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IMPROVEMENT OF *IN VITRO* BIOACCESSIBILITY OF PROVITAMIN A CAROTENOIDS BY FERMENTATION OF TROPICAL LEAFY VEGETABLES

*** Lessoy ZOUE[1], Sébastien NIAMKE[1]**
[1]*Faculty of Biosciences, Biotechnology Laboratory, Felix Houphouet Boigny University, Abidjan, Côte d'Ivoire, y.lessoy@yahoo.fr*
*Corresponding author
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Abstract: *The purpose of this study was to assess in vitro bioaccessibility of provitamin A carotenoids in fermented leafy vegetables. These fermented leafy vegetables are often used to prepare dishes in some parts of tropical Africa. Carotenoids were extracted from fermented leafy vegetables and analyzed by using High Performance Liquid Chromatography (HPLC). In order to simulate the human digestion tract, the in vitro bioaccessibility method known as simple, inexpensive and reproductible tool was performed. All leafy vegetables contained considerable amount of provitamin A carotenoids (26.61 ± 1.98 – 305.85 ± 14.38 µg/g dw) with the concentration varying from one source to another. Fermentation of leafy vegetables resulted in losses (24.5 – 78.22%) of provitamin A carotenoid contents. The in vitro bioaccessibility of provitamin A carotenoids of non fermented leafy vegetables ranged from 3.58 to 5.21% while that of fermented leafy vegetables was siginificantly different (P < 0.05) and ranged from 11.51 to 15.32%. The results also showed that fermented leafy vegetables may contribute between 9.48 and 111.31% of the vitamin A daily requirement (RDA) estimated to 550 µg for individuals in developing countries. This study highlighted that fermentation appears as a suitable non thermal processing to improve the bioaccessibility of provitamin A carotenoids of leafy vegetables. Thus, an increased consumption of fermented leafy vegetables could help combat vitamin A deficiency (VAD) which remains a major public health problem in developing countries.*

Keywords: *bioavailability, carotenoids, fermentation processing, leafy vegetables, vitamin A*

1. Introduction

Dietary carotenoids are fat-soluble pigments found in high concentrations in fruit and vegetables [1]. These bioactive components include carotenes (β-carotene, α-carotene and lycopene) and xanthophylls (zeaxanthin, lutein, α and β- cryptoxanthin) and they may prevent several diseases like cancer, cardiovascular disease and mainly blindness due to vitamin A deficiency (VAD) [2,3]. VAD remains a major public health problem in developing countries where children and pregnant women are usually affected. For these population groups, the largest contribution of vitamin A intake is provided by provitamin A carotenoids from plant foods, which may contribute up to 82% of the total vitamin A [4]. Therefore, food-based strategies for combating vitamin A deficiency in Africa include promotion and consumption of tropical leafy vegetables. Indeed, these plants are available, cheaper and provitamin A carotenoids-rich [5]. Commonly, tropical leafy vegetables are prepared at home by using different cooking methods (boiling, stewing, frying, and blanching) that have been reported to degrade 5 to 78% of β-carotene [6,7]. Considering that important amount of carotenoids needed by individuals may be lost during household cooking, the question of bioaccessibility and

bioavailability of provitamin A carotenoids from tropical leafy vegetables remains important. The term bioavailability refers to the fraction of an ingested nutrient available for utilization in normal physiological functions and storage. However, bioaccessibility is defined as the amount of an ingested nutrient that is available for absorption after digestion [8]. Many factors may affect the bioavailability of carotenoids and the term SLAMENGHI used to describe these factors was formulated as follow [9]: Species of carotenoid, molecular Linkage, Amount of carotenoids in a meal, Matrix in which the carotenoid is incorporated, Effectors of absorption and bioconversion, Nutrient status of the host, Genetic factors, Host-related factors, and mathematical Interactions. Absorption of carotenoids involves the release from the food matrix, dispersion and solubilization into mixed bile salt micelles, uptake by intestinal mucosal cells and circulation through the lymphatic system into the bloodstream [10]. For green leafy vegetables, the main factor that affects the bioaccessibility of carotenoids is the leaf matrix which may hinder the release of the carotenoids during digestion [11]. In order to reduce this matrix effect, traditional processing methods such as boiling, blanching and pounding are commonly used in households [12]. Some reports also indicated that boiling or blanching leafy vegetables resulted in 2 to 12% bioaccessibility of β-carotene [11]. Another scarce processing method used to reduce the leaf matrix effect is fermentation. Indeed, some populations of West and Central Africa ferment leafy vegetables for 3 to 4 days to prepare dishes with a distinct flavor. In addition, fermentation can enhance the nutritional quality of leafy vegetables by producing beneficial by-products. To the best of our knowledge, the bioaccessibility of provitamin A carotenoids in such relishes is not documented and little information were reported about the ability of such fermented tropical leafy vegetables to supply daily vitamin A requirement. *In vitro* model method is known as simple, inexpensive and reproductible tool to stimulate the human digestion tract [13]. Therefore, the aim of this study was to assess the impact of fermentation on the bioaccessibility of provitamin A carotenoids from tropical leafy vegetables by using *in vitro* model.

2. Materials and methods

2.1 Chemicals

The solvents (methanol, dichloroethane, methyltertiary-butyl diethyl ether and formic acid) were all HPLC grade and purchased from Fisher Scientific. The purified carotenoids standards (lutein, all-trans-β-carotene, 13-cis-β-carotene, 9-cis-β-carotene, α-carotene, β-apo-8-carotenal) and enzymes (porcine pepsin, pancreatin, bile extract) were from Sigma-Aldrich.

2.2 Fermentation processing of leafy vegetables

Twelve (12) leafy vegetables widely consumed in Côte d'Ivoire were selected (Table 1). All the leaves were collected at maturity from a periurban farmland (latitude: 5°19′14″ North; Longitude: 4°22′59″West) located in Abidjan District (Côte d'Ivoire). The collected leaves were authenticated by National Floristic Center (University Felix Houphouët-Boigny, Abidjan). The leaves were washed several times with distilled water, drained at ambient temperature, cut into small pieces and separated into two portions of 250 g each. The first portion was wrapped in clean papaya leaves for 4 days to induce natural fermentation in a covered plastic box. Afterwards, the fermented (F) leaves were oven-dried (50°C/3 days) and ground

into a powder with a laboratory crusher. The second portion was not subjected to the fermentation step but was used as control (non-fermented NF leaves). All the dried and powdered samples were stored at -18°C in airtight containers for further experimentation.

Table 1.

Common and local names of selected tropical leafy vegetables

Leafy vegetables	Common name	Local name
Abelmoschus esculentus	Okra	Gombo
Amaranthus hybridus	Green amaranth	Boronbrou
Basella alba	Indian spinach	Epinard
Celosia argentea	Common cockscomb	Soko
Colocasia esculenta	Taro	Taro
Corchorus olitorius	Jew's mallow	Kplala
Hibiscus sabdariffa	Roselle	Dah
Ipomea batatas	Sweet potato	Patate
Manihot esculenta	Cassava	Manioc
Myrianthus arboreus	Bush pineapple	Tikliti
Solanum melongena	Eggplant	Aubergine
Talinum triangulare	Ceylon spinach	Mamichou

2.3 Carotenoid analysis

2.3.1 Carotenoid extraction

Total carotenoids of F and NF leaves were extracted using a previously reported method [14] with slight modifications. Powdered samples (0.05 g) were mixed with 5 mL ethanol containing butylated hydroxytoluene (0.1%, w/v) and heated in a water bath at 85°C for 5 min. Then, 400 µL KOH in water (80% w/v) was added for saponification and the suspension was mixed using a vortex for 20 s and heated in a water bath at 85°C for 5 min. The tubes containing the reaction mixture were placed in ice after introducing 3 mL deionized water and carotenoids were extracted three times with 4 mL hexanes. β-*apo*-8'-carotenal was used as an internal standard and was added after saponification to account for mechanical losses. The combined extracts were dried under nitrogen and reconstituted in 1 mL 50:50 methanol-dichloroethane.

2.3.2 HPLC analysis

For the carotenoid identification and quantification, 25 µL extract was injected into an HPLC system (Waters Corporation; Milford, MA, USA) consisting of a 717 autosampler, 1525 binary pump, and a 2996 photo-diode array detector (PDA). The column used was a C_{30} YMC carotenoid column (4·6 x 250mm, 3mm). The HPLC solvent gradient included methanol-water (92:8, v/v) with 10 mM ammonium acetate (solvent A) and 100 % methyltertiary-butyl ether (solvent B). Samples were analyzed at 1 mL/min with a 30-min linear gradient from 70 to 40% solvent A. Lutein, β-carotene (including all-*trans*, 13-*cis*, and 9-*cis*), and α-carotene were identified and quantified using HPLC-purified standards. Chromatograms were generated at 450 nm.

2.4 *In vitro* bioaccessibility of provitamin A carotenoids

Determination of *in vitro* bioaccessibility of provitamin A carotenoids was carried out according to an *in vitro* digestion method [15] with slight modification. About 0.5 g of powdered vegetable sample was mixed with 10 mL ascorbic acid (1%, w/v). For the digestion, 5 mL of a 0.5%

(w/v) porcine pepsin solution in HCl 0.1M with physiological amounts of calcium (3.6 mmol), magnesium (1.5 mmol), sodium (49 mmol), potassium (12 mmol) and phosphate (6.4 mmol) were added. pH was adjusted to 2.0 with HCl 2 M and nitrogen gas was blown into the flask and the scraw capped test tube was incubated at 37°C in a shaking water bath at 250 rpm for 1 h. After the gastric digestion phase, the pH value was adjusted to 5 with NaOH 1M and 3 mL of pancreatin-bile solution (4 g/L bile extract and 25 g/L pancreatin dissolved in 0.1M $NaHCO_3$) was added. The pH value was further increased to 7.5 and the mixture was incubated again for 30 min. After intestinal digestion phase, the sample was centrifuged at 5000 *g* for 20 min. The supernatant was collected and mixed with 5 mL of 25% (w/v) NaCl and 5 mL of ethanol stabilised with 0.1% (w/v) BHT. Afterwards, carotenoids were extracted two times by centrifugation (5000 *g*; 20 min) with 5 mL hexane and the combined extracts were dried under nitrogen before reconstitution in 1 mL 50:50 methanol-dichloroethane. The released provitamin A carotenoids were analyzed and quantified by HPLC as described in 2.3.2.

2.5 Retinol activity equivalent determination

The calculation of retinol activity equivalent (RAE) of fermented leafy vegetables after *in vitro* bioaccessibility was based on the bioconversion factor defined as 2 µg accessible β-carotene to 1 µg of retinol (2:1)[16] .

2.6 Statistical analysis

Values were reported as means ± SD. Data were analyzed using XLStat 2016.2 (Addinsoft, NY, USA). Carotenoid composition and bioaccessibility values were compared using one-way ANOVA. Differences among processed leaves and treatment groups were determined using least significant differences (LSD) at $P < 0.05$.

3. Results and discussion

3.1 Carotenoid profile

Carotenoid contents and their chromatographic profile are given in Table 2 and Fig. 1, respectively. The identified carotenoids were lutein and provitamin A carotenoids (13-*cis*-β-carotene, α-carotene, all-*trans*-β-carotene, and 9-*cis*-β-carotene). All leafy vegetables contained considerable amount of provitamin A carotenoids (26.61 ± 1.98 – 305.85 ± 14.38 µg/g dw) with concentration varying from one source to another. Many factors affect carotenoid content of leafy vegetables, such as variety, location, cultivation, and post-harvest handling practices [17]. Considering non fermented (NF) leafy vegetables, the best sources of provitamin A carotenoids were *A. esculentus* (305.85 ± 14.38 µg/g dw), *C. olitorius* (200.56 ± 16.21 µg/g dw), *M. esculenta* (233.20 ± 13.13 µg/g dw) and *S. melongena* (220.71 ± 2.23 µg/g dw). Fermentation of leafy vegetables resulted in losses (24.5 – 78.22%) of provitamin A carotenoids contents. It's important indicating that the traditional fermentation of leafy vegetables involves lactic acid bacteria as main fermenting microorganisms [18]. The losses of carotenoids in this study is not surprising since carotenoids degradation occurs during lactic acid fermentation of vegetables as indicated by some authors [19].

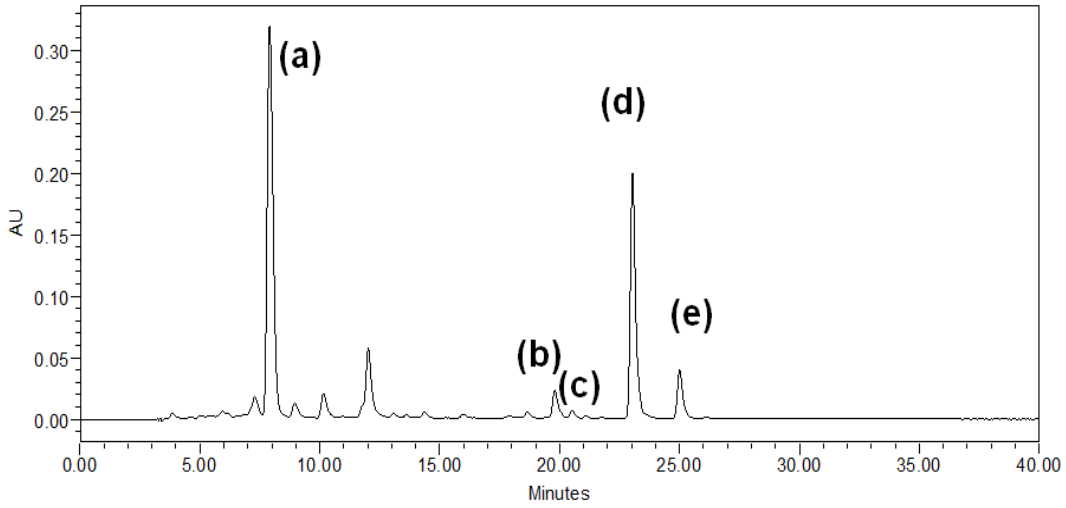

Fig. 1. Chromatrogram of carotenoids from selected tropical leafy vegetables
(a): lutein; (b): 13-cis-β-carotene; (c): α-carotene; (d): all-trans-β-carotene; and (e): 9-cis-β-carotene

Table 2.

Total provitamin A contents of fermented (F) and non fermented (NF) leafy vegetables

Leafy vegetables	Total provitamin A carotenoids (µg/g dw)	
	Non fermented (NF)	Fermented (F)
A. esculentus	305.85±14.38^{a}	193.90±3.07^{b}
A. hybridus	129.41±2.41^{a}	33.49±0.27^{b}
B. alba	83.07±3.01^{a}	62.71±6.55^{b}
C. argentea	148.46±5.55^{a}	32.33±1.48^{b}
C. esculenta	189.78±3.23^{a}	80.40±3.71^{b}
C. olitorius	200.56±16.21^{a}	122.88±3.21^{b}
H. sabdariffa	26.61±1.98^{a}	17.16±4.23^{b}
I. batatas	156.97±1.90^{a}	48.85±8.92^{b}
M. esculenta	233.20±13.13^{a}	140.78±1.88^{b}
M. arboreus	73.02±8.98^{a}	38.11±0.64^{b}
S. melongena	220.71±2.23^{a}	128.86±3.10^{b}
T. triangulare	178.25±1.62^{a}	45.98±0.43^{b}

Data are presented as means of triplicate analyses ± SD. Means with the same superscript letter in the same line for a single vegetable are not different at $P > 0.05$

3.2 *In vitro* bioaccessibility

Bioaccessibility of food components such as vitamins is an important feature to assess their role in human health. The *in vitro* bioaccessibility model has been developed as simple, inexpensive, and reproducible tools to study the human digestion of different food components (ascorbic acid, carotenoids, chlorophylls, polyphenols) [20]. The *in vitro* bioaccessibility of provitamin A carotenoids of non fermented leafy vegetables ranged from 3.58 to 5.21% while that of fermented leafy vegetables was siginificantly different ($P < 0.05$) and ranged from 11.51 to 15.32% (Table 3). The results for provitamin A carotenoids bioaccessibility of non fermented leafy

vegetables were consistent since less than 5% provitamin A carotenoids is generally released from unprocessed food matrix as indicated by some authors [21]. Indeed, the first step in digestion of carotenoid comprises disintegration of the food matrix in order to release carotenoids [22]. In green leaves, carotenoids components are organised in pigment–protein complexes located in cell chloroplasts, which have to be accessed before making carotenes available [23]. Contrary to non-fermented leaves, fermentation processing increased bioaccessibility of provitamin A carotenoids. The values of bioaccessibility of fermented leaves were similar to those (6-29%) of cooked or blanched leafy vegetables without oil [11,15]. Moreover, accessible percentage of provitamin A carotenoids from fermented leafy vegetables were slightly lower than the factor 1/6 (16,7%) proposed by the US Institute of Medicine (IOM) for the bioaccessibility of all-trans-*β*-carotene from vegetable food items [16]. Thus, fermentation of leafy vegetables appears as a suitable non thermal technique to improve the bioaccessibility of provitamin A carotenoids.

Table 3.

***In vitro* bioaccessibility of fermented (F) and non fermented (NF) leafy vegetables**

Leafy vegetables	**Bioaccessibility (%)**	
	Non fermented (NF)	**Fermented (F)**
A. esculentus	4.55±0.01^{a}	12.63±0.90^{b}
A. hybridus	5.21±0.02^{a}	14.33±0.01^{b}
B. alba	5.05±0.01^{a}	11.51±0.10^{b}
C. argentea	4.05±0.03^{a}	13.38±0.01^{b}
C. esculenta	4.16±0.78^{a}	12.11±0.69^{b}
C. olitorius	4.34±0.78^{a}	15.32±0.49^{b}
H. sabdariffa	4.31±0.05^{a}	12.16±0.01^{b}
I. batatas	4.69±0.01^{a}	14.58±0.02^{b}
M. esculenta	5.03±0.17^{a}	13.89±0.56^{b}
M. arboreus	5.02±0.15^{a}	14.21±0.05^{b}
S. melongena	3.58±0.20^{a}	12.76±0.35^{b}
T. triangulare	4.85±0.02^{a}	13.72±0.05^{b}

Data are presented as means of triplicate analyses ± SD. Means with the same superscript letter in the same line for a single vegetable are not different at $P > 0.05$

3.3 Contribution to vitamin A requirements

Assuming that the minimum intake of fermented leafy vegetable relish in a single meal is 50 g dry matter, the corresponding amount of accessible provitamin A carotenoids from the results of *in vitro* digestion was between 104.33 and 1224.47 µg (Table 4). The conversion factor adopted by IOM [16] assumes that one-sixth (1/6) of the total carotene content is absorbed into the mucosa, and that one-half (1/2) of absorbed *β*-carotene is converted to retinol. These conversion factors were used to estimate how much fermented leafy vegetables might contribute to the daily requirement (RDA) of vitamin A. In this study, the *in vitro* digestion data were used instead of the one-sixth (1/6) factor with assumption that 100% of the carotenes released after *in vitro* digestion is absorbed into the mucosa

[23]. Considering the one-half (1/2) factor of accessible provitamin A carotenoids, the retinol activity equivalent (RAE) varied from 52.16 µg (fermented *H. sabadariffa* leaves) to 612.23 µg for fermented *A. esculentus* leaves. The contribution to the RDA of retinol was calculated and the results showed that fermented leafy vegetables may contribute between 9.48 and 111.31% the RDA estimated to 550 µg vitamin A for individuals in developing countries [24]. Therefore, increasing consumption of fermented leafy vegetables could help alleviate vitamin A deficiency (VAD) which remains a major public health problem in developing countries.

Table 4.

Contribution of fermented leafy vegetables to daily vitamin A requirement

Fermented leafy vegetables	Accessible provitamin A carotenoids (µg)	Retinol activity equivalent (µg)	Contribution to daily vitamin A requirement (%)
A. esculentus	1224.47	612.23	111.31
A. hybridus	239.95	119.97	21.81
B. alba	360.89	180.44	32.80
C. argentea	216.28	108.14	19.66
C. esculenta	486.82	243.41	44.25
C. olitorius	941.26	470.63	85.56
H. sabdariffa	104.33	52.16	9.48
I. batatas	356.11	178.05	32.37
M. esculenta	977.71	488.85	88.88
M. arboreus	270.77	135.38	24.61
S. melongena	822.12	411.06	74.73
T. triangulare	315.42	157.71	28.67

4. Conclusion

In this study, the fermented leafy vegetables showed relatively important *in vitro* bioaccessibility of provitamin A carotenoids. Thus, they may contribute to the daily requirement of vitamin A. These findings could be useful in dietary intervention programmes to alleviate vitamin A deficiency (VAD) in developing countries. However, several food- and host-related factors may influence vitamin A bioavailability at different points. Thus, *in vitro* methodology for provitamin A bioaccessibility should be validated in different *in vivo* conditions.

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