Double W/O/W emulsion is effective for carrying calcium and vitamin D

Duplo W/O/W emulsão é eficaz para transportar cálcio e vitamina D

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Marina Carvalho Martins Madalão
PhD in Food Science and Technology
Institution: Universidade Federal de Viçosa
Address: Av. P H Rolfs, s/n, Campus Universitário, Viçosa - MG, CEP: 36570-900
E-mail: marina.madalao@ufv.br

Andréa Alves Simiqueli
PhD in Food Science and Technology
Institution: Universidade Federal de Juiz de Fora, campus Governador Valadares
Address: R. Manoel Byrro, 241, Vila Bretas, Governador Valadares - MG, CEP: 35032-620
E-mail: andrea.simiqueli@ufjf.br

Pedro Rodrigues Minim
Master in Dental Sciences
Institution: Faculdade de Odontologia de Bauru da Universidade de São Paulo
Address: Alameda Dr. Octávio Pinheiro Brisola 9-75, Bauru – SP, CEP: 17012-901
E-mail: pedro.minim@usp.br

Ana Alice da Silva Xavier Costa
Master in Food Technology
Institution: Universidade Federal da Paraíba
Address: Rua dos Escoteiros, s/nº, Mangabeira VII Distrito Industrial de Mangabeira,
João Pessoa - PB, CEP: 58058-600
E-mail: ana.a.xavier@ufv.br

Luis Antonio Minim
Doctor of Chemical Engineering
Institution: Universidade Federal de Viçosa
Address: Av. P H Rolfs, s/n, Campus Universitário, Viçosa - MG, CEP: 36570-900
E-mail: lminim@ufv.br
Valéria Paula Rodrigues Minim  
PhD in Food Science  
Institution: Universidade Federal de Viçosa  
Address: Av. P H Rolfs, s/n, Campus Universitário, Viçosa - MG, CEP: 36570-900  
E-mail: vprm@ufv.br

ABSTRACT
A double emulsion is a promising system to facilitate the fortification process of nutrients such as calcium and vitamin D, as it protects vitamin D from oxidation and calcium from interactions with food proteins. The aim of this work was to produce W/O/W type double emulsions carrying vitamin D and calcium. The influence of the proportion of aqueous phase W_i/W_e (10/60, 15/55 and 20/70) and the concentration of polyglycerol polyricinoleate (PGPR) emulsifier (2.0, 2.5 and 3.0% w/w) on the kinetic stability of the emulsions over 13 days of storage was evaluated. For this, the following were analyzed: zeta potential, rheological properties (flow curve and viscoelasticity), entrapment efficiency (EE), and kinetic stability (KS) of calcium localization, microstructure, and macroscopic stability of the W/O/W emulsions, as well as the interfacial tension of the primary emulsions (W_i/O). The highest EE and KS of calcium localization were observed in treatments with smaller proportions of internal aqueous phase, regardless of the PGPR concentration. Therefore, an aqueous phase proportion of 10/60 combined with 2% PGPR is recommended.

Keywords: double emulsion, nutrients, kinetic stability.

RESUMO
Uma dupla emulsão é um sistema promissor para facilitar o processo de fortificação de nutrientes como cálcio e vitamina D, pois protege a vitamina D da oxidação e o cálcio das interações com as proteínas alimentares. O objetivo deste trabalho foi produzir emulsões duplas tipo Wi/O/We que transportam vitamina D e cálcio. Foi avaliada a influência da proporção de Wi/W_e de fase aquosa (10/60, 15/55 e 20/70) e a concentração de polirricinoleato de poliglicerol (PGPR) emulsionante (2.0, 2.5 e 3.0% p/p) na estabilidade cinética das emulsões durante 13 dias de armazenamento. Para isso, foram analisados: potencial zeta, propriedades reológicas (curva de fluxo e viscoelasticidade), eficiência de aprisionamento (EE) e estabilidade cinética (KS) de localização de cálcio, microestrutura e estabilidade macroscópica das emulsões W/O/W, bem como a tensão interfacial das emulsões primárias (Wi/O). Os maiores EE e KS de localização de cálcio foram observados em tratamentos com menores proporções de fase aquosa interna, independentemente da concentração de PGPR. Portanto, recomenda-se uma proporção de fase aquosa de 10/60 combinada com 2% de PGPR.

Palavras-chave: emulsão dupla, nutrientes, estabilidade cinética.
1 INTRODUCTION

Calcium and vitamin D are nutrients that play various roles in the body, and their deficiencies have been associated with several diseases. An important factor to consider is the synergistic effect of calcium and vitamin D. This is because calcium homeostasis is regulated mainly by the parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [1,25(OH)2D], which is the active form of vitamin D (Bacchetta et al., 2022; Jiang et al., 2018).

The deficiency of these compounds is still very common among the population, hence the need for strategies based on the production of foods enriched with these compounds (Machado et al., 2023; Borba et al., 2022).

Several aspects must be considered in the food fortification process so that the addition of the nutrient is carried out without altering the final characteristics and the sensory quality of the product. Vitamin D, being a fat-soluble compound, is susceptible to oxidation, which can give the product an unpleasant taste. Calcium salts may interact with soy proteins, promoting their coagulation (Casé et al., 2005). Therefore, technologies for nutrient incorporation into foods need to be studied, among which double emulsion stands out.

Double emulsions are known as "emulsion within an emulsion," and the most common types are W/O/W and O/W/O (Heidari et al., 2022). By having phases with distinct characteristics, it allows the incorporation of both hydrophilic and lipophilic compounds into its structure (Rincón-Fontán et al., 2019; Shaddel et al., 2018). Among the various advantages of this system, it prevents the oxidation of compounds (Serdaroğlu & Urgu, 2016). This makes it a suitable alternative for enabling nutrient fortification (Simiqueli et al., 2019a; Simiqueli et al., 2019b), such as calcium and vitamin D in foods, as it protects vitamin D from oxidation and prevents the interaction of calcium with food matrix proteins, maintaining the stability of the system.

Double emulsion has been allied with sensory studies, as it is a technological strategy that can be used both to mask the unpleasant taste of the product resulting from the addition of some compound (Simiqueli et al., 2019ab), and to enhance flavor, allowing for the reduction of compounds harmful to health, such as salt (Paula et al., 2017).
In this context, the objective was to evaluate the influence of the proportion of aqueous phase (Wi/We) and the concentration of emulsifier (PGPR) on the production of double W/O/W emulsions carrying calcium and vitamin D, as well as the entrapment efficiency (EE) of calcium and kinetic stability (KS) of the localization of calcium ions.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN

The experiment was conducted in a completely randomized design (CRD) with subdivided plots, with a 3² factorial in the plots, and storage time (1, 4, 7, 10, and 13 days) in the subplots. The factors assessed were different proportions between the internal aqueous phase (10, 15, 20 per 100 g of double emulsion) and the PGPR concentration (2.0, 2.5 and 3.0%). The simple O/W emulsion system was considered as the control treatment, without the addition of calcium chloride and vitamin D, totaling 10 treatments. The experiments were performed in triplicate. The effect of the proportions of internal and external aqueous phase, PGPR concentration, and storage time on the physical-chemical parameters under study were analyzed through ANOVA followed by regression analysis, at a 5% significance level, using R software. A Dunnet's test was also performed to compare the control with the other treatments.

2.2 PREPARATION OF EMULSIONS

The treatments contained the same concentrations of calcium chloride and vitamin D (1000 mg of calcium chloride and 0.0225 mg of vitamin D per 100 g of double emulsion). The addition of 40% double emulsion in the product corresponds to 14.4% and 60% of the recommended daily intake (RDI) of calcium and vitamin D, respectively, which allows it to be considered a product "added" with calcium and "enriched" or "fortified" with vitamin D (Brazil, 1998). The oily fraction was 30% (w/w) for all treatments, while the aqueous fraction (70%; w/w) was divided between the internal and external phases in proportions of 10, 15, and 20% and 60, 55, and 50%, respectively. The preparation of the double emulsions (Wi/O/We) carrying calcium and vitamin D was carried out in two stages, through the two-step procedure, as adapted from Simiqueli et
al. (2019b). Initially, the primary W/O emulsion was prepared, and then this dispersion was added as a dispersed phase in a second external aqueous solution (Wₑ).

### 2.2.1 Preparation of W/O Emulsion

#### 2.2.1.1 Preparation of the Internal Aqueous Fraction (Wᵢ)

To obtain the internal aqueous solution, 1000 mg of calcium chloride was added, along with potassium sorbate (0.05%; w/w) and gelatin (0.75%; w/w), prepared with deionized water at 90 °C. The gelatin was pre-hydrated for ten minutes, followed by magnetic stirring for thirty minutes (Sapei et al., 2012; Simiqueli et al., 2019b).

#### 2.2.1.2 Preparation of the Oily Fraction (O)

PGPR at concentrations of (2.0, 2.5 and 3.0% w/w) was dissolved in sunflower oil, containing vitamin D (0.075% w/w) and Sudan red dye (0.02% w/w). This system was subjected to magnetic stirring for thirty minutes at a temperature of 90 °C.

#### 2.2.1.3 Preparation of the Wᵢ/O Emulsion

To obtain the Wᵢ/O emulsion, different masses of the internal aqueous solution (10, 15, or 20 g) previously prepared were slowly dispersed into 30 g of the oily fraction (O), under constant stirring, in a high-speed homogenizer (T18 Ultra Turrax – IKA) at 20,000 rpm for five minutes. The emulsions were then cooled to 4°C for one hour to promote gelation of the gelatin.

### 2.2.2 Preparation of Wᵢ/O/Wₑ Emulsion

#### 2.2.2.1 Preparation of the External Aqueous Fraction (Wₑ)

To obtain the external aqueous fraction, an aqueous solution containing 2% (w/w) Tween 80 and 0.5% (w/w) guar gum was prepared. This system was subjected to magnetic stirring for 30 minutes at 50 °C. Then, potassium sorbate (0.05% w/w) and sodium chloride were added to balance the osmotic pressure between the aqueous phases of the W/O/W emulsion. The concentrations of sodium chloride were 1.35, 0.90 and 0.68
mol/L, corresponding to treatments with 10, 15 and 20% internal aqueous phase, respectively.

2.2.2.2 Preparation of the Double Emulsion (W_i/O/W_e)

To obtain 100 g of the double emulsion (W_i/O/W_e), 40, 45, and 50% (w/w) of the primary emulsion (W_i/O) were slowly dispersed into 60, 55, and 50% (w/w) of the external aqueous solution (W_e), respectively. The process occurred under stirring in a high-speed homogenizer (T18 Ultra Turrax – IKA) operating at 18,000 rpm for 4.5 minutes. Afterwards, the emulsions were stored at 4 °C.

The control treatment, referring to the O/A emulsion, was prepared without the addition of calcium chloride and vitamin D, containing 30% oily fraction and 70% aqueous fraction, characterizing a simple A/O emulsion. Thus, 30 g of oil dyed with Sudan red dye was slowly dispersed into 70 g of an aqueous solution containing 2% (w/w) Tween 80, 0.5% (w/w) guar gum, and 0.05% (w/w) potassium sorbate. The system was agitated in a high-speed homogenizer operating at 18,000 rpm for 4.5 minutes and stored at 4 °C until instrumental analysis.

2.3 INSTRUMENTAL CHARACTERIZATION OF DOUBLE EMULSIONS

2.3.1 Microstructure

The emulsions were previously diluted (10 times) in the external aqueous phase and applied on specific slides covered by glass lamellas. They were then subjected to analysis under an optical microscope with 1000 x magnification (Olympus CX40).

2.3.2 Zeta Potential

To assess the Zeta potential (ζ), 1 mL of emulsion was previously diluted in 25 mL of deionized water following the methodology adapted from Wang et al. (2011). To determine the Zeta potential of the emulsions, a Zetasizer Nano-ZS (Malvern Instruments Inc., Southborough, MA) was used at a detection angle of 173°, conducted at 25 °C.
2.3.3 Rheological Properties

The determination of the rheological properties of the double emulsions was carried out using a rotational rheometer (Discovery Hybrid Rheometer 1, TA Instruments, USA), with a stainless steel parallel plate sensor (diameter = 25 mm; gap = 1 mm), at 25.0 ± 0.1 °C. The rheological behavior was obtained with a shear rate of 0.1 to 200 s⁻¹ (upward ramp, downward and upward) for 180s.

The viscoelasticity of the emulsions was determined through the dynamic oscillatory test. The linear viscoelasticity region was determined by applying a strain sweep test (0.001 to 1%) at a constant frequency of 1.0 Hz. Subsequently, the parameters of the elastic (G’) and viscous (G”) modules were obtained through a frequency sweep test (0.1 to 10.0 Hz) and constant stress of 0.5 Pa (according to the linear viscoelastic range determined).

2.3.4 Entrapment Efficiency and Kinetic Stability of Calcium Localization

The entrapment efficiency (EE) of calcium and kinetic stability (KS) of the localization of calcium ions were determined by quantifying the concentration of Ca²⁺ ions in the external aqueous phase. Thus, the Ca²⁺ concentration present in the external aqueous phase configures the concentration of Ca²⁺ not trapped in the internal aqueous phase. The EE was determined on the first day of the formation of the double emulsion. The KS was quantified over the storage days of the system (4; 7; 10, and 13 days).

The emulsions were centrifuged and filtered according to the methodology of Simiqueli, et al. (2019b). Ions calcium was determined by the atomic absorption spectroscopy methodology, as per Seeger et al. (2019), in an atomic absorption spectrophotometer (model 220FS; brand Varian). The EE and KS of the localization of calcium ions were determined using equations 1 and 2, respectively (Simiqueli et al., 2019b).

\[
EA \text{ (%) } = \left(1 - \frac{C_w}{C_t}\right) \times 100
\]

Eq 1.
\[ EC(\%) = \left[ \left( 1 - \frac{C_w}{C_t} \right) - \left( 1 - \frac{E_A}{100} \right) \right] \times 100 \]

Eq 2.

Where:

Cw is the concentration of calcium in the external aqueous phase and Ct is the total concentration of calcium

2.3.5 Macroscopic Stability

The macroscopic stability of the double emulsions was evaluated through the creaming index of the emulsified systems, according to Simiqueli et al. 2019b), over 13 days of storage at a temperature of 4 °C, with measurements on days 1, 4, 7, 10, and 13.

2.4 INTERFACIAL TENSION

The interfacial tension of the primary emulsions (W/O) was evaluated by drop profile analysis tensiometry PAT-1 (Sinterface Tensiometer eK, Berlin, Germany). The experiment was conducted at a temperature of 25.0 ± 0.1 °C and the area of the drop was adjusted to 14 mm². The interfacial tension was determined by adjusting the Laplace equation to the drop profile, using the equipment's software (Sinterface Tensiometer PAT 1 vers. 8.01).

3 RESULTS AND DISCUSSION

3.1 MICROSTRUCTURE

The microstructure of double (W/O/W) and control (O/A) emulsions at one day of storage time is presented in Figure 1. The formation of the double emulsion was confirmed in all treatments (Figure 1A), wherein it water droplets were observed dispersed within the oil droplets. The control treatment, as proposed, has a simple O/A emulsion structure, showing oil droplets dispersed in water (Figure 1B).
3.2 ZETA POTENTIAL

The zeta potential value of the double emulsions varied between -5.068 mV and -6.548 mV. Despite the low electric charge density of the droplets, the emulsions showed stability over 13 days of storage. This indicates that other factors contributed to the kinetic stability of the emulsions, such as the increase in the medium's viscosity through the addition of guar gum.

All emulsions showed negative zeta potential values. It is worth noting that the surfactant used in the second emulsification was Tween 80, which is non-ionic and therefore presents electrical neutrality. It is believed that the negative charges may be relative to the free fatty acids present in commercial sunflower oil, which has a pH close to 3.4, in addition to ions from guar gum, which, although non-ionic, may contain ions that contributed to the electrical charge of the droplet. This hypothesis was raised by Simiqueli et al. (2019b) after measuring the zeta potential of the guar gum solution (0.075%, w/w) and finding $\zeta = -16.3$ mV.

The zeta potential ($\zeta$) was not influenced by the storage time factor ($p>0.05$) (supplementary material), corroborating the results obtained by Paula et al. (2017) and Simiqueli et al. (2019b). The proportion of the aqueous phase and the concentration of PGPR also did not significantly influence the zeta potential of the emulsified systems (supplementary material). All double emulsions differed statistically from the control.
simple emulsion (p<0.05), which presented a higher zeta potential value (supplementary material). This may have occurred because only the double emulsions contained salts in their formulations (sodium chloride and calcium chloride), capable of altering the electric charge density present on the surface of the oil droplets. The calcium chloride that diffused from the internal aqueous phase to the external aqueous phase, and the sodium chloride added to the external aqueous phase. Thus, the cations from the dissociated salts likely interacted with the negative ions of the oil droplet, reducing the density of negative electric charges, and consequently, contributing to the reduction of the zeta potential.

3.3 RHEOLOGICAL PROPERTIES

The most suitable mathematical model (R² > 0.99) to describe the rheological behavior of the emulsions was that of Ostwald-de-Waele (n < 1). Therefore, the apparent viscosity (η) decreases with the increase in the shear rate. This result was expected, since at high oil concentrations in the dispersed phase (30%; w/w), emulsions tend to exhibit pseudoplastic behavior (Campanella et al., 1995).

The highest apparent viscosity was obtained when combining the lowest internal aqueous phase ratio (10/60) with the highest concentration of PGPR (Figure 2). It is suggested that the treatment containing the lowest proportion of internal aqueous phase and the highest concentration of PGPR formed a smaller number of water droplets and smaller size during the first emulsification. This is because the internal aqueous phase was the smallest, and, at higher concentrations, PGPR covered the entire surface of the droplets, resulting in smaller diameter drops and fewer in quantity. Therefore, performing the second stage of emulsification to form the W/O/W emulsion may have facilitated the formation of oil drops of smaller diameter and a greater number of drops. The greater number of oil drops confers greater apparent viscosity to the system (Simiqueli et al., 2019b).

Apparent viscosity changed significantly over storage time (p < 0.05). It is observed that between the first and fourth day there was an quick drop in viscosity, which remained constant until the 13th day (Figure 3). This may have occurred due to two factors: the first, related to the diffusion of water from the internal aqueous phase to the
external aqueous phase, causing the reduction of viscosity. The second, related to the probable coalescence of the oily droplets, which culminated in the increase of the diameter of the droplets and, therefore, a reduction in the number of oily droplets dispersed in the external aqueous phase. Consequently, the apparent viscosity of the double emulsion decreased. Simiqueli et al. (2019b) also observed a reduction in apparent viscosity over the storage time of W/O/W emulsions carrying ferrous sulfate.

Figure 2. Apparent viscosity as a function of water proportion and PGPR concentration.

\[ \hat{y} = -1.3834 + 0.1468 x_1 - 0.0003 x_1^2 + 1.4184 x_2 - 0.0873 x_2^2 - 0.0641 x_1 x_2 \]
\[ R^2 = 0.7782 \]

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Overall, the apparent viscosity of the control emulsion did not differ from that of the double emulsions (supplementary material), because the proportion of water and oil (70/30) and the concentration of guar gum (0.5% w/w) were the same for both emulsions.

Figure 3. Gradient graph for apparent viscosity as a function of water proportion and PGPR concentration.

The double emulsions were also analyzed for viscoelasticity parameters: storage modulus (G’) and loss modulus (G’’). G’ is related to the elastic characteristic of the material, and G’’, to the viscous nature. Tg δ is the relationship between the viscous and elastic components of the system (Tg δ = G’’/G’) which gives an indication of the elastic or viscous nature of the solution.
In Figure 4 are the graphs of both parameters as a function of frequency (Hz). It is observed that all emulsions presented $G'$ values higher than those of $G''$, indicating the predominance of the elastic character. This characteristic can also be proven by the value of $Tg$ ($Tg \delta < 1$, for the entire frequency range evaluated: $0.1 - 10$ Hz). Simiqueli et al. (2019b) also found a predominance of the elastic character in W/O/W emulsions carrying ferrous sulfate and justified this result due to the addition of the thickener guar gum, also used in the present study. The polysaccharide in contact with water presents a viscous aspect due to its ability to "drag" water molecules in its chain and, consequently, contributes to the physical structuring of the system.

Figure 4. Average values of viscoelasticity moduli ($G'$ and $G''$) and $Tg$ of emulsions as a function of frequency, at $25 \degree C$.

- ♦ 2 % PGPR
- ◇ 2.5 % PGPR
- ▼ 3 % PGPR
- ◈ Controle

Source: authors (2024).
For the same proportion of aqueous phase, there was an increase in the elastic and viscous moduli with the increase of frequency (Figure 4), for all PGPR proportions. However, the largest increase was observed in the elastic modulus. The simple emulsion exhibited behavior like that of the double emulsions, with predominance of the elastic character and elevation of both moduli with the increase in the frequency.

3.4 ENTRAPMENT EFFICIENCY AND KINETIC STABILITY OF CALCIUM LOCALIZATION

The entrapment efficiency (EE) and the kinetic stability of calcium localization (EC) were evaluated over the 13 days of storage of the double emulsions.

It is observed in Figure 5 that all treatments presented EE greater than 90% and EC greater than 75%. Only the proportion of the aqueous phase significantly influenced (p<0.05) the EE and EC of calcium (supplementary material). The treatment containing the aqueous phase ratio of 10/60 showed the highest EE and EC of calcium localization, regardless of the PGPR concentration. The EE was close to 95% (first day of storage), reaching the end of the 13 days of storage with EC greater than 90%. For Dickinson (2011), the EE of bioactive compounds through double emulsions is considered satisfactory when it occurs close to 95%, and EC close to 70% after a few weeks. Complete efficiency is not possible to be achieved, as during the second emulsification process some internal droplets break, and consequently, there is a loss of calcium to the external environment. Thus, all the double emulsions proposed in the present study meet the requirement of kinetic stability, confirming the high potential for use of these emulsions as calcium carriers. Considering the results presented, double W/O/W emulsions prepared with an aqueous phase ratio of 10/60 are the most recommended for carrying calcium.
3.5 MACROSCOPIC STABILITY

The kinetic stability of the emulsions was also assessed in macroscopic scale. Images of the emulsions were taken from the first day after their formation (day 1) to the end of the storage period (day 13) (Figure 6). It is observed that, regardless of the proportion of the aqueous phase and the concentration of PGPR, the emulsions remained stable during the 13 days of storage, with a creaming index close to 0%. Therefore, all formulations evaluated were sufficient to maintain the emulsion carrying calcium and vitamin D throughout the entire storage time.
3.6 INTERFACIAL TENSION

The decay of interfacial tension of the primary emulsions (W/O) over time was determined using a pendant drop tensiometer, with the results shown in Figure 7. A significant reduction in interfacial tension was observed in all systems evaluated. This indicates that the PGPR emulsifier is quickly adsorbed at the water/oil interface, reducing the interfacial tension and, consequently, favoring the emulsification process. Treatments containing the highest concentration of PGPR (3% w/w) showed a lower rate of reduction of tension, which was quickly reduced. The lowest PGPR concentration evaluated was 2% (w/w); at this concentration, regardless of the aqueous phase ratio, there was a higher initial interfacial tension.

It is worth mentioning that, even at the lowest emulsifier concentration evaluated (2%), the initial interfacial tension rapidly reduced over time, confirming the excellent emulsifying property of PGPR.
Figure 7. Interfacial tension decay profiles of the systems composing the primary emulsions W/O with different aqueous phase ratios.

Source: authors (2024).

4 CONCLUSION

All evaluated W/O/W double emulsions showed kinetic stability over the 13 days of storage. The highest entrapment efficiency (EE) and kinetic stability (EC) of calcium localization were observed in treatments with lower internal aqueous phase ratios,
regardless of the concentration of PGPR. Thus, considering the best performance and lowest production cost, an aqueous phase ratio of 10/60; 2% PGPR is suggested for obtaining a double W/O/W emulsion carrying calcium and vitamin D. The double emulsion system has demonstrated its potential for carrying calcium and vitamin D to be applied in food production.

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