ABSTRACT

Title of Document: FEASIBILITY OF SOLUBLE LEAF PROTEINS AS A CARRIER FOR VITAMIN D

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Resurgence of vitamin D deficiencies in recent years has ascribed the need for expansion of fortification strategies in food. Alluding to the fat soluble and sensitive chemical nature of vitamin D, existing fortification strategies invariably require using a substantial amount of fat as carriers for vitamin D. Though milk proteins have demonstrated good binding properties with vitamin D; allergen issues, lactose intolerance, and the need to cater to vegan population deter its extensive use. In this study, soluble leaf proteins extracted from low-alkaloid tobacco leaves were investigated as a possible carrier. Crude tobacco leaf proteins were extracted by a high-throughput mechanistic process, followed by a freeze-drying process to encapsulate vitamin D. Up to 84.68% (w/w) of vitamin D was successfully retained by tobacco leaf proteins using the process developed, indicating that crude leaf protein recovered from tobacco could be employed as an effective carrier for vitamin D.

FEASIBILITY OF SOLUBLE LEAF PROTEINS AS A CARRIER FOR VITAMIN D

By

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Chapter 1: Introduction

Vitamin D came into the limelight as that particular dietary constituent in cod liver oil that was responsible for eradicating rickets (McCollum et al., 1922), a nutritional disease occurring mostly in children and is characterized by skeletal deformities like bow legs, spinal deformities, impaired growth, muscle cramps, short stature (National Institutes of Health, 2008). Dietary sources such as fatty fish, liver (Bills, 1927), egg yolk (Koskinen and Valtonen, 1985), and wild mushrooms (Matilla et al., 2002) are known rich sources of vitamin D. Apart from the dietary sources, vitamin D can be synthesized in the body by the action of ultraviolet rays of the sunlight on skin. However, vitamin D synthesis is affected by factors including seasonal variation, pollution effects, latitudinal changes, as well as skin pigmentation. Due to the insufficient compensation from dietary sources and sunlight, vitamin D began to be fortified in food products as early as 1930 (Hollick, 2002). The two prominent metabolites used for vitamin D fortification are vitamin D_2 and D_3.

Recently, the resurgence of vitamin D deficiencies has led to a series of scientific discussions on the need for revision of fortification limits as well as expansion of fortification strategies (Calvo et al., 2004). In fact, the American Association for Pediatrics recently called for a revision of the existing fortification limits on vitamin D with a dramatic increase from the old standard 5 µg/day (200 IU) to 10 µg/day (400 IU) (Wagner et al., 2008). While the needs are evident, the means to incorporate vitamin D remain limited, mainly due to the fact that vitamin D is fat soluble and sensitive to acid,

oxygen, and light (Ball, 2006). To date, commercial fortification strategies revolve around emulsification and microencapsulation, which mostly require the use of emulsifiers and stabilizers in the formulations. An attempt made by Swaisgood and coworkers (2001) using β-lactoglobulin to bind with vitamin D was encouraging as the complex formed was shown to be soluble in aqueous solution, albeit the approach was cost-prohibitive for commercial applications. The study conducted by Semo et al. (2007) successfully demonstrated that pure casein micelles were capable of encapsulating up to 27% of vitamin D_2. Although their results remained inferior to those using emulsification technologies (Dewille et al., 1997), the feasibility of using protein-based matrix as a carrier for vitamin D was proven viable.

On the other hand, it is highly desirable that food products fortified with vitamin D could reach a much wider spectrum of population if the consumers do not have to worry about whether the products contain animal-origin or dairy-based ingredients. Therefore, plant leaf protein provides the perfect candidate over milk proteins and other allergen-containing protein sources. Besides, leaf proteins are advantageous if one could identify a fast-growing plant capable of synthesizing proteins at high yields, along with an effective processing technology to recover the protein from grown leaves in order to make leaf protein an attractive vitamin-D carrier for industrial applications.

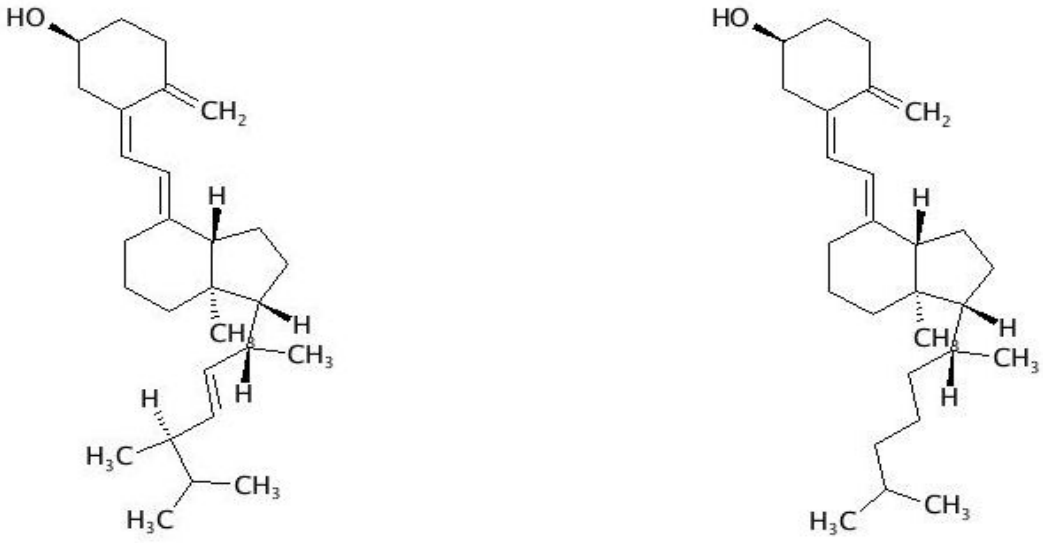
In the present study, crude protein powder extracted from the leaves of low-nicotine tobacco was employed to evaluate its ability to be a carrier for vitamin D. Various processing conditions, including pH and mixing strategies, were investigated to characterize their effects on retaining vitamin D using tobacco leaf protein powder. The

methodology to quantify vitamin D in the protein-vitamin D complex was also developed.

Chapter 2: Literature Review

2.1 Vitamin D

Vitamin D represents the group of steroid compounds that are antirachitic. Cholesterol acts as the parent compound of its steroid structure. The steroid structure of vitamin D is characterized by the opening of the B ring of the polycyclic ring system of cholesterol to give a conjugated triene system of double bonds. Thus Vitamin D_3, commonly named cholecalciferol, has the chemical name (*5Z,7E*)-9,10-seco-cholesta-5,7,10(19)-trien-3ß-ol, whereas vitamin D_2 is chemically named (*5Z,7E*)-9,10-seco-ergostate-5,7,10(19)22-trien-3ß-ol (Lytgoe et al., 1978). Structure-wise vitamin D_2 differs from vitamin D_3 by an additional double bond and an additional methyl group in the side chain.



Vitamin D_2 Vitamin D_3

Fig 2.1. Chemical structures of Vitamin D_2 and VitaminD_3 (Lytgoe et al., 1978)

Vitamin D is endogenously synthesized by the action of UV light in the range of 290-315 nm of sunlight on the precursor compounds present in the epithelial cells of plants and animals. These precursor compounds are in turn converted into the previtamin form which then isomerizes to the vitamin form. In the case of plants, fungi, and yeast the precursor is ergosterol, which on UV irradiation produces previtamin D_2 which isomerizes to ergocalciferol (Vitamin D_2). Likewise, in animals the precursor is 7-dehydrocholesterol which forms cholecalciferol (vitamin D_3). The intermediary metabolism of vitamin D in the human body is complex. However, the general overview is that a vitamin D binding protein transfers the vitamin D produced by sunlight exposure in the skin to the liver. In the liver the hydroxylation of the vitamin D to 25(OH)D takes place and further hydroxylated it to 1,25 dihydroxyvitamin D [$1,25(OH)_2D$] in the renal tissues (Hollick et al., 1980). The concentration of $25(OH)D_3$ in the serum is considered the accepted index of vitamin D status in humans. $1,25(OH)_2D$ is considered as the hormonally active form of vitamin D crucial in maintaining the Calcium Phosphate Homeostasis in the bone. In addition to bone health, vitamin D is associated with effects on myocardial development (Nibbelink et al., 2006), brain and fetal development, as well as reduced cancer risk (Garland et al., 1989).

The biopotency of vitamins are expressed in international units (IU). One IU of vitamin D is equivalent to 0.025 µg of crystalline vitamin D_3 or vitamin D_2 (WHO, 1949). Since Vitamin D_2 is easily and cheaply synthesized, it is the primary synthetic form of vitamin D used in pharmaceuticals (Eitenmiller and Landen Jr, 1999). However, it has been shown that the biopotency of vitamin D_3 is higher than vitamin D_2 based on the study conducted by Houghton and Vieth (2006) as they reported that "*differences in the*

efficacy of raising serum 25-hydroxy vitamin DD, diminished binding of vitamin D_2 metabolites to vitamin D binding protein in plasma and a non physiologic metabolism and shorter shelf life of vitamin D_2".

Moreover, fortification of vitamin D is challenging taking into consideration its sensitive chemical nature. The presence of conjugated double bonds provides an easy route for decomposition by oxidation (Eitenmiller and Landen Jr, 1999). Isomerization to 5,6 trans isomer and isotachysterol happen under acidic or light conditions. Temperature above 40°C and relative humidity above 85% have been shown to deteriorate vitamin D. Mild acidification isomerizes vitamin D to its inactive form. In general, in food products the stability of vitamin D is dependent on the stability of the fat matrix (Ball, 1983). Vitamin D_3 stored in solvents like ethanol are shown to exhibit better stability (Chen et al., 1965).

Since vitamin D is fat soluble, strict rules of fortification have been compiled to avoid effects of hypervitaminosis. In the United States, the recommended daily allowances range from 5 – 10 μg/day with regard to the guidelines established by the Institute of Medicine of the U.S. National Academy of Science. Based on this the U.S. Food and Drug Administration (FDA) has fixed the levels of fortification of vitamin D in food products (Table 2.1).

Table 2.1 Table 2.1: Recommended maximum levels of Vitamin D fortification in the U.S.

Forms	**Food products**	**Maximal levels**	**21 CFR* citation**
Vitamin D	Breakfast cereals	350 IU/100 g	137.305
	Grain products and Pastas	90 IU/100 g	137.350, 137.260, 139.115, 137.155
	Margarine	331 IU/100 g	166.110
	Milk	42 IU/100 g	131.110, 131.111, 131.112, 131.115, 13.127,131.130,131.147
	Yogurt	89 IU/100 g	131.200, 131.203, 131.206
Vitamin D_2	Soy based butter substitute spreads	330 IU/100 g	172.379
	Soy based cheese substitute and cheese substitute products	270 IU/100 g	172.379
	Soy beverages	50 IU/100 g	172.379
	Soy beverage products	89 IU/100 g	172.379
Vitamin D_3	Calcium fortified juices and drinks	100 IU/FDA regulatory serving size	172.380
	Cheese/ Cheese products	81 IU/30 g	172.380
	Meal Replacement bars	100 IU/40 g	172.380
	Soy Protein based meal replacement beverages	140 IU/240 ml	172.380

*Code of Federal Regulations

2.2 Fortification strategies

To expand the ability to fortify vitamin D in aqueous based formulations, various fortification strategies have been developed and are summarized in Table 2.2.

Table 2.2: Summary of Vitamin D fortified formulations

Fortification Strategy	Matrix	Form	Key Ingredients	Uses/ Remarks	Reference
Emulsification	Low pH beverage fortified with calcium	Vitamin D_3	Polysorbate , propylene glycol solution, cysteine, whey protein concentrate	Without homogenization Recovery: 43.4%	Dewille et al., 1997
		Vitamin D_3	Polysorbate, brominated vegetable oil, corn oil, gum Arabic	With 2 stage homogenization Recovery: 59.4%	Dewille et al., 1997
		Vitamin D_3	Corn oil, polysorbate , brominated vegetable oil	With 2 stage homogenization Recovery: 76.7%	Dewille et al., 1997
		Vitamin D_3	Hydrated heated and cooled gum arabic solution(20% by weight of gum in emulsion) sodium benzoate, citric acid (pH=4.0)	With 2 stage homogenization half life: 180 days	Dewille et al., 1997
		Vitamin D_3	Gum arabic, water, partially hydrogenated soybean oil, citric acid,	With 2 stage homogenization	Dewille et al., 1997

Fortification Strategy	Matrix	Form	Key Ingredients	Uses/ Remarks	Reference
			sodium benzoate	Recovery: 94.1%	
	Milk for cheddar cheese	Vitamin D_3	Polysorbate 80, propylene glycol,	Concentration decrease of 16% over 7 month ripening stage	Banville et al., 2000
Microencapsulation					
Spray Drying	Low pH beverage fortified with Calcium	Vitamin D_3 (cholecalciferol USP-FCC),	Dicalcium phosphate, gum acacia, coconut oil, BHT, lactose, silicon dioxide, sodium benzoate and sorbic acid	Average half life without homogenization was 12.6 days	Dewille et al., 1997
	Orange oil	Vitamin D_3	Sucrose, acacia gum, cornstarch modified, soy oil, alpha-tocopherol, maltodextrin, and vitamin C	Vitamin D stable for 8 weeks	Ling et al., 2003
	Dough for bread	Vitamin D_3	Edible fats, gelatin, sucrose	Recovery: 97.6% in wheat bread, 98.3% in rye bread	Natri et al., 2006

Fortification Strategy	Matrix	Form	Key Ingredients	Uses/ Remarks	Reference
Liposome	Milk for cheese	Emulsified Vitamin D	Polysorbate 80, propylene glycol proliposome suspension	Recovery better than the use of emulsified counterpart. Degradation 40% over 7 month ripening stage	Banville et al., 2000
Protein Binding					
Vitamin D_2 enriched casein micelles		Vitamin D_2 in ethanol	Sodium caseinate, Tri potassium citrate, Calcium chloride, Dipotassium hydrogen phosphate, homogenized, centrifuged to give pellet and serum	156 nm micelles Recovery: Pellet: 45 – 60%	Semo et al., 2007
Vitamin D bound ß-lactoglobulin complex		Vitamin D_2 in ethanol	β-Lactoglobulin extracted by affinity purification chromatography	Soluble in aqueous solution. Pure form of protein required	Swaisgood et al., 2001

2.2.1 Emulsification

Vitamin D_3 fortification in aqueous beverages is technically challenging because of its fat soluble nature. An oil-in-water emulsion is usually required as it involves dispersing the oil phase as fine droplets in water. From the physical chemistry point of view, a system always tends to the state of least energy. However, by creating multiple dispersed particles the surface energy of the system increases. So the system is always in a delicate balance of stability. Typical emulsification involves two steps, namely the selection of an appropriate emulsifier and the creation of a stable emulsion by homogenization. An emulsifier uses its hydrophobic part to adsorb around the part of oil droplets. In general, when creating an oil-in-water emulsion one would select an emulsifier with more oil binding parts as reflected by its hydrophilic-lipophilic balance (HLB) value (Shinoda and Frieberg, 1986). For example, propylene glycol is an emulsifier that has a long alcohol group that accounts for its solubility in water. Solution of vitamin D in propylene glycol when stored under proper storage conditions were found to maintain the potency better when compared with solutions of vitamin D in corn oil or sesame oil, whereas the rancidity of the oil was accounted for these differences (Huber and Barlow, 1943). However, vitamin D solutions of propylene glycol when diluted in water were found to deteriorate rapidly. Hence stronger emulsifiers such as polysorbates, a hydrophilic ester, has to be used. Unfortunately, polysorbate as an additive has restrictions on its recommended usage levels. Nevertheless, formulations of vitamin D in oil in conjunction with polysorbate and propylene glycol were found feasible (Dewille et al., 1997, Banville et al., 2000), as these emulsifiers were shown to attract and hold together two immiscible parts together. Moreover, brominated vegetable oil is a common option used in

emulsification to increase the specific gravity to prevent the fat content from coalescing and floating to the top.

The homogenization step helps in keeping the emulsion stage intact. The purpose of this step is to break up or evenly disperse the oil phase into the aqueous phase such that the particle size of the emulsion is sufficiently small to retard coalescence. Usually a two-stage homogenization is needed because the fine particles formed during the first stage can clump, hence the second stage set at a lower pressure is needed to break the clumps (Dewille et al., 1997).

2.2.2 Microencapsulation

Spray drying continues to be the most common microencapsulation technique used. New methods like liposome have also been used to encapsulate food, chemical, and pharmaceutical ingredients (Sharma and Sharma, 1997; Kirby et al., 1987). In the case of vitamin D encapsulation, the core ingredient (vitamin D) is first dissolved in a suitable medium and mixed with an emulsifying medium enriched with suitable antioxidants and humectants, and then homogenized. The mixture is then sprayed dried so that minute particles with wall coatings are formed (Klaui et al., 1970).

Lipid vesicles that contain an aqueous core are called liposomes, which are different from micelles structurally in that they have a bilayer membrane (Mozafari et al., 2008).The amphiphilic nature resulted from the bilayer structure makes liposomes an excellent carrier for bioactives. For instance, phospholipids have the property of forming lipid vesicles with required energy. Though generally larger compared with other encapsulating agents, liposomes have the advantage of being able to carry both fat

soluble and water soluble nutrients. Liposomes have been used in experimental studies on fortifying cheese (Banville et al., 2000). Polysorbate and propylene glycol emulsions of vitamin D have been incorporated into proliposome suspensions. In comparison with conventional polysorbate-propylene glycol emulsion, recovery of vitamin D was enhanced with liposomes. However, liposomes face the problem of destabilization if excess fat is present (Banville at al., 2000).

2.2.3 Protein Binding

By virtue of the amino acid residues, proteins possess hydrophobic and hydrophilic binding sites. The manner in which the protein conforms to the primary or secondary structure is dependent on its surrounding environment as they could exposes different sites based on the hydrophilicity or hydrophobicity of the environment.

Milk protein casein is known to exist as micelles in milk. Mimicking this formation has enabled formation of vitamin D-enriched casein micelles that are stable under high pressure homogenization and UV light exposure, reaching the retention power of up to 27% of vitamin D_2 (Semo et al., 2007).

Highly pure form of ß-lactoglobulin extracted from whey protein by bioselective affinity purification chromatography was reported to bind with vitamin D_2 as well as Vitamin D_3 dissolved in ethanol at the tryptophanyl residue of the protein. The binding capacities were observed to be dependent on pH and ionic condition with better retention at the pH of milk (Swaisgood et al., 2001, Forrest et al., 2005).

2.3 Soluble leaf protein in tobacco

Tobacco leaves are a rich source of leaf proteins. The two major proteins that characterize these leaves are the F1 (Fraction 1) protein popularly known as RUBISCO and the F2 protein. Studies on F1 protein showed that it has superior fat binding, water absorption/weight capabilities as compared to the leaf proteins from alfalfa, sugar beet, and soybean. F2 protein also shows similar functionality to F1 (Sheen, 1991). Both F1 and F2 proteins are found closely jointed to the chloroplast pigments in the leaves (Tso et al., 1983). To extract the proteins from tobacco, it is essential that the chloroplasts and cytoplasmic materials need to be separated or removed. Additionally, the extraction process has to be economically feasible while eliminating the concerns over residual nicotine contents inherent from tobacco plant.

A mechanistic high-throughput process developed by Fu et al. (2009) was shown capable of producing nicotine-free, spray dried crude protein powder using a phosphate buffer system. Based on the observation that pH 8.5 and pH 3 preparations of F1 crystal absorbed water better than the actual F1 protein itself (Sheen et al., 1985), it is reasonable to hypothesize that, by adjusting the pH value of the solutions containing F1 protein, one might be able to establish properties similar to the emulsifying/binding property commonly found in carriers for encapsulation of vitamin D.

Chapter 3: Research Objectives

The ultimate goal of this project was to identify a suitable form of soluble protein recovered from tobacco that could be used as a carrier of vitamin D. To achieve the goal, there are three specific objectives:

1. To identify suitable form of tobacco leaf protein that could be used as a carrier for vitamin D.
2. To characterize the properties of the tobacco protein and thereby develop a suitable analytical method to quantify vitamin D in the formulations
3. To evaluate the effectiveness of tobacco protein in retaining vitamin D in the formulations

Chapter 4: Materials and Methods

4.1 Recovery of tobacco protein

The crude tobacco protein powder was extracted by a patented (Lo and Fu, 2008) mechanistic process on a pilot scale. Leaves of low alkaloid tobacco variety (*Nicotiana tabacum* L. cv. MD-609LA) were separated from stalk of the tobacco plant and misted with water at high pressure to remove dirt and other particulates matter sticking to the leaves. These leaves were subsequently macerated in a hammer mill (Meadows #35, Meadows Mills Inc., North Wilkesboro, NC) with simultaneous pumping of buffer solution to create a semi-crushed mass. The buffer solution comprised of a mixture of phosphate buffer Na_2HPO_4-KH_2PO_4 (Sigma Aldrich, St. Louis, MO) at a concentration of 0.067 mol/l used in amounts of twice the weight of leaves. The macerated mass was then screw-pressed to separate the liquid ("green juice") from tobacco biomass. The "green juice" was stored at 4°C followed by subsequent centrifugation under 9000 ×*g* for 20 min at 4°C in a centrifuge (Beckman Coulter Avanti J-25I, Fullerton, CA) wherein the sedimented chloroplasts were separated from the tobacco juice leaving the greenish brown juice. The pH of the greenish brown juice was then adjusted to 4.6 by adding 85% Phosphoric acid (Sigma Aldrich, St. Louis, MO) to the brown juice under continuous stirring. The 4.6 pH juice was then centrifuged at 9000 ×*g* for 20 min at 4°C and the sediment re-dissolved in previously mentioned phosphate buffer solution. This solution was then subjected to spray- drying using a Mini Spray Drier Büchi B290 (Büchi Corporation, Newcastle, DE) at the inlet air temperature of 90°C and liquid feed rate 5%.

Fig 4.1: Low-alkaloid tobacco leaves were separated from stalks and rinsed upon arrival at the processing plant. Cleaned samples were placed evenly on the conveyor belt feeding into the hammer mill.

4.2 Solvent extraction of protein samples

Approximately 0.5 g of the crude tobacco leaf protein powder was weighed in a thimble and placed in a Soxhlet apparatus. Acetone, hexane and methanol were employed to assess their effectiveness in removing non-water-soluble compounds from the crude protein. The extraction was performed for 4 hours. After extraction the thimble with the

residue after solvent extraction was dried by purging nitrogen gas. The residual samples were then analyzed for their moisture content, yield, crude protein, and solubility.

The moisture content of the residual samples after Soxhlet extraction was determined by drying 0.1 g of the residual samples in an oven at 105°C for 2 hrs. The percent moisture was calculated by comparing the amount of water loss with the total weight of the sample. The nitrogen-dried unextracted residue was weighed to determine the yield of the extraction process

The protein content was calculated by analyzing the nitrogen content in the samples using the Leco analyzer (FP-528 model, Leco Corp., St. Joseph, MI). The instrument was calibrated using EDTA standard. The sample (0.2 g) was subjected to digestion at a furnace temperature of 950°F.

The percent protein content was calculated by multiplying the percent nitrogen content with a conversion factor of 5.79 (Knuckles, 1979). With the amount of moisture in the samples known, the protein content on a dry basis was calculated.

The solubility of the residual protein post extraction was determined by adding 0.1 g of the residual protein in 5 ml of distilled water under room temperature with constant stirring. The solution was then filtered (Whatmann Paper #1, Whatmann Inc., Piscataway, NJ) and dried in a furnace at 105 ^{o} C for 2 hrs. The retaining residues on the filter paper after drying were weighed to determine their respective solubility.

4.3 Retention of vitamin D using tobacco protein

To create a vitamin D-tobacco protein complex, 1 g of tobacco leaf protein powder was placed in a 300-ml freeze-drying glass flask (F05658000, Thermoscientific, Pittsburg, PA), followed by addition of 40 ml water and 4 ml of vitamin D_3 in 99% pure ethanol (1000 µg/ml). This mixture was magnetically stirred for 5 min before liquid nitrogen was added. Approximately 250 ml of liquid nitrogen was gradually poured into the glass flask with care until the mixture became completely solidified (by visual observation). The flask was immediately closed with the lid and carefully placed in a thermally insulated bag filled with dry ice. The connector end of the freeze-drying flask was connected to the freeze-dryer (RVT4104 model Refrigerated Vapor Trap, Thermo Electron Corporation, NY) at -110°F for 96 h. After freeze-drying the samples were weighed to determine the yield and stored until further analysis.

Various treatments were employed to optimize the vitamin D carrying capacity of tobacco protein, including the water content (40 ml vs. 20 ml), pH (4.3, 8.5, and 11.0), and mixing technique (stirring vs. sonication). For pH adjustment, 1 M sodium hydroxide solution was gradually added to the tobacco protein water solution prior to adding vitamin D. A Sonicator (28H ultrasonic bath, Neytech, Bloomfield, CT) at a frequency of 47±3 kHz for 5 min was employed as an alternative treatment for mixing.

4.4 Analysis of vitamin D

4.4.1 Analysis of crude protein properties

It is well recognized that pretreatments of samples containing protein and fat are needed in order to minimize variability and undefined peaks (Lumley, 1993). In the present study, a reverse-phase high performance liquid chromatography (HPLC) method was developed to quantify vitamin D in protein powders.

The fat content of the crude protein powder was determined by Soxhlet extraction using Petroleum ether (Sigma Aldrich, St. Louis, MO). The fat-rich solvent was subsequently evaporated under nitrogen to remove the solvent and the residual weight was noted for the calculation of percent fat in the sample.

The bulk density, an indicator of the porosity of the powder, was calculated by placing the crude protein powder in a 50-ml glass cylinder and constantly tapping it until the powder was tightly packed in the cylinder. The bulk density was calculated as the ratio between the weight of the protein powder and the volume of the cylinder. The denser the powder the more washes was required to remove the fat content in the sample.

4.4.2 Sample preparation and pretreatment

To prepare freeze-dried protein powder containing vitamin D for HPLC analysis, 0.1 g of each sample was weighed into a 20-ml test tube and 0.9 ml of 1 M aqueous potassium hydroxide (Sigma Aldrich) solution was added. The sample was then flushed with high purity nitrogen gas (NI HP 200, Airgas, Bladensburg, MD). The tubes were capped and gently shaken before placed in a 60°C water bath for 30 min. After 5 min of heating the

tubes were manually shaken to prevent adhesion of the powder onto the test tube due to temperature changes. The heated sample was transferred to an ice water bath for 10 min. Three ml of chloroform:methanol (2:1 v/v) solution was added to the test tube, as was 1.5 ml of chloroform, with each addition followed by vortexing for 30 s. The solution was then centrifuged at 1500 × *g* for 10 min at 4^{o}C in a Beckman TJ- 6 centrifuge (Beckman Coulter, Fullerton, CA). After centrifugation a clear separation was observed with the chloroform layer forming the bottom part. This bottom layer was aspirated out using a glass syringe. The chloroform layer was dried using nitrogen gas. Once dried, 4 ml of HPLC mobile phase was added and the solution was mixed and left to rest for 15 min. The reconstituted extract was filtered through a 0.45 µm Millex® HP (Millipore Corp., Billerica, MA) syringe-driven filter and sealed in a HPLC vial for analysis.

4.4.3 Preparation of standards

Vitamin D_3 crystal at 40,000,000 IU/g (99.6% purity) (PAT vitamins, Inc., City of Industry, CA) was employed as the internal vitamin D standard for HPLC. Pure vitamin D_3 (0.1 g) was dissolved in 100 ml volumetric flask with the HPLC mobile phase (as described below) to give a final concentration of 1000 µg/ml. Appropriate amounts of the above standard solution were dissolved in the mobile phase to 6 different concentrations ranging from 20-120 µg/ml, and were analyzed to give the calibration curve of peak areas versus the concentration. The retention time of the internal standard was further validated by running the Sigma-Aldrich vitamin D_3 standard (≥98%, Product No. C9756) following the same protocols. The standard solutions were stored in dark under cold conditions and were replenished every 3 weeks.

4.4.4 HPLC analysis

The quantification of vitamin D_3 was carried out in a Shimadzu LC-2010A (Columbia, MD) equipped with serial dual plunger pumps, an oven, an automated sampling injection unit, and an UV-VIS detector capable of detecting wavelength at 254 nm. Reverse phase chromatography (RP-HPLC) was performed using a C-18 column (10 µm particles, µ-Bondapak®, 3.9 mm ID, 300 mm length) (Waters Corporation, Milford, MA) attached to a guard column acting as the non-polar stationary phase and a polar mobile phase comprising of methanol:acetonitrile:water (volumetric ratio 49.5:49.5:1). The mobile phase flow rate was maintained at 0.5 ml/min and the injection volume was 10 µL.

4.5 Statistical Analysis

To investigate which of the freeze-dried formulations gave the best retention of vitamin D, ANOVA followed by Tukey's test was performed. Three replications of each sample were performed during HPLC analysis and three samples were analyzed for each of the six experimental treatments. The results were analyzed for statistical significance using SAS 9.1.3 software (SAS institute Inc., Cary, NC) with ANOVA followed by Tukey's test for mean separation. Complete statistical analysis is given in Appendix A.

4.6 Scanning electron microscopy Imaging

The sample was mounted on a conductive tape and then a gold coating was applied using a sputter coater (Hummer model, Anatech Ltd, Springfield, VA). Surfaces of samples were inspected using a scanning electron microscope (SU-70 model, Hitachi High-Technologies Corporation, Japan). The surfaces were surveyed at low magnification ranging from 300 to 1000 to observe the sample spread and then respective areas were zoomed at higher magnification. A common magnification of 4.5k was used for imaging for comparison of images. An accelerating voltage of 3 keV was used with a medium probe current.

Chapter 5: Results and Discussion

5.1 Effect of solvent extraction on protein samples

The crude tobacco leaf protein powder obtained from the pilot-scale operation was found to exhibit very little water solubility (Table 5.1). With the aim of increasing the water solubility of the protein, three different solvents, namely acetone, hexane, and methanol, were employed to remove any residual pigments, fat content, and polar impurities, respectively, that might exist in the crude protein powder. As seen in Table 5.1, before subjected to solvent extraction, the crude protein powder only gave an average solubility of 10.08 ± 0.15 g/l. Protein samples treated by acetone showed similar solubility before and after treatment, indicating that there was little effect of acetone on the configuration of protein in the powder.

Hexane treatment was found to slightly increase the solubility of the protein (10.82 g/l), whereas the samples treated by methanol showed a significant decrease in solubility (8.34 g/l). Demonstrated to effectively remove the neutral lipids in tobacco (Fiore, 1975), hexane however did not drastically change the solubility of protein powders, indicating that the protein powders obtained from the pilot process might form a structure that hindered the removal of neutral lipids and at the same time helped the retention of the moisture content. Contrarily, the high polarity of methanol (higher than acetone and hexane) (Schirmer, 1991) appeared to remove moisture from the protein samples, leaving the highest protein concentration among all treatments investigated (Table 5.1). With the low yield (61%) of the protein attainable, extraction of tobacco protein using methanol is

not an adequate operation, as evidenced by the low total protein recovered (24.83 g). Therefore, none of the solvent investigated was selected as a pretreatment for the crude protein powders prior to binding with vitamin D.

Table 5.1: Comparison of tobacco protein properties after Soxhlet solvent extraction

Solvent	**Solubility(g/l)**	**Yield (g)***	**Crude protein (%)**	**Total protein** (g)**	**Moisture (%)**
None	10.08±0.05	100 ± 0	27.8 ± 1.99	27.8 ± 1.99	13.4 ± 1.15
Acetone	10.33 ± 0.20	78.4 ± 2.75†	33.6 ± 4.76	26.40 ± 4.6	10.6 ± 0.85†
Hexane	10.82 ± 0.39 †	85 ± 0.35 †	30.7 ± 6.65	26.08 ± 5.56	13.3 ± 0.55
Methanol	8.34 ± 0.22 †	61 ± 1.4 †	40.7 ± 2.64†	24.81± 1.39	9.2 ± 0.75 †
P-value	<0.0001	<0.0001	0.0354	0.32	0.0007

Means ± S.D., n = 3. Values in the same column followed by '†' are significantly different from the control (no solvent).

*All data presented were on wet basis.

**Total protein (g) = Yield × crude protein

5.2 Optimization of HPLC method for vitamin D

5.2.1 Pretreatment of protein samples

As aforementioned, the properties of the protein in question are critical in determining the steps required to pre-treat the samples before HPLC analysis. With the inherent residual fat contents in the crude protein powder (Hudson and Karris, 1973), it is apparent that a mild saponification step needs to be employed to eliminate the peak interference caused

by the fat content. On the other hand, the bulk density of the crude protein was 0.36 ± 0.62 g/ml. Such low bulk density indicated that the protein particles could not be packed tightly together, leaving considerable spaces among protein powders. This is indicative that the protein powder has a substantial porosity which allows the solvent to effectively remove residual fat content with single wash.

To date, there is no published methodology tailored toward analyzing vitamin D in leaf protein samples. A well-documented method for determining vitamin D_3 in cheese, which involves three pretreatment steps (Kazmi et al., 2007), was employed as the base method for modification and optimization. In Kazmi's method, saponification under heat was first used to break the fat component adhering to the vitamin in cheese, followed by lipid extraction in a chloroform:methanol:water ternary system, and finally phase separation of the lipid rich chloroform layer, which was subsequently reconstituted in the mobile phase and analyzed. In the present study, since the protein sample contained less amount of fat – 5.1%, a milder saponification was used with 1 M aqueous KOH, which was sufficient for saponifying 0.1 g of the protein samples(Byrdwell, 2009).

Furthermore, in order to attain satisfactory phase separation, the ratio of chloroform:methanol:water needs to be modified. Based on the chloroform-methanol-water phase diagram (Bligh and Dyer, 1959), good lipid extraction could be performed under monophasic conditions. By changing the mixture into biphasic conditions, effective separation of lipid-rich layer could then be achieved.

As per the Bligh and Dyer method (1959), the volumetric ratio of chloroform:methanol :water at 1:2:0.8 was the best for lipid extraction in the monophasic region. To maintain

the ratio for treating crude protein samples, one must take into consideration the inherent moisture content in the sample as well as the moisture coming from the saponification solution (Table 5.2). As seen in Table 5.2, to maintain the chloroform:methanol:water ratio within monophase for 0.1 g of protein sample, the total of 0.80 ml of water was kept, with 0.008 ml of moisture came with the protein powder, and 0.80 ml moisture came from 0.9 ml of 1 M KOH.

Table 5.2: Calculation of the amount of 1 M KOH solution needed for 0.1 g of protein samples to maintain in the monophasic region in the chloroform:methanol:water phase diagram.

Moisture in system	
Moisture content	0.1 g has 8% moisture i.e. 0.008 ml is moisture
From aq. 1 M KOH	1M KOH = 5.6g/100 ml
.	Wt % of KOH = 5.6/105.6 x 100=5.3% w/w
	Wt% of moisture = 100-5.3 = 94.7
	0.9 ml ($\approx$ 0.9 g) of aq. 1 M KOH moisture = 94.7x0.9/100
	= 0.8523 g $\approx$ 0.8 ml water
Total	0.8+0.008=0.80081

In order to verify the exact location of the chloroform:methanol:water ratio on the phase diagram, the mass ratio of chloroform:methanol:water was identified to be 38.43:38.74:22.83 (Table 5.3), which is located in the monophase region of the ternary

phase diagram (Figure 5.1). Subsequent addition of chloroform should induce the biphasic condition. By adding 1.5 ml chloroform the mixture entered the biphasic region with the mass ratio of chloroform:methanol:water changed to 60.96:24.57:14.47, which fell into the biphasic region (Figure 5.1). A clear separation of the lipid-rich chloroform layer from the methanol-water layer was therefore achieved for the removal of residual fat content in the samples (Figure 5.2). The lipid-rich chloroform layer was harvested, dried, and dissolved in mobile phase before HPLC analysis.

Table 5.3: Verification of the mass ratio of chloroform:methanol:water in the ternary phase diagram.

	Monophase condition **Chloroform:methanol:water**	**Biphasic condition** **Chloroform:methanol:water**
Volume ml	1: 2: 0.8	2.5: 2: 0.8
Volume%	26.3: 52.63: 21.05	47.16:37.73:15.09
Density at 20 deg C*	1.3453: 0.6778: 0.9982	1.3453: 0.6778: 0.9982
Mass ratio	35.38: 35.67: 21.01(92.06)	63.44: 25.57: 15.06(104.07)
Mass %	38.43: 38.74: 22.83	60.96: 24.57: 14.47

*Source: calculated from (Perry's Chemical Engineers Handbook, 1997, table 2-91, 2-94)

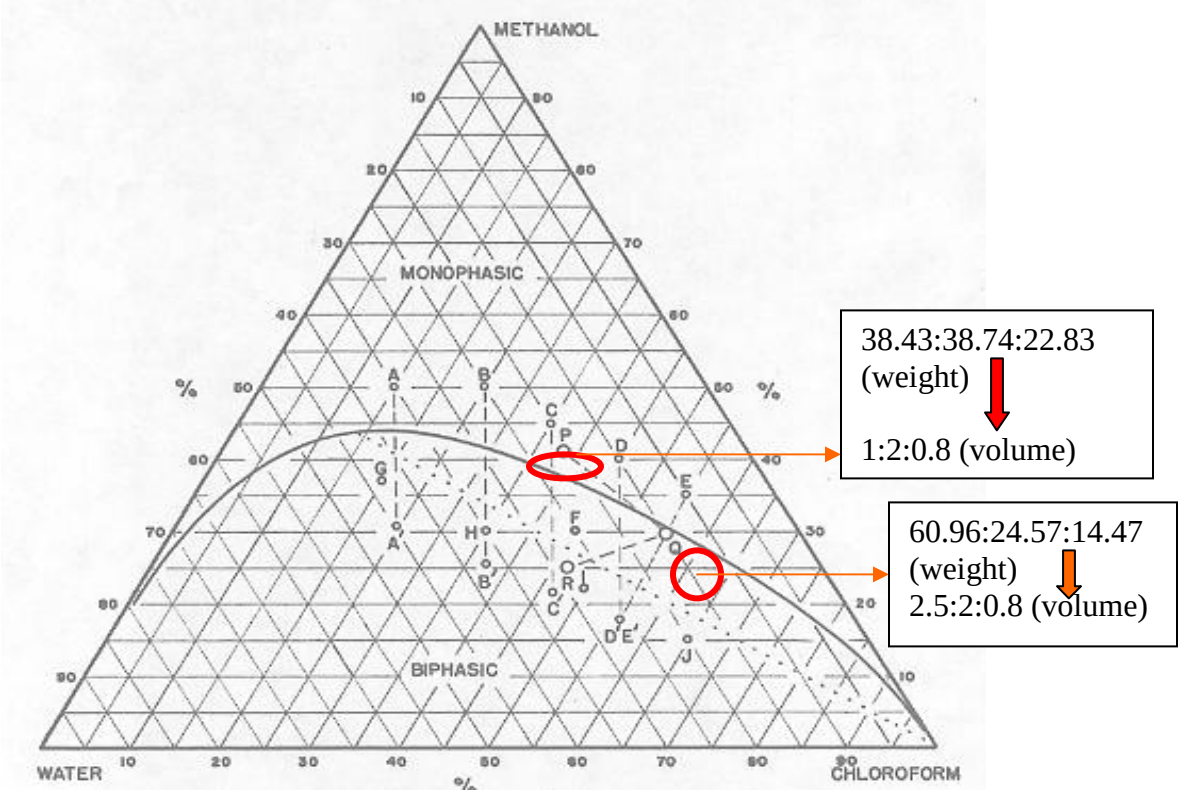


Fig 5.1: Ternary liquid equilibrium diagram of chloroform-methanol-water, % w/w 20 °C (Source: Bligh and Dyer, 1959)

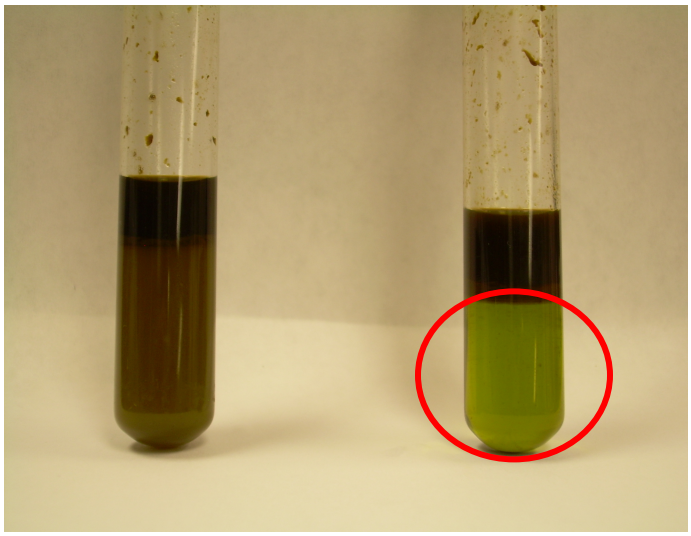
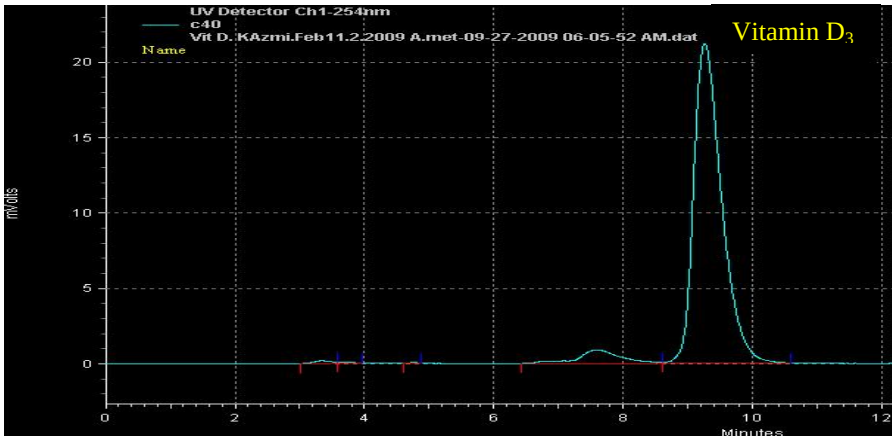


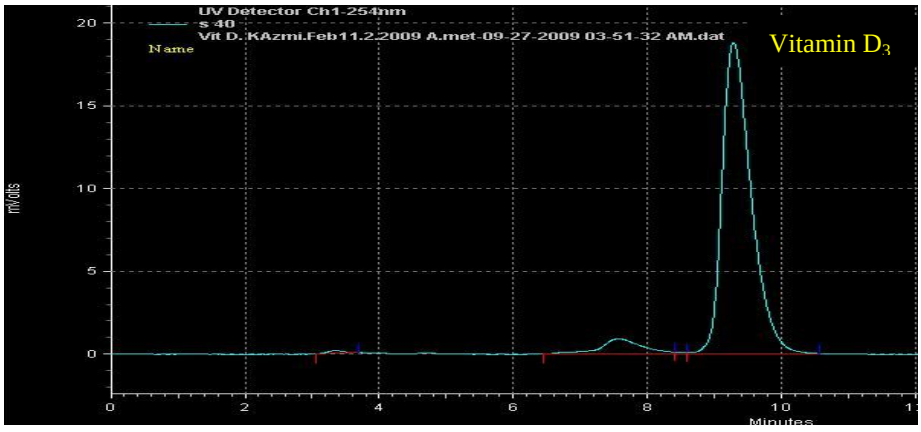
Fig 5.2: Comparison of phase separation of chloroform layer (bottom) from methanol-water layer (top) using the chloroform:methanol:water ratio (a) reported by Kazmi et al. (2007) and (b) optimized in the present study

5.2.2 HPLC calibration

By controlling the flow rate of the mobile phase, which was consisted of acetonitrile:methanol:water at the volumetric ratio of 49.5:49.5:1, a clear peak of vitamin D_3 acquired from PAT vitamins was attained at ca. 9.4 min retention time (Figure 5.3a). The same retention time was observed using the Sigma-Aldrich standard vitamin D_3 (Figure 5.3b). A linear calibration curve was developed using Vitamin D_3 at concentrations of 20, 40, 60, 80, 100, and 120 µg/ml with an R^2 of 0.9946 (Figure 5.4).



(a)



(b)

Figure 5.3: HPLC chromatogram of 40 µg/mL vitamin D_3 standard by (a) PAT Vitamins, CA and (b) Sigma Aldrich

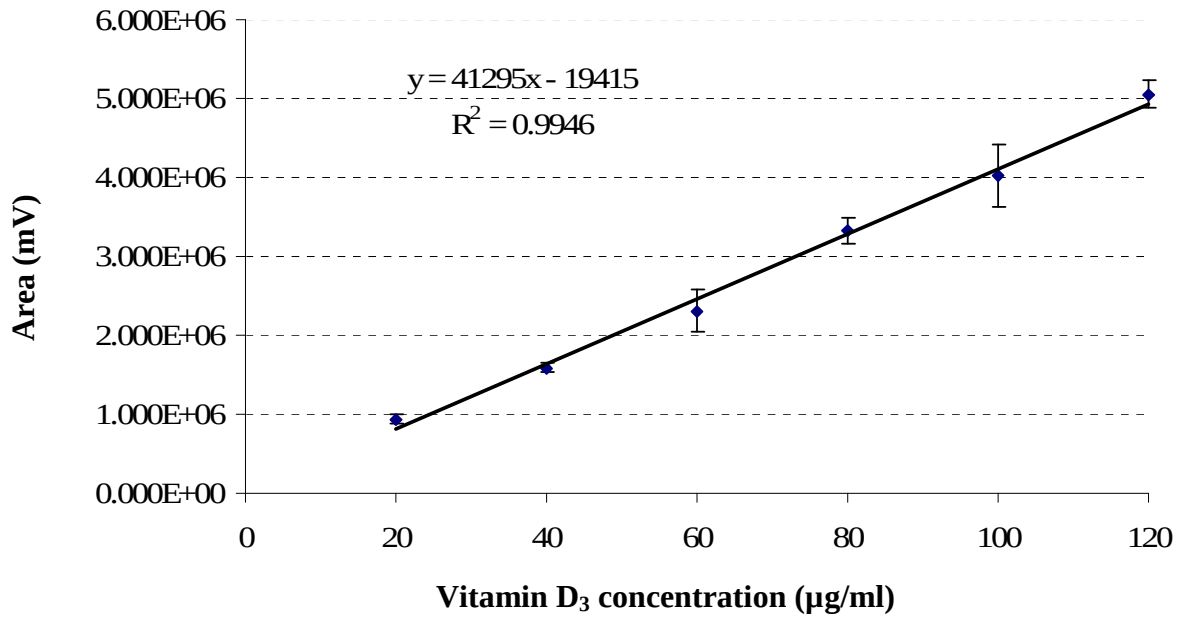


Figure 5.4: Vitamin calibration curve using a mobile phase acetonitrile:methanol:water of 49.5:49.5:1 (v/v) with a sample injection volume of 10 µL at 0.5 ml/min flow rate through a Waters C18 column (3.9x 300 mm) maintained at 25°C.

5.3 Formation of vitamin D-tobacco protein complex

By using the method described in Section 4.3, a vitamin D-tobacco protein complex was formed (Figure 5.5). An example of chromatographic analysis of vitamin D in the complex is shown in Figure 5.6. To examine the effect of freeze-drying, the original formulation (with leaf protein) when compared against the control (without leaf protein) showed significant difference in percent vitamin D recovery (fig 5.7) (84.68 ± 3.93 vs. 26 ± 4.51). Subsequent freeze-dried formulations were then compared and the formulation with 1 g tobacco powder, 40 ml water, vitamin D, and mixing proved to give a significant recovery of vitamin D over the formulations with pH change, lesser water, and sonication.

Figure 5.5 Vitamin D-tobacco protein complex formed in the present study.

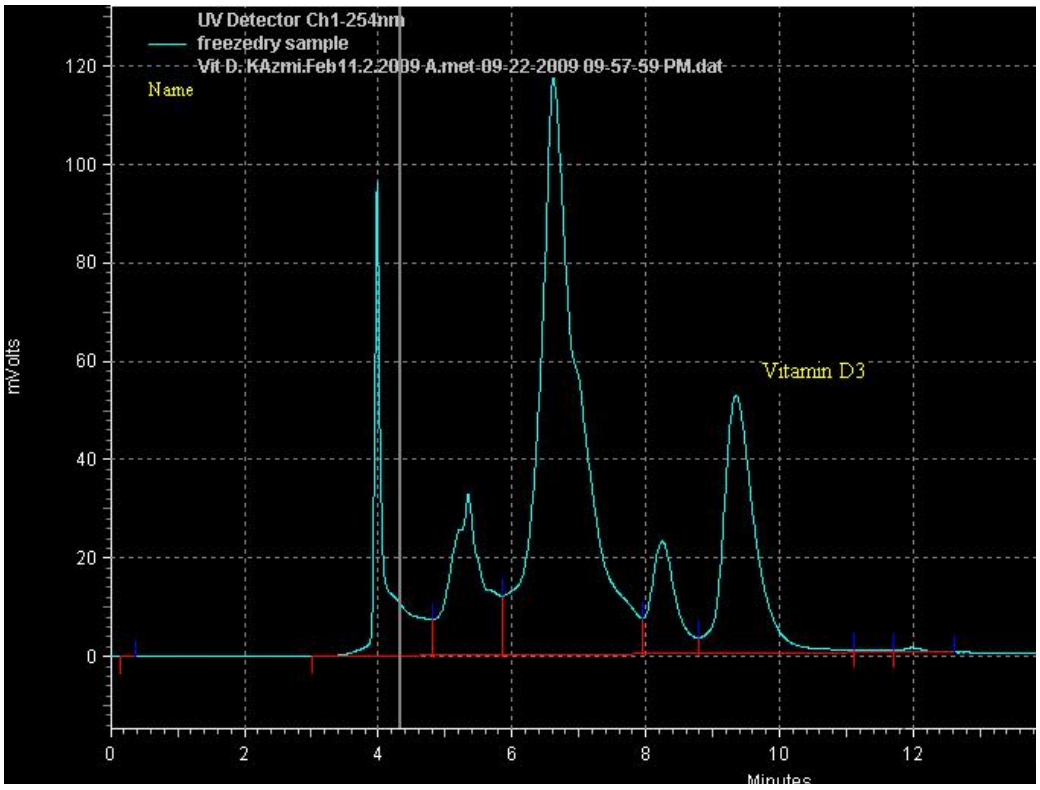


Figure 5.6 HPLC chromatogram showing vitamin D peak in the vitamin D-tobacco protein complex using a mobile phase acetonitrile:methanol:water of 49.5:49.5:1 (v/v) with a sample injection volume of 10 μL at 0.5 ml/min flow rate through a Waters C18 column (3.9x 300 mm) maintained at 25°C.

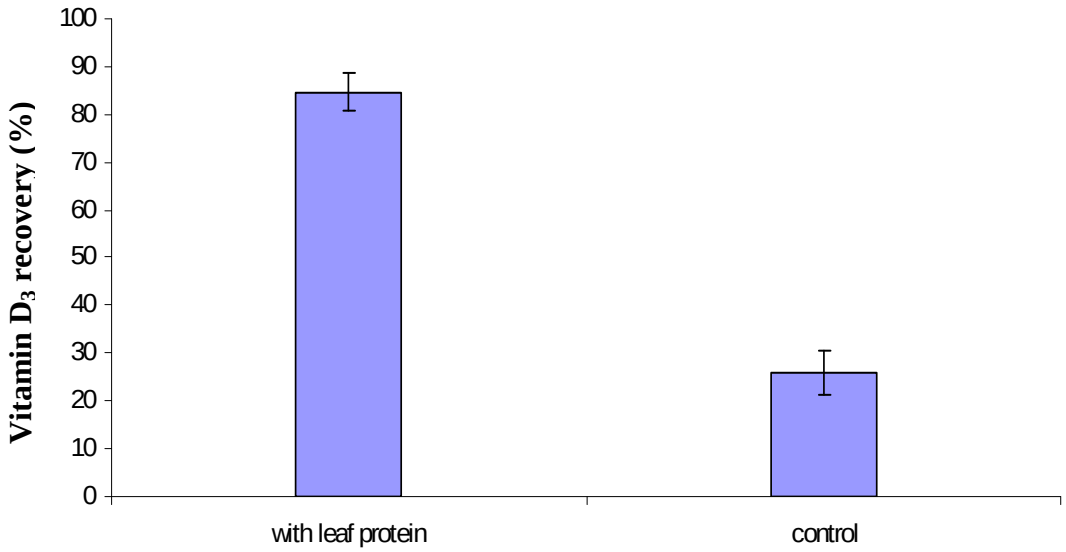


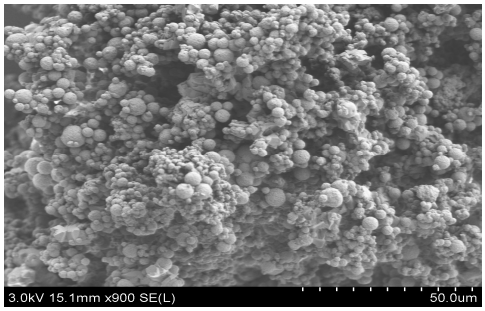
Figure 5.7: Comparison of vitamin D_3 recovery in freeze-dried formulation: with tobacco protein vs. control

5.4. Scanning Electron Microscopic imaging

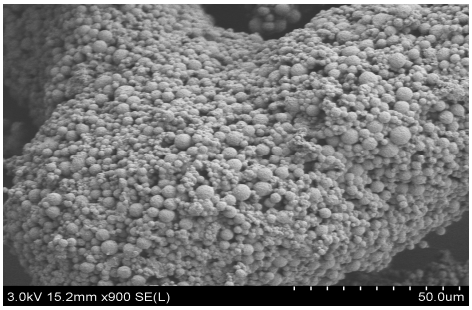
5.4.1 Effect of freeze-drying

The SEM image of the tobacco leaf crude protein powder at a low magnification of 900× showed a less packed structure of spherical molecules as compared to that of the freeze-dried protein powder (Figure 5.8a,b). Such a difference could be attributed to freeze-drying, which is known to induce undue stresses on the protein structure. It has been shown that, in the process of cooling during initial freezing, ice crystals are created and the size of the crystals determines the pore sizes that are created during subsequent drying (Wang, 2000). However, the vitamin D-protein complex showed lesser homogeneity (more gaps) in the aggregation compared to the freeze-dried protein powder (Figure 5.8c). The difference could be attributed to the presence of ethanol that is used to transfer

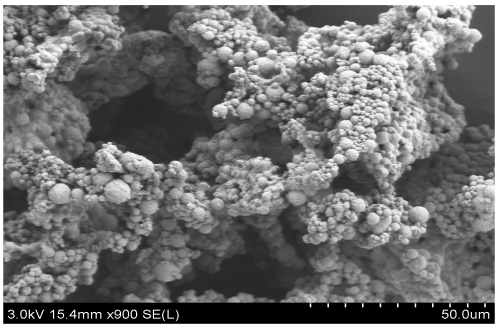
vitamin D, as it could possibly create a co-solvent system with the water in the formulation, leading to realignment of the water and consequently changing protein conformation (Yong et al., 2009). It is also intriguing that there was a slight increase in the water solubility of the vitamin D-protein complex from 10.08 to 10.78 g/l, which could be attributed to the increase in internal surface area (Hsu et al., 1995), as evidenced by the gaps and cracks observed in Figure 5.8c.



(a)



(b)



(c)

Figure 5.8: Scanning electron micrographs showing (a) the tobacco protein powder (b) tobacco protein powder after freeze-drying (c) the vitamin D-tobacco protein complex at 900× magnification.

5.4.2 Effect of pH change

On comparison of freeze-drying formulations prepared at pH of 4.3, 8.5, and 11.0 for vitamin D recovery, pH 4.3 showed the highest recovery (84.6 ± 3.92%) of vitamin D_3. The freeze-drying at higher pH of 8.5 and 11.0 showed no significant difference in vitamin D recovery (68.04 ± 4.89 % vs. 76.36 ± 7.32 %) between each other. The crude protein content showed no significant variability in all 3 samples, maintaining an approximate level at 30% (w/w) (Figure 5.9).

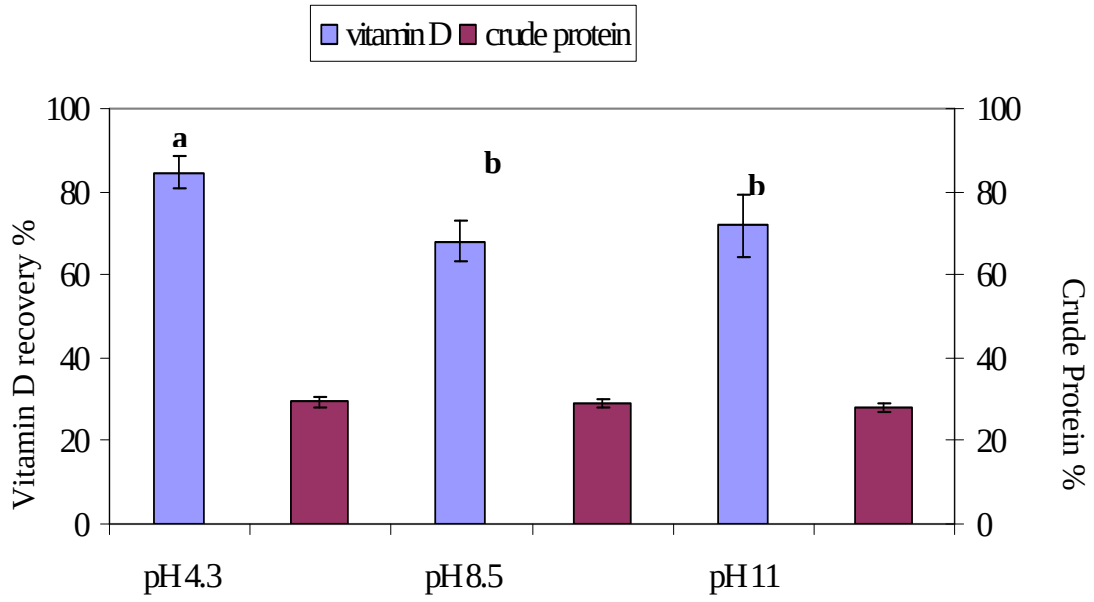
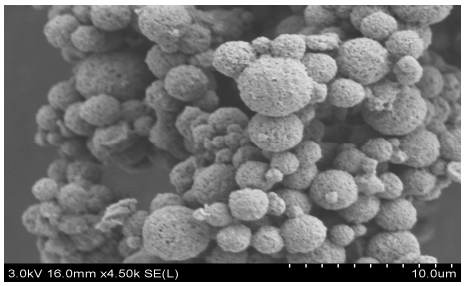


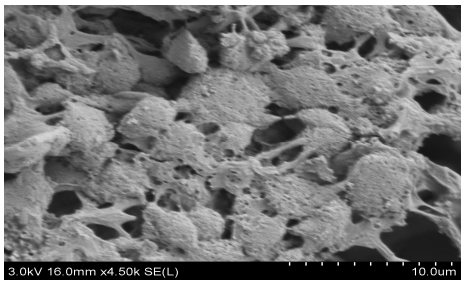
Figure 5.9: Comparison of vitamin D_3 recovery, and crude protein % in vitamin D-tobacco protein complex of varying pH

It is generally recognized that changes in pH could induce significant alterations in protein structure. At pH 4.3, the vitamin D-protein complex appeared to be spherical aggregates (Figure 5.10a). As the pH increased to 8.5, the spherical structure opened up and bridged with adjacent aggregates, forming an interwoven structure (Figure 5.10b). At pH 11.0, the spherical structure was completely disrupted, forming a continuous porous structure (Figure 5.10c). Such a structure change—with increased porosity—

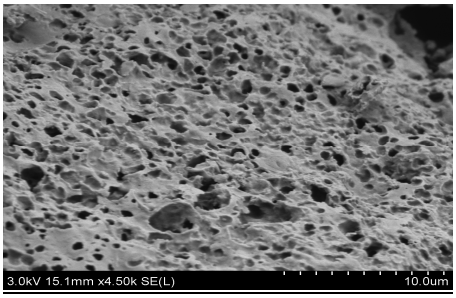
corresponded to the loss of vitamin D (Figure 5.9). Since freeze-drying at high pH in aqueous solutions is known to increase the β-sheet content and decrease the linkages within a protein (Carrasquillo et al., 2000), it could be postulated that some vitamin D was lost due to the change in protein structure. Although the water solubility of the vitamin D-protein complex was found to decrease with increasing pH values, no statistically significant difference was observed (Table 5.4). This suggests that the external hydrophobicity of the vitamin D-protein complex remained unchanged regardless of the structural change within the complex.



(a)



(b)



(c)

Figure 5.10: Scanning electron micrographs showing vitamin D-tobacco protein complex formulated at pH of (a) 4.3; (b) 8.5; (c) 11.0 at 45000× magnification.

Table 5.4: Solubility values of vitamin D – tobacco protein complex formulated at different pH (p value= 0.0709)

pH	Solubility
4.3	10.78±0.04
8.5	10.27±0.06
11	8.18 ± 2.01

5.4.3 Effect of mixing

As seen in Figure 5.11, different mixing of samples prior to freeze-drying led to differences in the vitamin D recovery. The highest vitamin D recovery was achieved when the samples were stir-mixed for 5 min, reaching 84.68 ± 3.92% (w/w). Sonication, a process known to enhance intermolecular mixing (Abismaïl et al., 1999) and is commonly used for cleaning or removal of proteinaceous materials on contact surfaces (Zips et al., 1990), was employed to assess its effectiveness in enhancing protein and vitamin D contacts during sample preparation. However, with sonication (47 kHz for 5 min) alone, a reduction in vitamin D recovery (62.53± 3.68%, w/w) was observed in comparison with stir-mixing (Figure 5.11). It was noted that there was a temperature increase by 20°C after sonication. Such a drastic change in temperature could cause degradation of vitamin D, hence the reduction in vitamin D recovery. The combination of sonication followed by mixing was also evaluated. Vitamin D recovery was the lowest when the samples were treated by sonication and mixing (56.32 ± 5.11%, w/w), mainly

due to the exposure of vitamin D under elevated temperature for an extension of 5 min during the mixing process. The crude protein content remained the statistically the same in all the three differentially mixed formulations. Nevertheless, the SEM pictures showed no perceptible difference among samples undergone different mixing treatments (Figure 5.12a,b,c).

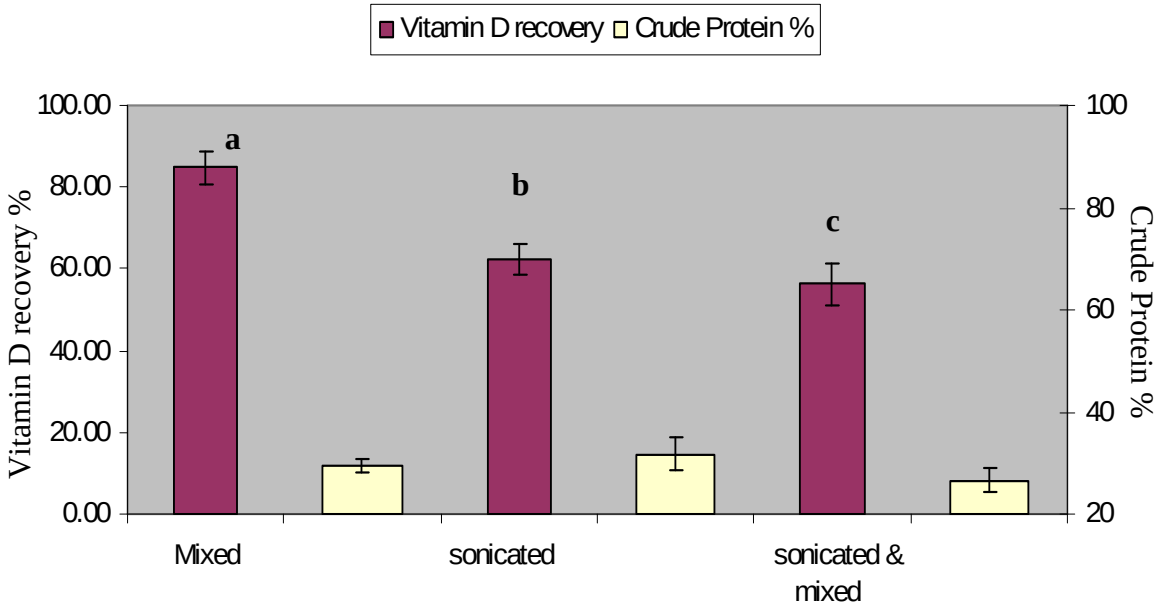
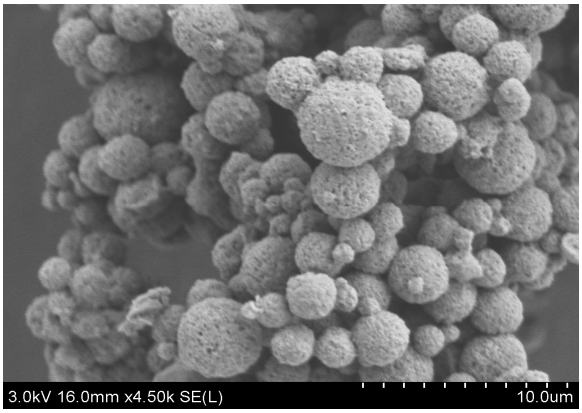
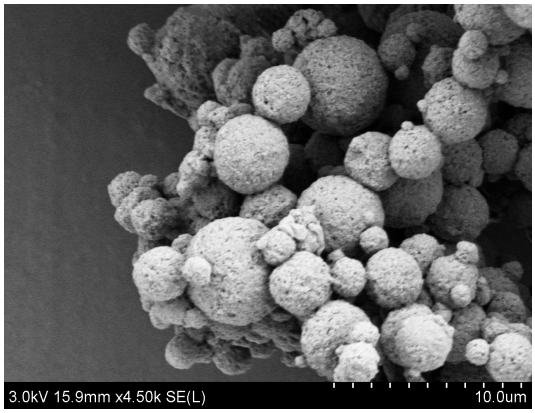


Figure 5.11: Comparison of vitamin D_3 recovery and crude protein % in the vitamin D-tobacco protein complex under different mixing conditions



(a)



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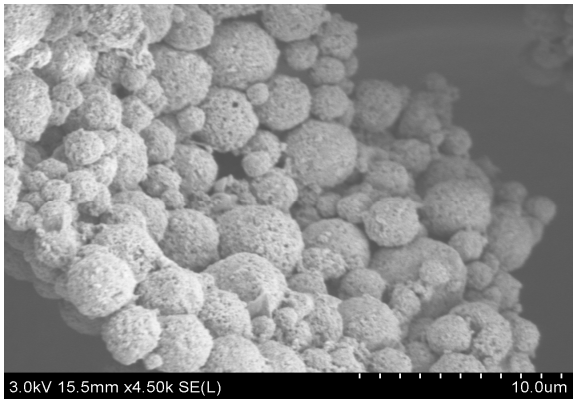
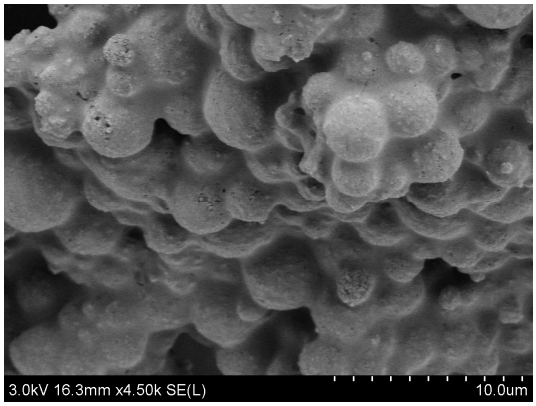


(c)



(d)

Figure 5.12: Scanning electron micrographs showing vitamin D-tobacco protein complex: formulated by (a) mixing with 40 ml water; (b) sonicated formulation; (c) sonicated and mixed formulation; and (d) mixing with 20 ml water at 45000× magnification

5.4.4 Effect of water content

As aforementioned, in the original formulation, 40 ml of water was employed to form the vitamin D-protein complex, reaching a final vitamin D recovery of 84.68 ± 3.92%. An attempt was made to reduce the water content to 20 ml to help gauge the possible effect of water content on protein conformation. As seen in Figure 5.13, with the protein content remained statistically similar in the complex, the vitamin D recovery in the 20 ml

water sample was significantly reduced (70.21 ± 8.92%), whereas the protein level in the complex remained consistent. Visually the spherical structure in the aggregates could not maintain when the water content was reduced by 50% (Figure 5.12 a, d), which could be attribute to the less amount of water in the formulation resulted in an increased concentration of protein. It is not uncommon that self stabilization of proteins could take place at increased protein concentrations where proteins tend to form bonds that interconnect adjacent proteins, leaving limited sites for vitamin D binding as well as surface area of ice/water interface during the freeze-drying process (Allison et. al., 1996).

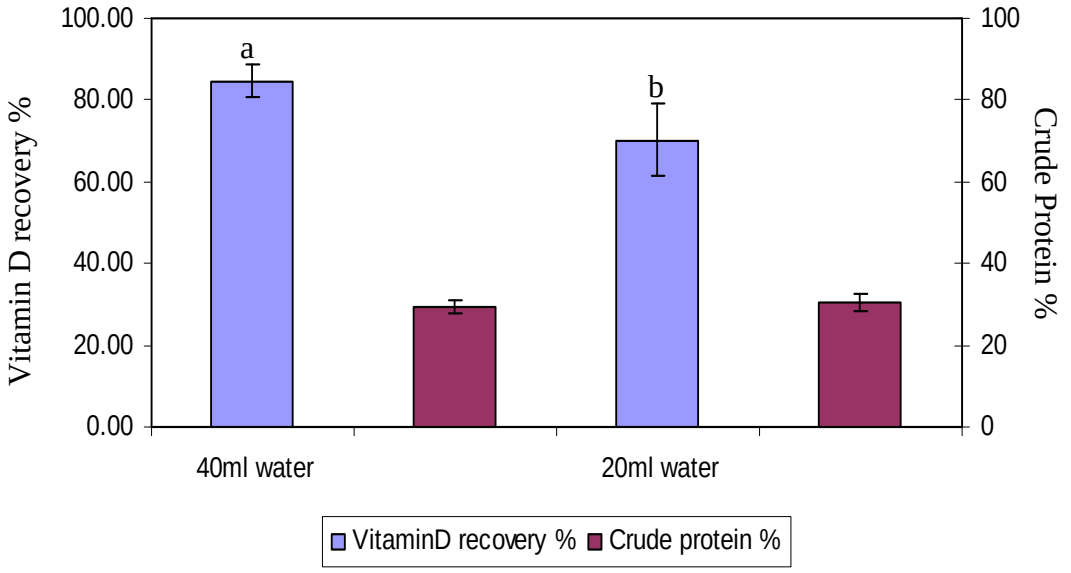


Figure 5.13: Comparison of vitamin D_3 recovery, and crude protein % in vitamin D-tobacco protein complex of different water content

In summary, crude tobacco protein powder was found to have higher retention of vitamin D as compared to the casein-vitamin D micelle (ca. 85% vs. 27%) (Semo et al., 2007). Additionally, the system developed in the present study requires fewer ingredients when compared to the emulsification and microencapsulation approaches (Dewille et al., 1997; Ling et al., 2003). Successful inclusion of vitamin D using a leafy protein could find applications beyond the current products fortified with vitamin D. While the ability of the complex to retain vitamin D is satisfactory and no distinct odor was detected, the appearance of the complex needs to be improved because the brownish green color of the complex could be a deterrent to consumer acceptability. Further studies should ascertain the shelf life of the vitamin D-protein complex with respect to its interaction with different food matrices. The bioavailability of vitamin D delivered by this complex should also be assessed to ensure its overall effectiveness.

Chapter 6: Conclusions

A vitamin D-protein complex was successfully formulated using crude protein extracted from low-alkaloid tobacco leaves without solvent treatment. By mixing aqueous suspension of the crude protein with a solution of vitamin D in 99% pure ethanol and immediately freezing it with liquid nitrogen prior to freeze-drying, a brownish-green, odorless powder capable of retaining up to 85% of vitamin D was produced. Processing conditions, including pH, mixing, and water content, was crucial in achieving the optimal formulation. The vitamin D retention power of the complex was superior to that of casein-vitamin D micelles. An HPLC method tailored for the analysis of vitamin D in the complex was also developed by preferentially separating vitamin D from the protein complex using chloroform/methanol/water ternary phase equilibrium.

Appendix A: Statistical Analysis

Comparison of solvent extracts with crude protein powder for solubility by dunnett's test (Table 5.1)

Dependent Variable: Solubility

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	10.5	3.5	57.07	<.0001
Error	8	0.49066667	0.613		
Corrected Total	11	10.99066667			

R-Square	Coeff Var	Root MSE	solubility mean
0.955356	2.503258	0.247656	9.893333

Source	DF	Anova SS	Mean Square	F Value	Pr > F
solvent	3	10.5	3.5	57.07	<0.0001

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.061333
Critical Value of Studentized Range	2.87966
Minimum Significant Difference	0.5823

Comparisons significant at 0.05 are indicated by ***

Solvent comparison	Difference between means	Simultaeneous 95% confidence limits	
hexane-none	0.74	0.1577	1.3223***
Acetone – none	0.2533	-0.329	0.8356
methanol-none	-1.74	-2.3223	-1.1577***

Comparison of solvent extracts with crude protein powder for crude protein% by dunnett's test (Table 5.1)

Dependent Variable: Crude protein%

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	275.46	91.82	4.71	<.0354
Error	8	156.02	19.5		
Corrected Total	11	431.48			

R-Square	Coeff Var	Root MSE	Crude protein% mean
0.638407	13.3017	4.416163	33.2

Source	DF	Anova SS	Mean Square	F Value	Pr > F
solvent	3	275.46	91.82	4.71	<0.0354

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	19.5025
Critical Value of Studentized Range	2.87966
Minimum Significant Difference	10.3833

Comparisons significant at 0.05 are indicated by **

Solvent comparison	Difference between means	Simultaeneous 95% confidence limits	
Methanol-none	5	2.517	23.283***
Acetone – none	5.8	-4.583	16.183
Hexane-none	2.9	-7.483	13.283

Comparison of solvent extracts with yield by dunnett's test (Table 5.1)

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2351.7025	783.900833	325.07	<0.0001
Error	8	19.291667	2.411458		
Corrected Total	11	2370.994167			

R-Square	Coeff Var	Root MSE	yield mean
0.991863	1.914977	1.552887	81.0967

Source	DF	Anova SS	Mean Square	F Value	Pr > F
solvent	3	2351.702500	783.900833	325.07	<0.0001

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	2.411458
Critical Value of Studentized Range	2.87966
Minimum Significant Difference	3.6512

Comparisons significant at 0.05 are indicated by ***

Solvent comparison	Difference between means	Simultaeneous 95% confidence limits	
hexane-none	-15.0	-18.651	-11.349***
acetone – none	-21.633	-25.285	-17.982***
methanol-none	-39.0	-42.651	-35.349***

Comparison of solvent extracts with total protein by dunnetts test (Table 5.1)

Dependent Variable: Total

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	13.68	4.56	0.32	0.8138
Error	8	115.5266667	14.4408333		
Corrected Total	11	129.2066667			

R-Square	Coeff Var	Root MSE	Total protein mean
0.105877	14.46742	3.80010	26.26667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
solvent	3	13.68	4.56	0.32	0.8138

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	14.44083
Critical Value of Studentized Range	2.87966
Minimum Significant Difference	8.9349

Comparisons significant at 0.05 are indicated by **

Solvent comparison	Difference between means	Simultaeneous 95% confidence limits	
Methanol-none	-1.4	-10.335	7.535
Acetone – none	-1.733	-10.668	7.202
Hexane-none	-3.00	-11.935	5.935

Comparison of solvent extracts with moisture by dunnetts test (Table 5.1)

Dependent Variable: moisture

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	38.6625	12.8875	17.53	0.0007
Error	8	5.88	0.735		
Corrected Total	11	44.5425			

R-Square	Coeff Var	Root MSE	Moisture mean
0.867991	7.374808	0.857321	11.62500

Source	DF	Anova SS	Mean Square	F Value	Pr > F
solvent	3	38.6625	12.8875	17.53	0.0007

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.735
Critical Value of Studentized Range	2.87966
Minimum Significant Difference	2.0158

Comparisons significant at 0.05 are indicated by ***

Solvent comparison	Difference between means	Simultaeneous 95% confidence limits	
hexane-none	-0.1	-2.1158	1.9158***
Acetone – none	-2.8	-4.8158	-0.7842***
methanol-none	-4.2	-6.2158	-2.1842***

Comparison of effect of pH with vitamin D recovery by tukey's test (Figure 5.9)

Dependent Variable: Vitamin D_3 recovery

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1370.558252	685.278126	22.10	<.0001
Error	24	744.176844	31.007369		
Corrected Total	26	2114.735096			

R-Square	Coeff Var	Root MSE	Vitamin D_3 recovery Mean
0.648099	7.440478	5.568426	74.83963

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Formulation	2	1370.558252	685.278126	22.10	<0.0001

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	31.00737
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	6.5553

Means with the same letter are not significantly different.

Tukey grouping	Mean	N	Formulation
A	84.681	9	pH 4.3
B	68.048	9	pH 8.5
B	71.790	9	pH 11

Comparison of effect of solubility with pH by tukey's test (Table 5.4)

Dependent Variable: Vitamin D_3 recovery

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	11.44026667	5.72013333	4.25	0.0709
Error	6	8.07813333	1.34635556		
Corrected Total	8	19.51840000			

R-Square	Coeff Var	Root MSE	Solubility Mean
0.586127	11.90485	1.160326	9.746667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
pH	2	11.44026667	5.72013333	4.25	0.0709

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.346356
Critical Value of Studentized Range	4.33920
Minimum Significant Difference	2.9069

Means with the same letter are not significantly different.

Tukey grouping	Mean	N	Ph
A	10.7867	3	pH 4.3
A	10.2733	3	pH 8.5
A	8.1800	3	pH 11

Comparison of effect of mixing with vitamin D recovery by tukey's test (Figure 5.11)

Dependent Variable: Vitamin D_3 recovery

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	4000.0562	2000.028100	108.95	<.0001
Error	24	440.555467	18.356478		
Corrected Total	26	4440.611667			

R-Square	Coeff Var	Root MSE	Vitamin D_3 recovery Mean
0.900789	6.315102	4.284446	67.84444

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Formulation	2	4000.0562	2000.0281	108.95	<0.0001

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	18.35648
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	5.0438

Means with the same letter are not significantly different.

Tukey grouping	Mean	N	Formulation
A	84.681	9	Mixed
B	62.528	9	Sonication
C	56.324	9	Sonication and mixed

Comparison of effect of all formulations with vitamin D recovery by tukey's test

Dependent Variable: Vitamin D_3 recovery

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4127.189520	825.437904	23.33	<.0001
Error	48	1697.979378	35.374570		
Corrected Total	53	5825.168898			

R-Square	Coeff Var	Root MSE	Vitamin D_3 recovery Mean
0.708510	8.628563	5.947653	68.92981

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Formulation	5	4127.189520	825.437904	23.33	<0.0001

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	35.37457
Critical Value of Studentized Range	4.19724
Minimum Significant Difference	8.3212

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Formulation
	A	84.681	9	Original
	B	71.790	9	pH 11
C	B	70.208	9	Hydration
C	B	68.048	9	pH 8.5
C	D	62.528	9	Sonication
	D	56.324	9	Sonication and mixed

Comparison of freezedried formulations for the highest crude protein% by Tukeys test:

Dependent Variable: Vitamin D_3 recovery

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	50.9650000	10.1930000	2.36	0.1032
Error	12	51.760000	4.31333333		
Corrected Total	17	102.7250000			

R-Square	Coeff Var	Root MSE	Vitamin D_3 recovery Mean
0.496130	7.092282	2.06857	29.2833

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Formulation	5	50.96500000	10.19300000	2.36	0.1032

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	4.313333
Critical Value of Studentized Range	4.75023
Minimum Significant Difference	5.6959

Means with the same letter are not significantly different.

Tukey grouping	Mean	N	Formulation
A	31.800	3	Hydration
A	30.700	3	Sonication
A	29.400	3	Original
A	29.100	3	Sonication and mixed
A	28.100	3	pH 11
A	26.600	3	pH 8.5

Bibliography

Abismaïl B, Canseiler JP, Wilhelm AM, Delmas H, Gourdon C (1999) Emulsification by ultrasound: drop size distribution and stability. Ultrasonics Sonochemistry, 6, 75–83

Allison SD, Dong A, Carpenter JF (1996) Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying and rehydration. Biophysical Journal. 71, 2022-2032

Ball GFM (ed) (2006) Vitamins in foods - analysis, bioavailability and stability: Vitamin D. CRC Press, Boca Raton, Florida.107 - 115

Ball GFM (ed) (1988) Fat soluble vitamin assays in food analysis: A comprehensive review. Elsevier Science Publishing Co., Inc., New York, USA. 24 - 35

Banville C, Villemard JC, Lacroix C (2000) Comparison of different methods for fortifying Cheddar cheese with Vitamin D. International Dairy Journal, 10, 375 – 382

Bills CE (1927) Antiricketic substances.VI. The distribution of vitamin D with some notes on its possible origin. Journal of Biological Chemistry, 72, 751-758

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911 – 917

Brannon PM, Yetley EA, Bailey RL (2008) Overview of the conference “Vitamin D and health in the 21st century: an update”. American Journal of Clinical Nutrition, 88(suppl), 483S-490S.

Brantley SL & Mellott NP (2000) Surface area and porosity of primary silicate minerals. American Mineralogist, 85, 1767- 1783.

Byrdwell WC (2009) Comparison of analysis of vitamin D_3 in foods using ultraviolet and mass spectrometric detection. Journal of Agricultural and Food Chemistry, 57, 2135 – 2146.

Calvo M, Whitling SJ, Barton CN (2004) Vitamin D fortification in the United States and Canada: current status and data needs. American Journal of Clinical Nutrition, 80(suppl), 1710S- 1716S

Carasquilo KG, Sanchez C, Griebenow K (2000) Relationship between conformational stability and lyophilisation induced structural changes in chymotrypsin. Biotechnology and Applied Biochemistry 31, 41-53

Chen PS, Jr, Terepka R, Lane K, Marsh A (1965) Studies of the stability and the ex tractability of Vitamin D. Analytical Biochemistry, 10, 421 – 434.

DeWille NT, Chandler MA, Mazer TB, Ragan RJ, Snowden GA, Geraghty ME, Johnson CD (1997) Low pH beverage fortified with Calcium and Vitamin D. US Patent 5597595.

Eitenmiller RR and Landen Jr. WO (1999) Vitamin Analysis for the health and food sciences: Vitamin D, CRC Press., Boca Raton, Florida.

Fiore JV (1975) Solvent extraction process. US patent 4144895.

Fleet JC (2008) Molecular actions of vitamin D contributing to cancer prevention. Molecular Aspects of Medicine, 29, 388 – 396.

Forrest SA, Yada RY, Rousseau D (2005) Interactions of vitamin D_3 with bovine β-lactoglobulin A and β-casein. Journal of Agricultural and Food Chemistry, 53, 8003- 8009.

Fu H, Machado PA, Hahm TS, Kratochvil RJ, Wei CI, Lo YM (2009) Recovery of nicotine-free proteins from tobacco leaves using phosphate buffer system under controlled conditions. BioResource Technology, doi:10.1016/j.biortech.2009. 10.045

Garland CF, Comstock GW, Garland CF, Helsing KJ, Shaw EK, Gorham ED (1989) Serum 25-hydroxyvitaminD and colon cancer: eight year prospective study, Lancet, 2, 1176-1178

Hoolick MF, MacLaughlin JA, Clark MB (1980) Photosynthesis of previtamin D_3 in human skin and the physiologic consequences. Science, 210, 203-205

Hollick MF (2002) Vitamin D: the underappreciated D-lightful hormone that is important for skeletal and cellular health. Current Opinion in Endocrinology**,** Diabetes and Obesity, 9, 87-98.

Houghton LA and Vieth R (2006) The case against ergocalciferol (vitamin D_2) as a vitamin supplement. American Journal of Clinical Nutrition, 84, 694 – 697.

Huber W and Barlow OW (1943) Chemical and biological stability of crystalline vitamin D_2 and D_3 and their derivatives. The Journal of Biological Chemistry, 149 (7), 125-137

Hudson BJF and Karis IG (1973) Aspects of vegetable structural lipids.I. The lipids of leaf protein concentrate. Journal of the Science of Food and Agriculture. 24, 1541-1550

Hsu CC, Nguyen HM, Yeung DA, Brooks DA, Koe GS, Bewley TA, Pearlman R (1995) Surface denaturation at solid-void interface--a possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. Pharm. Res. 12, 69–77

Kazmi SA, Vieth R, Rousseau D (2007) Vitamin D_3 fortification and quantification in processed dairy products. International Dairy Journal, 17(7): 753-759.

Kirby CJ, Brooker BE, Law BA (1987) Accelerated ripening of cheese using using liposome encapsulated enzyme. International Journal of Food Science and Technology, 22, 355-375

Klaui HM, Hausheer W, and Hushke G (1970) Technological aspects of the use of fat soluble vitamins and Carotenoids and the development of stablilised marketable forms. In: Fat Soluble Vitamins, Morton RA (Editor), first ed., Pergammon Press., New York, pp. 113 – 161.

Koskinen T and Valtonen P (1985) Identification and analysis of vitamin Ds forms in chicken eggs. In: Vitamin D. A chemical, biochemical and clinical update. Proceedings of the sixth Workshop on Vitamin D, Merano, Italy, March, 1985, Norman AW, Schaefer K, Grigoleit HG and Herrath DW (Eds.), Walter de Gruyter, New York, pp. 588-589

Knuckles BE, Kohler GO, deFremery D (1978) Procssing of fresh tobacco leaves for protein fractions. Journal of Agriculture and Food Chemistry, 27(2), 414-418.

Ling A, Wyland A, Williams K (2003) Vitamin fortification of foodstuffs. WIPO Patent WO/ 2003/ 026445.

Lo YM, Fu H (2008) Novel methods for recovery of leaf proteins. WIPO patent WO/ 2008/ 143914

Lumley ID (1993) Vitamin analysis in food. In: The technology of vitamins in food, Ottaway PB (ed.), Chapman & Hall Inc., New York, pp. 173 – 178

Lytgoe B, Moran TA, Nambudiry MEN, Tideswell J, Wright PW (1978) Calciferol and its relatives. Part 22.l A direct total synthesis of Vitamin D, and Vitamin D_3. Journal of Chemical Society, Perkin Transactions 1, 6, 590–595

Mattila P, Lampi AM, Ronkainen R, Toivo J, Piironen VI (2002) Sterol and vitamin D2 contents in some wild and cultivated mushrooms. Food Chemistry, 76, 293-298

McCollum EV, Simmonds N, Becker JE, Shipley PG (1922) Studies on experimental rickets. XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. Journal of Biological Chemistry, 53, 269-276

Mozafari MR, Johnson C, Hatziantoniou S, Demetzos C (2008) Nanoliposomes and their applications in food nanotechnology. Journal of Liposome Research, 18, 309-327

National Institutes of Health, National Library of Medicine, Medline Plus, Medical encyclopedia, Rickets Symptoms. Internet: http ://www.nlm.nih.gov/medlineplus /ency/article/000344.htm (accessed 6 December 2009)

Nibbelink KA, Tishkoff, DX, Hershey SD, Rahman, A, Simpson, RU (2007) 1, 25$(OH)_2$-vitamin D_3 actions on cell proliferation, size, gene expression, and receptor localization, in the HL-1 cardiac myocyte. The Journal of Steroid Biochemistry and Molecular Biology. 103(3-5), 533-537

Perry RH, Green DW, Maloney JO (eds) (1997) Perry's chemical engineers handbook. Mc-Graw Hill, NY, pp 2-91 – 2-98

Pike OA, (2003) Fat characterization. In: Food Analysis, Nielsen, S. S. (Editor), Third edition, Springer, New York, pp 227-246.

Renken SA, and Warthesen JJ (1993) Vitamin D stability in milk. Journal of Food Science, 58(3), 552 – 556.

Semo E, Kesselman E, Danino D, Livney D (2007) Casein micelles as a natural nano-capsular vehicle for nutraceuticals. Food hydrocolloids, 21, 936 – 942

Sharma A, Sharma US (1997) Liposomes in drug delivery: progress and limitations. International Journal of Pharmaceutics, 154, 123-140

Sheen JS (1991) Comparison of chemical and functional properties of soluble leaf proteins from four plant species. Journal of Agricultural and Food Chemistry, 39, 681 - 685.

Sheen JS and Sheen VL (1985) Functional properties of Fraction 1 Protein from tobacco Leaf. Journal of Agricultural and Food Chemistry, 33, 79 - 83.

Shinoda K, Friberg S (1986) Emulsions and Solubilization. John Wiley and Sons Inc., New York, pp 55-91

Swaisgood HE, Wang Q, Allen JC (2001) Protein ingredient for carrying lipophilic nutrients. US Patent 6290974.

Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine (1997) Vitamin D. In: Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D,and fluoride. Washington, DC: National Academy Press, 250–287.

Tso TC, Kung SD (1983) Soluble proteins in tobacco and their potential use. In: Leaf Protein Concentrates, Tehel L and Graham DG (eds.), Avi Publishing Company Inc., Connecticut, pp. 117 – 131.

Wagner CL, Greer FR; American Academy of Pediatrics, Section on Breastfeeding Medicine and Committee on Nutrition (2008) Prevention of Rickets and vitamin D deficiency in infants, children, and adolescents. Pediatrics, 122, 1142 - 1152.

Wang W (2000) Lyophilization and development of solid protein pharmaceuticals, International Journal of Pharmaceutics, 203, 1-60.

Wildman SG (1983) Plant: The Potentials for extracting protein, medicines, and other useful chemicals - Workshop Processings; US. Congress, Office of Technology Assessment: Washington, DC, OTA-BP-F-23, pp 63-77.

World Health Organization (1950) Expert committee on biological standardization report of the subcommittee on fat soluble vitamins. World Health Organization Technical Report Ser 3:7e

Yong Z, Yingjie D, Xueli W, Jinghua X, Zhengqiang L (2009) Conformational and bioactivity analysis of insulin: Freeze-drying TBA/water co-solvent system in the presence of surfactant and sugar. International Journal of Pharmaceutics, 371, 71-81

Zips A, Shaule G, Flemming HC (1990) Ultrasound as a means of detaching biofilms. Biofouling, 2, p. 322 - 325