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Effect of Traditional Cooking on *In Vitro* Bioaccessibility and Microstructure of β-Carotene and Starch, in Yellow-Fleshed *Manihot esculenta*

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Abstract: Identification of traditional cooking methods of food that can be used as dietary interventions helps improve quality of life. This study evaluates the effects of different traditional preparation methods on *in vitro* bioaccessibility and microstructure of β-carotene and starch in yellow-fleshed manioc (YFM) found in Sri Lanka. Preparation methods included, boiling with water and cooking with coconut milk. The content of β-carotene and *in vitro* bioaccessibility of β-carotene were determined by high-performance liquid chromatography with photo Diode array detection (HPLC-DAD). Estimated glycaemic index (EGI) was determined as a measure of bioaccessibility of starch, using *in vitro* digestion procedures. Under Bright field microscopy it was observed that cooking with coconut milk has made much vigorous cell disruption, causing the release of starch and β-carotene out of the cells through disrupted cell walls. According to results β-carotene content was 1.04 μg/g DW (dry weight) in 'Swarna' variety of raw YFM. Content of β-carotene for boiled YFM and YFM curry were 0.34 and 1.07 μg/g DW respectively, but *in vitro* accessible β-carotene content was not detectable. EGI of YFM curry was higher (50.3) than the EGI of boiled YFM (24.6). Thus among above mentioned preparations, the best method of preparation for obese and diabetic population is boiled YFM and there is no benefit of consuming any of the above mentioned preparations of YFM as a source of vitamin A.

Keywords: *In Vitro* Bioaccessibility, β-Carotene, Estimated Glycaemic Index, Microstructure, Starch, Yellow-Fleshed Manioc

1. Introduction

Vitamin A is required for several vital functions in the body including vision, immune function, gene transcription, reproduction, bone metabolism and skin & cellular health. Vitamin A deficiency, which can lead to many complications, has been identified as a major public health problem in more than half of all countries in the world and is the main cause of preventable blindness in children [1]. β-carotene is a pro-vitamin A carotenoid which can be found in a variety of orange, yellow and green fruits and vegetables such as pumpkins, carrots and sweet potatoes. Hence, foods rich in β-carotene are a good source of vitamin A for vitamin A deficient populations. In the food matrix, carotenes are usually associated with proteins and can be found in chromoplasts [2].

Starch is the main carbohydrate in human nutrition and is a major component in plant foods. It is a polysaccharide that is

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made up of glucose molecules. Starch in plant foods is generally organized into granules inside amyloplasts and can be visualized using staining techniques [3]. Consumption of starchy foods in higher amounts can lead to obesity and diabetes which are now considered as major health problems worldwide.

Study on some of West Indian vegetables has proven that the food processing methods have a significant impact on glycaemic index [4]. It has been found that food processing changes food microstructure and thereby facilitate gastrointestinal digestion of food [5].

Previous studies have shown that different thermal processing methods gives rise to different degrees of bioaccessibility for carotenoids [6, 7, 8]. This is mainly due to disruption of cellular barriers at the preparation step which leads to release of nutrients from food matrix.

Yellow-fleshed manioc which can be found in Sri Lanka are focused on in this study as it contains both β-carotene and starch. There is a need for a more complete database on β-carotene and starch concentrations of YFM in terms of food as consumed by individuals.

Hence, information about the β-carotene concentration and the starch concentration of raw and thermally processed YFM and the effect of thermal processing on them are needed to encourage the intake of food rich in pro-vitamin A carotenoids (β-carotene) by vitamin A deficient people and foods low in starch by obese and diabetic populations. Therefore the objective of this study was to evaluate the effects of different traditional preparation methods on the *in vitro* bioaccessibility and microstructure of β-carotene and starch in YFM found in Sri Lanka.

2. Methods

2.1. Sampling

YFM (*Manihot esculenta* / Variety: Swarna) roots at optimum harvesting age (8 months old) from three different plants were obtained from Horticultural Crop Research and Development Institute, Gannoruwa, Peradeniya, Sri Lanka.

For each cooking method, one sample from each plant (250 g × 3) was taken.

2.2. Thermal Processing

Thermal processing was done according to traditional cooking methods in different houses at different days randomly. The cooking methods used are hereunder presented.

2.2.1. Boiling of Vegetables with Water

A 250 g sample from each plant was boiled separately. Prior boiling the bark of the root (tuber) was peeled, washed thoroughly and cut into pieces of 1.5 × 1.5 × 1.5 cm^3 in size and allowed to boil in salty water for about 20 minutes in an uncovered pan.

2.2.2. Preparation of Coconut Milk Curry

A 250 g sample from each plant of YFM was cooked using coconut milk. Initially the bark of the root was peeled washed thoroughly with water. It was cut into pieces of 2 × 2 × 2 cm^3 in size. A mixture of spices and salt with coconut milk was added and cooked for about 20 minutes.

2.3. In Vitro Digestion Procedure for β-Carotene

The *in vitro* digestion procedure was done according to Chandrika et al., 2006 [7] and was performed on samples of YFM curry and boiled YFM. Then a 10.0 g sample from above mentioned 250 g cooked sample was homogenously crushed into pieces which were similar in size to chewed food and 10 mL of distilled water with 1% (w/v) ascorbic acid was added. Five milliliters of 0.5% porcine pepsin solution (Himedia RM 084) in 0.1 M HCl with physiological amounts of calcium (3.6 mM; 2.65 mg added as $CaCl_2.2H_2O$), Magnesium (1.5 mM; 1.52 mg added as $MgCl_2.6H_2O$), sodium (49 mM; 14.33 mg added as NaCl), potassium (12 mM; 4.47 mg added as KCl) and phosphate (6.4 mM; 4.35 mg added as KH_2PO_4) were added. The pH was adjusted to 2 using 2 M HCl if necessary, and transferred the solution into a 100 mL stoppered conical flask.

The flask was aerated with nitrogen and incubated at 37°C in a shaking incubator (IKA KSs 4000i) at 90 rpm for 1 hour. The pH was adjusted to 5 by adding 2 M NaOH. An amount of 3 mL of a mixture of pancreatin (Himedia RM 083) (4 g/L; 12 mg) and bile salt extract (HimediaRM 008) (25 g/L; 75 mg) both dissolved in 0.1 M $NaHCO_3$ and stabilized with 1% (w/v) D, L-α-tochopherol was added to it. The pH was further adjusted to 7.5 by adding 2 M NaOH. The flask was aerated with nitrogen and incubated at 37°C in a shaking incubator with orbital shaking at 90 rpm for 30 min.

The sample was centrifuged at 1000 ×g for 30 minutes and the supernatant was collected with a pipette. The supernatant was added into a separatory funnel containing 20 mL of 25% (w/v) NaCl. An amount of 20 mL 95% ethanol and 15 mL petroleum ether, both stabilized by 0.1% (w/v) Butylated Hydroxy Toluene (BHT), were added to it, shaken and allowed to separate. Lower layer was re-extracted by adding 25 mL petroleum ether and 10 mL 95% ethanol, both stabilized by 0.1% (w/v) BHT until petroleum ether layer become colourless. Petroleum ether layers were combined and evaporated to dryness using rotary vacuum evaporator at 35°C. The residue was dissolved in acetone stabilized by 0.1% (w/v) BHT and analyzed using HPLC. Procedure was performed in triplicate for each sample.

2.4. Reverse-Phase HPLC Analysis

Quantification was carried out using HPLC series 1260 (Agilent 1260Infinity) equipped with quaternary gradient pump, auto sampler, thermostatic column compartment and multiple detector. The gradient mobile phase was consisted of acetonitrile, methanol and ethyl acetate containing 0.05% triethylamine and used at a flow rate of 0.5 mL/min, using a monomeric C_{18} column (Eclipse Plus C_{18} Agilent 5 µm, 4.6 x 250 mm^2). A gradient was applied as 95:05:0 at 0 time to 60:20:20 at 20 min, maintaining this proportion until the

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end of the run. Injections volume was 10 µL. Detection was at 450 nm. All *trans* β-carotene was used as external standard.

2.5. Analysis of β-Carotene in Raw YFM and in Cooked YFM Before Digestion

Analysis was done according to Rodriguez-Amaya, 2001 [9]. For raw YFM, initially the bark of the roots (tuber) was peeled and the tubers were washed thoroughly. They were cut into small pieces and whole 250 g sample was crushed using mortar and pestle to obtain a homogenous mixture. Exactly 10.0 g of YFM was ground with 1 g of celite using a mortar and pestle while adding 10 mL of cold acetone. It was vacuum filtered through a sintered glass funnel. Grinding and filtration were repeated four more times for the residue, using 10 mL cold acetone each time, until the residue became white in colour.

Twenty milliliters of petroleum ether was transferred into a separatory funnel and the acetone extract obtained by above procedure was transferred to it along the wall and shaken well to mix. A small amount of distilled water was added along the wall and allowed to separate. After 30 minutes, the lower aqueous layer was discarded and upper organic layer was transferred into a 100 mL round bottom flask and it was evaporated to dryness using rotary vacuum evaporator at 35°C. The residue was dissolved in 1000µL of acetone and filtered through 0.45 µm pore size filter before HPLC analysis. The injection volume was 10 µl. Whole procedure was conducted under dim light and the sample containers were always covered with aluminium foil. Procedure was performed in triplicate for each sample.

2.6. Determination of Total Starch in Cooked YFM

The procedure was conducted according to the method established by McCleary et al 1997 [10]. From homogenously crushed samples of YFM curry and boiled YFM, 50.0 mg each were accurately weighed and 3 mL of distilled water was added carefully to moisten the sample followed by addition of 3 mL of 4 M KOH. It was shaken at room temperature for 30 minutes using a vortex. An amount of 3 mL of 0.4 M sodium acetate buffer (pH 4.75) was added to it and pH was adjusted to 4.75 with 1 M and 2 M HCl. An amount of 80 µL of amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich A7095) was added to it. It was mixed well and incubated at 60°C for 45 minutes in a shaking incubator.

The mixture was centrifuged at 3000 ×g for 15 min. The supernatant liquid was collected into a 500 mL volumetric flask using a pipette. Volume was made up to 500 mL by adding distilled water.

Absorbance value for each sample was determined using Glucose Oxidase - Peroxidase kit.

0.5 mL sample was mixed well with 1 mL of reagent from the Glucose Oxidase-Peroxidase kit and left for 30 minutes in a water bath at 37°C. The absorbance of the sample was read at 500 nm against a reagent blank (containing 0.5 mL distilled water instead of the sample) between 5 and 45 minutes after incubation using a spectrophotometer (Spectro UV-VIS Double beam PC scanning-UVD-2960 Labomed, Inc). Glucose concentrations of samples were determined using a standard curve prepared using glucose-water solutions prepared in the range of 10-60 ppm.

Procedure was done in triplicate for each sample. Same procedure was conducted in triplicate for a fresh white bread sample as the reference sample.

2.7. Determination of EGI of Cooked Samples by in Vitro Digestion Procedure

The procedure was conducted according to method established by Goni et al 1997 [11]. From homogenously crushed samples of YFM curry and boiled YFM, 50.0 mg was accurately weighed and were homogenized in 5 mL of HCl-KCl mixture (pH 1.5) for 1 minute using a homogenizer with controlled speed (level 4). The samples were incubated at 40°C for 60 minutes in a shaking incubator with 0.1 mL of pepsin (Himedia RM 084) solution (0.2 mL of HCl-KCl mixture containing 1 mg of pepsin from porcine gastric mucosa). An amount of 7.5 mL of Tris-malate buffer was added to make pH to 6.9. Then 2.5 mL alpha amylase solution (5 mL of Tris-Malate buffer containing 2.6 UI of alpha amylase from porcine pancreas) was added. The flasks were placed in a shaking incubator at 37°C. Aliquots (0.1 mL) were taken every 30 minutes from 0 to 3 hours. Alpha amylase was inactivated by immediately placing the tubes in a boiling water bath for 10 minutes with vigorous shaking every 30 seconds. Then 1 mL of 0.4 M sodium acetate buffer (pH= 4.75) and 30 µL of amyloglucosidase were added. The samples were incubated at 60°C for 45 minutes to hydrolyze the starch into glucose. Finally, the glucose concentration was measured using a Glucose Oxidase-Peroxidase kit and using a standard curve. The EGI for each sample was calculated using Microsoft excel 2013 and SPSS for windows 21.

Data analysis for EGI of thermally processed YFM and Squash was carried out as follows.

Glucose concentration for each sample was determined using the standard curve. Amount of starch in mg was calculated as glucose in mg × 0.9. The digestion curves were prepared according to following non-linear equation established by Goni et al 1997 [11].

$$C = C\infty\,(1 - e - kt) \qquad (1)$$

Where C is the percentage of starch hydrolyzed at time t (min); $C\infty$ is the equilibrium percentage of starch hydrolyzed after 180 min; and k is the kinetic constant. The variables $C\infty$ and k were estimated for each sample using SPSS for windows 21. The area under the hydrolysis curve (AUC) was calculated for each sample using the equation;

$$AUC = C\infty\,(tf - t0) - (C\infty/k)\,[1 - \exp\,[-k\,(tf - t0)]] \qquad (2)$$

Where tf is the final time (180 min) and t0 is the initial time (0 min).

Hydrolysis Index (HI) was obtained by dividing the AUC of each sample by corresponding AUC of reference sample (fresh white bread, GI=100). Finally EGI was predicted with the formula;

$$EGI = 39.71 + (0.549 \times HI) \quad (3)$$

Procedure was done in triplicate for each sample. Same procedure was conducted in triplicate for a fresh white bread sample as the reference sample.

2.8. Microscopy of Starch Granules

Bright field microscopy was done using FSX 100 Olympus microscope equipped with FSX-BSW software. Bright field microscopy images at × 20 and × 40 magnifications were obtained for raw and thermal processed samples of YFM. For visualization of starch granules, slides were stained with iodine and they were examined and photographed.

2.9. Determination of Moisture Content

Moisture contents of raw and thermally processed YFM were determined by drying triplicate samples from each vegetable sample in an oven at 60°C until a constant weight was obtained. The percentage moisture for each sample was calculated based on the average weight of the three dried samples.

3. Results

3.1. β-Carotene Content of Cooked YFM

The HPLC chromatogram for raw YFM had a peak of similar retention time and similar spectrum for the peak given by corresponding all-*trans*-β-carotene standard (Figure 1), by which the presence of β-carotene in the raw YFM sample was confirmed.

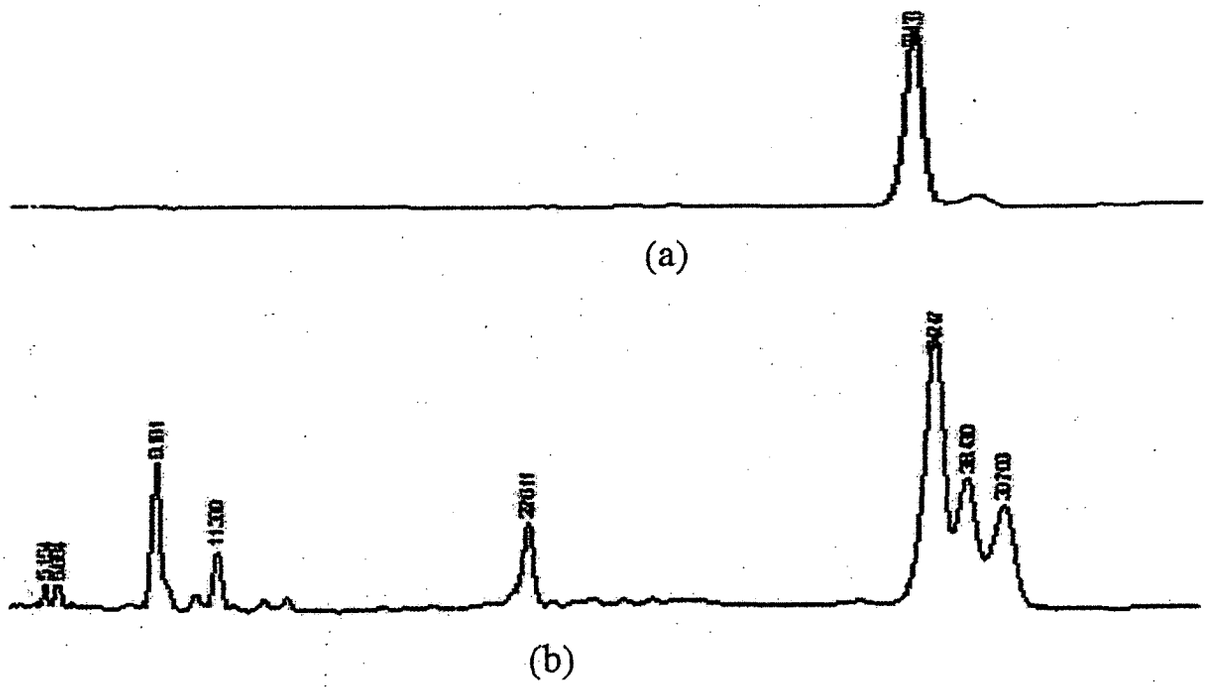

Figure 1. HPLC chromatograms for raw YFM.

(a) HPLC chromatogram of β-carotene standard used to assay raw YFM. (b) HPLC chromatogram of raw YFM; Peaks at 36.133 and 37.247 min. in (a) and (b) respectively are β-carotene.

A trace amount; 1.04 ± 0.15 μg/g DW of β-carotene was observed in raw YFM. Mean total β-carotene, in different preparations of YFM are shown in Table 1. The content of β-carotene for boiled YFM and YFM curry was 0.34 and 1.07 μg/g DW respectively.

Table 1. Total β-carotene contents of traditionally cooked vegetable preparations of YFM.

Vegetable preparation	Average moisture content (% w/w)	Total β-carotene (μg/g FW)[a]	Total β-carotene (μg/g DW)[a]
Raw YFM	47.92	0.54 ± 0.09	1.04 ± 0.15
Boiled YFM	68.65	0.10 ± 0.01	0.34 ± 0.09
YFM curry	69.81	0.32 ± 0.07	1.07 ± 0.34

[a]Mean ± standard deviation in three samples (n=3) in triplicate

3.2. In Vitro Bioaccessibility of β-Carotene in Cooked YFM

The bioaccessible β-carotene in both boiled YFM and YFM curry was not detectable in this study. This is attributed to the low content of total β-carotene present in the Swarna variety of YFM.

3.3. EGIs of Cooked YFM

The EGI of YFM curry (50.3 ± 6.4) was significantly higher ($P < 0.05$) when compared to that of boiled YFM (24.6 ± 3.3) (Table 2).

Table 2. EGIs of traditionally cooked vegetable preparations of YFM.

Vegetable preparation	Average moisture content[a] (% w/w)	EGI[a]
Boiled YFM	68.65	24.64 ± 3.33
YFM curry	69.81	50.25 ± 6.42

[a]Mean ± standard deviation in three samples (n=3) in triplicate

3.4. Effects of Traditional Cooking on Microstructure of YFM

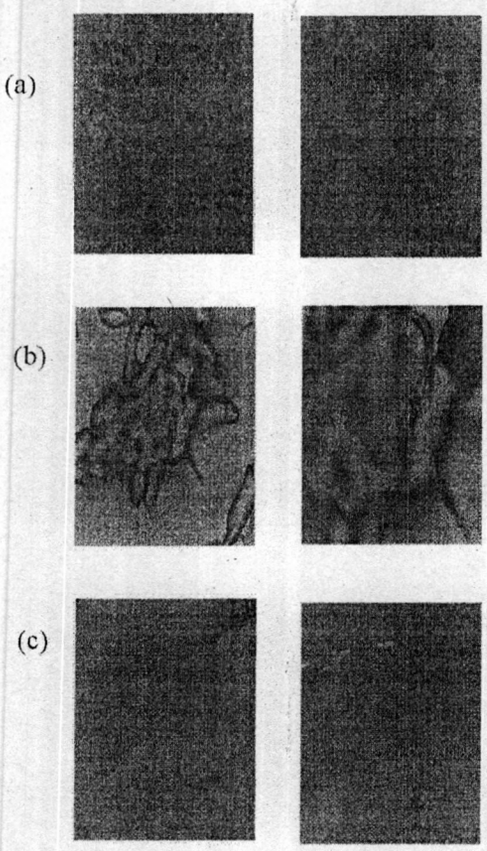

Figure 2. (a) Raw YFM sample shows cells which are closely bound to each other. β-carotene present in a limited area as a slight dispersion. (b) Boiled YFM sample shows two intact cells facing towards each other having prominent clusters of β-carotene along the middle lamella of each cell. (c) YFM curry sample shows cells with disrupted cell walls and slight dispersion of β-carotene through the cellular area. (Left column shows bright field images in ×20 magnification and right column shows same corresponding images in ×40 magnification)

Bright field microscopy images of β-carotene and starch in YFM preparations are shown in Figure 2 and Figure 3 respectively. Under microscopy β-carotene was identified by its characteristic yellow-orange colour and iodine stained starch granules were observed in blue-purple colour.

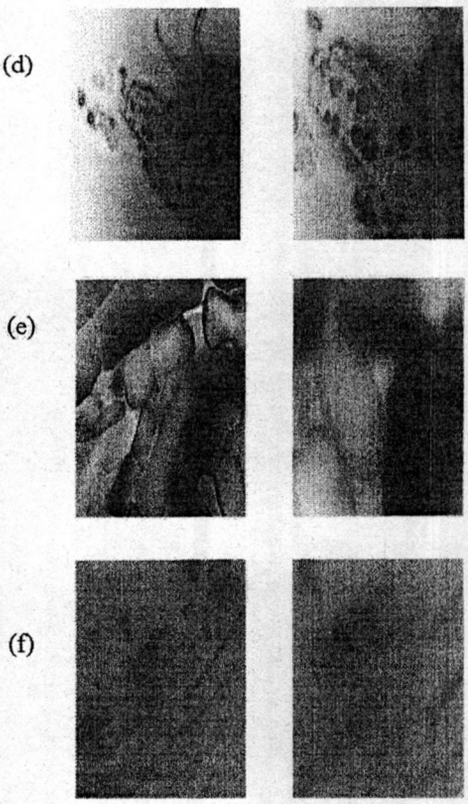

Figure 3. (d) Raw YFM sample shows intact cells with prominent cell walls which are closely bound to each other forming a hard tissue. Starch present as small globules within the cells. (e) Boiled YFM sample shows that intact cells are separated across middle lamella and has prominent dispersion of starch within each cell. (f) YFM curry sample shows cells with disrupted cell walls and slight dispersion of starch throughout the cellular area. (Left column shows brightfield images in ×20 magnification and right column shows same corresponding images in ×40 magnification)

Cooking of the vegetables has caused cell separation and disruption in YFM. Degree of tissue disruption seem to be proportional to degree of heat treatment.

Boiling has caused small carotene bodies within each cell to get together and form large aggregates whereas cooking with coconut milk has caused rupture of the aggregates making β-carotene to be dispersed within cells and also release of β-carotene out of cells into surrounding solution (Figure 2).

Cooking with coconut milk has made much vigorous cell disruption, causing the release of starch out of the cells through disrupted cell walls; hence, the density of starch within cells is low, whereas in boiled sample starch is present in high densities within each cell (Figure 3). Boiling has caused starch gelatinization where starch granules swell and rupture and starch become solubilized [12].

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4. Discussion

Although the *in vitro* bioaccessibility of β-carotene was not detectable in the present study, most studies show that *in vitro* bioaccessibility of β-carotene is higher when cooked with coconut milk than when boiled [13]. It has been found that the *in vitro* accessibility of β-carotene is more when boiled with coconut milk rather than boiled with water, in the case of Sri Lankan green leafy vegetables [7]. For most of the Sri Lankan non-leafy vegetables it has been found that the *in vitro* accessibility of β-carotene is more when prepared as a curry with coconut milk compared to other traditional cooking methods which includes boiling with water [6]. Studies to determine *in vitro* bioaccessibility of β-carotene with changes in microstructure due to different cooking methods have been done for vegetables such as orange-fleshed sweet potatoes in African countries. They conclude that the extent of cell wall rupture is the most important determinant of *in vitro* bioaccessibility of β-carotene [14]. Most of these studies have been done on vegetables that have been bio-fortified to produce more β-carotene, such as orange-fleshed sweet potatoes, yellow-fleshed manioc in African countries [14, 15, 16].

It has been found that cooking, which results in more efficient release of carotenoids from the food matrix by softening cell structures so that digestive enzymes can work more efficiently, resulting in higher β-carotene release from foods compared to the uncooked kind [17].

A study carried out in Sweden on orange fleshed sweet potatoes shows that there is no apparent effect of intestinal digestion on the plant cells that remains intact after food preparation and mastication. Thus, the preparation step is highly important to increase the *in vitro* bioaccessibility of nutrients [14]. Some nutrients found in plants are protected in nature against degradation inside cells by attaching to membranes, occluding inside cell organelles, or binding to cell walls, but this natural protection lowers bioavailability. Thermal and physical processing, mastication, and to limited extent digestion break down the cell walls, making the release of nutrients from the food matrix easier and rendering them available for absorption in the intestine [11].

Even though both boiled YFM and YFM curry showed detectable amounts of β-carotene, the bioaccessible β-carotene in both boiled YFM and YFM curry was not detectable in this study. It is unclear if this decrease in the content of β-carotene in digester was due to enhanced isomerization of all-*trans* –β-carotene during digestion of YFM in both boiled YFM and YFM curry. In the case of YFM curry there is a possibility that fat content is not in the bio accessible range. This is because the results from other investigations with humans on the effect of different amounts of fat on carotenoid absorption are also confusing. Some studies indicate that only a small amount of fat is required to substantially increase carotenoid bioavailability [18, 19] and that β-carotene bioavailability does not significantly improve when consumed with a high-fat compare to low fat meal [20]. Other studies show improved absorption of β-carotene with high fat compare to very low fat intake [21, 22]. Therefore further studies are necessary to elucidate the basis for the relatively poor bioaccessibility shown in the present study. Some losses in the recovery of β-carotene during prolonged processing procedures are to be expected, as this pro-vitamin A is highly susceptible to destruction by exposure to light and oxygen although the losses were minimized by taking some precautionary measures including performing analysis under dim light and using nitrogen gas whenever possible to minimize the contact with oxygen.

Studies on Glycaemic Index (GI) of vegetables such as cassava (staple food of African population) have been reported mostly from African countries. An in vivo study has shown that cassava paste gives relatively high GI (GI= 86) and another study from Tanzania has shown that cassava flour has a relatively low GI (GI=49.84) and thus good for treatment and management of diabetes [23, 24] These studies and another in vivo study on Jamaican sweet potatoes show that method of food processing has a significant impact on GI [25].

Hence, these results demonstrate that due to low EGI value, the boiled YFM will be beneficial to health than the YFM curry, especially to populations who are obese and suffering from diabetes. And also this study shows that there is no significance benefit on consuming any preparation of YFM as a source of vitamin A. These data on the β-carotene content and EGI in terms of food as eaten could be used in dietary intervention programmes to improve the quality of life of people.

5. Conclusions

This study shows that there is a significant effect of thermal processing on the *in vitro* bioaccessibility and microstructure of β-carotene and starch in YFM.

The degree of heat treatment determines the magnitude of changes in microstructure and thereby the degree of release of starch during digestion which represents by EGI values in different preparation methods of YFM. Boiled YFM is healthier for obese and diabetic population due to its low EGI when compared to YFM curry.

This study also shows that the degree of heat treatment determines the magnitude of changes in microstructure and thereby release of β-carotene in different preparation methods of YFM. But due to the fact that trace amount of β-carotene present in YFM, there is no significant benefit on consuming any preparation of YFM as a source of vitamin A. Thus dietary interventions to alleviate vitamin A status should take into account the possibility of non bioaccesibilty of β-carotene in different preparations of the same food when planning out such interventions. Furthermore different preparations of the same food with low EGI could be used in dietary intervention programmes to improve the quality of life of populations in Sri Lanka as well as in other developing countries.

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Declaration of Conflict Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

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