



Development of Chitosan-Stabilized Pickering Emulsions Loaded with Supercritical Fluid Extraction of Honey Bee Pollen for Functional Food Applications

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ABSTRACT

Honey bee pollen (HBP) is a nutritionally rich natural product containing phenolics and antioxidants; however, its rigid outer wall limits the bioavailability of these bioactive compounds. Therefore advanced extraction and delivery systems are required to enhance stability and functional performance for food applications. This study aimed to encapsulate a supercritical fluid extract (SFE) of honey bee pollen within chitosan-stabilized Pickering emulsions in order to improve encapsulation efficiency and antioxidant activity. Honey bee pollen obtained from Bingöl, Türkiye, was extracted with supercritical CO₂. Pickering emulsions were prepared via ultrasound-assisted emulsification in the presence of chitosan as a stabilizer, with SFE-HBP at 5%, 10%, and 15% (w/v). The prepared emulsions were characterized for their microstructure, particle size, zeta potential, and encapsulation efficiency. Total phenolic content (TPC) and antioxidant activities were assessed using the Folin-Ciocalteu and CUPRAC assays, respectively. All formulations yielded stable, uniformly distributed Pickering emulsions with spherical droplets and no visible phase separation. Indeed, encapsulation efficiency increased with increasing SFE-HBP concentration, from 73.96% to 87.06%. The particle size ranges (7.22–12.44 µm) and zeta potential values (+21.73 to +28.24 mV) indicate good colloidal stability. For both TPC and CUPRAC antioxidant activities, a concentration-dependent increase was observed, with the highest values in the 15% SFE-HBP-containing emulsions. Collectively, these findings indicate that a chitosan-based Pickering emulsion is an effective delivery system for SFE-derived bioactive compounds from honey bee pollen. The method provides enhanced encapsulation efficiency, antioxidant capacity, and physicochemical stability, hence exhibiting great potential for functional food and nutraceutical applications.

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Introduction

Honey bees collect pollen from a wide variety of flowers, which they then mix with nectar and enzymes (like amylase and catalase) from their salivary glands to create a pollen load (Campos et al., 2008; Denisow & Denisow-Pietrzyk, 2016). Honey bee pollen's composition is influenced by its geographical origin (Gardana et al., 2018), pollen diversity (Malagnini et al., 2022), and bee species (Köppler et al., 2007). Approximately 250 chemical compounds have been reported for honey bee pollen derived from various plant species (Komosinska-Vashev et al., 2015). The main components of honey bee pollen include proteins, amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, tryptophan, aspartic acid, serine, glutamic acid, proline, glycine, alanine, cystine, tyrosine, histidine, arginine),

lipids, fatty acids, carbohydrates (fructose, glucose, sucrose, maltose, melezitose, raffinose), vitamins, phenolics, minerals and volatile compounds (Ecem Bayram, 2021; Prđun et al., 2021; Sagona et al., 2017; Yang et al., 2013).

Honey bee pollen is recognized as a superfood due to its nutritional content, which can have health benefits (Alshallash et al., 2023; Baky et al., 2023; Xue et al., 2012). However, due to its outer wall layer, which is resistant to digestion by digestive enzymes and highly stable, the bioavailability of the bioactive compounds in its structure is limited (Zuluaga et al., 2015). Extraction methods significantly influence the bioactivity of honey bee pollen. Extraction parameters, including the solvent used, extraction time, temperature, and solid-to-liquid ratio, influence the bioactivity of honey bee pollen (Kim et al., 2015). Supercritical fluid technology is

promising for disrupting the two distinct layers of the bee pollen wall to release active ingredients (Erol, 2024; Xu et al., 2009). Additionally, incorporating bioactive compounds directly into food products poses several challenges due to the instability and low bioavailability of these compounds, as well as their high susceptibility to oxidation and sensitivity to heat and light, which limits their use in the food industry (Arserim-Uçar, 2020; Aguiar et al., 2016; de Souza Simões et al., 2017).

Encapsulation techniques can successfully address these challenges because encapsulation technologies provide notable benefits in stabilizing and enhancing the bioavailability of active compounds, including antioxidants, antimicrobials, and enzymes. This results in prolonged shelf life and enhances the delivery efficiency throughout a range of applications (Arserim-Uçar, 2024; Arserim-Uçar & Çabuk, 2020; Xu et al., 2024).

The methanolic extract of bee pollen, when combined with apple cider and encapsulated in chitosan particles, produced materials that exhibited antimicrobial and antioxidant activities (Baysal et al., 2022). Another study optimized spray-drying parameters to encapsulate bee pollen within milk powder, producing a functional milk powder (Thakur et al., 2020). The ethanolic extract of date palm pollen (*Phoenix dactylifera* L.) was encapsulated in sodium caseinate to produce yogurt fortified with pollen (El-kholy et al., 2019).

Chitosan is a cationic polymer produced by deacetylating chitin and is especially abundant in the exoskeletons of crustaceans, insects, and other arthropods, as well as molluscan shells. The manufacture of chitosan from shrimp shell waste commonly involves deacetylation at two distinct temperatures, namely 40 °C and 90 °C (Iber et al., 2022; Klinger et al., 2019). Chitosan is a valuable carrier for encapsulating and controlling the release of bioactive compounds because it is biodegradable, biocompatible, non-toxic, and generally recognized as safe (GRAS) (Aranaz et al., 2021).

Chitosan is used as a carrier for the encapsulating agent in different industrial applications, including the delivery of curcumin and β -caryophyllene (Hemmingsen et al., 2025), volatilized citronella oil (Hsieh et al., 2006), lime essential oil (Sotelo-Boyás et al., 2017), cuminum cyminum essential oils (Zhaveh et al., 2015), and mentha piperita essential oils (Beyki et al., 2014).

A simple definition of encapsulation would be the process that traps some substance, called an “active agent”, inside another substance, called “wall material” (Nedovic et al., 2011). Encapsulation is an effective method for delivering active agents, sensitive compounds, and drugs to their target sites (Klojdová et al., 2023). Encapsulation technology facilitates controlled and targeted release, improves physical stability and functionality, and masks off-flavors and odours of encapsulated active agents (Munin & Edwards-Lévy, 2011; Nedovic et al., 2011). Several encapsulation techniques are commonly employed, including extrusion, spray drying, spray cooling, fluidized bed coating, liposomes, co-preservation, ionic gelation, co-crystallization, electrohydrodynamic technologies, and emulsion (Arserim-Uçar, 2024; de Moura et al., 2018; Dewettinck & Huyghebaert, 1999; Hızır-Kadı et al., 2020; Ntoghian et al., 2018; Tackenberg et al., 2015).

Emulsions are heterogeneous with a homogeneous appearance and are thermodynamically unstable systems. Conventional emulsions, defined as dispersions of small spherical droplets of one liquid phase within another immiscible liquid phase, are used in various industrial applications (McClements, 2012). Pickering emulsions are stabilized by solid particles rather than by conventional emulsifiers. In these systems, oil droplets remain stable against destabilization by forming a barrier composed of solid particles smaller than the droplets (Zhang et al., 2023). Pickering emulsions are primarily stabilized using particles derived from polysaccharides and proteins, including cellulose nanocrystals and nanofibers, starch particles, chitosan, and various proteins, including zein, soy, peanut, pea proteins, as well as lignin extracted from food waste (Sarkar & Dickinson, 2020; Sharkawy et al., 2020). Various active agents, such as rutin, caffeine, thymol, and all-trans retinol, have been formulated in Pickering emulsion systems, as documented in the literature (Harman et al., 2019).

The present study aims to encapsulate a supercritical fluid extract (SFE) of honey bee pollen (HBP) using ultrasound-assisted emulsification to produce a Pickering emulsion stabilized by chitosan. The chitosan supercritical fluid extract (SFE) of honey bee pollen Pickering emulsion particles was characterized by analysis of their microstructure, encapsulation efficiency, zeta potential, and particle size. Additionally, the particles' antioxidant properties were evaluated to assess their potential for food applications.

Material and Methods

Materials

Honeybee pollen samples were obtained from Bingöl in Türkiye. Low-molecular-weight chitosan and acetic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Diacetyl tartaric acid ester of mono-diglycerides (DATEM) was kindly provided by DuPont (Denmark).

Supercritical Fluid Extraction (SFE) of Honey Bee Pollen

Honeybee raw pollen samples (Bingöl, Türkiye) were freeze-dried at -80 °C for 48 hours under 0.1 mPa pressure (Telstar, LyoQuest, Spain), then powdered using a laboratory blender. A Superex F-500 supercritical fluid extractor (Türkiye) was used to extract bee pollen at 50 °C and 300 bar, with a CO₂ flow rate of 2 L/min for one hour. After supercritical fluid extraction, supercritical fluid extract (SFE) of honey bee pollen (HBP) was obtained and stored at -20°C (Xu et al., 2009).

Preparation of Supercritical Fluid Extract of Honey Bee Pollen Chitosan Pickering Emulsion

Briefly, a 2.5% (w/w) chitosan solution was prepared by dissolving chitosan in 1% (v/v) acetic acid at room temperature with magnetic stirring overnight. SFE-HBP chitosan Pickering emulsions (SFE-HBP-CPE) were made at 5, 10, and 15 (w/v) % SFE-HBP, labeled as P1, P2, and P3, respectively (Table 1).

Table 1. SFE-HBP Chitosan Pickering Emulsions

Samples	Code
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Pickering emulsion with 5% (w/v) SFE-HBP	P1
Pickering emulsion with 10 % (w/v) SFE-HBP	P2
Pickering emulsion with 15 % (w/v) SFE-HBP	P3

In preparing the Pickering emulsion, 5% (v/v) DATEM was used, and the total ethanol concentration was maintained at 40%. Ultrasonication was performed for 30 seconds at 50% amplitude using a sonicator (BANDELIN electronic GmbH & Co. KG, Berlin, Germany) in each step (Arserim-Uçar, 2024; Baysal et al., 2022).

Size Distribution and Zeta-Potential Measurements of Pickering Emulsions

Accordingly, the particle size distribution and zeta (ζ) potential of the obtained SFE-HBP-CPE were determined following a modified method described by Song et al. (2022) using a Zetasizer Nano instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Optical Microscopy Images of Pickering Emulsions

The morphology of SFE-HBP-CPE was analyzed utilizing optical microscopy (Olympus BX43 SC180, Japan).

Encapsulation Efficiency (EE% %) of Pickering Emulsions

The encapsulation efficiency of the obtained SFE-HBP-CPE was evaluated using a slightly modified method described by Velderrain-Rodríguez et al. (2019). Encapsulation efficiency (EE) is calculated using the following equation (1)

$$EE = \left(\frac{TPE - TPP}{TPE} \right) \times 100 \quad (1)$$

TPE: The total polyphenol content in the honey bee pollen extract (SFE-HBP) dissolved in the aqueous phase solution

TPP: The total polyphenol content of Pickering emulsion formulations

Total Phenolic Content (TPC) of Pickering Emulsions

The total phenolic content (TPC) of the SFE-HBP-CPE samples was assessed using the Folin–Ciocalteu assay (Slinkard & Singleton, 1977). A volume of 50 μ L of the samples was mixed with 250 μ L of 0.2 N Folin–Ciocalteu reagent. Following that, 750 μ L of a 7.5% sodium carbonate solution was added to the mixtures. After two hours of incubation, the sample's absorbance was measured at a wavelength of 760 nm using a UV-VIS spectrophotometer (Shimadzu UV-1800, Japan). The total phenolic content of the samples was expressed as μ g of gallic acid equivalents mL⁻¹ emulsions (GAEs).

CUPRAC Assay of Pickering Emulsions

The antioxidant activity of the SFE-HBP-CPE samples was determined using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method (Apak et al., 2004). After adding 1 mL each of Cu²⁺ solution, Neocuproin solution, NH₄Ac buffer solution (pH = 7), and distilled water, the

solutions were mixed with 0.1 mL of the emulsion sample solution. After 30 min of incubation, absorbance was measured at 450 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). The results were expressed as μ g of Trolox equivalents mL⁻¹ emulsions.

Statistical Analysis

The statistical analysis of this research result was conducted using a one-way analysis of variance (ANOVA) and Tukey's comparison test using Minitab 17. A significance level of $p < 0.05$ was used. All experiments were performed in triplicate and the data were presented with mean $\pm$ standard deviation (n=3).

Results and Discussions

Stability of Chitosan Pickering Emulsions

Figure 1 displays images of Pickering emulsions stabilized by chitosan particles with SFE-HBP samples at concentrations of 5% (P1), 10% (P2), and 15% (P3) w/v. The formulated Pickering emulsions display a uniform appearance, with no visible phase separation, as illustrated in Figure 1.

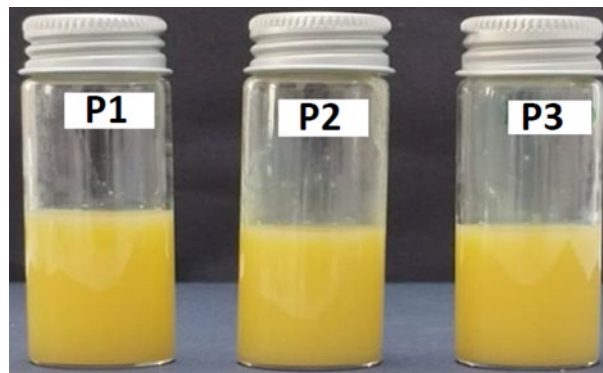


Figure 1. Photographs of chitosan stabilized Pickering emulsions with 5% (P1), 10% (P2) and 15% (P3) w/v SFE-HBP-CPE concentrations

Microstructures of Chitosan Pickering Emulsions

Figure 2 presents optical microscopy images that clearly illustrate the droplet structure of the prepared Pickering emulsions. The droplets were predominantly spherical and uniformly distributed throughout the continuous phase. No visible coalescence occurred, indicating that the chitosan– SFE-HBP particles were effectively adsorbed at the oil–water interface. This adsorption provided steric stabilization to the system. The optical microscopic images of Pickering emulsions confirm the successful development and stability of the Pickering emulsion system.

Determination of Encapsulation Efficiency (EE%)

The encapsulation efficiency of the Pickering emulsion formulations is displayed in Table 2. Encapsulation efficiencies for the Pickering emulsions containing 5% (P1), 10% (P2), and 15% (P3) w/v concentrations of SFE-HBP were recorded as $73.96 \pm 0.33\%$, $83.48 \pm 0.08\%$ and $87.06 \pm 0.06\%$, respectively. Increasing SFE-HBP concentration in Pickering emulsion formulations improved encapsulation efficiency.

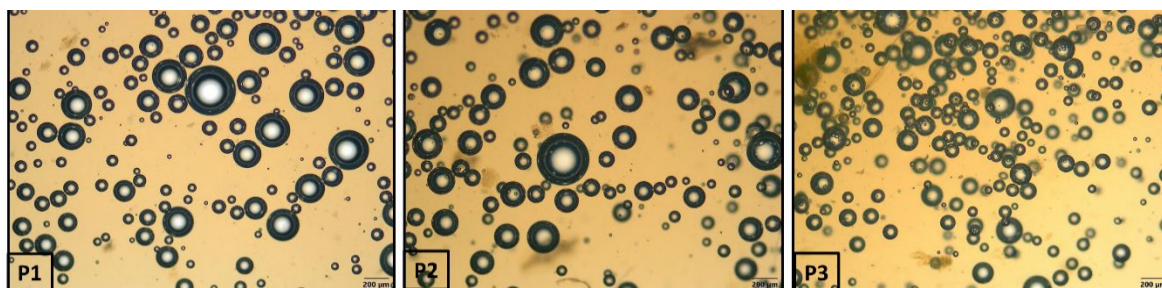


Figure 2. Optical microscopy images of freshly prepared chitosan stabilized Pickering emulsions with 5% (P1), 10% (P2) and 15% (P3) w/v SFE-HBP-CPE concentrations

Table 2. Pickering Emulsions Encapsulation Efficiency

Pickering Emulsion Formulations	Encapsulation Efficiency EE(%)
P1	73.96 ± 0.33 ^c
P2	83.48 ± 0.08 ^b
P3	87.06 ± 0.06 ^a

^{a,b,c} Different letters in the same column show a statistically significant difference ($p < 0.05$). All values are means ± SD, $n=3$.

Table 3. Particle Size and Zeta Potential of Pickering Emulsion Formulations

Pickering Emulsion Formulations	Zeta Potential (mV)	Particle Size Diameter(µm)
P1	21.73 ± 0.46 ^c	10.39 ± 0.29 ^a
P2	25.81 ± 0.64 ^b	12.44 ± 1.33 ^a
P3	28.24 ± 1.12 ^a	7.22 ± 0.38 ^b

^{a,b} Different letters in the same column show a statistically significant difference ($p < 0.05$). All values are means ± SD, $n=3$.

Table 4. CUPRAC Antioxidant Activity and Total Phenolic Content of Pickering Emulsions

Emulsion Formulations	CUPRAC (µg TE/mL Emulsion)	Total Phenol (µg GAE/ mL Emulsion)
P1	132.96 ± 2.18 ^c	59.62 ± 0.76 ^c
P2	164.78 ± 0.86 ^c	75.4 ± 0.66 ^b
P3	202.96 ± 1.73 ^a	88.51 ± 1.01 ^a

^{a,b,c} Different letters in the same column show a statistically significant difference ($p < 0.05$). All values are means ± SD, $n=3$.

In the work of Hızır-Kadı et al. (2020), a pollen extract was used to develop proliposomes (P-liposomes) and conventional liposomes (C-liposomes). P-liposomes and C-liposomes had encapsulation efficiencies of 75 % and 73 %, respectively. In a recent study, the ethanolic extract of bee pollen (BPE) and the thymol oil extract (TOE) were combined with chitosan nanoparticles (CSNPs) to develop a ternary nanoformulation. The EE of CSNPs for the bee pollen extract was reported as 77.2% (Alshehri & Abdella, 2023). Another investigation employed spray-drying to encapsulate bee pollen with maltodextrin, whey protein concentrate (WPC), and combinations thereof. Encapsulation efficiencies were reported as 82.51 ± 1.9% for the combination, 78.2 ± 2.3% for WPC, and 67.84 ± 3.35% for maltodextrin (Maqsoudlou et al., 2020).

Measurement of Zeta potential and Particle Size Distribution

Particle size and Zeta potential of developed Pickering emulsion formulations are given in Table 3. The zeta potential of SFE-HBP-CPE at 5%, 10% and 15% w/v was measured as 21.73 ± 0.46, 25.81 ± 0.64, and 28.24 ± 1.12 mV, respectively. Zeta potential results indicate that the obtained particles are well-stabilized. Bee pollen supercritical CO₂ extract with unsaturated fatty acids such as palmitic, oleic, linoleic, and linolenic acids (Aylanc et al., 2023; Çeksterytė et al., 2023; El Ghouizi et al., 2023), can exhibit surfactant behavior at the oil/water interface (Fameau et al., 2014).

Pickering emulsions are typically characterized in their initial state by dynamic light scattering (DLS) and light microscopy to determine the size, morphology, and aggregation state of emulsion droplets and stabilized particles (Fernandez Ainaga et al., 2025). The droplet diameter obtained via DLS is the average hydrodynamic diameter, which is defined as the actual diameter of the object being measured plus a strongly bonded water layer that moves with the object through the (Ahmad et al., 2014). The developed pickering emulsion formulations have particle sizes varying from 7.22 ± 0.38 to 12.44 ± 1.33 µm. The P2 (10%) emulsion had the largest particle size compared to P1 (5%) and P3 (15%). The increase in particle size observed in the P2 (10%) formulation is attributed to insufficient interfacial coverage by chitosan particles, where oleic, linoleic, and linolenic acids from the pollen SFE extract likely promoted droplet-droplet bridging at the interface, may lead to flocculations and aggregates and increase in hydrodynamic size (Schröder et al., 2018; Tiwari et al., 2024).

Zeta potential values and the particle size of SFE-HBP-CPE increased with SFE-HBP concentration. The zeta potential of the medium-chain triglyceride chitosan Pickering emulsions at pH 6.7 is 24 mV, according to the work of (Mwangi et al., 2016).

Hızır-Kadı et al. (2020) reported that the zeta potential of chitosan-coated liposomes without and with 0.2 % (w/v) concentration of pollen extract was measured at 694 nm and 734 nm; 54.13 ± 1.2 mV and 42.03 ± 1.5 mV,

respectively. According to Alshehri and Abdella (2023), the timol essential oil-bee pollen extract-chitosan has a high zeta potential of +46.28 mV. Ethanolic date palm pollen (DPP) extract encapsulated in sodium caseinate (NaCas) via homogenization and ultrasonication; increasing DPP extract concentration increased particle size from 198.30 to 274.90 nm, while Zeta potential remained between -10.50 to -10.70 (mV) (El-Kholy et al., 2019).

Determination of Total Phenolic Content (TPC)

In Table 4, the total phenolic content of chitosan–SFE-HBP containing Pickering emulsions was calculated as 59.62 ± 0.76 $\mu\text{g GAE ml}^{-1}$ emulsion for 5% (P1), 75.4 ± 0.66 $\mu\text{g GAE ml}^{-1}$ emulsion for 10% (P2), 88.51 ± 1.01 $\mu\text{g GAE ml}^{-1}$ emulsion for 15% (P3). The total phenolic content of the SFE-HBP extract is 4.58 ± 0.05 $\mu\text{g GAE g}^{-1}$ pollen dry weight. The results indicate that, with increasing concentration of the SFE-HBP extract, the TPC of the Pickering emulsions increased significantly ($p < 0.05$). In the study by Hızır-Kadı et al. (2020), pollen extract containing proliposomes and conventional liposomes exhibited total (surface and interior) total phenolic contents of 463.33 mg GAE L^{-1} and 592.5 mg GAE L^{-1} , respectively. Another study reported that, after optimizing spray-drying parameters to encapsulate bee pollen into milk powder, the 5% and 15 % pollen-containing samples showed total phenolic contents of 8.25 ± 0.09 and 12.33 ± 0.18 mg GAE g^{-1} , respectively (Thakur et al., 2020).

Determination of CUPRAC Antioxidant Activity

In Table 4, the CUPRAC antioxidant activity of chitosan SFE-HBP containing Pickering emulsions was determined to be 132.96 ± 2.18 $\mu\text{g TE mL}^{-1}$ emulsion for 5% (P1), 164.78 ± 0.86 $\mu\text{g TE mL}^{-1}$ emulsion for 10% (P2), 202.96 ± 1.73 $\mu\text{g TE mL}^{-1}$ emulsion for 15% (P3). Also, the CUPRAC antioxidant activity of the SFE-HBP extract is 54.64 ± 0.78 ($\mu\text{g TE mL}^{-1}$ extract). The CUPRAC assay results demonstrated that the antioxidant activity of Pickering emulsions was statistically significant ($p < 0.05$) and increased with increasing SFE-HBP extract concentration.

In the study by Hızır-Kadı et al. (2020), the pollen extract containing proliposomes and conventional liposomes exhibited total (surface and interior) CUPRAC antioxidant activity values of 525.74 $\mu\text{g TE mL}^{-1}$ and 557.9 $\mu\text{g TE mL}^{-1}$ in the emulsion, respectively. Baysal et al. (2022) found that, when the methanol extract of bee pollen was co-encapsulated with apple cider vinegar in chitosan carriers, antioxidant activity increased with pollen concentration.

Conclusion

In this work, chitosan-stabilized Pickering emulsions were successfully formulated and used as an efficient delivery carrier for supercritical CO_2 -extracted honey bee pollen. The ultrasound-assisted emulsification technique facilitated the production of stable emulsions with evenly distributed spherical droplets and excellent colloid properties. The increased concentration of honey bee pollen extract enhanced encapsulation efficiency, zeta potential, total phenolic content, and antioxidant activity,

thereby demonstrating the effectiveness of chitosan as a stabilizer carrier. The observed high encapsulation efficiency and positive zeta potentials underscore the strong interfacial adsorption of chitosan particles and the increased stability of the Pickering emulsion system. Moreover, the observed increase in both concentration-dependent relative content of phenolics and antioxidant activity in CUPRAC also validates that the bioactive constituents of honey bee pollen were preserved in the emulsion system. In summary, the results obtained demonstrate the effectiveness of chitosan emulsions in enhancing the functionality of bioactive compounds derived from pollen via supercritical fluid extraction, and these findings can be used to establish their importance in food technology. Future research can focus on digestibility and storage properties to validate such emulsions in food technology.

Declarations

Ethical Approval Certificate

Ethical committee approval and informed consent are not required for this study.

Author Contribution Statement

Esra Mesci Dindar: Data collection, literature search, investigation, writing original draft, writing – review and editing, *Dilhun Keriman Arserim-Uçar*: conceptualization, methodology, investigation, formal analysis, resources, writing – original draft, writing – review and editing, supervision, *Adnan Ayna*: conceptualization, writing original draft, review and editing, supervision.

Conflict of Interest

The author(s) declare no conflict of interest for this study

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