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ORIGINAL ARTICLE

Effect of temperature, pH and ionic strength on hydroxyapatite stabilised Pickering emulsions produced in batch and continuous mode

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Abstract
Oil-in-water (O/W) Pickering emulsions are attracting attention as carriers of lipophilic active compounds with clear advantages over traditional systems. Having in view their effective use it is important to study their stability against environmental stresses impacting manufacture, storage, and application conditions. In this work, hydroxyapatite nanoparticles (n-HAp) Pickering emulsions produced in continuous mode using a mesostructured reactor (average size ~ 7, 11 and 18 µm) and in batch mode using a rotor–stator device (average size ~ 18 µm) were studied concerning their behaviour at different temperatures (5–90 ºC), pH (2–10) and ionic strength (0–500 mM), conditions with relevance for food applications. Droplet size, morphology, and zeta-potential were analysed after 1 and 7 days under storage. In general, and despite the droplet size, the n-HAp Pickering emulsions were stable within the tested ionic strength range, at relatively high pH environments (6–10), and at temperatures up to 70 ºC. Pickering emulsions undergo complete phase separation at very low pH (2) due to n-HAp particle's disruption. A clear tendency to aggregation and coalescence was observed for high temperatures (70–90 ºC). Results indicate no significant differences related to the used production method. From an industrial perspective, this work also corroborates that the scale-up to a continuous process using a mesostructured reactor, NETmix, from a batch laboratorial process is feasible without impacting stability.

Keywords Pickering emulsions · Hydroxyapatite · Environmental stress · Stability

Introduction

Emulsification is considered a suitable method for preserving the innate characteristics of bioactive compounds over time, similarly to an encapsulation technique. Moreover, it facilitates the combination of highly lipophilic and hydrophilic components in the same product, which is an important strategy in the manufacture of important commercial food products [1, 2]. In this field, Pickering emulsions, due to their surfactant-free nature, are greener alternatives to conventional emulsions, offering advantages to protect bioactive compounds [3, 4]. Their stabilisation is ensured by solid particles, which due to their high attachment energy, bind almost irreversibly to the droplet's surface, ensuring stability, particularly against coalescence during storage. In fact, Pickering emulsions have good physical and chemical stability [5–7], which is important for food, cosmetics and pharmaceuticals applications, fulfilling sustainability and green label issues [6].

Oil-in-water (O/W) Pickering emulsions are suitable systems to encapsulate lipophilic active compounds [8], bringing advantages over traditional ones. They can improve the compatibility between components in a final product [6, 9], enhancing the stability, bioaccessibility and absorption of the lipophilic compounds in the human body [6, 10]. In this

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context, Pickering emulsions have received increased interest in recent years since they can contribute to the development of differentiated and innovative alternatives in fields such as food [11], cosmetics [10], and pharmaceutical [6].

The advantages and extensive applicability of Pickering emulsions, mainly in the development of fortified foods, functional cosmetics, and pharmaceuticals, is rising the interest for these systems. Of high importance are stability studies against environmental stresses such as temperature, pH, and ionic strength. Most reported data on Pickering emulsions stability focus factors influencing their production, such as the solid particles concentration, droplet size, oil content, and the stability to storage under controlled conditions. In this perspective, studying physical and chemical stability throughout manufacture, storage, and final application conditions (e.g., consumption) by simulating relevant conditions through environmental stress tests is of high interest [8]. Also, the incorporation of Pickering emulsions into final products (e.g., foods) should not adversely impact product's final physicochemical and organoleptic properties.

In this context, Mitbumrung, et al. [4] showed that Pickering emulsions stabilised by nanofibrillated mangosteen cellulose were stable under a wide range of ionic strength conditions and temperatures, becoming sensitive to low pH by showing droplet's aggregation and vitamin D_3 encapsulation efficiency loss. Zhou, et al. [12] evidenced that relatively high pH values (from 5 to 8) favour the aggregation of the nanochitin-stabilized Pickering emulsions. In another study, Bai, et al. [13] reported that Pickering emulsions stabilised by cellulose nanocrystals had quite good stability to pH changes (3 to 10), to the presence of electrolytes (100 mM NaCl), and to temperature (30 to 90 °C). However, cellulose nanocrystals-based Pickering emulsions showed droplet flocculation under acidic conditions (pH 2) and at high ionic strength media (200–500 mM NaCl). These observations corroborate the need to conduct these stability studies, which are significantly influenced by the nature of the solid particles. Furthermore, these studies can help to establish effective encapsulation procedures using emulsion-based carriers, e.g. for the protection and delivery of lipophilic vitamins in food products.

Most of the reported studies in the literature focus Pickering emulsions production in batch mode using rotor–stator or ultrasonic devices [14–16], and in continuous mode through microfluidic devices [17, 18]. However, these techniques have disadvantages associated with the used high shear conditions and limited production volumes [6]. To surpass these constraints, NETmix, a mesostructured reactor based on a static mixer enabling production in continuous mode, was recently proposed for Pickering emulsions production [19], showing to be a promising alternative.

NETmix consists of a 2D structured network of cylindrical chambers (mixing zones) connected by prismatic channels (segregation zones) repeated in various lines and columns. Due to geometric characteristics of the NETmix network, the flow inside the chambers evolves to a self-sustained oscillatory laminar flow regime inducing strong local dynamic mixing [20]. The mixing chambers enable successive and well-localized mixing points throughout the NETmix reactor, promoting an easily reproducible emulsification step [19]. NETmix proved to be a versatile technique that enables the control of the droplet size in a tailor-made way due to localized and well-defined mixing and generates small droplets at short times with high reproducibility [19]. Comparing the emergent microfluid devices and NETmix, both present similar size scales and capacity in terms of heat and mass transfer areas versus reactor volume and both operate in laminar regime. The main difference is that while in microfluidic devices the flow is parallel and heat and mass transfer are controlled by diffusion, in NETmix the flow is non-parallel, inducing chaotic mixing that facilitates heat and mass transfer [20, 21]. NETmix is a low energy device since it does not require external mixing input. NETmix is competitive with other existing mixers, delivering energy more efficiently into fluid mixing than stirred tanks, as shown by both experiments and CFD simulations [20]. Additionally, the space–time yield (STY), defined as the mass flow rate per reactor volume, can be used to compare the rotor–stator and NETmix in terms of production. Calculated values of STY for NETmix (3.0×10^7 kg/m^3/day) show that production can reach two orders of magnitude higher than the rotor–stator (2.4×10^5 kg/m^3/day) [19].

In this context, the purpose of the present work was to study the impact of different environmental stresses with impact on food products manufacturing, storage and digestion: temperature (5–90 ºC), pH (2–10), and ionic strength (0–500 mM) on the stability of nano-hydroxyapatite (n-HAp) stabilised Pickering emulsions produced by traditional (rotor–stator) and innovative (NETmix) methods. In this study, the morphology, droplet size, and zeta potential, were evaluated over a storage period of 7 days (sampling at 1 and 7 days) to understand Pickering emulsions' behaviour after the applied stresses, expanding the applicability of Pickering emulsions stabilised by n-HAp. HAp is widely used in biotechnological applications due to its excellent biocompatibility. Recently, n-HAp particles have been studied as Pickering stabilizers for achieving surfactant-free emulsions, which can contribute to the development of innovative products [11]. To the best of our knowledge, this work reports, for the first time, a systematic study analysing the influence of environmental stresses on the n-HAp Pickering stability, which might be useful considering production, storage and final application scenarios of these products.

Experimental methods

Materials

A hydroxyapatite aqueous paste, *nano*XIM-CarePaste, was supplied by Fluidinova S.A. (www.fluidinova.com). According to the technical information provided by Fluidinova, N*ano*XIM-Care Paste is composed by 15.5 ± 0.5 (wt%) of HAp nanoparticles (n-HAp) with typical size below 50 nm in a rod-like shape, and 4.5 ± 0.5 (wt%) of KCl. The water content is ≤ 81.0 (wt%). Sunflower oil was purchased from a local supermarket. Sodium oleate and fluorescent dye (Nile red) were obtained from Sigma-Aldrich. Isopropyl alcohol was purchased from Riedel–de Haen. Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was also used. All other chemicals were of analytical grade.

Pickering emulsions production

Oil-in-water (O/W) Pickering emulsions were prepared according to two previously reported procedures of the group, in batch mode using a rotor–stator device [14] (Fig. 1 – rotor–stator), and in continuous mode using the static mixer NETmix [19] (Fig. 1 – NETmix).

The Pickering emulsions were obtained using an oil to water ratio of 20:80 (*v/v*) and a solid particles content of 5 wt%. This formulation results in a stable emulsion as shown in previous work of the group [14, 19], being adequate to study the effect of environmental stresses in their stability. The aqueous phase corresponds to n-HAp (5 wt%) dispersed in water, and the oil phase to sunflower oil. In the batch procedure (Fig. 1 – rotor–stator), the aqueous phase was poured into the reactor and the sunflower oil injected dropwise using a peristaltic pump. The rotor–stator device was setup to operate at 11,000 rpm for 6 min. In the NETmix procedure, the aqueous and oil phases were firstly mixed to form a coarse emulsion (Fig. 1 – NETmix – configuration A). Then, the Pickering emulsion with the desired droplet size was obtained by recirculating the coarse emulsion inside the NETmix reactor during the needed number of cycles at the required Reynolds number (Re) (Fig. 1 – NETmix – configuration B). The production conditions in terms of Re were 300–400 and number of cycles 5–17. Three samples were produced with NETmix (average size ~ 7, 11 and 18 µm; to study the effect of the droplet size), and one with the rotor–stator device (average size ~ 18 µm; to compare with the NETmix and evaluate the effect of the used preparation method).

Pickering emulsions environmental stability tests

The produced Pickering emulsions were evaluated concerning the stability against typical stresses with interest for the production of commercial food products and upon ingestion (gastrointestinal tract passage). The range of the selected average droplet sizes took into account the typical sizes used in emulsion food products. Thus, all Pickering emulsions were submitted to different temperature conditions (5, 22, 30, 50, 70 and 90 ºC), pH (2, 4, 6, 8 and 10) and electrolyte (NaCl) concentrations (0, 100, 200, 300, 400 and 500 mM) aiming at evaluating their effect on emulsion properties (droplet size, shape, and surface charge). Additionally, the stability of the Pickering emulsions' subjected to the applied stresses was evaluated considering the importance of these parameters also during storage. For that, emulsions were stored for 7 days at room temperature and further evaluated. Each condition was tested in triplicate (independent assays).

Temperature effect

To evaluate the temperature effect, 10 mL of the Pickering emulsion were transferred into test tubes. The procedure was adapted according to the tested temperature. Namely, for low temperatures, the Pickering emulsions were stored in the fridge (5 ºC), and at room temperature (22 ºC). For higher temperatures, the Pickering emulsions were incubated in a water bath at the required value (30 to 90 ºC). After reaching the temperature equilibrium, samples were left under the chosen conditions for 30 min, then cooled to room temperature and stored for 1 and 7 days before analysis.

pH effect

To evaluate the pH effect, 10 mL of the Pickering emulsion were transferred to test tubes. The pH was adjusted to the required value (2, 4, 6, 8 and 10) with NaOH (0.1 M) or HCl (1 or 2.5 M) solutions, as needed. The pH was measured with a pH probe accompanied by gentle stirring. The emulsions were stored for 1 and 7 days, at room temperature, before analysis.

Ionic strength effect

An appropriated amount of anhydrous NaCl powder, namely the one needed to achieve the desired ionic strength (100 to 500 mM), was added directly into the freshly prepared Pickering emulsions that were then gently stirred to ensure NaCl solubilisation. The samples were stored at room temperature for 1 and 7 days until analysis.

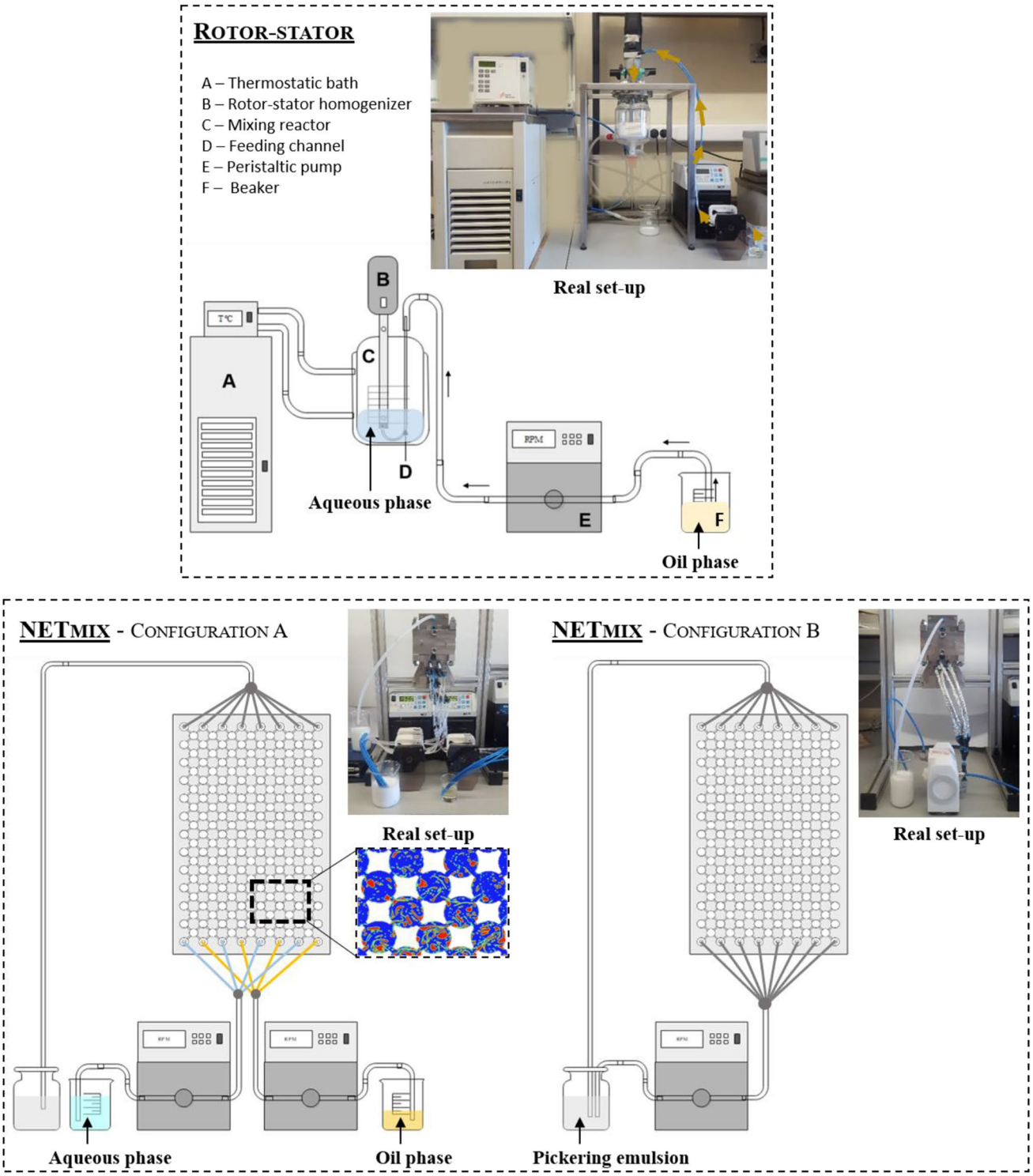

Fig. 1 Schematic representation of the rotor–stator homogenizer and NETmix set-up

Pickering emulsions characterisation

After submitting the Pickering emulsions to the different environmental stresses, the emulsion properties (droplet size, morphology, and surface charge) were evaluated. For that, diffraction particle size analyser, fluorescence optical microscopy and zeta potential analysis were conducted.

Droplet size analysis

Droplet size distributions (in volume) were obtained using a laser diffraction particle size analyser (Beckman Coulter LS230). The average size was determined from the obtained volume distributions and reported as the average volume diameter, $d_{4,3}$. These evaluations allow to inspect for possible coalescence and/or aggregation phenomena, reflected as an average diameter increase, which indicates signs of instability. The analysis was done in duplicate for each sample triplicate, and the data expressed as average ± standard deviation (SD).

Zeta potential analysis

The zeta potential, evaluated through the droplet surface charge, is an indicative of the emulsion stability. Thus, the sample can be considered stable for high positive (≥ 30) or high negative (≤ -30) values. In magnitude, zeta potential higher than 30 mV is obtained for stable emulsions meaning that, within this range, droplets develop enough electrostatic repulsion to avoid aggregations. The zeta potential was determined using an electrophoresis instrument, Zetaziser Nano ZS90 (Malvern Instruments). For the analysis, Pickering emulsions were diluted with distilled water as needed to avoid multiple scattering effects. The diluted sample was put into a folded capillary Zeta cell (ref: DS7010), and the determination carried out at 25 ºC. The analysis was performed in triplicate of each sample triplicate, and the data expressed as average ± SD.

Fluorescence microscopy analysis

Fluorescence microscopy was used to monitor the evolution of the oil droplets morphology and size after subjecting the Pickering emulsion to the environmental stresses. This technique enables a better observation of the oil droplets allowing to easily inspect the presence of the oil, namely the free oil, within the emulsion. Nile red stains the oil phase (individual oil droplets and lipid rich regions) that will appear as bright areas, whereas the aqueous phase (not dyed) will appear as dark regions in the micrographs. For fluorescence microscopy analysis, 100 µL of the Pickering emulsion was stained with 10 µL of a Nile red solution at 0.1% *w/v*. Then, an aliquot of each sample was placed on a slide and gently covered with a coverslip. The analysis was performed using a Nikon optical microscope fitted with a TRITC filter and a digital camera (Nikon DS-Q_i2). The acquisition and image processing was done using NIS-Elements Nikon software.

Statistical analysis

The experiments were performed in triplicate and expressed as average ± SD. The statistical analysis was performed using the commercial software IBM SPSS statistics (version 27.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyse the data of the environmental stresses studies, and significant differences between the average values ($p < 0.05$) evaluated using Tukey's test. Additionally, *T*-test was used to analyse size significant differences ($p < 0.05$) between 1 and 7 days under storage for each environmental stress.

Results and discussion

Pickering emulsions were produced in two ways: in batch mode using a rotor–stator homogenizer, and in continuous mode using NETmix. The NETmix device was chosen to overcome the common problems related to the traditional devices (rotor–stator) such as the lack of temperature and emulsification control, and limited volume scale productions. In this work, the samples were named according to the production method and droplet size of the Pickering emulsion: NET-low represents the Pickering emulsions produced in NETmix having lower droplet size (~ 7 µm), NET-middle the ones with intermediate (~ 11 µm), and the NET-high the ones with higher droplet size (~ 18 µm). The sample named as 'Batch' corresponds to the Pickering emulsions produced in batch mode using a rotor–stator homogenizer (~ 18 µm).

Initial Pickering emulsions

The Pickering emulsions produced according to the selected average sizes using the mesostructured reactor NETmix and the rotor–stator device are listed in Table 1. It puts in evidence the production conditions (Re and number of cycles) to achieve the required dimensions using

Table 1 NETmix and rotor–stator device productive conditions and respective Pickering emulsions characterisation (average diameter and zeta potential)

Sample name	Production conditions			Characterisation	
	Re	Cycles	Stirring (rpm)	Average diameter (µm)	Zeta potential (mV)
NET-low	400	17	-	7.11 ± 0.05	30.97 ± 0.41
NET-middle	400	5	-	11.34 ± 0.27	32.72 ± 1.09
NET-high	300	10	-	17.87 ± 0.58	32.00 ± 0.91
Batch	-	-	11,000	17.58 ± 0.11	31.53 ± 1.01

the NETmix, and stirring rate (rpm) for the rotor–stator device, together with the performed characterizations (average diameter and zeta potential). The influence of the production parameters in the chosen system, (O/W n-HAp stabilised Pickering emulsions prepared with sunflower oil) was previously studied by Ribeiro, et al. [19], where the feasibility of the NETmix technology to control the droplet size was reported. NETmix Pickering emulsions are referred as NET-low, NET-middle and NET-high, respectively for the samples holding proximate dimensions of 7, 11, and 18 µm. The sample produced with the rotor–stator, with proximate dimensions of 18 µm, is referred as Batch. Their typical size distributions are shown in Fig. 2—A. The produced Pickering emulsions were characterized by strong positive values of zeta potential (all over 30 mV), indicating stable emulsions with strong electrostatic repulsion inhibiting droplet aggregation. The produced Pickering emulsions were characterized by strong positive zeta potential values (all over 30 mV), indicating stable emulsions with strong electrostatic repulsion interactions, inhibiting droplet aggregation. This positive zeta potential value is related with the presence of the n-HAp solid particles at the oil surface. n-HAp particles are characterized by a zeta potential of 33.6 ± 2.63 mV, which is due to the presence of calcium ions in a peripheral position of the crystalline structure. Pickering emulsions macroscopic appearance was registered photographically after 1 day under storage for the samples not subject to any environmental stress (Fig. 2—B). For all cases, independently of the used productive method and droplet size, samples had a white appearance and good resistance to gravitational separation (data not shown), in accordance with the data previously reported by Ribeiro, et al. [14] and Ribeiro, et al. [19] which indicated a 2-months stability for this Pickering emulsion formulation.

Environmental stress stability studies

The produced Pickering emulsions were submitted to different environmental stresses, namely temperature (5–90 ºC), pH (2–10) and ionic strength (100–500 mM) to evaluate their stability under these conditions. The study was carried out using the three Pickering emulsions produced with NETmix and the one produced in Batch as listed in Table 1. The aim was to evaluate the effect of the droplet size, and the effect of using two distinct productive technologies. Pickering emulsion stability was evaluated under these environmental stresses over a storage period of 7 days with sampling at 1 and 7 days. These are typical storage times reported in the literature to evaluate emulsions after being subjected to environmental stresses, which mainly mimic what happens after opening or manipulate a food product. For each of the tested conditions size distributions of the Pickering emulsions were determined (data not shown), and from them the average size calculated. It was observed that, when the average size was maintained after 7 days, the same was observed for the corresponding size distributions. If modifications occur, this was also accompanied by changes in the size distribution.

Temperature effect

The produced n-HAp Pickering emulsions (NET-low, NET-middle, NET-high and Batch) were incubated at different temperatures, namely at 5, 22 (room temperature), 30, 50, 70 and 90 °C, and the effect on the average diameter, zeta

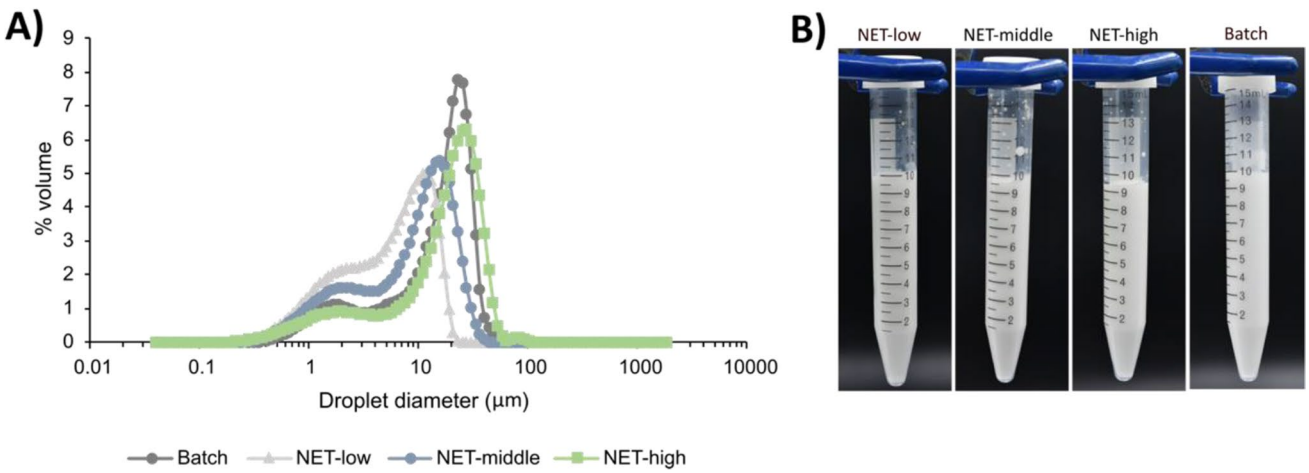

Fig. 2 Droplet size distribution (**A**) and photographic register after 1 day (**B**) of the Pickering emulsions produced by NETmix and rotor–stator device. The shown samples were not subjected to any environmental stress

potential and morphology evaluated considering two storage times: 1 and 7 days. The Pickering emulsions subjected to low temperatures (5 and 22 ºC) remained under these conditions during 7 days. For high temperature studies (30—90 ºC), the Pickering emulsions were subjected to the chosen temperature during 30 min, after which they were kept at room temperature. These conditions intended to simulate the usual storage conditions (5 and 22 ºC) and usual cooking conditions (30—90 ºC) followed by a storage period. Figure 3 shows the size expressed as average diameter and the zeta potential of the Pickering emulsions produced using the NETmix technology and the rotor–stator device, respectively, as a function of temperature for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was done for the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of emulsion sizes for all temperatures after 1 day, (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of emulsion sizes for all temperatures after 7 days, and (iii) for each tested temperature (5, 22, 30, 50, 70 and 90 °C) comparison of the emulsion size after 1 and 7 days storage. From the analysis of Fig. 3 it is possible to observe the impact of temperature on the Pickering emulsions with different sizes and produced by the two different technologies. Analysing the emulsion size after 1 day, there are changes for emulsions incubated at 90 ºC ($p < 0.05$), independently of the studied emulsion category. However, after 7 days of storage, differences start to be perceived for emulsions treated at 70 ºC during 30 min (or higher temperatures), with exception of the NET-low ($p < 0.05$). The emulsions start to develop instability under storage mainly those incubated at high temperatures. Additionally, it is possible to perceive that, temperatures in the range 5–50 ºC do not negatively impacted emulsion stability, which was preserved; the average diameters for these

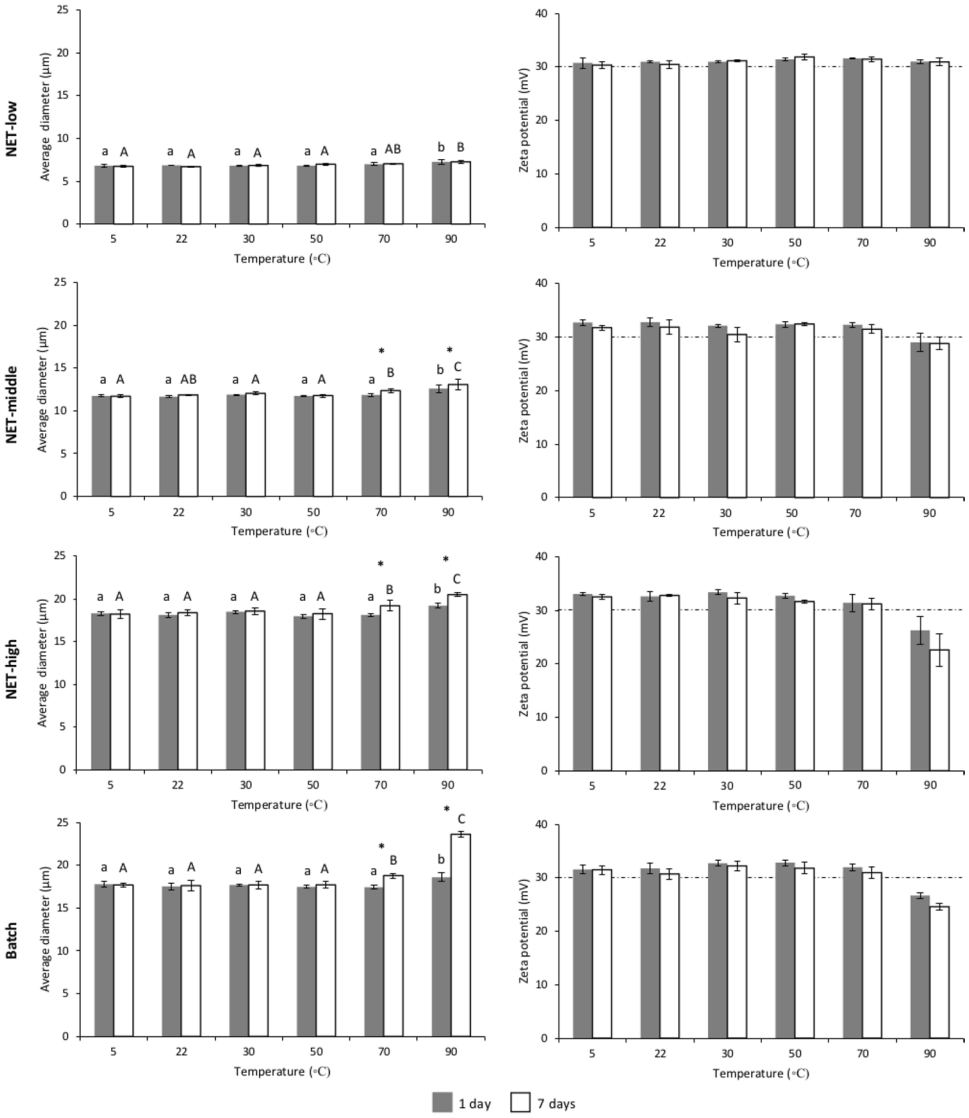

Fig. 3 Effect of temperature (5, 22, 30, 50, 70 and 90 ºC) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and Batch - 18 µm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all temperatures after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested temperatures, between 1 and 7 days of storage ($p < 0.05$)

samples were similar for both sampling times (1 and 7 days) without statistical difference ($p < 0.05$). However, for higher temperatures (70 and 90 ºC) some differences with time were registered, mainly for the NET-middle, NET-high and Batch samples. For the NET-low sample the size at high temperatures (70 and 90 ºC) was maintained constant with time.

Analysing the zeta potential, as registered in Fig. 3, it can be concluded that emulsions preserved the value higher than 30, which is an indicative of stability. The more relevant changes in the zeta potential values were observed for 90 ºC. In this case, the NET-low and NET-middle samples maintained the zeta potential value within the examined time period. However, the Batch and NET-high samples revealed a significant decrease in the zeta potential, which agrees with the observed average diameter increase for these samples. In fact, samples holding droplets of large size, are more prone to destabilization at high temperatures (90 ºC).

Figure 4 shows the fluorescence microscopy images of the studied Pickering emulsions, as a function of temperature, after 7 days under storage. The oil phase was stained with Nile red dye, and the droplets are visualised through emitted fluorescence in red colour. This technique enables the observation of the oil phase. After 1 day, the emulsions are characterized by a regular and uniform morphology, at different tested temperatures (see FigS1 of the supplementary material). Although the statistical differences of the size after 1 day at 90 ºC, in terms of optical observations no significant changes on emulsion morphology were observed. However, after 7 days (Fig. 4), particularly for the Pickering emulsions with large sizes (NET-middle, NET-high

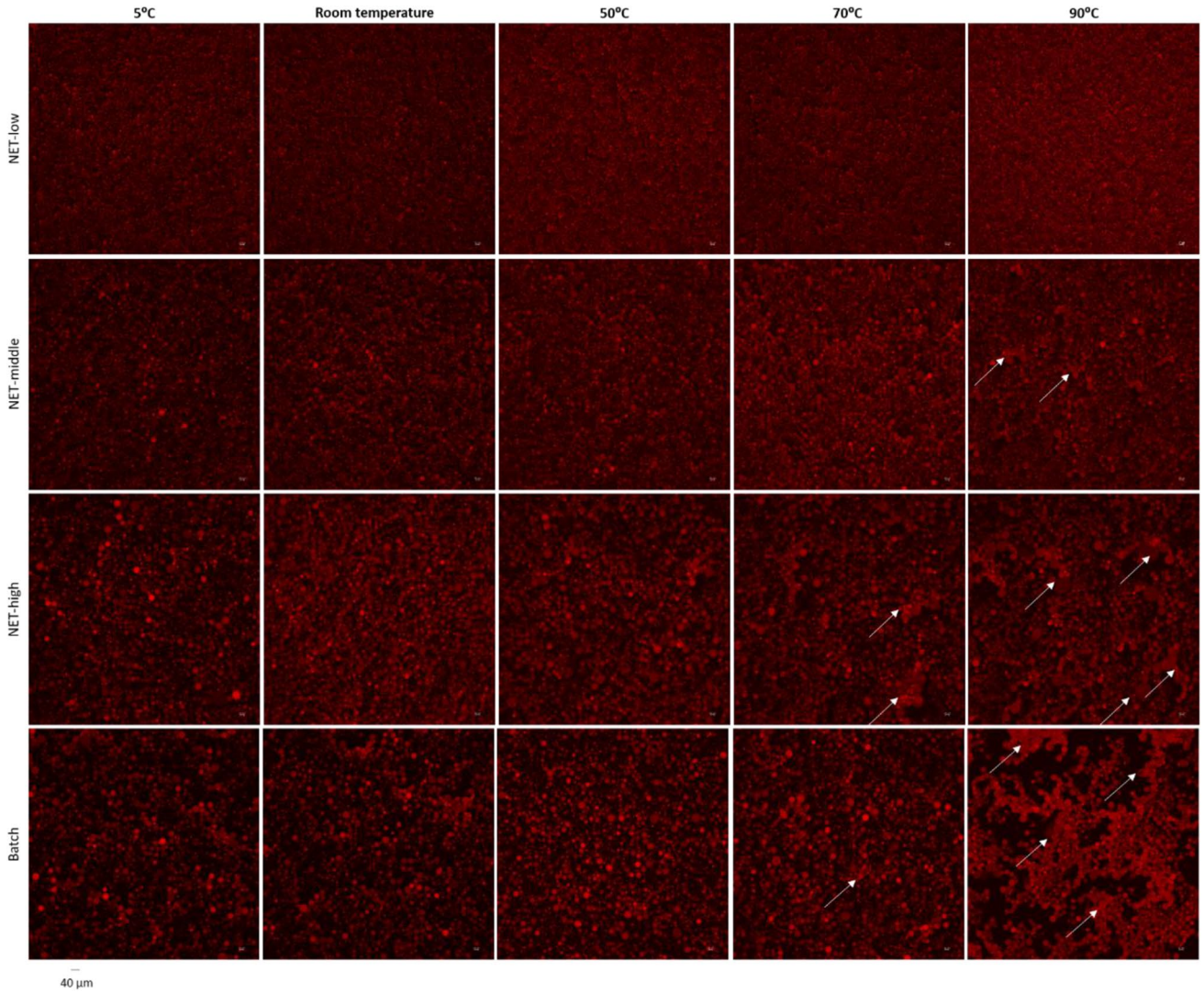

Fig. 4 Fluorescence microscopy images of the Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and Batch - 18 µm) at different temperatures after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x

and Batch) some droplet aggregations were observed for emulsions incubated at high temperatures during 30 min. Namely, the Pickering emulsions subjected to 70 and 90 °C presented clear signs of instability evidenced by small groups of droplet's aggregation and coalescence (indicated with an arrow). However, it is important to highlight that NET-middle, NET-high and Batch emulsions heated at 70 ºC present fewer aggregates, comparatively with the emulsion treated at 90 ºC. These aggregates occurring at 70 ºC agree with the slight, but significantly higher average diameter, as reported in Fig. 3 ($p < 0.05$). However, the recorded data for zeta potential (Fig. 3) indicates that they are, probably, in a reversible and weak state of aggregation since the achieved value indicated a stable emulsion.

The increase of the average diameter at higher temperatures can be related to Brownian motion, promoting particles' redistribution and detachment from the interface [22]. Thus, the increase in temperature leading to kinetic energy increase, could cause the exposure of the droplets' surface promoting droplet's collision and coalescence phenomena [22]. For example, Mwangi, et al. [22], who studied emulsions stabilised by chitosan with an average diameter of around 75 µm reported stable emulsions up to 50 ºC. Further temperature increase led to a considerable rise of the average droplet diameter and phase separation. On contrary, Bai, et al. [13] that studied emulsions stabilised by cellulose nanocrystals with an average diameter of around 1.2 µm reported emulsion stability up to 90 ºC (only minor average diameter and zeta potential changes were observed). Thus, previous reported literature results agree with the observed for the studied n-HAp stabilised Pickering emulsions. The temperature had adverse effects on emulsions with large droplet diameters, comparatively with the lower ones.

Comparing the behaviour of the n-HAp Pickering emulsions holding the same particle size but produced by different technologies (NET-high and Batch), it can be concluded that the productive method does not influences the emulsion behaviour in the range of studied temperatures since similar responses were detected.

pH effect

The influence of pH media on Pickering emulsions stability was studied. The pH ranged from 2 to 10, interspersed 2 in 2. The effect of pH on the average diameter and zeta potential of the produced emulsions is shown in Fig. 5. To complement this study, Fig. 6 shows the fluorescence microscopy images of the Pickering emulsions collected after 7 days under storage.

Figure 5 shows the average diameter size and the zeta potential as a function of pH for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was performed considering the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of all pH values after 1 day, (ii) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of all pH values after 7 days, and (iii) for each tested pH (2, 4, 6, 8 and 10) comparison for 1 and 7 days storage. Figure 5 evidences that all Pickering emulsions were sensitive for very low pH (pH 2), with no Pickering emulsion remaining due to the complete degradation of the n-HAp particles (detected as a transparent aqueous phase), accompanied by the oil phase at the top of the test tube; i.e. a complete phase separation (water and oil) have occurred, as shown in the photographic record. Accordingly, zeta potential and fluorescence microscopy were not determined.

At pH 4, an increase of the average droplet diameter was perceived for all the studied samples, when compared with the originally reported size in Table 1. Furthermore, the statistical analysis indicates that the emulsion at pH 4 had significant differences in size comparatively with the emulsions subjected to other pH values (6–10) after 1 day ($p < 0.05$). This fact can be related with the droplet aggregations observed in Fig. 6 (indicated by an arrow). After 7 days, the emulsions subjected to pH 6 showed slight differences from the other ones. Comparing the storage time (1 and 7 days), the statistical analysis indicates that the pH has a prompt effect, and no instabilities were further developed along the analysed storage period ($p < 0.05$).

Additionally, through zeta potential analysis (Fig. 5), it is possible to conclude that the pH highly impacts n-HAp Pickering emulsions stability. An increase of the Pickering emulsion stability is verified at high pH values, which was accompanied by a zeta potential rise. At these conditions ($pH > 10$), the n-HAp particles and droplets maintain their charge and electrostatic repulsion, and thus stability. For lower pH values ($4 < pH < 8$), n-HAp particles can undergo partial degradation, since these intermediate pH values can lead to the formation of Ca^{2+} and PO_4^{2-} ions derived from the n-HAp solubilization at acidic pH, reducing droplets' electrostatic repulsion. As a consequence, the zeta potential become lower and stability decreases. At pH 2, total dissolution of the n-HAp particles occurs, making zeta potential determination impossible. Nevertheless, Zhou, et al. [12], which also reported significant changes in nanochitin stabilised Pickering emulsions with pH variation, reported significant morphology changes with droplet's size increasing with pH rising.

Analysing the fluorescence microscopy images (Fig. 6), which shows the NET-low, NET-middle, NET-high and Batch emulsions, it is possible to observe the maintenance of the droplets shape from pH 6 to 10, meaning that they were stable against coalescence and aggregation phenomena within this pH range and during 7 days under storage. FigS2 (supplementary material) shows the fluorescence

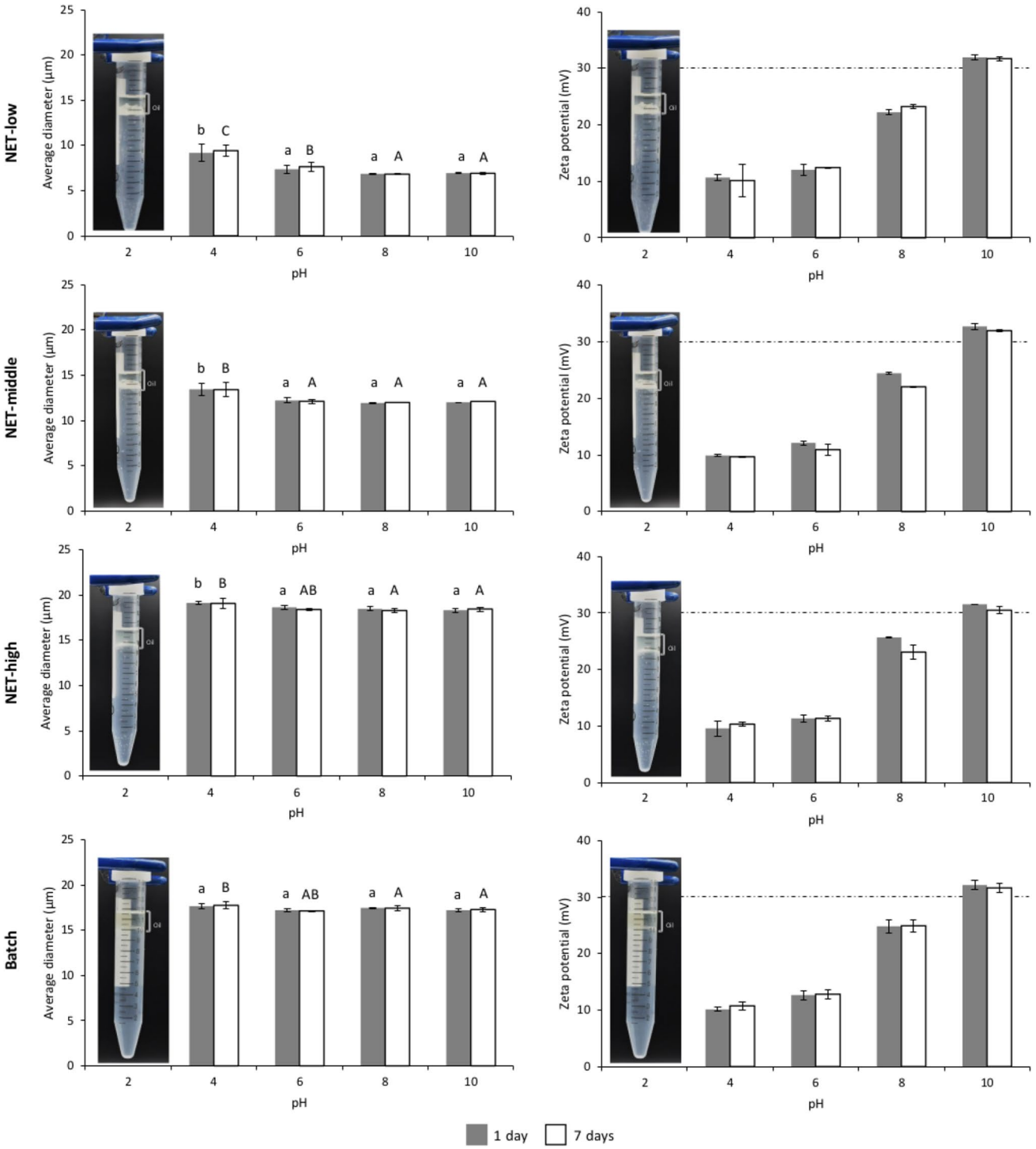

Fig. 5 Effect of pH (2, 4, 6, 8 and 10) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 μm, NET-middle - 11 μm, NET-high - 18 μm, and Batch - 18 μm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all pH values after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested pH values, between 1 and 7 days of storage ($p < 0.05$). Photographic register acquired after 1 day of the n-HAp Pickering emulsion at pH 2

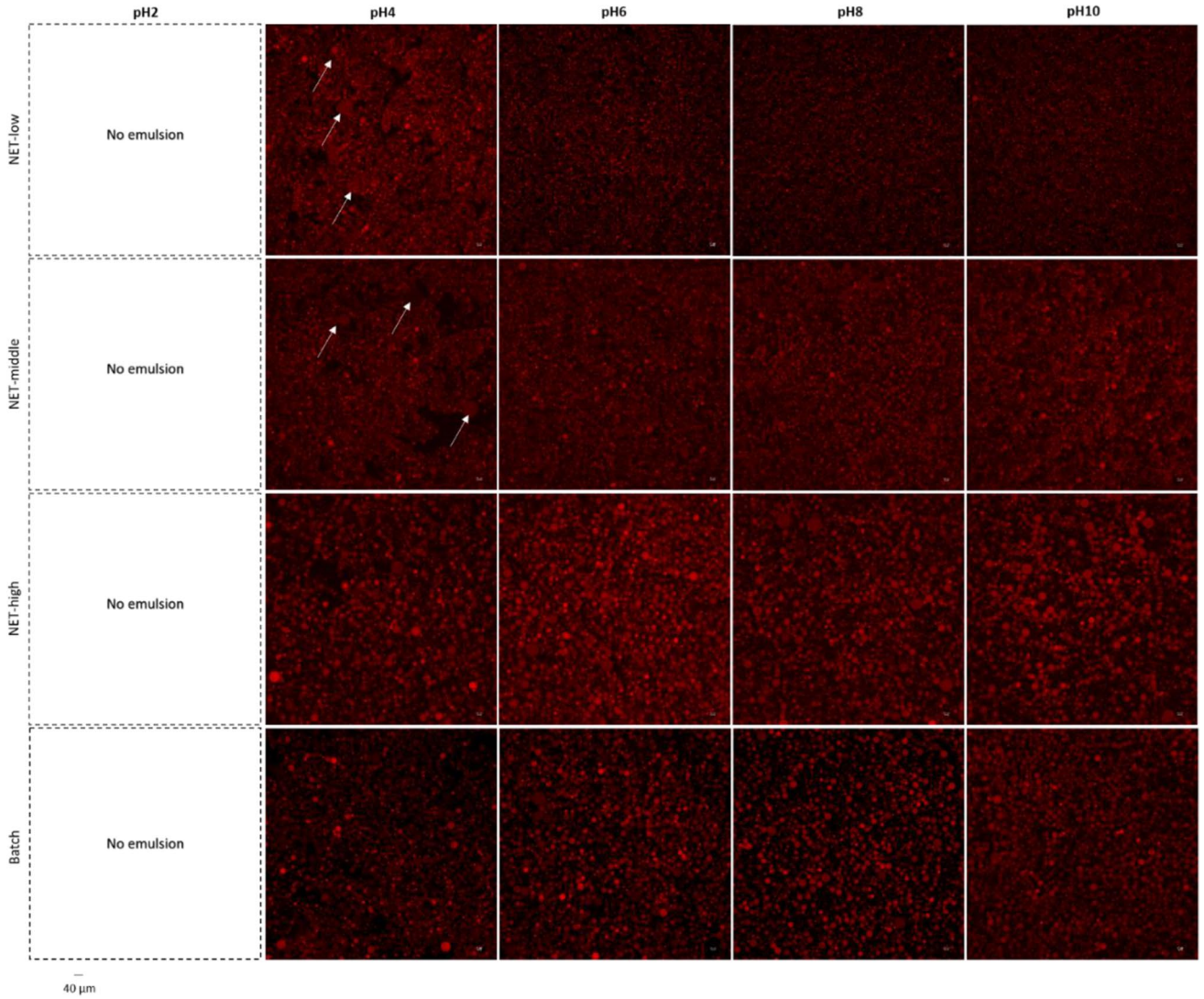

Fig. 6 Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and Batch - 18 µm) at different pH after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x

microscopy images of the Pickering emulsions after 1 day of storage. The emulsions subjected to low pH (4) tend to develop instabilities, mainly, aggregations. Although zeta potential values are lower for emulsions at pH 4, the observed instabilities and increased diameter were most noted for NET-low and NET-middle. In this case, there was a weaker electrostatic repulsion between the oil droplets; therefore, the repulsion generated by the adsorbed n-HAp layer was no longer enough compared to the van der Waals attractions, promoting droplet aggregation [13]. For the NET-high and Batch emulsions, the increase of the droplet size and aggregation formation was less noted. This fact may be related to the smaller size bar difference (in magnitude) registered for pH4 for these samples. For the other tested pH values (6 and 8), the fluorescence microscopy images evidenced a well-defined morphology without changes in the droplet size. Despite the zeta potential value being below 30, no instability phenomena were optically observed. n-HAp particles are stable at very specific conditions, and at pH 6 partial solubilization of the hydroxyapatite can occur forming other calcium phosphate particles, which can also promote the stabilization of the O/W emulsion, as was inferred by the fluorescence microscopy images (Fig. 5) showing droplets of regular shape and well dispersed in the continuous medium. However, the released ions decrease the zeta potential of the medium. Taking into account the achieved zeta potential value, it is expectable that emulsions at pH 6 are able to develop instability faster than emulsions at pH 8.

Results demonstrate that the stability of the n-HAp stabilised emulsions is strongly affected by pH, being these

emulsions more stable at high pH environments. Thus, the pH has a significant influence on Pickering emulsions stability, mainly due to its impact on HAp structure, i.e., low pH promotes HAp dissolution while at high pH, HAp maintains its structure.

Comparing the behaviour of the two n-HAp Pickering emulsions produced by different technologies (NET-high and Batch), no difference derived from the used productive method was observed. The two emulsions presented a similar behaviour at the different pH values.

Ionic strength effect

The ionic strength was tested on the produced emulsions (NET-low, NET-middle, NET-high, and Batch) by varying the electrolyte (NaCl) concentration from 0 to 500 mM. Figure 7 shows the average diameter and the zeta potential of the Pickering emulsions as a function of the ionic strength for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was performed considering the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of all ionic strengths after 1 day, (ii) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and Batch – 18 µm) comparison of all ionic strengths after 7 days, and (iii) for each ionic strength (0, 100, 200, 300, 400 and 500 mM) comparison for 1 and 7 days storage.

Pickering emulsions stabilized by n-HAp solid particles were stable in terms of droplet size without significant changes after 1 day for all tested NaCl concentrations ($p < 0.05$); however, very slight changes were statistically observed after 7 days (Fig. 7; $p < 0.05$). Except for the Batch Pickering emulsion subjected to 300 mM of NaCl, which showed differences between 1 and 7 days, it is possible to conclude that no significant changes occurred in

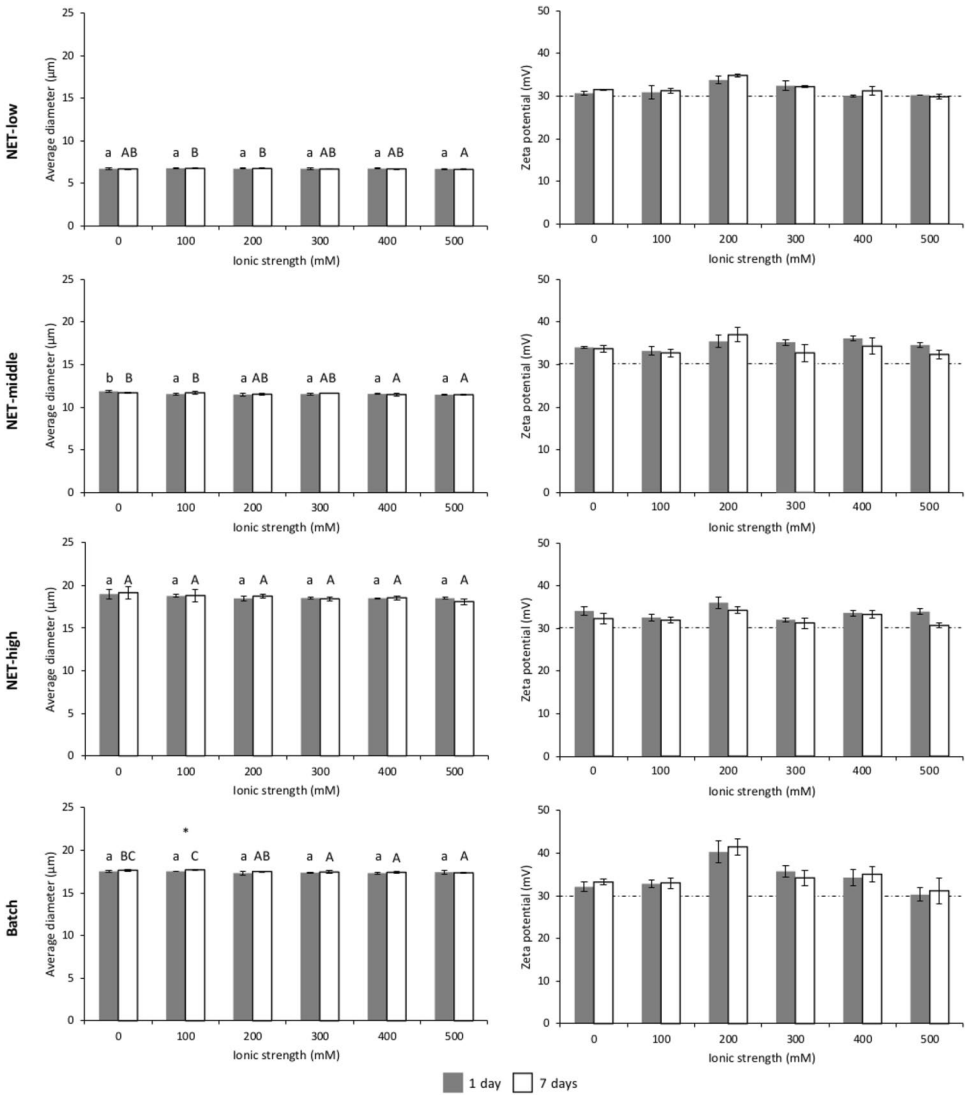

Fig. 7 Effect of ionic strength (100, 200, 300, 400 and 500 mM, NaCl) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and Batch - 18 µm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all electrolyte concentrations after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested electrolyte concentrations, between 1 and 7 days of storage ($p < 0.05$)

the average droplet diameter for all the other tested emulsions (Fig. 7), within the studied NaCl concentration range when comparing 1 and 7 days of the storage time. Overall, the emulsions maintained their size even at high electrolyte concentrations within the tested time-frame of 7 days. According to literature, different behaviours were observed for the Pickering emulsions face to NaCl adding. For example, similar results were reported by Xu, et al. [23], where protein stabilised emulsions were stable at high ionic strength. On the contrary, Mwangi, et al. [22] reported droplet's aggregation for the complete range of tested NaCl concentrations, when studying Pickering emulsions stabilised with chitosan particles, whereas Bai, et al. [13]reported emulsion creaming formation for concentrations higher than 200 mM.

In terms of zeta potential (Fig. 7), which indicates the emulsions' stability, it is possible to observe that all the emulsions revealed strong positive values (higher than 30 mV), and therefore can be considered stable after 1 and 7 days under storage; compatible with the existence of enough electrostatic repulsions between droplets ensuring emulsion's stability. Mitbumrung, et al. [4], who studied the influence of NaCl on nanofibrillated cellulose and whey protein Pickering emulsions, reported that the NaCl concentration increase (100 - 500 mM) promoted the accumulation of Na^+ ions at the negatively droplet surface, leading to a decrease of the zeta potential value.

The n-HAp stabilised Pickering emulsions were resistant to the presence of high NaCl concentrations. No coalescence and aggregation phenomena were observed after 7 days according to the fluorescence microscopy images, as shown in Fig. 8. The presence of NaCl does not compromise the O/W stabilizer role of the n-HAp particles within the time-frame of 7 days. The results for 1 day are presented in FigS3 of the supplementary material. The excellent stability of the n-HAp Pickering emulsions at different ionic strength can be attributed to the existing electrostatic repulsion between the droplets that overcome van der Waals attractions.

Similarly to the other studied environmental stresses, no significant differences were observed between the two used techniques (NETmix and Batch), corroborating the advantages of using the NETmix technology in the production of Pickering emulsions, as already discussed in a previous work of the group [19].

Conclusions

In this work, Pickering emulsions with different average droplet diameters were produced using the NETmix reactor (continuous mode; ~ 7, ~ 11, ~ 18 µm). For comparison a rotor–stator device was also used (batch mode; ~ 18 µm). In the NETmix reactor, the droplet size is controlled by the Reynolds number and the number of recirculation

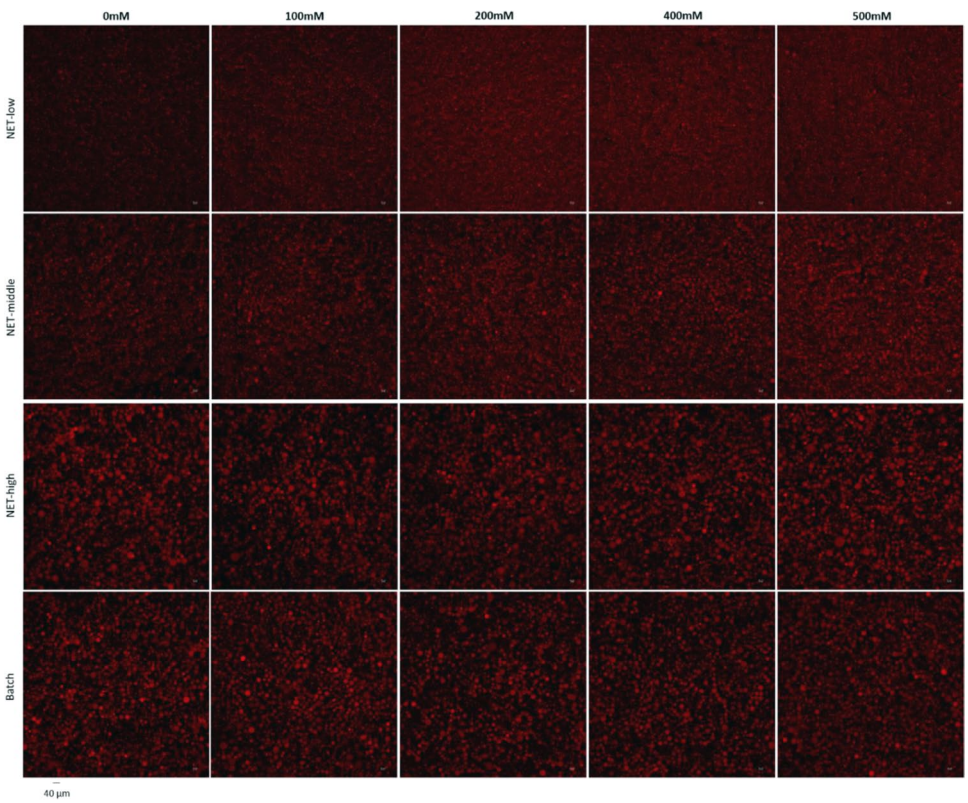

Fig. 8 Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and Batch - 18 µm) at different ionic strength after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x

cycles. NETmix is an attractive low-energy and versatile technique enabling continuous production, ensuring industrial scale-up of the process. The stability of the produced emulsions against different environmental stresses (temperature, pH and ionic strength) was studied. In this study, the temperature (5, 22, 30, 50, 70 and 90 ºC), pH (2, 4, 6, 8 and 10), and ionic strength (0, 100, 200, 300, 400 and 500 mM) were varied to cover a wide range of stress conditions with interest in food products production, storage and ingestion.

The n-HAp Pickering emulsions were stable within the full tested ionic strength range (0 to 500 mM), between 5 to 70 ºC, and in the pH interval of 6–10. A similar behaviour was observed regardless of the droplet size, even the ones with higher droplet sizes were more unstable at high temperatures (70–90 ºC), mainly after 7-days under storage. In this case, droplet's aggregations and coalescence were observed. The emulsions with lower diameters (~ 7 µm) were resistant to all the assayed temperatures, being more stable than the other tested samples. Additionally, all Pickering emulsions undergo phase separation at pH 2 due to n-HAp particle's disruption. Moreover, at low pH values ($4 \leq pH \leq 6$), the emulsions were characterized by low zeta potential values pointed out for droplet aggregation or instability development, justified by a partial solubilisation of the n-HAp particles at acidic medium. At these pH values the samples with lower average diameters tend to develop a prompt aggregation; for the ones with higher droplet sizes a tendency to become unstable, mainly after the 7-days of storage, was also observed.

Comparing the two emulsions produced by different techniques (NET-high and Batch; ~ 18 µm), identical behaviours under the tested stress conditions were observed, i.e., the used productive method have not influenced the emulsion behaviour, meaning that the scale-up by NETmix from a batch laboratorial scale is feasible, also reinforcing the previous achievements of this group [19] where NETmix technology was firstly introduced to produce Pickering emulsions.

Overall, the results presented in this work provide new insights into n-HAp based Pickering emulsions stability. It was observed that n-HAp solid particles provide good stability under strong adverse conditions (with the exception of pH 2 and 90 ºC), with droplets maintaining their size, morphology, and surface charge. These achievements provide useful insights into emulsion behaviour when incorporated in food products (manufacturing, storage or ingestion). It also supports and consolidates the prospectus of using n-HAp Pickering emulsions as carriers of bioactive principles, specifically lipophilic ones.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11483-022-09732-z.

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José Carlos B. Lopes: Conceptualization, Methodology.

Madalena M. Dias: Conceptualization, Supervision, Writing—Review & Editing.

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Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Conflicts of Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. For articles in life science journals that report the results of studies involving humans and/or animals.

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