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Effect of *cis*/*trans* Isomerism of β-Carotene on the Ratios of Volatile Compounds Produced during Oxidative Degradation

YVES WACHÉ,*,† AURÉLIE BOSSER-DERATULD,†,§ JEAN-CLAUDE LHUGUENOT,# AND JEAN-MARC BELIN†

Laboratoire de Microbiologie UMR UB/INRA 1082 and Laboratoire de Toxicologie alimentaire UMR UB/INRA 0938, ENSBANA, 1 esplanade Erasme, 21000 Dijon, France

β-Carotene is, when cleaved, an important source of flavor and aroma compounds in fruits and flowers. Among these aroma compounds, the main degradation products are β-ionone, 5,6-epoxy-β-ionone, and dihydroactinidiolide (DHA), which are associated by flavorists and perfumers with fruity, floral, and woody notes. These three species can be produced by degradation of β-carotene through an attack by enzyme-generated free radicals and a cleavage at the C9−C10 bond. This study investigated the influence of *cis/trans* isomerism at the C9−C10 bond on the production of β-carotene degradation compounds, first with a predictive approach and then experimentally with different isomer mixtures. β-Carotene solutions containing high ratios of 9-*cis*-isomers produced more DHA, suggesting a different pathway than for the transformation of *all-trans*-β-carotene to ionone and DHA. These results are important in the search for financially viable processes to produce natural carotene-derived aroma compounds.

KEYWORDS: **β-Carotene isomers; xanthine oxidase; β-ionone; dihydroactinidiolide**

INTRODUCTION

Carotenoids are important constituents of foods. Due to their color and the fact that their degradation leads to the production of aroma compounds (*1*), they play a major role in the sensorial properties of food products. Their structure and related physicochemical properties also result in a role of importance in biological systems. The biological functions of β-carotene **1** have been attributed to its ability to scavenge free radicals (*2*−*4*), physically quench singlet oxygen (*5*), and generate vitamin A (retinol) (*6*).

β-Carotene is present in nature in many isomeric forms. The *all-trans* **1** is the main form encountered, but some organisms contain large amounts of *cis*-isomers as for instance the halotolerant alga *Dunaliella bardawil*, which can have twice as much 9-*cis* **15** as *all-trans* (*7*). To date, most of the works investigating the characteristics of β-carotene have dealt with *all-trans*-β-carotene, but the 9-*cis*-isomer, mainly extracted from this algae, has also been studied. The behavior of this latter form in metabolism is rather different from that of the *all-trans* one. It is, for instance, not absorbed in the same way (*6*), but it seems to stimulate β-carotene absorption (*8*). Due probably to the higher reactivity of the *cis*-bond, it has a higher in vitro antioxidant activity (*9*) and in vivo antiperoxidative effect (*10*) than the *all-trans*-isomer. However, this latter form is a better inhibitor of low-density lipoprotein oxidation (*11*). The two forms seem also to have different roles in vitamin A synthesis (*12*), although 9-*cis*-β-carotene can also generate 9-*cis*-retinal **16**, -retinol, or -retinoic acids (*9*, *13*−*15*).

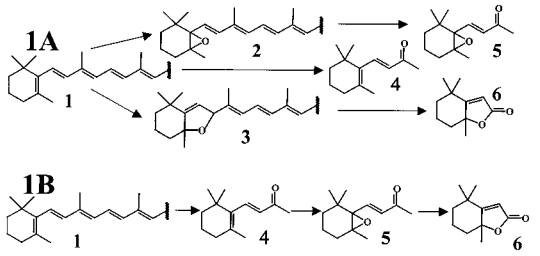

Figure 1. Pathways for the synthesis of β-ionone **4**, 5,6-epoxy-β-ionone **5**, and DHA **6** from β-carotene **1** and through 5,6-epoxy- **2** and 5,8-epoxy-β-carotene **3**: (A) pathway proposed in the literature (*16*–*18*); (B) pathway observed by Bosser et al. (*17*).

However, nothing is known about the effect of *cis*/*trans* isomerism on the generation of aroma compounds from carotenoids. β-Carotene, when cleaved, gives rise to various interesting compounds (*16*). Among them, β-ionone **4**, 5,6-epoxy-β-ionone **5**, and dihydroactinidiolide (DHA) **6** are often associated with fruity, floral, and woody notes to varying degrees. Two metabolic pathways originating the epoxy ionone and DHA have been proposed (**Figure 1**). In the first one suggested by the literature (*16*−*18*) (**Figure 1A**), β-carotene is first oxidized to 5,6-epoxy-β-carotene **2** and 5,8-epoxy-β-carotene **3**, and these compounds are cleaved to 5,6-epoxy-β-ionone and DHA, respectively. In the second one observed by Bosser et al. (*17*)

* Author to whom correspondence should be addressed (e-mail ywache@u-bourgogne.fr; fax ++33 3 80 39 66 41).
† Laboratoire de Microbiologie UMR UB/INRA 1082.
§ Present address: Diana Ingrédients, 56000 Vannes, France.
# Laboratoire de Toxicologie alimentaire UMR UB/INRA 0938.

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(**Figure 1B**), β-carotene is cleaved to β-ionone, which is oxidized to its 5,6-epoxy form and then lactonized to DHA. This cleavage can be caused by enzymatic activities or physicochemical conditions. This degradation has been and still is the subject of many studies (*19*, *20*) using enzymes (*21*−*23*) and physical (*24*) or chemical media (*25*).

Technology and particularly biotechnologies have tried to take advantage of this reaction to produce β-ionone and related volatiles using physicochemical processes (*26*, *27*) or enzyme-generated free radicals (*28*−*30*). This latter reaction is called co-oxidation because the enzyme (often lipoxygenase and xanthine oxidase in our case) catalyzes a reaction generating oxygen-derived radical species which, in turn, degrade carotenoids.

Unfortunately, yields are too low for industrial application partly because the cleavage site is not mastered and also because the production is divided into three interesting compounds. The influence of the structure of the carotenoid on the cleavage site and possible rearrangement has not been completely studied. Some workers (*31*, *32*) have investigated the relationship between structure and reactivity in the free radical mediated oxidation of carotenoids. Electron density along the polyene chain as well as the presence of functional groups appears to modify reactivity. Redox potentials of various carotenoids and their related cations have also been studied (*33*), but this work has not included the effect of *cis*-bonds in β-carotene on the degradation products.

We investigated in this work the effect on the cleavage site of β-carotene of *cis*/*trans* isomerism. The *cis*/*trans* isomerism was investigated first with a predictive approach, then, using isomer mixtures in the system used previously with *all-trans*-β-carotene (*29*).

EXPERIMENTAL PROCEDURES

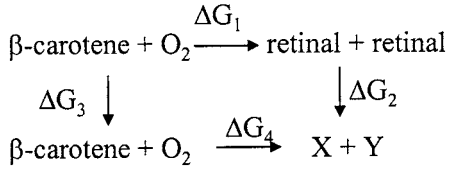
Degradation of β-Carotene in the Xanthine Oxidase/Acetaldehyde System. Reactions were carried out as previously described (*29*) at 37 °C in a 2-L enzymatic reactor with 250 rpm stirring. The reaction volume was 300 mL. The aqueous phase was composed of 27×10^{-3} units/mL xanthine oxidase (grade III from buttermilk), 48 mM acetaldehyde, and 50 mM phosphate buffer, pH 8.0.

β-Carotene (Fluka, Sigma-Aldrich, St Quentin Fallavier, France) was prepared according to the method of Ben Aziz et al. (*34*) as previously described (*29*). It was dissolved in chloroform with Tween 80 and evaporated to dryness under vacuum. The residue was then solubilized in a 0.25% EDTA solution and filtered through filter paper. Thereafter, sodium acetate buffer, pH 4.6, was added. The procedure was modified for iodine-catalyzed photoisomerization by adding ethanol-solubilized I_2 (20 mg per 500 mg of β-carotene) and exposing it for 7 min to a fluorescent lamp (20 W) according to the method of Tsukida (*35*). To avoid interaction with the enzyme, I_2 excess was reduced to I^- prior to the experiment by adding $Na^+HSO_3^-$. Initial β-carotene concentration was 90 mg/L in the reactor volume.

Analyses. β-Carotene isomers were quantified throughout the reaction with a Merck HPLC system with a Merck L-6200 pump, a 10-μL loop injector, a Merck L-3000 photodiode detector in the absorbance mode ($\lambda = 450$ nm), and a Merck Lichrocart 250-4 column, Purospher RP-18. The solvents used were methanol (95%) and tetrahydrofuran (5%) with a 1.2 mL/min flow. The reference β-carotene isomers were obtained from Roche (Basel, Switzerland), and each form was checked according to its spectrometric spectrum.

For volatile analysis, samples (18 mL) were extracted with CH_2Cl_2. After the addition of methyl isoeugenol as internal standard, they were concentrated under N_2 to a final volume of 3 mL. Analyses were carried out in a Varian 3400 gas chromatograph equipped with a CP Wax-58CB column (i.d., 0.25 mm; film thickness, 2 μm; length, 50 m) with a 4 °C/min temperature increase from 90 to 220 °C. Both injector and FID temperature were set to 250 °C. Details for analyses have been described previously (*29*). Qualitative identification of all volatiles was realized by GC-MS with the ion-trap system Finnigan Mat ITS 40 by comparing mass spectra from samples with those of pure products.

Molecular Modeling of the Oxidation Cleavage of β-Carotene. Molecular modeling, based on the method of free energy perturbation, was carried out using the software Molecular Advanced Design (MAD, Oxford Molecular) in an IBM Risc 6000 H-355 workstation equipped with a 24-plan graphic card. MAD is a molecular dynamic modeling software that uses the fields of Allinger force as the initial data. We calculated minimal free energies of the direct oxidation products of *all-trans*-β-carotene and 9-*cis*-β-carotene, taking as a reference the reaction of scission of one molecule of carotene to two retinals as follows



with $\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4$ and $\Delta G_3 = 0$. The reaction resulting in $X + Y$ is then compared to the reference by calculating $\Delta\Delta G = \Delta G_4 - \Delta G_1 = \Delta G_2$. The more negative $\Delta\Delta G$ is, the more the reaction resulting in $X + Y$ is favored.

RESULTS AND DISCUSSION

Prediction of the Oxidation Cleavage Site of *all-trans*- and 9-*cis*-β-Carotene. To predict the cleavage site of *all-trans*-β-carotene and that of its 9-*cis*-isomer, we calculated minimal free energies of the oxidation products and compared them to the formation of two molecules of retinal corresponding to a central cleavage. Results are given in **Figure 2**. $\Delta\Delta G = \Delta G_2$ (free energy variation of the reactions producing combination of compounds other than retinal) $- \Delta G_1$ (free energy variation of the reaction producing 2 retinals) shows the free energy difference between the two reactions; the more negative $\Delta\Delta G$ is, the more favored the noncentral scission reaction is compared to central scission. The results showed that, for both isomers, the lateral cleavage was favored. For the *all-trans*-isomer **1**, the favored cleavage was giving β-cyclocitral **14** and β-apo-8′-carotenal **13**, confirming the calculations using the method of molecular orbitals to characterize electronically *all-trans*-β-carotene (*36*). For the 9-*cis*-isomer **15**, the favored cleavage bond was the *cis*-bond (9−10) giving rise to β-ionone **4** and β-apo-10-carotenal **19**. This isomer was thus of potential interest for aroma compound production.

These calculations have been carried out for molecular oxygen oxidation without taking into account interference with the medium such as the solvent and the primary reactants. In the co-oxidation system we use, the degradation occurs through the action of radicals and β-carotenyl is formed before scission (*19*, *20*, *29*). Several reactions can thus be involved. The impact of high ratios of 9-*cis*-β-carotene on the degradation products was then investigated.

Degradation of β-Carotene Isomer Mixtures. From the different free energies of carotene isomers, we could expect different cleavage sites for *cis*/*trans*-isomers and, thus, differences in the ratios of degradation products. We compared the degradation of a mixture of isomers from photo-iodo-isomerized *all-trans*-β-carotene containing 54% of *all-trans*-isomer and 25% of 9-*cis*-isomer to *all-trans*-β-carotene (96% *all-trans* and 0.8% 9-*cis*). In both cases, a high degradation happened in the first 3 h (not shown) without any significant difference in the rates of degradation of the various isomers (**Table 1**). In **Figure 3** is presented the accumulation of β-ionone **4**, epoxy-β-ionone

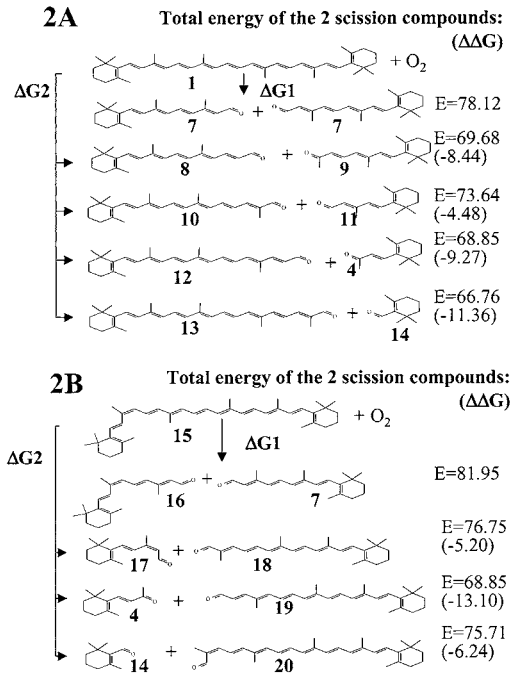


Figure 2. Oxidation reactions of *all-trans*-β-carotene (A) and 9-*cis*-β-carotene (B). Compounds [minimal energy] are **1**, *all-trans*-β-carotene [71.68]; **7**, retinal [39.06]; **8**, β-apo-14′-carotenal [37.60]; **9**, β-apo-13-carotenone [32.08]; **10**, β-apo-12′-carotenal [42.93]; **11**, β-ionylidene acetaldehyde [30.71]; **12**, β-apo-10′-carotenal [43.85]; **4**, β-ionone [25.00]; **13**, β-apo-8′-carotenal [49.87]; **14**, β-cyclocitral [16.89]; **15**, 9-*cis*-β-carotene [76.82]; **16**, 9-*cis*-retinal [42.89]; **17**, 9-*cis*-β-ionylidene acetaldehyde [33.82]; **18**, β-apo-12-carotenal [42.93]; **19**, β-apo-10-carotenal [43.85]; and **20**, 9-*cis*-β-apo-8-carotenal [58.82]. ΔG_1 is the free energy variation of the reaction producing two retinals (central scission), and ΔG_2 is the free energy variation of the reactions producing other combinations of compounds. $\Delta\Delta G = \Delta G_2 - \Delta G_1$ gives the free energy difference between the two reactions; the more negative $\Delta\Delta G$ is, the more favored the reaction is compared to central scission.

Table 1. Relative Percentage of the Various Isomers of β-Carotene from Isomerized β-Carotene during the Co-oxidation Experiment (±1%)

time (h)	*all-trans*	9-*cis*	13-*cis*	15-*cis*
0	55	24	19	3
1.5	54	23	18	4
4	53	24	19	4
6	53	23	17	5

5, and dihydroactinidiolide **6** during the degradation of β-carotene in the xanthine oxidase/acetaldehyde system from the different substrates: commercial β-carotene (96% *all-trans*) and photoisomerized β-carotene (24% 9-*cis*). The presence of β-cyclocitral **14** at trace level is not shown. With the *all-trans* system, β-ionone accumulated in the first 4 h to almost 3% and the concentrations decreased afterward to <2%. The profile of accumulation of its epoxide was similar but reached lower concentrations (maximum 2%). The lactone accumulated until 6 h, reaching a 3% concentration, and the concentration was stable afterward. With the isomer mixture, β-ionone accumulated in the first 2 h to only 2%, and its concentration then decreased. Its epoxide's concentration reached also a maximum after 2 h but at a higher concentration than with the *all-trans* preparation (2.8%). DHA accumulated very rapidly, reaching 6% after only 2 h and 8.5% after 24 h. By increasing the ratio of 9-*cis*-β-carotene, it was thus possible to increase the total amount of aroma compounds produced. However, the concentration of

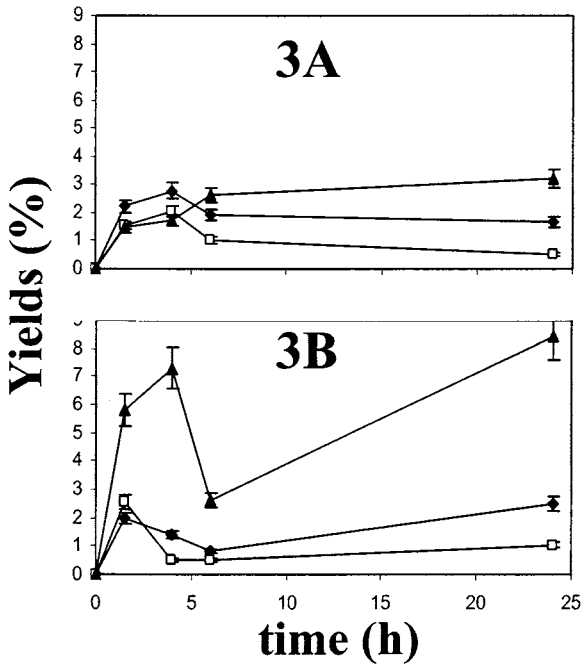


Figure 3. Yields of bioconversion of β-carotene to β-ionone (◆), 5,6-epoxy-β-ionone (□), and dihydroactinidiolide (▲) during the co-oxidation of 90 mg/L of commercial (96% *all-trans* and 0.8% 9-*cis*) (A) or iodine-catalyzed-photoisomerized (55% of *all-trans* and 24% of 9-*cis*) (B) β-carotene. Results are the mean of three independent experiments, and standard deviations are <10%.

β-ionone was not higher, and only the concentration of DHA was significantly increased. The rapid degradation of β-ionone in the reaction medium is possible as the radical species might also attack the products of degradation. Such a phenomenon has been already observed by Mordi et al. (*20*): they expected that the autoxidation of β-carotene would result in the major formation of β-cyclocitral **14** and β-apo-8′-carotenal **13**, but the β-cyclocitral appeared only after a few hours and they did not detect any β-apo-8′-carotenal at all. They explained these results by the possible rapid isomerization and oxidation of the degradation products in this highly reactive environment (*20*).

In our case, the "direct" expected oxidation product of *all-trans*-β-carotene **1**, β-ionone **4**, does not accumulate more with higher amounts of the 9-*cis*-isomer, whereas DHA accumulates more from the beginning of the experiment. In the pathway proposed by Bosser et al. (*17*), this product is more expected at the end of the reaction, after rearrangement of β-ionone. Our results suggest that 9-*cis*-β-carotene directly forms DHA without first forming β-ionone. The chemical pathway may thus involve the formation of 5,8-epoxy-β-carotene from 9-*cis*-β-carotene and the further cleavage into DHA (**Figure 1**).

In conclusion, higher ratios of 9-*cis*-β-carotene increase the formation of aroma compounds. However, we are not sure whether the 9−10 cleavage site is favored, as proposed by our prediction, or if the 9-*cis*-isomer is first transformed to its epoxide and then cleaved at the 8−9 level. Further work is necessary to determine the details of the mechanism.

This study shows once again that this cooxidation system developed by Bosser and Belin (29) is able to generate different carotenoid-derived degradation products from different substrates. After β-ionone was obtained from *all-trans*-β-carotene in the original study (*29*), the grasshopper ketone, a damascenone precursor, identified later from neoxanthin solutions (*37*), this study shows the rapid formation of dihydroactinidiolide from 9-*cis*-β-carotene-rich mixtures, a compound that appears

in higher ratios than from *all-trans*-β-carotene. In the conditions of this work, yields are relatively low but, as for the production of β-ionone, the system may be able to be improved to obtain higher yields (*28*).

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