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Design of a complex formulation for clinical nutrition applications

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Acknowledgments

Abstract / Resumo

The main research focus of this project was the development of a suitable enteral nutrition solution, to administer water insoluble nutrients overcoming their low bioavailability. To evaluate which ingredientes could produce the most effective formulation, solubility tests were performed to pre-selected oils and surfactants, followed by emulsifying capacity evaluation. The highest solubility results were obtained for MCT, EO (1:1). As for the emulsifying capacity, all of the four mixtures tested show some microemulsion. The full characterization of the emulsion and other tests are required to draw conclusions regarding the most efficient formulations.

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O principal foco de investigação deste projecto foi o desenvolvimento de uma solução de nutrição enteral, capaz de administrar nutrientes não solúveis em água e aumentar a biodisponibilidade dos mesmos. Para avaliar que ingredientes produzem a fórmula mais eficaz, foram efectuados testes de solubilidade a alguns óleos e surfactantes pré-selecionados, e uma avaliação à capacidade emulsificante. Os melhores resultados dos testes de solubilidade foram obtidos pela mistura MCT, EO (1:1). Quanto à capacidade emulsificante, todas as quatro misturas testadas formaram microemulsões. A caracterização total das emulsões e testes adicionais são necessários para tirar conclusões sobre quais das fórmulas são mais eficientes.

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Abbreviations

API – Active Pharmaceutical Ingredient

EO – Ethyl Oleate

GI - Gastrointestinal

GIT – Gastrointestinal Tract

HLB - Hydrophilic-Lipophilic Balance

LB – Labrasol

LBDDS – Lipid Based Drug Delivery Systems

LC – Long carbon chain

LFCS - Lipid Formulation Classification System

MC – Medium carbon chain

MCT – MCT Oil

ME – Microemulsion

Mig840 – Miglyol 840

NCEs – New Chemical Entities

PDMPD – Phase Diagram by Micro Plate Dilution

SEDDS – Self Emulsifying Drug Delivery System

SF – Sunflower Oil

SMEDDS – Self Microemulsifying Drug Delivery System

TG - Triglyceride

TW80 – Tween 80

Chapter 1

Preface: Context of the Project

At the beginning of 2012 I learned about the Erasmus Placements program, which sponsors students to apply for work placement in a European company, organization or research center.

I searched for biotech and pharmaceutical companies that offered university students the opportunity to do their master thesis in industrial research environment. My goal was to intern for one of these enterprises, during the following academic year, and write a master thesis about my work during that internship. The purpose of working in a large corporation was that I could both experience this type of a professional environment, and be part of an ongoing project aiming to develop a product that would eventually enter the market.

I contacted several companies and the one that offered me the most exciting project was Fresenius Kabi. I accepted their offer to join the Innovation and Development Team at their headquarters, in Germany.

Fresenius is a Fortune 500 company. The Fresenius Group has four business segments responsible for their operations worldwide: Fresenius Kabi, Fresenius Medical Care, Fresenius Helios and Fresenius Vamed. Kabi develops, produces and commercializes pharmaceuticals and medical devices. The main specialities are medicines, technologies for infusion and transfusion, and clinical nutrition.

The internship project consisted in designing a suitable lipid-based oral formulation to deliver lipophilic drugs to chronically ill patients. The formulation had to be produced using safe excipients and allow for an easy and affordable industrial production. The name of the drug used in the tests is not revealed for confidentiality reasons.

During the eight months I spent at Fresenius, I planned and carried out tests of solubility and emulsifying capacity, which provided me considerable

lab experience and valuable practical training. I attended several equipment training sessions and also had the chance to follow closely the work of other teams with whom I learned about business operations, regulations and marketing processes in the pharmaceutical field.

The experience was extremely enriching, and I am very grateful to the Innovation and Development team from Kabi for their teachings and exceptional internship program.

Chapter 2

Introduction

2.1. Importance this study

2.1.1. The low oral bioavailability of lipophilic compounds

Up to 70% of new chemical substances discovered by the pharmaceutical industry are poorly water soluble or lipophilic compounds. The low water solubility has been identified as the primary factor leading to poor oral bioavailability, high absorption variability, and issues in dose proportionality [1].

Very promising drug candidates are amongst these poorly soluble molecules. They commonly have a complex molecular structure, large size and molecular weight, high lipophilicity, inter and intramolecular H-bonding and other physicochemical properties that contribute to their low water solubility.

Increasing the bioavailability of these compounds is a real need, in order to afford the use of these drugs. For pharmaceutical companies, such as Fresenius Kabi, this is a promising challenge, and significant investments are being made in the design of new complex formulations capable of enhancing the bioavailability of selected molecules.

2.1.2. The enteral feeds market

The enteral feeds market in Europe is growing. The market leader for enteral feeds in Europe is Royal Numico NV. The other four firms that have the highest sales volume in this field are Novartis AG, Abbott Laboratories Ltd, Fresenius Kabi, and Nestle [2].

Fresenius Kabi has a broad portfolio of tube feeds, powder formulations and nutritional supplements. Most of these products are directed at dysphagia patients and come with different consistencies [3].

While general feeds are still important, the demand for enteral feeds designed for disease-specific solutions is foreseen to grow the most. Changes in lifestyle and an ageing population are the primary reasons for this scenario. Companies are now starting to focus on building pipelines of disease-specific products, mainly for cancer, diabetes, human immunodeficiency virus, Alzheimer and cardiovascular diseases [2].

As more technologically advanced enteral feeds and devices develop, enteral nutrition will be more frequently used in hospitals, and in the homecare segment as well. More parenteral nutrition treatments will be substituted by enteral nutrition solutions, with less invasion and side effects for the patients. And, at the same, time more clinical data will promote the market expansion.

2.2. Scope of the study

The main goal of the project was to design a complex formulation for clinical nutrition application. The development of a suitable enteral nutrition solution, to administer water insoluble nutrients overcoming their low bioavailability, constituted the base challenge.

This ambitious project continued after the end of this internship. This thesis covers the work done during eight-month of my stay at Fresenius, which comprises the method design, initial formulation models and first tests *in vitro*.

Following the chapters comprising the literature review, objectives, method design and materials used, the results of the *in vitro* studies performed are presented. They are divided into two main sections: dependence of solubility of a model substance and emulsifying capacity. The results obtained from testing the solubility of the model substance in different oils, surfactants and mixtures are discussed in the first part of the chapter. As for the second part, it focuses on the outcomes of the emulsifying capacity screening study, which was made through microemulsion assay evaluation and phase diagrams construction.

The formulation designed is lipid-based. This formulations are still a niche when in oral delivery of poorly soluble drugs. However, a bigger demand is increasing investment in research in this field.

The development of advanced lipid-based drug delivery systems is a suitable strategy to design successful pharmaceuticals with enhanced and more efficient therapeutic effects.

In particular the self-microemulsifying drug delivery system (SMEDDS), is a good alternative strategy to transport and deliver hydrophobic drugs [4].

SMEDDS are characterised as being physically stable and fairly easy to manufacture. They are isotropic mixtures of oils and surfactants, able to create a fine oil-in-water (O/W) emulsions when introduced in an aqueous system such as the human body. When using microemulsion systems as vehicles for bioactive molecules, the formulations should be passed on SMEDDS. The SMEDDS will then form an O/W upon dilution to a particular water content, commonly leading to better practical results in increasing the bioavailability of the molecule.

2.3. Objectives

This thesis aims to provide deeper insight on complex formulations that enhance absorption of poorly water-soluble substances, for clinical nutrition applications. More precisely, it describes and discusses the results obtained during the project of the designing and testing of a novel and innovative formulation aimed to improve bioavailability of lipophilic substances.

The purpose of the tests conducted was to create project ground for a new line of Fresenius Kabi oral products capable of delivering such substances to patients in need.

As shown in the literature review, several techniques to deliver lipophilic drugs are available, but not many are fully understood, practical, or adequate for critically or chronically ill patients. A model substance was used to test the hypothesis of a new formulation design that relies on a lipid-based delivery system to improve the bioavailability of lipophilic compounds.

Additionally, the interest relied in gaining a better understanding of the impact of the properties of excipients on the formation of these complex formulations.

The first part of this study will present data on the dependence of solubility of the model substance on ingredient properties. This data is crucial to evaluate which oils and surfactants show better results at solubilizing the drug and can become the best carrier. In order to explore and uncover the impact of ingredients on the formulation, there will be an analysis and discussion of the results, will be presented regarding the relationship between the properties of the mixture and components used.

In the second part, the focus is on the emulsifying capacity. Results are discussed with respect to the physicochemical properties of the ingredients. This second part is essential to identify the most promising microemulsion formulations that will on a later stage of the product development be characterized and tested before product release.

Chapter 3

Literature Review

3.1. Enhancing the bioavailability of poorly water-soluble substances

"These new compounds, like rocks, never dissolve in water" [5]

3.1.1. Introduction - the problem

Low water solubility is known to be one of the primary reasons for the poor absorption of new chemical entities (NCEs) by the human organism.

Around 40-70% of all NCEs developed are insufficiently soluble in water. Consequently, their absorption in the gastrointestinal tract (GIT) is small and inadequate [1]. A poorly soluble drug in aqueous media is one that has a longer dissolution time in the gastrointestinal (GI) fluids, than the time it takes for it to go through the portions of the GIT where absorption occurs [6].

These NCEs and potencial drug candidates are very promising, however they display some performance issues due to their own design and characteristics [7].

It is now possible to create artificially very complex compounds thanks to the fast technological innovation in the field. Several physicochemical properties contribute to the poor solubility of potential drug candidates. Among them are their typical complex structure; large size; ionic charge and pH. Molecular weight; and intra and inter molecular H-bonds are also characteristics that make these compounds poorly soluble on water [8], [9]. [10].

Because of the unique characteristics of these molecules, traditional methods and formulations are not suitable approaches. They fail to provide the necessary bioavailability [11], [12]. The most common traditional methods

are solid wet granulation, solid dry granulation and water-soluble liquid in a capsule.

Lipid formulation is a very interesting technology full of potential to address the issue of challenging new drugs design. Important advances have been made, however research is quite scarce in in this field. In fact, only a small percentage of currently marketed products uses lipids as the primary method of drug delivery [10]. Enhancing the bioavailability of these poorly soluble substances is of outmost importance.

3.1.2. Application of LBDDS – an opportunity

Humans have been using lipids to deliver drugs for a long time. Lipid-based creams, emulsions or suppositories have been on the market for long, and some of them were created in the ancient Egyptian times. However, only recently a more substantial evolution has been accomplished in new designs of oral lipid carriers for poorly soluble drug delivery [13].

The application of lipid-based drug delivery systems (LBDDS) is comprehensive and versatile as they can deliver different types of drugs, as well as proteins and peptides [14]. The primary objective of LBDDS is to increase the bioavailability of a component with low water solubility more than a traditional oral solid dosage form could ever achieve [15]. In this way, the lipid-based systems are can be used to create pharmaceutical dosage forms with a more promising therapeutic effect [16].

3.1.3. Oral drug delivery - the most convenient and accepted method

LBDDS can be administered to patients through several routes. Oral and parenteral are the most common, but there are products using nasal, dermal/transdermal, vaginal, ocular and pulmonar delivery methods. Oral is acknowledged the preferred way because it is a non-invasive method, less expensive and has fewer side effects – e.g. injection site reactions. It is particularly favorable in chronic therapies for the last reason stated [13].

Oral lipid-based formulations have not only proven their capability to improve gastrointestinal absorption of lipophilic drugs, but also to minimize reactions with food that sometimes make the absorption process less efficient

[1]. These products entered the market in 1981 and by 2007 they represented 3% of the oral formulations being commercialized [12]. They come in different levels of complexity, from one-excipient formulations to multiple-excipient self-emulsifying drug delivery systems [12].

Although, LBDDS have revealed more efficient results than the traditional oral formulations, in 2007 the marketed oral lipid-based formulations were still outnumbered 25 to 1 by the conventional formulations [1] [17] [18] [19]. The vast majority of oral formulations available in the market are still solid dosage forms, like tablets or capsules.

3.1.4. Advantages of Lipids – versatile excipients

The advantages of using lipid-based solutions to enhance bioavailability of lipophilic drug candidates and GI absorption is well documented with data in the literature [20] [21].

Lipids are considered to be an extremely versatile ingredient, that provides the formulation designer with many options for delivering and controlling the absorption of lipophilic drugs [15]. They can be manufactured in large scale and present many desirable features, such as being chemically compatibile and having self-emulsifying attributes [22].

3.1.5. Mechanisms by which lipids improve bioavailability

The principal mechanism by which oral LBDDS enhance the absorption of the bioactive molecules is making unecessary to solubilize the drug before absorption by the GIT. Other mechanisms include the protection from chemical and enzymatic degradation from gastric and environmental conditions; promotion of lymphatic drug transport and also creating a hydrophobic environment that causes the release of the drug to initiate later in time. This positive effect on drug absorption comes from several factors. The first is the stimulation of bile salts that leads to the emulsification of the drug in the GI fluid, enhancing solubility in vivo; the interaction with enterocyte-based transport and improving drug uptake and efflux; and the recruitment of lymphatic drug transport [20] [13].

3.1.6. Lipid Formulation Classification System

The Lipid Formulation Classification System (LFCS) was published for the first time in 2000 and it was modified in 2006. The modification made was the addition of a new 'type' of formulation [24]. The classification system - represented below in Table 1 – and research works like this one, will lead to a better understanding of key factors that determine the performance of LBDDS. The LFCS contributes to creating more efficient methods and evaluating their performance in a simpler way [13].

The LFCS Consortium develops research on LBDDS to orally administrate water insoluble drugs. It was created after the LFCS's publishing and since then integrates a scientific community of industrial and academic professionals. Its purpose includes developing guidelines that contribute to accelerating and promoting the development of drug delivery strategies for drug candidates. The goals of the Consortium are identifying the factors responsible for LBDDS performance and certifying operating procedures to assess this performance [25].

The LFCS has been discussing more in the last years towards deciding on a framework that can be adopted to compare the performance of lipid-based formulations. Group III has been divided into Type IIIA and Type IIIB, to make a distinction between formulations that contain a higher proportion of oils (Type IIIA) and the others, which are predominantly water-soluble (Type IIIB). The differentiation between Types IIIA and IIIB was based on the dimensions of excipients in formulations. Table 1 shows the differences between Type I, II, III and IV formulations. Table 2 displays the standard composition of several types of lipid formulations [26].

Formulation	Materials	Characteristics	Advantages	Disadvantages
Type I **Oils**	Oils with no surfactants (e.g. tri-, di- and monoglycerides).	Non-dispersing; requires digestion.	Recognized as safe (GRAS) status; simple; excellent capsule compatibility.	Formulation has weak solvent capacity unless drug is highly lipophilic.
Type II **SEDDS**	Oils and water-insoluble surfactants; no water-soluble components.	SEDDS formed without water-soluble components. Emulsion. Will be digested.	Unlikely to lose solvent capacity on dispersion.	Unclear O/W dispersion (particle size 0.25-2μm).
Type IIIA and IIIB **SEDDS/SMEDDS**	Oils, surfactants, co-solvents (both water-insoluble and water-soluble.	SEDDS/SMEDDS formed with water-soluble components. IIIA: Fine emulsion. IIIB: Transparent dispersion.	Transparent or mostly clear dispersion; drug absorption without digestion.	Possible loss of dissolving capacity on dispersion; less easily digested.
Type IV **Lipid-free**	Only water-soluble surfactants and cosolvents.	Formulation disperses and forms a micellar solution.	Adequate solvent capacity for most drugs.	Likely loss of the solvent capacity; may not be digestible.

Table 1 - Classification of Lipid-Based Formulations.

Source: [25] [26]

Excipients in formulation	Content of formulation (%, w/w)				
	Type I	Type II	Type IIIA	Type IIIB	Type IV
Triglycerides or mixed mono and diglycerides	100	40-80	40-80	<20	–
Water-insoluble surfactants (HLB < 12)	– –	20-60	--	--	0-20
Water-soluble surfactants (HLB > 12)	--	--	20-40	20-50	30-80
Hydrophilic cosolvents (e.g. PEG, Propylene glycol, transcutol)	--	--	0-40	20-50	0-50

Table 2 -Typical content of different types of lipid formulations.

Source: [25] [26]

3.1.7. Digestion and Absorption of Lipids

Using lipid-based formulations as a method of drug delivery can influence the absorption process of the drug. Most drugs that are delivered orally access systemic circulation via the portal blood. However, lipophilic drugs have a diferente path, they enter in systemic circulation via intestinal lymphatics, bypassing hepatic metabolism. Lipids can also have some consequences on digestion such as delays in the gastric transit period and increase passive permeability in the intestine [28].

Lipid digestion, illustrated in Fig.1, has three primary processes. Firstly, the fat globules dispersion that leads to the formation of an emulsion. Secondly, the enzymatic hydrolysis of triglycerides (TG) at the oil/water interface. Thirdly, the dispersion of the digestion products into an emulsion. This emulsion will have a high surface area from which absorption takes place.
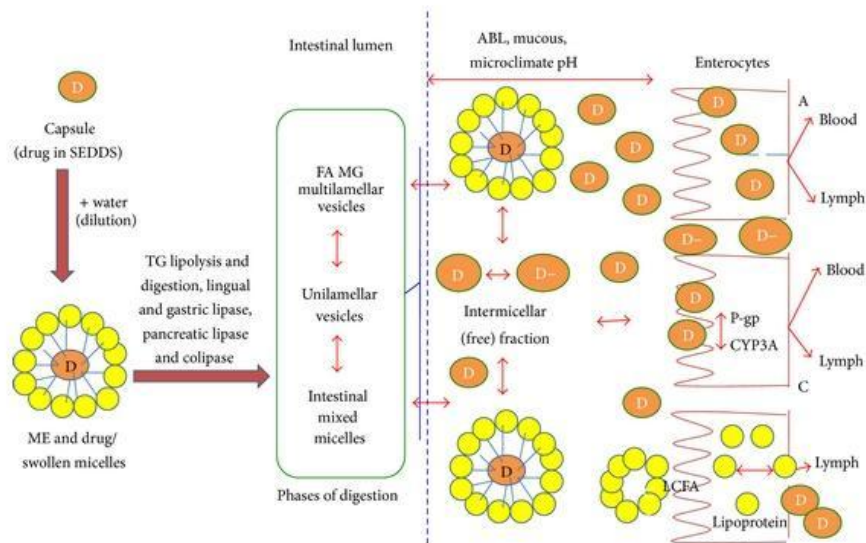
Figure 1 - Diagram of intestinal drug transportation with lipid-based systems.

(A) Increased membrane fluidity facilitating transcellular absorption, (B) opening of tight junctions to allow paracellular transport, (C) inhibition of P-gp and or CYP450 to increase intracellular concentration and residence time, and (D) stimulation of lipoprotein/chylomicron production. Abbreviations: aqueous boundary layer (ABL); drug (D); ionized drug substance (D-); fatty acid (FA); long-chain fatty acid (LCFA); microemulsion (ME); monoglyceride (MG); self-emulsifying drug delivery (SEDDS); triglyceride (TG); tight junction (TJ) .

Source: [29]

The dietary lipids start being digested as neutral TG in the stomach where gastric lipases begin the hydrolysis of the TG. They are decomposed into diglycerides and free fatty acids. The emulsion formed goes through the duodenum and there is an increase in the production of bile salts. Lipase enzymes are released from the pâncreas. There's an increase in surface area as droplets comprising the emulsion go through a reduction. This helps lipid hydrolysis to occur at the oil/water interface. The result is the production of one molecule of 2-monoglyceride and two molecules of fatty acids for each TG molecule hydrolyzed. These digestion products stay at the surface of the lipid droplets forming crystalline. The micelles produced from the interaction of the digestion products and bile salts will dissociate and release emulsified

lipid digestion products. When the proteins L-FABP and I-FABP get inside the enterocyte they bind to the fatty acids and help their solubilization [30].

3.2. Techniques to improve bioavailability

3.2.1. Introduction

Several methods have been designed and tested to enhance the bioavailability of lipophilic molecules. The most commonly used are particle size reduction (micronization or nano sizing), complexation with cyclodextrins, formation of salts and solubilization with cosolvents and surfactants. Changing physicochemical properties, through salt formation and particle size reduction can improve the dissolution rate of the drug. However, these approaches cannot sometimes be used, for example, salt formation of neutral compounds is not doable. Also, the salts of a weak acid and a weak base will most of the times go return to original base or acid forms, which can have adverse effects on the GIT. Particle size reduction may cause an increase of static charges, causing handling difficulties [31].

Only recently, focus has been turning to the technique of using lipid-based formulations to enhance the oral bioavailability of water insoluble drugs [32]. Lipid-based formulations are a physiologically well-tolerated and provide a vast choice of possibilities to formulate and to increase the bioavailability of water- insoluble drugs [13]. They are used for poorly soluble drugs in case the drug is an oil or when traditional formulations fail to enhance its bioavailability [12].

Lipid-based formulations include a vast group of formulations. From simple one-excipient triglyceride vehicle, such as corn oil, olive oil and soybean oil, to more complex formulations such as SEDDS [12]. The majority of lipid-based formulations are engineered to deliver the entire dose in solution so that the dissolution step does not happen in the GI tract. This characteristic has been considered to be an essential requisite for the good performance of these formulations [32].

LBDDS represent a considerable number of formulation options. They can be prepared using: solutions, emulsions, suspensions, microemulsions,

solid lipid nanoparticles, liposomes, SEDDS, SMEDDS, or dry emulsions [32]. Fig.2 displays a classification of self dispersing lipid formulations in four groups of lipid-based formulations based on the impact of dilution and digestion and also composition [33].
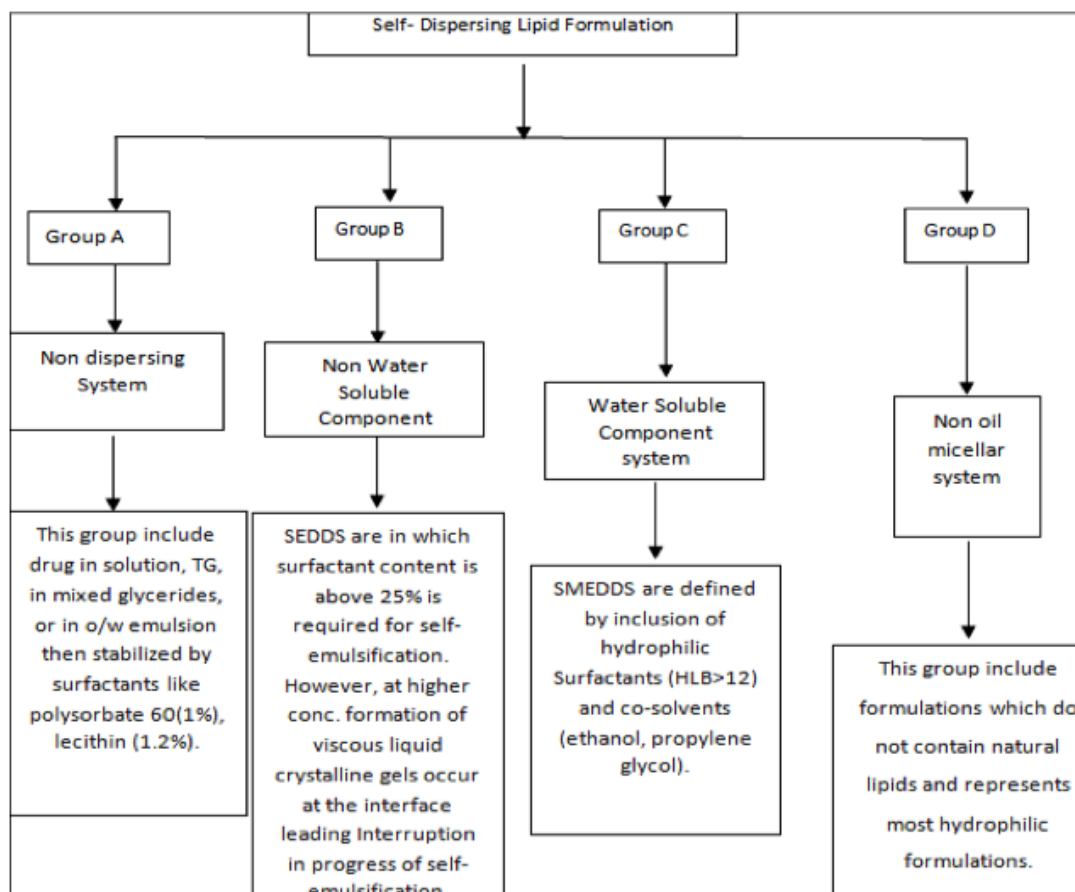
Figure 2 - Classification in four groups of lipid-based formulations based on composition and the effect of dilution and digestion.

Source: [33]

Microemulsions present intrinsic advantages, such as: being thermodynamically stable, optically clear and easy to prepare. Both water-soluble and oil-soluble compounds can be be solubilized thanks to the microdomains of opposite polarity in a one-phase solution [34].

3.2.2. Lipid-based Formulations – a focus on Microemulsions and SMEDDS

Microemulsions are mixtures of at least water, oil and a surfactant [35]. These systems are like a solution and have a stable inner structure of nanodroplets due to the action of the surfactants [36].

Microemulsions are very efficient carriers for a vast number of bioactive molecules, and when the ingredients are used in the proper ratio their formation is spontaneous, i.e. it does not require an input of energy [36].

In the 40s, Hoar and Shulman designed a clear single phase solution. This occured when they were making a titration with a milky emulsion containing hexanol. This experiment led to the introduction of the microemulsion concept [37]. The difference between emulsions and microemulsions are that emulsions are thermodynamically unstable and eventually the phases will separate, whilst microemulsions are stable [38].

Microemulsions are a cost effective technique to increase the bioavailability of poorly water soluble drugs. They have very low surface tension and small droplet size, leading to a high absorption. Interest in this systems are increasing, and microemulsion applications have now different administration methods. There have been significative results from the use of a microemulsion formulation of a poorly soluble drug, for example, immunosuppressants. Produced as a soft capsule, it contains a mixture of drug dissolved in oil and surfactant [31].

Even though, microemulsions have been studied and tested in depth from a physicochemical point of view, most of the systems investigated are not suitable for pharmaceutical use. The primary reason for this are the excipientes that need to be used [39]. The choice of ingredientes is critical when designing the formulation.

Studies show that for microemulsion systems to be used as vehicles for potential drugs, the formulations they should be passed on SMEDDS, that will go on creating a O/W solution when diluted to a specific water content [36].

SMEDDS and microemulsions are different, however they are considered to be a similar system. A SMEDDS is commonly a mixture of surfactant, oil and API that when is administered rapidly disperses and

creates droplets with identical diameter to those existent in microemulsions. They are manufactured easily and suitable for oral delivery given their self-emulsifying properties with the right choice and proportion of ingredients: lipid and surfactant [20]. The presence of the surfactant is necessary to obtain the hydrophilic-lipophilic balance needed for the emulsification to occur.

Dispersed in the GI tract they are exposed to movements from the stomach and intestines that initiates the emulsification process [20].

3.3. Selection of ingredientes

3.3.1. Introduction

The selection of ingredients is one of the most significant and challenging steps in the development of a suitable self-emulsifying lipid-based oral formulation.

An ideal excipient needs to be safe for human usage. It should be inert and not degrade during manufacturing or storage. It needs to be capable of solubilizing the dose of the API in the reduced boundaries of an oral capsule and have surface active properties that can allow self-emulsification or complete dissolution of the API. It must be reliable in delivering the transported drug and making it more bioavailable. Also, it should be compatible with a broad range of medicines, compounds, and other excipients. Manufacturing the product should be cheap and simple and allow ready scale-up from the lab to the industrial context [23].

3.3.2. Lipid components

Lipids are a resourceful class of ingredients capable of being used in many ways to enhance the delivery and absorption of chemical substances [15].

They are physically and chemically distinct substances between them. This class includes fatty acids, sterols, waxes, phospholipids, sphingolipids, glycerides and fat-soluble vitamins [20].

These ingredients can be used to deliver drugs orally as solutions, suspensions, emulsions, microemulsions, SEDDS or SMEDDS, solubilizing

the lipophilic compounds and facilitating self-emulsification and the absorption in the GIT [33].

The amount of this excipient will affect pancreatic secretion and consequently the absorption of the API so deciding the size of the dose is a vital step [20].

3.3.3. Surfactant

Most microemulsions and SMEDDS formulations need the addition of large quantities of surfactant.

The surfactants used in these formulations usually have high values of HLB. They provoque a quicker dispersion in the GIT and also decrease the risk of drug precipitation post the dilution in the gastro intestinal fluids [20].

Most surfactants have a polar head group and an apolar tail. The tail has the larger molecular volume especially in the case of ionic surfactants. During the dispersal process in water, surfactants self-associate due to intra and intermolecular forces [34].

Surfactants of biological origin are usually selected for higher safety, even though the synthetic alternative provides a more eficiente result in self emulsification. However, artificially synthesized surfactants tend to have higher values of toxicity for humans [20].

Upon the addition of surfactants to a mixture of oil and water, the molecules of the surfactant accumulate in the oil/water interface. Various phases can form resulting from it [34].

Fig.3 shows some association structures that can be created when surfactants are added to water, oil or a mixture of the two.
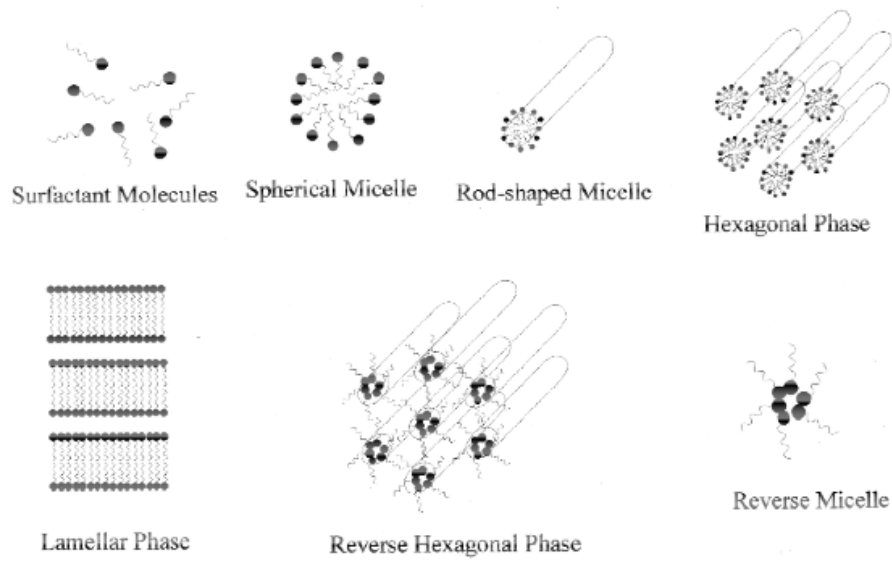
Figure 3 - Representation of some commonly observed self-association structures in water, oil or mixture of both.

Source: [34]

Fig.4 depicts the most recurrent types of microemulsions: oil-in-water, bicontinuous and water-in-oil.
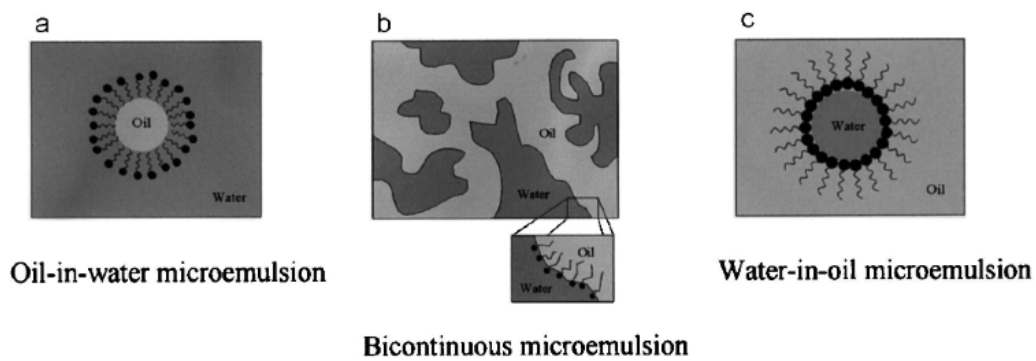
Figure 4 - Representation of three most common microemulsion microstructures: a) oil-in-water, b) bicontinuous, and c) water-in-oil.

Source: [34]

Chapter 4

Materials and methods to study formulation models

4.1. Materials

Oils and Surfactants:

Ethyl Oleate obtained from Sigma-Aldrich;

FK-Sunflower Oil obtained from Fresenius Kabi;

FK-MCT Oil obtained from Fresenius Kabi;

Miglyol 840 obtained from Sasol;

Tween 80 viscous liquid obtained from Sigma-Aldrich;

Labrasol obtained from Gattefossé;

Model API.

Devices:

Sartorius, Scale Extend, Model ED2245;

IKA RET basic, magnetic stirrer;

Thermo Electron Corporation, HERAEUS Pico17 centrifuge;

UV-spectrophotometer, Eppendorf BioSpectrometer, Kinetic.

Other Equipment:

Magnetic stir bars;

Disposable plastic eppis, Eppendorf, with volume 1.5ml;

Disposable plastic cuvettes, Plastibrand, 1.5ml semimicro (12.5 x 12.5 x 45mm);

Disposable plastic pipettes, Eppendorf 3ml;

Metal spatulas;

Glass beakers;

Glass bottles with lids;

Disposable latex gloves;

Protective glasses, shoes and lab coat.

<u>Specialized software:</u>

Origin Pro 8, by OriginLab Corporation.

4.2. Solubility tests

To evaluate which oils and surfactants present better results at forming microemulsions, four different oils and two different surfactants were pre-selected to perform solubility tests with our model API. The oils tested were Ethyl Oleate, FK-Sunflower Oil, FK-MCT Oil, and Miglyol 840. Moreover, the surfactants used were Tween 80 viscous liquid and Labrasol.

As shown in Fig. 5 solubility tests were performed using the following method:

Firstly, an excessive amount of our API was added with a metal spatula to a concentrate (oil, surfactant or mixture). The chemicals were precisely weighed, and the resulting suspension was mixed, at room temperature, for 16h at 480rpm, at 21ºC, using a magnetic stirrer. Secondly, the resulting mixed suspension was transferred to disposable plastic eppis and centrifuged at 10000 g for 10min. Thirdly, a new dilution was prepared using the supernatant that resulted from centrifugation. Lastly, the dilution was taken for analysis in a UV-spectrophotometer, where the absorbance values were measured at 425nm, using disposable plastic cuvettes.

The method was repeated three times for each oil, surfactant and mixture stock solution. The dilutions were also repeated three times for higher accuracy in the results.
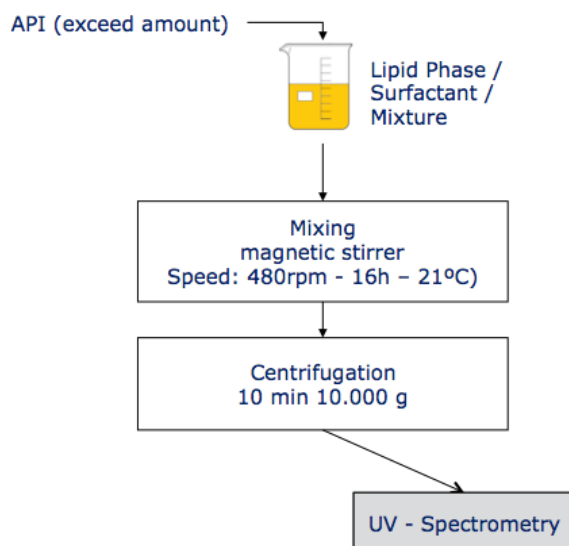
Figure 5 - Scheme showing the solubility test procedure.

In order to analyze the data, the maximum values of diluted API in the concentrate were calculated from a calibration line for each of the mixtures (API + concentrate) being tested. The UV-spectrometry measurements were repeated three times for more accurate results.

4.3. Emulsifying capacity evaluation by PDMPD method

In the second phase of our formulations study, emulsifying capacity was evaluated. The Phase Diagram by Micro Plate Dilution (PDMPD) method was used, and consists in gradually diluting the oil phase with the water phase in a microtitre plate.

The PDMPD method is an efficient and innovative approach that allows time and material savings while creating pseudo ternary phase diagrams for microemulsions and nanoemulsions.

Compared with the traditional titration method (drop method), the PDMPD method enables a more exact status description of mixtures in pseudo ternary diagrams. It also offers the possibility of examining the dilution stages simultaneously on just one microplate [40].

Microemulsion assays consisting of a water phase, an oil phase, and a surfactant phase were prepared on microtiter plates (96 wells) as shown in Fig. 6 and described by Maeder in "Hardware and software system for

automatic microemulsion assay evaluation by analysis of optical properties"
[41] with slight modifications.
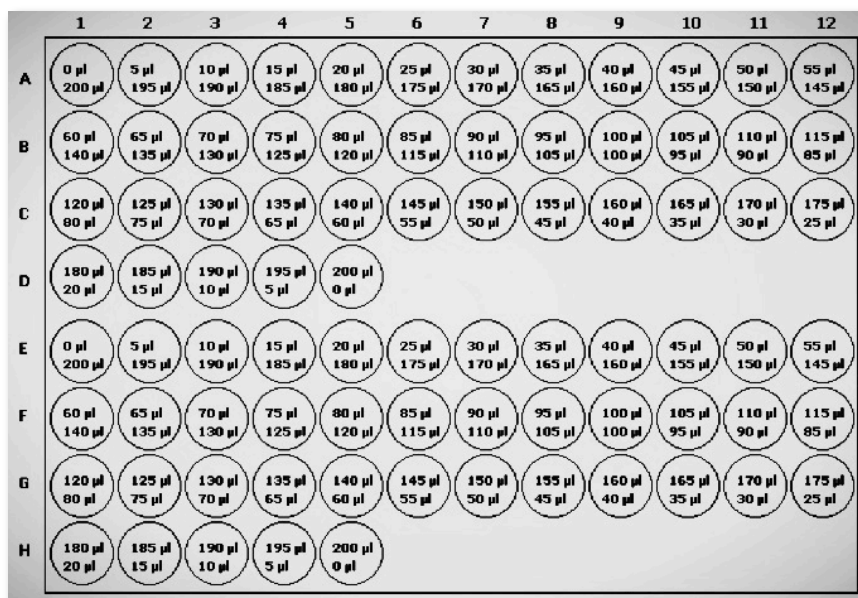
Figure 6 - Filling scheme for the microtitre plates.

Inside each well, the upper value corresponds to the water phase and the bottom value to the oil plus surfactant phase.

The preparation is described bellow:

Firstly, the mixtures of oil and surfactants were prepared using a magnetic stirrer, at speed 480rpm, for one hour, at 21°C.

To evaluate the five different ratios between one oil and one surfactant five different mixtures were prepared, as shown in Table 1. In total 20 mixtures were tested to assess the following mixtures: Tween80+EO; Tween80+MCT; Tween80+Mig840 and Tween80+(MCT,EO). For more accurate results, each mixture was prepared and tested three times making a total of sixty mixtures made.

	Oil 1 Phase %	Surfactant 1 Phase %
Mixture 1	50	50
Mixture 2	40	60
Mixture 3	30	70
Mixture 4	20	80
Mixture 5	10	90

Table 3 - Oil 1 /Surfactant 1 mixing ratios

Secondly, the wells were filled in two steps:

In the first phase, starting in A1 and finishing in D4 the mixture was gradually loaded in the wells using a Pipette Research Plus, 200µl, and disposable plastic pipette tips, Eppendorf, 200µl. The filling process must be done with care to avoid air bubbles, which is especially hard with the more viscous oils. If air bubbles are present, the plate is not valid for the study and must be thrown away.

In the second step, the aqueous phase is added, starting at D5 with 200µl up to A2 with 5µl. The microtitre plates used were Thermo Scientific* Nunc Flat Bottom 96-well polystyrene transparent plates with lids, 350µl/well.

The wells E1 to H5 of the same plate were loaded following the same procedure, but with a different mixture (different ratio of the surfactant and oil phase). Following this scheme, two fixed surfactant/oil-ratios can be placed on every plate. Table 2, below, illustrates the distribution.

Plates	Wells	Content
1	A1-D5	Mixture 1 + Water
1	E1-H5	Mixture 2 + Water
2	A1-D5	Mixture 3 + Water
2	E1-H5	Mixture 4 + Water
3	A1-D5	Mixture 5 + Water

Table 4 - Mixtures distribution by plates

Finally, the plates were sealed with their respective lids and were set in a Biometra, Rocking Platform, model WT15, for 16h, at maximum speed, with controlled temperature of 21ºC. At the end of the 16h, the plates were scanned using a RICOH Aficio, scanner, model MP-C2551 with a pre-prepared marked lid. Each plate was repeated a minimum of three times and in different days. From the analysis of the several repetitions, it was determined which combinations resulted in the formation of microemulsion. This study consisted of observing the scans and attributing a 0 when a well showed turbidity and a 1 when was transparent, and it was possible to see clearly the marked dot on the bottom of the well. Two observers did this analysis and the results were crossed checked. When the sum of the three test was 2 or 3, the preparation was considered an emulsion. When the sum was 0 or 1, it was not considered an emulsion as depicted in Table 3.

Well / Plate	16	21	25	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	0	0	0	NOT

Table 5 - Determination of emulsifying capacity of wells A1-A7 of plates 16, 21 and 25 containing a mixture of Tween80% and Mig840 (1:1).

After the determination of emulsifying capacity phase diagrams were plotted. The software used was Origin Pro 8, by OriginLab Corporation.

Fig. 7 shows one of the phase diagrams built. Each red point represents an emulsion formulation identified and each white point a non-emulsion. For each line in the diagram 3 plates were prepared and analyzed.
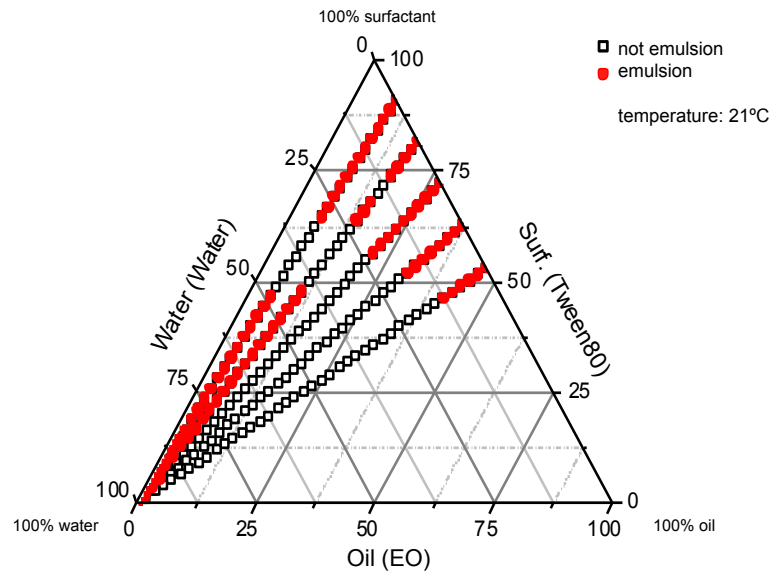
Figure 7 - Phase diagram

To develop this method, several pre-tests were made in different conditions. In the first experimental setup the vortex was used to shake 2 overlying plates, as shown on Fig. 8, at speeds 3, 2 and 1 and then one single plate at speeds 3, 2 and 1, for 16h. These pre-tests showed unrepeatable results and spilling. Therefore, the method was changed: the vortex was substituted a the rocking platform.

Different time periods were also pre-tested. Testing plates were set on a rocking platform for 8h, 9h, 16h, 18h, 20h and 22h. The selected mixing time was 16h, as the results for 18h, 20h and 22h were identical.
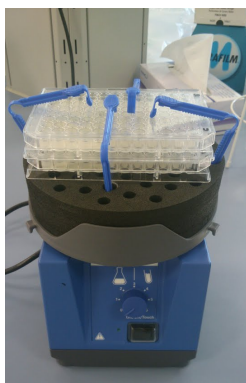
Figure 8 - Abandoned experimental setup using a vortex and two overlying plates

Chapter 5

Results and Discussion

5.1. Dependency of solubility of a model substance (1st part of the study)

5.1.1. Impact of the oil phase properties

The first part of the study tested the solubility behavior of the API chosen in the different oils and mixtures of oils selected. Different oils have different properties, such as carbon chain length, polarity, molecular weight and molecular structure and that results in a specific interaction with the API and specific solubilization performance.

Evaluating solubility behavior of the oils and mixtures is essential to identify the most suitable excipients to build a formulation capable of enhancing the absorption of the API by the human body.

The oils tested were Ethyl Oleate and Sunflower Oil - long carbon chain oils (LC) and MCT Oil and Miglyol 840 - medium carbon chain oils (MC). And the mixtures of oils tested were MCT Oil with Sunflower Oil (1:1), Miglyol 840 with Sunflower Oil (1:1), MCT Oil with Ethyl Oleate (1:1) and Miglyol 840 with Ethyl Oleate (1:1). Table 6, below, contains information about some of the properties of the four oils.

	FK MCT	Miglyol 840	FK Sunflower oil	Ethyl oleate
C-length	C8/C10	C8/C10	C18	C18
Molecular weight average	500.8	340	885	310.51
Density [g/cm³]	0.93-0.96	0.91- 0.93	0.91- 0.92	0.87
Viscosity at 20 °C [mPa·s]	25-33	9 – 12	60.8	6.89

Table 6 - Values of C-length, molecular weight average, density and viscosity of MCT, Miglyol 840, Sunflower Oil and Ethyl Oleate.

Fig. 9 shows the results from testing the solubilization behavior of the chosen API in the four tested oils and four mixtures of two oils.
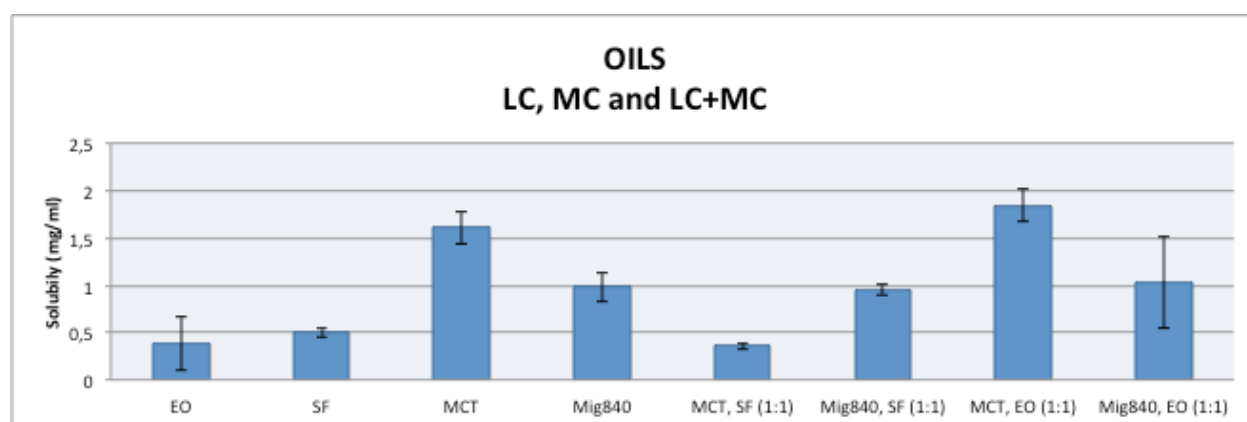
Figure 9 - Screening of oils and mixtures of two oils for the formulation at 21ºC.

Regarding the results obtained for the four oils, the MC oils (MCT and Mig840) presented higher values of solubility than the LC oils (EO and SF). The reason for which medium chain oils seem to be more efficient to solubilize the API used is the fact that the core part of the molecule has approximately the same size chain of carbons as the API. It can also be observed that oils with higher polarity (MCT and Mig840) seem to be more

suitable to prepare ME formulations than lower polarity oils (EO and SF), as typically they solubilize excipients better. These results were expected, and are in accordance with previous results from Kawakami [35].

Taking a more detailed look at molecular weight: the oil with higher molecular weight (SF, 885 g/mol) and the oil with lower molecular weight (EO, 310.5 g/mol) have shown poorer results than the Mig840 (340g/mol) and MCT (500.8 g/mol). The molecular weight of the tested API is approximately 370.0 g/mol, closer to the values of Mig840 and MCT.

Regarding the mixtures of oils they presented similar solubilizing power as the medium carbon chain oils. The addition of LCT oils to the MCT oils did not dramatically improve the results. The highest solubility results were obtained for MCT, EO (1:1) and the lowest solubility results were obtained for EO, SF (1:1). It was expected, however, that both mixtures presented higher solubility values, as according to Kawakami [35], the mixing of different types of oils enhanced the solubilization significantly. The oils used were different which is the most likely reason for this discrepancy.

It can also be observed that the standard deviation error is higher when EO is present. During the experimental procedure, it was noticed that EO provoked the plastic cuvettes to become turbid in just a few seconds after they were filled. This fact was considered the primary reason for the higher margin of error.

5.1.2. Impact of oil surfactant properties

Fig. 10 shows the solubilization behaviors of the API in the two tested surfactants Tween80 (20%) and Labraol (20%). The test with Labrasol was made later in time, after all, other solubility tests and emulsifying capacity screenings. Initially, Tween80 had been the only surfactant selected to be mixed with the oils and mixtures of oils.

We can clearly observe that Labrasol (20%), medium carbon chain, revealed a higher solubilizing capacity than Tween80 (20%), long carbon chain. Both surfactants are ME-forming surfactants. Their HLB values are similar, which indicates there is no significant difference between comparative sizes of the head group and tail group of the molecules.
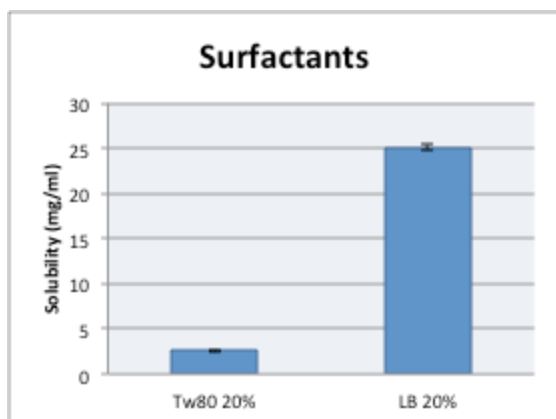
Figure 10 - Screening of surfactants for the ME formulation at 21ºC.

5.1.3. Impact of mixture properties

Fig.10 shows the solubilization behaviors of the API in mixtures of oils and a surfactant, Tween80. The values of solubilizing capacity of the surfactants alone are also in Fig.11 for convenience of comparison.

Both medium chain and long chain oils had a drastic enhancement in their solubilizing capacity when mixed with a surfactant. However, the combination of a medium chain oil with a long chain oil and surfactant showed the best results, which goes in accordance with results previously published by Kawakami [35].
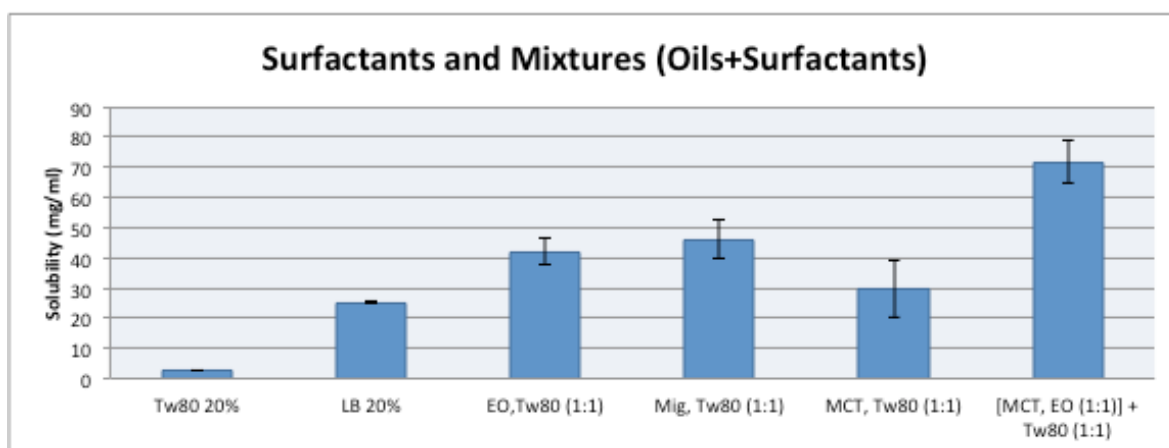
Figure 11 - Screening of surfactants and mixtures of oils and surfactants for the ME formulation at 21ºC.

5.2. Emulsifying capacity evaluation by PDMPD method (2nd part of the study)

As described in the methods chapter, in the second stage of the formulations study the emulsifying capacity of different formulations was evaluated. For that purpose the Phase Diagram by Micro Plate Dilution (PDMPD) method was used. It consists of gradually diluting the oil phase with the water phase in a microtitre plate and analyzing the results through the construction of phase diagrams.
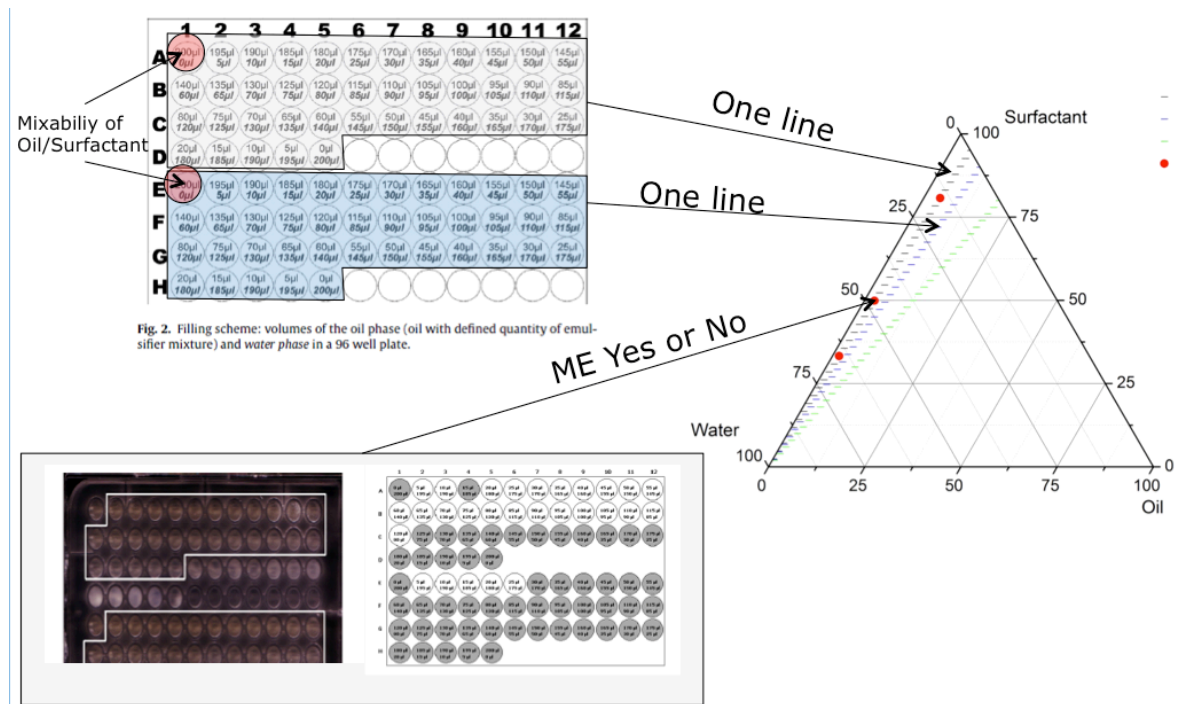
Figure 12 - Illustrative scheme of how phase diagrams are constructed.

After the dilution process, the microtitre plates are scanned, and these scans are in their turn analyzed. If a mixture is a microemulsion, it is represented by a red dot in the phase diagram. If the mixture is not a microemulsion, it is represented by a white dot.

The next page shows one of scans and microemulsion results obtained. All the other tables of results and scans are in annex section.

Microwell plates 15.L, 15.C, 15.R

Well / Plate	15.L	15.C	15.R	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	0	0	0	NOT
A8	0	0	0	NOT
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	0	0	0	NOT
D3	0	0	0	NOT
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion
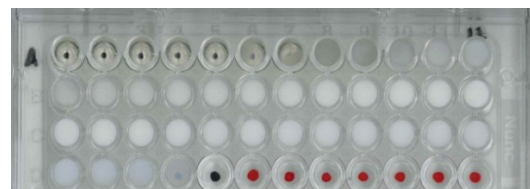
15.L   TW80(50%), EO(50%)
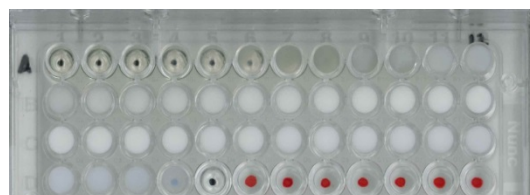
15.C   TW80(50%), EO(50%)
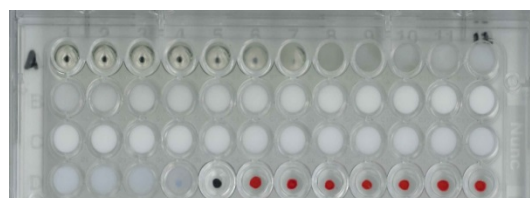
15.R   TW80(50%), EO(50%)

Figure 13 - Microwell plates 15.L, 15.C, 15.R, scans

Table 7 - Microwell plates 15.L, 15.C, 15.R, determination of emulsifying capacity.

With the results obtained from the scans analysis, the phase diagrams below were built. The software used was Origin Pro 8 and, as described previously, the mixtures that resulted in microemulsions are marked in the diagrams as red dots and the others as white dots. It is important to note that the first and the last dot in each line are not a formulation; they correspond to the wells that contain either 100% water or 100% mixture of oil and surfactant.

The PDMPD method allowed us to mix different ratios of the excipients in relatively few well plates and provided us with much more reliable data than the traditional drop method would. Looking at the scans, it is quite simple to identify the optically transparent microemulsions that allow the observer to see the dot in the bottom of the well.

The reason there are only lines present on the upper part of the diagram is that formulations that contain more oil than surfactant show very poor results, i.e., they do not form microemulsions. Therefore, for convenience of resources and time it was decided that only mixtures with surfactant percentage between 10 and 90 would be screened.

The most favorable scenario is to have a maximum number of red dots in the phase diagram. This represents a presence of microemulsion in most variation of aqueous states, meaning that the variation in content of water will have less influence in the maintenance of the microemulsion status.

Also important is to have a presence of red dots in the region of the phase diagram where surfactant percentage is not at its maximum. High concentrations of surfactant may lead to adverse side effects on patients due to toxicity.

Below are shown the four phase diagrams obtained. Figure 14 corresponds to the diagram of mixture EO and TW80; figure 15 corresponds to the mixture MCT and TW80; figure 16 corresponds to the mixture Mig840 and TW80 and figure 17 corresponds to the mixture of MCT and EO (1:1) and TW80.
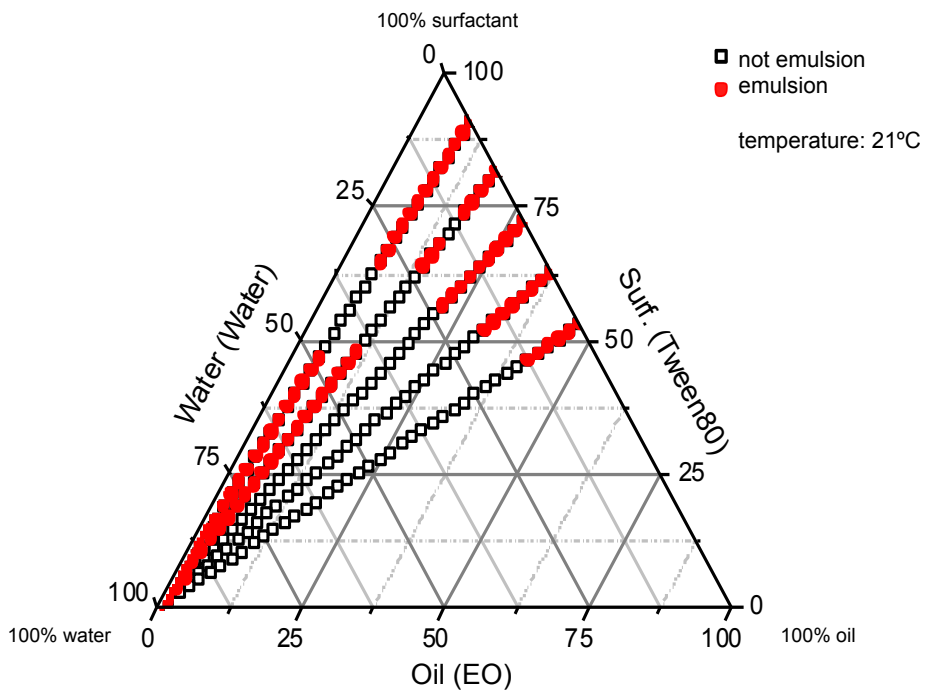
Figure 14 - Phase Diagram, Tween80 and EO.
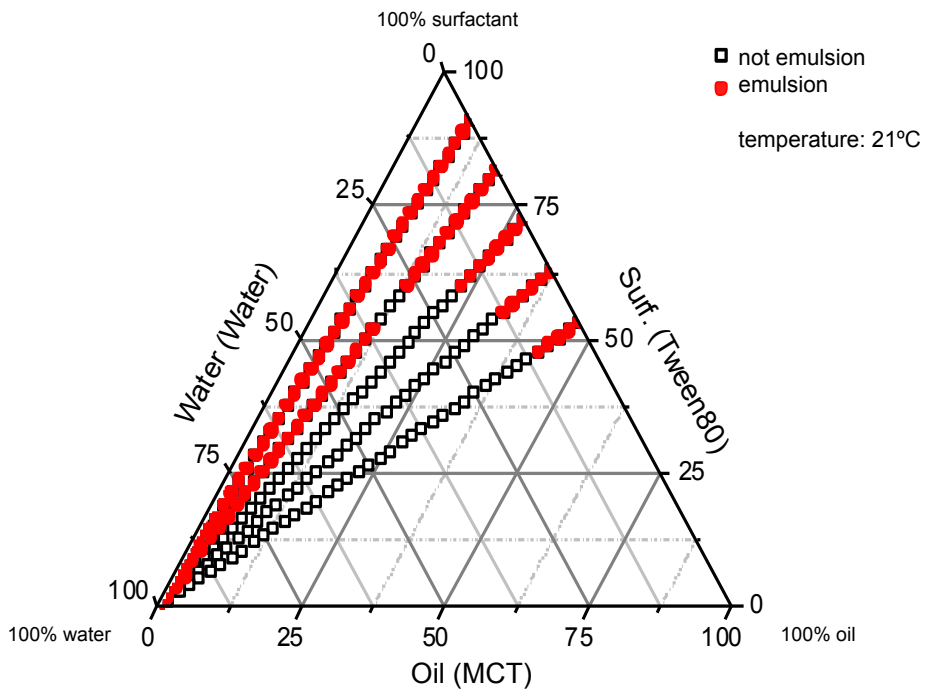
Figure 15 - Phase Diagram, Tween80 and MCT.
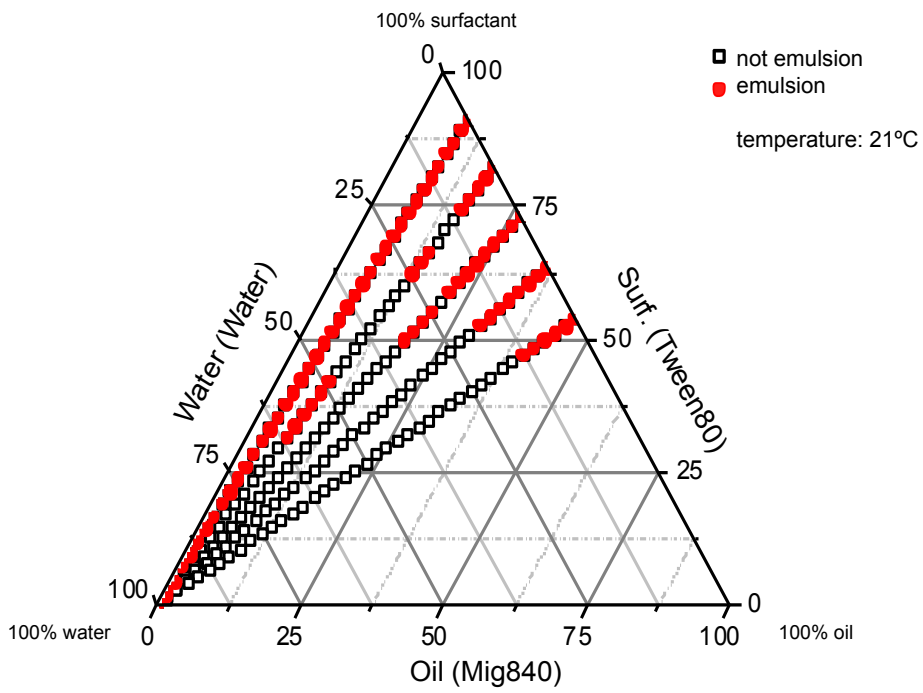
Figure 16 - Phase Diagram, Mig840.
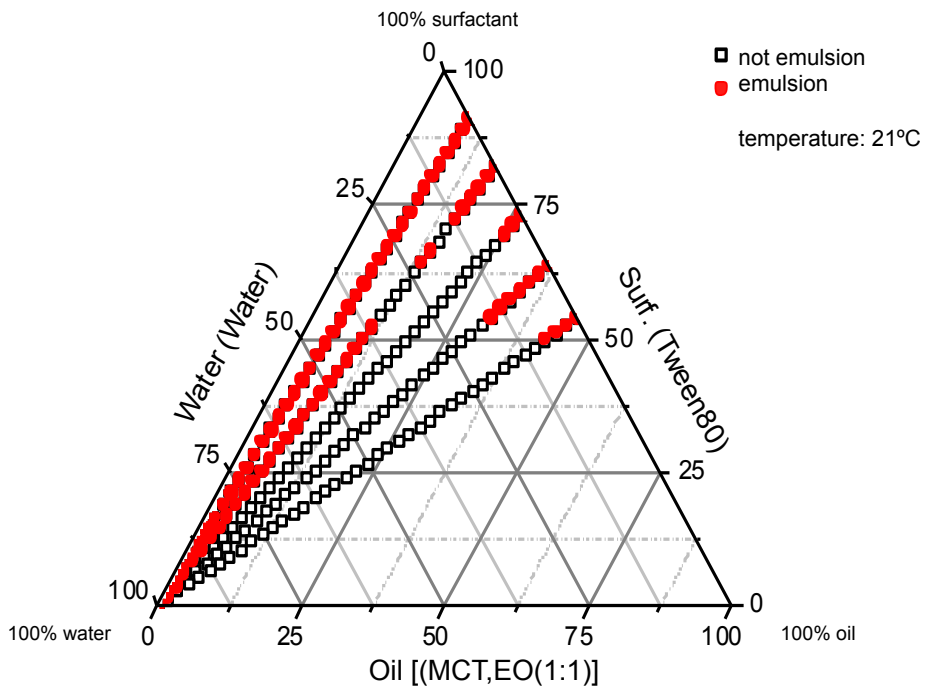
Figure 17 - Phase Diagram, Tween80 and MCT + EO (1:1)

At this stage, we can observe and compare the phase diagrams. It can be concluded that all of them present promising results, because microemulsions were formed at different variations of amount of the oils and surfactant.

All phase diagrams show some microemulsion formulations in the interval 50-70% of surfactant percentage, which is necessary for keeping toxicity levels low.

These results are not yet sufficient to choose the best formulation to be used. The full characterization of the emulsion and other tests are required to conclude about the most efficient formulations.

Chapter 6

Conclusion

To address the issue of low bioavailability, of some specific chemical compounds, several formulations were studied. The purpose of these formulations was to deliver these bioactive molecules and increase their bioavailability in the human organism. Various types of oils and mixtures are capable of forming microemulsions; the ones designed during this project revealed quite promising results.

The formulations tested can potentially improve the bioavailability of poorly soluble drugs, as for the results obtained from solubility and emulsifying screenings.

Mixing different oils, instead of combining one single oil with surfactant, did not enhance significantly solubility or the formation of microemulsions with the chosen API, contrary to what was expected. According to the literature mixing more than one oil with the surfactant would result in higher solubility of the chemical compound [35]. However, further tests using different excipients must be performed to confirm if the results are similar when using other mixture of oil and surfactant, or if the API is responsible for the difference.

The next steps of research will include further tests of solubility; test using Labrasol as the surfactant component instead of Tween 80 and doing emulsifying screenings to the remaining mixtures.

Furthermore, three other potential APIs will be studied. The objective is finding one formulation capable of delivering efficiently any of these four molecules.

After that stage the characterization of the emulsions will be carried ou, to analyse particle size and stability, a re-evaluation and optimization of the formulation will follow and finally the validation of the formulation in the artificial gut model.

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Annex

Phase Diagrams

Table 2. Microwell plates 15.L, 15.C, 15.R, determination of emulsifying capacity.

Well / Plate	15.L	15.C	15.R	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	1	1	Emulsion
E7	1	1	1	Emulsion
E8	1	1	1	Emulsion
E9	0	1	0	NOT
E10	0	0	0	NOT
E11	0	0	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	0	0	0	NOT
F6	0	0	0	NOT
F7	0	0	0	NOT
F8	0	0	0	NOT
F9	0	0	0	NOT
F10	0	0	0	NOT
F11	0	0	0	NOT
F12	0	0	0	NOT
G1	0	0	0	NOT
G2	0	0	0	NOT
G3	0	0	0	NOT
G4	0	0	0	NOT
G5	0	0	0	NOT
G6	0	0	0	NOT
G7	0	0	0	NOT
G8	0	0	0	NOT
G9	0	0	0	NOT
G10	0	0	0	NOT
G11	0	0	0	NOT
G12	0	0	0	NOT
H1	0	0	1	NOT
H2	0	0	1	NOT
H3	0	0	1	NOT
H4	1	0	1	Emulsion
H5	1	1	1	Emulsion
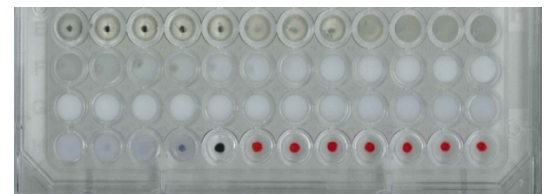
15.L   TW80(60%), EO(40%)
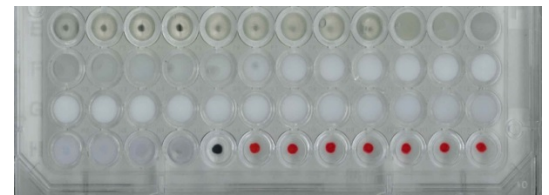
15.C   TW80(60%), EO(40%)
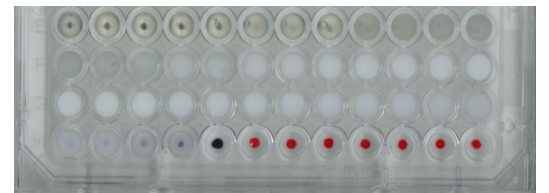
15.R   TW80(60%), EO(40%)

Fig.2. Microwell plates 15.L, 15.C, 15.R, scans

Table 3. Microwell plates 19,1, 19,2, 23, determination of emulsifying capacity.

Well / Plate	19,1	19,2	23	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	1	1	0	Emulsion
A8	1	0	1	Emulsion
A9	1	0	1	Emulsion
A10	1	0	1	Emulsion
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	1	1	1	Emulsion
D3	1	1	1	Emulsion
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion

19,1   TW80(70%), EO(30%)
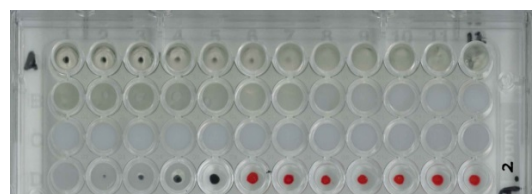
19,2   TW80(70%), EO(30%)

23   TW80(70%), EO(30%)

Fig.3. Microwell plates 19,1, 19,2, 23, scans

Table 4. Microwell plates 19,1, 19,2, 23, determination of emulsifying capacity.

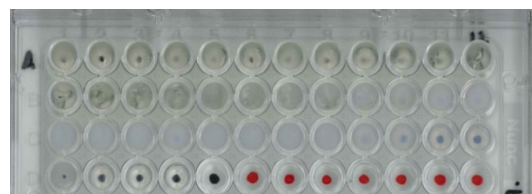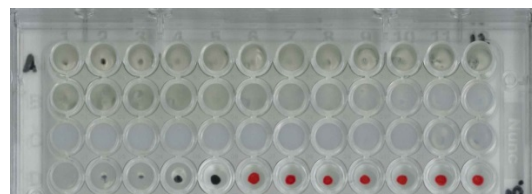| Well / Plate	19,1	19,2	23	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	0	0	0	NOT
E7	0	0	0	NOT
E8	1	1	0	Emulsion
E9	1	1	1	Emulsion
E10	1	1	0	Emulsion
E11	1	0	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	0	1	0	NOT
F5	0	1	0	NOT
F6	0	1	1	Emulsion
F7	1	1	1	Emulsion
F8	0	1	1	Emulsion
F9	1	1	1	Emulsion
F10	1	1	1	Emulsion
F11	1	1	1	Emulsion
F12	1	1	1	Emulsion
G1	1	1	1	Emulsion
G2	1	1	1	Emulsion
G3	1	1	1	Emulsion
G4	1	1	1	Emulsion
G5	1	1	1	Emulsion
G6	1	1	1	Emulsion
G7	1	1	1	Emulsion
G8	1	1	1	Emulsion
G9	1	1	1	Emulsion
G10	1	1	1	Emulsion
G11	1	1	1	Emulsion
G12	1	1	1	Emulsion
H1	1	1	1	Emulsion
H2	1	1	1	Emulsion
H3	1	1	1	Emulsion
H4	1	1	1	Emulsion
H5	1	1	1	Emulsion

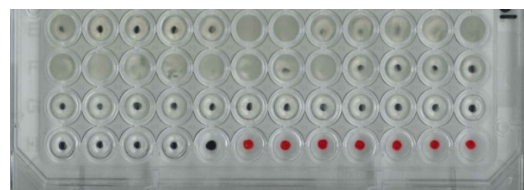19,1   TW80(80%), EO(20%)

19,2   TW80(80%), EO(20%)

23   TW80(80%), EO(20%)

Fig.4. Microwell plates 19,1, 19,2, 23, scans

Table 5. Microwell plates 18, 39, 40, determination of emulsifying capacity.

Well / Plate	18	39	40	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	1	1	Emulsion
E7	1	1	1	Emulsion
E8	1	1	1	Emulsion
E9	1	1	1	Emulsion
E10	1	1	1	Emulsion
E11	1	1	1	Emulsion
E12	1	1	1	Emulsion
F1	1	1	1	Emulsion
F2	0	0	1	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	0	0	0	NOT
F6	0	0	0	NOT
F7	0	0	0	NOT
F8	0	1	0	NOT
F9	1	1	1	Emulsion
F10	1	1	1	Emulsion
F11	1	1	1	Emulsion
F12	1	1	1	Emulsion
G1	1	1	1	Emulsion
G2	1	1	1	Emulsion
G3	1	1	1	Emulsion
G4	1	1	1	Emulsion
G5	1	1	1	Emulsion
G6	1	1	1	Emulsion
G7	1	1	1	Emulsion
G8	1	1	1	Emulsion
G9	1	1	1	Emulsion
G10	1	1	1	Emulsion
G11	1	1	1	Emulsion
G12	1	1	1	Emulsion
H1	1	1	1	Emulsion
H2	1	1	1	Emulsion
H3	1	1	1	Emulsion
H4	1	1	1	Emulsion
H5	1	1	1	Emulsion

18   TW80(90%), EO(10%)
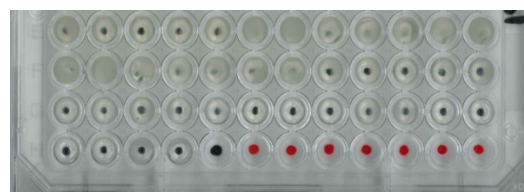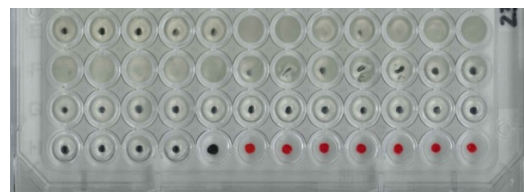

39   TW80(90%), EO(10%)

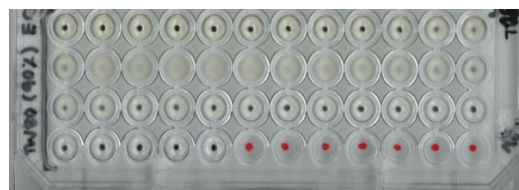40   TW80(90%), EO(10%)

Fig.5. Microwell plates 18, 39, 40, scans

Table 6. Microwell plates 16, 21, 25, determination of emulsifying capacity.

Well / Plate	16	21	25	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	0	0	0	NOT
A8	0	0	0	NOT
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	0	0	0	NOT
D3	0	0	0	NOT
D4	0	0	0	NOT
D5	1	1	1	Emulsion

16   TW80(50%), Mig(50%)

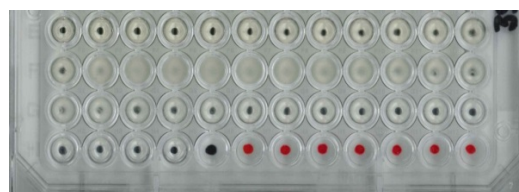

21   TW80(50%), Mig(50%)
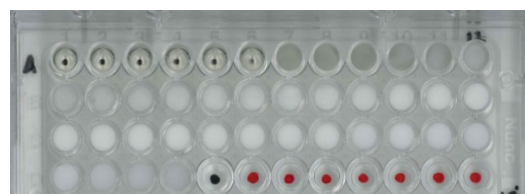
25   TW80(50%), Mig(50%)

Fig.6. Microwell plates 16, 21, 25, scans

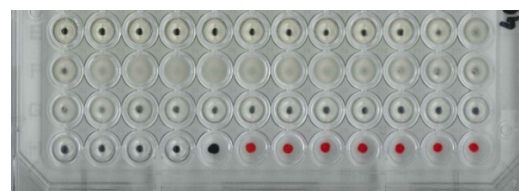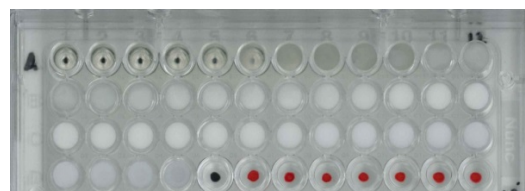Table 7. Microwell plates 16, 21, 25, determination of emulsifying capacity.

Well / Plate	16	21	25	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	1	1	Emulsion
E7	1	1	1	Emulsion
E8	1	1	1	Emulsion
E9	1	0	0	NOT
E10	0	0	0	NOT
E11	0	0	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	0	0	0	NOT
F6	0	0	0	NOT
F7	0	0	0	NOT
F8	0	0	0	NOT
F9	0	0	0	NOT
F10	0	0	0	NOT
F11	0	0	0	NOT
F12	0	0	0	NOT
G1	0	0	0	NOT
G2	0	0	0	NOT
G3	0	0	0	NOT
G4	0	0	0	NOT
G5	0	0	0	NOT
G6	0	0	0	NOT
G7	0	0	0	NOT
G8	0	0	0	NOT
G9	0	0	0	NOT
G10	0	0	0	NOT
G11	0	0	0	NOT
G12	0	0	0	NOT
H1	0	0	0	NOT
H2	0	0	0	NOT
H3	0	0	0	NOT
H4	0	0	0	NOT
H5	1	1	1	Emulsion

16   TW80(60%), Mig(40%)
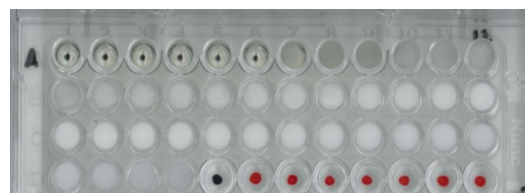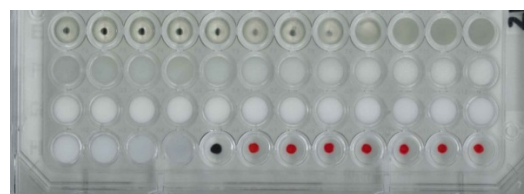

21   TW80(60%), Mig(40%)
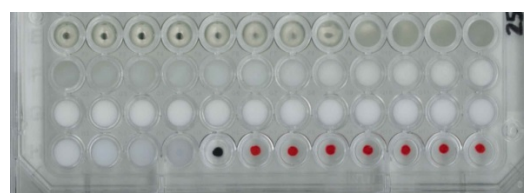
25   TW80(60%), Mig(40%)

Fig.7. Microwell plates 16, 21, 25, scans

Table 8. Microwell plates 17, 22, 26, determination of emulsifying capacity.
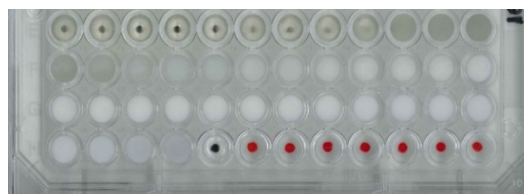
Well / Plate	17	22	26	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	1	1	1	Emulsion
A8	1	1	0	Emulsion
A9	1	1	1	Emulsion
A10	1	0	0	NOT
A11	1	1	1	Emulsion
A12	1	1	1	Emulsion
B1	1	0	1	Emulsion
B2	1	0	1	Emulsion
B3	1	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	1	0	NOT
B7	0	1	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	0	0	0	NOT
D3	0	1	0	NOT
D4	0	1	1	Emulsion
D5	1	1	1	Emulsion

17　　　TW80(70%), Mig(30%)

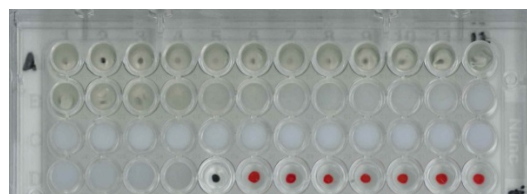

22　　　TW80(70%), Mig(30%)

26　　　TW80(70%), Mig(30%)
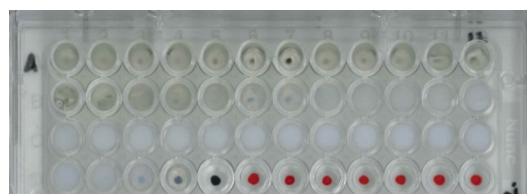
Fig.8. Microwell plates 17, 22, 26, scans

Table 9. Microwell plates 18, 39, 40, determination of emulsifying capacity.

Well / Plate	18	39	40	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	1	1	1	Emulsion
A8	1	1	1	Emulsion
A9	1	1	1	Emulsion
A10	1	1	1	Emulsion
A11	1	1	1	Emulsion
A12	1	1	1	Emulsion
B1	1	1	1	Emulsion
B2	1	1	1	Emulsion
B3	1	1	1	Emulsion
B4	1	1	1	Emulsion
B5	1	1	1	Emulsion
B6	1	1	1	Emulsion
B7	1	1	1	Emulsion
B8	1	1	1	Emulsion
B9	1	1	1	Emulsion
B10	1	1	1	Emulsion
B11	1	1	1	Emulsion
B12	1	1	1	Emulsion
C1	1	1	1	Emulsion
C2	1	1	1	Emulsion
C3	1	1	1	Emulsion
C4	1	1	1	Emulsion
C5	1	1	1	Emulsion
C6	1	1	1	Emulsion
C7	1	1	1	Emulsion
C8	1	1	1	Emulsion
C9	1	1	1	Emulsion
C10	1	1	1	Emulsion
C11	1	1	1	Emulsion
C12	1	1	1	Emulsion
D1	1	1	1	Emulsion
D2	1	1	1	Emulsion
D3	1	1	1	Emulsion
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion

18   TW80(90%), Mig(10%)

39   TW80(90%), Mig(10%)

40   TW80(90%), Mig(10%)

Fig.9. Microwell plates 18, 39, 40, scans

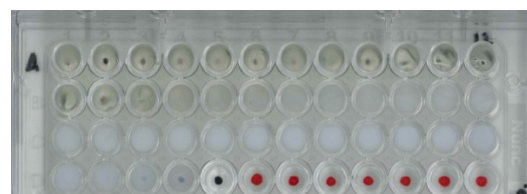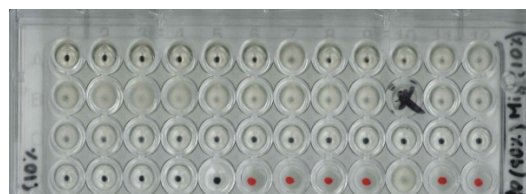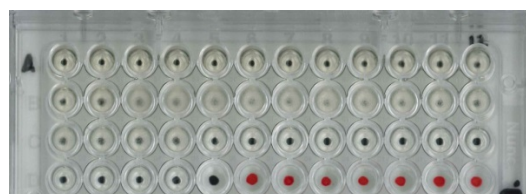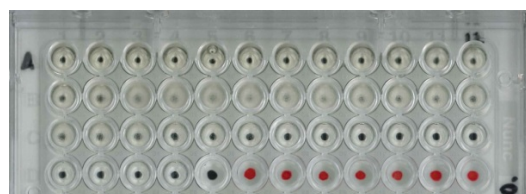Table 10. Microwell plates 20, 24, 34, determination of emulsifying capacity.

Well / Plate	20	24	34	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	0	0	0	NOT
A7	0	0	0	NOT
A8	0	0	0	NOT
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	0	0	0	NOT
D3	0	0	0	NOT
D4	0	0	0	NOT
D5	1	1	1	Emulsion

20   TW80(50%), MCT(50%)

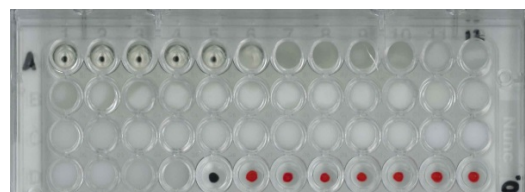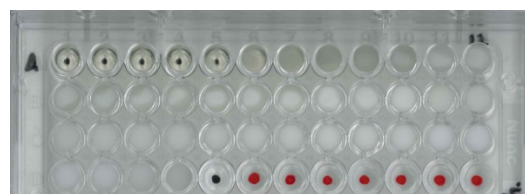24   TW80(50%), MCT(50%)

34   TW80(50%), MCT(50%)

Fig.10. Microwell plates 20, 24, 34, scans

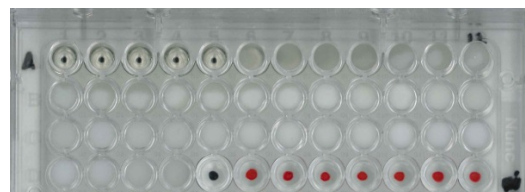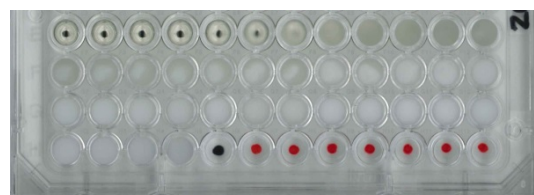Table 11. Microwell plates 20, 24, 34, determination of emulsifying capacity.

Well / Plate	20	24	34	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	1	1	Emulsion
E7	0	0	0	NOT
E8	0	0	0	NOT
E9	0	0	0	NOT
E10	0	0	0	NOT
E11	0	0	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	0	0	0	NOT
F6	0	0	0	NOT
F7	0	0	0	NOT
F8	0	0	0	NOT
F9	0	0	0	NOT
F10	0	0	0	NOT
F11	0	0	0	NOT
F12	0	0	0	NOT
G1	0	0	0	NOT
G2	0	0	0	NOT
G3	0	0	0	NOT
G4	0	0	0	NOT
G5	0	0	0	NOT
G6	0	0	0	NOT
G7	0	0	0	NOT
G8	0	0	0	NOT
G9	0	0	0	NOT
G10	0	0	0	NOT
G11	0	0	0	NOT
G12	0	0	0	NOT
H1	0	0	0	NOT
H2	0	0	0	NOT
H3	0	0	0	NOT
H4	0	0	0	NOT
H5	1	1	1	Emulsion

20   TW80(60%), MCT(40%)

24   TW80(60%), MCT(40%)

34   TW80(60%), MCT(40%)

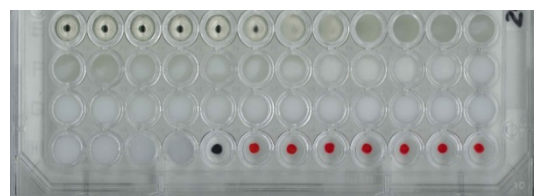Fig.11. Microwell plates 20, 24, 34, scans

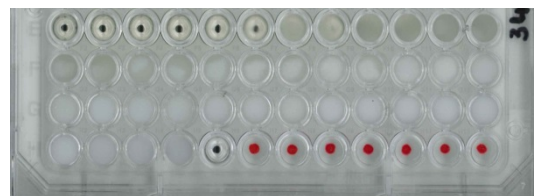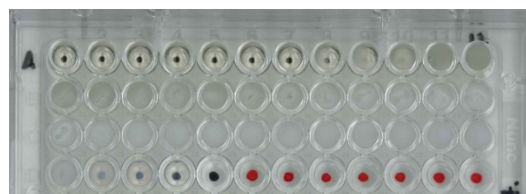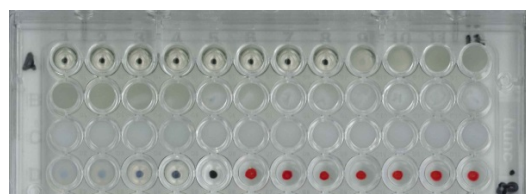Table 12. Microwell plates 27, 38, 41, determination of emulsifying capacity.

Well / Plate	27	38	41	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	1	1	1	Emulsion
A8	1	1	1	Emulsion
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	1	1	1	Emulsion
D3	1	1	1	Emulsion
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion

27   TW80(70%), MCT(30%)

38   TW80(70%), MCT(30%)

41   TW80(70%), MCT(30%)

Fig.12. Microwell plates 27, 38, 41, scans

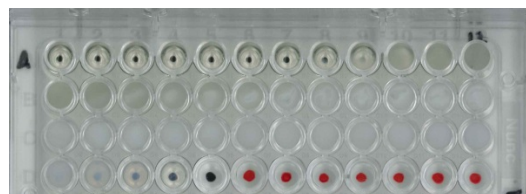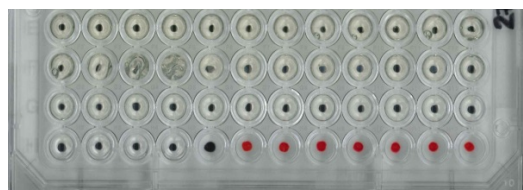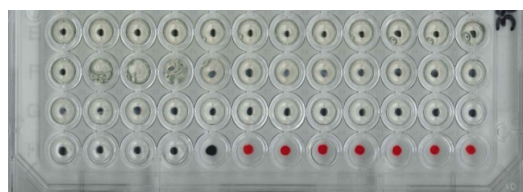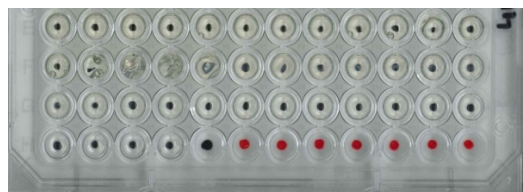Table 13. Microwell plates 27, 38, 41, determination of emulsifying capacity.

Well / Plate	27	38	41	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	1	1	Emulsion
E7	1	1	1	Emulsion
E8	1	1	1	Emulsion
E9	1	1	1	Emulsion
E10	1	1	1	Emulsion
E11	1	1	1	Emulsion
E12	1	1	1	Emulsion
F1	1	1	1	Emulsion
F2	1	0	0	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	1	1	0	Emulsion
F6	1	1	1	Emulsion
F7	1	1	1	Emulsion
F8	1	1	1	Emulsion
F9	1	1	1	Emulsion
F10	1	1	1	Emulsion
F11	1	1	1	Emulsion
F12	1	1	1	Emulsion
G1	1	1	1	Emulsion
G2	1	1	1	Emulsion
G3	1	1	1	Emulsion
G4	1	1	1	Emulsion
G5	1	1	1	Emulsion
G6	1	1	1	Emulsion
G7	1	1	1	Emulsion
G8	1	1	1	Emulsion
G9	1	1	1	Emulsion
G10	1	1	1	Emulsion
G11	1	1	1	Emulsion
G12	1	1	1	Emulsion
H1	1	1	1	Emulsion
H2	1	1	1	Emulsion
H3	1	1	1	Emulsion
H4	1	1	1	Emulsion
H5	1	1	1	Emulsion

27   TW80(80%), MCT(20%)

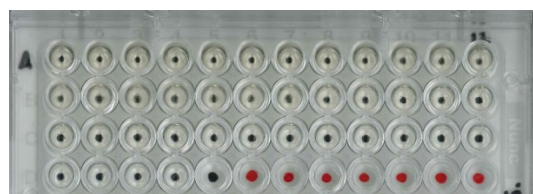38   TW80(80%), MCT(20%)

41   TW80(80%), MCT(20%)

Fig.13. Microwell plates 27, 38, 41, scans

Table 14. Microwell plates 28, 35, 36, determination of emulsifying capacity.

Well / Plate	28	35	36	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	1	1	Emulsion
A6	1	1	1	Emulsion
A7	1	1	1	Emulsion
A8	1	1	1	Emulsion
A9	1	1	1	Emulsion
A10	1	1	1	Emulsion
A11	1	1	1	Emulsion
A12	1	1	1	Emulsion
B1	1	1	1	Emulsion
B2	1	1	1	Emulsion
B3	1	1	1	Emulsion
B4	1	1	1	Emulsion
B5	1	1	1	Emulsion
B6	1	1	1	Emulsion
B7	1	1	1	Emulsion
B8	1	1	1	Emulsion
B9	1	1	1	Emulsion
B10	1	1	1	Emulsion
B11	1	1	1	Emulsion
B12	1	1	1	Emulsion
C1	1	1	1	Emulsion
C2	1	1	1	Emulsion
C3	1	1	1	Emulsion
C4	1	1	1	Emulsion
C5	1	1	1	Emulsion
C6	1	1	1	Emulsion
C7	1	1	1	Emulsion
C8	1	1	1	Emulsion
C9	1	1	1	Emulsion
C10	1	1	1	Emulsion
C11	1	1	1	Emulsion
C12	1	1	1	Emulsion
D1	1	1	1	Emulsion
D2	1	1	1	Emulsion
D3	1	1	1	Emulsion
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion

28   TW80(90%), MCT(10%)

35   TW80(90%), MCT(10%)
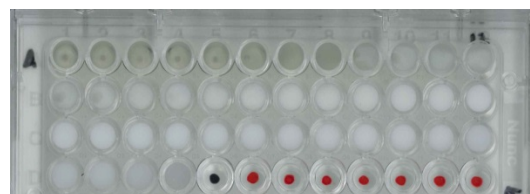

36   TW80(90%), MCT(10%)

Fig.14. Microwell plates 28, 35, 36, scans

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Table 15. Microwell plates 29, 31, 37, determination of emulsifying capacity.

Well / Plate	29	31	37	FINAL
A1	1	1	1	Emulsion
A2	1	1	1	Emulsion
A3	1	1	1	Emulsion
A4	1	1	1	Emulsion
A5	1	0	0	NOT
A6	0	0	0	NOT
A7	0	0	0	NOT
A8	0	0	0	NOT
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	0	0	0	NOT
B7	0	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	0	0	0	NOT
B12	0	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	0	0	0	NOT
C4	0	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	0	0	0	NOT
C10	0	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	0	0	0	NOT
D3	0	0	0	NOT
D4	0	0	0	NOT
D5	1	1	1	Emulsion

29   TW80(50%), [EO,MCT(1:1)] (50%)

31   TW80(50%), [EO,MCT(1:1)] (50%)
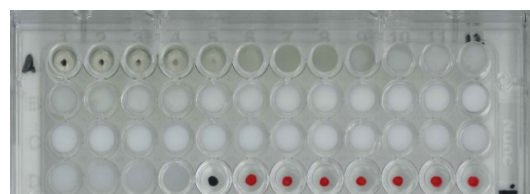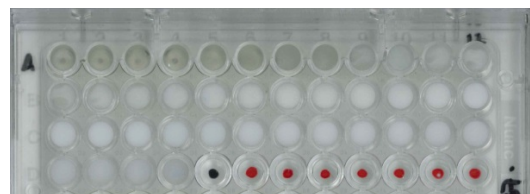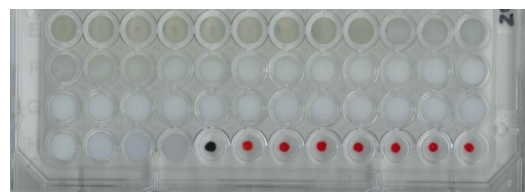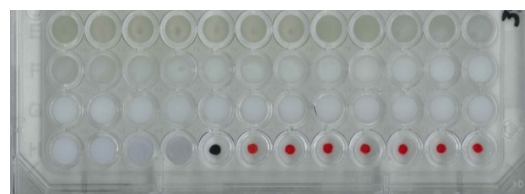

37   TW80(50%), [EO,MCT(1:1)] (50%)

Fig.15. Microwell plates 29, 31, 37, scans

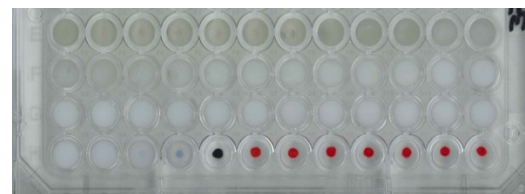Table 16. Microwell plates 29, 31, 37, determination of emulsifying capacity.

Well / Plate	29	31	37	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	0	1	1	Emulsion
E7	0	1	1	Emulsion
E8	0	0	0	NOT
E9	0	0	0	NOT
E10	0	0	0	NOT
E11	0	0	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	0	0	0	NOT
F5	0	0	0	NOT
F6	0	0	0	NOT
F7	0	0	0	NOT
F8	0	0	0	NOT
F9	0	0	0	NOT
F10	0	0	0	NOT
F11	0	0	0	NOT
F12	0	0	0	NOT
G1	0	0	0	NOT
G2	0	0	0	NOT
G3	0	0	0	NOT
G4	0	0	0	NOT
G5	0	0	0	NOT
G6	0	0	0	NOT
G7	0	0	0	NOT
G8	0	0	0	NOT
G9	0	0	0	NOT
G10	0	0	0	NOT
G11	0	0	0	NOT
G12	0	0	0	NOT
H1	0	0	0	NOT
H2	0	0	0	NOT
H3	0	0	0	NOT
H4	0	0	1	NOT
H5	1	1	1	Emulsion

29   TW80(60%), [EO,MCT(1:1)] (40%)
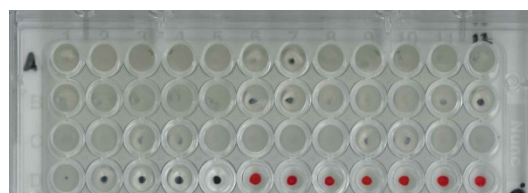

31   TW80(60%), [EO,MCT(1:1)] (40%)
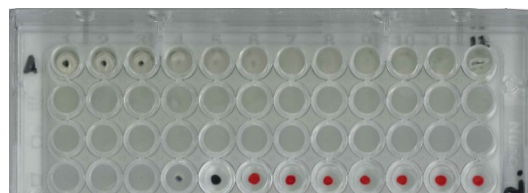

37   TW80(60%), [EO,MCT(1:1)] (40%)

Fig.16. Microwell plates 29, 31, 37, scans

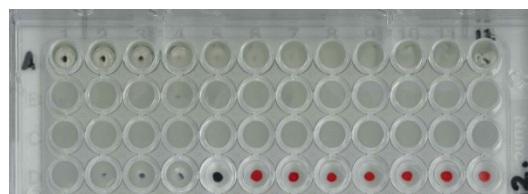Table 17. Microwell plates 30, 32, 42, determination of emulsifying capacity.

Well / Plate	30	32	42	FINAL
A1	1	1	1	Emulsion
A2	0	1	1	Emulsion
A3	0	1	1	Emulsion
A4	0	0	0	NOT
A5	0	0	0	NOT
A6	1	0	0	NOT
A7	1	0	0	NOT
A8	0	0	0	NOT
A9	0	0	0	NOT
A10	0	0	0	NOT
A11	0	0	0	NOT
A12	0	0	0	NOT
B1	0	0	0	NOT
B2	0	0	0	NOT
B3	0	0	0	NOT
B4	0	0	0	NOT
B5	0	0	0	NOT
B6	1	0	0	NOT
B7	1	0	0	NOT
B8	0	0	0	NOT
B9	0	0	0	NOT
B10	0	0	0	NOT
B11	1	0	0	NOT
B12	1	0	0	NOT
C1	0	0	0	NOT
C2	0	0	0	NOT
C3	1	0	0	NOT
C4	1	0	0	NOT
C5	0	0	0	NOT
C6	0	0	0	NOT
C7	0	0	0	NOT
C8	0	0	0	NOT
C9	1	0	0	NOT
C10	1	0	0	NOT
C11	0	0	0	NOT
C12	0	0	0	NOT
D1	0	0	0	NOT
D2	1	0	0	NOT
D3	1	0	1	Emulsion
D4	1	1	1	Emulsion
D5	1	1	1	Emulsion

30   TW80(70%), [EO,MCT(1:1)] (30%)
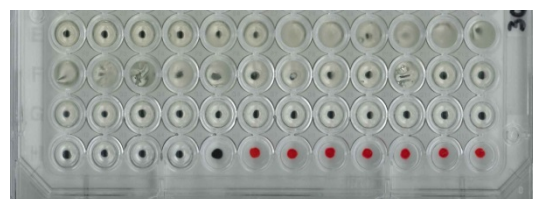

32   TW80(70%), [EO,MCT(1:1)] (30%)
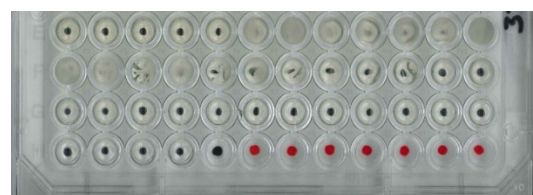

42   TW80(70%), [EO,MCT(1:1)] (30%)

Fig.17. Microwell plates 30, 32, 42, scans

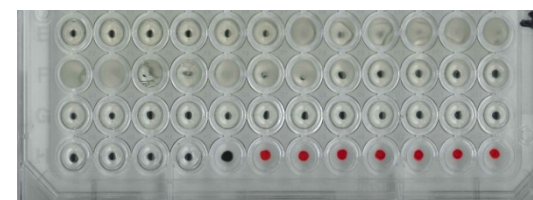Table 18. Microwell plates 30, 32, 42, determination of emulsifying capacity.

Well / Plate	30	32	42	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	0	1	Emulsion
E7	0	0	0	NOT
E8	0	0	1	NOT
E9	1	1	0	Emulsion
E10	1	1	0	Emulsion
E11	0	1	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	1	0	1	Emulsion
F5	1	1	0	Emulsion
F6	1	1	1	Emulsion
F7	1	1	1	Emulsion
F8	1	1	1	Emulsion
F9	1	1	1	Emulsion
F10	0	1	1	Emulsion
F11	1	1	1	Emulsion
F12	1	1	1	Emulsion
G1	1	1	1	Emulsion
G2	1	1	1	Emulsion
G3	1	1	1	Emulsion
G4	1	1	1	Emulsion
G5	1	1	1	Emulsion
G6	1	1	1	Emulsion
G7	1	1	1	Emulsion
G8	1	1	1	Emulsion
G9	1	1	1	Emulsion
G10	1	1	1	Emulsion
G11	1	1	1	Emulsion
G12	1	1	1	Emulsion
H1	1	1	1	Emulsion
H2	1	1	1	Emulsion
H3	1	1	1	Emulsion
H4	1	1	1	Emulsion
H5	1	1	1	Emulsion

30   TW80(80%), [EO,MCT(1:1)] (20%)

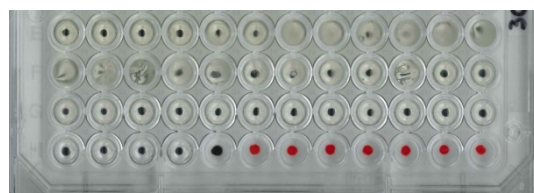32   TW80(80%), [EO,MCT(1:1)] (20%)

42   TW80(80%), [EO,MCT(1:1)] (20%)

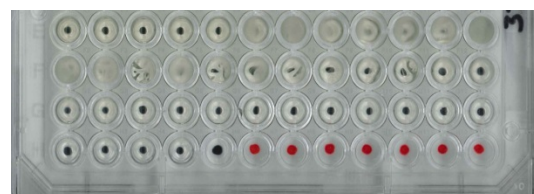Fig.18. Microwell plates 30, 32, 42, scans

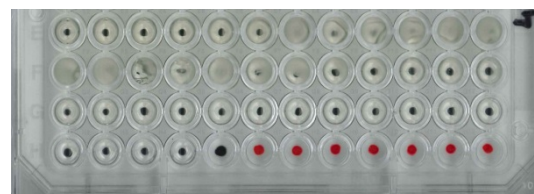Table 19. Microwell plates 30, 32, 42, determination of emulsifying capacity.

Well / Plate	30	32	42	FINAL
E1	1	1	1	Emulsion
E2	1	1	1	Emulsion
E3	1	1	1	Emulsion
E4	1	1	1	Emulsion
E5	1	1	1	Emulsion
E6	1	0	1	Emulsion
E7	0	0	0	NOT
E8	0	0	1	NOT
E9	1	1	0	Emulsion
E10	1	1	0	Emulsion
E11	0	1	0	NOT
E12	0	0	0	NOT
F1	0	0	0	NOT
F2	0	0	0	NOT
F3	0	0	0	NOT
F4	1	0	1	Emulsion
F5	1	1	0	Emulsion
F6	1	1	1	Emulsion
F7	1	1	1	Emulsion
F8	1	1	1	Emulsion
F9	1	1	1	Emulsion
F10	0	1	1	Emulsion
F11	1	1	1	Emulsion
F12	1	1	1	Emulsion
G1	1	1	1	Emulsion
G2	1	1	1	Emulsion
G3	1	1	1	Emulsion
G4	1	1	1	Emulsion
G5	1	1	1	Emulsion
G6	1	1	1	Emulsion
G7	1	1	1	Emulsion
G8	1	1	1	Emulsion
G9	1	1	1	Emulsion
G10	1	1	1	Emulsion
G11	1	1	1	Emulsion
G12	1	1	1	Emulsion
H1	1	1	1	Emulsion
H2	1	1	1	Emulsion
H3	1	1	1	Emulsion
H4	1	1	1	Emulsion
H5	1	1	1	Emulsion

30   TW80(80%), [EO,MCT(1:1)] (20%)

32   TW80(80%), [EO,MCT(1:1)] (20%)

42   TW80(80%), [EO,MCT(1:1)] (20%)

Fig.19. Microwell plates 30, 32, 42, scans

Calibration Lines

| concentrations:	2,3793E-08	1,84521E-06	4,30038E-06	4,27725E-06	5,9639E-06
Final ABS:	0,025000001	0,276000055	0,674000132	0,669000151	0,940000224
concentration (µg)	0,02379297	1,845209071	4,300378972	4,277249823	5,963897401

Calibration Line A

$y = 0.155015x + 0.008023$
$R^2 = 0.998931$
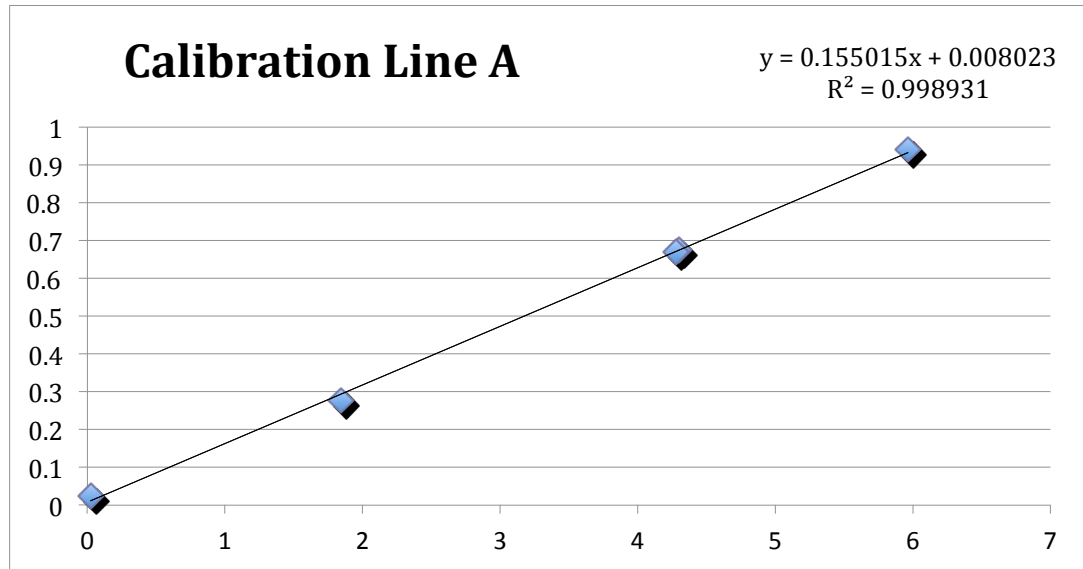
EO,Tw80 (1:1)

| concentrations:	5,79312E-07	1,28712E-06	1,03942E-06	1,86028E-06	1,52977E-06
Final ABS:	0,235000008	0,525000018	0,441000015	0,775000027	0,638000022
concentration (µg)	0,579311983	1,287120561	1,03942113	1,860275661	1,52977

Calibration Line B

$y = 0.419179x - 0.005022$
$R^2 = 0.998750$
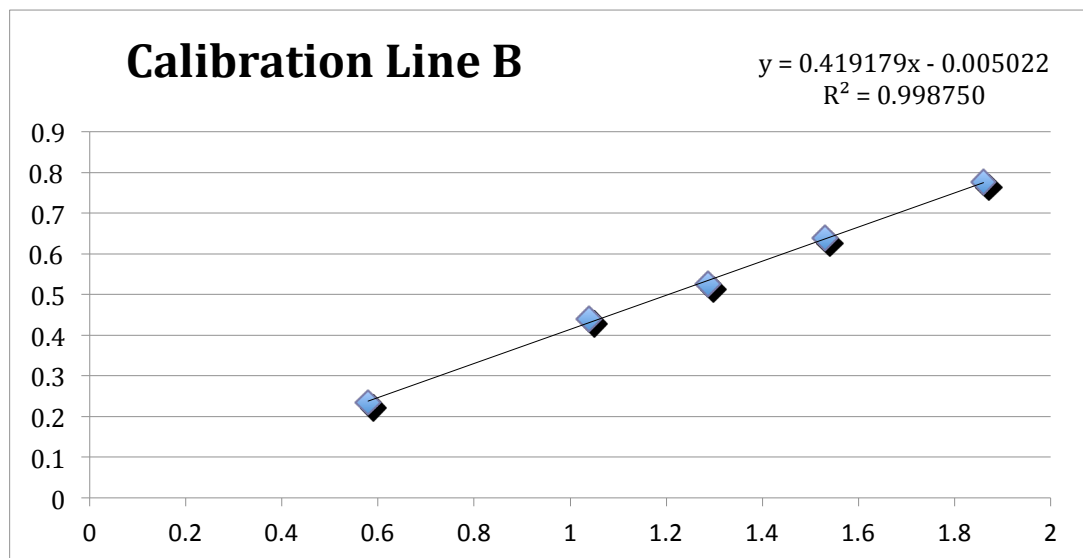
MCT, SF (1:1)

| concentrations (g):	3,808E-06	1,5284E-06	1,78699E-06	3,33445E-06	4,23086E-06
Final ABS:	0,394000028	0,131000011	0,163000014	0,340000024	0,446000031
concentration (µg/mL)	3,808000252	1,528399946	1,78698645	3,334447527	4,230864495

Calibration Line C

$y = 0.113703x - 0.039661$
$R^2 = 0.999908$
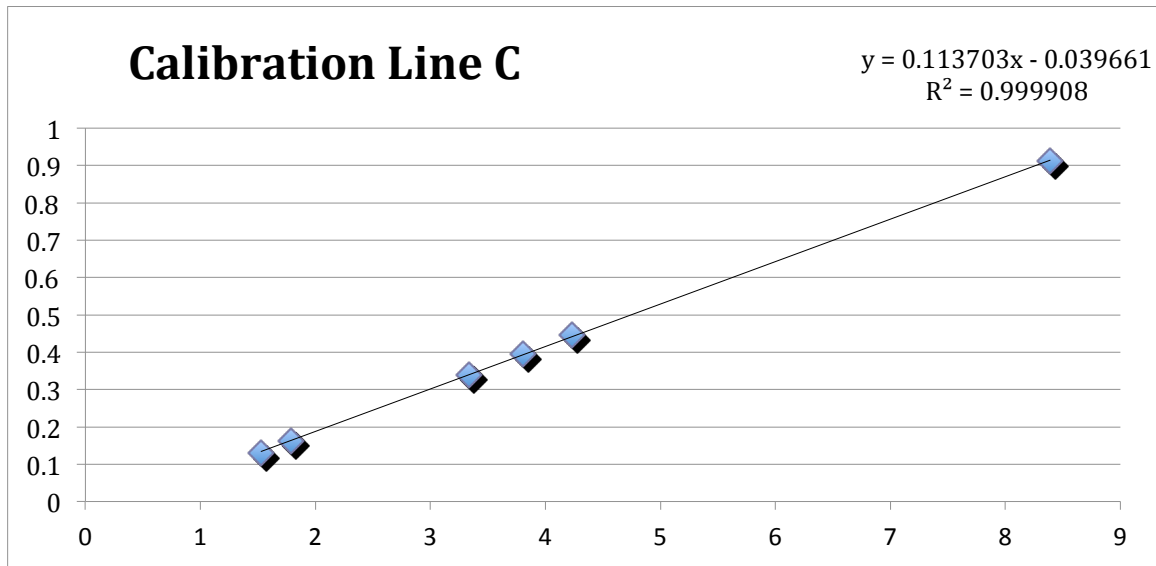
EO

| concentrations:	1,97424E-06	1,282E-06	2,79527E-06	2,36473E-06	3,24785E-06
Final ABS:	0,174000021	0,090000013	0,272000033	0,240000027	0,305000035
concentration (µg)	1,974240015	1,281996008	2,795265351	2,364734967	3,247846361

Calibration Line D

$y = 0.122371x - 0.068055$
$R^2 = 0.995728$

SF

83
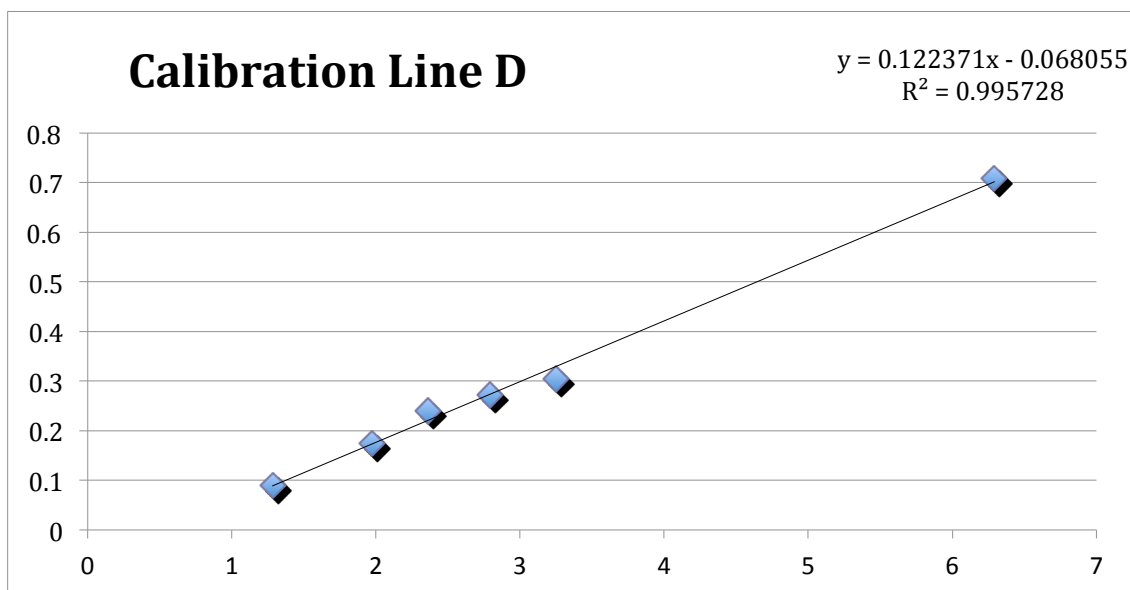
| concentrations:	1,83793E-06	5,46642E-06	2,86829E-06	3,55191E-06	4,53964E-06
Final ABS:	0,259000009	1,059000029	0,539000014	0,670000018	0,892000023
concentration (µg)	1,837926099	5,466424183	2,868293174	3,551914301	4,539641741

Calibration Line E

$y = 0.219016x - 0.116231$
$R^2 = 0.994233$
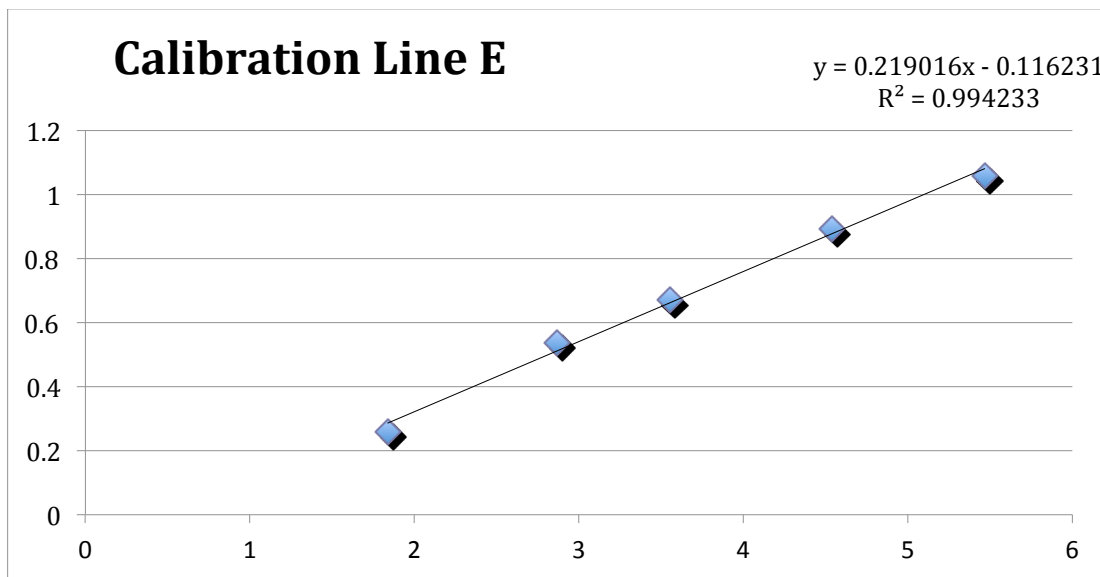
Mg840

| concentrations:	6,76269E-07	1,69852E-06	3,96871E-06	4,58894E-06
Final ABS:	0,054000011	0,201000033	0,518000061	0,566000075
concentration (µg)	0,676268762	1,698524155	3,968712444	4,588944945

Calibration Line F

$y = 0.133513x - 0.030156$
$R^2 = 0.996386$
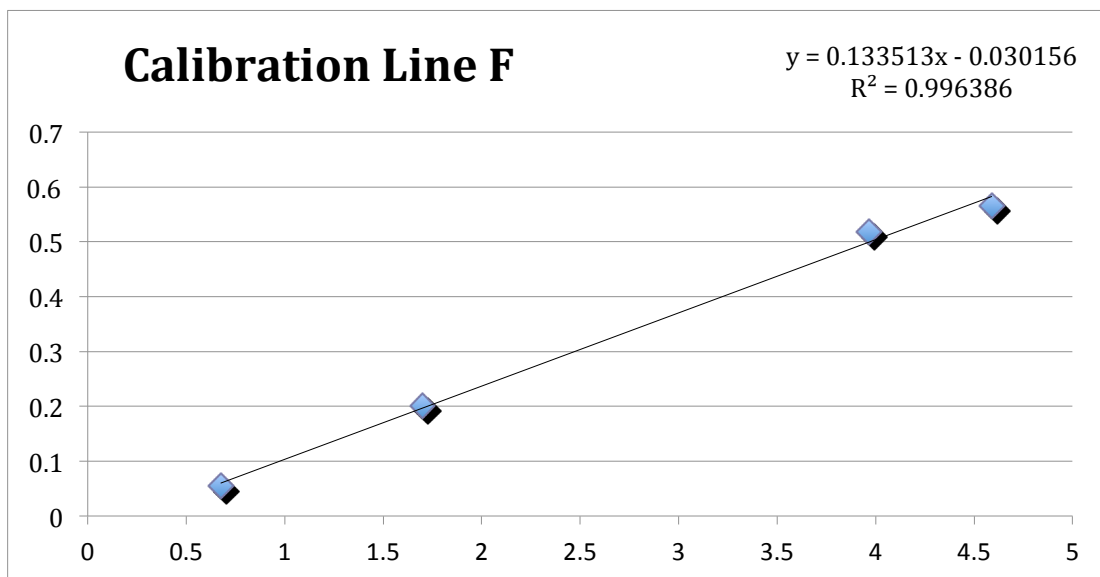
SF, Mg840 (1:1)

| concentrations:	2,94597E-06	4,30676E-06	1,63946E-06	2,53736E-06	1,32455E-06
Final ABS:	0,605000012	0,87800002	0,337000007	0,53300001	0,273000006
concentration (µg)	2,945971922	4,306755706	1,639456459	2,537362128	1,324549242

Calibration Line G

$y = 0.203035x + 0.007296$
$R^2 = 0.999368$
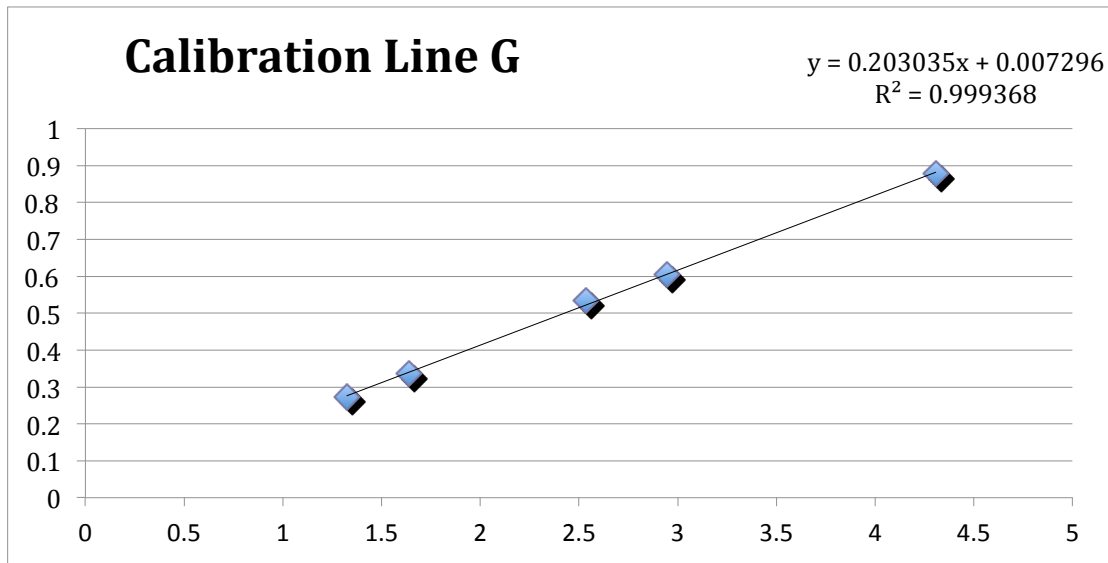
LB 20%

| concentrations:	6,68875E-07	1,17043E-06	2,36623E-06	8,95317E-07	6,6391E-07
Final ABS:	0,154000007	0,27200001	0,582000023	0,212000008	0,175000006
concentration (µg)	0,668874534	1,170430442	2,366232381	0,895316852	0,663909799

Calibration Line H

$y = 0.246560x - 0.005272$
$R^2 = 0.996241$
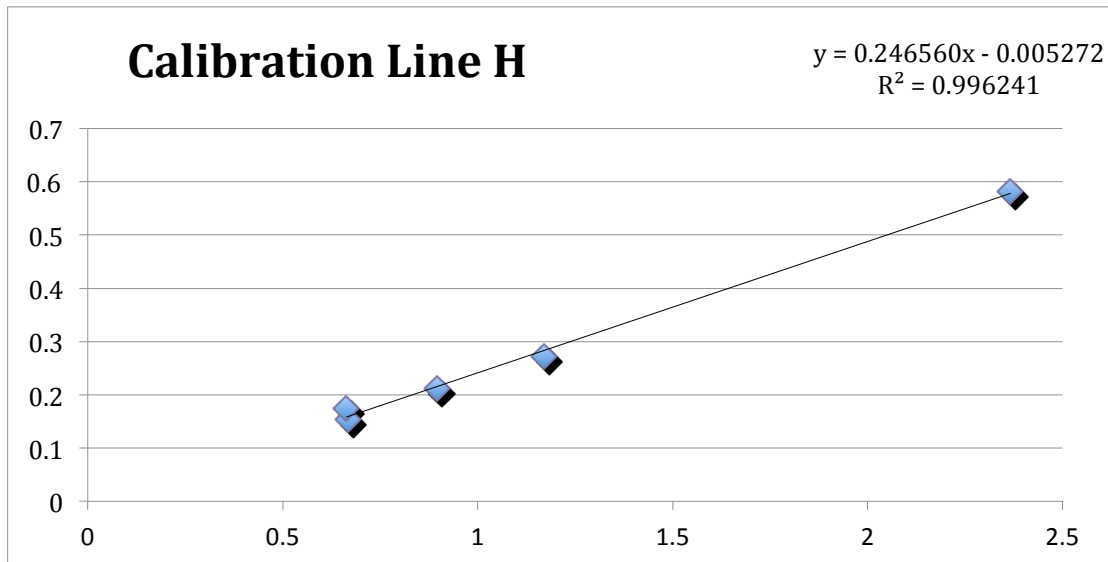
Tw80 20%

| concentrations:	5,99996E-07	2,3624E-06	1,28812E-06	2,71211E-06	1,92816E-06
Final ABS:	0,063000005	0,364000022	0,181000011	0,420000024	0,308000016
concentration (µg)	0,599995665	2,362397254	1,288117236	2,712109301	1,928155846

Calibration Line I

$y = 0.167938x - 0.032275$
$R^2 = 0.997698$
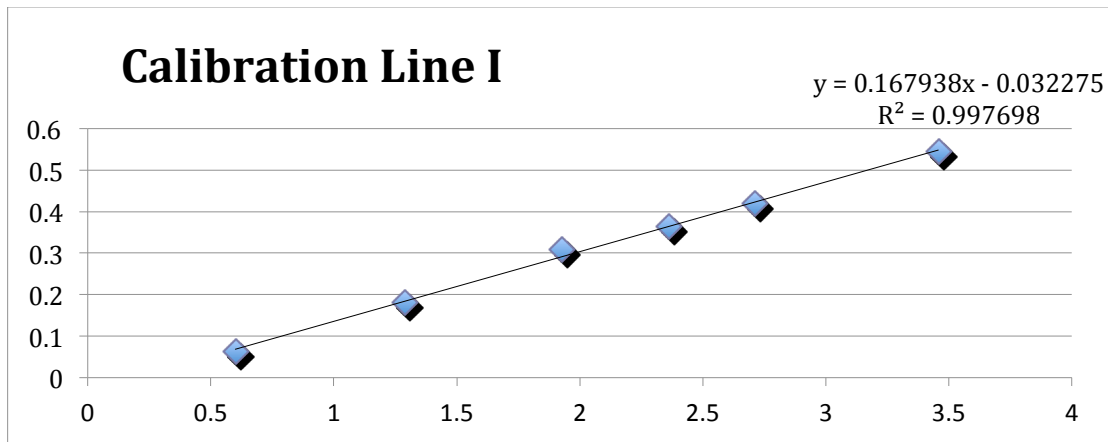
MCT

| concentrations:	3,12442E-06	5,75833E-06	2,23293E-06	3,25207E-06
Final ABS:	0,491000012	0,845000028	0,375000008	0,512000015
concentration (µg)	3,124421769	5,758334997	2,232933435	3,252067913

Calibration Line J

$y = 0.133456x + 0.076384$
$R^2 = 0.999930$
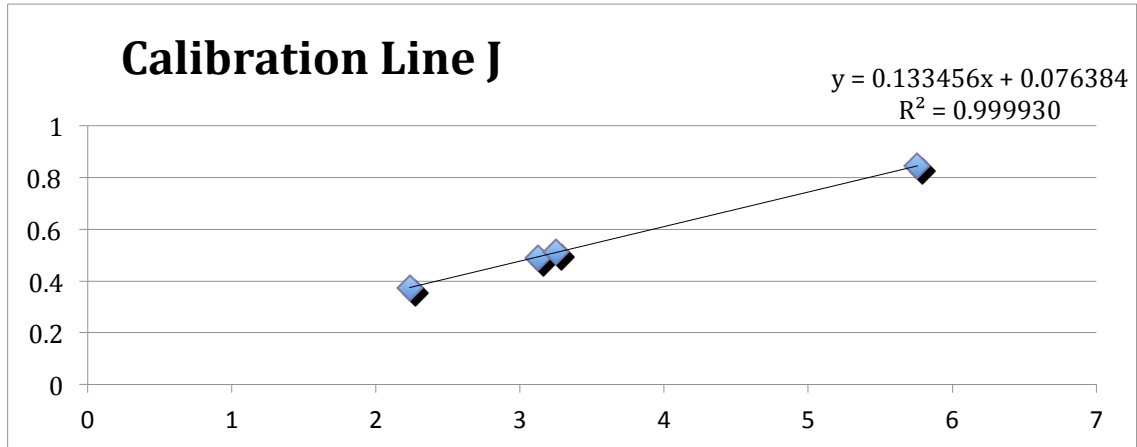
Tween+MCT (1:1)

| concentrations:	2,12377E-06	3,86116E-06	3,94083E-06	4,86286E-06
Final ABS:	0,296000006	0,464000013	0,453000011	0,561000019
concentration (µg)	2,123765011	3,861164283	3,940827206	4,862861896

Calibration Line K

$y = 0.095575x + 0.090143$
$R^2 = 0.992827$
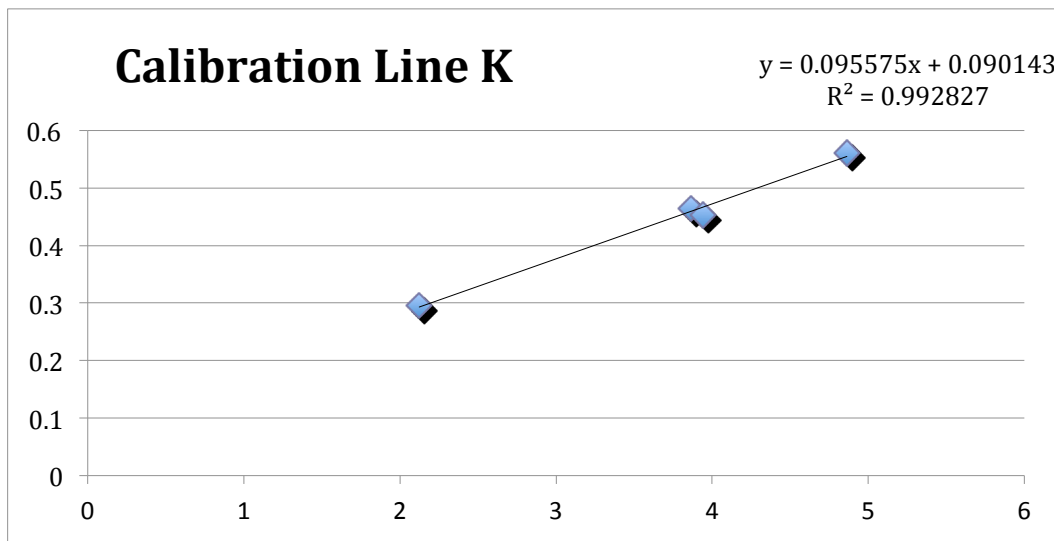
Tween+(EO,MCT (1:1)) (1:1)

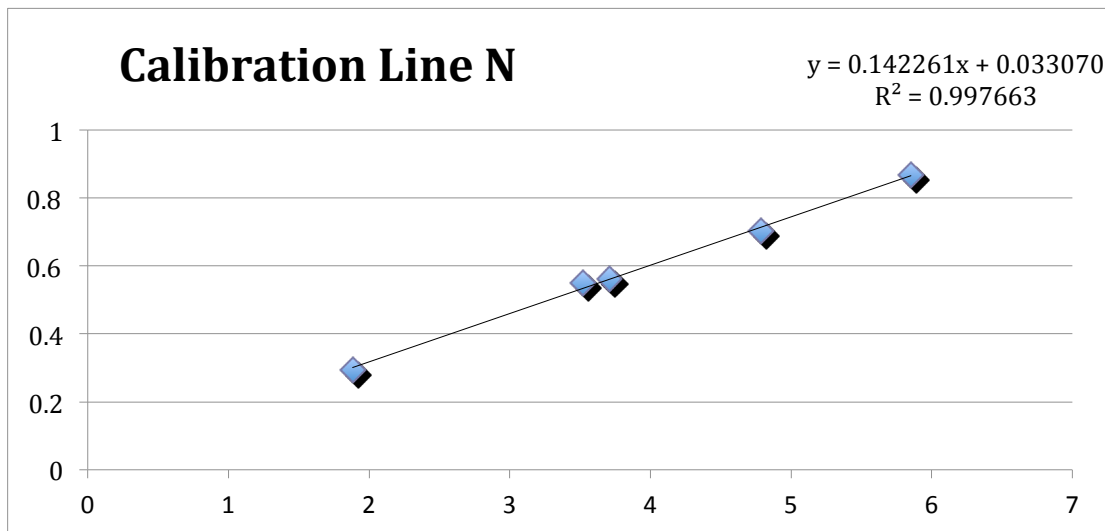| concentrations:	1,29823E-06	3,54818E-06	1,98991E-06	2,32774E-06
Final ABS:	0,275000004	0,584000012	0,383000006	0,429000007
concentration (µg)	1,298231711	3,548184559	1,989914305	2,327735842

Calibration Line L

$y = 0.136037x + 0.106087$
$R^2 = 0.996765$
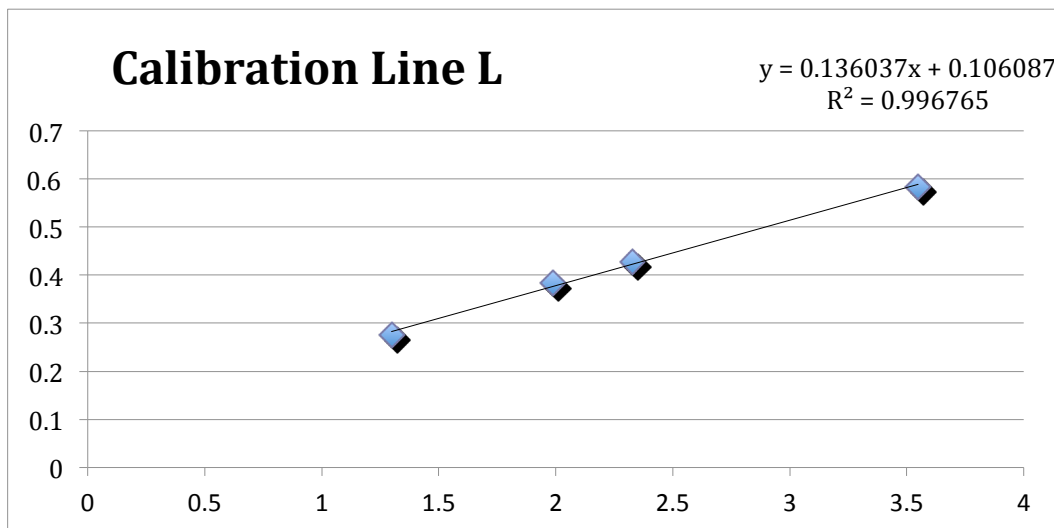
Tween+Mig840(1:1)

concentrations:	1,97504E-06	4,39417E-06	5,48021E-06	3,45089E-06	7,26029E-06
Final ABS:	0,216000008	0,46100002	0,580000025	0,373000025	0,747000033
concentration (µg)	1,975038644	4,394171654	5,480209488	3,450890467	7,260288762

Calibration Line M

$y = 0.100591x + 0.021520$
$R^2 = 0.999272$
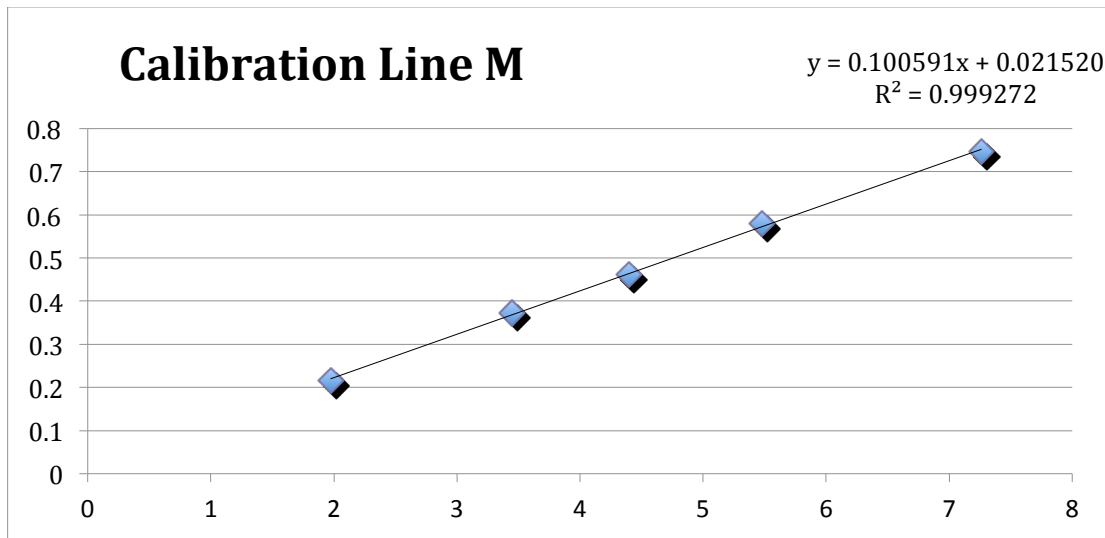
MCT, EO (1:1)

concentrations:	1,88314E-06	3,71093E-06	4,78935E-06	5,85161E-06	3,52189E-06
Final ABS:	0,295000018	0,562000038	0,702000049	0,868000064	0,549000039
concentration (µg)	1,883143314	3,710930063	4,789354802	5,85160852	3,521893686

Calibration Line N

$y = 0.142261x + 0.033070$
$R^2 = 0.997663$

Mig840, EO (1:1)