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PECTIN FROM APPLE POMACE: EXTRACTION, CHARACTERIZATION, AND UTILIZATION IN ENCAPSULATING ALPHA-TOCOPHEROL ACETATE

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PECTIN FROM APPLE POMACE: EXTRACTION, CHARACTERIZATION, AND UTILIZATION IN ENCAPSULATING α-TOCOPHEROL ACETATE

by

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PECTIN FROM APPLE POMACE: EXTRACTION, CHARACTERIZATION, AND UTILIZATION IN ENCAPSULATING α-TOCOPHEROL ACETATE

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Pomace is the main byproduct from apple juice and cider industry. Valuable compounds, such as pectin, can be obtained from apple pomace. Despite the wide utilization of pectin in the food industry, the available knowledge on the complicated extraction process and chemistry of pectin is limited. Pectin is a versatile food ingredient that can be utilized in the development of functional food ingredients. This research covers two main areas of importance on the production and utilization of pectin. The first study was aimed at optimizing process conditions for the acid extraction of pectin from apple pomace. Three extraction factors (hot acid extraction time, temperature, and pH) were investigated and optimized, based on pectin yield and purity, using response surface methodology. A linear model was developed to predict the pectin yield based on extraction conditions. The second study investigated the use of apple pectin as wall material component for the encapsulation of α-tocopherol acetate. α-tocopherol acetate microparticles were produced by spray drying, using selected levels of apple pectin and octenyl succinic anhydride (OSA) starch. The incorporation of small amounts of apple pectin in the wall material can increase the encapsulation efficiency of α-tocopherol acetate and reduce the particle size and distribution, without affecting the surface morphology of the particles.

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ABBREVIATIONS

2FI	Two factor interaction
AACCI	American Association of Cereal Chemists International
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
CPS	Counts per second
d.b.	dry basis
DE	Degree of esterification
DSC	Differential scanning calorimetry
FDA	Food and Drug Administration
HPSEC	High-performance size exclusion chromatography
R^2	Coefficient of determination
RSM	Response surface methodology
SEM	Scanning electron microscopy
USDA	United States Department of Agriculture
XRD	X-ray diffraction

INTRODUCTION

The processing of apples into juice and cider generates large amounts of residues, which are commonly known as apple pomace. In the United States, 2×10^5 tonnes of apple pomace were produced in 2010 (USDA 2012). Currently, most of this byproduct is underutilized and disposed into landfills. Apple pomace is a valuable source of fiber, especially pectin, and other micronutrients (Bhushan et al. 2008). Pomace could be utilized either for direct extraction of useful compounds or for the production of value added products. Various approaches have been tested and employed to effectively utilize apple pomace, from which pectin extraction have been recognized as an economically feasible process (Kennedy et al. 1999).

Pectin is one of the most structurally complex polysaccharides found in nature, and it is present in plants as a cell wall component. Pectin is mainly composed of α (1→4)-D-galacturonic residues, with various degrees of methyl esterification. Native pectins have up to ~60% galacturonic acid residues, whereas commercial pectins could be entirely made up of galacturonic acid residues (Voragen et al. 2009). Depending on the chemical characteristics of pectin, it is used in numerous food applications as a gelling agent, thickener, stabilizer, and emulsifier.

The commercial production of pectin generally involves the extraction of pectin from the plant material, at high temperatures under acidic conditions, followed by precipitation in an organic solvent (May 1990). The extraction conditions vary with the nature of raw material and process economics (Brejnholt 2010). The chemical characteristics of pectin, and therefore its properties, are influenced by the conditions of the extraction process. Therefore, it is important to select extraction conditions that allow high pectin yields

without compromising the quality. Even though the chemistry of pectin and its extraction have been widely studied (Thakur et al. 1997; Voragen et al. 2009), the gaps in knowledge still remain on the effects of the extraction conditions on yield, composition, physicochemistry, and functionality of pectin from apple pomace.

The traditional application of pectin is as a gelling agent in jams and jellies, but over the past few years pectin has been increasingly used in new applications, not only in the food industry, but also in the pharmaceutical industry. Various properties of pectin, such as gelling, emulsifying, and film forming abilities, in addition to its resistance to degradation in the upper gastro intestinal tract, has allowed the increasing use of pectin in the development of drug delivery systems through encapsulation (Desai 2005; Liu et al. 2003). Following the same rationale, pectin has the potential to be used in the encapsulation of unstable food ingredients.

α-tocopherol, which is the most abundant and active form of vitamin E, is commonly used by the food industry for food fortification and to inhibit lipid oxidation. However, the use of α-tocopherol is, sometimes, hindered by its sensitivity to heat, oxygen, and light, and high hydrophobicity (Eitenmiller and Lee 2004; Gregory III 2008). Pectin could be used as an ingredient in the development of a polymeric matrix for the encapsulation of α-tocopherol, to provide protection, enhance the stability, and to deliver α-tocopherol under specific conditions, or gastrointestinal environments.

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OBJECTIVES

Overall objective:

To optimize the extraction conditions of pectin from apple pomace and evaluate its use in the encapsulation of a lipophilic compound.

Specific objectives:

1. To optimize three extraction conditions (hot acid extraction time, temperature, and pH) of pectin from fresh apple pomace, based on pectin yield and uronic acid content.

Hypothesis:

Extraction conditions can be optimized to maximize the production yield of pectin, without compromising its desired quality attributes.

2. To use apple pectin as a wall material component for the encapsulation of α-tocopherol acetate.

Hypothesis:

Apple pectin with octenyl succinic anhydride (OSA) starch can be used to prepare microparticles, containing α-tocopherol acetate, for controlled delivery applications.

CHAPTER 1. LITERATURE REVIEW

1.1 Background

Apple (*Malus domestica* Borkh.) is a climacteric fruit cultivated in temperate regions (Luby 2003), and one of the most widely cultivated and consumed fruits worldwide (Sinha 2012). World production of apple was 7.5×10^7 tonnes in 2010, China and United States being the leading producers, with 47.6% and 5.6% respectively. About 68% of the United States production is utilized as fresh fruits for consumption. The remainder is processed mainly into canned, frozen, and dried apples, and apple juice and cider. Almost 45% of the apples destined to processing are used in the juice and cider production (Figure 1.1, p.45) (Sinha 2006; USDA 2012). Apple juice and cider, combined, occupied the second place in the juice production of United States with 1.1×10^9 gallons produced in 2011 (USDA 2013).

More than 7,500 apple varieties are grown around the world (Lopez-Fructuoso and Echeverria-Cortada 2010). Red Delicious, Gala, Golden Delicious, Fuji, and Granny Smith are the top apple varieties in the United States, constituting more than 60% of the production (USDA 2012). A blend of several varieties is used for the production of apple juice and cider (Sinha 2006). Blending varieties is practiced in order to obtain a product with better flavor, since a single variety of apple seldom has a satisfactory balance of sweetness, tartness, tang, and aromatic overtones.

Apple juice and apple cider are both products obtained by extraction of liquid portion from fresh apples. In the United States, "apple cider" refers to a non-alcoholic beverage, in contrast to most other regions in the world where it refers to a fermented alcoholic beverage, with an alcohol level in the range 0.5 to 8% (Ensminger 1994).

Generally, juice processing consists of following steps: 1) fruit selection and preparation (*e.g.*, quality inspection and cleaning), 2) milling or slicing, 3) juice extraction by pressing, 4) clarification and filtration, 5) pasteurization, and 6) packaging. While apple juice must be clarified by an enzyme treatment, followed by filtration to remove suspended particles (such as pectin, starch, and cellulose), apple cider is neither clarified nor filtered, resulting in a raw liquid with more solids and cloudy appearance. In addition this product is not pasteurized (Sinha 2006).

During the processing of apple fruits for juice or cider preparation, large amounts of solid residues (peel, core, seed, calyx, stem, and soft tissue) are generated (Kennedy et al. 1999). These residues, commonly known as apple pomace, account for approximately 25-30% of the weight of the original fresh fruit (Gullon et al. 2007). Currently a small proportion (~20%) of apple pomace is utilized in traditional ways, such as composting and low quality animal feed, while a large proportion (~80%) remains underutilized and goes to landfill or incinerator (Dhillon et al. 2013).

1.2 Utilization of apple pomace

Apple pomace is a well-known good source of carbohydrates, dietary fibers, vitamins, and minerals, among other functional components, such as polyphenols (Bhushan et al. 2008). For both economic and environmental reasons it is worth recovering these nutrients and developing value added products. Different approaches have been proposed to effectively utilize apple pomace; the most common applications are summarized in Table 1.1 (p.40).

Although numerous research studies have been conducted on the utilization of apple pomace, an economically feasible and efficient application is yet to be identified. High value products, such as aromas and polyphenols, could be obtained from apple pomace, but these compounds are usually present in small amounts (Kennedy et al. 1999). Thus, large amounts of apple pomace used as process raw material still remain after the extraction of these compounds. In addition, the production of these compounds, sometimes, requires expensive technologies that are not economical. Pectin production is considered as one of the most practical approaches for the utilization of apple pomace and also an economical utilization of the byproduct (Bhushan et al. 2008; Dilas et al. 2009; Fox et al. 1991).

1.3 Chemical structure and composition of pectin

1.3.1 Native pectin

Pectin is a cell wall structural carbohydrate present in plants. It is probably one of the most complex macromolecules found in nature (Bacic et al. 1988). Pectin is heterogeneous in both chemical structure and molecular weight (Thakur et al. 1997). Native pectin is composed of three major polysaccharides, all containing α (1→4)-D-galacturonic acid residues; usually referred to galacturonans (Ridley et al. 2001). These are homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II (Figure 1.2, p.46) (O'Neill et al. 1990; Vincken et al. 2003).

Homogalacturonan is a linear chain of α (1→4)-D-galacturonic acid residues with a variable degree of methyl esterification at the carboxyl group. It could be *O*-acetylated at C-2 or C-3 depending on the source (Vincken et al. 2003).

Rhamnogalacturonan-I consists of repeating units of the disaccharide α (1→2)-L-rhamnose-α (1→4)-D-galacturonic acid. Galacturonic acid residues can be *O*-acetylated at the C-2 or C-3, while 20-80% of the rhamnose residues can be substituted at C-4 or C-3 with neutral sugar side chains. The composition of the neutral sugars varies among plant sources, D-galactose, L-arabinose, and D-xylose being the most common. Other neutral sugars such as D-glucose, D-mannose, L-fucose, and D-glucuronic acid are found less frequently (O'Neill et al. 1990; Ridley et al. 2001).

Rhamnogalacturonan-II, despite its misleading identification, has a backbone of α (1→4)-D-galacturonic acid. Some of the side chains attached to the backbone that have been identified include 2-keto-3-deoxy-D-manno-octulosonic acid, 3-deoxy-D-lyxo-2-heptulosaric acid, apiose, and aceric acid (Spellman et al. 1983; York et al. 1985). All neutral sugars are located as side chains in the rhamnogalacturonan I and II domains, and therefore, these domains are often referred as "hairy regions" (Figure 1.2, p46) (Brejnholt 2010).

In addition to the three major domains described above, arabinogalactans, arabinans, and xylogalacturonans are also found in native pectin (Voragen et al. 1995), all lacking the galacturonan backbone.

The composition and chemical structures of the elements that constitute pectin depend on environmental conditions, plant source, and plant developmental stage, among others. In addition, pectin structure is modified by enzymatic and chemical reactions during plant growth, fruit ripening, and extraction (Schols and Voragen 2002). All of the above factors make the structure and molecular weight of pectin to vary based on plant source and even among tissues in the same plant (Willats et al. 2006).

1.3.2 Commercial pectins

Commercial pectins are structurally less complex due to the industrial extraction and purification process, which remove most of the neutral sugars (Figure 1.2, p. 46) (Schols and Voragen 2002). Commercial pectins consist mainly of a backbone of α (1→4)-D-galacturonic acid with partial methyl esterification of the carboxyl groups (May 1990). At least 65% of the extracted material must be galacturonic acid, in order for extracted material to be classified as commercial pectin (Committee on Food Chemicals Codex 1996; FAO 2009).

1.4 Commercial processing of pectin

Even though most plant tissues contain pectin, the major sources for the commercial production of pectin are citrus peel and apple pomace. This is mainly because of the availability of citrus peel and apple pomace from commercial processing operations. In addition, both sources have high contents of galacturonic acid and good gelling properties for food applications. Pectin is usually isolated from either wet or dry fruit residues. Pectin, with better quality characteristics, is obtained from wet fruit residues, since pectin is a heat labile material and quality is lost in pomace drying process. However, apple pomace and citrus peel, in the wet state, are very perishable, especially due to microbial activity. Molds infestations could produce pectin-degrading enzymes (*i.e.*, pectinases), which render pectin in the raw material unacceptable for food uses. Therefore, in order to allow the storage, transportation, and supply throughout the year the starting material is generally dried (Rolin 2002).

Commercial production of pectin, which consists of a series of processes and steps, is illustrated in Figure 1.3 (p. 47). First, the raw material is washed with water at low temperature (below 15ºC) to remove undesirable components, such as sugars, acids, glycosides, and pigments. Sometimes, this step is avoided because a certain amount of pectin can be lost with other solubles during the process (Turakhozhaev and Khodzhaev 1993). Next, insoluble pectin (protopectin) is hydrolyzed and extracted; in practice these two processes are collectively referred to as "pectin extraction". Pectin extraction is considered the most important process in the production process (Garna et al. 2007). It is a multi-stage physicochemical process where pectin molecules are hydrolyzed and solubilized from the cell walls and middle lamella of the plant tissue. Various extraction methods involving acids, alkalines, and enzymes, are available. All these methods primarily focus on breaking the bonds between pectin and other compounds, with minimum damage to the chemical structure of pectin (Andres et al. 1978). Commercial pectins are typically obtained by hot acid extraction, cheaper mineral acids, such as hydrochloric and sulfuric, are preferred for this process (Sandhu and Minhas 2006). The extraction conditions vary with raw material, desired type of pectin, and process economics. In general the hot acid extraction process conditions are employed in the ranges; pH 1-3, temperature 50-90ºC, and duration 3-12h (Rolin 2002).

Pectin is purified before and after precipitation. Foreign molecules, especially biopolymers, such as starch, are removed with an enzyme treatment, mineral salts are removed by dialysis or with the aid of ion-exchange resins, and undispersed solids are removed by filtration. Prior to pectin precipitation, the solution is concentrated by evaporation or membrane filtration (Rolin 2002; Turakhozhaev and Khodzhaev 1993).

Pectin is precipitated along with other low molecular weight compounds (*e.g.* ash), and therefore, it is necessary to "wash" the precipitate with additional alcohol to eliminate the remaining traces of contaminants. After precipitation, the extracted pectin is dehydrated by either drum or spray drying. Then the dried pectin is milled and sieved into a powder. Finally, the extracted pectin is submitted to a standardization process, in which different batches of pectins are combined in order to meet a set of specifications. Certain specifications are required by regulatory authorities, while others (such as degree of esterification) are to characterize the type of pectin. For "grading strength", which is the amount of pectin needed to impart a specific effect (*e.g.* gel firmness), pectin is further diluted to the specified strength using sugar, generally sucrose (Rolin 2002; Turakhozhaev and Khodzhaev 1993).

1.5 Functionalities and applications of pectin

Pectin is commonly utilized as a gel forming agent in food and pharmaceutical applications (Liu et al. 2003). The gel forming ability of pectin depends primarily on the degree of esterification (DE), which is the proportion of galacturonic acid units that are esterified with methanol (Sriamornsak 2003). Commercial pectins are classified based on their DE; high methoxyl pectins (HM, DE>50) and low methoxyl pectins (LM, DE <50).

A pectin gel is formed when portions of the homogalacturonan backbone are crossed-linked forming a continuous three-dimensional network, which is able to entrap water and solutes (Willats et al. 2006). The "joints" of homogalacturonan portions are often referred as "junction zones". Various forces may be involved in the formation of a junction zone (Thakur et al. 1997). In high methoxyl (HM) pectins junction zones are formed by hydrogen bonds and hydrophobic forces between methoxyl groups of pectin

molecules. To permit the formation of junction zones in HM pectins, low pH (<3.6) and soluble solids (usually sucrose) at a concentration higher than 55% (w/w) are required. In contrast, LM pectins require the presence of divalent metallic cations, such as calcium, to form junction zones by ionic cross-linking between non-esterified carboxyl groups (Willats et al. 2006). The rheology of pectin gels depends on properties of the pectin, such as molecular weight, attached neutral sugars, and degree of acetylation (Baïer et al. 1994).

Pectin, like most naturally occurring polysaccharides, can create viscous solutions; viscosity depends largely on the concentration of pectin and its molecular weight. At low concentrations (<0.5% w/w), pectin solutions exhibit Newtonian behavior, but as the concentration increases pectin solutions exhibit non-Newtonian, pseudoplastic characteristics. Pectins with higher molecular weight will exert higher viscosity. Other factors, such as degree of esterification, solvent, pH, soluble solids, and temperature also influence the viscosity of pectin (Rolin 2002; Voragen et al. 1995).

In addition to the gelling and thickening properties, certain pectins possess emulsifying and/or emulsion stabilizing properties. Studies have suggested that the emulsifying property of pectin could be originating from (a) the presence of acetyl groups, which enhance the hydrophobicity of pectin, (b) the protein residues present within the pectin, or (c) a combination of both (Drusch 2007; Leroux et al. 2003). Studies by Endress and Rentschler (1999) suggested that the emulsion stabilizing property of citrus and apple pectin were mainly based on the increase of viscosity of the emulsion, suggesting that pectins with a high degree of esterification were effective in stabilizing oil-in-water emulsions.

In addition, pectin is able to stabilize proteins in suspension. For example, pectin can stabilize milk protein under acidic conditions. It is thought that the homogalacturonan (HG) region of pectin, which is negatively charged, bind to the positively charged proteins in milk, preventing aggregation and sedimentation of casein (Willats et al. 2006).

1.5.1 Pharmaceutical applications of pectin

The use of pectin in the pharmaceutical industry is growing, but the amount used is much smaller compared to the food uses (Brejnholt 2010). Pectin is used in many pharmaceutical preparations as an excipient, thickener, stabilizer, film coating, and binding agent (Sriamornsak 2003). Due to its ability to bind positively charged heavy metal ions, pectin is used as a detoxifying agent. It has been used to remove toxic heavy metal ions, such as lead and mercury, from the gastrointestinal tract and respiratory organs of individuals who have been poisoned with heavy metals (Endress 1991). Since pectin is able to delay blood clotting, it is also used as hemostatic device to control hemorrhage or localized bleeding (Endress 1991).

Pectin has been widely utilized as a drug carrier, and as ingredient in controlled and sustained drug release formulations (Wong et al. 2011). Delivery systems designed to release drugs in the colon need to protect the drug during transit through the stomach and small intestine. The resistance of pectin to digestion in the upper gastrointestinal tract has allowed its use in the development of colon-specific drug delivery systems (Desai 2005; Liu et al. 2003). Tablets coated with a pectin film as well as pellets and microparticles made from pectin-based matrices are commonly used to successfully deliver drugs to the colon (Wakerly et al. 1996; Wong et al. 2011).

1.5.2 Food applications of pectin

Pectin is known for being the traditional gelling agent in jams and jellies (May 1990). Nowadays there is a large variety of commercial pectins, differing mainly in their degrees of esterification, available to meet the requirements of different jams or similar fruit containing, sugar rich, highly viscous systems (Voragen et al. 1995). Although the use of pectin in jams and jellies is still one of the largest markets for pectin (Brejnholt 2010), it is utilized in other foods as a thickener, texturizer, emulsifier, and stabilizer (FAO 2009).

Due to its stabilizing and thickening properties, pectin is widely used in the dairy industry. To prevent aggregation and precipitation of caseins, pectin is used in low acid (pH 3.5 - 4.2) milk products that are heat processed (Voragen et al. 1995). Pectin has various roles in yogurt depending on the type of product; as a thickener in spoonable yogurts; as a water binder in stirred yogurts; and as an emulsifier and to provide fat-like mouthfeel in low-fat yogurts. In addition, pectin is used in the production of fruit bases for yogurts to ensure uniform distribution of the fruit and to reduce color migration from the fruit to the yogurt (May 1990; Voragen et al. 1995).

Pectin has a variety of applications in the bakery industry. It is used to retain moisture and to improve volume, flexibility, and softness in breads. In frozen dough, pectin is used to delay the retrogradation of starch, while stabilizing the volume of the dough during freezing (Brejnholt 2010). The stabilizing and thickening properties of pectin have allowed its use in many other food products, such as mayonnaise, salad dressing, tomato ketchup, protein foams, and beverages (Pilnik and Voragen 1992).

New applications for pectin have emerged due to the increasing awareness of healthy life-styles of consumers, along with the increasing demand for functional food products. For example, pectin is commonly used in low-fat foods as a fat replacer (Min et al. 2010). Foods can be also coated with pectin and polyvalent cations prior to frying, to reduce the absorption of oil (Gerrish and Carosino 2001).

1.5.3 Unique properties of pectin for encapsulation of lipophilic compounds

Encapsulation of lipophilic compounds requires the preparation of emulsions, usually oil-in-water emulsions, which should be stable during the entire encapsulation process. Pectin is a polymer which is able to produce stable emulsions at low concentrations (Gharsallaoui et al. 2010). Sugar beet pectin has been used as an emulsifying wall component for the encapsulation of fish oil. The presence of acetyl and ferulic acid groups, as well as the high protein content of sugar beet pectin have been attributed to the enhancement of emulsion stability, and thus the improvement of the encapsulation of fish oil (Drusch 2007). Moreover, the negatively charged carboxylic side groups on pectin can electrostatically interact with the cationic groups of proteins (Syrbe et al. 1998). These interactions have been used to develop multilayered membranes around lipophilic compounds, which are utilized as encapsulation systems (Gharsallaoui et al. 2010; Humblet-Hua et al. 2011).

Pectin is a film-forming polymer. Fishman and Coffin (1995) conducted studies on the use of pectin and starch mixtures to produce flexible films with high modulus. The films were considered to be useful as coatings and controlled release carriers for food and pharmaceutical applications. Moreover, recent studies have shown that enteric coatings

and spray dried microparticles, both based on gelatinized high amylose starch and high methoxyl pectin, are stable in simulated gastro-intestinal environments (Desai 2005; Dimantov et al. 2004). Therefore, combinations of pectin and starch are useful, not only for microencapsulation, but also for targeted delivery of bioactive compounds, such as vitamin E, to the colon.

1.6 Use of pectin in encapsulation of vitamin E

1.6.1 Chemistry and functionality of vitamin E

Vitamin E represents a family of fat-soluble molecules composed by a chromanol ring and a phytol chain. There are eight vitamin E molecules known to occur in nature: four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). The prefixes α, β, γ, and δ indicate the position of methyl groups on the chromanol ring. Vitamin E molecules differ in the saturation of the side chain; tocopherols have a saturated side chain whereas tocotrienols have three conjugated double bonds (Bramley et al. 2000). In nature, α-tocopherol is the most abundant and the most biologically potent antioxidant form of vitamin E (Ball 2006; Eitenmiller and Lee 2004; Lin et al. 2006).

In humans vitamin E (α-tocopherol) functions as the principal tissue and plasma antioxidant (Traber and Atkinson 2007) and it is exclusively obtained from foods and supplements (Burton et al. 1983). Some free radicals are highly reactive and can attack proteins, nucleic acids, phospholipids, and other cellular macromolecules causing oxidative stress, resulting in the impairment of normal cellular activity (Bramley et al. 2000). Vitamin E (α-tocopherol) is capable in donating its phenolic hydrogen atom to react with and quench free radicals, thereby preventing lipid peroxidation and other

radical driven oxidative events (Bramley et al. 2000). In addition to its antioxidant function, vitamin E (α-tocopherol) is involved in other metabolic processes such as cellular signaling, regulation of gene expression, and enhancement of the immune system (Azzi and Stocker 2000; Traber and Sies 1996).

The recommended dietary allowance (RDA) for α-tocopherol is 15mg/day for males and females of 14 years and over (Food and Nutrition Board 2011). Although vitamin E (α-tocopherol) deficiency is generally observed in premature babies and individuals with either malabsorption syndromes or liver disease (Traber and Sies 1996), the intake of vitamin E has been related to health promotion and prevention of a variety of degenerative diseases, such as coronary heart diseases, cancer, inflammatory diseases, neurological disorders, and cataract and age-related macular degeneration (Bramley et al. 2000; Higdon et al. 2004). Despite the evidence that vitamin E may contribute to lower the risk of certain chronic diseases, the majority of men and women in the United States fail to meet the current recommendations for vitamin E intake (Maras et al. 2004).

The food industry has widely used vitamin E, as an antioxidant, to minimize oxidative reactions in products. For example, reactive oxygen species interact with functional molecules resulting in the formation of off-flavors and loss of nutritional value. The addition of vitamin E into food contributes to increased product stability and quality, and, therefore, an increased consumer acceptance (Faria et al. 2010). Vitamin E has also been used in food fortification to improve nutritional value. However, vitamin E decomposes easily in the presence of light, oxygen, alkaline pH, and transition metal ions (Ball 2006). Food is exposed to most of these destructive agents during processing and

storage. In addition, vitamin E is insoluble in water (Bramley et al. 2000), limiting its utilization in aqueous or hydrophilic food products.

Encapsulation could be applied as an appropriate mean to protect vitamin E from degradation during food processing, storage, transportation, and utilization. Encapsulation could increase the effectiveness and ensure optimal dosage of vitamin E. In addition, the encapsulation of the liposoluble vitamin would allow its incorporation into various food products with relatively hydrophilic matrices.

1.6.2 Microencapsulation in the food industry

Commercial food processing often requires the addition of functional compounds into food products, to obtain certain quality traits. These compounds may include preservatives and those that provide health benefits. However, adding these compounds into food presents many challenges, particularly with respect to the stability of the functional compounds during food processing and storage. Undesirable interactions could take place between the functional compounds and the food matrix (Champagne and Fustier 2007), causing loss of intended functionalities.

Microencapsulation, refers to a process by which one material or mixture of materials (core material, which carries the bioactive or functional properties) is coated with or entrapped within another material (wall material) (Finney et al. 2002). Microencapsulation has significant relevance to the food industry, since it is used to overcome critical issues in utilization of functional compounds. In addition, microencapsulation can be used to mask undesirable properties of the core material, such as off flavors, colors, etc., to reduce the transfer of the core material to the environment

or food matrix, to promote easier handling of the core material, and to control the release of the core material at the intended target upon ingestion (Shahidi and Han 1993).

There are certain requirements that an encapsulated delivery system is expected to meet: it should be prepared from food grade ingredients, cost-effective processing, be stable during various processing operations, deliver the core material efficiently, and improve the bioavailability of the core material (McClements et al. 2007; McClements and Li 2010).

Various techniques have been developed for microencapsulation of unstable compounds for food applications. These techniques can be classified into three groups: i) physical, ii) chemical, and iii) physicochemical (Table 1.2, p.42). The selection of a encapsulation technique depends on various factors, such as process economics, sensitivity of the core material, desired size of the microcapsule, properties of both the core and wall materials, and desired release mechanism (Shahidi and Han 1993).

1.6.2.1 Microencapsulation by spray drying

Despite the large number of available choices, spray drying is the most common technique of microencapsulation used in the food industry (Gharsallaoui et al. 2010). It is as a well-established and inexpensive processing operation that has been traditionally used in the food industry (Gouin 2004). In addition, spray drying can be utilized in the microencapsulation of heat sensitive materials including, but not limited to, flavors, vitamins, and fatty acids (Champagne and Fustier 2007).

The microencapsulation of lipophilic materials by spray drying generally starts with the formation of a stable emulsion (Figure 1.4, p. 48), which is prepared by the

dispersion of the core material in a solution of the wall material by homogenization of the dispersion (Gonnet et al. 2010). Then the emulsion is fed into a spray drier and atomized at the spray nozzle into a drying chamber. The atomization of the emulsion can be carried out by different types of atomizers, such as pressure nozzle, two-fluid nozzle, and three-fluid nozzle. As soon as the atomized particles come in contact with the drying air, the evaporation of the solvent (usually water) occurs instantaneously. The high water evaporation rate leads to the formation of a crust at the surface of the particle. Despite the high temperatures, generally used in the process, evaporative cooling, short time of exposure, and absorption of heat during solvent evaporation maintain the particles temperature sufficiently low, reducing the likelihood of heat damage to the core material. The dry particles are separated from the air using a cyclone (Gharsallaoui et al. 2010).

1.6.2.2 Wall materials for lipophilic core materials

Encapsulation efficiency and core material stability depend largely on the wall material composition and properties (Gharsallaoui et al. 2010). Generally, spray drying process is carried out using aqueous formulations. Therefore, the encapsulating agents should not only be compatible with the core material, but also should be water-soluble (Gouin 2004). In addition, wall materials must have good emulsification properties, film forming abilities, achieve high loading capacity of the core material, and provide protection to the core material against degradation (Reineccius 1988).

There are various wall materials derived from natural and synthetic sources available for the encapsulation of active ingredients for food uses. These include gums (gum arabic, alginates, etc), proteins (whey protein, sodium caseinate, gelatin, etc), and

modified starch derivatives (maltodextrin, OSA starch, etc) (Gouin 2004). Often times a combination of materials, from various groups, is used in order to obtain desired properties in an encapsulate a food ingredient (Madene et al. 2006). The selection of a wall material depends primarily on the characteristics of the core material, process of encapsulation, economics, trigger response to prompt the release of the encapsulated compound (*e.g.* pH change, mechanical stress, temperature, enzymatic activity, and time), and whether the encapsulating material is approved for food use by regulatory authorities (Gouin 2004; Madene et al. 2006).

Microencapsulation, of lipophilic ingredients by spray drying, requires wall materials that have good emulsifying and film forming properties. Gum arabic is one of the most commonly used encapsulating agents due to its high water solubility, low viscosity, and emulsification properties. However, the high cost and frequent shortages of gum arabic has resulted in demands for cheaper wall materials with consistent market supply (Gibbs et al. 1999). Table 1.3 (p.43) gives examples of various wall materials used for the microencapsulation of lipophilic compounds. There is a growing interest on the use of food starches for the development of delivery systems. Availability, low cost, film forming ability, and being inert (*i.e.*, does not react with food components) are some of the characteristics that make starch a promising material for encapsulation (Lay Ma 2010).

Certain properties of unmodified starches, such as high viscosity when mixed with water, and low affinity to lipophilic compounds, make them unsuitable for microencapsulation. Therefore, starch is modified by physicochemical processes, *e.g.* heat treatment, acid or enzymatic hydrolysis, esterification (Gibbs et al. 1999). Examples

of hydrolyzed starches include dextrin (a highly branched polymer, formed by the heating of starch with acid or base, to improve its water solubility and viscosity) and maltodextrin (glucose polymers of various lengths formed by the partial hydrolysis of cornstarch with acids or enzymes). Both dextrins and maltodextins have good film forming properties and are soluble at high solid concentrations. However, they are not good emulsifiers (Madene et al. 2006). Octenyl succinic anhydride (OSA) starch, is a commercially available modified starch prepared by a base catalyzed reaction of alkenyl succinic anhydride with granular starch in aqueous suspension (Tessler and Billmers 1996; Trubiano 1986). The incorporation of hydrophobic alkenyl groups into the hydrophilic starch molecule results in a starch with amphiphilic characteristics that are useful to stabilize oil-in-water emulsions and to encapsulate lipophilic material by spray drying (Trubiano 1986).

1.6.3 Pectin as a wall material for the encapsulation of lipophilic compounds

Various types of pectins have been successfully used in the encapsulation of certain functional food ingredients, to improve not only the encapsulation efficiency but also to protect the core material (Drusch 2007; Madziva et al. 2005; Sansone et al. 2011). Due to its functional properties, pectin may serve as an emulsion stabilizer and film forming agent in the development of food delivery systems (Li et al. 2008). Madziva et al. (2005) used low methoxyl pectin, in combination with alginate, to encapsulate folic acid, the encapsulation efficiency, retention, and stability of this vitamin was greater at the higher concentration of pectin (30%). Sansone et al. (2011) encapsulated nutraceutical extracts (from *Fadogia ancylantha, Melissa officinalis*, and *Tussilago farfara*) using a combination of maltodextrin with high methoxyl apple pectin to prepare particles with

smaller size, greater loading, and an acceptable level of protection. Drusch (2007) used a wall matrix of sugar beet and glucose syrup to encapsulate fish oil. The used of small proportions of sugar beet pectin (2.2%) allow the production of emulsion with a high content (50%) of fish oil, which was successfully encapsulated by spray drying.

Due to the film forming and emulsifying properties of pectin and the amphiphilic characteristics of OSA starch, a combination of the two could be used to develop a delivery system to encapsulate more efficiently lipophilic bioactive compounds, such as vitamin E. Since pectin is resistant to digestion by human gastrointestinal enzymes but degraded by colonic microflora (Liu et al. 2003), this delivery system have the potential to protect the bioactive compounds during transit through the gastrointestinal tract, while providing controlled release at targeted locations.

1.6.4 Use of pectin in controlled release applications

Encapsulation is a method used not only to protect unstable, functional food ingredients, but also to control their release under specific conditions. Controlled release is used in order to make the active ingredient available at a desired site and time, at a specific rate, while increasing the effectiveness of the active ingredient (Pothakamury and Barbosa-Cánovas 1995). The encapsulated active ingredient is released under the influence of a specific stimulus (or "trigger"), such as change in temperature, moisture or pH, pressure or shear, and enzymatic activity (Pothakamury and Barbosa-Cánovas 1995). Pectin-derived wall matrices have been extensively studied for colon-specific drug delivery (Liu et al. 2003). Colon is a site with pH near to neutral, longer transit time, and low enzymatic activity that make it a favorable environment for delivery and absorption

of drugs (Lorenzo-Lamosa et al. 1998). The use of pectin in control release is effective because pectin-based coatings or particles remain intact during transmit through the upper parts of the gastrointestinal tract. Pectin is biodegraded by colonic bacteria, providing promising opportunities for colonic drug delivery, using pectin-based matrices (Liu et al. 2003). This same rationale could be applied in the development of delivery systems for functional food ingredients.

1.7 Summary

Apple pomace is a byproduct from the juice and cider processing industry. Being a rich source of carbohydrates, fibers, and other minor nutrients, efforts have been made to utilize apple pomace in value added products. Pectin extraction has been considered as an economical and effective way to utilize apple pomace. Pectin is a cell wall structural carbohydrate abundant in apple pomace, and its extraction is a complex physicochemical process. Although many studies have been conducted on the extraction of pectin from various sources, the available knowledge-base on pectin extraction from apple pomace, especially the selection of process conditions, is still scarce.

Pectin is used by the food industry, for many applications, due to its gelling, thickening, emulsifying, and stabilizing properties. The pharmaceutical industry has also used pectin in various applications, in the development of drug delivery systems, specifically those directed to the colon. Pectin could be used in the development of delivery systems for lipophilic functional compounds, such as vitamin E. Pectin-based delivery systems could be very useful not only to enhance the quality of food products, but also to promote health benefits.

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Table 1.1 Common applications of apple pomace.

Application	Examples	References
Direct use[1]	Animal feed, compost, source of dietary fiber in bakery products, press aid in fruit juice preparation.	Singh and Narang (1992), Gassara et al. (2011), Masoodi et al. (2002), Roberts et al. (2004)
Extraction of bioactive compounds	Dietary fibers, antioxidants, pectin, oil.	Figuerola et al. (2005), Wijngaard and Brunton (2009), Schieber et al. (2003), Constenla et al. (2002), Tian et al. (2010)
Products obtained via fermentation	Organic acids, ethanol, aroma compounds, pigments, protein enriched animal feed, enzymes, heteropolysaccharides, baker's yeast.	Hang and Woodams (1984), Joshi and Sandhu (1996), Longo and Sanromán (2006), Attri and Joshi (2005) Villas-Bôas et al. (2003), Berovič and Ostroveršnik (1997), Stredansky and Conti (1999), Bhushan and Joshi (2006)

[1] Apple pomace is dried prior use for most direct uses, except for composting.

Table 1.1 *Continued.*

Application	Examples	References
Fuel production	Biogas, ethanol	Jewell and Cummings (1984), Hang (1987)
Substrate for edible mushrooms	Shiitake and oyster mushroom	Worrall and Yang (1992)

Table 1.2 Techniques used for food ingredient encapsulation, adapted from Shahidi and Han (1993)

Physical techniques	Chemical techniques	Physicochemical techniques
Spray drying	Molecular inclusion	Coacervation
Spray chilling	Interfacial polymerization	Liposome entrapment
Spray cooling		Organic phase separation
Fluidized bed coating		
Extrusion		
Centrifugal extrusion		
Freeze drying		
Cocrystallization		
Centrifugal suspension-separation		
Use of supercritical fluids		

Table 1.3 Wall materials used in the microencapsulation by spray drying of lipophilic compounds.

Wall material	Characteristics	Example	Core materials	References
Hydrolized starches	Oxygen barrier, low viscosity at high solid content, good solubility, film forming ability, limited emulsifying ability.	Maltodextrin	Cinnamon oleoresin; linoleic acid	Vaidya et al. (2006), Minemoto et al. (2002)
Modified starches	Good emulsifying ability, use subject to regulations.	OSA starch	Vitamin A; d-limonene	Xie et al. (2010), Paramita et al. (2012)
Gums	Film forming and emulsifying ability, retention of volatiles, quality and supply fluctuation.	Arabic gum, mesquite gum	Orange oil; cardamom oil;	Kim and Morr (1996), Beristain et al. (2001)
Cyclodextrins	Oxygen barrier, formation of inclusion complexes, relative expensive.	β-cyclodextrin	d-limonene; ethyl n-hexanoate; lemon oil	Shiga et al. (2001), Bhandari et al. (1999)

Table 1.3 *Continued.*

Wall material	Characteristics	Example	Core materials	References
Proteins	Film forming and emulsifying ability, expensive, dependent of pH and ionic strength, denaturation at high temperatures, potential to cause allergies.	Whey protein	Fish oil; avocado oil	Legako and Dunford (2010), Bae and Lee (2008)

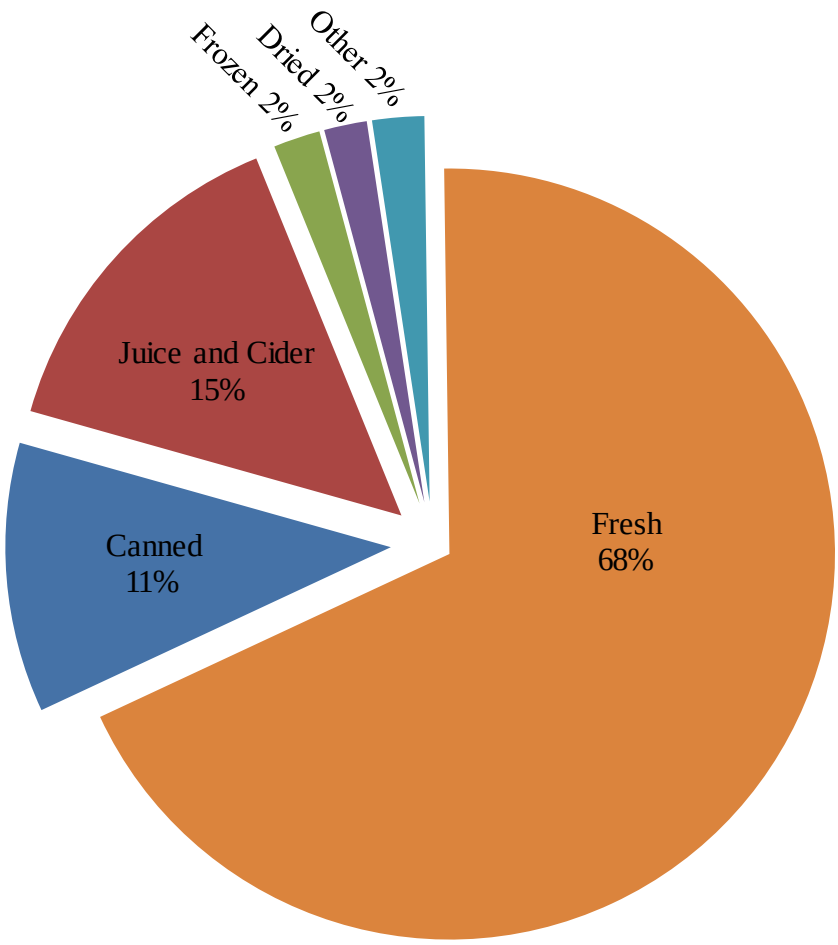

Figure 2.1 Utilization of the apple production in the United States in 2010, adapted from USDA database-Apple Statistics (USDA 2012).

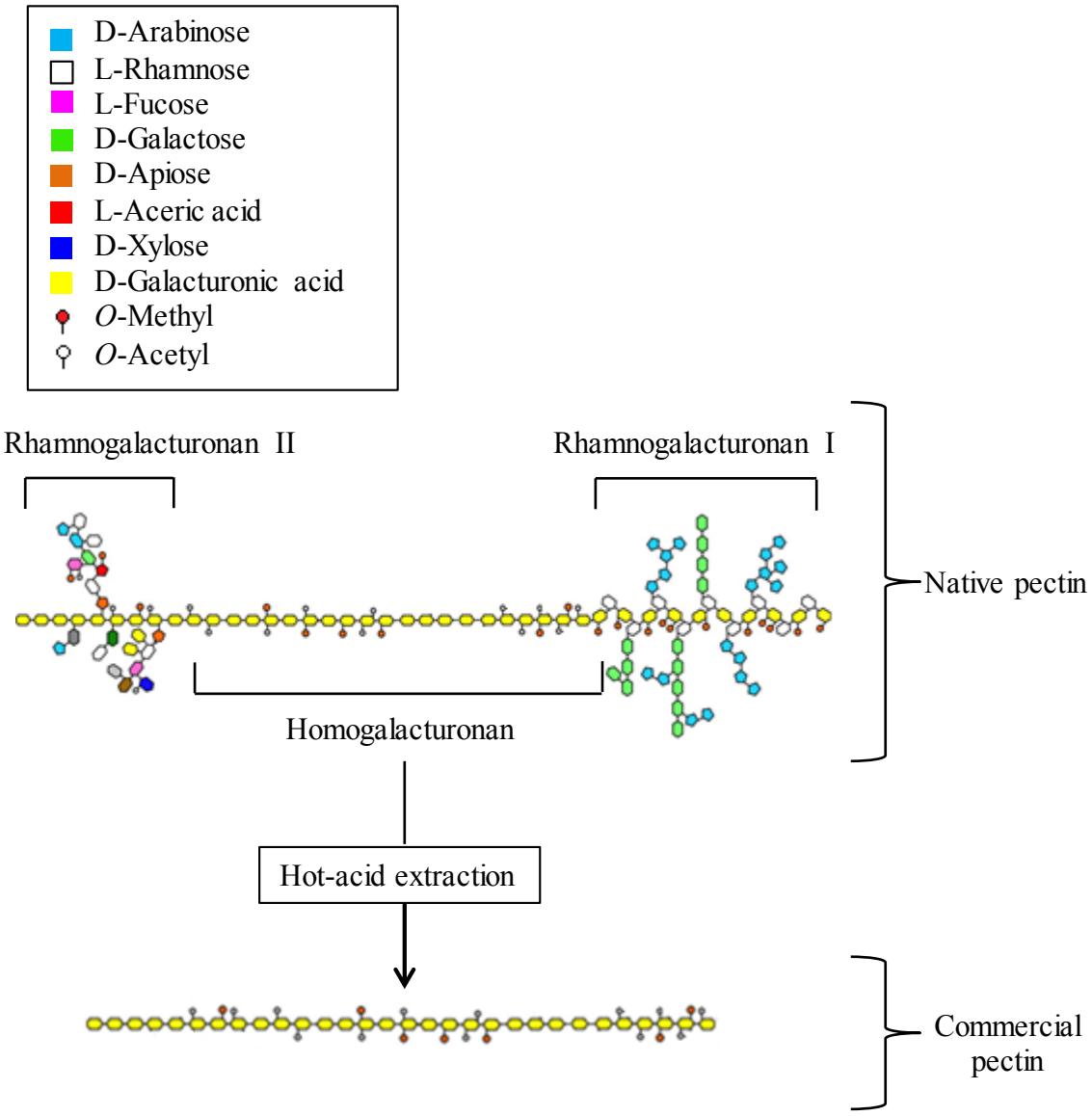

Figure 1.2. Schematic structure of native and commercial pectin, adapted from Harholt et al. (2010). With permission from American Society of Plant Biologists.

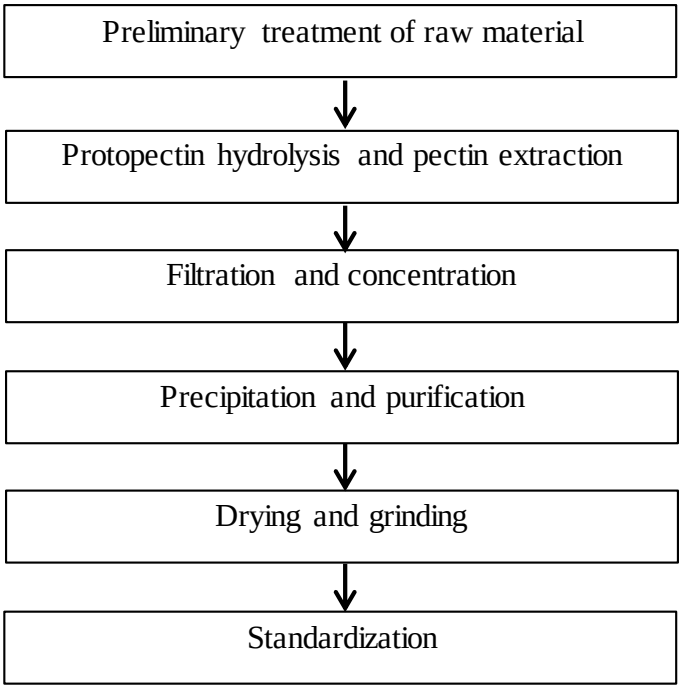

Figure 1.3 Typical process for the commercial production of pectin, adapted from Turakhozhaev and Khodzhaev (1993).

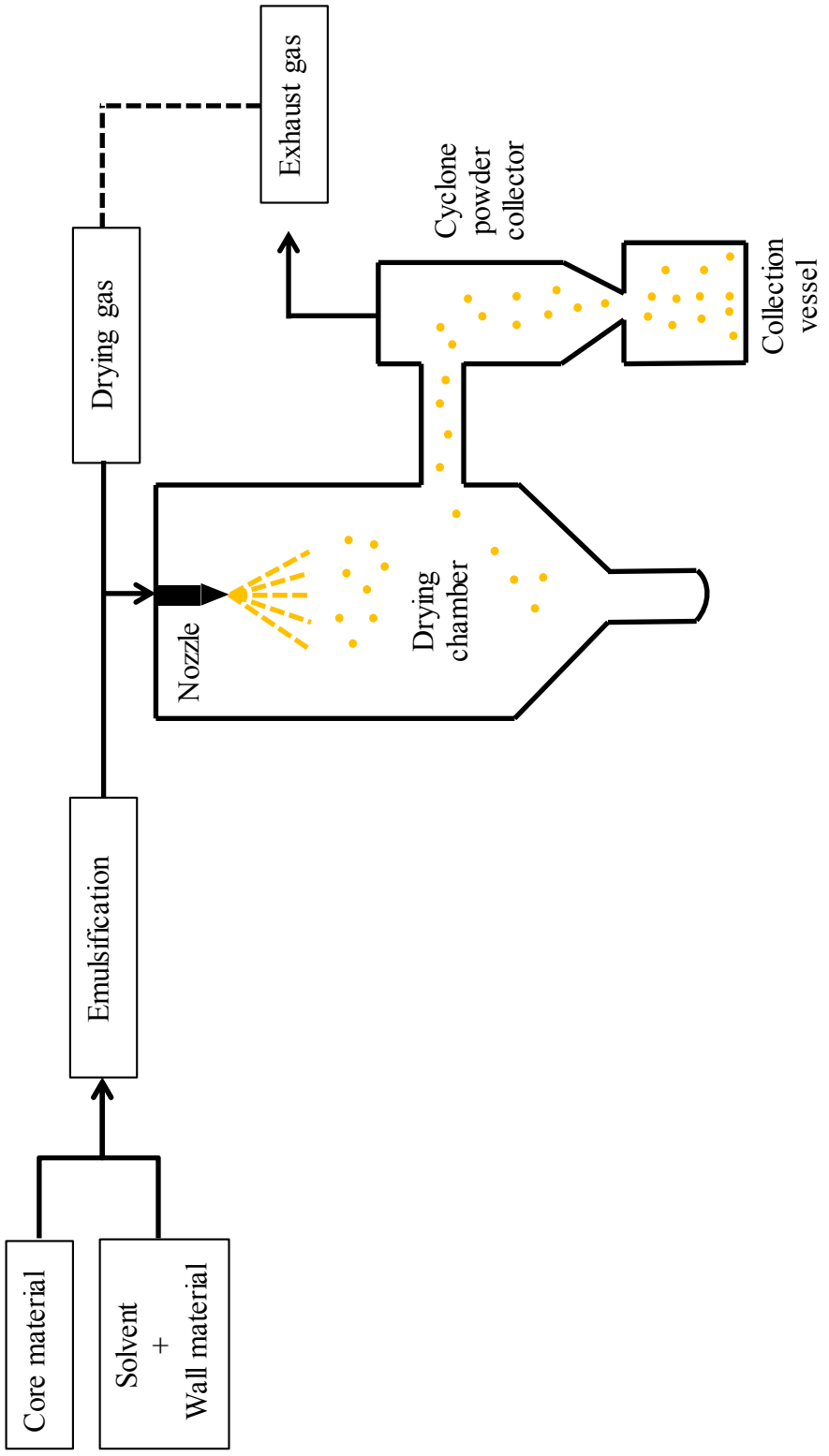

Figure 1.4 Preparation of encapsulated particles by spray drying under laboratory conditions.

CHAPTER 2. EXTRACTION OF PECTIN FROM FRESH APPLE POMACE: SELECTING OPTIMUM CONDITIONS TO MAXIMIZE YIELD.

ABSTRACT

Pectin is a cell wall polysaccharide widely used in the food industry as a gelling agent, stabilizer, emulsifier, and thickener. Pomace, the main byproduct of apple juice and cider processing, is known to be a good source of pectin. The aim of this study was to optimize process conditions for the hot acid extraction of pectin, from fresh apple pomace, to maximize yield. Three extraction conditions; pH (1.5-3.5), temperature (50-90°C), and hot acid extraction time (30-180 minutes) were selected and optimized based on pectin yield and uronic acid content, using a Box-Behnken experimental design. Process conditions were optimized, based on pectin yield, using a linear model. An amount of 8.6% (w/w d.b) yield was obtained, under selected conditions; pH 1.5, temperature 90°C, and time 120 minutes. A partial physicochemical characterization was performed on the extracted pectin. Pectin, extracted under optimum conditions, had a degree of esterification of 66.68%, molecular weight of 10^6 Da, uronic acid content of 64.67% (w/w d.b), and a neutral sugar content of 11.58% (w/w d.b).

2.1 INTRODUCTION

The processing of apple fruits for juice and cider making results in large amounts of solid byproducts, which are termed “apple pomace”. Apple pomace accounts for 25-35% (w/w) of the fresh apple fruits that are processed (Gullon et al. 2007). In 2010, approximately 2×10^5 tonnes of apple pomace were produced in the United States (USDA 2012).

Apple pomace is mainly composed of carbohydrates, fibers, proteins, and small quantities of minerals. It is considered a rich source of dietary fiber, especially pectin, with a content in the range of 10-15% (w/w d.b), depending on the source (Bushan and Gupta 2013).

Various approaches have been investigated to effectively utilize apple pomace. However, the production of pectin is considered the most reasonable utilization for apple pomace, according to previous studies (Bhushan et al. 2008; Dilas et al. 2009; Fox et al. 1991). The feasibility of the processing operations of pectin production and the wide use of pectin in various applications make pectin extraction an effective way to utilize apple pomace.

Pectin extraction is a multi-stage physicochemical process, where pectin molecules are hydrolyzed and solubilized from the cell wall and middle lamella of the plant tissue (Kertesz 1951). Extraction conditions used in the process vary depending on the raw material, desired type of pectin, and process economics. Commercial pectin is usually produced by hot acid extraction, followed by filtration, alcohol precipitation, drying, grinding, and standardization (May 1990).

Most recent advances in pectin extraction technology have not been published in the literature due to their proprietary nature. Many investigators have attempted to understand the effect of extraction conditions on yield, composition, physicochemistry, and functionality of pectin, from various sources, with limited success. The available literature on pectin extraction from apple pomace is scarce. Moreover, most published studies have focused on the extraction of pectin from dried plant material (Canteri-Schemin et al. 2005; Constenla et al. 2002; Hwang et al. 1998; Marcon et al. 2005),

whereas very few studies have used fresh plant material (Garna et al. 2007; Rascon-Chu et al. 2009) as the source.

Pectin production is sometimes considered an art rather than a science due to the multiple factors influencing the extraction process and their inter-dependencies. Extraction temperature, extraction pH, and hot acid extraction time are considered the most important factors in the pectin extraction process because they have shown significant effects on different pectin characteristics; *e.g.* molecular weight, degree of esterification, and yield (Canteri-Schemin et al. 2005; Garna et al. 2007; Kalapathy and Proctor 2001; Kulkarni and Vijayanand 2010; Marcon et al. 2005; Pagan et al. 2001; Vriesmann et al. 2012; Yapo et al. 2007). Generally, higher yields of pectin have been obtained at high extraction temperature, low extraction pH, and long hot acid extraction time. However, under these conditions the degradation of the pectin molecule is promoted, resulting in low molecular weights and low degree of esterification, which negatively affect the functional properties of pectin. Selecting the ideal combination of raw material and extraction conditions is critical in obtaining high yields of pectin, without compromising the desired quality. The aims of this study were; (1) to investigate the effect of three extraction factors (*i.e.*, extraction temperature, extraction pH, and hot acid extraction time) on pectin yield and (2) to establish optimum process conditions for pectin extraction from fresh apple pomace.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Apple pomace (from four varieties; Jonagold, Gala, Idared, and Golden delicious, from the September of 2012 harvest season) were obtained from the Kimmel Orchard &

Vineyard, Nebraska City, NE, USA. For this study a composite of pomace was made using equal proportions of each variety. Apple pomace samples were promptly collected after cider processing and stored at -22°C until use. All chemicals and solvents used for the experiments were of ACS certified grade.

2.2.2 Experimental design and statistical analysis

A response surface experimental design, Box-Behnken design with three independent variables, was used to determine the optimum processing conditions for pectin extraction from fresh apple pomace. The variables used were extraction temperature (30, 70, and 90°C), extraction pH (1.5, 2.5, and 3.5), and hot acid extraction time (30, 105, and 180 minutes). The levels of each variable were selected based on literature (Rolin 2002) and a series of preliminary tests (results are not reported here). Actual and coded values of variation levels are shown in Table 2.1 (p.72). Two responses (1) yield and (2) pectin content were measured. Yield was defined as the percentage of the extracted dried pectin to total dry matter of the apple pomace used for extraction; while pectin content was defined as the percentage of uronic acid in the extracted pectin (Vriesmann et al. 2012). The experimental design comprised of a total of 17 experiments with 5 center points and 12 factorial points (Table 2.3, p.74). Design and data analysis were carried out using Design Expert statistical software (version 7.1.3, Stat-Ease, Inc., Minneapolis, MN, USA).

The analysis of variance (ANOVA) was performed to validate the models for the process optimization. The optimal extraction conditions were estimated through

regression analysis and three-dimensional response surface plots of the independent variables and each dependent variable.

Physical and chemical characterizations were conducted, at least in triplicates. All numerical results are expressed as mean values and standard deviations (SD).

2.2.3 Proximate composition of apple pomace composite

Proximate composition of apple pomace composite was analyzed by standard AACC (AACC International 2002) and AOAC (AOAC International 1990) methods as follows: moisture – AOAC 920.151, crude protein – AOAC 990.03, total dietary fiber – AACC 32-25, fat – AOAC 920.39, and ash – AACC 08-01. Total sugars and starch were analyzed using YSI Select 2700 Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH, USA) (YSI 2000).

2.2.4 Pectin extraction

Garna et al. (2007) method, with minor modifications, was used for pectin extraction. Extraction conditions, *i.e.*, temperature, pH, and hot acid extraction time, were established based on the experimental design (Table 2.3, p.74).

Apple pomace (140g, from frozen sample) was blended with 500ml of distilled water for one minute using a laboratory blender (31BL91, Waring Commercial, Torrington, CT, USA) at high speed. The slurry was transferred to a 1L glass beaker and 500ml of distilled water were added and heated under continuous stirring (350rpm) using a digital hot plate stirrer with a thermostat control (HS40, Torrey Pines Scientific, Inc., San Marcos, CA, USA) until 20°C was reached. Hydrochloric acid was added to the

dispersion to set the pH to 1.5, 2.5, or 3.5; pH was monitored using and pH meter equipped with a temperature compensation probe (SA520, Orion, Boston, MA, USA). Dispersions were heated at the specific extraction temperature under continuous stirring (350rpm) using a digital hot plate stirrer.

The slurry was cooled to room temperature, within approximately 30 minutes, using an ice bath. To separate the liquid portion, the slurry was centrifuged at 4000*g* for 30 minutes in a centrifuge (Sorvall Legend XTR, Thermo Scientific, Madison, WI). The supernatants were filtered through a Buncher funnel with a Whatman No.4 filter paper connected to a vacuum filtration system (2515, Gardner Denver Welch Vacuum Technology, Inc., Niles, IL, USA) operated at a vacuum pressure of ~7.4psi. The remaining solids were centrifuged and filtered under the same conditions given above. The two filtrated supernatants were combined and the pH was adjusted to 3.5 with 1M sodium hydroxide. Then the extract was mixed with an equal volume of ethanol, and stirred for 10 minutes at 250rpm at room temperature. The precipitate was separated from alcohol by centrifugation, under the same conditions given above. Then the precipitate was dispersed in 500ml of 70% ethanol and stirred for 10 minutes at 250rpm at room temperature, and the alcohol was removed by centrifugation as explained above. The extracted pectin was freeze dried under -50°C and 0.22mbar by a bench top freeze drying system (FreeZone 4.5, Labconco Co., Kansas City, MO, USA) for approximately 60h. The dried pectin was ground into a fine powder on a laboratory hammer mill (Polymix PX-MFC 90D, Kinematica AG, Bohemia, NY, USA) equipped with a 1mm screen.

Production yield was gravimetrically determined and expressed as a weight percentage of the extracted dried pectin to total dry matter mass of the apple pomace used for extraction.

2.2.5 Determination of uronic acid content

The uronic acid content was estimated by the sulfamate/meta-hydroxydiphenyl method (Filisetticozzi and Carpita 1991). A standard curve was prepared by using α-D-galacturonic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Pectin samples (20mg d.b) were weighed into 100ml volumetric flasks. Distilled water (50ml) at 40ºC was added to the volumetric flasks and vortex mixed until samples were completely dispersed. The volumes were adjusted to 100ml using 40°C distilled water. Aliquots of 400µl from the pectin solutions were placed in 15ml screw capped glass tubes, followed by addition of 40µL of 4 M sulfamic acid at pH 1.6 (adjusted with a saturated solution of potassium hydroxide) and vortex mixed for 5s. Sulfuric acid, containing 75mM of sodium tetraborate (2.4ml), was then added and mixed for 5s using a fixed speed vortex mixer (Model 945410, Fisher Scientific, USA). The solutions were heated in a boiling water bath for 20 minutes, and then the tubes were placed in an ice bath for 10 minutes. After cooling, 80µl of 0.15% (w/v) *m*-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide was added and vortex mixed for 15s. Absorbance was read promptly (within 2 minutes) against the reagent control at 525nm using a UV-visible spectrophotometer (BioMate 3S, Thermo Scientific, Madison, WI, USA) using quartz cells.

2.2.6 Determination of neutral sugar composition

Neutral sugars were determined, as alditol acetates, by gas chromatography using AACC method 32-25 (AACC International 2002), with modifications as follows. Twenty milligrams of dried pectin were weighed in a 15ml glass tube. An internal standard, *myo*-inositol (3mg/ml), was added (1ml) to the pectin sample and vortex mixed for 5s. Then, 3.2ml of nanopure water and 0.15ml of 12M sulfuric acid were added to the glass tubes, which were covered with aluminum foil and loosely capped. Samples were hydrolyzed in a pressure cooker (Deni 9780, Keystone MFG. Co., Inc., Buffalo, NY, USA) at 15psi for 1h. An amount of 360µl of the hydrolysate syrup was transferred to a clean 15ml glass tube and 100µl of 12M ammonium hydroxide and 10µl of 2-octanol were added. After vortex mixing for 5s, sugars were converted to their corresponding alditols by adding 36µl of freshly prepared 3M ammonium hydroxide containing sodium borohydrate (150mg/ml). The mixture was incubated in a water bath at 40°C for 90 minutes. The reduction reaction was stopped by adding 36µl of glacial acetic acid. The solution was vortex mixed for 5s and 500µl of 1-methylimidazole was added and vortex mixed again for 5s; 5ml of acetic anhydride were added and vortex mixed for 5s. After that, acetylation was allowed to proceed for 10 minutes at room temperature. Then 1ml of ethanol was added, vortex mixed for 5s, and allowed to stand for a further 10minutes. The test tubes were moved to an ice bath and 5ml of 7.5M sodium hydroxide were slowly added, vortex mixed for 5s, and another 5ml of 7.5M sodium hydroxide were added without further mixing. The upper ethyl acetate layer was transferred to a 2ml polypropylene tube using a Pasteur pipette.

Alditol acetates derivatives were separated using a Clarus 580 Gas chromatograph (PerkinElmer, Inc., Waltham, MA, USA) fitted with a hydrogen flame ionization detector (FID) and a fused silica capillary column (SP-2380, Supelco Analytical, Bellefonte, PA, USA) with the following dimensions: 30m, 0.32mm I.D., and 0.2µm film thickness. Injection volume was 2µl. Injector (split ratio 1:20) and detector temperature were both at 240°C. Oven temperature was isocratic at 220°C for 20 minutes. Helium was used as carrier gas at a velocity of 1ml/min. Data was collected and analyzed by TotalChrom software (version 6.3.2, PerkinElmer, Inc., CT, USA).

A calibration solution with the internal standard (*myo*-inositol) was prepared using the following monosaccharides: L-(+)-arabinose, L-(+)-rhamnose, D-(+)-galactose, D-(+)-glucose, D-(+)-xylose, and D-(+)-mannose. Thirty milligrams of each sugar were weighed and placed into a 10ml volumetric flask, 5ml of nanopure water were added and vortex mixed for approximately 20s, finally the volume was adjusted with nanopure water.

Correction factors (CF_m) accounting for sugar losses during hydrolysis and derivatization and for different responses on chromatograph were calculated for each monosaccharide using the following equation.

$$CF_m = \frac{A_s \times W_m}{A_m \times W_s} \quad \text{.....Equation (2.1)}$$

Where A_m = peak area for the given monosaccharide, A_s = peak area for internal standard (*myo*-inositol), W_m = weight (mg) of monosaccharide in the 10 ml calibration solution W_s = weight (mg) of internal standard in the 10 ml calibration solution.

Neutral sugar residues were calculated using the following equation.

$$PR = \frac{CF_m \times A_m \times W_s \times F_m \times 100}{A_s \times S} \quad \text{.....Equation (2.2)}$$

Where PR = percentage of individual neutral polysaccharide residues in original sample, CF_m = correction factor, A_m = peak area for individual monosaccharide, W_s = weight (mg) of internal standard in the 10 ml calibration solution, F_m = correction factor for individual monosaccharides to polysaccharide residues (0.88 for pentoses and 0.90 for hexoses), A_s = peak area for internal standard (*myo*-inositol), S = sample weight (mg).

2.2.7 Determination of degree of esterification

The pectin degree of esterification (DE) was determined by the titrimetric method by Bochek et al. (2001), with minor modifications, as follows. Dried pectin (0.2g) was transfered to a 250ml Erlenmeyer flask; pectin was moistened with 5ml of ethanol and dispersed in 20ml of carbon dioxide free water at 40°C under continuous stirring (700rpm) for 2h. Then 10ml of the dispersed pectin was transferred into a 100ml volumetric flask and the volume was adjusted with carbon dioxide free water. An aliquot (20ml) was taken and transferred to a 250ml Erlenmeyer flask. The aliquot was titrated against sodium hydroxide (0.1N) in the presence of three drops of phenolphthalein solution and the result was recorded (V_1). Then, 10ml of sodium hydroxide (0.1N) were added and the solution was stirred at 300rpm at room temperature for 2h. After that, 10ml hydrochloric acid (0.1N) was added and hand shaken until the pink color disappeared. Three drops of phenolphthalein solution were added and the solution was titrated against sodium hydroxide (0.1N) and the result was recorded (V_2). The degree of esterification (DE) was calculated using the following equation.

$$DE = \frac{V_2}{V_1+V_2} \times 100 \quad \text{.....Equation (2.3)}$$

Where V_1 = volume (ml) of sodium hydroxide (0.1N) used in first titration, and V_2 = volume (ml) of sodium hydroxide (0.1N) used in second titration.

2.2.8 Determination of pectin molecular weight

Molecular weight of the extracted pectin was determined using High-performance size exclusion chromatography (HPSEC) following the method reported by Jung et al. (2013) with minor modifications, as follows. Samples of 0.05g (d.b) were dispersed in 10ml of 0.05M sodium nitrate by keeping at room temperature for one day on a multi tube rotator (4632Q, Thermo Scientific, WI, USA) with a fixed shaker speed at 30rpm. Dispersed samples were filtered through a 0.45μm polytetrafluoroethylene (PTFE) filter aided by a vacuum filtration system composed by a vacuum assisted filter (Samplicity Filtration System, EMD Millipore, Darmstadt, Germany) under 5psi vacuum pressure. Filtrate (100μl) was injected into HPSEC system, equipped with Shimadzu LC-20AD pump, Shimadzu CTO-20A column oven (Shimadzu Scientific Instruments Inc., Canby, OR, USA), Shodex DGU-20A Prominence Degasser, Shodex RI-101 detector (Shodex, Showa Denko K.K., Kanagawa, Japan), Size exclusion columns, Shodex OHpak SB-807G, SB-807 HQ, SB-806 M HQ, SB-804 HQ and SB-802.5 HQ (Shodex, Showa Denko K.K., Kanagawa, Japan) connected in series and maintained at 50°C. Degassed distilled water was used as a mobile phase at 1ml/min flow rate. Data were collected and analyzed by Chromatography Data Systems software (Shimadzu, Ezstart version 7.43, Shimadzu Scientific Instruments Inc., Canby, OR, USA). Pullulan standards (Standard P-

82, Showa Denko K.K., Kanagawa, Japan) P-5, P-10, P-50, P-100, P-200, P-400, and P-800 representing molecular weights 0.59×10^4, 0.96×10^4, 4.71×10^4, 10.7×10^4, 20.0×10^4, 34.4×10^4, 70.8×10^4, respectively, were used to create the molecular weight standard curve. The molecular weights of pectin samples were calculated using the standard equation given below.

$$\text{Log MW} = -0.2787 \times \text{RT} + 14.352 \quad \text{.....Equation (2.4)}$$

Where MW= Molecular Weight (Da), RT= Retention Time (minutes), with R^2=0.9983.

2.2.9 Determination of pectin protein content

Residual protein content in pectin was determined using AOAC Dumas combustion method 990.03 (AOAC International 1990).

2.2.10 Determination of pectin ash content

Ash content of extracted pectin was determined according to AOAC method 942.05 (AOAC International 1990).

2.3 RESULTS AND DISCUSSION

2.3.1 Proximate composition of apple pomace composite

Proximate composition of the apple pomace composite used in this study (Table 2.2, p.73) was comparable to compositions previously reported for other apple pomace (Carson et al. 1994; Figuerola et al. 2005; Sudha et al. 2007). The apple pomace composite, on dry basis, was mainly composed of total dietary fiber (54.82%), which includes pectin in the soluble fraction.

2.3.2 Optimization of pectin extraction

Pectin yield ranged from 0.96 to 8.61% (Table 2.3, p.74). The maximum yield was found under the experimental conditions of pH 1.5, temperature 90ºC, and hot acid extraction time 105 minutes. At similar extraction conditions, Bhalla et al. (1993) obtained a yield of 5.1%. This difference could be attributed to the nature of starting material used in the two studies, *i.e.*, dried apple pomace was used by Bhalla et al. (1993) whereas this study used wet frozen pomace. The drying process of apple pomace, commonly performed prior pectin extraction reduces the production yield of pectin. Constenla et al. (2002) studied the effect of apple pomace drying on the characteristics of pectin, finding a significant pectin yield reduction as the drying temperature increases.

Pectin content, expressed as uronic acid, ranged from 45.87 to 70.84% (Table 2.3, p.74). The maximum pectin content was found at the same extraction condition as the maximum yield, *i.e.* pH 1.5, extraction temperature 90ºC, and hot acid extraction time 105 minutes. Low extraction pH, high extraction temperature, and long hot acid extraction time did not seem to negatively affect the uronic acid content of the extracted pectin. In addition, the maximum pectin content, found in this study, is higher compared to what has been reported by others; Marcon et al. (2005) extracted pectin from dried apple pomace using a solution of 5% citric at 100°C for 80 minutes obtaining an uronic acid content of 42.3%. Garna et al. (2007) extracted pectin from fresh apple pomace using an extraction pH of 2 (pH adjusted with sulfuric acid), extraction temperature of 90°C for 2h obtaining a pectin content of 58.4%. Constenla et al. (2002) obtained a pectin content of 62.3% from dried apple pomace using an extraction pH of 2.5 (pH adjusted with nitric acid) and an extraction temperature of 80°C for 1h.

The regression analysis results are shown in Table 2.4 (p.75). The linear term was significant ($p < 0.0001$), and no improvement was observed in the model as additional terms were added. Therefore, the linear model was selected as the most appropriate for predicting pectin yield. Using the coefficient estimates obtained from the linear model the following equation was derived to model the pectin yield from fresh apple pomace.

$$Y_1 = 0.15 - 1.92X_1 + 0.12X_2 + 0.00675X_3 \quad \text{.....(Equation 2.6)}$$

Where Y_1 = pectin yield (%), X_1 = pH of the extraction solution, X_2 = extraction temperature (°C), and X_3 = hot acid extraction time (minutes), $R^2 = 0.9467$.

The linear model and corresponding response surface (Figure 2.1, p.79), revealed an increase in pectin yield with simultaneous increase in temperature and decrease in pH. A similar effect was obtained by Garna et al. (2007) and Pagan et al. (2001) in the extraction of pectin from fresh apple pomace and dried peach pomace, respectively. They found that extraction pH and extraction temperature had notable influences on the yield of extracted pectin, as high extraction temperature and low extraction pH combinations resulted in high pectin yields.

Even though the effect of hot acid extraction time on pectin yield seemed to be significant (Table 2.5, p.76), this was smaller compared to the effect of extraction temperature and extraction pH. This is supported by the response surfaces (Figure 2.1 and 2.2, pp.79 and 80), where the gradient of yield *vs*. time was lower compared to that of yield *vs*. pH and yield *vs*. temperature.

Modeling the uronic acid content of pectin was impossible; none of the evaluated potential models were found to be significant ($p > 0.05$) to fit the data (Table 2.6, p.77). This was caused primarily by the small variations among the results of uronic acid

content (Table 2.3, p.74). In addition, it is possible that the extraction conditions tested in this study do not have a significant effect on the uronic acid content of pectin. Studies by Vriesmann et al. (2012) on cacao pod husk also found that the extraction conditions did not influence the uronic acid content of pectin. Pectin content could be closely related to crop and biological factors, such as fruit variety and developmental stage. In addition, pectin composition is modified by enzymatic and chemical reactions during plant growth, fruit ripening, and storage (Schols and Voragen 2002).

2.3.3 Verification of predictive model

The mathematical model generated for the pectin yield was verified by extracting pectin from the apple pomace composite under a selected point of the optimum region; pH 1.5, temperature 90°C, and hot acid extraction time 120 minutes. These conditions yielded 8.6% pectin, which was within the range of 95% confidence interval predicted by the model. Thus, the predictive equation can be used to model the extraction process of pectin from fresh apple pomace, based on production yield.

It has been reported that 10-15% (w/w, d.b) of pectin can be obtained from apple pomace, on a dry matter basis (Rolin 2002). Garna et al. (2007) extracted 8.9% pectin from fresh apple pomace at 90°C using a sulfuric acid solution (pH 1.5) during 3h. The present study obtained yields comparable to Garna et al. (2007) process, with the advantage of a lower hot acid extraction time. Moreover, the pectin yield obtained in this study was higher compared to 3.9% obtained by Constenla et al. (2002) from dried apple pomace using a nitric acid solution (pH 2.5) for 1h at 80°C. However, the yield observed in the present study is lower compared to other studies that used organic acids,

specifically citric acid, for pectin extraction. Marcon et al. (2005) extracted pectin from dried apple pomace at 100°C for 80 minutes using 5% (w/v) citric acid, obtaining a pectin yield of 16.8%. Rascon-Chu et al. (2009) obtained a pectin yield of 16% from fresh apples at 100°C for 30 minutes when citric acid was used (6% w/v). These observations indicate that, in addition to pH, the type of acid also influences the pectin extraction yield. The lower pectin yields obtained, when mineral acids are used, could be related to a higher pectin hydrolysis, which leads to the production of soluble, smaller pectin molecules that cannot be precipitated by alcohol (Kalapathy and Proctor 2001).

2.3.4 Characterization of pectin extracted at the optimum region

2.3.4.1 Proximate composition

The pectin extracted in this study had an uronic acid content of 64.67% (Table 2.7, p.78), being the main component of the extracted material. Assuming that the entire uronic content found in the extract was galacturonic acid, the pectin content is very close to the legal standard (65%) to be considered as commercial pectin.

Based on the proximate composition analysis of the pectin extracted under the selected conditions, in addition to neutral sugars that are typically found in the composition of pectins (Kravtchenko et al. 1992), other components, such as protein and ash were also found in the extracted pectin (Table 2.7, p.78). Proteins could either be linked to pectin polysaccharides or exist in free form. According to studies on the fractionation of an industrial apple pectin, by ion exchange and size exclusion chromatography, proteins were eluted with the molecules of pectins (Kravtchenko et al. 1992). This could explain the amount of 6.3% of protein found in the extracted pectin. A

similar protein content (6%) was found by Marcon et al. (2005) in apple pectin extracted at 75°C for 55 minutes with 5% (w/v) citric acid. In addition to protein, 5.78% of ash was also found in the pectin extracted in this study. Constenla et al. (2002) extracted pectins at 80ºC, pH 2.5 for 1h from apple pomace, which were previously dried at selected temperatures (60-105ºC), and reported an ash content of 2.5-4.4% (w/w). The ash found in the pectin composition of the present study could be related not only to the minerals in the apple pomace, but also to the reagents, used during the extraction process, forming a salt (sodium chloride); hydrochloric acid used to acidify the apple pomace slurry and sodium hydroxide used to adjust the pH before alcohol precipitation.

In the extracted pectin, total amount of neutral sugars were found to be 11.58% (w/w, d.b) (Table 2.7, p.78). Glucose, galactose, and xylose were found in higher proportions than other neutral sugars. The amounts of neutral sugars found in this study were lower compared to those that have been reported by others; Rascon-Chu et al. (2009) obtained 33.5% neutral sugars from low quality apple fruits at 100°C for 30 minutes and 6% (w/v) citric acid; while Marcon et al. (2005) found 57.4-66.6% at selected extraction conditions (extraction temperature 50-100°C, hot acid extraction time 30-80 minutes, and 5% (w/v) citric acid). During the acid extraction of pectin, neutral sugar side chains and glycosidic linkages of some cell wall polymers can undergo partial hydrolysis and be converted into free sugars, which are not readily precipitated by ethanol (Garna et al. 2007). In addition, heat labile neutral sugars, such as arabinose, could undergo further hydrolysis during the extraction (Marcon et al. 2005). These explanations are applicable to the low neutral sugar levels observed in the pectin

extracted in this study, particularly the low contents of rhamnose, arabinose, and mannose.

It is noteworthy to mention that the presence of neutral sugars, proteins, and ash in pectin not only reduces the percentage of uronic acid, but also negatively affect the gelling properties of pectin (Thakur et al. 1997). Therefore, minimizing the levels of these "non-pectin" components, either during the extraction or purification, is important in obtaining good quality pectin.

2.3.4.2 Degree of Esterification

The proportion of galacturonic acid residues that are esterified with methanol defines the degree of esterification (DE). In this study, the DE of the extracted pectin was estimated to be 66.68%, and hence it can be considered as high methoxyl pectin. It has been reported that high extraction temperature, low extraction pH, and long hot acid extraction time lead to pectin de-esterification (Diaz et al. 2007; Garna et al. 2007). Despite the extraction conditions used in this study, the degree of esterification obtained was very close to the degree of esterification (70%) typically obtained by industrial processes (May 1990). Moreover, the DE observed in this study was higher compared to a previous study by Rascon-Chu et al. (2009), where pectin was extracted from fresh apple pomace at 100°C using 6% citric acid (w/v) for 30 minutes, obtaining a DE of 57%.

2.3.4.3 Molecular weight

The molecular weight of the pectin extracted at the selected conditions was estimated to be 10^6Da. The molecular weight of the extracted pectin in this study was

relatively higher compared to the average (~10^5Da) of commercial pectins and those previously reported for apple pectin (10^4-10^5 Da) (Constenla et al. 2002; Rascon-Chu et al. 2009; Rolin 2002). Molecular weights of pectins are expected to vary with the starting material and the extraction conditions. In addition, variation could be due to different techniques used for analysis, *e.g.* high-performance size exclusion chromatography in this study *vs.* viscosity measurements in Constenla et al. (2002) study.

2.4 CONCLUSIONS

This study investigated the effects of the principal extraction factors (*i.e.* temperature, pH, and hot acid extraction time) on the pectin yield and uronic acid content of pectin from fresh apple pomace. No specific patterns were observed in the uronic acid content caused by the tested extraction conditions. However, extraction yield increased significantly with increasing temperature, pH, and time. A linear model was developed to predict the pectin yield based on extraction conditions.

Pectin extracted at the selected conditions was a high methoxyl homogalacturonan with a large molecular weight and low content of neutral sugar residues. The proposed linear model can be used to predict the yield when fresh apple pomace is used as raw material for hot acid extraction of pectin. In addition, the findings and observations reported here contribute to the available, yet limited, knowledge-base on the complicated process of pectin extraction from apple pomace.

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Table 2.1 Box-Behnken experimental design and levels of factors used for optimization of pectin yield.

Variables	Symbol	Coded and actual levels		
		-1	0	+1
pH	X_1	1.5	2.5	3.5
Temperature (°C)	X_2	30	70	90
Time* (minutes)	X_3	30	105	180

*Time of heating and mixing with hot acid.

Table 2.2 Proximate composition of apple pomace composite.

	% Amount*
Moisture	78.49 ± 0.02
Total dietary fiber	54.82 ± 0.31
Total sugars (as invert)	35.86 ± 2.13
Starch	4.43 ± 0.40
Fat	2.47 ± 0.15
Crude protein	2.43 ± 0.25
Ash	1.80 ± 0.01

*All values, except moisture, are on dry basis. Freeze-dried pomace was used for other determinations.

Table 2.3 Box-Behnken experimental design and corresponding results for responses.

Experiment order	Variables*			Responses	
	pH	Temperature (°C)	Time (min)	% Yield	% Uronic acid in pectin
7	2.5 (0)	70 (0)	105 (0)	4.46	53.70
10	2.5 (0)	70 (0)	105 (0)	4.31	50.89
13	2.5 (0)	70 (0)	105 (0)	4.68	49.92
16	2.5 (0)	70 (0)	105 (0)	4.43	48.19
17	2.5 (0)	70 (0)	105 (0)	4.77	50.83
1	3.5 (+1)	70 (0)	30 (-1)	1.41	46.62
2	1.5 (-1)	70 (0)	180 (+1)	7.41	48.17
3	2.5 (0)	50 (-1)	30 (-1)	2.09	56.63
4	2.5 (0)	90 (+1)	30 (-1)	6.68	52.24
5	3.5 (+1)	70 (0)	180 (+1)	1.84	51.43
6	1.5 (-1)	70 (0)	30 (-1)	5.79	54.05
8	2.5 (0)	90 (+1)	180 (+1)	7.87	53.71
9	1.5 (-1)	50 (-1)	105 (0)	3.48	61.74
11	2.5 (0)	50 (-1)	180 (+1)	2.89	62.13
12	3.5 (+1)	90 (+1)	105 (0)	5.70	54.43
14	3.5 (+1)	50 (-1)	105 (0)	0.96	45.87
15	1.5 (-1)	90 (+1)	105 (0)	8.61	70.84

*Coded levels are given within parenthesis.

Table 2.4 Sequential model sum of squares for pectin yield.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	*p*-value
Mean *vs.* Total	352.27	1	352.27		
Linear *vs.* Mean	78.81	3	26.27	76.95	< 0.0001
2FI[*] *vs.* Linear	0.43	3	0.14	0.36	0.7820
Quadratic *vs.* 2FI	1.29	3	0.43	1.11	0.4075
Cubic *vs.* Quadratic[**]	2.57	3	0.86	23.49	0.0053[**]
Residual	0.15	4	0.036		
Total	435.52	17	25.62		

[*]Two factor interaction.

[**]The number of design points, in the Box-Behnken design, was insufficient to fit a cubic or higher order model.

Table 2.5 ANOVA for the selected linear model for pectin yield.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	*p*-value
Model	78.81	3	26.27	76.95	< 0.0001
pH	29.59	1	29.59	86.66	< 0.0001
Temperature	47.18	1	47.18	138.18	< 0.0001
Time	2.05	1	2.05	6.01	0.0292
Residual	4.44	13	0.34		
Lack of fit	4.29	9	0.48	13.09	0.0124
Pure Error	0.15	4	0.036		
Total	83.25	16			

Table 2.6 Sequential model sum of squares for uronic acid content.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	*p*-value
Mean *vs*. Total	48860.69	1	48860.69		
Linear *vs*. Mean	173.37	3	57.79	1.58	0.2417
2FI* *vs*. Linear	32.70	3	10.90	0.25	0.8621
Quadratic *vs*. 2FI*	203.74	3	67.91	1.99	0.2041
Cubic *vs*. Quadratic**	222.90	3	74.30	18.62	0.0082
Residual	15.96	4	3.99		
Total	49509.36	17	2912.32		

*Two factor interaction.

**The number of design points, in the Box-Behnken, design was insufficient to fit a cubic or higher order model.

Table 2.7 Proximate composition of pectin extracted at the optimum conditions* predicted by the model.

	% (w/w d.b)
Uronic acid	64.67 ± 0.02
Rhamnose	0.49 ± 0.02
Arabinose	0.49 ± 0.06
Xylose	2.12 ± 0.11
Mannose	0.83 ± 0.10
Galactose	2.15 ± 0.15
Glucose	5.48 ± 0.34
Protein	6.30 ± 0.00
Ash	5.78 ± 0.20

*Hot acid extraction time 120 minutes, pH 1.5, and temperature 90°C.

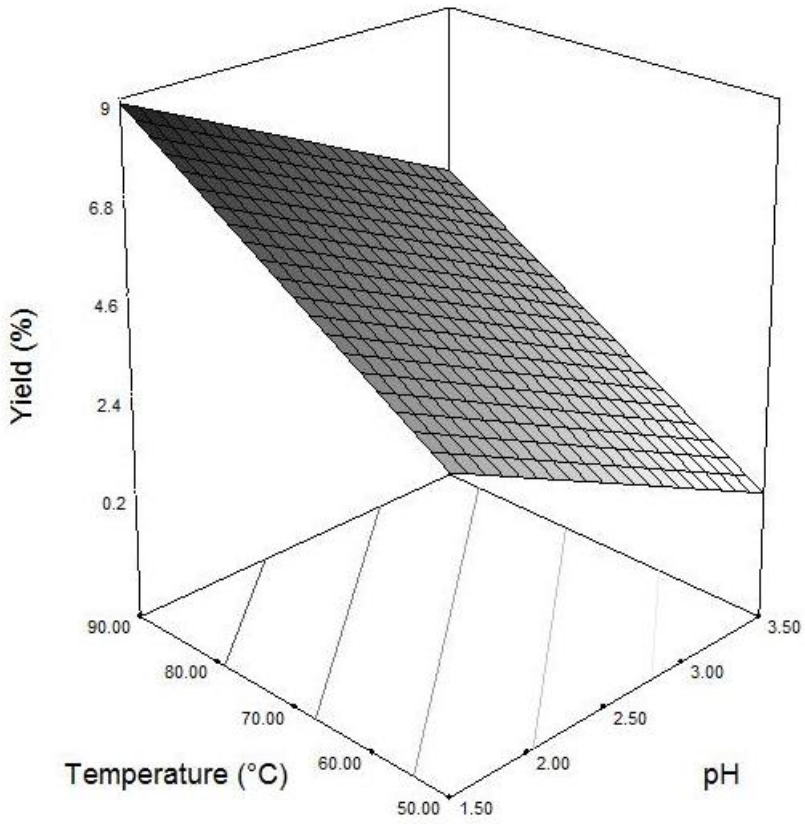

Figure 2.1 The response surface for pectin yield (%) as a function of extraction temperature (°C) and extraction pH at hot acid extraction time of 105 minutes.

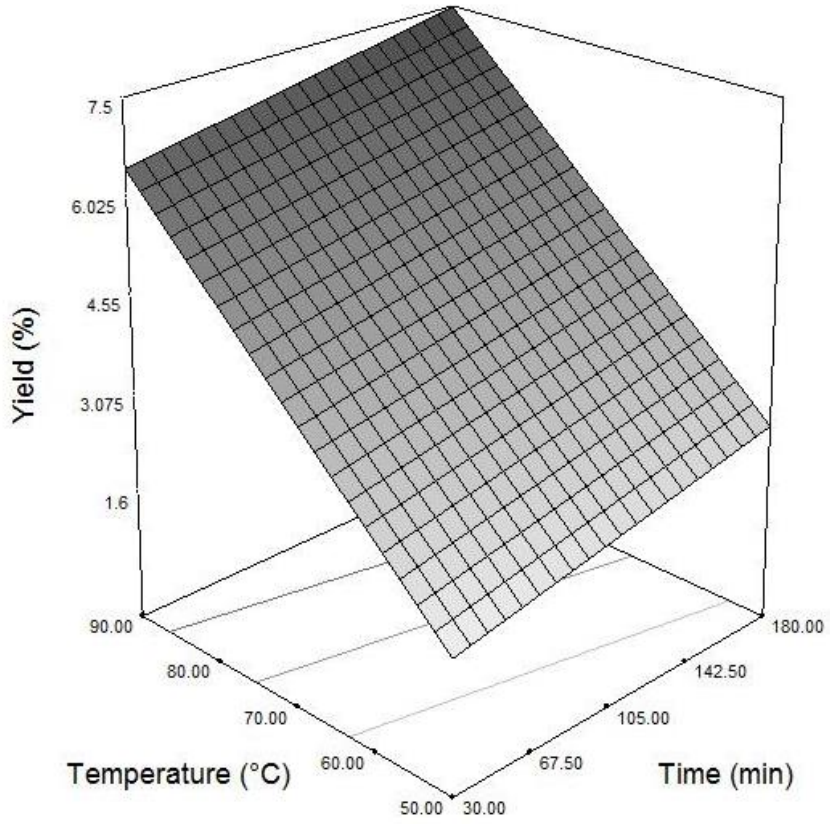

Figure 2.2 The response surface for pectin yield (%) as a function of extraction temperature (°C) and hot acid extraction time (minutes) at extraction pH of 2.5.

CHAPTER 3. ENCAPSULATION OF α-TOCOPHEROL ACETATE USING A PECTIN-OCTENYL SUCCINIC ANHYDRIDE STARCH MATRIX

ABSTRACT

α-tocopherol is a fat-soluble vitamin widely used in the food industry, primarily due to its antioxidant properties. The fortification of foods with α-tocopherol is limited due to its sensitivity to heat, oxygen, and light, and high hydrophobicity. Technologies that enhance the stability and facilitate the delivery of α-tocopherol acetate are important in functional food ingredient development. In this study, encapsulation was used as a means to protect and enhance the delivery of α-tocopherol acetate in microparticles. Apple pectin was investigated as an ingredient for preparing a wall material to encapsulate α-tocopherol acetate. Selected levels of apple pectin (0, 0.5, 1.0g to make up 24.0g of total solids with octenyl succinic anhydride (OSA) starch) were used in combination with OSA starch. Particles were prepared by emulsifying the wall materials with α-tocopherol acetate and spray drying the emulsion. Electron microscopy showed particles with generally spherical shape with surface indentations for all tested treatment combinations. Significant differences were observed among the treatments for the contents of encapsulated α-tocopherol acetate, which were in the range of 18.33 to 37.66%. The highest α-tocopherol acetate content was observed in the particles made with the highest level of apple pectin (1.0g). Particles with a smaller size and narrower distribution were obtained when 1.0g of pectin was used.

3.1 INTRODUCTION

Vitamin E is a term used to collectively identify a group of eight fat-soluble molecules; four tocopherols ($\alpha, \beta, \gamma,$ and δ) and four tocotrienols ($\alpha, \beta, \gamma,$ and δ), composed of a chromanol ring with a phytol chain (Eitenmiller and Lee 2004). All tocopherols and tocotrienols have the ability to act as antioxidants; quenching free radicals by donating the phenolic hydrogen. Among the existing forms of vitamin E, α-tocopherol is the most potent antioxidant with the highest biological activity (Gregory III 2008). Therefore, α-tocopherol is used as an additive to inhibit lipid oxidation and other radical driven oxidative events in food and biological systems (García et al. 2013).

The intake of α-tocopherol has been related in the prevention of chronic diseases associated with oxidative stress, such as cardiovascular diseases, atherosclerosis, and cancer (Brigelius-Flohe and Traber 1999). In addition, other disease prevention activities, such as hypertension, type 2 diabetes, and Alzheimer's, have been reported (Pierucci et al. 2007). In foods α-tocopherol is commonly used either as an antioxidant preservative or for fortification to improve nutritional value. However, the application of α-tocopherol in foods is limited due to its low stability in the presence of oxygen, heat, and light, and its poor aqueous solubility (García et al. 2013; Gregory III 2008). Therefore, there is a need to develop technologies to enhance the stability of vitamin E and to facilitate its efficient delivery.

Encapsulation is a process by which an active ingredient (core material) is coated with or entrapped within a wall material, providing protection against degradative reactions and loss of activity of the core material (Rosenberg et al. 1990). Encapsulation is also used for controlled release of active molecules, easier handling, and flavor and

taste masking (Shahidi and Han 1993). Among the various techniques available for the encapsulation of food ingredients, spray drying is the most commonly used technique in the food industry (Gharsallaoui et al. 2010). Spray drying is an operation that involves the atomization of a liquid product (solution, dispersion or emulsion), solvent evaporation by the hot air contacting the atomized material, dry product separation, and collection of the dry product (Ré 1998). This technique has been widely applied for encapsulating vitamins, fatty acids, flavors, and other unstable ingredients (Gharsallaoui et al. 2010; Jafari et al. 2008b).

One limitation of using spray drying in encapsulation for food applications is the limited number of available wall materials. Common wall materials, available for food applications, include carbohydrates (*e.g.* maltodextrin, modified starch), gums (*e.g.* guar gum, gum acacia), and proteins (*e.g.* whey protein, sodium caseinate, gelatin) (Gouin 2004). Each wall material has advantages and disadvantages, in terms of properties, cost, and encapsulation efficiency for certain applications (Sansone et al. 2011). Therefore, a combination of two or more wall materials is commonly used. Chemically modified starches have been used both as wall materials and emulsifiers for the encapsulation of a wide variety of lipophilic compounds, such as flavors, oils, and vitamins (Shogren et al. 2000). OSA starch is a chemically modified starch obtained by the esterification of granular starch with anhydrous octenyl succinic acid in an aqueous alkaline medium (Trubiano 1986). With the introduction of hydrophobic octenyl side chains to starch polymers, the modified starch obtains amphiphilic properties, which are useful in stabilizing oil-in-water emulsions (Shogren et al. 2000). OSA starch has been approved

by the FDA as a food additive, with a maximum degree of substitution of 0.02 (Code of Federal Regulations 1977).

Certain properties, such as encapsulation efficiency and stability, of microparticles could be improved by using pectin along with starch, rather than starch alone. Pectin is a structural plant polysaccharide consisting of a linear backbone of α (1→4)-D-galacturonic acid residues (Voragen and Pilnik 1995), which are esterified with methanol to different degrees. Commercial pectin is classified based on its degree of esterification (DE) as low methoxyl pectins (LM, DE<50) and high methoxyl pectins (HM, DE>50). Pectin is widely used as a gelling, thickening, and stabilizing agent in the food industry (May 1990). Due to its functional properties, such as pH-sensitivity, film forming ability, and resistance to degradation in the upper gastrointestinal tract, pectin is used in pharmaceutical applications (Liu et al. 2003). Controlled release drug formulations and colon-specific drug delivery vehicles have been developed using pectin (Liu et al. 2003; Sungthongjeen et al. 1999; Wakerly et al. 1996).

Studies on the encapsulation of α-tocopherol, using various techniques and wall materials, have been reported (Table 3.1, p.100). There are no published studies on the encapsulation of α-tocopherol using OSA starch and pectin containing wall materials.

This study was conducted to evaluate the use of apple pectin as a wall material for microencapsulation. Apple pectin was used in combination of OSA starch to encapsulate α-tocopherol acetate using spray drying. In order to evaluate the effect of apple pectin in encapsulation, microparticles, prepared with selected levels of apple pectin in wall material formulations, were evaluated for their properties.

3.2 MATERIALS AND METHODS

3.2.1 Materials

High methoxyl apple pectin (Yantai Andre Pectin Co. Ltd., Yantai, China) and a commercial octenyl succinic anhydride (OSA) corn starch, with a degree of substitution of 0.02, were used as wall materials. α-tocopherol acetate (TCI America, Portland, OR, USA) was used as core material. All other chemicals and solvents used for the experiments were of ACS certified grade.

3.2.2 Preparation of emulsions

Prior to microencapsulation, emulsions with selected levels of pectin were prepared (0, 0.5, and 1.0g), their compositions are listed in Table 3.2 (p.101). Based on preliminary tests (results are not reported here), pectin contents higher than 1.0g were not used because emulsions were excessively viscous for the proper processing in the spray dryer. Total solids content of feed emulsions were maintained at 40% (w/w). The emulsions were prepared following the method reported by Xie et al. (2010), with minor modifications, as follows. OSA starch was dissolved in 40ml of warm (70°C) distilled water, under constant stirring, at 400rpm, for 10 minutes using a programmable digital hot plate stirrer (HS40, Torrey Pines Scientific, Inc., San Marcos, CA, USA). The starch dispersion was covered with aluminum foil and kept overnight, at room temperature under constant stirring (200rpm). Apple pectin was dispersed by stirring, until a gel was formed, in 20ml of distilled water using a stainless steel flat spatula. The apple pectin gel was combined with the hydrated OSA starch in a 150ml beaker, followed by the addition of 4.8g of α-tocopherol acetate. The beaker was immersed in an ice bath and the mixture

was homogenized at 10,000rpm for 3 minutes, with a one minute interval after every minute of homogenization, using a Virtishear mechanical homogenizer (Model 225318, The VirTis Company, Inc., Gardiner, NY, USA). The homogenizer was equipped with a 20mm diameter shaft and a generator assembly, which consisted of a 20mm diameter slotted stator and a shearing rotor.

3.2.3 Microencapsulation by spray drying

The emulsions were spray dried in a Büchi B-290 mini spray dryer (Büchi Labortecknik AG, Flawil, Switzerland) equipped with a two-fluid pressure nozzle with a 0.7mm nozzle tip and a 1.5mm nozzle cap. The spray dryer was attached to a dehumidifier Büchi B-296. Experiments were conducted under the following spray dryer conditions: inlet and outlet temperature of 105°C and 70 ± 10°C, respectively, air flow rate of 473L/h, emulsion feed rate of 2ml/min, and aspiration of 90%. The emulsion was maintained under constant stirring (200rpm) while it was pumped into the spray dryer. The exhaust gas was monitored and humidity was maintained below 70% to ensure proper moisture removal. The particles were collected only from the collection vessel (*i.e.*, particles from drying chamber and filter were not collected) and stored at -20°C until analysis.

3.2.4 Determination of α-tocopherol acetate in microparticles

Total α-tocopherol acetate in the microparticles was determined as follows. Samples (50mg) were placed in a 50ml centrifuge tube and 30ml of ethanol were added. The tube was agitated for 1h at room temperature using a rotator (4632Q, Thermo

Scientific, WI, USA) at 30rpm speed. The tube was then centrifuged at 3000*g* for 15 minutes using a centrifuge (Sorvall Legend XTR, Thermo Scientific, Madison, WI). An aliquot (~1ml) of the supernatant was taken and absorbance was read against ethanol at 285nm using a UV-visible spectrophotometer (BioMate 3S, Thermo Scientific, Madison, WI, USA) using quartz cells, according to Dahot et al. (1990) method. Total α-tocopherol acetate content was calculated using the following equation.

$$\% \text{ α-tocopherol acetate} = \frac{\text{Calculated α-tocopherol acetate}}{\text{Theoretical α-tocopherol acetate}} \quad \text{.....Equation (3.1)}$$

To determine the encapsulated α-tocopherol acetate, 50mg of the microparticles were placed in a 50ml centrifuge tube and washed with 3ml of ethanol. The tube was placed in a rotator for 10s and then centrifuged at 1500*g* for 5min. The supernatant was discarded and 30ml of ethanol were added. Then the tube was agitated for 1h at room temperature on a rotator and centrifuged at 3000*g* for 15min. An aliquot of the supernatant was taken and absorbance was read against ethanol at 285nm using a UV-visible spectrophotometer and quartz cells. The encapsulated α-tocopherol acetate was calculated using equation 3.1 given above.

3.2.5 Size distribution analysis of microparticles

Particles size distributions were analyzed using a Malvern Mastersizer 3000 laser diffraction particle size analyzer equipped with an Aero S dry dispersion unit (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Sample was delivered into the system within the obscuration limit of 0.1-20% and an air pressure of 1×10^5Pa. Refractive index of 1.53, density of 1.5g/cm^3, and absorption index of 0.10 were used as the analytical

parameters. Data were collected and analyzed by Malvern software (Version 2.01, Malvern instruments Ltd., Malvern, UK).

3.2.6 Morphology analysis of the microparticles

The morphology of the particles was characterized using scanning electron microscopy (SEM). The samples were scattered on double sided stickers (Ted Pella, Inc., Redding, CA, USA), on top of metal stubs, and coated with gold-palladium alloy using a Hummer sputter coating system (Anatech Ltd., Union City, CA, USA). Coated specimens were observed under a Hitachi S-3000N variable pressure scanning electron microscope (Hitachi Science Systems, Tokyo, Japan) at an acceleration potential of 25kV. Images were recorded by image capturing software (Version 10-16-2266, Hitachi High-Technologies, Pleasanton, CA, USA) (Ratnayake and Jackson 2007).

3.2.7 Differential scanning calorimetry (DSC)

Microparticle samples (~10mg) were prepared and analyzed according to Ratnayake and Jackson (2007) method. Samples were scanned against a blank (empty pan) using a Perkin Elmer Pyris 1 DSC system (Perkin-Elmer Co., Norwalk, CT, USA) from 25 to 135ºC at a 10ºC/min scanning rate.

3.2.8 X-Ray diffraction (XRD)

X-ray powder diffraction profiles of microparticles were obtained by the procedures reported by Ratnayake and Jackson (2008). The samples were mounted on an aluminum sample plate with a small amount of ethanol applied at the bottom of the cavity

to hold the sample and slightly compressed using a spatula to obtain a smooth surface. A Bruker-AXS D8 Discover XRD system (Bruker AXS GmbH, Germany) with a general area detector diffraction system (GADDS), a Gobel mirror, a 0.5mm pinhole collimator, and a Bruker-Vantec-500 area detector was used. X-ray tube was set to 40kV and 40mA. Samples were scanned under the following: omega = 4°, detector swing angle = 18°, sample to detector distance = 20cm and exposure time = 180s. Bruker-AXS GADDS system software integrated area from 2Θ = 3 to 30°. Peak fitting software Origin (version 8.5, OriginLab Corporation, Northhampton, MA, U.S.A.) was used to calculate % relative crystallinities.

3.2.9 Statistical analysis

The study was conducted using completely randomized designs (CRD). For each experiment, determinations were replicated, at least, three times. Analysis of variance was performed and mean separations were performed by Tukey-Kramer HSD (honestly significant difference) test at $p < 0.05$, using JMP (Version 10.0.2, SAS Institute Inc. Cary, NC, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Particle morphology and size distribution

The morphology and size of spray dried particles influence the functionality, stability, and release pattern of the encapsulated material. Thus the production of small and homogeneous particles, without surface damage, are needed for a satisfactory delivery system (Pierucci et al. 2007). Scanning electron microscopy (SEM) images of

the spray dried α-tocopherol acetate particles from each of the tested emulsions showed no evidence of open pores or cracks on the surface (Figure 3.1, p.102). Surface damage can promote the deterioration and loss of the core material (Sheu and Rosenberg 1995). In addition, all the samples showed particles with uneven surfaces and indentations of generally spherical shape. Surface indentation of spray dried particles has been attributed to the shrinkage of the particles during the drying process (Gharsallaoui et al. 2010). The mechanism involved in the particle shrinkage is more pronounced when drying at low temperatures, since the solvent diffusion is slower, allowing more time for structures to deform, shrink, and collapse (Oakley 1997). Surface indentation has also been related to wall materials consisting of polysaccharides (Sheu and Rosenberg 1995). Similar spray dried microparticle morphologies have been reported previously (Alamilla-Beltran et al. 2005; Tan et al. 2009; Xie et al. 2010), in which starch based wall materials were used. The particles made from emulsions containing apple pectin (B and C), showed similar surface morphologies to particles made with only OSA starch (emulsion A) (Figure 3.1, p.102). Therefore the presence of pectin in the wall material appeared to have no influence on the surface morphology of the particles.

The size distributions of the spray dried α-tocopherol acetate particles spanned between approximately 0.2 to 80μm. Particles made with emulsion B and C showed an uni-modal peak at approximately 16μm, whereas particles made with emulsion A showed a bimodal peak. The detailed results for selected sizes classes are given in Table 3.3 (p.103). Generally, particles with smaller size were obtained in emulsion B and C, which contained 0.5 and 1.0g of pectin, respectively. Moreover, particles from emulsion C, showed a narrower size distribution (Figure 3.2, p.104). The properties of the feed

emulsion, such as viscosity and surface tension, influence the size of the emulsion droplet formation during atomization, and hence the size of the spray dried particle (Soottitantawat et al. 2003). According to Gharsallaoui et al. (2010), the size of the particles increases when both viscosity and surface tension are high. In this study, the addition of apple pectin to the emulsion formulations may have increased the apparent viscosity into a certain degree, but due to its emulsifying properties it may have also decreased the surface tension. Results suggest that particles made with apple pectin containing emulsions are better in terms of size distribution compared to particles made from emulsion containing solely OSA starch.

The sizes of the particles obtained in this study are larger compared to those reported in previous studies (Nesterenko et al. 2013; Pierucci et al. 2007). Differences in wall materials, emulsification, and spray drying conditions could have attributed to the observed differences.

3.3.2 Differential scanning calorimetry and X-ray diffraction analyses

The spray drying process involves certain conditions, such as high heat-moisture treatments and solvent evaporation, which could influence structural organization of the polymer matrices of the spray dried particles (Da Silva-Junior et al. 2009). DSC and XRD analyses on the wall materials and spray dried particles can provide information about the effects of spray drying on structural transitions and molecular interactions between the wall material components. These analyses also provide a good understanding on the overall structure, such as crystalline or amorphous natures, of the particles. DSC analysis did not reveal endothermic transitions on either the wall materials (OSA starch

and pectin) or on the spray dried particles produced with the three emulsions (detailed results are not reported here). Both OSA starch and spray dried particles showed completely amorphous matrices, as indicated by the X-ray powder diffraction patterns (Figure 3.3, p.105). X-ray diffraction pattern of pectin, however, revealed a major peak at approximately 21° 2θ (Figure 3.3, p.105). Lutz et al. (2009) reported similar results and XRD profiles for apple pectin. In this study, the main diffraction peak disappeared when pectin was combined with OSA starch and submitted to the encapsulation process. This could suggest a possible interaction between pectin and OSA starch in the polymer matrix.

3.3.3 Encapsulation efficiency

The total α-tocopherol acetate content found in the microparticles ranged from 45.66 to 65% (Table 3.4, p.106). Particles made only with OSA starch had the lowest content of α-tocopherol acetate, while higher contents were found in the particles made with pectin containing wall material mixtures. No significant differences ($p > 0.05$) were found in the α-tocopherol acetate contents between the particles containing 0.5 and 1.0g of pectin (Table 3.4, p.106).

In order to determine the encapsulated α-tocopherol acetate, the particles were washed with ethanol for 10s. The encapsulation efficiency was in the range of 18.33 to 37.66% (Table 3.4, p.106). α-tocopherol acetate contents were significantly different ($p < 0.05$) among the particles made with pectin; the highest encapsulation efficiency was observed in the particles that had the highest level of apple pectin (1.0g). The core material retention during spray-drying and the encapsulation efficiency are affected

primarily by the composition and characteristics of the emulsion and processing conditions (Gharsallaoui et al. 2010). In this study, all the emulsions were spray dried under the same conditions, therefore the differences observed in the α-tocopherol acetate contents must be related to the composition of the emulsions. Previous studies have obtained higher encapsulation efficiencies from emulsions with better stability (Danviriyakul et al. 2002; Minemoto et al. 2002). The use of pectin in the present study could have enhanced the emulsion stability, and therefore the encapsulation efficiency, by (1) increasing the viscosity of the emulsion, which reduces the movement and aggregation of α-tocopherol acetate droplets; and (2) reducing the surface tension and droplet size of the emulsion.

The α-tocopherol acetate contents in the particles obtained in this study were higher than those obtained by Yoo et al. (2006) (57.2%) and Somchue et al. (2009) (20-32%) using ionic gelation. The former used sodium alginate as wall material while the latter used two types of proteins (β-lactoglobulin and egg white protein). However, the tocopherol contents found in this study were lower compared to those obtained by Pierucci et al. (2007) (77.8-96%) and García et al. (2013) (82-94%), using pea protein and inulin, respectively, as wall materials and spray drying as the processing technique. The differences in encapsulation efficiency could be attributed to different wall materials and encapsulation conditions. In addition, previous studies have found that the emulsification method has an influence on the encapsulation efficiency (Jafari et al. 2008a). Emulsions with small oil droplets are more stable, during the spray drying process, and are enclosed more efficiently within the wall matrix. Jafari et al. (2008) reported that emulsions prepared with microfluidizers had a smaller droplet size and a

higher encapsulation efficiency compared to emulsions prepared with high shear mixers. In this study emulsions were prepared at 10,000rpm for 3 minutes using a high shear mixer, while the emulsions used by García et al. (2013) were homogenized at a higher speed (20 000rpm for 3 minutes), and the emulsions used by Pierucci et al. (2007) were prepared using an electronic agitator at 300rpm for 5 minutes. The differences observed in the encapsulation efficiency of α-tocopherol could be also attributed to the emulsification method.

3.4 CONCLUSIONS

Apple pectin and OSA starch were used as wall materials to successfully encapsulate α-tocopherol acetate through spray drying. The incorporation of apple pectin in the wall material blend can significantly increase the encapsulation efficiency of α-tocopherol acetate. Moreover, no visible differences were observed on the surface morphology of the particles as the pectin content was increased. Incorporation of pectin into wall material resulted in small particles with narrow size distribution.

The addition of apple pectin in to the wall material blend showed improved properties in the spray dried microparticles, and higher α-tocopherol encapsulation.

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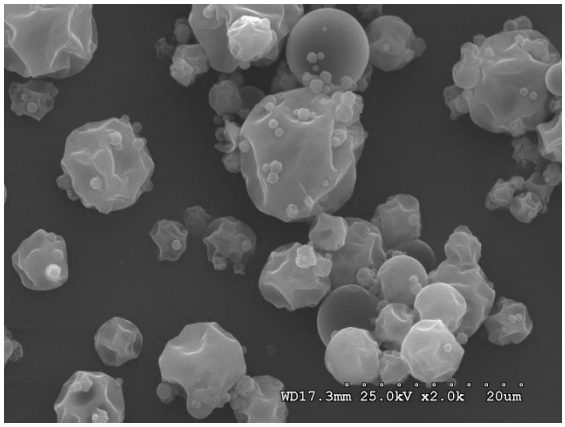
Table 3.1 Encapsulation techniques used to encapsulate α-tocopherol.

Technique	Wall material composition	Reference
Ionic gelation	Sodium alginate; β-lactoglobuline and egg white protein.	Yoo et al. (2006); Song et al. (2009)
Desolvation	Wheat gliadin	Duclairoir et al. (2002)
Freeze drying	Maltodextrin and gelatin	Farias et al. (2007)
Spray drying	Pea protein, carboxymethylcellulose, and maltodextrin; maltodextrin and sodium caseinate; sunflower protein; inulin and sodium caseinate	Pierucci et al. (2007); Selamat et al. (2009); Nesterenko et al. (2013); García et al. (2013)

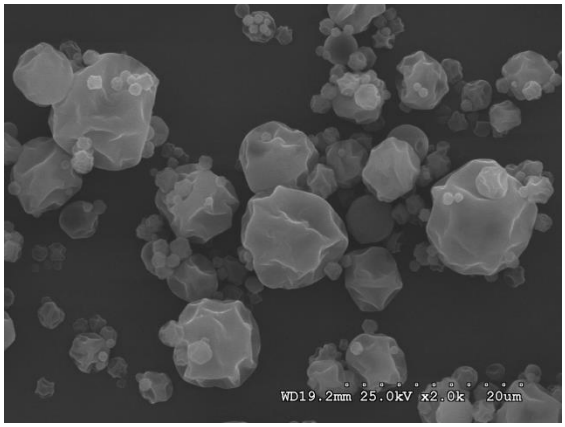
Table 3.2 Composition of feed emulsions.

Material	Emulsion A	Emulsion B	Emulsion C
OSA corn starch (g)*	24	23.5	23
Apple pectin (g)*	0	0.5	1
α-Tocopherol acetate (g)	4.8	4.8	4.8
Distilled water (ml)	60	60	60

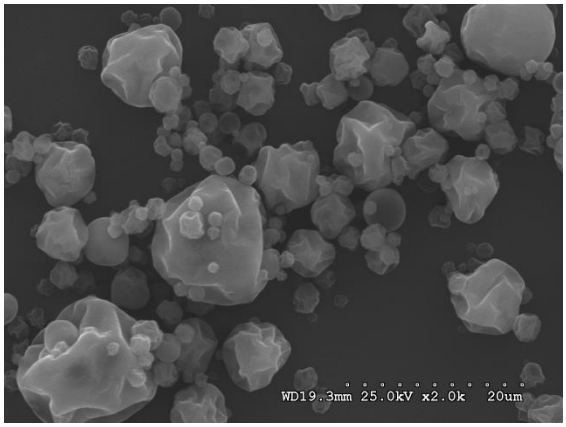
*[OSA starch + pectin] total solids was kept at 24g.



A



B



C

Figure 3.1 SEM images of spray dried α-tocopherol acetate microparticles from three emulsions (2000x): Emulsion A (A); Emulsion B (B); Emulsion C (C). Compositions of the emulsions are given in Table 3.2.

Table 3.3 Particle size distributions[1].

Emulsion[3]	Size[2] at 10% (μm)	Size at 50% (μm)	Size at 90% (μm)
A	6.09 ± 0.50 a	21.75 ± 1.74 a	79.95 ± 4.35 a
B	5.06 ± 0.19 b	19.37 ± 0.25 b	72.00 ± 3.73 b
C	4.71 ± 0.26 b	14.60 ± 0.22 c	48.37 ± 3.68 c

[1]Means followed by the same letter, within the same column are not significantly different ($p > 0.05$).

[2]Size at the upper limit of the specified % of the particle population.

[3]Compositions are given in Table 3.2.

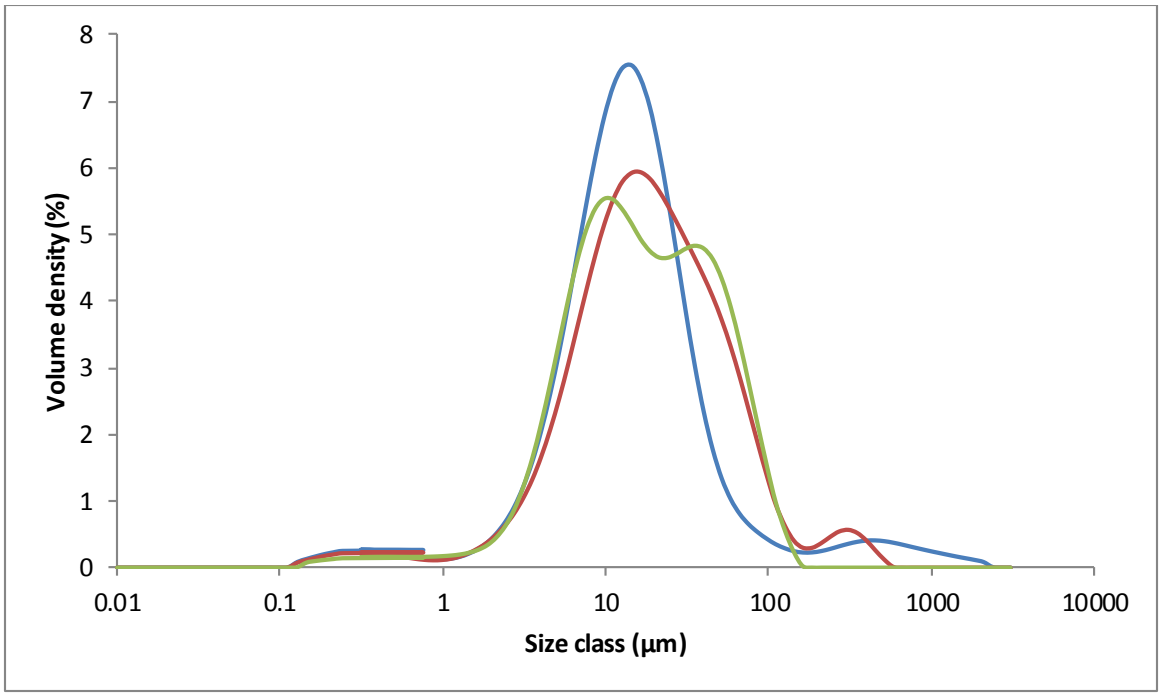


Figure 3.2 Size distributions of particles made with Emulsion A (—); Emulsion B (—); and Emulsion C (—). Compositions of the emulsions are given in Table 3.2.

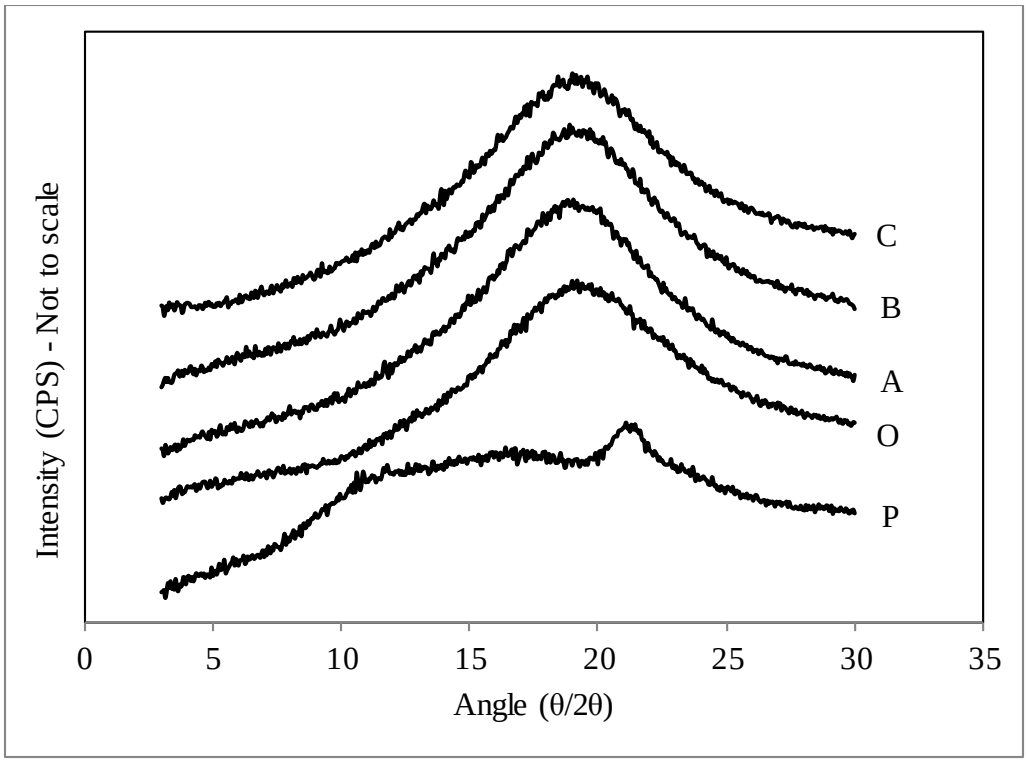


Figure 3.3 Representative X-ray diffraction patterns of walls materials and spray dried particles produced with the three emulsions: Pectin (P); OSA starch (O); Emulsion A (A); Emulsion B (B); Emulsion C (C). Compositions of the emulsions are given in Table 3.2.

Table 3.4 Total and encapsulated α-tocopherol in the microcapsules[1].

Emulsion[2]	Total α-tocopherol acetate (%)	Encapsulated α-tocopherol acetate (%)
A	45.66 ± 0.57 b	18.33 ± 0.57 a
B	63.00 ± 1.41a	25.33 ± 0.57 b
C	65.00 ± 0.00 a	37.66 ± 0.57 c

[1]Means followed by the same letters, within the same column, are not significantly different ($p > 0.05$).

[2]Compositions are given in Table 3.2.