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Nanoemulsion of Amber rice bran oil (*Oryza sativa* L.), characterization and properties

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ABSTRACT

A nanoemulsion of Iraqi Amber rice bran oil (NARBO) was prepared using an ultrasonic method. The nanoemulsions: NE1, NE2, and NE3 were formulated using an emulsifier (Tween 80 and sodium caseinate) in a ratio of 1:1 and at a concentration of (1.5, 2, 2.5%), and were examined to ensure their stability and physicochemical properties. All nanoemulsions carried a negative charge for zeta potential ranging between -31.51 and -18.52, with the highest value recorded in NE1 on day 7. The negative zeta potential increased on day 7 for all prepared nanoemulsions. and concerning droplet size, it ranged (from 89.4 to 144.2 nm), and the smallest size was recorded in NE3. Increasing the emulsifier concentration resulted in decreased droplet sizes. All nanoemulsions had good droplet size distribution, with a Polydispersity Index of less than 0.3. FE-SEM images revealed that the nanoemulsion droplets were spherical, uniformly distributed, and well-coated at all emulsifier concentrations. Viscosity values ranged between (3.5 to 6.2 cP) and increased with the concentration of emulsifier. From the results of the stability test, it can be concluded that NE3 was the most stable during the period studied, as the lipid index (CI) remained zero until the seventh day. The highest value of oil encapsulation efficiency (EE) (97.60%) was recorded in NE3. These results showed that a stable nanoemulsion was successfully prepared from Iraqi amber rice bran oil, which could represent a promising solution for improving the stability and bioavailability of the oil and the active and effective compounds it contains.

مستحلب نانوي من زيت نخالة أرز العنبر (*Oryza sativa* L.)، توصيفه وخواصه

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الخلاصة

تم تحضير مستحلب نانوي من زيت نخالة الأرز العنبر العراقي (NARBO) بطريقة الموجات فوق الصوتية، حيث تمت صياغة المستحلبات النانوية: NE1 ،NE2 ،NE3 باستخدام عامل استحلاب (توين 80 و كازينات الصوديوم) بنسبة 1:1 وبتركيز (1.5، 2، 2.5%)، وتم فحصها للتأكد من ثباتها وخصائصها الفيزيائية والكيميائية. حملت جميع المستحلبات النانوية شحنة سالبة لجهد زيتا تراوحت بين −31.51 و −18.52، سجلت أعلى قيمة لـ NE1 في اليوم السابع. وازداد جهد زيتا السالب في اليوم السابع ولجميع المستحلبات المحضرة، وفيما يتعلق بحجم القطرة، فقد تراوح (من 89.4 الى 144.2 نانومتر)، وسُجل أصغر حجم في NE3. أدت زيادة تركيز عامل الاستحلاب إلى انخفاض أحجام القطرات. تتمتع جميع المستحلبات النانوية بتوزيع جيد لحجم القطرات، مع قيمة PDI أقل من 0.3. كشفت صور FE-SEM أن قطرات المستحلب النانوي كانت كروية، وموزعة بشكل موحد، ومغلفة جيداً في جميع تركيزات عامل الاستحلاب. تراوحت قيم اللزوجة بين (3.5 إلى 6.2 سنتي بواز) وتزداد مع تركيز عوامل الاستحلاب. من نتائج اختبار الثبات يمكن استنتاج أن NE3 كان الأكثر ثباتاً خلال الفترة المدروسة، حيث بقي مؤشر الدهن (CI) صفر حتى اليوم السابع. تم تسجيل أعلى قيمة لكفاءة التغليف للزيت (EE) في NE3 وبنسبة (97.60%). أظهرت هذه النتائج انه تم النجاح في تحضير مستحلب نانوي مستقر من زيت نخالة أرز العنبر العراقي، والذي يمكن أن يمثل حلاً واعداً لتحسين الثبات والتوافر الحيوي للزيت والمركبات النشطة والفعالة التي يحتوي عليها.

الكلمات المفتاحية: الاستقرارية، جهد زيتا، مستحلب نانوي، مؤشر التشتت المتعدد، كازينات الصوديوم

INTRODUCTION

Consumer awareness of the substantial health benefits of vegetable oils has grown including rice bran oil (RBO). Recent years have seen an unprecedented level of interest in new research in this sector (Fraterrigo Garofalo *et al.,* 2021). Rice bran is a by-product of the rice milling process, it constitutes about 10-12% of the weight of the rice grain and is mainly used worldwide as animal feed and in the production of rice oil, it is also used in the preparation of baking products and functional foods (Nofan & Naji, 2021). RBO is considered the best among other vegetable oils due to its unique composition rich in antioxidant compounds such as tocopherol, tocotrienol, oryzanol, phytosterols, polyphenols, and squalene, in addition to unsaturated fatty acids, it is also known to be healthy for the heart due to the presence of γ-oryzanol, which helps reduce cholesterol levels and treat high cholesterol, blood fats and combating carcinogens and oxidants (Fraterrigo Garofalo *et al.,* 2023; Nugrahani *et al.,* 2024).

Amber rice, a type of rice cultivated in Iraq for centuries is known for its medium-sized grains that are not sticky and have a smooth texture when cooked Amber rice emits an aroma that sets it apart from other types of rice (Alawadi & Hague, 2018). Rice is considered the second food in Iraq (Aziz *et al.,* 2024). In the farming season of 2021-2022, around 384,926 dunums of rice were grown in Iraq yielding 422,463 tons of rice (CSO, 2022). Amber rice bran contains oil (18.70%), according to what was mentioned (Nofan & Naji, 2016). Although RBO contains bioactive compounds such as tocopherols, tocotrienols, and phytosterols, which contribute to its antioxidant properties, the oxidation of RBO limits its shelf life and use in various products.

Nanoemulsions offer a promising solution to improve the stability and bioavailability of RBO by reducing droplet size and increasing surface area. Nanoemulsions are dispersion mixtures of two phases that typically don't mix well, water and oil, brought together by surfactant molecules, with droplets sized between 5 to 200 nm (Chen *et al.,* 2022). These nanoemulsions can be categorized as either water based (oil in water) or oil based (water in oil) emulsions (Singh *et al.,* 2017). The size of the droplets affects the physical and chemical properties such as the optical property and maintaining stability, in addition to the release behavior and flow, so nanoemulsions are considered more suitable than microemulsions for many different applications (Nasr *et al.,* 2022). The droplet size in nanoemulsions can be controlled by process parameters such as emulsifier concentration, oil concentration, emulsification time, and energy input (Nakabayashi *et al.,* 2011; Hameed *et al*.,2023).

An emulsion's formulation and composition determine how stable it is, the type of emulsifier being crucial to the system's stabilization, the emulsifier kinetically stabilizes the nanoemulsion by lowering the surface tension between the water and oil phases and creating a film at the oil-water interface, thereby preventing the coalescence of dispersed fat droplets, the ability of surfactants to reduce surface tension usually comes through steric and electrostatic repulsion between emulsions (McClements & Jafari, 2018). In addition to the concepts of surface adsorption and reorganization at the oil-water interfaces, which result in a well-regulated emulsification process, selecting the appropriate emulsifier and its concentration in the oil phase depends on the necessary surface coverage to produce stable systems (Mushtaq *et al.,* 2023).

Ultrasonic emulsification is a high-energy method for preparing nanoemulsions, it has been proven to be an effective and rapid technique for formulating stable nanoemulsions, very small droplet sizes, and low polydispersity. Hence, it is widely used by researchers (Espinosa-Sandoval *et al.,* 2021; Lago *et al.,* 2019). Ultrasonic emulsification technology relies on two mechanisms, it operates by establishing a field that produces a wave leading to the formation of oil droplets that disperse throughout the system, the other mechanism used in this technology depends on utilizing ultrasound waves to improve cavitation, this process leads to the creation and subsequent collapse of bubbles due, to pressure changes induced by a sound wave, this causes intense local disturbances to be created, and small explosions to form within the liquid, eventually tearing up large-sized droplets into smaller droplets (Choradiya & Patil, 2021; W. Khan *et al.,* 2018). Ultrasonic emulsification technology was effective and successful in preparing nanoemulsions from rice bran oil (Bains *et al.,* 2022), (Razavi *et al.,* 2021), (Gao *et al.,* 2022), from soybean oil (Lehri *et al.,* 2021), and from a mixture of essential oils (Torres Neto *et al.,* 2024) and from D-limonene oil (Chiu *et al.,* 2024).

From this perspective, the main objective of this study is to manufacture a (NARBO) from a functional oil using ultrasound assistance. The characteristics and stability of the nanoemulsion will be studied, including the rheological behavior in addition to the droplet size, polydispersity index of the droplets (PDI), zeta potential, morphology, and stability of the nanoemulsion in storage.

MATERIAL AND METHODS

Materials

Rice bran was obtained from Al-Rawan Mill for Amber Rice in Najaf, Iraq. Absolute ethanol as a solvent was obtained from the German company (Honeywell), sodium caseinate was purchased from the Turkish company (Bensen), and Tween 80 was obtained from the German company (Wuerzteufel). Ultrasound probe device (LC-JY96-IIN,150W, probe6) China, while the homogenizer device (FTHMZ-20DN) is from MEDILAB Korea.

Stabilization of Rice Bran

Rice bran was stabilized following the method described by (Khan *et al.,* 2009), the bran was spread in a thin layer of 0.5 cm thickness on a tray and subjected to heating in a preheated oven at a temperature of 120 °C for 10 minutes, followed by cooling at room temperature. Subsequently, the stabilized bran was packed in specific weight aggregates in securely sealed bags made of polyethylene and kept in the freezer at a temperature of -27°C until use.

Rice Bran Oil Extraction

In the extraction process, the method used by (Amarante *et al.,* 2014) was followed, with some modifications. RBO was extracted using absolute ethanol as a solvent, a 1:3 ratio of bran to ethanol was used, and in most extraction batches, 135 g of rice bran and 405 ml of ethanol were added to a 1-liter beaker. The ethanol was preheated to 40 °C before adding it to the rice bran. The mixture was then thoroughly mixed, and the extraction was carried out at a temperature not exceeding 40°C with continuous stirring using a Magnetic stirrer for 90 minutes. After extraction, the rice bran was filtered through a cloth with fine pores and washed using an amount equal to 15% of the solvent quantity used in the extraction process. The filtrate was collected using centrifugation at ≈ 4032 ×g, 25°C for 7 min, the sediment was then discarded, and the filtrate containing the oil and solvent was evaporated using a rotary evaporator at 45 °C. The RBO was eventually obtained and kept refrigerated in a sealed dark glass container after the oil had been properly filtered via 0.45 μm filter paper.

Preparation of Nanoemulsion

The nanoemulsions were prepared according to the method (Cuomo *et al.,* 2019) with some modifications. The nanoemulsions were prepared with an oil content of 5% and three different concentrations of co-emulsifiers (sodium caseinate and Tween 80), and these concentrations were (3, 4, 5%) respectively, with a 1:1 (w/w) mixture ratio, which is equal to (1.5, 2.0, 2.5% w/w) for each, the concentrations of sodium caseinate and Tween 80 chosen were based on preliminary experiments that showed the instability of emulsions prepared with lower percentages of emulsifiers. As for water, it was (92, 91, and 90%), respectively (Table 1). Sodium caseinate and Tween 80 were dissolved in deionized water and the solution was left on the magnetic stirrer at a speed of 100 rpm for two hours at 25°C±1. Thus, the aqueous phase was prepared. After that, the Amber rice bran oil was added drop-by-drop to the aqueous phase while mixing with a hand mixer for five minutes. This process resulted in the formation of the primary emulsion. Subsequently, this emulsion was subjected to homogenization for 10 minutes at a speed of 10,000 rpm, while ensuring that the temperature of the emulsion did not rise during homogenization by placing the emulsion in an ice bath throughout the treatment process and monitoring the temperature. Then the emulsion was subjected to ultrasonication to achieve nano-sized droplets using an ultrasound device (LC-JY96-IIN, 150W, probe6). The device was set to 70% capacity for five minutes with a duty cycle of 5 seconds on and 5 seconds off and in the presence of an ice bath throughout the treatment period to prevent an increase in the nanoemulsion's temperature. The nanoemulsion was then placed in glass vials with an airtight seal and stored at 25°C±1until the examinations specific to characterizing the nanoemulsion (Figure 1).

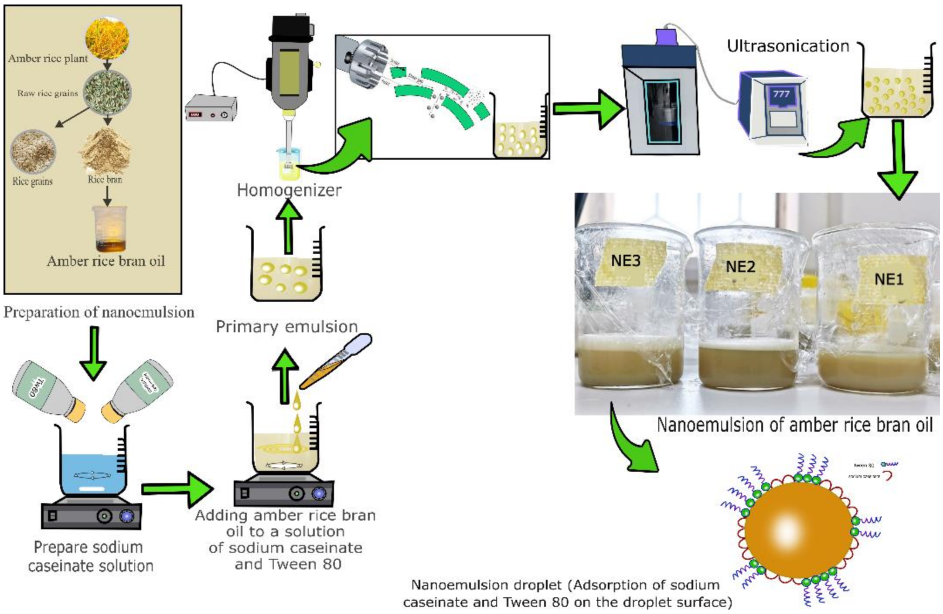

Figure 1: Schematic representation of nanoemulsion preparation from ARBO.

Table 1: Composition of materials used in the formation of the nanoemulsion.

Nanoemulsions sample	Oil%	Water%	Emulsifiers % Sodium caseinate %	Emulsifiers % Tween 80%
NE1	5%	92%	1.5%	1.5%
NE2	5%	91%	2%	2%
NE3	5%	90%	2.5%	2.5%

NE1=3%, NE2=4%, NE3=5%.

Nanoemulsion Characterization

pH Value

The pH of the nanoemulsion samples was measured 24 hours after preparation using a pH meter (PHS-.2C, SOONDA), of Chinese origin.

Viscosity

The viscosity of the newly prepared nanoemulsion samples was measured using a Brookfield DV-ETM viscometer. The device was set at 100 rpm and spindle number 1 was used at 25 °C (Choi *et al.,* 2011).

Encapsulation Efficiency (EE)

The amount of unencapsulated oil (also known as free or surface oil) present on the surface of the nanoemulsion powder was measured using the method described by (Tan *et al.,* 2005) with some modifications. A quantity of 5 ml of each of the prepared nanoemulsion samples, was dried at a temperature of 37 °C, for 24h and its weight was recorded after ensuring that it was entirely dry. The dried powder was then placed in a 40 ml glass bottle with a screw cap and 15 ml of hexane was added to the powder and shaken for two minutes at 25°C±1 to extract the free oil. The solvent mixture was then poured and filtered through Whatman filter paper No.1. The collected powder washed was rinsed on the filter paper three times with 20 ml of hexane each time by passing it through the powder. The remaining powder was then dried to evaporate all remaining solvents at 40°C until a constant weight was achieved. The free oil content was then calculated as a percentage of the weight difference in the powder before and after extraction and washing with hexane. The following formula was used to determine the encapsulation efficiency:

$$\text{Encapsulation efficiency (EE)\%} \frac{\text{total oil} - \text{free oil}}{\text{total oil}} \times 100$$

Studying the Morphological Characteristics of Droplets using a Field Emission-Scanning Electron Microscope (FE-SEM).

The morphological characteristics of the prepared nanoemulsion were examined using a Field Emission-Scanning Electron Microscope (FE-SEM) type (Inspect F50 FE-SEM) of Dutch origin. According to (Benito-Román *et al.,* 2020), the samples are sprayed with gold, inserted into the device, and observed at up to 120,000 x magnification.

Stability of Nanoemulsion

Storage stability

The stability test of the nanoemulsions was conducted by storing at room temperature is about 25°C±1 in the dark for 14 days.

Creaming Index (CI)

Creaming index was also used to confirm the stability of the nanoemulsions since it shows the extent of oil separates and creates a layer on top. After the NEs were prepared, 20 mL of each sample was put in sealed tubes, as stated in (Ferreira *et al.,* 2010). Keep at 25°C in the dark. CI was determined by measuring the heights of the vial's total emulsion (Ct) and cream layer (Cc) on the first, seventh, and fourteen storage days. This formula can be used to determine CI:

$$\text{Creaming Index (CI)} \frac{\text{total height of the cream layer (Cc)}}{\text{total height of nanoemulsion in the tube(Ct)}}$$

Zeta Potential

The zeta potential of the nanoemulsions was measured for the three periods (1, 7, and 14 days) using the (Zeta Plus) device (Brookhaven, USA) which was used to measure (Zeta Potential and Particle Size Analyzer), according to (Shakeel *et al.,* 2013) with some modifications. Samples were prepared for the zeta test by adding 1-3 drops of each sample to potassium chloride diluent, depending on the concentration sample. Then the formed sample is shaken well inside the glass container in preparation for measurement inside the device. Then approximately 2 ml of the sample is placed inside the cuvette, and the results are taken through the data processing unit. Measurements were performed in triplicate for each concentration.

Droplet Size and Poly Dispersity (PDI)

Dynamic light scattering (DLS) determined the drop size of nanoemulsions prepared with different concentrations and at three periods (1, 7, and 14 days) using Brookhaven, Zeta Plus (USA) according to (Shakeel *et al.,* 2013) with some modifications, where each model of nanoemulsion was diluted with potassium nitrate at a concentration of 10 mM, placed in a cell with a plug, and then measured. The polydispersity index (PDI), was also determined by DLS, the test was conducted in triplicate for each concentration.

Data Analysis

All measurements were conducted in triplicates, and the standard error (SE) was used. Data analysis was performed using the (SAS) program, and the data were analyzed (one-way ANOVA). Significant differences were determined at the probability level of ($P > 0.05$).

RESULTS AND DISCUSSION

The prepared nanoemulsions will be referred to as NE1, NE2, and NE3 relative to the concentrations of emulsifiers used (3,4,5%), respectively. This formulation was chosen because it yielded a stable nanoemulsion based on the preliminary experiments we conducted. Additionally, previous studies by (Perugini *et al.,* 2018) indicated that nanoemulsions prepared using a mixture of sodium caseinate and Tween 20 were stable and stable until at an acidic pH. (Nongnuan & Charnvanich, 2020) mentioned that nanoemulsions prepared using a mixture of sodium caseinate and Tween 80 had the smallest droplet size. It should be noted that the NEs had a slight transparency after preparation, which can be observed in (Figure 1).

It is crucial to consider pH changes or the presence of ions in the formulation of the nanoemulsion when designing the formulation since they can have intricate effects on the emulsion system. They can also affect the stability of the nanoemulsion during the preparation stage (Ganta *et al.,* 2014). The distribution and size of droplets are also impacted by pH, which influences the interfacial interactions between the emulsion and the oil/water phases (Goyal *et al.,* 2019). A pH close to (7) promotes optimal stability by maximizing the electrostatic repulsion between droplets, preventing aggregation and sedimentation (Onaizi, 2022). The pH of the nanoemulsions was tested 24 hours after preparation, pH was close for the three nanoemulsions, and there was no significant difference at the probability level

(0.05>P) (Figure 2), where it was as follows (NE1<NE3<NE2) and with a value of (5.94,6.04, 6.13) Respectively, this degree for the nanoemulsions was close to the neutral point, indicating that the nanoemulsions had stability properties. The specific stability ranges depend on the type of surfactant, the composition of the aqueous phase, and the oil phase (Chen *et al.,* 2022). Sodium caseinate used as one of the emulsifiers in the preparation of nanoemulsions is affected by the pH of the medium, as precipitation and aggregation of sodium caseinate and disintegration and collapse of the nanoemulsion structure can occur at the isoelectric point (pI) of sodium caseinate, which is equal to (pH = 4.6). The pH results of the nanoemulsion were close to the results of (Nongnuan & Charnvanich, 2020), who reported that the pH of their nanoemulsion was between (6.62-7.09). Bains et al. (2022), also indicated that the pH of the monolayer nanoemulsion was (7.08).

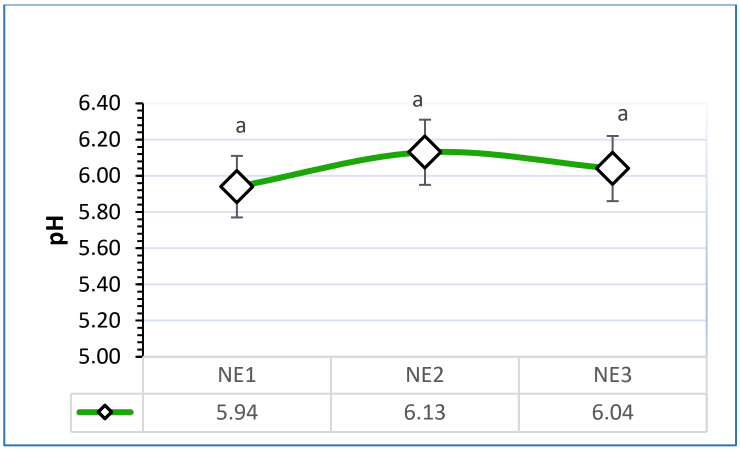

Figure 2: pH values for the nanoemulsion of Amber rice bran oil NE1, NE2, NE3; all data are presented with standard error (SE) and represent the means of three replicates (n=3). Similar letters indicate no significant differences at the probability level (P>0.05).

Viscosity, a crucial property of nanoemulsions, significantly impacts their stability. The viscosity values obtained for the formulations NE1, NE2, and NE3 were relatively low, ranging between 3.5 to 6.2 centipoise (cP), showing significant differences among the three nanoemulsions at a significant level of (0.05>P) (Figure 3). The relationship between the concentration of emulsifiers and viscosity can be observed, as an increase in the emulsifier concentration corresponds to a proportional increase in viscosity, it can be noted that as the concentration of emulsifiers increases, the viscosity value increases accordingly. Tween 80's structure is characterized by the presence of a large number of polyoxyethylene groups, which are hydrophilic in nature, with a water-oil balance of 15 (HLB=15) which gave Tween 80 the tendency to absorb the aqueous phase. As a result, increasing the concentration of Tween 80 reduces the free water in the nanoemulsion composition, leading to an increase in viscosity (Farooq *et al.,* 2021). The present findings align with those of Nongnuan & Charnvanich (2020), who used 10% RBO, and with Perugini et al. (2018), who used 5% RBO, in both studies, the viscosity of the nanoemulsion increased with increasing emulsifier concentration. Alfaro (2012) reported a viscosity of 20 (cP) for nanoemulsion prepared using 5% sodium caseinate and 10% oil.

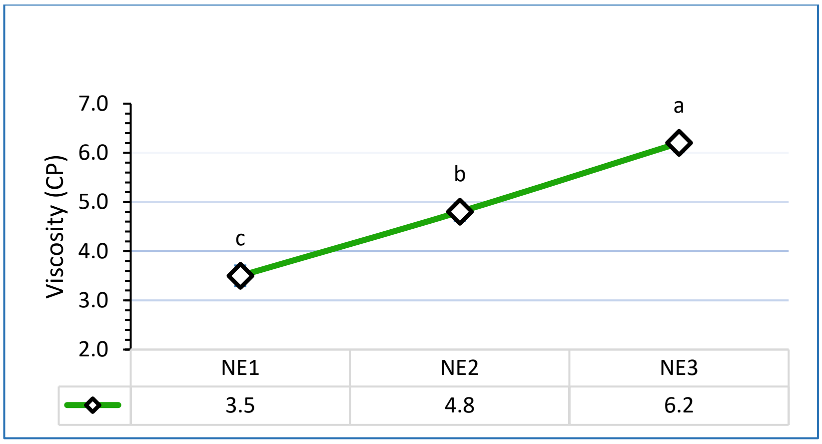

Figure 3: the viscosity value in the three formulations of nanoemulsions for Amber rice bran oil, NE1, NE2, NE3. All data were presented with standard error (SE) and equal to averages for three replicates (n=3). Different letters within the values of storage periods for each formulation indicate the presence of significant differences at the probability level (P>0.05).

Encapsulation efficiency (EE) is a critical parameter in nanoemulsion systems, as it determines the amount of material encapsulated within the nanoemulsion droplets. (Figure 4) illustrates the encapsulation efficiency of ARBO, where the highest efficiency encapsulation value was obtained in the NE3, which exhibited a significant difference at the probability level (0.05>P) from the nanoemulsions compared to NE1 and NE2, which did not differ significantly from each other. The order of nanoemulsions in terms of encapsulation efficiency was as follows (NE1<NE2<NE3) with values of (95.95, 96.60, and 97.60 %), respectively. The type and concentration of the emulsifier have a significant impact on the encapsulation efficiency of the nanoemulsion, as the increase in the concentration of the emulsifier led to an increase in the encapsulation efficiency because increasing the amount of encapsulating materials around the oil drop works to protects the oil more effectively and prevents its leakage (Ruengdech & Siripatrawan, 2022). Various procedures such as displacement of protein molecules, competitive adsorption, and strong surface protein interaction. As an outcome, protein molecules form a thick, viscoelastic, multilayered layer at the interface that contains tween between them, forming a layer around the oil droplet to prevent destabilization (Das *et al.,* 2023). Report (Perugini *et al.,* 2018) that the droplet surface coated with both emulsifiers benefited from two different stabilization mechanisms, the electrostatic repulsion of protein excipients in the formulation and the other being the static stabilization resulting from the presence of tween in the system.

Sodium caseinates are high molecular-weight protein emulsifiers that protect sensitive compounds against thermal degradation, oxidation, and the effects of free radicals by forming strong interlayers that inhibit physical processes. It also has molecular flexibility and surface hydrophobicity, the contact of protein biopolymer chain segments with the interfacial layer causes 100% coverage of the droplet interface, which enhances the effectiveness of protecting sensitive compounds (Marhamati *et al.,* 2021; Weigel *et al.,* 2018). The encapsulation efficiency of the nanoemulsion was higher than the encapsulation efficiency of nanoemulsions obtained by (Benito-Román *et al.,* 2020) when encapsulating

RBO using pea protein and maltodextrin, as the encapsulation efficiency was approximately 74%.

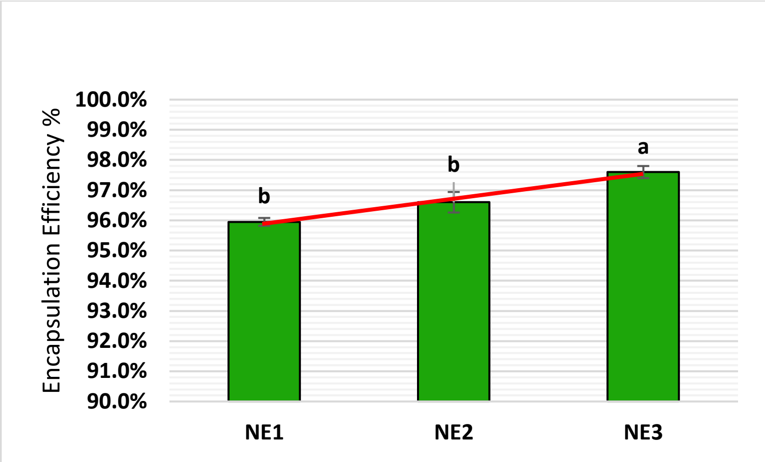

Figure 4: Encapsulation efficiency of ARBO in the three nanoemulsion formulations NE1, NE2, NE3; All data are presented with standard error and equal to averages for three replicates (n=3). Different letters indicate significant differences at the probability level (P>0.05).

One important component of nanoemulsions is the droplet shape. The emulsifier's capacity to adsorb at the oil-water interface and lower surface tension is critical to the stability of nanoemulsions, through the formation of viscoelastic interfacial multilayers around the droplets, proteins can improve the stability of the nanoemulsion against droplet coalescence (Salminen *et al.,* 2019). A morphological examination of the prepared nanoemulsions was conducted using a Field Emission-Scanning Electron Microscope (FE-SEM). The images revealed that the droplets of the nanoemulsions (NE3, NE2, NE3) were spherical and distributed uniformly. It is evident in the images that the oil droplets are well coated and All concentrations of emulsifiers were used (Figure 4). Thus, the FE-SEM micrograph corroborated the findings obtained from droplet size analysis using DLS, as the PDI results indicated that the nanoemulsion droplet had a uniform distribution. The image of NE2 image revealed the presence of merging and agglomeration in the nanoemulsion droplets. This explains what was observed in the change that occurred in the results of the tests for the size of the particles and their zeta potential, as the results indicated an increase in the particle size on the seventh day. The presence of small droplet aggregations in various areas of nanoemulsions is not significant and is common in nanoemulsions (Bains *et al.,* 2022; Galvão *et al.,* 2018), our results align with the latter, who indicated that the morphology of the nanoemulsion was smooth and the nanoemulsion droplets were spherical.

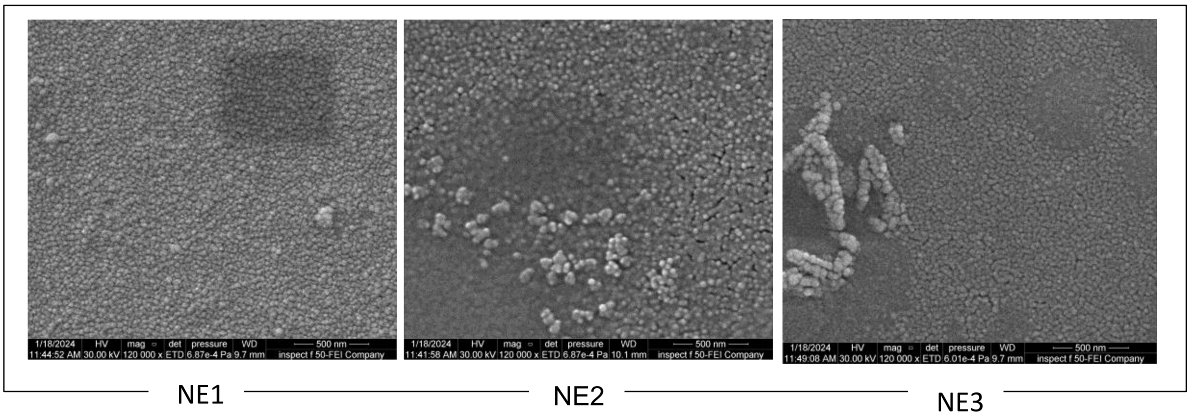

Figure 5: Microscope images (FE-SEM), showing the morphological characteristics of the nanoemulsions prepared from amber rice bran oil, formulated with a combination of Tween 80 and sodium caseinate (1:1) in three formulations NE1, NE2, NE3; at concentrations of 3%, 4%, and 5%, respectively.

The stability and Creaming index (CI) of the NEs were evaluated over three periods: Days 1, 7, and 14 (Figure 5). Based on the findings, the CI was zero for all nanoemulsions on the first day. After seven days, an increase (Creaming index) was observed for both NE1 and NE2, with a significant difference at a probability level ($P>0.05$), where it reaching 0.80% and 0.65% respectively, while the NE3 maintained a zero Creaming index (Figure 5-B), These results can be linked to the difference in droplet size of the oil, as increasing the concentration of the emulsifiers enhanced the oil droplets' electrostatic repulsion with one another and the aqueous medium, which in turn significantly improved the stability of the NE3 compared to NE1 and NE2 (Oliyaei *et al.,* 2022). On day 14, the Creaming index for all nanoemulsions increased significantly over day 7, as can be seen in the (Figure 5-A). The increase was slight for NE1 and NE2 compared to day 7, while the rise in the (CI) for NE3 can be attributed to the decrease in the negative charge of the nanoemulsion. This appeared in the (Zeta potential) test, which in turn led to a decrease in electrostatic repulsion, which affected the stability of the nanoemulsion. The Creaming index is inversely proportional to the increase in viscosity, which explains that as the concentration of the emulsifier (sodium caseinate) increases, the viscosity increases, leading to increased nanoemulsion stability (Cheong *et al.,* 2016). Droplet size is a critical factor in the stability of nanoemulsions and greatly affects their stability, studies have shown that nanoemulsions with small droplets, less than 200 nm in diameter, enhance the stability of these nanoemulsions against separation due to gravity, formation of droplet aggregates, and Ostwald ripening. Moreover, ultrasonic emulsification is a powerful method for preparing nanoemulsions; The mechanical effect of ultrasound waves, represented by shear stress, turbulence, and cavitation, contributes to reducing the size of the nanodrop and thus improving the stability of nanoemulsions (Liu *et al.,* 2019). In general, based on the results, the prepared nanoemulsions it was relatively stable during storage periods and at all concentrations of emulsifiers used. Our results agreed with (Perugini *et al.,* 2018) who indicated that RBO nanoemulsion prepared from a mixture of sodium caseinate and Tween 20 was stable even at the neutral point of sodium caseinate. Our results were better than those of (Benito-Román *et al.,* 2020), who indicated that the nanoemulsion prepared from RBO using pea protein and dextrin showed signs of instability in less than 24 hours of storage at 25 °C.

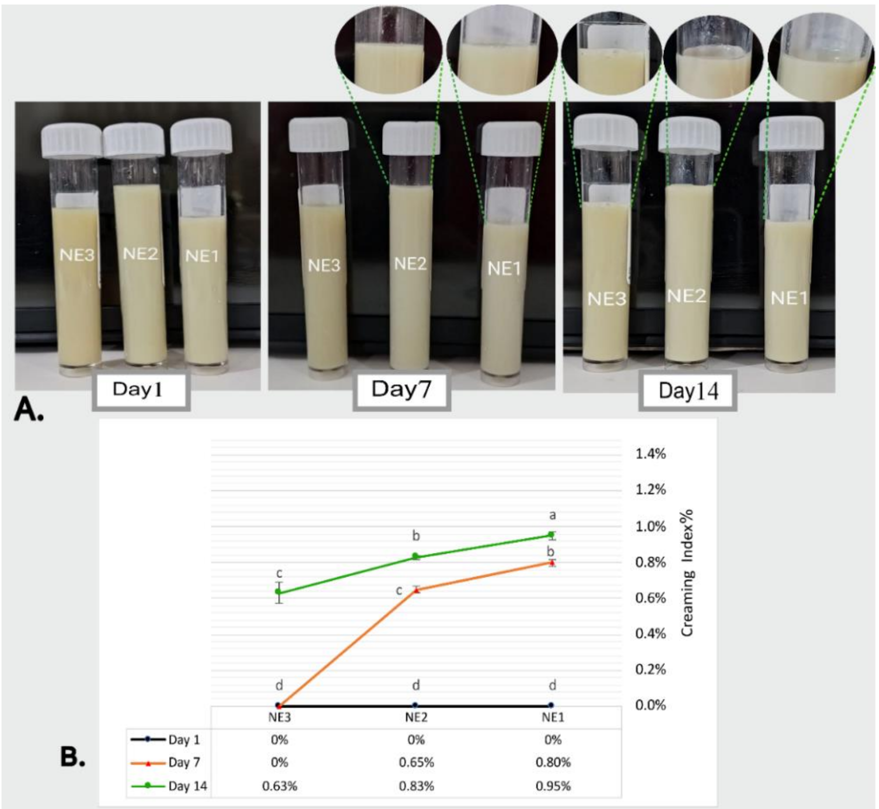

Figure 5: (A) Images of the stability of nanoemulsions (B) Creaming index in the three formulations of ARBO nanoemulsions NE1, NE2, NE3; during storage periods (Day1, Day7, Day14). All data are presented with standard error (SE) and represent the mean of three replicates (n=3). Different letters indicate significant differences at the probability level (P>0.05).

Zeta potential was used to measure the extent of electrical repulsion between molecules, aiming to ensure the stability of nanoemulsions. Based on the findings, all NEs contain a negative charge, as the zeta potential values of the prepared nanoemulsion formulations ranged from -18.52 to -31.51 mV. Since the force of repulsion between the droplets is greater than the force of attraction between them, the zeta potential values that are negative aid in the stability of the nanoemulsion during storage by preventing the agglomeration and coalescence of the dispersed phase of the nanoemulsions (McClements, 2012). The highest zeta potential value was recorded for the first nanoemulsion (NE1) on the seventh day of storage (figure 6). The results showed that, as the storage period increased, the value of the negative zeta potential increased on the seventh day for all prepared nanoemulsions. Although this increase was not significant in the two nanoemulsions (NE1, NE2) at a significance level of (0.05>P), it is an indicator of increased repulsion between the droplets and the nanoemulsions' good stability on the seventh day. On day 14, this increase decreased slightly in the nanoemulsions and was a significant decrease in the nanoemulsion (NE3) at a significance level (P>0.05). It was also observed that with increasing concentration of emulsifiers, the zeta potential value decreased, as in the two nanoemulsions (NE2, NE3) it was lower than its value for the first nanoemulsion on the first day. The reason can be attributed to the effect of Tween 80, as it works to replace the -OH group from the water adsorbed on the surface of the nanoemulsion droplet with the hydrophilic ether group of Tween 80, as increasing the concentration of Tween 80 reduces the value of the negative charge (Nongnuan & Charnvanich, 2020), who concluded in his study that the zeta potential of nanoemulsions was between (-19.97 to -26.09 mV), our results were close to his results. It was also close to the results of (Bains *et al.,* 2022), whose zeta potential value was between (-32.1 to -33.4), and who stated that the distribution of the negative charge and its variation in the nanoemulsion samples is due to the arrangement of the emulsifiers and oil. The zeta potential value for single-layered nanoemulsions prepared by (Razavi *et al.,* 2021) was around (-38.5 mV).

The physical stability of the nanoemulsion is revealed by the negative zeta potential value of nanoemulsions, as the strong electrostatic repulsion between molecules prevents droplet aggregation (Koo *et al.,* 2019). NE1 was the best among them, as there was no significant difference in its zeta potential values at the probability level (P>0.05) throughout the storage period. Despite the variation in the value of zeta potential between the three nanoemulsions, they all maintained negatively charged during storage, indicating good electrostatic repulsion.

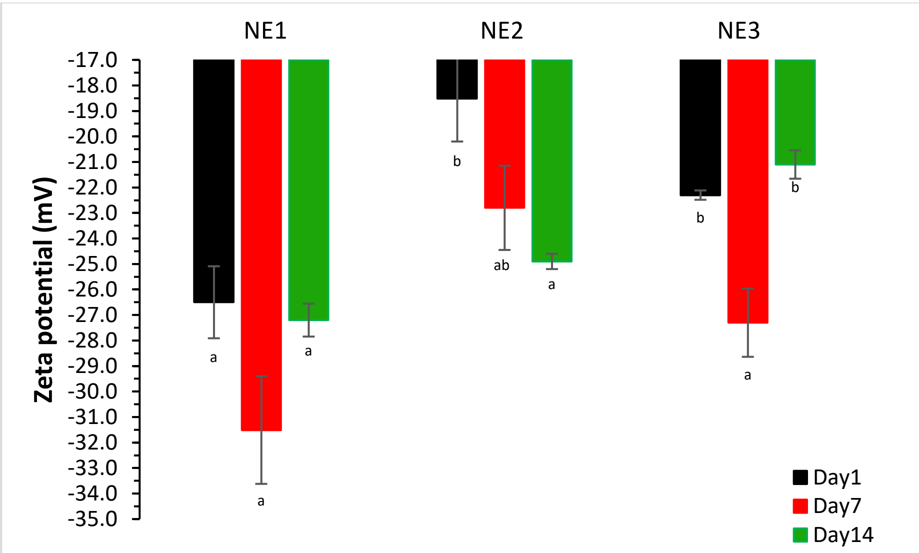

Figure 6: Zeta potential of rice bran oil nanoemulsions (NE1, NE2, NE3) and the change in it depending on storage periods over 14 days; Every data point is shown as the standard error (SE) and equal to averages for three replicates (n=3). Different letters within the storage periods of each nanoemulsion indicate the presence of significant differences at the probability level (P>0.05).

Particle Size and Polydispersity Index (PDI)

The droplet size of nanoemulsions is one of the critical parameters that must be considered because the destabilization of nanoemulsions is related to the size of their droplets due to the upward movement of the droplets, as smaller droplets size exhibits less tendency to coalesce or separate, thereby contributing to enhanced stability of the nanoemulsion (Fioramonti *et al.,* 2019). (Figure 7) illustrates the particle size and polydispersity index of nanoemulsions. The current study showed that on the first day, the droplet diameter of the ARBO nanoemulsions was as follows: NE3<NE2<NE1, with a size of 89.4, 93.3, and 118 nm, respectively, NE3 exhibited the smallest droplet size. From this result, it can be observed that an increase in the concentration of emulsifiers led to a decrease in the size of the droplets occurred, due to an increase in the thickness of the interface and its stability due to electrostatic repulsion interactions and reduced in the interfacial tension (Pal *et al.,* 2019). This is consistent with the findings (Cuomo *et al.,* 2019; Nongnuan & Charnvanich, 2020; Perugini *et al.,* 2018) pointed out in their work, as the amount of surfactants rises the size of the particles typically decreases because of the lower surface tension, at the boundary, between water and oil and the stabilized droplet sizes. This means that increasing the concentration of emulsifiers reduces the tension at the oil-water boundaries that occurs when these two liquids meet, which makes it difficult for larger droplets to combine and form larger droplets (Komaiko & McClements, 2015).

The effect of storage on the nanoemulsions, showed a significant increase in particle size for all nanoemulsions (NE1, NE2, NE3) at a probability level (P>0.05), and NE2 had the largest share in the increase on the seventh day in both particle size as well as PDI, the increase was from (93.3 nm to 118) and from (0.266 to 0.291), respectively. This can be explained by the zeta potential value of NE2 previously (Figure 6), as it had the lowest negative charge on the first day, which was an indication that it would have an increase in the size of the droplets as a result of the possibility of merging and coalescence of the nanoemulsion droplets due to the weak force of repulsion between them. Despite this increase, NE2 was the only nanoemulsion in which the droplet size was stable, as it remained consistent with the droplet size as well as the PDI, with no significant difference occurring between the two periods (day 7 and day 14) at the probability level (P>0.05). These indicators also return to the value of the negative charge that he was carrying during this period, as there was no significant difference between the value of his zeta potential between the two periods (day 7 and day 14) at the probability level (0.05>P).

The polydispersity index is used to evaluate the size distribution of particles in nanoemulsions, its value indicates the uniformity and stability of the size distribution of particles, PDI values typically range from 0 to 1, a Polydispersity Index (PDI) value below 0.3 indicates homogeneous size (the droplets in the emulsion are uniform in size and not significantly uneven), while a PDI value above 0.5 indicates large particles and an uneven distribution, signs of instability in the nanoemulsion (Nirmal *et al.,* 2023).

The PDI values of all the nanoemulsions (NE1, NE2, NE3) prepared were below 0.3 (Figure 7), indicating that all the nanoemulsions had good droplet size distribution. On day 1, the order of particle size distribution was as follows: NE2 < NE3 < NE1. Regarding the effect of storage on Polydispersity (PDI), there was no significant difference in the nanoemulsions (NE1, NE3) at the probability level (P>0.05), except for the change that occurred in NE2 on the seventh day mentioned earlier. The size of nanoemulsion particles decreases with increasing concentration of the emulsifier used, which in turn leads to obtaining particles with a homogeneous and uniform distribution. The outcomes aligned with the findings of (Nongnuan & Charnvanich, 2020), which indicated that as the concentration of the emulsifiers used increased, the particle size of the nanoemulsion decreased with the decreased value of the polydispersity index (PDI). The results also agreed with (Cuomo *et al.,* 2019), who found increasing the concentration of the emulsifiers reduced the size of the nanoemulsion droplets, stabilized, and the stability of the nanoemulsion increased.

The particle size and PDI of the RBO nanoemulsion prepared using 5% sodium caseinate as an emulsifier were (199.2nm) and (0.10), respectively (Alfaro, 2012). A study was conducted by (Razavi *et al.,* 2021) to prepare a double-layer nanoemulsion, the single-layer nanoemulsion prepared using 4.5% RBO, sodium caseinate, and Tween 80 had a particle size of about (65 nm) and a PDI of (0.277) and was consistent with our results, he pointed out that the small size of the particles is because used emulsifiers in a greater percentage than the percentage of oil used. Perugini et al. (2018) also used 5% RBO and Tween 20 with sodium caseinate, and the size of the nanoemulsion particles prepared with the mixed emulsifier was less than 350 nm.

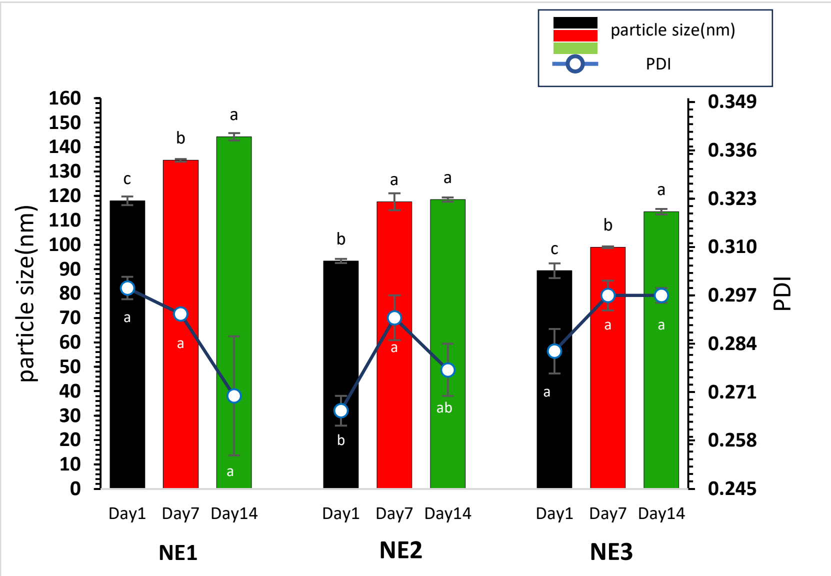

Figure 7: Illustrates the particle size and polydispersity index in the three formulations of nanoemulsions for Amber rice bran oil, NE1, NE2, and NE3, and their changes over storage periods. All data were presented with standard error (SE), and equal to averages for three replicates (n=3). Different letters within the values of storage periods for each formulation indicate the presence of significant differences at the probability level (P>0.05).

CONCLUSION

The current study focused on the preparation of nanoemulsion from Iraqi ARBO. A nanoemulsion was formulated and examined for its stability and properties. The NE3 emulsion was the most stable, as the honor index remained zero during the 7-day storage period and increased only slightly after 14 days. All nanoemulsions showed good particle size distribution. Droplets as small as 89.38 nm were obtained. The encapsulation efficiency of the droplets of oil was excellent. The pH of the nanoemulsions was close to the neutralization number, which indicates the stability of the nanoemulsions. Therefore, it can be said that the primary goal of our study has been achieved, which is to prepare a stable nanoemulsion of Iraqi ARBO, which could represent a promising solution to improve the bioavailability and stability of ARBO and the active and effective compounds it contains. The obtained results present in this study illustrated that encapsulating the oil using nanoemulsion technology that acts as a nano-delivery system can effectively help overcome the challenges faced by traditional delivery methods for these compounds which can be utilized in a variety of food, and pharmaceutical applications.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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