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Cellular Uptake of Carotenoid-Loaded Oil-in-Water Emulsions in Colon Carcinoma Cells in Vitro

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Oil-in-water emulsions allow the preparation of lipophilic compounds such as carotenoids in the liquid form. Here, the effect of a combination of some emulsifiers, such as two whey protein isolates (BiPro and BioZate), sucrose laurate (L-1695), and polyoxyethylene-20-sorbitan-monolaurate (Tween 20), on the stability of lycopene and astaxanthin in emulsions, droplet size, and cellular uptake of these carotenoids has been investigated. The degradation of lycopene was slightly more pronounced than that of astaxanthin in all emulsions. The concentration of lycopene and astaxanthin decreased by about 30% and 20%, respectively, in all emulsions after 3 weeks of storage in the dark at 4 °C. The kind of emulsifiers or their combinations have played an important role in the cellular uptake by the colon carcinoma cells line HT-29 and Caco-2.

KEYWORDS: Astaxanthin; lycopene; O/W emulsion; cellular uptake; chemical and physical stability

INTRODUCTION

Carotenoids are a class of natural pigments found in fruit and vegetables. Although animals appear to be incapable of synthesizing carotenoids, many animals obtain carotenoids from their diet. Carotenoids are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans. Carotenoids can be used as colorants for fat and water-based foods, such as margarine and beverages, respectively.

A number of beneficial health aspects of carotenoids have been reviewed and discussed in the literature (*1*−*3*). One important aspect is the antioxidant activity of carotenoids, and some carotenoids are effective singlet oxygen quenchers (*4*) and can inhibit the destructive effect of reactive oxygen species (*5*−*7*). In epidemiological studies, an increased intake of carotenoid-rich food was correlated with a decrease risk for some cancers, cardiovascular disease, age-related macular degeneration, and cataracts (*8*).

Lycopene is a carotenoid that gives vegetables and fruits, such as tomatoes, lycopene-carrot, pink grapefruit, guava, rosehip, red chilies, surinam cherry, and watermelon, their red color. It is an apolar, acyclic carotenoid highly unsaturated straight chain hydrocarbon with a total of 13 double bonds, 11 of which are conjugated (**Figure 1A**). Lycopene owes its ruby color to its extensively conjugated polyene structure. Because lycopene is a highly unsaturated molecule, it is very susceptible to oxidation (*9*). In the body, lycopene is deposited in the liver, lungs, prostate gland, colon, and skin. The ability of lycopene to function as an antioxidant contributes to a reduction in disease risks (*10*). In vitro experiments have demonstrated that lycopene quenches singlet oxygen and inhibits lipid peroxidation (*11*).

Astaxanthin is a red pigment occurring naturally in a wide variety of living organisms. It is the main carotenoid pigment, red-orange color, found in marine and aquatic animals (*12*). Astaxanthin is also contained in some food used in the human diet. Most crustaceans, including shrimp, crawfish, crabs and lobster, are tinted red by accumulated astaxanthin. Astaxanthin also occurs in fish eggs, e.g., salmon roe, and in some other fish species, e.g., red sea bream. It is a carotenoid belonging to the xanthophylls class. Xanthophylls are usually characterized by a hydroxylic group. It is mostly liposoluble, although its side rings have some polar substitute groups. Astaxanthin has a 40-carbon polyene chain with 13 double bonds, a hydroxylic group, and a ketonic group on its terminal rings (**Figure 1B**). It has been shown to exhibit potential health benefits, due to its high antioxidant capacity, and to be a powerful quencher of singlet oxygen activity in in vitro studies (*13*−*14*), and it is a strong scavenger of oxygen free radicals. Several studies have shown the effectiveness of astaxanthin as a cancer preventive in rats and mice. Dietary administration of it proved to significantly inhibit carcinogenesis in the mouse urinary bladder (*15*), rat oral cavity (*16*), and rat colon (*17*).

Data about bioavailability of carotenoids from vegetables range from 5% to 26% (*18*), and thus only a fraction of the

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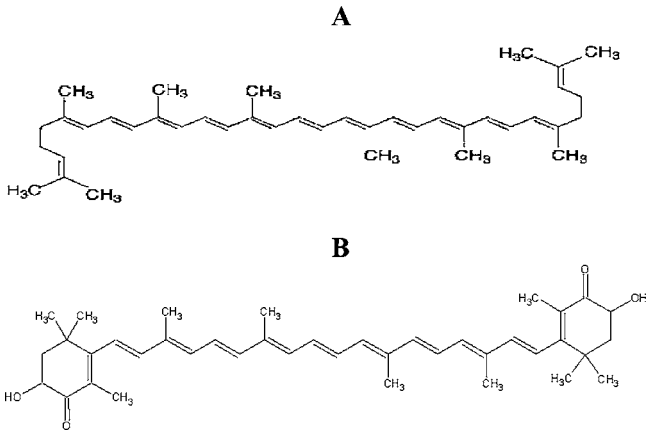

Figure 1. Structures of lycopene (**A**) and astaxanthin (**B**).

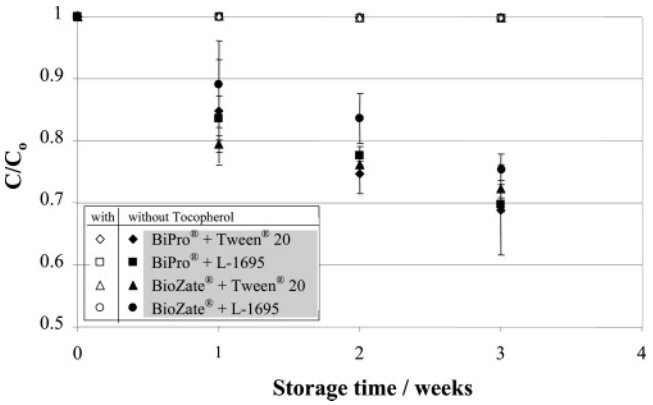

Figure 2. Degradation of lycopene in O/W emulsion. C = concentration, C_0 = initial concentration. Data are given as means ± SD.

carotenoids in food is available for utilization in physiological functions. This is probably due to the fact that carotenoids in fruit and vegetables may exist as crystals or bound in protein complexes (*19*). The bioavailability of solid carotenoids depends on their particle size and is only good for submicrometer particles (*20*). Carotenoids are insoluble in water. Their solubility in vegetable oil is usually less than 0.2 g/L at room temperature and depends on the presence of polar groups in the chemical structure of the carotenoid, and the triglyceride composition of the oil (*21*). Due to the system of conjugated double bonds, carotenoids are easily degraded by oxidation. Exposure to light or heat causes geometrical isomerization.

An oily solution is considered to be the ideal form of carotenoids regarding bioavailability (*18, 22*). Thermal processing improves dissolution of carotenoids into the lipid portion of the food matrix (*23*). Thermal and mechanical processing are convenient ways to enhance bioavailability. Oil-in-water (O/W) emulsions, in which carotenoids are dissolved in the disperse oil phase, combine the advantages of good bioavailability, applicability as a water-dispersible system, and high concentrations of carotenoids. Emulsions are thermodynamically unstable systems, but it is possible to form kinetically stable emulsions for a reasonable period of time—a few days, weeks, months, or years—by including adequate emulsifier prior to homogenization. Droplet size is of essential importance because of its great influence on physical stability.

For in vitro studies of carotenoid action, it is necessary to apply carotenoids in a formulation which leads to a good cellular uptake and is not toxic. In this work, cellular uptake of carotenoids from emulsions was investigated using colon carcinoma cells (HT-29 and Caco-2) as models for human colon epithelial cells. Cellular uptake can be used as a model for comparing the bioavailability of different carotenoids formulations.

MATERIALS AND METHODS

Preparation of Carotenoid-Enriched O/W Emulsions. In this study, eight different emulsions were prepared, each one with a combination (1:1) of two different emulsifiers. The emulsifiers used were whey protein isolate (BiPro, Davisco Foods International, Inc., Minnesota); hydrolyzed whey protein isolate (BioZate 1, Davisco Foods International, Inc., Minnesota), sucrose laurate (L-1695, Mitsubishi Chem., Düsseldorf, Germany), and polyoxyethylene-20-sorbitan-monolaurate (Tween 20, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to stabilize the oil droplets. Every experiment was repeated three times. Four emulsions were prepared with astaxanthin and the other four with lycopene as carotenoids with the same combination of emulsifiers. O/W emulsions containing either astaxanthin or lycopene with Tween 20 as emulsifier served as comparing systems. As antioxidant for a series of experiments, α-tocopherol (Carl Roth GmbH & Co.) was added to the oil phase at a concentration of 70 mg/L. O/W emulsions were prepared with bidestilled water as the continuous phase and fractionated palm oil (Bergabest MCT, 10 wt %, Schumann and Sohn, Karlsruhe, Germany) as the dispersed phase. The crystalline carotenoid, either astaxanthin (purity ≅ 90%) or lycopene (purity ≅ 95%) (BASF AG, Ludwigshafen, Germany), was dissolved in the oil phase. Carotenoid-loaded O/W emulsions were prepared according to Ax et al. (*24*). Afterward the O/W emulsions were homogenized at 120 MPa (Microfluidizer M-110Y, Microfluidics Corp., Newton, USA) to provide smaller droplets. The emulsion was kept in darkness and N_2 atmosphere at 4 °C.

Experiment on Chemical Stability. The concentration of each carotenoid was measured after preparation and once every week over 3 weeks. Analyses were carried out on the same weekday of each experiment to obtain the O/W emulsions. In order to measure the lycopene and astaxanthin concentration in the emulsions, they were extracted with n-hexane, ethanol, and dichloromethane (Merck KGaA, Darmstadt, Germany) and analyzed using a spectrophotometer (U2000, Hitachi Europe GmbH, Düsseldorf, Germany) (*25*).

Experiment on Physical Stability. Analyses of droplet size were carried out by laser diffraction with polarization intensity differential scattering (PIDS) technology (Couter LS230, Beckman-Coulter GmbH, Krefeld, Germany). The analysis of physical stability consisted of measuring the mean Sauter diameter ($d_{3,2}$) after the preparation and over 3 weeks. Samples were analyzed on the same weekday to control any coalescence from oil droplets.

In Vitro Tests of Carotenoid Uptake. For in vitro tests, human colon carcinoma cell lines HT-29 and Caco-2 were used as models for human colon epithelial cells. Cellular uptake of carotenoids from emulsions without α-tocopherol was investigated according to Briviba et al. (*26*).

RESULTS AND DISCUSSION

Chemical Stability of the O/W Emulsions Containing Carotenoids. Lycopene and astaxanthin concentration decreased in all emulsions without any antioxidant over three weeks (**Figures 2** and **3**). Probably such degradation was due to their oxidation, as could be observed by Ax (*27*). The combination of the BioZate and L-1695 as emulsifiers provided the most successful combination of those studied at ensuring lycopene stability (**Figure 2**). As can be seen in **Figure 3**, astaxanthin concentration decreased slower than lycopene concentration over 3 weeks, as expected because of its chemical structure. The combination of the BioZate and Tween 20 as emulsifiers showed the best combination of those studied to prevent astaxanthin degradation. Antioxidant mechanism of whey proteins seems to be free radical scavenging by amino acids. Whey proteins can inhibit lipid oxidation by several different mechanisms;

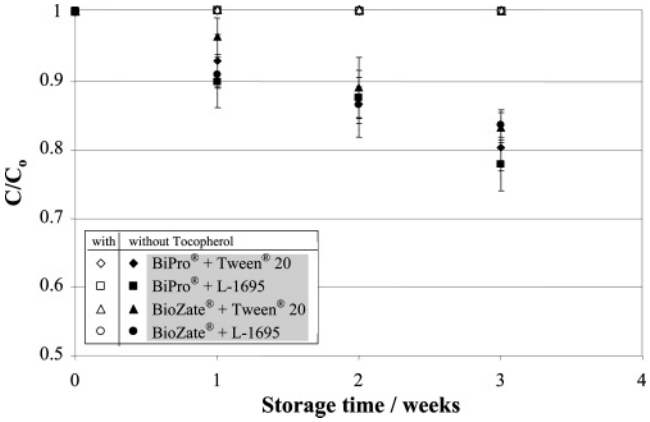

Figure 3. Degradation of astaxanthin in O/W emulsion. C = concentration, C_0 = initial concentration. Data are given as means ± SD.

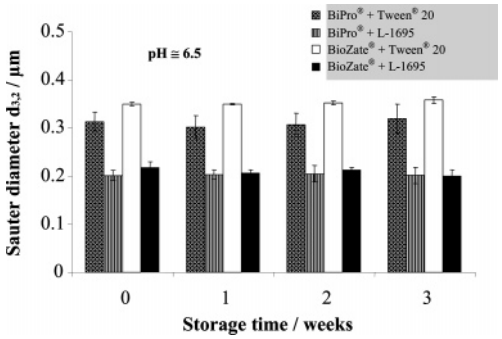

Figure 4. Stability of droplet size of lycopene-enriched O/W emulsions.

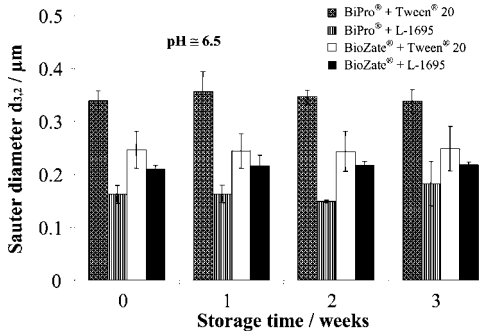

Figure 5. Stability of droplet size of astaxanthin-enriched O/W emulsions.

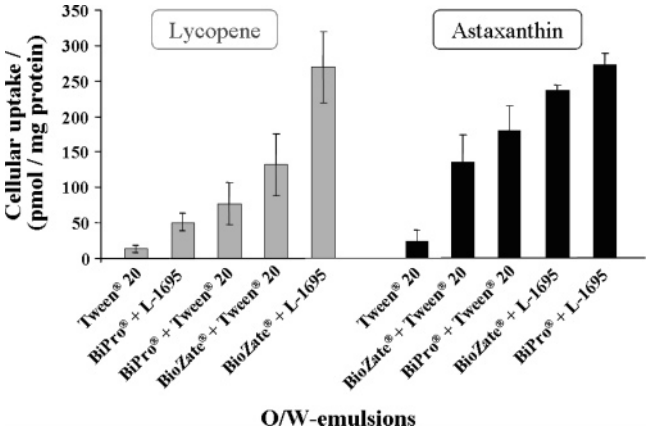

Figure 6. Absorption of lycopene and astaxanthin in colon carcinoma cells (HT-29).

sulfhydryl groups seem to be the most important antioxidant component (*28*). While these studies yielded interesting results concerning the antioxidant activity of whey proteins, the exact antioxidant mechanisms are still unclear. Donnelly et al. (*29*). observed a significant antioxidant effect when Tween 20 and whey proteins were added together as emulsifiers. The origin of this effect is currently not understood, but it may be due to the ability of surfactants to alter the conformation of proteins so that they expose more free radical scavenging or chelating amino acids (cysteine and tyrosine) or iron chelating amino acids.

Astaxanthin and lycopene concentrations remained constant in all emulsions containing α-tocopherol over 3 weeks (**Figures 2** and **3**). α-Tocopherol reacts with lipid peroxyl radicals to yield a relatively stable lipid hydroperoxide, and the tocopherolxyl radical interrupts the radical chain reaction (*10*). In this way, its addition protects astaxanthin and lycopene against oxidation (*27*, *30*).

Physical Stability of O/W Emulsions Containing Carotenoids. Figure 4 shows the physical stability of the droplet size of the emulsions that contain lycopene, over a period of 3 weeks. As can be observed there were no significant changes in it over the observed storage time at pH 6.5. Changes in droplet size in each emulsion over three weeks were not statistically significant. It means that no coalescence of the droplets was observed.

O/W emulsions stabilized with L-1695 combined with BiPro or BioZate showed smaller droplet size, about 0.2 μm. Similar results could be also observed for astaxanthin emulsions (**Figure 5**). Combination of BiPro and Tween 20 showed droplet sizes higher than 0.3 μm and BioZate and Tween 20 droplet sizes of approximately 0.25 μm.

Cellular Uptake. In **Figure 6**, results of the cellular uptake (HT-29 cell line) of carotenoids from O/W emulsions are shown. The highest cellular uptake of lycopene was observed from emulsions stabilized with combined BioZate and L-1695. For astaxanthin the combination of emulsifiers L-1695 and BiPro showed the highest cellular uptake. No significant differences between the cellular uptake of astaxanthin or lycopene from Caco-2 and HT-29 cells could be observed. Richelle et al. (*31*) studied the bioavailability of a formulation containing lycopene in which the carotenoid was entrapped with whey proteins. The study clearly showed that embedding lycopene into whey proteins enhances its bioavailability, so that it is equal to that from tomato paste.

The factor that is responsible for the enhanced bioavailability of lycopene in these kind of formulations is not yet clear. β-Lactoglobulin, a globular whey protein, has been shown to bind a variety of hydrophobic substances in vitro (*32*). The results presented suggest that O/W emulsions stabilized with whey protein could be an excellent vehicle to deliver carotenoids in vitro and in vivo. Papiz et al. (*33*) shows a drawing of the β-lactoglobulin fold binding a retinol molecule. According to their model binding, β-lactoglobulin could also accommodate astaxanthin or lycopene molecules.

Conclusions. This research showed that lycopene and astaxanthin in O/W emulsions without α-tocopherol degraded slightly after three weeks. Combination of BioZate and sucrose laurate (L-1695) as emulsifiers showed the most pronounced protection of lycopene. For astaxanthin stability, BioZate combined with Tween 20 showed the best effect. The nature of the interfacial membrane formed by these emulsifiers can have a large impact on the rate of carotenoid oxidation in O/W emulsions. Consequently, food scientists can enhance oxidation stability of carotenoids by manipulating interfacial characteristics using different emulsifiers. O/W emulsions stabilized with L-1695 and combined with BiPro or BioZate showed the smaller mean Sauter diameters for both emulsions containing lycopene and

those containing astaxanthin. Lycopene-loaded O/W emulsions stabilized with BioZate and L-1695 showed the highest cellular uptake. For astaxanthin, the combination of BioPro and L-1695 provided the best one.

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LITERATURE CITED

(1) Bendich, A.; Olson, J. A. Biological action of carotenoids. *FASEB J.* **1989**, *3*, 1927−1932.
(2) Krinsky, N. I. Actions of carotenoids in biological systems. *Annu. Rev. Nutr.* **1993**, *13*, 561−587.
(3) Mayne, S. T. Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J.* **1996**, *10*, 690−701.
(4) Conn, P. F.; Lambert, C.; Land, E. J.; Schalch, W.; Truscott, T. G. *Free Radicals Res. Commun.* **1992**, *16*, 401−408.
(5) Hiramaya, O.; Nakamura, K.; Hamada, S.; Kobayashi, Y. Singlet Oxygen Quenching Ability of Naturally Occuring Carotenoids. *Lipids* **1994**, *29*, 149−150.
(6) Stahl, W.; Sies, H. Lycopene: A Biological Important Carotenoid for Humans? *Arch. Biochem. Biophys.* **1996**, *336*, 1−9.
(7) Baltschun, D.; Beutner, S.; Briviba, K.; Martin, H. D.; Paust, J.; Peters, M.; Röver, S.; Sies, W.; Steigel, A.; Stenhorst, F. Singlet Oxygen Quenching Abilities of Carotenoids. *Liebigs Ann./Recueil* **1997**, 1887−1893.
(8) Gester, H. Anticarcinogenic Effect of Common Carotenoids. Internat. *J. Vit. Nutr. Res.* **1993**, *63*, 93−121.
(9) Henry, L. K.; Catignani, G. L.; Schwartz, S. J. Oxidative degradation kinetics of lycopene, lutein and 9-cis and all trans β-carotene. *JAOCS* **1998**, *75*, 823−829.
(10) Sies, H.; Stahl, W.; Sundquist, A. R. Antioxidant function of vitamins. Vitamins E and C, β-carotene and other carotenoids. *Ann. N.Y. Acad. Sci.* **1992**, *669*, 7−20.
(11) Di Mascio, P.; Kaiser, S.; Sies, H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochim. Biophys.* **1989**, *274*, 532−538.
(12) Alejung, P.; Wadstroem, T. Oral preparation for treatment of Helicobacter sp. Infections comprises xanthophylls, especially astaxanthin esterified with a fatty acid and derived from the alga Haematococcus sp. 1998, World Patent #9837874.
(13) Di Mascio, P.; Devasagayam, T. P. A.; Kaiser, S.; Sies, H. Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. *Trans. Biochem. Soc.* **1990**, *18*, 1054−1056.
(14) Miki, W. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **1991**, *63*, 141−146.
(15) Tanaka, T.; Morishita, Y.; Suzuki, M.; Kojima, T.; Okumura, A.; Mori, H. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. *Carcinogenesis* **1994**, *15*, 15−19.
(16) Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.; Hara, A. Chemoprevention of rat ral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res.* **1995a**, *55*, 4059−4064.
(17) Tanaka, T.; Kawamori, T.; Ohnishi, M.; Makita, H.; Mori, H.; Satoh, K.; Hara, A. Suppression of azomethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis* **1995b**, *16*, 2957−2963.
(18) West, C. E.; Castenmiller, J. Quantification of the "SLAMENGHI" Factors for Carotenoid Bioavailability and Bioconversion. *Int. J. Vit. Nutr.* **1998**, *68*, 371−377.
(19) Williams, A. W.; Boileau, T. W.; Erdman, J. W. Factors Influencing the Uptake and Absorption of Carotenoids. *Proc. Soc. Exp. Biol. Med.* **1998**, *218*, 106−108.
(20) Horn, D. Preparation and Characterization of Microdisperse Bioavailable Carotenoid. Hydrosols. *Angew. Makromol. Chem.* **1989**, *166/ 167*, 139−153.
(21) Borel, P.; Grolier, P.; Armand, M.; Partier, A.; Lafont, H.; Lairon, H.; Lairon, D.; Azais-Braesco, V. Carotenoids in biological emulsions: solubility, surface-to-core distribution, and release from lipids droplets. *J. Lipid Res.* **1996**, *37*, 250−261.
(22) Parker, R. S. Bioavailability of Carotenoids. *Eur. J. Clin. Nutr.* **1997**, *51*, 86−90.
(23) Gartner, C.; Stahl, W.; Sies, H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. *Am. J. Clin. Nutr.* **1997**, *66*, 116−122.
(24) Ax, K.; Schubert, H.; Briviba, K.; Rechkemmer, G.; Tevini, M. Oil-in-water emulsions as carriers of bioavailable carotenoids. In *Proceedings Partec 2001*, Nürnberg (in CD-ROM).
(25) Britton, G.; Liaaen-Jensen, S.; Pfander, H. *Spectroscopy*; Carotenoids, Vol. 1B; Birkhäuser Verlag: Basel, 1995.
(26) Briviba, K.; Schnäbele, K.; Schwertle, E.; Blockhaus, M.; Rechkemmer, G. β-Carotene inhibits growth of human colon carcinoma cells *in vitro* by induction of apoptosis. *Biol. Chem.* **2001**, *382*, 1663−1668.
(27) Ax, K. Emulsionen und Liposomen als Trägersysteme für Carotinoide. Ph.D. Thesis, Universität Karlsruhe (TH), Shaker Verlag, 2004.
(28) Phillips, L. G.; Whitehead, D. M.; Kinsella, J. *Structure-function properties of food proteins*; Academic Press: London, 1994.
(29) Donelly, J. L.; Decker, E. A.; McClements, D. J. Iron-catalyzed oxidation of Menhaden oil as affected by emulsifiers. *J. Food Sci.* **1998**, *63*, 997−1000.
(30) Ribeiro, H. S.; Ax, K.; Schubert, H. Stability of lycopene emulsions in food systems. *J. Food Sci.* **2003**, *68*, 2730−2734.
(31) Richelle, M.; Bortlik, K.; Liardet, S.; Hager, C.; Lambelet, P.; Baur, M.; Applegate, L. A.; Offord, E. A. A. food-based formulation provides lycopene with the same bioavailability to humans as that from tomato paste. *J. Nutr.* **2002**, *132*, 404−408.
(32) Narayan, M.; Berliner, L. J. Fatty acids and retinoids bind independently and simultaneously to β-lactoglobulin. *Biochemistry* **1997**, *36*, 1906−1911.
(33) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

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