Stability of β-Carotene in Protein-Stabilized Oil-in-Water Delivery Systems

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ABSTRACT: Inclusion of liposoluble bioactive compounds in fortified foods represents a complex challenge due to the labile nature of such compounds and the instability of oil-in-water emulsion-based delivery systems. In the present study, dispersions prepared with 10% (w/w) sunflower oil (SO) or hydrogenated palm kernel oil (HPKO) containing 0.05% (w/w) β-carotene were stabilized by various concentrations of whey protein isolate (WPI) or sodium caseinate (NaCas) (0.1 to 2.0% w/w) in 30% (w/w) sucrose aqueous solutions. Physicochemical characterization of emulsions was done considering the particle size, the particle surface protein coverage, and the physical state of continuous and dispersed phases. Physical stability of the systems and their protection properties on β-carotene were compared. The lipid carrier type and interfacial structure were investigated as the two key factors which regulate the stability of labile lipophilic bioactive molecules in food model systems. Our results showed high β-carotene stability when O/W systems were stable (protein concentration ≥ 0.8% w/w.) A (partially) solid lipid carrier (HPKO) enhanced protection compared to the liquid carrier (SO) as the bioactive molecules were entrapped in isolated domains within the solid lattice and kept apart from reactive species in the surroundings. NaCas provided a better barrier than WPI due to the different amino acid composition and interface structure which significantly reduced β-carotene degradation rate.

KEYWORDS: β-carotene, O/W emulsion, dispersion, sodium caseinate, whey protein isolate, hydrogenated palm kernel oil

■ INTRODUCTION

Fortification of foods by addition of bioactive compounds is a current trend for food industries. In particular, the scientific evidence supporting the health benefits related to dietary antioxidants[1−3] has induced food scientists to investigate efficient delivery systems for antioxidant fortification of liquid products.[4−6] Among antioxidants, bioactive lipids, such as carotenoids and especially β-carotene, are often the best model substances for a convenient scientific investigation, because they are cost-effective, easy to detect and chemically well characterized.

Inclusion of liposoluble bioactive compounds in water-based liquid food formulations is often difficult due to the poor solubility and the labile nature of such compounds which are sensitive to pH, temperature oxygen and light.[7] For this reason, these molecules need to be protected from the adverse effects of the environment which can cause structural modification (e.g., isomerization, introduction of oxygen and chain shortening) resulting in a loss of their beneficial biological activity.[8] The common degradation pathways for carotenoids are isomerization, oxidation and fragmentation. However, oxidation is the major cause of carotenoids degradation, and it is due to the interaction with oxygen-active species, such as singlet oxygen, superoxides, peroxides, hydroxyl radicals, and transition metals; but it is also stimulated by light and heat.[8,9] Conversely, the concurrent presence of antioxidants, such as tocopherols (vitamin E) and ascorbic acid (vitamin C), inhibits oxidation.[9] The general mechanism of β-carotene oxidation is similar to that of lipid peroxidation involving radicals.[10,11] According to the studies by El-Tinay and Chichester (1970),[12] the rate of loss of β-carotene in toluene by reaction with molecular oxygen followed zero-order reaction kinetics.

Food systems may contain sensitive components, such as lipophilic β-carotene, dissolved in dispersed lipid components with different physicochemical characteristics. However, studies on the effect of the physical state of the lipid phase and the interfacial structure on the degradation kinetics of encapsulated components are limited. In an earlier study, Okuda et al.[13] found that a liquid lipid carrier gave a better protection of the oxidation-sensitive methyl linolenate than a solid carrier, and Hu et al.[14] found an improved protection against corn oil oxidation when NaCas was used as emulsifier. In the present study, different possible factors which could improve the stability of oxygen sensitive compounds (i.e., β-carotene) in model food emulsions were investigated. Thus, β-carotene was incorporated into the dispersed phase of oil-in-water (O/W) emulsion systems, in which the lipid carrier was surrounded by a membrane of dairy proteins. Amphiphilic dairy proteins are widely used as food ingredients as they are readily adsorbed at the O/W interface and provide stability to dispersed systems.[15] The effects of the physical state of the lipid carrier and interfacial composition were evaluated for their impact on the bioactive and system stability. Hydrogenated palm kernel oil (HPKO) and sunflower oil (SO) were chosen as lipid carriers because of their totally different crystallization and melting temperature profiles. The stabilizing properties of different milk proteins were also considered. The protective properties of sodium caseinate (NaCas) and whey protein isolate (WPI) on β-carotene degradation at various concentrations was evaluated for the different structures at the O/W interface that they form.[16] Moreover, as emulsions are thermodynamically unstable systems and may undergo physical changes (e.g., gravitational separation, oiling-off, flocculation,

coalescence, Ostwald ripening and phase inversion),[17] their physical stability during storage was also determined.

■ MATERIALS AND METHODS

Materials. The WPI used was Bipro from Davisco Foods (Le Sueur, MN, USA). NaCas was purchased from Dairygold Food Ingredients Limited (Mitchelstown, Ireland). HPKO (SIL-CREAM 90) was donated by Trilby Trading (Drogheda, Ireland). SO (Flora Pure Sunflower Oil, Unilever) was purchased at the local market. Sucrose was purchased from Irish Sugar Ltd. (Carlow, Ireland). β-Carotene, analytical grade reagents for its analysis [n-hexane, butylated hydroxytoluene (BHT), sodium sulfate, methanol, potassium hydroxide], for protein determination [Folin−Ciocalteau reagent, sodium potassium tartrate, copper(II) sulfate pentahydrate] and for peroxide value (chloroform, acetic acid, potassium iodide, sodium thiosulfate, starch), preservatives (sodium azide, chloramphenicol), sodium hydroxide and hydrochloric acid were purchased from Sigma Chemical Co. (Sigma-Aldrich Ireland Ltd., Dublin, Ireland).

Emulsion Preparation. Proteins were prepared by dispersing 5% (w/w) protein powders in deionized water at room temperature under magnetic stirring for ∼3 h to ensure complete hydration and dispersion. HPKO or SO containing 0.05% (w/w) β-carotene was heated at 50 °C and stirred with a magnetic rod in the dark for at least 1 h to ensure that a homogeneous particle-free dispersion form was obtained (Figure 1). A 60% (w/w) sucrose syrup was also prepared and held at 50 °C. These sucrose and protein systems were mixed at the proper ratio and, after adjustment of the final weight by adding deionized water and of the pH to 7 using 1 M NaOH or 1 M HCl, blended to HPKO and SO to form a coarse pre-emulsion by using a high speed homogenizer (Ultra-Turrax T 25 Digital) for 30 s. The pre-emulsions (10% w/w oil, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0% w/w proteins; and 30% w/w sucrose in the aqueous phase) were then homogenized using a two stage valve homogenizer (APV-1000 high-pressure homogenizer, Wilmington, MA) with one cycle at 850 bar and cooled to room temperature, and ∼0.02% (w/w) sodium azide and ∼1 mg/kg chloramphenicol were then added. The final systems were cooled down to ∼4 °C overnight to ensure HPKO crystallization and then stored at 20 °C for 32 days.

Particle Size Analysis. The characterization of emulsified systems was done by measuring the particle size distribution and the average particle diameter by using a laser diffraction particle size analyzer (Malvern MasterSizer MSE, Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). The refractive index of the emulsion droplets chosen was 1.456, while the refractive index of the dispersion medium was 1.33. The absorbance value of the emulsion droplets was 0.1. Droplet size measurements are reported as particle size distribution and the surface-weighted, or Sauter, mean diameter, D[3,2].

Determination of Surface Protein Coverage. Surface protein coverage was determined using the method described by Ye,[18] with minor modifications. Emulsions (30 g) were centrifuged in a Beckman J2-21 high speed centrifuge at 15000 rpm (JA-20 fixed-angle rotor) at 4 °C for 1 h. The subnatant was removed by using a long needle syringe, then filtered sequentially through 0.45 and 0.20 μm filters (Minisart, Sartorius, Germany). The total protein concentration in the subnatant was determined spectrophotometrically by using the method of Lowry et al.[19]

The surface protein concentration (expressed in mg/m^2 of particle surface) was calculated from the difference between the amount of protein used to prepare the emulsion and that measured in the subnatant after centrifugation using the mean surface diameter, D[3,2] to calculate the specific surface area of the lipid particles as described by Tcholakova et al.[20]

Visual Observation. Samples were visually inspected in order to detect physical instability, such as phase separation, oiling-off, or creaming. After the preparation, emulsions containing liquid SO and solid HPKO were stored at 20 °C in glass test tubes (200 mm height, 20 mm internal diameter) and their appearance was evaluated over
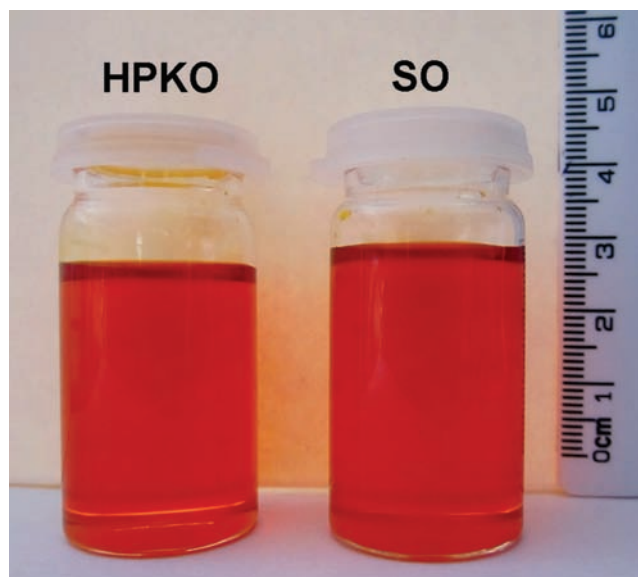

Figure 1. β-Carotene (0.05% w/w) in SO and HPKO after 1 h magnetic stirring in the dark.

32 days of storage. System instability was monitored by measuring the height of the lipid-rich layer on top (H_L) with a ruler and expressing it as creaming index, C.I. (%) by normalizing it for the height of the product in the test tube. C.I. = $(H_L/H_E) \times 100$ where H_L is the height of the lipid-rich layer on top and H_E is the height of the emulsion in the test tube.

Determination of Lipid Physical State. The thermal behavior of the bulk and dispersed lipid was studied by using differential scanning calorimetry, DSC, (Mettler Toledo 821e, Schwerzenbach, Switzerland) with liquid N$_2$ cooling. The data were analyzed using STARe thermal analysis software, version 6.0 (Mettler Toledo, Schwerzenbach, Switzerland). Samples (∼15 mg) were prepared in DSC aluminum pans (40 μL; Mettler Toledo, Schwerzenbach, Switzerland), and analyzed with a cooling scan from 50 to −40 °C for emulsions containing HPKO and from 50 °C to −60 °C for emulsions containing SO at −1.5 °C/min and with a heating scan over the same temperature range at 5 °C/min. Lipid crystallinity was also investigated by using X-ray diffraction. XRD data were collected on a Philips X'pert PW3719 diffractometer using Cu Kα radiation (λ = 1.540598 Å; voltage 40 kV and current 35 mA). Emulsified systems were placed on the XRD plate and as a thin layer then analyzed. Melted HPKO containing 0.05% β-carotene was poured on the flat XRD plate with cylindrical well, crystallized as a thin layer at ∼5 °C overnight and then incubated at 20 °C for one day before analysis. Ten repeated XRD analyses were performed in 0.015 steps, using a continuous scan over the 2 theta range of 10−55° for emulsified systems and 10−60° for bulk HPKO.

Determination of the Peroxide Value (PO Value). PO value, expressed in milliequivalents of active oxygen per kilogram of oil (mequiv/kg) was determined by iodometric titration with 0.01 N sodium thiosulfate as reported in the official EEC method no. 2568/91.[21] A weighted aliquot of approximately 1 g of oil was placed in a conical flask with a ground-glass stopper, and 25 mL of a mixture of chloroform and acetic acid (2:3) was added. After shaking to dissolve the substance, 1 mL of saturated potassium iodide solution was added and the mixture left to react in darkness for 5 min. The reaction was stopped by addition of 75 mL of distilled water, and the free iodine was then titrated with standard 0.01 N sodium thiosulfate solution.

Analysis of β-Carotene. A two step procedure was used for the extraction of β-carotene from emulsified samples. Emulsions were preliminarily destabilized by mixing with 1.5 mL ethanol. In the first
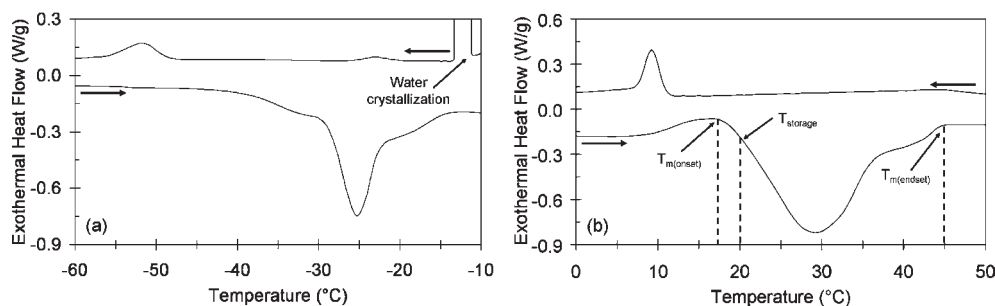
Figure 2. DSC thermograms showing crystallization and melting behavior of SO (a) and HPKO (b) containing 0.05% (w/w) β-carotene, emulsified by 1.2% (w/w) WPI. Cooling rate 1.5 °C/min, heating rate 5 °C/min.

step, weighted aliquots of ~1 g of product stored in 13 mL screw-capped polypropylene test tubes at 20 °C were saponified by adding and stirring, using a Vortex for 10 s, 1 mL of saturated potassium hydroxide in methanol in order to separate the lipid carrier (saponized fraction) from the β-carotene (unsaponized). The mix was placed in a thermostatic water bath and heated to 45 °C for 30 min to increase the reaction rate. In the second step, after cooling to room temperature, the unpolar β-carotene was extracted three times with 2 mL of n-hexane containing 0.1% (w/v) BHT. The organic solvent was added to the emulsion—KOH/methanol mix and stirred (Vortex for 30 s) and then left to stand for 10 min to obtain the separation of an organic layer containing β-carotene on top and a water layer on the bottom. The top layer was accurately removed with a glass Pasteur pipette and transferred in a glass vial containing a small amount (~0.5 g) of anhydrous sodium sulfate powder which adsorbed possible residual water. After 1:4 dilution with n-hexane containing 0.1% (w/v) BHT, extracts were analyzed spectrophotometrically (Varian Cary 300 Bio UV—visible spectrophotometer) at the maximum absorbance wavelength for β-carotene, λ_{max} = 450 nm.

Statistics. At least three aliquots of freshly prepared samples were observed in all the experimental procedures. All the analytical determinations were done in three replicates; therefore, data were reported as mean with standard deviation.

■ RESULTS AND DISCUSSION

Material Characterization. The physicochemical characterization of the materials used was carried out to develop systems for the protection and stabilization of β-carotene in aqueous dispersion based on solid or liquid lipids as carrier materials. The lipid carriers used were SO and HPKO. In the thermograms reported in Figure 2, the thermal behavior of dispersed systems emulsified by WPI was compared. Small differences in the crystallization of the dispersed droplets due to the action of the emulsifier type were found in agreement with Relkin at al.[22] However, variations in the crystallization were considered negligible for the purpose of this study, therefore, thermograms of NaCas-stabilized systems were not shown. As shown in Figure 2a, the DSC analyses of SO-containing systems showed exothermic peaks of water crystallization between −12 and −15 °C and lipid crystallization at −23 °C and −55 °C. The two peaks of SO crystallization were most likely due to the fact that SO was a complex mixture of triglycerides (TAGs) which crystallized as two independent fractions of high- and low-melting TAGs as we previously explained.[23] On the other side, emulsified HPKO showed a single peak of lipid crystallization at 8 °C (Figure 2b). Upon heating, emulsified SO was completely melted at ~−10 °C (Figure 2a), whereas HPKO showed a broad melting peak, ranging from ~17 °C to ~45 °C (Figure 2b). This hysteresis among the crystallization temperature
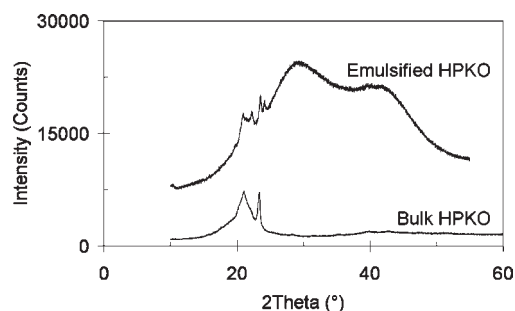
Figure 3. XRD patterns showing peaks of lipid crystallinity of bulk and 1.2% (w/w) WPI-stabilized HPKO-in-water emulsions containing 0.05% (w/w) β-carotene in the dispersed lipid phase, at 20 °C.
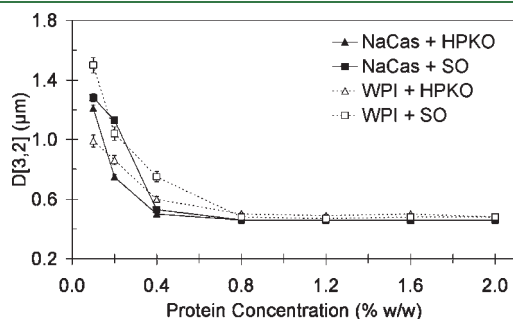
and the melting temperature was typical of emulsified systems.[24,25] Depending on particle size, the crystallization behavior of emulsified lipids typically required a high degree of supercooling for crystallization.[23,26−30] On this basis, delivery systems with liquid and (partially) solid lipid carriers could be produced by simply cooling the fresh emulsions to below the crystallization temperature of HPKO (~7 °C) and storing them at a temperature low enough to avoid HPKO melting (20 °C). These conditions were chosen to obtain systems similar to nanostructured lipid carriers (NLC) made of a solid matrix entrapping variable liquid lipid nanocompartments[31] which could allow an increased encapsulation efficacy.[32]

To verify the presence of a solid phase in the emulsified HPKO at 20 °C, X-ray diffraction analysis was done. As reported in Figure 3, bulk HPKO showed two main peaks of crystallinity at ~21 and ~23 °C. Emulsified HPKO showed a good correlation with the XRD patterns of bulk lipid, as peaks were detected between ~21 and ~24 °C (Figure 3) suggesting the presence of HPKO crystals at 20 °C. The differences of XRD peak positions could be due to the different crystal structure caused by the effects of the hydrophobic part of the proteinaceous emulsifier in the lipid crystal network, according to our earlier hypothesis.[30]

System Characterization and Development. According to the Stokes law,[33] one important parameter that helps to minimize the gravitational separation improving kinetic stability of O/W emulsions is the reduced size of the dispersed particles. Therefore, the first part of the work was done to evaluate the effectiveness of WPI and NaCas as emulsifiers by determining the concentration required to produce the minimum mean droplet size (maximum surface area per unit of volume of lipid) under the conditions of homogenization used. The dependence of the surface-weighted mean diameter, D[3,2], of the dispersed

droplets from protein concentration is reported in Figure 4. For all 4 different formulations (WPI + SO; WPI + HPKO; NaCas + SO; NaCas + HPKO), the emulsifier concentration was limiting below 0.8% (w/w) as the droplet size was reduced by increasing the concentration of the emulsifier. As explained by McClements,[34] under limiting concentration of an emulsifier, the particle size produced by homogenization was determined by the maximum amount of surface area that could be covered by the available emulsifier. Above this threshold, the particle size was independent of the emulsifier concentration, depending mainly on the energy input of the homogenizer. These latter conditions resulted in monomodal particle size distribution with a minimum $D[3,2]$ within the range of 0.46−0.50 μm (Figure 4). A similar particle size distribution allowed us to exclude the effect of the particle size when comparing similar systems. As previously reported by Relkin at al.,[35] a higher size resulted in a reduced degradation of α-tocopherol included in a milk fat carrier.

The functional behavior at the O/W interface of the disordered flexible NaCas and the compact globular WPI was studied to understand the particle-surface coverage and its impact on system and β-carotene stability. If the emulsifier was in excess and did not form multiple layers at the interface, the surface coverage (mg/m^2) remained constant. On the contrary, if the emulsifier formed multiple layers, the surface load increased as the concentration of emulsifier in the system was increased.[34] Results for the protein adsorption at the interface are summarized in Figure 5. In NaCas-stabilized emulsions containing SO or HPKO, the total surface protein concentration increased from 1.70 ± 0.05 and 1.55 ± 0.02 mg/m^2 to 3.64 ± 0.08 and 3.97 ± 0.24 mg/m^2, respectively, when protein concentration was increased from 0.1 to 0.8% (w/w). Above this concentration, when the main particle diameter remained constant, the surface
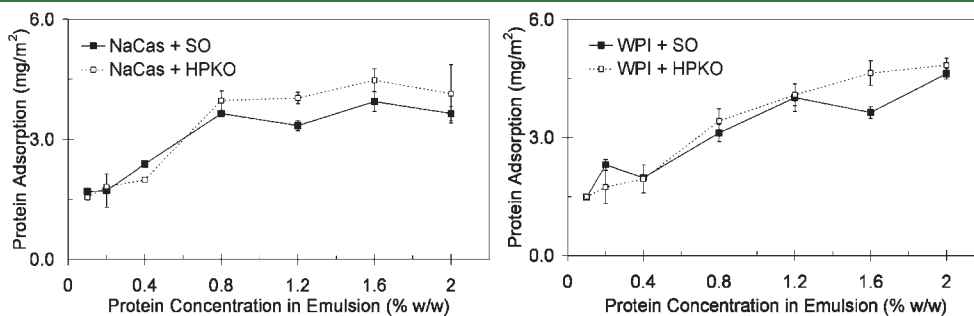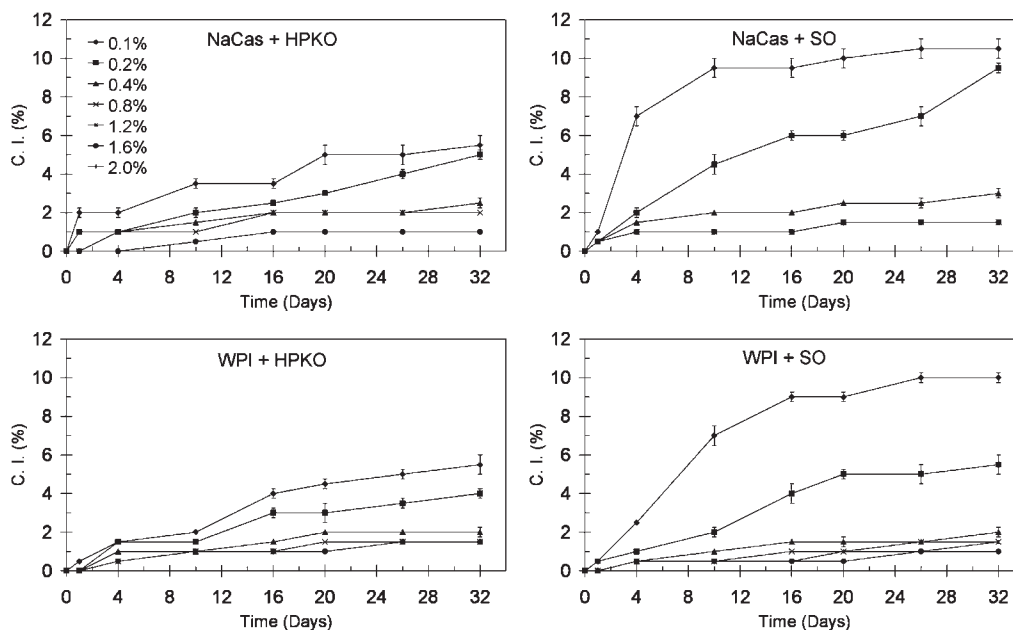
NaCas coverage remained roughly constant (3.34 ± 0.13, 3.94 ± 0.25, 3.64 ± 0.18 and 4.03 ± 0.14, 4.48 ± 0.29, 4.14 ± 0.73 mg/m^2 at 1.2, 1.6 and 2.0% (w/w) protein when SO and HPKO were used, respectively). This was most likely due to the formation of a single layer of proteins at the O/W whereas the exceeding protein remained in the aqueous phase. Conversely, the surface coverage of WPI progressively increased with increasing concentration in the aqueous phase, from 1.49 ± 0.01 to 4.62 ± 0.13 mg/m^2 for emulsions containing SO, and from 1.46 ± 0.01 to 4.84 ± 0.18 mg/m^2 for HPKO-containing systems. This different behavior was due to the different amino acid compositions of NaCas and WPI. On adsorption at the O/W interface, there was some macromolecular rearrangement and partial denaturation of ordered globular whey proteins (molten-globule state).[36] The partial unfolding of the structure upon adsorption caused an exposure of nonpolar groups and thiol and disulfide groups, which increased protein−protein hydrophobic interactions and cross-linking (via intermolecular disulfide bonding) through a thiol−disulfide exchange reaction or oxidation, respectively, leading to polymerization of the adsorbed protein layer.[37] Therefore, in the present study, the gradual increase of the surface protein concentration might be explained with the formation of a multilayer of WPI due to protein−protein interactions. On the other hand, NaCas rapidly saturated the O/W interface but its concentration remained roughly constant, because NaCas did not show extensive protein−protein cross-linking due to the fact that its two main components α_{s1} and β caseins ($\sim$75% of the total casein) contain no cysteine or free thiol groups.[38]

System Stability. The stability of systems with different formulations was followed over 32 days by observing gravitational separation phenomena and changes of particle size distribution. The impact of emulsifier concentration on emulsion particle size was very strong when both SO and HPKO were used as lipid carriers. Limiting concentration of emulsifier ($\leq$0.4% w/w) gave a bimodal particle size distribution with surface-weighted main particle diameter in the range of 1.28 ± 0.03 to 0.52 ± 0.01 μm for emulsions stabilized by NaCas, and 1.50 ± 0.05 to 0.6 ± 0.02 μm for emulsions stabilized by WPI (Figure 4). The particle size distribution and surface coverage influenced the physical stability of the emulsions. The large particle size distribution and the thin layer at the interface led to extensive particle−particle aggregation, which resulted in a visually detectable separation of a lipid-rich layer on the top and a lipid-depleted aqueous phase on the bottom (Figure 6). At 0.1% (w/w) protein concentration, systems containing SO were shown to complete phase separation (C.I. 10%) around 10−16 days, whereas HPKO-containing systems were partially stable (C.I. $\sim$6%). Emulsions containing HPKO were more stable than those containing SO probably

Figure 4. Sauter-average particle diameter, $D[3,2]$, of freshly prepared O/W emulsions containing 0.05% (w/w) β-carotene in the dispersed lipid phase as a function of the protein concentration in the system, after 1 homogenization cycle at 850 bar.

Figure 5. Particle surface protein coverage (mg/m^2) of NaCas- and WPI-stabilized 10% (w/w) O/W emulsions plotted as a function of the protein concentration in the system.

Figure 6. Gravitational separation phenomena of 10% (w/w) O/W emulsions containing 0.05% (w/w) β-carotene in the dispersed phase expressed as creaming index, C.I., (%). C.I. = $(H_L/H_E) \times 100$ where H_L is the height of the lipid-rich layer on top and H_E is the total height of the emulsion against observation time. Aqueous phase (pH = 7) contained 30% (w/w) sucrose.

because, in the latter, (real) coalescence accelerated the phase separation. An improved stability was progressively observed when the protein concentration was increased to 0.2% and 0.4% (w/w). Above this range of protein concentrations (i.e., 0.8−2.0% w/w), systems were visually stable and the particle size remained unchanged over the period of observation (D[3,2] = 0.46−0.5 μm).

β-Carotene Stability. β-Carotene concentration in the freshly prepared emulsions (i.e., time 0) was measured in order to obtain an understanding of the losses due to processing and efficacy of the extraction method. The results highlighted a reduced β-carotene concentration of a magnitude over the range of 3−4.5% (w/w) compared to the original concentration in the systems. During 32 days of storage at 20 °C, the changes of β-carotene concentration could be described by a zero-order kinetics reaction model, as shown in the example in Figure 7. According to this evidence, the comparison between the degradation rate of the various systems could be done by comparing the slopes of the curves of the linear plots of β-carotene concentration against time, as suggested by Labuza.[39]

The slopes of the linear plots describing β-carotene loss with changing formulation are summarized in Table 1. The first evidence was that the degradation rate of β-carotene was strictly dependent on system stability. When emulsifier concentration was insufficient to ensure stability of the system (0.1−0.2% w/w), the rate of loss was high (from −1.0821% w/w day^{-1} of 0.1% w/w WPI + SO to −0.4479% w/w day^{-1} for 0.2% w/w NaCas + HPKO). Partially stable systems (0.4% w/w) showed reduced rates of losses (−0.7777 to −0.4541% w/w day^{-1}) compared to unstable systems. These were still high, with the exception of HPKO + 0.4% (w/w) NaCas, when compared to stable systems for which values ranged from −0.7299 to −0.2190% w/w day^{-1}.

The impact of the lipid carrier type on β-carotene stability was significant. The solid lipid carrier, HPKO, greatly enhanced the stability of the bioactive compound. The rate of loss in emulsions containing HPKO was lower than in emulsion containing SO
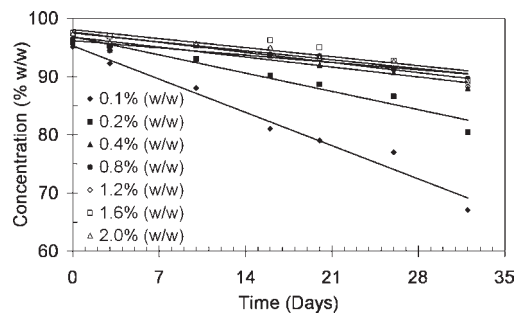
Figure 7. Kinetics of degradation of 0.05% (w/w) β-carotene included in the dispersed phase of 10% (w/w) HPKO-in-water emulsions prepared with various concentrations of NaCas.

both when systems were stable (protein concentration >0.4% w/w) and when they were unstable (protein concentration ≤0.4% w/w). This result confirmed the great attention and application of cosmetic and pharmaceutical preparations and food model trials, based on NLC.[40−43] According to these studies, the solid carrier enhanced stability by providing an effective barrier which protected the entrapped bioactive molecule for damages due to reactive species, and often enhanced bioavailability.[44] In the systems considered in the present study, β-carotene degradation was due to the oxygen in the surroundings and most likely to the presence of other pro-oxidant species (metals), such as impurities (metal ions) from sucrose and proteins. The partial crystallinity of the emulsified HPKO at 20 °C [melting temperature onset ($T_{m(onset)}$) = 17 °C] might have confined the bioactive compound in isolated liquid compartments surrounded by a protective barrier of solid HPKO network. This structure resembles that of a liquid lipid-in-solid lipid dispersion. Similar structures have been described for "multiple type" NLC in pharmaceutical studies.[31] This multiple type structure can be the explanation for the different results obtained by

Table 1. Kinetics of β-Carotene Loss (% w/w day^{-1}) in Partially Solid (HPKO) and Liquid (SO) Lipid Carriers Emulsified by Dairy Proteins during 32 Days Storage at 20° in the Dark

[Protein] (% w/w)	NaCas + HPKO	NaCas + SO	WPI + HPKO	WPI + SO
0.1	−0.8162	−0.8761	−0.6854	−1.0821
0.2	−0.4479	−0.7275	−0.6750	−0.9249
0.4	−0.2440	−0.6961	−0.4541	−0.7777
0.8	−0.1791	−0.5136	−0.4489	−0.7299
1.2	−0.2480	−0.4815	−0.5478	−0.7068
1.6	−0.2211	−0.4290	−0.5538	−0.7148
2.0	−0.2190	−0.4931	−0.5471	−0.6981

Okuda et al.,[13] who observed a higher degradation of methyl linolenate encapsulated in solid octadecane than in liquid octadecane. This was explained by the authors, with the exclusion of methyl linolenate from the crystal network toward the particle surface, where it could have reacted with oxidative species in the water phase.

The PO value (mequiv/kg) was also monitored for the lipids used as carriers. The PO value in HPKO was null whereas for SO it was found fairly constant (∼7 mequiv/kg) over the period of observation, as it was "buffered" by the presence of natural antioxidants (60 mg/100 mL vitamin E as stated by the producer). The constant PO value allowed us to postulate that the enhanced stability of β-carotene in HPKO was mainly due to the solid state of the carrier, which dramatically reduced interactions with the surroundings according to the Stokes—Einstein equation for diffusion $[D = (k_{B}T)/(6\pi\eta r)$ where D = diffusion constant, k_{B} = Boltzmann's constant, T = absolute temperature, η = viscosity, r = radius of the particle]. When β-carotene is present in a solid matrix of TAGs, lipid in the solid state is not available to act as medium for reactions, for which the molecular movement is essential, limiting transport and exchange of reactive species and products.

The effect of the emulsifier type on the kinetics of degradation of β-carotene was also remarkable. With the exception of systems containing 0.1% (w/w) protein and HPKO, NaCas showed a better protection of the bioactive than WPI, when both HPKO and SO were used as carriers. In protein-stabilized emulsions, it was hypothesized that the improved oxidative stability could be attributed to impact of the electrostatic interactions between ions and proteins at the interface. According to this, reactive cations were repelled by positively charged proteins below the isoelectric point. From studies on O/W emulsions stabilized by whey proteins, casein and soy proteins, the charge of emulsion droplets was not the only factor influencing the oxidative stability of the dispersed lipid phase.[14,45] Other factors such as ability of protein to chelate metal ions, scavenge free radicals, or react with reactive products of primary and secondary oxidation and the physical state of the lipid and the structural characteristics of the O/W interface (thickness, permeability and viscosity) can also modulate lipid oxidation.[46,47]

Even if the surface viscosity of caseins is lower than that of whey proteins,[48] according to Dickinson,[16] NaCas might have formed a protein layer which was thicker than the multilayer formed by WPI, in the range of concentrations used in this study. This reduced the permeation of reactive species from the aqueous phase to the lipid phase. As pointed out by Allen and Wrieden,[49] caseins are the most efficient antioxidant proteins in milk and provide a protective layer to milk fat after homogenization. Our result was coherent with a previous study by Hu et al.,[14] where the oxidative stability of the different protein-stabilized corn oil-in-water emulsions was in the order casein > WPI > soy protein isolate. These authors concluded that such a different behavior was most likely due to the differences in interfacial film thicknesses, protein chelating properties toward pro-oxidative metals in the water phase, and differences in the radical scavenging properties of amino acids.

In addition to this, we suggest that the free protein in the water phase might also have played a role in "neutralizing" reactive species. Above the surface saturation, the exceeding NaCas dispersed in the water phase as monomers or micellar aggregates might have formed complexes with reactive ionic species, thus preventing their contact with the sensitive bioactive compound.

In conclusion, stable dispersed systems suitable for β-carotene protection in food models were developed. Results highlighted two important pieces of evidence. First, the physical state of the lipid carrier deeply influenced the degradation kinetics of β-carotene. Particularly, HPKO protected the bioactive compound better than SO over the period of observation, with a degradation rate two times lower, due to the reduced exchange of reactive species with the surrounding and the lack of a reaction medium. Second, the emulsifier type played a role in modulating the kinetics of β-carotene degradation. NaCas showed a better protection efficacy compared to WPI, despite the fact that WPI formed a multilayer at the O/W interface. This difference may result from the different amino acid composition, which resulted in a different radical scavenging property, and the different thickness and structure of the interfacial membrane. Protein stabilized dispersions seems to be a promising strategy for the stabilization of improved food ingredients and foods containing lipophilic bioactive compounds, such as carotenoids and vitamins, but further research is necessary to clarify the crystal structure of β-carotene dispersed in a solid HPKO matrix and its implication on reaction rate, and the mechanisms by which proteinaceous interfacial membranes provide protection against oxidation.

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■ ABBREVIATIONS USED

SO, sunflower oil; HPKO, hydrogenated palm kernel oil; NaCas, sodium caseinate; WPI, whey protein isolate; XRD, X-ray diffraction;

BHT, butylated hydroxytoluene; O/W, oil-in-water; PO value, peroxide value; TAG, triglyceride (triacylglycerol or triacylglyceride); NLC, nanostructured lipid carrier

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7020

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