Comparative Analysis of Lipid Oxidation Stability and Bioaccessibility in Krill Oil Emulsions: Microfluidization vs. High-Pressure Homogenization

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September 28, 2023

Abstract

This study conducted a thorough investigation into the impact of two emulsification techniques, namely high-pressure homogenization (HPH) and microfluidization (MF), on the emulsification of krill oil. The comprehensive analysis encompassed various aspects, including particle size characterization, structural assessment, oxidative stability evaluation during storage, measurement of bioaccessibility, and in vitro simulated digestion analysis. Emulsions produced through MF exhibited several noteworthy advantages over those generated by HPH. Most prominently, MF-prepared emulsions featured smaller and more uniformly distributed particles, in stark contrast to the less uniform particles generated by HPH. Moreover, MF-based emulsions demonstrated significantly enhanced oxidative stability during storage, with astaxanthin degradation occurring at a substantially lower rate (38.11% for HPH compared to 89.44% for MF). When assessing emulsion behavior during in vitro simulated digestion, microfluidization formulations exhibited superior stability and markedly higher bioaccessibility in comparison to their HPH counterparts. Of particular significance was the remarkable increase in the release of free fatty acids observed during the intestinal phase of digestion in MF emulsions, indicating an improved lipid digestion process. This study firmly establishes microfluidization as the superior method for crafting emulsions of krill oil, especially within the context of the food industry. Microfluidization not only ensures the preservation of oil quality during storage but also significantly enhances emulsion stability and promotes improved digestibility. These findings hold substantial promise for the development of delivery systems for n-3 fatty acids, making them suitable for incorporation into a wide range of commercial food, beverage, and pharmaceutical products.

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K E Y W O R D S

krill oil Emulsions, microfluidization, high-pressure homogenization, oxidative Stability, astaxanthin degradation, bioaccessibility

INTRODUCTION

Krill Oil (KO) has garnered significant attention owing to its potential health benefits, stemming from its abundant reservoir of omega-3 polyunsaturated fatty acids (PUFAs) and the potent antioxidant astaxanthin. Nevertheless, the seamless integration of KO into diverse food products is beset with challenges, primarily stemming from its limited water solubility. This inherent challenge arises from KO's composition, rich in hydrophobic PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for human health but poorly soluble in water (Zhao et al., 2020). The hydrophobic nature of these components gives rise to hurdles in achieving a uniform dispersion of KO within aqueous matrices, ultimately leading to issues related to phase separation and the emergence of undesirable sensory attributes in the final product. Furthermore, KO remains highly susceptible to oxidative degradation, especially when exposed to oxygen during food processing and storage. The inherent oxidative instability of PUFAs can culminate in the development of off-flavors and a decline in the nutritional quality of food products containing KO (Zhou et al., 2020). Consequently, the resolution of both solubility and oxidative stability issues assumes paramount importance in fully realizing the potential of KO within the food industry.

To tackle the challenges associated with incorporating KO into food products, extensive research efforts have been dedicated to developing effective delivery systems capable of encapsulating and safeguarding KO. Among these systems, oil-in-water (O/W) emulsions have risen as a prominent and promising choice. These emulsions are composed of minuscule oil droplets dispersed within an aqueous phase, and their characteristics are intricately linked to the size and distribution of these droplets (Uluata et al., 2016). Traditionally, researchers have addressed KO's limited solubility and vulnerability to oxidation by introducing antioxidants or surfactants into the emulsion system. Antioxidants play a crucial role in mitigating lipid oxidation, while surfactants enhance emulsification and stabilize the resulting emulsion (Wang et al., 2020). While these additives have proven effective in extending the shelf life of KO- infused products, they have also attracted

significant attention and scrutiny from consumers. The modern consumer landscape increasingly favors clean label products characterized by straightforward and transparent ingredient lists. This trend aligns with food regulations that impose restrictions on the use of specific emulsifying agents or surfactants in many formulated food items. Consequently, there has been a growing preference for physical processing methods to create emulsion systems (Zhou et al., 2022), particularly those enriched with polyunsaturated fatty acids like KO. This evolving scenario has revitalized interest in physical processing techniques for crafting emulsions, particularly those offering a clean label appeal.

In the realm of emulsion preparation, physical processing methods exert a crucial influence by providing substantial energy input to the system, ultimately shaping the properties of the resulting emulsions. Two primary techniques, high-pressure homogenization (HPH) and microfluidization (MF), have garnered attention for their capacity to produce emulsions with minute droplets. These methods assume paramount importance in overcoming the challenges associated with encapsulating KO. HPH stands as a conventional technique widely employed for emulsion production (Kaya et al., 2021). In this process, the emulsion undergoes exposure to high pressures, inducing mechanical shear forces. These forces disrupt and reduce the oil droplets, leading to a reduction in their size. However, HPH exhibits a limitation in its potential to yield inconsistent droplet distributions, which can impact the overall emulsion stability. MF has emerged as an alternative and advanced method for emulsion preparation. It offers precise control over droplet size distribution, effectively addressing the non-uniformity issue observed in HPH. In the microfluidization process, the emulsion is compelled through a microchannel under high pressure, generating intense shear forces, turbulence, and cavitation (Zhao et al., 2021). These factors contribute significantly to a substantial reduction in droplet size. The precise control over these parameters in microfluidization allows for the production of emulsions characterized by remarkably consistent and small droplet sizes. During emulsion preparation, various factors come into play, exerting a significant impact on the stability, texture, and functional properties of the final product. These factors encompass the composition of the oil and water phases, the emulsifying properties of the aqueous phase, and the fatty acid composition of the oil phase. The choice of processing method, whether HPH or MF, can introduce variations in these factors, thereby influencing the overall performance of the emulsion. While prior studies have delved into the effects of different processing methods on emulsion properties, such as droplet size and distribution, these investigations have often been conducted using diverse oil-water compositions and emulsion systems. Consequently, a systematic comparison between HPH and MF in terms of their impact on the oxidative stability and digestive behavior of emulsions has been lacking. This knowledge gap underscores the significance of the present study, as it seeks to illuminate the potential advantages of homogenization in crafting KO emulsions suitable for a wide array of food applications.

This study aims to elucidate the impact of two homogenization methods, namely microfluidization (MF) and high-pressure homogenization (HPH), on the properties of KO emulsions. Its objective is to provide comprehensive insights by systematically evaluating various factors, including particle size, distribution, oxidative stability, controlled release, and bioaccessibility. The investigation addresses pressing challenges encountered by the food industry when incorporating krill oil into a diverse range of food products. By harnessing the advantages of emulsions, which enhance solubility and protect against oxidation, and by exploring innovative homogenization techniques like microfluidization, this study endeavors to offer valuable insights. These insights are expected to facilitate the development of krill oil-based food-grade emulsions characterized by superior stability and bioavailability. This, in turn, lays the foundation for the seamless integration of this beneficial marine oil into a wide array of culinary and dietary offerings. Ultimately, this research contributes to the sustainable and efficient utilization of marine functional lipids within the food industry.

MATERIALS AND METHODS

Materials

Antarctic krill oil was generously provided by Qingdao Seawit Life Science Co., Ltd (Qingdao, China), while all chemicals and reagents utilized in this study were of analytical grade.

Preparation of KO Emulsions

The creation of KO emulsions followed a two-phase procedure. In the aqueous phase, a 2.5% wt% stabilizer consisting of 65% sodium caseinate, 6% gum arabic, and 29% Tween-20 was added to a pH 7 phosphate buffer solution. This mixture was stirred for 2 h to ensure complete dissolution. Simultaneously, the oil phase was formed by dispersing 0.3g of KO in a 20 mL solution comprising dichloromethane and acetone in a 1:2 v/v ratio. The two phases were combined at a 1:9 ratio and stirred at 40 for 1 hour, resulting in the formation of a coarse emulsion. This crude emulsion was further sheared at speeds ranging from 800 to 1000 r/min for 5 to 7 minutes. Subsequently, a high-pressure homogenization (HPH) process was implemented, involving three rounds of homogenization at 800 bar (equivalent to 80 MPa). To remove any residual organic solvent, vacuum processing was employed (Kaya et al., 2021).

In parallel, a separate set of KO emulsions was produced using a (Langfang General Machinery Manufacturing Co. Ltd., Langfang, China) microfluidizer by following established protocols detailed in prior research. Essentially, this involved homogenizing a 10% w/w oil phase with a 90% w/w water phase. The oil and water phases were individually introduced into the dual-channel microfluidized bed through glass reservoirs. Upon convergence, they underwent high-pressure conditions of 80 MPa and were subjected to three cycles of this process (Zhao et al., 2021).

Characterization of KO Emulsions / Microcapsules

Particle Size and Zeta-Potential Analysis

Particle size distribution and zeta-potential measurements were conducted using a Laser particle size analyzer (Zetasizer 2000, Malvern Instruments, Worcestershire, UK). Prior to analysis, emulsions were diluted 20 times with deionized water. The measurements were performed at a constant temperature of 25 , with a fixed detection angle of 90°. Each measurement was replicated using a minimum of three freshly prepared samples (Francisco et al., 2023).

Scanning Electron Microscopy (SEM) Analysis

Before observation, the emulsion underwent spray drying using a pilot-scale spray drier (GEA-Niro, FSD-16-N, Germany). The feed rate was set at 20 g/min, and the inlet and outlet air temperatures were maintained at 135 and 95 , respectively. The resulting encapsulated sample was collected in a sterile glass vial directly from the cyclone and stored at -20 for subsequent analysis.

The morphology of the microcapsules obtained from the KO emulsion was examined using Scanning Electron Microscopy (SEM) (Hitachi, SU8010, Japan). SEM analysis was conducted at an accelerated voltage of 3 kV, and prior to examination, microcapsule samples were coated with a thin layer of sputtered gold to enhance imaging quality.

Fourier transform infrared spectroscopy analysis

The microcapsules, which had undergone pretreatment with HPH emulsion and MF emulsion, were analyzed using Fourier-transform infrared spectroscopy (FTIR) with a Thermo Nicolet instrument located in Waltham, Massachusetts, USA. To facilitate analysis, all samples were mixed with solid KBr powder, and the transmission of wave numbers within the range of 4000 to 500 cm^{-1} was recorded.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) analysis of the microcapsules were subjected to a heating process, ramping from 30 to 300degC at a rate of 10degC/min, utilizing a thermal analyzer (TA DSC25, TA Instruments, New Castle, DE, USA) within a nitrogen atmosphere maintained at 50 mL/min.

X-ray diffraction (XRD) analysis

XRD patterns were acquired using an XRD-7000S diffractometer (Shimadzu, Tokyo, Japan), following the methodology established by Wu et al. (2020)

Stability Assessment of KO Emulsion under Accelerated Oxidation Conditions

To assess the stability of the emulsion, lipid oxidation experiments were conducted by placing two sets of emulsions in sealed screw-cap glass tubes covered with aluminum foil. These samples were then incubated for 30 d in an incubator at a temperature of 50 degC to accelerate the oxidation process. At intervals of 5 d, samples were analyzed to monitor the production of primary lipid oxidation products. Measurements were based on the peroxide value (POV) and acid value (AV). The analysis followed the guidelines provided by the American Oil Chemists' Society (AOCS) official method AOCS Cd 8-53 (AOCS,1998).

Analytical Parameters for Component Evaluation during the Accelerated Storage Period

TAG and PLs Analysis

The lipid content in KO emulsions was determined using the MK-6S TLC-FID Analyzer (Iatroscan, Japan). Lipids were extracted using a chloroform-methanol mixture and dissolved in chloroform at concentrations ranging from 10 to 20 mg/mL. Separation was achieved using n-heptane, anhydrous ether, and formic acid (in a ratio of 42:28:0.3) as the developing agent. The analysis involved specific conditions such as air and hydrogen flow rates, as well as scanning speed, following the method outlined by Fu et al. (2023).

DHA and EPA Analysis

The fatty acid profiles of KO emulsions, with a specific focus on changes in EPA and DHA during accelerated storage, were determined using GC-MS (Agilent, America). This process involved the removal of unsaponifiable matter, extraction of saponifiable matter with hexane, and analysis under controlled conditions, including temperature gradients and injection volume, following the method described by Li et al. (2019).

PC Analysis

The quantification of PC content was carried out using HPLC with a Shimadzu LC-20AD Prominence HPLC system equipped with an Alltech ELSD 6000 detector. The analysis included precise control of gas flow rate and ELSD tube temperature. Separation was achieved using an Agilent Zorbax RX-SIL column, employing a specific mobile phase composition. PC content was calculated based on standard curve methods, using GPCho ($C_{16:0}/_{18:1}$) and GPEtn ($C_{16:0}/_{18:1}$) standards (Li et al., 2020).

PL Class Composition Analysis

PL class composition was determined using an Avance III 400 MHz NMR spectrometer (Bruker, Switzerland), following a previously reported method (Wan et al., 2022).

Astaxanthin Analysis

Quantification of astaxanthin content was conducted via HPLC (Waters, Mil-ford, MA, USA) employing a C18 column and a mobile phase consisting ofdichloromethane, methanol, acetonitrile, and water. A standard curve was established using astaxanthin standards ([?]97.1%) at various concentrations (Song et al., 2018).

In vitro **Simulated Digestion**

The *in vitro* digestion model was structured in three stages: mouth, stomach, and intestinal tract, each involving specific simulated digestive fluids. The compositions of these fluids, namely simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), were prepared in accordance with a previous study by El-Messery et al. (2020). The digestion experiments were conducted within a shaking water bath at 37degC.

In the simulated salivary stage, approximately 10 mL of samples were blended with an equal volume of SSF and gently stirred at 37degC and 120 rpm for 10 min.

Following salivary digestion, the simulated gastric stage involved the addition of 20 mL of SGF containing 3.2 mg/mL pepsin, 7 mL/L hydrochloric acid, and 2 g/L sodium chloride to the digestive fluid. The pH was

adjusted to 2.5 using 0.1M HCl. The digestive fluid was then stirred at 37degC and 120 rpm for 2 h.

In the simulated intestinal stage, after gastric digestion, the digest was promptly adjusted to pH 7 using a 0.1 M NaOH solution. Subsequently, 2.5 mL of pancreatic lipase (4.8 mg/mL), 4 mL of bile salt solution (5 mg/mL), 1 mL of $CaCl_2$ solution (750 mM), and 0.5 mL of glucosamine were added to the sample. The pH of the reaction system was monitored and maintained by titrating 0.1 M sodium hydroxide solution into the reaction vessel over a period of 2 h at 37degC.

Throughout the digestion process, the cumulative volume of added NaOH at different time points (20 min, 40 min, 60 min, 80 min, 100 min, and 120 min) was recorded, and the pH was maintained at 7.0. The corresponding release rate of free fatty acids (FFA) was calculated using the following equation:

$$\text{FFA}\,(\%) = \frac{VNaOH \times mNaOH \times Mlipid}{\text{Wlipid}} \times 100\%$$

where V_{NaOH} represents the volume (L) of sodium hydroxide required to neutralize the FFAs produced, m_{NaOH} is the molarity of the sodium hydroxide solution (0.1 M), W_{lipid} signifies the total weight of lipid initially present in the reaction vessel, and M_{lipid}denotes the molecular weight of KO.

Particle size and zeta-potential measurements

To track variations in particle size and zeta-potential distributions following *in vitro* digestion, a laser particle size analyzer (Zetasizer 2000, Malvern Instruments, Worcestershire, UK) was employed, applying the methods described previously.

Confocal laser scanning microscope (CLSM)

Modifications in the microstructure of emulsions, generated through different methods, throughout the *in vitro* digestion process, were visualized using a confocal laser scanning microscope (LEICA, SP8, Germany). Before observation, Nile red was utilized to label released oil, resulting in red fluorescence for the two different emulsions during *in vitro* digestion. Simultaneously, water phases were stained with Nile blue. The confocal images were captured using a 63x oil immersion objective (Rathnakumar et al., 2023).

Astaxanthin, DHA and EPA Content and Bioaccessibility

The content of astaxanthin, DHA, and EPA was individually determined following salivary, gastric, and intestinal digestion, employing the methods described previously. After *in vitro* digestion, a portion of the digesta was subjected to centrifugation at 8000 rpm/min at 25degC for 30 min. The resulting clear middle layer, formed after centrifugation, was assumed to contain the micellar fraction that solubilized astaxanthin, DHA, and EPA. This micellar phase was extracted using the wet oil extraction method and prepared for HPLC analysis.

The actual content of astaxanthin, DHA, and EPA was calculated based on their respective standard curves. Subsequently, the bioaccessibility was determined. The effective bioaccessibility (%) of astaxanthin, DHA, and EPA was calculated using the following formula: Astaxanthin/DHA/EPA bioaccessibility (%) = 100 x $C_{micelle}$ / $C_{initial}$, where $C_{micelle}$ represents the concentration of astaxanthin, DHA, and EPA in the micellar fraction, while $C_{initial}$ represents their initial concentrations in the samples.

Statistical Analysis

The experiments were conducted in triplicate, and the results are expressed as mean +- SD. Statistical analysis was performed using SPSS analytical software version 20.0 (SPSS Inc., Chicago, IL, USA). This analysis included One-way ANOVA and independent-samples T-test, with a significance level set at $p < 0.05$. To determine significant differences among the mean parameters, the Duncan test was employed ($p < 0.05$).

RESULTS AND DISCUSSION

Comparative Analysis of Microcapsule Properties between HPH and MF Methods

In Figure 1A, the droplet size distribution of emulsions prepared through two distinct methods, high-pressure homogenization (HPH) and microfluidization (MF), is depicted. It's worth noting that the curves representing the HPH samples displayed a bimodal distribution, while those of the MF samples exhibited a unimodal pattern. The unimodal distribution observed in the MF samples suggests a more controlled and precise emulsification process. In contrast, the bimodal distribution observed in the HPH samples may indicate a less uniform particle size distribution. The zeta-potential of the emulsion produced via microfluidization, as illustrated in Figure 1B, exhibited a significant increase. This elevated zeta-potential in the MF samples can be ascribed to the effective mixing and minimized particle aggregation facilitated by the microfluidization process. The consistent particle size distribution and reduced aggregation tendencies contribute to a heightened surface charge density, consequently leading to the observed increase in zeta-potential. Scanning electron microscopy (SEM) micrographs, as depicted in Figure 1C, provided valuable insights into the powders generated through spray drying. Notably, the HPH samples exhibited a higher degree of size variability, whereas the MF microcapsules displayed remarkable uniformity in both size and shape. This stark difference can be attributed to the microfluidization process, which subjects the emulsion to intense shear forces, turbulence, and cavitation, resulting in a more consistent droplet breakup and microcapsule formation. Furthermore, the diminished presence of surface oil in MF microcapsules signifies enhanced encapsulation efficiency.

Figure 1D exhibits the X-ray diffractograms of the microcapsules, revealing noteworthy insights. Both microcapsules displayed characteristic peaks at 2ϑ values of 20°, 27°, 32°, 45°, 57°, and 67°. However, microcapsules produced via microfluidization (MF) exhibited reduced peak intensities at 27°, 32°, 45°, 57°, and 67°, while displaying heightened intensity at $2\vartheta = 20°$. These distinctions suggest discernible alterations in the crystalline phases of microcapsules formed through different homogenization methods. The variations in peak intensities signify differences in the crystalline structures of these microcapsules. The increased peak intensity at $2\vartheta = 20°$ in MF microcapsules hints at a potentially distinct crystal form or arrangement, which may contribute to their enhanced stability and performance.

The Fourier-transform infrared spectra of the microcapsules, as depicted in Figure 1E, displayed prominent peaks in the ranges of 750-1000 cm^{-1}, 1450-1700 cm^{-1}, and 3000-3500 cm^{-1}. Remarkably, microcapsules produced *via* MF exhibited a shift toward higher wavenumbers. This shift might indicate alterations in chemical bonding or interactions within the microcapsule structure. The increased intensity of peaks in MF microcapsules could be attributed to a more densely packed and structured encapsulation matrix, thereby contributing to enhanced stability.

Figure 1F portrays the differential scanning calorimetry curve of KO microcapsules. It is noteworthy that both microcapsules exhibited an initial absorption peak around 42°C. However, with increasing temperature, the second absorption peak differed between HPH and MF microcapsules. HPH microcapsules showed a peak at 55°C, whereas MF microcapsules exhibited one at 62°C. This distinction suggests that MF microcapsules possess enhanced thermal stability. The higher second absorption peak temperature in MF microcapsules indicates their ability to endure higher temperatures before undergoing structural changes or degradation.

Thence, the comprehensive analysis of these results underscores the effectiveness of MF as a homogenization method for encapsulating KO. The observed advantages in MF-produced microcapsules, including droplet size distribution, zeta-potential, size uniformity, crystalline phase, structural stability, and thermal resistance, can be attributed to the controlled and efficient microfluidization process, establishing MF as the preferred technique for KO encapsulation.

Enhanced Oxidative Stability in MF-Prepared Emulsions

The Peroxide Value (POV) and Acid Value (AV) are critical indicators for assessing the quantity of hydroperoxide (the primary oxidation product in fats and oils) and the degree of oil hydrolysis, respectively. Monitoring these indices is crucial for maintaining food quality and safety. The results regarding POV and AV of emulsions after one month of storage at 25°C are depicted in Figure 2. Notably, MF-prepared emulsions consistently exhibited significantly lower POV and AV values ($p< 0.05$) compared to HPH-prepared emulsions. at the beginning of the storage period (day 0) with values of 3.70±0.03 vs. 4.05±0.36 mg POV/kg

oil and 18.50±3.54 vs. 6.49±1.31 mg AV/kg oil for MF and HPH, respectively. Similarly, after one month of storage (25), the values remained lower for MF-prepared emulsions, with values of 18.27+-0.92*vs.* 7.59+-0.94 mg POV/kg oil and 49.54+-2.96 *vs.* 16.35+-1.41 mg AV/kg oil for MF and HPH, respectively.

Figure 2 illustrates that the POV and AV of HPH-prepared emulsions exhibited a faster rate of increase compared to MF-prepared samples during the storage period. Several key mechanisms contribute to the superior oxidative stability observed in MF-prepared emulsions. Firstly, MF-prepared emulsions feature smaller droplet sizes and a more uniform distribution of droplets. This results in a higher surface area-to-volume ratio, facilitating greater interaction between the oil phase and the aqueous phase. This increased interfacial area enhances the encapsulation of pro-oxidants within the smaller oil droplets, reducing their contact with the bulk aqueous phase and, consequently, diminishing the initiation of oxidation reactions. Moreover, MF-prepared emulsions exhibit a remarkable resistance to aggregation. Aggregation can lead to localized areas of high oil concentration, making them particularly susceptible to oxidation. By preventing aggregation, MF emulsions hinder the propagation of oxidation reactions from one droplet to another. This isolation of oxidation events within individual droplets significantly contributes to the overall oxidative stability. Lastly, the uniform distribution of smaller oil droplets in MF-prepared emulsions ensures an even dispersion of oil throughout the emulsion. This uniformity minimizes the potential for localized oxidation events and further enhances the overall oxidative stability of the emulsion. The data presented in Figure 2 undeniably support the assertion that MF-prepared emulsions exhibit a substantial advantage in terms of oxidative stability compared to their HPH counterparts. These findings underscore the potential of MF-prepared emulsions to more effectively protect KO and other sensitive compounds during storage.

Evolution of Functional Compounds During Accelerated Storage

Figure 3 provides a comprehensive overview of the changes in various functional components during the accelerated storage period, shedding light on critical differences between emulsions prepared *via* HPH and MF.

Triglycerides (TAG) and Phospholipids (PL): The decrease rate of TAG and PL in HPH-prepared emulsions exhibited a rapid increase from the 5th day to the 30th day, indicative of substantial degradation. In contrast, the decrease rate in MF-prepared emulsions increased more gradually, with a notable change observed from the 15th day onwards. This disparity in the decrease rate of TAG and PL between HPH and MF emulsions was statistically significant.

The continuous and rapid increase in the decrease rate of EPA and DHA in HPH-prepared emulsions from the 5th day to the 30th day underscores their vulnerability to oxidation. In contrast, MF-prepared emulsions exhibited a slower increase in the decrease rate of these essential fatty acids, indicating better retention. This retention of EPA and DHA in MF emulsions corresponds with their lower POV and AV values compared to HPH emulsions, signifying a reduced rate of PUFA oxidation.

Astaxanthin: Astaxanthin, known for its strong antioxidant activity, displayed a stark contrast in behavior between HPH and MF emulsions. HPH emulsions experienced a consistently high decrease rate of astaxanthin throughout the storage period, while MF emulsions maintained a nearly constant decrease rate over 30 days. This variation in astaxanthin degradation aligns with the observations of POV and AV, where higher astaxanthin decrease rates correlated with elevated POV and AV due to the substantial loss of astaxanthin and a subsequent decrease in protective capacity.

Phosphatidylcholine (PC): The PC content in MF-prepared emulsions remained relatively higher throughout the storage period, and as time progressed, it decreased gradually. These results collectively indicate that MF emulsions exhibited better storage stability and were more effective in inhibiting chemical degradation compared to HPH emulsions.

The lower rates of functional component degradation in MF-prepared emulsions can be attributed to several key factors. MF emulsions possess superior stability characterized by smaller particle sizes, uniform distribution, and reduced aggregation or coalescence. These attributes collectively act as barriers that impede

oxidative transfer processes, forming a protective shield against deteriorative oxidation reactions. Consequently, KO emulsions produced through MF not only serve as a protective barrier against oxidative processes but also retain essential nutrients to a greater extent. The results presented in this section underscore the marked contrast in the behavior of functional compounds in emulsions prepared by HPH and MF during accelerated storage. MF emulsions consistently exhibit lower degradation rates of TAG, PL, EPA, DHA, astaxanthin, and better retention of PC, highlighting their superior storage stability and their capacity to preserve valuable nutrients. These findings emphasize the potential of MF-prepared emulsions for various applications that demand extended shelf life and nutrient retention.

Changes in Particle Size and Zeta-Potential during *In Vitro* Digestion

Changes in particle size and zeta-potential throughout the various stages of the gastrointestinal (GIT) model are illustrated in Figure 4. Initially, the MF-prepared emulsion exhibited a significantly smaller mean particle size, primarily attributable to the different homogenization method employed. However, as the emulsions progressed through the oral phase, the particle size of the HPH-prepared emulsion experienced a notable increase, whereas the MF emulsion exhibited minimal changes. Subsequent to the gastric phase, the MF emulsion demonstrated a substantial increase in mean particle diameter, suggesting a propensity for significant droplet aggregation. This observation aligns with prior studies (Li et al., 2020), which have reported that protein-stabilized emulsions tend to aggregate under gastric conditions. This aggregation can be attributed to factors such as low pH, hydrolysis of adsorbed proteins, weakening of electrostatic repulsion, and the occurrence of depletion or bridging flocculation induced by mucin. The results obtained from confocal microscopy supported and reinforced these findings. Zeta-potential, a critical indicator of colloidal suspension stability, was continuously monitored as the emulsions progressed through the various stages of the GIT model to assess alterations in interfacial properties. Initially, the MF-prepared emulsion exhibited a higher absolute value of zeta-potential, signifying better stability, which aligns with the previously obtained results. However, following the oral phase, there was a noticeable decrease in the magnitude of the negative charge on the MF emulsion. This reduction could potentially be attributed to electrostatic screening caused by the presence of mineral ions in simulated saliva or interactions between mucin molecules and the surfaces of oil droplets.

Further reductions in the absolute value of zeta-potential were observed as the emulsions encountered simulated stomach conditions. The low pH and high ionic strength of simulated gastric fluids could have led to alterations in the electrical properties of the droplets. Subsequently, after incubation in the small intestine phase, all samples displayed negative charges. This phenomenon may be attributed to the presence of anionic species from various types of particles, including undigested lipids, undigested proteins, micelles, vesicles, and calcium salts. Notably, the negative charges of the MF-prepared emulsion remained relatively higher throughout the gastrointestinal model, indicating its superior stability in the small intestine phase. Therefore, the emulsion produced by MF demonstrated enhanced stability across the different stages of the GIT model, highlighting its potential as a robust delivery system throughout the gastrointestinal tract.

Microstructural Changes During *In Vitro* Digestion

Figure 5 provides visual insights into the microstructural transformations occurring in KO emulsions produced by HPH and MF at different stages of digestion. These observations were made using confocal fluorescent microscopy, where the oil phase was stained red and the protein phase was stained green. The aim was to visually assess how emulsions formed by different homogenization methods offer protective effects during digestion. In the initial test group, fine and uniform droplet distributions were evident, with no signs of aggregation or coalescence, aligning with the findings presented in Figure 1 A and B. During the oral phase, minimal changes in droplet structure were observed. However, a slight degree of aggregation was noted in the stomach phase, likely due to the decrease in pH and protease-induced hydrolysis.

During the small intestine phase in the MF-prepared emulsion, at 0 minutes, there was a gradual release of lipids in response to bile extract and pancreatin digestion. Over time, an increasing number of lipids were observed, and by the small intestine phase at 120 minutes, complete lipid release had occurred. These findings

align with prior research by Zhao et al. (2021), which demonstrated that high oleic palm oil encapsulated in nanoliposomes *via* MF exhibited smaller particle sizes and polydispersity indices. This encapsulation effectively shielded the oil from the harsh acidic conditions of gastric digestion and allowed for the controlled release of core materials during *in vitro* digestion. In contrast, the control group exhibited extensive droplet aggregation or coalescence throughout the digestion stages, likely due to the inferior protective capabilities of the wall formed by HPH. This indicated that HPH-prepared emulsions were less stable compared to KO-in-water emulsions produced *via* MF. These findings underscore the superiority of MF as a method for generating uniform and stable emulsions that effectively protect KO from oxidation and hydrolysis, ensuring efficient absorption in the small intestine.

Changes in Content and Bioaccessibility of DHA, EPA, and Astaxanthin During *In Vitro* Digestion

The data in Figure 6 unequivocally demonstrates that the emulsion prepared by MF exhibited substantially higher levels of DHA, EPA, and astaxanthin after undergoing digestion in both the stomach and small intestine when compared to the emulsion prepared by HPH. This marked difference can be attributed to the robust electrostatic repulsion between oil droplets in the MF-prepared emulsion, which effectively reduces the transfer of oxidation processes within the simulated digestive tract. Additionally, the MF-prepared emulsion benefits from a more uniform and resilient wall material, which provides superior protection against KO degradation. Following the small intestine phase, the digesta resulting from oil lipolysis underwent centrifugation to separate the micelle phase, which contained the solubilized fraction of DHA, EPA, and astaxanthin. Bioaccessibility was determined by measuring their concentrations in both the micelle phase and the total digesta. Figure 6D clearly illustrates that the MF-prepared emulsion exhibited significantly higher bioaccessibility of DH5A, EPA, and astaxanthin. This enhancement can primarily be attributed to the emulsion's smaller particle size, which accelerates digestion due to its greater specific surface area. This observation aligns with previous research, such as the study by Salvia-Trujillo et al. (2017), which demonstrated a significant increase in carotenoid bioaccessibility with decreasing lipid droplet size in excipient emulsions. Additionally, the MF-prepared emulsion displayed greater stability with reduced droplet flocculation, further amplifying the surface area of lipids exposed to lipase.

Release of Free Fatty Acids (FFA) during *In Vitro*Digestion

The release of Free Fatty Acids (FFA) from both emulsions was closely monitored at various time points during intestinal digestion, and the results are depicted in Figure 7. The lipid digestion profiles of the emulsions produced by HPH and MF displayed similar patterns, characterized by an initial rapid increase in FFA levels followed by a more gradual rise over time until reaching a relatively stable final value.

Although there were no significant differences observed between the KO emulsions prepared by HPH and MF during the initial stages of digestion, some distinctions became apparent in the profiles of fatty acid release and the initial rates of lipid digestion. Notably, the MF-prepared emulsions exhibited a lower initial rate of lipid digestion. This difference can be attributed to alterations in the surface area of lipids exposed to digestive enzymes, a factor that is inversely proportional to the mean droplet diameter. As the digestion process advanced, the oil droplets within the homogenized emulsion tended to aggregate. This aggregation resulted in a reduction in the surface area of the oil droplets, limiting lipase's access to the droplet surfaces. This phenomenon aligns with findings from another study (Qin et al., 2016), which reported that the rate and extent of lipid digestion in aβ -carotene emulsion increased with decreasing mean droplet diameter (small [?] medium >> large). Moreover, it's worth noting that the MF-prepared emulsion exhibited a higher final extent of FFA release compared to the HPH-prepared emulsion. The final release of FFA from the MF-prepared emulsion reached approximately 80.72$\pm$1.05%, whereas the HPH-prepared emulsion released only 67.41$\pm$1.6% of FFAs. Several factors may contribute to this difference, including the resistance of colloidal particles to digestion and the potential hydrolysis of lipids before analysis due to emulsion instability. It is noteworthy that the HPH-prepared emulsion exhibited numerous non-digested lipid droplets, as observed through confocal microscopy (Figure 5A). This observation aligns with the relatively low extent of lipid digestion by the end of the small intestine phase. These findings emphasize the role of smaller particle size

in nanoemulsions in enhancing the bioavailability of encapsulated hydrophobic nutraceuticals by facilitating more rapid and complete digestion of the lipid phase.

CONCLUSION

This study underscores the substantial potential of microfluidization as an auspicious technique for the production of KO emulsions. Our research delves into the fabrication of emulsions using microfluidics, and it juxtaposes these findings with emulsions generated *via* the traditional high-pressure homogenization method. The KO emulsions created through microfluidization exhibit a gamut of superior attributes, prominently characterized by enhanced stability, reduced particle size, and a more uniform particle distribution. These commendable features are attributed to the meticulous design of the microfluidizer. Notably, these emulsions manifest improved oxidative stability over a month-long storage period at 25°C. This augmented stability is palpable through discernibly lower increments in peroxide value, anisidine value, and heightened retention of vital fatty acids such as EPA and DHA. The rationale behind this enhanced oxidative stability lies in the emulsion's reduced particle size, uniform distribution, and minimized aggregation or coalescence, collectively acting as impediments to oxidative processes. Furthermore, our *in vitro* digestion experiment serves to underscore the emulsion's exceptional stability and heightened bioaccessibility when crafted through microfluidization in comparison to those produced via high-pressure homogenization. Following exposure to various stages of a simulated gastrointestinal tract, this superior bioaccessibility can be attributed to the larger lipid surface area exposed to pancreatic lipase, stemming from the smaller droplet size inherent in microfluidized emulsions. Of particular note is the significant augmentation in the release of FFA within the intestinal phase for the microfluidized emulsion. This finding signifies an increased stability of the oil within the highly acidic gastric environment and an enhanced digestibility of lipids in the small intestine. In summation, these results carry considerable implications for the strategic design of O/W emulsion-based delivery systems, tailored for encapsulating, safeguarding, and proficiently delivering n-3 fatty acids across a spectrum of applications encompassing foods, pharmaceuticals, and assorted commercial products. The employment of microfluidization as the preferred method for producing KO emulsions exhibits remarkable potential in sustaining the quality of KO and, notably, emerges as a viable avenue for fortifying a multitude of food products with n-3 fatty acids.

ACKNOWLEDGMENTS

This work was financially supported by the Key Program of National Natural Science Foundation of China (Grant No.32230080), the Dalian Science and Technology Innovation Fund (Grant No.2022JJ11CG008) and the National Key R&D Program of China (Grant No. 2018YFC1406806).

CONFLICT OF INTEREST

The authors stated that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

AUTHORS CONTRIBUTIONS

Jia-rong Huang : Investigation, Original manuscript drafting; **Jian-run Zhang** : Methodology; **Jing Zhang** : Methodology; **Zhen-wen Shao** : Methodology; **Da-yong Zhou** : Supervision; **Liang Song** : Conceptualization, Securing funding, Project administration, Supervision, and Manuscript review and editing.

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Figure Captions

FIGURE 1 Comparative analysis of microcapsule properties between HPH and MF methods (A), zeta-potential (B), microphotographs captured (C), X-ray diffractograms (D), fourier-transform infrared spectra (E) and scanning calorimetry curve (F) of krill oil (KO) emulsions prepared by high-pressure homogenization (HPH) / microfluidization (MF)

FIGURE 2 Enhanced oxidative stability in MF-Prepared emulsions changes in AV. AV (A) and POV(B) of KO emulsion prepared by HPH / MF during an accelerated storage period. Different letters above the columns represent significant differences at $p < 0.05$. Bars represent standard deviations (n = 3)

FIGURE 3 Evolution of functional compounds during accelerated storage . TAG decrease rate (A), PL decrease rate (B), EPA decrease rate (C), DHA decrease rate (D), astaxanthin decrease rate (E), and PC content (F) of KO emulsion prepared by HPH / MF during accelerated storage. Different letters above the columns represent significant differences at $p < 0.05$. Bars represent standard deviations (n = 3)

FIGURE 4 Changes in particle size and Zeta-Potential during*In Vitro* digestion. Particle size distribution (A) and zeta-potentials (B) of emulsions prepared using HPH / MF after exposure to different stages of simulated gastrointestinal tract (GIT), $*p < 0.05$

FIGURE 5 Microstructural changes during *In Vitro*digestion. Confocal micrographs (A) of KO emulsions prepared by HPH / MF during *in vitro* digestion

FIGURE 6 Changes in content and bioaccessibility of DHA, EPA, and Astaxanthin during *In Vitro* digestion. DHA content (A), EPA content (B), and astaxanthin content (C) of KO emulsions prepared by HPH / MF after exposure to different regions of a simulated GIT. Bioaccessibility (D) of astaxanthin, DHA, and EPA in KO emulsions prepared by HPH / MF as they pass through different regions of a simulated GIT

FIGURE 7 Release of free fatty acids (FFA) during *In Vitro* digestion. The amount of free fatty acids released (A) from emulsions prepared by HPH / MF measured in pH-stat *in vitro*digestion model

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