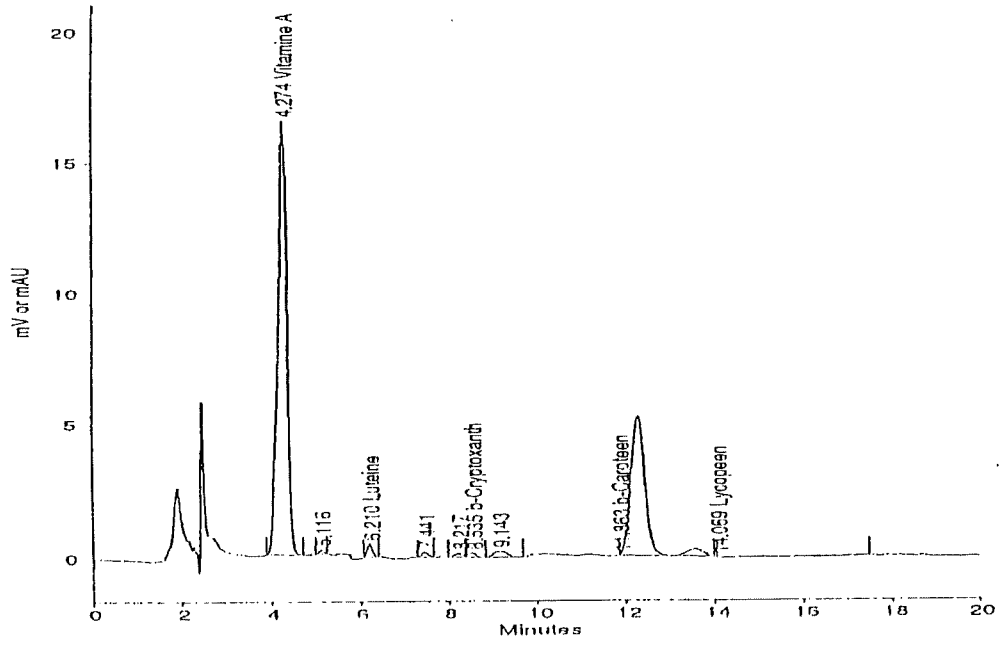


Figure 2 Chromatogram of a control sample of milk at 325 nm (0-5.75 min) and 450 nm (5.75-20 min).

3.3.2 Retinol and carotenoids in human milk (isocratic elution)

Note: *During the preparation of this manual, we were asked to include a Standard Operating Procedure based on isocratic elution. Therefore we have modified our previously described SOP* ***(3.3.1)****. The main difference is the choice of the column, mobile phase and elution technique. The description is based on the literature and on a personal communication from Neal E Craft (Craft Technologies, Inc., Wilson USA) but has NOT been tested in our laboratory.*

1 Title

Standard operating procedures for the determination of retinol and carotenoids in human milk using reversed-phase high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure.

3 Scope

The method described here is suitable for the measurement of retinol and carotenoids in human milk.

4 Principle

The fat in human milk is extracted with diethyl ether and petroleum ether, and saponified with ethanolic potassium hydroxide (based on the Roese-Gottlieb method, AOAC 1999). Retinol and carotenoids are extracted with hexane, and determined by reversed-phase high performance liquid chromatography (HPLC).

5 Precautions

The presence of highly conjugated double bonds renders retinol and carotenoids generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep samples capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes, 10 mL, with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL

6.3 Pipettes, Pasteur: 150 mm

6.4 Flasks, glass stoppered, volumetric: 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL and 2000 mL

6.5 Flasks, conical: 100 mL and 250 mL

6.6 Cylinders, measuring: 100 mL and 250 mL

6.7 Crimpvials, 2 mL amber coloured (Phasesep 403682)

6.8 Inserts, 200 µL, for crimpvials (Phasesep 403814)

6.9 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Shaker for tubes (IKA Vibrax VXR with VX2-V)

7.4 Dispenser for 0-10 mL

7.5 Laboratory shaking machine, to-and-fro with orbital motions (Edmund Buehler SM 25)

7.6 Centrifuge with adjustable speed up to 3000 g (Sigma 4-10)

7.7 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.8 Cuvettes, quartz glass, 3 cm light path for measurements between 200 and 500 nm

7.9 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl, see Remarks **14.1**.

7.10 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.11 Multiped, 25 mL (Eppendorf)

7.12 Combitips for multiped (Eppendorf 0030-048.440)

7.13 Dry-block heater (Techne) connected with an evaporating unit and nitrogen connection (Pierce Reacti-VAP evaporator 18785)

7.14 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.15 Rotary evaporator with nitrogen connection (with one way valve) and temperature adjustment (35°C)

7.16 Sonicator (Eurosonic 22, Wilten Woltil, NL)

7.17 HPLC – system (Thermoquest) with:

- SCM 400 Solvent degasser
- AS 3000 autosampler with cooling option at 4°C
- UV 2000 detector
- Data recording system
- Spherisorb ODS2 column (250 x 4.0 mm; 3 µm particle size; Ti frits) with Vydac replaceable guard-column 218GD54

8 Chemicals

8.1 Butylhydroxytoluene (BHT, Sigma B-1378)

8.2 Diethylether (Labscan A35095)

8.3 Potassium hydroxide (Merck 1.5033)

8.4 Ethanol 96%

8.5 Pyrogallol (Merck 100612)

8.6 Methanol, HPLC grade (Labscan C2517)

8.7 Tetrahydrofuran, HPLC grade (THF, Labscan C2520)

8.8 Distilled water

8.9 Triethylamine (Sigma T0886)

8.10 Ammonia solution 25% w/v (Merck 5432)

8.11 Petroleum ether, 40/60 (Merck 1.01775)

8.12 Nitrogen (highest purity and oxygen-free)

8.13 Hexane (Rathburn RH 1002)

8.14 Ethanol p.a. (Merck 1.00983)

8.15 Dichloromethane, HPLC grade (Labscan C2510)

8.16 Acetonitrile, HPLC grade

8.17 p-Dioxane, HPLC grade

8.18 Methanol, HPLC grade

8.19 Isopropanol, HPLC grade

8.20 Ammonium acetate p.a.

8.21 Ethyl acetate, HPLC grade

8.22 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Lutein (Fluka 95507 – 1 mg)
- Zeaxanthin (Roth 5672.1 – 1 mg)
- β-Cryptoxanthin (Roth 5675.1 – 1 mg)
- α-Carotene (CaroteNature 0007)
- β-Carotene (Sigma C4582 – 5 mg)
- Lycopene (Sigma L9879 – 1 mg)

9 Preparation of reagents and standards

9.1 Diethylether containing BHT (0.0025% w/v)

- Dissolve 25 mg butylhydroxytoluene (**8.1**) in diethylether (**8.2**) in a 1000 mL volumetric flask.
- Fill to the graduation mark with diethylether (**8.2**) and mix.

9.2 Potassium hydroxide (5% w/v) in ethanol containing pyrogallol (0.2% w/v)

- Dissolve 5 gram potassium hydroxide (**8.3**) in 100 mL ethanol (**8.4**) on a water bath with a temperature of 60°C.
- Add 200 mg pyrogallol (**8.5**), dissolve and mix.

The solution has a brown colour and needs to be prepared daily.

9.3 Eluent for HPLC: acetonitrile - p-dioxane - methanol/isopropanol 50:50 v/v% containing 150 mM ammoniumacetate – triethylamine (79.9:15:5:0.1 v/v/v/v)

- Transfer 750 mL p-dioxane (**8.17**) and 250 mL methanol/isopropanol (50:50 v/v%) (**8.18** and **8.19**) containing 2.5 g ammoniumacetate (**8.20**) into a 5000 mL volumetric flask.
- Add 5 mL triethylamine (**8.9**) with measuring cylinder.
- Fill to the graduation mark with acetonitrile (**8.16**) and mix.

9.4 Sampler eluent for HPLC: acetonitrile – isopropanol (50:50 v:v)

- Transfer 250 mL acetonitrile (**8.16**) in a 500 mL volumetric flask with a measuring cylinder.
- Fill to the graduation mark with isopropanol (**8.19**) and mix.

9.5 Preparation of standard solutions

9.5.1 Stock solutions

- Prepare the stock solutions by dissolving the specified quantity (Table 1) of the standards (**8.22**) with the appropriate solvent in the requested volumetric flasks.
- Fill to the graduation mark with solvent and mix.

Table 1 Stock solutions

Standard	Weight (in mg)	Volumetric flask (mL)	Solvent
Retinol	40	100	Ethanol
Lutein	1	50	Tetrahydrofuran*
Zeaxanthin	1	50	Tetrahydrofuran*
β-Cryptoxanthin	1	25	Tetrahydrofuran*
α-Carotene	1	50	Tetrahydrofuran*
β-Carotene	5	50	Tetrahydrofuran*
Lycopene	1	25	Tetrahydrofuran*

* Tetrahydrofuran containing 0.01% BHT

9.5.2 Diluted stock solutions

- Prepare the diluted stock solutions by bringing the specified quantities (Table 2) of the stock solutions (**9.5.1**) in the requested volumetric flasks (Table 2).
- The solvent of the stock solution needs to be evaporated under a nitrogen atmosphere before the volumetric flask is filled with the desired solvent (except for retinol).
- Fill to the graduation mark with the appropriate solvent (Table 2)

Table 2: Diluted stock solutions

Standard	Stock-solution (mL)	Volumetric flask (mL)	Solvent
Retinol	1	50	Ethanol
Lutein	2	25	Ethanol
Zeaxanthin	2	25	Ethanol
β-Cryptoxanthin	2	25	Hexane
α-Carotene	2	25	Hexane
β-Carotene	1	25	Hexane
Lycopene	3	25	Hexane

9.6 Determination of the concentration of diluted stock solutions

By measuring the absorbance of the diluted stock solutions (**9.5.2**) in the spectrophotometer, the concentration can be calculated with the *Lambert-Beer Law.*

- Ensure that the spectrophotometer is calibrated before analysis (Chapter 5).
- For greatest accuracy, use a cuvette with a 3 cm light path.
- Measure the absorbance at the specified wavelength against the solvent blank (Table 3).

Table 3: Wavelength and $E^{1\%}_{1cm}$ †

Standard	Wavelength (nm)	$E^{1\%}_{1cm}$
Retinol	325	1850
Lutein	445	2550
Zeaxanthin	450	2540
β-Cryptoxanthin	452	2386
α-Carotene	444	2800
β-Carotene	453	2592
Lycopene	472	3450

† $E^{1\%}_{1cm}$: Absorption coefficient in ethanol

Concentration: $c = (\text{absorbance} / \text{absorption coefficient}) \times 10^6$ (µg/dL)

For example: Measured absorbance of β-carotene: 0.706 ⇒

$c = 0.706 / 2592 * 10^6 = 272.4$ µg/dL

N.B. *To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see* ***14.2.***

9.7 Preparation of working standards

- Prepare the working standards by transferring the desired volumes (Table 4) of the diluted stock solutions (**9.5.2**) with a volumetric pipette into a conical flask of 100 mL.
- Evaporate the solvent using a rotary evaporator at 35°C under a nitrogen atmosphere.
- Dissolve the residue in mobile phase (**9.3**) and transfer quantitatively into a volumetric flask of 25 mL.
- Fill to the graduation mark with mobile phase (**9.3**), mix and close with stopper.

Table 4 Working standards

Diluted stock solution	Standard 1 (mL)	Standard 2 (mL)	Standard 3 (mL)	Standard 4 (mL)
Retinol	0	1	2	4
Lutein	0	1.0	2.0	4
Zeaxanthin	0	0.5	1	2
β-Cryptoxanthin	0	0.5	1	2
α-Carotene	0	1.0	2.0	4
β-Carotene	0	2	4	8
Lycopene	0	1.0	2.0	4

The approximate concentrations of the different components in the working standards are shown in Table 5.

Table 5 Concentration of components in working standards

Component	Standard 1 (µg/dL)	Standard 2 (µg/dL)	Standard 3 (µg/dL)	Standard 4 (µg/dL)
Retinol	0	30	60	120
Lutein	0	6	12	24
Zeaxanthin	0	2.5	5	10
β-Cryptoxanthin	0	5	10	20
α-Carotene	0	6	12	24
β-Carotene	0	25	50	100
Lycopene	0	20	40	80

In order to determine the exact concentration of the different components in the working standards, it is necessary to determine the purity of the different components with HPLC:

- Ensure that the HPLC is calibrated before analysis (Chapter 5).

- Transfer 1 mL of the diluted stock solutions (**9.5.2**) into the crimpvials and inject the samples in the HPLC system according to the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100 %.
With this correction for purity, the exact concentration of the different components in the standard solution can be calculated.

10 Procedure

10.1 Allow frozen milk samples thaw gently at room temperature (20-25°C) and mix well to disperse the cream in the milk prior to analysis. The sample can also be placed in an ultrasonic bath for five minutes to homogenise before starting the analysis.

10.2 Transfer 1.0 mL of homogenised milk or control sample into a Kimax tube using an adjustable pipette

10.3 Add 0.25 mL ammonia solution (**8.10**) with a multiped and mix for 30 seconds on a whirl mixer.

10.4 Add 1 mL ethanol (**8.4**) with a multiped with combitip.

10.5 Mix for 30 seconds on a vortex mixer.

10.6 Add 2 mL diethylether with BHT (**9.1**) with a dispenser.

10.7 Add 2 mL petroleum ether (**8.11**) with a dispenser and close firmly with screw cap.

10.8 Shake the tube horizontally in a laboratory-shaking machine for 5 minutes at 250 reciprocations per minute.

10.9 Centrifuge for 2 minutes at 3000 g.

10.10 Transfer as much as possible of the ether-phase with a Pasteur pipette into a clean Kimax tube.

10.11 Repeat the extraction steps **10.6 – 10.9**.

10.12 Transfer as much as possible of the ether-phase with a Pasteur pipette into the tube containing the first ether-fraction.

10.13 Evaporate the ether in a dry block heater at 35°C under nitrogen atmosphere (**8.12**).

10.14 Add 1.5 mL KOH/pyrogallol solution (**9.2**) with a multiped and combitip.

10.15 Purge several seconds nitrogen (**8.12**) through the tube, but not in the solution and close firmly with screw cap.

10.16 Place the tube in a tube shaker in the dark, and swing for three hours at 200 reciprocations per minute.

10.17 Add 1.5 mL distilled water (**8.8**) with a dispenser.

10.18 Add 3 mL hexane (**8.13**) with a dispenser and close tube firmly with screw cap.

10.19 Shake the tube horizontally in a laboratory-shaking machine for 5 minutes at 250 reciprocations per minute.

10.20 Centrifuge for 2 minutes at 3000 g.

10.21 Transfer as much as possible of the hexane-phase with a Pasteur pipette into a clean Kimax tube.

10.22 Repeat the extraction steps **10.18-10.20**.

10.23 Transfer as much as possible of the hexane-phase with a Pasteur pipette into the tube containing the first hexane-fraction.

10.24 Evaporate the hexane in a dry-block heater at 35°C under a nitrogen atmosphere (**8.12**).

10.25 Add, as quickly as possible after drying, 50 µL ethyl acetate (**8.21**) to dissolve the sample and add 200 µL mobile phase (**9.3**) with an adjustable pipette.

10.26 Close the tube immediately with screw cap and mix well with the whirl mixer.

10.27 Transfer the sample with a Pasteur pipette into a crimp vial with insert and close well. The sample is ready for HPLC analysis.

11 HPLC Analysis

11.1 Pump

The eluent (**9.3**) is pumped with a constant flow of 1.0 mL/min.

11.2 Detector

The UV2000 detector is used for measurement of retinol and retinyl acetate at 325 nm, and than switched to 450 nm for the measurement of carotenoids. Total run time: approximately 20 min.

11.3 Sampler

- If an automatic sampler is available keep the temperature of the sampler tray at 15°C.
- Use amber vials.
- Set the injection volume at 20 µL.
- The tip of the sampler is rinsed after each injection with acetonitrile:isopropanol (**9.4**).
- Add a solvent blank with each run.
- For analysis that are run overnight, the extracts can be kept for 18 hours in HPLC solvents without experiencing any change in the concentration of retinol or carotenoids (Craft et al. 1988).

11.4 Sequence

In a run of 48 samples, 2 control samples and three series of 4 standards are placed as follows (table 6):

Table 6 Sequence of samples and standards

4 standards
2 control samples
22 subject samples
4 standards
24 subject samples
4 standards

12 Data processing and reporting

For every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control

For the purpose of quality control (see Chapter 6), a control sample of human milk (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value by the conversion factor:

Table 7 Conversion factors for conventional and SI units

Analyte	Conventional units	Conversion factor: conventional→ S.I.	S.I. units
Retinol	µg/dL	0.0349	µmol/L
Retinyl acetate	µg/dL	0.0304	µmol/L
Lutein	µg/dL	0.0176	µmol/L
Zeaxanthin	µg/dL	0.0176	µmol/L
β-Cryptoxanthin	µg/dL	0.0181	µmol/L
α-Carotene	µg/dL	0.0186	µmol/L
β-Carotene	µg/dL	0.0186	µmol/L
Lycopene	µg/dL	0.0186	µmol/L

14.3 Ten percent of the samples are analysed in duplicate.

14.4 The retinol content can also be expressed per gram fat in human milk (Paragraph 2.2.1.1). Paragraph 3.7 provides additional information for the fat determination in human milk.

14.5 The pre-column needs to be replaced regularly, approximately after 150-200 samples, depending on the extent of contamination of the column. Less efficient separation can be recognised through an increase in pressure, and/or a decrease in peak symmetry, and/or a decrease in the separation, which is characterised, especially for vitamin A, by a 'shoulder peak'. Contaminated pre-columns can be rinsed for re-use. This can be done by reversing

the pre-column and rinsing with 10 mL dichloromethane (**8.15**) followed by 10 mL methanol (**8.18**), both with a flow rate of 1.0 mL/minute.

14.6 The working standards can be stored for about 6 weeks at –20°C without change in concentration

15 References

- *AOAC. Official methods of analysis. Vitamin A in milk, milk based infant formula, 992.04 50.1.02 – 50.1.03. Gaihersburg, Maryland: AOAC International, 1995.*
- *AOAC. Official methods of analysis of AOAC International. Sixteenth edition, volume 2: food composition, additives, and natural contaminants. Fat in milk, Roese-Gottlieb method, 905.02. Gaithersburg, Maryland: AOAC International, 1999.*
- *Arroyave G et al. Reprints of selected methods for the analysis of vitamin A and carotenoids in nutrition surveys. Washington DC: IVACG, 1982.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids. In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Retinoids and carotenoids. In: De Leenheer AP, Lambert WE, Nelis HJ (Eds). Modern chromatographic analysis of the vitamins. New York: Marcel Dekker, second edition, pp 1-72, 1992.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds.). The retinoids, biology, chemistry and medicine. New York: Raven Press, second edition, pp 179-210, 1994.*
- *ISO 12080-2, Dried skimmed milk – Determination of vitamin A content. International Organization for Standardization, 2000.*
- *Lucas A, Gibbs JAH, Lyster RLJ, Baum JD. Creamatocrit: simple clinical technique for estimating fat concentration and energy value of human milk. BMJ 1978; 1:1018-1020.*
- *Olson JA 1994, Vitamin A, retinoids and carotenoids. In: Shils ME (Eds.) Modern Nutrition in health and disease. Baltimore: Williams & Wilkins, eight edition, 1994.*
- *Sowell AL, Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multi wavelength detection. Clin Chem 1994; 40:411-416.*

3.4 Retinol in serum / plasma

1 Title

Standard operating procedure for the determination of retinol in serum/plasma using high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure

3 Scope

The method described here is suitable for the measurement of retinol in serum or plasma.

4 Principle

Serum/plasma is denatured with ethanol containing an internal standard (based on Cantilena and Nierenberg, 1989). The sample is extracted with hexane and the concentration of retinol in the extract is determined by high performance liquid chromatography (HPLC), based on Erhardt et al (2002).

5 Precautions

The presence of highly conjugated double bonds renders retinol generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep samples capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes (10 mL) with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL, 4.0 mL

6.3 Pipettes, Pasteur: 150 mm

6.4 Flasks, glass stoppered, volumetric: 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL and 2000 mL

6.5 Flasks, conical: 100 mL

6.6 Cylinders, measuring: 100 mL

6.7 Crimpvials, 2 ml amber coloured (Phasesep 403682)

6.8 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Dispenser for 0-10 mL

7.4 Laboratory shaking machine, to-and-fro with orbital motions (Edmund Buehler SM 25)

7.5 Centrifuge with adjustable speed up to 3000 g (Sigma 4-10)

7.6 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.7 Cuvettes, quartz glass, 1 cm path length for measurements between 200 and 500 nm

7.8 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl see **14.1**.

7.9 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.10 Multiped, 25 mL (Eppendorf)

7.11 Combitips for multiped (Eppendorf 0030-048.440)

7.12 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.13 HPLC – system (Thermo separation products) with:

- Pump
- Solvent de-gasser
- Autosampler with cooling option at 4°C
- UV 2000 detector
- Data recording system

Column: BDS Hypersil CN (length: 150 mm. diameter: 3 mm, particle size: 5µm) column (Keystone Scientific, Bellafonte PA) with a Javelin NH_2 guard column (Keystone Scientific, Bellafonte PA)

8 Chemicals

8.1 Sodium chloride (NaCl, Merck 1.06400)

8.2 Distilled water

8.3 Butylhydroxytoluene (BHT, Sigma B-1378)

8.4 Hexane, HPLC grade (Rathburn RH 1002)

8.5 Isopropanol, HPLC grade (Merck)

8.6 Ethanol p.a. (Merck 1.00983)

8.7 Nitrogen (highest purity and oxygen-free)

8.8 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Retinyl acetate (Fluka 95138 – 5 g)

9 Preparation of reagents and standards

9.1 Sodium chloride (0.9% w/v)

- Dissolve 0.9 gram NaCl (**8.1**) in distilled water (**8.2**) in a volumetric flask of 100 mL.
- Fill to the graduation mark with distilled water (**8.2**) and mix.

9.2 Hexane containing BHT (0.01% w/v)

- Dissolve 10 mg butylhydroxytoluene (**8.3**) in hexane (**8.4**) in a volumetric flask of 100 ml.
- Fill to the graduation mark with hexane (**8.4**) and mix.

9.3 HPLC eluent: hexane – isopropanol (98.5:1.5 v/v)

- Transfer 15 ml isopropanol **(8.5)** in a 1000 mL volumetric flask with a measuring cylinder.
- Fill to the graduation mark with hexane **(8.4)** and mix.

9.4 Preparation of internal standard solution

9.4.1 Stock solution

- Dissolve 50 mg retinyl acetate (**8.8**) in ethanol (**8.6**) in a 100 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.6**) and mix.

9.4.2 Diluted stock solution

- Transfer 10.0 mL stock solution of retinyl acetate (**9.4.1**) in a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.6**) and mix.

9.4.3 Internal standard solution

- Transfer 2 mL diluted stock solution of retinyl acetate (**9.4.2**) in a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.6**) and mix.

Prepare this internal standard of retinyl acetate daily from the diluted stock solution (**9.4.2**).

9.5 Preparation of standard solution

9.5.1 Stock solution

- Dissolve 50 mg retinol (**8.8**) in ethanol (**8.6**).and transfer to a 100 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.6**) and mix.

9.5.2 Diluted stock solution

- Transfer 1.0 mL stock solution of retinol (**9.5.1**) into a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.6**) and mix.

9.6 Determination of the concentration of internal standard and diluted stock solution

By measuring the absorbance of the internal standard solution of retinyl acetate (**9.4.3**) and the diluted stock solution of retinol (**9.5.2**) in the spectrophotometer, the exact concentration can be calculated with the *Lambert-Beer Law*.

- Ensure that the spectrophotometer is calibrated before analysis (Chapter 5).
- Use a cuvette with 1 cm light path.
- Measure the absorbance at the specified wavelength for retinol and retinyl acetate against the solvent blank.

Table 1 Wavelength and $E^{1\%}_{1cm}$ *

Standard	Wavelength (nm)	$E^{1\%}_{1cm}$
Retinol	325	1850
Retinyl acetate	325	1550

* $E^{1\%}_{1cm}$: Absorption coefficient (in ethanol)

Concentration: $c = (\text{absorbance} / \text{absorption coefficient}) \times 10^6$ (µg/dL)

For example: Measured absorbance of retinol: 0.204 $\Rightarrow$

$c = 0.204 / 1850 * 10^6 = 110.3$ µg/dL

N.B. To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see ***14.2****.*

9.7 Preparation of working standards

- Prepare the working standards by transferring the desired volumes (Table 2) of the diluted stock solutions (**9.4.2** and **9.5.2**) to 100 mL volumetric flasks with a volumetric pipette according the volumes shown in Table 2.
- Fill to the graduation mark with hexane (**9.2**) and mix, close well with a stopper.

Table 2 Working standards

Diluted stock solution	Standard 1 (mL)	Standard 2 (mL)	Standard 3 (mL)	Standard 4 (mL)	Standard 5 (mL)
Retinol	0	0.5	1.0	2.0	4.0
Retinyl acetate	0	1.0	1.0	1.0	1.0

The approximate concentrations of the different components in the working standards are shown in Table 3.

Table 3 Concentration of components in working standards

Component	Standard 1 (µg/dL)	standard 2 (µg/dL)	Standard 3 (µg/dL)	Standard 4 (µg/dL)	Standard 5 (µg/dL)
Retinol	0	2.5	5.0	10	20
Retinyl acetate	0	50	50	50	50

In order to determine the exact concentrations of different components in the working standards, it is necessary to determine the purity of the different components with high-performance liquid chromatography:

- Ensure that the HPLC is calibrated before analysis (Chapter 5).
- Transfer 1 mL of the internal standard of retinyl acetate (**9.4.3**) and 1 mL of the diluted stock solution of retinol (**9.5.2**) into crimp vials and inject the samples in the HPLC system according to the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100%

With this correction for purity, the exact concentration of the different components in the working standards can be calculated.

10 Procedure

10.1 Allow frozen serum or plasma samples to thaw gently at room temperature (20-25°C) prior to analysis and mix well. Bring 0.5 mL of serum, plasma, or control sample into a Kimax tube with a calibrated adjustable pipette.

10.2 Add 0.5 mL NaCl solution (**9.1**) with a multiped and combitip.

10.3 Add 1.0 mL internal standard (**9.4.3**) with an adjustable pipette.

10.4 Mix for 30 seconds on a vortex mixer.

10.5 Add 2,0 mL hexane (**9.2**) with a dispenser and close firmly with screw cap.

10.6 Shake the tube horizontally in the laboratory shaking machine for 5 minutes at 250 reciprocations per minute.

10.7 Centrifuge for 2 minutes at 3000 g.

10.8. Transfer 1 mL supernatant into a crimp vial with a Pasteur pipette and close crimp cap with hand crimper. The sample is ready to be injected into the HPLC.

11 HPLC Analysis

11.1 Pump

The HPLC eluent (**9.3**) is used with a constant flow of 0.7 mL/min.

11.2 Detector

The UV2000 detector is set at 325 nm.

11.3 Sampler

- If an automatic sampler is available keep the temperature of the sampler tray at 4°C.
- Use amber coloured vials
- Set the injection volume at 200 µL.
- The tip of the sampler is rinsed after each injection with the mobile phase.
- Include a solvent blank in each run.
- For analysis that are run overnight, the extracts can be kept for 18 hours in HPLC solvents without experiencing any change in the concentration of retinol or carotenoids (Craft et al. 1988).

11.4 Integration

An internal standard method is used to calibrate the working standards. A computerised programme is incorporated in order to facilitate automatic integration. Retinyl acetate is used as a reference standard. The concentration of the different components in the samples is reported in µg/dL. In the calculation model the 'bracketing' of standards is used.

11.5 Sequence

In a run of 48 samples, two control samples (**12.1**) and three series of 5 standards are placed as follows:

Table 4 Sequence of standards and samples

5 standards
2 control samples
22 subject samples
5 standards
24 subject samples
5 standards

12 Data processing and reporting

For every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control

For the purpose of quality control (see Chapter 6) a control sample of serum/plasma (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value by the conversion factor:

Table 5 Conversion factors for conventional and SI units

Analyte	Conventional units	Conversion factor: conventional→ S.I.	S.I. units
Retinol	µg/dL	0.0349	µmol/L
Retinyl acetate	µg/dL	0.0304	µmol/L

14.3 Ten percent of the samples are analysed in duplicate.

14.4 The working standards can be stored for about 6 weeks at –20°C without change in their concentration.

15 References

- *Cantilena LR, Nierenberg DW. Simultaneous analysis of five carotenoids in human plasma by isocratic high performance liquid chromatography. J Micronutr Anal 1989; 6: 127-145.*
- *Craft NE, Brown ED, Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988; 34:44-48.*
- *Erhardt JG, Craft NE, Heinrich F and Biesalski HK. Rapid and simple measurement of retinol in human dried whole blood spot. J. Nutr 2002; 132:318-321.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids, In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds). The retinoids, biology, chemistry and medicine. New York: Raven Press, second edition, pp 179-210, 1994.*

16 Annexes

Not applicable in this standard operating procedure

3.5 Retinol in dried blood spots

1 Title

Standard operating procedure for the determination of retinol in dried blood spots using high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure

3 Scope

The method described here is suitable for the measurement of retinol in dried blood spots.

4 Principle

Retinol in a dried blood spot sample is extracted with hexane and the concentration of retinol is determined by high performance liquid-chromatography (Erhardt et al., 2002).

5 Precautions

The presence of highly conjugated double bonds renders retinol generally unstable, and precautions should be taken to protect against losses. Prevent direct light, and work in subdued light, if available yellow-orange, to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Pipettes, volumetric: 1.0 mL

6.2 Flasks, glass stoppered, volumetric: 100 mL and 1000 mL

6.3 Cylinder, measuring: 100 mL

6.4 Micro centifuge tubes, 1.5 mL

6.5 Autosampler vials with 300 µl inserts

6.5 Filter paper cards (Schleicher and Schuell 903 specimen collection paper, Germany)

7 Apparatus and equipment

7.1 Analytical Balance 0.1 mg (Mettler Toledo AB 204)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Centrifuge with adjustable speed up to 8000 g (Sigma 4-10)

7.4 Adjustable pipette 1000 µl (Eppendorf). For volumes <200 µl see **14.1**.

7.5 Repeater pipette (Eppendorf).

7.6 Pipette tips

7.7 Sonicator (Eurosonic 22, Wilten Woltil, NL)

7.8 Hole punch (0.25 inch, 6.35 mm)

7.9 HPLC – system with:

- Pump
- Autosampler with cooling option at 4°C or mechanical injector
- UV detector
- Data recording system

Column: BDS Hypersil CN (length: 150 mm, diameter: 3 mm, particle size: 5µm) column (Keystone Scientific, Bellafonte PA) with a Javelin NH_2 guard column (Keystone Scientific, Bellafonte PA)

8 Chemicals

8.1 Ascorbic acid

8.2 Distilled water

8.3 Butylhydroxytoluene (BHT, Sigma B-1378)

8.4 Acetonitrile, HPLC grade (Merck)

8.5 Hexane, HPLC grade (Rathburn RH 1002)

8.6 Isopropyl alcohol, HPLC Grade (Merck)

8.7 Standards:

- Retinol (Fluka 95144 - 50 mg)
- Tocol (Matreya: www.matreya.com)

9 Preparation of reagents and standard

9.1 Distilled water / ascorbic acid (1% w/v)

- Dissolve 1 g ascorbic acid (**8.1**) in distilled water (**8.2**) into a 100 mL volumetric flask.
- Fill to the graduation mark with distilled water (**8.2**) and mix.

Prepare this reagents weekly and keep stored in the refrigerator.

9.2 Acetonitrile - BHT (0.5% w/v)

- Dissolve 500 mg butylhydroxytoluene (**8.3**) in acetonitrile (**8.4**) into a 100 mL volumetric flask.
- Fill to the graduation mark with acetonitrile (**8.4**) and mix.

9.3 Hexane - BHT (0.5% w/v)

- Dissolve 500 mg butylhydroxytoluene (**8.3**) in hexane (**8.5**) into a 100 mL volumetric flask.
- Fill to the graduation mark with hexane (**8.5**) and mix.

9.4 HPLC eluent: hexane – isopropanol (98.5:1.5 v/v)

- Transfer 15 ml isopropanol (**8.6**) into a 1000 mL volumetric flask with a measuring cylinder.

- Fill to the graduation mark with hexane (**8.5**) and mix.

9.5 Preparation of internal standard solution

9.5.1 Stock solution

- Dissolve 15 mg tocol in acetonitrile (**9.2**) into a 100 mL volumetric flask.
- Fill to the graduation mark with acetonitrile (**9.2**) and mix.

9.5.2 Internal standard solution

- Transfer 1.0 mL of tocol solution (**9.5.1**) into a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with acetonitrile (**9.2**) and mix.

The concentration of tocol in this internal standard solution is approximately 4 µmol/L. Store this solution in the refrigerator and prepare it fresh from the stock solution every week.

9.6 Preparation of standard spots

9.6.1 Collect whole blood into two heparin separator tubes.

9.6.2 Estimation of the hematocrit value: Draw well-mixed anticoagulated blood from one of two tubes into micro-hematocrit tubes. Fill the tube for about 3/4. Wipe off excess blood with gauze and seal one end with clay. Place sealed tubes with sealed end toward the outside in the micro-centrifuge. Centrifuge for approx. 5 min at 10 000-15 000 g. Promptly read the hematocrit value after the centrifugation has stopped (Hct %).

9.6.3 Prepare plasma from the blood sample out of the other heparin tube (Paragraph 2.1.3). Determine the retinol concentration in the plasma (Paragraph 3.2) in µmol/L or µg/dL. The retinol concentration in whole blood can be calculated:
Retinol in plasma (µmol/L or µg/dL) * (100-Hct) /100.

9.6.5 Prepare the standard dried blood spot samples as described in Paragraph 2.1.4 and 2.1.5 out of the well-mixed anticoagulated blood. The volume of the blood should be appoximately the same as used for the sample of blood to be tested.

9.6.6 Place cards in Ziploc bags and store as described in Paragraph 2.1.4 and 2.1.5.

9.6.7 Measure the retinol concentration in these spots.

10 Procedure

Extraction of retinol

10.1 Punch each blood spot (6.35 mm or ¼ inch diameter) out of the middle of each circle with a hole punch and transfer it into a 1.5 ml micro-centrifuge tube. In the same way, prepare a blank sample by punching the filterpaper away from a bloodspot.

10.2 Add 500 µL ascorbic acid solution (**9.1**) with a repeater pipette.

10.3 Close tube and mix, and incubate for 15 minutes in the dark at room temperature (20-25°C).

10.4 Place the tube in a sonicator for 5 minutes.

10.5 Add 400 µL internal standard (**9.5.2**) with a repeater pipette.

10.6 Mix briefly on a vortex mixer.

10.7 Add 400 µL hexane (**9.3**) with a repeater pipette.

10.8 Close the tube firmly.

10.9 Shake the tubes 2 minutes vigorously by hand.

10.10 Centrifuge for 1 minute at 8000 g.

10.11 Transfer approx. 300 µl of the supernatant to an autosampler vial or a tube for manual injection. The sample is now ready to be injected into the HPLC system.

11 HPLC Analysis

11.1 Pump

The mobile phase hexane/isopropanol (**9.4**) is used with a constant flow of 0.7 mL/min.

11.2 Detector

The UV detector is set for retinol (325 nm) and tocol (300 nm) for 4 minutes per run with a wavelength change from 325 to 300 in the middle between the retinol and tocol peak.

11.3 Sampler

- If an automatic sampler with refrigeration is available, keep the temperature of the sampler tray at 4°C.
- Use amber coloured vials otherwise cover against light.
- Set the injection volume at 200 µL.
- The tip of the sampler is rinsed after each injection with the mobile phase.
- For overnight analysis the serum/plasma extracts can be kept for 18 hours in HPLC solvents with no differences in retinol concentration (Craft et al. 1988).

11.4 Integration

For integration of the peak areas, a computerised programme should be used. The peak height of retinol can be used.

11.5 Sequence

In a run of 40 samples, 2 blanks, 2 quality control samples and 2 series each of 2 standards are placed as follows (table 1):

Table 1: sequence of standards and samples

1 blank DBS
1 quality control DBS
2 standard DBS samples
20 subject DBS samples
1 blank DBS
1 quality control DBS
2 standard DBS samples
20 subject DBS samples

12 Data processing and reporting

For each chromatogram, the area of the retinol peak is divided by the tocol peak. With this ratio, a calibration curve for the standards is made and the concentration of retinol in the subject samples directly calculated.

13 Quality control

Check for every batch the linearity of the standard DBS spots and the value for the quality control. For quality control (see Chapter 6), a control sample of dried blood spot (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for sample and internal standards to avoid poor precision.

14.2 Ten percent of the samples are analysed in duplicate.

15 References

- *Erhardt JG, Craft NE, Heinrich F and Biesalski HK. Rapid and simple measurement of retinol in human dried whole blood spot. J. Nutr 2002; 132:318-321.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids, In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition pp 181-233 , 1984.*

16 Annexes

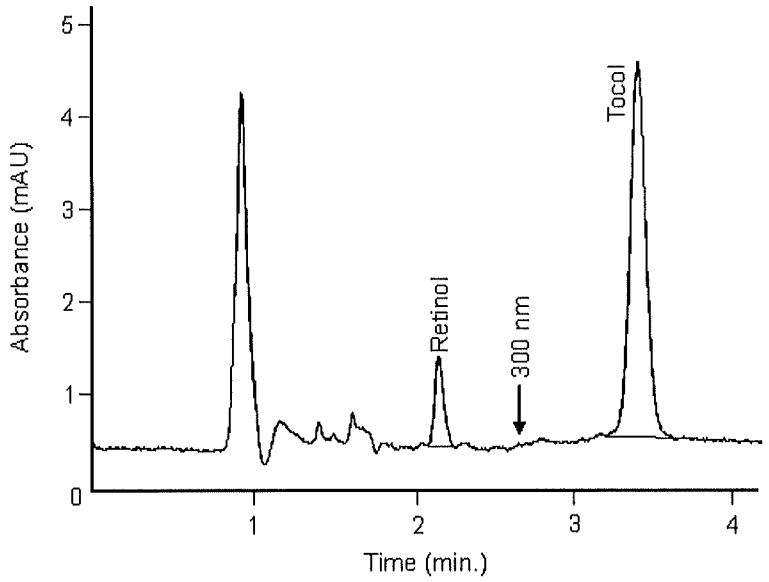


Fig 3.5 Chromatogram of retinol (325 nm) and tocol (300 nm) in a standard dried blood spot.

3.6 Retinol in human milk

1 Title

Standard operating procedure for the determination of retinol in human milk using high performance liquid chromatography.

2 Update and review summary

Not applicable in this standard operating procedure

3 Scope

The method described here is suitable for the measurement of retinol in human milk.

4 Principle

The fat in human milk is saponified with potassium hydroxide (modified AOAC procedure, 1995). The retinol is extracted with hexane and determined with high performance liquid chromatography (Erhardt et al, 2002).

5 Precautions

The presence of highly conjugated double bonds renders retinol generally unstable, and precaution should be taken to protect against losses:

- Prevent the sample and standards being exposed to air as much as possible. Flush with nitrogen if appropriate. Keep analyte in solution.
- Keep samples capped when not in use.
- Work in subdued, if available yellow-orange, light to prevent losses particularly in the UV region, 250-350 nm.

6 Materials

6.1 Kimax tubes, 10 mL, with PTFE seal screw cap. Check for smooth edges and proper fitting caps.

6.2 Flask, glass stoppered, volumetric: 50 mL, 100 mL, 500mL, and 1000 m

6.3 Pipettes, volumetric: 0.5 mL, 1.0 mL, 2.0 mL and 4.0 mL

6.4 Pipettes, Pasteur: 150 mm

6.5 Cylinder, measuring: 100 mL

6.6 Crimpvials, 2 mL amber coloured (Phasesep 403682)

6.7 Inserts, 200 µL for crimpvials (Phasesep 403814)

6.8 Crimpcaps with PTFE seal (Phasesep 412115)

7 Apparatus and equipment

7.1 Analytical Balance (Mettler Toledo AB 204; 0.1 mg)

7.2 Vortex mixer (Retch Mix TM01)

7.3 Laboratory shaking machine, to-and-fro and orbital motions (Edmund Buehler SM 25)

7.4 Centrifuge with adjustable speed up to 3000 g (Sigma 4-10)

7.5 Spectrophotometer (Varian, 50 Probe UV-Visible)

7.6 Cuvettes, quartz glass, 1 cm path length for measurements between 200 and 500 nm

7.7 Pipettes, calibrated adjustable: 250 µL, 500 µL, 1000 µL (Eppendorf). For volumes <200 µl see **14.1**.

7.8 Pipette tips, 1000 µL (Eppendorf, 0030-015.002)

7.9 Multiped, 25 mL (Eppendorf)

7.10 Combitips for multiped (Eppendorf 0030-048.440)

7.11 Hand crimper for closing crimpcap vials (HP 8710-0979)

7.12 Sonicator (Eurosonic 22, Wilton Woltil, NL)

7.13 Water bath, adjustable to 80 °C

7.14 HPLC – system (Thermo separation products) with:

- Pump
- Solvent de-gasser
- Autosampler with cooling option at 4°C
- UV 2000 detector
- Data recording system

Column: BDS Hypersil CN (length: 150 mm. diameter: 3 mm. particle size: 5-µm) column with a Javelin APS2 amino guard column (both from Keystone Scientific, Bellefonte PA).

8 Chemicals

8.1 Pyrogallol (Merck 100612)

8.2 Ethanol, 96%

8.3 Potassium hydroxide (Merck 1.5033)

8.4 Distilled water

8.5 Butylhydroxytoluene (BHT, Sigma B-1378)

8.6 Hexane (Rathburn RH 1002)

8.7 Isopropanol, HPLC grade (Merck)

8.8 Retinol (Fluka 95144 - 50 mg)

8.9 Ethanol, p.a. (Merck 1.00983)

8.10 Nitrogen (highest purity and oxygen-free)

9 Preparation of standards, reagents

9.1 Ethanol containing pyrogallol (0.04% w/v)

- Dissolve 400 mg pyrogallol (**8.1**) in ethanol (**8.2**) into a 1000 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.2**) and mix.

9.2 KOH (50% w/v)

- Dissolve 50 gram potassium hydroxide (**8.3**) in distilled water (**8.4**) in a volumetric flask (100 mL).
- Fill to the graduation mark with distilled water (**8.4**) and mix.

9.3 Hexane - BHT (0.01% w/v))

- Dissolve 10 mg butylhydroxytoluene (**8.5**) in hexane (**8.6**) in a volumetric flask of 100 ml.
- Fill to the graduation mark with hexane (**8.6**) and mix.

9.4 HPLC eluent: hexane – isopropanol (98.5:1.5 v/v)

- Transfer 15 ml isopropanol (**8.7**) into a 1000 mL volumetric flask with a measuring cylinder.
- Fill to the graduation mark with hexane (**8.6**) and mix.

9.5 Preparation of the standard solution

9.5.1 Stock solution

- Dissolve 50 mg retinol (**8.8**) in ethanol (**8.9**) into a 100 mL volumetric flask.
- Fill to the graduation mark with ethanol (**8.9**) and mix.

9.5.2 Diluted stock solution

- Transfer 1.0 mL stock solution of retinol (**9.5.1**) into a 100 mL volumetric flask with a volumetric pipette.
- Fill to the graduation mark with ethanol (**8.9**) and mix.

9.6 Determination of the concentration of diluted stock solution

By measuring the absorbance of the diluted stock solution of retinol (**9.5.2**) in the spectrophotometer, the concentration can be calculated with *Lambert-Beer Law.*

- Assure the spectrophotometer is calibrated before analysis (Chapter 5).
- Use a cuvette of 1 cm light path.
- Measure absorbance at the specified wavelength against the solvent blank: Retinol wavelength is set at 325 nm and $E^{1\%}_{1cm} = 1850$ (in ethanol).

Concentration: c = (absorbance / absorption coefficient) x 10^6 (µg/dL)

For example: Measured absorbance of retinol: 0.204 ⇒

c = 0.204 / 1850 * 10^6 = 110.3 µg/dL

*N.B. To convert values from conventional units (µg/dL) to S.I. units (µmol/L) see **14.2**.*

9.7 Preparation of the working standards

- Prepare working standards by transferring the desired volumes of the diluted stock solution (**9.5.2**) with a volumetric pipette to volumetric flasks of 100 mL according the following volumes:
  Standard 1: 0.5 mL diluted stock solution; Standard 2: 1.0 mL diluted stock solution;
  Standard 3: 2.0 mL diluted stock solution; Standard 4: 4.0 mL diluted stock solution.
- Fill to the graduation mark with hexane (**9.3**), mix and close well with stopper.

The approximate retinol concentrations in the different working standards are:

Standard 1: 2.5 µg/dL; Standard 2: 5 µg/dL;
Standard 3: 10.0 µg/dL; Standard 4: 20 µg/dL;

In order to determine the exact concentration of retinol in the working standards, it is necessary to determine the purity of retinol with high performance liquid chromatography (HPLC):

- Ensure that the HPLC is calibrated before analysis (Chapter 5)
- Transfer 1 mL of the diluted stock solution (**9.5.2**) in crimp vials and inject the samples into the HPLC system according the normal procedure.

Purity (%) = (Peak area component / Total peak area of the components within the standard) x 100%

With this correction for purity, the exact concentration of retinol in the standard solution can be calculated (µg/dL).

10 Procedure

10.1 Let frozen milk samples thaw gently at room temperature (20-25°C) and mix to disperse the cream into the milk prior to analysis. The sample can also be placed in a sonicator for 5 minutes to homogenise before starting the analysis.

10.2 Transfer 1.0 mL of homogenised milk or control sample into a Kimax tube with adjustable pipette.

10.3 Add 1.25 mL ethanol-pyrogallol solution (**9.1**) with a multiped and with combitip.

10.4 Mix for 30 seconds on a vortexl mixer.

10.5 Add 0.25 mL KOH solution (**9.2**) with a multiped and combitip.

10.6 Purge the tube above the solution with nitrogen (**8.10**) and close the tube firmly with screw cap.

10.7 Place the tubes for 30 minutes in a water bath at 80°C.

10.8 Cool down to room temperature (20-25°C).

10.9 Add 2.0 mL of hexane (**9.3**) with a 25 mL multiped and close firmly with screw cap.

10.10 Shake tubes horizontally in a laboratory shaking machine for about 5 minutes at 250 reciprocations per minute.

10.11 Centrifuge for 2 minutes at 3000 g.

10.12 Transfer as much as possible of the hexane-phase into a clean Kimax tube with a Pasteur pipette.

10.13 Repeat the extraction steps **10.9 - 10.11** twice.

10.14 Transfer as much as possible of the hexane-phase into the Kimax tube containing the first fraction with a Pasteur pipette.

10.15 Transfer the sample with a Pasteur pipette into a crimp vial with insert and close well. The sample is ready for HPLC analysis.

11 HPLC Analysis

11.1 Pump

The mobile phase hexane/isopropanol (**9.4**) is used with a constant flow of 0.7 mL/min.

11.2 Detector

The detector (UV 2000 nm) is set at 325 nm.

11.3 Sampler

- If an automatic sampler is available keep the temperature of the sampler tray at 4°C.
- Use amber coloured vials, otherwise cover against light.
- Set the injection volume at 50 µL.
- The tip of the sampler is rinsed after each injection with the mobile phase.
- Add a solvent blank with each run.

11.4 Integration

Calibration is executed with the working standards. A computerised programme is incorporated to facilitate automatic integration. The concentration of the different components in the samples is reported in µg/dL. In the calculation model the 'bracketing' of standards is used.

11.5 Sequence

In a run of 48 samples, 2 control samples (**12.1**) and three series of 5 standards are placed (table 1):

Table 1 Sequence of standards and samples

5 standards
2 control samples

22 subject samples
5 standards
24 subject samples
5 standards

12 Data processing and reporting

Of every sequence, a trend report is available after integration and re-integration. When the reports are being filed as 'csv'-files, the trend reports can be read in Excel. The results can be processed for presentation in tables.

13 Quality control

For the purpose of quality control (see Chapter 6), a control sample of human milk (Annex 6) is analysed in duplicate in the same run as the samples.

14 Remarks

14.1 Where the amount of available sample is small (e.g.< 200 µl) and the extraction volumes need to be scaled down accordingly, it is advisable to use positive displacement pipettes for samples and internal standards to avoid poor precision.

14.2 To convert values from conventional units (µg/dL) to S.I. units (µmol/L), multiply the conventional value by the conversion factor for retinol. This value is 0.0349.

14.3 The retinol and carotenoid content can also be expressed per gram fat in human milk (Paragraph 2.2.1.1). Paragraph 3.7 provides additional information for the fat determination in human milk.

14.4.Ten percent of the samples are analysed in duplicate.

14.5 The working standards can be stored for about 6 weeks at –20°C.

15 References

- *AOAC, Official methods of analysis. Vitamin A in milk, milk based infant formula, 992.04 50.1.02 – 50.1.03. Gaithersburg, Maryland: AOAC International, 1995.*
- *AOAC, Official methods of analysis of AOAC International. Fat in milk, Roese-Gottlieb method 905.02,. Gaithersburg, Maryland: AOAC International, 1999.*
- *Erhardt JG, Craft NE, Heinrich F and Biesalski HK. Rapid and simple measurement of retinol in human dried whole blood spot. J. Nutr 2002; 132:318-321.*
- *Frolik CA, Olson JA. Extraction, separation and chemical analysis of retinoids, In: Sporn MB, Roberts AB, Goodman DS (Eds.). The retinoids (volume 1). Orlando: Academic Press, first edition, pp 181-233, 1984.*
- *Furr HC, Barua AB, Olson JA. Analytical methods. In: Sporn MB, Robert AB, Goodman DS (Eds.). The retinoids, biology, chemistry and medicine. New York: Raven Press, second edition, pp 179-210, 1994.*
- *ISO 12080-2. Dried skimmed milk – Determination of vitamin A content. Geneva: International Organization for Standardization, first edition, 2000.*

16 Annexes *Not applicable in this standard operation procedures*

3.7 Fat determination in human milk

As mentioned previously (Paragraph 2.2.1.1) it may be necessary to express the retinol content in milk relative to the fat concentration in the milk. The fat concentration in milk can be estimated using the creamatocrit method (Lucas et al., 1978). The creamatocrit method is a useful tool when only small samples are available. A more extended/complex determination is the method of Roese-Gottlieb (AOAC 1999).

Calculation:

Fat (g/L) = (Creamatocrit (%) – 0.59) / 0.146).

References

- *AOAC, Official methods of analysis of AOAC International. Fat in milk, Roese-Gottlieb method, 905.02. Gaithersburg, Maryland: AOAC International, 1999.*
- *Lucas A, Gibbs JAH, Lyster RLJ, Baum JD. Creamatocrit: simple clinical technique for estimating fat concentration and energy value of human milk. BMJ 1978; 1:1018-1020.*

SECTION TWO: GOOD LABORATORY OPERATIONS, QUALITY CONTROL AND METHOD VALIDATION

CHAPTER 4: GOOD LABORATORY OPERATIONS

4.1 Introduction

The purpose of good laboratory operations is to identify critical points in laboratory procedures that need monitoring and for which corrections need to be made if necessary. These operations involve careful calibration and standardisation of the apparatus and procedures used, followed by scrupulous documentation and communication with all laboratory personnel involved in the analysis. Such an intensive interaction is essential as is shown in the figure below.

A standard operating procedure (SOP) is a central tool to reach the objectives of good laboratory operations. A well-designed SOP should ensure that:

- Each task is carefully planned and tested.
- Every time each individual performs the same task in the same manner with equipment calibrated in the same way.
- Each record can be tracked to verify the performance of the task and any irregularities.

In this Chapter, we describe how to prepare a SOP and we discuss practical aspects of some specific laboratory procedures.

4.2 Standard operating procedure

4.2.1 Format of a standard operating procedure

The standard operation procedure (SOP) should be adapted to the conditions in each laboratory and to any change in the analysis. Before a SOP is written, the following preparations should be made:

- Implement a coding system for all SOP's in the laboratory. This provides a way to trace a specific SOP over time.
- Design a specific format for the SOP if required. An example of a format is given in Annex 5.
- Establish a procedure for review and revision that should be in place before the SOP is made operational.
- Prepare an information system in which the latest updated SOP is accessible for everyone involved in laboratory analysis

- If any of the sections of the SOP format is not needed at that time state ' this section is not applicable in this SOP'.

4.2.2 How to prepare a standard operating procedure

4.2.2.1 Title

A consistent format should follow the following framework: Determination of {*analyte* or *measurant*} in the presence of B {*interference*} in C {*matrix*} using D {*principle*}.

4.2.2.2 Update and review summary

Every minor change in the text of a SOP should be written and authorised in the update table. All authorised changes should be made available in the current copies. Every method should be reviewed for fitness-for-purpose periodically. The summary serves as a record of the review and as a planning for the next review.

4.2.2.3 Scope

Describe the following aspects of the method if applicable and add others if appropriate:

- Analyte(s) to be determined
- Form in which the analyte(s) can be determined
- Sample matrix in which the analyte(s) should be determined
- The concentration range in which the outcome(s) of the analyte(s) are to be expected
- Known interference
- Minimum sample size needed for one analysis
- Essential techniques used by the method

4.2.2.4 Principle

Outline the principle by which the analytical technique should operate. An explanation of the principle of the calculations could be placed here.

4.2.2.5 Precautions

General precautions on existing hazards can be described here e.g.:

- Handling of samples that may contain contageous material
- Handling of solvents, reagents, standards or other risk containing material
- Handling of operating equipment
- Requirements for special handling environments (e.g. fume hood)

Detailed descriptions on safety measures or protection can also be given in relevant sections of the SOP, e.g. under 'Procedures' or 'Reagents and Materials'.

4.2.2.6 Materials

List all materials that are necessary for the determination. Number all items for later reference. Add any details such as specifications of supplier and product code if applicable.

4.2.2.7 Apparatus and equipment

The instruments should be mentioned with minimal performance requirements. Number all items to be used for later reference. Make a cross-reference to the quality control section, and if necessary to the instrument manuals of the manufacturers. For glassware, include grade where applicable.

4.2.2.8 Chemicals

All materials that are necessary for the determination should be listed. Number all items for later reference. Add any details if applicable such as:

- Specification of chemical (supplier, code number)
- Analytical grade
- Required concentration, (note w/v, w/w, v/v)
- Details of preparation
- Shell life of raw materials and prepared reagents
- Storage requirements
- Quality control materials coming from independent batches
- Labelling instructions
- Associate hazards and disposal hazards

4.2.2.9 Preparation of reagents, standards and samples

The preparation of reagents, standards and samples should be explained. All different items should be numbered for later reference. If the pre-analytical preparation of the samples appears to be extensive, a separate Chapter could be inserted. Describe the handling of the samples during the execution of the method.

4.2.2.10 Procedure

Describe the analytical procedure, cross-reference previous sections where applicable, including numbered reagents and instruments. When parameters such as time and temperature are expressed which are critical to the procedure, reference should be made to the relevant part of the quality control.

4.2.2.11 Analysis

Describe specific details on the analysis with the required apparatus and equipment.

4.2.2.12 Data processing and reporting

Lay out the formulae for calculating the results ensuring all terms are clearly defined and derived, indicate requirements for checking, cross-reference and quality control-requirements. Indicate how results should be reported, including e.g. rounding of numbers, final units, and confidence intervals.

4.2.2.13 Quality control

Explain what form the quality control takes, frequency of quality control checks during batch analysis, pass and fail criteria, and action to be taken in case of a failure. Refer to the relevant sections above. See Chapter 5 on the use of control samples in laboratory quality control. Include the equipment that needs to be calibrated, and refer to documentation of how the calibration is executed and what additional materials are needed and how often the calibration is performed.

4.2.2.14 Remarks

Add any specific remarks that cannot be placed under the other sections of the SOP.

4.2.2.15 References

Any references, which give fundamental background information on the method.

4.2.2.16 Annexes

Add annexes e.g. an example of a chromatogram of standards.

4.3 Specific laboratory procedures

4 3.1 Identification and labelling of samples

Confusing samples is a common way of getting wrong results. To prevent this type of avoidable mistake, all samples should be clearly marked with labels presenting the following information:

- Name or code of the project
- Location
- Vial number
- Type of sample (e.g. serum/plasma)
- Collection date
- Subject number
- Collection number (if more collections per subject are done)

Clearly, define before hand for each sample:

- Where to store and at which temperature
- How to protect from light, heat, vibration etc
- How long the different types of samples can be stored at the specific temperature

- Archive in a logbook, which can be computerised, where the samples should be stored and in which order

4.3.2 Calibration of pipettes

A fixed volume delivered by an adjustable pipette is of critical importance for the outcome of an analysis. Thus, a regular volume check of the used pipettes is unavoidable. The frequency of the volume check depends on the frequency of usage. The results of a calibration exercise should be entered in a logbook for each pipette present. An example of a standard operating procedure (SOP) for the calibration of adjustable pipettes is described below.

---

1 Standard operating procedure for the calibration of adjustable pipettes

2 Update and review summary

UPDATE

NR	Prepared by	Section	Nature of amendment	Date	Authorisation
(1)		3.4	Change flow rate	12-10-2001	

REVIEW

Date	Prepared or reviewed by	Outcome of review	Next review date	Authorisation
			25-03-2002	

3 Scope

This SOP covers the calibration of an adjustable pipette (250 µL).

4 Principle

A specific volume of water is taken with the pipette and weighted (W) on an analytical balance. The water temperature (T) is measured. With the Density (D) and weight (W) of the water the volume is calculated: V = W / D at that specific temperature. This calculated volume is compared with the expected volume.

5 Precautions

Eliminate draughts of air and temperature changes before the calibration of the pipettes.

6 Materials

Beaker

7 Apparatus and equipment and apparatus

- Analytical balance (0.1 mg)
- Thermometer
- Adjustable pipette (e.g. 50-250 µl)

8 Chemicals

Distilled water

9 Preparation of standards, reagents and samples

Not applicable in this standard operating procedure

10 Procedure

- Measure the temperature of a large volume of pure water to the nearest degree Celsius and record on the calibration form (Table 4.1)
- Place a beaker on the balance and tare the balance
- Note the number of the pipette on the calibration form (Table 4.1)
- Pipette an amount of water (50 µL) into the beaker, record weight (W), and tare again
- Repeat the previous steps 9 times
- Repeat previous steps for another volume: 250 µL

11 Analysis

Not applicable in this standard operating procedure

12 Data processing and reporting

- Obtain the Density of the water from Table 4.1 (g/mL)
- Calculate the volume from weight and Density as V = W / D (mL)
- Calculate the mean volume, standard deviation and coefficient of variation (%)
- Write the results on the calibration form

13 Quality Control

The pipette calibration form should be used to check if the deviation is acceptable. If the outcome is not within the limits, the pipette should be adjusted as instructed (see manufacturers' manual) or should be presented for check through outside services.

14 Remarks

Not applicable in this standard operating procedure

15 References

Not applicable in this standard operating procedure

16 Annex: Table 4.1 Example of a form for pipette calibration

CALIBRATION FORM

CALIBRATION OF PIPETTES - weight of volume
TYPE OF PIPETTE: EPPENDORF RV-PIPETTE 50-250 µL, SERIALNUMBER 061189.

TOLERANCE CHECK	MEASURED WEIGHT (mg)							
Date	Jun-95		Jan-96		Jul-96		Aug-97	
Temperature (°C)	24		19		24		22	
Contents pipette (µl)	50	250	50	250	50	250	50	250
1	50.2	248.6						
2	50.0	248.8						
3	50.0	247.9						
4	49.9	248.4						
5	49.8	247.9						
6	49.8	248.0						
7	50.1	248.8						
8	49.8	248.0						
9	49.9	248.4						
10	50.1	249.1						

HANDBOOK OF CHEMISTRY AND PHYSICS 80TH ED. 1999-2000.

Temperature (°C)	Density (g/ml)	Temperature (°C)	Density (g/ml)
15°C	0.9988	23°C	0.9975
16°C	0.9989	24°C	0.9973
17°C	0.9988	25°C	0.9970
18°C	0.9986	26°C	0.9968
19°C	0.9984	27°C	0.9965
20°C	0.9982	28°C	0.9962
21°C	0.9980	29°C	0.9959
22°C	0.9978		

TABLE 2: CALIBRATION OF PIPETTES - calculated volume
TYPE OF PIPETTE: EPPENDORF RV-PIPETTE 50-250 µL, SERIALNUMBER 061189.

TECHNICAL DATA	Imprecision	Inaccuracy
	50 µl : < 0.3%	± 1.4%
	250 µl : < 0.2%	± 0.6%

TOLERANCE CHECK	CALCULATED VOLUME (µL)							
Date	Jun-95		Jan-96		Jul-96		Aug-97	
Tempeature (°C)	24		19		24		22	
contents pipette (µl)	50	250	50	250	50	250	50	250
1	50.3	249.3						
2	50.1	249.5						
3	50.1	248.6						
4	50.0	249.1						
5	49.9	248.6						
6	49.9	248.7						
7	50.2	249.5						
8	49.9	248.7						
9	50.0	249.1						
10	50.2	249.8						
Number of measurements	10	10						
Mean (µl)	50.1	249.1						
SD	0.1	0.4						
% CV (Measured)	0.3	0.2						
% CV (Max acc).	0.3	0.2						
% System.Error (Measured)	0.2	0.4						
% System.Error (Max accept)	1.4	0.6						
TEST Precision	YES	YES						
TEST Accuracy	YES	YES						
ACTION Precision	NONE	NONE						
ACTION Accuracy	NONE	NONE						

4.3.3 Calibration of analytical balances

An analytical balance is used for the preparation of reagents or weighing of samples. In addition, it is also used to calibrate pipettes (**4.3.2**). Therefore, it is necessary to assure the balances are also checked. Calibration weights should be used to check the balance monthly or when the balance has been moved (Burtis et al. 1994). The results of the calibration exercise should be documented. An example of a standard operating procedure (SOP) for the calibration of analytical balances is described below.

---

1 Standard operating procedure for an analytical balance

2 Update and review summary

UPDATE

NR	Prepared by	Section	Nature of amendment	Date	Authorisation
(1)		1.0	Change of balance (type)	12-11-00	

REVIEW

Date	Prepared or reviewed by	Outcome of review	Next review date	Authorisation
			25-03-2002	

3 Scope

This standard operating procedure is used to check an analytical balance (0.1 mg), which is used for sample and standard weighing.

4 Principle

The analytical balance is checked monthly with the use of standard weights. The measured weight is compared with the expected weight for exceeding tolerance limits.

5 Precautions

Before proceeding the weighing any spilled substance, vibrations or temperature fluctuations should be eliminated. An anti-static brush should be used to reduce static electricity.

6 Materials

Not applicable in this standard operating procedure

7 Apparatus and equipment

- Standard weights: weight range for the use of the balance e.g. 100 mg and 500 mg
- Analytical balance

8 Chemicals

Not applicable in this standard operating procedure

9 Preparation of standards, reagents and samples

Not applicable in this standard operating procedure

10 Procedure

- Note the number of the analytical balance on a calibration form.
- Tare the balance.
- Place one of the two weights on the balance and record the weight on a calibration form.
- Repeat the above procedure nine times.
- Repeat previous two steps with a different calibration weight.

11 Analysis

Not applicable in this standard operating procedure

12 Data processing and reporting

- Calculate mean weight, standard deviation and coefficient of variation for each level.

13 Quality control

- Precision and bias should be within the manufacturers' specifications.
- If the deviation is not acceptable, arrange for the balance to be checked by an outside service.
- The balance and the standard weights should be checked annually by an external expert.
- The results of all weighing should be recorded in a (computerised) logbook of each balance

14 Remarks

Not applicable in this standard operating procedure

15 References

- *CRC Handbook of Chemistry and physics.* Lide DR (Eds.). CRC Press, 80th edition 1999-2000. Boca Raton

16 Annexes

Not applicable in this standard operating procedure

4.3.4 Freezers and refrigerators

To assure stability of samples during transport, handling or storage of samples, the temperature of freezers and refrigerators need to be monitored. By monitoring the temperature, appropriate action can be taken in case of an alarming situation.

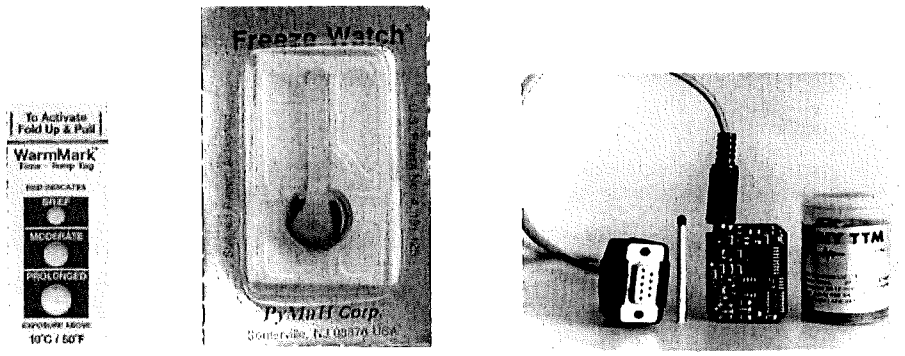


Figure 4.1: Temperature indicators: Warmmark indicator, Freeze watch indicator and Tiny TTM Auto data temperature logger.

Small temperature indicators (Figure 4.1) can be used to monitor the temperature of a cold chain (Hanjeet et al. 1996). An indicator should be placed together with samples in coolboxes. Limits should be set for acceptance or for refusal of samples which, have been submitted to an undesirable temperature change. Information on how to set up a monitoring system for temperature can be found at the WHO-web site:

- http://www.who.int/vaccines-documents/docspdf00/www518.pdf ***or***
- http://www.who.int/vaccines-documents/docspdf/www9804.pdf.

To avoid deterioration during storage of samples appropriate precautions should be taken:

- Monitor interior temperature of a freezer daily by plotting temperature against time in a graph
- Give limits and instructions on how to proceed when the temperature is out of range
- Prepare guidelines on how to proceed when the power supply of the freezer (or the refrigerator) is not stable
- Document any changes of temperature for samples on the work sheet for samples (Annex 4)
- A freezer-alarm system should be installed that will monitor undesirable temperature changes and report any irregularities in the power supply

4.3.5 Water bath and dry-block heater

The temperature of a water bath and dry-block heater needs to be monitored regularly for the adjusted temperature. If the temperature is too high, the tuning of the water bath or dry-block evaporator should be adapted. Check the temperature again before the start of each analysis. Document the temperature in the logbook of the apparatus.

4.3.6 Spectrophotometer

General recommendations for the use of a spectrophotometer:

- The spectrophotometer should be switched on at least 30 minutes before calibration.
- Cuvettes used for detection in the UV region (wavelength <380 nm) are very sensitive for fingerprints on the outside and scratches as these can absorb light significantly. Always check the cuvettes for scratches and fingerprints. Only hold cuvettes on their upper edge. The outside of cuvettes should be cleaned and wiped free of fingerprints before use.
  Fill all cuvettes with distilled water and measure the absorbance of each against the reference blank over the wavelength in use. This should be essentially zero.
- Maintenance and quality control procedures should be performed according to the manufacturer's instructions.
- The spectrophotometer should be covered with a plastic cover when not in use.

Several calibration procedures, provided by the manufacturer, for the spectrophotometer must be performed regularly to check its performance:

- Stray light calibration: Before use check the light flow in the spectrophotometer: The transmittance (%) should be zero when the light path is blocked. Adjust if necessary.
- For the verification and calibration of the wavelength scale of the spectrophotometer, a holmium oxide glass can be scanned over the wavelength of 280-650 nm. This Standard Reference Material is available from the National Institute of Standards and Technology (NIST SRM 2034), accessible at http://srmcatalog.nist.gov/srmcatalog/certificates/2034.pdf (Figure 4.2). Some companies sell holmium-oxide wavelength calibrators traceable to NIST (http://www.labsphere.com/products).

The spectrum measured should be compared with the certified values for tolerance limits and should be documented.

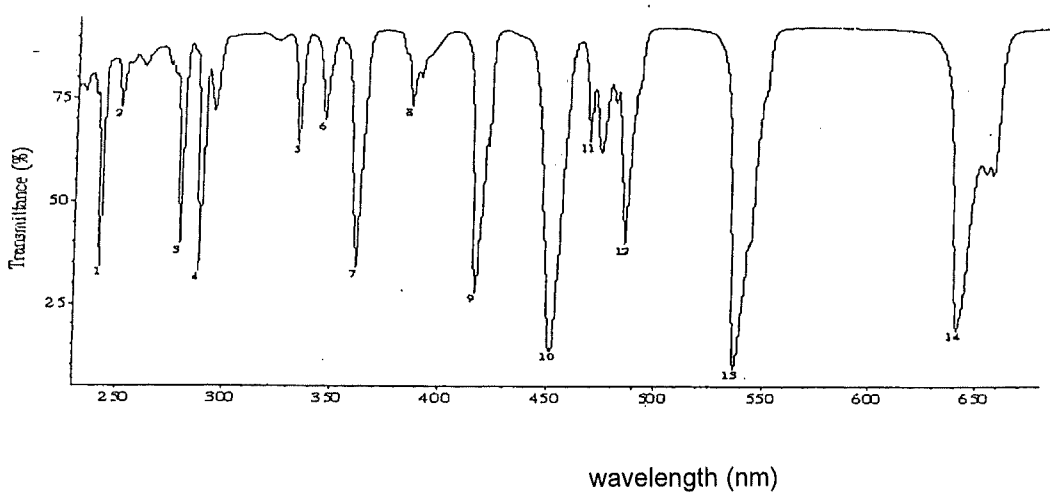


Figure 4.2 Spectral transmittance at 1 nm spectral bandpass of a 4% (w/v) solution of holmium oxide in 10% (w/v) perchloric acid (NIST SRM 2034).

4.3.7 High performance liquid chromatography

Calibration of HPLC systems depend on the type of system. Suitability of the system should be established before standards are analysed. Most HPLC systems have an automated system suitability test with detailed information in the manufacturers' manual.

A new column should be installed if the efficiency of the column resolution is no longer satisfactory. The efficiency of a new column can be assessed by measuring the number of theoretical plates (N). This should comply with the specifications provided by the manufacturer.

The number of theoretical plates (N) is calculated as follows:

$$N = 16 (t_R / w_b)^2$$

Where,

t_R = *Peak elution time,*

w_b = *Peak width at base,*

with the assumption that the capacity factor (k): 2< (k) < 10.

Before using a new column for sample analysis, check the efficiency of the separation of the new column for the compounds of interest with the peak resolution,

$$R_s = (t_{r2} - t_{r1}) / w_{b2}$$

Where,

R_s = *Peak resolution, which should be ≥ 1.5,*

t_{r2} = *Retention time peak 1,*

t_{r1} = *Retention time peak 2,*

w_{b2} = *Peak width at the base of peak 2,*

with the assumptions that $t_{r2} > t_{r1}$, $w_{b1} \approx w_{b2}$ and w_{b1} is peak at base 1 (Ettre 1993).

For troubleshooting of specific problems with the HPLC-system, check the web site http://kerouac.pharm.uky.edu/ASRG/HPLC/hplcmytry.html

4.4 Health and safety aspects

Working in a laboratory means exposure to risks. These risks bear upon the use of chemicals, contageous biological specimens, and materials such as glass that can cause injuries. In every laboratory, the staff should be acquaintanced with clear safety instructions.

General precautions in order to reduce risks include:

- Do not eat and drink in the laboratory
- Work neatly: use gloves, closed laboratory coat and safety glasses
- Clean working area after use
- Clean taps, centrifuges, handles of refrigerators etc. regularly
- Prevent inhalation of vapours: use fume-hood where applicable
- Do not pipette by mouth
- Use unbreakable materials where applicable
- Do not transport tubes or bottles in your hand but always in a tray
- Store and dispose of waste properly
- Do not work alone in the laboratory
- Know what to do in a hazardous situation
- Use appropriate disposal containers for broken or disposable glassware

4.4.1 Chemical hazards

Labels on containers provide some information regarding the properties of chemicals. Information on physical properties can be found on safety cards in several databases:

- http://www.cdc.gov/niosh/ipcs/icstart.html or
- http://www.camd.lsu.edu/msds/jssearch.htm

Use appropriate disposal containers for different types of chemicals.

4.4.2 Biological hazards

Working with biological materials can lead to risk of infection with hepatitis B or HIV or other known or unknown agents. Be aware of these risks and take necessary precautions:

- Avoid contact with all biological specimen specimens: always use gloves
- Wash hands regularly

- Use unbreakable materials where possible
- Use disinfectants (alcohol 70% v/v,) for disinfecting centrifuges, handles, refrigerators etc
- Avoid needle cuts; dispose needles in disposal container; never recap
- Collect infected disposal (such as cotton and plasters) in bags
- Collect remainders of specimen and dispose of safely
- Install and use a safe waste disposal system
- When working with biological specimens, protect yourself against hepatitis B infection through vaccination

References:

- *Burtis CA, Ashwood ER Eds. Tietz textbook of clinical chemistry. Second edition.Philadelphia, USA: WB Saunders Company, 1994.*
- *Tetra LS. IUPAC, International union of pure and applied chemistry. Nomenclature for chromatography. Pure & Applied Chem 1993; 65:819-72.*
- *Hanjeet K, Lye MS, Sinniah M, Schnur A. Evaluation of cold chain monitoring in Kelatan, Malaysia. Bulletin of the WHO 1996; 74:391-397.*

CHAPTER 6: METHOD VALIDATION

6.1 Introduction

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated:

- Before their introduction into routine use.
- Whenever the conditions, for which the method has been validated, change.
- Whenever the method is changed and when this change is outside the original scope (Hubert 1998).

A laboratory is responsible for ensuring that a method is adequately validated and that the validity is demonstrated in the environment of the laboratory. The laboratory should decide which method performance parameters (attributes) need to be characterised in order to validate the method. This decision should be based on relevant performance characteristics of the method. There is considerable agreement among minimal requirements with respect to the attributes of a method that need validation (Hubert 1998). In this Chapter, some of these attributes are illustrated with practical examples. The data in these examples are *fictive data* and are shown only for illustration of the calculation procedures. An extensive laboratory guide to method validation and related topics is available from: http://www.eurachem.bam.de.

6.2 Recovery

The failure to determine all of the analyte present in a sample may reflect an inherent problem in the method. This could be due to either problems in detecting the analyte or problems with extraction efficiency or sample cleanup. The fraction or percentage of an analyte added to a test sample that can be 'recovered' in the analysis is called recovery (Eurachem, 1998).

Recovery expressed as a percentage is calculated as follows:

$$R = ((CF-CU)/CA) \times 100\%$$

Where,

CF = concentration of analyte measured in the fortified sample

CU = concentration of analyte measured in the un-fortified sample

CA = concentration of analyte added in the fortified sample

Recovery should be measured for three levels of addition (50%, 100% and 200%) and should be replicated five or ten times for each level of addition. Consider the following data (Table 6.1), where 100% of the analyte is added. Average recovery (%R) at 100% level of addition is 94.2%.

Table 6.1 Recovery of analyte added to a sampple

Run	CU* (μmol/L)	CF (μmol/L)	CA (μmol/L)	R(%)
1	11.1	20.8	10.0	97.0
2	11.2	19.7	10.0	85.0
3	10.2	21.5	10.0	113.0
4	11.6	20.3	10.0	87.0
5	11.1	20.5	10.0	94.0
6	11.4	20.3	10.0	89.0
7	11.2	19.5	10.0	83.0
8	10.9	21.5	10.0	106.0
9	11.0	20.1	10.0	91.0
10	10.7	20.4	10.0	97.0

*) CU = concentration of analyte measured in un-fortified sample
C = concentration of analyte measured in fortified sample
CA = concentration of analyte added in fortified sample
R = recovery

6.3 Precision

Precision is the closeness of agreement between independent test results obtained under stipulated conditions (Eurachem, 1998). It depends only on the distribution of random errors and is not related to the true value. The measure of precision is usually expressed in terms of *imprecision* and computed as a standard deviation or coefficient of variation from the test result. Usually two types of precision are distinguished: repeatability or within laboratory precision, and reproducibility or between-laboratory precision. The latter term refers to agreement between test results obtained on identical samples in different laboratories with different operators using different equipment. Thus, reproducibility can be determined in proficiency studies (Paragraph 5.3.1).

Repeatability is determined by making replicate assays of the same analytical sample (e.g. a control sample) according to a stated replication design (e.g. between days, within series) under the same measurement conditions. Components of within laboratory precision are within-run (or within-day) and between-run (or between-day) variability.

The relationship among within-run and between-run variability is as follows:

$$s^2_{total} = s^2_{between} + (s^2_{within} / k)$$

Where,

s_{total}	=	total random measurement error (or imprecision)
$s_{between}$	=	between-run standard deviation
s_{within}	=	within-run standard deviation
k	=	number of replicates in a run

These components can be calculated from the analysis of control samples (Table 6.2) with N = 20 runs under repeatability conditions. These data are essentially the same as used to construct quality control charts in Chapter 5.

Table 6.2 Results of control samples

Run (day)	x_1*	x_2	$\bar{x}$	$s\ x$	$s^2\ x$
1	1.80	1.79	1.79	0.0049	0.0000
2	1.83	1.79	1.81	0.0241	0.0006
3	1.85	1.83	1.84	0.0101	0.0001
4	1.85	1.94	1.90	0.0570	0.0032
5	1.86	1.88	1.87	0.0115	0.0001
6	1.88	1.83	1.86	0.0385	0.0015
7	1.84	1.91	1.87	0.0441	0.0019
8	1.80	1.85	1.83	0.0301	0.0009
9	1.91	1.92	1.91	0.0115	0.0001
10	1.90	1.94	1.92	0.0287	0.0008
11	1.86	1.86	1.86	0.0000	0.0000
12	1.89	1.91	1.90	0.0115	0.0001
13	1.83	1.89	1.86	0.0441	0.0019
14	1.90	1.91	1.90	0.0129	0.0002
15	1.86	1.86	1.86	0.0014	0.0000
16	1.91	1.86	1.89	0.0325	0.0011
17	1.83	1.84	1.83	0.0119	0.0001
18	1.91	2.00	1.95	0.0629	0.0040
19	1.94	1.98	1.96	0.0311	0.0010
20	1.94	2.00	1.97	0.0395	0.0016

*) $x_{1,2}$ = control values, $\bar{x}$ = run or daily mean, s = variability

From the data presented in Table 6.2, the overall mean, $\bar{\bar{x}}$, the standard deviation of the run means, $s_{\bar{x}}$ = 0.05, the within-run standard deviation, s_{within}, and the between-run standard deviation, $s_{between}$, is calculated as follows.

$\bar{\bar{x}}$ $= \Sigma\ \bar{x} / N$ $= 1.88$

Where,

N = number of runs

$$s_{\xi} = \sqrt{\frac{\Sigma (\bar{x} - \bar{\bar{x}})^2}{N-1}}$$

$s_{within} = \sqrt{\Sigma s^2_x / N}$ $= 0.03$

$s_{between} = \sqrt{s^2 \bar{x} - (s^2_{within} / k)}$ $= 0.04$

k = number of controls in each run

The equation $s^2_{total} = s^2_{between} + (s^2_{within} / k)$, can be expressed as coefficients of variation:
$\%CV^2_{total} = \%CV^2_{between} + (\%CV^2_{within} / k)$.

Where,

$\%CV_{within}$ (within-run coefficient of variation) $= (s_{within} / \bar{\bar{x}}) \times 100\% = 1.6\%$

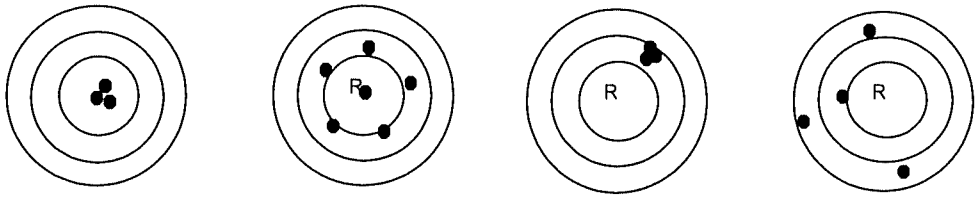
$\%CV_{between}$ (between-run coefficient of variation) $= (s_{between} / \bar{\bar{x}}) \times 100\% = 2.3\%$

The total random measurement error can now be calculated for single, duplicate or triplicate measurements:

- Imprecision based on single measurement of a sample $\sqrt{2.3^2 + 1.6^2} = 2.8\%$
- Imprecision based on duplicate measurement of a sample: $\sqrt{2.3^2 + (1.6^2)/2} = 2.6\%$

6.4 Accuracy

Accuracy is the closeness of the analytical value to the 'true value' (Eurachem 1998).
The difference between accuracy and precision is demonstrated in Figure 6.1.



I	II	III	IV
Accurate and precise	Accurate, not precise	Precise, not accurate	Not accurate, not precise

Figure 6.1 Accuracy and precision

The concept of 'true value' causes some difficulty, since some researchers argue that a true value can never be known. Terms often referred to as accuracy are 'trueness' and lack of 'bias'. Bias is the total systematic error and refers to lack of accuracy. In contrast, the random error refers to lack of precision (Paragraph 6.3). There may be one or more systematic error components contributing to the bias.

Analysing a sample with a known concentration, for example, a Certified Reference Material, can be used to assess accuracy. The measured value should be compared with the true value, supplied with the Certified Reference Material.

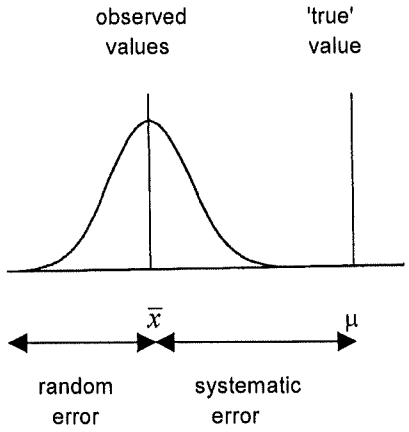


Figure 6.2 Random and systematic error ($\bar{x}$ = found mean, μ = expected mean).

A user-friendly approach, adapted from Jorhem et al. (2001), for the evaluation of results, derived from the analysis of Certified Reference Materials, is based on Z-scores. A Z-score shows directly how the laboratory result compares with the certified value of the Reference Material.

The formula for the Z-score is as follows: $$\text{Z-score} = \frac{(\bar{x}_{\text{found}} - \bar{x}_{\text{certified}})}{\sqrt{[s^2_b + (s^2_w / k)] / n}}$$

Where,

$\bar{x}_{\text{found}}$ = the mean result found by the analyst

$\bar{x}_{\text{certified}}$ = the certified mean

s^2_b = between-run variance (available from the analysis of in-house quality control

samples)

s^2_w = within run variance (available from the analysis of in-house quality control samples)

k = number of replicates of the CRM analysed per run

n = number of runs performed for the analysis of CRM

Table 6.3 gives the Z-scores for the analysis of three levels of retinol in SRM 968b. The following assumptions are made: s_b = 2.3%, s_w = 1.6%, k = 1, and n = 2. The parameter s_b and s_w were derived from Table 5.1. Z-scores smaller than 2 are usually considered acceptable, Z-scores between 2-3 are questionable, and Z-scores larger than 3 not satisfactory.

Table 6.3 Traceability of laboratory performance to Standard Reference Material (SRM 968b) provided by the National Institute of Standards and Technology

	Retinol		
	Certified value	Measured	Z-score
	(μmol/L)		
Low level retinol	1.03	1.01	-1.0
Medium level retinol	1.79	1.90	3.1
High level retinol	3.11	3.23	1.9

6.5 Sensitivity

Sensitivity is the change in the response of a measuring instrument divided by the corresponding change in stimulus. In high performance liquid chromatography analysis, the sensitivity is the signal output per unit concentration of a substance in the mobile phase entering the detector (Ettre, 1993). An example of the calculation of sensitivity (S) is shown in Table 6.4.

Table 6.4 Sensitivity (S) in a HPLC system

Concentration standard (A) (μmol/L)	Detector response (B) (μAU)	Sensitivity (B/A) (μAU/μmol/L)
1.0	162	162
2.0	322	161
3.0	459	153
4.0	696	174
5.0	804	161
6.0	1064	177
8.0	1134	142
10.0	1784	179
20.0	3702	185

Mean sensitivity (S) (μAU/μmol/L)	166

6.6 Limit of detection

The limit of detection (D) of a sample component in the mobile phase is a detector response that equals twice the noise level (Ettre, 1993). The detection limit can be calculated from the measured sensitivity (S) and noise (N). The noise (N) is the amplitude expressed in absorbance units of the envelope of the baseline that includes all random variations of the detector signal during one minute.

Thus: $D = 2N / S$.

Where,

N = 80 μAU (See Figure 6.3 for the measurement of the noise (N) from the baseline amplitude).

S = 166 μAU / μmol/L (See Table 6.4)

D = 2 x 80/166 = 0.96 μmol/L

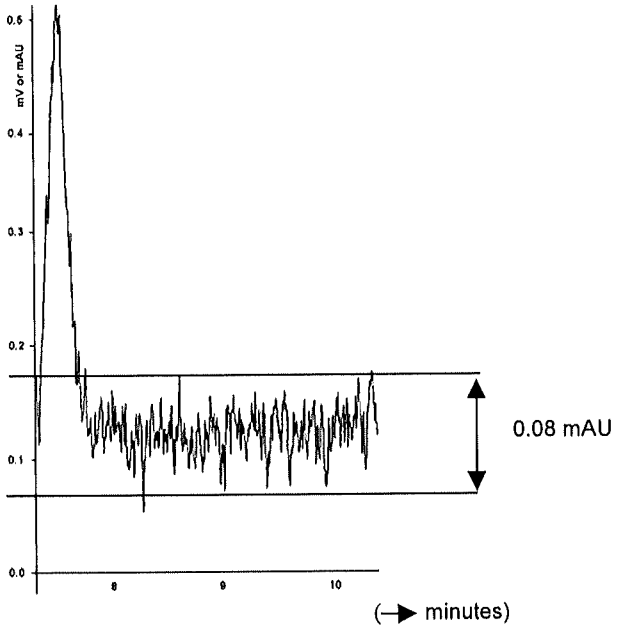


Figure 6.3 Noise measurement

6.7 Limit of quantitation

The limit of quantitation (Q) is the lowest level of the analyte that reliably can be quantified from zero (Wernimont and Spendly, 1987). As a rule of thumb in chromatography, the limit of quantitation is about twice the limit of detection, using the definition of detection limit described in Paragraph 6.6 (Ettre 1993). So the limit of quantitation in our example is: 2 x 0.96 = 1.92 μmol/L.

A slightly different approach makes use of the signal and standard deviation of a signal blank. Figure 6.4 represents the normal distribution of the blank signal, with expected mean μ_{bl}, and standard deviation σ_{bl}. The limit of detection in this example can be defined as $D = \mu_{bl} + 6\ \sigma_{bl}$ and the limit of quantitation as $Q = \mu_{bl} + 10\ \sigma_{bl}$ (Miller 1993).

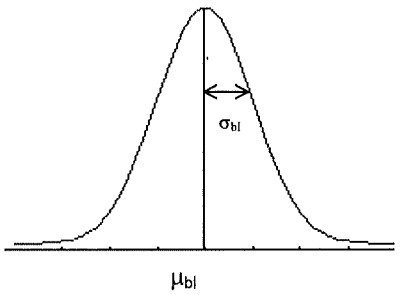


Figure 6.4: Normal distribution (μ_{bl} = blank signal and σ_{bl} = standard deviation).

6.8 Linearity

The linear range represents the range of concentrations of a substance for which the sensitivity of the detector is constant within a specified variation, usually ± 5 percent (Ettre, 1993). Linearity is determined by a series of injections of five or more standards whose concentrations span 80-120% of the expected concentration range.

Table 6.5: Detector response and concentration of standards

Concentration (μmol/L)	Detector response (μAu)	Sensitivity (μAu/(μmol/L)
2	0.077	0.039
6	0.241	0.040
10	0.392	0.039
15	0.602	0.040
20	0.783	0.039
25	1.013	0.040
30	1.187	0.040
35	1.372	0.039
40	1.599	0.040
45	1.802	0.040
50	2.110	0.040
60	2.280	0.038
70	2.590	0.037
80	2.800	0.035
90	3.060	0.034
100	3.240	0.032

There are several ways to present linearity of a detector's response, mathematical and graphical. Here we present a graph by plotting the detectors sensitivity against the amount injected. The upper limit of linearity can be graphically established as the amount at which the deviation exceeds the constant response by 5%. The level of constant response (Rc) and deviations can be calculated from the concentrations, areas and the calculated sensitivity (Table 6.5). The level of constant response (Rc) is 0.039 and the ± 5% deviations from the level of constant response are 0.037 and 0.041 respectively.

The data plotted in Figure 6.5 show a random pattern around the line of constant response. In this example, the concentration of 70 μmol/L exceeds the 5% level of deviation. Therefore, the detectors response is linear until 60 μmol/L.

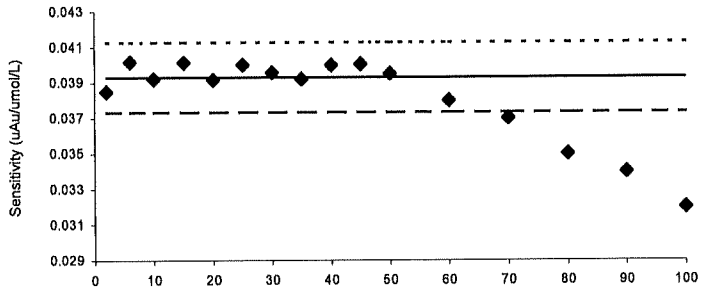


Figure 6.5 Linearity of the detector response
(Rc constant response; ♦ sensitivity; —— Rc; — — 0.95*Rc; - - - - - - 1.05*Rc)

References

- *Ettre LS. Nomenclature for chromatography. Pure and Appl Chem 1993; 65:819-872.*
- *Eurachem. The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. Teddington, Middlesex, UK: Eurachem, 1998.*
- *Huber L. Validation of analytical methods: review and strategy. LC-GC International 1998; 2:96-105.*
- *Jorhem L, Engman J, Schröder T. Evaluation of results derived from the analysis of certified reference materials –user-friendly approach based on simplicity. Fresenius J Anal Chem 2001; 370:178-82.*
- *Miller JC, Miller JN. Statistics for analytical chemistry. Ellis Horwood PTR Prentice Hall Analytical Chemistry Series. New York: Ellis Horwood, 1993.*
- *Wernimont GT, Spendley W. Use of statistics to develop and evaluate analytical methods. Arlington VA, USA: Association of Official Analytical Chemists, 1987.*

ANNEX 1:
EFFECT OF PRE-ANALYTICAL VARIATION ON VARIABILITY IN TEST RESULTS

The importance of standardised collection, handling, transport and storage of samples can be illustrated with the following example. Consider the following statistical relationship between the total variability in a test result and the biological variation, pre-analytical variation and analytical variation:

$$CV^2_t\ (\%) = CV^2_b\ (\%) + CV^2_p\ (\%) + CV^2_a\ (\%)$$

where,

$CV^2_t\ (\%)$ = total variation
$CV^2_b\ (\%)$ = biological variation
$CV^2_p\ (\%)$ = pre-analytical variation
$CV^2_a\ (\%)$ = analytical variation

Assume the retinol concentration in a blood sample of a subject is 0.50 µmol/L. Further assume an analytical variation of 6% and a biological within-subject variation, including pre-analytical variation, is 25% (de Pee et al.1997). The 95% confidence interval for this single test result equals: $0.50 \pm 2 \times 26\%$ (where, $26\% \approx \sqrt{[6^2 + 25^2]}$).

Biological variation perse is a given property of the metabolism of the nutrient and cannot be reduced analytically. It can be reduced by increasing the number of blood samples taken on different occasions. One type of variation, included in biological variation that can be reduced is pre-analytical variation. Rigid standardisation of the pre-analytical stage may reduce the combined biological within-subject and pre-analytical variation from 25% to approximately 15% (Ricos et al. 1999). Taking two independent blood samples, under the rigid standardised conditions, may give a 95% confidence interval of
$0.50 \pm 2 \times 12\%$ (where, $12\% \approx \sqrt{6^2 + 15^2/2}$). This is a drastic improvement compared with the previous result Note that reduction of the analytical variation, at this level of biological and pre-analytical variation, hardly affects precision of the measurement.

References

- *de Pee S, Yuniar Y, West CE, Muhilal. Evaluation of biochemical indicators of vitamin A status in breast-feeding and non-breast-feeding Indonesian women. Am J Clin Nutr 1997; 66:160-167.*
- *Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. Current databases on biologic variation: pros, cons and progress. Scand J Clin Lab Invest 1999; 59:491-500.*

ANNEX 2:

WORKSHEET STANDARD OPERATING PROCEDURE SERUM/PLASMA

Date: Project Name:

Name analyst: Project Number:

- τ Print worksheet
- τ Prepare standards
- τ Prepare reagents
- τ Switch on water bath at 35°C
- τ Pipette the sample
- τ Add sodium chloride
- τ Add internal standard with ethanol + BHT and mix
- τ Add hexane and shake for 5 minutes
- τ Centrifuge for 2 minutes
- τ Transfer hexane in clean tube
- τ Repeat hexane extraction
- τ Transfer hexane into tube with Hexane fraction
- τ Purge nitrogen to evaporate hexane at 35°C
- τ Add methanol-tetrahydrofuran and close and mix
- τ Transfer sample in crimpvial and close well
- τ Prepare HPLC for analysis
- τ Inject sample into HPLC
- τ Calculate and report results

ANNEX 3:

WORKSHEET STANDARD OPERATING PROCEDURE MILK

Date: Project Name:

Name analyst: Project Number:

- τ Print worksheet
- τ Prepare standards
- τ Prepare reagents
- τ Transfer the milk sample
- τ Add ammonia and mix
- τ Add ethanol and mix
- τ Switch on water bath at 35°C
- τ Add diethyl ether
- τ Add petroleum ether and shake for 5 minutes
- τ Centrifuge for 2 minutes
- τ Transfer sample into tube
- τ Repeat petroleum ether extraction
- τ Transfer sample into tube
- τ Add KOH/pyrogallol
- τ Purge with nitrogen, close tubes and mix for 3 hours
- τ Add water
- τ Add hexane
- τ Shake for 5 min
- τ Centrifuge for 3 min
- τ Transferring hexane in clean tube
- τ Repeat hexane extraction
- τ Add hexane in tube
- τ Evaporate under nitrogen at 35°C
- τ Add methanol/tetrahydrofuran (3:1)
- τ Close tube and mix
- τ Transfer sample into crimpvial and close
- τ Prepare HPLC for analysis
- τ Inject samples into HPLC
- τ Calculate and report the results

ANNEX 4

WORK SHEET OF SAMPLES

Run date
Name analyst

Project name
Project number

Notes

Tube/Sample number	Subject no	Tray nr/vial no	Remark
1	st	A01	
2	st	A02	
3	st	A03	
4	st	A04	
5	st	A04	
6	st	A05	
7	301	etc.	
8	302		
9	303		
10	304		
11	305		
12	etc.		
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ANNEX 5:

FORMAT OF A STANDARD OPERATING PROCEDURE

1. Title
2. Update and review summary

UPDATE

Nr	Prepared by	Section	Nature of amendment	Date	Authorisation
(1)		3.4	Change flow rate	12-10-00	

REVIEW

Date	Prepared or reviewed by	Outcome of review	Next review date	Authorisation
			25-03-2002	

3. Scope
4. Principle
5. Precautions
6. Materials
7. Apparatus and equipment
8. Chemicals
9. Preparation of standards, reagents and samples,
10. Procedure
11. Analysis
12. Data processing and reporting
13. Quality control
14. Remarks
15. References
16. Annexes

Annex 6:

PREPARATION OF CONTROL SAMPLES:

1) Serum/plasma control samples

- Determine the required concentration level(s) of the analyte in plasma/serum.
- Determine the amount of serum/plasma necessary for the determination
- Assess the amount of control sample needed for a longer period
- Estimate the amount of blood, that can be drawn from one person according to the medical ethical rules of the institute
- Find sufficient blood donors, the number depending on the above mentioned conditions
- Inform each donor about the reasons for blood collection
- Ask for signed informed consent from each donor
- Collect blood from each subject and prepare serum/plasma according to the protocol described in Chapter 2
- Gently mix the serum/plasma samples from each donor to make a homogeneous pool
- Determine the concentration of the analyte in the pool
- For low-level pools: dilute one volume of serum/plasma with two volumes of an aqueous solution containing sodium chloride (9 g/L) and bovine serum albumin (30 g/L)
- Aliquot the pool sample into cryogenic vials and freeze at $\leq -20°C$.

2) Dried blood spot control samples

- Determine the amount of dried blood spots necessary for the determination
- Assess the quantity needed for a longer period
- Estimate the amount of blood, that can be drawn from one person according to the medical ethical rules of the institute
- Find blood donors, the number depending on the above mentioned considerations
- Inform each donor about the reasons for blood collection
- Ask for signed informed consent from each donor
- Collect blood from each subject via venipuncture into lithium-heparin tubes according to the protocol described in Chapter 2.
- Transfer blood (20 µl) into every circle of the collection paper and allow to dry thoroughly according to the protocol described in Chapter 2.
- Store the dried blood spots as described in Chapter 2

3) Human milk control samples

- Determine the required concentration level in the milk
- Determine the amount of human milk necessary for the determination
- Assess the amount of control sample needed for a longer period
- Estimate the amount of human milk, that can be collected from one person according to medical ethical rules
- Find sufficient milk donors, the number depending on the above mentioned considerations
- Inform each donor about the reasons of the milk collection
- Ask for signed informed consent from each donor
- Collect milk from each subject according to the protocol described in Chapter 2
- Gently mix the milk samples from each donor to make a homogeneous pool
- Aliquot the pooled milk into freezer proof vials and freeze at $\leq$ -20°C