Food Research 1 (5) : 170 - 175 (October 2017)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Retention levels of vegetable extractable beta-carotene preserved in virgin coconut oil and unadulterated honey

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Article history:
Received: 16 May 2017
Received in revised form: 28 June 2017
Accepted: 29 June 2017
Available Online: 29 June 2017

Keywords:
Amaranthus,
Beta-carotene preservation,
Honey,
Coconut oil,
Antioxidant activity

DOI:
http://doi.org/10.26656/fr.2017.5.051

Abstract

Dark green leafy vegetables such as *Amaranthus* spp. are known to be good sources of beta -carotene, a pro-vitamin A carotenoid with highly potent antioxidant property. As an antioxidant, beta-carotene scavenges for electron and thus terminates chain reactions, prevents recurrence and formation of unstable oxygen which otherwise initiate a chain reaction leading to such cases as cancer. While beta-carotene is supplied from consuming vegetables to provide vitamin A, it is also given as a supplement in cases of deficiency. However, it is highly degraded in the presence of light, heat, and oxygen posing a challenge to the methods of its preservation. Coconut oil and honey are popularly consumed in many countries facing vitamin A deficiency as good antioxidants and yet their role in preservation is largely unknown. The study reports the retention of beta-carotene extracted from *Amaranthus* spp. and separately preserved in virgin coconut oil (VCO) and unadulterated honey for up to six months. HPLC and DPPH assay were used to determine beta-carotene and antioxidant activity respectively. Virgin coconut oil and honey had significantly different ($p<0.001$) antioxidant activities of 65.12±0.70 and 81.51±1.39 % radical scavenging activity respectively that compared well with those of BHT and ascorbic acid. The concentration of beta-carotene preserved in VCO and unadulterated honey degraded by 90%, though the final retention provided higher than the recommended daily allowance of retinol (0.216±0.001 and 0.312±0.003 retinol activity equivalent respectively) when 100 mg is consumed. Virgin coconut oil and unadulterated honey can preserve beta-carotene if high amounts are used.

1. Introduction

Vitamin A deficiency is a major issue in many developing countries where animal food is consumed minimally. Consumption of dark green leafy vegetables such as *Amaranthus* that are rich in the pro-vitamin A carotenoid, beta-carotene reduces vitamin A deficiency (Nyambaka and Ryley, 2001; Abukutsa-Onyango, 2002; Makombo *et al.,* 2010; Nawiri *et al.,* 2013). In places where dark green leafy vegetables are hardly available such as arid and semi-arid region, supplementation with retinol or beta-carotene is advised. Apart from being a pro-vitamin A carotenoid, beta-carotene is an antioxidant that scavenges for electrons, terminating the chain reaction which could otherwise be the genesis of myriad non-communicable diseases including cancer (Jordi and Andreu, 2000). Supplementation with beta-carotene not only addresses vitamin A deficiency but also provides the body with the much-needed antioxidant that scavenges for free radicals responsible for non-infectious diseases. Beta-carotene can be extracted from dark green vegetables when readily available during the rainy season.

The characteristic conjugated double bond system in beta-carotene is prone to isomerism and oxidation process that causes instability towards light, oxygen, heat, acid and alkaline conditions, thus losing its antioxidant and pro-vitamin A properties (Jordi and Andreu, 2000). Some efforts have been reported for the preservation of beta-carotene and these include encapsulation in lipid-based matrices and storing under

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eISSN: 2550-2166

vacuum or in an inert medium such as nitrogen gas (Nyambaka and Ryley, 2001; Moraes *et al.,* 2013). Such methods are expensive and thus hindering availability of beta-carotene to the needy in society. More investigations on the preservation of beta-carotene extracted from the vegetable matrix are therefore required.

Honey is an antioxidant rich in phenolic acids, carotenoids, and flavonoids. It also contains other antioxidants such as glucose, oxidase, catalase, ascorbic acid, carotenoid derivatives, organic acids, amino acids and proteins (Ferreira *et al.,* 2009; Khalil *et al.,* 2010). The preservative property of honey has been demonstrated by its prevention of lipid oxidation in ground poultry (McKibben and Engeseth, 2002). African traditional societies used honey in the preservation of pre -cooked meat by submerging it in honey. On the other hand, coconut oil, an immune-enhancer, an antibiotic and a drug that regulates body functions and defense mechanism have antioxidants properties and a longer shelf life that is attributed to phenolic compounds (Fife, 2005; Marina *et al.,* 2008; Seneviratne and Dissanayake, 2008). Coconut oil has been reported to increase the shelf-life of poultry meat submerged in it through reducing moisture content and bacteria colony (Aritonang *et al*., 2009).

The two matrices, coconut oil, and honey were envisaged to be good preservatives for beta-carotene based on their underlying properties. This was investigated in order that a scientific contribution towards addressing the challenge posed by the degradation of beta-carotene can be realized. In addition, vegetables are perishable and therefore strategies to extract beta-carotene from them for preservation is a key aspect towards addressing nutritional related conditions/ diseases.

2. Materials and methods

2.1 Chemical and reagents

All chemicals and reagents used were analytical grade purchased through Kobian Kenya limited. Ascorbic acid, 2,2-Diphenyl-1picrylhydrazyl (DPPH) and beta-carotene standard (Type 1) and solvents acetonitrile, dichloromethane and methanol (HPLC grades) were obtained from Sigma Chemicals Company (St Louis, USA).

2.2 HPLC instrumentation

Acetonitrile:methanol:dichloromethane mobile phase in the ratio of 70:10:20 was made. Using isocratic elution, and at 0.8 cm^3/min flow rate, 1µL of the sample was injected into a reverse-phase HPLC. A Column of 250 × 4.6 mm internal diameter and 5 µm particle size operated at 25-30 kgF and its oven had a temperature of 40^oC. Deuterium lamp was used as the detector to obtain UV spectra at 450 nm wavelength. The mobile phase was allowed to run through HPLC instrument for 20 minutes before injecting the sample.

2.3 Preparation of preservation matrices

Virgin coconut oil (VCO), obtained from three coconut vendors in Kongowea market (Mombasa County) was prepared by a wet process to retain its antioxidant properties (Fife, 2005). Fresh coconuts were mechanically grated from the kernel and the milk squeezed out using a clean piece of cloth. Coconut milk obtained was then boiled above 100^oC for an hour to allow water to evaporate. Honey in honey-combs, obtained from a farmer in Eldama-Ravine (Koibatek County). The honey-combs were placed in a hot water bath which allowed the honey to be squeezed out. The extracted matrices were then stored in air-tight bottle before used as a preservative for extracted beta-carotene.

2.4 Determination of antioxidant properties in preservation matrices

The method employed was as per Ferreira *et al*. (2009) but with slight modification. A blank was prepared using 0.002g of DPPH dissolved in methanol in a volumetric flask and the volume adjusted to 100 cm^3. The absorbance of this solution was determined in triplicates using Jenway 6300UV-Visibleat 517 nm. The value obtained was used as the standard value for antioxidant properties.

Methanolic extract of VCO and unadulterated honey were prepared by separately placing 5 g of the matrices in 20 cm^3 methanol (HPLC grade) and concentrated using a rotatory evaporator at 40^oC. Exactly 2 cm^3 of the extract was placed in a test tube, 4 cm^3 DPPH added and the mixture kept for 30 minutes in the dark to allow decolorization. The absorbance of the matrices was then determined in triplicates by UV-visible at a wavelength of 417nm.The absorbances were used to calculate % radical scavenging activities (%RSA). Determination of %RSA of synthetic antioxidants (butylated hydroxytoluene (BHT) and ascorbic acid) was determined using their absorbances. The above procedure was repeated using 5g of each synthetic antioxidant and the absorbance measured.

eISSN: 2550-2166

%RSA was determined as shown in Equation 1.

$$\% \text{ RSA} = [(A_{DPPH} - A_s) / A_{DPPH})] \times 100 \quad (1)$$

Where A_{DPPH} is the absorption of the blank and A_s is the absorption of the sample (Eurachem, 1998).

2.5 Extraction of beta-carotene

Approximately 1500g of *Amaranthus* spp. was obtained from Githurai market, Nairobi County and transported to Kenyatta University Chemistry Department laboratories and thoroughly washed with distilled water. Extraction of the carotenoid was done as described by Rodriguez-Amaya and Kimura (2005) with slight modification. The vegetables were blanched in water boiling at 90°C for two minutes. The leaves were blended with celite powder into a smooth paste.

Exactly 15 g of homogenous paste was placed in a conical flask and 50 cm^3 of cold acetone was added in small portions during sonication. The mixture was sonicated for about 10 minutes, the extract filtered and extraction repeated until there was no more color on the residue. The extract was then partitioned with 25 cm^3 of petroleum ether in a separating funnel and 10 g of BHT added. The lower chlorophyll layer was allowed to drain and the petroleum ether layer washed with 0.1% potassium hydroxide in ethanol. The mixture was allowed to stand for about 15 minutes lower layer drained and then washed (up to 5 times) with distilled water to remove any impurities. The carotenoid collected was dried using anhydrous sodium sulphate and then concentrated using a rotatory evaporator at 30°C.

2.6 Storage of beta-carotene in preservative matrices

The carotenoid extract was preserved in VCO and honey in the ratio of 30 mg to 15 g of VCO and homogenized by stirring in a stream of nitrogen gas. Using a syringe, The mixture, in 10 g portions was stored in an inert atmosphere at room temperature in brown air-tight vials that had dry nitrogen blown. Beta-carotene levels were monitored on the first day, after two weeks and then monthly for six months using RP-HPLC.

2.7 Determination of beta-carotene

Stock solution of beta-carotene standard was prepared by dissolving 1 mg in 2 cm^3 of analytical grade methanol and further diluted to obtain a series of solutions for the calibration curve. Method validation was determined using recovery test where 1 mg of beta-carotene standard was spiked in 50 mg of *Amaranthus* spp. vegetables. The accuracy of extraction was then determined using equation 2.

$$\% \text{ Recovery} = \frac{C_F - C_U}{C_A} \times 100 \quad (2)$$

Where C_u is the concentration in the unspiked sample, C_A is the concentration of spike (added solution) and C_F is the concentration determined in the spiked sample.

Using 1 cm^3 of HPLC grade methanol, reconstitution 1 mg of the unadulterated honey and VCO were done separately. By injecting 1 mL these solutions, the presence of beta-carotene was determined in the matrices.

The difference between the concentration of beta-carotene in the matrices and that which was already in matrices gave the amount of beta-carotene that was preserved. This is indicated by equation 3.

$$BC = BC(f) - BC(m) \quad (3)$$

Where, BC beta-carotene in honey or VCO, BC(f) beta-carotene in the formulation and BC(m) beta-carotene in the matrix

Equation 4 was used to calculate the retinol activity equivalent (RAE) of beta-carotenein the ratio of 1:12 (1 RAE = 12 µg beta-carotene).

$$1 \text{ RAE} = x_s \times \frac{1}{13} \quad (4)$$

Where X_s = concentration of beta-carotene in the sample

2.8 Data analysis

One way ANOVA was used to measure significant differences between means of concentration levels of beta-carotene at 95% and means separated using Student Newman Keul Test.

3. Results and discussion

3.1 Antioxidant activities of virgin coconut oil and honey

Table 1. Mean (± SD) antioxidant activities and levels of beta-carotene in unadulterated honey and virgin coconut oil

Matrix	% RSA	Concentration (mg/100g) DM n=3
VCO	65.12±0.70	Below detectable limits
Honey	81.51±1.39	11.6± 0.07
BHT	77.47 ± 0.10	
Ascorbic Acid	70± 0.17	
p-value	<0.001	

*VCO = virgin coconut oil; *BHT= butylated 4-hydroxytoluene

The antioxidant activity expressed as percentage radical scavenging activity (% RSA) for VCO and unadulterated honey is as shown in Table 1. Honey and VCO exhibited antioxidant activities that were not significantly different ($p<0.001$). A comparison of their % RSA with the synthetic antioxidants indicated that the two matrices have sufficient antioxidants which can scavenge radicals; attributed to the presence of phenolic compounds (Marina *et al*, 2008). In Table 1, the levels of beta-carotene of VCO and unadulterated honey prior to preservation are given. The results indicated that beta-carotene levels were below detectable limits (1.084 mg/L) in VCO while unadulterated honey had 11.6± 0.07 mg/100g (1.16 mg/kg) beta-carotene. The level of beta-carotene in honey was much lower compared to what was reported by Ferreira *et al*. (2009) who gave 84.98-90.78 mg/ml of methanolic extract of honey. The current study can attribute the differences to post-harvest handling Ferreira *et al*. (2009).

3.2 Levels of beta-carotene with preservation

A sample chromatograms of beta-carotene preserved in VCO is given in Figure 1. The carotenoid layer being a natural product gave several peak areas as shown by the chromatogram. Peak area representing beta-carotene was the one with retention time 3.564.

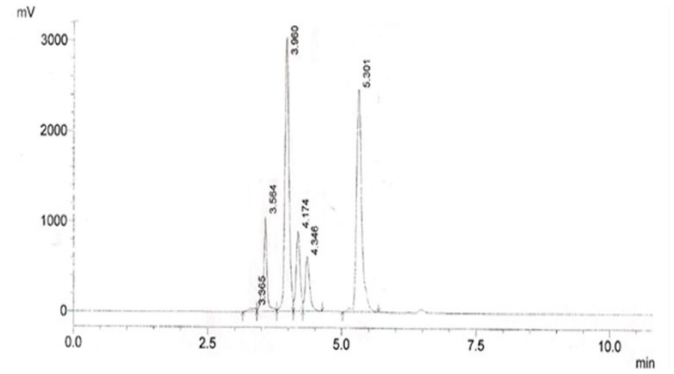

Figure 1. Chromatogram of beta-carotene preserved in VCO

Table 2. Mean levels of beta-carotene preserved in nitrogen, honey and coconut oil for 180 days

Days	Concentration mg/100g DM (Mean±SD), n=3		
	NBC	VCOBC	HBC
0	9.48±0.02[a]	30.33±0.08[a]	29.67±0.04[a]
14	9.67±0.17[a]	25.00±0.04[b]	28.76±0.12[b]
31	9.38±0.13[a]	17.00±0.02[c]	14.78±0.06[c]
60	9.41±0.01[a]	9.00±0.03[d]	8.74±0.03[d]
90	9.50±0.03[a]	9.00±0.01[e]	7.88±0.01[e]
120	9.48±0.02[a]	8.00±0.01[f]	6.31±0.01[f]
150	9.65±0.20[a]	3.00±0.02[g]	5.22±0.02[g]
180	9.45±0.08[a]	3.00±0.01[h]	5.10±0.02[h]
p-values	>0.05	<0.001	<0.001

*Mean values followed by different small letter in the same column are significantly different (α=0.05, One-way ANOVA, SNK-test)

VCOBC is beta-carotene in virgin coconut oil, HBC is beta-carotene in honey and NBC is beta-carotene in nitrogen

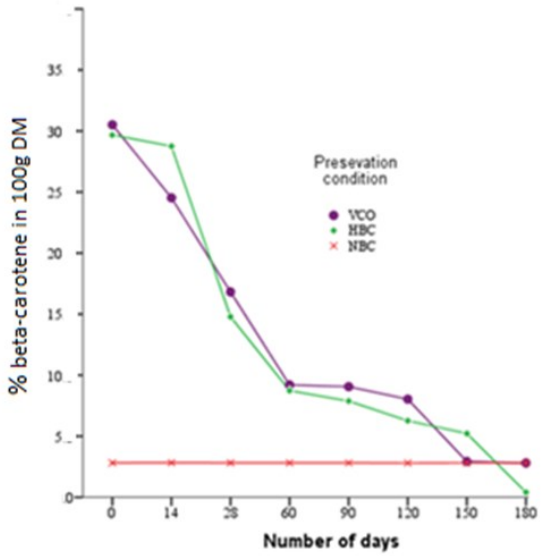

Figure 2. Beta-carotene degradation with storage

The level of beta-carotene from fresh vegetables was 59.97±0.04 mg/100g dry matter. Upon storage, the levels of beta-carotene reduced in both matrices. Table 2 shows the levels of beta-carotene preserved for 180 days while Figure 2 is the degradation pattern of beta-carotene in the two matrices with time. As shown in Table 2, the levels of beta-carotene in VCO significantly dropped ($p<0.5$) from 30.33±0.08 mg/100g to 3.00±0.01 mg/100g dry matter. A similar trend was seen in unadulterated honey where levels of beta-carotene dropped from 29.67±0.04 to 5.10±0.02 mg/100g dry matter ($p<0.5$). Rapid reduction within the first 3 months of storage by 21.33 mg (70.3%) in VCO, with the rest occurring 90-180 days (28.1%) (Figure 2). Similarly, 21.79 mg (73.4%) degraded in unadulterated honey within the first three months compared to 21.5% in the subsequent months. The reduced rate of degradation after three months may suggest the depletion of chemicals responsible for reacting with beta-carotene, suggesting that preserving higher amounts of beta-carotene in the matrices will result in lower degradation. The results imply 9.89% beta -carotene was preserved in VCO while 17.19% was retained in honey (Figure 2). The degradation pattern of the two matrices did not have a significant difference (P-value = 0.556) in their preservative properties. The presence of organic acids in the matrices of preservation and other compounds such as hydrogen peroxide contained in unadulterated honey can be attributed to the

degradation of beta-carotene. (McKibben and Engeseth, 2002; Bang *et al.,* 2003; Ferreira *et al*., 2009). Beta-carotene preserved in nitrogen (NBC), which was a control, did not show a significant difference in levels of preserved beta-carotene as an inert medium doesn't allow degradation (Nyambaka and Ryley, 2001; Moraes *et al*., 2013).

The amount of beta-carotene at each stage of storage was translated into available vitamin A as retinol activity equivalent (Table 3). The retinol activity equivalent of beta-carotene retained in VCO ranged from 2.347±0.006 to 0.216±0.001 and in unadulterated honey varied from 2.282±0.003 to 0.302±0.003. Daily requirements for vitamin A ranges from 400 µg for infants and 1300 µg for an adult (Canada Health, 2014) indicating that if one consumes 100 mg of beta-carotene in VCO or honey daily meets the vitamin A requirements (Canada Health, 2014; National Institute of Heath, 2013).

Table 3. Retinol activity equivalent from beta-carotene levels preserved in coconut oil and honey with storage

Days	Calculated RAE	
	VCOBC	HBC
0	2.347±0.006[a]	2.282±0.003[a]
14	1.887±0.001[b]	2.212±0.009[b]
31	1.294±0.001[c]	1.137±0.005[c]
60	0.708±0.003[d]	0.672±0.002[d]
90	0.697±0.002[e]	0.606±0.001[e]
120	0.618±0.002[f]	0.482±0.001[f]
150	0.226±0.001[g]	0.402±0.002[g]
180	0.216±0.001[h]	0.302±0.003[h]

VCOBC is Calculated retinol activity equivalent of beta-carotene in honey. HBC is calculated retinol activity of beta-carotene in honey

4. Conclusion

Virgin coconut oil and unadulterated honey have antioxidant properties that are sufficient. In the 180 days of preservation, the two matrices had preservative properties although degradation of beta-carotene occurred. By the end of preservation period degradation occurred, however, 10% of beta-carotene was preserved. Despite the degradation, the retained amount provides the required daily allowances for retinol. Virgin coconut oil and unadulterated honey can only retain high amounts when high amounts of beta-carotene are preserved.

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eISSN: 2550-2166