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ALPHA-TOCOPHEROL: EXTRACTION FROM RICE BRAN BY MICROWAVE-ASSISTED METHOD, AND ENTRAPMENT AND RELEASE FROM POLYMERIC NANOPARTICLES

A Thesis
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
Master of Science in
Biological and Agricultural Engineering

in

The Department of Biological and Agricultural Engineering

by
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December 2006

To whom made this possible, specially

My husband, Lucian Zigoneanu
and
My parents, Rodica and Csaba Papp

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ABSTRACT

The purpose of this study was two-fold, 1) to extract and quantify vitamin E components from rice bran using microwave extraction, to determine the antioxidant activity of the rice bran oil, and the effect of solvent and temperature on vitamin E components and oil yield, and 2) to entrap alpha-tocopherol into polymeric nanoparticles, to characterize the nanoparticles in terms of morphology, size and size distribution, zeta potential, entrapment efficiency, and amount of residual PVA associated with the nanoparticles, as well as to study the release of alpha-tocopherol from PLGA nanoparticles.

Microwave-assisted extraction was an efficient method for the extraction of oil and vitamin E components from rice bran. Hexane was a better solvent for rice bran oil extraction as compared to isopropanol at 40ºC. At higher temperature, isopropanol was a better solvent for oil extraction. Hexane extracted large amount of α-tocotrienol at 120ºC while the increase in temperature for isopropanol was more beneficial for the extraction of γ-tocopherol. No significant differences in the oil yield, total vitamin E, and antioxidant activity of rice bran oil was noticed between the conventional solvent and microwave-assisted extractions, at 40ºC.

For the second part of the study, emulsion evaporation method was used to synthesize spherical PLGA(αT) nanoparticles with SDS and PVA as surfactants. For SDS nanoparticles, the size of the nanoparticles decreased significantly with the entrapment of α-tocopherol in the PLGA matrix, while the size of PVA nanoparticles remained unchanged. The PDI after synthesis was under 0.100 for PVA nanoparticles and around 0.150 for SDS nanoparticles. Zeta potential was negative for all PVA nanoparticles. 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. The residual PVA associated to the nanoparticles after purification was approximately 6% (w/w relative to the nanoparticles). The release profile showed an initial burst followed by a slower release of the α-tocopherol entrapped inside the PLGA matrix. The release for nanoparticles with 8% α-tocopherol theoretical loading (86% released/first hour) was faster than the release for the nanoparticles with 16% α-tocopherol theoretical loading (34% released/first hour).

CHAPTER 1. INTRODUCTION

Many studies demonstrate the role of vitamin E vitamers in prevention of some chronic, age-related diseases such as cardiovascular diseases, atherosclerosis, cancer, arthritis, Alzheimer's, and Parkinson's (Brigelius-Flohe and Traber, 1999; Shui and Leong, 2005; Lloyd et al., 2000; Bramley et al., 2000, Wayner et al., 1987; Wang et al., 1996). These studies correlated the vitamin E activity with its function as a chain-breaking antioxidant that stops the free radical reactions (Brigelius-Flohe and Traber, 1999). The role of vitamers and, especially of α-tocopherol as an antioxidant is based on the high stability of α-tocopheroxyl radical, which is formed by loosing the phenolic hydrogen. The newly formed radical can bind another radical resulting in a non-radical product, or it can reconvert to α-tocopherol. Alpha-tocopheroxyl radical is quite stable because of the unpaired electron of the atom of oxygen which is delocalized in the aromatic ring (Bramley et al., 2000). The analytical methods used for detection of alpha-tocopherol are: spectrophotometry, fluorometry, electrochemistry, and light-scattering (Bramley et al., 2000).

Sources of vitamin E are wheat-germ oil, sunflower seed, almond, cereals, and others. Rice bran represents one of the rich sources of vitamin E components. Rice bran oil contains about 0.1-0.14% vitamin E components, concentrations that can vary substantially according to the origin of the rice bran (Diack and Saska, 1994; Lloyd et al., 2000; Hu et al., 1996). Several methods have been developed to extract vitamin E components from rice bran, namely supercritical fluid extraction, solvent extraction, and Soxhlet extraction. Microwave extraction is a newly developed extraction method which overcomes some drawbacks associated with the conventional extraction methods. Microwave-assisted extraction can be completed in minutes, polar or non-polar solvents can be used, a precise software-based control of all parameters of the extraction can be achieved, a higher analyte recovery can be obtained compared to other methods, and low amounts of solvent are needed (Ondruschka and Asghari, 2006; Eskilsson and Bjorklund, 2000).

Once extracted and purified, vitamin E vitamers can be delivered in several forms, free form, as a derivative (i.e. tocopheryl acetate), as an emulsion, or in nanoencapsulated form. Delivery of vitamins entrapped in polymeric nanoparticles has definite advantages over the delivery of non-entrapped vitamins. The release rate of the vitamins can be controlled and

consequently the dose frequency can be reduced. Furthermore, the bioactivity and stability of the active substance entrapped in the nanoparticle is protected (Lamprecht et al., 2001; Niwa et al., 1993; Uhrich et al., 1999; Mu and Feng, 2003). Alpha-tocopherol is sensitive mostly to oxygen, light, and temperature, and therefore the entrapment of alpha-tocopherol in a polymeric matrix may protect it from these environmental factors. Experimental results show that the half-life of compounds incorporated in nanoparticles is much longer than the half-life of non-encapsulated drugs. Another advantage of entrapment is that drugs are delivered more slowly and at a more constant rate, avoiding the high hepatotoxicity of the drug (Mainardes et al., 2005; Redhead et al., 2001). Also, drug bioavailability increases when the drugs are entrapped in nanoparticles (Lee et al., 2002), factor which is critical for lipophylic components such as vitamin E.

The goal of the present research was to extract vitamin E components from rice bran and to entrap the most biological active vitamin E vitamer, alpha-tocopherol, into a PLGA matrix in order to obtain small size nanoparticles. The thesis is divided into two main sections. Section one focuses on microwave-assisted and conventional solvent extraction of vitamin E components from rice bran oil; quantification of these components as well as determination of antioxidant activity of rice bran oil constitutes one of the main objectives of this research. The second section is oriented toward the entrapment of the most active vitamin E vitamers (alpha-tocopherol) into polymeric nanoparticles; characterization of the synthesized nanoparticles in terms of morphology, size and size distribution, zeta potential, entrapment efficiency, as well as the study of the sustained release of alpha-tocopherol from PLGA nanoparticles also make the objectives of this section.

The objectives of this research were:

Objective 1. Extraction of vitamin E components from rice bran using microwave extraction

a) Extraction of rice bran oil from rice bran by microwave-assisted method

b) Quantification of tocopherols and tocotrienols in the extracted oil

c) Determination of antioxidant activity of the rice bran oil

d) Study of the effect of solvent and temperature on alpha-tocopherol degradation.

Objective 2. Entrapment and release of alpha-tocopherol from polymeric nanoparticles

a) Synthesis of poly (D,L-lactide-co-glycolide) nanoparticles with entrapped α-tocopherol by emulsion evaporation method, with PVA and SDS as surfactants

b) Characterization of PLGA(αT) nanoparticles in terms of morphology (TEM), size, size distribution, and zeta potential (DLS), and entrapment efficiency (HPLC)

c) Study of the α-tocopherol release from PLGA(αT) nanoparticles.

1.1. Extraction of Vitamin E Components

Extraction of rice bran oil by microwave assisted extraction, as well as determination of its antioxidant activity and quantification of vitamin E components is included in Chapter 2. Many studies reported the use of microwave-assisted method for the extraction of antioxidants but only a few reported the use of microwave energy for the extraction of rice bran components (Duvernay et al., 2005). In this study, conventional solvent and microwave-assisted extractions were used as methods for the extraction of rice bran oil and rice bran components. The extractions temperatures were 40, 60, 80, 100, and 120ºC based on the similar conditions studied by Duvernay et al. (2005). For conventional solvent extraction 40ºC was chosen as the extraction temperature in order to compare the results with those obtained by microwave-assisted extraction. A polar solvent, isopropanol, and a non-polar solvent, hexane, were used for the extraction of rice bran oil. In this study, four vitamin E components, α- and γ- tocopherol and α- and γ- tocotrienol in the rice bran oil were quantified by normal-phase HPLC. The antioxidant activity of rice bran oil was tested by using DPPH radical scavenging capability. The α-tocopherol degradation study was performed in the same conditions as microwave-assisted extraction of rice bran oil.

1.2. Alpha-tocopherol Encapsulation and Release from PLGA Nanoparticles

The study on alpha-tocopherol encapsulation and release from PLGA nanoparticles is presented in Chapter 3. Emulsion-solvent evaporation was chosen as a method for the entrapment of alpha-tocopherol in the PLGA matrix. Many studies reported the use of emulsion evaporation method for nanoparticle preparation and entrapment of various drugs (Mainardes et al., 2005; Li et al., 2001; Mu and Feng, 2002; Muller,1990; Cascone et al., 2002; Birnbaum and Brannon-Peppas, 2003, Astete and Sabliov, 2005). The parameters which affect the size of the nanoparticles, and implicitly the release of the active component from the polymeric matrix are

presented in this section of the thesis. The parameters for synthesis of PLGA nanoparticles with entrapped α-tocopherol were selected based on the literature. Dichloromethane and ethyl acetate, in which both the polymer and the drug are soluble, were used as solvents for the preparation of the organic phase. The aqueous to organic ratio was chosen as 10:1, and Poly(DL-Lactide-co-Glycolide) (PLGA) was selected because of its bio-compatibility properties. PLGA 50:50 with a molecular weight of 45,000-75,000 was used as the matrix for drug entrapment. Another advantage of PLGA, besides the fact that it is degradable to non-toxic compounds, is that by changing the ratio of the monomers in the co-polymer the release rate of the drug can be controlled (Yoo, 2000). Hydrophilic and hydrophobic drugs can be entrapped by emulsion evaporation method (Mainardes et al., 2005, Redhead et al., 2001) with a higher entrapment efficiency for hydrophobic drugs than for hydrophilic components (Birnbaum and Brannon-Peppas, 2003). Alpha-tocopherol, a hydrophobic drug, was entrapped into the PLGA matrix using the single emulsion evaporation technique. A solution of 0.3% PVA (30,000-70,000 Da) and a solution of 2 mg/ml SDS was used as a surfactant in the aqueous phase. Sonication time was selected at 2 minutes in pulse mode with 38% amplitude at 750 W. Trehalose was added as a cryoprotectant to the particles before lyophilization.

The morphology of the nanoparticles was studied by Transmission Electron Microscopy (TEM). The size, size distribution, and zeta potential were measured by dynamic light scattering (DLS). The entrapment efficiency and release of alpha-tocopherol were studied by RP-HPLC.

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CHAPTER 2. DETERMINATION OF ANTIOXIDANT COMPONENTS IN RICE BRAN OIL EXTRACTED BY MICROWAVE-ASSISTED METHOD

2.1. Introduction

2.1.1. Rice Composition

Rice is a good source of calories provided by its high content of starch and high nutritional quality proteins; it is hypoallergenic and easily digested (Mazza, 1998). Besides the nutritional quality of rice, its antioxidant properties are also exceptional. The rice bran oil antioxidants are very efficient in reducing low density lipoprotein and total serum cholesterol (Hu et al., 1996; Mazza, 1998; Sugano and Tsuji, 1997; Kim, 2005; Rukmini and Raghuram, 1991). Almost all the oil of the rice grain is located in the bran and germ (Kim et al., 1999).

Clean rice bran contains 20-22% oil by weight (Mazza, 1998) but dilution of the rice bran during the milling process with other components such as hull and starch diminish the oil content to 15-20% (Shen et al., 1996; Mazza, 1998). Approximately 95-98% of the oil is extractable (Amarasinche and Gangodavilage, 2004). Rice bran oil contains a saponifiable fraction formed from saturated and unsaturated fatty acids and an unsaponifiable fraction containing tocopherols, γ-oryzanol, and squalene (Rukmini and Raghuram, 1991). The amount of rice components varies as a function of rice type, climatic conditions, storage conditions, rice bran stabilization, and processing methods (Amarasinche and Gangodavilage, 2004; Mazza, 1998), but it typically contains 88-89% neutral lipids, 3-4% waxes, 2-4% free fatty acids and approximately 4% unsaponifiables (Kim et al., 1999). Rice bran oil contains about 0.1-0.14% vitamin E components and 0.9-2.9% oryzanol; the concentrations can vary substantially according to the origin of the rice bran (Diack and Saska, 1994; Lloyd et al., 2000; Hu et al., 1996).

Vitamin E is a generic term for a group of four tocopherols (α-,β-,γ- and δ-) and four tocotrienols (α-,β-,γ- and δ-), of which α-tocopherol has the highest biological activity (Shin and Godber, 1993; Duvernay et al., 2005; Brigelius-Flohe and Traber, 1999; Bramley et al., 2000). These components are yellow, viscous liquids which are insoluble in water, but are readily soluble in organic solvents. A number of factors such as: oxygen, light, heat, alkali, trace minerals, and hydroperoxides can cause decomposition of vitamin E vitamers (Bramley, 2000). All components of vitamin E have an amphyphilic structure with a hydrophilic part (chromanol

ring) and a hydrophobic part (isoprenoid side chain) as shown in Figure 2.1. A number of studies showed that vitamin E functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions (Brigelius-Flohe and Traber, 1999; Becker et al., 2004; Huang et al., 2002). Because of its radical scavenging antioxidant properties, vitamin E inhibits lipid peroxidation in vitro and in vivo (Niki and Noguchi, 2004; Kim et al., 1999).

The other antioxidant found in rice bran oil, oryzanol is a mixture of esters of ferulic acid with sterols and triterpene alcohols and has similar antioxidant properties with vitamin E vitamers (Shin et al., 1997). Among the oryzanol components found in the rice bran cycloartenol, β-sitosterol, 24-methylene-cycloartanol, cyclobranol (cycloartenol), and campesterol (4-desmethysterols) are most prevalent (Lloyd et al., 2000). The oryzanol concentration in crude rice bran oil can reach 2% (v/v) (Lloyd et al., 2000).

2.1.2. Antioxidant Activity of Rice Bran Oil Components Found in Rice Bran Oil

It is believed that the implications of vitamin E vitamers and oryzanol in the prevention of some chronic age-related diseases such as cardiovascular diseases, atherosclerosis, cancer, arthritis, Alzheimer's, Parkinson's are related to their antioxidant function (Brigelius-Flohe and Traber, 1999; Shui and Leong, 2005; Lloyd et al., 2000; Bramley et al., 2000, Wayner et al., 1987; Wang et al., 1996).

Antioxidants can be classified as a function of their lipid or water solubility, as a function of their composition (proteins or small molecules) (Papas, 1996), or based on their function in plasma. In plasma, primary antioxidants can bind metal ions and as such they can reduce the initiation rate of lipid peroxidation; secondary antioxidants (i.e. α-tocopherol) act by reducing the chain propagation and amplification of lipid peroxidation (Ghiselli, 1995).

There are large numbers of oxidant capacity assays that use chemical chromogenic/fluorescent redox reactions to test antioxidant properties of different chemical components. Other methods are based on the properties of the biological systems; for example, the integrity of the cellular membrane is correlated with LDH (lactate dehydrogenase) leakage (Yu, 1999). Among the oxidative species used in vitro for testing the scavenging capacity of different antioxidants, hydrogen peroxide, peroxynitrite, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) or ABTS, and 1,1- diphenyl-2-picrylhydrazyl (DPPH) are most commonly used (Miller, 2001; Hoelzl et al., 2005; Miller et al., 1993). DPPH method was reported in the

literature as a suitable means to evaluate the antioxidant activity of different vegetable oils and it was selected as the method of choice in the present study to measure antioxidant activity of the rice bran oil (Espin et al., 2000; Parry et al., 2005; Oufnac et al., in press).

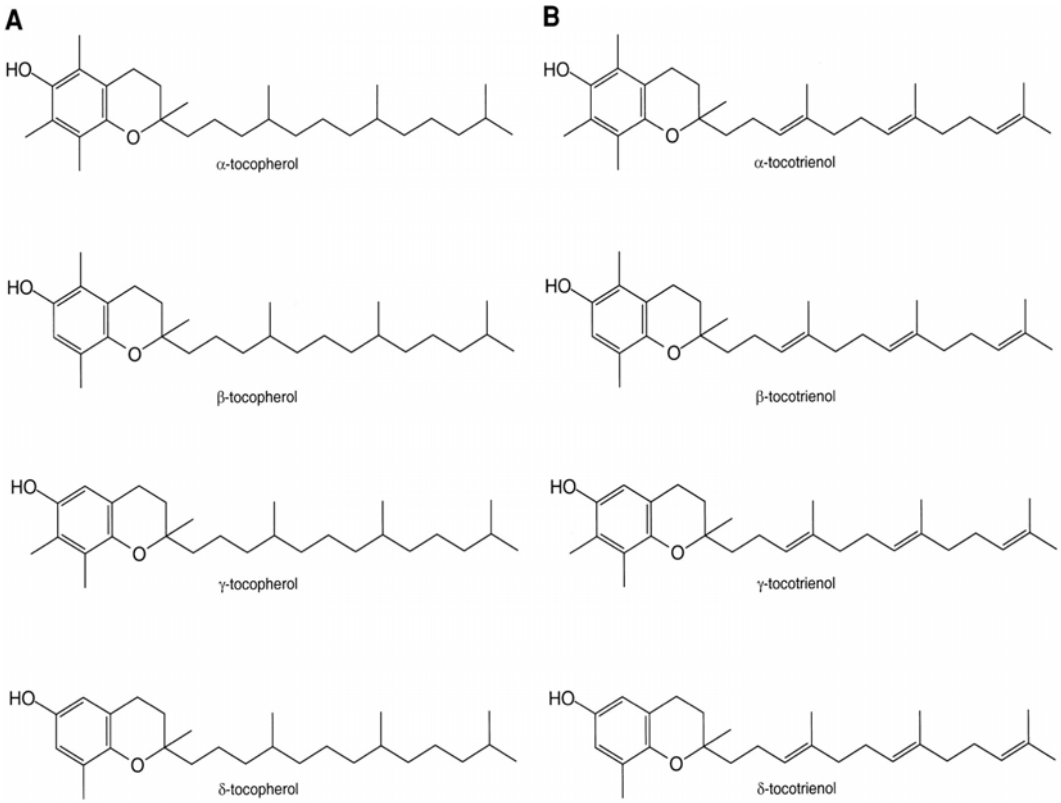

Figure 2.1. Chemical structure of vitamin E components (A-tocopherols, B-tocotrienols) (Shin and Godber, 1994)

2.1.3. Extraction Methods Used for Isolation of Rice Bran Oil Antioxidants

Various techniques used for extraction, isolation and purification of antioxidants from rice bran oil have been described in the literature (Shin, 1993, Xu, 1999, Rogers, 1993, Shen, 1997, Shin, 1994). It is commonly accepted that the avoidance of the oxidation of the compounds (Diack and Saska, 1994; Shin, 1997) is an important step during the process. For example, if rice bran is held at elevated temperature after extraction, the endogenous antioxidants can be degraded by temperature, especially during long term storage. Therefore, it is recommended that following extraction to work and to store the samples at the lowest possible temperature (Shin, 1997).

Rice bran is rapidly oxidized resulting in free fatty acids which affect the quality of rice bran oil (Amarasinche and Gangodavilage, 2004; Mazza, 1998). After milling, free fatty acids are formed at an initial rate of 5-7% by weight of oil per day as a result of lipase activity. Therefore, the stabilization of rice bran as soon as possible after milling is an important step in analyzing of rice bran oil components. Stabilization of rice bran can be achieved by controlling the lipase activity (Mazza, 1998). A widely used technique for bran stabilization is drying and steaming of bran, but other methods such as ohmic heating, refrigeration and chemical stabilization can also be used (Amarasinche and Gangodavilage, 2004; Lakkakula et al., 2004). Shin et al. (1997) studied the effect of storage conditions on retention of vitamin E vitamers and oryzanol. Raw rice bran was stored at ambient temperature and the variation of its components was monitored during one year period. In only 35 days the total vitamin E lost was 44% and the order of losses of vitamin E components and oryzanol was: $\alpha\text{-T} \geq \alpha\text{-T3} > \gamma\text{-T3} > \gamma\text{-T} \geq \delta\text{-T3} \geq \beta\text{-T} \geq \delta\text{-T} >$ oryzanol. Based on this order, α-tocopherol and α-tocotrienol proved to be the less stable among all vitamin E components during the first 35 days period. Mazza et al (2002) proved that tocopherols and tocotrienols were oxidized very slowly by atmospheric oxygen and in dark conditions. In a medium without oxygen tocopherols and tocotrienols could be heated up to 200ºC without showing any degradation.

Organic solvents, water, and supercritical carbon dioxide have been used for the extraction of rice bran oil (Amarasinche and Gangodavilage, 2004; Hu et al., 1996; Proctor and Bowen, 1996; Kuk and Dowd, 1998). The most common solvent used for extraction of edible oils is hexane (Bera, 2006; Mazza, 1998); hexane is the solvent of choice used to extract rice bran oil for commercial purposes (Hu, 1996). Isopropanol can be used as an alternative solvent when the hexane flammability is of concern (Proctor and Bowen, 1996). Bera et al. (2006) studied the effect of solvents on the extraction of some edible oils (flax seed oil and bahera oil) from seeds and found that solubility of the oils in isopropanol increased with temperature; at 30ºC the solubility of oils in isopropanol was 67% (w/w) and the two phases became miscible at 50ºC. For hexane, the oils and the solvent were miscible even at room temperature. The conclusion was that when polar solvents were used, because of the oil solubility change with temperature, the extraction yield was more significantly affected by temperature as compared to when non polar solvents were used.

The nature of the solvent is very important in extraction; polar short-chain alcohols seem to be a promising alternative to non-polar solvents for extraction of oil and antioxidants from rice bran (Hu et al., 1996; Proctor and Bowen, 1996). Alcohols extract more unsaponifiable components and phosphatides, but are poor solvents for triglycerides (Proctor and Bowen, 1996). Previous studies were reported on the solvent extraction of rice bran oil by using isopropanol to extract rice bran oil enriched in vitamins B and by using ethanol to obtain rice bran oil enriched in tocopherols and vitamins B (Hu et al., 1996). Hu et al. (1996) mentioned the use of isopropanol and hexane in the extraction of rice bran oil for the recovery of vitamin E and oryzanol components. Their studies demonstrated that the amount of crude oil, vitamin E, and oryzanol components increased with increasing the solvent-to-bran ratio from 2:1 to 3:1 and by rising the temperature from 40ºC to 60ºC. A study on the effect of the two solvents on the amount of oil, vitamin E and oryzanol showed that isopropanol extracted more vitamin E, less oil amount, and the same quantity of oryzanol as compared with hexane (Hu, 1996).

Proctor and Bowen (1996) studied the effect of hexane and isopropanol on the extraction of rice bran oil at room temperature. Oil yield extracted with hexane and isopropanol from 2 g rice bran was similar for both solvents and it was around 15% (Proctor and Bowen, 1996). Amarasinche and Gangodavilage (2004) studied the solvent extraction of rice bran oil using hexane for a number of rice bran varieties available in Sri Lanka. They found that for all types of bran, parboiling bran oil yield was higher compared with raw bran. The oxidative stability of rice bran oil under heat induced oxidation at 64ºC for 32 days was also studied by Proctor and Bowen (1996). The studies on oxidative stability of rice bran oil extracted with isopropanol and hexane showed that the oil extracted with isopropanol was more stable compared with the oil extracted with hexane. It was assumed that isopropanol extracted antioxidants which were not present in the oil extracted with hexane and those antioxidants protected the oil against oxidation. Since the level of phospholipids was the same in both type of oils, the difference in the antioxidant stability was attributed to tocopherols assumed to be found in higher concentration in the oil extracted with isopropanol than in the oil extracted with hexane (Proctor and Bowen, 1996).

Many studies were published in the literature related to the effect of various solvents on the extraction of rice bran oil using traditional heating methods (Hu et al., 1996; Proctor and Bowen, 1996). Microwave assisted solvent extraction is a relatively new extraction method that can be alternatively used for extraction of oils. The system is specially designed to operate at

elevated temperatures and pressures monitored by a fiber optic temperature probe and a pressure control side arm, respectively (Carro et al, 2000). The number of parameters needed for optimization of microwave extraction is lower and easier to set up as compared with supercritical fluid extraction. The disadvantages of supercritical fluid extraction and accelerated solvent extraction are higher cost of the equipments and the blockages in the systems as a result of the presence of water in the sample (Camel, 2000). The parameters usually studied for the optimization of microwave-assisted extraction are pressure or temperature, extraction time, microwave power, solvent composition, solvent volume, and matrix characteristic including water content (Camel, 2000; Eskilsson and Bjorklund, 2000; Bum, 2000). The selection of the solvent is an important step for obtaining optimal extraction results. The best solvent for microwave-assisted extraction should present a high selectivity to the analyte, good solvent-matrix interaction, and high microwave-absorbing properties (Eskilsson and Bjorklund, 2000). The highest dielectric constant of the solvent provides high thermal energy and consequently rapid heating. An example of solvent used for microwave-assisted extraction with low dielectric constant which does not absorb microwave energy, and therefore it does not heat is hexane (Eskilsson and Bjorklund, 2000). A low dielectric constant solvent such as hexane is used as a way to prevent the degradation of thermolabile components (Camel, 2000; Ondruschka and Asghari, 2006); in this case only the sample matrix is heated and the solute is released into the cold solvent.

In microwave-assisted extraction, the analyte migrates out from the matrix through the solvent while in classical solvent extractions the solvent diffuses into the sample matrix and solubilizes the analyte (Camel, 2000). The benefits of the microwave-assisted solvent extraction consist in that it can be completed in minutes, polar or non-polar solvents can be used, a precise software-based control of all parameters of the extraction can be achieved, a higher analyte recovery can be obtained compared to other methods, and low amounts of solvent are needed. The microwave-assisted method has further advantages including the fact that it is reproducible, the solvent can be heated over its boiling point resulting in the increase of extraction efficiency and speed, and simultaneous analysis of samples can be performed (Ondruschka and Asghari, 2006; Eskilsson and Bjorklund, 2000).

Microwave-assisted extraction was reported to be useful in extracting persistant organic pollutants, pesticides, phenols, metals, polymers, and pharmaceuticals and natural products

(Eskilsson and Bjorklund, 2000). Comparable efficiencies and reproducibility relative to conventional extraction methods were obtained in all reported studies. Microwave-assisted extraction of tanshinones from the roots of S. miltiorrhiza bunge (Pan et al., 2002) and of flavanoids from cultured cells of Saussurea medusa maxim (Gao and Liu, 2005) proved to be the most efficient technique as compared to the extraction of these compounds by heat reflux extraction, ultrasonic extraction, Soxhlet extraction, and extraction at room temperature (Pan et al., 2002). Better extraction efficiency in a short time of tea polyphenols and tea caffeine was obtained by microwave-assisted extraction as compared to ultrasonic, heat reflux and extraction at room temperature (Pan et al., 2003).

Duvernay et al. (2005) found that microwave extraction of rice bran oil was comparable to solvent extraction and suggested that a ratio solvent-to-bran of 3:1 and high temperatures increased the amount of oil and the quantity of antioxidants extracted with isopropanol from rice bran by using microwave extraction. A temperature increase from 80 to 110ºC produced an oil yield increase of 18% and a total vitamin E increase of 33%.

2.2. Objectives

The objectives of this study were: 1. to compare isopropanol and hexane as extraction solvents, by both microwave-assisted and conventional solvent methods; 2. to evaluate the oil yield and vitamin E yield as a function of extraction temperature; 3. to measure the effect of isopropanol and hexane as well as the effect of temperature on the antioxidant activity of the rice bran oil extracted; 4. to study the temperature and microwave effect on the degradation of alpha-tocopherol.

2.3. Materials and Methods

2.3.1. Chemicals and Materials

The rice bran used in the extraction studies was obtained from Cypress rice provided by Rice Research Station (Crowley, LA). HPLC grade methanol, hexane, and ethyl acetate were ordered from EMD chemicals (EMD chemicals Inc., Gibbstown, NJ), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was acquired from ScienceLab.com (ScienceLab.com, Inc., Houston, TX). Alpha-tocopherol, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were

purchased from Sigma-Aldrich (St. Louis, MO), isopropanol from Mallinckrodt Chemicals (Mallinckrodt Baker, Inc., Phillipsburg, NJ), and acetic acid from Fisher Scientific (Fisher Scientific, Springfield, NJ).

2.3.2. Rice Bran Preparation

Rice was milled using a Satake pilot scale rice mill (Satake Engineering, Co., Tokyo, Japan) located within the Biological and Agriculture Department at Louisiana State University; the mill was equipped with two operational units: shelling unit (Model GPS300A, Satake Engineering, Co., Tokyo, Japan) and whitening unit (Model VAF10AM, Satake Engineering, Co., Tokyo, Japan). After milling, rice bran was kept in the freezer at - 20ºC.

2.3.3. Microwave Assisted Solvent Extraction

In order to obtain crude rice bran oil the following procedure was pursued. Samples of 20 g of rice bran were weighed in each of the three pressure controlled Teflon vessels of the Ethos E Microwave Extraction System (Milestone, Inc. Monroe, CT). The Teflon vessels are designed to resist elevated pressures produced at temperatures higher than the boiling point of the solvent. As solvents for the extraction, a polar solvent (isopropanol) and a non-polar solvent (hexane) were used. The ratio of solvent to rice bran was 3:1 w/w. A magnetic stirring rod was added in each vessel. The three vessels were sealed and placed in the Microwave Extraction System. The Microwave Extraction System was set to operate in two steps. First, temperature gradually increased to the set extraction temperature for 5 min, using an energy level of 800 W maximum. At the end of the 5 minutes period the extraction temperature was reached. The second step was designed to hold the samples for a total period of 15 min at the extraction temperature by using an energy level of 500 W maximum. Five extraction temperatures, 40, 60, 80, 100, and 120ºC, respectively, were used for microwave assisted extraction. The temperature monitoring of the sample inside the vessels was accomplished by a fiber optic probe hooked up to the control system. After a ventilation period of 20 min in which the samples were cooled down, the vessels were unsealed and each sample was transferred to the filtering device. A vacuum pump (Thomas Compressors and Vacuum Pumps, Skokie, IL) was used to filter the solvent and oil mixture through a Whatman filter paper Φ=47 mm. After filtration, the mixture of the oil extracted and solvent was measured in a graduated cylinder and the volume recovered for each sample was recorded. Two milliliters of each sample were placed in a screw cap test tube previously

weighed. Two test tubes containing 2 ml mixture were prepared for each sample. These tubes were placed in a vacuum centrifuge evaporator (CentriVap Console Labconco, Kansas City, Missouri) and they were kept overnight in order to evaporate the solvent. The residual oil for each sample was weighed in order to determine the extraction yield. One of the test tubes for each sample was used immediately after evaporation to determine the vitamin E vitamers by HPLC. The other test tube was flashed with nitrogen in order to keep an inert atmosphere and to avoid possible degradation of the oil antioxidants and it was placed in the freezer under dark conditions for further antioxidant analysis.

2.3.4. Conventional Solvent Extraction

Isopropanol and hexane were the solvents used for the conventional solvent extraction. Twenty grams of rice bran was weighed in an Erlenmeyer flask and the volume of solvent respective to a 3:1 w/w solvent to bran ratio was added. Each flask was capped with a rubber stopper and each sample was shaken well for a better mixing of the bran and solvent. Each solvent extraction, with isopropanol and hexane, was done in triplicate. The flasks were placed in a 40ºC water bath under shaking for 15 min. The samples were allowed to cool for 20 min and were filtered through the filter device previously described. After filtration, the mixture of oil and solvent was measured and the volume recovered for each sample was recorded. Two milliliters of each sample were placed in a known weight screw cap test tube and two test tubes containing 2 ml mixture were prepared for each sample. These tubes were placed in a vacuum centrifuge evaporator (CentriVap Console Labconco, Kansas City, Missouri) for the evaporation of the solvent overnight. The residual oil for each sample was weighed in order to measure the extraction yield. One of the test tubes for each sample was used immediately after evaporation to determine the vitamin E components by normal phase HPLC. The other test tube was flashed with nitrogen and it was placed in the freezer under dark conditions for further analysis.

2.3.5. Determination of Vitamin E Components Using HPLC Method

After evaporation was completed, 2 ml hexane was added to each tube containing rice bran oil and each sample was vortexed for 10 sec. The test tubes were placed in a Hermle Labnet high capacity centrifuges (Denville Scientific Inc., South Plainfield, NJ) and were centrifuged for 20 min at 3500 rpm. One milliliter of the supernatant was transferred into a HPLC vial and 25 µL were injected into the HPLC system. The amount of vitamin E components in the samples

was determined by normal phase HPLC. The HPLC system consisted of Waters (Waters, Milford, MA) 510 pump, a 715 Ultra WISP injector, and a 470 fluorescence detector. Twenty five microliters from each vial were injected into a 25 cm x 4.6 cm diameter of 5 µm Supelcosil LC-Si (Supelco, Bellefonte, PA) column. The mobile phase had the following composition: 98.4% hexane, 0.8% ethyl acetate, and 0.8% acetic acid and the flow rate was 1.5 mL/min. The elutant was monitored using the fluorescence detector set at excitation wavelength 290 nm and emission wavelength 330 nm. Chromatograms were recorded and processed using Waters Millennium chromatography software. Four components of vitamin E, α and γ tocopherol and α and γ tocotrienol, respectively were determined and expressed as µg/g fresh rice bran. The total vitamin E content was expressed as the sum of all four vitamers.

2.3.6. The Effect of Microwaves and Heat on Alpha-tocopherol Degradation

Two samples of a volume of 750 ml alpha-tocopherol at a concentration of 0.10 g/L was prepared in isopropanol and hexane. A volume of 1 ml of α-tocopherol in hexane was sampled and 25 µl were injected into HPLC system as a control. Samples of 2 milliliters for both solvents were placed in two test tubes and were evaporated overnight in a vacuum centrifuge evaporator. After evaporation was completed, 2 mL of hexane was added to both test tubes, vortexed for 10 sec and analyzed by HPLC to assess degradation during evaporation process.

To study the effect of microwaves and heat on alpha-tocopherol degradation, 50 milliliters of sample were placed into the Teflon vessels of the Ethos E Microwave Extraction System; the experiments were done in triplicate. The vessels were sealed and placed in the Microwave Extraction System which was set to operate in two steps. The first step was the increase in temperature gradually using an energy level of a maximum 800 W for 5 minutes; at the end of the 5 minutes period the extraction temperature was reached. The second step was designed to hold the samples for a total period of 15 min at the extraction temperature by using an energy level of a maximum of 500 W. Five different temperatures, 40, 60, 80, 100, and 120ºC, respectively, were used to assess the effect of microwaves and temperature on the alpha-tocopherol degradation. Samples were allowed to cool down in the microwave for 20 min and two milliliters of each sample were withdrawn and placed in a screw cap test tube. Two milliliters of each samples were evaporated overnight in the vacuum centrifuge evaporator and 2 mL hexane was added to each one and vortexed for 10 sec. One milliliter from each sample was

transferred to a HPLC vial and 25 µL were injected into the normal phase HPLC previously described.

2.3.7. Antioxidant Activity of Rice Bran Oil

Determination of antioxidant activity of the rice bran oil was accomplished using DPPH radical scavenging method (Oufnac et al., in press). The experiments were performed in triplicates. An amount of 0.01 g oil from each sample was dissolved in 500 µL methanol and vortexed for 10 sec. Three different volumes of oil dissolved in methanol (40, 80, and 120 µL) were added to 2 mL of 25 mg/mL DPPH solution in methanol. The decrease in absorbance was monitored at 515 nm by using a UV-Visible Beckman Coulter spectrophotometer (Beckman Coulter, Fullerton, CA). The readings were done at time zero, after the oil mixed with the methanol was added to DPPH, and after 30 min of incubation at room temperature. The inhibition percentage was expressed using the following equation:

$$\text{Inhibition \%} = (Abs_{t=0} - Abs_{t=30\ min}) / Abs_{t=0} \times 100 \quad [1]$$

where $Abs_{t=0}$ was the absorbance of DPPH at time zero and $Abs_{t=30\ min}$ was the absorbance of DPPH after 30 min of incubation. The inhibition percentage was plotted against each quantity of rice bran oil solution to obtain a regression line. Trolox (0.5 mM) dissolved in methanol was used as a standard to express the inhibition capacity of rice bran oil solution as Trolox equivalent. The ratio between the slopes of the regression lines of rice bran oil solution and the Trolox solution was expressed as µmol Trolox Equivalent Antioxidant Activity.

2.3.8. Statistical Analysis

The means and standard deviations of the extraction yield, total vitamin E, vitamin E components, scavenging DPPH capability, and degradation of alpha-tocopherol were reported from triplicate determinations for each sample. Two-way ANOVA using Proc Mixed 2x5 factorial (SAS system, SAS Institute Inc., Cary, NC) was used to test significant differences among temperatures (40, 60, 80, 100, and 120ºC) and solvents (isopropanol and hexane) for microwave-assisted method. Two-way ANOVA using Proc Mixed 2x2 factorial was used to test significant differences among solvents (isopropanol and hexane) and extraction methods (microwave-assisted and conventional solvent extractions) at 40ºC. Multiple comparison tests were performed by using Tukey adjustment to determine the significant difference between treatments. Statistical significance was declared at $P < 0.05$.

2.4. Results and Discussions

2.4.1. Rice Bran Oil Yield Extracted by Conventional Solvent and Microwave-assisted Extractions

Rice bran oil yield extracted with isopropanol and hexane by conventional solvent and by microwave-assisted extractions is presented in Figure 2.2. The oil yield by microwave-assisted extraction at 40ºC was approximately 10% of the fresh rice bran for isopropanol and approximately 14% for hexane. By increasing the extraction temperature to 120ºC, the oil amount extracted was 50% higher when isopropanol was used as an extraction solvent (Table 2.1.). For hexane, no significant change in the amount of oil extracted was noticed by increasing the extraction temperature from 40 to 120ºC (Table 2.1.). It seems that at higher temperatures the solubility of rice bran oil in isopropanol increased and maybe more polar components were extracted such as alcohol soluble proteins and carbohydrates, increasing the amount of the oil extracted at these temperatures. The results are similar to those found by Duvernay et al. (2005), who showed a 54% increase in oil yield by increasing the temperature from 80 to 140ºC by microwave assisted method using isopropanol as extraction solvent. At 40ºC hexane extracted significant more amount of oil (0.14 g oil/g fresh rice bran) as compared with isopropanol (0.10 g oil/g fresh rice bran) by microwave-assisted method, data similar with the results found by Hu et al (1996). At higher temperatures (100 and 120ºC) isopropanol proved to be a significantly better solvent for the extraction of rice bran oil as compared with hexane.

The conventional solvent extraction at 40ºC for isopropanol yielded approximately 12% oil of the fresh rice bran, not significantly different from the oil yielded by microwave assisted extraction in the same conditions. The amount of oil extracted with hexane by conventional solvent extraction was approximately 14% which was similar with the amount of oil extracted with hexane by microwave-assisted extraction. These values were close to 14.95% oil yield extracted from rice bran by traditional solvent extraction using hexane as a solvent at ambient temperature by Proctor (1996). Hu et al. (1996) reported that approximately 19% oil from the fresh rice bran was extracted by hexane at 40 and 60ºC while isopropanol extracted 17 and 18% at 40 and 60ºC, respectively. The small differences in oil yield between different data reported in the literature and those found in this study can be explained by a number of factors that affect the rice and rice bran: storage conditions, rice, milling, rice bran stabilization, and the conditions used for extraction.

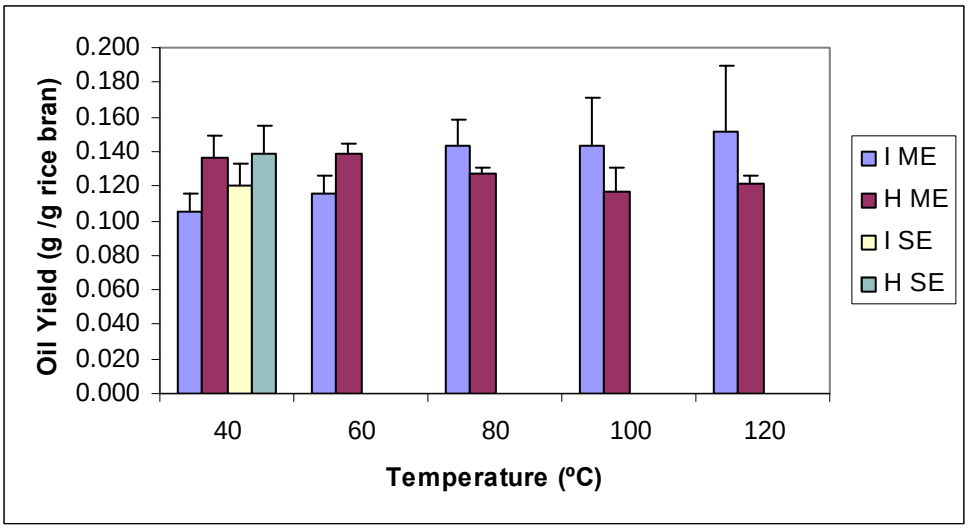

Figure 2.2. Oil yield obtained by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=6.

2.4.2. Total Vitamin E and Vitamin E Components of Rice Bran Oil Extracted by Conventional Solvent and Microwave-assisted Solvent Extractions

The amount of total vitamin E was expressed as the sum of all four components analyzed, α-tocopherol, α-tocotrienol, γ-tocopherol, γ-tocotrienol. By increasing the temperature from 40 to 120ºC the total vitamin E varied between 95.41 and 152.31 µg/ g fresh rice bran when isopropanol was used as a solvent, difference which was not found statistically different (Table 2.1.). When hexane was used as a solvent for the microwave extraction of vitamin E components, the total vitamin E increased, but not significantly from 56.22 to 98.19 µg/ g fresh rice bran by raising the temperature from 40 to 100ºC (Table 2.1.). A further increase in temperature to 120ºC provided a significant increase in the total vitamin E to 248.50 µg/ g fresh rice bran. The average total extracted vitamin E was significantly greater when hexane was used as a solvent as compared to isopropanol, for extractions performed at 120ºC. The total vitamin E obtained by conventional solvent extraction at 40ºC was 87.46 µg/ g fresh rice bran for isopropanol and 52.64 µg/ g fresh rice bran for hexane. These values are similar with the total vitamin E amount extracted in the same conditions by microwave-assisted extraction method (Figure 2.3.).

The variations of the amount of each vitamin E component with temperature for microwave-assisted extraction are presented in Table 2.1. and for conventional solvent extraction the data is presented in Table 2.2. For microwave-assisted extraction an increase in temperature

from 40 to 100ºC produced an increase of 47.4% in the extraction of α-tocopherol (not significant). For hexane, no significant difference was noticed in the microwave-assisted extraction of α-tocopherol by increasing the temperature (Figure 2.4.). At 80 and 100ºC, isopropanol extracted significantly more α-tocopherol as compared with hexane, 127.18% and 135.32% more α-tocopherol, respectively (Table 2.1.). No significant differences among the solvents and extraction methods were noticed related to α-tocopherol extracted (Table 2.2.).

Variation of α-tocotrienol with temperature and solvent is presented in Figure 2.5. When isopropanol was used as a solvent in the microwave-assisted extraction an increase in temperature from 40 to 120ºC produced an increase in the extraction amount of α-tocotrienol from 8.19 to 54.50 μg/ g fresh rice bran, which statistically is not significantly different (Table 2.1.). For hexane, the extraction amount of α-tocotrienol did not significantly increase between 40 and 100ºC (from 9.07 to 45.99 μg/ g fresh rice bran) but an increase in temperature to 120ºC significantly raised the amount of α-tocotrienol to 183.76 μg/ g fresh rice bran (Table 2.1.). Hexane extracted significantly more α-tocotrienol as compared with isopropanol at 120ºC. No significant differences among the solvents and extraction methods were noticed for the extraction of α-tocotrienol (Table 2.2.).

For γ-tocopherol, a significant change with temperature was noticed when isopropanol was used, from 5.37 to 7.29 μg/ g fresh rice bran for 40 and 120ºC, respectively (Figure 2.6.). When hexane was used as extraction solvent no significant change in the amount of γ-tocopherol was noticed as a function of temperature (Table 2.1.). In terms of the extraction method, at 40ºC, for both microwave-assisted and conventional solvent extractions, isopropanol extracted significantly more γ-tocopherol as compared to hexane (Table 2.2.). Also, for microwave-assisted extraction performed in the temperature range of 80 to 120ºC, isopropanol proved to be a significantly better extraction solvent for γ-tocopherol as compared with hexane (Table 2.1.).

The amount of γ-tocotrienol did not vary significantly with temperature when isopropanol was used as an extraction solvent. When hexane was used, a significant increase in γ-tocotrienol was noticed by raising the temperature in the range 40 to 60ºC, from 17.36 to 29.31 μg/ g fresh rice bran (Figure 2.7.). At 40ºC, both microwave-assisted and conventional solvent extractions with isopropanol extracted significantly more γ-tocotrienol as compared to hexane (Table 2.2.). The extraction performed in the temperature range 80 to 120ºC by microwave-

assisted method with isopropanol was proved to significantly extract more γ-tocotrienol as compared with microwave-assisted with hexane (Table 2.1.).

The optimum microwave-assisted extraction of α-tocopherol was accomplished at an extraction temperature of 100ºC when isopropanol was used as a solvent. Microwave-assisted extraction at 120ºC with hexane produced a very large yield of α-tocotrienol. The higher extraction temperature and microwave energy may release some free forms of tocopherols which were previously in esterified forms. This may explain the high extraction of α-tocotrienol at an elevated temperature by hexane.

Table 2.1. Oil yield, total vitamin E, vitamin E components, scavenging DPPH capability as a function of temperature using microwave-assisted extraction and solvent type

Temp (ºC)	Solvent	Oil[1]	Vitamin E[2]	α-Tocopherol[2]	α-Tocotrienol[2]	γ-Tocopherol[2]	γ-Tocotrienol[2]	Trolox Equivalent[3]
40	I	0.10 ± 0.01^{a}	$95.41 \pm 2.38^{a,b,c}$	$43.89 \pm 2.23^{a,b}$	8.19 ± 0.19^{a}	$5.37 \pm 0.16^{b,c,d}$	$37.96 \pm 1.21^{c,d}$	33.61 ± 7.35^{a}
60	I	$0.12 \pm 0.01^{a,b}$	$97.46 \pm 1.76^{a,b,c}$	$43.83 \pm 2.30^{a,b}$	8.27 ± 0.66^{a}	$5.44 \pm 0.09^{b,c,d,e}$	$39.93 \pm 0.50^{c,d}$	34.40 ± 1.80^{a}
80	I	$0.14 \pm 0.00^{c,d}$	$124.56 \pm 13.26^{a,b,c}$	59.16 ± 12.67^{b}	15.31 ± 1.72^{a}	$6.33 \pm 0.07^{c,d,e}$	43.76 ± 1.47^{d}	$45.49 \pm 6.13^{a,b,c}$
100	I	$0.14 \pm 0.03^{c,d}$	$137.80 \pm 15.47^{b,c}$	64.69 ± 9.26^{b}	22.74 ± 0.99^{a}	$6.90 \pm 0.98^{d,e}$	43.48 ± 5.34^{d}	$51.11 \pm 3.75^{b,c}$
120	I	0.15 ± 0.04^{d}	152.31 ± 11.35^{c}	$47.79 \pm 3.83^{a,b}$	54.50 ± 4.17^{a}	7.29 ± 0.50^{e}	42.73 ± 3.68^{d}	59.85 ± 6.41^{c}
40	H	$0.14 \pm 0.01^{b,c,d}$	56.22 ± 28.15^{a}	26.54 ± 15.23^{a}	9.07 ± 3.11^{a}	3.25 ± 1.11^{a}	17.36 ± 8.74^{a}	$41.38 \pm 3.93^{a,b}$
60	H	$0.14 \pm 0.01^{b,c,d}$	$81.49 \pm 7.62^{a,b,c}$	34.86 ± 6.28^{a}	12.90 ± 1.25^{a}	$4.42 \pm 0.17^{a,b}$	$29.31 \pm 0.67^{b,c}$	$42.58 \pm 1.45^{a,b}$
80	H	$0.13 \pm 0.00^{a,b,c,d}$	$66.40 \pm 10.67^{a,b}$	26.04 ± 4.45^{a}	14.90 ± 2.18^{a}	3.44 ± 0.75^{a}	$22.01 \pm 3.62^{a,b}$	$38.84 \pm 9.45^{a,b}$
100	H	$0.12 \pm 0.01^{a,b}$	$98.19 \pm 14.80^{a,b,c}$	27.49 ± 7.20^{a}	45.99 ± 5.44^{a}	$3.71 \pm 1.03^{a,b}$	$21.00 \pm 4.64^{a,b}$	$39.02 \pm 4.64^{a,b}$
120	H	$0.12 \pm 0.00^{a,b,c}$	248.50 ± 78.11^{d}	35.49 ± 3.62^{a}	183.76 ± 72.70^{b}	$4.77 \pm 0.41^{a,b,c}$	$24.49 \pm 3.10^{a,b}$	$43.55 \pm 2.73^{a,b}$

I= Isopropanol; H= Hexane;

1 Oil yield was expressed as g/ g fresh rice bran;

2 Total vitamin E and vitamin E components were expressed as µg/ g fresh rice bran;

3 Scavenging DPPH capability was expressed as Trolox Equivalent µmol/ g fresh rice bran. Significantly different values ($P<0.05$) of oil, vitamin E, vitamin E components, and scavenging DPPD capability in the same column are indicated by different letters ^{a,b,c,d}. n=3.

Table 2.2. Oil yield, total vitamin E, vitamin E components, scavenging DPPH capability as a function of solvent type using microwave-assisted and conventional solvent extractions at 40ºC

Solvent	Method	Oil[1]	Vitamin E[2]	α-Tocopherol[2]	α-Tocotrienol[2]	γ-Tocopherol[2]	γ-Tocotrienol[2]	Trolox Equivalent[3]
Isopropanol	ME	0.10 ± 0.01^{a}	95.41 ± 2.38^{a}	43.89 ± 2.23^{a}	8.19 ± 0.19^{a}	$5.37 \pm 0.16^{a,b}$	37.96 ± 1.21^{a}	33.61 ± 7.35^{a}
Hexane	ME	0.14 ± 0.01^{b}	$56.22 \pm 28.15^{a,b}$	26.54 ± 15.23^{a}	9.07 ± 3.11^{a}	3.25 ± 1.11^{c}	17.36 ± 8.74^{b}	41.38 ± 3.93^{a}
Isopropanol	SE	$0.12 \pm 0.01^{a,b}$	$87.46 \pm 7.41^{a,b}$	32.50 ± 5.03^{a}	8.48 ± 0.45^{a}	5.94 ± 0.13^{a}	40.54 ± 1.89^{a}	33.86 ± 3.52^{a}
Hexane	SE	0.14 ± 0.02^{b}	52.64 ± 9.08^{b}	20.73 ± 7.30^{a}	8.31 ± 0.99^{a}	$3.93 \pm 0.64^{b,c}$	19.68 ± 3.30^{b}	41.64 ± 5.04^{a}

1 Oil yield was expressed as g/ g fresh rice bran;

2 Total vitamin E and vitamin E components were expressed as µg/ g fresh rice bran;

3 Scavenging DPPH capability was expressed as Trolox Equivalent µmol/ g fresh rice bran. Significantly different values ($P<0.05$) of oil, vitamin E, vitamin E components, and scavenging DPPD capability in the same column are indicated by different letters ^{a,b,c,}. n=3.

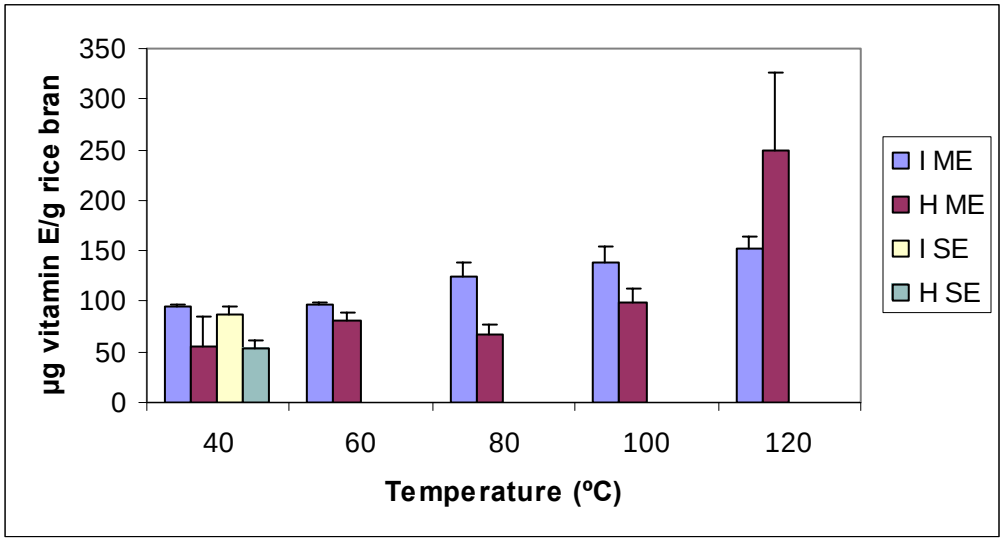

Figure 2.3. Total vitamin E extracted by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

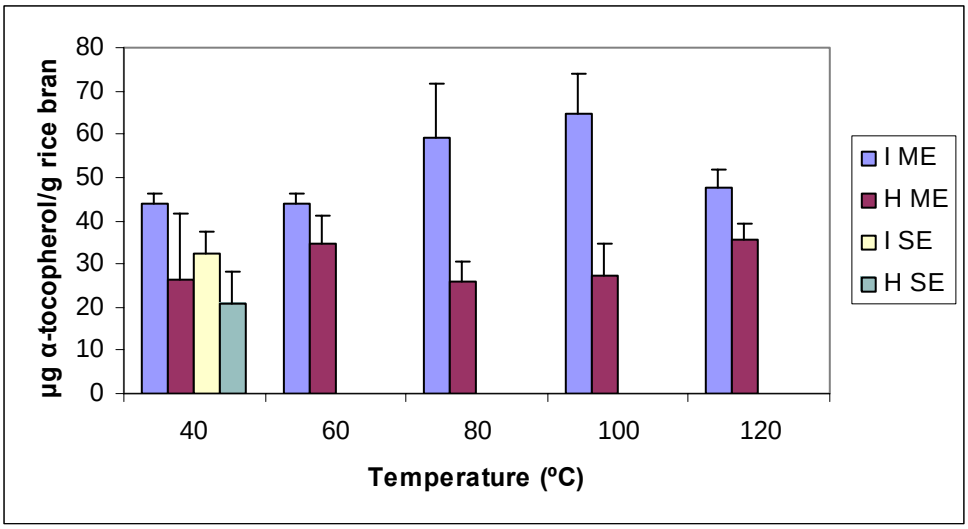

Figure 2.4. Alpha-tocopherol extracted by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

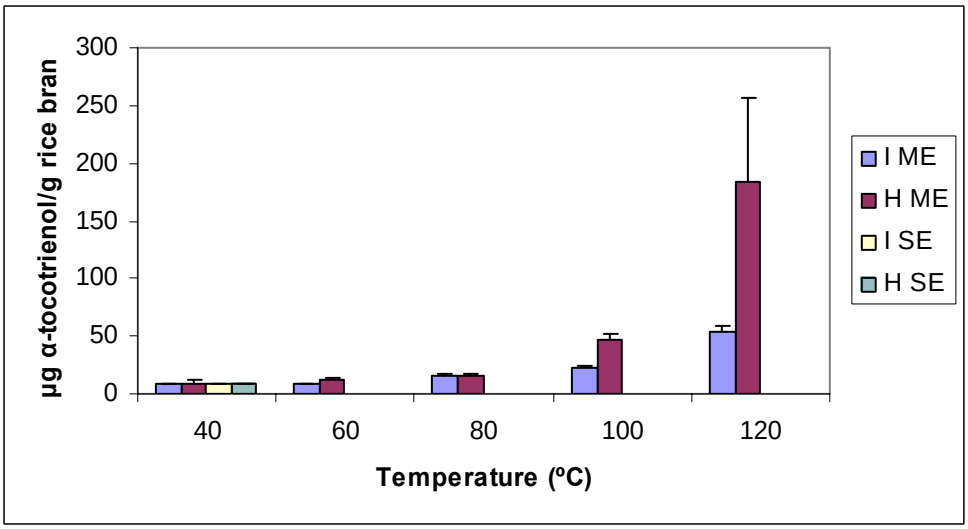

Figure 2.5. Alpha-tocotrienol extracted by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

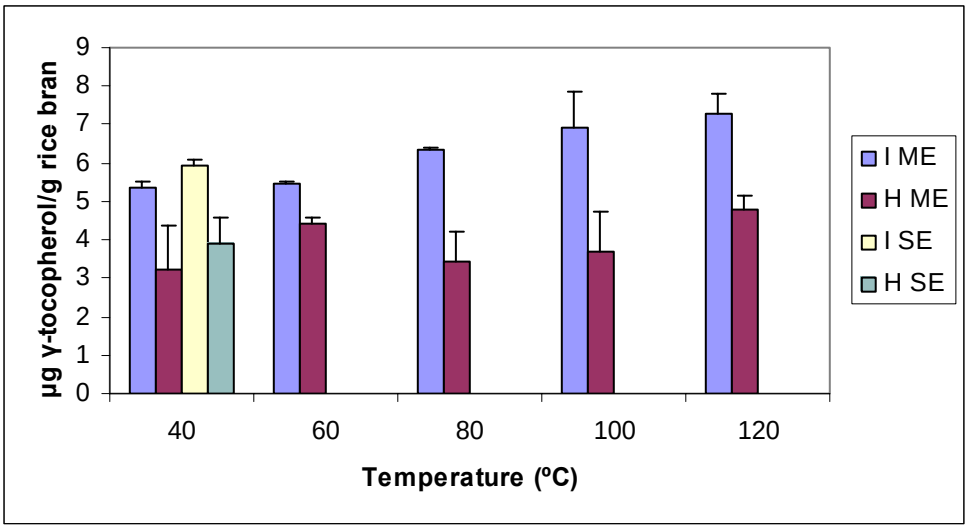

Figure 2.6. Gamma-tocopherol extracted by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

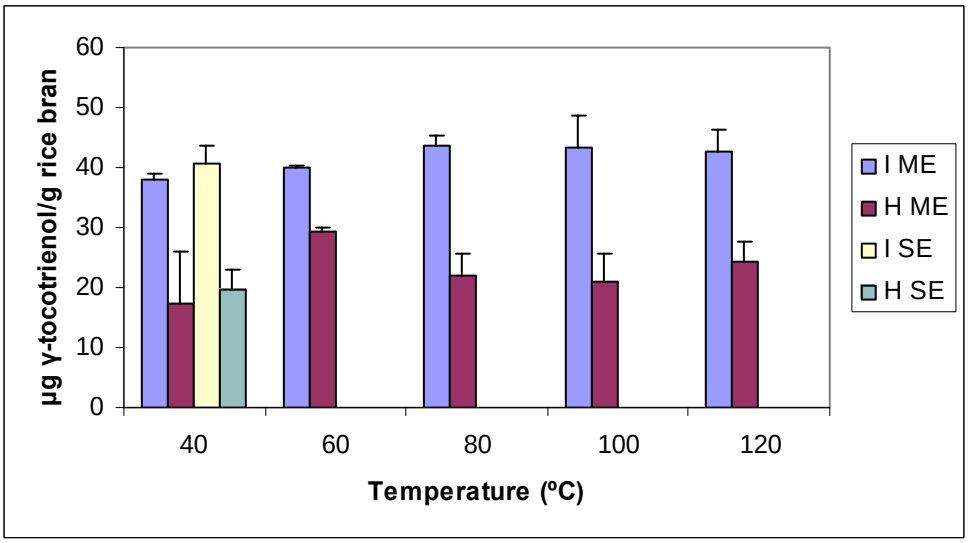

Figure 2.7. Gamma-tocotrienol extracted by conventional solvent and microwave-assisted methods using isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

2.4.3. Free Radical Quenching Capability of Rice Bran Oil Extracted by Conventional Solvent and Microwave-assisted Extractions

The results on free radical quenching capability of rice bran oil antioxidants obtained by conventional solvent and microwave-assisted extractions are presented in Figure 2.8. When isopropanol was used as a solvent, the free radical quenching capability increased significantly by increasing the temperature of microwave-assisted extraction, from 33.61 to 59.85 µmol

Trolox Equivalent per gram of fresh rice bran for 40 and 120ºC, respectively. The free radical quenching capability did not change significantly for the oil extracted with hexane by microwave-assisted extraction (Table 2.1.). The change in antioxidant activity of the rice bran oil followed a similar trend to that of the amount of oil extracted as a function of temperature. This suggests that the amount of total phenolic extracted compounds, which are soluble in methanol, may be correlated with the antioxidant activity. At 120ºC, the rice bran oil extracted with isopropanol presented a significant higher DPPH scavenging capacity in comparison with rice bran oil extracted with hexane. This can be explained by the higher miscibility of isopropanol with the rice bran oil at higher temperatures, which led to more antioxidants extracted at elevated temperatures. Since oryzanol is soluble in alcohols, maybe isopropanol at higher temperature extracted more oryzanol components in the rice bran oil which provided an increase in the antioxidant activity of the oil. No significant different DPPH scavenging capacity was noticed between the two solvent when both microwave-assisted and conventional solvent methods were compared (Table 2.2.). It is important to note that the DPPH radical scavenging capacity was determined in methanol so only the antioxidant capacity of the components soluble in methanol was tested.

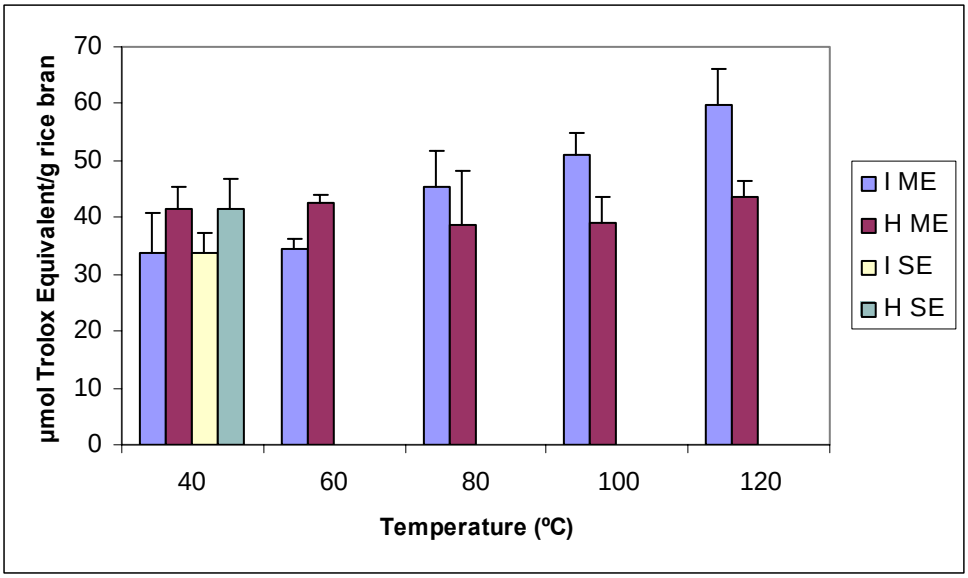

Figure 2.8. Capability of scavenging DPPH free radicals (Trolox Equivalent µmol/ g fresh rice bran) from rice bran extracted by conventional solvent and microwave-assisted methods isopropanol and hexane as extraction solvents (I= isopropanol, H= hexane, ME= microwave-assisted extraction, SE= conventional solvent extraction). n=3.

2.4.4. Study of Degradation of α-tocopherol in the Microwave Extractor System at Elevated Temperature

The results on degradation of α-tocopherol held at elevated temperature in the microwave extractor system is presented in Figure 2.9.a for α-tocopherol dissolved in isopropnaol and 2.9.b for α-tocopherol dissolved in hexane. No significant change in the amount of α-tocopherol during the process of microwave heating and evaporation was noticed. These findings are in agreement with the studies on the degradation of tocopherols and tocotrienols exposed to the environmental conditions (Mazza, 2002). Since microwave extraction and evaporation process were performed in dark conditions and in the presence of limited amount of oxygen for microwave extraction method, and under vacuum for the evaporation process, the exposure of the samples to light and oxygen was minimal. The most important factor in both processes (microwave heating and vacuum evaporation) was the elevated temperature. These results showed that during the whole extraction process α-tocopherol was not degraded.

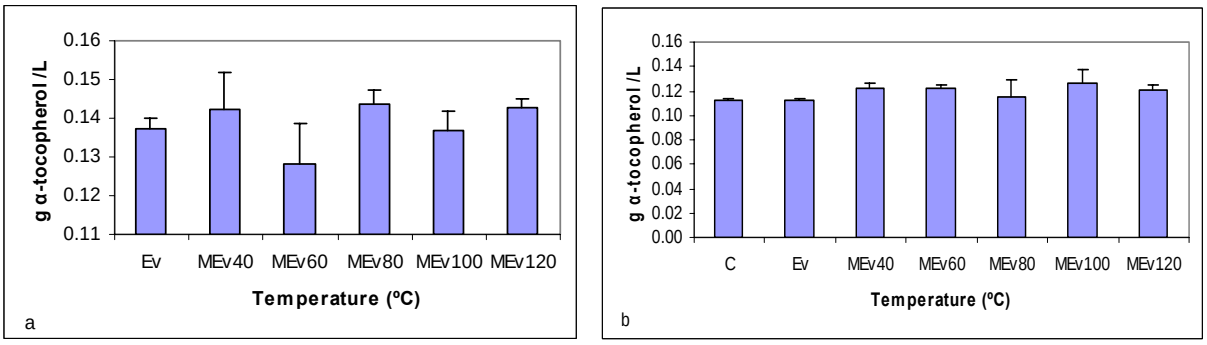

Figure 2.9. Study on degradation of α-tocopherol in the microwave extractor system at elevate temperature (a= isopropanol, b= hexane). Value C = control, Ev = evaporation effect, and MEv = microwave and evaporation effect. Error bars represent standard deviation of 3 replicates.

2.5. Conclusion

For microwave-assisted extraction, hexane proved to be a better solvent for rice bran oil extraction as compared to isopropanol for extractions performed at 40ºC; hexane extracted approximately 40% more oil than isopropanol. While by increasing the temperature, hexane did not extract significantly more amount of oil, isopropanol extracted about 25% more rice bran oil at 120ºC than hexane in the same conditions. At higher temperature, isopropanol proved to be a better solvent for rice bran oil. Hexane extracted large amount of α-tocotrienol at 120ºC while the increase in temperature for isopropanol was more beneficial for the extraction of γ-tocopherol. Also, isopropanol at higher temperature seems to extract more hydrophilic

antioxidants which were revealed in the 78% increase of DPPH scavenging capability by increasing the temperature from 40 to 120ºC.

No significant differences in the oil yield, total vitamin E, and antioxidant activity of rice bran oil was noticed between the two extraction methods, conventional solvent and microwave-assisted extractions, at 40ºC. The results showed that during the whole extraction process α-tocopherol was not degraded.

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CHAPTER 3. NANOPARTICLES WITH ENTRAPPED ALPHA-TOCOPHEROL; SYNTHESIS, CHARACTERIZATION, AND CONTROLLED RELEASE

3.1. Introduction

Encapsulation of alpha-tocopherol in polymeric nanoparticles can be achieved by a number of methods, including emulsion or microemulsion polymerization, interfacial or precipitation polymerization, emulsion evaporation, emulsion diffusion, solvent displacement and salting out. Many studies reported the use of emulsion evaporation method for nanoparticle preparation and entrapment of various drugs (Mainardes et al., 2005; Li et al., 2001; Mu and Feng, 2002; Muller,1990; Cascone et al., 2002; Birnbaum and Brannon-Peppas, 2003, Astete and Sabliov, 2006). The principle of this method is based on the emulsification of the organic phase in an aqueous phase; an oil in water (o/w) emulsion is formed with the organic phase (polymer and solvent) and the water phase, containing the surfactant. After the emulsification process, the solvent is evaporated resulting in the precipitation of the polymer and formation of nanoparticles. The emulsification process is a critical step in nanoparticles synthesis due to the direct relationship between the final size of particles and the emulsion droplets (Astete and Sabliov, 2006). Several synthesis parameters affect nanoparticle physical characteristics, which in turn will affect the release profile of the drug.

3.1.1. Parameters Affecting the Size of Nanoparticles

3.1.1.1. Solvent

Two major criteria are taken into consideration for choosing the organic solvent to be used in the nanoparticles preparation: the solubility of PLGA in this solvent and the immiscibility of the solvent with the aqueous phase (Astete and Sabliov, 2006; Birnbaum and Brannon-Peppas, 2003). The evaporation rate of the organic solvent also influences the formation of nanoparticles. It is very important that the evaporation of the solvent be performed in the shortest time; otherwise, a small decrease in solvent volume increases the viscosity of the droplets and causes coalescence of the particles. Vacuum rotative evaporator provided particles with smaller diameter than those obtained by magnetic stirring (Mainardes et al., 2005; Brannon-Peppas et al., 1995).

3.1.1.2. Organic to Aqueous Ratio

The increase of internal to external phases ratio (organic to aqueous ratio) provided a small decrease in nanoparticles size as a result of preventing the agglomeration of particles by using more organic solvent (Mainardes et al., 2005).

3.1.1.3. Polymer

In order to avoid chronic toxicity caused by the accumulation of nonbiodegradable polymers into the body, biodegradable polymers are preferred for synthesis of nanoparticles designed for pharmaceutical or food use (Niwa, 1993; Uhrich, 1999). Many biodegradable polymers can be used for micro- and nanoparticles synthesis, of which most frequently used are polylactic acid (PLA) and poly(lactic acid-co-glycolic acid) (PLGA). These polymers have been FDA approved for drug delivery (Birnbaum and Brannon-Peppas, 2003; Brannon-Peppas, 1995; Ghosh, 2004). High molecular weight nondegradable polymers with a molecular weight bigger than the capacity of glomerular filtration can not be excreted through the kidney (Yoo, 2000). Utilization of PLGA as drug delivery systems is based on its capacity to biodegrade into biologically safe products through natural biological processes (Mainardes, 2005; Birnbaum and Brannon-Peppas, 2003). An advantage of PLGA, besides the fact that is degradable into non-toxic compounds, is represented by the release rate of the drug which can be controlled by changing the ratio of the monomers in the co-polymer (Yoo, 2000). The amount of polymer used in synthesis of nanoparticles affects their size, shape, and agglomeration tendency. An increase in the amount of PLGA from 12.5 to 50 mg resulted in nanoparticles of a non-spherical shape of bigger size, which had the tendency to agglomerate (Mainardes et al., 2005).

3.1.1.4. Surfactant

The surfactant prevents the coalescence of particles and plays a crucial role in the emulsification step, and in droplet protection and stabilization. An increase in the surfactant concentration results in decreasing the particles diameter and narrowing the granulometric distribution. At low concentration of surfactant the stabilization of particles is difficult and agglomeration is present (Mainardes et al., 2005).

The type of surfactant is important for synthesis of stable and small nanoparticles. Surfactants such as PVA, SDS, Pluronic F68 are commonly used in the nanoparticles synthesis (Astete and Sabliov, 2006; Kim and Burgess, 2002; Lee et al., 2002; Mainardes and Evangelista,

2005). For the application of the nanoparticles in the biomedical field, the presence of a toxic surfactant residue on the particles surface must be avoided. For this reason, many research studies are concentrated on finding biodegradable and biocompatible surfactants that can be used in nanoparticles preparation (Astete and Sabliov, 2006).

PVA is widely used as an emulsifier in the synthesis of nanoparticles of a relatively small size and uniform size distribution. Residual PVA associated to the surface of particles, formed as a result of interconnected network between PVA and polymer, following purification could be up to 13% (w/w), relative to the weight of nanoparticles (Sahoo, 2002). The residual PVA associated to the surface of nanoparticles has a significant influence on nanoparticles size, zeta potential, polydispersity index, surface hydrophobicity, drug loading and drug release, and nanoparticles cellular uptake (Sahoo, 2002). The amount of residual PVA on the surface of particles is a function of the initial concentration of PVA and of the type of organic solvent used for the nanoparticle synthesis (Sahoo, 2002).

3.1.1.5. Drug Content

The increase in drug content causes an increase in particles size and size distribution due to higher viscosity of the dispersed phase (Mainardes et al., 2005). Redhead et al., (2001) studied the effect of the drug loaded on the physical characteristics of PLGA nanopaticles. The particles loaded with hydrophilic drugs (~ 150 nm) were slightly bigger than PLGA nanoparticles without entrapped drug (~ 135 nm). They confirmed that PLGA nanoparticles were more polar while polystyrene nanoparticles were more hydrophobic. This characteristic of the polymers can be used to design nanospheres with a desired released profile. The affinity of the drugs for different polymers provides the means to control the drug release rate from the polymer matrix.

3.1.1.6. Sonication

Sonication time is very important in making a microemulsion. If the phase is not sufficiently dispersed, and the emulsion droplets are big, bigger nanoparticles are obtained. The study of how shape and size distribution of nanoparticles changes as a function of sonication time revealed the following: the particles were spherical for all situations; while the sonication time increased, the mean diameter of nanoparticles was reduced, the granulometric distribution was narrower and the profile of distribution was monomodal for the highest sonication time (20 min) as per Mainardes et al. (2005). The explanation of this phenomenon was that the highest

energy released during sonication caused a fast dispersion of the organic phase, leading to the formation of small nanodroplets of a monomodal distribution. However, Lee et al. (2002) showed that sonication provided a lower encapsulation efficiency in comparison with that obtained using high-pressure homogenization.

3.1.1.7. Storage/ Resuspension

Following synthesis, the size of the nanoparticles is affected by aggregation. De and Robinson (2004) studied the effect of storage and temperature on morphology and stability of PLGA nanoparticles. Their interest was also to check if the particles aggregated after resuspension in water. The study was conducted over a period of 6 days at temperatures between 4 and 50ºC. Aggregation increased with an increase in storage temperature and it was more pronounced for the smaller nanoparticles. When the quantity of lactic acid in the copolymer increased, the extent of aggregation decreased. Three mechanisms were proposed to explain the aggregation of nanopaticles: a) after storage at elevated temperatures the PVA which forms a corona on the particles surface was affected by temperature and destabilized, resulting in aggregation; b) at high temperatures dichloromethane (the solvent) migrated to the surface dissolving the PLGA which was conducive to aggregation of particles; c) the type of drug entrapped did not affected the aggregation of nanoparticles (similar aggregation occurred for both Bodipy and Paclitaxel). All these suppositions were not confirmed, the only important recommendation to avoid irreversible aggregation was to store the formed nanospheres at 4ºC in desiccators.

3.2. Entrapment Efficiency

In general, the larger size nanoparticles present a higher drug loading capacity per particle (Muller, 1990). Hydrophobic drugs are entrapped easier and with much higher entrapment efficiency (usually greater than 90%) than hydrophilic drugs. This behavior is caused by the high solubility of the hydrophobic compound in the organic solvent (Birnbaum and Brannon-Peppas, 2003). The entrapment efficiency of hydrophilic drugs is low because of the drug lost in the aqueous phase during the emulsification process (Redhead, 2001; Birnbaum and Brannon-Peppas, 2003). Another reason for the low encapsulation efficiency of hydrophilic drugs is the smaller size of the particles and the short diffusion pathway (Redhead, 2001). The

entrapment efficiency of the drug can also be affected by the type of polymer in which the drug is incorporated. Li et al. (2001) reported that PEG-PLGA nanoparticles present a lower encapsulation efficiency of bovine serum albumin (48.6%) in comparison with PLGA nanoparticles (63.8%). Emulsifiers with a HLB (hydrophilic-lipophilic balance) value higher than 15 (tween 80 and tween 20) enhance the entrapment efficiency of naltrexone HCl by modifying the surface properties of the particles (Dinarvand et al., 2005).

3.3. Release Profile

In the past years, many studies were reported on the release profile of different hydrophobic drugs (Mainardes et al., 2005; Lee et al., 2002; Cascone et al., 2002; Yeo and Park, 2004; Dingler et al., 1999; Duclairoir et al., 2002; Mu and Feng, 2002) and hydrophilic drugs (Redhead et a., 2001; Niwa et al., 1993) encapsulated in PLGA. Two mechanisms by which the drug is released from nanoparticles were identified. One of these mechanisms was the diffusion of the drug through the polymer matrix, and the other was the erosion of the matrix as a result of the polymer degradation (Lee, 2002).

A comparison between the release profile of poorly water-soluble drugs and hydrophilic drugs revealed an initial burst in the release of water-soluble drugs, even when high-molecular-weight polymers were used for the drug entrapment. The fast release of water-soluble drug could be attributed to the higher affinity of the drug for the release medium, water (Niwa et al., 1993). An initial burst release of the drug from nanospheres is not optimum for utilization of nanoparticles in some medical applications, whereas in others it may be preferred (Niwa et al., 1993).

There are a number of factors that affect the release profile of the drugs entrapped in the nanoparticles:

3.3.1. Size of Nanoparticles

The size of the particles plays an important role in the release profile of the entrapped drugs. In general, the drugs entrapped in small particles (nanoparticles) were released faster and more constantly than those incorporated in bigger particles (microspheres) (Niwa et al., 1993). This was caused by the higher surface area per volume ratio of nanoparticles. Also, the small diffusion distance characteristic to nanoparticles is allowing for rapid release of the drug from

the core into the medium (Redhead et al., 2001; Niwa et al., 1993). This behavior was noticed for hydrophobic (Lee et al., 2002) and hydrophilic drugs (Redhead et al., 2001). Lee et al. (2002) studied the release profile of Cyclosporin A, a highly hydrophobic cyclic peptide, entrapped in PLGA matrix by solvent evaporation method. The drug release profile determined by RP-HPLC showed that the drug was released primarily as a result of diffusion as opposed to matrix erosion.

3.3.2. Characteristic, Molecular Weight, and Ratio of Monomers in the Polymer

The polymer characteristics used as a matrix for the entrapment of a drug influence the release rate of this compound (Birnbaum and Brannon-Peppas, 2003; Yeo and Park, 2004; Niwa et al., 1993). PLGA with a high molecular weight is degraded slower than the low molecular weight PLGA. For the drugs encapsulated into low-molecular-weigh polymer an initial burst was observed; increasing the molecular weight of the polymer resulted in a significantly lower burst. The rigid matrix of the high-molecular-weight polymer permits a slower diffusion of the drug through the matrix as compared to low-molecular-weight polymer (Niwa et al., 1993). Implicitly, the release rate is more sustained for high molecular weight PLGA.

It is well established in the literature that the degradation of PLGA is by bulk erosion. The release rate of the drug from the polymer matrix can be controlled by changing the ratio of the monomers in the co-polymer (Yoo, 2000). PLGA 85/15 and PLA are more hydrophobic than PLGA 50/50 (Lee, 2002) as a result of the higher hydrophilicity of the glycolil units in comparison with lactil units. Different diffusion rates of the aqueous phase into the polymer matrix were seen as a result of a different lactic to glycolic ratio in the co-polymers (Cascone et al., 2002). An increase in the glycolil units in the copolymer it will increase the absorption of water in the polymer matrix (Cascone, 2002). If the concentration of lactide is higher in the copolymer, the release of the drug is lower, possible due to the hydrophobic interaction between the polymer and the drug (Lee et al., 2002).

3.3.3. Type of the Drug Entrapped into the Nanoparticles

In general, the release profile of hydrophilic compounds shows a strong initial burst followed by a fast release of the drug, while for the hydrophobic compounds the release profile is much slower and more constant. The release of the drugs from nanoparticles depends on the nature of interactions between the drug and the polymer and on the affinity of the drug for the release medium (Birnbaum and Brannon-Peppas, 2003).

3.3.4. Ratio Polymer to Drug in the Nanoparticles

The amount of drug entrapped in the particles can affect the release rate. An increase in the drug amount in the nanoparticles results in an increase of the release rate for both hydrophobic (Mainardes et al. 2005) and hydrophilic drugs (Birnbaum and Brannon-Peppas, 2003). Mainardes et al. (2005) showed that the release of praziquantel (hydrophobic drug) in time was faster when the amount of drug entrapped was higher. The smaller the entrapped drug quantity the more uniformed the release was.

3.3.5. Freeze-drying

Yeo and Park (2004) studied the in vitro release profile of lysozyme after it was encapsulated in PLGA matrix with and without freeze-drying. The release test was performed for microcapsules suspended in the release medium. The microcapsules showed a different release profile depending on whether they were previous dried or not. Freeze-drying process appeared to induce damage, compromising the physical integrity of the microcapsules, and therefore affecting the release profile.

3.3.6. Release Medium

Many studies reported the use of phosphate buffered saline (PBS) as a release medium for PLGA loaded nanoparticles (Mu and Feng, 2002; Mu and Feng, 2003; Yoo et al., 2000; Mu and Feng, 2003a; Cascone et al., 2002; Redhead et al., 2001; Birnbaum and Brannon-Peppas, 2003; Sansdrap and Moes, 1997; Lamprecht et al., 2001; Niwa et al., 1993; Fonseca et al., 2002). Non-toxic surfactants were added to the release medium in order to increase the release rate of highly hydrophobic compounds. Tween 80 was added to the phosphate buffer release medium to increase the release of cyclosporin A from the PLA and PLGA nanoparticles (Lee et al., 2002) or of amoxicillin from PLGA microspheres (Kim and Burgess, 2002). Tween 80 was also added to PBS medium in which PEG-PLGA nanoparticles loaded with a protein were placed (Li et al., 2001).

3.3.7. Additives

A few methods for improving the release of drugs from PLGA microparticles were mentioned, especially for highly hydrophobic drugs. The addition of additives such as isopropyl myristate or sucrose, to the release medium accelerated the release of hydrophobic drugs. In vitro

release of taxol from PLGA matrix was increased by adding isopropyl myristate which changed the microparticle matrix allowing the formation of channels, which allowed for a faster diffusion of the drug from the microparticle (Birnbaum and Brannon-Peppas, 2003). Fatty acid esters added increased the release rate of hydrophobic drugs by solubilizing the drug and passing it trough these channels (Lee et al., 2002). Lee et al. (2002) studied the PLGA nanoparticles loaded with dexamethasone and entrapped into PVA hydrogels to establish the release profile of the drug. No important change in the release profile of the drug was observed by incorporating the nanoparticles into the PVA matrix.

Redhead et al., (2001) studied the release of Rose Bengal from PLGA nanoparticles. PLGA nanoparticles under 250 nm in size were coated with copolymers of the poloxamer and poloxamine series. Coating the particles with these copolymers minimized their capture by macrophages in vitro and in vivo, which lead to an increase in their life-time in the circulation system. The drug loading into these coated nanoparticles was a little higher than drug loading in uncoated particles. This behavior was attributed to the presence of the surfactant on the nanoparticles surfaces which adsorbed more drug into the matrix. The release profile observed was biphasic, showing an initial burst, as a result of the release of the drug present at the surface, followed by a slower release of the drug entrapped inside the PLGA matrix, by diffusion. Half of the drug entrapped in these coated particles was released in 10 hours.

3.4. Objectives

1. Synthesis of poly (D,L-lactide-co-glycolide) nanoparticles with entrapped α-tocopherol by emulsion evaporation method, with PVA as surfactant.

2. Characterization of PLGA(αT) nanoparticles in terms of morphology (TEM), size, size distribution, and zeta potential (DLS), and entrapment efficiency (HPLC).

3. Study of the release of α-tocopherol from the PLGA(αT) nanoparticles.

3.5. Materials and Methods

3.5.1. Chemicals and Materials

Poly(DL-Lactide-co-Glycolide) (PLGA) 50:50 with an average molecular weight of 45,000-75,000, poly(vinyl alcohol) (PVA) with an average molecular weight of 30,000-

70,000, α-tocopherol 95%, boric acid 99.99%, iodine puriss p.a., and potassium iodide, 99.99% were purchased from Sigma Aldrich (Sigma Chemical Co, St Louis, MO). Dichloromethane, ethyl acetate, and acetonitrile were obtained from EMD (EMD Chemicals Inc., Gibbstown, NJ). Sodium hydroxide and sodium chloride were acquired from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ), hydrochloric acid 1.0 N was obtained from VWR International (VWR International, West Chester, PA), and sodium dodecyl sulfate (SDS) (20% w/v) was purchased from Amresco (Amresco Inc., Solon, OH). Nanopure water was obtained using Nanopure Diamond (Barnsted International, Dubuque, Iowa) and 0.2 μm Barnsted D3750 Hollow Fibre Filter.

3.5.2. Synthesis of Nanoparticles with Entrapped α-tocopherol

Synthesis and characterization of nanoparticles were studied in triplicate and the release profiles of α-tocopherol in aqueous medium were studied in duplicate. Two concentrations of α-tocopherol, 8 and 16% w/w (relative to PLGA), were incorporated into the PLGA matrix. Unloaded PLGA nanoparticles were synthesized and they were used as control systems. Two types of nanoparticles were prepared using two surfactants, PVA and SDS, by emulsion evaporation method in the following ways:

a) Synthesis of Nanopaticles with SDS as a Surfactant

PLGA nanoparticles with entrapped α-tocopherol and SDS as an emulsifier were synthesized by emulsion evaporation method as follows. The organic phase was formed by dissolving 125 mg PLGA and α-tocopherol in 2.5 ml ethyl acetate. Two α-tocopherol concentrations were studied: 8% and 16% w/w (reported to PLGA). The organic phase was added to 2 mg/ml aqueous SDS solution (in nanopure water saturated with ethyl acetate) and emulsified with the homogenizer (Ultra Turrax T 18, IKA Works Inc., Wilmington, NC) for 3 min at 12,000 RPM. The emulsion was sonicated in an ice bath at 4˚C with a probe-type sonicator set at 750 W (VC505, Sonics & Materials Inc., Newtown, CT) for 10 min, in pulse mode and an amplitude of 38%. The ethyl acetate was evaporated under vacuum (40 mmHg) and nitrogen flow (100 ml/min) for 7 min in a rotovapor (Buchi R-124, Buchi Analytical Inc., New Castle, DE) (Astete and Sabliov, 2006). Unloaded nanoparticles were prepared following the same method, without adding the α-tocopherol.

b) Synthesis of Nanoparticles with PVA as a Surfactant

Synthesis of nanoparticles with entrapped α-tocopherol was accomplished following the guidelines presented in Figure 3.1.

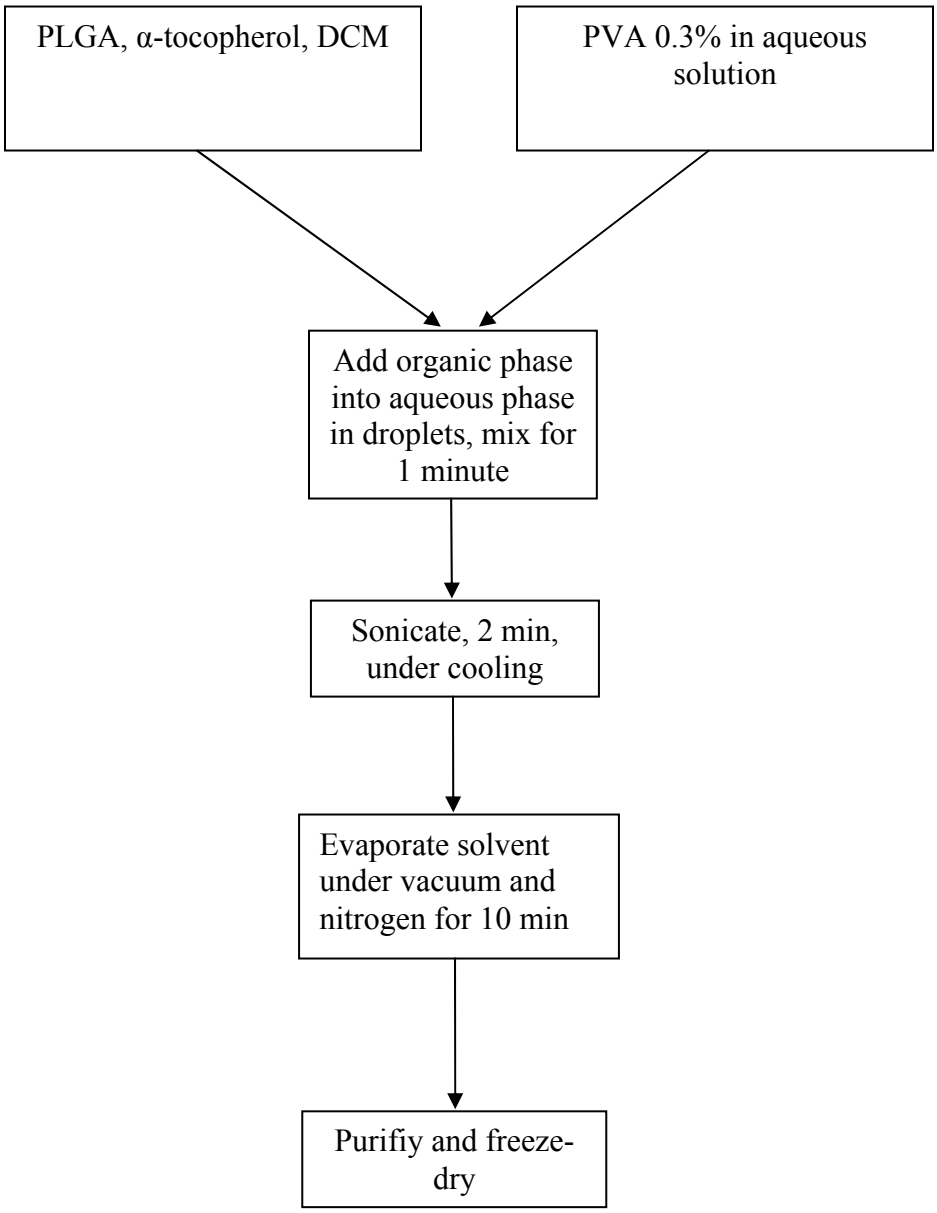

Figure 3.1. Schematic diagram for synthesis of nanoparticles with entrapped **α**-tocopherol using PVA as a surfactant

The organic phase was formed by dissolving 50 mg of PLGA in 2 ml dichloromethane. Two α-tocopherol concentrations were studied, 8% and 16% w/w (reported to PLGA); α-tocopherol at these concentrations was dissolved in dichloromethane. An aqueous phase was formed with 0.3% polyvinyl alcohol in nanopure water. The organic phase was added in droplets to the aqueous phase, and the emulsion was formed under homogenization (Ultra Turrax T 18, IKA Works Inc., Wilmington, NC) for 1 min at 10,000 RPM. The emulsion was sonicated in an ice bath at 4˚C with a probe-type sonicator of 750 W (VC505, Sonics & Materials Inc., Newtown, CT) for 2 min, in pulse mode at 38% amplitude. Dichloromethane was evaporated under vacuum (40 mmHg) and nitrogen flow (100 ml/min) for 10 min in a rotoevaporator (Buchi R-124, Buchi Analytical Inc., New Castle, DE) (Mainardes and Evangelista, 2005). Unloaded nanoparticles were prepared following the same method, without adding α-tocopherol to the organic solvent and they were used as a control system. Following synthesis, nanoparticles were purified by diafiltration to remove the excess α-tocopherol and surfactant. A GE Healthcare MidJet labscale membrane separations system with MidGee HOOP Cartrige H22LA of 1 μm pore size (Amersham Biosciences Corporation, Westborough, MA) was used for this purpose. The purification process was realized during one hour at 23 psgi inlet pressure and 18 psgi outlet pressure. After purification, nanoparticles were kept for two hours at - 80˚C and freeze-dried at - 41˚C under 110 mmHg of vacuum for 48 hours in a freezone 4.5 (Labconco Corporation, Kansas City, MO). Trehalose was added to the nanoparticles suspension before precooling at a ratio of 1:1 (w/w) relative to the amount of nanoparticles. Finally, the lyophilized samples were stored in desiccators placed in the refrigerator for further use.

3.5.3. Characterization of Nanoparticles with Entrapped α-tocopherol

Size, Size Distribution, and Zeta Potential

Nanoparticles were characterized in terms of size, size distribution, and zeta potential by dynamic light scattering (DLS) using the Malvern Zetasizer Nano ZS (Malvern Instruments Inc., Southborough, MA). Size and PDI measurements were made for freshly synthesized nanoparticles (prior to purification), for purified nanoparticles, and for resuspended nanoparticles. In all cases, a volume of 1.3 ml of each sample at a concentration of 0.3 mg/ml was placed in a polystyrene cuvette and the measurements were performed at 25ºC. The viscosity and refraction index was set to those specific to water. Zeta potential was measured

with a disposable capillary cell with a volume of 1 ml after purification. The mean values of size and PDI were determined using a mono-modal distribution.

Nanoparticle Morphology

The morphology of the nanoparticles was studied by Transmission Electron Microscopy (TEM) using a JEOL 100-CX (JEOL USA Inc., Peabody, MA) system. One droplet of the aqueous phase containing nanoparticles was placed on a copper grid of 400 mesh with carbon film. The excess of sample was removed with a filter paper. Uranium acetate 2% was used as a stain; the excess uranium acetate was removed after 1 min with a filter paper. The sample was dried before analysis by TEM.

Entrapment Efficiency

The entrapment efficiency of α-tocopherol in the PLGA nanoparticles was measured by reverse phase high-performance liquid chromatography (RP-HPLC). A sample of 2 mg lyophilized nanoparticles was dissolved in 2 ml acetonitrile:water (95:5). One milliliter of each sample was centrifuged with an Allegra 64R Centrifuge (Beckman Coulter, Fullerton, CA) at 30,000 RPM for 15 min in order to separate any solid residual. The entrapment efficiency of alpha-tocopherol was determined by injecting 25 µL of previous prepared sample into the HPLC column. The mobile phase used was acetonitrile: water: acetic acid in a ratio of 95:5:0.01 at a flow rate of 1.5 ml/min and a total run time of 20 min/sample. The column used was Discovery C18, 25 cm x 4.6 mm, 5 µm (Supelco, Sigma-Aldrich Corp., St. Louis, MO, USA), and the detector was a Waters 474 Scaning Fluorescence Detector (Waters Corporation, Milford, MA). The excitation wavelength was set at 290 nm and the emission wavelength at 320 nm. A standard curve was prepared under the same conditions. Chromatograms were recorded and processed using a Millennium 32 chromatography software. Alpha-tocopherol entrapment efficiency was expressed as percentage of the alpha-tocopherol entrapped into the nanoparticles reported to the initial amount of alpha-tocopherol used for nanoparticles preparation.

3.5.4. Determination of the Residual PVA Associated to the Nanoparticles Surface

The residual PVA associated with the nanoparticles was determined by a colorimetric method based on the formation of a colored complex between iodine and two adjacent hydroxyl groups of PVA (Sahoo et al., 2002). Typically, 3 mg of lyophilized nanoparticles were dissolved in 2 ml nanopure water. Two milliliters of sodium hydroxide 0.5 M were added and the mixture

was kept on a water bath at 60ºC for 15 min. For neutralization of each sample, 0.9 ml hydrochloric acid 1 N was added and the volume was adjusted to 7 ml with nanopure water. Three milliliters of 0.65M boric acid were added to each sample together with 0.5 ml of a I_2/KI solution (0.05M/0.15M). The final volume was adjusted to 12 ml with water. The absorbance of the samples was measured with a UV-Visible Beckman Coulter spectrophotometer (Beckman Coulter, Fullerton, CA) after 15 min of incubation at room temperature at 690 nm. A standard curve for PVA was prepared under the same conditions.

3.5.5. Controlled Release

Lyophilized nanoparticles were dissolved in the release medium, containing 0.1 M SDS/0.1 M NaCl, at a concentration of 1 mg/ml; the sample was divided in 10 samples of 1 ml. The controlled release study was performed at 37˚C and 100 RPM in an incubator shaker C25 KC (New Brunswick Scientific Inc., Edison, NJ). At predetermined time intervals, samples were withdrawn and centrifuged with an Allegra 64R Centrifuge (Beckman Coulter, Fullerton, CA) at 30,000 RPM for 15 min in order to settle the nanoparticles. After the removal of the supernatant which contained the alpha-tocopherol released, the nanoparticles were dissolved in the mobile phase and centrifuged at 30,000 rpm for 15 min. The supernatant containing the solvent with alpha-tocopherol was analyzed by reverse-phase HPLC. The mobile phase was acetonitrile:water:acid acetic in the ratio 95:5:0.01, the injected volume was 50 µl and the flow rate 1.5 ml/min. The column used was Discovery C18 25 cm x 4.6 mm, 5 µm (Supelco, Sigma-Aldrich Corp., St. Louis, MO, USA), and the detector was Waters 474 Scaning Fluorescence Detector (Waters Corporation, Milford, MA).

3.5.6. Statistical Analysis

The experimental data for size and PDI of nanoparticles was analyzed by two-way ANOVA using Proc Mixed procedure (SAS system, SAS Institute Inc., Cary, NC). The experiment was designed as a two-factor (alpha-tocopherol theoretical loading and the processing parameters after synthesis) treatment structure with three levels for each factor. Multiple comparison tests were done by Tukey's adjustment to determine the significant difference between treatments at $P<0.05$. The experimental data for residual PVA associated to nanoparticles, entrapment efficiency of alpha-tocopherol in the nanoparticles, and nanoparticles zeta potential were analyzed by one-way ANOVA using Proc Mixed procedure (SAS system,

SAS Institute Inc., Cary, NC). Multiple comparison tests were done by Tukey-Kramer adjustment to determine the significant difference between treatments at $P<0.05$.

3.6. Results and Discussion

3.6.1. Characterization of Nanoparticles

Morphology of Prepared Nanoparticles

The nanoparticles with entrapped alpha-tocopherol revealed a spherical shape with a narrow size distribution for nanoparticles prepared with different surfactants, SDS (Figure 3.2.) or PVA (Figure 3.5.), for the two alpha-tocopherol concentrations tested 8% (Figure 3.3, Figure 3.4.) and 16% (Figure 3.6., and Figure 3.7.).

The TEM pictures of 8 and 16% α-tocopherol theoretical loaded nanoparticles prepared with SDS as an emulsifier showed a good distribution and small nanoparticle sizes (Figure 3.3. and Figure 3.4.). The higher density of SDS synthesized nanoparticles observed in the pictures can be explained by their smaller size and higher amount of the initial components, as compared to the PVA synthesized nanoparticles. The initial amount of PLGA in the solvent was 50 mg/ml for SDS nanoparticles as compared with 25 mg/ml for PVA synthesized nanoparticles. Also, the difference between the two types of surfactants; SDS is a small anionic compound with a high affinity for water whereas PVA is a high MW polymer less prone to leave the nanoparticles for the water, also explains the higher density of the SDS synthesized nanoparticles.

The TEM pictures of 8 and 16% α-tocopherol theoretical loaded nanoparticles prepared with PVA as a surfactant showed a good size distribution and a small nanoparticle size (Figure 3.6. and Figure 3.7.); however, bigger nanoparticle sizes were observed as compared with those of SDS nanoparticles. A clear visual difference in size between SDS and PVA synthesized nanoparticles with entrapped α-tocopherol was noticed. No distinct difference was noticed however among the 0, 8, and 16% α-tocopherol theoretical loading for each surfactant. Also, the presence of PVA as a corona on the nanoprticles surface was visible in the TEM pictures; the corona was not noticed on the nanoparticles surface when prepared with SDS. The presence of α-tocopherol in the polymeric matrix could not be determined by TEM.

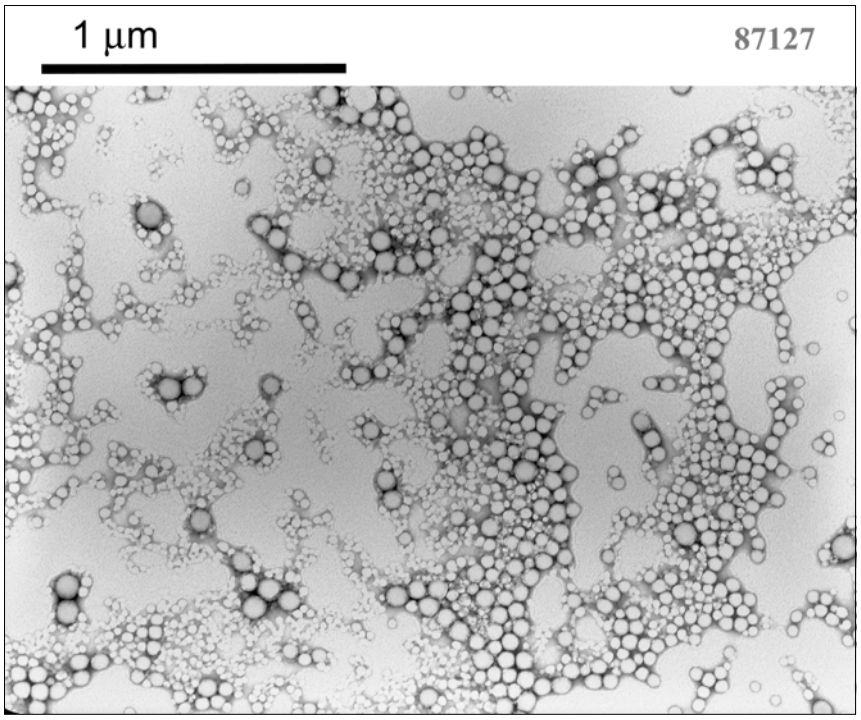

Figure 3.2. TEM picture of nanoparticles prepared with SDS as an emulsifier with 0% alpha-tocopherol theoretical loading

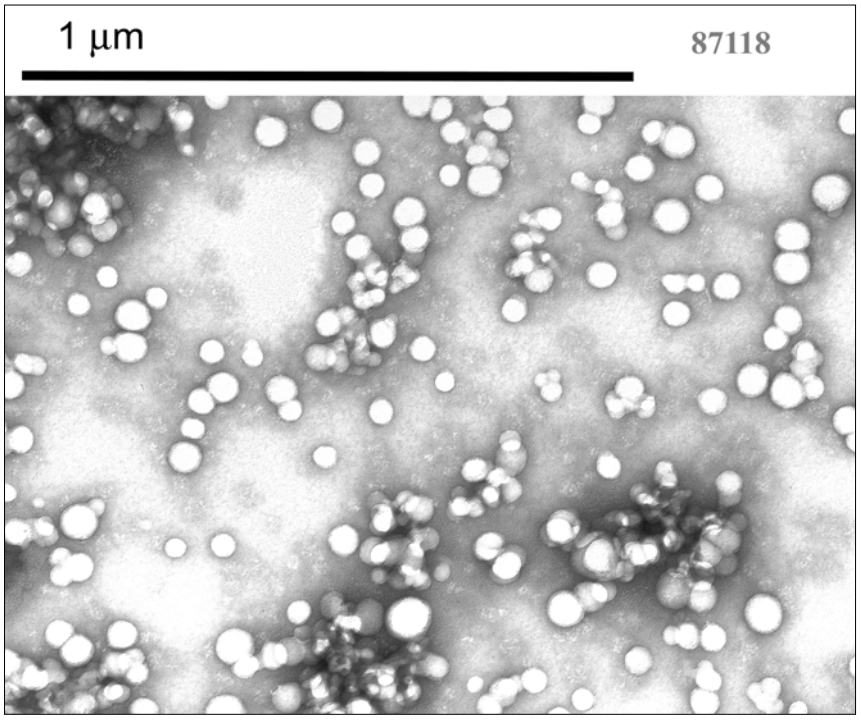

Figure 3.3. TEM picture of nanoparticles prepared with SDS as an emulsifier with 8% alpha-tocopherol theoretical loading

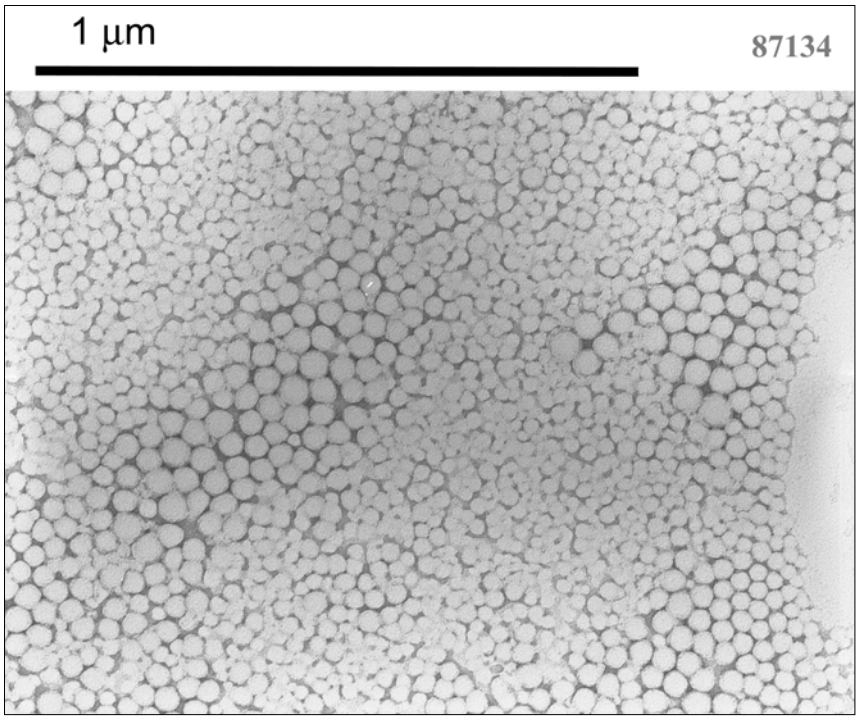

Figure 3.4. TEM picture of nanoparticles prepared with SDS as an emulsifier with 16% alpha-tocopherol theoretical loading

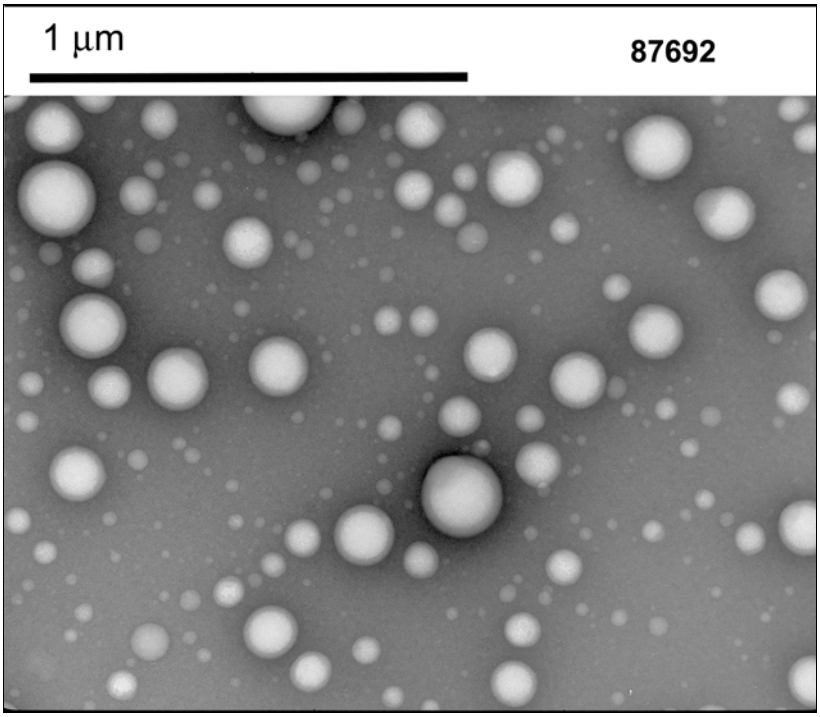

Figure 3.5. TEM picture of nanoparticles prepared with PVA as an emulsifier with 0% alpha-tocopherol theoretical loading

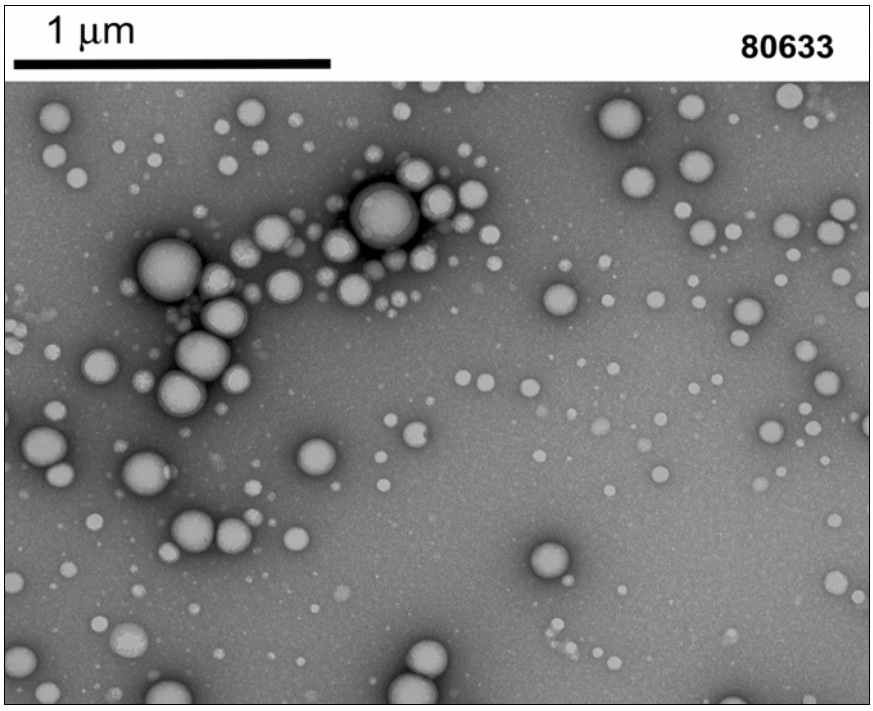

Figure 3.6. TEM picture for nanoparticles prepared with PVA as an emulsifier with 8% alpha-tocopherol theoretical loading

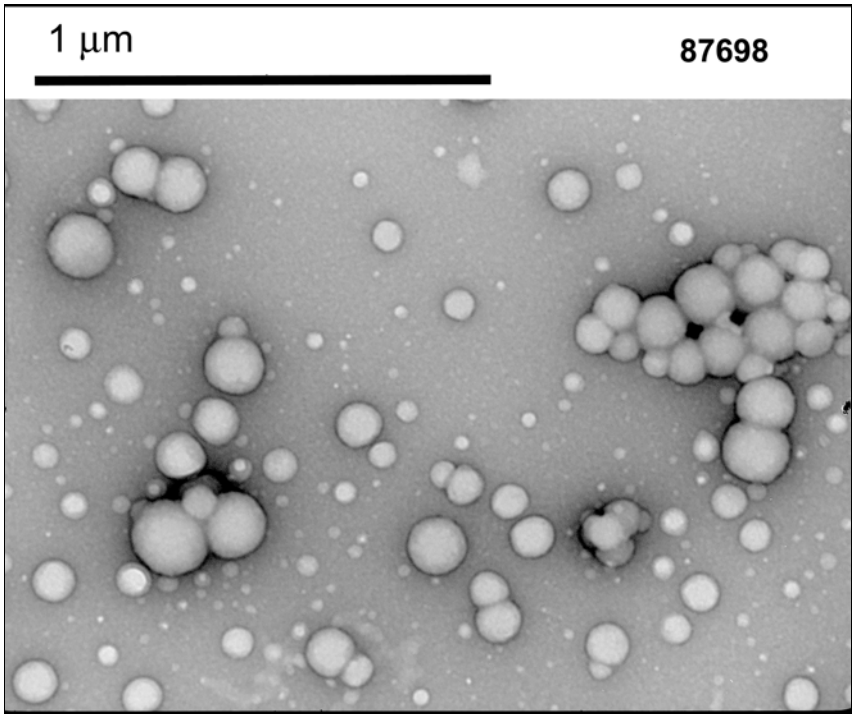

Figure 3.7. TEM picture of nanoparticles prepared with PVA as an emulsifier with 16% alpha-tocopherol theoretical loading

Size and PDI of Nanoparticles

Size and PDI (polydispersity index) for PVA nanoparticles was measured before and after purification, and after resuspension of lyophilized nanoparticles (Table 3.1.). For SDS nanoparticles, size and PDI was measured only after synthesis because of the difficulties in the purification step encountered as a result of the small size of the synthesized nanoparticles (Table 3.2.).

For PVA nanoparticles, the size of freshly synthesized nanoparticles before purification decreased but not significantly with the entrapment of α-tocopherol in the PLGA matrix, from 216.2 to 206.3 nm for 0% and 16% α-tocopherol theoretical loading, respectively (Table 3.1.). After purification by diafiltration, the size of nanoparticles increased slightly in all cases. The freeze-drying process caused an increase in the nanoparticles size with approximately 67 nm for nanoparticles with 8% α-tocopherol theoretical loading, and with approximately 32 nm for nanoparticles with 16% α-tocopherol theoretical loading. For unloaded nanoparticles, the increase in size after freeze-drying was not significant (Figure 3.8.). As compared with PVA nanoparticles, the size of SDS nanoparticles before purification was much smaller, 71 nm for SDS unloaded nanoparticles versus 216 nm for PVA unloaded nanoparticles. This high difference in size can be explained by differences in synthesis method, but mostly by the type of surfactant used. PVA is a big molecule as compared with SDS, so the residual PVA attached to the nanoparticles surface can cause this increase in size for PVA nanoparticles. Moreover, SDS is an ionic surfactant more efficient in reducing the size of the emulsion droplets directly correlated with the nanoparticles size as compared to PVA. An important change in size was noticed for SDS nanoparticles with alpha-tocopherol entrapment. With the entrapment of α-tocopherol in the PLGA matrix, the nanoparticles size decreased significantly from 71.2 nm for unloaded nanoparticles to 57.2 nm for nanoparticles with 8% α-tocopherol theoretical loading. By further increasing the amount of α-tocopherol theoretical loading to 16%, a further decrease in the size of nanoparticles was noticed (Table 3.2.). This behavior may be explained by the presence of α-tocopherol, which seems to accumulate on the nanoparticles surface and act as a co-surfactant together with SDS.

The PDI for PVA nanoparticles did not change significantly with the entrapment of α-tocopherol in the polymeric matrix (Table 3.1.). The PDI was lower than 0.100 in all cases for the samples before purification. After purification by diafiltration, the PDI increased to

approximately 0.150. Important amounts of PVA were removed during the purification process and this could cause some agglomeration of nanoparticles.

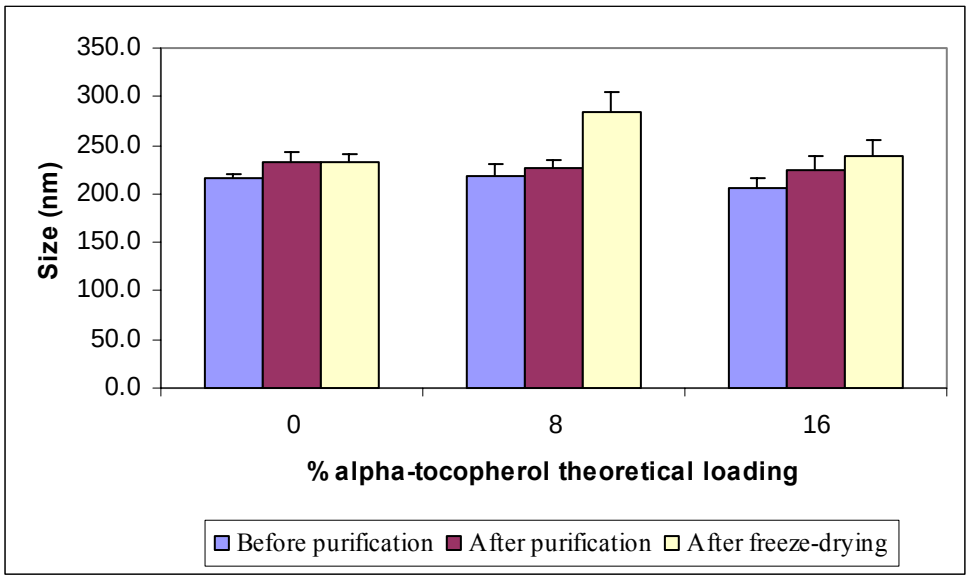

Figure 3.8. Variation of size of PVA nanoparticles with α-tocopherol theoretical loading and processing parameters after synthesis. n=3.

After the freeze-drying process, the PDI change was not significant as compared with the PDI of nanoparticles before purification for unloaded nanoparticles, but it increased for the nanoparticles with 8% α-tocopherol theoretical loading from 0.093 to 0.239, and for 16% α-tocopherol theoretical loading from 0.061 to 0.155 (Figure 3.9.). The PDI for SDS nanoparticles increased significantly with the entrapment of 8% α-tocopherol theoretical loading from 0.096 for 0% α-tocopherol theoretical loading to 0.136 for 8% α-tocopherol theoretical loading (Table 3.2.). A further increase of the α-tocopherol theoretical loading to 16% did not change the PDI significantly. Overall, the PDI for PVA nanoparticles before purification was smaller as compared with the PDI of SDS nanoparticles.

Table 3.1. Size and PDI of nanoparticles as a function of α-tocopherol theoretical loading and processing parameters after synthesis. n=3.

aT theoretical loading (% w/w relative to PLGA)	Size (nm)			PDI (au)		
	Before purification	After purification	After freeze-drying	Before purification	After purification	After freeze-drying
0	$216.2 \pm 3.8^{a,b}$	$231.8 \pm 10.3^{b,c}$	$232.8 \pm 8.0^{b,c}$	$0.083 \pm 0.023^{a,b}$	0.158 ± 0.056^{c}	$0.132 \pm 0.026^{b,c}$
8	$217.2 \pm 12.8^{a,b}$	$225.8 \pm 9.6^{b,c}$	284.0 ± 20.4^{d}	$0.093 \pm 0.012^{a,b}$	$0.130 \pm 0.044^{b,c}$	0.239 ± 0.031^{d}
16	206.3 ± 9.4^{a}	$223.6 \pm 14.7^{a,b,c}$	238.8 ± 16.5^{c}	0.061 ± 0.028^{a}	0.151 ± 0.059^{c}	0.155 ± 0.037^{c}

Significantly different values ($P<0.05$) of size and PDI are indicated by different letters a,b,c.

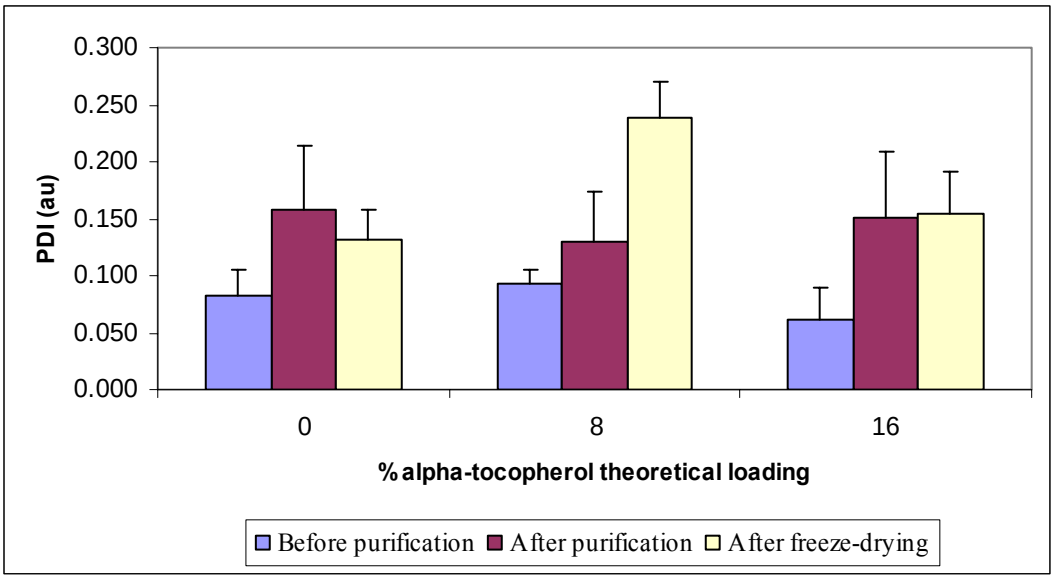

Figure 3.9. Variation of PDI of PVA nanoparticles with α-tocopherol theoretical loading and processing parameters after synthesis. n=3.

Table 3.2. Size and PDI of nanoparticles before purification as a function of α-tocopherol theoretical loading. n=3.

aT theoretical loading (% w/w relative to PLGA	SDS		PVA	
	Size (nm)	PDI (au)	Size (nm)	PDI (au)
0	71.2 ± 0.33[a]	0.096 ± 0.012[a]	216.2 ± 3.8[a,b]	0.083 ± 0.023[a,b]
8	57.2 ± 0.28[b]	0.136 ± 0.015[b]	217.2 ± 12.8[a,b]	0.093 ± 0.012[a,b]
16	53.6 ± 0.30[c]	0.150 ± 0.003[b]	206.3 ± 9.4[a]	0.061 ± 0.028[a]

Significantly different values ($P<0.05$) of size and PDI in the same column are indicated by different letters a,b,c,.

Zeta Potential of Nanoparticles

Zeta potential is another important parameter in characterization of the nanoparticles. A highly negative or positive zeta potential indicates high repulsive forces and implicitly low probability for nanoparticles to agglomerate. Under these circumstances, a long-term stability of the nanoparticles can be expected. Zeta potential of the nanoparticles was negative for unloaded nanoparticles and for nanoparticles with entrapped α-tocopherol (Table 3.3.). This negative value was expected based on the presence of PVA at the surface of nanoparticles. With the entrapment of α-tocopherol in the polymeric matrix, zeta potential became less negative. It increased significantly from -28.67 mV for unloaded nanoparticles to -13.56 mV for nanoparticles with 8% α-tocopherol theoretical loading. By further increasing the α-tocopherol theoretical loading to 16%, a not significant increase in zeta potential to -12.66 mV was noticed.

Table 3.3. Residual PVA associated with the nanoparticles, entrapment efficiency of α-tocopherol, and zeta potential of the resuspended nanoparticles. n=3.

aT theoretical loading (% w/w relative to PLGA)	Zeta potential (mV)	Entrapment efficiency (% of αT theoretical loading)	Residual PVA (% w/w from nanoparticles)
0	-28.67 ± 1.82[a]	0	5.09 ± 0.41[a]
8	-13.56 ± 2.33[b]	89.63 ± 11.63[a]	6.80 ± 0.75[a]
16	-12.66 ± 5.09[b]	95.59 ± 3.08[b]	7.16 ± 1.53[a]

Significantly different values (P<0.05) in the same column are indicated by different letters a,b,c,.

Entrapment Efficiency of α-tocopherol in the Nanoparticles

Entrapment efficiency is used to indicate the amount of drug entrapped into the polymeric matrix. The entrapment efficiency of 8% α-tocopherol theoretical loading was 89.63% and by a further increase in the initial α-tocopherol to 16%, the entrapment efficiency increased to 95.59% (Table 3.3.). The more α-tocopherol was added to the organic phase the higher the entrapment efficiency. This is in agreement with the data found in the literature about hydrophobic components whose entrapment efficiency is usually greater than 90% (Birnbaum and Brannon-Peppas, 2003).

Residual PVA Associated with the Nanoparticles

The residual PVA associated with the nanoparticles has an important significance for the stability of nanoparticles, by preventing the nanoparticles to aggregate; the residual PVA also affects the release profile of the drug from the polymeric particles. The residual PVA associated with the nanoparticles after purification was 5.09% (w/w relative to the nanoparticles) for unloaded nanoparticles. For nanoparticles with 8% α-tocopherol theoretical loading the residual PVA was 6.80%, and for 16% α-tocopherol theoretical loading the residual PVA was 7.16% (Table 3.3.).

3.6.2. Release Profile of α-tocopherol from PLGA Matrix

The release profile was studied for nanoparticles loaded with 8 and 16% α-tocopherol theoretical loading and prepared with PVA as an emulsifier. The release profile of α-tocopherol from PLGA matrix in 0.1 M SDS/0.1 M NaCl at 37ºC was biphasic, showing an initial burst followed by a slower release of the drug entrapped inside the PLGA matrix. The release profile showed an initial burst effect in the first hour for both, 8 and 16% α-tocopherol theoretical loading (Figure 3.10.). The burst effect was higher for 8% α-tocopherol theoretical loading when

approximately 86% was released in the first hour followed by a constant release in the next hours. In 24 hours approximately 97% of α-tocopherol was released with 100% released in 48 hours. The burst effect for 16% α-tocopherol theoretical loading was lower compared with that for 8% α-tocopherol theoretical loading. The burst effect noticed in the first hour was approximately 34% of the α-tocopherol determined at time zero. After the burst effect, the release profile for 16% α-tocopherol theoretical loading showed a uniform release in time. After 7 hours approximately 67% of α-tocopherol was released from nanoparticles with approximately 82% and 90% α-tocopherol released after 24 and 48 hours, respectively. The burst effect can be explained by the fast release of α-tocopherol found close to or attached to the surface of nanoparticles. The affinity of α-tocopherol to the release medium can be another reason for the fast release of α-tocopherol. The constant release profile that was noticed following the burst effect can be explained by the slow diffusion of α-tocopherol found inside the PLGA matrix. The release profile for nanoparticles prepared with SDS as an emulsifier was not studied because of the problems encountered during the purification step as a result of small nanoparticles sizes.

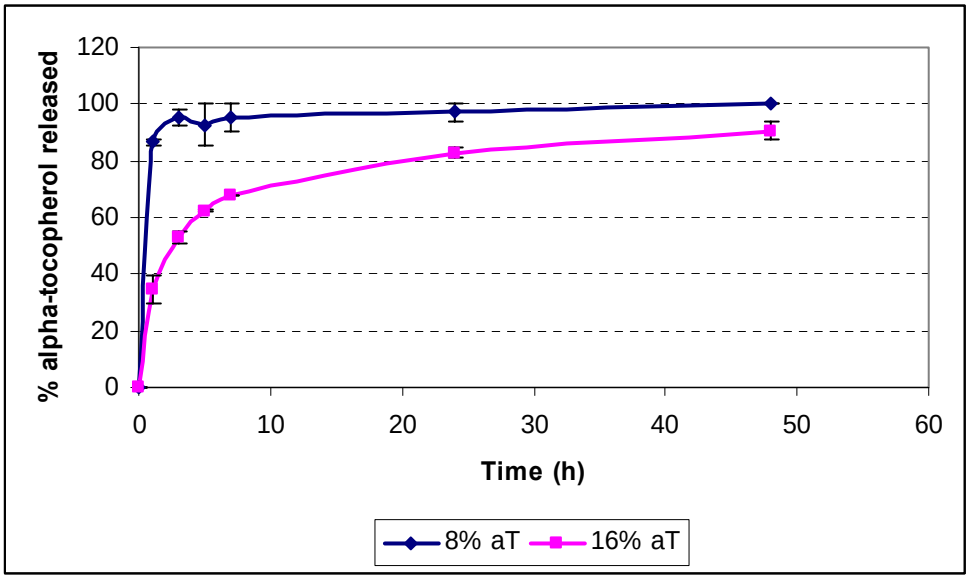

Figure 3.10. Release profile of α-tocopherol from PLGA matrix in 0.1 M SDS/0.1 M NaCl.

3.7. Conclusion

Emulsion evaporation method was a suitable technique for synthesis of spherical PLGA nanoparticles with a small size diameter, less than 100 nm for nanoparticles prepared with SDS as a surfactant, and about 200 nm for nanoparticles prepared with PVA as an emulsifier. For

nanoparticles prepared with SDS, the size of the nanoparticles significantly decreased with the entrapment of α-tocopherol in the PLGA matrix, from approximately 71 nm for unloaded nanoparticles to approximately 53 nm for nanoparticles with 16% α-tocopherol theoretical loading. For nanoparticles prepared with PVA, no significant change in the size of nanoparticles was noticed with the entrapment of α-tocopherol. The polydispersity index after synthesis was lower, under 0.100, for PVA nanoparticles, and around 0.150 for SDS nanoparticles. The polydispersity index for PVA nanoparticles increased during the purification step and during freeze-drying, but at all times it was not higher than 0.250. Zeta potential was negative for all nanoparticles prepared with PVA. The entrapment efficiency of α-tocopherol in the polymeric matrix was approximately 89% for nanoparticles with 8% α-tocopherol theoretical loading and approximately 95% for nanoparticles with 16% α-tocopherol theoretical loading. The residual PVA associated with the nanoparticles after purification was approximately 6% (w/w relative to the amount of nanoparticles) and it did not change with the amount of α-tocopherol entrapped. The release profile of α-tocopherol from PLGA matrix in the release media showed an initial burst effect followed by a more uniform release in the following hours. The release for nanoparticles with 8% α-tocopherol theoretical loading was faster with 86% release in one hour and 100% release in 48 hours. For the nanoparticles with 16% α-tocopherol theoretical loading, 34% of α-tocopherol was released in one hour and 90% in 48 hours.

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CHAPTER 4. CONCLUSION

The first part of this research was focused on microwave-assisted extraction method which proved to be an efficient method for the extraction of rice bran oil and vitamin E components from rice bran. Hexane extracted more rice bran oil as compared to isopropanol at 40ºC. At higher temperature, isopropanol proved to be a better solvent for rice bran oil extraction. Hexane extracted large amount of α-tocotrienol at 120ºC, while the increase in temperature for isopropanol was more beneficial for the extraction of γ-tocopherol. Also, antioxidant activity of isopropanol extracted oil was higher than that of hexane extracted oil; at higher temperature isopropanol seemed to extract more antioxidants. No significant differences in the oil yield, total vitamin E, and antioxidant activity of rice bran oil was noticed between the two extraction methods, conventional solvent and microwave-assisted extractions, at 40ºC.

Encapsulation of α-tocopherol in the Poly (DL-lactide-co-glycolide) (PLGA) matrix was the basis of the second part of this study. Emulsion evaporation method was a suitable technique for synthesis of spherical PLGA nanoparticles with entrapped alpha-tocopherol 100 nm in size when SDS was used as a surfactant, and less than 250 nm when PVA was used as a surfactant. For nanoparticles prepared with SDS, the size of the nanoparticles decreased significantly when α-tocopherol was entrapped in the PLGA matrix, while the size of the PVA nanoparticles remained unchanged. The polydispersity index after synthesis was lower, under 0.100 for PVA nanoparticles and around 0.150 for SDS nanoparticles, and it increased during the purification step and during freeze-drying to values as high as 0.250. Zeta potential was negative for all nanoparticles prepared with PVA. The entrapment efficiency of α-tocopherol in the polymeric matrix was approximately 89% for nanoparticles with 8% α-tocopherol theoretical loading and approximately 95% for nanoparticles with 16% α-tocopherol theoretical loading for PVA synthesized nanoparticles. The residual PVA associated to the nanoparticles after purification was approximately 6% (w/w relative to the amount of nanoparticles) and it was not a function of the amount of α-tocopherol entrapped. The release profile of α-tocopherol from PLGA matrix was biphasic, showing an initial burst followed by a slower release of the α-tocopherol entrapped inside the PLGA matrix. The release for nanoparticles with 8% α-tocopherol theoretical loading (86% α-tocopherol released /first hour) was faster than the release for the nanoparticles with 16% α-tocopherol theoretical loading (34% α-tocopherol released /first hour).

CHAPTER 5. FUTURE WORK

The main purpose of this thesis was divided into two parts: 1) to extract and quantify vitamin E components from the rice bran oil and to test their antioxidant activity and 2) to entrap α-tocopherol in the polymeric matrix and to characterize the prepared nanoparticles as well as to study the release profile of α-tocopherol from the PLGA matrix. The following future work is proposed to further the studied objectives:

Part I

- Detailed analysis of antioxidant components of rice bran oil extracted by microwave-assisted and conventional solvent extractions.
- Study of the effect of the extraction time on the oil yield and vitamin E components by microwave-assisted method.
- Determination of the extraction efficiency of different solvents in microwave-assisted extraction (specifically at high temperature).

Part II

- Study the controlled release of α-tocopherol from polymeric nanoparticles in different media. If the nanoparticles are used for parenteral administration, PBS is a recommended medium to study the release profile in, if the nanoparticles are used in a food application, the release profile of α-tocopherol in solutions with different pHs and chemical components should be tried.
- For medical and food applications the use of natural polymers and natural surfactants for the preparation of nanoparticles has a crucial importance.
- Study of alternative purification methods for the removal of the excess surfactant and drug, avoiding agglomeration or nanoparticles loss.
- Study the degradation of α-tocopherol in free form and encapsulated form under different environmental conditions.
- Elucidate the mechanism by which α-tocopherol is reducing the size of nanoparticles when it is entrapped in the nanoparticles prepared with SDS.
- Entrap and release other bioactive components (i.e. ω-3 PUFAs).
- Test the cellular uptake of PLGA(αT) nanoparticles and study the release profile of α-tocopherol in cell culture.

APPENDIX A. STANDARD CURVES

A.1. Standard Curve for α-tocopherol

Standard curve for α-tocopherol by reverse phase high-performance liquid chromatography (RP-HPLC), using the column Discovery C18, 25 cm x 4.6 mm, 5 µm (Supelco, Sigma-Aldrich Corp., St. Louis, MO, USA), and the detector Waters 474 Scaning Fluorescence Detector (Waters Corporation, Milford, MA) set at excitation wavelength 290 nm and emission wavelength 320 nm. The mobile phase was acetonitrile: water: acetic acid in a ratio of 95:5:0.01 at a flow rate of 1.5 ml/min. Standard curve was prepared with alpha-tocopherol 95% (Sigma Chemical Co, St Louis, MO).

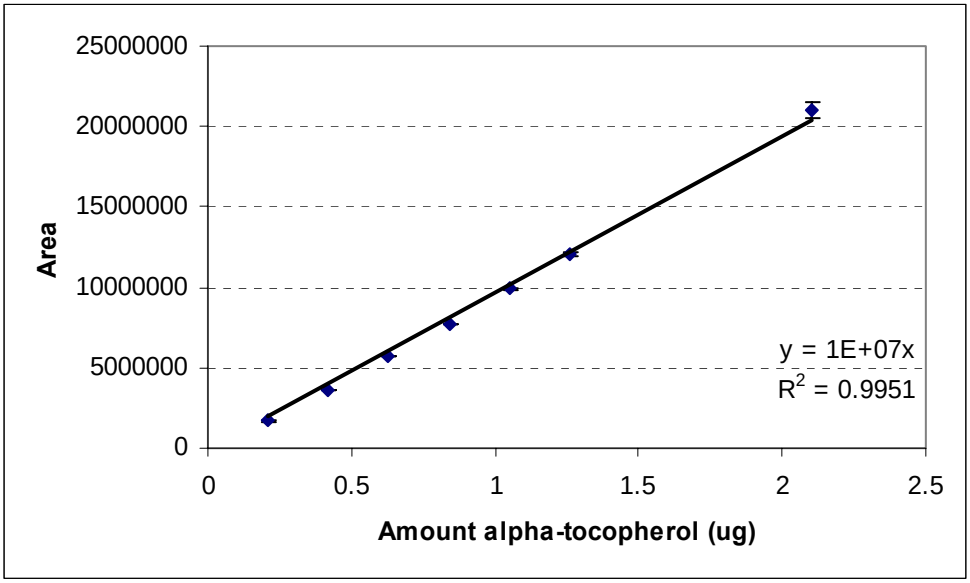

Figure A.1. Standard curve for alpha-tocopherol

A.2. Standard Curve for PVA

Standard curve for PVA was prepared using a colorimetric method based on the formation of a colored complex between iodine and two adjacent hydroxyl groups of PVA. The absorbance of the samples was measured with a UV-Visible Beckman Coulter spectrophotometer (Beckman Coulter, Fullerton, CA) at the wavelength 690 nm. Standard curve was prepared with PVA with an average molecular weight of 30,000-70,000 (Sigma Chemical Co, St Louis, MO).

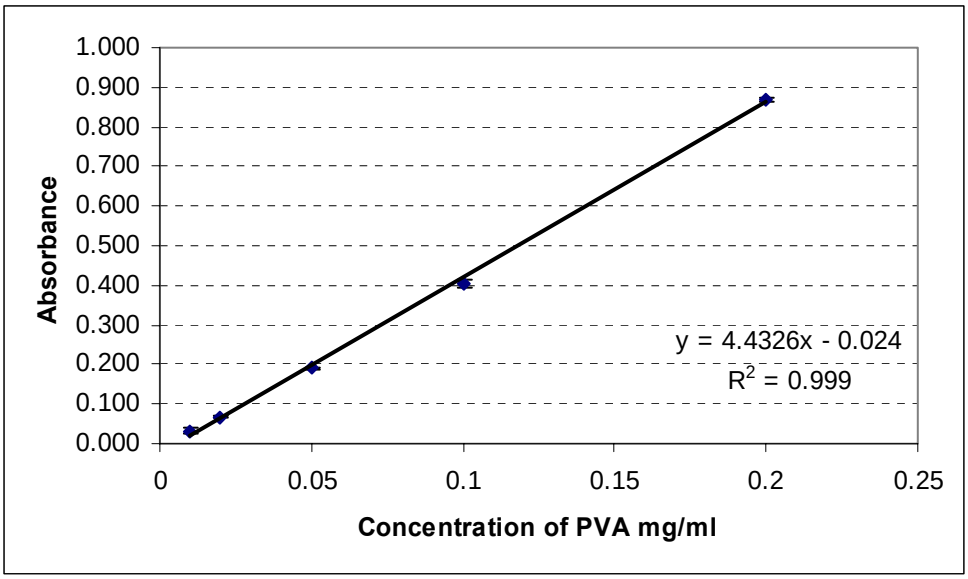

Figure A.2. Standard curve for PVA 30,000-70,000 Da

A.3. Standard Curve for Trolox

Trolox 0.5M was used for the preparation of the standard curve. The decrease in absorbance was monitored at 515 nm by using a UV-Visible Beckman Coulter spectrophotometer (Beckman Coulter, Fullerton, CA). The readings were done at time zero and after 30 min of incubation at room temperature. The inhibition percentage was expressed using the following equation: Inhibition % = $(Abs_{t=0} - Abs_{t=30\ min})/ Abs_{t=0} \times 100$;

$Abs_{t=0}$ was the absorbance of DPPH at time zero and $Abs_{t=30\ min}$ the absorbance after 30 min of incubation.

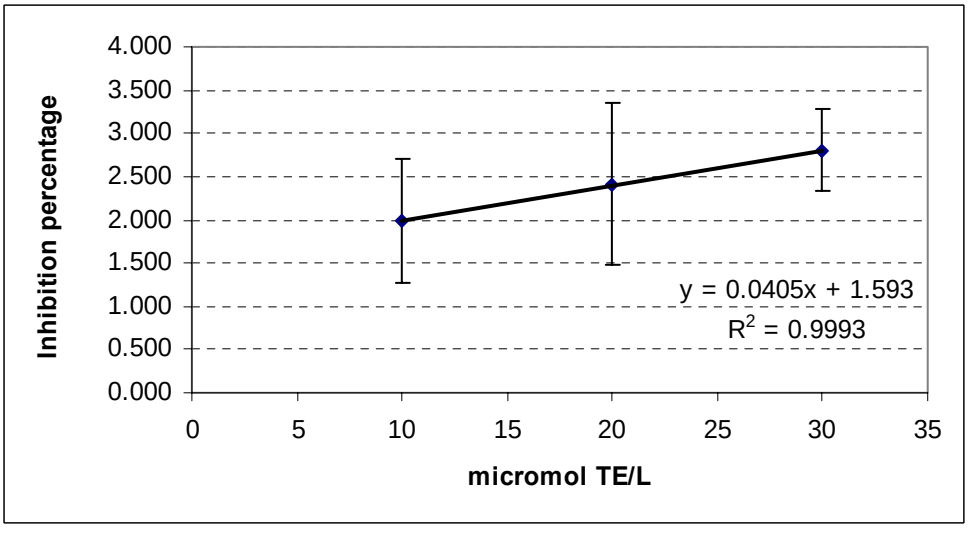

Figure A.3. Standard curve for Trolox

APPENDIX B. DATA FOR RICE BRAN OIL EXTRACTION

Table B.1. Amount of rice bran oil extracted

Sample	Empty tube	Tube +oil(from 2 ml)	Volume (ml)	g oil/g rice bran	AVG	SD
I 40 1A ME	8.63	8.75	29.2	0.088		
I 40 2A ME	8.66	8.77	40.5	0.111		
I 40 3A ME	8.67	8.76	53.5	0.120		
I 40 1B ME	8.66	8.8	29.2	0.102		
I 40 2B ME	8.67	8.77	40.5	0.101		
I 40 3B ME	8.72	8.8	53.5	0.107	0.105	0.011
I 60 1A ME	10.81	10.9	53	0.119		
I 60 2A ME	10.97	11.08	44	0.121		
I 60 3A ME	10.89	10.97	47	0.094		
I 60 1B ME	10.87	10.96	53	0.119		
I 60 2B ME	10.86	10.97	44	0.121		
I 60 3B ME	10.94	11.04	47	0.118	0.115	0.011
I 80 1A ME	8.69	8.8	60	0.165		
I 80 2A ME	8.67	8.78	50.9	0.140		
I 80 3A ME	10.8	10.91	58.1	0.160		
I 80 1B ME	8.62	8.71	60	0.135		
I 80 2B ME	10.95	11.05	50.9	0.127		
I 80 3B ME	10.92	11.01	58.1	0.131	0.143	0.016
I 100 1A ME	8.71	8.79	56.5	0.113		
I 100 2A ME	8.64	8.74	53	0.133		
I 100 3A ME	8.61	8.73	62.1	0.186		
I 100 1B ME	8.68	8.79	56.5	0.155		
I 100 2B ME	8.76	8.85	53	0.119		
I 100 3B ME	8.73	8.83	62.1	0.155	0.144	0.027
I 120 4A ME	8.65	8.79	34.5	0.121		
I 120 5A ME	8.62	8.8	47	0.212		
I 120 6A ME	8.73	8.88	34.1	0.128		
I 120 4B ME	8.78	8.93	34.5	0.129		
I 120 5B ME	8.68	8.84	47	0.188		
I 120 6B ME	8.74	8.89	34.1	0.128	0.151	0.039
H 40 1A ME	8.68	8.82	43.5	0.152		
H 40 2A ME	8.71	9.47	7	0.133		
H 40 3A ME	8.62	9.31	7.5	0.129		
H 40 1B ME	8.73	8.87	43.5	0.152		
H 40 2B ME	8.67	9.38	7	0.124		
H 40 3B ME	8.68	9.36	7.5	0.128	0.136	0.013
H 60 1 ME	8.7	8.83	43.5	0.141		
H 60 2 ME	8.72	8.87	38.5	0.144		
H 60 3 ME	8.7	8.83	40.2	0.131		
H 60 1 ME	8.63	8.76	43.5	0.141		
H 60 2 ME	8.75	8.89	38.5	0.135		
H 60 3 ME	8.66	8.8	40.2	0.141	0.139	0.005
H 80 1 ME	8.62	8.92	17	0.128		
H 80 2 ME	8.58	9.31	7	0.128		

						(table continued)
H 80 3 ME	8.76	9.04	18.9	0.132		
H 80 1 ME	8.71	9.01	17	0.128		
H 80 2 ME	8.59	9.31	7	0.126		
H 80 3 ME	8.56	8.82	18.9	0.123	0.127	0.003
H 100 1 ME	8.64	8.83	24.1	0.114		
H 100 2 ME	8.62	9.32	7.2	0.126		
H 100 3 ME	8.57	8.84	15.8	0.107		
H 100 1 ME	8.66	8.88	24.1	0.133		
H 100 2 ME	8.61	9.31	7.2	0.126		
H 100 3 ME	8.74	8.98	15.8	0.095	0.117	0.014
H 120 1 ME	8.65	8.82	29.2	0.124		
H 120 2 ME	8.65	8.98	14.5	0.120		
H 120 3 ME	8.66	8.85	26.7	0.127		
H 120 1 ME	8.72	8.89	29.2	0.124		
H 120 2 ME	8.69	9	14.5	0.112		
H 120 3 ME	8.67	8.85	26.7	0.120	0.121	0.005
I 40 1A SE	8.64	8.75	52	0.143		
I 40 2A SE	8.74	8.83	46	0.104		
I 40 3A SE	8.64	8.74	45.8	0.115		
I 40 1B SE	8.72	8.81	52	0.117		
I 40 2B SE	8.69	8.79	46	0.115		
I 40 3B SE	8.69	8.8	45.8	0.126	0.120	0.013
H 40 1A SE	8.61	9.28	8.9	0.149		
H 40 2A SE	8.67	8.97	20.2	0.152		
H 40 3A SE	8.62	8.72	53.8	0.135		
H 40 1B SE	8.68	9.35	8.9	0.149		
H 40 2B SE	8.7	8.98	20.2	0.141		
H 40 3B SE	8.71	8.79	53.8	0.108	0.139	0.017

ME = Microwave-assisted extraction
SE = Conventional solvent extraction
I = Isopropanol
H = Hexane

Table B.2. Vitamin E vitamers and total vitamin E as function of the extraction parameters

Sample	Volume (ml)	Area aT	Area aT3	Area gT	Area gT3	µg aT/g rb	µg aT3/g rb	µg gT/g rb	µg gT3/g rb	vitamin E µg/g rb
I 40 1A	29.2	7418962	1434130	1778214	12538069	43.327	8.375	5.192	36.611	93.506
I 40 2A	40.5	5723041	987815	1336372	9460141	46.357	8.001	5.412	38.314	98.084
I 40 3A	53.5	3925245	765098	1030765	7279818	42.000	8.187	5.515	38.947	94.648
I 60 1A	53	4350689	796131	1035969	7425463	46.12	8.44	5.49	39.35	99.40
I 60 2A	44	4985605	856225	1248186	9122834	43.87	7.53	5.49	40.14	97.04
I 60 3A	47	4416023	938597	1135010	8570997	41.51	8.82	5.33	40.28	95.95
I 80 1A	60	3722350	1111677	1068580	7477154	44.67	13.34	6.41	44.86	109.28
I 80 2A	50.9	6697725	1622204	1235192	8268190	68.18	16.51	6.29	42.09	133.07
I 80 3A	58.1	5561210	1384099	1083773	7629037	64.62	16.08	6.30	44.32	131.33
I 100 1B	56.5	5016648	2008043	1201043	7564828	56.69	22.69	6.79	42.74	128.91
I 100 2A	53	5899716	2053457	1128007	7274557	62.54	21.77	5.98	38.56	128.84
I 100 3A	62.1	6024991	1912393	1277260	7915675	74.83	23.75	7.93	49.16	155.67
I 120 1A	52.9	4816656	5361224	1448088	8868232	50.96	56.72	7.66	46.91	162.26
I 120 2A	43	5683442	6637368	1739888	9600193	48.88	57.08	7.48	41.28	154.72
I 120 3A	44.2	4925130	5621478	1521527	9046221	43.54	49.69	6.73	39.98	139.94
H 40 1A	43.5	5070762	1450136	1041857	6310852	44.116	12.616	4.532	27.452	88.716
H 40 2A	7	12456678	4868420	3720080	17585383	17.439	6.816	2.604	12.310	39.169
H 40 3A	7.5	12035387	5191047	3480764	16435112	18.053	7.787	2.611	12.326	40.777
H 60 1A	43.5	4478005	1648333	974042	6753995	38.959	14.340	4.237	29.380	86.916
H 60 2A	38.5	3588123	1572508	1152128	7429808	27.629	12.108	4.436	28.605	72.777
H 60 3B	40.2	4726772	1524895	1137970	7449888	38.003	12.260	4.575	29.949	84.787
H 80 1A	17	7606878	4548897	2197838	14192078	25.863	15.466	3.736	24.127	69.192
H 80 2A	7	3098353	1785059	737405	5097052	21.688	12.495	2.581	17.840	54.604
H 80 3A	18.9	8089764	4430009	2114134	12738918	30.579	16.745	3.996	24.077	75.397
H 100 1B	24.1	7406523	9912236	2034042	10930804	35.699	47.777	4.902	26.343	114.722
H 100 2A	7.2	3402493	5539277	869913	5175267	24.498	39.883	3.132	18.631	86.143
H 100 3A	15.8	1409646	3184843	391634	2280145	22.272	50.321	3.094	18.013	93.700
H 120 1A	29.2	6288136	6198078	1741218	9418341	36.72	180.98	5.08	27.50	250.29
H 120 2A	14.5	10832973	7757594	2971863	14690384	31.42	112.49	4.31	21.30	169.51
H 120 3A	26.7	7176490	9655556	1838541	9235920	38.32	257.80	4.91	24.66	325.69
I 40 1A SE	52	3499813	853032	1148177	7966010	36.398	8.872	5.971	41.423	92.663

										(table continued)
I 40 2A SE	46	3726624	931935	1317158	9093022	34.285	8.574	6.059	41.828	90.746
I 40 3A SE	45.8	2929159	871155	1266594	8376166	26.831	7.980	5.801	38.363	78.975
H 40 1A SE	8.9	12237471	4039393	3588461	17926509	21.783	7.190	3.194	15.955	48.121
H 40 2A SE	20.2	6794101	2240135	2157801	11009642	27.448	9.050	4.359	22.239	63.097
H 40 3A SE	53.8	1205204	807286	785670	3872609	12.968	8.686	4.227	20.835	46.716

SE = Conventional solvent extraction; all the other samples are extracted by microwave-assisted extraction; I = Isopropanol; H = Hexane; Area = peak area; aT = alpha-tocopherol; aT3 = alpha-tocotrienol; gT = gamma-tocopherol; gT3 = gamma-tocotrienol; rb = rice bran

Table B.3. Inhibition percentage as function of the extraction parameters

Sample	volume (µL)	g oil/L	A t=0	A t=30 min	Inhibition percentage
I 40 1B	40	0.4	0.5528	0.5141	7.00
I 40 1B	80	0.8	0.5109	0.4396	13.96
I 40 1B	120	1.2	0.466	0.3712	20.34
I 40 2B	40	0.4	0.5669	0.5315	6.24
I 40 2B	80	0.8	0.5195	0.4622	11.03
I 40 2B	120	1.2	0.4853	0.41	15.52
I 40 3B	40	0.4	0.5665	0.5409	4.52
I 40 3B	80	0.8	0.5289	0.481	9.06
I 40 3B	120	1.2	0.4936	0.4268	13.53
I 60 1B	40	0.4	0.5936	0.5586	5.90
I 60 1B	80	0.8	0.5484	0.487	11.20
I 60 1B	120	1.2	0.5144	0.4347	15.49
I 60 2B	40	0.4	0.597	0.5701	4.51
I 60 2B	80	0.8	0.5625	0.5152	8.41
I 60 2B	120	1.2	0.5255	0.4518	14.02
I 60 3B	40	0.4	0.604	0.5816	3.71
I 60 3B	80	0.8	0.5614	0.5121	8.78
I 60 3B	120	1.2	0.5293	0.4625	12.62
I 80 1B	40	0.4	0.5932	0.5607	5.48
I 80 1B	80	0.8	0.5554	0.494	11.06
I 80 1B	120	1.2	0.5192	0.4423	14.81
I 80 2B	40	0.4	0.5943	0.5501	7.44
I 80 2B	80	0.8	0.5458	0.458	16.09
I 80 2B	120	1.2	0.5126	0.4079	20.43
I 80 3B	40	0.4	0.5934	0.5538	6.67
I 80 3B	80	0.8	0.5537	0.4809	13.15
I 80 3B	120	1.2	0.5166	0.4225	18.22
I 100 1B	40	0.4	0.5927	0.5507	7.09
I 100 1B	80	0.8	0.5546	0.4816	13.16
I 100 1B	120	1.2	0.5226	0.4282	18.06
I 100 2B	40	0.4	0.5896	0.542	8.07
I 100 2B	80	0.8	0.5544	0.4653	16.07
I 100 2B	120	1.2	0.5116	0.4052	20.80
I 100 3B	40	0.4	0.5924	0.5515	6.90
I 100 3B	80	0.8	0.5539	0.4818	13.02
I 100 3B	120	1.2	0.5225	0.4277	18.14
I 120 1B	40	0.4	0.5424	0.5034	7.19
I 120 1B	80	0.8	0.5024	0.4226	15.88
I 120 1B	120	1.2	0.4622	0.3721	19.49
I 120 2B	40	0.4	0.5401	0.4869	9.85
I 120 2B	80	0.8	0.5054	0.4134	18.20
I 120 2B	120	1.2	0.4649	0.353	24.07
I 120 3B	40	0.4	0.548	0.507	7.48
I 120 3B	80	0.8	0.5037	0.4213	16.36
I 120 3B	120	1.2	0.4692	0.3675	21.68
H 40 1B	40	0.4	0.5687	0.5407	4.92

					(table continued)
H 40 1B	80	0.8	0.536	0.4842	9.66
H 40 1B	120	1.2	0.5021	0.4287	14.62
H 40 2B	40	0.4	0.568	0.5383	5.23
H 40 2B	80	0.8	0.5375	0.4791	10.87
H 40 2B	120	1.2	0.5054	0.4252	15.87
H 40 3B	40	0.4	0.5621	0.5389	4.13
H 40 3B	80	0.8	0.5274	0.476	9.75
H 40 3B	120	1.2	0.4925	0.4249	13.73
H 60 1B	40	0.4	0.5972	0.5562	6.87
H 60 1B	80	0.8	0.5628	0.4918	12.62
H 60 1B	120	1.2	0.5306	0.4405	16.98
H 60 2B	40	0.4	0.6022	0.5688	5.55
H 60 2B	80	0.8	0.5733	0.5102	11.01
H 60 2B	120	1.2	0.5456	0.4598	15.73
H 60 3A	40	0.4	0.5994	0.5649	5.76
H 60 3A	80	0.8	0.5681	0.5048	11.14
H 60 3A	120	1.2	0.5409	0.4544	15.99
H 80 1B	40	0.4	0.6062	0.5842	3.63
H 80 1B	80	0.8	0.588	0.546	7.14
H 80 1B	120	1.2	0.5576	0.4963	10.99
H 80 2B	40	0.4	0.5975	0.5603	6.23
H 80 2B	80	0.8	0.5639	0.5036	10.69
H 80 2B	120	1.2	0.5274	0.4297	18.52
H 80 3B	40	0.4	0.6006	0.5695	5.18
H 80 3B	80	0.8	0.5686	0.5082	10.62
H 80 3B	120	1.2	0.5336	0.4501	15.65
H 100 1A	40	0.4	0.5948	0.559	6.02
H 100 1A	80	0.8	0.5575	0.4925	11.66
H 100 1A	120	1.2	0.5244	0.4346	17.12
H 100 2B	40	0.4	0.5961	0.5594	6.16
H 100 2B	80	0.8	0.5603	0.4935	11.92
H 100 2B	120	1.2	0.5319	0.4396	17.35
H 100 3B	40	0.4	0.5931	0.5538	6.63
H 100 3B	80	0.8	0.5558	0.4862	12.52
H 100 3B	120	1.2	0.5224	0.4266	18.34
H 120 4B	40	0.4	0.5559	0.5166	7.07
H 120 4B	80	0.8	0.5166	0.4503	12.83
H 120 4B	120	1.2	0.4806	0.3918	18.48
H 120 5B	40	0.4	0.5577	0.518	7.12
H 120 5B	80	0.8	0.522	0.4479	14.20
H 120 5B	120	1.2	0.4829	0.3933	18.55
H 120 6B	40	0.4	0.5556	0.5157	7.18
H 120 6B	80	0.8	0.5151	0.4438	13.84
H 120 6B	120	1.2	0.4828	0.3877	19.70
I 40 1B SE	40	0.4	0.5565	0.5324	4.33
I 40 1B SE	80	0.8	0.5127	0.4689	8.54
I 40 1B SE	120	1.2	0.4714	0.4097	13.09
I 40 2B SE	40	0.4	0.5536	0.5277	4.68

					(table continued)
I 40 2B SE	80	0.8	0.5081	0.4607	9.33
I 40 2B SE	120	1.2	0.4664	0.4025	13.70
I 40 3B SE	40	0.4	0.5546	0.5294	4.54
I 40 3B SE	80	0.8	0.5116	0.4648	9.15
I 40 3B SE	120	1.2	0.4665	0.3998	14.30
H 40 1B SE	40	0.4	0.5637	0.539	4.38
H 40 1B SE	80	0.8	0.5263	0.4762	9.52
H 40 1B SE	120	1.2	0.494	0.4254	13.89
H 40 2B SE	40	0.4	0.5647	0.5269	6.69
H 40 2B SE	80	0.8	0.5319	0.4687	11.88
H 40 2B SE	120	1.2	0.5031	0.4172	17.07
H 40 3B SE	40	0.4	0.5666	0.5339	5.77
H 40 3B SE	80	0.8	0.5348	0.4708	11.97
H 40 3B SE	120	1.2	0.512	0.4271	16.58

SE = Conventional solvent extraction; all the other samples are extracted by microwave-assisted extraction; I = Isopropanol; H = Hexane.

Table B.4. Degradation of α-tocopherol by microwave-assisted method

Sample	Area	aT µg/L	aT g/L	AVG	SD
I1 TE only	6834129	136682.6	0.137		
I2 TE only	7011893	140237.9	0.140		
I3 TE 5	6765515	135310.3	0.135	0.137	0.003
I 40 1A	7546544	150930.9	0.151		
I 40 2A	7177580	143551.6	0.144		
I 40 3A	6609213	132184.3	0.132	0.142	0.009
I 60 1A	6329453	126589.1	0.127		
I 60 2A	5929483	118589.7	0.119		
I 60 3A	6974842	139496.8	0.139	0.128	0.011
I 80 1A	7167328	143346.6	0.143		
I 80 2A	7360587	147211.7	0.147		
I 80 3A	6996088	139921.8	0.140	0.143	0.004
I 100 1A	6907396	138147.9	0.138		
I 100 2A	6559570	131191.4	0.131		
I 100 3A	7047293	140945.9	0.141	0.137	0.005
I 120 1A	7014736	140294.7	0.140		
I 120 2A	7250917	145018.3	0.145		
I 120 3A	7139137	142782.7	0.143	0.143	0.002
H1 t=0 5	5462207	109244.1	0.109		
H2 t=0 5	5665997	113319.9	0.113		
H3 t=0 5	5654728	113094.6	0.113	0.112	0.002
H1 TE 5	5650688	113013.8	0.113		
H2 TE 5	5670571	113411.4	0.113		
H3 TE 5	5601455	112029.1	0.112	0.113	0.001
H 40 1A	5883153	117663.1	0.118		
H 40 2A	6344210	126884.2	0.127		
H 40 3A	5992039	119840.8	0.120	0.121	0.005

					(table continued)
H 60 1A	6240268	124805.4	0.125		
H 60 2A	5898742	117974.8	0.118		
H 60 3A	6161420	123228.4	0.123	0.122	0.004
H 80 1A	6213527	124270.5	0.124		
H 80 2A	6141728	122834.6	0.123		
H 80 3A	4950429	99008.58	0.099	0.115	0.014
H 100 1A	5752133	115042.7	0.115		
H 100 2A	6393477	127869.5	0.128		
H 100 3A	6825664	136513.3	0.137	0.126	0.011
H 120 1A	6219045	124380.9	0.124		
H 120 2A	6031576	120631.5	0.121		
H 120 3A	5863611	117272.2	0.117	0.121	0.004

I = Isopropanol; H = Hexane; TE = evaporation temperature.

APPENDIX C. DATA FOR CHARACTERIZATION AND REALEASE OF ALPHA-TOCOPHEROL ENTRAPPED IN THE PLGA MATRIX

Table C.1. Size, PDI, and zeta potential for nanoparticles as a function of alpha-tocopherol theoretical loading and processing parameters after synthesis

Record	Type	Sample Name	Measurement Date and Time	T (°C)	Z-Ave (d.nm)	PDI	ZP (mV)
4	Size	E1 repeated bp	Friday, October 20, 2006 3:30:43 PM	25	219	0.059	n/a
5	Size	E1 repeated bp	Friday, October 20, 2006 3:33:15 PM	25	219	0.103	n/a
6	Size	E1 repeated bp	Friday, October 20, 2006 3:35:46 PM	25	215	0.084	n/a
22	Size	E2 repeated bp	Saturday, October 21, 2006 6:54:59 PM	25	221	0.128	n/a
23	Size	E2 repeated bp	Saturday, October 21, 2006 6:57:30 PM	25	219	0.06	n/a
24	Size	E2 repeated bp	Saturday, October 21, 2006 7:00:01 PM	25	218	0.095	n/a
16	Size	E3bp	Saturday, October 21, 2006 3:01:08 PM	25	213	0.07	n/a
17	Size	E3bp	Saturday, October 21, 2006 3:03:39 PM	25	212	0.076	n/a
18	Size	E3bp	Saturday, October 21, 2006 3:06:11 PM	25	210	0.069	n/a
25	Size	S1 double repeated,aT8%bp	Wednesday, October 18, 2006 11:07:22 AM	25	200	0.078	n/a
26	Size	S1 double repeated,aT8%bp	Wednesday, October 18, 2006 11:09:54 AM	25	201	0.074	n/a
27	Size	S1 double repeated,aT8%bp	Wednesday, October 18, 2006 11:12:25 AM	25	200	0.103	n/a
7	Size	S2aT8%bp	Monday, October 16, 2006 3:54:11 PM	25	224	0.105	n/a
8	Size	S2aT8%bp	Monday, October 16, 2006 3:56:42 PM	25	226	0.094	n/a
9	Size	S2aT8%bp	Monday, October 16, 2006 3:59:14 PM	25	222	0.105	n/a
19	Size	S3,aT8%bp	Tuesday, October 17, 2006 6:33:30 PM	25	228	0.083	n/a
20	Size	S3,aT8%bp	Tuesday, October 17, 2006 6:36:02 PM	25	227	0.105	n/a
21	Size	S3,aT8%bp	Tuesday, October 17, 2006 6:38:33 PM	25	227	0.092	n/a
31	Size	D1aT16%bp	Wednesday, October 18, 2006 1:09:44 PM	25	216	0.102	n/a
32	Size	D1aT16%bp	Wednesday, October 18, 2006 1:12:17 PM	25	212	0.086	n/a
33	Size	D1aT16%bp	Wednesday, October 18, 2006 1:14:48 PM	25	215	0.051	n/a
55	Size	D2 double repeated aT16%bp	Friday, October 20, 2006 1:30:38 PM	25	212	0.069	n/a
56	Size	D2 double repeated aT16%bp	Friday, October 20, 2006 1:33:09 PM	25	211	0.092	n/a
57	Size	D2 double repeated aT16%bp	Friday, October 20, 2006 1:35:39 PM	25	208	0.025	n/a
43	Size	D3aT16%bp	Wednesday, October 18, 2006 5:25:09 PM	25	197	0.055	n/a
44	Size	D3aT16%bp	Wednesday, October 18, 2006 5:27:40 PM	25	193	0.036	n/a
45	Size	D3aT16%bp	Wednesday, October 18, 2006 5:30:10 PM	25	193	0.033	n/a
7	Size	E1 repeated ap	Friday, October 20, 2006 4:39:43 PM	25	229	0.15	n/a

(table continued)

8	Size	E1 repeated ap	Friday, October 20, 2006 4:42:14 PM	25	229	0.083	n/a
9	Size	E1 repeated ap	Friday, October 20, 2006 4:44:46 PM	25	226	0.114	n/a
25	Size	E2 repeated ap	Saturday, October 21, 2006 7:04:03 PM	25	243	0.191	n/a
26	Size	E2 repeated ap	Saturday, October 21, 2006 7:06:34 PM	25	251	0.277	n/a
27	Size	E2 repeated ap	Saturday, October 21, 2006 7:09:06 PM	25	238	0.173	n/a
19	Size	E3ap	Saturday, October 21, 2006 4:05:56 PM	25	229	0.177	n/a
20	Size	E3ap	Saturday, October 21, 2006 4:08:28 PM	25	222	0.121	n/a
21	Size	E3ap	Saturday, October 21, 2006 4:10:59 PM	25	219	0.135	n/a
28	Size	S1 double repeated,aT8%ap	Wednesday, October 18, 2006 12:59:38 PM	25	218	0.081	n/a
29	Size	S1 double repeated,aT8%ap	Wednesday, October 18, 2006 1:02:09 PM	25	211	0.121	n/a
30	Size	S1 double repeated,aT8%ap	Wednesday, October 18, 2006 1:04:40 PM	25	215	0.086	n/a
10	Size	S2aT8%ap	Monday, October 16, 2006 6:38:29 PM	25	227	0.121	n/a
11	Size	S2aT8%ap	Monday, October 16, 2006 6:41:00 PM	25	226	0.178	n/a
12	Size	S2aT8%ap	Monday, October 16, 2006 6:43:32 PM	25	227	0.106	n/a
22	Size	S3,aT8%ap	Tuesday, October 17, 2006 7:56:15 PM	25	240	0.182	n/a
23	Size	S3,aT8%ap	Tuesday, October 17, 2006 7:58:45 PM	25	235	0.197	n/a
24	Size	S3,aT8%ap	Tuesday, October 17, 2006 8:01:16 PM	25	233	0.099	n/a
34	Size	D1aT16%ap	Wednesday, October 18, 2006 3:00:26 PM	25	231	0.111	n/a
35	Size	D1aT16%ap	Wednesday, October 18, 2006 3:03:00 PM	25	230	0.168	n/a
36	Size	D1aT16%ap	Wednesday, October 18, 2006 3:05:31 PM	25	227	0.117	n/a
58	Size	D2 double repeated aT16%ap	Friday, October 20, 2006 3:20:37 PM	25	240	0.199	n/a
59	Size	D2 double repeated aT16%ap	Friday, October 20, 2006 3:23:08 PM	25	235	0.146	n/a
60	Size	D2 double repeated aT16%ap	Friday, October 20, 2006 3:25:39 PM	25	233	0.105	n/a
46	Size	D3aT16%ap	Wednesday, October 18, 2006 6:28:59 PM	25	215	0.269	n/a
47	Size	D3aT16%ap	Wednesday, October 18, 2006 6:31:31 PM	25	200	0.167	n/a
48	Size	D3aT16%ap	Wednesday, October 18, 2006 6:34:02 PM	25	201	0.074	n/a
40	Size/ZP	E1 after fd	Tuesday, October 24, 2006 5:49:19 PM	25	231	0.077	-26.7
41	Size/ZP	E1 after fd	Tuesday, October 24, 2006 5:51:50 PM	25	226	0.167	-27.2
42	Size/ZP	E1 after fd	Tuesday, October 24, 2006 5:54:21 PM	25	223	0.113	-27.1
43	Size/ZP	E2 after fd	Tuesday, October 24, 2006 5:58:41 PM	25	247	0.146	-30.5
44	Size/ZP	E2 after fd	Tuesday, October 24, 2006 6:01:11 PM	25	239	0.128	-31.5
45	Size/ZP	E2 after fd	Tuesday, October 24, 2006 6:03:41 PM	25	242	0.151	-30.4
46	Size/ZP	E3 after fd	Tuesday, October 24, 2006 6:10:10 PM	25	230	0.127	-29.6
47	Size/ZP	E3 after fd	Tuesday, October 24, 2006 6:12:41 PM	25	228	0.146	-27.5

(table continued)

48	Size/ZP	E3 after fd	Tuesday, October 24, 2006 6:15:12 PM	25	229	0.13	-27.5
1	Size/ZP	S1 after fd	Monday, October 23, 2006 8:00:42 PM	25	296	0.293	-14.9
2	Size/ZP	S1 after fd	Monday, October 23, 2006 8:03:13 PM	25	269	0.236	-16.7
3	Size/ZP	S1 after fd	Monday, October 23, 2006 8:05:44 PM	25	262	0.215	-16.4
4	Size/ZP	S2 after fd	Monday, October 23, 2006 8:09:13 PM	25	312	0.257	-14
5	Size/ZP	S2 after fd	Monday, October 23, 2006 8:11:45 PM	25	310	0.26	-14
6	Size/ZP	S2 after fd	Monday, October 23, 2006 8:14:16 PM	25	299	0.259	-13.5
7	Size/ZP	S3 after fd	Monday, October 23, 2006 8:24:43 PM	25	279	0.224	-10.8
8	Size/ZP	S3 after fd	Monday, October 23, 2006 8:27:13 PM	25	264	0.189	-10.2
9	Size/ZP	S3 after fd	Monday, October 23, 2006 8:29:46 PM	25	265	0.221	-11.5
10	Size/ZP	D1 after fd	Monday, October 23, 2006 8:33:27 PM	25	248	0.173	-9.58
11	Size/ZP	D1 after fd	Monday, October 23, 2006 8:36:21 PM	25	246	0.136	-10.6
12	Size/ZP	D1 after fd	Monday, October 23, 2006 8:38:52 PM	25	242	0.146	-10.7
13	Size/ZP	D2 after fd	Monday, October 23, 2006 8:42:38 PM	25	259	0.215	-19.4
14	Size/ZP	D2 after fd	Monday, October 23, 2006 8:45:09 PM	25	250	0.158	-19.6
15	Size/ZP	D2 after fd	Monday, October 23, 2006 8:47:39 PM	25	251	0.202	-19
16	Size/ZP	D3 after fd	Monday, October 23, 2006 8:50:45 PM	25	220	0.109	-8.34
17	Size/ZP	D3 after fd	Monday, October 23, 2006 8:53:16 PM	25	217	0.141	-8.51
18	Size/ZP	D3 after fd	Monday, October 23, 2006 8:55:47 PM	25	216	0.112	-8.19

E = unloaded nanoparticles; S = nanoparticles with 8% alpha-tocopherol theoretical loading; D = nanoparticles with 16% alpha-tocopherol theoretical loading; bp = before purification; ap = after purification; fd = freeze-drying; ZP = zeta potential; aT = alpha-tocopherol.

Table C.2. Entrapment efficiency of alpha-tocopherol entrapped in the polymeric matrix

Sample	Area	Amount sample (mg)	µg aT/ 25 µL	µg aT/ 1 mg sample	mg aT/sample	EE%	AVG	SD
S1 8% 25 µL R1	8258444	134.9	0.826	33.034	4.456	111.406		
S1 8% 25 µL R2	7600643	134.9	0.760	30.403	4.101	102.533		
S1 8% 25 µL R3	7290680	134.9	0.729	29.163	3.934	98.351		
S2 8% 25 µL R1	6775130	127.6	0.678	27.101	3.458	86.451		
S2 8% 25 µL R2	6669332	127.6	0.667	26.677	3.404	85.101		
S2 8% 25 µL R3	6599460	127.6	0.660	26.398	3.368	84.209		
S3 8% 25 µL R1	5827790	136.9	0.583	23.311	3.191	79.782		
S3 8% 25 µL R2	5818163	136.9	0.582	23.273	3.186	79.651		
S3 8% 25 µL R3	5787447	136.9	0.579	23.150	3.169	79.230	89.63	11.63
D1 16% 25 µL R1	13618551	135.6	1.362	54.474	7.387	92.334		
D1 16% 25 µL R2	13640790	135.6	1.364	54.563	7.399	92.485		
D1 16% 25 µL R3	13640648	135.6	1.364	54.563	7.399	92.484		
D2 16% 25 µL R1	15041241	135.5	1.504	60.165	8.152	101.904		
D2 16% 25 µL R2	14427252	135.5	1.443	57.709	7.820	97.745		
D2 16% 25 µL R3	14055900	135.5	1.406	56.224	7.618	95.229		
D3 16% 25 µL R1	14395105	132.9	1.440	57.580	7.652	95.655		
D3 16% 25 µL R2	14549037	132.9	1.455	58.196	7.734	96.678		
D3 16% 25 µL R3	14416846	132.9	1.442	57.667	7.664	95.800	95.59	3.08

S = nanoparticles with 8% alpha-tocopherol theoretical loading; D = nanoparticles with 16% alpha-tocopherol theoretical loading; R 1,2,3 = HPLC injection 1,2,3 (25 µL injected volume).

Table C.3. Residual PVA associated with nanoparticles as a function of alpha-tocopherol theoretical loading

Sample	Absorbance	PVA mg/ml	PVA mg/2 ml	PVA % (w/w) relative to n.p.	AVG	SD
E1 0%	0.3313	0.080	0.160	5.34		
E2 0%	0.2831	0.069	0.139	4.62		
E3 0%	0.329	0.080	0.159	5.31	5.09	0.41
S1 8%	0.428	0.102	0.204	6.80		
S2 8%	0.3785	0.091	0.182	6.05		
S3 8%	0.4784	0.113	0.227	7.56	6.80	0.75
D1 16%	0.3951	0.095	0.189	6.30		
D2 16%	0.3914	0.094	0.187	6.25		
D3 16%	0.5689	0.134	0.268	8.92	7.16	1.53

Table C.4. Alpha-tocopherol released from PLGA nanoparticles as a function of time

Sample	Area	µg aT/50 µL	µg aT/ 1 mL	µg aT/ 10 mg np	% alpha-tocopherol released	AVG	SD
S1 t=0	264782	0.0264782	0.529564	5.29564	0.00		
S2 t=0	384441	0.0384441	0.768882	7.68882	0.00	0.00	0.00
S1 t=1 h	37494	0.0037494	0.074988	0.74988	85.84		
S2 t=1 h	48044	0.0048044	0.096088	0.96088	87.50	86.67	1.18
S1 t=3 h	17636	0.0017636	0.035272	0.35272	93.34		
S2 t=3 h	11492	0.0011492	0.022984	0.22984	97.01	95.18	2.60
S1 t=5 h	32830	0.003283	0.06566	0.6566	87.60		
S2 t=5 h	7829	0.0007829	0.015658	0.15658	97.96	92.78	7.33
S1 t=7 h	21329	0.0021329	0.042658	0.42658	91.94		
S2 t=7 h	4586	0.0004586	0.009172	0.09172	98.81	95.38	4.85
S1 t=24 h	13463	0.0013463	0.026926	0.26926	94.92		
S2 t=24 h	2419	0.0002419	0.004838	0.04838	99.37	97.14	3.15
S1 t=48 h	0	0	0	0	100		
S2 t=48 h	0	0	0	0	100	100.00	0.00
D1 t=0	3234642	0.3234642	6.469284	64.69284	0.00		
D2 t=0	2405665	0.2405665	4.81133	48.1133	0.00	0.00	0.00
D1 t=1 h	1996070	0.199607	3.99214	39.9214	38.29		
D2 T=1 h	1657070	0.165707	3.31414	33.1414	31.12	34.70	5.07
D1 t=3 h	1479813	0.1479813	2.959626	29.59626	54.25		
D2 t=3 h	1163229	0.1163229	2.326458	23.26458	51.65	52.95	1.84
D1 t=5 h	1215957	0.1215957	2.431914	24.31914	62.41		
D2 t=5 h	915312	0.0915312	1.830624	18.30624	61.95	62.18	0.32
D1 t=7 h	1043636	0.1043636	2.087272	20.87272	67.74		
D2 t=7 h	778981	0.0778981	1.557962	15.57962	67.62	67.68	0.08
D1 t=24 h	517770	0.051777	1.03554	10.3554	83.99		
D2 t=24 h	443864	0.0443864	0.887728	8.87728	81.55	82.77	1.73
D1 t=48 h	223440	0.022344	0.44688	4.4688	93.09		
D2 t=48 h	281253	0.0281253	0.562506	5.62506	88.31	90.70	3.38

S = nanoparticles with 8% alpha-tocopherol theoretical loading; D = nanoparticles with 16% alpha-tocopherol theoretical loading

VITA

Imola Zigoneanu graduated from Babes-Bolyai University, Cluj-Napoca, Romania, in 1999. She received her bachelor degree in chemistry-physics and her master degree from the same university, in 2000, in organic chemistry. She worked for two years in a serological laboratory at Dermatovenereology Hospital where she became familiar with the techniques such as direct and indirect immunofluorescence, ELISA, and other immunotechniques. For another three years, she worked as a chemist in a biomedical laboratory at Infectious Diseases Hospital, Cluj-Napoca. There she was initiated in the biochemistry techniques commonly performed in a county hospital.

In spring 2005, she was accepted for a master's program in the Department of Biological and Agricultural Engineering at Louisiana State University, Baton Rouge, Louisiana. She is an active member of the Phi Kappa Phi and Gamma Sigma Delta honor societies, and of the Institute of Biological Engineering. She received the Graduate Student Merit Honor Roll from Gamma Sigma Delta Honor Society of Agriculture, Louisiana State University in spring 2006. Mrs. Imola Zigoneanu will be awarded the degree of Master of Science in Biological and Agricultural Engineering in December 2006, and she will start the doctoral studies at University of North Carolina at Chapel Hill in spring 2007 with the Francis P. Venable Fellowship.