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In vitro Bioaccessibility of Beta Carotene from Thermally Processed Leafy African Indigenous Vegetables

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Abstract

Beta carotene (BC), a pro-vitamin A carotenoid found in leafy African indigenous vegetables (LAIVs) and fruits, plays important biological roles towards protection against cardiovascular diseases, cancer and many others. Lack of vitamin A is a major challenge in many developing countries where its source is mainly vegetables. The carotenoid exists as a complex in different food matrices and has to be released from the food matrix for it to be bioaccessible. Different processing procedures affect bioacessibility, boiling and boil-frying being the main thermal processes used by many households in developing countries. The study assessed the bioaccessibility of BC in thermally processed (boiled and boiled-fried) spider plant, cowpeas, amaranth, vine spinach and pumpkin leaves using an *in vitro* method. After extraction and separation using column chromatography the levels of BC were determined using UV-Vis spectrophotometry. A static gastrointestinal digestion procedure was used to obtain bioaccessible levels of BC. The respective percentage bioaccessibility (%) of BC from spider plant, cowpeas, amaranth, vine spinach and pumpkin leaves were as follows: fresh 97.23±0.01, 81.60±0.36, 77.22±0.05, 61.36±1.87 and 94.48±0.57, boiled 78.80±1.86, 84.31±0.27, 92.28±0.46, 65.67±5.53 and 80.75±0.69, and boiled-fried [48.19±0.82, 31.02±3.09, 16.97±0.02, 23.15±2.82 and 23.33±2.89 respectively. Boiled LAIVs had higher percentage bioaccessibility than boiled-fried due to the effect of longer period of exposure to heat during processing. The knowledge on bioaccessibility of BC from the LAIVs reported in this study would play a key role in encouraging their consumption thus contributing to food security as well as curbing malnutrition.

Keywords: *In vitro* bioaccessibility; beta carotene; Leafy African Indigenous vegetables; thermal processing

1. Introduction

Leafy African indigenous vegetables (LAIVs) are valuable sources of macro and micro nutrients (vitamins, minerals, proteins and antioxidants) essential for human health (Palafox-Carlos *et al.,* 2011; Kamga *et al.,* 2013). Their utilization could greatly help in curbing malnutrition mainly in developing Countries (Kamga *et al.*, 2013). The levels of nutrients depend on factors such as species, climatic conditions, farming practices, age of the plant and post-harvest handling procedures (Rodriguez-Amaya and Kimura, 2004; Ahamed *et al.*, 2007). The LAIVs are resistant to pests and adverse environmental conditions and therefore can survive in unfavorable conditions (Kunyanga *et al.,* 2013). They are rich sources of vitamins to millions of people in both developed and developing countries and generally affordable (Kunyanga *et al.,* 2013; Bwembya *et al.,* 2014). Although there are more than 200 species of LAIVs in Kenya, most of these are underutilized. Leafy African indigenous vegetables are cultivated and produced in over 60% of households in rural and peri-urban parts of Kenya among the Luhya, Luo and Kisii communities (Abukutsa-Onyango 2007; Kebede and Bokelmann, 2017). Most of these vegetables, such as spider plant (*Cleome gynadra*), cowpeas (*Vigna unguiculata*), amaranth (*Amaranthus viridis),* vine spinach *(Basella alba)* and pumpkin leaves *(Cucurbita maxima)* are valuable sources of beta carotene. The World Health Organization recommends dietary allowances (RDAs) for vitamin A ranging from 0.3 to 1.3 mg/day (WHO, 1998).

Beta carotene (BC), a pro-vitamin A compound in vegetables and fruits, is important for eye health and good vision, metabolism, healthy skin and mucous membrane (You *et al.*, 2002; Zahra *et al.*, 2016). Vitamin A deficiency (VAD) is a major nutritional challenge among children aged below five years and women of child bearing age especially in sub-Saharan Africa (You *et al.,* 2002; Nawiri *et al.,* 2013). In Kenya, for example, the VAD prevalence is estimated at 76%

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in eleven districts (WHO, 1998). The deficiency affects immunity which damages the light sensitive receptors resulting in xerophthalmia and permanent blindness (Tan and Norhaizan, 2019). According to World Health Organization (WHO, 1998), 2.8 million pre-school children are at risk of VAD related blindness. To address the VAD challenge, nutritional approaches have been advocated for over and above supplementation and food fortification (Nawiri *et al.*, 2013; Shellack *et al.*, 2015). Nutritional approaches include promotion of vegetable consumption based on advantages of being resistant to pests and adverse environmental conditions, fast growing, affordable in addition to their nutritional value (Kunyanga *et al.,* 2013; Bwembya *et al.,* 2014). Leafy African indigenous vegetables are seasonal and their preservation by drying during large harvest periods will make them available during periods of scarcity (Opiyo *et al.,* 2015). In their consumption, a daily diet composed of 50% vegetables is enough to provide 4.0 mg/day for men and 4.4 mg/day for women (Ahamed *et al.*, 2007; Tan and Norhaizan, 2019). Amaranth has been reported to contain BC in the range from 47.82±1.32 to 64.35±1.46 mg/100g DW (fresh) and 4.97±1.22 to 48.04±2.18 mg/100g DW (freeze dried) (Cheptoo *et al.*, 2019). Veda *et al.*, (2006) reported levels of BC to be 1.90±0.27 mg/100g in fresh pumpkin leaves and 8.71±0.54 mg/100g in amaranth, while Kunyanga *et al.,* (2013), studying the same vegetables reported 2.66±0.04 mg/100g and 4.29±0.20 mg/100g respectively.

Bioaccessibility of BC from food is affected by factors such as diet composition (fat, fiber and protein) and thermal processing methods used like boiling and frying (Veda *et al.*, 2006). Thermal processing in the presence of oxygen and light decreases BC levels by degradation (Veda *et al.*, 2006; Nagao, 2014). Oxidation and isomerization are among the main reaction processes through which bioaccessibility of BC from vegetables is decreased (Veda *et al.*, 2006). Dietary fiber in vegetables also decreases bioaccessibility, it slows gastric emptying, digestion and absorption of nutrients mainly due to the water retained by pectin that forms viscous solution in the gut (Palafox-Carlos *et al.,* 2011). This they do by physically trapping nutrients within tissues and enhancing viscosity of gastric fluids thereby restricting the mixing process (Palafox-Carlos *et al.*, 2011). The fiber content of different vegetables has been reported; spider plant 0.8% (Lymio, 2003), cowpeas 19.45 % (Enyiukwu *et al.,* 2018), vine spinach 2.2% (Vicente *et al.*, 2009), pumpkin leaves, 12.04±0.02 % and amaranth, 8.64 ±0.04% (Kunyanga *et al.,* 2013). Aschoff *et al*., (2014) recorded an increased bioaccessibility with diminished fiber content in the sample.

The presence of fat/oil as well as mild cooking increases BC bioaccessibility (Tan and Norhaizan, 2019; Veda *et al.*, 2006; Óconnell *et al.,* 2007). Boiling and frying helps increase the surface area and interactions of hydrolytic enzymes and emulsifiers with food particles during the gastric and intestinal phases of digestion to release BC (Veda *et al.,* 2006; Nagao, 2014). The released BC is thus dispersed with the aid of bile, which contains bile salts and phosphatidylcholine before solubilized in mixed-micelles, formed through hydrolysis of lipids emulsified in digesta by lipolytic enzymes in pancreatic juice (Nagao, 2014). In *in vitro* bioaccessibility studies using gastrointestinal model, digestion process is mimicked in two consecutive steps, gastric and intestinal. Using a gastrointestinal model, Loh and Koh, (2018) recorded higher percent bioaccessibility of BC in boiled butternut (2.03±0.15%) and pumpkin (11.42±0.063%) compared to raw 1.65±0.04% and 10.56±0.44% respectively.

Adequate intake of vitamins and minerals among them beta carotene play a key role in reducing the prevalence of disease especially among the vulnerable groups, expectant mothers and children aged five years and below. Vitamin A deficiency is the leading cause of preventable childhood blindness and increases the risk of death from common childhood illnesses such as diarrhoea. Bioaccessibility of beta carotene from LAIVs has not been reported. Noting the underlying factors that affect bioaccesibility of BC, while advocating for consumption of LAIV's, this study employed an *in vitro* method to study bioaccessibility of pro-vitamin A carotenoid from thermally processed (boiled and boiled-fried) spider plant, cowpeas, amaranth, vine spinach and pumpkin leaves.

2. Materials and Methods

2.1 Equipment and chemicals

A UV-Vis spectrophotometer (Shimadzu model UV – 1601 PC, Kyoto, Japan) was used to quantify beta carotene. All the chemicals and reagents used were of analytical grade. Beta carotene standard (99.9 % pure) and enzymes; pepsin (porcine), pancreatin (porcine), lipase, bile salts, acetone, petroleum ether, silica gel, HCl, $NaHCO_3$ and NaCl were obtained from Sigma-Aldrich, Germany.

2.2 Sampling and sample preparation

Five leafy African indigenous vegetables (LAIVs) samples (spider plant (*Cleome gynadra*), cowpeas (*Vigna unguiculata*), amaranth (*Amaranthus blitum),* vine spinach *(Basella alba)* and pumpkin leaves *(Cucurbita maxima)* were collected from an open market in Kisii County, Kenya in the month of September which is a wet season. The five LAIVs were sampled (1 kg of each) and immediately sprayed with water to keep them moistened, packed in dark plastic polythene bags and transported to the Department of Food Science and Technology laboratory, Jomo Kenyatta University of Agriculture and Technology (JKUAT) for analysis.

Each of the types of vegetables was washed under tap water, rinsed with distilled water and flapped to remove water. Pumpkin leaves (*Cucurbita maxima),* which had broad leaves, were cut into small pieces after washing. Each vegetable type was divided into 2 portions, with the first portion of about 100 g used for fresh analysis and the second portion boiled. During boiling, 80 g of the fresh vegetables were boiled in 200 ml of distilled water for 10 minutes at 100^oC and then cooled to room temperature. For the fried samples, 40 g of the boiled vegetables were added to 40 ml of vegetable oil already heated in a cooking pan (100^oC). This was fried for 10 minutes and then cooled to room temperature. All the fresh, boiled and boiled-fried samples were placed in zip locked bags, frozen for five hours at -20^{o}C and then freeze dried at -50^{o}C for 96 hours. The freeze-dried samples were then wrapped in aluminium foil and kept in the refrigerator at 4^{o}C awaiting determination of BC.

2.3 Extraction and measurement of beta carotene

Extraction of beta carotene (BC) was performed according to the procedure in Rodriguez-Amaya and Kimura, (2004). Standard calibration curves were developed from BC standard series (0-10 ppm) after preparing a stock solution of 100 ppm. Approximately 2 grams of each sample was weighed in triplicates, placed in a mortar with about 10 mL of acetone. This was thoroughly ground and the acetone extract transferred into 100 mL volumetric flask. The residue was re-extracted with 10 mL acetone several times until the residue no longer gave color and the extracts added to the contents of the volumetric flask. The combined extract was made to a volume of 100 mL with acetone. Exactly 25 mL of the extract was evaporated to dryness using rotary evaporator. The residue was dissolved with 10 mL petroleum ether and the solution introduced into open chromatographic column packed with silica gel (70-230 mesh ASTM) and eluted with petroleum ether into a 25 mL volumetric flask, topped up to the mark and absorbance read at 440 nm in a UV-Vis spectrophotometer. The concentration of BC was determined based on regression analysis.

2.4 *In vitro* digestion and determination of bioaccessible beta carotene

Simulated *in vitro* digestion method was adapted from Veda *et al.*, 2006 and was done in triplicates. The process involved two phases; gastric phase and intestinal phase. The sample, 2 g, was subjected to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37^o C (16 g in 100 mL 0.1M HCl) for 2 hrs, followed by digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin) and 25 g by bile extract (porcine) in 1000 ml of 0.1 M $NaHCO_3$ pH 7.5 at 37^{o}C for 2 hrs. The micellar fraction containing the bioaccessible BC was separated by ultracentrifugation at 70 000 x g for 120 minutes using a Beck-man L7- 65 ultracentrifuge. The supernatant aliquot was filtered using 0.22 µm micro filter to obtain a micellar fraction, placed in an amber glass bottle and levels of BC measured as per section 2.3. To obtain percentage bioaccessibility (%), the bioaccessible levels were divided by the original levels then multiplied by one hundred.

2.5 Data analysis

One-way ANOVA was used to compare the mean levels of BC in vegetables prepared using different thermal processes, at p-values < 0.05 significant difference. Mean separations was done by standard error (Sawyer and Beebe, 2007).

3. Results and Discussion

The mean levels, bioaccessible levels and percentage bioaccessibility of BC in fresh, boiled and boiled-fried leafy African indigenous vegetables are given in **Table 1**.

The mean levels of BC ranged from 8.57±0.50 mg/100g (vine spinach) to 27.79±0.01 mg/100g (amaranth) in the fresh vegetables. According to WHO 1998, these range of levels of BC are sufficient to meet the RDA of 0.3 to 1.3 mg/day. These levels differ per vegetable based on a number of factors not limited to the species, maturity and fibre content (Rodriguez-Amaya and Kimura, 2004; Ahamed *et al*., 2007; Aschoff *et al*., 2014). The levels of BC in the fresh vegetables would determine the resultant levels when subjected to thermal processes.

The effect of thermal processing had a dual effect on the pro-vitamin A causing both an increase and decrease of BC. It was observed that these levels significantly decreased (p<0.001) in boiled-fried vegetable samples and significantly increased (p<0.001) in the boiled ones. Further, statistical test (ANOVA) showed that the effect of each thermal processes per the array of vegetables did not differ significantly (*p<0.002, **p=0.001, ***p<0.001). The mean levels of BC ranged from 5.40±0.41 mg/100g, (pumpkin leaves) to 10.36±1.20 mg/100g, (spider plant) in boiled-fried vegetables and from 14.22±2.30 mg/100g, (vine spinach) to 42.63±2.36 mg/100g, (amaranth) in boiled vegetables. The resultant levels are however sufficient to meet the RDA of 0.3 to 1.3 mg/day. The effect of thermal processing on the levels of BC in vegetables is known to occur depending factors such as presence of oxygen and light (Veda *et al.*, 2006; Nagao, 2014). Boiling for example, releases BC from the food matrix by breaking the cell wall and dissociating carotene-food matrix complexes; explaining the significant increase of BC in the boiled vegetables. On the contrary, boiled-fried vegetables exposed BC to high temperature for a longer period that resulted in the breakdown and ultimate decrease. Thermal processing of vegetables have reported both lower (Veda *et al.*, 2006; Bernhardt Schlich, 2006; Ahamed *et al.*, 2007) and higher (Cheptoo *et al.*, 2019) BC levels in comparison to those found in this study.

All the thermal processes resulted in reduced bioaccessible levels of BC (bioaccessible levels, Mean±SD, mg/100g DW) with over 16% bioaccessibility. The bioaccessible mean levels (mg/100g) of BC in fresh vegetables ranged from from 5.26±0.47 (vine spinach) to 27.02±1.00 (spider plant) which translated to 61.36±1.87 % to 97.23±0.06 %. Following thermal processing, the ranges were 9.97±0.03 (vine spinach) to 39.35±3.70 (amaranth) in the boiled and 1.13±0.12 (amaranth) to 5.40±1.32 (spider plant) in the boiled-fried vegetables implying ; 65.67±5.53 % to 92.28±0.46 % and 16.97±0.02 % to 48.19±0.82 % respectively.

There were varied effects on bioaccessibility of BC with processing of the LAIVs. Processing by boiling for example, led to an increase (3 % - 15%) of BC in cowpeas, amaranth and vine spinach but reduced in spider plant (19 %) and

Table 1: Mean, bioaccessible levels and percentage (%) bioaccessibility of BC in processed LAIVs

Vegetable	**Treatment**	**BC levels** (Mean±SD, mg/100g DW n=3)	**Bioaccessible levels** (Mean±SD mg/100g DW)	**% Bioaccessibility**
Spider plant	Fresh*	27.79±1.01^{b} 42.43±3.80^{c}	27.02±1.00^{b}	97.23±0.01^{c}
	Boiled**	10.36±1.27^{a}	33.48±3.79^{c}	78.80±1.86^{b}
	Boiled-fried***		5.40±1.32^{a}	48.19±0.82^{a}
	p-value	<0.001	<0.001	<0.001
Cowpeas	Fresh*	23.06±0.81^{b}	18.82±0.49^{b}	81.60±0.36^{b}
	Boiled**	25.63±0.51^{c}	21.61±0.47^{c}	84.31±0.27^{b}
	Boiled-fried***	7.51±0.46^{a}	2.33±0.21^{a}	31.02±3.09^{a}
	p-value	<0.001	<0.001	<0.001
Amaranth	Fresh*	29.99±3.80^{b}	21.47±0.42^{b}	77.22±0.05^{b}
	Boiled**	42.63±2.36^{c}	39.35±2.37^{c}	92.28±0.46^{c}
	Boiled-fried***	7.38±0.70^{a}	1.13±0.12^{a}	16.97±0.02^{a}
	p-value	<0.001	<0.001	<0.001
Vine spinach	Fresh*	8.57±0.50^{a}	5.26±0.47^{b}	61.36±1.87^{b}
	Boiled**	14.22±2.30^{b}	9.97±0.03^{c}	65.67±5.53^{b}
	Boiled-fried***	6.09±0.47^{a}	1.40±0.14^{a}	23.15±2.82^{a}
	p-value	<0.001	<0.001	<0.001
Pumpkin leaves	Fresh*	20.14±2.26^{b}	19.04±2.25^{b}	94.48±0.57^{c}
	Boiled**	25.70±0.92^{c}	20.76±0.92^{b}	80.75±0.69^{b}
	Boiled-fried***	5.40±0.41^{a}	1.26±0.16^{a}	23.33±2.89^{a}
	p-value	<0.001	<0.001	<0.001

Mean values of the same vegetable within the same column followed by the same letters are not significantly different (SNK, α=0.05); *p<0.002, **p=0.001, ***p<0.001

pumpkin leaves (14 %). It was noted that the converse effect was observed in all vegetables prior to the digestion process except in pumpkin leaves where a similar effect of reduction was noted for this thermal process. The bioaccessibility of BC following the process of boil-frying led to significant reduction in all vegetables (38 % - 71%). Factors including cooking/heating would explain the effect of processing vegetables on bioaccessibility of BC (Parada and Aguilera, 2007). Further, the chemistry of BC which includes being sensitive to heat and oxidation is also attributed to the observed reductions (Veda *et al.*, 2006; Nagao, 2014).

Further, the difference in bioaccessibility of BC could have been influenced by the plant cell wall (which has to be broken to release BC) made up of cellulose and pectin, the dietary fiber (Koh and Loh, 2018). Improved bioaccessibility as a result of thermal processing has also been reported by Koh and Loh (2018). On analyzing the vegetables amaranth, carrot, fenugreek leaves and pumpkin, an increase in percent bioaccessibility of BC as a result of frying was observed. Veda and co-workers (2006), however, observed a contradicting trend.

The microstructure of vegetables influences bioaccessibility of beta carotene (Svelander *et al.,* 2011). Pectin, a soluble fiber, affects lipid absorption as it can bind with fatty acids, cholesterol and bile acid. This could result in the formation of fatty acid-fiber complex thus losing the ability to form micelles (Koh and Loh, 2018). Aschoff *et al.,* 2014 for instance recorded an increased bioaccessibility with diminished fiber content in the sample. In this study, spider plant recorded higher bioaccessibility, 97% in the fresh and 48% in the boiled-fried, compared to vegetables cowpeas, amaranth, vine spinach and pumpkin leaves. The variations in bioaccessibility may be attributed to the differences in fiber content of the vegetables (Aschoff *et al.,* 2014). Further, the microstructure of vegetables is known to influence bioaccessibility of beta carotene, due to the difference in digestibility (Parada and Aguilera, 2007; Etchiverry *et al.,* 2012).

4. Conclusions

The levels of beta carotene in leafy African indigenous vegetables (spider plant, cowpeas, amaranth, vine spinach and pumpkin leaves) can significantly increase or decrease with thermal processing of the vegetable. The resultant levels are bioaccessible with the percent bioaccessibility enough to meet the recommended RDA for both children and adults.

Conflicts of interest

There are no conflicts of interest to declare.

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