2010

Antioxidant Nanoparticles as Delivery Systems

Carlos Ernesto Astete R.
Louisiana State University and Agricultural and Mechanical College

ANTIOXIDANT NANOPARTICLES AS DELIVERY SYSTEMS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in Engineering Science

by
Carlos Ernesto Astete R.
B.S., Catholic of Valparaiso, Chile, 1993
M.B.A., Adolfo Ibanez University, Chile, 2000
M.S., Louisiana State University, USA, 2005
May, 2010

To whom I love, specially

Sara, Felipe, and Camila, my lovely and
charming family
and
my parents, Carlos and Sara

ACKNOWLEDGEMENTS

After several classes, professors, laboratories, journals, articles, books, experiments, and friends I am able to finish my research. The endeavor was huge, and sensation of adventure, effort, journey, enthusiasm, accomplishment, also it is now time to give thanks to all people that in a direct or indirect way were related to the amazing quest that was my research.

When I came to Louisiana State University, I was apprehensive about the interdepartmental relationships, the academic environment, and the support of the professors. It was really gratifying to find the supporting and friendly environment that gave me the option to develop my research in a high-quality way.

The initial steps of a research journey need support of your advisor, and the further development it is only possible if you are able to form a team that permit share ideas, concepts, and continuous communication of advances and mistakes with a potential impact on the journey's fate. I am very thankful and grateful to my major professor Dr. Cristina Sabliov, in the Biological & Agricultural Department, at Louisiana State University. Her constant guidance, advice, feedback, cooperation, and support were a vital part for the completion of my research. Her constant feedback was a wonderful way to implement new ideas and to overcome the problems found in the path of my research. Along the same lines, all committee members were very helpful every time that I required their advice. In special, Dr. David Spivak, that helped with the VECAR synthesis. His feedback was vital to synthesize VECAR through different reaction steps like esterification, protection, de-protection, chromatography columns, and other techniques used in my research adventure. Dr. Debra Dolliver was vital for the synthesis of EC, I really appreciate her work, dedication, and support. Dr. Luis Escobar was always a source of advice and support. Thanks also go to Dr. Marybeth Lima which has been a source of inspiration and advice. Finally, I am pleased for the opportunity to explore, develop, initiate, and navigate in the turbulent sea of research, your continuous support and advice were fundamental to overcome the failures and find new ways to continue my journey through antioxidant nanoparticles and VECAR.

Dr. Rafael Cueto, in the Chemistry Department, at Louisiana State University, was essential in understanding the size characterization by dynamic light scattering (DLS) technique. He was always open to help me in the DLS characterization, and other issues related with my research. The constructive feedback was always really helpful.

I am delighted to thank Mrs. Cindy Henke in the Biological Science Department at Louisiana State University, for her help and advice in using the transmission electron microscope. Her constant preoccupation to find a way to get better pictures of the nanoparticles was very helpful.

Dr. Lavrent Khachatryan in the Chemistry Department, Louisiana State University, provided important information about oxidative behavior of nanoparticles through EPR data collected. I am really appreciative for his contribution and support.

I am really gratified for the contribution provided by Ms. Danielle Meador graduate student in Chemistry Department, Louisiana State University, through her hard work, feedback, and support which was vital to accomplish several objectives and overcome several details and problems through my research.

Many people were involved in different ways in my research, but I would like to mention especially Abitha Murugeshu, Nipur Patel, Taryn Leal, and Paige Chandler for their support and contribution to my research. Furthermore, the peaceful, stimulating, cooperating, and friendly atmosphere of Biological & Agricultural Department was important in the termination of my research and vital for a satisfying work.

Finally, I am very thankful and proud of my family, Sara my beautiful, persistent, and smart wife, Felipe my active, friendly, and clever boy, and Camila my dancer, curious, and lovely girl, for their constant support and understanding of my research. The long hours required in the information search, nanoparticles formation and characterization, and writing were always easier with their encouragement and cooperation. I am very gratified for my parents that continuously supported me in all my decisions and endeavors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The goal of the study was to develop antioxidant polymeric nanoparticles as a new delivery system for food and pharmaceutical applications. Natural antioxidants like alpha-tocopherol, vitamin C, and carnosine, are responsible to protect biological systems against free radicals attack. The conjugation of vitamin C-vitamin E (EC) and alpha-tocopherol-carnosine (VECAR) were performed to obtain antioxidant surfactants which were used for polymeric nanoparticle synthesis. The surfactant made of α-tocopherol (vitamin E) and ascorbic acid (vitamin C) of antioxidant properties dubbed as EC was used to make poly(lactic-co-glycolic) acid (PLGA) nanoparticles. Self-assembled EC nanostructures and PLGA-EC nanoparticles were made by nanoprecipitation, and the nanoparticle physical properties were studied at different salt concentrations, surfactant concentrations, and polymer:surfactant ratios. EC was soluble in polar solvents, and Span 80 was selected as a control due to its similar surfactant properties. The PLGA-EC nanoparticles and EC surfactant showed antioxidant activity based on DPPH technique and EPR study, which is not characteristics to commercially available Span80. The newly synthesized EC surfactant was found successful in forming uniform, small size polymeric nanoparticles of intrinsic antioxidant properties.

The next step was to synthesize VECAR, an antioxidant molecule made of carnosine and alpha-tocopherol derivative. The designed molecule looks for protection of low density lipoproteins (LDL) from oxidation by placing at the LDL interface two potent natural antioxidants. The LDL protection can impact the development and outcome of atherosclerosis. The approach followed was to use the phytyl chain as active site for coupling of carnosine. The synthesis process involved 8 steps starting with esterification of Trolox and finishing with the deprotection of the hydroxyl group of the alpha-tocopherol moiety. The antioxidant behavior of VECAR (0.0236 mM) was tested by DPPH assay which showed values similar to pure alpha-tocopherol (0.0229 mM). The values suggested full recovery of the active hydroxyl group of the alpha-tocopherol derivative. More studies are needed to test antioxidant behavior in the presence of lipids and ability of VECAR to protect LDL oxidation *in vitro*; *in vivo* analysis of LDL oxidation in the presence of VECAR is required to understand the fate of VECAR in more complex and real systems.

CHAPTER 1. INTRODUCTION

The present work focuses on surfactants, antioxidants, self-assembly, and polymeric nanoparticles as interactive entities for prevention and treatment of oxidative stress related diseases. Polymeric nanoparticles have been identified as a promising system for treatment of disease by delivery of active components at specific places in a controlled manner [1-7], which translates into a reduction in side effects, increased therapeutically effects, and higher treatment efficiency.

In general, there are two main approaches to synthesize polymeric nanoparticles: top-down and bottom-up techniques. The bottom-up techniques require monomers to build up nanostructures. This involves polymerization using initiators and surfactants (emulsion polymerization technique). The top-down techniques involves reduction of already formed structures by application of external forces like sonication or a physical-chemistry process (e.g. solvent diffusion, solvent-antisolvent properties). Examples of top-down techniques are emulsion evaporation, nanoprecipitation, and salting out [2]. All of these techniques involve surfactants that stabilize the nanostructures formed by application of external forces and reduce their aggregation when suspended in water [8,9]. Surfactant concentration commonly used in nanoparticles synthesis can reach up to 5-8 %wt. Safety of these surfactants is an issue. Sodium dodecyl sulfate (SDS) for example, is a good anionic surfactant but its use is restricted for food applications (e.g. less than 125 ppm is allowed in liquid & frozen egg whites). Polysorbates such as sorbitan monostearate is only allowed in finished whipped edible oil topping at concentrations lower than 0.4% [10]). To reduce the amount of surfactants present in the final formulations, washing steps are required. Centrifugation, ultra-filtration, and dialysis are examples of common techniques used, but they add extra cost and time to the synthesis. One approach to reduce or limit washing steps is to use bio-friendly and highly efficient surfactants in the polymeric nanoparticle synthesis.

The unique role of the polymeric nanoparticles is to act as carriers for the drug selected for treatment entrapped into the polymeric core. It is expected that the next generation of nanosystems will be more proactive; they will “sense” the environment and will react to the external stimuli to produce a controlled beneficial effect. Not only will they act as a carrier, but also as active agents. To accomplish this goal, the components of the system must possess characteristics that allow specific chemical and physical interactions with the environment. One

way to control these specific interactions of the nanoparticles with environment is to use a surfactant of desired properties in the nanoparticles synthesis. For example, functional surfactants with antioxidant action provide the option to synthesize colloidal nanostructures with inherent antioxidant activity. These structures could be useful in treating oxidative-stress related diseases such as atherosclerosis and cancer, among others, in addition to playing their obvious role as vehicles for drug transport and delivery. Polymeric nanoparticles formed with these surfactants would not require washing.

Antioxidants and vitamins are fundamental for human health. Natural antioxidants like vitamin E (α-tocopherol), vitamin C, flavonoids, and carotenoids are compounds that could potentially modulate oxidative stress. Antioxidants are defined as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate" by Halliwell & Gutteridge [11]. Oxidation of substrates is promoted by highly reactive species such as free radicals. Free radicals (i.e. superoxide anion, hydroxyl and peroxyl radical), and non free radicals (i.e. hydrogen peroxide, hypochlorous acid, and peroxynitrite) are present in all biological systems and can trigger oxidative processes with harmful consequences for the biological entity that result in aging, cancer, diabetes, cardiovascular disease, inflammatory responses, degenerative diseases, cataract, and others [12-20]. Hence, many of the principal diseases affecting human health that are related with the oxidative process can be prevented by antioxidants which limit cell damage produced by reactive oxygen and nitrogen species (ROS and NOS) [21-24].

Atherosclerosis has been related to the oxidative process, in that high levels of oxidant molecules or low levels of antioxidants were shown to be responsible for high oxidative stress triggering the atherosclerosis process. The oxidation theory of atherosclerosis attributes great importance to the oxidation process and deems free radicals as precursors for the first steps in the development of the disease [19,25-27]. In this context, great importance has been attributed to vitamin E, especially α-tocopherol, as the main lipophilic natural antioxidant present in the lipophilic phase of every cell. LDL is composed by a core of cholesterol and triglycerides surrounded by fatty acids, cholesteryl esters, apolipoproteins, and phospholipids [28-31]. Lipophilic antioxidants avoid oxidation of the lipid fraction of LDLs, but apolipoproteins are not protected from oxidation even in the presence of lipophilic antioxidants [32-36]. The limitations of lipophilic natural antioxidants can be overcome with an adequate design of an alternative

"natural" antioxidant formed by covalently linking the hydrophobic antioxidant (alpha-tocopherol) with a hydrophilic molecule with antioxidant properties (vitamin C or carnosine). The resulting amphiphilic molecule will insure that the antioxidant activity will be localized closer to apolipoproteins (present at the surface of the low density lipoproteins), for better antioxidant performance, with a potential synergetic behavior due to antioxidant-coantioxidant phenomena (vitamin E & vitamin C, vitamin E & carnosine). In addition, these new molecules (EC and VECAR) will have surfactant characteristics and could be used in synthesis of polymeric nanoparticles of inherent antioxidant characteristics.

The first approach followed to combat oxidative stress with polymeric nanoparticles was to synthesize particles with entrapped antioxidants such us superoxide dismutase, catalase, and melatonin into the polymeric matrix for the treatment of diseases related with oxidative damage [37-39]. The newer approach was to form antioxidant nanoparticles with inherent antioxidant activity due to the presence of antioxidant molecules (antioxidant surfactants) in the nanoparticle structure. Figure 1-1 depicts the concept of using antioxidant surfactants in the synthesis of polymeric nanoparticles with antioxidant properties.

The present dissertation presents three main chapters to show the current status and development of the concept of antioxidant nanoparticles as a new delivery system. Following the introduction, the second chapter shows a review on polymeric nanoparticles as delivery systems for antioxidants and vitamins. The information presented stresses the availability and importance of antioxidant molecules and the current status of nanosystems with entrapped antioxidants. Chapter 3 is focused on the development and use of a new surfactant (EC), a molecule synthesized by using natural antioxidant molecules: alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C). Alpha-tocopherol is hydrophobic, and vitamin C is hydrophilic. Thus, the amphiphilic EC surfactant results when the two are linked. The chapter presents work that describes the chemical vitamin E-vitamin C conjugation to form the EC surfactant, synthesis of nanostructures by self-assembly of the surfactant EC, and synthesis of polymeric nanoparticles by nanoprecipitation in the presence of EC. The nanostructures characterization (size, polydispersity, morphology, and antioxidant action) is presented relative to nanostructures formed under similar conditions in the presence of Span 80 as a surfactant. Chapter 4 presents information about synthesis of a new antioxidant molecule VECAR synthesized with vitamin E analog and carnosine (dipeptide). Detailed chemical synthesis of VECAR is presented step by

step, and study of the antioxidant action of VECAR is evaluated by DPPH and TBARS assay. The final two chapters present the conclusions and future work that could be pursuit based on concepts developed in my dissertation.

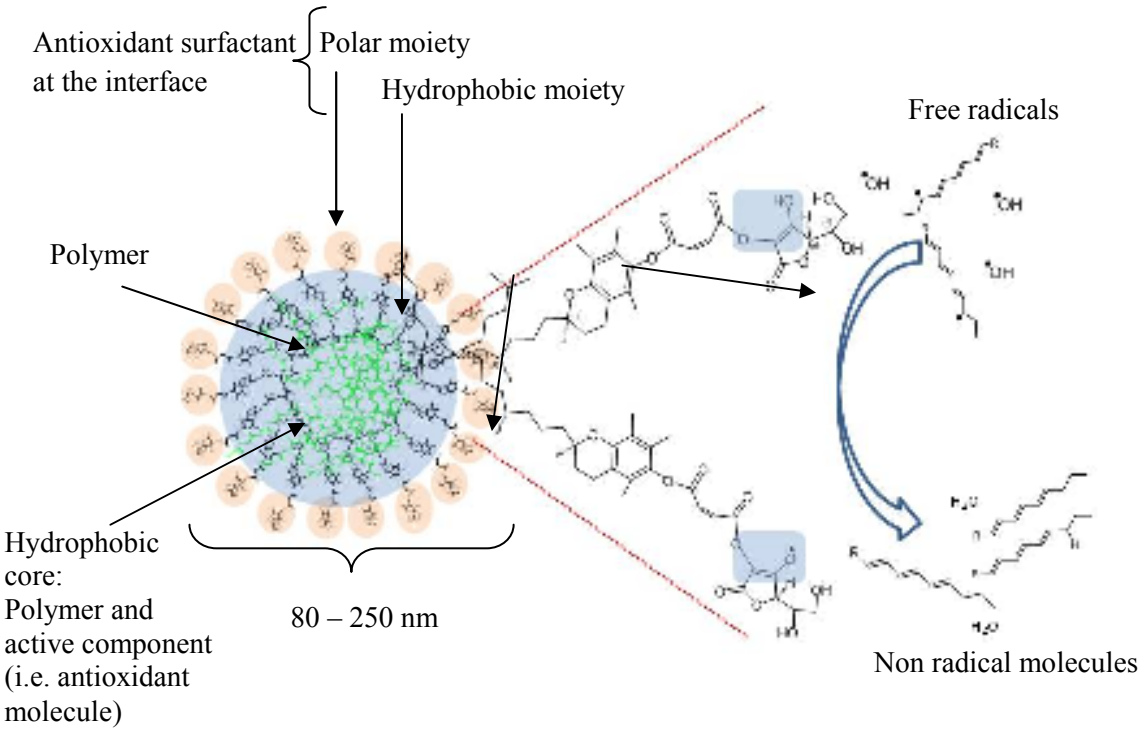


Figure 1-1: Antioxidant polymeric nanoparticles. Free radicals molecules can be scavenged by the antioxidant surfactant which is placed at the nanoparticle interface. Polymers like poly(lactide-co-glycolide acid) (PLGA) can be localized at the core with entrapped antioxidants or other drugs.

1.1 References

[1] Chiellini E, Chiellini EE, Chiellini F, Solaro R. Targeted administration of proteic drugs. I. Preparation of polymeric nanoparticles. Journal of Bioactive and Compatible Polymers 2001;16 (6):441-65.

[2] Astete CE, Sabliov CM. Synthesis and characterization of PLGA nanoparticles. Journal of Biomaterials Science-Polymer Edition 2006;17 (3):247-89.

[3] Kreuter J. Nanoparticles and microparticles for drug and vaccine delivery. Journal of Anatomy 1996;189:503-5.

[4] Damge C, Vranckx H, Balschmidt P, Couvreur P. Poly(alkyl cyanoacrylate) nanospheres for oral administration of insulin. Journal of Pharmaceutical Sciences 1997;86 (12):1403-9.

[5] Mukerjee A, Pruthi V. Oral insulin delivery by polymeric nanospheres. Journal of Biomedical Nanotechnology 2007;3 (1):68-74.

[6] Rawat M, Singh D, Saraf S, Saraf S. Nanocarriers: Promising vehicle for bioactive drugs. Biological & Pharmaceutical Bulletin 2006;29 (9):1790-8.

[7] Jeon HJ, Jeong JI, Jang MK, Park YH, Nah JW. Effect of solvent on the preparation of surfactant-free poly(DL-lactide-co-glycolide) nanoparticles and norfloxacin release characteristics. International Journal of Pharmaceutics 2000;207 (1-2):99-108.

[8] Israelachvili JN, Mitchell DJ, Ninham BW. Theory of Self-Assembly of Hydrocarbon Amphiphiles into Micelles and Bilayers. Journal of the Chemical Society-Faraday Transactions Ii 1976;72:1525-68.

[9] Sjostrom B, Kronberg B, Carlfors J. A Method for the Preparation of Submicron Particles of Sparingly Water-Soluble Drugs by Precipitation in Oil-in-Water Emulsions .1. Influence of Emulsification and Surfactant Concentration. Journal of Pharmaceutical Sciences 1993;82 (6):579-83.

[10] Administration FD. Listing of Food Additive Status. 2009. http://www.fda.gov/Food/FoodIngredientsPackaging/FoodAdditives/FoodAdditiveListings/ucm091048.htm.

[11] Halliwell B, Gutteridge JNC. The definition and measurement of antioxidants in biological systems. Free Radical Biology and Medicine 1995;18 (1):125-6.

[12] Young IS, Woodside JV. Antioxidants in health and disease. Journal of clinical pathology 2001;54 (3):176-86.

[13] Knight JA. Review: Free radicals, antioxidants, and the immune system. Annals of Clinical and Laboratory Science 2000;30 (2):145-58.

[14] Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free radical research 1999;31 (4):261-72.

[15] Vina J, Lloret A, Orti R, Alonso D. Molecular bases of the treatment of Alzheimer's disease with antioxidants: prevention of oxidative stress. Molecular Aspects of Medicine 2004;25 (1-2):117-23.

[16] Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radical Biology and Medicine 2000;28 (10):1456-62.

[17] Taylor A, Jacques PF, Epstein EM. Relations among aging, antioxidant status, and cataract. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1439S-47S.

[18] Cutler RG. Oxidative stress profiling: part I. Its potential importance in the optimization of human health. Annals of the New York Academy of Sciences 2005;1055:93-135.

[19] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radical Biology & Medicine 2000;28 (12):1815-26.

[20] Allen RG, Tresini M. Oxidative stress and gene regulation. Free Radical Biology and Medicine 2000;28 (3):463-99.

[21] Sabliov CM, Astete CE. Encapsulation and Controlled Release of Antioxidants and Vitamins. In: Garti N, editor. Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals. New York: CRC Press, 2008. pp. 297-330.

[22] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. Faseb Journal 1999;13 (10):1145-55.

[23] Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine 2007;43 (1):4-15.

[24] Halliwell B. Antioxidants in human health and disease. Annual Review of Nutrition 1996;16:33-50.

[25] Pryor WA. Vitamin E and heart disease: Basic science to clinical intervention trials. Free Radical Biology and Medicine 2000;28 (1):141-64.

[26] Morel DW, Dicorleto PE, Chisolm GM. Endothelial and Smooth-Muscle Cells Alter Low-Density Lipoprotein Invitro by Free-Radical Oxidation. Arteriosclerosis 1984;4 (4):357-64.

[27] Steinberg D, Berliner JA, Burton GW, Carew TE, Chait A, Chisolm GM, Esterbauer H, Fogelman AM, Fox PL, Furberg CD, Gaziano JM, Gey KF, Grundy SM, Harlan WR, Havel RJ, Hennekens CH, Hoff HF, Jackson RL, Kayden HJ, Keech A, Krinsky NI, Manson J, Parthasarathy S, Probstfield J, Pryor WA, Rifkind B, Stadtman ER, Wallace RB, Witztum JL, Ylaherttuala S, Yusuf S. Antioxidants in the Prevention of Human Atherosclerosis - Summary of the Proceedings of a National-Heart-Lung-and-Blood-Institute Workshop - September 5-6, 1991, Bethesda, Maryland. Circulation 1992;85 (6):2338-44.

[28] Bowry VW, Stocker R. Tocopherol-Mediated Peroxidation - the Prooxidant Effect of Vitamin-E on the Radical-Initiated Oxidation of Human Low-Density-Lipoprotein. Journal of the American Chemical Society 1993;115 (14):6029-44.

[29] Jialal I, Fuller CJ. Oxidized Ldl and Antioxidants. Clinical Cardiology 1993;16 (4):6-9.

[30] Sattler W, Christison J, Stocker R. Cholesterylester Hydroperoxide Reducing Activity Associated with Isolated High-Density and Low-Density Lipoproteins. Free Radical Biology and Medicine 1995;18 (3):421-9.

[31] Steinberg D. Oxidized low density lipoprotein - An extreme example of lipoprotein heterogeneity. Israel Journal of Medical Sciences 1996;32 (6):469-72.

[32] Noguchi N, Niki E. Apolipoprotein-B Protein Oxidation in Low-Density Lipoproteins. Oxygen Radicals in Biological Systems, Pt C 1994;233:490-4.

[33] Fuller CJ, Agil A, Lender D, Jialal I. Superoxide production and LDL oxidation by diabetic neutrophils. Journal of Diabetes and Its Complications 1996;10 (4):206-10.

[34] Mashima R, Yoshimura S, Yamamoto Y. Reduction of lipid hydroperoxides by apolipoprotein B-100. Biochemical and Biophysical Research Communications 1999;259 (1):185-9.

[35] Witting PK, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Eriksson AW, Stocker R. Inhibition by a coantioxidant of aortic lipoprotein lipid peroxidation and atherosclerosis in apolipoprotein E and low density lipoprotein receptor gene double knockout mice. Faseb Journal 1999;13 (6):667-75.

[36] Yang XP, Yan DG, Qiao CP, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC. Increased atherosclerotic lesions in ApoE mice with plasma phospholipid transfer protein overexpression. Arteriosclerosis Thrombosis and Vascular Biology 2003;23 (9):1601-7.

[37] Reddy MK, Wu L, Kou W, Ghorpade A, Labhasetwar V. Superoxide dismutase-loaded PLGA nanoparticles protect cultured human neurons under oxidative stress. Applied Biochemistry And Biotechnology 2008;151 (2-3):565-77.

[38] Schaffazick SR, Siqueira IR, Badejo AS, Jornada DS, Pohlmann AR, Netto CA, Guterres SS. Incorporation in polymeric nanocapsules improves the antioxidant effect of melatonin against lipid peroxidation in mice brain and liver. European Journal Of Pharmaceutics And Biopharmaceutics: Official Journal Of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E V 2008;69 (1):64-71.

[39] Simone EA, Dziubla TD, Arguiri E, Vardon V, Shuvaev VV, Christofidou-Solomidou M, Muzykantov VR. Loading PEG-catalase into filamentous and spherical polymer nanocarriers. Pharmaceutical Research 2009;26 (1):250-60.

CHAPTER 2. ENCAPSULATION AND CONTROLLED RELEASE OF ANTIOXIDANTS AND VITAMINS VIA POLYMERIC NANOPARTICLES[1]

2.1 Introduction

Antioxidants and vitamins are fundamental for human health. Natural antioxidants like vitamin E (α-tocopherol), vitamin C, flavonoids (i.e. quercitin, catequin, among others), are compounds which could potentially modulate oxidative stress. Many of the principal diseases affecting human health are related with the oxidative process, among which cardiovascular diseases, atherosclerosis, and cancer are the most prevalent. Antioxidants can prevent these diseases by reducing the reactive oxygen species (ROS) and thus limiting cell damage by ROS.

Natural antioxidants are prone to degradation and their bioavailability is limited by low absorption and degradation during delivery. The encapsulation of antioxidants and vitamins in polymeric nanoparticles is a promising way of improving the bioavailability of these components. Nanoparticles can be designed to deliver the antioxidant/vitamin to the specific site of action with minimal degradation of the bioactive component during delivery. The antioxidant/vitamin is then potentially released in a controlled manner by controlling the degradation profile of the polymer, or by using "smart" polymers which interact with the cell environment (temperature, pH, enzymes, ionic strength, and other) in a predictable way.

In this chapter, the importance of antioxidants to human health will be addressed, the advantages of nanoencapsulation of these components over more traditional delivery methods will be discussed, the top-down techniques available to entrap antioxidants and vitamins in biodegradable and biocompatible polymeric nanoparticles will be discussed, the methods available for polymeric nanoparticles characterization will be briefly mentioned, and some insights on the release profile of antioxidants and vitamins from polymeric nanoparticles will be presented. The chapter will close with conclusions and future research directions.

---

[1] Reprinted with permission from Woodhead Publishing Limited. Delivery and controlled release of bioactives in foods and nutraceuticals, edited by N. Garti, ISBN 1 84569 145 8, ISBN-13: 978 1 84569 145 5, Woodhead Publishing limited, UK.

2.2 Antioxidants and Vitamins in Protecting Human Health

2.2.1 Reactive Oxygen Species and Oxidation Process

Halliwell & Gutteridge [1] defined antioxidants as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate". Enzymatic and nonenzymatic antioxidants prevent cellular damage surged from chemical reactions that involve reactive oxygen species (ROS) [2-4]. The reactive oxygen species play beneficial roles in the cell metabolism influencing cell growth, energy production, and intracellular signaling, but ROS can be damaging to different tissues due to their involvement in lipid peroxidation, DNA modification, carbohydrates oxidation, and/or protein alteration. Damage caused by ROS has been related to cancer, diabetes, cardiovascular disease, inflammatory responses, degenerative diseases, aging, liver injury, cataract, and others [5-25].

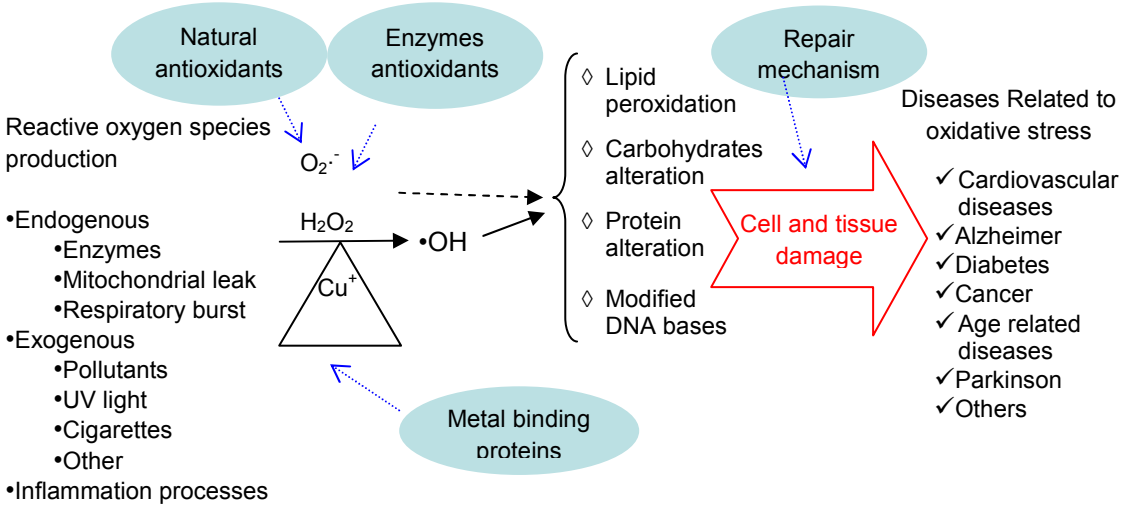


Figure 2-1: Schematic representation of oxidative damage and defense systems. The oval shapes represent the defense system against reactive oxygen species. Adapted from Young and Woodside (2001) [4].

Intracellular ROS are mainly generated by autooxidation of small molecules and from the activity of certain enzymes like oxidases, peroxidases, lipoxygenases, dehydrogenases, and cyclooxygenases; they can also be generated by exogenous sources (i.e. pollutants, cigarettes). The cellular sites of reactive oxygen species generation are mitochondria, peroxisomes, lysosomes, endoplasmic reticular membrane, nuclear membrane, and plasma membrane [26-29].

The state of a biological system exposed to excessive formation of ROS and limited antioxidant defense is defined as oxidative stress [30-32]. The human body has developed an internal defense against oxidative stress (Figure 2-1). Metal binding proteins control the concentration of free metal ions and reduce the potential risk of antioxidant molecules to act as pro-oxidants. Enzymatic and natural antioxidants act mainly as suppressors of radicals, protecting the cellular environment from oxidative damage by ROS which could otherwise result in oxidative stress related diseases. Enzymatic antioxidants and proteins are mainly hydrophilic and therefore act in a hydrophilic environment. The natural antioxidants (acquired from food) act in a hydrophilic or hydrophobic environment, based on their chemical makeup, which complement the enzymatic defensive system and enhance the protection of intracellular hydrophobic molecules. Lastly, if oxidative stress occurs, the repair system, consisting of different enzymes (i.e. lipases, DNA repair enzymes, proteases, and others) can repair the damage originated from the oxidation process [27].

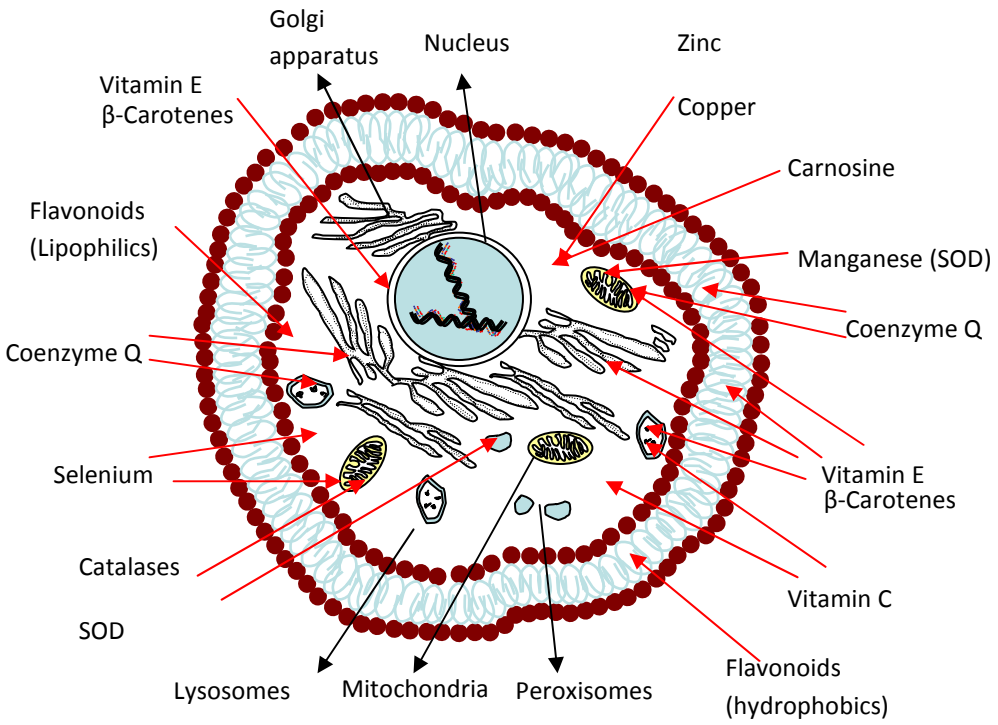


Figure 2-2: Localization of different antioxidant molecules in the cell. The distribution of natural antioxidants in the cell components is dictated by their chemical tendencies to be soluble in a hydrophilic (water) or a hydrophobic medium. Adapted from Machlin and Bendich (1987) [26].

A balanced food intake which includes vegetables, legumes, fruits and nuts has been suggested to ensure optimal tissue levels of antioxidants [33] necessary to maintain a balance between ROS formation and antioxidant defense. The distribution of natural antioxidant molecules in the cell is depicted in Figure 2-2. The different sites of action of natural antioxidants suggest that it is important to maintain adequate concentrations of both hydrophobic and hydrophilic antioxidant molecules to reduce the risk of oxidative stress in all cell compartments.

The understanding of the antioxidant characteristics is crucial for designing an adequate delivery and targeting system for the specific antioxidants of interest. In the next section, several chemical components of demonstrated antioxidant activity are described with emphasis on how nanoencapsulation can improve their delivery and function.

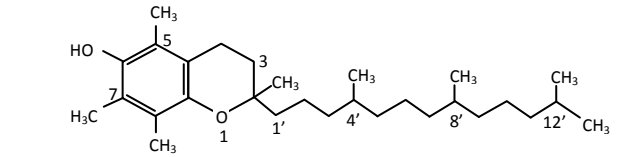
2.2.2 Natural Antioxidants in Biological Systems: Characteristics and Human Health

Natural antioxidants and vitamins can be classified based on their solubility in two classes, hydrophobic (water insoluble) and hydrophilic (water soluble). Examples of hydrophilic antioxidants are vitamin C and carnosine, whereas vitamin E, coenzyme Q_{10}, and carotenoids are examples of hydrophobic antioxidants.

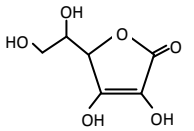
Vitamin E is a hydrophobic vitamin which has been intensively studied due to its antioxidant properties and non-antioxidant actions [34-38]. The vitamin E family is composed of four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ) (Figure 2-3). Sources of vitamin E are wheat-germ oil, sunflower seed, almond, cereals, and others. The daily recommended intake of α-tocopherol is 22.7 mg for adults and 18.7 mg for lactating women [39].

Vitamin E is absorbed in the intestine [40] to form chylomicron particles. Circulation of chylomicron particles (a structure formed with fatty acids, phospholipids, cholesterol, vitamin E and other hydrophobic molecules) in plasma is one mechanism for vitamin E transfer to muscles, adipose tissue and brain. Once delivered to the tissue, tocopherols are localized in the cellular membranes with the chromanol nucleus located in the phospholipid bilayer [41]. The role of vitamin E supplementation in prevention of several diseases such as cardiovascular diseases, atherosclerosis, cancer, arthritis, Alzheimer's, and Parkinson's, among others has been documented and linked to its ability to trap peroxyl radicals and to break the chain reaction of lipid peroxidation [34,42-49]. Targeted delivery of vitamin E to the cytoplsamatic compartments

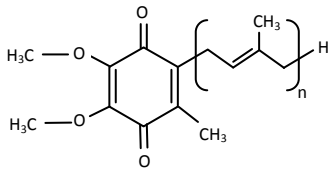
achieved with polymeric nanoparticles may increase its efficiency as an antioxidant and may aid in prevention and treatment of oxidative stress related diseases.



α-Tocopherol

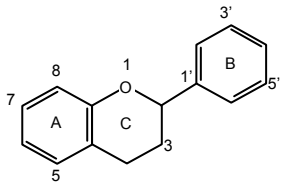


Dehydroascorbic acid

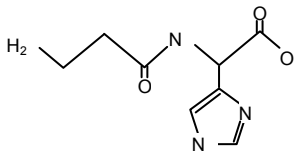


Coenzyme Q

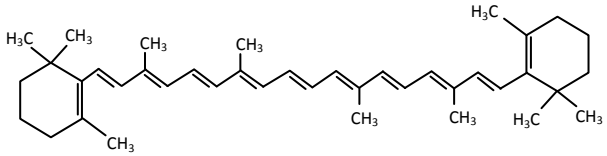
(Ubiquinone)



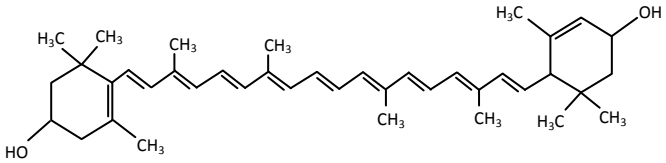
General flavonoid structure



Carnosine



β-carotene



Lutein

Figure 2-3: Chemical structure of selected natural antioxidants and vitamins.

Coenzyme Q_{10} is a hydrophobic molecule which is synthesized by humans (Figure 2-3). It can act as an electron carrier and proton translocator. Coenzyme Q_{10} is also found in plants, animals, and in most microorganisms. The amount of Coenzyme Q_{10} that can be provided by the diet is variable; for examples the coenzyme Q_{10} content of pork heart is 203 μg/g, 41 μg/g is

found in beef heart, 17 μg/g in chicken leg, 27 μg/g in herring fish, 2.2 μg/g in spinach, and 2.2 μg/g in oranges [50]. Coenzyme Q_{10} plays fundamental roles in energy conversion, regeneration of other antioxidant molecules, and cell death inhibition and cell growth regulation [50-53]. The intestinal absorption of coenzyme Q_{10} seems to be similar to the vitamin E absorption mainly due to its lipophilic characteristics [54]. The absorption is enhanced in the presence of lipids. In the small intestine, coenzyme Q_{10} is assimilated into chylomicrons to reach the plasma via the lymphatic system. The plasma concentration of coenzyme Q_{10} in healthy people can range from 0.4 to 0.6 (μmol/l) which is increased with supplementation [54-56]. The toxicity of coenzyme Q_{10} is extremely low. Supplementation of coenzyme Q_{10} up to 1200 mg/day has been used in trials without any adverse effects [54].

It should be noted that the plasma level of coenzyme Q_{10} decreases with aging [57] and supplementation or stimulation of Q_{10} biosynthesis are needed to increase the cellular levels of coenzyme Q_{10}. Supplements of Q_{10} have been used for their potential benefits against cardiovascular and neurodegenerative diseases, cancer, aging, migraine, and others [58-67]. Supplements of coenzyme Q_{10} administered orally have been shown however to have a low absorption efficiency (around 3-5%), mainly due to the hydrophobic characteristics of the bioactive component. Oil, emulsion, and powder formulations have been used in an attempt to increase the coenzyme Q_{10} absorption, with the solubilized forms being most effective [54,68,69]. Tikhaze et al. [70] found that the solubilized form of ubiquinone increased the antioxidant activity of this component by more than 36 times as opposed to its insoluble form. Nanoparticle formulation of coenzyme Q_{10} may show improved bioavailability characteristics yet as compared to the emulsion formulations.

Carotenoids are a class of antioxidants composed of more than 500 identified components (Figure 2-3). Carotenoids are abundant in vegetable pigments and are found mainly in carrots, broccoli, spinach, apricots, tomatoes, peas, and orange peppers among others [71]. The carotenoids level in the US population ranges between 0.05 to 0.5μg/ml [72]. The characteristic of carotenoids is the unsaturated 40 carbons skeleton which confers the hydrophobicity specific to this compound. The most abundant carotenoids found in humans are α- and β-carotene, lycopene, zeaxanthin, cryptoxanthin and lutein [73]. Absorption of carotenoids is passive by intestinal absorption and it is similar to the absorption of tocopherol and coenzyme Q_{10}. The

carotenoids plasma concentration should be around 0.4-0.5 μM for good health, as suggested by Albertini et al. [24].

The lipophilicity and structure of carotenoids affect their distribution in the cellular systems, *in vivo*. These components are poor water soluble and are usually associated with other components for improved absorption (i.e. lipoproteins). The lipophilic cell components are the main locations where carotenoids are found (i.e. plasma membranes, mitochondria membranes, Golgi membranes). Improved distribution of carotenoids in the cytoplasm or other cellular hydrophilic compartments can be achieved by nanoencapsulation and delivery with polymeric nanoparticles dispersable in aqueous mediums.

Polyphenols (i.e. flavonoids) are natural compounds found in many vegetables, fruits, nuts, cereals, and herbs [74-76]. Polyphenols is a family composed of more than 4000 identified molecules with the general structure depicted in Figure 2-3. The daily intake of polyphenols can reach 800 mg/day [77], with a mean intake of 23 mg/day in western countries [78]. In a normal diet, the plasma concentration of flavonoids is less than 1 μM [79]. The importance of flavonoids is represented by three characteristics, inhibitions of certain enzymes, potent antioxidant properties, and iron chelator ability [78,80-83].

The potential health benefits of flavonoids are related to anti-inflammatory, anti-hepatotoxic, antiviral, antimicrobial, anti-allergic and anti-tumor activities, and to vascular protection, regulation and control of diabetic cataracts, among others [75,84-88]. For those individuals less inclined to consume fruits and vegetables, sources of polyphenols, supplementation of the diet with nanoencapsulated flavonoids may be the lead to a healthier life.

Ascorbic acid is a water soluble vitamin fundamental in metabolic reactions, and as a cofactor of various enzymes involved in the hydroxylation process; it cannot be synthesized by humans **(**Figure 2-3). Different natural sources provide the necessary amounts of ascorbic acid, such as fruits (i.e. orange, papaya, strawberries) and vegetables (i.e. broccoli, red or green pepper, brussels) [32,89,90]. Albertini et al. [24] suggested that the optimal concentration of plasma vitamin C should be at least 40-50 μM for antioxidant purposes.

Vitamin C has been widely reported as a potent co-antioxidant with vitamin E, due to the synergism between the two antioxidants [91]. The process involves α-tocopherol regeneration from α-tocopheryl radicals in lipoproteins and membranes in the presence of vitamin C. A synergetic action has also been suggested between vitamin C, vitamin E, and β-carotene against

the oxidative damage generated by peroxynitrite anion and nitrogen dioxide [92]. Efficient co-delivery of synergistic antioxidants and vitamins can be achieved by entrapping and delivering these components in a controlled manner with polymeric nanoparticles.

Carnosine is a hydrophilic dipeptide (β-alanyl-L-histidine) which is found exclusively in animal tissue and in the brain (Figure 2-3). This histidine containing dipeptide is part of the ω-aminoacylamino acids family. Other compounds are anserine, homocarnosine, and homoanserine [93]. It has been shown by different studies that carnosine acts as an anti-oxidant, carbonyl scavenger (anti-glycating agent), and metal ion chelator [94-97]. Due to its hydrophilic nature, carnosine protects proteins from oxidation [96,98-102]. It has also been pointed out that carnosine could be beneficial for people with diabetes, especially to control the secondary effects of this disease [101,103]. Hipkiss [104] found that carnosine has a potential therapeutic effect on neurodegenerative diseases related with high levels of zinc such as Alzheimer's disease. Protection of carnosine against degradation during delivery to the site of action by entrapment in a polymeric matrix is a viable method to increase its bioavailability and efficiency in vivo.

To conclude this section, there is a wide variety of natural antioxidants and vitamins which have been shown to possess beneficial properties and could potentially improve human health. The antioxidant efficiency of these components *in vivo* is related not only to their chemical reactivity, but also to their localization and mobility. Bioavailability, synergetic inhibition of the oxidation process, and pro-oxidant effects of natural antioxidants are other important factors to consider in the evaluation of potential antioxidant activity of different compounds *in vivo* [105]. Scientific proof of the advantages offered by encapsulation and delivery of antioxidants/vitamins with polymeric nanoparticles are presented below.

2.3 Advantages of Encapsulation over Traditional Delivery Methods

Nanoparticles can offer several advantages over traditional delivery methods for antioxidants and vitamins, which include protection of the bioactive component, an increase in its bioavailability, delivery of the component to the site of action in a controlled manner, as well as the possibility to integrate the bioactive component in various foods for enhanced food quality (Figure 2-4).

2.3.1 Protection and Bioavailability

Bioavailability of antioxidants and vitamins can be enhancecd by delivering the bioactive components via polymeric nanoparticled due to several factors, the antioxidant is protected against harsh conditions (i.e. low stomach pH, enzymatic and microflora intestinal degradation), the extra-cellular interaction between the components of interest and other molecules is minimized, and low absorption of some antioxidants and vitamins (mainly hydrophobic) is improved.

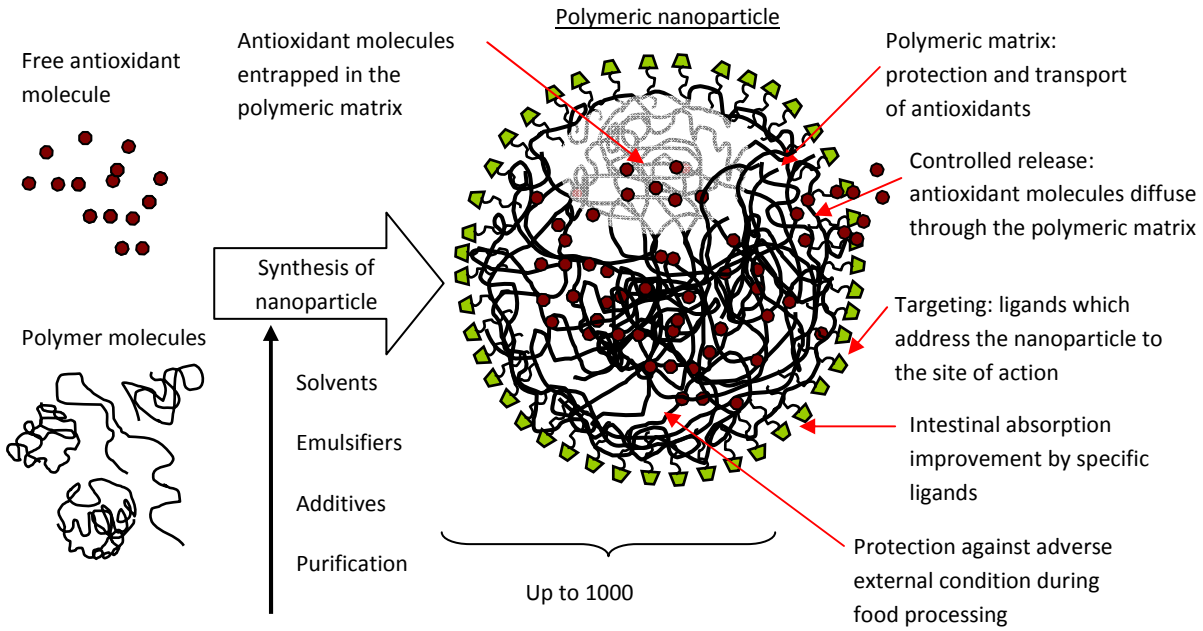


Figure 2-4: Description of the nanoparticle antioxidant system with emphasis on the advantages offered by nanoparticle delivery systems.

Several studies available in the literature are focused on protection of the bioactive component against natural degradation by nanoencapsulation. For example, the stability against UV and thermal degradation of nanoencapsulated Q_{10} was studied by Kwon et al. [106]. The polymer used to entrap Q_{10} was poly(methyl methacrylate) (PMMA), and the nanoparticles were prepared with different surfactants (i.e. poly(vinyl alcohol), sodium dodecyl sulfate). The entrapped coenzyme Q_{10} proved to be structurally more stable against UV and high temperature-induced inactivation as compared with an oil-based Q10 formulation.

Chen and Subirade [107] tested biodegradable nanoparticles made from chitosan and β-lactoglobulin designed for oral delivery of nutraceuticals. The protein, β-lactoglobulin protected the active component against pepsin and acid degradation in the gastric environment. The study suggested that these nanoparticles could protect the nutraceuticals entrapped against degradation by adverse conditions during food processing, or in the gastro-intestinal tract, and the absorption of these components could therefore be improved.

An improvement in the intestinal absorption of antioxidants is a means to improve their bioavailability. The attachment of ligands like lectins, invasins, folic acid, vitamin B12, biotin, glycoproteins (i.e. transferrin), membrane transduction sequences [108] was proved to improve intestinal uptake of nanoparticles with entrapped poor absorbable antioxidant molecules.

2.3.2 Targeting and Cellular Uptake

Polymeric nanoparticles can be surface functionalized to improve half life circulation and to minimize opsonins recognition of the nanoparticles (i.e. by addition of PEG), to improve the intestinal absorption (i.e. by using of a cationic polymer such as chitosan), to improve cellular uptake (i.e. by addition of a membrane disruption agent or agents which promote endosomal release), or to ensure that the antioxidant/vitamin reaches intra-cellular targets such as mitochondria (i.e. by attachment of lipophilic triphenylphosphonium).

Nanoparticle functionalization with vitamins is an interesting means to improve the cellular uptake of the nanoparticles. Vitamin H (biotin) was covalently attached to pullulan acetate, a hydrophobized polysaccharide by Na et al. [109] to form nanoparticles with a mean size of 99±52 nm (20 vitamin H groups per 100 anhydroglucose units of pullulan acetate), with entrapped adriamycin as a drug model. The release profile of adriamycin loaded nanoparticle was controlled by vitamin H content attached to pullulan; a specific interaction of the functionalized particle with hepatoma cell line (HepG2) by ligand-receptor interaction was proved and showed responsible of increased nanoparticle uptake.

The antioxidant action can be enhanced by targeting, when the antioxidant molecule is addressed to the specific site into the cell, such as mitochondria, nucleus, or endoplasmic reticulum, where oxidative stress occurs and causes damage associated to several diseases like Parkinson's disease, diabetes, ischemia reperfusion, and others. A significant decrease in heart dysfunction, cell death, and mitochondrial damage was shown after ischemia- reperfusion with oral administration of a target functionalized system (ubiquinol plus lipophilic

triphenylphosphonium) in rats. In comparison, ubiquinol delivered without targeted functionalization was ineffective for cardiac ischemia-reperfusion injury [13].

Muro et al. [110] entrapped an antioxidant molecule (catalase) in a functionalized nanoparticle, called anti-ICAM1. This system targeted endothelial cells and entered the cell via an amiloride-sensitive endocytic pathway. The anti-ICAM nanoparticle system was effective in offering protection to endothelial cells against oxidation for over 2 h after cellular uptake. It was suggested that due to the targeting ability of the nanoparticulate system, the oxidants were intercepted near their sites of generation or at sites where sensitive damage occurred and therefore a better protection against oxidative damage was possible.

2.3.3 Controlled Release

The polymeric matrix degradation as a result of interaction with different environmental parameters (temperature, enzymes, pH, ionic strength, and other) allows control of the amount of antioxidant released over time. Several studies published in the literature focus on the release of vitamin E, a model component for hydrophobic vitamins and antioxidants.

Shea et al. [111,112] entrapped vitamin E into a PEG-decanyl polymeric nanoparticle. The antioxidant property of the released alpha-tocopherol was tested in human neuroblastoma cells (SH-SY-5Y). The vitamin E added in the preparation was 20 mg, equivalent to 20% w/w (there is no information about the entrapment efficiency and size of the nanoparticle synthesized). The results showed that encapsulated vitamin E was more effective against ROS as compared to free vitamin E. The free vitamin E was effective only if administered before or during treatment with amyloid-β (a neurotoxic peptide which accumulates in Alzheimer's disease), but was not effective in protection against oxidation if administered 1 hr after the addition of amyloid-β to the cell culture. The encapsulated form of vitamin E was effective even when administered 1 hr after the amyloid-β treatment against ROS.

Duclairoir et al. [112] used wheat gliadin nanoparticles for vitamin E encapsulation by nanoprecipitation method. The mean size of the nanoparticle obtained was 900 nm. The entrapment efficiency ranged from 77% to 95%; the entrapment efficiency of vitamin E decreased as more vitamin E was added relative to the gliadin protein weight. The release profile showed an initial fast release (burst effect) and a further slower release. The release studies were perfomed for 100 h at 25 °C under nitrogen and in dark conditions.

Zigoneanu et al. [113] entrapped vitamin E in PLGA nanoparticles at two initial loadings of 8 and 16%. The nanoparticles synthesized by emulsion evaporation method measured less than 100 nm for nanoparticles prepared with SDS as a surfactant (Figure 2-5), and about 200 nm for nanoparticles prepared with PVA. The entrapment efficiency of α-tocopherol in the polymeric matrix was approximately 89% and 95% for nanoparticles with 8% and 16% α-tocopherol theoretical loading, respectively. The release profile showed an initial burst followed by a slower release of the α-tocopherol entrapped inside the PLGA matrix, similar to the findings of Shea et al. and Duclairoir et al. [111,112].

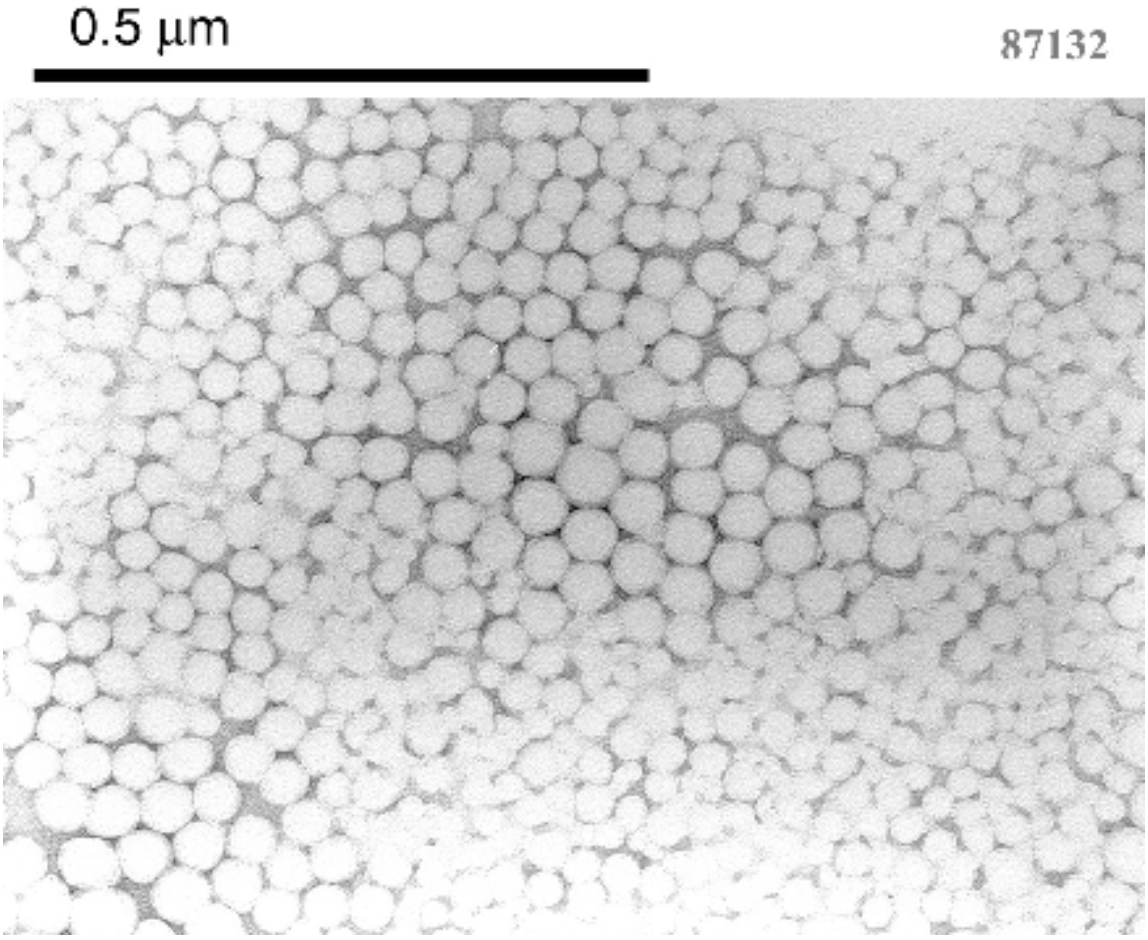


Figure 2-5: PLGA-SDS nanoparticles with entrapped vitamin E (16% w/w). The mean nanoparticle size as measured by DLS is 55 ± 3 nm.

2.3.4 Integration and Safety

The nanoencapsulated antioxidants and vitamins can be integrated into foods and beverages to enhance the quality (sensorial, chemical, and microbiological properties) of the food. Chen and Wagner [114] entrapped vitamin E in starch sodium octenyl succinate

nanoparticles designed for beverage applications. The mean size obtained was 112 nm which was dependent on the number of homogenization cycles used in the preparation. The vitamin E nanoparticles were more stable in terms of turbidity and aggregation over time as compared to the non-nanoparticle systems. The suspension with nanoparticles did not change in turbidity over 6 months and no perceptible creaming and/or ringing were observed over the same period of time. The stability of the nanoparticle suspension in apple juice also presented low turbidity without change over time (6 months). The vitamin E used was vitamin E acetate, and the stability studies were performed after pasteurization at 75 °C for 10 min. There is no information about the entrapment efficiency and release profiles of the vitamin.

It should be noted that prior to integration of nanoparticles in food systems it is necessary to develop a thorough understanding of the nanoparticle physico-chemical characteristics, and very importantly of their safety for human consumption. The safety of the nanoparticles is dictated by their components (polymer, surfactant, antioxidant, additives), by the properties of the nanoparticle (size, size distribution, surface characteristics), and by the nanoparticle bio-interaction (bioavailability, cellular uptake, targeting). Limited information is available in the literature regarding the safety of nanoparticles to human health, whether for pharmaceutical or food uses.

Several steps can be taken to insure nanoparticle safety for human consumption. Biocompatible, biodegradable components should be selected for nanoparticle synthesis. Synthetic polymers and natural macromolecules (i.e. albumin, chitosan) have been extensively researched as colloidal materials for nanoparticle production designed for drug delivery. Synthetic polymers have the advantage of high purity and reproducibility over natural polymers. Among the synthetic polymers, the polyesters family (i.e. poly(lactic acid) (PLA), poly(e-caprolactone) (PCL), poly(glycolic acid) (PGA)) are of interest in the biomedical area because of their biocompatibility and biodegradability properties. In particular, poly(lactide-co-glycolide) (PLGA) has been FDA approved for human therapy [115]. Food biopolymers (i.e. albumen, chitosan, dextran, alginate, etc) on the other hand are the alternative option when considering the safety of the nanoparticles for human food consumption.

The size and size distribution of the polymeric nanospheres used for delivery of antioxidants and vitamins, among other physical characteristics, are affected by the technique used for the nanoparticle production and the pertinent synthesis parameters, i.e. polymer used,

polymer molecular weight and concentration, the addition of active components, surfactants, solvents, and other additives, shear stress, evaporation rate, and other physical parameters [115-123]. These characteristics, such as size, size distribution and surface characteristics affect the behavior of the nanoparticles and their interaction with a biological system and ultimately their safety. It is important to understand how the physical characteristics relate to the efficiency of the delivery of the active components in question at the site of action. Entrapment and delivery of antioxidants with polymeric nanoparticles may be very efficient as compared to the delivery of the free component (i.e. non-nanoencapsulated), in which case the safety of the antioxidant delivered in high amounts becomes an issue which must be addressed.

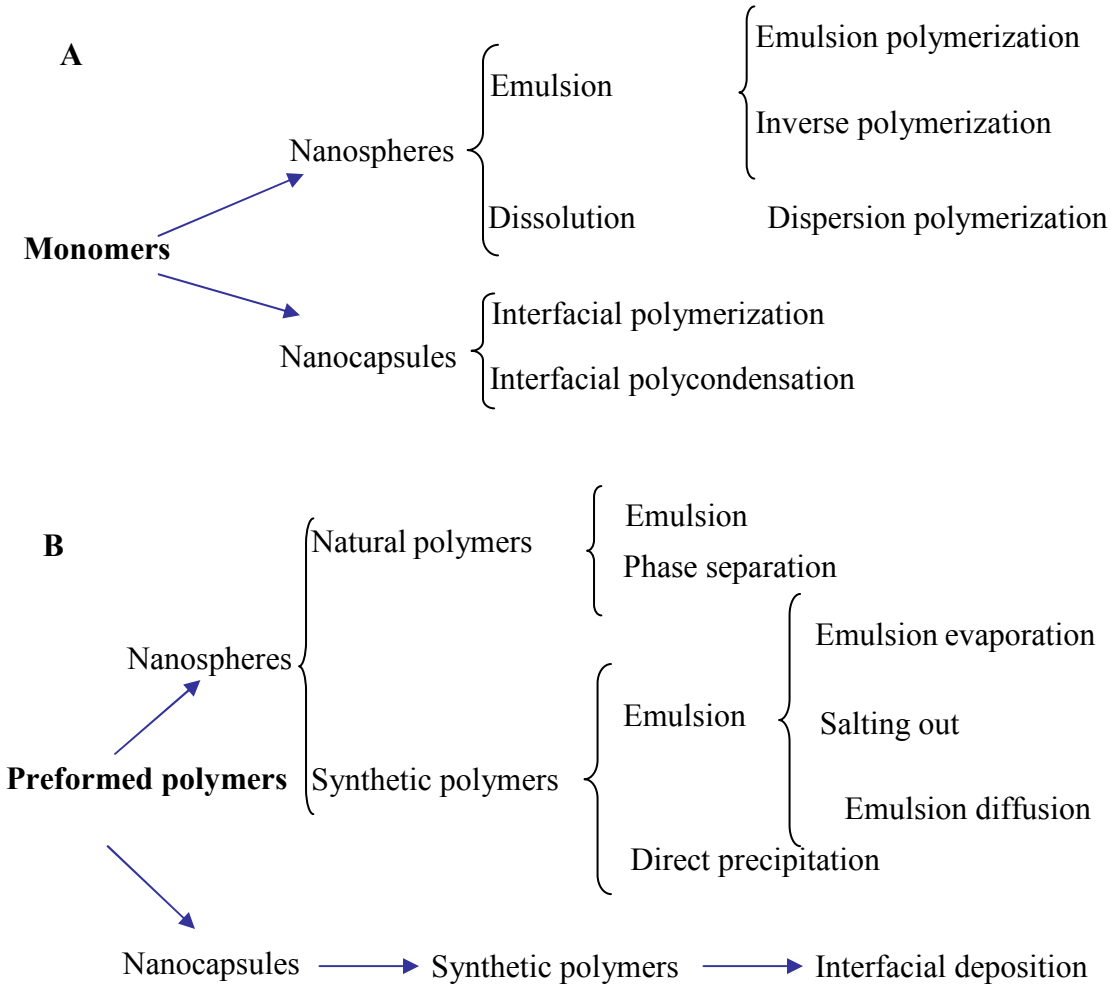


Figure 2-6: Polymeric nanoparticle synthesis techniques for encapsulation of antioxidants. A. bottom-up techniques, B. top-down techniques.

Synthesis of polymeric nanoparticles and the parameters that affect the nanoparticle physical characteristics have been intensively investigated during the last decade [121,124-131]. Methods available for nanoparticle synthesis can be divided into two classes: bottom-up and top-down techniques. The bottom-up techniques such as emulsion or microemulsion polymerization, interfacial polymerization, and precipitation polymerization, employ a monomer as a starting point (Figure 2-6). Polymerization methods require the addition of the antioxidant during polymerization, and cross-linking of the antioxidant with the polymer synthesized during the nanoparticle formation is possible. The cross-link modifies the antioxidant molecules with further loss of its antioxidant activity. The top-down techniques minimize the damage to the active components entrapped (vitamin E, flavonoids, carotenoids, and others) as compared to the polymerization methods. Some details on the top-down techniques are presented below.

2.4 Top-down Techniques Used for Encapsulation of Antioxidants and Vitamins in Polymeric Nanoparticles

Emulsion evaporation, emulsion diffusion, solvent displacement, and salting out are top-down techniques in which the nanoparticles are synthesized from pre-formed polymers. The characteristics of these methods are presented in Table 2-1.

2.4.1 Emulsion Diffusion Method

In this synthetic scheme, the polymer is dissolved in a suitable organic phase (e.g., benzyl alcohol, propylene carbonate, ethyl acetate), which must be partially miscible in water. The organic phase is then emulsified with an aqueous solution of a suitable surfactant (i.e. anionic sodium dodecyl sulfate (SDS), non-ionic polyvinyl alcohol (PVA), or cationic didodecyl dimethyl ammonium bromide (DMAB), under stirring. The diffusion of the organic solvent and the counter diffusion of water into the emulsion droplets induce polymer nanoparticle formation [132]. Important parameters that affect the nanoparticle size synthesized by emulsion evaporation are: copolymer ratio, polymer concentration, solvent nature, surfactant polymer molecular weight, viscosity, phase ratios, stirring rate, solvent nature, temperature and flow of water added [133-138].

2.4.2 Salting Out Method

In this method, the polymer is dissolved in the organic phase, which should be water-miscible, like acetone or tetrahydrofuran, ethanol. The organic phase is emulsified in an aqueous

phase, under strong mechanical shear stress. The aqueous phase contains the emulsifier and a high concentration of salts which are not soluble in the organic phase. Contrary to the emulsion diffusion method, there is no diffusion of the solvent due to the presence of salts. The fast addition of pure water to the o/w emulsion, under mild stirring, reduces the ionic strength and leads to the migration of the water-soluble organic solvent to the aqueous phase inducing nanosphere formation [120]. The final step is purification by cross flow filtration or centrifugation required to remove the salting out agent. Common salting out agents are electrolytes (sodium chloride, magnesium acetate, or magnesium chloride) or non-electrolytes, such as sucrose [129,139-141]. Important parameters to be considered to control the physical characteristics of the synthesized nanoparticles by this method are: polymer concentration and molecular weight, stirring rate and time, nature and concentration of surfactant and solvent, and cryoprotectans.

Table 2-1: Characteristics of top-down techniques used for antioxidants entrapment in polymeric nanoparticles

Characteristics	**Methods**			
	Emulsion diffusion	**Salting out**	**Nanoprecipitation**	**Emulsion evaporation**
Solvents availability	Low toxicity (benzyl alcohol)	Low toxicity	Low toxicity (acetone)	Low toxicity (ethyl acetate)
Energy consumption	Low	Low	Low	Medium
Water requirements	High	Medium	High	Low
Polymer concentration	Linear relation with Np size	Medium-high	Low to not affect Np size	Medium-high without increase Np size
Hydrophobic antioxidants	High entrapment	High entrapment	High entrapment	High entrapment
Hydrophilic antioxidants	Low entrapment	Low entrapment	Medium entrapment	Medium entrapment (by double emulsion)
Additives for size control	Recommended	Recommended	Recommended	Recommended
Process time	Medium-high	Medium	High	Reduced
Purification	Medium	High	Medium	Medium
Observations	Solvent limitation	Presence of salts	Polar solvents	Single emulsion or double emulsion

2.4.3 Nanoprecipitation (Solvent Diffusion, or Solvent Displacement) Method

This method can be mainly used for hydrophobic antioxidant entrapment. Polymer and drug are dissolved in a polar, water-miscible solvent such as acetone, acetonitrile, ethanol, or methanol. The solution is then poured in a controlled manner (i.e. drop-wise addition) into an aqueous solution with surfactant. Nanoparticles are formed instantaneously by rapid solvent diffusion. Finally, the solvent is removed under reduced pressure by evaporation. Important parameters to be considered in controlling the nanoparticle size, among other physical characteristics are: polymer/surfactant ratio, polymer concentration, surfactant nature and concentration, solvent nature, viscosity, additives, active component, and phase injection rate [142-151].

2.4.4 Emulsion Evaporation Method

Emulsion evaporation is the oldest method used to form polymeric nanoparticles from preformed polymers. The method is based on the emulsification of an organic solution of the polymer in an aqueous phase followed by the evaporation of the organic solvent. Normal emulsions of oil in water (o/w) or water in oil (w/o) and double emulsions (w/o/w) can be used to accommodate the entrapment of active components with different properties. The o/w emulsion can be used for entrapment of hydrophobic compounds, whereas w/o/w double emulsion is used for the entrapment of hydrophilic compounds. The method is widely used for microencapsulation because it is easy to scale up, it does not require high shear stress, and it can be adjusted (by use of the double emulsion method) to encapsulate water soluble drugs [152-160]. More details of single and double emulsion evaporation methods are presented below.

2.4.4.1 Single Emulsion Method (o/w)

The method is based on the emulsification of an organic solution which contains the polymer and the active component in an aqueous phase, followed by the evaporation of the organic solvent. Different surfactants such as poly(vinyl alcohol), sodium dodecyl sulfate, pluronic F68 can be dissolved in the aqueous phase. The size reduction of the emulsion droplet is accomplished by sonication or microfluidization for miniemulsion formation. The evaporation step is required to eliminate the organic solvent present in the organic phase. This leads to the precipitation of the polymer as nanoparticles with a diameter in the nanometers range. Important parameters which affect the nanoparticle size are: polymer molecular weight and concentration,

copolymer ratio and end groups, surfactant nature, phase ratio, solvent nature, evaporation rate, drug entrapment, additives, shear stress, and sterilization [160-165].

2.4.4.2 Double Emulsion Method (w/o/w)

The first step of the double emulsion method is the formation of a water in oil (w/o) emulsion where the aqueous solution contains the hydrophilic active component and the organic phase contains the polymer and a suitable surfactant (Span 80, pluronic F 68, and others) of a low HLB. The miniemulsion is formed under strong shear stress (i.e. sonication, microfluidization, high speed homogenization). Next, the (water in oil) in water (w/o/w) emulsion is formed and sonicated or homogenized for droplet size reduction. This second size reduction should be controlled to minimize the hydrophilic active component diffusion to the external aqueous phase. Evaporation, the final step, is used to remove the organic solvent. Evaporation is done under vacuum to avoid polymer and active component damage, and to promote final nanoparticle size reduction.

The main drawback of the double emulsion method is the large size of the nanoparticles formed and the leakage of the hydrophilic active component, responsible for low entrapment efficiencies. The coalescence and Ostwald ripening [166-168] are the two important mechanisms that destabilize the double emulsion droplets, and the diffusion through the organic phase of the hydrophilic active component is the main mechanism responsible for low levels of entrapped active component by this method [168]. The leakage effect can be reduced by using a high polymer concentration and a high polymer molecular weight, accompanied by an increase in the viscosity of the inner water phase, and an increase in the surfactant molecular weight [169]. Important parameters which affect nanoparticle size are: polymer/surfactants ratio, polymer concentration, surfactant nature, viscosity, solvent nature, shear stress, evaporation, additives, and first/second phase ratios [161,162,165,170-178].

Following the synthesis, the polymeric nanoparticles specifically designed for antioxidant and vitamins delivery are characterized in terms of morphology, size, size distribution, surface charge, surface characteristics and others.

2.5 Characterization Methods

The methods most broadly used to characterize nanoparticle morphology are transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic transmission

electron microscopy (cryo-TEM) and atomic force microscopy (AFM). Dynamic light scattering (DLS) is the most widely technique used to determine size and size distribution of the polymeric nanoparticles. Laser Doppler Anemometry is used to measure zeta potential, an important parameter when considering the stability of the nanoparticles in vitro. For chemical characterization, Fourier transform infrared spectroscopy (FT-IR) is used to analyze surface modification of the nanoparticles by the attachment of specific components. The quantitative determination of the active component entrapped in the nanoparticles is done by extraction of the drug and measurement of the drug concentration by spectroscopy at defined wavelengths (related to the active component) or by high pressure liquid chromatography (HPLC).

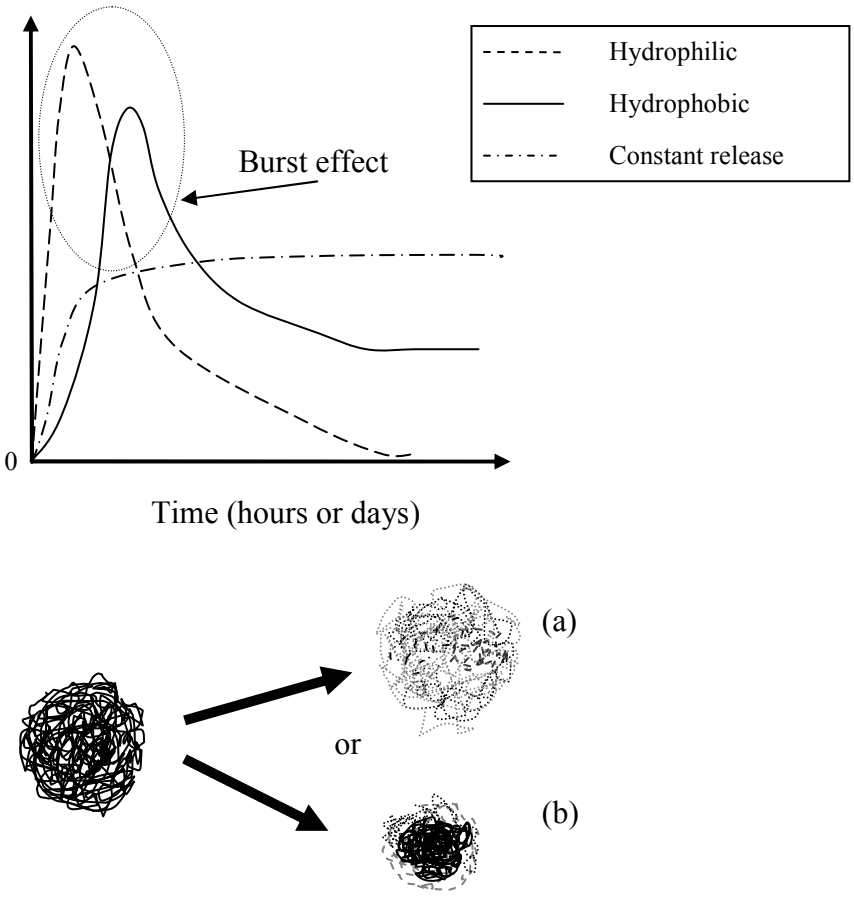


Figure 2-7: Typical release profile. In general, hydrophilic drugs release faster as compared with hydrophobic drugs. The diffusion forces are predominant initially, after which the degradation process is more predominant as the time elapses. The nanoparticle degradation can occur by bulk erosion (a), or surface erosion (b).

2.6 Controlled Release of Antioxidants and Vitamins and Parameters That Affect the Release

The release of the active components from the nanoparticles is critical in understanding the effects of the active components at the site of action. The release profiles of the antioxidant/vitamin will impact the availability, interactions, pharmacokinetics, and the formation of metabolites at the site of action. Different parameters affect the release profile of the antioxidant, which include the chemical structure of the active component, amount of drug entrapped, polymer hydrophobic/hydrophilic behavior, temperature, pH, presence of enzymes, sterilization, polymer structure (i.e. copolymer ratio, crystallinity), polymer molecular weight, and nanoparticle size, among others.

The common mechanisms which explain the drug release from polymeric nanoparticles are the natural degradation of the polymeric matrix (passive release), and diffusion of the drug through the matrix (active release). The diffusion process is dominant at the beginning of the active component release with minimum degradation of the particle. During this process, the active component diffuses from the polymeric matrix to the hydrophilic external environment and water molecules diffuse into the nanoparticle polymeric matrix. The diffusion rate is controlled by the hydrophilicity of the polymeric matrix and that of the active component. Diffusion is followed by a constant release of the drug over time which is controlled by the erosion process of the polymer (degradation of the whole matrix with final mass loss). These mechanisms will result in a typical release profile for active components entrapped in polymeric nanoparticles consisting of an initial fast release (burst effect), followed by a more constant release over time (Figure 2-7). The burst effect can be beneficial when an initial high release improves the action of the active component or detrimental when a constant release of the antioxidant over time is required.

The release studies are usually performed in vitro with buffer solutions incubated at 37°C. The main methods used to quantify the release studies are depicted in Figure 2-8. The main parameters that affect the release profile are summarized below.

2.6.1 Active Component (Type and Amount)

The drug entrapped interacts with the polymer matrix by hydrophobic-hydrophilic interaction, ionic interactions, van der Waals forces, steric effect, and others. The chemical and

physical interactions play a fundamental role in the degradation and diffusion of the entrapped antioxidant [143,172].

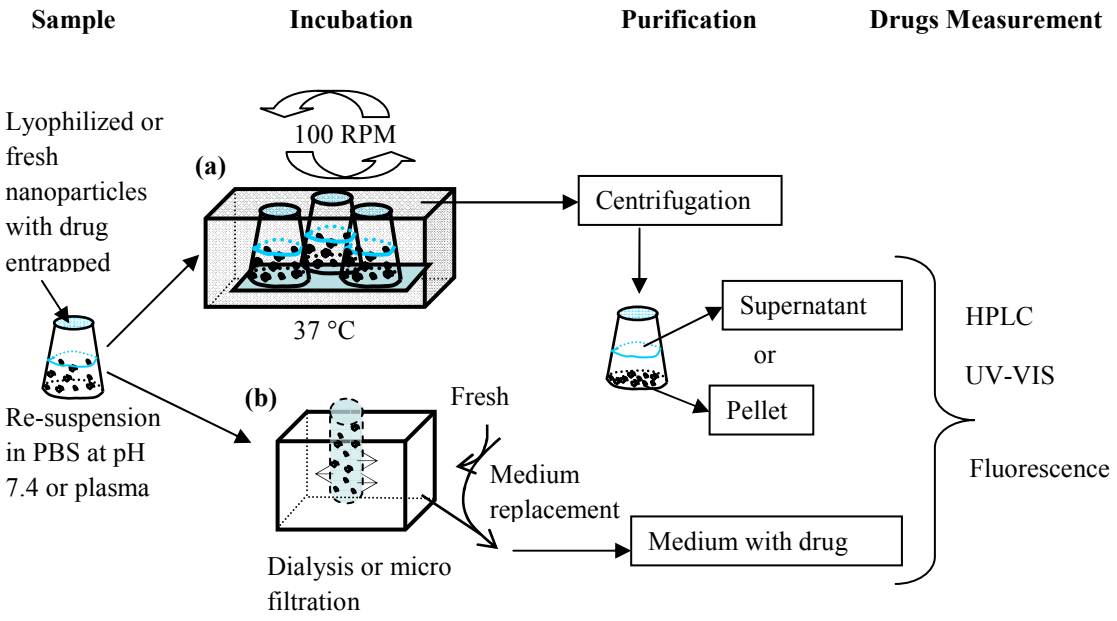


Figure 2-8: Summary of the common procedures used to quantify in vitro drug release profiles. The first method (a) uses centrifugation to separate the drug released from the nanoparticles suspension, the second one (b) uses filtration for separation. The suspension media usually is PBS (phosphate bovine serum). The temperature is maintained at 37°C with gentle agitation. The volume is maintained constant, in the first method, by addition of PBS in the same amount extracted for analysis. The other approach is to divide the sample in as many sub-samples as needed to study the release profile for the desired period of time. The second approach needs to maintain the concentration gradient necessary for the diffusion process to occur.

The amount of entrapped active component (i.e. antioxidant or vitamin) affects the release, in that the higher the initial amount of active component, the faster the release is. The amount of entrapped active component is expressed by two parameters, the entrapment efficiency and the drug content. The drug content is defined as mass of drug in the nanoparticles divided by mass of nanoparticles, usually expressed in % w/w. Normal value for the drug content ranges from 0.5 to 4 %w/w for hydrophilic drugs, and 10 to 15 %w/w for hydrophobic drugs. The entrapment efficiency is calculated by dividing the amount of drug entrapped by the theoretical amount of drug (the initial amount used in the nanoparticles formation). Entrapment efficiency for hydrophobic drugs ranges from 80 to 95%, and for hydrophilic drugs from 30 to 70 %.

2.6.2 Polymer (Type, Copolymer Ratio, MW)

Different polymers have been used for nanoparticles synthesis. Chitosan, dextran, albumin, pullulan, poly(lactic acid) (PLA), poly(lactide-co-glycolide acid), poly(ethyle oxide) (PEO), poly(caprolactone), poly(3-hydroxybutyrate) and others are natural and synthetic polymers used for nanoparticle synthesis. The degradation profiles of these components affect the drug release profile. For example, poly(lactic acid), poly(lactide-co-glycolide acid) (PLGA) and PEO-PLGA nanoparticles appear to degrade homogeneously in time without autocatalysis [140,179,180]. The degradation rate of PEO-PLGA nanoparticles is slightly higher than that of PLGA particles.

The hydrophilicity (hydrophilic-hydrophobic ratio) of the polymer impacts the release profile of the active component entrapped. An example is the PLGA polymer which is a hydrophobic copolymer formed with lactide and glycolide monomers. Changing the copolymer ratio, the PLGA copolymer can be more or less hydrophilic (50:50, 75:25, and 85:15 PLGA molar ratios are the most common copolymer ratios available commercially) which affects the polymer degradation profile. PLGA copolymers formed from D,L-PLA and PGA are amorphous in nature while L-PLA and PGA are crystalline [130,181]. The copolymer 75/25 showed faster drug release as compared with 85/15; after 15 hours 95 wt% was released from 75/25, and only 80 wt% for 85/15 copolymer ratio [178]. Nanoparticles with higher amount of lactide monomers showed a slower release rate. The more hydrophobic the polymer, the more intense hydrophobic interaction between polymer and drug entrapped, and thus a slower release profile [148,182-187].

The molecular weight of the polymer impacts the degradation process which affects the release profile, mainly during the degradation step. Polymer molecular weight ranges from a few thousands Daltons to over one hundred thousands of Daltons. Polymers with higher molecular weight present a slower degradation associated with a prolonged constant drug release over time. Diffusion processes are affected by the polymer molecular weight if the drug is mainly entrapped in the nanoparticle core [115,183,184,188].

2.6.3 Nanoparticle Size

Particle size is dictated by the polymer used, the method used, the drug entrapped, and the physical and chemical interactions between the drug and the polymer. The nanoparticle size, in turn can affect the release profile. Bigger nanoparticles degrade slower, and present slower active component diffusion through the polymeric matrix which affects the release profiles. The initial

burst effect is reduced with a further prolonged slow release due to slow nanoparticle degradation [189,190]. The drug released from microparticles is relatively slower than release from nanoparticles because of the higher surface area and smaller size of nanoparticles [130,178,191].

2.6.4 Environmental Conditions (pH, Temperature, Release Medium)

Temperature, pH, ionic strength, among other environmental conditions affects the drug release profile due an increase or decrease in polymeric degradation rates and changes in the diffusion process. The polymer behavior changes as a function of pH, temperature or other parameters. For example, poly(ortho esters) are stable at alkaline pH with increased degradation at acidic pH [192]. It would be ideal to use the internal cellular characteristics to release the drug in a controlled time dependent manner. Pullulan, hystidine base peptides, amine base polymers, poly(vinylpyrrolidone-co-dimethylmaleic anhydride) and others are examples of pH sensitive polymers that release the drug entrapped differently at different pHs. It is important to note that indeed physiological pH is around 7, but organelles have a different pH, endosomes are more acidic, and lysosomes have a pH as low as 5. At this pH, the nanoparticles suffer chemical alterations that activate degradation mechanisms or polymer reconfiguration which affect the release of the drug entrapped. Temperature can affect the stability of the nanoparticles, which in turn affects the release of the active component. The polymers poly(butyl methacrylate) and poly(N-isopropylacrylamide) are examples of temperature dependent drug release polymers [193-198].

The affinity between the drug and the release medium affect the release. Redhead et al. [199] worked with release of a hydrophilic component in different mediums. The release profiles performed in PBS showed an initial fast release, between 40 to 60 % of the hydrophilic active component was released in 24 hours. When serum was used, the release rates were faster with almost 50% of drug released in 1 hour. At 24 hours, almost 100 % was released. This difference was attributed to the presence of serum proteins and the affinity between the proteins and the drug [199]. The configuration that polymers can acquire (i.e. random coil, oval, rod, and other) in presence of good, ideal or bad solvents could also affect the drug release profile which adds more complexity to the solvent election as the organic phase in the synthesis process.

2.6.5 Complexation

Conjugation of the polymer with the active component causes a decrease in the diffusion process of the drug, and the degradation process becomes the dominant mechanism for the release of the active component. Yoo et al. [200] studied the anti-tumor activity of entrapped doxorubicin into nanoparticles. The non-conjugated drug entrapped in the matrix showed a fast release with almost 80% in the first day, and 100% of release was after 5 days. The sample with drug conjugated to PLGA (PLGA M.W of 5 kDa) showed slower release with 40% after one day which was continued up to 24 days, when 100 % of the drug was released.

2.7 Conclusions

Natural antioxidants and vitamins have been shown to possess beneficial properties that could potentially improve human health. Targeted delivery of vitamins and antioxidants via polymeric nanoparticles can offer several advantages over traditional delivery methods, which include protection of the bioactive component from degradation, an increase in the bioavailability of the vitamin, delivery of the component to the site of action in a controlled manner, as well as the possibility to integrate the bioactive component in various foods for enhanced food quality. Safety of the polymeric nanoparticles for antioxidant and vitamin delivery is a very important factor which must be considered in designing and building polymeric nanoparticles for targeted controlled delivery of antioxidants and vitamins.

Several methods are available to synthesize polymeric nanoparticles with entrapped antioxidants/vitamins, by starting with a preformed polymer. Each method has its advantages and disadvantages; the selection of the most suitable method should be based on the chemical characteristics of the active component and that of the polymer, as well as on the final use of the nanoparticles. The methods which are based on the diffusion of the organic solvent to form nanoparticles are limited to low polymer concentration for nanoparticle mean sizes of less than 200 nm. Methods that involve solvent evaporation are more time consuming and are more expensive, but are less sensitive to changes in the polymer concentration. Emulsion evaporation, in particular, can be used to entrap hydrophilic (w/o/w emulsion) or hydrophobic (w/o emulsion) antioxidants, which is an advantage. The salting-out method is suitable for formation of nanoparticles at higher polymer concentration, but the involved purification process is a limitation of this synthesis method. Surfactant concentration, polymer concentration, polymer

molecular weight, solvents, surfactant concentrations, and phase ratios play an important role in controlling the size of the nanoparticles in all methods available for nanoparticles formation.

The methods most broadly used to characterize nanoparticles in terms of morphology, size and size distribution, surface characteristics, entrapment efficiency, and release of the entrapped component, are the following, transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic transmission electron microscopy (cryo-TEM), atomic force microscopy (AFM), Dynamic light scattering (DLS), laser Doppler anemometry, Fourier transform infrared spectroscopy (FT-IR), spectroscopy, and high pressure liquid chromatography (HPLC).

The release of the active component consists of two steps, diffusion and polymer degradation. The diffusion process is predominant at the initial states of the release, followed by the release of the active component due to the polymer degradation. Different parameters affect the release profile, which include chemical structure of the active component, amount of drug entrapped, polymer hydrophobic/hydrophilic behavior, temperature, pH, presence of enzymes, method of sterilization, polymer structure (i.e. copolymer ratio, crystallinity), polymer molecular weight, and nanoparticle size, among others.

2.8 Future Trends

The entrapment and delivery of antioxidants and vitamins with polymeric nanoparticles is anticipated to become a significant research field in the near future, due to the extensive benefits that nanoencapsulation can offer. The single antioxidant entrapment approach will be enhanced by including multiple action antioxidants with potential synergetic effects in a single nanoparticle delivery system.

Nanoparticle size, among other physical characteristics, affects the release profiles of the bioactive component, and many researchers are continually attempting to decrease the average nanoparticle size with better control. There are important advances in the literature on understanding the mechanisms involved in manipulation and control of the nanoparticle characteristics and the improvement in the drug entrapment efficiency by carefully controlling these parameters.

The antioxidants entrapped into polymeric nanoparticle will present release profiles strictly modulated by carefully designing the particle to interact with the environment (i.e. pH, temperature) in a predictable way. Formation of nanoparticles that can interact with the human

body and can modify their responses based on changes in the environment is an important research area in the field. Several questions will be addressed to reach this goal, such as tailor-synthesis of new polymers for modulated responses and a better performance.

Very importantly, the understanding of the safety of the nanoparticle delivery system is critical for the final use of the system designed in food. It involves analysis of the complex interactions between the nanoparticles and the cellular environment, and the mechanism involved in the delivery of the antioxidant/vitamin at the site of action.

2.9 References

[1] Halliwell B, Gutteridge JNC. The definition and measurement of antioxidants in biological systems. Free Radical Biology and Medicine 1995;18 (1):125-6.

[2] Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free radical research 1999;31 (4):261-72.

[3] Knight JA. Review: Free radicals, antioxidants, and the immune system. Annals of Clinical and Laboratory Science 2000;30 (2):145-58.

[4] Young IS, Woodside JV. Antioxidants in health and disease. Journal of clinical pathology 2001;54 (3):176-86.

[5] Draper HH, Draper HH, Bird RP. Antioxidants and cancer. Journal of agricultural and food chemistry 1984;32:433-5.

[6] Zhang YH, Kramer TR, Taylor PR, Li JY, Blot WJ, Brown CC, Guo W, Dawsey SM, Li B. Possible immunologic involvement of antioxidants in cancer prevention. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1477S-82S.

[7] Vina J, Lloret A, Orti R, Alonso D. Molecular bases of the treatment of Alzheimer's disease with antioxidants: prevention of oxidative stress. Molecular Aspects of Medicine 2004;25 (1-2):117-23.

[8] Pani G, Colavitti R, Bedogni B, Fusco S, Ferraro D, Borrello S, Galeotti T. Mitochondrial superoxide dismutase: a promising target for new anticancer therapies. Current Medicinal Chemistry 2004;11 (10):1299-308.

[9] Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radical Biology and Medicine 2000;28 (10):1456-62.

[10] Taylor A, Jacques PF, Epstein EM. Relations among aging, antioxidant status, and cataract. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1439S-47S.

[11] Halliwell B. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? Cardiovascular research 2000;47 (3):410-8.

[12] Hazen SL. Oxidation and atherosclerosis. Free Radical Biology and Medicine 2000;28 (12):1683-4.

[13] Adlam VJ, Harrison JC, Porteous CM, James AM, Smith RAJ, Murphy MP, Sammut IA. Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. Faseb Journal 2005;19 (9):1088-95.

[14] Bergendi L, Benes L, Durackova Z, Ferencik M. Chemistry, physiology and pathology of free radicals. Life sciences 1999;65 (18-19):1865-74.

[15] Azzi A, Davies KJ, Kelly F. Free radical biology - terminology and critical thinking. FEBS letters 2004;558 (1-3):3-6.

[16] Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovascular Research 1999;43 (3):562-71.

[17] Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000;404 (6779):787-90.

[18] Ferrari CK. Functional foods, herbs and nutraceuticals: towards biochemical mechanisms of healthy aging. Biogerontology 2004;5 (5):275-89.

[19] Vitaglione P, Morisco F, Caporaso N, Fogliano V. Dietary antioxidant compounds and liver health. Critical Reviews in Food Science and Nutrition 2004;44 (7-8):575-86.

[20] Higuera-Ciapara I, Felix-Valenzuela L, Goycoolea FM. Astaxanthin: a review of its chemistry and applications. Critical Reviews in Food Science and Nutrition 2006;46 (2):185-96.

[21] Cutler RG. Oxidative stress profiling: part I. Its potential importance in the optimization of human health. Annals of the New York Academy of Sciences 2005;1055:93-135.

[22] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radical Biology & Medicine 2000;28 (12):1815-26.

[23] Yang TTC, Devaraj S, Jialal I. Oxidative stress and atherosclerosis. Journal of Clinical Ligand Assay 2001;24 (1):13-24.

[24] Albertini R, Moratti R, De Luca G. Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. Current molecular medicine 2002;2 (6):579-92.

[25] Allen RG, Tresini M. Oxidative stress and gene regulation. Free Radical Biology and Medicine 2000;28 (3):463-99.

[26] Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. Faseb Journal 1987;1 (6):441-5.

[27] Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Critical Reviews in Food Science and Nutrition 2004;44 (4):275-95.

[28] Nohl H, Gille L. Lysosomal ROS formation. Redox Report 2005;10 (4):199-205.

[29] Babior BM. Superoxide: A two-edged sword. Brazilian Journal of Medical and Biological Research 1997;30 (2):141-55.

[30] Turrens JF. Mitochondrial formation of reactive oxygen species. The Journal of Physiology 2003;552 (Pt 2):335-44.

[31] Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Progress in Lipid Research 2004;43 (3):200-27.

[32] Sagin FG, Sozmen EY. Anti-inflammatory Effects of Dietary Antioxidants. Current Medicinal Chemistry - Anti-Inflammatory & Anti-Allergy Agents; 2004;3 (1):19-30.

[33] Lachance PA, Nakat Z, Jeong WS. Antioxidants: an integrative approach. Nutrition 2001;17 (10):835-8.

[34] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. Faseb Journal 1999;13 (10):1145-55.

[35] Wagner KH, Isnardy B, Elmadfa I. gamma- and delta-tocopherols are more effective than alpha-tocopherol on the autoxidation of a 10% rapeseed oil triacylglycerol-in-water emulsion with and without a radical initiator. European Journal of Lipid Science and Technology 2004;106 (1):44-51.

[36] Wagner KH, Kamal-Eldin A, Elmadfa I. Gamma-tocopherol--an underestimated vitamin? Annals of Nutrition & Metabolism 2004;48 (3):169-88.

[37] Jiang Q, Christen S, Shigenaga MK, Ames BN. gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. The American Journal of Clinical Nutrition 2001;74 (6):714-22.

[38] Leonard SW, Paterson E, Atkinson JK, Ramakrishnan R, Cross CE, Traber MG. Studies in humans using deuterium-labeled alpha- and gamma-tocopherols demonstrate faster plasma gamma-tocopherol disappearance and greater gamma-metabolite production. Free Radical Biology & Medicine 2005;38 (7):857-66.

[39] Berman K, Brodaty H. Tocopherol (vitamin E) in Alzheimer's disease and other neurodegenerative disorders. CNS Drugs 2004;18 (12):807-25.

[40] Cohn W. Bioavailability of vitamin E. European Journal of Clinical Nutrition 1997;51:S80-S5.

[41] Wang X, Quinn PJ. Vitamin E and its function in membranes. Progress in Lipid Research 1999;38 (4):309-36.

[42] Kaul N, Devaraj S, Jialal I. Alpha-tocopherol and atherosclerosis. Experimental Biology and Medicine 2001;226 (1):5-12.

[43] Meydani SN, Wu D, Santos MS, Hayek MG. Antioxidants and immune response in aged persons: overview of present evidence. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1462S-76S.

[44] Harris A, Devaraj S, Jialal I. Oxidative stress, alpha-tocopherol therapy, and atherosclerosis. Current Atherosclerosis Reports 2002;4 (5):373-80.

[45] Upston JM, Kritharides L, Stocker R. The role of vitamin E in atherosclerosis. Progress in Lipid Research 2003;42 (5):405-22.

[46] Bramley PM, Elmadfa A, Kafatos FJ, Kelly Y, Manios HE, Roxborough W, Schuch PJ, Sheehy A, Wagner KH. Review vitamin E. Journal of the Science of Food and Agricultural 2000;80:913-38.

[47] Wayner DDM, Burton GW, Ingold KU, Barclay LRC, Locke SJ. The relative contribution of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochimica et Biophysica Acta (BBA) - General Subjects 1987;924 (3):408-19.

[48] Thomas SR, Stocker R. Molecular action of vitamin E in lipoprotein oxidation: implications for atherosclerosis. Free Radical Biology & Medicine 2000;28 (12):1795-805.

[49] Jialal I, Devaraj S. Scientific evidence to support a vitamin E and heart disease health claim: research needs. The Journal of Nutrition 2005;135 (2):348-53.

[50] Crane FL. Biochemical functions of coenzyme Q10. Journal of the American College of Nutrition 2001;20 (6):591-8.

[51] Quinn PJ, Fabisiak JP, Kagan VE. Expansion of antioxidant function of vitamin E by coenzyme Q. Biofactors 1999;9 (2-4):149-54.

[52] Kaikkonen J, Tuomainen TP, Nyyssonen K, Salonen JT. Coenzyme Q10: absorption, antioxidative properties, determinants, and plasma levels. Free Radical Research 2002;36 (4):389-97.

[53] Nohl H, Staniek K, Kozlov AV, Gille L. The biomolecule ubiquinone exerts a variety of biological functions. Biofactors 2003;18 (1-4):23-31.

[54] Bhagavan HN, Chopra RK. Coenzyme Q10: absorption, tissue uptake, metabolism and pharmacokinetics. Free radical research 2006;40 (5):445-53.

[55] Laaksonen R, Riihimaki A, Laitila J, Martensson K, Tikkanen MJ, Himberg JJ. Serum and muscle tissue ubiquinone levels in healthy subjects. The Journal of Laboratory and Clinical Medicine 1995;125 (4):517-21.

[56] Zita C, Overvad K, Mortensen SA, Sindberg CD, Moesgaard S, Hunter DA. Serum coenzyme Q10 concentrations in healthy men supplemented with 30 mg or 100 mg coenzyme Q10 for two months in a randomised controlled study. Biofactors 2003;18 (1-4):185-93.

[57] Turunen M, Sindelar P, Dallner G. Induction of endogenous coenzyme Q biosynthesis by administration of peroxisomal inducers. Biofactors 1999;9 (2-4):131-9.

[58] Overvad K, Diamant B, Holm L, Holmer G, Mortensen SA, Stender S. Coenzyme Q10 in health and disease. European Journal of Clinical Nutrition 1999;53 (10):764-70.

[59] Sarter B. Coenzyme Q10 and cardiovascular disease: a review. The Journal of Cardiovascular Nursing 2002;16 (4):9-20.

[60] Langsjoen PH, Langsjoen AM. Overview of the use of CoQ10 in cardiovascular disease. Biofactors 1999;9 (2-4):273-84.

[61] Beal MF. Coenzyme Q10 as a possible treatment for neurodegenerative diseases. Free radical research 2002;36 (4):455-60.

[62] Galati G, O'Brien PJ. Cytoprotective and anticancer properties of coenzyme Q versus capsaicin. Biofactors 2003;18 (1-4):195-205.

[63] Conklin KA. Coenzyme q10 for prevention of anthracycline-induced cardiotoxicity. Integrative Cancer Therapies 2005;4 (2):110-30.

[64] Mortensen SA. Overview on coenzyme Q10 as adjunctive therapy in chronic heart failure. Rationale, design and end-points of "Q-symbio"--a multinational trial. Biofactors 2003;18 (1-4):79-89.

[65] Genova ML, Pich MM, Biondi A, Bernacchia A, Falasca A, Bovina C, Formiggini G, Parenti Castelli G, Lenaz G. Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant. Experimental Biology and Medicine 2003;228 (5):506-13.

[66] Pich MM, Castagnoli A, Biondi A, Bernacchia A, Tazzari PL, D'Aurelio M, Castelli GP, Formiggini G, Conte R, Bovina C, Lenaz G. Ubiquinol and a Coenzyme Q reducing system protect platelet mitochondrial function of transfusional buffy coats from oxidative stress. Free Radical Research 2002;36 (4):429-36.

[67] Sandor PS, Di Clemente L, Coppola G, Saenger U, Fumal A, Magis D, Seidel L, Agosti RM, Schoenen J. Efficacy of coenzyme Q10 in migraine prophylaxis: a randomized controlled trial. Neurology 2005;64 (4):713-5.

[68] Miles MV, Horn P, Miles L, Tang P, Steele P, DeGrauw T. Bioequivalence of coenzyme Q(10) from over-the-counter supplements. Nutrition Research 2002;22 (8):919-29.

[69] Zaghloul AA, Gurley B, Khan M, Bhagavan H, Chopra R, Reddy I. Bioavailability assessment of oral coenzyme Q10 formulations in dogs. Drug Development and Industrial Pharmacy 2002;28 (10):1195-200.

[70] Tikhaze AK, Konovalova GG, Lankin VZ, Kaminnyi AI, Kaminnaja VI, Ruuge EK, Kukharchuk VV. Effect of ubiquinone Q(10) and antioxidant vitamins on free radical oxidation of phospholipids in biological membranes of rat liver. Bulletin of Experimental Biology and Medicine 2005;140 (2):181-3.

[71] Hughes DA. Dietary carotenoids and human immune function. Nutrition 2001;17 (10):823-7.

[72] Young AJ, Lowe GM. Antioxidant and prooxidant properties of carotenoids. Archives of Biochemistry and Biophysics 2001;385 (1):20-7.

[73] Cooke MS, Evans MD, Mistry N, Lunec J. Role of dietary antioxidants in the prevention of in vivo oxidative DNA damage. Nutrition Research Reviews 2002;15 (1):19-41.

[74] Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry 2001;49 (11):5165-70.

[75] Di Carlo G, Mascolo N, Izzo AA, Capasso F. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life sciences 1999;65 (4):337-53.

[76] Tapiero H, Tew KD, Ba GN, Mathe G. Polyphenols: do they play a role in the prevention of human pathologies? Biomedicine & pharmacotherapy 2002;56 (4):200-7.

[77] Pietta PG. Flavonoids as antioxidants. Journal of Natural Products 2000;63 (7):1035-42.

[78] van Acker SA, van den Berg DJ, Tromp MN, Griffioen DH, van Bennekom WP, van der Vijgh WJ, Bast A. Structural aspects of antioxidant activity of flavonoids. Free Radical Biology & Medicine 1996;20 (3):331-42.

[79] Hollman PCH. Absorption, bioavailability, and metabolism of flavonoids. Pharmaceutical Biology 2004;42:74-83.

[80] Cotelle N, Bernier JL, Catteau JP, Pommery J, Wallet JC, Gaydou EM. Antioxidant properties of hydroxy-flavones. Free Radical Biology & Medicine 1996;20 (1):35-43.

[81] Croft KD. The chemistry and biological effects of flavonoids and phenolic acids. Annals of the New York Academy of Sciences 1998;854:435-42.

[82] Karakaya S. Bioavailability of phenolic compounds. Critical Reviews in Food Science and Nutrition 2004;44 (6):453-64.

[83] Silva MM, Santos MR, Caroco G, Rocha R, Justino G, Mira L. Structure-antioxidant activity relationships of flavonoids: a re-examination. Free Radical Research 2002;36 (11):1219-27.

[84] Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free radical biology & medicine 2004;37 (3):287-303.

[85] Neuhouser ML. Flavonoids and cancer prevention: What is the evidence in humans? Pharmaceutical Biology 2004;42:36-45.

[86] Kaur C, Kapoor HC. Antioxidants in fruits and vegetables - the millennium's health. International Journal of Food Science and Technology 2001;36 (7):703-25.

[87] Kondratyuk TP, Pezzuto JM. Natural product polyphenols of relevance to human health. Pharmaceutical Biology 2004;42:46-63.

[88] Jeong WS, Kong ANT. Biological properties of monomeric and polymeric catechins: Green tea catechins and procyanidins. Pharmaceutical Biology 2004;42:84-93.

[89] Carr AC, Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. The American Journal of Clinical Nutrition 1999;69 (6):1086-107.

[90] Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK, Levine M. Vitamin C as an antioxidant: evaluation of its role in disease prevention. Journal of the American College of Nutrition 2003;22 (1):18-35.

[91] Coulter ID, Hardy ML, Morton SC, Hilton LG, Tu W, Valentine D, Shekelle PG. Antioxidants vitamin C and vitamin e for the prevention and treatment of cancer. Journal of General Internal Medicine 2006;21 (7):735-44.

[92] Bohm F, Edge R, McGarvey DJ, Truscott TG. Beta-carotene with vitamins E and C offers synergistic cell protection against NOx. FEBS letters 1998;436 (3):387-9.

[93] Bauer K. Carnosine and homocarnosine, the forgotten, enigmatic peptides of the brain. Neurochemical research 2005;30 (10):1339-45.

[94] Fontana M, Pinnen F, Lucente G, Pecci L. Prevention of peroxynitrite-dependent damage by carnosine and related sulphonamido pseudodipeptides. Cellular and Molecular Life Sciences 2002;59 (3):546-51.

[95] Hipkiss AR. Carnosine, a protective, anti-ageing peptide? The International Journal of Biochemistry & Cell Biology 1998;30 (8):863-8.

[96] Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. Mechanisms of Ageing and Development 2001;122 (13):1431-45.

[97] Baran EJ. Metal complexes of carnosine. Biochemistry (Moscow) 2000;65 (7):789-97.

[98] Boldyrev AA. Protection of proteins from oxidative stress: a new illusion or a novel strategy? Annals of the New York Academy of Sciences 2005;1057:193-205.

[99] Hipkiss AR, Brownson C. Carnosine reacts with protein carbonyl groups: another possible role for the anti-ageing peptide? Biogerontology 2000;1 (3):217-23.

[100] Hipkiss AR, Preston JE, Himsworth DT, Worthington VC, Keown M, Michaelis J, Lawrence J, Mateen A, Allende L, Eagles PA, Abbott NJ. Pluripotent protective effects of carnosine, a naturally occurring dipeptide. Annals of the New York Academy of Sciences 1998;854:37-53.

[101] Hipkiss AR. Glycation, ageing and carnosine: are carnivorous diets beneficial? Mechanisms of Ageing and Development 2005;126 (10):1034-9.

[102] Guiotto A, Calderan A, Ruzza P, Borin G. Carnosine and carnosine-related antioxidants: a review. Current Medicinal Chemistry 2005;12 (20):2293-315.

[103] Nagai K, Niijima A, Yamano T, Otani H, Okumra N, Tsuruoka N, Nakai M, Kiso Y. Possible role of L-carnosine in the regulation of blood glucose through controlling autonomic nerves. Experimental Biology and Medicine 2003;228 (10):1138-45.

[104] Hipkiss AR. Could carnosine suppress zinc-mediated proteasome inhibition and neurodegeneration? Therapeutic potential of a non-toxic but non-patentable dipeptide. Biogerontology 2005;6 (2):147-9.

[105] Niki E, Noguchi N. Dynamics of antioxidant action of vitamin E. Accounts of Chemical Research 2004;37 (1):45-51.

[106] Kwon SS, Nam YS, Lee JS, Ku BS, Han SH, Lee JY, Chang IS. Preparation and characterization of coenzyme Q(10)-loaded PMMA nanoparticles by a new emulsification process based on microfluidization. Colloids and Surfaces a-Physicochemical and Engineering Aspects 2002;210 (1):95-104.

[107] Chen L, Subirade M. Chitosan/beta-lactoglobulin core-shell nanoparticles as nutraceutical carriers. Biomaterials 2005;26 (30):6041-53.

[108] Russell-Jones GJ. Use of targeting agents to increase uptake and localization of drugs to the intestinal epithelium. Journal of Drug Targeting 2004;12 (2):113-23.

[109] Na K, Bum Lee T, Park KH, Shin EK, Lee YB, Choi HK. Self-assembled nanoparticles of hydrophobically-modified polysaccharide bearing vitamin H as a targeted anti-cancer drug delivery system. European Journal of Pharmaceutical Sciences 2003;18 (2):165-73.

[110] Muro S, Cui X, Gajewski C, Murciano JC, Muzykantov VR, Koval M. Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from

oxidative stress. American Journal of Physiology-Cell Physiology 2003;285 (5):C1339-C47.

[111] Shea TB, Ortiz D, Nicolosi RJ, Kumar R, Watterson AC. Nanosphere-mediated delivery of vitamin E increases its efficacy against oxidative stress resulting from exposure to amyloid beta. Journal of Alzheimer's Disease 2005;7 (4):297-301.

[112] Duclairoir C, Orecchioni AM, Depraetere P, Nakache E. Alpha-tocopherol encapsulation and in vitro release from wheat gliadin nanoparticles. Journal of microencapsulation 2002;19 (1):53-60.

[113] Zigoneanu IG, Astete CE, Sabliov CM. Nanoparticles with entrapped alpha-tocopherol: synthesis, characterization, and controlled release. Nanotechnology 2008;19 (10):- March 12.

[114] Chen CC, Wagner G. Vitamin E nanoparticle for beverage applications. Chemical Engineering Research & Design 2004;82 (A11):1432-7.

[115] Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Advanced Drug Delivery Reviews 1997;28 (1):5-24.

[116] Westesen K, Bunjes H, Hammer G, Siekmann B. Novel colloidal drug delivery systems. PDA Journal of Pharmaceutical Science and Technology / PDA 2001;55 (4):240-7.

[117] Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. Advanced Drug Delivery Reviews 2002;54 (5):631-51.

[118] Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. Journal Controlled Release 2002;83 (3):389-400.

[119] Barrat G, Courraze G, Couvreur C, Fattal E, Gref R, D. L, Legrand P, Ponchel G, Vauthier C. Polymeric Micro- and Nanoparticles as drug Carriers. In: Dumitriu S, editor. Polymeric Biomaterials. New York: Marcel Dekker Inc, 2000. pp. 753-81.

[120] Nakache E, Poulain N, Candau F, Orecchioni AM, Irache JM. Biopolymer and polymer nanopartciles and their biomedical applications. In: Nalwa HS, editor. Handbook of Nanostructured Materials and Nanotechnology. New York: Academic Press, 2000. pp. 577-635.

[121] Hans ML, Lowman AM. B iodegradable nanoparticles for drug delivery and targeting. Current Opinion in Solid State and Materials Science 2002;6 (4):319-27.

[122] Sekhara Rao GC, Kumar MS, Mathivanan N, Bhanoji Rao ME. Nanosuspensions as the most promising approach in nanoparticulate drug delivery systems. Pharmazie 2004;59 (1):5-9.

[123] Astete CE, Sabliov CM. Synthesis and characterization of PLGA nanoparticles. Journal of Biomaterial Sciences-Polymer Edition 2006;17 (3):247-89.

[124] Allemann E, Gurny R, Doelker E. Drug-Loaded Nanoparticles - Preparation Methods and Drug Targeting Issues. European Journal of Pharmaceutics and Biopharmaceutics 1993;39 (5):173-91.

[125] Hiremath SRR, Hota A. Nanoparticles as drug delivery systems. Indian Journal of Pharmaceutical Sciences 1999;61 (2):69-75.

[126] Quintanar-Guerrero D, Allemann E, Fessi H, Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. Drug Development and Industrial Pharmacy 1998;24 (12):1113-28.

[127] Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacological Reviews 2001;53 (2):283-318.

[128] Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Advanced Drug Delivery Reviews 2003;55 (3):329-47.

[129] Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Current drug delivery 2004;1 (4):321-33.

[130] Bala I, Hariharan S, Kumar MN. PLGA nanoparticles in drug delivery: the state of the art. Critical reviews in therapeutic drug carrier systems 2004;21 (5):387-422.

[131] Alexis F. Factors affecting the degradation and drug-release mechanism of poly(lactic acid) and poly[(lactic acid)-co-(glycolic acid)]. Polymer International 2005;54 (1):36-46.

[132] Quintanar-Guerrero D, Allemann E, Doelker E, Fessi H. A mechanistic study of the formation of polymer nanoparticles by the emulsification-diffusion technique. Colloid Polym Sci 1997;275 (7):640-7.

[133] Konan YN, Cerny R, Favet J, Berton M, Gurny R, Allemann E. Preparation and characterization of sterile sub-200 nm meso-tetra(4-hydroxylphenyl)porphyrin-loaded nanoparticles for photodynamic therapy. European Journal of Pharmaceutical Sciences 2003;55 (1):115-24.

[134] Lee SJ, Jeong JR, Shin SC, Kim JC, Chang YH, Lee KH, Kim JD. Magnetic enhancement of iron oxide nanoparticles encapsulated with poly(D,L-latide-co-glycolide). Colloids and Surfaces a-Physicochemical and Engineering Aspects 2005;255 (1-3):19-25.

[135] Ravi Kumar MN, Bakowsky U, Lehr CM. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials 2004;25 (10):1771-7.

[136] Kumar MN, Mohapatra SS, Kong X, Jena PK, Bakowsky U, Lehr CM. Cationic poly(lactide-co-glycolide) nanoparticles as efficient in vivo gene transfection agents. Journal of Nanoscience and Nanotechnology 2004;4 (8):990-4.

[137] Ahlin P, Kristl J, Kristl A, Vrecer F. Investigation of polymeric nanoparticles as carriers of enalaprilat for oral administration. International Journal of Pharmaceutics 2002;239 (1-2):113-20.

[138] Kwon HY, Lee JY, Choi SW, Jang YS, Kim JH. Preparation of PLGA nanoparticles containing estrogen by emulsification-diffusion method. Colloids and Surfaces a-Physicochemical and Engineering Aspects 2001;182 (1-3):123-30.

[139] Konan YN, Gurny R, Allemann E. Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. International Journal of Pharmaceutics 2002;233 (1-2):239-52.

[140] Zweers ML, Engbers GH, Grijpma DW, Feijen J. In vitro degradation of nanoparticles prepared from polymers based on DL-lactide, glycolide and poly(ethylene oxide). Journal of Controlled Release 2004;100 (3):347-56.

[141] Eley JG, Pujari VD, McLane J. Poly (lactide-co-glycolide) nanoparticles containing coumarin-6 for suppository delivery: in vitro release profile and in vivo tissue distribution. Drug Delivery 2004;11 (4):255-61.

[142] Prakobvaitayakit M, Nimmannit U. Optimization of polylactic-co-glycolic acid nanoparticles containing itraconazole using 2(3) factorial design. AAPS PharmSciTech 2003;4 (4):1-9.

[143] Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. Journal of Controlled Release 1999;57 (2):171-85.

[144] Csaba N, Caamano P, Sanchez A, Dominguez F, Alonso MJ. PLGA:poloxamer and PLGA:poloxamine blend nanoparticles: new carriers for gene delivery. Biomacromolecules 2005;6 (1):271-8.

[145] Niwa T, Takeuchi H, Hino T, Kunou N, Kawashima Y. Preparations of Biodegradable Nanospheres of Water-Soluble and Insoluble Drugs with D,L-Lactide Glycolide Copolymer by a Novel Spontaneous Emulsification Solvent Diffusion Method, and the Drug Release Behavior. Journal of Controlled Release 1993;25 (1-2):89-98.

[146] Ameller T, Marsaud V, Legrand P, Gref R, Renoir JM. Pure antiestrogen RU 58668-loaded nanospheres: morphology, cell activity and toxicity studies. European Journal of Pharmaceutical Sciences 2004;21 (2-3):361-70.

[147] Oster CG, Wittmar M, Unger F, Barbu-Tudoran L, Schaper AK, Kissel T. Design of amine-modified graft polyesters for effective gene delivery using DNA-loaded nanoparticles. Pharmaceutical Research 2004;21 (6):927-31.

[148] Saxena V, Sadoqi M, Shao J. Indocyanine green-loaded biodegradable nanoparticles: preparation, physicochemical characterization and in vitro release. International Journal of Pharmaceutics 2004;278 (2):293-301.

[149] Csaba N, Gonzalez L, Sanchez A, Alonso MJ. Design and characterisation of new nanoparticulate polymer blends for drug delivery. Journal of Biomaterials Science-Polymer Edition 2004;15 (9):1137-351.

[150] Panagi Z, Beletsi A, Evangelatos G, Livaniou E, Ithakissios DS, Avgoustakis K. Effect of dose on the biodistribution and pharmacokinetics of PLGA and PLGA-mPEG nanoparticles. International Journal of Pharmaceutics 2001;221 (1-2):143-52.

[151] De Jaeghere F, Doelker E, Gurny R. Nanoparticles. In: Mathiowitz E, editor. Encyclopedia of Control Drug Delivery. New York: John Wiley & Sons Inc, 1999. pp. 641-64.

[152] Alex R, Bodmeier R. Encapsulation of water-soluble drugs by a modified solvent evaporation method. I. Effect of process and formulation variables on drug entrapment. Journal of microencapsulation 1990;7 (3):347-55.

[153] Takada S, Yamagata Y, Misaki M, Taira K, Kurokawa T. Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique. Journal of Controlled Release 2003;88 (2):229-42.

[154] Obeidat WM, Price JC. Viscosity of polymer solution phase and other factors controlling the dissolution of theophylline microspheres prepared by the emulsion solvent evaporation method. Journal of Microencapsulation 2003;20 (1):57-65.

[155] Park SJ, Kim SH. Preparation and characterization of biodegradable poly(l-lactide)/poly(ethylene glycol) microcapsules containing erythromycin by emulsion solvent evaporation technique. Journal of Colloid and Interface Science 2004;271 (2):336-41.

[156] Mohammed FA, Hassan MA. Formulation and evaluation of ciprofloxacin hydrochloride and norfloxacin microspheres prepared by an enhanced emulsion-solvent evaporation process. Stp Pharma Sciences 2003;13 (5):319-27.

[157] Lopez-Quintela MA. Synthesis of nanomaterials in microemulsions: formation mechanisms and growth control. Current Opinion in Colloid & Interface Science 2003;8 (2):137-44.

[158] Bouchemal K, Briancon S, Perrier E, Fessi H. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. International Journal of Pharmaceutics 2004;280 (1-2):241-51.

[159] Schmitt V, Leal-Calderon F, Bibette J. Preparation of monodisperse particles and emulsions by controlled shear. Topics in Current Chemistry 2003;227:195-215.

[160] Zhao X, Goveas JL. Size selection in viscoelastic emulsions under shear. Langmuir 2001;17 (13):3788-91.

[161] Panyam J, Williams D, Dash A, Leslie-Pelecky D, Labhasetwar V. Solid-state solubility influences encapsulation and release of hydrophobic drugs from PLGA/PLA nanoparticles. Journal of Pharmaceutical Sciences 2004;93 (7):1804-14.

[162] Pietzonka P, Walter E, Duda-Johner S, Langguth P, Merkle HP. Compromised integrity of excised porcine intestinal epithelium obtained from the abattoir affects the outcome of in vitro particle uptake studies. European Journal of Pharmaceutical Sciences 2002;15 (1):39-47.

[163] Julienne MC, Alonso MJ, Amoza JLG, Benoit JP. Preparation of Poly(D,L-Lactide Glycolide) Nanoparticles of Controlled Particle-Size Distribution - Application of Experimental-Designs. Drug Development and Industrial Pharmacy 1992;18 (10):1063-77.

[164] Chung TW, Huang YY, Tsai YL, Liu YZ. Effects of solvent evaporation rate on the properties of protein-loaded PLLA and PDLLA microspheres fabricated by emulsion-solvent evaporation process. Journal of Microencapsulation 2002;19 (4):463-71.

[165] Song CX, Labhasetwar V, Murphy H, Qu X, Humphrey WR, Shebuski RJ, Levy RJ. Formulation and characterization of biodegradable nanoparticles for intravascular local drug delivery. Journal of Controlled Release 1997;43 (2-3):197-212.

[166] Pays K, Giermanska-Kahn J, Pouligny B, Bibette J, Leal-Calderon F. Coalescence in surfactant-stabilized double emulsions. Langmuir 2001;17 (25):7758-69.

[167] Pays K, Giermanska-Kahn J, Pouligny B, Bibette J, Leal-Calderon F. Double emulsions: how does release occur? Journal Controlled Release 2002;79 (1-3):193-205.

[168] Ficheux MF, Bonakdar L, Leal-Calderon F, Bibette J. Some stability criteria for double emulsions. Langmuir 1998;14 (10):2702-6.

[169] Domb AJ, Bergelson L, Amselem S. Lipospheres for controlled delivery of substances. In: Benita S, editor. Microencapsulation: Methods and Industrial Applications. New York: Marcel Dekker Inc, 1996. pp. 411-534.

[170] Aukunuru JV, Ayalasomayajula SP, Kompella UB. Nanoparticle formulation enhances the delivery and activity of a vascular endothelial growth factor antisense oligonucleotide in human retinal pigment epithelial cells. The Journal of pharmacy and pharmacology 2003;55 (9):1199-206.

[171] Vandervoort J, Ludwig A. Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study. International Journal of Pharmaceutics 2002;238 (1-2):77-92.

[172] Dillen K, Vandervoort J, Van den Mooter G, Verheyden L, Ludwig A. Factorial design, physicochemical characterisation and activity of ciprofloxacin-PLGA nanoparticles. International Journal of Pharmaceutics 2004;275 (1-2):171-87.

[173] Prabha S, Labhasetwar V. Critical determinants in PLGA/PLA nanoparticle-mediated gene expression. Pharmaceutical Research 2004;21 (2):354-64.

[174] Vandervoort J, Yoncheva K, Ludwig A. Influence of the homogenisation procedure on the physicochemical properties of PLGA nanoparticles. Chemical & Pharmaceutical Bulletin 2004;52 (11):1273-9.

[175] Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. Journal of Controlled Release 2002;82 (1):105-14.

[176] Dillen K, Weyenberg W, Vandervoort J, Ludwig A. The influence of the use of viscosifying agents as dispersion media on the drug release properties from PLGA nanoparticles. European Journal of Pharmceutics and Biopharmaceutics 2004;58 (3):539-49.

[177] Mu L, Feng SS. A novel controlled release formulation for the anticancer drug paclitaxel (Taxol): PLGA nanoparticles containing vitamin E TPGS. Journal of Controlled Release 2003;86 (1):33-48.

[178] Jeon HJ, Jeong YI, Jang MK, Park YH, Nah JW. Effect of solvent on the preparation of surfactant-free poly(DL-lactide-co-glycolide) nanoparticles and norfloxacin release characteristics. International Journal of Pharmaceutics 2000;207 (1-2):99-108.

[179] Lemarchand C, Couvreur P, Besnard M, Costantini D, Gref R. Novel polyester-polysaccharide nanoparticles. Pharmaceutical Research 2003;20 (8):1284-92.

[180] Li Y, Pei Y, Zhang X, Gu Z, Zhou Z, Yuan W, Zhou J, Zhu J, Gao X. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. Journal of Controlled Release 2001;71 (2):203-11.

[181] Beletsi A, Leontiadis L, Klepetsanis P, Ithakissios DS, Avgoustakis K. Effect of preparative variables on the properties of poly(dl-lactide-co-glycolide)-methoxypoly(ethyleneglycol) copolymers related to their application in controlled drug delivery. International Journal of Pharmaceutics 1999;182 (2):187-97.

[182] Fonseca C, Simoes S, Gaspar R. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. Journal of Controlled Release 2002;83 (2):273-86.

[183] Lamprecht A, Ubrich N, Yamamoto H, Schafer U, Takeuchi H, Maincent P, Kawashima Y, Lehr CM. Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. The Journal of Pharmacology and Experimental Therapeutics 2001;299 (2):775-81.

[184] Lee WK, Park JY, Yang EH, Suh H, Kim SH, Chung DS, Choi K, Yang CW, Park JS. Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer. Journal of Controlled Release 2002;84 (3):115-23.

[185] Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. International Journal of Pharmaceutics 2005;290 (1-2):137-44.

[186] Win KY, Feng SS. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials 2005;26 (15):2713-22.

[187] Zhang Y, Zhuo RX. Synthesis and drug release behavior of poly (trimethylene carbonate)-poly (ethylene glycol)-poly (trimethylene carbonate) nanoparticles. Biomaterials 2005;26 (14):2089-94.

[188] Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Karydas AG, Ithakissios DS. PLGA-mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in vivo drug residence in blood properties. Journal of Controlled Release 2002;79 (1-3):123-35.

[189] Allemann E, Leroux JC, Gurny R, Doelker E. In vitro extended-release properties of drug-loaded poly(DL-lactic acid) nanoparticles produced by a salting-out procedure. Pharmaceutical Research 1993;10 (12):1732-7.

[190] Prabha S, Zhou WZ, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. International Journal of Pharmaceutics 2002;244 (1-2):105-15.

[191] Panyam J, Dali MM, Sahoo SK, Ma W, Chakravarthi SS, Amidon GL, Levy RJ, Labhasetwar V. Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-co-glycolide) nano- and microparticles. Journal of Controlled Release 2003;92 (1-2):173-87.

[192] Gopferich A. Polymer degradation and erosion: Mechanisms and applications. European Journal of Pharmaceutics and Biopharmaceutics 1996;42 (1):1-11.

[193] Chung JE, Yokoyama M, Okano T. Inner core segment design for drug delivery control of thermo-responsive polymeric micelles. Journal of Controlled Release 2000;65 (1-2):93-103.

[194] Chung JE, Yokoyama M, Yamato M, Aoyagi T, Sakurai Y, Okano T. Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly(butylmethacrylate). Journal of Controlled Release 1999;62 (1-2):115-27.

[195] Giacomelli C, Le Men L, Borsali R, Lai-Kee-Him J, Brisson A, Armes SP, Lewis AL. Phosphorylcholine-based pH-responsive diblock copolymer micelles as drug delivery vehicles: light scattering, electron microscopy, and fluorescence experiments. Biomacromolecules 2006;7 (3):817-28.

[196] Gillies ER, Frechet JM. pH-Responsive copolymer assemblies for controlled release of doxorubicin. Bioconjugate chemistry 2005;16 (2):361-8.

[197] Kamada H, Tsutsumi Y, Yoshioka Y, Yamamoto Y, Kodaira H, Tsunoda S, Okamoto T, Mukai Y, Shibata H, Nakagawa S, Mayumi T. Design of a pH-sensitive polymeric carrier for drug release and its application in cancer therapy. Clinical Cancer Research 2004;10 (7):2545-50.

[198] Xu P, Van Kirk EA, Murdoch WJ, Zhan Y, Isaak DD, Radosz M, Shen Y. Anticancer efficacies of cisplatin-releasing pH-responsive nanoparticles. Biomacromolecules 2006;7 (3):829-35.

[199] Redhead HM, Davis SS, Illum L. Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: in vitro characterisation and in vivo evaluation. Journal of Controlled Release 2001;70 (3):353-63.

[200] Yoo HS, Lee KH, Oh JE, Park TG. In vitro and in vivo anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates. Journal Controlled Release 2000;68 (3):419-31.

CHAPTER 3. ANTIOXIDANT PLGA NANOPARTICLES MADE WITH NOVEL ALPHA-TOCOPHEROL-ASCORBIC ACID SURFACTANT

3.1 Introduction

Polymeric nanoparticles have been extensively researched as delivery systems of different active components such as anti-cancer drugs, vitamins, proteins, peptides, and others [1-6]. The advantages are numerous. For example, polymeric nanoparticles protect the drug against degradation while releasing it in a controlled manner which translates into lower toxicity and less side effects. Also, drug targeting can be achieved by surface attachment of moieties such as proteins, peptides, and other molecules (e. g. folic acid and lectins) of specific interaction with the target [7-11]. Despite all these advantages, polymeric nanoparticles, in and of themselves, do not provide any benefit in the treatment of disease, but rather, they act as carriers for the drug. Nanoparticles with inherent properties, such as antioxidant activity, could be useful in treating oxidative-stress related diseases such as atherosclerosis and cancer, in addition to playing their obvious role as vehicles for drug transport and delivery.

Antioxidant nanoparticles of promising characteristics in the treatment of diseases related with oxidative damage have been built already. The first approach was based on the entrapment of an antioxidant agent into the polymeric matrix to be protected and transported by the blood stream to the site of action. Superoxide dismutase (SOD) was entrapped, for example, into 291 nm PLGA nanoparticles synthesized by double emulsion method. The purpose was to protect human cultured neurons against oxidative damage promoted by hydrogen peroxide (H_2O_2). The results showed protection for 6 h compared to SOD in solution and pegylated SOD (PEG-SOD) at a dose of 100 U [12]. In another study, melatonin was entrapped in poly(methacrylic acid-*co*-methyl methacrylate) nanoparticles with Span 80© and Tween 80© as surfactants. The method used was nanoprecipitation, and the nanoparticle size was 241 ± 55 nm with a zeta potential of -33 ± 0.3 mV. The melatonin loaded nanoparticles showed better antioxidant protection compared with melatonin in solution at doses of 1 mg/kg and 10 mg/kg [13]. The entrapment of catalase (an enzyme that dissociates hydrogen peroxide into water and oxygen) into poly(ethylene grlycol (PEG) nanocarriers (spheres and filaments) by double emulsion was performed by Simone et al. with the purpose of promoting its resistance to proteases attack for an enhanced catalase activity.

The catalase entrapped in nanoparticles of 300 nm in size was more resistant to proteases attack, especially when catalase was conjugated to PEG [14].

A different approach was to provide antioxidant activity to the nanoparticles itself, by employing antioxidant materials in the making of the nanoparticle. The work of Williams *et al.* showed that conjugation of PEG with gluthatione (scavenger of hydroxyl radicals, superoxide radicals, singlet oxygen) self-assembled into nanoparticles. It was suggested that PEG formed the shell and glutathione formed the core by extended hydrogen bonding. The mean particle size as a function of PEG molecular weight ranged from 283 ± 23 nm to 315 ± 39 nm (M_n of 300 to 700 g/mol) measured by dynamic light scattering (DLS). When disulfide glutathione-PEG conjugate were used, the particle size increased to 400 nm approximately. The glutathione-PEG nanoparticle protected the human brain neuroblastoma cells against oxidative damage at comparable levels with free glutathione [15]. Nie *et al*. synthesized surface functionalized gold nanoparticles with synthetic antioxidant Trolox© (water soluble vitamin E analog). The data collected suggested that gold-Trolox nanoparticles with a mean size of 4.5 ± 0.7 nm were better antioxidants than Trolox in solution. The rate constant obtained by DPPH assay was 8 times higher for gold-Trolox nanoparticles than Trolox in solution [16]. In a different attempt to create nanoparticles of inherent antioxidant properties, the antioxidant α-lipoic acid was covalently conjugated to obtain molecules of different hydrophilicities which assembled into 200 to 600 nm nanoparticles (as a function of hydrophobicity) synthesized by nanoprecipitation. The nanoparticles showed an increased antioxidant activity as compared with α1-lipoic acid in solution [17].

Another approach, which was followed in this study, was to form polymeric nanoparticles by well established methods such as emulsion evaporation or in the presence of antioxidant surfactants. In this case, the final system consists of a polymeric core, a layer of antioxidant surfactant on the surface, and the active component (antioxidant or not) entrapped in the polymeric matrix. In this study, natural antioxidant molecules, alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C) were used to synthesize a new surfactant (EC) of antioxidant properties. Vitamin E is hydrophobic and vitamin C is hydrophilic, thus when the two are linked, an amphiphilic EC surfactant results. The selection of the two components was based on compelling information that vitamins E and C are some of the most potent natural antioxidants. Vitamin E has been syndicated as the main chain breaking agent of radical reactions (i.e. lipid

peroxidation) protecting hydrophobic molecules against reactive oxygen radicals (ROS) [18-21]; it has been documented that alpha-tocopherol acts mainly against peroxyl radicals and perhydroxyl radicals with rate constant of 2 x 10^5 l mol^{-1} s^{-1} [22]. On the other hand, at physiological pH, vitamin C is present in the ascorbate form. Ascorbate interacts with alpha-tocopheroxyl radical to regenerate alpha-tocopherol suggesting a synergetic effect between the two molecules (rate constant of 1.55 x 10^6 l mol^{-1} s^{-1}) [22]. Vitamin C reacts with perhydroxyl radicals at a rate constant of 1.6 x 10^4 l mol^{-1} s^{-1} [22]. It prevents damage by peroxynitrate, acts as a scavenger of hydroxyl radical, hypochlorous acid, thyl and sulphenyl radicals, and others [23]. Other natural antioxidant molecules such as polyphenols, carotenoids, coenzyme Q10, uric acid, albumins are important agents that defend biological systems against radicals attack [18] and could be further studied as viable components for synthesis of new surfactants of antioxidant properties.

While a literature review indicates that vitamin C and vitamin E derivatives have been synthesized as new synthetic antioxidants in an attempt to improve the antioxidant properties of the starting vitamin, or to form new surfactants [24-34], it was our goal to synthesize a novel surfactant of antioxidant properties from vitamin E and vitamin C to be used in nanoparticle synthesis. The present work describes the chemical vitamin E-vitamin C conjugation to form the EC surfactant, synthesis of nanostructures by self-assembly of the surfactant EC, as well as synthesis of polymeric nanoparticles by nanoprecipitation in the presence of EC. The nanostructures were characterized in terms of size, polydispersity, morphology, stability, and antioxidant activity were compared with nanostructures formed under similar conditions in the presence of Span80 as a surfactant.

3.2 Materials and Methods

3.2.1 Materials

Poly(lactide-co-glycolide acid) (PLGA) 50:50, molecular weight 30000-75000 g/mol, ascorbic acid, alpha-tocopherol, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 5,6-Isopropylideneascorbic acid, alpha-tocopherol, ethyl chloroformate, maleic anhydride, and anhydrous sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Acetone, ethanol, 1-propanol, hexane, acetonitrile, diethyl ether chloroform, ethyl acetate were HPLC grade (Mallinckrodt Baker, Pittsburgh, NJ).

3.2.2 Synthesis of EC Surfactant (Vitamin E-Vitamin C)

The synthesis of EC was performed in three steps (Figure 3-1), following a procedure similar to that described by Ogata and coworkers [35].

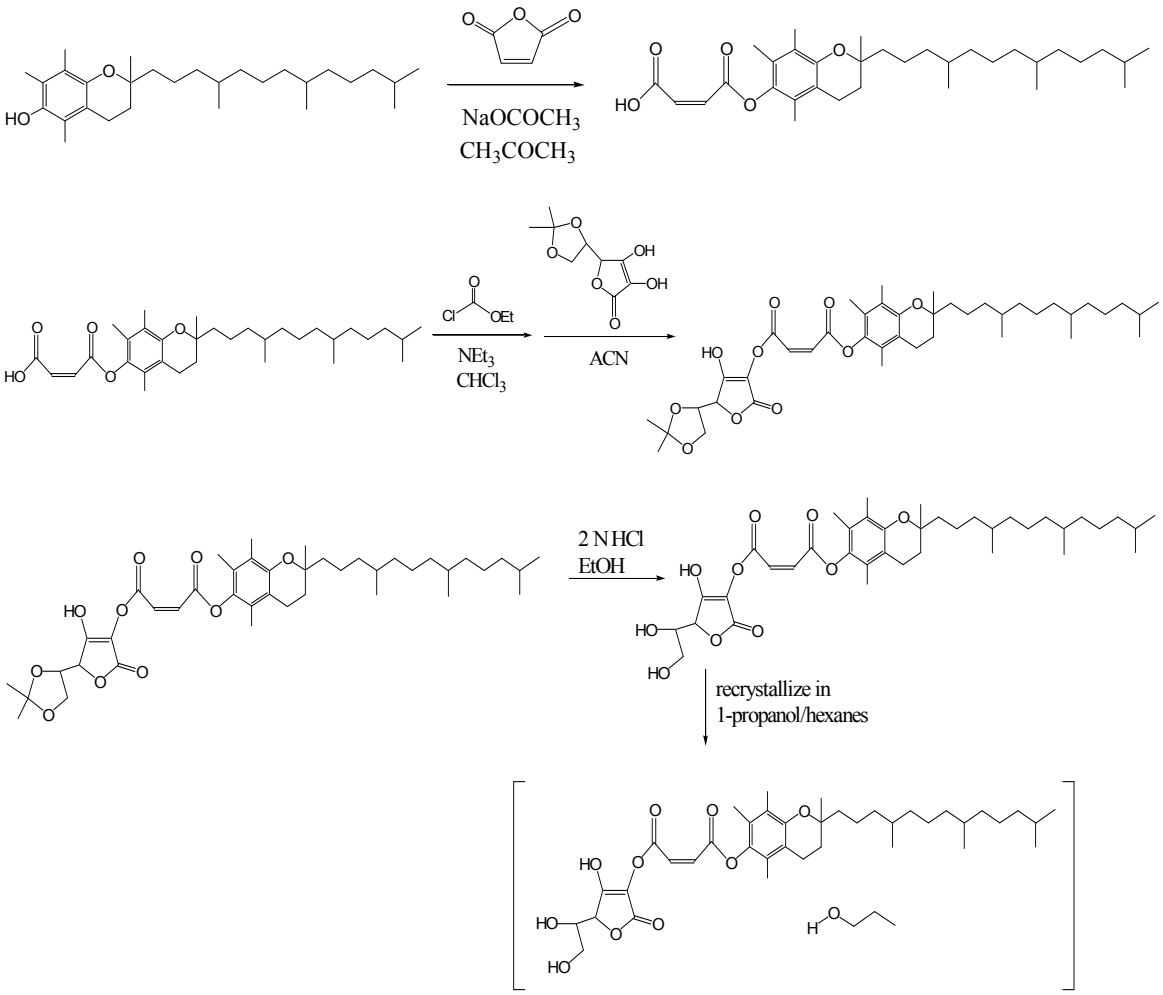


Figure 3-1: Reaction steps involved in EC synthesis

3.2.2.1 Synthesis of Maleic Acid Mono-Alpha-Tocopherol

Alpha-tocopherol (10.2 g, 0.024 mol), maleic anhydride (6.3 g, 0.064 mol), anhydrous sodium acetate (3.2 g, 0.39 mol) were placed in 65 ml of reagent grade acetone in a 100 ml round-bottomed flask and heated at reflux with stirring for 1 hour. The acetone was then removed by rotary evaporation. The reaction mixture was dissolved in 100 ml of diethyl ether and aqueous saturated ammonium chloride solution (50 ml) was added. This solution was stirred at room temperature for 20 minutes. The aqueous layer was removed and the organic layer was washed with water (2 X 100 ml). The ether layer was dried over $MgSO_4$, filtered and the ether

was removed by rotary evaporation to yield a golden-orange oil which slowly crystallizes (12.0 g, 96%). No further purification techniques were performed as the compound decomposes on silica gel.

^{1}H-NMR (300 MHz, CDCl3) δ0.84 (s), 0.87 (s), 0.88 (s), 0.89 (s), 0.97-1.18 (br m), 1.26 (br s), 1.28-1.40 (br m), 1.49-1.59 (br m), 1.70-1.90 (br m), 2.00 (s), 2.04 (s), 2.12 (s), 2.62 (br t, J = 9 Hz), 6.65 (1H, d, J = 13 Hz), 6.71 (1 H, d, J = 13 Hz).

^{13}C-NMR (75.5 MHz, CDCl3) δ11.91, 12.20, 13.04, 19.64, 19.71, 19.78, 20.61, 21.03, 22.66, 22.76, 24.46, 24.83, 28.00, 32.69, 32.80, 37.28, 37.35, 37.39, 37.45, 37.51, 39.37, 75.33, 117.69, 123.47, 124.37, 125.98, 128.07, 137.67, 139.74, 150.03, 164.68, 166.32.

3.2.2.2 Synthesis of 5,6-Isopropylideneascorbic Acid-2-O-Maleic Acid-Alpha-Tocopherol Diester

Maleic acid mono-alpha-tocopherol (6.3 g, 0.012 mol), triethylamine (4.1 g, 0.041 mol), and chloroform (33 ml) were combined in a 100 ml round-bottomed flask fitted with a septum and cooled to 0° C on an ice bath. Ethylchloroformate (2.0 g, 0.018 mol) was added dropwise. The reaction mixture was stirred at 0° C for 10 minutes. 5,6-Isopropylideneascorbic acid (3.6 g, 0.017 mol) in 45 ml acetonitrile was quickly added and the solution stirred at 0° C for 15 minutes and then warmed to 5° C over a 30 minute period with continued stirring. The solvent was then removed by rotary evaporation at room temperature. The reaction residue was then taken up in 40 ml of ethyl acetate, and this organic solution was washed with 2 N HCl (9.8 ml) and then with water (2 X 25 ml). The ethyl acetate solution was dried over $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. This yielded a golden oil (6.6 g, 76%). The compound was not further purified as it decomposed on contact with silica gel.

^{1}H-NMR on crude product (300 MHz, CDCl3) δ0.86 (s), 0.87 (s), 0.90 (s), 0.89 (s), 1.10-1.19 (br m), 1.24-1.27 (br m), 1.36 (s), 1.40 (s), 1.50-1.58 (br m), 1.74-1.86 (br m), 2.00 (s), 2.04 (s), 2.11 (s), 2.60 (br t, J = 6 Hz), 4.13 (sextet, J = 7 Hz), 4.38 (m), 4.70 (d, J = 3 Hz), 6.67 (s).

3.2.2.3 Synthesis of L-Ascorbic Acid-2-O-Maleic Acid-Alpha-Tocopherol Diester

5,6-isopropylideneascorbic acid-2-O-maleic acid-alpha-tocopherol diester (6.6 g, 0.0091 mol) was placed in 28 ml ethanol and 8 ml of 2N HCl solution. This solution was stirred at 60° C for 25 minutes. The ethanol was removed by rotary evaporator and the remaining residue was

dissolved in 35 ml of ethyl acetate. This was washed with water (2 X 10 ml). The ethyl acetate layer was dried with MgSO4, filtered and solvent was removed by rotary evaporation. The resulting oil was dissolved in 8.3 ml 1-propanol and 19 ml of hexanes. The solution was placed in the freezer overnight and crystals were collected by suction filtration. The crystals were recrystallized in 1:3 (v/v) 1-propanol: hexanes solution to yield a first crop of very light yellow solid which were dried for 7 hours under vacuum at room temperature (0.6388 g, 10 %).

^{1}H-NMR (300 MHz, d6-DMSO) δ0.80 (s), 0.82 (s), 0.83 (s), 0.84 (s), 0.85 (s), 0.95-1.13 (br m), 1.18-1.24 (br m), 1.35-1.55 (br m), 1.73 (br t), 1.95 (s), 1.97 (s), 2.01 (s), 2.55 (br t), 3.33 (t, J = 7 Hz), 3.81 (t, J = 7 Hz), 4.95 (s), 6.66 (d, J = 12 Hz), 7.08 (d, J = 12 Hz).

^{13}C-NMR (75.5 MHz, CDCl3) δ10.52, 11.64,12.02, 12.88, 19.54, 19.62, 19.65, 19.68, 20.00, 22.52, 22.61, 23.80, 24.25, 25.70, 28.85, 32.04, 32.09, 32.13, 36.67, 36.75, 36.78, 36.83, 38.84, 61.74, 62.52, 68.63, 74.84, 74.86, 75.54, 111.71, 117.09, 117.40, 121.89, 125.23, 126.56, 139.64, 148.82, 161.23, 163.42, 164.26, 167.41.

3.2.3 Nanoparticle Synthesis

EC and Span80 nanostructures in the absence or in the presence of poly(lactic-co-glycolic acid) were synthesized by a modified version of the nanoprecipitation method. An organic phase was made of acetone (0.5 ml) with dissolved PLGA (0 to 1.2 mg/ml) -when the polymer was used, and EC surfactant or Span80 (at various concentrations, 1 to 5 mg/ml). The organic phase was added to 5 ml of nanopure water (Thermo Scientific Barnstead Nanopure, Dubuque, IA) by a syringe under stirring on a magnetic stir plate (Corning, NY). Next, the sample was placed in a Buchi R-124 roto-evaporator (Buchi Corporation, New castle, DE) to remove the organic solvent, acetone. Evaporation was performed for 10 minutes under vacuum and nitrogen injection for fast solvent evaporation. The nanoparticle suspension was collected and stored for further analysis at 4 °C, under dark conditions.

3.2.4 Size, Size Distribution, and Zeta-Potential

The nanoparticle size and polydispersity index (PI) were evaluated by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Zeta potential measurements were obtained by Malvern Zetasizer Nano ZS with a MPT-2 autotritator required to study aggregation of particles against pH. The samples were diluted to a final concentration range of 0.1 mg/ml for all measurements.

3.2.5 Morphology of Antioxidant Nanoparticles

Transmission Electron Microscopy (TEM) with a JEOL 100-CX (JEOL USA Inc., Peabody, MA) microscope was used to study nanoparticle morphology. A droplet of the sample was mixed with a contrast agent (Uracyl acetate, 2%) and a carbon grid was passed on the surface of the droplet to create a film over the grid. The sample was placed in the TEM after 15 minutes for analysis.

3.2.6 DPPH Radical Scavenging Assay

A stock solution of DPPH in methanol at 0.4 mM was prepared and kept at -20 °C in the dark prior to use. The nanoparticle sample was diluted with acetic acid solution to 1 ml. The nanoparticle sample was added to dilute DPPH stock in methanol to obtain a final sample volume of 2 ml with a 0.1 mM DPPH concentration. The ratio between water and methanol was 1 to 1 v/v. The blank was prepared with 1 ml of acetic acid solution and pure methanol. The controls were prepared for each sample, with 1 ml of acetic solution and 1 ml of DPPH in methanol solution. Absorption readings were taken after 30 min at 518 nm using a Geminys 6 spectrophotometer (Thermo Scientific, Waltham, MA). The formula used to calculate the rate of oxidation was

$$\% \text{ change in activity} = ((Abs_{control} - Abs_{sample})/Abs_{control})*100 \quad [1]$$

The measurements were performed at 5 nanoparticle concentrations (0.025-0.1 mM). The data collected was used to obtain the best fit for the linear regression used to estimate the IC_{50}, which is the point at which 50% of the nanoparticles had been oxidized. Antioxidant activity of EC was measured at the same concentrations used for the nanoparticles synthesis.

3.2.7 DPPH Radical Scavenging Measurements by Electron Paramagnetic Resonance (EPR) Technique

The DPPH radical is widely used as a test radical to assess antioxidant capacity and activity of organic compounds. The EPR spectroscopy is one of the most sensitive techniques to detect and follow the behavior of the DPPH radical directly. Scavenging of the DPPH radical was followed by measuring the decrease in the EPR signal intensity.

EPR measurements were conducted on a Bruker EMX-20/2.7 EPR spectrometer (X-band) with dual cavities, modulation and microwave frequencies 100 kHz and 9.516 GHz, respectively. The typical parameters were: sweep width 100 G, EPR microwave power of 10 mW, and modulation amplitude 2.5 G. Time constant and sweep time were varied. Values of g-

factors were calculated using Bruker's WINEPR program which is a comprehensive line of software, allowing control of the Bruker EPR spectrometer, data-acquisition, automation routines, tuning, and calibration programs on a Window-based PC [http://us.bruker-biospin.com/brukerepr/winepr.html].

The samples were prepared as described in the DPPH assay method. The following solutions were subjected to the EPR examination; EC (0.1mM) mixed with DPPH (0.1mM) at ratio 1:1, EC–PLGA nanoparticles (NP) (0.1mM EC) mixed with DPPH (0.1mM) at ratio 1:1. As a reference, 0.1mM DPPH in 1:1 methanol:water solution was used. The solutions were prepared right before EPR measurements. A 30 µl sample was transferred into EPR capillary tube (i.d. ~ 1mm, o.d. 1.55mm) and the end was sealed by a critoseal (Fisherbrand). Then, the capillary was inserted in an ordinary 4 mm EPR tube and was placed into the EPR resonator to measure the EPR spectra of the DPPH radicals over time. The delay time was between 1 and 2 minutes after mixing of EC or NP with DPPH before the first EPR measurement was performed.

A kinetic measurement of DPPH radical scavenging upon mixing of EC (or NP) with DPPH was also performed. A volume of 30 µl sample was transferred into the EPR tube (o.d = 3mm, i.d. = 2mm), positioned in the cavity, and the same quantity of DPPH was injected rapidly to the solution by syringe through the tiny PVC hose (i.d =.0.3 mm) located directly in solution (close to the bottom of EPR tube).

The magnetic field was fixed at a specified value (at the strongest peak of the DPPH radical) and the EPR signal intensity was monitored as a function of time (vide infra, cf. Figure 3-12, top line).

3.2.8 Statistical Analysis

The statistical analysis was performed in SAS (Cary, NC). Anova proc mixed procedure with Tukey adjustment was used and significant differences were declared at a p value of 0.05. All the analyses were performed in triplicate.

3.3 Results and Discussions

EC exhibited poor water solubility, but it was soluble in polar solvents such as acetone. Hence, acetone was used to dissolve EC and/or surfactant Span80, as well the polymer PLGA. Upon addition of the organic phase to water and subsequent diffusion and evaporation of acetone, water dispersions of self-assembled EC and Span 80 formed. In the presence of PLGA,

polymeric nanoparticles stabilized by the surfactant (EC or Span80) were formed. The technique described is a modified version of nanoprecipitation, technique which allows formation of nanostructures by using diffusion properties of polar solvents and anti-solvent properties of polymers [36]. The effect of salts and surfactant concentration on the size of nanostructures formed from pure EC and Span 80 surfactants was studied. Next, polymeric particles with PLGA and each surfactant were made, and the effect of surfactant, PLGA ratio on nanoparticle size, and polydispersity was assessed. Finally, a mixture of EC and span 80 was used to analyze the effect of EC: span80 ratio on PLGA particle size and polydispersity. The antioxidant behavior was analyzed by DPPH and EPR.

3.3.1 Nanostructures Formed by Self-Assembly of EC and Span 80- Effect of Salt Type and Concentration

The effect of the type and concentration of salts present in the aqueous phase on the size and polydispersity of the nanostructures formed by EC and Span 80 surfactants was studied (Figure 3-2). The EC nanostructures formed in an aqueous solution of sodium bicarbonate showed a smaller particle size (Figure 3-2A) as compared to the size of the nanostructured formed in the presence of sodium chloride (statistically different). The EC nanoparticle size ranged between 12 and 30 nm for the salt concentrations studied (0-100 mM). In contrast, the polydispersity index (>0.5) was higher for these particles as compared to polydispersity of particles made in the presence of NaCl (Figure 3-2B).

The EC nanostructures synthesized in the presence of sodium chloride showed a mean size of 22 to 138 nm in diameter with a polydispersity index that ranged from 0.175 to 0.267. The tendency was for the size to increase as the salt concentration increased from 0 to 100 mM. The size increase was expected because the addition of salts increases the hydration of the electric double layer with a subsequent reduction of the electric repulsion by screening effect of the counter-coions which promoted aggregation. The aggregation number increases and critical micelle concentration (cmc) decreases with concentration of salt added [37,38]. Samples synthesized with sodium bicarbonate presented the smallest nanostructure size ranging from 11.6±1.6 to 30.9 ±6.9, with a minimum nanostructure size at 15 mM of sodium bicarbonate. The difference in nanostructure size formed in the presence of sodium chloride and sodium bicarbonate is explained by the counterions–coions localized in the electric double layer. The radii of chloride ions (1.8 Å) is bigger than OH^- and HCO_3^- ions (1.4, 1.6 Å) [39-41] with bigger

hydration size. When chloride ions are present in the electric double layer, the repulsion is smaller compared with the other ions, so bigger structures are formed by aggregation. The higher polydispersity index values for all nanostructures were at the lowest salt concentration tested (1.5 mM).

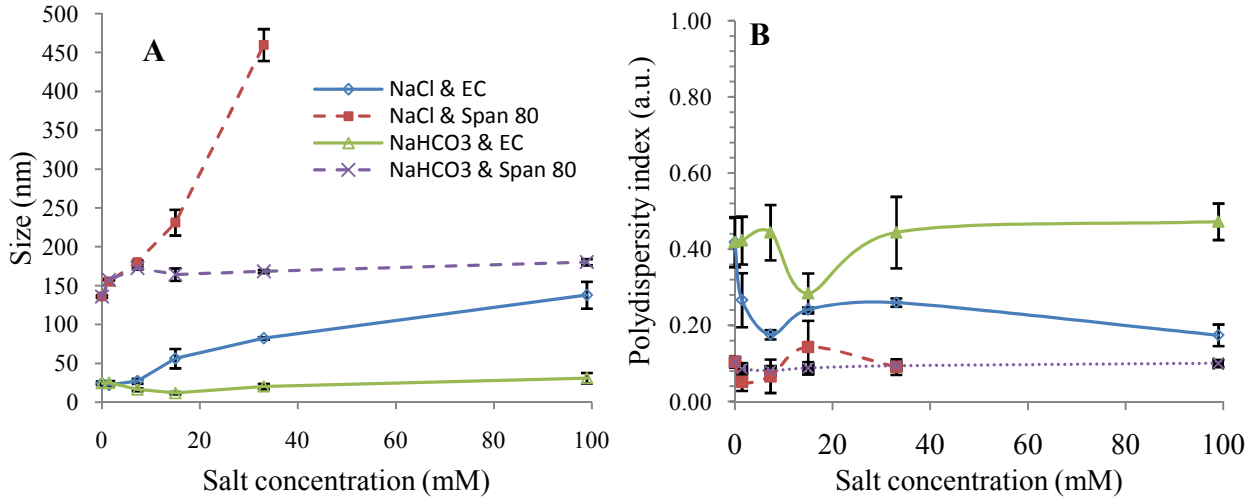


Figure 3-2: Effect of salt concentration on the size and polydispersity of Span 80 and EC nanostructures. (a) Nanostructure diameter and (b) Polydispersity index for Span 80 and EC self-assembled nanostructures in sodium bicarbonate and sodium chloride aqueous solutions.

The nonionic Span 80 self-assembled into bigger nanostructures as compared to EC (statistically different, Appendix B). When synthesized in an aqueous solution of sodium bicarbonate, the mean Span80 particle size ranged from 157 to 182 nm (Figure 3-2). The polydispersity index obtained varied between 0.052 and 0.144. At the lower salt concentration monodisperse Span80 nanostructures, represented by a polydispersity index value lower than 0.1, were formed. The Span80 samples synthesized in the presence of sodium chloride showed an increase in the particle size as a function of salt concentration. Moreover, the nanostructures synthesized with 99 mM sodium chloride in the aqueous phase presented phase separation following evaporation of acetone. The presence of chloride and sodium ions at high concentrations promoted less hydrogen bond interactions with the OH groups of Span 80 and the salting out process was responsible for the observed precipitation.

TEM pictures revealed that spherical shaped EC nanostructures were formed when sodium chloride was used in the aqueous phase (Figure 3-3). The size observed by TEM was in agreement with the DLS data and suggested a reduced aggregation and more uniform particle

size. Contrary to EC, hydrophobic Span 80 had a tendency to form bilayers similar to those formed by phospholipids, tendency which reflected in the bigger size of the Span 80 nanostructures and their smaller polydispersity index (Figure 3-4).

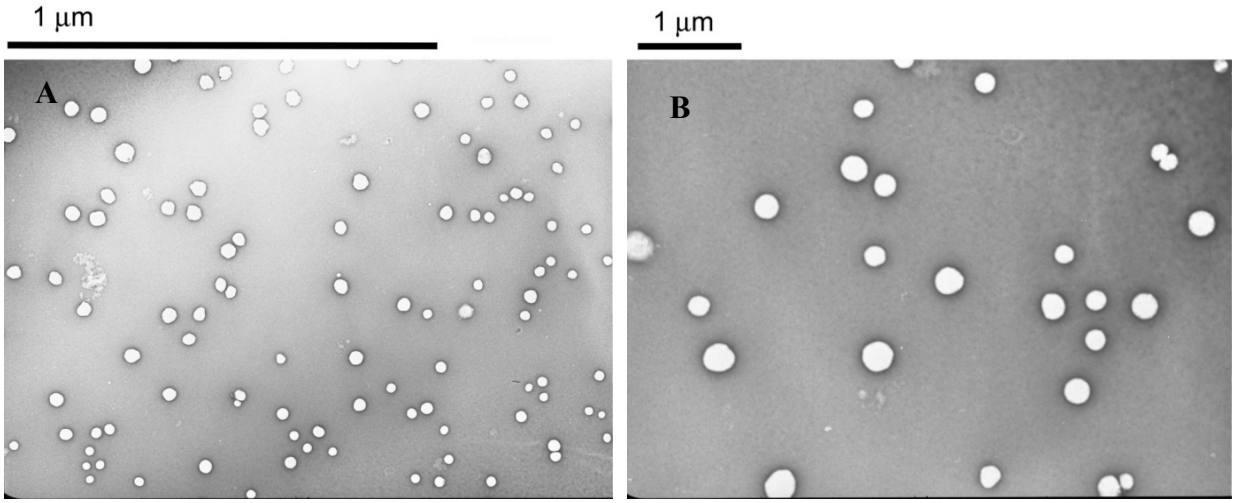


Figure 3-3: TEM pictures of EC surfactant in sodium chloride solution at 66x (A) and 16x (B) magnifications.

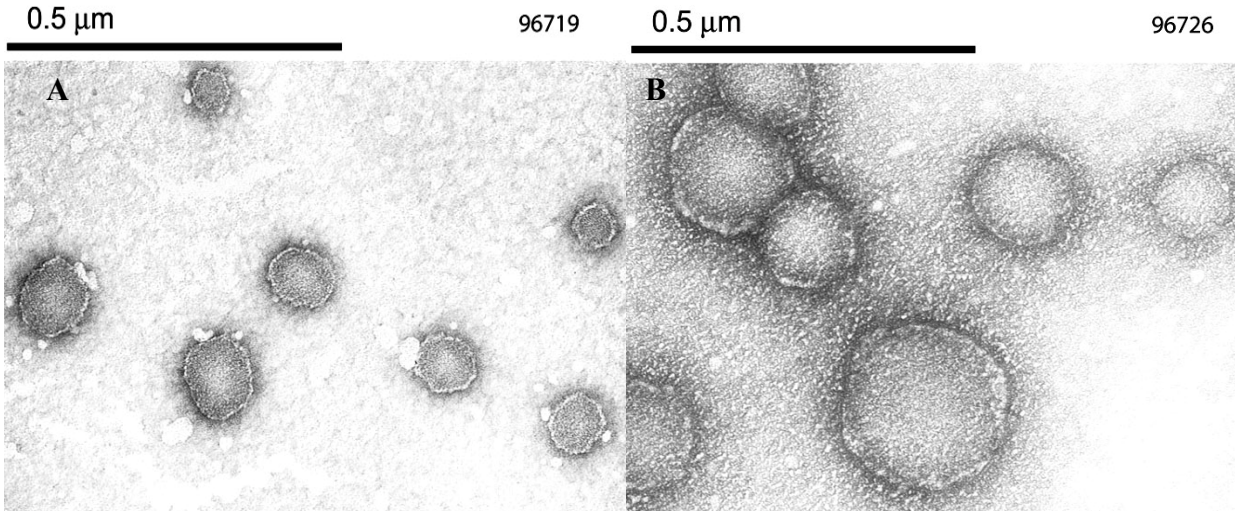


Figure 3-4: TEM pictures of Span 80 self-assembled nanostructure in sodium bicarbonate solution. Note: Contrast was enhanced by adding aqueous gold nanoparticles (4 nm Sigma Aldrich, 50 µL) to the organic phase in a concentration of 0.1 mg/ml prior to the TEM analysis as described in the methodology section.

3.3.2 Nanostructures Formed by Self-assembly of EC and Span 80- Effect of Surfactant Type and Concentration

Particle size increased with an increase in the concentration of the surfactant for both EC and Span 80. Polydispersity of self-assembled EC and Span 80 (non-ionic surfactant) increased as well, as the concentration of the components increased (Figure 3-5). The EC nanostructures showed a particle size that did not change at low surfactant concentrations (1-5 mM), but at concentrations exceeding 5 mM the size of the EC nanostructures increased sharply in acetic acid Samples formed with sodium bicarbonate were more stable, but the particle size increase was observed still at 7.28 mM EC. The increase in the amount of EC promoted aggregation which reflected in the high values of polydispersity index (Figure 3-5B) for the sample with acetic acid (0.749 for 7.3 mM of EC). For EC concentrations of 1.5 to 4.4 mM the polydispersity values were 0.149 to 0.171 which indicated formation of a monodisperse suspension. The particles synthesized in sodium bicarbonate presented a higher polydispersity value (0.516 to 0.241) in the 1.5 to 4.4 mM EC concentration range. At an EC concentration of 7.28 mM, the polydispersity was smaller (0.277) as compared to the sample formed in acetic acid (0.749).

Particles synthesized with Span80 without salt in the aqueous phase showed bigger particle size than the EC particles, with a stable particle size increase as the surfactant concentration was increased between 140.8 to 264.8 nm for 1.5 to 23.3 mM of Span 80, respectively. The polydispersity showed a stable increment from 0.074 to 0.335. Higher concentrations presented phase separation in which Span 80 was no longer able to form nanostructures.

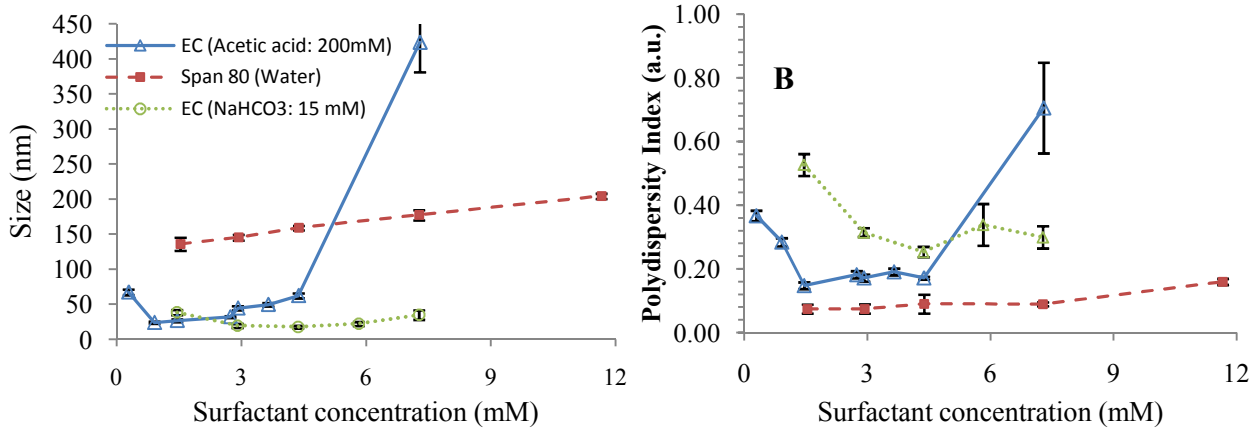


Figure 3-5: (A) Size and (B) polydispersity index of nanostructures synthesized at different EC and Span 80 surfactant concentrations.

3.3.3 Synthesis of Nanostructures with a Mixture of EC and Span 80

The effect of using a mixture of Span 80 and EC was evaluated on the nanostructures size and polydispersity (Figure 3-6). The tendency was for the particle size to increase when more Span 80 was added to the mixture (statistically different for each surfactant mixture concentration, appendix B). In the other side, the polydispersity tended to decrease to values of monodisperse suspensions (polydispersity < 0.1) with the addition of more Span 80. The data suggested that a reduction in the aggregation process promoted by the non-ionic surfactant Span 80 was achieved with the addition of the anionic EC surfactant, which promoted ionic repulsion and favored particle stabilization.

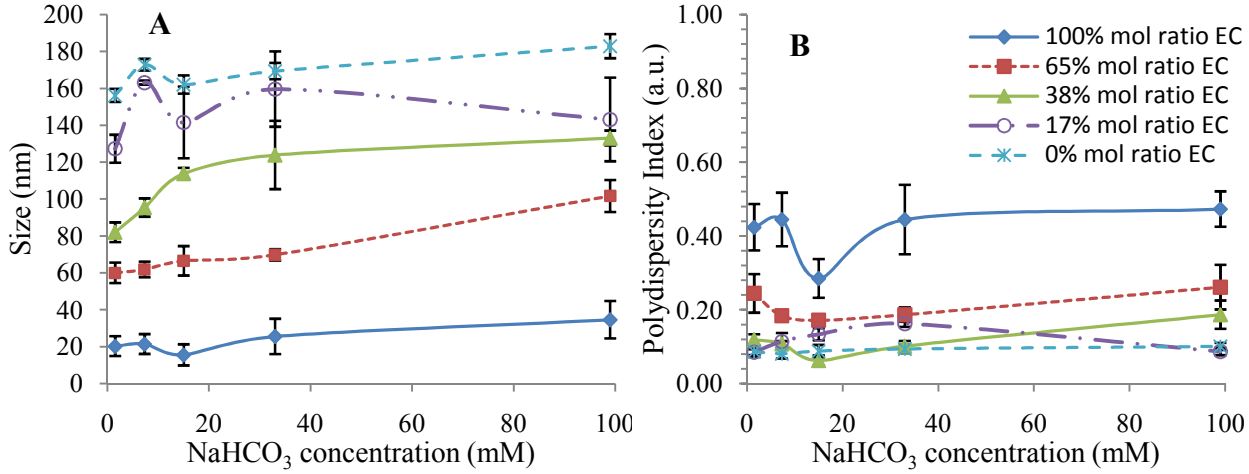


Figure 3-6: (A) Size and (B) polydispersity index values for nanostrucutres synthesized with a mixture of EC and Span80 surfactants.

3.3.4 PLGA Nanoparticles Synthesized with EC and Span 80 Surfactants- Effect of PLGA/Surfactant Ratio

The PLGA-EC nanoparticles presented a mean size ranging from 54 to 130 nm (Figure 3-7) as a function of the amount of PLGA entrapped in the core; the tendency was for the nanoparticle size to increase as the amount of PLGA increased. The size was relatively uniform over the different PLGA concentrations used. Addition of PLGA to the EC nanostructures did not increase aggregation, and the structures were more uniform compared with those formed by EC alone. The polydispersity index varied from 0.113 to 0.151. The reason of a more uniform PI compared to the nanostructures formed by EC alone is that PLGA promoted a more stable EC particle by adding a hydrophobic core which interacted with the hydrophobic tail of vitamin E present in the EC surfactant, so the hydrophobic interactions are stronger. The PLGA

nanoparticles synthesized with Span 80 presented a bigger size in comparison with the PLGA-EC nanoparticles, and ranged from 155 to 216 nm. The polydispersity index was similar to that of the PLGA-EC nanoparticles (0.115-0.165).

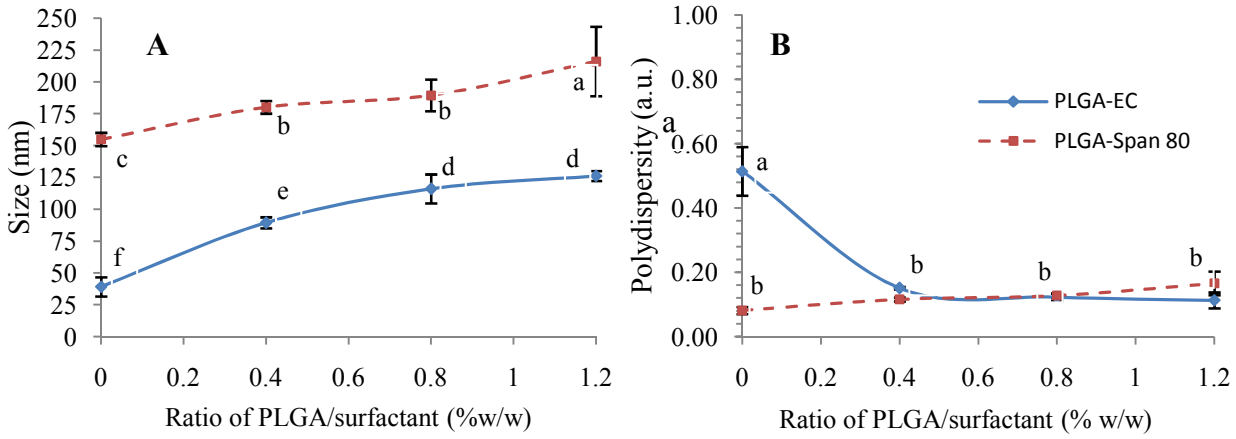


Figure 3-7: Size (A) and polydispersity (B) of polymeric PLGA nanoparticles synthesized with surfactant E-C and Span80.

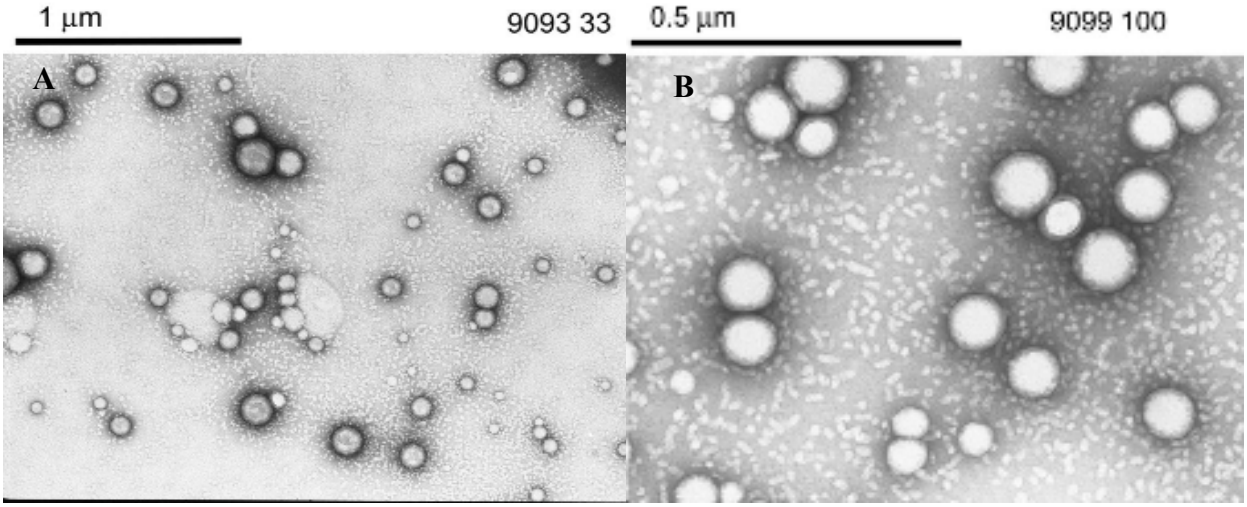


Figure 3-8: PLGA-EC nanoparticles (ratio PLGA to EC was 0.8 to 1) in sodium bicarbonate (15 mM) with a magnification (A) 33x and (B) 100x. The EC concentration used was 1 mg/ml)

TEM pictures (Figure 3-8 and Figure 3-9) of polymeric PLGA nanoparticles synthesized with EC and span 80 showed that the EC-PLGA nanoparticles presented a uniform size distribution with limited aggregation. The PLGA core promoted a uniform anchor to the hydrophobic vitamin E moiety of EC surfactant. The vitamin C moiety most likely placed itself at the interphase, facing the aqueous environment and allowing particle suspension in water. The PLGA-span 80 nanostructures were less uniform in size as exhibited by the TEM pictures

(Figure 3-9). A less uniform sample, with a tendency to aggregate was formed with PLGA and Span 80. The sample showed a mixture of two different structures, one formed of Span 80 alone, grey in appearance, in the form of vesicles (similar to those presented in Figure 3-4), and nanoparticles with PLGA and Span 80, depicted by the white circles.

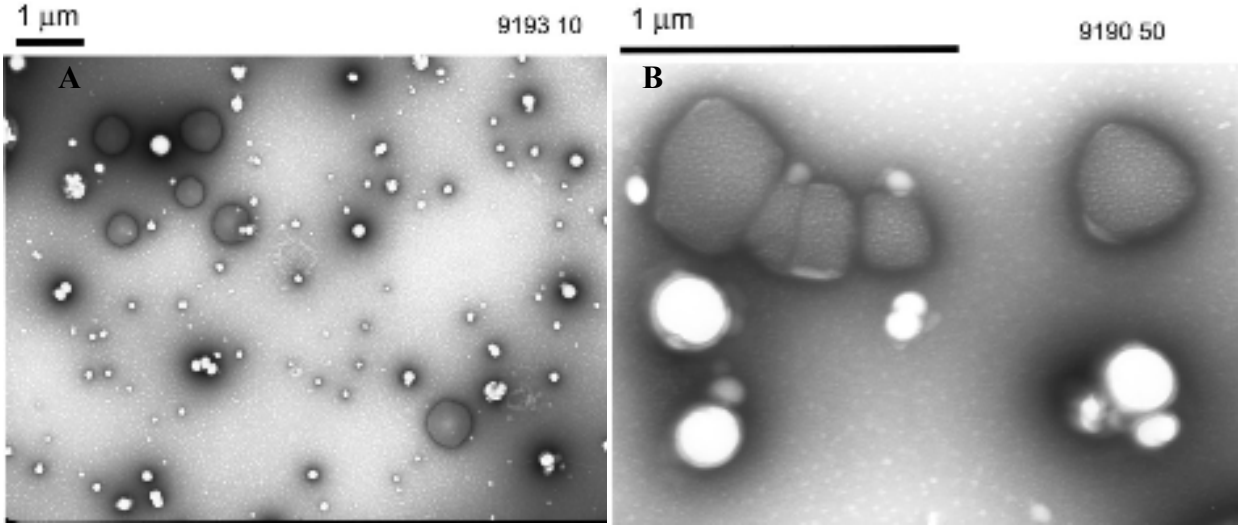


Figure 3-9: PLGA-Span80 nanoparticles (ratio PLGA to EC was 0.8 to 1) in $NaHCO_3$ (15 mM); two types of nanostructures are present, PLGA-Span 80 (white structures) and Span 80 vesicles (grey structures), (A) 10x and (B) 50x.

3.3.5 Antioxidant Properties of PLGA Nanoparticles Assessed by DPPH Assay

The DPPH technique was used to evaluate the antioxidant activity of PLGA-EC nanoparticles (PLGA-span80 particles had no antioxidant activity). Linear regression was used to calculate the 50 % inhibition of DPPH (Figure 3-10). The IC_{50} ranged from 30.6 to 37.4 µM for various PLGA concentrations, values which were not significantly different. The data suggested that the PLGA, size, and PI did not affect the antioxidant properties of the PLGA-EC nanoparticles with IC_{50} measuring 37.4 µM, 30.6 µM, 31.6 µM, and 33.4 µM for PLGA-EC particles made with polymer concentrations of 0 to 120% PLGA. The vitamin C moiety of the EC surfactant molecule contributed to the antioxidant activity of the polymeric antioxidant nanoparticle. The EC surfactant prepared with methanol water (1:1 volume ratio) showed similar IC_{50} to the nanoparticles synthesized with EC (30.1 µM). The pure vitamin C and E showed IC_{50} values of 21.7 µM and 17.5 µM, respectively. The differences in IC_{50} between pure vitamin E

and C with EC in solution and EC in nanoparticles were explained by the loss of an active OH group by the covalent link to vitamin C while rendered vitamin E moiety inactive.

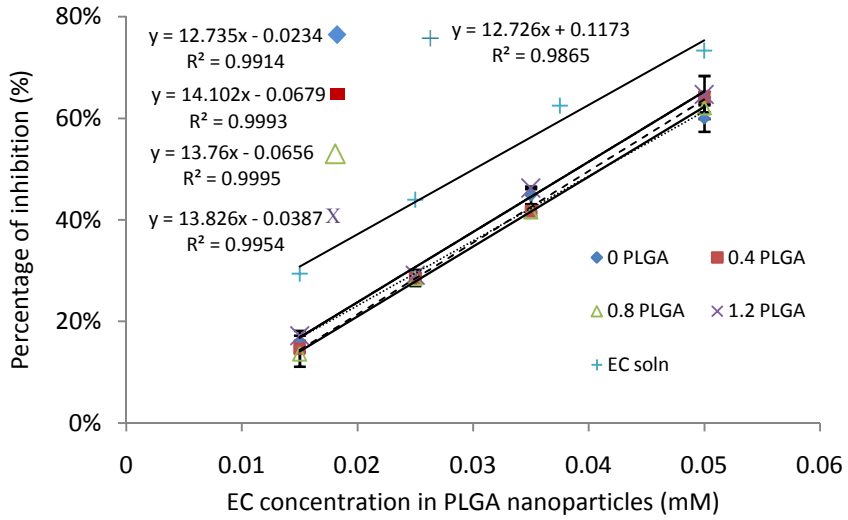


Figure 3-10: Rate of radical inhibition of PLGA-EC nanoparticles as a function of PLGA concentration as measured by the DPPH method

3.3.6 Electron Paramagnetic Resonance Measurements

Scavenging of DPPH radical by EC or EC-NPs was followed by measuring the decrease in the EPR signal intensity, DI/N, Figure 3-11 (green triangles for NP and black circles for EC). The free DPPH radicals degraded at a noticeable rate during first 30-40 minutes, stabilizing at value of DI/N = ~ 8, and then degraded at a slower rate, while the degradation of DPPH after 30 min was minimum (Figure 3-11). The degradation of DPPH radicals was remarkable in the presence of free EC or EC-NPs which was reflected in the strong decay in the first 1 to 2 minutes. The initial concentration of DPPH radicals dropped from DI/N = 12 units to ~ 7 units by scavenging either by free EC and EC-NPs. The DPPH radicals still interacted effectively with EC and EC-NPs after the initial 2 minutes of reaction time; degradation slowed down over time and ended in 50 - 60 minutes.

A remarkable degradation of DPPH radicals occurred not only in the first two minutes, but in fact during the first 5 - 6 seconds of mixing of EC or NP with DPPH. For instance, a rapid injection of DPPH solution into an EPR tube containing EC-NPs (or EC) solution showed a typical fast degradation curve, (Figure 3-12, top line), with ~ 40 % of initial DPPH degraded during first 5-6 seconds in diffusion regime. The comparison of antioxidant activity of organics

in this region, where the reaction time is measured in seconds, a fast mixing technique must be applied [16]. Another alternative is to compare scavenging activity of EC and EC-NPs over the time period, when the degradation rates of DPPH radicals are not fast and might be detected easily, for instance between 2 and 10 minutes after mixing of components (Figure 3-11). Using this approach, it was found that the antioxidant (scavenging) activity of EC was twice higher than that of EC-NPs (at the same EC concentration) when we compared the maximum slopes (at the beginning) for EC in relative units of intensity (0.21 units per minute) with the slope for EC-NPs (0.10 units per minute).

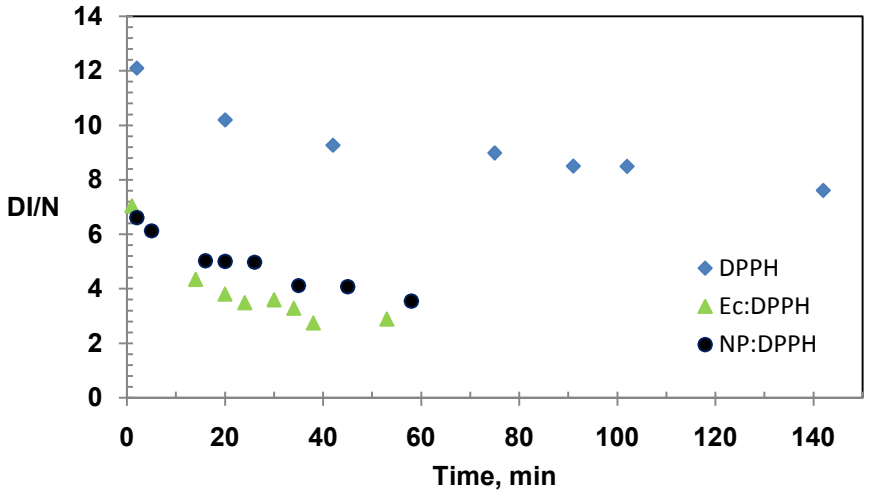


Figure 3-11: Scavenging of DPPH radicals by EC (triangles) and NP (black circles). Blue quadrates represent self degradation of DPPH radicals as a reference. (DI/N value is the double integrated (DI) intensity of the EPR spectrum that has been normalized (N) to account for the conversion time, receiver gain, number of data points and sweep width [http://www.bruker-biospin.com/winepr.html?&L=0]).

While EC was founded twice more potent than EC-NPs, EPR measurements clearly indicated that both EC and EC-NPs exhibited antioxidant activity. Three regions of interaction of DPPH with EC or EC-NPs were identified and used to account for different mechanisms of interaction between the antioxidant and DPPH over time; within seconds, the antioxidant activity of free EC or EC-NPs was similar. Within the intermediate region between 2 and 10 minutes incubation, the activity of EC was almost twice higher in comparison with EC- NPs. During the slow region, at times longer than 10 minutes incubation, the activity of EC in free EC and EC-NPs was comparable.

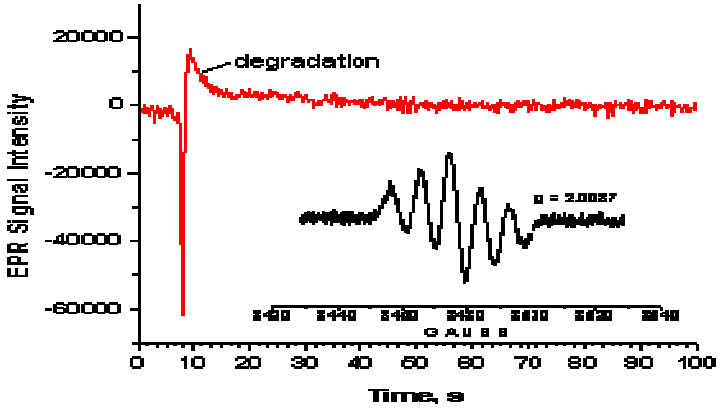


Figure 3-12: Time dependence of DPPH radical degradation (red line) in solution of NP (0.1mM): DPPH (0.1mM) = 1:1 in EPR tube (diffusion regime). The inset shows the EPR spectrum of DPPH radicals with g value 2.0037

3.4 Conclusions

A new antioxidant surfactant made of vitamin E and vitamin C was successfully synthesized and it was used to form PLGA polymeric nanoparticles with antioxidant activity. The EC surfactant was hydrophobic with a good solubility in polar solvents. Nanoprecipitation technique was used to synthesize self-assembled EC and Span 80 nanostructures, as well as polymeric PLGA-span 80 and PLGA-EC nanoparticles. The EC nanostructures were spherical and measured between 12 and 140 nm depending on the salt and surfactant concentration. Smaller EC nanostructures (11.6±1.6 nm) were obtained when sodium bicarbonate was used in the aqueous phase. In the presence of sodium chloride, bigger particles were formed, with particle sizes ranging from 22.0±1.3 to 137.8±17.4 nm at NaCl concentration of 1.5 to 99 mM. The polydispersity index was smaller for particles made in NaCl, and measured 0.267 to 0.175 at 1.5 to 99 mM NaCl concentration, as compared to values of 0.575 to 0.524 for the same concentration range in sodium bicarbonate. Span 80 formed vesicles of 136 to 460 nm, with a polydispersity index representative to a monodisperse suspension. The PLGA polymeric nanoparticle synthesized with the synthetic hydrophobic Span 80 surfactant showed a bigger particle size (180.1±12.4 to 216±27.3 nm) as compared to the PLGA-EC nanoparticles (89.6±4.5 to 126.2±3.9 nm). The polymeric PLGA-EC nanoparticles showed an antioxidant activity (IC_{50} of 36.3 to 42.1 mM as measured by DPPH assay). The capacity to provide antioxidant action was

provided by the vitamin C moiety of the EC surfactant which localized at the interface between the PLGA hydrophobic core and water. In comparison, the PLGA-span80 particles did not show any antioxidant activity. The antioxidant activity of EC and EC-PLGA nanoparticles has been confirmed by EPR measurements. Even though the scavenging activity of EC was about twice higher than that of EC-PLGA NPs, the antioxidant properties were in a similar range. The newly synthesized EC surfactant was therefore found successful in forming polymeric nanoparticles of intrinsic antioxidant properties.

3.5 References

[1] Chiellini E, Chiellini EE, Chiellini F, Solaro R. Targeted administration of proteic drugs. I. Preparation of polymeric nanoparticles. Journal of Bioactive and Compatible Polymers 2001;16 (6):441-65.

[2] Astete CE, Sabliov CM. Synthesis and characterization of PLGA nanoparticles. Journal of Biomaterials Science-Polymer Edition 2006;17 (3):247-89.

[3] Kreuter J. Nanoparticles and microparticles for drug and vaccine delivery. Journal of Anatomy 1996;189:503-5.

[4] Damge C, Vranckx H, Balschmidt P, Couvreur P. Poly(alkyl cyanoacrylate) nanospheres for oral administration of insulin. Journal of Pharmaceutical Sciences 1997;86 (12):1403-9.

[5] Mukerjee A, Pruthi V. Oral insulin delivery by polymeric nanospheres. Journal of Biomedical Nanotechnology 2007;3 (1):68-74.

[6] Rawat M, Singh D, Saraf S, Saraf S. Nanocarriers: Promising vehicle for bioactive drugs. Biological & Pharmaceutical Bulletin 2006;29 (9):1790-8.

[7] Agarwal A, Saraf S, Asthana A, Gupta U, Gajbhiye V, Jain NK. Ligand based dendritic systems for tumor targeting. International Journal of Pharmaceutics 2008;350 (1-2):3-13.

[8] Kreuter J. Drug Targeting with Nanoparticles. European Journal of Drug Metabolism and Pharmacokinetics 1994;19 (3):253-6.

[9] Rawat A, Vaidya B, Khatri K, Goyal AK, Gupta PN, Mahor S, Paliwal R, Rai S, Vyas SP. Targeted intracellular delivery of therapeutics: an overview. Pharmazie 2007;62 (9):643-58.

[10] Hammady T, Rabanel JM, Dhanikula RS, Leclair G, Hildgen P. Functionalized nanospheres loaded with anti-angiogenic drugs: Cellular uptake and angiosuppressive efficacy. European Journal of Pharmaceutics and Biopharmaceutics 2009;72 (2):418-27.

[11] Zhao XB, Li H, Lee RJ. Targeted drug delivery via folate receptors. Expert Opinion on Drug Delivery 2008;5 (3):309-19.

[12] Reddy MK, Wu L, Kou W, Ghorpade A, Labhasetwar V. Superoxide dismutase-loaded PLGA nanoparticles protect cultured human neurons under oxidative stress. Applied Biochemistry And Biotechnology 2008;151 (2-3):565-77.

[13] Schaffazick SR, Siqueira IR, Badejo AS, Jornada DS, Pohlmann AR, Netto CA, Guterres SS. Incorporation in polymeric nanocapsules improves the antioxidant effect of melatonin against lipid peroxidation in mice brain and liver. European Journal Of Pharmaceutics And Biopharmaceutics: Official Journal Of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E V 2008;69 (1):64-71.

[14] Simone EA, Dziubla TD, Arguiri E, Vardon V, Shuvaev VV, Christofidou-Solomidou M, Muzykantov VR. Loading PEG-catalase into filamentous and spherical polymer nanocarriers. Pharmaceutical Research 2009;26 (1):250-60.

[15] Williams SR, Lepene BS, Thatcher CD, Long TE. Synthesis and characterization of poly(ethylene glycol)-glutathione conjugate self-assembled nanoparticles for antioxidant delivery. Biomacromolecules 2009;10 (1):155-61.

[16] Nie Z, Liu KJ, Zhong CJ, Wang LF, Yang Y, Tian Q, Liu Y. Enhanced radical scavenging activity by antioxidant-functionalized gold nanoparticles: A novel inspiration for development of new artificial antioxidants. Free Radical Biology and Medicine 2007;43 (9):1243-54.

[17] Lee BS, Yuan X, Xu Q, McLafferty FS, Petersen BA, Collette JC, Black KL, Yu JS. Preparation and characterization of antioxidant nanospheres from multiple alpha-lipoic acid-containing compounds. Bioorganic & Medicinal Chemistry Letters 2009;19 (6):1678-81.

[18] Sabliov CM, Astete CE. Encapsulation and Controlled Release of Antioxidants and Vitamins. In: Garti N, editor. Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals. New York: CRC Press, 2008. pp. 297-330.

[19] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. Faseb Journal 1999;13 (10):1145-55.

[20] Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine 2007;43 (1):4-15.

[21] Halliwell B. Antioxidants in human health and disease. Annual Review of Nutrition 1996;16:33-50.

[22] Denisov ET, Afanas*ev IB. Oxidation and antioxidants in organic chemistry and biology. Boca Raton, FL: Taylor & Francis, 2005.

[23] Halliwell B. Vitamin C: Antioxidant or pro-oxidant in vivo? Free Radical Research 1996;25 (5):439-54.

[24] Noguchi N, Iwaki Y, Takahashi M, Komuro E, Kato Y, Tamura K, Cynshi O, Kodama T, Niki E. 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran: Design and evaluation as a novel radical-scavenging antioxidant against lipid peroxidation. Archives of Biochemistry and Biophysics 1997;342 (2):236-43.

[25] Chabrier PE, Auguet M, Spinnewyn B, Auvin S, Cornet S, Demerle-Pallardy C, Guilmard-Favre C, Marin JG, Pignol B, Gillard-Roubert V, Roussillot-Charnet C, Schulz J, Viossat I, Bigg D, Moncada S. BN 80933, a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation: a promising neuroprotective strategy. Proceedings Of The National Academy Of Sciences Of The United States Of America 1999;96 (19):10824-9.

[26] Capuzzi G, LoNostro P, Kulkarni K, Fernandez JE. Mixtures of stearoyl-6-O-ascorbic acid and alpha-tocopherol: A monolayer study at the gas/water interface. Langmuir 1996;12 (16):3957-63.

[27] Rezk RM, Haenen GRMM, van der Vijgh WJF, Bast A. The extraordinary antioxidant activity of vitamin E phosphate. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 2004;1683 (1-3):16-21.

[28] Iuliano L, Pedersen JZ, Camastra C, Bello V, Ceccarelli S, Violi F. Protection of low density lipoprotein oxidation by the antioxidant agent IRFI005, a new synthetic hydrophilic vitamin E analogue. Free Radical Biology and Medicine 1999;26 (7-8):858-68.

[29] Rosenau T, Adelwohrer C, Kloser E, Mereiter K, Netscher T. Bromination of alpha-tocopherol methano-dimer and ethano-dimer. Tetrahedron 2006;62 (8):1772-6.

[30] Rosenau T, Habicher WD. "Vitamin CE," a novel prodrug form of vitamin E. Chemical & Pharmaceutical Bulletin 1997;45 (6):1080-4.

[31] Tafazoli S, Wright JS, O'Brien PJ. Prooxidant and antioxidant activity of vitamin E analogues and troglitazone. Chemical Research in Toxicology 2005;18 (10):1567-74.

[32] Campo GM, Ceccarelli S, Squadrito F, Altavilla D, Dorigotti L, Caputi AP. Raxofelast (IRFI 016): A new hydrophilic vitamin E-like antioxidant agent. Cardiovascular Drug Reviews 1997;15 (2):157-73.

[33] Morley S, Cross V, Cecchini M, Nava P, Atkinson J, Manor D. Utility of a fluorescent vitamin E analogue as a probe for tocopherol transfer protein activity. Biochemistry 2006;45 (4):1075-81.

[34] Lo Nostro P, Ramsch R, Fratini E, Lagi M, Ridi F, Carretti E, Arnbrosi M, Ninham BW, Baglioni P. Organogels from a vitamin C-based surfactant. Journal of Physical Chemistry B 2007;111 (40):11714-21.

[35] Ogata K, Saito N, Yamada K, Maruyama G. L-ascorbic acid 2-O-maleic acid a-tocopherol diester 1-propanol adduct and process for producing the same. U.S. Patent 6,828,348 Dec 7, 2004.

[36] QuintanarGuerrero D, Allemann E, Doelker E, Fessi H. A mechanistic study of the formation of polymer nanoparticles by the emulsification-diffusion technique. Colloid and Polymer Science 1997;275 (7):640-7.

[37] Bohmer MR, Koopal LK, Lyklema J. Micellization of Ionic Surfactants - Calculations Based on a Self-Consistent Field Lattice Model. Journal of Physical Chemistry 1991;95 (23):9569-78.

[38] Griffiths PC, Paul A, Heenan RK, Penfold J, Ranganathan R, Bales BL. Role of counterion concentration in determining micelle aggregation: Evaluation of the combination of constraints from small-angle neutron scattering, electron paramagnetic resonance, and time-resolved fluorescence quenching. Journal of Physical Chemistry B 2004;108 (12):3810-6.

[39] Pau PCF, Berg JO, Mcmillan WG. Application of Stokes-Law to Ions in Aqueous-Solution. Journal of Physical Chemistry 1990;94 (6):2671-9.

[40] Keesee RG, Lee N, Castleman AW. Properties of Clusters in the Gas-Phase .3. Hydration Complexes of Co3- and Hco3-. Journal of the American Chemical Society 1979;101 (10):2599-604.

[41] Heindl A, Kohler HH. Rod formation of ionic surfactants: A thermodynamic model. Langmuir 1996;12 (10):2464-77.

CHAPTER 4. SYNTHESIS OF VITAMIN E-CARNOSINE (VECAR): NEW ANTIOXIDANT MOLECULE WITH POTENTIAL APPLICATION IN ATHEROSCLEROSIS

4.1 Introduction

The antioxidant theory links oxidative stress with cardiovascular diseases and major diseases such as cancer, acute inflammation, Parkinson's, and Alzheimer's [1-26]. In 2007, an estimated $430 billion were spent to treat around 80 million people suffering from some type of cardiovascular disease [27, 28]. Antioxidant agents play a vital role in the defense system against free radicals and oxidant molecules by avoiding radical propagation (chain reaction) via radical scavenging, metal ion chelation, and coantioxidant action (antioxidant regeneration) and may play a role in disease prevention and treatment. The balance between oxidative species and antioxidant systems (natural molecules, enzymes, and proteins) defines the oxidative stress of a living system, and the oxidative stress level determines if a biological system is under high risk for diseases [32].

Atherosclerosis in particular has been related to the oxidative process, in that high levels of oxidant molecules or low levels of antioxidants were shown to be responsible for high oxidative stress triggering the atherosclerosis process. The oxidation theory of atherosclerosis attributes great importance to the oxidation process and deems free radicals as precursors for the first steps in the development of the disease [33-36]. In this context, great importance has been attributed to vitamin E, especially α-tocopherol, as the main lipophilic natural antioxidant present in the lipophilic phase of every cell. Among the lipophilic antioxidants, α-tocopherol is more potent. To highlight the importance of α-tocopherol in humans, it has been shown that a specific enzyme α-tocopherol transfer protein (α-TTP) is responsible for α-tocopherol transport into lipoproteins. Lipoproteins then act as a main vehicle for α-tocopherol transport to different tissues [30, 37-52]. The transport protein α-TTP is responsible for the high levels of α-tocopherol (80% of the vitamin Es) in the cells as compared with the other seven isomers of vitamin E. Other important lipophilic antioxidants are coenzyme Q10 and carotenoids [8, 14, 53, 54].

The uptake of lipophilic molecules such us alpha-tocopherol from the diet occurs in the intestine through formation of structures called chylomicrons. Chylomicrons are mixtures of fatty acids, triglycerides, cholesterol, vitamin E, coenzyme Q_{10}, phospholipids, and bile salts.

Chylomicrons are in the micrometer range (1-10 μm) in size and are the precursors of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) produced in the liver. Several differences exist among these "lipophilic vehicles" based on their density, presence of protein components (apos), or size as the more predominant characteristics.

LDL is composed by a core of cholesterol and triglycerides surrounded by fatty acids, cholesteryl esters, apolipoproteins, and phospholipids. The outer layer is more hydrophilic due to the protein presence as compared to the core, which is more hydrophobic due to the presence of cholesterol and triglycerides [31, 55-60]. Oxidation of LDLs is associated with the development of atherosclerosis. The ox-LDL (i.e. the oxidized form of LDL) acts at different levels stimulating the host response and formation of foam cells. Lipophilic antioxidants avoid oxidation of the lipid fraction of LDLs, but apolipoproteins are not protected from oxidation even in the presence of lipophilic antioxidants [61-67].

The limitations of lipophilic natural antioxidants can be overcome with an adequate design of an alternative "natural" antioxidant formed by covalently linking the hydrophobic antioxidant with a naturally occurring hydrophilic molecule with antioxidant properties; the new structure will insure that the new component will be localized closer to apolipoproteins, have better antioxidant performance, and have potential synergetic behavior due to antioxidant-coantioxidant phenomena.

Hydrophilic antioxidants have the ability to protect proteins, DNA, and carbohydrates from oxidative molecules, and to regulate oxidative molecules for signaling purposes. Among hydrophilic molecules, vitamin C and carnosine play important roles in the protection of proteins and DNA from oxidative molecular attacks. Other molecules, such as flavonoids, uric acid, micronutrients, peptides, and proteins, have been researched for their potential antioxidant actions [54, 68-90].

The dipeptide carnosine (β-alanyl-L-histidine) has been researched as a protective agent against aging due to its antioxidant properties and ability to suppress protein glycation and crosslinking [26, 47, 54, 68, 72, 91-100]. Carnosine has also been suggested as an important agent in controlling secondary problems in diabetes [76, 82, 83, 101-120].

During the last 30 years, antioxidants and their impacts on biological systems have been heavily studied. Emphasis was placed on the understanding of antioxidant mechanisms in an

attempt to design better synthetic antioxidant compounds. There are several synthetic antioxidants (i.e. Trolox, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, and others) and vitamin E derivatives (i.e. Trolox, 3-oxa-chromanol type, brominated alpha-tocopherol methano-dimer, raxofelast, vitamin EC, probucol, etc.) used for different purposes [121-134]. Several synthetic antioxidant molecules reported in the literature use the OH group of α-tocopherol to attach other molecules to α-tocopherol, or modify the chromanol ring in the synthesis. These molecular changes of α-tocopherol decrease the specificity of α-TTP interaction for α-tocopherol. The α-tocopherol structure should be maintained as close as possible to the natural RRR conformation to protect the antioxidant properties of α-tocopherol and to insure that α-tocopherol is recognized and transported by α-TTP. Reactions should be performed in the phytyl chain, which is not a required moiety for the protein α-TTP to recognize α-tocopherol [49, 51, 135, 136]. In order to achieve this conjugation, the following criteria must be met: 1. the chromanol ring cannot be modified, 2. the phytyl chain should be long to confer hydrophobic behavior to the new molecule, and 3. the second natural antioxidant component should be covalently linked to the end of the phytyl chain. This new approach allows molecules such as carnosine or vitamin C to be linked to α-tocopherol, so they can be introduced into LDLs and lipophilic sections of cells (i.e. plasma membrane) by action of α-TTP. The new molecule can provide improved antioxidant properties as compared with initial natural antioxidants (α-tocopherol and carnosine), and offer an improved defense of LDL from oxidation, which has been postulated as an important event in atherosclerosis.

The main goal of this project was to synthesize and characterize a new antioxidant molecule based on α-tocopherol and carnosine (VECAR), with potential applications in prevention of atherosclerosis, but VECAR could be successfully used to prevent other oxidative stress related diseases.

4.2 Oxidation and Its Effect on Atherosclerosis

It is accepted that reactive oxygen species (ROS), which involves oxygen centered radicals and other oxidative molecules, such as reactive nitrogen species (RNS), and reactive sulfur species (RSS) act at different cellular levels and result in oxidation of native molecules. The oxidation process is associated with damage of proteins, lipids, DNA, and carbohydrates triggering different diseases.

4.2.1 Reactive Oxygen Species and Oxidation Process

The reactive oxygen species play significant roles in the cell metabolism such as cell growth, energy production, intracellular signaling, and antimicrobial action, but ROS can be very harmful by damaging different tissues due to lipid peroxidation, carbohydrates oxidation, or protein and DNA modification. The oxidative damage of all aforementioned molecules had been related to cancer disease, diabetes, cardiovascular disease, inflammatory responses, degenerative diseases, aging, liver injury, cataract, and others [2-4, 20, 54, 70, 76, 98, 111, 114, 137-159].

Antioxidants prevent cellular damage surged from chemical reactions that involve free radicals. Halliwell & Gutteridge defined antioxidants as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delay or inhibit the oxidation of that substrate". Free radicals are molecules that contain unpaired electrons [160]. There are several radical species with potential oxidative actions, such as reactive oxygen species (oxygen radical centered), reactive sulfuric species (sulfur radical centered), and reactive nitrogen species (nitrogen radical centered) (Table 4-1). All of these radical species or non radical molecules can act at cellular levels in metabolic, cellular signaling, and degenerative processes due to endogenous or exogenous sources of oxidant molecules (OM).

Intracellular free radicals are mainly generated by the autooxidation of small molecules and by activity of certain enzymes like oxidases, peroxidases, lipoxygenases, dehydrogenases, and cyclooxygenases. The cellular sites of reactive oxygen species generation are mitochondria, peroxisomes, lysosomes, endoplasmic reticular membranes, nuclear membranes, and plasma membranes [19, 161-163].

One important source of superoxide anions is the mitochondria (1 to 5 % of the oxygen used in the respiratory metabolism form superoxide anions [164]). ROS can result from the activity of several enzymes (e.g. cytochrome p450 oxidase), and can be formed by vascular endothelium, and phagocyte cells. Hydrogen peroxide is generated with superoxide anions by spontaneous dismutation and enzymatic reactions [17]. Figure 4-2 depicts the basic chemical reaction for biological aerobic system in which oxygen is reduced to water for energy supply. If the electrons are passed one at a time, ROS intermediates can be formed. Also, the reaction between metal ions and superoxide anions is responsible for radical formation (Figure 4-1).

Other important oxidizing molecules that are not free radicals are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxinitrate (ONOO-) species. The

singlet oxygen is associated with ionizing radiation (i.e. UV exposure) in which the oxygen electron reaches a higher energy state than the ground state.

Table 4-1: Reactive species with biological effect.

Name	Formula	Characteristics
Singlet oxygen	$^1O_2$	An electronically excited form of oxygen
Superoxide anion	$O_2•^-$	Oxygen is reduced by transfer of a single electron to its outer shell to form superoxide anion. $O_2•^-$ It can be produced by different cells like monocytes, macrophages, by autoxidation reaction and other mechanisms.
Hydroxyl radical	•OH	•OH reacts with most biomolecules at diffusion controlled rates with main action at the site of generation.
Hydrogen peroxide	H_2O_2	H_2O_2 is the main source of hydroxyl radicals in the presence of transition metal ions. It is also involved in the production of HOCl by neutrophils. The superoxide anion generated probably undergoes a dismutation reaction generating hydrogen peroxide. It is a very diffusible molecule in the cellular environment.
Hypochlorous acid	HOCl	HOCl formation is by myeloperoxidase action on chloride ions in the presence of H_2O_2. It can originate from chloramines and amino acid derived aldehydes. It can oxidize apolipoprotein B-100.
Peroxyl radical	ROO•	ROO• is formed by the addition of oxygen to alkyl radicals or from the breakdown of organic peroxides.
Alkoxyl radical	RO•	RO• can be formed by enzymes or chemical reactions.
Nitric oxide radical	NO•	NO• is a common gaseous free radical. It is produced by vascular endothelium, neutrophils and macrophages from arginine using the enzyme nitric oxide synthetase. Nitric oxide radical can form nitrogen dioxide radical ($NO_2•$) from reaction with O_2.
Peroxynitrite	$ONOO^-$	$ONOO^-$ is formed by the reaction of nitric oxide with superoxide anion. It can cause oxidation of proteins with –SH groups, and it can be decomposed to nitrogen dioxide ($NO_2•$). It can oxidize apolipoprotein B-100. It reacts with CO_2 forming nitrating, nitrosanting species.

ROS formation in cells is a process that under normal conditions reaches steady state; the imbalance of this process can generate diverse diseases due to oxidative stress. Oxidative stress describes a state of a biological system exposed to excessive formation of oxidative molecules (ROS, RSS, and RNS), and limited antioxidant defense, resulting in a harmful environment for proteins, DNA, and lipids [165]. In many diseases oxidative stress is a consequence and not a cause of the primary disease, but it is generally accepted that oxidative stress plays a vital role in tissue damage [18].

The damage generated by the action of oxidative molecules (OM) at the cellular level can be controlled by different defensive systems, which can be enzymes and non-enzyme systems [17,21,159]. The importance of understanding the interaction and potential unknown synergistic effect of the antioxidants against oxidative molecules (OM), especially under high levels of oxidative stress is critical.

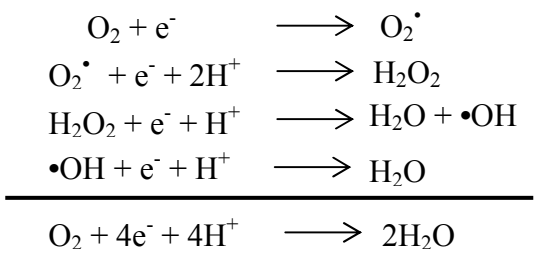


Figure 4-2: Reduction of an oxygen molecule. It requires four electrons. If the electrons are passed one at a time, it can form the intermediates (ROS).

The human system is a dynamic and an extremely complex system for studying antioxidants *in vivo*. As discussed before, oxidant molecules act at several levels, and not all the reactions have a negative impact on the body. The antioxidant mechanism of several natural molecules has been demonstrated in *vitro* and in *vivo* studies. Human studies showed unclear results for disease prevention related to oxidative stress, however in some studies the results were disappointing [166,167]. However, supplementation with antioxidant molecules from the diet is expected to positively impact human health based on the positive results shown in animal studies.

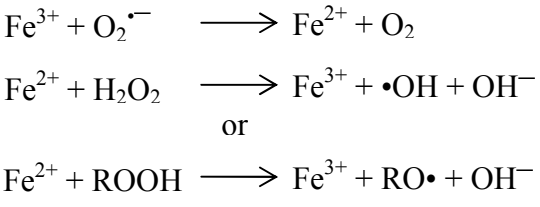


Figure 4-1: Superoxide anion also is involved in the Haber-Weiss reaction to form hydroxyl or alkoxyl radicals. The reaction is catalyzed by iron and copper ion metals.

This is a complex area of discussion, and several points of view have been developed to explain the problem. The antioxidant efficiency in *vivo* is related to their localization and not only the chemical reactivity of antioxidants. Mobility of lipophilic antioxidant molecules in the membrane, the presence in lipoproteins, fate of the radicals formed, synergetic inhibition of oxidation process, pro-oxidant effects, and pharmacokinetic of antioxidants, are important issues to consider in the antioxidant evaluation of potential antioxidant compounds in *vivo* [168]. The methods available to define the oxidative status of an individual are not well developed and standardized, and a combination of methods may show more accurate data than any individual method [169]. Moreover, it has been stressed that many cellular studies overestimate the exposure of cells for oxidative stress due to high levels of oxygen used (normally 21%). An

atmosphere of 3-4 % of oxygen should be used to work with the same physiological conditions [28,157,170].

There are several natural antioxidants vital to our metabolism and health, which can be acquired from food. For an optimum tissue level of antioxidants, a balanced food intake with vegetables, legumes, fruits, and nuts is recommended [171]. The different sites of action of natural antioxidants suggest that it is important to maintain adequate concentrations of both hydrophobic and hydrophilic antioxidant molecules to reduce the risk of oxidative stress in all cell compartments. A good antioxidant performance is achieved with a pool of different molecules acting at specific locations in the cell. The specificity and location play an important role in the balance between oxidants and antioxidants.

Many diseases, including atherosclerosis, have been related to the oxidative process. The oxidation theory of atherosclerosis is based on the presence of oxidized LDL when the disease is detected. The oxidized LDL can induce monocyte adhesion to endothelium, promote foam cell formation, induce migration and proliferation of smooth muscle cells, promote pro-coagulant properties of vascular cells, platelet adhesion, and other steps in atherogenesis [36,63,172,173]. This theory is supported by studies which show that antioxidants inhibit or delay lesions in animal models, as discussed in the next section.

4.2.2 Atherosclerosis Oxidation Theory

Free radicals and oxidative molecules are responsible for molecular modification of compounds by oxidation, which affects their normal behavior contributing to harmful consequences for human health. Atherosclerosis is a disease with a high impact on human health. The advances made in understanding the atherosclerosis process lately have been outstanding. The oxidation theory is one of the most popular theories developed; it attributes key roles to oxidation molecules in the initial steps of atherosclerosis.

The basis of this theory is that the oxidative processes change the artery walls with further monocyte macrophage recruitment from the bloodstream and excessive lipid deposition. In the process, the LDL is oxidized in the sub-endothelial space. A typical structure of LDLs is presented in Figure 4-3. The apoB-100 protein is located at the surface as well as more polar fatty acids, phospholipids, and unesterified cholesterol. The core is formed by neutral lipids such us triglycerides and cholesteryl ester. An average composition of LDLs is 20% phospholipids,

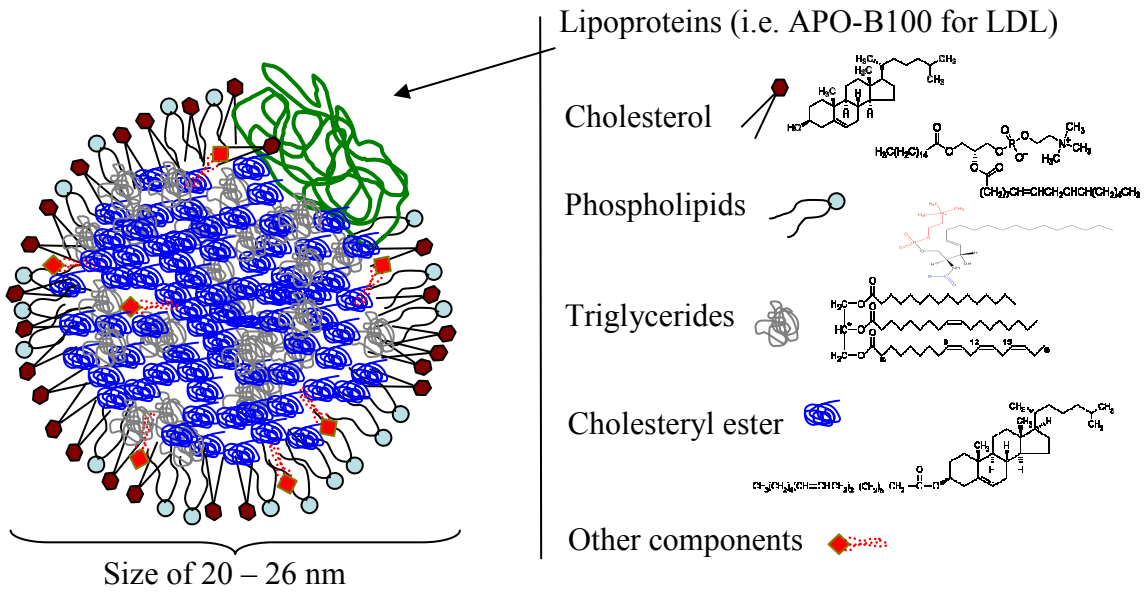


Figure 4-3: Sketch of low density lipoprotein (LDL) structure with main components. The center of LDL is formed of cholesterol and triglycerides, which are highly hydrophobic. There is a layer of more polar lipids formed mainly by phospholipids and polyunsaturated fatty acids on the surface. The other components can be α-tocopherol (6-12 per LDL molecule), γ-tocopherol (0.5 per LDL molecule), coenzyme Q10 (mean < 1 per LDL molecule), carotenoids (0.4 per LDL molecule), and others [31].

40%, cholesteryl esters, 20% protein, 10% unesterified cholesterol, 5% triglycerides, and 5% other lipophilic components .The LDLs range size is from around 20 to 26 nm [31, 55, 174-176]. The LDL molecule, in its oxidized form, can stimulate expression of adhesive molecules on endothelial cells, can be chemotactic, and can regulate cell scavenger receptors [36]. Figure 4-4 shows a sketch of LDL oxidative action in the sub-endothelial space. Wall thickening, vessel narrowing, and plaque formation can result in further thrombosis and plaque rupture.

In the 1980s, it was suggested that oxidized low density lipoproteins (ox-LDL) could promote cell damage [36]. The cell damage was studied by the incubation of ox-LDL with endothelial cells that presented a high uptake for macrophages with further modifications concluding with foam cell formation (Figure 4-4). The interesting observation was that the process described before, observed in the presence of ox-LDL, was not present when native LDL (not oxidized) was used [34, 59, 177-179].

The lipoprotein fractions isolated from atherosclerotic lesions have been shown to contain lipoprotein particles with some characteristics of oxidized LDL, such as increased electrophoretic mobility and reactivity with antibodies against oxidized LDL [56, 59, 63, 180,

181]. The results do not establish if these products arose from the atheroma or that they were present in the LDL before. The oxidation hypothesis is supported by studies using knockout mice. Suzuki and coworkers [182] reported that targeting scavenger receptor A (SRA) by homologous recombination reduced the rate of progression of atherosclerosis in apoE-deficient mice. This receptor recognized oxidatively modified LDL but not native LDL. It is suggested that the observed protection was due to a decrease in uptake of ox-LDL with further reduction in the accumulation of foam cells in the sub-endothelial space. The deficiency of macrophage scavenger receptors (CD36, SAR, SR-B1, and others) which binds to oxidized LDL has been associated with atherosclerosis events reduction [36]. There are other studies showing that other mechanisms can be involved in the protection observed when it was modified by the scavenger receptors because the genetic modifications can affect the presence of antibodies. The presence of circulating antibodies, which act against oxidized LDL, affects the atherogenesis, so the immune system can modulate atherogenesis. Data of experiments with immunization of hypercholesterolemic animals with ox-LDL had shown decrease of atherosclerotic lesion progression [183]. Studies suggest that LDL oxidation occurs through endothelial and smooth muscle cells, but other studies argue that LDL oxidation is mainly favored by monocyte-derived macrophages [34, 179, 184]. Physiological mechanisms of LDL oxidation *in vivo* are not clear, but studies suggest that superoxide, myeloperoxidase, peroxynitrite, thiols, and other molecules can participate in the oxidation process [17, 36, 172, 185, 186].

It has been suggested that α-tocopherol supplementation can decrease the low density lipoprotein (LDL) susceptibility to oxidation; however, it does not affect its glycation, which increases LDL oxidative susceptibility, or AGEs (advanced glycosilation end products) formation [63, 187-191]. Moreover, inhibitions of platelet adhesion, aggregation, and smooth cell proliferation have been related to α-tocopherol supplementation [192, 193]. It has been showed that α-tocopherol supplementation decreases CD36 expression (scavenger receptors that bind to oxidized LDL found in atherosclerotic lesions) and cholesteryl ester accumulation in human macrophages. Other tested antioxidants (β- or γ-tocopherol) failed to decrease CD36 [194].

The work developed by Stocker and colleagues [31, 55, 180, 188, 189, 195-209] suggested that α-tocopherol can be a pro-oxidant or antioxidant, and the switch in its behavior is related to the oxidative stress levels. At low oxidative stress, α-tocopherol acts as a prooxidant

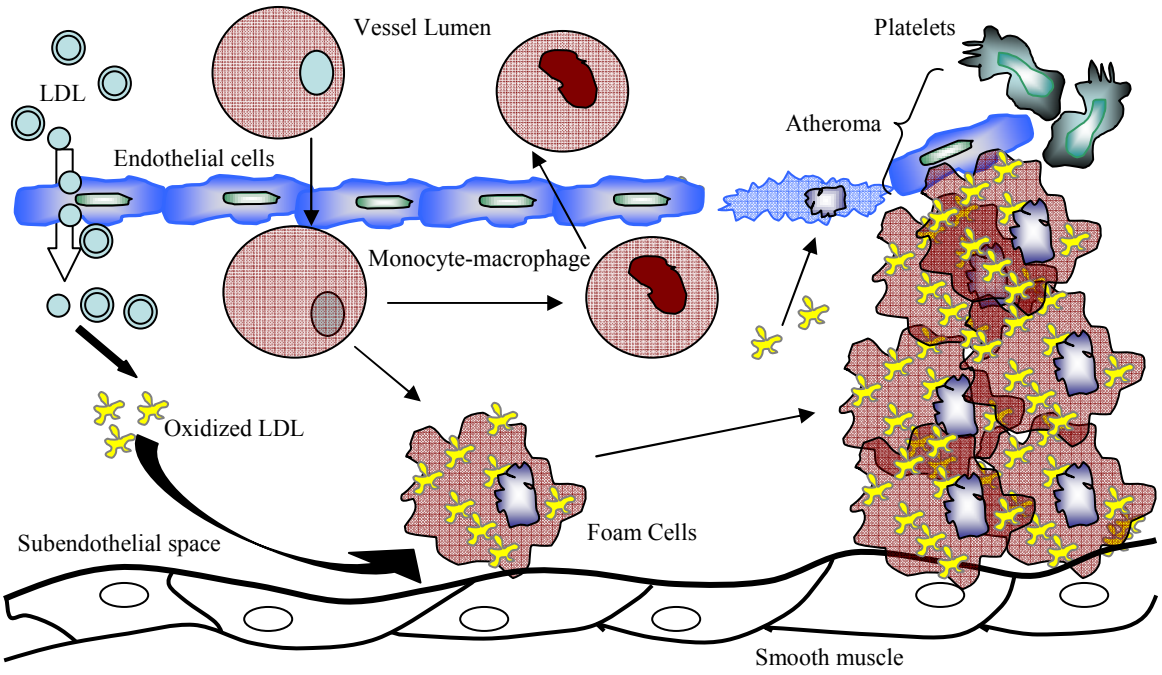


Figure 4-4: A sketch of arterial obstruction by atheroma from oxidized low density lipoproteins. Adapted from [29].

promoting lipid peroxidation; at high oxidative stress, α-tocopherol acts as an antioxidant. The occurrence of oxidative products in LDLs particles in the presence of α-tocopherol (antioxidant) supports this theory. The oxidized products that present a *cis*, *trans* configuration is favored when α-tocopherol is present in LDL particles. If the LDL is oxidized without the presence of vitamin E, the oxidized products obtained show a *trans* configuration. The animal LDL oxidized products tested were in the *cis, trans* configuration, which suggests that oxidation *in vivo* can occur in the presence of α-tocopherol [31, 189, 200, 208]. The tocopherol mediated peroxidation (TMP) theory mentions that the co-antioxidants are important to keep up the antioxidant behavior of α-tocopherol. The coenzyme Q-10 plays an important role as co-antioxidant and its supplementation can decrease atherosclerotic lesions [77, 84, 195, 210].

Moreover, it has been suggested that two electron redox (2e-redox) reactions could play a more important role than radical reactions in the oxidation processes of atherosclerosis [211]. The experiment was performed with probucol, probucol dithiobisphenol, and probucol bisphenol. The formulations with sulfur molecules (probucol and probucol dithiobisphenol) showed good results in avoiding macrophage accumulation, stimulating reendothelization, and inhibiting vascular smooth cell proliferation in mice and rabbits model. Also, it was suggested

that the induction of heme oxygenase-1 (OH-1) by probucol is an important factor for the protective effect showed by probucol and probucol dithiobisphenol. Phenolic compounds such as α-tocopherol act by 1 electron redox reaction. An example of 2e-oxidant is hypochlorite and peroxynitrite which are involved in atherogenesis process.

It should be noted that two other theories that explain the atherosclerosis process exist. The first theory is based on the response to injury hypothesis, which highlights that the initial event is injury to endothelial and smooth muscles. Some causes to the endothelial dysfunction are hypertension, diabetes mellitus, genetic alterations, elevated levels of LDL, infectious microorganisms, and others. The injuries increase the adhesiveness of the endothelium, its permeability, and inflammatory response, which finally produces the accumulation of different compounds in the growing injured area such as ox-LDLs [212]. The response to the retention hypothesis, second theory, establishes that the retention and accumulation of LDL and other atherogenic lipoproteins in the artery wall is the initial cause of atherosclerosis [213]. The second theory has been researched extensively in recent years because it offers a better explanation to the correlation of high levels of apoB-lipoproteins and higher incidence of cardiovascular disease. The interaction between glucosaminoglycans and apoB-100 of LDL and VLDL is responsible of the LDLs accumulation in the subendothelial wall. The interaction involves basic amino acids in apoB-100 (residue 3359-3369), which bind ionically to negatively charged sulphate groups of glucosaminoglycans. The oxidation of LDLs is a cause of the accumulation and other interactions [60, 214, 215]. These other two theories stress initial process for atherosclerosis, but they include oxidation of LDLs as important vehicle for atherogenesis.

Another interesting observation is that HDL can prevent atherogenesis. High levels of circulating HDL are associated with a lower risk of atherosclerosis. The studies related to HDL have focused on the apolipoprotein A-I (apoA-I), which acts by lowering the excess cholesterol from tissue and by transporting it to the liver for excretion. The identification of a mutation of this protein called apo A-I Milano has been a big step to use “HDL therapy in atherosclerosis” due to higher efficiency in cholesterol elimination from tissue. The studies performed with the synthetic drug showed a good performance; using a recombinant apoA-I conjugated with phospholipids lead to a significant reduction of coronary plaque burden after five weeks of treatment [216].

In *vivo* and in *vitro* assays have shown that Vitamin E is effective in protecting LDL from oxidation. This finding had triggered clinical trials and epidemiological studies in order to understand vitamin E effect in humans [33,217-219]. Unfortunately, These clinical trials showed dissimilar results, giving rise for more questions and theories. The clinical trial that showed positive results was the CHAOS trial [220], which used vitamin E doses of 400 to 800 IU daily in patients with angiographically-proven coronary artery disease. The study was followed over 5-years and showed 47% fewer myocardial infarcts (fatal plus nonfatal) for vitamin E supplemented patients, but the total mortality was not reduced. Other clinical trials showed no impact of vitamin E supplementation on atherosclerosis in the populations studied like the ATBC trial, which used 50 mg of vitamin E supplemented daily with no effect on cardiovascular end points [218]. The HOPE and GISSI trial used supplementation of 300 to 400 mg of vitamin E per day showed no effect on atherosclerosis [217,221].

These studies reflect that there is a need for more research to better understand the mechanisms involved in atherosclerosis, which would allow for a better design of drugs and systems for treatment of the disease possibly based on combinations of antioxidants.

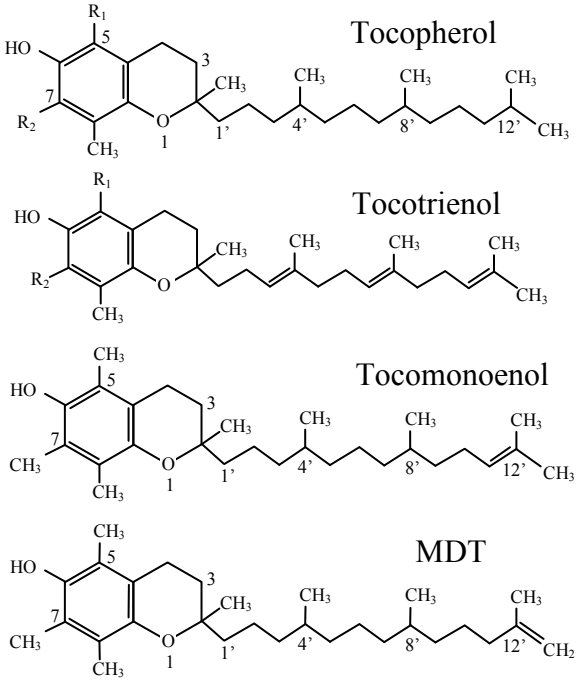
4.3 Natural Antioxidants

Oxidants and free radicals are associated with different diseases and cellular degeneration. Natural antioxidants together with enzymes, proteins, and genes play important roles as defense systems against oxidation. A balance between oxidants and antioxidants is necessary for adequate cellular survival and proliferation. In this framework of molecular interaction, natural antioxidant molecules act at different cellular levels. They can be localized in the cytoplasm, cellular membranes, nucleus, mitochondria, organelles, and in all types of tissues. The wide distribution of natural antioxidants is based on differences in their chemical structures that confer different properties such as hydrophilic-hydrophobic balance (HLB) to these components. The complete understanding of natural antioxidants action in the mammalian system is not a trivial pursuit; it is a challenging task that has been producing flourishing research in the last decade. Natural antioxidants such as flavonoids, uric acid, vitamin E, vitamin C, coenzyme Q10, retinol, and carnosine are molecules that can be synthesized by the mammalian body or come from food intake. One of the most important lipophilic antioxidant molecule studied is vitamin E, due to its major action in protection of lipids against lipid peroxidation, important for cell survival. Proteins, which are also oxidized, are vital components

of cellular machinery and are not protected against oxidation by lipophilic antioxidants such as alpha-tocopherol. Carnosine, on the other hand, has been highlighted as an important antioxidant for proteins in *vitro* and in animal models [118, 222-224]. Vitamin E and carnosine are adequate molecules, which through conjugation by covalent linkage allow the study of the potential benefits of a new antioxidant formed from a hydrophilic and a hydrophobic molecule. Information related to the structure, sources, mechanism, and roles of α-tocopherol and carnosine is presented in the following sections.

4.3.1 Vitamin E

Vitamin E is a lipophilic vitamin that has been the object of extensive research due to its antioxidant properties and non-antioxidant actions. The Vitamin E family is composed of four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ), the difference being that tocotrienols have unsaturated phytyl chains. The positions of the methyl group over the aromatic ring are different for each tocopherol and tocotrienol (Figure 4-5). In addition to the eight vitamin E components, other similar members have been identified in high levels in some fish species native to cold water such as marine derived tocopherol (MDT) or tocomonoenol, found in palm oil [225, 226] (Figure 4-5).



Tocopherol and tocotrienol	R_1	R_2
α	CH_3	CH_3
β	CH_3	H

Figure 4-5: Vitamin E family structures.

The main sources of vitamin E are wheat-germ oil, sunflower seed, almond, and cereal. Each natural source of vitamin E has different amounts of tocopherols, with soybean oil, corn oil, corn and soybean margarines, linseed oil, and others rich in γ-tocopherol [227]. The daily recommended intake of α-tocopherol is 22.7 mg/day for adults and 18.7 mg/day for lactating women [228], but it has been suggested

that higher values (i.e. 537 or 805 mg/day) are needed when supplementation of vitamin E addresses therapeutic issues [33]. Plasma concentration of α-tocopherol in healthy humans range from 20 to 40 μM [229]. The upper recommended limit intake of α-tocopherol is 1000 mg/day or 1500 IU/day [230].

Trapping peroxyl radicals and breaking the chain reactions of lipid peroxidation are the main functions of vitamin E as antioxidant. In addition, vitamin E quenches and reacts with superoxide anions ($O_2\bullet^-$), singlet oxygen ($^1O_2$), and hydroxyl radicals (•OH), and it prevents nitric oxide (NO) from reacting with $O_2\bullet^-$ [18].

Oxidation of α-tocopherol is a two electron process [231]. The non-radical oxidation process of α-tocopherol always forms para-quinoid and ortho-quinone methide, a transition state (Figure 4-6). The reactions occur in 5-position for all vitamin E members including γ-tocopherol, which does not have a methyl group in 7-position [232]. The antioxidant activity of vitamin E is related to the methylation pattern and amount of methyl groups present in the phenolic ring, which is higher for α > β > γ > δ tocopherols [233].

The reaction of α-tocopherol with peroxyl radicals forms α-tocopheroxyl radical, which is resonance stabilized (Figure 4-6). The final product can be a dimer formed by two α-tocopheroxyl radicals, tocopherol quinone, or the α-tocopheroxyl radical can react with another molecule (i.e. vitamin C) to regenerate α-tocopherol [234,235]. The metabolite of vitamin E is 2'-carboxyethyl)6-hydroxy chroman (CEHC). High α-tocopherol intakes are associated with an increase of α-CEHC in urine, which also holds true for the synthetic form of the vitamin [236]. The pathway involves oxidation of the phytyl chain to the corresponding hydrophilic metabolites without modification of the chromanol ring.

The pro-oxidant activity of vitamin E is based on the reduction of Cu^{2+} to Cu^{+} (or Fe^{3+} to Fe^{2+}), which generates tocopheryl radicals that can react with polyunsaturated fatty acids (PUFAs) at very low rates [18,237,238]. α-tocopherol, in the presence of Cu^{2+} ions, can induce oxidative damage to DNA *in vitro*. The oxidation of α-tocopherol produces Cu^{+}, which reacts with molecular oxygen to form superoxide anion, H_2O_2 by dismutation, and further $^{\bullet}OH$ which has been identified as responsible for DNA damage [239]. The pro-oxidant action of vitamin E has not yet been proven in mammals, most probably due to low levels of iron and copper ions which bind to proteins like ceruloplasmin and ferritin, respectively. It has been suggested by Dr. Stocker's research group that, according to the tocopherol mediate peroxidation (TMP) theory,

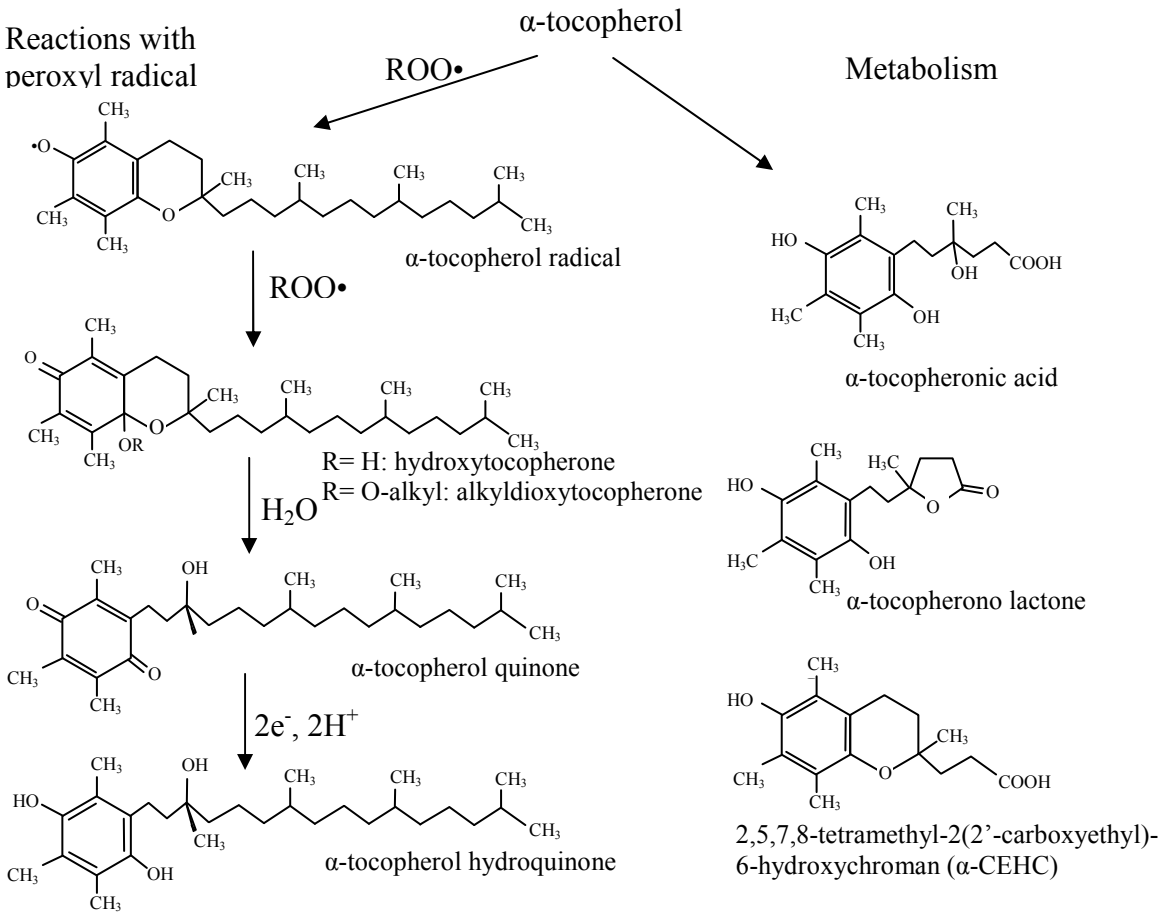


Figure 4-6: Chemical structures of α-tocopherol reaction products with peroxyl radicals, and α-tocopherol degradation products from metabolism [30].

α-tocopherol can act as a pro-oxidant upon mild oxidative stress and in the presence of low concentrations of co-antioxidant compounds (i.e. coenzyme Q-10, ascorbic acid). When the oxidative stress is higher, and co-antioxidants are present in high concentrations, α-tocopherol acts as an antioxidant [31, 55, 188, 189].

Cadenas and coworkers [240] suggested that higher doses of vitamin E provide enough protection against lipid peroxidation in guinea pigs (doses of 1500 mg/kg) under normal levels of vitamin C. In humans with a balanced diet, the normal intake of vitamin E is around 15-30 IU/d (10 to 20 mg/day) [33]. Researchers have suggested that moderate supplementation can be beneficial for heart-healthy behavior [33, 241], with doses of 400 UI/day (270 mg/day). The recommendation is based on doses used for animal studies and the safety of vitamin E. One drawback of α-tocopherol supplementation is the reduction of plasma and tissue levels of γ-

tocopherol [242]. Also, α-tocopherol supplementation is not recommended for patients taking anticoagulants, because tocopherols act synergistically with vitamin K antagonists and aspirin, resulting in an increased tendency to bleed [228,243].

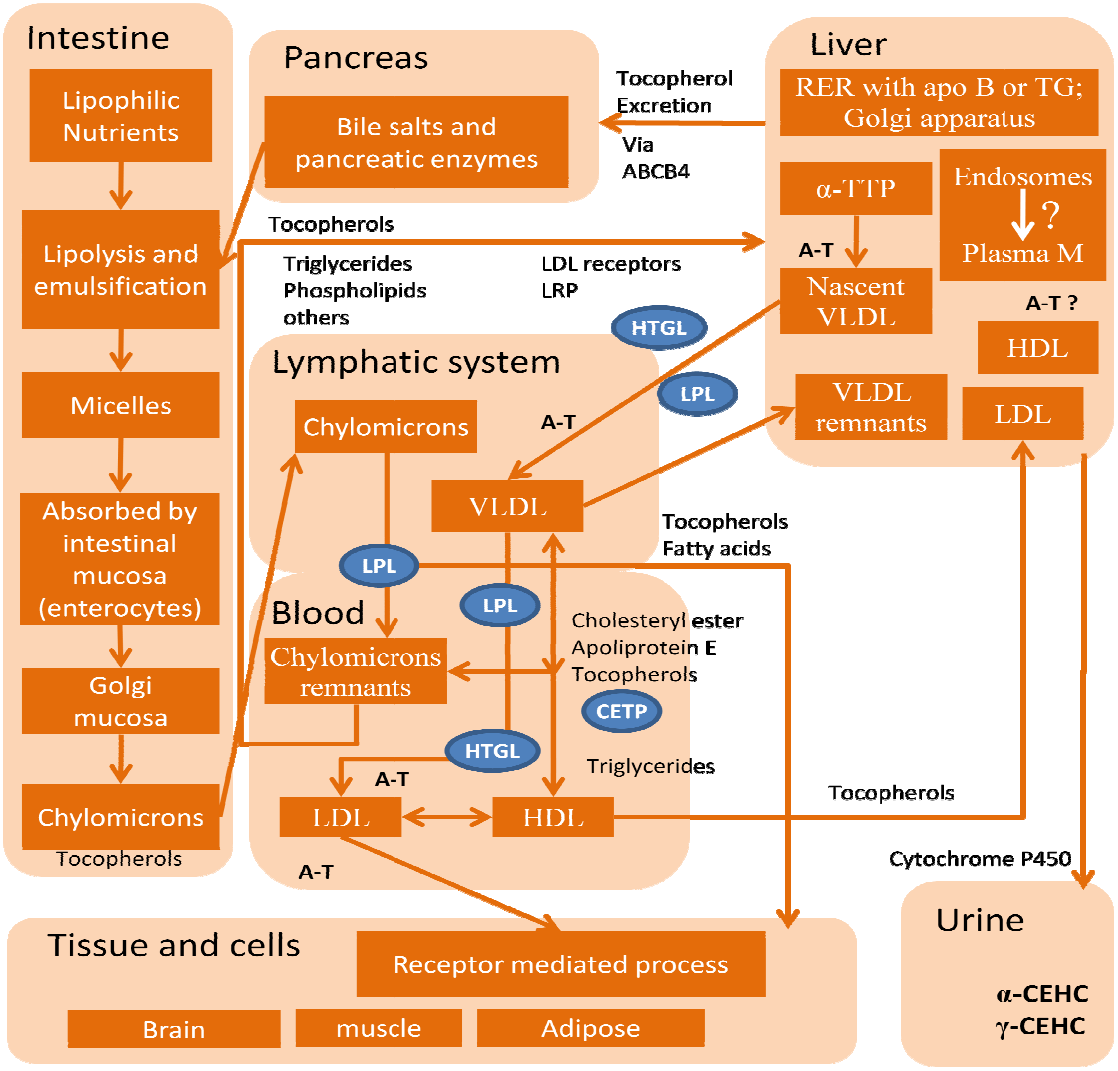


Figure 4-7: Absorption and transport of tocopherols (vitamin E). Tocopherol absorption and transport are linked to lipid absorption mechanisms. The small intestine is responsible for the formation of chylomicrons, which transports all tocopherols (not a selective mechanism). Chylomicrons are catabolized to chylomicron remnants by lipoprotein lipase (LPL) present in different tissues (endothelial-bound enzyme), which hydrolyze triglycerides and transfers tocopherols to different tissues. The chylomicrons remnants that reach the liver are transformed to nascent VLDL with incorporation of α-tocopherol by α-TTP. The VLDLs are catabolized in the circulation by LPL and hepatic triglyceride lipase (HTGL) to form VLDL remnants and LDL. The α-tocopherol is finally transported to different tissues by LDLs. The tocopherols excess is excreted by the liver to the pancreas or urine.

Supplementation with vitamin E has not shown conclusive results in the prevention and control of several diseases like cardiovascular disease, cancer, and atherosclerosis, but patients with pre-existing cardiovascular disease can benefit from supplementation of α-tocopherol (up to 800 IU/day or 537 mg/day) as suggested in some clinical trials [16]. Some studies with humans (small groups) have shown positive benefits of vitamin E supplementation with the addition of micronutrients like coantioxidants and selenium [33]. Elsayed research group showed that inhalation of oxidative air pollutants by rats was associated with a significant increase in vitamin E levels in lung tissue [244]. The theory is that oxidative stress could activate some signal transduction pathways, which mobilize antioxidants to the target. The location of vitamin E in cells and tissue differs; for example, vitamin E is present in neuronal membrane in unique locations, suggesting neurological function [245].

The potential of vitamin E is hopeful, but there is need for more research; the positive aspects are that treatment with vitamin E is not expensive and that there are no side effects due to the intake of vitamin E up to 400 IU/day [246]. Miller's research group performed a statistical study designed to find a relationship between vitamin E supplementation and total mortality (end point). The study was based on results published from 19 different clinical trials performed from 1966 to 2004 around the world on 135,000 participants suffering from diseases such as atherosclerosis, diabetes, Parkinson's, and others. It suggested that intakes of vitamin E supplements higher than 400 IU/day (approximately 180 mg/day) may increase mortality [246].

Cellular transport and absorption of vitamin E is by specific and nonspecific uptake. Figure 4-7 presents the main steps in the absorption and transport of tocopherols and α-tocopherols. The regulated uptake is specific for α-tocopherol. The non-specific uptake is for all other vitamin E family components [232]. The intestine takes up α-tocopherol and γ-tocopherol in a non-selective process in comparable proportions [247] via chylomicron particles. The first step is the micelle formation in the small intestine by bile salts, lipophilic compounds, and products of lipid hydrolysis by pancreatic lipases. The next step is formation of chylomicron particles in the Golgi apparatus of enterocytes (intestinal cells) [48, 248]. Chylomicrons particles are formed from different lipophilic compounds (tocopherols, cholesterols, fatty acids) together with apo proteins (Apo-B48), phospholipids and triglycerides. The chylomicrons are secreted to the plasma, where they are catabolized by endothelial-bound enzyme lipoprotein lipase (LPL) responsible for the formation of chylomicrons remnants [249]. Chylomicron circulation and

catabolism is one mechanism for vitamin E transfer to muscles, adipose tissue, brain tissue, and other tissues. Deficiency in LPL does not cause vitamin E deficiency in plasma, but it produces low levels of α-tocopherol in adipose tissues [234]. The cholesteryl ester transfer protein (CETP) acts at this level by exchanging cholesteryl esters from HDL to the remnants, and triglycerides from remnants to HDL [250]. Also, the chylomicrons remnants can acquire apolipoprotein E from HDL. Once the chylomicrons remnants reach the liver, they are used as substrate for secretion of very low density lipoproteins (VLDL).

VLDL and LDL act as a main transport pathway for α-tocopherol into the cells [40, 42, 188, 242, 251, 252].The protein α-tocopherol transfer protein (α-TTP) is responsible to add α-tocopherol to nascent VLDL. The steps of this process are not clear, but the animal studies and patients with deficiency in α-TTP showed that the protein is vital to achieve high levels of α-tocopherol in plasma. The work of Hosomi et al. evaluated α-TTP specificity for different vitamin E analogs and showed 100% affinity for RRR-α-tocopherol, 38% for β-tocopherol, 9% for γ-tocopherol, 2% for α-tocopherol acetate, 9% for Trolox [253]. VLDL is catabolized next by lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) in plasma. At this step, α-tocopherol can be transferred to HDL, or can continue with VLDL and ending into LDLs, or can return to the liver with the VLDL remnants (intermediate density lipoprotein, IDL). The liver can secret the vitamin E excess to the pancreas by the ATP binding cassette family of transporters (ABCB4) and p-glycoproteins, or the excess of vitamin E can be catabolized to carboxymethyl hydroxychroman (CEHC) by cytochrome p-540 [254-257].

4.3.2 Carnosine (β-alanyl-L-histidine)

Carnosine is a hydrophilic dipeptide (β-alanyl-L-histidine) synthesized exclusively by mammals. It is found in high concentrations in muscle and brain tissue, which suggest antioxidant activity considering the high oxidative stress in these tissues. A high oxidative stress is found in muscle tissues during anaerobic glycolysis (i.e. high levels of exercises) producing lactic acid, which reduces the pH in muscles [258]. Human skeletal muscle levels of carnosine are range from 2 to 20 mM. In the brain, carnosine is non-uniformly distributed with higher levels (up to 5 mM) in olfactory epithelium and bulbs [120,258]. The dipeptide carnosine and its analogs homocarnosine and anserine can be degraded by the enzyme carnosinase which is a group of intra- and extracellular dipeptidases [120].

This histidine containing dipeptide is part of the ω-aminoacylamino acids family (Figure 4-8). Other compounds are anserine, homocarnosine, and homoanserine [259]. It has been shown in different studies that carnosine acts as an anti-oxidant, carbonyl scavenger (anti-glycating), and metal ion chelator [115,260-266].

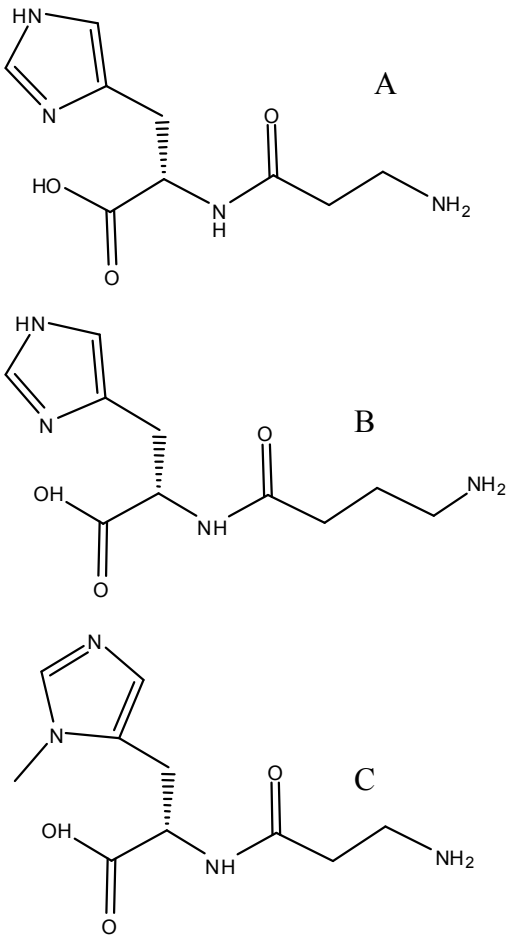


Figure 4-8: Structure of carnosine (A) Homocarnosine (B), and anserine (C)

The imidazole moiety of carnosine has been identified as responsible for its antioxidant properties. It was suggested that the protons on the nitrogen ring and on the methylene carbon next to the imidazole ring are required for antioxidant purposes. The imidazole molecule has higher antioxidant activity (around seven times higher) than 1-methylimidazole which lacks the proton on the ring. Homocarnosine, and anserine showed antioxidant activity comparable to carnosine [258]. The inhibition of deoxyguanosine oxidation, which was induced by ascorbic acid and copper ions in the presence of carnosine and anserine, suggested effective copper chelating properties. Another aspect studied was the effect of carnosine's buffering capacity on its antioxidant activity. A decrease in pH reduced the capacity of antioxidant molecules to act as antioxidants; carnosine maintains the pH at physiological values (7 - 7.2), so its antioxidant activity is not diminished [258].

An interesting property of carnosine is its ability to protect proteins from oxidation [102,103,118,120,263,267]. Reactive oxygen species can easily oxidize proteins, and the carbonyl groups of oxidized proteins can interact with lipids or carbohydrates to form compounds that can be inactive and resistant to degradation. Another hypothesis is that native proteins can be altered by products of lipid peroxidation and carbohydrates forming cross-linking stable products (AGEs, a group of glycated-end products) that accumulate in tissues [118,263]. It

is suggested that the rejuvenation phenomena observed in human fibroblast cells is promoted by carnosine, which regulates the proteins degradation and possibly involves other unknown mechanisms [118,266]. Also, an inverse relation between carnosine and diabetes has been reported [268]. Lee and coworkers [224] used diabetic Balb/cA mice to study the effect of dietary supplementation of histidine and carnosine. It has been suggested that carnosine could be beneficial for people with diabetes, especially to control the secondary effects of diabetes [267]. Diabetes can produce poor glycemic control, which causes LDL oxidation and glycation, and the results of Lee et al. showed that histidine and carnosine protect LDL against glucose-induced oxidation. The results showed that supplementation of 1 g/l of histidine or carnosine increased the insulin levels (from 6.8±1.4 nmol/l to 9.7±1 nmol/l), reduced trygliceride and cholesterol levels (hyperlipidemia; tryglicerides in heart from 54.6±4 mg/g to 37.6±3.1 mg/g, cholesterol in heart from 7.4±0.6 mg/g to 5.7±5.7 mg/g), enhanced catalase activity, and suppressed tumor necrosis factor (TNF)-alpha. Carnosine can also provide potential therapeutic effects on neurodegenerative diseases related to high levels of zinc (Alzheimer) [223,269,270]. Finally, it has been shown that low density lipoproteins (LDL) were protected against glucose-induced oxidation and glycation by a peroxynitrate (ONOO-) dependent process. The relative electrophoretic mobility of LDLs (peroxynitrate oxidation of LDLs increases its electrophoretic mobility due to more charged APOB-100) is reduced by 94% at 10 mM of carnosine concentration [260]. Moreover, carnosine can inhibit the oxidation of LDLs when the oxidation is catalyzed by copper *in vitro*. Carnosine can chelate copper making it unavailable to oxidize the protein apo B-100 of LDLs particles [83,271].

In summary, carnosine and its derivatives have potential benefits in several diseases related to oxidative processes, and it was selected as the hydrophilic antioxidant component for the proposed project.

4.4 General Characterization Techniques for Antioxidants

VECAR reactivity can be determined by several assays, which will provide a better understanding of the antioxidant behavior of the new molecules synthesized. These different assays provide information about the rate and time of protection, free radical scavenging properties, affinity to some radicals or oxidant molecules, pro-oxidant behavior, among others.

The free radical scavenging properties can be analyzed by several methods [272-278]. The main assays used are the DPPH and ABTS assay. DPPH assay: the 1,1-diphenyl-2-picryl

hydrazyl (DPPH) radical is a stable free radical of purple color. The addition of an antioxidant reduces the DPPH radical, which can be appreciated by color change (to yellow) measured in a spectrophotometer at 518 nm. ABTS assay: The mixture of 2,2-α-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide produces ABTS radical ($ABTS^{\bullet}$). Further incubation with peroxidase results in production of radical cation $ABTS^{\bullet+}$. The addition of VECAR antioxidant causes an attenuation of the color change, which can be measured at 734 nm. The activity is usually expressed as an equivalent of the millimolar of a standard trolox solution.

The superoxide anion scavenging properties can be evaluated by the following methods: Xanthine-xanthine oxidase system assay: The superoxide anion is formed by the reaction between xanthine and xanthine oxidase. The superoxide anion produced can be detected at 560 nm due to change in color of nitroblue tetrazolium (NBT) [279]. Alkaline DMSO assay: alkaline DMSO is used to form superoxide anion and the reduction of NBT is measured with and without the antioxidant molecule at 560 nm [280].

The hydroxyl radical scavenging properties of VECAR can be measured by the following methods: p-nitroso dimethylaniline (pNDA) bleaching assay: The hydroxyl radical formed by reaction of hydrogen peroxide with ferric chloride results in bleaching of pNDA. The addition of VECAR changes the bleaching profile and the absorbance is measured at 440 nm [280]. Deoxiribose assay: hydroxyl radical degrades deoxyribose, and it can be measured colorimetrically. The hydroxyl radical can be generated by Fenton's reaction of ferric chloride with hydrogen peroxide [280].

The nitric oxide scavenging performance of VECAR can be evaluated by using nitroprusside, which at physiological pH liberates nitric acid, which is converted to nitrous acid and further forms nitrite ions on contact with air. The final reaction forms a pink color measured at 546 nm [281].

The peroxynitrite scavenging performance can be determined by the following assays Tyrosine Nitration Assay: The peroxynitrite action produces nitration of tyrosine, and the addition of an antioxidant molecule generates some degree of inhibition, which can be measured [282]. Oxidation of dihydrorhodamine assay: The fluorescence of the oxidation of dihydrorhodamine can be used for peroxinitrate scavenging properties of antioxidants [283].

The action of VECAR against lipid peroxidation can be evaluated by several methods Thiobarbituric acid assay (TBARS): it is based on reaction of melondialdehyde (MDA), an end product of lipid peroxidation, with thiobarbituric acid [284]. Iodometric assay: This method can be used for PUFA peroxidation and protein peroxidation studies. Based on the I^- ability to reduce the hydroperoxides formed in lipid peroxidation process [10,285]. Other techniques available are: conjugated dienes, fluorescent techniques, protein fragmentation with SDS-PAGE, surface charges by electrophoretic mobility of Apo B-100, electron spin resonance (ESR), nuclear magnetic resonance (NMR), and others.

For *in vivo* measurements, different assays can be used to provide information on the VECAR reactivity of free radicals and oxidant molecules. The most used assays are totally reactive antioxidant potential (TRAP), total antioxidant activity (TAA), oxygen radical absorbing capacity (ORAC), and total antioxidant reactivity (TAR) [185,286,287].

4.5 Hypothesis

The cellular antioxidant defense system is vital for human life, responsible for balancing free radical species with antioxidant molecules such as natural antioxidant molecules, proteins, and enzymes. An imbalance between free radical species and antioxidant molecules is responsible for certain diseases as discussed in previous sections. Supplementation with natural antioxidants has been suggested as an important path for prevention and treatment of oxidative stress related diseases; however, some clinical trials performed in the last years showed poor results associated with supplementation of natural antioxidants for disease treatment in humans. The difference between results *in vitro* and in humans has been a topic of debate. In the quest for new insights to address the surfaced uncertainties, a new approach is proposed herein focused on conjugation of several natural antioxidants to form an antioxidant component of improved antioxidant performance when compared to that of the individual components due to synergetic effects between the components (Figure 4-9).

The hypothesis is:

> Conjugation of vitamin E with carnosine, another potent natural antioxidant, by covalent bonding results in a new molecule with potent antioxidant properties capable of protecting polyunsaturated fatty acids (PUFAs) and LDL from oxidation.

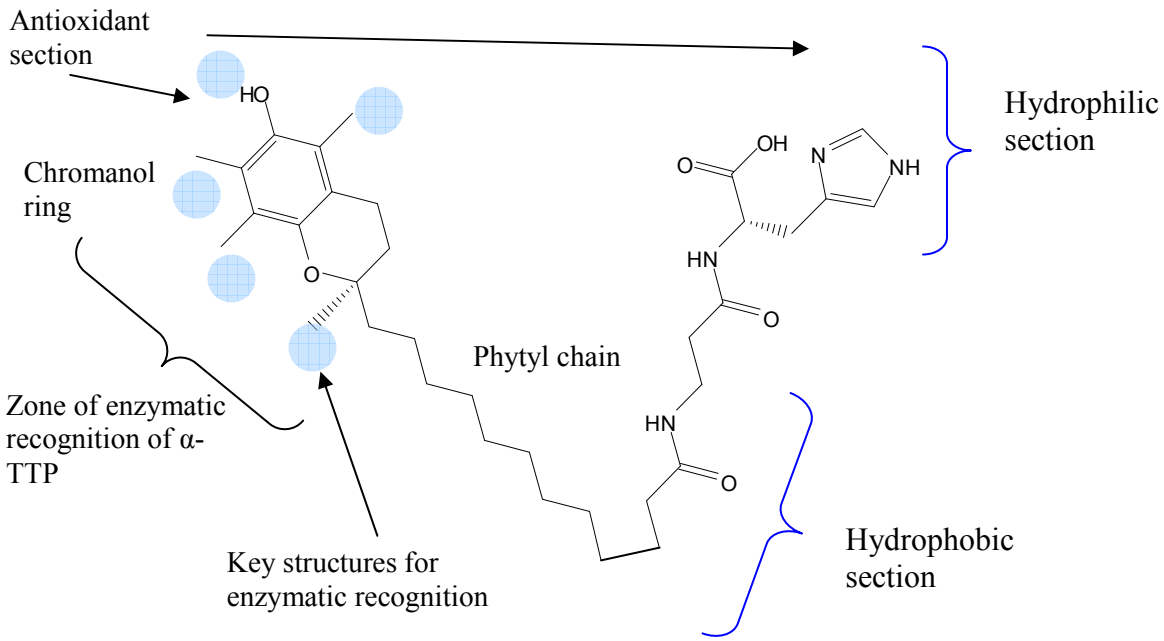


Figure 4-9: Schematic structure of VECAR. There is a hydrophilic section formed by the α-tocopherol chromanol ring and the carnosine dipeptide. The hydrophobic section is provided by the carbon tail that links polar heads to important sections. The circles around chromanol ring are depicted to represent the key zones for enzymatic recognition of VECAR by α-TTP enzyme.

4.6 Objectives

Several objectives were followed to assess the hypotheses previously established.

a. Synthesis of vitamin E-carnosine: Synthesis of VECAR (12 carbon chain lengths) VECAR) (Figure 4-9) based on the protection of OH group, activation of carbonyl group, Wittig reaction for ylide, attachment of carnosine, and de-protection of OH group.
b. Characterization of the molecular structure of VECAR by nuclear magnetic resonance (NMR) and analytic thin-layer chromatography (TLC)
c. Testing antioxidant properties of VECAR by DPPH and TBARS.

4.7 Materials

The following chemicals were purchased from Acros Organics: trolox 97%, tert-butylchlorodimethylsilane 98 %, and triethylamine 99%. Methanol anhydrous 99.8%, imidazole >99.5% (GC), dimethylformamide anhydrous 99.8%, diisobutylaluminum hydride (DIBAL) 1.0

M solution in hexane, 12-bromododecanoic acid 97%, acetonitrile anhydrous 99.8%, triphenylphosphine 99%, tetrahydrofuran <99.9%, lithium bis(trimethylsilyl)-amide (LiHMDS) 1.0 M solution in tetrahydrofuran, palladium (Pd/C) 10 wt. % (dry basis) on activated carbon, wet, Degussa type E101 NE/W, L-carnosine 99%, and 4-(dimethylamino) pyridine (DMAP) 99% were purchased from Sigma-Aldrich. Thionyl chloride 99+% and magnesium sulfate 99.5% were purchased from Alfa Aesar. The chemicals purchased from Mallinckrodt Chemicals were methanol, ethyl acetate, toluene, ammonium chloride, chloroform, sodium hydroxide, and sodium bicarbonate. Dichloromethane and hexanes 95% n-hexane were purchased from J.T. Baker. Silica gel Porosity: 60Å, Particle Size: 40-63 μm, Surface Area: 500-600 m^2/g, pH Range: 6.5-7.5, and silica gel of 300 Å, particle size of was purchased from Sorbent Technologies, and O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 99.58% was purchased from ChemPep Inc. The following chemicals were purchased from Fisher Chemical: 1-propanol and sodium chloride. Sodium bicarbonate was purchased from EMD Chemicals.

A stock solution of DPPH in methanol at 0.4 mM was prepared and kept at -20 °C in the dark prior to use. The sample was diluted with water (pH = 3.5 prepared by addition of acetic acid) to 1 ml. The sample was added to dilute DPPH stock in methanol to obtain a final sample volume of 2 ml with a 0.1 mM DPPH concentration. The ratio between water and methanol was 1 to 1 v/v. The blank was prepared with 1 ml of water (pH=3.5) and pure methanol. The controls were prepared for each sample, with 1 ml of water (pH = 3.5) and 1 ml of DPPH in methanol solution. Absorption readings were taken after 30 min at 518 nm using a Geminys 6 spectrophotometer (Thermo Scientific, Waltham, MA). The formula used to calculate the rate of oxidation was

$$\% \text{ change in activity} = ((Abs_{control} - Abs_{sample})/Abs_{control})*100 \quad [1]$$

4.8 Methods and Results

The first step was the synthesis of the new molecule (VECAR), and the second step was VECAR characterization to assess its antioxidant behavior.

4.8.1 Synthesis of α-tocopherol-carnosine (VECAR)

The synthesis of α-tocopherol-Carnosine (VECAR) was based on the protection of OH group, activation of carbonyl group, Wittig reaction for ylide, attachment of carnosine, and de-

protection. The method is based on Atkins and coworkers [288-299] and is described in more details below.

4.8.2 Trolox Methyl Ester

Synthesis of vitamin E-carnosine started with Trolox™. Trolox™ is a vitamin E derivative without the phytyl chain, which confers its hydrophilic behavior. S-trolox (3.5 g, 14 mmol) and dry methanol (26 mL) was stirred at 0°C for 15 minutes (Figure 4-10). Thionyl Chloride (1.26 mL, 16.9 mmol) was added to the solution and stirred at 0°C for an additional 10 minutes. The solution was refluxed for 1 hour at 70°C. Triethylamine (4 mL, 11 mmol) was added after 15 minutes. The solvent was evaporated and the crystals were re-dissolved in methanol. The re-crystallization from methanol yielded white crystals (2.89 g, 80%). ^{1}H ($CDCl_3$) NMR δ 4.24 (s, 1H, OH), 3.86 (s, 3H, OCH_3), 2.63 (m, 2H), 2.51 (m, 1H), 2.19 (s, 3H), 2.17 (s, 3H), 2.07 (s, 3H), 1.87 (m, 1H), 1.60 (s, 3H).

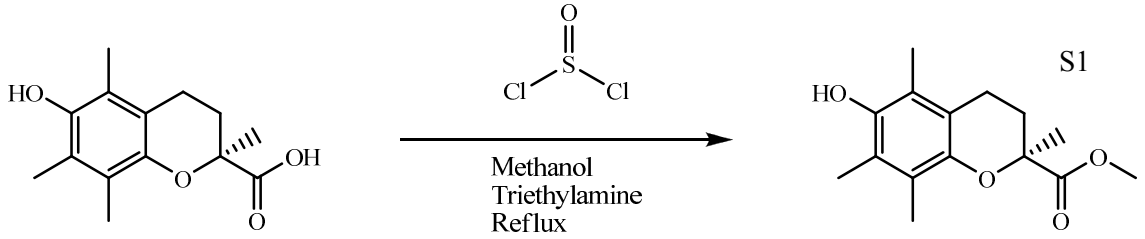


Figure 4-10: (S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) esterification S1

4.8.3 Protection of Hydroxyl Group

The product obtained in the first reaction, (S)-methyl-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate (S1) (1.254 g, 4.7 mmol), was mixed with tert-butyldimethylsilyl chloride (1.081g, 7.2mmol), and imidazole (1.339g, 19.7 mmol) in dry dimethylformamide (10 mL) (Figure 4-11). The mixture was stirred and heated in an oil bath under argon atmosphere at 85 °C for 5 h, at which point none of the starting material S1 was detected by TLC. The reaction mixture was poured into water and then extracted with ethyl acetate. The solution was dried over magnesium sulfate. Evaporation yielded a light yellow oil. The crude product was purified by column chromatography on silica gel using (CH_2Cl_2:hexane= 3:1) [299]. The yield of S2 was 1.62 g (90%). ^{1}H NMR ($CDCl_3$) δ 3.66 (s, 3H, OCH_3), 2.62 (m,

2H), 2.44 (m, 2H), 2.15 (s, 3H), 1.87 (m, 3H), 1.85 (m, 1H), 1.60 (s, 3H), 1.04 (s, 9H), 0.12 (s, 6H).

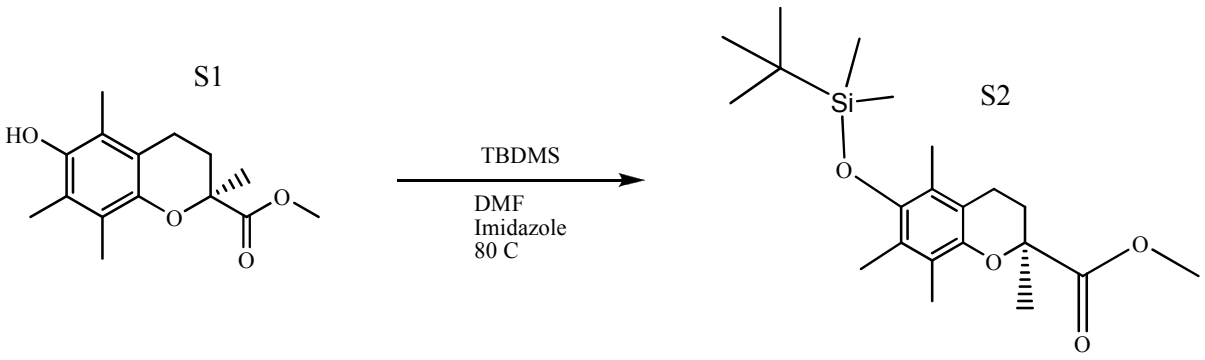


Figure 4-11: Synthesis of (S)-methyl 6-(*tert*-butyldimethylsilyloxy)-2,5,7,8-tetramethylchroman-2-carboxylate (S2) to protect the hydroxyl group in position 6.

4.8.4 Reduction of Ester to Aldehyde

S2 (3.282 g, 8.67 mmol) was added to dry hexane (35 mL), and was cooled in an acetone/dry ice bath to -75 °C (Figure 4-12). Diisobutylaluminum hydride (DIBAL-H, 1.0 M in hexane, 16 mL, 16 mmol) was added using a syringe as not to exceed -70 °C. After 2 h the reaction was quenched with dry methanol (10 mL) and stirred for 15 minutes. The solution was removed from the acetone/dry ice bath and was allowed to cool to -8°C. Water (15 mL) was added to the solution by syringe. The solution was then poured into water and extracted with hexane/ethyl acetate (2:1). The solution was dried over magnesium sulfate and the solvent was evaporated. The crude product was purified by column chromatography using CH_2Cl_2:hexane (1:1 to 3:1) [299]. The yield was of S3 was 2.33 g (77%). ^{1}H NMR ($CDCl_3$) δ 9.63 (s, 1H), 2.56 (m, 2H), 2.28 (m, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 1.84 (m, 1H), 1.39 (s, 3H), 1.05 (s, 9H), 0.12 (s, 6H).

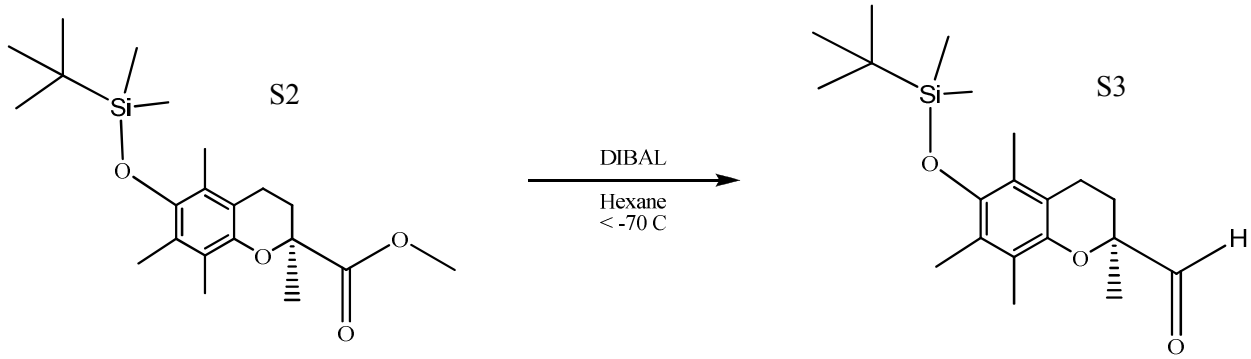


Figure 4-12**:** Reduction of S2 ester to synthesize (S)-6-(*tert*-butyldimethylsilyloxy)-2,5,7,8-tetramethylchroman-2-carbaldehyde (S3).

4.8.5 Ylide Synthesis

A solution of 12-bromododecanoic acid (1.66 g, 5.9 mmol) in dry acetonitrile (13 mL) was heated and stirred. Triphenylphosphine (1.637 g, 6.2 mmol) in dry acetonitrile (13 mL) was heated and stirred then added to the 12-bromododecanoic solution; the solution was refluxed overnight (Figure 4-13). The solvent was removed. The crystals were dissolved in DCM (14 mL). Toluene (40 mL) was added and the solution was stirred and heated to remove the DCM. The solution was cooled and the phosphonium salts crystallized where removed by filtration. The solution was decanted with toluene. The yield of S4 was 3.01 g (93.5%). ^{1}H NMR ($CDCl_3$) δ 7.84-7.68 (m, 15H), 3.68-3.50 (m, 4H), 2.36 (s, 2H), 1.61-1.19 (m, 16H).

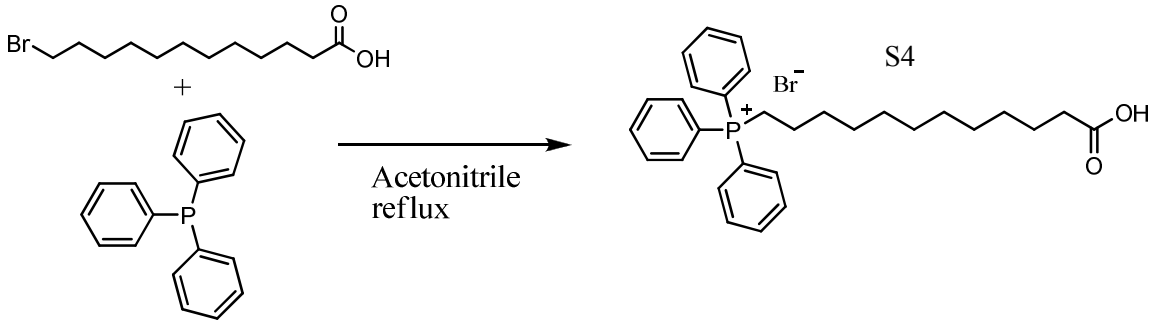


Figure 4-13: Reaction to synthesize (11-carboxyundecyl)triphosphonium bromide (S4) salt.

4.8.6 Wittig Reaction for Coupling the Phytyl Chain

The suspension of phosphonium salts S4 (2.927 g, 5.4 mmol) was dissolved in dry THF (55 mL) at room temperature under argon. A solution of LiHMDS in THF (1 M in THF, 16 mL, 13.4 mmol) was added dropwise via a syringe (Figure 4-14). The red ylide was stirred for 2 h under argon. A solution of S3 (1.85 g) in dry THF (8 mL) was added dropwise. The color changed from red to pale yellow. The suspension was stirred for an additional 3 h, until S3 cannot be found by TLC. The reaction was quenched with saturated NH_4Cl (80 mL) and water (80 mL) and then extracted with ethyl acetate. The solution was dried over magnesium sulfate. After solvent removal, trituration with cold hexane removed triphenylphosphine oxide. The hexane solution was evaporated, and the crude product was purified by column chromatography on silica gel using ethyl acetate:hexane (1:1) [299].Yield of S5 was 1.99 g (71%). ^{1}H NMR ($CDCl_3$) δ 5.32 (d, 2H), 2.55 (t, 2H), 2.35 (m, 1H), 2.11 (s, 8H), 2.09 (s, 2H), 2.04 (s, 3H), 1.64 (m, 2H), 1.46 (s, 4H), 1.24 (s, 9H), 1.04 (s, 9H), 0.11 (s, 6H).

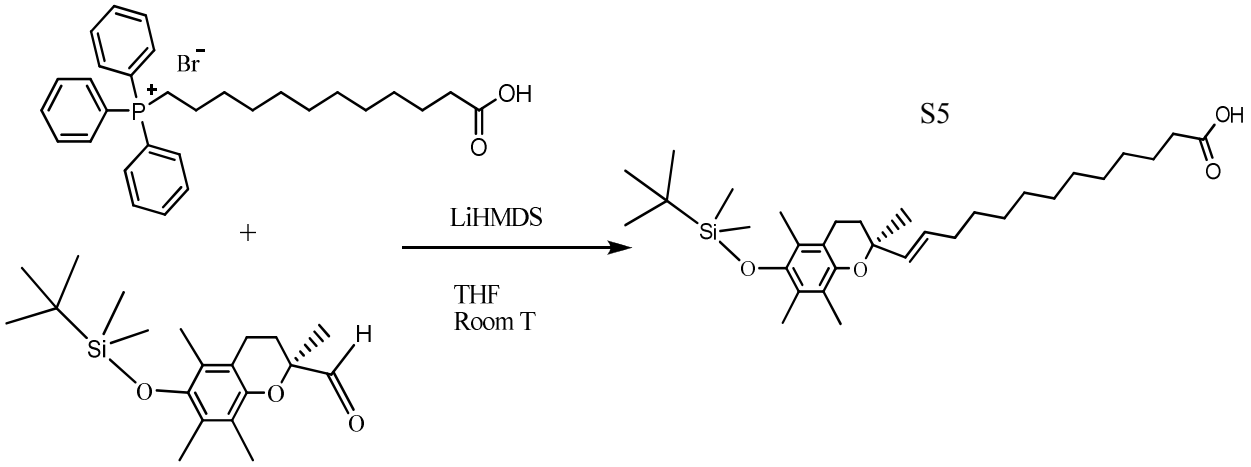


Figure 4-14: Wittig reaction to synthesize (S,E)-13-(6-(*tert*-butyldimethylsilyloxy)-2,5,7,8-tetramethylchroman-2-yl)tridec-12-enoic acid (S5).

4.8.7 Saturation of Phytyl Chain

Pd/C (0.505 g) was added to a solution of S5 (0.95 g, 3.67mmol) in ethyl acetate (85 mL). A hydrogen balloon was attached to the reaction mixture and it was stirred for 18 h at room temperature (Figure 4-15). The mixture was filtered and evaporated to obtain S6 as an oil. The crude product was purified by column chromatography using hexane:ethyl acetate (4:1). The Yield of S6 was 0.91 g (94%) [299]. ^{1}H NMR ($CDCl_3$) δ 2.55 (t, 2H), 2.37 (t, 2H), 2.07 (t, 9H), 1.80 (m, 2H), 1.58 (m, 2H), 1.22 (s, 18H), 1.05 (s, 9H), 0.12 (s, 6H).

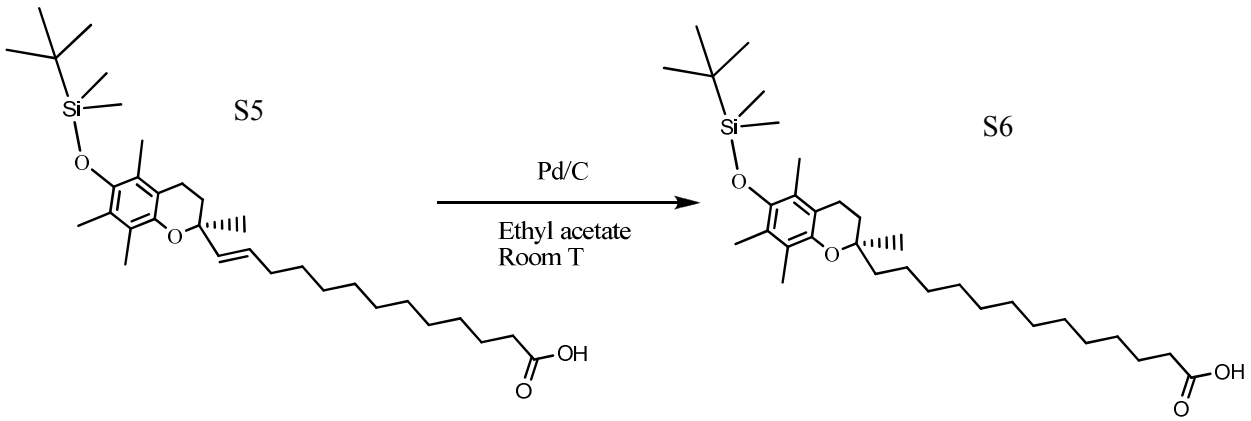


Figure 4-15: saturation reaction to synthesize (R)-13-(6-(*tert*-butyldimethylsilyloxy)-2,5,7,8-tetramethylchroman-2-yl)tridecanoic acid (S6).

4.8.8 Carnosine Methyl Ester Dihydrochloride

Thionyl chloride (0.25 mL, 3.3 mmol) was added dropwise to a suspension of carnosine (602.9 mg, 2.7mmol) in anhydrous methanol (24 mL) at 0 °C and was stirred for 10 minutes. The solution was refluxed at 75°C for 1 hour (Figure 4-16). After cooling down to room temperature, the mixture was concentrated. The product was used in the next step without further purification [300]. ^{1}H NMR (MeOH) δ 8.85 (s, 1H), 7.67 (s, 1H), 7.55 (s, 1H), 4.82 (m, 3H), 3.94 (s, 3H), 3.30 (m, 2H), 3.01 (m, 2H), 2.70 (m, 2H).

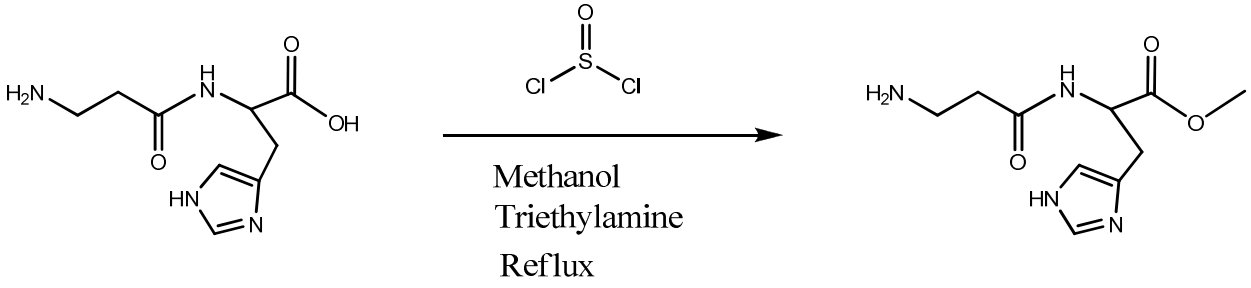


Figure 4-16: Carnosine esterification by acyl chlorides to synthesize methyl 2-(3-aminopropanamido)-3-(1H-imidazol-4yl)propanoate dihydrochloride (S7).

4.8.9 Coupling Carnosine Methyl Ester with Carboxylic Acid

The carboxylic acid compound S6 (840 mg, 1.59 mmol), DMAP (19 mg, 0.16 mmol), carnosine-methyl ester hydrochloride (S7) (662 mg, 2.39mmol), and triethylamine (1.67 mL, 5 equiv) were added to anhydrous DMF (13.4 ml) (Figure 4-17). The solution was cooled to 0°C in an ice bath. HBTU (721 mg, 1.9 mmol) in anhydrous DMF (3 mL) was added to the reaction mixture drop wise, and was stirred for an additional 20 minutes at 0°C. The reaction was stirred at room temperature over night. Next, the solvent was evaporated and dried under high vacuum. The washing and extraction steps were performed with chloroform and water without addition of magnesium sulfate. The organic phase was collected and evaporated under vacuum. The purification was performed with a reverse phase silica gel (C_{18}).The silica gel used was 300 Å pore size with a mixture of methanol:water (7:3) as eluent. The yield of S7 was 0.62 g (43%). [301-303]. ^{1}H NMR (MeOH) δ 8.52 (s, 1H), 7.24 (s, 1H), 3.74 (m, 3H), 2.57 (m, 2H), 2.41 (m, 2H), 2.09 (m, 13H), 1.78 (m, 2H), 1.58 (m, 4H), 1.52 (m, 4H), 1.27 (s, 15H), 1.22 (s, 4H), 1.04 (s, 9H), 0.11 (s, 6H)

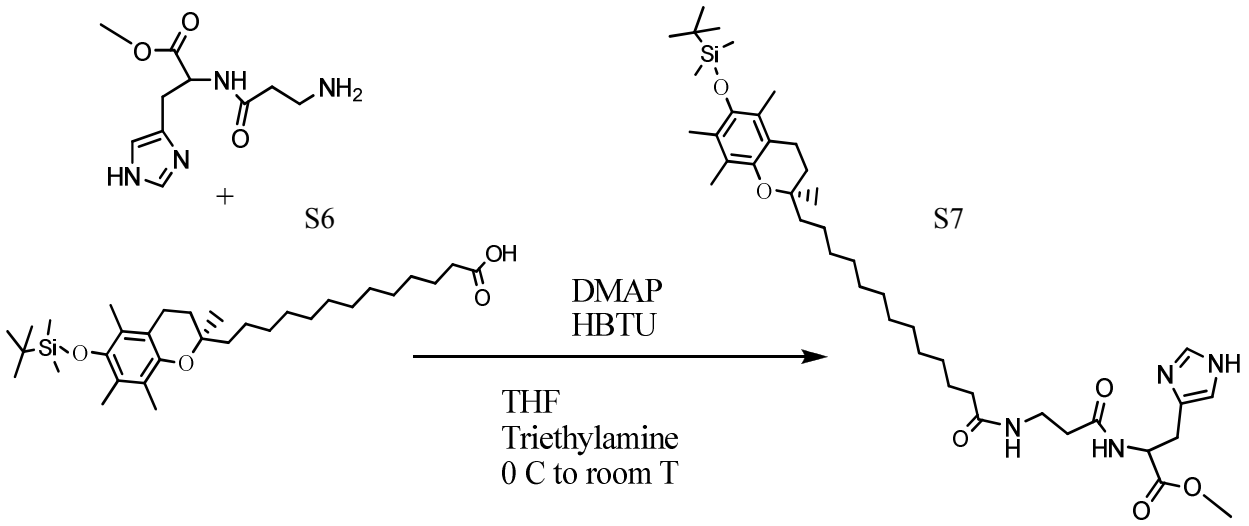


Figure 4-17: Coupling reaction with HBTU to synthesize methyl 2-(3-(13-((*R*)-6-(*tert*-butyldimethylsilyloxy)-2,5,7,8-tetramethylchroman-2-yl)tridecanamido)propanamido)-3-(1H-imidazol-4-yl)propanoate (S8).

4.8.10 De-protection of Hydroxyl Group of Chromanol Ring

The de-protection of (S8) was performed by dissolving S8 (260 mg), in dry THF (10 ml) and the addition of TBAF (2.9 mL of a 1 M THF solution) was performed drop wise via a syringe (Figure 4-18). The solution was stirred for 1.5 hour at room temperature. The THF was evaporated and replaced with chloroform (200 ml). The solution was washed with water three times. The crude VECAR product was purified by flash chromatography on reverse phase silica gel (60 Å) using gradient elution, methanol:water (30%) and methanol:water:ammonium hydroxide (8:1:1). The first two fractions collected the impurities and pure VECAR was collected in the last fraction. The yield of S8 was 130 mg (60%).

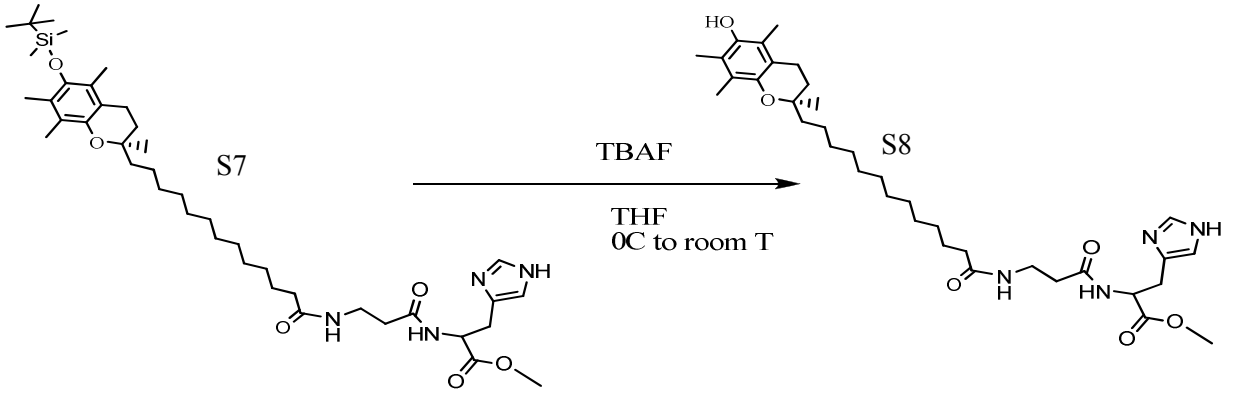


Figure 4-18: de-protection of S0 to synthesize methyl 2-(3-(13-((*R*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)tridecanamido)propanamido)-3-(1*H*-imidazol-4-yl)propanoate (VECAR).

4.8.11 Reverse Phase Silica Gel

The synthesis of reverse phase silica gel was performed to use it in the purification of VECAR after coupling reaction. Normal phase silica gel (100 mg) was suspended in toluene (500 ml), and 21 ml (0.52 mol) of octadecyltrichlorosilane (C_{18}, ODTS) was added drop wise. After 10 min, triethylamine (28 ml, 4 equiv.) was added to the suspension, and the final suspension was refluxed for 24 hr at 125 °C. Finally, the reverse phase silica gel was washed with toluene (1 L), DCM (1 L), ethyl acetate (1 L), and methanol (4 L). The product was dried under high vacuum and storage for use.

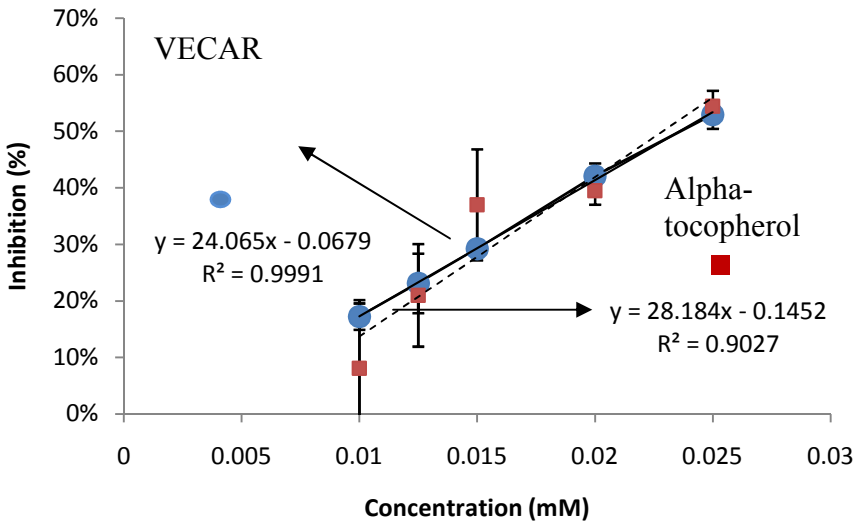


Figure 4-19: Rate of radical inhibition of VECAR by the DPPH method.

4.9 Antioxidant Activity of VECAR by DPPH

The antioxidant behavior of VECAR was studied with the DPPH assay to assess the antioxidant activity provided by the chromanol ring of VECAR. The deprotection of the hydroxyl group provides the antioxidant behavior of VECAR, similar to that of alpha-tocopherol.

The measurements were performed at 5 concentrations (Figure 4-19) to determine IC_{50}, which is the point at which 50% of DPPH has been reduced by VECAR. The IC_{50} was 24.9±1.4 µM for VECAR, the value for alpha-tocopherol was 24.4±1.5 µM. These values confirm that VECAR has antioxidant activities that are similar to pure alpha-tocopherol which is the main hydrophobic antioxidant used in the synthesis.

4.10 Conclusions

The synthesis of VECAR (carnosine plus alpha-tocopherol derivative) was accomplished in nine steps. The different steps followed to synthesize VECAR were designed to form a phytyl chain with a reactive end group (carboxylic acid). The first step (Trolox esterification) was performed by an acid chloride (thionyl chloride) reaction. The selection was based on the yields and reaction time compared with p-toluenesulfonic acid as catalyst. The yields were increased from 50% to 78% and the time was reduced from 18 hrs to less than 2 hrs when thionyl chloride was used as catalyst. The second step was the protection of the hydroxyl group present in the chromanol ring of Trolox by using tert-butyldimethylsilyl chloride (TBDMS) as protecting agent. The protection allowed the recovery of the hydroxyl group at the last reaction step. The third step was the reduction of the acid to an aldehyde by using DIBAL at -70 °C. The reaction was difficult because the separation of the un-reacted ester with the aldehyde required gradient elution of the column chromatography. The water present in the sample reduced the yields and increased the complexity during purification with flash chromatography. The reaction yields of the first three steps were improved by elimination of the residual water present in the samples after purification. Next, the Wittig reaction was used to add a phytyl chain of 12 carbons to the chromanol ring. The phytyl chain used was provided by 12-bromododecanoic acid, which allowed the synthesis of a phosphonium salt prior to the Wittig reaction. The product was then saturated in the presence of hydrogen. The coupling reaction of carnosine was complicated due to the hydrophobic/hydrophilic behavior of the new molecule. Hence, the carnosine was modified to carnosine ester to improves its solubility in polar solvents and avoid reactions between the carboxylic groups of carnosine and the alpha-tocopherol analog. Different coupling reactions such as dicyclohexylcarbodiimide (DCC), conjugated anhydrides, and (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) were attempted. The best results were achieved when HBTU was used as coupling reagent because the purification step was possible by using reverse phase column chromatography. Reverse phase silica gel was necessary because the molecule did not move through the normal silica gel. Finally, the de-protection was performed by tetrabutylammonium fluoride (TBAF) which is a standard protocol for elimination of TBDMS. The reaction times were reduced from over night (12 hrs) to 1.5 hr which improved the purification and final purity of VECAR. The reverse phase

silica was used in a flash column chromatography to purify VECAR after the coupling reaction and de-protection. The structure of VECAR was confirmed by H-NMR.

The active phytyl chain, which can be used to attach other molecules for therapeutic purposes in further experiments, allowed for the antioxidant properties of alpha-tocopherol to be maintained. VECAR presented an antioxidant behavior as confirmed by the DPPH assay. The VECAR IC50 value (24.9±1.4 μM) was close to that of pure alpha-tocopherol (24.4±1.5 μM) which confirmed the recovery of the hydroxyl group in the chromanol ring of VECAR. Diseases such as Parkinson's, Alzheimer's, and atherosclerosis are linked to oxidation process and could potentially benefit from VECAR.

4.11 References

[1] Halliwell B. Oxidative stress and neurodegeneration: where are we now? Journal of Neurochemistry 2006;97 (6):1634-58.

[2] Halliwell B. Oxidants and human disease: some new concepts. The FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 1987;1 (5):358-64.

[3] Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, McCord JM, Harman D. Oxygen radicals and human disease. Annals Of Internal Medicine 1987;107 (4):526-45.

[4] Halliwell B. Drug Antioxidant Effects - a Basis for Drug Selection. Drugs 1991;42 (4):569-605.

[5] Eiserich JP, van der Vliet A, Handelman GJ, Halliwell B, Cross CE. Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. The American Journal Of Clinical Nutrition 1995;62 (6 Suppl):1490S-500S.

[6] Jain SK, McVie R, Jaramillo JJ, Palmer M, Smith T, Meachum ZD, Little RL. The effect of modest vitamin E supplementation on lipid peroxidation products and other cardiovascular risk factors in diabetic patients. Lipids 1996;31 Suppl:S87-90.

[7] Spencer JP, Jenner A, Butler J, Aruoma OI, Dexter DT, Jenner P, Halliwell B. Evaluation of the pro-oxidant and antioxidant actions of L-DOPA and dopamine in vitro: implications for Parkinson's disease. Free Radical Research 1996;24 (2):95-105.

[8] Cooke JP. Nutriceuticals for cardiovascular health. The American Journal Of Cardiology 1998;82 (10A):43S-6S.

[9] Drury JA, Nycyk JA, Baines M, Cooke RW. Does total antioxidant status relate to outcome in very preterm infants? Clinical Science (London, England: 1979) 1998;94 (2):197-201.

[10] Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W, Vina-Ribes J. Functional food science and defence against reactive oxidative species. The British Journal Of Nutrition 1998;80 Suppl 1:S77-112.

[11] Traber MG, Jialal I. Measurement of lipid-soluble vitamins--further adjustment needed? Lancet 2000;355 (9220):2013-4.

[12] Traber MG, van der Vliet A, Reznick AZ, Cross CE. Tobacco-related diseases. Is there a role for antioxidant micronutrient supplementation? Clinics In Chest Medicine 2000;21 (1):173-87, x.

[13] Halliwell B, Zhao KC, Whiteman M. The gastrointestinal tract: A major site of antioxidant action? Free Radical Research 2000;33 (6):819-30.

[14] Pryor WA, Stahl W, Rock CL. Beta Carotene: From biochemistry to clinical trials. Nutrition Reviews 2000;58 (2):39-53.

[15] Halliwell B. Oral inflammation and reactive species: a missed opportunity? Oral Diseases 2000;6 (3):136-7.

[16] Jialal I, Traber M, Devaraj S. Is there a vitamin E paradox? Current Opinion In Lipidology 2001;12 (1):49-53.

[17] Young IS, Woodside JV. Antioxidants in health and disease. Journal of Clinical Pathology 2001;54 (3):176-86.

[18] Sagin FG, Sozmen EY. Anti-inflammatory Effects of Dietary Antioxidants. Current Medicinal Chemistry - Anti-Inflammatory & Anti-Allergy Agents; 2004;3 (1):19-30.

[19] Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Critical Reviews in Food Science and Nutrition 2004;44 (4):275-95.

[20] Halliwell B. Mechanisms involved in the generation of free radicals. Pathologie Biologie 1996;44 (1):6-13.

[21] Halliwell B. Antioxidant defence mechanisms: From the beginning to the end (of the beginning). Free Radical Research 1999;31 (4):261-72.

[22] Visioli F, Keaney JF, Halliwell B. Antioxidants and cardiovascular disease; panaceas or tonics for tired sheep? Cardiovascular Research 2000;47 (3):409.

[23] Halliwell B. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. Drugs & Aging 2001;18 (9):685-716.

[24] Evans P, Halliwell B. Micronutrients: oxidant/antioxidant status. British Journal of Nutrition 2001;85:S67-S74.

[25] O'Reilly JD, Mallet AI, McAnlis GT, Young IS, Halliwell B, Sanders TAB, Wiseman H. Consumption of flavonoids in onions and black tea: lack of effect on F-2-isoprostanes and autoantibodies to oxidized LDL in healthy humans. American Journal of Clinical Nutrition 2001;73 (6):1040-4.

[26] Jenner AM, Ruiz JE, Dunster C, Halliwell B, Mann GE, Siow RCM. Vitamin C protects against hypochlorous acid-induced glutathione depletion and DNA base and protein damage in human vascular smooth muscle cells. Arteriosclerosis Thrombosis and Vascular Biology 2002;22 (4):574-80.

[27] Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, Zheng ZJ, Flegal K, O'Donnell C, Kittner S, Lloyd-Jones D, Goff DC, Hong YL, Adams R, Friday G, Furie K, Gorelick P, Kissela B, Marler J, Meigs J, Roger V, Sidney S, Sorlie P, Steinberger J, Wasserthiel-Smoller S, Wilson M, Wolf P. Heart disease and stroke statistics - 2006 update - A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2006;113 (6):E85-E151.

[28] Davies KJ. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 2000;50 (4-5):279-89.

[29] Diaz MN, Frei B, Vita JA, Keaney JF. Mechanisms of disease - Antioxidants and atherosclerotic heart disease. New England Journal of Medicine 1997;337 (6):408-16.

[30] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. The FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 1999;13 (10):1145-55.

[31] Bowry VW, Stocker R. Tocopherol-Mediated Peroxidation - the Prooxidant Effect of Vitamin-E on the Radical-Initiated Oxidation of Human Low-Density-Lipoprotein. Journal of the American Chemical Society 1993;115 (14):6029-44.

[32] Pryor WA. Measurement of Oxidative Stress Status in Humans. Cancer Epidemiology Biomarkers & Prevention 1993;2 (3):289-92.

[33] Pryor WA. Vitamin E and heart disease: Basic science to clinical intervention trials. Free Radical Biology and Medicine 2000;28 (1):141-64.

[34] Morel DW, Dicorleto PE, Chisolm GM. Endothelial and Smooth-Muscle Cells Alter Low-Density Lipoprotein Invitro by Free-Radical Oxidation. Arteriosclerosis 1984;4 (4):357-64.

[35] Steinberg D, Berliner JA, Burton GW, Carew TE, Chait A, Chisolm GM, Esterbauer H, Fogelman AM, Fox PL, Furberg CD, Gaziano JM, Gey KF, Grundy SM, Harlan WR, Havel RJ, Hennekens CH, Hoff HF, Jackson RL, Kayden HJ, Keech A, Krinsky NI, Manson J, Parthasarathy S, Probstfield J, Pryor WA, Rifkind B, Stadtman ER, Wallace RB, Witztum JL, Ylaherttuala S, Yusuf S. Antioxidants in the Prevention of Human Atherosclerosis - Summary of the Proceedings of a National-Heart-Lung-and-Blood-

Institute Workshop - September 5-6, 1991, Bethesda, Maryland. Circulation 1992;85 (6):2338-44.

[36] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radical Biology & Medicine 2000;28 (12):1815-26.

[37] Kayden HJ, Traber MG. Clinical, nutritional and biochemical consequences of apolipoprotein B deficiency. Advances In Experimental Medicine And Biology 1986;201:67-81.

[38] Traber MG, Kayden HJ. Tocopherol distribution and intracellular localization in human adipose tissue. The American Journal Of Clinical Nutrition 1987;46 (3):488-95.

[39] Traber MG, Sokol RJ, Ringel SP, Neville HE, Thellman CA, Kayden HJ. Lack of tocopherol in peripheral nerves of vitamin E-deficient patients with peripheral neuropathy. The New England Journal Of Medicine 1987;317 (5):262-5.

[40] Traber MG, Ingold KU, Burton GW, Kayden HJ. Absorption and transport of deuterium-substituted 2R,4'R,8'R-alpha-tocopherol in human lipoproteins. Lipids 1988;23 (8):791-7.

[41] Traber MG, Kayden HJ. Alpha-tocopherol as compared with gamma-tocopherol is preferentially secreted in human lipoproteins. Annals Of The New York Academy Of Sciences 1989;570:95-108.

[42] Traber MG, Burton GW, Ingold KU, Kayden HJ. RRR- and SRR-alpha-tocopherols are secreted without discrimination in human chylomicrons, but RRR-alpha-tocopherol is preferentially secreted in very low density lipoproteins. Journal Of Lipid Research 1990;31 (4):675-85.

[43] Traber MG, Rudel LL, Burton GW, Hughes L, Ingold KU, Kayden HJ. Nascent VLDL from liver perfusions of cynomolgus monkeys are preferentially enriched in RRR- compared with SRR-alpha-tocopherol: studies using deuterated tocopherols. Journal Of Lipid Research 1990;31 (4):687-94.

[44] Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annual Review Of Nutrition 1990;10:357-82.

[45] Traber MG, Lane JC, Lagmay NR, Kayden HJ. Studies on the transfer of tocopherol between lipoproteins. Lipids 1992;27 (9):657-63.

[46] Traber MG. Determinants of plasma vitamin E concentrations. Free Radical Biology & Medicine 1994;16 (2):229-39.

[47] Traber MG, Packer L. Vitamin E: beyond antioxidant function. The American Journal Of Clinical Nutrition 1995;62 (6 Suppl):1501S-9S.

[48] Traber MG, Sies H. Vitamin E in humans: demand and delivery. Annual Review Of Nutrition 1996;16:321-47.

[49] Traber MG. Regulation of human plasma vitamin E. Advances In Pharmacology (San Diego, Calif) 1997;38:49-63.

[50] Traber MG. Utilization of vitamin E. Biofactors (Oxford, England) 1999;10 (2-3):115-20.

[51] Blatt DH, Leonard SW, Traber MG. Vitamin E kinetics and the function of tocopherol regulatory proteins. Nutrition (Burbank, Los Angeles County, Calif) 2001;17 (10):799-805.

[52] Traber MG. Vitamin E: too much or not enough? The American Journal Of Clinical Nutrition 2001;73 (6):997-8.

[53] Ibrahim W, Bhagavan HN, Chopra RK, Chow CK. Interaction between vitamin E (VE) and coenzyme Q10 (Q10) in rat tissues and mitochondria. Faseb Journal 1999;13 (4):A535-A.

[54] Halliwell B. Antioxidants in human health and disease. Annual Review of Nutrition 1996;16:33-50.

[55] Bowry VW, Ingold KU, Stocker R. Vitamin-E in Human Low-Density-Lipoprotein - When and How This Antioxidant Becomes a Prooxidant. Biochemical Journal 1992;288:341-4.

[56] Jialal I, Fuller CJ. Oxidized Ldl and Antioxidants. Clinical Cardiology 1993;16 (4):6-9.

[57] Sattler W, Christison J, Stocker R. Cholesterylester Hydroperoxide Reducing Activity Associated with Isolated High-Density and Low-Density Lipoproteins. Free Radical Biology and Medicine 1995;18 (3):421-9.

[58] Christison J, Karjalainen A, Brauman J, Bygrave F, Stocker R. Rapid reduction and removal of HDL- but not LDL-associated cholesteryl ester hydroperoxides by rat liver perfused in situ. Biochemical Journal 1996;314:739-42.

[59] Steinberg D. Oxidized low density lipoprotein - An extreme example of lipoprotein heterogeneity. Israel Journal of Medical Sciences 1996;32 (6):469-72.

[60] Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. Journal of Internal Medicine 2005;258 (5):395-410.

[61] Noguchi N, Niki E. Apolipoprotein-B Protein Oxidation in Low-Density Lipoproteins. Oxygen Radicals in Biological Systems, Pt C 1994;233:490-4.

[62] Fuller CJ, Agil A, Lender D, Jialal I. Superoxide production and LDL oxidation by diabetic neutrophils. Journal of Diabetes and Its Complications 1996;10 (4):206-10.

[63] Jialal I, Devaraj S. Low density lipoprotein oxidation, antioxidants, and atherosclerosis: A clinical biochemistry perspective. Clinical Chemistry 1996;42 (4):498-506.

[64] Mashima R, Yoshimura S, Yamamoto Y. Reduction of lipid hydroperoxides by apolipoprotein B-100. Biochemical and Biophysical Research Communications 1999;259 (1):185-9.

[65] Witting PK, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Eriksson AW, Stocker R. Inhibition by a coantioxidant of aortic lipoprotein lipid peroxidation and atherosclerosis in apolipoprotein E and low density lipoprotein receptor gene double knockout mice. Faseb Journal 1999;13 (6):667-75.

[66] Yang CY, Wang J, Krutchinsky AN, Chait BT, Morrisett JD, Smith CV. Selective oxidation in vitro by myeloperoxidase of the N-terminal amine in apolipoprotein B-100. Journal of Lipid Research 2001;42 (11):1891-6.

[67] Yang XP, Yan DG, Qiao CP, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC. Increased atherosclerotic lesions in ApoE mice with plasma phospholipid transfer protein overexpression. Arteriosclerosis Thrombosis and Vascular Biology 2003;23 (9):1601-7.

[68] Theriault A, Chao JT, Wang Q, Gapor A, Adeli K. Tocotrienol: A review of its therapeutic potential. Clinical Biochemistry 1999;32 (5):309-19.

[69] Wang WQ, Goodman MT. Antioxidant property of dietary phenolic agents in a human LDL-oxidation ex vivo model: Interaction of protein binding activity. Nutrition Research 1999;19 (2):191-202.

[70] Halliwell B, Zhao K, Whiteman M. Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good - A personal view of recent controversies. Free Radical Research 1999;31 (6):651-69.

[71] Aruoma OI, Deiana M, Halliwell B, Kaur H, Banni S, Corongiu FP, Dessi MA, Aeschbach R. Antioxidant action of hydroxytyrosol. Free Radical Biology and Medicine 1999;27:S31-S.

[72] Halliwell B. Vitamin C: poison, prophylactic or panacea? Trends in Biochemical Sciences 1999;24 (7):255-9.

[73] Shi HL, Noguchi N, Niki E. Dynamics of antioxidant action of ubiquinol: a reappraisal. Biofactors 1999;9 (2-4):141-8.

[74] Yamashita H, Nakamura A, Noguchi N, Niki E, Kuhn H. Oxidation of low density lipoprotein and plasma by 15-lipoxygenase and free radicals. Febs Letters 1999;445 (2-3):287-90.

[75] Deiana M, Aruoma OI, Bianchi ML, Spencer JP, Kaur H, Halliwell B, Aeschbach R, Banni S, Dessi MA, Corongiu FP. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. Free Radical Biology & Medicine 1999;26 (5-6):762-9.

[76] Decker EA, Livisay SA, Zhou S. A re-evaluation of the antioxidant activity of purified carnosine. Biochemistry Biokhimii︠A︡ 2000;65 (7):766-70.

[77] Witting PK, Petersson K, Letters J, Stocker R. Anti-atherogenic effect of coenzyme Q(10) in apolipoprotein E gene knockout mice. Free Radical Biology and Medicine 2000;29 (3-4):295-305.

[78] Ibrahim WH, Bhagavan HN, Chopra RK, Chow CK. Dietary coenzyme Q10 and vitamin E alter the status of these compounds in rat tissues and mitochondria. Journal of Nutrition 2000;130 (9):2343-8.

[79] Porkkala-Sarataho E, Salonen JT, Nyyssonen K, Kaikkonen J, Salonen R, Ristonmaa U, Diczfalusy U, Brigelius-Flohe R, Loft S, Poulsen HE. Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2 '-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arteriosclerosis Thrombosis and Vascular Biology 2000;20 (9):2087-93.

[80] Long LH, Halliwell B. Coffee drinking increases levels of urinary hydrogen peroxide detected in healthy human volunteers. Free Radical Research 2000;32 (5):463-7.

[81] Woodside JV, Denholm EE, Campbell MJ, Young IS, Honour J, Leathem A. The effect of phyto-oestrogen supplementation on antioxidant status. Proceedings of the Nutrition Society 2000;59:48a-a.

[82] Price DL, Rhett PM, Thorpe SR, Baynes JW. Chelating activity of advanced glycation end-product inhibitors. The Journal Of Biological Chemistry 2001;276 (52):48967-72.

[83] Decker EA, Ivanov V, Zhu BZ, Frei B. Inhibition of low-density lipoprotein oxidation by carnosine histidine. Journal Of Agricultural And Food Chemistry 2001;49 (1):511-6.

[84] Choy KJ, Deng YM, Hou JY, Wu B, Lau A, Witting PK, Stocker R. Coenzyme Q(10) supplementation inhibits aortic lipid oxidation but fails to attenuate intimal thickening in balloon-injured New Zealand white rabbits. Free Radical Biology and Medicine 2003;35 (3):300-9.

[85] Chai PC, Long LH, Halliwell B. Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines. Biochemical and Biophysical Research Communications 2003;304 (4):650-4.

[86] Niki E, Yoshida Y, Saito Y, Piga R, Noguchi N. Antioxidant action of vitamin E isoforms. Free Radical Research 2003;37:40-.

[87] Devaraj S, Traber MG. Gamma-tocopherol, the new vitamin E? The American Journal of Clinical Nutrition 2003;77 (3):530-1.

[88] Stocker R. Antioxidant activities of bile pigments. Antioxidants & Redox Signaling 2004;6 (5):841-9.

[89] Warren DL, Jenner P, Halliwell B. In vitro antioxidant actions of antiparkinsonian agents. British Journal of Pharmacology 1996;117:P187-P.

[90] Salgo MG, Pryor WA. Trolox inhibits peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes. Archives of Biochemistry and Biophysics 1996;333 (2):482-8.

[91] Harris WS. The Prevention of Atherosclerosis with Antioxidants. Clinical Cardiology 1992;15 (9):636-40.

[92] Traber MG, Diamond SR, Lane JC, Brody RI, Kayden HJ. beta-Carotene transport in human lipoproteins. Comparisons with a-tocopherol. Lipids 1994;29 (10):665-9.

[93] Pryor WA. Beta-Carotene, Vitamin-E, and Lung-Cancer. New England Journal of Medicine 1994;331 (9):612-.

[94] Halliwell B. Vitamin C: Antioxidant or pro-oxidant in vivo? Free Radical Research 1996;25 (5):439-54.

[95] Rehman A, Collis CS, Yang M, Kelly M, Diplock AT, Halliwell B, Rice-Evans C. The effects of iron and vitamin C co-supplementation on oxidative damage to DNA in healthy volunteers. Biochemical and Biophysical Research Communications 1998;246 (1):293-8.

[96] Halliwell B. Vitamin C and genomic stability. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 2001;475 (1-2):29-35.

[97] Azzi A, Kempna P, Ricciarelli R, Villacorta L, Visarius T, Zing JM. Cell signaling: Regulation by tocopherols. Free Radical Biology and Medicine 2002;33:S185-S.

[98] Vitaglione P, Morisco F, Caporaso N, Fogliano V. Dietary antioxidant compounds and liver health. Critical Reviews in Food Science and Nutrition 2004;44 (7-8):575-86.

[99] Cooke MS, Evans MD, Mistry N, Lunec J. Role of dietary antioxidants in the prevention of in vivo oxidative DNA damage. Nutrition Research Reviews 2002;15 (1):19-41.

[100] Engler MM, Engler MB, Malloy MJ, Chiu EY, Schloetter MC, Paul SM, Stuehlinger M, Lin KY, Cooke JP, Morrow JD, Ridker PM, Rifai N, Miller E, Witztum JL, Mietus-Snyder M. Antioxidant vitamins C and E improve endothelial function in children with hyperlipidemia: Endothelial Assessment of Risk from Lipids in Youth (EARLY) Trial. Circulation 2003;108 (9):1059-63.

[101] Hipkiss AR, Michaelis J, Syrris P, Kumar S, Lam Y. Carnosine Protects Proteins against in-Vitro Glycation and Cross-Linking. Biochemical Society Transactions 1994;22 (4):S399-S.

[102] Hipkiss AR. Carnosine, a protective, anti-ageing peptide? The International Journal Of Biochemistry & Cell Biology 1998;30 (8):863-8.

[103] Hipkiss AR, Brownson C. Carnosine reacts with protein carbonyl groups: another possible role for the anti-ageing peptide? Biogerontology 2000;1 (3):217-23.

[104] Hipkiss AR, Brownson C, Bertani MF, Ruiz E, Ferro A. Reaction of carnosine with aged proteins - Another protective process? Increasing Healthy Life Span: Conventional Measures and Slowing the Innate Aging Process 2002;959:285-94.

[105] Hipkiss AR. Is carnosine a naturally occurring suppressor of oxidative damage in olfactory neurones? Rejuvenation Research 2004;7 (4):253-5.

[106] Hipkiss AR. Would carnosine or a carnivorous diet help suppress aging and associated pathologies? Annals Of The New York Academy Of Sciences 2006;1067:369-74.

[107] Hipkiss AR. On the mechanisms of ageing suppression by dietary restriction - is persistent glycolysis the problem? Mechanisms of Ageing and Development 2006;127 (1):8-15.

[108] Nagradova NK. [Mechanism of action of carnosine on glycolytic oxidation reduction combined with phosphorylation.]. Biokhimii︠A︡ (Moscow, Russia) 1956;21 (1):17-25.

[109] Chasovnikova LV, Formazyuk VE, Sergienko VI, Boldyrev AA, Severin SE. The antioxidative properties of carnosine and other drugs. Biochemistry International 1990;20 (6):1097-103.

[110] Salim-Hanna M, Lissi E, Videla LA. Free radical scavenging activity of carnosine. Free Radical Research Communications 1991;14 (4):263-70.

[111] Pavlov AR, Revina AA, Dupin AM, Boldyrev AA, Yaropolov AI. The mechanism of interaction of carnosine with superoxide radicals in water solutions. Biochimica Et Biophysica Acta 1993;1157 (3):304-12.

[112] Boissonneault GA, Hardwick TA, Bogardus SL, Glauert HP, Chow CK, Decker EA. Interactions between Carnosine and Vitamin-E in Mammary-Cancer Risk. Faseb Journal 1994;8 (4):A425-A.

[113] Klebanov GI, Teselkin Yu O, Babenkova IV, Popov IN, Levin G, Tyulina OV, Boldyrev AA, Vladimirov Yu A. Evidence for a direct interaction of superoxide anion radical with carnosine. Biochemistry And Molecular Biology International 1997;43 (1):99-106.

[114] Wang AM, Ma C, Xie ZH, Shen F. Use of carnosine as a natural anti-senescence drug for human beings. Biochemistry 2000;65 (7):869-71.

[115] Brownson C, Hipkiss AR. Carnosine reacts with a glycated protein. Free Radical Biology & Medicine 2000;28 (10):1564-70.

[116] Boldyrev AA, Yuneva MO, Sorokina EV, Kramarenko GG, Fedorova TN, Konovalova GG, Lankin VZ. Antioxidant systems in tissues of senescence accelerated mice. Biochemistry Biokhimiia 2001;66 (10):1157-63.

[117] Hobart LJ, Seibel I, Yeargans GS, Seidler NW. Anti-crosslinking properties of carnosine: significance of histidine. Life Sciences 2004;75 (11):1379-89.

[118] Boldyrev AA. Protection of proteins from oxidative stress: a new illusion or a novel strategy? Annals Of The New York Academy Of Sciences 2005;1057:193-205.

[119] Yan H, Harding JJ. Carnosine protects against the inactivation of esterase induced by glycation and a steroid. Biochimica Et Biophysica Acta 2005;1741 (1-2):120-6.

[120] Guiotto A, Calderan A, Ruzza P, Borin G. Carnosine and carnosine-related antioxidants: a review. Current Medicinal Chemistry 2005;12 (20):2293-315.

[121] Rosenau T, Habicher WD. Novel tocopherol derivatives .5. The first organometallic derivative of vitamin E. Synlett 1996 (5):427-&.

[122] Rosenau T, Habicher WD, Chen CL. Novel tocopherol compounds .4. 5-tocopherylacetic acid and its derivatives. Heterocycles 1996;43 (4):787-98.

[123] Rosenau T, Habicher WD. Novel tocopherol compounds .7. gamma-tocopherol-5-carboxylic acid - A novel route to gamma-tocopherol. Synlett 1997 (2):208-&.

[124] Evans P, Halliwell B. Free radicals and hearing - Cause, consequence, and criteria. Ototoxicity: Basic Science and Clinical Applications 1999;884:19-40.

[125] Gille L, Gregor W, Rosenau T, Nohl H. Antioxidant properties of new vitamin E derivatives. Naunyn-Schmiedebergs Archives of Pharmacology 2004;369:R137-R.

[126] Campo GM, Ceccarelli S, Squadrito F, Altavilla D, Dorigotti L, Caputi AP. Raxofelast (IRFI 016): A new hydrophilic vitamin E-like antioxidant agent. Cardiovascular Drug Reviews 1997;15 (2):157-73.

[127] Cuzzocrea S, Costantino G, Mazzon E, Caputi AP. Beneficial effects of raxofelast (IRFI 016), a new hydrophilic vitamin E-like antioxidant, in carrageenan-induced pleurisy. British Journal of Pharmacology 1999;126 (2):407-14.

[128] Lee KH, Lim SY, Kang SM, Kim DH, Cho HK, Chung JH, Kwon HM, Chung KH, Lee H, Jang Y, Hwang KC. Antiproliferative mechanisms of raxofelast (IRFI-016) in H2O2-stimulated rat aortic smooth muscle cells. European Journal of Pharmacology 2004;484 (2-3):119-25.

[129] Altavilla D, Seminara P, Squadrito G, Minutoli L, Passaniti M, Bitto A, Calapai G, Calo M, Caputi AP, Squadrito F. Protective effects of antioxidant raxofelast in alcohol-induced liver disease in mice. Pharmacology 2005;74 (1):6-14.

[130] Watanabe A, Noguchi N, Fujisawa A, Kodama T, Tamura K, Cynshi O, Niki E. Stability and reactivity of aryloxyl radicals derived from a novel antioxidant BO-653 and related compounds. Effects of substituent and side chain in solution and membranes. Journal of the American Chemical Society 2000;122 (23):5438-42.

[131] Noguchi N, Okimoto Y, Tsuchiya J, Cynshi O, Kodama T, Niki E. Inhibition of oxidation of low-density lipoprotein by a novel antioxidant, BO-653, prepared by theoretical design. Archives of Biochemistry and Biophysics 1997;347 (1):141-7.

[132] Noguchi N, Sakai H, Kato Y, Tsuchiya J, Yamamoto Y, Niki E, Horikoshi H, Kodama T. Inhibition of oxidation of low density lipoprotein by troglitazone. Atherosclerosis 1996;123 (1-2):227-34.

[133] Rosenau T, Habicher WD. "Vitamin CE," a novel prodrug form of vitamin E. Chemical & Pharmaceutical Bulletin 1997;45 (6):1080-4.

[134] Cynshi O, Kawabe Y, Suzuki T, Takashima Y, Kaise H, Nakamura M, Ohba Y, Kato Y, Tamura K, Hayasaka A, Higashida A, Sakaguchi H, Takeya M, Takahashi K, Inoue K, Noguchi N, Niki E, Kodama T. Antiatherogenic effects of the antioxidant BO-653 in three different animal models. Proceedings of the National Academy of Sciences of the United States of America 1998;95 (17):10123-8.

[135] Parks E, Traber MG. Mechanisms of vitamin E regulation: research over the past decade and focus on the future. Antioxidants & Redox Signaling 2000;2 (3):405-12.

[136] Burton GW, Traber MG, Acuff RV, Walters DN, Kayden H, Hughes L, Ingold KU. Human plasma and tissue alpha-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. The American Journal Of Clinical Nutrition 1998;67 (4):669-84.

[137] Cutler RG. Oxidative stress profiling: part I. Its potential importance in the optimization of human health. Annals of the New York Academy of Sciences 2005;1055:93-135.

[138] Draper HH, Draper HH, Bird RP. Antioxidants and cancer. Journal of agricultural and food chemistry 1984;32:433-5.

[139] Zhang YH, Kramer TR, Taylor PR, Li JY, Blot WJ, Brown CC, Guo W, Dawsey SM, Li B. Possible immunologic involvement of antioxidants in cancer prevention. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1477S-82S.

[140] Taylor A, Jacques PF, Epstein EM. Relations among aging, antioxidant status, and cataract. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1439S-47S.

[141] Adlam VJ, Harrison JC, Porteous CM, James AM, Smith RAJ, Murphy MP, Sammut IA. Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. Faseb Journal 2005;19 (9):1088-95.

[142] Gutteridge JMC, Richmond R, Halliwell B. Oxygen Free-Radicals and Lipid-Peroxidation - Inhibition by the Protein Ceruloplasmin. Febs Letters 1980;112 (2):269-72.

[143] Halliwell B, Gutteridge JMC. Free-Radicals, Lipid-Peroxidation, and Cell-Damage. Lancet 1984;2 (8411):1095-.

[144] Halliwell B, Gutteridge JMC. Free-Radicals and Antioxidant Protection - Mechanisms and Significance in Toxicology and Disease. Human Toxicology 1988;7 (1):7-13.

[145] Tanigawa T, Yoshikawa T, Naito Y, Yoneta T, Ueda S, Oyamada H, Takemura T, Morita Y, Tainaka K, Yoshida N. Antioxidative action of zinc-carnosine compound Z-103. Advances In Experimental Medicine And Biology 1990;264:223-8.

[146] MacFarlane N, McMurray J, O'Dowd JJ, Dargie HJ, Miller DJ. Synergism of histidyl dipeptides as antioxidants. Journal Of Molecular And Cellular Cardiology 1991;23 (11):1205-7.

[147] Chow CK. Vitamin-E and Oxidative Stress. Free Radical Biology and Medicine 1991;11 (2):215-32.

[148] Halliwell B. The Role of Reactive Oxygen Species in Human-Disease. South African Journal of Science 1991;87 (11-12):576-84.

[149] Halliwell B. Drug antioxidant effects. A basis for drug selection? Drugs 1991;42 (4):569-605.

[150] Halliwell B. Reactive Oxygen Species and the Central-Nervous-System. Journal of Neurochemistry 1992;59 (5):1609-23.

[151] Scott BC, Butler J, Halliwell B, Aruoma OI. Evaluation of the Antioxidant Actions of Ferulic Acid and Catechins. Free Radical Research Communications 1993;19 (4):241-53.

[152] Halliwell B. The Chemistry of Free-Radicals. Toxicology and Industrial Health 1993;9 (1-2):1-21.

[153] Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344 (8924):721-4.

[154] Candlish JK, Das NP. Antioxidants in food and chronic degenerative diseases. Biomedical And Environmental Sciences: BES 1996;9 (2-3):117-23.

[155] DarleyUsmar V, Halliwell B. Blood radicals - Reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. Pharmaceutical Research 1996;13 (5):649-62.

[156] Fuller CJ, Grundy SM, Norkus EP, Jialal I. Effect of ascorbate supplementation on low density lipoprotein oxidation in smokers. Atherosclerosis 1996;119 (2):139-50.

[157] Davies KJ. An overview of oxidative stress. IUBMB Life 2000;50 (4-5):241-4.

[158] Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology & Medicine 2000;29 (3-4):222-30.

[159] Knight JA. Review: Free radicals, antioxidants, and the immune system. Annals of Clinical and Laboratory Science 2000;30 (2):145-58.

[160] Halliwell B, Gutteridge JMC. The Definition and Measurement of Antioxidants in Biological-Systems. Free Radical Biology and Medicine 1995;18 (1):125-6.

[161] Machlin LJ, Bendich A. Free-Radical Tissue-Damage - Protective Role of Antioxidant Nutrients. Faseb Journal 1987;1 (6):441-5.

[162] Nohl H, Kozlov AV, Gille L, Staniek K. Formation of reactive oxygen species by cell-organelles. Shock 2005;23:12-6.

[163] Nohl H, Gille L. Lysosomal ROS formation. Redox Report 2005;10 (4):199-205.

[164] Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. American Journal of Physiology-Cell Physiology 1996;40 (5):C1424-C37.

[165] Turrens JF. Mitochondrial formation of reactive oxygen species. Journal of Physiology-London 2003;552 (2):335-44.

[166] Coulter ID, Hardy ML, Morton SC, Hilton LG, Tu W, Valentine D, Shekelle PG. Antioxidants vitamin C and vitamin e for the prevention and treatment of cancer. Journal of General Internal Medicine 2006;21 (7):735-44.

[167] Stanner SA, Hughes J, Kelly CN, Buttriss J. A review of the epidemiological evidence for the 'antioxidant hypothesis'. Public Health Nutrition 2004;7 (3):407-22.

[168] Niki E, Noguchi N. Dynamics of antioxidant action of vitamin E. Accounts of Chemical Research 2004;37 (1):45-51.

[169] Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Progress in Lipid Research 2004;43 (3):200-27.

[170] Halliwell B. Oxidative stress in cell culture: an under-appreciated problem? Febs Letters 2003;540 (1-3):3-6.

[171] Lachance PA, Nakat Z, Jeong WS. Antioxidants: an integrative approach. Nutrition 2001;17 (10):835-8.

[172] Albertini R, Moratti R, De Luca G. Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. Current Molecular Medicine 2002;2 (6):579-92.

[173] Upston JM, Niu XW, Brown AJ, Mashima R, Wang HJ, Senthilmohan R, Kettle AJ, Dean RT, Stocker R. Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. American Journal of Pathology 2002;160 (2):701-10.

[174] Esterbauer H, Gebicki J, Puhl H, Jurgens G. The Role of Lipid-Peroxidation and Antioxidants in Oxidative Modification of Ldl. Free Radical Biology and Medicine 1992;13 (4):341-90.

[175] Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 2000;1488 (3):189-210.

[176] Wasan KM, Cassidy SM. Role of plasma lipoproteins in modifying the biological activity of hydrophobic drugs. Journal of Pharmaceutical Sciences 1998;87 (4):411-24.

[177] Henriksen T, Mahoney EM, Steinberg D. Enhanced Macrophage Degradation of Biologically Modified Low-Density Lipoprotein. Arteriosclerosis 1983;3 (2):149-59.

[178] Henriksen T, Mahoney EM, Steinberg D. Enhanced Macrophage Degradation of Low-Density Lipoprotein Previously Incubated with Cultured Endothelial-Cells - Recognition by Receptors for Acetylated Low-Density Lipoproteins. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 1981;78 (10):6499-503.

[179] Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of Low-Density Lipoprotein by Endothelial-Cells Involves Lipid-Peroxidation and Degradation of Low-Density Lipoprotein Phospholipids. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 1984;81 (12):3883-7.

[180] Hazell LJ, Stocker R. Oxidation of Low-Density-Lipoprotein with Hypochlorite Causes Transformation of the Lipoprotein into a High-Uptake Form for Macrophages. Biochemical Journal 1993;290:165-72.

[181] Noguchi N, Gotoh N, Niki E. Dynamics of the Oxidation of Low-Density-Lipoprotein Induced by Free-Radicals. Biochimica Et Biophysica Acta 1993;1168 (3):348-57.

[182] Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y, Horiuchi S, Takahashi K, Kruijt JK, vanBerkel TJC, Steinbrecher UP, Ishibashi S, Maeda N, Gordon S, Kodama T. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 1997;386 (6622):292-6.

[183] Horkko S, Binder CJ, Shaw PX, Chang MK, Silverman G, Palinski W, Witztum JL. Immunological responses to oxidized LDL. Free Radical Biology and Medicine 2000;28 (12):1771-9.

[184] Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. Macrophage Oxidation of Low-Density-Lipoprotein Generates a Modified Form Recognized by the Scavenger Receptor. Arteriosclerosis 1986;6 (5):505-10.

[185] Yang TTC, Devaraj S, Jialal I. Oxidative stress and atherosclerosis. Journal of Clinical Ligand Assay 2001;24 (1):13-24.

[186] Gerloczy F, Bencze B. [Vitamin E metabolism in young infants.]. Ceskoslovenska Pediatrie 1958;13 (7):608-9.

[187] Li YM, Mitsuhashi T, Wojciechowicz D, Shimizu N, Li J, Stitt A, He CJ, Banerjee D, Vlassara H. Molecular identity and cellular distribution of advanced glycation endproduct receptors: Relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. Proceedings of the National Academy of Sciences of the United States of America 1996;93 (20):11047-52.

[188] Upston JM, Kritharides L, Stocker R. The role of vitamin E in atherosclerosis. Progress in Lipid Research 2003;42 (5):405-22.

[189] Thomas SR, Stocker R. Molecular action of vitamin E in lipoprotein oxidation: Implications for atherosclerosis. Free Radical Biology and Medicine 2000;28 (12):1795-805.

[190] Jialal I, Devaraj S. Scientific evidence to support a vitamin E and heart disease health claim: Research needs. Journal of Nutrition 2005;135 (2):348-53.

[191] Singh U, Devaraj S, Jialal I. Vitamin E, oxidative stress, and inflammation. Annual Review of Nutrition 2005;25:151-74.

[192] Kaul N, Devaraj S, Jialal I. alpha-Tocopherol and Atherosclerosis. Experimental Biology and Medicine 2001;226 (1):5-12.

[193] Harris JD, Schepelmann S, Athanasopoulos T, Graham IR, Stannard AK, Mohri Z, Hill V, Hassall DG, Owen JS, Dickson G. Inhibition of atherosclerosis in apolipoprotein-E-deficient mice following muscle transduction with adeno-associated virus vectors encoding human apolipoprotein-E. Gene Therapy 2002;9 (1):21-9.

[194] Devaraj S, Hugou I, Jialal I. alpha-Tocopherol decreases CD36 expression in human monocyte-derived macrophages. Journal of Lipid Research 2001;42 (4):521-7.

[195] Stocker R. The ambivalence of vitamin E in atherogenesis. Trends in Biochemical Sciences 1999;24 (6):219-23.

[196] Suarna C, Dean RT, May J, Stocker R. Human Atherosclerotic Plaque Contains Both Oxidized Lipids and Relatively Large Amounts of Alpha-Tocopherol and Ascorbate. Arteriosclerosis Thrombosis and Vascular Biology 1995;15 (10):1616-24.

[197] Kohar I, Baca M, Suarna C, Stocker R, Southwellkeely PT. Is Alpha-Tocopherol a Reservoir for Alpha-Tocopheryl Hydroquinone. Free Radical Biology and Medicine 1995;19 (2):197-207.

[198] Lass A, Witting P, Stocker R, Esterbauer H. Inhibition of copper- and peroxyl radical-induced LDL lipid oxidation by ebselen: Antioxidant actions in addition to hydroperoxide-reducing activity. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism 1996;1303 (2):111-8.

[199] Waldeck AR, Stocker R. Radical-initiated lipid peroxidation in low density lipoproteins: Insights obtained from kinetic modeling. Chemical Research in Toxicology 1996;9 (6):954-64.

[200] Thomas SR, Davies MJ, Stocker R. Peroxynitrite oxidises LDL lipids via tocopherol-mediated peroxidation. Atherosclerosis 1997;134 (1-2):218-.

[201] Hazell LJ, Stocker R. alpha-tocopherol does not inhibit hypochlorite-induced oxidation of apolipoprotein B-100 of low-density lipoprotein. Febs Letters 1997;414 (3):541-4.

[202] Fu S, Davies MJ, Stocker R, Dean RT. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochemical Journal 1998;333:519-25.

[203] Terentis AC, Thomas SR, Burr JA, Liebler DC, Stocker R. Alpha-tocopherol oxidation products in atherosclerotic lesions. Free Radical Biology and Medicine 1999;27:S99-S.

[204] Thomas SR, Neuzil J, Mohr D, Stocker R. Coantioxidants Make Alpha-Tocopherol an Efficient Antioxidant for Low-Density-Lipoprotein. American Journal of Clinical Nutrition 1995;62 (6):S1357-S64.

[205] Azzi A, Boscoboinik D, Clement S, Ozer N, Ricciarelli R, Stocker A. Vitamin E mediated response of smooth muscle cell to oxidant stress. Diabetes Research and Clinical Practice 1999;45 (2-3):191-8.

[206] Simons LA, von Konigsmark M, Simons J, Stocker R, Celermajer DS. Vitamin E ingestion does not improve arterial endothelial dysfunction in older adults. Atherosclerosis 1999;143 (1):193-9.

[207] Mashima R, Witting PK, Stocker R. Oxidants and antioxidants in atherosclerosis. Current Opinion in Lipidology 2001;12 (4):411-8.

[208] Terentis AC, Thomas SR, Burr JA, Liebler DC, Stocker R. Vitamin E oxidation in human atherosclerotic lesions. Circulation Research 2002;90 (3):333-9.

[209] Stocker A. Molecular mechanisms of vitamin E transport. Vitamin E and Health 2004;1031:44-59.

[210] Brasen JH, Koenig K, Bach H, Kontush A, Heinle H, Witting PK, Yla-Herttuala S, Stocker R, Beisiegel U. Comparison of the effects of alpha-tocopherol, ubiquinone-10 and probucol at therapeutic doses on atherosclerosis in WHHL rabbits. Atherosclerosis 2002;163 (2):249-59.

[211] Wu BJ, Kathir K, Witting PK, Beck K, Choy K, Li C, Croft KD, Mori TA, Tanous D, Adams MR, Lau AK, Stocker R. Antioxidants protect from atherosclerosis by a heme oxygenase-1 pathway that is independent of free radical scavenging. Journal of Experimental Medicine 2006;203 (4):1117-27.

[212] Ross R. Mechanisms of disease - Atherosclerosis - An inflammatory disease. New England Journal of Medicine 1999;340 (2):115-26.

[213] Williams KJ, Tabas I. The Response-to-Retention Hypothesis of Early Atherogenesis. Arteriosclerosis Thrombosis and Vascular Biology 1995;15 (5):551-61.

[214] Skalen K, Gustafsson M, Innerarity TL, Boren J. Expression of "proteoglycan-binding defective LDL" in transgenic mice. Circulation 2000;102 (18):45-.

[215] Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL, Boren J. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 2002;417 (6890):750-4.

[216] Sirtori C, Marchesi M. High density lipoprotein administration: a new rherapeutic modality for the treatment of cardiovascular diseases. Current Medicinal Chemistry - Immunology, Endocrine and Metabolic Agents 2005;5:321-33.

[217] Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P, Study HOPE. Vitamin E supplementation and cardiovascular events in high-risk patients. New England Journal of Medicine 2000;342 (3):154-60.

[218] Leppala JM, Virtamo J, Fogelholm R, Albanes D, Taylor PR, Heinonen OP. Vitamin E and beta carotene supplementation in high risk for stroke - A subgroup analysis of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Archives of Neurology 2000;57 (10):1503-9.

[219] Blatt DH, Pryor WA, Mata JE, Rodriguez-Proteau R. Re-evaluation of the relative potency of synthetic and natural alpha-tocopherol: experimental and clinical observations. Journal of Nutritional Biochemistry 2004;15 (7):380-95.

[220] Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ, Brown MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). Lancet 1996;347 (9004):781-6.

[221] Fresco C, Carinci F, Maggioni AP, Ciampi A, Nicolucci A, Santoro E, Tavazzi L, Tognonia G, investigators G. Very early assessment of risk for in-hospital death among 11,483 patients with acute myocardial infarction. American Heart Journal 1999;138 (6):1058-64.

[222] Aldini G, Carini M, Beretta G, Bradamante S, Facino RM. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? Biochemical And Biophysical Research Communications 2002;298 (5):699-706.

[223] Dobrota D, Fedorova T, Stvolinsky S, Babusikova E, Likavcanova K, Drgova A, Strapkova A, Boldyrev A. Carnosine protects the brain of rats and Mongolian gerbils against ischemic injury: after-stroke-effect. Neurochemical Research 2005;30 (10):1283-8.

[224] Lee YT, Hsu CC, Lin MH, Liu KS, Yin MC. Histidine and carnosine delay diabetic deterioration in mice and protect human low density lipoprotein against oxidation and glycation. European Journal Of Pharmacology 2005;513 (1-2):145-50.

[225] Yamamoto Y, Maita N, Fujisawa A, Takashima J, Ishii Y, Dunlap WC. A new vitamin E (alpha-tocomonoenol) from eggs of the Pacific salmon Oncorhynchus keta. Journal of Natural Products 1999;62 (12):1685-7.

[226] Yamamoto Y, Fujisawa A, Hara A, Dunlap WC. An unusual vitamin E constituent (alpha-tocomonoenol) provides enhanced antioxidant protection in marine organisms adapted to cold-water environments. Proceedings of the National Academy of Sciences of the United States of America 2001;98 (23):13144-8.

[227] Wagner KH, Kamal-Eldin A, Elmadfa I. Gamma-tocopherol--an underestimated vitamin? Annals of Nutrition & Metabolism 2004;48 (3):169-88.

[228] Berman K, Brodaty H. Tocopherol (vitamin E) in Alzheimer's disease and other neurodegenerative disorders. CNS Drugs 2004;18 (12):807-25.

[229] Eggermont E. Recent advances in vitamin E metabolism and deficiency. European Journal Of Pediatrics 2006;165 (7):429-34.

[230] Bieri JG. Comments on the new dietary reference intake for vitamin E. American Journal of Clinical Nutrition 2002;75 (4):781-.

[231] Rosenau T, Habicher WD. Novel Tocopherol Compounds .1. Bromination of Alpha-Tocopherol-Reaction Mechanism and Synthetic Applications. Tetrahedron 1995;51 (29):7919-26.

[232] Azzi A, Stocker A. Vitamin E: non-antioxidant roles. Progress in Lipid Research 2000;39 (3):231-55.

[233] Vertuani S, Angusti A, Manfredini S. The antioxidants and pro-antioxidants network: An overview. Current Pharmaceutical Design 2004;10 (14):1677-94.

[234] Kayden HJ, Traber MG. Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. Journal Of Lipid Research 1993;34 (3):343-58.

[235] Thomas SR, Neuzil J, Mohr D, Stocker R. Coantioxidants make alpha-tocopherol an efficient antioxidant for low-density lipoprotein. The American Journal of Clinical Nutrition 1995;62 (6 Suppl):1357S-64S.

[236] Traber MG, Elsner A, Brigelius-Flohe R. Synthetic as compared with natural vitamin E is preferentially excreted as alpha-CEHC in human urine: studies using deuterated alpha-tocopheryl acetates. FEBS Letters 1998;437 (1-2):145-8.

[237] Proudfoot JM, Croft KD, Puddey IB, Beilin LJ. The role of copper reduction by alpha-tocopherol in low-density lipoprotein oxidation. Free Radical Biology and Medicine 1997;23 (5):720-8.

[238] Tafazoli S, Wright JS, O'Brien PJ. Prooxidant and antioxidant activity of vitamin E analogues and troglitazone. Chemical Research in Toxicology 2005;18 (10):1567-74.

[239] Yamashita N, Murata M, Inoue S, Burkitt MJ, Milne L, Kawanishi S. alpha-tocopherol induces oxidative damage to DNA in the presence of Copper(II) ions. Chemical Research in Toxicology 1998;11 (8):855-62.

[240] Cadenas S, Rojas C, Perezcampo R, Lopeztorres M, Barja G. Vitamin-E Protects Guinea-Pig Liver from Lipid-Peroxidation without Depressing Levels of Antioxidants. International Journal of Biochemistry & Cell Biology 1995;27 (11):1175-81.

[241] Traber MG. Does vitamin E decrease heart attack risk? summary and implications with respect to dietary recommendations. The Journal Of Nutrition 2001;131 (2):395S-7S.

[242] Jiang Q, Christen S, Shigenaga MK, Ames BN. Y-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. American Journal of Clinical Nutrition 2001;74 (6):714-22.

[243] Horwitt MK. Critique of the requirement for vitamin E. The American journal of clinical nutrition 2001;73 (6):1003-5.

[244] Elsayed NM. Antioxidant mobilization in response to oxidative stress: A dynamic environmental-nutritional interaction. Nutrition 2001;17 (10):828-34.

[245] Monroe EB, Jurchen JC, Lee J, Rubakhin SS, Sweedler JV. Vitamin E imaging and localization in the neuronal membrane. Journal of the American Chemical Society 2005;127 (35):12152-3.

[246] Miller ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. Annals of Internal Medicine 2005;142 (1):37-46.

[247] Cohn W. Bioavailability of vitamin E. European Journal of Clinical Nutrition 1997;51:S80-S5.

[248] Hacquebard M, Carpentier YA. Vitamin E: absorption, plasma transport and cell uptake. Current Opinion in Clinical Nutrition and Metabolic Care 2005;8 (2):133-8.

[249] Traber MG, Olivecrona T, Kayden HJ. Bovine milk lipoprotein lipase transfers tocopherol to human fibroblasts during triglyceride hydrolysis in vitro. The Journal Of Clinical Investigation 1985;75 (5):1729-34.

[250] Granot E, Tamir I, Deckelbaum RJ. Neutral Lipid Transfer Protein Does Not Regulate Alpha-Tocopherol Transfer between Human-Plasma Lipoproteins. Lipids 1988;23 (1):17-21.

[251] Traber MG, Goldberg I, Davidson E, Lagmay N, Kayden HJ. Vitamin E uptake by human intestinal cells during lipolysis in vitro. Gastroenterology 1990;98 (1):96-103.

[252] Traber MG. Vitamin E regulation. Current Opinion in Gastroenterology 2005;21 (2):223-7.

[253] Hosomi A, Arita M, Sato Y, Kiyose C, Ueda T, Igarashi O, Arai H, Inoue K. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. Febs Letters 1997;409 (1):105-8.

[254] Pfluger P, Kluth D, Landes N, Blumke-Vogt C, Brigelius-Flohe R. Vitamin E: underestimated as an antioxidant. Redox Report 2004;9 (5):249-54.

[255] Traber MG, Burton GW, Hamilton RL. Vitamin E trafficking. Annals Of The New York Academy Of Sciences 2004;1031:1-12.

[256] Sontag TJ, Parker RS. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. The Journal Of Biological Chemistry 2002;277 (28):25290-6.

[257] Birringer M, Drogan D, Brigelius-Flohe R. Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation. Free Radical Biology and Medicine 2001;31 (2):226-32.

[258] Kohen R, Yamamoto Y, Cundy KC, Ames BN. Antioxidant Activity of Carnosine, Homocarnosine, and Anserine Present in Muscle and Brain. Proceedings of the National Academy of Sciences of the United States of America 1988;85 (9):3175-9.

[259] Bauer K. Carnosine and homocarnosine, the forgotten, enigmatic peptides of the brain. Neurochemical Research 2005;30 (10):1339-45.

[260] Fontana M, Pinnen F, Lucente G, Pecci L. Prevention of peroxynitrite-dependent damage by carnosine and related sulphonamido pseudodipeptides. Cellular And Molecular Life Sciences: CMLS 2002;59 (3):546-51.

[261] Baran EJ. Metal complexes of carnosine. Biochemistry Biokhimii͡A 2000;65 (7):789-97.

[262] Hipkiss AR, Preston JE, Himsworth DTM, Worthington VC, Keown M, Michaelis J, Lawrence J, Mateen A, Allende L, Eagles PAM, Abbott NJ. Pluripotent protective effects of carnosine, a naturally occurring dipeptide. Towards Prolongation of the Healthy Life Span 1998;854:37-53.

[263] Hipkiss AR, Chana H. Carnosine protects proteins against methylglyoxal-mediated modifications. Biochemical and Biophysical Research Communications 1998;248 (1):28-32.

[264] Hipkiss AR, Worthington VC, Himsworth DTJ, Herwig W. Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite. Biochimica Et Biophysica Acta-General Subjects 1998;1380 (1):46-54.

[265] Hipkiss AR. Carnosine and protein carbonyl groups: A possible relationship. Biochemistry-Moscow 2000;65 (7):771-8.

[266] Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. Mechanisms of Ageing and Development 2001;122 (13):1431-45.

[267] Hipkiss AR. Glycation ageing and carnosine: Are carnivorous diets beneficial? Mechanisms of Ageing and Development 2005;126 (10):1034-9.

[268] Nagai K, Niijima A, Yamano T, Otani H, Okumra N, Tsuruoka N, Nakai M, Kiso Y. Possible role of L-carnosine in the regulation of blood glucose through controlling autonomic nerves. Experimental Biology and Medicine 2003;228 (10):1138-45.

[269] Hipkiss AR. Could carnosine suppress zinc-mediated proteasome inhibition and neurodegeneration? Therapeutic potential of a non-toxic but non-patentable dipeptide. Biogerontology 2005;6 (2):147-9.

[270] Hipkiss AR. Could carnosine be a naturally-occurring scavenger for acrolein and other reactive aldehydes in the brain? Neurobiology of Aging 2002;23 (4):645-6.

[271] Bogardus SL, Boissonneault GA. Carnosine inhibits in vitro low-density lipoprotein oxidation. Nutrition Research 2000;20 (7):967-76.

[272] Halliwell B, Aeschbach R, Loliger J, Aruoma OI. The Characterization of Antioxidants. Food and Chemical Toxicology 1995;33 (7):601-17.

[273] Kaur IP, Geetha T. Screening methods for antioxidants-a review. Mini Reviews In Medicinal Chemistry 2006;6 (3):305-12.

[274] Halliwell B. Antioxidant Characterization - Methodology and Mechanism. Biochemical Pharmacology 1995;49 (10):1341-8.

[275] Pankhurst G, Wang XL, Wilcken DE, Baernthaler G, Panzenbock U, Raftery M, Stocker R. Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein. Journal of Lipid Research 2003;44 (2):349-55.

[276] Yamauchi R, Southwell-Keely P, Suarna C, Ray S, Raftery M, Cynshi O, Stocker R. Characterization of the oxidation products of BO-653 formed during peroxyl radical-mediated oxidation of human plasma. Free Radical Biology and Medicine 2005;38 (1):32-40.

[277] Halliwell B. How to Characterize a Biological Antioxidant. Free Radical Research Communications 1990;9 (1):1-32.

[278] Miller NJ, RiceEvans CA. Spectrophotometric determination of antioxidant activity. Redox Report 1996;2 (3):161-71.

[279] Rodak L, Pospisil Z, Tomanek J, Vesely T, Obr T, Valicek L. Enzyme-Linked Immunosorbent-Assay (Elisa) Detection of Infectious Pancreatic Necrosis Virus (Ipnv) in Culture Fluids and Tissue-Homogenates of the Rainbow-Trout, Salmo-Gairdneri Richardson. Journal of Fish Diseases 1988;11 (3):225-35.

[280] Kunchandy E, Rao MNA. Oxygen Radical Scavenging Activity of Curcumin. International Journal of Pharmaceutics 1990;58 (3):237-40.

[281] Sreejayan, Rao MN. Nitric oxide scavenging by curcuminoids. The Journal Of Pharmacy And Pharmacology 1997;49 (1):105-7.

[282] Halliwell B, Evans P, Whiteman M. Assessment of peroxynitrite scavengers in vitro. Methods In Enzymology 1999;301:333-42.

[283] Haenen GR, Paquay JB, Korthouwer RE, Bast A. Peroxynitrite scavenging by flavonoids. Biochemical And Biophysical Research Communications 1997;236 (3):591-3.

[284] Osawa T, Ide A, Su JD, Namiki M. Inhibition of Lipid-Peroxidation by Ellagic Acid. Journal of Agricultural and Food Chemistry 1987;35 (5):808-12.

[285] Diplock AT. Defense against reactive oxygen species. Free Radical Research 1998;29 (6):463-7.

[286] Thomas SR, Leichtweis SB, Pettersson K, Croft KD, Mori TA, Brown AJ, Stocker R. Dietary cosupplementation with vitamin E and coenzyme Q(10) inhibits atherosclerosis in apolipoprotein E gene knockout mice. Arteriosclerosis Thrombosis and Vascular Biology 2001;21 (4):585-93.

[287] Letters JM, Witting PK, Christison JK, Eriksson AW, Pettersson K, Stocker R. Time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E knockout mice. Journal of Lipid Research 1999;40 (6):1104-12.

[288] Qian JH, Atkinson J, Manor D. Biochemical consequences of heritable mutations in the alpha-tocopherol transfer protein. Biochemistry 2006;45 (27):8236-42.

[289] Atkinson JK, Nava P, Frahm G, Curtis V, Manor D. Fluorescent tocopherols as probes of inter-vesicular transfer catalyzed by the alpha-tocopherol transfer protein. Vitamin E and Health 2004;1031:324-7.

[290] Qian J, Wilson K, Nava P, Morley S, Atkinson J, Manor D. Intracellular localization of alpha-tocopherol transfer protein and alpha-tocopherol. Vitamin E and Health 2004;1031:330-1.

[291] Morley S, Panagabko C, Stocker A, Atkinson J, Manor D. Structure-function relationship in the tocopherol transfer protein. Vitamin E and Health 2004;1031:332-3.

[292] Bradford A, Atkinson J, Fuller N, Rand RP. The effect of vitamin E on the structure of membrane lipid assemblies. Journal of Lipid Research 2003;44 (10):1940-5.

[293] Galli F, Lee R, Atkinson J, Floridi A, Kelly FJ. gamma-tocopherol biokinetics and transformation in humans. Free Radical Research 2003;37 (11):1225-33.

[294] Manor D, Atkinson J. Is tocopherol associated protein a misnomer? Journal of Nutritional Biochemistry 2003;14 (7):421-2.

[295] Traber MG, Paterson E, Atkinson J, Ramakrishnan R, Iacovoni V, Cross C. Studies in humans using deuterium-labeled alpha- and gamma-tocopherols demonstrate rapid plasma gamma-tocopherol disappearance. Faseb Journal 2003;17 (4):A279-A.

[296] Lawson KA, Anderson K, Menchaca M, Atkinson J, Sun LZ, Knight V, Gilbert BE, Conti C, Sanders BG, Kline K. Novel vitamin E analogue decreases syngeneic mouse mammary tumor burden and reduces lung metastasis. Molecular Cancer Therapeutics 2003;2 (5):437-44.

[297] Galli F, Lee R, Dunster C, Atkinson J, Floridi A, Kelly FJ. gamma-Tocopherol metabolism and its relationship with alpha-tocopherol in humans: A stable isotope supplementation study. Biofactors 2001;15 (2-4):65-9.

[298] Atkinson J, Nava P, Lei HS, Neely S, Panagabko C. Fluorescent analogs of tocopherol (vitamin E) for use in protein binding studies and cellular localization and distribution. Abstracts of Papers of the American Chemical Society 2001;222:U46-U.

[299] Lei HS, Atkinson J. Synthesis of phytyl- and chroman-derivatized photoaffinity labels based on alpha-tocopherol. Journal of Organic Chemistry 2000;65 (8):2560-7.

[300] Abdo MR, Joseph P, Boigegrain RA, Liautard JP, Montero JL, Kohler S, Winum JY. Brucella suis histidinol dehydrogenase: Synthesis and inhibition studies of a series of substituted benzylic ketones derived from histidine. Bioorganic & Medicinal Chemistry 2007;15 (13):4427-33.

[301] Zhao M, Bi LR, Bi W, Wang C, Yang Z, Ju JF, Peng SQ. Synthesis of new class dipeptide analogues with improved permeability and antithrombotic activity. Bioorganic & Medicinal Chemistry 2006;14 (14):4761-74.

[302] Wu G, Liu J, Bi L, Zhao M, Wang C, Baudy-Floc'h M, Ju J, Peng S. Toward breast cancer resistance protein (BCRP) inhibitors: design, synthesis of a series of new simplified fumitremorgin C analogues. Tetrahedron 2007;63:5510-28.

[303] Montalbetti CAGN, Falque V. Amide bond formation and peptide coupling. Tetrahedron 2005;61 (46):10827-52.

CHAPTER 5. CONCLUSIONS

Natural antioxidants and vitamins have been shown to possess properties that could potentially improve human health. Targeted delivery of vitamins and antioxidants via polymeric nanoparticles offers several advantages over traditional delivery methods, which include protection of the bioactive component from degradation, an increase in the bioavailability of the vitamin, delivery of the component to the site of action in a controlled manner, as well as the possibility to integrate the bioactive component in various foods for enhanced food quality. Safety of the polymeric nanoparticles for antioxidant and vitamin delivery is a very important factor which must be considered in designing and building polymeric nanoparticles for targeted controlled delivery of antioxidants and vitamins.

Several methods are available to synthesize polymeric nanoparticles with entrapped antioxidants/vitamins, by starting with a preformed polymer. Each method has its advantages and disadvantages; the selection of the most suitable method should be based on the chemical characteristics of the active component and that of the polymer, as well as on the final use of the nanoparticles. The methods which are based on the diffusion of the organic solvent to form nanoparticles are limited to low polymer concentration for nanoparticle mean sizes of less than 200 nm. Methods that involve solvent evaporation are more time consuming and are more expensive, but are less sensitive to changes in the polymer concentration. Emulsion evaporation, in particular, can be used to entrap hydrophilic (w/o/w emulsion) or hydrophobic (w/o emulsion) antioxidants, which is an advantage. The salting-out method is suitable for formation of nanoparticles at higher polymer concentration, but the involved purification process is a limitation of this synthesis method. Surfactant concentration, polymer concentration, polymer molecular weight, solvents, surfactant concentrations, and phase ratios play an important role in controlling the size of the nanoparticles in all methods available for nanoparticles formation. All methods involve the use of surfactants.

The next generation of antioxidant polymeric nanoparticles not only they will deliver antioxidants, but they will also provide intrinsic antioxidant action. One possibility of synthesizing polymeric nanoparticles of intrinsic antioxidant activity is to use antioxidant surfactants.

A new antioxidant surfactant made of vitamin E and vitamin C (EC) was successfully synthesized and it was used to form PLGA polymeric nanoparticles with antioxidant activity in this study. The EC surfactant was hydrophobic with a good solubility in polar solvents. Nanoprecipitation (self assembly) technique was used to synthesize self-assembled EC and Span 80 nanostructures, as well as polymeric PLGA-span 80 and PLGA-EC nanoparticles. The EC nanostructures were spherical and measured between 12 and 140 nm depending on the salt and surfactant concentration. The polymeric PLGA-EC nanoparticles showed an antioxidant activity (IC_{50} of 36.3 to 42.1 mM as measured by DPPH assay). The antioxidant action of the PLGA-EC nanoparticles was provided by the vitamin C moiety of the EC surfactant which localized at the interface between the PLGA hydrophobic core and water. In comparison, PLGA-span80 particles did not show any antioxidant activity (the antioxidant activity of α-tocopherol was lost in the synthesis). The antioxidant activity of EC and EC-PLGA nanoparticles has been confirmed by EPR measurements. Even though the scavenging activity of EC was about twice higher than that of EC-PLGA NPs, the antioxidant properties were in a similar range. The newly synthesized EC surfactant was therefore found successful in forming polymeric nanoparticles of intrinsic antioxidant properties.

The following step was to synthesize an amphiphilic molecule that maintained the intrinsic antioxidant activity of both antioxidants components used in the synthesis. Synthesis of VECAR (carnosine plus alpha-tocopherol derivative) was accomplished in eight steps. The approach developed was to form an active phytil chain which was used to attach carnosine. The H-NMR spectrum confirmed synthesis of VECAR. VECAR presented an antioxidant behavior as measured by DPPH (0.0236 mM) close to that of pure alpha-tocopherol (0.0229 mM) which confirmed the recovery of the hydroxyl group in the chromanol ring of VECAR. Diseases such as Parkinson's, Alzheimer's, and atherosclerosis are linked to oxidation process, and VECAR can prove as an effective antioxidant *in vitro* and *in vivo*, as well as potential new antioxidant surfactant for polymeric nanoparticles synthesis.

CHAPTER 6. FUTURE WORK

The antioxidant surfactants EC and VECAR were successfully synthesized and showed a good antioxidant activity. It is proposed that the next step for EC surfactant project should focus on covalent attachment of maleic acid to the OH groups that do not provide antioxidant action of vitamin E. The hydrophilic/lipophilic behavior of EC can be modified by attaching a molecule more hydrophilic than vitamin C, or the attachment of an additional ascorbic acid molecule. Another important area for EC surfactant is test the recovery of vitamin E antioxidant action after hydrolysis of EC into the components (vitamin E, vitamin C, and maleic acid) which occurs in the presence of esterases found in the body.

The antioxidant behavior of antioxidant surfactants synthesized (EC and VECAR) should be completely characterized. Assays with different initiators should be performed to detect the effectiveness of EC and VECAR against ROS, RNS, and RSS. The antioxidant activity of polymeric nanoparticles synthesized in the presence of EC/VECAR should be tested with the same protocols to understand their intrinsic antioxidant behavior and to detect potential differences between the antioxidant activity of the surfactant placed on the surface of the particles or in the bulk. The preliminary results obtained herein suggest that VECAR is a good antioxidant molecule. VECAR must be tested with lipids and LDL *in vitro* to understand its antioxidant behavior. Moreover, the *in vivo* analysis will be required to analyze the fate of VECAR in the lipid transport mechanism of biological entities. LDL should transport VECAR to different tissues, and animal studies will confirm the validity of that hypothesis. VECAR's ability to act as a surfactant should be tested, and VECAR should be used to make polymeric nanoparticles with inherent antioxidant activity. The method can be the same as the self assembly technique applied to make EC-PLGA polymeric nanoparticles. Additionally, other polymers besides PLGA can be used to study interactions between VECAR and polymer in formation of the nanoparticles.

Very importantly, the understanding of the safety of the nanoparticle delivery system is critical for the final use of the system in biological systems and others. It involves analysis of the complex interactions between the nanoparticles and the cellular environment, and the mechanism involved in the delivery of the antioxidant/vitamin at the site of action. The antioxidant surfactants toxicity and behavior in biological system should be studied both in bulk as well as

when it is part of a nanoparticle structure, to insure the safety of the materials for food or drug delivery applications.

APPENDIX A: AUTHORIZATION FOR REPRODUCTION

From: Julie Pleasance <Julie.Pleasance@woodheadpublishing.com>
Date: March 15, 2010 5:32:41 AM CDT
To: "Sabliov, Cristina M." <CSabliov@agcenter.lsu.edu>
Subject: RE: copyright permission

Dear Cristina
Thank you for your email below requesting permission to reproduce chapter 12 **"Encapsulation and controlled release of antioxidants and vitamins"** from the title **"Delivery and controlled release of bioactives in foods and nutraceuticals"** in a graduate student thesis.

I grant permission but ask that acknowledgement is given to the title, ISBN, author and publisher, Woodhead Publishing Limited, UK.

Kind regards

Julie Pleasance

Rights - Permissions Manager / Sales Administrator

Woodhead Publishing Limited
Abington Hall
Granta Park
Great Abington
Cambridge
CB21 6AH
Tel: +44 (0) 1223 891358 ext 139
Fax +44 (0) 1223 893694
Email Julie.Pleasance@woodheadpublishing.com
Website www.woodheadpublishing.com

Further your knowledge with Woodhead Publishing

APPENDIX B: SAS PROGRAMS AND OUTPUTS

SAS program for salt concentration in EC and Span 80 nanostructures. Two salts (Sodium chloride and sodium bicarbonate), six salt concentrations (0, 1.5, 7.3, 15, 33, and 99 mM), two surfactants (EC and Span80), triplicate data, and two independent experiments were evaluated with the following program.

```
dm 'log;clear;output;clear';
OPTIONS nodate nocenter pageno=1 ls=161 ps=512;
Title1 'Nanoparticle size with Span 80 and EC as surfactants';
Title2 'Carlos E. Astete';
ODS HTML style=minimal body='C:\Documents and Settings\Margarita\My Documents\Carlos\Dissertation for
PhD\EC\EC Data for SAS\Analysis01.html';
DATA EC; infile cards missover;
input OBS Su Sa Co Rep Exp Size PI;
cards; RUN;
1               1       1       1       1       1       26.15           0.492
2               1       1       1       2       1       27.13           0.502
3               1       1       1       3       1       27.83           0.378
4               1       1       2       1       1       21.02           0.203
5               1       1       2       2       1       20.9            0.204
6               1       1       2       3       1       20.9            0.199
7               1       1       3       1       1       29.93           0.161
8               1       1       3       2       1       29.77           0.175
9               1       1       3       3       1       29.46           0.172
10              1       1       4       1       1       67.71           0.238
11              1       1       4       2       1       67.71           0.24
12              1       1       4       3       1       67.07           0.247
13              1       1       5       1       1       82.48           0.254
14              1       1       5       2       1       82.36           0.252
15              1       1       5       3       1       81.51           0.261
16              1       1       6       1       1       153.8           0.148
17              1       1       6       2       1       153.1           0.16
18              1       1       6       3       1       154.1           0.146
19              1       2       1       1       1       26.15           0.492
20              1       2       1       2       1       27.13           0.502
21              1       2       1       3       1       27.82           0.378
22              1       2       2       1       1       23.98           0.661
23              1       2       2       2       1       22.99           0.712
24              1       2       2       3       1       23.17           0.713
25              1       2       3       1       1       16.68           0.411
26              1       2       3       2       1       17.17           0.427
27              1       2       3       3       1       16.44           0.472
28              1       2       4       1       1       10.74           0.28
29              1       2       4       2       1       10.76           0.275
30              1       2       4       3       1       10.6            0.252
31              1       2       5       1       1       16.99           0.46
32              1       2       5       2       1       17.36           0.465
33              1       2       5       3       1       17.37           0.464
34              1       2       6       1       1       24.14           0.629
35              1       2       6       2       1       25.87           0.486
36              1       2       6       3       1       25.84           0.489
```

37	2	1	1	1	1	134.6	0.118
38	2	1	1	2	1	135.3	0.122
39	2	1	1	3	1	134.1	0.098
40	2	1	2	1	1	160.1	0.022
41	2	1	2	2	1	159.0	0.028
42	2	1	2	3	1	153.9	0.074
43	2	1	3	1	1	180.3	0.055
44	2	1	3	2	1	184.0	0.124
45	2	1	3	3	1	180.9	0.119
46	2	1	4	1	1	225.3	0.209
47	2	1	4	2	1	215.6	0.179
48	2	1	4	3	1	226.5	0.206
49	2	1	5	1	1	446.5	0.092
50	2	1	5	2	1	439.7	0.127
51	2	1	5	3	1	438.2	0.093
52	2	1	6	1	1	.	.
53	2	1	6	2	1	.	.
54	2	1	6	3	1	.	.
55	2	2	1	1	1	134.6	0.118
56	2	2	1	2	1	135.3	0.122
57	2	2	1	3	1	134.1	0.098
58	2	2	2	1	1	157.0	0.09
59	2	2	2	2	1	156.6	0.111
60	2	2	2	3	1	156.9	0.066
61	2	2	3	1	1	173.4	0.092
62	2	2	3	2	1	173.9	0.092
63	2	2	3	3	1	174.3	0.084
64	2	2	4	1	1	170.6	0.108
65	2	2	4	2	1	167.1	0.098
66	2	2	4	3	1	163.0	0.098
67	2	2	5	1	1	166.8	0.087
68	2	2	5	2	1	170.0	0.101
69	2	2	5	3	1	171.5	0.085
70	2	2	6	1	1	184.5	0.101
71	2	2	6	2	1	182.9	0.092
72	2	2	6	3	1	182.7	0.102
73	1	1	1	1	2	22.99	0.411
74	1	1	1	2	2	23.46	0.394
75	1	1	1	3	2	22.1	0.337
76	1	1	2	1	2	24.08	0.332
77	1	1	2	2	2	23.13	0.337
78	1	1	2	3	2	22.07	0.326
79	1	1	3	1	2	24.16	0.174
80	1	1	3	2	2	24.17	0.198
81	1	1	3	3	2	24.22	0.177
82	1	1	4	1	2	44.52	0.235
83	1	1	4	2	2	44.44	0.25
84	1	1	4	3	2	45.16	0.242
85	1	1	5	1	2	82.76	0.28
86	1	1	5	2	2	84.39	0.264
87	1	1	5	3	2	82.49	0.251
88	1	1	6	1	2	122.5	0.202
89	1	1	6	2	2	121.5	0.177
90	1	1	6	3	2	121.9	0.214
91	1	2	1	1	2	22.99	0.411
92	1	2	1	2	2	23.46	0.394
93	1	2	1	3	2	22.1	0.337

```
94          1       2       2       1       2       26.15       0.456
95          1       2       2       2       2       29.4        0.412
96          1       2       2       3       2       23.57       0.498
97          1       2       3       1       2       18.06       0.384
98          1       2       3       2       2       14.56       0.356
99          1       2       3       3       2       13.56       0.308
100         1       2       4       1       2       10.68       0.241
101         1       2       4       2       2       12.11       0.204
102         1       2       4       3       2       14.77       0.237
103         1       2       5       1       2       23.76       0.397
104         1       2       5       2       2       24.56       0.423
105         1       2       5       3       2       21.09       0.405
106         1       2       6       1       2       38.07       0.498
107         1       2       6       2       2       31.06       0.533
108         1       2       6       3       2       40.21       0.51
109         2       1       1       1       2       137.2       0.092
110         2       1       1       2       2       137.3       0.106
111         2       1       1       3       2       137.1       0.088
112         2       1       2       1       2       153.5       0.045
113         2       1       2       2       2       148.9       0.075
114         2       1       2       3       2       155.4       0.069
115         2       1       3       1       2       180.0       0.02
116         2       1       3       2       2       176.6       0.042
117         2       1       3       3       2       179.5       0.041
118         2       1       4       1       2       216.3       0.15
119         2       1       4       2       2       253.6       0.055
120         2       1       4       3       2       249.6       0.062
121         2       1       5       1       2       481.7       0.084
122         2       1       5       2       2       475.3       0.063
123         2       1       5       3       2       478.0       0.089
124         2       1       6       1       2       .           .
125         2       1       6       2       2       .           .
126         2       1       6       3       2       .           .
127         2       2       1       1       2       137.2       0.092
128         2       2       1       2       2       137.3       0.106
129         2       2       1       3       2       137.1       0.088
130         2       2       2       1       2       157.3       0.081
131         2       2       2       2       2       158.7       0.081
132         2       2       2       3       2       157.9       0.091
133         2       2       3       1       2       171.7       0.07
134         2       2       3       2       2       172.5       0.059
135         2       2       3       3       2       169.8       0.087
136         2       2       4       1       2       158.2       0.085
137         2       2       4       2       2       158.8       0.072
138         2       2       4       3       2       157.7       0.066
139         2       2       5       1       2       167.3       0.112
140         2       2       5       2       2       166.9       0.093
141         2       2       5       3       2       167.4       0.084
142         2       2       6       1       2       180.2       0.1
143         2       2       6       2       2       179.1       0.091
144         2       2       6       3       2       177.4       0.117
;
Proc print data=EC;
title3 'raw data set';
run;
PROC CONTENTS data=EC;
  Title3 'Proc CONTENTS of data set EC';
```

```
RUN;
proc mixed data=EC CL method=type3;
        classes Su Sa Co Rep Exp ;
        Title3 'Two way ANOVA with PROC mixed for nanoparticle size (salt effect)';
        MODEL Size = Su Sa Co Su*Sa / htype=3 DDFM=Satterthwaite OUTp=res ;
        Random exp Exp*Su*Sa*Co;
        lsmeans Su Sa Co Su*Sa / pdiff adjust=tukey cl;
                ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;
RUN;
        TITLE4 'Post hoc adjustment with macro by Arnold Saxton For nanoparticle size (Salt effect)';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
           * converting mean separation output to letter groupings in Proc Mixed. ;
           * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
         %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
         %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc print data=mmm; run;
data new1; set mmm; if effect = 'Su*Sa'; run;
OPTIONS ls=132 ps=52;
proc plot data=new1; plot estimate*sa=su;
run;
OPTIONS ls=161 ps=512;
proc univariate data=res normal plot;        var resid;
title1 'Univariate Normality of Residuals for Size';
title2 'Carlos E data analysis';
run;
proc mixed data=EC CL;* method=type3;
        classes Su Sa Co Rep Exp ;
                Title3 'Two way ANOVA with PROC mixed for nanoparticle size (Salt effect)';
        MODEL Size = Su | Sa | Co @2 / htype=3 DDFM=Satterthwaite OUTp=res ;
                Random exp Exp*Su*Sa*Co;
                lsmeans Su | Sa | Co @2 / pdiff adjust=tukey;
                ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;* lsmeans;
RUN;
        TITLE4 'Post hoc adjustment with macro by Arnold Saxton For nanoparticle size';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
        * converting mean separation output to letter groupings in Proc Mixed. ;
        * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
        %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
                %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc univariate data=res normal plot;        var resid;
title1 'Univariate Normality of Residuals for Size (Salt effect)';
title2 'Carlos E data analysis';
run;
proc mixed data=EC CL;* method=type3;
        classes Su Sa Co Rep Exp ;
                Title3 'Two way ANOVA with PROC mixed for nanoparticle size';
        MODEL PI = Su | Sa | Co @2 / htype=3 DDFM=Satterthwaite OUTp=res1 ;
                Random exp Exp*Su*Sa*Co;
                lsmeans Su | Sa | Co @2 / pdiff adjust=tukey;
                ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;
RUN;
```

```
        TITLE4 'Post hoc adjustment with macro by Arnold Saxton For nanoparticle size';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
        * converting mean separation output to letter groupings in Proc Mixed. ;
        * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
        %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\\SAS\pdmix800.sas';
                *%include 'C:\pdmix800.sas';
                %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc univariate data=res1 normal plot;        var resid;
title1 'Univariate Normality of Residuals for PI (Salt effect)';
title2 'Carlos E data analysis';
run;
quit;
ODS HTML close;
```

The group estimation in SAS output:

Univariate Normality of Residuals for Size
Carlos E data analysis
Two way ANOVA with PROC mixed for nanoparticle size (Salt effect)
Post hoc adjustment with macro by Arnold Saxton For nanoparticle size

Effect=Su Method=Tukey-Kramer(P<0.05) Set=1

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
1	2	_	_	206.54	6.9031	A
2	1	_	_	39.9213	5.8342	B

Effect=Sa Method=Tukey-Kramer(P<0.05) Set=2

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
3	_	1	_	154.25	6.9031	A
4	_	2	_	92.2092	5.8342	B

Effect=Su*Sa Method=Tukey-Kramer(P<0.05) Set=3

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
5	2	1	_	250.09	11.0696	A
6	2	2	_	162.99	8.2508	B

7	1	1	_	58.4158	8.2508	C
8	1	2	_	21.4267	8.2508	D

Effect=Co Method=Tukey-Kramer(P<0.05) Set=4

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
9	_	_	5	182.77	10.1051	A
10	_	_	6	172.00	14.9883	A
11	_	_	4	115.36	10.1051	B
12	_	_	3	98.9617	10.1051	B
13	_	_	2	89.8567	10.1051	B
14	_	_	1	80.4379	10.1051	B

Effect=Su*Co Method=Tukey-Kramer(P<0.05) Set=5

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
15	2	_	5	314.11	14.2908	A
16	2	_	6	259.66	26.3509	AB
17	2	_	4	196.86	14.2908	BC
18	2	_	3	176.41	14.2908	BC
19	2	_	2	156.27	14.2908	BCD
20	2	_	1	135.93	14.2908	CD
21	1	_	6	84.3408	14.2908	DE
22	1	_	5	51.4267	14.2908	E
23	1	_	4	33.8558	14.2908	E
24	1	_	1	24.9425	14.2908	E
25	1	_	2	23.4467	14.2908	E

26	1	_	3	21.5150	14.2908	E

Effect=Sa*Co Method=Tukey-Kramer(P<0.05) Set=6

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
27	_	1	5	271.28	14.2908	A
28	_	1	6	238.00	26.3509	AB
29	_	1	4	143.63	14.2908	BC
30	_	2	6	106.00	14.2908	C
31	_	1	3	103.58	14.2908	C
32	_	2	3	94.3392	14.2908	C
33	_	2	5	94.2525	14.2908	C
34	_	2	2	91.1383	14.2908	C
35	_	1	2	88.5750	14.2908	C
36	_	2	4	87.0883	14.2908	C
37	_	1	1	80.4383	14.2908	C
38	_	2	1	80.4375	14.2908	C

Univariate Normality of Residuals for Size (Salt effect)

Carlos E data analysis

The UNIVARIATE Procedure

Variable: Resid (Residual)

Moments			
N	138	**Sum Weights**	138
Mean	0	**Sum Observations**	0
Std Deviation	2.94467668	**Variance**	8.67112072

Skewness	-2.8309443	**Kurtosis**	33.179654
Uncorrected SS	1187.94354	**Corrected SS**	1187.94354
Coeff Variation	.	**Std Error Mean**	0.25066753

Basic Statistical Measures			
Location		**Variability**	
Mean	0.000000	**Std Deviation**	2.94468
Median	0.036524	**Variance**	8.67112
Mode	0.019455	**Range**	37.30000
		Interquartile Range	1.07922

The mode displayed is the smallest of 2 modes with a count of 2.

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	7	**Pr >= |M|**	0.2684
Signed Rank	**S**	273.5	**Pr >= |S|**	0.5630

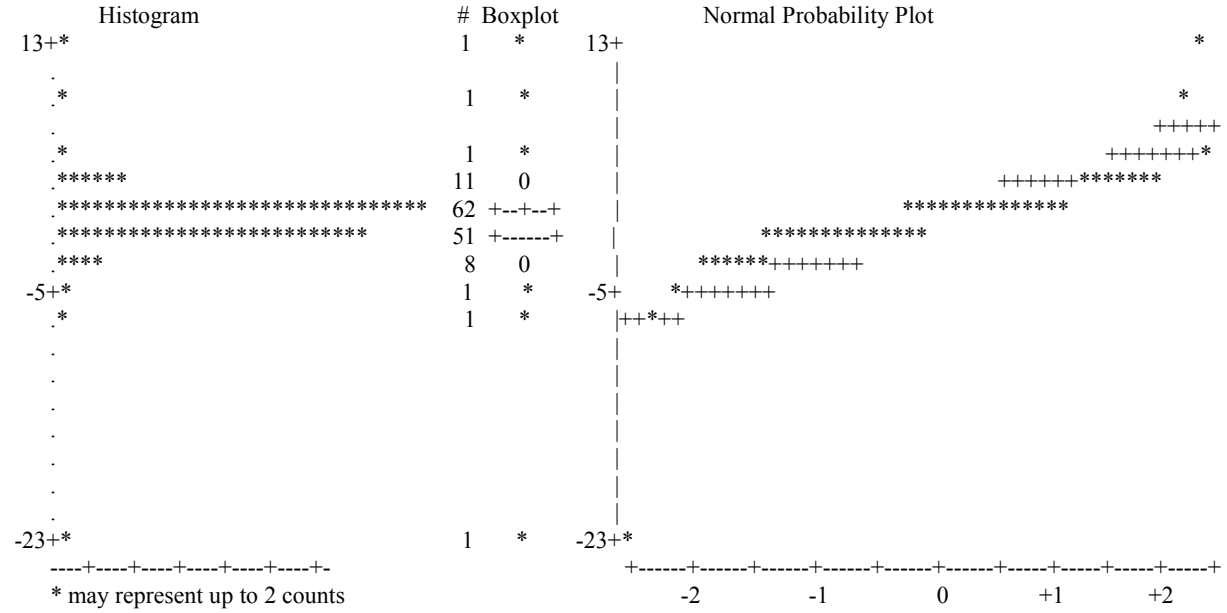
Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.61122	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.235058	**Pr > D**	<0.0100
Cramer-von Mises	**W-Sq**	2.667671	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	13.73822	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	13.7781329
99%	9.7781329
95%	3.6990568
90%	2.2044630
75% Q3	0.5573031
50% Median	0.0365239
25% Q1	-0.5219154
10%	-1.7957388
5%	-3.1282219
1%	-6.9465617
0% Min	-23.5218671

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-23.52187	118	3.79270	108
-6.94656	47	3.95344	48
-5.35730	107	5.17178	49
-3.84299	66	9.77813	120
-3.81846	42	13.77813	119

Missing Values

Missing Value	Count	Percent Of	
		All Obs	Missing Obs
.	6	4.17	100.00



Univariate Normality of Residuals for PI (Salt effect)

Carlos E data analysis

Two way ANOVA with PROC mixed for nanoparticle PI

Post hoc adjustment with macro by Arnold Saxton For nanoparticle size

Effect=Su Method=Tukey-Kramer(P<0.05) Set=1

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
1	1	_	_	0.3445	0.01604	A
2	2	_	_	0.07536	0.01748	B

Effect=Sa Method=Tukey-Kramer(P<0.05) Set=2

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
3	_	2	_	0.2624	0.01604	A

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
4	_	1	_	0.1575	0.01748	B

Effect=Su*Sa Method=Tukey-Kramer(P<0.05) Set=3

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
5	1	2	_	0.4326	0.01944	A
6	1	1	_	0.2565	0.01944	B
7	2	2	_	0.09222	0.01944	C
8	2	1	_	0.05851	0.02390	C

Effect=Co Method=Tukey-Kramer(P<0.05) Set=4

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
9	_	_	1	0.2615	0.02233	A
10	_	_	2	0.2453	0.02233	A
11	_	_	5	0.2203	0.02233	A
12	_	_	4	0.1804	0.02233	A
13	_	_	3	0.1792	0.02233	A
14	_	_	6	0.1731	0.03055	A

Effect=Su*Co Method=Tukey-Kramer(P<0.05) Set=5

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
15	1	_	2	0.4211	0.02934	A
16	1	_	1	0.4190	0.02934	AB
17	1	_	6	0.3493	0.02934	ABC

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
18	1	_	5	0.3480	0.02934	ABC
19	1	_	3	0.2846	0.02934	BC
20	1	_	4	0.2451	0.02934	CD
21	2	_	4	0.1157	0.02934	DE
22	2	_	1	0.1040	0.02934	E
23	2	_	5	0.09250	0.02934	E
24	2	_	3	0.07375	0.02934	E
25	2	_	2	0.06942	0.02934	E
26	2	_	6	-0.00315	0.05098	E

Effect=Sa*Co Method=Tukey-Kramer(P<0.05) Set=6

Obs	Su	Sa	Co	Estimate	Standard Error	Letter Group
27	_	2	2	0.3310	0.02934	A
28	_	2	6	0.3123	0.02934	AB
29	_	2	5	0.2647	0.02934	ABC
30	_	1	1	0.2615	0.02934	ABC
31	_	2	1	0.2615	0.02934	ABC
32	_	2	3	0.2368	0.02934	ABCD
33	_	1	4	0.1928	0.02934	BCDE
34	_	1	5	0.1758	0.02934	CDE
35	_	2	4	0.1680	0.02934	CDE
36	_	1	2	0.1595	0.02934	CDE
37	_	1	3	0.1215	0.02934	DE
38	_	1	6	0.03385	0.05098	E

Univariate Normality of Residuals for PI (Salt effect)
Carlos E data analysis
The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	138	**Sum Weights**	138
Mean	0	**Sum Observations**	0
Std Deviation	0.02280139	**Variance**	0.0005199
Skewness	-0.0562427	**Kurtosis**	3.2184454
Uncorrected SS	0.07122677	**Corrected SS**	0.07122677
Coeff Variation	.	**Std Error Mean**	0.00194098

Basic Statistical Measures			
Location		**Variability**	
Mean	0.00000	**Std Deviation**	0.02280
Median	-0.00060	**Variance**	0.0005199
Mode	-0.00556	**Range**	0.17434
		Interquartile Range	0.02098

The mode displayed is the smallest of 3 modes with a count of 2.

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	-3	**Pr >= |M|**	0.6705
Signed Rank	**S**	73.5	**Pr >= |S|**	0.8765

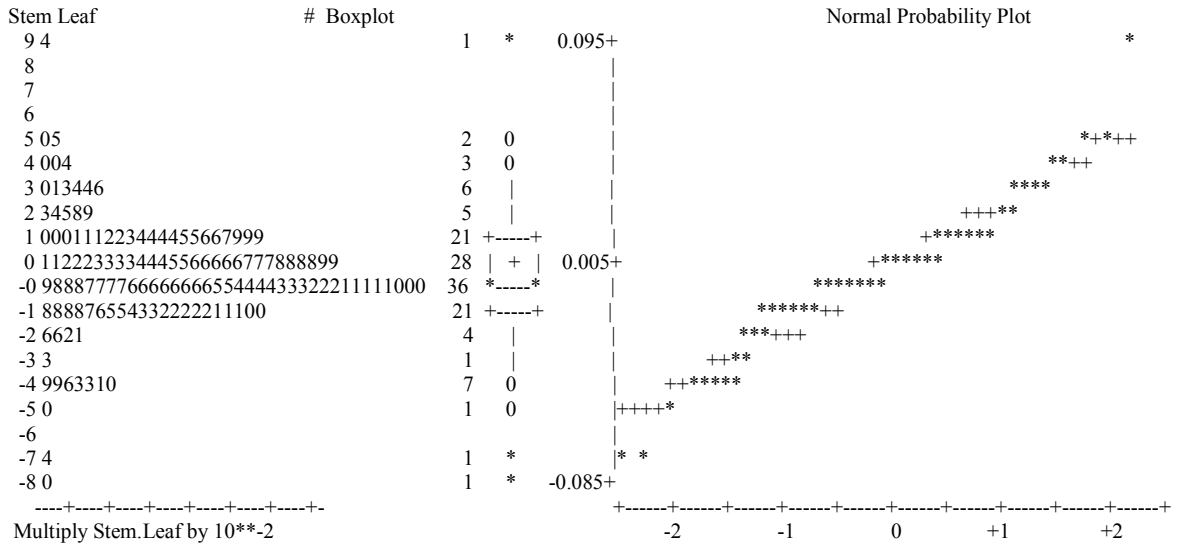
Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.941515	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.105816	**Pr > D**	<0.0100
Cramer-von Mises	**W-Sq**	0.426112	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	2.461663	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	0.094405133
99%	0.055353067
95%	0.035936727
90%	0.027968653
75% Q3	0.010643447
50% Median	-0.000602756
25% Q1	-0.010339757
10%	-0.021881928
5%	-0.043064032
1%	-0.073747976
0% Min	-0.079935968

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.0799360	21	0.0402520	1

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.0737480	3	0.0440640	20
-0.0500633	95	0.0502520	2
-0.0492520	93	0.0553531	118
-0.0485949	35	0.0944051	34

Missing Values			
Missing Value	**Count**	**Percent Of**	
		All Obs	**Missing Obs**
.	6	4.17	100.00



SAS program for effect of surfactant type and concentration. Two surfactants (EC and Span 80) dissolved in pure water (Span 80) or sodium bicarbonate (15 mM) or acetic acid solution 200 (mM) for EC, and 10 different surfactant concentrations (0, 1.5, 7.3, 15, 33, and 99 mM) were analyzed. The data was in triplicate with the following program:

```
dm 'log;clear;output;clear';
OPTIONS nodate nocenter pageno=1 ls=161 ps=512;
Title1 'Nanoparticle size of EC and Span 80 at different concentrations';
Title2 'Carlos E. Astete';
ODS HTML style=minimal body='C:\Documents and Settings\Margarita\My Documents\Carlos\Dissertation for
PhD\EC\EC Data for SAS\Analysis04.html';
DATA EC2; infile cards missover;
input obs Su Sa Co Rep Size PI;
cards; RUN;
1               1       1       1       1       .               .
2               1       1       1       2               .       .
3               1       1       1       3       .               .
4               1       1       2       1       .               .
5               1       1       2       2       .               .
6               1       1       2       3       .               .
7               1       1       3       1       143.0           0.06
8               1       1       3       2       125.2           0.074
9               1       1       3       3       139.1           0.088
10              1       1       4       1       .               .
11              1       1       4       2       .               .
12              1       1       4       3       .               .
13              1       1       5       1       149.3           0.058
14              1       1       5       2       145.4           0.079
15              1       1       5       3       141.1           0.086
16              1       1       6       1       .               .
17              1       1       6       2       .               .
18              1       1       6       3       .               .
19              1       1       7       1       161.9           0.059
20              1       1       7       2       159.2           0.092
21              1       1       7       3       155.4           0.118
22              1       1       8       1       .               .
23              1       1       8       2       .               .
24              1       1       8       3       .               .
25              1       1       9       1       171.5           0.083
26              1       1       9       2       185.5           0.09
27              1       1       9       3       174.3           0.095
28              1       1       10      1       207.8           0.159
29              1       1       10      2       200.4           0.169
30              1       1       10      3       204.5           0.15
31              2       2       1       1       65.76           0.383
32              2       2       1       2       71.63           0.35
33              2       2       1       3       64.15           0.367
34              2       2       2       1       25.13           0.281
35              2       2       2       2       22.1            0.298
36              2       2       2       3       24.55           0.273
37              2       2       3       1       25.53           0.145
38              2       2       3       2       28.97           0.138
39              2       2       3       3       25.93           0.159
40              2       2       4       1       33.04           0.185
41              2       2       4       2       30.46           0.191
42              2       2       4       3       32.39                   0.169
43              2       2       5       1       45.8            0.178
44              2       2       5       2       41.23           0.159
45              2       2       5       3       46.52           0.177
46              2       2       6       1       52.24           0.202
47              2       2       6       2       49.25           0.181
48              2       2       6       3       47.3            0.189
```

```
49              2       2       7       1       60.13           0.17
50              2       2       7       2       66.38           0.176
51              2       2       7       3       60.26           0.167
52              2       2       8       1       .               .
53              2       2       8       2       .               .
54              2       2       8       3       .               .
55              2       2       9       1       376.4           0.869
56              2       2       9       2       435.2           0.605
57              2       2       9       3       458.5           0.643
58              2       2       10      1       .               .
59              2       2       10      2       .               .
60              2       2       10      3       .               .
61              2       3       1       1       .               .
62              2       3       1       2       .               .
63              2       3       1       3       .               .
64              2       3       2       1       .               .
65              2       3       2       2       .               .
66              2       3       2       3       .               .
67              2       3       3       1       36.43           0.498
68              2       3       3       2       37.06           0.506
69              2       3       3       3       41.77           0.576
70              2       3       4       1       .               .
71              2       3       4       2       .               .
72              2       3       4       3       .               .
73              2       3       5       1       16.45           0.315
74              2       3       5       2       23.49           0.328
75              2       3       5       3       18.01           0.301
76              2       3       6       1       .               .
77              2       3       6       2       .               .
78              2       3       6       3       .               .
79              2       3       7       1       18.51           0.255
80              2       3       7       2       15.17           0.264
81              2       3       7       3       19.11           0.241
82              2       3       8       1       19.36           0.328
83              2       3       8       2       24.56           0.293
84              2       3       8       3       22.17           0.395
85              2       3       9       1       39.73           0.344
86              2       3       9       2       29.69           0.278
87              2       3       9       3       35.21           0.276
88              2       3       10      1       .               .
89              2       3       10      2       .               .
90              2       3       10      3       .               .
;
Proc print data=EC2;
title3 'raw data set';
run;
PROC CONTENTS data=EC2;
  Title3 'Proc CONTENTS of data set EC';
RUN;
proc mixed data=EC2 CL method=type3;
classes Sa Co Rep;
Title3 'Two way ANOVA with PROC mixed for Nanoparticle size of EC and Span 80 at different concentrations';
          MODEL Size = Sa Co Sa*Co/ htype=3 DDFM=Satterthwaite OUTp=res ;
          Random  rep;
          lsmeans Sa Co Sa*Co/ pdiff adjust=tukey cl;
          ods output diffs=ppp lsmeans=mmm;
```

```
      ods listing exclude diffs;
RUN;
TITLE4 'Post hoc adjustment with macro by Arnold Saxton for Nanoparticle size of EC and Span 80 at different
concentrations';
                        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
              * converting mean separation output to letter groupings in Proc Mixed. ;
              * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
            %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
            %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc print data=mmm; run;
data new1; set mmm; if effect = 'Sa*Co'; run;
OPTIONS ls=132 ps=52;
proc plot data=new1; plot estimate*Co=Sa;
run;
OPTIONS ls=161 ps=512;
proc univariate data=res normal plot;         var resid;
title1 'Univariate Normality of Residuals for Nanoparticle size (III) of EC and Span 80 at different concentrations';
title2 'Carlos E data analysis';
run;
proc mixed data=EC2 CL;* method=type3;
classes Sa Co Rep ;
Title3 'Two way ANOVA with PROC mixed for Nanoparticle size (III) of EC and Span 80 at different
concentrations';
            MODEL Size =  Sa | Co @2 / htype=3 DDFM=Satterthwaite OUTp=res ;
            Random  Sa*Co;
            lsmeans Sa | Co | Sa*Co @2 / pdiff adjust=tukey;
            ods output diffs=ppp lsmeans=mmm;
             ods listing exclude diffs;* lsmeans;
RUN;
TITLE4 'Post hoc adjustment with macro by Arnold Saxton for Nanoparticle size (III) of EC and Span 80 at
different concentrations';
            * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
            * converting mean separation output to letter groupings in Proc Mixed. ;
            * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
            %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
                        %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc univariate data=res normal plot;         var resid;
title1 'Univariate Normality of Residuals for Nanoparticle size (III)of EC and Span 80 at different concentrations';
title2 'Carlos E data analysis';
run;
proc mixed data=EC2 CL;* method=type3;
classes Sa Co Rep ;
Title3 'Two way ANOVA with PROC mixed for Nanoparticle PI of EC and Span 80 at different concentrations';
            MODEL PI =  Sa | Co @2 / htype=3 DDFM=Satterthwaite OUTp=res1 ;
            Random  Sa*Co;
            lsmeans  Sa | Co @2 / pdiff adjust=tukey;
            ods output diffs=ppp lsmeans=mmm;
             ods listing exclude diffs;
RUN;
TITLE4 'Post hoc adjustment with macro by Arnold Saxton for Nanoparticle size of EC and Span 80 at different
concentrations';
            * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
            * converting mean separation output to letter groupings in Proc Mixed. ;
            * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
```

```
%include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
*%include 'C:\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc univariate data=res1 normal plot;  var resid;
title1 'Univariate Normality of Residuals for Nanoparticle PI of EC and Span 80 at different concentrations';
title2 'Carlos E data analysis';
run;
quit;
ODS HTML close;
```

An important SAS output:

Univariate Normality of Residuals for Nanoparticle size (III) of EC and Span 80 at different concentrations

Carlos E data analysis

Two way ANOVA with PROC mixed for Nanoparticle size (III) of EC and Span 80 at different concentrations

Post hoc adjustment with macro by Arnold Saxton for Nanoparticle size (III) of EC and Span 80 at different concentrations

Effect=Sa Method=LSD(P<0.05) Set=1

Obs	Sa	Co	Estimate	Standard Error	Letter Group
1	1	_	.	.	
2	2	_	.	.	
3	3	_	.	.	

Effect=Co Method=LSD(P<0.05) Set=2

Obs	Sa	Co	Estimate	Standard Error	Letter Group
4	_	9	211.78	3.5859	ABC
5	_	7	79.5622	3.5859	DEF
6	_	5	69.7000	3.5859	DEF
7	_	3	66.9989	3.5859	DEF
8	_	1	.	.	
9	_	2	.	.	
10	_	4	.	.	

Obs	Sa	Co	Estimate	Standard Error	Letter Group
11	_	6	.	.	
12	_	8	.	.	
13	_	10	.	.	

Effect=Sa*Co Method=Tukey(P<0.05) Set=3

Obs	Sa	Co	Estimate	Standard Error	Letter Group
14	2	9	423.37	6.2110	A
15	1	10	204.23	6.2110	B
16	1	9	177.10	6.2110	BC
17	1	7	158.83	6.2110	CD
18	1	5	145.27	6.2110	CD
19	1	3	135.77	6.2110	D
20	2	1	67.1800	6.2110	E
21	2	7	62.2567	6.2110	EF
22	2	6	49.5967	6.2110	EFG
23	2	5	44.5167	6.2110	EFG
24	3	3	38.4200	6.2110	EFG
25	3	9	34.8767	6.2110	EFG
26	2	4	31.9633	6.2110	FG
27	2	3	26.8100	6.2110	G
28	2	2	23.9267	6.2110	G
29	3	8	22.0300	6.2110	G
30	3	5	19.3167	6.2110	G
31	3	7	17.5967	6.2110	G

Univariate Normality of Residuals for Nanoparticle size (III)of EC and Span 80 at different concentrations

Carlos E data analysis

The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	54	**Sum Weights**	54
Mean	0	**Sum Observations**	0
Std Deviation	8.86615982	**Variance**	78.6087899
Skewness	-1.6544175	**Kurtosis**	18.8256203
Uncorrected SS	4166.26587	**Corrected SS**	4166.26587
Coeff Variation	.	**Std Error Mean**	1.20653153

Basic Statistical Measures			
Location		**Variability**	
Mean	0.000000	**Std Deviation**	8.86616
Median	0.203333	**Variance**	78.60879
Mode	.	**Range**	82.10000
		Interquartile Range	4.94000

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	2	**Pr >= |M|**	0.6835
Signed Rank	**S**	27.5	**Pr >= |S|**	0.8153

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.622006	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.245121	**Pr > D**	<0.0100
Cramer-von Mises	**W-Sq**	0.99747	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	5.678334	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	35.133333
99%	35.133333
95%	8.400000
90%	4.450000
75% Q3	2.643333
50% Median	0.203333
25% Q1	-2.296667
10%	-3.833333
5%	-5.600000
1%	-46.966667
0% Min	-46.966667

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-46.96667	55	4.85333	85

Extreme Observations			
Lowest		Highest	
Value	Obs	Value	Obs
-10.56667	8	7.23333	7
-5.60000	25	8.40000	26
-5.18667	86	11.83333	56
-4.16667	15	35.13333	57

Missing Values			
Missing Value	Count	Percent Of	
		All Obs	Missing Obs
.	36	40.00	100.00

Univariate Normality of Residuals for Nanoparticle size (III)of EC and Span 80 at different concentrations

Carlos E data analysis

Two way ANOVA with PROC mixed for Nanoparticle PI of EC and Span 80 at different concentrations

Post hoc adjustment with macro by Arnold Saxton for Nanoparticle size of EC and Span 80 at different concentrations

Effect=Sa Method=LSD(P<0.05) Set=1

Obs	Sa	Co	Estimate	Standard Error	Letter Group
1	1	_	.	.	
2	2	_	.	.	
3	3	_	.	.	

Effect=Co Method=LSD(P<0.05) Set=2

Obs	Sa	Co	Estimate	Standard Error	Letter Group
4	_	9	0.3648	0.01339	ABC
5	_	3	0.2493	0.01339	DEF
6	_	5	0.1868	0.01339	GHI
7	_	7	0.1713	0.01339	GHI
8	_	1	.	.	
9	_	2	.	.	
10	_	4	.	.	
11	_	6	.	.	
12	_	8	.	.	
13	_	10	.	.	

Effect=Sa*Co Method=Tukey(P<0.05) Set=3

Obs	Sa	Co	Estimate	Standard Error	Letter Group
14	2	9	0.7057	0.02319	A
15	3	3	0.5267	0.02319	B
16	2	1	0.3667	0.02319	C
17	3	8	0.3387	0.02319	C
18	3	5	0.3147	0.02319	C
19	3	9	0.2993	0.02319	CD
20	2	2	0.2840	0.02319	CDE
21	3	7	0.2533	0.02319	CDEF
22	2	6	0.1907	0.02319	DEFG
23	2	4	0.1817	0.02319	DEFG
24	2	5	0.1713	0.02319	EFG

Obs	Sa	Co	Estimate	Standard Error	Letter Group
25	2	7	0.1710	0.02319	EFG
26	1	10	0.1593	0.02319	FG
27	2	3	0.1473	0.02319	FG
28	1	7	0.08967	0.02319	G
29	1	9	0.08933	0.02319	G
30	1	5	0.07433	0.02319	G
31	1	3	0.07400	0.02319	G

Univariate Normality of Residuals for Nanoparticle PI of EC and Span 80 at different concentrations

Carlos E data analysis

The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	54	**Sum Weights**	54
Mean	0	**Sum Observations**	0
Std Deviation	0.0331076	**Variance**	0.00109611
Skewness	1.79155778	**Kurtosis**	11.9060104
Uncorrected SS	0.058094	**Corrected SS**	0.058094
Coeff Variation	.	**Std Error Mean**	0.00450537

Basic Statistical Measures			
Location		**Variability**	
Mean	0.00000	**Std Deviation**	0.03311
Median	-0.00017	**Variance**	0.00110

Basic Statistical Measures			
Location		**Variability**	
Mode	.	**Range**	0.26400
		Interquartile Range	0.02200

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	-1	**Pr >= |M|**	0.8919
Signed Rank	**S**	-67	**Pr >= |S|**	0.5689

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.771385	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.225086	**Pr > D**	<0.0100
Cramer-von Mises	**W-Sq**	0.62033	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	3.42766	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	0.163333333
99%	0.163333333
95%	0.049333333
90%	0.016333333
75% Q3	0.009666667

Quantiles (Definition 5)	
Quantile	**Estimate**
50% Median	-0.000166667
25% Q1	-0.012333333
10%	-0.023333333
5%	-0.045666667
1%	-0.100666667
0% Min	-0.100666667

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.1006667	56	0.0283333	21
-0.0626667	57	0.0446667	85
-0.0456667	83	0.0493333	69
-0.0306667	19	0.0563333	84
-0.0286667	67	0.1633333	55

Missing Values			
Missing Value	**Count**	**Percent Of**	
		All Obs	**Missing Obs**
.	36	40.00	100.00

SAS program for PLGA nanoparticles synthesized with EC and Span 80 surfactants. The effect of PLGA/surfactant ratio was evaluated by using four concentration/ratios (0, 40%, 80%, and 120% w/w ratio of PLGA/surfactant). The surfactant mass was fixed at 1 mg/ml. The aqueous solution was with sodium bicarbonate 15 mM. The data was in triplicate with four independent samples (experiments).

```
dm 'log;clear;output;clear';
OPTIONS nodate nocenter pageno=1 ls=96 ps=512;
Title1 'PLGA Nanoparticle size with Span 80 and EC as surfactants';
Title2 'Carlos E. Astete';
ODS HTML style=minimal body='C:\Documents and Settings\Margarita\My Documents\Carlos\Dissertation for
PhD\EC\EC Data for SAS\Analysis02.html';
DATA ECP; infile cards missover;
input OBS Su PLGA Rep Exp Size PI;
cards; RUN;
1       1       1       1       1       45.88           0.577
2       1       1       2       1       48.19           0.57
3       1       1       3       1       48.73           0.65
4       1       1       1       2       17.36           0.41
5       1       1       2       2       19.84           0.394
6       1       1       3       2       21.26           0.365
7       1       1       1       3       33.47           0.53
8       1       1       2       3       29.94           0.436
9       1       1       3       3       35.45           0.401
10      1       1       1       4       37.97           0.428
11      1       1       2       4       31.21           0.486
12      1       1       3       4       41.26           0.547
13      1       2       1       1       89.61           0.166
14      1       2       2       1       90.98           0.15
15      1       2       3       1       92.37           0.143
16      1       2       1       2       85.11           0.15
17      1       2       2       2       84.18           0.165
18      1       2       3       2       82.75           0.169
19      1       2       1       3       85.51           0.134
20      1       2       2       3       84.32           0.161
21      1       2       3       3       83.56           0.143
22      1       2       1       4       93.28           0.171
23      1       2       2       4       92.96           0.142
24      1       2       3       4       91.96           0.146
25      1       3       1       1       107.3           0.072
26      1       3       2       1       104.8           0.176
27      1       3       3       1       105.0           0.128
28      1       3       1       2       113.9           0.085
29      1       3       2       2       112.7           0.117
30      1       3       3       2       111.5           0.13
31      1       3       1       3       129.3           0.107
32      1       3       2       3       127.0           0.129
33      1       3       3       3       128.2           0.095
34      1       3       1       4       114.6           0.158
35      1       3       2       4       115.0           0.116
36      1       3       3       4       113.5           0.122
37      1       4       1       1       122.0           0.176
38      1       4       2       1       121.6           0.124
```

39	1	4	3	1	121.4	0.114
40	1	4	1	2	121.8	0.09
41	1	4	2	2	120.0	0.115
42	1	4	3	2	119.9	0.118
43	1	4	1	3	130.1	0.133
44	1	4	2	3	130.0	0.104
45	1	4	3	3	128.8	0.095
46	1	4	1	4	128.2	0.099
47	1	4	2	4	128.7	0.069
48	1	4	3	4	127.3	0.099
49	2	1	1	1	150.6	0.062
50	2	1	2	1	151.2	0.073
51	2	1	3	1	151.1	0.12
52	2	1	1	2	155.3	0.074
53	2	1	2	2	155.1	0.078
54	2	1	3	2	154.5	0.052
55	2	1	1	3	162.2	0.074
56	2	1	2	3	159.1	0.1
57	2	1	3	3	160.9	0.094
58	2	1	1	4	153.2	0.065
59	2	1	2	4	152.6	0.092
60	2	1	3	4	152.3	0.049
61	2	2	1	1	172.7	0.136
62	2	2	2	1	173.7	0.123
63	2	2	3	1	177.3	0.092
64	2	2	1	2	178.5	0.084
65	2	2	2	2	178.8	0.064
66	2	2	3	2	178.6	0.116
67	2	2	1	3	182.1	0.123
68	2	2	2	3	181.2	0.108
69	2	2	3	3	180.8	0.095
70	2	2	1	4	184.3	0.133
71	2	2	2	4	184.5	0.092
72	2	2	3	4	183.9	0.136
73	2	3	1	1	200.6	0.13
74	2	3	2	1	204.6	0.12
75	2	3	3	1	205.4	0.114
76	2	3	1	2	193.1	0.131
77	2	3	2	2	191.7	0.139
78	2	3	3	2	190.5	0.108
79	2	3	1	3	179.8	0.138
80	2	3	2	3	181.8	0.127
81	2	3	3	3	179.6	0.136
82	2	3	1	4	184.0	0.107
83	2	3	2	4	186.3	0.137
84	2	3	3	4	182.6	0.137
85	2	4	1	1	178.9	0.171
86	2	4	2	1	188.4	0.147
87	2	4	3	1	187.7	0.06
88	2	4	1	2	197.4	0.138
89	2	4	2	2	190.5	0.135
90	2	4	3	2	190.9	0.129
91	2	4	1	3	239.4	0.218
92	2	4	2	3	236.1	0.189
93	2	4	3	3	233.3	0.19
94	2	4	1	4	227.3	0.163
95	2	4	2	4	227.9	0.172

```
96      2       4       3       4       224.9           0.178
;
Proc print data=ECP;
title3 'raw data set';
run;
PROC CONTENTS data=ECP;
  Title3 'Proc CONTENTS of data set EC';
RUN;
proc mixed data=ECP CL method=type3;
          classes Su PLGA Rep Exp ;
          Title3 'Two way ANOVA with PROC mixed for PLGA-surfactant Nanoparticle Size';
          MODEL Size = Su PLGA Su*PLGA / htype=3 DDFM=Satterthwaite OUTp=res ;
          Random exp Exp*Su*PLGA;
          lsmeans Su PLGA Su*PLGA / pdiff adjust=tukey cl;
          ods output diffs=ppp lsmeans=mmm;
           ods listing exclude diffs;
RUN;
          TITLE4 'Post hoc adjustment with macro by Arnold Saxton For PLGA-Surfactant Nanoparticle size';
          * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
             * converting mean separation output to letter groupings in Proc Mixed. ;
             * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
           %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
           %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc print data=mmm; run;
data new1; set mmm; if effect = 'Su*PLGA'; run;
OPTIONS ls=90 ps=30;
proc plot data=new1; plot estimate*PLGA=su;
run;
OPTIONS ls=105 ps=512;
proc univariate data=res normal plot;         var resid;
title1 'Univariate Normality of Residuals for PLGA-surfactant Nanoparticle Size';
title2 'Carlos E data analysis';
run;
OPTIONS ls=96 ps=512;
proc mixed data=ECP CL;* method=type3;
          classes Su PLGA Rep Exp ;
          Title3 'Two way ANOVA with PROC mixed for PLGA-Surfactant Nanoparticle size';
          MODEL Size = Su | PLGA @2 / htype=3 DDFM=Satterthwaite OUTp=res ;
                    Random exp Exp*Su*PLGA;
                    lsmeans Su | PLGA @2 / pdiff adjust=tukey;
                    ods output diffs=ppp lsmeans=mmm;
      ods listing exclude diffs;* lsmeans;
RUN;
          TITLE4 'Post hoc adjustment with macro by Arnold Saxton For PLGA-Surfactant Nanoparticle size';
          * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
          * converting mean separation output to letter groupings in Proc Mixed. ;
          * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
          %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
                    %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
OPTIONS ls=105 ps=512;
proc univariate data=res normal plot;         var resid;
title1 'Univariate Normality of Residuals for PLGA-Surfactant Nanoparticle Size';
title2 'Carlos E data analysis';
run;
```

```
OPTIONS ls=96 ps=512;
proc mixed data=ECP CL;* method=type3;
        classes Su PLGA Rep Exp ;
        Title3 'Two way ANOVA with PROC mixed for PLGA-Surfactant Nanoparticle PI';
        MODEL PI = Su | PLGA  @2 / htype=3 DDFM=Satterthwaite OUTp=res1 ;
        Random exp Exp*Su*PLGA;
        lsmeans Su | PLGA @2 / pdiff adjust=tukey;
        ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;
RUN;
        TITLE4 'Post hoc adjustment with macro by Arnold Saxton For PLGA-Surfactant Nanoparticle PI';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
        * converting mean separation output to letter groupings in Proc Mixed. ;
        * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
        %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
                *%include 'C:\pdmix800.sas';
                %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
OPTIONS ls=105 ps=512;
proc univariate data=res1 normal plot;       var resid;
title1 'Univariate Normality of Residuals for PLGA-Surfactant Nanoparticle PI ';
title2 'Carlos E data analysis';
run;
proc print data=mmm; run;
data new2; set mmm; if effect = 'Su*PLGA'; run;
OPTIONS ls=90 ps=30;
proc plot data=new2; plot estimate*PLGA=su;
run;
OPTIONS ls=96 ps=512;
quit;
ODS HTML close;
```

Univariate Normality of Residuals for PLGA-surfactant Nanoparticle Size

Carlos E data analysis

Two way ANOVA with PROC mixed for PLGA-Surfactant Nanoparticle size

Post hoc adjustment with macro by Arnold Saxton For PLGA-Surfactant Nanoparticle size

Effect=Su Method=Tukey-Kramer(P<0.05) Set=1

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
1	2	_	183.69	2.9996	A
2	1	_	90.6198	2.9996	B

Effect=PLGA Method=Tukey-Kramer(P<0.05) Set=2

Obs	Su	PLGA	Estimate	Standard Error	Letter Group

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
3	_	4	167.60	4.0908	A
4	_	3	152.62	4.0908	A
5	_	2	133.87	4.0908	B
6	_	1	94.5275	4.0908	C

Effect=Su*PLGA Method=Tukey-Kramer(P<0.05) Set=3

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
7	2	4	210.22	5.6753	A
8	2	3	190.00	5.6753	AB
9	2	2	179.70	5.6753	BC
10	2	1	154.84	5.6753	C
11	1	4	124.98	5.6753	D
12	1	3	115.23	5.6753	D
13	1	2	88.0492	5.6753	E
14	1	1	34.2133	5.6753	F

Univariate Normality of Residuals for PLGA-Surfactant Nanoparticle Size
Carlos E data analysis

The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	96	**Sum Weights**	96
Mean	0	**Sum Observations**	0
Std Deviation	1.65779626	**Variance**	2.74828843

Moments			
Skewness	-0.5748819	**Kurtosis**	3.0546504
Uncorrected SS	261.087401	**Corrected SS**	261.087401
Coeff Variation	.	**Std Error Mean**	0.16919812

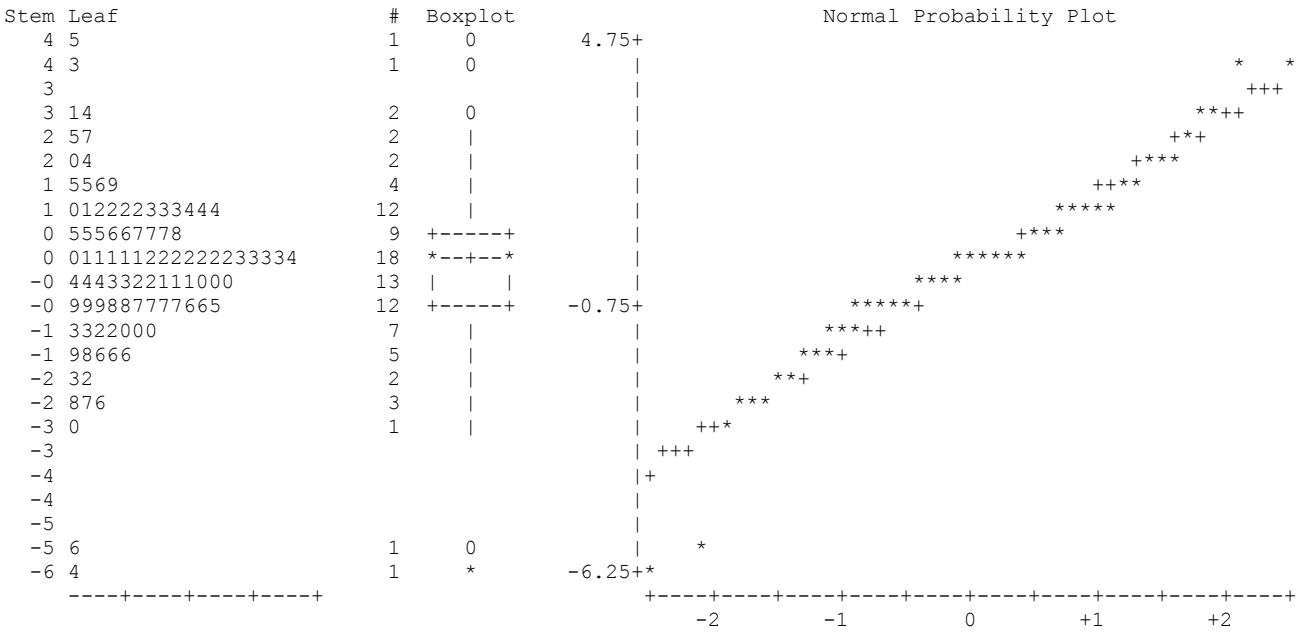
Basic Statistical Measures			
Location		**Variability**	
Mean	0.000000	**Std Deviation**	1.65780
Median	0.107279	**Variance**	2.74829
Mode	.	**Range**	10.83769
		Interquartile Range	1.68052

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	3	**Pr >= |M|**	0.6101
Signed Rank	**S**	88	**Pr >= |S|**	0.7496

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.945054	**Pr < W**	0.0005
Kolmogorov-Smirnov	**D**	0.088628	**Pr > D**	0.0630
Cramer-von Mises	**W-Sq**	0.218939	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	1.321885	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	4.467494
99%	4.467494
95%	2.682959
90%	1.626385
75% Q3	0.883860
50% Median	0.107279
25% Q1	-0.796661
10%	-1.769984
5%	-2.694090
1%	-6.370200
0% Min	-6.370200

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-6.37020	85	2.68296	63
-5.58251	11	3.12980	86
-3.03947	8	3.40591	91
-2.77947	73	4.29165	88
-2.69409	93	4.46749	12



Univariate Normality of Residuals for PLGA-Surfactant Nanoparticle Size

Carlos E data analysis

Two way ANOVA with PROC mixed for PLGA-Surfactant Nanoparticle PI

Post hoc adjustment with macro by Arnold Saxton For PLGA-Surfactant Nanoparticle PI

Effect=Su Method=Tukey-Kramer(P<0.05) Set=1

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
1	1	_	0.2168	0.009363	A
2	2	_	0.1177	0.009363	B

Effect=PLGA Method=Tukey-Kramer(P<0.05) Set=2

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
3	_	1	0.2803	0.01264	A
4	_	4	0.1344	0.01264	B
5	_	2	0.1309	0.01264	B
6	_	3	0.1233	0.01264	B

Effect=Su*PLGA Method=Tukey-Kramer(P<0.05) Set=3

Obs	Su	PLGA	Estimate	Standard Error	Letter Group
7	1	1	0.4828	0.01742	A
8	2	4	0.1575	0.01742	B
9	1	2	0.1533	0.01742	B
10	2	3	0.1270	0.01742	B
11	1	3	0.1196	0.01742	B
12	1	4	0.1113	0.01742	B
13	2	2	0.1085	0.01742	B
14	2	1	0.07775	0.01742	B

Univariate Normality of Residuals for PLGA-Surfactant Nanoparticle PI
Carlos E data analysis

The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	96	**Sum Weights**	96
Mean	0	**Sum Observations**	0
Std Deviation	0.02409855	**Variance**	0.00058074
Skewness	0.1314266	**Kurtosis**	2.16098655
Uncorrected SS	0.05517032	**Corrected SS**	0.05517032
Coeff Variation	.	**Std Error Mean**	0.00245955

Basic Statistical Measures	
Location	**Variability**

Basic Statistical Measures			
Location		**Variability**	
Mean	0.00000	**Std Deviation**	0.02410
Median	-0.00010	**Variance**	0.0005807
Mode	0.00477	**Range**	0.15101
		Interquartile Range	0.02434

The mode displayed is the smallest of 2 modes with a count of 2.

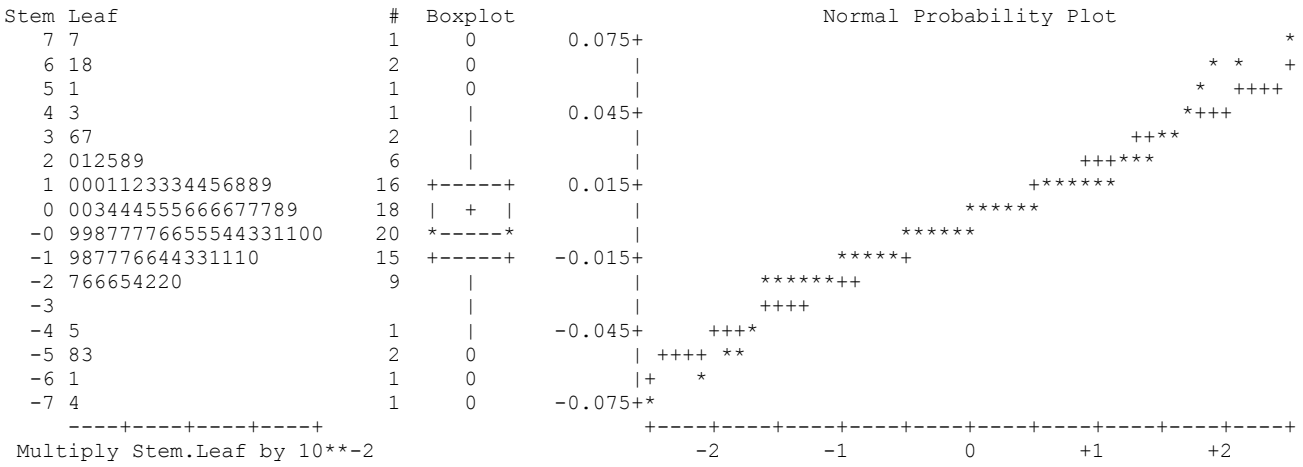
Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	-1	**Pr >= |M|**	0.9188
Signed Rank	**S**	-44	**Pr >= |S|**	0.8732

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.95262	**Pr < W**	0.0016
Kolmogorov-Smirnov	**D**	0.089843	**Pr > D**	0.0551
Cramer-von Mises	**W-Sq**	0.202487	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	1.402209	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	0.076649920
99%	0.076649920

Quantiles (Definition 5)	
Quantile	**Estimate**
95%	0.043034174
90%	0.024610563
75% Q3	0.011328460
50% Median	-0.000104772
25% Q1	-0.013014687
10%	-0.025233179
5%	-0.044794400
1%	-0.074364141
0% Min	-0.074364141

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.0743641	87	0.0430342	37
-0.0610598	9	0.0508828	26
-0.0581291	10	0.0608709	12
-0.0531172	25	0.0679402	7
-0.0447944	6	0.0766499	3



SAS program for nanoparticles synthesized with a mixture of EC and Span 80 surfactants. The effect of EC/Span 80 (% mol ratio) mixture on size and PI was evaluated by using five molar ratios (100%, 65%, 38%, 17%, and 0% of EC/PLGA). The aqueous solution was with sodium bicarbonate 15 mM. The data was in triplicate with two independent samples (experiments).

```
dm 'log;clear;output;clear';
OPTIONS nodate nocenter pageno=1 ls=96 ps=512;
Title1 'Mixture of EC and Span 80 on Nanostructures Size';
Title2 'Carlos E. Astete';
ODS HTML style=minimal body='C:\Documents and Settings\Margarita\My Documents\Carlos\Dissertation for
PhD\EC\EC Data for SAS\Analysis03.html';
DATA ECSpan; infile cards missover;
input OBS Su co Rep Exp Size PI;
cards; RUN;
1      1      1      1      1      23.98        0.501
2      1      1      2      1      26.99        0.398
3      1      1      3      1      24.17        0.413
4      1      2      1      1      16.68        0.411
5      1      2      2      1      17.17        0.327
6      1      2      3      1      16.44        0.472
7      1      3      1      1      10.74        0.28
8      1      3      2      1      10.76        0.325
9      1      3      3      1      10.6         0.252
10     1      4      1      1      16.99        0.46
11     1      4      2      1      17.36        0.274
12     1      4      3      1      17.37        0.464
13     1      5      1      1      24.14        0.629
14     1      5      2      1      25.87        0.386
15     1      5      3      1      25.84        0.489
16     2      1      1      1      66.65        0.294
17     2      1      2      1      60.01        0.288
18     2      1      3      1      66.85        0.293
19     2      2      1      1      57.01        0.173
20     2      2      2      1      59.09        0.198
```

21	2	2	3	1	57.97	0.195
22	2	3	1	1	75.9	0.156
23	2	3	2	1	72.94	0.169
24	2	3	3	1	72.07	0.167
25	2	4	1	1	73.4	0.183
26	2	4	2	1	71.78	0.214
27	2	4	3	1	68.2	0.207
28	2	5	1	1	109.4	0.319
29	2	5	2	1	112.3	0.318
30	2	5	3	1	106.3	0.309
31	3	1	1	1	79.52	0.121
32	3	1	2	1	76.93	0.107
33	3	1	3	1	75.12	0.14
34	3	2	1	1	92.69	0.107
35	3	2	2	1	91.01	0.118
36	3	2	3	1	89.2	0.112
37	3	3	1	1	113.2	0.064
38	3	3	2	1	109.2	0.067
39	3	3	3	1	110.8	0.038
40	3	4	1	1	110.3	0.09
41	3	4	2	1	103.0	0.105
42	3	4	3	1	107.8	0.1
43	3	5	1	1	138.9	0.236
44	3	5	2	1	135.3	0.212
45	3	5	3	1	135.0	0.212
46	4	1	1	1	134.1	0.087
47	4	1	2	1	134.3	0.075
48	4	1	3	1	133.8	0.086
49	4	2	1	1	162.6	0.146
50	4	2	2	1	161.6	0.136
51	4	2	3	1	162.7	0.117
52	4	3	1	1	158.5	0.147
53	4	3	2	1	159.1	0.109
54	4	3	3	1	159.9	0.118
55	4	4	1	1	177.8	0.157
56	4	4	2	1	178.2	0.158
57	4	4	3	1	178.5	0.151
58	4	5	1	1	164.3	0.089
59	4	5	2	1	163.6	0.073
60	4	5	3	1	163.2	0.095
61	5	1	1	1	149.0	0.09
62	5	1	2	1	156.6	0.111
63	5	1	3	1	156.9	0.066
64	5	2	1	1	173.4	0.092
65	5	2	2	1	170.3	0.092
66	5	2	3	1	178.5	0.084
67	5	3	1	1	166.8	0.108
68	5	3	2	1	169.3	0.098
69	5	3	3	1	161.1	0.098
70	5	4	1	1	165.2	0.087
71	5	4	2	1	177.3	0.101
72	5	4	3	1	171.5	0.085
73	5	5	1	1	184.5	0.101
74	5	5	2	1	195.2	0.092
75	5	5	3	1	180.1	0.102
76	1	1	1	2	14.84	0.272
77	1	1	2	2	14.96	0.278

78	1	1	3	2	16.49	0.295
79	1	2	1	2	23.88	0.473
80	1	2	2	2	29.35	0.568
81	1	2	3	2	24.62	0.544
82	1	3	1	2	21.66	0.153
83	1	3	2	2	16.15	0.348
84	1	3	3	2	23.06	0.324
85	1	4	1	2	38.99	0.142
86	1	4	2	2	28.74	0.561
87	1	4	3	2	33.45	0.514
88	1	5	1	2	44.63	0.5
89	1	5	2	2	43.5	0.474
90	1	5	3	2	43.37	0.477
91	2	1	1	2	56.02	0.194
92	2	1	2	2	54.57	0.206
93	2	1	3	2	55.67	0.19
94	2	2	1	2	66.39	0.177
95	2	2	2	2	65.47	0.169
96	2	2	3	2	64.83	0.188
97	2	3	1	2	59.5	0.186
98	2	3	2	2	59.11	0.165
99	2	3	3	2	59.26	0.185
100	2	4	1	2	69.37	0.172
101	2	4	2	2	68.52	0.171
102	2	4	3	2	68.11	0.171
103	2	5	1	2	93.74	0.193
104	2	5	2	2	94.08	0.218
105	2	5	3	2	93.72	0.208
106	3	1	1	2	86.73	0.105
107	3	1	2	2	86.85	0.132
108	3	1	3	2	86.34	0.108
109	3	2	1	2	100.4	0.106
110	3	2	2	2	99.35	0.107
111	3	2	3	2	99.38	0.098
112	3	3	1	2	116.6	0.075
113	3	3	2	2	115.9	0.06
114	3	3	3	2	116.5	0.072
115	3	4	1	2	139.9	0.102
116	3	4	2	2	140.6	0.087
117	3	4	3	2	141.2	0.125
118	3	5	1	2	130.4	0.163
119	3	5	2	2	129.4	0.145
120	3	5	3	2	129.0	0.15
121	4	1	1	2	120.9	0.095
122	4	1	2	2	120.1	0.091
123	4	1	3	2	119.8	0.076
124	4	2	1	2	164.8	0.096
125	4	2	2	2	163.9	0.098
126	4	2	3	2	162.9	0.091
127	4	3	1	2	124.7	0.147
128	4	3	2	2	123.7	0.148
129	4	3	3	2	122.8	0.132
130	4	4	1	2	141.5	0.165
131	4	4	2	2	140.0	0.176
132	4	4	3	2	141.2	0.167
133	4	5	1	2	122.2	0.092
134	4	5	2	2	123.1	0.097

```
135    4       5       3       2       121.8         0.075
136    5       1       1       2       157.3         0.081
137    5       1       2       2       158.7         0.081
138    5       1       3       2       157.9         0.091
139    5       2       1       2       171.7         0.07
140    5       2       2       2       172.5         0.059
141    5       2       3       2       169.8         0.087
142    5       3       1       2       158.2         0.085
143    5       3       2       2       158.8         0.072
144    5       3       3       2       157.7         0.066
145    5       4       1       2       167.3         0.112
146    5       4       2       2       166.9         0.093
147    5       4       3       2       167.4         0.084
148    5       5       1       2       180.2         0.1
149    5       5       2       2       179.1         0.091
150    5       5       3       2       177.4         0.117
;
Proc print data=ECSpan;
title3 'raw data set of Size and PI of nanostructures for Mixture of EC and Span 80';
run;
PROC CONTENTS data=ECSpan;
  Title3 'Proc CONTENTS of data set EC and Span 80 Mixtures';
RUN;
proc mixed data=ECSpan CL method=type3;
          classes Su co Rep Exp ;
          Title3 'Two way ANOVA with PROC mixed for EC-Span 80 Surfactants on Nanoparticle Size';
          MODEL Size = Su Co Su*Co / htype=3 DDFM=Satterthwaite OUTp=res ;
          Random exp Exp*Su*Co;
          lsmeans Su co Su*co / pdiff adjust=tukey cl;
          ods output diffs=ppp lsmeans=mmm;
      ods listing exclude diffs;
RUN;
TITLE4 'Post hoc adjustment with macro by Arnold Saxton For EC-Span 80 Surfactants on Nanoparticle Size';
          * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
          * converting mean separation output to letter groupings in Proc Mixed. ;
          * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
          %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
           %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
proc print data=mmm; run;
data new1; set mmm; if effect = 'Su*Co'; run;
OPTIONS ls=90 ps=30;
proc plot data=new1; plot estimate*Co=su;
run;
OPTIONS ls=105 ps=512;
proc univariate data=res normal plot;          var resid;
title1 'Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle Size';
title2 'Carlos E data analysis';
run;
OPTIONS ls=96 ps=512;
proc mixed data=ECSpan CL;* method=type3;
          classes Su Co Rep Exp ;
          Title3 'Two way ANOVA with PROC mixed for EC-Span 80 Surfactants on Nanoparticle Size';
          MODEL Size = Su | Co @2 / htype=3 DDFM=Satterthwaite OUTp=res ;
          Random exp Exp*Su*Co;
          lsmeans Su | Co @2 / pdiff adjust=tukey;
```

```
        ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;* lsmeans;
RUN;
TITLE4 'Post hoc adjustment with macro by Arnold Saxton For EC-Span 80 Surfactants on Nanoparticle Size';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
        * converting mean separation output to letter groupings in Proc Mixed. ;
        * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
        %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
        %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
OPTIONS ls=105 ps=512;
proc univariate data=res normal plot;        var resid;
title1 'Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle Size';
title2 'Carlos E data analysis';
run;
OPTIONS ls=96 ps=512;
proc mixed data=ECSpan CL;* method=type3;
        classes Su Co Rep Exp ;
                Title3 'Two way ANOVA with PROC mixed for EC-Span 80 Surfactants on Nanoparticle PI';
        MODEL PI = Su | Co  @2 / htype=3 DDFM=Satterthwaite OUTp=res1 ;
                Random exp Exp*Su*Co;
                lsmeans Su | Co @2 / pdiff adjust=tukey;
                ods output diffs=ppp lsmeans=mmm;
    ods listing exclude diffs;
RUN;
        TITLE4 'Post hoc adjustment with macro by Arnold Saxton EC-Span 80 Surfactants on Nanoparticle PI';
        * SAS Macro by Arnold Saxton: Saxton, A.M. 1998. A macro for ;
        * converting mean separation output to letter groupings in Proc Mixed. ;
        * In Proc. 23rd SAS Users Group Intl., SAS Inst., Cary, NC, pp1243-246.;
        %include 'C:\Documents and Settings\Margarita\My Documents\Carlos\SAS\pdmix800.sas';
        %pdmix800(ppp,mmm,alpha=0.05,sort=yes);
run;
OPTIONS ls=105 ps=512;
proc univariate data=res1 normal plot;        var resid;
title1 'Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle PI ';
title2 'Carlos E data analysis';
run;
proc print data=mmm; run;
data new2; set mmm; if effect = 'Su*Co'; run;
OPTIONS ls=90 ps=30;
proc plot data=new2; plot estimate*Co=su;
run;
OPTIONS ls=96 ps=512;
quit;
ODS HTML close;
```

Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle Size

Carlos E data analysis

Two way ANOVA with PROC mixed for EC-Span 80 Surfactants on Nanoparticle Size

Post hoc adjustment with macro by Arnold Saxton For EC-Span 80 Surfactants on Nanoparticle Size

Effect=Su Method=Tukey(P<0.05) Set=1

Obs	Su	co	Estimate	Standard Error	Letter Group
1	5	_	168.55	3.8833	A
2	4	_	146.85	3.8833	B
3	3	_	109.55	3.8833	C
4	2	_	71.9410	3.8833	D
5	1	_	23.4263	3.8833	E

Effect=co Method=Tukey(P<0.05) Set=2

Obs	Su	co	Estimate	Standard Error	Letter Group
6	_	5	118.99	3.8833	A
7	_	4	109.60	3.8833	AB
8	_	2	102.85	3.8833	BC
9	_	3	99.8183	3.8833	BC
10	_	1	89.0697	3.8833	C

Effect=Su*co Method=Tukey(P<0.05) Set=3

Obs	Su	co	Estimate	Standard Error	Letter Group
11	5	5	182.75	8.6834	A
12	5	2	172.70	8.6834	AB
13	5	4	169.27	8.6834	AB
14	4	2	163.08	8.6834	ABC
15	5	3	161.98	8.6834	ABC
16	4	4	159.53	8.6834	ABC
17	5	1	156.07	8.6834	ABC

Obs	Su	co	Estimate	Standard Error	Letter Group
18	4	5	143.03	8.6834	ABCD
19	4	3	141.45	8.6834	ABCD
20	3	5	133.00	8.6834	ABCD
21	4	1	127.17	8.6834	BCDE
22	3	4	123.80	8.6834	BCDE
23	3	3	113.70	8.6834	CDEF
24	2	5	101.59	8.6834	DEFG
25	3	2	95.3383	8.6834	DEFG
26	3	1	81.9150	8.6834	EFGH
27	2	4	69.8967	8.6834	FGHI
28	2	3	66.4633	8.6834	FGHI
29	2	2	61.7933	8.6834	GHIJ
30	2	1	59.9617	8.6834	GHIJ
31	1	5	34.5583	8.6834	HIJ
32	1	4	25.4833	8.6834	IJ
33	1	2	21.3567	8.6834	IJ
34	1	1	20.2383	8.6834	IJ
35	1	3	15.4950	8.6834	J

Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle Size
Carlos E data analysis
The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	150	**Sum Weights**	150
Mean	0	**Sum Observations**	0
Std Deviation	1.95880451	**Variance**	3.83691512
Skewness	0.15610324	**Kurtosis**	4.20019104
Uncorrected SS	571.700352	**Corrected SS**	571.700352
Coeff Variation	.	**Std Error Mean**	0.15993572

Basic Statistical Measures			
Location		**Variability**	
Mean	0.00000	**Std Deviation**	1.95880
Median	-0.01607	**Variance**	3.83692
Mode	.	**Range**	15.10000
		Interquartile Range	1.27513

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	-2	**Pr >= |M|**	0.8066
Signed Rank	**S**	-31	**Pr >= |S|**	0.9539

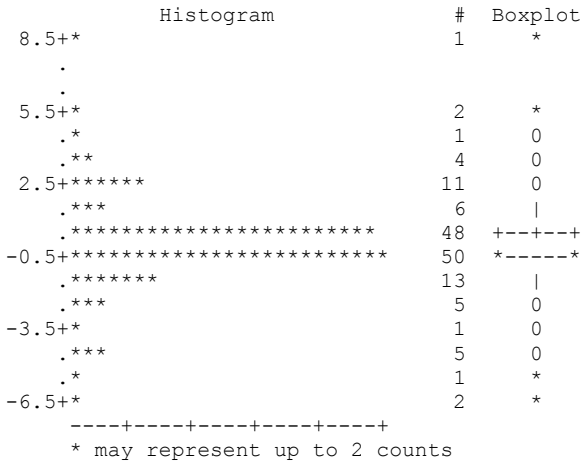
Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.89092	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.15186	**Pr > D**	<0.0100

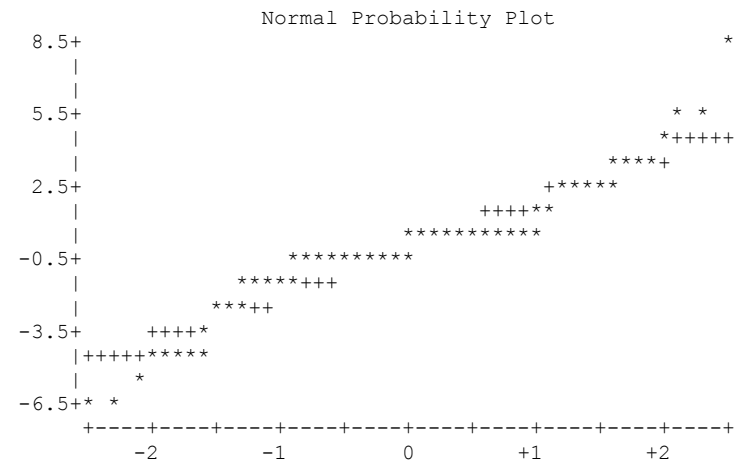
Tests for Normality				
Test	**Statistic**		**p Value**	
Cramer-von Mises	**W-Sq**	1.16582	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	6.038103	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	8.6484990
99%	5.9927008
95%	3.0554545
90%	2.2796532
75% Q3	0.6688278
50% Median	-0.0160702
25% Q1	-0.6063031
10%	-1.8607882
5%	-4.0795967
1%	-6.1072992
0% Min	-6.4515010

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-6.45150	75	3.61391	68
-6.10730	70	4.45055	66
-5.19060	61	5.36718	85

Extreme Observations			
Lowest		Highest	
Value	Obs	Value	Obs
-4.88282	86	5.99270	71
-4.58609	69	8.64850	74





Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle Size
Carlos E data analysis
Two way ANOVA with PROC mixed for EC-Span 80 Surfactants on Nanoparticle PI
Post hoc adjustment with macro by Arnold Saxton EC-Span 80 Surfactants on Nanoparticle PI

Effect=Su Method=Tukey-Kramer(P<0.05) Set=1

Obs	Su	co	Estimate	Standard Error	Letter Group
1	1	_	0.4001	0.01292	A
2	2	_	0.2092	0.01292	B
3	4	_	0.1163	0.01292	C
4	3	_	0.1155	0.01292	C
5	5	_	0.08987	0.01292	C

Effect=co Method=Tukey-Kramer(P<0.05) Set=2

Obs	Su	co	Estimate	Standard Error	Letter Group
6	_	5	0.2254	0.01292	A
7	_	2	0.1904	0.01292	AB
8	_	4	0.1893	0.01292	AB
9	_	1	0.1788	0.01292	AB
10	_	3	0.1471	0.01292	B

Effect=Su*co Method=Tukey-Kramer(P<0.05) Set=3

Obs	Su	co	Estimate	Standard Error	Letter Group
11	1	5	0.4925	0.02653	A
12	1	2	0.4658	0.02653	A
13	1	4	0.4025	0.02653	AB
14	1	1	0.3595	0.02653	ABC
15	1	3	0.2803	0.02653	BCD
16	2	5	0.2608	0.02653	BCDE
17	2	1	0.2442	0.02653	CDEF

Obs	Su	co	Estimate	Standard Error	Letter Group
18	3	5	0.1863	0.02653	DEFG
19	2	4	0.1863	0.02653	DEFG
20	2	2	0.1833	0.02653	DEFG
21	2	3	0.1713	0.02653	DEFG
22	4	4	0.1623	0.02653	DEFG
23	4	3	0.1335	0.02653	DEFG
24	3	1	0.1188	0.02653	EFG
25	4	2	0.1140	0.02653	EFG
26	3	2	0.1080	0.02653	FG
27	3	4	0.1015	0.02653	FG
28	5	5	0.1005	0.02653	FG
29	5	4	0.09367	0.02653	G
30	5	3	0.08783	0.02653	G
31	4	5	0.08683	0.02653	G
32	5	1	0.08667	0.02653	G
33	4	1	0.08500	0.02653	G
34	5	2	0.08067	0.02653	G
35	3	3	0.06267	0.02653	G

Univariate Normality of Residuals for EC-Span 80 Surfactants on Nanoparticle PI
Carlos E data analysis
The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	150	**Sum Weights**	150

Moments			
Mean	0	**Sum Observations**	0
Std Deviation	0.04055668	**Variance**	0.00164484
Skewness	-1.4606383	**Kurtosis**	14.2460802
Uncorrected SS	0.24508181	**Corrected SS**	0.24508181
Coeff Variation	.	**Std Error Mean**	0.00331144

Basic Statistical Measures			
Location		**Variability**	
Mean	0.00000	**Std Deviation**	0.04056
Median	-0.00024	**Variance**	0.00164
Mode	-0.00612	**Range**	0.41900
		Interquartile Range	0.02155

The mode displayed is the smallest of 5 modes with a count of 2.

Tests for Location: Mu0=0				
Test	**Statistic**		**p Value**	
Student's t	**t**	0	**Pr > |t|**	1.0000
Sign	**M**	-2	**Pr >= |M|**	0.8066
Signed Rank	**S**	28.5	**Pr >= |S|**	0.9576

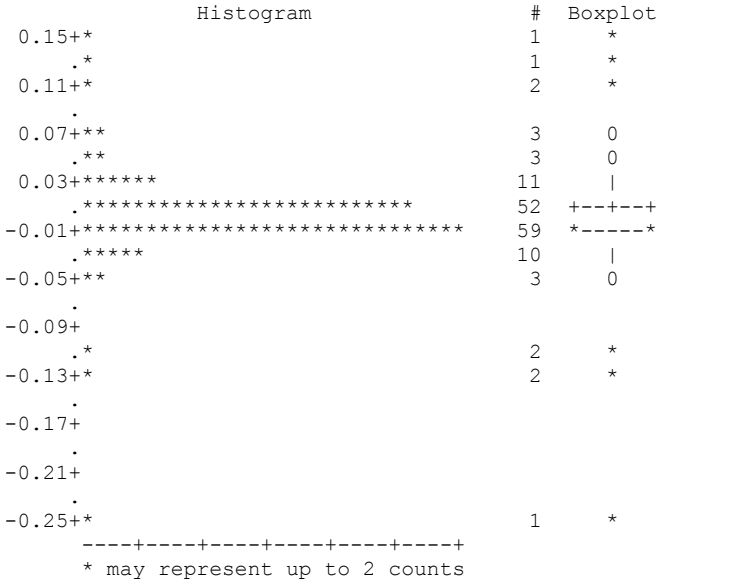
Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.732621	**Pr < W**	<0.0001
Kolmogorov-Smirnov	**D**	0.214304	**Pr > D**	<0.0100

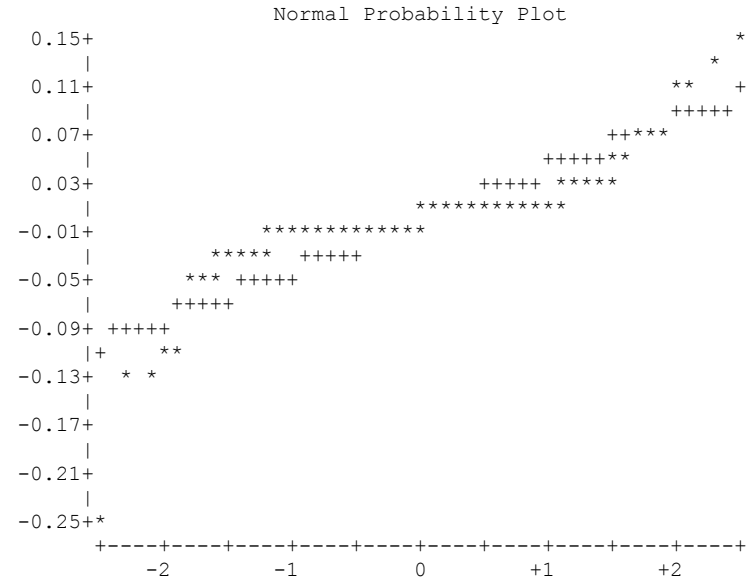
Tests for Normality				
Test	**Statistic**		**p Value**	
Cramer-von Mises	**W-Sq**	2.201264	**Pr > W-Sq**	<0.0050
Anderson-Darling	**A-Sq**	11.90277	**Pr > A-Sq**	<0.0050

Quantiles (Definition 5)	
Quantile	**Estimate**
100% Max	0.159317391
99%	0.130135863
95%	0.056682609
90%	0.027645215
75% Q3	0.011687000
50% Median	-0.000243345
25% Q1	-0.009864137
10%	-0.024023325
5%	-0.040360748
1%	-0.129317391
0% Min	-0.259682609

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.259683	85	0.0755585	80
-0.129317	11	0.1032421	1
-0.122587	82	0.1123174	87

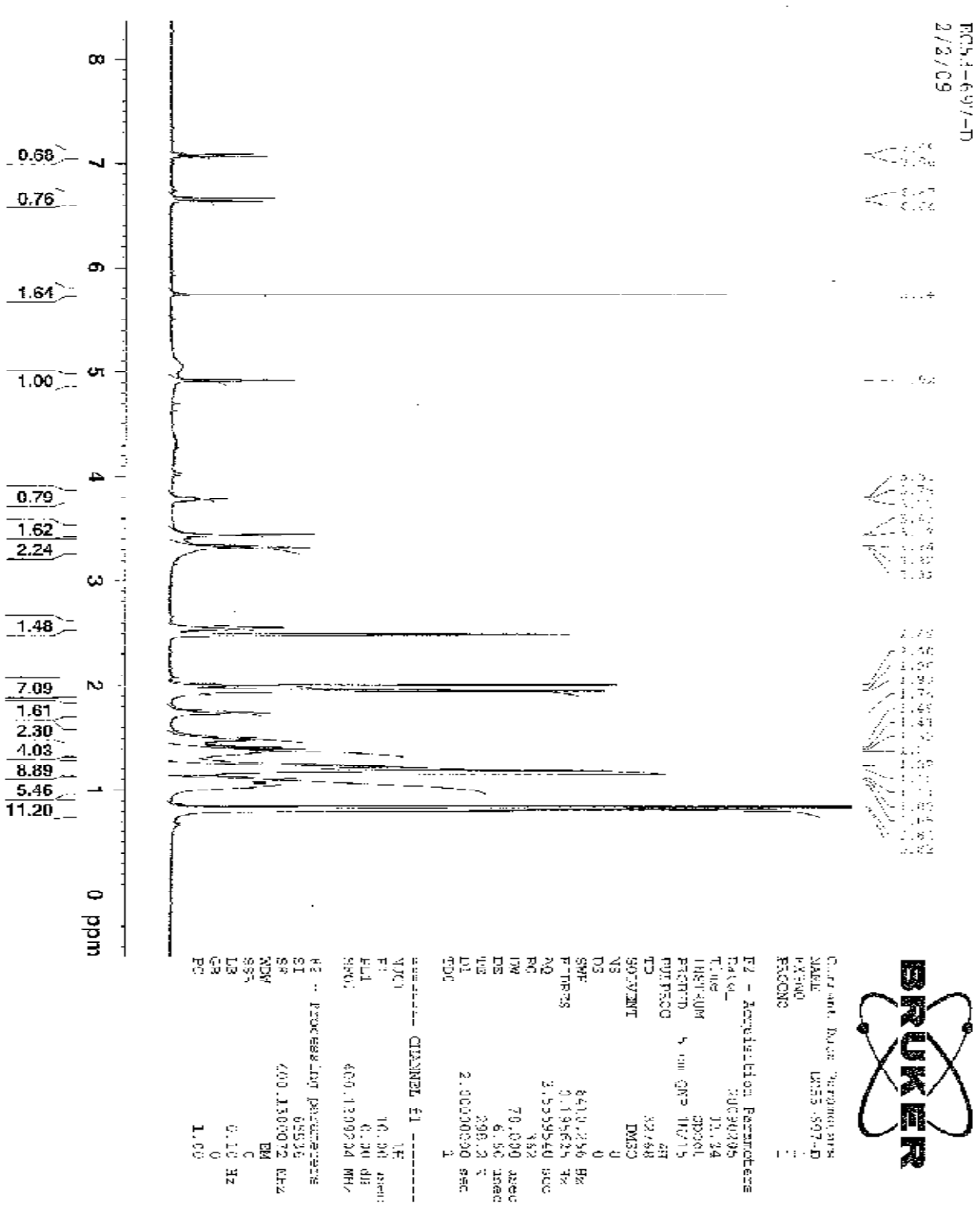
Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.112864	14	0.1301359	13
-0.112225	5	0.1593174	86



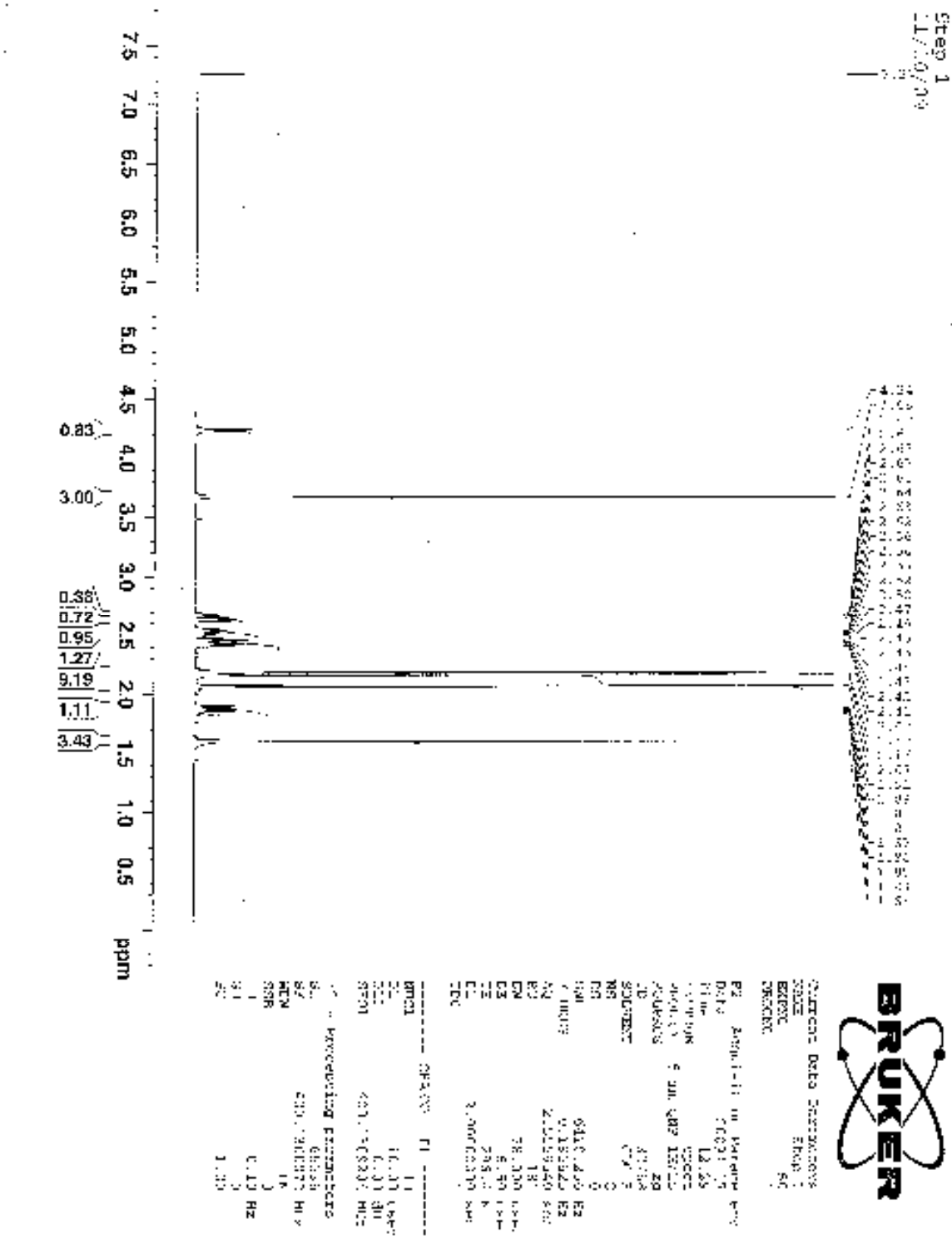


APPENDIX C: H-NMR

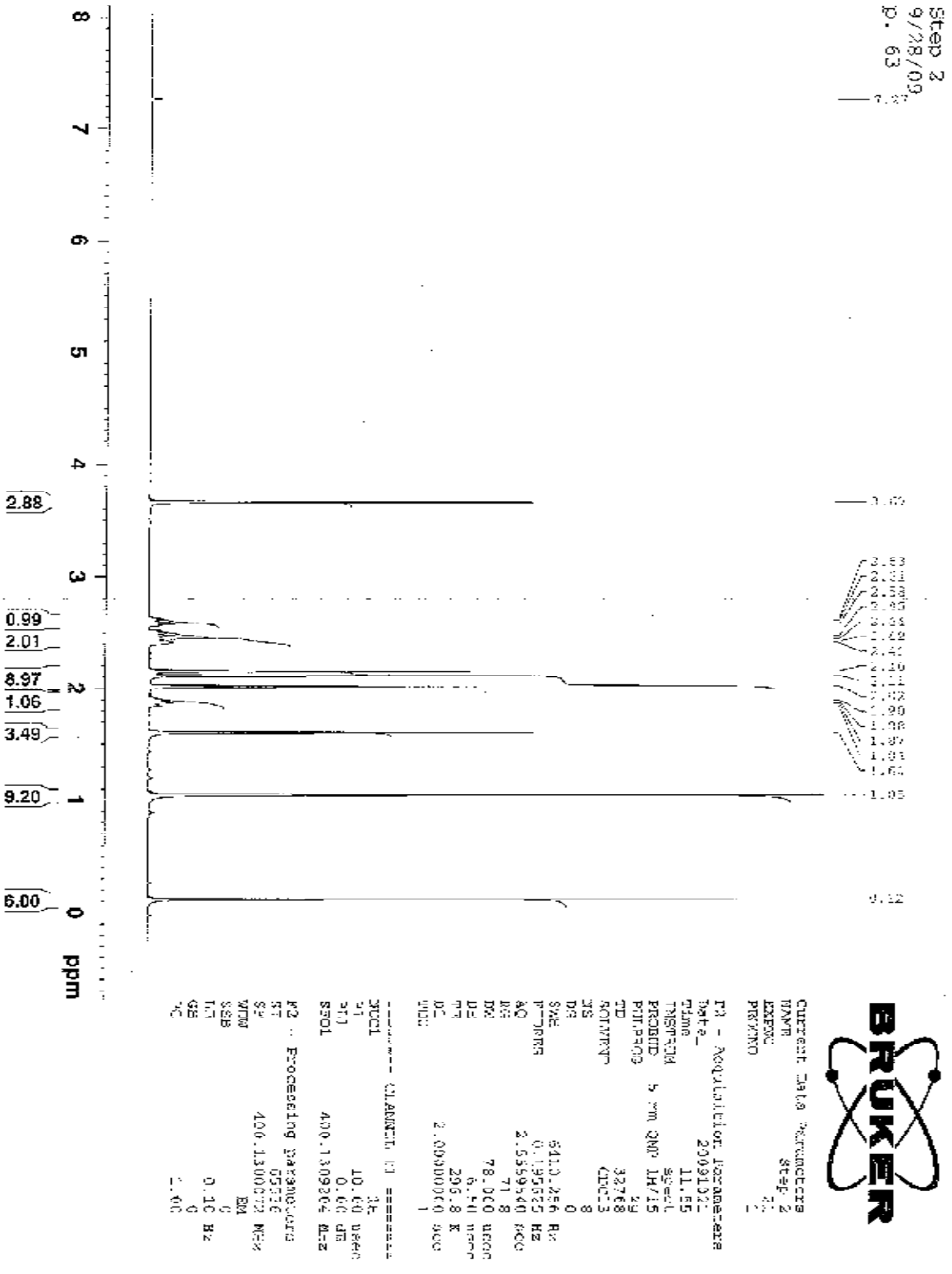
Surfactant EC H-NMR



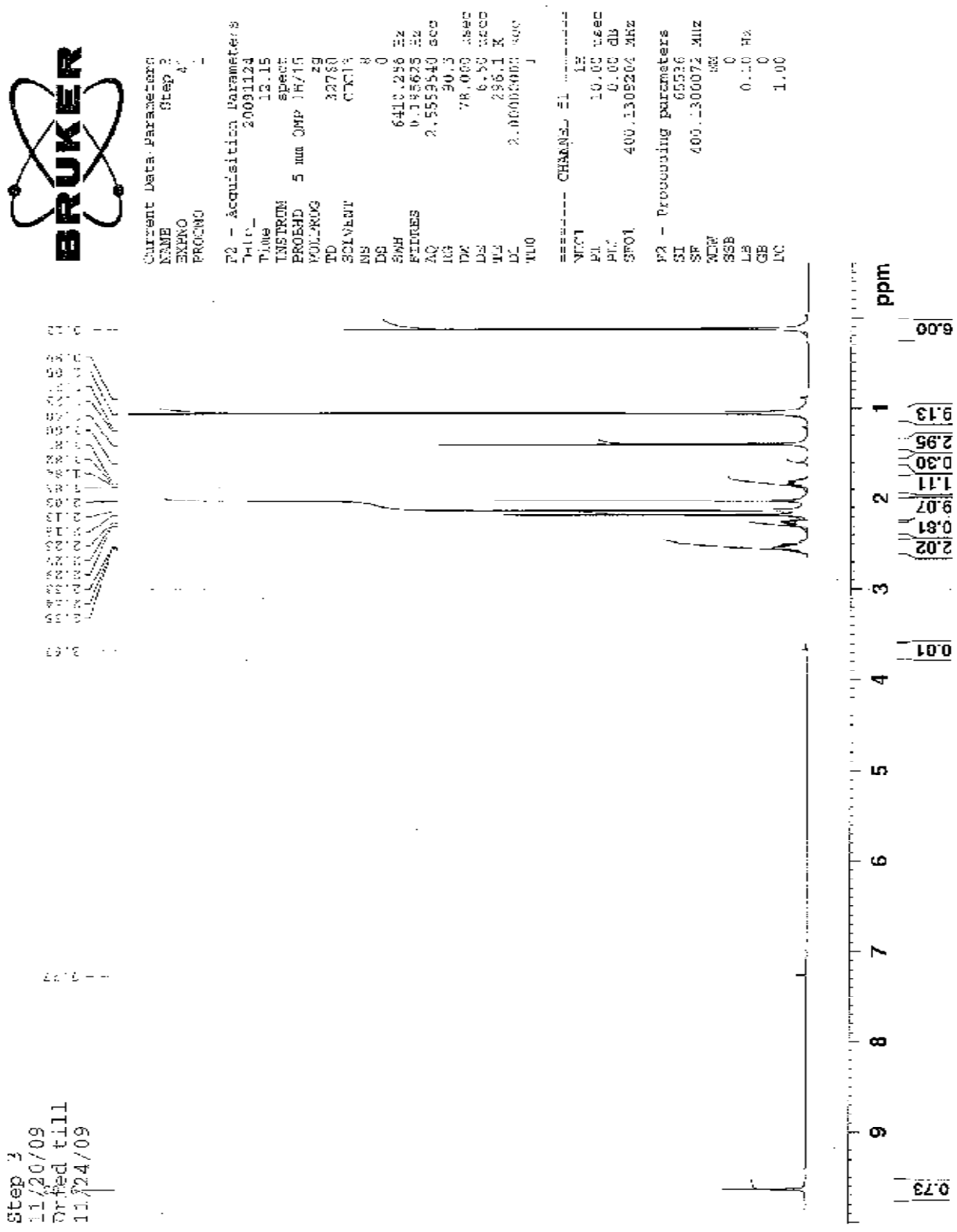
Step 1 H-NMR


Step 1
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
0.83
3.00
0.38
0.72
0.95
1.27
9.19
1.11
3.43
BRUKER

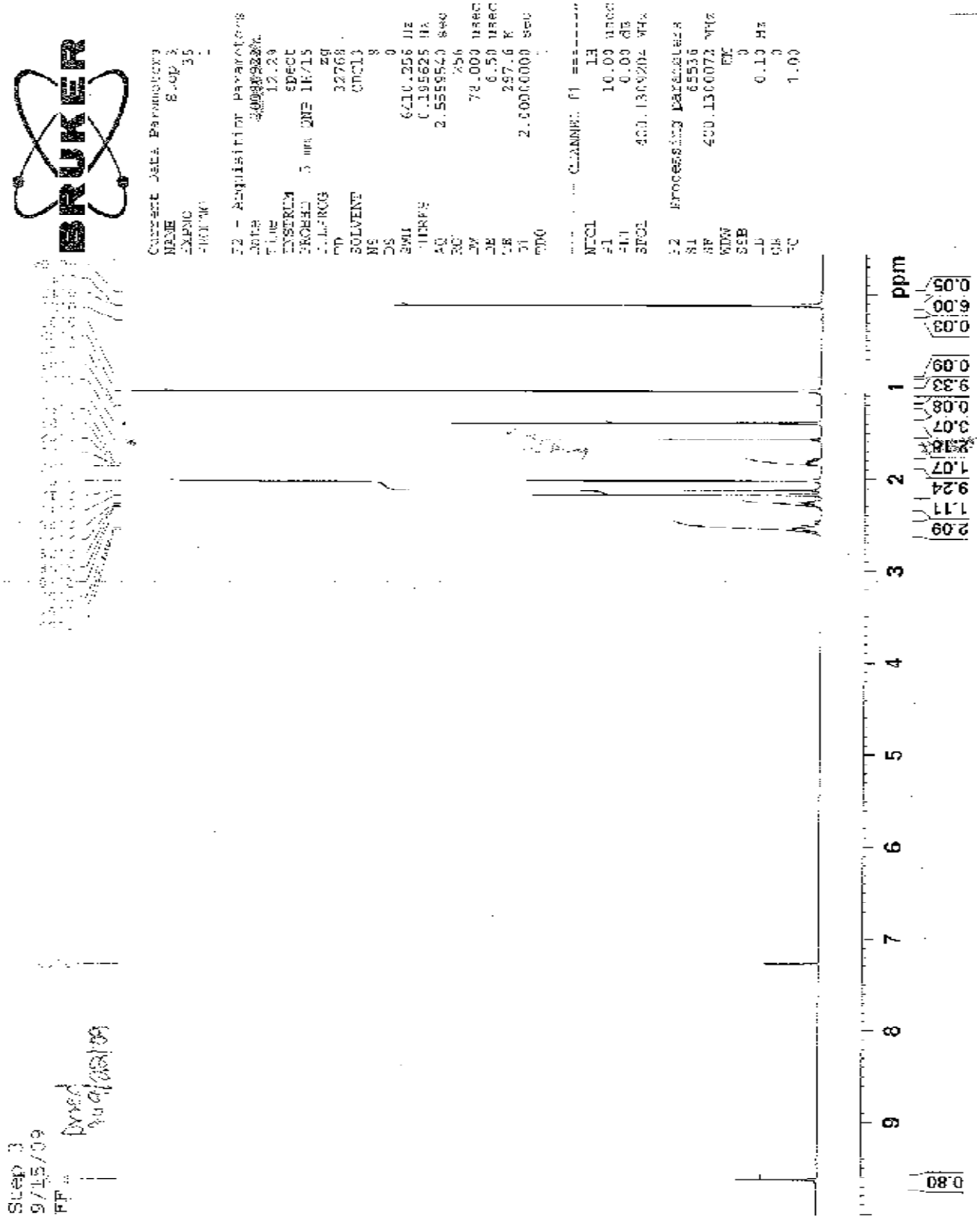
Step 2 HNMR



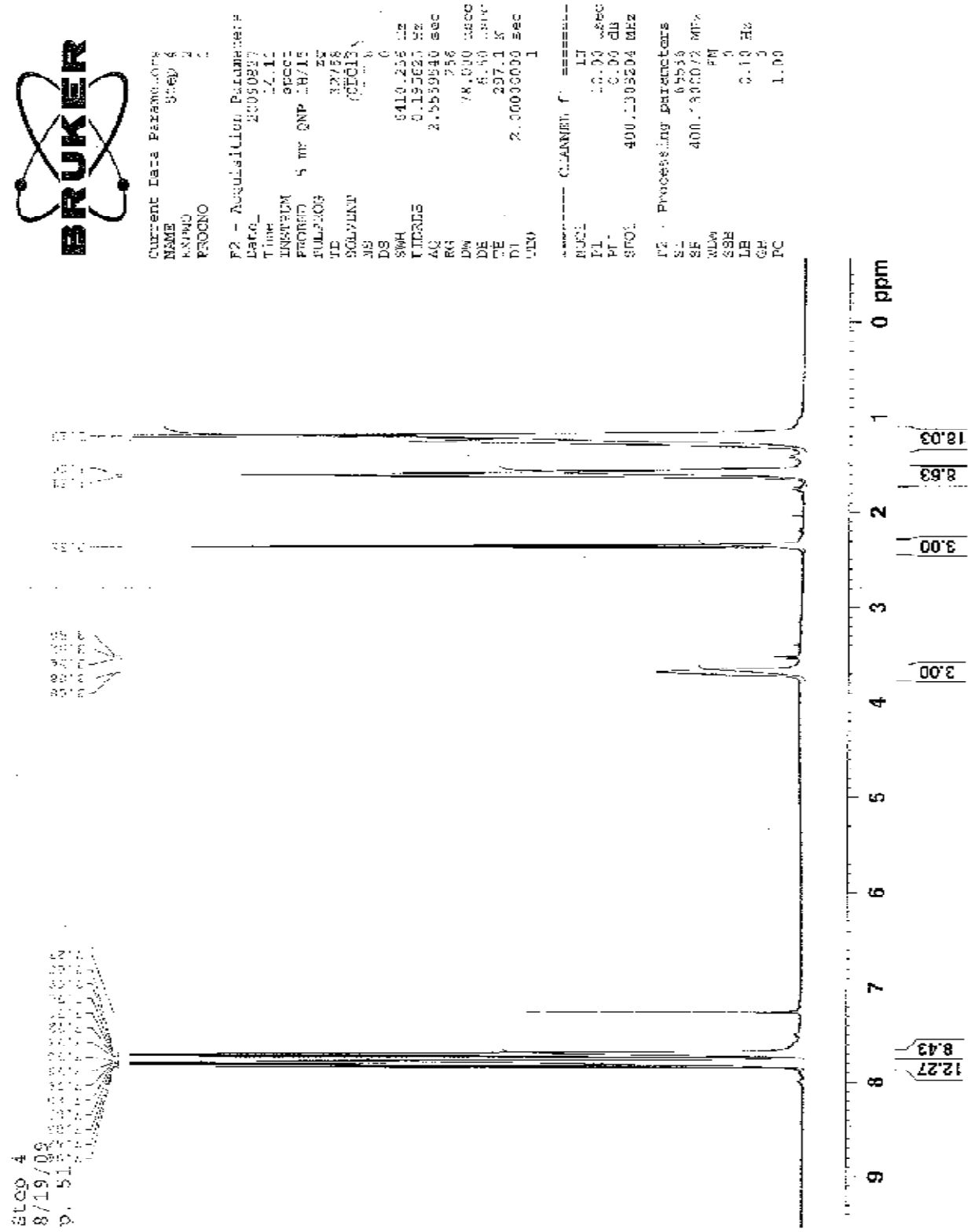
Step 3 H-NMR



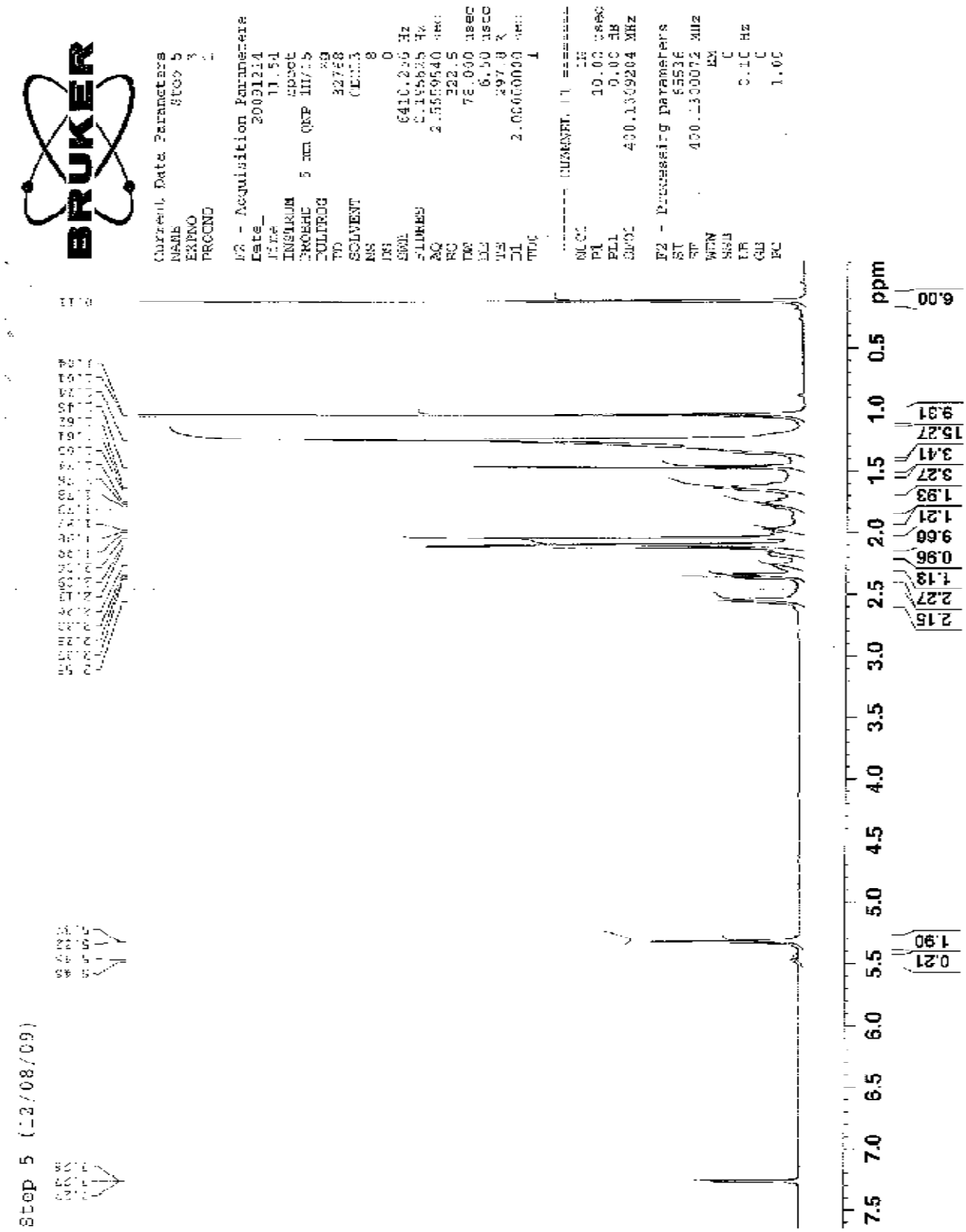
Step 3 H-NMR (sample with residual amount of water)



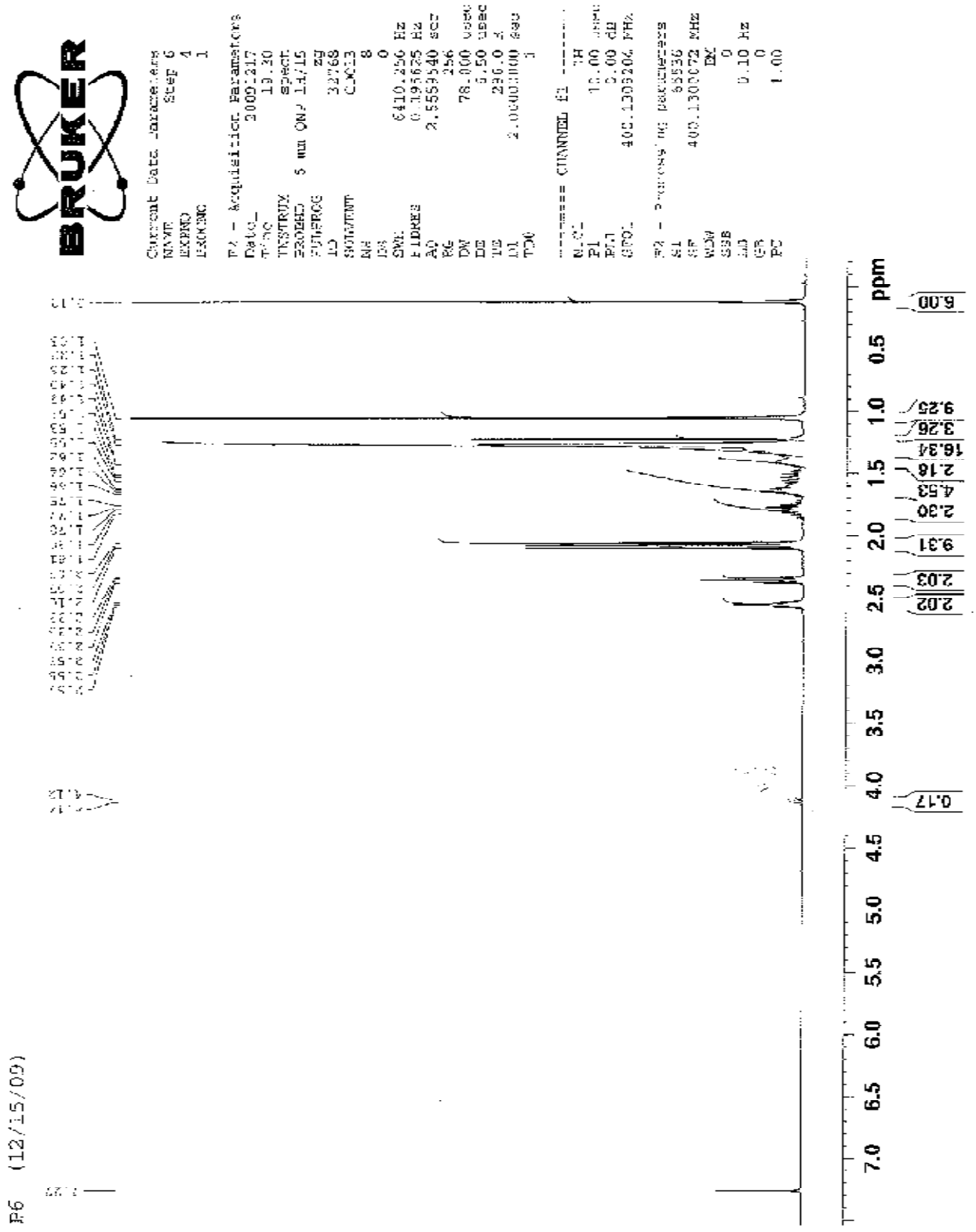
Step 4 H-NMR



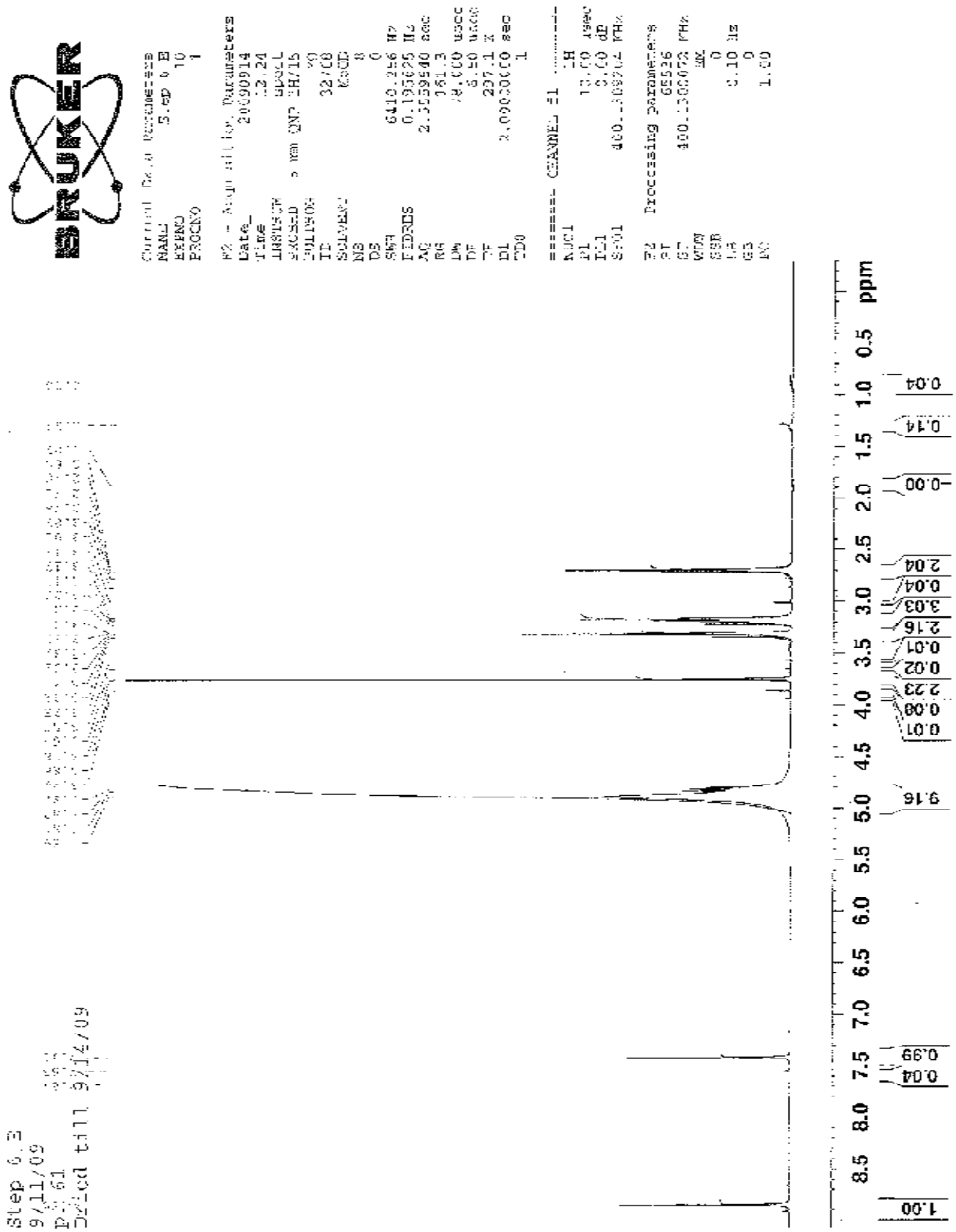
Step 5 H-NMR


Step 5 (12/08/09)
ppm
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
6.00
9.31
15.27
3.41
3.27
1.93
1.21
9.66
0.96
1.18
2.27
2.15
0.21
1.90

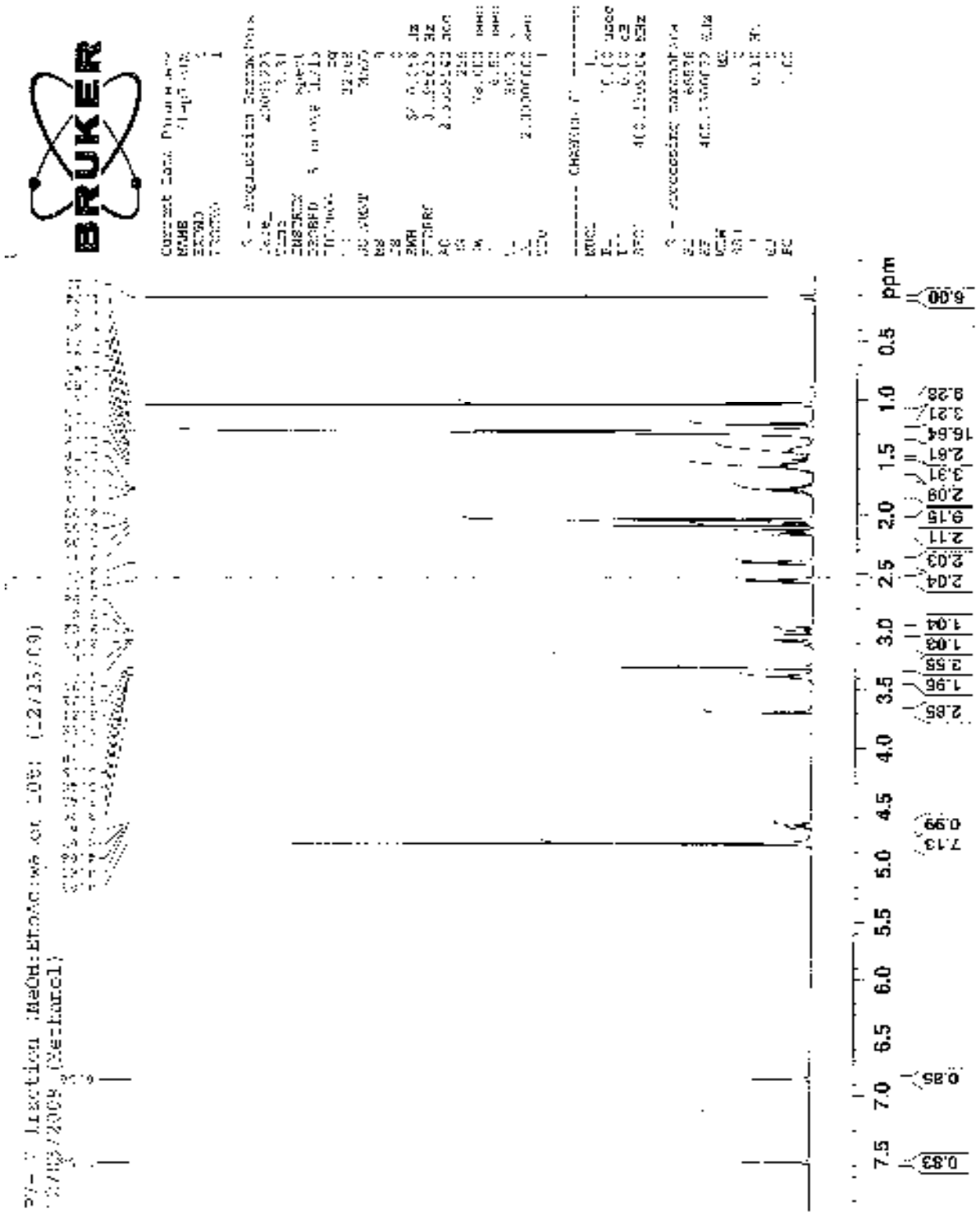
Step 6 H-NMR


BRUKER
P6 (12/15/09)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
6.00
9.25
3.26
16.34
2.16
4.53
2.30
9.31
2.03
2.02
0.17

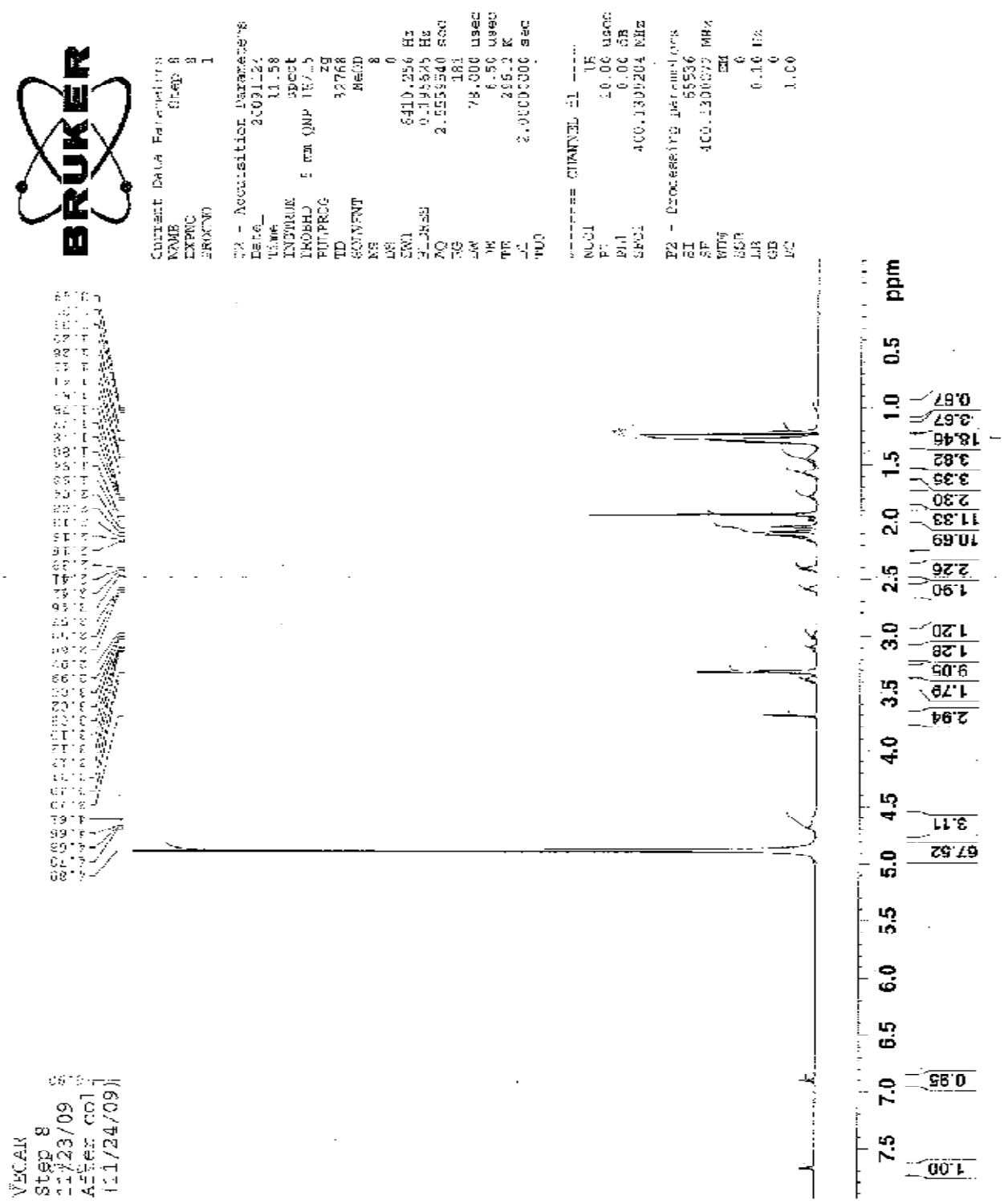
Step 6E H-NMR



Step 7 H-NMR



Step 8 H-NMR


BRUKER
ppm
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1.00
0.85
67.62
3.11
2.94
1.79
9.05
1.28
1.20
1.90
2.26
10.69
11.33
2.30
3.35
3.82
18.46
3.67
0.87

VITA

Carlos Astete graduated from Catholic of Valparaiso University in 1993, Chile. He received his bachelor's degree in biochemical engineering. He worked for three years in Watt's Foods as a research engineer. The position was focused on the impact of industrial contaminants on the environment, with emphasis on meeting national environmental regulations. The development of new products and improvement of old ones was another important research area as well. Next, Carlos Astete worked for three years in DAF S.A., a company oriented toward project development and informatics, as project manager of new accounts. In this position, Carlos Astete developed new interactions with the market by implementation of interactive platforms. In 2000, Carlos Astete received a degree of Master in Business and Administration from Adolfo Ibanez University, Chile. From 2000 to 2002, Carlos Astete continued working for DAF S.A., but in a regional manager position. The interaction and relationship with customers led by Carlos Astete was critical in enhancing the development of web-based technical support, and intranets. In 2003, Carlos Astete joined the Biological & Agricultural Department (BAE), Louisiana State University (LSU), as a graduate student. In fall 2005, Carlos Astete received the degree of Master in Science of Biological and Agricultural Engineering from Louisiana State University. In spring 2006, Carlos Astete was accepted in the Department of Biological & Agricultural Engineering to continue his studies towards a doctoral degree in engineering science. Since, Carlos Astete was an active member of the Gamma Sigma Delta, Gamma Beta Phi honor societies, and the National Society of Collegiate Scholars. Carlos Astete was awarded the Honored Student Award from American oil Chemist Society (AOCS) in 2010 and he was recognized by Gamma Sigma Delta as outstanding doctoral student in 2008. Carlos Astete already published 6 papers in important journals, a book chapter, a patent, and participated in several conferences related with polymeric nanoparticles. Mr. Carlos Astete will be awarded with the degree of Doctor of Philosophy in engineering science with minors in statistics and biological and agricultural engineering.