Encapsulating vitamin D3 dissolved in hemp seed oil for enhanced transdermal delivery, both protected by a Maillard reacted glycated lecithin matrix

Thesis

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Abstract

Vitamin D is a secosteroid with numerous benefits for human health, including its effects in skeletal health, immunomodulation, cell proliferation, and cellular differentiation. Despite these benefits, vitamin D deficiency is a global health concern. While supplementation is common in many countries and is commonly conducted by oral delivery, individuals with fat malabsorption face challenges with vitamin D absorption. Thus, transdermal delivery is therefore considered a suitable alternative for these individuals. However, the stability of vitamin D in most commercially available cosmetic products is questionable due to its sensitivity to temperature, light, pH, and oxygen. Therefore, this study aims at developing a vitamin D3 delivery system using hemp seed oil for enhanced transdermal delivery and Maillard reacted lecithin conjugates. Further the study focuses on characterizing and optimizing the encapsulation method to achieve thermal and oxidative stability of hemp seed oil and vitamin D3. Hemp seed oil was chosen as the carrier for vitamin D3 due to its excellent skin permeation ability and rich polyunsaturated fatty acid profile, which provides additional health benefits. According to results, moisture and water activity data conform with recommended values for low moisture food products whereas the p-anisidine test indicated good lipid quality during the period of storage. Thermogravimetric analysis and differential scanning calorimetry results demonstrate that the proposed wall matrix provides adequate thermal and oxidative stability for the encapsulated vitamin D3. The study did not identify a significant difference ($p < 0.05$) in retention of vitamin D3 based on the temperature treatment provided.

Moreover, 50 °C heat treatment achieved the best encapsulation efficiency of 59.5 % compared to 100 °C and the control. Overall, the use of Maillard-reacted glycated lecithin wall matrix appears to be a promising approach for protecting vitamin D3 from external stress factors that lead to degradation.

Dedication

To my family for all the love and support and my friends for making this journey worthwhile

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Fields of Study

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Chapter 01

Introduction

Vitamin D is a steroid hormone that exerts multiple benefits (Gorimanipalli et al., 2023). Literature highlights its diverse roles ranging from maintenance of skeletal health to other health benefits such as immunomodulation, cell proliferation, and cellular differentiation (Gorimanipalli et al., 2023; Santos et al., 2021). Accordingly, multiple studies have linked vitamin D deficiency with cancer, cardiovascular health, Keratoconus, multiple sclerosis and various other chronic conditions and infectious diseases (Gorimanipalli et al., 2023; Mahmoodani et al., 2017; M. B. Santos et al., 2021). However, despite its important role, deficiency of vitamin D remains a global issue (Santanatoglia et al., 2023; Yan et al., 2023). This stems from factors such as reduced sunlight exposure, especially during the winter season, reduced day time, and personal factors such as darker skin tones, age, and dietary patterns etc. (Mahmoodani et al., 2018; Passeron et al., 2019). Consequently, supplementation of vitamin D is practiced in many countries (Mahmoodani et al., 2018; Santanatoglia et al., 2023; Zareie et al., 2019).

There are two major vitamin D delivery pathways, namely oral and transdermal delivery (Alsaqr et al., 2015). The fortification of various food products, including bakery items, dairy products, and beverages, stands out as some of the most widely used approaches for oral delivery (Alsaqr et al., 2015; Mahmoodani et al., 2018). Vitamin D, when taken orally, is typically absorbed in the upper part of the small intestine with the help of bile acid (Alsaqr et al., 2015). However, Silva and Furlanetto (2018), supported by results of clinical trials, reported that individuals with conditions causing poor fat absorption may experience poor intestinal absorption of vitamin D, Such

conditions include cystic fibrosis, Crohn's disease, villous atrophy, scleroderma, and ulcerative colitis (Silva & Furlanetto, 2018).

Therefore, as an alternative approach, transdermal delivery could be considered useful for especially vulnerable populations having fat malabsorption. Moreover, vitamin D can be considered as an excellent candidate for transdermal delivery as it's a potent lipophilic drug and required only in small daily doses (400IU/ day) (Alsaqr et al., 2015). Therefore, a delivery system that ensures excellent skin permeation while preserving the integrity of the nutrient under oxidative conditions could serve as an effective method for supplementing vitamin D.

Formulating skin products for the current consumer market often poses challenges as consumers are increasingly leaning towards more "natural" products excluding synthetic chemicals (Amberg & Fogarassy, 2019). Therefore, the use of unsaturated fatty acids such as oleic acid opposed to synthetic chemicals to enhance skin permeation is becoming popular (Alsaqr et al., 2015). Moreover, literature reports that unsaturated fatty acids present in Hemp seed oil to be excellent skin penetrators and are reportedly used in numerous skin care products (Huang et al., 2020). This is attributed to its ability to temporary lower the skin barrier properties of the outer layers as detailed in section 2.5. On the other hand, preserving the integrity of vitamin D in any skin care supplement is vital to achieve maximum efficiency. However, vitamin D is highly prone to oxidation and isomerization under temperature, pH, light, time and harsh processing conditions (Zareie et al., 2019, 2021). This oxidative instability primarily arises from the presence of double bonds in the structure of vitamin D3 and D2 (Zareie et al., 2021).

Many recent studies on vitamin D retention have been conducted on fortified dairy products, vegetable oil or bakery products (Mahmoodani et al., 2018; Santanatoglia et al., 2023; Zareie et al., 2019, 2021). However, the drawback of using oils rich in unsaturated fatty acids is that oil

oxidation will consequently impact vitamin retention. A study by Mahmoodani et al., (2018) reports a similar occurrence in simulated whole milk powder as vitamin D retention is impacted by increase of lipid secondary oxidative products (Mahmoodani et al., 2018). Therefore, this finding underscores the importance of safeguarding vitamin D against degradation as well as protecting the lipid carrier from oxidation. Accordingly, researchers assert that any developed delivery system for vitamin D should have the capacity to protect both the vitamin and the oil in which it is solubilized from degradation, ensuring optimum efficacy.

Therefore, the present study holds promise as it aims to address the need for developing vitamin D delivery systems that can withstand processing and storage conditions without compromising its retention within the product matrix. Furthermore, this study focuses on creating a cost-effective delivery system through encapsulation.

Hemp seed oil was selected as the solvent for its multiple capabilities in terms of effectively solubilizing vitamin D, its reported skin permeation ability and potential health benefits derived from its rich polyunsaturated fatty acids (PUFAs) (Huang et al., 2020; Rezvankhah et al., 2022). Moreover, recent attention towards developing cosmetics utilizing hemp seed oil further supported the choice of lipid component for the encapsules (Huang et al., 2020; Rezvankhah et al., 2022). Furthermore, glycated soybean lecithin was used as the wall material as an attempt to valorize lecithin, which is a byproduct from soybean oil production. In addition, researchers have found that inducing Maillard reaction on a glycated lecithin wall could potentially exert antioxidant effects on its encapsuled core. This assertion is supported by the patent No. 20200197346A (Russi, 2022).

Therefore, the objective of this study was to develop a vitamin D3 delivery system using hemp seed oil as the solvent, and Maillard reacted lecithin conjugates as the wall material that could protect its internal core from oxidative stress, yielding enhanced shelf life.

Chapter 02

Literature Review

2.1 Introduction to Vitamin D

Vitamin D (VD) stands out as one of the oldest secosteroid and a prohormone in living organisms (Holick, 2003; Saponaro et al., 2020; Slominski et al., 2023). Many animals and plants, including phytoplankton and zooplankton, possess the ability to produce vitamin D when exposed to sunlight (Holick, 2003). There are different forms of vitamin D based on its chemical structure such as Vitamin D1, D2, D3 and D4. However, concerning human health, the essential forms of vitamin D are vitamin D3 (Cholecalciferol) (VD3) and vitamin D2 (Ergocalciferol) (VD2) (Gill et al., 2015). VD2 is exclusively present in plant-based foods, while VD3 is present in both animal-based foods and can be synthesized by the skin upon exposure to UV B radiation (290nm-350nm) from sun light (Gill et al., 2015; Jakobsen & Knuthsen, 2014). As illustrated in figure 2.1, VD2 differs structurally from VD3 by the presence of a methyl group at the carbon 24 (C24), along with a double bond at C22–C23 (Saponaro et al., 2020),. Although, VD2 and VD3 are structurally very similar, recent research have demonstrated that VD3 is more potent than VD2 in terms of elevating serum VD levels (Janoušek et al., 2022). Researchers have attributed the higher potency of VD3 to its different metabolic pathways and its higher affinity towards a Vitamin D Receptor (VDR) (Janoušek et al., 2022).

Moreover, according to the literature, 80 % - 90 % of vitamin D requirements are met through synthesis in the skin (Slominski et al., 2023). This underscores the critical role of the skin in the production of vitamin D3. Janoušek et al., 2022, recommended that a 20 minute long exposure to summer sun could yield about 250 µg of VD3 which is sufficient to provide the daily requirement of VD, > 30 ng /ml of serum (Janoušek et al., 2022)

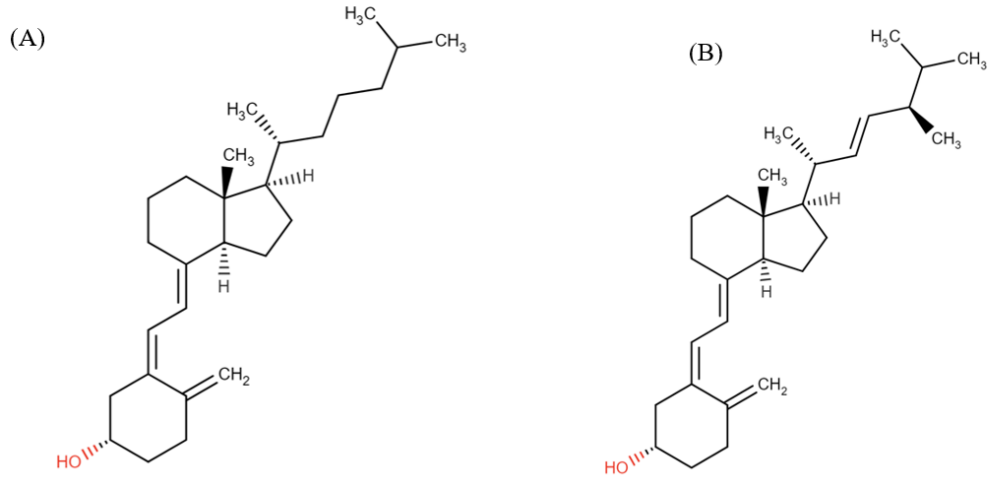


Figure 2.1: A, Structure of Vitamin D3 (C27H44O) and (B) Vitamin D2 (C28H44O)
Source: Human Metabolome database: Metabocard for VD3 and VD2 (Human Metabolome Database: Showing Metabocard for Ergocalciferol (HMDB0000900), n.d.)

As the present study primarily focuses on developing a stable encapsulated VD for transdermal delivery, this section will mainly focus on metabolism of VD synthesized through the skin.

Synthesis of VD3 through skin results from the photolysis of 7-dehydrocholesterol (7 DHC) located in the keratinocytes plasma membrane (Jodar et al., 2023). The process of photolysis is caused as the skin is exposed to sun light. Photons in UVB light penetrate through the epidermis and the energy of photons facilitate the photo transformation of 7 DHC resulting in the opening of the B ring to create Pre-VD3 (Janoušek et al., 2022; Slominski et al., 2023). However, Pre-VD3 is

thermally unstable, leading to further transformations yielding VD3 (Janoušek et al., 2022; Slominski et al., 2023). Janoušek et al., (2022), reported that at 37 ^{0}C, 80 % of pre-VD3 is isomerized into VD3. The generated VD3 is then be released into the extracellular space that will subsequently binds to a VD binding protein (VDBP) (Janoušek et al., 2022). Further, studies have revealed that VD3 metabolites have higher affinity towards VDBP compared to VD2 metabolites (Janoušek et al., 2022; Vieth, 2020). Accordingly, for the current study VD3 was selected due its association with skin synthesis and due to its proven efficacy compared to the other forms of VD in supplementation and fortification of products.

The efficacy of VD2 and VD3 in elevating human serum VD levels has been a subject of ongoing debate. Prior to the 1930s, it was widely assumed that both forms of calciferol were equally effective based on antirachitic bioassays (Armas et al., 2004). In a study conducted by Armas et al. in 2004, the focus was on comparing the potency of VD2 and VD3 (Armas et al., 2004). The study involved administering single doses of 50000 IU of both VD2 and VD3 to 20 healthy male individuals (Armas et al., 2004). Over the course of 28 days, the serum VD levels were measured following consumption. The findings of the study revealed that VD3 is three times more potent than VD2 in raising serum VD levels (Armas et al., 2004). Despite conflicting ideas in the literature regarding the potency of VD2 and VD3, systematic reviews and meta-analyses consistently favor the higher potency of VD3 over VD2 (Tripkovic et al., 2017). However, Tripkovic et al. (2017) noted a drawback in most of the available meta-analysis due to the heterogeneity of samples, emphasizing the need for more homogeneous samples with better statistical power. In response, a study using 25-hydroxyvitamin D [25(OH)D] as a marker similar to previous studies was conducted to conclude that VD3 raises serum VD levels by 74% - 75 %, whereas VD2 only

achieved a 33% - 34% increase (Tripkovic et al., 2017). These findings led to the identification of VD3 as a superior candidate for product development in the present study.

2.2 Importance of Vitamin D

VD is considered one of the most important micronutrients in the body (Pludowski et al., 2024). Initially it was believed that the function of VD is to maintain the homeostasis of serum calcium and phosphorous, i.e., having mainly an intra skeletal function (Durá-Travé & Gallinas-Victoriano, 2023). This understanding primarily arose from the role of VD in the prevention and treatment of rickets. Rickets is a condition that is caused by low levels of calcium and serum phosphate, which impair the mineralization of growth plates, leading to bone deformities (Pludowski et al., 2024). However, the discovery of VD receptors (VDR) in almost all human tissues changed this belief over time (Pludowski et al., 2024). Accordingly, VD is now considered as an important factor in maintaining both intraskeletal and extra skeletal health, a pleiotropic hormone that delivers multiple functions on different body organs (Durá-Travé & Gallinas-Victoriano, 2023).

Many studies have emphasized the importance of VD in terms of autoimmune diseases, and infectious diseases (Pludowski et al., 2024). VD is known to be important in maintaining gene expression and an important role in multiple metabolic pathways (Durá-Travé & Gallinas-Victoriano, 2023). Furthermore, numerous studies have underscored the significance of VD in extracellular chronic conditions such as cancer, autoimmune disorders, psychiatric diseases, cardiovascular diseases and infectious diseases (Durá-Travé & Gallinas-Victoriano, 2023; Pludowski et al., 2024).

Despite the emphasis placed on the significance of maintaining adequate levels of VD in the body, deficiency of VD remains a prevalent global health issue (Pludowski et al., 2024).

2.3 Deficiency of Vitamin D

Various literature sources present different guidelines and thresholds for defining vitamin D levels. However, a prevailing consensus among many authors suggests that a serum concentration below 75 nmol/L (or 30 ng/ml) indicates vitamin D deficiency (Janoušek et al., 2022; Nyakundi et al., 2023). It is important to note that a critical level below <25 or <30 nmol/L is associated with an increased risk of intra-skeletal diseases and is classified as severe vitamin D deficiency (Nyakundi et al., 2023).

The major cause for deficient VD levels is low sun exposure, as majority of the body VD is generated through the skin (Janoušek et al., 2022; Pludowski et al., 2024). Janoušek et al., (2022) reported that at high elevations, a 80 % of the UVB photons reaching the skin are reduced during the winter season. Moreover, deficiency symptoms are further exacerbated due to vegetarian diets, as most VD rich food are of animal origin (Durá-Travé & Gallinas-Victoriano, 2023). Apart from this, other factors that contribute towards VD deficiency are: clothing with limited skin exposure, high level of air pollution, having higher skin melanin concentration, and limited access to nutritious foods etc. (Mahmoodani et al., 2018; Nyakundi et al., 2023; Pludowski et al., 2024). In addition to this, with aging, available 7 DHC levels in skin is also reduced, which is the main precursor of VD3 (Janoušek et al., 2022).

Consequently, many countries such as USA, Canada, Finland and Denmark have initiated fortification of various food products, such as but not limited to margarine, milk, dairy drinks, cereals, biscuits, and fruit juices etc. (Mahmoodani et al., 2018; Nyakundi et al., 2023).

2.4 Vitamin D Supplementation

The specific form of VD used in fortification appears to vary in different studies (Vieth, 2020). Different forms such as cholecalciferol (VD3), ergocalciferol (VD2), calciriol (1,25-dihydroxyvitamin D) and calcidiol (25-hydroxyvitamin D) are mentioned in various studies (e.g., Vieth, 2020). In addition the recommended daily dose also varies with age, gender, body mass, prevailing health conditions and environmental factors (Sorrenti et al., 2023). Therefore, it is challenging to propose a definite dosage for everyone, as vitamin D supplementation needs to be tailored to the individual (Sorrenti et al., 2023). Additionally, any supplementation should be accompanied by monitoring vitamin D and calcium levels in the body to avoid potential toxicities (Sorrenti et al., 2023).

One of the most popular ways of delivering VD, is via the oral route through fortified food products (Janoušek et al., 2022; Mahmoodani et al., 2018). However, this could be considered as more of a preventive or a prophylactic measure rather than a therapeutic measure (Sawarkar & Ashtekar, 2020). Therapeutic VD is usually delivered through tablets, capsules and granules, etc. (Sawarkar & Ashtekar, 2020). Since VD is liposoluble, its absorption is believed to be similar than that of lipids (Janoušek et al., 2022). Vitamin D absorption begins in the stomach, where pepsin helps to separate it from its associated protein (Janoušek et al., 2022; Ramasamy, 2020). In the duodenum, enzymes like proteases, amylases, and lipases continue breaking down the food matrix to release vitamin D (Janoušek et al., 2022). Subsequently, bile acids initiate the emulsification process, forming mixed micelles that carry fat-soluble substances for absorption by enterocytes (Alsaqr et al., 2015; Janoušek et al., 2022).

However, there exists a few challenges associated with this mode of VD absorption. Drugs and health conditions that restrict fat absorption could lead to reduced VD bioavailability (Alsaqr et

al., 2015; Janoušek et al., 2022). Moreover, plant phytosterols and other fat soluble vitamins such as A,E, and K also could compete with VD for binding with micelles (Janoušek et al., 2022). Apart from these problems, several health conditions such as celiac disease, patients undergone gastric bypass surgery, Alzheimer's disease hinder the bioavailability of serum VD and its metabolites (Alsaqr et al., 2015). Therefore, supplementing VD through transdermal delivery has also being explored as a potential avenue for nutrient delivery in recent studies (Alsaqr et al., 2015; Sawarkar & Ashtekar, 2020).

2.5 Transdermal Delivery of Vitamin D

Skin is the largest organ of the body consisting of epidermis, dermis and subcutaneous layers (Fan et al., 2024). Based on the stage of development and cell morphology, epidermis can be further divided as stratum corneum, stratum granulosum, stratum spinosum and stratum basale, named from the outermost layer to inner most layer (Fan et al., 2024). The stratum corneum contains barrier properties that only allows the passage of smaller molecules (<600 Da) to diffuse into the body (Alsaqr et al., 2015; Fan et al., 2024). According to literature, the inherent characteristics of VD3 makes it ideal for transdermal delivery. Its low molecular weight (384.64 g/mol) and low required daily dosage (0.01 mg – 5 mg) are some of the reasons that warrant VD3 as excellent for transdermal delivery (Alsaqr et al., 2015; Fan et al., 2024; Sawarkar & Ashtekar, 2020). However, it has to be noted that the partition coefficient of VD3 in lipid/ water systems is around 10.2, indicating that VD3 is highly lipophilic (Sawarkar & Ashtekar, 2020). Thus, this property is advantageous for passing through the epidermis. The epidermis contains lipids, free fatty acids, and cholesterol, that makes the layer lipophilic thus it allows the penetration of low molecular weight lipophilic compounds (Fan et al., 2024). However, the dermis is more hydrophilic in nature (Fan et al., 2024). Therefore, the high hydrophobicity of VD3 could be problematic in passing

through the inner layers (Fan et al., 2024). A potential solution is to use materials such as proteins, peptides, and carbohydrates in combination with VD3, which could help overcome this issue (Fan et al., 2024, 2024). In addition to this, use of skin permeation enhancers further improve the penetration ability of the compound (Fan et al., 2024). These permeation enhancers are inactive compounds capable of partitioning into the stratum corneum by interacting with the lipids in the epidermis (Fan et al., 2024). This interaction allows the diffusion of the compound while temporarily reducing skin permeability barrier (Fan et al., 2024). Some examples for skin permeation enhancers are, alcohols, amides, terpenes and fatty acids (Alsaqr et al., 2015; Fan et al., 2024). For this study, hemp seed oil was used as the carrier for VD3. Therefore, the poly unsaturated fatty acids (PUFA) present in hemp seed oil could potentially improve the skin permeation ability of the developed product. Further, Fan et al., (2024) review that PUFA are more potent in reducing skin barrier compared to saturated fatty acids (SFA) due to its structural differences owing to double bonds. Moreover, these PUFA have the ability to alter the lipid organization of the stratum corneum and increase permeation through reversible fluidization (Alsaqr et al., 2015; Fan et al., 2024; Sawarkar & Ashtekar, 2020). Some of the PUFA in consideration are oleic, linoleic and linolenic acid, which are present in hemp seed oil (Fan et al., 2024; Huang et al., 2020). According to Huang et al., (2020), hemp seed oil contains about 90 % PUFA and is becoming popular in the cosmetic industry owing to its skin permeation ability.

2.6 Stability of Vitamin D3

Despite different methods of supplementing VD, it is imperative that the integrity of the VD3 molecule is preserved during processing and storage in order to gain targeted benefits. Unfortunately, literature reveal that VD is highly susceptible towards degradation, isomerization and loss of functionality upon exposure to temperature, light. pH, and oxygen (Jelić et al., 2024;

Mahmoodani et al., 2018; Mulrooney et al., 2021; Zareie et al., 2021). The presence of double bond in the structure of VD3 makes it highly vulnerable to processing and storage conditions (Zareie et al., 2021). In a study conducted by Tabibian et al.. (2017) revealed that the amounts of VD3 and VD2 present in bread reduced by 28 % over five days of storage (Tabibian et al., 2017). A similar occurrence was reported related to fortified vegetable oil, where a 16.9 % loss of VD3 was observed when heated up to 105 ^{0}C and 180 ^{0}C (Saghafi et al., 2018). Moreover a 67 % loss of fortified VD3 was reported in canola oil when heated at 180 ^{0}C or over (Ložnjak & Jakobsen, 2018). Therefore in order to address these issues related to VD stability different techniques have been proposed such as micro and nano emulsion preparation and encapsulation techniques, etc. (Mulrooney et al., 2021). Accordingly, the current study focuses on developing encapsulated VD3 by a Maillard reacted glycated lecithin matrix capable of preserving the integrity of VD3 while delivering adequate bioavailability and bio accessibility.

2.7 Encapsulation Techniques for Delivering Vitamin D3

Encapsulation techniques for VD are considered optimal by scientists due to its lipophilic nature (Bashir et al., 2024; Maurya et al., 2020). Encapsulation is expected to protect VD from physicochemical stress factors such as oxidation, pH changes, moisture, and temperature, while maintaining its bioavailability and improving release characteristics (Maurya et al., 2020). In the case of microencapsulation various techniques have been employed including spray drying, emulsification, use of nano-structured lipid carriers, and electrospinning techniques etc. (Maurya et al., 2020). The literature highlights that drying dried emulsions offer better stability than aqueous emulsions related to storage and oxidative stability (Rezvankhah et al., 2022).

Spray drying is a popular method used for developing dried microcapsules for bioactive compounds (Maurya et al., 2020). The usual procedure for encapsulation is based on the formation

of a dispersion of the polymeric material, that serves as the capsule wall. with the bioactive ingredient followed by the specific drying method (Bashir et al., 2024; Maurya et al., 2020).

As per the drying method a variety of techniques have been described in the literature such as spray drying, freeze drying, vacuum drying and oven drying (Bashir et al., 2024; Maurya et al., 2020). A study by Bashir et al., (2024), revealed that use of drying techniques such as spray drying and freeze drying could be effectively used in the encapsulation of VD3 with acceptable efficiency and release kinetics. However, a lack of research can be observed in terms of use of oven drying and vacuum drying for VD3 encapsulation. Ramos et al., (2021) demonstrated in their study that vacuum drying is more effective in the encapsulating fish oil with minimum oxidation and higher encapsulation efficiency.

Therefore, in this study, the encapsulation is achieved through oven drying followed by vacuum drying. The core material comprises of vitamin D3 (VD3) dispersed in hemp seed oil. The resulting emulsion was then combined with lecithin and dextrose, promoted by the Maillard reaction, which served as the wall material for encapsulation.

2.8 Hemp Seed Oil

Hemp seed oil was used as the VD3 dispersing medium for this study owing to its multiple benefits offered when compared with commercial available oils rich in PUFA (Rezvankhah et al., 2022). The oil, as denoted by its name, is derived from the Hemp plant (*Cannabis sativa L.)* that belongs to Cannabinaceae family (Spano et al., 2020). Cannabis plant has been popular over the history due to its drug abuse (marijuana) that led to banning of cultivation of all forms of *Cannabis Sativa L*. in the western world for many years (Cherney & Small, 2016). However, there are different cultivar types of Cannabis that contain less Δ^9-tetrahydrocannabinol (THC), which is the compound targeted in recreational use containing intoxicating effects (Spano et al., 2020). These

non- intoxicating bio types with less THC are known as Hemp or industrial Hemp (Cherney & Small, 2016). According to literature a minimum concentration of at least 1% THC is required to produce intoxication (Cherney & Small, 2016). Based on this, different values have been proposed in literature to distinguish Hemp from cannabis. In Canada and USA 0.3 % of THC is used as an arbitrary concentration to distinguish Hemp, where in Europe 0.2 % is the maximum threshold of THC to be considered legal Hemp (Cherney & Small, 2016; Spano et al., 2020). Even though a wide array of commercial products have been documented for Hemp, fiber, oil seed and pharmaceuticals appear to be the most promising product categories (Cherney & Small, 2016).

The shift towards Hemp based products in the western world is clearly reflected by its imports over the years. For instance, USA has imported hemp seed, oil and oil cake products worth of over $ 75 million USD in 2015 (Cherney & Small, 2016). This trend has only been increasing since.

One of the most important parts of the Hemp plant is the seed, which is denoted as suitable for human consumption (Cherney & Small, 2016). Hemp seed contains about 20 %- 25 % protein, which is a similar biological value to hen's egg white (Rehman et al., 2021). Therefore, literature identifies hemp seed as an excellent source of plant protein. Seeds also contain 20 % - 30 % of carbohydrate, 25 %- 35 % of lipids and a rich source of fiber and minerals (Rehman et al., 2021). Hemp seed oil has been of interest due to its rich PUSFA profile. Hemp seed oil contains about 7 % - 11 % SFA, about 90 % UFA and about 75 % of PUFA (Huang et al., 2020; Rezvankhah et al., 2022). The detailed benefits of Hemp seed oil in literature, range from improving cardiovascular health to development of functions of brain and cancer mitigation (Rezvankhah et al., 2022).

Based on these findings, an array of studies could be found in literature where the seed oil potential has been tested for food products, animal feed and cosmetic products. The known benefits of Hemp seed oil in presence of protein surfactant and for its ability to permeate the skin through the stratum

corneum makes this oil an excellent candidate for skin care products (Huang et al., 2020; Pei et al., 2020). However, having a higher percentage of PUFA, present challenges in terms of improving the product shelf life. The PUSFA present in Hemp seed oil are highly susceptible to oxidation upon exposure to light, temperature, oxygen, metals ions and moisture (Rezvankhah et al., 2022), which could result is negative effects as used as a vehicle to transfer VD. Therefore, the present study holds promise as it explores the possibility in not only protecting VD3 but also hemp seed oil from external stress factors.

2.9 Soy Lecithin

Lecithin is a generic term used to describe a mixture of lipids, with phospholipids (PL) making up more than 50 % of its composition (Alhajj et al., 2020; Robert et al., 2020). It can be sourced from either vegetable or animal origins (Robert et al., 2020). One of the ways of obtaining lecithin is through the valorization of byproducts of the oilseed industry (Wee et al., 2023). Moreover, lecithin obtained through these sources are widely utilized in different industries ranging from food, pharmaceutical, livestock, aquaculture and cosmetics etc. (Wee et al., 2023).

However, it is important to note that the physicochemical characteristics of lecithin varies depending on the source (Gupta & Gaur, 2023; Wee et al., 2023). Soybean lecithin contains the highest amount of PL among other plant lecithin sources reaching up to 65 % - 75 % (Wee et al., 2023). Therefore, soybean is considered one of the main sources of commercial lecithin (Wee et al., 2023). Moreover, de-oiling and refining of lecithin contribute towards increasing the purity of PL up to 97 % (Wee et al., 2023). However it must be noted that the purification process is costly and therefore the use of non-purified PLs could offer an economical solution for applications such as encapsulation of bio active components. Donmez et al. (2024) reported that highly purified or

synthetic PLs costs about $ 800/ 500 g, whereas refined soybean PL are available at $ 20- $ 30 per kg. Moreover, in north America non-purified soy lecithin can be found at a price low as $ 2.07/ kg (Donmez et al., 2024). Moreover, the fact that soy lecithin is a plant-based PL source makes it more suitable for modern applications in food and cosmetics, as contemporary consumers prefer plant-based ingredients over animal-based products (Kutzli et al., 2021). Furthermore, Kutzli et al., (2021) reported that global market prediction for sustainable and "cleaner labels" was USD 47.5 billion for the year 2023. Therefore, the use of soy lecithin for encapsulation purposes can be considered as an economical and futuristic means of protecting bioactive ingredients. However, it is challenging to produce an exact composition for soybean lecithin as different studies have reported different values in terms of lecithin composition based on purity, cultivars etc. Table 2.1 depicts the composition of soybean lecithin based on different studies.

Table 2.1:Phospholipid composition of soybean lecithin as reported by different studies.

Phospholipid composition of soybean lecithin as reported by different studies.

Reference	PC %	PE %	PI %	Other Phosphatides %
(Russi, 2022)	19-21	8-20	20-21	5-11
(Li et al., 2022) for 50 % purity lecithin	59.34	2.04	0.01	>30
(Robert et al., 2020)	12.7-16.7	6.5-13.6	6.5-11.8	Around 6

*PC, PE and PI represent Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylineositol respectively.

As indicated in Table 2.1, PCs are the major PLs found in soybean lecithin. Apart from this, PE, PI and LPC are also found in soybean lecithin (Wee et al., 2023). Figure 2.1 indicates the structures of most common PL found in soybean lecithin as reported by (Gupta & Gaur, 2023). Literature

reveals that the composition of soybean lecithin is highly depended on agronomic practices, environmental factors and plant genetic diversity (Robert et al., 2020).

2.10 Maillard Reacted Lecithin Conjugates

Maillard reaction as initially described by Luise Camille Maillard, is a series of non-enzymatic reactions between an amino group present in proteins and a carbonyl group of a reducing sugar (Kutzli et al., 2021). This is a naturally occurring reaction which requires no chemicals or enzymes (Kutzli et al., 2021). Moreover, research indicates that inducing Maillard reaction in protein products at increased temperatures aids in improving protein functionality (Kutzli et al., 2021). Accordingly, this can be considered as a clean and green way of improving food proteins functionality (Donmez et al., 2024; Kutzli et al., 2021).

Recently, Russi, (2022) demonstrated that Maillard reaction can also be initiated by reacting de-oiled lecithin with PUFA and a reducing sugar. The reaction requires elevated temperatures for a reasonable time at a sufficient moisture content (Russi, 2022). In this particular case the amino group is supplied through the PLs present in lecithin, namely PEs (Figure 2.2) . However, in order to obtain maximum results, de-oiling of lecithin is imperative. This could be performed through solvent extraction based on acetone (Russi, 2022). This step facilitate removal of residual soy oil and sterols (Russi, 2022).

Concerning Maillard reaction between phospholipids in soy lecithin and reducing sugars, Amadori-Phosphatidylethanolamine (Amadori-PE) (Figure 2.3) can be regarded as an early stable product (Han et al., 2022). This compound can also serve as a marker for the reaction (Donmez et al., 2024; Han et al., 2022). The product is generated when the amino group present in PE is reacted with the aldehyde group of the reducing sugar that leads to the formation of Schiff base (Donmez et al., 2024; Han et al., 2022).

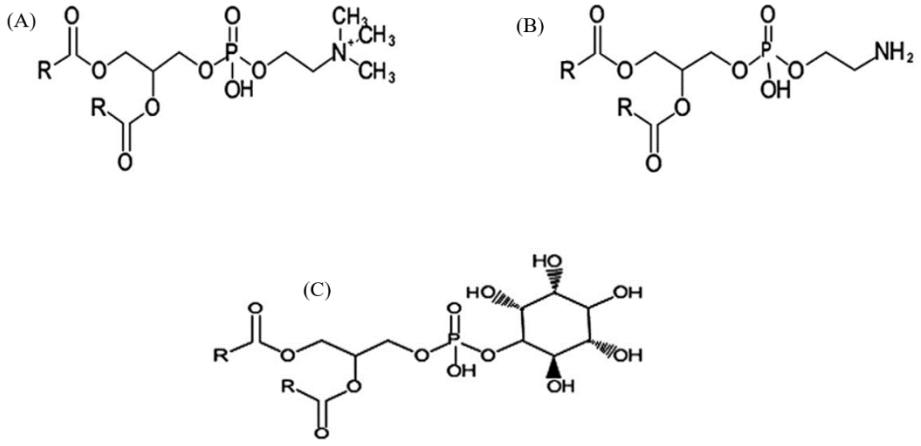


Figure 2.2: Structure of commonly observed Phospholipids in soybean lecithin.
(A) Phosphatidylcholine, (B) Phosphatidylethanolamine, (C) Phosphatidylineositol

However, a Schiff base is highly unstable, therefore the compounds undergo further isomerization creating initial stable products termed as Amadori-PE (Han et al., 2022). Additionally, this reaction could produce advanced glycation end products such as Carboxyethyl Phosphatidylethanolamine (CE-PE) and Carboxymethyl Phosphatidylethanolamine (CM-PE) (Han et al., 2022). The formation of these products is favored when samples are exposed to higher temperatures for extended periods (Han et al., 2022). Moreover, literature further indicate that providing thermal treatment under a vacuum could favor the Amadori reaction products (ARP) development instead of generating CM-PE (Han et al., 2022). This phenomenon is further elucidated by Le Chatelier's principle which can show that the removal of water drives the equilibrium towards the increased formation of Amadori-Phosphatidylethanolamine (ARP). (Cui et al., 2021; Donmez et al., 2024). These MRP are considered to be important as they offer a protective role against external stress factors while mitigating lipid oxidation (Cui et al., 2021; Donmez et al., 2024; Ixtaina et al., 2023). Therefore, development of encapsulated vitamin D, using Maillard reacted PLs conjugates could

be considered as a promising and economical way to reduce oxidation of both Hemp seed oil and VD3.

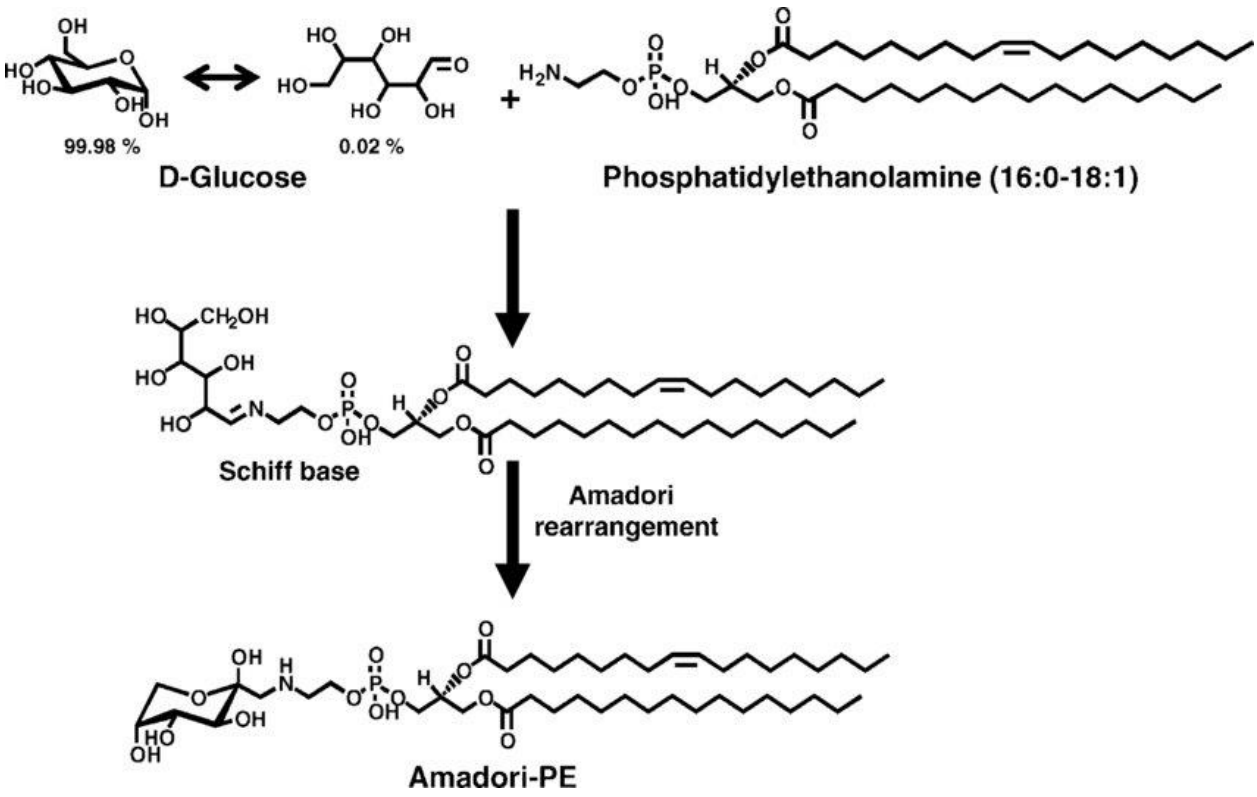


Figure 2.3: Maillard reaction between phospholipids and reducing sugars

Chapter 03

Materials and Methods

3.1 Materials

Hemp seed oil for the study was kindly provided by IND Hemp, Oil seed and Fiber, Montana, USA. The oil was produced from X-59 cultivar seeds, containing less than 0.3 % THC (*Agricultural Seed - IND HEMP*, 2024). Therefore, it is in compliance with the guidelines set by the United States Department of Agriculture (USDA) for the classification as commercially legal Hemp seed oil (*Agricultural Seed - IND HEMP*, 2024). Required de- oiled soy lecithin was kindly provided by Rusitec S.A, Buenos Aires, Argentina. Vitamin D3, 99 % was purchased from Fisher Scientific (Waltham, MA, USA). All other chemicals used for the study were of analytical grade and were purchased from either Fisher Scientific (Waltham, MA, USA) or Sigma Aldrich (St. Louis, MO, USA).

3.2 De-oiling of Soy Lecithin

The provided soy lecithin was further de-oiled to improve product quality, following a method published by Russi, (2022) with slight modifications. Initially, 15 g of lecithin was mixed with 5 g of acetone and vortexed for 2 minutes at 16000 g to homogenize the mixture. The mixture was then centrifuged for 30 minutes at 16,000 g at room temperature. After centrifugation, the top liquid layer was discarded. The remaining solid fraction was spread on aluminum foil and left to dry inside a fume hood overnight. The dried lecithin sample was ground to a fine powder the next day.

3.3 Emulsion Preparation

Sample preparation method was originally described by Russi, (2022), for encapsulating fish oil. For the study, the method was modified to suit encapsulating VD3. The product formula for sample preparation is mentioned in table 3.1. Initially a known amount of VD3 99 % was dispersed in hemp seed oil. Then, de oiled lecithin (as described in section 3.2) was added, and the mixture was stirred for 10 mins until the oil and lecithin appear to be well mixed. In a separate beaker, a dextrose solution was prepared by adding dextrose to warm water. The dextrose solution was then added to the lecithin-oil mixture and mixed for another 10 minutes. Final mixture was then heated for 1 hour at 100 ^{0}C and 50 ^{0}C using a hot air oven and the control was not provided with a heat treatment. Based on the heat treatment the samples were labeled as H100, H50 and NHT indicating 100 ^{0}C, 50 ^{0}C and no heat treatment respectively. Following the heat treatment, samples were vacuum dried at 50 ^{0}C, 60 mbar for 24 hours.

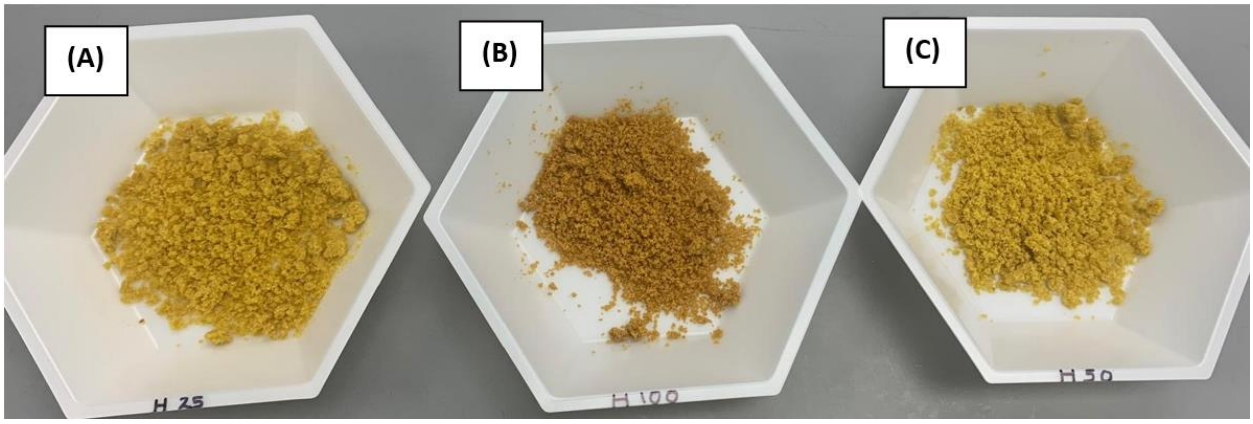


Figure 3.1: Vacuum dried samples. NHT, (B) H100 (C) H50

Prepared samples (Figure 3.1) were then transferred into airtight packages, labeled and stored at 4 ^{0}C until further analysis. All sample readings were taken using three biological replicates for each treatment.

3.4 Moisture Content, Water Activity (a_w) and Color

Moisture content was measured utilizing a halogen moisture meter (Moisture Analyzer HE53, Mettler Toledo, OH, USA) at 105 ^{0}C. For each measurement around 2 g of sample was measured and evenly distributed in the pan.

Table 3.1: Product Formula

Material	Amount % (W/W)
Vitamin D3 99 %	2
Hemp seed oil	18
Lecithin	55
Dextrose	5
Water	20

Water activity was determined using a water activity meter (AQUALAB® 4TE) at 24 ^{0}C. Color of each sample was determined based on the CIE L*(lightness) a* (redness) b* (yellowness) color values using bench top ColorQuest XE. Readings were obtained using D65 (day light) illumination with reflective specular included (RSI). Color differences between the samples were calculated based on the ΔE_{ab} values based on a formular by Commission of illumination (CIE). Following equation was utilized for the calculation.

$$\Delta E = \sqrt{\{(L*_{NHT} - L*_T)^2 + (a*_{NHT} - a*_{HT})^2 + (b*_{NHT} - b*_T)^2\}}$$

* NHT represents No heat treatment while T stands for the specific temperature (100/ 50)

3.4 Surface oil and Encapsulation Efficiency

Surface oil (SO) and Encapsulation Efficiency (EE) were determined based on a method described by Chen et al., (2020) and (Donmez et al., 2024) with slight modifications. 0.75 g of the samples were weighed and mixed with 7.5 ml of acetone. The mixture was then gently swirled for 30

seconds. The weight of a Whatman grade 1 filter paper was measured before filtering the solution through it. The filter paper with the sample was left to dry overnight under a fume hood to allow the acetone to evaporate. After 24 hours of drying, the final weight of the filter paper with the sample was measured until a constant weight was obtained. The SO and the EE of the samples were calculated based on the following equation.

$$Encapsulation\ Efficiency = \frac{Total\ oil - Surface\ oil}{Total\ oil} \times 100$$

3.5 Determination of Vitamin D3 Present in the Sample

Five grams of the sample were weighed into a 11 mL glass test tube. Subsequently, 5 mL of methanol was added, and the mixture was sonicated using a probe sonicator (Misonix XL-2000) for 30 seconds. After sonication, the cap was removed to allow the solid material to settle at the bottom, and the top methanol layer was decanted into a separatory funnel. To the remaining solid sample, 5 mL of a hexane: acetone (1:1 v/v) solvent was added and sonicated again for 30 seconds. The cap was removed to allow the solid material to settle, and the top hexane: acetone layer was added to the same separatory funnel. Approximately 10 mL of distilled water and 100 µL of saturated NaCl solution were added to the above mentioned separatory funnel promote phase separation. Once the solvents were settled, the lower polar phase was removed and discarded, while the upper non-polar phase was transferred to a new tube and filtered over anhydrous sodium sulfate in glass wool to remove any residual water. The filtrate was then topped off to a known volume of 25 mL using Methyl tert-butyl ether (MTBE) in a volumetric flask. Aliquots of 1 or 2 mL were immediately measured into glass tubes the samples were stored at -80°C until further analysis. Samples were then analyzed using UHPLC-MS triple quadruple (TSQ QUANTIVA) with electro spray ionization (ESI) source in positive ion mode. Compound detection was carried out

using product and precursor ion scan. Vitamin D2, was used as the internal standard for the quantitation. Injection volume of 10 µl was utilized while the load pump floor rate was maintained at 600 µl/ min.

3.6 Determination of Oxidative Stability

3.6.1 Extraction of oil

Extraction of both surface oil (SO) and encapsulated oil (EO) was done following a method by Linke et al. (2020)and Donmez et al. (2024) with modifications. Initially, 1 g of the sample was measured and mixed with double-distilled water. The mixture was placed in a water bath at 30 °C for 30 minutes, with vortexing at 5 minute intervals. Subsequently, 33.33 ml of an isooctane:2-propanol (3:1 v/v) mixture was added to the sample, and the mixture was vortexed. The sample was then centrifuged at 5000 g for 10 minutes. The upper organic phase was separated and filtered through Whatman grade 1 filter paper over anhydrous sodium sulfate (Na_2SO_4). The filtered solution was then used for the p-anisidine test. A blank sample was also prepared following the same method without the addition of encapsulated VD3 sample.

3.5.2 P- Anisidine value for secondary oxidative products

P- anisidine value (AV) was determined based on AOCS Official Methods Cd 18-90 (Donmez et al., 2024). The p-anisidine reagent was prepared by dissolving 0.25 g of p-anisidine in 100 ml of glacial acetic acid. Initially, 0.9 ml of the extracted upper layer, as described in Section 3.5.1, was placed in a cuvette, and the absorbance was measured at 350 nm using a spectrometer. The spectrometer was blanked with a reference cuvette containing the blank sample prepared in Section 3.5.1. Subsequently, 0.18 ml of the p-anisidine reagent was added to the 0.9 ml of the extracted upper layer from Section 3.5.1. The mixture was shaken vigorously and allowed to react for 10

minutes. A blank sample with 0.18 ml of p-anisidine reagent was used to blank the spectrometer. After waiting for 10 minutes, the absorbance of the samples was measured at 350 nm.

3.7 Thermal Stability of the Samples

3.7.1 Thermogravimetric Analysis (TGA)

Thermal stability in terms of oil oxidation and VD3 decomposition was studied using Thermo gravimetric analysis (TGA 550, TA instruments) following a method described by Jannasari et al., (2019). 10 mg of the sample was weighed using a microbalance and loaded into aluminum pans. Pans were hermetically sealed prior to loading into the instrument. Then the instrument was led to calibrate at 25 ^{0}C and temperature was ramped up at 10 ^{0}C/min until 500 ^{0}C. The onset temperature (T_{onset}) and the peak temperature (T_{peak}) were determined based on the thermal decomposition curve using analysis tool in TRIOS 5.7 software.

3.7.2 Differential Scanning Calorimetry

The thermal behaviors of Hemp seed oil and the micro encapsulated powder were observed using Differential Scanning Calorimetry (DSC 2500, TA Instruments), following a method by (Rezvankhah et al., 2022) with slight modifications. Samples in weight range of 5 mg – 10 mg were places into TGA aluminum pans and then hermitically sealed and loaded into the DSC instrument. An empty pan was used as the reference and the experiment was conducted under an inert gas environment containing N_2. Samples were equilibrated at – 50 ^{0}C and kept isothermal for 5 minutes. Then the sample was heated up to 120 ^{0}C using a temperature ramp of 10 ^{0}C/ min to identify the melting point (T_m) and the existence of a glass transition temperature (T_g).

3.8 Morphological Analysis

Morphology of the encapsulated VD3 samples were studied using Scanning Electron Microscopy (SEM) following a method by described by Donmez et al. (2024). Initially, the samples were mounted on stubs and then coated with gold using a sputter coater (PELCO, Model 3 sputter coater 91000). Then the samples were placed in the SEM microscope (Thermo Scientific Quattro ESEM) at 5kV voltage and different magnifications.

3.9 Statistical Analysis

All experiments were conducted with three replicates, and the results are reported as mean ± standard error mean. Initially, all results were subjected to Analysis of Variance (ANOVA) to identify treatment effects. Subsequently, multiple comparisons of means were performed using the Tukey HSD test to evaluate significant differences between treatments. All tests were conducted at a 0.05 level of significance. Statistical analyses were carried out using JMP Pro 17 and OriginPro 2020 software.

Chapter 04

Results and Discussion

4.1 Moisture Content and the Water Activity (a_w)

Moisture content and the water activity are important parameters in terms of shelf life of the developed product. Water activity indicates how water is bound within the wall matrix, whereas moisture content relates to the total available water content (Chaves & Pinho, 2020).

As indicated in Table 4.1, moisture content of all samples was below 4 %, which is the set moisture content value used for dried powders in the food industry (Klinkesorn et al., 2006). Furthermore, as depicted in table 4.1, all water activities reported are below 0.6. According to literature, products having water activity below 0.6 prevents the growth of pathogens and most microorganisms while bacteria growth is highly reduced at $a_w < 0.87$ (Chaves & Pinho, 2020). Moreover, both moisture content and water activity are significantly different ($p < 0.05$) for the H100 sample. This is attributed to the evaporation of moisture caused by the higher temperature treatment. Ideally, low water activity levels are expected to result in lower lipid oxidation in food systems. However, literature report that low moisture foods may behave differently according to the formulation and the type of lipid (Vu et al., 2020).

Table 4.1:Moisture Content % and Water Activity (a_w)

Sample	Moisture Content %	Water Activity
H 100	1.09 ± 0.18^{b}	0.23 ± 0.025^{d}
H 50	3.32 ± 0.25^{a}	0.42 ± 0.025^{c}
NHT	3.38 ± 0.15^{a}	0.44 ± 0.038^{c}

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$)

A study based on low moisture crackers reported that very low water activity could potentially increase the level of lipid oxidation in low moisture food products (Vu et al., 2020). In the study by Vu et al. (2020), authors reported an increase of lag phase in lipid oxidation with the increase of water activity levels. According to the study, $a_w > 0.4$ resulted in an increase of the lag phase for secondary oxidative products compared to $a_w < 0.2$. The authors further stated that water can bind to hydroperoxides, which are the result of primary lipid oxidation. This binding could delay the decomposition of hydroperoxides into secondary oxidative products that yield aldehydes, ketones, etc. in products (Vu et al., 2020).

Apart from lipid oxidation, water activity also influences the rate and extent of the Maillard reaction (Lund & Ray, 2017). Maillard reaction is reported to be at maximum at intermediate water activity levels ranging from 0.4 to 0.8 (Lund & Ray, 2017). This phenomenon is explained by the fact that at higher water activity levels, reactants have greater mobility. In contrast, at lower water activity levels, reactants become more concentrated, potentially increasing their interaction and enhancing initially the reaction rate (Lund & Ray, 2017). However, with further reduction of water activity, the reactants become so concentrated that diffusion is hindered, thereby lowering the rate of the Maillard reaction (Lund & Ray, 2017). Both NHT, and H50 samples falls within the range desirable for the Maillard reaction. However, the H100 sample is below the desirable water activity level (Lund & Ray, 2017).

4.2 Surface oil and Encapsulation Efficiency

Surface oil refers to the fraction of the oil that is non-embedded within the protective wall matrix (Linke et al., 2020). Therefore, the surface oil fraction negatively impact the shelf stability of the encapsulated powder as the surface oil is exposed to external environmental factors such as temperature, oxygen, light etc. (Donmez et al., 2024; Linke et al., 2020). Furthermore, the

encapsulation efficiency must include only the fraction of oil that is contained within the protective wall matrix, thus shielding the core of the encapsulated material from external stress factors. The table 4.2, summarizes the obtained percentage surface oil and the encapsulation efficiency.

Table 4.2: Surface oil and Encapsulation Efficiency

Sample	Surface Oil %	Encapsulation Efficiency %
H 100	17.3 ± 0.25^{a}	38.7 ± 0.87^{d}
H 50	11.4 ± 1.20^{b}	59.52 ± 4.26^{c}
NHT	12.6 ± 1.15^{b}	55.1 ± 4.08^{c}

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$)

As illustrated in table 4.2 and Figure 4.1, the highest encapsulation efficiency is reported for the H50 sample, while the lowest is reported for the H100 sample. Conversely, surface oil measurements indicate that the H100 sample had the highest amount of surface oil, while the H50 sample has the lowest. As depicted in Figure 4.1, encapsulation efficiency decreases with the increase in the amount of surface oil. Moreover, H100 sample was significantly different ($p < 0.05$) from the other two samples indicating that increase of temperature decreased the encapsulation efficiency of hemp seed oil. This effect can be attributed to the increased temperature, which facilitates the Maillard reaction and thus the resulting Maillard reaction products (MRPs) form at the interface, hindering the migration of oil inside the encapsulated product (Li et al., 2015). Consequently, this leads to a higher amount of surface oil compared to the oil within the matrix. Furthermore, Li et al., (2015), from spray drying experiments, reported that high temperatures can cause cracks in the wall matrix due to the rapid evaporation of moisture in the spray drying process.

A similar phenomenon is possible to occur ensuring the preparation of the H100 sample as higher temperature may have caused cracks that allowed hemp oil to seep out of the shell. However, the

use of lower drying temperature could avoid this effect. Moreover, in samples with higher moisture contents, microcapsules tend to agglomerate together to form larger particles (Li et al., 2015). Therefore, based on the results of encapsulation efficiency H50 treatment can be considered as the best treatment.

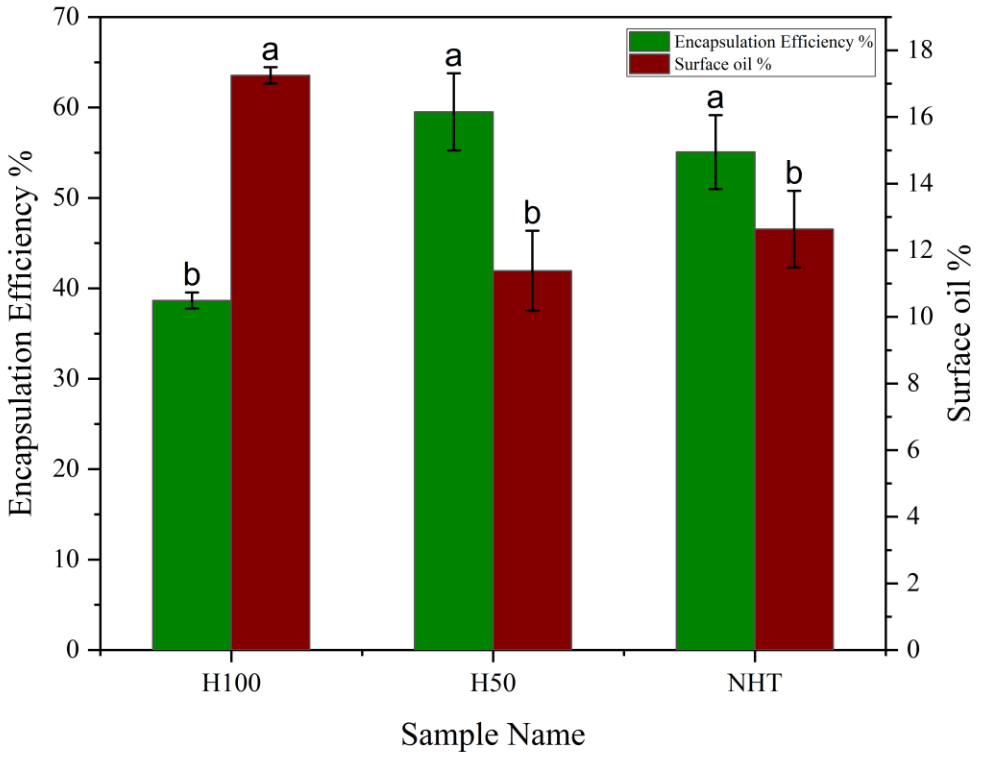


Figure 4.1:Effect of temperature on encapsulation efficiency and surface oil %. Samples with different superscripts are significantly different from each other ($p < 0.05$)

4.3 Total Vitamin D3

Table 4.3 presents the total amount of VD3 present in the samples, along with the percentage of VD3 remaining from the initial amount added during sample preparation. For the current study only total extractable VD3 was considered, as prolonged heat treatment during hot air drying and vacuum drying could potentially destroy VD3 present in the surface oil.

Table 4.3: Total vitamin D3 present in encapsulated powders

Sample Name	Vitamin D3 (µmol)	Available Vitamin D3 % after encapsulation
H100	110.5 ± 26.1 [a]	42.5 ± 5.1 [a]
H50	103.2 ± 13.2 [a]	39.7 ± 10.1 [a]
NHT	88.4 ± 9.7 [a]	34.1 ± 3.02 [a]

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$)

Percentage retention of VD3 after sample preparation is important as it directly relates with the success of the encapsulation process. Figure 4.2 graphically represents the amount of VD3 retained in encapsulated powders as a percentage of initial VD3 added. According to figure 4.2, the highest retention percentage was recorded by the H100 sample and the lowest was recorded by the control. This could be attributed to the protective effect exerted by the wall matrix of the encapsulated powder. In the case of the H100 sample, the Maillard-reacted wall material appears to provide more protection against VD3 degradation compared to H50 and the control. It is noteworthy that although H100 reported the highest retention of VD3, as shown in Figure 4.2, it also contained the highest amount of surface oil, which was significantly different from the other two powders. This high surface oil amount would typically suggest the lowest VD3 retention percentage. However, the high VD3 retention in the H100 sample may imply that the heat induced Maillard reaction in the wall matrix positively contributed to the retention of VD3. This could be further explained by the known antioxidant effect of Maillard Reaction Products (MRPs), which may have protected VD3 from further degradation while also acting as a barrier against heat and light transfer into the core (Donmez et al., 2024; F. Gao & Birch, 2016; Y. Gao et al., 2023). Based on the results the current approach of encapsulation can be considered promising in increasing shelf stability of VD3, with further improvements to encapsulation efficiency.

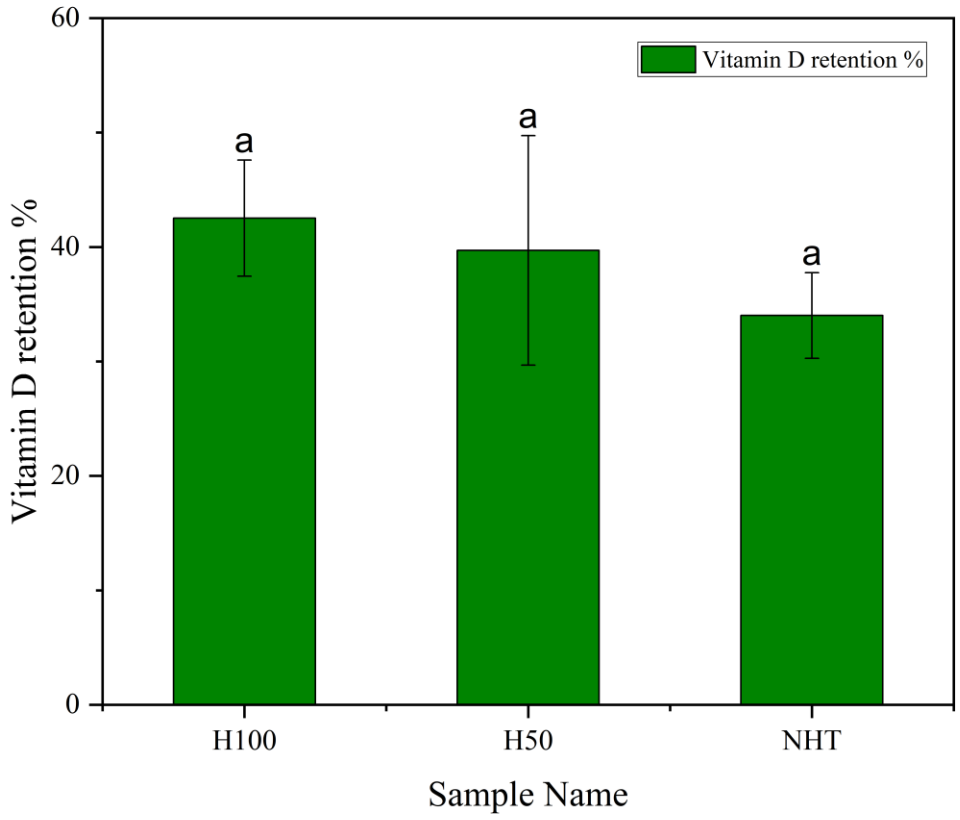


Figure 4.2:Effect of temperature on vitamin D retention. Samples with different superscripts are significantly different from each other ($p < 0.05$)

According to literature, VD3 is prone to thermal degradation. In a study using LC/MS for detection of the compound, authors observed reversible isomerization of VD3 to pre-VD3 upon exposure to heat above 65 ºC (Mahmoodani et al., 2017). Unfortunately, LC/MS cannot distinguish between the isomers unless it is coupled with a different detection technique, such as diode array detectors (DAD) or chemical derivatization of VD3 (Mahmoodani et al., 2017). Therefore, the present study was not able to distinguish between the VD3 isomers. However, it must be noted that pre-VD3 is an intermediate product generated during skin metabolism of VD (Ramasamy, 2020).

4.4 Effect of Temperature on Color

Table 4.4 and figure 4.3 indicate CIE L*a*b* color values obtained for the samples. For all three samples H100 was significantly different ($p < 0.05$) in color compared to the other two. Moreover, according to the ΔE_{ab} value the color difference of H100 is noticeable to the naked eye as it is above 5 based on guidelines by the International Commission on Illumination (CIE). Color difference between H50 and NHT was not distinguishable according to the ΔE value.

Table 4.4: Effect of temperature on color

Sample	L*	a*	b*	ΔE_{ab}
H100	56.4 ± 1.1 [b]	10.4 ± 0.1 [c]	24.95 ± 1.1 [f]	8.2
H50	61.4 ± 0.4[a]	8.1 ± 0.2 [d]	31.12 ± 0.4 [e]	0.9
NHT	60.7 ± 0.6 [a]	8.3 ± 0.1 [d]	31.68 ± 0.4 [e]	-----

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly differen`qt from each other ($p < 0.05$). ΔE_{ab} values are calculated relative to NHT (control) sample.

Based on table 4.4, significant difference in color of the H100 sample could be attributed to Maillard reaction (Augustin et al., 2006). Temperature is a key factor driving Maillard reaction as literature report varying activation energies based on reaction conditions ranging from 23 kJ/ mol to 238 kJ/ mol (Lund & Ray, 2017). Further, browning of H100 sample imply possible formation of MRPs (Donmez et al., 2024; Lund & Ray, 2017). A similar result is reported by Russi (2022) during the microencapsulation of fish oil using similar wall material.

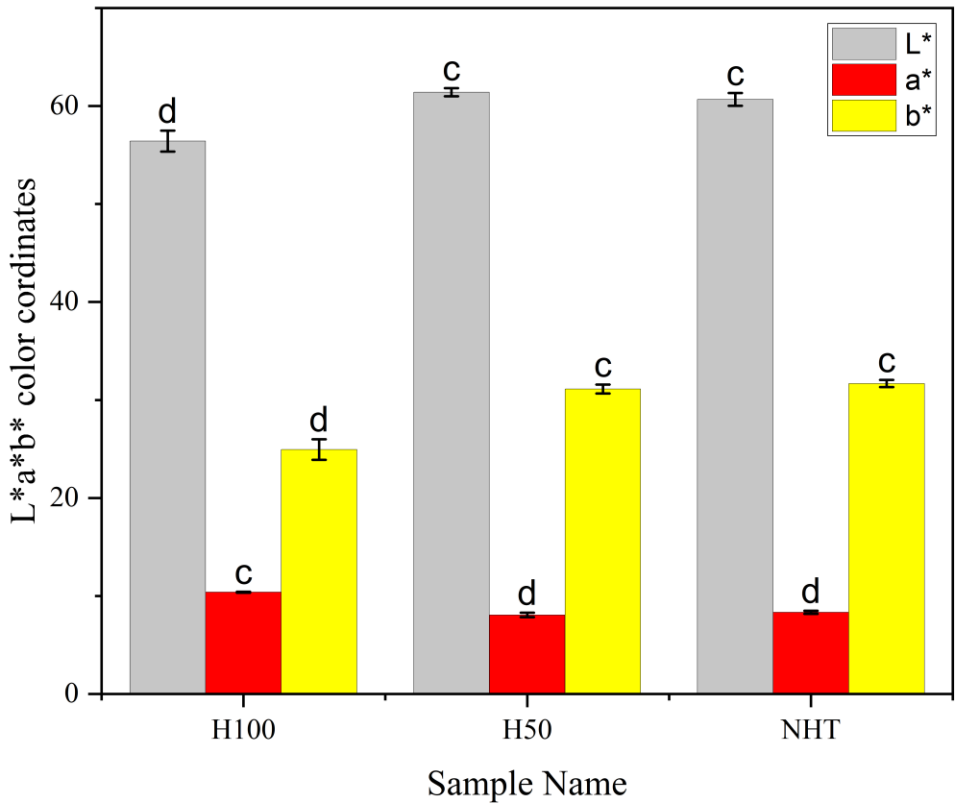


Figure 4.3: CIE L*a*b* color parameters for encapsulated VD3 powders. Samples with different superscripts are significantly different from each other ($p < 0.05$)

Furthermore, a study by Donmez et al., (2024), support this implication as the authors were able to detect Amadori products at 100ºC for a fish oil encapsulation process based on soy lecithin and dextrose at 20 % (w/w) moisture conditions. In the study, authors indicated that Lysophosphatidyl-ethanolamine (LPE) and phosphatidyl ethanolamine (PE) present in lecithin were able to react with the carbonyl group of dextrose yielding Amadori- PE and Amadori- LPE products (Donmez et al., 2024). Even though MRPs are known to possess antioxidant activity, Donmez et al., (2024) reported that a strong correlation between Amadori products and oxidative stability was not

observed (Donmez et al., 2024). Moreover, the LC/MS analysis showed that the presence of advanced glycation products was much less, compared Amadori products at similar processing conditions to the current study.

4.5 Oxidative Stability of the Encapsulated Oil

The oxidative stability of the encapsulated oil was evaluated based on secondary oxidative products using the p-anisidine test. Table 4.5 indicates the mean anisidine value obtained over the storage period and figure 4.4 illustrates the behavior of the secondary oxidative products during a storage period of 40 days.

Table 4.5: Anisidine value of encapsulated oil over 40 days of storage period

Sample Name	Day 0	Day 10	Day 20	Day 30	Day 40
H100	0.48 ± 0.12^{a}	0.94 ± 0.13^{b}	0.72 ± 0.06^{d}	1.14 ± 0.06^{f}	1.3 ± 0.04^{h}
H50	0.46 ± 0.13^{a}	0.4 ± 0.14^{c}	0.41 ± 0.04^{e}	0.71 ± 0.02^{g}	0.5 ± 0.18^{i}
NHT	0.65 ± 0.05^{a}	0.63 ± 0.11^{bc}	0.64 ± 0.09^{de}	0.63 ± 0.03^{g}	0.8 ± 0.11^{hi}

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$).

No significant difference in the anisidine value was observed on Day 0 for all samples ($p < 0.05$). However, over time, the anisidine value of the H100 sample increased significantly compared to the other two treatments ($p < 0.05$). According to Figure 4.3, the H100 sample exhibited the highest levels of secondary oxidative products. This could be attributed to the poor encapsulation efficiency observed for this treatment, as reported in Table 4.2. Poor encapsulation efficiency indicates that the H100 sample contained a high amount of surface oil. This is further evidenced by the microstructure imaging shown in Section 4.7. The surface oil, not being protected absorb oxygen from the air at high temperatures, leading to the formation of primary oxidative products, which over time degrade into secondary oxidative products such as aldehydes and ketones.

Moreover, literature reported that in low moisture food, water activity levels, below 0.2 contributes towards enhanced lipid oxidation as detailed in section 4.1 (Vu et al., 2020). Accordingly, contribution of all these factors may have led to comparatively high anisidine value reported for H100 sample. However, it must be noted that this value still well below the permissible anisidine value of 20 (Donmez et al., 2024).

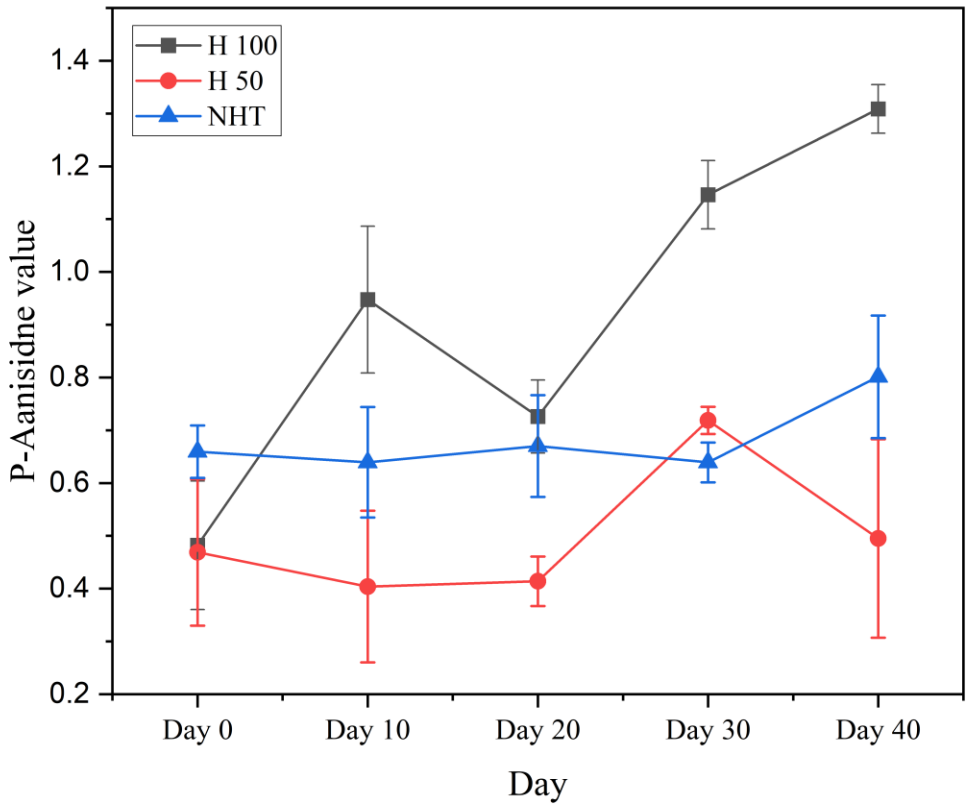


Figure 4.4: Oxidative stability of the encapsulated vitamin D3 powders over a 40 day storage period

At the end of the storage period, the lowest anisidine value was reported for the H50 sample. Based on the results, all samples recorded comparatively much lower anisidine values, indicating that the current approach is suitable for oil encapsulation. However, it should be noted that the reduced

presence of secondary oxidative products is also influenced by antioxidants present in commercial hemp seed oil, such as, zinc, and selenium. (*Agricultural Seed - IND HEMP*, 2024). Therefore, future studies for oxidative stability of hemp seed oil should involve pure hemp seed oil that does not contain significant amounts of antioxidants. This could be helpful in understanding the efficiency of the current encapsulation approach. Further, it would help to demonstrate that the approach could use Hemp oil without expensive antioxidant compounds such as vitamin E.

4.6 Thermal Stability

4.6.1 Thermogravimetric Analysis (TGA)

To evaluate the success of encapsulation to achieve better thermal stability, TGA was conducted. Improved thermal stability of encapsulated VD3 can be confirmed by observing the onset of a sudden drop in the thermogravimetric curve at a higher temperature when compared to pure VD3 (Bodbodak et al., 2024). Table 4.6 summarizes the onset temperature (T_{onset}) and the peak temperature (T_{peak}) measured at the maximum percentage weight loss. As indicated in the table, the degradation of pure VD3 occurred at 303.76 ± 1.43 °C. In contrast, all experimental treatments (H100, H50, and NHT samples) recorded significantly different ($p< 0.05$) higher T_{onset} values compared to pure VD3, confirming the successful encapsulation of VD3 with adequate thermal stability.

The improved thermal stability could be attributed to the fact that the wall matrix does not decompose easily (Bodbodak et al., 2024). Thus, it serves as a physical barrier to heat transfer, thereby prolonging the degradation of VD3 (Bodbodak et al., 2024). However, a significant treatment effect was not observed ($p > 0.05$) among the three encapsulated VD3 samples. Additionally, the initial mass loss in the encapsulated VD3 samples is due to moisture evaporation at high temperatures. Figure 4.5 shows the thermogravimetric curves for VD3, hemp seed oil, and

encapsulated VD3 samples. According to the graph, a slight decrease in weight can be observed around 241.2 °C for the pure VD3 sample. Literature indicates that this initial slight decrease in weight could be due to structural changes in VD3 caused by the elevated temperature, while the complete decomposition of VD3 occurs around 300 °C. (Jannasari et al., 2019).

Table 4.6: Thermal Degradation temperatures of vitamin D3 and encapsulated vitamin D3 samples

Sample Name	T_{onset} (°C)	T_{peak} (°C)
Vitamin D3	303.76 ± 1.43 [c]	339.26 ± 1.26 [d]
Hemp seed oil	388.48 ± 2.74 [a]	405.06 ± 0.14 [c]
H100	330.96 ± 3.32 [b]	399.38 ± 1.26 [c]
H50	335.43 ± 0.5 [b]	404.11 ± 1.02 [c]
NHT	329.62 ± 8.01[b]	401.16 ± 1.1 [c]

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$)

According to the thermogravimetric curve of the hemp seed oil, T_{onset} was observed at 388.48 ± 2.74 °C. Moreover, research indicates that the decrease in weight of hemp seed oil can be attributed to oil oxidation (F. Gao & Birch, 2016). Studies further reveal that with hemp seed oil, an initial subtle increase in weight is typically followed by a drop in the thermogravimetric curve (F. Gao & Birch, 2016). The initial weight gain is associated with the absorption of oxygen, leading to the formation of hydroperoxides (F. Gao & Birch, 2016). This is followed by the decomposition of these products into secondary oxidative products, such as aldehydes and ketones (F. Gao & Birch, 2016). The loss of mass is a result of evaporation of these secondary oxidative products (F. Gao & Birch, 2016).

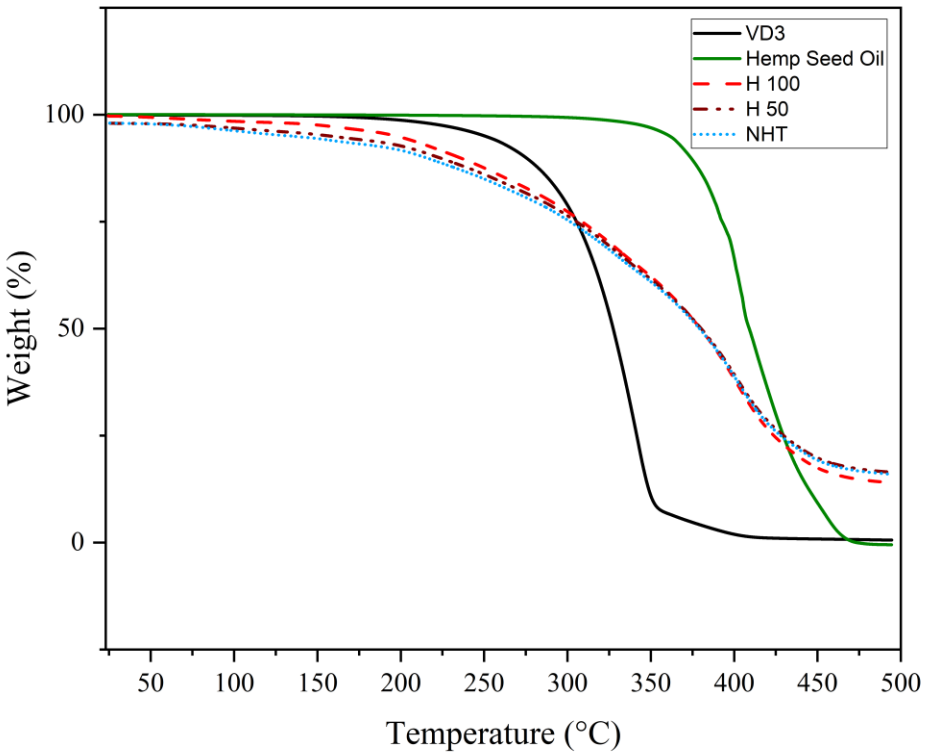


Figure 4.5: Thermogravimetric curve for Vitamin D3, Hemp seed oil and encapsulated vitamin D3 samples

Figure 4.6 illustrates the derivative of the thermogravimetric curve (DTG) for hemp seed oil and encapsulated VD3 powders. The figure indicates rate of mass loss due to thermal decomposition from 25 °C to 500 °C. According to the figure, thermal decomposition of hemp seed oil appears to be complex and follows a multistep process. Based on figure 4.6, distinctive 4 endothermic peaks can be observed for pure hemp seed oil. Literature reveals that these steps could be associated with decomposition of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) , saturated fatty acids (SFA) and finally volatilization of condensation and decomposed products (F. Gao & Birch, 2016; Santos et al., 2002). Studies further reveal that initial decomposition stages correspond with more unstable fatty acids, indicating PUFA followed

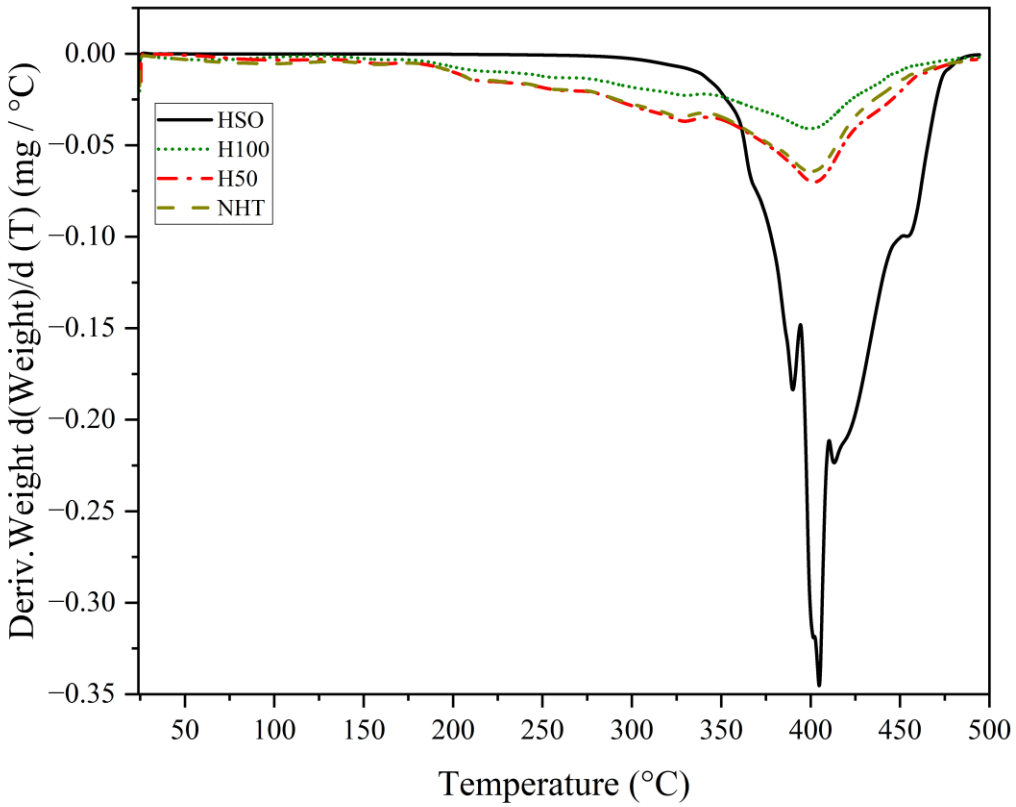


Figure 4.6:Derivative thermogravimetric (DTG) curve for Hemp seed oil and encapsulated vitamin D3 powders

by MUFA and finally SFA (F. Gao & Birch, 2016). Therefore, it can be inferred that thermal decomposition of hemp seed oil is highly dependent on its fatty acid profile. Moreover, according to figure 4.6, all encapsulated powders appear to demonstrate lower rates of mass change over temperature compared to that of pure hemp seed oil. The low rate of mass change can be attributed to successful encapsulation of the oil that hinder the heat transfer towards the encapsulated oil. Obviously, these are extreme temperature conditions to which the products will not be exposed during their application, thus warranting the absence of deleterious degradation that may affect their use.

4.6.2 Differential Scanning Calorimetry (DSC)

DSC was conducted to investigate the thermal behavior of the encapsulated hemp seed oil. As shown in table 4.7, hemp seed oil exhibited an endothermic peak at -31.185 ± 1.35 °C. According to literature, this peak is associated with the melting temperature of hemp seed oil (Rezvankhah et al., 2022).

Table 4.7: Melting point temperatures for hemp seed oil and encapsulated vitamin D3 powders

Sample Name	T_m onset (°C)	T_{peak} (°C)
Pure hemp seed oil	-31.185 ± 1.35 [b]	-25.925 ± 1.46 [c]
H100	-27.55 ± 0.56 [ab]	-23.19 ± 0.64 [c]
H50	-27.02 ± 0.03 [ab]	-23.77 ± 0.01 [c]
NHT	-26.15 ± 0.01 [a]	-16.46 ± 6.42 [c]

Results are reported as Mean ± Standard Error (SE). Values with different letters are significantly different from each other ($p < 0.05$)

A study by Rezvankhah et al., (2022) reported a melting point of -25.7 °C for hemp seed oil. Therefore, the value obtained in the current study is consistent with the literature. However, it has to be noted that different heating rates result in different peak shapes and sizes (Islam et al., 2023). This in turn result in obtaining different peak heights, temperatures and enthalpy values (Islam et al., 2023). This could be attributed to the fact that a small heating rate is sensitive towards small changes in the fatty acid profile (Islam et al., 2023). For the current study, a heating ramp of 10 °C / min was utilized, which is a relatively higher scanning rate suitable for profiling hemp seed oil (Islam et al., 2023).

The figure 4.7, represents the DSC thermogram of all encapsulated powders. As depicted in the graph, a narrow peak was not observed for the encapsulated powders. This is expected as hemp seed oil is not uniformly dispersed within the core matrix. Therefore, the heterogeneity of the oil distribution could be attributed to the wider peak observed for the encapsulated powders. However, based on table 4.7 and figure 4.7, it is evident that the melting point of the encapsulated powder is higher compared to that of naked hemp seed oil. This further emphasis successful encapsulation of the oil. Moreover, the delay in melting point for the encapsulated powders can be associated with wall matrix providing barrier properties in heat transfer as mentioned in section 4.6.1.

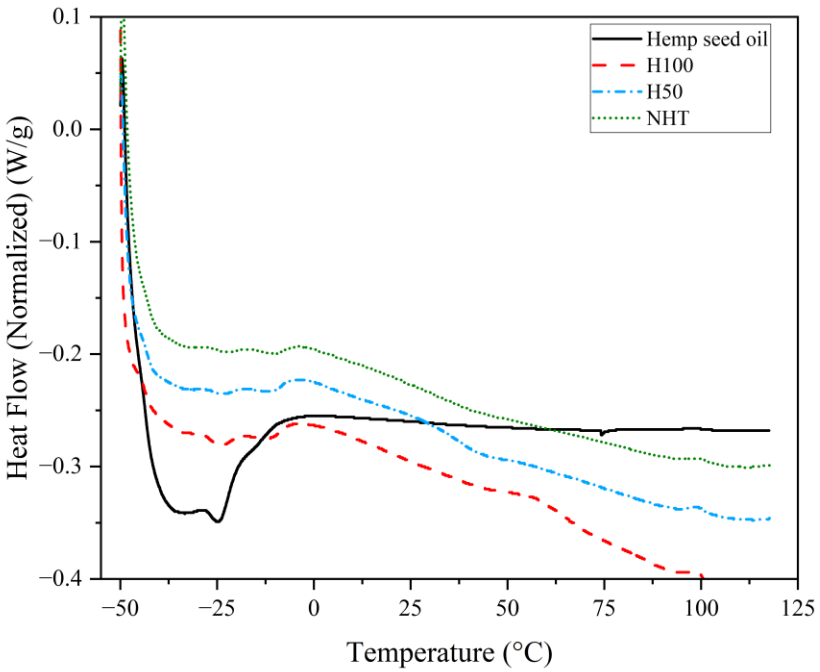
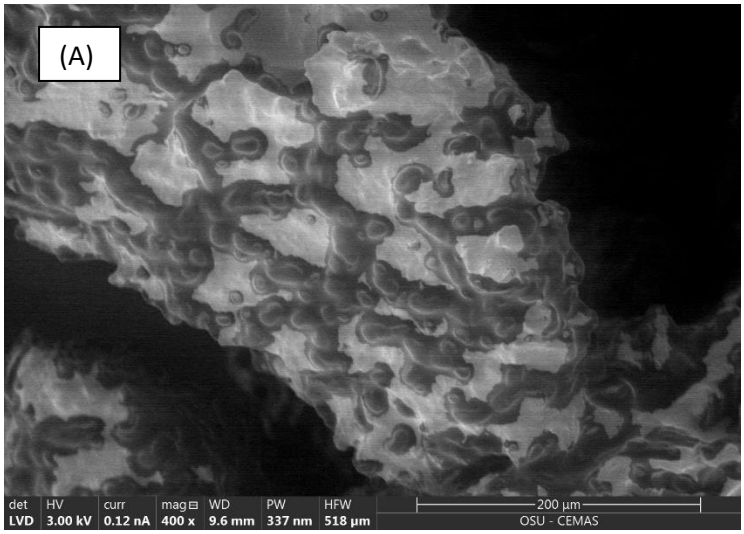
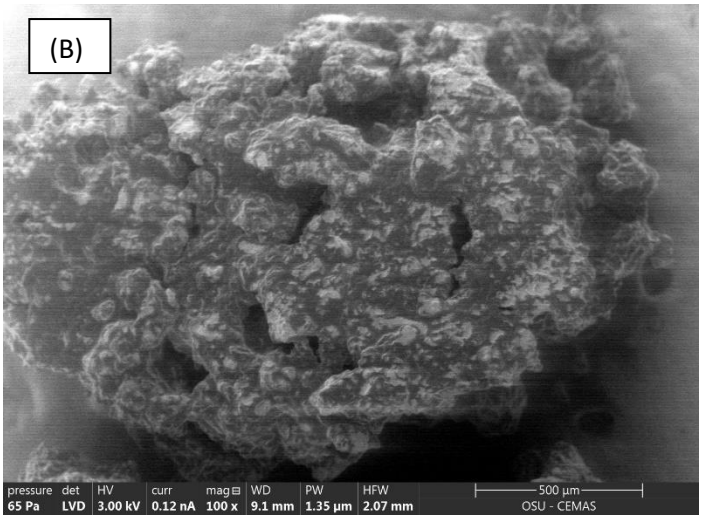


Figure 4.7:DSC thermograms for hemp seed oil and encapsulated VD3 samples

4.7 Morphological Analysis

Figure 4.8 illustrates SEM images of H100, H50 and NHT samples. According to the figure, encapsulates appeared to have an irregular and a rugged surface with varying shapes instead of the spherical shape obtained by spray drying. This shape is in agreement with similar studies (Donmez et al., 2024; Jannasari et al., 2019). The rugged surface can be attributed to the drying techniques utilized for the study. In the current study samples were dried initially in a hot air oven followed by the vacuum dryer. Therefore, the sudden moisture evaporation from the system may have resulted in the irregular surface morphology. Moreover, the powdered samples appear to contain agglomerates rather than individual spheres, this is associated with the moisture content of the samples. The white colored spots on the surface of the encapsulated powder are associated with the presence of surface oil. Compared to H50 and the control sample, H100 carries more white colored spots indicating presence of more surface oil. This observation aligns with the surface oil and encapsulation efficiency data reported in section 4.2. Additionally, these images provide clear evidence that help understand the vitamin retention data and the encapsulation efficiency data.





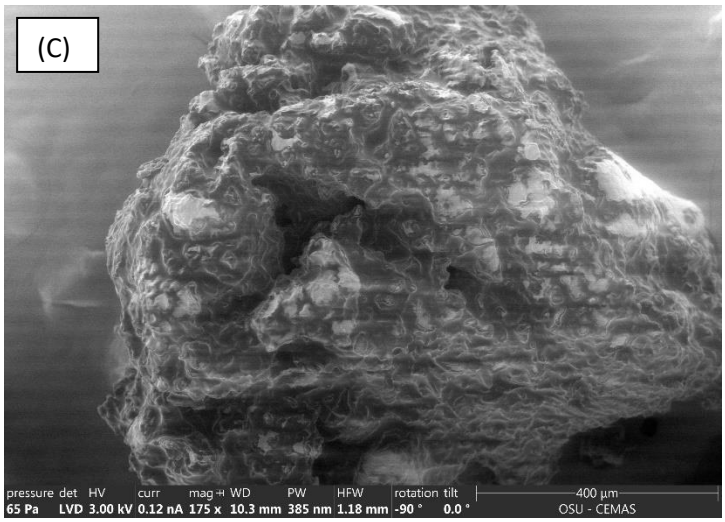


Figure 4.8: SEM images of the encapsulated vitamin D3 samples. H100, H50 and NHT samples are represnted by A,B and C images respectively.

Chapter 05

Conclusion

The proposed approach of encapsulating VD3 appears to be promising based on the thermal and oxidative stability data. Results suggest that the encapsulation method can protect VD against external stressor such as temperature as the protective wall effectively hindered heat transfer to the core material. Thus, the physical barrier created by the Maillard-reacted wall material successfully protected the encapsulated VD3 from temperature, light, and other external stress factors leading to degradation. The study showed that while the H50 treatment offered the best protection for the encapsulated sample in terms of lipid oxidation and encapsulation efficiency. The H100 treatment provided a slightly higher retention of VD3. Overall, considering all the results, the H50 treatment can be deemed the best temperature treatment for encapsulation using the current approach. The slightly higher VD3 retention must be counterbalanced against the lower encapsulation efficiency of the H100 samples and the presence of more surface oil, which would require additional and costly processing for its removal to achieve more efficient practical application.

Future studies focusing on the improvement of encapsulation efficiency could be highly beneficial for the development of this product for commercial applications. Specifically, enhancing encapsulation efficiency may address current issues related to the retention and stability of vitamin D3 , as well as reduce the presence of surface oil, thereby minimizing the need for additional and costly processing steps. Moreover, improved encapsulated VD3 ingredient could be further investigated by incorporating it into skin formulations to study its bioavailability. This would provide valuable insights into the potential dermatological applications of the product and its effectiveness in delivering VD3 through topical routes. Further analysis of VD3 oxidative products over time, abundance of Amadori product over time, concentration of lipid oxidative products over

time and relation to different water activity levels could provide important insights into the shelf stability of the product. Understanding these variables would be essential for optimizing storage protocols and ensuring consistent product quality. Therefore, studies focusing on the above areas could be highly valuable in terms of developing more robust encapsulation systems.

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