UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

TREBALL DE FI DE GRAU

FORMULATION AND CHARACTERIZATION OF BIFONAZOLE MULTIPLE EMULSION

Cecilia Callizo

(April 2016)

Main Field: Pharmaceutical Technology

Secondary Fields: Biopharmacy and Pharmacokinetics

Legislation and Ethics

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ABSTRACT

FORMULATION AND CHARACTERIZATION OF BIFONAZOLE MULTIPLE EMULSION

Superficial fungal infections of the skin (dermatomycoses) are among the most common diseases presented in our daily practice throughout the world. These infections are contagious diseases caused by either a human (anthropophilic) or animal (zoophilic) species of dermatophyte fungi. Nowadays, antifungal treatments are becoming even more important due to the increase of dermatomycoses prevalence. Nonetheless, antifungal clinical feasibility is limited by imidazole antifungal drug properties. As a consequence, new formulations, such as multiple emulsions, are being investigated in order to improve the antifungal drug bioavailability.

The aim of the present study is the formulation and characterization of different multiple emulsions which contain Bifonazole as antifungal drug. The last of these is performed straightaway after the preparation of the multiple emulsions and during the storage period.

To conclude the study, a comparison among the multiple emulsions formulationsis carried out and significant discrepancies analysed. As a result, the selection of the most suitable formulation for Bifonazole stability might be purposed.

FORMULACIÓ I CARACTERITZACIÓ D'EMULSIONS MÚLTIPLES DE BIFONAZOL

Les infeccions fúngiques superficials de la pell (dermatomicosis) es troben entre les malalties més comuns actualment arreu del món. Aquestes infeccions són malalties contagioses causades tant per l'home (antropofílic) com per animals (zoofílic). Actualment, els tractaments antifúngics estan adquirint molta rellevància degut a l'augment de la prevalença de les dermatomicosis. Tanmateix, l'eficàcia clínica dels fàrmacs antifúngics està limitada per les propietats dels seus principis actius. Com a conseqüència, noves formulacions, com les emulsions múltiples, estan sent investigades per tal de millorar la biodisponibilitat dels principis actius antifúngics.

L'objectiu principal d'aquest estudi és la formulació i caracterització de diferents emulsions múltiples que contenen Bifonazol com a principi actiu antifúngic. Aquesta caracterització es dur a terme immediatament després de la preparació de les emulsions múltiples i durant el període d'emmagatzemament.

Per concloure aquest estudi, es realitza una comparació entre les diferents emulsions múltiples i s'analitzen les discrepàncies significatives. Com a resultat, es proposa quina

és la formulació d'emulsió múltiple més estable utilitzant el Bifonazol com a principi actiu.

FORMULACIÓN Y CARACTERIZACIÓN DE EMULSIONES MÚLTIPLES DE BIFONAZOL

Las infecciones fúngicas superficiales de la piel (dermatomicosis) se encuentran entre las enfermedades más comunes actualmente en todo el mundo. Estas infecciones son enfermedades contagiosas causadas tanto por el ser humano (antropofílico) como por animales (zoofílico). Actualmente, los tratamientos antifúngicos están adquiriendo mucha relevancia debido al aumento de la prevalencia de las dermatomicosis. No obstante, la eficacia clínica de los fármacos antifúngicos está limitada por las propiedades de sus principios activos. Como consecuencia, nuevas formulaciones, como las emulsiones múltiples, están siendo investigadas con el fin de mejorar la biodisponibilidad de los principios activos antifúngicos.

El objetivo principal de este estudio es la formulación y caracterización de diferentes emulsiones múltiples que contienen Bifonazol como principio activo antifúngico. Esta caracterización se lleva a cabo inmediatamente después de la preparación de las emulsiones múltiples y durante el periodo de almacenamiento.

A modo de conclusión, se realiza una comparación entre las distintas emulsiones múltiples y se analizan las discrepancias significativas. Como resultado, se propone qué formulación de emulsión múltiple es más estable utilizando el Bifonazol como principio activo.

INTEGRATING EDUCATIONAL FIELDS

The present study integrates contributions from different educational fields. During its development knowledge of Pharmaceutical Technology, Biopharmacy and Pharmacokinetics and Legislation areas have been integrated.

- Pharmaceutical Technology: It is the main field of the study. The role of the Pharmaceutical Technology area of knowledge and skills were focused on the development of the multiple emulsions and its further characterization.

- Biopharmacy and Pharmacokinetics: To broad the knowledge about multiple emulsions beyond stability, is crucial to determinate the Bifonazole release properties and therefore, its pharmacokinetics characteristics.

- Legislation: The legislation area is basic to establish the normative framework. ICH guidelines have been followed during all the multiple emulsions development.

1. INTRODUCTION

1.1. SIMPLE EMULSIONS CONSIDERATIONS

A simple emulsion, also called just emulsion, consists of two immiscible liquids (usually oil and water), with one being dispersed as small spherical droplets inside the other. The substance that makes up the droplets in an emulsion is referred to as the dispersed, discontinuous or internal phase, whereas the one that surrounds is called the continuous or external phase (1).

Emulsions can be classified according to the relative spatial distribution of the oil and aqueous phase:

a) Oil-in-water emulsion (O/W): Oil droplets are dispersed in an aqueous phase *(Figure 1.1 a)*
b) Water-in-oil emulsion (W/O): Water droplets are dispersed in an oil phase *(Figure 1.1 b)*

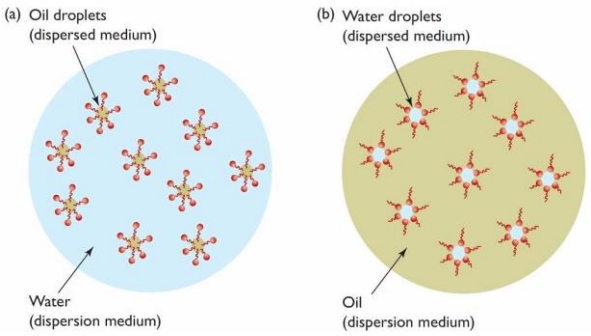

Figure 1: Oil-in-water emulsion (a) and water-in-oil emulsion (b) (2)

1.2. MULTIPLE EMULSIONS

Multiple emulsions are complex systems which many authors have called “emulsions of emulsions” (3,4). In 1925 *Seifriz* described multiple emulsions as the phase inversion of a simple emulsion(5).Multiple emulsions are formed from a dispersion of droplets which themselves contain smaller droplets of a liquid identical or similar to the external continuous phase. In multiple W/O/W emulsions, the internal and external phases are separated by an oil layer and require at least two surfactants for their common formation. The one with low HLB is used to form the primary w/o emulsion while the one with the high HLB is used to achieve secondary emulsification. The key factors affecting the formation of multiple emulsions are the chemical nature of various components, the concentration of the surfactants used in both steps of

emulsification, the volume fraction of the primary emulsion in the whole multiple emulsion and the mixing conditions (6,7).

The two major types are water-in-oil-in-water (w/o/w) and oil-in-water-in-oil (o/w/o) emulsions:

a) Water-in-oil-in-water emulsion: Small water droplets are dispersed in oil (water-oil primary emulsion (W/O)), and this primary emulsion is dispersed as large droplets inside an aqueous continuous phase *(Figure 2).*

b) Oil-in-water-in-oil emulsions: Small oil droplets are dispersed in water (oil-water primary emulsion (O/W)), and this primary emulsion is dispersed as large droplets in an oil continuous phase *(Figure 2).*

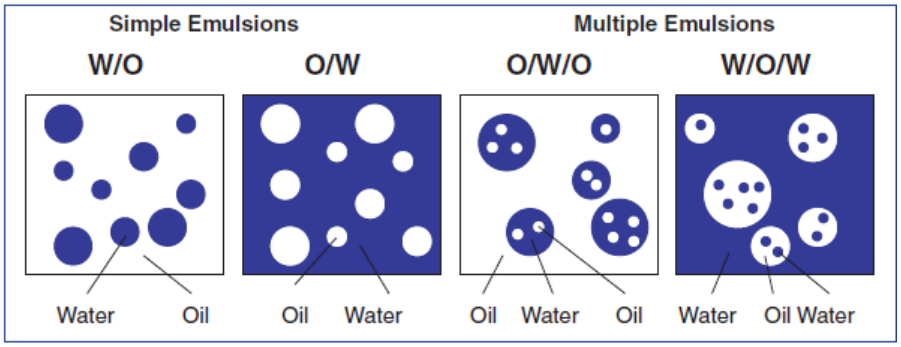

Figure 2: Schematic drawing of various types of emulsions (8)

Both types of double emulsions are often prepared through a two-step emulsification process. Firstly, the primary emulsion is prepared, followed by a re-emulsification in an oil solution or an aqueous solution in order to obtain the double emulsion.

Double emulsions have shown significant promises in many technological fields and for many applications because of their special structure. They highly suited in pharmaceutical, cosmetics and food industries (9,10). However, they only represent a little rate in the market (especially cosmetics products) due to the inherent instability of the preparation.

Their potential pharmaceutical applications include uses such as adjutant vaccines, prolonged and sustained drugs delivery systems or sorbent reservoirs of drug overdose treatments among others.

1.3. BIFONAZOLE PROPERTIES

Bifonazole ($C_{22}H_{18}N_2$) is a substituted imidazole antifungal agent structurally related to other drugs in this group as clotrimazole, econazole, miconazole, oxiconazole and

sulconazole. It is a white powder highly lipophilic with a very short half-life (1-2h) and is minimally absorbed following dermal application (0.6% of an applied dose) (11).

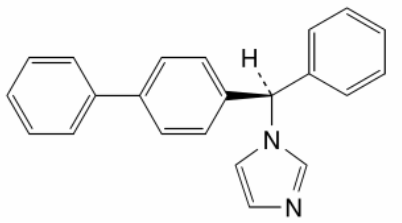

Figure 3: Bifonazole molecular structure (12)

It has demonstrated a broad spectrum of antifungal activity inhibiting the growth of moulds, yeasts, dermatophytes, dimorphic fungi and some Gram-positive bacteria(13).Bifonazole works by inhibiting ergosterol production, which is an essential component of fungal cell membranes. It destabilizes the fungal cytochrome P450 51 enzyme and its inhibition leads to cell lysis (14).

This antifungal drug is used for the treatment of superficial fungal skin infections such as dermatophytoses, cutaneous candidiasis and pityriasis versicolor (13).Bifonazole can be found in various solid dosage forms as cream, gel, solution or powders. However, all types of dosage forms are effective in formulations of Bifonazole 1% applied once daily.

2. OBJECTIVES

The aim of this academic work is to formulate different multiple emulsions for topical application containing Bifonazole as API. They have been developed according to the experience of the research group at Technology Department.

In order to achieve this objective, several specific goals have been proposed:

1. Formulation and preparation of different water-in-oil-in-water emulsions containing Bifonazole.
2. Characterization of these double emulsions by analysing morphology, droplet size, pH and conductivity values, viscosity, thermal stability and drug content.
3. Improve double emulsions stability by:
    a. Modifying the nature of the oil phase.
    b. Performing Ultra-Turrax method.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. API and excipients

The multiple emulsion components are of significant importance to ensure the stability and release properties desired.

The excipients used for the multiple emulsion formulation are detailed below *(Table 1).*The water used in all experiments was obtained from the MilliQ® Plus System located at SDM (Servei de Desenvolupament del Medicament) at the Pharmacy Faculty of Universitat de Barcelona.

		Name	Supplier	Lot	Expiry date
PRIMARY EMULSION	Oil Phase	Bifonazole	Acofarma	141824	30.11.2020
		Abil® EM 90 (cetyldimethiconecopolyol)	Evonik Industries	ES14700950	14.07.2016
		Span® 60 (sorbitan stearate)	Fagron	L14060137	04.2016
		Cetyl palmitate	Acofarma	140774	18.11.2016
		Isopropyl Myristateoil (IM)*	Acofarma	140952-N-1	18.04.2020
		Jojoba oil (YB)*	Acofarma	141311-N-1	01.04.2016
		Sweet almonds oil (SA)*	Acofarma	151389-P-1	01.07.2017
	Internal aqueous phase	Sodium chloride	Acofarma	130177	31.12.2016
		Purified water	SDM	---	---
GEL (EXTERNAL AQUEOUS PHASE)		Tego® Betain F (cocamidopropil betaine)	Evonik Industries	ES614907754	20.10.2016
		Tego® carbomer 341 ER (carbomer)	Evonik Industries	ES974DA010	05.2016
		Purified water	SDM	---	---

Table 1: Excipients' information

* Only one type of oil was used in each formulation

3.1.2. Instrumental

Name	Model	Supplier
Mettler Balance	PJ 300 Delta Range	Mettler Toledo, S.A.E, Spain
Analytical balance Kern®	ALJ 220-4	Karn&Sohn, GmH, Germany
Thermostatic bath Selecta	Selecta Precisterm S-138	JP Selecta, S.A., Spain
Mechanical shaker Schott	Schott RM 144D	Schott Ibérica, S.A., Spain
Ultra-Turrax J&K	TP 18/10 55	Janke& Kunkel. IKA, GmbH, Germany
Multi position magnetic stirrer + heating plate	Heidolph 50301. MR-1	Heidolph Instruments GmbH & Co, Germany
pH-MeterCrison	CrisonmicropH 2000	Crison Instruments, S.A., Spain
ConductimeterCrison	GLP 32	Crison Instruments, S.A., Spain
Optic Microscope Leica	DM 1000 LED	Leica Microsystems Ltd., Switzeland
Rotational Rheometer Thermo Scientific	HAAKE RheoStress 1	Thermo Fischer Scientific, GmbH, Germany
Turbiscan Lab®	Formulaction	Formulaction, L'Union, France
Mastersizer S	Malvern	Malvern Instruments Ltd., Malvern, UK
30ºC, 40ºC and 50ºC ovens	Heraeus B5042 E	Heraeus, S.A., Spain
Ultrasonic bath Selecta	Selecta 3000513	JP Selecta, S.A., Spain
Centrifuge Selecta	Centronic BLT	JP Selecta, S.A., Spain
Spectrophotometer ThermoSpectronic	Helios Beta	Thermo Fischer Scientific, GmbH, Germany

Table 2: Instrumentals information

3.2. METHODS

3.2.1. Water-in-oil-in-water (W/O/W) emulsions preparation

The most used method is to prepare a multiple emulsion in a two-step emulsification process (15). Both procedures consist in the preparation of a primary emulsion and its further emulsification with the external water phase (gel). In both emulsions a combination of surfactants is used to ensure the long-term stability of double emulsion (3,16). Therefore, multiple emulsions contain, at least, two surfactants:

a. Lipophilic surfactants: Abil® EM 90 and Span® 60 are used as low hydrophilic-lipophilic balance (HLB) surfactants to stabilize the interface of primary emulsions (W/O emulsions).

b. Hydrophilic surfactant: Tego® Betain F is used as high HLB surfactant in order to stabilize the external interface in W/O/W emulsions.

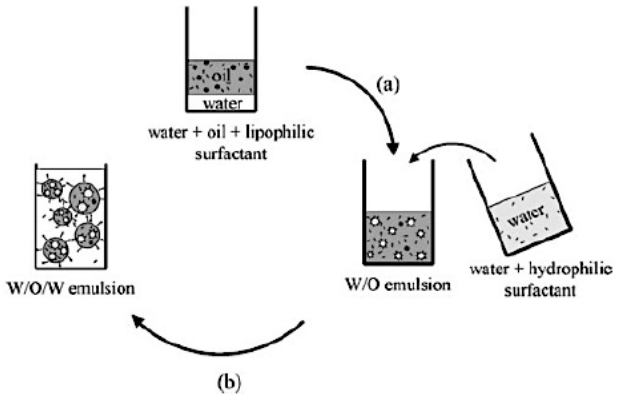

Figure 4: Two-step emulsification process (17)

Different multiple emulsion formulations were prepared using three different oils in its oil phase in order to compare the stability of Bifonazole. All multiple emulsions coding and composition is described in the table below *(Table 3).*

Name	Amount (%)				
	JMIM01B	JMIM01BT	JMYB01BT	JMSA01B	JMSA01BT
Oil phase					
Bifonazole	1,00	1,00	1,00	1,00	1,00
Abil® EM 90	1,50	1,50	1,50	1,50	1,50
Span® 60	2,00	2,00	2,00	2,00	2,00
Isopropyl Myristate Oil	11,00	11,00	--	--	--
Jojoba Oil	--	--	11,00	--	--
Sweet Almonds Oil	--	--	--	11,00	11,00
Cetyl palmitate	1,00	1,00	1,00	1,00	1,00
Internal Aqueous Phase					
Sodium Chloride	0,25	0,25	0,25	0,25	0,25
Purified water	32,25	32,25	32,25	32,25	32,25
Gel (External Aqueous Phase)					
Tego® Betain F	0,70	0,70	0,70	0,70	0,70
Tego® Carbomer 341 ER	0,20	0,20	0,20	0,20	0,20
Purified water	49,00	49,00	49,00	49,00	49,00

Table3: Multiple emulsions composition

With regard to the preparation of the primary emulsion, first it was necessary to prepare separately the water and oil phases. Water phase was prepared by dissolving 0,25g of NaCl in 32,35g of purified water. The oil phase was prepared by dissolving 1g of Bifonazole, 1,5 g of Abil® EM 90,2g of Span®60 and 1g of Cetyl palmitate in different oils depending on the formulation prepared. The oils used were Isopropyl Myristate (IM), Jojoba Oil (YB) and Sweet Almonds Oil (SA). In all cases 11g of oil was used.

Then, the two respective beakers with the oil and the water phase were stirred and heated until 70ºC (approximately during 8 minutes).

Once both phases are prepared, the water phase was poured into the oil phase under constant stirring of 300-400 rpm during 5 minutes. Thus, the primary emulsion (W/O) is obtained.

To prepare the external water phase (gel) which would be emulsified with the primary emulsion, 0,2g of Tego® Carbomer was dispersed in 49g of purified water containing 0,7g of Tego® Betain F. The gel was acquired by 24 hours of resting at room temperature. The following day, the dispersion was neutralized with a NaOH 10% solution to adjust the gel pH up to 6,5-7.

Finally, the water-in-oil-in-water emulsion was prepared by pouring the primary emulsion (W/O) into the external water phase (gel) under mixing using mechanical stirring during 15 minutes.

The multiple emulsions obtained were coded in function of its oil phase composition and considering if it was used the Ultra-Turrax method *(Table 4).*

Sample Code	Oil phase composition	Ultra-Turrax Method
JMIM01B	Isopropyl Myristate oil	No
JMIM01BT	Isopropyl Myristate oil	Yes
JMYB01BT	Jojoba oil	Yes
JMSA01B	Sweet almonds oil	No
JMSA01BT	Sweet almonds oil	Yes

Table 4: Samples codes of W/O/W prepared

3.2.2. Characterization of the water-in-oil-in-water emulsions

Once the W/O/W emulsion has been prepared, some tests have to be done in order to characterize it. It is crucial to determine the properties such as viscosity and also to examine its appearance to be able to compare different multiple emulsion samples.

All the measurements should be performed right after the preparation of the W/O/W emulsion, as well as after a certain period of time. The characterization of the sample includes visual appearance (organoleptic characteristics), microscopic observations, pH and conductivity determination, particle size analysis, stability studies and rheological measurements.

3.2.2.1. Microscopic analysis

Observations using a Leica Microsystems DM 1000 LED model were performed to observe the water-in-oil-in-water emulsions.

Firstly, multiple emulsion was diluted ten times with deionized water. Afterwards, using a pipette, one droplet of the diluted emulsion was brought on a 76 x 26 mm (Menzel-Glaser) microscope slide and it was covered with a 15 x 15 mm microscope slide cover (Menzel-Glaser). Finally, the sample was observed through microscope using 40x/0.25 Ph1 objective lens. Pictures were made using the Leica Microsystems EC model microscope camera.

3.2.2.2. PH determination

Straightaway the preparation of the multiple emulsion and after a certain period of time (every seven days), the pH value was measured in triplicate(18) by using a digital pH-Meter (CrisonmicropH 2000). All multiple emulsions were measured without any previous dilution and at room temperature (25ºC approximately).

3.2.2.3. Conductivity determination

The conductivity was measured in order to directly quantify the release of the entrapped electrolyte within the internal aqueous phase. All the multiple emulsion samples were diluted 1:20 with deionized water under magnetic stirring and were measured at 25±1ºC by using a GLP 31 Conductivity meter (Crison Instruments). Conductivity measures were performed immediately after the preparation of the multiple emulsions and also every seven days in triplicate (18).

3.2.2.4. Particle size analysis

Particle size and distribution analysis of multiple emulsions was performed using a Mastersizer S (Malvern Instruments). The instrument combines optic system, dynamic light scattering (DLS), with computer support in order to detect particles between 0.02 and 2000 µm. The results from the analysis were obtained using the derived diameter, an internationally agreed method of defining the mean of particle size.

All measurements were performed at 25ºC and sample particle size values, also known as volume-surface mean diameters, were obtained in triplicate and its standard deviation was also calculated.

3.2.2.5. Stability studies

1. *Thermal stability*

The goal of this study is to determinate the thermal stability at different storage temperatures through its macroscopic appearance in order to detect its further changes over time.

Identical transparent vials were filled with double emulsion formulations with the purpose of observing both surface and general aspect of the different samples through time.

This procedure was performed according to ICH Guidelines (19) and storage temperatures were: cold storage (5±1ºC), room temperature (23-26ºC), 30±1ºC, 40±1ºC and 50±1ºC. Samples were observed every week during the first month and afterwards every two weeks.

2. *Physical stability*

A technology based on the analysis of multiple light scattering was used for predicting the long-term physical stability of the developed formulations.

TurbiscanLab® (Formulaction) is used to characterise the stability of concentrated emulsions and dispersions. The principle of this measurement is based on using infrared light to detect the variation of the droplet volume fraction or mean size such as coalescence, flocculation, creaming and sedimentation.

The main advantage of Turbiscan Lab® is that detects destabilization phenomena up to 50 times quicker than naked eye, especially in case of opaque and concentrated systems (20,21).

Samples were not diluted and the test was performed at room temperature (23-26ºC) after one month of the double emulsion preparation.

3.2.2.6. Rheological studies

Rheological studies are crucial in the characterization of double emulsions; they can evaluate its stability (22).

All measurements of the rotational test were performed by triplicate using a rotational rheometer Thermo Scientific HaakeRheostress 1 (Thermo Fischer Scientific) equipped with a cone plate set-up with a fixed lower plate and a mobile upper cone Haake C60/2º Ti. The device was connected to a temperature control ThermoHaake Phoenix II + Haake C25P and to a computer provided with HaakeRheowin® Job Manager Software, used to perform the test, and HaakeRheowin® Data Manager, used as data analyser.

The shear rate ramp program included:

- Ramp-up period: from 0 to 100s^{-1} in 3min.
- Constant shear rate period: 100s^{-1} during 1min.
- Ramp-down period: from 100 to 0s^{-1} in 3min.

The rheological characterization of the samples was performed immediately after the preparation and during storage (once every two weeks). All data from the viscosity curves ($\eta=(\dot{\gamma})$) and the flow curves ($\tau=(\dot{\gamma})$) were recorded at 25ºC including viscosity values (Pa·s), apparent thixotropy (Pa/s) and adjusted flow curve-models (23).

3.2.3. Analysis of drug content in the double emulsion

3.2.3.1. Preparation of standard solutions for the calibration curve

The analytical method for the analysis of drug content has been previously validated; therefore, the linearity of the analytical procedure has been tested once determining the calibration curve.

With the purpose of preparing the phosphate solution, 8.709 g of K_2HPO_4 was diluted in 1 L of H_2O Milli Q. Consecutively; the dilution was sonicated in an ultrasound bath for 10 minutes. Once the phosphate solution was ready, the vehicle was prepared by adding 250 mL of phosphate solution to 750 mL of methanol. Finally, a swept-wavelength was performed in order to determinate in which wavelength Bifonazole had its peak.

On the other hand, standard stock solutions of Bifonazole were prepared by diluting 20 mg of drug in 10 mL of vehicle (methanol/phosphate solution 75:25). The final concentration obtained was 2.0 mg/mL. From these standard solution stocks of 10, 8, 6.4, 5.1, 4.08, 3.26 and 2.61 µg/mL were prepared with the same solvent.

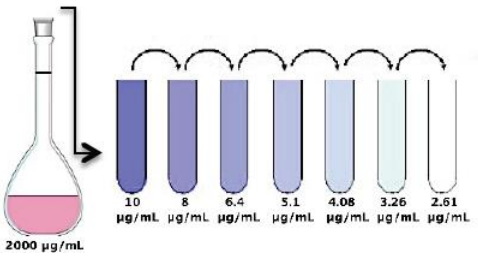

Figure 5: Standard solutions preparation

3.2.3.2. Preparation of double emulsion solutions

With the aim of analysing the Bifonazole content, all double emulsion samples were broken using the following procedure:

1. 500mg of double emulsion sample was diluted in 5 mL of methanol.
2. The dilution was sonicated in an ultrasound bath for 10 minutes.
3. The dilution was homogenised by centrifugation at 3000 rpm during 10 minutes.
4. The resulting supernatant was transferred to vial A.
5. 0.5 mL of vial A supernatant was diluted in 5mL of methanol in vial B.
6. 0.5mL of dilution of vial B was also diluted in 5mL of methanol/phosphate solution (75:25) in vial C.
7. The resulting solution in vial C was filtered and tested spectrophotometrically.

The spectrums of all the resulting solution were recorded twice using the methanol/phosphate solution (75:25) as blank.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF DOUBLE EMULSIONS

The multiple emulsion components are of significant importance to ensure the stability and release properties desired.

4.1.1. Organoleptic characteristics

All the multiple emulsion samples show strong similarities in their appearance. All water-in-oil-in-water emulsions are homogeneous, fluid, bright and white to pearly creams.

4.1.2. Microscopic analysis

Multiple emulsions microscopic structure can be observed in all samples *(Figures from 6 to 10),* therefore, small water droplets are dispersed in oil and those are dispersed as large droplets inside an aqueous continuous phase.

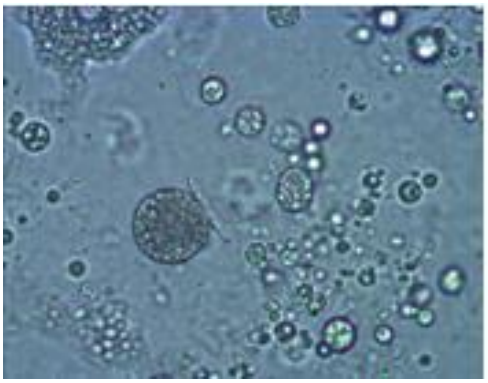

Figure 6: JMIM01B microscopic image

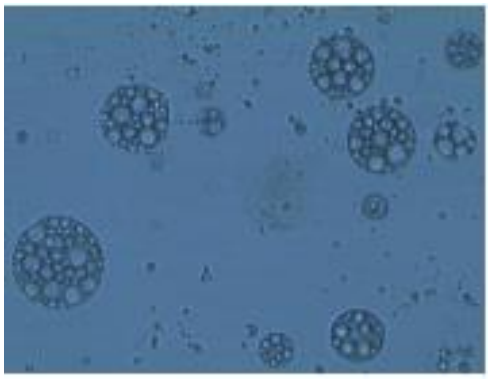

Figure 7: JMIM01BT microscopic image

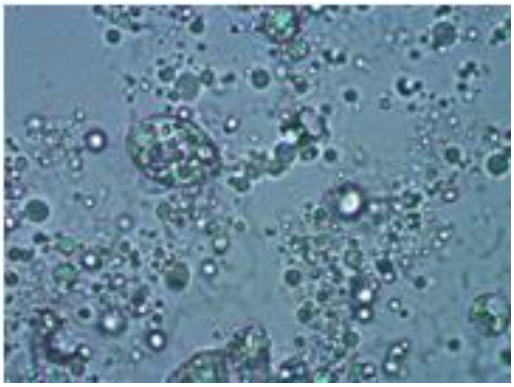

Figure 8: JMSA01B microscopic image

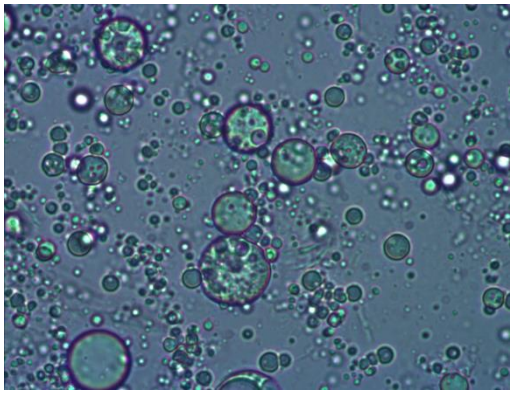

Figure 9: JMSA01BT microscopic image

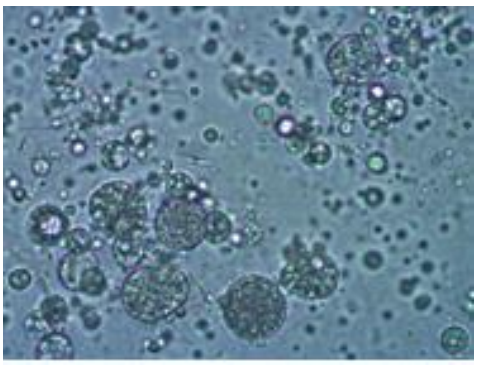

Figure 10: JMYB01BT microscopic image

Significant variations cannot be observed in photomicrographs above *(figures 6 to 10).* All samples show similar droplet size. These results are in accordance to the ones obtained from the Mastersizer analysis.

Finally, it could be affirmed that the multiplicity is maintained through storage time. Hence, all the multiple emulsions formulations are stable over time in its microscopic structure.

4.1.3. pH determination

After performing pH measurements during 10 weeks, figure 11 shows pH values evolution over storage time for multiple emulsion samples with different oil phase composition.

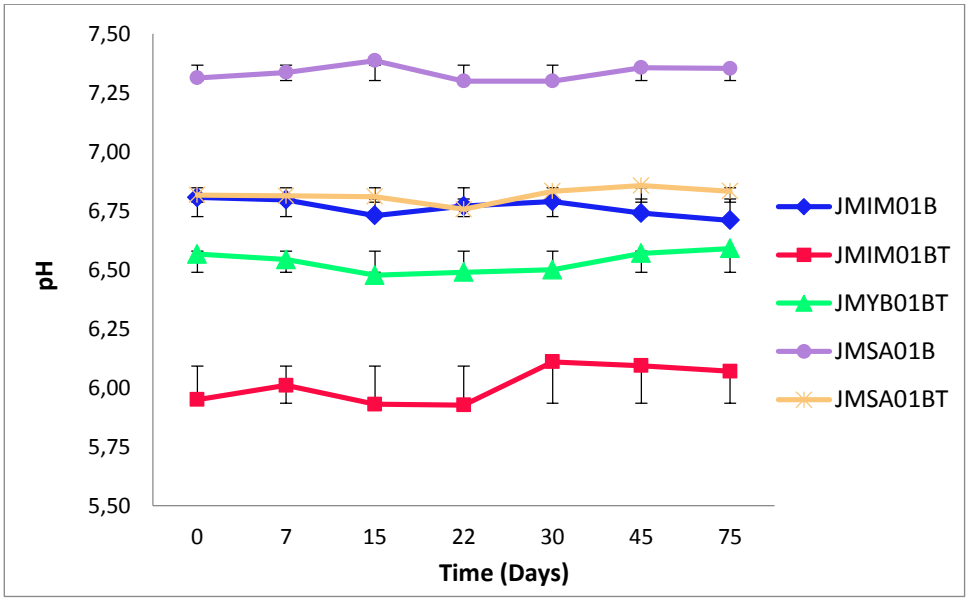

Figure 11: pH measurements as function of storage time

As we can see, all multiple emulsions have a pH between 5.8 and 7.3. According to the suitable pH for topical administration, which has been reported to be between 5.4 and 5.9 (24), only one sample would be accepted as a topical cream, the multiple emulsion formulated with Isopropyl Myristate which has been prepared using the Ultra-Turrax (JMIM01BT). The rest of the samples would have unreasonable basic pH for topical application.

Finally, it can be observed that all samples show constant pH values which could be a sign of stability through time.

4.1.4. Conductivity determination

After performing conductivity measurements for 10 weeks, figure 12 shows conductivity (µs/cm) evolution over time (days) for double emulsions with different oil phase composition.

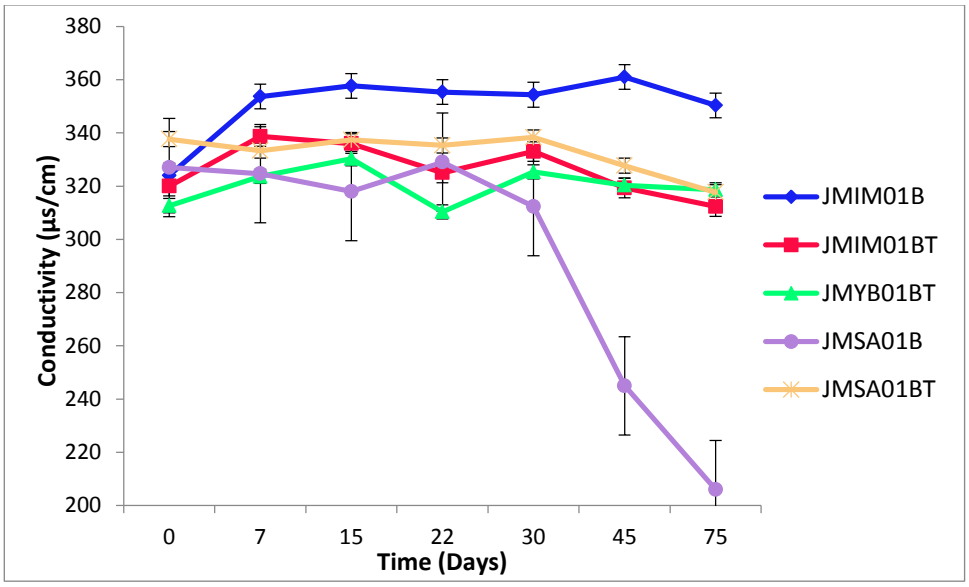

Figure 12: Conductivity values as function of storage time

Observing the graphic of the sample formulated with sweet almonds oil without Ultra-Turrax procedure (JMSA01B), it can be ensured that such a drastically decrease of conductivity over time means that it is an instable formulation, therefore, sweet almonds oil would not be a good candidate in the formulation of a water-in-oil-in-water emulsion of Bifonazole.

According to the rest of the samples, all the double emulsion formulation maintains its conductivity through time, a fact that could be interpreted as a sing of stability.

In case of Isopropyl Myristate formulation without Ultra-turrax procedure (JMIM01B) it can be seen a slight increase of conductivity. It could have been a phenomenon of water and NaCl migration from the inner water phase to the outer water phase (25).

4.1.5. Particle size analysis

After the droplet size analysis performed by Mastersizer S, important differences between formulations in reference to particle size distribution can be observed. While samples formulated with Isopropyl Myristate without Ultra-Turrax procedure (JMIM01B) *(figure 13)* and with Jojoba Oil (JMYB01BT) *(figure 14)* only have one size population, in the rest of the samples two size populations could be found *(figure 15 to 17).*

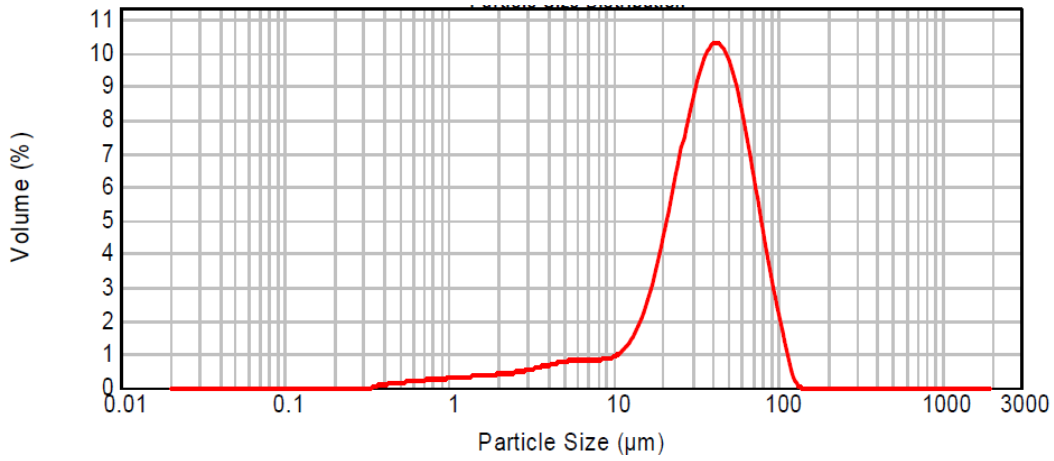

Figure 13: JMIM01B particle size distribution

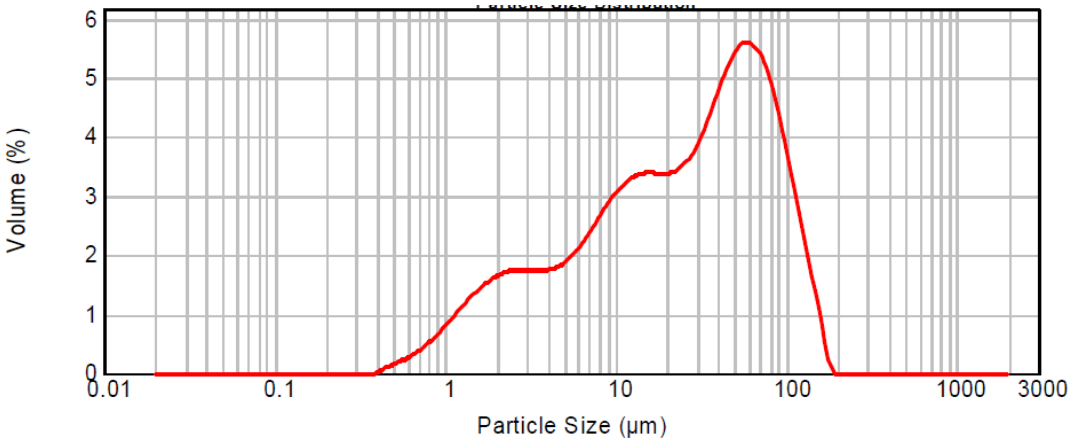

Figure 14: JMYB01BT particle size distribution

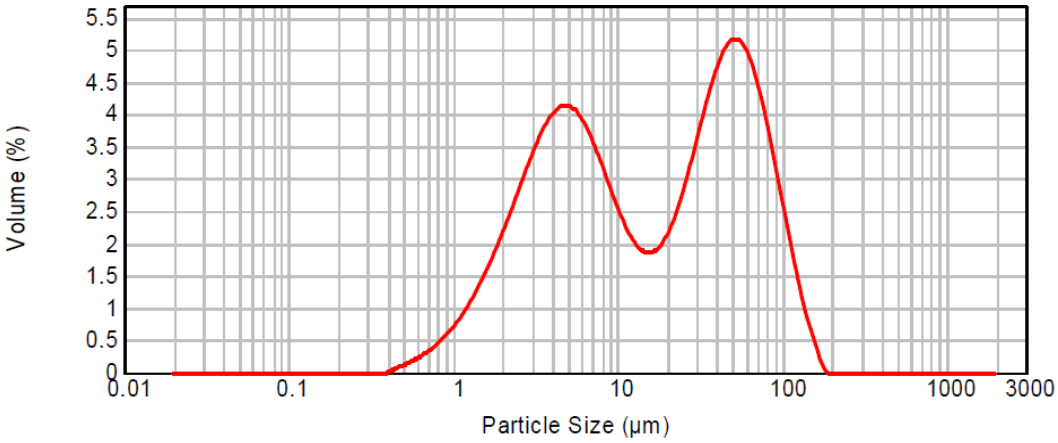

Figure 15: JMIM01BT particle size distribution

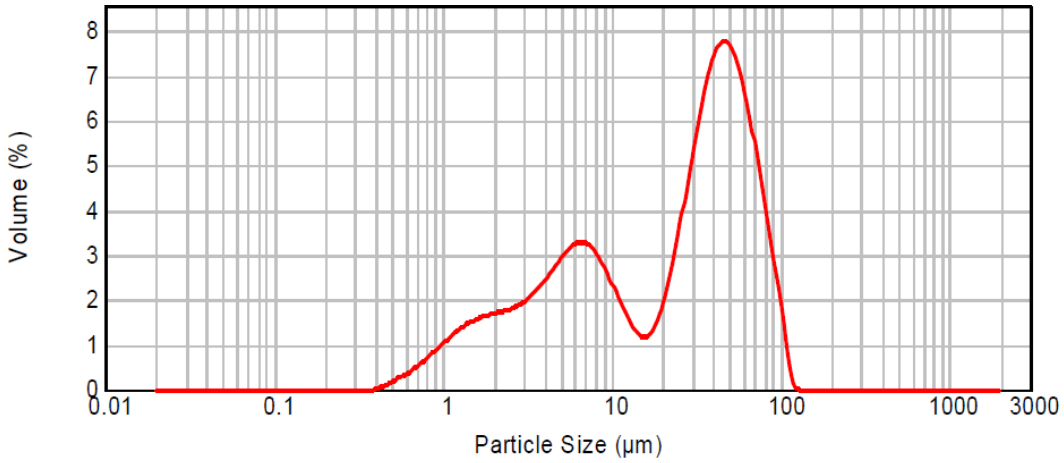

Figure 16: JMSA01B particle size distribution

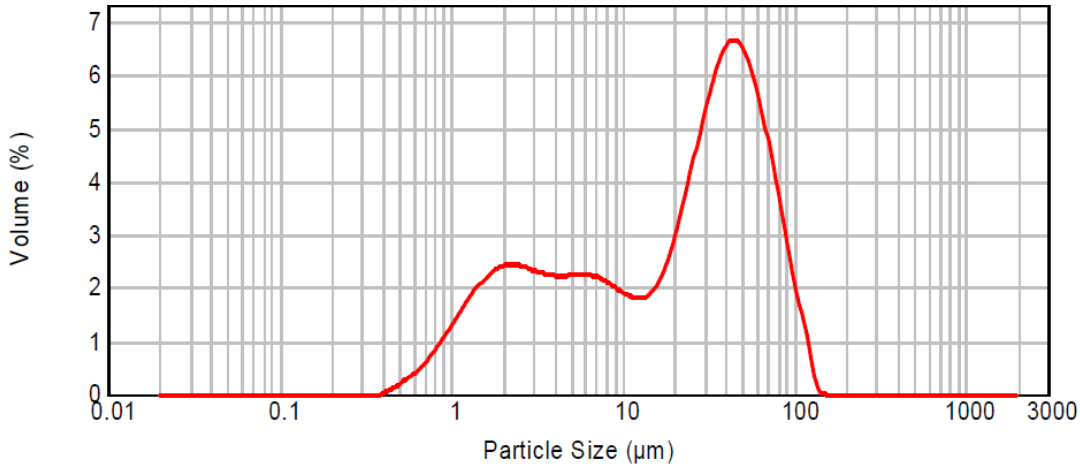

Figure 17: JMSA01BT particle size distribution

In addition to the droplet size distribution, it is worth analysing the droplet size of each formulation. Table 5 includes the particle size (volume-surface mean diameters) of all the multiple emulsions.**Faltacomentari sobre particle size table 5 quantingui els resultats**

	ParticleSize (µm)
JMIM01B	
JMIM01BT	
JMYB01BT	
JMSA01B	
JMSA01BT	

Table 5: Particle size values

In conclusion, several differences between samples can be highlighted in reference to droplet size distribution but regarding particle size analysis any important discrepancy can be emphasise.

4.1.6. Stability studies

For the selection of optimal multiple emulsions formulations, stability studies were performed in order to investigate the effect of storage.

4.1.6.1. Thermal stability

In order to simplify data analysis, a schematic stability table *(table 6)* was prepared with two colours (green for stable samples and red for instable ones) and with the following abbreviations:

- Stable (S): Unaltered sample. Its macroscopic appearance remains unchanged over time.
- Burned (B): Due to the density difference between the internal and the external phase, the sample has changed its appearance and two different phases can be observed.
- Exude (E): The sample is starting the emulsion-breaking process. Some oil droplets can be observed on the surface.

	DAYS	STABILITY				
		Cold store	RoomTª	30ºC	40ºC	50ºC
JMIM01B	1	S	S	S	S	S
	7	S	S	S	S	B
	15	S	S	S	S	B
	22	S	S	S	B	B
	30	S	S	S	B	B
	45	S	S	S	B	B
	75	S	S	S	B	B
JMIM01BT	1	S	S	S	S	S
	7	S	S	S	S	S
	15	S	S	S	S	B
	22	S	S	S	B	B
	30	S	S	S	B	B
	45	S	S	S	B	B
	75	S	S	S	B	B
JMYB01BT	1	S	S	S	S	S
	7	S	S	S	S	S
	15	S	S	S	S	B
	22	S	S	S	S	B
	30	S	S	S	S	B
	45	S	S	S	S	B
	75	S	S	S	S	B
JMSA01B	1	S	S	S	S	S
	7	S	S	S	S	B
	15	S	S	S	S	B
	22	S	S	S	S	B
	30	S	S	S	B	B
	45	S	S	S	B	B
	75	S	S	S	B	B
JMSA01BT	1	S	S	S	S	S
	7	S	S	S	S	B
	15	S	S	S	S	B
	22	S	S	S	S	B
	30	S	S	S	S	B
	45	S	S	S	B	B
	75	S	S	S	B	B

Table 6: Thermal stability

The data shown in the table above *(Table 6)* evince the different behaviour of the samples depending on its oil phase formulation. Exclusively the sample formulated with Jojoba oil (JMYB01BT) could have been storage during 10 weeks at 40ºC without destabilising *(Figure 18).*

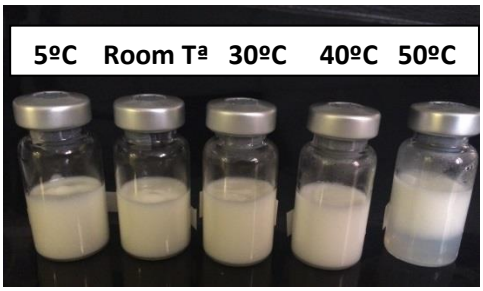

Figure 18: JMYB01BT samples

In conclusion, the results confirm that all samples can be storage during 10 weeks under cold and room temperatures and even at 30ºC and remain stables.

4.1.6.2. Physical stability

Figures 19 to 23 show multiple emulsions physical stability after performing Turbiscan Lab® analysis. No signals on the variation of the droplet volume fraction (migration) or mean size (coalescence) has been observed during 24 hours on all the multiple emulsions formulations. As a consequence, no significantly particle size variation (%ΔBS, <10%) phenomenon occurred over the analysed period.

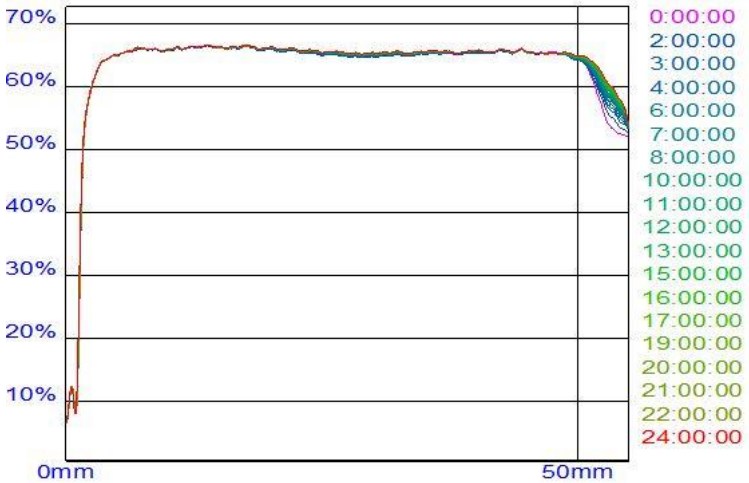

Figure 19: JMIM01B physical stability

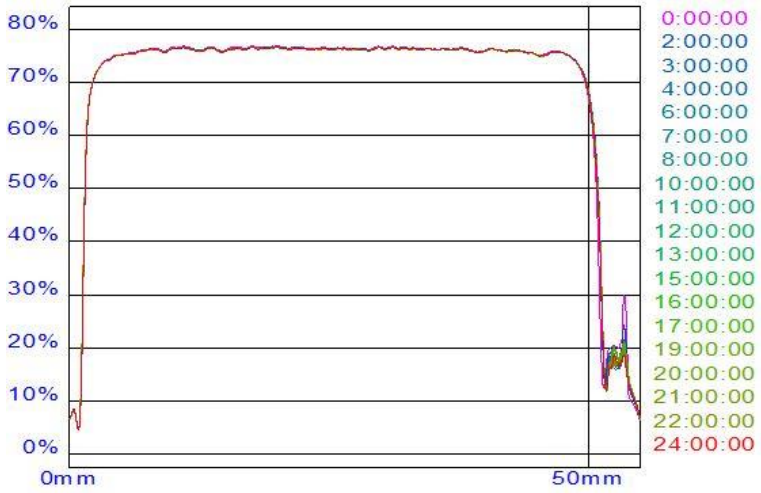

Figure 20: JMIM01BT physical stability

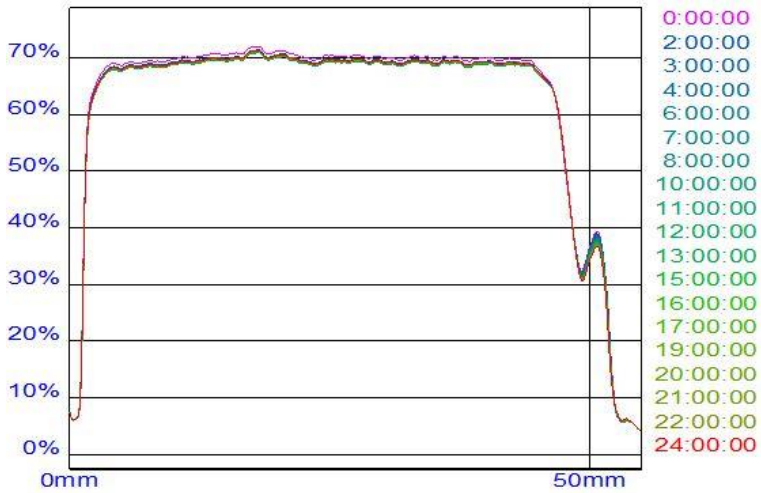

Figure 21:JMYB01BT physical stability

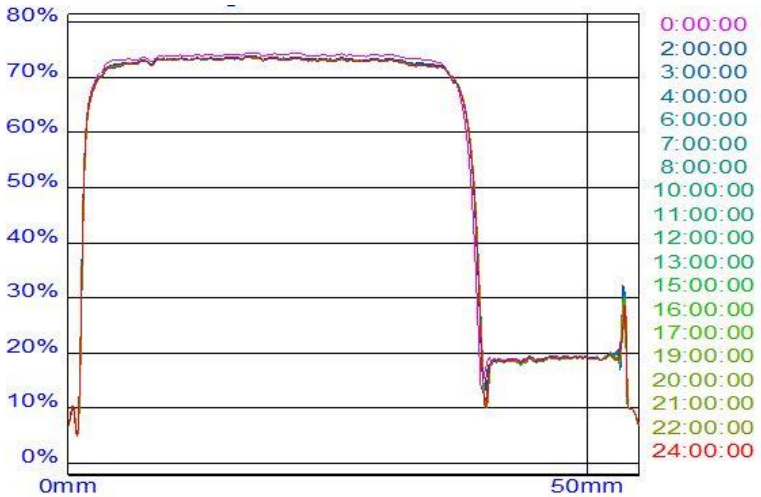

Figure 22: JMSA01B physical stability

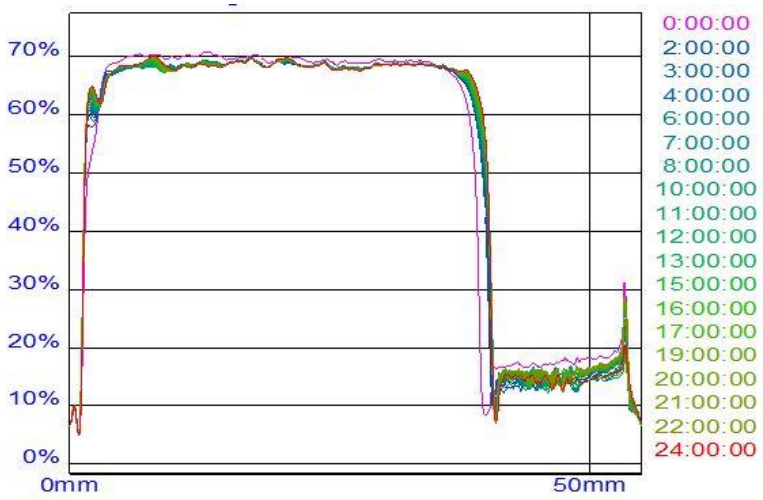

Figure 23: JMSA01BT physical stability

4.1.6.3. Rheological studies

Rheological data analysis was performed using HaakeRheowin® Data Manager.

Viscosity mean values (Pa·s) from all samples were determined from the constant share section at $100s^{-1}$ *(Figure 24).*

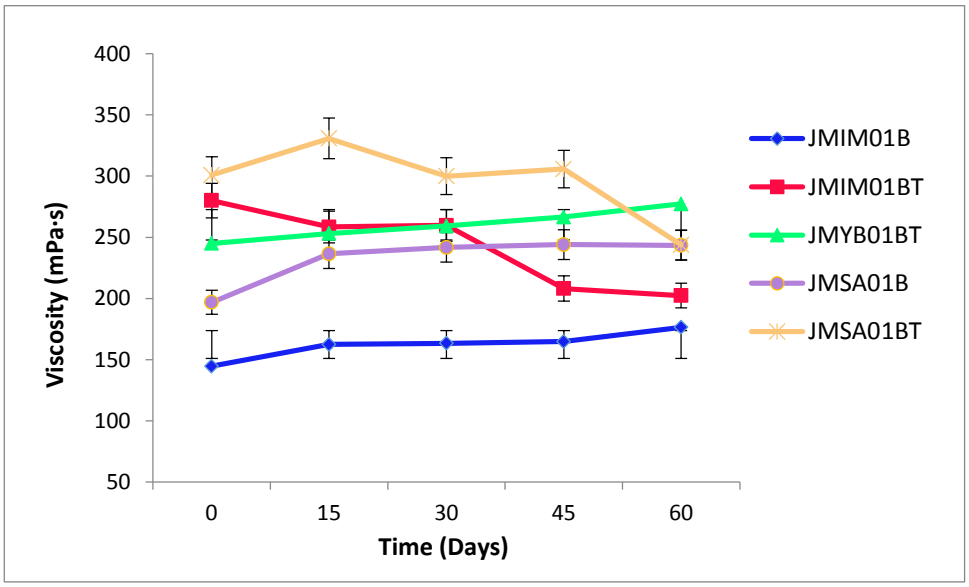

Figure 24: Viscosity mean values in function of time storage

As shown in Figure 24, viscosity evolution over storage time differs between multiple emulsions formulations. While samples JMIM01B, JMYB01BT and JMSA01B maintain its viscosity through time, the other two samples suffer a significant decrease over time. Thus could be due to the destabilization of the sample or the inner multiple emulsion properties which tend to destabilization.

Therefore, it can be assured that the best sample referring to viscosity determination is the one formulated with Jojoba oil (JMYB01BT). It perfectly maintains its high initial viscosity through time.

In reference to the determination of the apparent thixotropic (Pa/s), it was evaluated by determining the hysteresis loop area which can be described as the area between ramp-up and ramp-down periods *(Figures 25 to 29).*

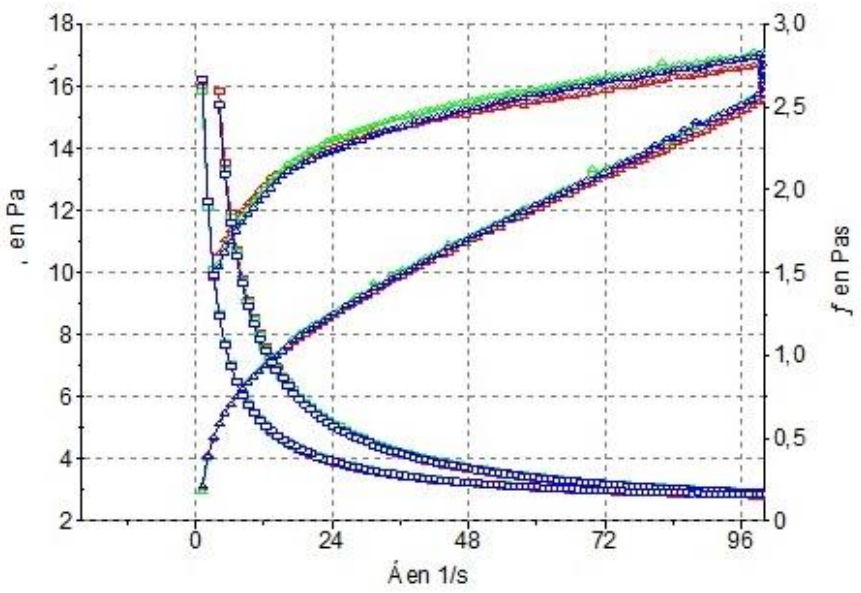

Figure 25: JMIM01B rheological study

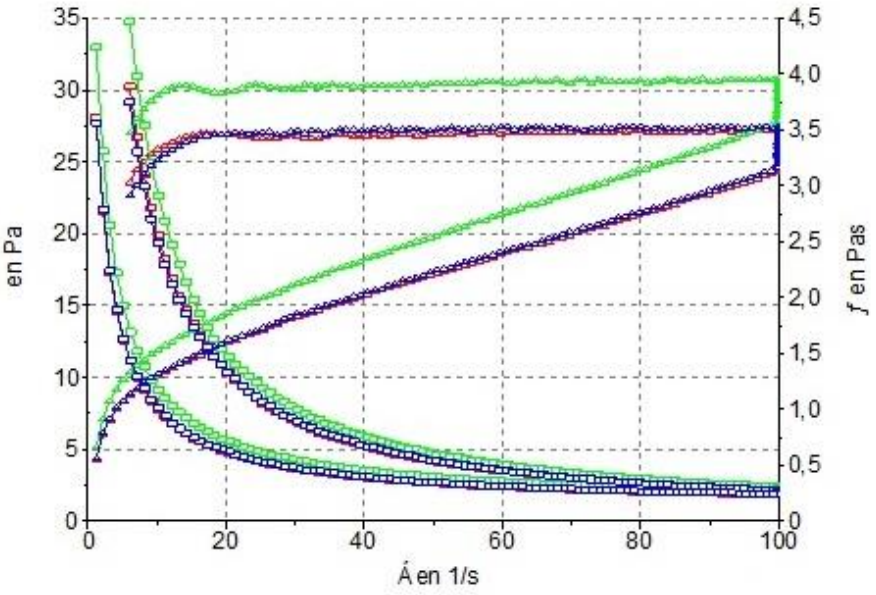

Figure 26: JMIM01BT rheological study

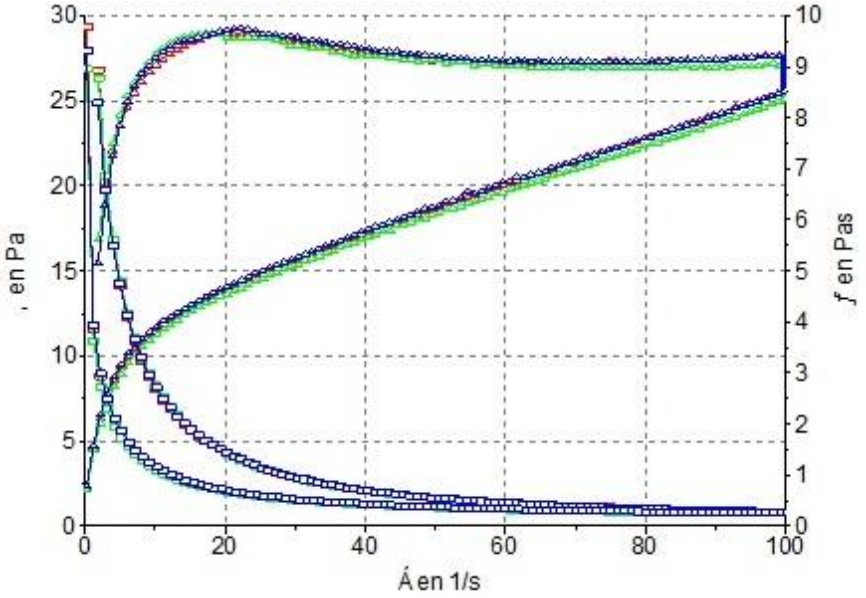

Figure 27: JMYB01BT rheological study

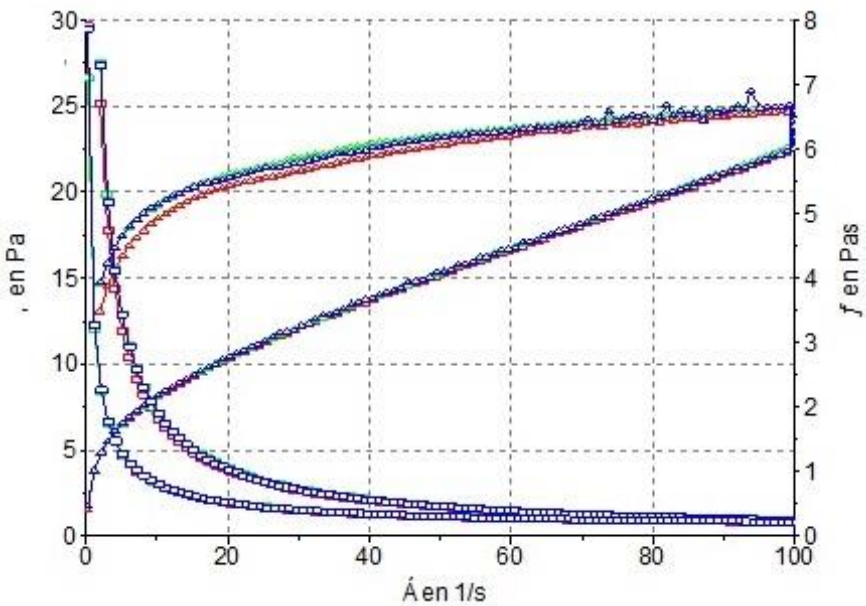

Figure 28: JMSA01B rheological study

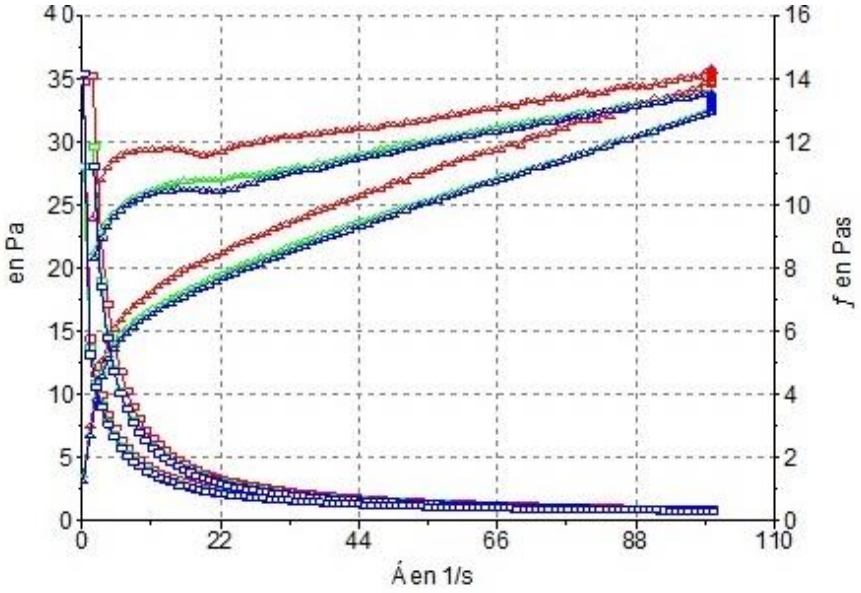

Figure 29: JMSA01B rheological study

As shown in pictures 25 to 29 all multiple emulsion samples present different hysteresis loop area which lead to show discrepancies in apparent thixotropic (Pa/s). None of those discrepancies are significant because are exclusively caused by different oil phase of the multiple emulsions formulations.

Finally, HaakeRheowin® Data Manager adjusted the data from the flow curve (τ=($\dot{\gamma}$)) to different mathematical models *(Table 7)* in order to determinate which model adjusted better to each sample.

Flow curve - models $(\tau = f(\dot{\gamma}))$	
Newton	$\tau = \eta \cdot \dot{\gamma}$
Bingham	$\tau = \tau_0 + (\eta_0 \cdot \dot{\gamma})$
Ostwald-de- Waele	$\tau = K \cdot \dot{\gamma}^n$
Herschel-Bulkley	$\tau = \tau_0 + K \cdot \dot{\gamma}^n$
Casson	$\tau = \sqrt[n]{(\tau_0^n + (\eta_0 \cdot \dot{\gamma})^n)}$
Cross	$\tau = \dot{\gamma} \cdot (\eta_\infty + (\eta_0 - \eta_\infty)/(1 + (\dot{\gamma}/\dot{\gamma}_0)^n)$

Table 7: Flow-curve models (26)

Table 8 includes the rheological behaviour analysis of all double emulsions samples at all times. It reveals that all formulations have a pseudoplastic behaviour fitted by Cross model with a good correlation coefficient (r).

As example, Figure 30 show how Cross flow-curve (green line) suits perfectly to JMIM01B sample behaviour.

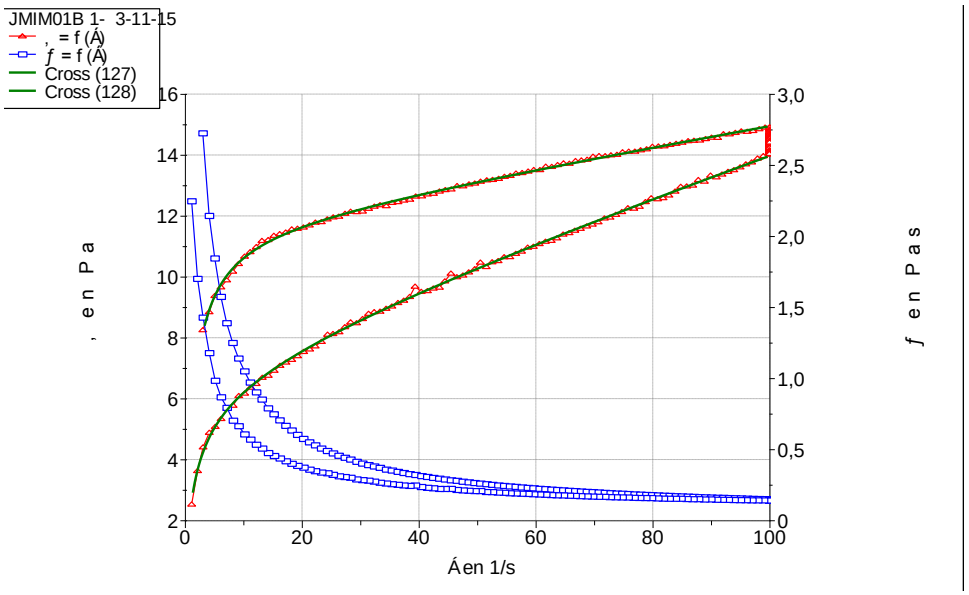

Figure 30: JMIM01B Cross flow-curve

	TIME (Days)	BETTER MATHEMATICAL MODEL FITTING		RHEOLOGICAL BEHAVIOUR
		Ramp-up section	Ramp-down section	
JMIM01B	0	r = 0,9997 Cross	r = 0,9998 Cross	Pseudoplastic
	15	r = 0,9994 Cross	r = 0,9999 Cross	Pseudoplastic
	30	r = 0,9989 Cross	r = 0,9999 Cross	Pseudoplastic
	45	r = 0,9991 Cross	r = 0,9998 Cross	Pseudoplastic
	60	r = 0,9990 Cross	r = 0,9999 Cross	Pseudoplastic
JMIM01BT	0	r = 0,9860 Cross	r = 0,9999 Cross	Pseudoplastic
	15	r = 0,9692 Cross	r = 0,9999 Cross	Pseudoplastic
	30	r = 0,9854 Cross	r = 0,9999 Cross	Pseudoplastic
	45	r = 0,9979 Cross	r = 0,9999 Cross	Pseudoplastic
	60	r = 0,9985 Cross	r = 0,9999 Cross	Pseudoplastic
JMYB01BT	0	r = 0,9976 Cross	r = 0,9997 Cross	Pseudoplastic
	15	r = 0,9890 Cross	r = 0,9998 Cross	Pseudoplastic
	30	r = 0,9885 Cross	r = 0,9998 Cross	Pseudoplastic
	45	r = 0,9809 Cross	r = 0,9999 Cross	Pseudoplastic
	60	r = 0,9871 Cross	r = 0,9999 Cross	Pseudoplastic
JMSA01B	0	r = 0,9999 Cross	r = 1 Cross	Pseudoplastic
	15	r = 0,9989 Cross	r= 0,9999 Cross	Pseudoplastic
	30	r = 0,9996 Cross	r = 0,9999 Cross	Pseudoplastic
	45	r = 0,9990 Cross	r = 0,9999 Cross	Pseudoplastic
	60	r = 0,9990 Cross	r = 0,9999 Cross	Pseudoplastic
JMSA01BT	0	r = 0,9990 Cross	r = 0,9995 Cross	Pseudoplastic
	15	r = 0,9973 Cross	r = 0,9997 Cross	Pseudoplastic
	30	r = 0,9943 Cross	r = 0,9997 Cross	Pseudoplastic
	45	r = 0,9918 Cross	r = 0,9997 Cross	Pseudoplastic
	60	r = 0,9951 Cross	r = 0,9997 Cross	Pseudoplastic

Table 8: Better mathematical model fitting

4.2. DRUG CONTENT

Firstly, the calibration curve was obtained *(Figure 31)* with the absorbance over the concentration results of the different standard solutions.

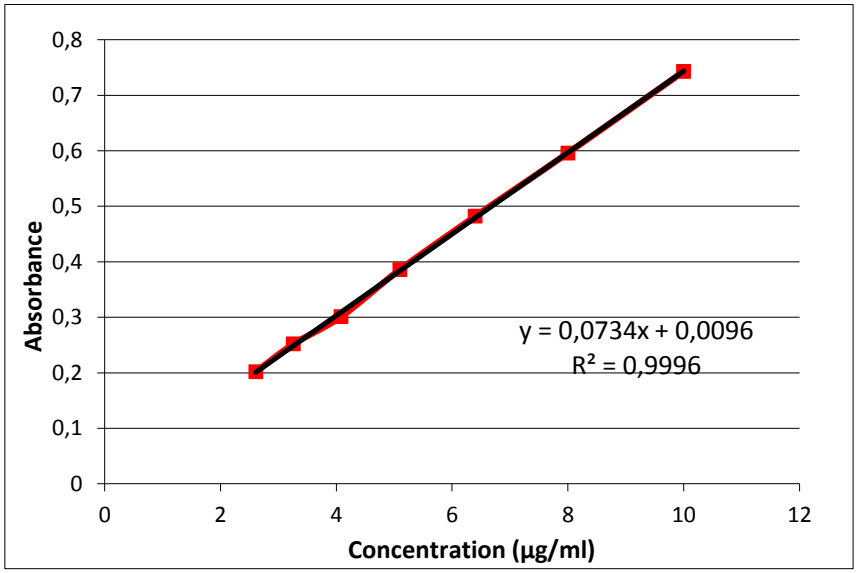

Figure 31: Calibration curve

Afterwards, the drug content analysis was performed and the results are shown in table 9.

	Absorbance	Concentration (µg/ml)	Drug Content (%)
JMIM01B	0,7175	0,964	96,4%
JMIM01BT	0,7465	1,004	100,4%
JMYB01BT	0,7740	1,041	104,1%
JMSA01B	0,6790	0,912	91,2%
JMSA01BT	0,7265	0,977	97,7%

Table 9: Bifonazole content

These values reveal that the experimental amounts extracted from the formula are very close to the theoretical ones which are 100% of Bifonazole. Therefore, the drug content of all different samples is indeed acceptable.

5. CONCLUSIONS

The study of the formulation and characterization of different multiple emulsions with 1% Bifonazole is contained in the present work. It describes the preparation of five formulations that differ only in the oil phase and in the Ultra-Turrax method preparation. The following conclusions may be drawn from this study:

1. In reference to organoleptic characteristics, all multiple emulsions had similar appearance in homogeneity, fluidity, brightly and white to pearly creams.

2. In all cases water-in-oil-in-water structure was confirmed by microscopic analysis which showed non-significant differences between samples that were prepared with our without the Ultra-Turrax method.

3. Concerning the pH analysis, only the sample JMIM01BT had suitable pH for topical administration.

4. Furthermore, conductivity analysis did not show relevant differences between samples, except JMSA01B that has dramatically decreased after 30 days.

5. Particle size analysis →Esperar a resultats de Mastersizer.

6. Regarding the stability studies it could be deduced that all the formulations are stable thermal and physically through storage time.

7. All rheological analysis reveals the good stability of the multiple emulsions formulations. Besides, it might be assured that the best viscosity stability through time is provided by the sample formulated with Jojoba oil (JMYB01BT).

8. In reference to drug content, all the samples are between the limits of drug content acceptance according to ICH (90-110%).

In conclusion, as it can be deduced from the characterization tests, the optimal multiple emulsion is the one formulated with Jojoba oil (JMYB01BT). Once it has been demonstrated which is the optimal oil phase for the multiple emulsion stability, the following step would be to perform biopharmaceutical studies in order to provide sustaining data about Bifonazole releasing properties.

BIBLIOGRAPHY

1. Pal R. Rheology of simple and multiple emulsions. Curr Opin Colloid Interface Sci. 2011 Feb;16(1):41–60.

2. Xie Y, Chen J, Zhang S, Fan K, Chen G, Zhuang Z, et al. The research about microscopic structure of emulsion membrane in O/W emulsion by NMR and its influence to emulsion stability. Int J Pharm. 2016;500(1-2):110–9.

3. Garti N. Double emulsions - Scope, limitations and new achievements. Colloids Surfaces A Physicochem Eng Asp. 1997;123-124:233–46.

4. Garti N, Bisperink C. Double emulsions: Progress and applications. Curr Opin Colloid Interface Sci. Current Chemistry Ltd. 1998;3(6):657–67.

5. Pal R. Rheology of double emulsions. J Colloid Interface Sci. 2007;307(2):509–15.

6. Matsumoto S, Kita Y, Yonezawa D. An attempt at preparing water-in-oil-in-water multiple-phase emulsions. J Colloid Interface Sci. 1976 Nov;57(2):353–61.

7. Florence AT, Whitehill D. The formulation and stability of multiple emulsions. Int J Pharm. 1982;11(4):277–308.

8. Daniels R, Knie U. Galenics of dermal products--vehicles, properties and drug release. J Dtsch Dermatol Ges. 2007;5(5):367–83.

9. Dickinson E. Food emulsions and foams: Stabilization by particles. Curr Opin Colloid Interface Sci. 2010;15(1-2):40–9.

10. Guzey D, McClements DJ. Formation, stability and properties of multilayer emulsions for application in the food industry. Adv Colloid Interface Sci. 2006;128-130(2006):227–48.

11. Karpe M, Kadam V, Patel V, Jadhav N. Formulation and Evaluation of Topical Delivery of Antifungal Drug Bifonazole Using Microemulsionbased Gel Formulations. 2006;16(2):64–22.

12. Pharmacopoeia BE, Bifonazole D, Adjust C. Bifonazolum BILBERRY FRUIT , DRIED Myrtilli fructus siccus BILBERRY FRUIT , FRESH. (impurity C):1098–9.

13. Lackner TE, Clissold SP. Bifonazole. Drugs. 2012;38(2):204–205.

14. Maertens J a. History of the development of azole derivatives. Clin Microbiol Infect. 2004;10 Suppl 1:1–10.

15. Matsumoto S, Kang WW. Formation and applications of multiple emulsions. J Dispers Sci Technol. 1989;10(4-5):455–82.

16. Pasquali RC, Sacco N, Bregni C. The studies on Hydrophilic-Lipophilic Balance (HLB): Sixty years after William C. Griffin's Pioneer Work (1949-2009). Lat Am J Pharm. 2009;28(2):313–7.

17. Van Der Graaf S, Schroën CGPH, Boom RM. Preparation of double emulsions by membrane emulsification - A review. J Memb Sci. 2005;251(1-2):7–15.

18. Raynal S, Grossiord JL, Seiller M, Clausse D. A topical W/O/W multiple emulsion containing several active substances: formulation, characterization and study of release. J Control Release. 1993 Aug;26(2):129–40.

19. Harmonisation IC on. Ich Harmonised Tripartite Guideline Validation of Analytical Procedures : ICH Harmon Tripart Guidel Valid Anal Proced TEXT Methodol Q2(R1) step4. 2005;

20. Liu J, Huang X, Lu L, Li M, Xu J, Deng H. Turbiscan Lab ® Expert analysis of the biological demulsification of a water-in-oil emulsion by two biodemulsifiers. J Hazard Mater. 2011 Jun 15;190(1-3):214–21.

21. Lemarchand C, Couvreur P, Vauthier C, Costantini D, Gref R. Study of emulsion stabilization by graft copolymers using the optical analyzer Turbiscan. Int J Pharm. 2003;254(1):77–82.

22. Vasiljevic D, Vuleta G, Primorac M. The characterization of the semi-solid W/O/W emulsions with low concentrations of the primary polymeric emulsifier. Int J Cosmet Sci. 2005;27(2):81–7.

23. Garakani K, Mostoufi AH, Sadeghi N, Hosseinzadeh F, Fatourechi M, Sarrafzadeh H, et al. Comparison between different models for rheological characterization of activated sludge. Iran J Environ Heal Sci Eng 8 (3), Koseoglu. 2011;255–64.

24. Paudel KS, Milewski M, Swadley CL, Brogden NK, Ghosh P, Stinchcomb AL. Challenges and opportunities in dermal/transdermal delivery. Ther Deliv. 2010;1(1):109–31.

25. Geiger S, Tokgoz S, Fructus A, Jager-Lezer N, Seiller M, Lacombe C, et al. Kinetics of swelling–breakdown of a W/O/W multiple emulsion: possible mechanisms for the lipophilic surfactant effect. J Control Release. 1998 Mar;52(1-2):99–107.

26. Baudez J-C, Ayol A, Coussot P. Practical determination of the rheological behavior of pasty biosolids. J Environ Manage. 2004 Sep;72(3):181–8.