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Co-encapsulation of vitamin D_3 and curcumin in plant protein-based nanoemulsions: formulation optimization, characterization, and *in vitro* digestion

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This study aimed to develop plant-based nanoemulsions (NE) for co-encapsulation of vitamin D_3 and curcumin, comparing the emulsifying properties of pea and potato proteins. Different oil and protein concentrations were tested, and formulations with the smallest particle size were characterized and submitted to harmonized static *in vitro* digestion. Formulations prepared with the highest protein concentration (10%) and lowest oil concentration (1%) led to NE with smallest particle size and lowest polydispersity index (PDI). Curcumin and vitamin D_3 were successfully co-encapsulated in both protein NE. During digestion, NE showed instability, particularly in the gastric phase, in which an increase in particle size was observed. Pea protein NE exhibited higher curcumin bioaccessibility (76.06 ± 12.05%) than potato protein NE (42.88 ± 3.58%), while the bioaccessibility of vitamin D_3 did not show significant differences. Curcumin stability was also higher in pea protein NE (21.44 ± 1.97%) compared to potato protein NE (12.38 ± 1.97%). These results showed that pea protein is more suitable for produced plant-based NE for co-encapsulation of these bioactive compounds. This work contributes to the development of plant-based NE as promising carriers for the co-encapsulation of bioactive compounds, envisaging the fortification of food products, especially plant-based foods, in response to the growing demand for sustainable alternatives.

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Sustainability spotlight

Micronutrient deficiencies remain a global challenge, particularly in populations relying on plant-based diets. This study introduces a sustainable nanoemulsion platform using renewable pea protein to co-encapsulate vitamin D_3 and curcumin, significantly enhancing their stability and bioaccessibility. By replacing synthetic or animal-derived emulsifiers with plant-based proteins, our work advances circular-bioeconomy principles and enables fortification of plant-based foods with a reduced environmental footprint. This innovation directly supports UN SDG 2 (Zero Hunger) by combating malnutrition, SDG 3 (Good Health and Well-being) through improved nutrient delivery, and SDG 12 (Responsible Consumption and Production) *via* sustainable ingredient sourcing.

1. Introduction

In recent decades, the food industry has increasingly fortified foods with bioactive compounds, such as vitamins, polyphenols, and probiotics to address potential nutritional deficiencies and promote public health.[1] However, many of these compounds are chemically unstable and prone to degradation, especially when exposed to oxygen, light, humidity, or heat. Furthermore, their direct incorporation into food products is often limited due to their low solubility and bioaccessibility.[2] Nanoencapsulation has emerged as an effective strategy to overcome these challenges, enhancing the solubility, stability, and bioaccessibility of compounds, while enabling controlled release and targeted delivery.[3,4] A variety of nanostructured delivery systems (NDS) are available for food applications, including biopolymer nanoparticles, nanohydrogels, nanoemulsions (NE), nanoliposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).[5] Plant-based ingredients, particularly proteins, are increasingly used for NDS due to their advantages in terms of sustainability, low cost, amphiphilic structure, high nutritional value, biocompatibility, biodegradability, and general safety (GRAS).[6]

For encapsulation of lipophilic compounds, such as vitamin D and curcumin, lipid-based systems such as nanoemulsions are particularly suitable, enhancing solubility, stability, and bioavailability ref. 7–10.

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Vitamin D is an essential micronutrient that plays a crucial role in bone mineralization and metabolic pathways.[11] Vitamin D has two forms: vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol). Vitamin D_2 is only synthesized by plants, whereas vitamin D_3 (vitD_3) is produced in the skin in response to UVB radiation from the sun and can also be obtained through diet (*e.g.*, oily fish, liver oil and eggs).[9] VitD_3 deficiency is one of the most common micronutrient malnutrition disorders, often associated to low sun exposure, constant use of sunscreen, and clothing type. Moreover, plant-based diet consumers may lack this micronutrient, since most food sources come from animal origin 11. VitD_3 deficiency has been associated with a wide range of diseases, including diabetes, cardiovascular, autoimmune, and inflammatory diseases, as well as cancers.[12] Despite efforts to increase vitD intake, fortifying foods with this micronutrient remains difficult due to its liposoluble nature and low water solubility.[13]

Curcumin, a natural phytochemical extracted from the rhizomes of *Curcuma longa* (also known as turmeric), is widely used in food as a spice, colorant, preservative, and antioxidant. Curcumin has been the subject of much attention due to its several benefits including antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic activities. Similar to vitD_3, curcumin is a hydrophobic molecule that is difficult to incorporate into foods due to its low water solubility, chemical instability, and low oral bioavailability.[14]

There is a growing interest in the encapsulation of multiple bioactive compounds within a single NDS, as this can enhance bioavailability and bioactivity through potential synergistic effects.[15] Combining vitD_3 and curcumin in lipid-based nanostructures can improve their individual stability, bioavailability, and potentiate synergistic biological effects. Recent studies demonstrate benefits of combined administration in pathologies such as osteoarthritis, type 2 diabetes and viral infections.[15–19] In fact, several dietary supplements already combine these compounds, reflecting strong market interest.[20–24]

In this study, we aimed to develop plant-based NE to co-encapsulate vitD_3 and curcumin. Despite the efforts that have been made by food researchers to replace animal-based ingredients with plant-based ingredients in the development of NDS, as far as we know, the available studies do not evaluate the nanostructures when subjected to the digestion process, which is very important to determine whether the formulations are suitable for food applications. On the other hand, the co-encapsulation of these two bioactive compounds has not been widely explored, yet they deserve all the attention since vitD_3 has been highly supplemented and fortified in food products (especially PB foods) and curcumin has numerous beneficial activities for health. Therefore, the emulsifying properties of two PB proteins, pea and potato to formulate the NE have been compared and once the optimum formulation was chosen, the bioactive compounds were co-encapsulated. NE were prepared at the proteins' optimal solubility pH (7 for pea, 3 for potato) to ensure a fair comparison. Then, the NE were characterized in terms of *z*-average diameter, polydispersity index (PDI), surface charge (ζ-potential), and encapsulation efficiency (EE), and submitted to a simulated *in vitro* digestion to evaluate which NE confers higher stability and bioaccessibility to the compounds. In this work, we take a closer look at the advances in nanoencapsulation techniques for bioactive compounds, with a specific focus on the co-encapsulation of vitD_3 and curcumin. The use of PB ingredients for nanoencapsulation is explored, which not only aligns with the growing trend of PB diets but also highlights the potential benefits and applications of these technologies in nutrition and health.

2. Materials and methods

2.1. Materials

Curcumin from *Curcuma longa* (Turmeric, purity of ≥ 65%), pepsin from porcine gastric mucosa (≥2500 U mg^{-1}, P7012), bile extract porcine (B3883), pancreatin from porcine pancreas (8X USP, P7545), Pefabloc® SC, salts used to prepare oral, gastric and intestinal electrolyte solutions, Nile red and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA). VitD_3 (purity of ≥98.5%) was purchased from Alfa Aesar by Thermo Scientific Chemicals (Massachusetts, USA). Corn Oil (Fula) was purchased from local supermarket. Potato protein isolate (Solanic 300, PP-300, containing *ca.* >90% of protein on dry mass) was kindly donated by Royal Avebe, U.A. (GK Veendam, Netherlands). Pea protein (Pisane® M9, containing *ca.* 86 ± 2% of protein on dry mass) was kindly supplied by Cosucra (COSUCRA Groupe Warcoing S.A., Pecq, Belgium). Acetonitrile (≥99.8%) and chloroform (≥99.5%) were obtained from Fisher Scientific (NJ, USA).

2.2. Plant-based nanoemulsions production

2.2.1. Protein solutions preparation. Pea protein and potato protein solutions were prepared according to ref. 25 with slight modifications. Proteins were dissolved in sodium phosphate buffer (10 mM) adjusted to pH 7 for pea protein and to pH 3 for potato protein and kept under stirring for 2 h. These pH values were chosen based on the solubility characteristics of the two proteins.[12,26] The protein solutions were kept overnight at 4 °C to ensure complete hydration. The protein solutions were then centrifuged at 15.000 g at 4 °C for 15 min to remove any insoluble particles remaining (Allegra 64R, Beckman Coulter Inc., USA).

2.2.2. Protein nanoemulsions preparation. NE were prepared according to ref. 10 with some modifications. The lipid phase (corn oil) was added to the aqueous phase (pea and potato proteins) and mixed in an Ultra-Turrax homogenizer (T18, Ika-Werke, Germany) at 15.000 rpm during 8 min. The NE solution was stored at 4 °C under dark conditions.

2.2.3. Experimental design (DOE). A central composite design (CCD) was used to select the formulation of pea and potato proteins NE. Therefore, it was established that an optimal formulation would be a small size and low polydispersity NE. The CCD was composed of two independent variables and a central point with three replicates, for a total of 7 experiments per design. The independent variables selected were oil concentration (X1) and protein concentration (X2). The

Table 1 Oil and protein concentration range correspondent to the different levels (−1, 0, 1) for pea and potato protein NE

Levels	−1	0	1
Oil concentration (%)	1	3	5
Protein concentration (%)	1	5.50	10

range for the different levels (−1, 0, 1) for pea and potato protein NE are shown in Table 1. The potato protein concentration was defined based on preliminary studies, and the pea protein concentration was according to their maximum solubility. Preliminary experiments indicated that ζ-potential did not vary significantly with changes in oil or protein concentration, therefore, only *z*-average diameter and PDI were selected as dependent variables for the CCD. Our aim with the DOE was to formulate particles with the smallest size and subsequently incorporate the maximum amount of compounds, considering their solubility limits and detection by UHPLC. Therefore, we considered that focusing on the *z*-average diameter and PDI would be the most appropriate way to optimize colloidal stability as part of our study. To validate the regression model of the CCD, an independent experiment was conducted with a non-tested combination of the independent variables, but still within the studied limits. This validation was performed with 2% of oil and 5% of protein for pea protein NE and 1.5% of oil and 9% of protein for potato protein NE.

2.3. Co-encapsulation of curcumin and vitD$_3$

The lipid phase consisted of 1% (w v^{-1}) corn oil, in which 0.1% (w/w) was first dissolved at 80 °C under magnetic stirring. Once filly dissolved, the temperature was reduced to 50 °C to dissolve 0.02% vitD$_3$ (w/w). After solubilization of both compounds, the lipid phase was stored in the dark at 4 °C. The aqueous phase consisted in 10% (w v^{-1}) protein solutions (pea or potato, selected from the DOE). For NE preparation, the lipid phase was added to the aqueous phase and homogenized using an Ultra-Turrax (T18, Ika-Werke, Germany) at 15.000 rpm during 8 min. The resulting NE were stored at 4 °C in the dark. Curcumin concentration was chosen based on previous studies,[27] while the vitD$_3$ concentration was selected according to its maximum solubility in the corn oil and according to the concentration required to allow quantification by UHPLC after digestion.

2.4. Nanoemulsions characterization

The *z*-average diameter and PDI of the NE were determined using a dynamic light scattering (DLS) instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd, Malvern Hills, UK). All samples were diluted (1 : 10) with a buffer solution of the same pH of the samples and analyzed in a disposable sizing cuvette. The ζ-potential was determined by electrophoresis (Zetasizer Nano-ZS, Malvern Instruments Ltd, Malvern Hills, UK.) using a folded capillary.

2.5. Curcumin and vitamin D$_3$ quantification

2.5.1. Curcumin extraction. Curcumin was extracted according to the procedure described by[10] with some modifications. Samples were mixed with chloroform and centrifuged at 2000 rpm at room temperature for 10 min. The extraction was repeated two times, and the obtained supernatant layers mixed and dried under nitrogen. The dried samples were resuspended in acetonitrile, filtered through a 0.22 μm filter, and analyzed in UHPLC as described in Section 2.5.3.

2.5.2. Vitamin D$_3$ extraction. VitD$_3$ was extracted according to the procedure described by[28] with some modifications. Samples were mixed with hexane:ethanol (1 : 1, v/v) and centrifuged at 4000 rpm at room temperature for 2 min. The extraction was repeated three times, and the obtained supernatant layers were mixed and dried under nitrogen. The dried samples were resuspended in acetonitrile, filtered through a 0.22 μm filter, and analyzed in UHPLC as described in Section 2.5.3.

2.5.3. UHPLC analysis. The quantification of curcumin and vitD$_3$ was conducted on a UHPLC system Shimadzu Nexera 40 Series (Japan) with a photo diode array and fluorescence detector (RF-20Axs). Curcumin was isocratically separated at 30 °C using the column YMC-pack ODS-AQ 5 μm (250 × 4.6 mm) and 2% acetic acid in H_2O:acetonitrile (47 : 53, v/v) pumped at a constant flow rate of 1 mL min^{-1}. Its detection was done at λ_{ex} = 420 nm and λ_{em} = 540 nm. The injection volume was 10 μL. A 6-point calibration curve was prepared at concentrations between 0.1 to 8 μg mL^{-1} (R^2 = 0.999). Retention times (RT) of bis-demethoxycurcumin (d-BMC), demethoxycurcumin (BMC) and curcumin were 11.6, 12.9 and 14.3 min, respectively. For vitD$_3$ separation was done at 40 °C using a Synergy 2.5 μm Hydro-RP 100 A (100 × 3 mm) column and acetonitrile : water (95 : 5, v/v) pumped at a flow rate of 0.8 mL min^{-1}. Detection was done at 265 nm. A 9-point calibration curve at concentrations between 0.05 to 50 μg mL^{-1} was prepared (R^2 = 0.999). RT of vitD$_3$ was 7.7 min. The injection volume was 10 μL. Compounds were quantified by comparing the peak areas with the respective calibration curves.

2.6. Encapsulation efficiency

The EE was determined according to the method described by[29] with some modifications, following eqn (1). The total amount of curcumin and vitD$_3$ were extracted from 500 μL of NE using the extraction procedure described on Section 2.5.1 and 2.5.2, respectively. The unloaded amount of curcumin and vitamin D$_3$ were extracted from the filtrated portion of 500 μL of NE, obtained after centrifugation for 10 min at 7000 rpm, using a 10 kDa Amicon® Ultra Centrifugal Filter (Millipore Corp., Ireland).

$$\mathrm{EE}(\%) = \frac{(\text{Total amount} - \text{Unloaded amount})}{\text{Total amount}} \times 100 \quad (1)$$

2.7. *In vitro* digestion

NE were subjected to an *in vitro* digestion procedure using the harmonized static *in vitro* digestion model described by.[30] In the oral phase, simulated salivary fluid (SSF) (KCl 15.1 mM, KH_2PO_4 3.7 mM, $NaHCO_3$ 13.6 mM, $MgCl_2(H_2O)_6$ 0.15 mM, $(NH_4)_2CO_3$ 0.06 mM and HCl 1.1 mM), $CaCl_2(H_2O)_2$ 0.3 M (in order to

achieve 0.75 mM at the final mixture) and Milli-Q water (in order to make up the final volume) were added to 5 mL of each sample. The samples were incubated at 37 °C under orbital agitation at 120 rpm for 2 min. α-Amylase was not used as samples did not contain starch.[31] In gastric phase, simulated gastric fluid (SGF) (KCl 6.9 mM, KH_2PO_4 0.9 mM, $NaHCO_3$ 25 mM, NaCl 47.2 mM, $MgCl_2(H_2O)_6$ 0.12 mM, $(NH_4)_2CO_3$ 0.5 mM and HCl 15.6 mM), $CaCl_2(H_2O)_2$ 0.3 M (in order to achieve 0.075 mM at the final mixture) and pepsin solution (with final activity of 2.000 U mL^{-1} in the final mixture) were added to the previous mixture. The pH was adjusted to 3.0 with HCl 1 M and Milli-Q water was added to make up the final volume. The samples were incubated at 37 °C under orbital agitation at 120 rpm for 2 h. Finally, the intestinal phase consisted of simulated intestinal fluid (SIF) (KCl 6.8 mM, KH_2PO_4 0.8 mM, $NaHCO_3$ 85 mM, NaCl 38.4 mM, $MgCl_2(H_2O)_6$ 0.33 mM and HCl 8.4 mM), $CaCl_2(H_2O)_2$ 0.3 M (in order to achieve 0.3 mM at the final mixture), bile salts (in order to achieve 10 mM at the final mixture) and pancreatin solution (with final activity of 100 U mL^{-1} in the final mixture). The pH was adjusted to 7.0 with NaOH 1 M or HCl 1 M and then Milli-Q water was added to achieve the final volume. The samples were incubated at 37 °C under orbital agitation at 120 rpm for 2 h. After each phase, samples were collected and placed in an ice bath for further particle's size and microscopy analysis.

2.8. Nanoemulsion characterization during *in vitro* digestion

The *z*-average diameter, PDI, and ζ-potential were determined at each digestion phase as described in topic 2.6., with some modifications. Before each measurement, all samples were diluted at 1 : 100 with a buffer solution of the same pH of the samples. The microstructure of the initial NE and after each digestion phase (oral, gastric, and intestinal) were observed using a fluorescence microscope (Olympus, BX51, Tokyo, Japan), according to other authors.[10] Samples were stained with Nile red solution (0.25 mg mL^{-1} DMSO) at a ratio of 1 : 10 (dye: sample, v/v) to visualize the oil droplets. The images were captured with a 100*x* oil immersion objective lens.

2.9. Bioaccessibility and stability

Bioaccessibility and stability have been determined at the end of the digestion based on the methodology described by.[10] At the end of the *in vitro* digestion, samples were collected and centrifuged (Allegra 64R, Beckman Coulter Inc., USA) at 18 500 g at 4 °C for 30 min to obtain the micellar phase. Curcumin and $vitD_3$ were extracted from digesta samples or micellar samples as described in Sections 2.6.1. and 2.6.2, respectively. After UHPLC quantification (Section 2.6.3), the bioaccessibility and stability were determined through the following equations (eqn (2) and (3)):[32]

$$\text{Bioaccessibility}(\%) = \frac{C_{\text{Micelle}}}{C_{\text{Digesta}}} \times 100 \quad (2)$$

$$\text{Stability}(\%) = \frac{C_{\text{Digesta}}}{C_{\text{Initial}}} \times 100 \quad (3)$$

where C_{Micelle} and C_{Digesta} are the curcumin and $vitD_3$ concentrations measured at the end of the digestion in micellar phase and raw digesta, respectively. C_{Initial} is the curcumin and $vitD_3$ concentration present in the NE at the beginning of digestion process.

2.10. Statistical analysis

The experimental designs' conditions were obtained using Protimiza Experimental Design web application. Statistically significant coefficients ($p \leq 0.05$) were considered for parametrization of regression models and respective curves.

The remaining data obtained in this work was analysed using Prism version 8.0.2 (GraphPad Software Inc., California USA). The statistical significance was determined by two-way ANOVA followed by post-hoc Tukey's honestly significant difference (HSD) test. Statistically significant differences were considered when *p*-value < 0.05. Results are presented as mean values of experiments performed at least in triplicate ± standard deviation (SD).

3. Results and discussion

3.1. DOE and formulation selection

The DOE was applied with the aim of formulating a plant-based protein NE with reduced *z*-average diameter and PDI. All experiments data results can be found in Fig. 1 and Tables 1 and 2 of the SI.

The regression models were able to modulate both the *z*-average diameter and PDI of pea and potato protein NE with a high determination coefficient. The NE formulations that presented the smallest *z*-average diameter and PDI (228.35 ± 9.49 nm and 0.25 ± 0.01 for pea protein and 271.67 ± 12.04 nm and 0.37 ± 0.01 for potato protein, respectively) were produced with the lowest oil concentration (1%) and the highest protein concentration (10%). It was also possible to observe that both oil concentration and protein concentration play a significant role in modulating the *z*-average diameter and PDI of NE. In general, higher oil concentrations resulted in larger particles, whereas higher protein concentrations provided more emulsifier to stabilize the oil droplets, resulting in smaller particles.

Model validation was performed with additional experiment (*i.e.*, pea protein X1 = −0.5; X2 = 0.46; potato protein X1 = −0.75; X2 = 0.78). Under these conditions, it is expected to obtain a *z*-average diameter and PDI of 314.10 ± 84.75 nm and 0.31 ± 0.07 for the pea protein NE and 301.16 ± 27.99 nm and 0.40 ± 0.02 for the potato protein NE, respectively. Experimentally, the results obtained were 322.40 ± 15.10 nm and 0.28 ± 0.01 for pea protein NE and 289.9 ± 18.2 nm and 0.4 ± 0.0 for potato protein NE, confirming the accuracy of the regression model.

The results of this study are consistent with previous studies, who also reported that lower oil concentrations and higher protein concentrations resulted in smaller particle sizes.[12] Also,[26] prepared NE with potato protein (PP-300) and observed that increasing the concentration of potato protein resulted in

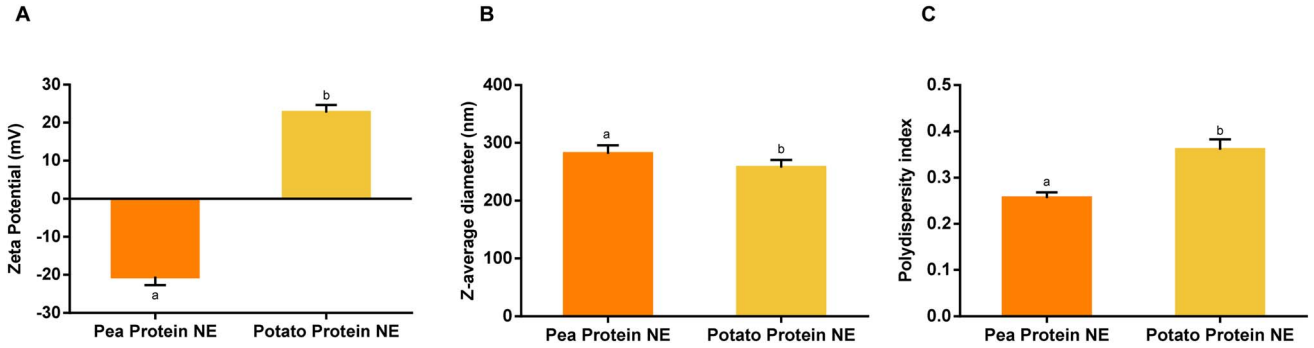

Fig. 1 Zeta potential (A), *z*-average diameter (B), and polydispersity index (C) of the pea and potato nanoemulsions. The results are presented as the mean ± SD. Different letters (a and b) represent statistically significant differences ($p < 0.05$) between samples. Samples were diluted (1 : 10) with sodium phosphate buffer (pH 3 for pea protein NE; pH 7 for potato protein NE) before *z*-average diameter and polydispersity index measurement.

a decrease in the particle size of the NE. Although the DOE did not allow to define an optimized formulation for each protein, clearly smaller particles were obtained with higher protein concentration (10%) and lower oil concentration (1%). Therefore, these conditions were selected for the co-encapsulation of bioactive compounds and subsequent evaluations.

3.2. Nanoemulsions' characterization

The *z*-average diameter, PDI, and ζ-potential of both NE co-encapsulating curcumin and vitD$_3$ are presented in Fig. 1. Pea protein NE presented higher ($p < 0.05$) *z*-average diameter (280.94 ± 14.98 nm) than potato protein NE (257.27 ± 12.91 nm) (Fig. 1B). Our particle size results are consistent with other authors who have also prepared NE with pea[7] and potato proteins.[26] The higher average *z*-diameter obtained for NE pea protein compared to those prepared with potato protein could be due to a variety of factors, including differences in molecular weight.

The polydispersity index (PDI) reflects the size distribution of particles within the sample, with value between 0.2 and 0.5 typically considered acceptable for stable droplet size in a NE.[33] Pea protein NE showed a significantly lower PDI value ($p < 0.05$) (0.26 ± 0.01) than potato protein NE (0.36 ± 0.02) (Fig. 1C). Although the potato protein NE presented with a smaller *z*-average diameter, their higher PDI value indicates a broader particle size distribution compared to pea protein NE. The PDI values observed in this study may be related to the preparation method. Ultra-turrax, despite being a cost-effective, practical, and easy-to-use tool for particles production, generally produces more heterogenous droplets than high-pressure homogenization.[34] Homogenizers are known to produce more uniform particles due to higher energy efficiency, although their cost and technical complexity may limit accessibility.

ζ-Potential measures the surface charge of the particles that can be positive or negative. NE are typically considered more stable when their ζ-potential value is around ±30 mV, because repulsion between the particles prevents their aggregation.[33] As expected, pea protein NE had a negative surface charge (−20.49 ± 2.26 mV) as it was prepared at a pH above its isoelectric point (pI) (around 4.3),[35] while potato protein NE had a positive surface charge (+22.67 ± 1.98 mV) since it was prepared at pH below its pI[8] (Fig. 1A).[26] The negative charge of the NE pea protein arises from the deprotonation of the carboxyl (–COOH) groups present in the acidic amino acid residues (glutamic or aspartic), while the positive charge of the NE potato protein results from the protonation of the amino groups in the basic amino acid residues (lysine, arginine, histidine).[12,26]

3.3. Encapsulation efficiency

Curcumin and vitD$_3$ were successfully co-encapsulated in both plant-based protein NE (Fig. 2). The EE of curcumin in pea protein NE and potato protein NE were 99.70 ± 0.21% and 98.00 ± 1.48%, respectively. Several studies have also reported high EE for curcumin, such as an EE of 90.56 ± 0.47% in NE prepared using whey protein and Tween-80 (Sari *et al.*, 2015)(Sari *et al.*, 2015); EE of 94.31 ± 0.77% in NE[36];[37] prepared NE stabilized by lecithin, tween 20 and sucrose monopalmitate and obtained EE over 75%; and[38] developed NE stabilized with Tween 20 and reported an EE between 64.30% to 93.60%.

The encapsulation efficiency of vitD$_3$ in pea protein NE and in potato protein NE were 100.00 ± 0.0% and 99.01 ± 1.71%, respectively. Several works also reported high EE of vitD$_3$ in different lipid-based nanosystems. For instance,[12] reported an EE of vitD$_3$ between 94.8 and 95.3% in pea protein NE;[7]

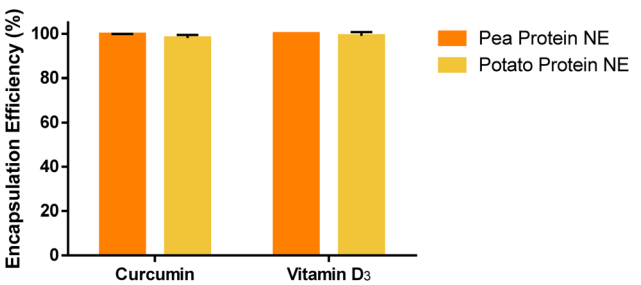

Fig. 2 Encapsulation efficiency (%) of curcumin and vitamin D$_3$ of pea and potato protein nanoemulsions. The results are presented as the mean ± SD.

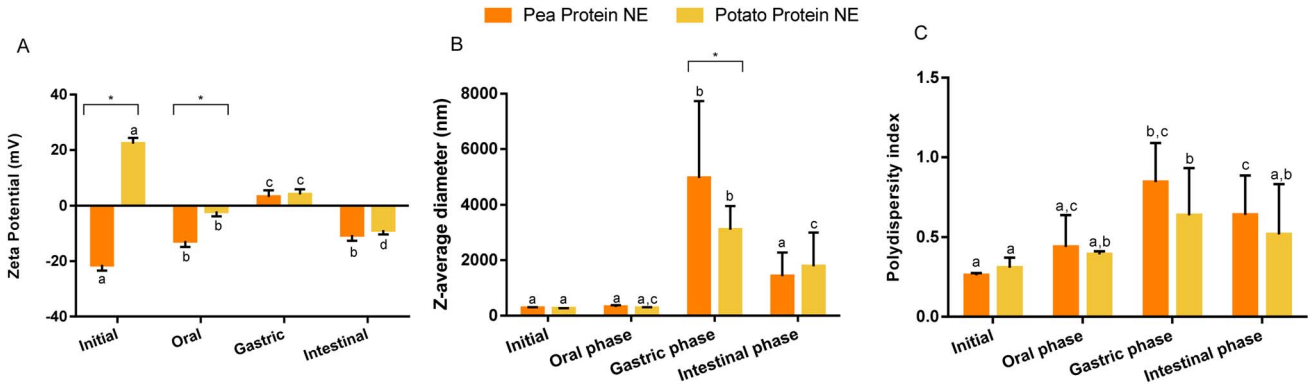

Fig. 3 Zeta potential (A), *z*-average diameter (B), and polydispersity index (C) of nanoemulsions in different *in vitro* gastrointestinal digestion phases. The results are presented as the mean ± SD. Different letters (a–d) represent statistically significant differences ($p < 0.05$) between the same sample in different digestion phases, whereas * represent statistically significant differences ($p < 0.05$) between samples on the same digestion phase.

obtained an EE of 89.3 ± 0.7% in pea protein-Tween 80 NE;[9] prepared NE stabilized by Tween 80 and Span 80 and observe an EE of 99.9%; and[39] observed an EE of 92.18% ± 4.027 in NE prepared with vegetable oils and Tween 80.

The EE obtained were higher than those reported in a previous study by,[40] which also co-encapsulated vitD$_3$ and curcumin in nanoliposomes and obtained encapsulation efficiencies between 70 ± 5% and 84 ± 5% for curcumin, and between 45 ± 2% and 74 ± 9% for vitD$_3$. The authors also characterized nanoliposomes encapsulated only with curcumin and observed that their EE were higher (77 ± 4%–95 ± 2%) compared to nanoliposomes co-encapsulated with both compounds. Although we did not evaluate NE encapsulated with curcumin and vitD$_3$ individually, we obtained EE values very close to 100% for both compounds, even when encapsulated simultaneously and at the solubility limit. This demonstrates the high efficiency of pea and potato proteins NE in co-encapsulating curcumin and vitD$_3$.

3.4. *In vitro* digestion

3.4.1. Influence of digestion on particle size and charge. Fig. 3A–C shows the *z*-average diameter, PDI, and ζ-potential, respectively, of the pea and potato protein NE before digestion (initial) and after each phase of the simulated *in vitro* digestion process (oral, gastric, and intestinal).

Before digestion, the pea and potato NE proteins had a spherical morphology (Fig. 4) and, during simulated digestion, a significant increase in the particle size of the NE was observed (Fig. 3B, C and 4). During simulated digestion, pea and potato protein NE exhibited changes in ζ-potential, particle size, and PDI, reflecting the combined effects of pH, enzymatic hydrolysis, and ionic interactions. In the oral phase, pea protein NE maintained a negative charge, since this phase occurs at the same pH[7] at which they were prepared, although ζ-potential significantly decreased ($p < 0.05$) from −21.27 ± 2.19 mV to −12.71 ± 0.21 mV, maybe due to the electrostatic screening effects of lipid particles and salt ions present in the SSF.[41] On the other hand, potato protein NE shifted from positive (22.32 ± 2.07 mV) to negative (−2.03 ± 1.77 mV), as this pH is near their isoelectric point. Under gastric conditions, ζ-potential of pea and potato protein NE became slightly positive, 3.10 ± 3.34 mV and 4.00 ± 1.84 mV, respectively, reflecting a reduction in electrostatic repulsion between droplets.[10] These decreases as well as protein hydrolysis mediated by pepsin may have facilitated aggregation (clearly visible in Fig. 4) as demonstrated by the increase in *z*-average diameter.[42] The pea NE increased from 317.47 ± 67.90 nm to 4960.73 ± 2779.03 nm, and the potato protein NE increased from 278.90 ± 22.89 nm to 3100.60 ± 855.95 nm. PDI of pea and potato protein also increased in the gastric phase, from 0.44 ± 0.20 to 0.84 ± 0.25 and 0.31 ± 0.06 to 0.64 ± 0.29, respectively. The higher PDI of pea NE compared to potato NE is consistent with fluorescence microscopy images (Fig. 4), which clearly show greater aggregation of pea NE droplets compared to potato protein NE. In the intestinal phase, ζ-potential shifted back to negative values (−10.57 ± 2.07 mV for pea protein NE and −8.78 ± 1.56 mV for potato protein NE) possibility due to the combined effects of adsorption of anionic substances (*i.e.*, bile salts present in the SIF), fatty acids production by lipid digestion, and pancreatin hydrolysis.[43] These interactions enhanced electrostatic and steric stabilization, preventing further aggregation and leading to a decrease in particle size (pea protein NE decrease to 1428.96 ± 855.65 nm and potato protein NE decrease to 1783.75 ± 1222.23 nm). Nevertheless, particle size remained larger than in the initial phase. Overall, the interplay of pH, enzymatic activity, and bile salt interactions explains the observed trends in NE stability, PDI, and aggregation, corroborated by both particle size analysis and fluorescence microscopy.

3.4.2. Stability and bioaccessibility. Bioaccessibility is defined as the fraction of the ingested bioactive compound that is released from the NE and solubilized in the micellar phase (after centrifugation of the resulting sample), whereas stability is defined as the fraction of non-transformed bioactive compound present in the entire digesta at the end of digestion.[44]

Fig. 5 shows the curcumin and vitD$_3$ bioaccessibility and stability measured at the end of the *in vitro* digestion. The curcumin's bioaccessibility was higher ($p < 0.05$) in pea protein

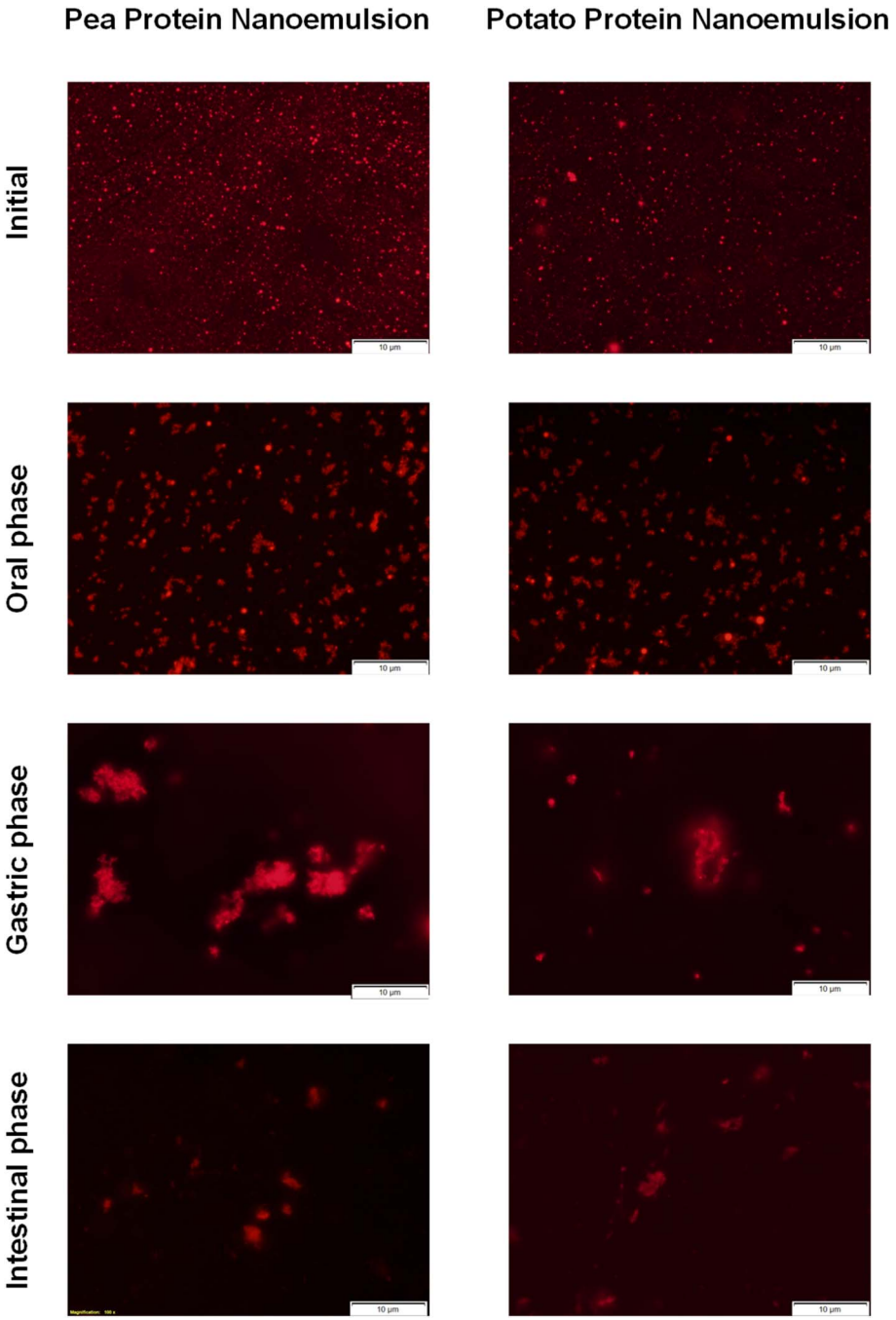

Fig. 4 Fluorescence microscopy images of nanoemulsions before and after each *in vitro* gastrointestinal digestion phase. The scale bar for all images is 10 µm.

NE (76.06 ± 12.05%) than in potato protein NE (42.88 ± 3.58%). In contrast, vitD$_3$ bioaccessibility did not show significant differences between pea protein NE (77.39 ± 14.19%) and potato protein NE (58.57 ± 11.26%). Our results are in line with previous studies, for instance,[10] encapsulated curcumin in NE and obtained a bioaccessibility of 71.7 ± 6.18%, and[45] developed pea protein NE for vitD$_3$ encapsulation and obtained a bioaccessibility of 62.9 ± 11.1%.

Curcumin's stability was also higher ($p < 0.05$) in pea protein NE (21.44 ± 1.97%) compared to potato protein NE (12.38 ± 1.97%), whereas vitD$_3$ stability was lower (6.14 ± 0.70% for pea protein NE and 5.35 ± 0.83% for potato protein NE), indicating that vitD$_3$ is more susceptible to degradation under digestive conditions, particularly acidic pH in the gastric phase.

Bioaccessibility was higher than stability at the end of digestion, which means that although the compounds were effectively released and ready for absorption, they were simultaneously exposed to conditions causing partial degradation. There are many factors that affect the bioaccessibility and stability of bioactive compounds, such as the composition and physical state of the lipid phase, particles size and charge, and the type of emulsifier.[46] Gonçalves 2021b evaluated the effects of

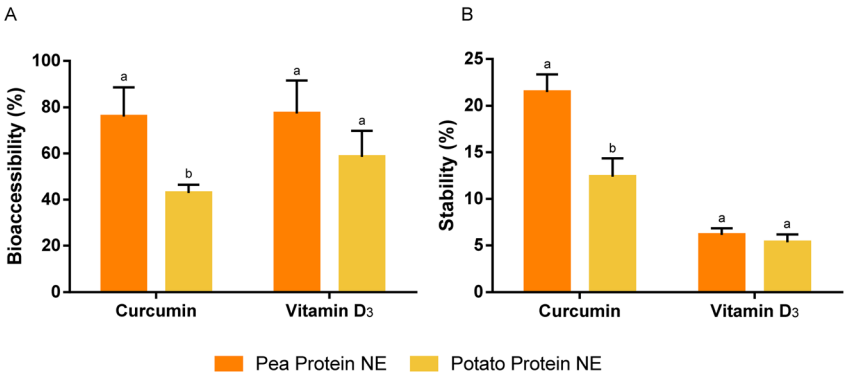

Fig. 5 Bioaccessibility (A) and stability (B) of curcumin and vitamin D_3 after *in vitro* digestion of nanoemulsions. The results are presented as the mean ± SD. Different letters (a and b) represent statistically significant differences ($p < 0.05$) between the same bioactive compound in different nanoemulsions.

different emulsifiers in NE encapsulating curcumin during *in vitro* digestion. The authors showed that the curcumin's stability was 27.6 ± 3.17%, 26.5 ± 1.67%, and 8.2 ± 0.66% for NE developed with lecithin, rhamnolipids, and Tween® 80, respectively.

Destabilization of NE during digestion, especially aggregation in the gastric phase,may have caused premature release of encapsulated compounds, exposing them to pH, ionic strength, and enzymatic degradation. In the intestinal phase, bile salts and fatty acids generated during lipid digestion may have contributed to the solubilization of the compounds, enhancing their incorporation into mixed micelles and improving bioaccessibility.

Overall, the pea protein NE showed superior bioaccessibility and stability for both encapsulated compounds. This may be partly due to the presence of a protein protease inhibitor (PP-300), which could have interfered with the action of digestive enzymes (*i.e.*, pepsin and pancreatin) during the digestion process. As a result, bioactive compounds may have been less accessible because they were trapped in the undigested protein, reducing their bioavailability. On the other hand, pea protein, being more stable at pH7 (intestinal phase conditions) than potato protein, likely provided better protection to the bioactive compound at the end of the digestion process by preventing early degradation.

These findings highlight the potential of pea protein NE as effective delivery systems for curcumin and vitD_3, supporting their application in food fortification to enhance nutritional value.

3. Conclusions

The DOE results showed that both the oil and protein concentrations have a significant effect on the *z*-average diameter and PDI of the pea and potato protein NE. The formulations selected to co-encapsulate the compounds contained the highest concentration of protein and the lowest concentration of corn oil. Curcumin and vitD_3 were successfully co-encapsulated in both proteins NE, in which the potato protein had the smallest *z*-average diameter. The pea and potato NE protein had a spherical morphology prior to digestion, but during simulated digestion, several changes occurred in the *z*-average diameter, PDI, and zeta potential of the NE, which can be attributed to various changes in pH, ionic strength conditions, as well as aggregation due to the action of digestive enzymes (particularly in the gastric phase). Pea protein NE showed higher bioaccessibility and stability to bioactive compounds co-encapsulated at the end of digestion compared to potato protein NE. Therefore, pea protein seems to be the most suitable plant-based ingredient to emulsify NE for enhancing the delivery of curcumin and vitD_3. Therefore, pea protein NE co-encapsulating curcumin and vitD_3 can be used to develop novel functional foods, especially plant-based foods, since there is an increasing demand for these products.

Author contributions

Márcia Marques: conceptualization, methodology, data curation, writing – original draft. Raquel F.S. Gonçalves: methodology, writing – review & editing. Daniel A. Madalena: methodology, writing – review & editing. Luís Abrunhosa: methodology, writing – review & editing. António A. Vicente: funding acquisition, supervision, writing – review & editing. Ana C. Pinheiro: conceptualization, funding acquisition, supervision, writing – review & editing, validation.

Conflicts of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

The data supporting this manuscript are available in this article and in the supplementary information (SI). Additional information can be made available from the corresponding author upon reasonable request. Supplementary information is available. See DOI: **https://doi.org/10.1039/d5fb00262a**.

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