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Nanoemulsions of β-carotene using a high-energy emulsification–evaporation technique

Hélder D. Silva [a], Miguel A. Cerqueira [a], Bartolomeu W.S. Souza [a], Clara Ribeiro [a,b], Maria C. Avides [a], Mafalda A.C. Quintas [a,c], Jane S.R. Coimbra [d], Maria G. Carneiro-da-Cunha [e], António A. Vicente [a,*]

[a] IBB, Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] Research and Development Department, Frulact, S.A., Rua do Outeiro, 589, Gemunde, 4475-150 Maia, Portugal
[c] CBQF/Escola Superior de Biotecnologia, Rua Dr Antonio Bernardino de Almeida, 4200-072 Porto, Portugal
[d] Department of Food Technology, Federal University of Viçosa (UFV), P. H. Rolfs Av., sn, Zip Code 36570-000 Viçosa, MG, Brazil
[e] Departamento de Bioquímica/Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Campus Universitário, s/n, Cidade Universitária, CEP 50670-901, Recife, PE, Brazil

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ABSTRACT

Nanoemulsions of β-carotene were prepared using a high-energy emulsification–evaporation technique based on a 2^3 level factorial design. Results show that it is possible to obtain dispersions at a nanoscale range. Process parameters such as time and shear rate of homogenization affected significantly particle size distribution in terms of volume-weighted mean diameter and surface-weighted mean diameter. The obtained nanoemulsions presented a volume-surface diameter ranging from 9 to 280 nm immediately after the production of particles, displaying in all cases a monomodal size distribution. Those nanoemulsions showed a good physical stability during 21 days storage. The stability was evaluated by the maintenance of size distribution. However, β-carotene retention inside the micelles and color were affected by storage. Processing conditions also influenced storage stability.

1. Introduction

Functional ingredients such as carotenoids, fatty acids, natural antioxidants and numerous other compounds, are being extensively used as active ingredients on a great variety of food products (Moraru et al., 2003). Carotenoids can be found in vegetables, fruits, leaves, fish and other sea products and are one of the most important groups of natural pigments. Recently, researchers have shown that carotenoids can be beneficial for human health, protecting and preventing against a number of health disorders such as cancer, cardiovascular disease, macular degeneration and cataracts (Albanes, 1999; Edge et al., 1997; Erhardt et al., 2003; Rock, 1997; Man and Tan, 2003).

β-Carotene is an important member of the carotenoids family found in many fruits and vegetables. It has a basic molecular structure made of eight isopropene units, which contains forty carbon atoms and two rings at the end of its conjugated double bond chain (Fig. 1). As a retinol precursor with a high conversion rate, β-carotene provides a substantial proportion of vitamin A in the human diet (Naves and Moreno, 1998; Omenn et al., 1996). For these reasons, there is a strong interest in using β-carotene and other carotenoids as functional ingredients in food products. However, β-carotene is insoluble in water and weakly soluble in oil at ambient temperature because of its crystalline form, thus making it difficult to incorporate in food products and with less bioavailability (Ribeiro and Cruz, 2005). Furthermore, β-carotene is sensitive to light, oxygen, and heat, which limit even more its applications in the food industry (Orset et al., 1999; Rodriguez-Huezo et al., 2004).

Recently, nanotechnology quickly emerged as one of the most promising and attractive research fields, with applications ranging from the aerospace to health industries (Jochen et al., 2006). Food Industry can also benefit from nanotechnology applications – e.g. this technology offers the potential to improve bioavailability and solubility of different functional ingredients (carotenoids, fatty acids, among others) (Quintanilla-Carvajal et al., 2010).

Nanodispersions (e.g. oil-in-water nanoemulsions) may contribute to improve β-carotene dispersibility in water, coloring strength potential (Horn and Rieger, 2001), and also to increase its bioavailability during gastro-intestinal passage (Deming and Erdman, 1999; Horn and Rieger, 2001; Yuan et al., 2008a).

Emulsifiers play a major role in the formation of nanoemulsions in aqueous solutions. They decrease the interfacial tension between the oil and water phases, reducing the amount of energy required to disrupt the droplets and leading to smaller size droplets. Moreover, they form a protective coating surrounding the droplets thus preventing coalescence (McClements, 1999). Tween 20 is a non-ionic emulsifier that adsorbs quickly at the oil–water

* Corresponding author. Tel.: +351 253 604419; fax: +351 253 678986.
E-mail address: avicente@deb.uminho.pt (A.A. Vicente).

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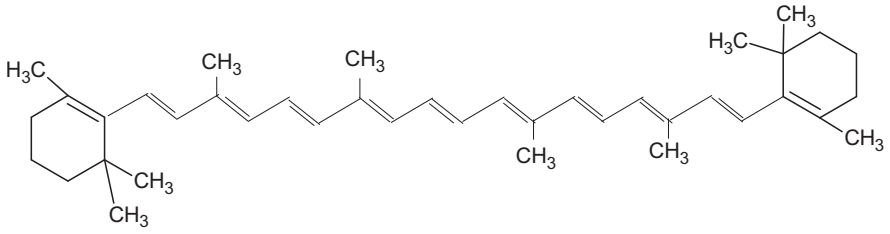

Fig. 1. Chemical structure of the β-carotene (adapted from Ribeiro et al., 2008).

interface, and has shown good results in small particles for various applications (Cheong et al., 2008) including nanoemulsions (Yuan et al., 2008a,b).

Tan and Nakajima (2005) have successfully prepared β-carotene in nanoemulsions using high-pressure homogenization. They studied influence of phase ratio and homogenization conditions on droplet size and β-carotene content.

This work focused on previous studies reported by Tan and Nakajima (2005), and aimed at replacing the high-pressure homogenization by conventional homogenization. The main objectives of this work were: (i) to obtain nanoemulsions of β-carotene using a high-energy emulsification–evaporation technique and to study the effect of processing variables such as homogenization time, shear rate and number of cycles on the size distribution of the obtained nanostructures; and (ii) to determine nanoemulsions' stability in terms of their size distribution, β-carotene concentration and colour during 21 days' storage.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Preparation of β-carotene nanoemulsions

Oil-in-water (O/W) emulsions were prepared according to Tan and Nakajima (2005) with some modifications. Briefly: 0.03% (w/w) β-carotene (higher than 97% purity, Fluka, USA) was dissolved at 40 °C in *n*-hexane (95% purity, Pronalab, Portugal). This organic solution was then added to an aqueous solution containing 0.5% (w/w) Tween 20 (97%, Sigma, USA) in Milli-Q water (Milli-Q synthesis, 18.2 MΩ cm). According to the results by Tan and Nakajima (2005) an organic/aqueous phase volume ratio of 1:9 was used. The emulsions were homogenized using an Ultra-Turrax homogenizer (T 25, Ika-Werke, Germany) according to the experimental design (Table 1). The *n*-hexane was then removed from the fine emulsion by rotary evaporation (Heidolph 2000, Germany). In order to simulate industrial production environment, no special care was taken to avoid contact with oxygen.

With the objective of assessing the effect of operational conditions on emulsion stability at the nanoscale, samples were analysed during a 21-day period of storage. The nanoemulsions obtained were stored at 4 °C in the absence of light, during the evaluation period, reproducing eventual commercial conditions.

2.1.2. Particle size analyses

The particle size in the emulsions was determined by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK). Each sample was analysed in a folded capillary cell. The measurements were made in triplicate, with three readings for each of them. The results are given as the average ± standard deviation of the nine values obtained.

Size distribution was evaluated based on the volume-weighted mean diameter ($D_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$) and the surface-weighted mean diameter ($D_{3,2} = \sum n_i d_i^3 / \sum n_i d_i^2$), being n_i the number of droplets in each size class and d_i the droplet diameter in the size class (Jafari et al., 2006, 2007 and Tan and Nakajima, 2005).

2.1.3. β-Carotene concentration

β-Carotene concentration was determined by measurement of the absorbance of the prepared emulsions at 479 nm using an UV–VIS spectrophotometer (Varian-UV–VIS Spectrophotometer, Germany) (Yuan et al., 2008a,b).

2.1.4. Colour evolution

Food colour and appearance are important visual factors that contribute to customer selection. The evaluation of colour changes during the storage period can be important for consumer's acceptance, being the maintenance of the original colour a key point. β-Carotene degradation reactions (e.g. oxidation) and particle size distribution can lead to changes in colour of nanoemulsions.

Emulsions were poured into a liquid sample (Holder mod. CR A-70) and colour parameters were determined using a tristimulus colorimeter (Minolta Chroma Meter CR400, Japan), programmed to use illuminant C as light source and the 2° observer for colour interpretation. A white tile (Minolta calibration plate) with

Table 1
Independent variables used in the 2^3 factorial design used: time of homogenization (t), shear rate of homogenization (expressed in terms of the rpm of the homogenizer) and number of cycles between each homogenization. Experimental values obtained for $D_{3,2}$ and $D_{4,3}$ immediately after processing and after 21 days of storage.

	Sample	rpm (min^{-1})	Cycles	t (min)	Storage time = 0 days		Storage time = 21 days	
					$D_{3,2}$ (nm)	$D_{4,3}$ (nm)	$D_{3,2}$ (nm)	$D_{4,3}$ (nm)
	1	3500	1	2	130.06 ± 0.45	146.60 ± 2.28	69.38 ± 3.65	72.94 ± 1.91
	2	3500	1	8	171.79 ± 8.37	200.68 ± 0.13	113.93 ± 0.32	126.07 ± 1.37
	3	3500	3	2	142.79 ± 0.36	166.89 ± 2.48	113.77 ± 6.30	136.91 ± 0.42
	4	3500	3	8	120.04 ± 6.60	153.05 ± 5.59	111.40 ± 3.89	127.35 ± 0.80
	5	6500	1	2	159.92 ± 10.51	200.95 ± 1.21	100.50 ± 8.34	108.33 ± 2.12
	6	6500	1	8	9.24 ± 0.16	9.78 ± 0.14	94.86 ± 39.59	102.87 ± 48.27
	7	6500	3	2	228.63 ± 0.01	276.77 ± 17.70	120.57 ± 29.69	180.22 ± 7.12
	8	6500	3	8	10.27 ± 1.85	10.78 ± 2.04	265.47 ± 120.86	381.20 ± 101.18
Central Point	9	5500	2	5				
	10	5500	2	5	161.98 ± 17.19	189.45 ± 22.69	115.88 ± 19.73	147.60 ± 8.62
	11	5500	2	5				

following standard value: $Y = 93.9$, $x = 0.3133$, $y = 0.3193$, was used to calibrate the equipment. Results were calculated by the equipment into the Hunter Lab colour scale. In this scale, L ranges from 0 (black) to 100 (white), a indicates degree of greenness (for negative a values) and degree of redness (for positive a results). Axis b also ranges from negative to positive values indicating, respectively, degree of blueness to yellowness. Colour changes were assessed using TCD (Francis, 1995).

$$\mathrm{TCD} = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

where L^*, a^* and b^* are the colour parameters at the end of the period under analysis and L_0^*, a_0^* and b_0^* are the colour parameters at the beginning of that period.

2.1.5. Microscopy

Confocal laser scanning microscopy (FV 1000 Fluoview, Olympus Europa GMBH, Germany) was used to confirm the presence of nanoemulsions of β-carotene. The fluorescence excitation wavelength used was 488 nm (green) with a 600× magnification (oil immersion). Samples were observed under the confocal laser scanning microscope by placing a drop of β-carotene nanoemulsion directly on a slide and no further treatment.

2.2. Statistical procedures

2.2.1. Experimental design

In order to evaluate the high-energy emulsion–evaporation technique for production and storage stability of β-carotene nanodispersions, these structures were prepared as described above, following a full factorial design 2^3 with a central point (Box et al., 2005) (see Table 1). The influence of process variables (homogenization time, shear rate and number of cycles) was tested on independent variables $D_{3,2}$ and $D_{4,3}$.

2.2.2. Data analyses

Data analyses were performed using Microsoft Windows Excel 2003 and STATISTICA 7.0 (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Effect of process conditions on β-carotene nanoemulsions

The combined action of surfactants with a high-energy homogenization–evaporation technique allowed the formation of stable β-carotene nanoemulsions. This was achieved using a high-speed homogenizer. Fig. 2 shows the solution containing the immiscible water and β-carotene phases (Fig. 2a) and the emulsion after the homogenization process and solvent evaporation (Fig. 2b).

The particle size of nanoemulsions, expressed in terms of the surface-weighted mean diameter ($D_{3,2}$) and volume-weighted mean diameter ($D_{4,3}$), are one of the most important and studied parameters of nanoemulsions (Tan and Nakajima, 2005; Ribeiro et al., 2008; Yuan et al., 2008a,b; Cheong et al., 2008). Table 1 shows the values of $D_{3,2}$ and $D_{4,3}$ for the nanoemulsions of β-carotene as determined by dynamic light scattering (DLS). Particle size distributions of the obtained samples ranged from 9.24 ± 0.16 to 276.77 ± 17.70 nm. In all cases a monomodal size distribution was obtained (e.g. Fig. 3). β-Carotene encapsulation in the nanostructures was confirmed by confocal laser scanning microscopy (Fig. 2c).

These observations confirm that it is possible to prepare emulsions of β-carotene and obtain structures in the nano range, without using high-pressure homogenization.

Typically, the droplet produced by high-speed blenders (such as the one used in this work) range between about 2000 and 10,000 nm in diameter (McClements, 1999). In the present work droplets in the range of 100 nm were obtained. This is due to the emulsification–evaporation technique applied for the formation of β-carotene particles: as hexane diffuses into the aqueous phase and evaporates at the water/air interface, droplets size reduces to the nano range level (Tan and Nakajima, 2005).

Table 1 also shows high standard deviation for diameters obtained at the central point (the only replicated condition of the

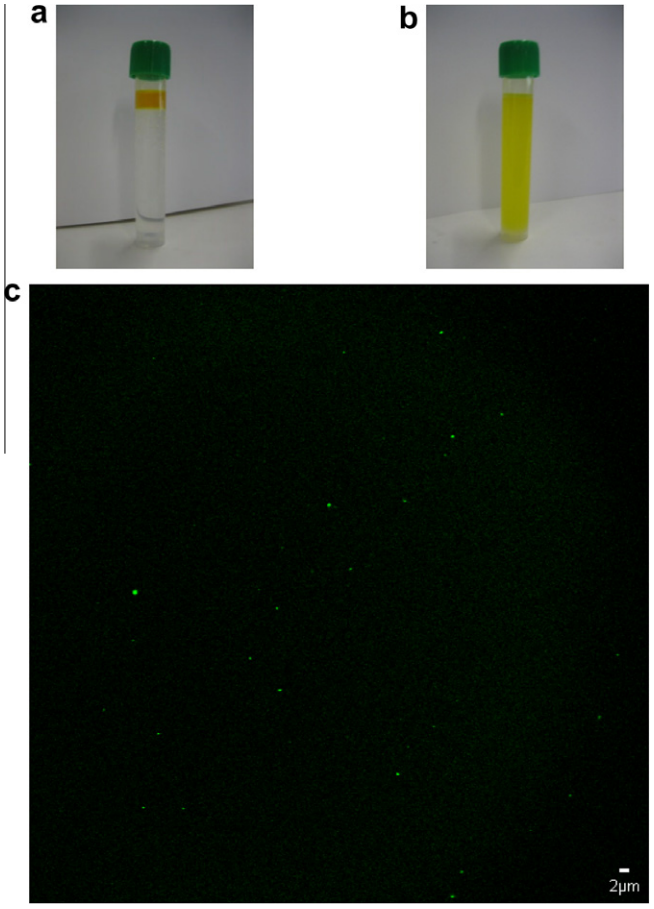

Fig. 2. Solution containing the immiscible phases: water and β-carotene solution (a); emulsion after the homogenization process and solvent evaporation (b); and confocal microphotographs of the final β-carotene nanodispersions (c).

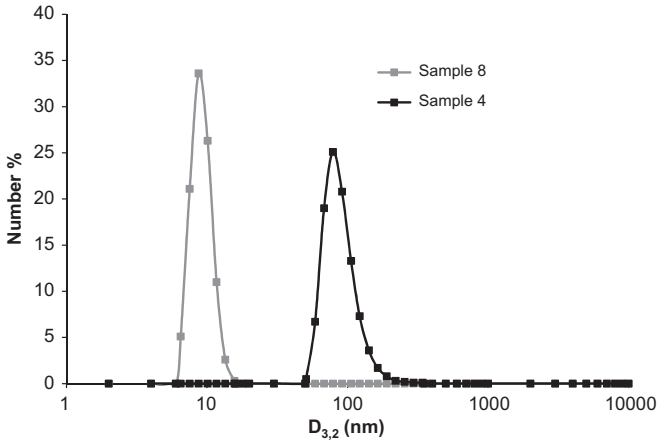

Fig. 3. Monomodal size distributions for nanoemulsions in the range of 10 nm (example is for sample 8) and 100 nm (example is for sample 4).

Table 2
ANOVA results for $D_{3,2}$ and $D_{4,3}$ estimation.

Diameter (nm)	independent variable	SS	df	*p*-Value
$D_{3,2}$	Time	15317.74	1	0.018744
	rpm * time	18820.22	1	0.015335
	Lack of fit	10406.21	6	0.152661
	Pure error	590.77	2	
	Total SS	45134.94	10	
$D_{4,3}$	Time	21728.53	1	0.022894
	rpm * time	30926.18	1	0.016250
	Lack of fit	11961.62	6	0.219510
	Pure error	1030.13	2	
	Total SS	65646.46	10	

experimental design). This indicates a large variability in the nanoemulsions size distribution. However, this variability did not impair the analyses of the results. In order to determine the most influent factors on particle size parameters ($D_{3,2}$, $D_{4,3}$) the experimental design results were analyzed using ANOVA (Table 2). Results show that both $D_{3,2}$ and $D_{4,3}$ are significantly influenced ($p < 0.05$) by homogenization time and by the interaction between the homogenization time and shear rate (expressed in terms of the number of rpm of the homogenizer). The influence of the number of cycles of homogenization in the properties of β-carotene nanoemulsions was also evaluated. However, number of cycles of homogenization did not affect significantly the nanodispersion size distribution.

As shown in Table 1 the mean diameter of the particles decreases with the increase of time and shear rate of homogenization. This has been explained by the high intensity of shear forces produced during the homogenization process (Tan and Nakajima 2005). Table 1 also shows that samples 6 and 8 have values of $D_{3,2}$ and $D_{4,3}$ closer to 10 nm; these values differ from those obtained for the other samples, which are within the 100 nm range. This great reduction is possibly due to the high-energy used to form the nanoemulsions in these two cases – the homogenization time (8 min) and shear rate (6500 rpm) were the highest used in the experimental design. This may indicate that further increases of the dispersion energy levels lead to even smaller particles.

3.2. Stability of β-carotene nanoemulsions during storage

The utilization of higher values of specific surface area of a given active ingredient generally leads to a higher dissolution rate and, possibly, to higher bioavailability. In many cases, particles in the nanometer range need to be chemically or physically stabilized (Tan and Nakajima 2005; Gutiérrez et al., 2008). As previously described, in this work the effect of operational conditions on emulsion stability at the nanoscale was assessed.

3.2.1. Size distribution

Table 1 shows values of $D_{3,2}$ and $D_{4,3}$ after 21 days' storage of the nanoemulsions. In general, it can be observed that size distribution parameters remain unchanged or with a slight decrease after 21 days' storage. This slight decrease is probably due to partial evaporation of residual *n*-hexane from the samples during storage: As the *n*-hexane is trapped in the nano droplets, the rotary evaporation step of this technique may not be sufficient and some amount of this compound may still be present in the droplets after production at residual level. Residual *n*-hexane may be admissible at different levels depending on the food product (EC Directive 2009-032). Hence, industrial application of this technique must be carefully analysed for each case.

Fig. 4 shows the evolution of samples' size during storage evaluated in terms of $D_{3,2}$. Samples 1 through 5 display a practically

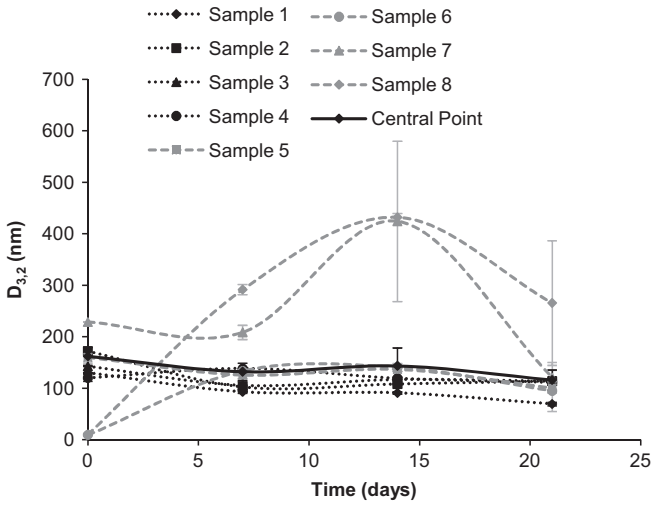

Fig. 4. $D_{3,2}$ evolution during 21 days' storage. Bars indicate standard deviation ($n = 3$). Lines are for readers' guidance and do not represent a model prediction.

constant size during time. However, for samples 6, 7 and 8, large variations in $D_{3,2}$ were observed: an initial large increase was followed by a decrease in diameter. These samples were also the ones that showed both smaller and larger diameters at day zero of analysis – around 10 nm (for samples 6 and 8) and 200 nm (for sample 7), as opposed to the other samples, which presented diameters in the 100 nm range. Smaller particles, as observed in samples 6 and 8, have a greater tendency to aggregate, since they are more numerous and more susceptible to the influence of Brownian motion, leading to a greater chance of collision and allowing aggregation to become a dominant mechanism for emulsion instability (Yuan et al., 2008b). As for sample 7, the presence of larger droplets in the dispersion may promote coalescence (Madrigal-Carballo et al., 2010). In the case of this sample, the incomplete evaporation of *n*-hexane may have also occurred during storage.

3.2.2. Retention of β-carotene

The degradation of some carotenoids can follow different order kinetic models, as a function of the type of β-carotene used, the solvent, the amount of light, and the oxygen available (Goldman et al., 1983; Limbo et al., 2007).

In the present study, β-carotene retention in the nanoemulsion was evaluated by the evolution of the normalized concentration (i.e., the actual concentration divided by the initial concentration value for each sample). Fig. 5 clearly shows that β-carotene degrades during storage time. Many factors may explain this observation, from which one may highlight the high surface area observed in the nano size range and the high degree of medium oxygenation occurring during homogenization. The high surface area surely contributes to improve the contact surface between β-carotene nanoemulsions and the surrounding dispersion medium, while oxygenation during homogenization will dissolve free radicals (Lander et al., 2000) in the surroundings of the nanostructures, thus promoting degradation reactions. Such reactions are mainly related with hydroxylation and oxidation of carotenoids, the later involving epoxidation with the consequent formation of volatile compounds such as apocarotenoids (Limbo et al., 2007).

The high values of standard deviation obtained in our results (Fig. 5) impair any significant conclusion on the effect of process parameters for β-carotene retention. However, some general observations can be made. Samples with lower shear rate of homogenization (1 through 4) present a slower degradation of β-carotene. This could possibly occur due to a lower solubilization of oxygen during samples preparation. Also, it is generally accepted

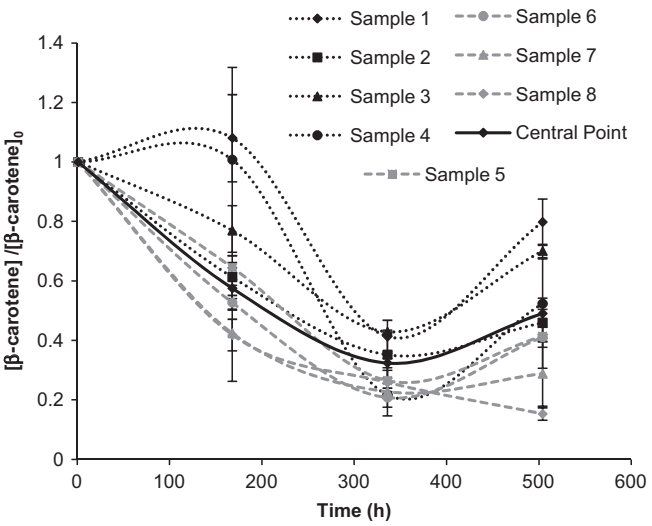

Fig. 5. β-Carotene retention during 21 days' storage. Bars indicate standard deviation ($n = 3$). Lines are for readers' guidance and do not represent a model prediction.

that the autoxidation of carotenoids is a free radical process, beginning with epoxidation and cleavage to apocarotenals (Rodriguez and Rodriguez-Amaya, 2007). Kennedy and Liebler (1991) identified the epoxy carotenoids and/or the apocarotenals formed from β-carotene. They identified as the main products formed by reaction of β-carotene with peroxyl radicals the 5,6-epoxy-β,β-carotene, 15,15'-epoxy-β,β-carotene and a major group containing a mixture of polar compounds (volatile and low molecular weight compounds). All these compounds are possibly involved in the autocatalytic reactions that may justify the behavior observed in samples 1 through 4.

As for the samples 6, 7 and 8, with higher oxygen solubilisation (all prepared with the same shear rate but with varying different size distribution (see Table 1)) a common tendency is also observed.

These results may indicate that depending on the processing variables, it is possible that the oxygenation of continuous phase may have a greater impact in β-carotene stability than the size distribution at the nanoscale.

3.2.3. Colour evaluation

During the evaluation period, the total colour difference (*TCD*) defined in Eq. (1), increased (Fig. 6). Fig. 6 shows that samples 6 and 8 presented the higher TCD values (differences comparing the reference values). Also, these were the samples that were more affected during storage in terms of size distribution and β-carotene retention. These results indicate that colour measurement is presumably a good complementary method to evaluate the stability of nanoemulsions. In our study β-carotene retention showed a lower decrease when compared with β-carotene retention in dispersions made in the presence of an oxygen partial pressure similar to that found in air, simulating packaging in air (Limbo et al., 2007). L^* parameter increased 1.3-fold (results not shown) between the initial value and that by the end of storage time (504 h). For similar conditions, Limbo et al. (2007) showed a rapid increase of L^* (during the first 50 h an increase of approximately twofold was registered). When comparing our results with the ones obtained for β-carotene dispersed in water (Limbo et al., 2007), it can be concluded that nanoemulsions can improve colour stability of β-carotene.

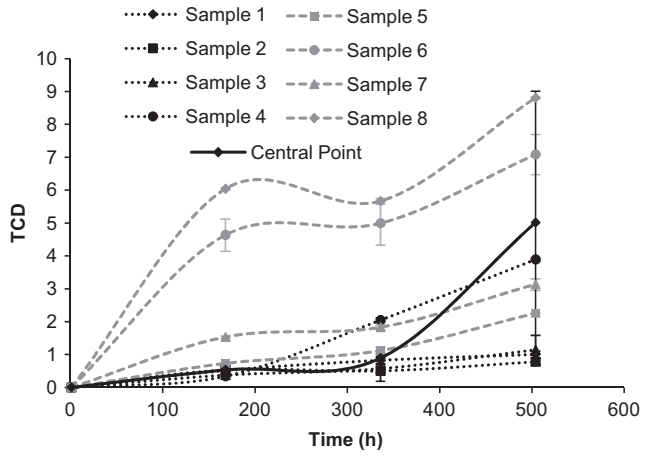

Fig. 6. Evolution of total color difference (TCD) during 21 days' storage. Bars indicate standard deviation ($n = 3$). Lines are for readers' guidance and do not represent a model prediction.

4. Conclusions

This work presents a proof-of-concept of the use of a high-energy emulsification–evaporation technique to produce oil-in-water nanoemulsions of β-carotene, without the need of using high-pressure homogenization. Results show that time and shear rate of homogenization are the most significant processing parameters influencing nanoemulsion size distribution.

β-carotene nanoemulsions showed a good physical stability in terms of size distribution by particle size analyser but are chemically unstable during storage (evaluated in terms of β-carotene retention). This chemical instability was readily observable through the color of nanoemulsions, which was affected by storage time. Results also show that processing conditions may influence the storage stability of nanoemulsions.

This work provides a low cost alternative for producing β-carotene nanoemulsions by the food industry. However, residual *n*-hexane concentrations need to be carefully reviewed in order to comply with current legislation.

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