

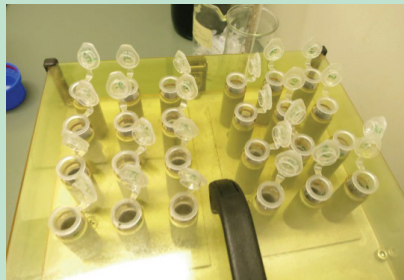
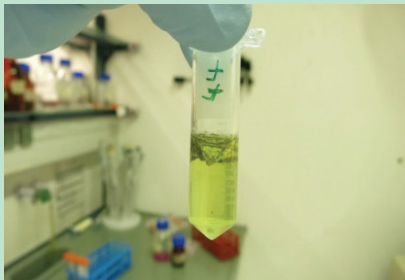
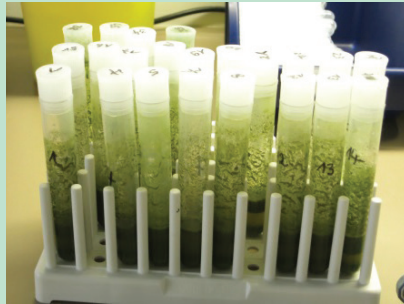
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Training Manual for the Analysis of Fe and Zn in Fresh Cassava Roots and Products



E.Y. Parkes, M. Steiner-Asiedu, K. Saalia, A. Acquatey-Mensah, O.O. Aina, P. Kulakow, R. Hanna, P.O. Iluebbey, A. Zhenchuk, F.J. Schweigert, S. Meyer, Y. Dingelstaedt, A. Hurtienne, and H. Kirscht

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A training manual

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Ibadan, Nigeria

To Headquarters from outside Nigeria:
IITA Ltd, Grosvenor House,
125 High Street,
Croydon CR0 9XP UK

Within Nigeria:
PMB 5320, Oyo Road
Ibadan, Oyo State

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Cover photo: Ground cassava leaves, Eppendorf tubes

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This manual is intended to serve as a repository of knowledge to facilitate laboratory analysis for micronutrient quantification and determination, and a reference for international and national research institutes, universities, and other educational Institutes. It is hoped that this manual will serve as a contribution to knowledge and further facilitate the efficiency and effectiveness of quantifying micronutrients (carotenoids, Fe, and Zinc) as a measure towards eradicating micronutrient deficiency and hidden hunger, thereby providing food and nutrition security for all.

The authors are indebted to everyone who contributed in diverse ways towards the successful compilation of this manual.

Protocol for the determination of carotenoids, iron, and zinc in cassava leaves and products

Determination of iron and zinc content of cassava roots, cassava leaves, and products made from cassava using the ICP-MS.

Sample preparation using the Wet Ashing Method

- Weigh about 5–10 mg of sample into 2 mL Eppendorf tubes
- Add 1 mL of a 1:1 mixture of 100% HNO_3 and 30% H_2O_2 to the sample and vortex to mix thoroughly.
- Heat the solution at 65 °C in a rotary evaporator for 30 min, and increase the temperature to 95 °C overnight.
- After wet ashing add 1 mL of a solution of 5% HNO_3 and 1.0 $\mu\text{g/L}$ Rhodium (Rh) to the contents of the Eppendorf tubes.
- Shake the tubes in a rotary device for 3 hours to ensure sample has dissolved in the solution.
- After 3 hours transfer 900 μL of a solution of 5% HNO_3 and 1.0 $\mu\text{g/L}$ Rhodium (Rh) into a 15 mL Corning tubes.
- Add 100 μL of the concentrated sample to the solution in the Corning tubes.
- Insert the tubes in the auto-sampler of the ICP-MS and run the experiment.

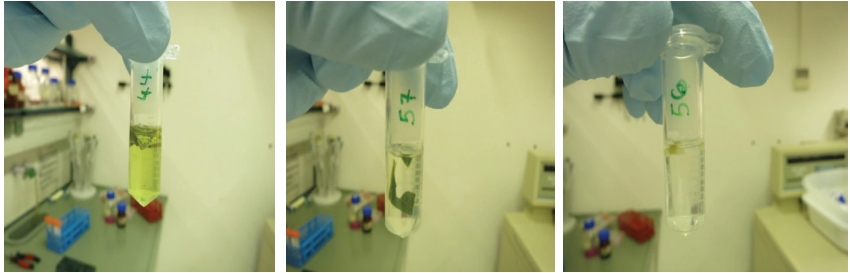


Figure 1. Samples in 2 mL Eppendorf tubes.

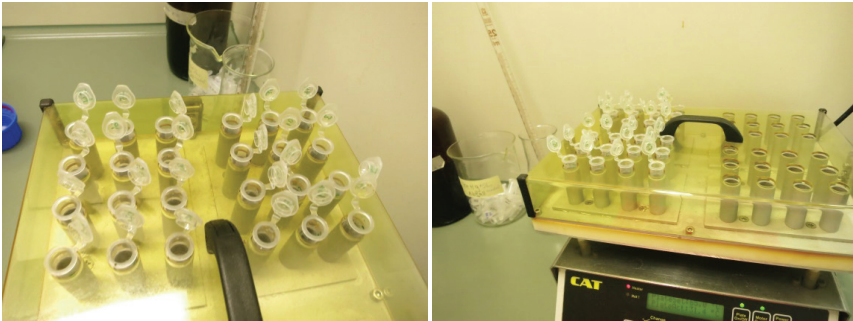


Figure 2. Samples in rotary evaporator.

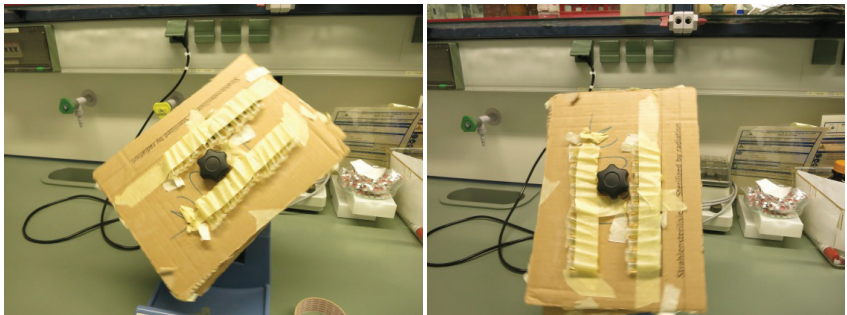


Figure 3. Samples in a rotary device.

Calculations:

$$\begin{aligned} \text{Fe Concentration } (\mu\text{g}/\text{mg weight of sample}) \\ = \frac{\text{Fe Conc. (ug/L)} \times \text{dilution factor} \times \text{total vol.}}{\text{Weight of sample (mg)}} \end{aligned}$$

$$\begin{aligned} \text{Zn Concentration } (\mu\text{g}/\text{mg weight of sample}) \\ = \frac{\text{Zn Conc. (ug/L)} \times \text{dilution factor} \times \text{total vol.}}{\text{Weight of sample (mg)}} \end{aligned}$$

$$\text{Dilution factor} = \frac{1000 \mu\text{L}}{100 \mu\text{L}} = 10$$

$$\text{Total volume} = 100 \mu\text{L} + 900 \mu\text{L} = 1000 \mu\text{L} \text{ or } 1 \text{ mL}$$

Sample preparation using the Microwave Ashing Method

- Weigh 50 mg of the samples into microwave tubes.
- Add a mixture of 1 mL 100% HNO₃, 200 μL Rhodium, and 800 μL distilled or bottled water to the mixture. (NB: Always add the water first to the sample before adding the acid).
- Cap the tube very well to prevent escape of solutions during ashing.

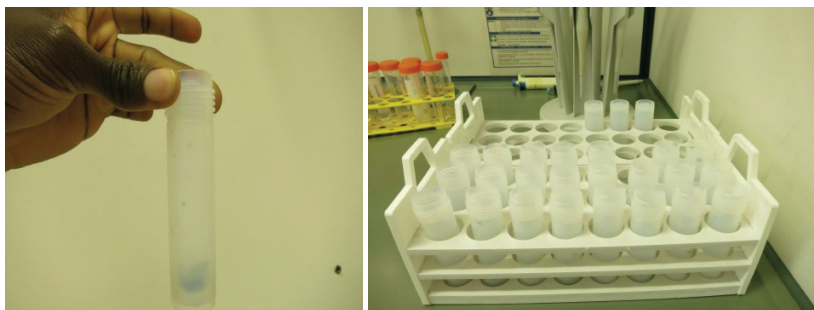


Figure 4. Samples in microwave tubes.

- Heat the sample in a microwave oven to a temperature of 200 °C for 15 min and allow the tubes to stay at 200 °C for 20 min.
- Allow the microwave oven to cool, remove the samples, and transfer the into 15 mL Corning tubes.
- Add 1800 µL of distilled water into different sets of Corning tubes and transfer 200 µL of the ashed sample into the tube. This is a dilution step.
- Add 2 mL of 20% HNO₃ cleaning reagent to the empty microwave tubes, cap them very well, and ash at 200 °C for 15 min, and allow tubes to stay at that temperature for 20 min. This step cleans the microwave tubes.
- Discard the acid in the tubes in a fume chamber and wash the tubes with distilled water.
- Insert the tubes in the auto-sampler of the ICP-MS and run the experiment.

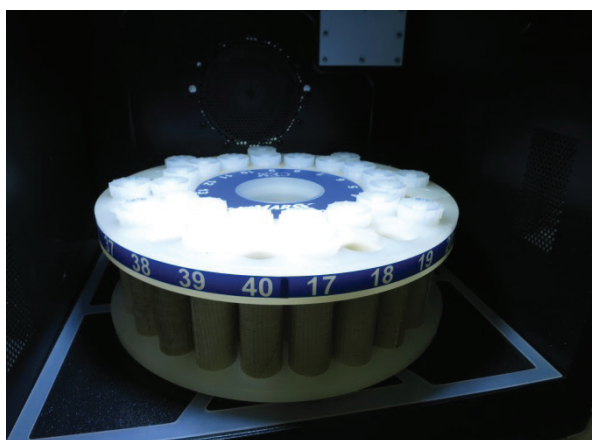


Figure 5. Samples in a microwave oven ready for ashing.

Calculations:

$$\begin{aligned} \text{Fe Concentration } (\mu\text{g}/\text{mg weight of sample}) \\ = \frac{\text{Fe Conc. } (\mu\text{g}/\text{L}) \times \text{dilution factor} \times \text{total vol.}}{\text{Weight of sample (mg)}} \end{aligned}$$

$$\begin{aligned} \text{Zn Concentration } (\mu\text{g}/\text{mg weight of sample}) \\ = \frac{\text{Zn Conc. } (\mu\text{g}/\text{L}) \times \text{dilution factor} \times \text{total vol.}}{\text{Weight of sample (mg)}} \end{aligned}$$

$$\text{Dilution factor} = \frac{2000 \mu\text{L}}{200 \mu\text{L}} = 10$$

$$\text{Total volume} = 200 \mu\text{L} + 1800 \mu\text{L} = 2000 \mu\text{L} \text{ or } 2 \text{ mL}$$

Preparation of standards for calibration curve:

Prepare concentrations of 5 $\mu\text{g}/\text{L}$, 10 $\mu\text{g}/\text{L}$, 50 $\mu\text{g}/\text{L}$, 100 $\mu\text{g}/\text{L}$, 150 $\mu\text{g}/\text{L}$, 200 $\mu\text{g}/\text{L}$, and 500 $\mu\text{g}/\text{L}$ from a standard of a mix of common elements, using 5% HNO_3 + 1 $\mu\text{g}/\text{L}$ Rhodium as diluent. Total volume of the standards should be 2 mL.

Sample calculation:

Preparing 5 $\mu\text{g}/\text{L}$ standard solution:

- Using a micropipette, transfer 10 μL of the mix of common elements into a 10 mL Corning tube
- Mix with 1990 μL of the diluent
- Vortex to mix the solution well



Figure 6. Samples in auto-sampler of ICP-MS.

Determination of the iron content using iCheck Iron



Figure 7. iCheck Iron Test Kit.

- Weigh approximately 10 g of the sample (i.e., wheat flour) into a 200 mL reagent bottle and add 90 mL distilled water to make a dilution factor of 10. Shake vigorously until the mixture is homogenous.
- Inject 3.3 mL of distilled water into a dry additive vial that comes with the iCheck Iron test kit to solubilize it.
- Inject 0.2 mL of the solubilized additive into the iCheck Iron reagent vial to activate it. A weak yellow color is observed in the vial after activation. Let the vial stand for about 5 minutes.
- Inject 0.4 mL of the sample solution into the activated vial. If iron is present a red color will develop. This

results from the reaction between iron in the sample and bathophenaltropin in the reagent vial.

- Shake the vial with sample vigorously for 10 seconds every 15 minutes.
- After one hour, centrifuge the vial for 1–2 minutes using manual centrifuge for complete separation of the organic and water phases inside the vial.
- Make sure the vial is clean and that there is a clear upper phase visible inside the vial. Place the vial into the iCheck Iron device for the measurement of the iron content in the diluted sample.
- The reading was multiplied by the dilution factor to obtain the iron concentration in the samples in mg/kg.
 - NB: Linear range of measurement of iCheck Iron = 1.5–12.0 mg/L
 - For detailed instructions see iCheck Iron User Manual and Measurement Step-by-step presentation

Determination of the zinc content using iCheck Zinc



Figure 8.:iCheck Zinc Test Kit.

- Pour into a 100 mL volumetric flask the zinc standard sample provided with iCheck Zinc case and top up to the 100 mL mark with distilled water. Shake until completely dissolved.
- Transfer the standard solution into a 250 mL reagent bottle.
- Add 2.4 g of preparation agent A (which comes with the iCheck Zinc Test Kit) to the standard solution and shake the bottle for about 10 seconds.
- Incubate the sample with preparation agent A for 30 minutes with vigorous shaking of the bottle and its contents every 10 minutes.

- Add 2.2 g of preparation agent B to the solution and swirl gently until completely dissolved. The solution is allowed to stand for 15 minutes. The cap of the bottle should be opened for 30 seconds to prevent the formation of foam.
- Switch on iCheck Zinc device and set it to “Standard Sample” mode.
 - NB: The iCheck Zinc vials must be stored at 4 °C. Bring the vials out to room temperature at least 30 minutes before the analysis.
- Insert new vial without the sample (blank vial) into iCheck Zinc device and press the measurement key. This is the blank.
- Remove the vial and inject 0.5 mL of the sample solution treated with preparation agents A and B. Shake for 10 seconds.
- Allow the vial to stand for 5 minutes after which it is inserted into the device and the reading for zinc determined.
- iCheck reading was multiplied by the dilution factor to obtain the Zinc concentration in mg/kg.
 - NB: Linear range of measurement of iCheck Zinc = 0.5–3.0 mg/L
 - For detailed instructions see iCheck Zinc User Manual

Modification of the sample ashing procedure

In order for the ashed samples to be tested with iCheck Iron and Zinc the expected concentration with ashed cassava leaves solutions must be in the iCheck measurement range. The initial procedure with 10 mg of the dry cassava leaves for the wet ashing method yields an expected iron concentration of < 1 mg/L. Therefore a bigger sample was tested:

First trial:

- Weigh 200 mg of sample into 2 ml Eppendorf tubes
- Add 1 ml of 1:1 mixture of 100% HNO₃ and 30% H₂O₂ to the sample and vortex (Rhodium was not added to prevent any potential interference with colorimetric reaction in the iron reagent vial).
- Heat the solution to 40 °C in a rotary evaporator
- Note: Samples absorb the solution so that no liquid is in the tube => no ashing possible.

Second trial:

- Weigh 200 mg of sample into 15 ml Falcon tubes.
- Add 10 ml of 1:1 mixture of 100% HNO₃ and 30% H₂O₂ to the sample and vortex.
- Heat the solution to 40 °C in a rotary evaporator => mixture foams strongly.
- Slowly increasing the temperature prevents foaming, but condensation is observed on the tip of the falcon

tube = > no ashing possible/ashing-process needs a very long period of time.

Third trial:

- Weigh 50 mg of sample into 2 ml Eppendorf tubes.
- Add 1 ml of 1:1 mixture of 100% HNO₃ and 30% H₂O₂ to the sample and vortex.
- Heat the solution at first at 40 °C in a rotary evaporator = > mixture begins to foam strongly = > it's necessary to supervise the samples permanently (take out samples, shake down, slowly increase the temperature).
- Increase the temperature to 95 °C overnight.
- After wet ashing add 1 ml of a solution of 5% HNO₃ to the content of the Eppendorf tubes.
- Shake the tubes in a rotary device for 3 hours to ensure dissolution of the sample in solution.
- Note: The mixture with the cassava leaves turns green-brown, the mixtures with cassava products turn light yellow.
- For ICP-MS: transfer 890 µl of 5% HNO₃ + 10µl Rhodium (1 µg/l Rh) and 100 µl of the concentrated sample into a 15 ml Falcon tubes. Insert the tubes in the auto-sampler of the ICP-MS and run the experiment.

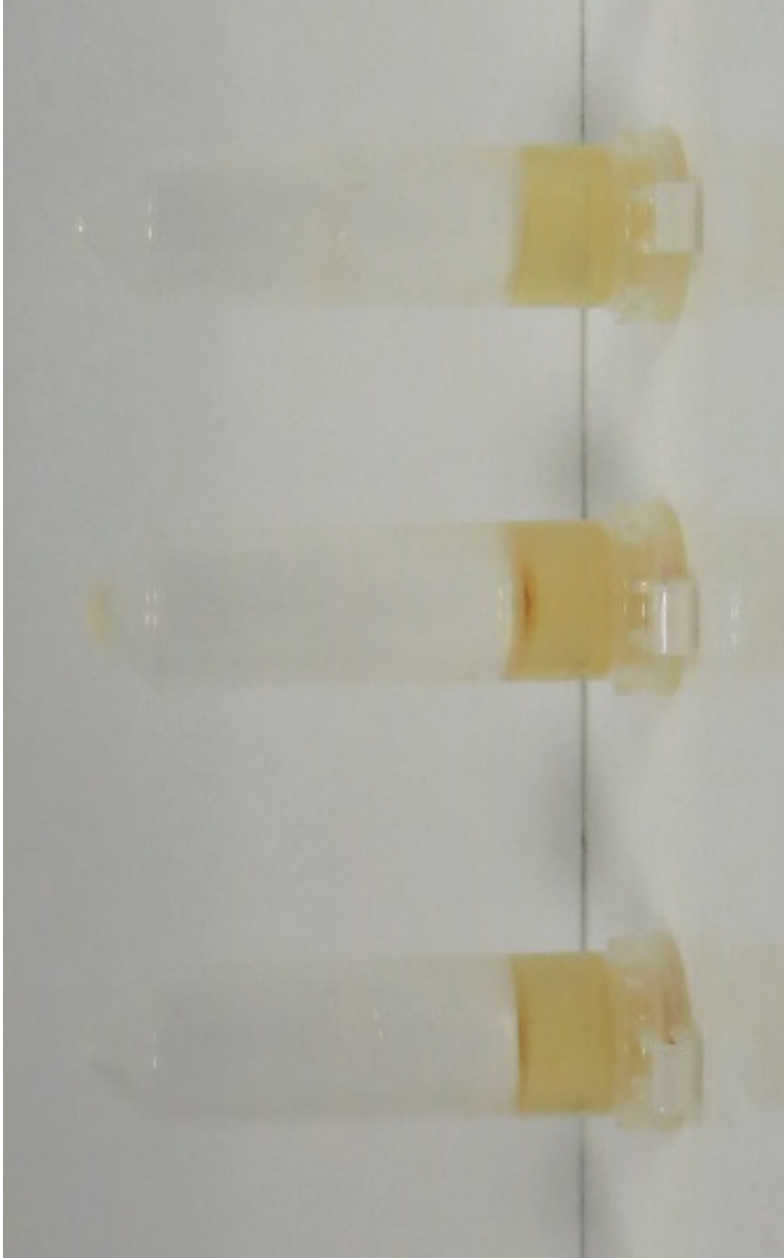


Figure 9. Vials after dissolution of the sample in solution.

Determination of iron content of cassava leaves, products, and the spinach standard from cassava using iCheck Iron

- Dilute the ashed concentrated sample solution (see Third Trial in the previous section) 1:1 with distilled water (i.e., 800 μ l of the concentrated sample + 800 μ l distilled water).
- Inject 0.4 ml of the diluted sample into the activated iCheck Iron vial, shake it for 10 sec and incubate for 5 min => measure.

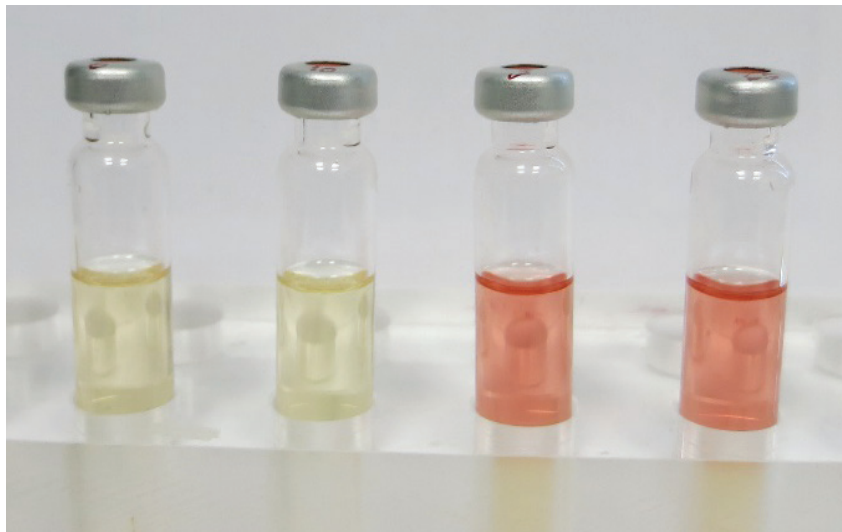


Figure 10: iCheck Iron vials with ashed cassava leaves and blank samples.

Determination of zinc content of cassava leaves, products, and the spinach standard using iCheck Zinc

Predilute preparation agents:

In order to adjust the preparation agents amounts to the low sample amounts that result from cassava leaves ashing of 1–2 mL; the agents were prediluted. (NB: the standard recommended sample volume used for iCheck Zinc is 100 mL).

- Prepare a solution of the Preparation Agents A+B:
 - 0.72 g A + 10ml H₂O
 - 0.88 g B + 10ml H₂O
- Take 0.5 ml of the diluted sample (see the section above for iCheck Iron) and add 0.25 ml of the Preparation A solution = > shake for 10 sec = > incubate for 30 min (shake every 10 min).
- Add 0.25 ml of the preparation agent B solution = > gentle swirl the sample tube (Caution: it will foam!) = > incubate for 15 min (repeat swirling until no new foam is formed).
- Inject 0.5 ml of the sample-additive-solution into the vial = > invert = > incubate for 5 min = > measure.
- The vial was measured after 5, 10, 15, 25, 30, 45 and 60 min of incubation. The red color is fully developed after 5 min and a background reaction is increasing with time leading to brown coloration. It is recommended to measure iron after 5 min.

Determination of carotenoids in cassava leaves using High Performance Liquid Chromatography (HPLC)

- Weigh approximately 0.1–0.4 g of ground sample into 10 mL propylene tubes and solubilize the cells of the sample in distilled water overnight under refrigerated conditions.
- Add 5 mL of a 3/2 v/v solution of n-hexane/ isopropanol to the samples in solution.
- NB: The n-hexane contains 0.05% Butylated hydroxytoluene (BHT), an antioxidant to enhance extraction of the carotenoids.
- Shake the tubes and the contents head-over-bottom in a rotary device for 15 min.
- Centrifuge the tubes at a speed of 3800 rpm for 5 minutes.
- After centrifuging use a dropping pipette to transfer the upper dark green organic layer (n-hexane) into new 20 mL extraction tubes ('A').
- Add 5 mL of n-hexane/isopropanol to the tubes containing the more hydrophilic phase in the propylene tubes and repeat earlier steps.
- Add 5 mL of 0.1M NaCl to the organic phase in the extraction tubes ('A').
- Shake the tubes vigorously for 2 min and cover the tubes with a thick towel or put in the dark for 30 min.
- After 30 minutes transfer the organic upper layer with a dropping pipette into new 20 mL extraction tubes ('B') and add 7.5 mL of n-hexane (containing 0.05% BHT) to the hydrophilic phase in the other extraction tubes ('A')

- Shake the tubes (A) vigorously and put the tubes in the dark for 30 minutes.
- After 30 minutes transfer the upper hexane/organic layer into extraction tubes “B”.
- Add 5 mL of n-hexane to extraction tube “A”, shake the tubes very well, and put the tubes in a dark environment for 30 minutes.
- After 30 minutes, transfer the upper organic phase to extraction tube “B”. At this point the volume of the n-hexane organic phase is approximately 17 mL.
- Top up the volume in the tube to the 20 mL mark with n-hexane containing 0.05% BHT; shake the tubes very well.

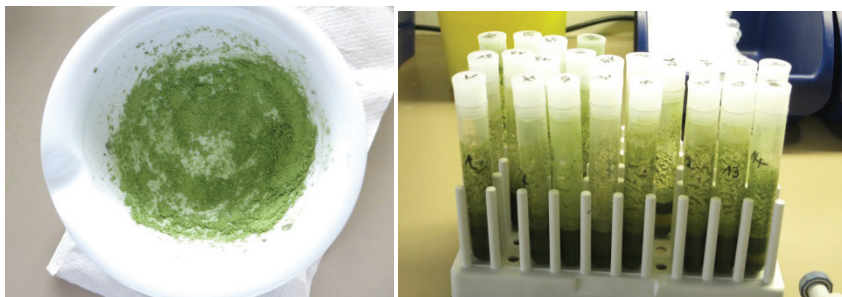


Figure 11. Preparation of cassava leaves for HPLC. Left: Ground cassava leaves. Right: Cassava leaves after solubilization.



Figure 12. Cassava leaves (in n-hexane/isopropanol) in rotary device.

- Transfer 200 μL aliquots of the extracted sample in the organic phase into 5 mL propylene tubes.
- Concentrate the samples in a sample concentrator by drying with nitrogen gas for about 10 min. A dry colored substance is obtained at the bottom of the tubes, with all the n-hexane gas evaporated.
- Add 200 μL of isopropanol to the dry concentrated samples and vortex. Put the tubes in a sonicator bath for 5 minutes to further ensure that the contents in the tube are mixed thoroughly.
- Centrifuge the tubes at 5000 rpm for 5 minutes.
- After centrifuging transfer the aliquots using a dropping pipette into the injection vials of the HPLC.
- NB: Care must be taken during the transfer so that air bubbles are not trapped in the cone of the vial. If air bubbles appear, tap the bottom of the vials gently to enable them escape.
- Put injection vials into the autosampler of the HPLC and the analysis of carotenoids are run. The results and chromatogram are displayed on the screen of the computer connected to the HPLC.

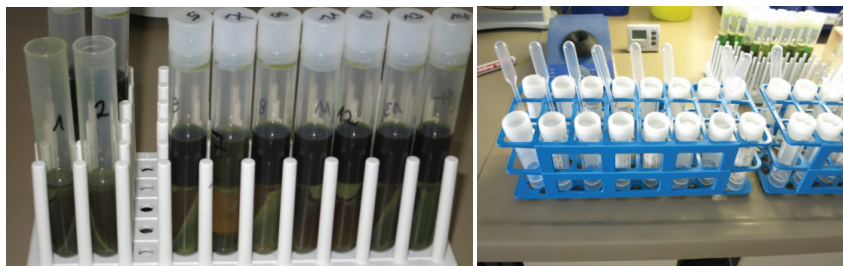


Figure 13. Upper dark green layer (left) being transferred into 20 mL extraction tubes (right).



Figure 14: Thick towel covering extraction tubes to enhance carotenoid extraction.



Figure 15. Extraction tubes 'A' (Red tube rack). Extraction tubes 'B' (Blue rack).

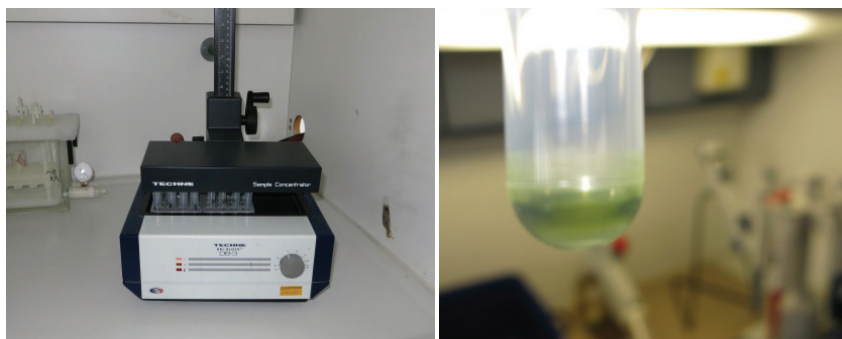
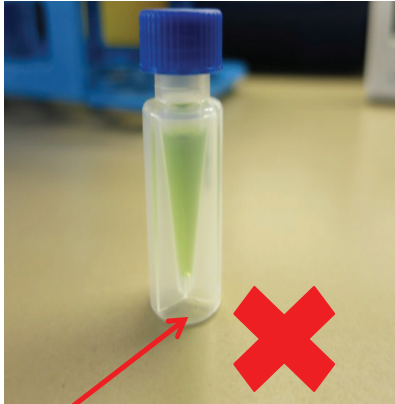


Figure 16. Samples in sample concentrator (Left). Dry colored sample after concentration (Right).



Air bubble trapped at the bottom of vial.

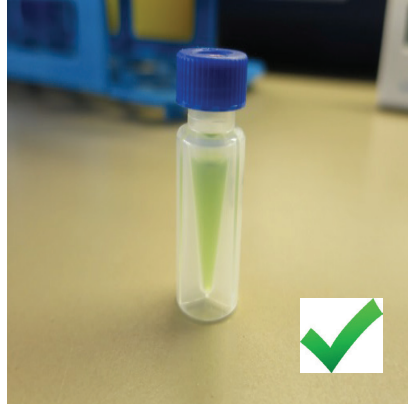


Figure 17. Precautions to take during transfer of aliquots into HPLC vials.



Figure 18. Diagram of HPLC.

Determination of carotenoids in products (e.g., gari, dry cassava roots) made from cassava

- Weigh approximately 1.0–1.5g of ground sample into 10 mL propylene tubes and solubilize the cells of the sample in distilled water overnight under refrigerated conditions.
- Add 5 mL of a 3/2 v/v solution of n-hexane/ isopropanol to the samples in solution.
- NB: The n-hexane contains 0.05% Butylated hydroxytoluene (BHT), an antioxidant to enhance extraction of the carotenoids.
- Shake the tubes and the contents head-over-bottom in a rotary device for 15 minutes.
- Centrifuge the tubes at a speed of 3800 rpm for 5 min.
- After centrifuging use a dropping pipette to transfer the upper organic layer (n-hexane) into new 20 mL extraction tubes ('A').
- Add 5 mL of n-hexane/isopropanol to the tubes containing the more hydrophilic phase in the propylene tubes and repeat earlier steps.
- Add 5 mL of 0.1M NaCl to the organic phase in the extraction tubes ('A').
- Shake the tubes vigorously for 2 min and cover the tubes with a thick towel or put in the dark for 30 min.
- After 30 min transfer the organic upper layer with a

dropping pipette into new 20 mL extraction tubes ('B') and add 7.5 mL of n-hexane (containing 0.05% BHT) to the hydrophilic phase in the other extraction tubes ('A').

- Shake the tubes (A) vigorously and put the tubes in the dark for 30 min.
- After 30 min transfer the upper hexane/organic layer into extraction tubes "B".
- Add 5 mL of n-hexane to extraction tube "A", shake the tubes very well, and put the tubes in a dark environment for 30 min.
- After 30 mins, transfer the upper organic phase to extraction tube "B". At this point the volume of the n-hexane organic phase is approximately 17 mL.
- Top up the volume in the tube to the 20 mL mark with n-hexane containing 0.05% BHT; shake the tubes very well.
- Transfer 1000 μ L or 1 mL aliquots of the extracted sample in the organic phase into 5 mL propylene tubes.
- Concentrate the samples in a sample concentrator by drying with nitrogen gas for about 10 min. A dry colored substance is obtained at the bottom of the tubes, with all the n-hexane gas evaporated.
- Add 200 μ L of n-hexane with 0.05% BHT to the dry concentrated samples and vortex. This makes a concentration factor of 5.
- Put the tubes in a sonicator bath for 5 min to further ensure that the contents of the tubes are thoroughly mixed.

- Centrifuge the tubes at 5000 rpm for 5 min.
- After centrifuging transfer the aliquots using a dropping pipette into the injection vials of the HPLC.
- NB: Care must be taken during the transfer so that air bubbles are not trapped in the cone of the vial. If air bubbles appear, tap the bottom of the vials gently to enable them escape.
- Put injection vials into the auto-sampler of the HPLC and the analysis of carotenoids are run. The results and chromatogram are displayed on the screen of the computer connected to the HPLC.

Determination of the total carotenoids content using iCheck Carotene



Figure 19. iCheck Carotene Test Kit.

- Weigh approximately 5 g of the sample (i.e., mashed cassava root) into a 50 mL falcon tube and add distilled water to 20 mL to make a dilution factor of 5. Shake vigorously until the mixture is homogenous.
- Inject 0.5 mL of the sample solution into the new iCheck Carotene reagent vial.
- Shake the vial with sample vigorously for 10 seconds and let it stand for at least 5 min.
- If necessary centrifuge the vial for 1–2 minutes using manual centrifuge for complete separation of the organic and water phases inside the vial.

- Make sure the vial is clean and that there is a clear upper phase visible inside the vial. Place the vial into the iCheck Carotene device for the measurement of the carotenoids content in the diluted sample.
- The reading was multiplied by the dilution factor to obtain the iron concentration in the samples in mg/kg.
 - NB: Linear range of measurement of iCheck Carotene = 0.25–25.0 mg/L
 - For detailed instructions see iCheck Carotene User Manual and Measurement Step-by-step presentation

Interpretation of the preliminary analytical results

Iron and Zinc in leaves:

- ICP-MS results with either wet or microwave ashing did not yield the concentrations expected in the reference material of spinach: wet weight Fe 12–5 mg/kg and dry weight 200–800 mg/kg. It is assumed that there is 94% water in the wet spinach sample.
- Repeating wet ashing with higher sample amount (increase from 8 mg to 50 mg) improved recovery to 11.5–12.8 mg/kg in wet spinach samples and 192–207 mg/kg in dry samples.
- iCheck Iron measurements of the ashed samples after 5 min on average measured 150% more than ICP-MS. Which bring the values closer to the expected. This shows potential for using iCheck Iron to quantify iron in cassava leaves upon digesting the organic matter with wet ashing.

Total carotenoids in leaves and cassava products:

- The reference sample of spinach expected TC content of 43–62 mg/kg of wet weight (717–1033 mg/kg dry weight) while mixed vegetables reference sample has expected TC content of 98–110 mg/kg in dry weight.
- HPLC showed low recovery for both reference samples: mixed vegetables: 42 mg/kg and wet spinach: 14–20 mg/kg.

- There is significant interference of chlorophylls with iCheck Carotene measurements in leaves leading to overestimated results.
- However iCheck Carotene measured results for reference samples are higher than for HPLC and come closer to the expected values.
- HPLC also has very low recovery of TC in cassava roots and products (ground and dry cassava): < 0.1–8 mg/kg. iCheck Carotene shows 3–12 mg/kg with same samples.

Background

- Cassava is grown in areas where mineral and vitamin deficiencies are widespread, especially in Africa. A marginal nutrient status increases the risk of morbidity and mortality.
- In sub-Saharan Africa, three million children under the age of five suffer total or partial blindness caused by vitamin A deficiency. In Nigeria, a nationwide food consumption and nutrition survey revealed that 29.5% of children under 5 years of age were vitamin A deficient. Vitamin A deficiency is a major public problem in over 75 countries in the developing world. Vitamin A deficiency in the early stage leads to night blindness and xerophthamia which may ultimately progress to blindness. Beta-carotene, the most potent and widespread form of pro-vitamin A, is the predominant carotenoid in cassava, occurring as a mixture of trans- and cis-forms. Trans- β -carotene acts as antioxidant that helps prevent heart attacks and cancer, lower cataract risks and muscular disorders and enhance the immune system.
- Breeding for high levels of beta carotene, require the use of methods for carotenoid quantification that require highly skilled and specialized personnel and expensive lab equipment with specific chemicals. Besides the qualitative screening method using the color chart, HarvestPlus has recommended HPLC as reference method and the spectrophotometer reading for Total Carotene Content (TCC). A cost-effective, simple, user-friendly, inexpensive, and rapid screening method introduced as BioAnalyt iCheck portable spectrophotometer iCheck has been used to effectively screen large sample sizes.

