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Stability of Nano-encapsulated Rice Bran Derived Bioactive Pentapeptide in Apple Juice

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Derived Bioactive Pentapeptide in Apple Juice

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ABSTRACT

Cereal grains and their components derived Bioactive compounds such as rice bran can promote health and can be derived from Rice bran contains 12-20 % protein and could be a good source for extracting bioactive peptides. A pentapeptide with a secqunce of amino acids Glu-Gln-Arg-Pro-Arg (EQRPR) has been prepared from heat stablized defatted rice bran (HDRB) and has demonstrated anti-cancer proprerties *in-vitro*. This bioactive pentapeptide can thus be used as a nutraceutical by incorporating it into a suitable food system. Fruit juces can be vehicles to incorporate this pentapeptide. Fruit juices contribute to about 60% of the consumed beverages in the U.S. However, the stability of the pentapeptide in beverages can be a problem due to possible interactions with other components. Nano-encapsulation is a novel and promising technique that can be used to deliver bioactive ingredients into food systems. This study involves the use of a nano-encapsulating technique to protect the bioactive pentapeptide, incorporating the encapsulated pentapeptide into apple juice (model system), and testing for the stability of the peptide. The null hypothesis of the study: The Nano-encapsulated pentapeptide shall degrade over time when incorporated in apple juice and the alternate hypothesis that the Nano-encapsulated pentapeptide incorporated apple juice shall be stable over a storage period of 6 months or more. The specific objectives of this research were to: (1) prepare nanoparticles using polylactic-co-glycolic acid (PLGA) to encapsulate the rice bran pentapeptide, (2) incorporate the encapsulated pentapeptide into apple juice, (3) evaluate the stability of incorporated pentapeptide at 4°C for 6 months. Nanoparticles that can deliver three different concentrations (200/ 400/and 600 µg/ml) of pentapeptide were prepared, and the particle size were measured using a laser particle size analyzer. Apple juice containing nanoparticles (loaded with pentapeptide) was ultra-centrifuged to separate nanoparticles, and the supernatant was analyzed by high performance

liquid chromatography (HPLC) C18 column reverse phase (RP) to test the stability of pentapeptide. Physical properties of the apple juice were studied which included the evaluation of color, microbial count total, acidity (pH), and soluble solid (TSS) during storage period of 60 days. A particle size ranging from 81 to 83 nm was observed, and the results indicated that there were no significant changes in the size over the storage period (0 – 60 days). There was no microbial growth observed in the prepared apple juice samples. Total Soluble Solids content was 11.0 ºBrix for the controls and 31.0 ºBrix for the Nano-encapsulated pentapeptide. The stability of pentapeptide at prepared concentrations: 200, 400 and 600µg/ml in water at pH of 3.7 at 0^{th} day was: 200ug/mL – 87 %, 400ug/mL – 97%, 600ug/mL – 91%) and in apple juice was: 200ug/mL – 96%, 400ug/mL – 98%, 600ug/mL – 94 %. The stability of pentapeptide at 60^{th} day in water was: 200ug/mL – 41%, 400ug/mL – 60%, 600ug/mL – 55%, and in apple juice was: 200ug/mL – 60%, 400ug/mL – 67%, 600ug/mL – 59%. The Nano-encapsulated pentapeptide in water at pH of 3.7 and apple juice was stable over the storage period of 60 days, which implies that the nanoparticles were effective in protecting the bioactive pentapeptide in the acidic environment of apple juice. The PLGA nanoparticles showed a remarkable effect in protecting and stabilizing the bioactive compounds (pentapeptide) during the shelf life at 4ºC. Polylactic-co-glycolic acid nanoparticles can thus be a promising carrier for the bioactive pentapeptide when incorporated into a juice medium.

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CHAPTER 1

INTRODUCTION

The International Markets Bureau (2011) documented in 2010 that fruit and vegetable juices are consumed at per capita consumption of about 30.3 liter/ person, and the retail market in the United States is 8.8 billion dollars. The US Department of Agriculture documented that 49% of Americans consume more than one glass (236.6 mL; 8 fluid oz.) of juice daily (Andon et al., 1996). The International Markets Bureau (2011) further asserts that consumers are increasingly becoming health conscious; therefore, the consumption of fruits and vegetables have increased in popularity with a larger proportion of the American population. Since consumers have become more health conscious and prefer to purchase 100% fruit juices with no additives, the manufacturers have improved the ingredients that are used in fruit juices. Fruit juices can offer health benefits, such as reducing the risk of cancers and cardiovascular disease. Some common ingredients in fruit juices are water, malic acid, sugar, fiber, and minerals. The interactions among native components in fruit juices and bioactive ingredients can be minimized, making juices suitable vehicles to incorporate bioactive compounds (peptides and proteins) (Day, et al., 2009; Tuorila & Cardello, 2002). Vehicles are food systems that can deliver bioactive ingredients so that consumers receive maximum health benefits. Apple juice can be a vehicle to incorporate peptides because it is the second most popular juice consumed in the U.S., making up 12.5% of total juice consumption preceded only by orange juice 60% of total juice consumption. Apple juice is rich in phytochemical compounds, especially flavonoids, and phenolic compounds (Boyer and Lui, 2004). Because of these beneficial components in fruit juices, apple and orange juices are fortified with calcium citrate malate (CCM) and show high calcium absorption from CCM. To mimic the composition of Ca fortified juices, the

concentrations of organic acid and carbohydrate in the test solution were manipulated. Apple juice has a high Ca absorption because of high fructose and low organic acid content (Andon et al., 1996). Also, orange juice is fortified with 1000 IU vitamin D3/236.6 mL, and the result shows more than 150 % increase in the serum 25- hydroxyvitamin D3 [25(OH)D] concentrations of adults over a 12-week period. Therefore, vitamin D intake can be increased by orange juice modification with vitamin D3 (Biancuzzo et al., 2011). Fruit juices such as apple and orange juice can be good vehicles for bioactive ingredients such as proteins, peptides, and vitamins due to their efficiency to impart health benefits.

Bioactive compounds have positive effects on human physiological health beyond their nutritional values. Examples of bioactives derived from cereal grains include oatmeal, rice bran, wheat bran, and protein rice. Rice bran (RB) is rich in protein; protein makes up approximately 12-20% of rice bran. A pentapeptide is a protein extracted from RB with a sequence of amino acids Glu-Gln-Arg-Pro-Arg (EQRPR), pentapeptide is prepared from heat stablized defatted rice bran (HDRB). EQRPR has demonstrated anti-cancer proprerties, and has the potential of being used as a drug or incorporated into sutitable food products such as orange juice (Kannan *et al*.,2008). For example, in a study conducted by Khairallah (2011), the stability of RB peptide fractions into orange juice environment was investigated for 6 months. The peptide fractions showed high stability at 4°C and pH 3.0- 4.5, which indicated that fruit juices can be an appropriate food system to incorporate bioactive compounds from rice bran. The pentapeptide EQRPR can be incorprated into several food aplications due to its health benefits, disease prevention, and higher stability.

It is likely that the bioactive peptides can interact with the components in juice matrix and influence the stability. The interaction between ingredients in fruit juices can initiate

chemical deterioration and changes in flavor and aroma of food products. To overcome this problem, the encapsulation technique can be an effective method to protect the bioactive peptide and prevent interactions with other compounds (Abhilash, 2010). Encapsulation method can be defined as "a process to entrap active agents within a carrier material" (Nedovic *et al*., 2011). The encapsulation technique contains two parts: a core or bioactives and a shell or coating materials (Abhilash, 2010), and it is an effective method that can be used to encapsulate the bioactive ingredients and impart their health benefits to specific sites of the human body.

Nano-encapsulation is a promising technique, and can be defined as the formation of particles loaded with ingredients in diameters ranging from1-100 nm (Reis *et al*., 2005). Nano-encapsulation is used in several areas of the food sector including, food processing, safety, and packaging (Garcia *et al*., 2010). Nanoparticles are solid submicron-sized particles that can carry functional food ingredients. Nanoparticles can be synthesized from natural or synthetic polymers such as poly d,l-lactide-co-glycolide acid (PLGA) (Janusz *et al.*, 2000). There is a need to investigate the stability of the bioactive pentapeptide in product applications since the consumer prefers bioactives in food products rather than in drug form.

The objectives of this study were to:

1. Prepare nanoparticle to encapsulate pentapeptide and test for stability for 6 months.
2. Incorporate the pentapeptide containing nanoparticles in apple juice.
3. Investigate shelf life stability of Nano-encapsulated pentapeptide in apple juice for 6 months at 4 °C.
4. Evaluate the physical attributes of apple juice with Nano-encapsulated pentapeptide for 6 months.

CHAPTER 2

LITERATURE REVIEW

A. Fruit juice consumption in the U.S.

The International Markets Bureau documented in 2010 that the fruit and vegetable juice industry was worth about 8.8 billion dollars in the United States. The International Markets Bureau (2011) further asserts that consumers are becoming more health conscious. On an average, about 12.3% of Americans consume apple juice, 60% orange juice, 9.6% mixed fruits, 3.2% cranberry, and 3.8% grape juice (International Markets Bureau, 2011). Consumers' perception of foods has changed from using food to satisfy hunger to seeing food as a tool for a healthy lifestyle. Flavors, aroma, price, and health benefits are the primary attributes that can attract consumers to purchase a food product (Urala and Lahteenmaki, 2007). Functional food can be defined as a food with "added technologically developed ingredients with specific health benefits" (Siro *et al*., 2008). The functional food market has changed to meet consumer demands, demonstrated by the invention of staple foods and beverages. Siro *et al*., (2008) reported that the United States has the largest market segments followed by Europe and Japan. American consumers contribute over 90% of the total sales. Functional food and beverages are new tools to receive additional health benefits (Siegrist *et al.,* 2008). The top 10 functional foods were investigated in a study conducted by Sloan (2010); it was found that the traditional foods, such as whole grains, topped the food choices list followed by fresh fruits and healthy snacks. People consume more natural fruit drinks since they are looking for products that are enriched in antioxidants, such as polyphenolics and flavonoids. Because consumers prefer juices that are rich in nutrients, low calorie, and have no added sugars, they will appreciate the idea of functional

foods. Therefore, food manufacturers focus on formulating improved, functional beverages to target physiological functions and attract consumers.

B. Suitability of vehicles for nutraceuticals and functional foods.

The health benefits of 100% fruit juices, such as low sugar and zero additives account for the increasing rate of consumption (Pollack, *et al.,* 2003). Gerhauser (2008) reportes "several lines of evidence suggesting that apples and apple products possess a wide range of biological activities which may contribute to health beneficial effects against cardiovascular disease, asthma and pulmonary dysfunction, diabetes, obesity, and cancer". In a study done by Duffey and Popkin (2006) on American adults, it was found that 20% of daily calories intake can be from beverages. Also, fruit juices are simple systems; for example, apple juice, mainly consisting of water, malic acid, sugar, fiber, and minerals; therefore, the possible interactions during the handling process or physical damage, which may initiate chemical and microbial spoilage can be monitored (Day *et al*., 2009; Tuorila and Cardello, 2002) by pasteurization of fruit juices (Polydera *et al*., 2003). For example, omega- 3 fatty acids, plant sterols, vitamins, and minerals have been used to fortify orange juice, such as *Tropicana* (Devaraj *et al.*, 2004). *Minute Maid* is another example of a beverage that is enriched with essential vitamins A, D, E, and B and minerals including zinc, calcium, magnesium, selenium, chromium, and frauctooligosaccharide (Tangpricha *et al*., 2003; Renuka *et al*., 2009). Apple juice a rich source of phytochemical compounds, especially phenolics. Apple juice is ranked as having the second highest amount of antioxidant and phenolics concentrations in comparison to other fruit juices in the U.S. Also, it has a high level of free phenolics (Boyer and Luis, 2004). Table 1 shows the average nutrients in apple juice and table 2 shows the polyphenol content in apple juice. The six classes of polyphenolic compounds in apple juice are: anthocyanins, flavonol glycosides, phenolic acids

(chlorogenic and p-coumaroylquinic acids) dihydrochalcones (phloretin glucoside and xyloglucoside), catechin (epicatechin, and procyanidins), and the procyanidins. A moderate amount of carbohydrate in the form of natural fructose and glucose are present in apple juice (Miller et al., 1996). These compounds can potentially reduce oxidative stress and regulate the immune system by reducing reactive oxygen species (ROS) (Boyer and Luis, 2004). Apple juice contains ascorbic acid (vitamin C), which can prevent degradation and oxidation of polyphenol compounds. Also, vitamin C can prevent a Millard reaction and maintain the flavor of apple juice, while also contributing to reduce the risk of cancers and cardiovascular disease (Chen and Sato, 1995). Apple juice is also rich in vitamin B complex including riboflavin, thiamin, and pyridoxine, which act as co-factors of enzymes involved in the functions in the body. Furthermore, apple juice contains a high amount of calcium, potassium, phosphorus, soluble fiber (pectin), and a low amount of sodium. Calcium is important for bone functions and blood clotting. Potassium is a major principle ion, which can maintain fluid and fluid electrolytic balance, maintain steady heart beat and intracellular pressure, and assist proteins metabolism. Apple juice can reduce sodium level in tissues because of high potassium content. Phosphorus plays a vital role in cell membrane structure and metabolic processes. Consuming apple juice can help detoxify liver, prevent gallstones, improve gut health, boost the immune system, and decrease the risk of diabetes (Jalili *et al.,* 2007). Since the diet is responsible for 80% of disease protection (Schaefer *et al*., 2005), consuming fruit juices that are high in phytochemical compounds and vitamins and minerals can promote health. Therefore, apple juice can be used as a vehicle to incorporate bioactives and peptides.

C. Vehicles to incorporate nutraceuticals, flavors, nutrients, and proteins.

Bioactive compounds can provide possible positive effects on human physiological health that transcend those offered by basic nutritional functions of food. Therefore, the constituents and derivatives of proteins and peptides play a potenitial role in treating as well as preventing diseases. The bioactive compounds, such as proteins and peptides, are known to impart several biological functions, such as anti-cancer, anti-obesity, anti-angiogenic, anti-hypertensive, antioxidants hypocholesterolemic, and immunomodulatory functions (Kannan *et al.,* 2008, Rayaprolu *et al.,* 2012). Soy and whey are rich sources of proteins that can produce bioactive compounds (Rayaprolu *et al.,* 2012). Oryzatensin is derived from rice albumin and demonstrates an immunostimulatory role. Also, soy bean proteolytic hydrolysis by alcalase and Proteinase S enzymes derived peptides have anticancer, antihypertensive and antioxidative effects (Kannan *et al.,* 2008, Rayaprolu *et al* 2012). Oatmeal, rice bran, cereal products, milk proteins (Wildman, 2007), fruit beverages, and nanomaterials are also good vehicles to deliver bioactive compounds. Preserving the active ingredients, bioavailability assures nutraceutical products' effectiveness in reducing the risk of diseases. Therefore, food manufacturers offer vehicles to deliver physiological health benefits to specific organisms. For example, these bioactive molecules acetylcholine, histamine, cortisone/hydrocortisone, and phenoxyacetic acids have gone through synthetic modifications of their parent compounds to get a specific activity (Kang *et al*., 2013). The nanoparticles were used to improve nuclear targeting efficiency in HepG2. "A 20 nm diameter modified gold particle by shell of bovine serum albumin (BSA) conjugated to various cellular targeting peptides" (Tkachenko *et al*., 2003). The results indicated that "Nanoparticles carrying peptides had a greater propensity for nuclear targeting than any other single peptide explored" (Tkachenko *et al*., 2003). Also, these vehicles can maintain their

active molecular form until consumption (Chen *et al.*, 2006). Since food proteins are safe, have high nutritional values, and have emulsification properties, they are widely used as vehicles for functional foods. For example, milk proteins, such as casein micelles, are ideal vehicles to transport micronutrients such as calcium and phosphate. Also, casein micelles can transport the components of the immune system, such as lactoferrin and immunoglobulin, and building blocks such as amino acids. For instance, a variety of small molecules can be bound by bovine albumin serum and B-lactoglobulin (Livney, 2010). Milk proteins are widely used because they are safe, inexpensive, natural, have high nutritional values, and contain a variety of structural and functionality attributes. Whey protein is a good vehicle to incorporate bioactives. For example, B- lactoglobulin is a whey protein found in cows' milk that can form nanoparticles linking the hydrophobic molecules. (−) Epigallocatechin-3-gallate EGCG is a water soluble catechin found in green tea and has a potential to reduce cancers, also neurodegenerative and cardiovascular disease. However, EGCG is sensitive to oxidation and its degradation can be affected by temperature, pH, and oxygen concentration. Also, EGCG can change the color of green tea to yellow or brown when it is degraded. Therefore, the B- lactogglobulin forms nanoparticles to encapsulate EGCG. This effectively protects against oxidation, and prevents unpleasant flavor and change in color (Shpigelman *et al*., 2010).

Zimet *et al.* (2011) found that nano-vehicles can be used to deliver hydrophobic nutraceuticals such as omega-3 fatty acid into beverages. Omega-3 fatty acids are polyunsaturated fatty acids such as docosahexaenoic acid (DHA), an important nutraceutical lipid which is known to provide protection against cardiovascular and other diseases. Since DHA is sensitive to oxidation, it was encapsulated by casein nanoparticles for a successful delivery (Zimet *et al.*, 2011). Also, milk proteins are capable of interacting with other biopolymers,

stabilizing emulsions, binding hydrophobic molecules, forming gel, and preventing oxidation. Casein micelles can reassemble in vitro, contributing functional properties while preventing oxidation, off flavor, and odor.

In the Garcia-Nebot *et al* (2009) study, caseinophosphopeptides (CPPs) were used as a vehicle for micronutrients such as iron (Fe). The CPPs were derived from casein by enzymatic hydrolysis. CPPs were added to fruit beverages such as grape, orange, and apricot puree. The Garcia-Nebot study concludes that the CPPs served as a good delivery system and contributed to a decrease in Fe deficiency and increased the bioavailability of minerals. Fruit beverages are suitable media for nutrients because of high nutrient solubility and low mineral absorption inhibitors concentrations. Nano vehicles which are part of nanotechnology are an excellent delivery system for bioatives such as peptides and nutrients. Also, fruit juices are a good food system for proteins and peptides.

D. Examples of peptides and proteins incorporated into beverages.

Beverages are ideal vehicles to incorporate nutraceuticals and bioactive ingredients, such as proteins, vitamins, minerals, and dietary fiber (Sharma *et al*., 1997). Sharma *et al*. (1997) formulated protein-fortified-fruit-based-beverages, such as orange juice, by adding whey protein, guar gum, sucrose, calcium lactate, citric acid, natural flavor, and color. Clarisoy is a plant protein isolate product that was developed by the Canadian company Burcon NutraScience. It is highly soluble, has low viscosity, heat-stable, transparent, has no off-flavor or color change, and has high stability in acidic conditions (2.5 - 4.2 pH). Therefore, it is a suitable protein to use in acidic beverages such as orange juice. Incorporation of soy protein isolates in acidic beverages has been demonstrated to have advantages in preventing coronary heart disease, osteoporosis, and some types of cancers. Soy protein isolates are also suitable for vegetarians or those that

have milk or dairy allergies (Segall, 2009). Also, fruit beverages, such as grape, orange, and apricot puree, were fortified with Caseinophosphopeptides (CPPs) to decrease Fe deficiency and increase the bioavailability of minerals.

Fruit beverages can also be good vehicles for peptides. Rice bran peptide fractions are water soluble; therefore, they are easily incorporated into beverages. Rice bran (RB) peptide fractions demonstrated anti-cancer, anti-obesity, and anti-Alzheimer properties (Kannan *et al* 2008, 2009 and 2011). Khairallah (2011) conducted a study in investigating the stability of rice bran peptide fractions incorporated into orange juice at pH 7.2 and 3.5, and 4°C for 42 days. The results indicated higher stability of the fractions at pH 3.5 in orange juice for 42 days than at pH 7.2, and the amount of peptide fractions decreased after 21 days. The color, pH, and vitamin C content of orange juice were stable.

E. Problems associated with incorporation of proteins and peptides into beverages.

A simple way to develop a novel functional food is incorporation of bioactive compounds such as, proteins, peptides, vitamins, and minerals in delivery systems. These bioactive compounds can possess health benefits. However, bioactive compounds' activity and health benefits can be limited due to instability during storage and processing such as pH, light, oxygen. Also, the interactions due to the presence of enzymes and other nutrients can affect the stability of bioactives (Chen *et al*., 2005). In Shimonia's (2004) study, the soy isoflavones shows browning activity due to genistein loss during storage at room temperature for more than two years. The main reasons for the addition of bioactive compounds in food system are to improve nutrition, texture, appearance, and flavor; however, the stability of food products can be affected by the addition of bioactive compounds. The chemical treatments during processing such as alkaline, acidic treatments can have an impact on stability of bioactives. Chemical modifications

(acylation, glycosylation, phosphorylation, reductive alkylation, succinylation, and lipophilization), have an impact on improved functionality. However, these chemical modifications can cause negative effects because of the possibility of some residual chemicals; therefore, food industry practices the chemical modifications with caution (Korhonen *et al.*, 2003). Heat treatment which is the oldest and the most common preservative method is used in the food industry. The bioactivity can be reduced due to the exposure of high temperature (60-90C^{o}), which might denature the protein. For example, whey proteins retained their bioactivity when pasteurized at a standard temperature 72C^{o} for 15 seconds. Furthermore, heat treatment can induce a Millard reaction when lysine residues in proteins interact with reducing sugar in the food matrix. Ultra high pressure is used in food products under isostatic pressure at room temperature, which may cause conformational changes on proteins (Korhonen *et al.,* 2003). The physical and chemical characteristics of the food products can have an impact on nutrition.

Most epidemiological studies suggest increasing the intake of fruits and vegetables to reduce the risk of a number of chronic diseases (Nicoli *et al*.1999). Fruit and vegetables provide multiple phytochemical, fibers, and bioactive compounds, which possess antioxidant activity and can prevent cellular damage *in vitro*. However, people rarely consume fruit and vegetables in their raw state, and they need to be proceeded before consumption due to safety and economic issues. In general preservatives are known to reduce naturally occurring antioxidant compounds in food. The recent approach to improve the antioxidant content in fruits and vegetables is to minimize processing damage, and increase the shelf life. However, the addition of antioxidants does not offer an effect on human health that can be achieved from naturally occurring antioxidants (Nicoli *et al*.1999). Nicoli *et al*. (1999) reported that the antioxidant amounts declined in thermally treated fruits and vegetables, which resulted in lower post-consumption

intake of ascorbic acid and polyphenols in humans. A study conducted by Khairallah (2011) on testing the stability of peptide fractions in orange juice indicated that the peptide fractions were stable for 42 days. Peptide fractions in the orange juice reached zero after 42 days, which implies that the acidic environment of orange juice affect the stability of peptide fractions. In Patras *et al.,* (2011) study, on the stability of strawberry juice during storage, the results indicated that antioxidant capacity of the juice changed when stored for more than 28 days. Lightness value (L) of the juice decreased considerably when stored for more than 28 days and at 15° C. It was also observed that kinetics (k) constantly increased with increase in temperature. Rate of reaction of (k) for anthocyanins changed from $0.95x10^{-2}$ day^{-1} to 1.71×10^{-2} day^{-1} at 4 °C 15 °C. Thus, the overall stability was affected. The stability is the main challenge in incorporating bioactive compounds into beverages. This challenge can potentially decrease the health benefits and physiological functions of bioactive compounds. Food manufacturers have invented new techniques to encounter these challenges, such as encapsulation and Nano-encapsulation techniques.

F. Processes of overcoming the issues associated with incorporation of bioactives.

Encapsulation is defined as the process employed to entrap the substance using a secondary substance. Encapsulation also has been used in the food industry to coat bioactive ingredients creating new food structures with unique functionalities (Sekhon, 2010). Encapsulation composed of two parts: a core or coated material and a shell or coating material (Abhilash, 2010). Bioactive substances are packaged by secondary materials, which are known as encapsulants, to form microcapsulates. These encapsulants, or shells, act as preservative coatings of the functional ingredients to avoid degradation by undesirable chemical reactions, which might influence human health or the flavor, color, and aroma of food products.

Encapsulation promotes the transport of bioactive molecules, such as minerals, antioxidants, vitamins, fatty acids, and phytosterols including molecules like lycopene and lutein. Proteins and polysaccharides also have been used to encapsulate, deliver, and protect lipophilic components such as u-3 rich oils, conjugated linoleic acid (CLA) (Matalanis *et al*., 2011). Encapsulation also transports living cells, such as probiotics, into foods (Smith and Charter, 2010). Also, it increases the stability, as it prolongs shelf life, prevents interactions of flavors with other compounds and off flavor, facilitates handling processes, and reduces vitamin loss in functional ingredients (Gibbs and Kermasha, 1999). Encapsulation can carry the functional ingredient to the appropriate site in the body or organism, maintain the functional materials in their active state during processing and storage by protecting them from chemical and biochemical degradation, and control the release rate under certain conditions such as pH, temperature, and ionic strength (Weiss at al., 2006). Encapsulation is an effective delivery system that can maintain the stability and bioavailability of bioactive compounds into fruit juices.

Several techniques can be applied to encapsulate bioactives, such as spray-dry chilling or cooling, extrusion, and emulsification. Spray drying is widely employed for flavors and dehydration of materials such as powdered milk. It is an economical method and does not require specialized equipment. Spray drying can be designed to the required capacity and the process is very rapid. There is various available spray drying designs for each specification of products (Leak, 1989). Spray drying can be employed with either heat sensitive or heat resistant products. However, the main two disadvantages of spray drying are that the equipment is very bulky and the ancillary equipment is expensive. The overall thermal efficiency is low, as the large volumes of heated air pass through the chamber without contacting a particle, thus not contributing

directly to the drying (Leak, 1989). Spray chilling or spray cooling is another employed method of encapsulation. In spray cooling, vegetable oil is usually used as an outer material, while spray chilling uses fractionated or hydrogenated vegetable oil. Spray cooling requires special handling and storage conditions (Gibbs *et al*., 1999). Extrusion is a method of encapsulation, which is primarily applied to visible flavor pieces, colors and vitamin C (Lebovka *et al*., 2011). Extrusion can provide thinner walls by increasing the pressure with low operation cost. Extrusion has flexibility in the design of the product. The main disadvantages of this method are the low speed of extrusion and low production, while cost of tools is high. The extrusion process has high amount of wastes (Gibbs *et al.*, 1999). However, liposomes, nanoliposomes, and nanoparticles are the new encapsulation techniques in food industry. Liposomes are a superior example of encapsulation. Liposomes and nanoliposmes are made of phospholipid bilayers, and hence they contain both polar and nonpolar regions. “Liposomes are closed, continuous, vesicular structures composed mainly of phospholipid layers” (Mozafari *et al*., 2008). The hydrophobic groups interact with hydrophobic groups that are in other lipid molecules, whereas the hydrophilic groups of the lipids will align the aqueous phase to form the phospholipids or liposomes outer layers. The lipid sheet will be folded into a spherical shape and will form a stable capsule because there are no interactions between water and lipids. Liposomes are mainly used to encapsulate flavor agents, and range from a few nanometers to microns. The liposome encapsulation method is used in the cheese-making application to decrease the ripening time and increase shelf life. Nanoliposomes are liposomes’ nanometric form, and they can act as nanocarriers to deliver bioactives. Egg, soy, and milk economically produce insoluble phospholipids in lecithin. These proteins can be used to form liposomes and nanoliposomes. They contribute greatly to human health (Gibbs *et al*., 1999). Takahashi *et al* (2009) conducted a

study in examining the feasibility of using liposomes to encapsulate *curcuma longa* L. *Curcuma longa* L. The main component in rhizomes, and *Curcuma longa* L has demonstrated anticancer properties and other activities. Liposomes were used to encapsulate *curcuma longa* L to protect and deliver the bioactives. The study indicated that adsorption of *curcuma longa* L in the GI and the antioxidant activity of plasma were enhanced by using liposomes. However, large molecules such as proteins permeate very slowly through the liposome bilayer, while small hydrophilic molecules permeate more quickly. If large molecules are soluble in the lipids that form the outside of liposomes, then they can permeate through a liposome bilayer (Gibbs *et al.*, 1999). Liposome encapsulation requires restricted conditions to maintain their stability. For example, freshly prepared lipids and solvents, averting exposure to oxygen and extreme temperature are required for increasing stability. Using appropriate handling conditions and avoiding charge neutralization by adding metal chelators are also required. The liposomes will aggregate because of van der Waals interactions; therefore, the aggregation can be reduced by the addition of phosphatidic acid (Anal&Singh, 2007).

Recently a new technology has emerged in encapsulating to deliver bioactive such as nanotechnology. Nanotechnology "involves creating and manipulation of organic and inorganic matter at the nanscale" (Luykx, *et al*., 2008). Nanotechnology provides potential benefits for both producers and consumers. It improves flavor, color, and all other properties of food products. Nano-encapsulation defined as "forming particles loaded with ingredients in diameters of 100 nm" (Reis *et al*., 2005). Also Weiss *et al*., (2006) defined Nano-encapsulation as a technique that is used to alter and coat biological and non-biological structures that are less than 100 nm in size. Furthermore, nanoparticles have shown increased shelf life stability. Nano-encapsulation is more efficient in comparison to liposomes and nanoliposomes (Hans and

Lowman, 2002). Nano-encapsulation is involved in several areas of the food sector, such as food processing, safety, and packaging (Garcia *et al*., 2010). Nano-encapsulation is a powerful technology, which can tolerate the properties and functionalities of bioactives, and potentially lead to improving the stability and delivery of bioactive ingredients. Functional ingredients including, vitamins, flavorings, colorings, and antioxidants are varied in their physical and molecular properties. They are never used in their pure forms; therefore, a delivery system is required (Fakruddin *et al.,* 2012). For instance, the most important groups of natural pigments are carotenoids, which contribute approximately 70 % of vitamin A to the human diet. Carotenoids provide protection against cancers and cardiovascular diseases; however, they are insoluble in water which potentially reduces its incorporation in foods. In a study conducted by Yuan *et al*., (2008) 'beta carotenoids were incorporated into micro emulsion prepared by using a series of polyoxythylene sorbitan esters of fatty acids as emulsifiers". The stability of beta carotenoids was investigated during storage for four weeks at 4C^{o}. The results indicated that beta carotenoids were gradually degraded and the loss was only 14% by the end of the study.

In addition to increased stability, the nanostructure has been used in food industry with claims that it provides better texture and consistency such as low fat nanostructure mayonnaise and ice cream. They were produced as healthy alternatives for consumers by providing a creamy texture as full fat products (Chaudhry *et al*., 2008). Nanoparticles are also used to encapsulate broad ranges of proteins such as tetanus toxoid, lysozyme, and insulin. In a study conducted by Bilati *et al* (2005), poly (D, L-lactic acid) and poly (D, L-lactic-co-glycolic acid) were used to form nanoparticles to encapsulate tetanus toxoid, lysozyme, and insulin. The results showed that the encapsulation improved protein loading and stability. In another study gold nanoparticles have been used to encapsulate a pentapeptide (Cys, Ala, Leu, Asp, and Asp) CALNN and it

formed extremely stable nanoparticles in water (Lévy, *et al.*, 2004). Nanotechnology can contribute a high quality system for bioactive ingredients and nutraceuticals such as proteins and peptides.

G. Nano-encapsulation of bioactives.

Nanoparticles have potential in overcoming issues associated with the incorporation of peptides into beverages. Nano-encapsulation has been used in several applications in food industry including manufacture and processing (Luykx, *et al*., 2008). Small particle size makes nanoparticles more effective than other encapsulation approaches such as the use of liposomes. The small particle size, chemical composition, surface structure, and toxicological properties give nanoparticles their unique features (Luykx, *et al.*, 2008). Furthermore, the small particle size provides advantages, such as ease in ingesting particles into the circulatory system. It also has large surface areas, which can provide different physical and chemical effects on food products. Subsequently, various chemical interactions can be prevented (Garcia *et al*., 2010). In the nano delivery system, the nutraceuticals, bioactive peptides, and antioxidants can be absorbed, incorporated, and dispersed. The Nano delivery system can prevent nutraceutical degradation, and can enhance the stability. Thus, the bioavailability and delivery of bioactive peptides and nutraceuticals to specific cells or tissues in the body can be increased.

Sekhon, (2010) defined the nanofoods as “The term ‘nanofood’ describes food that has been cultivated, produced, processed or packaged using nanotechnology techniques or tools, or to which manufactured nanomaterials have been added”. Nano-food products mostly use engineered nanomaterials (ENMs) that include three categories: inorganic, organic, and surface-functionalized nanomaterials (Sekhon, 2010).

In-organic nanomaterials can be used in food additives and food packaging applications. The ENMs in these applications include transition metals such as silver and iron, non-metal such as selenium and silicates, and alkali metals such as calcium and magnesium. Nanosilver is used in several food applications such as functional foods and water. Functional foods and water provide antimicrobial, antioxidant, and supplements. Nanoselenium is also being used in green tea as a food additive and promotes health due to increasing selenium uptake. Nanocalcuim, nanomagnesium salts, and nanoiron are used as health supplements (Sekhon, 2010).

Organic nanomaterials are used in food applications as food additives or health supplements. These materials include benzoic, citric, and ascorbic acids; vitamins A and E; isoflavones; omega-3 fatty acid; lutein; and beta-carotein. These organic nanomaterials can increase the uptake and absorption of bioactive compounds and, hence, enhance bioavailability (Sekhon, 2010). Third, surface functionalized nanomaterials display a specific functionality when they are added to the matrix like antimicrobial and preservative activity. Functionalized ENMs can offer mechanical strength or a barrier against gas and volatile compounds movement and moisture when binding to the matrix. Nanoclay metal is a natural nanomaterial that is organically modified and used in food packaging to enhance gas properties. Furthermore, functionalized nanomaterials can deliver bioactive compounds and nutrients. The natural nano-vehicle casein missiles are used to deliver hydrophobic bioactive compounds. A novel encapsulation to deliver heat-sensitive bioactive compounds such as probiotics is developed. Delivery of hydrophobic nutraceuticals through B-lactoglobulin–pectin nanocomplexes in clear acid beverages is anther innovation of nanotechnology (Sekhon, 2010).

Sekhon (2010) reports that word ‘nano’ comes from the Greek for dwarf, and one nanometer is about 60,000 times smaller than a human hair in diameter or the size of a virus. It is

important to note that nanoparticles are very small, less than 100nm (Sekhon, 2010). Food nanotechnology potentials are unlimited and have a positive impact on science of food such as new food product invention in texture, taste, and storage stability. It is reported by nanotechnology analyst that 150-600 nano-foods are available in the market (Sekhon, 2010). Nanoparticles deliver bioactive ingredients to the desirable site of function, provide protection during processing or from chemical or biochemical degradation, and increase the capability of controlling the release of bioactives in specific environmental conditions, such as pH, ionic strength, etc. (Weiss et al., 2006). The small nanoparticle size enhances adhesive forces, prolongs transit time of bioactives in the gastrointestinal tract and increase bioavailability (Luykx *et al.,* 2008). The small nanoparticle size has different bio-distribution from organ to organ; therefore, it is necessary to control the particle size for each application (Cheng, 2007). The application of nano-encapsulation process has various advantages. It is responsible in making food processing faster and more efficient. Nano-encapsulation improves stability of bioactive compounds, especially during storage and processing to avoid any undesirable effects with the food matrix, and controls the release (Luykx *et al.,* 2008).

Proteins, lipids, polysaccharides, and polymeric networks based are the types of nanoparticles that exist in food applications. Food grade polymers (copolymers) are extensively used in controlled release carriers for protein preparation due to their biodegradability and compatibility, especially those that are derived from poly (lactic acid) and poly (glycolic acid) such as a biodegradable copolymer poly D, L-lactide-co-glycolide acid (PLGA). PLGA is an aliphatic polyester polymer (Luykx *et al.,* 2008). PLGA polymer can be degraded into two biocompatible by-products, lactic and glycolic acids, through hydrolytic cleavage of ester bonds. The copolymer PLGA is excreted from the body as carbon dioxide and water. The PLGA

degradation rate is essential to regulate the release rate of the coated ingredients based on molecular weight and hydrophobicity of PLGA. PLGA biopolymer is rich in glycolic acid and has a high degradation rate. The degradation rate increases as the molecular weight decreases due to the carboxyl groups. The acid-catalyzed degradation accelerates due to the carboxyl groups that are toward the end of PLGA structure. The hydrophobicity of PLGA biopolymer and acid catalysis potentially affect the stability of proteins and peptides (Park *et al*., 2002). PLGA was used to encapsulate haloperidol, which is a drug to treat schizophrenia. It showed great influence on haloperidol, controlling release and stability. To protect the integrity during formulation and delivery, PLGA nanoparticle was used to load and dispense insulin molecule (Kumari *et al.*, 2010). In Ravichandran's *et al.,* (2011) study, PLGA nanoparticles have been used to encapsulate phenolics (benzoic acid) to prevent microbial growth in meat systems. Nano-encapsulated phenolics were effective in preserving the meat for a longer storage period. In Ganea's et al., (2010) study, a lipid soluble benzoquinone-based phytochemical (Thymoquinone) was encapsulated by poly (D,L lactide-co- glycolide) PLGA nanoparticles to protect antioxidant and anticancer activities. In another study, the poly-D, L-lactide (PLA) has been used to encapsulate the antioxidant (quercitrin), and it has the potential to deliver the antioxidant (quercitrin) (Kumari et al., 2011).

Using polymers such PLGA in forming nanoparticles is a great promise in delivering functional ingredients such as pentapeptide because it has unique characteristics in encapsulating, loading, and releasing. The outcome of nanoparticle materials can be controlled because this polymer can be processed with numerous functionalities and properties (Schubert *et al.,* 2010). PLGA polymers have the most favorable degradation properties and have shown potential immense in delivering proteins and peptides. The degradation properties of this

polymer can be tuned by dominating the molecular weight, lactide and glycolide ratio, and concentrations of peptides (Makadia, *et al.,* 2011). Degree of PLGA crystallinity directly influences the biodegradation rate. For instant, when the PLA is co-polymerized with crystalline PGA, the degree of PLGA crystallinity is reduced. Thus, the hydration and hydrolysis rate increase. Makadia, *et al.,* (2011) reported that the ratio of 50:50 PLA/PGA showed the fastest degradation rate. PLGA polymer has been used as an ideal delivery carrier in delivering protein and peptides because of its biodegradability, compatibility, and the ease of it to be elaborated with desired characteristics and functionalities (Singh, *et al.,* 2009).

Other polymers such as polylactic acid PLA polymer were used in several encapsulation applications. A steroid hormone progesterone C-21, which involves in the menstrual cycle and pregnancy, was loaded in PLA–PEG–PLA nanoparticles. It shows that the PLA nanoparticles encapsulated effectively with 70±5%, while PEG increased release in vitro (Kumari *et al.,* 2010). Furthermore, BSA was encapsulated by a tadpole-shaped polymer mono (6-(2-aminoethyl) amino-6- deoxy)-cyclodextrin-PLA (CDen-PLA). It showed 71.6% encapsulation efficiency and high stability after release of BSA from nanoparticles (Kumari *et al.,* 2010). Poly-caprolactone PCL is a polymer that can be degraded under physiological conditions by hydrolysis of its ester linkages. The copolymeric nanospheres containing taxol Polyethylene glycol–PCL are reported as promising anticancer properties owning to their efficiency of loading drug by 20%. Also, copolymeric nanospheres systems such as mPEG/PCL can be a great delivery system ad can also possess anticancer properties (Kumari *et al.,* 2010).

H. Nano-encapsulation of pentapeptide in apple juice.

Peptides provide unique features more than proteins and lipids; thus, they become attractive for therapeutic delivery applications. Most of anticancer agents can lead to systemic

toxicity and adverse effect because they cannot distinguish between cancerous and normal cells; therefore, decreasing the drug dose is required (Sinha et al., 2006). Furthermore, those anticancer agents are not economical and result undesirable toxicity because large quantity are needed for rapid elimination and widespread distribution into targeted organs and tissues. Low specificity of some anticancer drugs leads to harmful effects on healthy cells and tissues (Sinha et al., 2006). Peptides afford high valency due to their small size, which minimizes the radius of the resulting peptides-nanoparticles conjugate. The immunogenicity *in vivo* can be also reduced by the small size of peptides. Peptides can be easily sequenced and synthesized in the laboratory; therefore, they are economical. Peptides can be very specific and bind with high affinity to their cognate receptors, since they are naturally occurring from protein precursors. Furthermore, multifunctional groups of peptides can be incorporated into the nanoparticles (NP_S) to produce a 'value-added' material that serves multiple purposes in one NP **(**Delehanty *et al.*, 2010). Peptides are very attractive and useful molecules for the evolution of NPs.

Biologically active peptides can be derived from dietary protein with several physyological functions. Rice bran (RB) can be a good example of dietary protein that is enzymatically hydrolyzed to release the bioactives. Rice bran has significant amounts of protein consisting of approximately 12-20 %. Pure peptide (pentapeptide) that is derived from heat-stablized deffated rice bran has been demonstrated to have anti-cancer properties. It inhibits colon cancer cells by 84%, breast cancer cells by 80%, liver cancer cells by 84%, and lung cancer cells by 69% (Kannan et al., 2010). Rice bran constitutes 10% of the rough rice grain and is an excellent source of vitamin E and B (Parrado et al., 2008). Rice bran is produced by removing the pericarp and germ from the outer layer of brown rice. Abrasively milling removes the brown outer layer and results in white rice grain (Saunders, 1990). Hemicelluloses and

glucofractuctans are fibers that constitute rice bran in addition to ash, enzymes, vitamins, antioxidants, and protein (Ali *et al*., 2010). According to Herbst and Herbst (2007) rice bran peptides can also lower cholesterol levels.

Kannan *et al* (2008) conducted a study to investgate the antidisease properties such as liver and colon cancer of rice bran peptides. Kannan *et al* (2008) generated bioactive peptides through enzymatic hydrolysis of rice bran. The peptides were fractionated into > 50, 10-50, and <5 KDa sizes and tested for anticancer properties. The results indicate that the bioactive peptides <5 and 5-10 KDa sized fractions supressesd colon cancer cells (Caco-2) growth by 80%. The <5 KDa fractions inhibited liver cancer cell (HepG2) growth by 50%. Bioactive peptides can be readily prepared, however, maintaining bioactivity and stability of peptides are two main challenges facing the food industry. Nano-encapsulation of these bioactives can potentially maintain the stability and prevent degration of bioactive peptides during storage period (Allémann *et al*., 1998).

I. Shelf life stability of Nano-encapsulated pentapeptide in apple juice.

A wide variety of food products are manufactured in a location that is far away from the places where they are consumed. Current manufacturing and distribution practices may delay the consumption of food products for a few weeks or months from production. Hence, food products may undergo a series of chemical reactions which may cause change in desirable flavor, aroma, appearance and texture of food products (Kepplinger *et al.,* 2001). The overall quality of food products will be affected and subsequently the consumer acceptance and purchase of food products will decrease and may cause food product wastage. The deterioration of food products' quality will significantly influence the profits of a company.

Typically, manufactures attempt to enhance the quality of food products by adding preservative agents (Kepplinger *et al.,* 2001). However, the addition of preservative agents may lead to problems including initiation of additional chemical reactions with other components in the food matrix and producing change in food product characteristics (Kepplinger *et al.,* 2001). Consumers demand high quality food products with less chemical additives and hence, manufactures look for alternatives from natural sources to enhance food quality and shelf life.

After a long storage period, all food products including fruit juices are subjected to spoilage, leading to quality degradation. The main target of scientists and manufacturers is to produce and deliver natural, safe, and healthy products with satisfactory shelf life. Physical, chemical, enzymatic, and microbial reactions can all cause spoilage (Could, 1996). However, all types of food spoilage can be prevented by preservative techniques, such as encapsulation, which can be applied to provide high quality food products. Various other preservative techniques that can be applied to food products to inhibit microbial growth are chilling, freezing, drying, curing, and vacuuming. Microbial growth can also be inactivated by irradiation and pasteurization and can be restricted by packaging and aseptic processes. Also, multiple techniques have been developed, such as ultrahigh pressure, bacteriolytic enzymes, electroporation, and nano-thermosonication to prevent spoilage (Could, 1996).

Fruits and vegetables products can undergo physical degradation during storage since they do not receive heat treatment. Physical damage can trigger chemical or microbiological spoilage, which can be enhanced in opened containers and dented cans. Various types of oxidation can result in chemical degradation. Chemical degradation can affect the color of products, such as forming yellow and brown pigments, haze, and sediments (Spanos *et al.,* 1990). Siebert *et al* (1996) conducted a study proposing that turbidmetric methods used to

measure haze-active proteins and polyphenols in beverages also affect the stabilization procedures, so proteins that lack proline were found to have little or no haze when polyphenols were added. In fruit juices, polysaccharides increase haze. Beverages such as orange, grapefruit and apple juice have higher proline, which is an active polyphenol. an active polyphenol is highest in fruit juices, which may cause beverage destabilization. Proline is an important component of proteins that bind to polyphenols. Incidentally, free proline competes with haze-active protein in binding to polyphenols. Free amino acid-polyphenol complexes are smaller and are capable of being more soluble than protein-polyphenol complexes (Siebert *et al*., 1996). Haze-forming polyphenols contain two binding groups. Each has two hydroxy groups on an aromatic ring. The ratio of protein and polyphenol strongly influences the amount of haze formed. When there is a large amount of proteins, the numbers of polyphenol binding ends and protein binding sites become almost equal; thus, it is important to stabilize beverages to delay haze formation (Siebert, 1999). Normally, these beverages are stabilized to delay the onset of protein-polyphenol haze formation. As a result, the shelf life of food products can be limited when certain compounds are formed or flavor and nutrients are lost. It is essential to use a pasteurization technique to prolong the shelf life of fruit beverages and prevent the formation of off-flavor, odor, and loss of nutritional value.

Lactic acid bacterium and *Lactobacillus* spp. and *Leuconostoc* spp. causes spoilage and undesirable flavor and odor in some fruit juice. *Lactobacillus* spp and *Leuconostoc* spp cannot flourish in such high sugar concentrations or at low temperature (for example,45% sucrose and 5°C), even though the pH range of apple juice is between 3.0 and 4.5 which is an ideal range for *Lactobacillus* spp. and *Leuconostoc* spp to survive. Additionally, apple juice can become spoiled by *E. coli* O157:H7 bacteria, mold, and yeast, if it is held at improper temperatures. Also, apple

juice can become spoiled by yeast even if it is chilled. Therefore, pasteurization of apple juice is important to prevent microbiological degradation, although pasteurization processes may discolor apple juice (Evrendilek *et al.*, 2000). Nano-encapsulation provides protection of proteins and peptide under extreme temperatures and pH (Yin *et al.*, 2004).

Nano-encapsulation can prolong the shelf life of food products and significantly enhance proteins and peptide performance. Furthermore, the natural deterioration of products can be prevented since the encapsulated products can withstand harsh environmental conditions such as 30 to 60ºC and pH 1 to 12 (Yin *et al.*, 2004). Furthermore, Nano-encapsulated proteins and peptides display great shelf life stability and long blood stream' circulation time in vivo, which provide a control manner release and site specific delivery of proteins and peptides (Yin *et al.*, 2004). Nano-encapsulation of bioactives such as proteins and peptides can enhance the delivery of bioactives, increase shelf life stability, and prevent deterioration of food products during storage or as a result of the effect of extreme temperatures and pH.

Table 1. Average nutrient content in apple juice (per 100 g)

Composition	Apple juice(g)
Water (g)	88.1
Energy (kcal/kg)	48/203
Protein (g)	0.07
Carbohydrates (g)	11.1
Fiber (g)	0.77
Pectin (g)	0.032
Potassium (g)	116
Calcium (g)	4.2
Magnesium (g)	6.9
Phosphorus (g)	7.0
Organic fruit acids	0.74

(Source: Gerhauser, 2008).

Table 2. Polyphenol content of apple juice

Phenolics	Apple juice (concentrate) per100g
Total polyphenols	110 – 173
Hydroxycinnamic acids	69 – 122
Dihydrochalcones	9 – 54
Flavan-3-ols: Mono- and dimers	14 – 32
Flavonols (quercetin-glycosides)	4 – 7

(Source: Gerhauser, 2008).

CHAPTER 3

Preparation of nanoparticle, and investigation of the physical attributes and stability pentapeptide in apple juice.

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INTRODUCTION

Nanotechnology is a technique that provides potential benefits for both producers and consumers by the delivery of bioactive, antimicrobials, and improving flavor and color of food products (Onwulata, 2012; Zemit *et al.,* 2011; Mukha *et al.,* 2013). Nano-encapsulation is defined as “forming particles loaded with ingredients in diameters of 100 nm” (Reis *et al*., 2005). Nanoparticles are an ideal encapsulation approach for functional foods and recently has been used in several food applications including manufacturing and processing (Luykx, *et al*., 2008). Nanoparticles can increase the stability and controlled release of bioactives due to their small particle size in comparison to particles in micrometer sizes (Hans and Lowman, 2002). Food grade polymers (copolymers) are extensively used as controlled release carriers for protein preparation because of their biodegradability and compatibility; especially Poly lactic glycolic acid (PLGA), which is a copolymer of polylactic acid (PLA) and polyglycolic acid (PGA) (Hans and Lowman, 2002). Poly lactic glycolic acid (PLGA) is the most common food grade polymer used in the preparation of nanoparticles. It dissolves at low concentrations in organic solvents such as dimethyl sulfoxide (DMSO) and is insoluble in water.

A nono-precipitation method used in this study can be defined as a simple method of preparation and fabrication of polymeric nanoparticles such as PLGA. The formation of nanoparticles by nono-precipitation does not require any sophisticated equipment. It is a sensitive procedure with low energy cost and modest equipment in comparison with other

methods such as emulsion/solvent diffusion, and a variety of solvents can be used such as DMSO. Furthermore, this method aids in preparing nanodispersion in one step, decreasing energy input, and increasing encapsulation yield (Boon-Seang Chue *et al*., 2007). The nanoprecipitation technique is the simplest method used to prepare nanoparticles, which involves two phases: the aqueous and organic phase. Since the polymer (PLGA) is insoluble in water, nanoprecipitation occurs as soon as the PLGA solution comes in contact with the dispersing phase. The emulsifier in the aqueous phase can stabilize and prevent aggregation of nanoparticles.

Particle size is the most important characteristic, which can determine the success of encapsulating, loading, and releasing the pentapeptide. Peptides' loading and release, and nanoparticles' stability can be affected by particle size. Singh *et al.,* (2009) reported that "The small size of nanoparticles allows for efficient uptake by a variety of cells type and selective drug accumulation at target sites". A laser particle size analyzer is an instrument that is widely used for particle size analysis. A laser particle size analyzer is a useful instrument to measure the particle size of nanoparticles since it includes a laser device. This instrument is sophisticated but used friendly and has the capability to analyze a broad range of particles in a variety of dispersion media. It requires only three minutes for the measurement and analysis of particle size (A guidebook to particle size analysis, 2012). The particles first will pass through a laser beam, and the light that scattered will be collected in the forward direction over a range of angle. To yield the distribution of particle size, the scattered intensity distribution is analyzed by computer. The particles size is important to be measured because it can potentially affect the stability of nanoparticles (A guidebook to particle size analysis, 2012).

The HPLC reversed phase chromatography (RP) has been used to test the stability of bioactives, and it has been widely used in the area of biochemical separation and purification. For instance, proteins and peptides possess some degree of hydrophobic character; therefore, they can be separated by reversed phase column chromatography with excellent recovery and resolution properties. According to Amersham Biosciences (1999) the mechanism of reverse phase HPLC involves binding hydrophobic molecules in the solute during the mobile phase and the hydrophobic ligand in the stationary phase.

Responding to the consumer demand for high quality attributes is a crucial step in maintaining the product's success in the market. It was found in several studies that consumers are first attracted by the appearance of food products before the health benefits of products (Tuorila and Cardello, 2002; Urala and Lahteenmaki, 2007). Therefore, conducting an analysis of physical attributes is crucial to assess the quality of apple juice after the addition of pentapeptide and nanoparticles. PH and color, in apple juice are important parameters that should be monitored during storage with and without nanoparticles and could affect the physical attributes of apple juice.

Experiments were conducted to investigate the stability of Nano-encapsulated pentapeptide incorporated apple juice for 6 month at 4° C.

MATERIALS

The following materials were used to prepare nanoparticles. Apple juice concentrate was purchased from a local grocery store. Poly-lactic-glycolic acid (PLGA) was purchased from Boehringer Ingelheim Chemicals, Inc. (Ingelheim, Germany). Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ). Polyvinyl alcohol (PVA) was purchased from Kuraray (NewYork, NY).

To investigate the shelf life stability of Nano-encapsulated pentapeptide incorporated in apple juice, the following instruments were used: particle size analyzer Model BI-9000ATDigital Correlator, Brookhaven Instruments Corporation (Holtsville, NY) was used at Dr. Surendra Singh's laboratory Department of Physics University of Arkansas, Fayetteville, AR. Scan electron microscopy FEI NOVA Nanolab Department of Nanotechnology University of Arkansas, Fayetteville, AR was used for scanning the nanoparticles. A biopore C-18 preparative high–performance liquid chromatography HPLC column (Biopore Prep ID 22 XL 250 nm part # 34955) was used to analyze the nanoparticles and pentapeptide. The chemicals for HPLC were purchased from Sigma (St.Louis, MO). Ultra-centrifuge Model J2-21 from Beckman Inc.(Brea, CA) was used to separate the nanoparticles at Dr. Denise Greathouse's laboratory Department of Chemistry University of Arkansas, Fayetteville, AR. The ultracentrifuge tubes to separate the nanoparticles were purchased from Beckman Coulter (Brea, CA).

To evaluate the physiochemical properties of nanoparticles and Nano-encapsulated pentapeptide in apple juice, the following instruments were used: pH meter from Orion Research Inc. (Boston, USA), refractometer from Atago Inc. (Osaka, Japan), Chroma- meter from Minolta Inc. (Osaka, Japan), Tryptic Soy Agar for total plate count (TPC) and Potato Dextrose Agar (PDA) for mold and yeast were procured from Becton Dickinson and company (Spark, MD).

METHODS

Preparation of nanoparticle using PLGA polymer and incorporation in apple juice.

The procedure developed in Dr. Hettiarachchy's lab was followed to prepare the nanoparticles. Nano-precipitation method consisted of two phases: organic and aqueous phase. Initially, the organic phase of nanoparticle were prepared by slowly adding 0.75g PLGA 503 H monomer to 15.0 ml DMSO while stirring using magnetic stirrer until all the contents were

dissolved. Then, the aqueous phase was prepared by adding 0.05g polyvinyl alcohol (PVA) to 10 ml of de-ionized (DI) water pH (3.7) while stirring by magnetic stirrer until all contents were dissolved. While the organic phase was stirring, 3.0 ml were taken by syringe (3cc, 23GI syringe and precision glide needle) from the organic phase and slowly injected into the aqueous phase to form nanoparticles. The nanoparticles were formed in contact with aqueous phase due to the immiscibility of organic phase consisting of pentapeptide and PLGA in water. The particle size of nanoparticles was measured by a laser particle size analyzer.

The nano-precipitation method was used as described above to encapsulate the pentapeptide. The organic phase of the nanoparticles was prepared by slowly adding 0.75g PLGA 503 H monomer to 15.0 ml DMSO while stirring until all the contents were dissolved. Pentapeptide at concentrations (200/400/ 600µg/ml) were added to the organic phase while stirring. These three concentrations were calculated based on an inhibitory concentration of cancer cell lines by 50% (IC 50) from a study conducted by Kannan *et al* (2009). The aqueous phase was prepared by adding 0.05g polyvinyl alcohol (PVA) to 10 ml of apple juice, which was prepared by mixing one part apple juice concentrate and three parts water to achieve the total soluble solids of 11 ºBrix (determines the consistency and solubility of the food product) and stirred until all contents were dissolved. Three milliliters of the organic phase were taken using a syringe and were slowly injected into the aqueous phase to form nanoparticles. Three controls of apple juice were also prepared: 1. 10.0 ml apple juice, 2. 10.0 ml of apple juice and nanoparticles, 3. 10.0 ml water with nanoparticles only. The samples were pasteurized at 71°C for 15 seconds as per FDA recommendation (Korhonen *et al.*, 1998), and stored at 4 °C. The particle size and the stability of pentapeptide were tested before and after pasteurization using a

particle size analyzer. The products were stored at 4 °C for the duration of the study and evaluated at periodic intervals from 0, 2, 7, 14, 30 days, and monthly up to 6 months.

Preparation of a standard curve to determine pentapeptide concentrations.

A series of dilution of pentapeptide from 200, 400, 600, 1000, 1200, and 1600 µg/ml was prepared. The pH was adjusted to 3.7 to mimic the pH of apple juice. High performance liquid chromatography with C18 column was used. The solvents used were 0.1 %TFA in and acetonitrile 50: 50 and 0.1 %TFA, at a flow rate 1ml/min with 10µl injection volume for 45min at 215 nm.

Stability of Nano-encapsulated pentapeptide in pasteurized apple juice by HPLC.

HPLC method (Kannan *et al.,* 2009) was followed to investigate the stability of the pentapeptide. The samples of pentapeptide in water, pentapeptide in apple juice, Nano-encapsulated pentapeptide in DI water at pH 3.7 and in apple juice with 200,400, and 600µg/ml and apple juice were analyzed by HPLC (RP) C18 column. The samples of Nano-encapsulated pentapeptide in water and apple juice with three different concentrations of pentapeptide (200/400/600µg/ml), the controls of nanoparticles in water were ultra-centrifuged at 60,000 rpm (120,000x g) for 30 min and 4 ºC to separate the nanoparticles from the solution. The supernatants were collected and filtered through 20µm filter before sequencing to the HPLC analysis. The solvents used were 0.1 %TFA in acetonitrile 50: 50 and 0.1 %TFA in water, flow rate 1.00 ml/min, wavelength 215nm.

Statistical analysis.

The statistical analysis was conducted using the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC) and the stability of the Nano-encapsulated pentapeptide was studied using the 3 factor factorial completely randomized design.

Measurement of the particle size of Nano-encapsulated pentapeptide in pasteurized apple juice.

The particle size was measured after pasteurization to determine the effect of pasteurization on the nanoparticles stability. The mean particle diameter of nanoparticles prepared for delivering bioactives was measured by a laser particle size analyzer. In a culture tube, 50.0 µl of the Nano-encapsulated pentapeptide incorporated into apple juice with three concentrations of pentapeptide (200/400/600µg/ml)) was added to 10.0 ml of DI water separately. The solution was taken from the culture tube and added to the sample holder that was housed in the instrument. The sample holder was placed in the chamber of the laser particle size analyzer and the lid was closed. While testing the samples, the first window of the detection kept at 400 µm. The second window adjusted to 633 nm, and the time of running the samples was adjusted to 2 minutes. The resulting measurements of particle size were recorded.

Scan electron microscopy (SEM) in Nano-encapsulated pentapeptide in pasteurized apple juice.

SEM was used to image nanoparticles because it provides morphological and chemical information which is important in determining the stability of nanoparticles (Utsunomiy and Ewing, 2003, Musumeci et al., 2006, and Hafner, 2007). Vacuum conditions and electrons are used in SEM to form a magnified image. The sample of Nano-encapsulated pentapeptide in apple juice with 400µg/ml was dried by placing a drop of the sample on a double-sided tape attached on a metallic sample stand for 48 h at room temperature before the analysis. The double-sided tape attached on a metallic sample stand coated under argon atmosphere with a thin layer of gold. The main components to form an image in SEM are shown in Figure 1; the microscope column, specimen camber, and the vacuum system. The black box of the SEM

consists of an electron gun, which was the source of the electron beam, a series of lenses including condenser and objective, which was used to control the diameter of the beam and focus the beam on the specimen. Specimen position controllers were used to expose an area of beam/specimen interaction, which produced the image of nanoparticles by generating several detectable signals.

Testing physical properties of pasteurized apple juice with nanoparticles (contain pentapeptide).

The samples of pentapeptide in water, pentapeptide in apple juice, apple juice, Nano-encapsulated pentapeptide in DI water (pH 3.7) and in apple juice at 200/400/ 600µg/ml, were tested for color and microbiological attributes. All the measurements were conducted for triplicate samples. The procedures of the physical properties are as follow.

a. Color

A Chroma-meter was used to measure the color using white tile to calibrate the instrument. The apple juice samples were measured in triplicate. The color values *L*, *a*, and *b* were recorded. The *L* is a measure of brightness or whiteness, which ranges from 0 to 100 (If L =100, the sample will be white, and if L=0, the sample will be black). The parameter *a* is a measure of redness, which ranges from – *a* to + *a* (green= - *a* and red= + *a*). *b* is a parameter to measure the yellowness, which ranges from – *b* to + *b (*blue= - *b* and yellow= + *b).* The differences of color were calculated by following equations:

$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$

$Chroma = (a^{\wedge 2} + b^{\wedge 2})^{1/2}$

$Hue = \arctan(a/b)$

b. Enumeration of microbial survivors

The microbiology test was conducted to determine the effect of pasteurization (Evrendilek et al., 2000). Microbial inactivation and growth were determined during the storage period using the total plate count (TPC), and plate count for mold and yeast. Trypticase soy agar pour plate (TSA) to determine the total plate count (TPC). Sterile peptone water (0.1 % w/v) was used to dilute the samples, which were plated total plate count agar. Potato dextrose agar (PDA) was used for yeast and mold counts. The colonies were counted after incubating TSA plates at 30 °C for 48 hours, and at 22 °C for 5 days for PDA plates (Evrendilek et al., 2000).

c. (Brix) Total soluble solid

Total soluble solids (TSS) is an important parameter that defined the consistency and solubility of apple juice. A refractometer was used to measure total soluble solids of apple juice at ambient temperature. The recommended TSS for apple juice is 11.00 ºBrix.

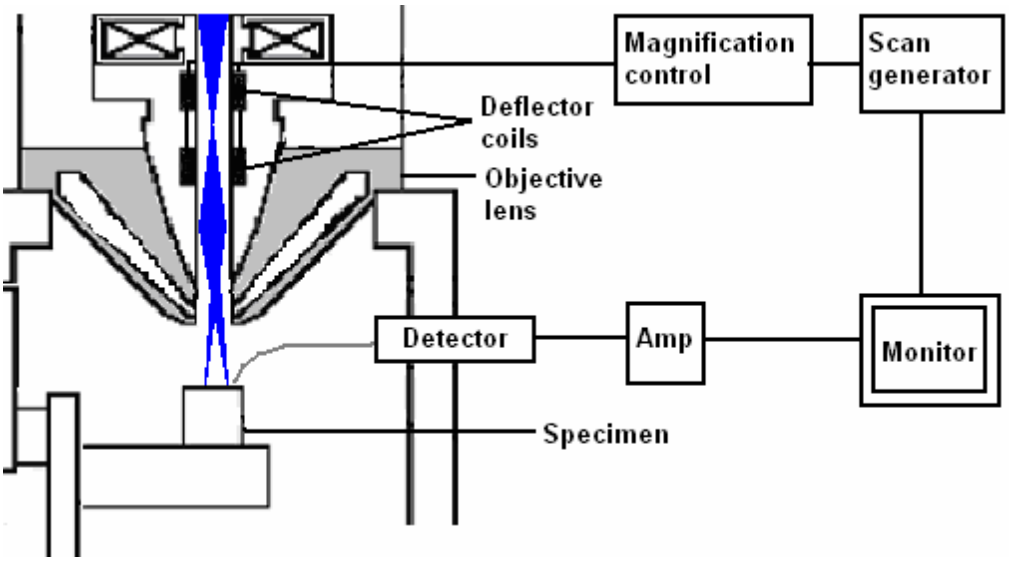

Figure 1: The main elements of Scan Elements Microscopy.

(Source: Hafner, 2007)

RESULTS AND DISCUSSION

Stability of pentapeptide incorporated nanoparticles in pasteurized apple juice by HPLC.

The pH of DI water (pH 6.9) was adjusted to a 3.7 to match that of apple juice for comparison of the stability of Nano-encapsulated pentapeptide over the storage period. The percentage degradation of pentapeptide at three different concentrations (in water and apple juice) over the storage period was calculated using the equation derived from the standard curve plot (R^2 = 0.99) (Figure 2). All the samples were conducted for duplicates. Figure 2 shows the standard curve with the following equation

Concentration (µg/ml) = (area+ 47.421)/ 1.539

Pure apple juice (reconstituted from concentrate) was spiked on the HPLC column to show the absence of any peptides that have the same retention time as the pentapeptide. The apple juice containing known concentrations of pentapeptide (200/400/600µg/ml) was spiked to confirm the retention time of the pentapeptide which was between 11 and 13 minutes from the start of the run. Figures 3 to 5 (a - f) are the HPLC profiles of the non-encapsulated pentapeptide in water (pH of 3.7) showing degradation over time. The HPLC analysis showed significant degradation (P value <0.001) of non-encapsulated pentapeptide (at three different concentrations) in water from the 0 day: (200ug/mL – 87.8%, 400ug/mL – 97.4%, 600ug/mL – 91.9%) to the 60th day: (200ug/mL – 41.8%, 400ug/mL – 60.8%, 600ug/mL – 55.8%) of storage. This could be due to the low pH (3.7) of the solution and possible interactions between the pentapeptide and water molecules which are confirmed by similar previous studies (Toll *e t al*., 2005).

Figure 6 (a-f) shows the HPLC profile of apple juice alone, which is composed of organic acids including quinic acid, citric acid, galacturonic acid, and malic acid, and amino acids

including aspartic, asparagine and glutamic acids (Timberlake, 1957). Previous studies have shown similar retention times for organic / amino acids as in Figure 6 (Nour *et al.,* 2010). The HPLC profiles Figures 7 to 9 (a - f) show the non-encapsulated pentapeptide at three different concentrations in apple juice with significant degradation over storage period 0 day: (200ug/mL – 96.6%, 400ug/mL – 98.1%, 600ug/mL – 94.5%) to the 60^{th} day: (200ug/mL – 60.6%, 400ug/mL – 67.5%, 600ug/mL – 59.1%). The effect of pH and the possible interactions with the components of apple juice (organic and amino acids) are the possible reasons for the degradation of the pentapeptide which is shown in Figures 10 and 11. It was observed that the pentapeptide degraded over the storage period of 60 days in both water and apple juice at pH of 3.7 and temperature 4ºC. The results imply that the degradation of pentapeptide in apple juice over the storage period decreased in a way similar to the degradation of pentapeptide in water. However, the IC50 concentration (400µg/ml) shows better stability in apple juice than the lowest and highest concentrations of pentapeptide (200/600µg/ml) respectively. This might be due to the acidic environment of apple juice, concentration of pentapeptide, and interactions between bioactive pentapeptide and apple juice matrix.

In present study the nanoparticles was used to encapsulate the bioactive pentapeptide in apple juice. This study also aimed to evaluate the enhancement of shelf life and delivery of pentapeptide in apple juice over a period of 60 days. Figures 12-19 (a-f) show the HPLC profiles of Nano-encapsulated pentapeptide incorporated in water at pH of 3.7 and apple juice are stable over the storage period of 60 days. The absence of pentapeptide peak implies that the pentapeptide remained encapsulated within the PLGA nanoparticles during the storage period. In this case, Figure 20 shows an illustration of nanoparticles and pentapeptide attached through electrostatic interaction. The electrostatic interactions occur when the nanoparticles that have the

negatively/positively charged surface attach to the peptide which has the opposite charge. In a study conducted by Khairallah (2011), peptide fractions derived from rice bran were incorporated in orange juice to test the storage stability. The peptide fractions' were found to degrade significantly during the 0 to 42 day storage period. The researcher indicated that the degradation might have occurred due to interactions between peptide fractions and the orange juice components. Therefore, encapsulating the pentapeptide can considerably reduce the interactions and potentially lead to prolonged stability.

Particle size of Nano-encapsulated pentapeptide in pasteurized apple juice.

Particle size is the most important characteristic that should be monitored for successful encapsulation, loading, and release of pentapeptide. This can also affect nanoparticle stability and the delivery of bioactives. Particle size of pasteurized Nano-encapsulated pentapeptide in water (pH of 3.7) at varying concentrations of pentapeptide (200/400/600µg/ml) was measured from day 0 to day 60 using particle size analyzer. Figure 21 shows the particle size of the nanoparticles which ranged from 82 to 83nm and remained stable during the storage period with (P value <0.05%). The uniformity of the particle diameter indicated that the prepared nanoparticles were of even size and the pasteurization had no deleterious effect. Particle size for Nano-encapsulated pentapeptide in apple juice with varying concentrations of pentapeptide (200/400/600µg/ml) was measured from 0 to 60 days, and the diameter of particle size ranged from 82 to 83nm. The results from the particle size analysis showed successful stability of pentapeptide since they had large surface area to the volume ratio. Similar observations were recorded on Ravichandran *et al* (2011) and Ganea *et al.,* (2010) studies.

Scan electron microscopy (SEM) of Nano-encapsulated pentapeptide in pasteurized apple juice

SEM was used to determine the form and stability of nanoparticles during the storage period. Images of the Nano-encapsulated pentapeptide (400µg/ml) in apple juice were taken at 0, 2, 7, 14, 30, and 60 days of storage period using the SEM. Figure 22 shows the SEM images of the Nano-encapsulated pentapeptide in apple juice at IC50 (400µg/ml) from 0 day to 60 day storage. The nanoparticles showed spheroidal shape, which was stable over the storage period. Thus, it can be concluded that the Nano-encapsulation provided protection from degradation to the pentapeptide in the apple juice environment. Similar images were observed on Bilati *et al* (2005).and Ganea *et al.,* (2010) studies.

Physical properties of Nano-encapsulated pentapeptide in pasteurized apple juice

a. Color

Color is an important parameter which can provide an image for the consumer about the quality and acceptance of a food product. The analysis of the apple juice color was based on the lightness (L), redness (a*), and yellowness (b*). Chroma (Cr) is a significant parameter of color which indicates the intensity and relates to consumer appeal. Hue of a food product indicates the actual color of the material and contributes to the overall color expectation from the product. Total color difference, ΔE, is the magnitude of overall color difference compared between the apple juice alone (control) and the Nano-encapsulated pentapeptide in apple juice (Lee and Coates, 1999). Figures 23, 24 and 25 show Chroma, Hue, and ΔE respectively for the two beverage samples over the storage period of 0 to 60 days.

Chroma of apple juice alone did not show any significant changes during the storage period. For the apple juice incorporated with varying concentrations of pentapeptide

(200/400/600µg/ml) Cr measurements over the storage period (0-60 days) ranged from 0.5 to 0.3. The results indicated that there was no significant change in the Cr values among the three concentrations of pentapeptide in apple juice (controls). The Cr of Nano-encapsulated pentapeptide in apple juice during the storage period ranged from 3.2 to 2.8, which indicated no significant change of the Cr value during storage period of 60 days. The pasteurization of the nanoparticles did not affect the Cr during storage. However, there was significant difference between the Chroma of non-encapsulated pentapeptide in apple juice and the Nano-encapsulated pentapeptide and confirmed with (p- value 0.001).

The Hue angle of the apple juice alone was stable over the storage period of 60 days, and it was intensely colored (Yellow). The Hue angle of apple juice containing varying concentrations of pentapeptide (200/400/600µg/ml) ranged from -0.1 to 0.1. There is no difference in the Hue among the three concentrations of pentapeptide incorporated apple juice. In a previous study investigating the stability of peptide fractions in orange juice, the changes in the color of orange juice control samples and the orange juice with rice bran peptide fractions was similar over the storage time of the study (Khairallah, 2011). These results are consistent with our findings. The results indicated that the Hue of the Nano-encapsulated pentapeptide in apple juice was stable over the storage period which showed Light yellow color. The negative values for Hue angles might be due to the negative values of a* since the Hue angle is a function of a* and b* values.

Total color difference, ΔE, during the storage period of 60 days between the apple juice and the Nano-encapsulated pentapeptide in apple juice ranged from 15.2 to 14.2. The results from the color studies indicated significant differences between the apple juice alone (control) and apple juice with the Nano-encapsulated pentapeptide. The difference in color remained

consistent during the storage period, days 0 to 60 which is attributed to the addition of nanoparticles which were white in colors. These changes in color are significant between the treatments (apple juice and Nano-encapsulated pentapeptide); while there was no significant changes over the storage time. The pasteurization temperature of 71ºC for 30 seconds did not affect the color during the storage period. Pasteurization and low storage temperature of 4ºC were effective in preventing significant changes or deterioration of color during storage which is consistent with published research (Burdurlu *et al*., 2003).

b. Enumeration of microbial survivors

The microbial growth was determined during the storage period using Tryptic Soy Agar (TSA) pour plates used for the total plate count (TPC). The Potato Dextrose Agar (PDA) was used to count the yeast and mold colonies. The TPC and PDA agar plates were prepared to enumerate the microbial colonies during the entire shelf life of the control samples, pentapeptide incorporated in water and apple juice, and Nano-encapsulated pentapeptide in apple juice. No microbial growth was observed on both TSA and PDA plates during the storage period of 60 days, which indicated that the pasteurization of apple juice at 71ºC was effective in inhibiting microbial growth. Furthermore, apple juice is rich in phytochemicals, which can be defined as non-nutrient plant components including polyphenols, flavonoids, hydroxycinnamic acids, dihydrochalcones, flavonols (quercetin glycosides), catechins and oligomeric procyanidins which are known for antioxidant activities (Gerhauser, 2008). These phytochemicals have also shown significant antimicrobial activity against pathogenic microorganisms including E.coli, L.monocytogenes, and S. aureus that cause diseases in human (Alberto *et al*., 2008).

c. Acidity (pH)

The pH of apple juice is an important factor that can determine the stability of pentapeptide. The pH of the Nano-encapsulated pentapeptide in apple juice samples and the controls from zero to 30 days did not show any significant change. The results indicated that the pH remained stable for the samples at 3.7, which is within the range of apple juice pH (3.00 to 4.00) that prevented any degradation of the nutritional components. After 60 days the pH of the (controls) pentapeptide in apple juice with varying concentrations (200/400/600µg/ml) dropped to 3.3, which was not significant, since it is still in acceptable range pH 3.0- 4.0. This change in pH might be due to the glutamic acid in the pentapeptide, which is negatively charged that maintained the pH in the acidic range (Khairallah, 2011). In the previous study on the stability of peptide fractions in orange juice at pH 3.5 the peptide fractions showed stability throughout the study in comparison to peptide fractions in orange juice stored at pH 7.2 (Khairallah, 2011). The addition of pentapeptide did not affect the pH of apple juice since there was no significant difference between apple juice and pentapeptide incorporated apple juice. Similar observations were recorded by previous researchers on the stability of proteins and protein hydrolysates in acidic beverages (Khairallah 2011 and Kazmerski *et al.*, 2003).

d. Total soluble solid (Brix)

Total soluble solid (TSS) is a significant factor to determine the consistency of color, clarity and solubility of apple juice. The TSS of apple juice was adjusted to 11 ºBrix which is consistent with that of commercially available apple juice. The total soluble solids of the control samples, apple juice alone and pentapeptide incorporated apple juice, at 0, 2, 7, 14, 30, and 60 days were 11 and 11.3 ºbrix respectively. This implies that the incorporation of pentapeptide in the apple juice did not significantly affect the TSS over time. The consistency of apple juice

(control) was not visibly different from the pentapeptide incorporated apple juice with three various concentrations. The TSS of Nano-encapsulated pentapeptide in apple juice at 0, 2,7,14, 30 and 60 days was 31 ºBrix. Hence, there was a significant difference in TSS between the control samples and the Nano- encapsulated pentapeptide in apple juice which was due to the addition of PLGA nanoparticles, which requires addition of PLGA (0.75g) to (10 ml) of apple juice. However, the consistency of Nano-encapsulated apple juice is not visibly different from some of the commercially apple juice (manufacture name: nudie and Pip organic).

Conclusion

Encapsulation technique used in the food industry has increased because it provides protection for the encapsulated materials from moisture, heat, and other extreme conditions in food environment. Hence, the encapsulation can potentially enhance the stability and maintain viability of bioactive compounds (Gibbs, *et al.,* 1999). Encapsulation is defined as an incorporation of bioactive compounds in a small capsule. In general, encapsulation technique involved three steps: (1) forming the wall around the bioactive compounds (2) preventing the leakage of the bioactive (3) removing undesirable materials out of the capsule (Gibbs, *et al.,* 1999). The most common copolymer are used in the food industry is PLGA due to its biodegradability and compatibility. PLGA has been extensively used to deliver proteins and peptide and it is approved by FDA (Makadia *et al*, 2011). PLGA can be degraded in vivo enzymatically or non-enzymatically producing tow toxicologically safe monomers (Makadia *et al*, 2011).

Nano-precipitation method was used to prepare Nano-encapsulated pentapeptide since it is a simple method of preparation and fabrication of polymeric nanoparticles such as PLGA (Schubert *et al*., 2011). The formation of nanoparticles by nano-precipitation does not require

any external input such as high shearing, sonication, or homogenization (Schubert *et al*., 2011). It is a sensitive procedure with low energy cost and modest equipment, and a variety of solvents can be used such as DMSO or acetone. Furthermore, this method aids in preparing nanodispersion in one step, decreasing energy input, and increasing encapsulation yield (Schubert *et al*., 2011).

Recently, role of proteins in a diet as physiologically active compounds have arisen (Korhonen and Pihlanto, 2003)). Proteins are the main source of bioactive compounds such as active peptides. Peptides provide several unique features in comparison to proteins and lipids; thus, they are more efficient for therapeutic delivery applications. They exhibit various activities such as anti-cancer, anti-hypertension, and antioxidant (Korhonen and Pihlanto, 2003). Peptides can be easily sequenced and synthesized in the laboratory; therefore, they are economical. Peptides can be very specific and bind with high affinity to their cognate receptors, since they are naturally occurring from protein precursors (Delehanty *et al*., 2010). Active peptides derived from cereal grains have potentially impacted on cancer prevention and treatment. For example, bioactive pentapeptide derived from rice bran has shown to inhibit colon cell proliferation (Kannan *et al.*, 2008). Multifunctional groups of peptides can be incorporated into the nanoparticles (NP_S) to produce a 'value-added' material that serves multiple purposes in one NP (Delehanty *et al*., 2010). Peptides are very attractive and useful molecules for the evolution of NPs. however, maintaining biactivity and stability of peptides are two main challenges facing the food industry. Nano-encapsulation of these bioactives can potentially maintain the stability and prevent degration of bioactive peptides during storage period (Allémann *et al*., 1998). The demand on nanocomposution has increased based on global market report. In 2010, the consumption of nanocompsution was over $ 800 milion, increased to $ 920 milion in 2011, and it

is expected to reach $ 2.4 milion by 2016 (BCC research). Nano-encapsulation of anticancer pentapeptide will have a great impact on improving public health and wellness since cancer one of the most leading causes of death in the U.S.

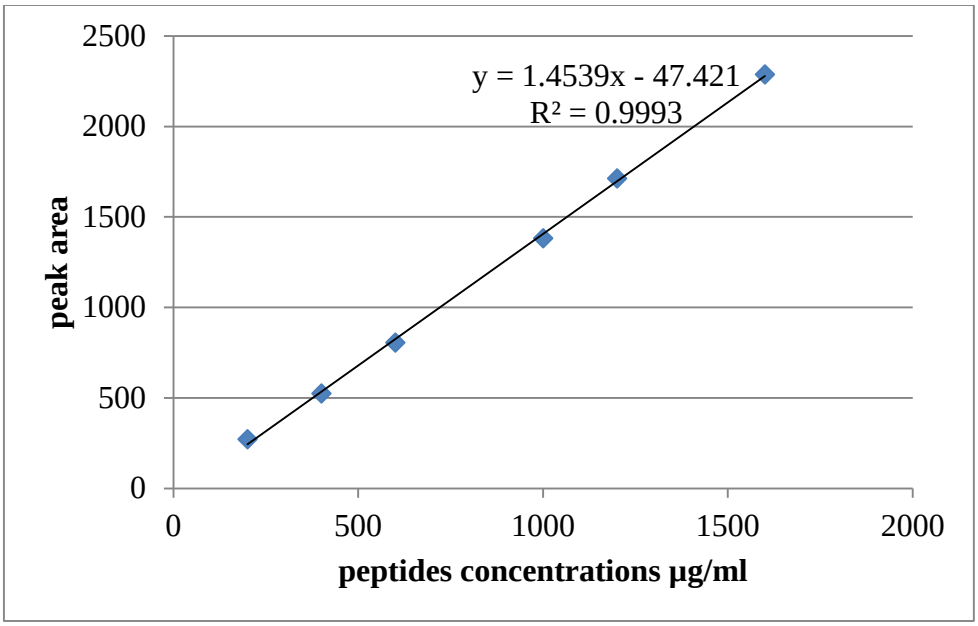

Figure 2: Standard curve of pentapeptide at increasing concentrations based on peak areas from retention times on an affinity HPLC column.

Figure 3-a day 0

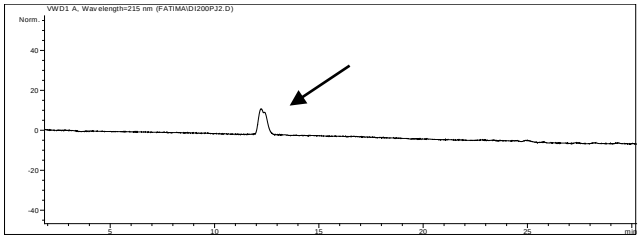

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Figure 3-b day 2

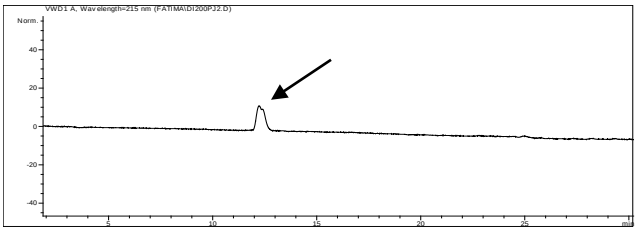


Figure 3-c day 7

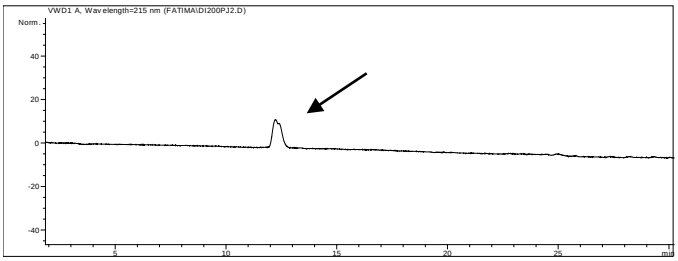


Figure 3-d day 14

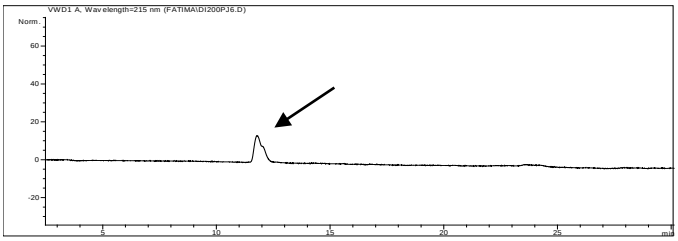


Figure 3-e day 30

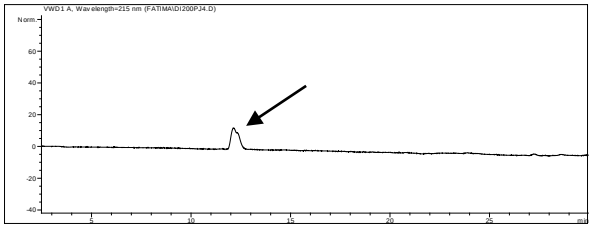


Figure 3-f day 60

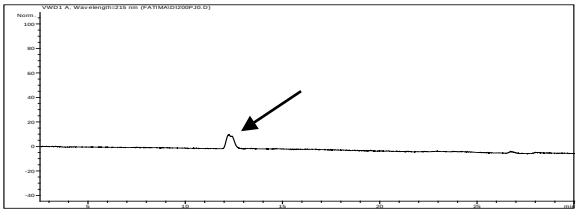


Figure 3(a-f): HPLC profiles of the pentapeptide (200µg/ml) incorporated in water at pH of 3.7

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. retention time; 12.3, 12.3, 12.3, 11.9, 12.3, and 12.2 respectively. Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 4-a day 0

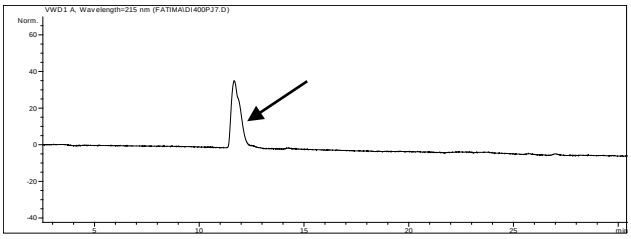


Figure4- b day 2

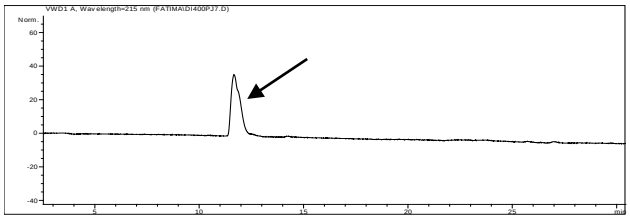


Figure 4-c day 7

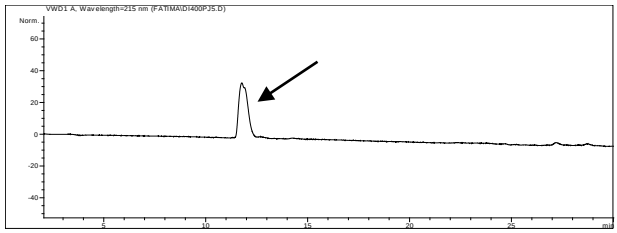
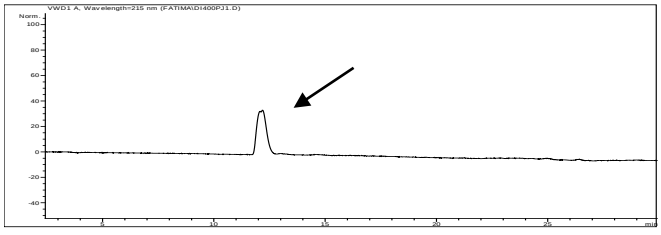


Figure4-d day 14



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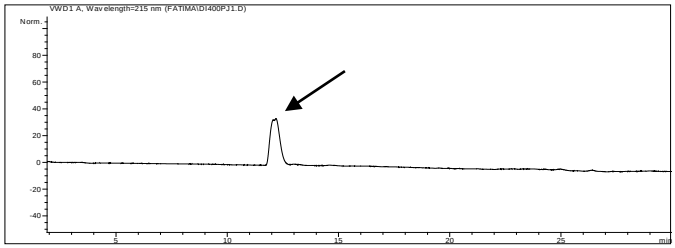


Figure 4-f day 60

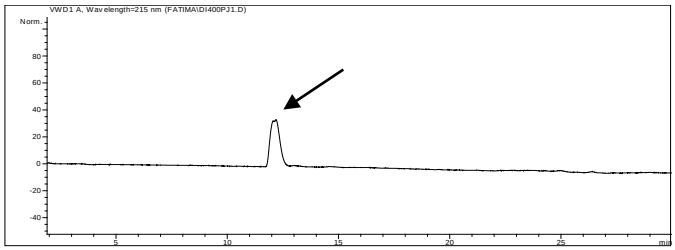
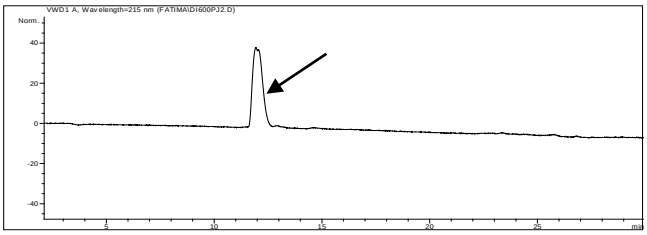


Figure 4(a-f): HPLC profiles of the pentapeptide (400µg/ml) incorporated in water at pH of 3.7

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm .retention time; 12.1, 12.1, 12.1, 12.0, 12.0, and 11.7 respectively Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 5-a day0



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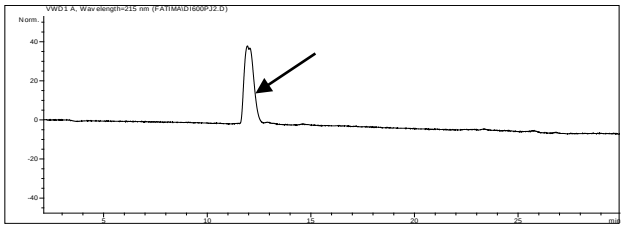

Figure5-c day 7

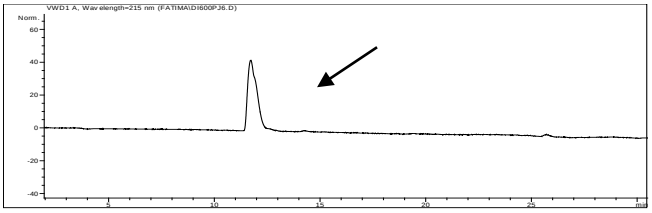

Figure 5-d day 14s

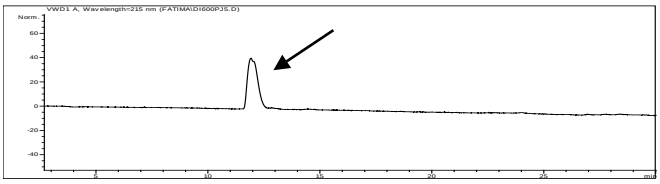

Figure5-e day 30

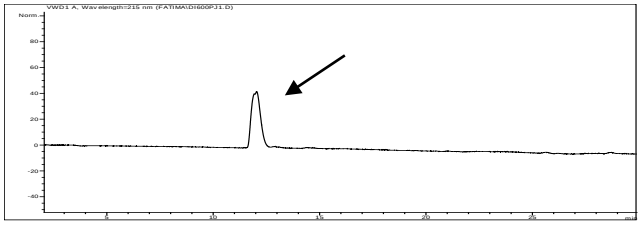

Figure 5-f day 60

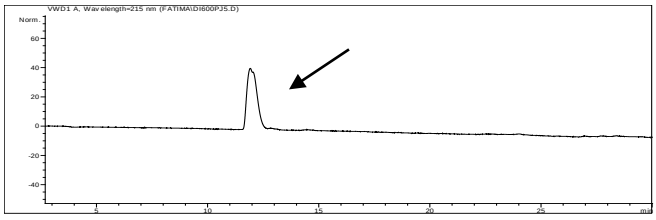

Figure 5(a-f): HPLC profiles of the pentapeptide (600µg/ml) incorporated in water at pH of 3.7.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Retention time of pentapeptide: 12.0, 12.0, 12.0, 11.7 , 11.9, and 12.4 respectively ; Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 6- a day 0

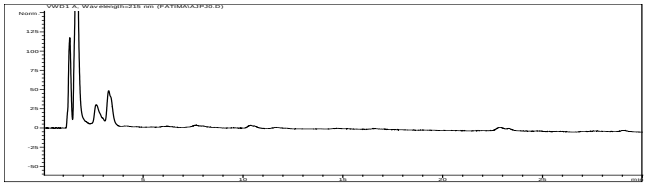

Figure 6-b day 2

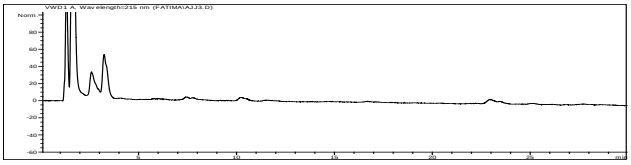

Figure 6-c day 7

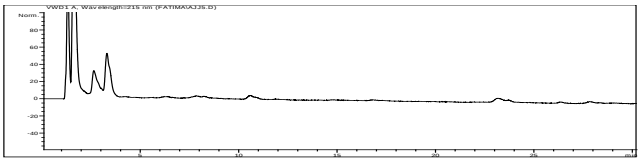

Figure 6-d day 14

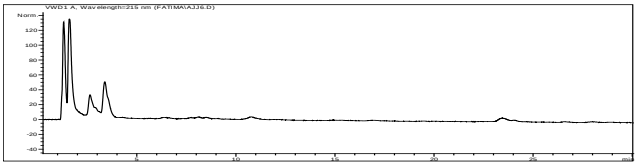

Figure 6-e day 30

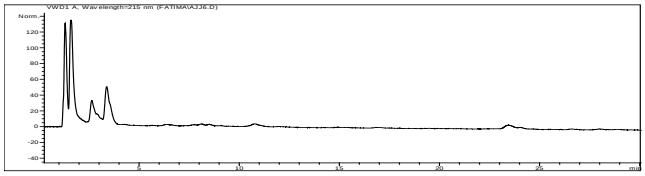

Figure 6-f day 60

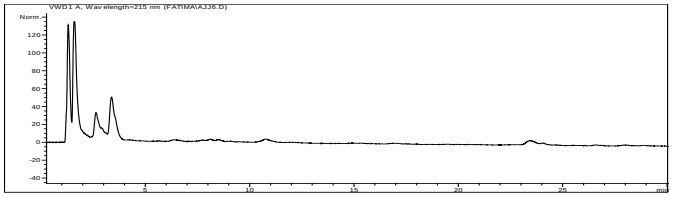

Figure 6(a-F): HPLC profiles of apple juice alone.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Y-axis represents the absorbance units and the X-axis represents retention times

Figure7-a day 0

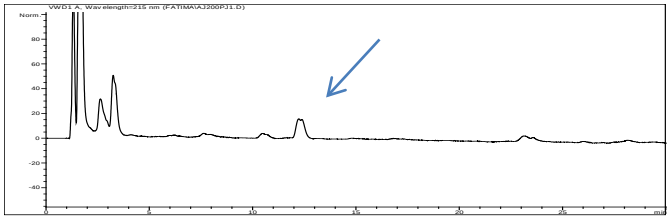

Figure7-b day 2

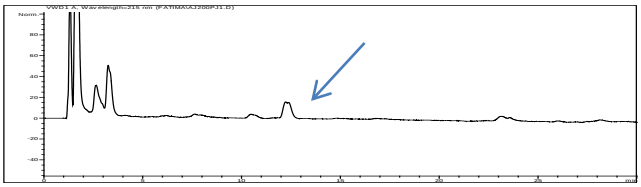

Figure 7-c day 7

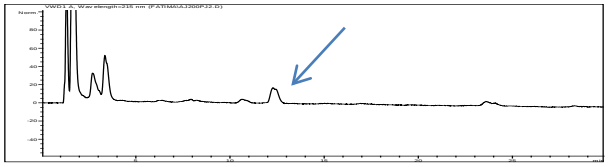

Figure 7-d day 14

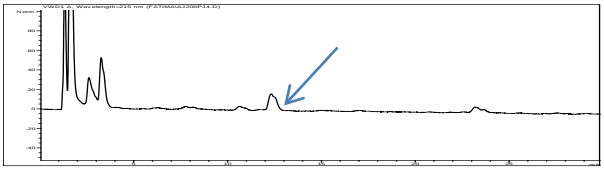

Figure 7-e day 30

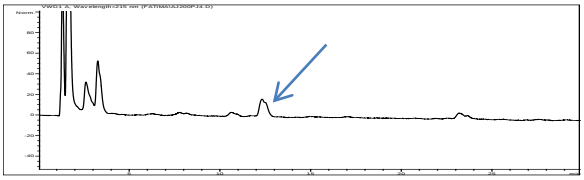

Figure 7-f day 60

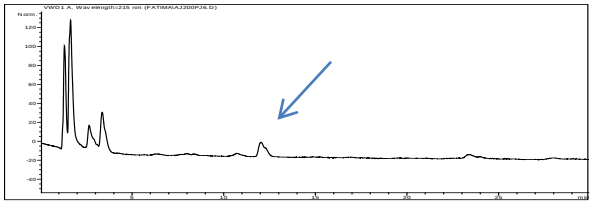

Figure 7(a-f): HPLC profiles of the pentapeptide (200µg/ml) incorporated in apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm; Retention time of pentapeptide: 12.2, 12.2, 12.2,12.3 ,12.2, and 11.7 respectively ; Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 8-a day 0

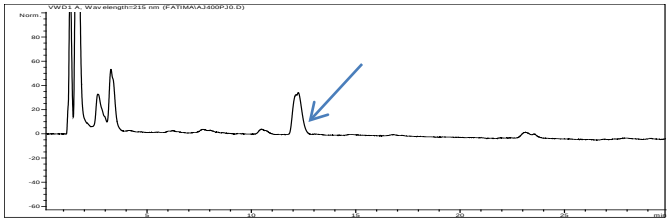

Figure 8-b day 2

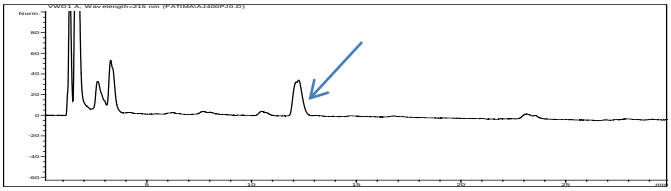

Figure 8-c day 7

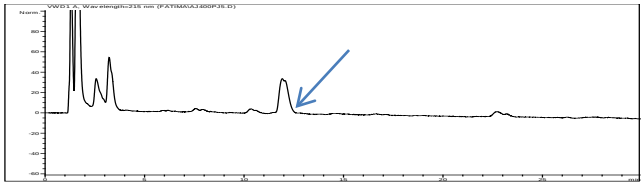

Figure 8-d day 14

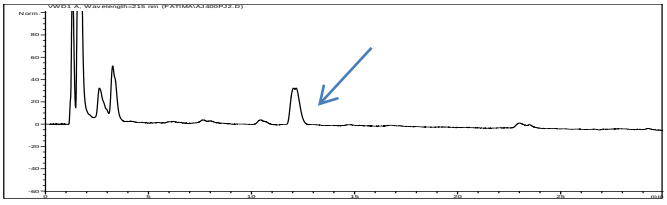

Figure 8-e day30

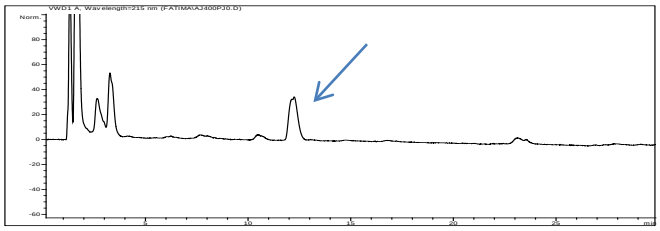

Figure 8-f day 60

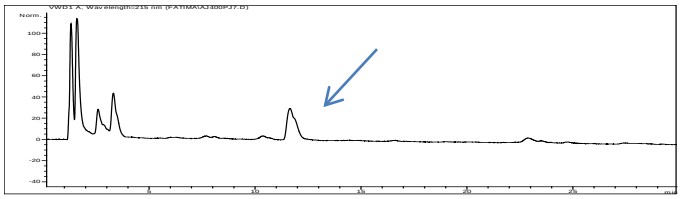

Figure 8(a-f): HPLC profiles of the pentapeptide (400µg/ml) incorporated in apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm; Retention time of pentapeptide: 12.1, 12.1, 12.1, 12.0, 12.2, and 12.0 respectively**;** Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 9-a day 0

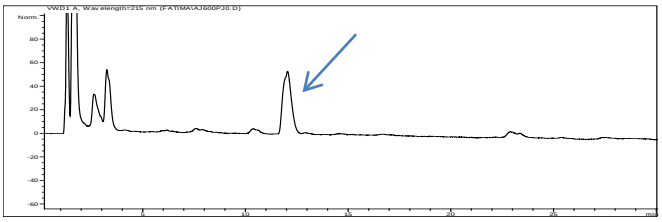

Figure 9-b day 2

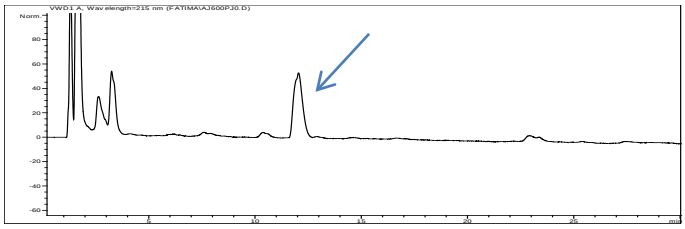

Figure 9-c day 7

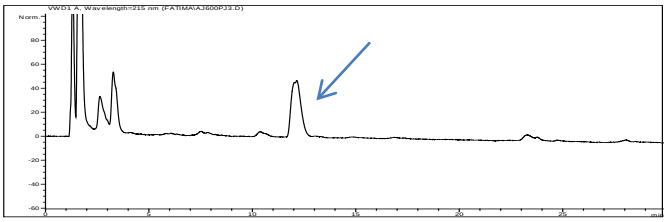

Figure 9-d day 14

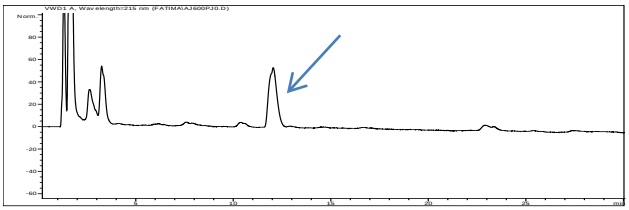

Figure 9-e day 30

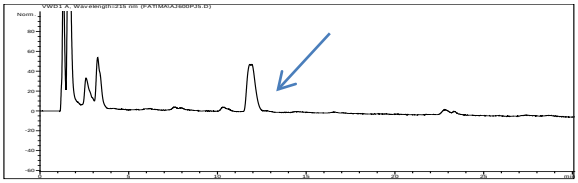

Figure 9-f day 60

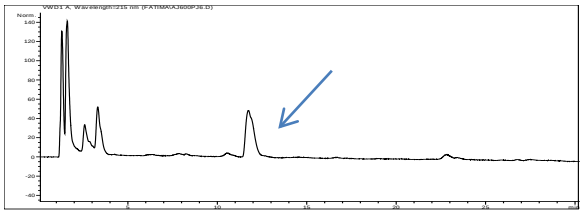

Figure 9(a-f): HPLC profiles of the pentapeptide (600µg/ml) incorporated in apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm; Retention time of pentapeptide: 12.0, 12.0, 12.0,12.3,12.0 , and 11.9 respectively ; Arrow a head shows the peak of the pentapeptide. Y-axis represents the absorbance units and the X-axis represents retention times

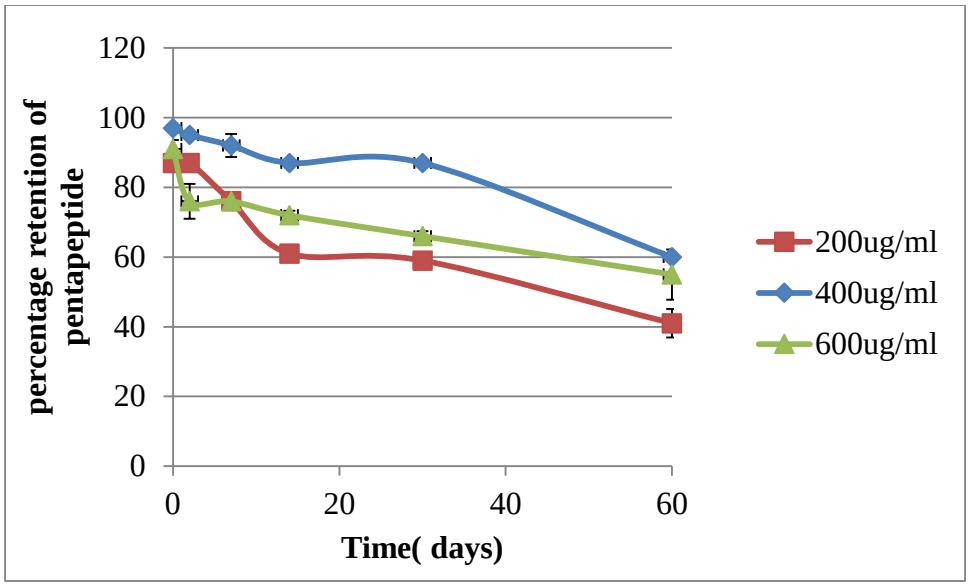

Figure 10: The stability of varying concentrations of pentapeptide in water at a pH of 3.7 based on the percentage of pentapeptide degraded over the storage period.

The values are represented as means of replicate analysis ± standard deviation with (P value <0.00).

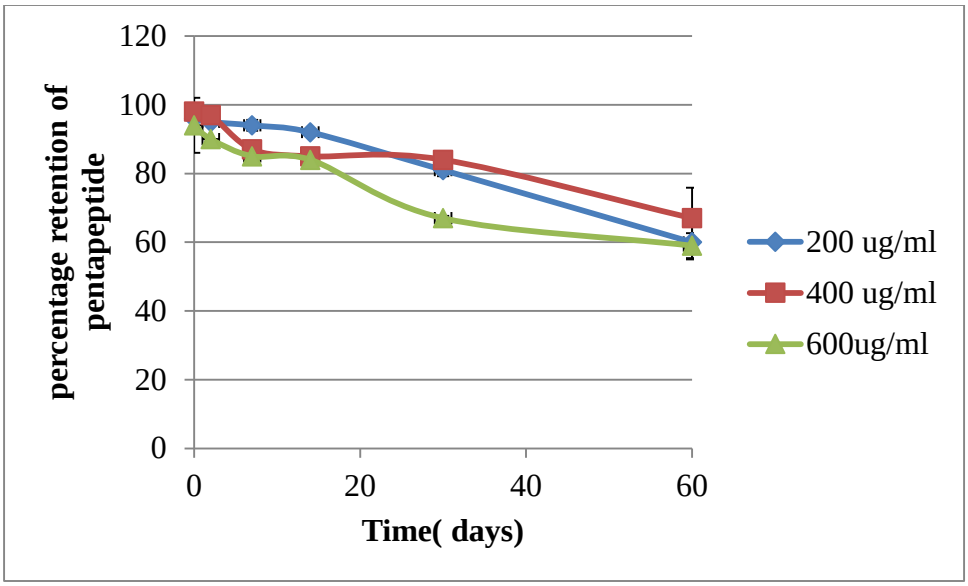

Figure 11: The stability of varying concentrations of pentapeptide in apple juice based on the percentage of pentapeptide degraded over the storage period.

The values are represented as means of replicate analysis ± standard deviation (P value <0.0001).

Obs	Treatment	Days	Concentration	Peakarea	Peptide	Percentage
1	AJ	0	200	230.10	190.880	95.4400
2	W	0	200	207.10	175.060	87.3000
3	AJ	0	200	237.30	195.830	97.9150
4	W	0	200	209.00	176.360	88.1800
5	AJ	0	400	522.80	392.200	98.0500
6	W	0	400	523.00	392.330	98.0825
7	AJ	0	400	524.20	393.160	98.2900
8	W	0	400	515.50	387.180	96.7950
9	AJ	0	600	777.70	567.500	94.5833
10	W	0	600	705.30	517.700	86.2833
11	W	0	600	805.00	586.290	97.7150
12	AJ	2	200	230.10	190.880	95.4400
13	AJ	2	200	230.10	190.880	95.4400
14	W	2	200	207.10	175.060	87.5300
15	AJ	2	400	521.90	391.580	97.8950
16	W	2	200	209.00	176.360	88.1800
17	AJ	2	400	518.70	389.380	97.3450
18	W	2	400	501.20	377.340	94.3350
19	AJ	2	600	739.35	541.100	90.1833
20	W	2	400	515.50	387.180	96.7950
21	W	2	600	633.80	460.840	76.8067
22	W	2	600	625.40	460.294	76.7156
23	AJ	7	200	225.90	187.990	93.9950
24	AJ	7	200	230.10	190.880	95.4400
25	W	7	200	172.20	151.050	75.5250
26	AJ	7	400	446.80	339.920	84.9800
27	W	7	200	178.80	155.590	77.7950
28	AJ	7	400	474.60	359.040	89.7600
29	W	7	400	495.10	373.140	93.2850
30	AJ	7	600	702.00	515.450	85.9083
31	W	7	400	488.40	368.540	92.1350
32	AJ	7	600	702.00	515.450	85.9083
33	W	7	600	622.60	468.540	78.0900
34	W	7	600	621.80	462.760	77.1267
35	AJ	14	200	220.90	184.550	92.2750
36	AJ	14	200	219.90	183.860	91.9300
37	W	14	200	132.70	123.880	61.9400
38	AJ	14	400	446.80	335.730	83.9325
39	W	14	200	130.20	122.160	61.0800
40	AJ	14	400	440.70	331.600	82.9000
41	W	14	400	468.60	342.810	85.7025
42	AJ	14	600	685.60	504.170	84.0283
43	W	14	400	450.00	354.920	88.7300
44	AJ	14	600	702.00	515.450	85.9083

45	W	14	600	583.50	433.900	72.3167
46	W	14	600	627.55	438.500	73.0833
47	AJ	30	200	187.30	161.440	80.7200
48	AJ	30	200	192.50	165.010	82.5050
49	W	30	200	130.20	122.160	61.0800
50	AJ	30	400	440.70	339.920	84.9800
51	W	30	200	122.60	116.940	58.4700
52	AJ	30	400	434.70	335.730	83.9325
53	W	30	400	451.00	354.920	88.7300
54	AJ	30	600	426.90	326.240	54.3733
55	W	30	400	468.60	342.120	85.5300
56	AJ	30	600	652.80	481.610	80.2683
57	W	30	600	531.80	398.390	66.3983
58	W	30	600	540.80	404.580	67.4300
59	AJ	60	200	.	95.700	47.8500
60	W	60	200	64.00	76.600	38.3000
61	AJ	60	200	166.30	147.021	73.5107
62	W	60	200	84.60	90.851	45.4253
63	AJ	60	400	346.00	270.597	67.6493
64	W	60	400	269.30	217.865	54.4663
65	AJ	60	400	345.00	269.909	67.4773
66	W	60	400	343.30	268.763	67.1907
67	AJ	60	600	513.60	385.919	64.3198
68	W	60	600	417.60	319.890	53.3150
69	AJ	60	600	423.60	324.017	54.0028
70	W	60	600	462.30	350.612	58.4353

Table 3: statistical analysis of pentapeptide in water at Ph of 3.7 and apple juice during storage period of 60 days.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	35	14861.88322	424.62523	15.27	<.0001
Error	34	945.21308	27.80038		
Corrected Total	69	15807.09630			

Table 4: ANOVA table of pentapeptide in water at pH of 3.7 and apple juice.

Figure 12-a day 0

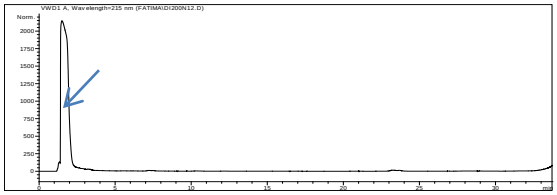

Figure 12-b day 2

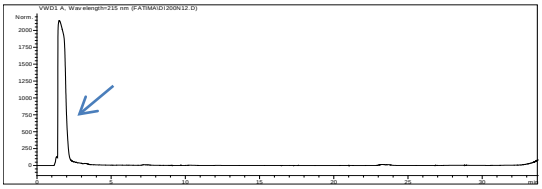

Figure12-c day 7

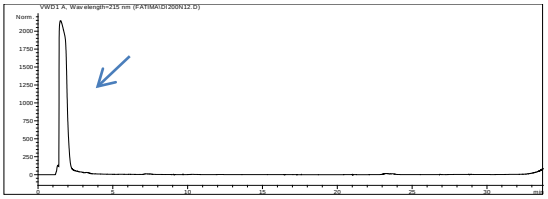

Figure 12-d day 14

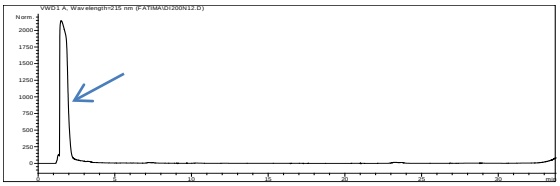

Figure12 -e day 30

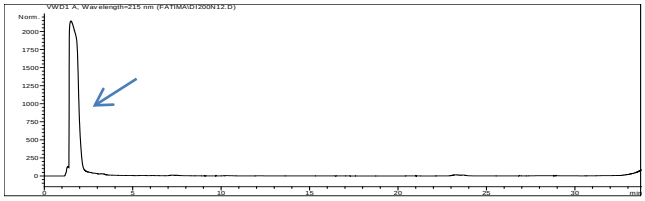

Figure 12-f day 60

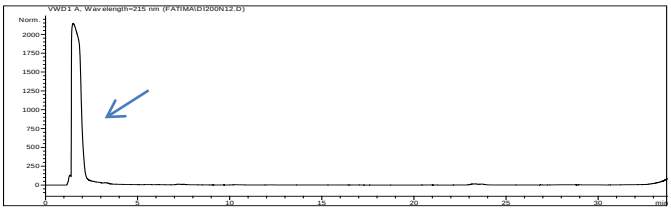

Figure 12(a-f): HPLC profiles of Nano-encapsulated pentapeptide (200µg/ml) incorporated water at pH of 3.7.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 13-a day 0

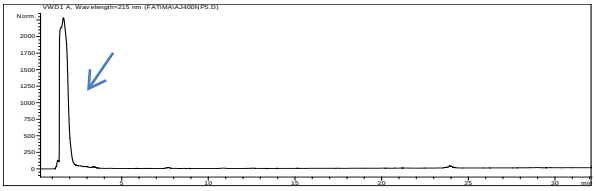

Figure 13-b day 2

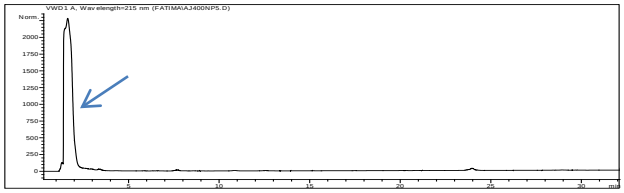

Figure 13-c day 7

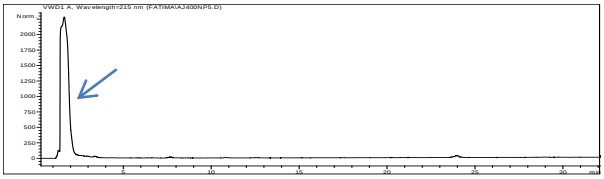

Figure13-d day 14

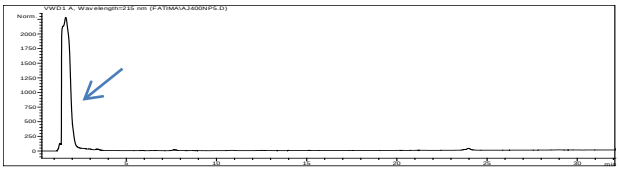

Figure 13-e day 30

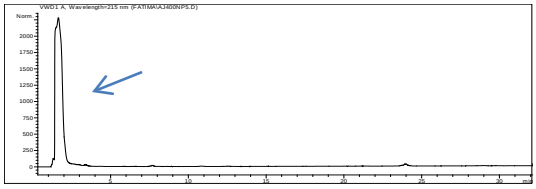

Figure 13-f day 60

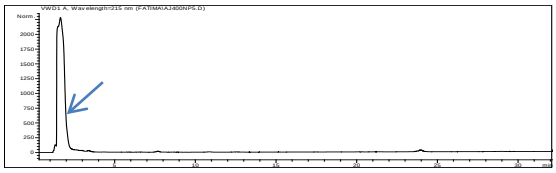

Figure 13(a-f): HPLC profiles of Nano-encapsulated pentapeptide (400µg/ml) incorporated water at pH of 3.7.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 14-a day 0

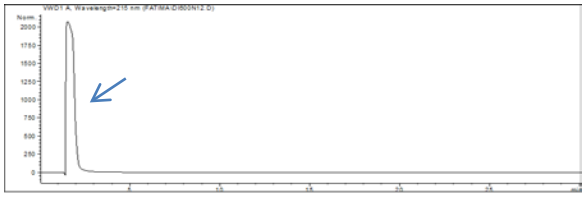

Figure 14-b day2

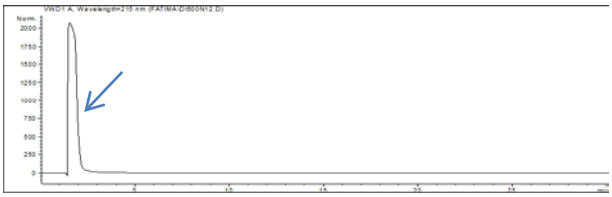

Figure 14-c day 7

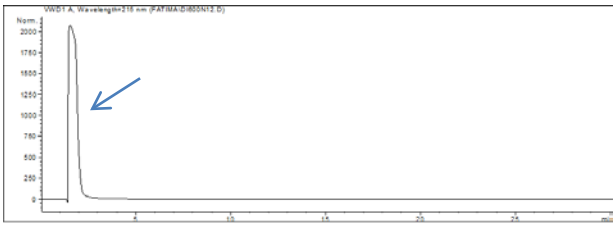

Figure 14-d day 14

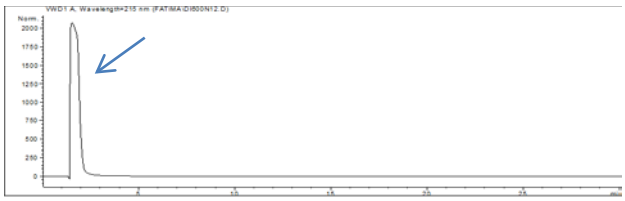

Figure 14-e day 30

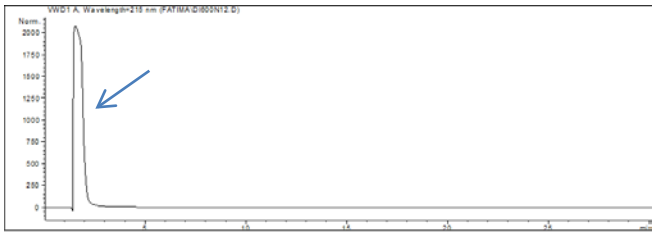

Figure-f day 60

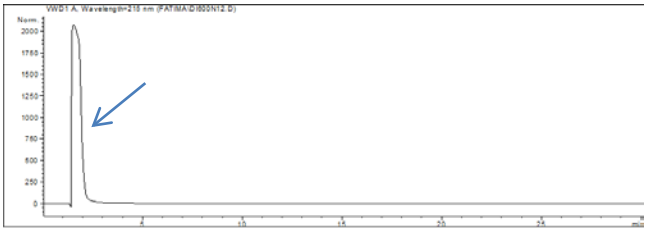

Figure 14: HPLC profiles of Nano-encapsulated pentapeptide (600µg/ml) incorporated water at pH of 3.7.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 15-a day 0

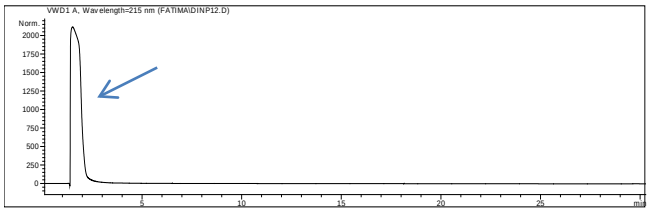

Figure 15-b day 2

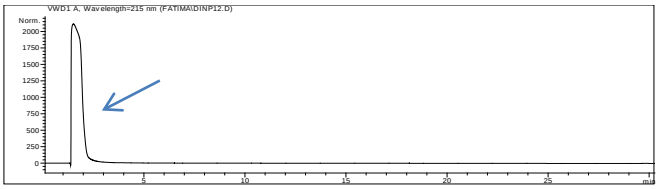

Figure 15-c day 7

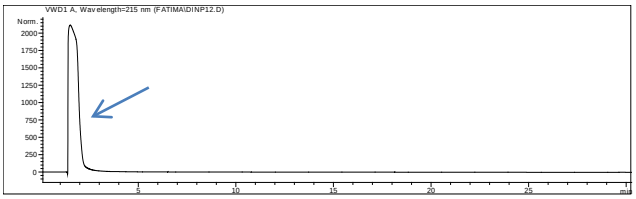

Figure 15-d day 14

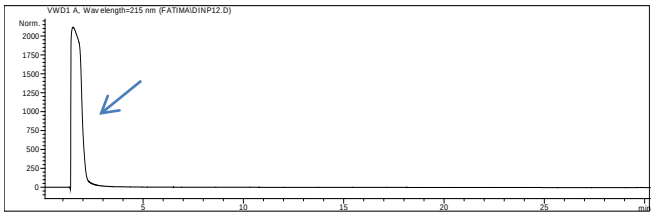

Figure 15-e day 30

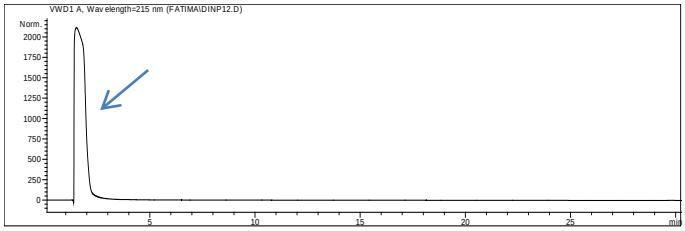

Figure 15-f day 60

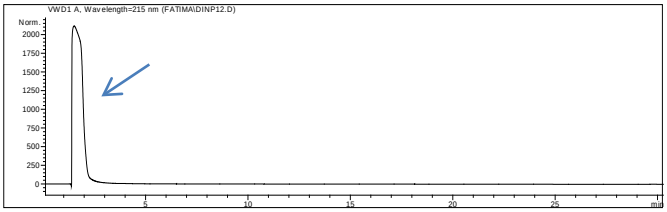

Figure 15(a-f): HPLC profiles of Nanoparticles in water at pH of 3.7.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 16-a day 0

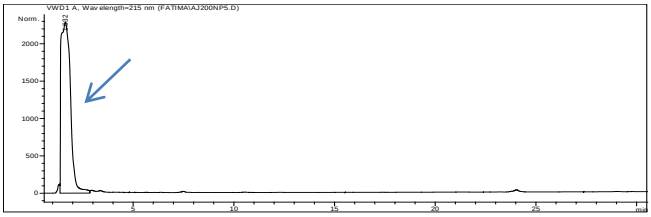

Figure 16-b day 2

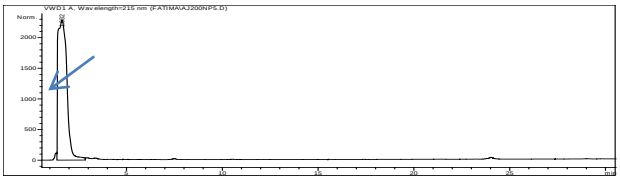

Figure 16-c day 7

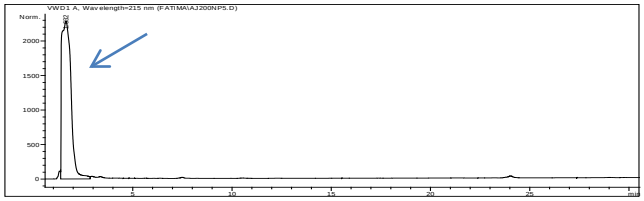

Figure 16-d day 14

Figure 16-e day 30

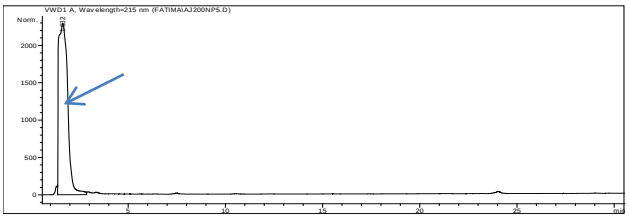

Figure 16-f day 60

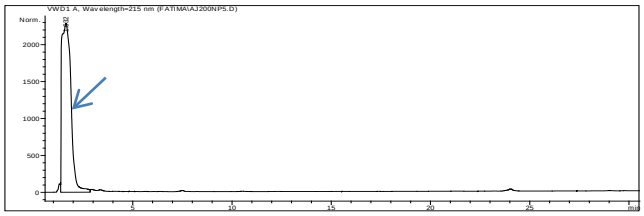

Figure 16(a-f): HPLC profiles of Nano-encapsulated pentapeptide (200µg/ml) incorporated apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 17-a 0 day

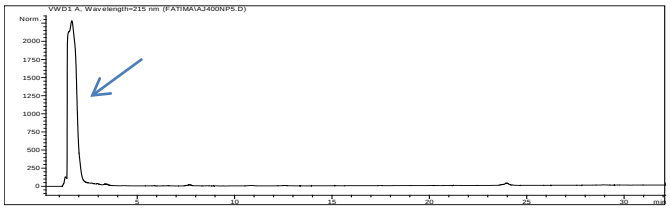

Figure 17-b day 2

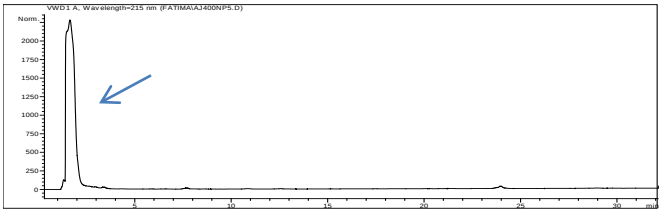

Figure 17-c day 7

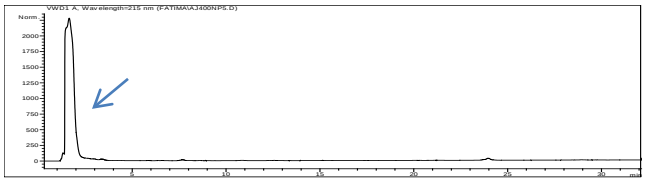

Figure 17-d day 14

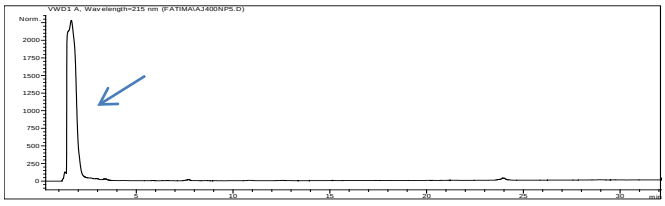

Figure 17-e day 30

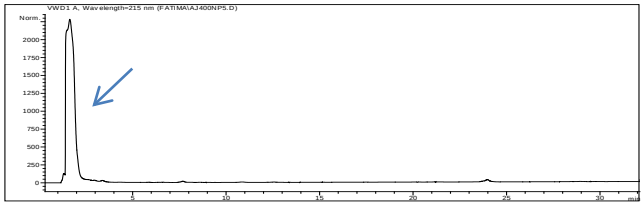

Figure 17-f day 60

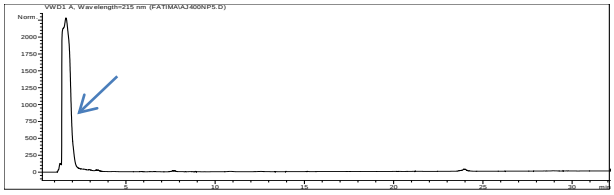

Figure 17: HPLC profiles of Nano-encapsulated pentapeptide (400µg/ml) incorporated apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 18-a day 0

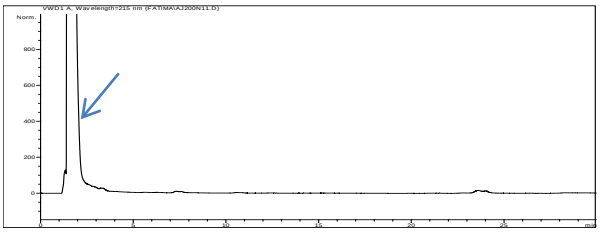

Figure 18-b day 2

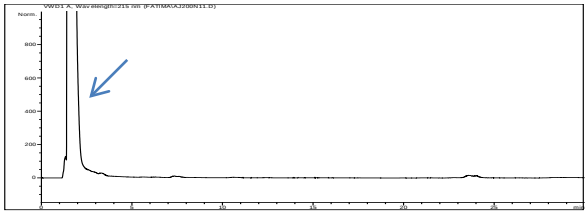

Figure 18-c day 7

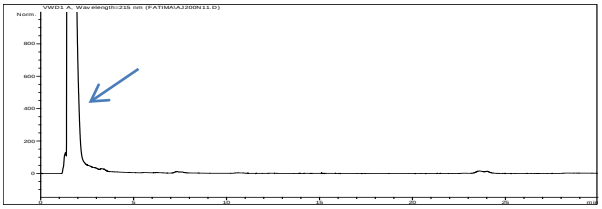

Figure 18-d day 14

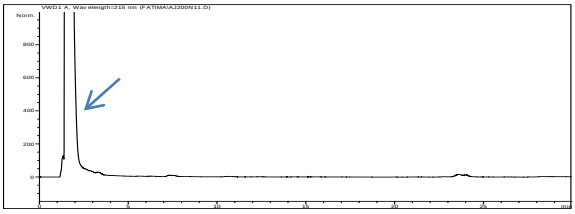

Figure 18-e day 30

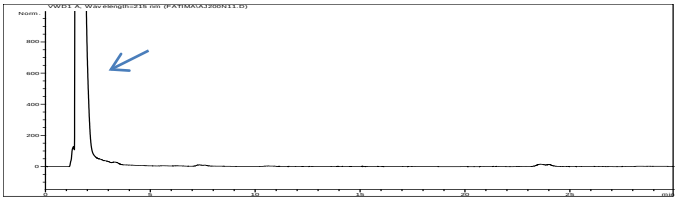

Figure 18-f day 60

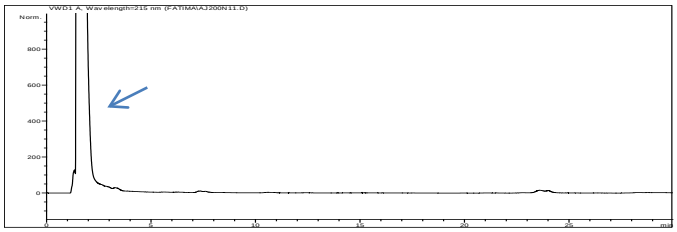

Figure 18(a-f): HPLC profiles of Nano-encapsulated pentapeptide (600µg/ml) incorporated apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

Figure 19-a day 0

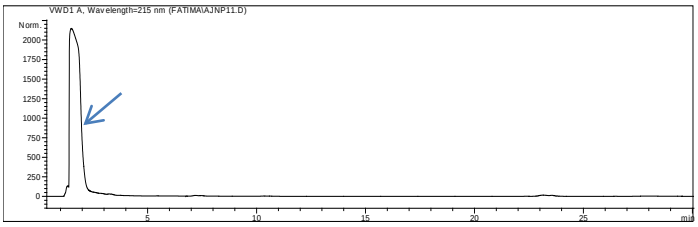

Figure 19-b day 2

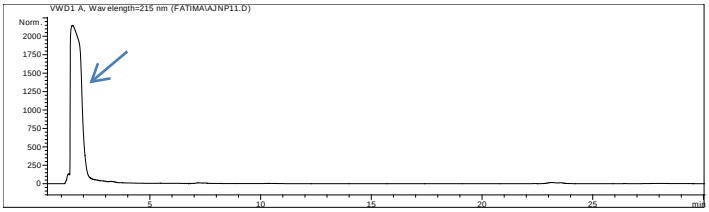

Figure 19-c day 7

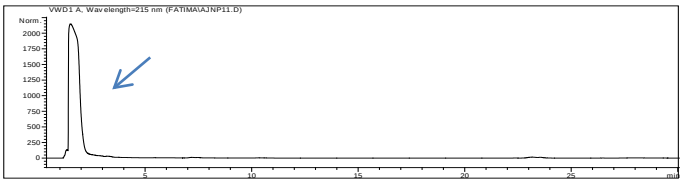

Figure 19-d day 14

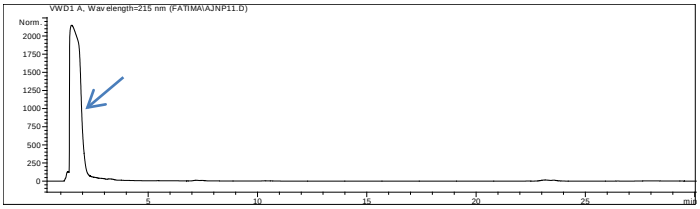

Figure 19-e day 30

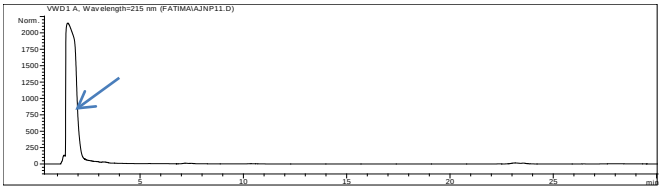

Figure 19-f day 60

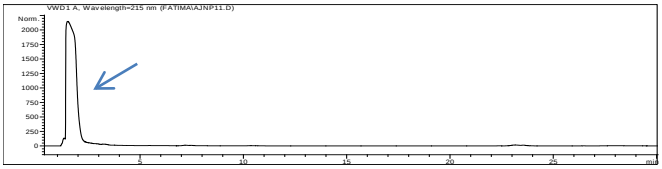

Figure 19(a-f): HPLC profiles of Nanoparticles in apple juice.

Solvent system: 0.1 % TFA in water and 0.1 %TFA in acetonitrile in water (50%); Flow rate: 1ml/min; Injection volume: 10µl; Elution time: 45min; Absorbance measured: 215 nm. Arrow a head shows the peak of the solvent DMSO. Y-axis represents the absorbance units and the X-axis represents retention times

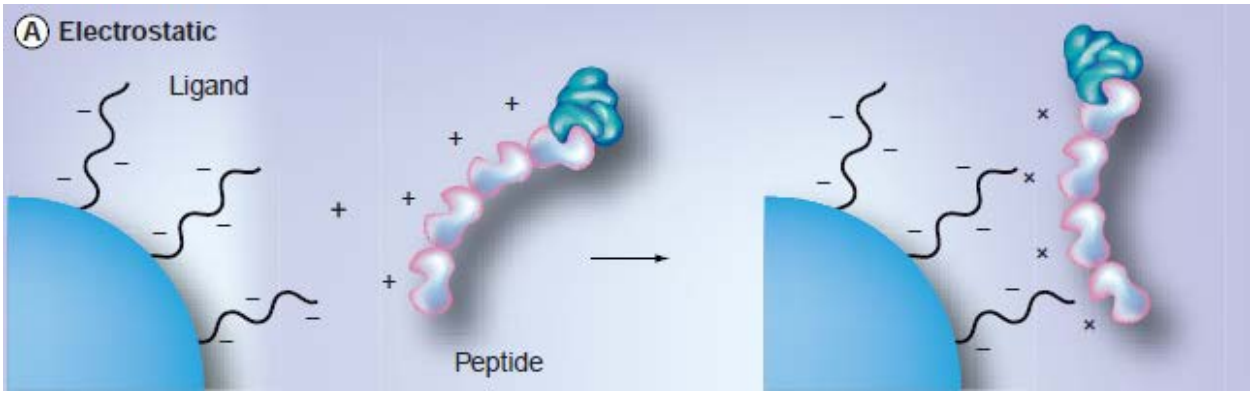

Figure 20: Illustration of electrostatic interactions between nanoparticles and peptide

(Source: Delehanty *et al.*, 2010).

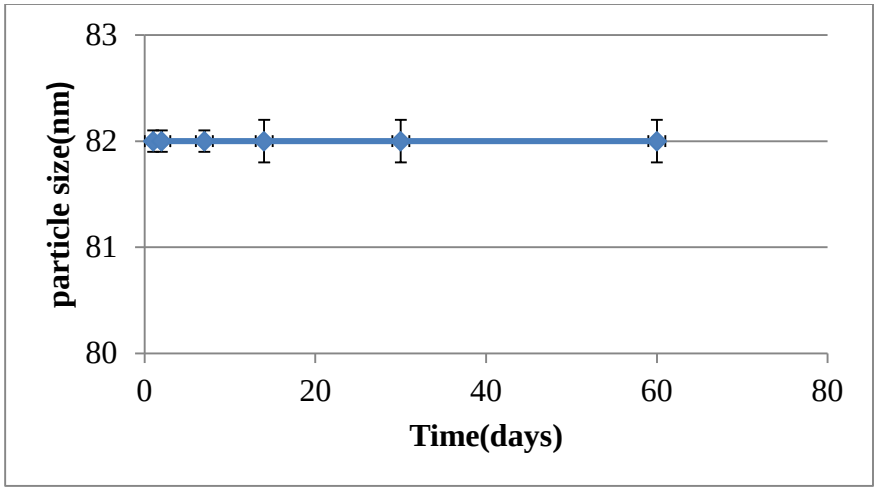

Figure 21: The particle size stability of Nano-encapsulated pentapeptide in apple juice (200/400/600µg/ml) over the storage period (0 to 60 days).

The values are represented as means of replicate analysis ± standard deviation. (P value <0.05).

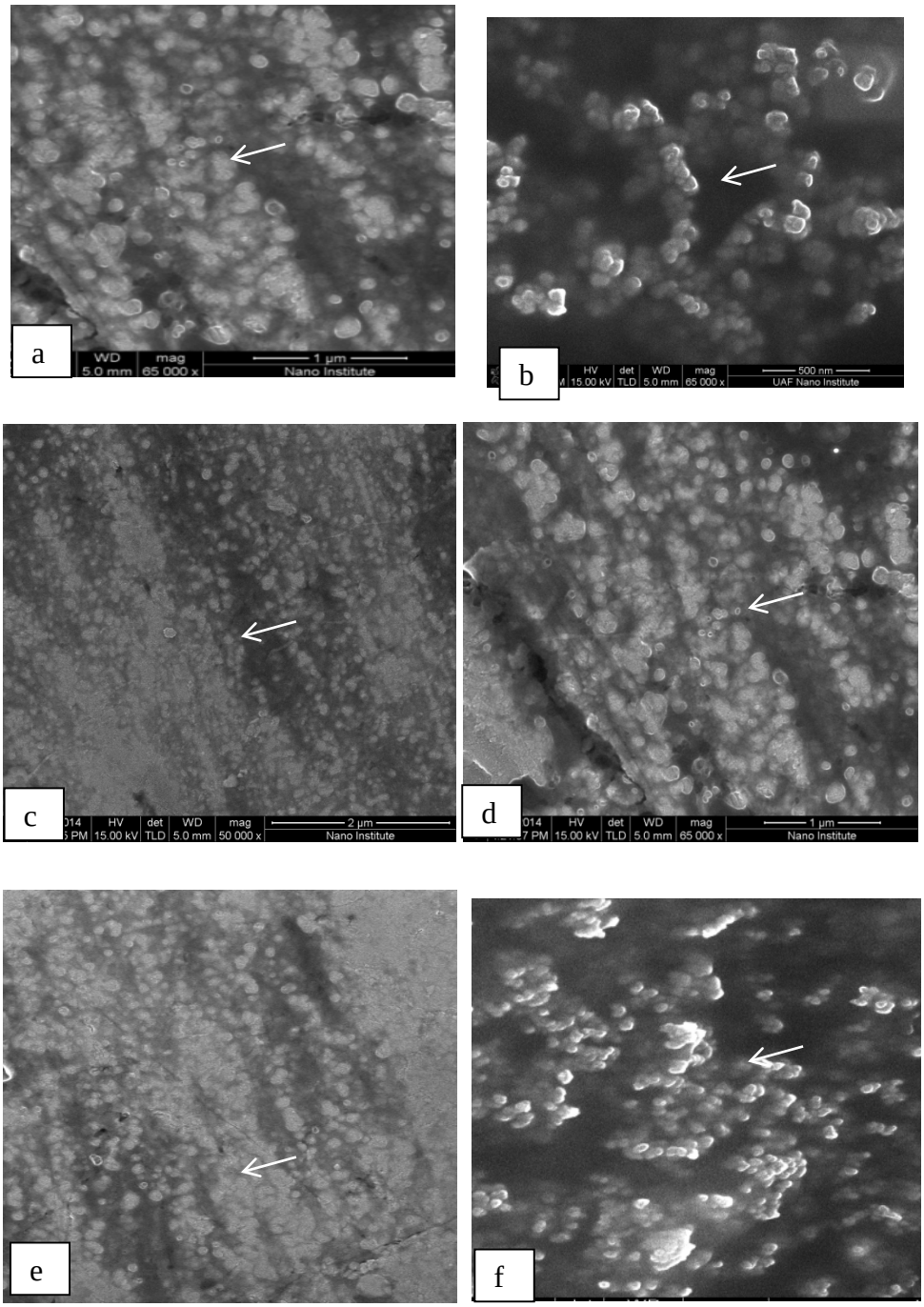

Figure 22: SEM image of Nano-encapsulated pentapeptide in apple juice.

Images labeled a, b, c, d, e, f were taken at 0, 2, 7, 14, 30, and 60 days of storage respectively. Magnification: image a - 65.000x, image b - 65,000x, image c 50,000 x, image d 65,000x, image e 50,000x, and image f 65.000x respectively. The nanoparticles are indicated by arrows in all the images.

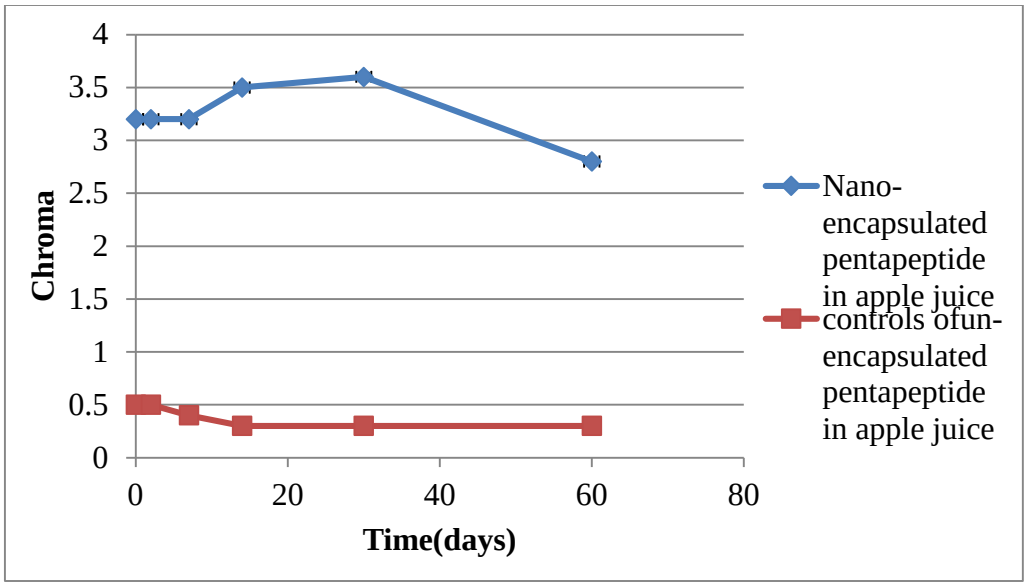

Figure 23: The Chroma changes of the Nano-encapsulated and non-encapsulated pentapeptide incorporated apple juice in storage.

The values are represented as means of replicate analysis ± standard deviation with (P value <0.0001).

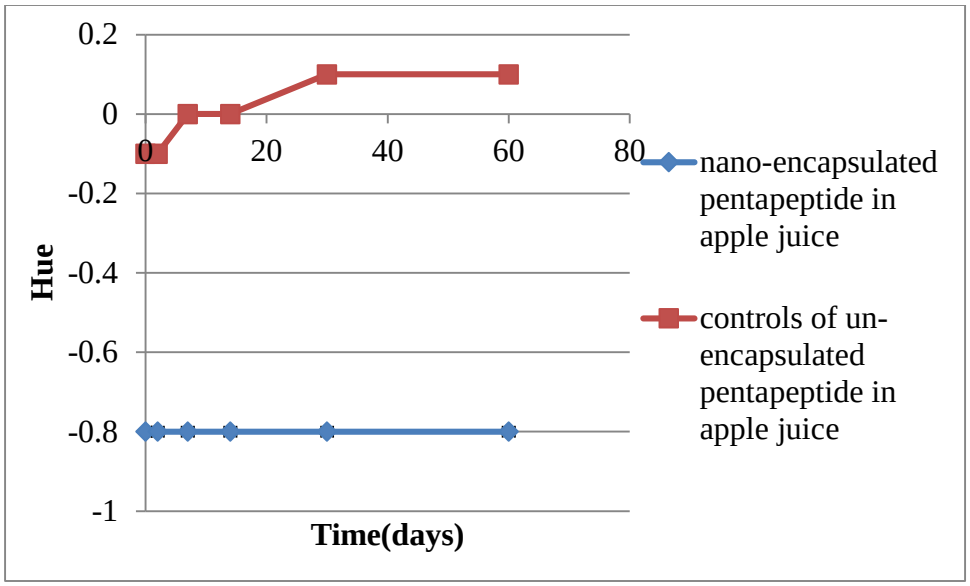

Figure 24: The Hue Change of Nano-encapsulated and non-encapsulated pentapeptide incorporated apple juice in storage.

The values are represented as means of replicate analysis ± standard deviation with (P value< 0.001).

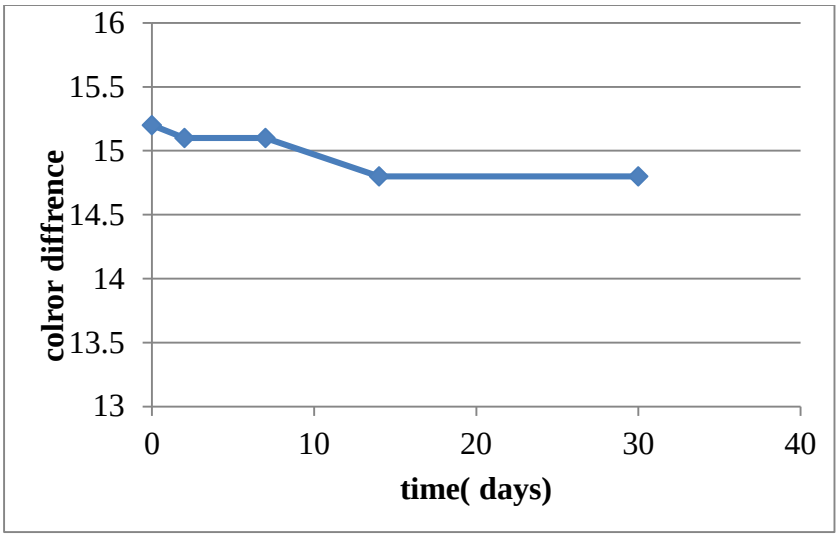

Figure 25: The color change of Nano-encapsulated pentapeptide incorporated apple juice and control (apple juice) in storage.

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04/22/2014

Dear Sir/Ma'am,

This is to inform you that Fatima Alessa is the first author of the manuscript produced from her M.S. thesis Chapter 3 and completed more than 51% of the work of the manuscript submitted to Journal of Food Processing and Technology.

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