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Assessment of Carotenoid Bioavailability of Whole Foods Using a Caco-2 Cell Culture Model Coupled with an in Vitro Digestion

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Epidemiological studies have shown that consumption of carotenoid-rich fruits and vegetables is associated with a reduced risk of developing chronic diseases. β-Carotene, α-carotene, and β-cryptoxanthin are precursors of vitamin A, a nutrient essential for human health. However, little is known about the bioavailability of carotenoids from whole foods. This study characterized the intestinal uptake performance of carotenoids using monolayers of differentiated Caco-2 human intestinal cells and mimicked human digestion to assess carotenoid absorption from carrots and corn. Results showed that Caco-2 cellular uptake of β-carotene and zeaxanthin was higher than that of lutein. Uptake performances of pure carotenoids and carotenoids from whole foods by Caco-2 cells were both curvilinear, reaching saturated levels after 4 h of incubation. The time kinetics and dose response of carotenoid uptake presented a similar pattern in Caco-2 cells after plating for 2 and 14 days. Furthermore, the applicability of this new model was verified with whole grain corn, showing that cooked corn grain significantly enhanced carotenoid bioavailability. These results support the feasibility of the in vitro digestion cell model for assessing carotenoid absorption from whole foods as a suitable and cost-effective physiological alternative to current methodologies.

KEYWORDS: Carotenoids; β-carotene; bioavailability; Caco-2; cell culture

INTRODUCTION

Epidemiological studies have consistently shown that the consumption of carotenoid-rich foods is associated with a reduced risk of developing several chronic diseases such as cardiovascular disease and cancer (*1*−*5*). It has been reported that carotenoids have specific biological activities such as free radical scavenging (*6*), singlet oxygen quenching (*7*), and lipid peroxidation inhibition (*8*). Carotenoids may prevent the development of atherosclerosis and preserve vascular function by inhibiting the oxidation of low-density lipoprotein (LDL) cholesterol (*9*−*11*). Certain carotenoids such as lutein and zeaxanthin have been associated with a reduced risk of cataract development (*12*) and have been shown to play a protective role in the progression of early atherosclerosis (*13*).

Carotenoids, such as β-carotene, α-carotene, and β-cryptoxanthin, are precursors of vitamin A (*14*). Vitamin A deficiency is a global health problem responsible for growth failure and increased susceptibility to infection, blindness, and death (*15*). It has been estimated that over 3 million children worldwide suffer from xerophthalmia and that 250−500 million children go blind annually due to vitamin A deficiency (*16*). This situation occurs most commonly in India, Africa, Latin America, and the Caribbean. Grains such as rice and wheat are the predominant foods in these regions, and these grains are typically milled to remove the aleurone layer prior to further processing into foods. This milling results in a food product that is deficient in provitamin A carotenoids. Breeding and genetic recombinant technologies to produce biofortified foods such as provitamin A enhanced rice endosperm are current approaches used to increase the levels of carotenoids in natural foods (*17*, *18*).

Humans are unable to biosynthesize carotenoids and must acquire these essential compounds through the diet. Research to understand and enhance carotenoid bioavailability is also important, as much is still unknown; a majority of the work to understand bioavailability has focused on dietary supplements, not natural food sources of carotenoids. Additionally, many factors have been found to affect carotenoid absorption once ingested (*19*−*22*), and a better understanding of these factors and their interactions is necessary.

Currently there are no validated methods for the quantitative estimation of carotenoid bioavailability from dietary sources or synthetic supplements (*23*). Carotenoid bioavailability is usually

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assessed primarily by monitoring the change of carotenoid concentration in plasma following either acute/chronic administration or the ingestion of the purified carotenoids or a carotenoid-rich meal (*24*, *25*). However, in these studies individuals have typically shown a wide variation in plasma carotenoid content after the consumption of carotenoid-rich diets, suggesting that absorption of carotenoids had been influenced by genetic, physiological, and metabolic factors (*26*). Additionally, the levels of ingested carotenoids that have actually been absorbed and metabolized are difficult to measure (*24*). Some animal models for the assessment of human carotenoid bioavailability have tried to solve this problem (*27*), but such models are limited in application; the efficiency of carotenoid absorption, the conversion ratio of carotenoids to retinol, and the metabolic pathway of carotenoids are known to be species dependent (*28*). Approaches using isotopic labeling coupled with mass spectral analysis for the measurement of the absorption of carotenoids from foods give the most direct measurement of carotenoid bioavailability (*29*); however, these methods are costly and complex.

Given the limitation of using animal or human subjects, a simple alternative model for studying human intestinal carotenoid absorption would be useful. Parker (*30*) suggested that the development of in vitro Caco-2 cell culture models for assessing carotenoid bioavailability from foods could be a cost-effective alternative. Garrett et al. (*31*, *32*) reported a Caco-2 cell culture model for the assessment of carotenoid bioavailability from fresh and cooked vegetables and meals. They used a digestion procedure in which 2-monoacylglycerol, oleic acid, phosphatidylcholine, vegetable oil, and animal fat were added to the meals, and micellarized carotenoids were separated via ultracentrifugation. The amount of micellarized carotenoids present was used as an estimation of carotenoid bioavailability. During et al. (*33*) reported a Caco-2 cell model used to characterize carotenoid absorption and secretion. In the presence of microsomes from rat liver, Tween 40, oleate, and taurocholate, cells could assemble and secrete chylomicrons, which enhanced cellular absorption and secretion of carotenoids. A potential limitation of these approaches to mimic human physiological absorption of carotenoids is that the models incorporated additional emulsifiers, which are not ingested along with carotenoid-rich fruits and vegetables as part of the human diet. Therefore, it is necessary to develop an alternative Caco-2 cell model that mimics human physiological absorption and more closely parallels the human diet.

It has been found that the consumption of heat-processed carrots and spinach can significantly increase the β-carotene concentration in human plasma (*34*), and heat treatment of tomatoes can result in enhanced lycopene bioavailability in humans (*35*). Although previous research has shown that processing of carotenoid-rich fruits and vegetables can increase carotenoid bioavailability, few reported studies have examined the effects of processing on carotenoid bioavailability from whole grains or have fully characterized grain carotenoid profiles. It is urgent and worthy to establish a database of carotenoid bioavailability from whole grains because staple foods such as wheat and corn can provide provitamin A to developing countries where vitamin A deficiency is a serious problem. A model that can provide rapid results and multiple assessments would be quite useful and economical for this purpose.

Many Caco-2 cell culture models utilize cells plated 2 weeks postconfluency (*31*, *33*, *36*) because it has been considered that maximum cellular differentiation was exhibited after this time period (*37*). To our knowledge, no study has examined the involvement of carotenoid receptors, carriers, or functional enzymes in the transmission of carotenoids across cell membranes. Parker (*30*) suggested that the uptake of carotenoids by duodenal mucosal cells was associated with passive diffusion of carotenoids driven by concentration gradients. Therefore, it is reasonable to propose that when a short-term assay is used to screen for carotenoid bioavailability from foods, it is possible to use cells plated after 48 h instead of 14 days or more. Although Caco-2 cells plated at different times, for example, 48 h versus 14 days, may exhibit differences in intracellular enzyme activities (*37*), it is not known whether this results in differences in rate or efficiency of carotenoid uptake.

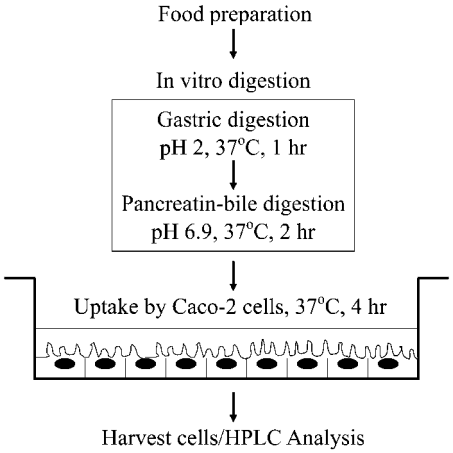

Figure 1. In vitro digestion/Caco-2 cell culture model for carotenoid bioavailability.

The objectives of this study were (1) to develop a Caco-2 cell culture model coupled with an in vitro digestion process to assess bioavailability of carotenoids, (2) to assess the uptake of carotenoids from whole foods including carrots and whole grains, and (3) to compare efficacy of carotenoid bioavailability in Caco-2 cell cultures plated after 48 h and 14 days. Our overall goal is to provide researchers with a suitable, rapid, and cost-effective Caco-2 cell culture model for whole food carotenoid bioavailability screening that is particularly useful for the assessment of whole grains (**Figure 1**).

EXPERIMENTAL PROCEDURES

Chemicals. Butylated hydroxytoluene (BHT), β-carotene, lutein, carotene (2:1, β:α), bile extract (from porcine), pepsin (from porcine stomach mucosa), and pancreatin (from porcine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). Zeaxanthin was purchased from Indofine Chemical Co. (Somerville, NJ). All chemicals were of analytical grade quality. Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), methanol (MeOH), and methl *tert*-butyl ether (MtBE) were purchased from Fisher Scientific (Pittsburgh, PA). Williams' Medium E (WME), Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY).

Cell Culture. The Caco-2 human colon cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and stock cultures were maintained in DMEM supplemented with 5% FBS, 10 mM HEPES, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. In this study, we performed two different Caco-2 cell culture models: culture 1, with cells cultured for 2 days; and culture 2, with cells cultured for 2 weeks (14 days).

Culture 1 (2-Day Cells). Cells were seeded in six-well flat-bottom plates at a density of 1×10^6 cells per well. The cells were used at 48 h after plating, and the quantity of cellular protein and cell numbers per well were determined.

Culture 2 (2-Week Cells). Cells were seeded in six-well flat-bottom plates at a density of 5×10^5 cells per well. The cells were used at 14 days after plating, and the quantity of cellular protein and cell numbers per well were determined.

Sample Preparation. All procedures with carrots and corn were performed under subdued lighting to minimize the destruction of carotenoids. Carrots, purchased from a local supermarket, were cleaned with distilled water, wiped dry, and finely chopped. The diced carrots were mixed with an equal weight of saline (140 mM NaCl and 5 mM KCl) including 150 μM BHT and homogenized cold (−5 °C) to a pureed consistency with a blender for 3 × 3 min. The homogenized sample was transferred to a 50 mL screw-capped tube wrapped with aluminum foil and stored at −70 °C until use for further experiments. The thawed carrot slurry that was subjected to a cooking procedure was mixed with saline in a 1:1 w/w ratio before cooking in a 100 °C water bath for 15 min. After the sample had cooled to room temperature, the in vitro digestion procedure was performed as described below.

The corn samples were milled in a grinder to a fine powder (60 mesh), mixed thoroughly, and stored at −40 °C for further experiments. In this study, we assessed the carotenoid bioavailability of raw/digested corn as well as that of cooked/digested corn. For raw/digested corn, the in vitro digestion was directly performed as described below. For cooked/digested corn, the sample was mixed with saline (1:4 w/w ratio corn/saline) before cooking in a 100 °C water bath for 15 min.

In Vitro Digestion. The in vitro digestion method used was a modification of that previously described by Miller et al. (*38*). Briefly, the sample was mixed with saline (140 mM NaCl, 5 mM KCl, and 150 μM BHT) to create a final volume of 18 mL and acidified to pH 2 with 0.1 M/1 M HCl. Then the sample was mixed with 0.5 mL of pepsin solution (0.2 g of pepsin in 5 mL of 0.1 M HCl) and incubated in a shaking water bath at 37 °C for 1 h. After gastric digestion, the pH of the digestate was increased to 6.9 with 0.1 M/1 M $NaHCO_3$. Further intestinal digestion was performed with the addition of 2.5 mL of pancreatin−bile solution (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M $NaHCO_3$) and incubated in a shaking water bath at 37 °C for 2 h. The total digestate weight was adjusted to 28 g with saline. The digest was stored at −70 °C for further experiments.

Uptake of Carotenoids by Caco-2 Cells. In this study, we developed two model designs (designs 1 and 2) to assess the uptake of carotenoids by both culture 1 and culture 2. Design 1 targeted treatments of pure carotenoid compounds. Design 2 targeted treatments of whole foods. The two model designs are described below.

Design 1 (Pure Compounds). Lutein, zeaxanthin, and β-carotene were used as pure compounds for cell treatments. Carotenoids were dissolved in DMSO and diluted to an average concentration of 1−8 μM (final concentration of DMSO was 2%) in WME medium. All stock solutions in WME contained 4 μM BHT. Upon treatment initiation, the medium was removed, and the cells were washed three times with PBS. The cells were treated with 1 mL of prepared carotenoid solution and incubated at 37 °C. Carotenoid uptake was terminated at indicated times by removing the treatment medium and washing the cells three times with washing solution (1:2:3, v/v/v, ether/MeOH/PBS). Washed cells were collected/harvested three times with 1 mL of cold PBS containing 10% (v/v) methanol. Samples were stored at −40 °C under a blanket of nitrogen until analysis.

Design 2 (Whole Foods). For the whole foods, cooked/digested carrots and corn were used as whole food treatments. Digestion solutions were centrifuged at 12000*g* for 5 min. The collected supernatant was diluted 1:4 v/v with WME and added to the Caco-2 cell culture. Cells were treated as in design 1 described above.

HPLC Analysis of Carotenoids. The carotenoid content of samples was determined using HPLC according to a modified method previously described (*39*). Frozen samples were thawed, and the cell homogenates were sonicated for 2 min on ice. Aliquots were extracted three times with 1 mL of hexane, dried under a stream of nitrogen, and redissolved in 0.4 mL of MeOH/THF (1:1, v/v). The carotenoid content of each sample was quantified using an RP-HPLC procedure employing a 250 × 4.6 mm YMC C_{30} column, particle size = 3 μm (Waters Inc., Wilmington, NC). The mobile phase of MeOH/water (95:5, v/v) (solvent A) and methyl *tert*-butyl ether (solvent B) was used at a flow rate of 1.5 mL/min. The gradient procedure was as follows: (1) initial conditions of 75% solvent A and 25% solvent B, (2) increasing solvent B to 30% between 11 and 13 min, (3) maintaining 70% solvent A and 30% solvent B for 9 min between 13 and 22 min, (4) decreasing solvent B from 30 to 25% between 22 and 23 min, (5) maintaining 75% solvent A and 25% solvent B for at least 5 min before the next injection would occur. Lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-carotene concentrations in the carrot sample, corn sample, and cellular extracts were extrapolated from the pure carotenoid standard curves. Carotenoid recoveries from the incubation were 94 ± 5.5% for lutein, 100 ± 5.0% for zeaxanthin, 96 ± 2.5% for β-cryptoxanthin, 96 ± 7.5% for β-carotene, and 96 ± 7.5% for α-carotene.

Cytotoxicity. The methylene blue assay was used to assess the potential toxicity of diluted sample digests toward Caco-2 cells, following the procedure of Oliver et al. (*40*).

Statistical Analysis of Data. All data were reported as mean ± standard deviation (SD) for three replicates of each treatment. All analyses of data were performed using the SAS software program (SAS Institute, Cary, NC). Means were considered to be significantly different if p values were <0.05.

RESULTS

Pure Carotenoid Compound Uptake by Caco-2 Cells. Time kinetics of carotenoid uptake by Caco-2 monolayers after different incubation time periods are shown in **Figure 2**. For the 2-day cell model treated with 4 μM lutein, zeaxanthin, and β-carotene (**Figure 2a**), lutein, zeaxanthin, and β-carotene uptake could be detected after 1 h and reached a stable level after 4 h, when the concentrations of lutein, zeaxanthin and β-carotene were 272 ± 90, 1009 ± 316, and 1094 ± 120 pmol, respectively. The amounts of lutein, zeaxanthin, and β-carotene uptake did not show a significant change ($p < 0.05$) after 4 h of incubation. Within a 12 h period, uptake of zeaxanthin and β-carotene by 2-day cells was higher than that of lutein. For the 2-week cell model treated with 4 μM lutein, zeaxanthin, and β-carotene (**Figure 2b**), carotenoids reached a stable level in the Caco-2 cells after 3 h, when the concentrations of lutein, zeaxanthin, and β-carotene were 125 ± 50, 586 ± 198, and 616 ± 112 pmol, respectively. The amounts of zeaxanthin and β-carotene uptake did not show a significant change ($p < 0.05$) after 3 h of incubation. Uptake of zeaxanthin and β-carotene was also higher than that of lutein within a 12 h period. In general, the time kinetics of carotenoid uptake by 2-day cells and 2-week cells were quite similar.

Dose responses of carotenoid uptake by Caco-2 monolayers after different treatment doses are shown in **Figure 3**. Lutein, zeaxanthin, and β-carotene uptake by the 2-day cell model after 6 h of incubation (**Figure 3a**) increased with increasing carotenoid concentrations and reached a stable level when carotenoid concentrations were 4 μM; the amounts of lutein, zeaxanthin, and β-carotene uptake by the 2-day cells were 397 ± 108, 1483 ± 413, and 1340 ± 162 pmol, respectively. The amounts of lutein, zeaxanthin, and β-carotene uptake did not show a significant change ($p < 0.05$) between carotenoid concentrations of 4 and 8 μM. In the 8 μM treatment range, uptake of zeaxanthin and β-carotene by 2-day cells was higher than that of lutein. For the 2-week cell model treated for 6 h of incubation (**Figure 3b**), the amounts of lutein and β-carotene uptake were directly related to the dose of carotenoids added to the medium and reached a stable level when carotenoid concentrations were 4 μM, when the concentrations of lutein and β-carotene were 165 ± 12 and 303 ± 51 pmol, respectively. The amounts of lutein and β-carotene taken up did not show a significant change ($p < 0.05$) between carotenoid concentrations of 4 and 8 μM. The uptake of zeaxanthin and β-carotene by 2-day cells was also higher than that of lutein in the 8 μM

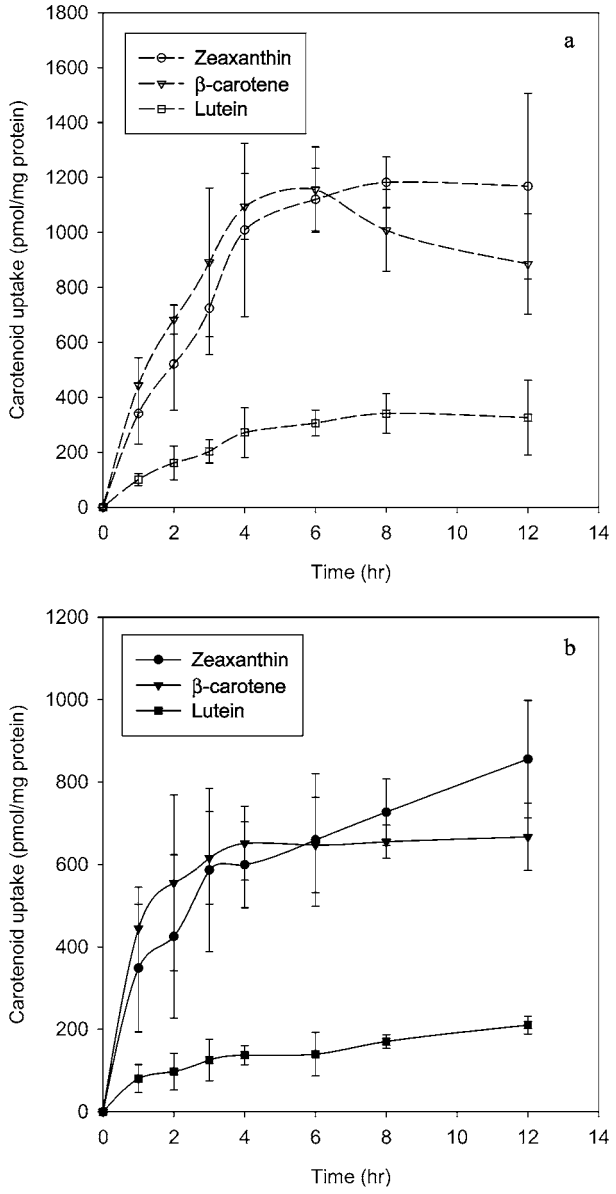

Figure 2. Time kinetics of pure carotenoid compound uptake by Caco-2 cells, (**a**) 2 and (**b**) 14 days after plating. Caco-2 cells were incubated with 1 mL of 4 μM lutein, zeaxanthin, and β-carotene in WME including 2% DMSO for indicated times. Each point represents the mean ± SD of triplicate observations.

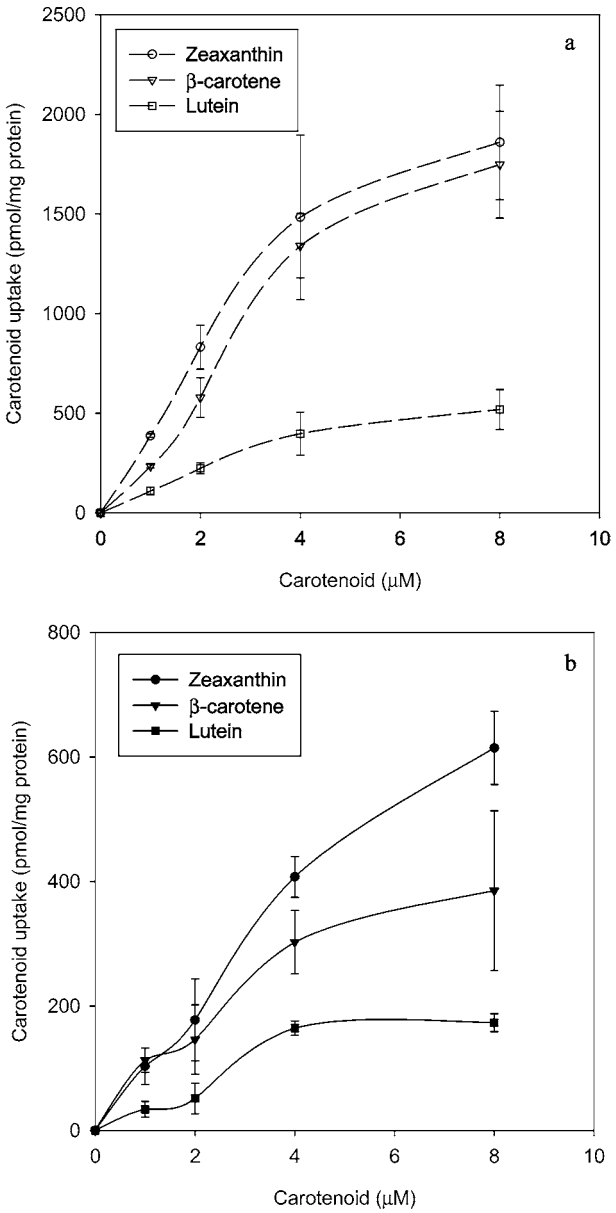

Figure 3. Dose response of pure carotenoid compound uptake by Caco-2 cells, (**a**) 2 and (**b**) 14 days after plating. Indicated amounts of lutein, zeaxanthin, and β-carotene in 1 mL of WME including 2% DMSO were applied to Caco-2 cell monolayers and incubated for 6 h. Each point represents the mean ± SD of triplicate observations.

treatment range. Generally speaking, the dose response of carotenoid uptake by 2-day cells was similar to that of 2-week cells.

Characterization of Carotenoid Uptake by Caco-2 Cells from Whole-Food Carrots. As can be seen in **Figure 4**, the α-carotene and β-carotene from the whole-food carrots were taken up in a time-dependent manner by the Caco-2 monolayers. For the 2-day cell model treated with 4 g of cooked and digested carrots (**Figure 4a**), uptake of α-carotene and β-carotene reached a saturated level in the Caco-2 cells after 4 h, when the concentrations of α-carotene and β-carotene were 247 ± 29 and 409 ± 54 pmol, respectively. The amounts of α-carotene and β-carotene taken up did not show a significant change ($p < 0.05$) after 4 h of incubation. For the 2-week cell model treated with 4 g of cooked and digested carrots (**Figure 4b**), carotenoids reached a saturated level after 2 h, when the uptake of concentrations of α-carotene and β-carotene were 331 ± 2 and 566 ± 7 pmol, respectively. The amounts of α-carotene and β-carotene taken up did not show a significant change ($p < 0.05$) between 2 and 8 h of incubation. Generally speaking, the patterns of time kinetics of carotenoid uptake from the whole-food carrots by 2-day and 2-week cells were similar.

The amounts of α-carotene and β-carotene from the whole-food carrots taken up by the Caco-2 monolayers over a range of doses can be seen in **Figure 5**. For the 2-day cell model treated for a 4 h of incubation with 1 g of carrots (**Figure 5a**), uptake of α-carotene and β-carotene by cells was not detected. Uptake of these compounds was detected at a treatment of 2 g of carrots and reached a saturated level when the treatment was increased to 4 g of carrots, when the concentrations of α-carotene and β-carotene in the cells were 261 ± 37 and 397 ± 55 pmol, respectively. The amounts of α-carotene and β-carotene taken up did not show a significant change ($p < 0.05$) between 4 and 6 g of carrots. For the 2-week cell model

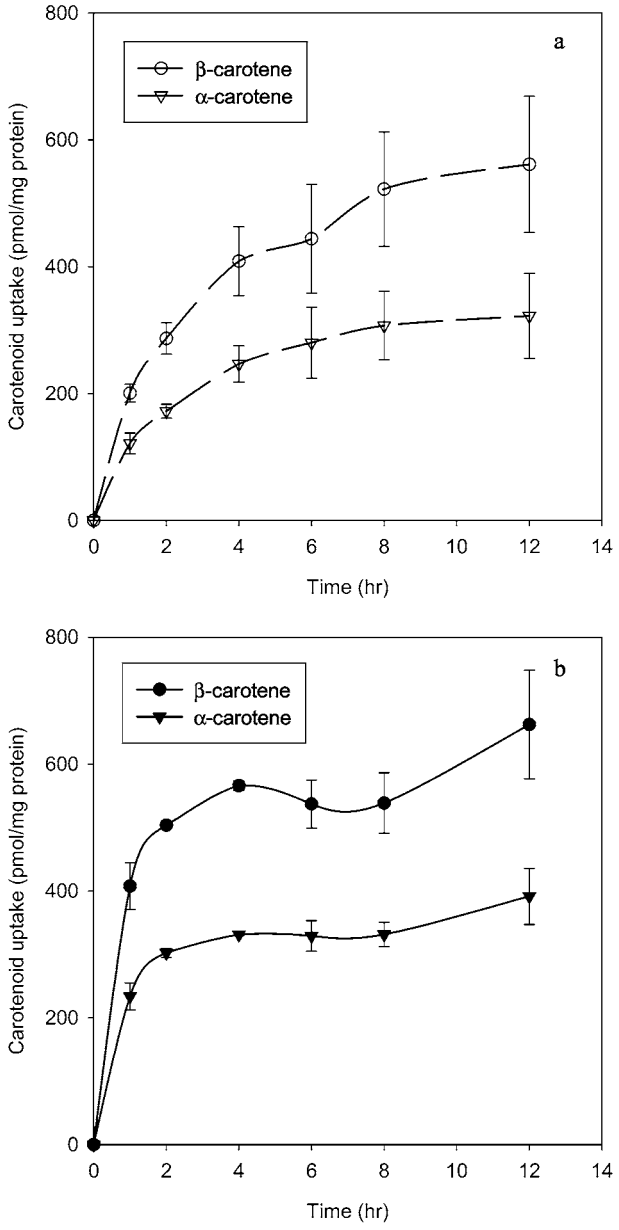

Figure 4. Time kinetics of carotenoid uptake from carrots by Caco-2 cells, (**a**) 2 and (**b**) 14 days after plating. Caco-2 cells were incubated for indicated times with 1 mL of diluted (1:4) digestion solution in WME. The digestion solution was prepared from 4 g of carrots with cooking at 100 °C for 15 min and in vitro digestion. Each point represents the mean ± SD of triplicate observations.

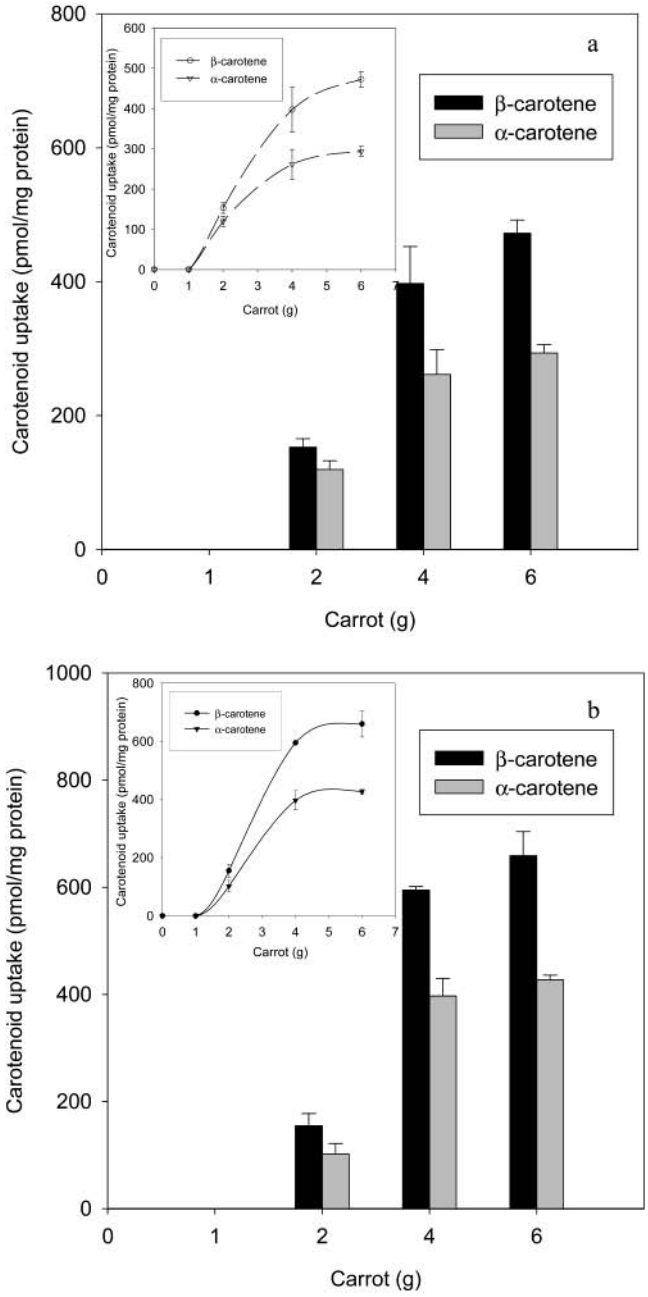

Figure 5. Dose response of carotenoid uptake from carrots by Caco-2 cells, (**a**) 2 and (**b**) 14 days after plating. Caco-2 cells were incubated for 4 h with 1 mL of five different concentrations of diluted (1:4) digestion solutions in WME. The five digestion solutions were prepared from 0, 1, 2, 4, and 6 g of carrots with cooking at 100 °C for 15 min and in vitro digestion. Each point represents the mean ± SD of triplicate observations.

incubated for 4 h and treated with 1 g of carrots (**Figure 5b**), uptake of α-carotene and β-carotene by cells was not detected. At a treatment of 2 g of carrots, uptake of these carotenoids was detected and further reached a saturated level in the Caco-2 cells at a treatment of 4 g of carrots, when the concentrations of α-carotene and β-carotene were 397 ± 33 and 595 ± 7 pmol, respectively. The amounts of α-carotene and β-carotene taken up did not show a significant change ($p < 0.05$) between 4 and 6 g of carrots. Obviously, the dose response patterns of carotenoid uptake by 2-day and 2-week cells from the whole-food carrots were similar.

Bioavailability of Carotenoids from Corn. Figure 6 shows the uptake of lutein, zeaxanthin, and β-cryptoxanthin by Caco-2 cell monolayers from raw/digested and cooked/digested corn. For the 2-day cell model treated with 2 g of corn for a 4 h incubation (**Figure 6a**), uptake of carotenoids by cells from cooked corn was higher than that from raw corn. Uptake of lutein and zeaxanthin from cooked/digested corn by cells was 0.9- and 1.2-fold, respectively, significantly higher than that from raw/digested corn ($p < 0.05$). In addition, for the 2-week cell model treated with 2 g of corn for a 4 h incubation (**Figure 6b**), similar results were obtained: cooking enhanced the amount of carotenoids taken up by cells. Uptake of zeaxanthin from cooked corn by cells was 1.1-fold, significantly higher than that from raw corn ($p < 0.05$). The uptake of carotenoids from whole-food corn by 2-day and 2-week cells was similar in performance, as illustrated in **Figure 5**.

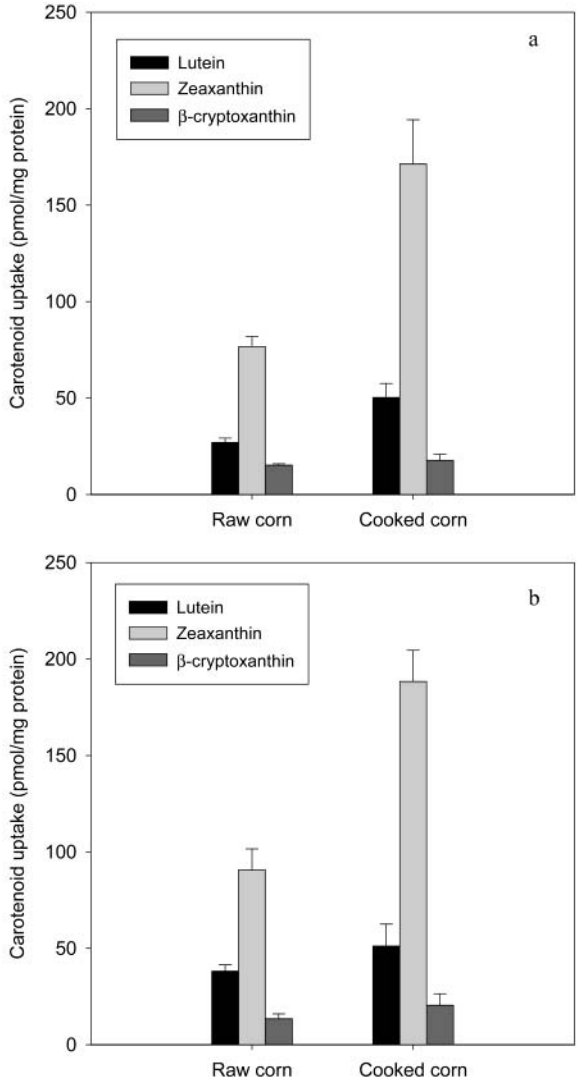

Figure 6. Uptake of carotenoid by Caco-2 cells from corn 2 (**a**) and 14 days after plating (**b**). Caco-2 cells were incubated for 4 h with 1 mL of diluted (1:4) digestion solutions in WME. The digestion solutions were prepared from 2 g of raw or cooked corn at 100 °C for 15 min with in vitro digestion. Each point represents the mean ± SD of triplicate observations.

DISCUSSION

The Caco-2 human intestinal cell line is a potentially useful model for studying the transport and metabolism of dietary phytochemicals such as carotenoids (*30*). This cell line has been used to assess the performance of intestinal absorption of lipophilic compounds such as vitamin E and retinol (*41*, *42*). We conducted a series of studies to characterize the intestinal uptake of pure carotenoid compounds and dietary carotenoids from whole-food matrices using monolayers of the differentiated Caco-2 human intestinal cell line. The model we built in this study showed a consistent characterization of absorption of pure carotenoid compounds and dietary carotenoids from whole-food carrots and corn by Caco-2 human intestinal cells. In our study, dietary carotenoids from whole foods were absorbed by Caco-2 cells in a manner similar to that exhibited by the pure compounds. These data support the feasibility of using a Caco-2 cell culture model coupled with an in vitro digestion procedure to assess carotenoid bioavailability of whole foods.

Several studies (*31*, *33*, *43*) have also reported cell culture models to assess carotenoid bioavailability from pure carotenoid compounds; however, the results of absorption performance from these studies do not match our observations in this study. Scita et al. (*43*) treated rat small intestinal cells (hBRIE 380) with β-carotene solubilized in THF/DMSO and showed that β-carotene cellular accumulation was linear up to a concentration of 25 μM. Garrett and his colleagues (*31*) used a composite solution of carotenoids, 2-monoacylglycerol, oleic acid, and phosphatidylcholine to measure the uptake of pure carotenoid compounds by Caco-2 cells. This study showed that the maximum accumulation of β-carotene in Caco-2 cells occurred when the carotenoid concentration was 18 μM. Additionally, cells accumulated β-carotene linearly from the medium during 20 h of incubation. During et al. (*33*) conducted an emulsification cell model with the addition of Tween, oleate, and taurocholate to assess cellular uptake and secretion of pure carotenoid compounds into chylomicrons. Using this model, it was found that intracellular β-carotene had not reached saturated levels even after 16 h of incubation. Previous researchers have successfully used DMSO to process transepithelial transport of lipophilic drugs in Caco-2 cell cultures (*44*, *45*). In the present study, we used 2% DMSO as a carrier of pure carotenoid compounds and showed that after treatment, accumulation of intracellular carotenoids was curvilinear, reaching maximum accumulation at a carotenoid concentration of 4 μM. We also found that uptake of pure carotenoids by 2-day and 2-week cells reached a stable maximum/saturated level after 3−4 h of incubation. Our different observations of the performance of carotenoid uptake could have been due to the use of different cell types and delivery vehicles. Previous literature and our study support this opinion (*23*, *31*).

Previous work has suggested that the transfer of lutein, a polar carotenoid, from the lumen across the brush border was more efficient than that of β-carotene (*46*). However, our results indicated that in 2-day and 2-week cells intracellular lutein concentrations were lower than that of intracellular β-carotene. Garrett and his colleagues (*47*) reported similar results. In both studies, the emulsified forms of carotenoids were used to deliver carotenoids to Caco-2 cells. Borel et al. (*48*) suggested that the emulsified form of β-carotene was more efficient than that of lutein, a more polar carotenoid, due to the stability of the emulsified compound. This proposal directly supports the results from our present study. Interactions between carotenoids may also explain the differences in absorption performance between β-carotene and lutein. Kostic et al. (*25*) examined serum responses after single doses of β-carotene and lutein, both alone and as an equimolar mixture. They found that when combined, β-carotene significantly reduced the serum responses for lutein to 53−61% of control values. O'Neill and Thurnham (*49*) similarly found that the absorption of β-carotene and lycopene was significantly lower than that of lutein. A similar effect was also reported by Paetau et al. (*50*) in their study of canthaxanthin. We further propose that the difference in metabolism, retention, and oxidation of individual carotenoids must be considered. Some carotenoids may be transferred to retinol in cells, whereas other carotenoids may be metabolically processed or oxidized. Garrett et al. (*47*) has suggested that ~30% of lutein may be metabolized or spontaneously oxidized by cells. To date, the research to fully describe the cellular metabolism and secretion of individual carotenoids is not complete, and further extensive study is necessary.

Garrett et al. (*32*, *47*) used the Caco-2 cell model to study carotenoid bioavailability from foods. Vegetable oil and animal fat were added to baby food and stir-fried vegetables and further used in the model as a food matrix. To prepare non-cytotoxic

carotenoid treatment/medium for Caco-2 cells, ultracentrifugation (167000*g* for 95 min) and filtration (0.22 μm) were used to separate the aqueous and digestate/micellar fractions. It was reported (*47*) that this two-step separation methodology resulted in the significant loss of carotenoids such as lycopene ($p < 0.01$) due to micellar instability. We suggest that this micellar model, although useful, does not adequately represent the full scope of human physiological digestion and absorption. Most commonly, when humans eat carotenoid-rich fresh fruits and vegetables, additional oils, fat, or emulsifiers may be not consumed at the same time. Taking this dietary pattern into consideration, to more realistically mimic the absorption behavior of human intestinal cells, we used cooked and in vitro digested carrot dilution treatments to directly treat Caco-2 cells without the addition of extra oils or emulsifiers.

Furr and Clark (*51*) reported that in the human digestive system, lipid soluble compounds such as carotenoids might be emulsified with bile salts from the gall bladder to produce a micellar suspension, which is absorbed across the epithelial cell membrane. We found in our model that the performance of carotenoid uptake by 2-day and 2-week Caco-2 cells can reach a saturated level after a 2−4 h treatment. To date, few reports have indicated actual time periods required for carotenoids to cross human enterocyte membranes. On the basis of our findings, we suggest that following the consumption of a carotenoid-rich meal, only 2−4 h may be required for carotenoids to enter intestinal cells from the digested system. As is typical of the daily human diet, food is ingested on average every 5−7 h; foods are digested in the stomach and intestine within 3−4 h. Thus, it seems logical that carotenoid uptake would require ~2−4 h for completion, coinciding with our results. Thus, our Caco-2 cell culture model coupled with in vitro digestion for measuring carotenoid absorption from whole foods may be used as a suitable as well as rapid, simple, and cost-effective physiological alternative.

Our study characterized and compared intracellular carotenoids in Caco-2 cells that had been previously cultured for 2 and 14 days. Caco-2 cells plated for 2 days were seeded in a six-well plate at a density of 1.0×10^6 cells/well, whereas the 14-day cells were seeded at a density of 5×10^5 cells/well. After plating for 2 and 14 days, the cell numbers and protein amounts of Caco-2 cells were measured. After the respective incubation periods, our results showed that both reached at least 95% confluence and that no significant differences in cell number and protein content were measurable. Additionally, Caco-2 cells plated for 2 and 14 days exhibited similar trends in carotenoid uptake, with similarities in saturation time, curvilinear shape, and rate. These results meaningfully supported our initial assumptions and the premise that the uptake of carotenoids by human intestinal cells is by passive diffusion across a concentration gradient of carotenoids (*52*). Possible differences existing in the activities of intracellular enzymes between Caco-2 cells plated for 2 and 14 days would not have affected passive diffusion of carotenoids and absorption measurement. As a tool to screen carotenoid bioavailability from whole foods, we suggest that the use of a 2-day Caco-2 cell culture is equally as valid as an 11−14-day culture. As an in vitro cell model for accessing the bioavailability of carotenoids, the primary consideration above other factors should be the point at which cells reach confluency.

Heat-processed foods have long been perceived to have lower nutritional value than fresh commodities due to the possible heat degradation of some nutrients. However, it is possible that cooking and other food processing steps variably affect carotenoid content, depending on the different carotenoid compounds and foods due to differences and complexities in carotenoid binding formation. Some common household cooking methods, such as microwave cooking, steaming, or boiling in a small amount of water, do not drastically change the carotenoid content of fruits and vegetables. Mild heat treatment of yellow-orange vegetables, such as carrots, sweet potato, and pumpkin, has been shown to result in a loss of only ~8−10% of the α-carotene and β-carotene (*52*). In our study, we showed that the cooking process increased the bioavailability of carotenoids from corn. Several researchers support our findings with similar study results. Rock et al. (*34*) showed that the consumption of heat-processed carrots and spinach significantly enhanced β-carotene concentration in human plasma, whereas others have shown that heat treatment of tomato enhanced lycopene bioavailability in humans (*35*, *53*, *54*). These findings do not support the concept that heat-processed foods have lower nutritional values than fresh products, but suggest that processing may actually be nutritionally beneficial in some products. Thus, such research can significantly affect consumer food choices, resulting in an increased consumption of certain processed fruits, vegetables, and whole grains.

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