IJFANS
International Journal of Food And Nutritional Sciences

Volume 6, Issue 3, July 2017, www.ijfans.com e-ISSN: 2320-7876

INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

IMPACT FACTOR ~ 1.021

Official Journal of IIFANS

IJFANS International Journal of Food and Nutritional Science

e-ISSN 2320 -7876 www.ijfans.com
Vol. 6, No. 3, July 2017

Research Paper ***Open Access***

THE PIGMENTS OF SORGHUM PERICARP ARE ASSOCIATED WITH THE CONTENTS OF CAROTENOIDS AND PRO-VITAMIN A

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Received on: 3rd March, 2017 Accepted on: 1st May, 2017

Objectives and Methods: Sorghum is a staple crop consumed in certain regions of Africa and Asia, where vitamin A deficiency is prevalent. However, the correlation of sorghum intake and vitamin A deficiency is contradictory. The objective of this study was to identify and quantify the carotenoids and pro-vitamin A in the selected sorghum accessions with various pericarp pigments by using LC-MS. Results: Among five carotenoids (α-carotene, β-carotene, lutein, zeaxanthin, and β-cryptoxanthin) identified and quantitated, three (α-carotene, β-carotene and β-cryptoxanthin) were precursors of vitamin A. The highest content of total carotenoids was found in the sorghum accessions with yellow pericarp (PI656096, PI585374, PI563448 and PI585351), whereas the highest β-carotene content was found in the accessions with brown or yellow pericarp (PI655996, PI656096, PI585374, PI563448 and PI585351). The lowest carotenoids were found in the accessions with white pericarp (PI533943, PI656112, PI565121 and PI560493). The pro-vitamin A was 584.9±38.9 ng/g DW in the accessions with yellow pericarp, 250.6±28.9 ng/g DW in brown pericarp, and 89.0±12.3 ng/g DW in white pericarp, respectively. Conclusion: The results suggest, for the first time, a possible prevention of vitamin A deficiency by consuming selected sorghum varieties with yellow pericarp pigments.

Keywords: Carotenoids, Pro-vitamin A, Sorghum, Pericarp pigments, Vitamin A deficiency

INTRODUCTION

Grain sorghum (*Sorghum bicolor*) is a staple food in Southern Asia and Africa where the climate is too hot and dry for other grains to thrive (Maunder, 2005; and FAO, 2017). Sorghum is the 5th most important cereal, the average consumption per capita in Africa is 23 kg/person/year (FAO, 2017). The United States is the largest producer of sorghum in the world, followed by Nigeria, Sudan, Mexico, and India. As the main dietary foods (Anglani, 1998; and Ramatoulaye *et al.*, 2016), sorghum provides some advantages such as phytochemical anthocyanins- and/or phenolic acids-enriched antioxidant activity related to chronic disease prevention (Awika and Rooney, 2004). Furthermore, sorghum is a gluten-free product. Therefore, is an alternative grain for individuals with gluten sensitivities or celiac disease (De Mesa-Stonestreet *et al.*, 2010). Sorghum grain contains high fiber content with a lower glycemic index than other grains, which may assist to reduce obesity and Type-2 diabetes. On the other hand, compared to wheat, corn, and rice, there are some drawbacks to sorghum use in food such as lower sensory quality and reduced protein digestion due to the cross-linked protein properties when cooked (Duodu

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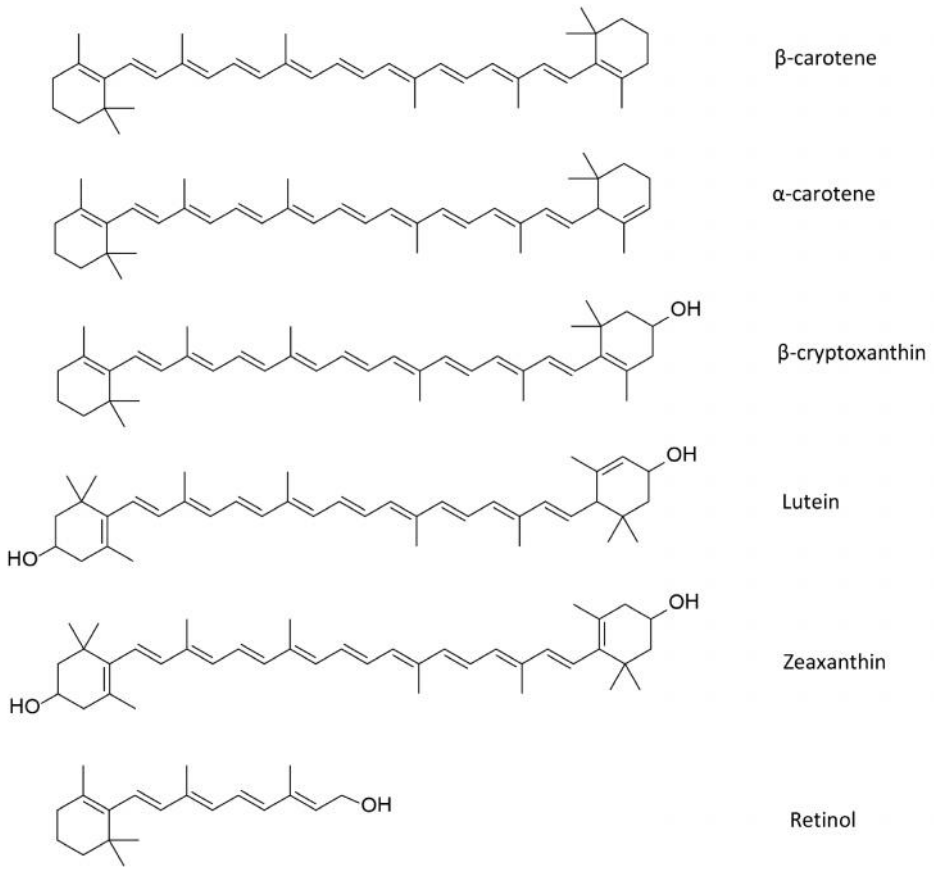
et al., 2003). The high levels of fiber tannins in sorghum products have also been concerned regarding anti-nutrient absorption, although sorghum containing tannins are not grown in the United States.

According to the World Health Organization, there are estimated 75-140 million children with vitamin A deficiency. Among of this group 4.4 million of children have developed the symptoms of xerophtalmia, and 6 million of pregnant women have night blindness during pregnancies every year (FAO, 1995). In South and Southeast Asia, there are about 45% of the children and pregnant women with vitamin A deficiency. In Africa, more than half of this group of population are affected (West, 2002). The severity of vitamin A deficiency in these areas may be related to sorghum consumption.

In addition to animal-based food sources, dietary vitamin A is available in provitamin A carotenoids. Carotenoids are one of the phytochemicals that include carotenes (β-carotene, α-carotene, and lycopene) and xanthophyll (lutein, zeaxanthin, and β-cryptoxanthin). The chemical structures of the common carotenoids found in sorghum are shown in Figure 1. When compared with vitamin A retinol structure, three carotenoids, i.e., β-carotene, α-carotene, and β-cryptoxanthin, have nutritional value of provitamin A (Bendich, 1993; Paiva and Russell, 1999; Rao and Rao, 2007; and Fiedor and Burda, 2014). Conversion of β-carotene to vitamin A involves cleavage of the central double bond of β-carotene molecule to yield two 20-carbon molecules of vitamin A. In contrast, α-carotene or β-cryptoxanthin generates just one molecule of retinol (Graham and Rosser, 2000), although much lower conversion rates in human body have been reported, i.e., only 1/12 for β-carotene and 1/24 for α-carotene or β-cryptoxanthin (Tang, 2010; and Institute of Medicine, 2000). The Recommended Dietary Intake (RDI) of vitamin A for women and men has been established as 700 µg and 600 µg, respectively (Olson, 1987), since vitamin A deficiency or inadequacy has induced many problems

Figure 1: Chemical Structures of the Common Carotenoids Found in Sorghum versus Vitamin A Retinol



such as blindness, anemia, immune dysfunction, infection, xerophthalmia, growth retardation, and some chronic diseases (Olson, 1987; and West and Darnton-Hill, 2008).

Sorghum usually contains provitamin A carotenoids, but the association of sorghum intake and vitamin A deficiency is contradictory. Some studies conducted in Africans have indicated that various types of malnutrition including vitamin A deficiency were observed when sorghum was the principle grain in the diet (Lipkie *et al.*, 2013; and Adegboye *et al.*, 2016). Other studies, however, suggested that the sorghum consumed in Africa and Asia be a critical source of dietary carotenoids that might provide the needed pro-vitamin A's (Kean *et al.*, 2007; and Kean *et al.*, 2011). We hypothesized these inconsistent association might be due to the different sorghum varieties consumed. The different varieties could differ in the colors of the endosperm and/or pericarp which were contributed by the pigmented carotenoids (Fernandez *et al.*, 2009).

The phenotypic color of sorghum endosperm and pericarp is determined by genotypic factor related to carotenoid biosynthesis (Rhodes *et al.*, 2014). Endosperm usually exhibits yellow or white color. According to the USDA-National Plant Gemplasm System, only 381 accessions of sorghum exhibited yellow endosperm from a total of 42,869 sorghum accessions. When compared with white, yellow endosperm showed the higher levels of carotenoids (Cardoso *et al.*, 2015). For example, Fernandez *et al.* (2008) found that total carotenoid contents in yellow and white endosperm were around 1.68 and 0.20 μg/g DW, respectively. When compared with endosperm, carotenoids are enriched at the pericarp. However, relatively little data have been published documenting the carotenoid contents in the sorghum pericarp, and virtually nothing is known about the relationship of the pigments of sorghum pericarp with pro-vitamin A status.

The objective of this study was to identify and quantify the carotenoids and pro-vitamin A status in the selected sorghum accessions with various pigments of endosperm and pericarp. These innovative results could be significant in identifying particular sorghum varieties that might be benefit in prevention of vitamin A deficiency.

MATERIALS AND METHODS

Reagents and Chemicals

Methanol, ethyl acetate, acetone and petroleum ether at HPLC grade were purchased from Thermal Fisher Scientific (Suwanee, GA). Sodium hydroxide was purchased from Thermal Fisher Scientific (Suwanee, GA), and anhydrous ammonium acetate was purchased from MP Biomedicals, LLC (Solon, OH). Water used in all preparation and analysis was purified through Barnstead E-Pure Deionization System (Dubuque, IA) and filtered using Millipore 0.45 um membrane (Bedford, MA). The internal standard of trans-β-apo-8'-carotenal was obtained from Sigma-Aldrich (St. Louis, MO).

Sample Preparation

Nine sorghum accessions were selected on the basis of their endosperm and pericarp colors. Yellow endosperm/brown pericarp (PI 655996), yellow endosperm/yellow pericarp (PI 656096, PI 585374, PI 563448, and PI 585351), yellow endosperm/white pericarp (PI 533943 and PI560493), and white endosperm/white pericarp (PI 656112 and PI565121) were grown in Manhattan, KS during the 2015 summer crop season. All the sorghum was harvested in November 2015, the panicles were threshed, and the seeds were ground to flour by using an Udy Mill. Sorghum flour was placed in centrifuge tubes and wrapped with aluminum-foil paper to minimize carotenoid photooxidantion reaction and immediately stored at -40 °C. Final carotenoid contents are quantitated and expressed as nanograms per gram of dry weight.

Carotenoid Extractions

The carotenoids were extracted using the methods described by Kean *et al.* (2007), Kean *et al.* (2008) and Lipkie *et al.* (2013) with some modifications. All sample preparations and extractions were performed under amber light to minimize carotenoid degradation. Approximately 2 grams of each dry flour sample were dispersed in 8 mL of double-distilled water which containing 0.1% BHT to prevent carotenoid oxidation. The slurry was saponified at 50 °C in a water bath for 30 min upon adding 6 mL of 80% NaOH and 2 mL of methanol with vortexing. After saponification, the carotenoids were extracted with 6 mL of petroleum ether and 2 mL of acetone. Samples were vortexed for 30 sec and then centrifuged at 3,500 g for 5 min to insure complete phase separation. The petroleum ether layer was collected and the residue was then re-extracted by two more times. The petroleum ether fractions were combined and dried under nitrogen gas, re-dissolved in 1,200 μL of 1:1 methanol/ethyl acetate for yellow endosperm/yellow pericarp sorghum. Other extracts from yellow endosperm/brown pericarp, yellow endosperm/white pericarp, or white endosperm/white pericarp sorghum was re-dissolved in 600 μL of 1:1 methanol/ethyl acetate. Then

the dissolved solutions were filtered by a 0.45 µm filter prior to HPLC analysis. A known concentration of the internal standard, i.e., trans-β-apo-8'-carotenal, was added at the beginning of the extraction to account for extraction recovery and quantification equivalence. All the samples were repeated in triplicate.

HPLC Analysis

Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic separation. This system was employed by a DGU-20A3 built-in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A YMC waters (Milford, MA) C30 reversed phase column (250 mm length, 4.6 mm diameter, 3.0 µm particle size) was used for the carotenoids separations. HPLC separation method was previous reported referred to Kean *et al.* (2007) with some modifications. Elution was performed with mobile phase A (methanol in 1 M ammonium acetate at 98:2 v/v) and mobile phase B (ethyl acetate) by a gradient of mobile phase B at 5-35% for 20 min and 35% for 10 min before returning to 5%. The flow rate was maintained at 1 mL/min and the column temperature was maintained at 40 °C. The detector performed a full spectrum scan between 190-800 nm where 450 nm was used for monitoring carotenoids. Trans-β-apo-8'-carotenal was used as an internal standard for adjustment of extraction recovery and quantitation of carotenoid equivalence. The average recovery was around 60% in this study. Data was analyzed using the LC solution software (Kyoto, Japan).

TOF / MS Analysis

Bruker UltraFlexII MALDI-TOF mass spectrometry in Linear negative mode was used to carry out carotenoid identification. The samples were analyzed using 30 mg/mL DHB (2, 5-Dihydroxybenzoic acid) matrix solution in ACN (Acetonitrile) and 0.1% TFA (Trifluoroacetic acid). Carotenoid compounds were confirmed by HPLC retention time, monoisotopic mass and absorbance spectra pattern according to previous publication (Britton, 2009; and Rivera, and Canela-Garayoa, 2012).

Statistical Analysis

Data were analyzed using SAS statistical software, version 9.3 (SAS Institute, Cary, NC, USA). Results were evaluated by one-way ANOVA. Tukey's post-hoc test was used to assess the differences of individual carotenoids content in various sorghum accessions. The results were presented as means ± SD, and $p < 0.05$ was considered significant.

RESULTS

Chromatographic Separation

The profile of the carotenoid HPLC chromatograms from the representative sorghum accessions with various pericarp pigments were shown in Figure 2. Total five carotenoids (lutein, zeaxanthin, β-carotene, α-carotene, and β-cryptoxanthin) and internal standard (trans-β-apo8'-carotenal) were eluted within 30 min.

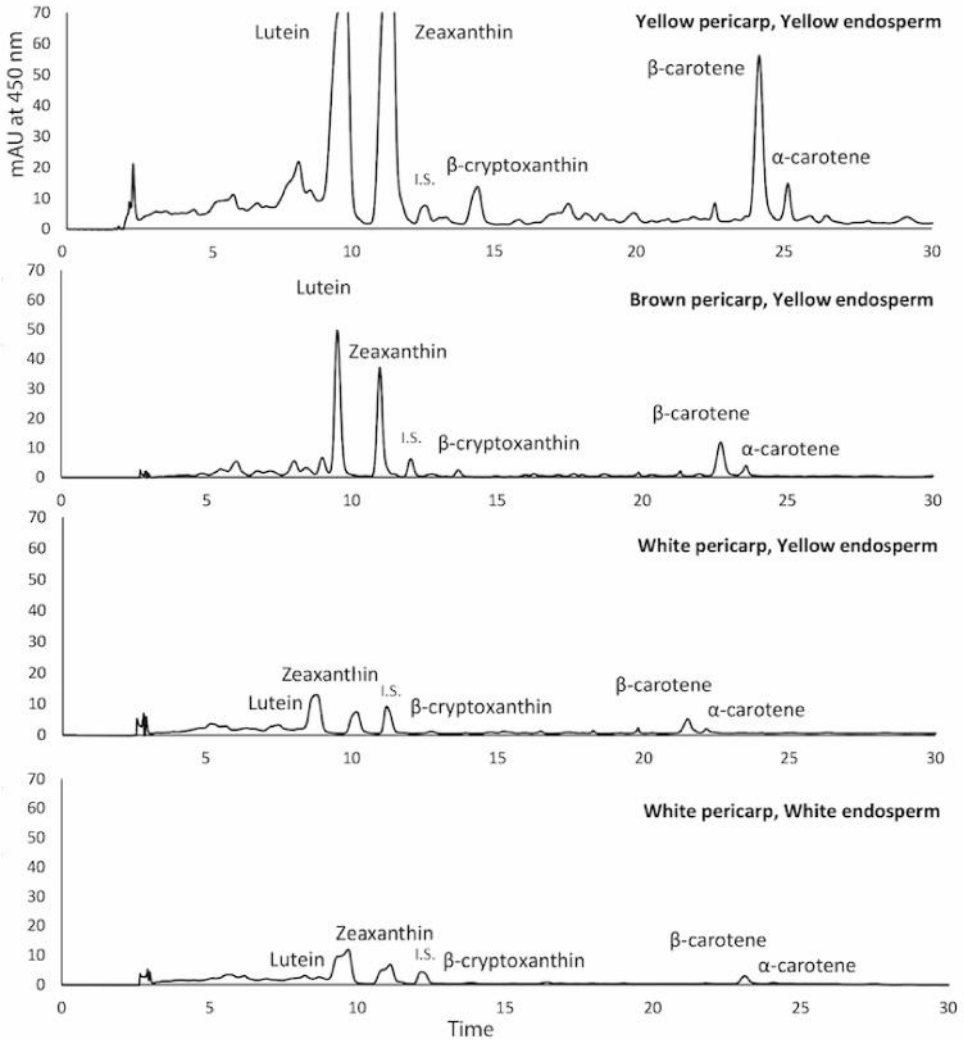
Mass Spectrometric Identification

Following HPLC separation, MALDI-MS data was generated by monitoring the negative ion spectra from 300-1000 m/z. The m/z ratio of each parent mass was listed in Figure 4. Totally three peaks were identified including lutein and zeaxanthin (m/z 567), α-carotene and β-carotene (m/z 535) and β-cryptoxanthin (m/z 552). As shown in Figure 3, β-carotene (m/z 535), lutein (m/z 567), and zeaxanthin (m/z 567) are three major carotenoids in sorghum accessions. MALDI-MS did not separate α-carotene from β-carotene or lutein from zeaxanthin because of identical molecular weights. However, they could be separated well by HPLC based on their different retention times (Figure 2).

Quantification

The highest contents of total carotenoids were found in sorghum accessions with yellow pericarp/yellow endosperm (PI 656096, PI 585374, PI563448, and PI585351), followed by brown pericarp/yellow endosperm (PI 655996). PI 585351 contained a total carotenoid content of 2343.2±96.5 ng/g DW, while PI 655996 was 762.5±103.9 ng/g DW. White pericarp/yellow endosperm (PI 533943 and PI 560493) and white pericarp/white endosperm (PI 656112 and PI565121) showed the lowest carotenoid contents at 241.2±19.0 and 284.6±25.6 ng/g DW, respectively. As shown in Figure 4, the contents of individual carotenoid in different pericarp colors demonstrated the similar pattern as the total contents of carotenoids. Top three carotenoids (lutein, zeaxanthin, and β-carotene) accounted for more than half of the total carotenoids. The contents of pro-vitamin A calculated based upon the converted contents of α-carotene, β-carotene, and β-cryptoxanthin were 584.9±38.9 ng/g DW in yellow pericarp/yellow endosperm, 250.6±28.9 ng/g DW in brown pericarp/yellow endosperm, 89.0±12.3 ng/g DW in white pericarp/yellow endosperm,

Figure 2: Chemical Structures of the Common Carotenoids Found in Sorghum versus Vitamin A Retinol



and 79.0±7.3 ng/g DW in white pericarp/white endosperm, respectively.

DISCUSSION

To verify the relationship of the pigments of sorghum pericarp with the contents of carotenoids and pro-vitamin A, we conducted this pilot study and quantitated carotenoids in nine selected sorghum accessions with different pericarp colors. Total five carotenoids including lutein, zeaxanthin, β-carotene, α-carotene, and β-cryptoxanthin were identified and quantitated. Among of which, lutein, zeaxanthin, and β-carotene were three major carotenoids and α-carotene, β-carotene and β-cryptoxanthin belonged to precursors of vitamin A. Yellow pericarp/yellow endosperm sorghum accessions showed the highest contents of carotenoids and vitamin A equivalent, followed by brown pericarp/yellow endosperm and white pericarp/yellow endosperm. The white pericarp/white endosperm displayed the least.

It should be noted that the HPLC quantitation method applied in this study demonstrated a better separation and sensitivity than the reported studies by others. Three previous studies could detect two to three carotenoids only, while five carotenoids were separated and quantitated in this study. Furthermore, the contents of carotenoids detected in this study were much higher than the studies reported by others. For example, the contents of lutein and zeaxanthin in the yellow pericarp/yellow endosperm found in this study

Figure 3: Representative HPLC Chromatograms of Carotenoids Detected in the Sorghum Accessions with Various Pigments of Pericarp and Endosperm

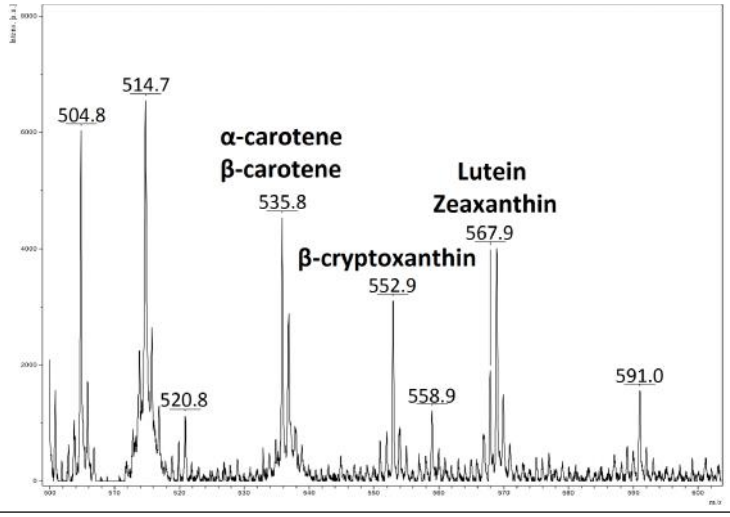
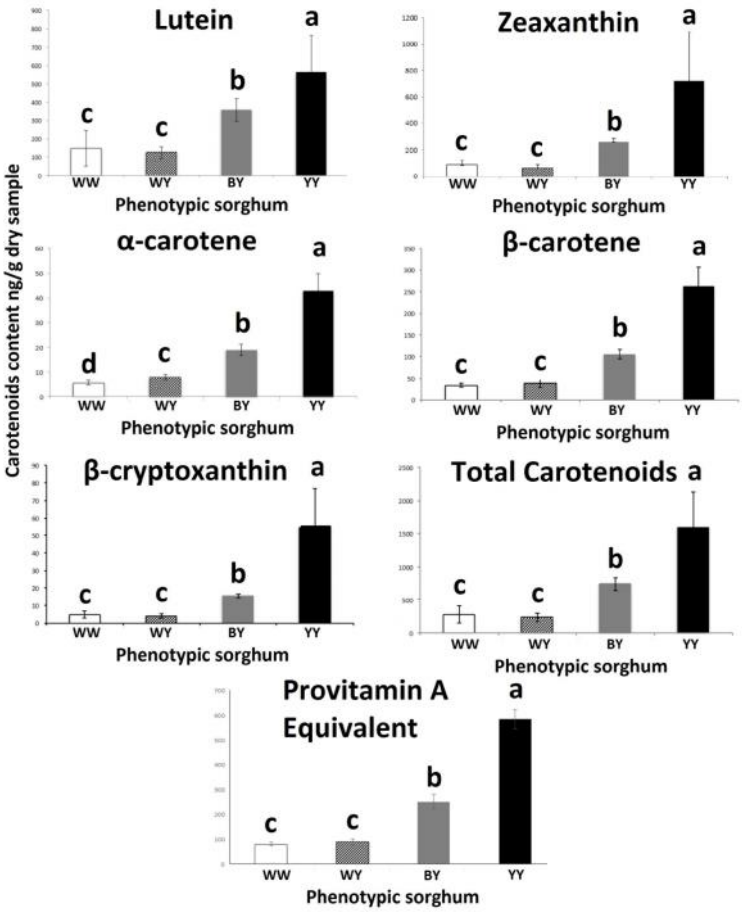


Figure 4: Representative TOF-MS of Carotenoids Identified in the Sorghum Accessions



were higher than the study reported by Kean *et al.* (2007) and Cardoso *et al.* (2015). The content of β-carotene in yellow endosperm sorghum varieties was also higher than the study reported by Kean *et al.* (2007). The reason why more and higher contents of carotenoids could be detected in this study appeared to be related to our improved method by using C30 column rather than a traditional C18 column. When compared with C18, C30 column was much more hydrophobic and thus had better resolution and separation of different geometrical isomers (Rimmer *et al*., 2005). In addition, the increased concentration and time of ethyl acetate might help enhance carotenoid interaction and elute a sharp peak. Higher column temperature and less light exposure conducted in this study might also help to release the pressure and lessen the oxidative degradation.

Based on the results of this study, five carotenoids were identified. Three of them (β-carotene, α-carotene, and β-cryptoxanthin) were precursors of vitamin A. To the best our knowledge, this is the first study showing the association of the sorghum pericarp pigment with carotenoid-induced vitamin A contents. The data of pro-vitamin A equivalence might be valuable, given the highest equivalence of pro-vitamin A was found in yellow pericarp and then followed by brown pericarp of sorghum varieties. In addition to endosperm color, pericarp color could be a visual marker for carotenoid contents, this experiment could be thus more significant in helping breeders to select sorghum varieties which contain high pro-vitamin A.

Besides of the pro-vitamin A status provided by β-carotene, α-carotene, and β-cryptoxanthin, the other two abundant carotenoids, i.e., lutein and zeaxanthin, may offer some biological functions. For example, both of them were only carotenoids that found in the retina and lens (Johnson, 2002). According to NIH National Eye Institute, these two antioxidant carotenoids may play an important role in the protection against eye disease such as age-related macular degeneration (Eye, 2001; Clemons *et al.*, 2003; and San Giovanni *et al.*, 2007).

CONCLUSION

Total five carotenoids including three pro-vitamin A carotenoids were identified and quantified in the selected sorghum accessions with various pericarp pigments by using LC-MS. The highest contents of total carotenoids and pro-vitamin A were detected in the sorghum accessions with yellow pericarp/yellow endosperm, followed by brown pericarp/yellow endosperm and white pericarp/yellow endosperm. The lowest carotenoids and pro-vitamin A were found in the accessions with white pericarp/white endosperm. It appeared that the phenotypic diversity of sorghum pericarp colors was associated with the content of carotenoids and pro-vitamin A, indicating a different impact of various sorghum varieties on vitamin A deficiency and suggesting a possible prevention of vitamin A deficiency by breeding certain sorghum varieties with pericarp pigments.

ACKNOWLEDGMENT

We thank Mr. Benjamin Katz and Dr. John M. Tomich, Kansas State University Biotechnology Core Facility, for conducting MS analysis. This study was supported in part by a USDA Cooperative Project KS511-1001903 from the Agricultural Experiment Station, Kansas State University (Contribution #17-073-J from the Kansas Agricultural Experiment Station).

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