The effect of microfluidization pressure on the physical stability of vitamin A in oil-in-water emulsions

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Abstract
In this study, vitamin A was encapsulated within oil-in-water emulsions by high-pressure microfluidization prepared using phosphate buffer (90%), corn oil (10%), and whey protein isolate (2%) as an emulsifier. The influence of microfluidization pressure (10, 50, 100, 200 MPa) on the particle size, zeta potential, and the physical and chemical stability of emulsions was evaluated. The physical stability of emulsion was determined by multiple light scattering technique. The content of vitamin A was measured by HPLC–DAD during an accelerated storage test at 40 °C during 4 weeks. The color of the samples was monitored using a colorimeter. The results showed that the lowest particle size distribution and the highest absolute value of zeta potential on the droplets' surface charge were obtained by applying a pressure of 100 MPa. Nanoemulsions prepared at 100 MPa also showed the highest colloidal stability. However, higher microfluidization pressure (up to 200 MPa) had a negative impact on the prepared emulsion’s stability. The results of chemical stability by HPLC measurements during storage time were in agreement with the results of physical stability and color change.

Keywords Encapsulation · Emulsion · Physical stability · Vitamin A · Shelf-life study

Introduction
Vitamin A is a nutrient necessary for human growth, and it has a key function in multiple biological functions, such as vision. Previous studies showed that vitamin A is important for gene transcription, epithelial cell growth, immune system development, and neurological functions [1, 2]. Vitamin A deficiency can cause visual health problems. One solution to prevent vitamin A deficiency is food fortification using delivery systems, such as emulsions.

Vitamin A is poorly soluble in water and unstable in the presence of light and oxygen and when exposed to heat. Microencapsulation is a common strategy to increase the dispersibility of lipophilic bioactive compounds. However, the sensory properties, color, and flavor of food products should not be modified [3, 4]. Emulsion-based delivery systems are suitable and viable alternatives for the protection, encapsulation, and delivery of bioactive compounds [5, 6].

Previous studies showed that emulsions have great potential in protecting, stabilizing, and delivering the lipophilic bioactive compounds by encapsulating them in the oil core [7]. In recent years, they have been highly considered because of their different applications, particularly in the field of food and pharmaceuticals. Different types of colloidal systems have been used, such as oil-in-water (O/W) and water-in-oil (W/O) emulsions [8, 9]. Various approaches can be used in the preparation of oil-in-water emulsions, which can modify the structure or composition to increase the physical and chemical stability of encapsulated delivery systems, such as: antioxidant addition, particle size, and zeta potential control [10]. As regards the application of emulsion to vitamins, many studies concluded that emulsion encapsulation can enhance vitamin A bioavailability. Tanglao et al. used whey proteins to encapsulate vitamin A in virgin coconut O/W emulsions. The results indicated that encapsulation could improve the stability of vitamin A [11, 12].

Emulsions can be prepared using various types of equipment and methods, including a microfluidizer, colloidal mills and ultra-high-pressure homogenization (UHPH). In the UHPH method, the fluid accelerates to a very high velocity over a very short distance. This can disrupt oil droplets and produce particles with a smaller size. In this method,
the increase in the pressure and the number of passages can reduce emulsion particle size and increase stability against creaming and sedimentation [13, 14]. On the other hand, the smaller particle size distribution increases the emulsion stability against flocculation and/or coalescence, because attractive forces among droplets can be reduced by acting on the droplet size distribution [15]. Moreover, the emulsifying properties of some proteins can be increased by UHPH by improving the formation of a more rigid interfacial layer [16].

In the previous work of the authors, the effect of microfluidization pressure in presence of α-tocopherol content on the retention of vitamin A in O/W emulsions was studied. The results indicated that in the absence of α-tocopherol, the percentage loss of encapsulated vitamin A linearly decreases by increasing pressure from 10 to 100 MPa during 5 weeks of storage at 40 °C [17]. Although previous research works studied the effect of ultra-high-pressure homogenization on the chemical stability of O/W emulsions, the effect of microfluidization pressure on the physical stability of vitamin A encapsulated in O/W emulsion has not been deeply investigated. The aim of this work is to monitor the effect of a high pressure microfluidizer (10–200 MPa) on the physical stability of prepared emulsions over time (4 weeks at 40 °C). During the shelf-life study, the physical properties of the emulsions were measured by light scattering techniques and with parameters, such as particle size, zeta potential, color and vitamin A retention.

**Materials and methods**

**Chemicals**

Corn oil was purchased from the company Sigma Aldrich (Milano, Italy). Whey protein isolate was purchased from Fonterra Coöperatie U.A. and vitamin A was obtained from the DSM company (Nutritional products holding AG, Bazel, Switzerland). All reagents used were of chemical grade. Phosphate buffer (10 mM, pH = 7) was used to prepare all solutions and emulsions.

**Emulsion preparation**

An oil phase was prepared by adding 0.5% (w/w) of vitamin A to corn oil to obtain a final concentration of 520 ± 8 µM with mild heating (≤ 5 min at 50 °C) and then stirring for 1 h at room temperature to ensure it was fully dissolved. To avoid oxidation, each sample was flushed with nitrogen during dissolution. An aqueous phase was prepared by dissolving 2% (w/w) whey protein isolate (WPI) in an aqueous buffer solution (10.0 mM phosphate buffer, 0.01% (w/w) sodium azide, pH 7.0). Oil-in-Water emulsions were prepared by homogenizing 10% (w/w) oil phase with 90% (w/w) aqueous phase at ambient temperature (25 °C). A coarse emulsion premix was prepared using an Ultra-Turrax (Model T25 digital, IKA, Königswinter, Germany) homogenizer for 2 min at 18,000 rpm, which was then passed through a high-pressure microfluidizer (Model 101, Microfluidics, Newton, MA) equipped with an auxiliary processing diamond channel of 200 μm followed by a Z-type diamond cell of 87 μm channel diameter (H30Z 200 µM and G10Z 87 µM). For each preparation, four levels of pressure were applied (10 MPa, 50 MPa, 100 MPa, 200 MPa) in three cycles. The freshly prepared emulsions (about 100 mL) were instantly divided into two aliquots, placed in falcon tubes, covered by aluminum foil and then stored at 40 °C. This storage temperature was used to accelerate the degradation of vitamin A.

**Quantitative analysis of vitamin A by high-performance liquid chromatography**

The vitamin A content during the accelerated storage test was determined by HPLC according to the method of Gatti et al. (2000) with some modifications [18]. In brief, the amounts (500 mg) of all samples were accurately weighed into separated and labelled falcon tubes. The extraction was done using 4 mL of a solvent solution composed of acetonitrile–ethanol–acetic acid (70:20:10, v:v:v) followed by ultrasound extraction at (35 kHz) for 5 min. The supernatants’ separation was done by centrifugation at 3,500 rpm for 6 min and filtration with a 0.45 µm syringe filter. 100 µL of the filtered solution was diluted with 990 µL volume of ethanol (98%, HPLC grade) as a mobile phase to obtain the final concentration for the HPLC determination performed with an Agilent 1260 Infinity Binary LC system. The HPLC system was controlled by ChemStation software. The separation was carried out using Thermo Fischer ODS Hypersil 125 × 4 mm column (pore size 5 µm). The flow rate was set at 1 mL/min and the injection volume was 20 µL. The column temperature was set at 25 °C, the detection wavelength was set at 326 nm and the overall time was 10 min.

**Colloidal stability**

The colloidal stability of emulsions was tested with multiple light scattering measurement performed with an optical analyser Turbiscan® Lab Tower (Formulation, L’Union, France), which consists of six stations. The reading head was composed of a pulsed near-infrared light source (λ = 880 nm) and two synchronous transmission (T) and backscattering (BS) detectors. The transmission detector received the light, which passed through the sample at 0° and 180° from the incident beam. The back scattering detector received the light, which scattered backwards by...
the sample at 135° and 45° from the incident beam [16, 19]. The detection head measured the entire sample vial height (40 mm), which was filled with around 20 mL of each sample in 20-min intervals for 120 h at 25 °C. The principle of the measurement was based on the particle migration, which caused a change in sample backscattered light (BS) as a function of time. This was taken as a measure of emulsion colloidal stability. The overall stability of the emulsions was evaluated using the TSI (Turbiscan stability index), which gave information regarding the general behavior of the obtained emulsion. The TSI was calculated as the sum of all destabilization processes occurring in the sample using the following equation [20, 21]:

\[
\text{TSI} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - x_{BS})^2}{n - 1}},
\]

where \(x_i\) was the average value of backscattering for each minute, \(x_{BS}\) was the average value of \(x_i\) and \(n\) was the number of scans.

**Determination of droplet size distribution**

The particle size distribution of the prepared emulsions was determined by a light scattering technique using a Mastersizer 3000 (Malvern Instruments, Malvern, UK). Emulsion samples were diluted in distilled water until an appropriate obscuration in the diffractometer cell (9–10%) was obtained. The refractive index was set at 1.52 and the adsorption index at 0.01. The measurement was done on fresh emulsions and during storage time. The surface mean diameter \(D_{[4,3]}\) and the volume mean diameter \(D_{[3,2]}\) were reported as mean and standard deviation from the total number of nine measurements.

**Zeta potential measurement**

Zeta potential is a measurement of both net and potential surface charge distribution at the interface. The zeta potential of the obtained emulsions was measured using a Malvern Zetasizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Samples were diluted prior to analysis with the same buffer used for emulsion preparation (pH≈7) at a ratio of 1:100 (v/v). The temperature was set to 25 °C for all analyses to avoid multiple scattering effects and using the Smoluchowski-Kramers approximation to convert electrophoretic mobility to zeta potential. The average and standard deviation of the measurement of three replicates were reported at constant ionic pH values [22, 23].

**Colour**

Color change is a typical indicator of an oxidation process of carotenoids [24]. Therefore, the color change of the emulsions during storage was measured using a colorimeter. Briefly, the chemical degradation of vitamin A was monitored by measuring changes in emulsion color. Banasaz et al. reported in the absence of antioxidant in emulsions, the percentage loss of vitamin A linearly decreasing by increasing pressure from 10 to 100 MPa [17]. In current study, there is a linear correlation between color and vitamin A degradation. The more vitamin A degraded over time, the color became more intense. The tristimulus color coordinates \((L^*, a^*, b^*)\) were measured using the HunterLab method (Hunter Associates Laboratory, Inc Virginia, US). \(L^*\) is a measure of lightness (higher value indicates a lighter color), \(a^*\) values are a measure of redness/greenness and \(b^*\) values are an indication of yellowness (higher positive values indicates a more yellow color). Before each measurement, the emulsions were placed into a transparent flat-faced petri dish. The petri dish surface was placed on the colorimeter measuring cell and the values were measured. All measurements were done in triplicate.

**Results and discussion**

**Turbiscan analysis**

Emulsions are thermodynamically unstable systems with a mean diameter of droplets between 20 and 100 nm, or between 20 and 500 nm as reported in some of the studies [25]. After formation, they can undergo undesirable phase separation phenomena, such as coalescence, sedimentation and flocculation [12]. The Turbiscan stability index (TSI) can provide overall stability change over time. Figure 1 shows the TSI for all emulsions containing 2% WPI prepared at 10 MPa, 50 MPa, 100 MPa and 200 MPa. The results showed that the emulsions prepared at 10 and 50 MPa were the most unstable. Their TSI index increased with a great variation during the storage time from 0.05 to 13 for 10 MPa emulsions and from 0.04 to 11.57 for those prepared at 50 MPa. Over time, the migration of dispersed particles to the surface of the emulsion, referred to as creaming, was observed in these samples. The visual inspection of samples prepared at 10 MPa confirmed the separation of phases after 5 days of storage at 24 °C. Backscattering signals decreased at the bottom of the sample vial while they increased at the top due to an increase in concentration of the dispersed phase. This was due to a larger particle size and higher interfacial tension between oil droplets and the continuous phase [13, 26]. In comparison, the TSI of emulsions prepared at 100 and
200 MPa increased from 0.04 to 7.4 (100 MPa) and from 0.04 to 5.9 (200 MPa), respectively. These emulsions were more stable against phase separation and showed better physical stability over the storage time.

Measurement of particle size distribution

The results obtained by multiple light scattering were confirmed by measurements of particle size during the storage period at 40 °C. Surface mean diameters D[3,2] and volume mean diameters D[4,3] were measured every week as it shown in Fig. 2. Just after preparation, all emulsion samples obtained at different pressures had a D[3,2] lower than 2 µM. During the first week of storage, the changes in D[3,2] values of emulsion prepared at 100 MPa and 200 MPa were insignificant and increased from 70 to 110 nm for the emulsion prepared at 100 MPa and from 360 to 450 nm for those prepared at 200 MPa. Concerning the D[4,3] values, emulsions prepared at 10 and 50 MPa showed a significant change in particle size distribution over time. The D[4,3] of the samples prepared at 10 MPa significantly increased from 1.9 µm measured on the day of preparation to 150 µm at week 4 (t test, p < 0.05). However, the major droplet size changes in prepared emulsions were observed from week 3 to week 4 of the storage [27]. This can be due to phase separation of prepared emulsions and instability phenomenon such as creaming. The results confirmed the works of Atarés et al. and Hebishy et al., who showed that droplet size reduction by a high-pressure homogenizer stabilized emulsions against both creaming and lipid oxidation. The reason for the higher physical stability of emulsions by particle size reduction can be correlated to the whey protein quantity directly in contact with the oil phase preventing close and long-time contacting of emulsion droplets. This can consequently result in a higher interfacial area resulting from particle size reduction [16, 28].
Measurements of zeta-potential

To further investigate the emulsion samples prepared at different pressures, the zeta potential was measured as an indication of the electrical repulsion and attraction at the surface of suspended particles. Zeta potential changes of emulsions over 4 weeks of storage ranged from $-4$ mV to $-30$ mV. As can be seen in Fig. 3, the effect of microfluidizer pressure (10, 50, 100, 200 MPa) on the zeta potential of the emulsions during the storage was significant. Emulsions prepared at 10 MPa and 50 MPa had the lowest electrostatic repulsion, ranging from $-19$ mV to $-4$ mV for 10 MPa and from $-25$ mV to $-20$ mV for those prepared at 50 MPa. When coagulation among particles occurred, the repulsive forces between particles decreased and therefore zeta potential values decreased [29–31]. This happened in the samples prepared at 10 MPa. Instead, the emulsions prepared at 100 MPa and 200 MPa had the highest electrostatic repulsion and showed negative zeta potential values ranging between $-26$ and $-30$ mV, indicating high stability of the emulsion. Indeed, a high absolute value of zeta potential was generally required to ensure stability without agglomeration. A further increase of pressure up to 200 MPa led to a slight increase in the potential values. However, the statistical analysis showed that the zeta potential values in the first 2 weeks of storage were not significantly different for the samples prepared at 100 and 200 MPa ($t$ test, $p > 0.05$). In general, the results confirmed the previous data obtained from the droplet size distribution, showing that higher particle size reduction led to a higher emulsion stability.

Color analysis (CIE Lab)

Color change is a typical indicator of an oxidation process of carotenoids including vitamin A. The influence of storage on the color changes of prepared emulsions was measured to obtain an indication of the chemical stability of vitamin A. Freshly prepared emulsions had a white color similar to milk, however, the color tended to fade and change to a yellowish color when vitamin A underwent chemical degradation. The color fading of emulsions was monitored by measuring the color for 4 weeks at 40 °C. The total color difference ($\Delta E$) of emulsions was measured to quantify overall color changes during the accelerated storage test [10, 24].

$$\Delta E^* = \left[ (L^* - L_{0*})^2 + (a^* - a_{0*})^2 + (b^* - b_{0*})^2 \right]^{1/2},$$

(2)

where $L^*$, $a^*$ and $b^*$ were the values obtained by measurements of color during storage time and $L_{0*}$, $a_{0*}$ and $b_{0*}$ were the initial color coordinates of the prepared emulsions. The effect of microfluidizer pressure on the total color difference during storage time is shown in Fig. 4. In general, there was a linear increase in the total color difference ($\Delta E^*$) during the accelerated storage test for all emulsions. However, the figure clearly shows a significant increase in the rate of color

![Fig. 3 Zeta potential obtained from the emulsion formulations prepared at a 10 MPa; b 50 MPa; c 200 MPa; d 100 MPa microfluidization pressure](image)

![Fig. 4 $\Delta E$ obtained from the emulsion formulations prepared at a 10 MPa; b 50 MPa; c 200 MPa; d 100 MPa microfluidization pressure](image)
change for the emulsions prepared at 10 MPa and 50 MPa. This can be a good indicator for the rate of vitamin A degradation during shelf-life with a lower level of microfluidizer pressure.

**Chemical stability**

Finally, the influence of the four microfluidization pressures (10, 50, 100 and 200 MPa) on the degradation of the encapsulated vitamin A in the emulsion samples was quantified by HPLC–DAD. The degradation of vitamin A in emulsion formulations is displayed in Fig. 5. The initial concentration of the vitamin A in the emulsions was gradually decreased during the storage period. The ANOVA analysis (Tukey LSD test, $p < 0.05$) showed that the level of applied pressure was significant for the retention of the vitamin A. The emulsions prepared with the pressure of 100 MPa lost only 25 ± 2% of the vitamin A, whereas in the other emulsion samples, the vitamin A concentration decreased by 35 ± 3% (200 MPa), 39 ± 2% (50 MPa) and 49 ± 2% (10 MPa). Accordingly, the most stable emulsion formulations were those prepared with the higher microfluidization pressure. The following pressures retained from the highest to the lowest concentration of vitamin A in the samples after 4 weeks of storage: 100 MPa > 200 MPa > 50 MPa > 10 MPa.

**Conclusions**

The results of this study showed the significant effect of different level of microfluidizer pressure on the physical and chemical stability parameters of vitamin A encapsulated in Oil-in-Water emulsions during an accelerated storage test of 4 weeks at 40 °C. The analysis performed by a light scattering technique showed that the effect of the applied pressure was significant on the droplet size distribution, zeta potential and colloidal stability of the prepared emulsions. Vitamin A loss was linearly reduced by increasing the pressure from 10 to 100 MPa. However, further increases in microfluidization pressure up to 200 MPa did not result in a positive impact, either on Vitamin A loss or colloidal stability. Regarding the physical stability, similar trends were observed for the emulsions prepared at 100 and 200 MPa. In summary, the best physical stability and vitamin A retention over the shelf-life study was obtained for the emulsions prepared at 100 MPa.

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**Declarations**

**Conflicts of interest** The authors declare no conflict of interest.

**Compliance with ethics requirements** This article does not contain any studies with human participants or animals performed by any of the authors.

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