PROCESSING HIBISCUS BEVERAGE USING DENSE PHASE CARBON DIOXIDE

By

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To my parents and sister

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PROCESSING OF A HIBISCUS BEVERAGE
USING DENSE PHASE CARBON DIOXIDE

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Consumer demand for natural beverages with health promoting properties that offer fresh-like sensory attributes and changes in U.S. demographics have created the opportunity for the development of new products that would target new market segments.

Hibiscus sabdariffa (family Malvaceae) red calyces are rich in anthocyanins and other phenolic compounds. Fresh and dried hibiscus is used to prepare cold and hot beverages and their preparation includes an extraction step followed by a pasteurization method. Although thermal preservation of foods is effective in reducing microbial loads it can also lead to organoleptic and nutritional changes. Nonthermal processes like dense phase carbon dioxide (DPCD) are an alternative which may help preserve the color, flavor, and nutrients of food.

Equivalent cold and hot water conditions were found for anthocyanins extraction of dried hibiscus in this research. Likewise, similar polyphenolic profiles and chemical composition of aroma compounds were observed between fresh and dried hibiscus.

Solubility of CO_2 in a hibiscus beverage (5.06 g CO_2/mL at 31.0 MPa) and optimal processing conditions to inactivate yeasts and molds (Y&M) were 34.5 MPa and 6.5 min. DPCD was a viable technology for processing hibiscus beverage since it extended its shelf life for 14 weeks of refrigerated storage. Quality attributes were maintained during storage. Lower losses of anthocyanins were observed in the DPCD (9%) hibiscus beverage as compared to thermally treatment process (14%) and no major changes in total phenolics content and antioxidant capacity occurred during storage. Changes in hibiscus aroma volatiles during storage did not affect untrained panelists overall likeability of the product.

Findings in this research can help in the development and marketing of hibiscus beverage.

CHAPTER 1
INTRODUCTION

Justification

Anthocyanins are water-soluble pigments responsible for the red to purple to blue colors in many fruit, vegetables, flowers, and cereal grains. The interest in anthocyanin pigments has intensified in recent years because of their possible health benefits. Thus in addition to their functional role as colorants, anthocyanin extracts may improve the nutritional quality of foods and beverages (Wrolstad 2004).

Consumer demands for natural beverages with health promoting properties that offer fresh-like sensory attributes and changes in U.S. demographics with Hispanics and Blacks as important growth-driving demographics (Mintel 2008) have created the opportunity for the development of new products that would target these market segments.

Hibiscus sabdariffa (family Malvaceae) is a short-day annual shrub that grows in many tropical and subtropical countries and is one of the highest volume specialty botanical products in international commerce (Plotto 1999). The red calyces are the part of the plant with commercial interest and are rich in organic acids, minerals, anthocyanins, and other phenolic compounds.

Fresh and dried hibiscus calyces are used to prepare cold and hot beverages which are commonly mixed with a sweetener and are characterized by an intense red color and acidic flavor which provides a sensation of freshness. The preparation of a hibiscus beverage includes an extraction step followed by a pasteurization method. Although thermal preservation of foods is effective in reducing microbial loads it can also lead to organoleptic and nutritional changes. Nonthermal processes are an

alternative which may help preserve the color, flavor, and nutrients of food. Dense phase carbon dioxide (DPCD) is a cold pasteurization method that uses pressures below 90 MPa in combination with carbon dioxide (CO_2) to inactivate microorganisms. This non-thermal technology is mainly used in liquid foods and since the food is not exposed to the adverse effect of heat, its fresh-like physical, nutritional, and sensory qualities are maintained.

Hypothesis

The combination of a cold extraction process with dense phase carbon dioxide (DPCD) processing will help prevent the degradation of anthocyanins present in a hibiscus beverage, and thus provide a product with enhanced quality and phytochemical activity.

Specific Objectives

1. To compare the effects of cold and hot water extraction on the physicochemical and phytochemical properties of hibiscus extracts and to identify and quantify the anthocyanins and major polyphenolics present in extracts obtained from fresh and dried hibiscus by equivalent cold and hot water extraction conditions.
2. To determine the aroma profile differences between four extracts obtained from fresh and dried hibiscus extracted at two different conditions (22 °C for 4 h and 98 °C for 16 min), by GC-MS and GC-olfactometry.
3. To determine the solubility of CO_2 in a hibiscus beverage, to optimize DPCD processing parameters based on microbial reduction, and to monitor during refrigerated storage the microbial, physicochemical, and phytochemcial changes of DPCD processed hibiscus beverage compared to thermally treated and control (untreated) beverages.

4. To determine the effect of DPCD processing on the sensory attributes and aroma compounds of hibiscus beverage when compared to a thermally treated and a control (untreated) and to monitor the changes in these attributes during refrigerated storage.

CHAPTER 2
LITERATURE REVIEW

The Beverage Market

The global beverage market is comprised of four sectors: 1) hot drinks, 2) milk drinks, 3) soft drinks, and 4) alcoholic drinks. Hot drinks include tea, coffee, and hot-malt based products; milk drinks include white drinking milk and flavored milk products; soft drinks are divided into five main subcategories: (bottled water; carbonated soft drinks; dilutables including powder and liquid concentrates; 100% fruit juice and nectars with 25-99% juice content; still drinks including ready to drink (RTD) teas, sports drinks, and other non carbonated products with less than 25% fruit juice, and alcoholic drinks which include beer, wine, sprits, cider, sake and flavored alcoholic beverages (Roethenbaugh 2005). A diagram of these sectors is presented in Figure 2-1.

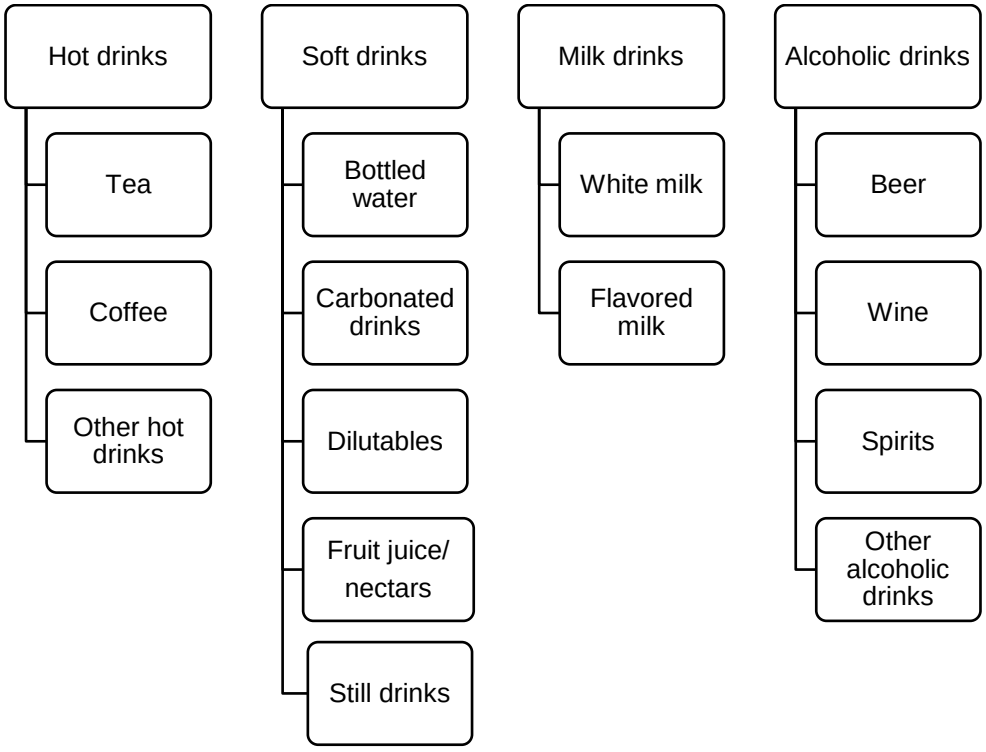


Figure 2-1. Beverage sectors and segments. (Source: Roethenbaugh 2005).

Soft drinks are normally defined as sweetened water-based beverages, usually having a balanced acidity. Flavor, color, fruit juice or fruit pulp are often added in their formulation. The main ingredient in soft drinks is water, and thus their primary function is hydration. There are two basic types of soft drinks: ready-to-drink (RTD) products and concentrates or dilute-to-taste products. The RTD sector is divided into products that are carbonated and those that are non-carbonated (Ashurst 2005).

The market of ready to drink (RTD) non-carbonated beverages can be divided in four segments: 1) bottled water, 2) sports/energy drinks, 3) fruit juice/juice drinks, and 4) RTD teas and coffees.

Market Performance and Competitive Context of U.S. Ready to Drink Non-Carbonated Beverages

Sales for ready to drink non-carbonated beverages reached $38.6 billion in 2007, exhibiting a 35% growth, measured in current prices, during the period 2002-2007. The market is projected to grow 33% in current prices from 2008-12, or the equivalent of 16% when considering the impact of inflation (Figure 2-2). Enhanced bottled waters, energy drinks, and RTD teas are the categories that have driven this growth (Mintel 2008).

Fruit juice and juice drinks, bottled water, sports and energy drinks, and RTD tea and coffee accounted for 37.4, 28.6, 26.0, and 8.1% or the RTD non-carbonated beverage market in 2007. While the sales for fruit juice and juice drinks are forecast to decline in the period 2007-2012, the other three categories will have increasing sales over this period (Figure 2-3).

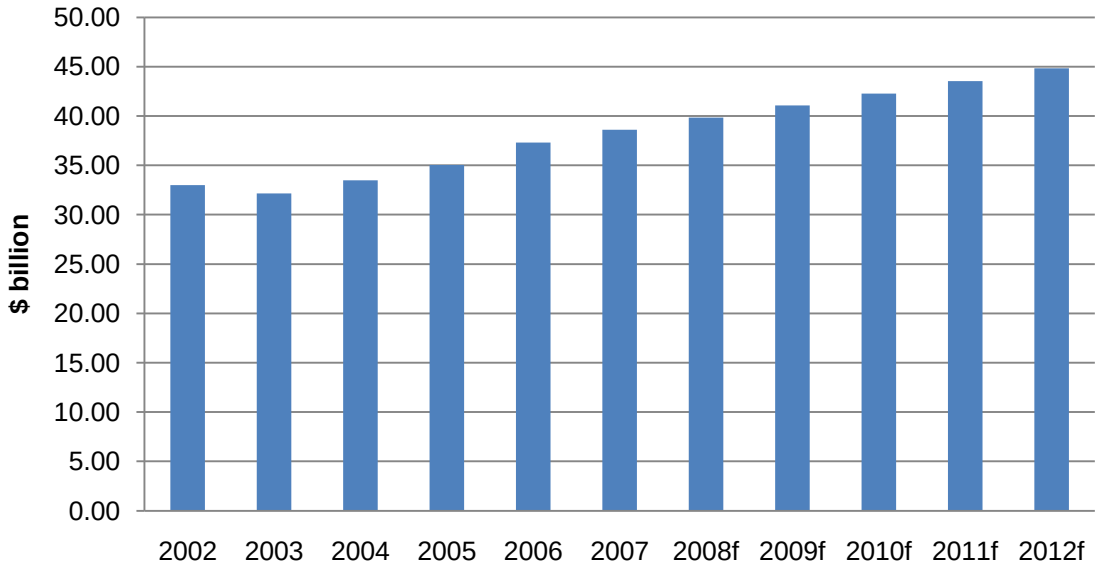


Figure 2-2. Total U.S. sales and forecast (f) of RTD non-carbonated beverages at inflation adjusted prices, 2002-12. (Source: Mintel 2008).

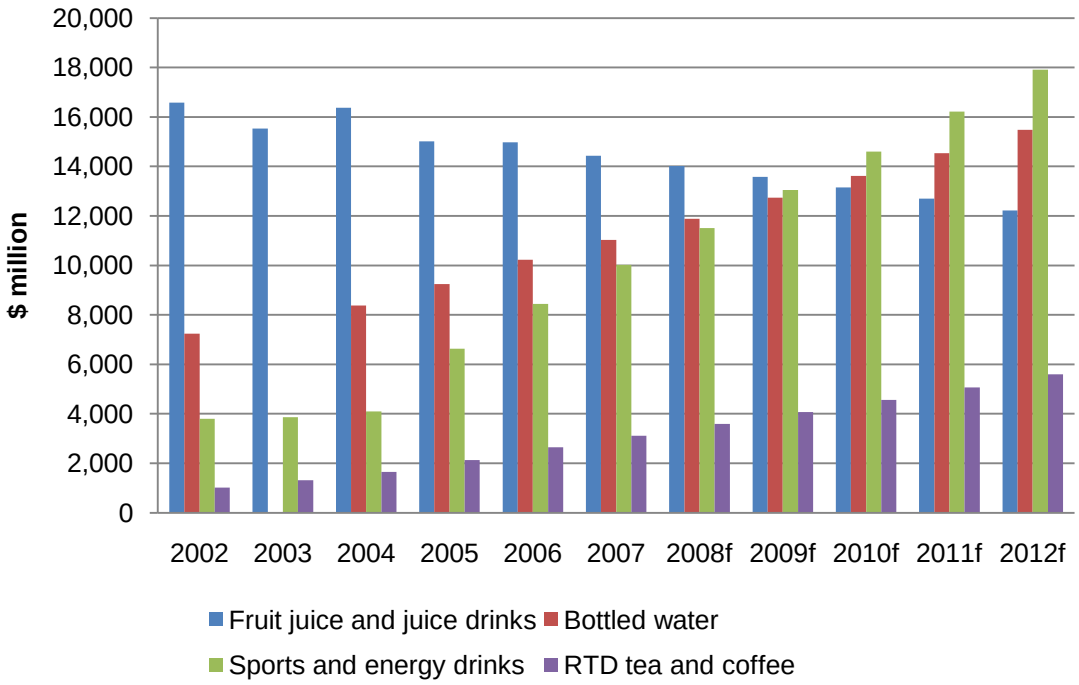


Figure 2-3. U.S. sales and forecast (f) of RTD non-carbonated beverages at current prices, by segment, 2002-12. (Source: Mintel 2008).

PepsiCo dominates brand sales in this category, its RTD non-carbonated beverage portfolio includes the top juice (Tropicana), bottled water (Aquafina), sports drink (Gatorade), and tea (Lipton) brands. Because PepsiCo is such a strong player in every segment, its total market share as of February, 2008 was 25.3% according to FDMx (Food, drug and mass merchandisers excluding Wal-Mart) sales (Figure 2-4). However, sales growth has been slow, allowing other beverage suppliers to gain a market share (Mintel 2008).

Coca-Cola is well-positioned to gain a market share with its Glacéau, Fuze, and Powerade brands. Its sales increased nearly 14% during 2007-08, adding 1.1% to Coca-Cola's share in the market. The company experienced solid sales growth in every segment (Mintel 2008).

While major beverage companies dominate the RTD non-carbonated beverage category, mid-level entrants are defined by their strong representation in a single segment, such as Ocean Spray in the juice segment, or Nestlé in water sales. Smaller (under the category "Other" in Figure 2-4) participants in the market have found their niche, and are defined mostly by a single brand targeting a specific demographic, like those in the energy/sports drink segment like Red Bull and Rockstar (Mintel 2008).

Many small, high-growth companies present alliance opportunities for big players. These include, Hansen's Natural (natural soda and fruit juice, and energy drinks), Jumex (Hispanic-targeted beverages), Jones Soda (unique soda flavors), Tampico (Hispanic-targeted beverages) and AriZona Tea Co. (RTD tea) (Mintel 2008).

Private label is becoming a more important player in the RTD non-carbonated beverage category. While its presence is strongest in commodity segments such as

juice and water, it is growing fastest in trendy beverages (sports/energy and RTD teas and coffees) (Mintel 2008).

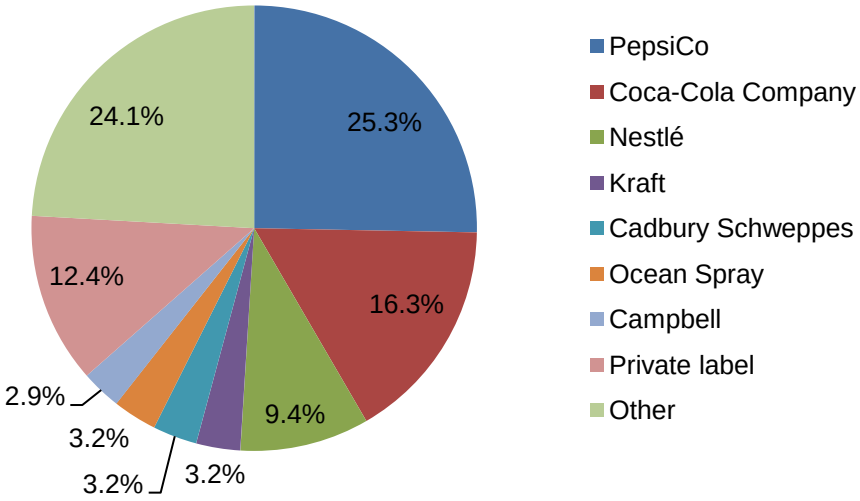


Figure 2-4. Market share according to FDMx (Food, drug and mass merchandisers excluding Wal-Mart) sales of leading RTD non-carbonated beverage companies, February, 2008. (Source: Mintel 2008).

Consumption and Demographic Trends

The two biggest market trends are health/wellness and convenience. Consumers are demanding more from their beverages. Drinks should not only be thirst-quenchers but also provide added benefits. Health and wellness increasingly plays an influential role in consumer choices on the beverage aisle. Consumers are seeking products that add value to their diet; however, not only must products deliver nutrition conveniently, but the packaging must carry a convenient format (Mintel 2008).

Hispanics and blacks are important growth-driving demographics, not only because these groups are projected to exhibit an above-average population growth, but also because they display an above-average incidence of juice consumption. Additionally, both groups are the key consumer in high-growth sports and energy drinks markets (Mintel 2008).

According of the U.S. Census Bureau projection for 2050, non-Hispanic whites will no longer make up the majority of the population. Today non-Hispanic whites make up about 68% of the population. This is expected to fall to 46% in 2050 as a result of a much older white population relative to minorities. Hispanic population is projected to change from 15% to 30% of the total U.S. population while African American and Asian Americas will reach 15 and 9% of the population by 2050 (Table 2-1). The U.S. has nearly 305 million people today. The population is projected to reach 400 million by 2039 and 439 million in 2050 (U.S. Census Bureau 2009).

Table 2-1. U.S. population projections

	2008	2050
Non-Hispanic whites	68%	46%
Hispanic	15 %	30%
African Americans	12%	15%
Asian American	5%	9%

(Source*:* U. S. Census Bureau 2009).

Hibiscus (*Hibiscus sabdariffa*)

Characteristics and Economic Importance

There are more than 300 species of hibiscus around the world. One of them is *Hibiscus sabdariffa*, Linn, which is a member of the Malvaceae family. The origin of *H. sabdariffa* is not fully known but it is believed to be native to India and Malaysia and to have been carried at an early date to Africa. It is widely grown in tropical and subtropical regions including Africa, South East Asia and some countries of America. Seeds are said to have been brought to the New World by African slaves. It is know by different synonyms and vernacular names such as “roselle” in the U.S and England, “l’oiselle” in France, “jamaica” or “flor de jamaica” in Mexico and Spain, “karkade” in Sudan and

Arabia, “sorrel” in the Caribbean and “byssap” in Senegal (Morton 1987; Stephens 2003). In this study the word “hibiscus” will be used to refer to *Hibiscus sabdariffa*.

Hibiscus (Figure 2-5) is a short-day annual shrub and can grow to a height of 1–3 m, depending on variety. The green leaves are about 8–12 cm long and the stems, branches, leaf veins and petioles are reddish purple. Flowers are up to 12.5 cm wide, they are yellow with a rose or maroon eye, and are made up of five petals. After the flowers fall apart, the calyx which is a red cup-like structure consisting of 5 large sepals with a collar (epicalyx) of 8 to 12 slim pointed bracts around the base, begins to enlarge, becomes fleshy, crisp but juicy (3.2-5.7 cm long), and fully encloses the velvety capsule, (1.25-2 cm long), which is green when immature, 5-valved, with each valve containing 3 to 4 kidney-shaped light-brown-seeds, (3-5 mm long). The capsule turns brown and splits open when mature and dry (Morton 1987; De Castro and others 2004).

A **B** **C** **D**

Figure 2-5. Hibiscus pictures. A: hibiscus plant, B: hibiscus flower, C: hibiscus calyxes, and D: opened hibiscus calyx with velvety capsule in the center.

Usually, hibiscus is propagated by seeds or cuttings and grows on sandy soil. The ideal planting time in North America is from April to May, blooming occurs in September and October, and calyces are ready for harvest in November and

December. The "fruits" should be gathered before any woody tissue develops in the calyx. They should be tender, crisp, and plump (Stephens 2003).

Hibiscus has several uses. Its calyces, which is the part of the plant of commercial interest, are used either fresh or dehydrated in the processing of preserves, jellies, jams and sauces for their rich pectin content, to prepare hot and cold beverages which are commonly mixed with a sweetener and are characterized by an intense red color and acidic flavor which provides a sensation of freshness, in the production of wine, and color and flavor extracts. They are also a source of soluble and insoluble fiber. The leaves are used extensively for animal fodder and fiber and are also used in salads, and the seeds are a source of protein and lipids and constitute a byproduct in hibiscus production (Al-Wandawi and others 1984; El-Adawy and Khalil 1994; Mounigan and Badrie 2007; Sáyago-Ayerdi and others 2007; Hainida and others 20008).

Traditionally fresh hibiscus calyces are harvested by hand and are either frozen, dried in the sun or artificially preserved and are either sold into the herbal tea and beverage industry, or local and regional markets. Five kilograms of fresh calyces dehydrate to 0.45 kg of dried hibiscus. Industrial scale operations that use hibiscus include production of vacuum concentrated extract, spray drying of extracts, beverages, natural food colorant and natural food flavor (Al-Kahtani and Hassan 1990).

Hibiscus is one of the highest volume specialty botanical products in the international commerce and demand has steadily increased over the past decades. Approximately 15,000 metric tons of dried hibiscus enter international trade each year. Many countries produce hibiscus but the quality markedly differs. China and Thailand are the largest producers and control much of the world supply. Mexico, Egypt,

Senegal, Tanzania, Mali, Sudan, and Jamaica are also important suppliers but production is mostly used domestically (Plotto 1999).

Germany and the U.S. are the main countries importing hibiscus. The biggest German buyer is Martin Bauer, one of the oldest and largest companies in the herb industry. They use hibiscus in numerous products including herbal teas, herbal medicines, syrups and food coloring. Main importers in the U.S are Celestial Seasonings and Lipton, both tea companies. Hibiscus is also used in ready to serve beverages made by Knudson, Whole Foods and other food and beverage manufacturers (Plotto 1999).

Commercial Hibiscus Products

Hibiscus' striking red color, refreshing properties and associated health benefits has attracted the interest of several entrepreneurs to start a business around the idea of manufacturing hibiscus based beverages. There are four ready to drink commercial products that use hibiscus as the main ingredient. Following is a brief description of these products as well as their marketing approach.

Hibiscus Lemon Bissap

Produced by the company Adina for Life Inc. (located in California) and established in 2005, this product is marketed as a New Age beverage, with all-natural ingredients, refreshing, and with good-for-you appeal. The label is bright, colorful, and folksy. The company sources its hibiscus using fair trade arrangements from independent farmers in Senegal. Adina's president is also from Senegal and considers this product to help rescue traditional beverage mixes from his country (Anon 2006).

Cañita Aguas Frescas (jamaica (hibiscus) flavor)

Produced by the company Eat Inc. (located in North Carolina) and established in 2003, this product is marketed as 100% natural that provides health benefits and targets mainly Hispanic consumers. It has the intent of bringing a traditional Mexican beverage known as “Agua de jamaica” to the Hispanic population in the U.S. (Anon 2006).

Squish Hibiscus Pressé

Produced by the company Squish Hibiscus Pressé located in New Zealand, this product is marketed as a beverage with unique exotic floral fruity flavor that has beneficial properties. This is a new product in the New Zealand market that consumers are not familiar with. The market segments to which this product is targeted are women between 18 and 35 years old and kids (Anon 2006).

Simply Hibi

Produced by Ibis Organica, a UK based company; this company sources its raw material from Uganda and has established a program to help improve living conditions in that country. The product contains 87% hibiscus extract and 13% grape juice concentrate and it is marketed as 100% natural and high in antioxidants.

The ingredients and nutritional facts for these four products are presented in Table 2-2.

Composition and Associated Health Benefits

Hibiscus calyces are rich in organic acids including succinic, oxalic, tartaric, and malic acids (Wong and others 2002), hibiscus acid which is a lactone form of (2S,3R)-(+)-2-hydroxycitric acid and its 6-methyl ester (Hansawasdi and others 2000), ascrobic acid, β-carotene, and lycopene (Wong and others 2002). It is also high in phenolic compounds such as protocatechuic acid (3,4-dihydroxybenzoic acid) (Tseng and others

1998; Liu and others 2002; Lin and others 2003) and chlorogenic acid (Segura-Carretero and others 2008) (Figure 2-6), minerals (aluminum, chromium, copper and iron) (Wrobel and others 2000), sugars (glucose, fructose, sucrose and xylose) (Pouget and others 1990a; Wong and others 2002), water-soluble polysaccharides (Muller and Franz 1992) and anthocyanins (Du and Francis 1973; Wong and others 2002). There can be composition variations depending on variety, soil, climate and growing conditions, and post harvest handling and processing. The nutritional composition of fresh hibiscus calyces is presented in Table 2-3.

For many years, hibiscus has been used in different countries as a medicinal herb for therapeutic purposes. According to different ethnobotanical studies, some traditional medicines use the aqueous extract of the plant as a diuretic, for treating gastrointestinal disorders and hypercholesterolemia, and as a diaphoretic and antihypertensive drug (Herrera-Arellano and others 2004).

Many biological activities have been reported in aqueous extracts of *Hibiscus sabdariffa*. Animal experiments have shown that the consumption of this extract has antihypertensive (Odigie and others 2003), antiatherosclerotic (Chen and others 2003), lipid profile reduction (Carvajal-Zarrabal and others 2005), and antioxidant properties (Suboh and others 2004; Hirunpanich and others 2006; Liu and others 2006). Studies with human patients have also shown that the regular consumption of hibiscus extract has an antihypertensive effect (Haji Faraji and Haji Tarkhani 1999; Herrera-Arellano and others 2004) and reduces serum cholesterol in men and women (Lin and others 2007).

Several compounds isolated from hibiscus extracts also possess pharmacological activities.

Table 2-2. Ingredients and nutritional facts of four hibiscus commercial products

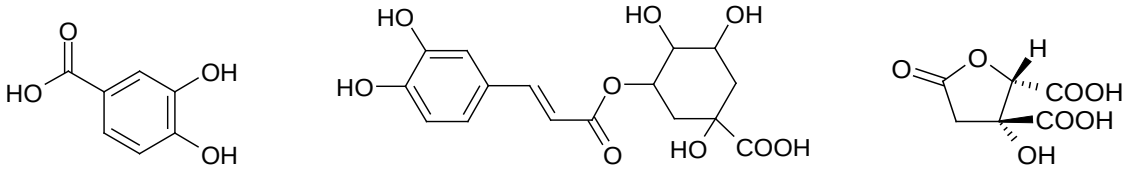
				
	Hibiscus Lemon Bissap	Cañita Aguas Frescas (jamaica flavor)	Squish Hibiscus Pressé	Simply Hibi*
Ingredients				
Water	•	•	•	•
Hibiscus		•	•	•
Sugar		•		
Fructose			•	
Organic evaporated cane juice	•			
Concentrated pineapple juice	•			
Lemon juice	•			
Camu-camu	•			
Organic rosehips	•			
Acerola	•			
Ascorbic acid			•	
Nutritional Facts				
Serving size	236 mL	236 mL	375 mL	
Servings per container	1.75	2	1	
Calories	80	135	124	
Sodium	15 mg		11 mg	
Total carbohydrates	28 g	34 g	28.9 g	
Sugars	19 g	34 g	28.9 g	
Vitamin C	4%			
Magnesium		2%		
Potassium		2%		
Calcium	4%	4%		
Camu-camu	50 mg			
Rosehips (organic)	50 mg			
Acerola	167 mg			
Lemon bioflavonoids	83 mg			

* Nutritional data for this product is not available. Pictures were taken from the actual products by Milena Ramirez. Nutritional data was retrieved from the bottle labels or from *New nutrition business 12(1):19-22).*

Table 2-3. Nutritional composition of fresh hibiscus calyces

	g/100g		mg/100g		mg/100g
Water	86.58	Calcium	215	Vitamin C	12
Protein	0.96	Phosphorus	37	Riboflavin	0.028
Lipids	0.64	Iron	1.48	Niacin	0.31
Carbohydrates	11.13	Sodium	6	Thiamin	0.011
Ash	0.51	Potassium	208	Vitamin A	287 UI
		Magnesium	51	Energy	49 kcal

(Source: USDA 2009)



1 2 3

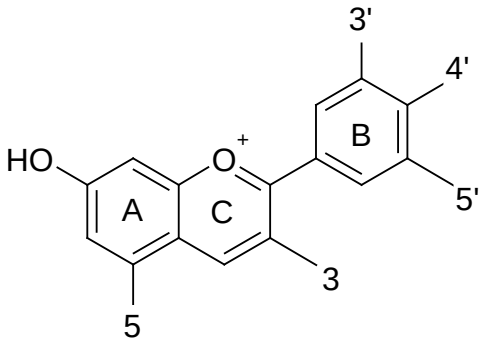
Figure 2-6. Compounds found in some hibiscus extracts: 1 = protocatechuic acid, 2 = chlorogenic acid and 3 = hibiscus or hibiscic acid

Several compounds isolated from hibiscus extracts also possess pharmacological activities. Protocatechuic acid has antiatherosclerosis (Lee and others 2002), antitumor promotion (Tseng and others 1998; Lin and others 2003; Olvera-García and others 2008), antioxidant (Lin and others 2003), and anti-inflammatory (Liu and others 2002) activities. Anthocyanins isolated from hibiscus exhibited antioxidant (Wang and others 2000) and anticancer (Chang and others 2005; Hou and others 2005) activities while hibiscus acid and its 6-methyl ester have shown to be *α*-amylase inhibitors (Hansawasdi and others 2000).

Hibiscus Anthocyanins

Recently there has been a market interest in hibiscus anthocyanins due to their beneficial health effects and high antioxidant properties which have been extensively evaluated (Tee and others 2002; Tsai and others 2002; Tsai and Huang 2004; Prenesti

and others 2007: Sáyago-Ayerdi and others 2007) and as a potential source of natural food colorant. The two major anthocyanins present in hibiscus are: delphinidin-3-sambubioside also known as delphinidin-3-xylosylglucoside or hibiscin and cyanidin-3-sambubioside also known as cyaniding-3-xylosylglucoside or gossypicyanin. They account for approximately 70 and 30% of total anthocyanins, respectively. Other anthocyanins like delphinidin-3-glucoside, delphinidin-3-(feruloyl)rhamnoside, cyanidin-3-glucoside, cyaniding-3-O-rutinoside, and cyaniding-3,5-diglucoside have been found in minor concentrations in some varieties (Figure 2-7) (Du and Francis 1973; Pouget and others 1990b; Tsai and others 2002; Wong and others 2002; Mourtzinos and others 2008; Segura-Carretero and others 2008).



Anthocyanin	3’	4’	5’	3	5
Cyanidin-3-sambubiioside	OH	OH	H	2-O-*β*-xylosyl-D-glucose	OH
Cyanidin-3-glucoside	OH	OH	H	Glucosyl	OH
Cyanidin-3,5-diglucoside	OH	OH	H	3,5-diglucosyl	OH
Cyanidin-3-rutinoside	OH	OH	H	O-rutinosyl	OH
Delphinidin-3-sambubioside	OH	OH	OH	2-O-*β*-xylosyl-D-glucose	OH
Delphinidin-3-glucoside	OH	OH	OH	Glucosyl	OH
Delphinidin-3-(feruloyl)rhamnoside	OH	OH	OH	(feruloyl)rhamnoside	OH

Figure 2-7. Chemical structure of anthocyanins present in hibiscus

The stability of hibiscus anthocyanins has been studied in model systems testing the effect of different chemical compounds (ascorbic acid, BHA, propyl gallate, disodium EDTA, sodium sulfite) (Pouget and others 1990a), temperature (Gradinaru and others 2003; Dominguez-López and others 2008; Cisse and others 2009), sugar type and concentration (Tsai and others 2004), copigmentation and polymerization (Tsai and Huang 2004) as well as their stability in various foods including jellies, beverages, gelatin desserts, and freeze dried products. Color stability during storage has also been tested (Esselen and Sammy 1975; Clydesdale and others 1979). Heat, light, and humidity were all found to be detrimental to anthocyanin stability.

Some studies have shown that thermal degradation of hibiscus anthocyanins follow first-order reaction kinetics (Gradinaru and others 2003; Dominguez-López and others 2008; Mourtzinos and others 2008). Thermal stability of hibiscus anthocyanins in the temperature range of 60-90 °C in the presence or absence of *β*-cyclodextrin was studied. The temperature-dependent degradation was modeled by the Arrhenius equation and the activation energy for the degradation of hibiscus anthocyanins was ~54 kJ/mol. The presence of *β*-cyclodextrin improved thermal stability of nutraceutical antioxidants present in hibiscus extracts both in solution and solid state (Mourtzinos and others 2008). Another study showed that the activation energy for the degradation of hibiscus anthcoyanins was 66.22 kJ/mol (Duangmal and others 2008) while a third study found that copigmentation with chlorogenic acid didn't improve their stability in solution and activation energies for their degradation were between 55.68 and 63.22 kJ/mol (Gradinaru and others 2003).

Hibiscus Extraction Process

Some researchers have focused on hibiscus water extracts while others have employed organic solvents to extract possible bioactive compounds. Indeed the different extraction techniques (extraction time and temperature) make comparison among studies difficult. Moreover different varieties have been used. Table 2-4 summarizes some of the conditions used for hibiscus extraction found in the literature.

Some research has been done regarding the optimization of hibiscus extraction process. One study tested three different hibiscus to water ratios (1:52, 1:67, 1:62 w/v) at three extraction times (20, 25, 30 min) in a hot extraction at 100 °C. They found that optimum conditions based on color and taste were 1:62 w/v for 30 min (Bolade and others 2009). Wong and others (2003) found that optimum condition for hibiscus extraction was 3.5 h at 60 °C based on anthocyanins content and color.

Hibiscus Flavor

Hibiscus flavor is a combination of sweet and tart, similar to cranberry. Few studies have been done related to hibiscus flavor. Gonzalez-Palomares and others (2009) identified 20 volatile compounds in a hibiscus extract using SPME and GC-MS, including terpenoids, esters, hydrocarbons, and aldehydes. They also found 14 compounds in reconstituted spray dried extracts from which only 10 were present in the original extract and the other four were products of degradation. Thermally generated volatiles from untreated, frozen, hot-air-dried at 50 °C, and hot-air-dried at 75 °C hibiscus by steam distillation were analyzed by GC and GC-MS (Chen and others 1998). They characterized more than 37 compounds including fatty acid derivatives, sugar derivatives, phenol derivatives, and terpenes.

Table 2-4. Hibiscus extraction conditions found in the literature

Country of origin	F[a]	D[b]	Solvent	Hibiscus: solvent ratio	Extraction time	Extraction temperature	A[c]	Reference
		•	MeOH with 0.125% citric acid	1:1.65 w/v	48 h			Pouget and others 1990a
Sudan		•	Water	1:10 w/w	40 min	60 °C		Al-Kahtani and Hassan 1990; Hassan and Hobani 1998
Mexico			Water	1:8 w/v	15 min	60 °C		Beristain and others 1994
Taiwan		•	Water	1:30 w/v	10 min	Boiling		Duh and Yen 1997
Malasya	•		MeOH	1:10 w/v	24 h	25 °C	•	Tee and others 2002
Taiwan		•	Water	1:100 w/v	3 min	Boiling		Tsai and others 2002
Malaysia	•		Water	1:5 w/v	1 h	Boiling		Wong and others 2002
Egypt		•	3% Formic acid in MeOH		24 h	4 °C		Gradinaru and others 2003
Malaysia	•		Water	1:40 w/w	30-300 min	30-90 °C		Wong and others 2003
Mexico	•		Water	1:8 w/v	128 min	Ambient		Andrade and Flores 2004
Mexico		•	Water	1:50 w/v	10 min	Boiling		Herrera-Arellano and others 2004
Nigeria	•		Water	1:30 w/v	30 min	Boiling		Oboh and Elusiyan 2004
Taiwan		•	Acidified EtOH (1.5 mol/L HCl)	1:50 w/v				Tsai and Huang 2004
Mexico		•	Water	1:10 w/v	5 min	Boiling		Dominguez-Lopez and others 2008
Singapore		•	Water	1:50 w/v	1 h	Ambient	•[d]	Wong and others 2006
Egypt		•	Water	1:50 w/v	5-930 min	Ambient		Prenesti and others 2007
Egypt		•	Water	1:50 w/v	3 min	100 °C		Prenesti and others 2007
Egypt		•	12% v/v EtOH in water	1:50 w/v	30 min	Ambient		Prenesti and others 2007
Mexico		•	Water	1:20 w/v	5 min	Boiling		Sáyago-Ayerdi and others 2007
Mexico		•	Water	1:50 w/v	10 min	Boiling		Olvera-García and others 2008

a = fresh hibiscus, b = dried hibiscus, c = agitation, d = occasional, e = sonication.

Table 2-4. Continued

Country of origin	F[a]	D[b]	Solvent	Hibiscus: solvent ratio	Extraction time	Extraction temperature	A[c]	Reference
Mexico		•	Water	1:50 w/v	Overnight	Ambient		Olvera-García and others 2008
		•	Acidified MeOH (MeOH/HCl (99:1 v/v))	1:10 w/v	4 h	Ambient	•	Segura-Carretero and others 2008
		•	Acidified MeOH (MeOH/HCl (99:1 v/v))	1:10 w/v	30 min	Ambient	•[e]	Segura-Carretero and others 2008
		•	Acetic acid (15% v/v)	1:40 w/v	48 h	Ambient	•	Segura-Carretero and others 2008
Senegal		•	Water/MeOH/HCl, 50:50:2	1:125 w/v	30 min		•[e]	Juliani and others 2009
Senegal		•	Water	1:62.5 w/v	15 min		•[e]	Juliani and others 2009
Mexico	•		30% v/v EtOH in water	1:12.5 w/v	168 h	Ambient	•[d]	Gonzalez-Palomares and others 2009
Nigeria		•	Water	1:52-1:62 w/v	20-30 min	100 °C		Bolade and others 2009
Taiwan		•	Water	1:40 w/v	2 h	95 °C		Lin and others 2007
Guatemala and Senegal		•	Water	1:10 w/v	10 h	25 °C		Cisse and others 2009

a = fresh hibiscus, b = dried hibiscus, c = agitation, d = occasional, e = sonication.

They concluded that hibiscus aroma was a combination of terpene derivatives with fragrance notes and sugar derivatives with a caramel like odor.

Phenolic compounds

Phenolic compounds are products of the secondary metabolism of plants. Biogenetically they originate from two main synthetic pathways: the shikimate pathway and the acetate pathway. Chemically, phenolics can be defined as substances that have an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives (Bravo 1998).

Many properties of plant products are associated with the presence, type, and content of their phenolic compounds. Of significance to producers and consumers of foods are the astringency of foods, the beneficial health effects of certain phenolics or their potential antinutritional properties when present in large quantities (Shahidi and Naczk 2004).

Classification

Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. They occur mainly in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. The associated sugars can be present as monosaccharides, disaccharides, or even oligosaccharides. The most common sugar residue is glucose, but galactose, rhamnose, xylose, and arabinose can also be found, as well as glucuronic and galacturonic acids among others. They can also be associated with carboxylic and organic acids, amines, lipids, and other phenols (Bravo 1998).Polyphenols can be divided into at least 10 different

classes depending on their basic chemical structure (Table 2-5). Flavonoids, which are the most important single group, can be further subdivided into 13 classes (Table 2-6).

Phenolic Compounds Attributes

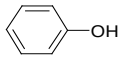
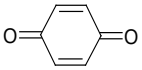
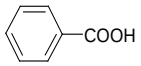
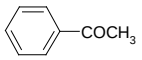
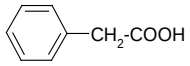
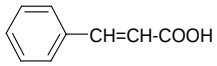
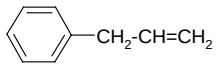
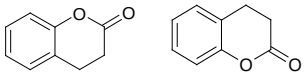
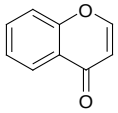
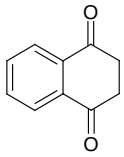
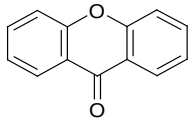
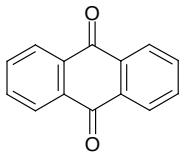
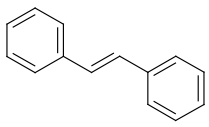
Positive attributes of phenolic compounds include: contribution to flavor and astringency, natural pigments, antimicrobial and antiviral properties, anti-inflammatory activity, antitumor and anticancer activity, antimutagenicity, antioxidant potential, and reduction of coronary heart disease risk (Lule and Xia 2005).

There are also some negative attributes of phenolic compounds that include: off-flavor and taste contribution, discoloration due to enzymatic and nonenzymatic reactions, and antinutritional activity because of interactions with proteins, carbohydrates, minerals, and vitamins (Lule and Xia 2005).

Contribution to flavor

Phenolic compounds may contribute to the aroma and taste of numerous food products of animal and plant origin. The presence of chlorogenic acid can be related to the bitterness of wine, cider, and beer while hydroxycinnamates and their derivatives are responsible for the sour-bitter taste of cranberries. Phenolic substances also contribute to the flavor of vanilla pod and vanilla extracts. Vanillin, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl methyl ester have been found to be the most abundant volatiles but simple phenolics such as *p*-cresol, eugenol, *p*-vinylguaiacol, and *p*-vinylphenol as well as aromatic acids such as vanillic and salicylic acids are also present. Ripe bananas contain volatile phenolics such as eugenol, methyleugenol, elimicin, and vanillin. Strawberry volatiles contain esters of some phenolic acids such as ethyl salicylic, methyl cinnamic, and ethyl benzoic acids.

Table 2-5. Main classes of polyphenolic compounds

Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	see Table 2-6
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

(Source: (Bravo, 1998).

Table 2-6. Classification of food flavonoids

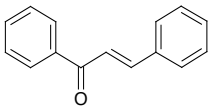
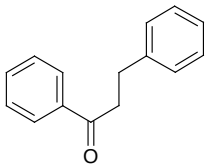
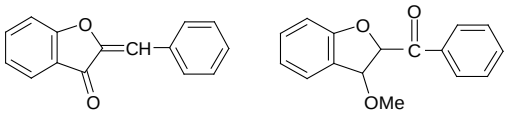
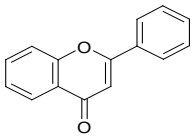
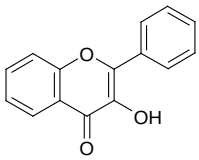
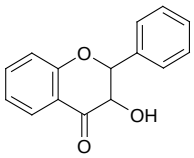
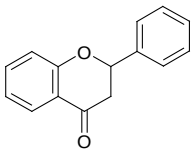
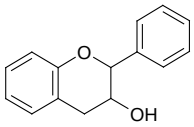
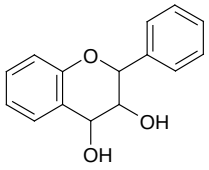
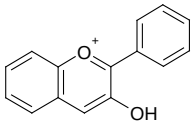
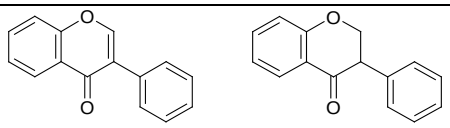
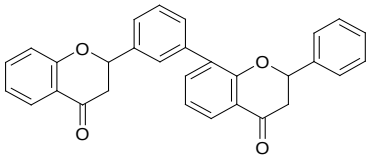
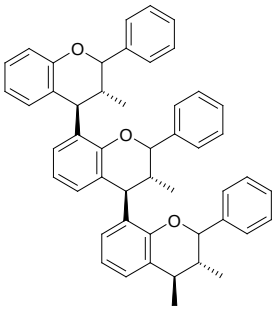
Flavonoid	Basic Structure
Chalcones	
Dihydrochalcones	
Aurones	
Flavones	
Flavonols	
Dihydroflavonol	
Flavanones	
Flavanol	
Flavandiol or leucoanthocyanidin	
Anthocyanidin	

Table 2-6. Continued

Flavonoid	Basic Structure
Isoflavonoids	
Bioflavonoids	
Proanthocyanidins or condensed tannins	

(Source: (Bravo 1998).

Thymol also is a major contributor to the flavor of essential oils from tangerine and mandarin. Phenolic substances may be responsible for the flavor of a number of spices and herbs (Shahidi and Naczk 2004; Lule and Xia 2005).

Antioxidant potential

One of the principal roles that have been proposed as part of the actions of phenolics is that of an antioxidant. Their antioxidant action can arise from a combination of several chemical events, which include enzyme inhibition, metal chelation, hydrogen donation from suitable groups and oxidation to a nonpropagating radical. The health implications of an antioxidant depend on how well it is absorbed by the body and how it is metabolized, in addition to partition effects (Parr and Bolwell 2000).

Anticarcinogenic action

The possible mechanism of action of anticarcinogens can be classified into two groups, blocking and suppressing, depending on the point of action. Some compounds can both block and suppress. The main action of blocking agents is to stimulate the carcinogen-detoxifying enzymes and to inhibit enzymes which have the potential to activate precarcinogens into carcinogens (Parr and Bolwell 2000).

Anthocyanins

Anthocyanins are water-soluble pigments responsible for the red to purple to blue colors in many fruit, vegetables, flowers, and cereal grains. In general, its concentration in most fruits and vegetables goes from 0.1 to 1% dw. The total content of anthocyanins varies among fruits and vegetables, their different cultivars and is also affected by genetic make-up, light, temperature and agronomic factors (Shahidi and Naczk 2004; Wrolstad 2004).

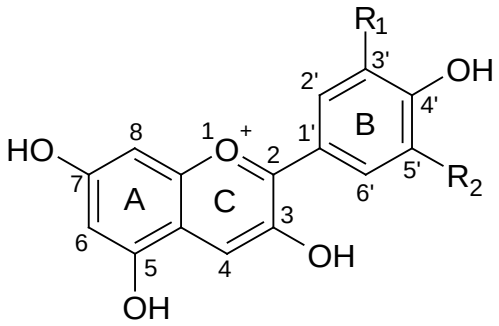
Since color is one of the most important quality attributes in food, anthocyanin-rich plant extracts might have a potential use as a natural alternative to food colorants. Anthocyanins-based colorants are manufactured for food use from horticultural crops grown for that specific purpose as well as from processing wastes. The interest in anthocyanins pigments has intensified in recent years because of their possible health benefits. Thus in addition to their functional role as colorants, anthocyanins extracts may improve the nutritional quality of foods and beverages (Wrolstad 2004).

Classification

Chemically, anthocyanins are flavonoids and are based on a C15 skeleton. The anthcyanidins (aglycones) are the basic structure of anthocyanins. They consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen which is also

bonded by a carbon-carbon bond to a third aromatic ring B (C6-C3-C6) (i.e. anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins)) (Figure 2-8) (Delgado-Vargas and others 2000; Gradinaru and others 2003; Castañeda-Ovando and others 2009).

The differences in color and stability between anthocyanins are related to the number of hydroxyl and methoxyl groups, the nature, position, and number of sugars attached to the molecule, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong and others 2003).



Aglycon	Substitution pattern R_1	R_2	λ_{max} (nm) Visible spectra
Pelargonidin	H	H	494 (orange)
Cyanidin	OH	H	506 (orange-red)
Delphinidin	OH	OH	508 (blue-red)
Peonidin	OCH_3	H	506 (orange-red)
Petunidin	OCH_3	OH	508 (blue-red)
Malvidin	OCH_3	OCH_3	510 (blue-red)

Figure 2-8. Structural and spectral characteristics of the major naturally occurring aglycons. (Source: Rodriguez-Saona and Wrolstad 2005).

An increase in the number of hydroxyl groups tends to deepen the color to a more bluish shade while an increase in methoxyl groups increase redness. Glucose, galactose, rhamnose, and arabinose are the sugars most commonly found in

anthocyanins, usually as 3-glycosides or 3,5-diglycosides. Rutinosides (2-O-*α*-L-rhamnosyl-D-glucosides), sophorosides (6-O-*β*-D-glucosyl-D-glucosides), and sambubiosides (2-O-*β*-D-xylosyl-D-glucosides) also occur as well as some 3,7-diglycosides, and 3-triosides. The most common acylating agents include cinnamic acids (caffeic, *p*-coumaric, ferulic, and synaptic) and aliphatic acids (acetic, malic, malonic, oxalic, and succinic) (Clifford 2000; Delgado-Vargas and others 2000).

There are 17 known naturally occurring anthocyanidins but only six are common in higher plants: pelargonidin (Pg), peonidin (Pn), cyaniding (Cy), malvidin (Mv), petunidin (Pt), and delphinidin (Dp). The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) (Kong and others 2003).

Stability

Anthocyanin pigments can be destroyed easily during processing and storage. A number of factors influence their stability including pH, temperature, humidity, light, oxygen, enzymes, as well as the presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions and copigments. The study of anthcoyanins characteristics can help develop products and processing conditions that will yield high-quality products (Jackman and others 1987; Clifford 2000; Delgado-Vargas and others 2000; Gradinaru and others 2003; Mazza and others 2004).

The effective pH range for most anthocyanins colorants is limited to acidic foods because of the color changes and instability that occur above pH 4 ((Wrolstad, 2004). At

a given pH, equilibrium exists between four different anthocyanin structures: a quinoidal (anhydro) base, a flavylium cation, and the colorless carbinol pseudo-base and chalcone (Figure 2-9).

Copigments are substances that contribute to anthocyanins coloration by protecting the anthocyanin molecule; this mechanism is unique to the anthcyanin family. Usually copigments have no color by themselves but when added to an anthocyanin solution they greatly enhance its color (Mazza and Brouillard 1990).

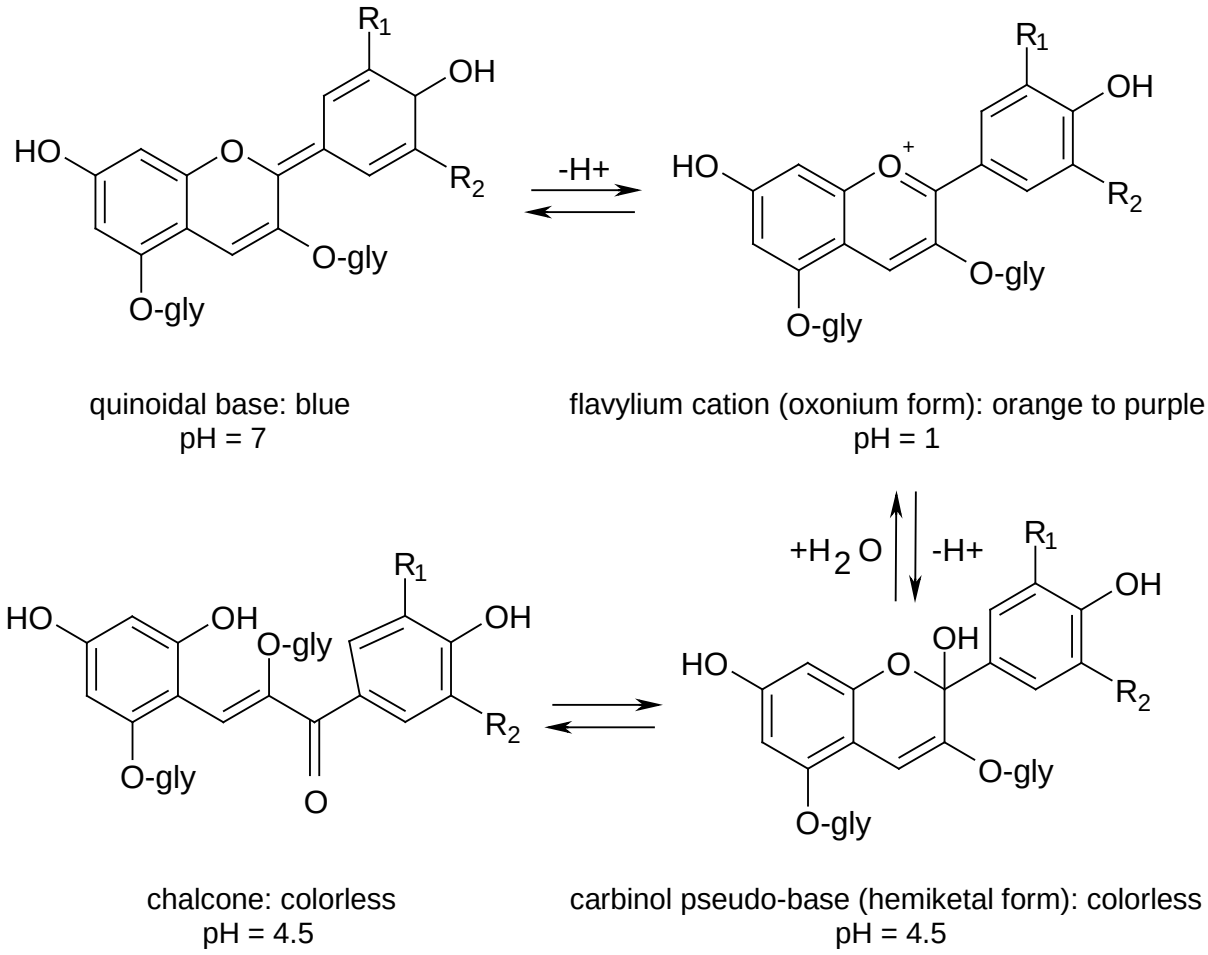


Figure 2-9. Predominant structural forms of anthcoaynins present at different pH levels. (Source: Giusti and Wrolstad 2005).

Anthcoyanins react with flavones, alkaloids, amino acids, benzoic acids, coumarin, cinnamic acids, and a wide variety of other flavilyum compounds. This weak hydrophobically-driven interaction (van der Waals interactions) is known as intermolecular copigmentation. Intramolecular copigmentation occurs with the acylation of the molecule and is more effective than the intermolecular one. The basic role of copigments is to protect the colored flavylium cation from the nucleophilic attack of the water molecule (Delgado-Vargas and others 2000).

The association between anthcoyanins and copigments leads to an absorbance increase in the visible range (hyperchromic effect) and a shift of the λ_{max} toward higher wavelengths (bathochromic effect) (Gradinaru and others 2003).

Health Benefits

In plants, anthocyanins serve as attractants for pollination and seed dispersal, give protection against the harmful effects of UV irradiation, and provide anti-viral and anti-microbial activities (Wrolstad 2004).

Anthocyanins could exhibit multiple biological effects, e.g. reduced risk of coronary heart disease and stroke, anticarcinogen activity, antioxidant/antiradical activity, anti-inflammatory action, inhibition of blood platelet aggregation and antimicrobial activity, treatment of diabetic retinopathy and prevention of cholesterol induced atherosclerosis (Wang and others 1997; Espin and others 2000; Wrolstad 2004).

Beverage Processing

Beverage processing typically involves an extraction step (juices and teas extraction), followed by blending were they can be mixed with other ingredients like water, sweeteners, acidulants, flavorings, colors, and preservatives among others.

Beverages then go into processing, filling, and packaging. The purpose of the processing and packaging steps is to produce a product that is wholesome and safe for the consumer (Ashurst 2005).

Thermal Processing

Traditionally beverages are thermally processed. There are five main processes that use heat as a way to assure microbial safety in juices and soft drinks:

1. Flash pasteurization
2. Hot filling
3. In-pack pasteurization
4. Aseptic filling
5. Chilled distribution

Process selection depends on the level of microbial contamination of the raw materials and packaging, whether the product composition will favor the growth of microorganisms, the ability of the product to resist heat, and the desired shelf life (Ashurst 2005).

Flash pasteurization

Normally the juice is passed through a balance tank or feed tank before being fed to the pasteurizer. The liquid is generally heated by hot water in a plate or tubular (spiral) heat exchanger to the desired pasteurization temperature and held at that temperature for a specified time in a holding tube before being cooled to the filling temperature (usually ambient) using chilled water. Flash pasteurizers usually have a regeneration section. The pasteurized product is then sent to a filling machine.

Hot filling

In hot filling, the product is heated (in a heat exchanger), sent to the filler hot and filled into containers. The containers are closed and are held at or above the desired temperature for a specified time prior before being cooled. This is usually done in a

tunnel with water sprays. In this system, not only the product but also the container is heat treated.

In-pack pasteurization

This type of process is generally the most severe and microbiologically most secure form of heat treatment. The filled closed pack is put into a tunnel pasteurizer were the treatment is given by means of water sprays at various controlled temperatures. The pasteurizer is dived into zones. First there is a heating zone where the temperature of the container and the product is raised, next there is the pasteurizing zone where the product is held to the pasteurizing temperature for a specified time and finally there is a cooling zone where the product is cooled below 30 °C.

Aseptic filling

Aseptic filling is a special case of flash pasteurization that often uses a higher temperature profile. For successful aseptic filling, clean containers, clean product, clean headspace, and clean closures should be brought together in an environment that prevents recontamination. This operation normally takes place in a closed space over pressure with sterile air.

Chilled distribution

Flash pasteurized product is filled cold into clean bottles on clear fillers and then is stored in refrigerated warehouses and is sold to costumers from chill cabinets (Ashurst 2005).

Dense Phase CO_2 Processing (DPCD)

Dense phase carbon dioxide (DPCD) is a cold pasteurization method that uses CO_2 under pressures below 50 MPa. This non-thermal technology is mainly used in

liquid foods. Since the food is not exposed to the adverse effect of heat, its fresh-like physical, nutritional, and sensory qualities are maintained (Damar and Balaban 2006).

Mechanisms of microorganisms' inactivation by DPCD

The exact means of microbial inactivation are not clear but several mechanisms may be involved:

1. **pH lowering effect**

CO_2 can lower the pH of the aqueous parts of the food by dissolving and forming carbonic acid (H_2CO_3), which further dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{-2}) and hydrogen (H^+) ions lowering extracellular pH, by the following equilibrium:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{-2} \quad (1)$$

CO_2 penetrates the microbial cell membrane and lowers its internal pH by exceeding the cell's buffering capacity. This change in internal pH may inactivate microorganisms by inhibiting essential metabolic systems such as enzymes.

2. **Inhibitory effect of molecular CO_2 and bicarbonate ion**

Bacterial enzymes may be inhibited by CO_2 by formation of a bicarbonate complex, excess CO_2, pH lowering by dissolved CO_2, sorption/interaction of CO_2 into the enzyme molecules, and precipitation of intracellular Ca^{+2} and Mg^{+2} carbonates.

3. **Physical disruption of cells**

The disruption of physical cells was the first mechanisms proposed for microorganisms' inactivation and suggests that microbial cells' bursting' is due to the rapid pressure release and the expansion of CO_2 within the cell.

4. **Modification of cell membrane and extraction of cellular components**

This mechanism is based on the lipo- and hydrophilicity and solvent characteristics of CO_2. Extraction of intracellular substances and their transfer out of the

cell during pressure release may lead to microbial inactivation (Damar and Balaban 2006).

Factors affecting microbial inactivation

Several factors may influence microbial inactivation including:

1. **Water activity and water content**

DPCD is more effective as a_w increases. A higher water content of the treated product increase CO_2 solubility and enhances microbial inactivation.

2. **Pressure**

Since CO_2 solubility increases with increasing pressure this can help in microorganisms' inactivation.

3. **Temperature (T)**

Although solubility of CO_2 decreases with increasing temperature, higher T can increase the diffusivity of CO_2 and the fluidity of the cell membrane which can facilitate the penetration of CO_2 into cells. T can also affect the change of CO_2 from subcritical to supercritical phase (T_c = 31.1 °C) (Figure 2-10). Under supercritical conditions the penetration power of CO_2 is higher and at the near-critical region there is a rapid change in solubility and density of CO_2 (Damar and Balaban 2006).

4. **Initial pH**

Low pH facilitates penetration of carbonic acid through the cell membrane leading to more inactivation.

5. **Cell growth phase and age**

Young cells are more sensitive and are easier to inactivate than mature cells.

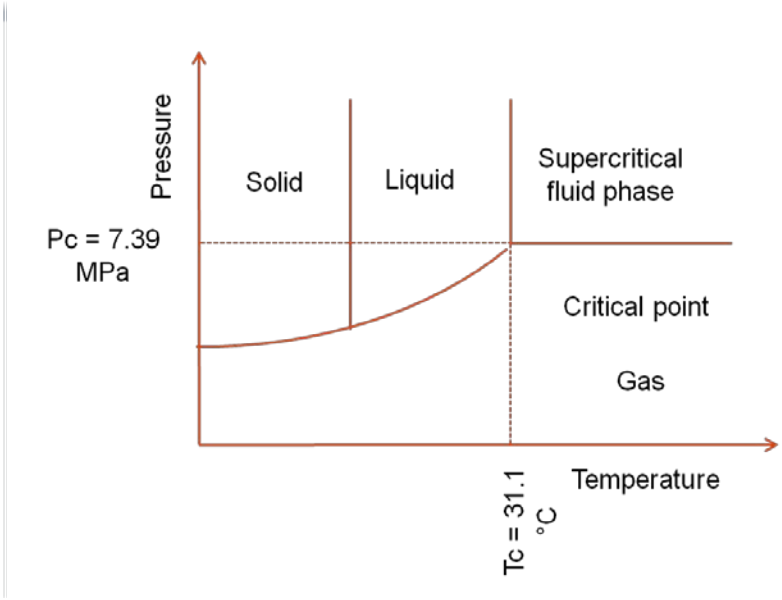


Figure 2-10. Phase diagram of carbon dioxide

6. Type of microorganism

Different bacteria have different susceptibilities to DPCD. It has been suggested that the nature of the cell wall could be important in the difference in sensitivity between G(+) and G(-) bacteria. Since G(-) bacteria have thin cell walls, they are expected to be more sensitive and their cells walls could be more easily ruptured as compared to G(+) bacteria.

7. Type of treatment system

The system used for DPCD treatment can affect the microbial inactivation rate. Systems that allow better contact of CO_2 with the food are more effective in microbial reduction because of the more rapid saturation of the solution with CO_2. Usually batch systems require longer treatment times for microbial inactivation as compared to continuous systems. Inactivation rate can be increased in batch systems by using agitation (Damar and Balaban 2006).

Solubility of CO_2

In a continuous flow DPCD system, several variables are controlled during processing: pressure, temperature, residence time, and %CO_2. The amount of CO_2 used should assure a complete saturation of the liquid but since its solubility at processing conditions is not known this can lead to the use of excess CO_2 elevating production costs.

CO_2 solubility in liquid foods can be affected by pressure, temperature, and food composition. Pressure has a direct effect on CO_2 solubility meaning that as pressure increases, CO_2 solubility increases while as temperature increases, solubility of CO_2 decreases. Other substances present in the food (composition) either increase or decrease the solubility of CO_2 (Calix and others 2008).

Recent studies have focused on the measurement of CO_2 solubility in liquid model systems and fruit juices. These experiments were done using an experimental apparatus designed and built at the University of Florida (Calix and others 2008). This system is designed to saturate a known amount of liquid by bubbling CO_2 from the bottom of a vessel under controlled pressure and temperature and afterwards the CO_2 gas is expanded and measured at ambient pressure.

Table 2-7 presents solubility of CO_2 for water, orange, apple, and grapefruit juice measured at different pressures in these studies.

Solubility of CO_2 in fruit juices is lower than that of pure water because of the presence of solutes such as sugars and acids which lowers the amount of CO_2 that can dissolve (Calix and others 2008; Ferrentino and others 2009).

Table 2-7. CO_2 solubility of liquid foods measured at 40 °C

Liquid	Pressure (MPa)	Solubility*	Reference
Water	7.58 – 31.0	4.71 – 6.32	Calix and others 2008
Orange juice	7.58 – 15.86	4.10 – 4.98	Calix and others 2008
Apple juice	7.58 – 15.86	3.95 – 5.01	Calix and others 2008
Grapefruit juice	7.58 – 31.0	3.97 – 4.70	Ferrentino and others 2009

* g of CO_2/100 g of liquid.

DPCD treatment systems

Batch, semi-continuous, and continuous systems have been developed for DPCD applications. In a batch system, CO_2 and the food to be treated are stationary in a container during treatment. A semi-continuous system allows a continuous flow of CO_2 through the chamber while a continuous system allows flow of both CO_2 and the liquid food through the system.

A typical batch system has a CO_2 gas cylinder, a pressure regulator, a pressure vessel, a water bath or heater, and a CO_2 release valve. The sample is placed into the pressure vessel and temperature is set to the desired value. CO_2 is then introduced into the vessel until the sample is saturated at the desired pressure and temperature. The sample is left in the vessel for a period of time and then the CO_2 outlet valve is opened to release the gas. Some systems contain an agitator to decrease the time to saturate the sample with CO_2 (Damar and Balaban 2006).

A continuous flow DPCD system was developed in 1999 by Praxair (Chicago, III., U.S.A.) (Figure 2-11).

CO_2 and the product are pumped through the system and are mixed before entering the high pressure pump, which increases the pressure to the processing levels.

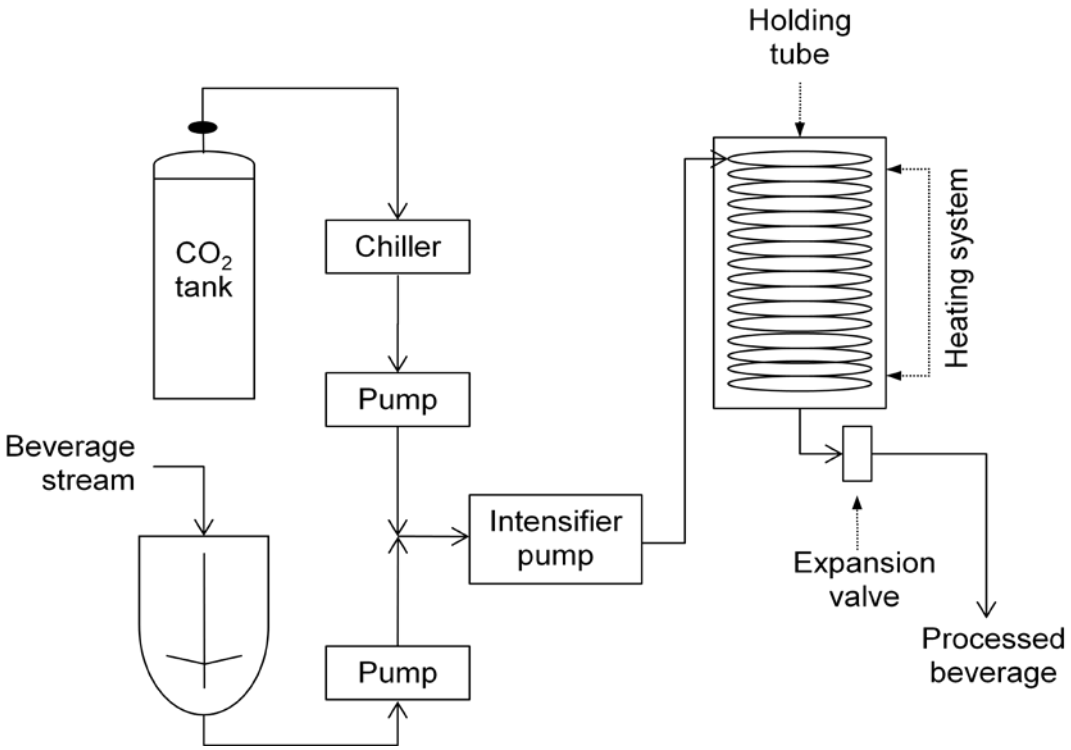


Figure 2-11. Schematic diagram of the continuous flow dense phase CO_2 system Product temperature is controlled in holding coils. Residence time is adjusted by setting the flow rate of the product. At the end of the process, CO_2 is released by means of an expansion valve (Damar and Balaban 2006).

DPCD food applications

DPCD has been applied mainly to liquid foods. This technology has been tested in several products at the University of Florida, Food Science and Human Nutrition Department using the continuous flow system presented in Figure 2-11, and includes: orange, mandarin, and grapefruit juice, beer, grape juice, and coconut water among others.

Orange juice

Several studies with orange juice (OJ) showed that DPCD treatment can improve some physical, nutritional and quality attributes such as cloud formation and stability,

color, and ascorbic acid retention (Damar and Balaban 2006).

Kincal and others (2005) tested the capacity of the DPCD system to reduce both natural and inoculated microbial loads of pulp-free Valencia OJ at different pressures (38, 72, and 107 MPa), temperatures (25 and 34.5 °C), residence times (3-10 min), and CO_2/juice ratios (0.1-1.0). A storage study was conducted at 1.7 °C with juice processed at 107 MPa, CO_2/juice ratio of 1.0 and residence time of 10 minutes. Residence time had the greater influence on microbial reduction, followed by pressure. The CO_2/juice ratio and temperature showed not to be the driving forces on microbial load reduction in this system. They proved that the system was able to achieve a 5-log reduction of the natural flora in spoiled juice (38, 72, and 107 MPa at 25 and 34.5°C, CO_2/juice ratio of 1.0 and residence time of 10 min), and 5-log decrease of pathogenic *Escherichia coli* O157:H7 (107 MPa and residence time of 10 min), *Salmonella typhimurium* (38, 72, and 107 MPa and residence time of 10 min), and *Listeria monocytogenes* (38, 72, and 107 MPa and residence time of 10 min). During the refrigerated storage study, they observed an increase in the bacterial number possibly because of an injury/repair mechanism of some of the microorganisms or due to post-contamination.

A study was performed by Kincal and others (2006) to treat pulp-free Valencia OJ at pressures of 38, 72, and 107 MPa, and CO_2/juice (w/w) ratios from 0.40 to 1.18 with a constant residence time of 10 min.

The highest PE inactivation (46.3%) was obtained when the pressure was 107 MPa and no heat was applied. PE activity decreased with storage time. Cloud increased between 446 and 846% after treatments and remained 4 times higher than the control during storage. They found no significant changes in pH and °Brix of treated samples.

TA increased slightly after treatment and remained constant throughout storage. Small but insignificant increase in L^* and a^* values occurred after treatment. Juice color did not change drastically during storage. Sensory evaluations of DPCD-treated and untreated OJ were not significantly different after 2 weeks of refrigerated storage at 1.7^oC (Balaban and others 2008).

Mandarin juice

Lim and others (2006) processed mandarin juice. The process variables were temperature (25-45°C), pressure (13.8-41.4 MPa), residence time (5-9 minutes) and $\%CO_2$ (2-12). They found that temperature and $\%CO_2$ had a significant effect in log reduction of total aerobic plate count while pressure and residence time were not significant. The maximum log reduction (3.47) was observed at 35 °C, 41.4 MPa, 9 min and 7 $\%CO_2$. PE inactivation ranged from 6.1 to 50.7% and maximum inactivation was achieved at 45°C, 41.4 MPa, 7 min and 7% CO_2. Cloud was not only retained but enhanced. The highest cloud increase was 38.4% at 45°C, 27.6 MPa, 7 min, and 2% CO_2. Lightness and yellowness increased and redness decreased after treatment. pH and °Brix didn't change after treatment while titratable acidity of treated samples was higher than the untreated juice (Balaban and others 2008).

Grapefruit juice

Red grapefruit juice was processed using DPCD at pressures of 13.8, 24.1, and 34.5 MPa and residence time of 5, 7, and 9 min at a constant temperature of 40 °C and CO_2 level of 5.7% to evaluate the effect of treatments on yeasts and molds and total aerobic bacteria inactivation. A five log reduction for yeasts and molds and total aerobic

bacteria was achieved at 34.5 MPa and 7 min of treatment (Ferrentino and others 2009).

Ferrentino and others (2009) also conducted a storage study with DPCD processed red grapefruit juice for 6 wk at 4 °C. No growth of total aerobic bacteria and yeasts and molds was observed in the DPCD treated juice. Cloud in the juice increased 91% while PE inactivation was partial (69.17%). No significant differences in °Brix, pH, and TA were detected between treated and untreated samples while the treated juice had a higher lightness and redness and lower yellowness. Total phenolics content was not affected by treatment and storage and slight differences were detected for ascorbic acid content and antioxidant capacity.

Beer

Dagan and Balaban (2006) studied the effect of DPCD on beer quality. They predicted a maximum log reduction in yeast population of 7.4 logs at processing conditions of 26.5 MPa, 21 °C, 9.6% CO_2, and residence time of 4.77 min. DPCD pasteurization reduced haze from 146 nephelometric turbidity units (NTU) to 95 NTU. Aroma and flavor of beer at these same conditions was not significantly different when compared to a fresh beer sample in a difference from control test. Foam capacity and stability of beer were minimally affected by the process; however these changes were unnoticed by consumers.

Grape juice

Several studies were conducted with muscadine grape juice testing the effect of DPCD on microbial reduction, physicochemical, phytochemical and quality changes after treatment and during storage. Different processing conditions of pressure (1.2-40.2

MPa), CO_2 levels (0-15.7%), and constant residence time (6.25 min) and temperature 30 °C) were evaluated by Del Pozo-Insfran and others (2006a). Results showed that processing pressure was a significant factor affecting microbial inactivation but that CO_2 content was the processing parameter that had the major influence in microbial log reduction.

Subsequent storage stability for 10 wks at 4 °C with two treatments that achieved >5 log reduction (34.5 MPa at 8 and 16% CO_2) were evaluated and compared to a heat pasteurized juice (75 °C, 15 s). Results showed that thermal pasteurization decreased anthocyanins (16%), soluble phenolics (26%), and antioxidant capacity (10%) whereas no changes were observed for both DPCD treated juices. DPCD juices also retained higher anthocyanins (335 mg/L), polyphenolics (473 mg/L), and antioxidant capacity (10.9 μmol of Trolox equivalents/mL) than thermally pasteurized juices at the end of storage (Del-Pozo-Insfran and others 2006a).

Insignificant differences in sensory attributes (color, flavor, aroma, and overall likeability) were observed between unprocessed and DPCD juices, while significant differences were observed between unprocessed and heat-pasteurized juices. Panelists preferred DPCD over heat-pasteurized juices throughout the first 6 weeks of storage but afterwards the growth of yeast and mold adversely affected juice aroma. Comparable microbial counts were observed between DPCD and thermally pasteurized juices during the first 5 weeks of storage (Del-Pozo-Insfran and others 2006a).

Another study evaluated the phytochemical stability and organoleptic attributes of an ascorbic acid fortified muscadine grape juice as affected by DPCD processing and

addition of thyme polyphenolic cofactors (*Thymus vulgaris*; 1:100 anthocyanin-to-cofactor molar ratio) (Del-Pozo-Insfran and others 2006b).

DPCD processing insignificantly altered initial juice phytochemical and antioxidant content, whereas thermal pasteurization reduced anthocyanins (263 mg/L), ascorbic acid (42 mg/L), soluble phenolics (266 mg/L), and antioxidant capacity (6 μmol of Trolox equivalents/mL). Similar trends were observed during storage, and data showed that increasing the CO_2 level from 8 to 16% during DPCD processing contributed to the reduction of juice phytochemical and antioxidant degradation. Copigmentation helped retain higher anthocyanins, soluble phenolics, and antioxidant capacity during storage without affecting initial juice aroma and flavor characteristics (Del-Pozo-Insfran and others 2006b).

A third study by Del Pozo-Insfran and others (2007) assessed the effect of DPCD processing on polyphenol oxidase (PPO) activity, polyphenolic and antioxidant changes in muscadine grape juice under different processing pressures (27.6, 38.3, and 48.3 MPa), CO_2 levels (0, 7.5, and 15%), and constant residence time (6.25 min) and temperature (30 °C). Pressure alone was responsible for a 40% decrease in PPO activity that resulted in 16–40% polyphenolic and antioxidant losses. Increasing CO_2 from 0 to 7.5% was responsible for an additional 35% decrease in enzyme activity and a 2-fold greater polyphenolic retention. However, insignificant changes in PPO activity or polyphenolic retention were observed when CO_2 was increased to 15%.

Subsequently two DPCD conditions (48.3MPa at 0 and 15% CO_2) were evaluated for polyphenolic and antioxidant changes during storage (4 °C, 4 wks). Juices with residual PPO activity following processing resulted in greater polyphenolic (8-10-

fold) and antioxidant capacity (4-fold) degradation compared to control juices with no PPO activity.

Coconut water

The effects of DPCD on microbial, physical, chemical and sensorial quality of a coconut water beverage were evaluated by Damar and others (2009). Different processing conditions of pressure (13.8, 24.1, and 34.5 MPa), temperature (20, 30, and 40 °C), %CO_2 level (7, 10, 13 gCO_2/100g of beverage), and constant residence time of 6 min were tested. Pressure was not significant in microbial reduction whereas temperature and %CO_2 levels were significant.

In the same study, DPCD-treated (34.5 MPa, 25 °C, 13% CO_2, 6 min), heat-pasteurized (74 °C, 15 s) and untreated coconut beverages were evaluated during 9 wks of refrigerated storage (4 °C). Total aerobic bacteria of DPCD and heat-treated samples decreased while that of untreated samples increased to >10^5 CFU/mL after 9 wks. DPCD increased titratable acidity but did not change pH (4.20) and °Brix (6.0).

Likeability of DPCD-treated coconut water was similar to untreated. Heat treated samples were less liked at the beginning of storage. Off flavor and taste-differences-from control scores of heated samples were higher than DPCD during the first two weeks (Damar and others 2009).

Sensory Evaluation

The attributes of a food item are typically perceived in the following order: appearance (color, size and shape, surface texture), odor/aroma/fragrance, consistency and texture, and flavor (aromatics, chemical feelings, taste). However in the process of perception most or all of the attributes overlap (Meilgaard and others 2007).

Sensory tests provide useful information about the human perception of product changes due to ingredients, processing, packaging, or shelf-life. Sensory evaluation includes a set of test methods with guidelines and established techniques for product presentation and well-defined responses**,** statistical methods, and guidelines for interpretation of results. There are three primary sensory tests: discrimination tests (focus on the existence of overall differences among products), descriptive analysis (specification of attributes), and affective or hedonic testing (measuring consumers likes and dislikes) (Lawless and Heymann 1998).

Difference-from-Control-Test

A difference-from-control-test is used to determine whether a difference exists between one or more samples and a control, and to estimate the size of any such difference. One sample is designated the “control”, “reference”, or “standard”, and all other samples are evaluated with respect of how different each is from the control (Meilgaard and others 2007).

Flavor Analysis

Flavor perception depends of the combined responses or our senses and the cognitive processing of these inputs (Reineccius 2006).

Taste is the combined sensation that derives from specialized taste receptor cells located in the mouth. It is primarily limited to the tongue and is divided into the sensations of sweet, sour, salty, bitter, and umami while olfaction is the sensory component that results from the interaction of volatile food components with olfactory receptors in the nasal cavity. The stimulus for the aroma of a food can be orthonasal (the odor stimulus enters the olfactory region directly from the nose as we sniff the food)

or retronasal (the stimulus enters from the oral cavity as we eat a food) (Reineccius 2006).

Most of the techniques used in aroma isolation take advantage of either solubility or volatility of the aroma compounds (Reineccius 2006).

Solid Phase Micro Extraction (SPME)

SPME is a relatively new technique for the isolation of food aromas. An inert fiber is coated with an adsorbent. The adsorbent coated fiber is placed in the headspace of a sample, or the sample itself if liquid, and allowed to adsorb volatiles. The loaded fiber is then thermally desorbed into a GC carrier gas flow, and the released volatiles are analyzed. Since SPME is an equilibrium technique, the volatile profile obtained is strongly dependant of the sample composition and careful control of all sampling parameters is required (Reineccius 2006).

The effectiveness of SPME techniques depends on many parameters such as : type of fiber, sample volume, temperature and extraction time, and desorption of analytes from the fiber (Waldemar and others 2004).

Gas chromatography-Olfactometry (GC-O)

GC-O is a technique only applied to aroma studies. In olfactometric techniques, the nose is used as a GC detector. The GC system may be set up in such a way that the column effluent is split so that a portion of the effluent goes to a sniffing port and the reminder goes to a GC detector (flame ionization (FID) or an MS detector). The GC-O produces what is called an aromagram, which is a listing of the odor character of each peak in a GC run. Mass spectroscopy is generally used in the flavor area to either determine the identity of an unknown or to act as a mass-selective GC detector. The

GC-MS as an identification tool is unique because of its high sensitivity (10-100pg) (Reineccius 2006).

With consumer demands for natural beverages with health promoting properties that offer fresh-like sensory attributes, *Hibiscus sabdariffa* may offer an additional market in this arena. Previous research has focused on a hot beverage or extraction process while new technologies focus on minimal to nonthermal processing. Dense phase carbon dioxide processing may offer an alternative to the traditional hibiscus processing and provide consumers a product with fresh-like quality and health benefits. This research focused on three main areas: 1) finding alternatives to the water extraction conditions that do not involve heat but suitable for nonthermal processing, 2) comparing DPCD to heat pasteurization by evaluating the physicochemical, phytochemical, and sensory properties during processing and storage, and 3) evaluating aroma and phytochemical profiles of water hibiscus extracts obtained from fresh and dried hibiscus.

CHAPTER 3
EFFECT OF COLD AND HOT WATER EXTRACTION ON THE PHYSICOCHEMICAL AND PHYTOCHEMICAL PROPERTIES OF *HIBISCUS SABDARIFFA* EXTRACTS

Introduction

Hibiscus sabdariffa L (family Malvaceae) is a tropical annual shrub. China, Thailand, Mexico, Egypt, Senegal and Tanzania are among the main producing countries. In Mexico, this plant is known as “flor de jamaica” or simply “jamaica”. The red calyces are the part of the plant with commercial interest and are rich in organic acids, minerals, anthocyanins, and other phenolic compounds.

Hibiscus extracts contain two major anthocyanins, delphinidin-3-sambubioside and cyanidin-3-sambubioside. Their spectral characteristics (Degenhardt and others 2000), MS fragmentation patterns (Giusti and others 1999), and potential antioxidant (Wang and others 2000) and anticancer (Chang and others 2005; Hou and others 2005) activities have been previously studied. Similarly, other polyphenolic compounds including protocatechuic acid (Lee and others 2002; Olvera-Garcia and others 2008), hibiscus acid and its 6-methyl ester (Hansawasdi and others 2000) have also been found to be present in hibiscus extracts and have been associated with pharmacological activities. Differences in variety and extraction conditions (type of solvent, concentration, time and temperature) potentially affect the polyphenolic profile of the extracts and makes comparison between studies difficult.

Traditionally, fresh hibiscus is either frozen or dried in the sun for preservation and used in the production of natural color, flavor extracts and/or beverages. Preparation of a hibiscus beverage includes an extraction step followed by a pasteurization method. The use of non-thermal technologies such as dense phase carbon dioxide, pulsed UV light, high hydrostatic pressure, and pulsed electric fields as

a preservation method does not justify an extraction step that involves high temperature, and an alternative extraction at a lower temperature should be considered.

The objectives of this study were: (1) to compare the effects of cold (25°C) and hot (90°C) water extraction on the physicochemical and phytochemical properties of hibiscus extracts and (2) to identify and quantify the anthocyanins and major polyphenolics present in extracts obtained from fresh and dried hibiscus by equivalent cold and hot water extraction conditions.

Materials and Methods

Chemicals and Standards

Commercial standards of chlorogenic acid, gallic acid, protocatechuic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Delphinidin-3-glucoside and cyanidin-3-glucoside were purchased from Polyphenols Laboratories AS (Sandnes, Norway). AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), fluorescein (free acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Folin-Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.).

Extracts Preparation

Fresh and sun dried *Hibiscus sabdariffa (cv. "Criollo")* were obtained from Puebla, Mexico. Hibiscus samples were stored in glass jars, flushed with nitrogen and kept frozen at -22 °C until used. For the first part of the experiment, dried hibiscus was mixed with distilled water at a ratio of 1:40 (w/v) and maintained at 25 °C (cold extraction, CE) or 90 °C (hot extraction, HE) for four different times (30, 60, 120, and 240 min for CE and 2, 4, 8, and 16 min for HE). Eight treatments (TRT's) were tested. For the second part of the experiment, four extracts were prepared using equivalent

cold and hot water extraction conditions. Fresh (F) and dried (D) hibiscus were mixed with distilled water at a ratio of 1:4 and 1:40 (w/v) respectively, and extracted at both 25°C for 240 min (CE) and 90°C for 16 min (HE). For CE, temperature was controlled using a Constant Temperature Circulator Bath, Model 900 (Fisher Scientific; Pittsburg, Pa., U.S.A.) and stirring was applied using a stirrer plate Model PC-353 (Corning, Lowell, Mass., U.S.A.) at speed #4. For HE, a Microprocessor Controlled Water Bath, Series 280 (Precision Scientific, Winchester, Va., U.S.A.) was used to control temperature. All the obtained extracts were filtered under vacuum (Whatman filter paper #4) and their physicochemical and phytochemical properties were measured using the methods described below.

pH, Total Solids, and Titratable Acidity

pH was measured using a pH meter EA920 (Orion Research, Boston, Mass., U.S.A.) and total solids (TS) were determined by difference in weight after drying the samples at 105 °C for 24 h in an oven (Precision Scientific, Winchester Va., U.S.A.). A Brinkmann Instrument (Brinkmann Instruments Co., Westbury, N.Y., U.S.A.) consisting of a Metrohm 655 Disomat, Metrohm 614 Impulsomat, and Metrohm 632 pH meter was used to measure titratable acidity (TA). Samples of 10 mL were used and TA was determined by titration with 0.1 N NaOH until pH 8.1 and expressed as % malic acid (g/100 mL).

Color, Color Density and Hue Tint

Color was measured using a ColorQuest XE colorimeter (HunterLab, Reston, Va., U.S.A.). Samples (40 mL) were placed in a 20 mm cell and L^*, a^*, and b^* parameters were recorded in total transmittance mode, illuminant D65, 10° observer angle.. Color density and hue tint were determined by measuring the absorbance (A) at

420, 520, and 700 nm for samples (200 μL) using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale Calif., U.S.A.) and calculated as:

$$\text{Color density} = (A_{420\ nm} - A_{700\ nm}) + (A_{520\ nm} - A_{700\ nm}) \quad (1)$$

$$\text{Hue tint} = (A_{420\ nm} - A_{700\ nm})/(A_{520\ nm} - A_{700\ nm}) \quad (2)$$

as described by Giusti and Wrolstad (2005).

Anthocyanin Content, Total Phenolics and Antioxidant Capacity

Anthocyanin content was determined by the pH differential method ($A_{510\ nm}$ and $A_{700\ nm}$ at pH 1.0 and 4.5, dilution factor (DF) of 4) and expressed in mg/L of delphinidin-3-glucoside (MW = 465.2, ε = 23700) as described by Giusti and Wrolstad (2005). Total phenolics were measured using the Folin-Ciocalteu assay ($A_{765\ nm}$, DF of 4) and quantified as gallic acid equivalents (mg/L) (Waterhouse 2005). Absorbance measurements for anthocyanin content and total phenolics were made using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale Calif., U.S.A.).

Antioxidant capacity was evaluated using the oxygen radical absorbance capacity (ORAC) assay and results were expressed as Trolox equivalents (TE) per milliliter (μmol of TE/mL) as described by Huang and others (2002) using a SpectraMax Gemini XPS microplate sprectrofluorometer (Molecular Devices, Sunnyvale, Calif., U.S.A.). Data was acquired and analyzed using SoftMax Pro 5.2 software (Molecular Devices, Sunnyvale, Calif., U.S.A.).

Characterization of Major Polyphenolics

Equivalent cold and hot water extraction conditions (25 °C for 240 min (CE) and 90 °C for 16 min (HE)) were selected based on the first part of this study. Four hibiscus extracts were prepared: dried hibiscus cold water extract (DCE), dried hibiscus hot

water extract (DHE), fresh hibiscus cold water extract (FCE), and fresh hibiscus hot water extract (FHE). LC-MS and HPLC analysis were performed in order to identify the major polyphenolic compounds including anthocyanins present in these extracts.

LC-MS identification

Chromatographic analyses were performed on an Agilent 1200 series HPLC (Agilent, Palo Alto, Calif., U.S.A.) equipped with an autosampler/injector and diode array detector. A Dionex C18 5 μm 120A column (250 x 4.6 mm) was used for compound separation (Dionex, Sunnyvale, Calif., U.S.A.). Mobile phases consisted of water (phase A) and 60% methanol in water (phase B), both adjusted to pH 2.4 with formic acid. A gradient solvent program ran phase B from 0% to 60% in 20 min; 60% to 100% in 20 min; 100% for 7 min; 100% to 0% in 3 min and final conditions were held for 2 min. The flow rate was 0.8 mL/min, and detection was done at 260, 280, 320, 360 and 520 nm.

Electrospray ionization mass spectrometry (ESI-MS) was performed with a HCT series ion trap mass spectrometer (Bruker Daltonics, Billerica, Mass., U.S.A.). Column effluent was monitored in positive and negative ion mode of the MS in an alternative manner during the same run. Other experimental conditions on the mass spectrometer were as follows: nebulizer, 45 psi; dry gas (nitrogen), 11.0 L/min; dry temperature, 350 °C; ion trap, scan from m/z 90 to 1000; smart parameter setting (SPS), compound stability, 50%; trap drive level, 60%. The mass spectrometer was operated in Auto MS^2 mode. MS^2 was used to capture and fragment the most abundant ion in full scan mass spectra.

Polyphenolics were identified by comparison of UV/vis (190-660 nm) spectral interpretation, retention time, comparison to standards, and MS fragmentation patterns.

HPLC quantification

Anthocaynins and polyphenolics were quantified using a Dionex HPLC system equipped with an autosampler/injector and diode array (PDA 100) detector (Dionex, Sunnyvale, Calif., U.S.A.). Compounds were separated on a 250 x 4.6 mm Dionex C18 5 μm 120A column (Dionex, Sunnyvale, Calif., U.S.A.). Mobile phases consisted of water (phase A) and 60% methanol in water (phase B), both adjusted to pH 2.4 with *o*-phosphoric acid. A gradient solvent program ran phase B from 0% to 60% in 20 min; 60% to 100% in 20 min; 100% for 7 min; 100% to 0% in 3 min and final conditions were held for 2 min. The flow rate was 0.8 mL/min, and detection was done at 260, 280, 320, 360 and 520 nm.

Statistical Analysis

Each treatment condition was repeated in triplicate. Analysis of variance (ANOVA) and mean separation using Tukey's test (α=0.05) were performed to evaluate the differences between extraction times, temperatures, and treatments using SAS 9.0 Statistical software (SAS Institute Inc., Cary, N.C., U.S.A.).

Results and Discussion

Effect of Extraction Conditions

The physiochemical and phytochemcial parameters measured for the eight hibiscus treatments analyzed are presented in Table 3-1 and Figures 3-1, 3-2, and 3-3.

Color density (CD), anthocyanins content (AC), total phenolics (TP), and antioxidant capacity (AOX) increased with increasing time for both extraction temperatures (25 and 90 °C) while L^* values decreased with time in both cases. There were no significant differences between the first two (30 and 60 min, and 2 and 4 min)

and last two (120 and 240 min, and 8 and 16 min) extraction times at both temperatures for hue tint (HT) values; although the latter times were higher.

For cold water extraction (25 °C), time had a significant effect ($p < 0.0001$) in all the parameters measured but pH. Total solids (TS) and b^* increased from 30 to 60 min and afterwards remained constant (measurements at times 60, 120, and 240 min were not significantly different). Titratable acidity (TA) increased from 30 to 120 min and remained constant at 240 min (measurements at 120 and 240 min were not significantly different) while a^* values increased from 30 to 60 min, remained constant from 60 to 120 min and decreased at 240 min. For hot water extraction (90 °C), time had a significant effect ($p < 0.0335$) in all the measured parameters. pH increased from 2 to for 4 min and remained constant until 16 min. TS and TA increased until 8 min and remained constant until 16 min. a^* values were constant for times 2 and 4 min and decreased at 8 and 16 min while b^* values were constant at 2, 4, and 8 min, and decreased at 16 min.

There was a significant effect ($p < 0.0001$) of treatment conditions (temperature + time) in all the measured parameters but pH. Treatments CE1 and HE1 were equivalent in TS and TA (Table 3-1), treatments CE2 and HE2 were equivalent in TS, TA, b*, CD, and AC (Figure 3-1). Treatments CE3 and HE3 were equivalent in TS, TA, and AC while treatments CE4 and HE4 were equivalent in TS, TA, CD, and AC.

L^* values were significantly lower (darker color) in hot water extracts as compared to cold water ones while a^* values were slightly higher in the cold water extracts. Hue tint (HT) is a measurement of color degradation in anthocyanin containing products. From Table 3-1, it can be observed that the extracts obtained with cold water have lower values than the ones obtained with hot water. This indicates that

temperature had an effect on hibiscus extract's color, and thus anthocyanins. A higher hue tint value is associated with an increase in absorbance at 420 nm (yellow tones) in relation to that at 520 nm (red tones); this is undesirable because it is an indication of anthocyanins degradation

Anthocyanin content was not significantly different between treatments CE2 and HE2, CE3 and HE3, and CE4 and HE4 (Figure 3-1) so equivalent cold and hot water extraction conditions for anthocyanins were found. As can be seen from Figure 3-2, TP extraction was better with hot water (90 °C) than with cold water (25 °C). Prenesti and others (2007) also found that hot water (100 °C for 3 min) extracted a higher phenolic content compared to cold water hibiscus extracts. The higher concentration of polyphenolic compounds other than anthocyanins in hot water extracts may have contributed to a higher antioxidant activity in these extracts as compared to cold water extracts (Figure 3-3). Tsai and others (2002) found that hibiscus anthocyanins contributed to 51% of total antioxidant capacity and that other phenolic compounds were responsible for the remainder of activity.

Qualitative differences were observed between the cold and hot water hibiscus extracts. Cold extracts had a clear appearance and bright red color whereas the hot extracts presented a more opaque red color and some haze possibly associated to a higher concentration of phenolic compounds other than anthocyanins.

Parameters Correlations

Several correlations between the parameters measured were found in both extraction processes with $r^2 > 0.9$. Linear regression parameters and correlation coefficients are presented in Table 3-2. This linear behavior will only be valid before reaching equilibrium during the extraction process, after which, the values of the

measured parameters will remain almost constant. Similarly the equilibrium for the extraction of anthocyanins and other polypheolic compounds could be reached at different times. Since some of the methods used in this study can be easier or faster than others, these equations could be used to predict parameters measured with more time consuming methods within the range of studied extraction conditions.

L^* and CD values were inversely correlated. A high L^* value (lighter color) will be associated with a low CD value. Color density, anthocyanins content, total phenolics and antioxidant capacity were all directly correlated.

Polyphenolics Identification

Anthocyanins and other polyphenolics identified in hibiscus extracts are presented in Figure 3-4 and Tables 3-3 and 3-4. Compounds were identified on the basis of their retention time, absorption spectrum, MS fragmentation pattern, and where possible by comparison to an authentic standard.

Peak 1 (t_R, 13.4 min; λ_{max}, 271 nm) was identified as gallic acid by comparison of the absorption spectrum with a standard. This was confirmed by MS-MS analysis that showed the presence of a negatively charged molecule ion ($[M-H]^-$) at m/z 169 which fragmented to produce a secondary fragment ion (MS^2) at m/z 125 (see Table 3-4). The presence of gallic acid in hibiscus extract was measured previously by GC-MS (Mourtzinos and others (2008).

Peak 2 (t_R, 17.1 min; λ_{max}, 259 nm) was identified as protocatechuic acid glucoside. The absorption spectrum was compared to a protocatechuic acid standard; the presence of the glucose molecule slightly shifted the retention time. MS analysis of the peak reveled a $[M-H]^-$ at m/z 315 that fragmented to yield the ion m/z 153 which

corresponds to protocatechuic acid. The difference between ions 315 and 153 gave an ion with m/z 162 which corresponds to glucose. The same MS fragmentation patterns for protocatechuic acid glucoside were reported in dried plum (Fang and others 2002). Protocatechuic acid isolated from hibiscus extracts was demonstrated to have anti-atherosclerosis (Lee and others 2002), antitumor (Olvera-Garcia and others 2008; Tseng and others 1998), antioxidant (Lin and others 2003), and anti-inflammatory (Liu and others 2002) activities.

Peaks 3 (t_R, 18.7 min; λ_{max}, 326 nm), 4 (t_R, 23.0 min; λ_{max}, 327 nm), 5 (t_R, 23.6 min; λ_{max}, 327 nm), and 7 (t_R, 24.3 min; λ_{max}, 331 nm) were identified as caffeoylquinic acids which are esters formed between caffeic and quinic acid. Their identification was based on previously developed structure-diagnostic hierarchical keys (Clifford and others 2003), UV-vis spectrum and retention time was compared relative to a commercial 5-caffeoylquinic acid (chlorogenic acid) standard. Peaks 3, 4, 5, and 7 produced a $[M-H]^-$ at m/z 353 and MS^2 ions at m/z 191 (corresponds to quinic acid), 179 (corresponds to caffeic acid), 173, and 135, (peak 7 only had MS^2 ions at m/z 191 and 173). Peak 4 was identified as 5-caffeoylquinic acid (5-CQA) by comparison with an authentic standard. According to Clifford and others (2003) 5-CQA is characterized by an intense base peak at m/z 191 and a weak secondary ion at m/z 179. Peak 3 was identified as 3-caffeoylquinic (3-CQA) acid since it is characterized by a base peak at m/z 191 and a relatively intense secondary ion at m/z 179 while peak 5 was identified as 4-caffeoylquinic (4-CQA) acid with a characteristic base peak at m/z 173 (Clifford and others 2003). Peak 7 was tentatively identified as a caffeoylquinic acid isomer from its absorption spectrum and fragmentation patterns (see Table 3-4). The presence of 5-

CQA has been previously reported in hibiscus extracts (Mourtzinos and others 2008; Segura-Carretero and others 2008).

Peaks 6 (t_R, 24.2 min; λ_{max}, 529 nm) and 8 (t_R, 26.2 min; λ_{max}, 521 nm) were identified as delphynidin-3-sambubioside (D3S) and cyanidin-3-sambubioside (C3S) which are the two major anthocyanins present in hibiscus (Table 3-3). Identification was based on their absorption spectrum and MS fragmentation patterns which have been previously reported (Juliani and others 2009; Degenhardt and others 2000; Giusti and others 1999). The difference between the MS of the molecule (597) and the aglycone (303) for D3S gave a m/z of 294 which corresponds to xylose-glucose (132+162) known as sambubiose. Similarly, the MS for the C3S molecule (581) and the aglycone (287) yields the sambubiose disaccharide.

Peaks 9 (t_R, 29.0 min; λ_{max}, 359 nm), 10 (t_R, 30.9 min; λ_{max}, 348 nm), and 11 (t_R, 32.0 min; λ_{max}, 356 nm) were tentatively identified as flavonols for their characteristic absorption spectrum with λ_{max} ~360 nm. Peak 12 (t_R, 35.7 min; λ_{max}, 355 nm) was also tentatively identified as quercetin-3-rutinoside by its absorption spectrum and MS fragmentation patterns which revealed a base peak at m/z 609 and MS^2 at m/z 301. The difference between m/z 609 and 301 gave a m/z of 308 that corresponds to the disaccharide rutinose formed between rhamnose (m/z 146) and glucose.(m/z 162).The presence of rutinose has been previously reported in hibiscus extract as part of an anthocaynin (cyanidin-3-rutinoside) by Segura-Carretero and others (2008). Quercetin-3-rutinose with the same MS fragmentation patterns was found in black and green tea (Del Rio and others 2004) and in pear skins (Lin and Harnly 2008).

Polyphenolics Quantification

Polyphenolics were quantified in the four hibiscus extracts studied (dried hibiscus cold water extract (DCE), dried hibiscus hot water extract (DHE), fresh hibiscus cold water extract (FCE), and fresh hibiscus hot water extract (FHE) (Table 3-5). Results were expressed in milligrams per L of extract. Hydroxybenzoic acids accounted for ~2% of the total polyphenolics quantified in the dried hibiscus extracts and ~0.5% in the fresh hibiscus extracts. Caffeoylquinic acids accounted for ~45% and ~38% of total in dried and fresh extracts, respectively while flavonols accounted for ~10% of the total in all four extracts. Anthocyanins accounted for ~45% and ~50% of the total in the dried and fresh hibiscus extracts, respectively.

As seen in Table 3-5, the DHE sample had the highest concentration of total polyphenols followed by DCE, FCE, and FHE. Gallic acid was not detected in the fresh extracts and its presence in the dried hibiscus extracts could be attributed to a breakdown of another phenolic compound during the drying process. The concentration of protocatechuic acid glucoside was higher in fresh hibiscus extracts and a significantly lower concentration of caffeoylquinic acids was also observed compared with the dried extracts.

Hibiscus anthocyanins distribution was ~68% and 64% of the total for D3S and 32 and 36% for C3S in dried and fresh extracts, respectively. This indicated that a significantly higher concentration of C3S was found in the fresh hibiscus extracts as compared to dried extracts. Delphinidin-3-sambubioside was present in a significantly higher concentration in the hot water extracts as compared to the cold water ones but no significant differences were found in the concentration of cyanidin-3-sambubioside in the cold and hot water extracts for both fresh and dried hibiscus.

Conclusions

Equivalent cold and hot water conditions were found for anthocyanins extraction of dried hibiscus. Similar polyphenolic profiles were observed between fresh and dried hibiscus extracts although differences were found in the concentration of compounds. Hydroxybenxoic acids, caffeoylquinic acids, flavonols and anthocyanins constituted the polyphenolic compounds identified in hibiscus extracts. Findings of this research can provide more flexibility to hibiscus processing. Extraction process selection for industrial applications should consider availability of raw material (fresh or dried hibiscus), processing technology, time, and economic considerations.

Table 3-1. Measured pH, total solids (TS) (g of solids/100 mL of extract), titratable acidity (TA) (g of malic acid/100 mL of extract), and color (L*, a*, b* values, color density (CD) and hue tint (HT)) for the extracts.

TRT	T (°C)	time (min)	pH	TS	TA	L*	a*	b*	CD	HT
CE1	25	30	2.37^{a*}	0.68^{d}	0.28^{d}	54.18^{a}	65.85^{d}	45.39^{e}	1.04^{f}	0.35^{cd}
CE2	25	60	2.32^{a}	0.92^{bc}	0.38^{abc}	46.29^{b}	67.65^{a}	66.92^{abc}	1.82^{de}	0.35^{d}
CE3	25	120	2.32^{a}	0.97^{ab}	0.40^{ab}	43.78^{cd}	67.50^{a}	68.76^{a}	2.05^{c}	0.36^{c}
CE4	25	240	2.31^{a}	1.00^{ab}	0.44^{a}	40.79^{ef}	67.16^{ab}	68.22^{ab}	2.55^{ab}	0.36^{c}
HE1	90	2	2.37^{a}	0.79^{cd}	0.33^{cd}	44.82^{bc}	66.73^{bc}	65.19^{c}	1.73^{e}	0.38^{b}
HE2	90	4	2.37^{a}	0.90^{bc}	0.37^{bc}	42.13^{de}	66.39^{c}	67.03^{abc}	2.00^{cd}	0.38^{b}
HE3	90	8	2.36^{a}	0.95^{ab}	0.39^{ab}	39.34^{f}	65.65^{d}	65.70^{bc}	2.34^{b}	0.39^{a}
HE4	90	16	2.33^{a}	1.08^{a}	0.43^{a}	35.26^{g}	63.93^{e}	60.29^{d}	2.70^{a}	0.39^{a}

CE = Cold extraction, HE = Hot extraction. Data represents the mean of n=9. * Values with similar letters within columns are not significantly different (Tukey's HSD, p > 0.05).

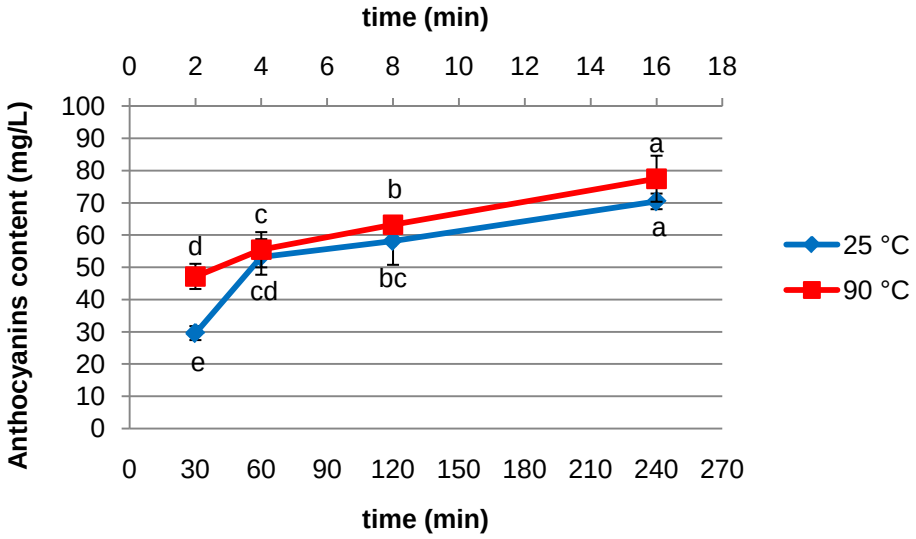


Figure 3-1. Total anthocyanins content expressed as delphinidin-3-glucoside (mg/L) for the extracts. The upper time scale belongs to the 90 °C curve and the lower time scale belongs to the 25 °C curve. Data represents the mean of n=9. Values with similar letters within the figure are not significantly different (Tukey's HSD, p > 0.05).

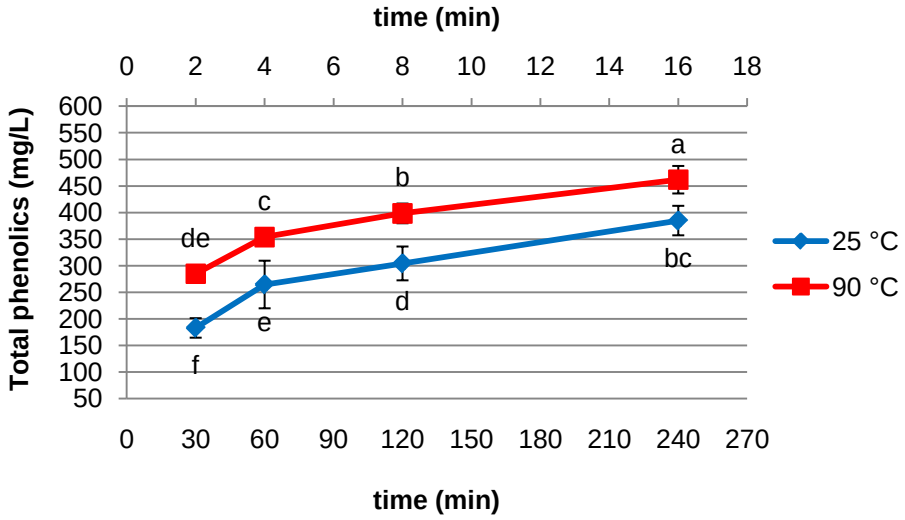


Figure 3-2. Total phenolics content expressed as gallic acid equivalents (mg/L) for the extracts. The upper time scale belongs to the 90 °C curve and the lower time scale belongs to the 25 °C curve. Data represents the mean of n=9. Values with similar letters within the figure are not significantly different (Tukey's HSD, $p > 0.05$).

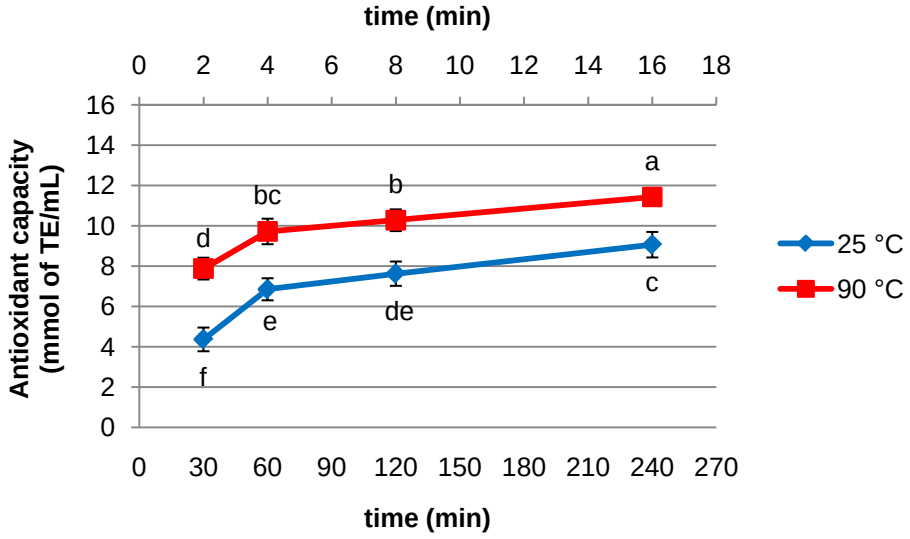


Figure 3-3. Antioxidant capacity (μmol of TE/mL) L) for the extracts. The upper time scale belongs to the 90 °C curve and the lower time scale belongs to the 25 °C curve. Data represents the mean of n=9. Values with similar letters within the figure are not significantly different (Tukey's HSD, $p > 0.05$).

Table 3-2. Linear regression and correlation coefficients between measured parameters for cold and hot water extraction processes.

	Cold Extraction			Hot Extraction		
	m	b	r^2	m	b	r^2
L^* vs color density	-8.77	62.62	-0.99	-9.61	61.48	-0.99
Color density vs anthocyanins content	0.04	-0.04	0.96	0.03	0.34	0.96
Anthocyanins content vs total phenolics	0.18	2.56	0.95	0.17	-3.65	0.95
Total phenolics vs antioxidant capacity	38.85	13.66	0.92	44.96	13.66	0.93

m = equation slope, b = equation intercept.

Table 3-3. Identification of anthcocyanins present in hibiscus using their spectral characteristics with HPLC-DAD and positive ions in LC-MS and MS^2.

		HPLC-DAD Data		LC-MS Data (m/z)	
Peak	Compound[a]	t_R (min)	λ_{max} (nm)	MS (molecule)	MS^2 (aglycone)
6	Dpd-3-sambubioside	24.2	529	597	303
8	Cyd-3-sambubioside	26.2	521	581	287

[a] Abbrevaitions: Dpd, delphinidin; Cyd, cyanidin.

Table 3-4. Identification of polyphenolics present in hibiscus using their spectral characteristics with HPLC-DAD and negative ions in LC-MS and MS^2, and respective standards.

		HPLC-DAD Data		LC-MS Data (m/z)		
					MS^2	
Peak	Compound	t_R (min)	λ_{max} (nm)	MS	base peak	other peaks
1	Gallic acid[a]	13.4	271	169	125 (100)[d]	
2	Protocatechuic acid glucoside[b]	18.2	260	315	153 (100)	
3	3-caffeoylquinic acid	18.7	326	353	191 (100)	179 (58), 173 (7), 135 (14)
4	5-caffeoylquinic acid[a]	23.0	327	353	191 (100)	179 (2), 173 (0.4), 135 (0.70)
5	4-caffeoylquinic acid	23.6	327	353	173 (100)	191 (20), 179 (39), 135 (14)
7	Caffeoylquinic acid isomer[c]	24.3	331	353	191 (100)	173 (3)
12	Quercetin-3-rutinoside[c]	35.7	355	609	301 (100)	

[a] Confirmed with authentic standards. [b] Confirmed with the standard of the acid. [c] Tentatively identified.
[d] Values in parenthesis indicate the intensity of the ion.

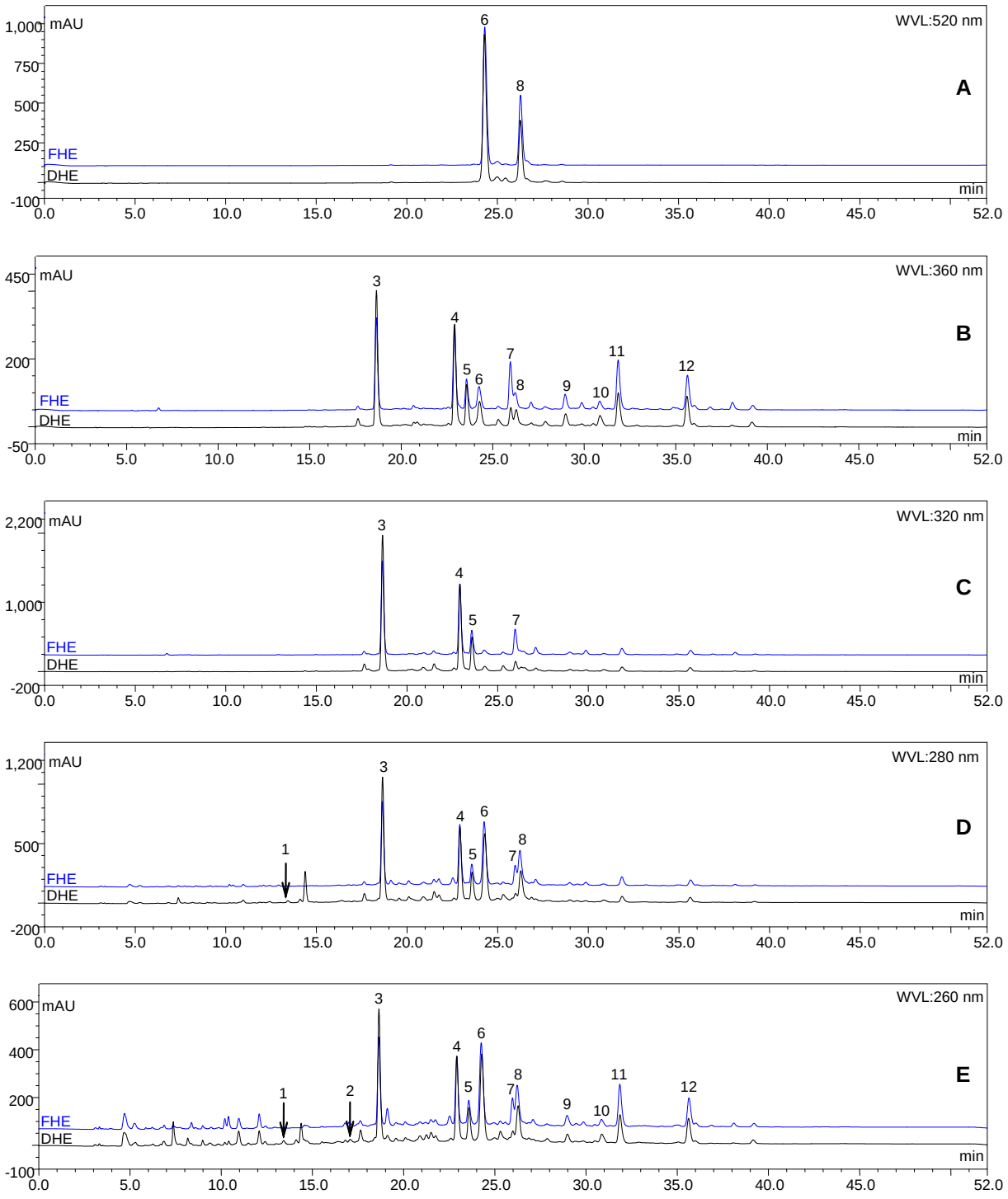


Figure 3-4. HPLC chromatograms of dried hibiscus (DHE) and fresh hibiscus (FHE) hot water extracts: (A) 520 nm, (B) 360 nm, (C) 320 nm, (D) 280 nm, and (E) 260 nm. For peak identification see Tables 3-3 and 3-4.

Table 3-5. Polyphenolics content (mg/L) of hibiscus samples analyzed in this study[d].

Compound (peak)[e]	DCE	DHE	FCE	FHE
Hydroxybenzoic acids				
Gallic acid (1)[f]	0.65[a]	0.58[a]	nd[l]	nd
Protocatechuic acid glucoside (2)[g]	0.06[b]	0.05[b]	0.19[a]	0.13[ab]
Total	0.71	0.63	0.19	0.13
Caffeoylquinic acids[h]				
3-caffeoylquinic acid (3)	67.53[b]	73.01[a]	51.97[c]	49.70[c]
5-caffeoylquinic acid (4)	43.64[b]	46.23[a]	39.05[c]	38.15[c]
4-caffeoylquinic acid 5)	17.52[b]	18.81[a]	14.12[c]	13.51[c]
Caffeoylquinic acid isomer (7)	3.93[b]	4.13[b]	10.86[a]	12.09[a]
Total	132.62	142.18	116.00	99.94
Flavonols[i]				
Unidentified (9)	5.56[b]	5.70[ab]	5.55[b]	5.84[a]
Unidentified (10)	5.52[a]	5.58[a]	5.11[b]	4.86[c]
Unidentified (11)	10.21[b]	9.99[b]	12.29[a]	12.10[a]
Quercetin-3-rutinoside (12)	8.45[a]	9.20[a]	9.38[a]	9.21[a]
Total	29.74	30.47	32.33	32.01
Anthcoyanins				
Delphinidin-3-sambubioside (6)[j]	87.32[c]	100.90[a]	87.76[c]	96.16[b]
Cyanidin-3-sambubioside (8)*k*	41.62[b]	44.88[b]	50.30[a]	50.89[a]
Total	128.94	145.78	138.06	147.05
Total phenolic compounds	292.01	319.06	286.58	279.13

Data represents the mean of n=6. [d] Values with similar letters within rows are not significantly different (Tukey's HSD, $p > 0.05$). e Peak numbers refer to the compounds identified in Tables 3-3 and 3-4. [f,g,h,I,j,k] Quantified with gallic acid, protocatechuic acid, chlorogenic acid, quercetin, delphinidin-3-glucoside, and cyanidin-3-glucoside standards, respectively. [l] Abbreviations: nd, not detected.

CHAPTER 4
AROMA PROFILES OF BEVERAGES OBTAINED FROM FRESH AND DRIED HIBISCUS

Introduction

Hibiscus sabdariffa commonly known as hibiscus or roselle, grows in many tropical and subtropical countries and is one of the highest volume specialty botanical products in international commerce (Plotto 1999). Hibiscus is an annual herbaceous shrub and is a member of the Malvaceae family. The swollen calyces, which are red and cup-like, are the part of the plant of commercial interest (Morton 1987; De Castro and others 2004). Fresh and dried hibiscus calyxes are used to prepare cold and hot beverages. Sweeteners and spices can be added depending on the region where it is consumed.

Extensive work has been done in the area of hibiscus anthocyanins due to their beneficial health effects, high antioxidant properties, and potential source as a food colorant (Tee and others 2002; Tsai and others 2002; Tsai and Huang 2004; Prenesti and others 2007: Sáyago-Ayerdi and others 2007). Studies with human patients have also shown that the regular consumption of hibiscus extract has an antihypertensive effect (Haji Faraji and Haji Tarkhani 1999; Herrera-Arellano and others 2004) and reduces serum cholesterol in men and women (Lin and others 2007).

Hibiscus flavor is a combination of sweet and tart. Few studies have been done related to hibiscus flavor. Gonzalez-Palomares and others (2009) identified 20 volatile compounds in hibiscus extract using SPME and GC-MS, including terpenoids, esters, hydrocarbons, and aldehydes. They also found 14 compounds in reconstituted spray dried extracts from which only 10 were present in the original extract and the other 4 were products of degradation. Thermally generated volatiles from untreated, frozen, hot-

air-dried at 50 °C, and hot-air-dried at 75 °C hibiscus by steam distillation were analyzed by GC and GC-MS (Chen and others 1998). They characterized more than 37 compounds including fatty acid derivatives, sugar derivatives, phenol derivatives, and terpenes.

The objective of this study was to determine the aroma profile differences between four extracts obtained from fresh and dried hibiscus extracted at two different conditions, by GC-MS and GC-olfactometry.

Materials and Methods

Sample Preparation

Fresh and sun dried hibiscus (*Hibiscus sabdariffa* cv. “Criollo”) were obtained from the same harvest (November 2006 – January 2007) from Puebla, Mexico. Hibiscus samples were stored in glass jars, flushed with nitrogen and kept frozen at -20 °C until used. Four different extracts were prepared; fresh (F) and dried (D) hibiscus were mixed with distilled water in a ratio of 1:4 and 1:40 (w/v) respectively and extracted at 22 °C for 240 min (cold extraction (CE)) and 98 °C for 16 min (hot extraction (HE)). Extraction ratios (hibiscus: water) were determined based on moisture content of fresh (90%) and dried (9%) hibiscus (measured at 105°C for 24 h in an oven (Precision Scientific, Winchester, Va., U.S.A.)). Stirring at low speed was applied for cold extraction and no stirring was applied for hot extraction. After extraction, samples were filtered under vacuum using Whatman filter paper #4.

The pH of the samples was measured using a pH meter EA920 (Orion Research; Boston, Mass., U.S.A.) and °Brix was determined with an ABBE Mark II refractometer (Leica Inc.; Buffalo, N.Y., U.S.A.).

Headspace Volatiles Sampling

Headspace volatiles were extracted and concentrated using SPME. Ten milliliters of hibiscus extracts were added to a 22 mL screw cap amber glass vial PTFE/silicone septa containing a small stir bar. Samples were equilibrated for 20 min in a water bath at 40 °C, and hibiscus headspace volatiles were extracted for 30 min using a 1 cm 50/30 mm DVB/Carboxen/PDMS SPME fiber (Supelco, Bellefonte, Pa., U.S.A.). Before each exposure the fiber was cleaned for 5 min in the injection port (200 °C) of the GC-O or GC-MS instruments.

GC-O Analysis

GC-O analysis was carried out using a HP 5890 Series II Plus GC (Palo Alto, Calif., U.S.A) with a sniffing port and a flame ionization detector (FID). Hibiscus volatiles from the SPME fiber were desorbed in the GC injection port. A SPME injector liner (SPME injection sleeve, 0.75 mm i.d., Supleco; Bellefonte, Pa., U.S.A.) was used. The GC column effluent was split between the FID and the olfactometer. The injector temperature was 200 °C, and the detector temperature was 250 °C. Helium was used as the carrier gas at 1.67 mL/min. The oven was programmed from 35 °C (held for 5 min) to 250 °C at 6 °C/min with a final hold of 10 min. Volatiles were separated using a DB-5 (30 m x 0.32 mm. i.d. x 0.5 μm, J&W Scientific; Folsom, Calif., U.S.A.) or a DB-Wax (30 m x 0.32 mm. i.d. x 0.5 μm, Restek; Bellefonte, Pa., U.S.A.) column. Two olfactory assessors were employed. Samples were sniffed two times by each assessor in each column.

Aroma descriptions and approximate times were recorded for every sample. Assessors indicated the intensity of each aroma peak using a linear potentiometer with

a 0-1 V signal. Aromagrams and FID chromatograms were recorded and integrated using Chrom Perfect 4.4.23 software (Justin Innovations, Inc.; Palo Alto, Calif., U.S.A.). A peak was considered aroma active only if at least half the panel found it at the same time with a similar description. Linear retention index values were determined for both columns using a series of alkanes (C5-C25) run under identical conditions.

GC-MS Analysis

Mass Spectrometry (GC-MS) was used to identify the odor-active volatiles detected in the GC-O experiment. GC-MS analysis was conducted using a HP 6890 GC coupled with a MSD 5973 (Agilent Technologies; Palo Alto, Calif., U.S.A.). Hibiscus volatiles from the SPME fiber were desorbed in the GC injection port (splitless mode) at 200 °C. The fiber was removed after 5 min exposure in the injection port. Volatiles were separated using a DB-5 (30 m x 0.32 mm. i.d. x 0.5 μm, J&W Scientific; Folsom, Calif., U.S.A.) or a DB-Wax (30 m x 0.32 mm. i.d. x 0.5 μm, Restek; Bellefonte, Pa., U.S.A.) column. The oven was programmed from 35 °C (held for 5 min) to 250 °C at 6 °C/min with a final hold of 10 min. Helium was used as the carrier gas at 1.67 mL/min. The mass spectrometer was operated in the total ion chromatogram (TIC) at 70 eV. Data were collected from 35 m/z to 400 m/z. All samples were run in duplicate in each column. Chromatograms were recorded and integrated using Enhanced Chemstation (version 01.00) software (Agilent Technologies; Palo Alto, Calif., U.S.A.). Mass spectral matches were made by comparison with NIST 98.1 (NIST; Gaithersburg, Md., U.S.A.) and WILEY 8.1 (Wiley; New York, N.Y., U.S.A.) mass spectral libraries. Only those compounds with spectral fit values equal to or greater than 850 were considered

positive identifications. Linear retention index values were determined for both columns using a series of alkanes (C5-C25) run under identical conditions.

Identification Procedures

Identifications were based on the combined matching of retention indices (LRI values) from DB-5 and DB-Wax columns, matches made from spectra in the NIST and WILEY libraries, aroma descriptors, and linear retention index matches from literature.

Statistical Analysis

Analysis of variance (ANOVA) and mean separation using Tukey's test (α=0.05) were performed to evaluate differences in pH and °Brix between the analyzed samples using SAS 9.0 Statistical software (SAS Institute Inc., Cary, N.C., U.S.A.).

Results and Discussion

Four samples using fresh frozen and sun dried hibiscus were prepared: DHE (dried hibiscus hot water extraction), DCE (dried hibiscus cold water extraction, FHE (fresh hibiscus hot water extraction), and FCE (fresh hibiscus cold water extraction). Extraction conditions and measured pH and °Brix values are presented in Table 4-1. There was not a significant difference (p = 0.0581) in °Brix between the four samples. Samples prepared with dried hibiscus had a significantly (p = 0.0003) lower pH as compared to those prepared with fresh hibiscus, reason unknown.

Hibiscus Volatiles Composition

Hibiscus volatiles were divided into five chemical groups. A total of 14 aldehydes, 10 alcohols, 5 ketones, 2 terpenes, and 1 acid were identified. The relative difference in total volatiles in terms of peak area was normalized to total peak area of DHE (dried hibiscus hot water extraction) which was 590. Results are shown in Figure 4-1. In relation to DHE, total peak area was reduced by 40, 59, and 98% for DCE, FHE, and

FCE samples, respectively. In both fresh and dried hibiscus, hot water extraction gave a higher concentration of volatiles as compared with cold extraction which indicates that temperature facilitates the extraction process but it can also lead to undesirable degradation reactions of hibiscus aromas. In the same way, dried hibiscus extracts had a higher concentration of volatiles as compared to fresh hibiscus. A bigger gradient in moisture content between the dried hibiscus (9% moisture) and the extraction solvent (water (100% moisture)) as compared to the gradient between the fresh hibiscus (90% moisture) and water (100% moisture) may have helped make the extraction process faster, and thus increased the concentration of volatiles extracted from the dried hibiscus. Composition of the four samples was similar but there were major quantitative differences. Aldehydes comprised the largest group of volatiles contributing between 57 and 63% of the total in the hot water extracts and from 37 to 45% in the cold water extracts, followed by alcohols (23 to 24% in hot water extracts and 28 to 36% in the cold water extracts), ketones (7-12%), acids (4-8%), and terpenes (2-3%). In the case of FCE, acids (19%) were higher than ketones. Aldehydes were present in a higher proportion in hot water extracts while alcohols were present in a higher proportion in cold water extracts. This may indicate that extraction temperature could influence the aroma profiles of the obtained extracts by accelerating the degradation or formation of compounds.

GC-MS Identifications

A total of 32 volatiles were identified using GC-MS in hibiscus samples, 15 of which were not identified before in hibiscus. Limonene, linalool, *α*-terpineol, eugenol, and furfural were previously identified in two studies (Gonzalez-Palomares and others 2009; Chen and others 1998) while nine other compounds (hexanal, heptanal, octanal,

nonanal, 2-heptenal (E), 5-methyl-furfural, 1-hexanol, dehydroxylinalool oxide b, acetic acid) and two other compounds (decanal and benzaldehyde) were also found in hibiscus extracts by Chen and others (1998).and Gonzalez-Palomares and others (2009).respectively. Differences in volatile profiles among studies can be attributed to the hibiscus variety used and the extraction methods. The extraction solvent polarity as well as the extraction conditions (time, solute concentration, and temperature) may impact the aroma profile of the final product.

Table 4-2 lists the 32 volatiles detected in this study. To compare the volatiles in the four extracts, peak areas were normalized on the single largest peak found in all samples. This peak was the nonanal peak in the DHE sample. It was assigned a value of 100 and the remaining peaks in all four samples were normalized to it. Twenty eight, 25, 17, and 16 volatiles were found in the DHE, DCE, FHE, and FCE respectively. Thirteen compounds (hexanal, heptanal, limonene, octanal, 6-mehtyl-5-hepten-2-one, nonanal, 1-octen-3-ol, acetic acid, decanal, bornylene, 2-nonenal (E), 1-octanol, and geranylacetone) were present in all four samples and their concentration was lower in the fresh and the cold water extracted samples. Nonanal (100) and decanal (99) were the volatiles present in highest concentration in DHE and were also among the three compounds present in highest concentration for the other three samples. Nonanal was 36 and decanal was 39 for DCE, 43 and 75 for FHE and 3 and 2 for FCE. Nonanal and decanal are aldehydes that may form as a product of lipid oxidation.

Dehydroxylinalool oxide a and b were only present in extracts from dried hibiscus. Since there are similar amounts of both compounds in the extracts obtained with cold and hot water, these could be degradation products of linalool formed during

the drying process. Furfural and 5-methyl furfural were also only detected in the extracts from dried hibiscus. These compounds are sugar degradation products and may also have developed during hibiscus drying.

On the other hand, linalool and 1-hexanol-2-ethyl were only detected in the extracts from fresh hibiscus. Their absence in the dried samples may be attributed to the fact that these compounds may have degraded during drying and led to the formation of other compounds.

GC-O Aroma Profiles

A total of 22 aroma compounds were found in hibiscus extracts and are listed in Table 4-3. Peak heights were normalized to the most intense peak in all four samples. This peak was 6-methyl-5-hepten-2-one in the DHE sample. It was assigned a value of 100 and the remaining peaks in all four samples were normalized to it. Seventeen, 16, 13, and 10 aroma active compounds were detected for DHE, DCE, FHE, and FCE samples respectively. Seven compounds were detected in all four samples and were confirmed with GC-MS (hexanal, 3-octanone, octanal, 6-methyl-5-hepten-2-one, nonanal, 2,4-nonadienal (E,E), and geranylacetone).

The most intense odorants were 6-mehtyl-5-hepten-2-one and nonanal in all four extracts followed by geranylacetone, eugenol, and 2-Nonenal (E) in the DHE sample, geranylacetone, 2-Nonenal (E), and an unidentified compound for the DCE sample, and linalool, geranylacetone, and octanal for the fresh hibiscus extracts,

The compound 6-methyl-5-hepten-2-one was present in all four samples and had the highest intensity in all of them. This compound was described to have a mushroom, dirt, green aroma and has previously been reported in tomatoes (Buttery and others 1987) and Rooibos tea (Kawakami and others 2003). Nonanal was the second highest

in aroma intensity in all four samples with a descriptor of fruity, green. Geranylacetone was present in all samples, was among the five highest intensity compounds, and was described as fruit-like, apple sauce smell. Geranylacetone has been found previously in Merlot and Cabernet wines (Gurbuz and others 2006) and is one of the major components of Rooibos tea.

In the fresh hibiscus extracts, the compounds linalool (floral, woody, citrus) and octanal (lemon, citrus) were among the highest intensity aroma compounds. As mentioned before, linalool was not detected in dried hibiscus extracts while octanal is present in dried hibiscus samples and is the sixth highest intensity peak in both cold and hot water extracts. Linalool is a compound associated with floral notes and has previously been reported to be present in jasmine green tea (Ito and others 2002) and citrus blossom (Jabalpurwala and others 2009) among others. Octanal has been described to have a fruity, citrus aroma in lychee (Mahattanatawee and others 2007).

The compound 2-Nonenal (E) (cucumber, green, floral) was present in dried hibiscus samples as one of the highest intensity peaks. Eugenol (sweet spices) was only detected in the DHE sample while an unidentified (anise) compound was present in the DCE sample with a high intensity.

The five highest intensity peaks for all four samples were: 2 ketones, 2 aldehydes and 1 alcohol. The compounds 6-mehtyl-5-hepten-2-one, geranylacetone, and 2-Nonenal (E) which are important aroma impact compounds present in hibiscus extracts were identified for the first time in hibiscus.

Conclusion

The four hibiscus extracts studied had a similar chemical composition of aroma compounds with hot extracted hibiscus samples having a slightly higher aldehyde

concentration and cold extracted samples a slightly higher alcohol concentration. Total peaks concentration was the highest for the dried hibiscus hot water extract, and decreased in both cold water extracts and fresh hibiscus extracts. There were some differences in aroma peak intensities in the four hibiscus samples with the dried hibiscus hot water extraction having the highest intensity. In general, hibiscus aroma is a combination of earthy, green, floral, and fruity notes but the final flavor profile is affected by the preservation and extraction process.

Table 4-1. Extraction conditions and measured pH and °Brix values for hibiscus samples included in this study.

Sample	Extraction temperature (°C)	Extraction time (min)	Hibiscus: water ratio	pH	°Brix
DHE	98	16	1:40 w/v	2.48 ± 0.01[b]	1.25 ± 0.07[a]
DCE	22	240	1:40 w/v	2.49 ± 0.00[b]	1.25 ± 0.07[a]
FHE	98	16	1:4 w/v	2.55 ± 0.01[a]	1.10 ± 0.00[a]
FCE	22	240	1:4 w/v	2.57 ± 0.01[a]	1.10 ± 0.00[a]

DHE = dried hibiscus hot water extraction. DCE = dried hibiscus cold water extraction. FHE = fresh hibiscus hot water extraction. FCE = fresh hibiscus cold water extraction.

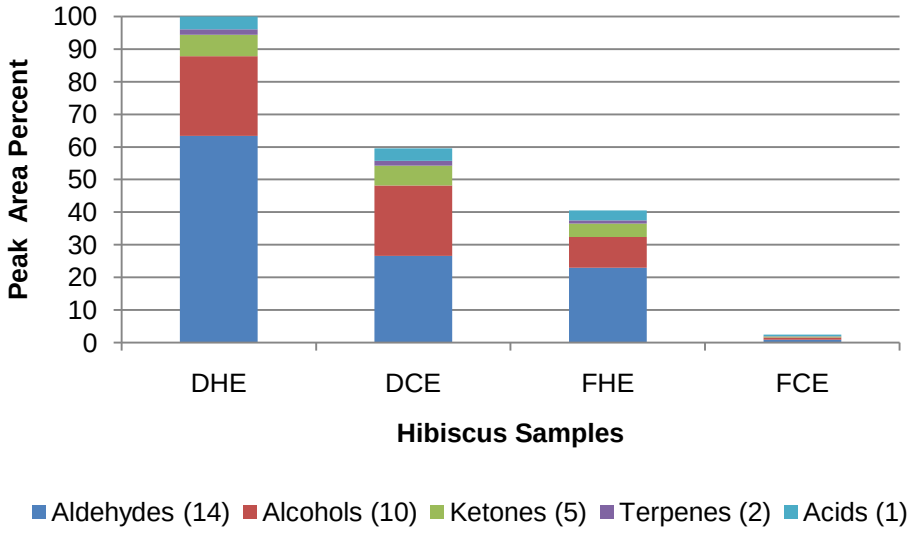


Figure 4-1. Chemical composition of hibiscus headspace volatiles. Total number of compounds for each class is put in parentheses. All four samples were normalized to the total peak area of DHE (dried hibiscus hot extraction). DCE = dried hibiscus cold extraction, FHE = fresh hibiscus hot extraction, FCE = fresh hibiscus cold extraction.

Table 4-2. MS identification of hibiscus volatiles. Peak areas were normalized (100) to the largest peak in all four samples.

#	Name	CAS #	LRI		Normalized peak area (%)			
			CW	DB5	DHE	DCE	FHE	FCE
1	Hexanal[a]	66-25-1	1100	793	55.42	25.93	3.77	0.33
2	Heptanal[a]	111-71-7	1195	903	4.99	0.29	0.21	0.09
3	Limonene[a,b,c]	138-86-3		1026	0.22	0.07	0.18	0.15
4	Dehydroxylinalool oxide a	13679-86-2	1210	993	37.86	35.04	-	-
5	Dehydroxylinalool oxide b[a]	13679-86-2	1246	1007	26.35	21.26	-	-
6	3-Octanone	106-68-3	1264		2.90	5.60	-	-
7	Octanal[a]	124-13-0	1299	1002	36.27	14.00	12.60	0.31
8	2,2,6-Trimethylcyclohexanone	2408-37-9	1329	1031	7.14	4.54	-	-
9	2-Heptenal, (E)[a]	18829-55-5	1342	958	4.73	-	-	-
10	6-methyl-5-Hepten-2-one	110-93-0	1355	989	21.70	16.73	19.33	1.09
11	1-Hexanol[a]	111-27-3	1373		-	7.66	4.80	0.48
12	Nonanal[a]	124-19-6	1405	1100	100.00	35.63	42.97	2.56
13	Octenal	2548-87-0	1448		5.81	1.05	-	-
14	1-Octen-3-ol	3391-86-4	1468	983	46.08	39.75	7.40	0.34
15	Acetic acid[a]	64-19-7	1485		23.21	22.40	17.94	2.85
16	Furfural[a,b]	98-01-1	1496	832	24.33	19.16	-	-
17	1-Hexanol-2-ethyl	104-76-7	1508	1030	-	-	6.66	1.89
18	Decanal[b]	112-31-2	1513	1204	98.71	39.45	74.76	1.96
19	Bornylene	464-17-5	1541	1227	9.47	8.63	5.24	0.34
20	Benzaldehyde[b,c]	100-52-7		961	0.07	-	-	-
21	2-Nonenal (E)	18829-56-6	1555	1159	11.53	3.67	1.35	0.13
22	Linalool[a,b]	78-70-6	1568	1098	-	-	23.13	1.04
23	1-Octanol	111-87-5	1577	1071	17.93	9.94	10.64	0.42
24	5-Methyl furfural[a]	620-02-2	1608		4.14	3.85	-	-
25	2-Nonanone[c]	821-55-6		1089	0.13	0.13	-	-
26	1-Nonanol	143-08-8	1678	1172	15.78	13.78	-	-
27	α-terpineol[a,b]	98-55-5	1725		-	-	2.63	-
28	(E,E)-2,4-Nonadienal	5910-87-2	1728	1213	13.69	8.87	-	-
29	2-Undecenal	53448-07-0	1772	1362	8.58	-	-	-
30	(E,E)-2,4-Decadienal	25152-84-5	1836	1315	5.67	4.87	-	-
31	Geranylacetone	3796-70-1	1876	1440	7.36	9.03	5.47	0.72
32	Eugenol[a,b,c]	97-53-0		1356	0.07	-	-	-
	Total normalized peak area				590.1	351.3	239.1	14.7

DHE = dried hibiscus hot water extraction. DCE = dried hibiscus cold water extraction. FHE = fresh hibiscus hot water extraction. FCE = fresh hibiscus cold water extraction. [a] Compounds previously reported in *H. sabdariffa* by Chen and others (1998). [b] Compounds previously reported in *H. sabdariffa* by Gonzalez-Palomares and others (2009). [c] LRI values for this compounds were calculated using peak areas from DB-5 column.

Table 4-3. Hibiscus aroma active compounds. Peak heights were normalized (100) to the most intense peak in all four samples.

#	Name	LRI CW	LRI DB5	Aroma descriptor	Normalized peak height (%) DHE	DCE	FHE	FCE
1	Unknown	1054		sweet, fruity	-	-	33.33	32.17
2	Hexanal[a]	1102		green, grass, nutty	47.50	43.33	30.00	16.67
3	Unknown	1200		sweet, fruity	44.17	41.67	-	-
4	3-Octanone[a]	1270		butter, cookie, baked	33.33	30.00	26.67	16.67
5	Octanal[a]	1304	1003	lemon, citrus	58.33	57.50	50.00	33.33
6	6-methyl-5-Hepten-2-one[a]	1349	980	mushroom, dirt, green	100.00	83.33	91.67	58.33
7	Nonanal[a]	1410	1103	fruity, green	95.83	82.50	83.33	54.17
8	Octenal[a]	1452		rancid nuts	25.00	16.67	-	-
9	1-Octen-3-ol[a]	1477	975	mushroom, dirt, metallic	56.67	50.00	-	-
10	Furfural[a]	1497		sweet, baked bread	52.92	-	-	-
11	Decanal[a]	1520		sweet, nutty	33.33	25.00	-	-
12	2-Nonenal (E)[a]	1562	1154	cucumber, green, floral	61.67	66.67	45.00	-
13	Linalool[a]	1570		floral, woody, citrus	-	-	58.33	52.50
14	1-Octanol[a]	1579		fresh leather, chemical	20.83	16.67	-	-
15	1-Nonanol[a]	1674		chemical, painty	25.00	-	-	-
16	2,4-Nonadienal, (E,E)[a]	1735	1215	rancid nuts, citrus, green	49.17	44.17	25.00	20.00
17	Unknown	1754	1244	anise	-	63.33	41.67	33.33
18	2-Undecenal[a]	1780		green, grass	35.83	31.67	-	-
19	Unknown	1850		rancid nuts	-	33.33	26.67	-
20	Geranylacetone[a]	1870	1430	fruit-like, apple sauce	66.67	75.00	58.33	45.83
21	Unknown	1940		sweet spices, floral	-	-	41.67	-
22	Eugenol[a]	2100	1350	sweet spices	66.67	-	-	-
	Total intensity				872.9	760.8	611.7	363.0

DHE = dried hibiscus hot water extraction. DCE = dried hibiscus cold water extraction. FHE = fresh hibiscus hot water extraction. FCE = fresh hibiscus cold water extraction.
[a] Compounds confirmed with GC-MS.

CHAPTER 5
PROCESSING HIBSCUS BEVERAGE USING DENSE PHASE CARBON DIOXIDE: MICROBIAL AND PHYTOHCEMICAL STABILITY

Introduction

Juices and beverages are traditionally preserved by thermal methods which are effective in reducing microbial loads but can also lead to organoleptic and nutritional changes. Nonthermal processes are an alternative that may help preserve the color, flavor, and nutrients of food, and thus address consumers' demands for high quality, fresh-like products with extended shelf life.

Dense phase carbon dioxide (DPCD) is a continuous nonthermal processing system for liquid foods that uses pressure (≤90 MPa) in combination with carbon dioxide (CO_2) to inactivate microorganisms. In a continuous flow DPCD system, several variables are controlled during processing: pressure, temperature, residence time, and %CO_2 in the liquid food. The amount of CO_2 used should assure a complete saturation of the liquid but since its solubility at processing conditions is not known this can lead to the use of excess CO_2 elevating production costs. Previous studies with muscadine grape juice showed that DPCD was more effective than pasteurization in retaining anthocyanins and other phenolic compounds during processing and storage (Del Pozo-Insfran and others 2006a; 2006b). Furthermore, DPCD was effective in extending the shelf life of coconut water (Damar and others 2009) and red grapefruit juice (Ferrentino and others 2009) for up to 9 and 6 weeks of refrigerated storage, respectively.

Hibiscus sabdariffa, a member of the Malvaceae family, is an annual shrub widely grown in tropical and subtropical regions including Africa, South East Asia and some countries of America. The calyces contain anthocyanins and other phenolics and are of commercial interest. They are used either fresh or dehydrated to prepare hot and

cold beverages which are commonly mixed with a sweetener, and are characterized by an intense red color, acidic flavor, and a sensation of freshness. Recently there has been increasing interest in hibiscus anthocyanins due to their beneficial health effects and high antioxidant properties which have been extensively evaluated (Tee and others 2002; Tsai and others 2002; Tsai and Huang 2004; Prenesti and others 2007: Sáyago-Ayerdi and others 2007) and as a potential source of natural food colorant.

The objectives of this study were (1) to determine the solubility of CO_2 in a hibiscus beverage, (2) to optimize DPCD processing parameters (pressure and residence time) based on microbial reduction, and (3) to monitor during 14 weeks of refrigerated storage the microbial, physicochemical, and phytochemcial changes of DPCD processed hibiscus beverage compared to thermally treated and control (untreated) beverages.

Materials and Methods

Chemicals and Standards

Commercial standards of gallic acid, chlorogenic acid, and quercetin were purchased from Sigma-Aldrich (St. Lous, Mo., U.S.A.). Caffeic acid was purchased from ACROS Organics (Geel, Belgium). Delphinidin-3-glucoside and cyanidin-3-glucoside were purchased from Polyphenols Laboratories AS (Sandnes, Norway). AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), fluorescein (free acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2carboxilic acid) and Folin-Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Lous, Mo., U.S.A.).

Beverage Preparation

Dried *Hibiscus sabdariffa* (cv. "Criollo") (9% moisture content) obtained from Puebla, Mexico was mixed with water (1:40 w/v) using a 200 L stainless steel mixing

tank Model UAMS (Cherry-Burrell, Iowa, U.S.A.) and maintained at 25°C for 1 h. Mixing was applied intermittently by alternating intervals of 10 min mixing and 10 min rest. The extract was then filtered using four layers of cheesecloth. A beverage was prepared by adding sucrose to a concentration of 100 g sucrose/L of extract and then was placed in 3 gallon sealable buckets. For the DPCD process optimization, the beverage was incubated at 25 °C for 4 days to obtain a high initial microbial load. The spoiled beverage was placed in the refrigerator at 4 °C for 24 h before processing with DPCD. For the solubility and storage experiments, the beverage was prepared as mentioned above, without incubation, one day before processing and refrigerated.

Solubility Experiment

CO_2 solubility in the hibiscus beverage was measured between 6.9 and 31.0 MPa at 40 °C using an apparatus designed and built at the University of Florida, Food Science and Human Nutrition department (Gainesville, Fla., U.S.A.) as previously described by Ferrentino and others (2009). In this batch system, a known volume of sample was saturated by bubbling CO_2 at the desired experimental conditions and then dissolved CO_2 was measured at atmospheric pressure. Solubility of CO_2 in water at the same experimental conditions was also measured for comparison. Experiments were done in duplicate and results were expressed as g of CO_2/100 g of liquid sample.

Dense Phase CO_2 Equipment

The DPCD equipment located at the University of Florida (Gainesville, Fla., U.S.A) was constructed by APV (Chicago, Ill., U.S.A.) for Praxair (Chicago, Ill., U.S.A.). A schematic diagram of the equipment is presented in Figure 2-11. In this continuous flow equipment, CO_2 and the hibiscus beverage were pumped through the system and mixed before entering the high-pressure pump (intensifier pump). Processing pressure

levels were controlled by this pump while the desired temperature was maintained in the holding coil (79.2 m, 0.635 cm i.d.). Turbulent flow and mixing were reached at the entrance of the coil by passing the mixture through a static mixer and a small diameter tube (length of about 180 cm). Residence time was adjusted by setting the flow rate of the mixture. An expansion valve was used at the end of the process to release the CO_2 from the mixture and the beverage was collected into 1 L sterile bottles as previously described by Damar and others (2009).

DPCD Process Optimization

Optimal processing parameters to achieve a microbial log reduction of 5 were determined by using response surface methodology. A central composite design (Table 5-1) consisting of 11 experiments with 4 factorial points, 4 star points, and 3 central points in which the independent variables were pressure (P) (13.8-34.5 MPa) and residence time (RT) (5-8 min), and the dependent variables were yeasts and molds (Y&M) and aerobic plate counts (APC) was used. With this response surface design we were able to reduce the volume of beverage required and were able to prepare it in one batch. The total volume needed for the 11 experiments was ~ 160L. Processing parameters were selected based on previous research results and equipment specifications.

Hibiscus beverage with an initial microbial load of 3.0×10^7 CFU/mL for Y&M and 4.9×10^3 CFU/mlL for APC was processed at the different experimental conditions at a constant temperature (40 °C) and constant CO_2 level (8%) which was selected based on the minimum flow that could be handled by the CO_2 pump. Microbial counts from each experimental condition were made in duplicate by serially diluting ($1x10^{-1}$ to $1x10^{-5}$) 1 mL of beverage in 9 mL sterile Butterfield's phosphate buffer (Weber Scientific,

Hamilton, N.J., U.S.A.). Microbial counts were determined by plating 1 mL of each dilution in duplicate for yeasts and molds and aerobic count plates (3M Petrifilm Microbiology, St. Paul, Minn., U.S.A.) and enumerating after 48 h at 35 °C and 5 d at 24 °C respectively according to the manufacturer's guidelines.

Thermal Processing Conditions

For thermal processing, hibiscus beverage was pumped by a peristaltic pump (Cole Parmer, Chicago Ill., U.S.A.) through two stainless steel tube sections (3.2 m, 0.457 cm i.d. ea.) placed inside a temperature controlled water bath (Precision Scientific, Chicago Ill., U.S.A.). In the first section, the beverage was heated to 75 °C (temperature was measured using a thermocouple) and then entered the second section where it was held at 75 °C for 15 s. The beverage was then passed through a cooling stainless steel tube (5.2 m, 0.457 cm i.d.) in a water/ice bath and chilled to ~15 °C before it was collected into 1 L sterile glass jars. Platinum-cured silicone tubing (0.635 cm i.d.; Nalgene, Rochester, N.Y., U.S.A.) was used to connect the pump to the stainless steel heating, holding, and cooling sections. A schematic diagram of the setup used for the hibiscus beverage thermal processing is presented in Figure 5-2.

Storage Experiment

Fresh prepared hibiscus beverage was divided into three parts. One part was kept as control and did not receive any treatment; the second part was processed using DPCD at 34.5 MPa, 8% CO_2, 6.5 min, and 40 °C while the third part was thermally treated at 75 °C for 15 s. Each processing condition was repeated in triplicate. DPCD processing parameters were determined based on the solubility and optimization studies described above. Both the control and treated samples were stored in 1 L glass jars. Microbial, physicochemical and phytochemical analyses were performed at weeks

0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 of refrigerated storage at 4 °C. To quantify individual anthocyanins and other phenolic compounds present in hibiscus, HPLC analysis was done at weeks 0, 2, 5, 8, and 14 of storage. The methods used in all the analyses are described as follows.

Microbial Analysis

Total aerobic plate count (APC) and yeasts and molds (Y&M) were measured as described in the optimization section.

pH, °Brix, and Titratable Acidity

pH and °Brix were measured using a pH meter EA920 (Orion Research; Boston, Mass., U.S.A.) and a ABBE Mark II refractometer (Leica Inc.; Buffalo, N.Y., U.S.A.). A Brinkmann Instrument (Brinkmann Instruments Co., Westbury, N.Y., U.S.A.) consisting of a Metrohm 655 Disomat, Metrohm 614 Impulsomat, and Metrohm 632 pH meter was used to measure titratable acidity (TA). Samples of 10 mL were used and TA was determined by titration with 0.1 N NaOH until pH 8.1 and expressed as % malic acid (g/100 mL).

Color Density and Hue Tint

Color density and hue tint were determined by measuring the absorbance (A) at 420, 520, and 700 nm of 200 μL samples using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale Calif., U.S.A.) and calculated as:

$$\text{Color density} = [(A_{420\ nm} - A_{700\ nm}) + (A_{520\ nm} - A_{700\ nm})]$$

$$\text{Hue tint} = (A_{420\ nm} - A_{700\ nm})/(A_{520\ nm} - A_{700\ nm})$$

as described by Giusti and Wrolstad (2005).

Anthcyanin Content, Total Phenolics and Antioxidant capacity

Anthocyanin content was determined by pH differential method ($A_{510\ nm}$ and $A_{700\ nm}$ at pH 1.0 and 4.5, dilution factor (DF) of 4) and expressed in mg/L of delphinidin-3-glucoside (MW = 465.2, ε = 23700) (Giusti and Wrolstad 2005). Total phenolics were measured using the Folin-Ciocalteu assay ($A_{765\ nm}$, DF of 4) and quantified as gallic acid equivalents (mg/L) (Waterhouse 2005). Absorbance measurements for anthocyanin content and total phenolics were made using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale Calif., U.S.A.).

Antioxidant capacity was evaluated using the oxygen radical absorbance capacity (ORAC) assay and results were expressed as Trolox equivalents (TE) per milliliter (μmol of TE/mL) as described by Huang and others (2002) using a SpectraMax Gemini XPS microplate sprectrofluorometer (Molecular Devices, Sunnyvale, Ca., U.S.A.). Data was acquired and analyzed using SoftMax Pro 5.2 software (Molecular Devices, Sunnyvale, Calif., U.S.A.).

HPLC Quantification of Polyphenolics

Polyphenolics were identified by comparison of UV/vis (190-660 nm) spectral interpretation, retention time, and comparison to standards. Anthocyanins and polyphenolics were quantified using a Dionex HPLC system equipped with an autosampler/injector and diode array (PDA 100) detector (Dionex, Sunnyvale, Calif., U.S.A.). Compounds were separated on a 250 x 4.6 mm Dionex C18 5 μm 120A column (Dionex, Sunnyvale, Calif., U.S.A.). Mobile phases consisted of water (phase A) and 60% methanol in water (phase B), both adjusted to pH 2.4 with *o*-phosphoric acid. A gradient solvent program ran phase B from 0% to 60% in 20 min; 60% to 100% in 20

min; 100% for 7 min; 100% to 0% in 3 min and final conditions were held for 2 min. The flow rate was 0.8 mL/min, and detection was done at 260, 280, 320, 360 and 520 nm.

Statistical Analysis

Analysis of variance (ANOVA) and mean separation using Tukey's test (α=0.05) were performed in the solubility study to evaluate the effect of pressure on CO_2 solubility. Response surface methodology was used in the DPCD optimization study to determine optimal processing conditions. Repeated measures ANOVA and mean separation using Tukey's test (α=0.05) was performed to evaluate the effect of treatment (fresh (CONTROL), thermal (HTST), and DPCD processed) and storage time (0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 weeks) on the dependent variables measured. All statistical analyses were conducted using SAS statistical software (SAS Institute Inc., Cary, N.C., U.S.A.).

Results and Discussion

Solubility Measurements

CO_2 solubility in a hibiscus beverage and water was measured between 6.9 and 31.0 MPa at 40 °C. Pressure had a significant effect on solubility of CO_2 in both the hibiscus beverage (4.16 to 5.06 g CO_2/100 mL from 6.9 to 31.0 MPa) and water (4.50 to 6.32 g CO_2/100 mL from 6.9 to 31.0 MPa). After 17.2 MPa, CO_2 solubility remained almost constant in both the hibiscus beverage and water (Figure 5-1). CO_2 solubility in water was significantly higher than the hibiscus beverages at all pressures tested but 6.9 MPa. The presence of solutes such as sugars and acids in the hibiscus beverage lowered the amount of CO_2 that could be dissolved. Previous studies have shown that solubility of CO_2 in fruit juices is lower than that of pure water because of the presence of solutes. CO_2 solubility in orange and apple juice measured at 15.9 MPa was around

5% (Calix and others 2008) while that of grapefruit juice at 31.0 MPa was 4.97% (Ferrentino and others 2009). These values are comparable to those obtained for hibiscus beverage (5.06%).

Based on the CO_2 solubility results, a first attempt to use 6% CO_2 for the DPCD experiments was tried. This concentration of CO_2 (1% higher) would assure a complete saturation of CO_2 in the beverage. This decision was made to account for the fact that the solubility test was performed using a batch system with long contact time between the CO_2 and the beverage whereas the DPCD processing equipment is a continuous system in which lower contact times are used. After processing the hibiscus beverage using 6% CO_2 and acquiring data, the DPCD system showed that there were fluctuations in the CO_2 flow during the process because the CO_2 pump was not designed to handle such a low flow. Our second attempt was to find the minimum flow that would assure a constant reading throughout the process. After several attempts, it was found that the CO_2 pump could maintain a steady flow of 8% CO_2 and we used this value for our DPCD processing experiments.

Microbial Inactivation Study

Initial microbial loads in the beverage obtained after incubation for 4 days were 3 x 10^7 CFU/mL for Y&M and 4.9 x 10^3 CFU/mL for APC. The APC population reached is not very high which can be a result of microflora competition in which the low pH (2.43) of the beverage and high sugar concentration (9.7 °Brix) favored the growth of Y&M. The response surface experimental design and achieved log reductions for each of the treatments tested is presented in Table 5-1. A minimum of 5 log reduction for Y&M and 0.85 log reduction for APC was achieved for all DPCD treatments.

Several mechanisms have been proposed for DPCD inactivation of microorganisms (Damar and Balaban 2006). One of the main factors that lead to microbial inactivation is the pH lowering effect when CO_2 is dissolved. Since the hibiscus beverage has an initial low pH, this reduces the lowering pH effect of CO_2 and this may be the reason for low bacteria inactivation in the hibiscus beverage. Another mechanism for microbial inactivation is the effect caused in the microorganisms' cells during the decompression process. This can be the mechanisms by which Y&M were inactivated.

Two quadratic equations, (1) and (2), were obtained from the central composite design solution to describe Y&M ($r^2 = 0.81$) and APC ($r^2 = 0.55$) log reduction (LR) as a function of pressure (P) and residence time (RT). Both quadratic models were not statistically significant and were not suitable to predict the inactivation of microorganisms present in the hibiscus beverage within pressures and residence times ranges studied.

$$\text{Log reduction (Y\&M)} = 3.8745 + 0.0155 \cdot P + 0.2200 \cdot t + 0.0007 \cdot P^2 - 0.0037 \cdot P \cdot t + 0.0042 \cdot t^2 \quad (1)$$

$$\text{Log reduction (APC)} = -0.6804 + 0.0187 \cdot P + 0.4493 \cdot t - 0.0001 \cdot P^2 - 0.0019 \cdot P \cdot t - 0.0311 \cdot t^2 \quad (2)$$

As can be seen from Table 5-1, treatment 8 (24.1 MPa, 8 min) showed the highest LR for Y&M followed by treatments 10 (34.5 MPa, 6.5 min) and 11 (34.5 MPa, 8 min). On the other hand, treatment 5 (24.1 MPa, 6.5 min) had the highest log reduction for APC and treatment 10 was among the second highest APC log reduction treatments while treatment 11 was among the lowest APC log reduction treatments. Based on these results, our approach was to select treatment 10 for further DPCD processing experiments. This treatment conditions consists of the upper level pressure within our experimental range studied (34.5 MPa) which will assure a complete solubility of CO_2

during processing and the middle level residence time of 6.5 min which is more feasible for industrial applications than longer times.

Microbial Stability during Storage

Microbial stability of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD), and thermally treated (HTST) hibiscus beverages during storage is presented in Figures 5-2 and 5-3. Aerobic plate counts in all three beverages (Figure 5-2) remained constant between 2 and 3 logs during the 14 weeks of storage. The HTST beverage showed slightly lower counts when compared to the other two beverages. Neither the DPCD nor the HTST treatments reduced the initial bacteria population possibly because it was difficult to observe microbial reductions when starting with a low population. In the case of yeast and molds (Figure 5-3), the DPCD and HTST treatments reduced the initial population by around 3 logs and both beverages where very stable since both treatments were effective in inactivating the initial Y&M population and there was no growth during storage. For the CONTROL, a maximum of 5 logs at week 6 was reached and declined afterwards possibly associated with the death stage of the microorganisms. The sensory characteristics of the CONTROL beverage indicated that fermentation was taking place. Overall the DPCD and HTST beverages were microbiologically stable during the 14 weeks of storage favored by the beverage low pH and storage temperature (4 °C).

Physicochemical Stability during Storage

Physicochemical changes in the studied hibiscus beverages during storage are shown in Table 5-2. There were no significant differences between treatments (CONTROL, DPCD, HTST) over time for pH and °Brix. There was a significant effect of treatment over time for all other parameters measured. Titratable acidity in the DPCD

treated beverage was significantly higher when compared with the CONTROL and HTST beverages which can be due to the presence of residual CO_2 remaining in solution in the beverage after depressurization. A similar behavior was observed by Calix and others (2008) in orange and apple juices.

Color density significantly decreased over time for all three treatments (CONTROL, DPCD, and HTST). This indicates that there is a decline in the absorbance at 520 nm which can be associated with degradation of anthocyanins. At time 14 weeks of storage, the HTST beverage showed a significantly lower value of color density as compared to the CONTROL and DCPD beverages. Moreover, the hue tint values (Figure 5-4) significantly increased for all three treatments during storage which also indicates some loss of red color in the samples.

Phytochemical Stability during Storage

Phytochemical changes during storage for the three hibiscus beverages studied are presented in Tables 5-2 and Figure 5-5. Several polyphenolic changes during storage were measured using authentic standards and their concentration was expressed in mg/L of beverage (Table 5-3). This included gallic and caffeic acid, caffeoylquinic acids which were quantified using a chlorogenic acid standard and were identified based on their characteristic absorption spectrum at λ_{max} 320 nm, delphinidin-3-sambubioside and cyanidin-3-sambubioside that are the main anthocyanins present in hibiscus extracts, and flavonols which were quantified using quercetin and identified by their characteristic absorption spectrum at λ_{max} 360 nm.

There was a significant effect of treatment (CONTROL, DCPD, HTST) in anthocyanins content, total phenolics, antioxidant capacity, gallic acid, caffeic acid, and

flavonols content. Anthocaynins content (Figure 5-6) significantly decreased during storage for all three treatments. A loss of 11, 9, and 14% in anthocyanins was observed for CONTROL, DPCD, and HTST beverages respectively. At time 14 weeks, the concentration of anthocyanins in all three treatments was significantly different, with the CONTROL having the highest and HTST beverage the lowest concentration. There were no major changes in total phenolics and antioxidant capacity during storage for all three treatments. There were some slight differences between storage times possibly related to the breakdown and formation of polyphenolic compounds. A previous study with muscadine grape juice (Del Pozo and others 2006a) showed that losses in anthocyanins during processing and storage were around 78% for a pasteurized juice and only 35% for a DPCD processed juice. A similar behavior in total phenolic and antioxidant capacity was also found. The greater losses and differences between treatments in the grape juice as compared to the hibiscus beverage can be attributed to a higher initial concentration of polyphenolics and higher pH of the grape juice.

As shown in Table 5-3, the concentration of gallic acid increased with increasing storage time for the DPCD beverages and to a greater extent for the CONTROL. Similarly, the presence of caffeic acid in the CONTROL and DPCD beverages at time 14 weeks was detected and can be a breakdown product of the caffeoylquinic acids present in the beverage. Both phenomena could be related to polyphenolic compounds breaking down due to microbial activity. There were no major changes in the caffeoylquinic acids and flavonols content during storage, although at time 14 weeks there was a significantly lower concentration of both polyphenolics in the CONTROL beverage as compared to the other two treatments. There was a significant but small

decrease in the concentration of delphinidin-3-sambubioside and cyanidin-3-sambubioside for all the three beverages during storage. Overall there were no big phytochemical losses during storage for any of the three treatments. This can be attributed to the low pH of the beverage, the low storage temperature and the presence of sucrose in the beverage. A previous study (Tsai and others 2004) has shown that sucrose solutions favored the stability of hibiscus anthocyanins by decreasing the availability of water that is needed for the anthocyanins degradation process.

Conclusions

CO_2 solubility in a hibiscus beverage and optimal processing conditions to inactivate microorganisms (Y&M and APC) were determined. DPCD was found to be a viable technology for extending the hibiscus beverage shelf life since it showed to be microbiologically stable during the 14 weeks of refrigerated storage. Quality attributes such as pH and °Brix were not affected by DPCD whereas TA increased. A loss of only 9% anthocyanins during storage was observed for the DPCD processed hibiscus beverage which was lower when compared to a heat pasteurization process and no major changes in total phenolics content and antioxidant capacity occurred during storage

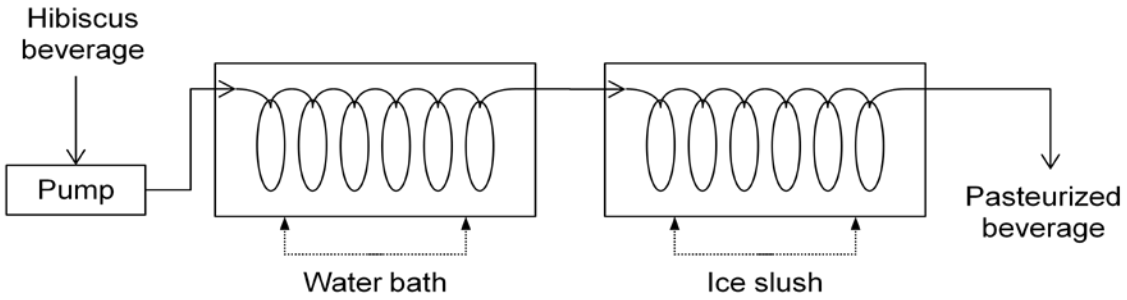


Figure 5-1. Schematic diagram of the setup used for the hibiscus beverage thermal treatment (75 °C for 15 s).

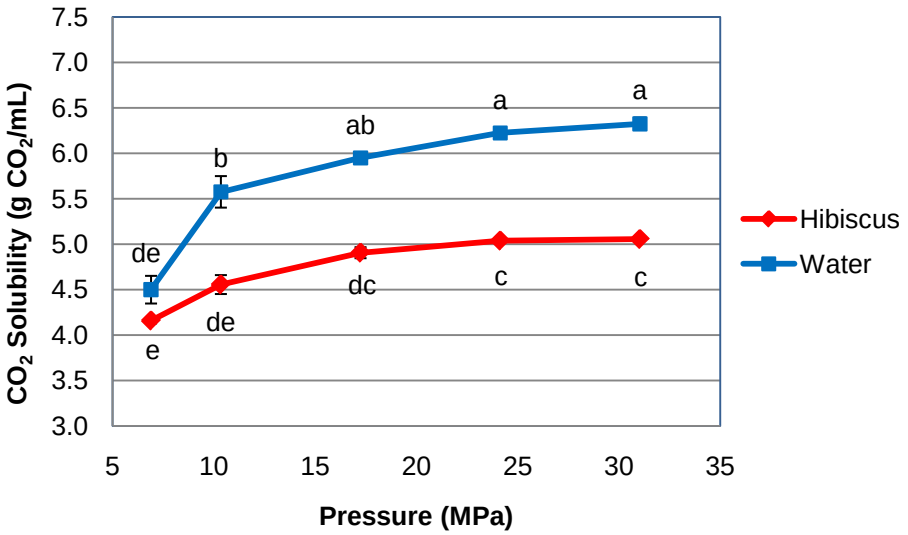


Figure 5-2. CO_2 solubility in water and a hibiscus beverage as a function of pressure measured at 40 °C. Data represents the mean of n=3. Values with similar letters within the figure are not significantly different (Tukey's HSD, $p > 0.05$).

Table 5-1. Response surface design used to test the effect of pressure and residence time on microbial reduction (log_{10}) at 40 °C and 8% CO_2.

Run	Pressure (Mpa)	Residence time (min)	Beverage flow rate (g/min)	CO_2 flow rate (g/min)	LR* (APC)	LR (Y&M)
1	13.8	5.0	500.0	40.0	$0.93^{abc} \pm 0.03$	$5.20^{e} \pm 0.08$
2	13.8	6.5	384.6	30.8	$0.99^{abc} \pm 0.03$	$5.63^{d} \pm 0.06$
3	13.8	8.0	312.5	25.0	$0.92^{bc} \pm 0.03$	$5.67^{d} \pm 0.04$
4	24.1	5.0	500.0	40.0	$0.88^{c} \pm 0.05$	$5.30^{e} \pm 0.05$
5	24.1	6.5	384.6	30.8	$1.05^{a} \pm 0.07$	$5.56^{d} \pm 0.08$
6	24.1	6.5	384.6	30.8	$0.99^{abc} \pm 0.09$	$5.62^{d} \pm 0.09$
7	24.1	6.5	384.6	30.8	$1.04^{ab} \pm 0.06$	$5.65^{d} \pm 0.06$
8	24.1	8.0	312.5	25.0	$1.02^{ab} \pm 0.04$	$6.26^{a} \pm 0.11$
9	34.5	5.0	500.0	40.0	$1.04^{ab} \pm 0.06$	$5.85^{c} \pm 0.06$
10	34.5	6.5	384.6	30.8	$1.03^{ab} \pm 0.02$	$6.07^{b} \pm 0.01$
11	34.5	8.0	312.5	25.0	$0.91^{bc} \pm 0.08$	$6.09^{b} \pm 0.06$

Y&M = yeasts and molds, APC = aerobic plate count. * LR = log_{10} reduction.

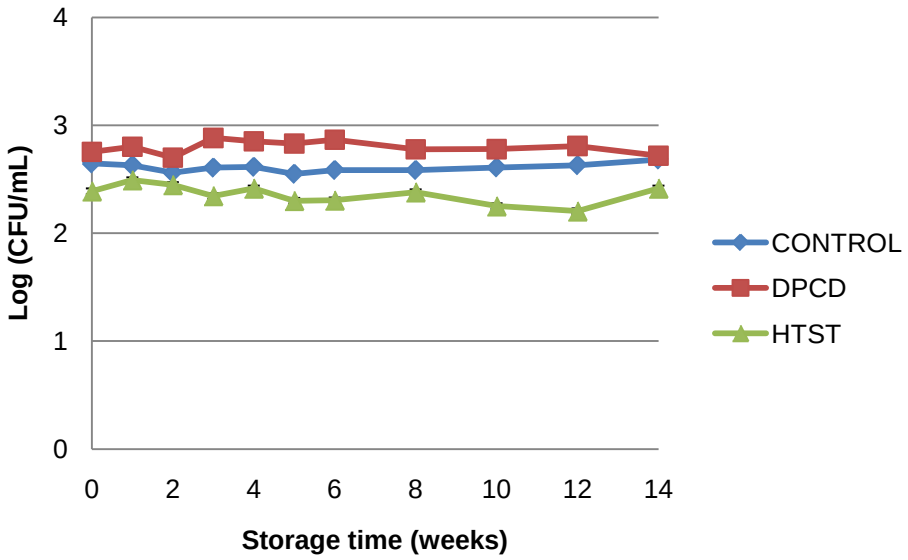


Figure 5-3. Aerobic plate counts of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

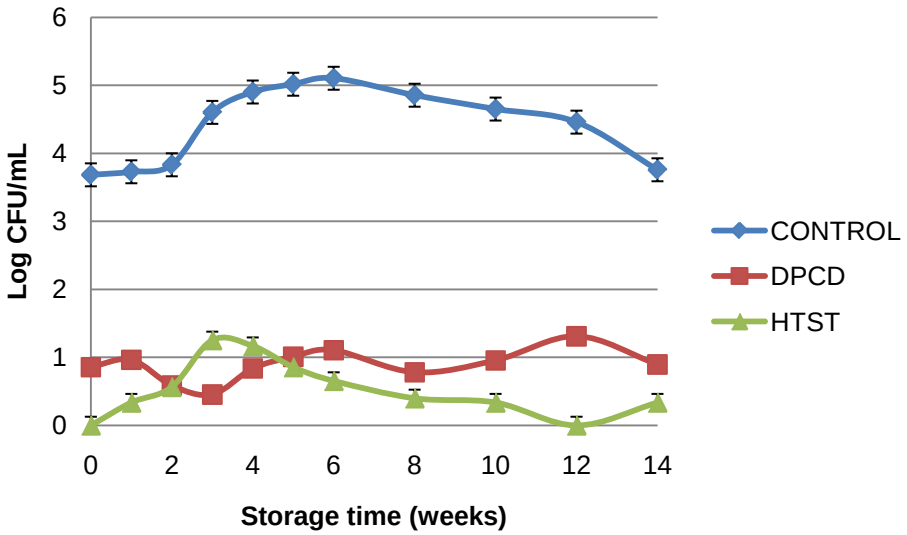


Figure 5-4. Yeast/mold counts of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

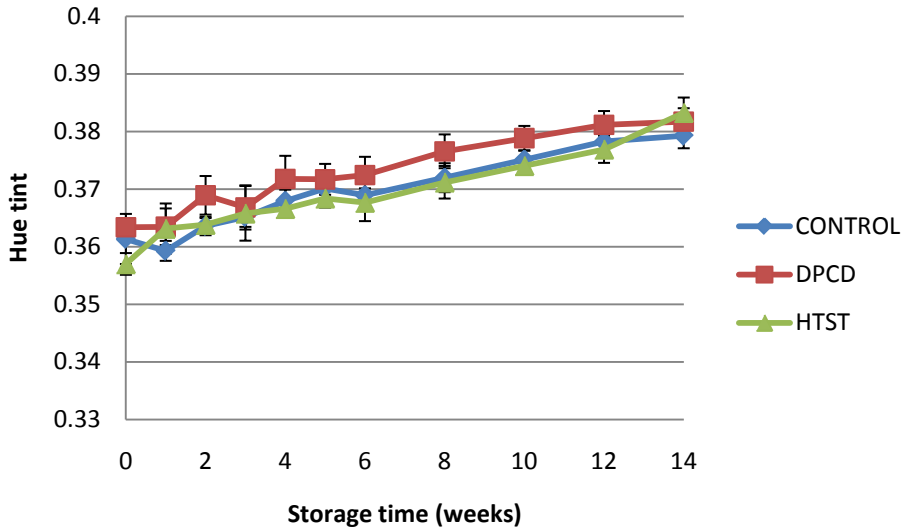


Figure 5-5. Hue tint values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

Table 5-2. Physicochemical and phytochemical changes of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD), and thermally treated (HTST) hibiscus beverages during refrigerated storage at 4 °C.

	Storage time										
	week 0	week 1	week 2	week 3	week 4	week 5	week 6	week 8	week 10	week 12	week 14
pH											
CONTROL	2.43^{b*}	2.45^{a}	2.47^{a}	2.45^{b}	2.45^{c}	2.47^{b}	2.47^{b}	2.47^{a}	2.48^{a}	2.48^{b}	2.48^{a}
DPCD	2.45^{a}	2.44^{b}	2.46^{b}	2.46^{a}	2.47^{a}	2.48^{a}	2.48^{a}	2.48^{a}	2.48^{a}	2.49^{a}	2.49^{a}
HTST	2.45^{a}	2.45^{a}	2.45^{b}	2.45^{b}	2.47^{b}	2.48^{a}	2.48^{a}	2.48^{a}	2.48^{a}	2.49^{a}	2.49^{a}
°Brix											
CONTROL	9.70^{ab}	9.70^{a}	9.70^{b}	9.87^{b}	9.70^{b}	9.67^{a}	9.90^{a}	10.20^{a}	10.07^{a}	9.93^{a}	9.70^{b}
DPCD	9.77^{a}	9.70^{a}	9.90^{a}	9.93^{a}	9.87^{a}	9.70^{a}	9.63^{b}	10.10^{b}	9.83^{b}	9.57^{b}	9.77^{ab}
HTST	9.63^{b}	9.67^{a}	9.90^{a}	9.73^{c}	9.90^{a}	9.60^{b}	9.97^{a}	10.00^{c}	9.82^{b}	9.63^{b}	9.90^{a}
Titratable acidity (g of malic acid/100 mL)											
CONTROL	0.37^{b}	0.37^{b}	0.38^{b}	0.38^{b}	0.37^{b}	0.38^{b}	0.38^{b}	0.38^{b}	0.37^{ab}	0.38^{b}	0.38^{b}
DPCD	0.40^{a}	0.41^{a}	0.40^{a}	0.41^{a}	0.41^{a}	0.40^{a}	0.41^{a}	0.41^{a}	0.40^{a}	0.41^{a}	0.40^{a}
HTST	0.37^{b}	0.36^{b}	0.38^{b}	0.37^{b}	0.38^{b}	0.38^{b}	0.38^{b}	0.38^{b}	0.38^{b}	0.37^{b}	0.38^{b}
Color density											
CONTROL	1.78^{ab}	1.78^{b}	1.75^{ab}	1.74^{a}	1.71^{b}	1.72^{a}	1.73^{a}	1.74^{a}	1.70^{a}	1.66^{a}	1.67^{a}
DPCD	1.80^{a}	1.80^{a}	1.76^{a}	1.75^{a}	1.76^{a}	1.73^{a}	1.74^{a}	1.74^{a}	1.69^{a}	1.64^{a}	1.66^{a}
HTST	1.76^{b}	1.79^{ab}	1.73^{b}	1.75^{a}	1.72^{ab}	1.69^{a}	1.72^{a}	1.68^{b}	1.63^{b}	1.58^{b}	1.58^{b}
Total phenolics (mg/L)											
CONTROL	263.86^{a}	259.01^{a}	247.97^{b}	248.38^{a}	251.13^{a}	246.43^{ab}	249.13^{a}	250.54^{a}	251.99^{a}	253.44^{a}	264.05^{a}
DPCD	259.62^{ab}	256.86^{a}	252.26^{a}	253.22^{a}	248.52^{a}	245.03^{b}	253.91^{a}	252.32^{ab}	252.25^{a}	252.17^{a}	255.65^{b}
HTST	254.73^{b}	259.39^{a}	245.04^{b}	251.09^{a}	241.64^{b}	248.36^{a}	241.61^{b}	248.36^{b}	247.15^{b}	245.94^{b}	250.06^{c}
Antioxidant capacity (μmol of TE/mL)											
CONTROL	5.93^{a}	6.11^{b}	6.62^{a}	5.77^{a}	6.04^{a}	5.96^{a}	6.64^{a}	6.53^{a}	6.27^{a}	6.01^{a}	5.85^{b}
DPCD	5.66^{a}	6.10^{b}	5.43^{b}	6.19^{a}	5.90^{a}	6.08^{a}	6.31^{a}	5.59^{b}	5.76^{a}	5.92^{a}	6.80^{a}
HTST	6.38^{a}	7.00^{a}	5.84^{b}	6.34^{a}	6.42^{a}	6.53^{a}	6.11^{a}	5.27^{b}	5.81^{a}	6.35^{a}	6.92^{a}

Data represents the mean of n=9. * Values with similar letters within columns are not significantly different (Tukey's HSD, $p > 0.05$).

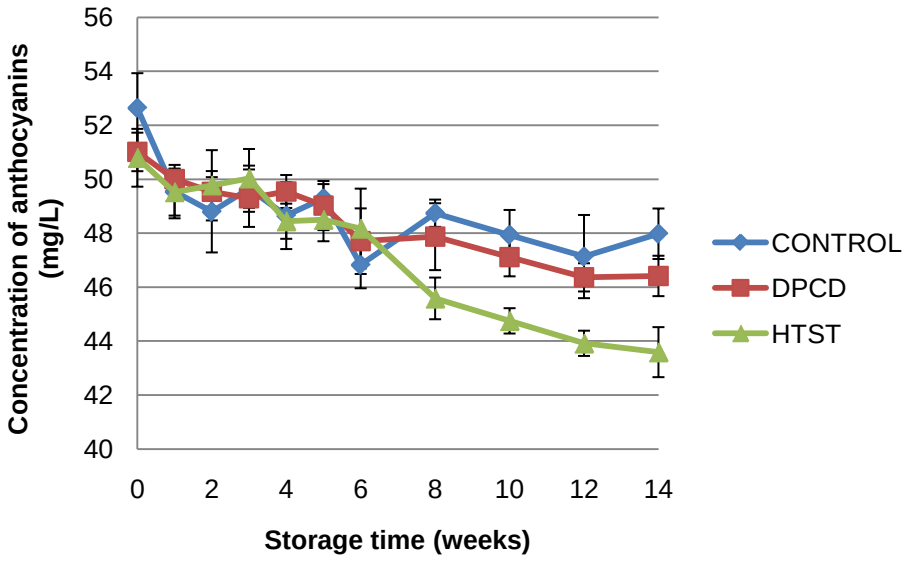


Figure 5-6. Concentration of anthocyanins of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

Table 5-3. Polyphenolics content (mg/L) of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD), and thermally treated (HTST) hibiscus beverages during refrigerated storage at 4 °C.

	Storage time				
	week 0	week 2	week 5	week 8	week 14
Galllic acid[d]					
CONTROL	0.79[a*]	2.53[a]	3.25[a]	3.89[a]	4.03[a]
DPCD	0.50[b]	0.77[b]	1.03[b]	1.27[b]	1.57[b]
HTST	0.57[b]	0.68[b]	0.63[c]	0.71[c]	0.71[c]
Caffeoylquinic acids[e]					
CONTROL	93.59[a]	98.13[a]	94.05[a]	94.11[a]	89.88[b]
DPCD	91.90[a]	96.78[a]	95.74[a]	95.28[a]	94.50[a]
HTST	92.51[a]	98.36[a]	93.31[a]	95.10[a]	94.73[a]
Caffeic acid[f]					
CONTROL	nd[j]	nd	nd	nd	5.73[a]
DPCD	nd	nd	nd	nd	4.26[b]
HTST	nd	nd	nd	nd	nd
Delphinidin-3-sambubioside[g]					
CONTROL	65.26[a]	64.64[a]	61.96[ab]	62.14[a]	58.07[ab]
DPCD	63.12[b]	61.75[b]	62.53[a]	61.15[ab]	59.23[a]
HTST	64.79[ab]	62.20[ab]	59.12[b]	58.72[b]	56.78[b]
Cyanidin-3-sambubioside[h]					
CONTROL	29.98[a]	29.60[a]	28.23[a]	28.10[a]	26.61[a]
DPCD	28.78[a]	28.46[a]	28.49[a]	27.95[a]	27.43[a]
HTST	29.99[a]	29.03[a]	27.76[a]	27.74[a]	27.40[a]
Flavonols[i]					
CONTROL	23.05[b]	23.55[a]	22.44[b]	22.79[b]	22.65[b]
DPCD	25.22[a]	24.96[a]	25.02[a]	25.18[a]	24.23[ab]
HTST	25.08[a]	25.15[a]	24.64[a]	25.45[a]	24.78[a]

Data represents the mean of n=6. * Values with similar letters within columns of each polyphneolic category are not significantly different (Tukey's HSD, $p > 0.05$). [d,e,f,g,h,i] Quantified with gallic acid, chlorogenic acid, delphinidin-3-glucoside, cyanidin-3-glucoside, and quercetin standards respectively. [j] Abbreviations: nd, not detected.

CHAPTER 6
PROCESSING HIBISCUS BEVERAGE USING DENSE PHASE CARBON DIOXIDE: SENSORY ATRIBUTES AND AROMA COMPOUNDS STABILITY

Introduction

Hibiscus sabdariffa (family Malvaceae) is a short-day annual shrub that grows in many tropical and subtropical countries and is known by different synonyms and vernacular names such as “roselle” in the U.S. and England, “l’oiselle” in France, “jamaica” or “flor de jamaica” in Mexico and Spain, “karkade” in Sudan and Arabia, “sorrel” in the Caribbean and “byssap” in Senegal (Morton 1987; Stephens 2003).

Traditionally fresh hibiscus calyces are harvested by hand and are either frozen or dried, in the sun or artificially, for preservation. They are typically sold into the herbal tea and beverage industry or in local and regional markets where they are used in the preparation of beverages, and color and flavor extracts (Plotto 1990). Studies with human patients have shown that the regular consumption of hibiscus extract has an antihypertensive effect (Haji Faraji and Haji Tarkhani 1999; Herrera-Arellano and others 2004) and reduces serum cholesterol in men and women (Lin and others 2007).

The preparation of a hibiscus beverage includes an extraction step followed by a pasteurization method. Although thermal preservation of foods is effective in reducing microbial loads it can also lead to organoleptic and nutritional changes. Nonthermal processes are an alternative which may help preserve the color, flavor, and nutrients of food. Dense phase carbon dioxide (DPCD) is a cold pasteurization method that uses pressures below 90 MPa in combination with carbon dioxide (CO_2) to inactivate microorganisms. This non-thermal technology is mainly used in liquid foods and since the food is not exposed to the adverse effect of heat, its fresh-like physical, nutritional, and sensory qualities are maintained.

Previous studies have shown that DPCD processed beverages keep their fresh-like characteristics after processing and storage. Likeability of DPCD-treated coconut water was similar to untreated samples while heat treated samples were less appealing (Damar and others 2009). Similarly, no differences in sensory attributes (color, flavor, aroma, and overall likeability) were observed between unprocessed and DPCD muscadine grape juices but there were differences when compared to a heat-pasteurized juice (Del-Pozo-Insfran and others 2006a).

The objectives of this study were (1) to determine the effect of DPCD processing on the sensory attributes and aroma compounds of hibiscus beverage when compared to a thermally treated and a control (untreated), and (2) to monitor the changes in these attributes during refrigerated storage.

Materials and Methods

Beverage Preparation

Dried *Hibiscus sabdariffa* (cv. “Criollo”) (moisture content of 9%) obtained from Puebla, Mexico was mixed with water (1:40 w/v) using a 200 L stainless steel mixing tank Model UAMS (Cherry-Burrell, Iowa, U.S.A.) and maintained at 25°C for 1 h. Mixing was applied intermittently by alternating intervals of 10 min mixing and 10 min rest. The extract was then filtered using four layers of cheesecloth. A beverage was prepared by adding sucrose to a concentration of 100 g sucrose/L of extract and then was placed in 3 gallon sealable buckets and refrigerated before processing.

Processing and Storage Conditions

Fresh prepared hibiscus beverage was divided into three parts. One part was kept as CONTROL and didn’t receive any treatment; the second part was processed using DPCD at 34.5 MPa, 8% CO_2, 6.5 min, and 40 °C while the third part was pasteurized at

75 °C for 15 s (HTST). The DPCD processing conditions were confirmed to achieve >5 log reduction of yeasts/molds according to previous experiments. Both the control and treated samples were stored in 1 L glass jars. Physicochemical, sensory and aroma compound analysis were done at weeks 0 and 5 of refrigerated storage at 4 °C .Color analysis was performed at weeks 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 of storage.

Dense Phase CO_2 Equipment

The DPCD equipment located at the University of Florida (Gainesville, Fla., U.S.A.) was constructed by APV (Chicago, Ill., U.S.A.) for Praxair (Chicago, Ill., U.S.A.). It is a continuous flow equipment in which CO_2 and the hibiscus beverage were pumped through the system and mixed before entering a high-pressure pump. Processing pressure was controlled by this pump while the desired temperature was maintained in the holding coil (79.2 m, 0.635 cm i.d.). Turbulent flow and mixing were reached at the entrance of the coil by passing the mixture through a static mixer and a small diameter tube (length of about 180 cm). Residence time was adjusted by setting the flow rate of the mixture. An expansion valve was used at the end of the process to release the CO_2 from the mixture and the beverage was collected into 1 L sterile bottles as previously described by Damar and others (2009).

Thermal Processing Conditions

For thermal processing, the hibiscus beverage was pumped by a peristaltic pump (Cole Parmer, Chicago Ill., U.S.A.) through two stainless steel tube sections (3.2 m, 0.457 cm i.d. ea.) placed inside a temperature controlled water bath (Precision Scientific, Chicago Ill., U.S.A.). In the first section the beverage was heated to 75 °C (temperature was measured using a thermocouple) and then entered the second section where it was held at 75 °C for 15 s. The beverage was then passed through a

cooling stainless steel tube (5.2 m, 0.457 cm i.d.) in a water/ice bath and chilled to ~15 °C before it was collected into 1 L sterile glass jars. Platinum-cured silicone tubing (0.635 cm i.d.; Nalgene, Rochester, N.Y., U.S.A.) was used to connect the pump to the stainless steel heating, holding, and cooling sections. A schematic diagram of the setup used for the hibiscus beverage pasteurization is presented in Figure 5-2.

Physicochemical Analysis

pH and °Brix were measured using a pH meter EA920 (Orion Research; Boston, Mass., U.S.A.) and a ABBE Mark II refractometer (Leica Inc.; Buffalo, N.Y., U.S.A.). A Brinkmann Instrument (Brinkmann Instruments Co., Westbury, N.Y., U.S.A.) consisting of a Metrohm 655 Disomat, Metrohm 614 Impulsomat, and Metrohm 632 pH meter was used to measure titratable acidity (TA Samples of 10 mL were used and TA was determined by titration with 0.1 N NaOH until pH 8.1 and expressed as % malic acid (g/100 mL).

Sensory Evaluation

Flavor and overall likeability of fresh and processed hibiscus beverages were compared using a difference from control test at weeks 0 and 5 of storage. A randomized complete block design was used, and differences from control values were recorded on a line scale with anchors at 0 and 10 that represented “no difference” to “extremely different” in flavor. Panelists compared the flavor of the reference (fresh/unprocessed beverage (CONTROL)) with that of a hidden reference (fresh beverage (CONTROL)), the thermally (HTST), and the DPCD processed beverages. A 9-point hedonic scale was also conducted in order to compare the overall likeability of fresh (hidden reference) and processed hibiscus beverages. For the taste panel at week 5, the reference was fresh hibiscus beverage that was kept frozen at -20 °C.

Before sensory analysis all beverages (fresh, thermally, and DPCD processed) were degassed in order to have equal carbonation levels by placing them in 2 L sterile glass bottles on a stir plate with continuous stirring and vacuum (15” Hg) was pulled for 20 min using a Gast vacuum pump (Model DOA-P104-AA; Beonton Harbor, Mich., U.S.A.). All samples were chilled and kept in ice at a temperature of ~4°C before serving. They were then served on a tray in numbered plastic cups containing ~30 mL of sample. A cup of deionized water and non salted crackers were also provided to the panelists to cleanse their palate between evaluations. Both sensory tests were performed at the University of Florida taste panel facility using 75 untrained panelists in each test.

Headspace Volatiles Sampling

Headspace volatiles were extracted and concentrated using Solid Phase Micro Extraction (SPME) technique. Ten milliliters of hibiscus beverage were added to a 22 mL screw cap amber glass vial PTFE/silicone septa containing a small stir bar. Samples were equilibrated for 20 min in a water bath at 40 °C and afterwards hibiscus headspace volatiles were extracted for 30 min using a 1 cm 50/30 mm DVB/Carboxen/PDMS SPME fiber (Supelco, Bellefonte, Pa., U.S.A.). Before each exposure the fiber was cleaned for 5 min at 200 °C in the GC-MS injection port.

GC-MS Analysis

GC-MS analysis was conducted using a HP 6890 GC coupled with a MSD 5973 (Agilent Technologies; Palo Alto, Calif., U.S.A.). Hibiscus volatiles from the SPME fiber were desorbed into the GC injection port (splitless mode) at 200 °C. A SPME injector liner (SPME injection sleeve, 0.75 mm i.d., Supleco; Bellefonte, Pa., U.S.A.) was used.

The fiber was removed after 5 min exposure in the injection port. Volatiles were separated on both a DB-5 (30 m x 0.32 mm. i.d. x 0.5 μm, J&W Scientific; Folsom, Calif., U.S.A.) and a DB-Wax (30 m x 0.32 mm. i.d. x 0.5 μm, Restek; Bellefonte, Pa., U.S.A.) column. The oven was programmed from 35 °C (held for 5 min) to 250 °C at 6 °C/min with a final hold of 10 min. Helium was used as the carrier gas at 1.67 mL/min. The mass spectrometer was operated in the total ion chromatogram (TIC) at 70 eV. Data were collected from 35 m/z to 400 m/z. All samples were run in duplicate in each column. Chromatograms were recorded and integrated using Enhanced Chemstation (version 01.00) software (Agilent Technologies; Palo Alto, Calif., U.S.A.). Mass spectral matches were made by comparison with NIST 98.1 (NIST; Gaithersburg, Md., U.S.A.) and WILEY 8.1 (Wiley; New York, N.Y., U.S.A.) mass spectral libraries. Only those compounds with spectral fit values equal to or greater than 850 were considered positive identifications. Linear retention index values were determined for both columns using a series of alkanes (C5-C25) run under identical conditions.

Identification Procedures

Identifications were based on the combined matching of retention indices (LRI values) from DB-5 and DB-Wax columns, matches made from spectra in the NIST and WILEY libraries and linear retention index matches from literature.

Color Analysis

Color was measured using a ColorQuest XE colorimeter (HunterLab, Reston, Va., U.S.A.). Samples of 40 mL where placed in a 20 mm cell and L^*, a^*, and b^* parameters were recorded in total transmittance mode, illuminant D65, 10° observer angle. Chroma $(a^{*2} + b^{*2})^{1/2}$ and hue angle (arctan b^*/a^*) were calculated from the

measured a^* and b^* values. Chroma provides a measure of color intensity, while hue angle (0° = red-purple, 90° = yellow, 180° = bluish-green, 270° = blue) indicates the sample color itself (McGuire 1992).

Color difference (ΔE) values were also calculated using the following formula:

$$\Delta E = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2} \quad (1)$$

Where L_0, a_0, and b_0 are the reference values at storage time 0 week for each of the treatments (CONTROL, DPCD, and HTST) and L^*, a^*, and b^* are the values at time t = 1,2,3,… weeks of storage.

Statistical Analysis

Sensory data was recorded and analyzed using Compusense five (Compusense, Guelph, Ontario, Canada). Analysis of variance (ANOVA) and mean comparisons using t-test and Tukey's test were conducted at the 5% significance level. Repeated measures ANOVA and mean separation using Tukey's test (α=0.05) was performed to evaluate the effect of treatment (fresh (CONTROL), thermal (HTST), and DPCD processed) and storage time.(0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 weeks) on color parameters using SAS statistical software (SAS Institute Inc., Cary, N.C., U.S.A.).

Results and Discussion

Physicochemical Analysis

The measured pH, °Brix, and TA for the CONTROL, DPCD, and HTST beverages at weeks 0 and 5 of refrigerated storage are presented in Table 6-1. It is important to notice that the CONTROL at week 5 was kept frozen in order to be used as reference in the second taste panel. No significant differences in pH were observed between the DPCD and HTST beverages at both week 0 and 5. Significant differences

in TA were found between DPCD and the CONTROL, and HTST beverages at both storage times. A higher TA in the DPCD beverage can be a result of residual CO_2 in the beverage. For the same reason, a significantly higher °Brix value was found in the DPCD beverage when compared with HTST. This higher value can also be a result of residual carbonic acid. An increase in TA of DPCD treated coconut water (Damar and others 2009) and orange juice (Kincal and others 2006) was also observed in previous DPCD studies.

Sensory Evaluation

Two taste panels were conducted during storage. In the first test (week 0), 55% of panelists were females, 91% of males and 83% of females were in the 18-30 age range, while for the second panel (week 5), 48% of panelist were females, 74% of males and 94% of females were in the 18-30 age range.

For the taste panel at week 0, there were no significant differences between the CONTROL (hidden reference) and HTST beverages. However, significant differences were detected by panelists between the CONTROL and DPCD beverages. The ranking for overall likeability for the three tested beverages were not significantly different (Table 6-2) which indicates that regardless of treatment panelists preference remained the same. For the taste panel at week 5 there were not significant differences between the CONTROL (hidden reference) and HTST beverages. However, the DPCD was rated as significantly different from the other two by panelists. Similarly, the ranking for overall likeability showed no significant differences between the hidden reference and the HTST beverage but the DPCD beverage was ranked significantly lower than the other two beverages as shown in Table 6-2. Previous studies on muscadine grape juice and coconut water showed greater differences in flavor and overall likeability between the

DPCD and thermally treated samples. They also found that the DPCD sample was very similar to the hidden reference (Damar and others 2009; Del Pozo and others 2006a). This may indicate that thermal processing affected more the organoleptic characteristics of the grape juice and coconut water than in the hibiscus beverage.

In both taste panels, the DPCD beverage was different from the hidden reference and at week 5, it was also significantly different from the HTST beverage. However, despite these differences panelist ranking for overall likeability showed no significant differences in the taste panel at week 0 and overall likeability values where even higher in the taste panel at week 5. DPCD differences from the other two beverages can be attributed to two possible factors. First, even when the DPCD beverage was degasified before sensory analysis there could still be residual CO_2 remaining which would result in a carbonated beverage mouth feel which may have also affected the acidity/sweetness balance causing the panelists to perceive the beverage as less sweet. This can be confirmed by a higher TA in the DPCD beverage as described previously. Second, flavor compounds in the beverage will have dissolved in the residual CO_2 and lost during degasification.

Aroma Compounds

Stability of aroma compounds were monitored during weeks 0 and 5 of storage. The chemical composition of hibiscus beverages headspace volatiles are presented in Figure 6-1. Total peak areas for all analyzed beverages were normalized to the total peak area of CW0 (CONTROL week 0). Alcohols and aldehydes constituted the major fractions of hibiscus beverages aroma volatiles. A total of 4 aldehydes, 6 alcohols, 2 ketones, and 1 acid were considered for analysis. A slight decrease (21%) in alcohols and ketones was observed in the DPCD beverage after processing (week 0) while there

was considerable loss (88%) in all volatiles in the thermally treated (HTST) beverage which may indicate that there was decomposition of compounds due to heating and possible formation of other compounds that were not studied in this experiment or that volatiles were lost by evaporation during pasteurization. At week 5 of storage, there was a considerable loss of all volatiles in the DPCD beverage (70%), a slight decrease in alcohols and ketones (17%) for the CONTROL, and no changes in the volatiles for the HTST beverage. The loss of DPCD volatiles at week 5 can be a result of decomposition or formation of compounds or possibly they could be lost during degasification as discussed previously. A previous study with melon juice also showed that DPCD retained more volatile compounds as compared to the pasteurized juice (Chen and others 2009).

The volatile compounds used in this analysis were identified by GC-MS and are presented in Table 6-3. To compare the volatiles in the hibiscus beverages at weeks 0 and 5 of storage, peak areas were normalized (100) to the largest peak (1-Octen-3-ol) in the CONTROL (C) week 0 sample. A total of 13 compounds were considered for analysis, 6 of which (dehydroxylinalool oxide b, octanal, 1-hexanol, acetic acid, furfural, and decanal) have been previously identified in hibiscus extracts (Gonzalez-Palomares and others 2009; Chen and others 1998). The compounds present in highest concentration in all six samples were 1-octen-3-ol, decanal, octanal, 1-hexanol, and nonanal. A mushroom-like, fruity, citrus, and fruity aroma were associated with 1-octen-3-ol, octanal, and nonanal respectively in lychee (Mahattanatawee and others 2007), decanal was described as sweet, waxy, orange in merlot and cabernet wines (Gurbuz

and others 2006) while 1-hexanol was related to green, sweet notes in both lychee and red wines.

Color Analysis

Color stability was assessed over 14 weeks of refrigerated storage. Treatment (CONTROL, DPCD, HTST) had a significant effect ($p < 0.0001$) over storage time for all the color parameters measured and calculated (L^*, a^*, b^*, and hue angle, chroma, and ΔE). There were slight changes for the L^* values in all three treatments over storage but the trend was almost constant (Figure 6-2). At time 14 weeks of storage, the DPCD beverage had a significantly lower L^* value as compared with HTST and CONTROL. As can be seen in Figure 6-3, the a^* values decreased slightly over time for all the treatments and after 14 weeks of storage there were no significant differences between the DPCD and HTST beverages. The parameter that showed the most change was b^* (Figure 6-4). In the three treatments, there was a slight but significant decrease of the b^* values with time. There were significant differences between treatments at time 14 with the CONTROL having the highest and the HTST beverage the lowest b^* value. Hue angle slightly decreased over storage time for the CONTROL, DPCD, and HTST beverages (Figure 6-5). These changes indicate that the beverages will follow a color degradation pattern from a bright red color to a red-purple color. At time 14 weeks, there were no significant differences in hue angle between the CONTROL and DPCD beverages, the HTST sample was significantly lower. In the same way, chroma decreased (the beverages became less intense in color) for all three samples (Figure 6-6) and at time 14 weeks they were significantly different with the CONTROL having the highest and HTST the lowest value. A decrease in hue angle and chroma during

storage (25 °C) was previously reported in a radish anthocyanin extract (Giusti and Wrolstad 1996). The calculated ΔE value showed a significant increase over storage time. Since the b^* value was the parameter that contributed the most to this difference in color, the HTST beverage showed a significantly higher value after 14 weeks of storage followed by the DPCD and CONTROL beverages as sown in Figure 6-7. Changes in color during storage can be attributed to the degradation of anthocyanins which are the pigments responsible for the red color in the hibiscus beverages with the HTST beverage showing a higher change in color when compared to the DPCD beverage.

Conclusions

Changes in hibiscus aroma volatiles during storage did not affect panelists overall likeability of the product. DPCD was found to be a viable technology for processing hibiscus beverages since it maintained its characteristic red color over 14 weeks of storage and retained more aroma volatiles than the heat pasteurized beverage. Possible losses of aroma volatiles during the degasification process can be prevented by recovering them and adding them back to the beverage. Further studies are needed to better understand the chemistry of hibiscus aroma compounds and to reduce their loss during storage.

Table 6-1. Measured pH, °Brix, and titratable acidity (TA) (g of malic acid/100 mL of beverage) at weeks 0 and 5 of refrigerated storage (4 °C).

	week 0			week 5		
	pH	° Brix	TA	pH	° Brix	TA
CONTROL	2.43^{b}	9.70^{ab}	0.37^{b}	2.47^{b}	9.67^{a}	0.38^{b}
DPCD	2.45^{a}	9.77^{a}	0.40^{a}	2.48^{a}	9.70^{a}	0.41^{a}
HTST	2.45^{a}	9.63^{b}	0.37^{b}	2.48^{a}	9.60^{b}	0.38^{b}

Data represents the mean of n=9. * Values with similar letters within columns are not significantly different (Tukey's HSD, $p > 0.05$).

Table 6-2. Difference in flavor and overall likeability between fresh (reference and hidden reference), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverages detected by untrained panelists (n = 75) at weeks 0 and 5 of refrigerated storage (4 °C)

	Week 0		Week 5	
	Difference in flavor*	Overall likeability	Difference in flavor	Overall likeability
Hidden reference	2.78^{b**}	5.23^{a}	3.06^{b}	5.89^{a}
DPCD	3.75^{a}	5.01^{a}	5.16^{a}	5.27^{b}
HTST	3.34^{ab}	5.23^{a}	3.63^{b}	5.93^{a}

* Difference observed when compared to given reference (difference from control test). ** Values with similar letters within columns are not significantly different (Tukey's HSD, $p > 0.05$).

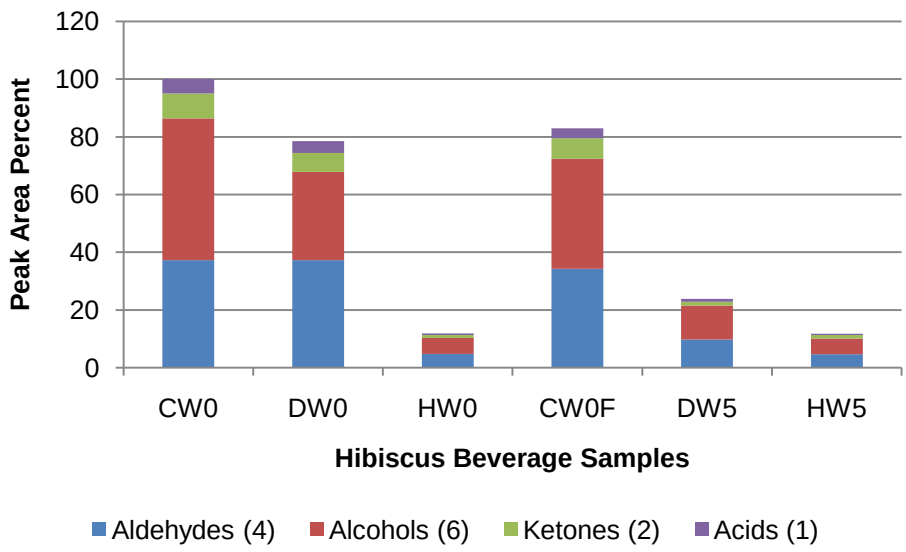


Figure 6-1. Chemical composition of hibiscus beverage headspace volatiles during storage. Total number of compounds for each class is put in parenthesis. All six samples were normalized to total peak area of the sample CW0 (CONTROL week 0). C = CONTROL, D = DPCD, H = HTST, W = week.

Table 6-3. MS identification of hibiscus beverage volatiles during storage. Peak areas were normalized (100) to the largest peak (1-Octen-3-ol) in the CONTROL (C) week 0 sample.

					Normalized peak areas (%)					
			LRI		week 0			week 5		
#	Name	CAS #	DB-Wax	DB5	C	D	H	C	D	H
1	Dehydroxylinalool oxide a	13679-86-2	1210	993	48.9	14.4	5.2	29.7	5.96	5.1
2	Dehydroxylinalool oxide b[a]	13679-86-2	1246	1007	43.9	11.6	4.9	19.8	5.79	4.3
3	3-Octanone	106-68-3	1264		16.3	9.5	2.1	11.9	1.24	2.1
4	Octanal[a]	124-13-0	1299	1002	83.5	49.6	8.5	70.9	8.52	8.8
5	6-methyl-5-Hepten-2-one	110-93-0	1355	989	45.5	36.7	5.5	38.7	8.13	6.3
6	1-Hexanol[a]	111-27-3	1373	873	80.6	62.5	9.0	72.9	11.97	9.5
7	Nonanal	124-19-6	1405	1100	67.2	66.6	6.4	54.9	9.69	7.3
8	1-Octen-3-ol	3391-86-4	1468	983	100.0	78.8	11.7	81.6	44.10	11.9
9	Acetic acid[a]	64-19-7	1485		35.4	29.6	3.6	24.5	7.66	3.2
10	Furfural[a,b]	98-01-1	1496	832	15.9	26.0	3.4	7.5	7.50	2.9
11	Decanal[b]	112-31-2	1513	1204	99.5	123.5	15.9	111.0	43.80	14.0
12	1-Octanol	111-87-5	1577	1071	30.9	20.6	3.6	28.8	8.19	3.7
13	1-Nonanol	143-08-8	1678	1172	45.2	30.3	4.9	39.3	7.88	4.9
	Total normalized peak area				712.7	559.7	84.6	591.3	170.4	84.0

C = CONTROL, D = DPCD, H = HTST. [a] Compounds previously reported in H. sabdariffa by Chen and others (1998). [b] Compounds previously reported in H. sabdariffa by Gonzalez-Palomares and others (2009).

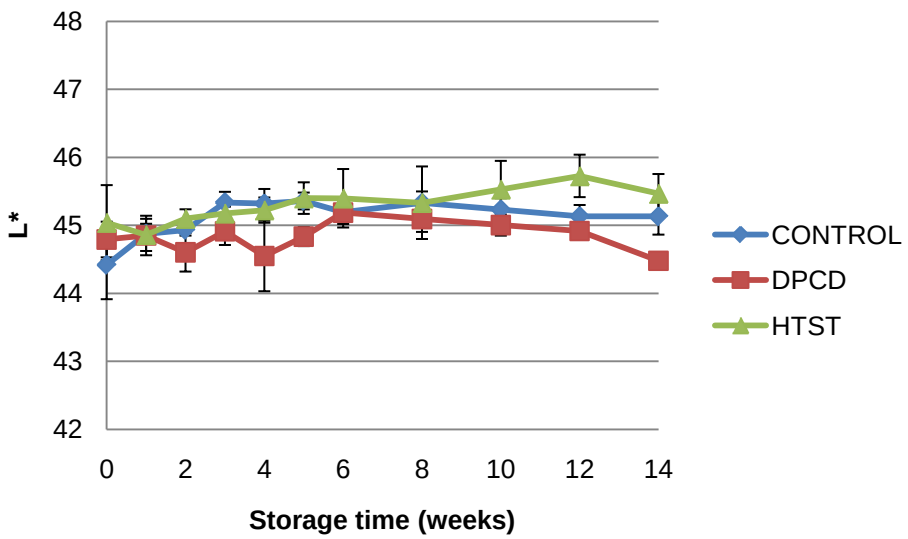


Figure 6-2. L* values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

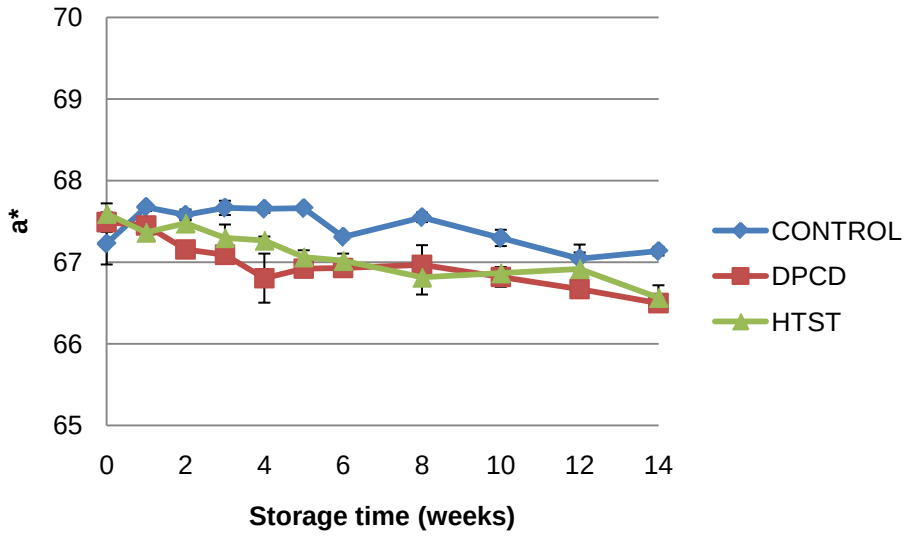


Figure 6-3. a* values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

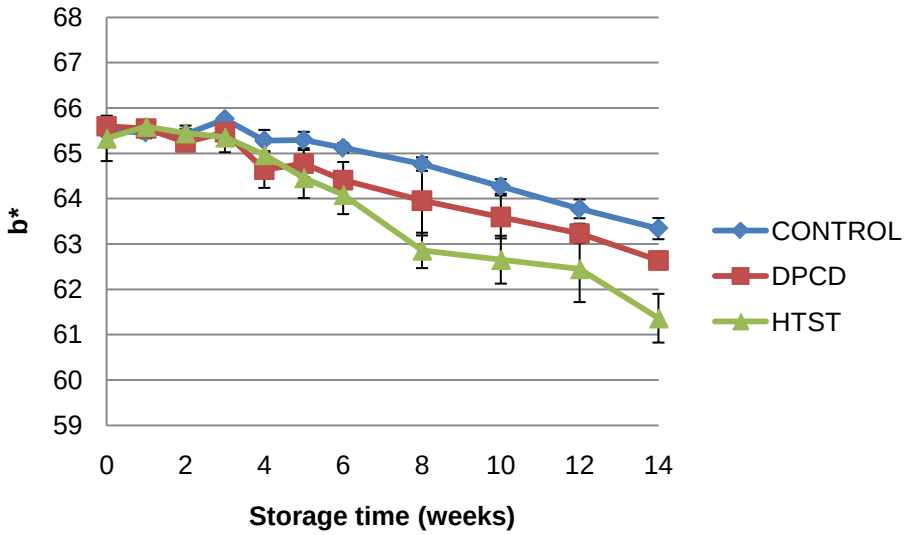


Figure 6-4. b^* values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

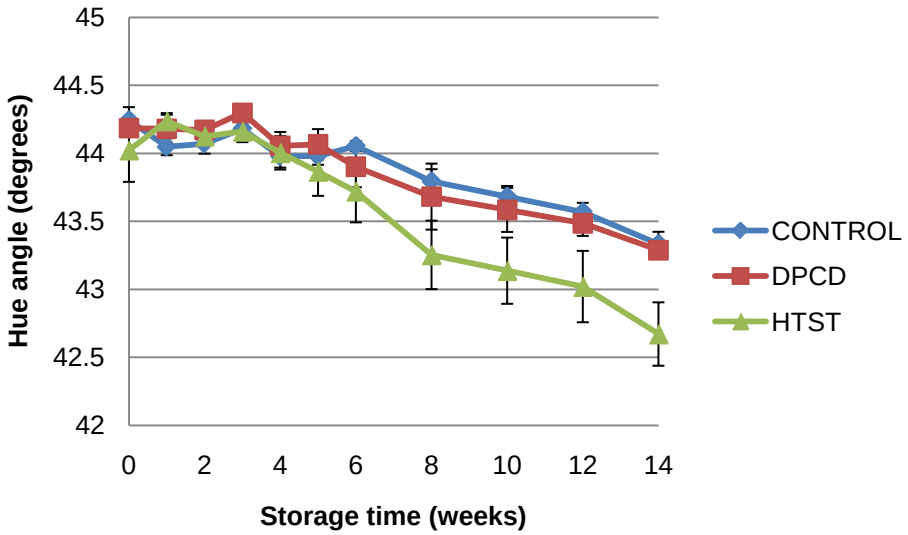


Figure 6-5. Hue angle values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

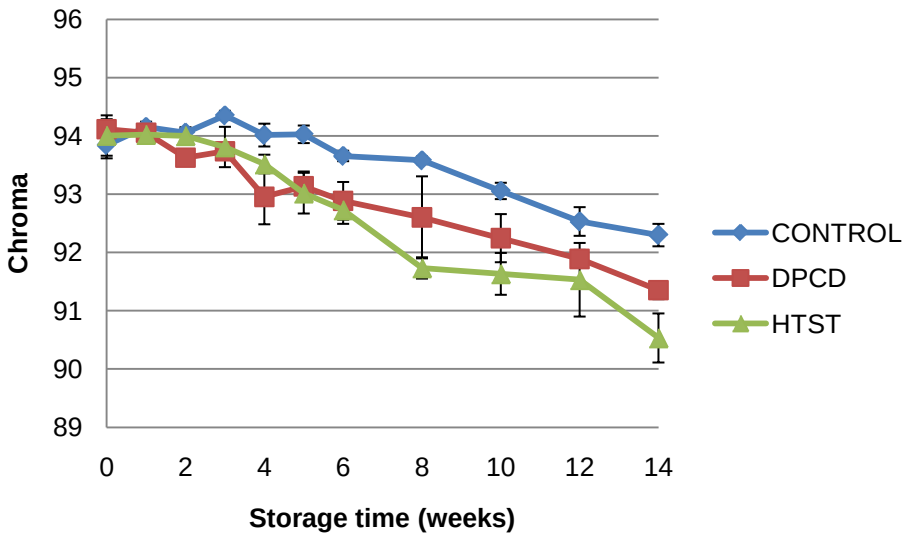


Figure 6-6. Chroma values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

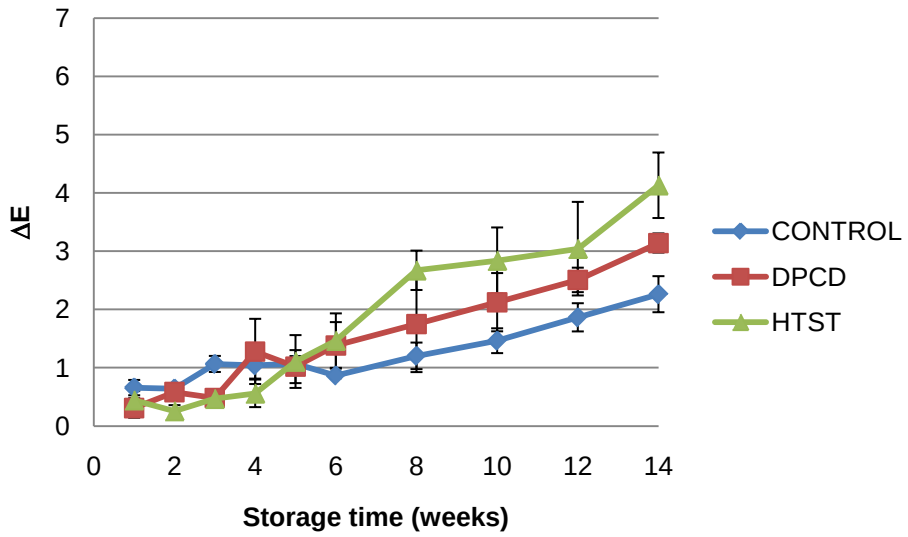


Figure 6-7. ΔE values of unprocessed (CONTROL), dense phase-CO_2 processed (DPCD; 34.5 MPa, 8% CO_2, 6.5 min, 40 °C) and thermally treated (HTST; 75 °C, 15 s) hibiscus beverage during refrigerated storage (4 °C).

CHAPTER 7
SUMMARY AND CONCLUSIONS

Findings of this research can provide more flexibility to hibiscus processing. Extraction and process selection for industrial applications should consider availability of raw material (fresh or dried hibiscus), final product quality and phytochemical characteristics, and economic considerations

Equivalent cold and hot water conditions (240 min at 25 °C and 16 min at 90 °C) were found for anthocyanins extraction of dried hibiscus. Similar polyphenolic profiles and chemical composition of aroma compounds were observed between fresh and dried hibiscus extracts although differences in concentration were found. Fifteen aroma compounds were identified for the first time. In general, hibiscus aroma is a combination of earthy, green, floral, and fruity notes but the final flavor profile is affected by the preservation and extraction process.

Solubility of CO_2 in a hibiscus beverage (5.06 g CO_2lmL at 31.0 MPa) and optimal processing conditions to inactivate microorganisms (34.5 MPa and 6.5 min for a Y&M log reduction of 6.1) were determined. DPCD was found to be a viable technology for processing hibiscus beverages since it extended its shelf life and maintained the characteristic red color for 14 weeks of refrigerated storage. Quality attributes such as pH an °Brix were not affected by DPCD whereas TA increased. A loss of only 9% of anthocyanins during storage was observed in the DPCD processed hibiscus beverage which was lower as compared to a heat pasteurization process and no major changes in total phenolics content and antioxidant capacity occurred during storage. Changes in hibiscus aroma volatiles during storage did not affect panelists overall likeability of the product.

APPENDIX A
EXTRACTION EXPERIMENT STATISTICAL ANALYSIS

Table A-1. SAS software output of statistical analysis for the anthocyanins concentration data (AC) perfumed in the hibiscus extraction experiment (Chapter 3).

EXTRACTION
The GLM Procedure

Class Level Information

Class	Levels	Values
Treatment	8	25-120 25-240 25-30 25-60 90-16 90-2 90-4 90-8

Number of observations 72

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	13655.68417	1950.81202	78.77	<.0001
Error	64	1585.00755	24.76574		
Corrected Total	71	15240.69172			

R-Square	Coeff Var	Root MSE	AC Mean
0.896002	8.748260	4.976519	56.88582

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	7	13655.68417	1950.81202	78.77	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	7	13655.68417	1950.81202	78.77	<.0001

Tukey's Studentized Range (HSD) Test for AC

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	64
Error Mean Square	24.76574
Critical Value of Studentized Range	4.43126
Minimum Significant Difference	7.3507

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	77.464	9	90-16
A	70.885	9	25-240
B	63.211	9	90-8
C B	58.159	9	25-120
C	55.436	9	90-4
C D	53.197	9	25-60
D	47.160	9	90-2
E	29.574	9	25-30

---------- Temperature=25 ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
time	4	30 60 120 240

Number of observations 36

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	8057.681364	2685.893788	112.12	<.0001
Error	32	766.575146	23.955473		
Corrected Total	35	8824.256510			

R-Square	Coeff Var	Root MSE	AC Mean
0.913129	9.242845	4.894433	52.95375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	3	8057.681364	2685.893788	112.12	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	3	8057.681364	2685.893788	112.12	<.0001

Tukey's Studentized Range (HSD) Test for AC

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	32
Error Mean Square	23.95547
Critical Value of Studentized Range	3.83162
Minimum Significant Difference	6.2512

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	time
A	70.885	9	240
B	58.159	9	120
B	53.197	9	60
C	29.574	9	30

---------- Temperature=90 ----------

The GLM Procedure

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4484.797153	1494.932384	58.45	<.0001
Error	32	818.432403	25.576013		
Corrected Total	35	5303.229555			

R-Square	Coeff Var	Root MSE	AC Mean
0.845673	8.315437	5.057273	60.81789

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	3	4484.797153	1494.932384	58.45	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	3	4484.797153	1494.932384	58.45	<.0001

Tukey's Studentized Range (HSD) Test for AC

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	32
Error Mean Square	25.57601
Critical Value of Studentized Range	3.83162
Minimum Significant Difference	6.4592

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	time
A	77.464	9	16
B	63.211	9	8
C	55.436	9	4
D	47.160	9	2

APPENDIX B
STORAGE EXPERIMENT STATISTICAL ANALYSIS

Table B-1. SAS software output of statistical analysis for the anthocyanins concentration data (AC) perfumed in the hibiscus storage experiment (Chapter 5).

STORAGE
The GLM Procedure

Class Level Information

Class	Levels	Values
time	11	0 1 2 3 4 5 6 8 10 12 14
Treatment	3	C D T

Number of observations 297

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	56.9747574	28.4873787	9.43	0.0001
Error	294	888.2431136	3.0212351		
Corrected Total	296	945.2178710			

R-Square	Coeff Var	Root MSE	AC Mean
0.060277	4.330435	1.738170	40.13847

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	56.97475742	28.48737871	9.43	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	56.97475742	28.48737871	9.43	0.0001

Repeated Measures Analysis of Variance

Repeated Measures Level Information

Level of time	1	2	3	4	5	6	7	8	9	10

MANOVA Test Criteria and Exact F Statistics for the Hypothesis of no time Effect
H = Type III SSCP Matrix for time
E = Error SSCP Matrix

S=1 M=3.5 N=142

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.00000	6839662	9	286	<.0001
Pillai's Trace	1.00000	6839662	9	286	<.0001
Hotelling-Lawley Trace	215234.11193	6839662	9	286	<.0001
Roy's Greatest Root	215234.11193	6839662	9	286	<.0001

MANOVA Test Criteria and F Approximations for the Hypothesis of no time*Treatment Effect
H = Type III SSCP Matrix for time*Treatment
E = Error SSCP Matrix

S=2 M=3 N=142

Statistic	Value	F Value	Num DF	Den DF	Pr > F

Wilks' Lambda	0.37623085	20.03	18	572	<.0001
Pillai's Trace	0.74964003	19.12	18	574	<.0001
Hotelling-Lawley Trace	1.32338497	20.97	18	472.9	<.0001
Roy's Greatest Root	0.98306355	31.35	9	287	<.0001

NOTE: F Statistic for Roy's Greatest Root is an upper bound.
NOTE: F Statistic for Wilks' Lambda is exact.

Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	237.565234	118.782617	23.57	<.0001
Error	294	1481.873250	5.040385		

Univariate Tests of Hypotheses for Within Subject Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F	Adj Pr > F G - G	Adj Pr > F H-F
time	9	15393156.09	1710350.68	384657	<.0001	<.0001	<.0001
time*Treatment	18	1280.52	71.14	16.00	<.0001	<.0001	<.0001
Error(time)	2646	11765.26	4.45				

Greenhouse-Geisser Epsilon	0.1367
Huynh-Feldt Epsilon	0.1379

---------- time=0 ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
Treatment	3	C D T

Number of observations 27

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	12.57264263	6.28632131	8.18	0.0020
Error	24	18.44201807	0.76841742		
Corrected Total	26	31.01466070			

R-Square	Coeff Var	Root MSE	AC Mean
0.405377	2.048539	0.876594	42.79120

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	12.57264263	6.28632131	8.18	0.0020
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	12.57264263	6.28632131	8.18	0.0020

Tukey's Studentized Range (HSD) Test for AC

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.768417
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	1.032

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	43.7506	9	C
B	42.4015	9	D
B	42.2215	9	T

---------- time=14 ----------

The GLM Procedure

Dependent Variable: AC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	56.96992981	28.48496491	58.59	<.0001
Error	24	11.66832881	0.48618037		
Corrected Total	26	68.63825862			

R-Square	Coeff Var	Root MSE	AC Mean
0.830003	1.824962	0.697266	38.20717

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	56.96992981	28.48496491	58.59	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	56.96992981	28.48496491	58.59	<.0001

Tukey's Studentized Range (HSD) Test for AC

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.48618
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	0.8208

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	39.7695	9	C
B	38.5811	9	D
C	36.2709	9	T

APPENDIX C
HIBISCUS SABDARIFFA PICTURES

Figure B-1. Pictures of dried hibiscus (A), dried hibiscus extraction process (B), hibiscus beverage (C), hibiscus beverage in the dense phase carbon dioxide (DPCD) feed tank (D), DPCD processing equipment (E), DPCD processed hibiscus beverage (F), hibiscus beverage samples for analysis (G), hibiscus beverage under refrigerated storage (H), and DPCD processed hibiscus beverage after 14 weeks of storage at 4 °C (I). Photos by Milena Ramirez.

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BIOGRAPHICAL SKETCH

Milena M. Ramirez Rodrigues was born in Puebla, Mexico. After graduating from high school (American School of Puebla) in July 1998, she enrolled in the Food Engineering program of the Universidad de las Americas-Puebla (UDLA). Before finishing her bachelor's she was offered an assistantship to pursue a master's in food science. In July 2005 she was awarded a scholarship from CONACyT (National Mexican Council of Science and Technology) and had the opportunity to pursue her Ph.D. in Food Science at the University of Florida. While at UF she decided to enroll in the agribusiness master's program, from which she graduated in May 2009. After finishing her Ph.D., Milena hopes to continue exploring her interests in new product development and marketing.