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 determinations) 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.

![Diagram of venipuncture sites](image)

**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 \( \leq -20^\circ\text{C} \), 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 \( \leq -20^\circ\text{C} \) 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 \( \leq -70^\circ\text{C} \) 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 years 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: at 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 \( \leq -20^\circ C \) 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 \( \beta \)-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, pooled 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 ºC 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 Woltit, 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).
Collection, handling, transport and storage of samples

1. Collect the milk from the breast in a collection container (25-50 mL)

2. Use freezer-proof storage tubes to store 2-5 mL homogeneous milk.

**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 ≤ -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 ≤ -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 ≤ -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

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