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# Improvement of the content in bioaccessible lipophilic micronutrients in raw and processed drumstick leaves (*Moringa oleifera* Lam.)

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**ABSTRACT:** Our objective was to evaluate the influence of cooking (boiling, steaming, frying) and processing (freezing, freeze-drying, sun drying, oven drying, sterilization or powdering) on the micronutrient profile of drumstick leaves ( $\beta$ -carotene, lutein,  $\alpha$ -tocopherol) and the bioaccessibility of the latter after in vitro digestion. Fresh drumstick leaves contain a high level of carotenoids and  $\alpha$ -tocopherol with a very low bioaccessibility (<1%). Freezing, steaming and sterilization preserve the more the micronutrients and most of the processing methods improve the contents in bioaccessible  $\beta$ -carotene, lutein and  $\alpha$ -tocopherol. Among the cooking practices, only steaming was efficient to increase the compound release. The structural changes of the leaf tissue during processing partially explain the modulation of the bioactive compound release during the course of in vitro digestion. After boiling and steaming, the cells of epidermis and mesophyll were slightly disorganized while frying induced a deep deconstruction of the overall tissue with cell wall rupture.

**Keywords:** cooking, vitamin retention, in vitro digestion, food deconstruction, diffusion.

## 1. Introduction

The potential of indigenous trees to combat desertification and contribute to the well-being and food security of local population was studied since decades (Nesbitt, McBurney, Broin, & Beentje, 2010). The drumstick tree (*Moringa Oleifera* Lam.) is interesting because it provides environmental services and could bring other added values for human well-being. The integration of *Moringa* tree in farming and pastoral systems could improve the sustainability of local food systems by providing livelihoods and diet diversification (Morton, 1991).

In traditional medicine, Drumstick is known for its effect on the digestive tract (bactericide, antidiarrheal, laxative), kidney function (diuretic) and anti-inflammatory effect (Arun Giridhari, Malathi, & Geetha, 2011; Paliwal, Sharma, Sharma, & Sharma, 2011; Sharma, Paliwal, Janmeda, & Sharma, 2012; Coppin et al., 2013). Several human studies have highlighted the anti-hyperglycemic, anti-dyslipidemic and antioxidant activities properties of drumstick (William, Lakshminarayanan, & Chegu, 1993; Kumari, 2010; Nambiar, Guin, Parnami, & Daniel, 2010; Arun Giridhari et al., 2011; Kushwaha, Chawla, & Kochhar, 2014). In northern countries, encapsulated drumstick powder is already commercialized by the pharmaceutical industry for its antioxidant capacity, anti-hyperglycemic as well as lipid and cholesterol lowering effect (William et al., 1993; Kumari, 2010; Nambiar et al., 2010; Arun Giridhari et al., 2011; Kushwaha et al., 2014).

In Africa and Asia, distinct parts of the tree are consumed such as flowers, leaves, fruits, bark, grains or roots. The leaves can be integrated in salad, traditional sauces, or soups in raw or processed form; young leaves can also be steam cooked (Sallau, Mada, & Ibrahim, 2012). The

drumstick leaves have a quantifiable amounts of proteins, essential fatty acids, minerals, vitamins (B, C, E, provitamin A) and a wide variety of polyphenols (flavonoids, chlorogenic acid) (Ramachandran, Peter, & Gopalakrishnan, 1980; Makkar & Becker, 1996; Aslam et al., 2005; Amaglo et al., 2010; Coppin et al., 2013). The consumption of the leaves could fulfill the recommended daily requirements of several macro and micronutrients. In fresh or dried forms, the addition of drumstick leaves increases the content in iron, proteins and vitamin A of traditional dishes (Ejoh, Dever, Mills, & Tanumihardjo, 2010). In animal models (gerbil, mouse), the carotene bioavailability was high with raw and dehydrated drumstick leaf consumption (Nambiar & Seshadri, 2001; Ejoh et al., 2010). After few weeks of depletion, the addition of tropical leafy vegetables to the gerbil's diet increased the vitamin A liver stores while serum retinol was not affected by the treatment. In the latter study, the high carotenoid bioavailability was attributed to the small particle size of powdered leaves (Ejoh et al., 2010). Vitamin deficiencies are public health issues in Africa and South-East Asia and still concern an estimated 190 million preschool-age children and 19.1 million pregnant women (WHO, 2009). Populations with low incomes have insufficient micronutrient intakes especially in vitamin A. Some carotenoids with provitamin A activity ( $\beta$ -carotene) and vitamin E ( $\alpha$ -tocopherol) have antioxidant properties and contribute to several functions such as cell growth and integrity, tissue differentiation, immunological defense and vision. The drumstick leaves are rich in these bioactive compounds but their biological effects are still controversial. Leafy vegetables could have a low nutritional efficacy because of their structure and biochemical composition (rich in fibers and phenolic compounds).

The effect of processing on the nutritional value of drumstick leaves has been investigated for several years but the capacity of the leaves to deliver their micronutrient during digestion is not documented. The aim of the present work was to assess the influence of several treatments on the content in total and bioaccessible  $\beta$ -carotene, lutein and  $\alpha$ -tocopherol of drumstick leaves.

## **2. Materials and methods**

### **2.1. Materials and chemicals**

Solvents, HPLC standards ( $\beta$ -carotene,  $\alpha$ -carotene, lutein,  $\alpha$ -tocopherol,  $\delta$ -tocopherol) and enzymes (pepsin 800-2,500 U/mg, pancreatin B1750, bile extract porcine B8631) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). The polytetrafluoroethylene (PTFE) membranes were obtained from Sartorius (Palaiseau, France). Capsule in hydroxypropyl methylcellulose (HPMC) were bought in a local drugstore.

Drumstick leaves (1 kg) were purchased from Gopal and Co supermarket (Paris) and leaves with same color and size were chosen to limit experimental variations. Vegetables were cleaned with distilled water and the stem and other inedible parts were removed. Raw leaves ( $5 \times 1$  g) were directly submitted to *in vitro* digestion to assess the content in bioaccessible micronutrients. Then, treatments were applied to 11 batches of leaves (10 g) for further analysis.

### **2.2. Dry matter assessment**

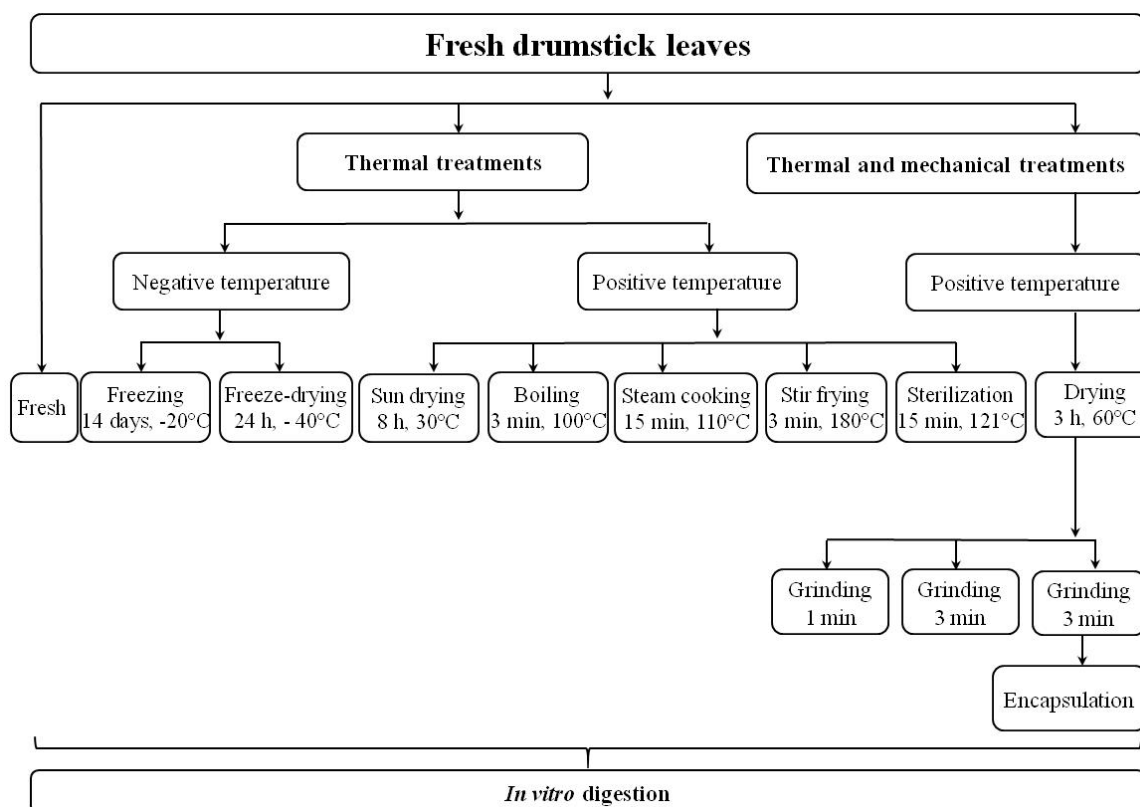
The dry matter (DM) contents of the raw and processed leaves were determined by drying the leaves in oven (24 h at 105 °C). Dry matter was then weighed precisely on a Mettler AE166 balance (Viroflay, France).

### **2.3. Processing methods and culinary treatments**

Ten treatments were applied to samples of fresh drumstick leaves (2 g) with 5 repetitions for each one (Fig. 1). Some treatments correspond to Thai cooking and the others are preservation methods. Boiling was realized in milli-Q water, stir-frying in colza oil, and the equipment used for the treatments were as follows:

- freeze-drying Alpha 1-2 LD plus, Christ (Osterode, Germany)
- oven dryer (Radnor, Pennsylvania, USA)
- grinder from IKA (Staufen, Germany). Grinding for 1 and 3 min provides particle size of 1 mm and 200  $\mu\text{m}$  respectively

For encapsulation, 0.5 g of fine powdered leaves was introduced in hydroxypropyl methylcellulose (HPMC) capsules. After treatments, the samples were weighed to determine the cooking yields.



**Figure 1.** The treatments applied to drumstick leaves before *in vitro* digestion.

#### 2.4. *In vitro* digestion protocol (IVD)

The IVD protocol was adapted from a published procedure with the addition of 80  $\mu\text{L}$  of colza oil (diluted in acetone 32%) at the beginning of digestion to enhance carotenoid micellarisation (Hedrén, Mulokozi, & Svanberg, 2002; Reboul et al., 2006). Samples (2 g) were mixed in an amber screw-capped bottle with 15 mL of oral solution (pyrogallol 1.3% in NaCl 0.9%). Samples were incubated for 10 min at 37 °C with gentle stirring (IKAMAG® RT 15 power, IKA, Staufen, Germany). The gastric phase started then by adding 500  $\mu\text{L}$  of 1 M HCl and adjusting the pH to  $4.0 \pm 0.1$ , and then 2 mL of porcine pepsin (4% in 0.1 M HCL). The mixture was incubated at 37 °C for 30 min with gentle stirring. Thereafter, the pH was raised to  $6.0 \pm 0.1$  by adding 800  $\mu\text{L}$  of 0.9 M of sodium bicarbonate buffer before the addition of 9 mL of the intestinal solution: porcine pancreatin (0.2%) and porcine bile extract (1.2%) dissolved in 0.1 M of trisodium citrate, pH 6. Then, 4 mL of porcine bile extract at 0.1 g/mL were added. The samples were incubated for 30 min in a stirring water bath at 37 °C. Digesta were centrifuged at 4000 g for 1 h at 10 °C (Heraeus Multifuge X1R, Thermo Fisher Scientific, Villebon sur Yvette, France). Finally, 10 mL was collected with a needle fitted to a syringe and filtered through 0.2  $\mu\text{m}$  cellulose acetate membrane to recover the micellar fraction. Lipophilic compounds were then extracted.

## 2.5. Determination of total and bioaccessible carotenoids and tocopherols

Carotenoids and tocopherols were extracted from 50 mg of raw or processed leaves. Samples were ground (Turrax, Prolabo, Coueron, France) for 1 min with 15 mL of ethanol/hexane (4:3, v/v) and then centrifuged for 30 min at 10 °C and 4000 g (Heraeus Multifuge, Thermo Fisher Scientific, Villebon sur Yvette, France) (Taungbodhitham, Jones, Wahlqvist, & Briggs, 1998). The supernatant was transferred into a glass funnel and 10 mL of NaCl 10% was added (p/v). After 10 min, the lower phase was discarded and the organic phase was washed with 10 mL of distilled water. Organic extracts were recovered, dried under nitrogen and residues were dissolved in 2 mL of acetone. After filtration on 0.2 µm PTFE membranes (minisart SRP4, Sartorius), sample (20 µL) was injected into the HPLC.

After *in vitro* digestion, the micronutrients were extracted on 10 mL of digesta by adding 20 mL of ethanol/hexane (4:3, v/v). Samples were homogenized and 20 mL of NaCl 25% were added (p/v). After 10 min, the organic phase was re-extracted with 20 mL of ethanol/hexane (4:3, v/v) and washed with 15 mL of distilled water. The organic phase was dried under nitrogen and residues were dissolved in 400 µL of acetone. The extracts were filtered through 0.2 µm PTFE membranes and analyzed with HPLC.

Elution was performed with a Dionex P680 pump. The column was a polymeric C30 (4.6 mm i.d × 250 mm, 5 µm particle size, YMC Europe, Schermbeck, Germany). The mobile phase comprised of two mixes at a flow rate of 1 mL min<sup>-1</sup>. Mix A constituted of methanol and milli-Q water, 60:40 (v/v), and B mix constituted of methanol, methyl tert-butyl-ether and milli-Q water, 28.5:67.5:4 (v/v/v). The gradient applied progressed from 100% to 0% (A/B) over a period of 31 min and the temperature was approximately 25 °C. A UV-visible photodiode array detector (Dionex UVD 340U) was used and chromatograms were analyzed at the wavelength of maximum absorption of the carotenoids and α-tocopherol in the mobile phase (450 and 292 nm respectively). External calibration was used in the range of 0.5 to 25 mg/L.

Retinol equivalents (RE) were calculated using a conversion factor of 12 for β-carotene and of 24 for the other provitamins A (α-carotene) for 1 retinol and expressed in mg RE/100 g (Murphy, 2002).

The content in bioaccessible β-carotene and α-tocopherol corresponds to the amounts of compounds transferred to the aqueous micellar phase at the end of IVD. The bioaccessibility (in %) corresponds to the amount of compounds transferred to the micellar phase compared to the initial contents in the fresh leaves.

## 2.6 Light microscopy

Samples (raw or processed leaves) were fixed for 2 weeks in 0.2 M phosphate buffer at pH 7.2, supplemented with 2% (v/v) paraformaldehyde, 1% (w/v) caffeine, and 1.5% (v/v) glutaraldehyde at 4°C (Buffard-Morel et al., 1992). Progressive dehydration of samples was realized with ethanol from 50 to 100%, transfer to butanol/ethanol 1:1 (v/v), and then 100% butanol. Each sample was impregnated in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrhirm/Ts, Germany). Semi-thin sections of 4 µm were cut using a Historange microtome (LKB Broma, Uppsala, Sweden). Sections were stained with specific reagents as follow: i) periodic acid-Schiff's reagent (Sigma-Aldrich, Lyon, France) and with naphtol blue black (NBB) to specifically reveal polysaccharides (in pink) and proteins (in dark blue) (Buffard-Morel et al., 1992); ii) ruthenium red to stain pectins (in pink) (Sterling, 1970); iii) toluidine blue to reveal tannins in greenish blue (Brillouet et al., 2012). Observations and microphotographs were performed with a Leica camera (Wetzlar, Germany) DMRB 301-370 light microscope (Evolution MP 5.0 color media Cybernetics Cooled RTV 32-0040C-140).

## 2.7. Statistical analysis

The micronutrient content of the moringa leaves was compared after 3 culinary treatments and 7 preservation methods applied with 5 repetitions. Data were assessed by F-test in one-way analysis of variance (ANOVA) using Statgraphics plus 5.1 (Virginia, USA). Significance was accepted at probability  $P < 0.05$ .

### 3. Results and discussion

#### 3.1. Effects of cooking and processing methods on the dry matter content and micronutrient profile

In the raw leaves, the major lipophilic compounds were  $\beta$ -carotene, lutein and  $\alpha$ -tocopherol and their respective quantities were 63, 32, and 57 mg for 100 g DM (Table 1). These results are in accordance with previously published data (Subadra, Monica, & Dhabhai, 1997; Nambiar & Seshadri, 1998; Saini, Shetty, Prakash, & Giridhar, 2014). The DM was rather high 24 % as well as the RE content (5.3 mg for 100 g DM) as previously observed (2.5 mg for 100 g DM) (Kidmose, Yang, Thilsted, Christensen, & Brandt, 2006).

**Table 1.** The contents in dry matter, carotenoids,  $\alpha$ -tocopherol and retinol equivalent of raw and processed drumstick leaves for 100 g DM.

Transformation	n	DM (g/100g)	$\beta$ -carotene (mg/100g)	Lutein (mg/100g)	$\alpha$ -tocopherol (mg/100g)	RE (mg/100g)
Raw	5	24.2 $\pm$ 0.7 <sup>c</sup>	63.6 $\pm$ 3.0 <sup>a</sup>	32.5 $\pm$ 4.5 <sup>a,b</sup>	57.6 $\pm$ 12.1 <sup>a</sup>	5.3 $\pm$ 0.3 <sup>a</sup>
Freezing	5	22.2 $\pm$ 1.8 <sup>c</sup>	46.2 $\pm$ 4.5 <sup>b</sup>	34.8 $\pm$ 3.0 <sup>a,b</sup>	58.4 $\pm$ 12.1 <sup>a</sup>	3.8 $\pm$ 0.4 <sup>b</sup>
Freeze-drying	5	93.8 $\pm$ 1.2 <sup>b</sup>	30.6 $\pm$ 3.9 <sup>e</sup>	31.3 $\pm$ 6.0 <sup>b</sup>	56.0 $\pm$ 6.9 <sup>a,b</sup>	2.6 $\pm$ 0.3 <sup>e</sup>
Boiling	5	8.5 $\pm$ 0.7 <sup>a</sup>	21.8 $\pm$ 3.1 <sup>f</sup>	21.9 $\pm$ 2.6 <sup>c</sup>	42.2 $\pm$ 3.9 <sup>c</sup>	1.8 $\pm$ 0.3 <sup>f</sup>
Steaming	5	15.2 $\pm$ 3.2 <sup>d</sup>	51.2 $\pm$ 5.1 <sup>b</sup>	37.8 $\pm$ 2.9 <sup>a</sup>	45.8 $\pm$ 11.2 <sup>c,b</sup>	4.3 $\pm$ 0.4 <sup>b</sup>
Frying	5	97.8 $\pm$ 4.4 <sup>a</sup>	10.4 $\pm$ 0.4 <sup>g</sup>	9.9 $\pm$ 1.0 <sup>d</sup>	6.4 $\pm$ 1.3 <sup>e</sup>	0.9 $\pm$ 0.0 <sup>g</sup>
Sterilization	5	8.5 $\pm$ 0.7 <sup>e</sup>	45.8 $\pm$ 2.5 <sup>b,c</sup>	32.5 $\pm$ 1.4 <sup>a,b</sup>	27.1 $\pm$ 1.0 <sup>d</sup>	3.8 $\pm$ 0.2 <sup>c,b</sup>
Sun drying	5	93.4 $\pm$ 0.6 <sup>b</sup>	15.2 $\pm$ 3.3 <sup>g</sup>	20.2 $\pm$ 2.2 <sup>c</sup>	14.6 $\pm$ 2.0 <sup>e</sup>	1.3 $\pm$ 0.3 <sup>g</sup>
OvD-CG	5	96.0 $\pm$ 1.8 <sup>a,b</sup>	34.6 $\pm$ 6.1 <sup>e</sup>	34.6 $\pm$ 4.9 <sup>a,b</sup>	49.3 $\pm$ 10.4 <sup>a,b,c</sup>	2.9 $\pm$ 0.5 <sup>e</sup>
OvD-FG	5	96.0 $\pm$ 1.8 <sup>a,b</sup>	40.1 $\pm$ 3.7 <sup>c,d</sup>	37.5 $\pm$ 6.8 <sup>a</sup>	41.0 $\pm$ 9.0 <sup>c</sup>	3.3 $\pm$ 0.3 <sup>c,d</sup>
OvD-FGE	5	96.0 $\pm$ 1.8 <sup>a,b</sup>	35.4 $\pm$ 4.9 <sup>d,e</sup>	35.8 $\pm$ 4.7 <sup>a,b</sup>	27.5 $\pm$ 1.1 <sup>d</sup>	2.9 $\pm$ 0.4 <sup>d,e</sup>

n: number of repetitions.

DM: dry matter.

RE: retinol equivalent.

Data are presented as the mean  $\pm$  standard deviation.

Means with distinct lowercase superscript letters in the same column are significantly different

( $p \leq 0.05$ ).

OvD-CG: oven drying and coarse grinding.

OvD-FG: oven drying and fine grinding.

OvD-FGE: oven drying, fine grinding and encapsulation.

The DM in cooked leaves ranged from 8.5-22.2 % and drying led to dehydrated products with low residual water contents (4 to 6 %). The steam cooking, freezing and sterilization were the treatments which preserve the best  $\beta$ -carotene and retinol equivalent (RE), with respective loss ranging from 19-27 % and 16-37 %. In contrast, drastic losses of  $\beta$ -carotene and RE were observed after frying and sun drying.

Thermal and mechanical treatments (oven drying-fine grinding and encapsulation) also induced compound loss (>45%). During the leaf grinding and encapsulation, the surface area in contact with atmosphere and light was increased which caused the micronutrients loss by oxidation reactions. As observed in our work, sun drying (with light exposures) can induce drastic loss reaching 80 % (Park, 1987; Craft, Wise, & Soares, 2002; Saini et al., 2014; Knecht, Sandfuchs, Kulling, & Bunzel, 2015).

Steaming preserved more carotenoids than cooking in water where losses ranged from 24 to 83% (Jayarajan, Reddy, & Mohanram, 1980; Dikshit, Udipi, Rao, & Manohar, 1988; Grimme, Horst, & Brown, 1984; Dietz, Kantha, & Erdman, 1988; Mazzeo et al., 2011). Frying was the treatment that degraded the more the biochemical compounds.

### 3.2. Influence of cooking and processing on the contents in bioaccessible micronutrients

Once the IVD was completed, the lipophilic bioaccessible compounds are released from the food matrix and transferred to the micelles of the digestive medium. In raw leaves, the contents in bioaccessible  $\beta$ -carotene, lutein and  $\alpha$ -tocopherol were low ( $< 2$  mg/100 g DM) as well as their relative bioaccessibility ( $< 3$  %) (Fig. 2, 3 and 4).

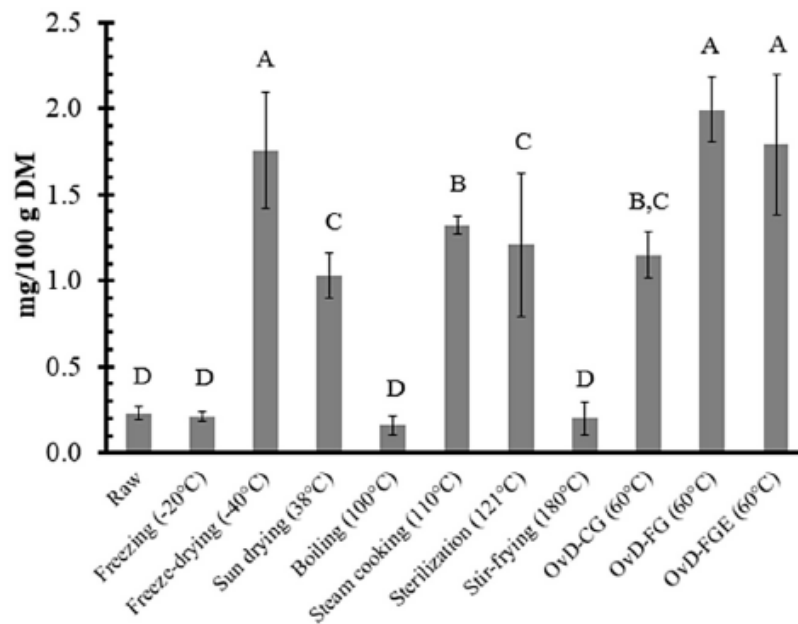
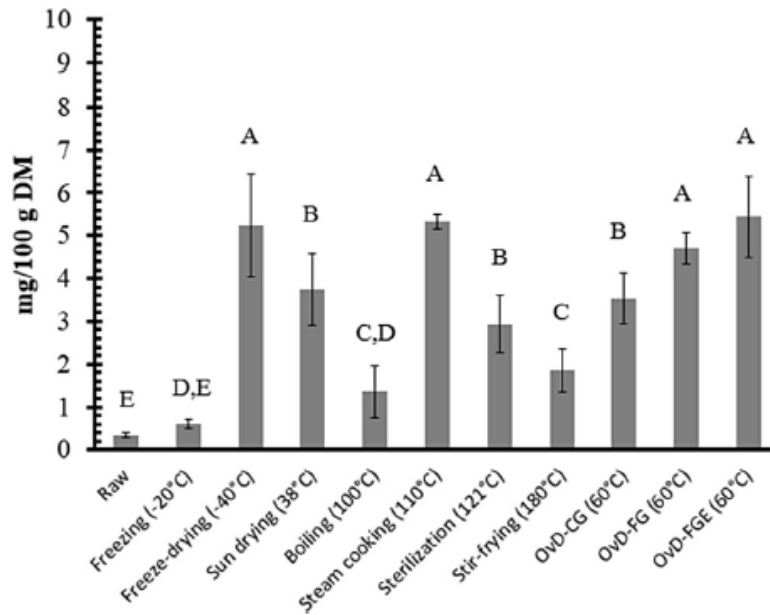
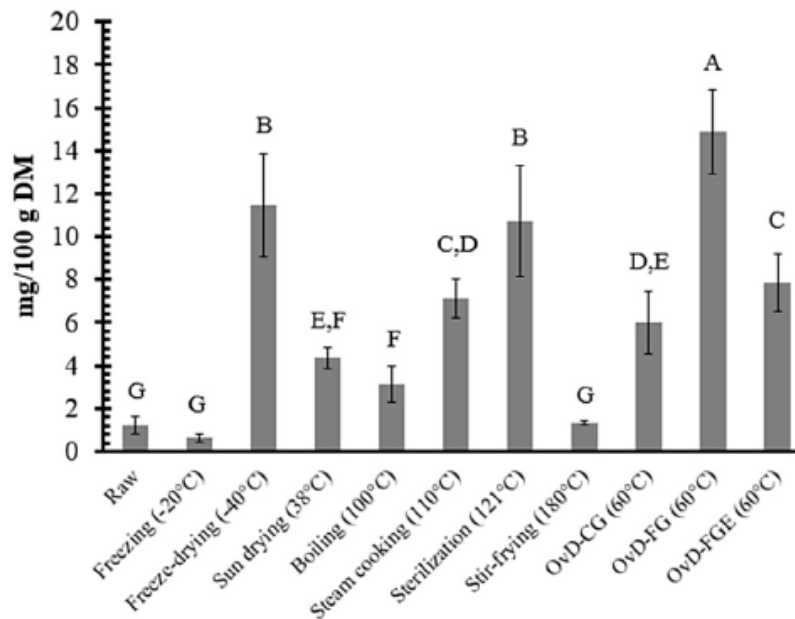


Fig. 2. The contents in bioaccessible  $\beta$ -carotene in the raw and processed drumstick leaves (mg/100 g DM). Different letters above each bar indicate significant difference ( $p < 0.05$ ). OVD-CG: oven drying and coarse grinding. OVD-FG: oven drying and fine grinding. OVD-FGE: oven drying, fine grinding and encapsulation.



**Fig. 3.** The contents in bioaccessible lutein in the raw and processed drumstick leaves (mg/100 g DM). Different letters above each bar indicate significant difference ( $p < 0.05$ ). OVD-CG: oven drying and coarse grinding. OVD-FG: oven drying and fine grinding. OVD-FGE: oven drying, fine grinding and encapsulation.

Xanthophylls were more bioaccessible than carotenes as observed in numerous studies (Pérez-Gálvez & Mínguez-Mosquera, 2005; Reboul et al., 2006; Granado-Lorencio et al., 2007; Palmero, Lemmens, Hendrickx, & Van Loey, 2014). The content in bioaccessible  $\alpha$ -tocopherol was always higher than carotenoids whatever the treatment. Most of the preservation methods (freeze drying, sterilization, sun drying, oven drying and encapsulation) increased significantly the contents in bioaccessible micronutrients and the effect of the preservation step was always higher for lutein (12-fold increase) than  $\beta$ -carotene (4-fold increase).



**Fig. 4.** The contents in bioaccessible  $\alpha$ -tocopherol in the raw and processed drumstick leaves (mg/100 g DM). Different letters above each bar indicate significant difference ( $p < 0.05$ ). OVD-CG: oven drying and coarse grinding. OVD-FG: oven drying and fine grinding. OVD-FGE: oven drying, fine grinding and encapsulation.



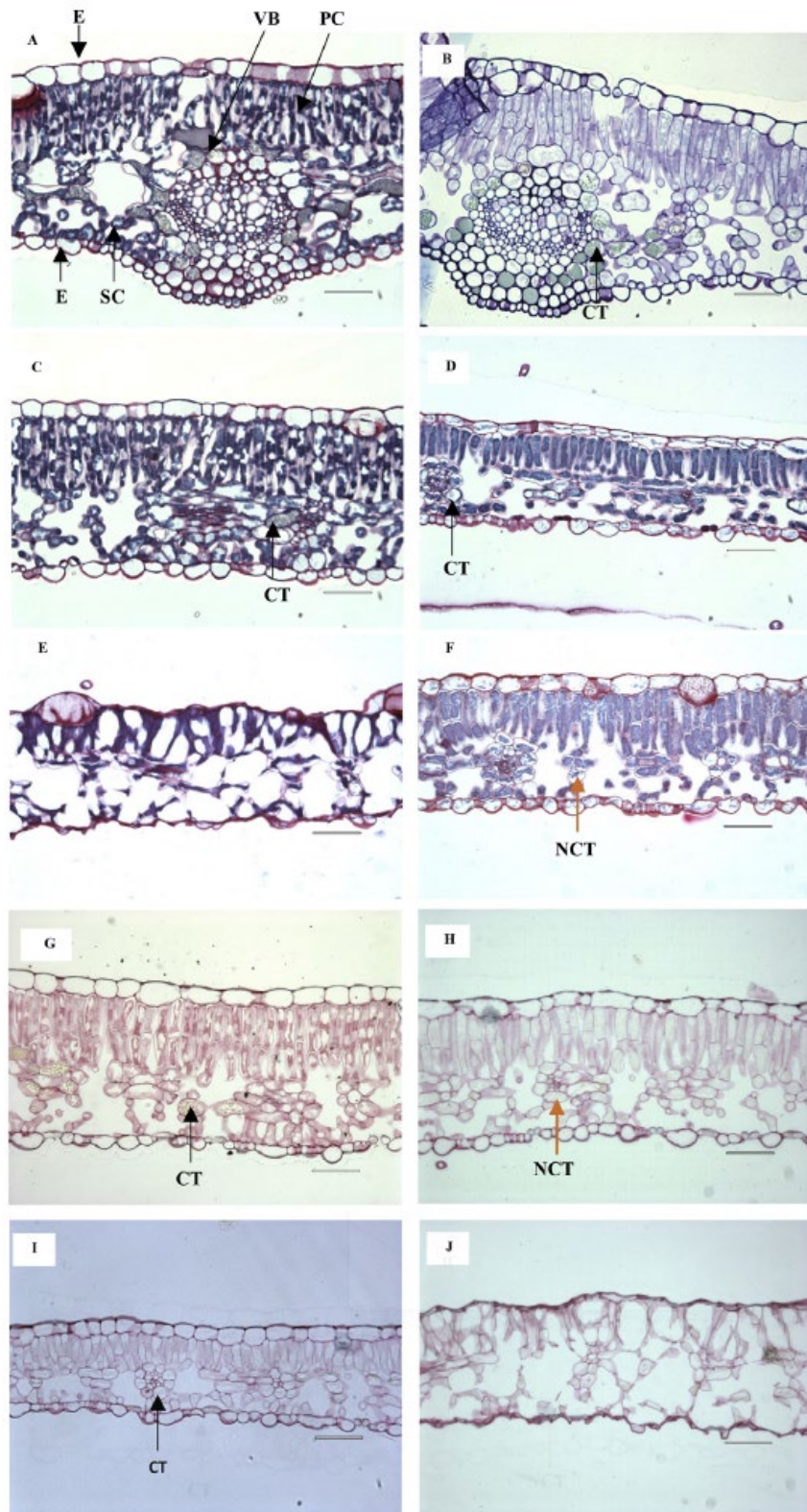
When drying was combined with grinding, the contents in bioaccessible micronutrients were improved. Grinding improved the diffusion of the compounds from the cells to the digestive medium; it opened the cells by breaking their walls and reducing the particle size to less than 1 mm or 200  $\mu\text{m}$  according to the grinding time. It was previously demonstrated in wheat, that fine grinding allows disruption of plant cell walls and improves phenolic acid bioaccessibility (Hemery et al., 2010).

After steaming and sterilization, the leaves became softer than the raw ones (data not shown) probably due to the long duration of the thermal treatment (15 min). Improvement of carotenoid and  $\alpha$ -tocopherol micellarisation was already observed after boiling in water (Nambiar & Seshadri, 2001; Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Moelants et al., 2012). Frying did not improve the content in bioaccessible compounds probably because the treatment induces important losses of carotenoids by chemical reactions (Achir, Randrianatoandro, Bohuon, Laffargue, & Avallone, 2010).

Encapsulation of dried and powdered leaves was an efficient way to deliver bioaccessible compounds. The HPMC capsule was not a physico-chemical barrier for the micronutrient release; experimentally, it was dissolved in 5 min in the digestive medium.

### **3.3. Structural changes after culinary treatments**

To improve the micronutrient bioaccessibility, the cells have to be opened by destructuration. The light micrographs clearly show that the leaf was organized with an upper and lower epidermis, and a mesophyll tissue comprised of palisade and spongy cells. The vascular bundles were also visible at the middle of the cross section (Fig. 5A-B). The palisade mesophyll was composed of elongated cells perpendicular to the upper epidermis; the contents of palisade cells stain blue with periodic acid-Schiff's reagent/NBB suggesting a high amount of protein (Fig. 5A). Tannin containing cells appeared greenish blue with toluidine blue, green with ruthenium red, but were also discernible as distinct cells with periodic acid-Schiff's reagent/NBB within the spongy mesophyll (Fig. 5A and G). According to the scale, we clearly see that the cells have a size (length and width) lower than 100  $\mu\text{m}$ . Grinding the leaves to 1 mm or 200  $\mu\text{m}$  particle only opened a fraction of the total leaf cells.



**Figure 5.** Light micrographs of drumstick leaves (cross section, x20): raw leaves (A – C and G) and cooked leaves (D and I: boiled, E and J: fried, F and H: steamed). Periodic acid-Schiff's and naphthol blue black staining (A, C, D, E and F), toluidine blue staining (B), ruthenium red staining (G, H, I, J). E: epidermis. PC: palisade cells; SC: spongy cells; VB: vascular bundle, CT: condensed tannins, NCT: none contain condensed tannins (orange arrow pointing). Scale bars = 100  $\mu$ m.

Morphological and qualitative changes were observed after all the treatments. In boiled and steamed leaves, epidermal and palisade mesophyll cells appeared intact and were similar to untreated leaves (Fig. 5C, D, F, H and I). Some spongy cells appeared slightly disorganized after boiling and steaming. Frying promoted a loss of integrity of both epidermal and mesophyll cells, and a loss of protein and tannin. Upper and lower epidermal layers were no longer visible while the palisade and spongy cells appeared ruptured and disorganized. In contrast, both boiling and steaming did not appear to rupture the cells, while the blue staining pattern showed the protein content was more diffuse (Fig. 5C, D and F). No apparent structural difference was observed between the boiled and steamed that could explain the higher bioaccessibility measured in the latter. However, a comparison of sections of boiled and steamed samples stained with Ruthenium Red and NBB indicated a loss of tannin from cells in the steamed samples, while the tannins were still observed in the boiled samples (Fig. 5C, D, F).

In raw leaves, cell wall and plastid substructure are a physicochemical barrier for bioaccessibility (Vishnevetsky, Ovadis, & Vainstein, 1999; Palmero et al., 2013). In general, cooking such as boiling improves the carotene bioaccessibility by changing cell morphology and softness (De Belie, Herppich, & De Baerdemaeker, 2000; De Belie et al., 2000; Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008; Palmero et al., 2014). This was not observed in our case maybe because drumstick leaves are tree leaves with a higher mechanical resistance; their cell wall thickness is probably higher than other soft leafy vegetables.

Cooking (boiling, steaming) with moist heat (with water or steam) involves gentle heat transfer from a liquid or vapor to food; the temperatures used are rather moderate (<110°C) and the vitamins are rather well retained by the leaves. On the contrary, during frying, the heat transfer is more intense because the oil temperature was maintained at 180°C; in these conditions, the compounds are highly degraded by chemical reactions (isomerisation and cleavage) and the food matrix is destructured. As a consequence, the vitamin bioaccessibility in % is increased because some cells were opened by frying but the overall content in bioaccessible vitamins is very low because most of them were degraded by chemical reactions.

The methods of preservation tested are based on water activity decrease (drying, lyophilization), microorganism destruction (sterilization) or conservation in negative temperatures (freezing, lyophilization). Except for sterilization, the temperatures applied during the operation units were moderate and the vitamins were well retained. The combination of thermal and mechanical treatments by grinding is the best way to increase bioaccessible carotenoid and tocopherol contents because it induces some cell ruptures. The sterilization at 121°C under pressure also favors the leaf destructuration; they are easily dispersed in small pieces during the *in vitro* digestion (data not shown).

## **5. Conclusion**

As other leafy vegetables, drumstick leaves contain a high level of carotenoids and vitamin E ( $\alpha$ -tocopherol) but, the contents in bioaccessible micronutrients are very low in the fresh product. The operation units which preserve the more the micronutrient and RE contents were freezing, steaming and sterilization while frying and sun-drying cause drastic loss. Drying leaves at moderate temperature (60°C) and grinding them was the best way to increase the content in bioaccessible  $\beta$ -carotene, lutein and tocopherol. Encapsulation of dried-powdered leaves was also an efficient way to deliver bioaccessible compounds during *in vitro* digestion. Even with the best combination of operation units (drying plus grinding), a great proportion of the bioactive compounds are still not released from the moringa leaves; grinding in smaller particle sizes (< 200  $\mu$ m) would allow to better functionalize drumstick leaf powder.

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