

Uptake and Metabolism of β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone in Caco-2 cells

Boluwatiwi O. Durojaye¹, Kenneth M. Riedl², Robert W. Curley, Jr.³, Earl H. Harrison¹

¹Department of Human Sciences, ²Department of Food Science and Technology, and ³College of Pharmacy, The Ohio State University, Columbus OH 43210

Corresponding author:

Earl H. Harrison

1787 Neil Avenue, 314 Campbell Hall,

Columbus, OH 43210 USA.

614-292-8189; harrison.304@osu.edu

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Abbreviations: BCO1: β -carotene 15, 15'-oxygenase, BCO2: β -carotene 9', 10'-oxygenase, DAD: diode array detector, MTBE: methyl *tert*-butyl ether, QTOF: quadrupole time-of-flight, RA: retinoic acid, RAR: retinoic acid receptor, RT: retention time, RXR α : retinoid X receptor alpha.

ABSTRACT

β -Apocarotenoids are eccentric cleavage products of carotenoids formed by chemical and enzymatic oxidations. They occur in foods containing carotenoids and thus might be directly absorbed from the diet. However, there is limited information about their intestinal absorption. The present research examined the kinetics of uptake and metabolism of β -apocarotenoids. Caco-2 cells were grown on six-well plastic plates until a differentiated cell monolayer was achieved. β -Apocarotenoids were prepared in Tween 40 micelles, delivered to differentiated cells in serum-free media, and incubated at 37 °C for up to 8 hours. There was rapid uptake of β -apo-8'-carotenal into cells and β -apo-8'-carotenal was largely converted to β -apo-8'-carotenoic acid and a minor metabolite that we identified as 5, 6-epoxy- β -apo-8'-carotenol. There was also rapid uptake of β -apo-10'-carotenal into cells and β -apo-10'-carotenal was converted into a major metabolite identified as 5, 6-epoxy- β -apo-10'-carotenol and a minor metabolite that is likely a dihydro- β -apo-10'-carotenol. Finally, there was rapid cellular uptake of β -apo-13-carotenone and this compound was extensively degraded. These results suggest that dietary β -apocarotenals are extensively metabolized in intestinal cells via pathways similar to the metabolism of retinal. Thus, they are likely not absorbed directly from the diet.

Keywords: β -carotene, Absorption, Cell uptake, Carotenoid metabolism, Dietary lipids, Intestine, Nutrition/Lipids, Vitamin A

INTRODUCTION

Vitamin A is a general term for structurally related compounds that have the functional activity of retinol (1). De novo synthesis of provitamin A carotenoids occurs almost exclusively in plants and some microorganisms such as algae and fungi (2, 3). Dietary vitamin A can be obtained in two major forms: preformed vitamin A (retinol and retinyl esters) and provitamin A carotenoids (β -carotene, α -carotene, and β -cryptoxanthin) (1, 2). Preformed vitamin A is present in animal derived products such as liver, eggs, and dairy products. In contrast, plant food sources (mostly fruits and vegetables) account for significant intake of dietary provitamin A carotenoids (2, 3). Sweet potatoes and pumpkin, for example, provide high amounts of α -carotene; oranges, papaya, and peppers are excellent sources of β -cryptoxanthin; and carrots, squash, and dark-leafy vegetables are important sources of β -carotene (3, 4).

β -Carotene is an excellent precursor of vitamin A and exhibits this provitamin A activity because it has two unsubstituted β -ionone rings as well as the correct number and position of methyl groups in its long hydrocarbon chain (2). Two important enzymes, β -carotene 15, 15'-oxygenase (BCO1) and β -carotene 9', 10'-oxygenase (BCO2), catalyze the oxidative cleavage of β -carotene to produce biologically active metabolites (Figure 1) (5). BCO1, localized in the cytosol (6), cleaves β -carotene to give two molecules of retinal, which can be converted to retinoic acid (RA) or retinol (2, 7). On the other hand, BCO2, localized in the mitochondria (6, 8), cleaves β -carotene to yield β -ionone and β -apo-10'-carotenal, which can be converted to retinal by BCO1 (2, 9). Peroxidases and lipoxygenases have been suggested as enzymes that could catalyze the formation of β -apocarotenoids other than β -apo-10'-carotenal (10). Apocarotenoids are also formed non-enzymatically via chemical oxidation during food processing and cooking or in tissues under conditions of oxidative stress.

β -Apocarotenoids have biological activity and previous studies suggest that they may elicit their functions through gene regulation (5, 7). Retinoic acid receptors (RAR) are a family of transcription factors that activate genes with retinoic acid response elements as part of their promoter sequence (7, 11). A number of studies have investigated the binding affinity and effects of β -apocarotenoids on these transcription

factors. Work by Eroglu et al. (7) demonstrated that some β -apocarotenoids are potent antagonists of α , β , and γ isoforms of RARs. β -Apo-14'-carotenoic acid, β -apo-14'-carotenal, and β -apo-13-carotenone were high affinity ligands for RARs α , β , and γ and significantly antagonized the transactivation of all three RAR isoforms by RA. Also, these β -apocarotenoids effectively decreased the RA-induced upregulation of RAR β and cytochrome P450-26A1 in HepG2 cells. In addition, Eroglu et al. (12) examined the effects of β -apocarotenoids on the activation and signaling of retinoid X receptor alpha (RXR α). β -Apo-13-carotenone significantly inhibited the 9-cis-RA induced activation of RXR α and its transcriptional response. Molecular modeling and ligand binding studies showed that β -apo-13-carotenone directly binds to the ligand binding domain of RXR α .

The exact mechanisms of formation of β -apocarotenoids, other than β -apo-10'-carotenal and retinal, from β -carotene and other carotenoids consumed in the diet by humans are not fully understood (10, 13). However, these eccentric cleavage products are present in a carotenoid-rich diet. Harrison and colleagues (14) identified several β -apocarotenoids in cantaloupe and orange-fleshed honeydew melons. The quantities of β -apo-8'-carotenal, β -apo-10'-carotenal, β -apo-12'-carotenal, β -apo-14'-carotenal, and β -apo-13-carotenone detected using HPLC-MS were approximately 1.5% of β -carotene in both melons. In addition, our laboratory has evidence that these β -apocarotenoids are present in pumpkin, sweet potato, and yellow squash at about 2.5%, 0.4%, and 3% of β -carotene respectively (Fleshman, M.K. and Harrison, E.H., Unpublished data). Thus, it is possible that β -apocarotenoids might be directly absorbed from the diet (13). However, there is limited knowledge about their bioavailability and intestinal absorption.

The Caco-2 intestinal cell line is a well-established *in vitro* model that has been used to study the absorption of the C40 carotenes and xanthophylls (15, 16). Differentiated cells have morphological and functional characteristics comparable to enterocytes (17, 18) and these include very well developed brush border membranes and tight junctions that decrease the probability of transport of molecules between cells (19). Thus, Caco-2 cells are a good model to study the apical uptake and metabolism of β -apocarotenoids. In the present study, we investigated the uptake and metabolism of β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone using Caco-2 cells as a model. We focused specifically on these β -apocarotenoids

because β -apo-8'-carotenal is commercially available and is routinely utilized as a food colorant; β -apo-10'-carotenal is the major product of BCO2 cleavage of β -carotene; and β -apo-13-carotenone, with a similar structure to retinol, has been shown to be a potent antagonist of vitamin A signaling.

MATERIALS AND METHODS

Chemicals and Supplies

Unless otherwise stated, all chemicals and supplies were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Cell culture reagents were obtained from Gibco® Life Technologies Corporation (Grand Island, NY). TLC plates were purchased from Analtech (Newark, DE). Plates were 20 cm by 20 cm glass backed with a 1000 micron layer of silica gel containing a fluorescent indicator. The synthesis of β -apo-8'-carotenoic acid, β -apo-10'-carotenal, β -apo-10'-carotenoic acid, β -apo-12'-carotenal, and β -apo-13-carotenone have been described previously (7). In addition, β -apo-10'-carotenol was synthesized as described elsewhere (20). Finally, β -apo-8'-carotenol and 5, 6-epoxy- β -apo-8'-carotenol were synthesized according to the methods described below.

Synthesis of β -Apocarotenoids

Synthesis of β -apo-8'-carotenol

5 mg of β -apo-8'-carotenal, 15 mg (3.3 equiv.) of sodium borohydride, 5 mL of absolute ethanol, and 2 mL of toluene were mixed in a dry round bottom flask under argon with magnetic stirring. After 30 minutes, the color of the mixture had not changed. After 1 hour, the mixture became a little lighter colored. More sodium borohydride was added, the reaction mixture stirred for 30 minutes, and 1 mL of 4 °C saturated ammonium chloride was added. The mixture was now still lighter in color and was partitioned between saturated sodium chloride and ethyl acetate. The ethyl acetate layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The dried residue was resolubilized in ethyl acetate, spread onto a preparative TLC plate, and allowed to develop for 40 minutes using 20% ethyl acetate/hexanes as mobile phase. The lower band, tentatively identified as β -apo-8'-carotenol was suspended and stirred in acetone for 20 minutes, filtered, and concentrated under reduced pressure. The newly synthesized compound was then characterized by NMR and HPLC (see HPLC analysis - method 1).

Synthesis of 5, 6-epoxy- β -apo-8'-carotenol

13.6 mg of previously synthesized β -apo-8'-carotenol was dissolved in approximately 10 mL of dichloromethane and placed in an oven dried round bottom flask with a magnetic stirrer and an argon inlet. After cooling to 4 °C, 8.9 mg (1.2 equiv.) of *meta*-chloroperoxybenzoic acid was added. The mixture lost its color immediately. The ice bath was removed and stirring continued for 4 hours. The solution was washed twice with saturated sodium bicarbonate, then with distilled water followed by saturated sodium chloride, and dried over sodium sulfate. Filtration and concentration under reduced pressure afforded crude product, which was purified by preparative TLC and characterized as for β -apo-8'-carotenol.

Cell Culture Conditions

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown and maintained as described elsewhere (21). Briefly, 75 cm² flasks were seeded with 0.4×10^6 cells and grown in DMEM containing 25 mM glucose, 15% heat-inactivated FBS, 1% penicillin-streptomycin, 1% L-glutamine (200 mM), 1% non-essential amino acids, and 1% fungizone. Cells were incubated at 37 °C in an atmosphere of air/CO₂ (95:5 v/v) and cells were passaged every 7 days or when cells were at 75–80% confluency.

Preparation of β -Apocarotenoids for Uptake Studies

We prepared β -apocarotenoids in Tween 40 as previously described (15) with some changes and delivered them in serum-free media to cells (see description below). Briefly, β -apocarotenoids were solubilized in either hexane or ethanol to prepare a stock solution of the pure compounds. The required volume of each β -apocarotenoid dissolved in appropriate solvent, 22.5 μ L of 10% v/v Tween 40 (in ethanol), and 400 μ L of acetone were placed into a 4 mL glass vial. The resulting mixture was dried under stream of nitrogen gas at 30–40 °C, resolubilized in 200 μ L of acetone, and dried again. Dried residues were solubilized in 1 mL of serum-free media, vortexed briefly, and sonicated for 1 minute. The resulting mixture was dissolved in additional serum-free media to yield the desired test concentrations of the clear micellar solutions.

Stability of β -Apocarotenoids during Incubation in Cell Culture Conditions

Previous studies have shown that carotenoids are susceptible to spontaneous chemical oxidation and degradation during incubation in cell culture environment (22, 23). Thus, we investigated the stability of the β -apocarotenoids in the same cell culture conditions as were used for the cell uptake experiments. The method for preparing β -apocarotenoids in Tween 40 has been described above. For the stability experiments, 2 mL of serum-free media, containing the individual β -apocarotenoids (β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone), was added to six-well plates without Caco-2 cells and incubated at 37 °C for time points up to 8 hours. Aliquots of media at 0, 1, 4, and 8 hours were collected and stored at -80 °C before extraction and HPLC analysis.

Uptake of β -Apocarotenoids into Caco-2 Cells

For experiments, six-well plates were seeded with 0.15×10^6 cells and maintained in cell culture conditions described above until a confluent monolayer was achieved. Once confluent, the amount of FBS used in media was decreased to 7.5% and the media was regularly replaced after 2 days. Cell cultures used for experiments were between passages 22–40 when monolayers were 10–14 days post-confluent (i.e. fully differentiated). At the start of each experiment, cell monolayers were washed once with basal DMEM. 2 mL of serum-free media, containing a known concentration of each β -apocarotenoid (β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone), was added to each well and incubated at 37 °C.

After incubation, the integrity of the monolayer was examined by phase contrast microscopy and media containing the test compound was collected into a 15 mL centrifuge tube. There were no observed changes in morphology of cell monolayers after treatment with any β -apocarotenoids. Cell monolayers were washed once with 2 mL of ice-cold PBS containing 2 g/L bovine serum albumin and twice with 2 mL of ice-cold PBS to remove extracellular β -apocarotenoids. 2 mL of ice-cold PBS was added to each well and cells were harvested into 15 mL centrifuge tubes using a plastic scraper. Tubes were then centrifuged

at $1000 \times g$ for 10 minutes at $4\text{ }^{\circ}\text{C}$. The supernatant was discarded and the cell pellets were stored under nitrogen gas at $-80\text{ }^{\circ}\text{C}$ before extraction and HPLC analysis (24).

Extraction of β -Apocarotenoids

Extraction from media

Cell culture media samples before and after incubation were extracted according to the method of Kopec et al. (25) with slight modifications. Briefly, 1 mL of media and 1 mL of acetone were placed in a 15 mL centrifuge tube and vortexed for 1 minute. 4 mL of hexanes was added and the mixture was vortexed again for 1 minute. The resulting mixture was then centrifuged at $3000 \times g$ for 10 minutes at $4\text{ }^{\circ}\text{C}$. The upper layer was transferred into an 11 mL screw cap glass vial and solvent was dried under a stream of nitrogen gas at $30\text{--}40\text{ }^{\circ}\text{C}$. Extracts were then stored at $-80\text{ }^{\circ}\text{C}$ before HPLC analysis.

Extraction from cell monolayer

Cell pellets were resuspended in 1 mL of PBS and probe sonicated for 20 seconds. 1 mL of acetone was added and the tube was vortexed for 1 minute. 4 mL of hexanes was added and the mixture was vortexed again for 1 minute. The resulting solution was then centrifuged at $3000 \times g$ for 10 minutes at $4\text{ }^{\circ}\text{C}$. The upper layer was transferred into an 11 mL screw cap glass vial and solvent was dried under a stream of nitrogen gas at $30\text{--}40\text{ }^{\circ}\text{C}$. Extracts were stored at $-80\text{ }^{\circ}\text{C}$ before HPLC analysis.

HPLC Analysis

Method 1: Analysis of β -apo-8'-carotenal and β -apo-10'-carotenal

Sample extracts were solubilized in 200 μL of 1:1 v/v methanol/methyl *tert*-butyl ether (MTBE), syringe filtered (0.22 μm pores; Millipore), and 20 μL of the extract was injected for analysis. Separation of β -apocarotenoids was achieved using a YMC C30 column ($4.6 \times 150\text{ mm}$, 5 μm ; Waters, Ireland) on an Agilent 1200 series HPLC system coupled to a diode array detector (DAD) (Agilent Technologies, Santa Clara, CA) with a mobile phase of 98:2 v/v methanol/1 M ammonium acetate pH 4.6 (solvent A) and MTBE (solvent B). The flow rate was set at 0.8 mL/minute and a column temperature of $35\text{ }^{\circ}\text{C}$ was maintained.

Analytes were separated with a linear gradient from 15% B to 40% B over 10 minutes, followed by elution at 40% B for 10 minutes before re-equilibrating to the initial conditions for 4.1 minutes. Elution was monitored at 380 nm, 450 nm, and 460 nm for β -apo-8'-carotenal and at 380 nm and 438 nm for β -apo-10'-carotenal. β -Apocarotenoids in samples were quantified by comparing the peak area against a standard curve prepared with pure standard compounds.

Method 2: Analysis of β -apo-13-carotenone

Sample extracts were dissolved in 200 μ L of 1:1 v/v methanol/HPLC-MS grade water, syringe filtered (0.22 μ m pores; Millipore), and 20 μ L of the extract was injected for analysis. The HPLC system and column used for this analysis were described in method 1. The composition of the mobile phase was methanol (solvent A) and HPLC-MS grade water (solvent B). The flow rate was set at 0.8 mL/minute and a column temperature of 35 °C was maintained. Analytes were separated with 15% B for 15 minutes and elution was monitored at 345 nm. β -Apo-13-carotenone in samples was quantified by comparing the peak area against a standard curve prepared with known concentrations of pure β -apo-13-carotenone.

UHPLC-MS Analysis of Cellular Metabolites

Method 1: Analysis of cellular metabolites of β -apo-8'-carotenal

Cellular metabolites of β -apo-8'-carotenal were separated by an Agilent 1290 Infinity I UHPLC (Agilent Technologies, Santa Clara, CA) using a YMC C30 column (2.0 \times 150 mm, S3; Waters Corporation, Milford, MA) with a mobile phase of 80% methanol/20% water (solvent A) and 78% MTBE/20% methanol/2% water (solvent B) where both mobile phases contained 2% (w/v) ammonium acetate. The flow rate was set at 0.4 mL/minute and ramped up linearly from 0% B to 60% B over 7 minutes and re-equilibrated over the subsequent 5 minutes. The column temperature was maintained at 45 °C. The entire eluent flow was introduced to an Agilent 6550 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Santa Clara, CA) with positive ion electrospray source parameters of 400 °C sheath gas at 12 L/minute, 150 °C drying gas at 18 L/minute, 30 psi nebulizer, 3 kV Vcap, and 1.5 kV nozzle. Ion

funnel settings were 90 V low pressure funnel, 200 V high pressure funnel. Also, there was a DAD inline to collect UV-vis spectra from 230 nm through 640 nm.

Method 2: Analysis of cellular metabolites of β -apo-10'-carotenal

Cellular metabolites of β -apo-10'-carotenal were analyzed by an Agilent 1290 Infinity I UHPLC (Agilent Technologies, Santa Clara, CA) using an ACE super C18 column (2.1×150 mm, $2 \mu\text{m}$; Advanced Chromatography Technologies, Scotland) with a mobile phase of 80% methanol/20% water (solvent A) and 78% MTBE/20% methanol/2% water (solvent B) where both mobile phases contained 2% (w/v) ammonium acetate. The flow rate was set at 0.5 mL/minute and a column temperature of 45°C was maintained. Analytes were separated with 10% B for 1.5 minutes, followed by a linear gradient over 7 minutes to 65% B and subsequently to 100% B over 2 minutes before re-equilibrating to the initial conditions for 2 minutes. The entire eluent flow was introduced to an Agilent 6550 QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA) with positive ion electrospray source parameters of 400°C sheath gas at 12 L/minute, 150°C drying gas at 18 L/minute, 30 psi nebulizer, 3 kV Vcap, and 1.5 kV nozzle. Ion funnel settings were 90 V low pressure funnel, 200 V high pressure funnel. Also, there was a DAD inline to collect UV-vis spectra from 230 nm through 640 nm.

Statistical Analyses

All data are presented as means and standard deviations. Means and standard deviations were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA). Graphs were produced using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Stability of β -Apo-8'-carotenal, β -Apo-10'-carotenal, and β -Apo-13-carotenone

Given that β -apocarotenoids can undergo chemical oxidation and degradation during incubation in cell culture, we investigated the stability of these compounds in the same conditions as were used for the cell uptake experiments. Serum-free media containing 1 μ M or 5 μ M of individual β -apocarotenoids (β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone) were incubated in six-well plates without cells for up to 8 hours. Percentage recovery of β -apocarotenoids was calculated relative to the concentration of β -apocarotenoids in serum-free media at the start of the experiment. Figure 2 summarizes the results for the stability of β -apocarotenoids in cell culture conditions. After 1 hour of incubation, the recovery of 1 μ M β -apo-8'-carotenal in serum-free media was 97.9% whereas the recovery of 5 μ M β -apo-8'-carotenal was 93.3% at the same incubation time. There was a slight degradation of β -apo-8'-carotenal as incubation time increased but our recovery of this β -apocarotenal was still good. Specifically, the recovery of 1 μ M β -apo-8'-carotenal in serum-free media was 86.1% after 8 hours of incubation. We obtained similar results for the stability of 5 μ M β -apo-8'-carotenal after 8 hours of incubation.

Similarly, the recovery of β -apo-10'-carotenal at early time points was very good. The recovery of 1 μ M β -apo-10'-carotenal in serum-free media was 106.0% after 1 hour of incubation while the recovery of 5 μ M β -apo-10'-carotenal was 102.2% after 1 hour of incubation. As with β -apo-8'-carotenal, there was also minimal degradation of β -apo-10'-carotenal as incubation time increased. The recovery of 1 μ M β -apo-10'-carotenal in serum-free media was 73.7% after 8 hours of incubation. On the other hand, we obtained a higher recovery (85.2%) after 8 hours of incubation for 5 μ M β -apo-10'-carotenal. Furthermore, after 1 hour of incubation, the recovery of 1 μ M β -apo-13-carotenone in serum-free media was 91.3% while the recovery of 5 μ M β -apo-13-carotenone was 93.9% at the same incubation time. There was some degradation of β -apo-13-carotenone in serum-free media as incubation time increased. Specifically, the recovery of 1 μ M β -apo-13-carotenone in media was 76.6% after 8 hours of incubation. In contrast, the recovery of 5 μ M β -apo-13-carotenone was 86.8%. The results of these stability experiments are important

because they established that these compounds are very stable under cell culture conditions and do not rapidly degrade before we incubate them with cells.

Kinetics of Uptake of β -Apo-8'-carotenal: Effect of Incubation Time and Initial β -Apo-8'-carotenal Concentration

We investigated the kinetics of uptake of β -apo-8'-carotenal as a function of incubation time and concentration. Figures 3 - 6 summarize the results. Figure 3a shows a representative chromatogram of the uptake of β -apo-8'-carotenal into Caco-2 cells. The UV absorption spectra of the indicated peaks are also given (Figure 3b). When cells were incubated with β -apo-8'-carotenal for 1 hour, we detected two peaks (initially named compounds X and Y) in addition to β -apo-8'-carotenal. Compound X was the major peak detected while compound Y and β -apo-8'-carotenal were present in small amounts. Our HPLC conditions enabled us to resolve both cellular metabolites from the parent apocarotenal. We also observed that compounds X and Y do not get released into the media after they are formed within cells. We then determined the identities of the cellular metabolites detected. The components of cell extracts obtained after incubation with β -apo-8'-carotenal were separated by UHPLC (Figure 4a) and the accurate masses of the cellular metabolites were determined using a high resolution mass spectrometer. The major peak, compound X, differed from β -apo-8'-carotenal by a mass of +16 while the minor peak, compound Y, differed from β -apo-8'-carotenal by a mass of +18 (Figure 4b, Table 1).

Based on this UHPLC-MS analysis, we proposed putative compounds that could be metabolites X and Y. The proposed compounds were synthesized, purified by TLC, and characterized by NMR spectroscopy. Subsequently, we analyzed the synthetic compounds by HPLC-DAD (see HPLC analysis - method 1) and compared their retention times (RT) and UV absorption spectra to those of the cellular metabolites of β -apo-8'-carotenal. Based on accurate mass as well as identical RT and UV absorption spectrum of an authentic standard, compound X was confirmed as β -apo-8'-carotenoic acid. In addition, we identified the minor peak (Y) as 5, 6-epoxy- β -apo-8'-carotenol based on its accurate mass, RT, and UV absorption spectrum showing fine structure consistent with an alcohol and a hypsochromic shift in

absorption maximum consistent with the loss of a double bond (Figure 3b, Table 1). Figure 5a shows the kinetics of uptake of β -apo-8'-carotenal for an individual experiment. Fully differentiated Caco-2 cells were incubated with 1 μ M β -apo-8'-carotenal for 30 minutes - 8 hours. As mentioned previously, we observed the rapid accumulation of β -apo-8'-carotenal in cells and detected two metabolites, the major one of which was confirmed as β -apo-8'-carotenoic acid and the other we identified as 5, 6-epoxy- β -apo-8'-carotenol. We observed that the formation of β -apo-8'-carotenoic acid in cells increased as a function of incubation time and this corresponds with a decrease in the concentration of β -apo-8'-carotenal that accumulated within cells. In addition, we observed the disappearance of β -apo-8'-carotenal from media as a function of incubation time (Figure 5b).

Taken together with the cell uptake data, we calculated the percent recovery of β -apo-8'-carotenal at each incubation time point. In preliminary experiments, we observed the rapid uptake of β -apo-8'-carotenal into cells but we could only account for about 10% of this apocarotenal after 24 hours of incubation (data not shown). Thus, we reduced our maximum incubation time to 8 hours. β -Apo-8'-carotenal and β -apo-8'-carotenoic acid accounted for 97.3% of the initial β -apo-8'-carotenal concentration after 30 minutes of incubation and both of those compounds accounted for 83.2% of the initial β -apo-8'-carotenal concentration after 1 hour of incubation. These recovery values were expected because there is some degradation of β -apo-8'-carotenal with increasing incubation time. Also, the amount of 5, 6-epoxy- β -apo-8'-carotenol that accumulated in Caco-2 cells was much lower than that of β -apo-8'-carotenoic acid based on peak height comparisons (Figure 3a). We also studied the uptake of β -apo-8'-carotenal as a function of concentration. Figure 6 describes the dose dependent accumulation of β -apo-8'-carotenal into Caco-2 cells. When differentiated monolayers were incubated with β -apo-8'-carotenal (1–5 μ M) for 4 hours, we observed that the amount of β -apo-8'-carotenal in cells increased with increasing initial concentration of β -apo-8'-carotenal. Also, we observed that the formation of β -apo-8'-carotenoic acid in cells increased linearly. Similarly to the time course experiments, the amount of 5, 6-epoxy- β -apo-8'-carotenol formed within cells was much lower than that of β -apo-8'-carotenoic acid based on peak height comparisons for the different levels of concentration we studied (data not shown).

Kinetics of Uptake of β -Apo-10'-carotenal: Effects of Incubation Time

We investigated the kinetics of uptake of β -apo-10'-carotenal into Caco-2 cells as a function of incubation time. Figure 7a is a representative chromatogram of the uptake and metabolism of β -apo-10'-carotenal in Caco-2 cells. The UV absorption spectra of the indicated peaks are also shown (Figure 7b). When cells were incubated with β -apo-10'-carotenal for 30 minutes, two peaks (initially named compounds A and B) were detected along with β -apo-10'-carotenal. Compound A was the major peak detected whereas compound B and β -apo-10'-carotenal were present in minor amounts. We also observed that compounds A and B do not get released into the media after they are formed within cells. We next established the identities of cellular metabolites A and B. Compound A elutes at 3.63 minutes and its UV absorption spectrum has a fine structure with absorption maxima at 378 nm and 394 nm. On the other hand, compound B elutes at 4.01 minutes and its UV absorption spectrum has a fine structure with absorption maxima at 379 nm and 395 nm. This indicates that both compounds are more hydrophilic than β -apo-10'-carotenal. We initially thought that the cellular metabolites of β -apo-10'-carotenal would be similar to those observed with β -apo-8'-carotenal. Hence, we analyzed several short-chain β -apocarotenoids in the same HPLC-DAD conditions used to separate β -apo-10'-carotenal and its metabolites. The standard compounds examined were β -apo-13-carotenone, retinal, retinoic acid, β -apo-12'-carotenal, β -apo-10'-carotenoic acid, and β -apo-10'-carotenol. However, none of these compounds eluted at the same RT as A or B (Figure 8).

We then determined the accurate masses of both metabolites using a high resolution mass spectrometer. The major peak, compound A, differed from β -apo-10'-carotenal by a mass of +18 whereas the minor peak, compound B, differed from β -apo-10'-carotenal by a mass of +4 (Table 2). Based on accurate mass, RT, and UV absorption spectra showing fine structure consistent with an alcohol and a hypsochromic shift in absorption maximum consistent with the loss of a double bond, compound A was identified as 5, 6-epoxy- β -apo-10'-carotenol while compound B is likely a dihydro- β -apo-10'-carotenol (Figure 7b, Table 2). Figure 9a shows the kinetics of uptake of β -apo-10'-carotenal for an individual experiment. We observed the rapid uptake of β -apo-10'-carotenal into cells up to 1 hour. There was a

subsequent decrease in cellular accumulation of β -apo-10'-carotenal with increasing incubation time (data not shown). Also, we observed the disappearance of β -apo-10'-carotenal from media as a function of time (Figure 9b). We then determined the recovery of β -apo-10'-carotenal in cells and media after incubation. The percent recovery of β -apo-10'-carotenal after incubation was about 75.5% at 15 minutes, 71.0% at 30 minutes, 73.6% at 45 minutes, and 57.6% at 1 hour.

Effects of Incubation Time and Initial β -Apo-13-carotenone Concentration on the Uptake and Metabolism of β -Apo-13-carotenone

We investigated the kinetics of uptake of β -apo-13-carotenone into Caco-2 cells as a function of incubation time and concentration. Figure 10a is a representative chromatogram of the uptake of β -apo-13-carotenone into Caco-2 cells. The UV absorption spectra of the indicated peaks are also given (Figure 10b). In contrast to the β -apocarotenals, we did not detect any UV-absorbing metabolites when cells were incubated with 5 μ M β -apo-13-carotenone for 30 minutes or 8 hours. Figure 11a summarizes the kinetics of uptake of β -apo-13-carotenone as a function of incubation time. We observed the rapid uptake of β -apo-13-carotenone into cells but subsequent extensive degradation with increasing incubation time. Also, we observed the disappearance of β -apo-13-carotenone from media as a function of time (Figure 11b). In addition, we determined the recovery of β -apo-13-carotenone in cells after incubation. Initial experiments had shown that β -apo-13-carotenone recovery in cells after 4 hours of incubation was less than 5% (data not shown). Thus, we reduced our maximum incubation time to 1 hour. The percent recovery of β -apo-13-carotenone after incubation was about 62.1% at 15 minutes, 59.2% at 30 minutes, 53.1% at 45 minutes, and 49.6% at 1 hour. These recovery values were expected because there is some degradation of β -apo-13-carotenone with increasing incubation time. Figure 12 summarizes the dose dependent accumulation of β -apo-13-carotenone into Caco-2 cells. Fully differentiated monolayers of Caco-2 cells were incubated for 45 minutes with β -apo-13-carotenone (1–5 μ M). We observed that the cellular accumulation of β -apo-13-carotenone increased with increasing initial concentration of β -apo-13-carotenone.

DISCUSSION

β -Apocarotenoids are eccentric cleavage products of carotenoids formed by chemical and enzymatic oxidations. These cleavage products demonstrate potent bioactivity in different experimental models. β -Apocarotenoids inhibit the cell proliferation of breast tumor cells via an RAR-independent mechanism (26) and human leukemia cell line HL-60 (27). These compounds have also been shown to upregulate the gene expression of adipocyte markers during the differentiation of 3T3-L1 cells (28). In addition, β -apocarotenoids can bind to retinoid- and/or lipid-responsive transcription factors and may modulate these proteins' abilities to stimulate transcription of target genes (7, 11, 12). The presence of β -apocarotenoids in carotenoid-rich fruits, vegetables, and other plant food items has been reported by our laboratory (14) and others (29-31). Furthermore, numerous lines of evidence demonstrate that these eccentric cleavage products are detected in the serum or tissues of WT (32-34) and BCO knockout animals (34, 35) as well as the plasma of humans consuming diets containing carotenoids or given a bolus of β -carotene (7, 36). Collectively, these data suggest that β -apocarotenoids might be directly absorbed from the diet. Here, we have examined the uptake of β -apo-8'-carotenal, β -apo-10'-carotenal, and β -apo-13-carotenone into Caco-2 cells. Also, we have demonstrated the metabolism of these eccentric cleavage products after cellular uptake.

When cells were incubated with β -apo-8'-carotenal for 1 hour, we detected two peaks (initially named compounds X and Y) in addition to β -apo-8'-carotenal. Compound X was the major peak detected while compound Y and β -apo-8'-carotenal were present in minor amounts (Figure 3a). Based on accurate mass as well as identical RT and UV absorption spectrum of an authentic standard, compound X was confirmed as β -apo-8'-carotenoic acid. Compound Y was identified as 5, 6-epoxy- β -apo-8'-carotenol based on its accurate mass, RT, and UV absorption spectrum showing fine structure consistent with an alcohol and a hypsochromic shift in absorption maximum consistent with the loss of a double bond (Figure 3b, Table 1). The results presented here are in agreement with previous studies that examined the absorption of β -apo-8'-carotenal in different species. Early work detected β -apo-8'-carotenoic acid as a urinary metabolite in dogs given a daily dose of β -apo-8'-carotenal (37). Subsequent work by multiple research groups also

corroborate that this apocarotenoic acid is a major metabolite present in the serum and tissues of animals fed β -apo-8'-carotenal (38, 39). In one of those studies, the intact aldehyde and β -apo-8'-carotenol were also present in minor amounts (38). This is similar to what is observed in the present study - the difference being that we detected 5, 6-epoxy- β -apo-8'-carotenol.

Although there is limited evidence, it is not too far-fetched to suggest that β -apo-8'-carotenal is similarly converted to β -apo-8'-carotenoic acid in humans. Olson and colleagues (40) examined the uptake and metabolism of a single dose of β -apo-8'-carotenal in healthy male volunteers with adequate vitamin A status. β -Apo-8'-carotenal was rapidly absorbed and was extensively metabolized in all subjects. Serum metabolites observed were β -apo-8'-carotenoic acid, β -apo-8'-carotenol, β -apo-8'-carotenyl palmitate, β -apo-10'-carotenol, and β -apo-12'-carotenal. Unsurprisingly, the unchanged aldehyde was only detected in minor amounts. Taken together, these data suggest that the major proportion of β -apo-8'-carotenal is metabolized to the corresponding β -apocarotenoic acid (38). This metabolic conversion is similarly observed with retinal, which can be converted to retinol or retinoic acid. Previous studies also demonstrate the formation of epoxy metabolites of vitamin A *in vivo* (41, 42). Thus, we propose that β -apo-8'-carotenal taken up from carotenoid-rich foods is metabolized by intestinal cells according to the pathway shown in Figure 13.

We next investigated the uptake and metabolism of β -apo-10'-carotenal in cells. *In vitro* enzymatic assays show that β -apo-10'-carotenal is the major product of eccentric cleavage of β -carotene by BCO2 (43, 44). The unchanged aldehyde has been detected in the plasma and tissues of mice on a β -carotene containing diet (45). The corresponding alcohol and acid are also generated *in vivo* (35). WT and BCO1^{-/-} but not BCO2^{-/-} and BCO1^{-/-}/BCO2^{-/-} mice accumulate β -apo-10'-carotenol, mainly in its esterified form, and β -apo-10'-carotenoic acid in their livers when given a diet with β -carotene as the only vitamin A source (35, 44). There is renewed interest in β -apo-10'-carotenoids because of emerging reports about the role these compounds play in embryogenesis. Costabile et al. (20) showed that β -apo-10'-carotenal and β -apo-10'-carotenol are biologically active metabolites that regulate placental lipoprotein biosynthesis and allow for effective transport of β -carotene from the maternal circulation to the embryo independent of changes to the

mRNA expression of RA target genes. Instead, these β -apocarotenoids activate hepatic nuclear factor 4α , which in turn upregulates the gene expression and activity of the placental microsomal triglyceride transfer protein (20), which is important for the assembly and secretion of apoB containing lipoproteins (46, 47). Recently, Quadro and colleagues showed that β -apo-10'-carotenal can support normal embryonic development during periods of vitamin A deficiency (48).

Caco-2 cells incubated with β -apo-10'-carotenal for 30 minutes produced two peaks detected by HPLC-DAD (Figure 7a). The two metabolites, appearing at RT 3.63 minutes (compound A) and 4.01 minutes (compound B) both have UV absorption spectra with fine structure. Compound A has absorption maxima at 378 nm and 394 nm whereas compound B has absorption maxima at 379 nm and 395 nm. We initially thought that the cellular metabolites of β -apo-10'-carotenal would be similar to those observed with β -apo-8'-carotenal. Thus, we analyzed β -apo-13-carotenone, retinal, retinoic acid, β -apo-12'-carotenal, β -apo-10'-carotenoic acid, and β -apo-10'-carotenol by HPLC-DAD. However, none of the compounds tested had the RT or absorption maxima of the metabolites (Figure 8). We then determined the accurate masses of both metabolites using a high resolution mass spectrometer. Based on accurate mass, RT, and UV absorption spectrum showing fine structure consistent with an alcohol and a hypsochromic shift in absorption maximum consistent with the loss of a double bond, compound A was identified as 5, 6-epoxy- β -apo-10'-carotenol whereas compound B is likely a dihydro- β -apo-10'-carotenol (Figure 7b, Table 2).

Several studies suggest that β -oxidation, involving the stepwise cleavage of two carbons, is a possible pathway for the metabolism of β -apocarotenoids to shorter chain β -apocarotenoids, retinal, and retinoic acid. Wang et al. (49) showed that β -apo-10'-carotenoic acid, β -apo-12'-carotenoic acid, and β -apo-14'-carotenoic acid are produced following *in vitro* incubation of β -apo-8'-carotenoic acid with mitochondrial fractions isolated from rabbit livers. Similarly, Al-Hasani et al. (39) reported the formation of β -apo-10'-carotenoic acid and β -apo-12'-carotenoic acid in the serum and tissues of vitamin A-depleted quails maintained on a diet with β -apo-10'-carotenal as the only source of provitamin A activity. We have now proposed two additional metabolites (5, 6-epoxy- β -apo-10'-carotenol and a dihydro- β -apo-10'-

carotenol) that may be formed when β -apo-10'-carotenal taken up from carotenoid-rich foods is metabolized by intestinal cells. The chemical structures for the newly proposed metabolites are shown in Figure 14.

We also examined the uptake and metabolism of β -apo-13-carotenone. Caco-2 cells incubated with β -apo-13-carotenone for 30 minutes produced an individual peak with RT and absorption maximum equivalent to authentic β -apo-13-carotenone standard. On the other hand, when cells were incubated with β -apo-13-carotenone for 4 or 8 hours, no peaks were detected (Figure 10). One possible explanation is that the β -apo-13-carotenone might be metabolized to short chain metabolites with limited UV absorption. We also cannot rule out the possibility that the compound was converted into metabolites with UV absorption but that once the metabolites were generated, they were rapidly degraded.

In summary, our results are consistent with a recent clinical trial showing a lack of direct absorption of β -apocarotenals in healthy volunteers consuming a daily dose of high β -carotene tomato juice, containing precisely quantitated amounts of β -apocarotenals, for 4 weeks (31). The findings from that study also support our conclusions that β -apocarotenals are rapidly metabolized in the intestine. In contrast to the β -apocarotenals, β -apo-13-carotenone was quantitatively detected in all subjects including the control group consuming a carotenoid-free juice. Thus, the authors of that study concluded that β -apo-13-carotenone could possibly be formed from the oxidative cleavage of preformed vitamin A. Further research is required to understand the mechanisms of absorption and metabolism of β -apocarotenoids in animal models and humans.

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TABLES

Table 1. Mass for β -apo-8'-carotenal and the cellular metabolites of β -apo-8'-carotenal.

Compound	Mass (g/mol)
β -apo-8'-carotenal	416.6490 ^a
Compound X	433.3101 ^b
Compound Y	435.3258 ^b

^a Value indicates nominal mass.

^b Value indicates accurate mass as determined by UHPLC-MS.

Table 2. Mass for β -apo-10'-carotenal and the cellular metabolites of β -apo-10'-carotenal.

Compound	Mass (g/mol)
β -apo-10'-carotenal	376.5840 ^a
Compound A	394.2866 ^b
	395.2935 ^b
Compound B	380.3072 ^b

^a Value indicates nominal mass.

^b Value indicates accurate mass as determined by UHPLC-MS.

FIGURES

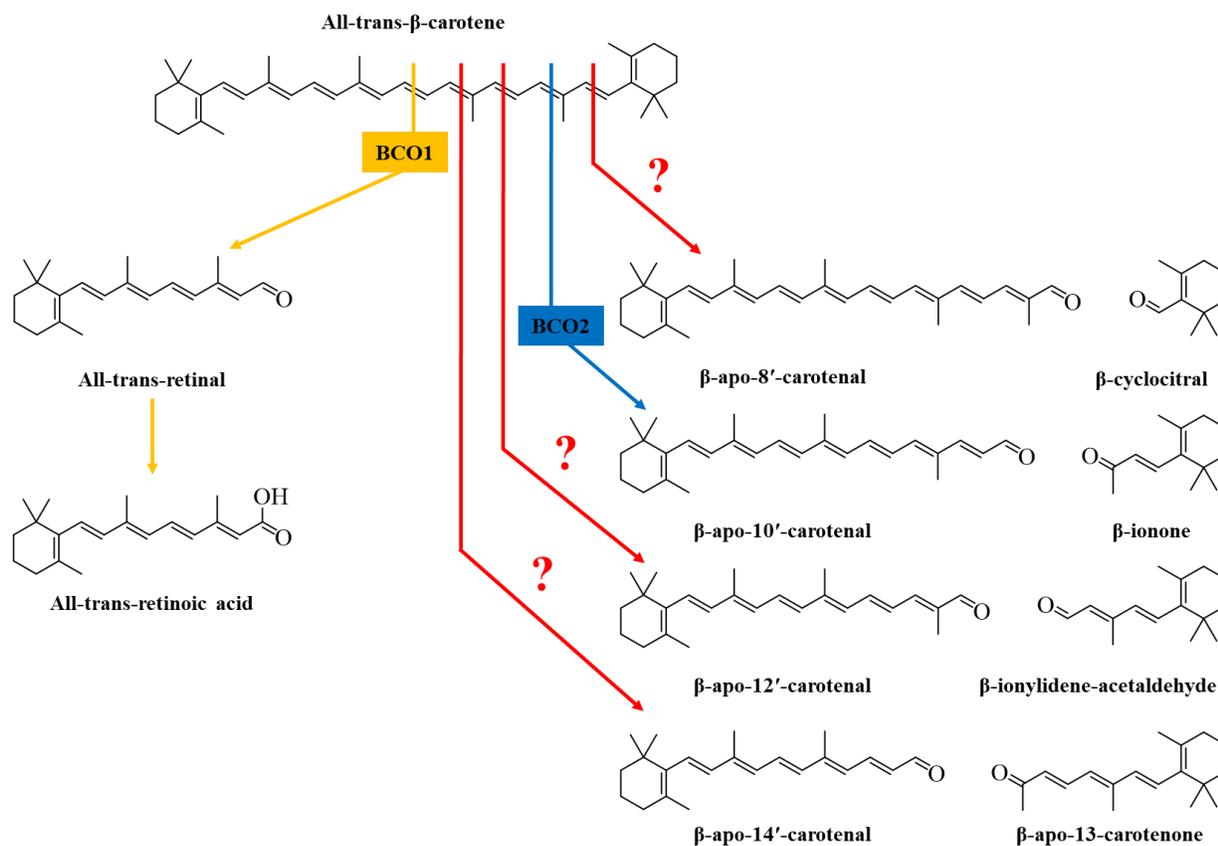


Figure 1. Structures of the products of the central and eccentric cleavage of β -carotene

All-trans- β -carotene can be cleaved by β -carotene 15, 15'-oxygenase (BCO1) to yield two molecules of retinal, which can be converted to retinol or retinoic acid. On the other hand, the main product of the eccentric cleavage pathway of β -carotene is β -apo-10'-carotenal and this reaction is catalyzed by β -carotene 9', 10'-oxygenase (BCO2). Also, β -carotene can undergo chemical and enzymatic oxidations *in vivo* and in foods to yield other long-chain and short-chain β -apocarotenoids. The exact mechanisms and enzymes responsible for these products are unknown.

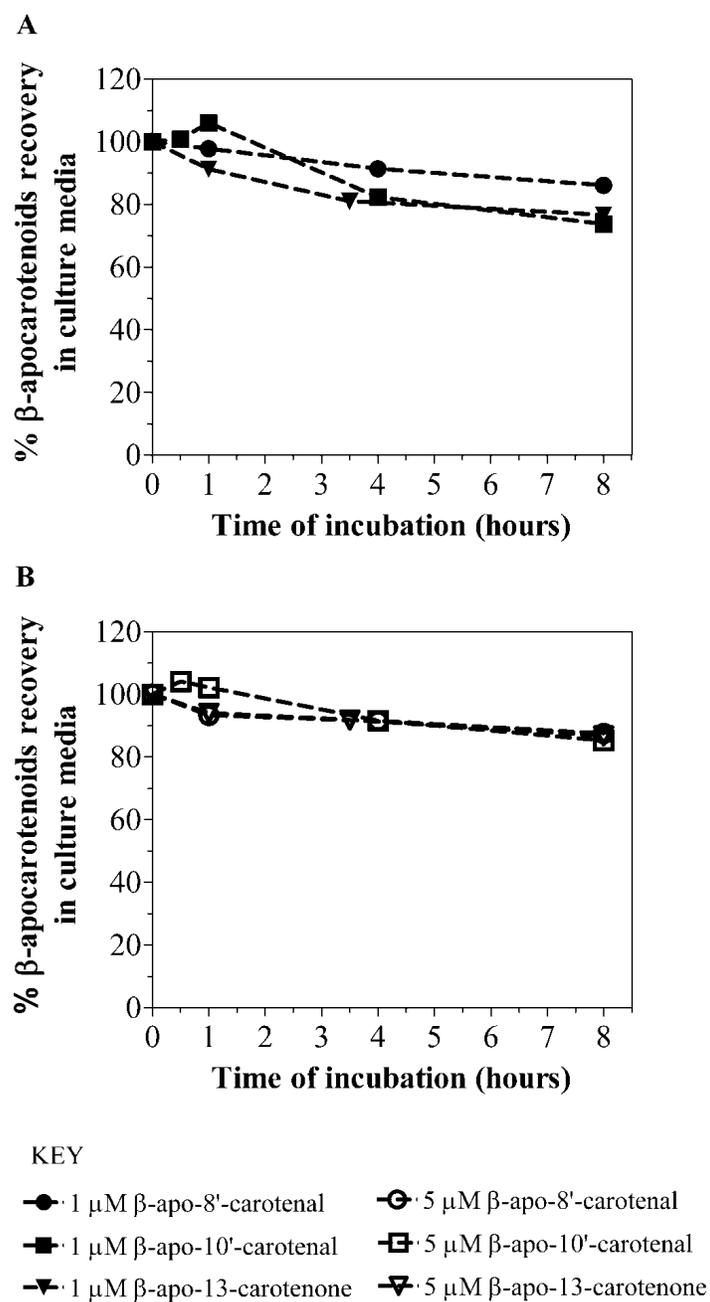


Figure 2. Stability of β-apocarotenoids in cell culture conditions

Serum-free media containing Tween 40 micelles of 1 μM (**A**) or 5 μM (**B**) of individual β-apocarotenoids (β-apo-8'-carotenal, β-apo-10'-carotenal, and β-apo-13-carotenone) were incubated at 37 °C for time points up to 8 hours. Following incubation, β-apocarotenoids were extracted from the media and analyzed by

HPLC-DAD. Percent recovery at each time point was calculated relative to the initial concentration of β -apocarotenoids in cell culture media at the start of the stability experiment.

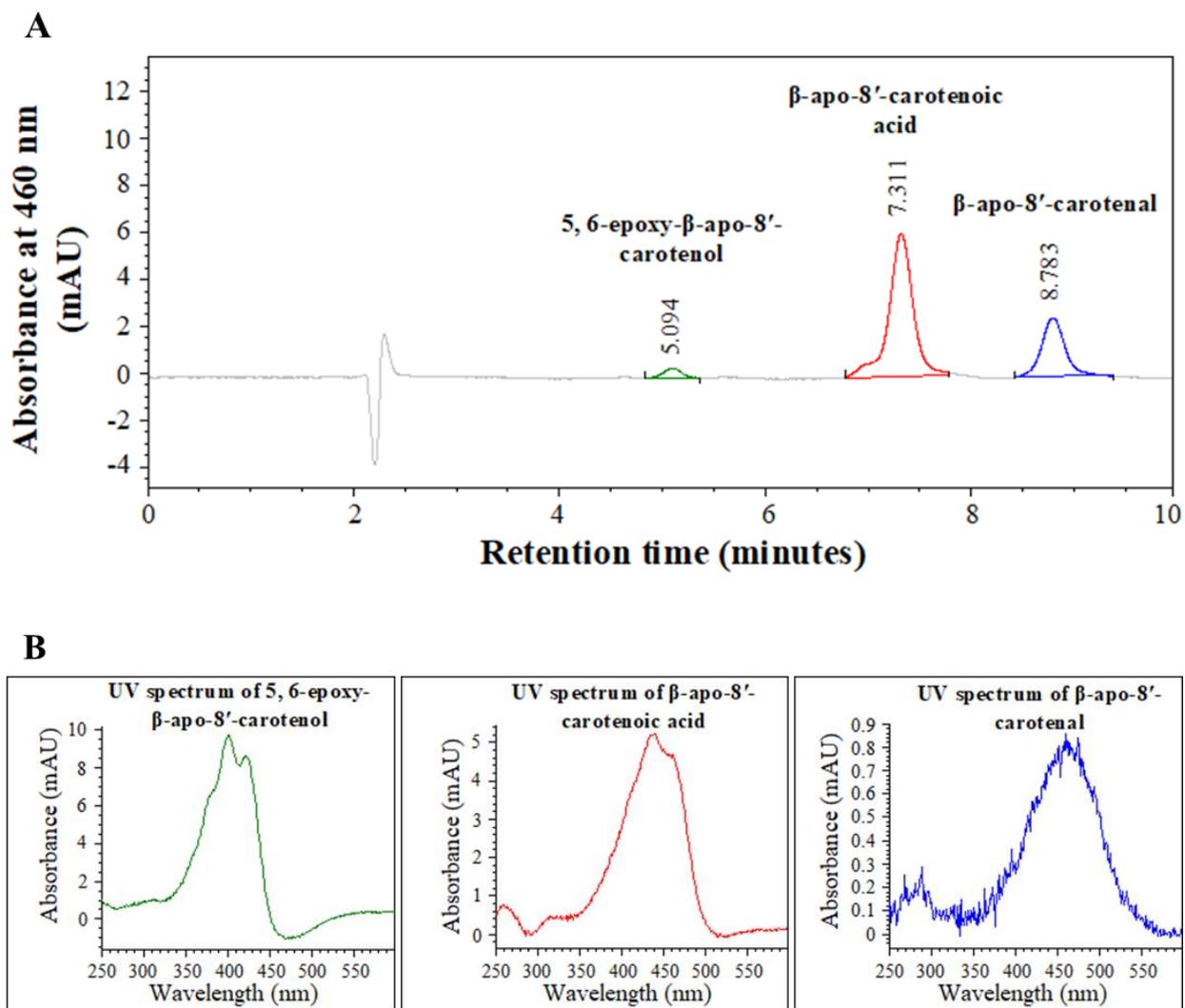


Figure 3. HPLC-DAD analysis of cell extract after incubation with β -apo-8'-carotenol

(A): Representative HPLC-DAD chromatogram of the uptake of 1 μ M β -apo-8'-carotenol into fully differentiated Caco-2 cells after incubation at 37 $^{\circ}$ C for 1 hour. (B): UV-vis spectra of β -apo-8'-carotenol and its cellular metabolites, the major one of which was confirmed as β -apo-8'-carotenoic acid (compound X) and the other we identified as 5, 6-epoxy- β -apo-8'-carotenol (compound Y).

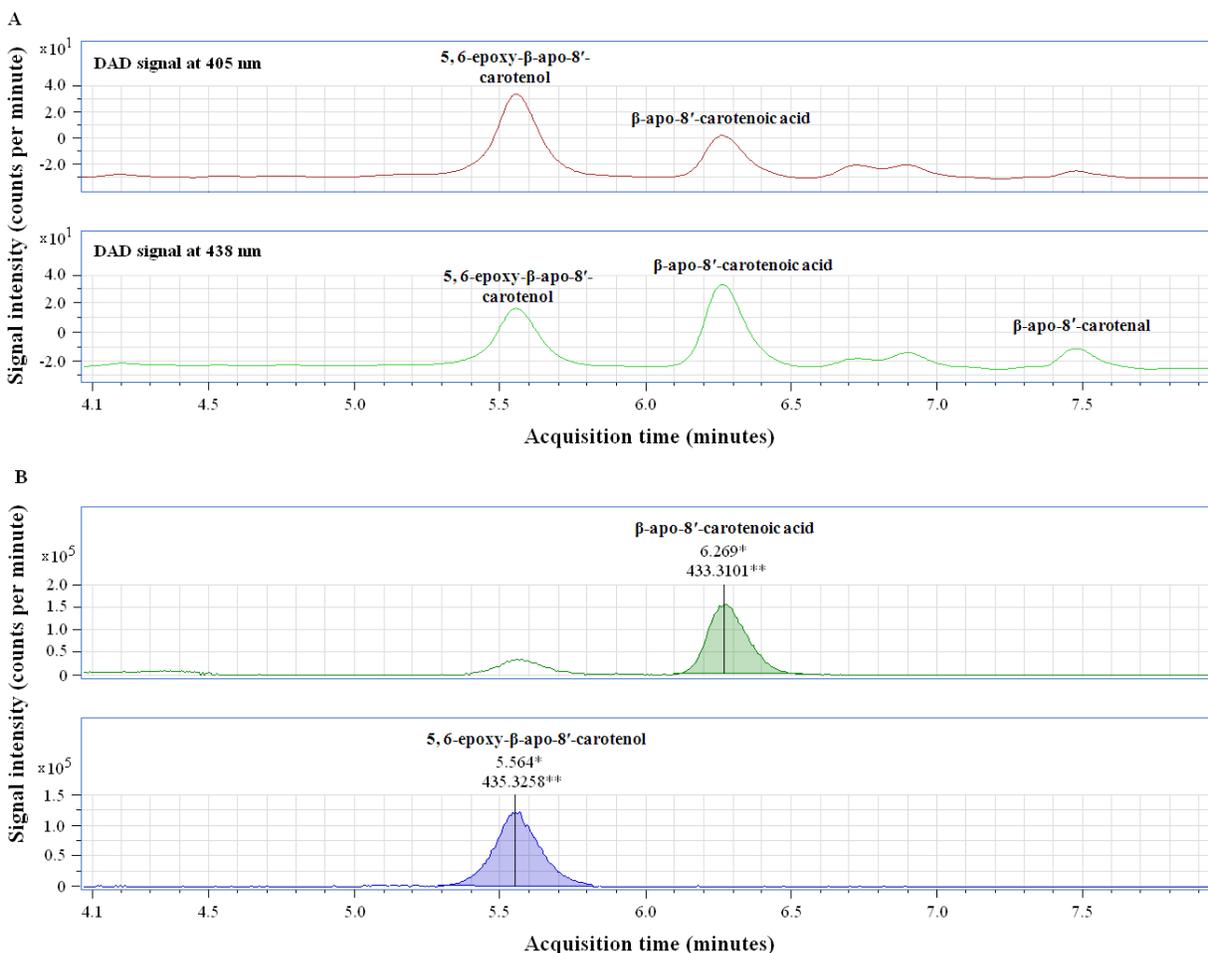


Figure 4. UHPLC-MS analysis of cellular metabolites of β -apo-8'-carotenol

(A): UHPLC separation of molecules in Caco-2 cells incubated with 1 μ M β -apo-8'-carotenol for 1 hour prior to accurate mass analysis. (B): Determination of the accurate masses for the cellular metabolites of β -apo-8'-carotenol by quadrupole time-of-flight (QTOF) mass spectrometry. The major peak, compound X, differed from β -apo-8'-carotenol by a mass of +16 while the minor peak, compound Y, differed from β -apo-8'-carotenol by a mass of +18. Based on accurate mass, RT, and UV absorption spectra, compound X was identified as β -apo-8'-carotenoic acid while compound Y is 5, 6-epoxy- β -apo-8'-carotenol. Note: * indicates acquisition time while ** indicates accurate mass for each peak shown.

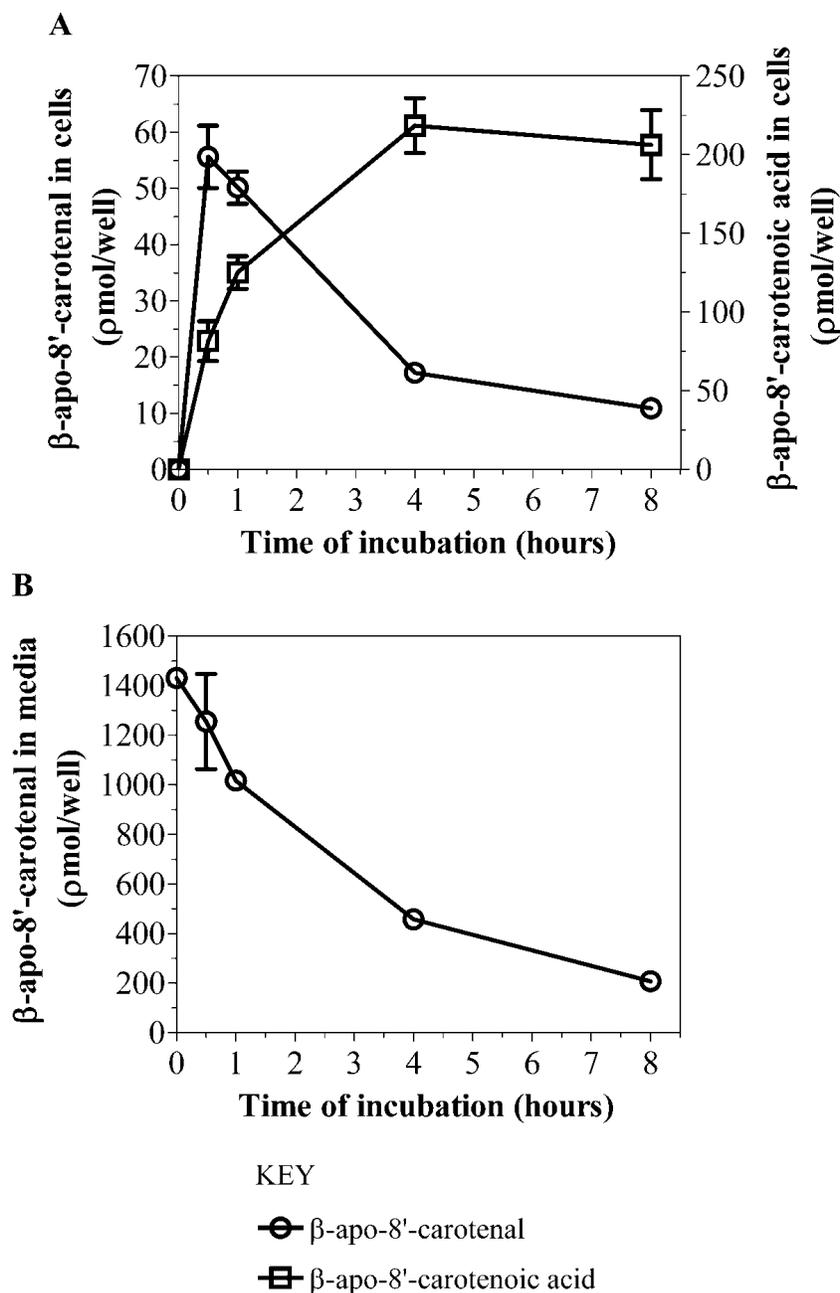


Figure 5. (A): Time course of uptake and metabolism of β -apo-8'-carotenal in Caco-2 cells. (B): Effect of incubation time on the disappearance of β -apo-8'-carotenal from media.

Differentiated Caco-2 cells were incubated with serum-free media containing 1 μ M β -apo-8'-carotenal at 37 °C for 30 minutes, 1, 4, and 8 hours. After incubation, the extracts from media and cells were analyzed using HPLC-DAD. The results shown represent an individual experiment and the points are means \pm

standard deviations of 3 or more wells. Furthermore, the data shown in (A) have been confirmed with two independent experiments.

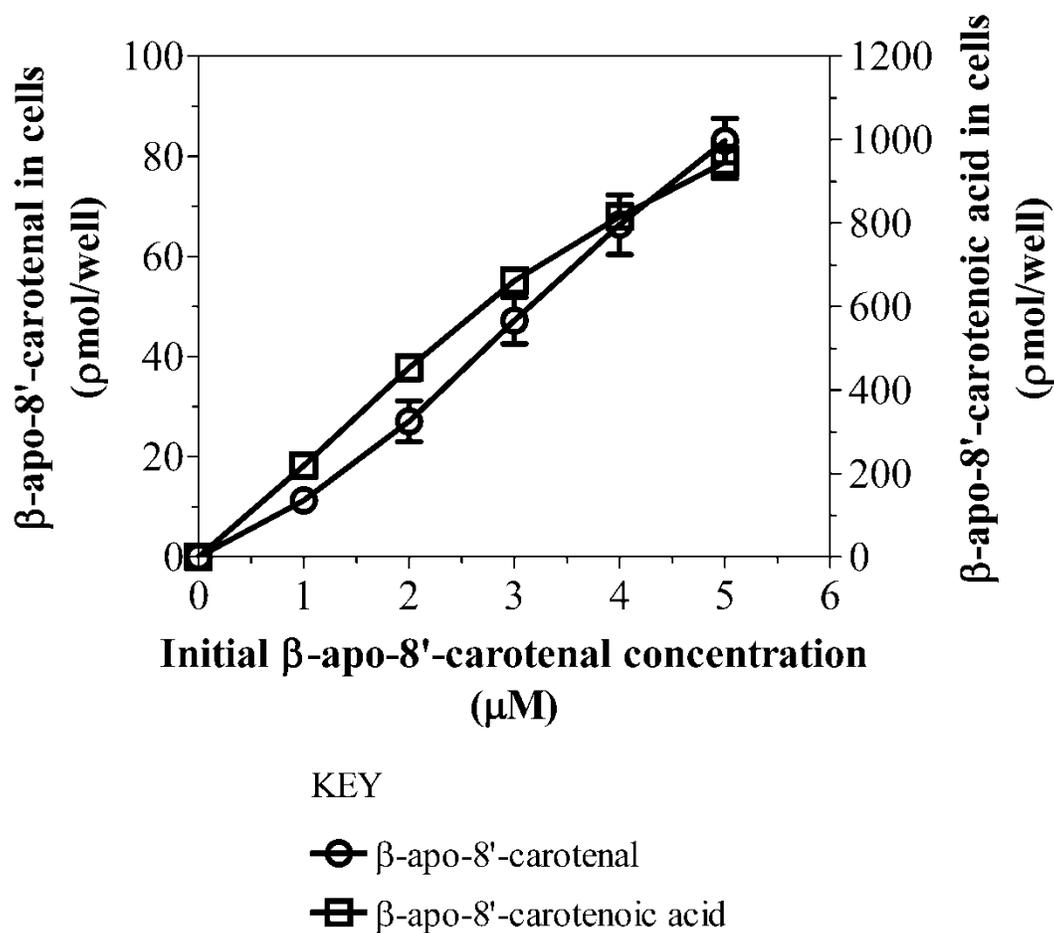


Figure 6. Dose dependent uptake and metabolism of β -apo-8'-carotenal in Caco-2 cells

Differentiated Caco-2 cells were incubated with serum-free media containing 1, 2, 3, 4, or 5 μ M β -apo-8'-carotenal at 37 °C for 4 hours. After incubation, the extracts from cells were analyzed using HPLC-DAD. The results shown represent an individual experiment and the points are means \pm standard deviations of 3 or more wells. Furthermore, the data shown above have been confirmed with three independent experiments.

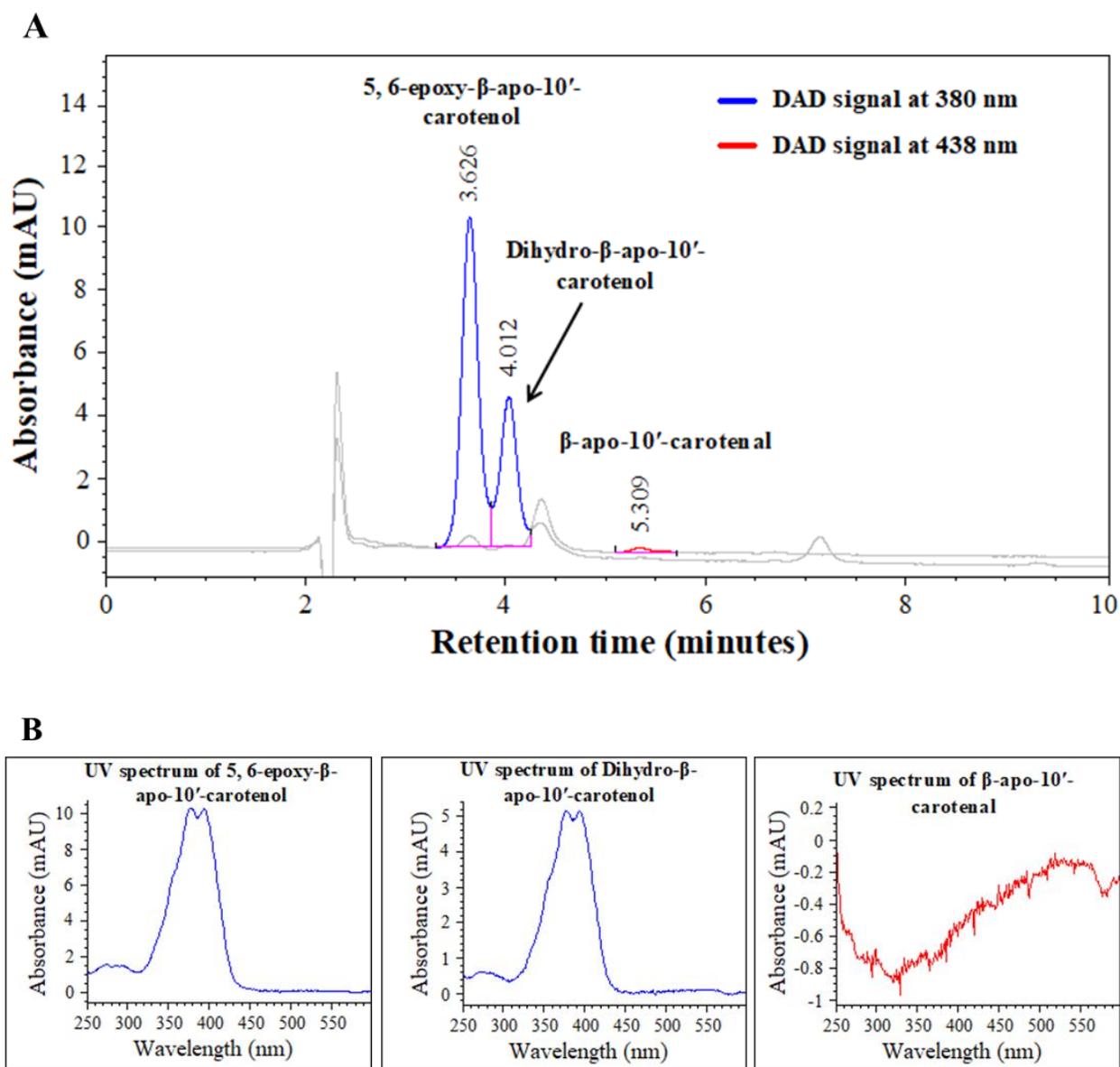
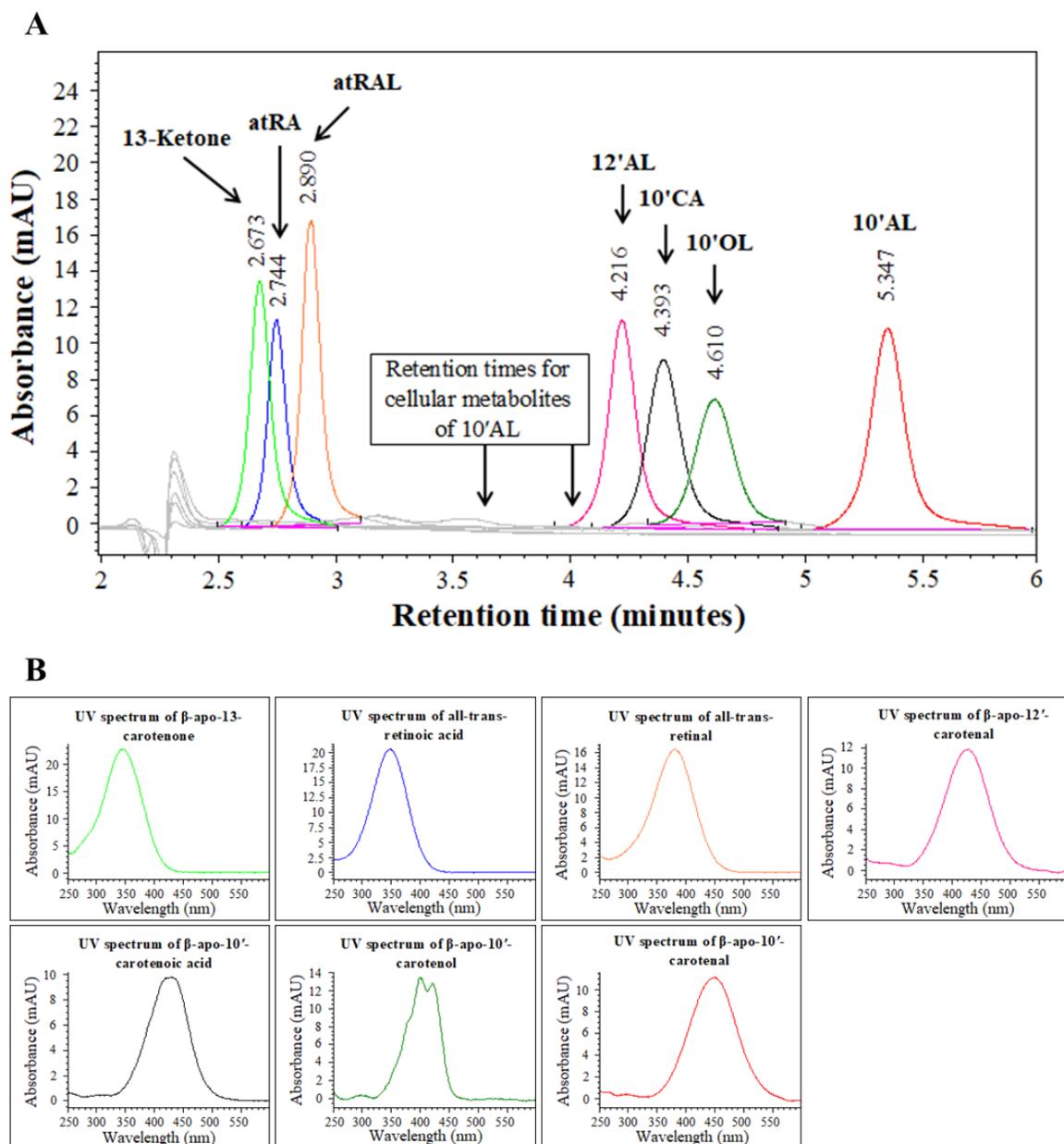


Figure 7. HPLC-DAD analysis of cell extract after incubation with β -apo-10'-carotenol

(A): Representative HPLC-DAD chromatogram of the uptake of 1 μ M β -apo-10'-carotenol into fully differentiated Caco-2 cells after incubation at 37 $^{\circ}$ C for 4 hours. (B): UV-vis spectra of β -apo-10'-carotenol and its cellular metabolites, the major one of which was identified as 5, 6-epoxy- β -apo-10'-carotenol (compound A) and the other is likely a dihydro- β -apo-10'-carotenol (compound B).



Abbreviations: 13-ketone means β -apo-13-carotenone; atRA means all-trans-retinoic acid; atRAL means all-trans-retinal; 12'AL means β -apo-12'-carotenal; 10'CA means β -apo-10'-carotenoic acid; 10'OL means β -apo-10'-carotenol; 10'AL means β -apo-10'-carotenal

Figure 8. HPLC-DAD analysis of possible cellular metabolites of β -apo-10'-carotenal

(A): Overlap of HPLC-DAD chromatograms for reference compounds of known retinoids and β -apocarotenoids. (B): UV-vis spectra for the retinoids and β -apocarotenoids analyzed by HPLC-DAD.

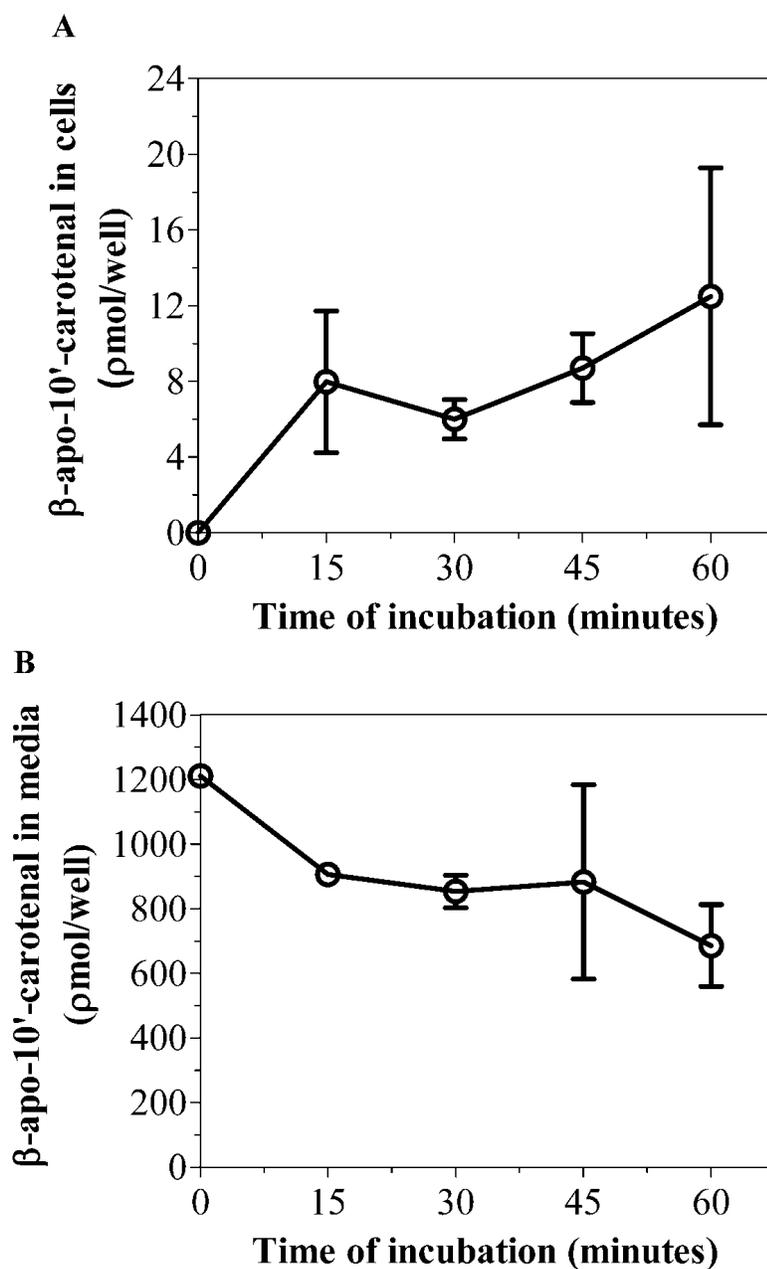


Figure 9. (A): Time course of uptake and metabolism of β -apo-10'-carotenal in Caco-2 cells. (B): Effect of incubation time on the disappearance of β -apo-10'-carotenal from media.

Differentiated Caco-2 cells were incubated with serum-free media containing 1 μ M β -apo-10'-carotenal at 37 $^{\circ}$ C for 15, 30, 45, and 60 minutes. After incubation, the extracts from media and cells were analyzed by HPLC-DAD. The results shown represent an individual experiment and the points are means \pm standard

deviations of 3 or more wells. Furthermore, the data shown in (A) have been confirmed with two independent experiments.

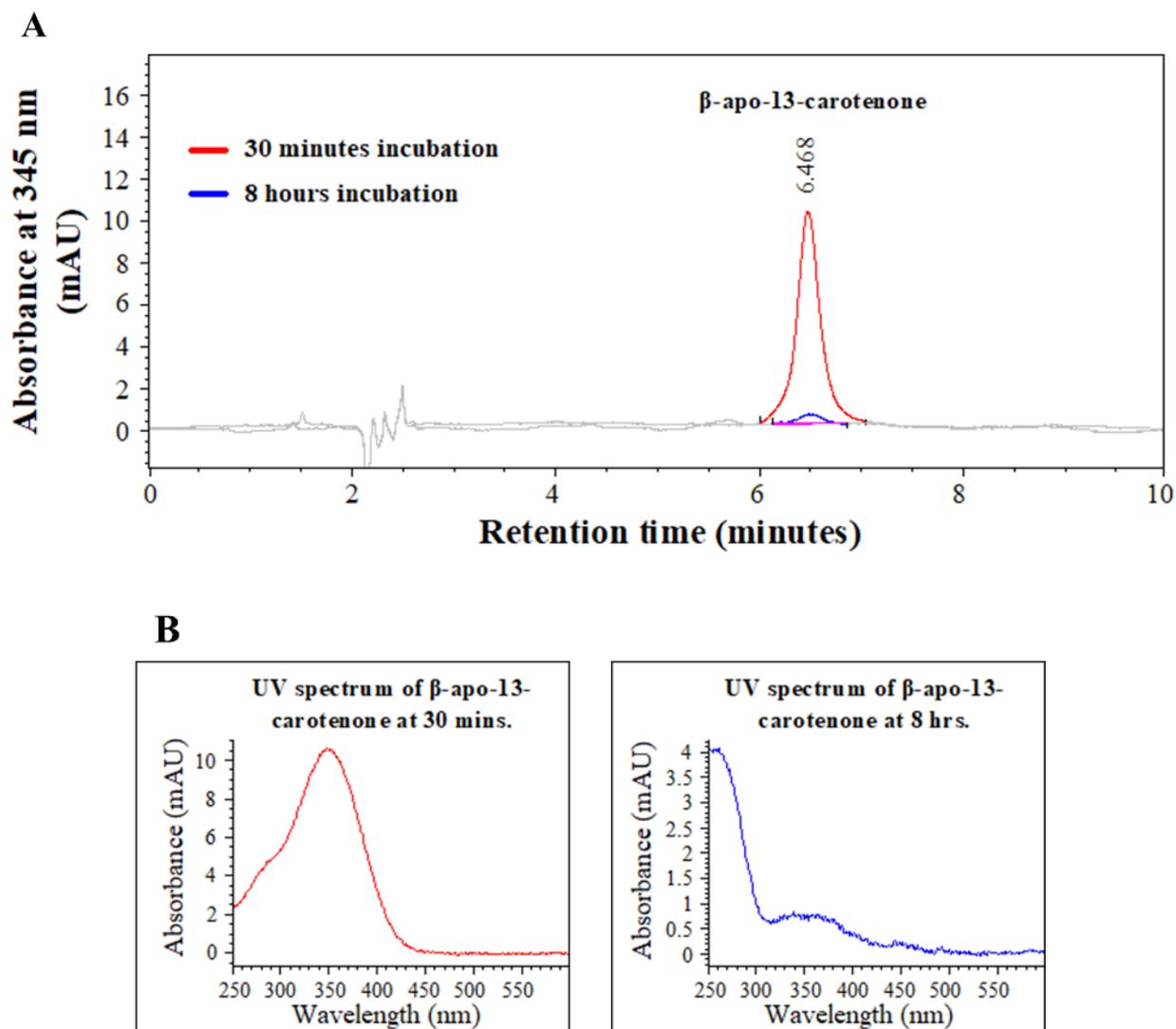


Figure 10. HPLC-DAD analysis of cell extract after incubation with β -apo-13-carotenone

(A): Representative HPLC-DAD chromatogram of the uptake of 5 μ M β -apo-13-carotenone into fully differentiated Caco-2 cells after incubation at 37 $^{\circ}$ C for either 30 minutes or 8 hours. (B): UV-vis spectra of β -apo-13-carotenone at both incubation time points. No cellular metabolites were detected.

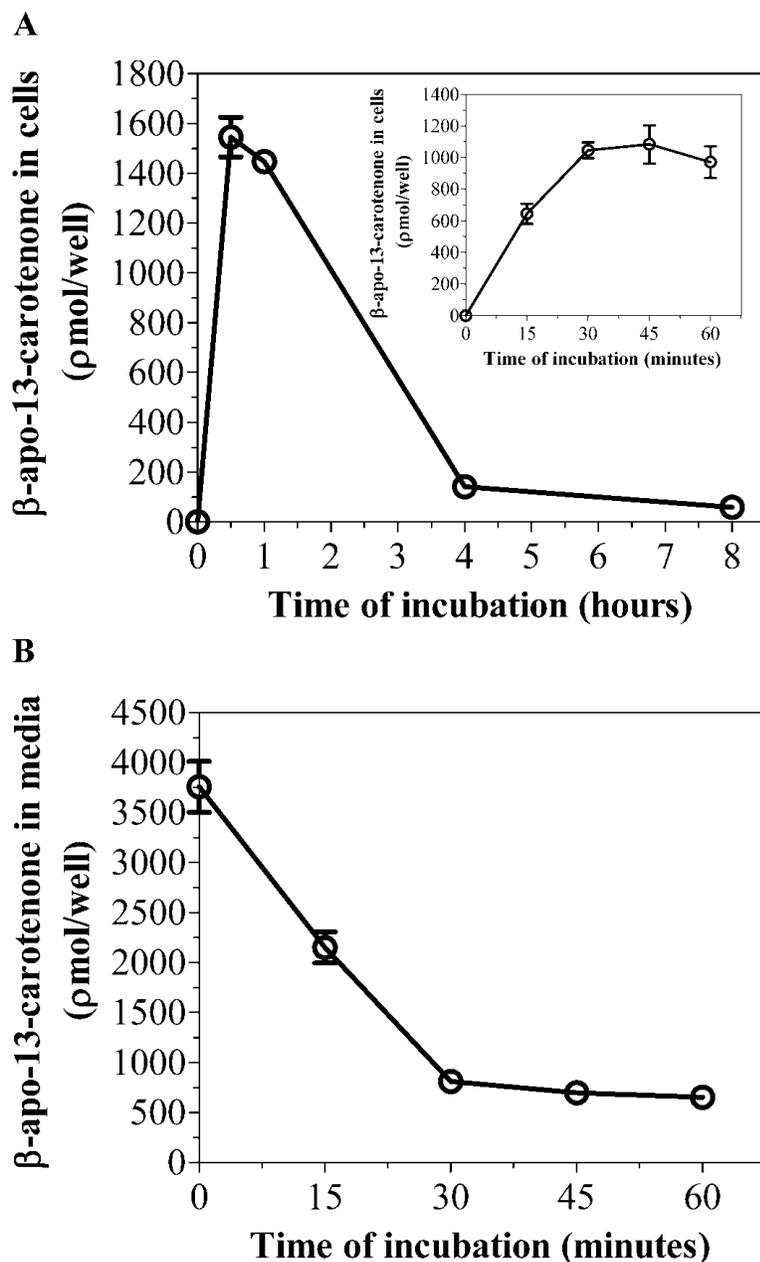


Figure 11. (A): Time course of uptake of β -apo-13-carotenone into Caco-2 cells. **(B):** Effect of incubation time on the disappearance of β -apo-13-carotenone from media.

Differentiated Caco-2 cells were incubated with serum-free media containing $5 \mu\text{M}$ β -apo-13-carotenone at 37°C for time points up to 8 hours. After incubation, the extracts from media and cells were analyzed by HPLC-DAD. The inset graph in (A) shows the uptake of β -apo-13-carotenone into cells at time points

up to 1 hour. No peaks were detected in media after 4 or 8 hours of incubation. The results shown represent an individual experiment and the points are means \pm standard deviations of 3 or more wells. Furthermore, the data shown in (A) have been confirmed with two independent experiments.

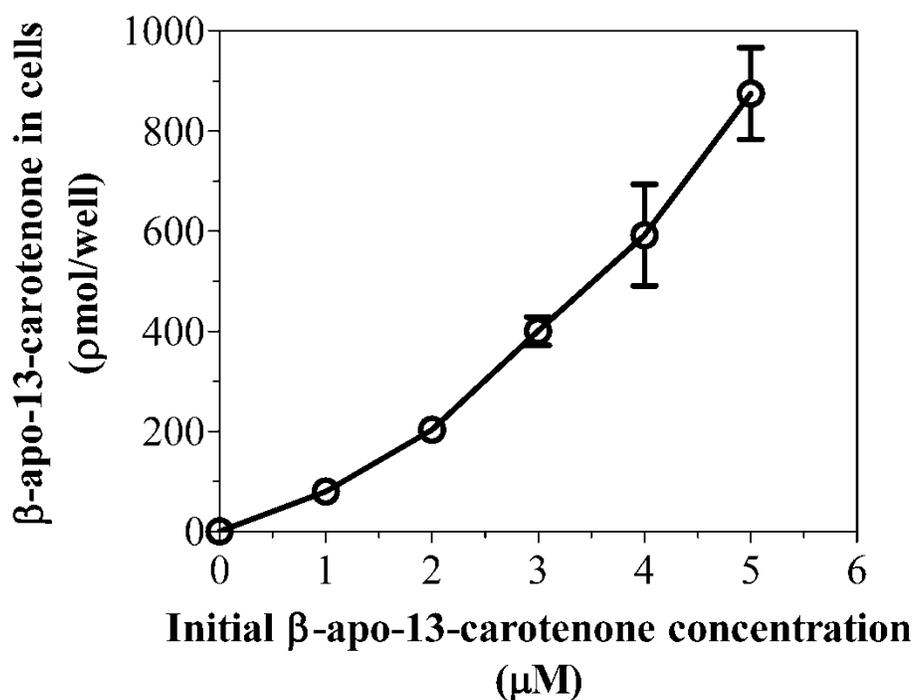


Figure 12. Dose dependent uptake of β -apo-13-carotenone into Caco-2 cells

Differentiated Caco-2 cells were incubated with serum-free media containing 1, 2, 3, 4, or 5 μM β -apo-13-carotenone at 37 °C for 45 minutes. After incubation, the extracts from cells were analyzed using HPLC-DAD. The results shown represent an individual experiment and the points are means \pm standard deviations of 3 or more wells. Furthermore, the data shown above have been confirmed with two independent experiments.

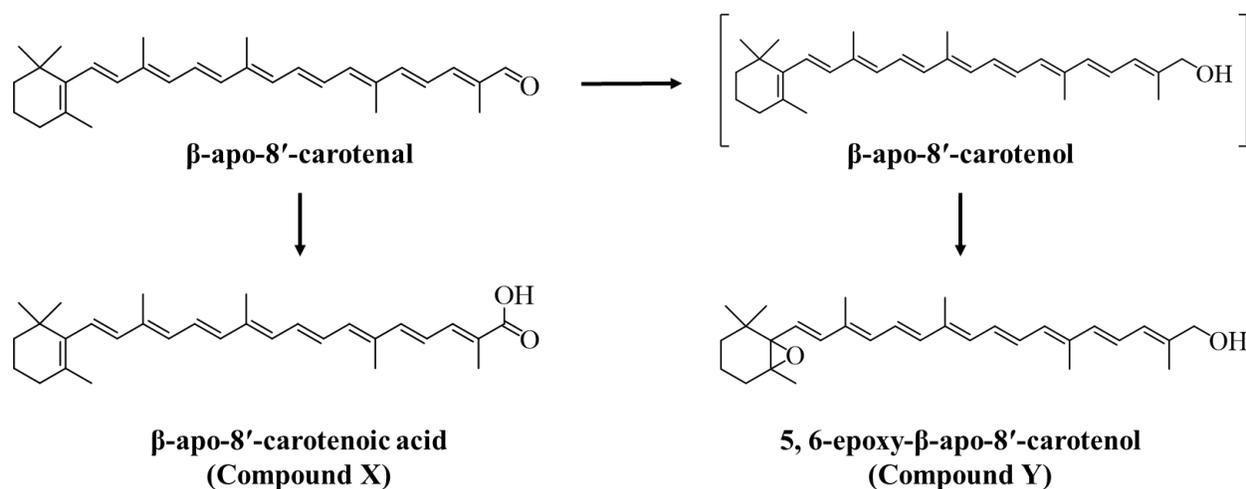


Figure 13. Structures of the metabolites in the metabolic pathway of β -apo-8'-carotenal in Caco-2 cells

There was rapid uptake of β -apo-8'-carotenal into cells and β -apo-8'-carotenol was largely converted to β -apo-8'-carotenoic acid. Several studies show that this apocarotenoic acid is a major metabolite present in the serum and tissues of animals fed β -apo-8'-carotenal (38, 39). This metabolic conversion is similar to what is observed with retinal, which can be converted to retinol or retinoic acid. β -Apo-8'-carotenal was also converted to a minor metabolite that was identified as 5,6-epoxy- β -apo-8'-carotenol, which is analogous to the formation of epoxy metabolites of vitamin A *in vivo* (41, 42).

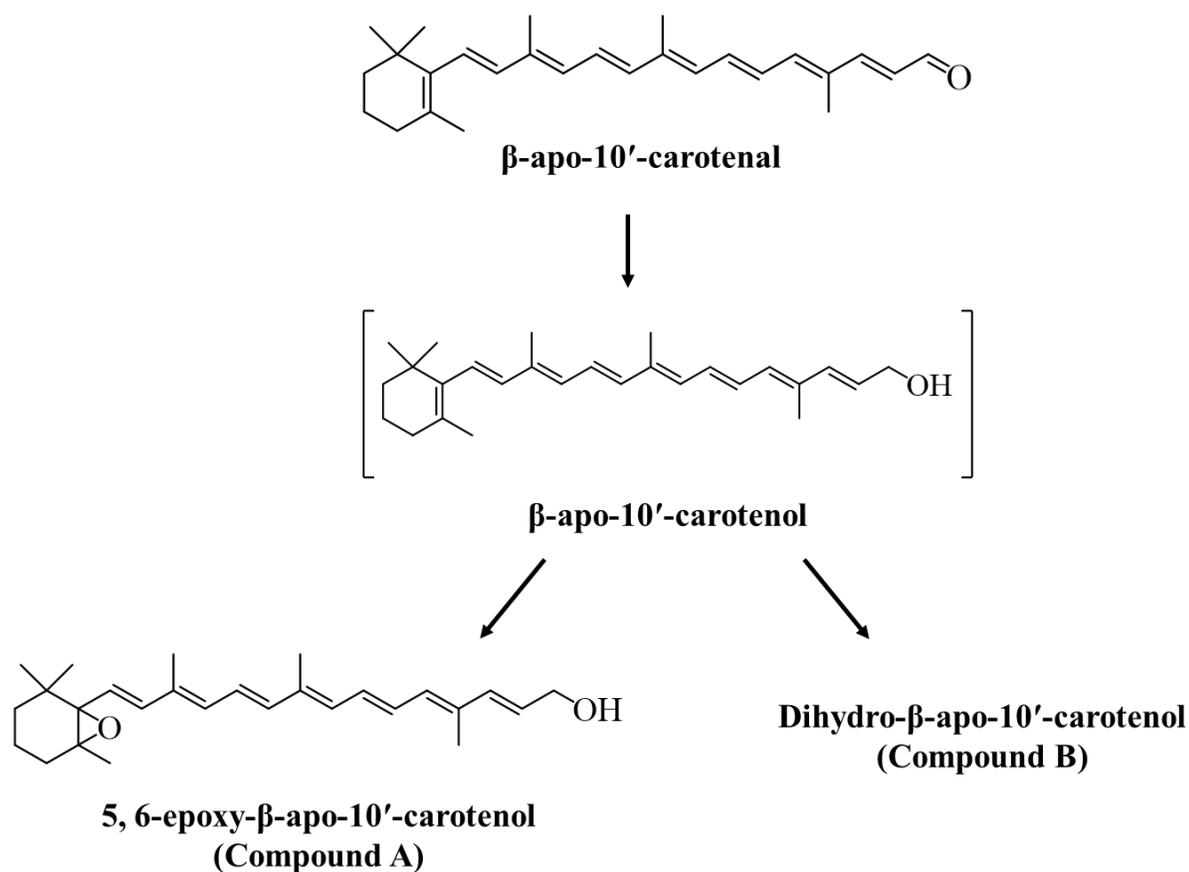


Figure 14. Structures of the metabolites in the metabolic pathway of β -apo-10'-carotenal in Caco-2 cells

There was rapid uptake of β -apo-10'-carotenal into cells and β -apo-10'-carotenal was converted to a major metabolite that we identified as 5,6-epoxy- β -apo-10'-carotenol and a minor metabolite that is likely a dihydro- β -apo-10'-carotenol. Previous studies have suggested that β -apo-10'-carotenol, β -apo-10'-carotenoic acid, and β -apo-12'-carotenoic acid are detected in serum and tissues of animals given β -apo-10'-carotenal as the only source of provitamin A activity (39). However, in the present study, we did not detect any of these metabolites.