THESIS

DEVELOPMENT OF A HIGH-THROUGHPUT PHENOTYPING METHOD FOR MEASURING SORGHUM CAROTENOIDS

Submitted by

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ABSTRACT

BRIDGING THE GAP BETWEEN BIOFORTIFICATION AND CONSUMPTION: DEVELOPING HIGH-THROUGHPUT PHENOTYPING METHODS TO FACILITATE SORGHUM BREEDING.

Vitamin A deficiency is the leading cause of preventable blindness in young children, and also leads to infertility and decreased immune function. Humans cannot synthesize vitamin A, so it must be consumed in the diet, either as preformed vitamin A or as provitamin carotenoid (PVACs) in plant foods. Access to PVACs is limited in sub-Saharan Africa where many diets are less diverse and primarily consist of cereals, such as sorghum, with intrinsically low concentrations of PVACs. Therefore, biofortification breeding efforts aim to increase PVACs to biologically relevant levels to reduce global vitamin A deficiency.

In order to select and breed high carotenoid varieties, thousands of progeny in a breeding program must be phenotyped. High-performance liquid chromatography (HPLC) is the gold standard carotenoid phenotyping method; however, it is expensive and time-consuming, making it impractical for large-scale screening. We hypothesized that a high-throughput phenotyping (HTP) method using UV-VIS spectrophotometry can identify high carotenoid sorghum lines for selection during breeding.

In this study, a simple and rapid method for carotenoid extraction and UV-VIS spectrophotometric detection in a 96-well plate format was developed and validated. To develop the HTP method, we measured 60 samples using both HPLC and UV-VIS, identifying a strong correlation (R^2=0.62, p-value<$4.51x10^{-14}$) between total carotenoid concentrations measured with the HTP method and the gold standard HPLC method. To validate the HTP method, we measured carotenoids in 249 lines in a biparental breeding family, using both HPLC and

UV-VIS, and again identified a strong correlation (R^2=0.61, p-value<$2.2x10^{-16}$). We also compared the predictability of the UV-VIS method to that of a simple visual inspection of grain color and found that the UV-VIS method performed significantly better. This promising HTP method will enable rapid screening of a large number of samples, helping breeders more efficiently make selections for carotenoid biofortification.

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1. Micronutrient deficiency

Malnutrition and food insecurity are public health problems that persist around the world, particularly in low-income countries. They are responsible for more ill health than any other cause. Despite global efforts to alleviate the problem, malnutrition has been increasing in the last few years, partly due to the 2019 pandemic, global drought, inequitable distribution of food resources, and a growing population in regions that are most at risk. It is estimated that around one-third of the global population suffers from micronutrient deficiencies (Dhaliwal et al., 2022; Wali et al., 2022), primarily in regions where staple cereals such as rice, maize, and sorghum are consumed. These staples provide high-caloric content, but are intrinsically limited in essential micronutrients, such as iron, zinc, and provitamin A carotenoids (Lowe, 2021).

Carotenoids are an important class of compounds for both plants and humans. In plants, carotenoids have a major role in photosynthesis and photooxidative protection. In humans, carotenoids reduce the risk of several chronic diseases, contribute to vision health, and some can be converted to vitamin A (provitamin A carotenoids or PVACs). It is estimated that 46 countries have severe rates of vitamin A deficiency (VAD), predominantly in Africa and Southeast Asia (World Health Organization, 2009), with millions of preschool-age children and pregnant women the most affected. In these regions, plant carotenoids are often the primary source of vitamin A (Global Nutrition Report, 2021), and although they are good sources of calories, they are generally low in provitamin A carotenoids (World Health Organization, 2009).

Sorghum (*Sorghum bicolor*) is the fifth most cultivated cereal crop in the world (de Morais Cardoso et al., 2017), and an integral component of the diets of those living in many West and East African countries (National Sorghum Producers, 2021). Its resilience to harsh growing conditions has made sorghum a critical food source in arid and semi-arid regions (Khalifa & Eltahir, 2023). Sorghum grains, like other cereals, accumulate low concentrations of

PVACs. Thus, sorghum biofortification—the process of improving the nutritional quality of a crop variety—offers a possibility to alleviate vitamin A deficiency. Breeding, biotechnology, and agronomic modification have been used for biofortification, but the breeding approach is the most feasible and accepted tool to meet nutrition requirements (Ofori et al., 2022).

Phenotyping is crucial in sorghum breeding programs as it helps breeders select germplasm with the desired traits. However, nutrient phenotyping is often a bottleneck during the breeding process due to the (1) lack of standardized methods of extraction and analysis, (2) high cost and long run time of existing methods, (4) the need for qualified personnel, (5), and (6) particularly in low-income countries, limited access to companies selling lab supplies such as equipment and chemicals. Therefore, developing a high-throughput carotenoid phenotyping method is essential to helping breeders speed up the decision-making process.

2. Understanding the Structure and Biosynthesis of Carotenoids in Plants for analytical method development

2.1. Carotenoid biosynthesis

A thorough comprehension of each compound's synthesis and breakdown stage is fundamental for sorghum biofortification and selecting the appropriate breeding approach (Zhai et al., 2016). The carotenoid pathway has been extensively documented in Arabidopsis and maize, giving insights into the genes, proteins, and metabolites that drive the accumulation of provitamin A carotenoids in the grain (Figure 1.1). However, most of the regulatory pathway controls are unknown (Cruet-Burgos et al., 2020a).

The carotenoind biosythesis pathway is highly conserved across plant species (Trono, 2019). The methylerythritol 4-phosphate (MEP) pathway produces the precursor compounds for the carotenoid biosynthesis pathway, starting with the condensation of glyceraldehyde-3-phosphate (GAP) and pyruvate and ending with the synthesis of geranylgeranyl pyrophosphate (GGPP), the precursor compound that is used in the carotenoid

pathway (Farré et al., 2010; Rodriguez-Concepcion, 2010). As a result of the action of phytoene synthase (PSY), two molecules of GGPP are condensed, producing 15-cis-phytoene. Phytoene is transformed into lycopene by two desaturation reactions catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). The poly-cis compounds that these enzymes originate are converted to the all-trans form by ζ-carotene isomerase (ZISO) and CRTISO via a photo-isomerization reaction (Zhai et al., 2016).

After lycopene synthesis, the pathway splits into two branches. When lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) act complementary on the two ends of lycopene (β, ε-branch), α-carotene is produced. But when LCYB acts by itself (β, β-branch), β-carotene is synthesized (Trono, 2019). The hydroxylation of α-carotene and β-carotene produces lutein and zeaxanthin, respectively. These reactions are catalyzed by carotenoid hydroxylase enzymes specific to the β-ring (HYDB or CYP97A in plants) and the ε-ring (CYP97C) (Cazzonelli, 2011).

Lutein represents the endpoint of the β, ε-branch, whereas the β, β-branch continues with the epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP). This reaction produces violaxanthin via antheraxanthin and could be reversed by violaxanthin deepoxidase (VDE) when the plants have high light stress (Demmig-Adams & Adams, 2002). Finally, the β, β-branch pathways end when violaxanthin is converted into neoxanthin by the action of neoxanthin synthase (NXS). In some plant species, the carotenoid biosynthesis pathway can continue further to synthesize specialized carotenoids such as keto-carotenoids (Gómez-García & Ochoa-Alejo, 2013)

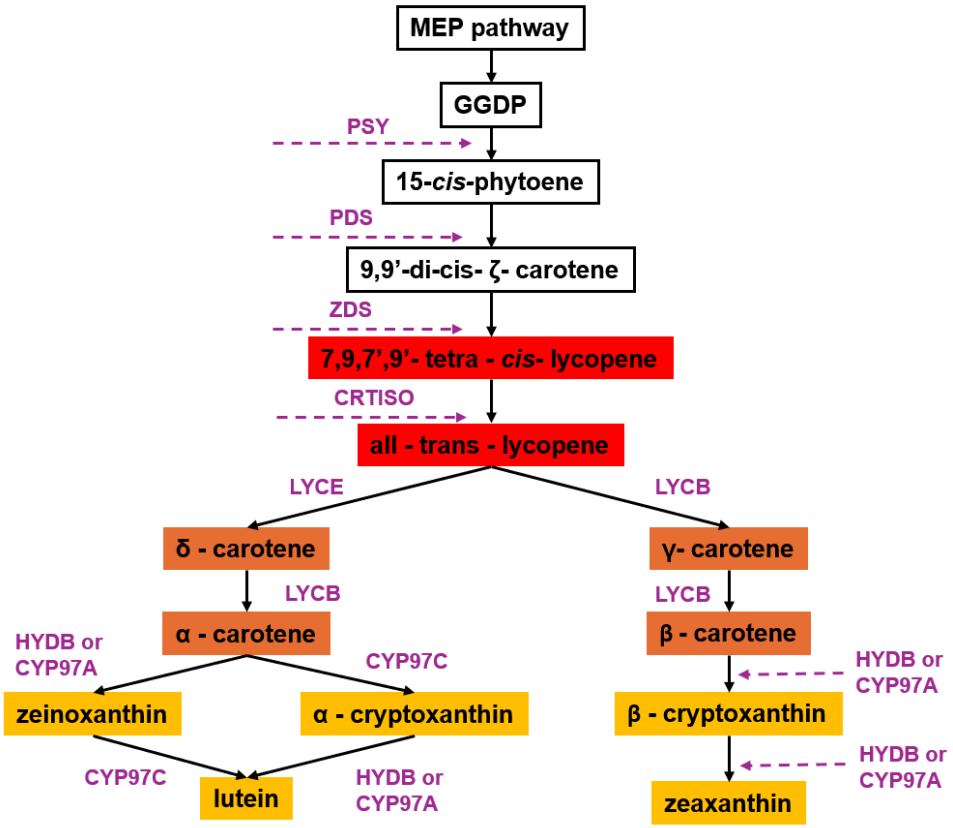


Figure 1.1. Scheme of Carotenoid Pathways. Biosynthesis and degradation occur within plastids of plant cells, and degradation products (apocarotenoids) are sequestered to other plant tissues

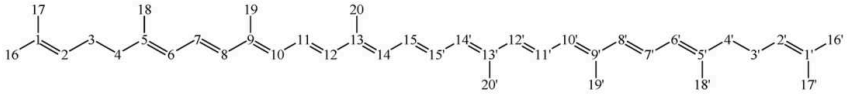
2.2. Molecular structure of carotenoids

Carotenoids are widespread pigments that provide distinctive yellow, orange, and red colors in plants (Cazzonelli, 2011). According to the Carotenoid DataBase (http://carotenoiddb.jp), around 1,158 carotenoids found in 691 organisms have been discovered (Yabuzaki, 2017), all synthesized by photosynthetic organisms and some bacteria and fungi (Trono, 2019). Besides providing striking bright colors, carotenoids are essential for photosynthesis, photoprotection, and phytohormone production, including ABA and strigolactone (Meléndez-Martínez et al., 2019). Furthermore, animals and humans cannot synthesize carotenoids; however, they can modify them by the action of two

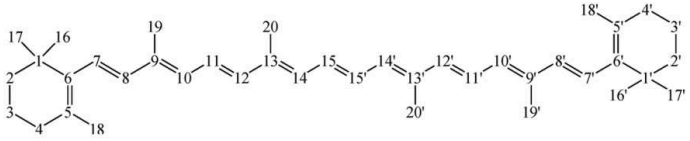
carotenoid-cleavage oxygenases (β,β-carotene 15,15′-monooxygenase, and β,β-carotene 9′,10′-dioxygenase) into oxidized derivatives as retinoids and apocarotenoids (Oxley et al., 2014).

It has been discovered that only 40 carotenoids are part of the human diet, with 20 identified in blood and tissues (Rivera-Madrid et al., 2020). The majority is comprised of β-carotene, α-carotene, and β-cryptoxanthin, which can be converted to vitamin A due to the presence of an unsubstituted β-ionone ring. Lutein, zeaxanthin, and lycopene contribute to overall human health (Carilho et al., 2014; Rao & Rao, 2007).

With some exceptions, carotenoids have a standard structure consisting of 40 carbon atoms arranged in 4 terpene units joined in a reverse isoprenoid fashion at the molecule's center. The carbons are counted from the ends to the molecule's center, from 1 to 15 on one side of the molecule and from 1′ to 15′ on the other. Meanwhile, the carotenoids containing methyl groups are numbered 16 to 20 and 16′ to 20′, respectively (Figure 1.2). Similarly, the alternating single and double bonds serve as the light-absorbing chromophore and determine the absorption of particular wavelengths in the visible spectrum. The carotenoids with more double bonds are rich in reactive electrons, so are more susceptible to oxidation and isomerization.



Lycopene



β-Carotene

Figure 1.2. Numbering of carbon atoms in an acyclic (lycopene) and a cyclic (β-carotene) carotenoid.

In terms of structure, carotenoids can be classified by several features: (1) the presence of oxygen molecules in the structure; (2) the presence of hydrogenation in the carbon polyene chain; (3) the presence of β-ionine rings at one or both ends of the structure; and (4) length of the polyene chain. Naturally, carotenoids appear in the all-trans form as long as they do not undergo structural modifications due to oxidation and isomerization (Nagarajan et al., 2017). When the carotenoid structure is rearranged into different isomeric forms, they are represented with the annotation of alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), and zeta (ζ) (Khoo et al., 2011; Rodriguez-Concepcion et al., 2018). The isomerization confers the different physicochemical characteristics, including solubility and absorbability. The all-trans-isomeric forms are more thermodynamically stable than other isomers, but more prone to cyclization and aggregation. On the other hand, cis-isomers are more readily solubilized and readily available to be absorbed in the body (Kirti et al., 2014).

Carotenoids are molecules that are not found alone in nature; they can form aggregate interactions with other molecules, such as fatty acids, carbohydrates, and proteins, as a result of reversible bonding by hydrogen bonds, van der Waals interactions, dipole forces and the hydrophobic effects of hydrophobic molecules (Meléndez-Martínez et al., 2019; Rodriguez-Concepcion et al., 2018). Due to their oxygenated structure, xanthophylls are found primarily esterified with fatty acids in different plant structures or coupled with sugar moieties. Similarly, some carotenoids can form more water-soluble structures with proteins known as carotenoproteins (Bhosale & Bernstein, 2007).

2.3. Classification of carotenoids

Carotenoids can be classified by their structure's presence or absence of oxygen (Figure 1.3). When their structure exclusively contains carbon and hydrogen, they are known as hydrocarbon carotenoids, or carotenes, such as α-carotene, β-carotene, γ-carotene, ζ-carotene, lycopene, neurosporene, phytoene or phytofluene (Meléndez-Martínez et al., 2019).

Carotenoids containing oxygen as a functional group are called xanthophylls (Figure 1.3). The most common form of oxygenation is in the form of hydroxyl groups, present, for instance, in β-cryptoxanthin, lutein, and zeaxanthin (Nagarajan et al., 2017). Other oxygenated moieties in xanthophylls are epoxides, which can be found in compounds such as antheraxanthin, neoxanthin, violaxanthin, and auroxanthin. There are also carotenoids found in nature that contain carbonyl groups (e.g., astaxanthin) and others that display oxygenated groups in the form of carboxylic, acetate, lactone, or sulfate groups (Meléndez-Martínez et al., 2019).

Besides these general classifications, we can find some minor carotenoids in nature: those that have one or two additional isoprenoid units, presenting 45 or 50 atoms of carbon in their structure, and those subgroups of carotenoids that contain fewer than 40 atoms of carbon, such as norcarotenoids and apocarotenoids (Rodriguez-Concepcion et al., 2018).

Additionally, carotenoids can also be classified as primary or secondary carotenoids. Primary carotenoids play a crucial role in photosynthesis and act as antioxidants. Since they absorb the light in the orange-to-red spectrum, they transmit light energy from the sunlight absorbed by chlorophyll. On the other hand, secondary carotenoids are xanthophylls that do not get involved in photosynthesis, but serve as accessory pigments in plants (Amorim-Carrilho et al., 2014).

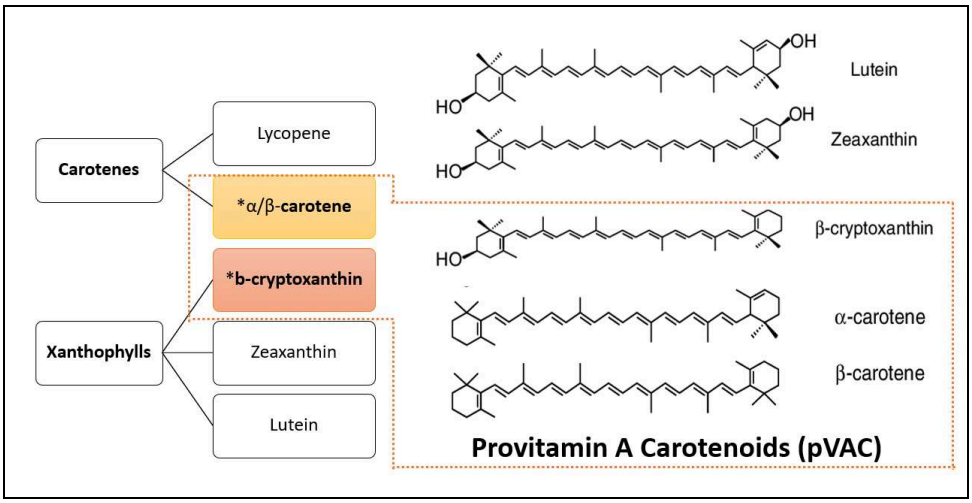
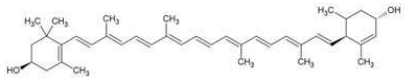
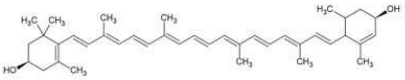
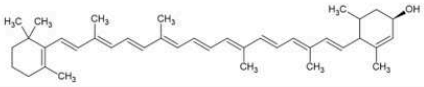
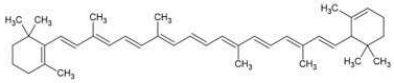
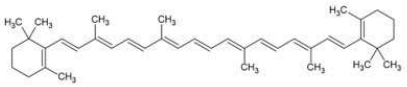


Figure 1.4. Chemical structures of major carotenoid species found in human plasma

2.4. Physicochemical characteristics

Carotenoids are characterized by their extensive network of conjugated double bonds, which primarily contribute to their general qualities and properties, such as color, reactivity, shape, absorbance, solubility, and stability (Meléndez-Martínez et al., 2019) .With a few exceptions, carotenoids are generally lipophilic compounds soluble in organic solvents such as acetone, methanol, diethyl ether, hexane, and others (Table 1.1) (Britton, 2022). In biological systems, they are predominantly located within cell membranes, although interactions with biomolecules such as proteins and sugars can also lead to their presence in hydrophilic environments. Carotenes interact with membranes due to their hydrocarbonated structure, while xanthophylls, with hydroxyl groups, are distributed across various cellular locations (Scott, 2001). Their hydrophobic nature causes most carotenoids to aggregate and crystallize within chromoplasts in aqueous environments, with melting points typically ranging between 130-220°C (Meléndez-Martínez et al., 2007).

Table 1.1. Physicochemical properties and structure of main carotenoids found in Sorghum grain (Structures made in ACD/ChemSketch)

Carotenoid	Formula	Structure	Solvent	W/length absorbed, λmax (nm)
Lutein	$C_{40}H_{56}O_2$		Chloroform Ethanol Petroleum ether	435,458,485, 422,445,475, 421,445,474
Zeaxanthin	$C_{40}H_{56}O_2$		Chloroform Ethanol Petroleum ether Acetone	433,452,479, 430,452,479, 428,450,478, 424,449,476
β-cryptoxanthin	$C_{40}H_{56}O$		Chloroform Ethanol Petroleum ether	435,459,485 428,450,478 425,449,476
α- carotene	$C4_0H_{56}$		Chloroform Ethanol Petroleum ether Hexane	433,457,484 424,448, 478 423,444,473 422,445473
β -carotene	$C_{40}H_{56}$		Chloroform Ethanol Petroleum ether Hexane	435,461,485 429,452,478 429,452,478 425,470,477

The intense color of carotenoid molecules is the product of their double bonds, with a minimum of seven necessary for perceptible coloration (Meléndez-Martínez et al., 2007). For instance, α-carotene has seven double bonds, resulting in a pale yellow color, while phytoene, containing three double bonds, remains colorless. This coloration arises from the oscillation of electrons along the unsaturated hydrocarbon chain. When light is absorbed, the molecules change from their basal energy state to a higher or excited state (Britton, 1995).

Carotenoids predominantly absorb light within the visible spectrum at 400 to 500 nm range due to delocalized π-electrons, often called "chromophores," within the conjugated unsaturated region of the molecule. While minor variations exist in the spectral profiles of individual carotenoids, these differences play an essential role in their differentiation. Similarly, the spectra of cis- or Z-isomers closely resemble those of the all-trans or all-E forms, though with some alterations in the maximum absorbance values and fine structure (Šesták, 2004).

The shape or fine structure of the spectrum can be noted as proportion III/II, as seen in Figure 1.4. Fine structure also gives information for the differentiation of carotenoids. However, there are carotenoids with the same chromophore, like β-carotene and its hydroxy-derivative zeaxanthin, that have identical fine structures. III is the longest wavelength peak, while II is the medium absorption peak. The height of the peaks is measured taking into account the baseline lowest distance between the two peaks (Britton, 2022).

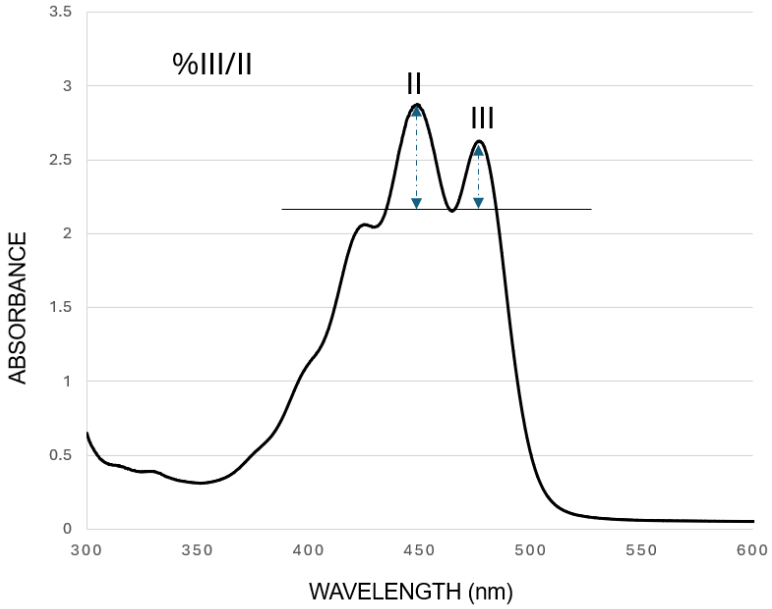


Figure 1.4. The calculation of fine structure %III/II of β-carotene in hexane

2.5. Current methods for carotenoid phenotyping in breeding programs: Challenges and Limitations

Quantifying carotenoids in plant breeding programs is critical for provitamin A biofortification. The specific carotenoids of interest, equipment availability and affordability, desired sensitivity, and sample matrix all influence the phenotyping approach to be selected. Various tools are commonly employed simultaneously in large-scale plant breeding programs to cross-validate data and provide a thorough understanding of carotenoid profiles.

However, smallholder breeding programs that are pivotal to improving and introducing locally adapted varieties face several limitations. Currently, breeders select superior lines based on field-based visual selections (whiteness and yellowness), which is not the best practice since yellowness is an indicator of presence or absence of carotenoids but does not predict the concentrations. For example, (Reynolds et al., 2020) describe visual phenotyping as a convenient manner to select traits. Still, it is subjective and potentially provides low repeatability depending on the observer's judgments. There is no publications related to the visual assessment of carotenoids in sorghum grain but for other traits Bowman et al., 2004 report that breeders with more than 30 years of expertise tended to select cotton lines with higher lint yields than new breeders (Bowman et al., 2004). Furthermore, when phenotyping large populations is necessary, visual selection becomes a limitation, so more reliable tools should be introduced into resource-limited breeding programs (Cudjoe et al., 2023).

Today, the top method for identifying and quantitating carotenoids and derivatives is high-performance liquid chromatography (HPLC), combined with UV–VIS absorption detection or a diode array detector (DAD) that allows recording of each carotenoid's complete UV absorption spectrum. HPLC is considered the “gold standard” (Jaramillo et al., 2018) since it accurately separates individual carotenoids and isomers based on their polarities. Similarly, HPLC has been extensively used in scientific research, and its reliability and reproducibility make it a trusted analysis method.

Carotenoid separation can be carried out using either normal or reverse-phase HPLC. However, normal-phase HPLC is not recommended when non-polar carotenoids need to be separated. Conversely, reverse-phase (RP) HPLC enables the enhanced resolution of carotenoids due to the significant interaction between the molecules and the non-polar stationary phase. The HPLC columns most used for reverse-phase chromatography are the ones packed with hydrophobic (C18) matrices, having the compounds eluting in order of polarity, with more water-soluble compounds eluting first and more non-polar compounds eluting

later. However, the C18 column inefficiently resolves geometrical and positional isomers, primarily lutein and zeaxanthin (Gupta et al., 2015). Sometimes, separations have shown improvement when gradient elution with mixtures of various organic solvents is utilized, but this could be improved when an RP-HPLC column packed with C30 ligands is used (Rajendran et al., 2005; Sander et al., 1994). This enables the separation of geometrical isomers but requires longer run times, resulting in low throughput (Gupta et al., 2015).

Although HPLC provides the best data, its application in large-scale selection can be problematic. Besides being a labor-intensive method that requires specialized training, it is a technique that requires expensive high-purity reagents and standards. Also, lab supplies needed throughout the process can affect the total cost per sample. Additionally, for data analysis and interpretation, the process requires replicates of the same sample, which is not feasible when many breeding lines must be evaluated.

An alternative to HPLC-PDA is using UV-VIS spectrophotometry alone. This straightforward detection method is particularly suitable for analyzing large sample sets (Kopec et al., 2012; Biswas, Sahoo, & Chatli, 2011). Unlike HPLC, which combines separation and UV-VIS detection, UV-VIS spectrophotometers measure the absorption or transmission of light passing through a sample directly, without separating its components. This method relies on Beer–Lambert's law, where absorbance correlates linearly with concentration, making it useful for quantifying carotenoids. Despite its drawbacks compared to HPLC (Bulduk & Akbel, 2021), UV-VIS spectrophotometry is widely used due to its affordability and convenience. For instance, it has been employed to measure carotenoids in crops like pumpkin, where β-carotene predominates, simplifying calculations by focusing on the most concentrated carotenoid (Hagos et al., 2022).

However, there are disadvantages associated with using spectrophotometry to measure carotenoids (Table 1.2). For instance, spectrophotometers cannot measure individual carotenoids, making it challenging to target PVACs and other specific carotenoids of interest. As

with HPLC analysis, spectrophotometry UV-VIS also requires long extraction steps, which is detrimental to carotenoids due to factors such as heat, light, oxygen, and metal ions that can break down their structures (Philip and Francis, 1971; Scita, 1992; Henry et al., 1998).

Another HTP technique used in breeding programs is near-infrared spectroscopy (NIRS; Table 1.2). However, it has not been used for carotenoid measurements in sorghum grain, because the predictive ability of NIRS is hampered by the low carotenoid concentrations in the grain. This method is often non-destructive to the sample (so seeds can be measured and subsequently used in planting or for additional phenotyping), and that allows testing several quality traits simultaneously, as reported in cassava roots and sweet potatoes (Belalcazar et al., 2016; Jaramillo et al., 2018). NIRS technology is also advantageous since it requires little or no sample preparation, making it less time-consuming than HPLC and UV-VIS spectrophotometry (Nkouaya Mbanjo et al., 2022). In addition, this technology offers reliable results for TCC and β-carotene with a high correlation to those obtained through HPLC (Jaramillo et al., 2018).

Prediction with NIRS depends entirely on the reliability of primary calibration data, and a long calibration procedure and validation based on laboratory reference methods is required (Berardo et al., 2004). In an experiment conducted by Jaramillo et al., 2018 in cassava, the NIRS method generated less precise agreement with HPLC at the higher range for TCC and β-carotene concentrations since the calibration range was shorter than the range of the analyzed samples. Therefore, when NIRS is used, it is essential to include samples from all ranges of possible concentrations and their corresponding laboratory data to enable accurate predictions. Otherwise, the results will be underestimated or overestimated. However, as sorghum carotenoid concentrations become higher due to biofortification breeding, NIRS predictions can likely be improved and could be an effective HTP phenotyping method.

Table 1.2. Techniques for carotenoid quantification in breeding programs

Technique	Extraction considerations	Parameters measured		Instrument cost
High-Performance Liquid Chromatography (HPLC)	Filters, nitrogen, ultra-pure reagents, and solvents. Additional instrumentation	The absolute concentration of individual carotenoid		Very High
Visual assessment	None	Presence or absence of carotenoids		None
Spectrophotometry UV-VIS	Filters, nitrogen, ultra-pure reagents, and solvents Additional instrumentation	Total Carotenoid Concentration (TCC)		Moderate
Near-infrared spectroscopy (NIRS)	None Whole or ground grain, with no extraction	TCC	β-carotene	Moderate

3. Value of high-throughput phenotyping

3.1. High-throughput phenotyping: Breaking through the bottleneck in crop breeding

Plant breeding is defined as changing plant species' genetic patterns to increase their value and utility for human well-being (Bhadouria et al., 2019). Plant breeding started when humans first began farming. However, its scientific foundation was fully established after Mendel's work on genetics at the end of the nineteenth century (Caligari & Brown, 2017),

followed by integration of advanced biotechnological methodologies, such as cisgenesis, genome editing, and speed breeding (Abdul Aziz & Masmoudi, 2024). Although relevant progress has been accomplished, plant breeding still depends on a traditional evaluation of phenotypes.

Plant phenotyping is a crucial aspect of plant breeding programs. "Phenotyping" is commonly defined as methods and protocols for measuring traits that come from the interactions between a specific genotype and its environment (Pieruschka & Poorter, 2012). Phenotyping aims to precisely and adequately assess features such as yield, resistance, or nutritional content (Fiorani and Schurr, 2013). Unfortunately, existing phenotyping approaches are frequently inefficient, time-consuming, labor-demanding, and costly (Araus et al., 2018). Despite the tremendous advances over the years, there is still a need to achieve the throughput required for fast trait evaluation and plant improvement (Jin et al., 2021).

Breakthroughs in genomics for plant improvement have contributed to speeding up plant breeding programs, integrating molecular markers to enable researchers to identify quantitative trait loci (QTL) linked with traits of interest. Similarly, genome-wide association studies (GWAS) have also been included to rapidly identify candidate genes associated with a trait of interest, such as carotenoid biosynthesis (Cruet-Burgos et al., 2020a). Although the genotyping tools have begun to outpace phenotyping capabilities in terms of speed and accuracy, recent advances in HTP are allowing us to close this gap. In fact, phenotypic information is essential to enable the construction of prediction models to accelerate the selection process (Gris, 2012).

In recent years, efforts have been made to develop phenotyping approaches to rapidly assess numerous plant features without sacrificing accuracy or precision while lowering human subjectivity, cost, and labor (Sankaran et al., 2019; Yu et al., 2016). While significant progress has been made, many methodologies are still in the validation process and assessment of scaling potential. Researchers are also working to establish standardized protocols and criteria for evaluating the performance of these methods across various crops and traits.

3.2. Innovations with low innovation readiness and use constitute bottlenecks for scaling

According to Krishna et al., 2023, breeding crops for a specific trait is limited by several aspects, including length of time to develop new varieties (10-20 years), the high cost, and lack of high throughput phenotyping tools for selection (Krishna et al., 2023). Therefore, to accomplish the goal of breeding for a specific trait (i.e., carotenoids), it is necessary to know the availability of resources that will contribute to obtaining that attribute and assess which factors constrain its development. Another consideration is that the scalability of an innovation, such as sorghum varieties with high concentrations of carotenoids, is limited by the least developed core or complementary innovations within the innovation group (also known as a bottleneck) (Sartas et al., 2020). To understand the dynamic, Fig 1.5 depicts an adapted version of ‘Liebig's barrel’ (Whitson and Walster, 1912). Every innovation can be represented as a wooden stave in a barrel. Just as the shortest stave or staves of unequal lengths limit a barrel's capacity, the scaling potential of an innovation package is limited by the innovation with the lowest readiness and use (Sartas et al., 2020). As part of an innovation package, different staves are joined together by a hoop. Each stave's length corresponds to innovation readiness, while the width corresponds to innovation utilization. The taller and wider the staves, the more water the barrel can contain. In other words, increased innovation readiness and use lead to more significant effect at scale. For example, the scaling of sorghum varieties with high concentrations of carotenoids may be limited by the absence of molecular markers or phenotyping methods to select high-carotene sorghum varieties. One can continue to invest in plant breeding to improve high-nutrient varieties, but only as long as appropriate phenotyping tools are available. When we understand that innovations scale as part of innovation packages, the next step is to determine which innovations limit the scaling of the innovation package and the most resource-efficient technique for overcoming such bottlenecks (Sartas et al., 2020).

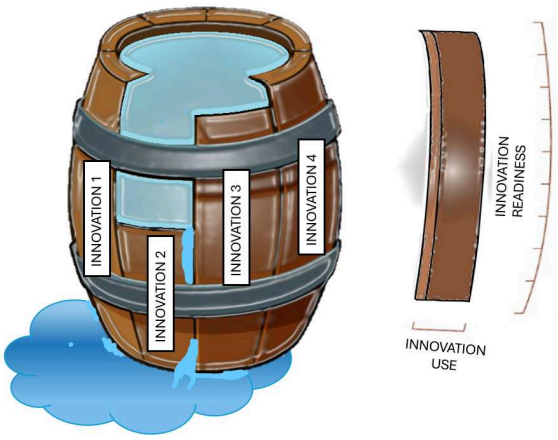


Figure 1.5. Scaling Readiness Barrel to illustrate how innovation(s) with the lowest readiness limit an innovation package's capacity to achieve impact at scale.

Conclusion and chapter overview

Improving PVAC content in sorghum is possible through natural genetic variation that can be achieved over time. Accelerating this process is particularly essential in breeding programs that aim to contribute to nutritional improvement in countries with a high prevalence of vitamin A deficiency. Efforts have focused on developing molecular, prediction, and phenotyping techniques that, when used synergistically, can lead to sorghum varieties with higher PVAC content. However, many available tools do not offer the speed to accelerate the selection process. Currently, breeders, particularly those in small breeding programs, do not have efficient and accessible tools, so their conclusions are based on rudimentary techniques such as visual inspection of the grain. This practice is useful in certain cases, but the results are likely unreliable, leaving room for bias.

The quantification of carotenoids is primarily based on analytical techniques involving extraction, quantification, and reporting. HTP methodologies exist for the quantification of vitamin A in different plant species. However, few reports focus on plant breeding programs. The quantification of carotenoids is challenging in sorghum grains due to the inherent difficulty in the

analysis of these samples. This is because grains have naturally low concentrations of carotenoids, and their solid structure requires lengthy extraction processes.

In this era where malnutrition continues to be a global problem affecting millions, access to fast and affordable technologies to aid the decision-making process in breeding is imperative. That is why we present in the following chapters the optimization of an HPLC method that allows the quantification of individual carotenoids in a relatively short time and the development of a 96-well-plate quantification method for the quantification of TCC. The validation results show that the methods are reliable, accurate, and relatively fast in accelerating the breeding process. Although the HTP method does not allow specific quantification of provitamin A beta carotene, we hypothesized that TCC provides sufficient information to differentiate lines with low, medium, and high concentrations of PVAC. This is because there is a significant correlation between TCC and PVAC concentration. About 700 accession numbers were tested for the two methods, followed by correlation analysis to test the hypothesis.

Chapter 1. Literature Cited

Abdul Aziz, M., & Masmoudi, K. (2024). Molecular Breakthroughs in Modern Plant Breeding Techniques. *Horticultural Plant Journal*. https://doi.org/10.1016/j.hpj.2024.01.004https://doi.org/10.1186/s13007-022-00892-0

Abdul-Hammed, M., Bello, I. A., & Oladoye, S. O. (2013). Simultaneous Spectrophotometric Determination of Lycopene and Beta-Carotene Concentrations in Carotenoid Mixtures of the Extracts from Tomatoes, Papaya and Orange Juice: Lycopene and ?-Carotene Determination. *Biological Sciences - PJSIR*, *56*(2), Article 2. https://doi.org/10.52763/PJSIR.BIOL.SCI.56.2.2013.90.97

Alamu, E. O., Nuwamanya, E., Cornet, D., Meghar, K., Adesokan, M., Tran, T., Belalcazar, J., Desfontaines, L., & Davrieux, F. (2021). Near-infrared spectroscopy applications for high-throughput phenotyping for cassava and yam: A review. *International Journal of Food Science & Technology*, *56*(3), 1491–1501. https://doi.org/10.1111/ijfs.14773

Amorim-Carrilho, K. T., Cepeda, A., Fente, C., & Regal, P. (2014). Review of methods for analysis of carotenoids. *TrAC Trends in Analytical Chemistry*, *56*, 49–73. https://doi.org/10.1016/j.trac.2013.12.011

Araus, J. L., Kefauver, S. C., Zaman-Allah, M., Olsen, M. S., & Cairns, J. E. (2018). Translating High-Throughput Phenotyping into Genetic Gain. *Trends in Plant Science*, *23*(5), 451–466. https://doi.org/10.1016/j.tplants.2018.02.001

Barba, A. I. O., Hurtado, M. C., Mata, M. C. S., Ruiz, V. F., & Tejada, M. L. S. de. (2006). Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry*, *95*(2), 328–336. https://doi.org/10.1016/j.foodchem.2005.02.028

Belalcazar, J., Dufour, D., Andersson, M. S., Pizarro, M., Luna, J., Londoño, L., Morante, N., Jaramillo, A. M., Pino, L., Becerra López-Lavalle, L. A., Davrieux, F., Talsma, E. F., & Ceballos, H. (2016). High-Throughput Phenotyping and Improvements in Breeding Cassava for Increased Carotenoids in the Roots. *Crop Science*, *56*(6), 2916–2925. https://doi.org/10.2135/cropsci2015.11.0701

Berardo, N., Brenna, O. V., Amato, A., Valoti, P., Pisacane, V., & Motto, M. (2004). Carotenoids concentration among maize genotypes measured by near infrared reflectance spectroscopy (NIRS). *Innovative Food Science & Emerging Technologies*, *5*(3), 393–398. https://doi.org/10.1016/j.ifset.2004.03.001

Bhadouria, R., Singh, R., Singh, V. K., Borthakur, A., Ahamad, A., Kumar, G., & Singh, P. (2019). Chapter 1 - Agriculture in the Era of Climate Change: Consequences and Effects. In K. K. Choudhary, A. Kumar, & A. K. Singh (Eds.), *Climate Change and Agricultural Ecosystems* (pp. 1–23). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-816483-9.00001-3

Bhosale, P., & Bernstein, P. S. (2007). Vertebrate and invertebrate carotenoid-binding proteins. *Archives of Biochemistry and Biophysics*, *458*(2), 121–127. https://doi.org/10.1016/j.abb.2006.10.005

Bowman, D., Bourland, F., Myers, G., Wallace, T., & Caldwell, D. (2004). *BREEDING AND GENETICS Visual Selection for Yield in Cotton Breeding Programs*. https://www.semanticscholar.org/paper/BREEDING-AND-GENETICS-Visual-Selection-for-Yield-in-Bowman-Bourland/20ac1d72ec39e405d7111fa0d6583339fd198b10

Britton, G. (1995). ChemInform Abstract: UV/Visible Spectroscopy. *ChemInform*, *26*(32). https://doi.org/10.1002/chin.199532313

Britton, G. (2022). Chapter One—Getting to know carotenoids. In E. T. Wurtzel (Ed.), *Methods in Enzymology* (Vol. 670, pp. 1–56). Academic Press. https://doi.org/10.1016/bs.mie.2022.04.005

Bulduk, I., & Akbel, E. (2021). A comparative study of HPLC and UV spectrophotometric methods for remdesivir quantification in pharmaceutical formulations. *Journal of Taibah University for Science*, *15*(1), 507–513. https://doi.org/10.1080/16583655.2021.1991737

Caligari, P. D. S., & Brown, J. (2017). Plant Breeding, Practice. In B. Thomas, B. G. Murray, & D. J. Murphy (Eds.), *Encyclopedia of Applied Plant Sciences (Second Edition)* (pp. 229–235). Academic Press. https://doi.org/10.1016/B978-0-12-394807-6.00195-7

Cazzonelli, C. I. (2011). Carotenoids in nature: Insights from plants and beyond. *Functional Plant Biology: FPB*, *38*(11), 833–847. https://doi.org/10.1071/FP11192

Cernelic, K., Prosek, M., Golc-Wondra, A., Rodic, Z., Simonovska, B., & Puklavec, M. (2013). *Influence of Synthetic Antioxidants on Extraction of All*-trans-*Lutein from Spinach under Air and Nitrogen Atmosphere*. *2013*. https://doi.org/10.4236/fns.2013.42027

Chan, C. C. (2011). Principles and Practices of Analytical Method Validation: Validation of Analytical Methods is Time-consuming but Essential. *The Quality Assurance Journal*, *14*(3–4), 61–64. https://doi.org/10.1002/qaj.477

Cruet-Burgos, C., Cox, S., Ioerger, B. P., Perumal, R., Hu, Z., Herald, T. J., Bean, S. R., & Rhodes, D. H. (2020a). Advancing provitamin A biofortification in sorghum: Genome-wide association studies of grain carotenoids in global germplasm. *The Plant Genome*, *13*(1), e20013. https://doi.org/10.1002/tpg2.20013

Cruet-Burgos, C., Cox, S., Ioerger, B. P., Perumal, R., Hu, Z., Herald, T. J., Bean, S. R., & Rhodes, D. H. (2020b). Advancing provitamin A biofortification in sorghum: Genome-wide association studies of grain carotenoids in global germplasm. *The Plant Genome*, *13*(1), e20013. https://doi.org/10.1002/tpg2.20013

Cudjoe, D. K., Virlet, N., Castle, M., Riche, A. B., Mhada, M., Waine, T. W., Mohareb, F., & Hawkesford, M. J. (2023). Field phenotyping for African crops: Overview and perspectives. *Frontiers in Plant Science*, *14*. https://www.frontiersin.org/articles/10.3389/fpls.2023.1219673

de Morais Cardoso, L., Pinheiro, S. S., Martino, H. S. D., & Pinheiro-Sant'Ana, H. M. (2017). Sorghum (Sorghum bicolor L.): Nutrients, bioactive compounds, and potential impact on human health. *Critical Reviews in Food Science and Nutrition*, *57*(2), 372–390. https://doi.org/10.1080/10408398.2014.887057

Demmig-Adams, B., & Adams, W. W. (2002). Antioxidants in Photosynthesis and Human Nutrition. *Science*, *298*(5601), 2149–2153. https://doi.org/10.1126/science.1078002

Dhaliwal, S. S., Sharma, V., Shukla, A. K., Verma, V., Kaur, M., Shivay, Y. S., Nisar, S., Gaber, A., Brestic, M., Barek, V., Skalicky, M., Ondrisik, P., & Hossain, A. (2022). Biofortification—A Frontier Novel Approach to Enrich Micronutrients in Field Crops to Encounter the Nutritional Security. *Molecules*, *27*(4), 1340. https://doi.org/10.3390/molecules27041340

Doebley, J. F., Gaut, B. S., & Smith, B. D. (2006). The Molecular Genetics of Crop Domestication. *Cell*, *127*(7), 1309–1321. https://doi.org/10.1016/j.cell.2006.12.006

Dunn, W. B., Kuligowski, J., Lewis, M., Mosley, J. D., Schock, T., Ulmer Holland, C., Zanetti, K. A., Vuckovic, D., & Metabolomics Quality Assurance and Quality Control Consortium (mQACC). (2023). Metabolomics 2022 workshop report: State of QA/QC best practices in LC–MS-based untargeted metabolomics, informed through mQACC community engagement initiatives. *Metabolomics*, *19*(11), 93. https://doi.org/10.1007/s11306-023-02060-4

Galli, G., Horne, D. W., Collins, S. D., Jung, J., Chang, A., Fritsche-Neto, R., & Rooney, W. L. (2020). Optimization of UAS-based high-throughput phenotyping to estimate plant health and grain yield in sorghum. *The Plant Phenome Journal*, *3*(1), e20010. https://doi.org/10.1002/ppj2.20010

Global Nutrition Report. (2021). *The state of global nutrition—Global Nutrition Report*. https://globalnutritionreport.org/reports/2021-global-nutrition-report/

Gómez-García, M. D. R., & Ochoa-Alejo, N. (2013). Biochemistry and Molecular Biology of Carotenoid Biosynthesis in Chili Peppers (Capsicum spp.). *International Journal of Molecular Sciences*, *14*(9), Article 9. https://doi.org/10.3390/ijms140919025

Gris, D. (2012). *IMPLEMENTING HIGH-THROUGHPUT PHENOTYPING AT THE NDSU DRY BEAN (PHASEOLUS VULGARIS L.) BREEDING PROGRAM USING UNMANNED AERIAL SYSTEMS*.

Gupta, P., Sreelakshmi, Y., & Sharma, R. (2015). A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant Methods*, *11*(1), 5. https://doi.org/10.1186/s13007-015-0051-0

Hagos, M., Redi-Abshiro, M., Chandravanshi, B. S., & Yaya, E. E. (2022). Development of Analytical Methods for Determination of β-Carotene in Pumpkin (Cucurbita maxima) Flesh, Peel, and Seed Powder Samples. *International Journal of Analytical Chemistry*, *2022*, 9363692. https://doi.org/10.1155/2022/9363692

Hunter, M. C., Smith, R. G., Schipanski, M. E., Atwood, L. W., & Mortensen, D. A. (2017). Agriculture in 2050: Recalibrating Targets for Sustainable Intensification. *BioScience*, *67*(4), 386–391. https://doi.org/10.1093/biosci/bix010

ICH. (2024). *ICH Q2(R2) Validation of analytical procedures—Scientific guideline | European Medicines Agency*. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline

Inbaraj, B. S., Lu, H., Hung, C. F., Wu, W. B., Lin, C. L., & Chen, B. H. (2008). Determination of carotenoids and their esters in fruits of *Lycium barbarum* Linnaeus by HPLC–DAD–APCI–MS. *Journal of Pharmaceutical and Biomedical Analysis*, *47*(4),

812–818. https://doi.org/10.1016/j.jpba.2008.04.001

Jaramillo, A. M., Londoño, L. F., Orozco, J. C., Patiño, G., Belalcazar, J., Davrieux, F., & Talsma, E. F. (2018). A comparison study of five different methods to measure carotenoids in biofortified yellow cassava (Manihot esculenta). *PLOS ONE*, *13*(12), e0209702. https://doi.org/10.1371/journal.pone.0209702

Jin, X., Zarco-Tejada, P. J., Schmidhalter, U., Reynolds, M. P., Hawkesford, M. J., Varshney, R. K., Yang, T., Nie, C., Li, Z., Ming, B., Xiao, Y., Xie, Y., & Li, S. (2021). High-Throughput Estimation of Crop Traits: A Review of Ground and Aerial Phenotyping Platforms. *IEEE Geoscience and Remote Sensing Magazine*, *9*(1), 200–231. https://doi.org/10.1109/MGRS.2020.2998816

Khalifa, M., & Eltahir, E. A. B. (2023). Assessment of global sorghum production, tolerance, and climate risk. *Frontiers in Sustainable Food Systems*, *7*. https://doi.org/10.3389/fsufs.2023.1184373

Khoo, H.-E., Prasad, K. N., Kong, K.-W., Jiang, Y., & Ismail, A. (2011). Carotenoids and Their Isomers: Color Pigments in Fruits and Vegetables. *Molecules*, *16*(2), Article 2. https://doi.org/10.3390/molecules16021710

Kirti, K., Amita, S., Priti, S., Mukesh Kumar, A., & Jyoti, S. (2014). Colorful World of Microbes: Carotenoids and Their Applications. *Advances in Biology*, *2014*, e837891. https://doi.org/10.1155/2014/837891

Kopec, R. E., Schweiggert, R. M., Riedl, K. M., Carle, R., & Schwartz, S. J. (2013). Comparison of high-performance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quantitation of carotenoids, retinyl esters, α-tocopherol and phylloquinone in chylomicron-rich fractions of human plasma. *Rapid Communications in Mass Spectrometry : RCM*, *27*(12), 10.1002/rcm.6576. https://doi.org/10.1002/rcm.6576

Krishna, T. P. A., Veeramuthu, D., Maharajan, T., & Soosaimanickam, M. (2023). The Era of Plant Breeding: Conventional Breeding to Genomics-assisted Breeding for Crop Improvement. *Current Genomics*, *24*(1), 24–35. https://doi.org/10.2174/1389202924666230517115912

Lowe, N. M. (2021). The global challenge of hidden hunger: Perspectives from the field. *Proceedings of the Nutrition Society*, *80*(3), 283–289. https://doi.org/10.1017/S0029665121000902

Meléndez-Martínez, A. J., Mapelli-Brahm, P., Hornero-Méndez, D., & Vicario, I. M. (2019). *Structures, Nomenclature and General Chemistry of Carotenoids and Their Esters*. https://doi.org/10.1039/9781788015851-00001

Meléndez-Martínez, A. J., Vicario, I. M., & Heredia, F. J. (2007). Pigmentos carotenoides: Consideraciones estructurales y fisicoquímicas. *Archivos Latinoamericanos de Nutrición*, *57*(2), 109–117.

Ménard, G., Biais, B., Prodhomme, D., Ballias, P., Petit, J., Just, D., Rothan, C., Rolin, D., & Gibon, Y. (2013). Chapter Nine—High-Throughput Biochemical Phenotyping for Plants. In D. Rolin (Ed.), *Advances in Botanical Research* (Vol. 67, pp. 407–439). Academic Press. https://doi.org/10.1016/B978-0-12-397922-3.00009-5

Monk, R., Franks, C., & Dahlberg, J. (2014). Sorghum. In *Yield Gains in Major U.S. Field Crops* (pp. 293–310). John Wiley & Sons, Ltd. https://doi.org/10.2135/cssaspecpub33.c11

Nagarajan, J., Ramanan, R. N., Raghunandan, M. E., Galanakis, C. M., & Krishnamurthy, N. P. (2017). Chapter 8—Carotenoids. In C. M. Galanakis (Ed.), *Nutraceutical and Functional Food Components* (pp. 259–296). Academic Press. https://doi.org/10.1016/B978-0-12-805257-0.00008-9

National Sorghum Producers. (2021). *The Global Significance of Sorghum—Africa*. National Sorghum Producers. https://sorghumgrowers.com/magazine/the-global-signficance-of-sorghum-africa/

Nkouaya Mbanjo, E. G., Hershberger, J., Peteti, P., Agbona, A., Ikpan, A., Ogunpaimo, K., Kayondo, S. I., Abioye, R. S., Nafiu, K., Alamu, E. O., Adesokan, M., Maziya-Dixon, B., Parkes, E., Kulakow, P., Gore, M. A., Egesi, C., & Rabbi, I. Y. (2022). Predicting starch content in cassava fresh roots using near-infrared spectroscopy. *Frontiers in Plant Science*, *13*, 990250. https://doi.org/10.3389/fpls.2022.990250

Ofori, K. F., Antoniello, S., English, M. M., & Aryee, A. N. A. (2022). Improving nutrition through biofortification–A systematic review. *Frontiers in Nutrition*, *9*, 1043655. https://doi.org/10.3389/fnut.2022.1043655

Ortiz, D., & Ferruzzi, M. G. (2019). Identification and Quantification of Carotenoids and Tocochromanols in Sorghum Grain by High-Performance Liquid Chromatography. *Methods in Molecular Biology (Clifton, N.J.)*, *1931*, 141–151. https://doi.org/10.1007/978-1-4939-9039-9_10

Oxley, A., Berry, P., Taylor, G. A., Cowell, J., Hall, M. J., Hesketh, J., Lietz, G., & Boddy, A. V. (2014). An LC/MS/MS method for stable isotope dilution studies of β-carotene bioavailability, bioconversion, and vitamin A status in humans[S]. *Journal of Lipid Research*, *55*(2), 319–328. https://doi.org/10.1194/jlr.D040204

Pieruschka, R., & Poorter, H. (2012). Phenotyping plants: Genes, phenes and machines. *Functional Plant Biology: FPB*, *39*(11), 813–820. https://doi.org/10.1071/FPv39n11_IN

Popescu, M., Iancu, P., Pleșu, V., Bîldea, C. S., & Todasca, C. M. (2022). Different spectrophotometric methods for simultaneous quantification of lycopene and β-carotene from a binary mixture. *LWT*, *160*, 113238. https://doi.org/10.1016/j.lwt.2022.113238

Rajendran, V., Pu, Y. S., & Chen, B. H. (2005). An improved HPLC method for determination of carotenoids in human serum. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *824*(1–2), 99–106. https://doi.org/10.1016/j.jchromb.2005.07.004

Reynolds, M., Chapman, S., Crespo-Herrera, L., Molero, G., Mondal, S., Pequeno, D. N. L., Pinto, F., Pinera-Chavez, F. J., Poland, J., Rivera-Amado, C., Saint Pierre, C., & Sukumaran, S. (2020). Breeder friendly phenotyping. *Plant Science*, *295*, 110396. https://doi.org/10.1016/j.plantsci.2019.110396

Riggi, E. (2010). Recent Patents on the Extraction of Carotenoids. *Recent Patents on Food, Nutrition & Agriculture*, *2*(1), 75–82.

Rivera-Madrid, R., Carballo-Uicab, V. M., Cárdenas-Conejo, Y., Aguilar-Espinosa, M., & Siva, R.

(2020). 1—Overview of carotenoids and beneficial effects on human health. In C. M. Galanakis (Ed.), *Carotenoids: Properties, Processing and Applications* (pp. 1–40). Academic Press. https://doi.org/10.1016/B978-0-12-817067-0.00001-4

Rodriguez-Concepcion, M., Avalos, J., Bonet, M. L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, D., Limon, M. C., Meléndez-Martínez, A. J., Olmedilla-Alonso, B., Palou, A., Ribot, J., Rodrigo, M. J., Zacarias, L., & Zhu, C. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Progress in Lipid Research*, *70*, 62–93. https://doi.org/10.1016/j.plipres.2018.04.004

Saini, R. K., & Keum, Y.-S. (2018a). Carotenoid extraction methods: A review of recent developments. *Food Chemistry*, *240*, 90–103. https://doi.org/10.1016/j.foodchem.2017.07.099

Saini, R. K., & Keum, Y.-S. (2018b). Carotenoid extraction methods: A review of recent developments. *Food Chemistry*, *240*, 90–103. https://doi.org/10.1016/j.foodchem.2017.07.099

Sander, L. C., Sharpless, K. E., Craft, N. E., & Wise, S. A. (1994). Development of engineered stationary phases for the separation of carotenoid isomers. *Analytical Chemistry*, *66*(10), 1667–1674. https://doi.org/10.1021/ac00082a012

Sankaran, S., Quirós, J. J., & Miklas, P. N. (2019). Unmanned aerial system and satellite-based high resolution imagery for high-throughput phenotyping in dry bean. *Computers and Electronics in Agriculture*, *165*, 104965. https://doi.org/10.1016/j.compag.2019.104965

Sartas, M., Schut, M., Proietti, C., Thiele, G., & Leeuwis, C. (2020). Scaling Readiness: Science and practice of an approach to enhance impact of research for development. *Agricultural Systems*, *183*, 102874. https://doi.org/10.1016/j.agsy.2020.102874

Scott, K. J. (2001). Detection and Measurement of Carotenoids by UV/VIS Spectrophotometry. *Current Protocols in Food Analytical Chemistry*, *00*(1), F2.2.1-F2.2.10. https://doi.org/10.1002/0471142913.faf0202s00

Šesták, Z. (2004). Britton, G., Liaaen-Jensen, S., Pfander, H. (ed.): Carotenoids. Handbook. *Photosynthetica*, *42*(2), 186–186. https://doi.org/10.1023/B:PHOT.0000040641.40049.19

Shi, C., Zhao, L., Zhang, X., Lv, G., Pan, Y., & Chen, F. (2019). Gene regulatory network and abundant genetic variation play critical roles in heading stage of polyploidy wheat. *BMC Plant Biology*, *19*(1), 6. https://doi.org/10.1186/s12870-018-1591-z

Somegowda, V. K., Diwakar Reddy, S. E., Gaddameedi, A., Kiranmayee, K. N. S. U., Naravula, J., Kavi Kishor, P. B., & Penna, S. (2024). Genomics breeding approaches for developing *Sorghum bicolor* lines with stress resilience and other agronomic traits. *Current Plant Biology*, *37*, 100314. https://doi.org/10.1016/j.cpb.2023.100314

Strati, I. F., & Oreopoulou, V. (2011). Effect of extraction parameters on the carotenoid recovery from tomato waste. *International Journal of Food Science & Technology*, *46*(1), 23–29. https://doi.org/10.1111/j.1365-2621.2010.02496.x

Sun, T., Rao, S., Zhou, X., & Li, L. (2022). Plant carotenoids: Recent advances and future perspectives. *Molecular Horticulture*, *2*(1), 3. https://doi.org/10.1186/s43897-022-00023-2

Tang, J., Li, X., Zhang, Y., Yang, Y., Sun, R., Li, Y., Gao, J., & Han, Y. (2022). Differential Flavonoids and Carotenoids Profiles in Grains of Six Poaceae Crops. *Foods*, *11*(14), 2068. https://doi.org/10.3390/foods11142068

Tanwar, R., Panghal, A., Chaudhary, G., Kumari, A., & Chhikara, N. (2023). Nutritional, phytochemical and functional potential of sorghum: A review. *Food Chemistry Advances*, *3*, 100501. https://doi.org/10.1016/j.focha.2023.100501

Trono, D. (2019). Carotenoids in Cereal Food Crops: Composition and Retention throughout Grain Storage and Food Processing. *Plants*, *8*(12), 551. https://doi.org/10.3390/plants8120551

Wali, N., Agho, K., & Renzaho, A. M. (2022). Hidden hunger and child undernutrition in South Asia: A meta-ethnographic systematic review. *Asia Pacific Journal of Clinical Nutrition*, *31*(4), 713–739. https://doi.org/10.3316/informit.814683734292935

Wiseman, E. M., Bar-El Dadon, S., & Reifen, R. (2017). The vicious cycle of vitamin a deficiency: A review. *Critical Reviews in Food Science and Nutrition*, *57*(17), 3703–3714. https://doi.org/10.1080/10408398.2016.1160362

World Health Organization. (2009). *Global prevalence of vitamin A deficiency in populations at risk 1995-2005: WHO global database on vitamin A deficiency*. https://www.who.int/publications-detail-redirect/9789241598019

Xu, J., Lin, J., Peng, S., Zhao, H., Wang, Y., Rao, L., Liao, X., & Zhao, L. (2023). Development of an HPLC-PDA Method for the Determination of Capsanthin, Zeaxanthin, Lutein, β-Cryptoxanthin and β-Carotene Simultaneously in Chili Peppers and Products. *Molecules*, *28*(5), 2362. https://doi.org/10.3390/molecules28052362

Yabuzaki, J. (2017). Carotenoids Database: Structures, chemical fingerprints and distribution among organisms. *Database*, *2017*, bax004. https://doi.org/10.1093/database/bax004

Yao, W., Li, G., Yu, Y., & Ouyang, Y. (2018). funRiceGenes dataset for comprehensive understanding and application of rice functional genes. *GigaScience*, *7*(1), gix119. https://doi.org/10.1093/gigascience/gix119

Yu, N., Li, L., Schmitz, N., Tian, L. F., Greenberg, J. A., & Diers, B. W. (2016). Development of methods to improve soybean yield estimation and predict plant maturity with an unmanned aerial vehicle based platform. *Remote Sensing of Environment*, *187*, 91–101. https://doi.org/10.1016/j.rse.2016.10.005

Zhai, S., Xia, X., & He, Z. (2016). Carotenoids in Staple Cereals: Metabolism, Regulation, and Genetic Manipulation. *Frontiers in Plant Science*, *7*. https://doi.org/10.3389/fpls.2016.01197

Zhang, H., Ge, Y., Xie, X., Atefi, A., Wijewardane, N. K., & Thapa, S. (2022). High throughput analysis of leaf chlorophyll content in sorghum using RGB, hyperspectral, and fluorescence imaging and sensor fusion. *Plant Methods*, *18*(1), 60. https://doi.org/10.1186/s13007-022-00892-0

Chapter 2: Accelerating Sorghum Carotenoid Quantification: Innovations in High-Throughput Methodology Development.

Introduction

The world population is projected to grow exponentially to around 10 billion by 2050; consequently, to fulfill the growing demand levels, crop yield and nutrient quality need to be improved (Hunter et al., 2017). This is especially essential in addressing Vitamin A deficiency that afflicts millions worldwide.

Genetic improvement in sorghum through breeding is the most accepted way to increase PVACs to biologically relevant levels, and this has progressed with the rapid advancement of functional genomics tools. This has allowed the sequencing and identification of genes that influence traits of interest in an increasing number of crop genomes (Shi et al., 2019; Yao et al., 2018). However, current information cannot be used efficiently to understand gene associations underlying specific traits due to inefficient phenotyping methods (Jin et al., 2021). On the other hand, at the field level, phenotyping is also a limiting factor for progeny identification and selection because existing techniques are often too expensive and require specialized equipment and personnel that limit their adoption, as well as do not allow for large-scale sample analysis (Cudjoe et al., 2023; Galli et al., 2020; Zhang et al., 2022).

Regardless of the phenotyping method, the critical consideration is whether the data generated is informative and meets the requirements for advancing genetic improvement. In this context, there is no one-size-fits-all approach or ideal methodology. The ultimate goal is to select methods that provide accurate and reliable data, enabling breeders to make informed decisions and accelerate crop improvement.

Different methodologies have been tested for quantifying carotenoids in various crops (Alamu et al., 2021; Jaramillo et al., 2018) but have yet to be introduced in a sorghum breeding

program. This may be because many of the techniques are in the validation process or require adjustments in the prediction models. Also, breeders have yet to enter the era of change, and transitioning from conventional to new developments has often been challenging.

Many reasons exist for the need to validate analytical procedures, including the quality of science and ensuring that the methods to be released correctly work for their intended use (ICH, 2024). Although this task is time-consuming, validation is required for all analytical techniques to be released (Chan, 2011). Several parameters must be considered and documented, including accuracy, sensitivity, specificity, reproducibility, and robustness.

Additionally, during the validation process, it is vital to pay close attention to the extraction methods to maintain the quality of the final quantification (Bijttebier et al., 2016). Compared to other analytes, carotenoid extraction is not generally standardized (Riggi, 2010), and their recovery in sorghum grain is limited because of several factors, including physical barriers of the samples and the presence of carotenoids with varying degrees of polarity. Furthermore, carotenoids are molecules that require careful handling because they are prone to be degraded to heat, light, and acids (Saini & Keum, 2018a)

During the extraction process, samples require grinding and homogenization to facilitate the access of organic solvents in the matrix (Saini & Keum, 2018). Selecting the appropriate solvent option is critical for an efficient extraction (Strati & Oreopoulou, 2011). For nonpolar or esterified carotenoids, nonpolar and medium-polar solvents are usually selected. Meanwhile, polar solvents are usually the best option for extracting carotenoids that contain oxygenated groups in their structure (Amorim-Carrilho et al., 2014). Some reports mention that simultaneous extraction of polar and nonpolar can be achieved when a mixture of acetone, ethanol/methanol, and hexane is used as the solvent. On the other hand, ethanol and acetone are efficient when high water is present (Saini & Keum, 2018a).

In addition to validation, phenotyping methodologies must be simple and accessible. This requires ingenuity and strategy to adapt complex techniques to more user-friendly and high-throughput versions. The more intuitive and easy to use, the more the end user will accept the method. For instance, microplate technology could help with the low-cost measurement of the concentration of carotenoids in sorghum breeding programs at a large scale (Ménard et al., 2013). A microplate reader enables the measurement of the concentration of a sample in each well of the plate as you perform a series of experiments without having to set up each one individually.

In this study, we aimed to develop and validate a simple and rapid method for carotenoid extraction and UV-VIS spectrophotometric detection in a 96-well plate format. We aimed to facilitate the phenotyping of thousands of progeny in a breeding program to efficiently select and breed high carotenoid varieties. We hypothesized that our high-throughput (HTP) method would effectively extract and measure TCC. Additionally, we optimized an HPLC-PDA method to quantify lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene in sorghum grain. This method will be the gold standard for comparison with our HTP phenotyping method. This promising HTP method holds the potential to rapidly screen a large number of samples, thereby assisting breeders in making more efficient selections for carotenoid biofortification.

Methods

Determination of initial concentration of carotenoid standards stock solution

Since carotenoids are light-sensitive, all the procedures were carried out under yellow light to avoid photoisomerization reactions, and all the glassware was amber. The vials containing the standards were removed from the freezer and allowed to reach room temperature. Commercially available standards of lutein (Sigma), zeaxanthin (Sigma),

β-cryptoxanthin (Sigma), and α-carotene (Sigma) were reconstituted and mixed by vortex individually with 1 mL of ethyl acetate: ethanol (50:50 v/v) HPLC grade until getting a final concentration of 1 mg/mL. This procedure was carried out with extreme care since each vial contains only 1 mg of standard. The β-carotene (Sigma), which came already resuspended in THF: Ethanol with 0.1% BHT (70:30) (w/v) at a final 100 ppm concentration, was transferred to an appropriately labeled vial. All the standards were kept in the freezer at - 80 C for future analysis.

To determine the actual initial concentration of the stock solutions, 200 µL of each was resuspended in 3000 µL of the solvent indicated in Table 2.1, and 1.5 mL of the solution was transferred to a 1 cm quartz cuvette. The absorbance of the solutions was recorded using a single-beam spectrophotometer (Jasco V-730) at the wavelength indicated in Table 2.1 for each standard (Rodriguez-Amaya et al. 2001).

Table 2.1. ελ the molar absorption coefficient (Lmol−1cm−1)

Carotenoid	Solvent/Blank	Wavelength λ (nm)	ελ
Lutein	Ethanol	445	2550
Zeaxanthin	Ethanol	449	2540
β-cryptoxanthin	Hexane	450	2460
α- carotene	Hexane	445	2710
β- carotene	Hexane	450	2620

The concentration of the standards was calculated using the formula:

$$A = \varepsilon\lambda \, x \, C \, x \, D$$

Where:

A = Absorbance registered in the spectrophotometer,

ελ = Molar absorption coefficient ($Lmol^{-1}cm^{-1}$) of the carotenoids at specific wavelength λ (Refer to table 2.1),

C = Molar concentration (mol/L) of the carotenoid,

D = Width of the cuvette.

After the concentration was determined, the standards were stored at -80 °C until the calibration curve was prepared. The standard stock initial concentration was calculated every time a new calibration curve was constructed.

Preparation of Internal Standard (IS)

The commercially available retinyl palmitate 46959-U (Sigma) was utilized as the internal standard. It was reconstituted and mixed in an ultrasonic bath containing ice water to a final concentration of 20 mg/mL in ethyl acetate: ethanol (50:50 v/v). The stock solution was stored at - 80 °C in the freezer for future dilutions. A 20 ppm solution (IS 20 ppm) was prepared using ethyl acetate: ethanol (50:50 v/v) from the stock solution. The solution was stored at - 80 °C in the freezer for further analysis.

Preparation of calibration curve including Internal Standard (IS)

Using the stock concentrations determined in "*Determination of standard stock initial concentration,"* the standards lutein, zeaxanthin, β-cryptoxanthin, and α- carotene were resuspended to a final concentration of 100 ppm. A 100 ppm Master Mix (MM100) of the four standards was prepared by resuspending 50 μL of each stock solution of lutein, zeaxanthin, β-cryptoxanthin, and α-carotene in 300 μL of the solvent containing ethyl acetate: ethanol (50:50 v/v). Reaching a final volume of 500 μL. A calibration curve with eight points using MM100, β-carotene 100 ppm, and IS 20 ppm was constructed, as shown in Table 2.2. The working

standard solutions were stored in pre-labe amber HPLC vials with the corresponding standard number. The working standard solutions at different concentrations were prepared every time an experiment was conducted.

Table 2.2. Preparation of working standards for calibration curves

STD #	MM100 (µL)	β-carotene 100 ppm (µL)	Internal standard (µL)	Solvent (µL)	STD 1 (µL)	Final Concentration (ppm)	
						lutein, zeaxanthin, β-cryptoxanthin, and α-carotene	β-carotene
1	200	100	0	1700	0	10	5
2	50	25	40	885	0	5	2.5
3	25	10	40	925	0	2.5	1
4	10	5	40	945	0	1.0	0.5
5	0	0	40	910	50	0.5	0.25
6	0	0	40	935	25	0.25	0.1
7	0	0	40	950	10	0.1	0.05
8	0	0	40	955	5	0.05	0.025

Extraction of carotenoids

Extraction with saponification

Extraction was based on a method modified by Gupta, Sreelakshmi, and Sharma (2015) (Gupta et al., 2015). Dry whole-grain sorghum was ground into fine powder flour in Omni Bead Ruptor 24 Elite. To reduce carotenoid degradation, extraction was carried out under a yellow-light environment. For extraction, 100 ± 0.5 mg of flour and 200 mg of ascorbic acid were carefully weighed in a 1.5 mL tube. Then, 400 µL of the extraction buffer was added (absolute ethanol, 1% BHT), vortexed for 1 min, and the tubes were placed in a thermal block heater at 85 °C for 5 min. The tubes were removed from the heat, and 20 µL KOH (80% w/v in H2O) was

added and vortexed for 1 min. Then, the tubes were returned to the thermal block for 15 minutes with intermittent shaking every 5 minutes.

The samples were cooled to room temperature and centrifuged for 5 min at 1900 x g. The supernatant was transferred to a clean, new 1.5 mL tube.

Another extraction from the pellet was performed, and the supernatants were combined. The combined supernatant was centrifuged at 5000 x g to separate the solvent containing carotenoids from the remaining particles (Figure 2.1).

The samples were dried at room temperature with a gentle stream of N_2 for ~35 minutes, and finally, the carotenoids were re-suspended in 100 µL of a solution of ethanol: ethyl acetate (50:50 v/v). The mixture was shaken for 3 minutes to ensure complete dissolution. After the resuspension, 60 µL of the samples were carefully transferred into an amber HPLC vial containing glass inserts.

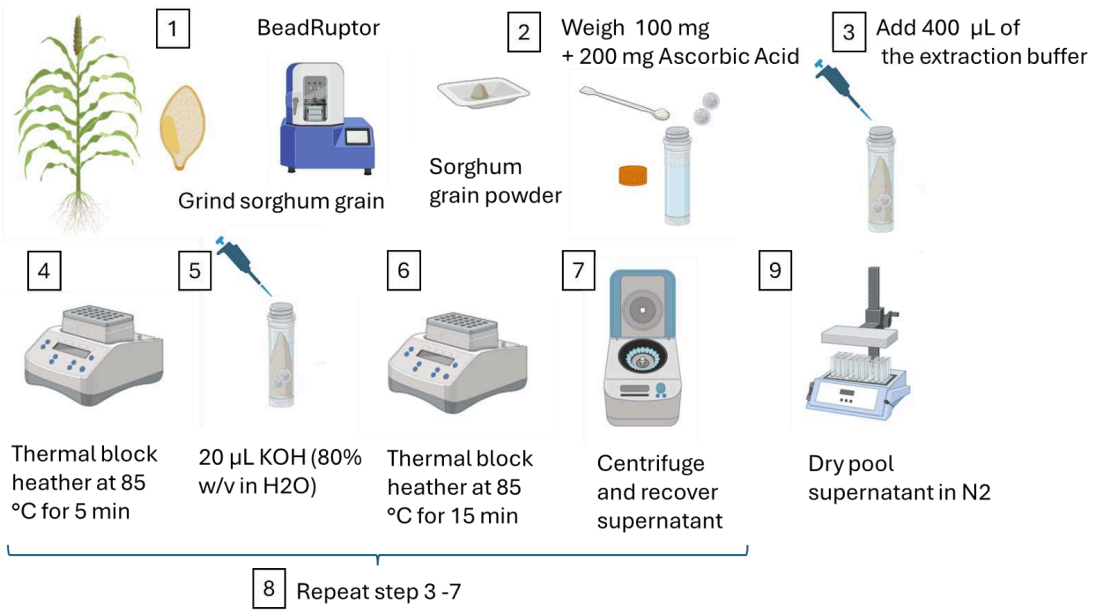


Figure 2.1. Workflow for the saponification extraction method

Solid-liquid High-throughput Extraction

The dried sample was ground into powder using a stainless steel analytical mill (IKA A11 basic) and then stored in 5 mL screw-cap self-standing tubes at −80 °C until needed. The carotenoids were extracted using a modified method previously described by Tang et al. 2022 for Poaceae crops (Tang et al., 2022)

Briefly, 300 ± 0.05 mg of powder of each sample was weighed in a 2 mL Screw-Cap Self-Standing microtube, and then 1000 µL of the extraction solvent 1 containing 0.2 ppm of IS and 0.01% BHT in ethyl acetate: acetone: ethanol (1:1:1, v/v/v)) and two stainless steel beads were added.

The tubes were carefully placed into the homogenizer Omni Bead Ruptor 24 Elite for 5 minutes at 2.5 m/s, having a dwelling time of 25 sec after the first 2.5 min. The samples were taken out from the homogenizer and centrifuged for 4 min at 13000 x g, and the supernatant was transferred into a 1.5 mL Eppendorf tube, which was correctly labeled. The extraction from the pellet was carried out one more time with 700 µL of MTBE. The combined supernatant was centrifuged for 30 seconds at maximum speed and transferred into a new 2 mL Screw-Cap Self-Standing microtube. The samples were reconcentrated using a stream of N_2 for 35 minutes at room temperature until an orange pellet was seen in the bottom of the tube. The dried sample can be kept at - 80 °C.

The concentrated samples were dissolved with 250 µL of a mixed ethyl acetate solution and ethanol (50:50 v/v) for HPLC analysis and the 96-well plate reader method, respectively. The samples were vortexed for 10 sec and then centrifuged for 30 sec at maximum speed. Around 50 - 60 µL of the solution was transferred into a clean glass insert housed in an amber HPLC vial and 150 µL in each well of the plate (Figure 2.2).

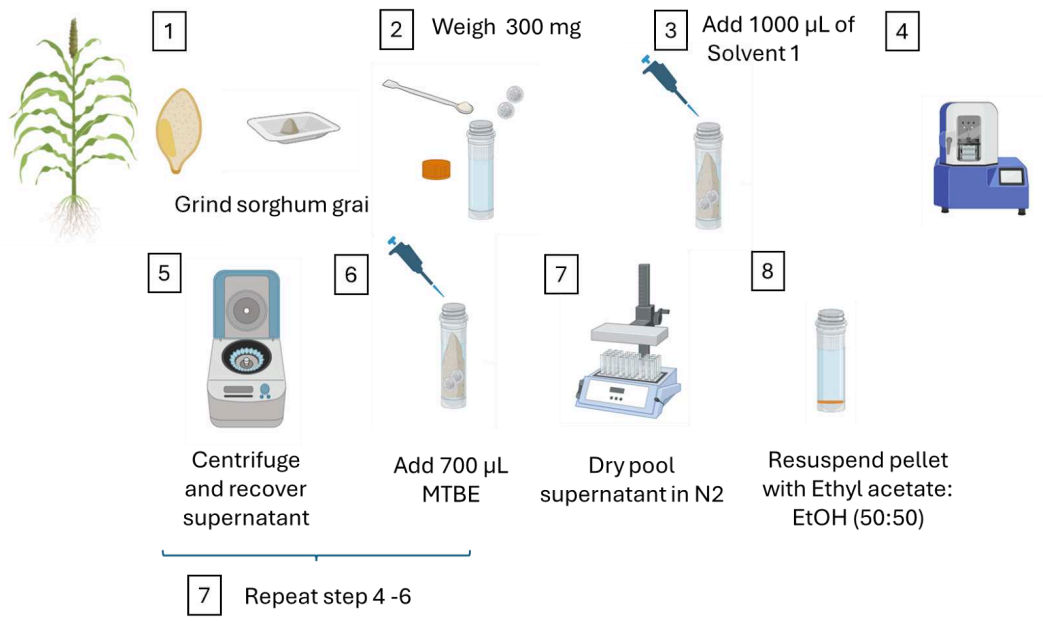


Figure 2.2. Workflow for the solid-extraction method

HPLC-PDA analysis of carotenoids

The carotenoids were quantified using reverse phase chromatography RP-HPLC Flexar (PerkinElmer, United States), consisting of a quaternary pump and a photodiode array detector (PDA). A C30 column (150 x 2 mm I.D. S-3 µm; YMC American, Inc.) was utilized for the carotenoid separation, having 7 µL of the sample injected. The mobile phase A was 95% methanol: 1.0 M ammonium acetate (98:2 v/v), and mobile phase B consisted of methyl tertbutyl ether, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v). Carotenoids were resolved using a gradient as described in Table 2.3.

Table 2.3. HPLC gradient conditions

Step	Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (mL/min)	Curve
1	**1.0**	100	0	0.48	0.0
2	**3.0**	20	80	0.48	1.0
3	**1.5**	0	100	0.48	1.0
4	**1.5**	0	100	0.48	0.0
5	**1.0**	100	0	0.48	1.0
6	**6.0**	100	0	0.48	0.0

The column temperature was maintained at 35°C. The eluting peaks were detected at 450 nm for the carotenoids and 325 nm for the internal standard. Quantification was performed using TotalChrom Navigator Version 6.3.4.

Identification and quantification of carotenoid in samples

Peaks were identified by comparing the retention times and UV–Vis spectral data with those of the corresponding standards when possible. Samples and standard data were normalized using the area obtained from the IS, and the concentration of each analyte was calculated using the calibration curve of the corresponding standard as a reference. All standard solutions were prepared as described above in the standard preparation section. Five-point external standard curves were constructed for the standard mix. Carotenoid concentrations were then calculated using a linear regression $y = mx + b$, where y = concentration and x = area of the five-point standard curve/areas of IS. The regression equation and correlation coefficient (R^2) were obtained.

Evaluation of HPLC methods performance

Assay performance was evaluated in selectivity, linearity, percentage of recovery, inter-day and intra-day reproducibility, limits of detection (LOD), and quantitation (LOQ). For these, three freeze-dried low-concentration carotenoid line samples (LCCS) (accession numbers: PI511015, PI511018, PI510951) previously analyzed by Cruet-Burgos et al., 2019, were selected. The samples were milled and mixed together in a ratio of 1:1:1 and kept at -80 °C for further analysis.

Percent of recovery and extraction reproducibility were compared between (1) saponification and (2) solid and liquid extraction methods using LCCS spiked with 0.25, 0.5, 1.0, and 2.5 ppm for lutein, zeaxanthin, β-cryptoxanthin, and α-carotene ppm and 0.1, 0.25, 0.5, and 1.0 ppm for β-carotene.

The percentage of recovery of each carotenoid was calculated by $R(\%) = [(Ct - Ci)/C] \times 100$, where R(%) is the percent of recovery, Ct is the total carotenoid content in the spiked sample, Ci is the initial carotenoid content in the sample, and C is the amount of carotenoid standard added to the sample. Extraction reproducibility (% CV) was evaluated on the spike LCCs pooled samples at the same concentrations using three technical replicates. The accepted parameters for the study were 80 - 125% for R(%) and < 20 for % CV, respectively.

Interday and intraday variation (% CV) were evaluated over four consecutive days at 0.25, 0.5, 1.0, and 2.5 ppm for lutein, zeaxanthin, β-cryptoxanthin, and α-carotene, whereas for β-carotene at 0.1, 0.25, 0.5, 1.0, and 2.5 ppm using the solid-liquid method. Four technical replicates were analyzed for the different concentrations in the interday variation analysis. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated for each compound with concentrations ranging from 0.05 to 0.5 ppm for lutein, zeaxanthin, β-cryptoxanthin, and α-carotene and from 0.025 to 0.25 ppm for β-carotene as follows: LOD = standard deviation (blank) * 3/slope of the regression line and LOQ = standard deviation (blank) * 10/slope of the

regression line. Similarly, a selectivity test was conducted to evaluate that the solvents do not interfere with the ability to see the carotenoids on the chromatogram.

Preparation and Evaluation of Quality Control (QC) Sample

A QC sample was prepared by pooling previously analyzed high carotenoid line samples (HCS): PI585348 and PI585347 (Cruet-Burgos et al., 2019). For its preparation, 5 g of each HCS was weighed into a 50 mL Falcon tube protected with aluminum foil. The mixture was shaken for 5 min and then kept at -80 °C for future analysis. The QCs were injected every ten samples throughout the sequence created during the HPLC method performance evaluation. The following metrics were monitored: concentration (CV < 20%) and retention time (±1%). If QC samples failed, the analysis was stopped until adequate QC was achieved—similarly, a principal component analysis (PCA) was used to evaluate the similarity of the QC injections.

Evaluation of HTP method performance

For the assay performance, the pool samples of LCCS from the "E*valuation of extraction and HPLC methods performance"* section were selected.

Using three technical replicates, extraction reproducibility (% CV) was evaluated on the spiked samples at the same concentrations (2.5 ppm). The accepted parameters for the study were 80 - 125% for R(%) and >20 for % CV, respectively.

Interday and intraday variation (% CV) were evaluated over four consecutive days, having three technical replicates per day, as described in the previous section for the HPLC method. In addition, LOD and LOQ were calculated for the total concentration of carotenoids (TCC). Polypropylene 96-well plates were used to evaluate the performance of the HTP method.

HTP analysis of carotenoids

The samples were quantified using a 96-well UV-VIS plate reader BIOTEK PowerWave XS2 at 450 nm, having 150 µL of the sample in each well. The absorbance of the samples was corrected using ethyl acetate: ethanol (50:50 v/v) as a blank. The TCC of each sample was calculated using the calibration curve generated from the five standards as a reference. The standard solutions were prepared as described above in the standard preparation section. Five-point external standard curves (ranging from 0.25, 0.5, 1.0, 2.5, and 5 ppm) were constructed for the standard mix. Carotenoid concentrations were then calculated using a linear regression y = ax + b, where y = TCC and x =Absorbance. The regression equation and correlation coefficient (R^2) were calculated.

Results

HPLC method Validation for carotenoids in sorghum grain

The HPLC conditions were evaluated regarding column type, temperature, and mobile phases to obtain the best chromatographic conditions. Five methods were adapted and tested to separate the carotenoids efficiently—different columns, such as C18 and C30, and various mobile phases (Table 2.4). The most efficient separation conditions were achieved with a YMC C30 3 µm 2.0 mm × 150 mm column at 35 °C and a gradient elution of methanol (95%): 1.0 M ammonium acetate (98:2 v/v) and methyl tertbutyl ether, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v). The run time per sample was significantly reduced to 13 min. The UV wavelength of the PDA for the quantification of each compound was selected at 450 nm based on retention time and UV spectra (190 nm - 700 nm) compared with those of the standards. Using the optimized method, the five compounds were separated without coelution from other components. A typical chromatogram of standard solutions of carotenoids is shown in Figure 2.4.

Table 2.4. Methods tested

Method	Cruet-Burgos et al., 2019	Jin et al., 2017	Marinova et al., 2006	Tan et al., 2017
Run time	20 min	12 min	14 min	18 min
Column	Eclipse Plus C18 column (1.8 µm, 2.6 mm × 50 mm)	C18 column (4.6 × 250 mm, 5 µm)	C18 column (4.6 × 250 mm, 5 µm) column	YMC C30 3 µm 2.0 mm × 150 mm column, with a YMC carotenoid guard column (2.0 × 23 mm)
Mobile phase A	Methanol/H20 (98:2, v/v)	Isopropanol (65%)	0.1 % BHT and 0.05 % triethylamine (TEA) in acetonitrile: methanol (95:5 v/v)	Methanol: 1.5% ammonium acetate (98:2 v/v)
Mobile phase B	Methanol/H2O (95:5, v/v)	Methanol (35%)	0.1 % BHT and 0.05 % triethylamine (TEA) in acetonitrile:methanol: ethyl acetate (60:20:20 v/v/v)	Methyl tertbutyl ether, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v)
Mobile phase C	Methyl tert-butyl ether (MTBE)			
Elution	Gradient	Isocratic	Gradient	Gradient
Column Temperature	25 °C	30 °C	28 °C	35 °C
Sample Temperature	10 °C	10 °C	7 °C	7°C
Detection	450 nm	450 nm	450 nm	450 nm
Injection volume	10 µl	10 µl	10 µL	7 µL

Linearity was evaluated using a calibration curve to check the ability of the analytical method to obtain a proportional response to the analyte concentration in the sample. A

five-point calibration curve was developed, based on five concentrations from 0.25 to 5 ppm for lutein, zeaxanthin, β-cryptoxanthin, and α-carotene, and from 0.1 to 2.5 ppm for β-carotene. Calibration curves for all compounds have an $R^2 > 0.99$ (see standards calibration curve, Figure. 2.3).

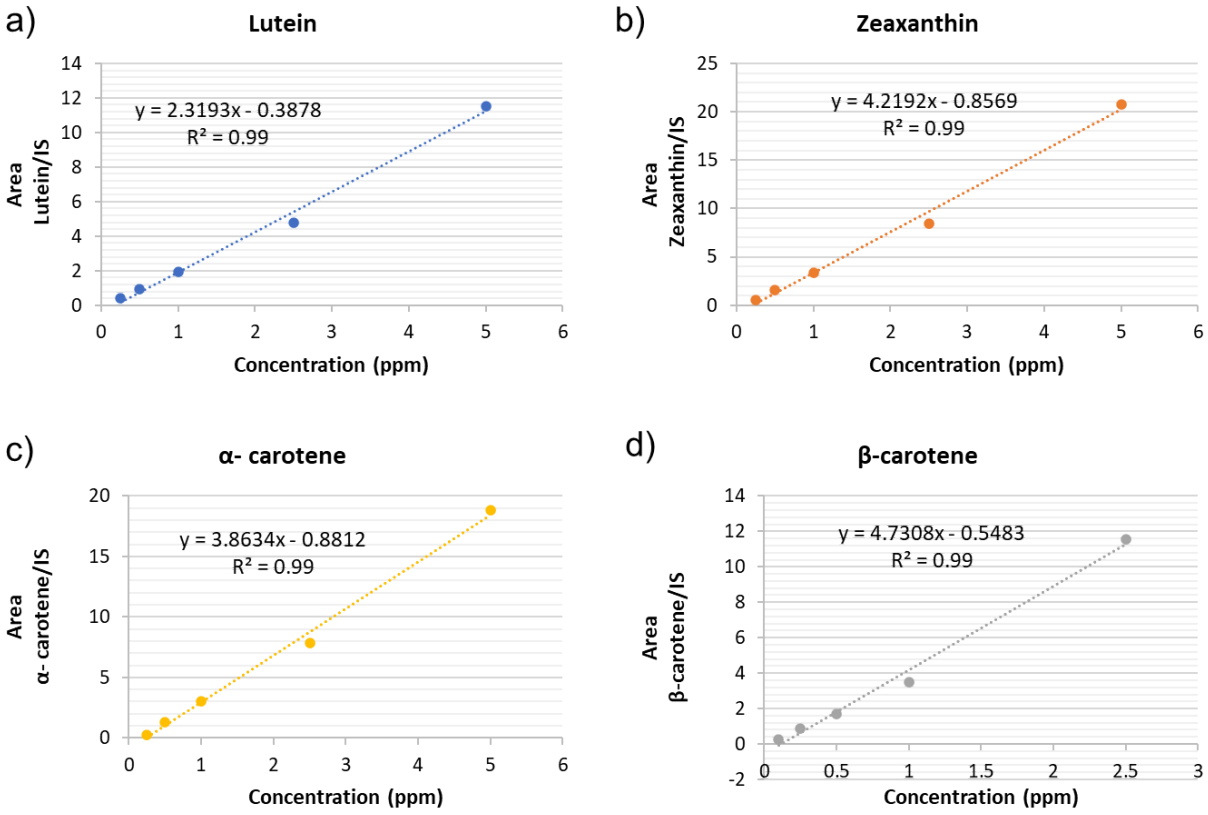


Figure 2.3. Standard calibration curves developed for the HPLC method showed high linearity. Standard calibration curve for a) lutein, b) zeaxanthin, c) α-carotene, and d) β-carotene.

To test the method specificity, blank, mobile phase A, mobile phase B, and extraction solvents were injected and analyzed. The chromatogram obtained was compared to the chromatogram from the standards (Figure 2.4) and a grain sample extract. Results for the grain sample extract showed the characteristic carotenoid peaks (Figure 2.5), which agreed with

those obtained from the standards. The specificity analysis revealed no peak at the retention when the carotenoids eluted, indicating that the solvents and their components do not interfere with or enhance the quantitative determination of carotenoids.

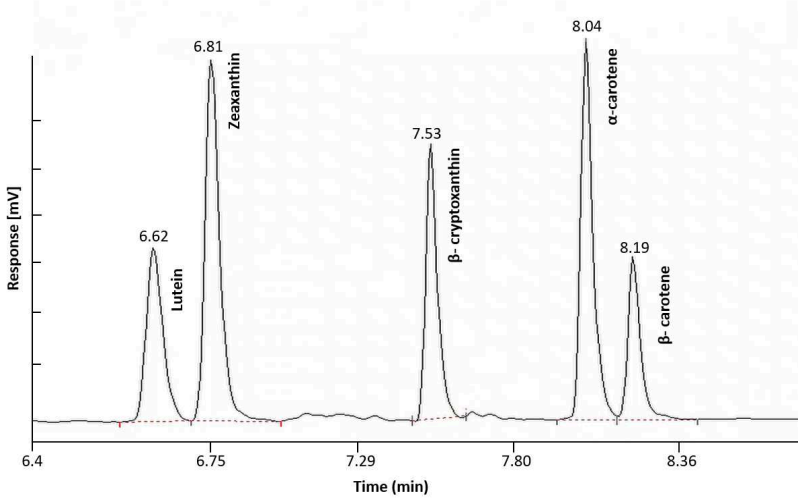


Figure 2.4. HPLC profile of carotenoid standards (5 ppm) recorded at 450 nm. The compounds are (6.62 min) lutein, (6.81 min) zeaxanthin, (7.53 min) β-cryptoxanthin, (8.04 min) α-carotene, (8.19 min) β-carotene.

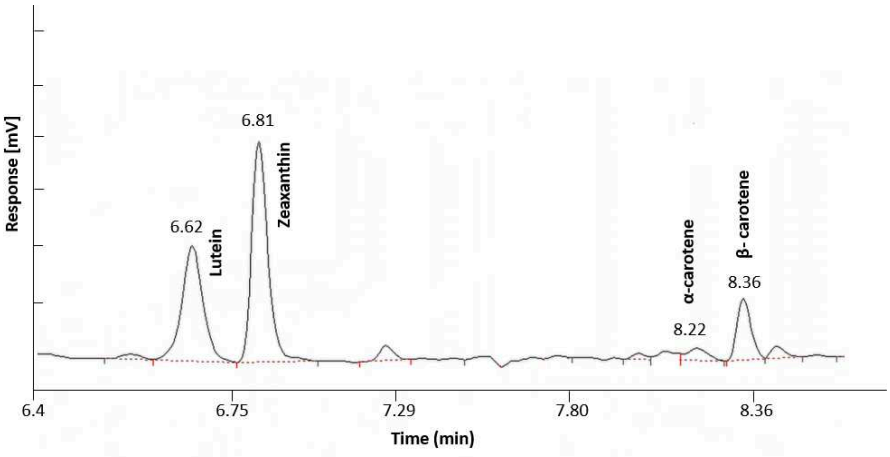


Figure 2.5. HPLC profile of carotenoids from sorghum grain extracts show characteristic peaks. HPLC profile of sample recorded at 450 nm. The compounds are (6.62 min) lutein, (6.81 min) zeaxanthin, β-cryptoxanthin (not present); (8.22 min) α-carotene, (8.36 min) β-carotene.

To assess the reproducibility of carotenoid quantification, both intraday and interday variations were examined using saponification and solid-liquid methods. This evaluation also aimed to identify the method with the highest efficiency in extracting target carotenoids from sorghum grain.

Our results revealed that solid-liquid extraction yielded superior extraction efficiency than saponification, as determined by HPLC analysis. Based on these findings, solid-liquid extraction was chosen as the preferred method for carotenoid extraction in subsequent analyses.

Percent recovery after saponification extraction ranged from 36% to 106% for all compounds, with lutein and zeaxanthin with the highest recovery at 0.5 and 1.0 ppm (106% and 86.5%), as well as β-carotene with more than 90% at 0.25 and 0.5 ppm. For β-cryptoxanthin and α-carotene, the recovery rates were the lowest at the three different concentrations among the compounds. The extraction reproducibility ranged from 5% to 71% CV for all the carotenoids tested at different concentrations. Most samples had a % CV less than 25% at a concentration of 0.5 ppm and 1.0 ppm for lutein, zeaxanthin, and α-carotene, except for β-cryptoxanthin, which had 47.2% at 0.5 ppm. At 0.1 ppm and 0.25 ppm lutein, zeaxanthin, α-carotene, and β-cryptoxanthin and, the % CV was either not measurable (NA) or > 25%. Similar results were obtained with β-carotene when the recovery rate was evaluated at 0.05 and 0.125 ppm (Table 2.5).

Within-run/day (intraday) and between-run/day (interday) % CVs were measured at 0.25, 0.5, and 1 ppm for lutein, zeaxanthin, β-cryptoxanthin and α-carotene, and 0.125, and 0.25 and 0.5 for β-carotene (Table 2.6). Intraday variation ranged from 4% to 158% CV for all the compounds. The two lowest concentrations at which the carotenoids were analyzed had a higher % CV. The interday variation was assessed for four consecutive days, with three technical replicates conducted per concentration each day. The results show that the CV calculated for the analytes ranged from 13% to 46%, with lower variation at the highest

concentrations evaluated. Despite the lower variation, some of the CV% were still >20%; hence, the analysis is not reproducible after the samples underwent saponification extraction.

Table 2.5. Recovery rate R(%) and extraction reproducibility % CVs for saponification extraction (n = 3)

Compound	**0.1 ppm**	**0.25 ppm**	**0.5 ppm**	**1.0 ppm**
Lutein	N/A	103	106	106
%CV	N/A	71	19	13
Zeaxanthin	N/A	82	86.5	86.5
%CV	N/A	81	7	9
β-cryptoxanthin	N/A	36	47.2	47
%CV	N/A	71	48	5
α-carotene	N/A	39	49	49
%CV	N/A	26	24	12
Compound	**0.05 ppm**	**0.125 ppm**	**0.25 ppm**	**0.5 ppm**
β-carotene	N/A	54	92	92
%CV	N/A	92	24	28

Table 2.6. Interday and intraday variation (% CV) were evaluated over 4 consecutive days (n = 3 per day) at 0.25 ppm, 0.5 ppm, and 1 ppm for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and 0.125 ppm, 0.25 ppm, and 0.5 ppm for β-carotene using the saponification method.

No	Analyte	Concentration ppm	Intraday (%CV)	Interday (%CV)
1	**Lutein**	0.25	60	47
		0.5	8	13
		1	19	32
2	**Zeaxanthin**	0.25	7	35
		0.5	22	18
		1	11	34
3	**β-cryptoxanthin**	0.25	34	46
		0.5	15	15
		1	24	28
4	**α-carotene**	0.25	158	NA
		0.5	36	33
		1	4	14
5	**β-carotene**	0.125	NA	NA
		0.25	24	43
		0.5	28	43

The percent of recovery and extraction reproducibility after solid-liquid extraction results are shown in Table 2.7. The recovery percentage ranged from 33% to 109% for all compounds, with lutein, zeaxanthin, and β-carotene with the highest recovery rate. β-cryptoxanthin overall had the lowest percent of the recovery, from 65% to 85% at 0.5 ppm and 1 ppm, respectively. The extraction reproducibility ranged from 2% to 13% CV, with most carotenoids having a % CV less than 20% at the different concentrations tested.

Within-run/day (intraday) and between-run/day (interday) % CVs were measured at 0.25 0.5, and 1.0 for lutein, zeaxanthin, β-crytoxanthin and α-carotene, and 0.125, 0.25, and 0.5 for β-carotene (Table 2.7). Intraday and interday variation ranged from 4% to 15% CV for all compounds, indicating reproducible results (CV<20%) after solid-liquid extraction. In addition, the detection limit (LOD) and quantification limit (LOQ) were calculated for every carotenoid, as described in Table 2.8. The results are comparable with those reported by (Gupta et al., 2015)

Table 2.7. Recovery rate R(%) and extraction reproducibility % CVs for solid-liquid extraction (n = 3)

Compound	0.1 ppm	0.25 ppm	0.5 ppm	1.0 ppm
Lutein	N/A	61	103	106
%CV	N/A	8	7	13
Zeaxanthin	N/A	66	109	96.5
%CV	N/A	2	9	6
β-cryptoxanthin	N/A	58	65	87
%CV	N/A	NA	NA	5
α-carotene	N/A	33	70	89
%CV	N/A	6	13	6
β-carotene	N/A	102	108	92
%CV	N/A	2	6	2

Table 2.8. Limits of detection and quantitation of HPLC method. All values from samples prepared via the solid-liquid extraction method

Analyte	LoD (ppm)	LoQ (ppm)	Reported LoD*	Reported LoQ*
Lutein	0.08	0.26	0.075	0.25
Zeaxanthin	0.04	0.12	0.075	0.25
β-cryptoxanthin	0.06	0.17	0.075	0.25
α-carotene	0.04	0.11	0.12	0.4
β-carotene	0.05	0.16	0.75	0.25

* Gupta et al 2015

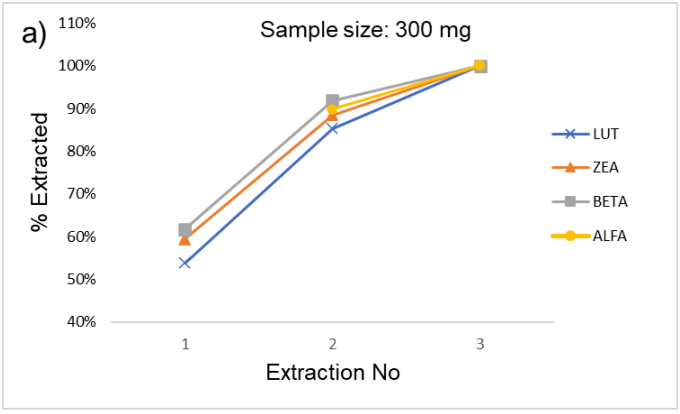
Optimization of solid-liquid extraction method

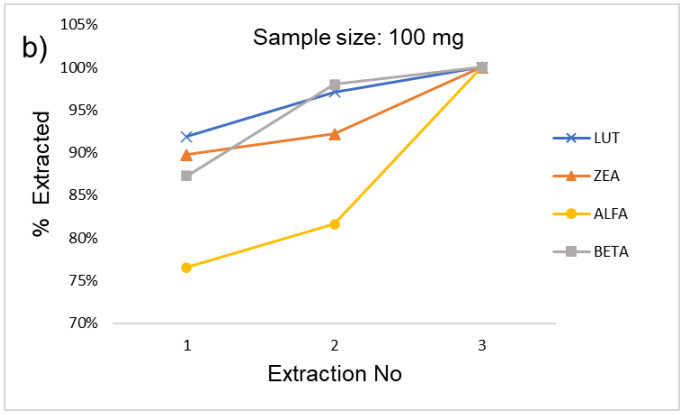
The solid and liquid extraction method was chosen as the most suitable approach primarily because it significantly reduced extraction time and ensured reproducibility (CV>20%). To optimize the research outcomes while minimizing extraction duration, the recovery rate was examined following 1, 2, and 3 washing steps to assess variances. Furthermore, given the utilization of one extract for both HPLC and the HTP method, the sample size was increased from 100 mg to 300 mg. and resuspended in a volume of 250 µL, necessitating the assessment of recovery rates using 300 mg of the sample after 1, 2, and 3 extraction cycles. Extraction efficiency as a function of the number of extraction steps for 100 mg and 300 mg of sorghum is shown in Figure 2.6.

The most accurate results were obtained for samples with high carotenoid content if three successive extractions were performed independently of the sample size. However, for rapid routine analyses, the number of extraction steps could be reduced to one when 100 mg is used, but a reduction of α-carotene recovery is the tradeoff for efficiency (Figure 2.6b). The recovery rate for lutein, zeaxanthin, β-cryptoxanthin, and β-carotene was consistent regardless

of the number of extractions when 100 mg of sample was used (80%-100% CV), indicating that one extraction step is enough for most carotenoids when 100 mg of sample is used for the solid-liquid extraction method.

When 300 mg was utilized, the percentage of extracted carotenoids was lower (50%—60%) when one extraction step was performed, and α-carotene could not be recovered. However, after adding more steps, the percentage recovered increased to 80%—100% (Figure 2.6a), and α-carotene was quantifiable in the HPLC. This confirms that at least two extraction steps were needed when the sample size became larger. Therefore, in order to analyze extracts with both HPLC and UV-VIS, the extractions were performed with 300 mg and two wash steps. Interestingly, variation in yellowness in the final extracts were easily visible (Figure 2.6c).





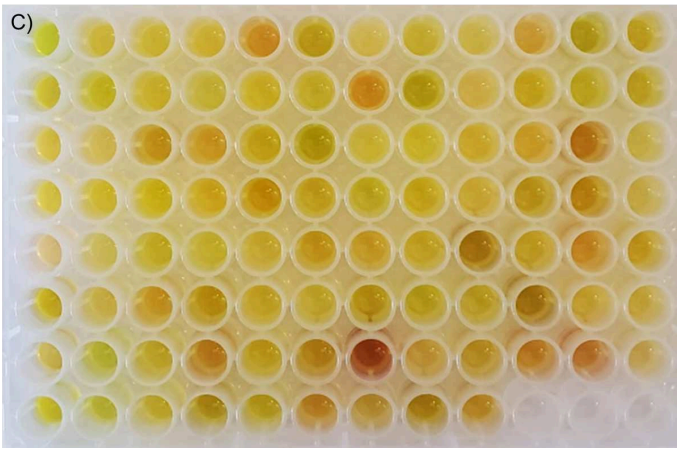


Figure 2.6. Larger starting sample and two washes were ideal for comparing HPLC and UV-VIS. Extraction efficiency as a function of the number of extractions for a) solid-liquid extraction with 300 mg of sample (n = 5), b) solid-liquid extraction with 100 mg of sample, c) carotenoids extracts.

Development of a high-throughput phenotyping (HTP) method for carotenoid quantification

To establish a high-throughput method in a 96-well format for carotenoid quantitation, three different plastic compositions were tested to identify which plastic 1) better withstands the organic solvent in which the samples are resuspended, and 2) creates lower background noise. Commercially available polypropylene, cyclolefin, and polystyrene 96-well plates were tested. The characteristics of the material are described in Table 2.9. Parameters such as melting time, plastic-carotenoid interference at 450 nm, and linearity were evaluated.

Table 2.9. Comparison of material resistance between polypropylene (PP), cycloolefin (COC/COP) and polystyrene (PS)

	Polypropylene (PP)	**Cycloolefin (COC/COP)**	**Polystyrene (PS)**
Temperature stability	High	Moderate	Low
Breakage resistance	High	High	Low
Chemical resistance	High	High	Low
Transparency	Low	High	High

The plastic composition of the tested 96-well plates did not affect the quantification of carotenoids at 450 nm, regardless of the material's properties (Figure 2.7). However, the polystyrene exhibited limited resistance after 20 seconds of being exposed to the solvent used for resuspending carotenoids, so only polypropylene (PS) and cycloolefin (CO/COP) were subjected to further testing.

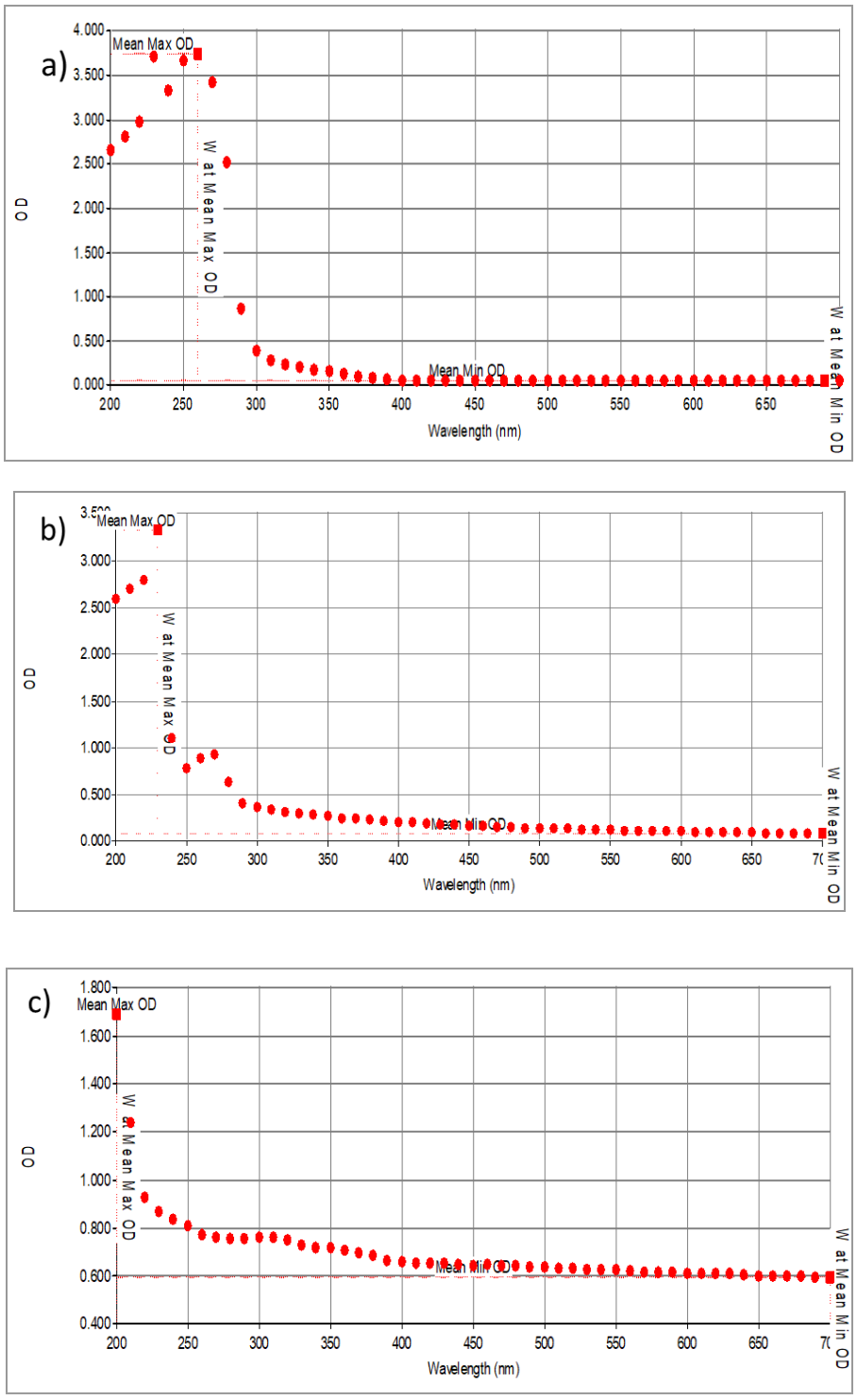


Figure 2.7. Polypropylene plates demonstrated overall superior characteristics for use in the HTP method. UV full spectra of the 96-well plate material: a) polystyrene, b) cycloolefin, and c) polypropylene.

Cycloolefin demonstrated superior characteristics in terms of transparency and chemical resistance. However, the required number of 96-well plates made from this material was not commercially available during the experiment. Despite polypropylene's reduced transparency, it was selected for use in the HTP method due to its chemical resistance, durability, compatibility with organic solvents, and minimal absorbance at 450 nm (Figure 2.7c). A linearity test was conducted using carotenoid standards at various concentrations, resulting in R^2 values exceeding 0.99, regardless of the opacity (Figure 2.8a)

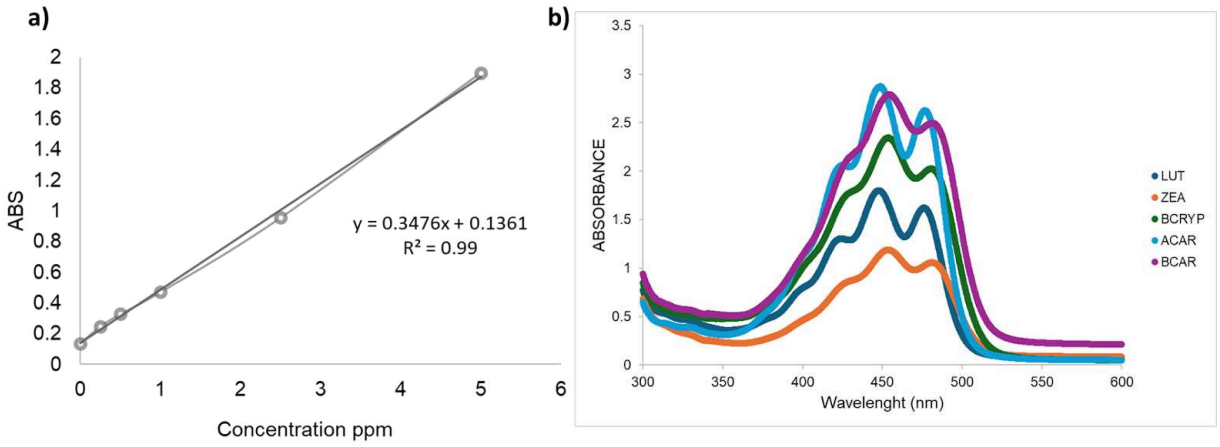


Figure 2.8. Polypropylene plates produce carotenoid results with high linearity. a) Example of a standard calibration curve for the total concentration of carotenoids using PP 96-well plates, and b) UV spectra of individual carotenoids

Method Validation for UV-VIS.

Following solid-liquid extraction, the complete set of carotenoids was isolated. Thus, individual targeting of β-carotene using UV-VIS spectrophotometry was not feasible. Full-spectrum analysis spanning 300-600 nm showed overlapping carotenoid spectra with a distinctive absorption peak approximately at 450 nm (Figure 2.8b). Therefore, the total concentration of carotenoids (TCC) was measured through the UV-VIS method. The calibration curve for this method was performed using the five standards at different concentrations described in Table 2.2. The concentration results of the samples obtained after the linear

regression were multiplied by 5, corresponding to lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene for the calculation of TCC.

The method's linearity was performed in a range of 0.1 to 5 ppm, and it showed good linearity with a regression of y = 0.3476 x + 0.1361 (R^2 = 0.999) (Figure 2.8a). In addition, the detection and quantification limits for TCC were calculated to be 0.31 ppm and 0.95 ppm, respectively. Intraday and interday precision were CV 4 % to 10 % and 1.8 % to 20%, respectively. Method repeatability showed CV % of 4%. Method accuracy was evaluated by standard addition to the sample, and the results obtained showed good percent recovery (%R) of 85% to 103%.

Discussion

HPLC method optimization: C30 column; mobile phases with ammonium acetate, MTBE, and low water content; and flow rate of 0.48 mL/min

This chapter presents the development and validation of an HPLC-PDA method for quantifying lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene. All carotenoids, including the IS, were quantified in a single analytical run with gradient elution conditions at 450 and 325 nm, respectively. The interday and intraday repeatability were acceptable after the samples were extracted using the solid-liquid extraction and the normalization with internal standards. The LOQ of this method fitted in the low concentration range for all evaluated carotenoids, enabling the determination of intrinsic low carotenoid levels that some accession numbers have. Additionally, the method can apply to the study of bioavailability and bioaccessibility, where the carotenoid ranges tend to be very low.

We tested different methods with different mobile phase compositions and stationary phases. The composition of mobile phase A: 95% methanol: 1.0 M ammonium acetate (98:2 v/v), and mobile phase B: methyl tertbutyl ether, methanol, and 1.5% ammonium acetate

(90:8:2, v/v/v) allowed the best separation of the analytes in our method. All compounds were eluted in sharp peaks. However, the peak of β-cryptoxanthin was affected at the lowest concentration tested when the IS was introduced to the sample, increasing the noise at the retention time where β-cryptoxanthin eluted. This could happen because both compounds coeluted at the same retention time but at different wavelengths (450 nm and 325). This effect was not visible when no IS was added to the samples. We kept the IS for the study even though the β-cryptoxanthin peak was affected at the lowest levels tested.

During the separation, we noticed that the resolution of the carotenoids was more efficient when the mobile phase A was supplemented with a minimal fraction of water. The initial conditions we used for mobile phase A included 98% methanol (Ortiz & Ferruzzi, 2019). According to (Gupta et al., 2015) carotenoids are sparingly soluble in water, but some carotenoids, such as the free xanthophylls, contain hydroxyl groups that can bind to the water structure. In the initial optimization phase, we did not use ammonium acetate in the mobile phase; however, the separation of some isomers was better after it was added. Kopec et al., 2013 assert that adding ammonium acetate enhanced the results of carotenoids and retinyl esters in human plasma compared to the effects of just adding water (Kopec et al., 2013). For the mobile phase B, ethyl acetate was initially used, but the separation of α-carotene and β-carotene was not achieved. After replacing ethyl acetate with MTBE, these isomers were resolved in around 8.22 and 8.36 min, respectively. This is because α-carotene and β-carotene are soluble in non-polar solvents such as MTBE.

Once the composition of the mobile phases was determined, we proceeded to optimize the run time of the method. The first strategy was to increase the pumping flow rate; however, the system back pressure exceeded the permitted limits at flow rates higher than 0.48 mL/min. With a flow rate of 0.48 mL/min, it was possible to shorten the running time of the method. Additionally, we optimized the stabilization time in step 6 in the gradient (Table 2.3). This was

achieved by determining the exact time the system back pressure returns from step 6 to the initial conditions in step 1. Step 6 could be reduced from 8 to 6 min.

Regarding the stationary phase, C18 and C30 columns were tested. When using the C18 columns, the resolution of some carotenoids was not obtained. For example, after using the method proposed by Jin et al., 2017 lutein and zeaxanthin co-eluted in a single peak as well as α-carotene and β-carotene. Similarly, when using the chromatographic conditions proposed by Cruet-Burgos et al., 2019 and Marinova et al., 2006 using columns with C18 ligands, the targeted carotenoids were not resolved, with peak splitting observed between α-carotene and β-carotene. (Gupta et al., 2015) point out that C18 columns are not thick enough, leading to a weak interaction between carotenoid molecules and the stationary phase; as a result, poor isomer separation could be observed. The targeted carotenoids were separated after using a C30 column at 35 °C.

Extraction method optimization: a combination of solvents and two wash steps is necessary for efficient extraction of both polar and nonpolar carotenoids

Several methods have been published about carotenoid extraction, comparing extraction effectiveness and solvents. Currently, efforts are put into developing efficient, quick, and economical methods (Saini & Keum, 2018b). This is especially important when high-throughput methods are needed for sorghum breeding programs. As high-throughput as the analytical method should be, so should the extraction method.

This study compared two extraction methods used to extract carotenoids in sorghum grains. The parameters investigated were based on the percentage recovery and reproducibility of individual carotenoids after extraction and the extraction time per batch. We observed that the results obtained after the saponification process do not meet the parameters initially established in the study, which were 80 to 125% for recovery (%) and less than 20% for CV (%), respectively. The recovery percentages for lutein, zeaxanthin, and β-carotene were acceptable;

however, the CV was higher than 20% for the other carotenoids. Regarding reproducibility in extraction, we observed that the results are acceptable at higher concentrations but not reproducible at lower. Usually, the error in quantification tends to be more significant when working at concentrations closer to the detection and quantification limits.

On the other hand, the interday and intraday reproducibility analysis indicate that some results exceed the permitted reproducibility limits, with 158% and 46% variation in the intra-day and interday analysis, respectively. This may be due to the method involving sample heating. Despite using heat-resistant tubes, we observed that some burst and spilled, which agrees with Inbaraj et al., 2008, who indicate that during saponification, losses and degradation of carotenoids occur, reducing the yield in the extraction process. In addition, (Saini & Keum, 2018b) point out that saponification is optimal for samples with high lipid and chlorophyll content. Since sorghum grains contain a reduced percentage of lipids (~3%) (Tanwar et al., 2023), this type of extraction may not be necessary.

We found that organic solvent extraction yielded the highest efficiency for extracting carotenoids from sorghum grains. The selection of solvents plays a critical role in this process, with the choice determined by their compatibility with the specific carotenoids targeted for extraction. Considering the polar nature of specific carotenoids such as lutein, zeaxanthin, and β-cryptoxanthin, we opted for ethanol, acetone, and ethyl acetate as the extraction solvents. Conversely, MTBE was selected for non-polar compounds such as α-carotene and β-carotene (Saini & Keum, 2018). These solvents were combined to enable simultaneous extraction of multiple carotenoids, resulting in highly efficient extraction with recovery percentages falling within the predetermined limits for all targeted carotenoids. Analysis of %CV for intraday and interday variations revealed consistently reproducible results. This reproducibility can be attributed to stringent control over extraction variables and significantly shorter extraction times than saponification, thereby minimizing molecule degradation.

Initially, the extraction was conducted using 100 mg of the sample, accompanied by a washing step involving proportional amounts of ethanol, acetone, ethyl acetate, and MTBE spiked with BHT. However, recovery percentages deviated from the initial values upon increasing the sample weight to 300 mg to ensure sufficient extract for both methods. Subsequently, by implementing a two-step extraction process involving (1) 0.1% BHT in ethanol, acetone, and ethyl acetate (1:1:1 v/v/v) and (2) MTBE, efficient recovery of carotenoids was achieved. To prevent oxidation and degradation of the molecules, butylated hydroxytoluene (BHT) was added at a concentration of approximately 0.1% (w/v), as recommended in the literature (Cernelic et al., 2013). A solvent mixture of ethanol and ethyl acetate (1:1 v/v) was employed as a resuspension solvent because the carotenoid separation needs compatibility between the injection solvent and mobile phase. Ideally, the injection solvent should be compatible with the mobile phase or more polar than the reverse phase to provide an on-column concentration of samples (Gupta et al., 2015).

UV-VIS method optimization: polypropylene 96-well plates are well-suited for TCC quantification

Most UV-VIS spectrophotometric methods used for carotenoid quantification rely on single-beam spectrophotometers with a 1 cm quartz cuvette (Abdul-Hammed et al., 2013; Barba et al., 2006; Kopec et al., 2013). Depending on the sample, results may be reported based on the highest carotenoid concentration or Total Carotenoid Concentration (TCC). However, for our study's objective of developing a rapid carotenoid detection method, the single-beam spectrophotometer is unsuitable for quantifying a significant number of samples simultaneously. Therefore, we propose a rapid method in a 96-well plate format for TCC quantification.

Since organic solvents are required for extraction, the plate material must be resistant and non-interfering with quantification. After testing several materials, polypropylene was the

most suitable choice despite its lack of transparency. Polypropylene results show absorbance in the lower UV wavelengths, specifically at 290- 370 nm. Although there are no reports of using 96-well polypropylene plates for carotenoid quantification, they have been utilized in some spectrophotometry applications.

Validation parameters revealed good linearity ($R^2 > 0.9$) and repeatability within established parameters, confirming the suitability of 96-well polypropylene plates for this assay. Wavelength selection was based on scanning the entire UV-VIS spectrum, revealing that carotenoids exhibit the highest absorbance at ~450 nm without interference from the 96-well plate plastic. This agrees with what was reported in the literature (Hagos et al., 2022; Ortiz & Ferruzzi, 2019; Xu et al., 2023).

Initially, we aimed to quantify the concentration of β-carotene. However, full spectra plotting indicated that all carotenoids absorb light at the same wavelength, resulting in overlap. Although differentiated PVAC extraction could be performed for sorghum grains, naturally low concentrations (e.g., α-carotene and β-cryptoxanthin) might lead to misleading results. Furthermore, there are reports of quantifying two carotenoids individually (lycopene and β-carotene) using synthetic mixtures based on physical models from spectral data in tomatoes (Popescu et al., 2022); further investigation is needed to determine if these methods can be applied to quantify more than two carotenoids, particularly in sorghum grain.

Conclusion

This study introduces a solid-liquid extraction method coupled with HPLC-DAD for the qualitative and quantitative analysis of carotenoids. We have successfully developed and validated a method capable of simultaneously quantifying five major carotenoids in sorghum grain. Optimal chromatography separation was achieved using a C30 column, significantly

reducing the run time per sample. The method exhibited high reproducibility, with low LOD and LOQ, making it suitable for investigating carotenoid levels in sorghum grain samples when employing solid-liquid extraction.

Our findings suggest that saponification is unsuitable for carotenoid extraction in sorghum grain, leading to significant losses during extraction. Conversely, solid-liquid extraction yielded more consistent results, as evidenced by improved reproducibility in interday and intraday analyses. Additionally, we developed a 96-well plate method for quantifying total carotenoid concentration using polypropylene plastic. The method proved to be a high-throughput, time-saving, and cost-effective alternative to HPLC with acceptable reproducibility and validation parameters. In conclusion, our study provides essential tools for breeders by offering valuable methodologies to accelerate carotenoid biofortification in sorghum grain.

Chapter 2. Literature Cited

Abdul-Hammed, M., Bello, I. A., & Oladoye, S. O. (2013). Simultaneous Spectrophotometric Determination of Lycopene and Beta-Carotene Concentrations in Carotenoid Mixtures of the Extracts from Tomatoes, Papaya and Orange Juice: Lycopene and ?-Carotene Determination. *Biological Sciences - PJSIR*, *56*(2), Article 2. https://doi.org/10.52763/PJSIR.BIOL.SCI.56.2.2013.90.97

Alamu, E. O., Nuwamanya, E., Cornet, D., Meghar, K., Adesokan, M., Tran, T., Belalcazar, J., Desfontaines, L., & Davrieux, F. (2021). Near-infrared spectroscopy applications for high-throughput phenotyping for cassava and yam: A review. *International Journal of Food Science & Technology*, *56*(3), 1491–1501. https://doi.org/10.1111/ijfs.14773

Amorim-Carrilho, K. T., Cepeda, A., Fente, C., & Regal, P. (2014). Review of methods for analysis of carotenoids. *TrAC Trends in Analytical Chemistry*, *56*, 49–73. https://doi.org/10.1016/j.trac.2013.12.011

Barba, A. I. O., Hurtado, M. C., Mata, M. C. S., Ruiz, V. F., & Tejada, M. L. S. de. (2006). Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry*, *95*(2), 328–336. https://doi.org/10.1016/j.foodchem.2005.02.028

Cernelic, K., Prosek, M., Golc-Wondra, A., Rodic, Z., Simonovska, B., & Puklavec, M. (2013). *Influence of Synthetic Antioxidants on Extraction of All*-trans*-Lutein from Spinach under Air and Nitrogen Atmosphere*. *2013*. https://doi.org/10.4236/fns.2013.42027

Chan, C. C. (2011). Principles and Practices of Analytical Method Validation: Validation of Analytical Methods is Time-consuming but Essential. *The Quality Assurance Journal*, *14*(3–4), 61–64. https://doi.org/10.1002/qaj.477

Cudjoe, D. K., Virlet, N., Castle, M., Riche, A. B., Mhada, M., Waine, T. W., Mohareb, F., & Hawkesford, M. J. (2023). Field phenotyping for African crops: Overview and perspectives. *Frontiers in Plant Science*, *14*. https://www.frontiersin.org/articles/10.3389/fpls.2023.1219673

Galli, G., Horne, D. W., Collins, S. D., Jung, J., Chang, A., Fritsche-Neto, R., & Rooney, W. L. (2020). Optimization of UAS-based high-throughput phenotyping to estimate plant health and grain yield in sorghum. *The Plant Phenome Journal*, *3*(1), e20010. https://doi.org/10.1002/ppj2.20010

Gupta, P., Sreelakshmi, Y., & Sharma, R. (2015). A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant Methods*, *11*(1), 5. https://doi.org/10.1186/s13007-015-0051-0

Hagos, M., Redi-Abshiro, M., Chandravanshi, B. S., & Yaya, E. E. (2022). Development of Analytical Methods for Determination of β-Carotene in Pumpkin (Cucurbita maxima) Flesh, Peel, and Seed Powder Samples. *International Journal of Analytical Chemistry*, *2022*, 9363692. https://doi.org/10.1155/2022/9363692

Hunter, M. C., Smith, R. G., Schipanski, M. E., Atwood, L. W., & Mortensen, D. A. (2017).

Agriculture in 2050: Recalibrating Targets for Sustainable Intensification. *BioScience*, *67*(4), 386–391. https://doi.org/10.1093/biosci/bix010

ICH. (2024). *ICH Q2(R2) Validation of analytical procedures—Scientific guideline | European Medicines Agency*. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline

Inbaraj, B. S., Lu, H., Hung, C. F., Wu, W. B., Lin, C. L., & Chen, B. H. (2008). Determination of carotenoids and their esters in fruits of *Lycium barbarum* Linnaeus by HPLC–DAD–APCI–MS. *Journal of Pharmaceutical and Biomedical Analysis*, *47*(4), 812–818. https://doi.org/10.1016/j.jpba.2008.04.001

Jaramillo, A. M., Londoño, L. F., Orozco, J. C., Patiño, G., Belalcazar, J., Davrieux, F., & Talsma, E. F. (2018). A comparison study of five different methods to measure carotenoids in biofortified yellow cassava (Manihot esculenta). *PLOS ONE*, *13*(12), e0209702. https://doi.org/10.1371/journal.pone.0209702

Jin, X., Zarco-Tejada, P. J., Schmidhalter, U., Reynolds, M. P., Hawkesford, M. J., Varshney, R. K., Yang, T., Nie, C., Li, Z., Ming, B., Xiao, Y., Xie, Y., & Li, S. (2021). High-Throughput Estimation of Crop Traits: A Review of Ground and Aerial Phenotyping Platforms. *IEEE Geoscience and Remote Sensing Magazine*, *9*(1), 200–231. https://doi.org/10.1109/MGRS.2020.2998816

Kopec, R. E., Schweiggert, R. M., Riedl, K. M., Carle, R., & Schwartz, S. J. (2013). Comparison of high-performance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quantitation of carotenoids, retinyl esters, α-tocopherol and phylloquinone in chylomicron-rich fractions of human plasma. *Rapid Communications in Mass Spectrometry : RCM*, *27*(12), 10.1002/rcm.6576. https://doi.org/10.1002/rcm.6576

Ménard, G., Biais, B., Prodhomme, D., Ballias, P., Petit, J., Just, D., Rothan, C., Rolin, D., & Gibon, Y. (2013). Chapter Nine—High-Throughput Biochemical Phenotyping for Plants. In D. Rolin (Ed.), *Advances in Botanical Research* (Vol. 67, pp. 407–439). Academic Press. https://doi.org/10.1016/B978-0-12-397922-3.00009-5

Ortiz, D., & Ferruzzi, M. G. (2019). Identification and Quantification of Carotenoids and Tocochromanols in Sorghum Grain by High-Performance Liquid Chromatography. *Methods in Molecular Biology (Clifton, N.J.)*, *1931*, 141–151. https://doi.org/10.1007/978-1-4939-9039-9_10

Popescu, M., Iancu, P., Pleșu, V., Bîldea, C. S., & Todasca, C. M. (2022). Different spectrophotometric methods for simultaneous quantification of lycopene and β-carotene from a binary mixture. *LWT*, *160*, 113238. https://doi.org/10.1016/j.lwt.2022.113238

Riggi, E. (2010). Recent Patents on the Extraction of Carotenoids. *Recent Patents on Food, Nutrition & Agriculture*, *2*(1), 75–82.

Saini, R. K., & Keum, Y.-S. (2018a). Carotenoid extraction methods: A review of recent developments. *Food Chemistry*, *240*, 90–103. https://doi.org/10.1016/j.foodchem.2017.07.099

Saini, R. K., & Keum, Y.-S. (2018b). Carotenoid extraction methods: A review of recent

developments. *Food Chemistry*, *240*, 90–103. https://doi.org/10.1016/j.foodchem.2017.07.099

Shi, C., Zhao, L., Zhang, X., Lv, G., Pan, Y., & Chen, F. (2019). Gene regulatory network and abundant genetic variation play critical roles in heading stage of polyploidy wheat. *BMC Plant Biology*, *19*(1), 6. https://doi.org/10.1186/s12870-018-1591-z

Strati, I. F., & Oreopoulou, V. (2011). Effect of extraction parameters on the carotenoid recovery from tomato waste. *International Journal of Food Science & Technology*, *46*(1), 23–29. https://doi.org/10.1111/j.1365-2621.2010.02496.x

Tang, J., Li, X., Zhang, Y., Yang, Y., Sun, R., Li, Y., Gao, J., & Han, Y. (2022). Differential Flavonoids and Carotenoids Profiles in Grains of Six Poaceae Crops. *Foods*, *11*(14), 2068. https://doi.org/10.3390/foods11142068

Tanwar, R., Panghal, A., Chaudhary, G., Kumari, A., & Chhikara, N. (2023). Nutritional, phytochemical and functional potential of sorghum: A review. *Food Chemistry Advances*, *3*, 100501. https://doi.org/10.1016/j.focha.2023.100501

Xu, J., Lin, J., Peng, S., Zhao, H., Wang, Y., Rao, L., Liao, X., & Zhao, L. (2023). Development of an HPLC-PDA Method for the Determination of Capsanthin, Zeaxanthin, Lutein, β-Cryptoxanthin and β-Carotene Simultaneously in Chili Peppers and Products. *Molecules*, *28*(5), 2362. https://doi.org/10.3390/molecules28052362

Yao, W., Li, G., Yu, Y., & Ouyang, Y. (2018). funRiceGenes dataset for comprehensive understanding and application of rice functional genes. *GigaScience*, *7*(1), gix119. https://doi.org/10.1093/gigascience/gix119

Zhang, H., Ge, Y., Xie, X., Atefi, A., Wijewardane, N. K., & Thapa, S. (2022). High throughput analysis of leaf chlorophyll content in sorghum using RGB, hyperspectral, and fluorescence imaging and sensor fusion. *Plant Methods*, *18*(1), 60. https://doi.org/10.1186/s13007-022-00892-0

Chapter 3. Evaluating a High-Throughput Spectrophotometric Approach for Carotenoid Analysis in a Sorghum Breeding Population

Introduction

Carotenoids are natural pigments involved in several plant functions (photoprotection, antioxidant properties, hormone precursor) (Sun et al., 2022), while in humans, once obtained through diet, they can be transformed into vitamin A (Rivera-Madrid et al., 2020). Carotenoids that can be transformed into vitamin A—including α-carotene, β-carotene, and β-cryptoxanthin—are known as provitamin A carotenoids (PVACs). In some cases, these compounds are the only source of vitamin A (Global Nutrition Report, 2021). This occurs mainly in regions lacking dietary diversity, with limited access to products containing preformed vitamin A (retinol) (Wiseman et al., 2017). Their diet is primarily based on consuming foods rich in calories but substantially poor in PVAC content. As a result, these regions have a high prevalence of vitamin A deficiency (VAD) (World Health Organization, 2009).

Among the most consumed crops is sorghum, a staple cereal that represents a food source for millions of people in West and East African countries (National Sorghum Producers, 2021). Its resilience to extreme conditions, genetic and phenotypic diversity, and cultural importance make sorghum a good candidate for biofortification (Monk et al., 2014; Somegowda et al., 2024). Biofortification through breeding aims to identify and cultivate sorghum germplasm with higher PVAC content and thus offers a solution to alleviate cases of VAD.

However, the selection process for high PVAC germplasm in breeding is costly and time-consuming, representing a bottleneck in biofortification (Doebley et al., 2006). HPLC, the gold standard for carotenoid qualification, is a sensitive instrument with many analytical advantages (Jaramillo et al., 2018). However, its use is not feasible in a breeding program when thousands of samples have to be evaluated at early stages of selection, and instrument

availability is limited. Currently, breeders base their decisions on the visual evaluation of sorghum grain. Color is a moderate predictor of carotenoid presence. Although this technique is potentially cheaper and more convenient, it has limitations associated primarily with the analyst's subjectivity. In addition, this technique only allows for the differentiation of the presence or absence of carotenoids (McDowell et al., 2024, in review), not the categorization between sorghum lines with low, medium, and high carotenoid content. Therefore, to address this problem, developing fast, efficient, accessible, reliable and high throughput (HTP) techniques is imperative to accelerate the decision-making process in the breeding workflow.

UV-VIS spectrophotometry has been widely used for the rapid quantification of carotenoids. This technique is one of the cheapest and easiest to use for carotenoid quantification compared to HPLC (Kopec et al., 2012; Biswas, Sahoo, & Chatli, 2011). For example, β-carotene was evaluated in cassava, sweet potato and pumpkin using UV VIS spectrophotometry (Hagos et al., 2022; Jaramillo et al., 2018). There are also reports of differential quantification of lycopene and β-carotene in tomatoes using physical models obtained from spectral data (Abdul-Hammed et al., 2013). The use of spectrophotometers has been so recurrent that even portable spectrophotometers are now available for the specific quantification of carotenoids such as iCheck Carotene (BioAnalyt). However, since the concentrations of carotenoids in sorghum grain are very low, these "off the shelf" methods are not suitable for sorghum phenotyping.

In most cases, UV-VIS methods have been carried out using the single beam spectrophotometer version with quartz cuvettes, allowing the analysis of one sample at a time. This results in a disadvantage in large-scale analysis. This is due to the use of organic solvents in the extraction of the molecules, which tend to be incompatible with plastic supports used for rapid spectrophotometric applications. Despite the limitations, UV VIS spectrophotometry is still an economical and faster alternative to HPLC, providing accurate results.

Another limitation of UV-VIS spectrophotometry is that it does not allow the differentiation of individual carotenoids, particularly those that can be transformed into vitamin A. However, studies show that individual carotenoid concentration correlates with total carotenoid concentration (TCC) (Cruet-Burgos et al., 2020b). Thus, TCC obtained via UV-VIS could be a good predictor of the concentration of PVACs in sorghum grains.

The goal of this research was to 1) validate an HTP method for the quantification of TCC in sorghum grains using a 96-well plate format in a biparental breeding population containing 249 sorghum lines, and 2) compare our HTP method with conventional visual inspection. The HTP-determined TCCs were compared with the PVACs and TCCs obtained via HPLC. We hypothesized that our HTP method provides sufficient phenotypic information to differentiate low, mid, and high PVAC carotenoid sorghum lines for selection during breeding. Using the HTP method, we determined that the TCC is highly correlated with PVACs; hence, it allows differentiation between lines with high and low PVACs but not medium PVAC lines. Similarly, the predictive ability of the HTP method was also compared with the predictive ability of a visual assessment method using 75 randomly selected sorghum lines. The results reveal that the HTP method is more predictive of PVAC content than a simple visual assessment. This promising HTP method will enable rapid screening of a large number of samples, helping breeders to more efficiently make selections for carotenoid biofortification.

Materials and Methods

Description of sorghum breeding lines used in the study

For the study, 249 F3 progeny in a biparental family were analyzed (Cruet-Burgos et al., 2019). The parental lines (PI585348 x PI585369) were selected for their high PVAC content. The family was grown at Kansas State University Agronomy North Farm during the Summer of

2020. The F2 plants were self-pollinated, and the resulting F2:3 grain was harvested and stored at -80 ℃ until carotenoid extraction. Samples were randomly run using the two methods in batches of 84 daily samples.

Extraction methods for pro-vitamin A carotenoids

Carotenoid extractions of the F2:3 family were carried out under yellow light to avoid photodegradation, using a modified solid-phase method described in Chapter 2. In short, the dried samples were ground into powder using a stainless steel analytical mill (IKA A11 basic), and 300 ± 0.05 mg were weighed out in a 2 mL Screw-Cap Self-Standing microtube. Then, 1000 µL of the extraction solvent 1 containing 0.2 ppm of IS and 0.01% Butylated hydroxytoluene (BHT) in ethyl acetate: acetone: ethanol (1:1:1, v/v/v) and two stainless steel beads were added to the microtube. Three separate extractions per line were conducted (technical replicates) to demonstrate reproducibility of the method.

The tubes were placed into the homogenizer (Omni Bead Ruptor 24 Elite), and processed for 5 minutes at 2.5 m/s, with a brief pause of 25 sec after the first 2.5 min. The samples were centrifuged for 4 min at 13000 x g, and the supernatant was transferred into a 1.5 mL Eppendorf tube. The extraction was repeated one more time from the pellet, with 700 µL of Methyl tert-butyl ether (MTBE). The combined supernatant was centrifuged for 30 seconds at maximum speed and transferred into a new 2 mL Screw-Cap Self-Standing microtube. The samples were concentrated using a stream of N_2 for 35 minutes at room temperature or until an orange pellet was seen in the bottom of the tube.

The concentrated samples were dissolved with 250 µL of a mixed ethyl acetate solution and ethanol (50:50 v/v). The samples were vortexed for 10 sec and then centrifuged for 30 sec at 13 000 xg. Around 50 - 60 µL of the solution was transferred into a clean glass insert housed in an amber HPLC vial and 150 µL in each well of the plate.

Instrumentation and analytical techniques employed for quantitation

HPLC analysis

The carotenoids were quantified at 450 nm using reverse phase chromatography RP-HPLC Flexar (PerkinElmer, United States), consisting of a quaternary pump and a photodiode array detector (PDA). A C30 column (150 x 2 mm I.D. S-3 µm; YMC American, Inc.) at 35 °C was utilized for the carotenoid separation,with a sample injection volume of 7µL. The autosampler was maintained at 10° C. The mobile phase A consisted of 95% methanol: 1.0 M ammonium acetate (98:2 v/v), and mobile phase B consisted of MTBE, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v). Carotenoids were resolved using the following gradient at a fixed flow of 0.48 mL/min: 100% A to 20% A over 3 minutes, 20% A to 0% A over 1.5 minutes, 0% A held for 1.5 minutes, a return to 100% A over 1.0 minute, and a hold on 100% A for 6.0 minutes to recondition the column. The internal standard retinyl palmitate was quantified at 325 nm. Quantification was performed using TotalChrom Navigator Version 6.3.4.

Identification and quantification of carotenoid in samples

Peaks were identified by comparing the retention times and UV–Vis spectral data with those of the corresponding standards when possible. Samples and standard data were normalized using the area obtained from the IS, and the concentration of each analyte was calculated using the calibration curve of the corresponding standard as a reference. Five-point external standard curves were constructed from the standard mix ranging from 0.25 to 5 ppm for lutein, zeaxanthin, β-cryptoxanthin, and α-carotene, whereas from 0.1 to 2.5 ppm for β-cryptoxanthin. Carotenoid concentrations were then calculated using a linear regression $y = mx + b$, where y = concentration and x = area of the five-point standard curve/areas of IS. The regression equation and correlation coefficient (R^2) were obtained.

For the quantification, a quality control (QC) was injected after every ten samples throughout the sequence created for the HPLC method. The following metrics were monitored: concentration (CV < 20%) and retention time (±1%). If QC samples failed, the analysis was stopped until adequate QC was achieved—similarly, a principal component analysis (PCA) was used to evaluate the similarity of the QC injections (Figure 3.1).

HTP quantification of carotenoids

The samples were quantified using a 96-well UV-VIS plate reader (BIOTEK PowerWave XS2) at 450 nm and the software Gene5. 150 µL of the sample was placed in each well. The absorbance of the samples was corrected using ethyl acetate: ethanol (50:50 v/v) as a blank. The TCC of each sample was calculated using the calibration curve generated from the five standards as a reference. Five-point external standard curves ranging from 0.25 to 5 ppm were constructed for the standard mix. Carotenoid concentrations were then calculated using a linear regression $y = ax + b$, where y = TCC and x =Absorbance. The regression equation and correlation coefficient (R^2) were calculated.

Visual assessment of carotenoids

Four participants visually evaluated seventy-five randomly selected sorghum lines for grain yellowness. The samples were selected based on the β-carotene concentration obtained through HPLC. Samples with low, medium, and high concentrations were included. Each sample was ranked from 1 to 3 based on the level of yellow coloration: samples with no coloration were assigned a value of 1, those with faint yellow coloration were assigned a value of 2, and samples with striking yellow coloration were assigned a value of 3. A 2.5 mL tube was filled with the grain from individual lines, and each line was assessed in triplicate. Replicates were randomly distributed, rather than placed next to each other, to avoid bias. Results were compared with those from the HPLC and HTP methods.

Statistical analysis of data

Pearson's correlation analysis was performed between the TCC data obtained with the HTP method versus the individual concentration of carotenoids obtained with HPLC analysis in order to verify the association and direction of the correlation between parameters. Similarly, the correlation among TCC - HTP, TCC - HPLC and visual assessment methods was evaluated. Qualitative analysis was based on a PCA to evaluate the presence of some data grouping and, as a result, to identify separated clusters in the principal components plot. The coefficient of determination (R^2) was calculated to assess the model's ability to predict TCC-HPLC and PVAC concetration in a linear regression setting, with TCC-HTP as the independent variable.

Bland Altman plot analysis was used to assess the agreement between the HTP and HPLC methods. The Bland Altman plot analysis illustrates the agreement or interchangeability of methods by plotting the methods' mean on the X-axis and the difference of the methods on the Y-axis. Limits of agreement are defined as the mean difference ± 1.96 standard deviations of the differences

Results

We first wanted to ensure the accuracy of the HPLC data collected when working with very large sample sets (n = 249) To ensure the data acquired were suitable for analysis and could produce valid and robust datasets, QC samples were included in the assay. We initially hypothesized that the pooled QC samples would perform appropriately, predicting low variation compared to the experimental samples. After processing the data, we observed that the QC samples were closely clustered in the center of the PCA plot (Fig. 3.1, red oval), indicating consistency and low technical variation between replicates. This result suggests the samples remained stable throughout the run, with no significant sample-instrument interaction. In contrast, the samples exhibited greater variation, as shown by the scattered points within the

green oval. We also calculated the %CVs between the technical replicates of the target carotenoids: 11% for lutein and zeaxanthin, and 9% and 8% for α-carotene and β-carotene, respectively. These results confirm that the method met the predefined metrics established at the beginning of the experiment.

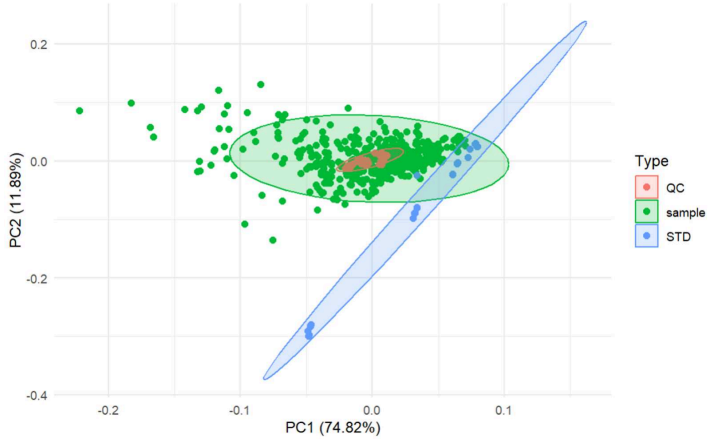


Figure 3.1. Replicate HPLC measurements were consistent and had low variation. PCA score plot for the samples. Samples in different groups are presented in different colors: (red) pooled QC, (green) sorghum samples, and (blue) standards. Circles represent the 95% confidence interval.

We next sought to determine the range and means of concentrations for β-carotene, β-cryptoxanthin, zeaxanthin,α-carotene, lutein, and total carotenoid concentration (TCC) in the grain of 249 sorghum lines in the biparental breeding family. The TCC in the samples, measured on a dry weight basis, ranged from 1.7 µg/g to 27.0 µg/g with the HPLC method, while with the HTP method, the TCC values ranged from 3.4 µg/g to 30.3 µg/g. The average TCC of the samples screened by HPLC was 8.6 µg/g, whereas the average for the high throughput method was higher at 12.5 µg/g. This difference in means is because, in the HPLC analysis, only the four targeted carotenoids were considered for the quantification. In contrast, the HTP method measures all molecules that absorb light at 450 nm, creating an overestimation of the values. Among the individual carotenoids measured by HPLC, lutein and zeaxanthin were found in the

highest concentrations in sorghum grain, with ranges of 0.7-16.4 µg/g and 0.5-9.0 µg/g, respectively. These were followed by β-carotene, which ranged from 0.1 to 3.0 µg/g, and α-carotene, which ranged from 0.1 to 0.6 µg/g. The values for β-cryptoxanthin were either unmeasurable or under the quantitation limit of the method (>0.17 µg/g).

Table 3.1. Quantification of Carotenoids in Fresh Sorghum Grain Using HPLC and HTP Methods

Parameter	Lutein µg/g DM	Zeaxanthin µg/g DM	α-carotene µg/g DM	β-carotene µg/g DM	TCC-HPLC µg/g DM	TCC-HTP µg/g DM
Min	0.7	0.5	0.1	0.1	1.7	3.4
Mean	4.1	3.4	0.2	0.9	8.6	12.5
Max	16.4	9.0	0.6	3.0	27.0	30.3

*DM: Dry matter

Next, we wanted to determine the degree to which total carotenoid content reflects PVAC content, so we assessed the relationships between the traits. Pearson correlation coefficient (R) estimates were conducted between the TCC-HTP method and individual carotenoids and their correlation with the HPLC method (Figure 3.2, upper corner), and scatter plots with regression lines were constructed to illustrate these relationships (Figure 3.2, lower corner), The center diagonal contains histograms showing the distribution of the concentrations of each trait. We found strong positive correlations between PVAC and non-PVAC: lutein and β-carotene (R=0.77; $P<2.2\times10^{-16}$), and zeaxanthin and β-carotene (R=0.63; $P<2.2\times10^{-16}$). Additionally, there was a positive correlation between PVAC TCC for both HPLC and UV-VIS methods: TCC HPLC and β-carotene (R=0.82; $P<2.2\times10^{-16}$) and TCC UV-VIS and β-carotene (R=0.64; $P<2.2\times10^{-16}$). In contrast, α-carotene exhibited a low correlation (R=0.23; $P<2.2\times10^{-16}$ and R=0.27; $P<2.2\times10^{-16}$), likely due to the limited number of samples with detectable levels of α-carotene

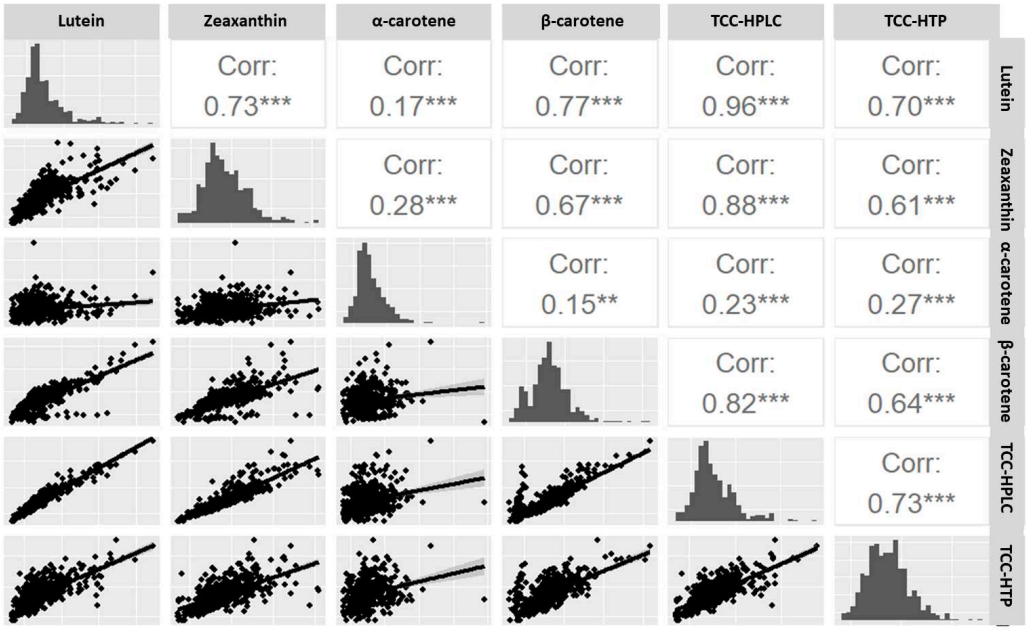


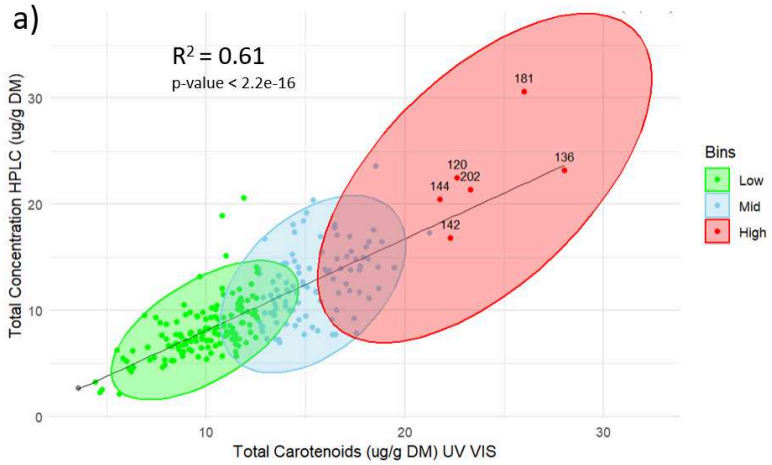
Figure 3.2. Most carotenoid compounds are highly correlated with TCC measured through both HPLC and UV-VIS. Relationship among grain carotenoid traits in the sorghum germplasm collection: center diagonal contains histograms of the concentrations of each trait; upper diagonal contains correlations and significance; and lower diagonal contains scatter plots with regression lines (n=249). ***Significance at the .001 level. Units are µg/g

Next, we hypothesized that the HTP method would sufficiently differentiate between high, medium, and low TCC and β-carotene in a breeding population. We performed a simple linear regression model to test this hypothesis with the selected independent variable (TCC-HTP) and the dependent variables (β-carotene and TCC-HPLC). The X-axis was divided into three main concentration categories—low (< 10 µg/g), mid (10-20 µg/g), and high (>20 µg/g). The prediction accuracy (R^2) and statistical significance (p-value) were estimated. The results showed a moderate prediction ability with R^2 = 0.61 with a high statistical significance (p-value <0.001) between TCC-HTP and TCC-HPLC (Figure 3.4a).

Considering the highly significant correlation between the β-carotene and HTP method, we also performed a linear regression between both variables to determine how predictive the

β-carotene concentration is based on TCC. The results indicate that there is also a moderate prediction with $R^2 = 0.5$ and a high statistical significance (p-value <0.001) (Figure 3.4b). Although the correlation is moderate, the data obtained by the HTP method is sufficient to distinguish clearly between sorghum lines with high and low PVAC concentrations. There is partial overlap for sorghum lines with medium PVAC concentrations and lines with higher and lower concentrations (Figure 3.4b). This suggests that while there may be instances in which classification is challenging, the overall differences are sufficient to distinguish the groups in most cases. For the breeding goal of selecting sorghum lines with exceptionally high PVAC concentrations, the high lines distinctly grouped within the red ellipse can easily be identified and used to introgress high carotenoid alleles into elite breeding lines.

Furthermore, in the linear regression analysis of TCC-HPLC and TCC-HTP, six sorghum lines (120, 136, 142, 144, 181, 202) with the highest concentrations of carotenoids were clearly differentiated. The same lines were also identified in the regression analysis between β-carotene and TCC-HTP (120, 136, 142, 181, 202). However, additional sample IDs (15, 1, 177) appear with higher β-carotene content, which were not observed in the first regression. Conversely, sample 144, which shows high TCC, is found to have a low β-carotene content in this analysis.



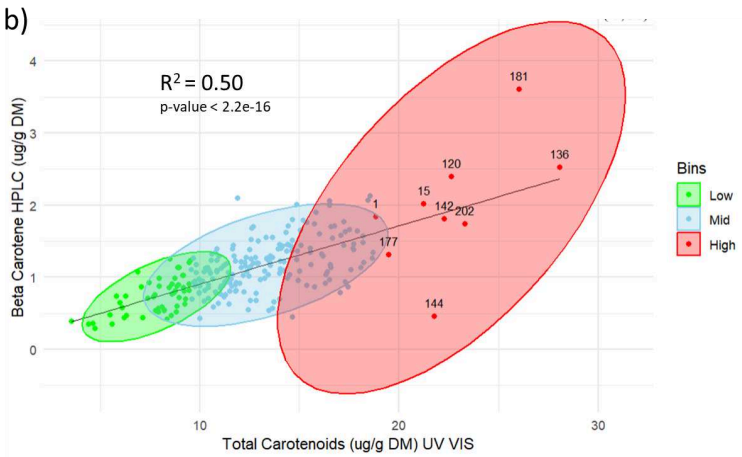


Figure 3.4. Strong positive relationship between HPLC and UV-VIS methods. Scatterplots between TCC measured by HPLC versus spectrophotometer (a) and β-carotene versus spectrophotometer (b). The X-axes are divided into three TCC categories—low (< 10 µg/g, green), mid (10-20 µg/g, blue), and high (>20 µg/g, red).The black lines indicate linear regression lines. The ellipses represent the 95% confidence intervals for each bin group.

The data was also presented in a Bland Altman plot, which shows the relationship between the mean TCC measured via the HTP method and the HPLC method on the x-axis and the percent difference of the HTP method values from HPLC on the y-axis (Fig. 3.5a). The results of comparing carotenoid concentrations using HPLC and the HTP spectrophotometer method show both overestimation and underestimation instances. In the Bland-Altman plot, points above the zero difference line indicate that the HTP method predicted higher carotenoid values than the HPLC method for those samples. In contrast, points below this line indicate that the HTP method predicted lower carotenoid values for those samples. This pattern of overestimations and underestimations suggests that the spectrophotometer does not consistently measure higher or lower than the HPLC; the differences between the methods vary depending on the sample.

The Bland Altman plot also showed that the HPLC and the spectrophotometer method agreed within the range 3.0 to -7.5. This indicated the confidence limits at 95% of the

differences between the two methods. Some samples fell outside this range, meaning there are outliers where the difference between the methods is larger than expected based on the overall agreement. However, most sample differences lay within this range, indicating that the methods can be considered to have an acceptable agreement for our purposes.

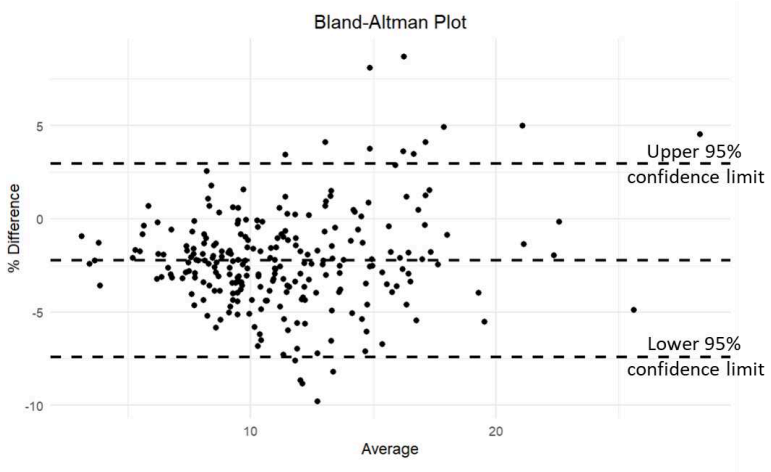


Figure 3.5. Comparison of TCC by HPLC and HTP. Bland Altman plot of percent differences in TCC (HTP minus HPLC results) against the average of the two methods. The dashed lines represent the upper and lower 95% confidence limits.

Next, we hypothesized that the HTP UV-VIS method could more accurately predict PVAC content than the current method of visual selection used by breeders. To test this hypothesis, we visually assessed yellowness in 75 sorghum grain samples with four independent observers and conducted a Pearson correlation analysis to explore the relationship between the two methods. Findings from the visual assessment method indicate a notably weak relationship with the HPLC results. Specifically, there was no significant correlation between TCC-HPLC and the visual assessment (Table 3.2). Interestingly, there was a low but significant correlation between the TCC-UV VIS method and the visual assessment. There was no significant correlation between the visual assessment and lutein, β-carotene, or α-carotene, suggesting that the visual method is inadequate for estimating concentrations of most carotenoids. However, a moderately significant correlation ($R = 0.35^*$, $p < 0.001$) was identified between the visual assessment and zeaxanthin content. Notably, a statistically significant

moderate positive correlation (R = 0.27*) was found between the visual assessment and the TCC high-throughput method.

Table 3.2. Carotenoid compounds have a low correlation with the visual assessment method. ***Significance at the .001 level.

	Lutein	Zeaxanthin	α-carotene	β-carotene	TCC-HPLC	TCC-HTP
Visual	Corr: -0.00	Corr: 0.35**	Corr: 0.19	Corr: 0.15	Corr: 0.13	Corr: 0.27*

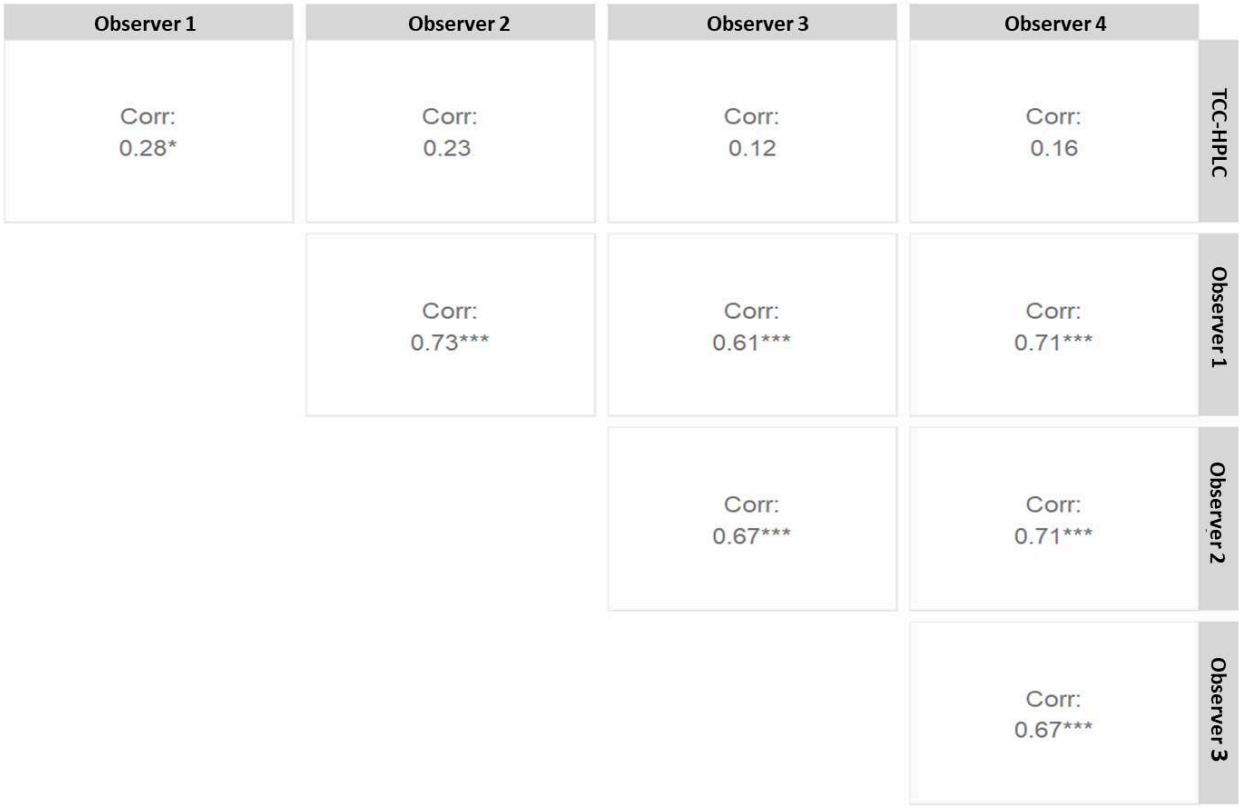


Figure 3.6. Observers' visual scores scores have a low correlation with TCC-HPLC but are high with each other. Relationship among visual assessments in the sorghum germplasm collection: Values indicate correlations and significance (n=75). ***Significance at the .001 level.

To further assess the consistency and reliability of the visual assessment method, we analyzed the correlations between each observer's evaluations and the actual concentrations as

measured by HPLC, as well as the correlations among the observers (Figure 3.6). Only 1 observer had results that were significantly correlated with the HPLC data. In contrast, the correlations among the observers' results were significantly correlated. These results highlight that while the visual assessment may not closely predict the carotenoid concentrations, there is a moderate degree of consistency among the observers in assessing yellowness.

Discussion

Sorghum breeding programs aim to develop elite breeding varieties with high PVAC concentrations. However, this goal has not been achieved, partly due to lack of fast and low-cost methods for screening germplasm in breeding programs. While it is true that the conventional HPLC quantification of carotenoids is the gold standard, this is expensive and time-consuming and requires exhaustive controls. For example, a pool QC was included in every 10 injected samples to monitor potential degradation throughout the HPLC run (Dunn et al., 2023). Therefore, the potential of spectrophotometry UV - VIS in a 96-well plate format was evaluated as a simple and affordable screening method for sorghum breeding programs.

HPLC vs HTP quantification of carotenoids

HPLC analysis of sorghum grain indicates that the provitamin A carotenoids (pVACs) present in the grain consist primarily of α-carotene and β-carotene. In most samples, β-cryptoxanthin concentration was negligible; hence, it was not considered in the quantification of TCC. We found that the major carotenoids detected were lutein and zeaxanthin, as was previously reported by Burgos-Cruet et al., 2020. The total carotenoid content was calculated by adding the concentrations of the individual carotenoid (TCC-HPLC). The results also show a significant correlation between the evaluated carotenoids; however, it was found that α-carotene had the lowest correlation with others, but still had a statistically significant relationship. The high degree of correlation between the individual carotenoids is probably because these

compounds share a common biosynthetic pathway (Davey et al., 2009). Due to the high correlation between the carotenoids and TC-HPLC, we hypothesized that HTP-TCC values provide enough phenotyping information to separate sorghum lines between high, mid, and low concentrations.

UV Vis spectrophotometry has been used extensively as a high-throughput method to quantify TCC without the same complications involved in liquid chromatography. In most cases, using a single-beam spectrophotometer with a quartz cuvette for quantification is reported, which results in a lengthy method for our purposes. For this reason, a 96-well plate method with polypropylene plastic was adapted to analyze samples on a large scale and to be accessible to breeders. The TCC-HTP results obtained by this method correlate significantly with the TCC results obtained by HPLC, as was observed in other UV-VIS spectrophotometry analyses (Davey et al., 2009; Hagos et al., 2022; Jaramillo et al., 2018).

Likewise, the maximum, minimum, and average values with the HTP method are higher. This can be explained by the fact that only 5 carotenoids of interest were considered to quantify TCC via HPLC. Unlike the HTP method, all the compounds in the sample were read at 450 nm, including some impurities that can overestimate the quantification (Wang et al., 2019).

Strengths and limitations of TCC-HTP as a predictor of carotenoid concentrations

The correlation between the TCC HPLC method and TCC UV VIS method of 0.61 represents a moderate and reasonable agreement between the two methods. The plot 3.4a reveals a linear relationship between the TCC values obtained from both methods, suggesting that, in general, as the TCC measured by the HTP method increases, the TCC measured by HPLC also increases. This finding is consistent with the results reported by Jaramillo et al. (2019), where the R^2 value was the highest compared to other tested techniques evaluated for carotenoids. While a higher R^2 value was expected in this study, impurities in the sample may have increased the absorbance in UV-Vis, thereby affecting the correlation value.

Furthermore, to better understand the performance of the HTP method, the TCC-HTP measured on the X-axis is binned into three main concentration categories: low, mid, and high. This division helps classify and identify sorghum lines based on their carotenoid content. Despite the moderate correlation, the model effectively distinguishes between high and low carotenoid concentrations. However, samples with medium concentrations can sometimes be misclassified into other categories. For our purposes, precise quantification is less critical; what matters most is the effective separation of breeding lines with the highestt carotenoid concentrations, which can be achieved via our HTP method.

Since β-carotene is the most relevant PVAC in sorghum grains, we evaluated the ability to predict it based on TCC-HTP results. The R^2 value obtained shows a statistically significant correlation between both parameters. The samples with higher β-carotene concentrations correspond to those with high TCC. This is particularly important when selecting samples for breeding since samples with high TCC content would intrinsically have high PVAC values. This fact should be confirmed with more sorghum populations to support our hypothesis that our HTP method can be used as a proxy for PVAC carotenoid content.

The agreement between methods was evaluated using a Bland-Attman plot. This indicates a small systematic bias between HPLC and the spectrophotometer, with HPLC generally reading 0.7 μg/g lower than the spectrophotometer on average. While ideally, the bias would be zero, a bias of -0.7 μg/g is relatively small and may be considered acceptable for our purposes. Bland Altman plots showed that HPLC had the best agreement with the limits of agreement -7.5 and 3 μg/g—this spread variability between the two methods was expected. These systematic differences are because only the carotenoids of interest were considered in the HPLC analysis.

Occasionally, there are large disagreements between methods. Investigating these outliers could reveal specific issues with either measurement method under certain conditions. This could be solved by repeating the analysis of the samples with higher discrepancies.

Furthermore, both overestimations and underestimations were observed within the data, indicating inconsistent differences between methods. Previous studies also showed overestimations of the spectrophotometer when comparing carotenoid concentration with HPLC at the highest concentrations (Islam & Schweigert, 2015).

Strengths and limitations of grain yellowness as a predictor of carotenoid concentrations

Biofortified sorghum could be developed when more high-throughput selection methods are available. Currently, breeders rely on the qualitative visual assessment of carotenoids. Sorghum grain yellowness has been shown to be a predictor of carotenoid presence, but can not effectively differentiate carotenoid concentrations between lines (McDowell et al., 2024, in review). Four participants were included in the study to test our hypothesis that the HTP method is more reliable than the visual evaluation of sorghum grain. The evaluation criteria were based on sorghum sorting experience and the ability to differentiate yellowness within a population. Results indicate a strong correlation among observers but no significant correlation with the actual concentrations as measured by HPLC. This suggests that the accurate visual quantification of carotenoids can be difficult (Chandler et al., 2013); therefore, it is necessary for a more analytical tool to evaluate yellowness (McDowell et al., 2024, in review). Similarly, this technique is subjective and provides low repeatability depending on the observer's judgments. Bowman et al., 2004 report that breeders with more than 30 years of expertise tended to select cotton lines with higher lint yields than new breeders (Bowman et al., 2004), so it may be that breeder's with more experience will more accurately use visual assessments to predict grain carotenoid content, as was demonstrated by the significant correlation between observer 1's visual assessment and HPLC values. Observer 1 had the greatest experience with grain phenotyping compared to the other observers. However, despite its significance, the correlation was still quite low.

Visual assessment as a phenotyping tool presents challenges, because grain yellowness can be influenced by the presence of other compounds in the pericarp, such as anthocyanins, condensed tannins, flavanones, and chlorophylls, which can either enhance or mask the yellow coloration (McDowell et al., 2024, in review). Furthermore, during visual inspections, some of the highest carotenoid lines displayed green areas around the pericarp, potentially leading to misleading evaluations. This observation aligns with findings by Stange and Flores (2012), who reported that total carotenoid levels tend to follow the same decline/increase pattern observed in total chlorophylls. The intense presence of chlorophylls in some lines with high carotenoid content could also be attributable to the developmental stage at which the grain was harvested.

The visual assessment scores were also compared with the concentration of the individual carotenoids, and a statistically significant positive correlation with zeaxanthin was found. This relationship could be because zeaxanthin is one of the highest concentrated carotenoids in sorghum grain and may be the major contributor of the yellow-orange pigment (Minguez-Mosquera et al., 1990), which can be more visible than the other pigments. It suggests that visual color evaluation has some utility in estimating the presence of zeaxanthin. In contrast, the other carotenoids showed no significant correlation, suggesting that the other carotenoids did not contribute to the grain color during the visual inspection. Further color assessments in other sorghum families need to be conducted to confirm these results. Similarly, the quality of the results could be enhanced if sorghum breeders participated in the experiment.

Conclusion

Our results highlight the challenges in grain color phenotyping when using HPLC and visual assessment. While both techniques can be employed for selecting high-carotenoid germplasm during pre-breeding and early selection cycles of biofortification, there is a pressing need for faster, more accurate, and more reliable methods to differentiate carotenoid

concentrations within sorghum grain germplasm. The HTP method based on UV-VIS spectrophotometry demonstrates the ability to categorize and distinguish sorghum lines based on their total carotenoid and PVAC content, facilitating targeted breeding and selection. This method offers a significantly faster and more efficient way to screen large numbers of samples compared to HPLC and visual inspection, despite being somewhat less accurate. By focusing on extremes (high and low TCC), the HTP method effectively identifies the most significant differences, leveraging its strengths to ensure reliable selection. Our findings support the hypothesis that the HTP method is more efficient and reliable than visual assessment for selecting sorghum lines with high carotenoid concentrations. By utilizing the HTP method, breeders can achieve more accurate and consistent selection for high carotenoid germplasm, leading to biofortified sorghum varieties with enhanced nutritional value.

Chapter 3. Literature Cited

Abdul-Hammed, M., Bello, I. A., & Oladoye, S. O. (2013). Simultaneous Spectrophotometric Determination of Lycopene and Beta-Carotene Concentrations in Carotenoid Mixtures of the Extracts from Tomatoes, Papaya and Orange Juice: Lycopene and ?-Carotene Determination. Biological Sciences - PJSIR, 56(2), Article 2. https://doi.org/10.52763/PJSIR.BIOL.SCI.56.2.2013.90.97

Bowman, D., Bourland, F., Myers, G., Wallace, T., & Caldwell, D. (2004). BREEDING AND GENETICS Visual Selection for Yield in Cotton Breeding Programs. https://www.semanticscholar.org/paper/BREEDING-AND-GENETICS-Visual-Selection-for-Yield-in-Bowman-Bourland/20ac1d72ec39e405d7111fa0d6583339fd198b10

Chandler, K., Lipka, A. E., Owens, B. F., Li, H., Buckler, E. S., Rocheford, T., & Gore, M. A. (2013). Genetic Analysis of Visually Scored Orange Kernel Color in Maize. Crop Science, 53(1), 189–200. https://doi.org/10.2135/cropsci2012.02.0129

Cruet-Burgos, C., Cox, S., Ioerger, B. P., Perumal, R., Hu, Z., Herald, T. J., Bean, S. R., & Rhodes, D. H. (2020). Advancing provitamin A biofortification in sorghum: Genome-wide association studies of grain carotenoids in global germplasm. The Plant Genome, 13(1), e20013. https://doi.org/10.1002/tpg2.20013

Davey, M. W., Saeys, W., Hof, E., Ramon, H., Swennen, R. L., & Keulemans, J. (2009). Application of Visible and Near-Infrared Reflectance Spectroscopy (Vis/NIRS) to Determine Carotenoid Contents in Banana (Musa spp.) Fruit Pulp. Journal of Agricultural and Food Chemistry, 57(5), 1742–1751. https://doi.org/10.1021/jf803137d

Doebley, J. F., Gaut, B. S., & Smith, B. D. (2006). The Molecular Genetics of Crop Domestication. Cell, 127(7), 1309–1321. https://doi.org/10.1016/j.cell.2006.12.006

Dunn, W. B., Kuligowski, J., Lewis, M., Mosley, J. D., Schock, T., Ulmer Holland, C., Zanetti, K. A., Vuckovic, D., & Metabolomics Quality Assurance and Quality Control Consortium (mQACC). (2023). Metabolomics 2022 workshop report: State of QA/QC best practices in LC–MS-based untargeted metabolomics, informed through mQACC community engagement initiatives. Metabolomics, 19(11), 93. https://doi.org/10.1007/s11306-023-02060-4

Global Nutrition Report. (2021). The state of global nutrition—Global Nutrition Report. https://globalnutritionreport.org/reports/2021-global-nutrition-report/

Hagos, M., Redi-Abshiro, M., Chandravanshi, B. S., & Yaya, E. E. (2022). Development of Analytical Methods for Determination of β-Carotene in Pumpkin (Cucurbita maxima) Flesh, Peel, and Seed Powder Samples. International Journal of Analytical Chemistry, 2022, 9363692. https://doi.org/10.1155/2022/9363692

Islam, K. M. S., & Schweigert, F. J. (2015). Comparison of three spectrophotometric methods for analysis of egg yolk carotenoids. Food Chemistry, 172, 233–237. https://doi.org/10.1016/j.foodchem.2014.09.045

Jaramillo, A. M., Londoño, L. F., Orozco, J. C., Patiño, G., Belalcazar, J., Davrieux, F., & Talsma, E. F. (2018). A comparison study of five different methods to measure carotenoids in biofortified yellow cassava (Manihot esculenta). PLOS ONE, 13(12), e0209702. https://doi.org/10.1371/journal.pone.0209702

Minguez-Mosquera, M. I., Gandul-Rojas, B., Garrido-Fernandez, J., & Gallardo-Guerrero, L. (1990). Pigments present in virgin olive oil. Journal of the American Oil Chemists' Society, 67(3), 192–196. https://doi.org/10.1007/BF02539624

Monk, R., Franks, C., & Dahlberg, J. (2014). Sorghum. In Yield Gains in Major U.S. Field Crops (pp. 293–310). John Wiley & Sons, Ltd. https://doi.org/10.2135/cssaspecpub33.c11

National Sorghum Producers. (2021). The Global Significance of Sorghum—Africa. National Sorghum Producers. https://sorghumgrowers.com/magazine/the-global-signficance-of-sorghum-africa/

Rivera-Madrid, R., Carballo-Uicab, V. M., Cárdenas-Conejo, Y., Aguilar-Espinosa, M., & Siva, R. (2020). 1—Overview of carotenoids and beneficial effects on human health. In C. M. Galanakis (Ed.), Carotenoids: Properties, Processing and Applications (pp. 1–40). Academic Press. https://doi.org/10.1016/B978-0-12-817067-0.00001-4

Somegowda, V. K., Diwakar Reddy, S. E., Gaddameedi, A., Kiranmayee, K. N. S. U., Naravula, J., Kavi Kishor, P. B., & Penna, S. (2024). Genomics breeding approaches for developing Sorghum bicolor lines with stress resilience and other agronomic traits. Current Plant Biology, 37, 100314. https://doi.org/10.1016/j.cpb.2023.100314

Sun, T., Rao, S., Zhou, X., & Li, L. (2022). Plant carotenoids: Recent advances and future perspectives. Molecular Horticulture, 2(1), 3. https://doi.org/10.1186/s43897-022-00023-2

Wang, Q., Wang, G., Xie, S., Zhao, X., & Zhang, Y. (2019). Comparison of high-performance liquid chromatography and ultraviolet-visible spectrophotometry to determine the best method to assess Levofloxacin released from mesoporous silica microspheres/nano-hydroxyapatite composite scaffolds. Experimental and Therapeutic Medicine, 17(4), 2694–2702. https://doi.org/10.3892/etm.2019.7238

Wiseman, E. M., Bar-El Dadon, S., & Reifen, R. (2017). The vicious cycle of vitamin a deficiency: A review. Critical Reviews in Food Science and Nutrition, 57(17), 3703–3714. https://doi.org/10.1080/10408398.2016.1160362

World Health Organization. (2009). Global prevalence of vitamin A deficiency in populations at risk 1995-2005: WHO global database on vitamin A deficiency. https://www.who.int/publications-detail-redirect/9789241598019