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In vitro digestion and bioaccessibility studies of vitamin E-loaded nanohydroxyapatite Pickering emulsions and derived fortified foods

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Abstract

Vitamin E is a lipophilic vitamin playing an essential role in human health. Due to oxidative instability, it presents fast degradation and bioactivity loss. In this study, vitamin E-loaded Pickering emulsions stabilized by nano-hydroxyapatite (n-HAp) were produced using a static mixer (NETmix), a technique enabling continuous production and droplet size tailoring. Thus, oil-in-water (O/W) emulsions containing vitamin E at a content of 1mg/mL were produced with different droplet sizes (7.53, 11.56 and 17.72 μm) using an O/W ratio of 20/80 (v/v). Their stability during in vitro gastrointestinal digestion and vitamin E bioaccessibility were investigated. It was observed that n-HAp particles disrupt in the stomach and subsequently aggregate as random calcium phosphates in the small intestine, leading to low vitamin E bioaccessibility due to oil entrapment. The emulsion showing the highest vitamin E bioaccessibility (3.29±0.57%, sample with the larger average droplet size) was used to produce fortified gelatine and milk, resulting in an increased bioaccessibility (10.87±1.04% and 18.07±2.90%, respectively). This fact was associated with the presence of macronutrients and the lower n-HAp content. Overall, n-HAp Pickering emulsions offer advantages for vitamin E encapsulation directed to fortified foods development, a process able to be extended to other lipophilic vitamins.

Keywords: NETmix; Pickering emulsions; Lipophilic vitamin; *in vitro* digestion; Bioaccessibility.

1 Introduction

Vitamin E is a lipophilic vitamin comprising eight compounds, namely: α-, β-, γ- and δ-tocopherol and tocotrienol. Among them, α-tocopherol is the most abundant and biologically active form (Borel, Preveraud, & Desmarchelier, 2013; Burton, 1994; Yang & McClements, 2013). Vitamin E can be naturally found in fresh fruits (kiwi, mango, tomato), vegetables (broccoli, lettuce) and dry fruits (sunflower seeds, hazelnuts, peanuts, almonds) (Borel et al., 2013). Vitamin E intake can avoid cellular ageing and reduce diseases such as dementia, cancer and cardiovascular disorders (Katouzian & Jafari, 2016; Niki & Noguchi, 2020; Yang, Decker, Xiao, & McClements, 2015).

Vitamin E is currently added to fortified foods, beverages and supplements (Lv, Zhang, Tan, Zhang, & McClements, 2019). However, its effectiveness is not easy to achieve due to its high lipophilic character. Vitamin E cannot be effectively incorporated into aqueous systems and presents oxidative instability, degrading fast and losing bioactivity (Hategekimana & Zhong, 2015; Katouzian & Jafari, 2016). For these reasons, vitamin E is often encapsulated to increase stability, bioavailability, and compatibility with hydrophilic food matrices (Hategekimana, Masamba, Ma, & Zhong, 2015; Lv et al., 2019; Mujica-Álvarez et al., 2020; Yang & McClements, 2013).

Among the reported encapsulation systems, Pickering emulsions (PEs), which are emulsifier-free systems, start to be proposed as solutions to increase vitamins bioavailability (Hategekimana et al., 2015; Mitbumrung, Suphantharika, McClements, & Winuprasith, 2019), even some challenges still need to be surpassed. In the work of Lv et al. (2019) dealing with different Pickering stabilizers, vitamin E bioaccessibility was found to be more effective with whey protein systems than with gum Arabic and Quillaja saponin. Comparatively with Tween 80 stabilized emulsions, Zhou et al. (2020) reported a lower vitamin D_3 bioaccessibility using nanochitin PEs, fact associated with the droplet

aggregation in the gastrointestinal tract (GIT). Moreover, Winuprasith et al. (2018) who studied vitamin D_3-loaded nanofibrillated cellulose PEs, reported a decreased bioaccessibility rate as the cellulose concentration increased.

The reported studies evidence that the bioaccessibility of lipophilic vitamins-loaded PEs depends on several factors. These can be related with the emulsion itself (type of particles, oil phase composition, O/W ratio, and O/W interfacial properties), or with the gastrointestinal environment (presence of calcium ions, bile salts, and phospholipids), which can affect (positively or negatively) the bioaccessibility (Yang et al., 2015). Namely, Zhou et al. (2021) showed that titanium dioxide and nanochitin, when added to milk conjunctly with vitamin D_3, did not significantly affect vitamin bioaccessibility. In another study, Tan et al. (2020) reported that chitosan reduced vitamin D_3 bioaccessibility by binding to the mixed micelles. Summarizing, these results highlight the importance to proceed with the testing of different Pickering systems and their impact on bioaccessibility.

In this context, the purpose of the present work was to study vitamin E-loaded n-HAp Pickering emulsions, which were produced using NETmix technology (a static mixer enabling continuous mode), trying to evidence the impact of their droplet size on vitamin E stability, bioaccessibility and bioavailability. HAp is a biocompatible inorganic material mostly used in biomedical and biotechnological applications. This scenario is changing with some food-grade HAps emerging commercially. Thus, following some reported results pointed out Pickering emulsions as promising systems to improve vitamins bioaccessibility, n-HAp stabilized Pickering emulsions were firstly tested as vitamin E carriers, then used to produce fortified milk gelatine and milk. To the best of authors knowledge, this work reports, for the first time, the use of n-HAp Pickering

emulsions as vitamin E carriers and the study of emulsion droplet size effect on bioaccessibility.

2 Experimental methods

2.1 Materials

The *nano*XIM-CarePaste, hydroxyapatite aqueous paste supplied by Fluidinova S.A., is composed of 15.5±0.5 (wt%) of HAp nanoparticles (n-HAp) with particle size <50 nm, 4.5±0.5 (wt%) KCl and a water content ≤81.0 (wt%). This n-HAp has a composition similar to the one commercialized as food grade (*nano*XIM-FoodPaste). Sunflower oil, a 100% vegetable oil obtained from sunflower seeds with a fatty acids composition per 100g of 10 g, 28 g and 53 g of saturated, monosaturated and polysaturated, respectively, milk (semi-skimmed) and neutral gelatine (in powder) were purchased from a local supermarket. Other technical properties of the oil include, refractive index of 1.473 (determined by refractometry, BOECO Digital, Germany), acid value of 0.72 mg KOH/g, free fatty acids in oleic acid equivalents of 0.36% (Ca 5a-40, according to AOCS (2003)), viscosity of 0.06 Pa·s (determined by rheometry, Anton Paar, GmbH, Austria), and density of 923 kg/m3 (determined by pycnometry). Fluorescent dyes (Nile red - technical grade, and Nile blue A - ≥75%) and α-tocopherol (98%), further referred as “vitamin E”, were obtained from Sigma-Aldrich. Isopropyl alcohol (99.7%) was purchased from Riedel-de Haen. Pepsin from porcine gastric mucosa (≥2500 U·mg-1), bile extract porcine, pancreatin from porcine pancreas (8x USP), Pefabloc® SC and the salts used for the preparation of oral, gastric and intestinal electrolyte solutions were purchased from Sigma-Aldrich. Ethanol (99.8%) and methanol (95%) were purchased from Fisher Scientific, and hexane obtained from VWR Chemicals. Distilled water, treated in a Milli-

Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was used. All other chemicals were of analytical grade.

2.2 Vitamin E-loaded Pickering emulsions production and characterization

Oil-in-water (O/W) PEs were prepared, in continuous mode, according to a previously reported technology, NETmix (Ribeiro, Manrique, Barreiro, Lopes, & Dias, 2021). Briefly, the PEs were obtained using an O/W ratio of 20:80 (*v/v*). The aqueous phase corresponds to n-HAp (5 wt%) dispersed in water, and the oil phase to a mixture of sunflower oil and vitamin E. Vitamin E was mixed directly in the sunflower oil at a content of 5 mg/mL (oil phase). For each sample 230 mL have been produced.

NETmix (**Figure 1**) comprises consecutive rows of mixing chambers interlinked by channels, forming a network. The mixing chambers enable successive and well-localized mixing points along the reactor, promoting an easily reproducible emulsification step. To produce the PEs, the NETmix procedure comprised a first step where the aqueous and oil phases are mixed using two peristaltic pumps (Ismatec, Germany) to form a coarse emulsion (pre-emulsion, configuration A). Then, droplet size reduction is achieved by recirculating the pre-emulsion in the NETmix using a diaphragm pump (Almatec, Germany) (configuration B). Recirculation increases the residence time in the NETmix, leading to droplet size reduction. The production conditions in terms of Reynolds number (300 and 400) and the number of cycles (5, 10 and 17) varied according to the required droplet size, as described in **Table 1**. To avoid vitamin E degradation a cooling bath (15 ºC, Paar physica viscotherm VT2, The Netherlands) was coupled to the NETmix to control emulsion temperature (18-20 ºC). PEs with a proximate droplet size of 7, 11 and 18 µm were produced and named as NET-low, NET-middle and NET-high, respectively. The obtained PEs were characterized concerning particle size using a laser diffraction

particle size analyser (Beckman Coulter LS230; California) and zeta potential using a Zetaziser Nano ZS90 (Malvern Instruments, United Kingdom).

Table 1: NETmix conditions used to produce the n-HAp stabilized Pickering emulsions.

SAMPLE NAME	Re	CYCLES
NET-low	400	17
NET-middle	400	5
NET-high	300	10

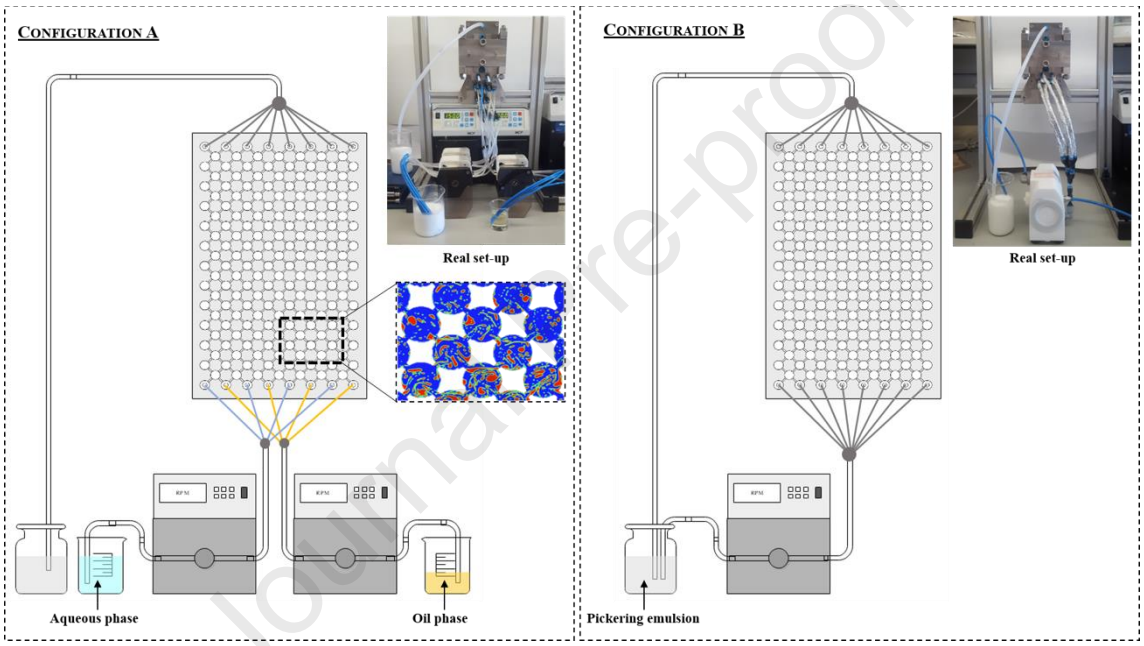


Figure 1: Experimental NETmix set-up used to produce PEs: configuration for the first pass (pre-emulsion preparation (A), and configuration for the recirculation mode (B).

2.3 Vitamin E-fortified foods production

To produce the vitamin E-fortified foods, vitamin E-loaded PEs were incorporated in neutral gelatine and semi-skimmed milk. A ratio of 1:2 of PE to the food matrix was used. The final concentration of vitamin E in the food matrix was 0.33 mg/mL, higher than the daily reference intakes of Regulation (EU) no 1169/2011 (1.8 mg or 0.9 mg of vitamin E per 100 g of food or 100 mL of beverages, respectively). This value was used to facilitate the quantification of vitamin E by ultra-performance liquid chromatography (UPLC) after digestion. For the fortified milk, 25 mL of PE were added to 50 mL of milk. The mixture

153 was stirred at 50 rpm for 1 min and stored in the fridge. For the fortified gelatine, 1.5 g
154 of powder gelatine were hydrated with 25 mL of distilled water under stirring, completed
155 with 25 mL at 50 ºC (total water 50 mL), then added with 25 mL of the PE. After stirring
156 (50 rpm) for 1 min the final mixture was stored in the fridge to solidify.

157 **2.4 *In vitro* gastrointestinal (GI) digestion studies**

158 The produced vitamin E-loaded PEs (NET-low, NET-middle and NET-high) and fortified
159 foods (milk and gelatine with vitamin E-loaded PEs) were digested *in vitro* to check
160 emulsions' behaviour in case of vitamin E-loaded PEs, and vitamin E bioaccessibility for
161 emulsions and fortified food samples. The harmonized static *in vitro* digestion model
162 described by Minekus et al. (2014) was used. This is a three-stage model comprising the
163 simulation of the mouth, stomach and small intestine conditions. The composition of the
164 simulated digestion fluids is presented in **Table 2**. All fluids and samples (vitamin E-
165 loaded PEs, fortified milk and gelatine) were pre-heated at 37 ºC during 5 min.
166 For the oral stage, simulated salivary fluid (SSF), $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.75
167 mM at the final mixture) and Milli-Q water were added to the tested sample (5 mL). The
168 mixture was incubated at 37 ºC for 2 min under orbital stirring at 120 rpm. A final ratio
169 of food to SSF of 1:1 (*v/v*) was targeted. Since the samples did not have starch, α-amylase
170 was not used (Brodkorb et al., 2019). In the gastric stage, simulated gastric fluid (SGF),
171 $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.075 mM at the final mixture) and pepsin solution (with
172 the final activity of 2000 U/mL in the final mixture) were added to the previous mixture.
173 The pH was adjusted to 3.0 with HCl 1 M, and Milli-Q water was added to make up the
174 final volume, and the mixture incubated for 2 h at 37 ºC under orbital stirring at 120 rpm.
175 A final ratio of oral sample to SGF of 1:1 (*v/v*) was targeted. The intestinal stage consisted
176 of simulated intestinal fluid (SIF), $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.3 mM at the final
177 mixture), bile salts (to reach 10 mM at the final mixture) and pancreatin solution (with

the final activity of 100 U/mL in the final mixture). The pH was adjusted to 7.0 with NaOH 1 M or HCl 1 M, then Milli-Q water was added to achieve the final volume. The samples were incubated for 2 h at 37 ºC under orbital stirring at 120 rpm. A final ratio of gastric sample to SIF of 1:1 (*v/v*) was targeted.

After each stage, samples were collected to check the morphology and stability of vitamin E-loaded PEs along the simulated gastrointestinal tract. The samples collected at the end of the gastric stage were maintained in an ice bath to decrease the pepsin activity until analysis. After complete digestion, the reaction was stopped by adding the enzyme inhibitor Pefabloc® (1 mM) (10 µL for each 1 mL of the sample). The sample provided after the intestinal stage was used to analyse bioaccessibility and stability of vitamin E-loaded PEs and fortified foods. All the samples were tested at least in triplicate.

Table 2: Composition of simulated digestion fluids.

SALT SOLUTION	SSF (mM)	SGF (mM)	SIF (mM)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H2O)_6$	0.15	0.12	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
HCl	1.1	15.6	8.4

SSF: simulated salivary fluid; SGF: simulated gastric fluid; SIF: simulated intestinal fluid.

2.5 Pickering emulsions digesta characterization

Zeta potential can reveal emulsion stability. The emulsion can be considered stable for high positive (≥30) or high negative (≤-30) values. The determination was done with a Zetaziser Nano ZS90 (Malvern Instruments, United Kingdom). For that, the PEs were diluted with distilled water to avoid multiple scattering effects, then placed in a folded

197 capillary Zeta cell (ref: DS7010). The measurement was conducted at 25 ºC. Each sample
198 was measured in triplicate and the data expressed as average±standard deviation (SD).
199 To check PEs morphology along digestion, the samples collected at each GIT stage were
200 analysed by CLSM using a Leica TCS-SP5 AOBS (Leica Microsystems, Germany).
201 CLSM is a microscopic technique allowing to observe the particles and oil phase. The
202 digesta sample (100µL) was stained with a mixture of Nile red at 0.1% (*w/v*) and Nile
203 blue A at 0.1% *w/v* dissolved in isopropyl alcohol (10µL). Nile blue and Nile red are able
204 to dye the solid particles and the oil phase, respectively. The dyed digesta PE was placed
205 on a slide, and fluorescent dyes excited at 488 nm (Nile red) and at 633 nm (Nile blue A).
206 The images were digitally recorded and processed with a LASX software. The initial PE
207 was also analysed.

208 **2.6 Vitamin E bioaccessibility, stability and bioavailability evaluation**

209 After passing through the three simulated GIT stages, vitamin E concentration was
210 determined according to the procedure described by Lv et al. (2019) with some
211 modifications. Briefly, the digesta obtained after the small intestine stage was centrifuged
212 (Allegra 64R, Beckman Coulter Inc., USA) at 18700 *g* and 4 °C for 30 min to obtain the
213 micellar phase. Before UPLC (Nexera X2, Shimadzu, Japan) analysis, vitamin E was
214 extracted from digesta and micellar phase samples. For that, 3 mL of the sample were
215 mixed with 3 mL of a hexane/ethanol mixture (1/1, *v/v*), then centrifuged at 4000 rpm for
216 2 min (Multifuge X3R, Heraeus, Germany) to obtain a supernatant layer. This extraction
217 was repeated three times, and the obtained supernatant layers mixed and dried under
218 nitrogen. The dried samples were dissolved in methanol and filtered through a 0.22 µm
219 filter. The vitamin E concentration was determined using a UPLC with a C18 column
220 (150×2.1 mm, 1.7 µm, Kinetex®) using a mixture of 95% methanol and 5% double-
221 distilled water as the mobile phase. An isocratic elution mode at 0.4 mL/min, with a

column temperature of 40 ºC and a 10 µL injection volume were used. The detection wavelength for vitamin E was 295 nm.

Vitamin bioaccessibility was determined by measuring the concentration of vitamin E in the mixed micelle ($C_{micelle}$) and digesta phases ($C_{digesta}$) using the following equation:

$$Bioaccessibility = \frac{C_{micelle}}{C_{digesta}} \times 100 \quad (1)$$

Stability was calculated as the vitamin E fraction remaining untransformed in the small intestine and calculated as

$$Stability = \frac{C_{digesta}}{C_{initial}} \times 100 \quad (2)$$

where, $C_{initial}$ represents the vitamin E concentration in the initial stage, i.e., the vitamin E concentration of the PE (1 mg/mL).

The bioavailability of the vitamin E was then estimated as the product of bioaccessibility and stability

$$Bioavailability = Bioaccessibility \times Stability \quad (3)$$

The calculated bioavailability corresponds to an estimative of vitamin E absorption.

2.7 Statistical analysis

The digestion and bioaccessibility studies of PEs and fortified foods were done in triplicate and duplicate, respectively. Data is presented 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 of independent variables followed by Tukey's test was carried out on the digesta PEs measurements to establish significant differences (p<0.05). For fortified foods measurements, a *t*-test was used to determine the significant difference among the tested samples (p<0.05).

3 Results and discussion

3.1 Vitamin E-loaded Pickering emulsions characterization

The vitamin E-loaded PEs with tailored droplet average sizes were produced using NETmix according to the parameters described in **Table 1**. The influence of these parameters in the average droplet size of the emulsion was previously reported by Ribeiro et al. (2021), and adopted in this study since the used vitamin E concentration did not impacted the sunflower oil viscosity (0.06 Pa·s).

Figure 2 shows the optical microscopy images of NET-low, NET-middle and NET-high samples with the determined droplet size distributions, average droplet size (7.53, 11.56 and 17.72 µm, respectively) and zeta potential (+31.18, +32.20, and +32.05 mV, respectively). In terms of the zeta potential, similar values were obtained for the 3 samples; namely, the produced PEs were characterized by a strong positive zeta potential, indicating that droplet aggregation is inhibited resulting in stable emulsions due to an electrostatic repulsion mechanism.

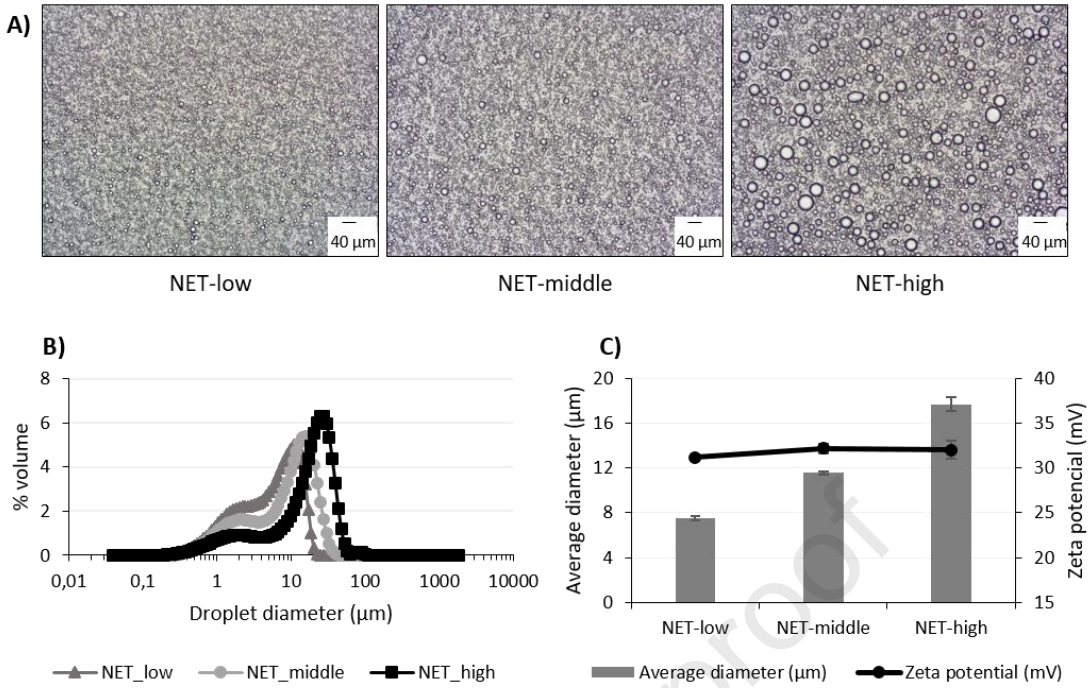


Figure 2: Optical images (A), droplet size distributions (B), average size distributions (µm) and zeta potential (mV) (C) of the produced vitamin E-loaded Pickering emulsions produced using NETmix technology.

3.2 Vitamin E-loaded Pickering emulsions gastrointestinal digestion

The vitamin E-loaded PEs were digested *in vitro* through a simulated GIT. After each stage (mouth, gastric and intestine phases), a sample was collected for analysis, namely concerning for microstructure (CLSM), zeta potential, vitamin E bioaccessibility and stability analysis.

3.2.1 Influence on microstructure morphology

Figure 3 shows the CLSM analysis of the studied PEs (NET-low, NET-middle, and NET-high) in the initial stage and throughout the simulated GIT. The red fluorescence colour represents n-HAp particles (dyed by Nile blue), whereas the green fluorescence colour represents the oil phase (stained by Nile red).

At the initial stage, a packed n-HAp layer is observed around the oil droplets for all tested PEs, confirming the efficient stabilization role of the nanoparticles. After exposure to the mouth stage, the number of oil droplets decreased, justified by the dilution applied in the

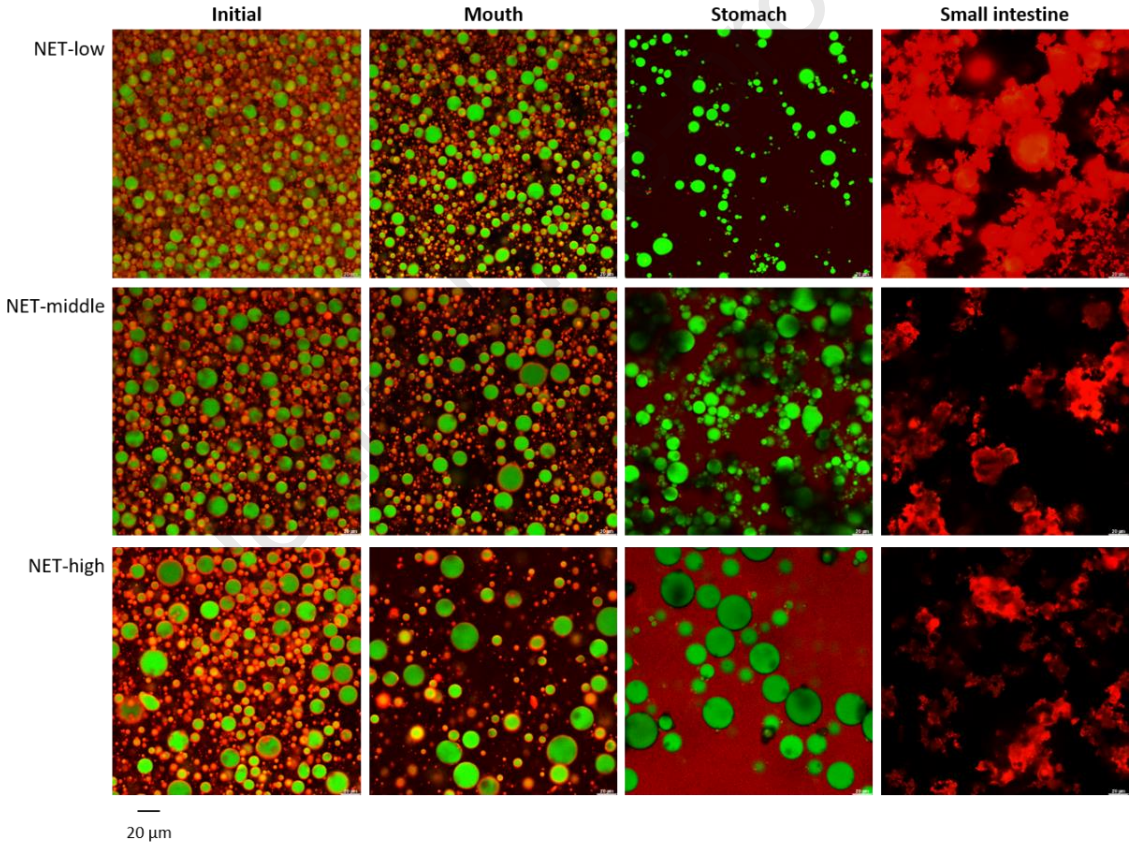
digestion model. In this stage, no changes in the n-HAp stabilizing role were perceived, i.e., the n-HAp particles maintained their tight position at the oil surface. Also, for all the tested samples, no evident changes in the droplet size were observed, and no agglomerates were detected.

In the stomach, the PEs face stringent conditions, namely they contact with gastric fluids characterized by a highly acidic pH (around 1-3), relatively high ionic strength (around 150 mM), and a high enzymes content (McClements, 2016). The acquired CLSM images (**Figure 3**) revealed that this is the first local in GIT where the PEs undergo strong changes. Namely, it is evident that the n-HAp particles are not covering the oil droplets, i.e., they are not acting anymore as Pickering stabilizers in all tested PEs. The n-HAp particles seem to have disrupted, becoming dissolved in the aqueous medium, fact compatible with the observation of a quite uniform red background. In fact, the Nile blue, which dyes the hydrophilic compounds, might be staining the Ca^{2+} and PO_4^{2-} species of the disrupted n-HAp particles, and other molecules resulting from the two digestive stages. These results are in agreement with Ramis, Coelho, Córdoba, Quadros, and Monjo (2018) that studied the n-HAp paste behaviour in simulated gastric fluid, identifying its rapid and complete dissolution under gastric environment after 7.5 min at 37 ºC. In this work, together with particle's disruption, a slight oil droplet size increase was observed after exposure to gastric conditions, which may also be justified by the n-HAp detachment from the oil droplets facilitating coalescence.

After passing the stomach, medium in which the n-HAp particles become completely dissolved, the digestion proceeds to the small intestine. Within this phase, the environment conditions change, namely the pH turn to neutral. Under these conditions, and as shown in the CLSM images taken after the intestine phase (**Figure 3**), appreciable changes were noticed. The material of the dissolved n-HAp particles coming from the

297 stomach phase forms large and random calcium phosphate clusters upon in contact with
298 neutral pH. This result corroborates the observations made by Epple (2018) that reported
299 the precipitation of HAp in random calcium phosphate clusters at neutral pH after
300 contacting acidic pH.

301 The PEs tailored with different sizes showed similar behaviour during GI digestion with
302 apparent stability in mouth stage, n-HAp disruption in gastric conditions and aggregates
303 formation in intestinal conditions. However, NET-low PE has larger aggregates than the
304 other samples after intestinal phase (**Figure 3**).



305

306 Figure 3: Overlapped CLMS images of the vitamin E loaded Pickering emulsions with different sizes
307 (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) after each stage of digestion (mouth,
308 stomach and small intestine), comparatively with the initial emulsion. The n-HAp particles and the oil
309 droplets are presented as red and green colour, respectively. For interpretation of the references to the
310 colour in this figure legend, the reader is referred to the web version of the article. The images were taken
311 with a magnification of 20x and zoom of 3x.

3.2.2 Influence on zeta potential

Figure 4 shows the zeta potential of the studied PEs (NET-low, NET-middle, NET-high) throughout the simulated GIT. For comparison purposes, the zeta potential of the initial emulsions is included. The statistical analysis was done for each stage by comparing the zeta potential value of the three tested emulsions with different droplet size. It was observed that the value changes throughout the GIT, including the surface charge shifts from positive to negative between the mouth and stomach. The three emulsions presented similar behaviour for all GIT stages (p<0.05).

The contact with the mouth promoted an appreciable decrease in the zeta potential magnitude (from approximately from 32 mV to 16 mV), probably associated with the neutral pH of this stage and the adsorption of ionic species at the droplets' surface. After mouth stage, no statistical differences were observed between the three emulsions (p<0.05) meaning a similar behaviour for the PEs regardless the size. These results suggest that PEs become more prone to instabilities after the mouth stage. In accordance, other authors working with PEs stabilized with nanofibrillated cellulose observed small changes in the droplet size and morphology after this first digestion phase, which was related to mucin presence (Winuprasith et al., 2018).

Upon in the stomach, the PEs zeta potential becomes low in magnitude and negative (around -3 mV) and once again no statistical differences were observed between the three emulsions (p<0.05). This change can be attributed to the highly acidic pH of the gastric fluids together with the presence of enzymes (i.e., pepsin), which can lead to the adsorption of negatively charged molecules neutralizing the positive charge (Zhou et al., 2020). Also, the high ionic strength medium can have contributed to the emulsion destabilization. These observations agree with the CLSM images acquired after the stomach stage (**Figure 3**).

In the small intestine, all the tested PEs presented a relatively strong negative zeta potential (but not high enough to be considered stable), which can be attributed to the presence of bile acids, phospholipids, and free fatty acids generated from the digestion of the emulsified oil (triglycerides). These molecules are negatively charged, leading to a zeta potential decrease. This observation is in accordance with the work of Zhou et al. (2020) that reported a strong zeta potential decrease for emulsions after the small intestine stage, fact associated with the presence of bile acids and peptides. Comparing the three emulsions, after small intestine stage there was significant differences (p<0.05), namely between NET-low and NET-high. NET-high had stronger negative zeta potential value, possibly indicating more effective adherence of the bile acids and others species at the cluster's surface.

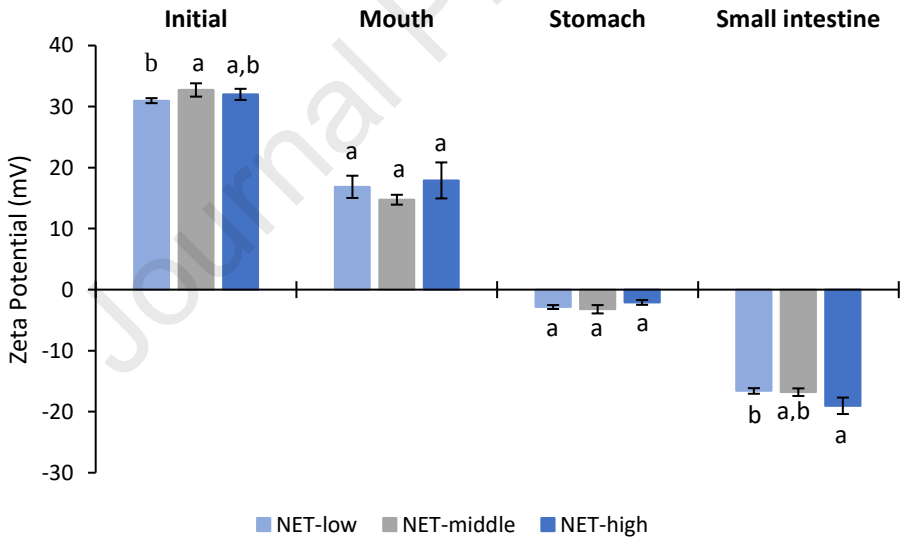


Figure 4: Zeta potential of the vitamin E-loaded Pickering emulsions (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) along the gastrointestinal tract (mouth, stomach and small intestine), comparatively with the initial emulsion. In each GIT stage, different letters represent significant differences between the Pickering emulsions (p<0.05).

3.2.3 Influence on vitamin E bioaccessibility and stability

In this section, the influence of the GI digestion on vitamin E stability and bioaccessibility was evaluated (**Figure 5**), and the statistical analysis was done to compare the three

emulsions for each evaluated parameter. In general, all PEs presented a high vitamin E stability. The NET-high sample showed the lowest and statistically different vitamin E stability followed by NET-low and NET-middle, respectively, 28.9±2.59%, 32.1±1.43% and 33.8±1.53% (p<0.05). However, all emulsions exhibited low vitamin E bioaccessibility, with NET-high sample presenting a slightly higher and statistically different value, comparatively with NET-low and NET-middle, respectively, 3.29±0.57%, 2.15±0.15% and 2.03±0.12% (p<0.05). Therefore, NET-high sample presented the highest value of calculated bioavailability, followed by NET-middle and NET-low, 0.94±0.15%, 0.70±0.03% and 0.69±0.03%, respectively (p<0.05).

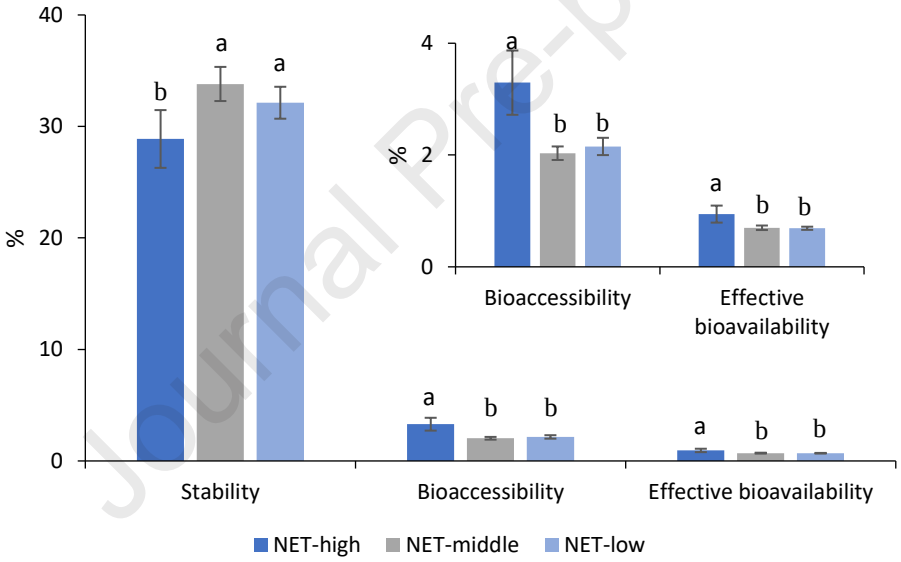


Figure 5: Bioaccessibility, stability and effective bioavailability of vitamin E loaded Pickering emulsions with different particle's sizes (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) after *in vitro* digestion. Different letters in each evaluated parameter represent significant differences between the tested Pickering emulsions (p<0.05; one-way ANOVA): $^{a-b}$ for bioaccessibility, $^{A-B}$ for stability, and $^{x-y}$ for effective bioavailability.

The low observed bioaccessibility for the tested PEs can be attributed to the high amount of free Ca^{2+} ions derived from the n-HAp particles disruption in the gastric phase. Ca^{2+} ions can form insoluble aggregates with anionic species, such as bile salts and free fatty acids, decreasing the mixed micelle's formation. Additionally, Ca^{2+} can also form

aggregates with PO_4^{2-} species (random calcium phosphates). These aggregates can entrap the lipidic phase, hindering its digestion and, consequently, the micellar phase formation. The CLSM images taken after the intestinal phase (**Figure 6**) support this hypothesis. In fact, the insoluble aggregates (stained in red) and the lipidic phase (stained in green) are superimposed, meaning that some lipidic phase remained entrapped inside these structures. In addition, the insoluble aggregates can also entrap the mixed micelles containing the vitamin E, fact in accordance with the observed high stability and low bioaccessibility, as also reported by (Yang et al., 2015) in a work addressing vitamin E. Zhou et al. (2020), which compared the effect of using PEs produced with nanochitin particles with emulsions produced with Tween 80 on vitamin D_3 bioaccessibility, reported a lower bioaccessibility for PEs. The authors justified these results by the precipitation of the mixed micelles in the presence of the nanochitin together with the presence of the non-digested vitamin D_3 lipid phase carrier. Additionally, Tan et al. (2020) examined the impact of using chitosan on vitamin D_3 bioaccessibility with results showing that it promoted severe droplet flocculation in the small intestine, reducing the amount of free fatty acids and leading vitamin D_3 bioaccessibility decrease. This effect was justified by chitosan ability to bound with vitamin-loaded mixed micelles promoting their precipitation.

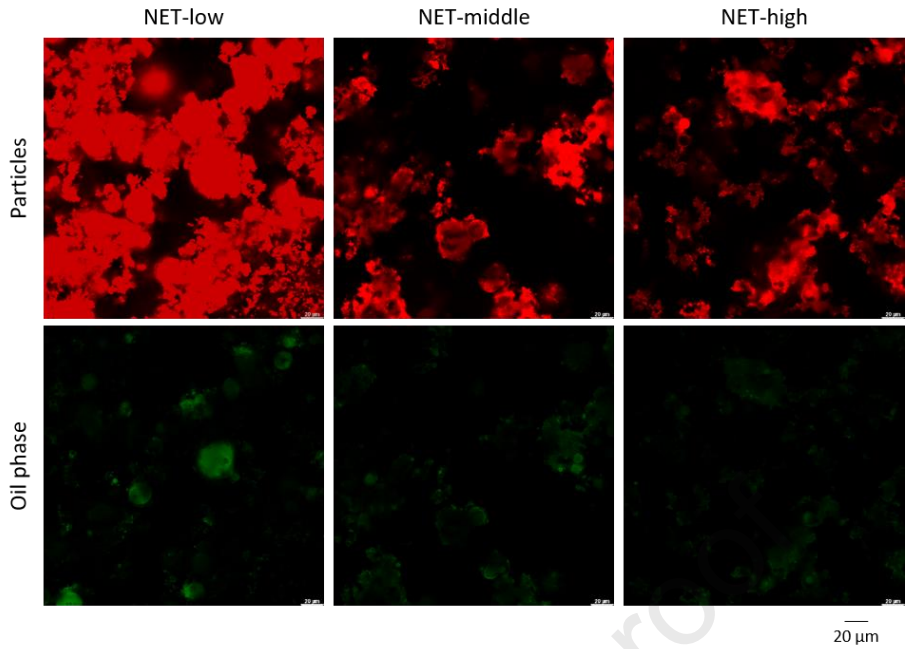


Figure 6: CLSM images Pickering emulsions (NET-low, NET-middle and NET-high) after intestinal phase.

The NET-high sample presented a slightly, but significantly higher vitamin E bioaccessibility, comparatively with the other tested emulsions (p<0.05). This can be associated to the observed droplet size for this emulsion after the gastric phase. Once the NET-high sample had a higher droplet size, the lipid digestion rate at the intestinal phase was, probably, slower than the ones of other emulsions, meaning that the formation of the mixed micelles delayed the Ca^{2+} ions binding to other ionic species. Therefore, the presence of Ca^{2+} ions showed to have a high impact on vitamin E bioaccessibility and stability. Moreover, the PE produced with the larger particle size (NET-high) lead to better results in terms of vitamin E bioaccessibility.

3.3 Vitamin E fortified foods

The PE presenting the best vitamin E bioaccessibility after digestion (NET-high) was incorporated in gelatine and milk to study the effect of the food matrix in this parameter. Gelatine is constituted only by proteins and milk comprises fat, proteins and

carbohydrates. Both foods are widely consumed. Inorganic solid particles are usually used in the food industry to modify properties such as viscosity, brightness, and whiteness (Weir, Westerhoff, Fabricius, Hristovski, & von Goetz, 2012; Zhou et al., 2021). In this work, the n-HAp solid particles were used as stabilizers in PEs for vitamin E carriers, thus contributing to the organoleptic properties and to add functional properties to foods. **Figure 7** shows the photos of the used food matrices, gelatine (**Figure 7**-A) and milk (**Figure 7**-B) without incorporation (control samples) and after the incorporation of the vitamin E-loaded PE (fortified foods). In gelatine, the emulsion addition changes the sample from transparent to white, effect clearly observed. Regarding milk, no significant macroscopic changes in terms of colour were observed. In **Figure 7**-A1 and A2, and **Figure 7**-B1 and B2 the optical microscopy images of gelatine and milk (control sample and fortified food, respectively) are shown. It is possible to observe a clean and homogeneous image for the control food matrices, whereas for the fortified foods the presence of the PE is noticeable. To note that the Pickering droplets remained with their typical morphology and size after incorporation.

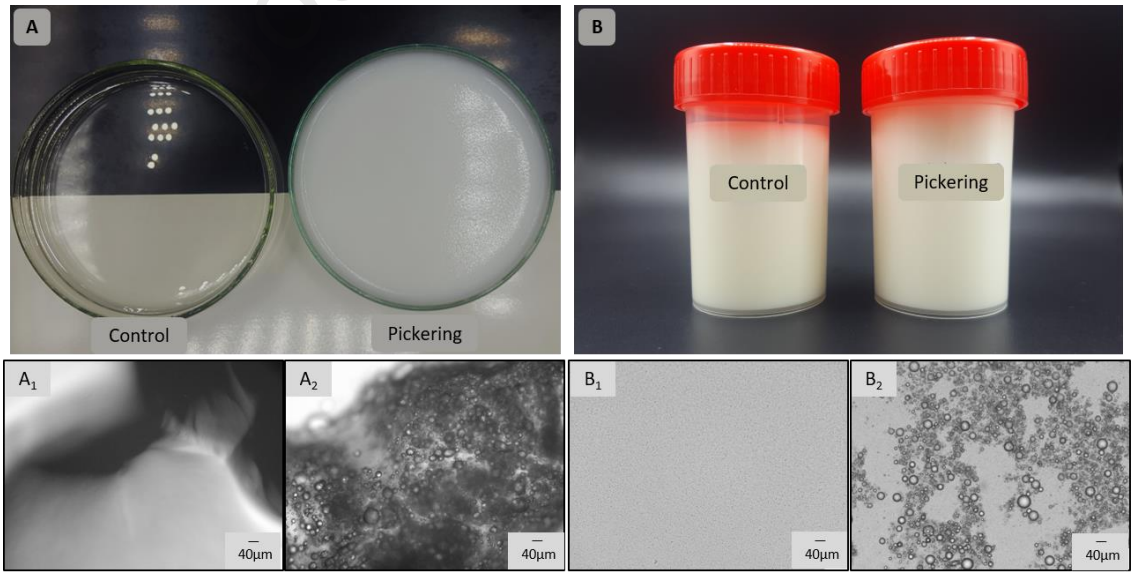


Figure 7: Photos of control samples and fortified foods: gelatine (A) and milk (B). Optical microscopy images for gelatine and milk controls, (A_1 and B_1, respectively), and fortified gelatine and milk (A_2 and B_2, respectively). Images were acquired at a magnification of 10x.

After emulsion incorporation, the fortified foods (fortified milk and fortified gelatine) were digested to access vitamin E stability and bioaccessibility. To check for any food matrix or PE materials effects, control samples (foods without incorporation, and foods added with non-loaded PEs) were also *in vitro* digested to determine vitamin E presence. **Figure 8** shows the stability, bioaccessibility and bioavailability of the vitamin E after fortified gelatine and fortified milk digestion, the statistical analysis was done to compare the fortified foods for each evaluated parameter. In general, the two fortified foods lead to good vitamin E stability, with fortified gelatine (gelatine_VitE-PE) showing higher and statistically different vitamin E stability, comparatively with fortified milk (milk_vitE-PE), namely 49.2±2.28% and 38.4±1.10%, respectively (p<0.05). Comparing the two fortified foods, the milk_vitE-PE sample presented higher and statistically different bioaccessibility, 18.1±2.90%, in comparison with 10.9±1.04% for gelatine_VitE-PE (p<0.05). In addition, milk_vitE-PE presented higher and statistically different bioavailability than gelatine_VitE-PE (6.93±1.09% and 5.33±0.36%, respectively) (p<0.05). To note that the control samples (foods without incorporation) presented no vitamin E (data not shown). In the case of foods added with non-loaded PEs, only a very residual vitamin E content, attributed to the sunflower oil, was detected (data not shown). Thus, the quantified vitamin E after digestion of the fortified foods was related to the incorporated vitamin E-loaded PEs.

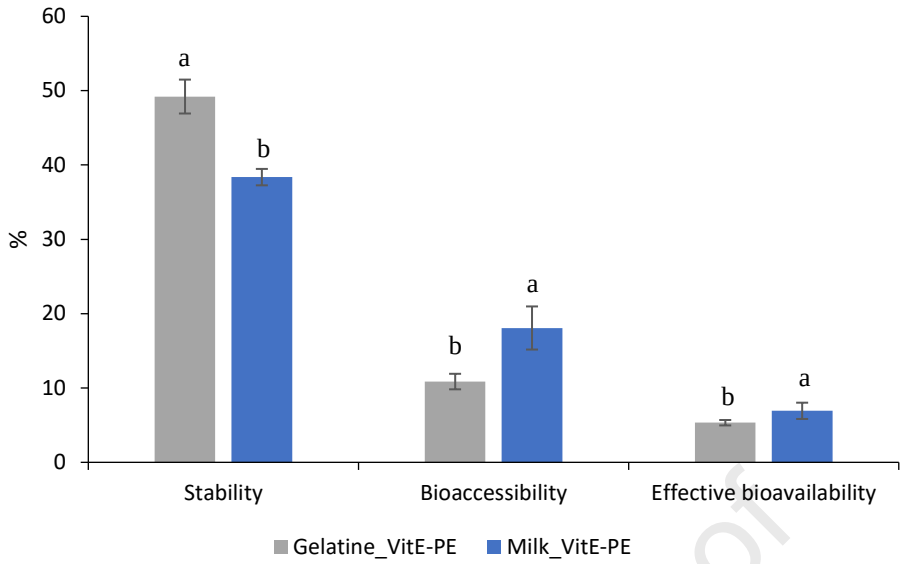


Figure 8: Bioaccessibility, stability and adequate bioavailability of fortified food with vitamin E loaded Pickering emulsions (NET-high: ~18 µm) after *in vitro* digestion. Different letters represent significant differences between fortified food matrices in each parameter (p<0.05; *t*-test): $^{a-b}$ for bioaccessibility, $^{A-B}$ for stability, and $^{x-y}$ for adequate bioavailability.

One important point to highlight is the fact that fortified foods lead to better vitamin E bioaccessibility and stability in comparison with the non-incorporated vitamin E-loaded PEs. Namely, for vitamin E-loaded PEs, the maximum achieved bioaccessibility and stability values were 3.29±0.57% and 28.9±2.59%, receptively, for the sample NET-high, which is much lower than the values obtained with fortified milk (18.1±2.90% and 38.4±1.10% for bioaccessibility and stability, receptively) and fortified gelatine (10.9±1.04% and 49.2±2.28% for bioaccessibility and stability, respectively). The natural presence of macronutrients in the fortified foods led to a vitamin E bioaccessibility increase, possibly associated with their ability to displaced some n-HAp particles from the oil droplet's surface, facilitating lipid digestion, vitamin E release and solubilisation, and mixed micelles formation (Zhou et al., 2020). Furthermore, fortified milk presented a slightly higher bioaccessibility than gelatine, which can be related to fat presence in this food matrix, which can improve the micellar phase formation. This can be explained because the mixed micelles generated during the lipid digestion had more free-fatty acids

available for their formation. Therefore, if a higher number of mixed micelles can be formed, more vitamin E can be incorporated leading to an increased bioaccessibility. Another fact contributing positively to vitamin E bioaccessibility is the lower amount of n-HAp particles presented in the fortified food. This leads to the formation of less Ca^{2+} species, decreasing aggregates formation. Additionally, the incorporation of vitamin E-loaded PE in gelatine and milk also lead to an increased vitamin E stability, which indicates lower vitamin E loss during GI digestion.

Although considering different lipophilic vitamins, the results reported in the present work for bioaccessibility agree with those published by Zhou et al. (2021). The plant-based milk fortified with a vitamin D_3-loaded emulsion together with nanocellulose or titanium dioxide particles showed a vitamin D_3 bioaccessibility around 20% (Zhou et al., 2021).

4 Conclusion

The interest in using PEs as carriers of lipophilic vitamins, foreseeing their use in fortified foods, is a topic gathering interest within the scientific community. This implies the digestion study of both PEs and fortified foods to have a more complete image of the parameters impacting vitamin bioaccessibility. In this study, n-HAp Pickering emulsions are proposed as vitamin E carriers, and the impact of droplet size (7.53, 11.56 and 17.72 µm) in vitamin E stability, bioaccessibility and bioavailability was studied. For that, emulsions with tailored droplet size were produced by NETmix, a static mixer enabling continuous production at controlled emulsifying conditions.

In terms of digestion, the tested vitamin E-loaded PEs presented similar behaviour along the GIT, with n-HAp particles being disrupted in gastric conditions with subsequent formation of aggregates under intestinal environment. The droplet average size impacted vitamin E bioaccessibility, with the NET-high sample, i.e., the sample with the higher

droplet size (17.72 µm), leading to the higher vitamin E bioaccessibility results (3.29±0.57%). The achieved low values were associated with the entrapment of the oil phase containing vitamin E within the formed random calcium aggregates (intestinal phase). When the vitamin E-loaded PE NET-high was incorporated in food matrices (gelatine and milk), vitamin E bioaccessibility increased significantly (10.87±1.04% for gelatine and 18.07±2.90% for milk), putting in evidence the positive effect of the food matrix in the bioaccessibility.

Overall, n-HAp Pickering emulsions offer advantages for vitamin E encapsulation directed to fortified foods development, a process able to be extended to other lipophilic vitamins, and to other Pickering stabilizers. The obtained results also pointed out the interest proceed with further studies, namely to understand the effect of the food matrix composition in the achieved bioaccessibility. Moreover, it also highlights the importance of combining the study of PEs with their final applications to evaluate more accurately the real potential of these innovative solutions.

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Highlights

NETmix was successfully used to produce vitamin E-loaded Pickering emulsions.

Hydroxyapatite disrupts in the stomach solubilizing in the aqueous medium.

The formation of calcium aggregates in the intestine hinder vitamin E bioaccessibility.

Higher droplet size Pickering emulsions enhanced vitamin E bioaccessibility.

Incorporation of Pickering emulsions in foods improved vitamin E bioaccessibility.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: