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DEGRADATIVE PRO-VITAMIN A ACTIVE COMPOUNDS OF ALL-*TRANS*-β-CAROTENE IN DEHYDRATED DARK GREEN LEAFY VEGETABLES

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ABSTRACT. Dark green leafy vegetables (DGLV) are rich source of pro-vitamin A carotenoids, with all-*trans*-β-carotene as the main compound contributing over 90% of the vitamin A content. The other pro-vitamin A carotenoids present in DGLV are the *cis* isomers of β-carotene; the 9-*cis* and the 13-*cis*, and α-carotene in some vegetables. The dehydration processes of freeze-, solar- and sun-drying resulted in all-*trans*-β-carotene undergoing isomerization and oxidation to produce *cis* isomers and monoepoxides of β-carotene, which are pro-vitamin A active, and some volatile compounds. The isomerization process results in the reduction of the relative proportions of all-*trans* isomer and an increase in the relative proportion of the *cis* isomers. Oxidation of all-*trans*-β-carotene induced the formation of vitamin A active epoxides; 5,6- and 5,8-monoepoxides of β-carotene as intermediate products that decompose to smaller volatile compounds. The epoxides were detected in low but sometimes in measurable amounts on some dehydrated and/or stored vegetable samples. The vitamin A active degradative compounds of all-*trans*-β-carotene were monitored using isocratic HPLC procedures. The factors influencing degradation of pro-vitamin A carotenoids during dehydration are discussed.

KEY WORDS: Degradative pro-vitamin A active compounds, Pro-vitamin A carotenoids, All-*trans*-β-carotene, Dark green leafy vegetables

INTRODUCTION

In most developing countries dietary vitamin A is derived from pro-vitamin A carotenoids in fruits and vegetables. In the African diet dark green leafy vegetables are the most variable vegetables as they contain carotene, vitamin C, protein, calcium and iron. However, the availability and consumption of these vegetables depend on the seasonal changes and this affects the nutritional status of the people. During rainy season fruits and vegetables are consumed fresh in large quantities but become scarce or not available during dry season. Because of their high moisture content these foods are highly perishable and can only be made available in times of need by preservation. Dehydration especially solar-drying is the best preservation method suited for developing countries where facilities for other methods are poorly established [1]. However, dehydration is accompanied by many changes, including chemical reaction and physical and structural changes which affect both nutritional and sensory qualities.

β-Carotene is the carotenoid that has the highest vitamin A content and occurs most abundantly in fruits and vegetables. It exists in nature in the more stable all-*trans* configuration, although some *cis* isomers exist naturally in some fruits and vegetables [2, 3]. During food processing procedures such as cooking, canning and drying, some of the *trans* carotenes are converted to either *cis* isomers with lower pro-vitamin A activity or oxidative products which are either pro-vitamin A inactive or have lower activity [2-7].

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Changes in pro-vitamin A compounds in foods during processing and storage follow a variety of pathways depending on reaction conditions. In the absence of oxygen, thermal treatment results in isomerization that give noticeable increase of up to 24% in the levels of *cis* isomers and a corresponding decrease in the *trans* isomers [7-8]. In the presence of oxygen various oxidative compounds are produced that are enhanced by the presence of light, heat, humidity, metal ions and enzymes through free radical reaction and produce epoxides, apocarotenals and other hydrocarbon derivatives of lower carbon chain [9]. Such processes result in high loss of pro-vitamin A activity in the affected foods. Some of the degradative compounds are vitamin A active but are never included when compiling food composition tables. Similarly, the need for vitamin A intervention strategies in developing countries through programs aimed at promoting preservation and distribution practices require that individual pro-vitamin A compounds be determined. In the tropical countries sun drying and in recent times solar dehydration are used in the preservation of vegetables. Sun drying is the oldest and easiest dehydration method that involves drying substances by open-air. However, many shortcomings such as contamination by dirt or rodents, infestation by insects, easy spoilage from exposure to weather elements and animals, and uncontrolled drying conditions leading to low quality products has enabled considerable interest be focused on solar dehydration. Solar dehydration, which uses solar energy for hot air dehydration and results in higher quality products, provides an appropriate preservation technique. This study therefore reports pro-vitamin A active compounds that are produced during sun and solar dehydration and storage of dark green leafy vegetables.

EXPERIMENTAL

Materials and reagents. Spring cabbage (*Brassica oleracea* L. var. *acephala* L.) and Italian spinach (*Spinacia oleracea* L.) were obtained from the local markets at Leeds, UK. Cowpea leaves (*Vigna unguiculata* L.) and African herb (*Gynandropsis gynandra*) were grown in a temperature-controlled greenhouse at Leeds, while in Kenya cowpea leaves were purchased from local markets in Nairobi. The β-carotene (type IV) and α-carotene (type V) standards were obtained from Sigma Chem. Co. (UK).

Acetonitrile, dichloromethane and water used for chromatography were HPLC grade (Fisons Scientific apparatus, Loughborough, and Rathburn Co., UK) and all other solvents and chemical reagents were of Analar grade (Vickers Laboratories, West York; BDH, or Fisions, UK).

HPLC methodology. The HPLC system consisted of high pressure pump (Series 300) from Applied Chromatography Systems (UK) connected to a 250 mm x 4.6 mm (id) stainless steel reversed phase column (Chromspher PAH for epoxides and Vydack TP-201 for isomers, each of 5 μm particle size) and guard column from Chrompack (Middelburg, The Netherlands). Samples were introduced into the column through a 10 μL Rheodyne injection valve (Model 7125) and the separated compounds monitored at 450 nm using 0.05 absorbence unit full-scale detector sensitivity by a variable wavelength detector (Varian UV-50). A Servogor 460 recorder using a chart speed of 12 cm/min recorded the chromatograms.

Separation was done at room temperature using a mobile phase consisting of acetonitrile, dichloromethane and water in the ratio 39:9:2 and pumped at the rate of 1.0 mL/min for the epoxides. The isomers were separated using methanol, dichloromethane and water in the ratio 79:15:6 for the mixture of α- and β-carotene isomers and in the ratio 80:15.2:4.8 for sample

extracts, and pumped at 0.8 and 1.0 mL/min, respectively. The mixtures were always freshly prepared and degassed by passing helium for 5 min before use.

Spectrophotometry. Ultraviolet-visible (UV-VIS) spectra were recorded in hexane solution for the epoxies and petroleum ether solution for isomers using a Pye Unicam spectrophotometer, model SP 8-100 (Cambridge, UK). The concentrations of standard solutions were confirmed using Cecil Digital Spectrophotometer (Cambridge, UK).

Preparation and extraction of vegetables. The preparation and extraction of fresh samples were made as described by Nyambaka and Ryley [10]. The samples were freeze-dried, simulated solar-dried, solar-dried and sun dried for the study. Samples for freeze-drying were blast frozen for one hour at –30 °C and then dried with a laboratory freeze drier (S B Freeze Driers Ltd., Folkedtone, UK) to a vacuum pressure of 50 mm Hg. Simulated solar dehydration was done in UK. Samples for simulated solar-drying were spread in wire mesh trays in loads of less than 2.0 kg m^{-2} and allowed to dry for 6-8 hours in a simulated solar dryer design constructed in the Procter Department of Food Science, University of Leeds.

An indirect solar dryer was used to solar dry the samples in Kenya following a similar process as that of simulated solar drying. Sun drying was also done using trays and loading density similar to those of solar dehydration whereby the trays were placed adjacent to the solar dryer and exposed to the sun's rays.

In the extraction of dry samples, 0.5 g of the finely ground material was used. 5 mL of distilled water was added to the sample, allowed to stand for 10 min and then extracted as given in the procedure by Nyambaka and Ryley [10]. The extracts were filtered through a membrane filter (0.44 μm pore size) before being injected into the HPLC column. All samples were extracted and analyzed in duplicate. Peak height measurement was used for calculations.

Epoxidation of β-carotene. Epoxide compounds of β-carotene were prepared following the method given by Marty and Berset [11]. 1.0 mL of *m*-chloroperoxybenzoic acid solution (100 μg/mL) in dichloromethane was carefully introduced through one neck of a twin necked round-bottomed flask containing 10 mL of β-carotene solution (100 μg/mL) in dichloromethane and immersed in ice. The other neck had a $CaCl_2$ guard column. The mixture was magnetically stirred for one hour and the resultant solution concentrated and separated on TLC plates.

The separation and collection of the β-carotene epoxides was achieved on alumina plates (aluminum oxide 60 F_{254} neutral, layer thickness, 0.2 mm) with 10% diethyl ether in hexane as solvent. Each colored band was recovered in dichloromethane, concentrated by evaporation under a flow of nitrogen and rechromatographed to obtain pure compounds.

Isomerisation of β-carotene. The preparation of β-carotene isomers and the TLC separation and purification of these isomers was carried out according to the procedure given by Nyambaka and Ryley [10].

RESULTS AND DISCUSSION

The major isomers of β-carotene found in blanched and dehydrated dark green leafy vegetables and the changes in relative proportions of the *cis* isomers relative to all-*trans* isomer are shown in Table 1. The relative proportions were obtained using chromatographic peak heights as percentage of the total peak height absorbance at 450 nm. The proportions of the *cis* isomers,

especially the 9-*cis* isomer increased on dehydration, with its extent depending on the type of dehydration used and the type of vegetable. The increase of the *cis* isomers was accompanied by a corresponding decrease in the *trans* isomer. The reduction in the proportion of all-*trans* isomers and the increase in the proportion of *cis* isomers suggest that *cis* isomers were formed during the drying process. Higher levels of *cis* isomers in simulated solar-, solar- and sun-dried products suggest that the processing conditions employed were more severe than in freeze-drying. Sweeney and Marsh [12] observed that no formation of stereoisomers of carotene in freeze-dried carrots while Chandler and Schwartz [7] reported the formation of *cis* isomers during the dehydration of sweet potatoes. Italian spinach and spring cabbage experienced the highest formation of *cis* isomers, suggesting that their leaf tissues were affected most by heat. Blanching causes wilting and damage to tissues of these plants, resulting in increased isomerization and oxidation in subsequent processing.

Table 1. Changes in the percentage proportions of the *cis* isomers of β-carotene relative to all-*trans* isomer in blanched and dehydrated dark green leafy vegetables.

Vegetables	% Proportion		% Difference from blanched samples	
	9-*cis*	13-*cis*	9- *cis*	13- *cis*
Kenyan samples				
Cowpea leaves - blanched	14.5	7.1	-	-
- solar-dried	17.5	9.5	3.0	2.4
- sun-dried	20.7	8.1	6.2	1.0
UK samples				
Cowpea leaves - blanched	8.4	6.5	-	-
- freeze-dried	13.3	6.8	4.9	0.3
- simulated solar-dried	14.5	6.9	6.1	0.4
Spring cabbage - blanched	11.9	5.2	-	-
- freeze-dried	15.4	5.3	3.5	0.1
- simulated solar-dried	21.5	7.2	9.6	2.0
African herb - blanched	14.1	6.9	-	-
- freeze-dried	20.2	7.5	6.1	0.6
- simulated solar-dried	20.8	8.6	6.7	1.7
Italian herb - blanched	14.1	5.8	-	-
- freeze-dried	19.3	7.9	5.2	2.1
- simulated solar-dried	21.9	7.8	7.8	2.0

The isomers were separated by a HPLC procedure and identified by their behavior in the UV-visible absorbance spectra and the elution order from the TLC plate [10]. Oxidation compounds were noted as new peaks in chromatographic profiles of the carotene isomers of some dehydrated and stored samples. Blanched and freeze-dried samples did not show any peak prior to storage while simulated solar-dried, solar- and sun-dried samples indicated some levels of the oxidized compounds prior to storage. This showed that freeze-drying conditions did not encourage oxidation of β-carotene. However, freeze-dried samples after storage had their profiles indicating the presence of oxidized compounds.

The oxidative compounds were separated on a TLC plate and the UV-visible spectra of the major oxidation products obtained are shown in Figure 1. The spectra had the maximum absorbance shifting to the lower wavelength (hypsochromatic shift), indicating that the compounds were the epoxides. Epoxidation of β-carotene at the cyclic double bonds results in loss of double bonds so that when one bond is lost in 5,6-mono- and di-epoxides, a

hypsochromatic shift of some 5 and 7 nm, respectively, is experienced. Loss of two double bonds in 5,8-mono- and di-epoxides results in the hypsochromatic shift of some 20-25 and 50 nm [13]. The spectral properties were noted in the spectra corresponding to various epoxides isolated from TLC plates. The separation and identification of these epoxides will be reported separately.

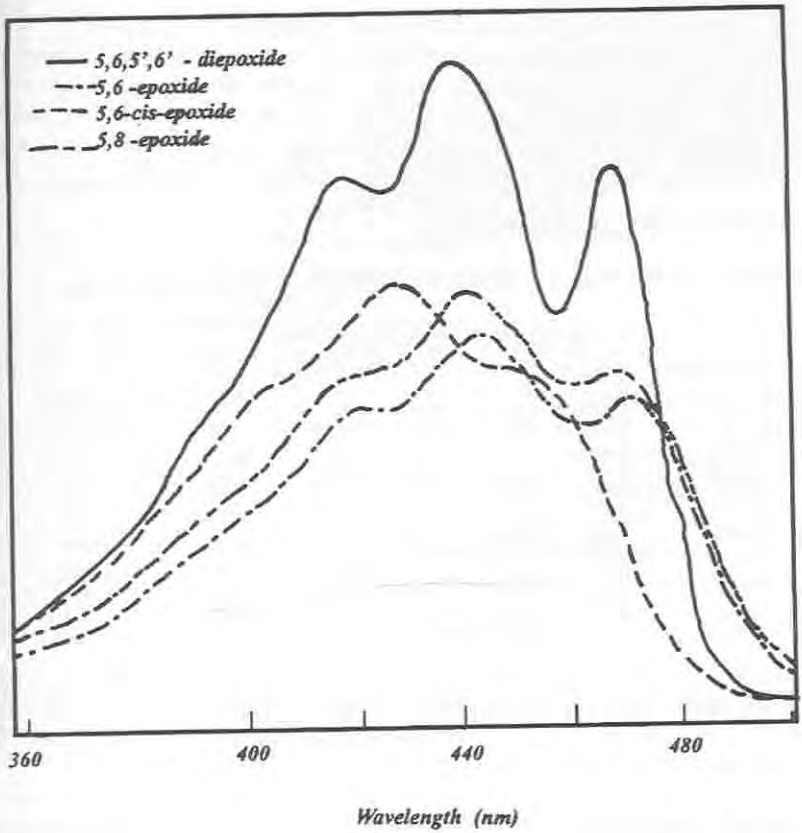

Figure 1. Overlay of absorption spectra of β-carotene epoxides separated by TLC.

The pro-vitamin A concentrations were determined by calculating the content isolated using the extinction coefficients of individual compounds (Table 2). The concentration of the pro-vitamin A compounds found in blanched and dehydrated dark green leafy vegetables and the vitamin A contents are shown in Table 3. The vitamin A content, as all-*trans*-β-carotene equivalent, were obtained from the content of individual compound and their percentage activity. Although all-*trans*-β-carotene equivalent contributes over 90% of the vitamin A content, the contribution of other pro-vitamin A compounds should not be underestimated. The levels of the epoxides however, did not show noticeable increase with storage, suggesting that they decomposed as soon as they were formed.

Table 2. Vitamin A activities (%) and absorptivities at maximum absorbance wavelengths of provitamin A compounds in blanched and dehydrated dark green leafy vegetables*.

Compound	Activity	$E^{1\%}_{1cm}$	Solvent	λ_{max} (nm)
All-*trans*-β-carotene	100	2592	P.E.	453
All-*trans*-α-carotene	53	2800	P.E.	444
9-*cis*-β-Carotene	38	2360	P.E.	449
13-*cis*-β-Carotene	53	1930	P.E.	444
β-Carotene 5,6-epoxide	21	2670	H	446
β-Carotene 5,8-epoxide	50	2520	H	427

*Source: [12-14]. P.E. = petroleum ether, H = hexane.

Table 3. Pro-vitamin A and the vitamin A content[1] of dehydrated and stored cowpea leaves.

Product	Pro-vitamin A content (μg/g DM)[2]						Vitamin A content (μg/g DM)
	All-*trans*-β-	9-*cis*-	13-*cis*	5,6-E	5,8-E	All-*trans*-α-	
F-dried 0	776	95	39	nd	nd	50	860
F-dried 16	148	16	7	7	18	12	173
S.s-dried 0	646	91	39	nd	5	52	731
S.s-dried 16	167	17	8	8	14	8	190
S-dried 0	599	78	30	nd	7	26	660
S-dried 5	252	27	16	7	12	10	293
Sun-dried 0	520	68	36	6	18	18	585
Sun-dried 5	250	30	20	7	16	7	286

F-dried = freeze-dried; S.s-dried = simulated solar-dried; S-dried = solar-dried. 0 = immediately after dehydration; 5 = 5 months storage in laminated bags at room temperature; 16 = 16 months storage in vacuum tins at 5 °C; nd = not detected; DM = dry matter. [1]Expressed as all *trans*-equivalent. [2]Mean of three samples from same batch.

The carotenoids are unstable to many processing procedures because of their conjugated system of double bonds. Drying and extrusion cooking are particularly destructive processing steps which experience high losses of the pro-vitamin A carotenoids [14]. The destructive processes, which result in lowered activity, are basically by oxidation and to some extent isomerization of all-*trans* isomers to the *cis* isomers.

The formation of *cis* isomers during dehydration was demonstrated by the increase in the proportions of the dominant *cis* isomers of β-carotene and the corresponding decrease of the all-*trans* isomers in the dehydrated products. The dehydration temperature and the low pH in dry products may have caused the observed increase in the *cis* isomers. The rate of formation of *cis* isomers from all-*trans* carotenoids is directly proportional to the intensity of light, particularly light at the wavelength of the main absorption bands, increase in temperature and the presence of catalysts such as acids and iodine [15].

Vegetables contain many organic acids including ascorbic acid, which make the pH of the vegetables range between 5 and 5.6 [16]. Drying the vegetables reduces the buffering action of cell fluid and results in low pH which causes many changes in the food product including isomerization of the pro-vitamin A. Isomerization is also increased in elevated temperatures, explaining why sun-, solar- and simulated solar-dried products had higher proportions of *cis* isomers. Various thermal processes of vegetables have been reported to cause isomerization of

carotenoids [7-8, 12]. Chandler and Schwartz [7] observed higher formation of *cis* isomers (28.7%) in dehydrated flakes of sweet potato relative to other processed products from canning, microwaving and baking, and the quantity formed was related to the severity and length of heat treatment.

The destruction of β-carotene in dehydrated products may be attributed largely to oxidative degradation through a free-radical process. The presence of trace metals such as iron and copper in plant foods promote this oxidation process [17]. During food processing cellular disruption increases exposure of carotenoids to trace metals that initiate and propagate free-radical oxidation [9]. Many fruits and vegetables contain small quantities of lipids, less than 1%, which are responsible for the development of off-flavour and the loss of fat-soluble vitamins and pigments through co-oxidation reactions [17]. The presence of metal ions and enzymes influences oxidation of β-carotene by promoting the production of free radicals, either directly or indirectly through the action β-carotene of lipids [9]. Green leafy vegetables contain high levels of trace metals that on processing are free to participate on various chemical reactions.

Studies on autoxidation conditions of β-carotene have postulated that the initial step in its breakdown occurs through the formation of epoxides [18-19]. The terminal 5-6 double bond possesses the highest electron density for easy attack and generates the formation of the 5,6-epoxide as the initial product which then decomposes to give the furanoid oxide (5,8-epoxide), diepoxides and carbonyl compounds in a sequence of consecutive reactions. The evidence for the high oxygen consumption supports this sequence of consecutive reactions in the degradation of β-carotene during oxidation [20-21].

Other studies have shown that both epoxides and carbonyl compounds are produced at the same proportional rates during oxidation process, suggesting that the oxygen attach occurs at multiple sites on the double bonds [22-23]. Autoxidation of β-carotene is a peroxyl radical mediated reaction in which the radical species formed from β-carotene combine with oxygen to form carotene peroxyl radicals that initiate attach on other β-carotene molecules in a chain reaction [23-25]. In this process the peroxyl radical is added to the polyene chain followed by a unimolecular decomposition of the carbon-centered radicals to give carbonyl compounds or epoxides and alkoxyl radicals [24].

The amounts of the main epoxides of β-carotene isolated from dehydrated and stored samples of dark green leafy vegetables were low, 5-18 μg/g dry matter. Since the proportions were not increasing in response to the degradation of β-carotene with storage the epoxides were intermediate compounds, which then decompose to smaller compounds (Table 3). The epoxides together with volatile carbonyl compounds have been identified in various oxidation processes including those involving heat treatment, photooxidation and spontaneous autoxidation of homogenous solutions of β-carotene, and in biological products submitted to drying [13, 18-20].

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