Luís Miguel Costa Marques

Degree in Biochemistry

Natural antioxidants extraction and their incorporation into model pharmaceutical systems

Dissertation to obtain a Master Degree in Biotechnology

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

O presente trabalho está divido em duas partes englobando áreas científicas distintas, que se juntam com um propósito funcional: estudo da composição polifenólica em folhas de três genótipos de café e suas respostas aos stresses de frio e seca; análise do efeito da incorporação de dois antioxidantes e um fármaco num sistema de microemulsões modelo.

Três genótipos de café, Icatu, Obatã e Apoatã, foram submetidos a condições de stress de temperatura e teor hídrico, investigando-se a resistência dos cafeeiros aos tratamentos impostos. As respostas foram analisadas relativamente à fracção fenólica das folhas dos cafeeiros. Verificou-se que os polifenóis estão de facto envolvidos na resposta aos stresses abióticos impostos, sobressaindo o 4.5-diCQA que parece ter um papel essencial na recuperação ao frio na Obatã. Foram identificados e quantificados vários compostos fenólicos sendo os maioritários: 5-CQA, epicatequina, procianidina, 3,4-diCQA, 3.5-diCQA, 4.5-diCQA, 5-FQA e ainda a mangiferina como novidade em folhas de *C. arabica* e biomarcador de distinção entre *C. arabica* e *C. canephora* Corroborou-se que a Apoatã (*Coffea canephora*) tem maior quantidade de polifenóis do que Icatú e Obatã (*Coffea arabica*). Determinou-se que a Icatu e Obatã parecem ser mais resistentes ao stress de frio mas mais sensíveis à seca comparadas com Apoatã.

As microemulsões modelo são constituídas por um surfactante não-iónico, $C_{12}E_5$, decano e água, estando este sistema muito bem caracterizado. Incorporaram-se dois antioxidantes, 5-CQA e α-tocoferol, e um fármaco, a lidocaína e estudou-se o seu efeito através dos diagramas de fases e DLS. Propôs-se um modelo geral, que defende que a adição de uma molécula hidrofílica aumenta as temperaturas da fase microemulsão (micelas) enquanto que uma hidrofóbica diminui, e que tem aplicações directas na indústria farmacêutica. A actividade antioxidante não é significativamente afectada dentro e fora da microemulsão e verificou-se um efeito sinérgico resultante da mistura 5-CQA e α-tocoferol no sistema.

Palavras chave: Polifenóis do café; stress de frio e seca; microemulsões; antioxidantes em microemulsões.

Abstract

This work is divided in two parts comprising two distinct areas of science that are bonded with a functional purpose: study of the polyphenolic composition in leaves of three coffee genotypes and their responses to cold and drought stresses; and analysis of the effect of incorporation of some polyphenolic compounds in a microemulsion model system.

Three genotypes of coffee, Icatu Obatã and Apoatã, were subjected to temperature and water content stress conditions and their sensitivity or resistance to the treatments imposed was investigated. Responses were analyzed from phenolic fraction point of view in the leaves of coffee plants. It was found that polyphenols are actually involved in the response to these abiotic stresses and that the 4.5-diCQA seems to have an essential role in the recovery from cold in Obatã. Various phenolic compounds were identified and quantified, being the majority: 5-CQA, epicatechin, procyanidin, 3,4-diCQA, 3.5-diCQA, 4.5-diCQA, 5-FQA and mangiferin that was discovery as a new polyphenol in *C. arabica* and also works as a biomarker to differentiate between *C. arabica* and *C. canephora*.

It was confirmed that Apoatã (*Coffea canephora*) possesses a greater amount of polyphenols than Obatã and Icatu (*Coffea arabica*). It was ascertained that Icatu and Obatã seem to be more resistant to cold stress than Apoatã, but more sensitive to drought than Apoatã.

The model microemulsions is build up from a nonionic surfactant, $C_{12}E_5$, decane and water, a system already well characterized. Two antioxidants, 5-CQA and α-tocopherol, and a drug, lidocaine, were incorporated in microemulsions, being the effect of its addition studied through phase diagrams and DLS. A general model was proposed, arguing that the addition of a hydrophilic molecule increases the temperature of the microemulsion phase (micelles), while a hydrophobic decreases, which is of most importance for pharmaceutical applications. The antioxidant activity is not significantly affected within and outside the microemulsion and was determined the existence of synergistic effect when mixing 5-CQA and α-tocopherol in this system.

Keywords: Coffee polyphenols; cold and drought stresses; microemulsions; antioxidants in microemulsions.

Index

Copyright III

Acknowledgments V

Resumo VII

Abstract IX

Index XI

Figures index XIII

Tables index XIX

List of abbreviations, acronyms and symbols XXI

Objectives XXIII

1. Introduction 1

1.1 Coffee history 1

1.2 Botanical and environmental aspects of coffee plant 2

1.3 Drought and low temperatures effects on coffee plant and control of oxidative stress 3

1.4 Polyphenols in coffee – biological activities, properties and role 6

1.5 Impact of polyphenols on human health 12

1.6 Microemulsions - history and definition 17

1.7 Microemulsions properties 18

1.8 Components and properties 19

1.9 Microemulsions structures 24

1.10 Phase diagrams 26

1.11 Industrial Applications 29

2. Materials and methods 33

3. Results and discussion 39

3.1 Identification of polyphenols 39

3.2 Response of polyphenols to drought and cold treatments 43

3.3 Antioxidant activity of coffee leaves extracts (DPPH method) 52

3.4 Quantification some phenolic compounds in coffee leaves 53

3.5 Standard microemulsion ($C_{12}E_5$ + decane + water) 60

3.6 Chlorogenic acid addition 70

3.7 Lidocaine addition 80

3.8 α-Tocopherol 84

3.9 Antioxidant activity - microemulsions 89

3.10 Synergistic effect 96
4. Conclusions 99
5. Future directions 101
6. Bibliography 103

Figures index

Figure 1.1 Major coffee producing countries in the world. 3

Figure 1.2 Main families and biosynthetic pathways of plants polyphenols. 7

Figure 1.3 General chemical structure of hydroxybenzoic acids. 9

Figure 1.4 Chemical structure of hydroxycinnamic acids. 9

Figure 1.5 Chemical structures of: A – Caffeic acid; B – Quinic acid and C – Chlorogenic acid. 10

Figure 1.6 General chemical structures of flavonols. 11

Figure 1.7 Chemical structures of flavanols (above) and an example of a trimeric procyanidin. 12

Figure 1.8 Polyphenols bioactivities 13

Figure 1.9 Chemical structure of 4.5-dicaffeoylquinic acid (4.5-diCQA). 14

Figure 1.10 Chemical structure of mangiferin. 14

Figure 1.11 α-tocopherol chemical structure. 15

Figure 1.11 Chemical structures of anionic surfactants. 20

Figure 1.12 Chemical structure of a cationic surfactant, ... 21

Figure 1.13 Chemical structure of a zwitterionic surfactant 21

Figure 1.14 Chemical structures of a non-ionic surfactant 22

Figure 1.15 Chemical structure of two different oils 23

Figure 1.16 Low (A) and high (B) curvature of the surfactant interface, corresponding to bigger and smaller droplets 25

Figure 1.17 A few structures in which microemulsions may self-assembly 25

Figure 1.18 Schematic representation of two microemulsions: A - Oil-in-water (O/W) and B - water-in-oil (W/O) microemulsions. 25

Figure 1.19 A hypothetical ternary phase diagram of an oil, surfactant and water system. 27

Figure 1.20 A - Phase diagram of the microemulsion system with $C_{12}E_5$/decane/water with a fixed surfactant to oil ratio (volume fraction, Φ). 28

Figure 1.21 A simpler phase diagram can be presented in the form of a triangular prism..... 28

Figure 1.22 Chemical structure of the drug lidocaine. 29

Figure 1.23 Comparison between the effectiveness of large conventional skin care systems (A) and microemulsions formulations (B)..... 30

Figure 3.1 Representative spectra of some phenolic compounds. 5-CQA 39

Figure 3.2. Chromatogram of extract of Icatu leaves at 280 nm and 325 nm..... 40

Figure 3.3 Chromatogram of extract of Obatã leaves at 280 nm and 325 nm 41

Figure 3.4 Chromatogram of extract of Apoatã leaves at 280 nm and 325 nm. . 42

Figure 3.5 Calibration curve, total phenolic content (mg with gallic acid equivalents per liter) versus absorbance (at 765 nm)..... 44

Figure 3.6 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Icatu coffee leaves. 46

Figure 3.7 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Obatã coffee leaves..... 48

Figure 3.8 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Apoatã coffee leaves..... 50

Figure 3.9 Total phenolic content (percentage per leaves in dry weight) and antioxidant activity (percentage DPPH reduction) versus temperature treatment, for Obatã genotype in Control condition of drought..... 52

Figure 3.10 Total phenolic content and antioxidant activity versus temperature treatment, for Apoatã genotype in mild drought condition. 52

Figure 3.11 Total phenolic content and antioxidant activity versus temperature treatment, for Icatu genotype in severe drought condition..... 53

Figure 3.12. Calibration curve of 5-CQA at 325 nm. 54

Figure 3.12 Calibration curve of epicatechin at 275 nm..... 54

Figure 3.13 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Icatu (at severe drought) 55

Figure 3.14 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Obatã (at severe drought)..... 55

Figure 3.15 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Apoatã (at severe drought). 56

Figure 3.16 Comparison between 4.5-diCQA and total phenolic content profile in response to cold treatment in Obatã (at control condition). 58

Figure 3.17 Microemulsion solutions as a function of temperature, showing different macroscopic phases..... 60

Figure 3.18 Phase diagram of the standard system (decane + C12E5 + water) with fixed surfactant to oil volume ration (0.815:1), varying water dilution. 61

Figure 3.19 Diffusion (left axis) and polydispersity index (right axis) versus temperature for the sample Φ = 0.05 62

Figure 3.20 Diffusion (left axis) and polydispersity index (right axis) versus temperature for the sample Φ = 0.15. 63

Figure 3.21 Size distribution by intensity, varying with temperature from 25 °C to 31 °C of the Φ = 0.05 sample. 64

Figure 3.22 Histogram of the size distribution by intensity of the Φ 5% sample at the lower phase boundary (25 °C). 65

Figure 3.23 Histogram of the size distribution by intensity of the Φ 5% sample near the upper phase boundary (29.5 °C). 65

Figure 3.24 Correlation function versus time. It is represented Φ = 0.05 sample, within the temperature trend, from 25 °C to 31 °C. 66

Figure 3.25 Diffusion versus temperature, of the standard system 68

Figure 3.26 Diffusion coefficient versus volume fraction 69

Figure 3.27 Relative diffusion coefficient values (Dc/D0) and the hard spheres behavior prediction, versus volume fraction. 69

Figure 3.28 Phase diagram of the microemulsions with 5-CQA addition (in percentage by volume) 71

Figure 3.29 Phase diagram of the microemulsions with the addition of 5-CQA.72

Figure 3.30 Size distribution by intensity, showing the Φ = 0.05 with 0.25% 5-CQA addition sample. 73

Figure 3.31 Correlation function versus time, showing the Φ = 0.05 with 0.25% 5-CQA addition sample..... 73

Figure 3.32 Diffusion versus temperature of the, Φ = 0.05 with 1% NaCl addition microemulsion sample and comparing with Standard Φ = 0.05 microemulsion. 74

Figure 3.33 Diffusion versus temperature of the, Φ = 0.15 with 1% NaCl addition microemulsion sample and comparing with Standard Φ = 0.15 microemulsion. 75

Figure 3.34 Zeta potential distribution, total counts versus Zeta Potential 76

Figure 3.35 Zeta potential versus percentage (in volume) of 5-CQA addition of the Φ = 0.05 microemulsion. 76

Figure 3.36 Zeta potential versus volume fraction with a 1% 5-CQA addition in microemulsions samples. 77

Figure 3.38 Diffusion versus temperature, where are represented for the Φ = 0.15 microemulsion with different percentages (in volume) of 5-CQA addition. 78

Figure 3.37 Proposed model for the effect of a hydrophilic antioxidant addition to a non-ionic microemulsion 79

Figure 3.38 Phase diagram of microemulsions with lidocaine addition..... 80

Figure 3.39 Diffusion versus temperature, of the standard system and Φ = 0.05 samples, with 1%, 5% and 10% lidocaine addition. 81

Figure 3.40 Size distribution by intensity, showing the Φ = 0.05 sample, with 5% lidocaine addition, between 22.5 °C and 28.5 °C. 82

Figure 3.41 Correlation function versus time. It is represented the Φ = 0.05 sample with 5% lidocaine addition, between 22.5 °C and 28.5 °C. 82

Figure 3.43 Proposed model for the effect of a hydrophobic drug addition to a non-ionic microemulsion..... 83

Figure 3.44 Phase diagram of the microemulsions with the α-tocopherol addition. 84

Figure 3.45 Diffusion versus temperature of the Φ = 0.05 and Φ = 0.15 microemulsion samples with 5% of α-tocopherol addition..... 85

Figure 3.46 Diffusion versus temperature of the Φ = 0.05 and Φ = 0.15 microemulsion samples with 5% of α-tocopherol addition, Φ = 0.15 with 5% and 10% of lidocaine addition and also the standard Φ = 0.15 microemulsion..... 86

Figure 3.47 Size distributed by intensity, showing the behavior of the Φ = 0.15 microemulsion sample with 5% α-tocopherol addition, between 17 °C and 22 °C. 87

Figure 3.48 Correlation function (%) versus time (μs)..... 87

Figure 3.49 Proposed model for the effect of a hydrophobic antioxidant addition to a non-ionic microemulsion..... 88

Figure 3.50 Calibration curve of DPPH in methanol 89

Figure 3.51 Reduction of DPPH with 5-CQA addition. 90

Figure 3.52 Calibration curve of DPPH in decane. 91

Figure 3.53 Reduction of DPPH with α-tocopherol addition..... 91

Figure 3.54 EC50% (g antioxidant/kg DPPH) versus chlorogenic acid and α-tocopherol. 92

Figure 3.55 Calibration curve of DPPH in Φ = 0.05 microemulsion. 93

Figure 3.56 Reduction of DPPH with 5-CQA addition in Φ = 0.05 microemulsion. 93

Figure 3.57 Reduction of DPPH with 5-CQA addition both in bulk and in Φ = 0.05 microemulsion..... 94

Figure 3.58 Reduction of DPPH with α-tocopherol addition..... 95

Figure 3.59 Absorbance (at 515 nm) versus antioxidant concentration (μmol/dm3). 96

Figure 3.60 Percentage of DPPH consumption when mixture the two antioxidants (5-CQA and α-tocopherol) and theoretical consumption (according to individually antioxidant activities). 97

Tables index

Table 1.1 Main differences between arabica and robusta coffee plant species 2

Table 1.2 Major differences between microemulsions and emulsions. 18

Table 3.1 Identification of phenolic compounds for Icatu 40

Table 3.2 Identification of phenolic compounds for Obatã 41

Table 3.3 Identification of phenolic compounds for Apoatã 42

Table 3.4 – Changes in total phenolic content of coffee leaves in relation to cold and drought treatments for Icatu genotype 45

Table 3.5. Changes in total phenolic of coffee leaves in relation to cold and drought treatments for Obatã genotype 47

Table 3.6 Changes in total phenolic content of coffee leaves in relation to cold and drought treatments for Apoatã genotype.. 49

Table. 3.7 5-CQA content in coffee leaves (mg/g dry weight leaves). 56

Table 3.8 Average of the total values (mg/g leaves dry weight) of the remaining phenolic compounds identified for the three water content conditions and for three genotypes.. 57

Table 3.9 DPPH consumption (%) of 5-CQA in microemulsion (ME), α-tocopherol in microemulsion (ME) and the mixture of the two antioxidants in microemulsion.. 97

List of abbreviations, acronyms and symbols

Abbreviation	Full form
5-CQA	5-caffeoylquinic acid
APX	Ascorbate peroxidase
$C_{12}E_5$	Pentaethylene glycol monododecyl ether
CFQA	Caffeoylferuloylquinic acid
CGA	Chlorogenic acids
CMC	Critical Micelle Concentration
Ctr	Control
DAD	Diode Array Detector
diCQA	Dicaffeoylquinic acid
diFQA	Diferuloylquinic acid
di-p-CoQA	Di-p-coumaroylquinic acids
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E-O	Ethylene oxide
FQA	Feruloylquinic acid
GAE	Gallic acid equivalents
GR	Glutathione reductase
HIV	Human immunodeficiency virus
ICO	International Coffee Organization
L_3	Bicontinuous structure
LDL	Low Density Lipoprotein
Lα	Lamellar phase
MD	Mild Drought
O/W	Oil-in-water
PAL	Phenylalanine ammonia-lyase
p-CoQA	p-coumaroylquinic acids
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase High Performance Liquid Chromatography

SOD	Superoxide dismutase
TPC	Total Phenolic Content
UV	Ultra-violet
W/O	Water-in-oil
γ	Surface Tension
ΔA	Surface Area
ΔG	Gibbs free energy
ΔS	Entropy change
Φ	Volume Fraction

Objectives

I. Study the variation of the polyphenolic composition in coffee tree leaves and relate it with temperature (cold) and water availability (drought) stresses.

Most of the existing literature is made for coffee beans; hence this study will be a novelty because it will be made in leaves, contributing to a broader analysis of the whole coffee plant. Coffee is an economically valuable product (worldwide), and by relating polyphenols to abiotic stresses resistance, may contribute in the future, to higher product yields and also to make uso of the whole plant.

II. Make an extensive qualitative and quantitative analysis of the polyphenolic composition, using three different coffee plant genotypes (already used in the market): Icatu, Obata and Apoatã.

III. Identify a molecule that works as a biomarker to relate the status of temperature and drought stress imposed.

IV. Characterize a model microemulsion ($C_{12}E_5$/decane/water) through phase diagrams construction and Dynamic Light Scattering.

V. Develop formulations with antioxidants incorporated (α-tocopherol and 5-caffeoylquinic acid) and a drug, lidocaine and evaluate the effect of the incorporation of this components in the system.

VI. Evaluate the antioxidant capacity of the formulations and verify if there is a synergistic effect between the antioxidants in this system.

The conclusions of these experiments may have a major impact on the cosmetics and pharmaceutical industry since they will provide an *a priori* knowledge of the effect, on microemulsion phase temperatures, that a component will have when added to the formulations.

1. Introduction

1.1 Coffee history

Historically, coffee has been a desired product for more than a millennium. As a legend, it is said that coffee was probably originated in Kaffa, province of Ethiopia, around the year 800 A.D., when a shepherd was very surprised with the vividness of his goats after they ate this red coffee beans. It was allegedly here that coffee was born.

Apart from this one and other legends that may have arisen, what is really known is that, around the year 1000. after a few monks had religiously drunk coffee and exploited its stimulating effect, as other properties such as, better concentration, increase of attention and keeping them awake, the desire for the coffee drink began to expand across the entire world. Arabia was the main exporter by using infertile beans that kept them in first place as traders, until around 1600. From this point on, the expansion of the coffee plant trade had evolved through successive deviations (stealing) from country to country, as the stimulating feeling caused by this drink was enjoyed by all who were experiencing it. Finally when coffee crossed the Atlantic (1727-1800) it bloomed in Brazil where it carefully began to be mass-produced and protected for its value, until the present day, being now considered an everyday elixir, a pleasure drink for the people (*1-3*).

Nowadays coffee is one of the most economically valuable basic products (or natural commodities) in world trade, being only surpassed in value by oil. It is used as a source of foreign exchange for many developing countries. Cultivation, processing, trading and transportation, create employment for millions of people around the world. In least developed countries, export of coffee can account, in some cases, for about a portion of 80 % of their foreign exchange earnings (*4*). Only in 2010 a world consumption of 134 million bags (60 kg), was estimated, according to the Monthly Coffee Market Report of July 2011, provided by International Coffee Organization (ICO) (*5*). Not exclusively because of its economical importance, but also by the pleasure provided by the coffee drink, coffee plant has been extensively studied, giving emphasis on the development of more resistant plants to environmental stresses.

1.2 Botanical and environmental aspects of coffee plant

Coffee belongs to the botanical family Rubiaceae, which has some 500 genera and over 6.000 species. Coffee plants are a member of *Coffea* genus and there are about 90 species. From these, the ones that economically dominate the world coffee trade, include *C. arabica*, *C. canephora* (being *Coffea robusta* the most known variety) as the majority and also, *C. liberica* and *C. dewevrei*.

From all the coffee species, the two most important (trade and consumed) are *Coffea arabica* L. and *Coffea canephora* Pierre (also known as *Coffea robusta*), which accounts for over 60% of world production. Arabica coffee is the more consumed, accounting for about 62% and robusta practically for the rest. When comparing the two species, arabica and robusta, several differences are found being some presented in table 1.1. For instance although robusta coffee plants appear to be more vigorous, productive, robust ("giving is name to use") and have a higher caffeine content, the beverage derived from its beans is considered to be of lower quality than the one from arabica coffee (*6*).

Table 1.1 Main differences between arabica and robusta coffee plant species (*7, 8*).

Characteristic	**arabica**	**robusta**
Date species described	1753	1895
Chromosomes (2n)	44	22
Time from flower to ripe cherry	9 months	10-11 months
Flowering	after rain	irregular
Ripe cherries	fall	stay
Yield (kg beans/ha)	1500-3000	2300-4000
Root system	deep	shallow
Optimum temperature (yearly average)	15-24° C	24-30° C
Optimal rainfall	1500-2000 mm	2000-3000 mm
Optimum altitude	1000-2000 m	0-700 m
Caffeine content of beans	0.8-1.4%	1.7-4.0%
Typical brew characteristics	acidity	bitterness, full
Body	average 1.2%	average 2.0%

The coffee crop is confined to the inter-tropical zone (figure 1.1), due to its required climatic conditions such as temperature and humidity. Major changes that occur in these two parameters may affect the coffee yields, turning them into the main environmental "enemies" for the coffee plant, being drought the more frequent followed by cold and frost. Frost occurs from time to time damaging or even killing the coffee plant. In the case of drought, episodes are

more frequent, thus this environmental stress highly affects the coffee production, causing prices to rise due to decrease of yield, that may fall to 80% in very dry years (*9*). According to these confined environmental growth conditions, it is possible to understand why countries like Brazil and Colombia are leaders in coffee production. In fact, according to the "Coffee Monthly Market Report" of July 2011 (*5*), obtained from ICO, when analyzing the market fundamentals, the main exporting countries were (figure 1.1), Brazil, Vietnam, Colombia, Indonesia, Ethiopia, India and Mexico (in descending order).

Figure 1.1 Major coffee producing countries in the world. The top ten coffee producers are shown in yellow (*1*).

Knowing the concepts behind the coffee plant ecophysiology has been one of the main aims attempted to achieve, since it is one of the most consumed and traded products (commodities) in the world (*10*). The major key issues to be studied not only by scientific areas as biotechnology, biochemistry, genetics, agriculture industry but also, more indirectly by economy, have been the improvement of: coffee plant production conditions, performance, growth, development and understand what influences the acclimation ability to environmental stresses.

1.3 Drought and low temperatures effects on coffee plant and control of oxidative stress

Long term breeding programs carried out at many institutions such as the Genetic Section of the Agronomic Institute of Campinas (IAC) and the Federal University of Viçosa (UFV, Brazil) in collaboration with the Investigation Center of Coffee Rusts (CIFC) have offered new high yielding quality cultivars with resistance to *Hemileia vastatrix* (Bettencourt and Rodrigues, 1988), and continues to improve certain species with regard to resistance to cold

and drought. A few cultivars have been released from some research centers, such as Icatu Vermelho, Icatu Amarelo, Obatã and Catucaí. These cultivars and other genotypes have shown the high profitability level of the investments being made by genetic breeding research and are being prepared to be released in the market (*11*).

Examples of some studied genotypes are:

1. Icatu

This genotype, hybrid of *C. canephora x C. arabica* - IAC 2944, is very similar to arabica, since it was backcrossed so many times with arabica, that some researchers consider it as such. It is a high stature plant, vigorous, medium to late fruit maturing, rust resistant and gives an excellent beverage quality. Icatu species seem to tolerate cold better, especially at their initial development stages (*12*).

2. Obatã

The genotype IAC Obatã 1669-20 is derived from the crossing of Villa Sarchi (*C. arabica*) coffee with the Timor Hybrid (*C. arabica x C. canephora*), conducted by the "Centro de Investigação das Ferrugens do Cafeeiro" (CIFC), in Oeiras, Portugal. It is a cultivar of low stature, vigorous, with high resistance to rust, large fruits and seeds and are highly productive plants, with yields averaging between 60 and 70 bags of green coffee per hectare. It is demanding in irrigation and nutrition. Have shown excellent production and quality of the coffee beverage is good, which is why the use of this genotype has rapidly increased (*12-14*).

3. Apoatã

Apoatã is different when compared to the two mentioned above, since it is a cultivar of *Coffea canephora* (*C. canephora cv. Apoatã* IAC 2258). Normally used as rootstocks, it is resistant to some nematodes species and rust and it is a vigorous plant, although it has a much more developed root system than arabica coffee, it shows to be more sensitive to cold than the other one, as also indicated in table 1.1 (*12*).

1.3.1 Plant responses to drought and cold stresses involving oxidative stress

Abiotic stresses such as drought and extreme temperatures pose a greater threat, more than the reduction of yield for major crop species (*15*). In the two last decades, reviews focused on the physiology of coffee, for both arabica and robusta (*16-18*) water relations (*19, 20*) and drought tolerance (*21, 22*) in coffee plants.

Drought and low temperatures effects on coffee plant and control of oxidative stress

The responses of plants to abiotic stresses such as drought and cold are very complex to interpret, showing different modifications in conjunction with the soil drought. All the dynamics involved in soil water depletion, plant growth and different temperatures are major sources of variation in responses to these types of stress that may often be a result of multidimensional stress (*23*).

The primary effects regarding abiotic stress, such as drought and cold, are mostly related to: cellular damage and enzyme inactivation, resulting for example from oxidative and osmotic stress, leading to the breakdown of homeostasis, proteins and membranes damage. An inevitable consequence is the breaking of electron transport in chloroplasts to molecular oxygen. When this happens there is a decrease in energy consumption without a significant reduction in energy capture and this will contribute to a higher possibility of transfer the excited state of chlorophyll to O_2, producing $O_2^{\cdot -}$, which in turn can lead to •OH and H_2O_2 formation. These molecules are known as reactive oxygen species (ROS) and are responsible for damage of the cellular machinery, causing lipid peroxidation and consequently membrane injury, enzyme inactivation, protein degradation and disruption of DNA strands (*24-29*).

All this imbalance triggers several protection mechanisms (controlled by downstream signaling and transcriptional control), as:

- osmoprotection (proline accumulation) and antifreezing proteins (*30*) (chitinase, heatshock proteins and coldrelated genes);
- control of highly reactive oxygen species by, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as ascorbate and α-tocopherol molecules (*10*);
- promotion of the reduction of energy capture, mainly in leaves by its movements and rolling, and also by chloroplasts movements to the opposite side where the light is focused, thus decreasing the absorption of incident radiation by until 40% (*31, 32*).

All these processes prevent the over-reduction of the electron transport chain and the over-acidification of the thylakoid lumen (which would photodamage the photosystem II), and contribute to the decreasing of highly reactive compounds that are generated during the induced plant stress (*28*).

Much research has been done with regard to all this "classic" components of oxidative stress regulation and indeed much is already known. Even so, there are other components - secondary metabolites - in plants, with emphasis to those derived from the phenylpropanoid biosynthetic pathway, which also have been reported with an important role in response to

abiotic stresses, including cold, drought, and pathogen defense by acting as free radicals scavengers (*33*).

1.4 Polyphenols in coffee – biological activities, properties and role

As previously stated, coffee has always been a very popular drink due to its stimulant effects, enjoyed by all who have tasted it, but during the last decades, there has been a new interest related to one of its major constituents, these secondary metabolites: polyphenols.

Nowadays, polyphenols have gained a major interest by the community due to associated benefits to human health. They can be found in fruits, vegetables, cup of tea or coffee and even in a glass of red wine.

Plants synthesize a wide range of organic compounds, and those with fundamental functions associated with photosynthesis, growth and development of the plant, are considered primary metabolites. On the other hand, there are the secondary metabolites that despite having been ignored for too long are now becoming attractive. They have been related to have a major role in plant protection, from UV light, pathogens attack and protection responses for other biotic and abiotic stresses (*34*). In addition, secondary metabolites may have diverse interests, since they can be used as dyes, flavoring agents, drugs, and also viewed as potential sources of new natural drugs. However, these are distinguished from the classical vitamins or pharmaceutical drugs, once they are not used for short-term well being. Instead there have been multiple evidences that the long-term administration of them may have favorable effects on various types of diseases: cancer, chronic diseases, especially cardiovascular and diabetes type II (*35-37*).

1.4.1 Phenylpropanoid pathway

Before taking a closer look at the polyphenols existant in coffee plant, one need to understand how these compounds are synthesized, what regulates their production, that is, its biosynthesis (figure 1.2).

Briefly, the entrance to the phenylpropanoids pathway is the shikimate pathway that generates (besides the shikimate), three aromatic amino acids: phenylalanine, tyrosine, and tryptophan. From this point on the formation of phenylpropanoids (polyphenolic compounds), occurs through the phenylpropanoid pathway starting with the deamination of L-phenylalanine into trans-cinnamic acid, catalized by the enzyme phenylalanine ammonia-lyase (PAL).

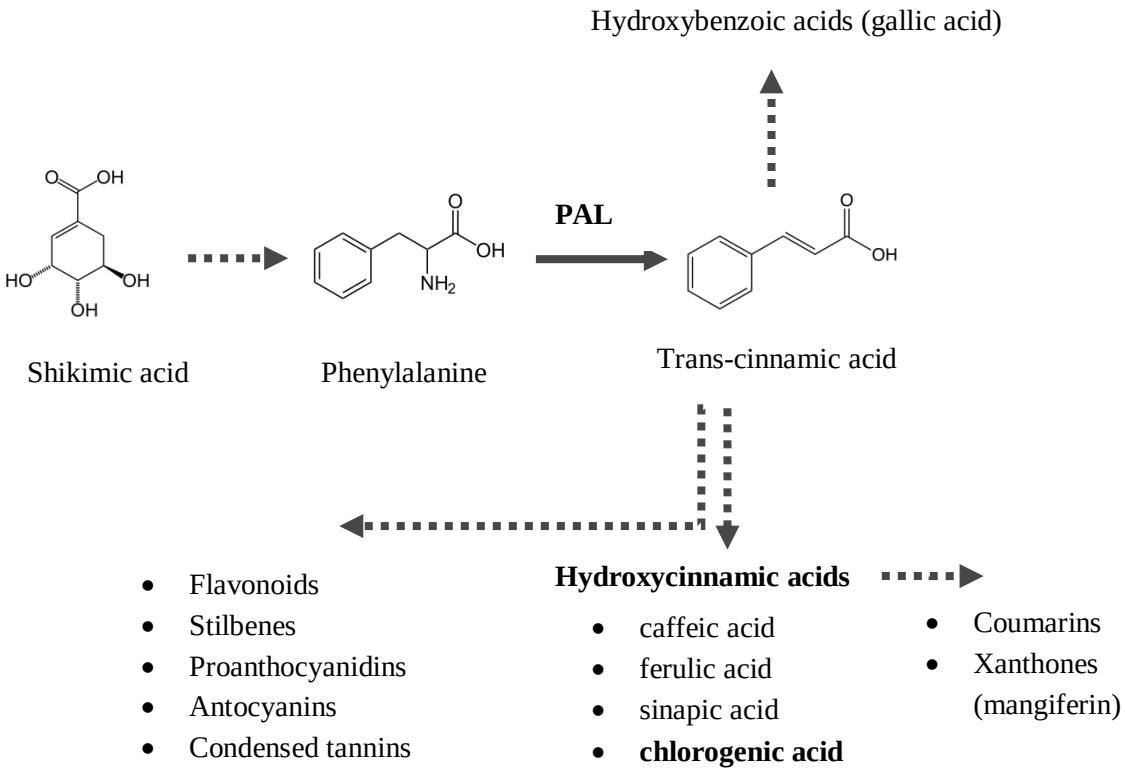


Figure 1.2 Main families and biosynthetic pathways of plants polyphenols.

From trans-cinnamic acid, two major groups can be formed: hydroxybenzoic acids (gallic acid and protocatechuic acid, for example) and hydroxycinammic acids (caffeic, ferulic acid and chlorogenic acid for example). Hydroxybenzoic acids usually appear in very low amounts in plants. Otherwise, hydroxycinnamic acids, are found in much larger quantities in plants, especially in coffee plant, hence they will be more covered in this chapter.

Hydroxycinnamic acids are produced, from trans-cinnamic acid, via several reactions such as hydroxylation, methylation and dehydratation. Some examples are: p-coumaric, caffeic, ferulic, sinapic acids and chlorogenic acid. Finally, from this hydroxicinnamic acids, hydroxicinnamoyl alcohol derivatives can be formed, that through oxidative polymerization become the units lignin polymers (*33, 38*).

The subcellular localization (intracellular, plastidial) of these pathways has been investigated for more than a decade. They may be produced near the site of infection in case of a pathogen attack, accumulation in the epidermis in case of increased UV radiation, and accumulation in vacuoles as glycosides or other conjugates for later use (*39*), thus it often depends on the stress to which the plant is imposed. However it is not known whether the compounds are synthesized *in situ* or are transported by adjacent cells (*40, 41*).

1.4.2 Polyphenols classification

Secondary metabolites of plants can be divided into three major groups:

1) Phenolic acids and flavonoids;

2) Terpenoids;

3) Nitrogen-containing alkaloids and sulfur-containing compounds.

For the purpose of this work, only the first group will be detailed: flavonoids and phenolic acids. Even with more detail in phenolic acids rather than flavonoids, despite both being present in coffee, coffee leaves are much richer in phenolic acids than flavonoids (*42*).

Polyphenolic compounds are ubiquitous constituents of higher plants and therefore found in many commonly consumed beverages, such as wine, tea and coffee (*42-44*). They are characterized to have at least one aromatic ring with one or more hydroxyl groups attached. Depending on their structure (more than 8.000 structures have been reported) (*45*), they can be classified in different ways, based on the number and arrangement of carbon atoms.

1.4.3 Phenolic acids

There are two major classes of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids.

- The first group, hydroxybenzoic acids, have a general skeleton of C1-C6 compounds (figure 1.3) and with the exception of gallic acid that may be present in significant amounts in tea leaves (up to 4.5 g / kg fresh weight), the amount of hydroxybenzoic acids in edible plants is usually very low. Other examples include, protocatechuic acid, syringic acid and salycilic acid,

being the latter one best known as a plant hormone or a compound that can be used for the preparation of aspirin (acetylsalicylic acid) (*46*).

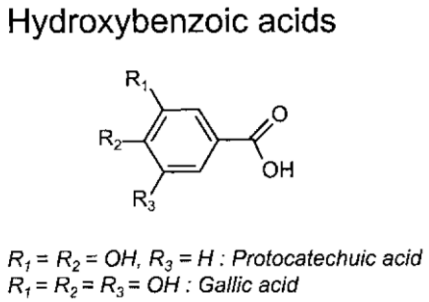


Figure 1.3 General chemical structure of hydroxybenzoic acids (*44*).

- Hydroxycinnamic acids are much more common in plants than hydroxybenzoic acids. They have a C6-C3 skeleton (figure 1.4) being p-coumaric, caffeic, ferulic and sinapic acids, the most common.

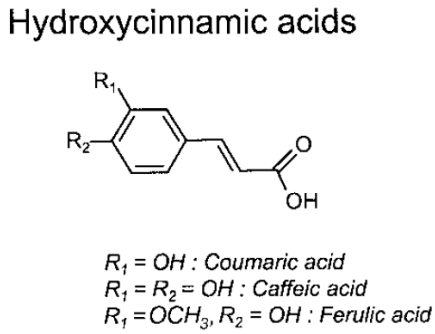
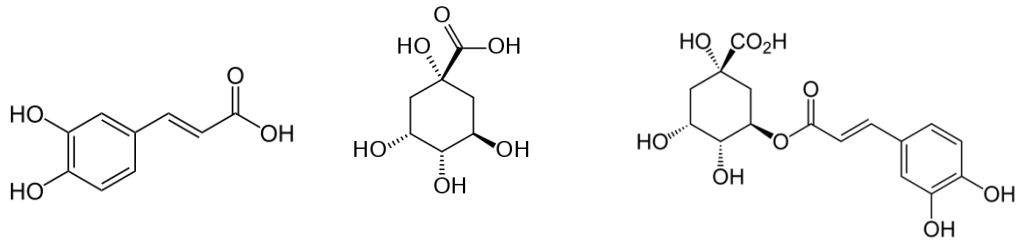


Figure 1.4 Chemical structure of hydroxycinnamic acids (*44*).

One of the best known compounds of this subclass is 5-caffeoylquinic acid (5-CQA), commonly referred to as chlorogenic acid, gives name to another great family related to, trans-cinnamic and quinic acids conjugates.

1.4.4 Chlorogenic acids

Chlorogenic acids (CGA) are esters formed between quinic acid (1,3,4.5-Tetrahydroxycyclohexanecarboxylic acid) and trans-cinnamic acids (figure 1.4). In general, the values described in the literature for total CGA in regular coffee beans, on dry matter basis, may vary from 4 to 8,4 % for *Coffea arabica*, and from 7 to 14,4 % for *Coffea canephora*, with some hybrids presenting intermediate levels (*47-50*).



A B C

Figure 1.5 Chemical structures of: A – Caffeic acid; B – Quinic acid and C – Chlorogenic acid.

Depending on the nature, number and position of cinnamic acid and on the cyclohexane ring of quinic acid substituints, chlorogenic acids may be divided into (*42*):

- Caffeoylquinic acids: the most often reported are the isomers, 3-CQA, 4-CQA and 5-CQA, with 5-CQA being the most abundant compound found in coffee beans, responsible for about 56-62 % of total chlorogenic acids;

- Dicaffeoylquinic acids (diCQA): existing isomers include, 3,4-diCQA, 3.5-diCQA and 4.5-diCQA. DiCQA isomers account for about 15-20% of total chlorogenic acids in coffee beans;

- Feruloylquinic acids (FQA): existing isomers include 5-FQA, 4-FQA and 3-FQA, which may count for about 5-13% of total chlorogenic acids;

- p-coumaroylquinic acids (p-CoQA) and caffeoylferuloylquinic acids (CFQA): 3-p-CoQA, 4-p-CoQA and 5-p-CoQA are recently identified isomers and are present in smaller amounts (remaining percentage of the ones referred above).

Chlorogenic acid is found in many plants (*51-53*), but it is in coffee plant that they are present in greater amounts, ranging from 4% to 14% (dry matter basis) of phenolic content in coffee beans (*42, 47*), having already been identified 45 different CGA compounds , including minor classes (less than 1% of total chlorogenic acids content), such as diferuloylquinic acids (diFQA), di-p-coumaroylquinic acids (di-p-CoQA) and dimethoxycinnamoylquinic acids, that have been recently identified (*54, 55*).

Besides having an important role in quality (flavor, acidity) of coffee beverage (*56*) chlorogenic acids are the phenolic compounds in plants that have been most studied in applications to improve human health. Due to its high antioxidant potential, many health benefits have been attributed to chlorogenic acids, both *in vivo* and *in vitro*, being hepatoprotective, immunoprotective, hypoglycemic and antiviral activities (*57*).

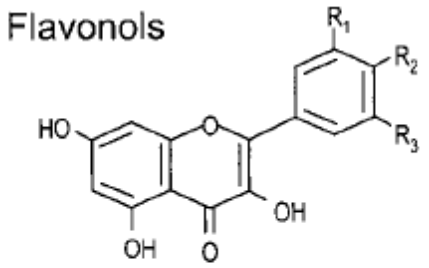
1.4.5 Flavonoids

Flavonoids, even with less impact on coffee plant, due to their lower presence, also contribute to the antioxidant potential of coffee (*44*), thus some families will also be presented.

The flavonoids are characterized by having 15 carbons, consisting of two aromatic rings connected by three carbons (pyran ring) with a basic structure, C6-C3-C6 (Figure 1.5). Depending on the substituents, ususally hydroxyl groups, methyl or sugar and the level of oxidation in the C3 ring, there are several major subclasses: flavonols, flavones, flavan-3-ols (or flavanols), isoflavones, flavanones and anthocyanidins.

Flavonols

Flavonols are the subclass of flavonoids most present in foods, having the basic structure shown in figure 1.6 and the most representative compounds are quercetin and kaempferol. Flavonols are normally present in small amounts (15 to 30 mg/kg fresh weight), and are often associated with a sugar moiety such as glucose or rhamnose. Usually, they are accumulated in the outermost tissues (leaves) once their biosynthesis is stimulated by light. One of the richest sources is onions that can go up to 1,2 g / kg fresh weight (*44*).



$R_2 = OH; R_1 = R_3 = H$: *Kaempferol*
$R_1 = R_2 = OH; R_3 = H$: *Quercetin*
$R_1 = R_2 = R_3 = OH$: *Myricetin*

Figure 1.6 General chemical structures of flavonols

.

In coffee flavonols can be considered as minor class, representing about 1,6% (dry weight) of total polyphenol concentration in *C. arabica* (*58*).

Flavanols (flavan-3-ols)

Flavanols or flavan-3-ols, comprise monomeric (catechins) or polymeric (proanthocyanidins, which are also known as condensed tannins) molecules, making this subclass, one of the most complex of the flavonoids. Flavanol molecules, have two chiral centers and therefore are four possible isomers depending on the level of hydroxylation in the B-ring (middle ring). For example, both (+)-catechin, (-)-epicatechin and vice versa can exist, being some forms more or less present in certain plants.

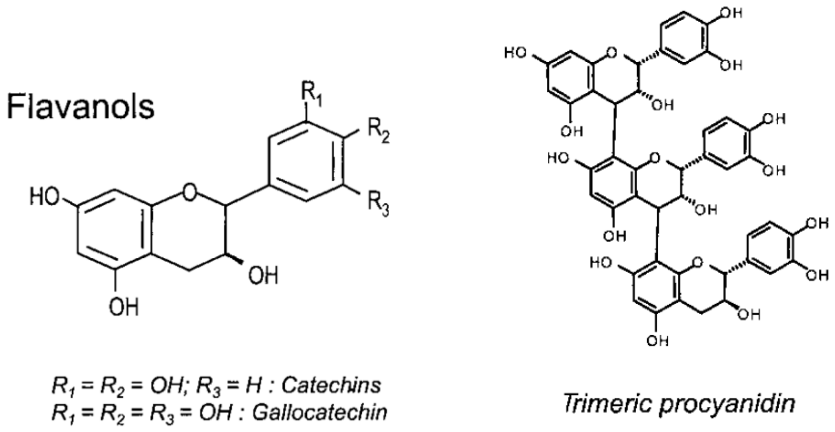


Figure 1.7 Chemical structures of flavonols (left) and an example of a trimeric procyanidin (right).

For instance, in fresh coffee pulp an average of 21,6% of epicatechin and 2,2% of catechin content was found (*59*). Other families of flavonoids are not covered because they are not present in coffee or their amount is very low.

1.5 Impact of polyphenols on human health

The main reason that drew attention comes from antioxidant properties of polyphenols. Besides that, polyphenols shows specific roles, such as in help treating diseases such as cancer, cardiovascular and neurodegenerative diseases and inhibition of low-density lipoprotein (LDL) cholesterol from oxidation (*60, 61*). A wide range of roles that polyphenols can exhibit is presented in figure 1.8.

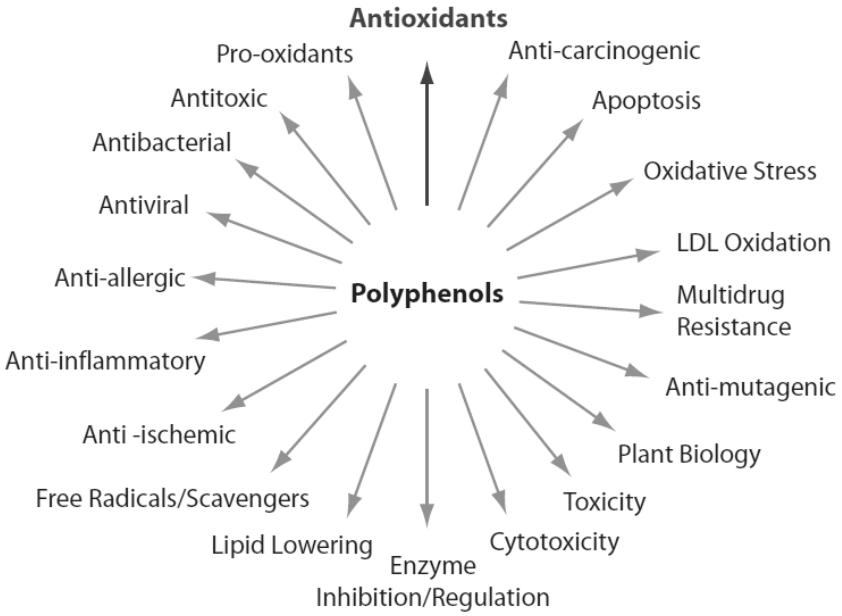


Figure 1.8 Polyphenols bioactivities (62).

Fruits, vegetables, cereals, chocolate and beverages such as wine, tea and coffee are the main sources of polyphenols. Dietary intake of polyphenols is estimated to be about 1 g/day (*44*, *61*) and these compounds are extensively metabolized by intestinal and hepatic enzymes, and also by the intestinal microflora.

Polyphenols found in the coffee plant present general properties, such as:

5-CQA:

Is the most abundant compound in the coffee plant, either in beans or in leaves (*42*, *63*) and some of its properties besides protection against oxidative damage include, an increase in resistance of LDL from oxidation (*60, 64*), there is quantitative evidence of an inverse relation between coffee drinking and liver cancer (*65, 66*) and a role in inhibiting the formation of potent mutagenic and carcinogenic N-nitroso compounds in vivo (*67*).

Di-CQA (dicaffeoylquinic acids)

Despite di-CQA (figure 1.9) isomers not being present in significant amounts in coffee, they have been reported as having a key role in helping treating several diseases, including rust resistance (to *Hemileia vastatrix*, for example) in coffee plants. In humans, they have shown

that they can contribute to the inhibition of HIV replication (*in vitro*) (*68, 69*) and have shown a protective effect in *in vitro* assays involving the Alzheimer's disease (*70*).

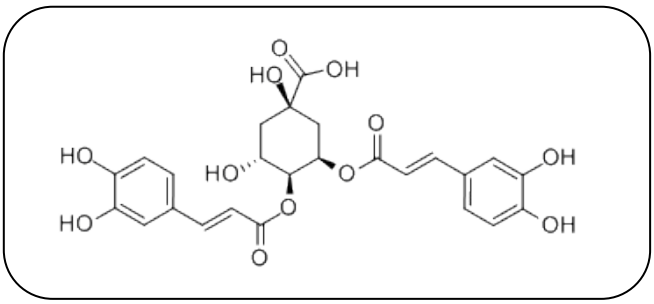


Figure 1.9 Example of a diCQA chemical structure, in this case is 4.5-dicaffeoylquinic acid (4.5-diCQA).

Mangiferin

Mangiferin (figure1.10) has been reported very rarely in the coffee plant, there is indeed only one reference (*71*) and (now) another one will be made with this work, showing that this is a major component in coffee leaves and a possible biomarker to distinguish *C. arabica* from *C. canephora*, thus an interesting compound to study further in the future.

This polyphenol has been reported to possess antidiabetic properties, thus it is suggested to be used in treating type-2 diabetes (*72-74*).

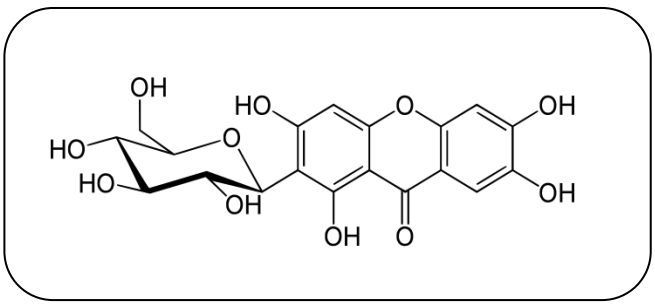


Figure 1.10 Chemical structure of mangiferin.

Epicatechin and procyanidins

Diets rich in flavonoids, particularly beverages and foods (as tea, wine and apples) that are rich in epicatechin and procyanidins, are associated with cardiovascular disease prevention (*75*). There are many suggestions that epicatechin is an important mediator in positive cardiovascular effects (*76, 77*) and in future directions, by knowing the bioavailability and

consumed values of this polyphenol, it could be used for therapeutic interventions aimed to improve and maintain vascular health.

Procyanidins were found as the main vasoactive polyphenols in red wine (*78*), chocolate and cocoa (*79*) and is also reported due to its ability in inhibiting LDL oxidation *in vitro*, however the mechanisms by which these flavonoids enhance cardiovascular health are still to be established.

α-tocopherol

Vitamin E or one of its forms, α-tocopherol (figure 1.11) will be approached here, since it was one of the antioxidants (lipophilic one) that were used to study in microemulsions formulations in this work.

α-tocopherol is the form of vitamin E that is preferentially absorbed and accumulated in humans (*80*) and is widely used as an inexpensive antioxidant in cosmetics and food supplements.

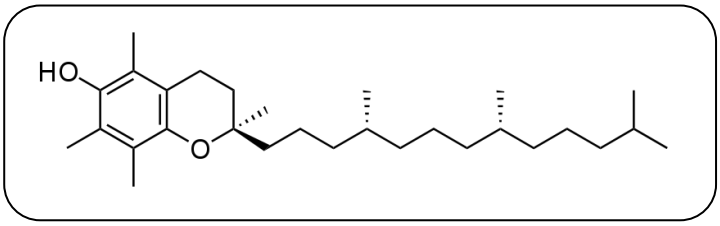


Figure 1.11 α-tocopherol chemical structure.

Products containing α-tocopherol are commonly used in the belief that it (or generally vitamin E) is good for the skin, and many cosmetics include it, often labeled as tocopherol acetate, tocopheryl linoleate or tocopheryl nicotinate (*81*). Some studies have shown that applying vitamin E before UV exposure significantly reduces acute responses such as edema and eythema (*82*), as well as that the topical application is related to decrease of the incidence of ultraviolet UV-induced skin cancer (*83, 84*).

Due to all these beneficial properties for human health, a great bet on the market, has been the use of these natural compounds for applications in the pharmaceutical and cosmetic industry, mainly in drug delivery systems (*85, 86*) and topical skin applications (*87-89*). A system that can be used for these applications, that has been progressing for decades, is microemulsions.

1.6 Microemulsions - history and definition

It is well established that two liquids like water and oil do not mix, but by addition of an appropriate amount of surfactant, they can be brought into a single phase (macroscopically homogeneous but microscopically heterogeneous). When these three components, water, oil and surfactant are mixed, the determinant factor lies in the surfactant molecule. In a very brief explanation, the surfactant molecule has a polar (head) and an apolar (tail) region, thus hydrophilic and hydrophobic, which make it possible to form structures where water and oil are together in the same solution separated by a surfactant film. Depending on the amount and type of surfactant (and the other components as well), this unique class of optically clear solutions that is currently known as, microemulsions, can be made which comprise the colloidal systems that have attracted much scientific and technological interest over the past decades (*90*).

This interest stems from their unique characteristic properties as, ultralow interfacial tension, large interfacial area, solubilization for both water and oil-soluble compounds, and that they are spontaneously forming and thermodynamically stable (*91*). These and other properties insures that microemulsion systems have been studied from a fundamental point of view and make them attractive and suitable for many industrial and technological applications, including, personal care product formulations (cosmetics), novel drug delivery systems, enhanced oil recovery, nanoparticles formation, chemical reaction media and environmental protection (*90*).

The microemulsion concept was introduced in 1943 by Hoar e Schulman, who generated a clear single phase solution by titrating a milky emulsion with hexanol (*92*) but it was Schulman and his coworkers who first proposed the word “microemulsion” in 1959 (*93*).

A general definition of the term “microemulsion”, used nowadays, was given later by Danielsson and Lindman, who described it as a “system, composed of water, oil and an amphiphilic component, being an optically isotropic and thermodynamically stable liquid solution” (*94*)

In the late 1970's and early 1980's, interest in microemulsions increased when it was recognized that these systems could improve oil recovery. Comparing to other surfactant solutions, microemulsions have a ultralow interfacial tension, lower than 10^{-3} mN/m (minimum for this process is 10^{-2} mN/m), that allows them to remove successfully the residual oil in the tertiary recovery (*90, 95*). While in the late 80's most citations on microemulsions where focused more on physical and colloid chemistry, this pattern has now changed to work dealing with more applied aspects of health sciences, in particular, in the pharmaceutical sciences (*91*). In fact, the areas where microemulsions have increased dramatically during the past decades, major ones being the, pharmaceutical and cosmetic industries, oil recovery, food industry,

pollution control, agrochemicals and textile dyeing, and a few will be in detail later in this chapter (*90*).

1.7 Microemulsions properties

Other colloidal systems such as emulsions can be used as well, when compared with, microemulsions. However, microemulsions have much more advantages and some are presented in table 1.2 (*85*, *96*).

Table 1.2 Major differences between microemulsions and emulsions.

Microemulsions	Low energy required for preparation	Thermodinamically stable	Isotropic/clear appearance (aesthetic property for consumer products)	Dynamic microstructure (fluctuating surfaces)	Small aggregates (less than 100 nm) important in drug delivery
Emulsions	Large input of energy	Unstable, eventually separate	Cloudy/turbid	Static structure (until coalescence)	Large droplets (0.1 to 10 µm)

All these advantages stem from the fact that the surface tension in microemulsions has very low values. In order to have a better understanding and a more detailed explanation, we must look at it in a thermodynamic point of view.

Taking one step back, the scientific process of formulating the otherwise immiscible oil and water by adding an amphiphile is itself an art, however behind this art there is always a great amount of science. The immiscibility arises due to very high interfacial tension between oil and water. For a mixture to be thermodynamically stable, Gibbs free energy (ΔG), must be at a minimum value and the change in ΔG is dependent on the surface tension of the oil-water interface (*91*), according to equation 1.1:

$$\gamma = \frac{\Delta G}{\Delta A}$$, at constant pressure and temperature, equation 1.1

γ, surface tension; ΔG, Gibbs free energy; ΔA, surface area

If the surface tension is higher between two solvents, Gibbs free energy increases as well and hence the mixing fails. Therefore to change the ΔG to very low values, surface tension requires to be reduced to very low values as well and there, begins the role of surfactant. A simplified thermodynamic model has been proposed (*97*) to explain the formation of a microemulsions system as shown in equation 1.2:

$$\Delta G = \gamma \Delta A - T\Delta S, \quad \text{equation 1.2}$$

where ΔG is Gibbs free energy, γ is surface tension, ΔA surface area, T is temperature and ΔS is entropy change (related to dispersion entropy of the system).

In microemulsions formation, the changes in ΔA are very large due to the large number of very small droplets formed. In the beginning it was proposed that in order for a microemulsion to be formed a (temporary) negative value of γ was required (*98*), but it is now recognized that while value of γ is always positive (*99*), it is very low (of the order of fractions of mN/m), due to the migration of surfactant molecules to the interface of the two immiscible phases. Besides that, the ΔS, entropy change of the system, offsets the other component, because when mixing one phase in the other in the form of a large number of small droplets, there is a positive change in entropy ($T\Delta S >> \gamma\Delta A$) thus, Gibbs free energy becomes negative and so the microemulsification process is spontaneous resulting in a thermodynamically stable dispersion.

1.8 Components and properties

To be able to understand how microemulsions are formed and what can possibly influence them, one must perfectly know their components and its properties. In other words, we must know the properties of the surfactant, of the oil molecules and also of the water that is not always just water.

Microemulsions consist of three parts, a hydrophilic compound, a hydrophobic compound and an amphiphilic compound. The hydrophilic molecule is in most of the cases water, but it can also have salts, for example, potassium phosphate buffer or sodium chloride (*89, 97*). The hydrophobic molecule is often an oil (or a mixture of them), and the amphiphilic

part is a surfactant. Additives to microemulsions can be co-surfactants, salt and polymers, but here we will not go into detail on that.

1.8.1 Surfactant

The term surfactant comes from *Surface Active Agent*, and surfactants lower the surface tension of a liquid or the interfacial tension between two liquids. Conventional surfactant molecules comprise of polar head group (hydrophilic region) and an apolar tail region (hydrophobic region).

There are mainly four types of surfactants: anionic, cationic, zwitterionic and non-ionic. The first three (anionic, cationic and zwitterionic) are charged (in the head group region), and the charged counterpart is called a counter ion, and the last one, non-ionic, does not have charge.

Anionic surfactants (figure 1.11) are negatively charged in the head group, usually with, sulfates, sulfonates, phosphates and carboxylates. Examples of anionic surfactants are:

- Sodium dodecyl sulfate (SDS);
- Sodium lauryl ether sulfate (SLES);
- Carboxylates (Sodium stearate);
- Sodium bis-2-ethylhexylsulphosuccinate (AOT).

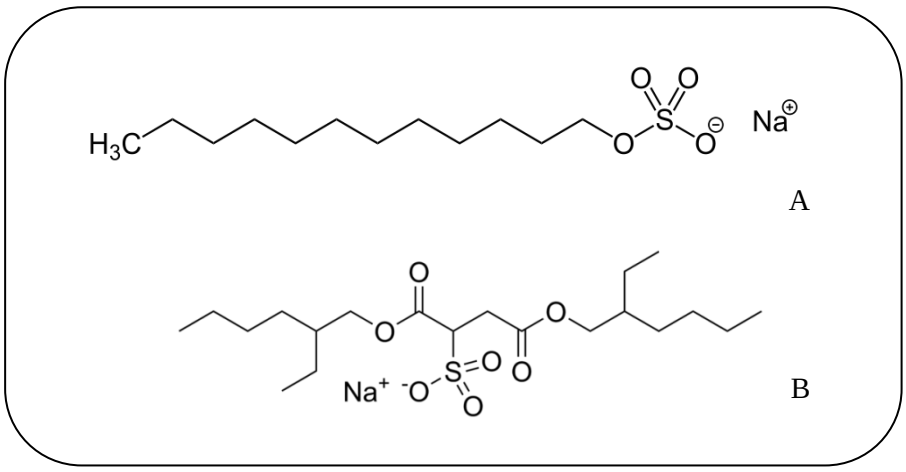


Figure 1.11. Chemical structures of anionic surfactants, SDS (A) and AOT (B).

Cationic surfactants (figure 1.12) are positively charged in the head group, and in this case the more used counter-ions are, bromides and chlorides. Examples are:

- Cetyl trimethylammonium bromide (CTAB) and cetyl trimethylammonium chloride (CTAC);
- Decyltrimethylammonium bromide (DETAB).

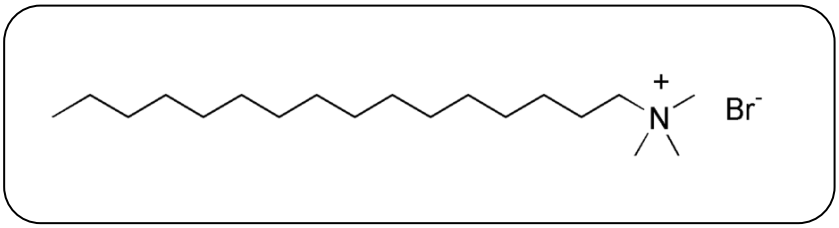


Figure 1.12. Chemical structure of a cationic surfactant, CTAB.

Zwitterionic surfactants (figure 1.13) have both positive and negative charges in the polar region but are neutral overall. Some examples of this type of surfactant are:

- 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS);
- Lecithin (mixture of several fatty substances, from a variety of sources like soybean and egg yolk, with its major constituent is diacylphosphatidylcholine);
- Phospholipids are also an example of zwitterionic surfactants and exhibit excellent biocompatibility.

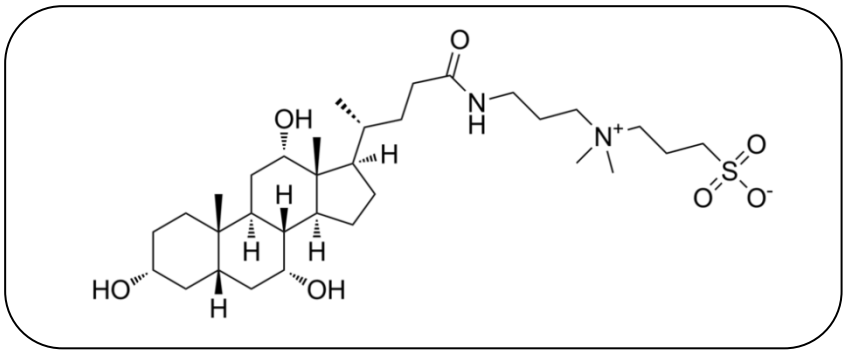


Figure 1.13. Chemical structure of a zwitterionic surfactant, CHAPS.

Non-ionic surfactants (figure 1.14) do not have charge in the polar group, but a polar headgroup like an ethylene oxide (E-O) chain or a sugar group. They are not as influenced by addition of salts as the other groups and the ones with E-O groups are temperature sensitive.

Amongst the most widely used non-ionic surfactants are:

- Polyoxyethylene alkyl ethers (C_mE_n, where m is hydrocarbon chain length and n is the number of oxyethylene units), like $C_{12}E_5$ (pentaethylene glycol monododecyl ether), Brij 35 ($C_{12}E_{23}$) and Triton X-100;
- Sucrose esters, such as sorbitan monooleate (Span 80);
- Glucosyde alkyl ethers, as lauryl glucoside;
- Cocamide MEA - cocamide monoethanolamine (derived from coconut oil).
- Some are registered trademark, as Transcutol® (diethylene glycol monoethyl ether) and Plurol Isostearique® (isostearic acid ester of polyglycerols mixture).

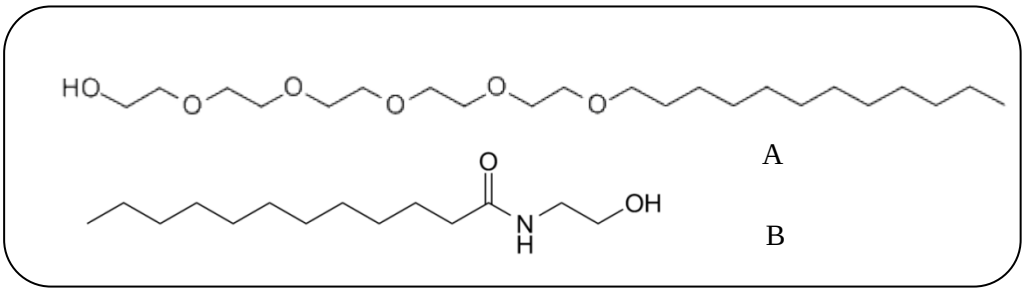


Figure 1.14 Chemical structures of a non-ionic surfactant, $C_{12}E_5$ (A) and cocamide MEA (B).

Polyoxyethylene alkyl ethers (C_mE_n) have some distinguishing properties that make them good to use as model systems (*100*). They are particularly attractive because it is possible to create a microemulsion by just varying temperature and diluting with water, without the use of a co-surfactant or co-solvents (*101*). These systems have also been extensively studied, and there are detailed information available so they are ideal as a model system, in particular microemulsions formed with $C_{12}E_5$ (*85, 101-104*).

In most formulations the presence of a co-surfactant or co-solvents such as a short-chain alcohol, is required to further decrease the interfacial tension, to be able to have many different structures. Some of these additives are irritating and many co-surfactants are not pharmaceutically acceptable. Most of non-ionic surfactants are generally are considered safe for human applications, and are generally regarded as less irritant than ionic surfactants.

The critical micelle concentration (CMC) is very low, therefore low amounts of this type of surfactants are needed to form aggregates.

The temperature sensitivity comes from the ethylene oxide group (hydrophilic part) as it becomes less soluble in water with increasing temperature (*105*) (most compounds have the opposite pattern). This way by changing the temperature it is possible to "regulate" the transition from one phase to another, thus making the studies much easier. In ionic surfactants case, this could be done with the addition of a co-surfactant or co-solvent or possibly salt, which requires several samples.

1.8.2 Oil

Compounds that can be used as an oil for formulating microemulsions, generally can go from, fatty acids, esters of fatty acids and alcohols, medium chain triglycerides to hydrocarbons.

The most common used, range from, corn oil (mostly triglyceride), capric acid (decanoic acid), ethyl oleate (ester of oleic acid and ethanol), isopropyl myristate, an ester of isopropanol and myristic acid (figure 1.15) and aromatic hydrocarbons (benzene, toluene) or aliphatic hydrocarbons, such as alkanes like, decane and hexadecane (figure 1.15). There are also some cases of using terpentenes like, limonene and menthol to exploit their penetration enhancement abilities (*89*) and take advantage of its odor.

The choice of oil depends on the purpose with which the microemulsion is made. For instance, if the aim is large-scale use (example in enhanced oil recovery), one wants an economic oil (preferably with vegetable origin, even if it is not a pure solution but a of mixture similar oils). If one wants to study a system for pharmaceutical application, as drug delivery for example, the oil must be biocompatible, i.e, one already approved by regulatory bodies for pharmaceutical use (not toluene for instance). In general one should look to price, biocompatibility, solubility, viscosity, depending on the purpose for which microemulsion is used. On the other hand, most of the fundamental work and research focuses on using hydrocarbons, such as decane, hexane, dodecane, cyclohexane, as the oil component, because they have similar structures (*101, 104*) (although can differ in some properties). Changing from a decane to a dodedecane, is useful to study the effect of different oil in the system, if we know exactly that this is the only thing changing, thus have more control and can predict the behaviour of the microemulsion. However, these last oils are not suitable pharmaceutical ingredients. Different oils have different properties such as, density, vapor pressure, solubility in water, among others. Even though the last one (solubility in water), may be very low for oils in general, the differences between being, extremely low soluble or just low soluble, can influence the kinetics, so reactions may occur more or less rapidly and thus influencing the choice.

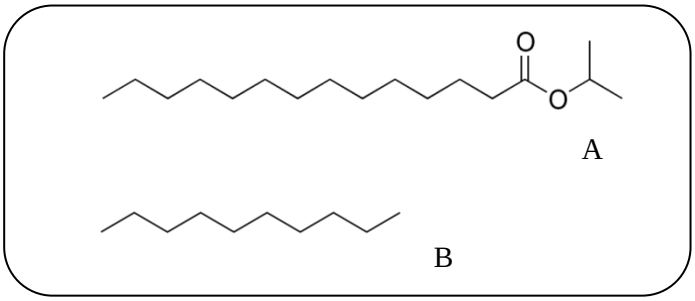


Figure 1.15 Chemical structure of two different oils, isopropyl myristate (A) and decane (B).

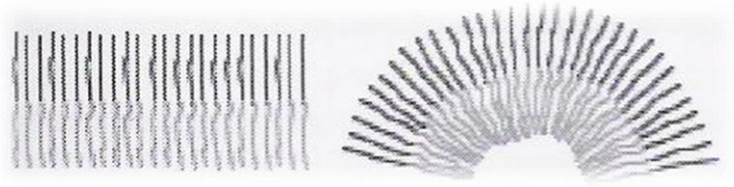
1.9 Microemulsions structures

When dispersed in water or in non-aqueous solvents (such as alkanes), surfactants self-assemble into a variety of equilibrium phases, which depend from the interplay of various molecular forces and also of entropy considerations in order to minimize the free energy of the overall system (*97*). In other words, surfactant molecules locate at the oil/water interface and orientate the hydrophilic region towards the water and the hydrophobic region for oil. When there are enough surfactant molecules (when it reaches the CMC) they will start to aggregate into what is called micelles.

A number of phases can result of the assembling of surfactant molecules, which are structured on the microscopic and sometimes noticed on a macroscopic scale. They are related with the curvature of the structures, and with the one that is thermodynamically more stable.

1.9.1 Spheres (micelles) growth

Microemulsions with non-ionic surfactants of E-O type are temperature dependent, meaning that the structures change with increase or decrease of temperature. When the system is in the microemulsion phase temperatures, it is considered to be mainly composed of spherical micelles at least at the lower temperatures (*101*, *104*). In non-ionic surfactants of EO-type, like $C_{12}E_5$, the higher the temperature the less solubility in water do ethylene oxide groups (hydrophilic heads) have (*105*). Thus, when the temperature increases, the water will cover less space between the hydrophilic regions of the surfactant promoting the curvature reduction of the interfacial film (figure 1.16). Knowing this and that the, interfacial area and enclosed volume can be considered to be conserved quantities, starting from a spherical shape, a micellar growth has to involve a shape change of the micelles. The balance between the curvature of the film, energy and entropy in the system and interaggregate interactions, regulates the size of the micelles. From a detailed analysis of self-diffusion data and relaxation data in the decane system (*104*) it was concluded that the micelles in that system become elongated (prolate shape) as the temperature is increased in the microemulsion phase. The temperature-dependent growth of the micelles, involving curvature changes is illustrated in figure 1.16.

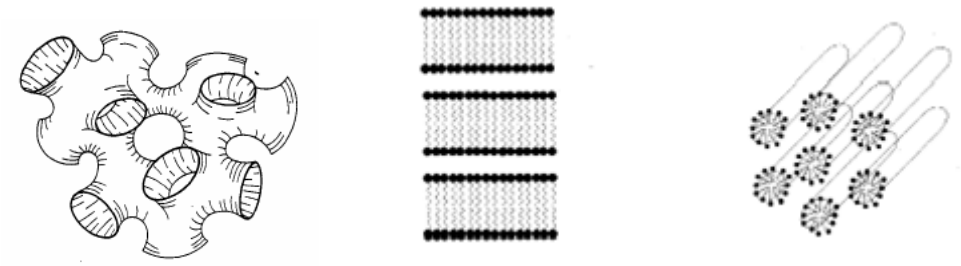


A B

Figure 1.16 Low (A) and high (B) curvature of the surfactant interface, corresponding to bigger and smaller droplets. Showing that it is a balance between how "tight" surfactant molecules are (more or less curvature) as well as the penetration of water and oil in the hydrophilic and hydrophobic chains of the surfactant, respectively. This will determine the curvature and size of the micelles (*106*).

1.9.2 Different structures formed

Besides spheres there are many more structures (figure 1.17) in which microemulsions can self-assembly being all idealized as a set of interfaces dividing polar and apolar domains.



A B C

Figure 1.17 A few structures in which microemulsions may self-assembly. A – bicontinuous structure (L_3); B – Lamellar structure (Lα) and C – Hexagonal structure (*97*).

Depending on the amount of the three compounds, two types of microstructures can be most commonly found, as we can see in figure 1.18.

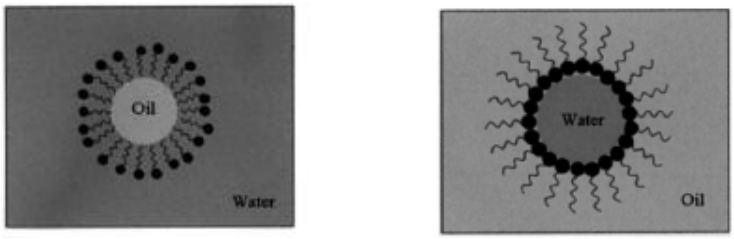


Figure 1.18 Schematic representation of two microemulsions: A - Oil-in-water (O/W) and B - water-in-oil (W/O) microemulsions (*97*).

Oil-in-water (O/W) microemulsions are formed, when the volume fraction of oil is low. On the contrary, we have water-in-oil (W/O) microemulsions or reverse micelles, when the volume fraction of oil is high compared to the water and surfactant fractions.

1.10 Phase diagrams

In order to characterize microemulsions there are several methods and techniques, as Dynamic Light Scattering (DLS), Nuclear Magnetic Ressonanse (NMR), Small Angle X-ray Scattering (SAXS) and Small Angle Neutron Scattering (SANS), mainly used to study unknown systems (*103*). The need to use one or another depends on the characterization that we want to do and the availability of the equipment as some are lab-scale while others are large scale facilities with the need to apply in order to get access. If it were to determine more precisely a type of structure or to evaluate the influence of the addition of a certain incorporated compound, it would require different techniques.

Despite all these techinques, the phase diagram construction is the first characterization made to obtain a good and general approximation to the behavior of the system. The construction of phase diagrams provides information on the boundaries of different phases, depending on the components (surfactant, oil and water) and temperature (in case of temperature sensitive non-ionic surfactants). Plus it also can be used to infer about the structure organization. It is through them that we can compare different microemulsion systems with different surfactants, oils and other components, and evaluate the system for a particular application. Note that in order to construct phase diagrams, the measurements and equipment needed are quite simple. In this case it is only necessary to make a visual inspection of the microemulsion samples, since the changes are in the range of clear samples, to turbid, opaque, two phases, and some other mixtures between these. For other systems the phase separation might be slower and the aggregates bigger so that there is no clear phase and in this case other techniques are needed. We may also take advantage of the fact that these colloidal systems are isotropic, but they can also show birefringence (be anisotropic) and thus we can use polarized light, to aid its construction, since there are phases besides microemulsions that may be formed that can vary with the orientation of the incident light (polarizer) such as lamellar, hexagonal or reverse hexagonal. The only drawback with the determination of phase boundaries is that, in order to achieve the determination of several phases, or in other words, to have accurate values

of temperature where changes occur, this process may become very time consuming, that is why it is also usual to make cuts and fix ratios of the components.

The phase diagram can be represented by a ternary diagram (figure 1.19) (three-component system with fixed temperature and pressure) or by a triangular prism if the temperature is considered as a variable (figure 1.21). Another useful way of representing the phase diagram of a microemulsion system is to keep the concentration or ratio between one or two components constant and then just have different concentrations versus temperature (figure 1.20).

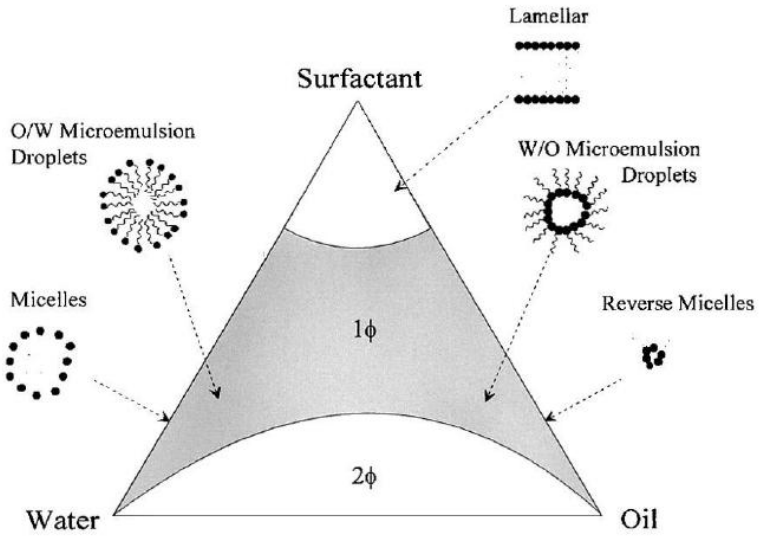


Figure 1.19 A hypothetical ternary phase diagram of an oil, surfactant and water system. There are shown several structures in which microemulsions can self-assembly: micelles, reverse micelles, and lamellar structure. At very high surfactant concentrations two phase systems are observed (*97*).

The representation above (figure 1.19) does not take into account the effect of temperature. In the case of microemulsions with non-ionic temperature sensitive surfactants, this is a very important parameter. Thus in figures 1.20 and 1.21, there is another way of representing phase diagrams where temperature is taken into consideration. Here the ratio surfactant to oil is fixed and varied amount of water that is added.

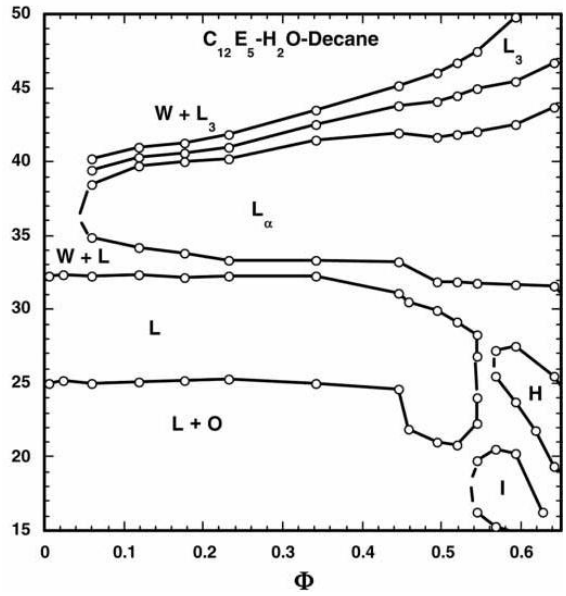


Figure 1.20. A - Phase diagram of the microemulsion system with $C_{12}E_5$/decane/water with a fixed surfactant to oil ratio (volume fraction, Φ), showing dependence on temperature (Y-axis) and several structures: L+ O (oil); Sample below lower phase boundary (oil on top, surfactant and water in the bottom) where there is a macroscopic phase separation; L phase; Sample in the microemulsion temperature range where the structure corresponds mainly to spheres (nanomicelles), where oil, surfactant and water are in only one macroscopically homogeneous phase; Lα, Lamellar phase, where is seen birefrangence glowing on the right image using polarized light; L_3 phase corresponds to bicontinuous; H phase corresponds to hexagonal and I to cubic phase (*101*).

A different phase diagram is represented in Figure 1.21, by a triangular prism, which evidence the fixed ratio between surfactant and oil, and changes in the amount of water, in the system.

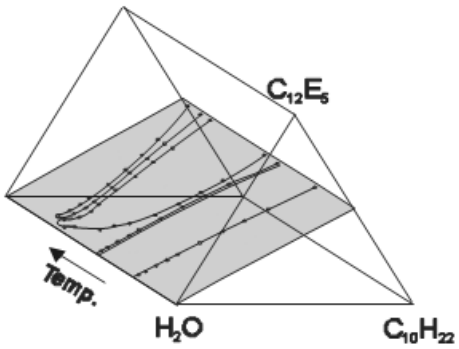


Figure 1.21. A simpler phase diagram can be presented in the form of a triangular prism, which has the temperature varying and also the three main components (oil, surfactant and water). In this case the system is the same as above, decane/$C_{12}E_5$/water with fixed oil to surfactant ratio (1:0.815).

1.11 Industrial Applications

Since the discoveries of Hoar and Schulman (*92*), microemulsions have evolved in so many fields that now can range from chemistry to biology and also to oil industry, in which then branched out in numerous applications. Examples include:

- Pharmaceutical industry (drug delivery) (*107*);
- Cosmetics industry (*96*);
- Foods industry (nutraceutical carriers) (*108, 109*);
- Agrochemicals (pesticides) (*110*);
- Dyeing processes (*111, 112*);
- Microemulsions in enhanced oil recovery, among others (*113-115*).

Regarding the first topic, in drug delivery systems, during drugs administration a major problem that exists with lipophilic drugs is related to the compromised absorption due to its low aqueous solubility. Some part is absorbed and reaches the pharmacological site of interest where it is effective. However the remaining part that is not absorbed can cause toxicity and side effects. Moreover, another drawback with some methods currently used for drug administration, is the time it takes, after the application, until the full effect being noted. Enhancement of the efficacy and toxicity reduction can be achieved by encapsulation of the drug in mainly aqueous drug delivery systems, such as microemulsions. An example of a long-term used drug (*116*) in topical applications is lidocaine (figure 1.22). Briefly, this drug is a local anesthetic and antiarrhythmic that has a rapid onset and intermediate duration of action of efficacy (*96*).

There are several studies on the use of lidocaine in microemulsions, where it is proved that this system promotes increased cutaneous penetration (*87, 117*), the capacity of lidocaine solubility in microemulsion was analyzed**,** using microemulsion as vehicle increases dermal drug delivery of lipophilic drugs in humans (lidocaine) (*118*) as well as fundamental studies about incorporation of lidocaine in microemulsions systems (*119, 120*).

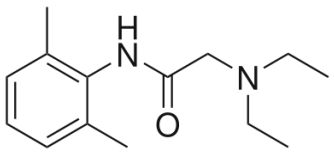


Figure 1.22. Chemical structure of the drug lidocaine.

On the second topic, the cosmetic industry, more and more studies using microemulsions have been appearing due to their advantages over existing conventional formulations. Increasing knowledge of the unique properties of microemulsions (referred previously), such as very small dimensions (nanoscale) of the particles and high thermodynamic stability, contributes to the progress of this technology (*121*). It is also known that it helps in visual appearance and in commercial fields that microemulsions are transparent. This is part of the reasons why they are now being widely investigated for preparing personal care products with superior features such as having improved product efficiency, stability, appearance and minimal irritation (*122*).

Antioxidants have been largely used in dermatological and cosmetic products owing to its property of scavenging free radicals and oxidative agents that form due to many unfavorable conditions to which we are exposed every day. One of the most widely used antioxidants is vitamin E (α-tocopherol). It has been used in O/W microemulsions due to enhanced capability of this system to delivering the antioxidant predominantly in the epidermis or penetrate further, preventing the accumulation in other organs than the skin.

There are already some products on the market, as the case of trademark Agera® Rx, which uses microemulsions formulation, where it includes among other components, vitamin E.

Comparing this formulation with conventional ones, it is claimed that the treatment with microemulsions provides significant protection deep within the skin (*123*), where fibroblasts produce new collagen and elastin thus playing a critical role in wound healing and skin health (figure 1.23).

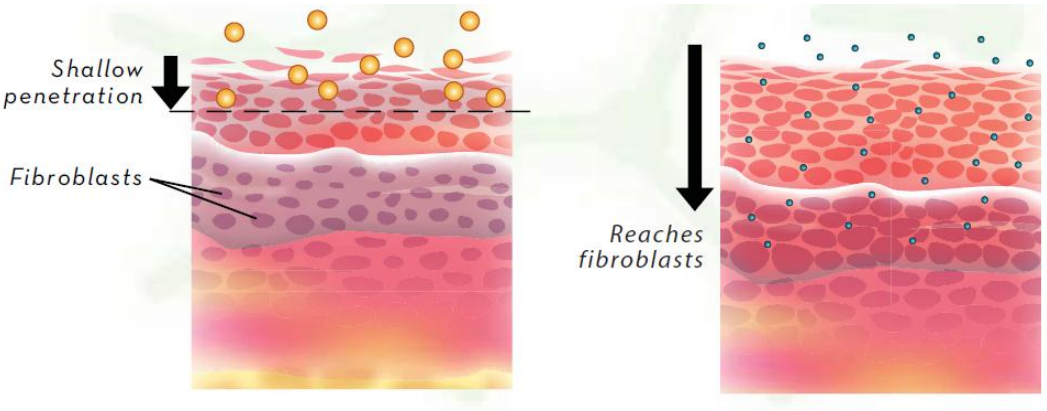


Figure 1.23. Comparison between the effectiveness of large conventional skin care systems (A) and microemulsions formulations (B) (*124*)

Besides vitamin E, other antioxidant compounds have been studied in microemulsions systems, as the case of reveratrol (*125*), lycopene (*88*) and quercetin (*126*) where it was demonstrated that w/o microemulsions increased the skin penetration of quercetin both *in vitro* and *in vivo*, and did not cause skin irritation (*127*).

Regarding the most abundant antioxidant in coffee plant, chlorogenic acid, there are some recent studies with the combination of this polyphenol and microemulsions (*128*). The finding was quite different from previous findings on relatively to hydrophobic polyphenols, once here there was a pretreatment of the skin with chlorogenic acid containing microemulsion and it prevented erythema formation induced by UV irradiation.

Finally it has been seen that when mixing different polyphenols in microemulsions they can exhibit a synergistic effect. As the case of chlorogenic acid with vitamin E (*129*) and ferulic acid with vitamin C and E (*130*), where it was seen the synergistic protection against oxidative stress in skin, and thus this application could be useful for protection against photoaging and skin cancer.

2. Materials and methods

Coffee plant materials and growth conditions

The experiments were carried using 1.5-year-old plants from three different genotypes: Icatu (IAC 2944 - *C. canephora x C. arabica*), Apoatã (*C. canephora cv. Apoatã*) and Obatã (IAC 1669-20 - *Villa Sarchi x Timor Hybrid*). Potted plants were transferred into walk-in growth chambers (10.000 EHHF, ARALAB, Portugal), and maintained with a 12 hours photoperiod, a relative humidity of 65-70% and an irradiance of 750-850 mmol $m^{-2}.s^{-1}$. After the acclimatization period, plants were successively submitted to: a gradual temperature decrease (0.5 ºC/day), from 25/20 ºC to 13/8 ºC (day/night), over 24 days, to allow the expression of acclimation ability; a three day chilling cycle at 13/4 ºC (3x13/4 ºC), where the plants were subjected to 4 ºC during the night and in the first 4 hours of the morning (thus, with light), followed by a gradual rise up to 13 ºC, applied throughout the rest of the diurnal period. After that, plants were allowed to recover from cold for seven days at 25/20 ºC, and then more seven days to recover from drought. In addition to being divided by temperature treatment, plants were also divided into three sets of conditions with different water content (drought treatment): control (no lack of water), mild drought (about 50% water content) and severe drought (about 25% water content). Measurements were made from the two top pairs of recent mature leaves from each branch of 8-10 plants per genotype. Material was frozen in liquid N_2 and stored at -80 ºC until use, and for further analysis, leaves tissue extractions were performed using an ice-cold mortar and pestle.

Phenolic extraction and chromatographic conditions

Leaf samples (about 100 mg fresh weight) were homogenized for 30 minutes (under vigorous shaking, Variomag®Poly15) in 5 mL of a of 70% (v/v) methanol (99,9% Sigma) solution. After filtration (0.45 µm, Millex®, PVDF Durapore) the samples were instantly analyzed through RP-HPLC. For separation, a 100 µL sample was injected in a system with a RP-18, 5 µm column (Purospher® STAR RP-18 endcapped), maintained at 37°C, in a HPLC system (Merck – Hitachi Model D-7000), with autosampler L-7200. L-6200A Intelligent Pump, L-5025 Column Thermostat and Diode Array detector (DAD) L-4500. The elution was performed with methanol and 0.5% trifluoracetic acid (99% Sigma) (w/v) in a gradient grade - 0 to 2 minutes (10% methanol), 2 to 12 minutes (methanol 90% to 60%), 12 to 30 minutes (methanol 60% to 50%), 30 to 35 minutes (methanol 50% to 20%), 35 to 40 minutes (methanol

20%), 40 to 45 minutes (methanol 20% to 90%) and finally 45 to 50 minutes (90% methanol) - using a flow of 0.7 mL min^{-1}. Two extractions were made and analyzed in duplicate.

Data analysis was performed using the D-7000 HSM System Manager - *Chromatography Data Station Software* (version 3.1.1) to integrate the peak areas at any wavelength (200-700 nm). For quantification of some polyphenols, it was used as standards, 5-CQA (≥ 95% Fluka) and Epicatechin (≥ 90% Sigma-Aldrich).

Mass spectrometry conditions

Mass spectrometry was carried out using an HPLC system (Alliance, Waters 2695 Separation Module) with a photodiode detector (DAD, Waters 2996) coupled to mass spectrometer (Micromass Quattro Micro API) with a triple quadrupole (TQ) and an electrospray ionization (ESI) source operating mode negative. The separation was performed on a sample dC 18 column (5µm) 150x21 mm i.d. Atlantis™ (Waters) using a solution of water and formic acid (≥ 96% Sigma-Aldrich) (A) (100:0.5% v/v) and acetonitrile (≥ 99,9% Sigma-Aldrich) (ACN). The flow rate of eluent used was 0.2 mL/min in a gradient grade - 0 to 30 minutes (5% ACN), 30 to 45 minutes (40% ACN), 45 to 47 minutes (90% ACN) and 47 to 60 minutes (5% ACN) and column temperature maintained at 35 °C.

Total phenolic content – Folin-Ciocalteau method

Total phenolic content was determined according the Folin-Ciocalteau colorimetric method (*131*). Briefly, 20 µL of the appropriate dilutions of extracts were added to 1480 µL of distilled water and oxidezed with Folin Ciocalteau reagent (Sigma-Aldrich) (100 µL). The reaction was neutralized with 300 µL of sodium carbonate, and thereafter 30 minutes at 40 ºC of incubation the absorbance of samples was measured at 765 nm in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA). Gallic acid was used as a standard, and the results were expressed as means of triplicates (mg Gallic acid equivalents per g of leaves extract dry weight; mg GAE/g leaves extract dry weight).

Antioxidant activity of coffee leaves extracts and microemulsions – DPPH method

Antioxidant activity of coffee leaves extracts was determined according to the original method (*132*)**,** though being aware of some improvements and changes that have been made regarding this method. For instance: 1) the possible decrease in DPPH concentration was taken into account during the measurements, therefore the control solution was monitored periodically and any decrease in absorbance corrected; 2) instead of waiting a certain time after the mixture of the antioxidant solution and DPPH, the reaction was monitored kinetically by UV/vis spectroscopy until it reached the plateau of the reaction (where it stabilizes), where data was

taken, as suggested by other authors (*133*); 3) Before doing the tests for antioxidant activity in leaf extract and coffee in microemulsions, a range of concentrations of the antioxidant solutions were tested, to avoid the total consumption of the free radical DPPH.

In the case of coffee leaves extracts, 10 µL were added to 1 mL of a 60 µM DPPH (Sigma-Aldrich) solution prepared in methanol, briefly stirred and the kinetics of the reaction was followed at 515 nm in a Shimadzu UV-1800 spectrophotometer (Shimadzu Europe GmbH, Germany), using the data when the reaction stabilized (plateau).

Microemulsion samples preparation and support equipment

A stock solution was made at a fixed ratio of 0.815:1 (surfactant:oil, by volume), to which was added milli-Q water in order to obtain desired various volume fractions (Φ, percentage in volume of surfactant plus oil in solution), Φ = 0.005; Φ = 0.01, Φ = 0.02, Φ = 0.05, Φ = 0.07, Φ = 0.1, Φ = 0.15, Φ = 0.2. Thus the preparation of these many solutions is facilitated and it is as if one worked with a system of only "two components". After the solution is prepared, it was necessary to stir and heat to about 5 ºC above the limit of stability of the microemulsion phase (upper phase boundary), and finally stirring a little while it is cooling down. Microemulsions were prepared in glass tubes (1x10 cm) with Teflon screw caps. Surfactant was, pentaethylene glycol monondodecyl ether ($C_{12}E_5$) (Nikkol BL-5SY); Decane (Sigma-Aldrich) was used as oil and water milli-Q, without any prior treatment to any one of them.

In order to determine the phase diagram, the samples were placed in a water bath reservoir (Lauda® A100), with thermometer and temperature controller (Lauda®), and phase changes were verified by visual inspection. To stabilize the temperature, the water reservoir has four external connections for incoming and outgoing of water, in order to have a continuous flow of water into the reservoir, which causes no significant temperature changes. In order to make an accurate determination of phase boundaries, the temperature variation was done very slowly (from 0.3 °C to 1.5 °C per hour), since the system needs time to stabilize the self-assembly structures. On the determination of the phase changes it was needed to be more accurate by having extra waiting time between the measurements and also to have high reproducibility in determination. A bluish solution means close to phase change, as the micelles scatter more light with increasing size; At temperatures below the microemulsion phase there is white on top (oil), transparent on bottom (water and surfactant in the middle) that corresponds to a phase separation ("L+O") and with no stirring it separates into two phases; Transparent solution, correspond to liquid microemulsion phase ("L_1"); A one-phase solution that is between turbid and opaque (white) means it is in the phase above (in temperature) of the microemulsion,

which can be of type bicontinuous (L_3 or "sponge phase") or lamellar liquid crystalline ("L_α") being the latter one verifiable through observation of glowing when viewed through a polarimeter.

Microemulsions with salt addition

Microemulsions of volume fractions $\Phi = 0.05$ and $\Phi = 0.15$, with salt equivalent of the charge (ionic strength) of 0.5-1% CQA charge, were made. The preparation of these microemulsions was identical to the standard ones, but instead of using pure milli-Q water, a water solution containing NaCl was used.

Microemulsion samples with Lidocaine, 5-CQA and α-tocopherol

Stock solutions of oil plus surfactant were made with 1%, 5% and 10% addition of lidocaine (percentage in the oil by volume) respecting the constant ratio of 0.815:1 (surfactant: oil), to which was added milli-Q water in order to obtain the desired volume fractions (from $\Phi = 0.005$ to $\Phi = 0.2$).

As for microemulsions with 5-CQA added, two batches of solutions were made: one that remained constant concentration of 5-CQA to 1% (by water volume), and another which remained constant the volume fraction ($\Phi = 0.05$ and $\Phi = 0.15$) and varying the addition of 5-CQA in 0.01%, 0.025%, 0.05%, 0.1%, 0.25%, 0.5%, 1% and 2%.

Regarding α-tocopherol addition preparation was similar to lidocaine (once this is added to the oil as well). $\Phi = 0.05$ and $\Phi = 0.15$ solutions with 5% and 10% addition were made. The subsequent procedure treatments for all solutions were the same to that of standard solutions.

Microemulsion samples with 5-CQA, α-tocopherol and DPPH

Regarding the antioxidant activity measurements in microemulsions, two equal volume fraction microemulsion solutions were mixed. DPPH was added to oil (1% in volume, in order to not change the phase diagram) and that concentration took into account the further dilution, as well as for α-tocopherol microemulsions preparation. Microemulsions with 5-CQA added were performed using 5-CQA (Fluka) in water solution, taking into account in concentration further dilutions. 5-CQA and DPPH (Sigma-Aldrich) were weighted in a OHAUS® microbalance, and α-tocopherol (Fluka) in a OHAUS® balance (since it is liquid solution at room temperature). The experiments were made with DPPH as soon as possible to prevent the degradation (reduction), yet once more, a control solution was made to monitor the absorbance

decrease, which was taken into account in calculations of antioxidant activity. Similarly to antioxidant activity in measurements of coffee leaves extracts, here a range of concentrations were tested for not to have a total reduction of free radical DPPH, being these used for the antioxidant activity determination. Kinetics of the reaction was followed at 515 nm in a Shimadzu UV-1800 spectrophotometer, using the data when it stabilized (plateau). Calibration curves were made for systems with compounds in and out of microemulsions, and the calibration curve with the two compounds together, was only possible in a microemulsion system.

Dynamic Light Scattering

Dynamic light scattering measurements were made on a Zetasizer Nano ZS (Malvern Instruments), using a Low volume disposable sizing cuvettes. In the case of zeta potential were used Universal Dip Cell (ZEN 1002) and the Smoluckowski approach model was used for zeta potential. Data was analyzed using the Zetasizer Software version 6.20.

The temperature trend of microemulsions was made using the temperature controller (sensibility of 0.1 ºC) in the machine, and the diffusion, size, viscosity, polydispersity and zeta potential were acquired. Temperature runs were made in triplicate with 0.5 ºC intervals and waiting periods of 6 minutes between the temperature change. An essential step in this determination is to confirm the correct temperature of the microemulsion at the beginning of the measurement. Since it is not possible to use a thermometer inside, several measurements at the first temperature were made, until obtain a high reproducibility of diffusion values and only then began the temperature trend.

3. Results and discussion

3.1 Identification of polyphenols

Chromatograms were followed at 325 and 280 nm, as these are the characteristic maximum wavelengths of polyphenolic compounds. Phenolic acids with the benzoic acid carbon framework have their maximum wavelength in the 230 to 280 nm range, but it can also extend to about 325 nm, due to the additional conjugation (*134*), as in chlorogenic acids subclass. Nevertheless, other maximum wavelengths which appeared on the peaks were also analyzed due to the coupling of HPLC to a diode array detector, multi-wavelength detector.

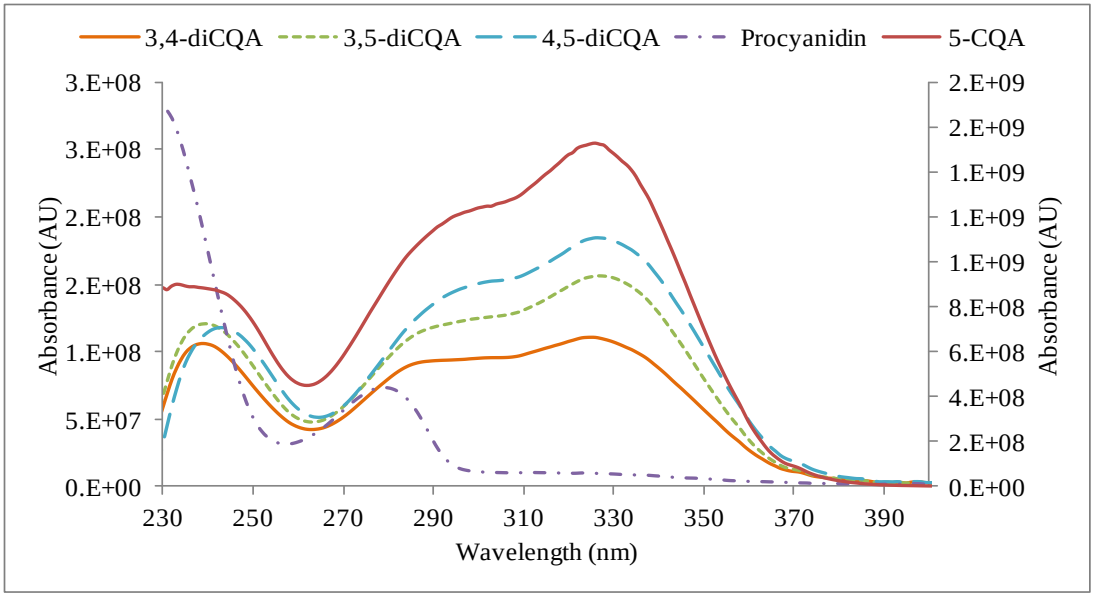


Figure 3.1 Representative spectra of some phenolic compounds. 5-CQA is represented on the y-axis on the right, and the rest of the compounds on the left. The remaining identified compounds have very similar spectra to 5-CQA, maximum at about 325 nm or only at 280 nm (as procyanidin).

The chromatograms in figure 3.2 show the peaks corresponding to the phenolic compounds present in the coffee leaves extract. Since the spectra of the analyzed compounds (figure 3.1) are very similar (belong to the same family), in order to achieve a more effective identification, mass spectrometry analysis was also used.

First standard addition was tried to help identification of the peaks, however the risk of having overlapped peaks became an additional error in identification, whereas with mass spectrometry a mass-to-charge ratio of charged particles is measured, thus the identification of a particular peak is more accurate (tables 3.1, 3.2 and 3.3).

Since the results involve three coffee plants genotypes, each one subjected to six and three conditions of temperature and water content (54 values), respectively, the chromatograms below are representative.

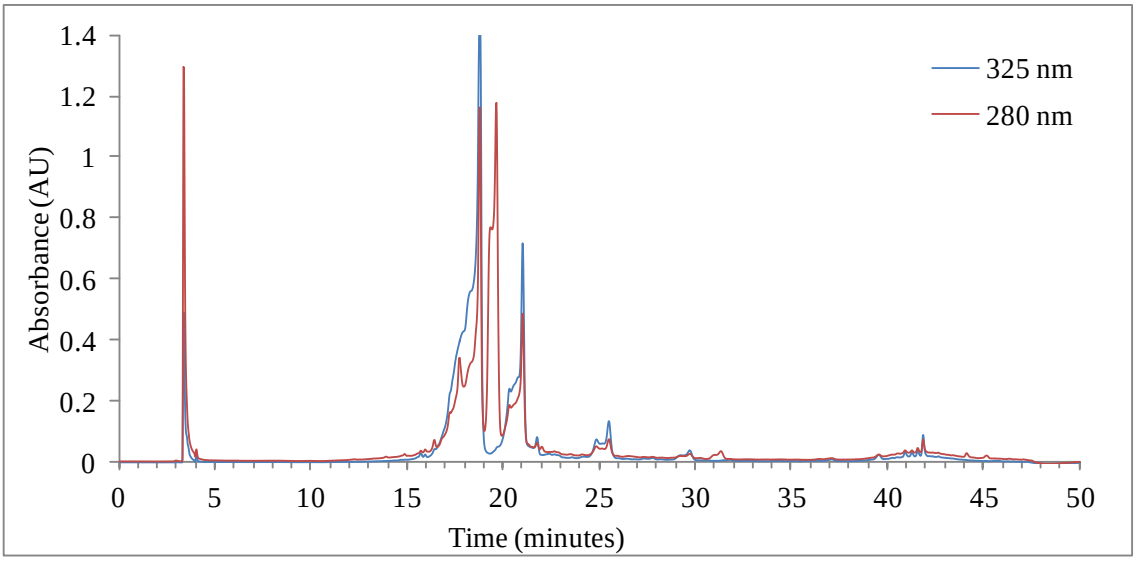


Figure 3.2 Chromatogram of extract of Icatu leaves at 280 nm and 325 nm. This is a representative chromato gram of this variety, and particularly corresponds to Control (Ctr) condition of water content and 25/20 ºC of temperature.

Table 3.1 Identification of phenolic compounds for Icatu with, retention time (RT), maximum wavelength (λ_{max}) and mass of predominant precursor ions and fragments ([M-H]). Underlined values correspond to the compound identification. The remaining values were also obtained but in smaller amounts and have not yet been identified.

RT(minutes)	λ_{max}				Identification	[M- H-]		
17.71			285	230	Catechin	**289**	641	407
18.77		325	287	233	5-CQA	**353**	707	191
19.63			279	230	Procyanidin	577		
20.99	363	317	257	232	Mangiferin	**421**	289	
21.73		324	283	235	5-FQA	367		
24.83		325	285	240	3,4-diCQA	515		
25.49		327	287	242	3.5-diCQA	515		
29.68		325	285	244	4.5-diCQA	515		
31.31			281	230	unidentified	641		
37.09		316	274	246	unidentified	541		
39.55		316	282	245	quercetin 3-O-rhamnoside	**447**	543	

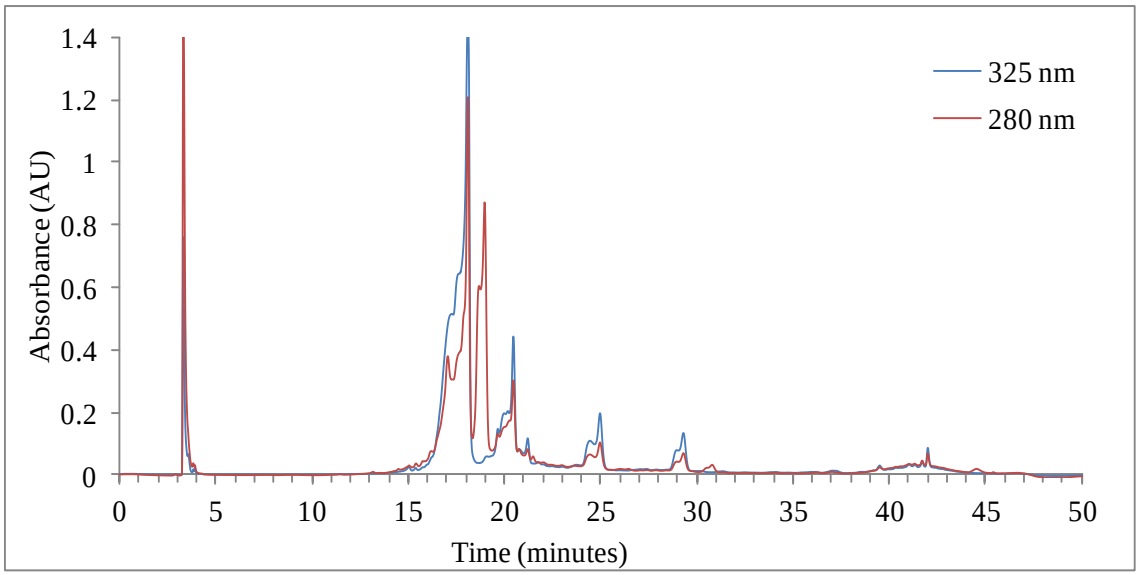


Figure 3.3 Chromatogram of extract of Obatã leaves at 280 nm and 325 nm. This is a representative chromatogram of this variety, and particularly corresponds to Ctr condition of water content and 25/20 ºC of temperature.

Table 3.2 Identification of phenolic compounds for Obatã with, retention time (RT), maximum wavelength (λ_{max}) and mass of predominant precursor ions and fragments ([M-H]). Underlined values correspond to the compound identification. The remaining values were also obtained but in smaller amounts and have not yet been identified.

RT (minuts)	**λ_{max}**				**Identification**	**[M- H-]**	**fragments**	
17.71			285	230	Catechin	**289**	641	407
18.77		325	287	233	5-CQA	**353**	707	191
19.63			279	230	Procyanidin	577		
20.99	363	317	257	232	Mangiferin	**421**	289	
21.73		324	283	235	5-FQA	367		
24.83		325	285	240	3,4-diCQA	515		
25.49		327	287	242	3.5-diCQA	515		
29.68		325	285	244	4.5-diCQA	515		
31.31			281	230	unidentified	641		
37.09		316	274	246	unidentified	541		
39.55		316	282	245	quercetin 3-O-rhamnoside	**447**	543	

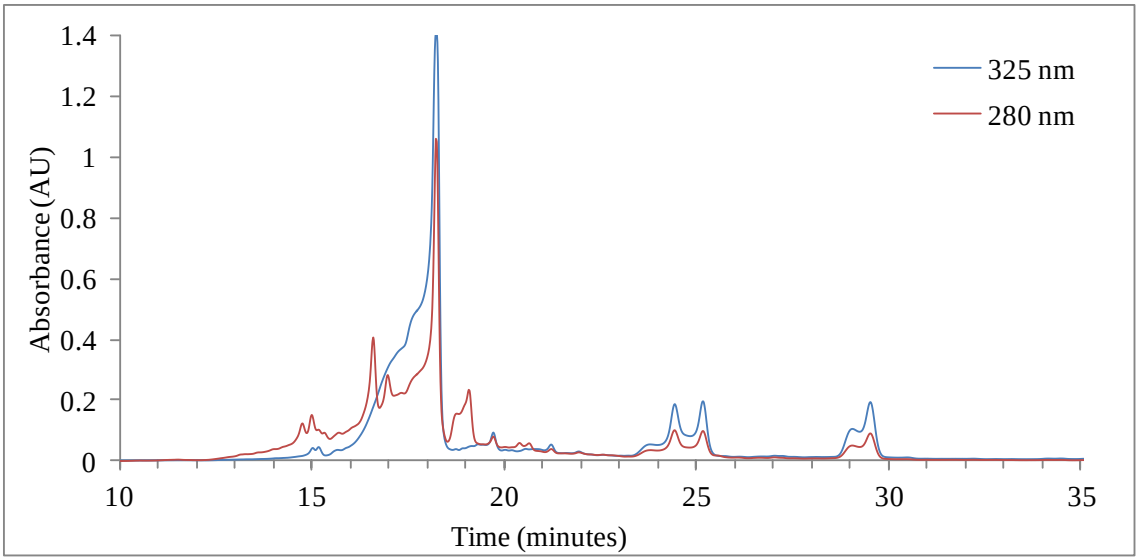


Figure 3.4 Chromatogram of extract of Apoatã leaves at 280 nm and 325 nm. This is a representative chromatogram of this variety, and particularly corresponds to Ctr condition of water content and 25/20 ºC of temperature.

Table 3.3 Identification of phenolic compounds for Apoatã with, retention time (RT), maximum wavelength (λmax) and mass of predominant precursor ions and fragments ([M-H]). Underlined values correspond to the compound identification. The remaining values were also obtained but in smaller amounts and have not yet been identified.

RT (minuts)	λmax			Identification	[M- H-] fragments		
16.59		279	230	catechin	289		
16.91		279	230	procyanidin	577		
18.21	325	287	233	5-CQA	**353**	707	191
19.07		279	230	Procyanidin	577		
19.71	317	281	230	catechin	289		
21.20	324	283	235	5-FQA	367		
24.43	325	285	240	3,4-diCQA	515		
25.17	327	287	242	3.5-diCQA	515		
29.52	325	285	244	4.5-diCQA	**515**	455	

Analyzing the chromatograms of the three coffee leaves extracts (figures 3.2, 3.3 and 3.4), it is seen that they share many of the major compounds. More particularly, Icatu and Obatã are qualitatively more similar to each other, with Apoatã as the one that differs most. This is due to the fact that Icatu and Obatã are hybrids of *C. arabica* with *C. canephora*, but often backcrossed with arabica, so that they are almost assumed as an arabica variety (this is even more valid for Icatu rather than Obatã), while Apoatã is a variety of *Coffea canephora*, thus not having any contribution from *C. arabica.*

The major compound in either case is undoubtedly the 5-CQA, it can go from 13% to 22% of total phenolic content and from 1,9% to 2,3% of dry weight leaf content, depending on coffee genotype (Icatu, Obatã or Apoatã).

Compounds that are present in greater amount after 5-CQA are: 3,4-diCQA, 3.5-diCQA, 4.5-diCQA, 5-FQA, catechin and procyanidin. Both the procyanidin and catechin have yet not been identified as to its isomer ((+)- catechin or (-) - epicatechin; proantocyanidin B1, B2, C1, C2, among others). For future work, the identification would be performed in case they show to have an important role in the response to stresses imposed after analysis.

As a novelty in coffee and in large quantity, another compound identified, was mangiferin. This polyphenol is classified as a xanthone, appearing in greater amounts in mangoes (hence its name) and as mentioned before in the introduction chapter, has several properties, especially antidiabetic (*72*). There is only one reference to the existence of mangiferin on coffee, and yet it is in a wild coffee variety (*71*), not very well documented and besides, there they have not studied how this polyphenol varied with stress or even within other coffee varieties. In contrast, here it was found that mangiferin may be a biomarker compound for distinguishing *Coffea arabica* from *Coffea canephora*, since it was only present in Icatu and Obatã, and no significant content was found in Apoatã.

In addition, the presence of other compounds found in very low amounts were verified (not quantified, since the signal to noise ratio was not very favorable for further analysis), including: 5-pCoQA and rutin. Although we have not analyzed the differences of these polyphenols with the treatments of temperature and water content, it does not mean they cannot play an important role in coffee plant defenses from oxidative stress; however this was done in order to not analyze results with very large errors. A future analysis could be to detail the changes of these compounds in minority (however this would require new optimizations methodology).

3.2 Response of polyphenols to drought and cold treatments

It is always difficult to state that a particular response (increase, decrease or maintaining) of a certain polyphenolic compound is due to a particular imposed stress. In most cases, a response depends on a combination of several factors. Thus total phenolic content of coffee leaves will be evaluated in the first place in order to have a general idea of its common response to the imposed stresses.

Data will be analyzed by doing a generalization of the response of the coffee plants using three temperature phases: acclimatization period (from 25/20 ºC to 13/8 ºC), chilling (for 3x13/4 ºC), and recovery phases (recovery from cold and recovery from drought); and three water content conditions: control (Ctr), mild drought (MD) and severe drought (SD).

3.2.1 Total phenolic content (TPC)

Before starting the analysis it is important to keep in mind that robusta varieties developed in a typical equatorial climate, with abundant rainfall distributed to a period of ten months and enough wetness, while arabica evolved in a region marked by dry seasons (until five months) and low air humidity (*21*).

A calibration curve with gallic acid was performed to quantify the TPC (figure 3.5).

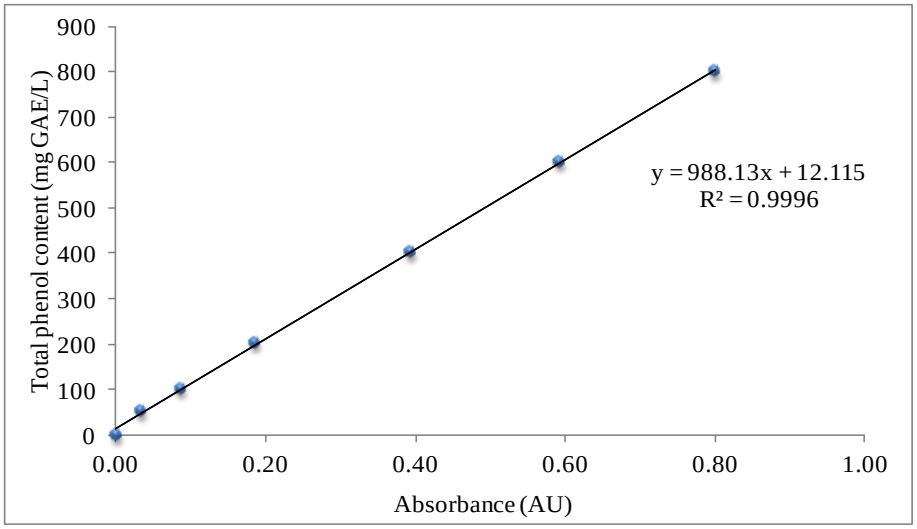


Figure 3.5 Calibration curve, total phenolic content (mg with gallic acid equivalents per liter) versus absorbance (at 765 nm).

Values of TPC (in mg GAE/L), were converted to mg of gallic acid equivalents per g (dry weight) of leaves extract (mg GAE/g leaves extract), through dry weight:fresh weight ratios.

3.2.2 Individual responses of the three coffee genotypes

Icatu

Analyzing the responses of phenolic compounds regarding the cold treatment (table 3.4), a general response was observed, which comprises an initial decrease in the amount of TPC and then an increase from 13/8 °C (final phase of acclimation) to 3x13/4 °C (chilling), regardless the drought treatment (water content).

As for the drought treatment, the amount of phenolic compounds increases from the control condition to mild drought, and have the lower values at severe drought.

Table 3.4 – Total phenolic content of coffee leaves changes regarding cold and drought treatments for Icatu genotype. Data is given in percentage of total phenolic content (per leaves in dry weight) and each value is the mean of triplicate measurements and the respective standard deviation (SD). "Rec cold" = recovery from cold and "rec drought" = recovery from drought.

Cold treatment/Drought treatment	Icatu - Total phenolic content % per coffee leaves					
	Severe drought	SD	Mild drought	SD	Control	SD
25/20 °C	9.85	± 0.68	15.29	± 1.05	13.38	± 0.92
18/13 °C	4.28	± 0.29	10.78	± 0.74	6.95	± 0.48
13/8 °C	10.18	± 0.70	16.73	± 1.15	10.71	± 0.74
3x13/4 °C	11.60	± 0.80	14.04	± 0.97	13.52	± 0.93
rec cold	5.50	± 0.38	11.75	± 0.81	10.33	± 0.71
rec drought	8.66	± 0.60	12.46	± 0.86	9.72	± 0.67

A better and more general visualization of the response profile of phenolic compounds can be ascertained from the graphic in figure 3.6.

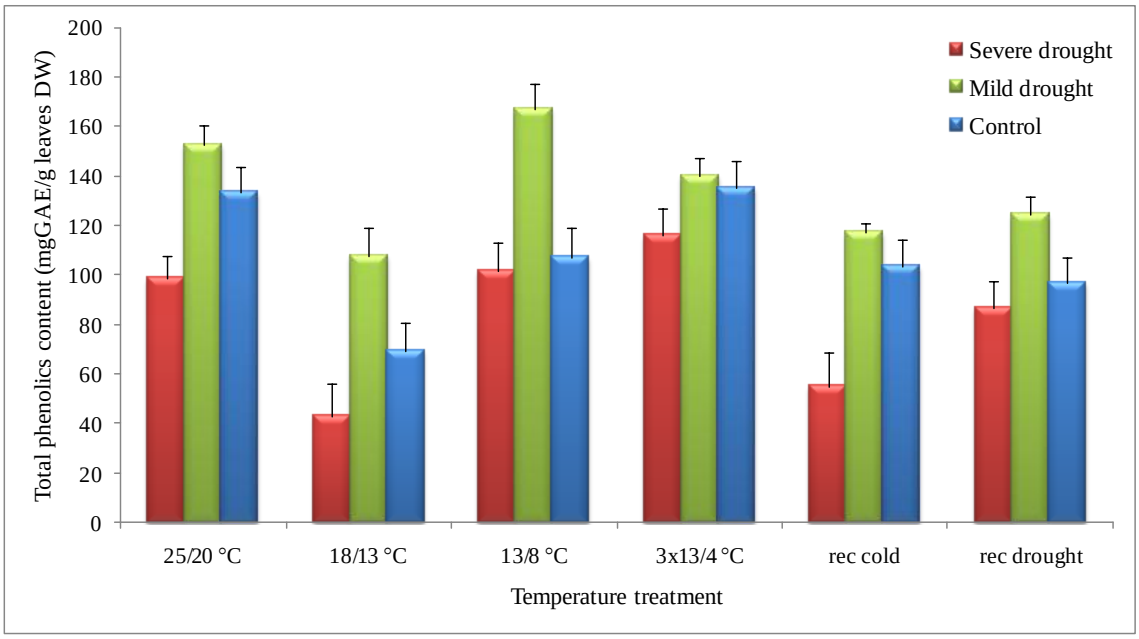


Figure 3.6 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Icatu coffee leaves. Three conditions of water content severe drought, mild drought and control are represented in red, green and blue, respectively. Rec cold = recovery from cold; rec drought = recovery from drought.

By doing a more detailed analysis, it is seen that during acclimatization period (25/20 °C to 13/8 °C), there is a decrease of TPC followed by an increase to near the initial values, indicating that the plant probably recognizes the drop in temperature but the phenolic response only begins at 13/8 °C. In particular, the plant in severe drought condition reveals a major difference in this decrease (about 56%), meaning that here the stress to the plant might be greater due to the "multiple" (double) stress conditions, cold plus drought.

Under chilling, the values are getting close to the initial ones (25/20 °C), or in some cases even higher (in severe drought and control). Upon the recovery phase, there is a slight decrease and finally stabilization in recovery from drought to around the initial values. This way Icatu generally shows that it is able to respond to the cold stress to which it is submitted but not so good to the drought stress (from the polyphenolic point of view).

In general, TPC of Icatu coffee leaves ranged from 167.3 and 43.8 mg GAE/g leaves dry weight (particularly to the conditions of MD - 13/8 ° C and SD - 18/13 ° C, respectively). These amounts of TPC, are in agreement with the observations made by other authors (*63*, *135*), who also found this range of concentrations of TPC in coffee leaves.

Icatu seems to be resistant to cold, although somewhat sensitive to drought, since the values increase from control to mild drought, meaning that the oxidative stress control mechanisms were triggered. However, at severe stress condition, the plant might have started to be unable to respond so straightforwardly, most likely because it was getting more and more injured.

Phenolic compounds were found to be involved in the oxidative stress response, in this case caused by cold and drought in this coffee genotype, since their concentrations varied, according to the response profile of the plant in the more critical conditions.

Obatã

Analyzing the values for TPC in Obatã genotype (table 3.5 and figure 3.7), it appears that with temperature treatment they increase gradually from the beginning until the recovery from drought phase, and decrease in the last condition to (about) the initial levels. Regarding different water content conditions (drought), it is seen that, the differences between them are not as relevant as in Icatu case, although being in average higher in control and mild drought followed by severe drought.

Table 3.5 Total phenolic content of coffee leaves changes regarding cold and drought treatments for Obatã genotype. Data is given in percentage of total phenolic content (per leaves in dry weight) and each value is the mean of triplicate measurements and the respective standard deviation (SD). "Rec cold" means recovery from cold and "rec drought", recovery from drought.

Cold treatment/Drought treatment	Obatã - Total phenolic content % per coffee leaves					
	Severe drought	SD	Mild drought	SD	Control	SD
25/20 °C	6.36	± 0.44	8.12	± 0.56	6.69	± 0.46
18/13 °C	9.19	± 0.63	6.72	± 0.46	12.99	± 0.90
13/8 °C	12.18	± 0.84	9.39	± 0.65	9.42	± 0.65
3x13/4 °C	8.71	± 0.60	10.59	± 0.73	10.25	± 0.71
rec cold	12.17	± 0.84	16.83	± 1.16	11.97	± 0.82
rec drought	8.94	± 0.61	8.48	± 0.58	12.93	± 0.89

In a more particular approach, it is seen that there is a tendency for gradual increase of TPC during both the acclimatization phase and in the chilling (25/20 ° C to 3x13/4 ° C), lasting

until the recovery from cold condition. There is in fact an increase of the TPC of about 50%, for all drought conditions from 25/20 °C to "rec cold". Finally in the recovery from drought condition there is decrease to almost the initial values.

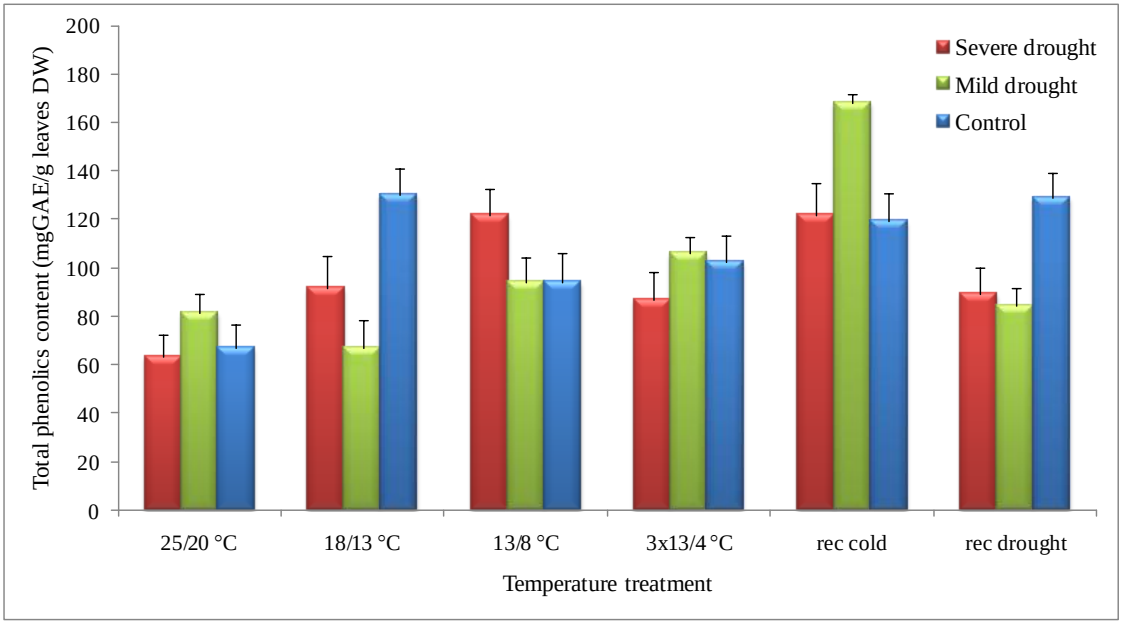


Figure 3.7 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Obatã coffee leaves. Three conditions of water content severe drought (SD), mild drought (MD) and control (Ctr) are represented in red, green and blue, respectively. Rec cold = recovery from cold; rec drought = recovery from drought.

When comparing the drought treatments, there is not a clear difference between the three conditions, which may imply that Obatã genotype is able to respond to this stress, and also more efficiently than Icatu. On the contrary, regarding cold, there is a more pronounced increase of TPC when lowering the temperature, meaning that Obatã is suffering a greater stress with low temperatures.

Once again by analyzing the changes in TPC profile, we can assess indirectly the situation of the plant (if more or less injured), indicating that phenolic compounds seem to be indeed involved in protecting the plant. Whether they act first, after or during the main mechanisms (e.g. enzymatic regulation) it is not known. That work is being evaluated in parallel to this, and is not yet published.

In overall, TPC of Obatã ranged from 168,4 and 63,6 mg GAE/g leaves dry weight (in particular to the conditions of mild drought – recovery from cold and severe drought – 25/20 ° C) respectively, therefore similar to those found in Icatu.

Apoatã

On the present variety, it is immediately noticed that the TPC, is much higher compared with Icatu and Obata (table 3.6). This is related to the fact that they belong to different species. Thus, total phenolic content becomes a differentiating factor between the two species, meaning that the *C. canephora* species (as Apoatã) are richer in phenolic compounds than *C. arabica* species (as Icatu and Obatã). The same type of results have been published by other authors (*42*, *136*)

Table 3.6 Total phenolic content of coffee leaves changes regarding cold and drought treatments for Apoatã genotype. Data is given in percentage of total phenolic content (per leaves in dry weight) and each value is the mean of triplicate measurements and the respective standard deviation (SD). "Rec cold" means recovery from cold and "rec drought", recovery from drought.

Cold treatment/Drought treatment	Apoatã - Total phenolic content % per coffee leaves					
	Severe drought	SD	Mild drought	SD	Control	SD
25/20 °C	13.03	± 0.9	11.28	± 0.78	14.00	± 0.96
18/13 °C	19.28	± 1.33	16.41	± 1.13	16.37	± 1.13
13/8 °C	15.99	± 1.1	14.71	± 1.01	17.61	± 1.22
3x13/4 °C	16.16	± 1.11	9.79	± 0.67	15.60	± 1.08
rec cold	19.54	± 1.35	5.23	± 0.36	15.92	± 1.10
rec drought	15.40	± 1.06	9.99	± 0.69	14.01	± 0.97

It is seen that the TPC values are higher in severe drought condition compared with control and mild drought. This difference may indicate that there was trigger response regarding water content and therefore polyphenols may have a role in defense against this stress here. Regarding temperature conditions, it appears that there is (in general) an increase of TPC during acclimatization phase, but a gradual decrease during chilling, and recovery from cold and from drought phases (better seen in figure 3.8). This analysis, can be used to support results obtained by other authors (*137*) about Apoatã being more sensitive to cold, since no significant increase with decreasing temperature was seen.

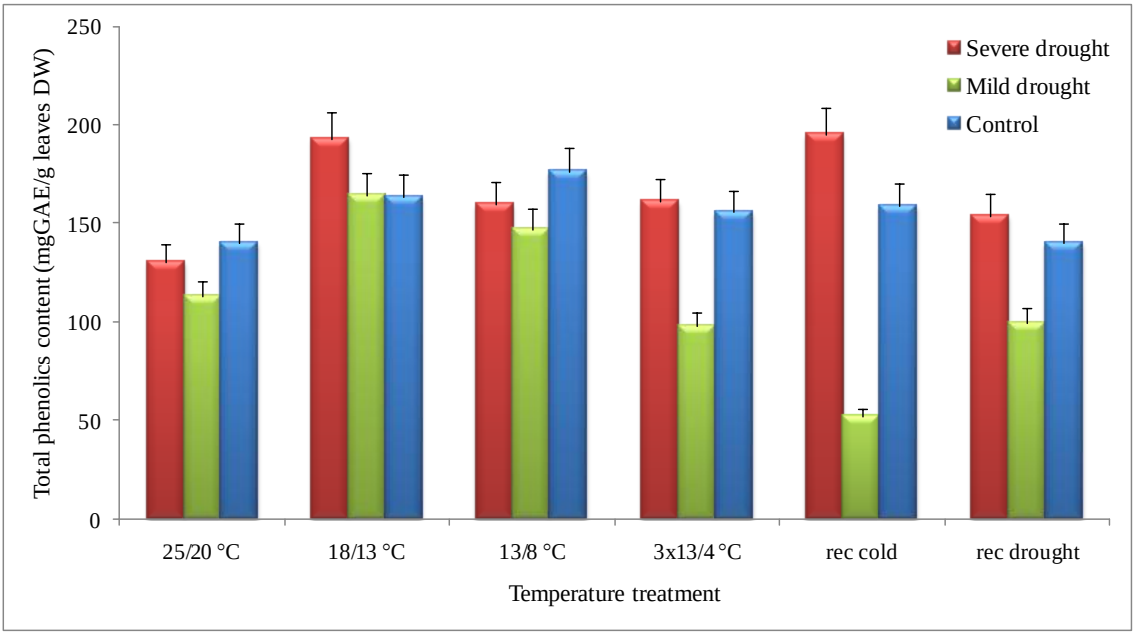


Figure 3.8 Total phenolic content (mg GAE/g leaves dry matter) versus temperature treatment for Apoatã coffee leaves. Three conditions of water content severe drought (SD), mild drought (MD) and control (Ctr) are represented in red, green and blue, respectively. Rec cold = recovery from cold; rec drought = recovery from drought.

Apart from the values for mild drought conditions (where the plant visually demonstrated severe damage), it is seen that the total phenolic content in control and severe stress conditions do not vary much with the temperature treatment.

Higher values of TPC are seen in severe stress condition, at 18/13 °C and recovery from cold. When compared to the initial temperature condition it corresponds to an increase of about 33% for both. Therefore, by not having a noticeable phenolic response this suggests that Apoatã has less efficient control of oxidative stress induced by cold, which is consistent with their greater cold sensitivity previously reported (*137*). In the case of mild drought, it is verified that the defense of the plant was triggered during the acclimation period, however it did not resist and started to get so damaged, that possibly the biosynthetic pathways (phenylpropanoid) were not a priority in that case. Yet it appears that in the last condition values increase again, which may mean that the plant could have been able to recover from the stress induced.

With regard to the amount of TPC in Apoatã, values ranged from, 195,4 and 52,3 mg GAE/g leaves dry weight, although this is the value for mild drought – recovery from cold condition, the lower value without counting with this one, was 97,9 mg GAE/g leaves dry weight in mild drought – 3x13/4 ° C (to the conditions of severe drought – recovery from cold and mild drought – recovery from cold).

The conclusions regarding the greater or lesser sensitivity to both stresses imposed were supported by visual observation of plants, i.e., the yellowing and rolling of leaves, the plant showing more or less wilt, in cases where plants were more damaged.

The difference in the triggering of antioxidative profiles supports the hypothesis of its importance to cold (and photoinhibition) tolerance in *Coffea sp*, and could therefore provide a useful probe to identify tolerant genotypes.

Some earlier publications (*9, 137, 138*), have shown that there is some evidence that the genotype Icatu is more resistant to cold and Apoatã more sensitive to cold. Yet there are numerous studies that focus on other cold stress regulators, such as Chloroplastic Cu, Zn-SOD, APX, ascorbate, α-tocopherol, ROS quantification (hydrogen peroxide, hydroxyl radical), transcription factors and cold-regulated genes, which show for example that the stomatal aperture in Apoatã is less responsive to decreasing water availability than in arabica genotypes, and that is more sensitive to cold (*9*).

3.3 Antioxidant activity of coffee leaves extracts (DPPH method)

Antioxidant activity of extracts of coffee leaves was determined, by a matter of realizing that the phenolic profile of plant response to induced stresses was in agreement to the antioxidant activity of the coffee leaves extracts, and results were expressed in terms of percentage of DPPH reduction by coffee leaves extracts (figures 3.9, 3.10 and 3.11).

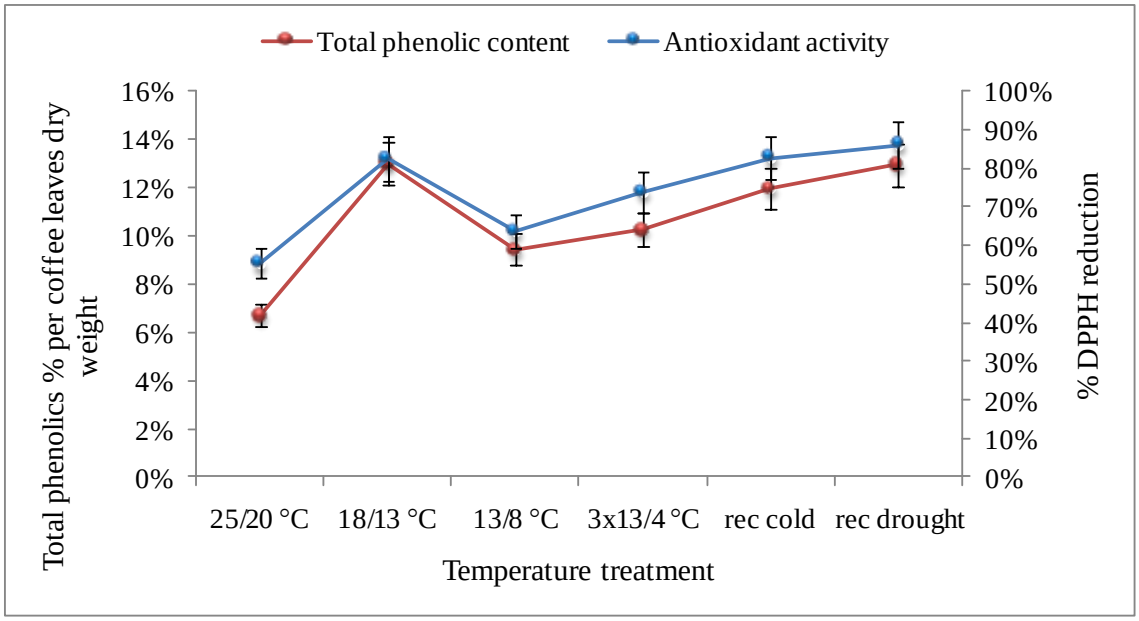


Figure 3.9 Total phenolic content (percentage per leaves in dry weight) and antioxidant activity (percentage DPPH reduction) versus temperature treatment, for Obatã genotype in Control condition of drought.

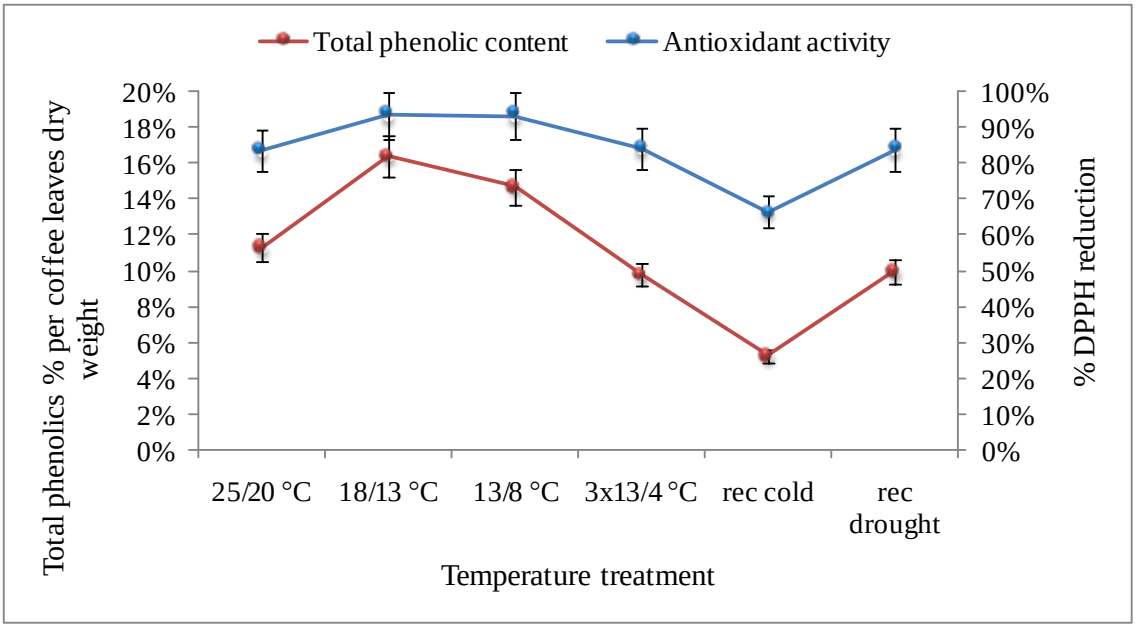


Figure 3.10 Total phenolic content (percentage per leaves in dry weight) and antioxidant activity (percentage DPPH reduction) versus temperature treatment, for Apoatã genotype in mild drought condition.

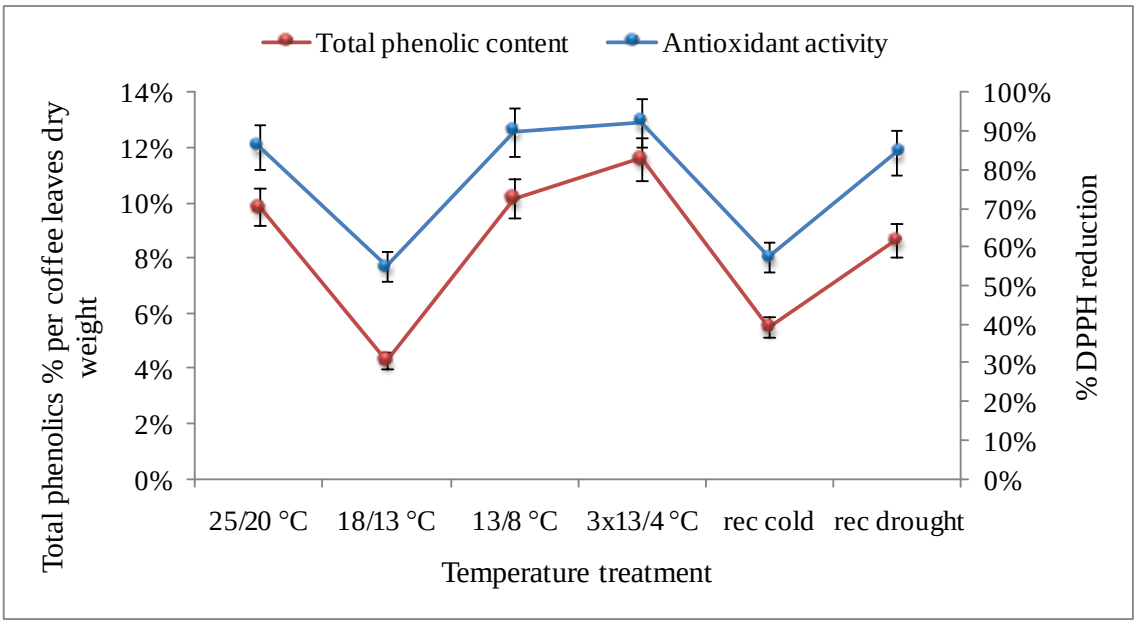


Figure 3.11 Total phenolic content (percentage per leaves in dry weight) and antioxidant activity (percentage DPPH reduction) versus temperature treatment, for Icatu genotype in severe drought condition.

The graphics of the figures above are representative of the antioxidant activity determined for extracts of coffee leaves.

Antioxidant activity was analyzed for all imposed conditions, however, since there were no significant variations when comparing the total phenolic content profiles with the antioxidant activity (graphics from figures 3.9, 3.10 and 3.11), it is concluded that there was no contamination (for example with other antioxidants) in the extracts.

3.4 Quantification some phenolic compounds in coffee leaves

Phenolic compounds identified in the coffee leaves extracts were quantified and their particular response profiles were compared with the TPC profile obtained by Folin-Ciocalteau, in order to analyze any possible individual response.

Quantification was performed by peak area and comparison with 5-CQA as standard. It was possible to quantify the isomers of chlorogenic acids individually using its molar absorptivity, according to the equation 3.1 previously used by other authors (*47*)

$$C = \frac{RF.\varepsilon(1).M(2).A}{\varepsilon(2).M(1)}, \quad equation\ 3.1$$

where, C is the concentration of the isomer in mg/ml; RF is the response factor of 5-CQA standard (mg.ml^{-1}/area); ε(1) is the molar absorptivity of 5-CQA; ε(2) is the molar absorptivity of the isomer in question; M(1) is the molecular mass of 5-CQA; M(2) is the molecular mass of the isomer in question and A is the area of the peak corresponding to the isomer in question.
Molar absorptivities (x10^4 mol^{-1}.cm^{-1}) are as follows (at 325 nm): 5-CQA = 1.95; 3.4-diCQA = 3.18; 3.5-diCQA = 3.16; 4.5-diCQA = 3.32; 5-FQA = 1.93.

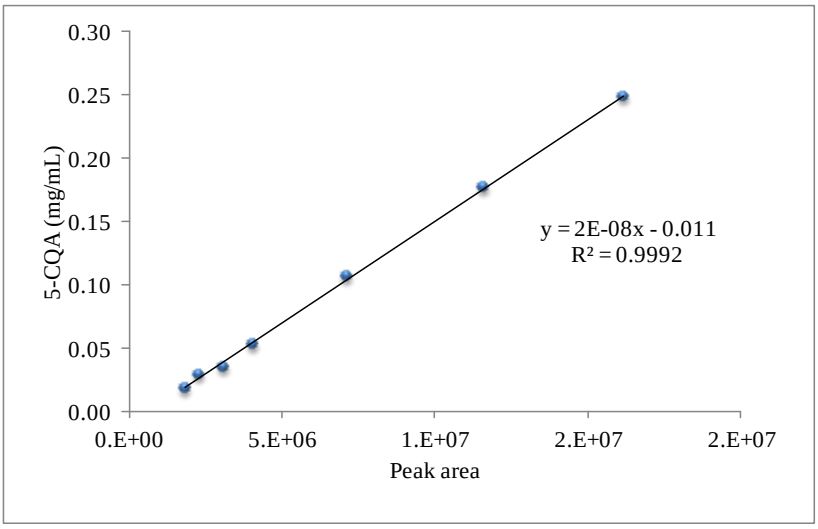


Figure 3.12. Calibration curve of 5-CQA at 325 nm. Area of the peak versus 5-CQA concentration (mol/dm^3).

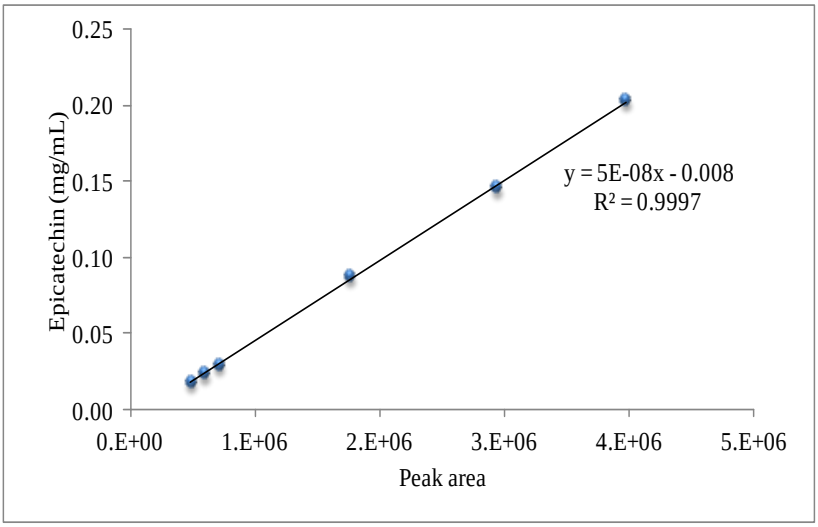


Figure 3.12. Calibration curve of epicatechin at 275 nm. Area of the peak versus epicatechin concentration (mol/dm^3).

5-CQA

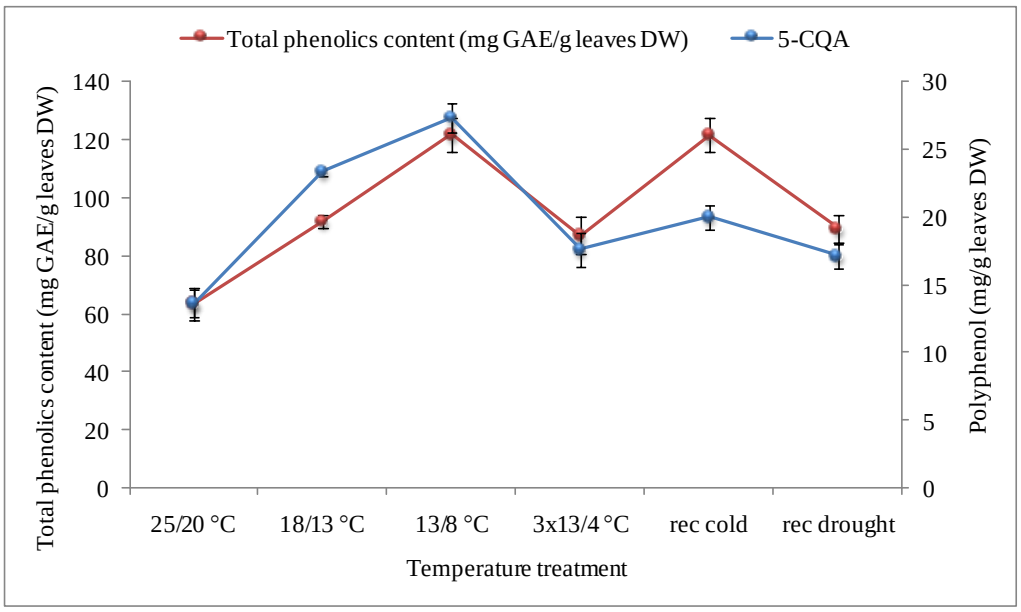


Figure 3.13 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Icatu (at severe drought)

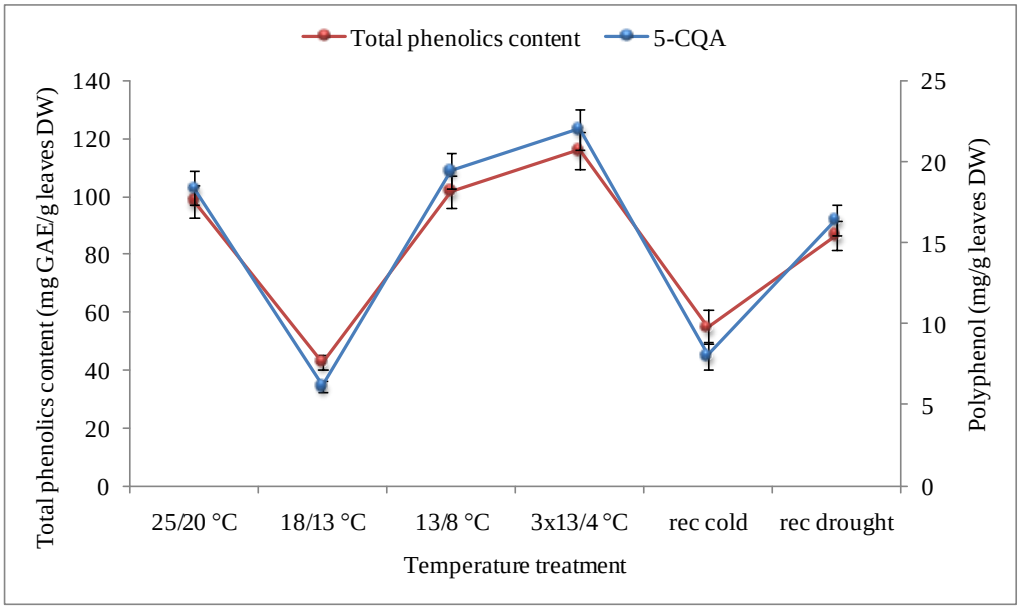


Figure 3.14 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Obatã (at severe drought).

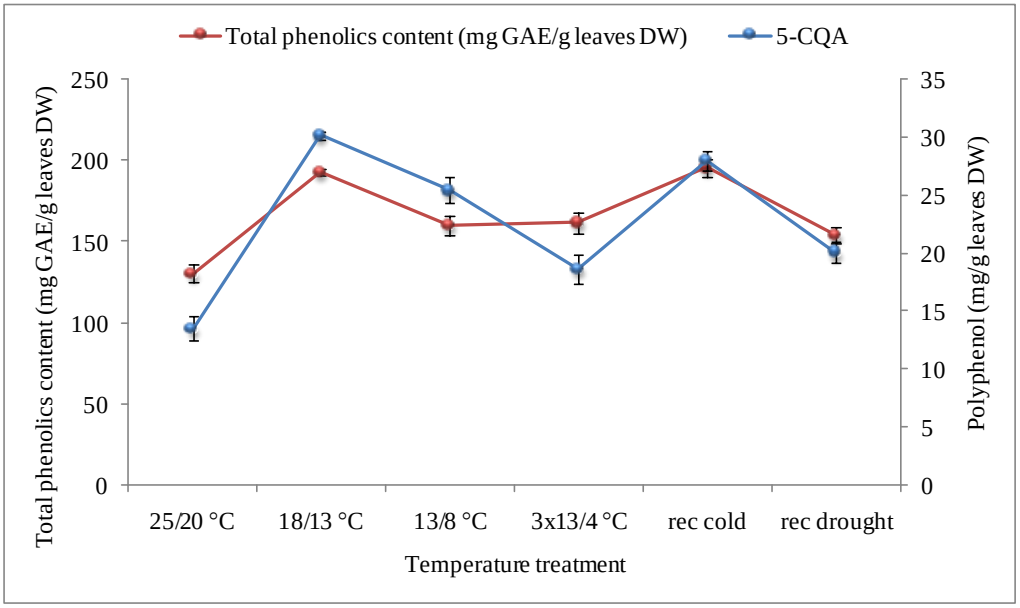


Figure 3.15 Comparison between 5-CQA and total phenolic content profile in response to cold treatment in Apoatã (at severe drought).

Regarding 5-CQA, no major differences were observed when compared to the profile of TPC, three coffee genotypes.

As the data is very extensive, and, there are still other drought conditions to represent, the graphics from figures above are representative for all conditions, since no major differences between the profiles of TPC and 5-CQA were observed.

Table 3.7 5-CQA content in coffee leaves (mg/g dry weight leaves). Each value is the mean of the six temperature treatments thus, higher and lower (H/L) values are represented as well for each water content condition with the respective standard deviation.

Water content/Genotype	Icatu	H/L	Obatã	H/L	Apoatã	H/L
Control	28.0 ± 1.9	39.7 ± 2.7 18.4 ± 1.2	24.5 ± 1.7	32.3 ± 2.2 16.9 ± 1.1	21.6 ± 1.5	25.2 ± 1.7 13.6 ± 0.9
Mild drought	25.8 ± 1.7	31.7 ± 2.2 21.3 ± 1.4	19.4 ± 1.3	26.6 ± 1.8 12.3 ± 0.9	12.4 ± 0.8	21.5 ± 1.4 1.9 ± 0.1
Severe drought	15.0 ± 1	22 ± 1.5 6.1 ± 0.4	19.8 ± 1.4	27.3 ± 1.8 13.6 ± 0.9	22.6 ± 1.5	30.1 ± 2 13.4 ± 0.9

Since the detailed analysis of TPC has been made before (in section 3.2) and knowing that the response profile of the identified compounds is consistent with the TPC, there is no need to detail again for all the identified polyphenols, thus the quantitative analysis will be summarized.

Analyzing the table 3.7 it can be seen that the quantity (in average) of 5-CQA decreased since the control until severe drought in Icatu and Obatã, but regarding Apoatã although has decreased in mild drought (because the plant was heavily damaged) it has increased from control to severe drought. These results are consistent with those already analyzed regarding TPC, that is, Icatu and Obatã are more sensitive to drought, but on the other hand Apoatã seems to be more resistant. This conclusion is in terms of phenolic analysis, once there is a triggered response and consequently an increase of 5-CQA from control to severe drought.

It is also seen that the genotypes that are richer in 5-CQA are: Icatu, Obatã and Apoatã, in descending order. However, particularly in severe drought, Apoatã is richer in this polyphenol (30.1 mg/g 5-CQA dry weight leaves). The previous analysis of 5-CQA is representative for the other identified compounds, because once again, the stress responses for the remaining polyphenols were as in agreement with the general response of TPC as 5-CQA.

For the remaining compounds, in order to have an overview of the amount present in leaves of three genotypes, the table 3.8 presents the average quantities in the three different genotypes.

Table 3.8 Average of the total values (mg/g leaves dry weight) of the remaining phenolic compounds identified for the three water content conditions and for three genotypes. There are also represented the highest and lowest values of total values, since this is a mean of many values.

			Higher	Lower
3,4-diCQA	Icatu	**0.60**	1.50	0.18
	Obatã	**0.76**	1.58	0.41
	Apoatã	**2.76**	7.20	0.69
3.5-diCQA	Icatu	**0.04**	0.10	0.02
	Obatã	**0.06**	0.13	0.02
	Apoatã	**0.12**	0.28	0.04
4.5-diCQA	Icatu	**0.010**	0.019	0.004
	Obatã	**0.023**	0.080	0.009
	Apoatã	**0.15**	0.36	0.04
5-FQA	Icatu	**0.036**	0.062	0.020
	Obatã	**0.058**	0.086	0.033
	Apoatã	**0.02**	0.03	0.01
Epicatechin	Icatu	**4.51**	8.92	0.85
	Obatã	**5.53**	11.12	1.33
	Apoatã	**2.61**	4.70	0.13

In a brief analysis, it appears that Apoatã has a greater amount of diCQA isomers than Icatu and Obatã. On the other hand, Icatu and Obatã are richer regarding the flavonol epicatechin. The 3,4-diCQA value of 7,2 mg/g in Apoatã is referred to the recovery from cold phase (in severe drought), indicating that this isomer may have a more relevant role in responses to this type of stress in this genotype.

In fact, regarding the response profiles, most of the phenolic compounds identified showed no major changes compared to the TPC, including 3,4-diCQA, 3.5-diCQA, 5-FQA, mangiferin and procyanidin. However there was a case where this similarity was not so linearly observed, indicating that probably an individual response was triggered by this polyphenol in particular - 4.5-diCQA.

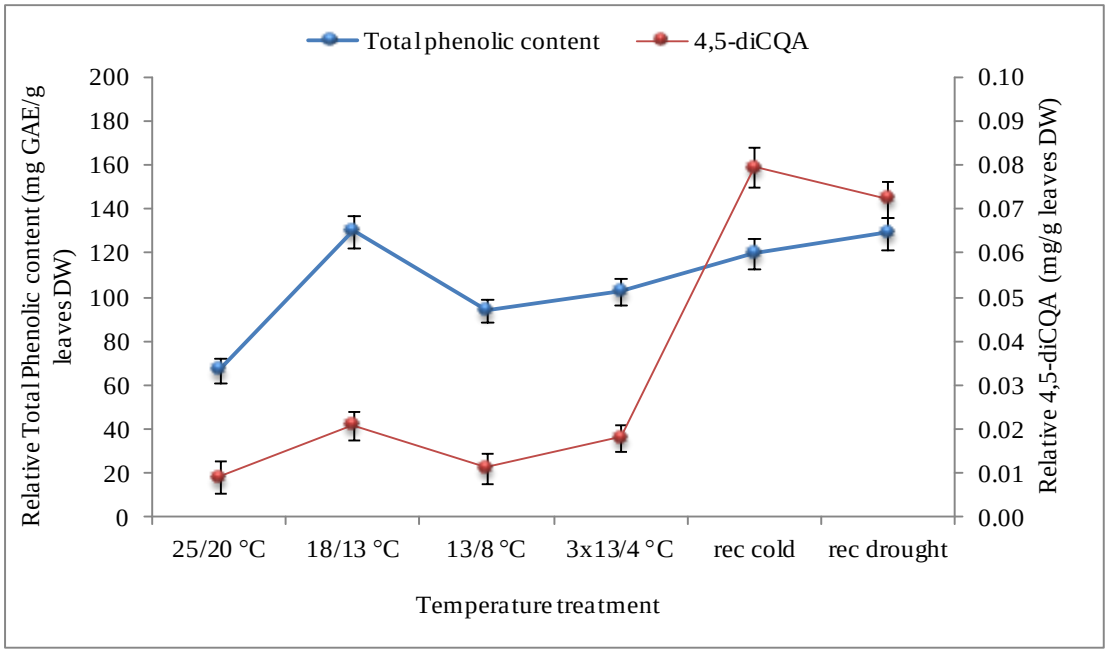


Figure 3.16 Comparison between 4.5-diCQA and total phenolic content profile in response to cold treatment in Obatã (at control condition).

It is seen that with 4.5-diCQA case in Obatã, which can be seen in figure 3.16, it has increased much more sharply than the phenolic compounds in general (total polyphenol content), in the recovery from cold condition. This was also found in mild and severe drought conditions, but not so significantly as here.

Interestingly this is a compound that has been appearing related to some relevant roles in plant defense, such as the possible involvement in the response of resistance of coffee to

Hemileia vastatrix (*139*) and also in the appearance of russet spotting in leaves (brownish) due to their accumulation and subsequent oxidation (*140*). This way one can make the analogy and affirm that regarding the response to cold in Obatã, 4.5-diCQA may play an more essential role in defense against oxidative stress.

3.5 Standard microemulsion ($C_{12}E_5$ + decane + water)

3.5.1 Visual inspection

The first analysis that was done to microemulsions solutions, after its preparation, was the visual inspection, to determine the phase diagram of the system.

Without being overemphasized that this system uses a nonionic surfactant of E-O type, and therefore is temperature dependent, the phase diagram was obtained by varying the temperature of the samples, while it was observed the phase changes.

Phase boundaries determination was made upward and downward in temperature. Although the disadvantage in the latter case is that, it takes more time to determine the phase changes since when it reaches and passes the lower phase boundary (where the oil does not fit in the micelles and therefore must go to the water phase). This is not as a favorable process as the opposite (going up in temperature) in which the oil is already in the aqueous phase and tends to go into the micelles (oil phase).

The figure below is a representative picture of macroscopic appearance of the microemulsions systems in different phases, and hence different structures.

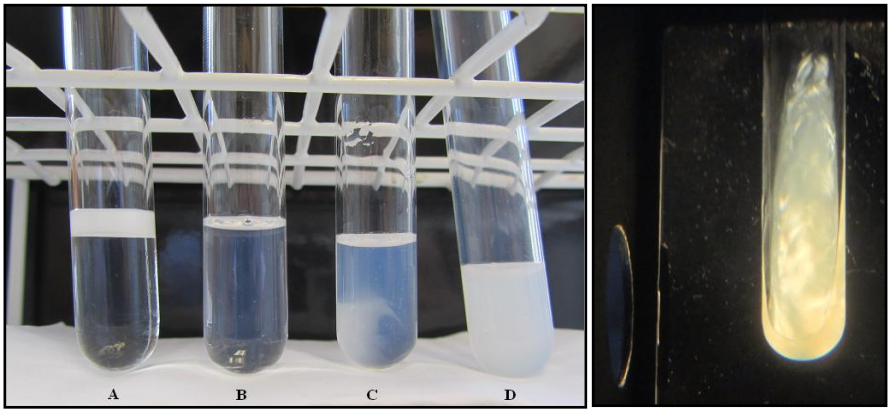


Figure 3.17 Microemulsion solutions as a function of temperature, showing different macroscopic phases. A – L1+ Oil; Sample below lower phase boundary (oil on top, surfactant and water in the bottom) where there is a macroscopic phase separation. B – L or microemulsion phase; Sample in the microemulsion temperature range where the structure corresponds mainly to spheres (nanomicelles), where oil, surfactant and water are in only one macroscopically homogeneous phase. C – Bicontinuous or L_3; Sample near the upper phase boundary, where sample is cloudy and starting to be turbid. D – Lamellar phase ($L\alpha$); Sample above the upper phase boundary, where is seen birefringence glowing on the right image using polarized light.

Although not being the scope of this study to analyze other phases that may assembly in microemulsion systems, the phase that appears more to the right in figure 3.17, which corresponds to lamellar phase, consists, very briefly of parallel and plane layers of surfactant bilayers separated by layers of solvent forming a one-dimensional lattice that exhibits optical anisotropy, a fact resulting and proven by brightness of the sample when observed through polarized light, due to deviation of the polarized light plane.

3.5.2 Phase diagram

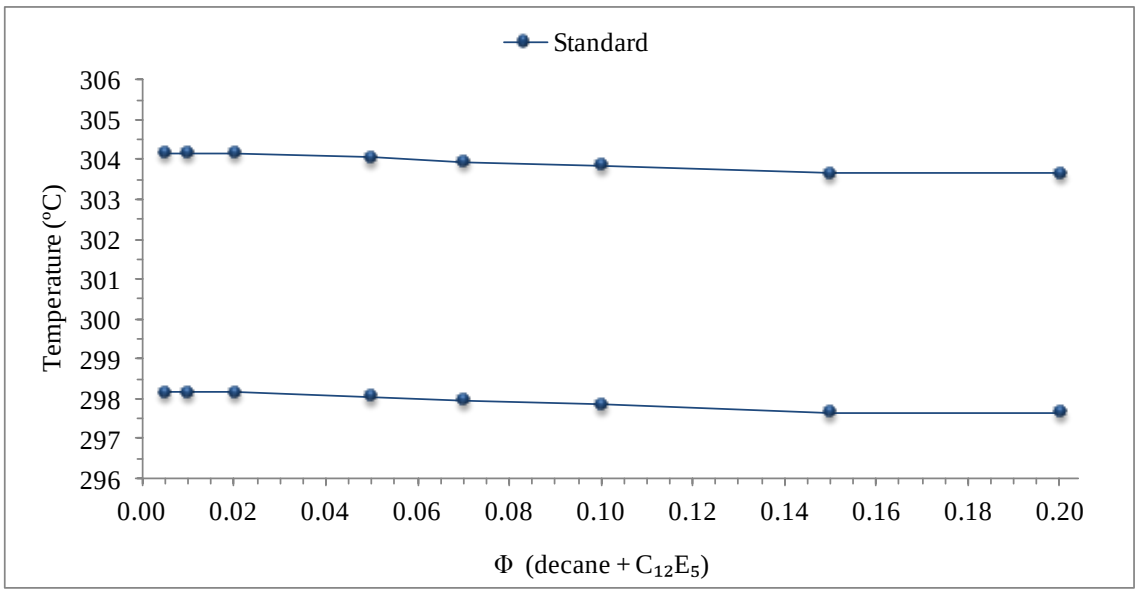


Figure 3.18 Phase diagram of the standard system (decane + $C_{12}E_5$ + water) with fixed surfactant to oil volume ration (0.815:1), varying water dilution. The phase diagram is shown as temperature versus the volume fraction. The microemulsion phase for these volume fractions is stable (and also clear) over a temperature range of approximately 6 °C, from 25 °C (lower phase boundary) to 31°C (upper phase boundary).

Only volume fractions up to $\Phi = 0.2$ were studied. since beyond $\Phi = 0.3$ or $\Phi = 0.4$ other structures, than the droplet microemulsion (spheres), such as the (micellar) cubic phase (I_1) and hexagonal phase (H_1) begin to appear (*104*).

3.5.3 Dynamic Light Scattering

Through visual inspection it is possible to analyze the changes that occur at the macroscopic level with the temperature variation, however in order to obtain information about the changes on a smaller scale, the nanometer scale, it is necessary to use other techniques, such as DLS. DLS was used to study the (collective) diffusion and through that, indirectly size, shape and polydispersity, of the microemulsion.

3.5.4 Diffusion and polydispersity

A temperature trend using *Zetasizer nano ZS* apparatus was ascertained, where all the needed parameters have been calibrated before measuring the samples (temperature, time and number of measurements). Since these microemulsions are temperature dependent, and once in this case (collective) diffusion is really what is being measured, it is crucial to ensure that the temperature at which the sample begins to be measured is actually the desired temperature, in other words, needs be the lower phase boundary temperature (as it was previously described in materials and methods chapter).

All samples were measured, but to simplify the analysis (and data presentation) only the $\Phi = 0.05$ and $\Phi = 0.15$ samples will be displayed with more detail, as representatives for the rest of the samples (since the behavior is quite similar, between them).

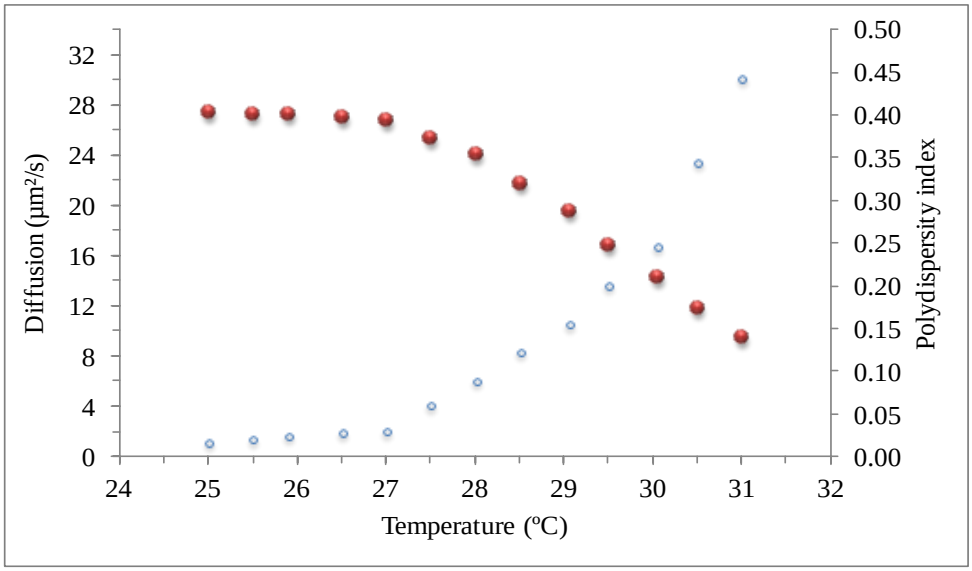


Figure 3.19 Diffusion (left axis) and polydispersity index (right axis) versus temperature for the sample $\Phi = 0.05$. Diffusion is represented in red spheres and polydispersity index blue circles.

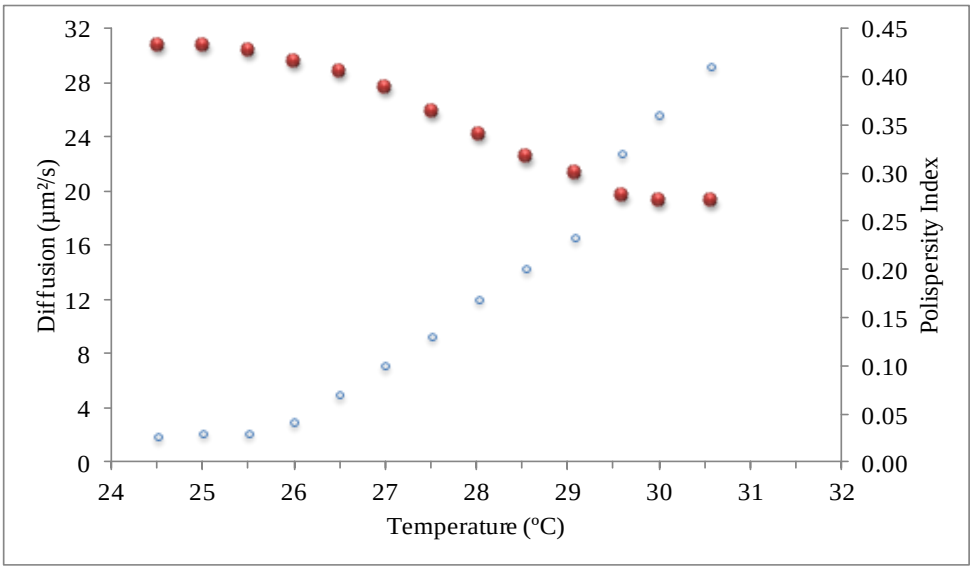


Figure 3.20 Diffusion (left axis) and polydispersity index (right axis) versus temperature for the sample Φ = 0.15. Diffusion is represented in red spheres and polydispersity index in blue circles.

In both figures 3.19 and 3.20. the diffusion behavior of the microemulsion droplets plotted against the temperature increasing from, 25 °C to 31 °C (microemulsion temperature range) is represented. As can be seen, diffusion decreases with increasing of temperature. The direct explanation for this is that the aggregates grow. The explanation for the growth is also simple. The curvature of the surfactant film changes and gives increasing size of the aggregates with increasing temperature. The explanation for the change of curvature in not that simple, but in order to attempt to explain and demonstrate it in a simplified way, it has been suggested that this may be due to the decreasing of solubility of the ethylene glycol group (hydrophilic region) of the surfactant when temperature increases (hydrogen bonds tend to be less effective). In thermodynamic terms with the increasing of the temperature there is a breakdown of the water structure surrounding the ethylene oxide group, thus the curvature of the surfactant interface is affected, and tends to decrease, thereby contributes to increase the size of the droplets, leading to spheres becoming either just larger (if there is more oil as is the case below lower phase boundary) or ellipsoids as it is just above lower phase boundary. Since the area per head group of surfactant is constant (constant amount of surfactant), as well as the amount of oil, when the temperature increases and the size of droplets becomes bigger, there is a time when the oil cannot fill the total volume of the spheres because the surface area (filled by the surfactant head groups) increases to power of 2, ($4\pi r^2$), while the volume of the sphere (filled with oil) increases to the power of 3, ($\frac{4}{3}\pi r^3$). Thus a balance between curvature (allowed), entropy and thermodynamic stability regulates the size of micelles, or other formed structures.

Regarding the polydispersity, it should always be measured, as it helps in following the behavior of the system giving indication of the variety of sizes. To be able to rely on the results, polydispersity should never be greater than 0.4, and even from 0.3 much discrimination is present in the existing structures. This parameter is especially used for following the behavior of the micelles, by knowing that, near the lower phase boundary the polydispersity is very low for this system (around 0.01) which can be an indirect aid to be sure that the initial temperature is correct and the system was well made. When the temperature starts to be closer to the upper phase boundary, polydispersity becomes very high (above 0.35), indicating that possibly the structure of the microemulsion has changed to bicontinuous –like (figures 3.19 and 3.20).

3.5.5 Size

The size of the micelles was also analyzed, although it is an indirect measure, since it is calculated by the DLS with various assumptions of model equations (for instance assuming a hard spheres growth behaviour). From the Stokes-Einstein equation, by the relationship between speed of particles due to Brownian motion, the perceived size is obtained.

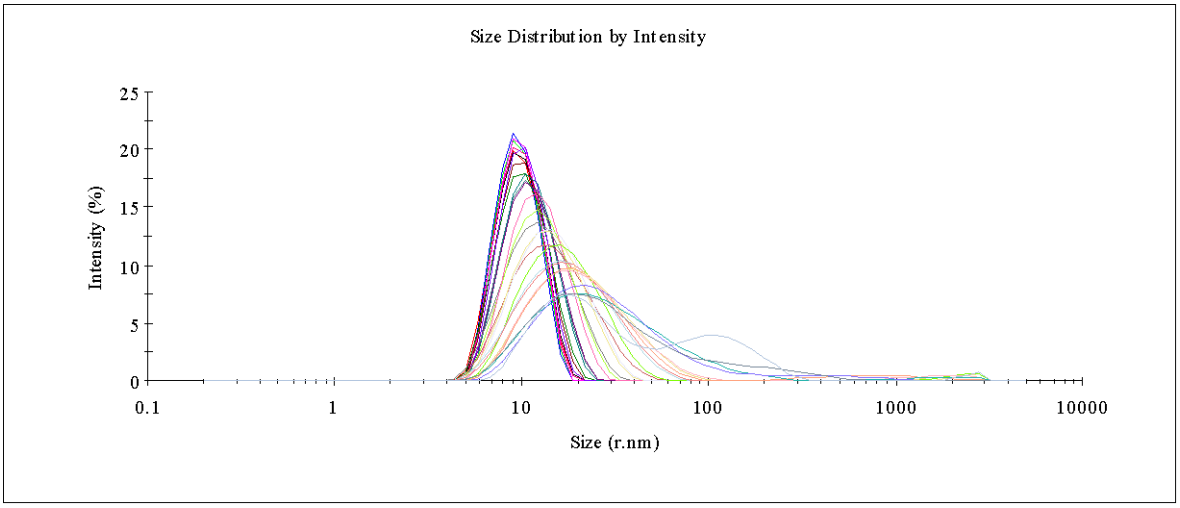


Figure 3.21 – Size distribution by intensity, varying with temperature (from left to right) from 25 °C to 31 °C of the Φ = 0.05 sample.

Observing the graphic of the figure 3.21, where is represented the size of the micelles with increasing temperature, it is seen that the size starts at around 8 to 9 nm (8.58 nm in particular) and as the temperature increases, the size also increases, as explained earlier in the diffusion graphic (figure 3.19) and in the introduction chapter. An important feature is that the width of the peak also increases, which is further evidence that the variety of sizes (polydispersity) also increases. This is a reason for us to confine (only) at the size data near the

lower phase boundary, since there the micelles have a good approximation to spheres, while at higher temperatures this approach becomes less valid.

Size given by the machine, is calculated assuming that the micelles grow as hard spheres (such as polystyrene latex), when these structures are indeed very dynamic. It is important to note that it helps to realize that what prevails in solution at higher temperatures is no longer a sphere like micelle but possibly a bicontinuous-like structure, thus one should be careful when analyzing data.

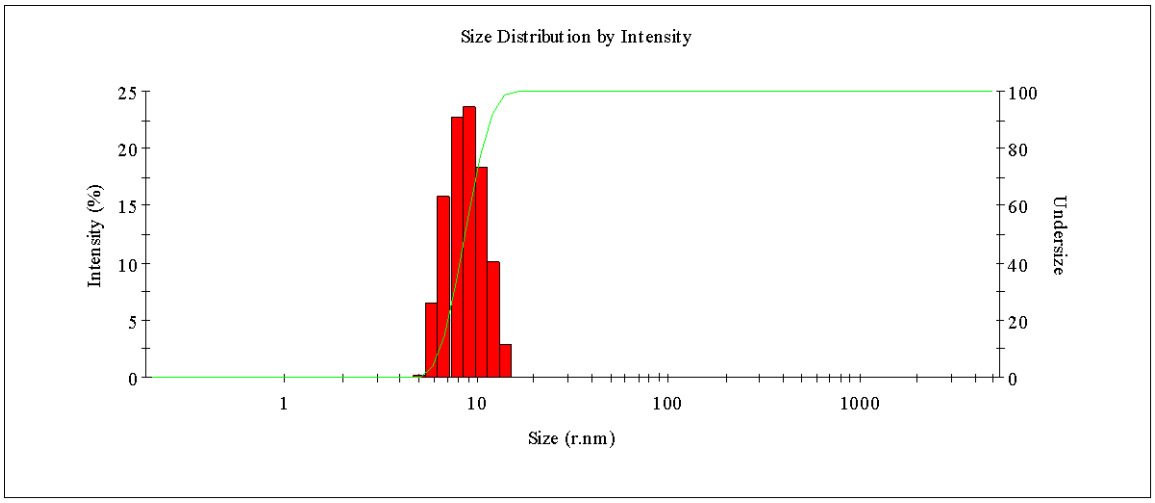


Figure 3.22 Histogram of the size distribution by intensity of the Φ = 0.05 sample at the lower phase boundary (25 °C).

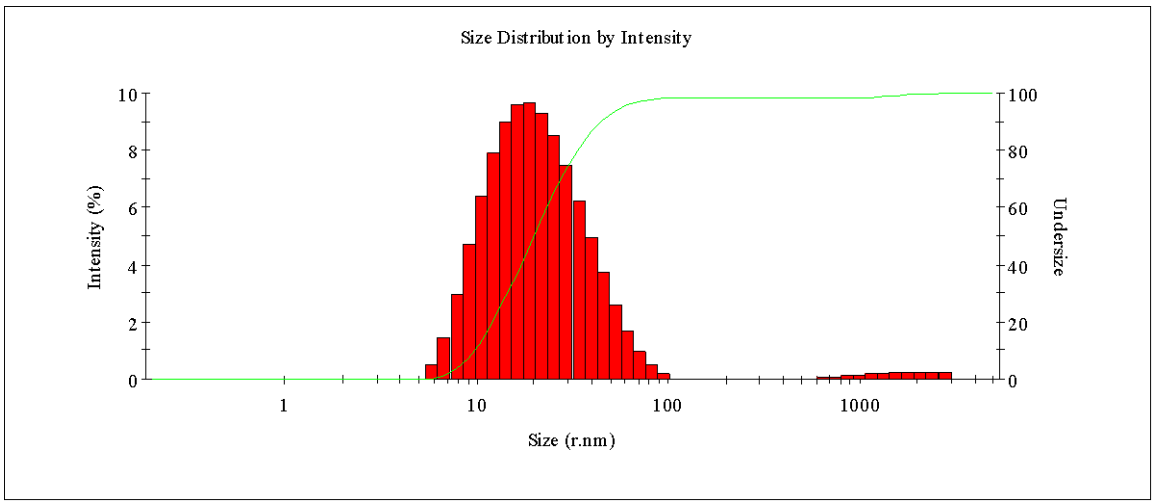


Figure 3.23 Histogram of the size distribution by intensity of the Φ 0.05 sample near the upper phase boundary (29.5 °C).

Analyzing the above histograms it is clear that the initial size of the droplets is much more well-defined than the size showed near the upper boundary phase (29.5 °C). Here the

structure is no longer spherical but more probably a bicontinuous-like structure, and the solution starts to become not as clear as initially (a bluish color in the samples is seen, known as Tyndall effect, meaning the phase boundary is approaching). Another way to see that the size is not as well defined at the end, is to note that the intensity in the first histogram (lower phase boundary) is greater than the second (near upper phase boundary), in other words, in the beginning most of the particles have the same shape (spherical) and as the temperature increases, they assume different shapes, leading to the intensity of diffusion being more distributed. This was further supported by previous studies (*141*)

Correlation function is another type of data that helps to visualize the behavior of microemulsions. This comes from the principle that large particles move slowly, while smaller particles move quickly. If large particles are being measured, as they are moving slowly, the intensity of the pattern of acquiring data will also fluctuate slowly, and similarly if small particles are being measured then, as they are moving quickly, the intensity of the pattern will also fluctuate quickly.

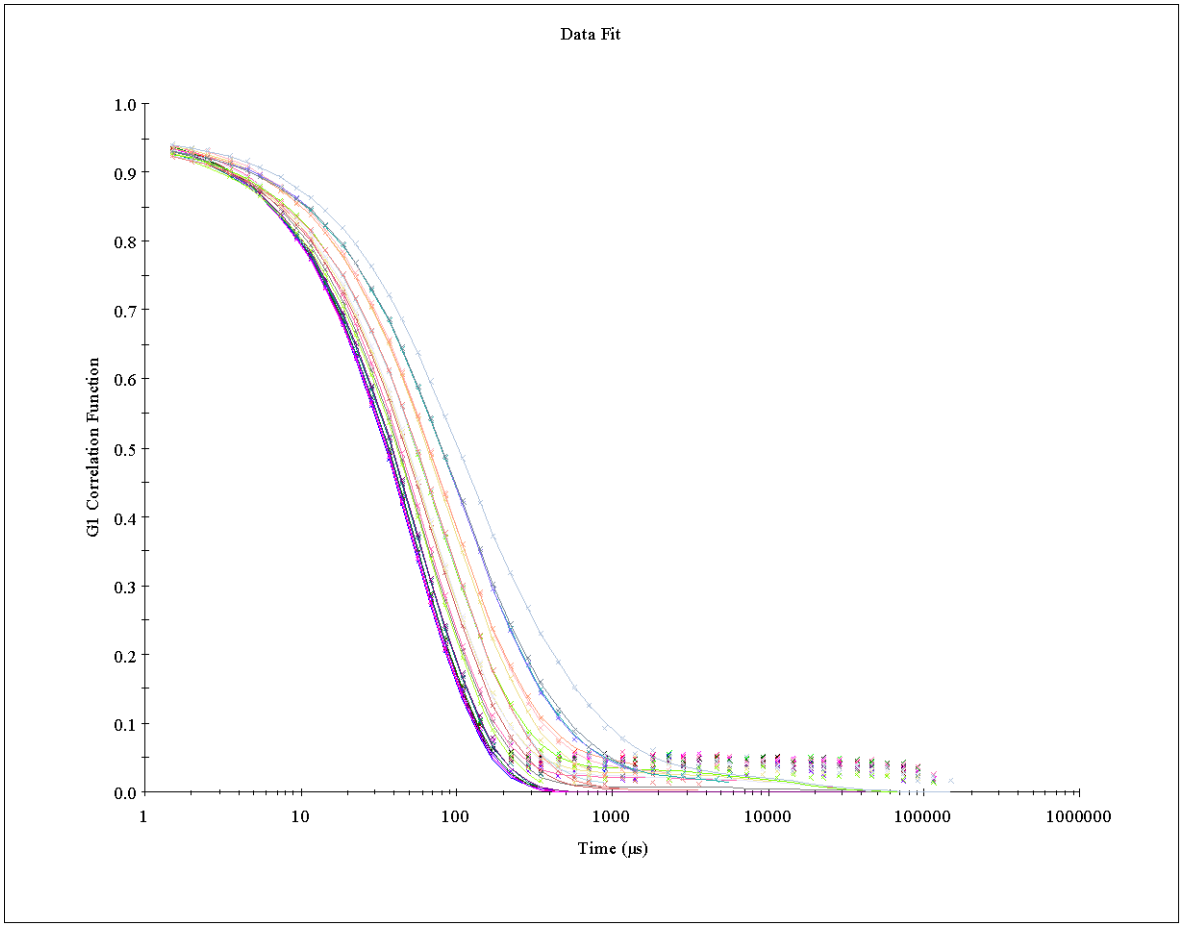


Figure 3.24 Correlation function versus time. It is represented Φ = 0.05 sample, within the temperature trend, from 25 °C to 31 °C (from left to right).

The graphic of the figure 3.24, shows the correlation function for large and small particles or in other words, from left to right, the temperature increases, thus the size also increases. It can be seen that, the rate of decay for the correlation function is related to particle size as the rate of decay is much faster for small particles than it is for large.

The same behavior was seen in all the other volume fractions (Φ), with only minor differences in the growth temperatures (also seen in the phase diagram), so they are not included here.

Interpretation of diffusion between several volume fractions (Φ)

According to the Stokes-Einstein equation (equation 3.2), as the diffusion decreases the radius of the particle increases,

$$D0 = \frac{kBT}{6\pi\eta r}, \quad equation\ 3.2$$

where, D_0 is diffusion constant; k_B is Boltzmann's constant; T is absolute temperature; η is the viscosity and r is the radius of the spherical particle.

If one looks at the plots of the diffusion versus temperature of several volume fractions (figure 3.25), we find that there is a slight increase in diffusion when increasing volume fraction (from Φ = 0.05). Although the data are within the parameters of required precision for a good measure, one should not compare results of different volume fractions. This is due to the fact that the way these results are ascertained do not take into account the concentration effect of the sample. Moreover, what is being measured is actually the collective diffusion and the size is then calculated using the modified Stokes-Einstein equation, assuming that the particles are at infinite dilution (where $D = D_0$), thus behave as hard spheres

When the data from different volume fractions are put together, it appears that there is a slight increase in diffusion (figure 3.25), although this is only verified from Φ= 0.05 onwards.

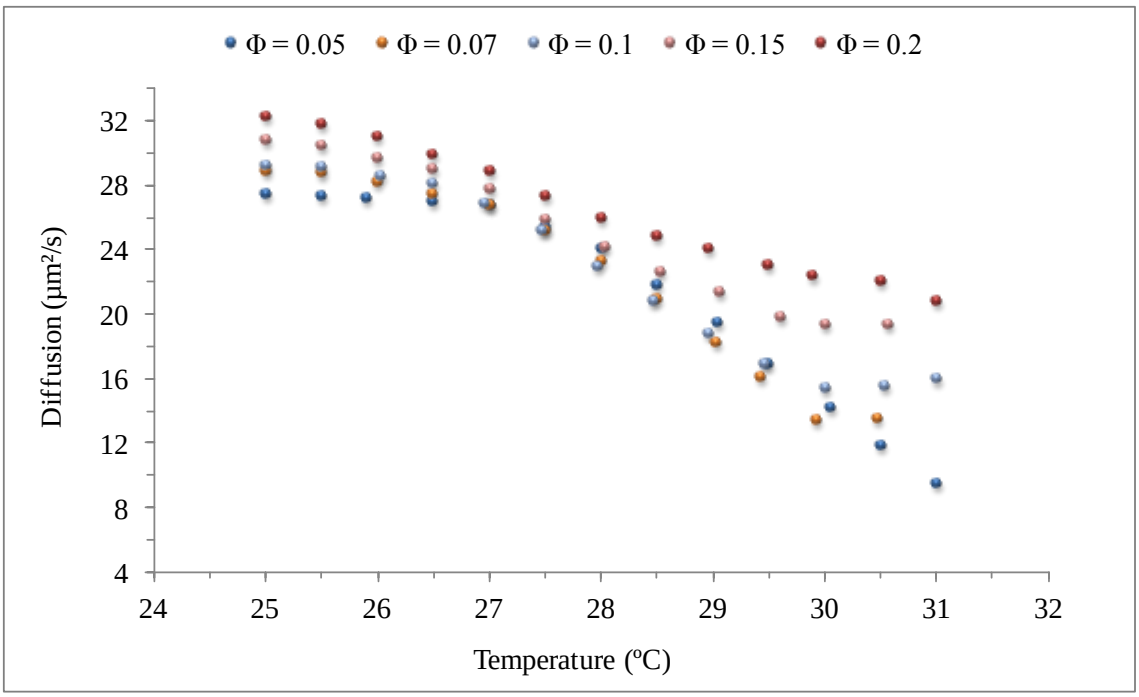


Figure 3.25 Diffusion versus temperature, of the standard system showing the Φ = 0.05, Φ = 0.07, Φ = 0.1, Φ = 0.15 and Φ = 0.2 samples.

The increase of diffusion values with higher volume fractions, might be explained by the fact that we measure collective diffusion and hence the higher number of particles in solution, will contribute to the increase of Brownian motion (and perhaps a greater sterical and electrostatic repulsion), therefore leading to diffusion become higher.

When comparing collective diffusion from different volume fractions one need to take the concentration dependence into account and for hard spheres this is known. Having the relative diffusion coefficient (Dc/D0), where Dc is collective diffusion coefficient at a certain temperature, and D0 is diffusion coefficient at infinite dilution (which is obtained by extrapolating the diffusion data to zero concentration), it is possible to obtain an approximation to the behavior of hard spheres (figure 3.27). We use D0 because there we know that the micelles are as has close to hard spheres as possible. The curve of hard spheres is then obtained through equation 3.3 (*142*)

$$\frac{Dc}{D0} = 1 + 1.45\Phi_{Hs} \quad \text{, equation 3.3}$$

where, Dc is the (collective) diffusion coefficient, D0 is the diffusion coefficient at infinite dilution, 1.45 is a coefficient for monodisperse hard spheres (*143*) and Φ_{Hs} is hard sphere volume fraction and equals to 1.14Φ (*142*)

The way size is obtained does not take into account the concentration effect, so it should be interpreted more like a perceived size (in case the particles are not spherical) rather than the absolute size. In this regard an extrapolation of the data towards zero volume fraction (where micelles are considered to be spheres (*101*, *104*)) (figure 3.26) allowed us to obtain the value of D0.

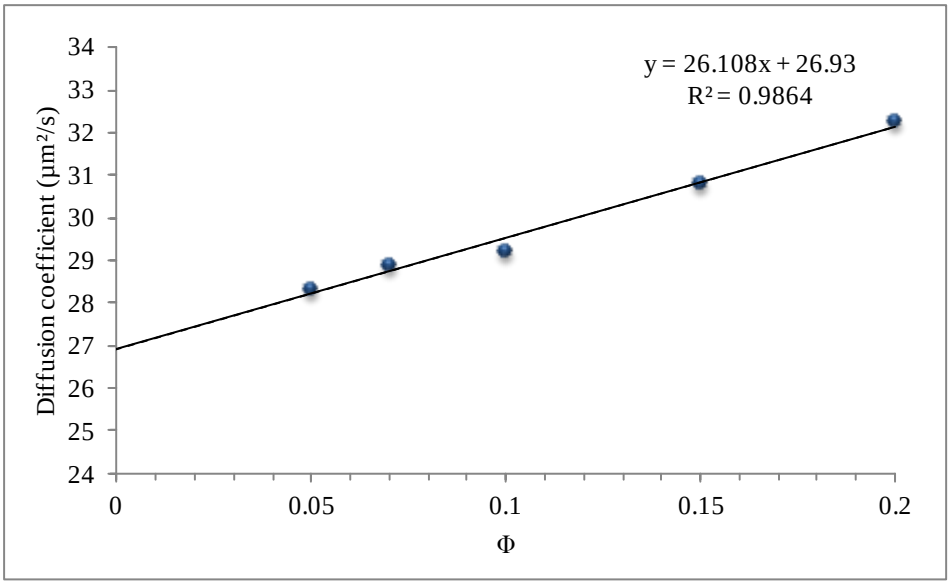


Figure 3.26 Diffusion coefficient versus volume fraction at 25 °C. A tendency line used to calculate the D0 value (D0 = 26,93 µm²/s) is represented.

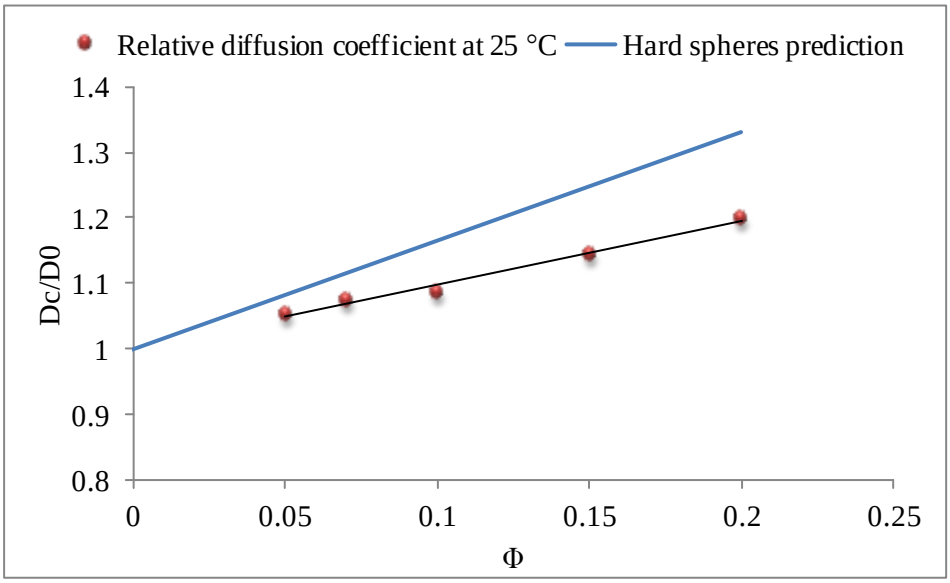


Figure 3.27 Relative diffusion coefficient values (Dc/D0) and the hard spheres behavior prediction, versus volume fraction.

Analyzing the graphic in figure 3.27, it is possible to observe that the curve of relative diffusion coefficient (Dc/D0) obtained from the experimental values, is lower than the one corresponding hard spheres behavior prediction, and that this difference increases with the

volume fraction. As the relative diffusion coefficients values are lower than the ones of the prediction of hard spheres, as it is seen in graphic of figure 3.27, means that the size of the droplets is in fact bigger with increasing the volume fraction (since diffusion is smaller than the prediction), and not smaller, as might have been suggested only by plotting the values of diffusion of various volume fractions. Although the results data do not match perfectly the curve of the behavior of hard spheres, these are very close to it, indicating that the droplets of this system are actually very close to spheres. For instance, if we look at other references (*102-104*), using longer oils like hexadecane, the behavior deviates more from hard spheres behavior compared to decane. To further analyze which would be the droplet shape assumed, as ellipsoid for instance (prolate or oblate), it would require further detailed studies. Yet, it has been suggested (*141, 144*), that for this system, is one-dimensional growth that then changes into a bicontinuous system without macroscopic phase separation and that the micelles take a more elongated form (close to a prolate) as the temperature increases.

We are able to see here using DLS technique that this is in line with what has been previously seen, that the aggregates have a hard sphere behavior but deviates from it at higher volume fractions as one-dimensional growth that then grows into a bicontinuous microemulsion without macroscopic phase transition at even higher temperatures. Phase diagram temperatures obtained are also in line with earlier measurements (*101*). Having shown that it is possible to follow the standard system (a previously well characterized system) made it possible to start to investigate unknown systems.

Subsequently, the influence of addition of other components to the system will be examined, in particular: a drug, lidocaine and two antioxidants will be examined. One of the antioxidants is hydrophilic, chlorogenic acid (found in large amounts in coffee) and other hydrophobic, α-tocopherol (also present in coffee).

3.6 Chlorogenic acid addition

Among all the phenolic compounds identified in the coffee leaves of the three varieties (first part of this chapter), the one chosen to be studied in this microemulsion system was 5-CQA. In addition to be chosen due to its antioxidant potential, this was the compound found in higher abundance in coffee, from 1.9 to 2.3% as dry weight leaves content and accounting for 12.6 to 21.5% of the TPC, depending on the genotype. Hence its study and use in microemulsions for pharmaceutical or cosmetic use, becomes extremely important since it could act as protector against cell damage caused by reactive oxygen species and also might act as

having the potential to increase the lifetime of an incorporated drug that would be administered via microemulsion formulation, protecting it from oxidation.

As described on the materials and methods chapter, several microemulsions were made, varying the 5-CQA concentration in microemulsions, thus giving the possibility of having a broad spectrum of analysis.

3.6.1 Phase diagram

Once again, the phase diagram (figure 3.28) was first analyzed in order to get an (macroscopic) overview of any changes to the system.

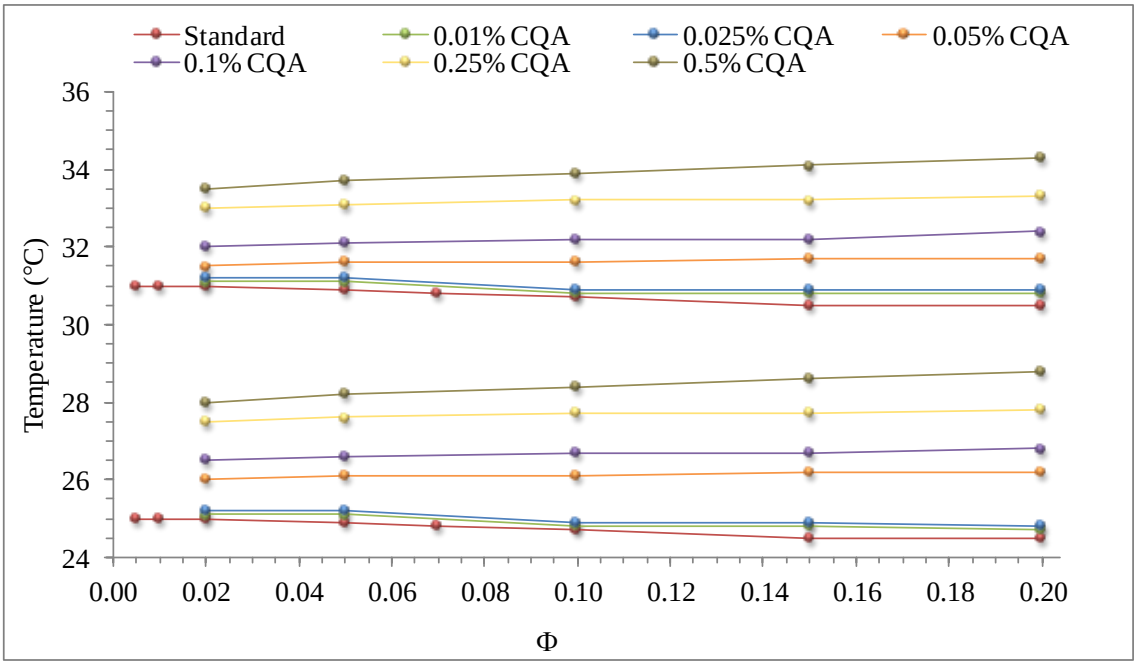


Figure 3.28 Phase diagram of the microemulsions with 5-CQA addition (in percentage by volume). The phase diagram is shown as temperature versus volume fraction. In different colors are represented several percentages (by volume), of the microemulsions with 5-CQA addition, and also the system without addition (standard).

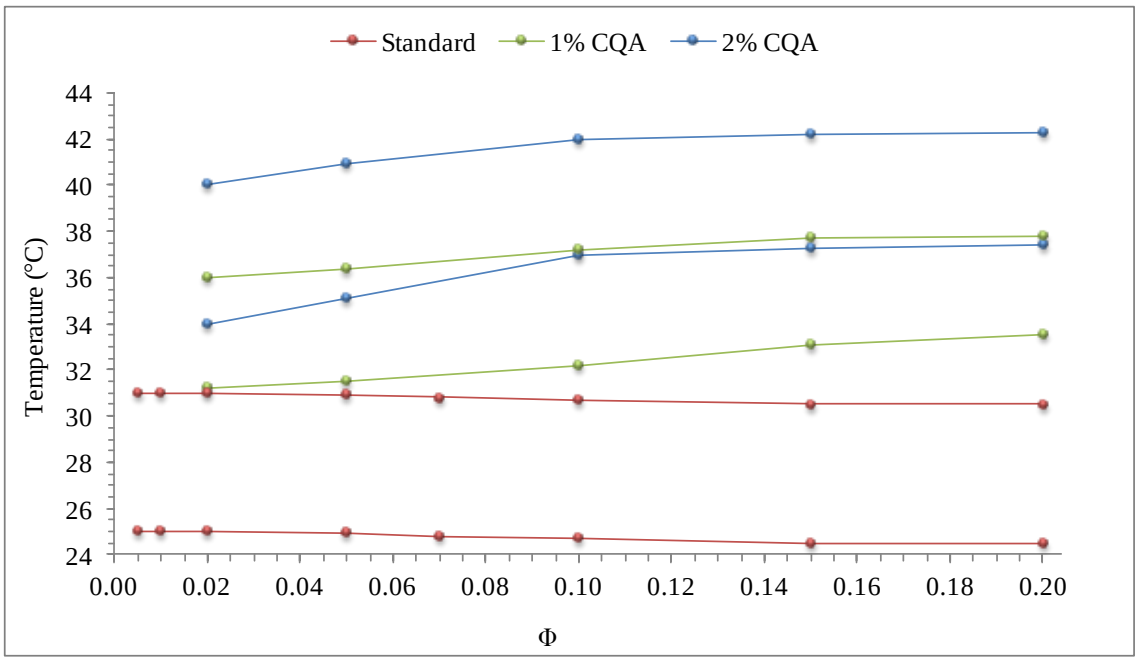


Figure 3.29 Phase diagram of the microemulsions with the addition of 5-CQA. The phase diagram is shown as temperature versus volume fraction. In different colors are represented several percentages (by volume), of the samples with 5-CQA addition, and also the system without addition (standard).

Note that the two figures above (figures 3.28 and 3.29) correspond to the same phase diagram and are separated to improve the presentation.

Doing a first analysis it is noted that the addition of 5-CQA to this microemulsions, changes the phase diagram by increasing the temperature of phase boundaries. Depending on the amount of 5-CQA added, the temperature phase boundaries (both lower and upper phase boundary) may increase up to maximum of 10 °C. However it is shown that until 0.1% addition, temperature does not vary substantially (up to about 1.5 °C). The value of 0.1%, which corresponds to 1 mg/ml (or 1000 ppm), is already a very significant concentration of 5-CQA. In practical terms, by comparing this concentration with the average of 5-CQA amount present in a cup of coffee (about 400 to 700 ppm, depending on the species, arabica or robusta (*145*)), this would be a non harmful dose that could be used without any risk of toxicity.

3.6.2 Dynamic light scattering

Since there is a vast number of samples and data from these experiments is therefore extensive, it will be represented again the $\Phi = 0.05$ microemulsion sample as representative for all.

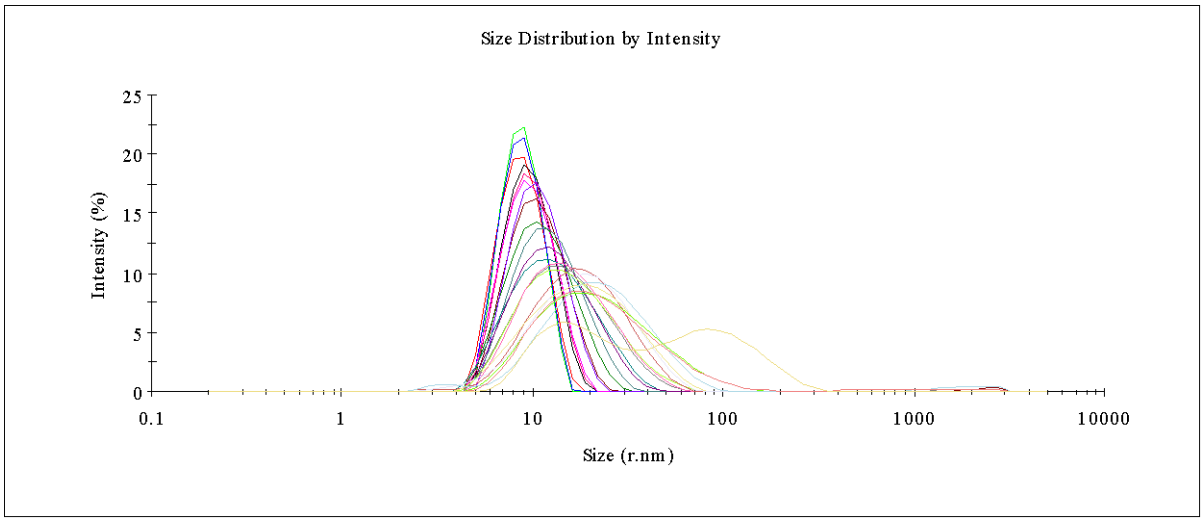


Figure 3.30 Size distribution by intensity, showing the Φ = 0.05 with 0.25% 5-CQA addition sample. There is represented the temperature trend from 27.5 °C to 33.5 °C.

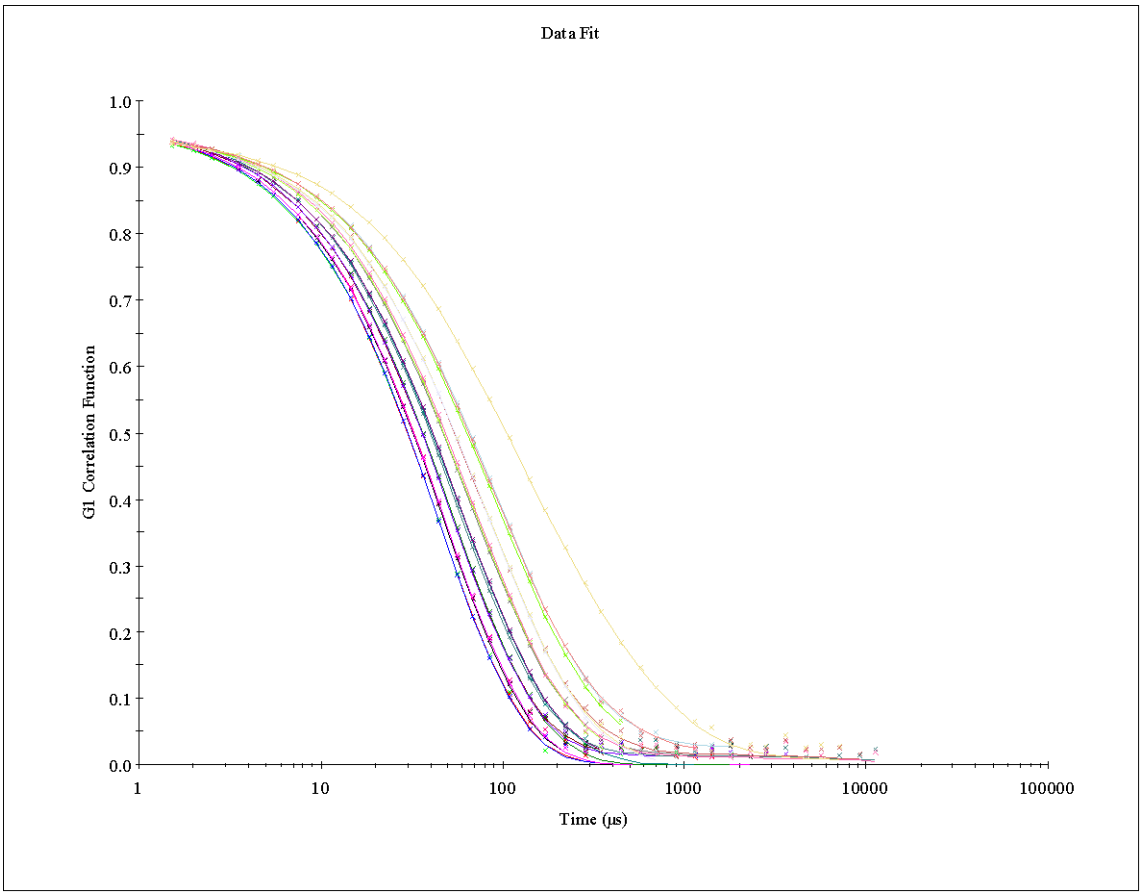


Figure 3.31 Correlation function versus time, showing the Φ = 0.05 with 0.25% 5-CQA addition sample. There is represented the temperature trend from 27.5 °C to 33.5 °C.

Once the graphics in figures 3.30 and 3.31 have proved to be very similar to those presented earlier in the case of the standard microemulsion, the explanation given is the same that was given in that case, thus avoiding repetition here.

Nevertheless, the diffusion versus temperature plots for some of the microemulsion samples made with 5-CQA addition, will be shown later.

In order to determine the possible cause of the changes in temperature to the system with 5-CQA addition, two following tests were made: the addition of a salt (NaCl) with the same ionic strength as the 5-CQA addition, to determine the bulk charge influence, and zeta potential determination, to determine if the aggregates get charged by 5-CQA.

3.6.3 Salt (NaCl) addition

As can be concluded from figure (3.32), by adding NaCl with an equivalent charge (ionic strength) as to fully charged 1% CQA solution, which raised the temperature phase boundaries in the phase diagram, about 7.5 ºC, in this case there were no significant temperature changes to the system.

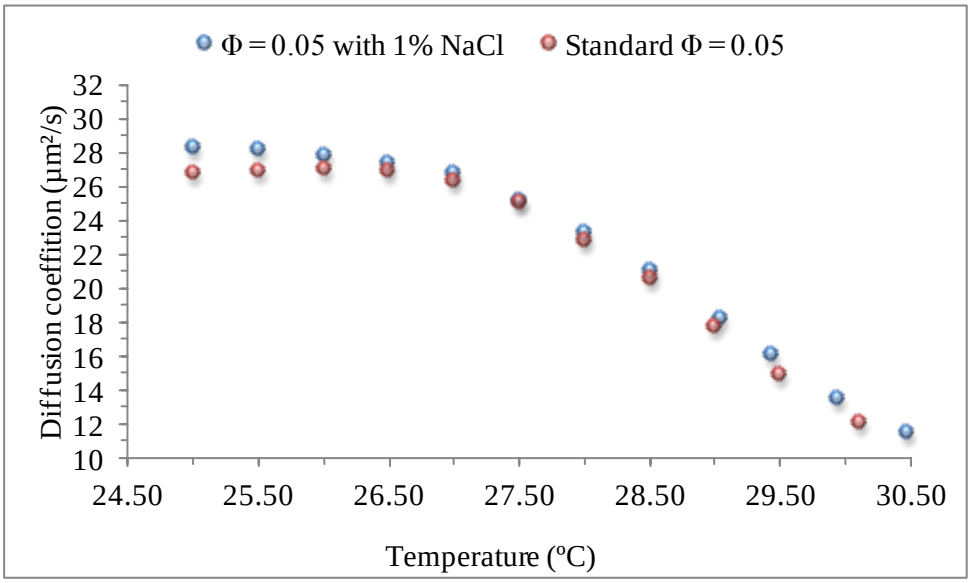


Figure 3.32 Diffusion versus temperature of the, Φ = 0.05 with 1% NaCl addition microemulsion sample and comparing with Standard Φ = 0.05 microemulsion.

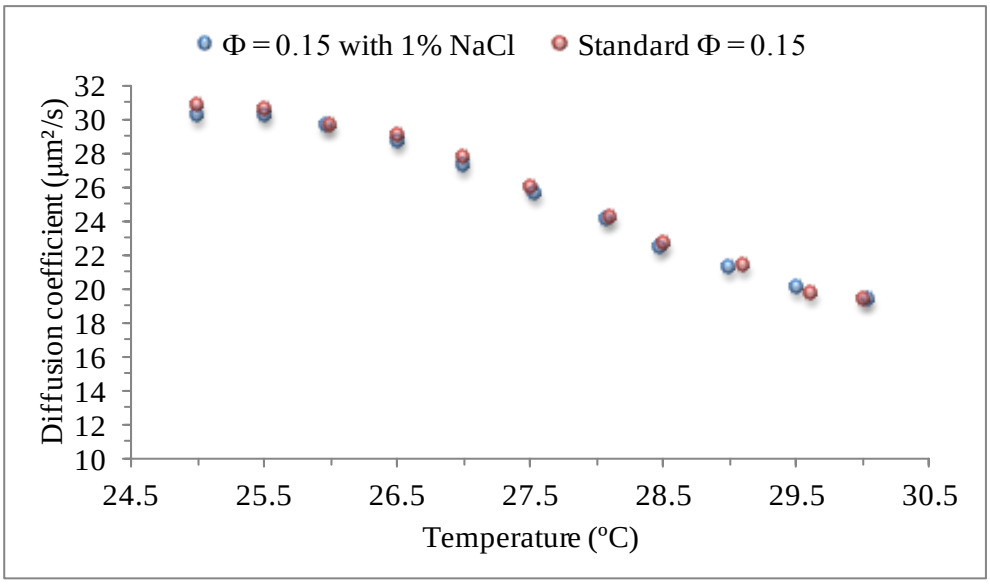


Figure 3.33 Diffusion versus temperature of the, Φ = 0.15 with 1% NaCl addition microemulsion sample and comparing with Standard Φ = 0.15 microemulsion.

It is evident that there were no significant changes to the system, macroscopically or microscopically, when adding a salt (NaCl). Smaller percentages of added salt (not shown here) were also tried, and likewise there were no significant changes.

No bulk charge effect was seen, thus whether it is the penetration effect or the charge effect of the 5-CQA molecule when interacting with the surfactant interface as if it would be a co-surfactant or being just close to the film as a co-solvent (or even a combination of these), that may be the reason for the observed effect of the 5-CQA addition to microemulsion.

3.6.4 Zeta Potential

In order to analyze the distribution of the 5-CQA molecules in the microemulsion, and to comprove that it may be really distributed (at least partially) in the external region of the interface of the surfactant, zeta potential was evaluated.

Observing the graphics in figure 3.34, it is clear that there are in fact charges surrounding the droplets, although the potential is low (about 5 mV), it is not zero compared to the system without 5-CQA addition.

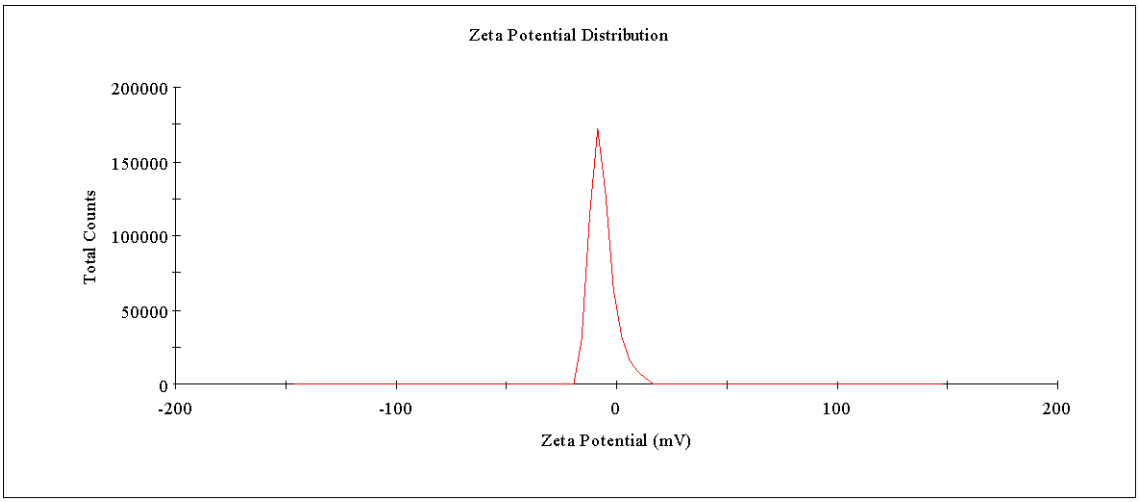


Figure 3.34 Zeta potential distribution, total counts versus Zeta Potential (mV), showing a peak near 5 mV (5.13 mV) of the sample Φ =0.05 with 1% 5-CQA addition.

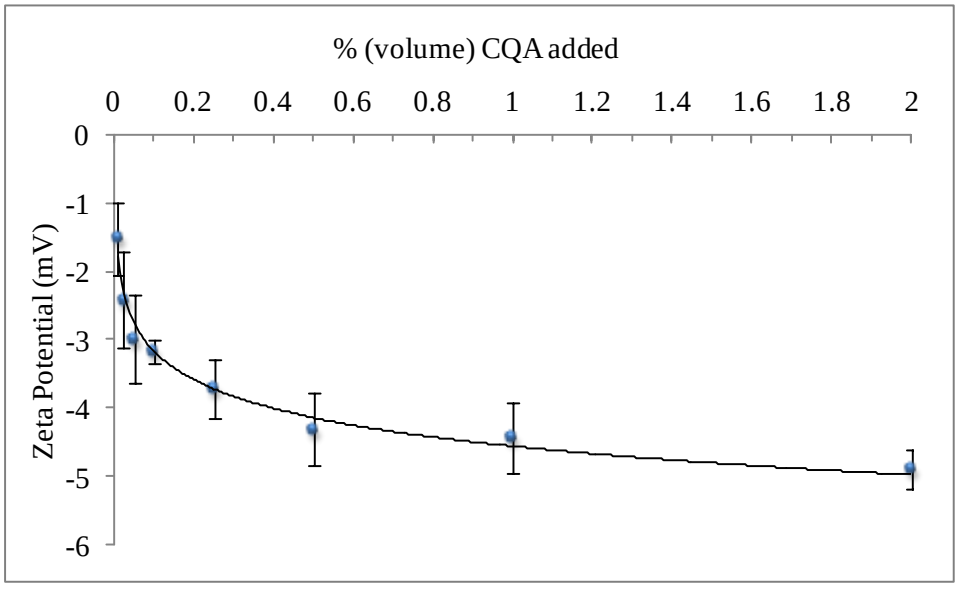


Figure 3.35 Zeta potential versus percentage (in volume) of 5-CQA addition of the Φ = 0.05 microemulsion.

The graphic of the figure 3.34, confirms that the system with 5-CQA addition has zeta potential values different from zero and that it increases with the addition of antioxidant molecules. Analyzing the curve, there seems to be a tendency to saturation of the potential when reaching higher percentages of 5-CQA, that may mean, that is the "saturation point" which

corresponds to the value where it is no longer possible to have more charges per droplet (particle), which is reached near the 2% addition of 5-CQA.

On the other hand, analyzing all volume fractions with the addition of 1% of 5-CQA (figure 3.36), it is seen that the potential is lower as higher is the volume fraction. This is due to the fact that the higher is the volume fraction (higher surfactant plus oil amount), the less water is in the system. As the 5-CQA is rather in the aqueous phase of the microemulsion, means that when the concentration of droplets increases, the lower is the amount of 5-CQA in the solution thus zeta potential is nearest to zero.

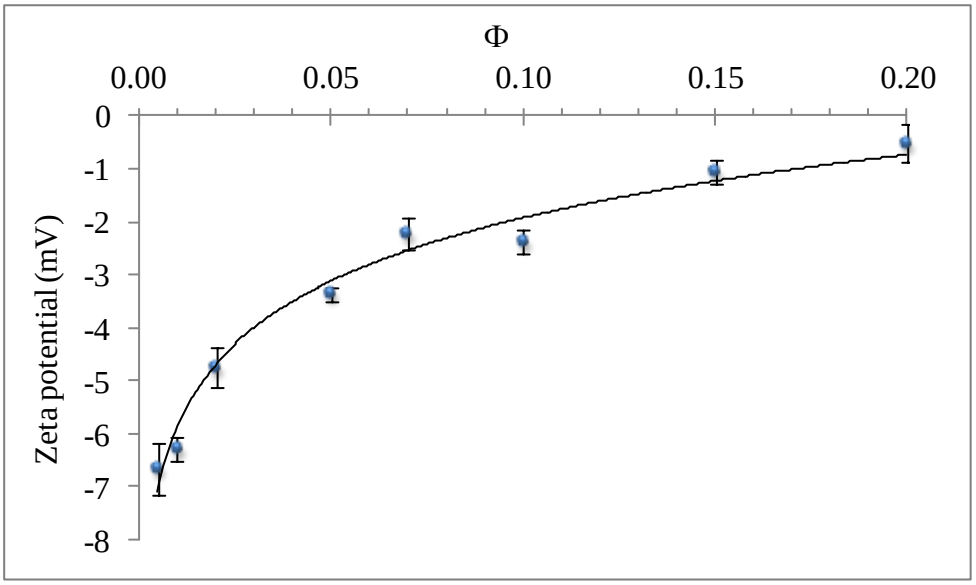


Figure 3.36 Zeta potential versus volume fraction with a 1% 5-CQA addition in microemulsions samples.

Of all the volume fractions, the maximum zeta potential value measured was 6.7 mV (in average) for the lower volume fraction (Φ = 0.05). 1% of 5-CQA addition was the concentration chosen to study zeta potential, since the values were reasonable and measurable, comparing to 2% addition that required higher temperatures (between 36 °C and 41 °C) had difficulty in dissolution (hence a greater error associated), and as for lower amounts of addition (below 1%) it showed very low zeta potential values, which are also associated with a higher error.

From zeta potential results we conclude that 5-CQA is close enough to the aggregates for them to behave as if they are charged. If 5-CQA is loosely attached or if it is part of the surfactant film we cannot determine. Just the bulk charge from NaCl did not make the aggregates behave as if they were charged. The effect is small but is concentration dependent.

It was proved that the addition of a hydrophilic molecule, in this specific case the chlorogenic acid, shifts the phase diagram, increasing the temperature phase boundary, at least in the studied region, where the droplets are considered discrete aggregates.

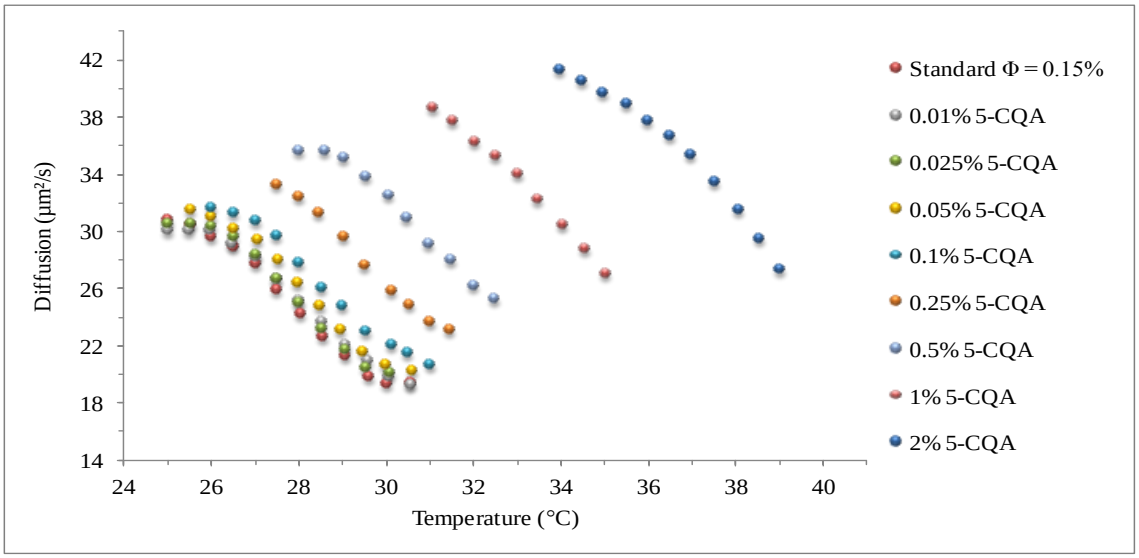


Figure 3.38 Diffusion versus temperature, where are represented for the Φ = 0.15 microemulsion with different percentages (in volume) of 5-CQA addition.

Finally, by analyzing all samples with different percentages of 5-CQA addition, it is seen that the rate of growth of the micelles with temperature does not vary significantly with the amount of 5-CQA, since the slope of the curves is similar. It is observed that the initial and final diffusion values increase with 5-CQA addition, and that this behavior is explained by the effect noted in figure 3.37. In other words, as the amount of 5-CQA increases, the more visible is the effect of increased curvature contributing to the particles having a smaller size and therefore a higher diffusion.

3.6.5 Explanatory model for the addition of a hydrophilic molecule to microemulsion

After all these studies and analysis of results from data of several used techniques, it was proposed an explanatory model for the behavior of microemulsions with the addition of a hydrophilic compound, in this case an antioxidant.

One of the plausible explanations for this behavior may be related to the influence that 5-CQA molecule has on the curvature of the droplets in the microemulsion. First 5-CQA is a hydrophilic polar molecule, so it is standing mostly in the aqueous phase of microemulsion but as it also has hydrophobic parts, it is (more) located in the surfactant interface region, as proven

by the fact that the aggregates acted as if they had a charge. It will help increase the curvature, causing the micelles to have a smaller size, and consequently the temperature required for droplet formation (the lower phase boundary) increases.

The figure 3.37 illustrates an explanatory model for the behavior showed upon the addition of the 5-CQA to microemulsions.

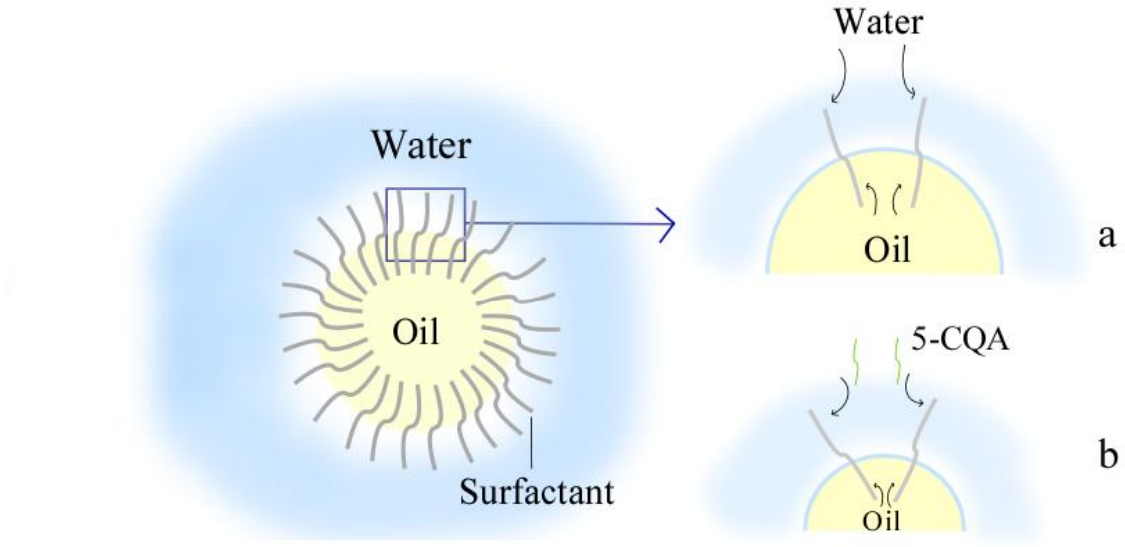


Figure 3.37 Proposed model for the effect of a hydrophilic antioxidant addition to a non-ionic microemulsion. On the left is represented the structure of microemulsions micelle (spheres) in the temperature range of microemulsion phase. On the right, there is a representative illustration of the effect of 5-CQA addition to the microemulsion system. The arrows in picture **a** represent the balance between tendencies or forces applied by water and oil molecules on the surfactant layer, while in **b**, with the presence of 5-CQA, represented in green (in the external surface of the droplets or surfactant film) there is an increase of the curvature of the surfactant layer, thus contributing to decrease the size. Note that this is a representative illustration, so the sizes of the molecules are not in scale.

3.7 Lidocaine addition

One of the many applications that microemulsions have is working as a drug delivery system, where a drug is encapsulated within the micelles in order to increase its availability, protection, solubility, among others. Previously it was seen how a molecule that is preferably in the aqueous phase (such as 5-CQA), influences the microemulsion system, and then it will be analyzed the effect of another molecule (in this case a drug, lidocaine), which is preferentially located in the oil phase.

3.7.1 Phase diagram

All volume fractions with three different amounts of lidocaine additions, 1%, 5% and 10% were investigated. The phase diagram, once again, was the first step of the analysis of this system of microemulsions (figure 3.38).

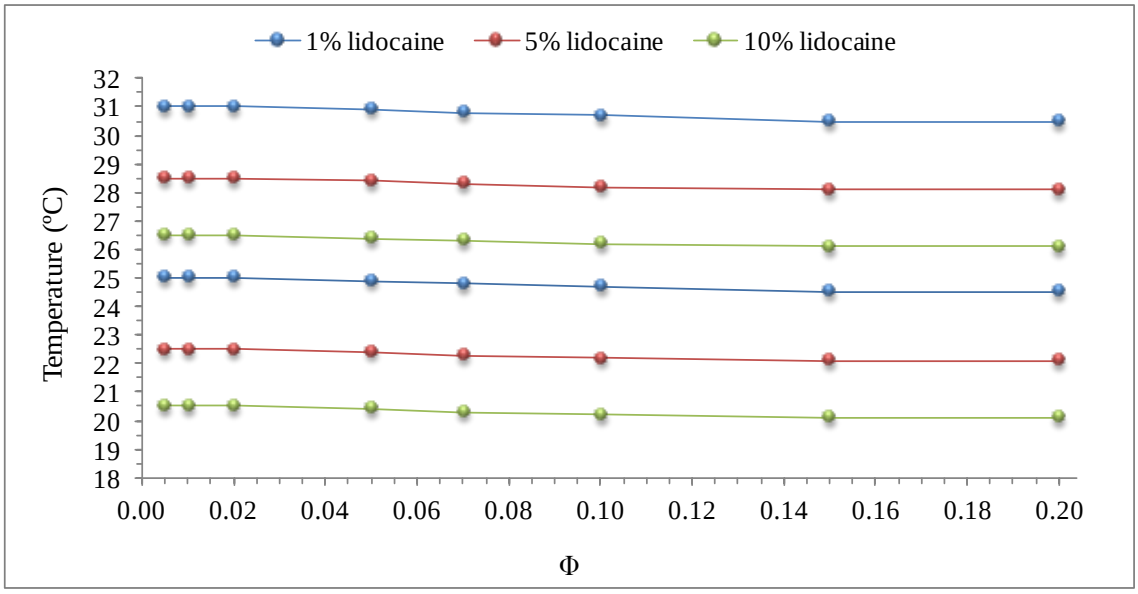


Figure 3.38 Phase diagram of microemulsions with lidocaine addition. The phase diagram is shown as temperature versus the total volume fraction. Lower and upper phase boundary of the several volume fractions are represented with different percentages of lidocaine addition (volume percentage in oil): 1%, 5% and 10% addition. Phase boundaries: 25 °C to 31 °C for 1% lidocaine addition; 22.5 °C to 28.5 °C for 5% lidocaine addition and 20.5 °C to 26.5 °C for 10% lidocaine addition.

Observing the phase diagram of the microemulsions with lidocaine addition, it is seen that the initial temperature of the microemulsion region (L_1) decreases with the increasing of

lidocaine and can go down to 20.5 ℃ in the lower phase boundary in the case of 10% addition (of oil volume).

3.7.2 Dynamic light scattering

By measuring the dynamic light scattering of the several samples with different volume fractions and lidocaine additions, it is seen in figure 3.39, (with the Φ = 0.05 representative for all volume fractions) that the growth of particles seems to follow the same slope compared with the standard system (without addition).

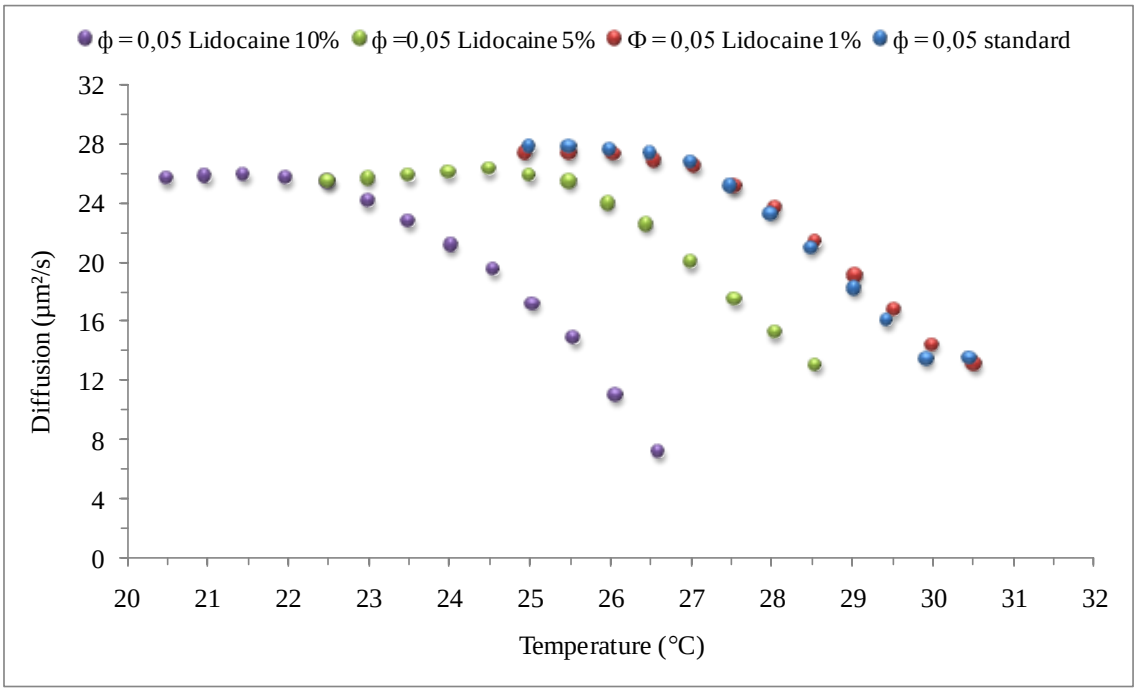


Figure 3.39 Diffusion versus temperature, of the standard system and Φ = 0.05 samples, with 1%, 5% and 10% lidocaine addition.

A slight difference is also noticed in the sense that the more is the amount of lidocaine added, the lower the diffusion obtained (meaning a larger size). This is due to the fact that the higher is the amount of lidocaine, the greater is the effect in decreasing temperature and thus lower diffusion, as it is temperature dependent, but the size is the same, if calculated.

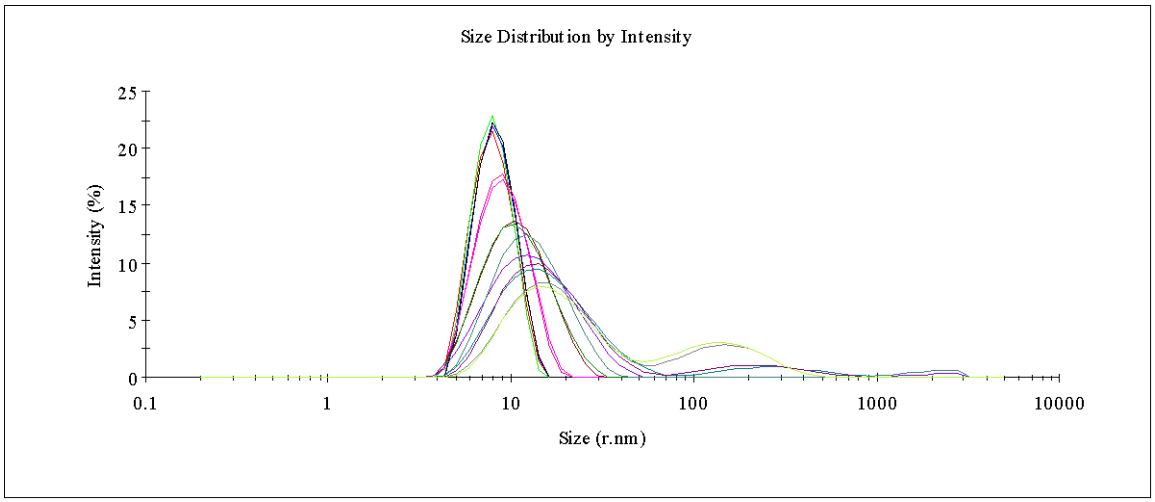


Figure 3.40 Size distribution by intensity, showing the Φ = 0.05 sample, with 5% lidocaine addition, between 22.5 °C and 28.5 °C.

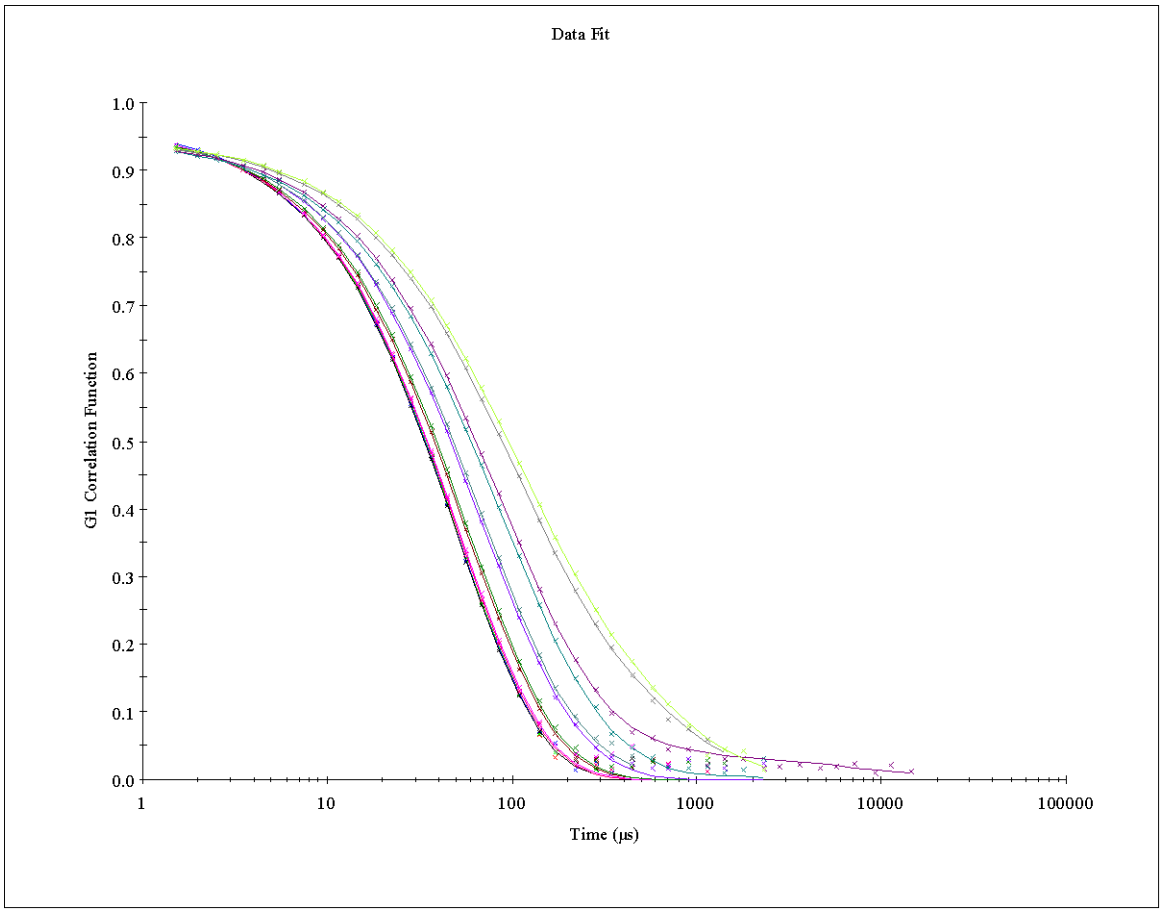


Figure 3.41 Correlation function versus time. It is represented the Φ = 0.05 sample with 5% lidocaine addition, between 22.5 °C and 28.5 °C.

As can be seen, the graphics of figures 3.40 and 3.41, of size of the micelles and correlation function with the temperature trend are identical to those obtained for the standard system and 5-CQA addition, being the explanation for this profile the same as that given to other cases.

In this case the explanation is opposite to the chlorogenic acid one, in other words, when lidocaine is incorporated into micelles (mostly in the oil phase), it will promote the decrease of the curvature of the surfactant interface. Moreover, since these solutions "have less oil," because 5% or 10% of the oil is replaced with lidocaine (which is not genuine oil), the differences will be more pronounced. Also the fact that a change in the oil (thus in the oil to surfactant ratio) will change the droplet size, whereas the dilution of water does not change the size so directly due to the dilution effect.

Following the model previously proposed for the 5-CQA behavior, in this case we have a lidocaine molecule located (at least partially) in the surfactant interface (in oil phase) that will be penetrating or pressing the hydrophobic tails of surfactant, contributing to reduce the curvature of the interface layer. The smaller the curvature, the larger is the size, thus the temperature must be lowered to micelles to have a smaller size allowing to be enough oil to fit inside the micelles (figure 3.43).

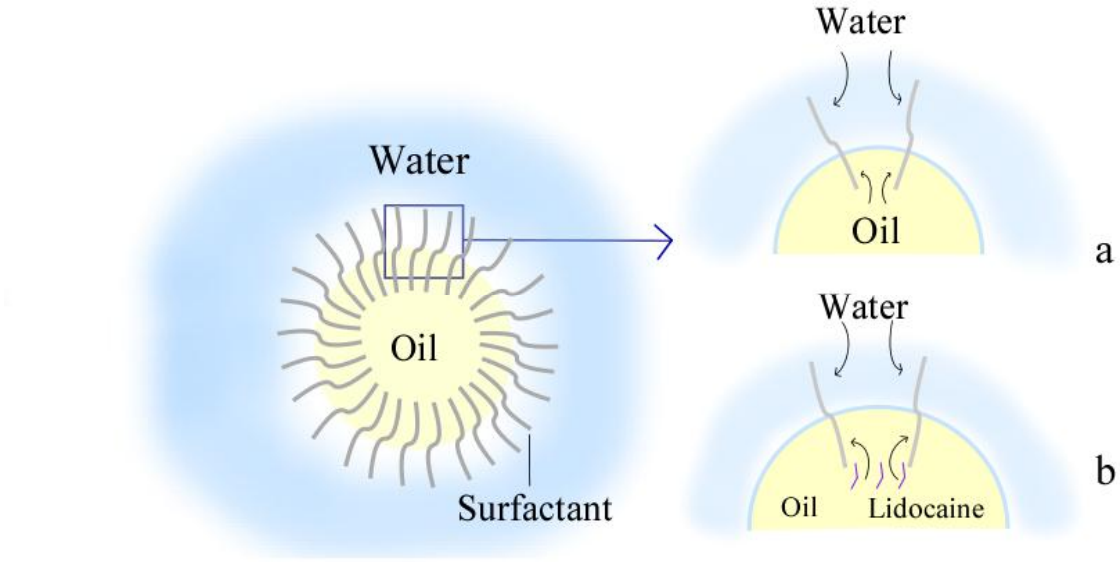


Figure 3.43 Proposed model for the effect of a hydrophobic drug addition to a non-ionic microemulsion. On the left is represented the structure of microemulsions micelles (spheres) in the temperature range of microemulsion phase. On the right, there is a representative illustration of the effect of lidocaine addition to the microemulsion system. The arrows in picture **a** represent the tendencies or forces applied by water and oil molecules on the surfactant layer, while in **b**, with the presence of lidocaine represented in purple (in the internal surface of the droplets or surfactant film) there is an decrease of the curvature of the surfactant layer, thus contributing to increase the size. Note that this is a representative illustration, so the sizes of the molecules are not in scale.

3.8 α-Tocopherol

α-tocopherol (also known as vitamin E) was chosen as another antioxidant to study in microemulsions, because it is one of the more used compounds in cosmetic and food (supplements) industry today due to its properties (referred in introduction chapter). Another reason is that it is mainly distributed in the oil phase and thus allows to study the antioxidant effect inside the micelles and aftermost the combination of two antioxidants (one inside and the other outside, 5-CQA) at the same time.

In addition, regarding future applications for this kind of system (with one antioxidant or even with two antioxidants), it could be used to protect an incorporated drug, making possible to double the protection, as it would be protected both in the oil and aqueous phase.

3.8.1 Phase diagram

The phase diagram of the system with the addition of α-tocopherol (figure 3.44), revealed in this case that, the phase boundary's temperature decreased more than the system with the lidocaine addition. It is shown that the lower phase boundary went down to about 17,2 ° C for 5% of α-tocopherol addition and 11,3 °C for 10% addition (in oil volume).

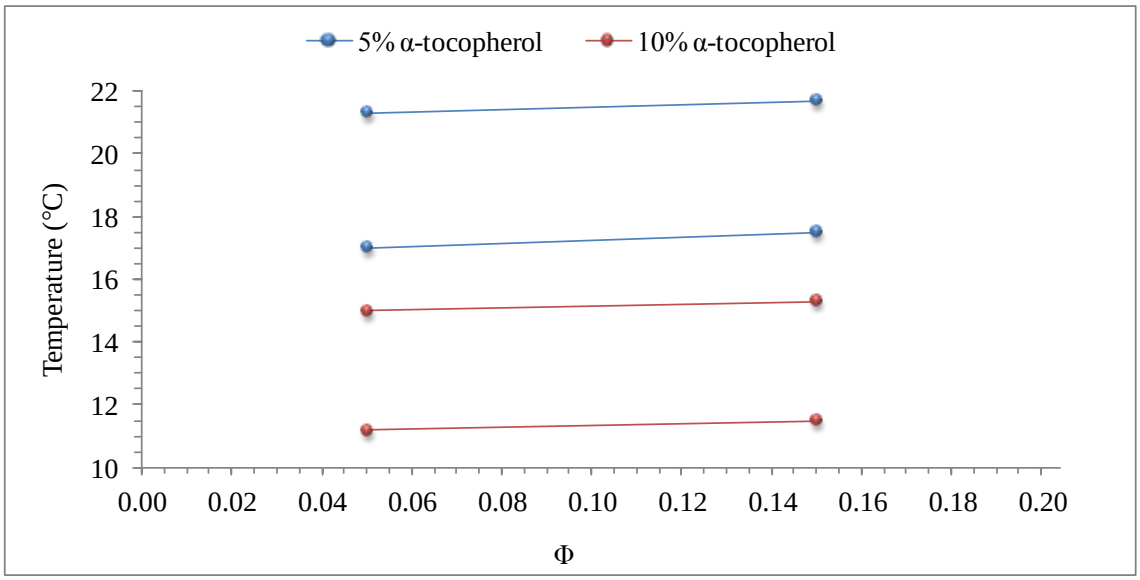


Figure 3.44 Phase diagram of the microemulsions with the α-tocopherol addition. The phase diagram is shown as temperature versus volume fraction (surfactant plus oil). In blue and red are represented the 5% and 10% α-tocopherol additions respectively, of Φ = 0.05 and Φ = 0.15 samples.

These lower temperatures may be due to the fact that α-tocopherol has higher hydrophobicity than lidocaine, leading to behave much more like an oil than lidocaine. In fact

we compared the solubility of α-tocopherol and lidocaine on decane and water, and it was found that the solubility ratio is 20:1 for lidocaine in decane and water, whereas α-tocopherol was practically insoluble in water. The effect of lidocaine on the curvature might be from both penetration into the tails, and penetration into the heads of the surfactant, from the part of lidocaine that is in oil and the water part respectively, and by that partially cancel each other. That is why the effect is not as strong as in α-tocopherol case. The bulkier carbon ring of α-tocopherol may increase the effect caused on the tail compared with just decane penetrating the tail.

3.8.2 Dynamic light scattering

When added to the microemulsion, α-tocopherol showed to have more influence than lidocaine, as it has been seen so far by the phase diagram. The figures 3.45 and 3.46 show the measurements of dynamic light scattering of microemulsions for some samples with the addition of this antioxidant and also are compared with lidocaine.

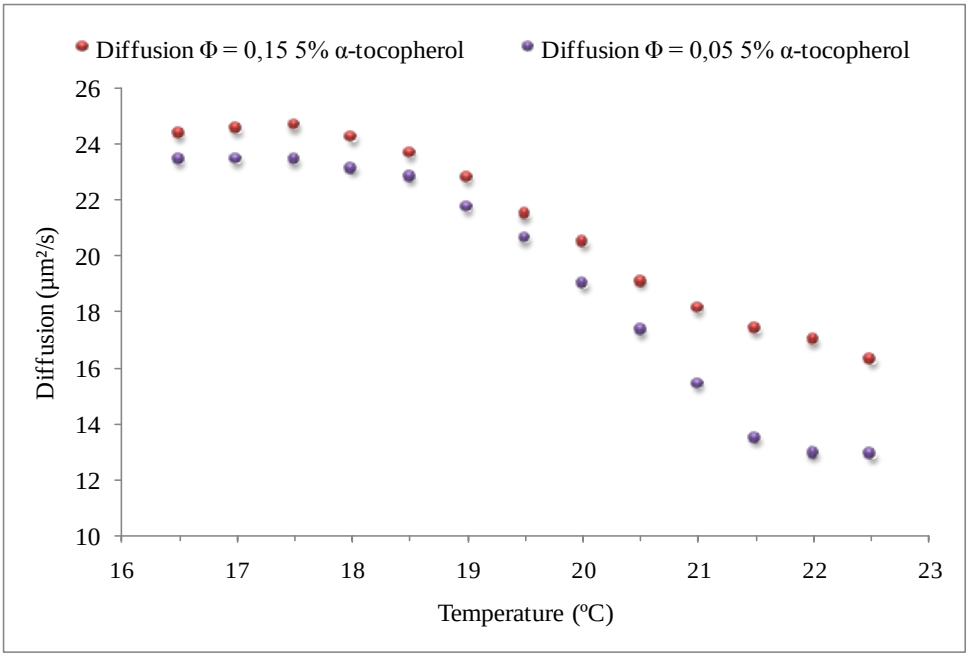


Figure 3.45 Diffusion versus temperature of the Φ = 0.05 and Φ = 0.15 microemulsion samples with 5% of α-tocopherol addition.

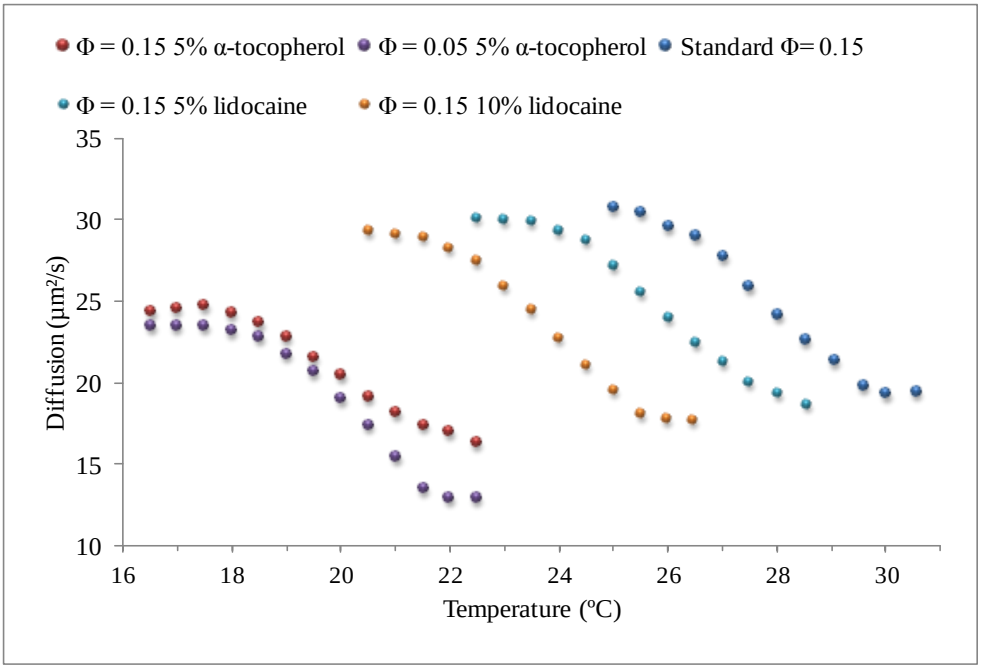


Figure 3.46 Diffusion versus temperature of the Φ = 0.05 and Φ = 0.15 microemulsion samples with 5% of α-tocopherol addition, Φ = 0.15 with 5% and 10% of lidocaine addition and also the standard Φ = 0.15 microemulsion.

Through the DLS results was possible to confirm that the "growth curves" of the micelles with temperature are similar in terms of inclination. However they grow at different temperatures.

A conclusion that can be made of these experiments is that one can take into account the hydrophobicity of the molecule that is being added to the microemulsion formulation to predict the greater or lesser effect on the temperature stability of the microemulsion phase.

As representative data, below (figures 3.47 and 3.48) are the size measures taken by DLS of one volume fraction (Φ = 0.15) with α-tocopherol addition over the temperature trend. The growth trends are similar to those obtained previously for lidocaine and 5-CQA addition and also for the standard system, thus the explanation is the same.

3.8.3 Size

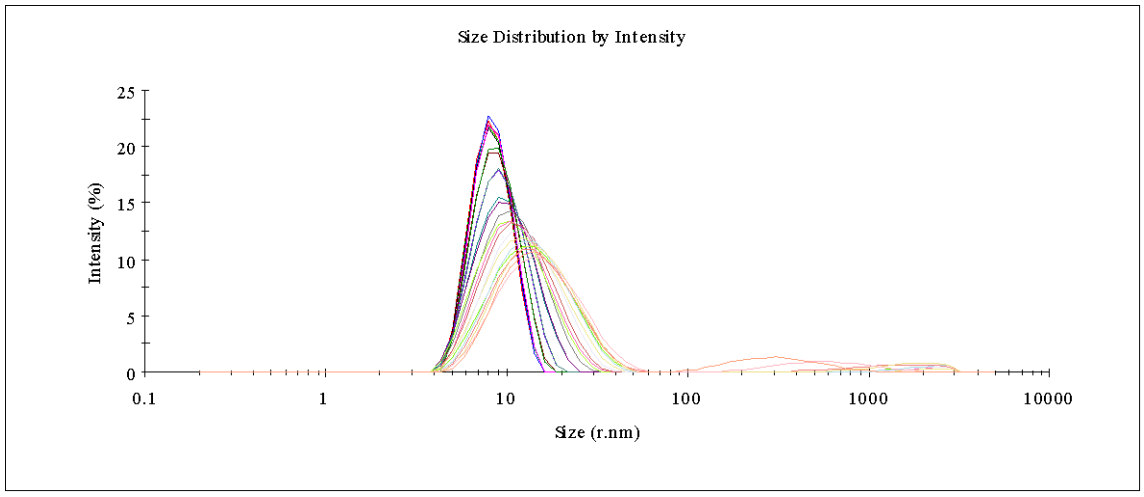


Figure 3.47 Size distributed by intensity, showing the behavior of the Φ = 0.15 microemulsion sample with 5% α-tocopherol addition, between 17 °C and 22 °C.

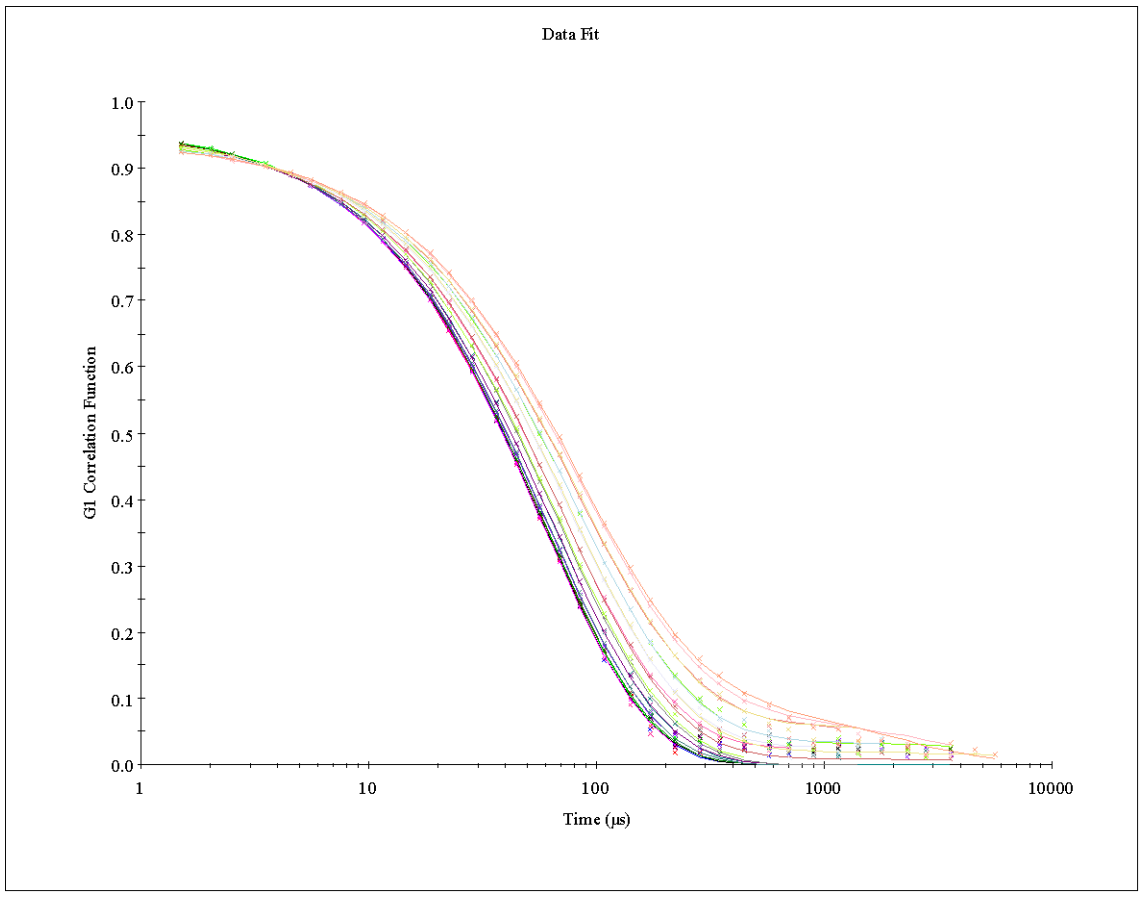


Figure 3.48 Correlation function (%) versus time (µs), showing the data fit of the Φ = 0.05 α-tocopherol 5% sample with increasing temperature.

As it was seen previously in the proposed model for the behavior of hydrophobic compounds addition (figure 3.49), an identical model is proposed for the effect of α-tocopherol addition to a microemulsion in figure below.

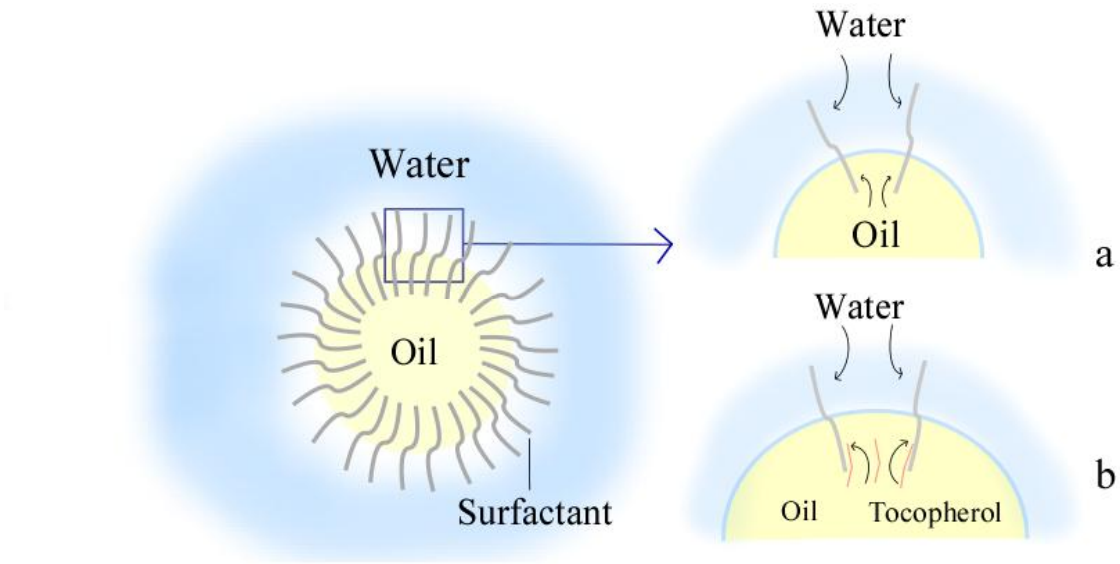


Figure 3.49 Proposed model for the effect of a hydrophobic antioxidant addition to a non-ionic microemulsion. On the left is represented the structure of microemulsions micelles (spheres) in the temperature range of microemulsion phase. On the right, there is a representative illustration of the effect of α-tocopherol addition to the microemulsion system. The arrows in picture **a** represent the tendencies or forces applied by water and oil molecules on the surfactant layer, while in **b**, with the presence of α-tocopherol represented in orange (in the internal surface of the droplets or surfactant film) there is big decrease of the curvature of the surfactant layer, thus contributing to increase the size. Note that this is a representative illustration, so the sizes of the molecules are not in scale.

3.9 Antioxidant activity - microemulsions

One of the future aims of this (model) microemulsion system is its development for use in biological systems, particularly in topical, dermal or transdermal application (in order to help protect the skin damages caused by free radicals or as an additive to protect another active compound). Thus it is of enormous interest to evaluate their antioxidant potential. This way we evaluated the antioxidant activity of both compounds used (chlorogenic acid and α-tocopherol) when incorporated into the microemulsion.

To evaluate the antioxidant activity, the capacity of an antioxidant to reduce the free radical *2,2-diphenyl-1-picrylhydrazyl* (DPPH method), was investigated.
Note: although several calibration curves were made, what we need to take into account is the exact initial value of DPPH concentration and the percentage that was reduced by an antioxidant. Since the DPPH and α-tocopherol have hydrophobic character, and 5-CQA is a hydrophilic molecule, means that the determination of single α-tocopherol and 5-CQA was made in different solvents. In this sense the beauty of the microemulsion system is to be able to join in, hydrophobic and hydrophilic compounds in one solution, thus its antioxidant capacity was evaluated.

3.9.1 5-CQA in bulk

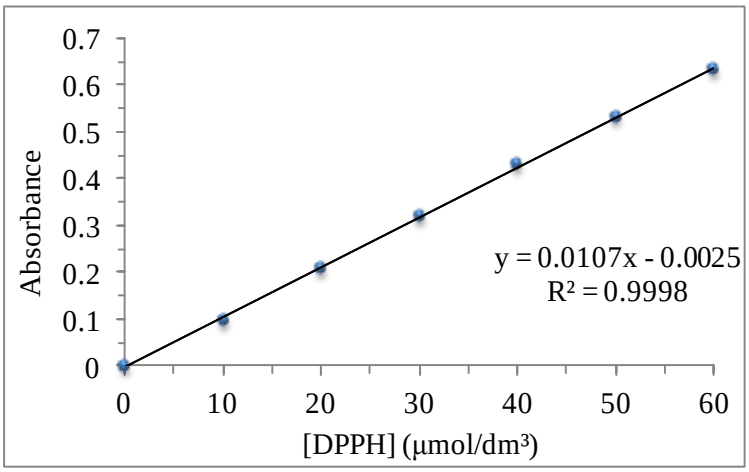


Figure 3.50 Calibration curve of DPPH in methanol. Absorbance (at 515 nm) versus DPPH concentration in µmol/dm^3. Each value is the mean of triplicate measurements.

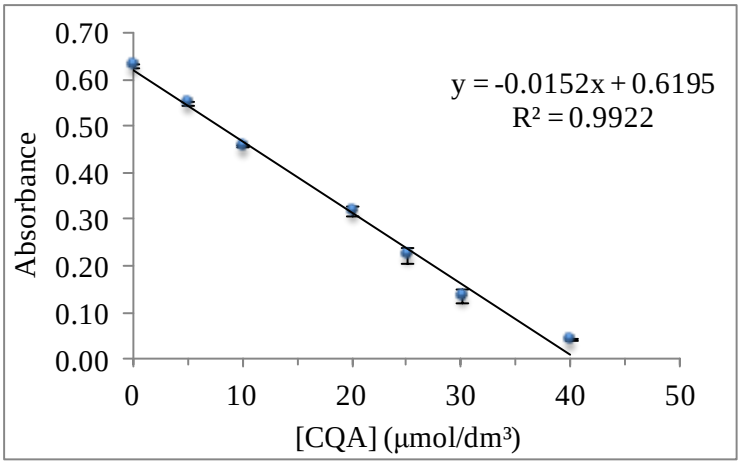


Figure 3.51 Reduction of DPPH with 5-CQA addition. Absorbance (at 515 nm) versus 5-CQA concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

Methods of expressing antioxidant activity appear to be as varied as the methods of measurement (*146*), thus for scientific comparison, the parameter that was chosen to express the antioxidant potential was EC50%, which corresponds to the required effective concentration (EC) of antioxidant molecule or extract, needed to reduce the DPPH concentration by half, and it was chosen to be expressed in g of antioxidant per kg of DPPH.

In order to calculate the EC50% value for 5-CQA in bulk it is needed:

$[DPPH]_{initial}$ = 60 µmol/dm^3.
$Absorbance_{inicial}$ = 0.6305 (± 0.019).
$Absorbance_{50\%}$ = 0.3153 (± 0.013)

From equation taken from the graphic of figure 3.51, it is possible to calculate the amount of 5-CQA that reduced 50% of DPPH:

$$[CQA]_{50\%} = \frac{0.6195 - Absorbance_{50\%}}{0.0152}$$

$[5\text{-}CQA]_{50\%}$ = 20 µmol/dm^3
$[5\text{-}CQA]_{50\%}$ = 0.0071 g/L
$[DPPH]_{initial}$ = 0.0237 g/L
By standardizing the units for comparison (g antioxidant/kg DPPH), we have,

$$EC50\% = \frac{[CQA]_{50\%}}{[DPPH]_{initial}}$$

✓ EC50% = 299,4 g 5-CQA/Kg DPPH.

Similar results were obtained by other authors (*147*)

3.9.2 α-tocopherol in bulk

The determination of antioxidant activity of α-tocopherol (in bulk) was made in decane, as it is the oil used in microemulsions.

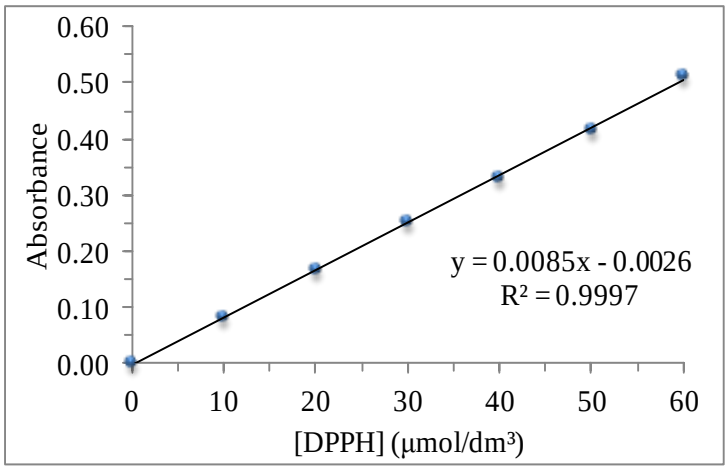


Figure 3.52. Calibration curve of DPPH in decane. Absorbance (at 515 nm) versus DPPH concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

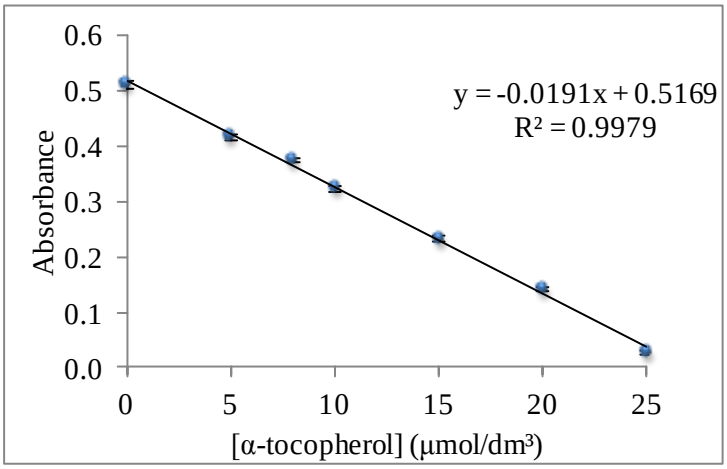


Figure 3.53 Reduction of DPPH with α-tocopherol addition. Absorbance (at 515 nm) versus α-tocopherol concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

Following the same reasoning made for CQA, we have,

$Absorbance_{inicial}$ = 0.5105 (± 0.021).

Absorbance$_{50\%}$ = 0.2553 (± 0.015).

$$[tocopherol]_{50\%} = \frac{0.5186 - Absorbance_{50\%}}{0.0192}$$

[α-tocopherol]$_{50\%}$ = 13,47 μmol/dm^3

[α-tocopherol]$_{50\%}$ = 0.0058 g/L

$$EC50\% = \frac{[\alpha\text{-}tocopherol]_{50\%}}{[DPPH]_{initial}}$$

- ✓ EC50% = 245,2 g α-tocopherol/Kg DPPH.

Similar results were obtained by other authors (*148*)

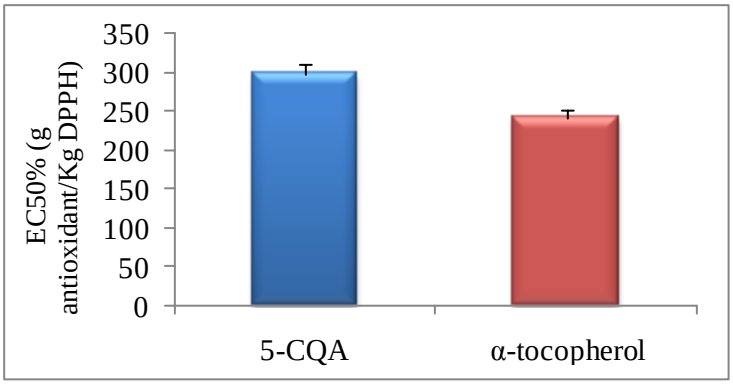


Figure 3.54 EC50% (g antioxidant/kg DPPH) versus chlorogenic acid and α-tocopherol. Each value is the mean of triplicate measurements.

Comparing the EC50% values of the two antioxidants obtained until now (in bulk), it is seen that the compound with the highest antioxidant activity is α-tocopherol, since it requires a smaller amount to reduce 50% of DPPH free radical, compared with the 5-CQA.

3.9.3 5-CQA in microemulsion

Note: The volume fraction chosen to study this antioxidant activity part was Φ = 0.05, since it showed the best results in general when comparing all samples. The diffusion results were the ones within the values that followed the more similar behavior to (hard) spheres; zeta potential showed higher and more reliable values, comparing the highest volume fractions. Thus the next responses given to the studies of antioxidant activity will be taken as model response.

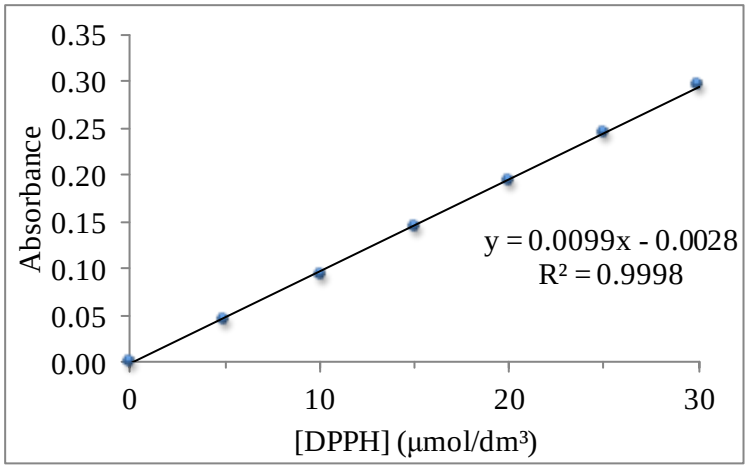


Figure 3.55 Calibration curve of DPPH in Φ = 0.05 microemulsion. Absorbance (at 515 nm) versus DPPH concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

In microemulsions, it was not possible to dissolve the amount of DPPH necessary to obtain the 60 µM in Φ = 0.05 microemulsion. Due to that fact the concentration of DPPH was reduced to half (and accordingly the amounts of 5-CQA and α-tocopherol).

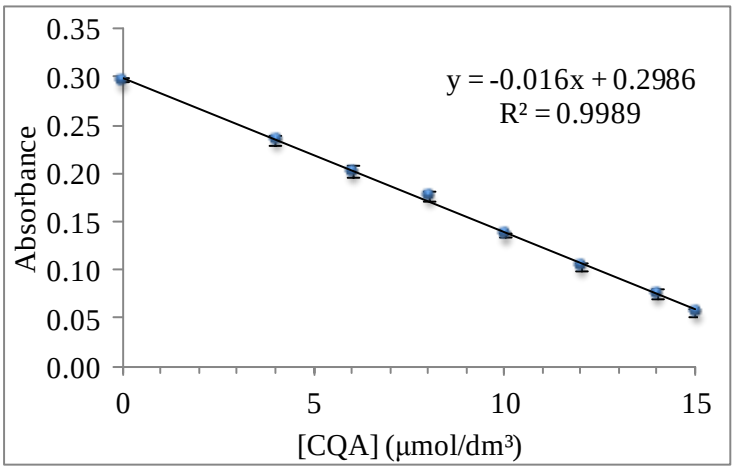


Figure 3.56 Reduction of DPPH with 5-CQA addition in Φ = 0.05 microemulsion. Absorbance (at 515 nm) versus 5-CQA concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

$Absorbance_{inicial}$ = 0.2986 (± 0.013).

$Absorbance_{50\%}$ = 0.1493 (± 0.017).

$$[CQA]_{50\%} = \frac{0.2986 - Absorbance_{50\%}}{0.016}$$

$[5\text{-CQA}]_{50\%}$ = 9,37 µmol/dm^3

$[5\text{-CQA}]_{50\%}$ = 0.0033 g/L

$[DPPH]_{initial}$ = 0.0118 g/L

$$EC50\% = \frac{[CQA]_{50\%}}{[DPPH]_{initial}}$$

✓ EC50% = 280.6 g 5-CQA/Kg DPPH.

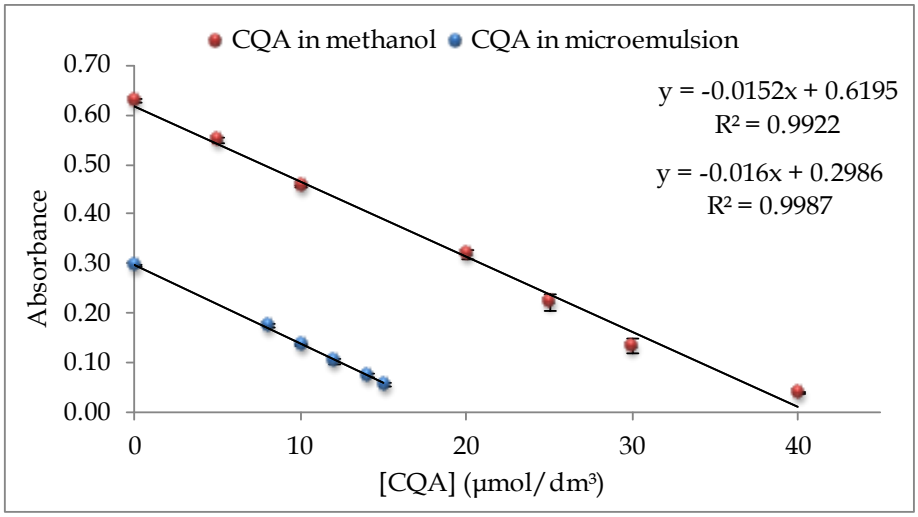


Figure 3.57 Reduction of DPPH with 5-CQA addition both in bulk and in Φ = 0.05 microemulsion. Absorbance (at 515 nm) versus 5-CQA concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

Observing the EC50% values for 5-CQA in bulk and in microemulsion, it appears that has not changed that much (increased 6,3%), so we can conclude that the antioxidant activity of 5-CQA when microemulsion undergoes a small positive increase (although close to the error, about 5%).

3.9.4 α-tocopherol in microemulsion

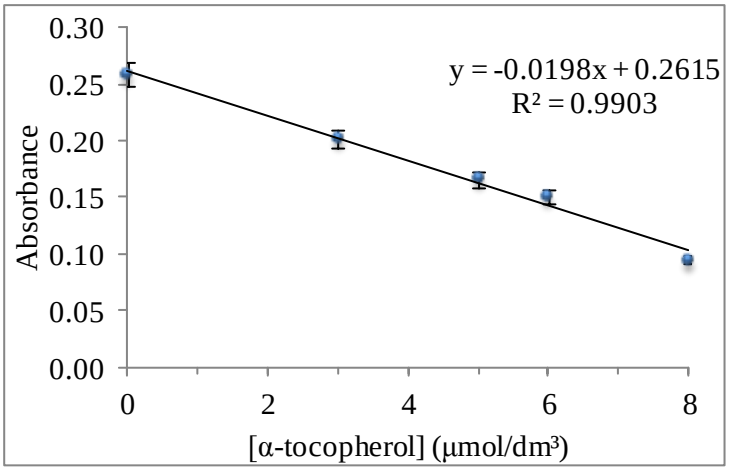


Figure 3.58 Reduction of DPPH with α-tocopherol addition. Absorbance (at 515 nm) versus α-tocopherol concentration (µmol/dm^3). Each value is the mean of triplicate measurements.

Following the same steps used for the previous calculations:

α-tocopherol concentration needed to reduce DPPH to Absorbance$_{50\%}$:

$$[CQA]_{50\%} = \frac{0.2615 - Absorbance_{50\%}}{0.0198}$$

[α-tocopherol]$_{50\%}$ = 6,68 µmol/dm^3

[α-tocopherol]$_{50\%}$ = 0.0029 g/L

[DPPH] $_{initial}$ = 0.0118 g/L

$$EC50\% = \frac{[\alpha\text{-}tocopherol]_{50\%}}{[DPPH]_{initial}}$$

- EC50% = 243,1 g α-tocopherol/kg DPPH.

Once again the EC50% values for α-tocopherol in bulk and in microemulsion are similar, meaning that being in microemulsion does not affect the antioxidant activity significantly (increased 0.1%).

3.10 Synergistic effect

3.10.1 5-CQA + α-tocopherol in microemulsion

Finally two antioxidants were used together in the microemulsion and the antioxidant potential of the whole system was evaluated. One of the advantages with this system can be the double protection from oxidation of a possible incorporated drug both within the oil or the aqueous phase. Other advantage (which might be explored in future studies) is the possibility of increasing the amounts of antioxidants and rationalize them, so that we can have the maximum amount of each and still have the microemulsion stable at temperatures close to room temperature, since it is now know that one antioxidant changes the temperature boundaries up (5-CQA) and the other down (α-tocopherol).

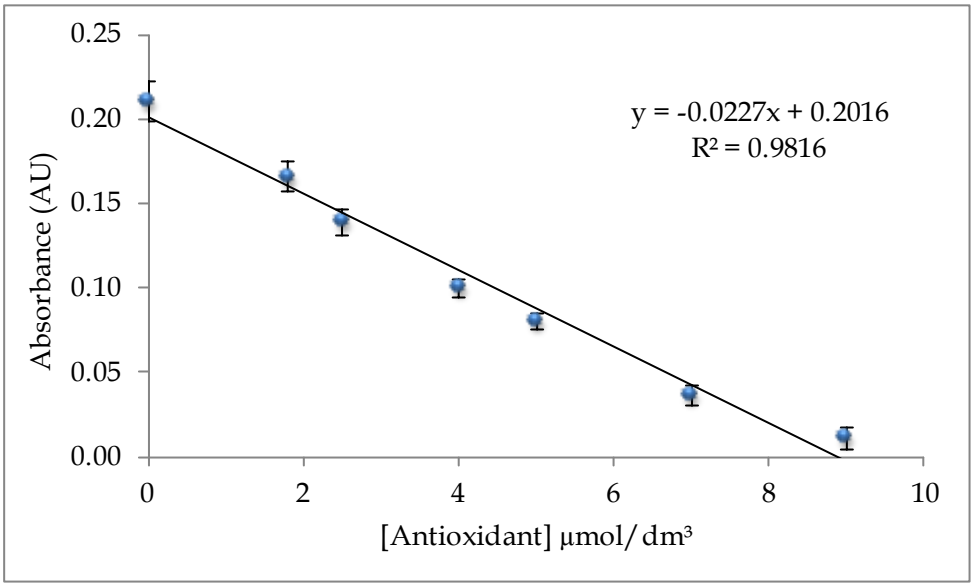


Figure 3.59 Absorbance (at 515 nm) versus antioxidant concentration (µmol/dm3). Reduction of DPPH with α-tocopherol plus 5-CQA addition (equimolar addition). Each value is the mean of triplicate measurements.

The antioxidant activity of individual antioxidants and in the system with both incorporated in microemulsion were related in order analyze if there is any synergistic effect, as previously reported with mixtures of polyphenols (*129, 149, 150*)

The synergistic effect occurs when the value of antioxidant activity obtained by the mixture is greater than the sum of the antioxidant activity values of the individual antioxidants. Thus, we examined the DPPH consumption of the mixture and compared the values with those that would be obtained from the sum of antioxidants individually.

Table 3.9 DPPH consumption (%) of 5-CQA in microemulsion (ME), α-tocopherol in microemulsion (ME) and the mixture of the two antioxidants in microemulsion. Concentration of the antioxidants is expressed in μmol/dm3, and percentage of DPPH consumption was taken from the initial and final absorbance values. Theoretical consumption equals to the sum of the two antioxidants individually and synergistic effect is the ratio between theoretical consumption and real mixture consumption. Each value is the mean of triplicate measurements and the standard deviation is represented in synergistic values.

	[5-CQA] in ME			
	1	2	2.5	3.5
DPPH consumption %	4.8%	10.2%	12.9%	18.3%
	[α-tocopherol] in ME			
	1	2	2.5	3.5
DPPH consumption %	6.4%	14.1%	17.9%	25.6%
	[5-CQA + α-tocopherol]			
	2	4	5	7
Theoretical consumption %	11.2%	24.3%	30.8%	43.9%
Mixture consumption %	26.1%	52.5%	61.9%	82.5%
Synergistic effect	42.9 ± 4.2%	46.2 ± 4.5%	49.8 ± 4.3%	53.2 ± 4.7%

As can be seen by the values of table 3.7, there is indeed a synergistic effect between this two antioxidants, 5-CQA and α-tocopherol, in about 46%, being this value also previously observed in a microemulsion system, not exactly the same, but similar (*129*).

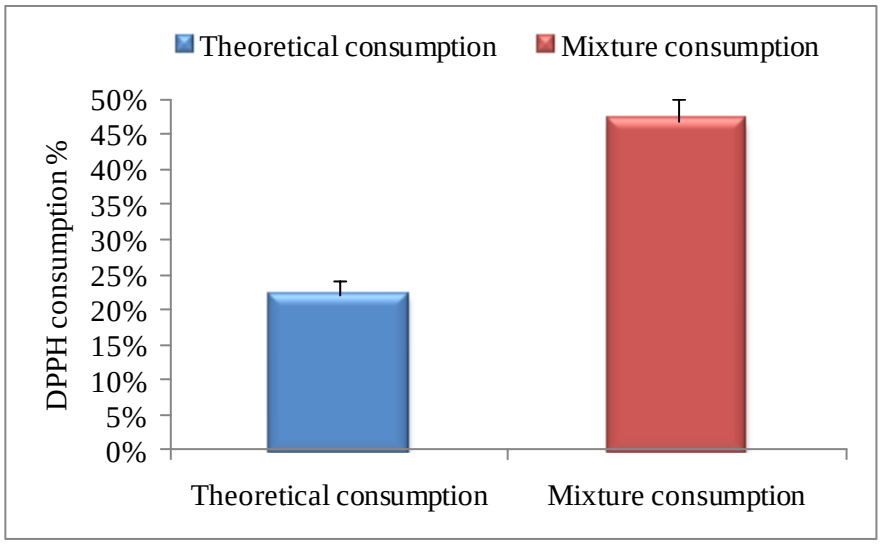


Figure 3.60 Percentage of DPPH consumption when mixture the two antioxidants (5-CQA and α-tocopherol) and theoretical consumption (according to individually antioxidant activities). Note that, it was used an average of the values of lower concentrations (2, 4 and 5 μmol/dm^3) of the mixture, since with higher concentrations the behavior is not so linear.

Observing the graphic in figure 3.60. it is possible to verify a synergistic effect (on average) resulting from the mixture of these two antioxidants in microemulsion system. With this result, one can affirm that this system may become even more effective by using these two antioxidants:

- ✓ Being one hydrophobic and the other lipophilic, they shift the temperature of the phase diagram in opposite directions, allowing dosing them rationally in order to get a stable microemulsion at room temperature and the maximum antioxidant potential;
- ✓ Using the two antioxidants together, we obtain a synergistic effect leading the system to have even greater antioxidant potential;
- ✓ Being one in the aqueous phase and the other in the oil phase (mostly), it is possible to obtain a double protection on a hypothetical case of incorporating a drug to use this system in drug delivery applications.

4. Conclusions

✓ With this long-term study (plants take months to grow) it was possible to analyze the responses to cold and drought stress imposed on coffee plants in terms of their phenolic composition. Although it was most noticeable for the Icatu and Obata genotypes (arabica), it was shown that, in fact, polyphenols have a clear role in the response to oxidative stress, since this interconnection was not yet fully clarified in other studies.

✓ It was restated the differentiation between *Coffea robusta* (Apoatã) and *Coffea arabica* (Icatu and Obatã) as to their percentage of phenolic compounds, ie, *Coffea robusta* is richer in polyphenols than *Coffea arabica*.

✓ It was contributed to the evidence that the Icatu and Obatã genotypes are more resistant to cold than Apoatã. However regarding the new factor studied (not previously studied in these varieties), the drought, it was found that in this case Icatu is more sensitive than Obatã and Apoatã.

✓ We conducted a comprehensive overview for identifying polyphenols existing in coffee leaves, since most previous studies were done on coffee beans.

✓ Although it has already been mentioned (only one reference) the presence of mangiferin in a certain coffee leaves specie (*Coffea pseudozanguebariae*), here it was discovered this new phenolic compound in these varieties. In addition and as a novelty of the work, it was also noted that mangiferin can be a possible biomarker to distinguish arabica from robusta (that are in wider use as they are grown commercially).

✓ The detailed analysis of the response profile of polyphenolic compounds showed that each individual profile is generally similar among all, meaning that they all work together in the defense to stress response. However some differences were reported regarding the 4.5-diCQA, that seems to have an essential role in Obatã in recovery from cold stress.

✓ It was made an interconnection study between the antioxidants present in the phenolic extract of coffee leaves and its possible incorporation into microemulsions systems, which can be used for applications in the cosmetic and pharmaceutical fields.

✓ It was concluded that the $C_{12}E_5$/decane/water system is suitable to study the influence of the addition of bioactive compounds or drugs in microemulsions. Works great as a model, since its composition is extremely well known and characterized, which is proven by the number of references. Although not a biocompatible system, it serves as a knowledge base to understand *a priori* what is the expected effect of adding a certain drug or antioxidant to a nonionic microemulsion.

✓ A general model for the effect of adding hydrophobic and hydrophilic components to the microemulsions system was proposed:

- A hydrophilic molecule (such as 5-CQA) shifts the temperature of the micelles phase upwards, by contributing to the increase of the micelles curvature.
- A lipophilic molecule (such as α-tocopherol and licocaine) shifts the temperature of the micelles phase down due to the decrease of the micelles curvature.
- The temperature change depends on the hydrophobicity or lipophilicity as well as other properties such as the effect it has on the tails of the surfactant of added components.

✓ The antioxidant activity of 5-CQA and α-tocopherol in and out of microemulsions was studied and discovered that this antioxidant potential is not changed significantly when comparing the two conditions.

✓ It was concluded that when mixed in, α-tocopherol and 5-CQA (and this is only possible in microemulsion) there is a synergistic effect (about 46%), which is a significant result if one wants to make a cosmetic formulation or drug delivery system, since now it is know that the antioxidant potential of this antioxidants is greater when they are mixed.

✓ This Master Dissertation resulted in a book chapter, a scientific article and in a presentation in poster at a national conference.

5. Future directions

Coffee is already a functional food (or rather drink), but in a futuristic perspective, we could study the possibility of using coffee leaves (taking advantage that this part is not used to make the drink) to extract these beneficial phenolic compounds and use them in several applications, as in the cosmetic area or as a supplementing for therapeutic uses. Moreover, genetically modified plants could be created with regard to polyphenols, maintaining their organoleptic properties, but increasing, both the plants defenses and the presence of bioactive compounds (similar study has been done) (*151*).

Knowing now what is the expected effect of adding a certain hydrophilic or lipophilic molecule to a system of microemulsions, the next step is to create a formulation with biocompatible components (surfactant and oil), and rationalize the balance between them to have the maximum amount of antioxidants. This way a much more effective formulation could be obtained with regard to protection from free radicals which we are all exposed to in everyday, thereby delaying the cellular aging process.

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