CHANGES IN ANTIOXIDANT CAPACITY AND COLOUR ASSOCIATED TO THE FORMATION OF β-CAROTENE EPOXIDES AND OXIDATIVE CLEAVAGE DERIVATIVES

Running title: Oxidation-derived chemical and colour changes in β-carotene

Poliana D. Gurak[1,3], Adriana Z. Mercadante[1], M.L. González-Miret[2], Francisco J. Heredia[2], Antonio J. Meléndez-Martínez[2*]

[1]Department of Food Science, Faculty of Food Engineering, University of Campinas, Postal code 13083-862, Campinas, Brazil.

[2]Laboratory of Food Colour and Quality, Department of Nutrition and Food Science, Faculty of Pharmacy, Universidad de Sevilla, 41012, Sevilla, Spain.

[3]Present address: Chemical Engineering Department, Federal University of Rio Grande do Sul, Postal code 90040-000, Porto Alegre, Brazil.

*Corresponding author: tel.: +34 954556495; fax: +34 954557017. e-mail address: ajmelendez@us.es (A. J. M-M)

Abstract

In this study, HPLC-DAD-MS/MS was applied for the identification of compounds derived from all-*trans*-β-carotene following epoxidation and oxidative cleavage. The consequences on both the CIELAB colour parameters and *in vitro* antioxidant capacity were also evaluated. Five apocarotenoids, three secocarotenoids, seven *cis* isomers and two epoxides were detected as a result of the oxidative cleavage. On the other hand, four epoxides and three *cis* isomers were detected as a consequence of the epoxidation reaction. Some compounds were detected for the first time as a result of oxidation reactions. Both reactions led to a marked decrease in the b^* and C^*_{ab} values, indicating that these paremeters can be used as a tool for the rapid assessment of β-carotene oxidation. Moreover, the oxidative cleavage of β-carotene resulted in increased capacity to both scavenge $ABTS^{\bullet+}$ and quench singlet oxygen. These results suggest that the study of the antioxidant capacity of these oxidative derivatives and their possible usefulness as food ingredients deserves further attention.

Keywords: antioxidant capacity; apocarotenoids; carotenoid epoxides; secocarotenoids; CIELAB; colour; epoxidation; oxidation; oxidative cleavage.

1. Introduction

Fruits and vegetables constitute the major sources of carotenoids in the human diet. More than 700 carotenoids have been identified so far in Nature of which approximately 50 are commonly present in human diets. However, only a few, including in some cases different geometrical isomers, are commonly found in human plasma (Khachik, Spangler & Smith, 1997; Meléndez-Martínez, Stinco, Liu & Wang, 2013). Among these carotenoids, β-carotene is widely distributed in foods and an important additive in foods, beverages, cosmetics and feeds. In addition to being the most efficient precursor of vitamin A, β-carotene is also a potential antioxidant that may have other biological functions (Lin, Chang, Yang, Chen, Wang, Chang, 2012).

The same physical-chemical characteristics that are responsible for the antioxidant properties and the intense colour of β-carotene make this pigment susceptible to chemical changes promoted by external agents, such as heat, light and oxidants, among others (Britton, 1995). These changes can have an impact on its in colour due to *cis/trans* isomerisation or formation of degradation compounds, such as epoxides, short chain products and, in some cases, volatile compounds (Mercadante, 2008). Several studies have reported the effects of processing, storage and heating on the stability of carotenoids, especially β-carotene. In this sense, some studies have facilitated the identification of minor oxidative products of carotenoids in foods (Rodriguez & Rodriguez-Amaya, 2007; Rodriguez & Rodriguez-Amaya, 2009).

The study of the oxidative metabolites of carotenoids is also important from a nutritional point of view as they may be biologically active (Lobo, Amengual, Palczewski, Babino & von Lintig, 2012; Sharoni et al., 2012; Mein, Lian & Wang, 2008). Typical examples are retinol, retinal and retinoic acid, which are formed upon the oxidative cleavage of provitamin A carotenoids. In fact, the intact carotenoids

absorbed into the body, can also be enzymatically converted into other oxidative derivatives, which may have diverse biological functions (Mein et al., 2008; Lobo et al., 2012). Likewise, oxidative metabolites of lycopene chemically obtained by cleavage with $KMnO_4$ or by synthesis are being studied as potential bioactive compounds (Caris-Veyrat, Schmid, Carail & Bohm, 2003; Reynaud, Aydemir, Rühl, Dangles & Caris-Veyrat, 2011).

As far as products derived from the oxidation of β-carotene are concerned, previous studies have reported that they could be effective inhibitors of breast tumour cell proliferation (Tibaduiza, Fleet, Russell & Krinsky, 2002). The cleavage products of β-carotene have also been postulated to induce oxidative *in vitro* stress under some circumstances (Augustin, Siems, Sommerburg, Langhans, Schild & Wiswedel, 2002), inhibit intracellular communication junctions (Yeh & Hu, 2003), and show cytotoxic (Hurst, Saini, Jin, Awasthi & van Kuijk, 2005; Kalariya, Ramana, Srivastava & van Kuijk, 2008) and genotoxic effects (Alija, Bresgen, Sommerburg, Langhans, Siems & Eckl, 2006; Kalariya, Ramana, Srivastava & van Kuijk, 2009).

The study of the oxidation of carotenoids is therefore important as it has an impact on the nutritional and sensory quality of food products. More specifically, the study of the formation of oxidative derivatives of carotenoids over time is interesting as their detection in products can provide valuable information for quality control purposes. For instance, the presence of some of these compounds can be used as markers to assess the extent of oxidation that the product has undergone. Besides, colour changes associated to oxidation can be used likewise, as the instrumental colour measurements offer a series of advantages that make it amenable for quality control purposes (Meléndez-Martínez, Ayala, Echávarri, Negueruela, Escudero-Gilete, González-Miret, Vicario & Heredia, 2011). In this regard, it is expected that

their oxidation affect also the antioxidant protection they could impart. However the information on the changes in the *in vitro* antioxidant activity associated to the oxidation of carotenoids is scarce despite the undeniable interest it could have for the industry.

In relation to these topics, the main objectives of this study were three: 1) to study the time-course formation of oxidative derivatives of β-carotene by epoxidation with MCPBA and oxidative cleavage with $KMnO_4$, 2) to assess the colour changes associated to the oxidations in terms of CIELAB colour parameters and 3) to evaluate the changes in the *in vitro* antioxidant capacity during the oxidative cleavage with $KMnO_4$ by measuring the capacity of the products to scavenge the $ABTS^{\bullet+}$ radical and to quench singlet oxygen ($^1O_2$).

2. Material and Methods

2.1. Materials

The standard of synthetic all-*trans*-β-carotene was acquired from Sigma Chemical Company (St. Louis, USA), 15-*cis*-β-carotene, 13-*cis*-β-carotene, 9-*cis*-β-carotene, β-apo-12'-carotenal, β-apo-10'-carotenal and β-apo-8'-carotenal were donated by DSM Nutritional Products (Basel, Switzerland). All of the standards showed at least 95 % purity as determined by HPLC-DAD and used as received. The *m*-chloroperbenzoic acid (MCPBA) (77 %), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), methylene blue (MB), and dimethylanthracene (DMA) reagents were purchased from Sigma–Aldrich, and the permanganate potassium ($KMnO_4$) was supplied by Merck (Darmstadt, Germany). The solvents and salts used were pro analysis grade and were purchased from

Labsynth (Diadema, Brazil). The solvents for HPLC were obtained from Merck or Mallinckrodt Baker (Philipsburg, USA). For chromatographic analysis, the samples and solvents were filtered using 0.22 and 0.45 μm membranes, respectively, from Millipore (Bedford, USA).

2.2. Reactions of β-carotene with potassium permanganate (oxidative cleavage) and m-chloroperbenzoic acid (epoxidation).

The oxidative cleavage was carried out according to the methodology described by Caris-Veyrat et al. (2003) and Rodriguez et al. (2007), with modifications. An aqueous solution of $KMnO_4$ was added to an ice-cold solution of β-carotene in dichloromethane, in a proportion of 2.6 mol equivalent $KMnO_4$ to 1 mol equivalent of β-carotene. The reaction mixture was stirred at room temperature and 1 mL aliquots of the organic phase were taken at 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50 and 60 min. Each aliquot was washed five times with distilled water. The organic layer, separated by centrifugation, was dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated to dryness under a stream of nitrogen.

The epoxidation methodology described by Rodriguez et al. (2007) was slightly modified. A saturated aqueous solution of sodium bicarbonate ($NaHCO_3$) was added to an ice-cold solution of β-carotene in dichloromethane (1 mol equivalent). To the resulting two-layered mixture, a solution of MCPBA (1.5 mol equivalent) in dichloromethane was added and the reaction mixture was immediately stirred for 60 min. One-mL of organic phase from the reaction mixture were taken at 0, 5, 7, 10, 15, 20, 30, 40, 50 and 60 min. The organic layer was separated from the reaction mixture by successive washing with 20 % sodium thiosulfate ($Na_2S_2O_3$), saturated aqueous

143 $NaHCO_3$ and water. The organic layer, separated by centrifugation, was dried over
144 anhydrous sodium sulphate (Na_2SO_4) and concentrated to dryness under nitrogen
145 stream.

146 In both oxidation reactions aliquots were taken at short intervals in order to
147 maximize the number of intermediate compounds formed, which is interesting to
148 obtain more information on the mechanism of the reactions.

149

150 *2.3. Analysis of derived-β-carotene compounds by HPLC-DAD-MS/MS*

151 The analysis of carotenoids was performed with a Shimadzu HPLC (Kyoto,
152 Japan) connected in series to a diode array detector (DAD) (Shimadzu, model SPD-
153 M20A) and a mass spectrometer (MS) with an ion-trap analyser and atmospheric
154 pressure chemical ionisation (APCI) source from Bruker Daltonics (model Esquire
155 4000, Bremen, Germany). The UV/Vis spectra were obtained between 250 and 600
156 nm, and the chromatograms were processed at 450 nm. The MS parameters were
157 set as previously reported (De Rosso & Mercadante, 2007). The carotenoids were
158 separated on a C_{30} YMC column (5 µm, 250 mm x 4.6 mm i.d.; Waters, Wilmington,
159 USA) using a mobile phase with a linear gradient of MeOH/MTBE from 95:5 to 70:30
160 in 30 min followed by a linear gradient to 50:50 in 20 min at 0.9 mL/min and column
161 temperature set at 32 °C (Zepka & Mercadante, 2009a). When quantitation was
162 carried out, 0.1% triethylamine (TEA) was added to the mobile phase to enhance
163 carotenoid recovery (Emenhiser, Simunovic, Sander, & Schwartz, 1996). On the
164 other hand, TEA was excluded from the mobile phase when the MS detector was
165 used because it is easily ionized in the APCI source, and as a result the carotenoid
166 ion signals decrease (De Rosso et al., 2007).

The carotenoids were quantified using an external calibration curve of all-*trans*-β-carotene constructed with seven concentration levels (1 – 100 μg/mL), chosen to span those of all the isomers of β-carotene in the injected samples. The β-carotene-derived compounds were quantified using the all-*trans*-β-carotene and identified by considering the following parameters: elution order on the C_{30} column, UV/Vis spectrum features (maximum absorption wavelength, (λ_{max}), spectral fine structure (% III/II) and *cis* peak intensity (% A_B/A_{II})), and MS spectrum characteristics (protonated molecule and MS/MS fragments). These parameters were compared to those of the available standards analysed under the same conditions and to data reported in the literature (De Rosso et al., 2007; van Breemen, Dong, & Pajkovic, 2012).

2.4. Measurement of colour

Colour was assessed on an HP8452 UV/Vis diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA). A 10 nm path length glass cuvette was used for the measurements, and the whole visible spectrum (380 - 770 nm) was registered ($\Delta\lambda$ = 2 nm). The colour parameters of the uniform colour space CIELAB (CIE, 1978) under CIE Illuminant D_{65} and 1964 Standard Colourimetric Observer were obtained by means of the CromaLab® software (Heredia, Álvarez, González-Miret & Ramírez, 2004).

Due to influence of the solvent on carotenoid spectra, all the samples were dissolved in petroleum ether for the colour measurements. To minimize the influence of concentration on the colour coordinates, all samples were diluted considering the same dilution factor so that the absorbance readings were within the range 0.1-1.0.

The CIELAB space includes an index of lightness (L^*) and two colour coordinates (a^* and b^*). L^* is related to the luminosity, a property according to which each colour can be considered as equivalent to a member of the grey scale between black ($L^* = 0$) and white ($L^* = 100$). The parameter a^* has negative values for greenish colours and positive values for reddish ones, whilst b^* has positive values for yellowish colours and negative values for bluish colours. The total colour difference (ΔE^*), which is important for evaluating the relationship between the visual and numerical analyses, was calculated as the Euclidean distance between two points in the three-dimensional space defined by L^*, a^* and b^* (equation 1). Chroma (C^*_{ab}), calculated according to equation 2, is considered the quantitative attribute of colourfulness, and hue (h_{ab}) is used as a qualitative attribute of colourfulness (equation 3). In other words, chroma gives information about how vivid a colour is, and hue is the attribute according to which colours are traditionally considered as reddish, orange, and yellowish (Heredia et al., 2004).

$$\Delta E^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{1/2} \quad \text{(equation 1)}$$

$$C^*_{ab} = \left[(a^*)^2 + (b^*)^2\right]^{1/2} \quad \text{(equation 2)}$$

$$h_{ab} = 180 + \arctan(b^*/a^*) \quad \text{when a* < 0} \quad \text{(equation 3)}$$

2.5. Antioxidant capacity

To evaluate the capacity of β-carotene and its oxidative derivatives to scavenge the $ABTS^{\bullet+}$, the method described by Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans (1999) and adapted and validated to microplates in the Brazilian laboratory was used. The ABTS radical cation (7 mM solution) was formed by chemical reaction with potassium persulphate (2.45 mM). To a 96-well microplate,

270 μL of the $ABTS^{\bullet+}$ solution and 30 μL of the β-carotene or chemical derived-compounds or trolox solutions in ethyl acetate/ethanol (1:1) were added.

The absorbance was measured at 750 nm each 10 s, during 15 min, in a microplate reader (Synergy Mx, BioTek, Vermont, USA), equipped with a thermostat set at at 30 °C and dual reagent dispenser. The results were obtained by relating the percentage of inhibition to the concentration of the samples (1-80 μM quantified as all-*trans*-β-carotene) or to the trolox concentrations (2.5-20 μM). According to Re et al. (1999), the free radical scavenging capacity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC), calculated as slope carotenoid curve/slope trolox curve.

The percentage of protection against $^1O_2$ was evaluated according to the method described by Montenegro, Rios, Mercadante, Nazareno & Borsarelli (2004), with modifications. The reaction was carried out at 25±1 ˚C using 50 μL of DMA in ethyl acetate as the actinometer, 1420 μL of MB in ethanol as the sensitizer and 50 μL of four solutions of β-carotene or chemical derived-compounds (1-80 μM quantified as all-*trans*-β-carotene) in ethyl acetate/ethanol (1:1) with moderate agitation under an air atmosphere. The blank was done replacing the carotenoid solution by 50 μL of ethanol/ethyl acetate (1:1). The excitation source was a 150 W filament lamp coupled with red and orange cut-off filters to avoid direct excitation of the carotenoids. The excitation light (> 570 nm) was focused into the cell providing the excitation of the sensitiser (MB) and generating singlet oxygen, which reacted with DMA. The intensity decay of the absorbance of the DMA (measured at 375 nm) was monitored at intervals of 30 s during 5 min using a diode array UV/Vis spectrophotometer (Agilent). The kinetics data obtained from the intensity decay of the absorbance of

DMA were fitted to a first-order reaction to calculate the rate constants. For this purpose the Origin Pro 8 software (OriginLab Corporation, Northampton, MA, USA) was used The percentage of protection that β-carotene and its chemical derived-compounds offered to the actinometer (DMA) was calculated with Equation 4.

$$\text{protection}(\%) = \frac{k_{obs}^{DMA} - k_{obs}^{DMA+EXT}}{k_{obs}^{DMA}} x100 \qquad \text{(equation 4)}$$

where k_{obs}^{DMA} is the observed first-order rate constant fitted to the DMA decay curve (obtained in the blank experiment); and $k_{obs}^{DMA+EXT}$ is the observed first-order rate constant fitted to the DMA decay curve in the presence of β-carotene and its chemical derived-compounds.

All measurements were performed in duplicate.

3. Results and Discussion

3.1. Compounds formed upon the chemical epoxidation and oxidative cleavage of β-carotene

Carotenoid degradation pathways are highly influenced by the agents and conditions involved. The degradation may occur by autooxidation or be catalysed by other chemical species or conditions to form a plethora of compounds, such as *cis* isomers, epoxides, apocarotenoids, seco-carotenoids, volatiles and polymers (Caris-Veyrat et al., 2003; Zepka et al., 2009a; Knockaert, Pulissery, Lemmens, Buggenhout, Hendrickx, & Van Loey, 2013).

The $KMnO_4$ can react with carbon-carbon double bonds by different mechanisms. Thence seventeen compounds, along with β-carotene, were detected via the chemical reaction induced by $KMnO_4$ (**Table 1)**.

The reaction mechanism for the oxidative cleavage of β-carotene is thought to involve the isomerization of a *trans*- to *cis*- double bond so that *syn*-addition of the permanganate ion could take place to form the well-established cyclic permanganate ester at different sites. This gives rise to the expected series of β-apo-carotenals with 6 to 10 conjugated double bonds, due to oxidative cleavage at the double bonds of the polyene backbone. In addition, oxidative cleavage at the double bond in the β-ring formed semi-β-carotenone and at C-5,C-5' gave rise to β-carotenone. Further oxidation also occurred to form apo-8'-semi-β-carotenone.

To the best of our knowledge the formation of β-carotenone, apo-8'-semi-β-carotenone and di-*cis*-isomers of β-carotene (probably 9,15-di-*cis*-β-carotene, 9,13'-di-*cis*-β-carotene or 13,15-di-*cis*-β-carotene) was not previously reported during the chemical reaction with $KMnO_4$.

The mechanism of the reaction with MCPBA is accomplished by electrophilic attack on the C=C bonds. Due to the presence of even traces of acids, 5,6-epoxides can undergo rearrangements to form the corresponding furanoid (5,8-epoxide) isomer. Moreover, the protons present in the medium electrophilic attack the oxygen of the epoxide group and the double bond between C-7 and C-8 undergoes resonance, moving to C-6 and C-7 (Eugster, 1995). From the chemical reaction induced by MCPBA, at least 7 derived-β-carotene-compounds were formed (**Table 1**). A series of mono- and di-epoxides of β-carotene were formed; however, the reaction time and/or conditions were not sufficient to favour the formation of 5,8:5',8'-diepoxy-β-carotene. To the best of our knowledge 9-*cis*-5,8-epoxy-β-carotene and 9-*cis*-β-carotene were detected for the first time in the reaction with MCPBA.

Most of the compounds identified in the present study as a result of the chemical oxidations were already detected in processed foods or model systems mimicking the processes in the industry, such as heating of simulated fruit juices (Zepka et al., 2009a), heating of solid β-carotene (Qiu, Chen & Li, 2009), and oxidation by ozone (Benevides, Veloso, Pereira & Andrade, 2011). The structure of the carotenoids formed as a result of the chemical reactions induced by $KMnO_4$ and MCPBA and their proposed sequence of formation are shown in **Supplementary Fig. S1.**

3.2. Quantitative changes over time

The β-carotene standard used in both chemical reactions consisted of 99 % of the all-*trans* isomer and 1% of *cis* isomers at time zero. **Fig. 1** shows the evolution of the peak area observed for β-carotene degradation and formation of derivatives during the chemical reaction with $KMnO_4$. During the first 10 min of oxidative cleavage, the concentration of all-*trans*-β-carotene decreased 70 %, with the concomitant formation of *cis* isomers of β-carotene (15-*cis*, 13-*cis* and 9-*cis*), secocarotenoids, apocarotenoids and small amounts of epoxides. At 20 min of reaction, all-*trans*-β-carotene and its *cis* isomers were completely consumed (**Fig. 1A** and **Fig. 1B**). At this time the highest amounts of semi-β-carotenone (**Fig. 1C**) and apocarotenoids (**Fig. 1D.**) (primarily β-apo-8'-carotenal, followed by β-apo-10'-carotenal and β-apo-12'-carotenal) were detected. In the course of the reaction, the highest amount of β-carotenone was detected at 30 min. Due to further oxidation, the highest concentrations of apo-8'-semi-β-carotenone, β-apo-14'-carotenal and β-apo-15-carotenal, the final oxidation products, were noticed at 60 min.

Taken together, these data seem to indicate that the isomerisation of all-*trans*-β-carotene into *cis* isomers is an important step for the oxidative break of carbon-carbon double bonds, at least to give primary oxidative metabolites like semi-β-carotenone and β-apo-8'-carotenal, that can be considered intermediates for the formation of β-carotenone and shorter apocarotenoids. The fact that a *cis* isomer of the latter has been detected is also noteworthy in this respect. This observation can be important and deserve further study since, interestingly, it has been reported that some members of the carotenoid cleavage oxygenases (CCOs) family (which catalyse the cleavage of carotenoids at specific positions to produce biologically relevant apocarotenoids) may have preference for *cis* isomers as substrates (Alder et al., 2012, Hu, Liu, Ernst, Krinsky, Russell & Wang, 2006). In other words, evidence is accumulating that the formation of some oxidative cleavage products of carotenoids occurs via the formation of *cis* isomers.

The evolution of the levels of β-apo-8'-carotenal and semi-β-carotenone were very similar. Between 20 min to 30 min their peak areas decreased 60 % and 80 %, respectively. On the other hand, the amounts of apo-carotenals with shorter chains, β-apo-14'-carotenal and β-apo-15-carotenal, continuously increased until the end of reaction.

In the reaction with MCPBA, β-carotene was not entirely consumed until 60 min. After 10 min, the electrophilic attack of MCPBA caused 80 % loss of all-*trans*-β-carotene with a slight increased amount of 13-*cis* and decreased of 9-*cis*-β-carotene (**Fig. 2A**). Unlike the reaction with $KMnO_4$, the presence of *cis* isomers of β-carotene was noted until 20 minutes of reaction (**Fig. 2B**). The highest amounts of epoxides were observed at 10 minutes and then they continuously decreased up to 60 min (**Fig. 2C** and **2D)**.

334 The epoxidation at positions 5,6 or 5',6' was favoured by the presence of a β-
335 ionone ring where the terminal double bonds have a higher electron density than the
336 polyenic chain and, consequently, favour the attack by MCPBA (Rodriguez et al.,
337 2007). Therefore, from 20 minutes onwards little changes were observed in the levels
338 of both mono- and diepoxides of β-carotene. In the present study, 5,6-epoxy-β-
339 carotene was found in the largest amount probably due to the fact that the MCPBA
340 was added all at once in the present study and not dropwise as in the work of
341 Rodriguez et al. (2007), where 5,6:5',6'-diepoxy-β-carotene as the major epoxide.
342 The addition at once may have caused saturation of the medium. In addition, the fact
343 that only minor amounts of 5,8-furanoid derivatives were detected (that is, that the
344 5,6-epoxides were not totally converted into their 5,8-furanoid counterparts by the
345 presence of acid) could be due to the addition of $NaHCO_3$ to the reaction medium,
346 which could have neutralised the acid released by MCPBA.

347 The amounts of all-*trans*-β-carotene lost in both chemical-catalyzed reactions
348 were not compensated by those of the new compounds formed. Therefore, only a
349 fraction of the derived-β-carotene-compounds was detected by HPLC-DAD-MS/MS.
350 This fact also occurred when degradation of carotenoids was catalyzed by heat
351 (Zepka et al., 2009a), light (Pesek & Warthesen, 1990), atmospheric oxygen
352 catalyzed by metalloporphyrin (Caris-Veyrat et al., 2003), among other factors. This
353 could be attributed to the generation of low molecular weight compounds that were
354 not detected by the HPLC-DAD-MS/MS system, although the formation of high
355 molecular weight compounds has also been reported (Qiu et al., 2009).

356

357 *3.3. Associated colour changes*

The influence of the chemical structure of carotenoids on their visible absorption spectra characteristics is well established but little is still known concerning its relationship with objective colour coordinates. All the samples were located on the 2nd quadrant of the CIELAB *a***b** plane (values of *a** ranging from -10.2 to -1.7 and values of *b** ranging from 83.2 to 10.1, **Supplementary 2 and 3),** i.e., all samples were classified as yellowish, regardless of the reaction time. Meléndez-Martínez, Britton, Vicario & Heredia (2007) also classified an acetone solution of β-carotene as yellowish (*a** = -3.1 and *b** = 44.9 CIELAB units). However, it is important to highlight that the colour of carotenoids does not only depend on their chemical structure, but also on their concentration and interaction with other molecules, among other factors. Thus, β-carotene crystals are orange, whilst solutions with absorbances at 450 nm within the 0-1 range appear yellowish.

The *b** values showed the greatest decrease (about 2.5-fold in the case of the cleavage and 8.2-fold in the case of the epoxidation) as a consequence of both chemical reactions. The *a** coordinate (greeness-redness) showed a shift towards positive values, although the changes were not as large numerically (**Fig. 3**). The changes in C^*_{ab} values were very similar to those described for *b**, indicating that β-carotene oxidation led to a decrease in colour vividness, which was more pronounced in the case of the epoxidation reaction. In both reactions, the values of *L** increased slightly, indicating that the samples appeared slightly brighter as both reactions progressed. On the other hand, it was observed that the oxidative cleavage led to small changes in hue (< 2°), in contrast with the changes noticed in the epoxidation reaction (≈ 15°). In the latter case, a more pronounced shift away from orange hues and towards yellowish hues was observed. The pronounced colour

change that was observed immediately after the addition of the reagent in the epoxidation reaction is noteworthy (**Supl. S3**).

As the reaction with $KMnO_4$ progressed a hypochromic effect took place. Between 0 and 5 min the λ_{max} remained at 450 nm (due to the presence of all-*trans*-β-carotene as the predominant compound) and there was a clear decrease in absorbance, which continued during the rest of the reaction. After 30 min of reaction, all-*trans*-β-carotene was completely consumed, and there was a loss of fine structure relative to the spectrum at t = 0 min, including the disappearance of the *cis* peak and the appearance of bands at approximately 460 and 470 nm. These were associated to the formation of oxidation compounds, such as β-apo-8'-carotenal, semi-β-carotenone, β-carotenone and apo-8'-β-carotenone, which absorb maximally at longer λ than all-*trans*-β-carotene.

As a result of the reaction between β-carotene and MCPBA, hypochromic and hypsocromic effects were observed. In addition, an increase in the fine structure in the UV/Vis spectrum was observed as the reaction progressed (**Fig. 4b**). Although the reaction medium was a mixture of carotenoids, the fine structure of the spectra increased between 20 and 60 min due to the increased amounts of epoxides and total disappearance of all-*trans*-β-carotene.

The colour differences (ΔE^*) were greater than 3 at 3 min reaction with $KMnO_4$ ($\Delta E^* = 3.5$) and just after the addition of the oxidant reagent for the reaction with MCPBA ($\Delta E^* = 18.3$) (**Supplementary S2**). From an industrial point of view, the ranges of colour differences 1.1–2.8 and 2.8–5.6 CIELAB units correspond to rigorous and normal tolerances, respectively, whereas colour differences over 5.6

CIELAB units ought to be easily distinguished (Lozano, 1978; Melgosa, Pérez, Yebra, Huertas & Hita, 2001).

At the end (60 min) of the reaction with MCPBA, the value of ΔE^* was 73.4, whilst for oxidation with $KMnO_4$, ΔE^* was 48.1. (**Supplementary S2**). These results indicated that the epoxidation of β-carotene led to a much higher (ca. 1.5-fold) colour difference than its oxidative cleavage.

Altogether, it can be concluded that as a result of both reactions, most of the compounds formed had shorter chromophores and, therefore, shorter λ_{max} as compared to those of β-carotene (450 nm). As a result of these chemical changes a clear hypochromic effect was observed in the UV/Vis absorption spectra as well as a noticeable hypsochromic shift in the case of the oxidation with MCPBA. In terms of colour, at the end of the reactions the samples appeared both less yellow (above all in the case of the epoxidation) and vivid, as indicated by the marked decreases in b^* and C^*_{ab}, respectively. Indeed, visually noticeable colour differences in the samples were observed very early in the oxidation reactions. Therefore, b^* and C^*_{ab} are promising colour parameters for the rapid assessment of the formation of oxidative derivatives. More importantly, because C^*_{ab} is the quantitative attribute of colourfulness, visual changes in colour vividness can be used to monitor the degradation of β-carotene. During the measurements of colour, different dilutions were performed. The variations of chroma (C^*_{ab}) were similar between the different dilutions. In another study with thermal degradation, the colour parameters also proved to be good predictors of carotenoid contents (all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene) in the model system of cashew apple juice heated to 60 and 90 °C (Zepka, Borsarelli, Silva & Mercadante, 2009b).

3.4. Influence of the oxidative cleavage on the antioxidant capacity of β-carotene

The capacity to scavenge the $ABTS^{\bullet+}$ radical was evaluated at different times of the oxidative cleavage reaction with $KMnO_4$ (0, 10, 20, 30 and 60 min) and at least five concentrations were tested at each time point. The mean TEAC values, calculated as slope carotenoid/slope Trolox curves, and total carotenoid contents (estimated by the sum of peak areas detected in the HPLC-DAD) found at different reaction time are shown in **Fig. 5**. At zero time, all-*trans*-β-carotene had an average TEAC of 2.3, which agreed well with the value reported by Re et al. (1999). After 10 min of oxidative cleavage, β-carotene was still the major product (60.7 % of all-*trans* and *cis* isomers) and the mixture showed similar TEAC value (2.4) to that at zero time. From this time on, as the reaction time increased, the TEAC values also increased, reaching over 3 times higher value at 60 min when compared to time zero. This increased capacity to scavenge $ABTS^{\bullet+}$ can be attributed to the disappearance of β-carotene and appearance of oxidative cleavage compounds with c.d.b. systems of different length, all of them containing at least one carbonyl group. Another important consideration is that these analyses were conducted on the reaction mixture immediately after the oxidative cleavage, and the products were not purified due to their low stability. In other words, at time zero $ABTS^{\bullet+}$ could only react with one antioxidant (β-carotene), whilst later on several potential antioxidant compounds could have reacted with this radical and be involved in the observed increased antioxidant activity. In addition, the possibility of the existence of synergistic effects cannot be ruled out.

Since the antioxidant capacity of these mixtures of carotenoids has not yet been reported in the literature, the results obtained were compared to a series of

carotenoids analysed separately. Mueller & Boehm (2011) reported that oxidative derivative compounds of β-carotene, such as β-apo-8'-carotenal, show lower TEAC values than that of β-carotene. On the other hand, Rodrigues, Mariutti, Faria & Mercadante (2012) studying the antioxidant capacities of gum arabic and maltodextrin microcapsules containing carotenoids (β-carotene, β-apo-8'-carotenal and β-apo-12'-carotenal) against reactive oxygen and nitrogen species, observed that β-apo-8'-carotenal led to the highest increase in the scavenging capacity when incorporated into both microcapsules. It was hypothesized that the carbonyl group (CHO) in β-apo-8'-carotenal probably allows this carotenoid to hold strategic positions in the microcapsules facilitating the interaction with the ROS and RNS, and that, in addition, its conjugated double bonds system, simultaneously facilitates electron donation.

The mixtures of oxidative cleavage compounds were also evaluated for their capacity to protect against $^1O_2$ because carotenoids are widely known as efficient physical quenching quencher of this reactive species. Four different concentrations of carotenoids from each reaction time were used to calculate the IC_{50}, which was based on the concentration of the antioxidant, in μg/mL, required to obtain 50 % DMA protection. The results showed IC_{50} values ranging from 1.84 to 4.49 μg/mL (**Fig. 5**), with decreased IC_{50} values as the reaction time increased. Thus, the antioxidant capacity against $^1O_2$ of the mixture formed by the chemical reaction with $KMnO_4$ increased with increased reaction time, as also verified for the capacity to scavenge $ABTS^{\bullet+}$.

In summary, the changes in the antioxidant capacity against a radical and a non-radical species as a result of the oxidative cleavage of β-carotene had similar

trends, although different antioxidant mechanisms were involved, electron transfer for $ABTS^{\bullet+}$ and energy transfer for $^1O_2$. In general, these results show that the mixture of oxidation-derived compounds had higher ability to scavenge $ABTS^{\bullet+}$ and to protect against $^1O_2$ as compared to intact all-*trans*-β-carotene, and that this fact may be, at least partially, related to the incorporation of at least one oxygen atom in the carotenoid structure of the oxidation products.

4 Conclusions

The major compounds formed during the treatment of β-carotene with MCPBA were as follows: 5,6-epoxy-β-carotene, 5,6:5',6'-diepoxy-β-carotene, 5,6:5',8'-diepoxy-β-carotene, and 5,8-epoxy-β-carotene. The major products in the oxidative cleavage reaction of β-carotene with $KMnO_4$ were: apo-8'-β-carotenone, β-apo-8'-carotenal, semi-β-carotenone, β-carotenone, 10'-apo-β-carotenal, 12'-apo-β-carotenal, 14'-apo-β-carotenal, and 15-apo-β-carotenal. In this reaction several *cis* isomers were detected.

The formation of compounds with shorter chromophores as compared to that of β-carotene led to a clear hypsochromic effect on the UV/Vis absorption spectra, along with a hypochromic displacement in the case of the epoxidation reaction. In terms of colour, this fact led to marked decrease in yellowness and vividness, with visually noticeable colour differences. In addition, $b^*$and C^*_{ab} values are promising colour parameters for the rapid assessment of the formation of oxidative derivatives.

On the other hand, both the capacity to scavenge $ABTS^{\bullet+}$ and to protect against $^1O_2$ increased over time as the chemical reaction of β-carotene and $KMnO_4$ proceeded. Thus, the evaluation of the *in vitro* antioxidant capacity of the oxidation

derivatives detected in this study (all of them containing carbonyl groups in the c.d.b. system) both individually and in combinations appears as an interesting research topic in the carotenoid field in order to assess possible synergistic effects.

The results of this study are interesting in relation to the possible use of oxidative derivatives of β-carotene not only as colorants, but rather as versatile ingredients that could also contribute to protect foods from oxidation reactions and, maybe, provide health benefits.

Acknowledgments

The Brazilian authors thank the Foundation for the Support of Research of the State of São Paulo (FAPESP) for their financial support. AJMM acknowledges funding from the European Union (Marie Curie Reintegration Grants, FP7-224789 INTERCAROTEN) and the Spanish programs Ramón y Cajal (ref. RYC-2010-07115) and Juan de la Cierva (ref JCI-2008-1767). The authors are members of the IBERCAROT network, funded by CYTED (ref. 112RT0445).

Appendix A Supplementary data

Supplementary 1. Figure S1. Proposed sequence of formation of β-carotene-derived compounds by chemical reactions with $KMnO_4$ and MCPBA.

Supplementary 2. **Table S2.** CIELAB colour coordinates of the mixtures obtained by chemical reaction of β-carotene with $KMnO_4$.

Supplementary 3. **Table S3.** CIELAB colour coordinates of the mixtures obtained by chemical reaction of β-carotene with MCPBA.

5 References

Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., Ghisla, S., Bouwmeester, H., Beyer, P., & Al-Babili, S. (2012). The path from β-carotene to carlactone, a strigolactone-like plant hormone. *Science*, 335, 1348-1351.

Alija, A.J., Bresgen, N., Sommerburg, O., Langhans, C.D., Siems, W., & Eckl, P.M. (2006). Beta-carotene breakdown products enhance genotoxic effects of oxidative stress in primary rat hepatocytes. *Carcinogenesis*, 27, 1128-1133.

Augustin, W., Siems, W., Sommerburg, O., Langhans, C.D., Schild, L., & Wiswedel, I. (2002). Beta-carotene cleavage products induce oxidative stress by impairing mitochondrial functions: brain mitochondria are more sensitive than liver mitochondria. *Free Radical Biology & Medicine*, 33, S326.

Benevides, C. M. J., Veloso, M. C. C., Pereira, P. A. P., & Andrade, J. B. (2011). A chemical study of β-carotene oxidation by ozone in an organic model system and the identification of the resulting products. *Food Chemistry*, 126, 927-934.

Britton, G., (1995). Structure and properties of carotenoids in relation to function. *The Journal of the Federation of American Societies for Experimental Biology,*9, 1551-1558.

Caris-Veyrat, C., Schmid, A., Carail, M., & Böhm, V. (2003). Cleavage products of lycopene produced by *in vitro* oxidations: characterization and mechanisms of formation. *Journal of Agricultural and Food Chemistry, 51*, 7318-7325.

CIE (1978). *Recommendations on uniform color spaces, color-difference equations, psychometric color terms, CIE Publication No. 15 (E-1.3.1) 1971, Supplement* 2. Viena: Bureau Central de la CIE.

549 De Rosso, V., & Mercadante, A. Z. (2007). Identification and quantification of
550 carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *Journal of Agricultural*
551 *and Food Chemistry*, *55*, 5062-5072.
552 Emenhiser, C., Simunovic, N., Sander, L. C., & Schwartz, S. J. (1996). Separation of
553 geometrical carotenoid isomers in biological extracts using a polymeric C_{30} column in
554 reversed-phase liquid chromatography. *Journal of Agricultural and Food Chemistry,*
555 *44*, 3887- 3893.
556 Eugster C.H. (1995). Chemical derivatization: microscale tests for the presence of
557 common functional groups in carotenoids. In: Britton, G., Liaaen-Jensen, S., Pfander,
558 H, (eds), Carotenoids: isolation and analysis, vol 1A. Birkhäuser Verlag, Basel, pp
559 71-80.
560 Heredia, F. J., Álvarez, C., González-Miret, M. L., & Ramírez, A. (2004).
561 CromaLab®, análisis de colour, Registro General de la Propiedad Intelectual SE-
562 1052-04: Sevilla, Spain.
563 Hu, K. Q., Liu, C., Ernst, H., Krinsky, N. I., Russell, R. M., & Wang, X. D. (2006). The
564 biochemical characterization of ferret carotene-90, 100-monooxygenase catalyzing
565 cleavage of carotenoids in vitro and in vivo. *Journal of Biological Chemistry*, *281*,
566 19327–19338.
567 Hurst, J. S., Saini, M. K., Jin, G-F., Awasthi, Y. C., & Kuijk, F. J. G. M. van. (2005).
568 Toxicity of oxidized β-carotene to cultured human cells. *Experimental Eye Research*,
569 *81*, 239-243.
570 Kalariya, N.M., Ramana, K.V., Srivastava, S.K., & Kuijk F.J.G.M. van. (2008).
571 Carotenoid derived aldehydes-induced oxidative stress causes apoptotic cell death in
572 human retinal pigment epithelial cells. *Experimental Eye Research*, *86*, 70-80.

573 Kalariya, N.M., Ramana, K.V., Srivastava, S.K., & Kuijk, F.J.G.M. van. (2009).
574 Genotoxic effects of carotenoid breakdown products in human retinal pigment
575 epithelial cells. *Current Eye Research*, *34*, 737-747.
576 Khachik, F., Spangler, C. J., & Smith, C. J. Jr. (1997). Identification, quantification,
577 and relative concentrations of carotenoids and their metabolites in human milk and
578 serum. *Analytical Chemistry*, *69*, 1873-1881.
579 Knockaert, G., Pulissery, S. K., Lemmens, L., Buggenhout, S. V., Hendrickx, M., &
580 Van Loey, A. (2013). Isomerisation of carrot β-carotene in presence of oil during
581 thermal and combined thermal/high pressure processing. *Food Chemistry*, *138*,
582 1515–1520.
583 Lin, H-W., Chang, T-J., Yang, D-J., Chen, Y-C., Wang, M., & Chang Y-Y. (2012).
584 Regulation of virus-induced inflammatory response by β-carotene in RAW264.7 cells.
585 *Food Chemistry*, *134*, 2169–2175.
586 Lobo, G. P., Amengual, J., Palczewski, G., Babino, D., & von Lintig, J. (2012).
587 Mammalian carotenoid-oxygenases: key players for carotenoid function and
588 homeostasis. *Biochimica et Biophysica Acta*, *1821*, 78–87.
589 Lozano, R. D. (1978). El color y su medición. Americalee, Buenos Aires. 640 p.
590 Mein, J. R., Lian, F., & Wang, X-D. (2008). Biological activity of lycopene metabolites:
591 implications for cancer prevention. *Nutrition Reviews*, *66*, 667-683.
592 Meléndez-Martínez, A. J., Ayala, F., Echávarri, J. F., Negueruela, A.I., Escudero-
593 Gilete, M. L.,González-Miret, M. L., Vicario, I. M., & Heredia, F. J. (2011). A novel
594 and enhanced approach for the assessment of the total carotenoid content of foods
595 based on multipoint spectroscopic measurements. *Food Chemistry*, *126*, 1862–1869.

596 Meléndez-Martínez, A. J., Britton, G., Vicario, I. M., & Heredia, F. J. (2007).
597 Relationship between the colour and the chemical structure of carotenoid pigments.
598 *Food Chemistry*, *101*, 1145–1150.
599 Meléndez-Martínez, A. J., Stinco, C. M., Liu, C., & Wang, X-D. (2013). A simple
600 HPLC method for the comprehensive analysis of *cis/trans* (*Z/E*) geometrical isomers
601 of carotenoids for nutritional studies. *Food Chemistry*, *138*, 1341-1350.
602 Melgosa, M., Pérez, M. M., Yebra, A., Huertas, R. & Hita, E. (2001). Algunas
603 reflexiones y recientes recomendaciones internacionales sobre evaluación de
604 diferencias de color. *Optica Pura y Aplicada*, *34*, 1–10.
605 Mercadante, A. Z. (2008). Food Colorants - Chemical and Functional Properties. In
606 C. Socaciu (Ed.), Carotenoids in Foods: Sources and Stability during Processing and
607 Storage (pp. 213 – 235), New York: CRC Press.
608 Montenegro, M.A., Rios, A. de O., Mercadante, A. Z., Nazareno, M. A., & Borsarelli,
609 C. D. (2004). Model studies on the photosensitized isomerization of bixin. *Journal of*
610 *Agricultural and Food Chemistry*, *52*, 367- 373.
611 Mueller, L., & Boehm, V. (2011). Antioxidant activity of β-carotene compounds in
612 different *in vitro* assays. *Molecules*, *16*:1055-1069.
613 Pesek, C. A. & Warthesen, J.J. (1990). Kinetic model for photoisomerization and
614 concomitant photodegradation of β-carotenes. *Journal of Agriculture and Food*
615 *Chemistry 38*, 1313-1315.
616 Qiu, D., Chen, Z-R., & Li, H-R. (2009). Effect of heating on solid β-carotene. *Food*
617 *Chemistry*, *112*:344-349.
618 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C.
619 (1999). Antioxidant activity applying an improved ABTS radical cation decolorization
620 assay. *Free Radical Biology & Medicine*, *26*, 1231-1237.

621 Reynaud,E., Aydemir, G., Rühl, R., Dangles, O., & Caris-Veyrat, C. (2011). Organic
622 Synthesis of New Putative Lycopene Metabolites and Preliminary Investigation of
623 Their Cell-Signaling Effects. *Journal of Agricultural and Food Chemistry, 59*, 1457-
624 1463.
625 Rodrigues, E.; Mariutti, L. R.; Faria, A. F.; & Mercadante, A. Z. (2012). Microcapsules
626 containing antioxidant molecules as scavengers of reactive oxygen and nitrogen
627 species. *Food Chemistry*, *134*, 704-711.
628 Rodriguez, E.B., & Rodriguez-Amaya, D.B. (2007). Formation of apocarotenals and
629 epoxycarotenoids from β-carotene by chemical reactions and by autoxidation in
630 model systems and processed foods. *Food Chemistry*, *101*, 563–572.
631 Rodriguez, E.B., & Rodriguez-Amaya, D.B. (2009). Lycopene epoxides and apo-
632 licopenals formed by chemical reactions and autoxidation in model systems and
633 processed foods. *Journal of Food Science*, *74*, 674-682.
634 Tibaduiza, E. C., Fleet, J. C., Russell, R. M., & Krinsky, N. I. (2002). Excentric
635 cleavage products of β-carotene inhibit estrogen receptor positive and negative
636 breast tumor cell growth *in vitro* and inhibit activator protein-1-mediated
637 transcriptional activation. *Journal of Nutrition*, *132*, 1368-1375.
638 van Breemen, R. B., Dong, L., & Pajkovic, N,. D. (2012). Atmospheric pressure
639 chemical ionization tandem mass spectrometry of carotenoids. *International Journal*
640 *of Mass Spectrometry*, *312*, 163– 172.
641 Yeh, S. L., & Hu, M. L. (2003). Oxidized [beta]-carotene inhibits gap junction
642 intercellular communication in the human lung adenocarcinoma cell line A549, *Food*
643 *Chemistry Toxicology*, *41*, 1677.

644 Zepka, L. Q., & Mercadante, A. Z. (2009a). Degradation compounds of carotenoids
645 formed during heating of a simulated cashew Apple juice. *Food Chemistry*, *117*, 28–
646 34.
647 Zepka, L. Q., Borsarelli, C. D., Silva, M. A. Z. P. de, & Mercadante, A. Z. (2009b).
648 Thermal degradation kinetics of carotenoids in a cashew apple juice model and its
649 impact on the system color. *Journal of Agriculture and Food Chemistry*, *57*, 7841-
650 7845.

651

652 **Figure captions**

653 **Figure 1.** Evolution with time of peak area of (A) all-*trans*-β-carotene; (B) *cis* isomers
654 of β-carotene ● and 5,6-epoxy-β-carotene ∇; (C) semi-β-carotenone ★; β-
655 carotenone ο; peak 01 ◇ and (D) *cis*+*trans* isomers of 8-apo-carotenal ☆, β-apo-
656 10'-carotenal ▼, β-apo-12'-carotenal □, β-apo-14'-carotenal ♦, β-apo-15-carotenal Δ,
657 during chemical reaction with $KMnO_4$.

658

659 **Figure 2.** Evolution with time of peak area of (A) all-*trans*-β-carotene; (B) *cis* isomers
660 of β-carotene; (C) 5,6-epoxy-β-carotene ★ and 5,8-epoxy-β-carotene ☆; (D)
661 5,6:5',6'-diepoxy-β-carotene ▼ and 5,6:5',8-'diepoxy-β-carotene ∇, during chemical
662 reaction with MCPBA.

663

664 **Figure 3.** Representation of the samples in the *a*b** colour plane.

665

666 **Figure 4.** UV/Vis spectra measured during the chemical degradation of β-carotene
667 with $KMnO_4$ (a) and MCPBA (b).

Figure 5. Capacity to scavenge $ABTS^{\bullet+}$ (TEAC, bar graphic) and protection against $^1O_2$ (IC_{50} in μg/mL, bar graphic), and total carotenoid contents (mM, continued line), detected in the solutions from the chemical reaction of β-carotene with $KMnO_4$. No measurements were made at 40 and 50 minutes.

Table 1. Derived-β-carotene compounds formed by epoxidation with MCPBA and oxidative cleavage with $KMnO_4$, detected by HPLC-DAD-MS/MS.

Highlights

Changes in colour and antioxidant capacity over the oxidation of β-carotene were studied.

Nineteen different carotenoids were detected during the oxidation reactions.

Some colour parameters appeared useful to detect the formation of oxidation products.

Some mixtures of oxidation products showed more antioxidant capacity than β-carotene.