Standard Operating Procedure (SOP) for Maize Carotenoid PVA Analysis

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1. Introduction

Plant phenotyping refers to **a quantitative description of the plant's anatomical, ontogenetical, physiological and biochemical properties**. Today, rapid developments are taking place in the field of quality analysis using High Performance Liquid Chromatography (HPLC) in order to quantify the carotenoid contents in maize kernels that allow for the characterization of plant traits in high-throughput.

2. Purpose

The purpose of this document is to outline the roles, responsibilities and procedures to be followed in high throughput phenotyping in maize.

3. Scope

This document contains carotenoid analysis procedure in maize breeding. It covers steps from sample collection to HPLC analysis.

4. Definition of terms

HPLC: High Performance Liquid Chromatography

Chromatogram: The output of a HPLC run, a graph depicting HPLC detector response for concentration of carotenoids and time.

Saponification: An extraction step aimed at removing lipids from a sample to eventually release carotenoids from fatty acids and lipids that interfere with separation in HPLC.

5. Roles and Responsibilities

All staff involved in implementing carotenoid analysis in the maize improvement program at IITA must use the high throughput phenotyping SOP. No alteration should be made to the procedures unless approved exceptionally by the program leaders. The list of individuals responsible for each section of the high throughput phenotyping SOP is listed below.

Crop Lead (CL) Responsible for the overall management of the trials and for delegating team responsibilities. The CL is the lead breeder and coordinator of Maize Improvement Program at IITA.

Breeder (B): Coordinate the field layout of experiments, planting and checks on the implementation of defined protocols on the different experimental sites. Ensures all trials are established in the on-station and out-stations respectively. This includes trial management and data collection.

Trial Manager (TM): Oversees trial preparations and management protocols, land acquisitions, oversees planting in the outstations. Also supervises planning of inputs and other planting logistics for the various stations.

Research Supervisor (RS): Coordinates the activities of the Research Technician to ensure that assigned tasks are carried out correctly. The RS involves in planting, field

management and post-trial management practices as well as coordinates fertilizer application in on station and outstation experimental fields. S/he involves in the Nursery and seed increase protocols as delegated by the CL and B respectively.

Research Technician (RT): The Research Technician performs field tasks as defined in the trial protocols such as field data collection or field management practices. RT's responsibility is to perform assigned tasks including the use of digital tools defined in the protocol for capturing, storing, transmitting, and ensuring quality of data within defined time periods.

Laboratory Manger (LM): S/He is responsible for arranging for the performance servicing of the HPLC and acquisition of HPLC columns and reagents required for analysis. Ensures that laboratory protocols are duly followed. He also validates all results computed before it is forwarded to the breeder.

Laboratory Research Supervisor (LS): Receives and properly store all samples for carotenoid analysis in Food and Nutrition Science laboratory. Prepare samples for extraction and analysis, operate the lab mills for crushing sample to 0.1mm sample size. Supervise the extraction of carotenoids from maize samples. Operate the HPLC machine to run samples for major carotenoids and identify carotenoids from the chromatograms and compute results.

Laboratory Technician (LT): assist in sample extraction and carries out duties assigned by the LS and LM respectively.

6. Procedure

CAROTENOID ANALYSIS

Field procedures:

- Dry maize ears for carotenoid analysis are harvested directly into dirt-free paper bags and shelled immediately after harvest.
- Maize ears that are not dry (>12% Moisture Content) should be dried in shades (not directly in the sun or in oven).
- 10g of clean maize kernels (about 150 kernels) are sampled in clean seed envelops.
- Samples for analysis are stored in 4c refrigerators.

MILLING

- Samples are grinded to <0.5mm particle size using Perten 500 laboratory mill.
- Milled samples are analyzed immediately or stored in -20c freezer for analysis within 18hours.

Carotenoids are extracted from milled maize samples using ethanol and hexane and carotenoid separation and identification using HPLC system.

CAROTENOID EXTRACTION

- **Apparatus**: Screw cap tubes, weighing balance, vortex mixer, water bath, centrifuge, concentrator tubes, evaporator, N2 gas
- **Solvents and Reagents for Extraction**: 0.1% butylhydroxytoluene ethanol, 80% Pottassium Hydroxide (KOH) for Saponification, n-Hexane, ice distilled water.

PROTOCOL FOR REHYDRATION AND EXTRACTION

- Weigh 0.6g of ground maize in a screw cap tube

- Add 6ml of ethanol containing 0.1% BHT.
- Vortex for 1 min
- Allow to cool for 5mins in a water bath at 85^0C
- Take out of water bath and 0.5ml of 80% KOH
- Vortex for 30secs.
- Allow to stand for 5mins in a water bath at 85^0C
- Repeat steps 6&7.
- Take out of water bath and put into ice bath
- Add 3ml of cold H_20
- Add 3ml of Hexane
- Vortex for 10secs
- Centrifuge for 10secs at 1000RPM.
- Pipette the upper phase into a 50ml concentrator tube
- Repeat steps 11-14 for 3 times
- Concentrate the extract in the evaporator at 40^0C using N_2 gas for 25mins.

INTRODUCTION INTO REVERSE HPLC SYSTEM

i. **Solvents and Reagents:** (50:50) Dichloromethane: Methanol, 100% methyl-tert-butyl ether (MTBE) HPLC grade MEOH: H_20 (92%:8%).

ii. **Protocol:**

- Immediately before injection, dissolve in 1ml of 50:50 DCE: MEOH.
- Vortex for 10secs
- Transfer into HPLC vial and run for major carotenoids using HPLC machine

- Extract the chromatogram, identify major carotenoid and calculate value of each carotenoid.

iii. HPLC CHROMATOGRAPHIC CONDITIONS

Carotenoid analysis is carried out using Waters Alliance 2695 Quaternary pump systems with an autosampler and a photodiode array detector 2996 Detector. Carotenoid is separated using 5-lm C30 Carotenoid Column (4.6 _ 250 mm; 3 ll). The HPLC system is operated in a gradient mode starting with 70% methanol/water (92:8 v/ v) with 10 mM ammonium acetate and 30% methyl tertiary butyl ether (100%) to 40% methanol/water and 60% methyl tertiary butyl ether. The flow rate is 1.0 ml/min and the solvents were HPLC grade. To maximize detection of carotenoids, absorbance is measured at 450 nm. Provitamin A is defined as the sum of b-carotene, b cryptoxanthin and a-carotene, with a-carotene and b-cryptoxanthin contributing 50% of the value of b-carotene.

CALCULATION OF CAROTENOIDS

$$\mathbf{Cx(ug/g)} = \frac{\mathbf{Ax} \times C_s(ug/mL) \times \text{total volume of extract (mL)}}{A_s \times \text{sample weight (g)}}$$

where Cx = concentration of carotenoid X;

Ax = peak area of carotenoid X;

Cs = concentration of the standard.

As = peak area of the standard.

QUALITY CONTROL:

- Commercial Standards: For preparation and validation of calibration curves.

- Standard Reference Materials: To check the suitability of the chromatographic systems as well as a test for the accuracy of the analytical data.
- Selected maize varieties with high B carotene.
- Replicate analysis: Duplicate runs of each sample to test for precision.
- Performance Maintenance (PM) of HPLC systems: Annual PM is done to ensure optimum system performance.
- Purity of Solvents: Only HPLC grade solvents are used for analysis.

1. Centrifuge the mixture for 5 mins at 15000 RPM and the supernatant was discarded.
2. Wash the pellets with 70% alcohol and dried.
3. Dissolve the pellets in 50 µL TE buffer and stored at -20^0 C.
4. Samples ready for analysis

7. Appendix

7.1 Contacts for support

For Issues relating to high throughput phenotyping, you can contact Udo, Enoobong (IITA) E.Udo@cgiar.org

8. References

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