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In vitro bioaccessibility of β-carotene in pumpkin and butternut squash subjected to different cooking methods

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Abstract

β-carotene, a type of provitamin A, is beneficial to our health. However, the compound needs to be released from its food matrix before being utilised by the body. Thus, understanding the bioaccessibility of β-carotene in the food consumed is a crucial step. The objective of this study was to determine the effect of various cooking methods on bioaccessibility of β-carotene in pumpkin and butternut squash. *In vitro* digestion was carried out on raw and cooked (steamed, boiled, and deep-fried) pumpkin and butternut squash. β-carotene was identified using RP-HPLC. Generally, butternut squash (4.99±0.02mg/100g) had higher β-carotene content than pumpkin (4.34±0.04mg/100g). Thermal processing resulted in lower β-carotene content in pumpkin samples; however, it increased the β-carotene content in butternut squash samples. In term of bioaccessibility, thermal processes increased the percentage of bioaccessible β-carotene in both pumpkin and butternut squash samples. Raw pumpkin had 10.56±0.44% of bioaccessible β-carotene while raw butternut squash had only 1.65±0.04%. Bioaccessibility of β-carotene in deep-fried pumpkin and butternut squash were significantly higher than their raw sample with 68.86±0.86% ($p<0.001$) and 22.32±2.12% ($p<0.05$) of bioaccessible β-carotene respectively. The deep-frying method was found to enhance the bioaccessibility of β-carotene significantly in both of these samples but not boiling and steaming methods.

Introduction

Pumpkin (Figure 1) is a cultivar of squash plant and from the family of Cucurbitaceae, genus of *Cucurbita*. The commonly consumed pumpkin species in Malaysia is *Cucurbita moschata* (Zuhanis, 2014). There is 3100 µg of β–carotene found in every 100g of pumpkin according to United States Department of Agriculture (USDA) (2015a). Butternut squash (*Cucurbita moschata* Duchesne) (Figure 2) is in the same family and genus as pumpkin with higher β–carotene content, 4226µg in every 100g (USDA, 2015b). It also has yellow and orange coloured peel and pulp and tastes sweet and nutty which is similar with pumpkin. However, it is in the shape of a pear which differentiates butternut squash from the pumpkin.

Pumpkin and butternut squash are good sources of β-carotene for human consumption. Although studies found that pumpkin and butternut squash have a high level of β-carotene concentration, there are limited studies on bioaccessibility of β-carotene in these two fruits, specifically, a study on the effect of the cooking method on the bioaccessibility of

Figure 1. Pumpkin (Source: Norshazila *et al*., 2014)

Figure 2. Butternut Squash (Source: Zaccari and Galietta, 2015)

β-carotene. β-carotene, a type of carotenoids that appears mostly in the range of orange coloured fleshed fruits and vegetables, has antioxidant properties which are able to reduce the occurrence of some chronic diseases such as cardiovascular disease and cancer due to its oxygen quenching

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capacities of the compound (Edge *et al*., 1997; Fraser and Bramley, 2004; Chen, 2015). β-carotene is also one of the provitamin A carotenoids, other than α-carotene and β-cryptoxanthin, which helps in the acquisition of vitamin A that is crucial for the human vision, immune function, and normal development (Chen, 2015). A study found that consumption of β-carotene rich food has the ability to increase the vitamin A intake among women and children who are always at risk of vitamin A deficiency (Hotz *et al*., 2012).

Vitamin A deficiency had been recognised as one of the serious health problems in developing countries with the poorer setting of economic deprivation. Malaysia was considered to have mild subclinical vitamin A deficiency with the vitamin A deficiency prevalence of 2.5% in male and 4.5% in female (Ministry of Health/ UNICEF, 2000). Poor bioavailability has a predominant role in the development of vitamin A deficiency in low-income countries as the community relies heavily on plant sources for meeting the requirement of vitamin A. The major source of vitamin A in these countries is from dietary intake of β-carotene (a form of provitamin A).

In order for the β-carotene to be utilised by our body and converted to vitamin A, it must first be accessible. Bioaccessibility of β-carotene is often defined as the fraction of a compound that is able to release from the food matrix and transfer to mixed micelles to make uptake by the intestinal mucosa possible (Bengtsson *et al*., 2009). There are some factors that could influence the bioaccessibility of β-carotene to mixed micelles to be uptaken by the intestinal mucosa, such as matrix in which the compound is present, presence and type of lipid, heat treatment and degree of homogenisation during food processing (Rock *et al*., 1998; van den Berg, 2000). Generally, dietary fat and heat treatment were found to be associated with the increase in bioaccessibility of β-carotene (Ornelas-Paz *et al*., 2008; Carvalho *et al*., 2014). Different cooking methods to prepare pumpkin and butternut squash dishes would produce different effects on bioaccessibility of β-carotene and it is worth for study. Limited study on the effect of the cooking method on bioaccessibility of β-carotene in pumpkin and butternut squash in Malaysia leads to the need to carry out this study. The aim of this research, therefore, was to determine the *in vitro* bioaccessibility of β-carotene in pumpkin and butternut squash subjected to different cooking methods (boiling, steaming and deep-frying)

Materials and Methods

Sample selection and preparation

Pumpkin (locally grown) and butternut squash (imported from Australia) samples were conveniently selected from the local market and stored in dark at room temperature 26-32°C. Sample preparation was carried out according to the method by Tumuhimbise *et al*. (2009). All the samples were cut into the size of 15-20mm thickness prior to different cooking methods (steam, boil, deep-fry). Raw and cooked samples were then homogenised and grounded with mortar and pestle prior to analysis (Tumuhimbise *et al*., 2009).

Reagents and chemicals

All chemicals used were of analytical grade. β-carotene standard was obtained from Sigma-Aldrich (Schnelldorf, Germany). Enzymes such as pepsin (porcine), α-amylase, pancreatin (porcine), and bile extract (porcine) were purchased from Sigma-Aldrich (Schnelldorf, Germany) as well.

In vitro *digestion of raw and processed pumpkin and butternut squash samples*

Bioaccessibility of β-carotene was determined by using *in vitro* digestion according to the method described by Garrett *et al*. (1999). Triplicate samples (~1 g) were weighed subjected to stimulated gastric digestion in the presence of 2 mL porcine pepsin (Sigma P 6887) (40mg/mL in 0.1M HCl) after acidification to pH 2.0 with 1M HCl. Samples were then put into orbital shaker at 95 rpm with 37°C for 1h.

Intestinal digestion was stimulated by increasing the pH of sample to 5.3 with 1.0-1.3 mL of 0.9 M $NaHCO_3$ followed by the addition of 9 mL pancreatin/bile extract solution (2mg/mL pancreatin (Sigma P 1750), 12 mg/mL bile extract (Sigma B 8631) in 100 mM $NaHCO_3$). The final concentration of pancreatin was 0.4mg/mL while bile extract was 2.4mg/mL in the reaction mixture (Garrett *et al*., 1999). pH was then being adjusted to 7.5 by the addition of 1M of NaOH before being blanketed with nitrogen gas. Samples were then incubated at 37°C for 2 h in an orbital shaker (95 rpm) for completion of intestinal digestion.

After the completion of *in vitro* digestion, samples were centrifuged at 5000 x g for 20 minutes in order to obtain the supernatant fraction. Then, an aliquot of the supernatant went undergone membrane filtration by using Millipore membrane of 0.45 µm pore size, 20 mm diameter to obtain the micellar fraction into a clean amber glass (Tumuhimbise *et al*., 2009).

Extraction of β-carotene from homogenised pumpkin and butternut squash samples

Extraction of β-carotene was carried out according to the method described by Bengtsson *et al.* (2008). About 1 g of sample was triplicated with test tubes. Before being vortexed and centrifuged at 4750 x g for 3 minutes, samples were added with 2 mL of acetone containing 0.1% (w/v) butylated hydroxytoluene. The supernatant was saved into a new test tube. Residues in the samples were being extracted with 2 mL of acetone repeatedly and centrifuged. The process took around 5 times until the residues turned colourless.

For the acetone extract (supernatant), 3 mL of petroleum ether (40-60°C) was added with deionised water (5 mL) to facilitate the separation of phases. The two phases (organic phase and water phase) were separated by centrifugation at 4750 x g for 4 minutes. After the centrifugation, the organic phase was pipetted into a new test tube. This step was repeated for one time. The organic phases that obtained were dried in a rotary evaporator at 35°C under a stream of nitrogen. The residue was then dissolved in 5 mL of mobile phase consisting of methanol/methyl tert-butyl ether (60:40, v/v).

Extraction of β-carotene from in vitro *digested pumpkin and butternut squash samples*

The β-carotene in a micellar fraction from the in vitro digested samples were extracted with hexane/acetone/ethanol (50:25:25) containing 0.1% (w/v) butylated hydroxytoluene. The process was repeated twice. Next, samples were vortexed and centrifuged at 4750 x g for 3 minutes to aid in phase separation. After centrifugation, the organic phase was obtained and combined before dried in a rotary evaporator at 35°C under a stream of nitrogen. The residues obtained was dissolved in 1 mL of mobile phase consisting of methanol/methyl tert-butyl ether (60:40, v/v) (Bengtsson *et al*., 2008).

β-carotene analysis by HPLC

Reserved phase HPLC was employed to analyse β-carotene content in the samples. HPLC system was equipped with a pump, a degasser and a diode array detector operating at 450nm. Absorption spectra were recorded between 250 and 500 nm. Data were stored and processed by PC1000 Version 3.5 Software. Separations were carried out on Prontosil C18 carotenoid column. The injection volume was 20 µL and flow rate was 1.3 mL/min. The mobile phase used for isocratic elution consisted of methanol: methyl tert-butyl ether: water (55:41:4, v/v/v). β-carotene was identified by using β-carotene standard and comparing the spectra data with reported values (Tumuhimbise *et al*., 2009).

Data analysis

Data collected were being analysed by IBM SPSS 21.0. Percentage of bioaccessibility of β-carotene in pumpkin and butternut squash in the three replicate measurements were expressed in mean and standard deviation. Bioaccessibility of β-carotene was calculated using the formula as follow:

$$\text{Bioaccessibility (\%)} = 100 \times (a / b)$$

where,

a= element content of the bio-accessible fraction (mg mineral element / 100 g of sample)

b= total β-carotene content (mg mineral element / 100g)

Comparison in the mean percentages of bioaccessibility of β-carotene in pumpkin and butternut squash was subjected to analysis of variance (one-way ANOVA) with Games-Howell post hoc test. Games-Howell post hoc test was used as the homogeneity of variances assumption was violated. P values <0.05 were considered significant.

Results and Discussion

β-carotene content in pumpkin and butternut squash

Raw pumpkin (PR) was found to contain 4.34±0.04 mg/100g of β-carotene in this study while 4.99±0.02 mg/100g of β-carotene was found in raw butternut squash (BR) (Table 1). Thermal processes had led to a decrease of β-carotene content which resulted in significantly lower β-carotene content in cooked pumpkin as compared to PR. Among the cooked pumpkin, steamed pumpkin (PS) retained the highest amount of β-carotene content, followed by boiled pumpkin (PB) and the least retained β-carotene content in the pumpkin was deep-fried pumpkin (PD).

PS and PB had 3.82±0.04 mg/100g and 3.57±0.05mg/100g of β-carotene respectively. PS was significantly ($p<0.05$) lower by 12.0% as compared to PR while reduction of β-carotene content in PB was significantly ($p<0.001$) lower by 17.8% as compared to PR. The beta-carotene content of PS was significantly ($p<0.05$) higher than the amount of β-carotene in PB. The findings in this study were in agreement with the previous study that reported a reduction of β-carotene in pumpkin pulp after boiling and steaming processes (Tumuhimbise *et al*., 2009; Maria *et al*., 2015). Oxidisation, isomerisation and degradation of β-carotene (the presence of double bonds in the carbon chain) can occur due to heat, light, and acidity (Mercadante, 2007). Processing of samples can further expose samples to oxidation. PS was found to retain a higher amount of β-carotene than PB. A plausible explanation was the indirect contact of boiling water on the PS whereas PB was directly immersed into the boiling water. Besides, a decrease in β-carotene in PB might also be possible due to the absorption of water during boiling which resulted in dilution of the compound, as reported by Podşedek (2007).

PD had 3.08±0.04 mg/100g of β-carotene,

Table 1. β-carotene content in pumpkin and butternut squash

Samples	β-carotene content (mg/100g)
Raw Pumpkin (PR)	4.34±0.04[a]
Boiled Pumpkin (PB)	3.57±0.05[b]
Steamed Pumpkin (PS)	3.82±0.04[c]
Deep-Fried Pumpkin (PD)	3.08±0.04[d]
Raw Butternut Squash (BR)	4.99±0.02[e]
Boiled Butternut Squash (BB)	10.23±0.05[f]
Steamed Butternut Squash (BS)	10.58±0.38[f]
Deep-Fried Butternut Squash (BD)	5.67±0.08[g]

*Values were expressed as mean ± standard deviation of three replicates (n=3) Means followed by different letters in the same column were significantly different ($p<0.05$)

29.0% lesser than β-carotene content in PR and was significantly ($p<0.001$) lower than PR. The least retained β-carotene content in PD was possibly due to the lipid soluble properties of the β-carotene compounds which were readily solubilised in oil during frying (Azizah *et al*., 2009). Leaching of β-carotene during soaking and degradation of β-carotene during frying might also occur (Tian *et al*., 2016).

Interestingly, cooked butternut squash showed an increase in β-carotene content which was in contrast with the findings for cooked pumpkin. β-carotene content in BS increased significantly ($p<0.05$) to 10.58±0.38mg/100g which equivalent to approximately 2.1 folds of β-carotene content in raw butternut squash (BR) while boiled butternut squash (BB) had β-carotene content of 10.23±0.05mg/100g, significantly ($p<0.001$) higher than β-carotene content in BR. Deep-fried butternut squash (BD) had 5.67±0.08mg/100g of β carotene which was 13.5% significantly ($p<0.05$) higher as compared to BR. However, the increase of β- carotene content in BD was lower as compared to steamed butternut squash (BS) and BB.

From the findings in this study, it showed that thermal processes decreased β-carotene content in the pumpkin but increased β-carotene content in butternut squash. Studies had shown that stability and content of carotenoids might be different even if the same process was applied to the sample (van den Berg *et al*., 2000; Chandrika *et al*., 2006; Reboul *et al*., 2006). Dietz *et al.* (1988) and Khachik *et al.* (1992) found an increase in the carotenoid content of cooked vegetables, probably due to an enhanced extractability of carotenoids from the vegetable matrix. This was also in agreement with Azizah *et al*. (2009) who found that boiling and stir-frying increased β-carotene content. Softening of plant tissues due to the swelling of the cell wall as a result of thermal processing could be one of the reasons (Parada and Aguilera, 2007). Thus, the compound can be extracted easier as compared to a compound in the non-disrupted cell wall. As the same treatments resulted in a varied trend (increasing and decreasing of β-carotene content) on pumpkin and butternut squash, further study on the microstructure of cooked pumpkin and butternut squash is warranted to understand the rationale behind such findings.

Bioaccessibility of β-carotene in pumpkin and butternut squash

Thermal processes generally increased bioaccessibility of β-carotene in both pumpkin and butternut squash with the percentages of bioaccessible β-carotene in butternut squash samples were lower than that in pumpkin samples (Table 2). Bioaccessibility of β-carotene in PD was significantly ($p<0.001$) higher than PR. However, there was no significant difference ($p\geq0.05$) between bioaccessibility of β-carotene in PR with PB and PS in this study. In BR, there was only a low percentage of β-carotene that was bioaccessible, 1.65±0.04%. The percentage of bioaccessible β-carotene in BD was significantly ($p<0.05$) higher than BR. There was also no significant difference ($p\geq0.05$) between BR with BB and BS in this study.

The lower percentage of bioaccessible β-carotene in PR and BR might be due to the hardened surface of samples that limited the disruption of the matrix for the release of compound (Tumuhimbise *et al*., 2009). It was well established that bioaccessibility of β-carotene in the raw sample was the lowest as compared to processed samples, as shown in some studies (Veda *et al.*, 2006; Tumuhimbise *et al*., 2009; Maria *et al.*, 2015). A study by Jeffery *et al*. (2012) concluded that the cell wall and chromoplast substructure which creates structural barriers for the release of carotenoids during digestion were

Table 2. Bioaccessibility of β-carotene in Pumpkin and Butternut Squash

Samples	Bioaccessibility (%)
Raw Pumpkin (PR)	10.56±0.44[a]
Boiled Pumpkin (PB)	11.42±0.63[a]
Steamed Pumpkin (PS)	12.76±0.80[a,d]
Deep-Fried Pumpkin (PD)	68.86±0.86[b]
Raw Butternut Squash (BR)	1.65±0.04[c]
Boiled Butternut Squash (BB)	2.03±0.15[c]
Steamed Butternut Squash (BS)	2.38±0.22[c]
Deep-Fried Butternut Squash (BD)	22.32±2.12[d]

*Values were expressed as mean ± standard deviation of three replicates (n=3). Means followed by different letters in the same column were significantly different ($p<0.05$)

important factors that could affect the bioaccessibility of β-carotene. The cell wall in the cooked sample was thinner as compared to raw sample as it was not destructed by any heat treatment (Tumuhimbise *et al*., 2009).

The highest bioaccessibility of β-carotene among pumpkin samples was from PD (68.86±0.86%) and the highest among butternut squash was from BD (22.32±2.12%). The addition of fat and heat to the sample had increased the percentage of bioaccessible β-carotene by approximately 6.5 folds and 1.1 folds as compared to bioaccessibility of β-carotene in PR and BR respectively.

Tumuhimbise *et al*. (2009) reported the highest bioaccessibility of β-carotene in sweet potatoes was prepared by the deep-fried method. The high bioaccessibility of β-carotene was not only increased through disruption of the matrix but also the incorporation of fat (Priyadarshani, 2015). As compared to other phytonutrients such as lutein and xanthophylls, highly lipophilic carotenes were found to be significantly affected by the addition of oil (Huo *et al.*, 2007; Nagao *et al*., 2013; Victoria-Campos *et al*., 2013). Fat gives a hydrophobic environment for the release of lipid soluble β-carotene and promotes micelles formation and absorption, as reported by Parada and Aguilera (2007). Palm olein oil was used in this study. It contains no β-carotene as reported by Ducreux *et al.* (2005), thus did not affect the β-carotene content in the samples. According to Malaysian Palm Oil Board (MPOB) (2011), palm olein oil majority is made up of oleic acid (C18:1) (monounsaturated fatty acid) and palmitic acid (C16:0) (saturated fatty acid). Monounsaturated fatty acid had been shown to significantly increase carotenoid bioaccessibility. Besides, long chain triglycerol (oleic acid) was more efficient in enhancing β-carotene bioaccessibility compared to medium chain triglycerol (C6 to C10) (Nagao *et al*., 2013). This can be explained by the micellar solubilisation capacity which was required to make carotenoids more bioaccessible. A longer chain of triglycerides can be hydrolysed easier resulting in swollen micelles which led to an increase in carotenoid bioaccessibility (Lemmens *et al*., 2014).

In this study, PB had 11.42±0.63% of bioaccessible β-carotene while 12.76±0.80% of bioaccessible β-carotene found in PS. However, bioaccessibility of β-carotene in PB and PS showed no significant differences as compared to PR. The similar trend was also shown in BB and BS with bioaccessibility of β-carotene in BB (2.034±0.15%) and BS (2.38±0.22%), both showing no significant differences as compared to BR.

Tydeman *et al.* (2010) and Victoria-Campos *et al.* (2013) also did not observe any significant increase in carotenoid micellarisation upon heat processing. Tydeman *et al.* (2010) explained that heating processing (boiling and steaming) separated the intact cells which still encapsulated the carotenoid during the digestion process instead of broke the cells, which is one of the influential factors for carotenoids bioaccessibility. Variations during samples processing such as of particle size reduction and duration of heat treatment might affect the bioaccessibility of β-carotene as well, making the results obtained in different studies varied greatly (Tydeman *et al*., 2010).

Comparison of β-carotene bioaccessibility in pumpkin and butternut squash

Although pumpkin and butternut squash are from the same family and genus, the bioaccessibility of β-carotene in both the samples with the same heat processing varied greatly (Table 2). Bioaccessibility

of β-carotene pumpkin ranged from 10.56±0.44% to 68.86±0.86% while there was only 2.03±0.15% to 22.32±2.12% of bioaccessible β-carotene in butternut squash. When comparing between each of the heating processes (boiled, steamed and deep-fried), the percentage of bioaccessible β-carotene in pumpkin were significantly higher ($p<0.05$) than butternut squash although the β-carotene content was generally higher in butternut squash than pumpkin.

Dietary fibre (cellulose and pectin), which made up the plant cell wall, was believed to be one of the factors influencing the bioaccessibility of β- carotene in this context (Tydeman *et al*., 2003; Waldron *et al*., 2003; Ellis *et al*., 2004). According to USDA Nutrient Database (2015a,b), the fibre content in pumpkin (0.5 g/100g) is lower as compared to butternut squash (2.0 g/100g). Pectin as a soluble fibre can bind or interact with fatty acids, cholesterol and bile acid thus affecting lipid absorption. Fatty acid could form complex with fibre and thus it could lose the ability to form micelles for micellarisation of lipid soluble compound (Phillips, 1986; Anderson *et al*., 2009; Gropper *et al.*, 2009). A diminished fibre content in the sample was found to have a correlation with an increased (as much as 2.6 folds) bioaccessibility of β-carotene, as demonstrated in a study by Aschoff *et al.* (2014).

Conclusion

In a conclusion, thermal processes only significantly increased the bioaccessibility of β-carotene in deep fried pumpkin and butternut squash. There were no significant differences between steamed and boiled pumpkin and butternut squash with their raw samples. It was suggested that incorporation of oil in deep-fried samples played a role in the increase of β-carotene bioaccessibility in pumpkin and butternut squash. Besides, bioaccessibility of β-carotene was generally higher in pumpkin than butternut squash. It is recommended that inclusion of the study on the microstructure of the pumpkin and butternut squash cell after heat treatment is essential to have a clearer picture of the effect of heat towards the plant cell wall which acted as the major barrier for the bioaccessibility of β-carotene.

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