UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Entrapment of *Rosmarinus officinalis* polyphenols in redispersible lipid-based systems

Encapsulação de polifenóis de *Rosmarinus officinalis* em sistemas lipídicos redispersíveis

VICTOR OLORUNTOBA BANKOLE

Ribeirão Preto

2020

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Concentration Area: Medicaments and Cosmetics.

Supervisor: Prof. Dr. Wanderley Pereira Oliveira
Co-supervisor: Dr. Claudia Regina F. Souza

Versão corrigida da Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas no dia 28/04/2020. A versão original encontra-se disponível na Faculdade de Ciências Farmacêuticas de Ribeirão Preto/USP.

Ribeirão Preto
2020

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Victor Oloruntoba Bankole

Entrapment of *Rosmarinus officinalis* polyphenols in redispersible lipid-based systems. Ribeirão Preto, 2020.
179 p.; 30 cm.

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1. Proliposome 2. Encapsulation 3. Spray drying 4. *Rosmarinus officinalis* 5. Polyphenols 6. Antioxidant and antimicrobial activity 7. Stability studies

APPROVAL PAGE

Name: Victor Oloruntoba Bankole

Title: Entrapment of *Rosmarinus officinalis* polyphenols in redispersible lipid-based systems.

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Approved on: 28/04/2020

Examiners

Prof. Dr.: _____

Institution: _____ Signature: _____

Prof. Dr.: _____

Institution: _____ Signature: _____

Prof. Dr.: _____

Institution: _____ Signature: _____

Prof. Dr.: _____

Institution: _____ Signature: _____

This work was carried out in the Department of Pharmaceutical Sciences of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, in the Pharmaceutical Process Research and Development Laboratory (LAPROFAR)

Financial Support

CAPES

This thesis is dedicated to my parents; Chief Samuel Arinde Bankole and Mrs. Lydia Dupeola Bankole, for their very many sacrifices and labours, both physical and spiritual.

ACKNOWLEDGEMENT

I acknowledge God Almighty for His sure protection, provision, and presence in all things that I have laid my hands on till this day. I am so grateful because He took me from nowhere, brought me here, and has plans to take me somewhere much greater. He is the pillar of my life.

Many thanks to my supervisor, Prof. Dr. Wanderley Pereira Oliveira, who did not only offer me a space in his laboratory, but also guided me through the realization of this project. I deeply appreciate his dedication, patience, friendship, mentorship, understanding, words of encouragement, and the support given me from the first day.

To Dr. Cláudia Regina Fernandes de Souza my co-supervisor for her teachings, encouragement, support, and valuable friendship during the period of being together.

To Marcelo Luis Lombardi Martinez, the technician at LAPROFAR, for his constant help, support and friendship. His work was of great importance for carrying out this study.

To my colleagues and friends at the LAPROFAR group – Iara, Debora, Luciane, Andressa, Sirlene, Yan, and Leonardo; for the team work, inspiration, and mutual assistance during these years.

To the School of Pharmaceutical Sciences of Ribeirão Preto (FCFRP) – USP, for the opportunity to carry out this research project.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

To Prof. Dr. Sergio Luiz Salvador and Prof. Dr. Carolina Patricia Aires Garbellini both of FCFRP-USP; Prof. Dr. Luciano Bachmann of FFCLRP-USP; and their research groups for productive partnerships and collaborative work.

To Prof. Dr. Kamilla Swiech Antonietto, Prof. Dr. Fabio Bentes Freire, and Prof. Dr. Gustavo César Dacanal for their valuable contributions to this work during the qualification exam.

To the coordinators of the Graduate Program and the Committees, for encouraging academic excellence; to all members of staff at the graduate office of the FCFRP-USP for their attention and dedication always.

Special thanks to the Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria and all members of staff, for granting me this opportunity of study leave.

Very deep gratitude to my father, Chief S. A. Bankole, for being an inspiration all through my days; to my mother, Mrs. L. D. Bankole, from whom I have learnt to be who I am; and to my siblings for being a constant source of joy.

Special thanks to Pastor Marcelo Sampaio, his family and the entire church (*Igreja Essência Biblica*) for the brotherly love.

To all dear friends and family members without whom this venture would have been almost impossible - Abayomi Ogunjimi, Lakunle Jaiyesimi, Seni Akinrinade, Michael Osungunna, Price Udo Price, Temitayo Price, Ayoola Ajibola, Eduardo Moises Silva, Roberta Soares, Paulin Sonon, Ibrahim and Sally, Bright Anim.

To my lovely wife, Mrs. Yetunde Odunayo Bankole, for her support, understanding, encouragement, patience and prayers, and for believing in me; and to my kids: Aramitope Olamide, Arawande Olaoluwa, and Marcelo Akinola Oluwole, for having to bear the brunt of an “absent daddy”.

To all those whose names are not mentioned here, but who contributed in one way or the other to the success story related in the pages of this thesis.

My very sincere appreciation!

“A horse is prepared for the day of battle,
but victory comes from the Lord”
Proverbs 21:31 (HCSB)

RESUMO

BANKOLE, V.O. **Encapsulação de polifenóis de *Rosmarinus officinalis* em sistemas lipídicos redispersíveis.** 2020. 179 p. Tese de Doutorado. Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2020.

Rosmarinus officinalis L. (alecrim) compreende compostos polifenólicos, principalmente ácidos fenólicos e diterpenos que possuem excelentes propriedades antioxidantes e antimicrobianas. No entanto, obter o máximo de benefícios desse material é limitado por várias desvantagens; incluindo baixa solubilidade em água, biodisponibilidade e estabilidade, entre outros problemas. O encapsulamento de extratos vegetais em diferentes materiais é uma maneira confiável de melhorar suas propriedades físico-químicas e contornar os desafios. O encapsulamento em prolipossomas é particularmente interessante, tendo outras vantagens de incorporar múltiplos componentes de polaridade variável com estabilidade relativamente maior em comparação com formulações líquidas. Portanto, este estudo tem como objetivo preparar prolipossomas de polifenóis de alecrim com o desejo de encapsular compostos bioativos de polaridade variável e melhorar o escopo de sua aplicabilidade. Os polifenóis das folhas secas e moídas de alecrim foram extraídos por maceração dinâmica, filtrados, concentrados e liofilizados. Após estudos preliminares, as composições lipossômicas (usando fosfatidilcolina hidrogenada de soja e colesterol) encapsulando polifenóis de alecrim (ácidos cafeico, rosmarínico e carnósico e carnosol como marcadores) foram preparadas pelo método de substituição de solvente. As composições foram secas em um *spray dryer* em escala de laboratório a uma vazão de 4,0 g/min e temperatura de 100 °C, usando a lactose como adjuvante de secagem. As formulações de prolipossomas foram otimizadas por planejamento experimental, utilizando o Planejamento Composto Central, e validadas pela correlação de valores experimentais de atributos críticos de qualidade com valores preditos. Prolipossomas secas por *spray drying* (SDP) foram caracterizados pelo teor de umidade, atividade da água, retenção e conteúdo total dos polifenóis marcadores, densidade e propriedades de fluxo, cristalinidade, morfologia, espectroscopia no infravermelho, e redispersibilidade – incluindo tamanho da vesícula e potencial zeta após a hidratação. O desempenho de secagem foi caracterizado pela determinação da recuperação do pó. O SDP otimidado e o extrato liofilizado (LE) foram avaliados quanto às propriedades antioxidantes (método $DPPH^{\bullet}$) e antimicrobiano (antibacteriano e antifúngico). Um estudo de estabilidade foi realizado para avaliar o efeito da umidade e temperatura relativa no SDP e LE. As amostras de armazenamento foram analisadas de forma semelhante quanto a alterações nas propriedades físico-químicas. Os resultados das execuções experimentais mostraram que o SDP exibiu retenção de polifenóis, variando de 62,0 – 100,0% p/p; sendo dependente das variáveis de composição e lipofilicidade dos polifenois. A recuperação do SDP variou de 20,1 a 45,8 %, com teor de umidade e atividade da água entre 1,7 ± 0,14 - 2,5 ± 0,23% p/p e 0,30 ± 0,004 - 0,47 ± 0,003, respectivamente. As variáveis de composição influenciaram as propriedades do prolipossomas, com combinações ótimas de 4,26% p/p, 4,48% p/p e 7,55% p/p para a concentração de lipídeos, concentração de LE e a razão de adjuvante de secagem:(lipídio+extrato), respectivamente, em base úmida. Os resultados mostraram concordância entre os valores preditos e experimentais, exceto a retenção de carnosol, que foi 22 % menor. O SDP ideal apresentou alta atividade antioxidante com IC_{50} de 9,2 ± 0,2 µg/mL, superior aos resultados obtidos para LE (10,8 µg/mL) e hidroxitolueno butilado (BHT), um antioxidante sintético (12,5 µg/mL). MIC e MFC contra *Candida albicans* (ATCC1023) foram 312,5 µg/mL e 1.250 µg/mL, respectivamente, abaixo de valores obtidos para várias cepas de bactérias também avaliadas. A estabilidade do produto sofreu maior influência da umidade de armazenagem (em relação à temperatura), indicando a necessidade de armazenamento em embalagem impermeável. O SDP é mostrado como um excelente método para encapsular polifenóis hidrofílicos e lipofílicos de alecrim, gerando um produto inovador com propriedades físico-químicas e biológicas aprimoradas.

Palavras chave: Polifenois de alecrim, *Spray drying*, Prolipossomas, Atividade antioxidante, Atividade antimicrobiana, Estudos de estabilidade.

ABSTRACT

BANKOLE, V.O. **Entrapment of *Rosmarinus officinalis* polyphenols in redispersible lipid-based systems.** 2020. 179 p. Thesis (Doctoral). School of Pharmaceutical Sciences of Ribeirão Preto - Universidade of São Paulo, Ribeirão Preto, 2020.

Rosmarinus officinalis L. (rosemary) comprise polyphenolic compounds, principally phenolic acids and diterpenes which possess excellent antioxidant and antimicrobial properties. However, deriving maximum benefits from this material is limited by several drawbacks; including low solubility, bioavailability, and stability among others issues. Encapsulation of plant extracts in different materials is a credible way to improve their physicochemical properties and circumvent these challenges. Encapsulation in proliposomes is particularly interesting, having further advantages of incorporating multiple components of varying polarity with relatively higher stability compared to liquid formulations. Hence, this study aims at preparing proliposomes of rosemary polyphenols with a view to encapsulating bioactive compounds of varying polarity, thus enhancing the scope of their applicability. Polyphenol-rich extract from dried and milled rosemary leaves was obtained by dynamic maceration, filtered, concentrated and freeze-dried. Following preliminary studies, liposomal compositions (using hydrogenated soyphosphatidylcholine and cholesterol) encapsulating rosemary polyphenols (caffeic, rosmarinic and carnosic acids, and carnosol as markers) were prepared by a modified solvent replacement method. The compositions were dried in a lab-scale spray dryer at flow rate of 4.0 g/min and temperature of 100 °C, using lactose as the drying aid to obtain proliposomes. The proliposome formulations were optimized by experimental design, using the Central Composite Design, and validated by correlating experimental values of critical quality attributes with the predicted. Spray dried proliposomes (SDP) were characterized by moisture content, water activity, retention and total content of marker polyphenols, density and flow properties, crystallinity, morphology, infrared spectroscopy, and redispersibility – including vesicle size and zeta potential on hydration. The spray drying performance was characterized by determination of the powder recovery. The optimal SDP and lyophilized extract (LE) were evaluated for antioxidant ($DPPH^{\bullet}$ method) and antimicrobial (antibacterial and antifungal) properties. Stability study was carried out to evaluate the effect of relative humidity and temperature on SDP and LE. Storage samples were similarly analysed for changes in physicochemical properties. Results of experimental runs showed that SDP exhibited polyphenol retention, ranging from 62.0 – 100.0% w/w; showing dependence on composition variables and polyphenol lipophilicity. SDP recovery ranged from 20.1 to 45.8 %, with moisture content and water activity of 1.7±0.14 – 2.5±0.23 %w/w and 0.30±0.004 – 0.47±0.003, respectively. Composition variables influenced proliposome properties with optimal combinations being 4.26% w/w, 4.48% w/w, and 7.55% w/w for lipid concentration, LE concentration, and drying aid:(lipid+extract) ratio, respectively on wet basis. Results showed concurrence between predicted and experimental values except carnosol retention, being 22 % lower. Optimal SDP showed high antioxidant activity with IC_{50} of 9.2±0.2 µg/mL, superior to results obtained for LE (10.8 µg/mL) and Butylated Hydroxytoluene, a synthetic antioxidant (12.5 µg/mL). MIC and MFC against *Candida albicans* (ATCC1023) were 312.5 µg/mL and 1,250 µg/mL, respectively; lower than values obtained for bacteria strains used. The product stability was more affected by storage humidity (compared to temperature), indicating need for waterproof packaging. SDP is shown as a veritable tool to encapsulate hydrophilic and lipophilic rosemary polyphenols generating a product with improved physicochemical and biological properties.

Keywords: Rosemary polyphenols, Spray drying, Proliposomes, Antioxidant activity, Antimicrobial activity, Stability studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABS	Absorbance
ANOVA	Analysis of Variance
ANVISA	Agencia Nacional de Vigilancia Sanitaria (National Sanitary Surveillance Agency)
AS	Analytical Standard
AST	Accelerated Stability Testing
ATCC	American Type Culture Collection
A_w	Water Activity
BHA	Butylated Hydroxy Anisole
BHT	Butylated Hydroxy Toluene
CAF	Caffeic Acid
CAR	Carnosol
CCD	Central Composite Design
CH	Cholesterol
CIE	Comission Internacionale l'Eclairage
CLSI	Clinical and Laboratory Standards Institute
CMC	Carboxymethylcellulose
CNA	Carnosic Acid
C_s	Solid Content
CVA	Carvacrol
DLS	Dynamic Light Scattering
DoE	Design of Experiments
$DPPH^{\bullet}$	1,1-Diphenyl-2-picrylhydrazyl free radica
EO	Essential Oil
EU	European Union
FD	Factor of Dilution
FTIR	Fourier Transform Infrared
GAE	Gallic Acid Equivalent
GRAS	Generally Regarded as Safe
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
HPMC	Hydroxypropyl Methylcellulose
HSDP	Hydrated Spray Dried Proliposome

HSDP	Hydrated Spray Dried Proliposomes
HSPC	Hydrogenated Soy Phosphatidyl Choline
IC_{50}	50 % Inhibition Concentration
I_{Carr}	Carr's Index
$I_{Hausner}$	Hausner's Factor
KF	Karl Fischer
LA	Lactose
L_D	Loss on Drying
LE	Lyophilized Extract
LLF	Liquid Liposomal Formulation
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
NLC	Nanostructured Lipid Carriers
PDI	Polydispersibility Índex
PG	Propyl Gallate
PL	Phospholipid
PVP	Polyvinylpyrrolidone
QbD	Quality by Design
QE	Quercetin Equivalent
R_{EC}	Powder Recuperation
RH	Relative Humidity
ROA	Rosmarinic Acid
RSD	Relative Standard Deviation
RSM	Response Surface Methodology
SAS	Supercritical Anti-solvent
SD	Spray Drying
SDP	Spray Dried Proliposomes
SEM	Scanning Electron Microscopy
SLN	Solid Lipid Nanoparticles
T_E	Total Extractive Content
T_F	Total Flavonoid
Tg	Glass Transition Temperature
Tgi	Inlet Drying Gas Temperature

T_p	Total Polyphenol
US/USA	United States of America
USP	United States Pharmacopoeia
UV	Ultraviolet
Ws/Wmax	Feed Rate to Maximum Capacity Ratio
X_P	Moisture Content
XRD	X-Ray Diffraction
ZP	Zeta Potential
ρ_a	Apparent Density
ρ_c	Compaction Density

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1. Introduction

1 INTRODUCTION

Antioxidants are substances that, when present in low concentrations compared to that of an oxidisable substrate, significantly delay and/or inhibit the oxidation of that substrate or removes oxidative damage to a target molecule (YADAV et al, 2016; DAUQAN; ABDULLAH; SANI, 2011). They are capable of preventing the damaging effects of free radicals both in the human body and in various synthetic materials (IONITA et al., 2015). As such, they are suggested to play the physiological role of preserving the integrity of cellular components which may be compromised as a consequence of chemical reactions involving free radicals (BREWER, 2011; NIMSE; PAL, 2015).

A substantial body of evidence has been gathered to support claims that free radicals are involved in key roles during fundamental cellular reactions, suggesting that oxidative stress might be important in the pathophysiology of common diseases including atherosclerosis, chronic renal failure, and diabetes mellitus (DEVASAGAYAM et al., 2004; PERCIVAL, 1998). Antioxidants have also been found very useful in the food industry to prevent lipid peroxidation, the mechanism by which lipid components of nutrients become rancid (NEDOVIC et al., 2011). The general mechanism of an antioxidant action involves two stages: (i) the radical trapping stage and (ii) the radical termination stage (ANTOLOVICH et al., 2002; MASUDA et al., 2001; THATOI; PATRA; DAS, 2014).

With respect to how they are derived, antioxidants can be classified as either endogenous or exogenous. The former refers to those that are produced in the body (living tissues) as a component of innate defense mechanism; being largely composed of enzymes. The latter, however, refers to those that are incorporated from the external environment; they can either be synthetically derived or naturally sourced (YADAV et al., 2016). Although synthetic antioxidant compounds enjoyed patronage in the recent past, there is currently increasing interest in the use of natural antioxidants, such as tocopherols, flavonoids and plant polyphenols as additives in foods and pharmaceutical systems (FRUTOS; HERNÁNDEZ-HERRERO, 2005; HERNÁNDEZ et al., 2009; KAMKAR et al., 2014; WILLIAMS; SPENCER; RICE-EVANS, 2004).

Like other naturally sourced antioxidants, extracts of some aromatic plants have been favourably applied as functional materials in these systems due to their biocompatibility, multifunctionality, relative affordability and/or availability (CHEN et al., 2011; CHRISTAKI et al., 2012; OZSOY ET AL., 2017; SHAIKH et al., 2014), as well as avoidance of toxicity problems associated with the use of synthetic antioxidants, such as butylated hydroxy anisole

(BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG) which are now known to form hazardous quinones (AMAROWICZ; NACZK; SHAHIDI, 2000; IONITA et al., 2015; KAMKAR et al., 2014). The market for antioxidants of vegetable origin is expected to experience a huge growth just as demands for natural products are generally on the increase (LACATUSU et al., 2010a). This preference may be sustained following evidence that antioxidant effects of natural extracts are often more effective than many other individual antioxidants (RUKTANONCHAI et al., 2009).

Research into polyphenols, a class of antioxidants of natural origin, started more recently than others (SCALBERT et al., 2005) but has garnered much momentum over the years (CORTÉS-ROJAS; SOUZA; OLIVEIRA, 2016; SECOLIN; SOUZA; OLIVEIRA, 2017). Currently, plant polyphenols have received high attention of the pharmaceutical, nutraceutical, cosmetic, and food sectors mainly due to their attributed biological activities (HABTEMARIAM, 2016a). They have indeed proven to be very worthy antioxidants with evidence of protection of cell constituents against oxidative damage, therefore, limiting the risk of various degenerative diseases associated to oxidative stress such as cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, age-related skin damage, and osteoporosis (ARTS; HOLLMAN, 2005; SCALBERT et al., 2005; VITA, 2005).

One of such plants whose extract has been studied and found to be rich in polyphenols is *Rosmarinus officinalis* L. (Lamiaceae) commonly called rosemary or romero. The rosemary is a polyphenol-rich herb, supporting its use as preservative and antioxidant in cosmetics, foods and other multi-component systems, as well as herbal remedy in protection from and management of various diseases.

Native to the Mediterranean, rosemary is cultivated in many parts of the world as a valuable household spice for flavoring and preserving foods, and as herbal drug in folk medicine (MORENO et al., 2012; SÁNCHEZ-CAMARGO; HERRERO, 2017). While folkloric uses include as antispasmodic and hair growth stimulant, the plant has been scientifically evaluated for a wide range of activities such as neurologic, anti-inflammatory, antidiabetic, hepatoprotective, antitumerogenic, antimicrobial (including antiviral) and antioxidant activity, as well as its potential to relax the smooth muscles of the trachea and intestine (HASSANI; SHIRANI; HOSSEINZADEH, 2016). Its biological activities are linked to high concentrations of phenolic compounds in three main classes, among others: phenolic diterpenes – e.g. carnosic acid, carnosol, rosmanol, epirosmanol, and methyl carnosate; flavonoids – e.g. cirsimaritin, genkwanin; and phenolic acids – e.g. rosmarinic and caffeic acids (ANDRADE et al., 2018; GENENA et al., 2008; LAURA; GARZON; VICENTE, 2010;

MORENO et al., 2006; SOUZA et al., 2008; ZHANG et al., 2012). Specifically, its high antioxidant activity is attributable to carnosic, caffeic, and rosmarinic acids; carnosol; and the flavonoids (LUIS; JOHNSON, 2005; TAVASSOLI; DJOMEH, 2011; THORSEN; HILDEBRANDT, 2003). In fact, it is suggested that rosemary has enjoyed the greatest level of attention among herbs and spices as source of antioxidants (ERKAN; AYRANCI; AYRANCI, 2008; HASSANI et al., 2016; MORENO et al., 2012). Indeed, the rosemary extracts are commercially available for use as a natural antioxidant for foods, being considered safe and effective (NIETO; ROS; CASTILLO, 2018).

It is known that the extraction procedure (e.g. extraction method, duration, temperature, solvent type, and so on) has significant influence on the composition of a plant extract and, consequently, its antioxidant – and other biological – activity (DELFANIAN et al., 2015; DO et al., 2014; PIETRZAK; NOWAK; OLECH, 2014; TIR; DUTTA; BADJAH-HADJ-AHMED, 2012). Biological activity may further be influenced by loading plant extracts in different materials, causing a reduction or an outright prevention of degradation of the active principles as well as controling their availability in biological systems (IONITA et al., 2015; VISENTIN et al., 2012a). This observation is particularly true for polyphenols whose high scavenging properties towards radical oxygen species make them susceptible to degradation reactions during storage due to several factors such as heat, humidity exposure and processing conditions, thereby impairing their long-term stability (DENG et al., 2018; GAFNER; BERGERON, 2005; VOLF et al., 2013; ZHANG et al., 2012).

Similar to other bioactive substances, encapsulation of plant extracts in different materials is a credible way to improve their physicochemical properties and to slow down the degradation rates of their main active constituents. The improvement of bioavailability of the bioactive compounds in biological systems has also been reported (IONITA et al., 2015; VISENTIN et al., 2012a). In the literature, polyphenols of rosemary and other plants have been encapsulated in solid lipid nanoparticles and similar systems (CAMPOS et al., 2017; GUPTA; SHARMA, 2006; SECOLIN et al., 2017; SECOLIN, 2014), mostly with primary focus on encapsulation of carnosic acid rather than various compounds (VISENTIN et al., 2012). Because food and pharmaceutical systems are often complex in nature, ensuring incorporation of multiple bioactive compounds for their synergistic activity is essential to optimal protection and activity. However, these previously developed systems have the limitation of failure to accommodate compounds of varying polarity; a situation which might impair product activity and stability, among other considerations.

Proliposome is an innovative approach to retain these compounds of varying lipo/hydrophilicity in the same formulation system. Proliposomes are dry, free-flowing powders usually developed from phospholipids together with cholesterol and other excipients, compositions that can immediately form liposome suspension through simple redispersion of these systems in aqueous medium (GANGISHETTY; EEDARA; BANDARI, 2015; XU et al., 2009). Their solid properties confer an improvement on the otherwise challenging physical stability of liposomes without influencing their intrinsic characteristics (KARN et al., 2014; NEKKANTI et al., 2016).

An attempt to encapsulate plant polyphenols in this type of structure is an attractive exercise following from many considerations. Firstly, encapsulation is a promising approach towards protecting polyphenols as well as improving their physicochemical properties and their functionality (KUMARI et al., 2014; MUNEER et al., 2017). Secondly, compounds of varying polarity can be retained in the liposomal system. While the hydrophilic core provides suitable ambient and protection for more polar compounds, the hydrocarbon complex in the liposomal wall can be explored to accommodate polyphenol compounds that exhibit lipophilicity (MUNEER et al., 2017). Moreover, polyphenols often present a poor bioavailability mainly due to low water solubility (BELŠČAK-CVITANOVIĆ et al., 2018; PICCOLELLA; PACIFICO, 2017). Lastly, many of these molecules possess a very astringent and bitter taste, which might limit their use in food or in oral medications (FANG; BHANDARI, 2010; KALOGEROPOULOS et al., 2010; MUNIN; EDWARDS-LEVY, 2011; NEDOVIC et al., 2011). The proliposome encapsulation approach had been employed in the formulation of different compounds of natural origin (CHU et al., 2011; HAO et al., 2015; JAISWAL, 2013; SILVA et al., 2017; WANG et al., 2015; ZHENG et al., 2015).

To this end, spray drying has been widely used both on bench-top scale and industrially, because in addition to producing stable systems in the form of powders; reducing the risks of microbial contamination; and transport and storage costs, it is a robust method that allows the optimization of particle characteristics, which can be used in thermo-sensitive products, in addition to having low operating costs (OLIVEIRA; FREITAS; FREIRE, 2009; INGVARSSON et al., 2011; INGVARSSON et al., 2013). It is therefore, considered suitable for the preparation of proliposomes encapsulating rosemary polyphenols.

Nevertheless, the production of proliposomes by spray drying is a multivariate process. The physicochemical properties of product are affected by composition variables and spray drying operating conditions (PATIL-GADHE; POKHARKAR, 2014). Understanding the effects of these multiple input variables on product properties is an important step towards

consistently engineering a product with preset requirements (LIONBERGER et al., 2008; TELFORD, 2007; VERMA et al., 2009). Rather than assessing the effect of each input variable on desirable outcomes per time, the Design of Experiment (DoE) is an efficient methodology usually adopted to simultaneously determine the effects of multiple variables on product properties. (CZITROM, 1999; JAIN et al., 2015; KASINATHAN; VOLETY; JOSYULA, 2014). This way, products with optimal physicochemical and biological properties can be prepared in a rapid, cost effective and reproducible manner (BAN et al., 2017; KURHAJEC et al, 2017).

This work presents a systematic study that aimed to stabilize and improve the functionality of rosemary polyphenols by employing the spray drying method to develop an optimal proliposome product. This is with a view to preparing an innovative product which simultaneously retain polyphenols of varying polarity contained in the rosemary extract. Antioxidant and antimicrobial properties were evaluated to highlight the potential for application of developed proliposome in food, pharmaceutical, nutraceutical and cosmetic products.

2. Literature Review

2 LITERATURE REVIEW

2.1 Products of natural origin for medicinal purposes: current realities

Currently, the demand for herbal or medicinal plant products throughout developed and developing countries has increased significantly. According to a World Health Organization survey, about 70 – 80 % of the world population in developing countries, due to the poverty and lack of access to modern medicine, rely essentially on non-conventional medicine in their primary healthcare (SASIDHARAN et al., 2011). This strategy is mostly based on the use of medicinal plant and their derivatives, known with various names such as: botanicals, herbal medicines, phytomedicines, etc (CALIXTO, 2000). Presently, about 25 – 30 % of all drugs available as therapeutics are derived from natural products (plants, microbes and animals).

Although only few plants have been scientifically studied for the assessment of their quality, safety and efficacy, the development of standardized phytomedicines demands considerably lower funds and seems to be perfectly feasible in underdeveloped countries compared to developing a new drug by synthesis, or even from the prototype of a natural source which require huge resources. In certain countries such as Brazil, China, India, and Nigeria, phytomedicines are registered as drugs, albeit the companies need to prove their safety, quality and efficacy based on scientific information (CALIXTO, 2000).

Nowadays, the premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation (SASIDHARAN et al., 2011). In the light of this, whole plants, animal parts, and even microorganisms initially employed in unmodified form have largely been replaced by extract concentrates (liquid or solid form) of active substances or chemical precursors and attenuated organism to improve their intensity, uniformity of action and lower possibility of unwanted effects.

2.2 Plant extracts: classification, composition, and potential applications

Bioactive compounds in plants are described as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Secondary metabolites are produced within the plants alongside the primary biosynthetic and metabolic routes which produce compounds associated with plant growth and development. Some primary plant metabolites such as vitamins and minerals elicit pharmacological or toxicological effects when

ingested at high dosages. They are regarded as products of biochemical "side tracks" in the plant cells and not needed for the daily functioning of the plant (PAULSEN, 2010).

These phytoactive components are classified according to different criteria, one of which is by their pharmacological effects. In this sense, classes of bioactive compounds include glycosides, alkaloids terpenoids, anthraquinones, tannins and flavonoids. Although these phytochemicals are capable of eliciting changes in physiological functions of man and animals, most of them hold important functions in the living plants, including in plant protection, attraction and chemical signalling. For example, terpenoids may attract pollinators or seed dispersers, or inhibit competing plants; alkaloids usually ward off herbivore animals or insect attacks (phytoalexins); while flavonoids can protect against free radicals generated during photosynthesis (PAULSEN, 2010).

Flavonoids are a family of polyphenolic compounds which are widespread in vegetable materials and consumed as part of the human diet in significant amounts. Dietary polyphenols are a main source of antioxidants, possessing varieties of biological activities, ranging from anti-ageing, anti-inflamatory, anticancer, to lowering of blood cholesterol level and improving bone strength. These polyphenolic compounds are richly distributed in a wide species of plants, one of which is *Rosmarinus officinalis*. The relative abundance of polyphenolic compounds in this plant specie is established in the literature.

To obtain bioactive phytopharmaceutical ingredient from herbal sources, the raw material needs to be submitted to an extraction process. Several extraction procedures can be employed, ranging from percolation, maceration, dynamic maceration, to modern techniques such as ultrasound or microwave assisted, and supercritical fluid extractions. These processes generate an extractive solution containing the compounds of interest.

2.3 Rosmarinus officinalis

Rosmarinus officinalis L. (Lamiaceae) commonly known as rosemary or romero is a perennial shrubby herb, natural to the Mediterranean basin; including countries like Portugal, Spain, Morocco, Tunisia and Italy. For thousands of years, it was primarily used as a culinary spice for flavouring due to its aromatic nature, but now widely cultivated and applied in alternative medicine and natural therapies, cosmetics and pharmaceuticals (ANDRADE et al., 2018; HUSSAIN et al., 2010; ZU et al., 2012; FADEL et al., 2011).

Rosemary has been widely studied as a natural source of bioactive compounds with diverse biological activities and potential applications (VISENTIN et al., 2012). In addition to continuous use of rosemary in food flavouring, its extracts, essential oils and isolated chemical constituents have demonstrated interesting biological activities, including antioxidant, antimicrobial, anti-inflammatory, anti-tumorigenic, anti-allergic, metal chelating, chemo-preventive and cytoprotective activities (BORRAS-LINARES et al., 2014; COUTO et al., 2012; SÁNCHEZ-CAMARGO; HERRERO, 2017; SUI et al., 2012; LIU et al., 2011; WANG et al., 2004). This make them suitable candidates as bioactive ingredients to develop functional cosmetics, pharmaceutical and food products (VISENTIN et al., 2012; ZU et al., 2012; PSOTOVA et al., 2006).

The extract of rosemary is an established source of various bioactive compounds, the most prominent class arguably being polyphenols (ERKAN et al., 2008; COUTO et al., 2012; MORENO et al., 2012; HASSANI et al., 2016). Other group of compounds found in the extract include tannins, polysaccharides, triterpenic acids, volatiles, as well as some protein matter and lipophilic substances. Polyphenols are a large group of at least 10,000 different compounds with phenolic structural features i.e. one or more aromatic rings with one or more hydroxyl groups attached to them. They are among the most widespread classes of secondary plant metabolites in nature with almost ubiquitous distribution and abundance in majority of fruits and vegetables.

The major compounds found in rosemary extracts are phenolic acids such as rosmarinic acid and caffeic acid; phenolic diterpenes such as: carnosic acid, carnosol or rosmanol; flavonoids such as genkwanin, cirsimaritin or homoplantaginin; and triterpenes such as ursolic acid (LUIS; JOHNSON, 2005; TAVASSOLI; DJOMEH, 2011; THORSEN; HILDEBRANDT, 2003).

Like many other drugs of vegetable origin, variations exist in the constituent compounds of rosemary arising from difference in geographical location, seasonal considerations, abiotic stress, phenological stages of the plants, processing and handling, among others (REZA; MOGHADAM, 2015). For example, the concentration of carnosic acid and other diterpenes in rosemary could vary due to a number of environmental factors (e.g. sun light intensity and water stress) and growth conditions as well as genetic factors. These differences in the content and concentration of constituent compounds in a plant, together with the varying extraction and processing techniques used by different manufacturers, lead to variability in the quality of phytopharmaceuticals.

Evidence of bioactivity (CHRISTAKI et al., 2012) and potential applicability of such extracts in the agricultural (OZCAN, 2003), food (AGUILAR et al., 2008; PEREIRA et al., 2017), nutraceutical (CELIKTAS; BEDIR; SUKAN, 2007; MORENO et al., 2012; GAD; SAYD, 2015), cosmetic (LACATUSU et al., 2010), and pharmaceutical industries (BREWER, 2011; GIRD et al., 2017) is well documented. There are evidences supporting application of rosemary extracts for wound healing (ALIZARGAR et al., 2012) and other biological uses (RIBEIRO-SANTOS et al., 2015; SANCHEZ-CAMARGO; HERRERO, 2017).

2.4 Biological activities of rosemary

Rosemary is considered an interesting aromatic plant for its content of bioactive compounds that have putative benefits on human health. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction (essential oil component) and the phenolic constituents (ARRANZ et al., 2015; BABOVIC et al., 2010; TEIXEIRA et al., 2013). The whole plant possesses various lines of biological activities which have been variously evaluated by workers.

2.4.1 Antioxidant activity

It is suggested that rosemary has enjoyed the greatest level of attention among herbs and spices as a source of antioxidants (HASSANI et al., 2016) among its many other related activities. Scientific data puts rosemary among the spices with highest antioxidant activities of all the herbs and spices previously investigated (WANG et al., 2008). This relative superiority of antioxidant activity of rosemary extract is related to its high content of polyphenolic compounds (COUTO et al., 2012; DEL BANO et al., 2003), being chiefly the phenolic diterpenes; carnosic acid and carnosol, and the phenolic acids such as rosmarinic acid and caffeic acid, albeit to a lesser degree (ZIBETTI et al., 2013; CARVALHO et al., 2005; DEL BANO et al., 2003).

Due to its safety profile and efficacy, the European Union (EU) has approved the use of rosemary extract (E392) as a natural antioxidant for food preservation (Source: www.food.gov.uk/science/additives/enumberlist - Current EU approved additives and their E Numbers, Food Standards Agency 2016). Owing to its antioxidant activity, rosemary extract has been used as a natural preservative to improve the shelf life of perishable foods (HABTEMARIAM, 2016b), with current applications including dairy products (GAD; SAYD, 2015), vegetable oils (DIAS; MENIS; JORGE, 2015), other food materials and products (de RAADT et al., 2015; PEREIRA et al., 2017), cosmetics and phytomedicines (ALIZARGAR et

al., 2012; CELIKTAS et al., 2007; GAD; SAYD, 2015; GIRD et al., 2017; OZAROWSKI et al., 2013). The total content of the phenolic diterpenes (carnosic acid and carnosol), being the principal constituents responsible for the antioxidative properties have been described as the distinct feature of any commercially available extract of rosemary intended for use as antioxidant (SRINIVASAN; KAWAMURA, 2016).

Several *in vitro* studies regarding the antioxidant activity of the whole extract of rosemary and its main isolated compounds have been carried out using different methods, including the 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), the thiobarbituric acid, superoxide anion, lipid free radicals scavenging activity assays, and Rancimat – determination of oxidative stability of fat (ANDRADE et al., 2018; SECOLIN, 2014). Such studies have shown the antioxidant potential of rosemary phytochemicals, often determined as ability to neutralize reactive oxygen species, a property closely related to other biological activities, such as cytoprotective and anticancer properties.

Rosemary extract rich in carnosic acid and its derivative carnosol are capable of interfering with lipid oxidation by donating hydrogen atoms to lipid free radicals. These materials have shown intrinsic superior capacity to donate hydrogen atoms (H$^{\bullet}$) to lipid free radicals compared to synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT). This is due to the fact that each molecule of the phenolic diterpenes has two O-phenolic hydroxyl groups, located at C11 and C12 positions of catechol moiety, serving as better hydrogen donors comparted to their synthetic counterparts, each with a single hydroxyl group (SENANAYAKE, 2018). Moreover, the hydroxyl groups present in carnosic acid and carnosol molecules have the ability to chelate prooxidant metal ions, and thus help delay lipid oxidation via a secondary mechanism. Studies have also shown that phenolic diterpenes of rosemary are capable of scavenging other types of free radicals including peroxyl, hydroxyl and DPPH$^{\bullet}$ radicals.

It is known that the antioxidant activity of carnosic acid against lipid free radicals stretches to its second-step decomposition product, rosmanol, indicating a long lasting activity and thus making rosemary extract a viable alternative in lipid based food systems, cosmetics products, and pharmaceutical preparations of preventive and treatment medicines (ANDRADE et al., 2018; SENANAYAKE, 2018). Rosmarinic acid is also found in abundance in many prepared extracts and is well noted for various biological properties, including antioxidant activity (SUI et al., 2012; LIU et al., 2011; LUIS et al., 2007; COUTO et al., 2012; PSOTOVA

et al., 2006; WANG et al., 2004). Thus, it is also considered an important chemical marker of this species.

2.4.2 Antimicrobial activity

Rosemary is also known for its powerful antimicrobial - activity and is widely used today as food preservative (PAULI; SCHILCHER, 2010). Besides the antibacterial properties, its essential oil also has insecticidal, antiparasitic and antifungal activities, making it an important tool in microbial diseases and against infective agents.

In vitro studies measuring minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill dynamic processes exists in the literature. For example, Andrade and coworkers reported some individual antimicrobial activity and a possible synergistic effect between the bioactive compounds – carnosic acid, carnosol, rosmarinic acid, oleanolic acid, ursolic acid – of the extract and the essential oil, against Gram-positive bacteria (e.g. *Staphylococcus* and *Bacillus species*), Gram-negative bacteria (*Proteus vulgaris*, *Pseudomonas aeruginosa* and *Escherichia coli*), and fungi (*Candida albicans* and *Aspergillus niger*) (ANDRADE et al., 2018). Carnosic acid showed antiviral activity against human respiratory syncytial virus (SHIN et al., 2013).

Whereas aqueous extract of rosemary, containing exclusively rosmarinic acid, showed a low efficacy against bacteria strains (MORENO et al., 2006), extracts of organic solvent such as methanol and acetone (MORENO et al., 2006), and supercritical carbon dioxide (GENENA et al., 2008) showed good antimicrobial activity against both Gram positive and Gram negative bacteria, and yeast in correlation to its carnosic acid/carnosol content.

2.4.3 Other activities

Several biological activities of whole rosemary plant, its parts and isolated compounds against several diseases (e.g. cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, age-related skin damage, and osteoporosis) have been evaluated by many workers and extensively reviewed by RIBEIRO-SANTOS et al. (2015).

2.5 Previous and current studies on development and processing of rosemary

The conventional solid-liquid extraction techniques have been used to extract bioactive compounds such as flavonoids, phenolic diterpenes and triterpenes from dried milled leaves of rosemary, as for several other plants. Although newer extraction methods have been developed, the solvent extraction method remains relevant when considerations are given to ease of

manipulation, cost-efficiency ratio, and access to basic infrastructure in developing areas. It is also true that solvent extraction remains a common practice amongst the population for routine exploitation of these plant benefits.

For industrial purposes, liquid extracts are usually concentrated and dried, resulting in powder product with higher concentration of active compounds. Dried extracts have several advantages over the liquid forms: including higher stability and relative ease of handling, standardization, transportation, and storage (OLIVEIRA; BOTT; SOUZA, 2006). Moreover, dried extracts allow the manufacture of solid dosage forms, like tablets and capsules, which represent most of the medicines used worldwide (LEUENBERGER; LANZ, 2005). In the light of the above advantages, among others, rosemary extracts have been prepared and processed by various methods to obtain dry, free flowing powder concentrates of its bioactive compounds (BENELLI; SOUZA; OLIVEIRA, 2013; CHAUL et al.; 2017; de FREITAS, 2019; IONITA et al., 2015; KASPARAVIČIENĖ et al., 2013; SOUZA et al., 2008; SOUZA; OLIVEIRA, 2009; SOUZA; BOTT; OLIVEIRA, 2007).

Production of dried extracts can be achieved by various methods, including freeze drying, spray drying, and spouted and fluidized beds, with spray drying being the method most commonly used in the herbal processing industries (SOUZA; OLIVEIRA, 2005). Thermal processing of bioactive products such as herbal medicinal materials may have an effect on the physicochemical properties of product as well as cost (SOUZA; OLIVEIRA, 2005; SOUZA et al., 2008). Thus, such studies are often carried out using statistical design of experiments (DoE) to optimize such variables that may influence the properties of the final product (CHAUL et al., 2017; COUTO et al., 2012). Various studies involving processing methods and conditions have been conducted towards improving the physiochemical properties of rosemary extract while retaining its functional properties.

For example, SOUZA et al (2008) evaluated dryer performance and the physicochemical product properties during manufacture of spray and spouted bed dried extracts of rosemary. This is with a view to elucidating the effects of the drying processes/methods as well as operating conditions on system performance and product properties, paying particular attention to loss or degradation of active compounds occasioned by the process employed towards obtaining standardized dried extracts. In general, the total flavonoid degradation ratio tended to increase proportionally with both the ratio of feed material to maximum capacity (Ws/Wmax) and inlet drying gas temperature (Tgi), whereas the increase in drying gas temperature resulted in a reduction in total phenol concentration. Irrespective of inclusion of

drying aids (a mixture of colloidal silicon dioxide (Tixosil 333®) and maltodextrin DE 14), severe performance problems were observed with the spouted bed drying of the extracts, principally being excessive product accumulation in the bed and low product recovery ratio, together with marked losses of active markers (SOUZA et al., 2008).

A similar study was carried out by COUTO et al (2012) to elucidate the effects of processing factors on product properties during the manufacture of standardized dried rosemary extracts. Response surface methodology (RSM) was employed to verify the effect of processing parameters on the chemical markers' contents and *in vitro* antioxidant activities of rosemary extracts obtained via spray drying. Results indicate that an extract feed rate of 6 mL/min, a drying air inlet temperature of 140 °C and a spray nozzle air flow rate of 50 L/min are optimal drying conditions required to guarantee the physicochemical and functional quality of the products.

In another study, CHAUL and coworkers investigated the effect of extract feed rate, drying air inlet temperature, and spray nozzle airflow rate on several physicomechanical properties of a spray dried extract of rosemary using a 3^3 Box–Behnken design and Response surface methodology (RSM). The study showed that spray drying technique is viable for engineering intermediate products of rosemary with improved physicomechanical characteristics. Drying air inlet temperature was recognized as the main factor affecting quality attributes with low levels of moisture content and water activity indicated as the best set of conditions to achieve a greater powder extract recovery while suitable flowability and compressibility require low level extract feed rate (2 mL/min), low drying air inlet temperature (80 ^{o}C), and intermediate spray nozzle airflow rate (40 L/min) (CHAUL et al., 2017).

2.6 Limitations to the use of crude extracts of rosemary

Despite medicinal and commercial importance, issues of physicochemical instability (such as effect of pH, moisture, heat, and light), low bioavailability, non-susceptibility to controlled and targeted delivery, reactivity with components of the matrix, and unpleasant organoleptic properties, among other considerations, have limited the direct application of bioactive materials of herbal origin. In the case of rosemary extract, for example, some of the challenges hindering direct application in use systems and products include low aqueous solubility, low bioavailability, and rapid metabolism of its polyphenols which, incidentally, are the components responsible for bioactivity of the extract. These limitations are more significant when the multi-polar nature and high reactivity of polyphenols of rosemary are considered.

A pragmatic way to circumvent some of the aforementioned limitations is the use of mixtures of various polyphenols that bring synergistic effects, resulting in lower effective dose required and facilitating action on multiple targets. The combination of polyphenols with existing drugs and therapies also shows promising results and significantly reduces their toxicity (MOJZER et al., 2016).

A more promising solution involves the use of excipients to process polyphenols into stable and easy-to-use formulations, thereby improving desirable properties. This approach not only aid dissolution and absorption of polyphenols but also prevent their degradation and thus enables significantly higher concentrations to reach the target sites. Recently, the use of excipients has been in the form of micro- and nano-encapsulation of bioactive components.

2.7 Encapsulation of rosemary polyphenols

Encapsulation technology, widely used in various industrial sectors, enables the protection of bioactive materials including natural antioxidants, yet permitting their controlled release and other benefits of particulate systems (EZHILARASI; KARTHIK; CHHANWAL, 2013). Hence, this concept is adopted by many as a way to circumvent the limitations encountered with use of materials of natural origin. The process involves incorporating bioactive compounds (polyphenols in the case of rosemary) within protective capsules that insulate the stock from the external environment (various environmental and physiologic conditions such as moisture, heat, light, and pH), thus avoiding unwanted reactions and product degradation (CAMPELO, 2018).

Although the extraction procedure has significant influence on the composition of a plant extract and, consequently, its biological activity (TIR et al., 2012; DO et al., 2014; PIETRZAK et al., 2014; DELFANIAN et al., 2015), encapsulation of plant extracts in different materials might improve physicochemical properties of the active principles and also slow down degradation, which can improve the availability of the bioactive compounds in biological systems (VISENTIN et al., 2012; IONITA et al., 2015). In the literature, polyphenols of rosemary and other plants have been encapsulated in solid lipid nanoparticles and similar systems (GUPTA; SHARMA 2014; SECOLIN, 2014; CAMPOS et al., 2017; SECOLIN et al., 2017) with a greater focus on carnosic acid encapsulation rather than multiple compounds (VISENTIN et al., 2012). Pharmaceutical and cosmetic systems, often complex in nature, require incorporation of multiple bioactive compounds present in the extracts for synergism of activity and optimal protection. However, these previously developed systems have as major

limitation the inefficiency to accommodate compounds of varying polarity for possibility of synergism and extended spectrum, among other considerations.

Lipid-based dried product encapsulating rosemary polyphenols have been mostly prepared and evaluated for improved physicochemical properties and stability enhancement. Various attempts have been made to encapsulate whole extracts of rosemary as well as pure polyphenol compounds in lipid-based delivery systems. For example, LACATUSU et al. developed solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) loaded with rosemary extract with a view to evaluating the viability of SLN/NLC in preserving and improving antioxidant activity of rosemary extract compared to native methanolic extract. Results indicated almost two-fold increment in antioxidant activity following encapsulation and can be enhanced further by a careful selection of suitable surfactant, lipid and proper preparation conditions (LACATUSU et al., 2010).

Some other studies on encapsulation of whole extract of rosemary found in the literature include: use of supercritical antisolvent process (SAS) to precipitate compounds with antioxidant property from ethanolic solution, then encapsulating them in polymers (VISENTIN et al., 2012); comparison of antioxidant activity of rosemary extract in solution to that embedded in polymeric systems (IONITA et al., 2015); fluidized bed encapsulation of rosemary extract components (BENELLI et al., 2014; BENELLI; OLIVEIRA, 2019); encapsulation of supercritical rosemary extract in different delivery systems following *in vitro* digestion (ARRANZ et al., 2015); evaluation of the intestinal permeability of free form and liposome preparation of rosemary extract polyphenols and terpenoids in Caco-2 cell monolayers (BORRA et al., 2017); microencapsulation of rosemary polyphenols in whey protein and maltodextrin by combined emulsification and spray drying techniques (BUŠIĆ et al., 2018); and coating of microcrystalline cellulose cores (Celphere) with a lipid-based system loaded with polyphenol-rich extract (BENELLI; OLIVEIRA, 2019). In all the studies, results showed encapsulation as an effective way of improving the solubility, efficacy, and stability of rosemary extract as well as physicochemical properties that influence activity.

In other studies, appropriate carriers have been used for the protection, delivery and release of isolated polyphenolic compounds. Previous investigation include: encapsulation of rosmarinic acid in chitosan and modified chitosan for topical delivery (CASANOVA; ESTEVINHO; SANTOS, 2016); use of chitosan nanoparticles as a delivery system for rosmarinic acid investigating antitumor activity against hepatocellular carcinoma cells (Darwish et al., 2018) and for ocular activity (da SILVA et al., 2015; da SILVA et al., 2016);

microencapsulation of rosmarinic acid in polycaprolactone for application in cosmetics (KIM et al., 2010); carnosic acid encapsulation in lecithin-based nanoemulsion system (ZHENG, 2018); and carnosic acid-loaded chitosan nanoparticles for intranasal delivery (VAKA et al., 2013), all showed improved bioavailability, bioefficacy and/or chemical stability compared to free dispersion or powders of the respective isolated compound.

2.8 Encapsulation in lipid systems

Encapsulation of bioactive compounds may be effected in various materials, including solid and liquid lipids, phospholipids and sterols, surface active agents, polymer systems, dextrins and dextrans, and complexes. Of these wall materials, lipid carriers have received particular attention for encapsulation of phytoactive compounds in the last few decades due to many advantages they confer including higher solubility and bioavailability, increase in stability, sustained release, drug targeting and prevention of degradation resulting from physical and chemical factors (MORAES et al., 2013). They are also preferred due to their higher drug loading capacity, economic viability, suitability for large scale production and versatility; being useful in topical, oral and parenteral formulations (FATHI; MOZAFARI; MOHEBBI, 2012; SALA et al., 2018).

Lipid capsules vary not only in composition but also their structures and the methods employed in obtaining them. Lipid micro/nanocapsules may be prepared by various methods including homogenization (high pressure or high shear), extrusion, supercritical fluid technology, spray drying or spray congealing, etc. On structural basis and physical state, they have been classified into various types including liquid emulsions (microemulsions and nanoemulsions), dried emulsions and auto-emulsifying systems, solid dispersions, solid lipid nanoparticles, nanostructured lipid carriers, lipid microparticles, liquid crystals, liposomes and proliposomes, among others (EZHILARASI et al., 2013).

Of these, phospholipid-based systems are especially suitable for simultaneous encapsulation of hydrophilic and lipophilic compounds owing to their structure. Thus, they are considered ideal for formulating materials containing bioactive compounds with different properties such as vegetable extracts.

2.8.1 Liposome and proliposome formulations

Liposomes are phospholipid-based vesicles spontaneously generated from interaction of the hydrophilic and hydrophobic parts of the phospholipid, typically lecithin, in an aqueous environment. These vesicles possess either one or several bilayers delimiting one or several

aqueous compartments (Figure 2.1). Hence, they are classified as either unilamelar or multilamellar vesicles (SALA et al., 2018). Their lamellarity and respective sizes has been used in their categorisation (LAOUINI et al., 2012). Although they are mostly spherical in structure, some studies have reported an elongated structure, dependent on process, osmotic, and lipid properties (EDWARDS; BAEUMNER, 2006). Thus, an analysis method which incorporates information on both size and morphology is often desirable.

As carrier systems, liposomes have been explored more than any other system due to their versatility and capacity for simultaneous encapsulation of amphiphilic, hydrophilic (in the aqueous compartments), and lipophilic (within the lipid bilayers) micro- and macromolecules (ALAVI; KARIMI; SAFAEI, 2017; HUA; WU, 2013; MOZAFARI, 2010). Various materials have been utilized to prepare different liposome forms such as archaeosomes (vesicles formed from lipids sourced from archaeobacterial membrane); niosomes (prepared from nonionic surfactants); novasomes (prepared from a mixture of polyoxyethylene fatty acids, free fatty acids and cholesterol); emulsomes (containing a phospholipid bilayer with a solid fat core); cryptosome or stealth liposome (prepared from polyethylene glycol with the objective of increasing particle circulation time); vesosomes (a liposome-derived multi-compartmental structure of lipid vesicles with great potential for drug delivery); transfersomes (liposomes capable of crossing the stratum corneum and penetrate deeply into the skin; and ethosomes (lipid vesicles composed of phospholipids and 20–45 % ethanol) (ALAVI et al., 2017; SALA et al., 2018; WAGNER; VORAUER-UHL, 2011).

Liposome preparations have many advantages over other lipid-based structures for encapsulation. Besides ensuring encapsulation of compounds of differing polarity, they are beneficial for stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake due to their structural similarity to physiologic cells, and improving biodistribution of compounds to target sites *in vivo*, thereby enabling effective delivery of encapsulated compounds to target sites while minimizing systemic toxicity (SERCOMBE et al., 2015). They have also found application in cosmetics, food, and agricultural sectors where they present an attractive delivery system due to their flexible physicochemical and biophysical properties, which allow easy manipulation to address different delivery considerations. A variety of procedures for the preparation of different types of liposomes have been developed and adequately categorized, with their respective drawbacks described (CHEN et al., 2010; TURÁNEK et al., 2003; WALKER, 2010).

Although liposomes have been widely used for drug delivery, they have a major setback which limit their application for medicinal purposes; i.e. their chemical instability associated with tendency of phospholipids in liposomal formulations to suffer hydrolysis and oxidation (EDWARDS; BAEUMNER, 2006). Oxidation and hydrolysis of lipids may lead to the formation of short-chain lipids, resulting in compromised vesicle structures and leakage, an occurrence which may be minimized by either storing liposome preparations away from light and/or inclusion of antioxidants, storing the liposome preparation under an atmosphere of nitrogen or argon, or ensuring that peroxide forming solvents (e.g. isopropyl ether, diethyl ether) are completely removed from the preparation prior to storage (EDWARDS; BAEUMNER, 2006; RITA; MOREIRA, 2015).

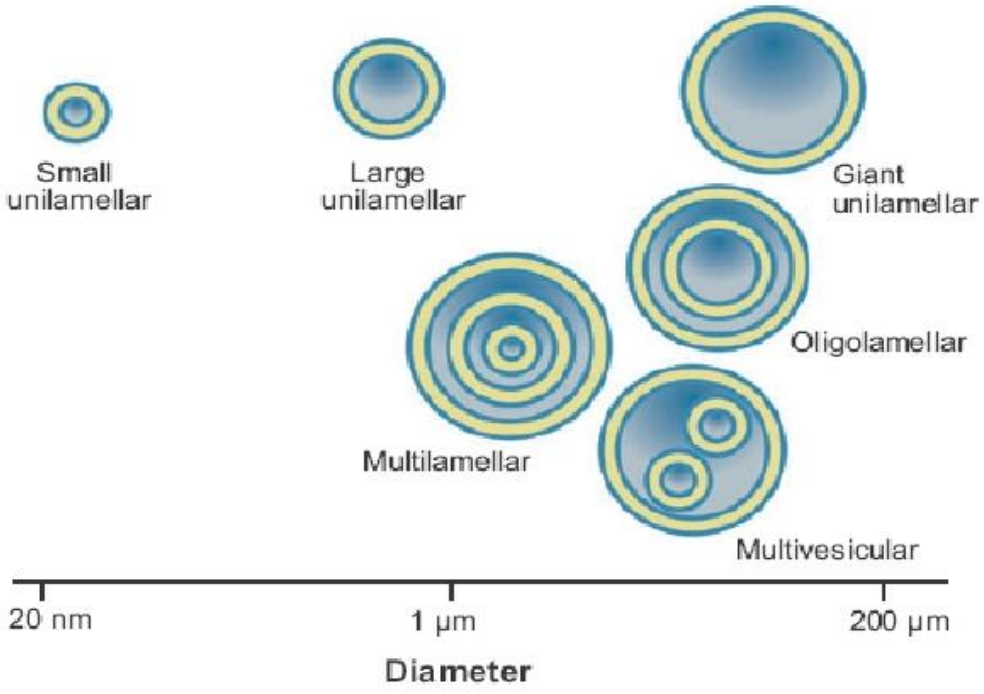


Figure 2.1: Schematic representation of commonly applied classification for liposomes. Small unilamellar vesicles, SUV (~0.02 µm to ~0.2 µm), large unilamellar vesicles, LUV (~0.2 µm to ~1 µm), and giant unilamellar vesicles (>1 µm). Drawings not to scale.
(**SOURCE:** JESORKA; ORWAR, 2008)

To be functionally viable, stability of liposome systems is required not only during production but also on storage and administration. In a bid to overcome the instability inherent with liposomes, PAYNE et al. introduced, a dry phospholipid formulation (proliposome) as an alternative to conventional aqueous liposomes (PAYNE et al., 1986). The proliposome–

liposome method, based on the conversion of an initial proliposome preparation into a liposome dispersion by dilution with an aqueous phase, is considered one of the most satisfactory (MORAES et al., 2013). Proliposomes are dry, free-flowing powders developed from phospholipids, usually together with cholesterol and other excipients; compositions that can immediately form a liposomal suspension *in situ* when in contact with water (XU et al., 2009). Their solid properties confer an improvement on the otherwise challenging physical stability of ready-made liquid liposome preparations – lower storage and transportation costs, higher concentration power, and improved stability – without influencing their intrinsic characteristics (INGVARSSON et al., 2011; NEKKANTI et al., 2016).

The proliposome-liposome method is suitable for the encapsulation of a wide range of drugs with varying water and alcohol solubilities and has extremely high encapsulation efficiencies when compared with other methods based on passive entrapment. It further has advantages of simplicity, high reproducibility, easy scale up, ease of product transportation, and useful for preparation of sterile liposome (MORAES et al., 2013; TURÁNEK et al., 2003; WAGNER; VORAUER-UHL, 2011), thus improving on identified limitations of other methods for liposome preparation (GANGISHETTY et al., 2015).

The proliposome approach has been employed in formulating different compounds of natural origin (CHU et al., 2011; HAO et al., 2015; JAISWAL, 2013). An attempt to encapsulate plant polyphenols such as those of rosemary in this type of structure is attractive from two major considerations; the first being that compounds of varying polarity can be retained in the liposomal system. While the hydrophilic core provides a suitable ambient for polar compounds, the hydrocarbon complex in the liposomal wall can be explored to accommodate polyphenol compounds that exhibit lipophilicity (MUNEER et al., 2017). Secondly, this encapsulation approach is highly promising for protecting bioactive components and increasing their solubility and functionality (KUMARI et al., 2014; MUNEER et al., 2017). This is particularly desirable for polyphenols which themselves possess scavenging properties towards radical oxygen species, a property which unfortunately is also responsible for a lack in long-term stability, making these natural compounds very sensitive to moisture, light and heat (VOLF et al. 2013; DENG et al. 2018). Moreover, polyphenols often present a poor bioavailability mainly due to low water solubility (PICCOLELLA; PACIFICO, 2017; BELŠČAK-CVITANOVIĆ et al., 2018), and a very astringent and bitter taste, which limit their use in, for example, oral medications and products (FANG; BHANDARI, 2010;

KALOGEROPOULOS et al., 2010; MUNIN; EDWARDS-LEVY, 2011; NEDOVIC et al., 2011), thereby necessitating encapsulation.

Proliposomes are obtained by drying compositions required for the formation of liposomes, being basically composed of phospholipids, cholesterol, and the bioactive compound to be encapsulated. Because drying is involved, they also require addition of drying aids among other excipients, the properties of which depends on compatibility with other components as well as the dehydration methods of choice. They are basically made from the following ingredients:

- Phospolipids

Phospholipids are amphoteric organic molecules composed of a glycerol moiety substituted by one or two fatty acids and an additional polar group - this latter group may be choline (phosphatidylcholine or lecithin), ethanolamine, serine or inositol (DAVID; MEDVEDOVICI; SANDRA, 2000). Their properties and multifunctional application as technological excipients (as solubilizer; wetting agent; emulsifier; and encapsulating agent in microemulsions, mixed micelles, liquid crystals, and liposomes) have been discussed (LI et al., 2014; SINGH; GANGADHARAPPA; MRUTHUNJAYA, 2017).

Although the term "lecithin" is sometimes used as a synonym for phospholipids, clarifications on these terminologies their sources, physicochemical properties and the effects of these properties on formulation processing and physiologic interactions are available in the literature (van HOOGEVEST, 2017a; van HOOGEVEST, 2017b; KOYNOVA; CAFFREY, 1998; CHEN et al., 2010).

- Sterols

In addition to phospholipids, liposomes and proliposome systems usually contain sterols in their structure, the most commonly used being cholesterol. Cholesterol is an essential constituent of the physiologic biomembrane of eukaryotes, one of its primary functions being modulation of physiochemical properties of the lipid bilayers. It does not by itself form bilayer structures but incorporated into phospholipid membranes as a fluidity buffer of the lipid membrane, with a potential for membrane permeability control and alteration of transition temperature of the phospholipid membrane (CHEN et al., 2010). Its main function in liposomal systems is to improve the stability of the vesicle bilayers – in the presence of biological fluids – by modulating their fluidity, thus, reducing permeability and leakage of the active payload (MOZAFARI et al., 2017).

Ergosterol, the major sterol component present in lower eukaryotes (such as certain protozoa, yeasts and and other fungi), and insects (such as drosophila), have been tested as a component of liposomal system and compared with cholesterol (ARORA; RAGHURAMAN; CHATTOPADHYAY, 2004). The required concentrations of sterols are largely dependent on intended application of the formulation (MOZAFARI, 2010).

- Drying aids

Drying aids, otherwise known as drying carriers or lyoprotectants (for dehydration methods involving freezing temperatures), are often solid materials applied to support drying of lipid-based preparations. Their mechanism of action is either by acting as a direct adsorbent for liquid excipients or by encapsulating the dispersed lipid colloids from the continuous phase prior to drying (TAN; RAO; PRESTIDGE, 2013). For proliposome preparation, it is important that the drying aids are water solubility to facilitate the formation of liposome vesicles following redispersion in aqueous medium.

Commonly used drying aids in proliposome formulations fall in different categories and have been tested as drying aid in proliposome preparation: disaccharides and other sugars e.g. sorbitol, mannitol, glucose, trehalose, sucrose, lactose (CROWE; CARPENTER; CROWE, 1998; INGVARSSON et al., 2011; AHAMMED et al., 2017; SECOLIN et al., 2017; XU et al., 2009); polysaccharides and oligosaccharides e.g. maltodextrins, cyclodextrins, gum acacia, and starch sodium octenyl succinate, inulin and dextran (INGVARSSON et al., 2011); amphiphilic polymers e.g. poloxamers (Pluronics/Kolliphor), hydroxypropyl methylcellulose (HPMC), carboxymethylcellulose (CMC) sodium, and polyvinylpyrrolidone (PVP) (TAN; RAO; PRESTIDGE, 2013); and proteins and amino acids (CHEN et al., 2010; POPOVA; HINCHA, 2004).

- Solvents

Organic solvents are often used in the preparation of proliposomes to solubilize the lipid (and sometimes the drug too) components. They also function in providing softness to the vesicle membrane (SINGH et al., 2019) and are used either individually or as a solvent mixture. Volatility is an essential property of solvents for use in proliposomes since they are eventually evaporated from the formulation system. The most commonly used volatile organic solvent are ethanol, methanol, ether and chloroform; the choice is usually influenced by toxicity and compatibility issues.

❖ Other components

i. Stabilizers: gums (e.g. xanthan gum) and other polysaccharides are often used as a thickening agent to prevent phase separation following rehydration of proliposome powder (MORAES et al., 2013).

ii. Neutral pH buffers and antioxidants such as sodium ascorbate could also be incorporated in the composition to limit phospholipid oxidation in the presence of moisture (SALA et al., 2018).

2.8.2 Proliposomes of phytoconstituents and products

The proliposome method has been applied in various studies for encapsulation of both natural and synthetic material, including whole extracts and isolated compounds. Such products are proposed for direct application in various industries or as intermediates to be developed into a final dosage form (KHAN et al., 2015).

Proliposome powders of silymarin, the main active flavonoid extract in the dried fruits of *Silybum marianum*, was prepared towards improving its poor bioavailability and hepatoprotective effects. On rehydration, the prepared silymarin proliposome formed multilayer liposome structure encapsulating silymarin with improved intestinal transport and better hepatoprotective effects compared to silymarin tablets (WANG et al., 2015). In a similar study, CHU et al formulated proliposomes of dehydrosilymarin (oxidized form of silymarin) with a view to improving its oral bioavailability (CHU et al., 2011). Following oral administration in rabbits, the relative bioavailability of proliposomes versus suspension was 228.85 %. Results obtained suggested that proliposomes may be a useful vehicle for oral delivery of dehydrosilymarin, a drug with poor aqueous solubility.

Zheng and coworkers successfully developed proliposomes containing a bile salt to improve the oral bioavailability of *Ginkgo biloba* extract. The extract-loaded proliposomes were prepared by spray drying method, optimized by response surface methodology, physicochemically characterized, and assessed for *in vitro* and *in vivo* bioavailability. Results showed that proliposome formulation delayed release and enhanced dissolution of Ginkgo flavonoids and terpene lactones, with up to 333 % increment in absorption of bioactive compounds in the gastrointestinal tract while decreasing its elimination. Proliposomes were further shown to selectively deliver Ginkgo extract to critical target tissues, including the brain (ZHENG et al., 2015).

Proliposome delivery system has also been used to improve the oral availability of ginseng fruit saponins compared to its commercially available tablet formulation (HAO et al., 2015). *In vitro* drug release study indicated that developed proliposome has a controlled drug release profile. The controlled release and enzymatic stability offered by the developed proliposomes may significantly decrease elimination of the fruit saponins and enhance intestinal absorption. In other studies, liposomes of beta-carotene (MORAES et al., 2013), *Momordica charantia* extract (JAISWAL, 2013), and curcumin (SILVA et al., 2017) were prepared using the proliposome method to improve solubility and stability. These studies suggest proliposome formulations have application in foods and pharmaceuticals, among other uses.

2.9 Methods for proliposome preparation

Dry phospholipid particles can be produced by several methods, such as modified thin film deposition, freeze drying, crystal-film method, power bed grinding, super-critical techniques, and spray drying (FAN et al., 2007; KHAN et al., 2015; XIA et al., 2012).

- *Film deposition method:* deposition of lipid film on to a carrier was the original proliposome preparation method, developed by PAYNE et.al (1986) and has been reported with many modifications including pan coating, fluid bed coating or using a rotary flash evaporator (KATARE; VYAS; DIXIT, 1990; KUMAR; GUPTA; BETEGERI, 2001; POTLURI; BETAGERI, 2006). This method affords a high surfactant to carrier mass ratio in the preparation of proliposomes, but the possibility of over wetting of the matrix and inefficiency due to frequent discontinuation step of solvent addition and evaporation leading to high processing time are major drawbacks.

- *Film dispersion-freeze drying method:* This method involves the combination of film dispersion with freeze-drying techniques to prepare proliposomes and has been applied for encapsulation of different compounds including phytoactive materials (CHU et al., 2011; FEI et al., 2009).

- *Supercritical anti-solvent (SAS) method:* this method has been used in controlled crystallization of various drugs from dispersion in supercritical fluids, carbon dioxide. (HUANG et al., 2014; XIA et al., 2012; LIU et al., 2014). This technique, considered as environment friendly due to the low volume of solvent used in the process, typically leads to formation of uniformly sized proliposomes. In addition, the low operating temperature helps in

the preparation of proliposomes for temperature sensitive drugs (COCERO et al., 2009; NEKKANTI; VENKATESAN; BETAGERI, 2015).

- *Spray drying**:*** The use of spray drying is of special interest in proliposome production on industrial scale as it is known for ease of scale-up and possibility of particle engineering. In many cases, particle formation and drying are carried out in a continuous single step, thus allowing control of particle size and shape (MUNEER et al., 2017; NEDOVIC et al., 2011).

Other methods: there are other methods for preparation of proliposomes including the fluidised bed method among others (MUNEER et al., 2017; GUPTA; BARUPAL; RAMTEKE, 2008).

Although rosemary extract has been extensively studied and reports on encapsulation of its polyphenols in various matrices exist in the literature, such studies generally focus on concentrating and/or encapsulating a selected compound, usually carnosic acid. There is no known study in which simultaneous encapsulation of multiple polyphenol compounds contained in the rosemary extract is put into perspective. This current work, therefore, conceptualized the encapsulation of multiple polyphenols found in the extract of rosemary using the proliposome-liposome technology. This is towards taking advantage of potential synergism of activity that exists between these components, thus improving efficacy and widening spectrum of activity as well as improving physicochemical properties and polyphenol stability. In this manner, the functionality of the polyphenol-rich extract can be improved for various applications.

3. Objectives

3 OBJECTIVES

3.1 General objective

This work had as general objective the encapsulation of polyphenol-rich extract of rosemary in lipid carriers, using compositions based on soy phosphatidylcholine and cholesterol, followed by spray drying; with emphasis on the preparation processes, formulation optimization using Design of Experiment approach, procedures used for physical-chemical characterization, evaluation of product stability and potential biological activities.

3.2 Specific objectives

In order to achieve the proposed general objective of this work, the following steps were developed:

- Bibliographic review and updating;
- Milling and characterization of dried leaves of rosemary;
- Preparation of dry extract rich in bioactive compounds of interest (polyphenols) from milled rosemary leaves – involving preparation of solution extract, concentrated extract, and freeze drying;
- Development/adaptation/revalidation of analytical methods for physico-chemical characterization of crude extracts (concentrated and lyophilized) and the encapsulation systems to be prepared;
- Preliminary studies on rosemary polyphenol encapsulation in liposomal composition – method of preparing the formulations, selection of materials (adjuvant types and ratios, screening of solvents, etc);
- Preparation of proliposomes encapsulating polyphenol-rich freeze-dried extract of rosemary by spray drying method, using established operational parameters;
- Optimization of composition variables using design of experiment – statistical analyses and results validation;
- Standardization of methodologies to characterize the physical properties of the formulations obtained (macroscopic analysis, microscopy, centrifugation, thermal stress, zeta potential and particle size).

- Evaluation of the physical-chemical properties of spray dried proliposome (moisture content, water activity, redispersity, morphology, powder flowability, x-ray diffraction, polyphenol content and retention in product matrix, and drying performance);
- Evaluation of potential biological activities – antioxidant (DPPH method), antimicrobial (antibacterial and antifungal);
- Assessment of stability of lyophilized extract and spray dried proliposome – influence of storage temperature and relative humidity on physico-chemical properties including polyphenol degradation, moisture content and powder flowability, colour changes, surface morphology, crystalline/amorphous characteristics, molecular interactions.

4. Material and Methods

4 MATERIAL AND METHODS

4.1 Reagents, solutions, and reference substances

- Absolute ethanol (Labsynth);
- Methanol – HPLC grade (Sigma-Aldrich Chemical Co);
- Acetonitrile – HPLC grade (Sigma-Aldrich Chemical Co);
- Anhydrous sodium carbonato (Labsynth);
- Aluminium chloride (Labsynth);
- Sodium tungstate (Vetec química fina)
- DPPH (Sigma-Aldrich Chemical Co);
- Resazurin (Sigma-Aldrich Chemical Co);
- Strains of bacteria and fungi: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (25923), *Bacillus subtilis* (ATCC 21332), *Klebsiela pneumoniae* (ATCC 23357), *Pseudomonas aeruginosa* (ATCC 2327), *Candida albicans* (ATCC 1023);
- Culture media: Mueller-Hinton Broth and Agar (Sigma-Aldrich), Sabouraud Dextrose Broth and Agar (BD Difco™ USA);
- Buffer solutions (phosphate – pH = 7.2 and acetate – pH = 4.0);
- Physiologic solution (NaCl; 0.15 mol/L);
- Reference substances: quercetin (Sigma-Aldrich Chemical Co), pirogalol (Sigma-Aldrich Chemical Co); caffeic acid (Sigma-Aldrich Chemical Co); rosmarinic acid (Sigma-Aldrich Chemical Co); carnosic acid (Sigma-Aldrich Chemical Co); carnosol (Sigma-Aldrich Chemical Co); Phosphomolibdic acid (Vetec química fina); Terbinafine (Fagron, China - donated by the Pharmacy Education Unit-FCFRP/ USP).

4.2 Adjuvants

- Phospholipon® 90H - hydrogenated soy phosphatidylcholine (Lipoid GMBH);
- Cholesterol (Sigma-Aldrich Chemical Co);
- Lactose monohydrate (Natural Pharma).

4.3 Equipments

The following equipments were used in the development of this work:

- Mechanical sieve shaker (Bertel) with stack of sieves;
- Multichannel magnetic stirrer (Marte mod. Mag-Multi);
- Single channel magnetic stirrer (Nova Ética mod. 119);
- Mechanical agitator/stirrer (Fisatom mod. 713D);
- Alcoholmeter (Incoterm);
- Analytical and semi-analytical weighing balances (Marte and Mettler Toledo);
- Moisture analyzer/balance (Sartorius MA35, Goettingen, Germany);
- Water bath (Fisatom mod. 550);
- Bath sonicator (Odontobrás mod. 802 and Eco-Sonics mod. ultronique);
- Vacuum pump (Prismatec - mod. 131);
- Peristaltic pump (COLE-PARMER mod. 7553-75);
- Climatic chambers (Nova Etica mod. B.O.D 411D and mod. 420E);
- Eppendorf centrifuge (Eppendorf Instruments INC mod. 5810R);
- ColourQuest XE colorimeter (HunterLab);
- High Performance Liquid Chromatograph (Shimadzu Prominence CBM-20, equipped with two high pressure pumps mod. LC-10 AD, an automatic sampler mod. SIL-10ADVP, a photodiode array spectrophotometric detector mod. SPD-M20A, chromatographic column C18 (250 mm x 4.6 mm d. I. Shimadzu Shim-pack CLC (M) with particle size of 5 µm, and controlled by LC Solution software);

- Zetasizer (Malvern Nano ZS90);
- Heating mantle (Fisatom mod. 52);
- Refrigerators - Electrolux Air flow system DC38, -80 ^{o}C Ultra freezer;
- X-ray diffractometer (Billerica mod. D2 Phaser, Bruker-AXS coupled with a Lynxeye mode 1 scanner detector);
- Infra-red spectrophotometer (Nicolet 380 coupled to a Totally Attenuated Reflection (TAR) Durascope);
- Scanning electron microscope (Carl Zeiss mod EVO 50 scanning electron microscope; sample preparation using a Bal-Tec mod. SCD-050 sputter coater);
- Probe sonicator and accessories (Sonics mod. Vibra-cell);
- Tapped density tester (Caleva mod. TDT 22);
- AquaLab 4TEV water activity analyzer (Decagon Devices Inc.);
- Bench top lyophilizer (SNL 108, Thermo Fischer Scientific);
- Bench top spray dryer (Lab-Plant mod. SD-05);
- UV-Vis spectrophotometer (HP 8453 operating the HP *Chem-Station software)*;
- Drying oven and sterilizer (Fanem, mod. 315 SE);
- Rotary evaporator (Fisatom, mod. 802);
- Knife mill (Marconi, MA 680);
- Vortex machine (Reax top, Heidolph);
- MilliQ water dispensing system (Millipore Direct-Q 3);
- Water distiller (Gehaka CG-200);
- Clevenger apparatus system;
- pH-metro Micronal (mod. B474);
- Stainless steel extraction tank (height: 34 cm, diameter: 22 cm) with 12 L total capacity;

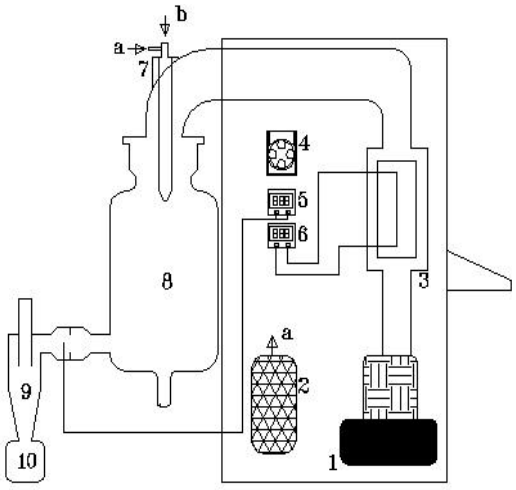
- Karl Fischer titrator (Titrino plus 870 Methrohm);
- Other materials – dessicators, micropipettes, filters, buchner funnels, spatulas;
- Glass wares – pycnometer, beakers, volumetric flasks, test tubes, pipettes, bottles, stirrers;

Other equipment necessary for the development of the work.

4.3.1 Drying equipments

Spray dryer

For this work, the spray dryer model SD-05, manufactured by Lab-Plant (UK), was used. The drying chamber has a diameter of 215 mm and a height of 500 mm. The main components of the system are the drying suspension feed system, consisting of a peristaltic pump (COLE-PARMER, model 7553-75), a double fluid type atomizer with compressed air and an air compressor; drying air supply system, consisting of an air blower and air filter; drying gas temperature control system and sample collecting system (cyclone) – presented in Figure 4.1.



1. Blower + air filter
2. Air compressor
3. Heater
4. Peristaltic pump
5. Temperature ontrol
6. Control (PID)
7. Atomizing nozzle: (a) drying suspension feed; (b) compressed air supply
8. Drying chamber
9. Cyclone
10. Dry product/powder collector

Figure 4.1: Schematic diagram of the spray dryer utilized.

SOURCE: Souza et al. (2008).

Lyophilizer (*Freeze dryer*)

Freeze drying can present high quality dry products, including plant extracts, although high cost of production limits its wide application. This system was used to dehydrate the concentrated rosemary extract; it was considered that low temperature drying (such as lyophilization) will preserve the integrity of the otherwise thermolabile polyphenols contained in the extract which are linked to its bioactivity.

The concentrated extract was lyophilized in a Thermo Fischer Scientific model SNL 108 (Figure 4.2) containing a freeze-drying unit – Micromodulyo 1.5 L (305x330x432), stainless steel condenser; 1/4 hp compressor and 0.30 kw power; ultra-vacuum pump VLP 195 FD-115, freeze-drying vials with independent valves. No cryoprotectant or other adjuvant was added to the liquid extract prior to dehydration.

Figure 4.2: Photograph of the Thermo Fisher Scientific SNL 108B freeze dryer.

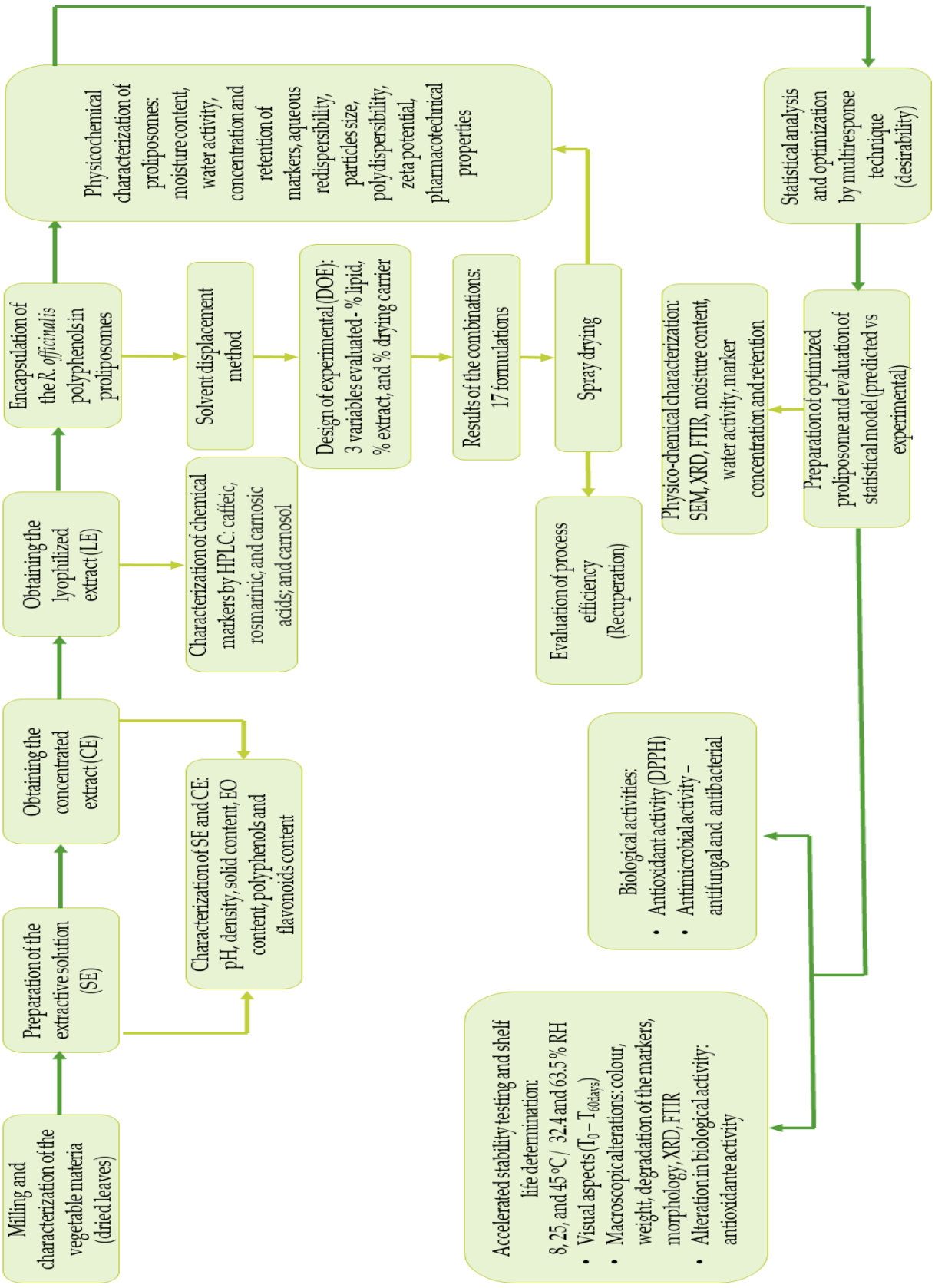


Figure 4.3: Simplified project flowchart.

4.4 Experimental methods

4.4.1 Herbal material processing

4.4.1.1 *Milling and characterization*

Dried leaves of *Rosmarinus officinalis* were acquired from Santos Flora, Mairiporã, São Paulo (LOT: 1505080153). The dried vegetable material was milled in a knife mill (MARCONI MODEL MA 680) using a sieve with mesh 20 (833 μm). The powdered vegetable obtained was collected in airtight plastic bag and stored in a tightly closed opaque plastic container. The resulting powder was characterized by determination of the granulometric distribution, loss on drying (L_D), total extractive content (T_E), and essential oil (EO) content, according to the methods presented afterhand.

4.4.1.2 *Granulometric distribution*

Analysis of particles size and size distribution of milled vegetable raw material was carried out according to the Brazilian Pharmacopoeia (2010) using a stack of standardized sieves. 100 g sample material was weighed into the uppermost sieve on a stack with aperture size between 0.5 and 0.0375 mm (top to bottom) and a collector at the base. The stack was subjected to mechanical agitation using a sieve shaker (Bertel®) for 20 minutes and mass of deposited fraction in each sieve was obtained. The result was determined as percentage accumulated fraction for subsequent particle classification.

4.4.1.3 *Determination of loss on drying (L_D)*

The loss on drying was determined by gravimetric analysis using the method described by SOUZA (2007). Using a moisture analyzer Sartorius MA35 (Goettingen, Germany), about 2 g sample of milled vegetable material was evenly spread on the weighing plate of the balance, previously tarred. The balance was operated at 105 ºC for the required time to determine the loss on drying. The determination was done in triplicates. The oven method was also used for comparison with the results obtained by using the moisture balance. In this method, 2 g of the powdered vegetable material was placed in a Petri dish of known weight. The content of the Petri dish was evenly spread before it was placed in an oven at 105 ºC and dried for sufficient time until a constant weight is obtained (24 h). The result is taken as an average of three determinations and expressed in percentages (SECOLIN, 2014; ZAMPIÉR, 2012), as:

$$L_D = \frac{p-m}{m} \cdot 100 \qquad (4.1)$$

where: L_D = Loss on drying (% m/m); m = mass of dried residue (g); p = mass of sample (g).

4.4.1.4 *Determination of total extractive content (T_E)*

A sample of 1 g of the milled vegetable material was subjected to decoction (without agitation) in 100 g of 70 %v/v ethanol for 10 minutes (the initial weight before decoction process was noted). After cooling, the flask was made up to the initial weight with 70 %v/v ethanol. The content of the flask is filtered using a filter paper; the initial 20 mL filtrate is discarded. Of the remaining filtrate, about 2 g was placed on the plate of the moisture analyzer (Sartorius MA35) which has previously been heated using the standard procedure presented before. The balance was tarred, following which the 2 g sample was introduced and evaporated to dryness. The result was an average of three determinations, using equation 4.2:

$$T_E = \frac{m \cdot FD \cdot 100}{p} \qquad (4.2)$$

where: T_E = total extractives content (% m/m); m = mass of dried residue (g); p = mass of sample (g); FD = dilution factor.

4.4.1.5 *Determination of essential oil (EO) content*

50 g of the milled vegetable material was placed in a 1 L round bottom flask to which 300 mL water was added. The essential oil was extracted using a Clevenger apparatus system. The distillate was placed in a freezer (-20 ^{o}C) to separate the essential oil from water. The volume of the essential oil was determined and the percentage EO yield calculated, the result being an average of three determinations.

4.4.1.6 *Quantification of total flavonoid (T_F) in herbal material*

The spectrophotometric method described by SOUZA (2007) was used in the determination of the total flavonoid (T_F) content of the powdered dried leaves of *Rosmarinus officinalis*. A weight of 2 g of the dried milled leaves was placed into a 250 mL flat-bottom flask containing 30 mL of 40 %v/v ethanol. The flask was placed on the heating mantle and connected to a reflux. The

material was refluxed for 15 minutes after which the content was filtered into a measuring cylinder using a cotton wool plug. After filtration, the residue together with the cotton plug (which is already impregnated with the extract from the leaves) was transferred into another flask and a further 30 mL of 40 %v/v ethanol was added to the 250 mL flask together with the cotton plug. The procedure was repeated two more times. After the final 15 minute-period of reflux, the extract was filtered into the measuring cylinder and the cotton wool plug carefully pressed to express the absorbed content. The content of the measuring cylinder was transferred into a 250 mL volumetric flask and made up to volume using 40 %v/v ethanol. 10 mL of the content of the flask was withdrawn and transferred into a 100 mL flask where the volume was completed with 40 %v/v ethanol. 10 mL of the resulting dilute solution was transferred into each of four 25 mL flasks labeled as "blank" and samples 1, 2, and 3. The flask labeled blank was made to volume (25 mL) with 40 %v/v ethanol while 2 mL of aluminum chloride solution 0.5 %w/v was added to each of the samples 1, 2 and 3 before completing the volume to 25 mL with 40 %v/v ethanol. The samples were rested for 30 minutes after which spectrophotometric readings were taken at 425 nm.

In order to quantify flavonoid content, calibration curve for a representative standard flavonoid, quercetin was built. To build the flavonoids calibration curve, solution of quercetin standard of different concentrations (1 – 24 μg/mL) was prepared in ethanol (40 %v/v) and the absorbance value determined spectrophotometrically at 425 nm. Blank samples were also prepared (same as corresponding concentration but without inclusion of $AlCl_3$). The experiment was carried out in triplicates.

4.4.1.7 *Quantification of total polyphenol (T_P) in herbal material*

The procedure used is based on the Folin-Denis test which consists of reduction of the phosphomolibdic-phosphotungstic acid by the phenolic compounds in alkaline medium producing an intense blue colour measured spectrophotometrically at 760 nm (BOTT, 2008; SOUZA et al., 2008). 0.75 g of powdered vegetable drug was weighed into a 500 mL flask and 100 mL distilled water was added. The mixture was refluxed for 30 minutes and then cooled with running water. The supernatant was then transferred into a 250 mL flask and made up to volume with purified water. The solution was then filtered with a filter paper into a measuring cylinder; discarding the first 20 mL. The solution obtained is referred to as the standard solution.

To determine the total polyphenols content, 5 mL of the standard solution prepared was measured into a 25 mL volumetric flask and made to volume with purified water. 2 mL of this solution was transferred into each of five 30 mL amber-coloured test tube (the test is to be performed in triplicates but five solutions are prepared and measured, the three closest results being those of choice). Afterwards, 2 mL of Folin-Denis reagent and 16 mL of saturated solution of sodium carbonate were subsequently added under a fume cupboard and shaken with a mechanical agitator. After two minutes of addition of sodium carbonate, the content of the tube was read with the spectrophotometer at 750 nm. Purified water was used as blank and all sample tubes were read with the three closest values selected. The results were expressed as pirogalol equivalent.

The calibration curve for the quantification was made by preparing dilute solutions (2.5 μg/mL - 40 μg/mL) of pirogalol, a chemical standard for polyphenols. The Folin-Denis reagent was added to each tube per time followed by sodium carbonate (Na_2CO_3) to provide the alkaline medium required for the reaction. The absorbance of samples was determined spectrophotometrically at 750 nm and the calibration curve constructed. Purified water was used as blank samples. The experiment was carried out in triplicates.

4.4.2 Preparation and physicochemical characterization of liquid rosemary extract

Hydroalcoholic extract of milled vegetable material was obtained by dynamic maceration (200 rpm) at 1:10 (w/v) plant/solvent ratio and 70 ^oC for one hour using 70 %v/v ethanol as solvent. The extract obtained was vacuum filtered (filter paper) and concentrated to about 10 % solid content by rotary evaporation at 50 ^oC and vacuum pressure of 600 mmHg. The extractive solution and concentrated extract were both characterized by determination of the pH, density, solids content, and total flavonoids and polyphenolic contents, according to the procedures presented following:

4.4.2.1 *Determination of pH of extractive solution and concentrated liquid extracts*

The pH of extractive solutions and concentrated extract were determined at 25 ^oC using a pH meter previously calibrated with standard phosphate and acetate buffers, pH 7.0 and 4.0, respectively. The results are average of three determinations (ZAMPIÉR, 2012).

4.4.2.2 *Density of ethanolic solution and concentrated liquid extract*

The relative density of extractive solution and concentrated extract was determined at 25 ºC using a 10 mL pycnometer. The sample density is calculated using Equation 4.3. The result is an average of three determinations (ZAMPIÉR, 2012).

$$\rho = \frac{m_{se}}{m_{water}} \times \rho_{water}^{25\,ºC} \qquad (4.3)$$

where: ρ = density of the extractive solution (g/cm^3); m_{se} = mass of liquid extract in the pycnometer (g); m_{water} = mass of distilled water contained in the pycnometer (g).

4.4.2.3 *Solid content of the extractive solution and concentrated extract*

The determination of the solids content (extractable matter) of the extractive solution and concentrated extract was carried out by gravimetric analysis (SOUZA, 2007). About 2 g of liquid extract was evaporated to constant weight on a moisture analysis balance operated at 105 ºC and the solid content determined according to Equation 4.4. The result is an average of three determinations.

$$C_s = \frac{m}{p} \cdot 100 \qquad (4.4)$$

where: C_s = solids content (% m/m); m = mass of dried residue (g); p = mass of sample (g).

4.4.2.4 *Quantification of total flavonoids (T_F) in extractive solution and concentrated liquid extracts*

Total flavonoid content of both extractive solution and concentrated extract was carried out in accordance with the method described in Section 4.4.1.6. Respective quantities of extractive solutions and concentrated extract corresponding to about 500 mg of solid content, were used in the determination of their total flavonoid content. The total flavonoid content was expressed in relation to quercetin, the result being an average of six determinations.

4.4.2.5 *Quantification of total polyphenols (T_P) in extractive solution and concentrated liquid extracts*

Determination of total polyphenol in both extractive solution and concentrated extract was carried out in accordance with the method described in Section 4.4.1.7. Respective quantities of hydroalcoholic extractive solution and of concentrated extract equivalent to about 100 mg of solid mass was used in the determination of their total polyphenols content. The absorbance of samples was measured spectrophotometrically, with purified water used as blank samples. Polyphenol concentrations were determined on the pirogalol calibration curve and expressed in relation to pirogalol (gallic acid equivalent). Results are an average of six determinations.

4.4.3 Preparation and chemical characterization of the freeze-dried rosemary extract

Powdered rosemary extract was obtained from the concentrated liquid extract by freeze drying. About 25 mL of concentrated extract was dispensed into each of 50 mL falcon tubes and congealed at −20 °C, being at a slant position, over 24 h. Afterwards, the tubes were transferred into a −80 °C ultra-freezer for further 2 h. The congealed sample was freeze-dried in a bench-top lyophilizer at condensation temperature of −40 °C for 48 h. The freeze-dried product was placed in airtight amber bottles and stored at −20 °C until use.

4.4.3.1 *Quantification method for rosemary polyphenol markers in the freeze-dried extract*

The biological properties of rosemary are usually linked to the high content of polyphenolic compounds, mainly rosmarinic acid (ROA), carnosol (CAR) and carnosic acid (CNA), and mildly caffeic acid (CFA). The concentrations of these compounds in the freeze-dried extract was determined by HPLC-DAD according to the method proposed by WELLWOOD and COLE (2004), with some modifications (BENELLI; OLIVEIRA, 2019).

Concentrations of the polyphenolic markers in the lyophilized extract were determined with the aid of calibration curves built for ROA, CAR, CNA, and CFA using serial dilutions of reference standards. Quantification of each polyphenol marker in the freeze-dried extract was done by integration of the area under the respective peak and comparing same with that of the external standard.

4.4.3.2 *Method revalidation*

The revalidation of the HPLC method developed previously in our lab was performed by determination of the linearity, precision, and accuracy (ANVISA, 2017).

Linearity was determined from solution of each of the marker compounds (in methanol) at different concentrations being; caffeic acid (2, 5, 10, 20, 30, and 40 μg/mL), rosmarinic acid (10, 50, 100, 250, and 500 μg/mL), carnosol, and carnosic acid (30, 60, 60, 150, 300, and 600 μg/mL). Preparations were done in quadruplicates. Each batch concentration was prepared by diluting separate stock solutions and evaluated using the HPLC chromatographic conditions. Analytical curves of integrated area (Shimadzu® LC Solution software) for each compound were plotted. Curves were subjected to linear regression based on Equation 4.5.

$$y = mx + c \quad (4.5)$$

where: y is area, x is concentration of respective compound, m is the slope of the curve and c is the intercept. Linearity was ascertained by values of $R^2 \geq 0.999$. Determinations were carried out in quadruplicates and calculations done using the Microsoft Excel software (Microsoft, USA).

Intra-day and inter-day precision were determined for all compounds using low, medium and high concentrations levels for each of the marker compounds. Four replicates were used in the determination of precision. Intra-day precision was determined within a single day by back-calculating the concentration of each marker compound using the linearity equation. Results were calculated as relative standard deviation using Microsoft Excel software.

The accuracy of the method was verified using low, medium and high concentrations levels for each of the marker compounds. Four different replicates of each compound were used to determine the accuracy of the method. The results were expressed in percent ± relative error according to the following Equation 4.6:

$$P = \frac{C_{exp}}{C_{theo}} \cdot 100 \quad (4.6)$$

where: P is accuracy (%); C_{exp} is experimental concentration; and C_{theo} is the theoretical concentration.

4.4.4 Encapsulation of rosemary polyphenols in proliposomes

4.4.4.1 *Preparation of liposomal compositions encapsulating rosemary polyphenols*

The proliposomes production starts with the encapsulation of the rosemary polyphenols in liposomal compositions, using the solvent replacement method (WAGNER; VORAUER-Uhl, 2011) with some modifications. The lipid phase consisted of preset quantities of hydrogenated soy phosphatidylcholine (Phospholipon® 90H) and cholesterol (9:1) dissolved in 50 mL of ethanol 90 %v/v at 65 ^{o}C, while aqueous phase consisted of a dispersion of lyophilized rosemary extract in purified water. The two phases were brought to the same temperature before the lipid phase was injected into the aqueous phase under agitation. Residual alcohol in the system was vacuum removed at 48 ^{o}C/600 mmHg. The liposomal composition obtained was put to rest at 8 ^{o}C (refrigerator) for 24 h to allow for complete stabilization; and then mixed with lactose (drying aid) before submission to spray drying to produce the proliposomes.

To adequately select the control parameters that may be critical to physicochemical characteristics of proliposome powders, a Quality by Design (QbD) approach was employed. Based on literature study, potential factors that could interfere with desired quality properties of proliposomes were enumerated. Composition variables were selected and their effects on proliposome properties studied using the Design of Experiment (DoE) approach (Section 4.4.5). The concentration of solids in the feed was maintained constant at 10.9 % w/w for all formulations developed according to the experimental design, using distilled water (APPENDICES I and II). The liquid liposomal formulations were submitted to spray drying to generate proliposomes.

4.4.4.2 Spray drying procedure

The drying conditions were set according to previous works developed by our group (SECOLIN *et al.* 2017; CORTÉS-ROJAS et al., 2012; GUEDES; SOUZA; OLIVEIRA, 2009). Drying runs were carried out in a bench top SD-05 spray dryer (Lab-Plant UK Ltd, Huddersfield, UK) with a concurrent flow regime, having a drying chamber of 215 mm diameter and 500 mm height. The spray dryer was previously stabilized with the feed of distilled water at same drying conditions. The outlet drying temperature was monitored at intervals of 5 minutes to ascertain steady state. When this condition was reached, feeding with liposomal formulation (liposomal composition including lactose) into the spray dryer commenced. The room temperature and relative humidity were monitored with the aid of a digital thermo-hygrometer (Minipa MTH – 1361) during

each drying run. The drying formulation was fed at a flowrate of 4 g/min (provided by a peristaltic pump) through a twin fluid atomizer (1.0 mm of orifice diameter) with internal mixing connected to a compressed air line. The flowrate of atomizing air was maintained at 17 L/min at pressure of 1.5 kgf/cm^2. The inlet gas temperature and flow rate were maintained for all experiments at 100 ^{o}C and 60 m^3/h, respectively. The inlet and outlet spray drying temperatures, environment/room temperature and relative humidity were monitored during spray drying runs in order to certify the reproducibility of the drying conditions.

4.4.5 Design of Experiments

The effects of three composition variables on physicochemical properties of spray dried proliposomes were studied by using a completely randomized 2^3 Box-Wilson Central Composite Design (CCD). The composition variables studied were the total lipid concentration, concentration of rosemary extract, and the drying aid concentration ratio, i.e. drying aid:(lipid+extract). The variables were studied at three main levels, having α as ± 1.682 and with three replicates at the central points. Table 4.1 presents the levels (values) of coded and uncoded variables, and Table 4.2 the resulting experimental design. The total concentration and retention of markers content in the proliposomes, moisture content, water activity, and product recuperation, were the experimental responses analyzed.

The CCD design allows the determination of linear, quadratic and interaction effects of variables, which can be expressed by the following model (Equation 4.7), which can be fitted to experimental data by regression analysis.

$$Y_i = a_0 + a_1.X_1 + a_2.X_2 + a_3.X_3 + a_{11}.X_1^2 + a_{22}.X_2^2 + a_{33}.X_3^2 + a_{12}.X_1.X_2 + a_{13}.X_1.X_3 + a_{23}.X_2.X_3 + \varepsilon \quad (4.7)$$

where: Y_i is the expected response associated with the combination factors, a_0 to a_{33} are the regression coefficients, X_1 to X_3 denotes the factors, and ε represents the experimental error. The statistical significance of the effects was tested by analysis of variance (ANOVA).

The statistical significance at 90, 95, and 99 % confidence levels of the linear, quadratic and interaction effects of the investigated variables on selected proliposomes properties (moisture content, water activity, total marker retention, relative marker content, product recovery) was

assessed through variance analysis (ANOVA) and regression analysis using the software Statistica® 13.0 (StatSoft Inc., USA).

Table 4.1. Uncoded variables and their respective values.

Coded variable	Uncoded variables	Levels				
		-1.682	-1	0	+1	+1.682
A	Lipid concentration (%)[w.b]	2.0	4.0	7.0	10.0	12.0
B	Extract concentration (%)[w.b]	0.5	1.5	3.0	4.5	5.5
C	Drying aid concentration ratio i.e. drying aid:(lipid+extract)[d.b]	0.86	1.0	1.2	1.4	1.54

w.b: wet basis; d.b: dry basis

Table 4.2. Nonrandomized CCD used in proliposomes preparation (coded variables).

FORMULATIONS	CODED VARIABLES		
	A[a]	B[b]	C[c]
F1	-1.000	-1.000	-1.000
F2	1.000	-1.000	-1.000
F3	-1.000	1.000	-1.000
F4	1.000	1.000	-1.000
F5	-1.000	-1.000	1.000
F6	1.000	-1.000	1.000
F7	-1.000	1.000	1.000
F8	1.000	1.000	1.000
F9	-1.682	0.000	0.000
F10	1.682	0.000	0.000
F11	0.000	-1.682	0.000
F12	0.000	1.682	0.000
F13	0.000	0.000	-1.682
F14	0.000	0.000	1.682
F15	0.000	0.000	0.000
F16	0.000	0.000	0.000
F17	0.000	0.000	0.000

[a]Lipid concentration (% w/w, w.b.); [b]Extract concentration (% w/w, w.b.);
[c]Drying aid concentration ratio (%w/w, d.b.), i.e. drying aid:(lipid+extract).

The regression models fitted to experimental results allowed the determination of the best condition for proliposome production, by using the multiresponse optimization (desirability approach). Desirability values for responses evaluated showed significant dependence on composition variables studied. By using the desirability function in the Statistica® 13.0 software, respective values of each coded variable to reach the desired outcomes were established. Fresh proliposome batch was prepared at the optimum conditions determined, and its properties

compared to those estimated by the regression model. The differences were reported as percentile relative error.

4.4.6 Physicochemical characterization of the proliposomes and drying performance

Moisture content, water activity, total content of polyphenol markers, percentage retention of marker compounds, zeta potential and size distribution of the redispersed powder, flow properties (apparent and compaction density, Carr index, and Hausner factor) were the properties used for proliposomes characterization.

In addition, the proliposome obtained at optimized condition, were submitted to S.E.M. (scanning electronic microscopy), x-ray diffraction and fourier transformed infra-red (FTIR) analyses to determine its morphology, crystalline characteristics, and chemical interaction between the proliposome constituents, respectively.

Spray drying performance was evaluated by determination of the powder production ratio, referred to as product recuperation (R_{EC}).

The experimental methods used in these characterizations are presented following:

4.4.6.1 *Moisture content (X_p) and water activity (A_w)*

The moisture content of the spray dried powder was determined by gravimetric method in a moisture analyzer Sartorius MA35 (Goettingen, Germany). Water activity was measured in an AquaLab 4TEV instrument (Decagon Devices Inc., Pullman, WA) at 25 ºC, using the dew point sensor. Results are expressed as mean and standard deviation of triplicate measurements.

4.4.6.2 *Concentration and retention of polyphenol markers in proliposomes*

The concentration of selected rosemary polyphenol markers in proliposomes was evaluated by the HPLC-DAD method described in Section 4.4.3.1. The retention of markers polyphenols in the dried powder was calculated as the concentration of the compound in the proliposome powder (Q_f) relative to the original amount added to the liposomes composition (Q_i) by using Equation 4.8. Total content of marker in bulk quantity was determined as concentration (w/w) of each compound in any taken sample of proliposome powder, results being mean and standard deviation of triplicate determinations.

$$Retention\ (\%) = Q_f / Q_i \cdot 100 \qquad (4.8)$$

4.4.6.3 *Proliposome redispersity, particle size, polydispersity index and zeta potential*

The capability of the powdered product to spontaneously form liposome vesicles when redispersed in water was evaluated. Samples of spray dried proliposomes were redispersed in purified water at the same solid concentration (10.9 % w/w) of the initial liposomal composition feed to the spray dryer. The mixture was placed under mild agitation for 60 minutes using a magnetic stirrer (IKA – Werke mod. RT 15, Germany). The particle size, polydispersity index (PDI) and zeta potential of the reconstituted composition were measured in a zetasizer (Malvern Nano ZS90, UK) using the principle of Dynamic Light Scattering, and compared with the values obtained for initial liposome formulations.

4.4.6.4 *Evaluation of pharmacotechnical properties of proliposomes*

- Apparent and compaction densities

The apparent density (ρ_a) was determined by gently placing an exactly weighed amount of proliposome (~1 g) into a 10 mL beaker and measuring the volume occupied. To determine the compaction density (ρ_c), the proliposome sample in the beaker was subjected to 1250 taps according to FDA's recommendations (USP, 2011) using a tapped density tester (Caleva mod. TDT 22). The results were expressed by the mean (standard deviation) of three determinations, defined according to Equation 4.9.

$$\rho = \frac{\text{mass}_{proliposome}}{\text{volume occupied}_{proliposome}} \quad (4.9)$$

- Carr's compressibility index and Hausner factor

The experimental values obtained for apparent density (ρ_a) and compaction density (ρ_c) were used for estimation of the Carr index (I_{carr}) and Hausner ratio ($I_{Hausner}$), by applying the Equations 4.10 and 4.11, respectively:

$$I_{Carr} = \frac{(\rho_c - \rho_a)}{\rho_c} \times 100 \quad (4.10)$$

$$I_{Hausner} = \frac{\rho_c}{\rho_a} \quad (4.11)$$

4.4.6.5 *Spray drying performance*

The product recovery (R_{EC}) during drying, the percentage amount of proliposome collected from the cyclone relative to solid content of feed formulation (CORTÉS-ROJAS *et al.* 2015), was assumed as a measure of system performance. R_{EC} was determined by mass balance in the spray dryer, according to Equation 4.12.

$$R_{EC} = \frac{M_C\,(1-X_P)}{W_S \cdot C_S \cdot T} \cdot 100 \qquad (4.12)$$

where: R_{EC} is the product recuperation (%); M_C, the mass of proliposome product collected by the cyclone (g); X_P, product moisture content (g); Ws, the liquid liposomal composition feed rate (g/min); C_S, the solid content of the feed liposomal composition (g/g); and T, the processing time (min).

4.4.7 Evaluation of physico-pharmaceutical characteristics of optimized proliposome

The proliposome obtained at optimized condition and the individual and physical mixture of its main constituents were submitted to scanning electronic microscopy (S.E.M.), x-ray diffraction and fourier transform infra-red spectroscopy (FTIR) analyses to characterize its morphology, crystalline characteristics, and chemical interaction between the constituents.

4.4.7.1 *Particle morphology and surface characteristics*

A small quantity of powdered samples (proliposome, isolated constituents and physical mixture) were placed on sample supports (aluminium stub) with the aid of a two-phase adhesive carbon tape (Electron Microscopy Sciences). Each sample was metalized by covering it with a very thin film of carbon and gold by evaporation and cathodic pulverization process, respectively, using a Bal-Tec model SCD-050 (Furstentum, Liechtenstein) Sputter Coater under pressure of 0.1 mbar.

Morphological characterization of samples was carried out using a scanning electron microscope (Carl Zeiss model EVO 50), operated at vacuum pressure of 10^{-5} Torr and acceleration voltage of 20 kV. All sample images were generated in a secondary electrons (SE) contrast mode at 20 kV (secondary electron detector).

4.4.7.2 *Crystalline properties*

X-ray diffraction analyses were carried out with the objective to determine the crystalline or amorphous state of the starting materials and of proliposomes, and the effect of production process on these properties, as well. Samples analysed included pure samples of each constituent of the proliposome – lecithin, cholesterol, lyophilized extract, lactose – as well as the optimized spray dried proliposome product. In this manner, the state of each component part before and after processing may be observed.

Analyses were carried out by putting each dry powder sample on a sample support and the surface shut by a glass slip. X-ray diffractograms were obtained in an automatic X-ray diffractometer (D2 PHASER, Bruker-AXS, Billerica, Massachusetts, USA), with CuKa radiation at a wavelength of 1.54060 Å, voltage of 30 kV, 10 mA current, and a θ/2θ coupled scanner using Lynxeye (mode 1) detector. Operations were carried out at scanning speed of 6 °/min; scanning range 2θ between 5 and 50°; step size of 0.025°; and a step time rate of 2.4°/min. The angular positions of the intense peak from the individual excipients, proliposome formulation and physical mixture were examined and compared to the ones of the pure lyophilized extract.

4.4.7.3 *Fourier Transform Infrared Spectroscopy (FTIR)*

FTIR analyses were carried out in a Nicolet 380 spectrophotometer (Thermo Nicolet, USA) coupled to an accessory known as attenuated total reflection (ATR) Durascope (Smiths Detection, USA). The spectra were acquired with a resolution of 4 cm^{-1} around the spectral region of 4000 – 700 cm^{-1}. The Durascope, is equipped with a diamond crystal whose spectra absorption is between 4000 – 400 cm^{-1}, although the spectral range of 700 – 400 cm^{-1} exhibit high absorption, more difficult to analyse. Initially, measurement of *background* absorbance was made, being without any sample placed in the ATR crystal. Subsequently, the sample was deposited on the platform (analysis area with a diameter of 250 μm); completely covering the ATR crystal and the spectra was collected. In order to obtain good contact between the diamond crystal and the sample, pressure was applied on the sample as provided by the equipment. Acquisition of absorption spectra was carried out by utilizing steps of 4 cm^{-1} and 32 repetitions per spectrum. All samples were analyzed in triplicates.

4.4.8 Biological activities

The rosemary plant is known for many traditional uses, and aspects of phytochemistry and biological activities of its extracts have been carried out extensively. In this work, emphasis was given to the antioxidant and antimicrobial activities of the plant polyphenols, activities for which the plant is specifically recognized. The methods hereafter presented were also used to measure the potential bioactivity of lyophilized extract (LE) and demonstrate that prepared spray dried proliposome (SDP) retained the measured bioactivity, and in certain cases showed superior activity.

4.4.8.1 *Antioxidant assay*

The extract of rosemary is a known rich source of compounds that sequester free radicals, such as flavonoids and polyphenols. The antioxidant activity of the LE and optimized SDP were determined by the DPPH$^{\bullet}$ (1,1-Diphenyl-2-picrylhydrazyl free radical) scavenging method (FERNANDES et al., 2014). This method has been widely used in several studies, including screening of plant extracts as potential antioxidants. In the presence of an antioxidant molecule, the DPPH$^{\bullet}$ radical is reduced due to its capability of accepting a hydrogen atom supplied by the antioxidant compound. The reduction might be monitored by measuring the concentration-dependent decrease in absorbance at 517 nm, observable as colour change from violet to pale yellow.

Stock solution of lyophilized extract and spray dried proliposome (equivalent to 70 mg/mL of LE) were prepared by reconstituting the powders with absolute ethanol (analytical standard, A.S). The stock solution was subjected to bath sonication for 30 minutes to ensure complete dissolution of bioactive compounds. From these stock solutions, varying volume of stock solution was thereafter taken and diluted with ethanol (A.S), resulting in different concentrations for each powder sample. In this manner, LE and SDP samples were evaluated at 2.8 – 55.8 µg/mL (LE basis). 1 mL of 0.1 M acetate buffer (pH 5.5), 1 mL of ethanol, 0.5 mL of 250 µM ethanolic solution of DPPH$^{\bullet}$ were mixed in a test tube, to which 10 µL of dilute sample solution being evaluated was added. The change in absorbance was measured at 517 nm after 20 minutes of reaction time at room temperature. A blank solution containing 1 mL of the acetate buffer and 1.5 mL absolute ethanol (A.S) was used as the blank while a solution containing 1 mL of the acetate buffer, 1 mL of absolute ethanol (A.S), and 0.5 mL of DPPH$^{\bullet}$ solution with concentration of 250 µM in absolute ethanol but without DPPH$^{\bullet}$ solution, was used as negative control. Quercetin (0.4 – 3.0 µg/mL)

and the synthetic antioxidants butylated hydroxytoluene (BHT) (5.0 – 50.0 µg/mL) and butylated hydroxyanisole (BHA) (1.0 – 10.0 µg/mL), were used as the reference antioxidants (positive controls). Results were obtained and expressed as percentage inhibition and IC_{50}. The percentage $DPPH^{\bullet}$ inhibition/reduction was calculated according to Equation 4.13 while IC_{50} was taken as the sample concentration in µg/mL required to reduce 50 % of the $DPPH^{\bullet}$ free radicals added to the reaction medium. All determinations were performed in triplicate and results expressed as mean (standard deviation).

$$\text{DPPH reduced (\%)} = \frac{Abs_{inicial} - Abs_{final}}{Abs_{inicial}} \times 100 \qquad (4.13)$$

where: Abs = Absorbance

4.4.8.2 *Antimicrobial assay*

Antimicrobial tests were carried out in order to highlight the potential uses of the product as a preservative or antimicrobial agent for pharmaceutical, nutraceutical and cosmetic products. The assays were done with the lyophilized extract (LE) and the spray dried proliposome (SDP) product obtained at the optimal conditions. Bacteria and yeast used were standard strains of the American Type Culture Collection (ATCC).

For the antibacterial tests, stock solutions of LE and SDP (equivalent to 5 mg/mL of LE) were prepared as follows:

Sample I (LE): 25 mg of LE was weighed into a 5 mL volumetric flask and the volume completed with 50% methanol. The dispersion was subjected to bath sonication at 30 ^{o}C for 60 minutes to guarantee complete dissolution of the bioactive compounds of interest. The resulting solution/dispersion was transferred into a 10 mL beaker and subjected to magnetic stirring for 30 minutes.

Sample II (SDP): a sample mass of SDP (equivalent to 25 mg of LE) was weighed into a 5 mL volumetric flask and the volume completed with 50 % methanol. The dispersion was subjected to bath sonication at 30 ^{o}C for 60 minutes for dissolution of bioactive compounds of interest. The resulting solution/dispersion was transferred into a 10 mL beaker and subjected to magnetic stirring for 30 minutes.

Sample III (SDP): a sample mass of SDP (equivalent to 25 mg of LE) was weighed into a 5 mL volumetric flask and the volume completed with 50 % methanol. The dispersion was transferred into a 10 mL beaker and subjected to magnetic stirring for 90 minutes.

4.4.8.2.1 *Evaluation of bacterial and fungal sensitivity*

The antimicrobial activity of the extract and SDP were assayed by the broth microdilution test. The assays were performed on a 96-well flat-bottom plates, which consist of columns numbered from 1 to 12 and rows from A to H (Figure 4.4).

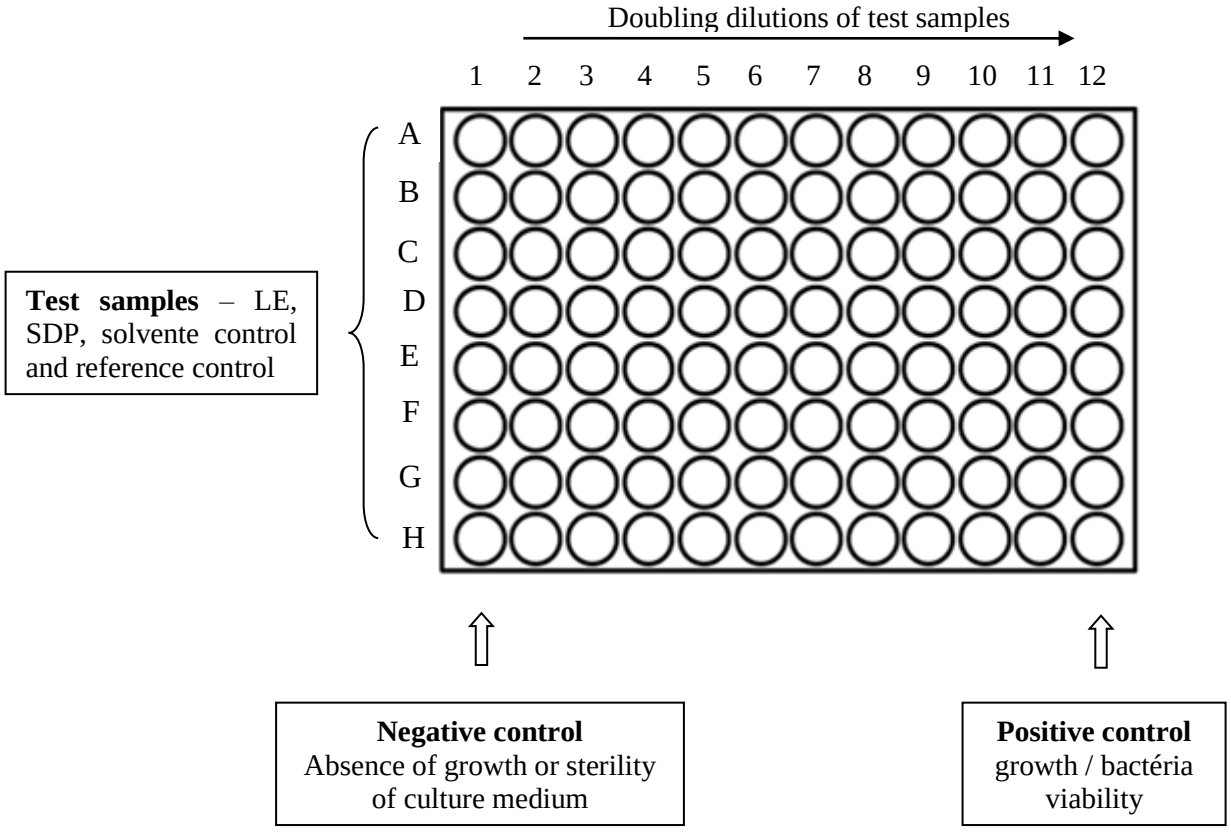


Figure 4.4: Scheme of the microplate prepared for each organism in the antimicrobial assays.

4.4.8.2.2 *Assessment of bacterial sensitivity*

Antibacterial activity of prepared samples were evaluated by determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the broth microdilution technique according to the guidelines of Clinical and Standard Laboratory Institute (CLSI, 2012) with very slight modifications. The assay was carried out against strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 21332), *Pseudomonas aeruginosa* (ATCC 2327), and *Klebsiella pneumoniae* (ATCC 23357).

Briefly, each test organism was streaked on Mueller-Hinton Agar and incubated for 24 h at 35 ^{o}C. Five colonies of approximately 1 mm diameter were picked and suspended in 5 mL sterile 0.9 %w/v saline solution. The resulting suspension was vortexed for 15 seconds and the cell density was spectrophotometrically adjusted to 0.5 McFarland standard at 530 nm wavelength in 0.9 %w/v saline solution, resulting in an inoculum containing 1.5 x 10^8 CFU/mL. A working suspension of 1.5 x 10^6 cells per mL was made in Mueller-Hinton Broth by 1:100 dilution. Exactly 10 µL of the final bacterial inoculum was added to each well containing 100 µL each of Mueller Hinton Broth and doubling dilution of the respective test sample.

In this manner, the MIC of samples were determined with final bacterial density of 0.75 x 10^5 cells per mL in each well. The test and control samples, and their respective concentration range were as follows: 2.5 mg/mL – 4.883 µg/mL for both pure LE and SDP (LE basis); 0.6 mg/mL – 1.17 µg/mL for the positive control, chlorhexidine; 2.5 mg/mL – 4.883 µg/mL for the polyphenol standard, rosmarinic acid; 25 – 0.0488 % for sovent control, methanol; physiologic saline as negative control (with growth); and plain medium as growth control (no growth, to confirms aseptic methodology).

The plates were incubated for 48 h at 35 ^{o}C after which it was visually examined for the presence or otherwise of bacterial growth. Confirmatory test was carried out by adding 20 µL of 0.02% resazurin to each well and further incubating for 1 h at 35^{o}C. Presence of bacterial growth was indicated by a change from bluish-purple to pink colour; where bluish-purple colour indicate the absence of bacterial growth. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth.

The MBC of all test samples and standards were determined by taking a 10 µL-sample with the aid of a pipette from the MIC well and wells with higher concentrations (respective wells

without growth, for each test sample) and placing it on already prepared and marked Mueller Hinto Agar plates. The plates were then incubated at 35 °C for 24 h. The MBC against the respective bacteria was determined as the lowest concentration of each test sample able to kill 100 % of the bacteria i.e. without any visible growth Assays were made in triplicates with duplicate controls.

4.4.8.2.3 *Assessment of fungal sensitivity*

This assay was carried out by using a *Candida albicans* strain (ATCC1023) as a model. Stock solutions of pure LE and SDP at 10 mg/mL (LE basis) were prepared in 50 % methanol and sonicated for 60 minutes in an ultrasonic bath, and thereafter maintained under mild agitation for 30 minutes using a magnetic stirrer. A second SDP solution was prepared by using only mild agitation on the magnetic stirrer for 90 minutes. The determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were performed by the broth dilution technique according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI 2017) with slight modifications.

Briefly, *Candida albicans* was streaked on Sabouraud Dextrose Agar and incubated for 24 h at 35 ^{o}C. Five colonies of approximately 1 mm diameter were picked and suspended in 5 mL sterile 0.9 %w/v saline solution. The resulting suspension was vortexed for 15 seconds and the cell density was spectrophotometrically adjusted to 0.5 McFarland standard at 530 nm wavelength in 0.9 %w/v saline solution, resulting in a suspension containing 1.5 x 10^8 CFU/mL. A working suspension of 0.75 x 10^5 cells per mL was made in Sabouraud Dextrose Broth by 1:100 dilution followed by 1:20 dilution. Exactly 100 µL of the final fungal suspension was added to each well containing 100 µL of a doubling diluted test sample. MIC of samples were determined with final fungal density in each well of 0.75 x 10^4 cells per mL while the concentration ranges of test samples were as follows: 2,500 µg/mL – 4.883 µg/mL for both pure LE and SDP (LE basis); 1,250 – 2.441 µg/mL for the polyphenol standard, rosmarinic acid; 250 – 0.488 µg/mL for terbinafine, as positive control; 12.5 – 0.024 % for methanol, as solvent control; 0.9 %w/v saline solution, as negative control (with growth); and plain medium as growth control (no growth, to confirms aseptic methodology).

The plates were incubated for 48 h at 35 ^{o}C after which it was visually examined for the presence or otherwise of fungal growth. Confirmatory test was carried out by adding 20 µL of 0.02 % resazurin to each well and further incubating for 1 h at 35 ^{o}C. Presence of fungal growth was

indicated by a change from bluish-purple to pink colour; where bluish-purple colour indicate the absence of fungal growth. The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. 10 μL of the broth from MIC well were then incubated on Sabouraud Dextrose Agar at 35 °C for 24 h for the determination of MFC, the lowest concentration able to kill 100 % of the yeasts. Assays were made in triplicates with duplicate controls.

4.4.9 Stability testing of lyophilized extract and proliposome product

During the developmental stages and prior to product registration, regulatory agencies require that phytomedicines (and other bioactive materials) be submitted and approved in various tests, including stability testing protocols such as those described by ANVISA (2012). Since most herbal-based bioactive systems contain multiple components with source-to-source variations, they often differ in their chemical composition and physicochemical properties. As such, assessment of stability of phytopharmaceuticals is individualized and carried out within the ambits of established standard protocols for stability testing.

In this work, accelerated stability testing (AST) was carried out in order to assess the effects of storage temperature (^{o}C) and relative humidity (RH) on the physical and chemical properties of lyophilized extract (LE) and spray dried proliposome (SDP), including the evaluation of the visual appearance, physicochemical properties, and degradation of the polyphenol marker compounds (caffeic, rosmarinic and carnosic acids, and carnosol) along the storage time. Testing was carried out under controlled storage conditions (Table 4.3) achieved in climatic chambers (Nova ética® B.O.D 411D and Nova ética® 420E).

Table 4.3: Stability test conditions and sampling time for quantification of polyphenol in stored samples

Temperature (^{o}C)	Relative Humidity (%)	Code	Sampling time (T_{days})								
			0	5	10	15	20	30	40	50	60
25	32.4	A	-	×	×	×	×	×	×	×	×
	63.5	B	-	×	×	×	×	×	×	×	×
45	32.4	C	-	×	×	×	×	×	×	×	×
	63.5	D	-	×	×	×	×	×	×	×	×
8*	~50.0	R	×	×	×	×	×	×	×	×	×

*Samples stored in hermetically sealed packages.

Samples of 0.35 g of proliposome or 0.25 g pure lyophilized extract were exactly weighed on an analytical balance and placed into open, amber coloured glass bottles (2 cm diameter and 4 cm height). These amounts guarantee a thin layer of samples of the spray dried proliposome and lyophilized extract in their respective containers, allowing a high surface area between surrounding air and powder during storage. The following storage conditions were studied: Temperature of 25 and 45 °C at relative humidity (RH) conditions of 32.4 % and 63.5 % (Table 4.3). The desired relative humidity values were achieved in airtight desiccators containing saturated salt solutions of $MgCl_2 \cdot 6H_2O$ salt to achieve relative humidity of 32.4 % and $NaNO_2$ salt for relative humidity of 63.5 %.

The saturated salt solutions were prepared with high purity salts and freshly distilled water. Solutions obtained were very viscous, having a large number of crystals present, thus guaranteeing the saturation and consequently the desired environment relative humidity (LABUZA, 1984). Another care taken was to ensure the presence of a thin layer of water (0.5 cm) above the salt layer inside the desiccator. During the storage period the saturated solutions were periodically stirred to ensure saturation and maintenance of the preset testing conditions. The experimental set up is represented in Figure 4.5.

Further, storage samples of SDP and LE were weighed amd placed into moisture-proof storage envelope. The envelopes were hermetically sealed, and stored in a refrigerator at 8 ^oC. The stability test was carried out during 60 days and samples for analysis were withdrawn at predetermined intervals (Table 4.3).

The effect of the storage conditions on the physical properties of the powder was evaluated by monitoring macroscopic alterations in powder properties (visual examination of powder flow, agglomeration and caking, colour changes, changes in weight, ease of redispersibility, and properties of liposomal vesicles obtained on aqueous redispersion of proliposome powder).

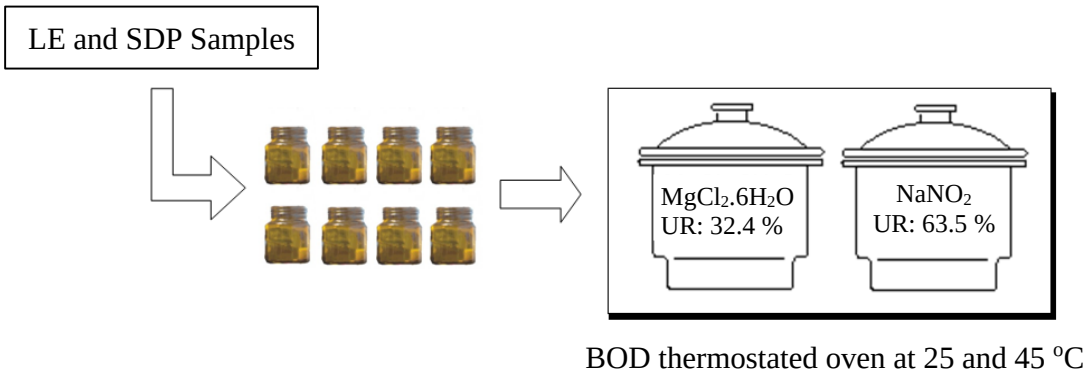


Figure 4.5: Sample layout and desiccator preparation scheme.

Samples of LE and SDP were withdrawn at preset periods and analysed by various techniques in order to determine changes in their visual appearance and physicochemical properties (water sorption, potential degradation of bioactive markers, analysis of powders morphology by S.E.M., evaluation of crystalline or amorphous state of powders by XRD analysis, and observing the fate of chemical groups present with the aid of FTIR). Quantitative analysis of colour changes and evaluation of antioxidant activity of samples by the DPPH• radical sequestering assay were also carried out. The methods employed are described as follows:

4.4.9.1 *Changes in weight and visual appearance*

About 1.5 g of pure lyophilized extract and spray dried proliposome were weighed into respective open, amber-coloured Petrit plates. The initial weight (time = zero day; T_{0day}) of the plates with their content were noted and the plates were thereafter stored at their respective conditions of storage used in this study. Sample weight was monitored throughout the period of experiment by gravimetric analysis and reported as mean absolute percentage of the ratio of the difference in weight to initial weight, Equation 4.14. Weight change was mainly caused by water desorption or absorption during storage period.

$$\text{Change in weight (\%)} = \frac{W_{T60days} - W_{T0day}}{W_{T60days}} \times 100 \qquad (4.14)$$

Physical examination of powder flow, agglomeration and caking was carried out using the same plates utilized for monitoring weight changes. Powder flow was described as flowable (F);

semi-flowable (SF); or non-flowable (NF), depending on their behavior when the plates were gradually tilted to about 45^{o}. Powder agglomeration was noted as presence of lumps and granular materials in the powder mass, while caking was considered as impeded or total lack of flow in which the powder grains/particles are stuck to each other and to the walls of the container. The extent of agglomeration and caking were visually observed and described as either absent (N); present (Y); or conspicuously present (YY). Changes in colour during storage were also visually observed and registered with picture images.

4.4.9.2 *Quantification of bioactive polyphenols markers*

The amount of the marker compounds – caffeic acid, rosmarinic acid, carnosol, and carnosic acid – remaining at each test point was determined, relative to their respective concentration in the powder samples at zero time (T_{0day}). The amount of each polyphenol marker compound in the LE and SDP samples was quantified by HPLC (using the method described in Sections 4.4.3.1 and 4.4.6.2 Assays were carried out in quadruplicates and results expressed as mean and standard deviation. Graphs of degradation pattern (i.e. Concentration of polyphenol marker compounds vs time) were thereafter derived.

- *Degradation kinetics of marker compounds*

Kinetics of each polyphenol compound degradation was evaluated by adjusting zero order and first order models to degradation data according to equations 4.15 and 4.16, respectively (BANKOLE; OLADIMEJI, 2017). Models were assessed by degradation rate constants (k_0 and k_1) and adjusted correlation coefficient (R^2_adj) in order to select the best fitting models for predicting shelf life at each storage condition.

$$C_t = C_0 + k_0 t \quad (4.15)$$

$$\ln C_t = \ln C_0 - k_1 t \quad (4.16)$$

where: C_0 – initial concentration of polyphenols compound; C_t – concentration of polyphenols compound at time t; k_0 and k_1 – release rate constant for zero-order and first-order, respectively.

4.4.9.3 *Physicochemical characterization of the samples submitted to stability testing*

The LE and SDP samples at time zero (T_{0day}) and after 60 days of storage (T_{60days}) at the conditions indicated in Table 4.3 were submitted to S.E.M., XRD and FTIR analysis in order to detect changes in their physicochemical characteristics, namely: surface properties and morphology, crystalline or amorphous state, interaction between the chemical groups. These analyses were done following the procedures presented in Section 4.4.7.

The samples were also submitted to colorimetric analysis to measure color changes during stabilility testing, which can be linked to degradation reactions (e.g. hydrolysis, occurrence of Maillard reactions, lipid oxidation, and so on). The LE and SDP were monitored throughout the storage period at all storage conditions. Dispersions of LE and SDP samples were prepared in 50 mL of methanol 50 %. The dispersion was subjected to bath sonication for 15 minutes and made up to volume with the solvent. It was thereafter transferred into a 50 ml falcon tube and centrifuged at 7500 rpm for 25 minutes. 20 mL of the supernatant was measured into the quartz cuvette of the colorimeter and analyzed (ZAMPIER, 2012; CORTES-ROJAS, 2015). The colorimetric analysis was done in a Color Quest XE colorimeter (Hunter Lab, Reston, Virginia, USA). The measurements were carried out using the D65 illuminant and an observation angle of 10^o. The colorimeter was operated at the Total Transmittance (TTRAN) mode with the filter at nominal position and through an area view of 1.0 inch, to measure three parameters; "L-a-b" being: luminosity (L*), red saturation index (a*), and yellow saturation index (b*) as proposed by the Comission Internacionale l'Eclairage (CIE).

Results were expressed in terms of luminosity, L*, being black for L* = 0 and white for L* = 100; and parameters of chromaticity defined as a* (+a* = red and –a* = green) and b* (+b* = yellow and –b* = blue). These parameters were used to calculate the Chroma (C*), and Hue angle (H^o), otherwise known as tone, based on Equations 4.17 and 4.18, respectively.

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (4.17)$$

$$H^o = tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (4.18)$$

4.4.9.4 *Antioxidant activity of lyophilized extract and spray dried proliposome*

The effects of storage period and condition on biological activity were determined by evaluation of antioxidant activity of LE and SDP samples submitted to stability testing. The evaluation of antioxidant activity was carried out by the DPPH$^{\bullet}$ (1,1-Diphenyl-2-picrylhydrazyl) free radical scavenging method, following procedure described in 4.4.8.1. Activity is expressed as IC_{50} and percentage of inhibition values. Results were compared to the standard antioxidants; BHA and BHT, and with the polyphenol quercetin. These compounds are generally recognized for their potent antioxidant activity. Statistical differences between the experimental results were evaluated by the Two-Way ANOVA followed by Bonferroni post-hoc test, adopting a significance level of 5 % ($p < 0.05$).

5. Results and Discussion

5 RESULTS AND DISCUSSION

5.1 Granulometric analysis of milled leaves of *R. officinalis*

The purpose of milling is to mechanically reduce the plant material to small fragments, thus preparing it for the extraction procedure. Size reduction by milling will increase the surface area of the material thereby enhancing the extraction efficiency (SIMÕES et al., 2010).

The dried leaves of rosemary, after milling, were screened in a stack of sieves with the aid of a sieve shaker for the determination of particle size distribution. Figure 5.1 presents the results of particle size distribution (accumulated fraction against mean particle size) of the milled rosemary leaves used in this study. The average particle diameter was found to be 426.3 µm, classified according to the Brazilian Pharmacopoeia as moderately granular to semi-fine. This size range is considered optimum for extraction of bioactive compounds from herbal materials. Size reduction of biomass before extraction offers greater surface area for mass transfer, which enhances the diffusion of active principles into the solvent. However, there is a critical size limit below which extraction is impaired. Very fine particles may remain at the solvent surface during the extraction process and/or adsorb solvent on its surface, reducing the extraction efficiency (PĂTRĂUȚANU et al., 2019).

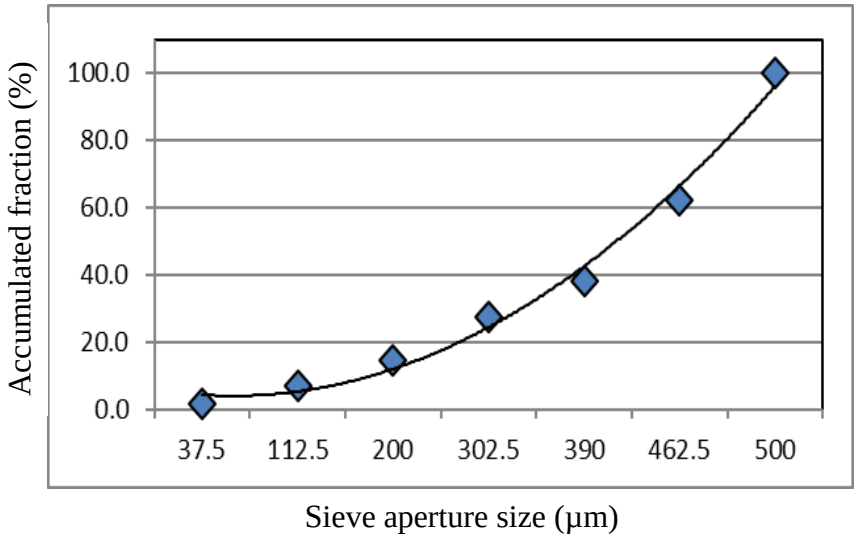


Figure 5.1: Granulometric distribution of dried milled leaves of *R. officinalis*

5.2 Characterization of milled dried leaves of *Rosmarinus officinalis*

Milled herbal material was also evaluated for total content of volatile material (loss on drying), total extractive material, essential oil content, total flavonoid and total polyphenol content. These preliminary tests are essential to determine suitability of the starting material as well as determine the influence of processing methods and handling (during project development) on constituent compounds of interest.

Excess of moisture content may result in degradation of principal compounds of interest (polyphenol) via hydrolysis and proliferation of microbial contaminants. The loss on drying was determined by gravimetric analysis using the open tray oven and the moisture analyzer. Results obtained were similar for open tray oven being 6.19 ± 0.02 % and moisture analyzer (balance) being 6.20 ± 0.12 % (Table 5.1). The values obtained are below 10 % limit recommended by the Brazilian Pharmacopoeia, indicating suitability of herbal material for project development.

Total extractive material obtained was 0.4 ± 0.05 % (Table 5.1). This is a reflection of the plant part soluble in the solvent system used i.e. 70 %v/v ethanol. This solvent system, intended to extract polyphenols of interest, has been successfully utilized in previous studies for extraction of the major polyphenols of rosemary and other plants (BENELLI et al., 2014; BENELLI; OLIVEIRA, 2019; SECOLIN; SOUZA; OLIVEIRA, 2017).

Rosemary is an aromatic plant, its leaves being a source of essential oils. Its essential oil has been evaluated and proven to exhibit various biological properties (RAŠKOVIĆ et al., 2014). Rosemary leaves used in this study was found to contain 1.27 ± 0.07 % (dry leaves basis) essential oil (Table 5.1), a value that is within the concentration range reported in the literature (BENSEBIA; ALLIA, 2015; HUSSAIN et al., 2010; JAMSHIDI; AFZALI; AFZALI, 2009). This affirms the suitability of the starting material and its potential for exhibiting biological activities.

5.3 Quantification of total flavonoids in milled dried rosemary leaves

The term "plant phenolic compounds" encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans and lignins (STALIKAS, 2007). These compounds occur as a complex mixture with a wide range of polarity and hydrophobicity.

Flavonoids are constituents that can be considered as markers due to their great structural diversity as well as their chemical stability (STALIKAS, 2007). The total content of flavonoids and polyphenols in the plant raw material were analyzed according to the procedures indicated in Sections 4.4.1.6 and 4.4.1.7, respectively.

Quantification of total flavonoids was based on the reaction of flavonoids with aluminum chloride ($AlCl_3$) resulting in the bathochromic shift of absorption wavelength of flavonoids. In this manner, concentration of flavones and flavonols in the extractive materials can be determined spectrophotometrically at λ_{max} = 425 nm and differentiated from other phenolic compounds which equally complex with $AlCl_3$ but show λ_{max} at 434 nm (ZAMPIÉR, 2012).

Total flavonoid (T_F) content was determined based on an analytical curve (APPENDIX III) constructed for quercetin, used as a chemical standard. The flavonoid content was calculated using the equation of the analytical curve for quercetin (APPENDIX IV) and results indicated as a quercetin equivalent – QE (Table 5.1). Results obtained showed that the rosemary dried leaves contained 6.94 ± 0.034 (mg QE/g).

5.4 Quantification of total polyphenols in milled dried rosemary leaves

The Folin-Denis method was used for quantification of total polyphenols. This method is based on the specific reaction between phenolics and Folin-Denis reagent in alkaline medium causing the reduction of the phosphomolibdic-phosphotungstic acid producing an intense blue colour which is measured spectrophotometrically at wavelength of 750 nm (BOTT, 2008; SOUZA, 2007).

Polyphenol content was quantified from a calibration curve constructed for pirogalol, used as a chemical standard. Folin-Denis reagent was added to each tube per time followed by sodium carbonate (Na_2CO_3) to provide the alkaline medium required for the reaction. The absorbance of samples was determined spectrophotometrically and the calibration curve constructed (APPENDIX V).

The polyphenol content (T_P) was calculated using the equation of the analytical curve for the pirogalol standard (APPENDIX IV) and results indicated as gallic acid equivalent – GAE (Table 5.1). Results obtained showed that the rosemary dried leaves contained 27.80 ± 2.11 (mg GAE/g) where GAE: gallic acid equivalent.

Table 5.1: Physicochemical characterization of crude vegetable drug and liquid extracts.

MATERIAL	PARAMETERS DETERMINED							
	Loss on drying (%)	$Extractive_{total}$ (% w/w)	Solid content (% w/v)	EO content (%)	pH (-)	Density (g/cm^3)	$Flavonoid_{total}$ (mg QE/g)	$Polyphenol_{total}$ (mg GAE/g)
Herbal material	[a]6.20 ± 0.12 [b]6.19 ± 0.02	0.4 ± 0.05	-	1.27 ± 0.07	-	-	6.94 ± 0.03	27.80 ± 2.11
Extractive solution	-	-	3.29 ± 0.05	-	5.75 ± 0.01	0.91912 ± 0.0013	15.28 ± 0.77	145.82 ± 3.58
Concentrated liquid extract	-	-	10.25 ± 0.11	-	5.01 ± 0.04	1.0102 ± 0.0006	14.17 ± 0.29	127.86 ± 8.90

[a] and [b] are determination by gravimetric analysis using the humidity balance and circulated tray oven, respectively
QE: quercetin equivalent; GAE: gallic acid equivalent

5.5 Preparation of extractive solution and concentrated extract of rosemary

Solvent extraction by maceration has been used by various authors to obtain different phytoactive compounds from their respective plant sources. Although there are newer methods for extraction of bioactive compounds from plant sources (MOJZER et al., 2016), solvent extraction remains relevant due to low cost, simplicity and ease of handling, and versatility (SIMÕES et al., 2010).

Since the polyphenols of rosemary vary in their polarity, it is logical to use a solvent system that facilitates extraction across the range of polarity of desirable compounds. Ethanol offers this advantage as it aids the extraction of less polar compounds which are not extractable with water. Also, ethanol is more volatile and has a lower boiling point than water, thus it has a greater tendency to be removed from hydroalcoholic extracts during concentration relative to water, leading to a reduction in the alcohol content of the concentrated extract. In addition, ethanol is known to have very little toxicity with acceptable safety prolife (OLIVEIRA; BOTT; SOUZA, 2006). Polyphenols are generally more hydrophilic than lipophilic due to their phenolic nature. Therefore, free polyphenols are readily extractable by solvents such as methanol, ethanol, acetonitrile, and acetone, or by their mixtures with water (MOJZER et al., 2016).

In a bid to ascertain extraction of both polar and non-polar polyphenolic compounds, therefore, a mixture of ethanol and water (70:30 v/v) was used in this study as extraction solvent (Section 4.4.2). This solvent system acceptable for the extraction of phenolic compounds and flavonoids (ZAMPIÉR, 2012) and has been successfully utilized in different studies by our group (BOTT; LABUZA; OLIVEIRA, 2010; GUEDES et al., 2006b; SOUZA; BOTT; OLIVEIRA, 2007). The use of ethanol in the extractive solution, associated with the temperature used during the extraction process aids chemical stabilization, preventing enzymatic oxidation of polyphenols which may occur during the extraction process (HARBORNE, 1984).

The extraction conditions utilized and procedure for obtaining concentrated extracts were selected from previous works developed by our group – LAPROFAR (SOUZA; OLIVEIRA, 2006; SOUZA, 2007; BOTT, 2008; CORTÉZ-ROJAS et al., 2012, ZAMPIER, 2012).

5.5.1 Characterization of extractive solution and concentrated extract of rosemary

The extractive solution and concentrated extract obtained from it were characterized by determination of pH (Section 4.4.2.1), relative density (Section 4.4.2.2), solids content (Section

4.4.2.3), total flavonoids (Section 4.4.2.4), and total polyphenols (Section 4.4.2.5). The experimental results are presented in Table 5.1.

The extractive solution gave acidic pH of 5.75 ± 0.01, reducing marginally to 5.01 ± 0.04 after the solution was concentrated. Phenolic acids, diterpenes, and flavonoids are the principal components of the rosemary extract, known to be acidic in nature (TSAO, 2010).

Concentrated extract displays greater acidity occasioned by solvent removal – resulting in the increase in percentage solid content for concentrated extract. Zampier showed correlation between total solid content of the extracts of *Dalbergia ecastaphyllum* and both total flavonoid and polypohenol content as well as antioxidant activity (ZAMPIÉR, 2012).

Higher percentages of total flavonoid and polyphenols corresponds to higher antioxidant activity represented as lower IC_{50} value, the extract concentration required to obtain 50% of its maximum antioxidant activity. The concentration step is thus relevant, firstly to increase the relative proportion of bioactive compounds and also to facilitate the next step, freeze drying of the extract.

Results showed that concentrated extract has slightly lower quantities of both flavonoid and polyphenol compared to those found in the extractive solution (Table 5.1). This could be a resultant effect of removal of ethanol during the concentration process. Higher water content in concentrated extract might have facilitated degradation of bioactive compounds, principally by hydrolysis, prior to the lyophilization process. This demonstrates the necessity of dehydration of extracts of phytoactive constituents to avoid potential degradation and loss of desirable bioactivity or even transformation to toxic compounds.

5.6 Preparation and characterization of lyophilized rosemary extract

Dehydration of concentrated liquid extract is necessary to retain biological activity by limiting degradation of bioactive compounds, among other considerations. Freeze drying was considered an appropriate method to obtain dried rosemary extract since low temperature drying (such as lyophilization) is known to preserve the integrity of otherwise thermolabile polyphenols contained in the extract.

5.6.1 Selection of polyphenol markers and quantification in lyophilized extract

5.6.1.1 *Revalidation of quantification method*

Efficacy and safety data from various medicinal plants are still insufficient to support their use, sometimes due to the lack of appropriate assessment methodologies. In many instances, it is either the bioactive principle has not been established or there is no sufficient data to support their range of activity (pharmacological actions), degree of efficacy (regarding dosage), and their toxicity profile (SOUZA-MOREIRA; SALGADO; PIETRO, 2010).

However, the rosemary plant has been successfully characterized and used for culinary and curative purposes for ages without any known toxicity. Rosemary extracts are commercially available for use as a natural antioxidant for foods in Europe and USA, and has received GRAS status (Generally Recognized as Safe), being considered safe and effective (AGUILAR et al., 2008; ANDRADE et al., 2018; NIETO; ROS; CASTILLO, 2018; PETIWALA; JOHNSON, 2015). It is for these reasons that rosemary extract has been suggested as a safe alternative to synthetic antioxidant, which have deleterious effects on humans (de RAADT et al., 2015; TAGHVAEI; JAFARI, 2015; WANG et al., 2008; XIE et al., 2017).

It is commonly opined that phytoactive materials derive their efficacy from fingerprint compounds which are usually monitored during the development and processing of such materials. Importantly, the search for markers is based on the assumption that any plant has one or more constituents responsible for its activity and that, in theory, they would replace the use of the plant in its entirety. However, there are several examples that bioactivity may be the result of synergism between the constituents of a particular plant. Cases of constituents reported as active have been reported which, when isolated, were toxic or ineffective for human use (SPRINGFIELD; EAGLES; SCOTT, 2005).

Several studies have shown rosemary as a polyphenol-rich herb, principal mong which are phenolic diterpenes (e.g. carnosic acid, carnosol), phenolic acids (e.g. rosmarinic and caffeic acids) besides others (ANDRADE et al., 2018; GENENA et al., 2008; LAURA et al., 2010; MORENO et al., 2006; SANCHEZ-CAMARGO; HERRERO, 2017; SOUZA et al., 2008; ZHANG et al., 2012).

The biological activity of rosemary has been associated to its major polyphenols; mainly rosmarinic acid (ROA), carnosol (CAR), carnosic acid (CAN), and mildly to caffeic acid (CFA)

(MOJZER et al., 2016; XIE et al., 2017; ZHANG et al., 2012). Therefore, these compounds were selected as chemical markers and quantified in the lyophilized extract to serve as a baseline in the determination of their concentration in the spray dried proliposomes developed.

The quantification method is as described under Section 4.4.3.1. The method was revalidated before being used in this work. Method revalidation was performed by determination of the linearity, accuracy, and precision parameters (Section 4.4.3.2).

Results of linearity experiments (Table 5.2) show that the method was linear within the concentration range utilized for each of the marker compounds with $R^2 > 0.999$ for all samples. The regression equations obtained were used in the quantification on respective compounds, where y is the peak area corresponding to concentration, x.

The accuracy of an analytical procedure is a measure of closeness or agreement between a theoretical concentration of a test material and the experimental value, usually expressed as relative error. The utilized method showed accuracy for the simultaneous quantification of the marker compounds at different test levels with relative errors lower than 5 % (Table 5.3).

Precision is a measure of the agreement of measures of a single analyte to the repeated aliquot measures of the same homogeneous sample usually expressed as a variance, standard deviation or variation coefficient. According to ANVISA (2017), it is regarded as repeatability (intra-day) or reproducibility/intermediate precision (inter-day). The precision of the method was confirmed with relative standard deviation (RSD) values of less than 5 % for intra- and inter-day analysis of all marker compounds (Table 5.4).

Table 5.2: Regression equations and coefficients for the marker compounds derived from plots of chromatogram area against respective concentration of each compound.

Marker compounds	**Evaluation parameters**	
	Equation	**R^2**
Caffeic acid	y = 40225x - 9742.1	0.9994
Rosmarinic acid	y = 21493x - 97583	0.9999
Carnosol	y = 2202x - 14101	0.9998
Carnosic acid	y = 2794.2x - 47708	0.9995

Table 5.3: Accuracy parameters for method utilized to simultaneously quantify marker compounds.

Accuracy parameters	***Biomarker Compounds Used at Varying Concentration (Levels)**				
	Caffeic acid (CFA)	Rosmarinic acid (ROA)	Carnosol (CAR)	Carnosic acid (CNA)	**Carvacrol (CVA)
Recovery (%)	98.1 - 99.5	98.7 - 100.3	-	98.9 – 100.8	99.4 – 100.1
Relative error	≤ 1.9	≤ 1.3	-	≤ 1.1	≤ 0.6

* Three concentration levels were utilized for each compound (*See method*)
** The same method was validated for carvacrol (here used as internal standard with detection at 284 nm)

Table 5.4: Precision parameters for HPLC method used to quantify the marker compounds.

Precision parameter		***Biomarker compounds at varying concentration (levels)**				
		CFA	ROA	CAR	CNA	Carvacrol
% RSD	**Day 1**	≤ 2.71	≤ 1.15	-	≤ 1.85	≤ 2.25
	Day 2	≤ 1.99	≤ 1.43	-	≤ 2.51	≤ 1.36

* Three concentration levels were utilized for each compound (*See method*)
RSD: relative standard deviation**Carvacrol
CFA: caffeic acid; ROA: rosmarinic acid; CAR: carnosol; CNA: carnosic acid

5.6.1.2 *Quantification of selected polyphenol markers in lyophilized extract*

Chromatography, in combination with other analytical techniques, allows for the separation and quantification of constituent compounds of a plant extract. This permits the knowledge of the chemical composition of a plant extract – being the active ingredient or the toxic compound – and quantification of a compound or group of compounds serving as markers of the species.

In this maner, qualitative and quantitative control is achievable, enabling the standardization of plant material and their products. With the aid of high-performance liquid chromatography (HPLC), for example, profiles of marker compounds can be obtained by application of validated methods.

Figures 5.2 (a – e) and 5.3. (a – d) show the chromatographic profiles for the lyophilized extract and the four selected markers of *Rosmarinus officinalis* quantified. Table 5.5 show the experimental values of the concentration of CFA, ROA, CAR, and CNA in the lyophilized extract.

Table 5.5: Concentration of polyphenol markers in lyophilized extract of rosemary.

	Concentration of marker polyphenols (%w/w)			
	CFA[a]	ROA[b]	CAR[c]	CNA[d]
Lyophilized extract	0.06±0.005	4.38±0.02	3.69±0.06	3.37±0.06

[a]Caffeic acid; [b]Rosmarinic acid; [c]Carnosol; [d]Carnosic acid.

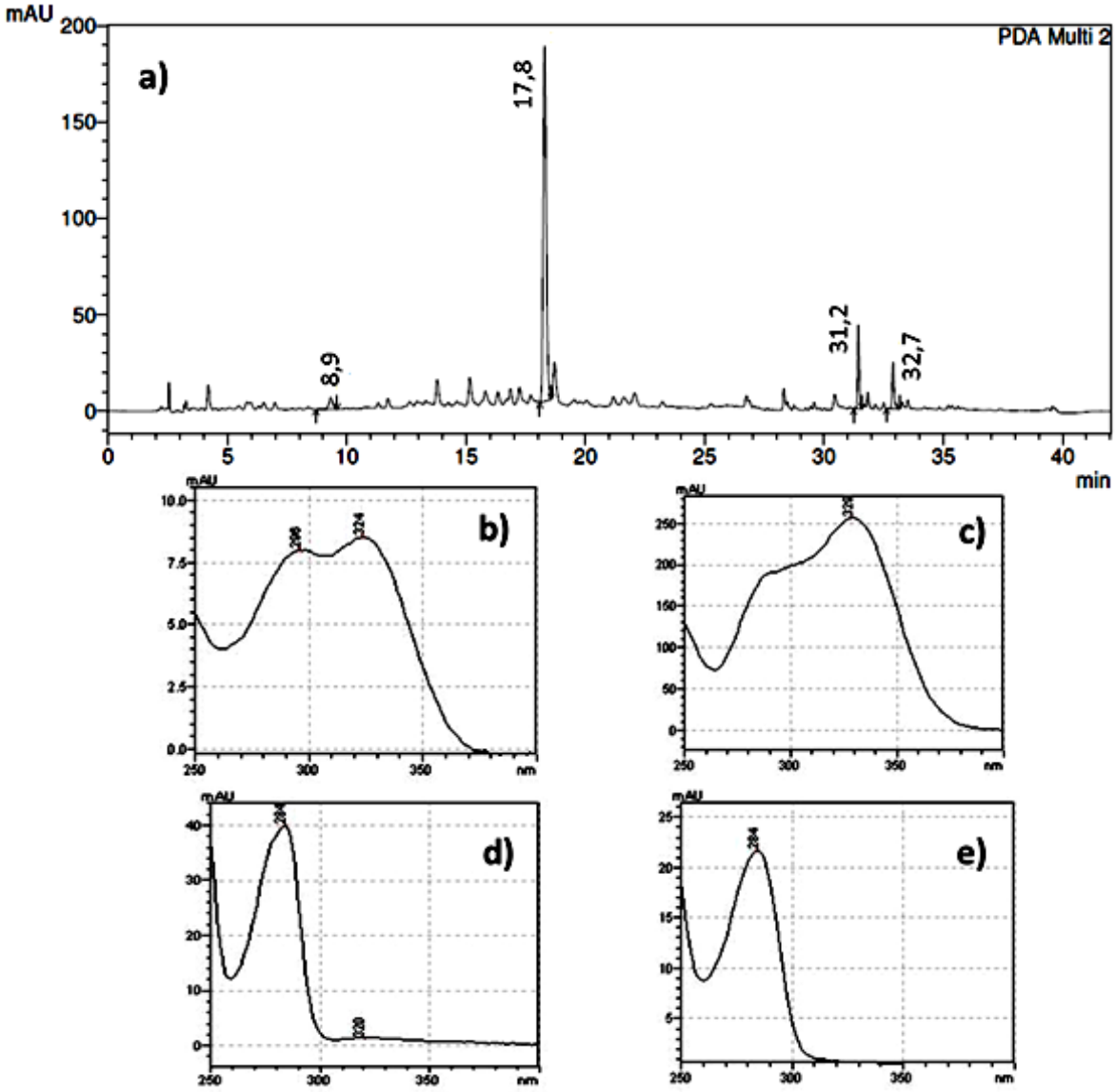


Figure 5.2: a) Chromatogram of the extract of *Rosmarinus officinalis* in 70 % ethanol; b) UV spectra showing the peaks with retention time of 8.9 min; c) UV spectra showing the peaks with retention time of 17.8 min; d) UV spectra showing the peaks with retention time of 31.2 min; e) UV spectra showing the peaks with retention time of 32.7 min.

(SOURCE: BENELLI, 2015).

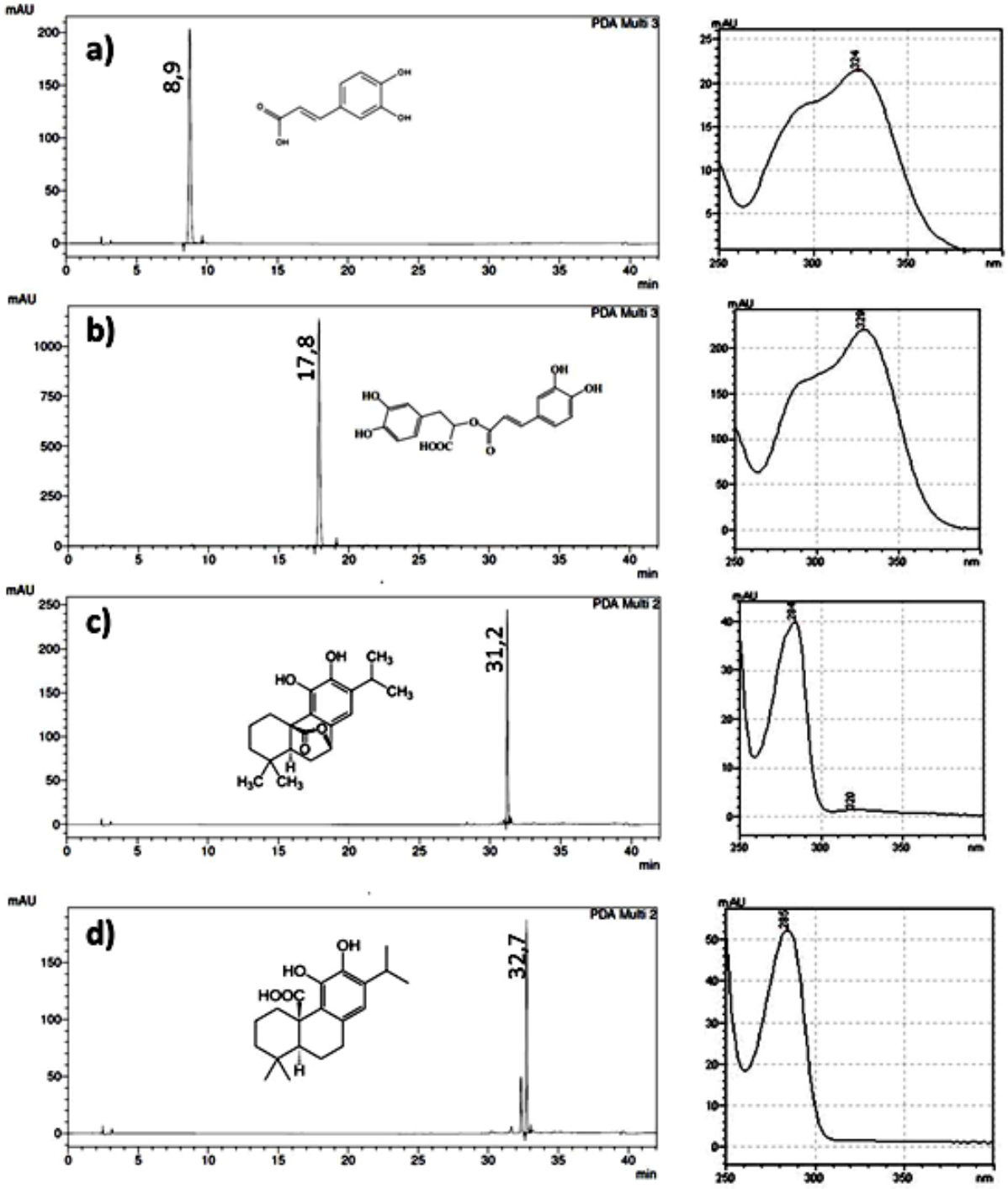


Figure 5.3. Chromatograms and UV profiles of the standards of: a) caffeic acid; b) rosmarinic acid; c) carnosol; and d) carnosic acid.

(SOURCE: BENELLI, 2015).

5.7 Encapsulation of rosemary polyphenols in proliposomes by experimental design

Encapsulation in the pharmaceutical and food industry is a process in which one or more ingredients or additives are coated with an edible layer. Encapsulation of plant extracts in different materials is a credible way to improve the physicochemical properties and to slow down the degradation rates of their main active constituents. The improvement of bioavailability of the bioactive compounds in biological systems has also been reported (IONITA et al., 2015; VISENTIN et al., 2012).

Liposome is one system among many for encapsulation of food and pharmaceutical ingredients and additives/excipients. The microscopic lipid vesicles, due to the lipophilic and hydrophilic portions of their constituents, are convenient for accommodation of substances of various natures. While the hydrophilic substances stay in the aqueous compartment, the lipophilic are inserted or adsorbed on the membrane (MACHADO et al., 2015). Literature reports revealed that the encapsulation efficiency of phenolic compounds depends on the morphology of the liposome, itself dependent on the method implemented for their preparation (FAN et al., 2007). There exist various methods for liposome preparation, one of the most viable ones being the proliposome method in which liposome vesicles are spontaneously prepared by dispersion of powder proliposome products in water.

The production of proliposomes by spray drying is a multivariate process. The physicochemical properties of product are affected by composition variables (ingredients selected and their relative proportions) and spray drying operating conditions (PATIL-GADHE; POKHARKAR, 2014). The Design of Experiment (DoE) is an efficient methodology usually adopted to determine the effects of multiple variables on product properties.

In this work, liposomal compositions encapsulating polyphenols of rosemary were firstly prepared by the method described in Section 4.4.4.1 and subjected to spray drying according to section 4.4.4.2. Design of experiment approach was used to observe the effect of composition variables on product properties. The central composite design (CCD) was employed to study the effects of selected variables (Sections 4.4.6) on characteristics of the resultant powder formulations.

5.7.1 Proliposome redispersibility and particle properties of prepared liposomes

A powdered product is essentially referred to as a proliposome if it has the capacity to easily form liposomes when hydrated. In order to validate the propensity of the developed spray dried proliposome (SDP) to easily redisperse to liposomes, dried products from the experimental runs were redispersed in purified water at the concentration of original liquid compositions. Proliposomes were readily redispersible in aqueous medium at same original concentration by mildly mixing the dispersion on a magnetic stirrer, promptly forming vesicles loaded with the rosemary polyphenols encapsulated in the dried system.

The mean particle size, polydispersity index (PDI), and zeta potential of vesicles resulting from SDP redispersion were compared with the values obtained for initially prepared liquid liposomal formulation (LLF) prior to spray drying (Table 5.6). Higher particle sizes were observed from hydrated spray dried proliposome (HSDP) compared to the corresponding liquid liposomal formulation (LLF) from which it was derived. While particle size ranged between 668 and 3006 nm for LLF, the sizes of vesicles obtained from redispersed SDP ranged between 1478 to 4530 nm.

No specific pattern of change was observed; however, the noted differences might have resulted from process influences. The increment in particle size observed might be due to association of the drying aid (lactose) with vesicles following spray drying, increasing the mean particle diameter. The observed increment in particle size might also be due to lamellarity of obtained liposome vesicles following aqueous redispersion. The proliposome method is known to generate multilamellar liposome vesicles with diameter greater than small unilamellar vesicles produced by the ethanol injection liposome preparation method (FONG et al., 2016).

However, the proliposome method is suitable for encapsulation of a wide range of drugs with varying alcohol/water solubility, and is known for extremely high encapsulation efficiency compared with other methods that are based on passive entrapment (TURÁNEK et al., 2003; ZOGHI; KHOSRAVI-DARANI; OMRI, 2018). The multilamellar or large unilamellar may be reduced by some physical methods, principally extrusion and sonication (NAVA et al., 2011; SHARMA; SHARMA, 1997; WANG, 2017; ZYLBERBERG; MATOSEVIC, 2016). In a further assay, for instance, the particle size of hydrated proliposomes was successfully reduced by up to 60 % by bath sonication of samples for 60 minutes, without attendant disruption of vesicle stability

(Sections 4.4.8.2 and 5.11.2). Statistical analyses revealed that vesicle sizes obtained are dependent on use concentration of extract and lipid (results of analysis not shown in this report).

Polydispersibility values obtained were generally ≥ 0.5 while zeta potential was below -20 mV for all formulations. While the zeta potential values indicate a potential thermodynamic stability of both the liquid formulation (LLF) and hydrated SDP, their PDI values can be considered adequate since the formulations developed have not been proposed for parenteral medical uses in which such disparity in particle sizes is unacceptable. Vesicles from hydrated SDP showed higher zeta potentials than those of corresponding LLF, perhaps due to the addition of the drying aid to liposomal composition.

Table 5.6: Particle properties of liquid liposome formulation (LLF) vs hydrated spray dried proliposome (HSDP) encapsulating rosemary polyphenols (nonrandomized CCD).

Formulation runs	Particle diameter (nm)		Polydispersity index, PDI (-)		Zeta potential, ZP (mV)	
	LLF	HSDP	LLF	HSDP	LLF	HSDP
F1	1818±350	2531±521	1.00±0.00	0.70±0.13	-38.4±1.1	-28.5±1.2
F2	1307±49	1478±94	0.89±0.05	0.96±0.04	-43.6±0.8	-24.7±2.1
F3	1045±223	2398±418	1.00±0.00	1.00±0.00	-34±4.3	-20.7±1.6
F4	1724±113	4530±774	0.88±0.03	0.34±0.07	-33.1±2.2	-24.0±1.8
F5	2909±478	3299±406	1.00±0.00	0.53±0.06	-35.1±2.7	-22.7±3.2
F6	836±27	1661±153	0.65±0.09	0.83±0.27	-37.8±0.9	-24.4±0.9
F7	874±124	3144±287	1.00±0.00	0.69±0.04	-27.8±3.1	-20.6±0.8
F8	1835±202	3150±387	0.92±0.08	0.46±0.04	-31.4±1.5	-20.2±0.7
F9	831±130	2878±176	0.88±0.09	0.64±0.14	-31.6±2.7	-27.9±2.2
F10	1776±191	1721±309	0.63±0.20	0.83±0.19	-37.5±0.4	-25.7±1.0
F11	668±122	1692±254	0.92±0.08	1.00±0.00	-34.0±1.6	-26.2±1.0
F12	734±90	2578±330	0.98±0.03	1.00±0.00	-33.5±2.4	-28.4±0.4
F13	2371±195	3922±732	0.93±0.13	0.58±0.06	-35.8±2.6	-32.2±1.0
F14	1750±180	2872±321	0.86±0.06	0.88±0.21	-34.2±3.1	-31.3±1.3
F15	3006±297	4166±308	1.00±0.00	0.97±0.05	-40.5±2.5	-29.3±1.6
F16	2541±199	4292±457	1.00±0.00	0.86±0.24	-33.8±1.5	-30.9±0.6
F17	2646±408	4064±589	0.61±0.02	0.89±0.12	-41.7±1.7	-27.9±1.5

LLF: liquid liposome formulation; HSDP: hydrated spray dried proliposome; F1 – F17: experimental runs

5.7.2 Density and flow properties of developed proliposomes

Spray dried proliposomes were characterized for powder density, compressibility and flow properties according to Sections 4.4.5.4. Experimental data for bulk and tapped density (ρ_b and ρ_t) were obtained for all spray dried proliposomes. Results obtained showed that the proliposome products presented rather high values for ρ_b and ρ_t ranging from 0.211 to 0.298 and 0.294 to 0.394, respectively. From the density data, the $I_{Hausner}$ and I_{carr} were derived (Table 5.7), being indirect measures of flow and compression behavior of powders, respectively. These parameters are very critical for characterizing powders in industrial processes such as capsules filling and tablet manufacture (GARCIA; PEREIRA; DIAS, 2012; SECOLIN et al., 2017).

Table 5.7: Density and flow properties of spray dried proliposomes.

Formulations	Density properties		Flow properties	
	Bulk density (g/cm^3)	Tapped density (g/cm^3)	$I_{Hausner}$ (-)	I_{carr} (-)
F1	0.252±0.003	0.350±0.030	1.43±0.07	30.1±3.6
F2	0.292±0.005	0.394±0.002	1.35±0.03	25.7±1.7
F3	0.249±0.002	0.337±0.015	1.35±0.05	26.0±2.8
F4	0.260±0.006	0.340±0.005	1.31±0.05	23.5±2.8
F5	0.252±0.016	0.332±0.004	1.32±0.10	24.0±5.7
F6	0.284±0.011	0.390±0.001	1.38±0.06	27.2±3.1
F7	0.211±0.002	0.279±0.022	1.32±0.09	24.2±5.4
F8	0.298±0.001	0.384±0.010	1.29±0.04	22.4±2.3
F9	0.211±0.008	0.283±0.017	1.34±0.03	25.2±1.9
F10	0.277±0.009	0.358±0.022	1.29±0.04	22.4±2.2
F11	0.267±0.012	0.367±0.013	1.38±0.01	27.5±0.8
F12	0.257±0.017	0.346±0.017	1.35±0.02	25.8±1.1
F13	0.214±0.036	0.294±0.059	1.37±0.05	26.8±2.5
F14	0.261±0.001	0.360±0.012	1.38±0.05	27.3±2.6
F15	0.252±0.006	0.335±0.004	1.33±0.02	24.7±1.1
F16	0.277±0.008	0.375±0.025	1.35±0.05	26.0±2.8
F17	0.242±0.021	0.332±0.022	1.37±0.03	27.0±1.4

$I_{Hausner}$ and I_{carr} values obtained differed across the formulations in the experimental design being in the range 1.29±0.04 – 1.43±0.07 and 22.4±2.2 – 30.1±3.6 %, respectively. These values show that SDP prepared generally showed passable to poor flowability and are compressible in nature. Although the Carr index is sometimes specifically indicated in compressibility property of

a powder, it is also a measure of flow, and vice versa. This is because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow.

The values obtained may be linked to interaction between particle-particle (cohesion) and/or particle-surface (adhesion). Materials processed by spray drying have been described as usually presenting poor flowability due to the small particle diameters obtained, resulting in highly compressible powders (CORTÉS-ROJAS; OLIVEIRA, 2012; ONWULATA; KONSTANCE; HOLSINGER, 1996; QUISPE-CONDORI; SALDAÑA; TEMELLI, 2011). Friction between small particles is higher, resulting in increased resistance to flow. With respect to flow, powders indicated as "passable" may hang up during flow and therefore need addition of flow aids (lubricants/glidants). For powders showing "poor" comportment, on the other hand, flow is generally obtained by inducing vibration (USP, 2014), principally via the processing equipment.

SDP obtained in this study showed better flow properties compared to those of products obtained during spray drying of lipid compositions encapsulating the extract or rosemary in various wall materials: Compritol®/Labrasol®/Gelucire®/Acacia gum + colloidal silicon dioxide (Aerosil® 200); Acacia gum/Maltodextrin/Capsul® + low ratio carboxymethylcellulose (CMC) as viscosity impacting agent; Acacia gum/Maltodextrin/Capsul® + high ratio carboxymethylcellulose (CMC) (BENELLI et al., 2014).

Statistical analyses (not included in this report) of the results show that the flow and compressibility parameters evaluated ($I_{Hausner}$ and I_{Carr}) are dependent on, and can be improved by altering the lyophilized extract and lipid content of the powders. The independence of these characteristics on carrier was discussed by SECOLIN et al (2017) in a study using lactose and trehalose as carrier. Materials such as silica and silicates have also been suggested as flow conditioners and contribute toward reducing the density of finely compacted powders (CORTÉS-ROJAS; OLIVEIRA, 2012).

5.8 Critical proliposomes properties and drying performance

The proliposome method was adopted for use in this study since it offers various advantages over other known methods for liposome preparation. However, certain factors are considered

critical to the physicochemical properties of proliposome powders as well as of liposome vesicles obtained when they are redispersed in aqueous medium (PATEL; SHELAT; LALWANI, 2016).

The selection of study variables depends largely on the desirable properties of the proliposome product. These desirable properties (product quality attributes) may in turn be formulated by considering the intended uses of the proliposome, either as a final product or its suitability for further processing and packaging.

Study variables of interest to this work are the composition variables which have previously been shown to influence quality properties. Quality attributes of proliposome product evaluated in this study are the moisture content and water activity, total concentration and retention of marker compounds, and powder recuperation form the spray drying process.

Even at a favorable drying condition, for example, lipid, drug and drying carrier concentrations varied in this study showed significant linear, quadratic and interaction effects on evaluated properties, albeit to varying degrees. This suggests that the formulation composition is critical in determining proliposome properties. Results and findings are discussed further in the following sections.

Table 5.8 presents the results of proliposomes properties and powder production (product recovery, R_{EC}), for all experimental runs carried out as detailed in Section 4.4.5.

Regression analyses were applied to the experimental data to find the statistically significant effects of composition variables on the responses evaluated. Hence, the linear, quadratic and interaction regression coefficients and their statistical significance at different levels were derived (Table 5.9). The ANOVA and effect estimates were based on assumptions of normal and independent residuals distribution, with mean zero and constant variance (MONTGOMERY, 2012).

5.8.1 Moisture content and water activity

Moisture content (X_p) of powder samples provides information regarding efficiency of solvent removal during drying, and can be linked to their physicochemical and microbiological stability, solubility, morphology and flowability. The SDP show very low values of X_p, in the range 1.7±0.14 % – 2.5±0.23 %, evidencing a slight effect of composition variables.

Water activity (A_w), being a measure of the energy state of water present in a system, is a property independent of sample quantity. The water activity has significant effect on several degradation reactions such as lipid oxidation, nonenzymatic browning, and so on. Values lower than 0.5 are usually recommended to avoid microbial growth, guaranteeing the microbiologic product stability (TAPIA; ALZAMORA; CHIRIFE, 2008). However, the lipid oxidation generally shows a minimum in the A_w range of 0.2 to 0.35, and increase below and above this range (LABUZA; DUGAN, 1971). Although this range is generally recognized, it is desirable to determine optimal water activity for each product. In this work the values of A_w were in the range of 0.301 to 0.472, above the lower limit value and slightly above the upper value; guaranteeing the low rates of lipid oxidation of the powdered proliposomes.

Therefore, it can be partially concluded that the drying condition and composition proportions of the liposomal formulation used are suitable for preparing products having potential stability to intra-matrix chemical reactions and microbial proliferation.

Table 5.8: Physicochemical properties of spray dried proliposomes and product recovery (R_{EC}) according to DoE.

			Concentration of Marker Compounds			Retention of Marker Compounds			
EXP. RUNS	X_p (-)	A_w (-)	ROA (mg/100g)	CAR (mg/100g)	CNA (mg/100g)	ROA (%)	CAR (%)	CNA (%)	R_{EC} (%)
F1	2.92±1.03	0.401±0.004	579.9±16.6	443.7±9.0	355.7±8.0	97.2±2.8	88.1±1.8	77.4±1.7	38.6
F2	2.68±0.90	0.377±0.006	245.9±2.2	244.2±5.8	180.2±2.0	86.1±0.8	101.3±2.4	82.0±0.9	45.8
F3	2.06±0.02	0.404±0.006	1191.6±25.8	814.1±19.5	634.4±4.2	102.9±2.2	83.2±2.0	71.1±0.5	38.2
F4	3.90±0.03	0.453±0.006	664.2±6.8	479.9±6.0	406.7±2.6	97.8±1.0	83.7±1.0	77.8±0.5	28.5
F5	2.71±1.07	0.365±0.009	481.9±3.4	432.6±6.5	321.8±3.3	96.9±0.7	103.0±1.6	84.0±0.9	43.0
F6	2.50±0.78	0.472±0.003	215.1±4.6	193.9±4.9	168.3±6.4	90.5±1.9	96.6±2.4	91.9±3.5	40.9
F7	1.98±0.04	0.430±0.004	975.1±11.3	611.8±7.0	551.5±2.0	101.0±1.2	75.1±0.9	74.2±0.3	40.7
F8	2.06±0.04	0.349±0.002	549.3±3.9	395.1±8.3	371.8±6.6	97.1±0.7	82.7±1.7	85.3±1.5	28.1
F9	2.05±0.00	0.301±0.004	1281.9±18.1	630.8±3.9	614.1±9.9	106.4±1.5	62.0±0.4	66.2±1.1	34.3
F10	3.12±0.22	0.397±0.007	369.0±3.1	309.3±4.5	265.0±1.5	93.0±0.8	92.4±1.3	86.8±0.5	20.1
F11	3.04±0.17	0.402±0.004	80.9.0±3.3	150.7±0.9	81.7±2.2	63.8±2.6	104.6±0.9	83.6±2.2	24.1
F12	1.96±0.01	0.370±0.004	916.0±16.0	546.3±3.9	487.0±5.2	104.4±1.8	73.8±0.5	72.1±0.8	29.5
F13	1.95±0.04	0.429±0.015	699.6±5.1	519.5±4.8	459.6±5.5	99.3±0.1	87.4±0.1	84.7±1.0	39.0
F14	2.50±0.19	0.411±0.015	510.1±12.0	368.7±4.8	343.8±7.3	98.5±2.3	84.4±1.1	86.3±1.8	40.1
F15	1.83±0.13	0.372±0.020	585.4±6.5	425.9±.3	364.1±7.0	98.1±1.1	88.5±1.4	79.2±1.5	33.5
F16	1.94±0.07	0.370±0.014	580.9±7.2	465.6±6.2	369.3±2.6	97.3±1.2	88.4±1.2	80.4±0.6	32.5
F17	1.77±0.16	0.395±0.00	578.8±6.0	441.8±3.3	381.3±2.5	97.0±1.0	87.7±0.7	81.0±0.5	32.7

R_{EC}: product (SDP) recovery from spray drying process; X_p: moisture content; A_w: water activity
ROA: rosmarinic acid; CAR: carnosol; CNA: carnosic acid

Table 5.9: Regression coefficients and their statistical significance levels for product properties and product recovery (R_{EC}).

Input factors (Lone/interacting)	X_p	A_w	Concentration of Marker Compounds			Retention of Marker Compounds			R_{EC}
	(-)	(-)	ROA (mg/100g)	CAR (mg/100g)	CNA (mg/100g)	ROA (%)	CAR (%)	CNA (%)	(%)
a_0 - Mean/Interc.	1.837*	0.402*	116.776*	88.472*	73.005*	97.342*	88.284*	80.157*	32.451*
a_1 - $A_{(L)}$	0.239***	-0.001	-45.242*	-22.403*	-19.385*	-3.587***	4.827***	4.741*	-3.011
a_{11} - $A_{(Q)}$	0.290***	0.009	15.738*	3.249	5.187*	1.268	-4.116	-1.192	-0.479
a_2 - $B_{(L)}$	-0.193	-0.008	47.765*	24.194*	23.725*	7.058*	-12.941*	-3.383*	-1.744
a_{22} - $B_{(Q)}$	0.260***	0.004	-7.387***	-5.343**	-5.788*	-4.261**	6.494***	-0.715	-0.624
a_3 - $C_{(L)}$)	-0.101	0.000	-11.404*	-8.819*	-5.249*	0.013	-0.292	2.175**	0.249
a_{33} - $C_{(Q)}$	0.161	-0.023**	0.136	1.418	2.513**	0.983	-1.050	1.994**	3.877***
a_{12} - $A_{(L)} \times B_{(L)}$	0.296***	0.023**	-8.809***	-2.817	-1.958***	1.061	0.162	0.674	-3.408
a_{13} - $A_{(L)} \times C_{(L)}$	-0.216	-0.015	4.220	1.963	1.748***	0.710	-1.565	0.965	-1.515
a_{23} - $B_{(L)} \times C_{(L)}$	-0.192	0.025**	-5.070	-5.643***	-1.801***	-0.832	-2.424	-0.740	0.321
Adj._R^2	0.751	0.824	0.967	0.951	0.992	0.744	0.720	0.853	0.645

*Effect significant at $p \leq 0.01$; **Effect significant at $p \leq 0.05$; ***Effect significant at $p \leq 0.1$.
A: Lipid concentration (%w/w), B: Extract concentration (%w/w); C: Drying aid:(lipid+extract) ratio
R_{EC}: product (SDP) recovery from spray drying process; X_p: moisture content; A_w: water activity
ROA: rosmarinic acid; CAR: carnosol; CNA: carnosic acid

Moisture content (X_p) and water activity (A_w) of SDP are expected to be linked to drying conditions and formulation composition, since drying promotes changes in water binding and dissociation. The regression analyses (Table 5.9) show that X_p is slightly influenced by the linear and quadratic effects of the variable Lipid concentration (A), quadratic effect of the lyophilized extract concentration (B), and of interaction A×B ($p \leq 0.1$). The drying aid ratio did not show statistical significance on SDP moisture content. However, the observed effects on X_p were not relevant from an engineering point of view, since the changes in X_p were small (perhaps due to the identical spray drying condition used).

On the other hand, the influence of the composition variables investigated were more pronounced on A_w. The regression analyses presented in Table 5.9 indicates significant effects of the drying aid ratio (quadratic effect), as well as of interaction between lipid vs extract concentration and extract vs drying aid ratio (A×B and B×C), at $p \leq 0.05$. These results are expected since the water binding capacity of a dried powder is directly correlated to its composition and structure.

Indeed, the SDP structure and propensity for water absorption is intrinsically linked to composition variables investigated. For example, although lactose has been previously used as drying aid in lipid systems encapsulating polyphenols (SECOLIN et al., 2017), it has been reported that lactose monohydrate loses its water of hydration at 100 ^{o}C, the drying temperature used in this study (RAUT et al., 2011).

5.8.2 Concentration and retention of marker compounds in proliposomes

The content of major rosemary polyphenols in the SDP is highly linked to the composition variables investigated, since all factors in high or small degree affects the relative quantity of bioactive compounds added to the original liposomal composition. Following the logical reasoning, the amount of ROA, CAR and CNA is positively correlated with the amount of lyophilized extract added to original liposomal composition; and conversely with the lipid and/or drying aid concentration.

On the other hand, the retention of the bioactive marker compounds in the SDP correlates with composition variables in a more complex way. Figure 5.4 shows a comparison between the retention efficiency of ROA, CAR and CNA in the SDP. As can be seen in Figure 5.4, the percentage retention of ROA was higher than the ones observed for CAR and CNA, with average

values of: ROA = 95.7 ± 9.5 %; CAR = 87.2 ± 10.9 %; and CNA = 80.2 ± 6.6 %. Interestingly, the effects of composition variables on retention of CAR and CNA showed similar trends, while ROA shows an opposite behavior for most of the experimental runs. These behaviors can be linked with the intrinsic chemical properties of the specific marker compound (ROA, CAR, or CNA). For example, rosmarinic acid is a more hydrophilic compounds (log P ~1.1 – 1.8), while the diterpenes carnosic acid and carnosol are liposoluble (log P ~4.1 – 4.8).

Hence, these compounds should be partitioned in the aqueous and lipid phases of the encapsulating composition in different ways, affecting their retention efficiency in the SDP. Moreover, carnosic acid is relatively unstable, mainly in solvent, and the air might induce its degradation reaction; and carnosol is justly one of its degradation products (SCHWARZ; TERNES, 1992). Rosmarinic acid, on the other hand is relatively more stable than CAR and CNA (ZHANG et al., 2012).

The retention extremes observed in Figure 5.4 are in agreement with the lipophilicity of each compound i.e. the more lipophilic compounds such as CAR and CNA exhibited higher retention values at F11 (highest lipid ratio), while ROA (the less hydrophobic compound) showed better retention at F9 (lowest lipid ratio), and vice versa. The method for SDP preparation here presented could also be used for simultaneous encapsulation of multiconstituents materials (natural or synthetic), having varied polarity.

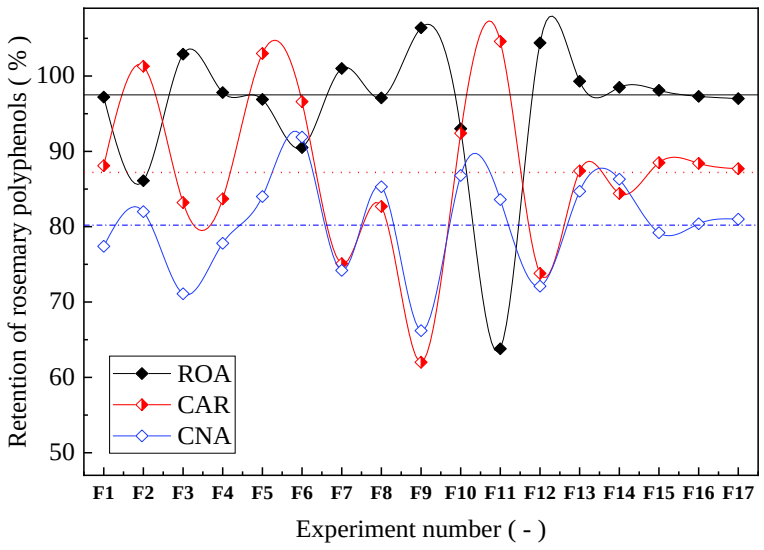


Figure 5.4: Poliphenol retention of ROA, CAR, and CNA for all experimental runs.

The regression analyses performed for concentration and retention of ROA, CAR and CNA (Table 5.9), adequately describe the behavior physically expected, and show an acceptable agreement with the experimental data ($0.951 \leq R^2 \leq 0.992$ and $0.720 \leq R^2 \leq 0.853$), respectively.

A convenient way to view the magnitude of the effects of each factor over the dependent variables is through the construction of Pareto chart of the standardized effects. Figures 5.5 and 5.6 show the resulting Pareto Charts of the effects of investigated factors for concentration and retention of ROA, CAR and CNA in the formed SDP, respectively (A: Lipid concentration; B: LE concentration; and C: Drying aid ratio). The increase in extract concentration was positive for the retention of ROA, but detrimental to the retention of CAR and CNA (effects highly significant, $p \leq 0.01$). These behaviors are also linked to compounds stability and lipophilicity, as discussed beforehand.

ZHANG et al. (2012) suggested a first order, concentration dependent degradation pattern for CAR, similar to what is observed for the compound in this system during processing and possibly giving rise to decreased retention as concentration increases. Its relatively higher lipophilicity also suggests favored partitioning into lipidic wall of the proliposomes rather than the aqueous core. Hence, higher ratio of extract to lipid presumably led to greater retention of hydrophilic compounds apparently protected in the aqueous vesicle core rather than lipophilic components which are no longer efficiently encapsulated, and therefore exposed to degradation at the vesicle periphery(NAKAYAMA et al., 2000).

The linear effect of lipid concentration further demonstrates this relationship, although showing a quasi-significance ($p \leq 0.10$) for both ROA and CAR retention with negative and positive effects, respectively. Increasing the lipid concentration in the liposomal composition bring benefits for retention of the nonpolar compounds CAR and CNA (positive signals in the higher regression coefficients, a_1), but is disadvantageous for the retention of ROA.

Regarding the carnosic acid (CNA) retention, evaluated lone factors ranged from being significant ($p \leq 0.05$) to highly significant ($p \leq 0.01$). Lipid and drying aid concentrations showed positive effects on CNA, similar to observed for CAR and in line with their lipophilicity. Notwithstanding, the drying aid ratio showed statistically significant effect only for the retention of CNA ($p \leq 0.05$); a positive effect. Since the degradation of CAR is concentration dependent as we previously suggested, the reaction is skewed away from buildup of its concentration, hence,

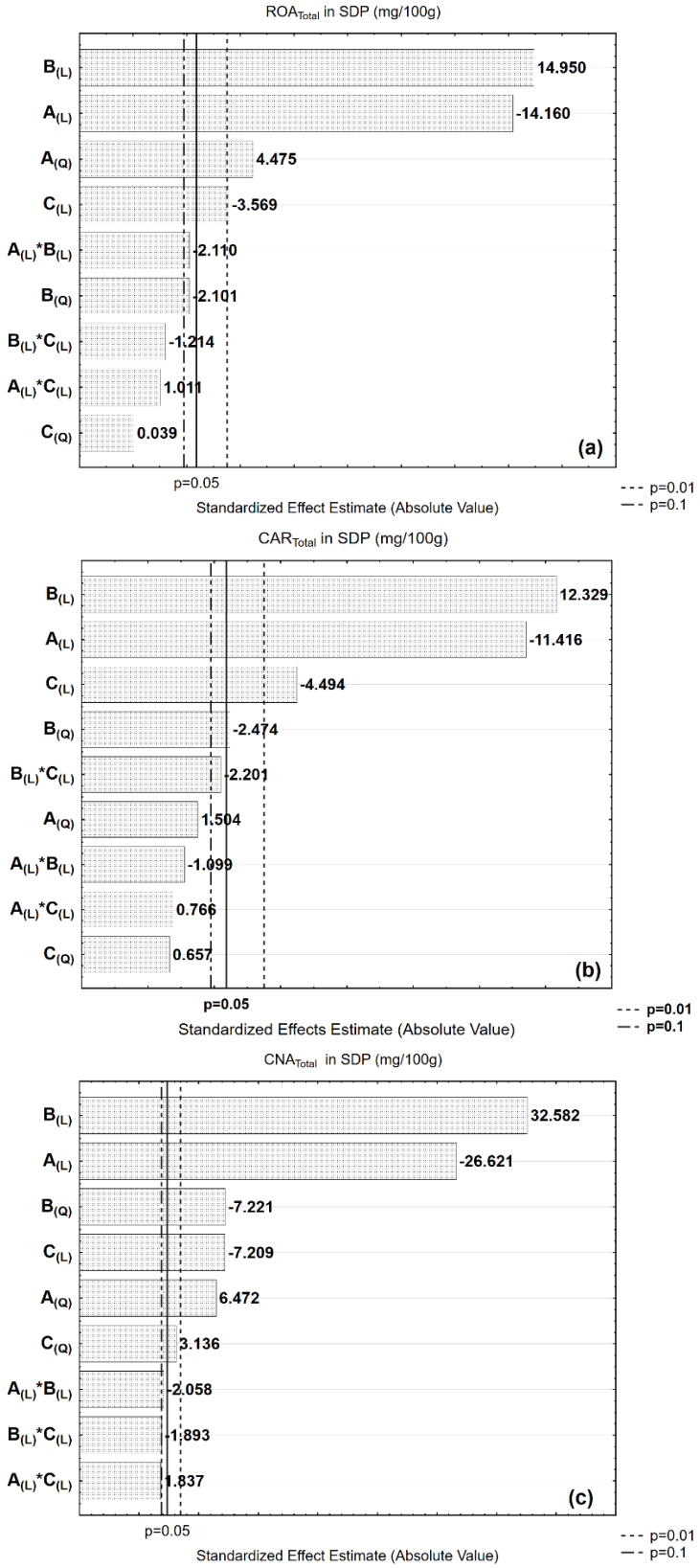


Figure 5.5: Standardized Pareto Charts of studied variables' effects, respectively for total content of **a:** ROA; **b:** CAR; and **c:** CNA in the SDP.

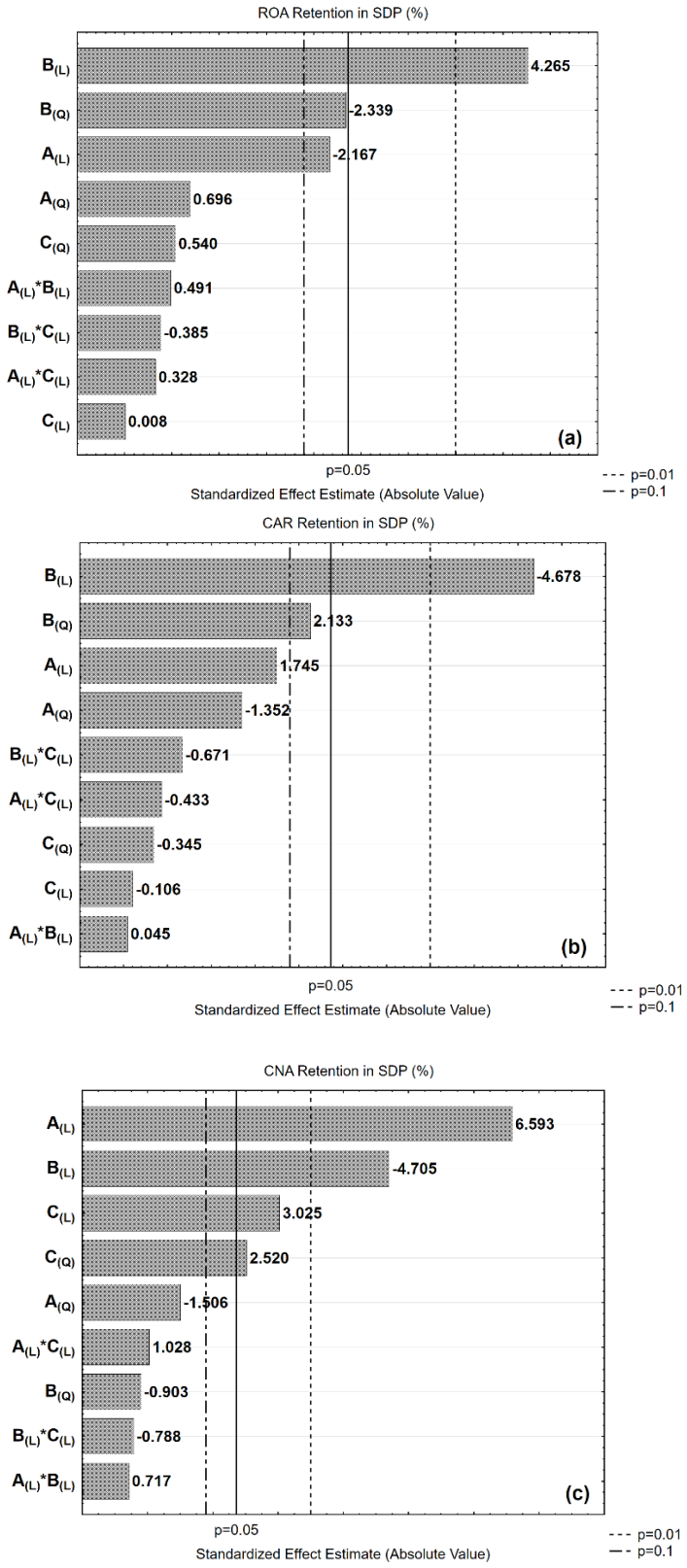


Figure 5.6: Standardized Pareto Charts of studied variables' effects, respectively for retention of **a:** ROA; **b:** CAR; and **c:** CNA in SDP.

further degradation of CNA (BANKOLE; SOUZA; OLIVEIRA, 2018). The significant positive effect attributed to drying aid concentration on CNA retention might be due to protective effect offered by lactose molecules, thereby slowing down or preventing degradation of the compound (LO; TSAI; KUO, 2004; SECOLIN et al., 2017). Evaluated factors showed no interaction effect on retention of bioactive compounds in the SDP. Integrity of the bioactive compounds was largely preserved exhibiting retention greater than 60.0 % of each compound for all SDP batches.

Figures 5.7A and 5.7B present response surfaces showing the effects of the most significant variables (extract and lipid concentrations) on the concentration and retention of ROA, CAR and CNA in the SDP, respectively. The plots were obtained for the drying aid ratio at midpoint (0.0), which are representative of those obtained at both lowest (-1.682) and highest (+1.682) drying aid levels. As can be seen in the graphs presented in Figure 5.7A, the effects of the composition variables on the concentration of ROA, CAR, and CNA in the SDP, exhibit high similarity, evidencing the predominance of "dilution" effects on these responses.

Similarly, relationship between studied variables and markers retention response revealed that retention patterns of polyphenols compounds at 0.0 (mean) level of lactose concentration (Figure 5.7B) are similar to those obtained at -1.682 (low), and +1.682 (high) levels. While ROA retention is facilitated at higher extract concentration, both CAR and CNA are favorably retained at high lipid concentration and low extract concentration levels.

Figures 5.7A and 5.7B are representative response surface plots for biomarker content and retention, respectively. Visual effects of significant variables – % extract and % lipid – are displayed for zero level of drying aid (lactose) only. The response surfaces plots are similar to those obtained at low and high drying aid levels.

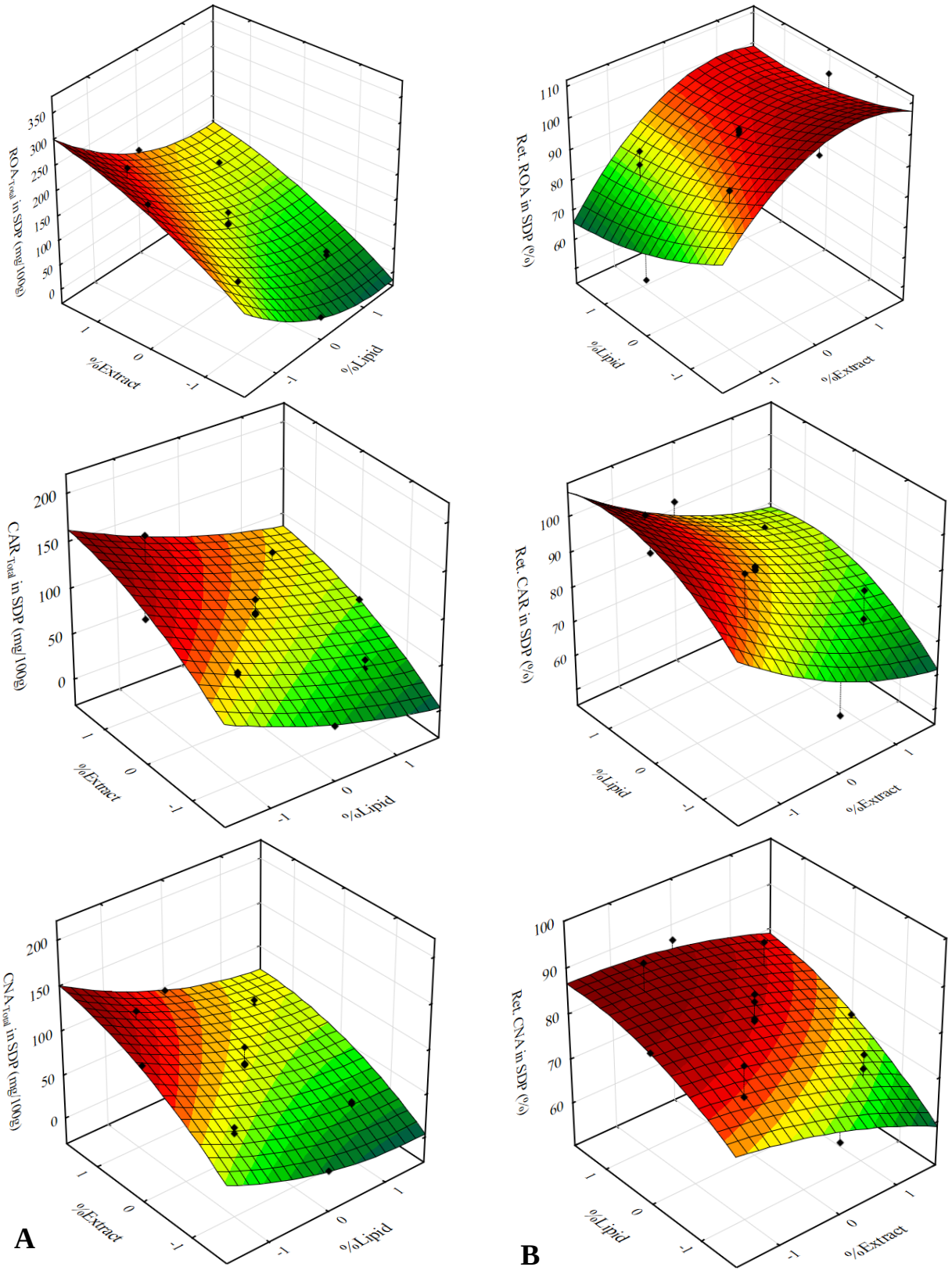


Figure 5.7: Response surface plots for the content and retention of ROA, CAR and CNA in SDP as a function of the significant variables, respectively Figures (**A**) and (**B**).

5.8.3 Powder recuperation from spray drying operation

The SDP recovery (R_{EC}) from the spray dryer ranged between 20.1 and 45.8 % (Table 5.8). This relatively low percentage of product recuperation might be attributed to losses by elutriation of the fine particles generated during the spray drying, a common occurrence in bench-top spray dryers using cyclone as unique powder collection system. Another cause is the stickiness of a parcel of the atomized product on dryer wall, which has been shown to be dependent on the glass transition temperature of the feed composition and drying temperature used (CORTÉS-ROJAS; OLIVEIRA, 2012).

This is really critical during spray drying of lipid compositions due to the low phase transition point of lipid constituents. For example, the phospholipon 90H has melting temperature between 55 and 67 ° C (TELANGE et al., 2016); with a glass transition temperature below this value. Hence, both the lipid and the extract tended to reduce the glass transition temperature of SDP, while the drying aid increases (data not shown). Carrier materials are essential for the spray drying of materials such as lipids with low glass transition temperatures (BARBOSA et al., 2015). Nature and concentration of drying carrier have been previously shown to have some significant effect on drying process and powder recuperation from spray drying (CORTES-ROJAS; SOUZA; OLIVEIRA, 2014).

In fact, the results of the regression analysis performed for R_{EC} (Table 5.9) evidenced a negative tendency of the concentration of the lipid and of rosemary lyophilized extract added to the liposomal formulation (although not significant statistically), while the effect of the drying aid was positive ($p \leq 0.1$). It is known that though the composition may exhibit some influence on the drying process, the yield of processed materials using the spray dryer are largely dependent on the conditions utilized in the drying process (KRISHNAIAH; NITHYANANDAM; SARBATLY, 2014; TONTUL; TOPUZ, 2017).

Product recovery might be a critical issue, especially in industrial applications where it may be employed as a measure of process efficiency and in analysis of cost implications (CHAUL et al., 2017). Since the proliposomes approach to encapsulate rosemary polyphenols and others phytopharmaceuticals holds a great potential for application in large industrial production scale, it is important to put product recovery and process efficiency in perspective during research and developmental stages.

5.9 Determination of the optimum condition for proliposome preparation by multiresponse optimization – The desirability approach

The multiresponse optimization (the desirability approach) was applied to the mathematical models fitted to the experimental results to determine the best formulation composition ratios that will generate SDP with acceptable values for water activity and moisture content, high retention and concentration of polyphenols marker compounds, and adequate powder recovery at the spray drying condition utilized. The optimization procedure was implemented in the software Statistica® 13.0 (StatSoft Inc.), by using predefined ranges of each response.

Table 5.10 presents the estimated optimum processing conditions. The ratio of drying aid (lactose to total liposomal solid) was predicted as 0.86, corresponding to 7.55 %w/w on wet basis. In order to confirm the validity of the optimization procedure, an additional experiment was carried out using the optimum formulation composition ratios determined. Table 5.11 shows the comparison between experimental and predicted SDP properties obtained at optimum processing conditions. It can be observed that relative errors between the experimental and predicted values showed concurrence for all responses except for the percentage retention of carnosol, which was 22 % lower than the predicted value. This might be either solely due to the mathematical model used in the optimization of CAR, which presented the lowest R^2 (0.720). This lack of good fit might have resulted from the fact that the amount of CAR is not only dependent on its initial and final concentration but also on degradation of CNA. Experimental error incurred during quantification of this compound might have also contributed to the difference observed.

Table 5.10: Optimized processing conditions for SDP production, coded and uncoded values.

Factor	Coded value	Uncoded value
Lipid concentration (% w/w,$^{w.b.}$)	-0.841	4.26
Extract concentration (% w/w,$^{w.b.}$)	+0.841	4.48
Drying aid concentration ratio (% w/w,$^{d.b.}$) i.e. lactose:(lipid+extract)	-1.682	0.86

w.b: wet basis; d.b: dry basis

Table 5.11: Predicted and experimental values of quality attributes of SDP at optimum points.

Quality attribute	Experimental value	Predicted value	Relative error (%)
Water activity (-)	0.387 ± 0.012	0.402	-3.9
Moisture content (% w/w)	2.03 ± 0.14	1.84	9.4
ROA retention (% w/w)	100.0 ± 2.5	97.3	2.7
CAR retention (% w/w)	72.0 ± 6.6	88.3	-22.7
CNA retention (% w/w)	83.1 ± 4.4	80.2	3.5
ROA content (mg/100 g)	615 + 23	583.9	5.1
CAR content (mg/100 g)	431.0 + 9.5	442.3	-2.6
CNA content (mg/100 g)	375 + 13	371.7	1.0

ROA: rosmarinic acid; CAR: carnosol; CNA: carnosic acid

5.10 Physico-pharmaceutical characterization of optimized proliposome and its constituents

The physicochemical characterization of proliposomes were carried out by determination of their morphological aspects by SEM, crystalline characteristics by x-ray diffraction (XRD), and aspects linked to their composition by Fourier Transform Infrared Spectroscopy (FT-IR).

5.10.1 Scanning Electron Microscopy (SEM)

The morphological aspects of microparticles are mainly related to the drying kinetics and formulation composition (TEWA-TAGNEA; BRIANC; FESSI, 2007; CUT-ROJES, 2011). Scanning electron microscopy (SEM) is an important tool for characterization of physical properties of powders and dry materials, being able to visualize structures and show details of surfaces and morphology. The importance of this analysis is that the functionality and flow of powders – among other properties such as solubility, hygroscopicity, storage – are directly related to the particle structure (AULTON, 2005).

Figure 5.8 shows the photomicrographs of the ordinary mixture of the constituent ingredients and the optimized spray-dried proliposome product, obtained by scanning electron microscopy (SEM) with magnifications of 200x, 1.00kx, and 3.00kx.

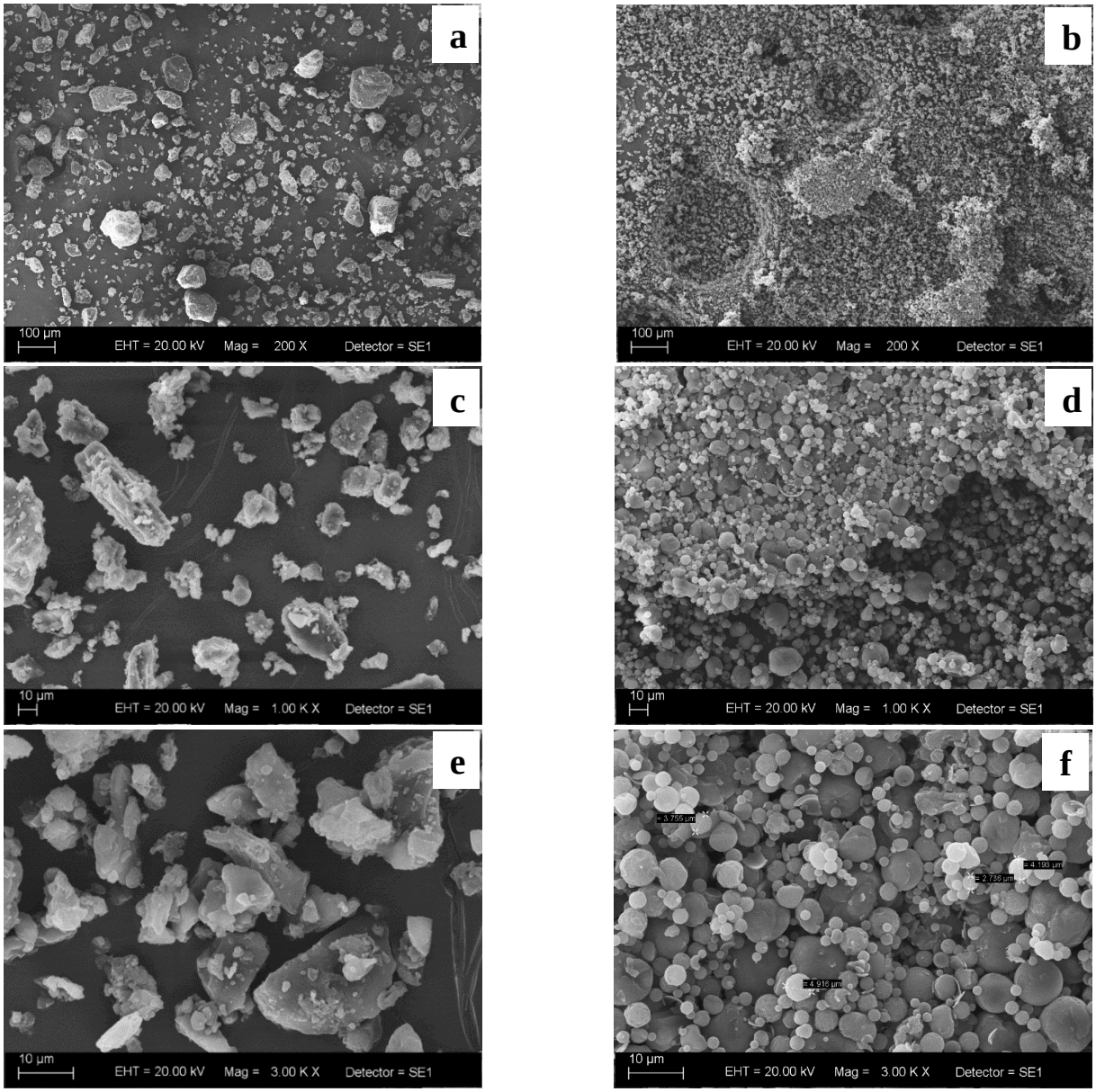


Figure 5.8: SEM photomicrographs of physical mixture of proliposome component (a, c, and e) and SDP (b, d, and f) at different magnifications.

The individual components of the physical mixture are shown as free individual particles randomly distributed within each other whereas proliposome particles are predominantly smooth spherical microcapsules, without visible porosity. The SDP product show mostly non-agglomerated free particles, suggesting a product with low moisture content. They exhibit the characteristic profiles of atomized particles: spherical particles of various sizes and agglomerates, the characteristics of which depend on adjuvant used. In the spray drying, atomized drops are exposed to a hot stream of air and are rapidly dehydrated. Thus, dried particles generally retain the shape of the misty drops with only a reduction in particle size due

to shrinkage. As the outer surface is more exposed to heat, there is usually the formation of a plastic crust porosity (OLIVEIRA; PETROVICK, 2010).

5.10.2 X-Ray Diffraction (XRD)

XRD is typically used for studying the crystallinity of pharmaceutical powder ingredients and products. Figure 5.9 presents information obtained by x-ray diffraction on the crystallinity of the spray dried proliposome, physical mixture of the constituent ingredients, and individual component – phospholipid, cholesterol, lactose, and lyophilized rosemary extract. The lyophilized extract (LE) showed few crystalline peaks, characteristic of powder materials obtained by freeze drying. On the other hand, lactose (LA) and cholesterol (CH) showed multiple high intensity peaks indicating crystallinity. The physical mixture of the ingredients, containing the same proportion as in the spray dried proliposome product, showed multiple peaks which are representative of the individual component of the mixture. These peaks observed for individual ingredients and their physical mixture were absent in the spray-dried proliposome. The diffractogram of the SDP presents rather large, nondefined peaks with abundant noise, indicating formation of amorphous structures. These findings suggest that in pure form, the lyophilized extract exhibits some degree of crystallinity (although very slight); however, upon its incorporation into proliposome formulation, the crystalline structure transformed into amorphous. The same is true for the other components making up the proliposome product. A similar phenomenon previously described by KHAN et al (2017) during encapsulation of beclometasone dipropionate in proliposomes.

It is probable that the drying process and properties of the drying aid in a feed composition would significantly impact product characteristics, including the degree of crystallization; the extent of crystallinity of spray dried powders being largely dependent on the glass transition temperature of the constituents as well as the drying conditions and properties of the feed composition. In a specific drying environment, the difference between the temperature of drying gas and glass transition temperature is proportional to the propensity for crystallization (CORTES-ROJAS; OLIVEIRA, 2012). As such, lower drying temperatures, relative to the Tg of the drying aid favours generation of amorphous powders. This might be the case with the proliposome product containing lactose with a Tg of around 101 ^{o}C, spray dried at 100 ^{o}C. It has also been suggested that amorphous products are more likely to be generated during drying of complex mixtures such as those containing herbal extracts, since constituents might hinder the occurrence of crystallization (LANGRISH; WANG, 2009; CORTES-ROJAS; OLIVEIRA, 2012).

Transformation from crystalline to amorphous forms observed is interesting from the point of view of redispersibility of the product, and eventual solubility and bioavailability of the encapsulated polyphenols. It is known that amorphous powders have higher solubility and greater biovailability compared to their crystalline counterparts (MORAES et al., 2013; SILVA et al., 2017a; TONY et al., 2015; CORTES-ROJAS; OLIVEIRA, 2012). As such, there is the advantage of enhanced solubility for polyphenols following proliposome encapsulation, bringing a solution to a challenge of bioavailability with which polyphenols have been identified.

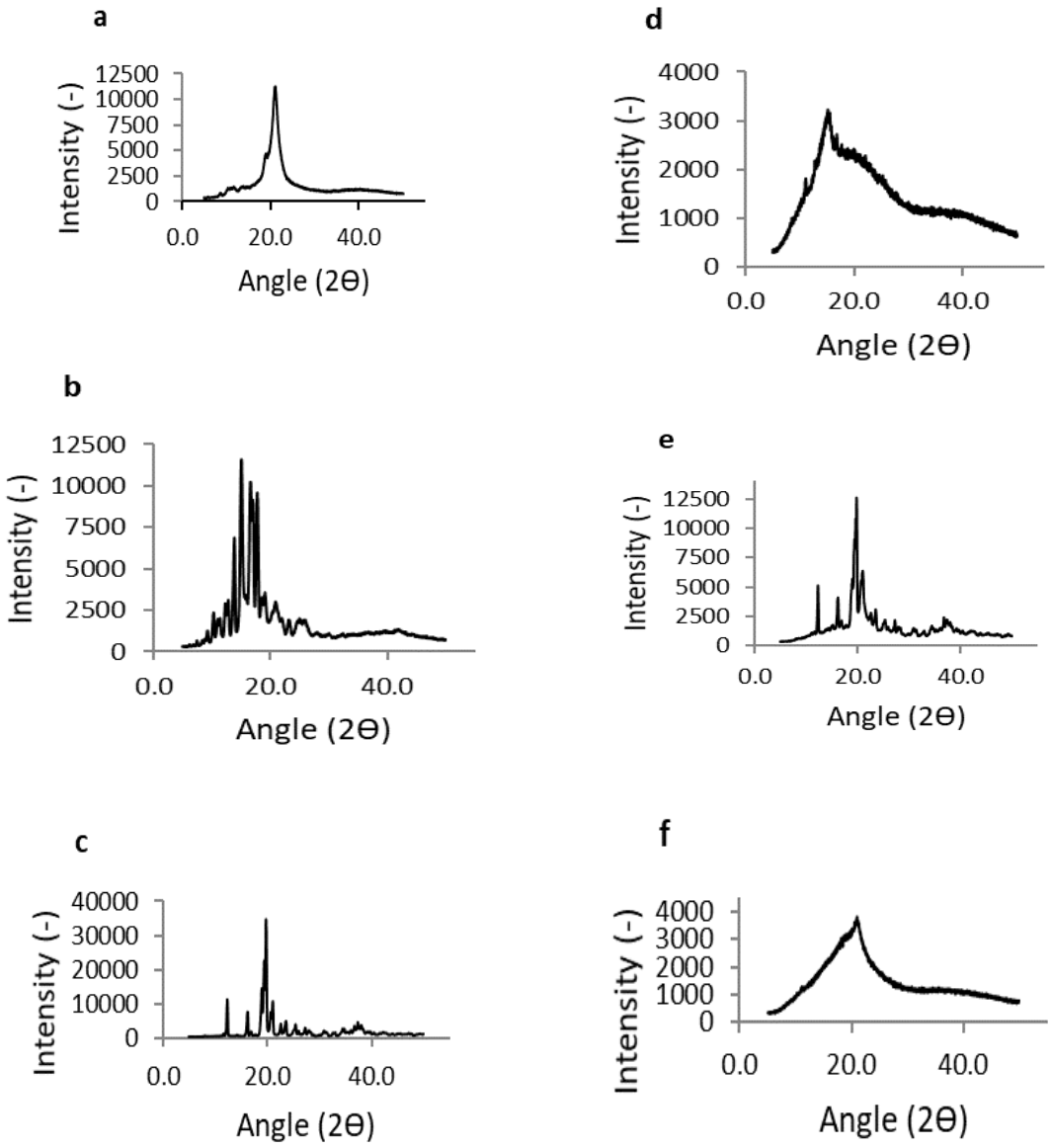


Figure 5.9: X-ray diffractograms for phospholipid (a); cholesterol (b); lactose (c); lyophilized extract (d); their physical mixture (e); and spray dried proliposome (f).

5.10.3 Fourier Transform Infra-red Spectroscopy (FT-IR)

FTIR studies were conducted to investigate the relationships between the components of the proliposomes. Figure 5.10 shows the spectra obtained by infrared spectroscopy analyses of the spray dried proliposome (SDP), individual components of the system (phospholipid – PL; lactose – LA; and lyophilized extract – LE), and their physical mixture. The physical mixture showed representative peaks of all constituent ingredients, suggesting absence of chemical interaction between the component. However, a comparison of the spectra of both the lyophilized extract and physical mixture to that of the SDP indicate that several peaks characteristic of the lyophilized extract obtained between 1187 and 1685 cm^{-1} wavenumbers were present in the physical mixure but missing in the SDP spectra. This observation might have resulted from encapsulation of the components of the lyophilized extract in the SDP, rather than the formation of new bond from chemical interaction. Proliposome formulations prepared by spray drying are known for high encapsulation efficiency in which the extract components are effectively retained within the lipid matrix and coated by the drying aid, thus limiting the appearance of peaks.

SILVA et al. (2017) identified the peak at wavenumber of about 1200 cm^{-1} to the asymmetrical stretching of a P=O group in Phospholipon 90H, visible in our spectra for pure phospholipid, physical mixture, and SDP at 1251 cm^{-1}. This peak is known to shift its position to higher wavenumbers when involved in hydrogen bonding (POPOVA; HINCHA, 2003), an occurrence which was not noticed in our physical mixture and SDP samples.

It can also be observed that the peaks associated to the phospholipid at 2847 and 2911 cm^{-1} are retained in both the physical mixture and the SDP with respect to their location. Since spectra intensity are concentration based, more intense peaks were obtained for pure phospholipid compared to the physical mixture and SDP. These peaks were ascribed to stretching vibration of CH_2 group by SILVA et al., (2017), and the higher its intensity, the more ordered are the acyl chains of the phospholipids. This observation further confirms that no chemical interaction between the lipid and other components. Absence of additional peaks in the FT-IR spectra is also considered an indication that there was no chemical interaction between the drug and formulation ingredients (JANGA et al., 2012).

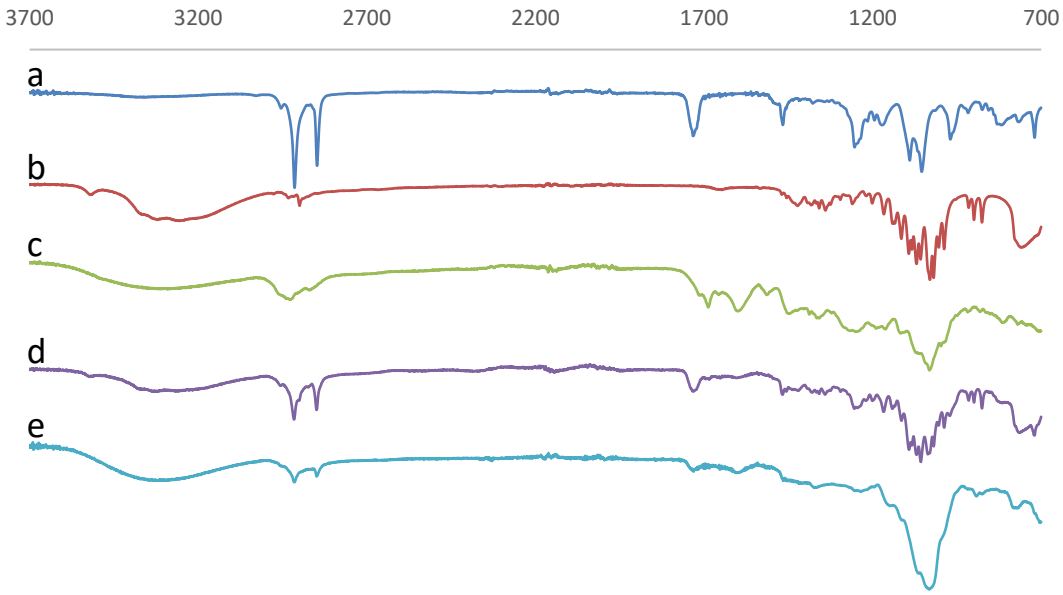


Figure 5.10: FT-IR spectra for phospholipid (a); lactose (b); lyophilized extract (c); their physical mixture (d); and spray dried proliposome (e); to evaluate the effect of processing on molecular interactions.

5.11 Biological assays

Assays were carried out to demonstrate the viability of prepared SDP as an alternative delivery system, bearing in mind the advantages associated with it. Antioxidant and antimicrobial activities of the SDP and LE were measured as representative tests to confirm retention of the plant polyphenols in SDP, and demonstrate its superior bioactivity to ordinary LE samples.

5.11.1 Antioxidant assay

The molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) is characteristically stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol at about 517 nm. When a solution of DPPH$^{\bullet}$ is mixed with that of a substrate that can donate a hydrogen atom, the DPPH molecule becomes reduced, thereby losing its violet coloration. In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored (ALAM; BRISTI; RAFIQUZZAMAN, 2013).

Results of DPPH$^{\bullet}$ reduction produced by pure LE, optimized SDP, synthetic antioxidants (BHT and BHA), and quercetin are shown in Table 5.12. BHT and BHA have been used as antioxidants in foods and personal care product/cosmetic ingredient, among other applications. However, deleterious effects to humans linked to them have stimulated the search of viable and safe alternatives (de RAADT et al., 2015; TAGHVAEI; JAFARI, 2015; WANG et al., 2008; XIE et al., 2017). Rosemary extracts are commercially available for use as a natural antioxidant for foods in Europe and USA, and has received GRAS status (Generally Recognized as Safe), being considered safe and effective (AGUILAR et al., 2008; NIETO et al., 2018; PETIWALA; JOHNSON, 2015).

Results here reported showed that our lyophilized rosemary extract (IC_{50} = 10.7 µg/mL) and proliposome (IC_{50} = 9.2 µg/mL – LE basis) has slight superior antioxidant activity than BHT (12.5 µg/mL), see Table 5.12. Antioxidant activity of rosemary polyphenols was observed to be similar to that obtained for quercetin (comparison on rosmarinic and carnosic acids, and carnosol concentration basis). As can be seen, the encapsulation of lyophilized extract in phospholipid-based proliposome enhanced the antioxidant activity of LE (lower IC_{50}). This is in accordance with previous studies showing improved antioxidant activity of natural compounds by complexation and encapsulation (CHOI; KANG; MOK, 2018; LIU et al., 2017).

Pinsuwan et al demonstrated enhanced antioxidant activity of liposomes encapsulating extract of *Hibiscus sabdarifa* using an *in vitro* skin model (PINSUWAN et al., 2010). Similar *in vitro* activity enhancement using liposome-based encapsulation of natural products has been reported by other researchers using different methods (CHOI et al., 2018; YUAN et al., 2017). FENG et al evaluated the *in vivo* effect of liposomal encapsulation of chlorogenic acid following oral administration in mice. They observed that administration of free chlorogenic acid and liposome-encapsulated significantly decreased the relative liver weight hitherto induced by tetrachloromethane. However, there was significantly higher increase in the activities of antioxidant liver enzymes GSH-Px and T-SOD for animals that received liposome encapsulated chlorogenic acid compared to free compound (FENG et al., 2016). These improvements observed has been associated with increase in solubility upon liposome formulation which in turn improves interaction with free radicals (LIU et al., 2017; PAN; ZHONG; BAEK, 2013).

Table 5.12: IC_{50} values and DPPH• inhibition capacity of the SDP, compared to LE, the synthetic antioxidants (BHT and BHA), and quercetin.

Sample	IC_{50} (µg/mL)[a]	Inhibition (%)
LE	10.8 ± 0.3*	89.0 ± 0.1
SDP	9.2 ± 0.2*	83.1 ± 0.9
BHT	12.5 ± 0.6*	88.2 ± 0.2
BHA	3.0 ± 0.2*	85.8 ± 0.8
QCT	1.0 ± 0.1*	86.3 ± 0.9

[a] Antioxidant activity by the DPPH• method, expressed as IC_{50}. *: $p < 0.05$. Statistical difference determined by One-Way ANOVA followed by Tukey post-hoc test. LE: lyophilized rosemary extract; SDP: spray dried proliposome; BHT: butylated hydroxyltoluene; BHA: butylated hydroxylanisole; QCT: Quercetin.

5.11.2 Antimicrobial assay

Several studies report potent antimicrobial activity of rosemary extracts obtained by different methods (BEGUM et al., 2013; NIETO et al., 2018; RIBEIRO-SANTOS et al., 2015; TAVASSOLI; DJOMEH, 2011) and suggested to be dependent on polyphenolic compounds (IONITA et al., 2015). Hence, it was decided to investigate the antimicrobial activity of SDP (obtained at optimum conditions) and the lyophilized extract (LE), using a yeast strain and five strains of selected bacteria (comprising gram positive and negative, spore-forming organisms) as model for antimicrobial property; aiming to emphasize possible SDP applications in pharmaceutical or food compositions.

Samples of LE and SDP dispersions to be used in the antibacterial activity testing were prepared in 50 % methanol solvent. SDP, on redispersion converts to liposomal vesicles encapsulating both hydrophilic and lipophilic compounds in their inner core and wall material, respectively. The effect of dispersion process on properties of the vesicles formed and its attendant effect on bioactivity was evaluated by preparing sonicated (HSDP-A) and unsonicated (HSDP-B) samples, containing similar SDP concentrations.

These samples were characterized for vesicle size, their size variation and hydrodynamic stability (Table 5.13). Sonicated dispersion showed about 65 % reduction in size of vesicles encapsulating the polyphenols without adversely affecting their content and hydrodynamic stability. This is desirable as it increases the surface area and facilitates contact between the drug carrier and the microbial cells.

Table 5.13: Physical properties of liposome vesicles obtained from hydrated spray-dried proliposome (HSDP).

PROPERTY	HSDP-A	HSDP-B
Particle size (nm)	537.27	1509.68
Polydispersibility index (-)	0.766	0.986
Zeta potential (mV)	-40.3	-27.5

HSDP-A: hydrated spray-dried proliposome, sonicated;
HSDP-B: hydrated spray-dried proliposome, unsonicated

5.11.2.1 *Antifungal assay*

Table 5.14 shows the experimental results of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against *C. albicans* (ATCC1023) for pure lyophilized extract, hydrated SDP (HSDP), rosmarinic acid and terbinafine (positive control) using the microdilution method. Rosmarinic acid, the most abundant polyphenol in the extract, was used as an internal control to detect if it contributes to the antifungal activity while methanol was included as solvent control.

All test samples showed antifungal activity within concentration ranges tested except rosmarinic acid and methanol. HSDP samples showed similar level of activity to pure LE, indicating that bioactive compounds remain intact and successfully retained during proliposome preparation. It was observed that HSDP sample sonication did not have any effect on its antifungal activity. This suggests that prepared proliposomes may be used as antifungal agent without any complicated process of redispersion. This is particularly desirable since prepared products are considered to enhance dispersibility in aqueous medium and improve stability of bioactive compounds compared to pure extract.

LE and HSDP samples gave MIC of 312.5 μg/mL, well below ≤ 1000 μg/mL, the value suggested for plant extracts (BALDIM et al., 2019; HOLETZ et al., 2002), hence, were considered effective against the test microorganism. Rosmarinic acid gave no activity at used concentration range which is several folds higher than its concentration in the extract. This suggests that antifungal activity observed is perhaps due to carnosol and carnosic acid rather than rosmarinic acid, an observation in concordance with previous studies on antimicrobial activity of rosemary extracts (BIRTIC et al., 2015; GENENA et al., 2008 CAMPO; AMIOT; NGUYEN-THE, 2000).

A closer look at obtained results for retention of bioactive compounds revealed that although both LE and HSDP showed similar MIC values against *C. albicans*, the concentration

of carnosol and carnosic acid in HSDP was lower (as shown by percentage retention) by 28.0 % and 16.9%, respectively. Since these compounds are here suggested to be responsible for the bioactivity under discussion, it is supposed that HSDP system, in reality, has a higher efficiency for the delivery of these compounds against *C. albicans.* Terbinafine is a broad-spectrum antifungal agent, applicable for both oral and topical routes of administration. Although the MIC obtained for terbinafine appears far lower than those of HSDP, it should be borne in mind that the values quoted for HSDP were based on whole extract. Dilute methanol, used as solvent for samples based on lyophilized extract gave no activity against *C. albicans*, confirming that antifungal effects observed are due to bioactive compounds present in the extract.

Table 5.14: *In vitro* sensitivity of *Candida albicans* to rosemary lyophilized extract, processed SDP, rosmarinic acid and terbinafine (positive controls), methanol (solvent control) and 0.9 w/v saline solution (negative controls), determined by broth microdilution method.

TEST SAMPLE	MIC[d] (μg/mL)	MFC[e] (μg/mL)
Pure LE[a]	312.5	1250
HSDP-A[b]	312.5	1250
HSDP-B[c]	312.5	1250
Rosmarinic acid	>1250	>1250
Terbinafine	≤0.4883	0.9766
Methanol	>12.5%	>12.5%
0.9 w/v saline solution	na*	na*

[a]Lyophilized extract of rosemary;
[b]Hydrated spray dried proliposome, sonicated;
[c]Hydrated spray dried proliposome, unsonicated;
[d]Minimum inhibitory concentration; [e]Minimum fungicidal concentration;
*na: "no activity" observed against tested microorganism.

5.11.2.2 *Antibacterial assay*

Antibacterial activity was measured using typed organisms in which the MIC and MBC were determined and results presented in Table 5.15. The MIC, a measure of bacterial sensitivity to antimicrobial agents, was determined for redispersed LE and SDP samples against strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (25923), *Bacillus subtilis* (ATCC 21332), *Pseudomonas aeruginosa* (ATCC 2327), and *Klebsiela pneumoniae* (ATCC 23357).

Gram-negative microorganisms such as *E. coli* show higher resistance to antimicrobial agents due to the complexity of the cell membrane structure which impairs interaction and penetration of lethal materials, hence the higher MIC value recorded compared to *S. aureus* which is a gram-positive bacterium. However, all three test samples showed equal MIC value of 0.3125 mg/mL against *B. subtilis* and *E. coli*. The former, a Gram-positive bacterium exhibits sporulation which confers resistance on bacteria and may account for similar susceptibility observed for both *B. subtilis* and *E. coli*. MIC values of 0.0781, 0.0781 and 0.1563 mg/mL were obtained for LE, HSDP-A, and HSDP-B, respectively, against *S. aureus*, a non-sporulating Gram-positive bacterium. The difference in activity between HSDP samples may be attributed to sonication process, resulting in homogenization, higher biomarker solubility and reduced particle size for HSDP-A, consequently leading to improved cellular interaction and eventual penetration into bacterial cell greater than HSDP-B.

P. aeruginosa and *K. pneumoniae* showed higher resistance to LE and HSDP samples with MIC values of 0.625 and 1.250 mg/mL, respectively. The gram-negative nature of these microorganisms may be responsible for higher resistance observed due to cellular complexity earlier mentioned. Besides this, their pathologic versatility, highly adaptive capacity, and intrinsic resistance contribute to difficulty in treatment with many antimicrobials thus making them of serious health concern. Whereas particle size reduction does not influence the MIC of SDP against *P. aeruginosa*, a MIC-lowering effect of size reduction was observed against *K. pneumoniae* (HSDP-A vs HSDP-B).

MIC values ≤ 1000 μg/mL was obtained using LE and HSDP samples against all test organisms except *K. pneumoniae*; hence were considered effective against the test microorganism (BALDIM et al, 2019; HOLETZ et al., 2002). The inferior antimicrobial activity of rosmarinic acid (pure compound) to the rosemary extract and prepared proliposome is further demonstrated in the antibacterial test. The results obtained might confirm that antimicrobial activity observed is likely due to carnosol and carnosic acid as earlier suggested.

The potential enhancement of antibacterial activity of polyphenols using the SDP is obvious when retention of compounds principally responsible for the antibacterial activity, carnosol and carnosic acid, is considered. Similar to observation made for activity against candida, the SDP exerts same effect (similar MIC) as those of pure LE on all bacteria strains used, though containing lower concentration of the compounds supposedly responsible for observed activity. Retention of 72.0 and 83.1 for carnosol and carnosic acid in SDP, respectively, translates to between 20 and 30 % increase in activity with respect to LE as baseline.

On the other hand, LE, HSDP-A, and HSDP-B gave a uniform MBC value of 0.3125 mg/mL against *E. coli* and 0.6250 mg/mL against *B. subtilis*. This could be associated with the spore-forming properties of *B. subtilis* on exposure to the antimicrobial agents. While LE and HSDP-A gave equal MBC value of 0.15625 mg/mL against *S. aureus*, HSDP-B showed a higher MBC of 0.3125 mg/mL against the same orgnaism. This difference in MBC values may also be attributed to the difference in bacteria nature as well as the difference in physicochemical properties of samples. MBC value for *P. aeruginosa* and *K. pneumoniae* could not be determined as they apparently fall outside the concentration range of test samples.

Table 5.15: *In vitro* sensitivity of strains of selected bacteria to rosemary lyophilized extract, spray dried proliposome, rosmarinic acid chlorhexidine (positive controls), methanol (solvent control), and saline solution (negative controls), determined by broth microdilution method.

SAMPLES	MIC (µg/mL)					MBC (µg/mL)				
	E. coli ATCC25922	*S. aureus* ATCC25923	*B. subtilis* ATCC21332	*P. aeruginosa* ATCC2327	*K. pneumoniae* ATCC23357	*E. coli* ATCC25922	*S. aureus* ATCC25923	*B. subtilis* ATCC21332	*P. aeruginosa* ATCC2327	*K. pneumoniae* ATCC23357
LE	312.50	78.13	312.50	625.0	1250.0	312.5	156.25	625.0	>2500.0	>2500.0
HSDP-A	312.50	78.13	312.50	625.0	1250.0	312.5	156.25	625.0	>2500.0	>2500.0
HSDP-B	312.50	156.25	312.50	625.0	1250.0	312.5	312.5	625.0	>2500.0	>2500.0
ROA	-	-	-	625.0	1250.0	-	-	-	>2.500	>2.500
CHX	4.69	2.34	4.69	9.38	≤1.17	18.75	9.38	18.75	37.5	4.6875
MeOH	>25%	25%	>25%	12.5%	>25%	-	-	-	-	-
0.9% NaCl	na*	na*	na*	na*	na*	na*	na*	na*	na*	na*

MIC: Minimum inhibitory concentration;
MBC: Minimum bactericidal concentration;
LE: Lyophilized extract of rosemary;
HSDP-A: Hydrated spray dried proliposome, sonicated;
HSDP-B: Hydrated spray dried proliposome, unsonicated;
ROA: Rosmarinic acid
CHX: Chlorhexidine
MeOH: Methanol
0.9 % NaCl: physiologic saline
*na: "no activity" observed against tested microorganism.

5.12 Stability studies

The stability study represents an important consideration during product development. During storage, instability manifesting as changes in physicochemical properties of products are usually encountered with time. Appropriate packaging and optimal storage conditions are often applied measures to limit physical reactions and microbial growth – often resulting from water absorption and temperature effects – thereby ensuring that product properties are maintained intact for as long as possible. Quality changes in herbal materials and their products can be evaluated as a function of time through stability tests involving stressing climatic comditions, especially temperature, relative humidity and oxygen (VEIT, 2002). Information obtained from such studies are also useful in determination of optimal storage condition, appropriate packaging material, and product shelf life.

Generally, the degradation of a bioactive compound in solid form or product is much slower than when the same is contained in an aqueous-based product in which components might be exposed to various degradation reactions including hydrolysis, Maillard reaction, oxidation, and so on. This enhancement of stability in the solid phase is especialy true for materials which exist in the crystalline state (SOUZA et al., 2013). However, it is often desirable to have an insight into environmental conditions that possibly affect stability of these solid products and the extent of these effects. This is to forestall product damage and economic loses during transportation and storage of products, particularly at tropical climates. It is, therefore, usual to choose intense storage conditions using higher temperatures and humidity in order to accelerate the stability forecast (GIL-ALEGRE et al., 2001). The data obtained during the accelerated stability study can then be used to assess the impact of adverse conditions that may occur during shipping.

The current study aims to provide a better understanding of how humidity and temperature influence the physical and chemical properties of lyophilized extract (LE) and spray dried proliposome (SDP), with special attention to degradation of the marker compounds (caffeic, rosmarinic and carnosic acids, and carnosol) present in the dry extracts of rosemary and the effect of encapsulation in proliposomes on their stability.

For these tests, sample materials were stored in temperature-controlled chambers set at 25±0.5 and 45±0.5 ºC. Relative humidity control was performed using desiccators containing saturated salt solutions of $MgCl_2.6H_2O$ and $NaNO_2$ to confer ambient relative humidity equal to 32.4 and 63.5 %, respectively. The relative humidity values obtained using the saline

solutions were confirmed with a portable hygrometer, results indicating variation of only about 1.0 % at both storage temperatures and humidities during the stability testing.

Samples were collected and analyzed at predetermined time, with results compared to initial samples at the start of the experiment (T_{0day}) for macroscopic differences, variation in levels of polyphenols markers, variation in physicochemical characteristics, and changes in antioxidant activity.

5.12.1 Macroscopic changes

5.12.1.1 *Weight changes in powder samples*

The residual moisture of dried products is fundamental to their stability and directly contributes to chemical degradation. Many solid bioactive substances can absorb moisture during storage because commonly used packaging materials are water permeable. For this reason, moisture content can be used as a criterion for judging the quality of products in which degradation is dependent on water present (BOTT, 2008). Hence, the moisture content of the LE and SDP samples was monitored by weighing sample containers at each sampling time during the 60-day storage period.

Results obtained for changes in the weight of LE and SDP samples at the different storage conditions is presented in Figure 5.11. It was observed that SDP samples had increase in weight significantly higher than LE samples at corresponding storage condition. This could be attributed to higher moisture absorption capacity (hygroscopicity) of the excipients used for proliposome preparation. It could also be noted that combined effects of high humidity and temperature resulted in doubling weight gain for SDP samples while LE samples stored at this condition are significantly different from others.

5.12.1.2 *Visual appearance*

Color is an important attribute for the acceptance of food and nutraceutical products by consumers. Although alterations in chemical composition often attracts the most attention during product development and analysis, changes in physical and organoleptic properties such as colour, odour and taste may indicate significant degradation, resulting in loss of confidence in the product and non-acceptance by its users.

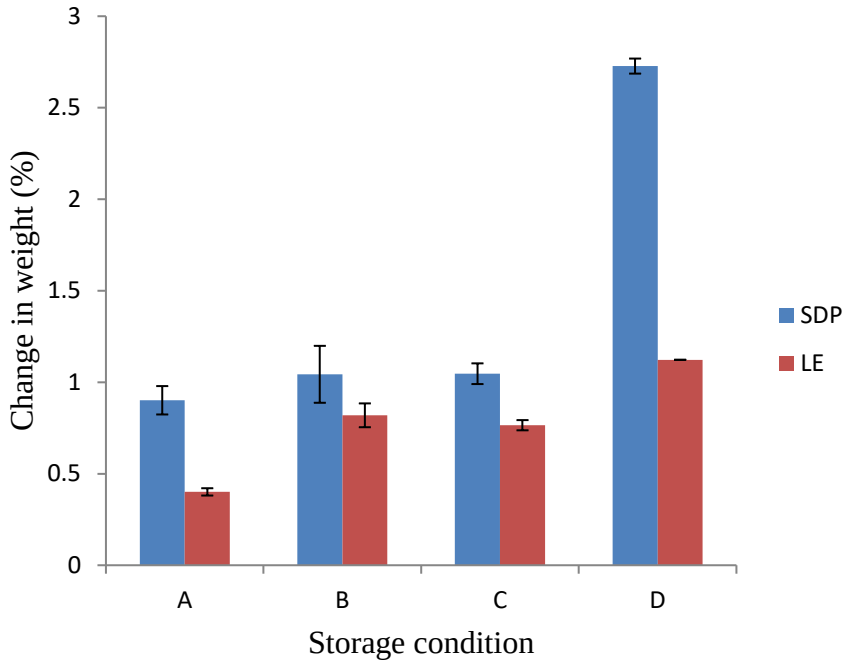


Figure 5.11: Changes in weight of SDP and LE samples following accelerated stability study (T_{60days}) at storage conditions – 25°C/32.4%RH (A); 25°C/63.5%RH (B); 45°C/32.4%RH (C); and 45°C/63.5%RH (D).

Figure 5.12 presents visual images (camera photos) of the rosemary extract and spray-dried proliposome during storage at different conditions. There was noticeable colour changes in both materials following storage. Although storage temperature and relative humidity both influenced changes in colour of SDP and LE samples, it can be observed that relative humidity influences colour change to a greater degree than storage temperature. Indeed, samples placed at higher relative humidity – and having higher moisture uptake as reported – showed higher degree of darkening (browning) as a function of the storage time.

The changes in colour observed might be an effect of enzymatic browning and degradation, resulting from enzyme-catalysed chemical reactions that are linked to moisture presence. The oxidation type reaction involves the action of polyphenol oxidase, catechol oxidase, and other enzymes present in the extract to generate melanins and benzoquinones from natural phenols. The quinones are then polymerized, resulting in brown pigment formation known as melanosis.

A quantitative colorimetric analysis was separately carried out for more adequate description and discussion using established parameter for samples stored at different conditions (Section 5.12.3.4).

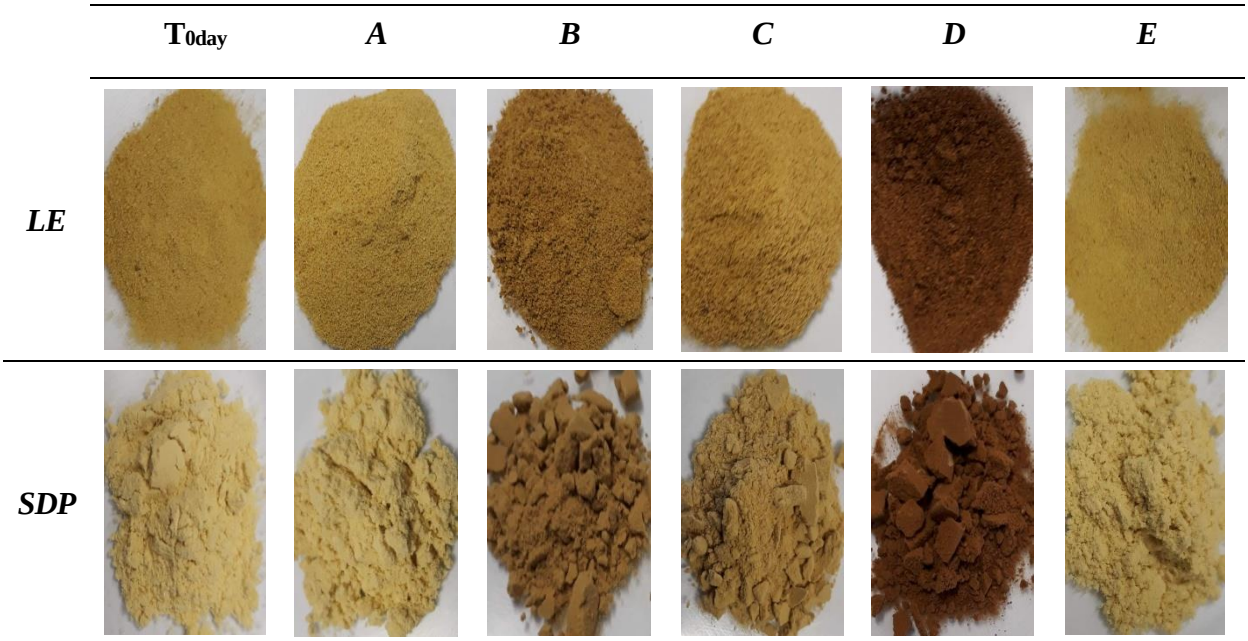


Figure 5.12: Images of: LE at T_{0day} and T_{60days} for storage conditions A to D and Refrigerator (E). SDP at T_{0day} and T_{60days} for storage conditions A to D and Refrigerator (E).

5.12.1.3 *Powder flow, agglomeration and caking*

The flowability of the dried extracts and SDP was carried out in duplicate plates at each storage condition, except in the refrigerator (since the package was sealed). Results observed are presented in APPENDIX VI.

SDP powders showed fluidity at the beginning of the experiment (T_{0day}). However, flowability was lost at varying sampling time, depending on the storage condition. Although flowability was maintained throughout the storage period at condition A, there was a gradual loss of powder flowability at the other conditions. Perhaps this behavior can be linked to the increase in product moisture during storage. Even at higher temperature but lower humidity, powder flowability was maintained for a longer period compared to storage at low temperature and high humidity (B vs C). Storage at high humidity conditions resulted in faster impedance to powder flow than lone effect of temperature, evidently by comparison of A vs B and C vs D. Significant reduction in powder flow was noticed after 5 days of storage at conditions B and D, and powder flow was totally lost after only 10 days of storage at both conditions. This observation might be due to moisture absorption by the powder, resulting in particle aggregation and stickiness.

The phenomenon was more significant for SDP samples probably due to the presence of adjuvants such as lactose used in the proliposome preparation. It could be that during drying, lactose monohydrate lost its associated water molecule but on exposure to high humidity,

lactose reverted to its hydrated form by water adsorption (LISTIOHADI et al, 2008). The same might be the case for lipids contained in the SDP which are known to have capacity for adsorption of water molecules.

On the other hand, lyophilized extract without adjuvants showed loss of flow at a later time compared to SDP at corresponding storage condition. A combined effect of temperature and humidity resulted in significant loss of flow at condition D after 30 days, ahead of condition B which became noticeable at T_{40days}. There was eventual loss of LE powder flow at T_{50days} for conditions B and D, an occurrence which was delayed until T_{60days} at condition C. These observations might have resulted from moisture adsorption, evidenced by possible water catalyzed reactions resulting in browning and weight changes as shown above.

Particle agglomeration follows similar patterns to those observed for powder flow, with effect of storage more pronounced on the SDP than LE samples (APPENDIX VII). SDP samples showed particle agglomeration after 5 days of exposure to relative humidity of 63.5 %, an observation which was delayed until T_{10days} at humidity of 32.4 %. After 10 days of storage it was possible to observe the formation of clusters and the tendency of caking of the SDP samples. LE samples, however, showed milder agglomeration with relative humidity being more significant factor than storage temperature. At the end of the experiment (T_{60days}), particle agglomeration was noticed in all LE samples except those stored at 25 ^{o}C/32.4 % (condition A). These observations are corroborated by Figure 5.12.

Caking can be considered as an instantaneous agglomeration phenomenon and is usually caused by wetting of the particle surface which causes plasticity and sometimes even the dissolution of substances present in the region. This phenomenon is often attributed to powders that are amorphous in nature (BOTT, 2008). This behavior is undesirable for powder products due not only to chemical degradation (loss of pharmacological activity and/or formation of toxic products) but also to material storage problems due to agglomeration and product packaging. Pattern of powder caking observed are presented in APPENDIX VIII. Caking was conspicuous after 30 days storage of SDP at all other conditions except at 25 ^{o}C/32.4 % (condition A). A combined effect of high temperature and relative humidity (45 ^{o}C/63.5 %RH, condition D) resulted in presence of caking after 20 days storage of LE. Higher degree of agglomeration and caking phenomena observed in SDP relative to LE on storage are largely associated with the additives utilized, perhaps principally due to lactose (HUPPERTZ; GAZI, 2015).

5.12.2 Degradation profile of polyphenols markers and kinetics

5.12.2.1 *Quantification of bioactive polyphenols markers*

Chromatograms obtained by HPLC for LE and freshly prepared SDP samples are shown in Figures 5.13a and 5.13b, respectively, while those obtained after storage (T_{60days}) at different conditions are presented in APPENDIX IX (a – l). HPLC assays showed that the original profile was maintained during the whole storage period, without the appearance of new peaks or disappearance of peaks presenting in the original chromatogram. However, reduction in peak areas corresponding to the concentration of the polyphenols markers was observed following storage. The extent of this concentration reduction is dependent on storage condition.

The degradation pattern of each polyphenol marker was obtained by quantifying the amount of the respective compound retained in the sample at intervals during storage using the HPLC method ealier described. Analysis was carried out on samples and the effects of storage conditions and sample type (LE or SDP) on degradation of polyphenol markers during storage are show in Figures 5.14 to 5.17. It is evident that storage condition significantly influences degradation of all the compounds, the extent of degradation of each marker dependent on its stability and sample type – being either pure lyophilized extract or prepared proliposome.

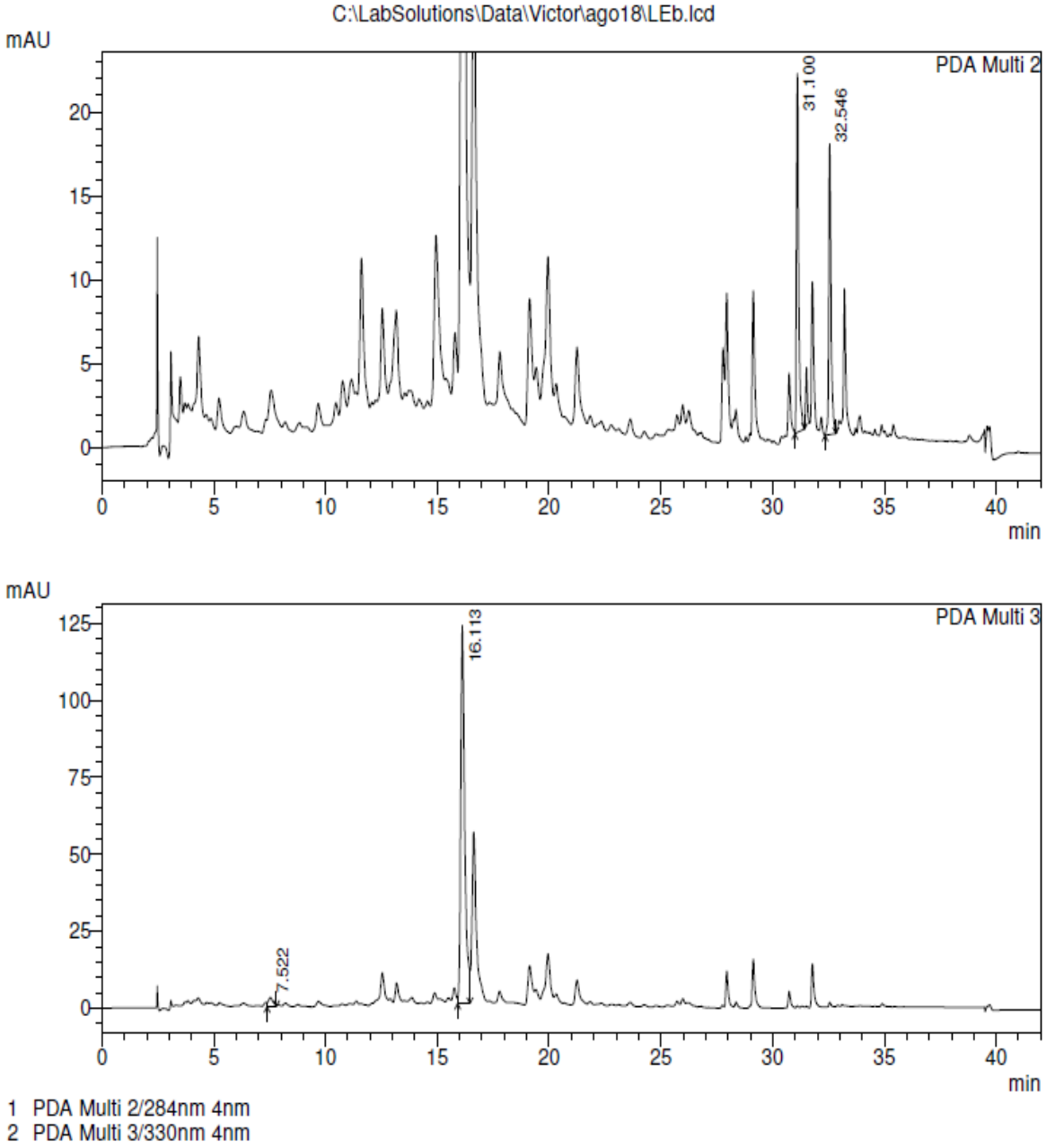


Figure 5.13a: HPLC chromatograms of freshly prepared lyophilized extract (LE).

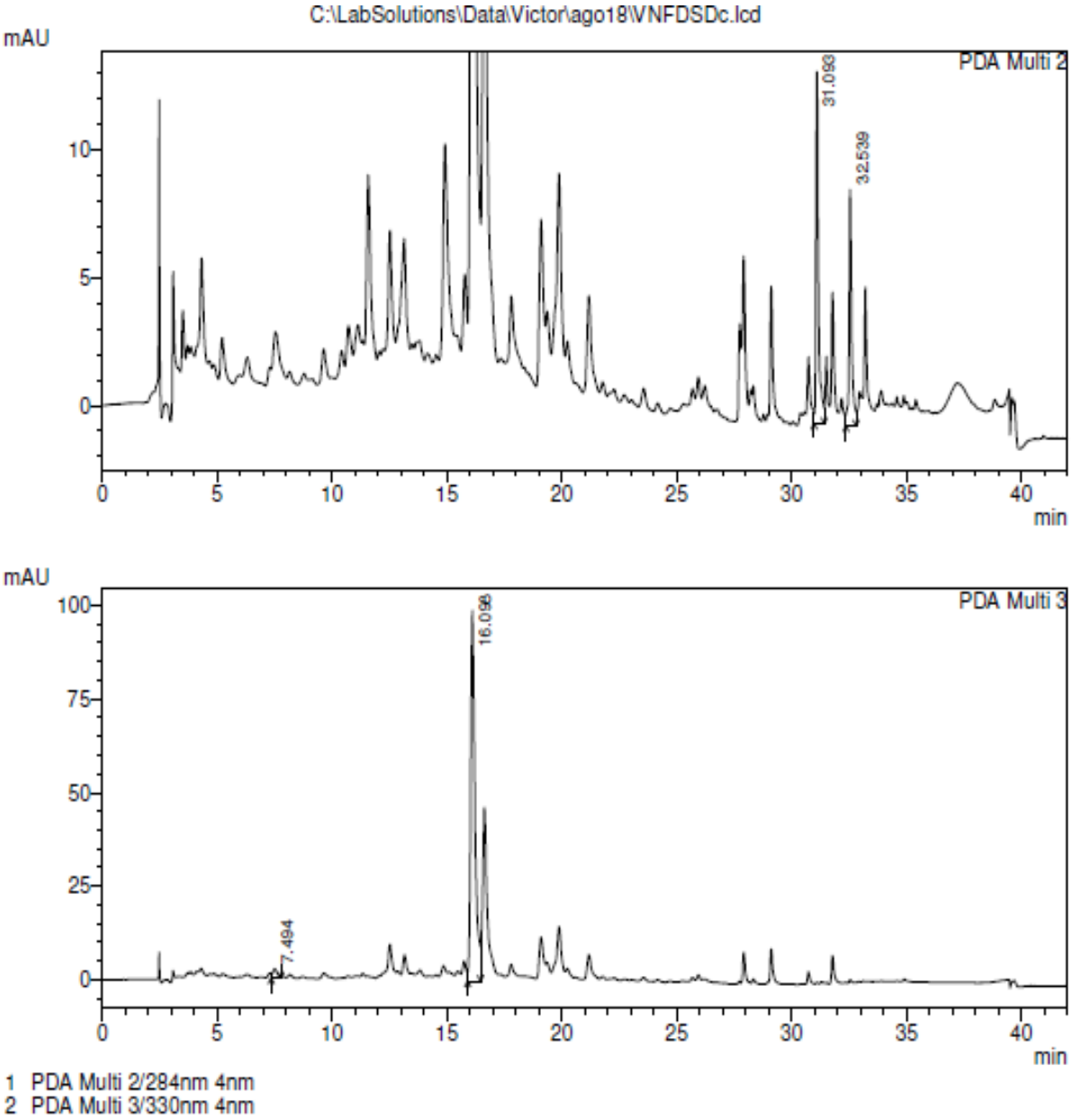


Figure 5.13b: HPLC chromatograms of freshly prepared spraydried proliposome (SDP).

The degradation profile obtained for caffeic acid at the different storage conditions is shown in Figure 5.14. Although present in relatively smaller concentration in the extract, caffeic acid showed greatest resistance to degradation at room temperature with average retention of between 80 and 90 % even at elevated humidity condition. According to SUN et al (2013) the thermal degradation temperature of caffeic acid is greater than 65 ºC hence, the minimal degradation of the compound at storage temperatures used in this study. The degradation observed at the storage temperatures might, therefore, be due to the single or combined effect of relative humidity and temperature. Storage condition with simultaneously high storage temperature and relative humidity resulted in reduced concentration of caffeic acid by almost one half during 60 days (Figure 5.14). In any case, there was no significant alteration in caffeic acid content of SDP samples stored in the refrigerator during 60 days but a marginal concentration decline was noticed in LE samples stored at the same condition. This difference in comportment is possibly due to the effectiveness of encapsulation of the compound in the proliposome system.

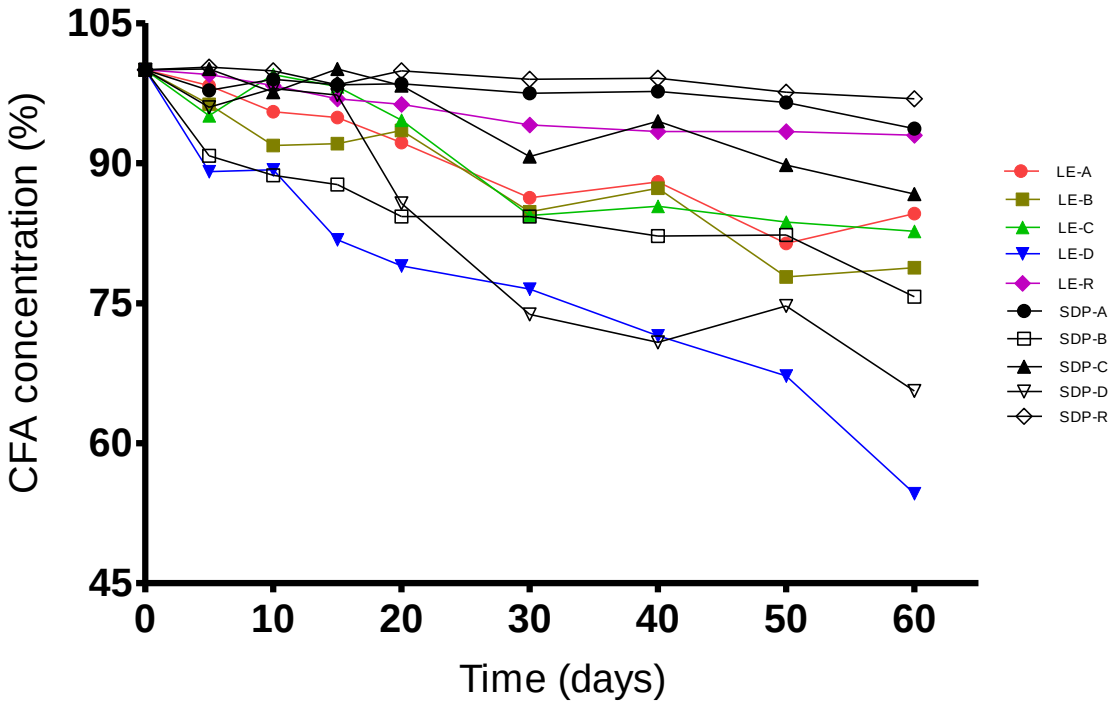


Figure 5.14: Degradation profile of caffeic acid (CFA) contained in lyophilized extract (LE) and spray-dried proliposome (SDP) during storage at different conditions – 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R) i.e. refrigerator.

The single effect of humidity appears to be greatest in relation to degradation of rosmarinic acid. Figure 5.15 shows that rosmarinic acid remained stable at low humidity conditions and in the regrigerator with a retention above 90 % during 60 days storage. However, its stability is noticeably compromised at higher relative humidity (63.5 %RH) conditions, falling below 75 % at 25 ^{o}C and about 40 % at 45 ^{o}C. During an evaluation of photosensitivity and effect of temperature differences on degradation of storage samples of rosemary polyphenols individually and in mixtures, Zhang and his coworkers reported relative stability for rosmarinic acid at storage temperatures ranging from -10 to 40 ^{o}C with retention above 90 % (ZHANG et al., 2012). Those results are similar to those obtained in this current study. However, the previous study did not evaluate the effect of relative humidity to stability of rosmarinic as carried out in this current study.

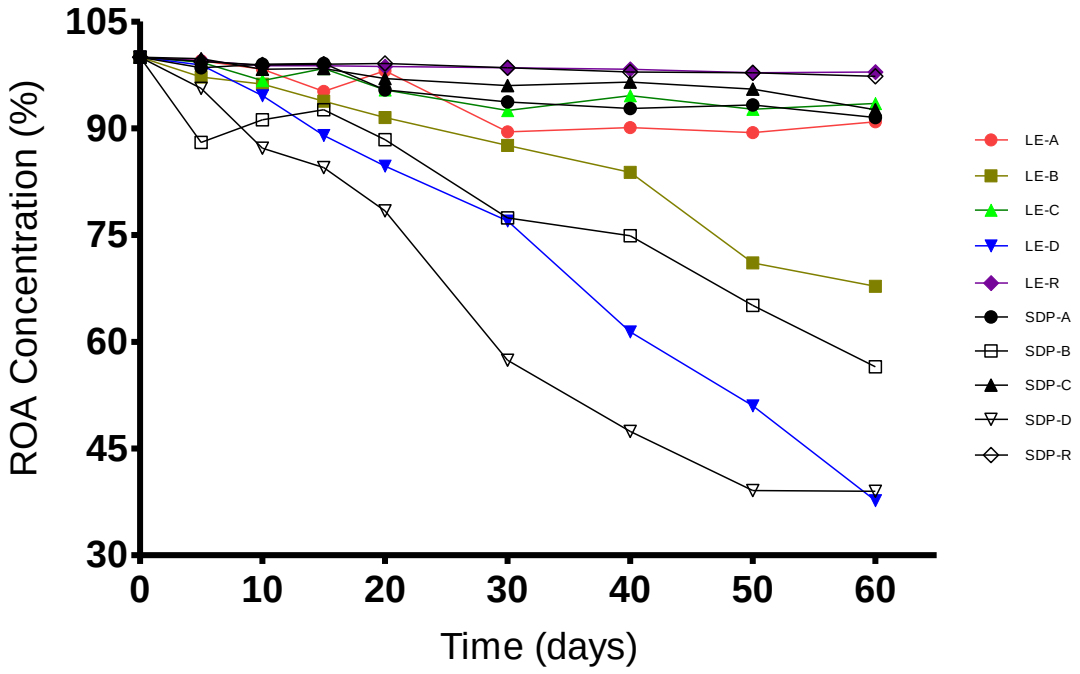


Figure 5.15: Degradation profile of rosmarinic acid (ROA) contained in lyophilized extract (LE) and spray-dried proliposome (SDP) during storage at different conditions – 25^{o}C/32.4%RH (A); 25^{o}C/63.5%RH (B); 45^{o}C/32.4%RH (C); 45^{o}C/63.5%RH (D); and 8 ^{o}C/55 %RH (R) i.e. refrigerator.

Figures 5.16 and 5.17 show the degradation profile of carnosol and carnosic acid, respectively, for both LE and SDP samples during storage. Generally, high relative humidity condition was observed to have more pronounced effect on the degradation of all polyphenol markers than temperature variation. Comparison of polyphenol marker retention between SDP and LE samples stored at similar condition showed significant difference for caffeic acid,

carnosol, and carnosic acid but not rosmarinic acid. While SDP showed superior retention of caffeic and carnosic acids, the reverse was the case with carnosol.

Encapsulation of carnosic acid, the most susceptible of the polyphenols, in spray dried proliposome effectively prevented degradation evidenced by higher retention compared to corresponding LE sample at similar storage conditions. A similar phenomenon was observed for caffeic acid retention at all storage conditions. Carnosol is a known decomposition product of carnosic acid (BIRTIC et al., 2015; ZHANG et al., 2012). Thus, higher concentration of carnosol observe in LE on storage could be a result of higher degradation of carnosic acid in the LE, leading to build-up of carnosol concentration in LE samples compared to SDP.

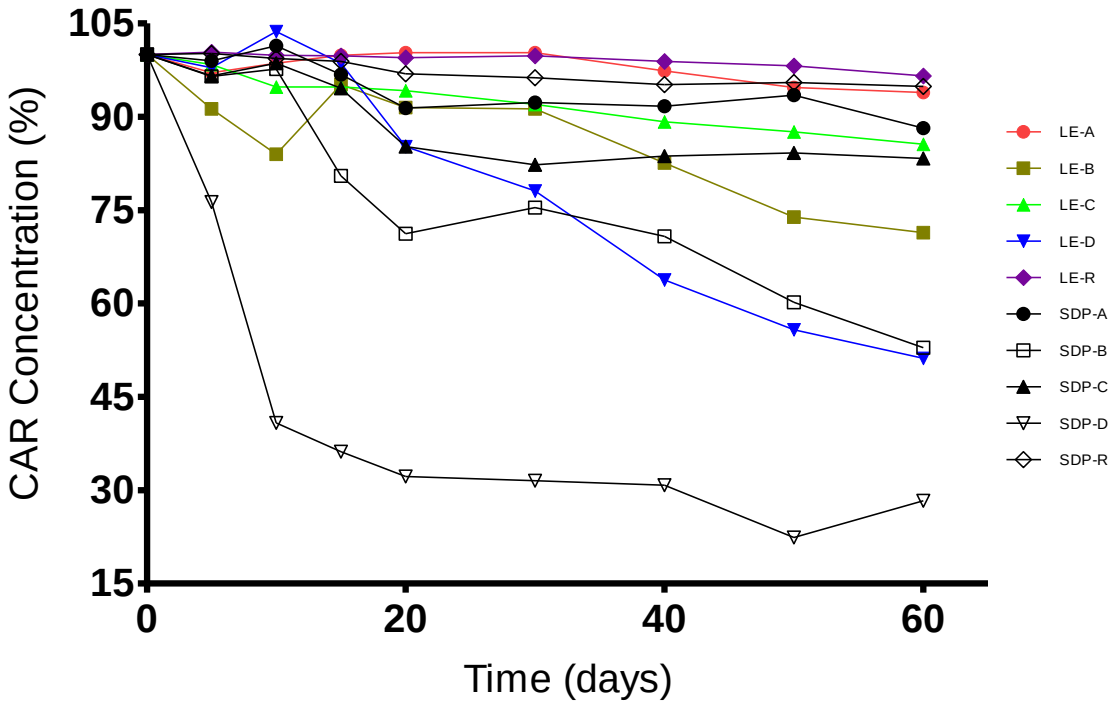


Figure 5.16: Degradation profile of carnosol (CAR) contained in lyophilized extract (LE) and spray-dried proliposome (SDP) during storage at different conditions – 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R) i.e. refrigerator.

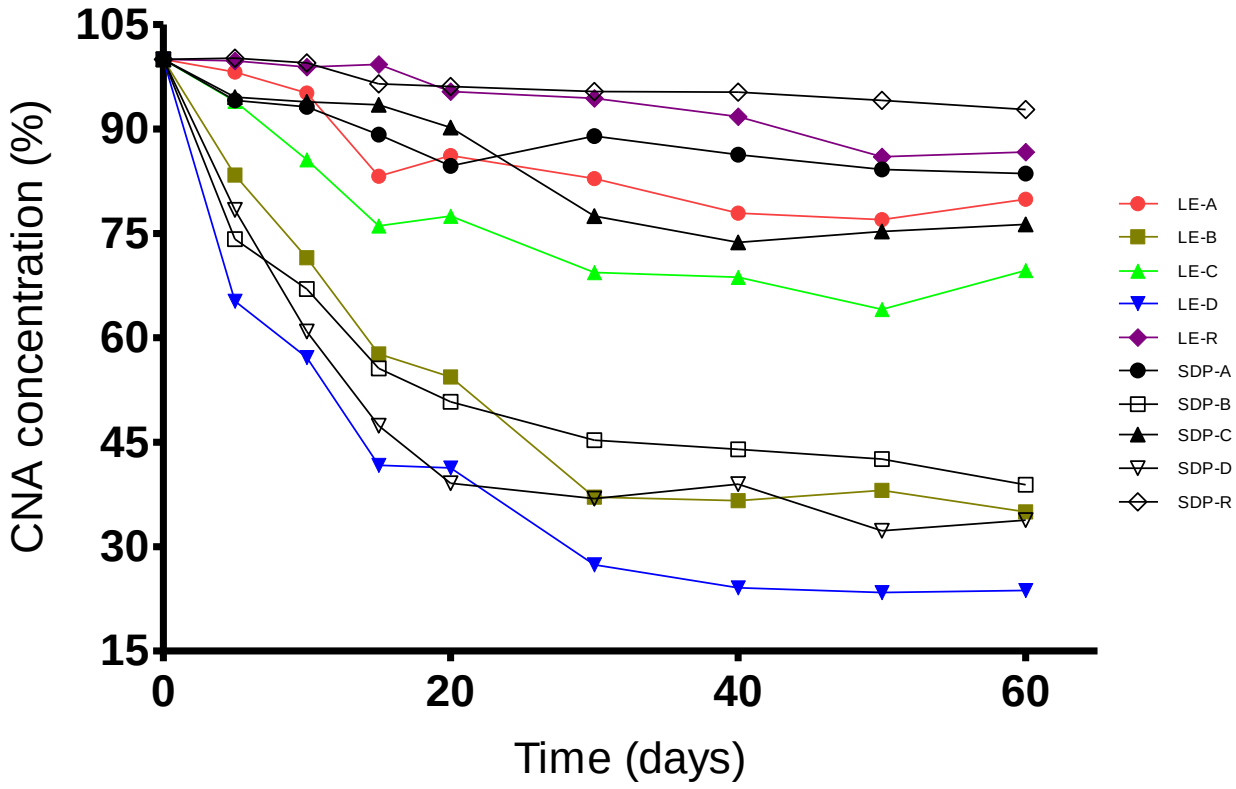


Figure 5.17: Degradation profile of carnosic acid (CNA) contained in lyophilized extract (LE) and spray-dried proliposome (SDP) during storage at different conditions – 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R) i.e. refrigerator.

The extent of degradation for each compound at each storage conditions after 60 days storage is shown on Figure 5.18 (a – d). It can be observed that caffeic acid was the least affected by storage, with 54.6 and 65.6 % retained at storage condition D (45 ºC/63.4 % RH, being the high extremes of temperature and humidity). On the other hand, carnosic acid, though being of relative abundance at the beginning of the experiment, degraded to 23.7 and 35.0 % at similar storage condition, making it the least stable of all marker compounds. This is typical of diterpenic compounds, known for their instability and sensitivity to many different factors, being rapidly decomposed or transformed into other compounds (RAZBORSEK; IVANOVIC, 2016). The instability of carnosic acid, especially in solvents, has been previously highlighted (BIRTIC et al., 2015). It is confirmed that carnosic acid is more stable in non-polar organic solvents such as DMSO and less so in polar ones such as MeOH and water (THORSEN; HILDEBRANDT, 2003).

Whereas the relative stability observed for caffeic acid at high relative humidity (63.5 %) may be due to *in situ* stability, carnosol concentration obtained might have resulted as a

degradation product of carnosic acid (LOUSSOUARN et al., 2017; ZHANG et al., 2012) rather than its intrinsic stability at elevated moisture levels.

It is also observable that lower RH values tend to favour the retention of rosmarinic acid. In fact, above 90 % rosmarinic acid was retained at other conditions except those with high relative humidity. Zhang et al reported that rosmarinic acid in ethanolic solution, either by itself or in a mixture of other polyphenolic compounds, did not degrade appreciably, even when exposed to light at temperature extremes (ZHANG et al, 2012).

Overall, results obtained revealed that rosemary extract and its proliposome product are better preserved in airtight containers or packaging materials where they are not exposed to the adverse effect of oxygen and moisture. Although the effect of temperature on polyphenol degradation may not be collateral, especially at low humidity conditions, it is advisable to limit product exposure to high temperature during shipping and avoid storage at elevated temperatures.

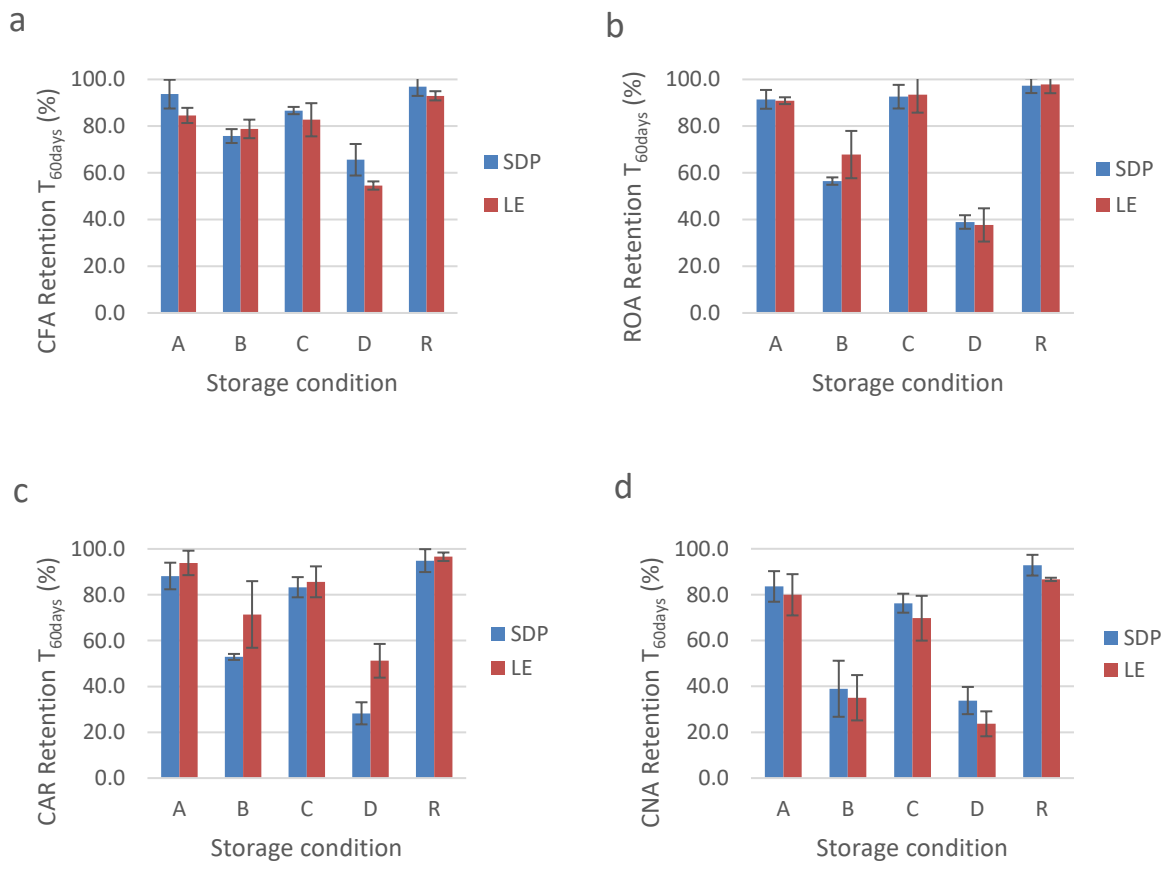


Figure 5.18: Percentage retention of caffeic acid (a); rosmarinic acid (b); carnosol(c); and carnosic acid (d); in SDP and LE samples following 60-day storage at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R).

5.12.2.2 *Degradation kinetics*

Chemical compounds are known to undergo degradation over time, the rate and extent dependent on the storage conditions and factors that influence the decomposition of each compound. Temperature and relative humidity are relevant storage condition variables that influence degradation and their effects are often shown on degradation profile graphs. Such profiles are useful in predicting degradation rates through kinetics study and analysis of best-fit model. This approach permits the construction of knowledge regarding optimal storage conditions and product shelf life. To achieve this objective for a multi-component system such as plant extracts and their products, experimental results of degradation profile of each bioactive marker are considered.

In this work, the HPLC method previously described was used to determine concentration of polyphenols marker compounds at different storage conditions during the storage period (represented in Figures 5.14 – 5.17). Results of biological assays carried out (antifungal and antibacterial, Sections 5.11.2.1 and 5.11.2.2) suggest that carnosol and/or carnosic acid are, perhaps principally responsible for the evaluated biological activity. Therefore, degradation kinetics study was carried out for both compounds in the spray dried proliposome samples.

Zero-order and first-order kinetic models were fitted to experimental results of both carnosol and carnosic acid degradation. Tables 5.16 and 5.17 presents the kinetic parameters (±95% confidence level) and regression coefficients (R^2) for carnosol and carnosic acid, respectively. Table 5.16 reveals that both zero- and first-order kinetic models fitted adequately the experimental results of carnosol degradation, with slight superiority of the first-order model (having higher R^2). The shape of the graphs of carnosol degradation as a function of time also indicates the comportment of a first-order model; a somewhat concave orientation. Similar results were obtained for carnosic acid with higher R^2 values obtained for first-order kinetics at all storage conditions except condition C (45 ºC 32.4 % RH) which presented very similar R^2 values for both models.

It was observed that the first-order degradation constant (k_1) – and indeed the zero-order degradation constant (k_0) – was higher for samples at high relative humidity for both compounds, indicating collateral effect of the factor on stability of both compounds. The influence of RH (or water activity, a_w) on degradation of herbal medicinal products has been observed in various studies (CORTES-ROJAS; SOUZA; OLIVEIRA, 2016; SOUZA et al., 2013). BOTT et al reported that the degradation of vitexin, the chemical marker in the extract

of *Passiflora alata*, is dependent on the relative humidity (or the % RH of storage, which relates to the water activity of the product), while several changes in the sennosides of senna leaves were noted at low temperatures and high relative humidities by another group of workers (BOTT; LABUZA; OLIVEIRA, 2010; GOPPEL; FRANZ, 2004). Goppel and Franz further observed that the flavonoid compounds used as chemical markers in senna leaves showed a distinct degradation pattern when high temperature and RH are combined (40 ^{o}C and 75 % RH), leading to a remarkable reduction of the flavonoids. The reduction in the concentration of the glycoside flavonoids was dependent on the packing used (GOPPEL; FRANZ, 2004).

SOUZA *et al* (2013) also noted that the catechin of M*aytenus ilicifolia* product stored at higher RH shows losses in the catechin content greater than the samples stored at lower RH. Although they reported that increase in storage temperature also led to an increase in the catechin degradation rate, this behaviour is more pronounced at high RH. They concluded that catechin degradation was higher at high RH, and less dependent on temperature.

It is however, important to note that stability is also adversely affected at elevated temperature. This is evident considering the degradation rate (indicated by the calculated degradation constant) observed for carnosic acid which doubles for sample at high temperature, even at low relative humidity (condition C). The degradation rate of carnosol also increased by about 70 % for the same storage condition, compared to low values of both temperature and relative humidity (condition A).

Table 5.16: Kinetic parameters (±95% confidence level) and regression coefficients (R^2) of the zero-order and first-order models fitted to the experimental data of carnosol degradation in spray dried proliposome.

			Model parameters			
Polyphenol marker			Zero-order model		First-order model	
	T (^{o}C)	RH (%)	k_0±CL (% day^{-1})	R^2	k_1±CL (% day^{-1})	R^2
Carnosol	25	32.4	-1.0853	0.7125	-0.0019±0.0010	0.7161
	25	63.5	-4.5009	0.8916	-0.0102±0.0025	0.9131
	45	32.4	-1.7715	0.7154	-0.0033±0.0017	0.7160
	45	63.5	-5.4509	0.5474	-0.0189±0.0109	0.6569
	8	≈ 50	-0.5741	0.8915	-0.0010±0.0003	0.8936

T: temperature; RH: relative humidity; CL: confidence level.

Table 5.17: Kinetic parameters (±95% confidence level) and regression coefficients (R^2) of the zero-order and first-order models fitted to the experimental data of carnosic acid degradation in spray dried proliposome.

			Model parameters			
Polyphenol marker			Zero-order model		First-order model	
	T (°C)	RH (%)	k_0±CL (% day^{-1})	R^2	k_1±CL (% day^{-1})	R^2
Carnosic acid	25	32.4	-1.4026	0.71	-0.0025±0.0012	0.7187
	25	63.5	-5.0545	0.71	-0.0134±0.0051	0.8119
	45	32.4	-2.862	0.85	-0.0053±0.0018	0.8460
	45	63.5	-5.7405	0.65	-0.0165±0.0077	0.7419
	8	≈ 50	-0.7606	0.88	-0.0012±0.0004	0.8874

T: temperature; RH: relative humidity; CL: confidence level.

Using the first-order kinetic equations fitted to the experimental data of carnosol and carnosic acid degradation, the shelf life of the spray dried proliposome could be suggested. Table 5.18 shows the time to 20 % loss of carnosol and carnosic acid, considered an acceptable estimate of shelf life. Although regulatory standards such as in the United States prescribe lower percentage drug loss of 10 % for drugs, higher limits may be applicable to dietary supplements and botanicals which usually contain more than one bioactive constituent, often exerting their biological activity through synergism. The time to reach 80 % retention for carnosic acid may be considered the shelf life of the proliposome product at the respective storage condition, being generally lower than duration obtained for carnosol; about 180 days at 8 °C (refrigerator) or 91 days at 25 °C and 32.4 %RH.

Table 5.18: Time (days) to 80% retention of carnosol and carnosic acid in spray dried proliposome at different storage conditions according to First-order degradation kinetics.

T (°C)	RH (%)	Time to 80 % retention (days)	
		CAR	CNA
25	32.4	114.8	90.9
	63.5	21.9	16.7
45	32.4	67.2	42.1
	63.5	11.8	13.5
8	≈ 50	223.0	179.3

T: temperature; RH: relative humidity; CAR: carnosol ; CNA: carnosic acid

5.12.3 Physicochemical characteristics of storage samples

Storage samples were analysed by physical methods to determine the effect of storage conditions on surface morphology, crystal properties, colour changes, and possible chemical interactions within the sample matrix.

5.12.3.1 *Surface morphology by Scanning Electron Microscopy (SEM)*

Photomicrographs of storage samples of lyophilized extract after 60 days at different conditions are presented in Figure 5.19, with a magnification of 500× (for A) and 1000× (for B–D). Particle analysis shows irregularly shaped, highly porous powder with different particle sizes. Following storage, tiny needle-like projections are observed on sample surfaces which are absent in freshly prepared extract. The appearance and extent of these needle are influenced by storage condition; whereas very scanty are seen on samples stored at low temperature and RH (condition A), it is much more conspicuous on all other samples, especially those stored at high RH (Conditions B and D). These needle-like structures, perhaps represent manifestation of crystallization of some compounds. This possibility may be buttressed by the appearance of very small crystallization peaks observed for sample D during XRD analysis (Figure 5.21).

Photomicrographs of storage samples of spray-dried proliposome after 60 days at different conditions are presented in Figure 5.20, with a magnification of 10,000× (for A and C) and 5000× (for B and D). The particles presented the characteristic profile of atomized products: spherical shaped, highly porous powder with different particle sizes. Surface analysis of particles show rough, porous surfaces, characteristics which are considered dependent on the adjuvants used in spray dried powder materials and the drying kinetics (CORTES-ROJAS, 2011; SECOLIN, 2014). The effect of drying adjuvant, including lactose, was summarized by Secolin, citing several studies (SECOLIN, 2014). Adjuvants with low encapsulation properties give rise to microcapsules with very porous, rough and brittle surfaces, which is in accordance with the results here presented.

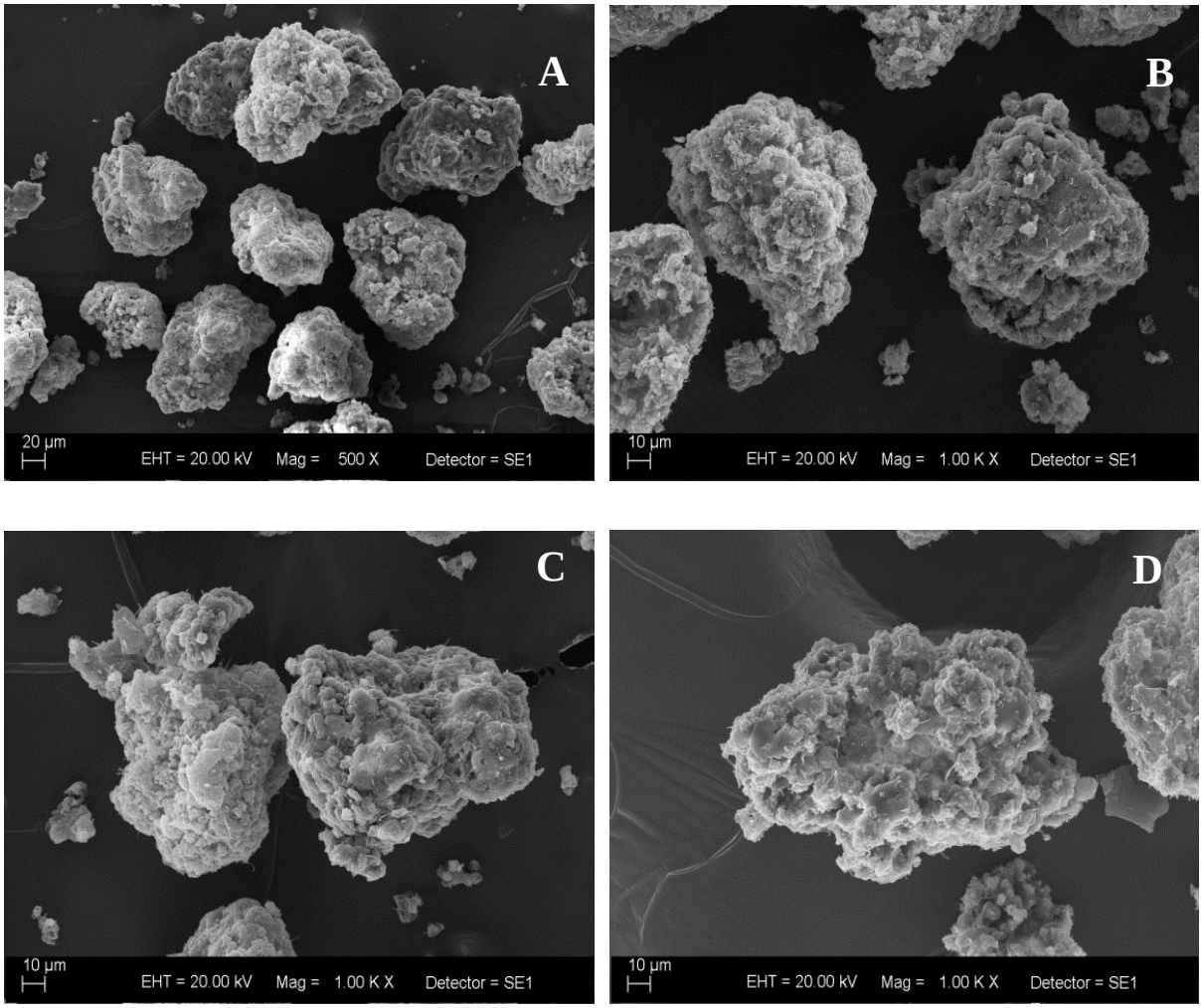


Figure 5.19 – Scanning electron micrographs of lyophilized extract samples after 60-day storage (T_{60days}) at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH; and (D).

Storage at high humidity conditions altered surface properties from rough and porous characteristics observed at conditions A and C to smooth and shiny, more pronounced at storage condition D > B. Spherical and amorphous proliposome particles prepared (T_0) retained their rugosity during storage at lower temperatures but became less wrinkled and laminar upon stressing at elevated temperature (condition C), elevated relative humidity (condition B), or a combination of both.

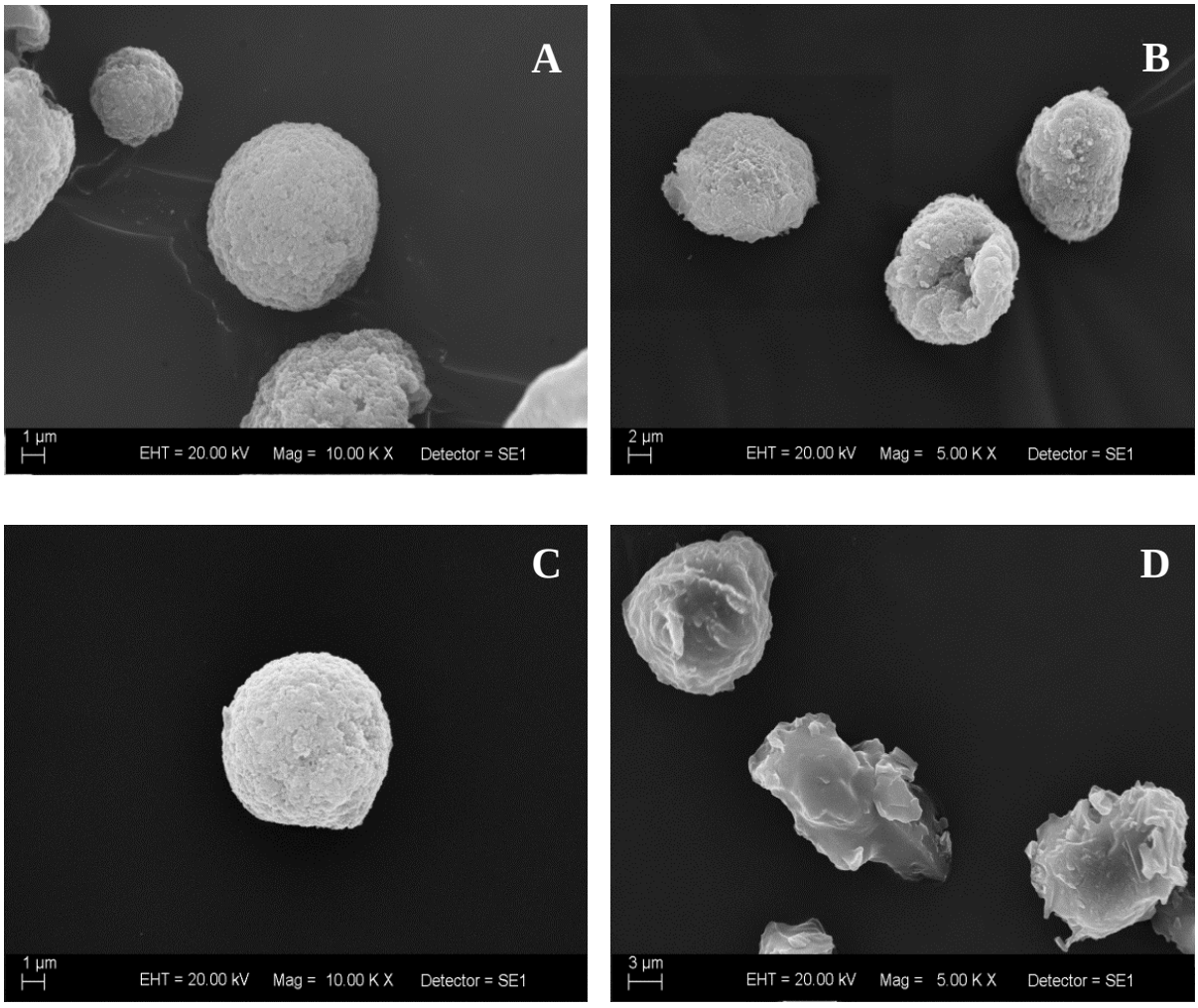


Figure 5.20 – Scanning electron micrographs of SDP samples after 60 day-storage (T_{60days}) at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); and 45ºC/63.5%RH (D).

5.12.3.2 *Crystal properties by x-ray diffraction*

X-ray diffraction analysis is of great importance in determining the physical state (crystalline or amorphous form) of a powder, which is fundamental to the stability of the product. Drying conditions influence the degree of crystallinity which is in turn related to various physical properties of powders such as porosity, adhesion, solubility and fluidity (TONELI et al., 2010). As shown in a previous section, XRD results are presented as diffractograms, where the peaks, detected at different angles, have different relative intensities (Section 5.12.2). Wide intensity angles mean that molecules are in the amorphous solid state, randomly oriented and thus diffract x-rays in all directions resulting in a typical amorphous pattern of "halo" which has been reported by our group and other authors (BOTT; LABUZA; OLIVEIRA, 2010). On the other hand, crystalline solids have sharp and defined peaks as the molecules are in a state of high ordering (CANO-CHAUCA et al., 2005).

Since the crystalline state is characterized by ordered three-dimensional arrangement of molecules, it is therefore a state of high stability. Amorphous solids, on the other hand, are characterized by the disordered arrangement of molecules with a metastable configuration thus making them generally more soluble and less stable than their corresponding crystals (SUN et al., 2012; YU, 2001).

The objective of these tests was to verify if physical transformations such as change in the degree of crystallinity occurred during the storage period of the lyophilized extract (LE) and spray-dried proliposome (SDP). X-ray diffraction spectra of LE and SDP samples at different storage conditions were obtained by the previously described method (Section 4.4.8.2) and the results are shown in Figures 5.21 and 5.22.

The diffraction profiles observed for LE samples (Figure 5.21) show that the freshly prepared (T_{0day}) lyophilized extract of rosemary is amorphous in nature. It is often thought that obtaining powders through the lyopilization process often results in amorphous rather than crystalline materials. For example, ZAMPIÉR (2012) presented diffractograms obtained from fresh lyophilized extract of D*albergia ecastaphyllum* showing characteristics of totally amorphous powder. However, crystalline powders have been obtained from plant extracts processed in similar manner as is the case with *Psidium guajava* lyophilized extract, showing peaks corresponding to crystallinity around 15 º. This type of variation suggests that crystalline or amorphous state of powders is not only dependent on dehydration method but also on the composition of the dry extract itself and adjuvants used.

The amorphous nature of LE was largely maintained during the storage period. Pure LE gave diffraction profiles showing that products stored at RH of 32.4 % for 60 days maintained their amorphous form. On the contrary, small peaks characteristic of crystallization appeared at condition RH of 63.5 % (conditions B and D) (Figures 5.21b and 5.21d). It might be said that a combination of temperature and RH seems to give more pronounced crystallization at T_{60days} since no obvious crystallization peak was observed at storage condition C (25 ºC/63.5 %). The appearance of crystallization peaks on exposure to higher temperatures and relative humidity suggest that some of the components can crystallize upon stressing over time. Samples stored in the refrigerator shows spectra very similar to that obtained at the beginning of the experiments (T_{0day}).

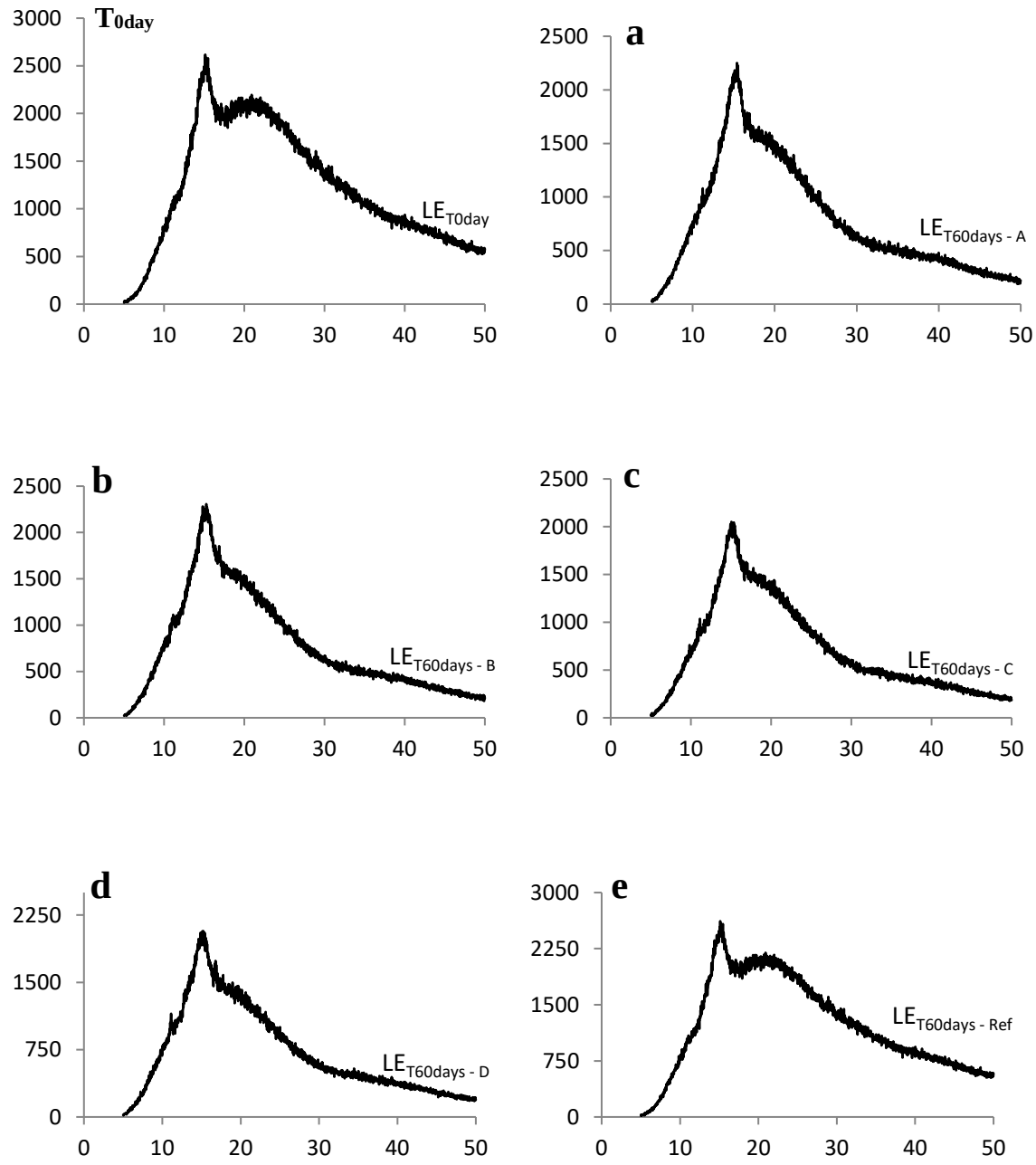


Figure 5.21 - X-ray diffraction spectra for lyophilized extract (LE) of rosemary pre- and post storage at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R).

SDP samples showed amorphous characteristics at the beginning of the experiments; indicated by the abundant noise pattern without any distinct peak observed. Similar pattern was observed for samples stored in the refrigerator as well as storage condition with low temperature and humidity (25ºC/32.4 %). Contrary to observations for ordinary LE, however, storage of SDP at elevated temperature and/or humidity levels resulted in very intense characteristic crystallization peaks with peak intensity dependent on storage condition (Figure 5.22).

Lone effect of high relative humidity value (63.5 %) resulted in higher degree of crystallinity compared to the lone effect of temperature. Samples stored at 63.5 % relative humidity showed similar crystalline pattern, with peak intensity higher for 45 ºC than those obtained at 25 ºC. Samples stored at elevated temperature also showed crystallinity, even at reduced humidity levels (45 ºC/32.4 %), but with fewer peaks and lower intensity than those obtained at elevated humidity level. These observations may be due to crystallization of certain constituents of the product on storage, especially sugars (e.g. lactose used as drying aid) and lipid materials (such as lecithin and cholesterol used as wall material) which are known to experience changes in crystal properties when stored under stress conditions, including elevated temperatures and relative humidity.

Transformation of amorphous molecules into crystals highly influences the pharmacological activity of powders containing bioactive materials. Crystalline and amorphous forms have different dissolution profiles with the latter favouring solubility, a step which often necessarily precedes exhibition of biological activity. Crystallization also interferes with the stability and flowability of the powder (BOTT, 2008).

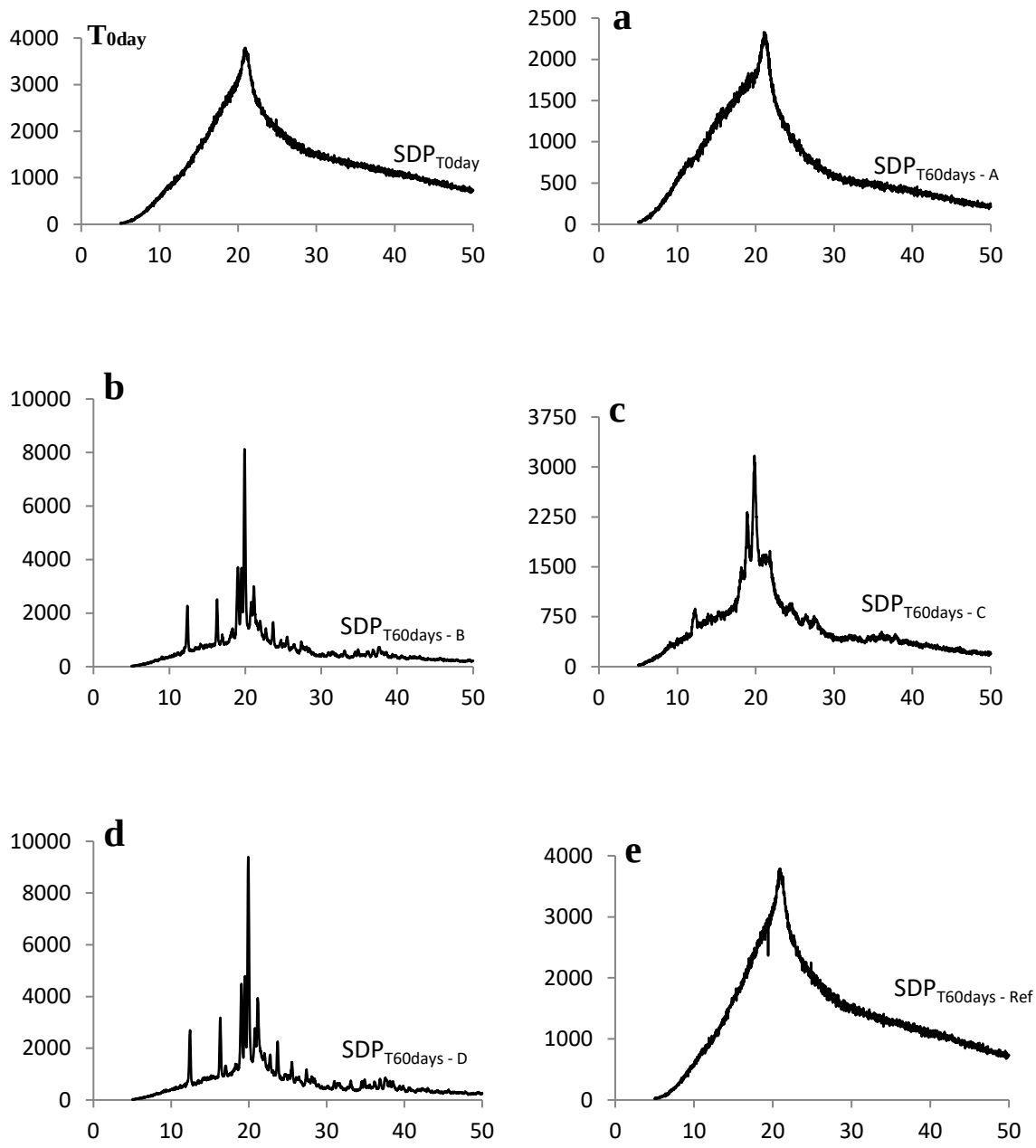


Figure 5.22 - X-ray diffraction spectra for lyophilized spray dried proliposome (SDP) pre- and post storage at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R).

5.12.3.3 *Molecular interactions by infrared (FTIR) spectroscopy*

Figures 5.23a and 5.23b show the infrared spectra of fresh and storage samples for LE and SDP samples, respectively. Irrespective of storage condition, LE samples at T_{60days} showed spectra characteristics that are very similar to those obtained prior to storage (T_{0day}). This suggests that constituent compounds are retained throughout the storage period, without obvious deletion of peaks.

On the other hand, it was observed that spectra characteristics obtained at the initial time (T_{0day}) only shared similarities with those of samples stored at 25 ºC/32.4 % RH (condition A) but different from those obtained at other conditions. A closer look between wavenumbers 778 and 1457 cm^{-1} revelead that the large band present in the original sample, associated to encapsulation of LE compnents are replaced by various peaks in this range with the introduction of new peaks hitherto missing in the original sample. These peaks are similar to those earlier described for pure LE and lactose (Section 5.12.3), suggesting that these storage conditions adversely affected stability of SDP.

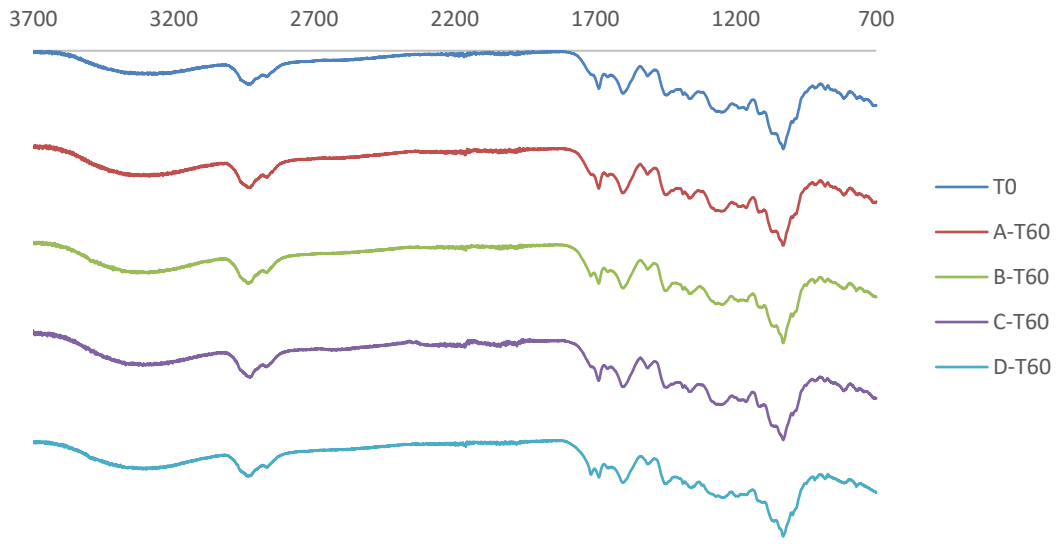


Figure 5.23a: FTIR spectra for freshly prepared lyophilized extract (a); and following storage for 60 days at 25ºC/32.4%RH (b); 25ºC/63.5%RH (c); 45ºC/32.4%RH (d); and 45ºC/63.5%RH (e); to evaluate the effect of storage condition on molecular interactions.

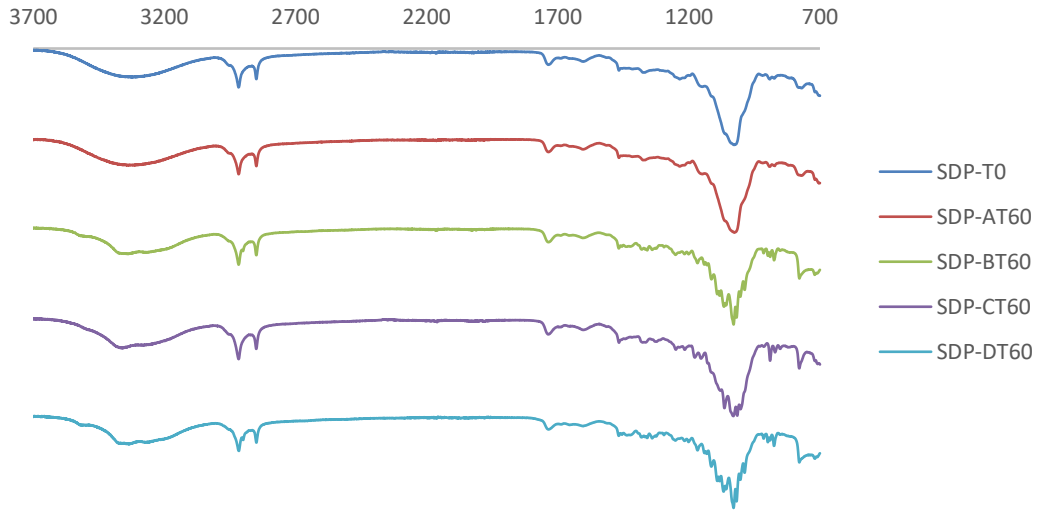


Figure 5.23b: FTIR spectra for freshly prepared proliposome (a); and following storage for 60 days at 25ºC/32.4%RH (b); 25ºC/63.5%RH (c); 45ºC/32.4%RH (d); and 45ºC/63.5%RH (e); to evaluate the effect of storage condition on molecular interactions.

5.12.3.4 *Colorimetric assay*

The complexity of plant extracts and their products, like other plant materials, predisposes them to a wide range of chemical reactions such as oxidation and hydrolysis thereby leading to instability (CORTÉS-ROJAS et al., 2016). Manifest instability and changes in chemical properties are often accompanied by alterations in physical properties such as sample colour which are visible to the eyes.

Since the perception of colour is highly subjective, a quantitative approach is desirable to adequately measure relative changes expressed with respect to colour (hue), lightness (brightness or brilhance), and saturation (vividness or intensity). This quantitative approach to measure slight changes in physical appearance of samples is necessary as part of a comprehensive quality assurance protocol. Such quantitative measurements ensure that slight changes that might go unnoticed are taken into consideration for reasons of safety and consumer confidence (PATHARE; OPARA; AL-SAID, 2013).

Standard parameters are involved in the colour quantification process, the most important ones being the hue angle and the chroma. The hue is described as the attribute of color perception by means of which an object is judged to be red, yellow, green, blue, or purple while colour chroma is a consideration of the purity of a color, or its freedom from white or grey (HAROLD, 2001; PATHARE; OPARA, 2013).

The extent of changes in the hue angle and the chroma during storage are presented for the lyophilized extract (Figures 5.24a and 5.24b) and proliposome (Figures 5.25a and 5.25b). LE and SDP samples both showed changes in colour (hue) during storage, the extent dependent on the storage condition. RH of 63.5 % changed the colour of both LE and SDP from the light beige to darker beige colour.

As earlier observed, colour changes are more pronounced at high RH conditions i.e. higher environmental moisture. The moisture content could affect the mobility of the molecular system which is directly related to the velocity of the degradation reactions. It is thought that these reactions are responsible for the colour changes observed. It has been postulated that the molecular mobility of bioactive compounds is significantly higher in amorphous state, resulting in an increase in the rate of occurrence of caking, agglomeration and browning reactions among others (CORTÉS-ROJAS; SOUZA; OLIVEIRA, 2016). Since our samples are completely amorphous at the beginning of the experiment, these occurences proceeded more rapidly at high RH. This may be mitigated by conserving the glassy state of the amorphous matrix through maintenance of critical water content and critical water activity in the powder, thus increasing product stability (MORAGA et al., 2012).

Besides the enzymatic browning of LE and SDP previously postulated (Section 5.12.1.2), colour changes in samples might also include the occurrence of non-enzymatic browning reaction, often referred to as Maillard reaction. Lactose is a reducing sugar and may react with certain amine/amino acid components in the extract or lipid materials used. Although it is known that Maillard reaction takes place rapidly at temperatures above 100 ^{o}C (HUPPERTZ; GAZI, 2015), such type of reaction has been suggested to influence colour changes in phytopharmaceutical products during storage at lower temperatures (SOUZA et al, 2013). It could be that this reaction is not only depend on temperature and other storage conditions but also time dependent, especially at lower temperatures.

The hue angle of the SDP was higher than LE for freshly prepared samples (beginning of the experiments). This is because color parameters of original proliposome come from the addition of carrier, hence higher than those of LE. However, SDP suffered greater colour change compared to LE stored at the same condition, probably due to effect of additives that promote reactions earlier described. A similar trend was observed for colour chroma of both LE and SDP samples.

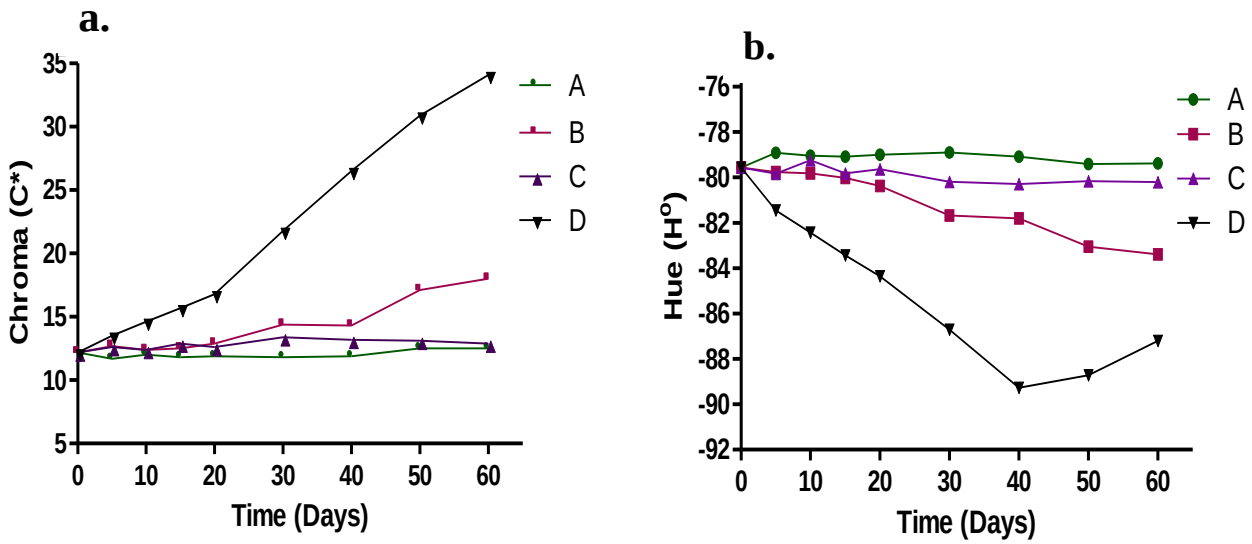


Figure 5.24: Chroma and Hue angle parameters for quantification of colour changes in lyophilized extract (LE) during storage at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); and 45ºC/63.5%RH (D).

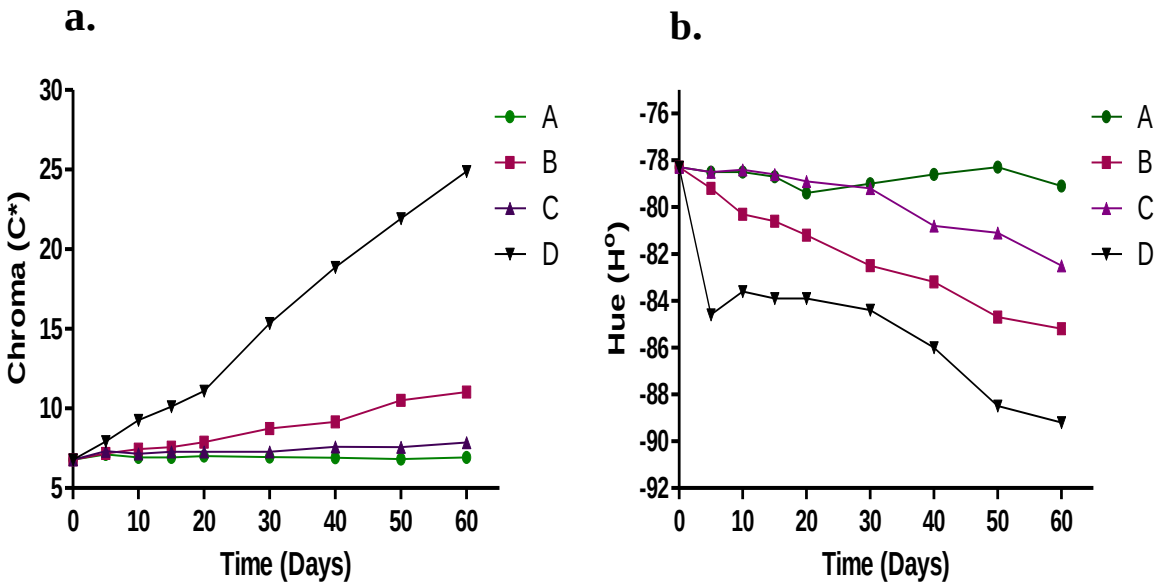


Figure 5.25: Chroma and Hue angle parameters for quantification of colour changes in spray dried proliposome (SDP) during storage at 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); and 8 ºC/55 %RH (R).

5.12.4 Antioxidant assay of storage samples – DPPH radical sequestering test

Antioxidant activity of storage samples of LE and SDP was carried out after the stability study in order to assess the impact of variation in the concentration of active substances,

occasioned by storage condition, on antioxidant activity. The DPPH• radical sequestering method was used for this evaluation. Table 5.19 shows the results obtained for LE and SDP samples before and after storage at different conditions.

It can be observed that antioxidant activity of both LE and SDP samples decreased following storage, the extent dependent on the storage condition. Relative humidity influenced activity to a higher degree than temperature, similar to observations for retention of polyphenol marker.

The reduction in activity may be due to significant degradation of bioactive constituents. Results earlier presented showed that carnosol and carnosic acid were the most susceptible polyphenol constituents, in some cases reducing below one-half of initial concentration. Aruoma et al. reported that approximately 5 % of the dry weight of rosemary leaves is made up of carnosol and carnosic acid, although the same fraction was estimated to account for > 90 % of the antioxidant activity (ARUOMA et al, 1992; JORDÁN et al, 2012). Hence, significant degradation of these constituents is mostly responsible for loss of activity, especially in lyophilized extract.

Table 5.19: IC_{50} values and DPPH• inhibition capacity of SDP and LE samples obtained before and after stability testing experiments.

Samples	LE		SDP	
	IC_{50} (µg/mL)[a]	Inhibition (%)	IC_{50} (µg/mL) [a #]	Inhibition (%)
T_{0day}	$10.8\pm0.3^{**}$	89.0±0.1	$9.2\pm0.2^{**}$	83.1±1.0
T_{60days} –A	$10.9\pm0.1^{**}$	88.8±0.1	$9.5\pm0.3^{**}$	83.7±1.1
T_{60days} –B	$11.8\pm0.1^{***}$	87.6±0.3	$16.3\pm0.5^{***}$	84.4±0.1
T_{60days} –C	11.2 ± 0.4^{ns}	86.7±0.3	11.5 ± 0.1^{ns}	85.8±0.9
T_{60days} –D	$18.6\pm1.2^{***}$	86.4±0.1	$22.4\pm0.6^{***}$	84.5±0.2
T_{60days} –R	$10.8\pm0.3^{**}$	88.6±0.5	$9.3\pm0.2^{**}$	85.8±0.8

[#] IC_{50} obtained on LE basis
[a] Antioxidant activity by the DPPH• method, expressed as IC_{50}.
Statistical difference determined by Two-Way ANOVA followed by Bonferroni post-hoc test.
$^{***}p < 0.001$; $^{**}p < 0.01$; $^{ns}p > 0.05$ (not significant).
LE: lyophilized rosemary extract; SDP: spray dried proliposome;
Storage: 25ºC/32.4%RH (A); 25ºC/63.5%RH (B); 45ºC/32.4%RH (C); 45ºC/63.5%RH (D); 8ºC/55 %RH (R)

Furthermore, crystallization of samples usually leads to reduction of solubility which possibly impairs bioactivity. This is particularly true for SDP samples which was initially amorphous in nature but converted to crystalline, with degree of crystallinity depending on storage condition. This possibility may be confirmed when antioxidant activity of LE and SDP samples are compared at similar storage condition. Although SDP samples stored at high RH condition (conditions B and D) gave higher concentrations of these polyphenols after storage, LE samples showed lower IC_{50} at both conditions. This could be due to reduced solubility of SDP samples resulting from crystallization whereas the amorphous nature of LE samples was largely retained after storage.

Antioxidant activity of both LE and SDP samples remained unchanged following storage in the refrigerator using moisture proof packaging material. Also, there was no significant change in antioxidant activity at low temperature and RH (25 ^{o}C/32.4 % RH, condition A) even when samples were stored in open containers. Despite reduced concentration of individual bioactive compound at these conditions, retained activity may be due to synergism between polyphenols.

6. Conclusions

6 CONCLUSIONS

In this study, a review of the literature was carried out to establish the level of research and options available and already used in encapsulating polyphenols contained in the extract of rosemary towards improving their stability and functionality. Materials and methods were carefully selected to meet the objectives of the work; bearing in mind compatibility and relative toxicity of ingredients, simplicity and reproducibility of methods, as well as product affordability.

6.1 Preliminary studies

Proliposome was considered in this study for effective encapsulation of different polyphenols of rosemary. Following initial tests of various solvents, ethanol was found appropriate for both polyphenol extraction and preparation of liposomal composition, being volatile and providing solubility for the lipids and polyphenol components. From an array of drying carriers that have been explored in related studies by our group and others, lactose was selected from the point of view of efficiency and cost. Spray drying was carried out at conditions used in previous studies by our group, bearing in mind the transition temperature of lipids used in this study. The performance of the process was adequate and the results confirmed that lactose has potential for use as a drying aid for lipid carriers.

6.2 Formulation preparation and optimization

The possibilities of changes in product properties resulting from variations in input factors underscore the importance of Quality by Design strategy for preparing products with predetermined properties and fit for intended use. This is achievable by Design of Experiments, a systematic approach to determine the relationship between factors involved in a process and the effects of those factors on process output. The Central Composite Design was demonstrated as an efficient approach in which the effects of changes in composition variables were assessed within a workable number of experiments to arrive at the targeted product quality.

The optimization of the processing variables using multi-response analysis was successfully validated. The experimental responses determined at optimum processing condition exhibited good agreement with estimated values. It was shown that relative concentration and retention of each rosemary polyphenol in spray dried proliposomes was a function of its own polarity and composition variables. Whereas retention of rosmarinic acid is largely dependent on concentration of the extract, values for carnosol and carnosic acid were

shown to be influenced by lipid, extract and drying aid concentrations. Water activity was shown to depend on the drying aid (lactose) concentration while moisture content was only slightly influenced by both lipid and extract concentrations. Although spray dried proliposome products obtained did not show excellent flow and compressibility properties, these parameters could be improved by inclusion of certain other excipients with acceptable characteristics.

6.3 Potential biological activities

Lyophilized extract of rosemary showed good antimicrobial activity against yeast, and both Gram-positive and Gram-negative organisms. It was therefore, considered suitable for application as therapeutic pharmaceutical agent or as preservatives in various industries. The antimicrobial activity demonstrated is in addition to the antioxidant activity established by the DPPH free radical sequestering method.

These potentials of biological activity could be enhanced by preparing the extract in systems that aid their biaoctivity and stability such as proliposome-liposome systems. The optimized SDP loaded with the rosemary polyphenols showed an enhancement of the antioxidant activity and improved efficacy against the test microorganisms when compared to pure lyophilized extract. In this manner, lower concentrations could be applied to achieve the desired activity due to higher potency of the polyphenols encapsulated in the proliposome system; possibly by enhancing solubility of bioactive compounds as well as improving their ability to penetrate cellular barriers.

6.4 Stability studies

Stability studies carried out for pure lyophilized extract and proliposome product revealed not only changes in visual and pharmacotechnical properties, but also degradation of polyphenol components; the extent of which was shown to be dependent on the storage condition. This degradation adversely affected the antioxidant activity of the test materials, reducing the potency by up to 50 %. Although storage temperature adversely affected evaluated product properties, relative humidity was shown as the more critical factor responsible for changes observed in both the extract and proliposome. Although encapsulation of proliposomes offered protection and higher stability for the polyphenol, this gain might be limited by the environmental condition to which the product is exposed. While degradation of polyphenols is slower in proliposomes compared to unencapsulated extract stored at similar conditions, significant polyphenol degradation was observed for both ordinary extract and proliposome product exposed to unfavourable environmental conditions.

The First-order kinetics best fitted the degradation of both carnosol and carnosic acid. Based on these components, the shelf life of the proliposome product was determined as 12 days at 45 ^{o}C/63.5 % relative humidity, 90 days at 25 ^{o}C/32.4 % relative humidity, and 180 days when stored in a sealed packaging material inside a refrigerator at 8 ^{o}C. Hence, it is important to ensure a storage condition adequate enough to preserve the encapsulated polyphenol and prolong product shelf life.

6.5 General conclusions

In this study, proliposome was shown as a viable system for encapsulation of rosemary polyphenols through a systematic study of the relationships between composition variables and their effects on desirable responses, guided by experimental design. The proliposome powder obtained presented adequate retention and concentration of bioactive polyphenols, and other physicochemical properties following spray drying of liposomal composition. The product displayed ready redispersibility in aqueous medium to form liposomes with acceptable properties including particle size, and improved stability. It is projected that these advantages over ordinary lyophilized extract adequately justify the costs incurred with respect to materials and process of proliposome production. The increased efficacy achieved will mean lower quantities of bioactive required for desired effects, while the enhanced efficiency of redispersion compensates for the cost of intense mixing and solubilization operations.

Results obtained in this work furnish strong evidences that the proliposome product, having improved physicochemical properties and superior bioactivity, might be applicable as a natural antioxidant or as a phytopharmaceutical agent in treatment and prevention of several acute/chronic diseases in humans, either singly or as a component of a pharmaceutical dosage form. It might also be used as natural preservative in several categories of products such as foods, nutraceuticals, cosmetics and skin care preparations where microorganisms remain a source of contamination and degradation. However, storage condition is very critical to maintenance of these activities. It is important that the proliposome product is stored in an environment of low relative humidity and low temperature. This ensures prevention of degradation of the polyphenol compounds while maintaining the physicochemical and organoleptic properties.

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Appendices

APPENDIX I

Formulation composition by Central Composite Design (non-randomised)

Formulation	A (% w/w)	B (% w/w)	C (-)	$m_{carrier}$ (100g) (g)	CS (-)	H20 (Cs = 10.9) (g)	m_{final} (g)
F1	4	1.5	1.0	5.5	11.0	0.9	100.9
F2	10	1.5	1.0	11.5	23.0	111.0	211.0
F3	4	4.5	1.0	8.5	17.0	56.0	156.0
F4	10	4.5	1.0	14.5	29.0	166.1	266.1
F5	4	1.5	1.4	7.7	13.2	21.1	121.1
F6	10	1.5	1.4	16.1	27.6	153.2	253.2
F7	4	4.5	1.4	11.9	20.4	87.2	187.2
F8	10	4.5	1.4	20.3	34.8	219.3	319.3
F9	2	3.0	1.2	5.9	10.9	0.0	100.0
F10	12	3.0	1.2	18.1	33.1	203.7	303.7
F11	7	0.5	1.2	9.0	16.4	50.9	150.9
F12	7	5.5	1.2	15.0	27.6	152.8	252.8
F13	7	3.0	0.9	8.6	18.6	71.0	171.0
F14	7	3.0	1.5	15.4	25.4	132.7	232.7
F15	7	3.0	1.2	12.0	22.0	101.9	201.9
F16	7	3.0	1.2	12.0	22.0	101.9	201.9
F17	7	3.0	1.2	12.0	22.0	101.9	201.9

A: % Lipid; **B**: % Extract; **C**: %Carrier/%LIP

APPENDIX II

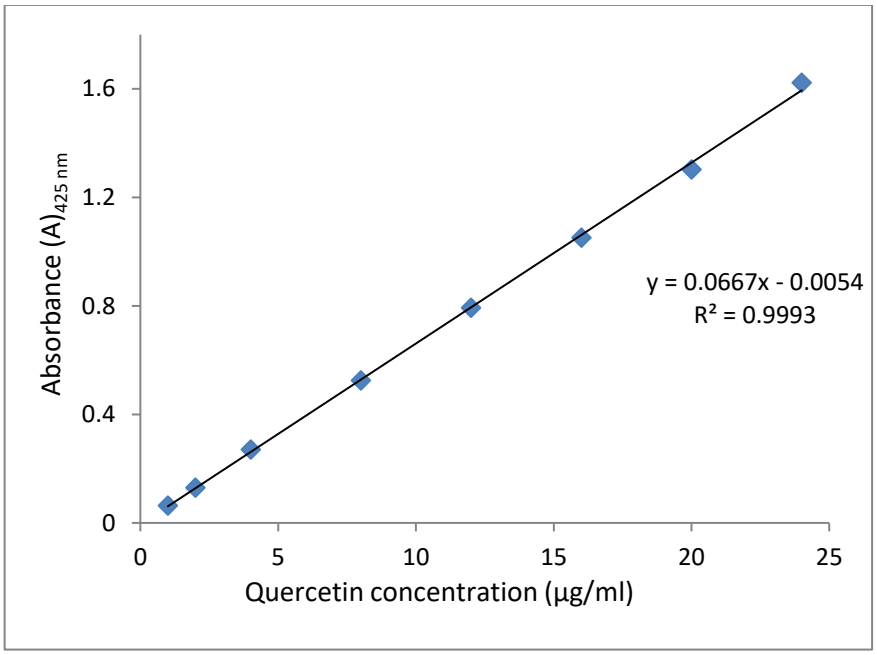
Formulation composition by non-randomised Central Composite Experimental Design and drying conditions

Formulation	Lipid Phase			Aqueous Phase		Amounts					
	Lecithin (g)	Cholesterol (g)	$Ethanol_{90\%}$ (g)	FD extract (%)	Purified water (g)	Liposomal solid (g)	Total mass (g)	Lactose (g)	$C_{total\ solid}$ (-)	Water added (g)	Final mass (g)
F1	3.6	0.4	50.0	1.5	94.5	5.5	100.0	5.5	11.0	0.9	100.9
F2	9.0	1.0	50.0	1.5	88.5	11.5	100.0	11.5	23.0	111.0	211.0
F3	3.6	0.4	50.0	4.5	91.5	8.5	100.0	8.5	17.0	56.0	156.0
F4	9.0	1.0	50.0	4.5	85.5	14.5	100.0	14.5	29.0	166.1	266.1
F5	3.6	0.4	50.0	1.5	94.5	5.5	100.0	7.7	13.2	21.1	121.1
F6	9.0	1.0	50.0	1.5	88.5	11.5	100.0	16.1	27.6	153.2	253.2
F7	3.6	0.4	50.0	4.5	91.5	8.5	100.0	11.9	20.4	87.2	187.2
F8	9.0	1.0	50.0	4.5	85.5	14.5	100.0	20.3	34.8	219.3	319.3
F9	1.8	0.2	50.0	3.0	95.1	5.0	100.0	5.9	10.9	0.0	100.0
F10	10.8	1.2	50.0	3.0	85.0	15.1	100.0	18.1	33.1	203.7	303.7
F11	6.3	0.7	50.0	0.5	92.5	7.5	100.0	9.0	16.4	50.9	150.9
F12	6.3	0.7	50.0	5.5	87.5	12.5	100.0	15.0	27.6	152.8	252.8
F13	6.3	0.7	50.0	3.0	90.0	10.0	100.0	8.6	18.6	71.0	171.0
F14	6.3	0.7	50.0	3.0	90.0	10.0	100.0	15.4	25.4	132.7	232.7
F15	6.3	0.7	50.0	3.0	90.0	10.0	100.0	12.0	22.0	101.9	201.9
F16	6.3	0.7	50.0	3.0	90.0	10.0	100.0	12.0	22.0	101.9	201.9
F17	6.3	0.7	50.0	3.0	90.0	10.0	100.0	12.0	22.0	101.9	201.9

Note: *Proliposomes were generated by spray drying. Spray drying condition utilized are as follows: inlet gas temperature = 100 ^oC; gas feed rate = 60 Lpm; formulation feed rate = g/min; atomizer = 1mm; atomizer pressure = 1.5 kgf/cm^2; gas atomizer = 17 Lpm*

APPENDIX III

Calibration curve of quercetin standard for the determination of total flavonoid



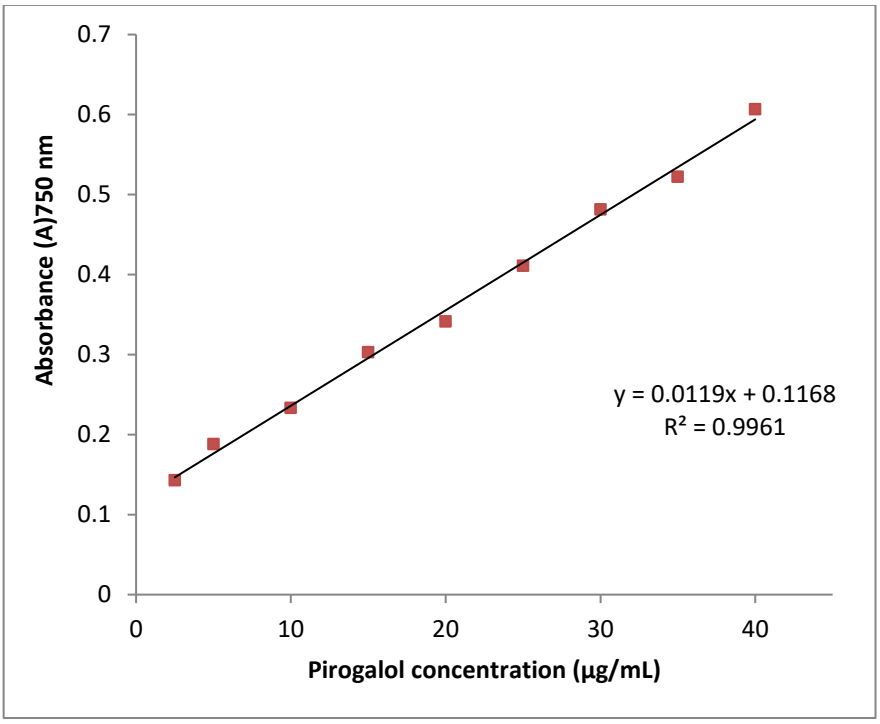
APPENDIX IV

Equations and regression coefficients for calibration curves of quercetin and pirogalol.

STANDARDS	RESULTS	
	Equation	R^2
Quercetin	0.0667x – 0.0054	0.9993
Pirogalol	0.0119x + 0.1168	0.9961

APPENDIX V

Calibration curve of pirogalol standard for the determination of total polyphenol



APPENDIX VI

Flow characteristics of lyophilized rosemary extract (LE) and spray-dried proliposome (SDP) powders following storage at different conditions

					SAMPLING TIME (h)								
SPECIMEN	T (°C)	RH (%)	CODE	SAMPLES	T_0	T_5	T_{10}	T_{15}	T_{20}	T_{30}	T_{40}	T_{50}	T_{60}
SDP	25	32.4	A	*1*	F	F	F	F	F	F	F	F	F
				2	F	F	F	F	F	F	F	F	F
		63.5	B	*1*	F	SF	NF	NF	NF	NF	NF	NF	NF
				2	F	SF	NF	NF	NF	NF	NF	NF	NF
	45	32.4	C	*1*	F	F	SF	SF	NF	NF	NF	NF	NF
				2	F	F	SF	SF	NF	NF	NF	NF	NF
		63.5	D	*1*	F	SF	NF	NF	NF	NF	NF	NF	NF
				2	F	SF	NF	NF	NF	NF	NF	NF	NF
LE	25	32.4	A	*1*	F	F	F	F	F	F	F	F	F
				2	F	F	F	F	F	F	F	F	F
		63.5	B	*1*	F	F	F	F	F	F	SF	NF	NF
				2	F	F	F	F	F	F	SF	NF	NF
	45	32.4	C	*1*	F	F	F	F	F	F	SF	SF	NF
				2	F	F	F	F	F	F	SF	SF	NF
		63.5	D	*1*	F	F	F	F	F	SF	SF	NF	NF
				2	F	F	F	F	F	SF	SF	NF	NF

F: flowable; SF: semi-flowable; NF: non-flowable
SDP: spray dried proliposomes; LE: lyophilized extract
T: temperature; RH: relative humidity

APPENDIX VII

Agglomeration of lyophilized rosemary extracts (LE) and spray-dried proliposome (SDP) particles following storage at different conditions

					SAMPLING TIME (h)								
SPECIMEN	TEMP. (°C)	RH (%)	CODE	SAMPLES	T_0	T_5	T_{10}	T_{15}	T_{20}	T_{30}	T_{40}	T_{50}	T_{60}
SDP	25	32.4	A	*1*	N	N	Y	Y	Y	Y	Y	Y	Y
				2	N	N	Y	Y	Y	Y	Y	Y	Y
		63.5	B	*1*	N	Y	YY	YY	YY	YY	YY	YY	YY
				2	N	Y	YY	YY	YY	YY	YY	YY	YY
	45	32.4	C	*1*	N	N	YY	YY	YY	YY	YY	YY	YY
				2	N	N	YY	YY	YY	YY	YY	YY	YY
		63.5	D	*1*	N	Y	YY	YY	YY	YY	YY	YY	YY
				2	N	Y	YY	YY	YY	YY	YY	YY	YY
LE	25	32.4	A	*1*	N	N	N	N	N	N	N	N	N
				2	N	N	N	N	N	N	N	N	N
		63.5	B	*1*	N	N	Y	Y	Y	Y	Y	Y	Y
				2	N	N	Y	Y	Y	Y	Y	Y	Y
	45	32.4	C	*1*	N	N	N	N	N	N	Y	Y	Y
				2	N	N	N	N	N	N	Y	Y	Y
		63.5	D	*1*	N	N	Y	Y	Y	Y	Y	Y	Y
				2	N	N	Y	Y	Y	Y	Y	Y	Y

N: absent; Y: present; YY: conspicuously present
SDP: spray dried proliposomes; LE: lyophilized extract
T: temperature; RH: relative humidity

APPENDIX VIII

Caking of lyophilized rosemary extract (LE) and spray-dried proliposome (SDP) powders following storage at different conditions.

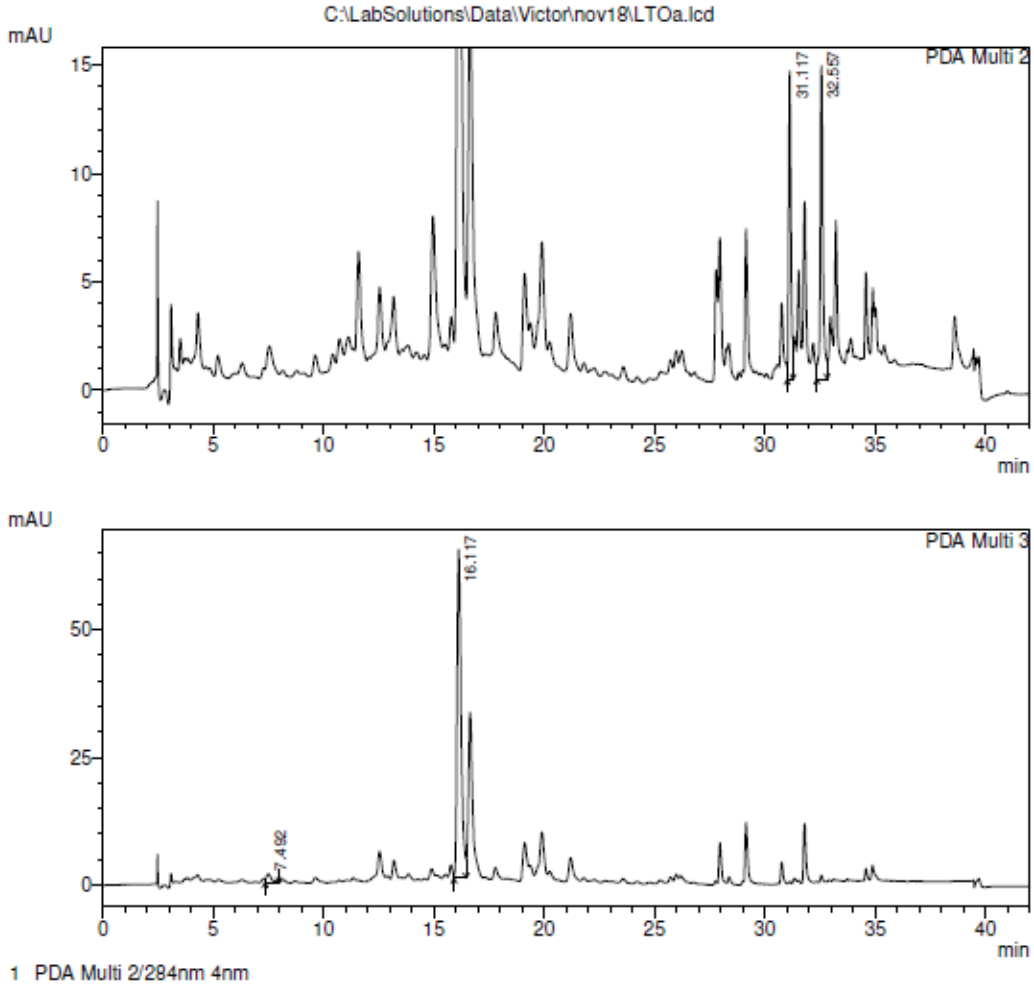
					SAMPLING TIME (h)								
SPECIMEN	TEMP. (°C)	RH (%)	CODE	SAMPLES	T_0	T_5	T_{10}	T_{15}	T_{20}	T_{30}	T_{40}	T_{50}	T_{60}
SDP	25	32.4	A	*1*	N	N	N	N	N	N	N	N	Y
				2	N	N	N	N	N	N	N	N	Y
		63.5	B	*1*	N	N	Y	Y	Y	YY	YY	YY	YY
				2	N	N	Y	Y	Y	YY	YY	YY	YY
	45	32.4	C	*1*	N	N	N	N	Y	YY	YY	YY	YY
				2	N	N	N	N	Y	YY	YY	YY	YY
		63.5	D	*1*	N	N	Y	Y	YY	YY	YY	YY	YY
				2	N	N	Y	Y	YY	YY	YY	YY	YY
LE	25	32.4	A	*1*	N	N	N	N	N	N	N	N	N
				2	N	N	N	N	N	N	N	N	N
		63.5	B	*1*	N	N	N	N	N	N	N	Y	Y
				2	N	N	N	N	N	N	N	Y	Y
	45	32.4	C	*1*	N	N	N	N	N	N	Y	Y	Y
				2	N	N	N	N	N	N	Y	Y	Y
		63.5	D	*1*	N	N	N	N	Y	Y	Y	Y	Y
				2	N	N	N	N	Y	Y	Y	Y	Y

N: absent; Y: present; YY: conspicuously present
SDP: spray dried proliposomes; LE: lyophilized extract
T: temperature; RH: relative humidity

APPENDIX IX

Chromatogram of LE and SDP samples at different storage conditions after 60 days (T_{60days})

<Chromatogram>



1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

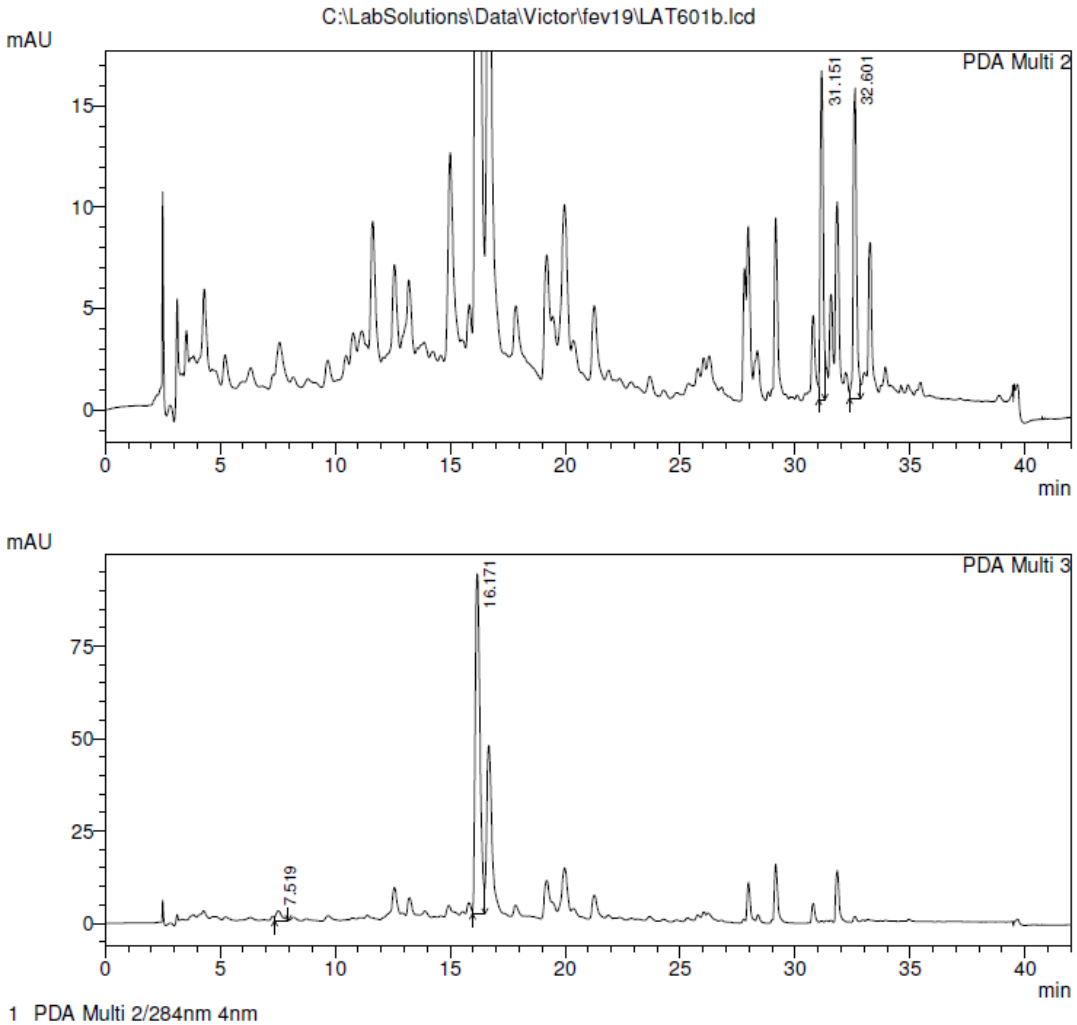
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.117	103408	14220	0.000	
2		32.557	123023	14440	0.000	
Total			226431	28660		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.492	27657	1724	0.000	
2		16.117	824980	64317	0.000	
Total			852638	66041		

a. Lyophilized extract at T_{0day}

<Chromatogram>



<Results>

PDA Ch2 284nm 4nm

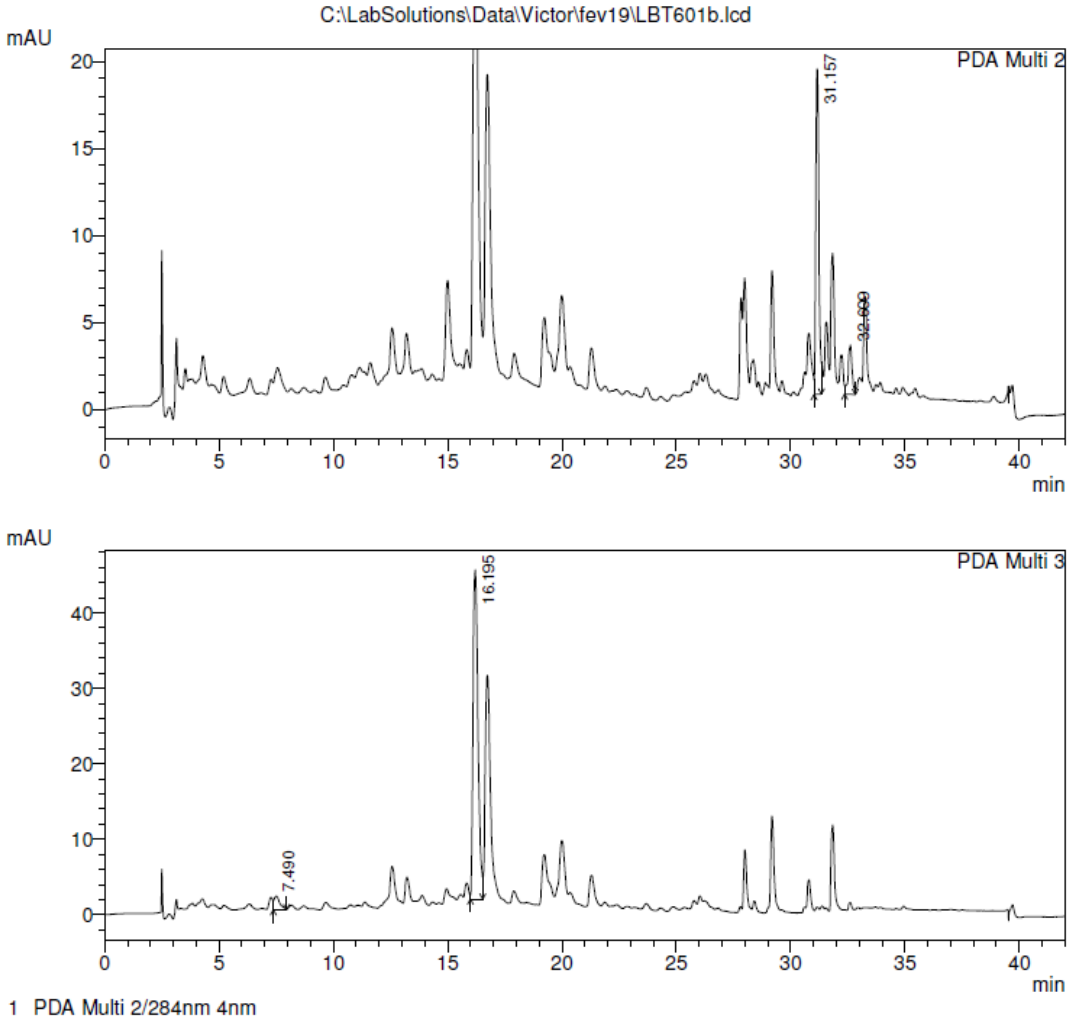
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.151	145801	16237	0.000	
2		32.601	157689	15329	0.000	
Total			303489	31566		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.519	47821	2660	0.000	
2		16.171	1354488	91964	0.000	
Total			1402309	94624		

b. Lyophilized extract after 60 days (T_{60days}) storage at 25 ºC / 32.4 % RH

<Chromatogram>



1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

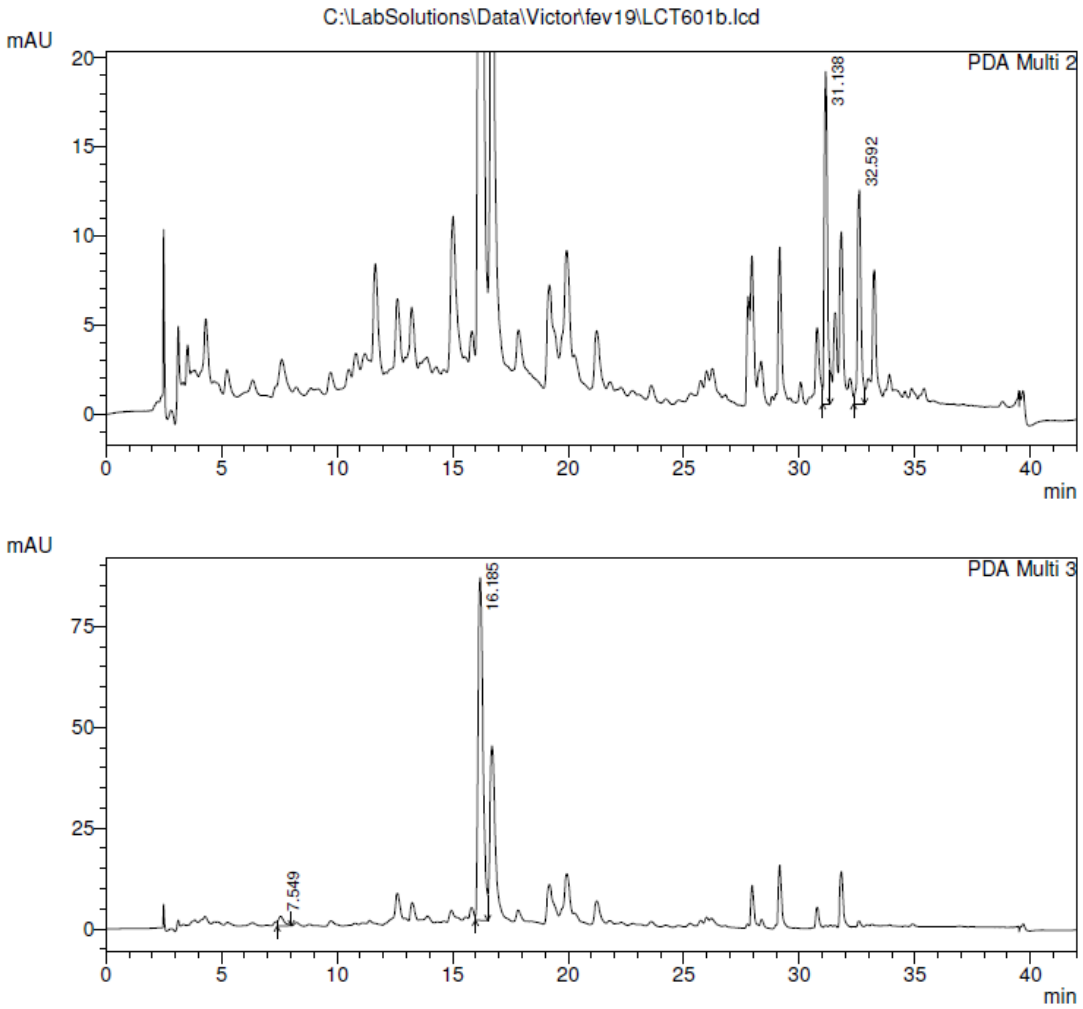
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.157	177878	18712	0.000	
2		32.609	34215	2833	0.000	
Total			212094	21545		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.490	31309	1791	0.000	
2		16.195	659808	43610	0.000	
Total			691116	45401		

c. Lyophilized extract after 60 days (T_{60days}) storage at 25 ºC / 63.5 % RH

<Chromatogram>



1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.138	177000	18656	0.000	
2		32.592	128382	12019	0.000	
Total			305382	30675		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.549	40617	2342	0.000	
2		16.185	1275377	84951	0.000	
Total			1315994	87293		

d. Lyophilized extract after 60 days (T_{60days}) storage at 45 ºC / 32.4 % RH

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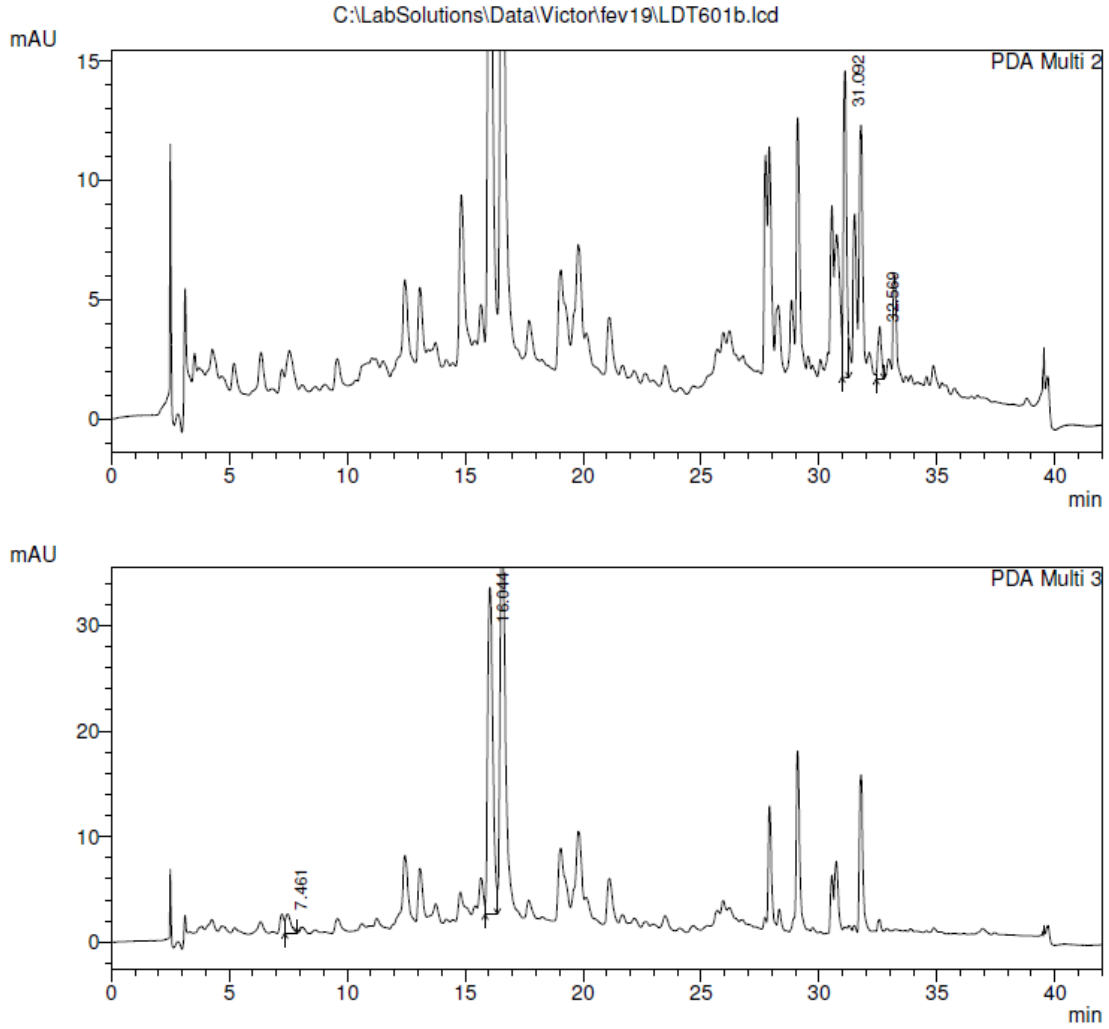


1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

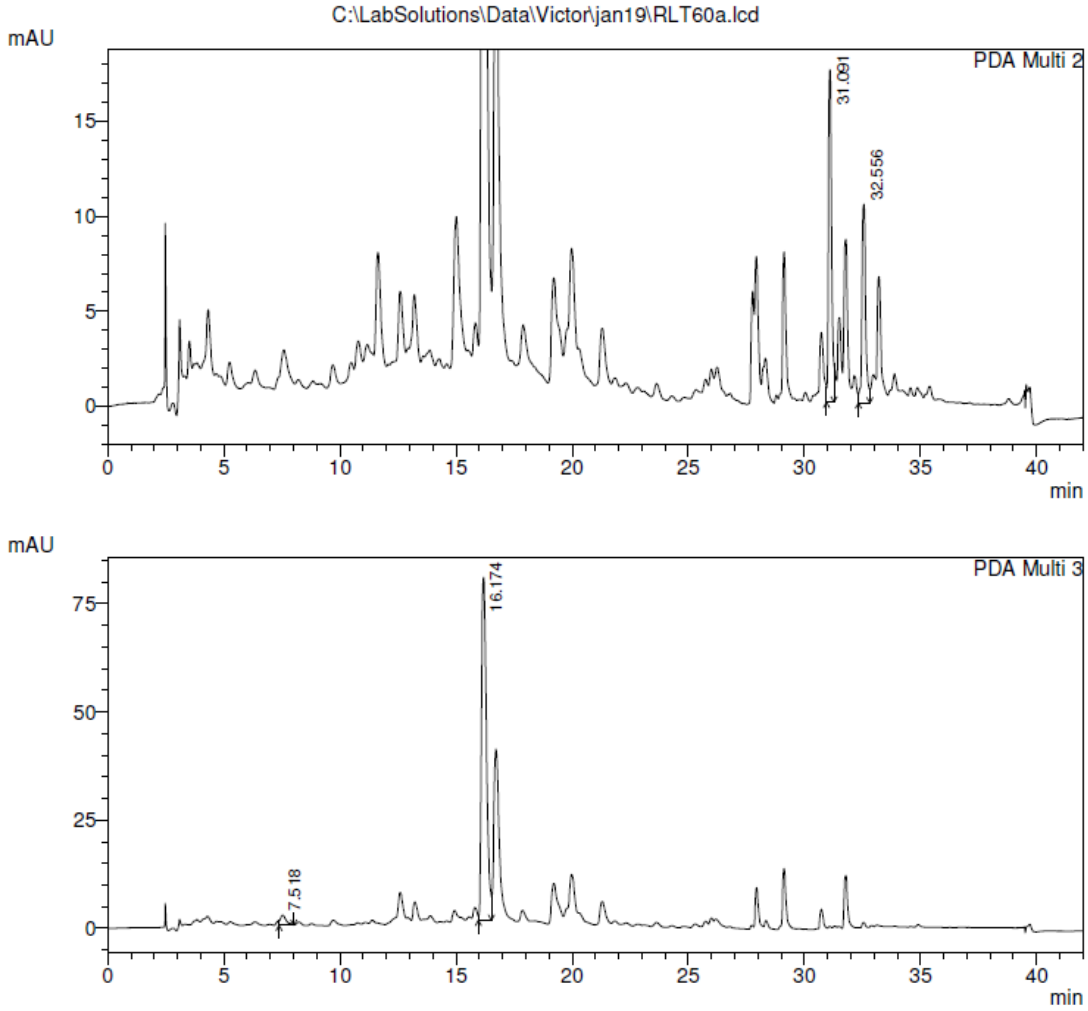
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.092	122561	12861	0.000	
2		32.569	22663	2207	0.000	
Total			145224	15068		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.461	29074	1802	0.000	
2		16.044	469313	30954	0.000	
Total			498387	32756		

e. Lyophilized extract after 60 days (T_{60days}) storage at 45 ºC / 63.5 % RH

<Chromatogram>



<Results>

PDA Ch2 284nm 4nm

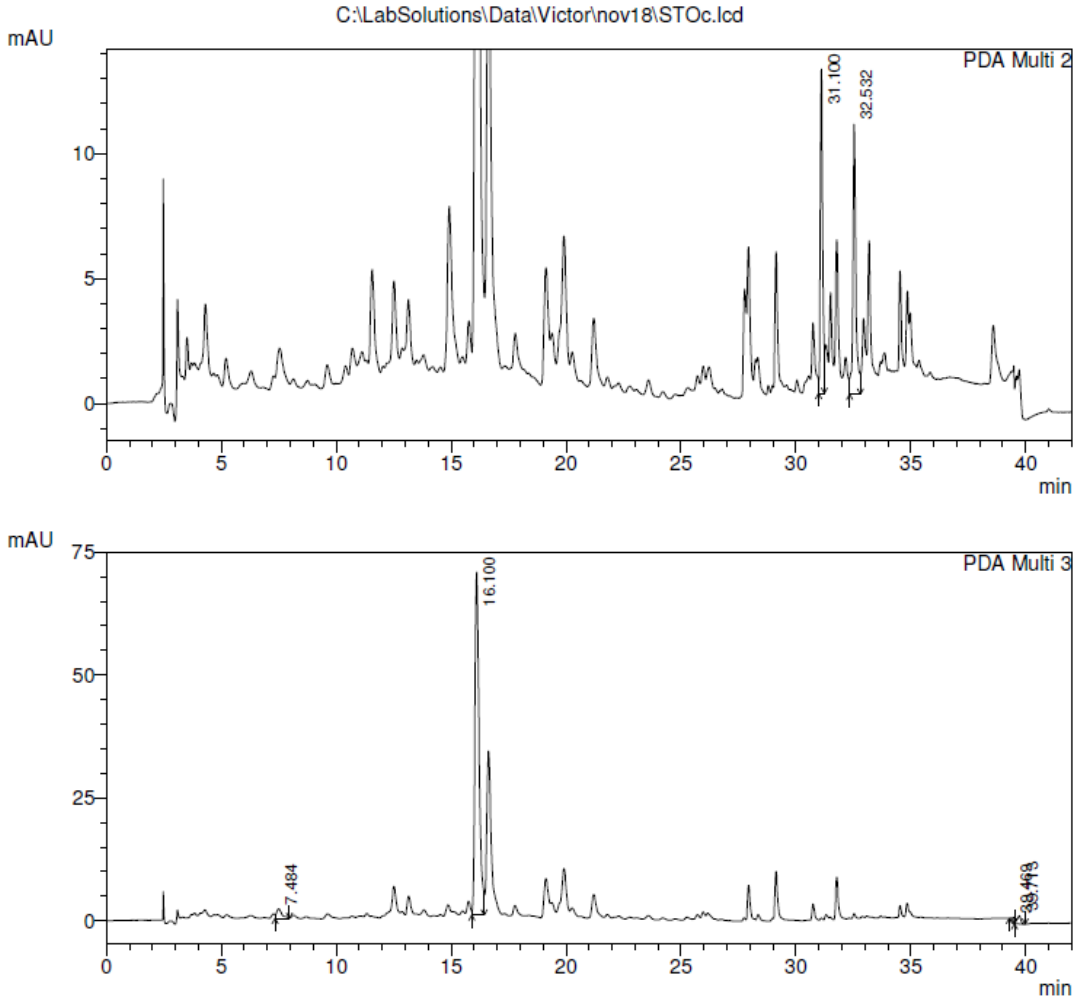
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.091	166931	17515	0.000	
2		32.556	116197	10462	0.000	
Total			283128	27976		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.518	43292	2366	0.000	
2		16.174	1200278	79259	0.000	
Total			1243571	81625		

f. Lyophilized extract after 60 days (T_{60days}) storage in refrigerator (hermetically sealed package)

<Chromatogram>



<Results>

PDA Ch2 284nm 4nm

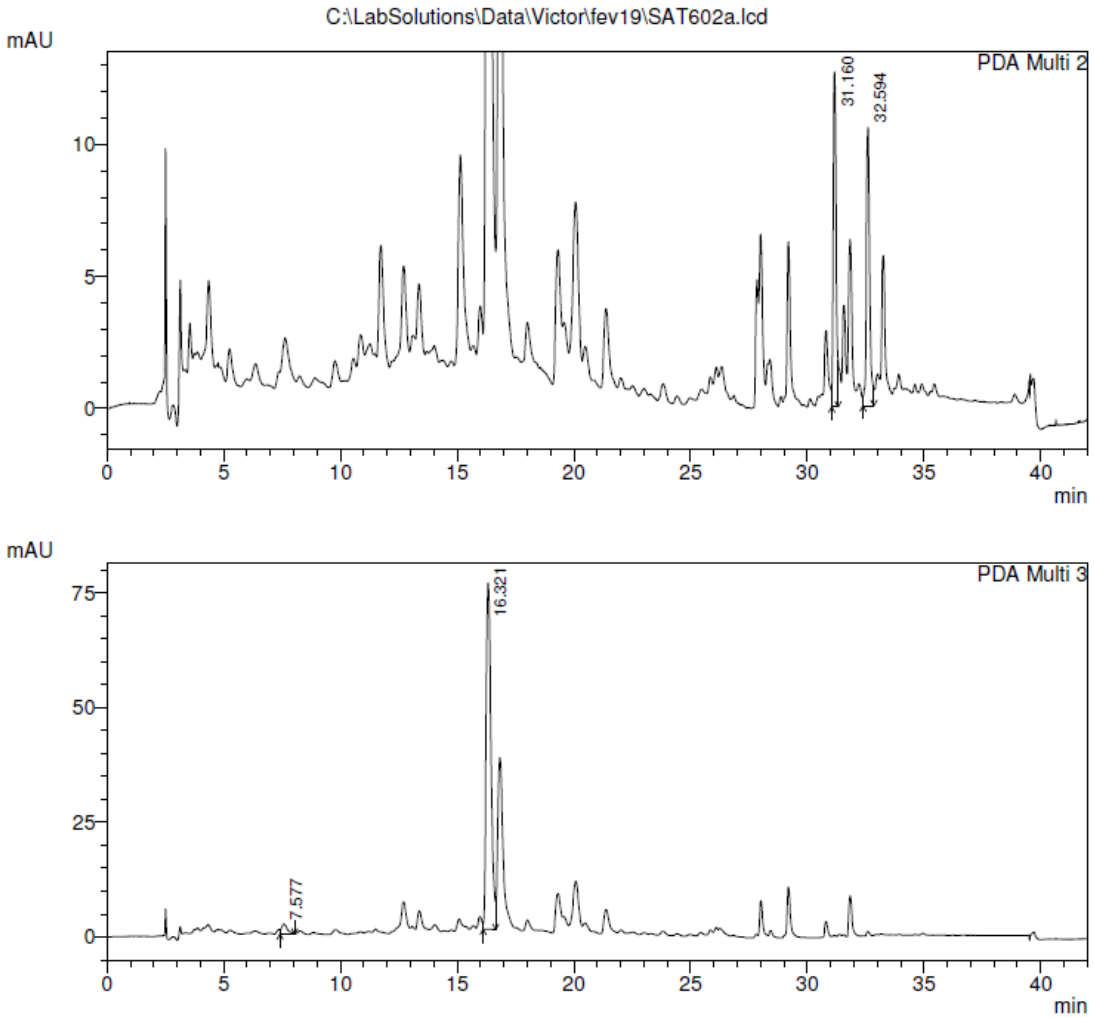
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.100	92244	12991	0.000	
2		32.532	95672	10815	0.000	
Total			187916	23806		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.484	31854	1947	0.000	
2		16.100	891772	69785	0.000	
3		39.469	7087	1139	0.000	
4		39.713	19804	1663	0.000	
Total			950517	74535		

g. Spray dried proliposome at beginning of stability study (T_{0day})

<Chromatogram>



1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.160	108978	12683	0.000	
2		32.594	105713	10530	0.000	
Total			214691	23213		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.577	40448	2179	0.000	
2		16.321	1109744	75489	0.000	
Total			1150192	77668		

h. Spray dried proliposome after 60 days (T_{60days}) storage at 25 ºC / 32.4 % RH

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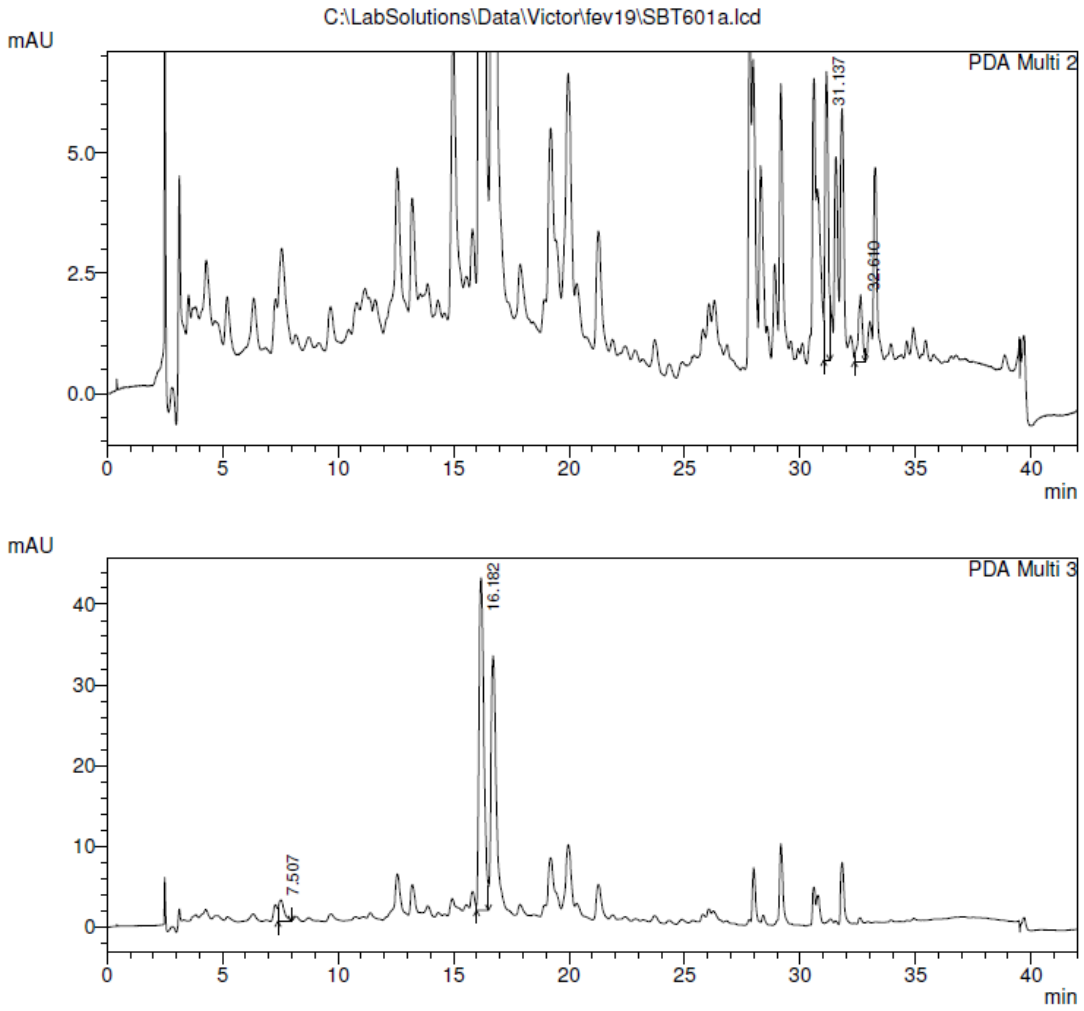

1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.137	57479	6003	0.000	
2		32.610	16769	1387	0.000	
Total			74248	7390		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.507	43787	2645	0.000	
2		16.182	615607	41211	0.000	
Total			659394	43856		

i. Spray dried proliposome after 60 days (T_{60days}) storage at 25 ºC / 63.5 % RH

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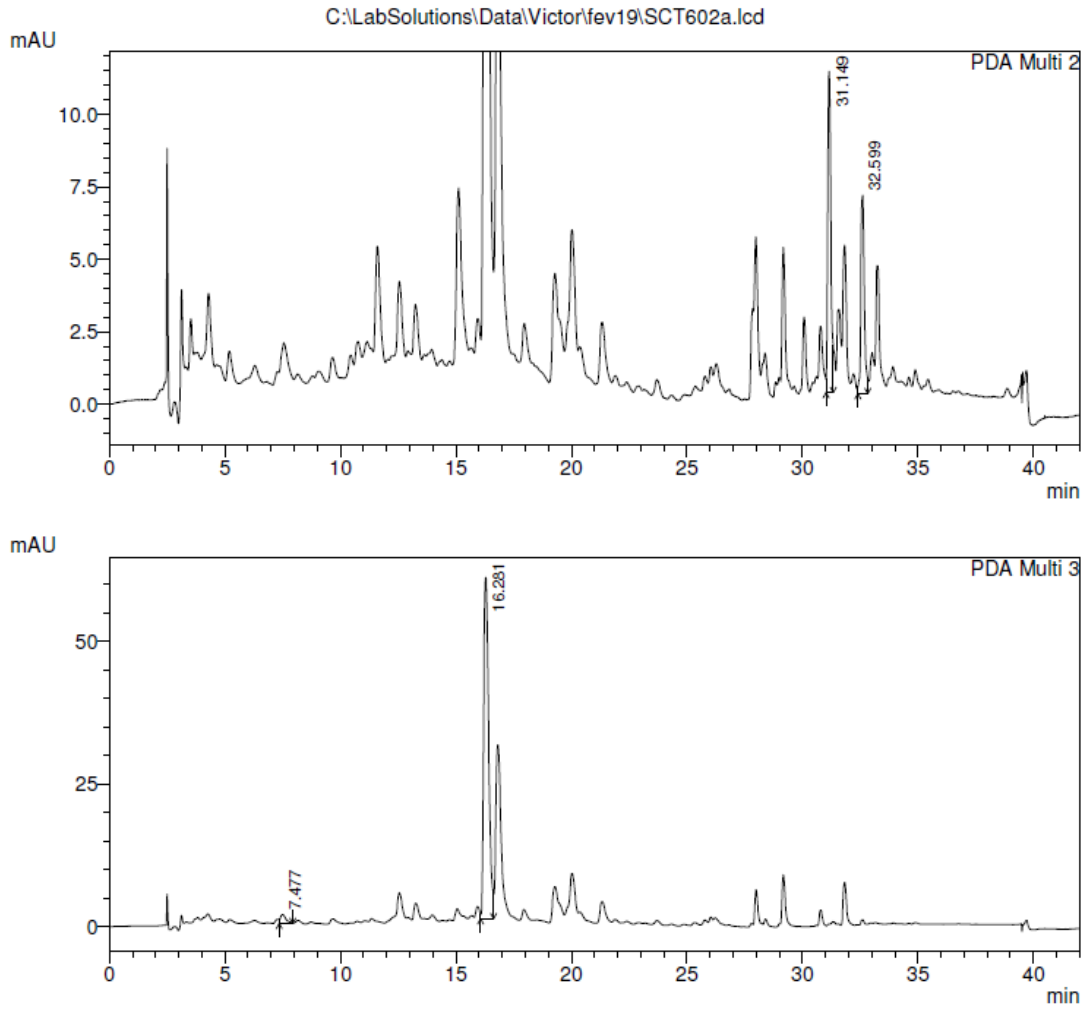

1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.149	104529	11088	0.000	
2		32.599	72617	6846	0.000	
Total			177146	17934		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.477	26558	1542	0.000	
2		16.281	904671	59876	0.000	
Total			931228	61418		

j. Spray dried proliposome after 60 days (T_{60days}) storage at 45 ºC / 32.4 % RH

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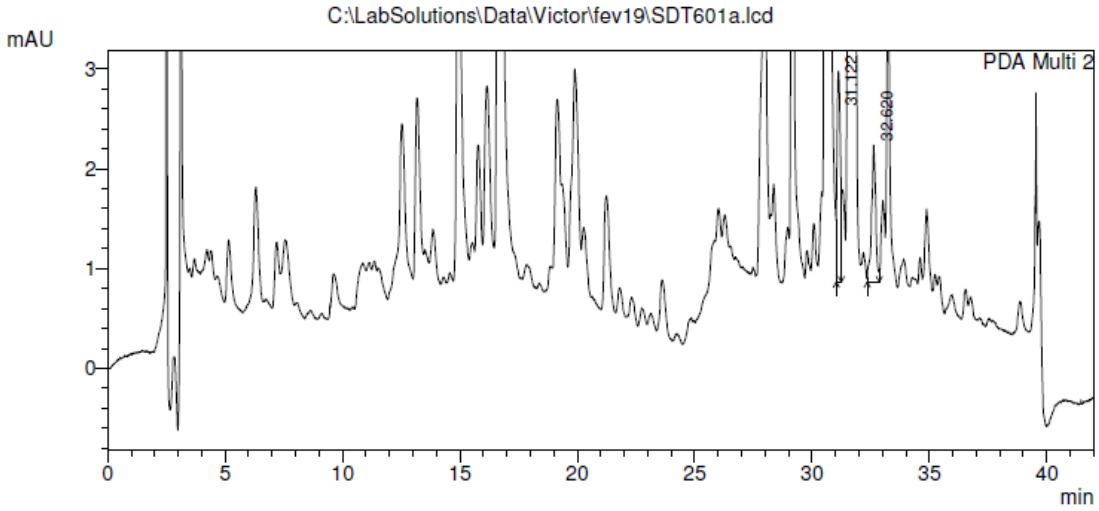
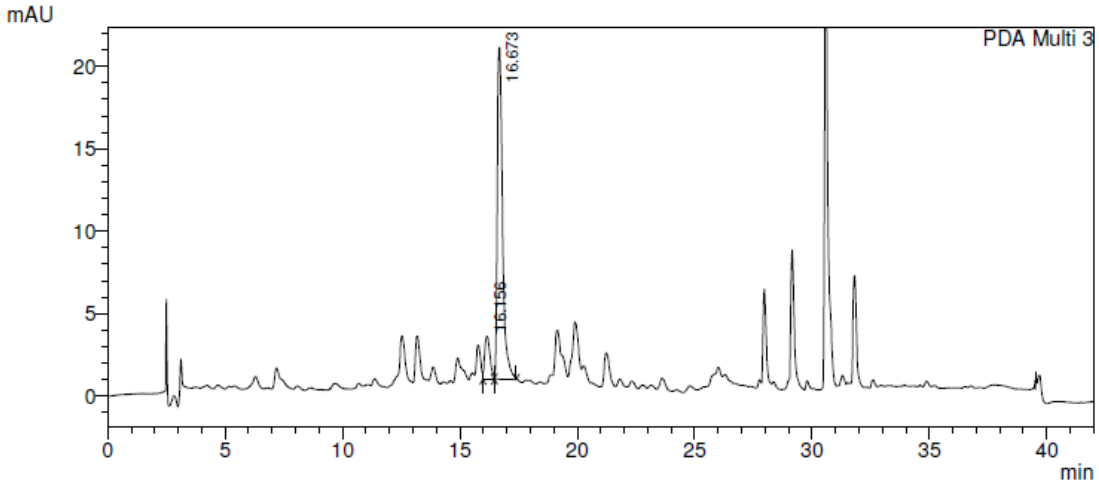




1 PDA Multi 2/284nm 4nm
2 PDA Multi 3/330nm 4nm

<Results>

PDA Ch2 284nm 4nm

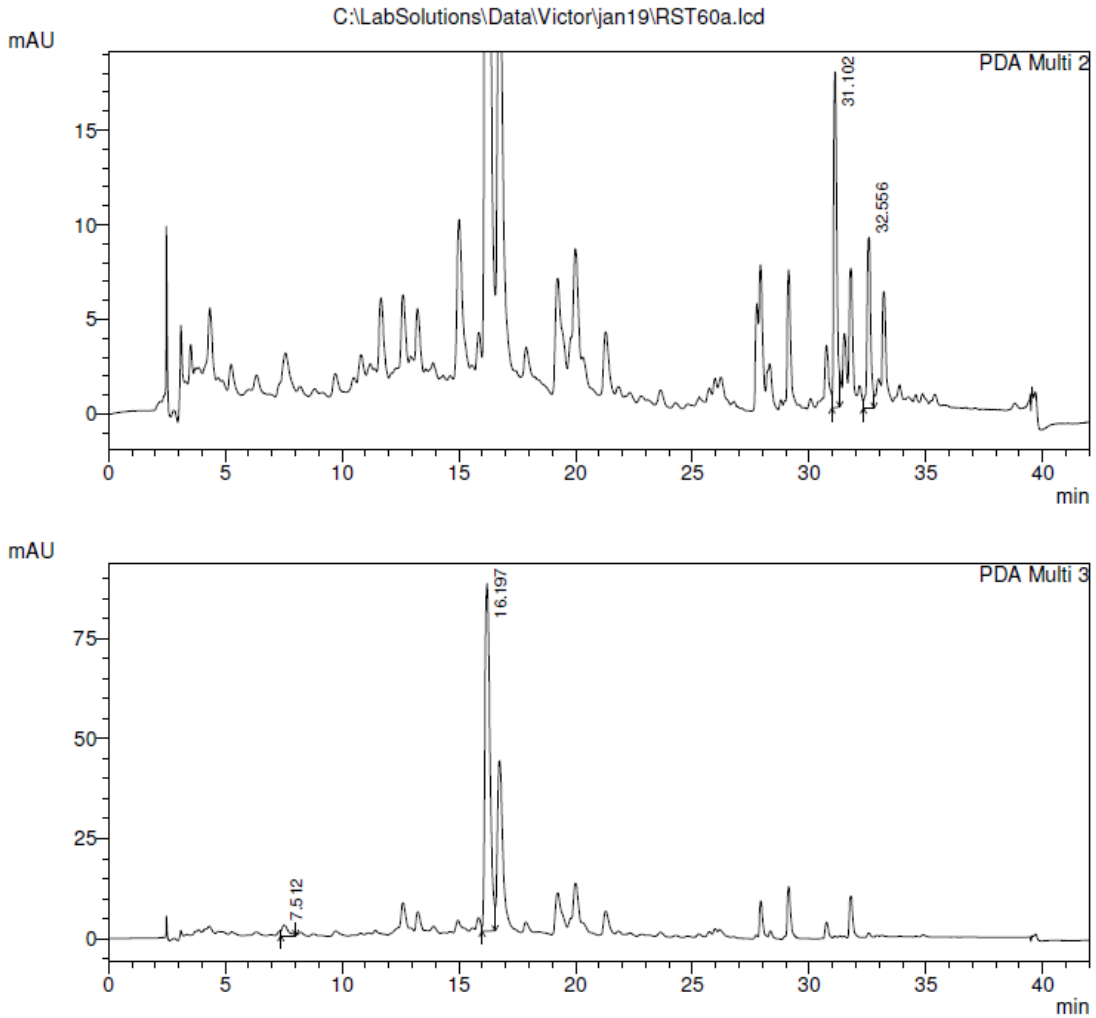
Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1	RT31.122	31.122	20682	2115	0.000	ug/mL
2	RT32.620	32.620	16648	1369	0.000	ug/mL
Total			37330	3485		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1	RT16.156	16.156	41086	2599	0.000	ug/mL
2		16.673	313733	20137	0.000	
Total			354819	22736		

k. Spray dried proliposome after 60 days (T_{60days}) storage at 45 ^{o}C / 63.5 % RH

<Chromatogram>



<Results>

PDA Ch2 284nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		31.102	169170	17751	0.000	
2		32.556	98999	9021	0.000	
Total			268169	26772		

PDA Ch3 330nm 4nm

Peak #	Name	Ret. Time	Area	Height	Conc.	Units
1		7.512	46860	2612	0.000	
2		16.197	1318761	86826	0.000	
Total			1365621	89439		

l. Spray dried proliposome after 60 days (T_{60days}) storage in refrigerator (hermetically sealed package)