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Laboratory Experiment on Emulsions: Study of the Effect of Osmotic Pressure on Double Emulsions Preparation

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Abstract: Double emulsions are ternary systems commonly used in several disciplines in areas such as food technology, applied chemistry, chemical engineering, materials science, pharmacology and environmental science. In several courses related to these areas, the implementation of laboratory experiment is required to strengthen the knowledge acquired by students during the theoretical lessons. However, it is difficult to find published practical experiments in this field. This work presents a four-hour hands-on laboratory experiment in which students can easily formulate and prepare water-in-oil-in-water double emulsions for vitamin B_{12} encapsulation. In this experiment, students can analyze the effect of the osmotic pressure produced by the addition of different NaCl concentrations in each aqueous phase, which could lead to the swelling and deswelling phenomena of the inner aqueous droplets and, therefore, affect the encapsulation efficiency of the formulated systems. The double emulsions are analyzed by the students in terms of size and encapsulation efficiency.

Keywords: lab experiment; learning theories; colloids courses; double emulsions; encapsulation; osmotic pressure; droplet size distribution

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1. Introduction

In science education, laboratory experimentation plays a vital role in students' learning, enriching their knowledge and potential by increasing their interest, attention, and critical capacity, while validating the theoretical knowledge they have acquired during their lessons [1,2]. Laboratory activities have a central role in science curriculum, as they are many benefits from engaging students in a science laboratory. Since the early 1960s, laboratory experiments in science education have been used to engage students in investigation, discovery, inquiry, and problem-solving activities [3]. In this sense, in some chemistry and technical degrees, a final degree project is also carried out in a research laboratory [4]. This type of methodology represents a source of promising new insights on research in science [5]. Furthermore, laboratory lessons can also contribute to the development of students' manipulation, observation, cooperation, and social communication skills [6]. Moreover, a laboratory environment also offers a more pleasant environment, which is often less monotonous and less tense than that of a theoretical class environment.

Due to the wide applications of emulsions and other typical colloidal systems such as vesicles, hydrogels, colloidosomes, and hollow particles, the preparation and formulation of these systems are studied in several types of degrees and master's programs related to chemistry, chemical engineering, food technology, and pharmacology.

One of the eligible subjects of the Master in Chemical Engineering at the University of Oviedo (Spain) is *Emulsions and Suspensions Technology*, in which general knowledge about the synthesis and preparation of emulsions is explained during theoretical lessons. This is intended to strengthen the connection between the formulations learned in class and the manual skills acquired in the lab, by developing one of the three experiments performed in

the 10 h laboratory classes as part of the 34 h design of the eligible subject. Moreover, one of the theoretical hours is used for an introduction to the lab experiments, explaining the theoretical background, materials, and methods.

Due to the scarce information regarding the didactic material for colloidal systems, it is difficult to find practical experimental lab designs [7]. This article presents one of the laboratory experiments designed for students to acquire practical knowledge about the formulation of water-oil-water ($W_1/O/W_2$) double emulsions for the encapsulation of a bioactive compound (vitamin B_{12}) and the stability/instability mechanisms involved.

Different formulations were tested using sodium chloride (NaCl) to study the importance of the osmotic pressure equilibrium between both aqueous phases for the stability and release of the formulated $W_1/O/W_2$ double emulsions. Each formulation contains exactly the same number of stabilizers and water/oil ratios. However, the NaCl concentration in each aqueous phase differs from one formulation to another, which affects the biocompound encapsulation efficiency of the formulated double emulsions.

2. Theoretical Background

An emulsion is defined as a two-phase system consisting of at least two immiscible liquids with different compositions: one of them dispersed as drops in the other [8,9]. The drop-shaped liquid is called the dispersed phase or inner phase, whereas the liquid in which these drops are suspended is called the continuous phase or external phase. The structure of an emulsion is highly dependent on the volume fraction of the water, the oil, and a third component called the emulsifying agent or emulsifier, as well as the nature of the interfacial film. The most common situation corresponds to the simple emulsions formed by two liquids, one hydrophilic (water) and the other hydrophobic (oil).

Emulsions play an important role in the formulation of foods for the production of simple oil-in-water (O/W) emulsions (e.g., dressings or artificial milks) as well as for the preparation of water-in-oil (W/O) emulsions (e.g., margarine and low-fat spreads). Some emulsions are final products (e.g., coffee creamers), and other emulsions can be used as ingredients, helping to form the structures of more complex products (e.g., yogurts that must interact with other food ingredients but should not be destabilized in the process). However, emulsion droplets can also create new structures in a product (i.e., ice creams) where emulsion destabilization is required for this purpose [9].

Emulsions also have the ability to transport or solubilize hydrophilic or hydrophobic substances in a water phase, in which it is interesting to encapsulate bioactive compounds since these substances (vitamins and antioxidants, among others), presented as natural constituents in food, provide health benefits beyond the basic nutritional value of the product.

One of the most common type of emulsions used for encapsulation are multiple emulsions, which were first reported in 1925 by Seifriz [10]. The simplest multiple emulsions are double emulsions: they are ternary systems where the dispersed droplets contain smaller droplets of a different phase. They have either a water-in-oil-in-water ($W_1/O/W_2$) structure, where W_1 is the internal aqueous phase, O is the oily phase, and W_2 is the external aqueous phase, or an oil-in-water-in-oil ($O_1/W/O_2$) structure [8], where O_1 is the internal oily phase, W is the intermediate water phase, and O_2 is the external oily phase. The former ($W_1/O/W_2$) is the most commonly used type of double emulsions.

Double emulsions are complex systems with higher instabilities than simple emulsions due to their large and different interfacial areas. In addition to the conventional Ostwald ripening and the coalescence or creaming phenomena observed in simple emulsions, other instabilities related to swelling or deswelling can be observed in double emulsions. The swelling phenomena is related to the fact that the inner aqueous drops grow due to the migration of the external aqueous phase through the oil drops [11,12]. On the contrary, the deswelling phenomena is related to the migration of the inner water drops to the external aqueous phase, which decreases the concentration of the internal aqueous droplets and reduces their size (Figure 1).

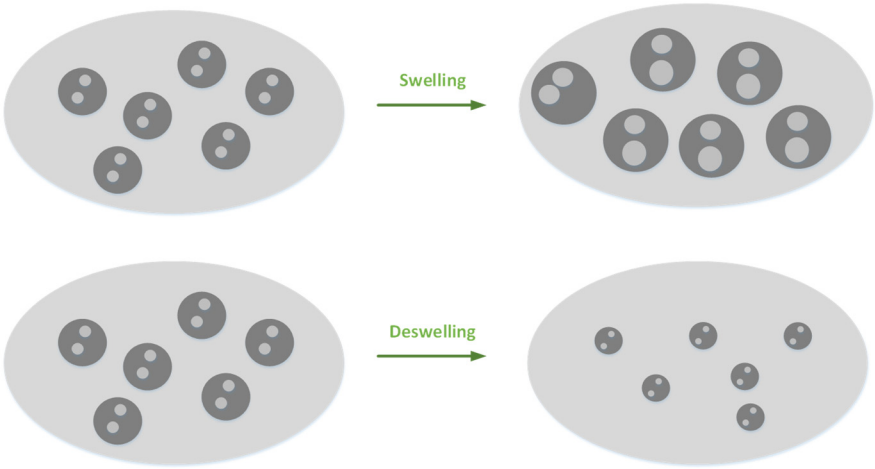

Figure 1. Schematic diagram of swelling and deswelling phenomena in double emulsions.

3. Lab Experiment

This lab experiment was performed in a 4 h session. The work was done in groups of two or three students each. Before the 4 h session, a lesson on emulsion theory was explained. Moreover, a one-hour session was included, where practical information was presented about the lab experiment.

3.1. Emulsions Preparation

The procedures used to prepare double emulsions and the selection of the components are similar to those used in previous research works [13–15], where satisfactory results were obtained in terms of stability, particle size, and encapsulation efficiency (EE). First, the three separate phases must be prepared: the internal aqueous phase (W_1), the oily phase (O), and finally the external aqueous phase (W_2). Vitamin B_{12} was selected as the biocompound to be encapsulated, since it is not an expensive material, it is feasible to be detected by conventional spectrophotometry techniques, and satisfactory results were obtained when it was encapsulated with these types of colloidal systems in previous studies [15]. The experimental protocol is described in detail in the student experimental handout (Appendix A).

3.1.1. Dispersed and Continuous Phases Preparation

W_1 phase (100 mL) was prepared with 0.5 M NaCl and 2500 mg/L vitamin B_{12} in MilliQ water. The oily phase (250 g) was composed of Miglyol® 812 (density 945 kg/m^3 at 20 °C, Sasol GmbH, Hamburg, Germany) and 5% *w/w* of polyglycerol polyricinoleate (PGPR, $C_{21}H_{42}O_6$, Brenntag AG, Essen, Germany), which acts as stabilizer of the W_1/O emulsion. Miglyol® 812 is a neutral oil formed by esters of caprylic and capric fatty acids and glycerol. PGPR was prepared by the esterification of condensed castor oil fatty acids and is a powerful water-in-oil emulsifying agent commonly used with limited concentration in food formulations; it is highly effective for stabilizing W_1/O emulsions, as demonstrated by several studies [11–16]. The critical micelle concentration (CMC) value for PGPR was estimated in previous studies by the Wilhelmy plate method and was found to be between 0.76 and 1.5% *w/w* in the oily phase [17]. PGPR and Miglyol® 812 were previously mixed under magnetic stirring for 30 min to ensure total solubilization of the stabilizer in the oil.

The W_2 phase (100 mL) consisted of MilliQ water and 2% *w/v* of Tween 20 nonionic surfactant (polyoxyethylene sorbitan monolaurate, Sigma-Aldrich, St. Louis, MO, USA) that acts as stabilizer of the O/W_2 interface. Tween 20 is a polyoxyethylene sorbitol ester that belongs to the polysorbate family, and its CMC is 60 mg/L.

Three different salt (NaCl) concentrations were used in the external aqueous phase W_2 to study the effect of osmotic pressure equilibrium between both aqueous phases: 0.1,

0.5, and 1 M. Each laboratory group of two students prepared a different concentration. All W_2 components were previously mixed under magnetic stirring for 10 min.

3.1.2. W_1/O Emulsion Preparation

The preparation of the internal emulsion (W_1/O) was performed in glass vessels by high-shear mixing (Silentcrusher M Homogenizer, Heidolph, Schwabach, Germany) using a 6 mm dispersing tool at 20,000 rpm for 5 min. The final emulsion had a total volume of 30 mL, with a volumetric W_1/O ratio of 20/80.

3.1.3. $W_1/O/W_2$ Double Emulsions Preparation

The primary W_1/O emulsion was used as the internal phase of the final double emulsion by redispersing it in an external aqueous phase (W_2). The ratio between W_1/O internal emulsion and W_2 was 20/80. For this second emulsification step, the Silentcrusher M homogenizer was also used but at a lower speed: 5000 rpm for 2 min. Emulsification was carried out in 15 mL graduated tubes.

Figure 2 summarizes the procedure described to prepare double emulsions by mechanical agitation in a two-step process.

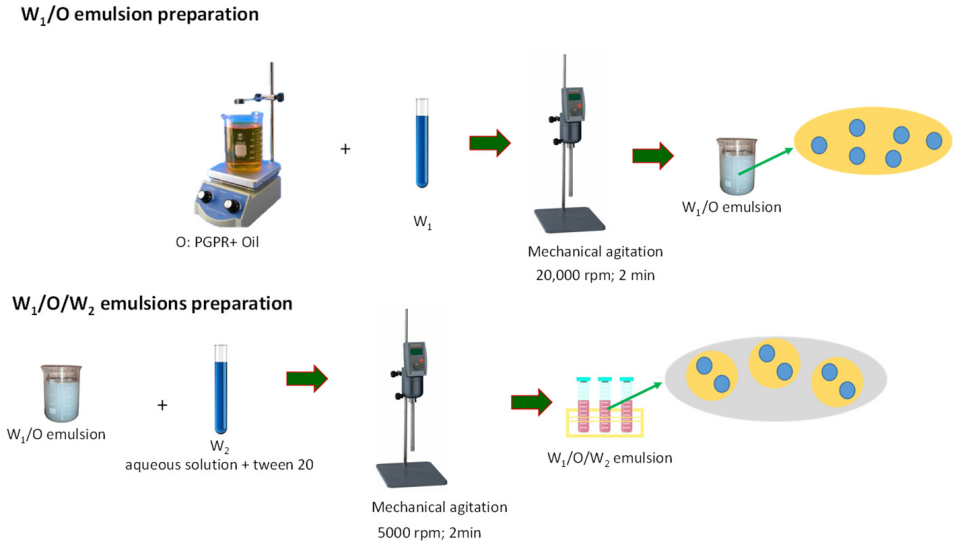

Figure 2. Double emulsion preparation by a two-step emulsification process.

3.2. Particle Size Characterization

Droplet size distributions of the prepared double emulsions were analyzed by laser light scattering using a Mastersizer S long bench apparatus (Malvern Instruments, Ltd., Malvern, UK). A refractive index of 1.54 was used. Samples were first diluted with deionized water to prevent multiple scattering effects.

Micrographs of the emulsions were obtained with an Olympus BX50 light microscope (Olympus, Tokyo, Japan) at 10–100× magnification using UV–Vis and fluorescence lamps, in order to confirm the particle sizes measured by the laser light scattering technique.

3.3. Measurement of the Clarification Layer

The clarification layer that appeared at the bottom of the graduated tube used for the second emulsification step was measured to determine the amount of W_2 present in the double emulsion and, therefore, whether a portion of W_1 was migrating to W_2. Samples were centrifuged at 10,000 rpm for 10 min to improve phase separation. This observation allowed students to evaluate the effect of the osmotic pressure on the difference in salt concentration between W_1 and W_2.

3.4. Encapsulation Efficiency Determination

The encapsulation efficiency (EE) of double emulsions is defined as the percentage of compound of interest in W_1 that remains in the primary emulsion (W_1/O) after the second emulsification step [18,19]. It can be calculated using Equation (1) [20].

$$EE = 100 - \frac{C_{recovered} \times 100}{C_0 R_y} \quad (1)$$

The encapsulation (or entrapment) efficiency of the bioactive compound should be determined after separation of the external aqueous phase. To do this, the external aqueous phase must be previously recovered by centrifugation at low speed (1000 rpm for 20 min) and filtration with a 0.22 µm polyvinylidene difluoride (PVDF) syringe filter, to eliminate all the cream oily phase still present. Then, the concentration of biocompound of interest in the recovered aqueous phase ($C_{recovered}$) is determined by measuring the absorbance in the external phase (W_2), which is correlated with its concentration using a calibration curve. An increase in the absorbance indicates the release of the inner phase to the external phase. It is necessary to have previously prepared a calibration curve of absorbance vs. biocompound concentration (Appendix B).

However, the recovery yield (R_y) must be determined to measure if any amount of the biocompound of interest was retained (loss) during the centrifugation and filtration processes, to avoid an overestimation of the EE values obtained [18]. Therefore, a standard emulsion, where 100% of the W_1 is present in W_2, was required for its determination. For this purpose, an oil-in-water emulsion (O/W_2) was prepared using the same formulation as in the experiments. This O/W_2 emulsion was then diluted at the same ratio with W_1, which contained the appropriate amount of biocompound of interest. For this analysis, a blank reference also needed to be prepared, consisting of an O/W_2 emulsion diluted with W_1, in which the marker (biocompound) was not present. Finally, the R_y was calculated by measuring the $C_{recovered}$ in the external aqueous phases using Equation (2):

$$R_y(\%) = \frac{C_{recovered} \times 100}{C_0} \quad (2)$$

where C_0 is the maximum concentration of biocompound expected in the external aqueous phase.

Vitamin B_{12} concentration in the aqueous phase, $C_{recovered}$, was determined at 361 nm wavelength using a T80 UV/VIS spectrophotometer (PG Instruments Ltd., Wibtoft, UK). A calibration curve was previously calculated following the instructions detailed in Appendix B.

Differences in NaCl concentration of W_1 and W_2 phases affect the EE and the corresponding release [20,21]. Therefore, several formulations were prepared by modifying the salt content in both aqueous phases, and then EE for vitamin B_{12} of the corresponding $W_1/O/W_2$ double emulsion was determined.

3.5. Hazardous

No hazardous materials are used in this lab experiment, so no additional requirement is needed.

4. Running the Experiment

The laboratory experiment is performed after 20 h of theoretical and practical exercise course so that the students have a background on the theoretical aspects of the formulation, preparation, and characterization of the emulsions. In addition to the theoretical concepts, the experiment is easy to run, and only basic lab skills are required. The student experimental handout used and the corresponding data collection sheet are detailed in Appendices A and C.

In the prelab hour, the procedure to be followed during the experiment is explained in detail to the students, indicating the instruments used, the concentration of each chemical, and the final amounts of the prepared simple and double emulsions.

Hence, before starting the lab experiment, the students must calculate the amounts of each chemical needed to prepare the W_1/O and $W_1/O/W_2$ emulsions. The experimental handout (Appendix A) is supplied to the students, as well as a data collection sheet (Appendix C) where they should indicate the amount of each component used for the preparation of each phase and emulsion.

Once each group has prepared the three necessary phases, the simple and double emulsions are formulated following the indicated procedure.

Double emulsions are characterized by laser light scattering in terms of mean droplet size and droplet size distribution. Microscopy is also used to assess the feasibility of the laser technique for droplet size measurement.

The formulated double emulsions are observed to evaluate the clarification layer of each emulsion after centrifugation takes place, to promote the migration of the oil droplets to the top of the graduated tube. This information could give the students an idea of the amount of W_1 that had migrated to W_2.

Once both aqueous phases are ready, each laboratory group should obtain the calibration curve for vitamin B_{12} concentration by using spectrophotometry. Figure 3 shows an example of a calibration curve obtained at 361 nm.

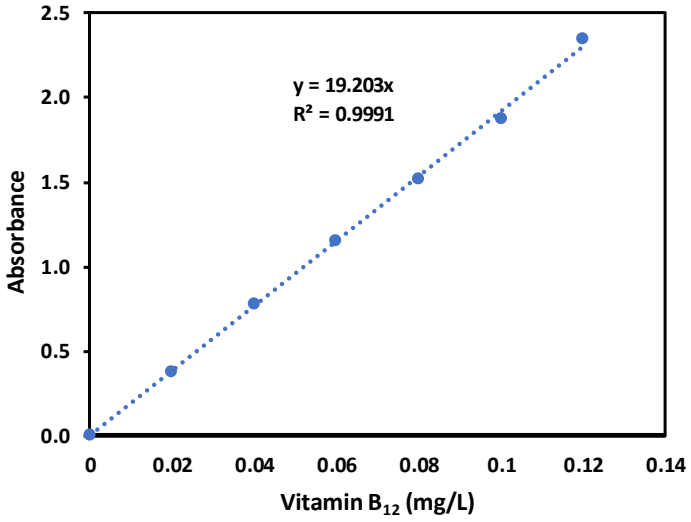

Figure 3. Calibration curve for vitamin B_{12} concentration in the continuous W_2 phase.

Each group evaluated the concentration of vitamin B_{12} in the external continuous phase (W_2) of the emulsion they had prepared. Centrifuged samples were used for this purpose. To ensure that the clarification aqueous layer was collected without any oily phase droplets, plastic tubes that can be easily pierced with a pin from the bottom were used to ensure no contamination.

The calibration curve was used to calculate the amount of free vitamin B_{12} that was not encapsulated in the emulsion droplets. Figure 4 indicates the schematic protocol.

The Ry obtained after averaging the results achieved by all the groups of students had a value of 99.5 $\pm$ 1; being close to 100%, this value was similar to those previously reported [16,18].

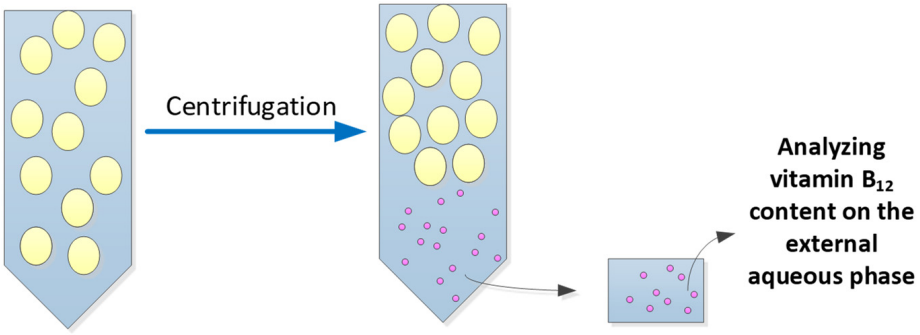

Figure 4. Centrifugation step to obtain continuous phase free of oil droplets.

5. Interpreting Student Observations

All of the experimental results obtained by each lab group were evaluated in the final report of each student, despite the fact that each group prepared one of the emulsions with a different salt concentration in the external aqueous phase.

The students used particle size measurements to study the effect of the difference in the salt concentration between both aqueous phases to evaluate the swelling and deswelling phenomena that occurred in the emulsions prepared by all the groups. Emulsions with a higher salt concentration in W_2 presented a lower mean droplet size value, caused by the deswelling of the W_1 droplets.

Figure 5 shows an example of the particle size distribution of the three double emulsions that were prepared. Table 1 shows an example of the volume mean ($D_{[4,3]}$) and median ($d_{(0.5)}$) diameters of the oil droplets in the formulated double emulsions. $D_{[4,3]}$ is the volume-weighted mean diameter, which is calculated by Equation (3):

$$D_{[4,3]} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (3)$$

where d_i is the droplet diameter, and n_i the number of droplets with diameter di; $d_{(0.5)}$ means that 50% of the total droplets are smaller than this size, i.e., it is the median particle size.

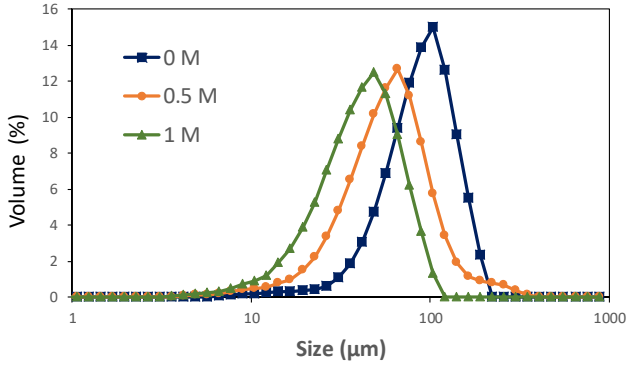

Figure 5. Particle size distribution of double emulsions formulated with different NaCl concentrations in W_2.

Table 1. Mean particle size of oil droplets in double emulsions and absorbances obtained by analyzing the external aqueous phase in each case.

NaCl Concentration	$D_{[4,3]}$	$d_{(0.5)}$	Absorbance in W_2
0 M	485.83 µm	83.81 µm	0.080
0.5 M	61.64 µm	54.49 µm	0.197
1 M	44.02 µm	43.97 µm	0.339

The students could observe that the gently centrifuged samples (Figure 6) showed appreciable differences in their clarification layers. Furthermore, the students were able to easily identify the migration of W_2 to W_1 and vice versa, which was produced by the osmotic pressure induced by the salt concentration difference between both aqueous phases and was responsible for the swelling and deswelling phenomena.

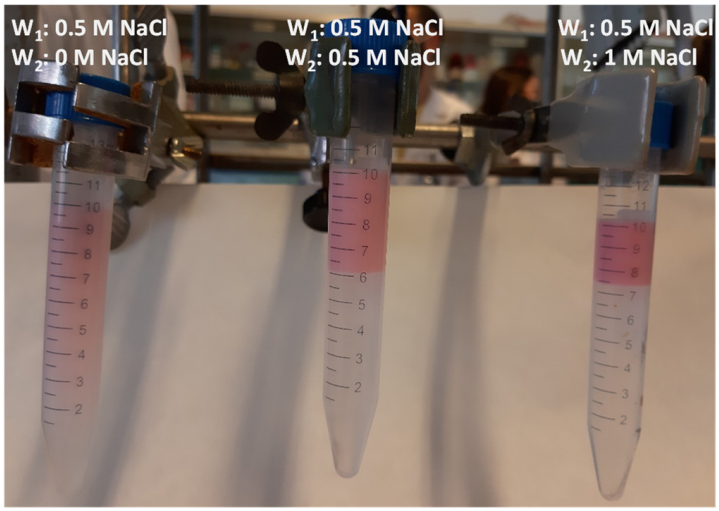

Figure 6. Visual inspection of the osmotic pressure balance acting on the swelling and deswelling in double emulsions.

Pierced tubes were used to collect W_2 from each sample and analyze it spectrophotometrically, in order to further calculate the concentration of vitamin B_{12}. Table 1 shows an example of the obtained results, where the increasing absorbance values were obtained as the concentration of NaCl on the continuous phase increased, indicating a lower EE of vitamin B_{12}.

6. Evaluation

After one week of running this experiment, each student presented an individual report, so their comprehension of the data obtained in the lab can be evaluated. The final report must contain the following items: (i) the goal of the experiment, (ii) a materials and methods section, (iii) the results and a discussion, and (iv) the conclusions. In this final report, the results of the particle size, particle size distribution, and calculations regarding the migration of W_1 to W_2 (or of W_2 to W_1) and the vitamin B_{12} EE should be presented, analyzed, and discussed.

The work carried out by each student was evaluated in three sections: (i) pre-lab work regarding the calculations of the amounts of chemicals required for the preparation of emulsions (10% of the final grade); (ii) laboratory work, followed with care and precision, and demonstrated teamwork (40%); (iii) an individual report by the student (50%).

7. Conclusions

The implementation of lab experiments and interactive science laboratory experiments such as the one presented in this work can enrich the students' interests in emulsions, suspensions, and colloidal fields. In addition, with this type of practical class, the knowledge of the concepts introduced in the theoretical sessions is enriched.

This experiment serves as an ideal candidate for providing students with laboratory skills training on colloidal systems.

Moreover, the presented experiment can be used as an open laboratory experiment that could also enrich the students' critical ability, by using the problem-based learning model. In this case, the students must select the appropriate compounds to use in order to create

osmotic pressure differences between both aqueous phases. Further, they must determine the optimal conditions to optimize the encapsulation efficiency of the biocompound.

Author Contributions: Conceptualization, J.M.B. and Á.C.; methodology, G.G. and M.M.; software, G.G. and M.M.; validation, G.G., M.M. and J.M.B.; formal analysis, Á.C. and G.G.; investigation, M.M. and G.G.; resources, M.M. and G.G.; data curation, M.M. and J.M.B.; writing—original draft preparation, M.M. and G.G.; writing—review and editing, Á.C. and J.M.B.; visualization, Á.C. and M.M.; supervision, Á.C., G.G. and M.M.; project administration, G.G. and M.M.; funding acquisition, G.G. and M.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Student Experimental Handout

Vitamin B_{12} encapsulation in double emulsions: Effect of osmotic pressure.

Appendix A.1. Objectives

1. To prepare water-in-oil-in-water emulsions containing the biocompound of interest.
2. To study the encapsulation efficiency (EE) and the effect of the osmotic pressure on it.

Appendix A.2. Theoretical Considerations

Multiple emulsions were first reported in 1925. The simplest multiple emulsions are double emulsions, which are ternary systems where the dispersed droplets contain smaller droplets of a different phase. They have either a water-in-oil-in-water ($W_1/O/W_2$) or an oil-in-water-in-oil ($O_1/W/O_2$) structure, as shown schematically in Figure A1.

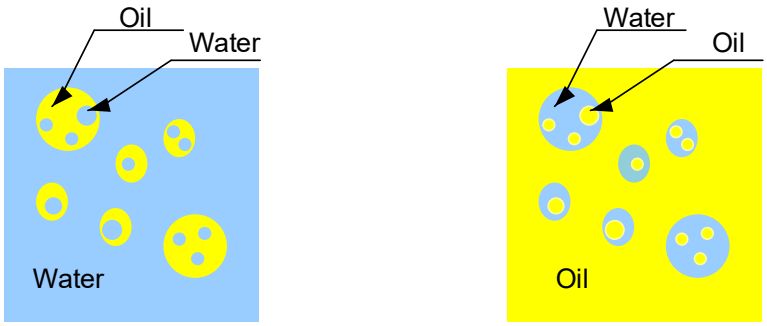

Figure A1. Structure of double emulsions: (**a**) water-in-oil-in-water emulsion ($W_1/O/W_2$) and (**b**) oil-in-water-in-oil emulsion ($O_1/W/O_2$).

To formulate a $W_1/O/W_2$ double emulsion, at least two stabilizers are introduced into the system: one lipophilic, to form the primary W_1/O emulsion, and the second hydrophobic, to form the final multiple emulsion.

Due to their complex structure, multiple emulsions can be viewed as systems that control the transport of molecules from an external to an internal phase or vice versa. Their major potential is found in pharmaceutical, food, and cosmetic applications. To obtain a

stable double emulsion, the stability of the single W_1/O emulsion must be ensured. This stability depends on droplet size (normally around 1 μm), the amounts of the dispersed and continuous phases (water is usually in the range of 20%–30% *v/v*), and the emulsifier affinity for both phases (HLB).

The encapsulation efficiency (EE) of the double emulsions is defined as the percentage of the marker/compound of interest in W_1 that remains in the primary emulsion (W_1/O) after the second emulsification step, which can be calculated by the following equation (Equation A1):

$$EE(\%) = 100 - \frac{C_{recoverd} \times 100}{C_0 R_y} \quad \text{(A1)}$$

The encapsulation (or entrapment) efficiency of the bioactive compound must be determined after the separation of the external aqueous phase. For this purpose, the external aqueous phase must be previously recovered by centrifugation at a low speed (1000 rpm for 20 min) and filtration with a 0.22 μm polyvinylidene difluoride (PVDF) syringe filter, to eliminate all the cream oily phase that is still present. Then, the concentration of the biocompound of interest in the recovered aqueous phase ($C_{recovered}$) is determined by measuring the absorbance in the external phase (W_2), which is correlated with its concentration using a calibration curve. An increase in the absorbance indicates the release of the inner phase to the external phase. It is necessary to have previously prepared a calibration curve of the absorbance vs. the biocompound concentration (Appendix B).

However, the recovery yield (R_y) must be determined, to measure if any amount of the biocompound of interest is retained (loss) during the centrifugation and filtration processes, in order to avoid an overestimation of the EE values that are obtained. Therefore, a standard emulsion, where 100% of the W_1 is present in W_2, is required for its determination. For this purpose, an oil-in-water emulsion (O/W_2) is prepared using the same formulation as in the experiments. This O/W_2 emulsion is then diluted at the same ratio with W_1, which contains the appropriate amount of the biocompound of interest. For this analysis, a blank reference also needs to be prepared consisting of an O/W_2 emulsion diluted with W_1, in which the marker/biocompound is not present. Finally, the R_y is calculated by measuring the $C_{recovered}$ in the external aqueous phases using the following equation Equation A2):

$$R_y(\%) = \frac{C_{recovered} \times 100}{C_0} \quad \text{(A2)}$$

where C_0 is the maximum concentration of the biocompound expected in the external aqueous phase.

Differences in the NaCl concentrations of the W_1 and W_2 phases affect the EE and the corresponding release. Therefore, several formulations are prepared by modifying the salt content in both aqueous phases, and then the EE for the vitamin B_{12} (the biocompound of interest) of the corresponding $W_1/O/W_2$ double emulsion is determined.

Appendix A.3. Chemicals and Equipment

50 mL beakers
100 mL beakers
100 mL flasks
High-speed stirrer (Silentcrusher M Homogenizer, Heidolph, Germany)
Organic phase:
Miglyol® 812 (density 945 kg/m^3 at 20 °C, Sasol GmbH, Germany),
Surfactants:
PGPR (polyglycerol polyricinoleate, $C_{21}H_{42}O_6$, Brenntag AG, Germany)
Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma-Aldrich, USA)
NaCl (Panreac, Castellar del Vallès, Spain)
Deionized water
Biocompound:

Vitamin B_{12} (Sigma Aldrich, USA)
Spectrophotometer (T80 UV/VIS Spectrophotometer, PG Instruments Ltd., UK)

Appendix A.4. Experimental Procedure

Appendix A.4.1. Preparation of Primary W_1/O Emulsion

- Prepare the internal aqueous phase (W_1), which consists of 100 mL of an aqueous solution containing 0.5 M of NaCl and 2500 mg/L of vitamin B_{12}. Calculate the required amount (250 g approximately), given that each emulsion has a final volume of 30 mL.
- Prepare the oily phase by dissolving 5% *w/w* of PGPR in Miglyol 812.
- Weigh the proper amounts of the continuous and dispersed phases to prepare the primary W_1/O emulsion using 20% *w/v* of the internal phase.
- Prepare 30 mL of the primary emulsion using the high-speed stirrer at 20,000 rpm for 5 min.
- Measure the size using the Mastersizer and optical microscope. This primary W_1/O emulsion should have a mean droplet size around 1 micron (only visible at 100× magnification by microscopy). When observed by a probe, this emulsion, prepared using PGPR as the emulsifier, is very stable against creaming or coalescence.

Appendix A.4.2. Preparation of Double $W_1/O/W_2$ Emulsion

- The primary W_1/O emulsion is used as an internal phase of the final double emulsion by re-dispersing it in an external aqueous phase (W_2). Thus, prepare 100 mL of the external aqueous solution (W_2), which contains 2% *w/v* of Tween 20 and the corresponding amount of NaCl that is required. In order to study the effect of the osmotic pressure on the amount of NaCl in the W_2 should be different for each group of students. Therefore, each group prepares the following solutions:

  Group 1: Prepare a W_2 solution with 0 M NaCl;
  Group 2: Prepare a W_2 solution with 0.5 M NaCl;
  Group 3: Prepare a W_2 solution with 1 M NaCl.

- Weigh the appropriate amount of the dispersed phase (W_1/O) and continuous phase (W_2) to prepare the $W_1/O/W_2$ double emulsion with a 20/80 ratio of W_1/O in W_2.
- Prepare 30 mL of the $W_1/O/W_2$ double emulsion by using the high-speed stirrer at 5000 rpm for 2 min.
- Measure the final droplet size of the double $W_1/O/W_2$ emulsions with the Mastersizer and by visual inspection under the microscope.

Appendix A.4.3. Determination of the Ry

Complete the calibration curve of the absorbance vs. the vitamin B_{12} concentration by following the instructions in Appendix B.

Prepare a standard emulsion, in which 100% of W_1 is present in W_2, to simulate an emulsion where 0% EE has been obtained. For this purpose, weigh the same amount of O and W_2 previously calculated for a 30 mL of $W_1/O/W_2$ double emulsion and prepare a simple oil-in-water emulsion (O/W_2) using the high-speed stirrer at 5000 rpm for 2 min. Then, dilute this O/W_2 emulsion by adding the corresponding amount of W_1 containing vitamin B_{12}.

In addition, prepare the blank reference consisting of an O/W_2 emulsion diluted with W_1, in which no vitamin B_{12} was present. This formulation simulates an emulsion in which 100% EE has been obtained.

Centrifuge the samples to recover the external water phase by low-speed centrifugation (1000 rpm for 20 min) and filter with a 0.22 µm polyvinylidene difluoride (PVDF) syringe filter.

Measure the absorbance of W_2 with the spectrophotometer and determine the corresponding $C_{recovered.}$

Calculate the R_y using Equation (A2).

Appendix A.4.4. Determination of the EE

Centrifuge the samples to recover the external water phase by low-speed centrifugation (1000 rpm for 20 min) and filter with a 0.22 μm polyvinylidene difluoride (PVDF) syringe filter.

Measure the absorbance of W_2 with the spectrophotometer.

Calculate the EE using Equation (A1).

Appendix A.4.5. Effect of the Osmotic Pressure

Discuss the results obtained by the different groups regarding the effect of the NaCl concentration present in both aqueous phases.

Appendix A.5. CALCULATIONS

1. Plot the calibration curve of the absorbance vs. the vitamin B_{12} concentration in mg/L.
2. Make a table summarizing the mean diameters obtained for both the primary W_1/O and double $W_1/O/W_2$ emulsions that have been formulated. Compare the mean sizes obtained from the Malvern Mastersizer and micrographs.
3. Calculate the Ry (%) obtained.
4. Estimate the EE values for the tested formulations.
5. Analyze the effect of the osmotic pressure regarding the data of the different groups.

Appendix A.6. REFERENCES

- Aserin, A. *Multiple Emulsions: Technology and Applications*; Wiley Interscience: Hoboken, NJ, USA, 2008.
- Charcosset, C. Preparation of emulsions and particles by membrane emulsification for the food processing industry. *J. Food Eng.* **2009**, *92*, 241–249.
- Díaz-Ruiz, R.; Martínez-Rey, L.; Laca, A.; Álvarez, J.R.; Gutiérrez, G.; Matos, M. Enhancing trans-Resveratrol loading capacity by forcing $W_1/O/W_2$ emulsions up to its colloidal stability limit. *Colloid Surf B-Biointerfaces* **2020**, *193*, 111130.
- Díaz-Ruiz, R.; Valdeón, I.; Álvarez, J.R.; Matos, M.; Gutiérrez, G. Simultaneously encapsulation of trans-resveratrol and vitamin D_3 in highly concentrated double emulsions. *J. Sci. Food Agric.* **2020**, *101*, 3654–3664.
- Dickinson, E. Double emulsions stabilized by food biopolymers. *Food Biophys.* **2011**, *6*, 1–11.
- Frasch-Melnik, S.; Norton, I.T.; Spyropoulos, S. Fat-crystal stabilised w/o emulsions for controlled salt release. *J. Food Eng.* **2010**, *98*, 437–442.
- Frasch-Melnik, S.; Spyropoulos, F.; Norton, I.T. $W_1/O/W_2$ double emulsions stabilised by fat crystals—Formulation, stability and salt release. *J. Colloid Interface Sci.* **2010**, *350*, 178–185.
- Khadem, B.; Khellaf, M.; Sheibat-Othman, N. Investigating swelling-breakdown in double emulsions. *Colloid Surf. A-Physicochem. Eng. Asp.* **2020**, *585*, 124181.
- Matos, M.; Gutiérrez, G.; Coca, J.; Pazos, C. Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions containing trans-resveratrol. *Colloid Surf. A-Physicochem. Eng. Asp.* **2014**, *442*, 69–79.
- Matos, M.; Gutiérrez, G.; Iglesias, O.; Coca, J.; Pazos, C. Enhancing encapsulation efficiency of food-grade double emulsions containing resveratrol or vitamin B_{12} by membrane emulsification. *J. Food Eng.* **2015**, *166*, 212–220.
- O'Regan, J.; Mulvihill, D.M. Water soluble inner aqueous phase markers as indicators of the encapsulation properties of water-in-oil-in-water emulsions stabilized with sodium caseinate. *Food Hydrocoll.* **2009**, *23*, 2339–2345.
- Seifriz, W. Studies in emulsions. III-V. *J. Phys. Chem.* **1925**, *29*, 738–749.

Appendix B. Instructions for Performing the Calibration of the Absorbance of the Vitamin B_{12} Present in the External Water Phase (W_2) of $W_1/O/W_2$ Double Emulsions

Appendix B.1. Aim

The aim of this appendix is to provide instructions on how to perform the calibration of the absorbance of the external water phase (W_2) of a double ($W_1/O/W_2$) emulsion containing vitamin B_{12}.

Appendix B.2. Extent

The purpose of this calibration is to determine the vitamin B_{12} encapsulation efficiency (EE) over time of the double emulsions that are prepared with both the aqueous phases (W_1 and W_2) used for this calibration. A new calibration must be performed for each aqueous solution prepared. The instructions for performing the calibration of the absorbance of the external water phase (W_2) with several vitamin B_{12} concentrations in the internal water phase (W_1) are as follows.

Appendix B.3. Instructions for Performing Calibration with the Spectrophotometer

(1) Do a scan with the spectrophotometer to select the appropiate absorbance wavelength (λ, nm). A maximum of absorbance of around 361 nm should be observed for the prepared vitamin B_{12} stock solution.
(2) Calculate the amounts of W_2 and W_1 required for 30 mL of double emulsion. Add the corresponding mL of W_2 to a vessel and place 1.5 mL in a disposable cuvette to measure the absorbance (A_1). Use this solution as the blank solution.
(3) Add increasing amounts of W_1 in gradual steps (0.2 mL), until the maximum amount required (1.4 mL) is added to the vessel containing the known volume of W_2 (24 mL) with adequate mixing, and measure the absorbance (A_2). Perform each measurement in triplicate, checking between each measurement that the absorbance of the blank solution is zero.
(4) Repeat step 3 four more times, until the total amount of W_1 required is added.
(5) Represent the data of the absorbance values vs. the concentration in Excel and calculate the lineal regression.

Table A1. Data of the absorbance values vs. concentration of vitamin B12.

Absorbance	Concentration (mg/L)
A_1	C_1
A_2	C_2
A_3	C_3
A_4	C_4
A_5	C_5
A_6	C_6

Appendix C. Data Collection Sheet

Table A2. Preparation of W_1/O emulsion.

W_1 Weight (g)	O Weight (g)	Agitation (rpm)	Time (s)	Mastersizer Size (μm)	Microscope Size (μm)

Table A3. Preparation of $W_1/O/W_2$ emulsion.

W_1/O Weight (g)	W_2 Weight (g)	Agitation (rpm)	Time (s)	Mastersizer Size (μm)	Microscope Size (μm)

Table A4. Calibration curve.

Absorbance	Vitamin B_{12} Concentration (mg/L)	Linear Regression: y = ax + b y: Absorbance x: Vitamin B_{12} Concentration (mg/L) r^2

Table A5. Determination of the recovery yield (Ry).

Group	Absorbance of Simulated 100% EE Emulsions	$C_{recovered}$ (mg/L)	C_0 (mg/L)	Ry (%)

Table A6. Determination of the encapsulation efficiency (EE).

Group	$W_1/O/W_2$ Formulation	Absorbance of W_2 Recovered	EE (%)

Pictures of visual inspection of emulsions prepared and micrographs obtained.

References

1. McCutchan, A.L.; Johnson, B.A. Laboratory Experiments on Ice Melting: A Need for Understanding Dynamics at the Ice-Water Interface. *J. Mar. Sci. Eng.* **2022**, *10*, 1008. [CrossRef]
2. Ali, N.; Ullah, S.; Khan, D. Interactive Laboratories for Science Education: A Subjective Study and Systematic Literature Review. *Multimodal Technol. Interact.* **2022**, *6*, 85. [CrossRef]
3. Hofstein, A. The laboratory in chemistry education: Thirty years of experience with developments, implementation, and research. *Chem. Educ. Res. Pract.* **2004**, *5*, 247–264. [CrossRef]
4. Nogales-Delgado, S.; Encinar Martín, J.M.; Román Suero, S. Teaching How to Research: A Case Study on Chemical and Industrial Engineering Degrees. *Educ. Sci.* **2022**, *12*, 673. [CrossRef]
5. Brüggemann, J.; Bizer, K. Laboratory experiments in innovation research: A methodological overview and a review of the current literature. *J. Innov. Entrep.* **2016**, *5*, 24. [CrossRef]
6. Hassan, J.; Devi, A.; Ray, B. Virtual Laboratories in Tertiary Education: Case Study Analysis by Learning Theories. *Educ. Sci.* **2022**, *12*, 554. [CrossRef]
7. Yin, D.; Chen, J.; Gu, B.; Geng, W. Investigating the Phase Inversion of Pickering Emulsions: An Experiment to Explore Colloid and Interface Chemistry Concepts. *J. Chem. Educ.* **2018**, *95*, 662–665. [CrossRef]
8. Aserin, A. *Multiple Emulsions: Technology and Applications*; Wiley Interscience: Hoboken, NJ, USA, 2008.
9. Charcosset, C. Preparation of emulsions and particles by membrane emulsification for the food processing industry. *J. Food Eng.* **2009**, *92*, 241–249. [CrossRef]
10. Seifriz, W. Studies in Emulsions. III-V. *J. Phys. Chem.* **1925**, *29*, 738–749. [CrossRef]
11. Díaz-Ruiz, R.; Valdeón, I.; Álvarez, J.R.; Matos, M.; Gutiérrez, G. Simultaneously encapsulation of *trans*-resveratrol and vitamin D_3 in highly concentrated double emulsions. *J. Sci. Food. Agric.* **2021**, *101*, 3654–3664. [CrossRef] [PubMed]
12. Khadem, B.; Khellaf, M.; Sheibat-Othman, N. Investigating swelling-breakdown in double emulsions. *Colloids Surf. A Physicochem. Eng. Asp.* **2020**, *585*, 124181. [CrossRef]
13. Díaz-Ruiz, R.; Martínez-Rey, L.; Laca, A.; Álvarez, J.R.; Gutiérrez, G.; Matos, M. Enhancing *trans*-Resveratrol loading capacity by forcing $W_1/O/W_2$ emulsions up to its colloidal stability limit. *Colloids Surf. B Biointerfaces* **2020**, *193*, 111130. [CrossRef] [PubMed]
14. Matos, M.; Gutiérrez, G.; Coca, J.; Pazos, C. Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions containing *trans*-resveratrol. *Colloids Surf. A Physicochem. Eng. Asp.* **2014**, *442*, 69–79. [CrossRef]
15. Matos, M.; Gutiérrez, G.; Iglesias, O.; Coca, J.; Pazos, C. Enhancing encapsulation efficiency of food-grade double emulsions containing resveratrol or vitamin B_{12} by membrane emulsification. *J. Food Eng.* **2015**, *166*, 212–220. [CrossRef]

16. Wilson, R.; Van Schie, B.J.; Howes, D. Overview of the preparation, use and biological studies on polyglycerol polyricinoleate (PGPR). *Food Chem. Toxicol.* **1998**, *36*, 711–718. [CrossRef] [PubMed]
17. Pawlik, A.; Cox, P.W.; Norton, I.T. Food grade duplex emulsions designed and stabilised with different osmotic pressures. *J. Colloid Interface Sci.* **2010**, *352*, 59–67. [CrossRef] [PubMed]
18. Dickinson, E. Double Emulsions Stabilized by Food Biopolymers. *Food Biophys.* **2011**, *6*, 1–11. [CrossRef]
19. O´Regan, J.; Mulvihill, D.M. Water soluble inner aqueous phase markers as indicators of the encapsulation properties of water-in-oil-in-water emulsions stabilized with sodium caseinate. *Food Hydrocolloids* **2009**, *23*, 2339–2345. [CrossRef]
20. Frasch-Melnik, S.; Spyropoulos, F.; Norton, I.T. $W_1/O/W_2$ double emulsions stabilised by fat crystals—Formulation, stability and salt release. *J. Colloid Interface Sci.* **2010**, *350*, 178–185. [CrossRef] [PubMed]
21. Frasch-Melnik, S.; Norton, I.T.; Spyropoulos, F. Fat-crystal stabilised w/o emulsions for controlled salt release. *J. Food Eng.* **2010**, *98*, 437–442. [CrossRef]