PHENOLICS, ANTHOCYANINS AND ANTIOXIDANT ACTIVITY IN RED RASPBERRY MUFFINS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of MARIA U. ROSALES SOTO find it satisfactory and recommend that it be accepted.

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Chair

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PHENOLICS, ANTHOCYANINS AND ANTIOXIDANT ACTIVITY

IN RED RASPBERRY MUFFINS

Abstract

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Raspberries are a rich source of phenolic compounds, including flavonoids. Anthocyanins are the major group among flavonoids. Mixing and temperature baking influences on phenolics, antioxidant activity and anthocyanins from raspberry fruit were evaluated during the preparation of muffins. Frozen red raspberries (cv. Meeker) were processed to obtain raspberry juice. Red raspberry juice, control and raspberry batter and muffin samples were extracted using three solvents: 100% methanol, methanol:HCl and ethanol:HCl and analyzed for phenolics, antioxidant activity, and anthocyanins. Anthocyanins were also identified using HPLC with diode array detector. Change of the original pH of the fruit due to mixing with other muffin ingredients affected the final color of the batter and baked muffin product. Acidified methanol was selected as the solvent giving a greater amount of the parameters selected considering raspberry muffin as the product of interest. The largest phenolic content was obtained for raspberry batter (0.70 mg gallic acid equivalent (GAE)/g DM) compared to the phenolics given by the 2.4% solids of raspberry juice present in the raspberry batter dry sample (0.56 mg GAE/g DM). Raspberry muffin had a lower amount of phenolics (0.44 mg GAE/g DM) in

comparison to the raspberry batter. On the contrary, antioxidant activity increased in the raspberry muffin (0.023 μmol Trolox equivalent (TE)/g DM) compared to the raspberry batter (0.012 μmol TE/g DM). Total anthocyanins were determined using a spectrophotometric method. Anthocyanin content decreased 42.5% from 0.080 mg of cyanidin-3-glucoside (CGE)/g DM present in the 2.4% solids of red raspberry juice to 0.046 mg of CGE/g DM found in raspberry batter. HPLC analysis showed the presence of four anthocyanins in red raspberry juice, cyanidin-3-sophoroside, cyanidin-3-glucoside, cyanidin-3-glucosylrutinoside, and pelargonidin-3-sophoroside. Pelargonidin-3-sophoroside was undetectable in the raspberry batter and muffin products.

This study confirmed that baking temperatures and batter mixing affect antioxidant activity, phenolic and anthocyanin content of red raspberry products. Possible approaches to protecting health benefiting phenolics in raspberry include using whole fruits with coatings.

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Dedication

This thesis is dedicated to my wonderful parents, Rolando and Flor, who have raised me to be the person I am today. You have been with me every step of the way, through good times and bad. Thank you for all your unconditional love, guidance, and endless support that you have always given me, especially throughout this tremendous endeavor. Thank you for helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to.

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CHAPTER I

INTRODUCTION

Raspberries continue to rank as the third most popular berry in the United States for fresh use after strawberries and blueberries. The United States is the world's third-largest producer of raspberries. Raspberries come in red, black, purple and yellow varieties (Geisler 2007). Consumption has grown for all types of these berries over the last several years, but annual per capita consumption increase for raspberries averaged 3 to 7 percent higher than for strawberries and blueberries from 2000 to 2005 (USDA 2006). More than 80% of raspberries produced in Oregon and Washington are red raspberries with the remainder being black raspberries (Hui and others 2006a).

The leading producing states for red raspberries are Washington, Oregon and California. As reported in 2004, Washington accounted for approximately 60% of the United States production of red raspberries, at nearly 70 million pounds per year (WRRC 2008). In 2006, that value decreased when Washington raised 57.6 million pounds of red raspberries valued at $20.5 million. Oregon leads the United States in black raspberry (*Rubus occidentalis*) production with 4 million pounds grown in 2006, valued at $9.8 million (Geisler 2007). In Washington State, the leading red raspberry variety is "Meeker", which is a late season, summer fruiting raspberry.

US consumption of fresh raspberries has tripled since the early 1990s to an estimated 0.33 pound per person in 2005. While up sharply from earlier in the decade,

much of the growth in fresh-market consumption occurred in recent years. Meanwhile, US frozen raspberry consumption has fluctuated between 0.10 pound and 0.30 pound per person, fresh-weight equivalent basis, during 1992-2005 (USDA 2006).

A possible cause for this increase in demand is the widely touted health benefits of raspberries. Raspberries are known to have potential antioxidant and anticancer components due to the fact they are rich in phenolics and show high radical-scavenging activity. The fruit contains high levels of anthocyanins derived from cyanidin and pelargonidin (Mullen and others 2002b; Proteggente and others 2002; Kim and Padilla-Zakour 2004). Also, raspberries are a good source of dietary fiber and potassium (Hui and others 2006a).

Fresh raspberries have a very short shelf life, and are generally only readily available in the summer. For this reason, raspberries are commonly sold in the form of frozen fruits, or in jams and sauces (Beekwilder and others 2005a). As mentioned by Kim and Padilla-Zakour (2004), jam is the most popular shelf stable product made from raspberry fruit.

Raspberries are also a popular component of various processed foods including cereal-based products. Popular chemically leavened baked goods that include muffins constitute traditional wheat-based breakfast foods (Yu 2008). Among these muffins, blueberry muffins are a popular breakfast and snack favorite of thousands of people. They are especially good if made with fresh wild blueberries but these fruit are not

available the whole year and overall, demand for wild blueberries exceeds supply. This is one reason that fabricated blueberries using apple or pectin gel as a base were developed and are often included in blueberry-containing baking mixes and cereal products including bars. The current need for increasing the availability and suitability of blueberries for use in muffin mixes, breakfast cereals, and the like, and ensuring a year around source of blueberries suitable for a variety of food applications (Phillips 2001) has led to consideration of other berries to meet demand. Because of the popularity of blueberry products, and the shortage of blueberries to meet demand, other berries, including raspberries, are viewed as an alternative for meeting demand for berry containing baked goods and cereals and also for extending product lines and increasing consumer choice.

Because of the health benefits of berries, there is a demand for their inclusion in other types of processed foods. The market demand is for natural materials rather than chemical antioxidants to be added to extruded products or baked products has increased the demand for raspberries and raspberry based products. Particularly with higher value added products, the market requirement for a 'clean' ingredient statement with a focus on 'natural' ingredients is important. Raspberries contain high contents of phenolic compounds (anthocyanins, ellagic acid, quercetin, etc.) and these compounds have antioxidant function that could provide additional benefit in our diets by inclusion in popular food items for adults and children. Foods with high levels of anthocyanins also provide attractive colors, primarily red and purple to a food (Camire and others 2007), characteristics that can contribute enormously to the creation of a visually appealing

product to consumers interested in healthy foods. There is a concern that food processes such as baking cause a loss of some phytochemicals, so it is crucial to consider the chemical and nutritional changes occur during processing.

Incorporation of antioxidant containing berries to muffins provides a popular vehicle to potentially increase the antioxidant intake. Although muffins are not the largest contributor to sales in an in-store bakery department, this popular breakfast and snack food item has maintained steady sales throughout 2006 and outpaced bakery department growth by 4 percent. Nationally, muffin sales made up 4.4 percent of total bakery sales in 2006, up 0.2 percentage points from the previous year. In 2006, muffin dollar sales were lowest in early January and again in December indicating that competition from other holiday breakfast foods that impacted everyday muffin sales. In 2007, muffins sales decreased 0.1% in relation to 2006 in the Eastern Region of the US, and a decrease of 0.3% was observed in the Western Region. As is shown in **Figure 1,** muffin sales as a percent of total sales in the East were double that of other regions (Anonymous 2006, Perishables Group 2008). In this context and considering that muffin sales are stable or increasing slightly, inclusion of red raspberries into this product could provide an attractive means of introducing the healthful benefits of phytochemicals to a food that normally would not be a significant dietary source of antioxidants.

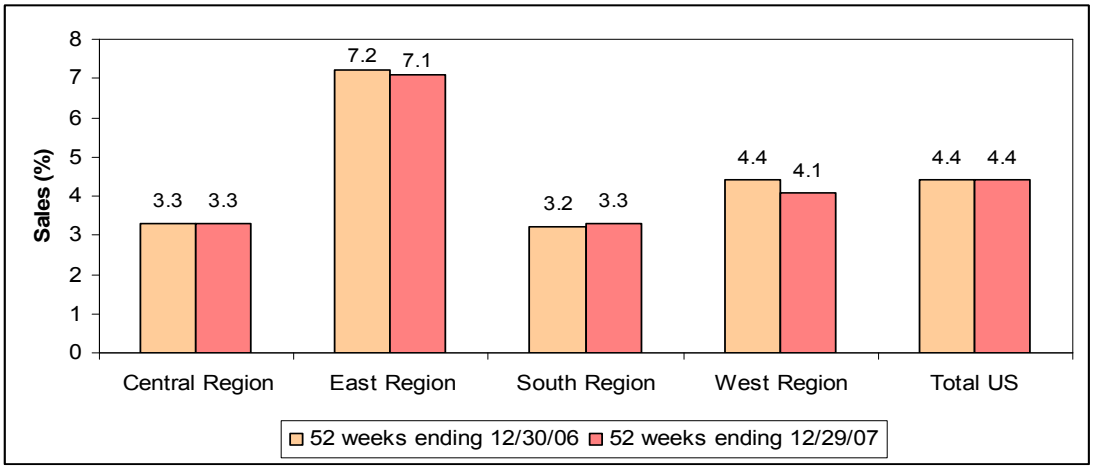

Figure 1- Muffin percentage contribution to total bakery sales in United States (US) 2006-2007 (Perishables Group 2008)

Raspberries are an excellent source of phenolic compounds and by adequately controlling the parameters during the baking process, it should be possible to retain these heat labile compounds, taking advantage of the potential benefits that those compounds can bring us, as natural antioxidants, and in the long run to improve quality of life.

The objective of this study was to determine whether changes occur in the phenolic content, antioxidant activity and anthocyanins of red raspberry juice used as an ingredient for the preparation of muffins and during the baking process. Measurements were made in the batter before thermal treatment and in the final product after the baking process. The possible influence of temperature and pH of the heating media on changes in phenolic content and antioxidant activity is discussed.

LITERATURE REVIEW

1. RED RASPBERRY (*Rubus idaeus L.)*

Origin

Raspberries were first introduced into cultivation in Europe nearly 450 years ago. The red raspberry, which is native to Asia Minor and largely grown in Europe, is popular for commercial production in the United States because it is generally cold-tolerant, high yielding, and resistant to diseases. By the early nineteenth century, more than 20 cultivars of red raspberry were grown in both England and the US. English cultivars were then exported to the US (Pritts 2006; USDA 2006).

Botanical features

Raspberries, known as bramble fruits, belong to the rose family, Rosaceae, genus *Rubus,* and subgenus *Idaeobatus.* Cultivated raspberries in North America are derived mainly from two species, the red raspberry (*Rubus idaeus*) and the black raspberries (*Rubus occidentalis*) the only types grown on a large scale (Mazza and Miniati 1993; Barret and others 2005).

Plant

Raspberries are erect, semi-erect, or trailing, generally thorny shrubs, bush types, producing renewal shoots from the ground called "canes". The plants are perennial, composed of biennial canes, which overlap in age and roots living for many years. Individual canes grow vegetatively for one year, initiate flower buds in late summer; fruit

the following summer, then die. Canes are upright or semi-erect and may reach a height of 8 feet or more. The first year canes are called "primocanes" (syn. autumn fruiting) and in the second year when they flower, "floricanes". Stems may have sharp, strong thorns or spines, have scattered, weak prickles, or be thornless. Leaves are mostly trifoliate and ovate. After the entire cane dies to the ground it should be pruned out to leave space for newly developing vegetative canes for next year's fruit (Scheer and Garren 1981; Hartmann and others 1988; Anonymous 2006; USDA 2006).

Flower

For raspberries, inflorescences are cymose, and some flowers are borne singly in axils of leaves on fruiting laterals. Flowers are initiated in late summer in biennial types, early to mid-summer in primocane fruiting types. The gynoecium consists of 60-100 ovaries, each of which develops into a drupelet (Anonymous 2006).

Fruit

In all brambles, the fruit is an aggregate of drupelets composed of 75 to 125 bright red drupelets that are fleshy and contain seeds. Drupelets are held together by their geometrical symmetry and microscopic, interlacing epidermal hairs. The fruit separates from the receptacle, producing a hollow fruit with no core. Fruiting begins in the second year of the planting, and continues for more than 10 years if properly managed. Fruit development occurs rapidly, taking only 30-50 days for most raspberries (Scheer and Garren 1981; Robbins 1987; Barret and others 2005; Anonymous 2006).

Parts used: Fruit and leaves.

Raspberries have been eaten fresh for thousands for years, mainly the fruit, but medicinal uses of raspberries are related to raspberry leaf tea dating to the 16th century. Today, raspberry fruits are either harvested by hand and eaten fresh or machine-harvested and processed. The major products of processing are individual quick frozen (IQF) (with the best quality of fruit), block frozen or frozen in bulk for institutional use, puree frozen (8 – 15°Brix), juice (about 9°Brix), concentrate, canned, aseptic packs, and preserves. Some of the fruits are combined with sugar and packaged in retail-sized containers. Raspberry juice, made with the lower quality fruit, is usually blended with apple, pear, or grape juice because the flavor is too intense for direct consumption. Recently, the demand for fruit wines has increased, and raspberries make one of the better wines. Some wineries add raspberry juice to grape wine to obtain a less expensive raspberry-flavored wine. Raspberry beer is also made at breweries and meaderies (Pritts 2006; USDA 2006).

Varieties

The main varieties in the northwest US and in the British Columbia area of Canada are Willamettte and Meeker (**Table 1)** (Barret and others 2005). The Willamette is a darker red berry than the Meeker. Its use is decreasing in favor of the Meeker, which is a thimble-shaped, bright red fruit with an excellent raspberry flavor. It is preferred as a freezing berry.

Table 1 - Characteristics of Northwest US Raspberry Cultivars (Barret and others 2005)

Variety	Characteristics	Strengths	Weaknesses
Meeker	Leading variety, suitable for processing and fresh market; midseason	Medium yield Medium size Medium solids Consistent productivity Machine harvestable Good flavor	Soft
Willamette	Dark fruit preferred for processing but darkens too rapidly for fresh market; early season	High fruit color Fruit flavor Machine harvestable Early	Fruit color Moderate productivity Soft Small Low yield Low solids

Producers

The three major raspberry production regions are (1) Russia, (2) Europe (mostly in Poland, Hungary, Serbia, Germany, and the UK), and (3) the Pacific Coast of North America (British Columbia, Washington, California and Oregon) (**Figure 1**) (Poincelot 1980; Hartmann and others 1988; Pritts 2006).

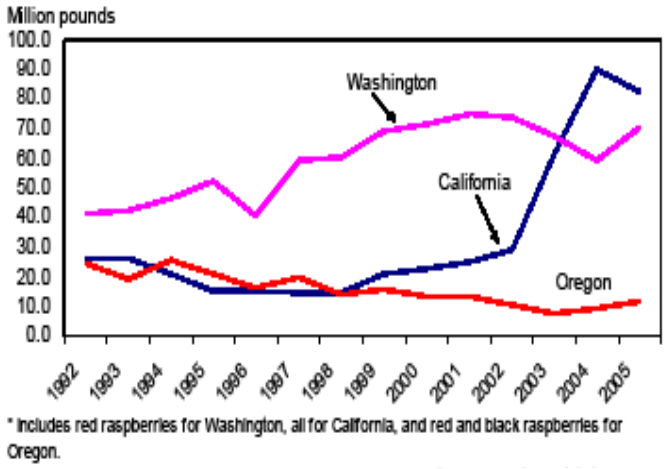

Figure 1- Raspberry production in three major states (USDA 2006)

Many other countries, such as Chile, New Zealand, and Australia, have significant production as they supply the fresh market during winter in the northern hemisphere. World production is estimated at more than 400 000 t (Pritts 2006).

The United States is the world's third-largest producer of raspberries (USDA 2006). Russia, Serbia and Montenegro surpass US production as is shown in **Table 2**. **Table 3** shows red raspberry production in Washington.

Table 2 - Main producers of raspberries in the world (2002) (Anonymous 2006)

Top 10 Countries (% of world raspberry production)	
1. Russia (24%)	6. Ukraine (5%)
2. Serbia & Montenegro (23%)	7. Hungary (4%)
3. United States (13%)	8. Canada (3%)
4. Poland (11%)	9. UK (2%)
5. Germany (7%)	10. France (2%)

Market

The demand for raspberry products is increasing worldwide (Barret and others 2005). The major consumer of red raspberry is the processing sector (USDA 2006). Farmer's markets are included in the group of consumers of fresh raspberries.

The best quality fruits can be easily marketed to white tablecloth restaurants, but it is more difficult to market second quality fruits that are bruised or small. These fruits are used to produce a number of value-added products such as raspberry jellies, pies and muffins (Radoutcheva and others 2006).

Table 3 - Red raspberry production - Washington (US) (2001 – 2006) (WRRC 2007)

Year	Total crop						Fresh Market			Processing		
	Harvested Acres	Yield per acre (lbs)	Total production (000 lbs)	Utilized production (000 lbs)	Price per lb (cents)	Value of utilized prod ($000)	Production (000 lbs)	Price per lb (cents)	Value ($000)	Production (000 lbs)	Price per lb (cents)	Value ($000)
2001	9500	7900	76050	75050	50.3	$37784	3550	158	$5609	71500	45.0	$32175
2002	9500	7800	74100	74100	49.9	$36985	5200	115	$5980	68900	45.0	$31005
2003	9200	7300	67700	67200	54.4	$36554	1400	167	$2338	65800	52.0	$34216
2004	9000	6600	59400	59400	77.4	$45960	1500	169	$2535	57900	75.0	$43425
2005	9500	7400	70300	70300	55.9	$39275	1400	197	$2758	68900	53.0	$36517
2006	9600	6000	57600	57600	35.6	$20530	850	212	$1802	56750	33.0	$18728

Over the last 5 years (2001-2005) an average of 96 percent of Washington's red raspberry production was marketed to processors while in Oregon, this share averaged 90 percent of the State's combined red and black raspberry output. The National Agricultural Statistics Service (NASS) does not breakdown California's production into fresh market and processing. However, approximately 95 percent of California's raspberry crop is sold in the fresh market. Under this assumption, it is estimated that California growers produced about 78.4 million pounds of raspberries for the fresh market in 2005 and approximately 4.12 million pounds for the processing sector. By far, Washington remains the top supplier of raspberries for processing in the United States, accounting for an estimated over 80 percent of total processing volume. Oregon ranks second (USDA 2006).

Barret and others (2005) mentioned that demand in the US has increased the imports of raspberry products to over 5 million pounds. New red raspberry products increased 10% in 1994 to 330 new products. Most of the introductions were in the baking and dairy sectors. In baked and confectionary products, raspberry and chocolate are often found in combination. US studies reported in 1994 showed that raspberry was the most popular flavor behind chocolate, vanilla and strawberry for dairy desserts.

Market research indicated that raspberry is preferred mostly by 25-to-44-year-old women. Kids, especially of Caucasian descent, prefer "red" flavor and will choose fruits such as raspberries. In relation to Hispanic, Asian and Black populations, there is a less preference for the raspberry flavor (Barret and others 2005). Several new products have been introduced in European markets: truffles, milk drinks, puddings, and conserves.

According to the WRRC (2004), muffins, cakes and bread are also prepared and included in the breakfast treats section.

2. PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY IN RED RASPBERRIES

Raspberries (*Rubus idaeus*) are among the fruits containing the highest antioxidant levels. Raspberries have an antioxidant activity that can be attributed to ellagitannins, anthocyanins, and vitamin C (Mullen and others 2002b). In addition to vitamin C, the antioxidant activity of raspberries is primarily due to two classes of compounds: anthocyanins and ellagitannins. Anthocyanins (Greek *anthos*, flower and kyanos, blue), which are red pigment polyphenols, are mainly found in berry fruits and grapes (Beekwilder and others 2005b, Mazza 1997). Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium of flavylium salts (Mazza 1997). These have been implicated in the protection against coronary heart disease and certain types of cancer (Middleton and others 2000; Nichenametla and others 2006). Ellagitannins, which are complex derivatives of ellagic acid units arranged around glucose moieties (Beekwilder and others 2005a), have been identified in tea, many medicinal plants, and several fruits, including raspberries and others berries (Clifford and Scalbert 2000; Kähkönen and others 2001). In addition to their vasorelaxation properties (Mullen and others 2002a), ellagitannins have been described to have general antioxidant effects (Beekwilder and others 2005b). Raspberry could therefore be considered as a model fruit source for a variety of potentially healthy compounds. In view of the potential

health-related activities of polyphenolic antioxidants such as anthocyanins and tannins, these can be regarded as markers for fruit quality.

Phenolics in Food

Phenolics can be defined as secondary metabolites, substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives (esters, methyl esters, glycosides, etc.). Phenolic compounds include simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Phenolics are recognized by their contribution to the pigmentation of plant foods. Many properties of plant foods are associated with the presence, type, and content of their phenolic compounds such as astringency of foods, beneficial effects or antinutritional properties (Ho and others 1992; Macheix and others 1990; Shahidi and Naczk 2004). The major phenolic compounds found in fruits are listed in **Table 4**:

Flavonoids make up the most important single group of phenolics in foods consisting mainly of flavones, isoflavones and anthocyanidins. The flavonoids are formed via condensation of phenylpropane (C6-C3) compound via participation of three molecules of malonyl coenzyme A, which leads to the formation of chalcones that subsequently cyclize under acidic conditions (Shahidi and Naczk 2004).

Table 4 - The major classes of phenolics in fruits (Macheix and others 1990)

Number of carbon atoms	Basic skeleton	Class	Example	Fruit (example)
7	C_6-C_1	Hydroxybenzoic acids	*p*-Hydroxybenzoic	Strawberry
9	C_6-C_3	Hydroxycinnamic acids	Caffeic	Apple
		Coumarins	Scopolin	Citrus
10	C_6-C_4	Naphthoquinones	Juglone	Walnut
13	C_6-C_1-C_6	Xanthones	Mangiferin	Mango
14	C_6-C_2-C_6	Stilbenes	Resveratrol	Grape
15	C_6-C_3-C_6	Flavonoids	Quercetin, Cyanidin	Cherry
		Isoflavonoids	Daidzein	French bean
N		Lignins		Stone fruits
		Tannins		Persimmon

Among flavonoids, anthocyanins are known as flavans because of lack of the carbonyl group in the 3-position. Anthocyanins are glycosidically bound anthocyanidins present in many flowers and fruits. Each anthocyanidin may be glycosylated and acylated by different sugars (glucose, galactose, rhamnose, and arabinose) and acids at different positions, therefore the number of anthocyanins is 15-20 times greater than the number of anthocyanidins. Anthocyanins are responsible for the bright red, blue, and violet colors of many fruits and flowers (Mazza 1997; Sikorski 1997; Shahidi and Naczk 2004).

Antioxidant activity

The antioxidant activity is probably the most extensively studied aspect of the bioactivity of phenolic compounds (Proteggente and others 2003). A great variety of free-radical scavenging molecules including phenolic compounds can be found in plants. Radicals are normally produced by a number of mechanisms such as aerobic metabolism. Free radicals can induce oxidative damage in lipids, proteins and nucleic acids and can induce or contribute to atherosclerosis, cancer, diabetes mellitus, and neurodegenerative disorders. Diets rich in plant-based products are thought to have a protective effect on cells and tissues and may offer some protection from these degenerative diseases (Manosroi and others 2005; Salta and others 2007) although quantitation of effects in whole animal systems is difficult and may be quite different than what is observed with *in vitro* assays or in cell culture. According to Eberhardt and others (2000), a strong inhibition of tumour-cells *in vitro* was shown, probably due to the combination of phytochemicals in apples.

There is some evidence regarding the role that fruit and vegetable consumption can play in maintaining human health and reducing the risk of cancer, cardiovascular disorders, and other degenerative and chronic diseases caused by oxidative stress (Wang and Jiao 2000; Liu and others 2002; Andersen and others 2003; Kalt 2005). The evidence from numerous *in vitro*, *in vivo*, and clinical studies focused on mainly how certain phytochemicals (vitamin C, carotenoids and phenolics) in many fruits and vegetables can affect specific physiological processes and provide benefits to human health (**Table 5**). Antioxidant content of most foods, mainly of plant origin, is almost exclusively from phenolic substances. In this respect, phytochemicals that possess antioxidant characteristics result from their electron-rich structure characterized by oxidizable double bonds and hydroxyl groups. These particular chemical moieties are believed to contribute to the overall health-protective effects of fruits and vegetables because antioxidants may mitigate oxidative stress in cells, possibly suppressing disease development and the aging process (Pokorný 2003; Kalt 2005). Free radical induced oxidative stress has been associated with several cellular toxic processes including oxidative damage to protein and DNA, membrane lipid oxidation, enzyme inactivation, and gene mutation that could result in cell death and may lead to carcinogenesis (Wang and Jiao 2000; Liu and others 2002).

The powerful free radical scavenger *in vitro* property of pure phenolic compounds has been demonstrated both with synthetic free radicals such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and (2,2-dyphenyl-1-picrylhydrazyl)

Table 5 - Properties of phytochemical antioxidants in plant tissues (Kalt 2005)

Feature	Vitamin C	Carotenoids	Phenolics
Subgroups	Ascorbic acid and dehydroascorbic acid	Numerous, for example, lutein, lycopene, α-carotene, β-carotene, zeaxanthin; cryptoxanthin	Numerous, for example, phenolic acids, hydroxycinnamates, flavonoids inc. flavonols, catechins
Solubility	Water	Lipid	Water
Cellular localization	Dissolved in apoplast, cytosol, chloroplast, mitochondria, and vacuole	Associated with membrane protein complexes in chloroplast or chromoplast	Dissolved in vacuole and apoplast
Structural localization	Uniformly distributed	Some types (for example, tomato lycopene) preferentially in surface tissues like peel and outer pericarp	Anthocyanins preferentially in peel; proanthocyanidins in peel and seed; hydroxycinnamates in flesh
Changes with ripening	Differs with species	Pigmented forms change	Pigmented forms increase

(DPPH) assays and with physiologically relevant peroxyl radical, hydroxyl radicals, and superoxide determinations (Proteggente and others 2003).

The DPPH assay is an easy and accurate method for measuring the antioxidant activity of fruit and vegetable juices or tissue extracts (Al-Dabbas and others 2007). The Trolox equivalent antioxidant (TEAC) capacity assay developed by Rice-Evans and co-workers as was mentioned by Huang and others (2005) has been broadly applied in assaying food samples. This assay is based on the scavenging of the ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt by hydrogen-donating compounds (Proteggente and others 2003). Another method is the oxygen radical absorbance capacity (ORAC), which measures the antioxidant capacity of botanical samples and biological samples (Huang and others 2005; Wu and others 2008). The total radical-trapping antioxidant parameter (TRAP) assay that is a quantitative measure of the total secondary antioxidant content of a biological fluid according to Sies (2007) has also been widely used. The main differences among assays are the substrates, probes, reaction conditions, and quantitation. It is extremely difficult to compare the results from these different assays or to determine how the results of these assays would predict the biological activity at a cellular or tissue level. Some of the methods are summarized in **Table 6** (Huang and others 2005).

Table 6 – *In vitro* antioxidant capacity assays (Huang and others 2005)

Assays involving hydrogen atom transfer reactions $ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$ $ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$	ORAC (oxygen radical absorbance capacity) TRAP (total radical trapping antioxidant parameter) Crocin bleaching assay IOU (inhibited oxygen uptake) Inhibition of linoleic acid oxidation Inhibition of LDL oxidation
Assays by electron – transfer reaction $M(n) + e \text{ (from AH)} \rightarrow AH^{\bullet} + M(n-1)$	TEAC (Trolox equivalent antioxidant capacity) FRAP (ferric ion reducing antioxidant parameter) DPPH (diphenyl-1-picrylhydrazyl) Copper (II) reduction capacity Total phenols assay by Folin-Ciocalteu reagent
Other assays	TOSC (total antioxidant scavenging capacity) inhibition of Briggs-Rauscher oscillation reaction chemiluminescence electrochemiluminescence

Regarding the DPPH method, the antioxidant properties of a given analyte may be determined by monitoring the reaction of the species with a model free radical, 2,2-diphenyl-1-picrylhydrazyl or DPPH. Although Frankel and Meyer (2000) pointed out that there are no approved, standardized methods, the DPPH method is included among the free radical-trapping methods that measure the ability of antioxidants to react with radicals. DPPH (**Figure 2**) is a synthetic "stable" free radical because of delocalization of the spare electron throughout the molecule, which is used to study structural effects on the activity of phenolic antioxidants (Frankel and Meyer 2000). This delocalization gives rise to a deep violet color when DPPH is dissolved in methanol, with an absorption maximum at 515 nm. When a solution of DPPH is mixed with an antioxidant, absorbance

decreases when the odd electron (free radical) of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants (AH) (**Equation 1**) (Ozcelik and others 2003). A subsequent loss of the violet color is observed, although a residual pale yellow may remain due to the picryl group present on the DPPH molecule.

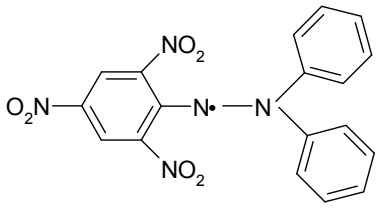

Figure 2 – DPPH structure

$$\mathbf{DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

This assay is limited because DPPH radicals interact with other radicals (alkyl), and the time response curve to reach the steady state is not linear with different ratios of antioxidant/DPPH (Brand-Williams and others 1995; Sánchez-Moreno and others 1998; Frankel and Meyer 2000)

3. THERMAL TREATMENT, pH AND TREATMENT OF RASPBERRY JUICE

To maximize juice yield and color-flavor extraction, a hot break process is often used in juice processing. This method is common for grape and tomato juice and also for berries. Increased interest in highly colored juices, rich in phenolic compounds with associated health benefits, is driving the development of better techniques to preserve the functional components while maximizing extraction (Barret and others 2005).

Effect of baking treatment on phenolic compounds and antioxidant activity

Processing can alter and often damage fruit and vegetable antioxidants. Changes in the antioxidant functionality depend not only on the energy requirement, but also on other factors such as air access, temperature, food composition, time and exposure to light (Pokorný 2003). Maceration, heating, and various separation steps can result in oxidation, thermal degradation, leaching, and other events that lead to lower levels of antioxidants in processed food compared with fresh. This is particularly true in the case of vitamin C and phenolic antioxidants. However, in the case of carotenoids, processing can lead to a dissociation of antioxidants from plant matrix components, an increase in carotenoid antioxidants, and improved digestive absorption (Shi and Le Maguer 2000). The localization of components within plant materials becomes important when tissues such as peels and seeds are separated from other components during processing, as in juice and wine production (Waterhouse and Walzem 1998). This can reduce the level of certain antioxidant components from processed food products and yield processing byproducts that still contain substantial levels of antioxidant compounds (Waterhouse and Walzem 1998; Skrede and others 2000).

Phenolic antioxidants are subject to degradation during processing. In the same study in which vitamin C, β-carotene, and α -carotene were examined in carrots, spinach, potatoes, and several brassicas, losses in total phenolic content after blanching and long-term frozen storage ranged from 20% to 30% (Puupponen-Pimiä and others 2003). In this study, total phenolic content was positively correlated with the total antioxidant activity of these foods, suggesting that among vitamin C, carotene, and phenolics, phenolics make an important contribution to the antioxidant activity of these foods. Like vitamin C,

phenolic antioxidants are water-soluble and can be leached from fruit and vegetable tissues by processing in water (Kalt 2005).

Some phenolic antioxidants, especially flavonoids, are present as esters or glycosides. They are partially hydrolyzed during boiling, and these hydrolytic changes influence both their distribution between the lipid and aqueous phases and their reaction with lipid free radicals (Pokorný 2003). After fresh spinach was boiled in water, approximately half of the flavonoid content was found in the cooking water and the other half in the cooked tissue. In the case of broccoli, one-fifth of flavonol glycosides was retained after the boiling process, the anti-radical activity of mushroom juice was reduced and no effect was observed in onions and yellow bell peppers (Gil and others 1999; Pokorný 2003).

In processing blueberries into juice, substantial losses of phenolics occurred; the recovery of anthocyanins, procyanidins, and chlorogenic acid were 32%, 43%, and 53%, respectively. Heat-labile enzymes in blueberry fruit (for example, polyphenol oxidase) made a large contribution to the loss in anthocyanins. Approximately 20% of the anthocyanins in blueberries were retained in the press cake after juicing (Skrede and others 2000; Kalt 2005). In the case of quince, jellies contained lower concentrations of chlorogenic acid than jams, because of the more severe thermal treatment that the fruit is subjected during the preparation of jelly. An increase of ellagic acid, explained by a release of ellagitannins, was also observed during the thermal treatment of red raspberry to prepare jams, whereas freezing and long-term frozen storage of raspberries induced a significant decrease in the ellagic content (Fleuriet and Macheix 2003).

Flavonoids are reduced by thermal processes such as boiling, frying and microwave cooking (Buchner and others 2006). Among them, limitations of anthocyanins have been reported due to their poor stability, which depends on their chemical structure and concentration of the pigment, pH, self-association, temperature, oxygen, light intensity, polymeric forms, metallic ions, enzymes, ascorbic acid, sugars and their degradation products and sulfur dioxide, and presence of cofactors or copigments, among others (Rodriguez-Saona and others 1999; Cevallos-Casals and Cisneros-Cevallos 2004; Brenes and others 2005). According to Garcia-Viguera and others (1999), boiling produced the greatest rate of anthocyanin destruction in strawberry jams, 16% when the boiling time was 10 min, and 81% when heating was from 16 to 17 min. Kalt and others (1999) showed freezing (-20°C) to have little effect on anthocyanin levels. Temperatures between freezing and boiling allow gradual loss of anthocyanins (Kalt and others 1999; Seeram and others 2001).

According to Havlíková and Míková (1985), the decomposition of anthocyanins in elderberries (*Sambucus nigra* L.) follows a rational exponent-order kinetics values ranging from 1.1 to 1.7 at the pH range 2.2 – 4.0. Anthocyanins structure depends on the pH of the medium. The most stable cationic forms of anthocyanins in an acid medium can be used for coloring food.

Many studies have been conducted on color, appearance, and pigment composition of processed raspberry products such as preserves and juices. Diemair and Schormüeller (1934) cited by Mazza and Miniati (1993), studied the effect of addition of water on color and its thermal stability in raspberry juice. Factors which positively influence stability of anthocyanins are: absence of oxygen, low pH, and low processing

and storage temperatures. Temperature also plays a role in the stability of anthocyanins of frozen raspberry products. The losses of anthocyanins during 12 months storage were lower when juice was stored at -18 ºC than at -2ºC.

As a rule, Macheix and others (1990) determined that anthocyanins are transformed into colorless forms with the exception of pigments acylated by hydroxycinnamic acids at a temperature of around 25ºC, and in an acidic medium close to natural conditions. The great reactivity of flavylium cation towards nucleophilic reagents such as the water molecule is the main factor that causes the fading process.

As mentioned by Barret and others (2005), berry flavor and color degrades with exposure to prolonged elevated temperatures. Also, the juices of both strawberries and raspberries tend to drip with heating and progression of ripening.

Effect of pH on phenolic compounds and antioxidant activity

pH affects anthocyanin stability. In acidic solutions, four anthocyanin species exist; base, flavylium cation, hemiketal pseudo-base and chalcone (Brouillard 1982). At pH levels from 1 to 3, the stable flavylium cation is predominant with minor amounts of the colorless hemiketal form present (Mazza 1997; Brouillard 1982; Sarni-Manchado and others 1997). The flavylium cation is the most stable form of anthocyanin, so low pH levels are preferable for anthocyanin retention. Anthocyanin color is pH dependent, at 3.5 is usually red, becoming colorless and then shifting to blue as the pH increases (Hui and others 2006b).

Coffey and others (1981) investigated the possibility of stabilizing the raspberry anthocyanins by the formation of complexes with Sn^{2+} and Al^{3+} ions. Their results

indicate that complex formation occurs with cyanidin 3-glucoside and Al^{+3} at pH 2 and 3 and with Sn^{+2} at pH 3 and 4. A limited application of these complexes is expected considering that may be unstable and involve more degradation of the anthocyanins because of the production of less stable intermediates.

4. PHENOLIC COMPOUNDS OF RED RASPBERRIES

Raspberries (*Rubus idaeus*) are high in polyphenolic phytochemicals, particularly flavonoids such as anthocyanin pigments, which gives raspberries their characteristic color (Weber and Liu 2002). Raspberries contain glycosides of the flavonols quercetin and kaempferol. Ellagic acid is released from cell walls through hydrolysis of ellagitannins to hexahydroxydiphenic acid, which forms an inner dilactone spontaneously, called ellagic acid. Numerous derivatives of ellagic acid exist, formed through methylation, glycosylation and methoxylation. Ellagic acid is a possible chemopreventive agent in human carcinogenesis. Nonetheless, there is still conflicting evidence as to the anticarcinogenic effectiveness of ellagic acid. Furthermore, it has not been resolved how much ellagic acid and quercetin are absorbed into the body from dietary sources. There is little information about the influence of juice concentrate processing on the phenolic composition of red raspberry juices (Ho and others 1992).

Maza and Miniati (1993) reported that cyanidin 3-glucoside, cyanidin 3-sophoroside, cyanidin 3-rutinoside, and cyanidin 3-glucosylrutinoside were identified in the raspberry cv. Meeker. This cultivar also contained traces of cyanidin 3,5-diglucoside and 3-sophoroside pelargonidin derivative. The synthesis of anthocyanins in fruits of raspberry depends on ecological and physiological factors, but certain quantitative

differences within a species are more or less characteristics of the cultivar. In some cultivars, anthocyanin synthesis proceeds uniformly with ripening, while in others there is a maximum in the first or second phase of ripening. As cited by Hui and others (2006b), accumulation of anthocyanins tends to be higher at the end of the ripening, and there is an influence of sugar levels, light, temperature, ethylene, and increased metabolite translocation from leaves to fruits. In addition, over ripening in raspberry causes an undesirable dark color.

Postharvest storage of raspberry fruits generally produces an increase in anthocyanin concentration and a loss in titratable acidity, but the increase in anthocyanin content is influenced by the degree of fruit ripeness at harvest. "Meeker" red raspberry fruit harvested at the inception stage of maturity (25 - 75% of the surface is green) and at the ripe stage, shows an increase of anthocyanin concentration higher than in fruit harvested when 100% of the surface is red to purplish-red. In addition to anthocyanin, red raspberries also contain high contents of flavonols (0.072 to 0.102 mg/g FW), flavan-3-ols (0.032 to 0.049 mg/g FW) and phenolic acids. Those compounds are known to interact with anthocyanins to produce an increase in color intensity and a bathochromic shift in the spectrum of the anthocyanin to give purple to blue colors (Mazza and Miniati 1993).

It is evident that raspberries are rich in phenolic compounds, particularly flavonoids (anthocyanins), which have demonstrated a wide range of biochemical and pharmacological effects including antioxidant activities. The available information suggests that regular consumption of raspberries should have a long-term health benefit. However, for increased availability of health compounds besides the benefits provided by

the fresh fruit, new food products such as muffins rich in these phytochemicals need to be developed. Also, there is a need of a better understanding of the chemistry of raspberry phenolics not only to define mechanisms of those compounds, but also to determine their stability under thermal treatment and a different pH condition in the food system. Therefore, this study will help to understand raspberry potential benefit when added to a food system such as muffins.

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CHAPTER TWO

TOTAL PHENOLIC AND ANTIOXIDANT ACTIVITY OF RED RASPBERRY (cv. MEEKER) MUFFINS

ABSTRACT

Red raspberry fruits, a rich source of phenolic compounds, were evaluated as part of a model food, specifically muffins. The effect of the baking process and the pH of the batter on the phenolic content and recovery of total antioxidant activity were investigated in muffins prepared with red raspberry juice (RRJ) incorporated as a value-added food ingredient. The initial phenolic content of freeze-dried RRJ extracted with acidified methanolic solution was 23.20 mg Gallic Acid Equivalent (GAE)/g DM and a total antioxidant activity of 2.49 μmol Trolox Equivalent (TE)/g DM after extraction in 100% methanol. Raspberry batter (RB) contained 0.70 mg GAE/g DM. A decrease in total phenolic content was found after baking the raspberry muffins (RM) (0.44 mg GAE/g DM) following recovery of phenolic compounds in acidified methanolic solution as previously described. Baking did not appear to result in a significant loss of antioxidant activity (0.023 μmol TE/g DM) compared to RB (0.020 μmol TE/g DM) ($p<0.05$). The total phenolic content and antioxidant activity of RRJ, batter and muffins were positively correlated ($r=0.99$). The mean percent recovery of phenolic compounds in RB and RM extracted with acidified methanolic solution were 47% and 34%, respectively.

KEYWORDS: Phenolic compounds, antioxidant activity, DDPH, muffin.

INTRODUCTION

Substantial epidemiological evidence shows that diets rich in fruit and vegetables could be more effective than a dietary supplement in part because of the form and manner in which they are consumed, as a major component in the diet rather than as a drug. Foods high in antioxidants have been associated with reduced risk of chronic health disorders including cancer and coronary heart diseases (Eberhardt and others 2000; Huang and others 2005). That protective role is partly attributed to constituents such as vitamins C and E, flavonoids, carotenoids, lycopene, selenium and dietary fiber. Flavonoids, the most important group of phenolics, are a class of secondary metabolites showing antioxidant activity in both *in vivo* and *in vitro* systems (Mazza and others 2004; Robards and Antolovich 1997).

Phenolic compounds are bioactive substances occurring widely in food plants. Phenolic compounds are closely associated with the sensory and nutritional quality of fresh and processed plant foods. Many plants are good sources of natural antioxidants including phenolic compounds (Ho and others 1992). Phenolic compounds are abundant in highly colored berry fruits and include hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins) and hydrolyzable tannins (Seeram and others 2006). Anthocyanins, flavonols and their conjugates, and ellagitannins are the major phenolic compounds present in raspberries. The content of these compounds may be influenced by cultivar, maturity, processing and geographic area of origin. The level of anthocyanins in red raspberry is 0.52 mg/g according to Shahidi and Naczk (2004).

Red raspberry (*Rubus idaeus)* is one of the richest dietary sources of anthocyanins, which are present in the form of glycoside-anthocyanin complexes, or anthocyanidins which are water-soluble pigments present in many fruits, and are responsible for the bright red color of the fruit (Shahidi and Naczk 2004). Anthocyanins are one of the most important group of plant pigments (Sikorski 1997) and these are receiving increasing attention for their possible health benefits. Anthocyanins have been used as natural pigments. Anthocyanin content is a major factor in fruit juice quality (Lee and others 2005).

Beekwilder and others (2005) pointed out that fresh raspberries have a very short shelf life, and are generally only readily available during the summer. For this reason, raspberries are sold in the form of frozen fruits, or in jams and sauces. When raspberries are quick-frozen in liquid nitrogen, and subsequently stored at -20°C for a year, vitamin C content decreases by up to 50%. Other parameters, such as the antioxidant activity and anthocyanin content, seem to be unaltered. A variety of short-term treatments (3 days at room temperature, 4°C or -30°C) seem to have an effect no greater than 20% on the antioxidant activity, anthocyanin or ellagitannin content. Kalt and others (1999) reported a doubling of phenolic content and antioxidant activity upon storage of fresh raspberries at room temperature for 8 days at 20°C. Raspberry (cv. Nova) was stored in the dark in a stainless steel chamber which was sealed with an airtight Plexiglass lid and the vapor pressure controlled. Synthesis of phenolics may be due to the carbon skeletons provided by the organic acids present because of the increase of the titratable acidity of raspberries during storage. However, such prolonged storage can only be achieved without quality loss when the raspberry fruits are harvested at early ripening stages. Jam making has a

more significant effect than unprocessed storage. Processing of jam involves several steps and the addition of other ingredients such as sugar, acid and pectin, followed by a heating process that can affect the anthocyanin stability. Jams are acidified to pH~3 by addition of citric acid. At this pH, anthocyanins are slowly deglycosylated by acid hydrolysis, and the resulting anthocyanin aglycones are less stable. Due to the number of process steps, differences in practices, and the potential effect of process steps on antioxidant activity, data on the effect of jam making on antioxidant activity are sometimes contradictory. Some studies reported that jam making doubled the total antioxidant activity of raspberry. Greater antioxidant activity was also observed in raspberry jam due to the production of hydroxyl groups (aglycons) induced from the conjugated forms of phenolics after an acid hydrolysis (Amakura and others 2000). In another report, no change in antioxidant activity of raspberry jam was observed, in comparison to fresh raspberries (Kim and Padilla-Zakour 2004). According to Beekwilder and others (2005), the amount of anthocyanins was slightly reduced by the jam-making process. This reduction can be attributed to molecular complex formation by the anthocyanins that is still not fully understood. According to those studies, thermal processing seems to cause some changes to anthocyanin content. In relation to that, a similar phenomenon may be likely for baked products, for example if raspberries are included as ingredient in muffins.

As reported by Phillips (2001), muffins are a breakfast and snack favorite of thousands of people. They are especially good if made with fresh wild blueberries but they are not available the whole year. According to Phillips (2001), there is a current need for improving the quality of blueberries for use in muffin mixes, breakfast cereals,

and the like and ensuring a year around source of blueberries suitable for a variety of food applications.

Identification of ways to incorporate red raspberry fruit in muffins, as health food ingredients in human diet could provide many health benefits. Andersen and others (2003) pointed out that a major challenge for the modern food industry is to make products, which combine convenience with freshness and healthy eating. Wolfe and Liu (2003) suggested the incorporation of apple skin in muffin as a way of increasing both the phenolic content and the dietary fiber content of the food. Considering the amount of apple skin generated as a result of apple processing, this could be a food ingredient available in high volumes. However, there is limited information on the potential for incorporation of red raspberries into bakery products such as muffins and their contribution to total phenolic content (TPC) and antioxidant activity (AA) of the final product.

Despite the very well known benefits of red raspberries, there is limited information about the potential human health benefits that baked products could have and how certain types of food processing operations could affect the content and biological activity of antioxidant constituents. Therefore, determination of phenolic content and antioxidant activity of the raw material and its comparison to the content in baked products will help to determine how healthful these new food products might be, and assist consumers to make decisions related to their purchasing preferences. In addition, researchers would orient their studies to determine how to best retain nutritive properties of red raspberries, considering their use as a whole fruit, before their inclusion in a baked product to avoid possible loss of their health promoting constituents.

The objective of this study was to determine the impact of baking and pH on phenolic content and antioxidant activity of muffins made with red raspberry juice (RRJ) (cv. "Meeker") using biochemical assays. Effects of different solvent systems on the extraction of phenolics and antioxidant activity were also investigated and addressed in this study.

MATERIALS AND METHODS

Materials

Frozen red raspberries (FRR) cv. Meeker were provided by the Washington Red Raspberry Commission (WRRC). Folin-Ciocalteu reagent, 2,2 Diphenyl 1-1 picrylhydrazyl (DPPH) and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO). Sodium carbonate, methanol, sodium hydroxide and hydrochloric acid were purchased from J.T. Baker Inc. (Phillipsburg, NJ). Ingredients for muffin preparation were purchased from the local market. Precision fine wire thermocouples were model 5TC-TT-T-20-72 (Omega Engineering, Inc., Stamford, CT). Microplates were 96-well BD Falcon™ 353915 (Fisher Scientific, Pittsburgh, PA) and the sealing tape for multiwell plates was Nunc® (Sigma-Aldrich, Milwaukee, WI).

Sampling procedure

One variety of red raspberry (*Rubus idaeus*, cv. Meeker) grown in Washington (US) was selected for this study.

Juice preparation

Seven Kg of frozen red raspberries (FRR) were processed into juice in the pilot plant of the School of Food Science at Washington State University (WSU) using a Champion World's Finest Juicer (Plastaket MFG, Co., Lodi, CA). A screen with openings with a diameter of ca. 0.51 mm was used in this juicer to retain and eliminate the seeds present in red raspberries. Juice was packaged in polyethylene bags (Freezer bags double zipper, 16.5 cm x 14.9 cm, Ziploc®) and stored frozen at -35°C for 5 days until the preparation of the extracts.

Muffin production

Muffins contained the following ingredients: All purpose enriched, bleached, presifted wheat flour (General Mills, Inc. Minneapolis, MN), pure granulated white sugar (Domino Foods, Inc., Yonkers, NY), salt (IGA brand, IGA Inc., Chicago, IL), baking powder double acting (Clabber Girl, Co., Terre Haute, IN), 100% Pure Canola Oil (Safeway brand, Safeway Inc., Pleasanton, CA), natural nonfat dry milk (Safeway brand, Safeway Inc., Pleasanton, CA), and egg white P-110 (Henningsen Foods, Inc., Omaha, NE) (**Table 1)**.

Considering the average of red raspberry fruits added to baked products is 13% (WRRC, 2004), 10% RRJ was determined to be a comparable level for addition to the batter. Two muffin formulations were evaluated: 10% (w/w) replacement of wheat flour with RRJ (raspberry muffin, RM), and a control with 0% flour replacement (control muffin, CM). The ingredients were mixed for 10 min using a hand mixer (Hamilton Beach/Proctor-Silex, Inc, Washington, NC) and then batter was massed (80 g) into paper

muffin cups (6 cm top diameter x 3 cm depth) (Reynolds Metals Company, Richmond, VA) and baked in a preheated oven (Frigidaire, Pittsburgh, PA, USA) at 176.6°C for 25 min.

K-type thermocouples probes (Omega Engineering, Inc., Stamford, CT) connected to a USB-based 8-channel temperature measurement module (Measurement Computing Corporation, Norton, MA) were inserted in five samples of control muffins. Both oven and internal muffin temperature were recorded every min with TracerDAQ™ 1.9 Thermocouple Software (Measurement Computing Corporation, Norton, MA).

After baking, muffins were loosely covered with a cotton cloth and cooled at room temperature (23°C) for 2 h.

Batter and muffins samples for chemical analysis were frozen, freeze-dried and subsequently ground into a fine powder using a mortar and pestle and stored at -18°C in airtight bottles (Rupasinghe and others 2008). Experiment was performed in triplicate.

Physical and chemical analysis

Muffin ingredients were analyzed for moisture content in triplicate according to the AOAC Official Method 931.04 using a vacuum oven (National Appliance Company, Portland, OR) (AOAC 2000b). Dry matter (DM) of ingredients was determined by a weight difference calculation.

Titratable acidity was determined for RRJ according to AOAC glass electrode method 942.15, sec. 37.1.37B (AOAC 2000a) and using a titration unit TitroLine easy Schott Instruments GmbH (Mainz, Deutschland, Germany). Results were expressed in terms of grams of anhydrous citric acid per 100 g of RRJ on a fresh weight basis.

pH of the batter and muffins was measured. An electronic pH-meter Digital Ionalizer®/501 (Orion Research Incorporated, Cambridge, MA) was used with a pH electrode Orion 910500 (Thermo Electron Corporation, Whaltman, MA). After calibration, using standard solutions at pH = 4 and pH = 7, pH of RRJ was measured. The batter and muffin pH were measured at a 1:10 (w/w) dilution in deionized water. Muffin samples for pH were collected from the center using a stainless steel cork borer (0.85 cm inner diameter) (Shearer and Davies 2005; Doulia and others 2006).

Muffins were evaluated for mass, height and volume. The volume of five muffins was measured by a modified rapeseed displacement method **(Figure 1)** (AACC 2000).

Preparation of the extract for phenolic and antioxidant activity assays

Three different solvent systems were used for extraction of the finely ground samples. One g of dry raspberry juice was weighed into a 50 mL polyethylene centrifuge tube and the sample was extracted with 20 mL of 100% methanol (M), methanol:HCl (36.5-38%) (99:1, v/v) (MH) or ethanol (95%) acidified with 1N HCl (85:15) (EH). Batter and muffin samples (4 g) were extracted using the M and MH solvents, and 1 g was used for EH solvent. Samples were mixed using an Omni mixer homogenizer (Omni International, Waterbury, CT) at speed control 3 for 1 min at room temperature (20°C). Extraction continued for 3 h at room temperature for M and MH solutions and 1 h for the EH solutions without further agitation. The supernatant was recovered by centrifugation at 5°C, 11872 x g for 15 min. The supernatant fluids were kept at -76°C in airtight bottles and filtered through a Whatman N°4 filter paper before analysis (Wada and Ou 2002; Li and others 2007). Samples were analyzed in triplicate.

Measurement of phenolic compounds and antioxidant activity

Determination of Total Phenolic Content (TPC)

Total phenolic content was determined using the Folin-Ciocalteau procedure described by Singleton and Rossi (1965) and modified by Chaovanalikit and Wrolstad (2004). A 0.5 mL sample of the aqueous extract or a series of gallic acid standards (0, 50, 100, 150, 200 ppm) were mixed with 0.5 mL of the Folin-Ciocalteau reagent 2N and 7.5 mL of deionized water. The mixture was held at room temperature (20°C) from 30 sec to 8 min before adding 1.5 mL of 20% sodium carbonate (w/v), and kept at the same temperature for 2 h before measuring the absorbance at 765 nm. Results were expressed on a dry weight basis as mg gallic acid equivalents (GAE)/g DM.

The % recovery of TPC from RRJ incorporated muffin was calculated based on:

% Recovery =

$$\frac{\text{mg/g DM of TPC detected in RM} - \text{mg/g DM of TPC detected in CM}}{\text{mg of TPC added from RRJ estimated for g of raspberry muffin on DM basis}} \times 100 \quad \text{(1)}$$

The estimated concentration in muffins on a dry matter basis was calculated as:

Estimated amount =

concentration of TPC of RRJ x 2.4% RRJ in raspberry muffin on DM basis …… (2)

Determination of Antioxidant activity (AA)

The antioxidant activity of the extracts was measured using a relative $DPPH^{\bullet}$ scavenging capacity (RDSC) method of Cheng and others (2006) using the free radical

2,2 diphenyl-1-picrylhydrazyl (DPPH). A microplate scanning spectrophotometer PowerWave X-I with a KC4 v.3.0 PowerReports^{TM} Software (Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to determine the concentration of DPPH. Two-hundred µL of methanol solvent was added to blank wells, 100 µL to control wells and 100 µL of sample extracts and Trolox solutions in methanol were put into the appropriate wells in 96- well microplates, clear, flat-bottom, with no lid using an eight-channel pipetter. The RRJ methanolic extract (30 µL) was diluted with 100% methanol (970 µL) before analysis to obtain a 3% solution. One-hundred µL of 0.2 mM DPPH was added to control, standard and sample wells and microplates were covered with microplate sealing tapes (Cheng and others 2006; Yu 2008). Plate was shaken at 1 level (low) for 5 sec before every reading. The decrease in absorbance was determined at 515 nm every min for 2 hours or until the absorbance became steady. DPPH (quenched) was calculated as follows **(3)**:

$$\%DPPH^{\bullet}\ quenched = [1- ((A_{sample} - A_{blank})/(A_{control} - A_{blank}))] \times 100 \quad \ldots\ldots\ldots \quad (3)$$

Where: **A** represents absorbance at 515 nm

For the determination of antioxidant activity, the area under the curve (AUC) of %DPPH$^{\bullet}$ versus antioxidant-DPPH reaction time for each sample was calculated. The AUC was used because this determination takes into account the kinetic and the thermodynamic measurements of the radical-antioxidant reactions (Huang and others 2005; Cheng and others 2006). An example of AUC is shown in **Figure 2** and is mathematically expressed as follows **(4)**:

$$A = \int_0^b f(x)\,dx \quad \text{..... (4)}$$

Where: **A**: Area; **x**: time

The above integral for the raspberry juice, products and Trolox standard (6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid) at concentrations of 0, 25, 50, 75 and 100 µM, was expressed numerically using trapezoidal rule for calculation, equation **(5)** (Yu 2008):

$$\mathbf{AUC = 0.5\,X_o + (X_1 + X_2 + X_3 + X_4 + X_5 + \ldots\ldots + X_{y-1}) + 0.5X_y \quad \text{..... (5)}}$$

Where:

X_o: %DPPH quenched at 0 time

X_1, X_2, etc: %DPPH quenched at each minute to y= 40 min

X_y: %DPPH quenched when the steady time was reached

The standard curve was generated by plotting AUC values for Trolox at different concentrations. Antioxidant activity was calculated using the standard curve prepared and expressed as µmol Trolox Equivalent (TE) per g sample dry matter using the following equation **(6)**:

$$\mu\text{moles TE/g} = (\mu\text{moles/L}) \times DF \times (L_{solvent}/g_{sample}) \quad \text{..... (6)}$$

Where:

DF: Dilution factor for sample extract

$L_{solvent}$: Volume of solvent used for extraction of the sample

g_{sample}: Amount of sample used for extraction

Statistical Analysis

Data were reported as mean ± standard deviation (SD). A Randomized Complete Block Split Plot design was analyzed using a mixed model (SAS Institute Inc. v. 9.1, Cary, NC, USA). An analysis of variance procedure was used to determine significant differences ($p<0.05$) among treatments. In addition, Tukey's Honestly Significant Difference (HSD) test was used for pairwise comparisons of treatments. Regression analysis was conducted using Microsoft Office Excel 2003 (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

Batter and Muffin properties

Temperature values recorded for oven and muffin samples are shown in **Figure 3**. A decrease in the volume of the raspberry muffin (131.5 ± 19.4) mL was observed relative to the control sample (169.8 ± 25.3) mL (**Figures 4 and 5).** This decrease of volume could be attributed to suppression of carbon dioxide (CO_2) formation due to the lower pH by interaction of RRJ and other ingredients (Shearer and Davies 2005) since double acting baking powder was used as a chemical leavening. As a consequence, CO_2 was released early in the baking process resulting in a lower product volume (**Figure 5**). Double acting means the leavening reaction occurs twice due to the presence of two acid

materials that are sodium aluminum sulfate and monocalcium phosphate. One of them acts first with the baking soda and, the action of the second acid takes place during the baking process (Navy Department 1961). Moreover, the addition of raspberry juice to baked products could be associated with a decrease of volume, due in part to dilution of gluten (Pomeranz and others 1977). On the other hand, a larger volume was observed for the control muffin (**Figure 4**) due to lack of excess of acid present besides the acidity provided by the acid components of the baking powder.

Moreover, the addition of raspberry juice, that meant presence of additional acid in the batter, caused the presence of holes (airpockets) in the internal part of the RM (**Figures 6 and 7**). After baking, water migrates from crumb to crust and some water evaporates (Giovanelli and others 1997). In RM formulation, ca. 24% was wheat flour, which when combined with water formed batter suitable for making bakery products such as muffins. The major storage protein of wheat is gluten, which traps gas during fermentation (Damodaran 2008). According to Wong (1989), flour ingredients (protein, starch and lipid) are integrated into the gluten network and as a result, a starch-protein-lipid complex matrix is formed. During muffin preparation, development of gluten is not desired because this development produces peaks and tunnels (Griswold 1962). In addition, microfibrils within the gliadin fraction, which is one of the protein components of gluten, dissociate into monomers when the pH decreases (Wong 1989) as could have been the case in our study. The CM observed in **Figure 6** was large and symmetrical without peaks or tunnels, and had a pebbled crust, even grain and was tender. On the other hand, RM (**Figure 7**) seemed to have been mixed improperly or for an excessive amount of time, which caused a decrease of the tenderness of the product. As was

mentioned by Pyler (1952), duration of mixing is more important than the speed of mixing since flour reacts markedly to variations in mixing times, and variations in mixing times will influence volume of the baked product. In addition, RM looked more dense (compact) that was confirmed by the low volume (131.5 mL) and height (3.4 cm); and the greater weight loss (9.1%) in comparison with the values obtained for the CM (volume, 169.8 mL; height, 4.9 cm; weight loss, 8.5%) (**Table 2**). CM rose evenly and well during the early part of the baking period because the batter contained many carbon dioxide cells in readily extensible gluten.

As was mentioned before, a probable release of CO_2 , because of the addition of RRJ (1.9 ± 0.17 g citric acid/100 g FW), occurred during mixing. Griswold (1962) added that when CO_2 is lost, batter is an initially lumpy mixture, later is smooth, and pours in strands from the spoon, assuring gluten development. Fewer tunnels will be formed if extra CO_2 is present owing to additional baking powder. Arora and Camire (1994) determined that the addition of potato peels increased density of muffins and reduced the number of airpockets. Peaks and tunnels observed in RM **(Figure 7**) were not completely understood since it is uncertain whether other chemical reactions, including additional release of CO_2, could have taken place during baking.

The CM mass decreased approximately 8.5% after baking, lower than the 9.1% weight loss determined for RM (**Table 2**). As reported by Shearer and Davies (2005), mass changes in this case were due to moisture evaporation after the baking process at 176.6°C for 25 min with an average internal temperature of 111°C. Pyler (1952) reported that 100°C was the internal temperature of muffins when the oven temperature was 204°C and at the same baking time of 25 min.

Determination of total phenolics in red raspberry juice, batter and muffin

Total phenolics of raspberry juice, batter and baked product are presented in **Table 3** as gallic acid equivalents (GAE). RB showed a higher amount of phenolics in comparison to the control batter (CB) regardless of the extraction method used for recovery of the phenolic compounds. The case was similar for the muffin products where higher values were determined for the RM in relation to the CM. Moreover, values found for RB were higher than the values reported for RM.

The results shown in **Table 3** indicated marked effects of solvent systems on the estimation of TPC. Although ethanol:HCl (EH) presented a high extraction efficiency similar to methanol:HCl (MH), the solvent was chosen based on the high TPC found in the product of interest, raspberry muffin (RM), which showed the highest amount of TPC with MH (99:1, v/v) solvent (0.44 ± 0.020 mg GAE/g DM). TPC of M, MH, and EH RRJ extracts were 13, 23.2 and 22.2 mg GAE/g DM, respectively. Li and others (2007) determined that EH solvent showed the highest efficiency when used with purple wheat bran and muffin samples. In our study, EH showed the highest values of TPC for CB and RB. Rupasinghe and others (2008) also detected TPC in CB that demonstrated that wheat flour contains phenolics, mainly phenolic acids where ferulic acid is the principal compound. MH was also a good extractant compared to M for all the samples evaluated except for CM.

Considering that 10% of RRJ was used in the batter and muffin samples, a calculation was done to determine the amount of RRJ solids found in the dry samples analyzed. DM content of the muffin ingredients was used and was found that 24 mg of RRJ solids were contained in 1 g of dry solids sample. Normalization of the TPC of RRJ

was done by multiplying by 0.024. TPC of all the samples were compared after normalization as can be observed in **Figure 8.**

As mentioned before, the impact of baking was evaluated in this study. Regarding the RB and RM, which contained 10% of RRJ, the thermal treatment affected the % recovery of TPC. TPC of RRJ was normalized by multiplying the value by 0.024 to obtain the recovery of RRJ from the batter and, as a consequence from the muffin (**Table 4**). Based on the recovery of original phenolic compounds of RRJ from the RM, the baking process affected their content. One of the very interesting findings of this study was that an increase of TPC was detected in RB using the three different extraction solvents. Presence of other ingredients with phenolic content, such as wheat in flour, added this constituent to the actual content of phenolics provided by RRJ. Looking at **Table 3,** TPC of RRJ obtained using the MH solvent shows an increase of 25% that was produced at the time of preparing the RB. Thus, TPC of RRJ (normalized value) increased 25% from 0.56 to 0.70 mg GAE/g DM in batter. Then, a reduction of 37% was found when comparing RB (0.70 mg GAE/g DM) and RM (0.44 mg GAE/g DM). In addition, RRJ (normalized value) (0.56 mg GAE/g DM) showed 21.4% more TPC than RM (0.44 mg GAE/g DM). It seems that baking effect played an important role on the reduction of TPC. Comparing CB and CM, a decrease of 44% of the TPC was also observed from 0.45 mg GAE/g DM) found in CB to 0.25 mg GAE/g DM in CM.

Figure 9 shows that there were significant differences ($p<0.05$) in phenolic recovery for different extraction solvents in RB. Considering that RB and RM were the samples where RRJ was added, the recovery of total phenolics provided by the red raspberry juice before and after baking process was determined. The highest TPC

recovery in RB was determined with M solvent (87%) where only phenolics provided by RRJ were considered. In RM, the effect of M (12%) was lower in comparison to MH (34%) and EH (46%) ($p<0.05$) (**Table 4**). **Figure 10** shows that significant differences were found on the TPC of RB and RM when MH solution was used ($p<0.05$). In addition, significant differences were found for the following pair comparisons: RRJ vs. RB, RRJ vs. RM, CB vs. RB, CB vs. CM, and CM vs. RM at the 0.05 simultaneous level of significance.

As shown in **Figure 3**, the average internal temperature reached in the internal area of the muffin at 25 min of baking was 111°C. According to Kalt and others (1999), raspberries among all the small fruits evaluated, were most affected by storage that included different time and temperature. Raspberries contain glycosides of the flavonols quercetin and kaempferol (Ho and others 1992). As was reported by Buchner and others (2006) flavonoids such as quercetin underwent degradation on heating at 100°C. In addition, Rupasinghe and others (2008) determined that baking process affected all phenolic compounds of muffins made with apples skin.

The concentration of total phenolics for dried RRJ extracted with MH in this study (23.2 mg GAE/g DM) was in good agreement with the value reported by Pantelidis and others (2007) for the same cv. (21.2 mg GAE/g) when an aqueous methanolic solution was used. Heinonen and others (1998) cited that phenolics in raspberry extracts (cv. Tulameen) were 2.65 and 3.03 mg GAE/g FW when the extraction solvents were 70% acetone and 60% methanol, respectively. Some reasons for differences among values are season of harvest, maturity level, extraction solvents and method of analysis (Proteggenete and others 2003).

Thermal treatment effect on phenolic content in berries was reported by Amakura and others (2000) who found that phenolic content was not changed significantly by jam processing. Kim and Padilla-Zakour (2004) supported this observation when they did not find significant differences in total phenolics between fresh fruits (cherries, plums and raspberries) and jams processed at the temperature range of 104 to 105°C. In contrast, Piga and others (2003) determined that hot air dehydration of plums (max. T = 85°C) to obtain prunes reduced the TPC of this functional food.

Antioxidant activity of red raspberry juice, batter and muffin

Antioxidants, including radical scavengers, may protect important molecules from radical attacks and consequently reduce the risk of aging-associated health problems, such as cancer and heart disease. Natural antioxidants are in high demand for preparing functional foods and supplements because of their possible health benefits. Samples were evaluated by their capacities to directly react with and quench the stable radical $DPPH^{\bullet}$. The color of methanolic DPPH solutions changed from purple to yellow, due to the formation of diphenylpicryl-hydrazyl when a reduction was produced by either a hydrogen radical or electron –donation process (Kaur and Arora 2008).

Methanol (100%) extraction solvent was determined to be the preferred system for the DPPH assay. As mentioned by Yu (2008), the same solvent used for DPPH dilution is recommended for use in samples in order to get the absorbance of the sample-DPPH reactions in the linear range of Trolox standard curve. Some interferences due to the pH of RRJ (3.5 ± 0.087) and/or other solvent systems that included HCl were found in preliminary experiments. Methanol extracts (**Figure 11**) were able to reduce the DPPH

radical to the yellow-colored diphenylpicrylhydrazyne and absorbance at λ 515 nm decreased, but the acidic extracts (**Figures 12 and 13**) were not able to reach a final yellow color in the extracts containing raspberry juice (RRJ, RB and RM) and instead a light color was observed. It is probable that the red/pink color observed in the MH and EH extracts interfered with the DPPH absorbance. Arnao (2000) mentioned that in the visible region, a greater interference on the part of the sample can be expected at low wavelengths, so the more color of the sample, the smaller the absorbance decrease and the less antioxidant activity is measured. As was mentioned before, antioxidant activity of the samples was measured at λ 515 nm. DPPH is purple and the maximum absorbance is at λ 515 nm; meanwhile anthocyanins, contained in the sample analyzed, are red in color and absorb at λ_{max} 520 nm. Therefore, underestimation of antioxidant activity values could have occurred because of sample interferences considering that anthocyanins absorbance overlaps at 515 nm (Arnao 2000; Muñoz-Espada and others 2004). To this respect, RRJ, RB and RM, showed the highest %DPPH quenched when 100% methanol extracts were combined with DPPH solution (**Figure 11**). On the other hand, small antioxidant activity was found for CB and CM.

Ozcelik and others (2003) reported the absorbance of DPPH decreased under light exposure, oxygen, and varies with pH and solvent. DPPH was found to be stable in pH 7 and pH 9 buffer solutions in an acetone system. On the contrary, DPPH decreased by 70% in the dark and 80% with light in a pH 10 potassium carbonate-potassium borate-potassium hydroxide buffer in acetone. In methanol solutions, a significant change of the absorbance of DPPH was not observed at pH 10. DPPH decreased by 50% in the dark and 55% under light in pH 4 potassium biphthalate in methanol. Ozcelik and others

(2003) determined that methanol buffer systems offered better stability of DPPH and that the stability will depend on the type of buffer. Huang and others (2005) pointed out that acids or bases present in the solvent can influence the ionization equilibrium of phenols and cause a reduction or enhancement of the measurements.

The measurement of the consumption of $DPPH^{\bullet}$ radical allows one to determine the intrinsic ability of a substance to donate hydrogen atoms or electrons to this reactive species in a homogeneous system. The method is based on the reduction of alcoholic $DPPH^{\bullet}$ solution in the presence of a hydrogen-donating antioxidant due to the formation of non-radical form DPPH-H (Duan and others 2006). A blue-violet color changes gradually to green and yellow and a decrease in absorbance at 515 nm is monitored during the reaction. The kinetic behaviour of the radical disappearance in the presence of Trolox expressed as absorbance at 515 nm vs. time is presented in **Figure 14**.

The DPPH kinetic behavior of RRJ, batter and muffin samples extracted in methanol solvent is shown in **Figure 11.** The %DPPH quenched followed this order: RM>RRJ>RB>CM>CB. As is shown in **Figure 11**, the "steady state" for the antioxidant –$DPPH^{\bullet}$ reactions was reached at 40 min. This time was used to compare the samples of interest using the area under the curve (AUC) of %DPPH quenched vs. antioxidant-$DPPH^{\bullet}$ reaction time in a cycle of 40 min. AUC utilizes both inhibition time and degree that reflects the different reaction kinetics. In consequence, results obtained using AUC provide better data than methods using a fixed time or inhibition degree (Huang and others 2002; Prior and others 2005).

RRJ appeared to have lower radical scavenging activity in comparison to RM due to a 3% dilution that was used so not to exceed the concentration needed to quench more than 70% of DPPH and avoid experimental artifacts during the study (Košmerl 2007). No dilution was needed in the other samples since the antioxidants contained in RRJ were already diluted in the mix of ingredients.

Considering that 10% of RRJ was used in the batter and muffin samples, a calculation was done to determine the amount of RRJ solids found in the dry samples analyzed. DM content of the muffin ingredients was used to obtain a normalized value and was found that 24 mg of RRJ solids were contained in 1 g of dry solids sample.

After adjusting the AA of RRJ using the corresponding dilution factor, the AA was 2.49 μmol TE/g DM (before normalization) that was higher than the AA obtained for RM (0.023 μmol TE/g DM) (**Table 5**). After normalization of the AA of RRJ by multiplying by 2.4%, AA of RRJ (0.060 μmol TE/g DM) was still higher than the AA of RM. Rupasinghe and others (2008) determined the AA of muffin made with 32% apple skin (AS) using FRAP and ORAC assays. The values found were 4.9 and 5.8-fold, respectively, greater than that of muffins without AS. In this raspberry study, the AA was 1.5-fold greater in the RM than CM when 10% RRJ was used. Jakobek and others (2007) found in red raspberry 19 μmol TE/g FW using the conventional DPPH method, but different cultivar, extraction conditions and solvent system (methanol/HCl 2% (95:5, v/v) were used.

AA in RM was 1.2-fold greater than RB but statistically not significant differences were found in their capacities to quench DPPH radicals ($p<0.05$). This suggested that the thermal treatment did not greatly affect the radical scavenging

properties of the products. Also, no significant differences were observed in CB vs. CM. On the other hand, significant differences were found between the AA of RRJ and the other samples evaluated ($p<0.05$) (**Table 5**). Jakobek and others (2007) used the conventional colorimetric analysis and found in red raspberries 18.61 μmol TE/g fruit. When evaluating the antioxidant activity of red raspberries, vitamin C content has to be considered as mentioned by Proteggente and others (2003). Vitamin C (ascorbic acid) is a very effective scavenger of free radicals both *in vitro* and *in vivo*. Proteggenete and others (2002) reported the content of vitamin C in raspberries was 0.26 mg/g FW. A reduction of the antioxidant activity could have been due to the losses of vitamin C produced during the freeze-drying process in the preparation of the material (Proteggente and others 2003).

Beekwilder and others (2005) cited ellagitannins as the biggest contributors (more than 50%) to antioxidant activity in raspberry. Ellagitannins are present in raspberries in the range of 0.01-0.02 mg/g DM. Meanwhile, anthocyanins contribute about 25%. According to Clifford and Scalbert (2000), ellagitannins are largely contained in the seeds, which were not used in this experiment. Absence of seeds is another reason that could have influenced the low antioxidant activity of the samples evaluated in our study.

Influence of the solvent in which the reaction takes place is an important factor that affects mechanism of reaction (Pérez-Jiménez and others 2008). Therefore, to compare results obtained from this study with others, attention had to be paid to the solvents and methods used.

The correlation plot of TPC and AA shows a poor positive correlation exists between these measured parameters (r=0.32) (**Figure 15**). RRJ (normalized value) had TPC of 0.56 mg GAE/g DM with an AA of 0.060 μmol TE/g DM; meanwhile, RB had TPC of 0.70 mg GAE/g DM and AA of 0.020 μmol TE/g DM. On the contrary, Rupasinghe and others (2008) showed a strong correlation between TPC and AA determined using FRAP (r=0.94) and ORAC (r=0.95) assays of muffins incorporated with different levels of apple skin. Possible interferences of the chemistry involved in the DDPH method could have been influenced the poor correlation found in this experiment. Liu and others (2002), who studied different cultivars of raspberry (Heritage, Kiwigold, Goldie and Anne), also found that a higher phenolic concentration resulted in a greater antioxidant activity determined using the total oxyradical scavenging capacity (TOSC) assay.

CONCLUSIONS

Consumption of baked products constitutes an important part of a daily breakfast considering that people are continually grabbing meals on the go. Among baked products, muffins ranks third in bakery breakfast products and attract a broad range of consumers of all ages. Incorporation of fruits to regular muffins to add an extra value is not as easy as seems to be, if the objective is to provide health benefits to the consumers. The inclusion of red raspberries to muffins to increase antioxidant content such as phenolic compounds has to be further studied to avoid losses of those compounds during thermal treatment and mixing with the other ingredients.

RRJ added to muffins at the level of 10% provided consumers a total phenolic content of 0.44 mg GAE/g DM and a total antioxidant activity of 0.023 μmol TE/g DM. Those values were lower than the values provided regularly by the fresh fruit. Therefore, red raspberries must be protected from the aggressive thermal treatment applied and pH modifications of the medium to preserve the inherent quality and benefits of these compounds, and of the final product by avoiding leakage of the raspberry pigments.

Addition of RRJ to muffin increased the content of TPC and AA present in a regular muffin (CM). AA provided by RRJ was decreased during the muffin preparation, but no significant differences were found for TPC.

FUTURE RESEARCH

Considering the antioxidant activity and phenolic content found in red raspberry juice, future research could be focused on the evaluation of the effect of these compounds on delaying rancidity of muffins. This can be assessed because muffin formulation has a small amount of oil in its composition that can decrease shelf life of the final product. In addition, new techniques such as microencapsulation of the fruit or fruit components could be implemented before the inclusion of this fruit into a baked product. In this way, a juice loss and therefore, color dispersion can be avoided in the final product, and probably, some antioxidant components would be protected from thermal processing and detrimental impact on baking properties from pH changes to the batter from addition of the raspberry juice.

Regarding the underestimation of results for the antioxidant activity test due to the interference of other compounds present, such as anthocyanins, modifications of the method have to be implemented. Measurement of the absorbance of anthocyanins at 515 nm could be measured and values for interfering subtracted from the DPPH absorbance to adjust for the effect of the interfering compounds.

Evaluation of loss of individual phenolic compounds is an interesting topic to be explored to quantify and determine which antioxidant structures are more stable to thermal treatment or to mixing. In addition, inclusion of a sensory evaluation could contribute to determining the acceptance of the products developed through the collection of consumer opinions that will lead to improvement of the product.

Future research focused on the characterization and quantification of individual phenolic compounds should therefore be conducted using available analytical standards.

The nature and content of specific ellagitannins in processed products should be clarified. Those compounds belong to the group of hydrolyzable tannins and some authors confirmed that they may be the biggest contributor to antioxidant activity in raspberry.

Table 1- Muffin formulation

Ingredients	Percentage by weight (%)	
	Control Muffin	**Raspberry Muffin**
Flour	34.05	24.05
Sugar	15.42	15.42
Salt	0.13	0.13
Baking Powder (double acting)	1.29	1.29
Water	32.13	32.13
Vegetable oil	13.88	13.88
Skim milk powder	2.57	2.57
Whole egg powder	0.53	0.53
Red raspberry juice	-	10.00
Total	**100**	**100**

Table 2- Properties of control batter and muffin and with the addition of 10% of red raspberry juice

Samples/Properties	Control	10% RRJ
Batter		
pH	7.5	7.1
Muffin		
pH	7.6	6.3
Weight loss (g)*	6.9 ± 0.45 (8.5)**	7.3 ± 0.41 (9.1)**
Volume (mL)*	169.8 ± 25.28	131.5 ± 19.44
Height (cm)*	4.9 ± 0.43	3.4 ± 0.28

* Results reported are mean values of three replications ± standard deviation.

** Numbers in parenthesis are the percentage of weight loss of samples after baking.

Table 3-Total phenolic* content of red raspberry juice and juice-containing batter and muffins using different solvent extraction systems (N=3)

Product	Solvent System**		
	M	**MH**	**EH**
Red raspberry juice***	0.31 ± 0.024^{a}	0.56 ± 0.029^{b}	0.53 ± 0.025^{b}
Control Batter	0.17 ± 0.0058^{a}	0.45 ± 0.015^{b}	0.53 ± 0.068^{c}
Raspberry Batter	0.44 ± 0.020^{a}	0.70 ± 0.015^{b}	0.87 ± 0.090^{c}
Control Muffin	0.27 ± 0.023^{a}	0.25 ± 0.0058^{a}	0.086 ± 0.057^{b}
Raspberry Muffin	0.30 ± 0.015^{a}	0.44 ± 0.020^{b}	0.34 ± 0.079^{a}

* Results reported are mean values of three determinations ± standard deviation expressed as mg GAE/g DM. Sample means containing different letters in the same row are significantly different from one another ($p<0.05$).

** M, extracts in 100% methanol, MH, extracts in methanol 100%:HCl, EH, extracts in ethanol 95%:HCl 1 N.

*** A normalization was done by multiplying TPC values of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

Table 4- The mean percent recovery of phenolic compounds of red raspberry juice before and after baking in a model system muffin (N=3)

		Total Phenolic Content* **(mg GAE/g DM)**		**Recovery of phenolic compounds (%)****
		Estimated amount**	Detected amount***	
Raspberry batter	100% MeOH	0.31 ± 0.024	0.27 ± 0.015	87
	MeOH:HCl	0.56 ± 0.029	0.26 ± 0.023	47
	EtOH:HCl	0.53 ± 0.025	0.34 ± 0.057	63
Raspberry muffin	100% MeOH	0.31 ± 0.024	0.04 ± 0.015	12
	MeOH:HCl	0.56 ± 0.029	0.19 ± 0.015	34
	EtOH:HCl	0.53 ± 0.025	0.25 ± 0.087	46

* Results reported are mean values of three determinations ± standard deviation.

** Estimated amount is based on the amount of phenolics in RRJ added to the muffin mixture. A normalization was done by multiplying TPC values of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

*** Detected amount for Raspberry Batter = TPC RB – TPC CB

Detected amount for Raspberry Muffin = TPC RM – TPC CM

**** %Recovery of phenolics measured is based on all the phenolics originated from RRJ

Table 5- Antioxidant activity of red raspberry juice and juice-containing batter and muffins (N=3)

Product	**AA (µmol TE/g DM)***
Red raspberry juice**	0.060 ± 0.004^{a}
Control Batter	0.012 ± 0.001^{b}
Raspberry Batter	0.020 ± 0.001^{ce}
Control Muffin	0.015 ± 0.001^{bc}
Raspberry Muffin	0.023 ± 0.001^{de}

* Results reported are mean values of three determinations ± standard deviation expressed as µmol TE/g DM. Sample means containing different letters in the same column are significantly different from one another ($p<0.05$).

** A normalization was done by multiplying AA value of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

Figure 1- Apparatus for measuring volume by rapeseed displacement

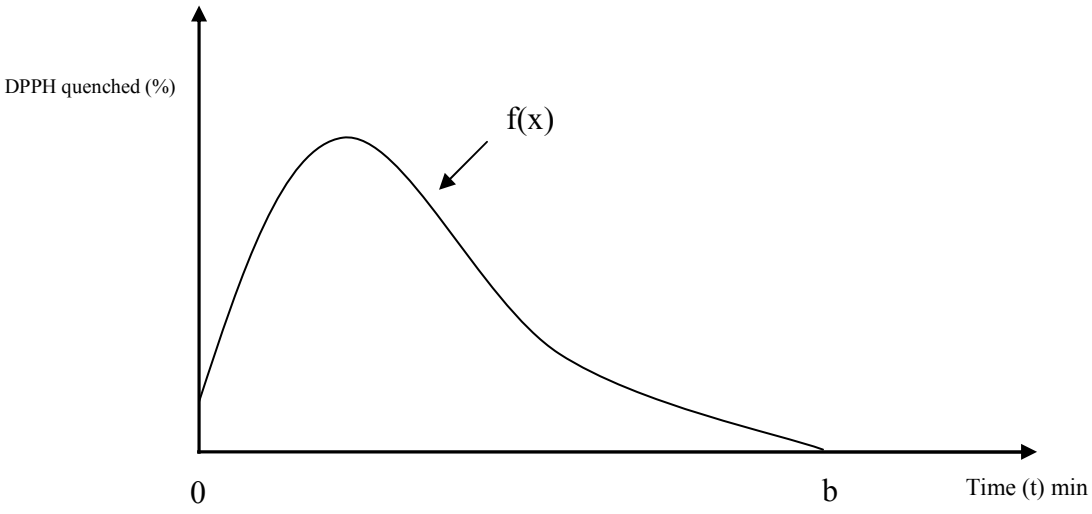

Figure 2- Area under the curve (AUC)

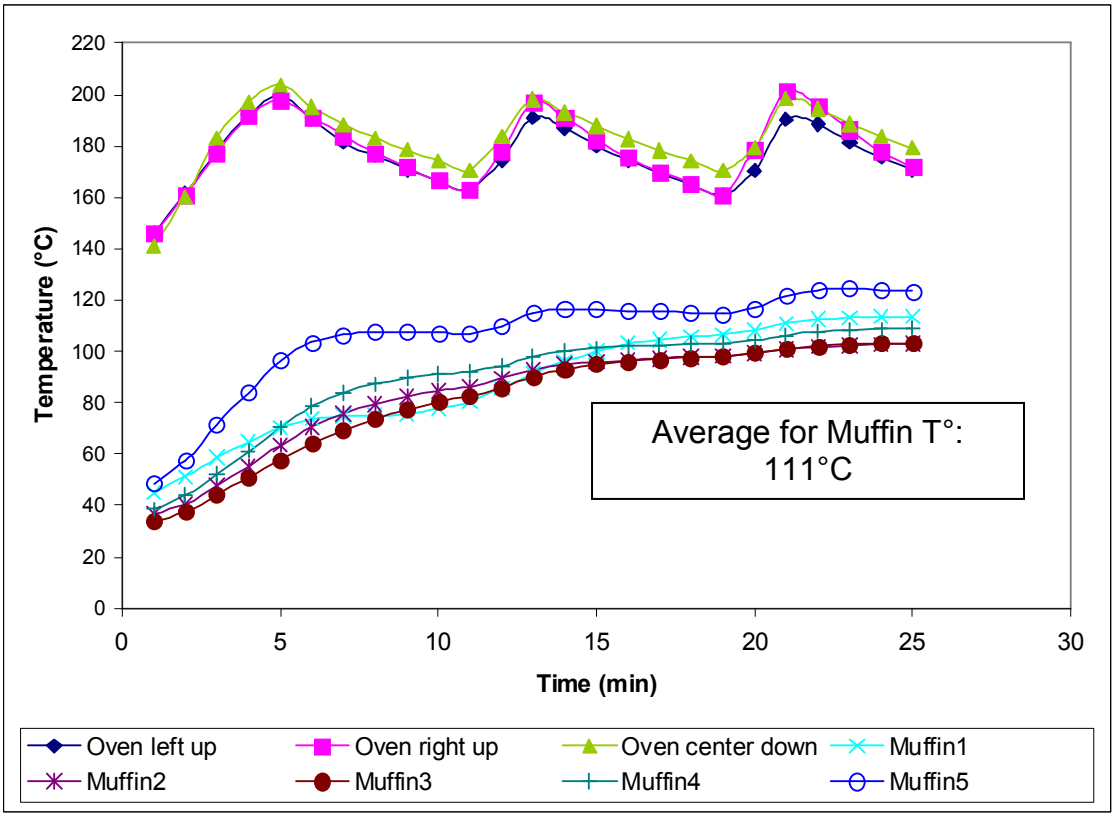

Figure 3- Muffins and oven temperature control during baking of muffins

Figure 4- Control Muffin (CM)

Figure 5- Raspberry muffin (RM)

Figure 6- Transversal cut of CM

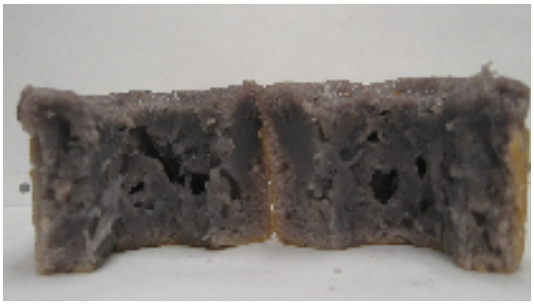

Figure 7- Transversal cut of RM

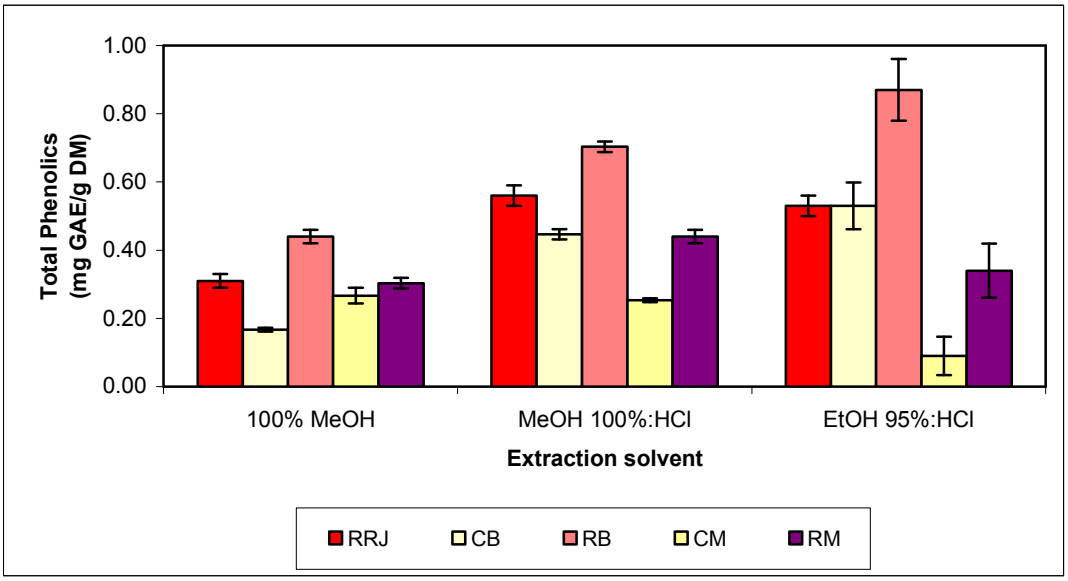

Figure 8- Total phenolic content of red raspberry juice normalized to reflect amount used in batter/muffin formulation using different solvent systems

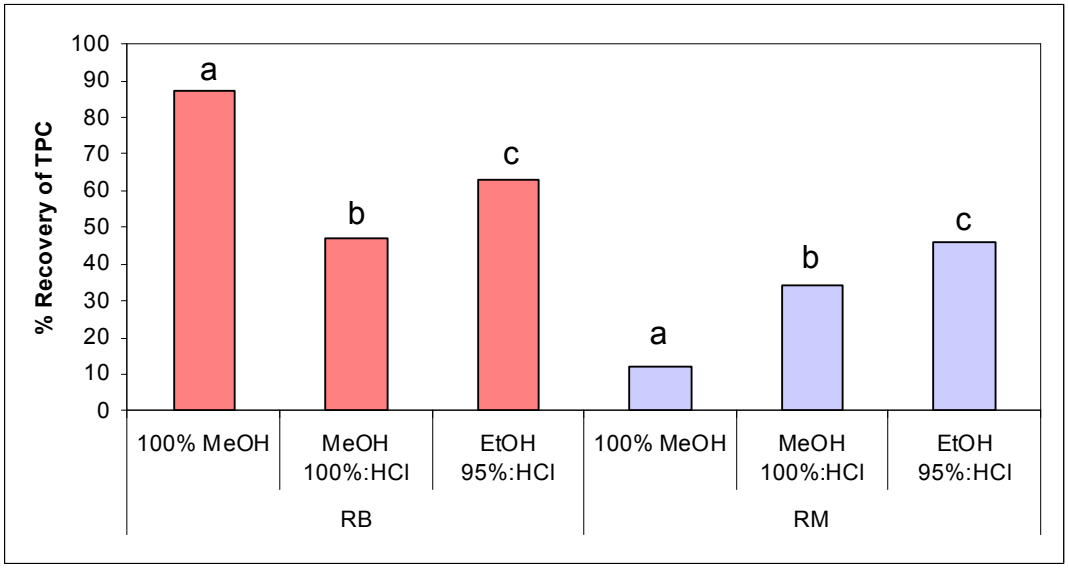

Figure 9- The relationship between extraction solvent and the recovery of the total phenolic content from red raspberry juice extract in raspberry batter and muffin. Same letters in a specific product means that values are not significantly different ($p < 0.05$).

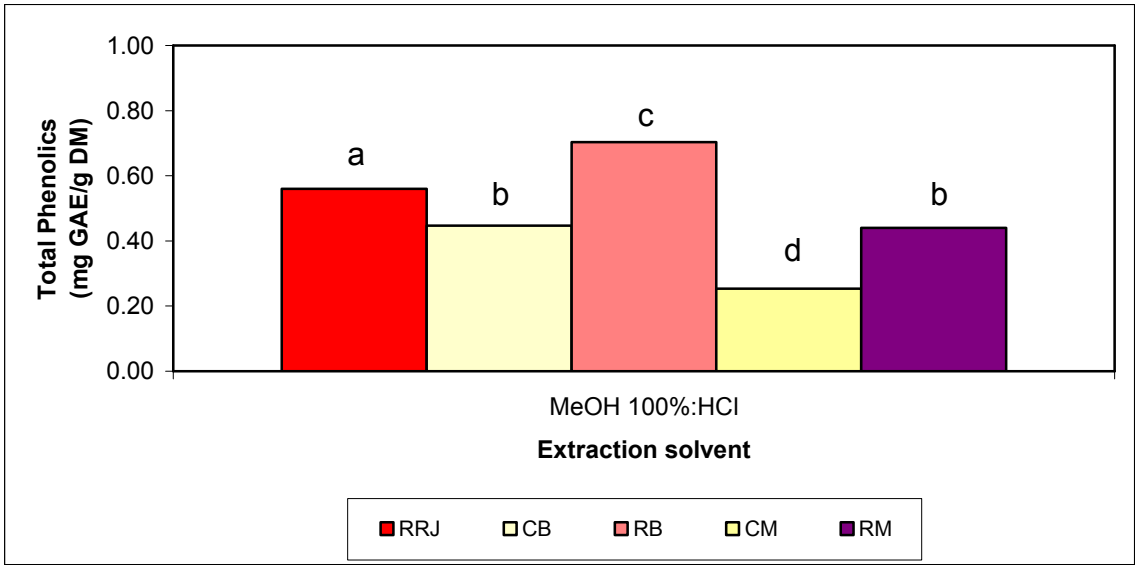

Figure 10 – Total phenolic content of red raspberry juice normalized to reflect amount used in batter/muffin formulation using methanol:HCl solvent system. Same letters mean that values are not significantly different ($p < 0.05$).

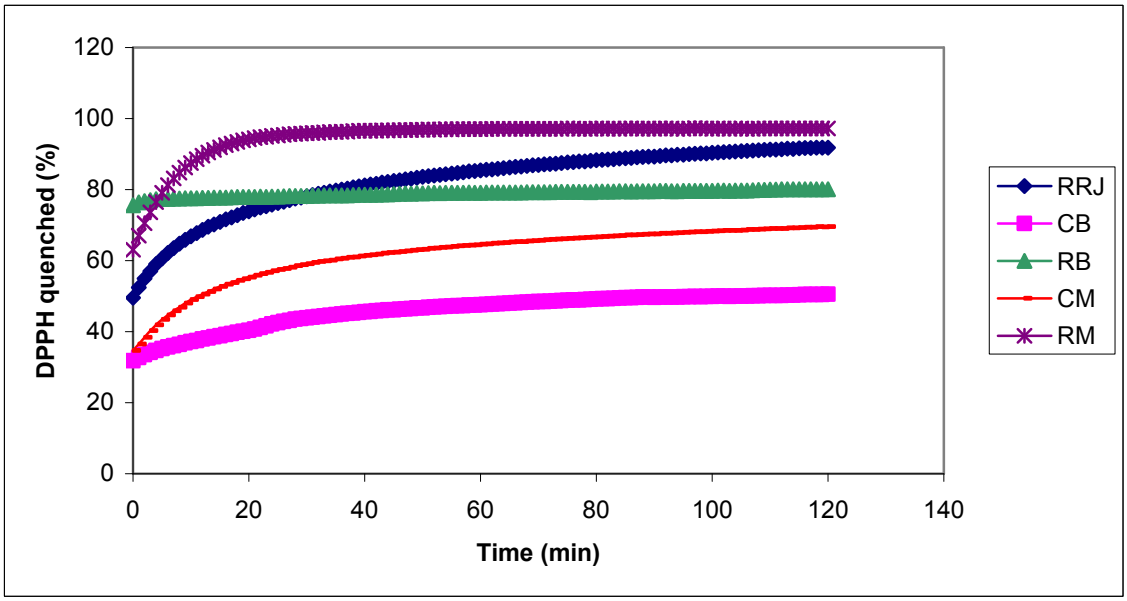

Figure 11 – Quenching of DPPH by methanol extracts of raspberry juice and juice-containing batter and muffins

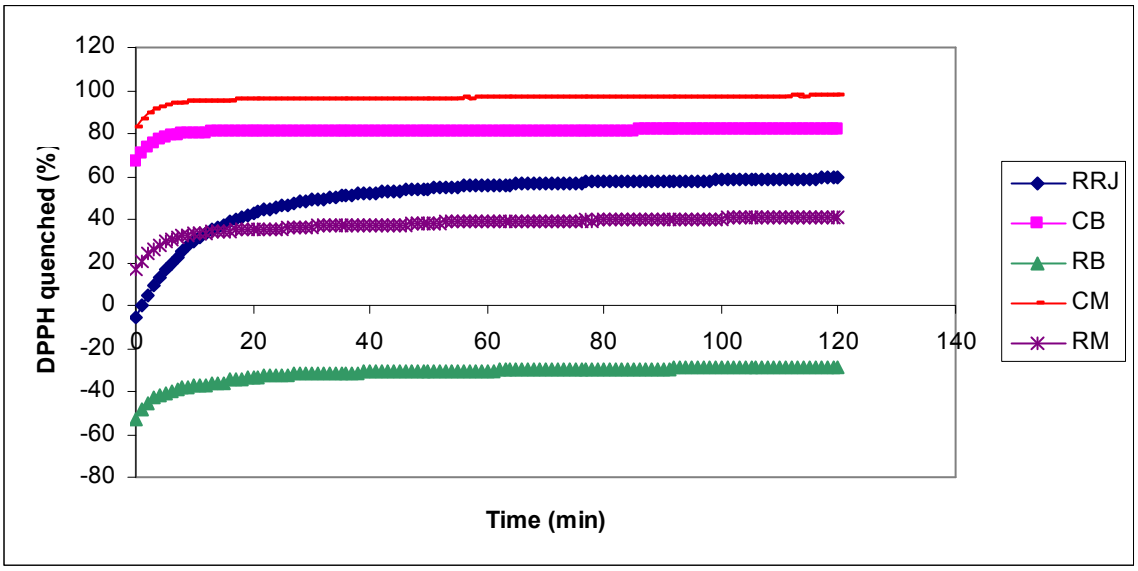

Figure 12 – Quenching of DPPH by methanol:HCl extracts of raspberry juice and juice-containing batter and muffins

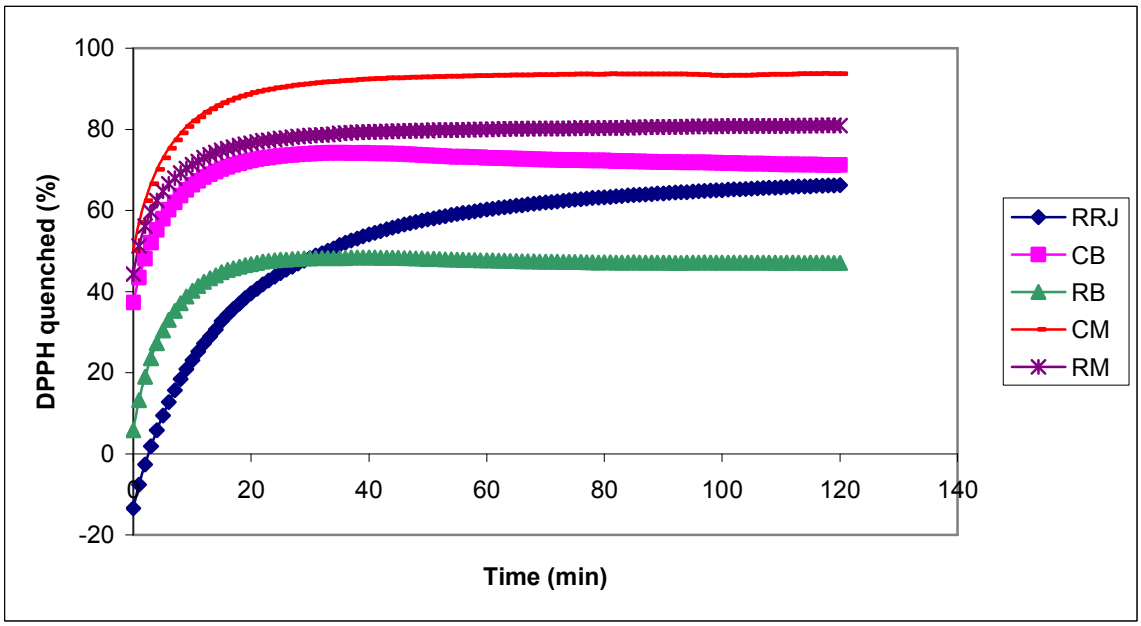

Figure 13 – Quenching of DPPH by ethanol:HCl extracts of raspberry juice and juice-containing batter and muffins

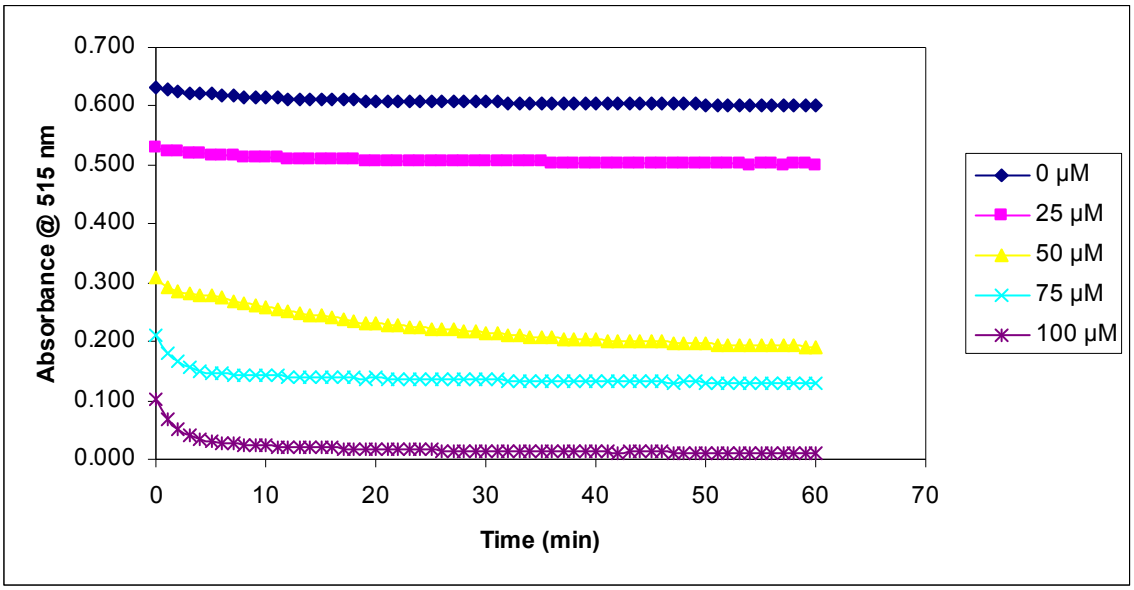

Figure 14- DPPH behavior in the presence of Trolox antioxidant standard solution

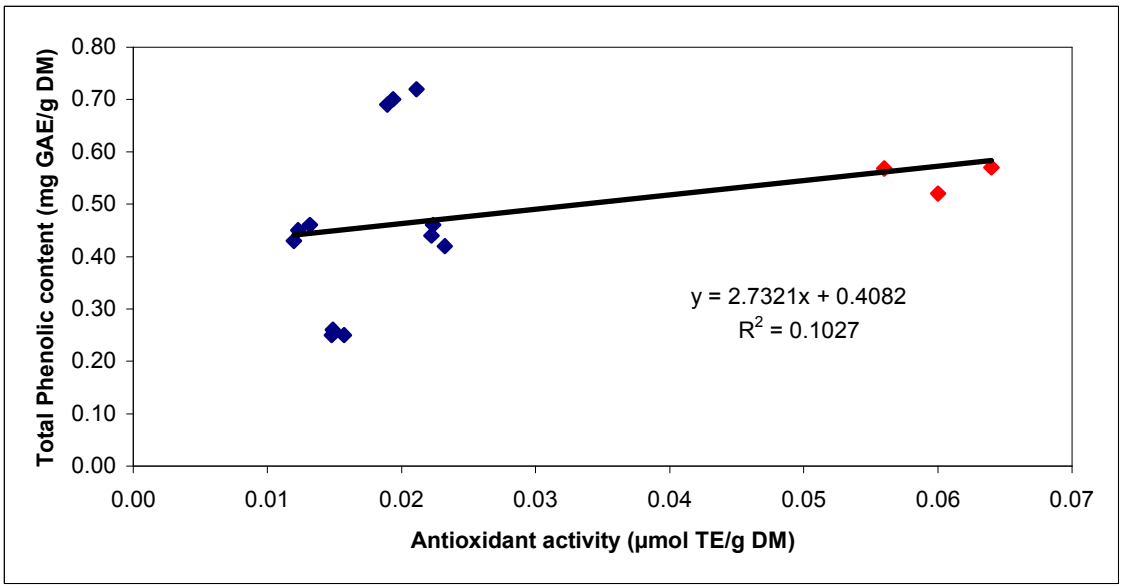

Figure 15- Correlation plot of TPC versus AA of red raspberry juice and samples

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CHAPTER THREE

ANTHOCYANIN COMPOUNDS IN RED RASPBERRY (cv. MEEKER) CONTAINING MUFFINS

ABSTRACT

Red raspberry juice (RRJ) cv. Meeker containing muffins were analyzed for total anthocyanin content (TACY) using a pH differential method. Identification of certain anthocyanins present in RRJ and in the muffins was done using a reversed-phase high performance liquid chromatography (HPLC) method coupled with a diode array detector (DAD). Cyanidin-3-sophoroside was identified as the major anthocyanin, followed by cyanidin-3-glucoside and cyanidin-3-glucosylrutinoside in the red raspberry juice, batter and muffins. The total anthocyanin content of raspberry containing products was measured using a spectrophotometric method and expressed as mg cyanidin-3-glucoside/g DM. The recovery of anthocyanins using three different solvent extraction systems was evaluated and a significant difference was found among the extraction capacity of methanol, methanol:HCl and ethanol:HCl 1N. Methanol:HCl RRJ extracts contained the highest amount of ACY in comparison to the other treatments and that solvent was selected for further experiments. RRJ had 3.3 cyanidin-3-glucoside mg/g DM, followed by RB (0.05 mg/g DM). No anthocyanins were detected in RM by the spectrophotometric method.

Keywords: Anthocyanins, HPLC, red raspberries, muffin.

INTRODUCTION

Raspberries, as well as products made from them, owe their attractive color to the presence of anthocyanin (ACY) pigments (Fuleki and Francis 1968). Anthocyanins are the largest group of water-soluble pigments in the plant kingdom and belong to the class of phenolics called flavonoids. Flavonoids, secondary plant phenolics, are synthesized in plant tissues, provide flavor and color in flowers, fruits and leaves (Bermudez-Soto and Tomas-Barberan 2004). Anthocyanins, the glycosides and acylglycosides of anthocyanidins, are responsible for the bright colors such as orange, red and blue of many flowers, fruits and vegetables (Markakis 1982; Mazza and others 2004; Wu and Prior 2005). They are distinguished from other flavonoids as a separate class by virtue of their ability to form flavylium (2-phenylbenzopyrylium) cations (1-3) (Mazza and others 2004).

Considering that each anthocyanidin may be glycosylated and acylated by different sugars and acids, at different positions, the number of anthocyanins is greater than the number of anthocyanidins (Mazza and Miniati 1993). There are 6 common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin), whose structures can vary by glycosidic substitution at the 3 and 5 positions. Additional variations occur by acylation of the sugar groups with organic acids. The basic structure of an anthocyanin pigment is shown in **Figure 1** (Mazza and others 2004; Lee and others 2005). The most commonly anthocyanidin in nature is cyanidin (Mazza and others 2004).

The total anthocyanin content of raspberries and other fruits such as cranberries varies depending on factors such as species, variety, growth conditions, physiological

state of the plant and fruit, size, position of the fruit on the plant, application of chemicals, etc. (Fuleki and Francis 1968).

According to Wu and Prior (2005), anthocyanins are believed to play an important role in plant function because they are a major group of secondary metabolites. Anthocyanins are considered important in the food industry and in human nutrition. There is an increasing interest in anthocyanins due to their probable health benefits in preventing chronic and degenerative diseases including heart disease and cancer (Mazza and Miniatti 1993; Wu and Prior 2005).

Distribution and actual chemical structures of anthocyanins in foods, such as muffins, is critical to know in order to promote their consumption. However, major anthocyanins may not necessarily be the most active compounds biologically (Wu and Prior 2005).

The extraction of ACY is the first step in the determination of total as well as individual ACYs in any type of plant tissue. The extraction amount should be such that a maximum amount of ACY will be recovered with a minimum of adjuncts and the loss of ACY due to enzymatic and non-enzymatic changes will be kept at the minimum. This is usually accomplished by repeatedly extracting the macerated plant material with cold 1% hydrochloric acid in methanol (Fuleki and Francis 1968).

For the determination of total anthocyanins (TACY), a pH differential method was used. This method is based on the structural change of the anthocyanin chromophore between pH 1 and pH 4.5. The assumption is that monomeric, or “pure”, anthocyanins have little or no absorbance in pH 4.5 buffer and undergo a reversible structural transformation as a function of pH (colored oxonium or flavylium form at pH 1, and

colorless hemiketal form at pH 4.5). Meanwhile, polymeric or degraded anthocyanins are assumed to absorb at pH 4.5. Although nearly all monomeric anthocyanins are in the hemiketal form at pH 4.5, small portion are in the form or the flavylium form, which will make a small contribution to the absorbance. The difference in absorbance of the pigments at 520 nm is proportional to the monomer concentration (Wrolstad 1976; Lee and others 2005).

The objectives of this study were to determine and identify the anthocyanins present in red raspberry juice cv. Meeker and baked products (muffins) made with red raspberry juice using a spectrophotometric measurement and HPLC with diode array detector. The intent of this study was to provide information about how several factors such as temperature and pH influence the total anthocyanin content and their presence in raspberry muffins suggested as a healthy alternative for consumption during breakfast.

MATERIALS AND METHODS

Materials

Ethanol was purchased from Washington State University Central Stores. Hydrochloric acid, formic acid, ethyl ether, ammonium sulfate, HPLC grade ethyl acetate, phosphoric acid, HPLC grade methanol, potassium chloride and sodium acetate trihydrate were obtained from J.T. Baker Inc. (Phillipsburg, NJ). Metaphosphoric acid was purchased from Sigma-Aldrich (St. Louis, MO). Ideain (cyanidin-3-galactoside) was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ).

Sampling procedure

One variety of red raspberry (*Rubus idaeus*, cv. Meeker) grown in Washington was selected for the elaboration of juice. Cranberries, grown in Washington, and blackberries from a commercial store were also analyzed.

Juice preparation

This procedure was followed as specified in Chapter II.

pH

This procedure was followed as specified in Chapter II.

Soluble solids

Soluble solids were measured from red raspberry juice in order to get a single strength-solution around 10°Brix for measurement of anthocyanins. 1:10 dilutions (sample:distilled water) were used for all samples. A Pocket Refractometer PAL-1 (Atago US, Inc.) was used at 20°C and the results were reported as degrees Brix.

Anthocyanins assay

Preparation of the extracts

Three different solvent systems were used for extraction of the finely ground samples. One g of freeze dried raspberry juice was weighed into a 50 mL polyethylene centrifuge tube and the sample was extracted with 20 mL of methanol (100%) (M),

methanol:HCl (36.5-38%) (99:1, v/v) (MH) or ethanol (95%) acidified with 1N HCl (EH). Freeze dried batter and muffin samples (4 g) were prepared and extracted using the same solvents as mentioned in previous Chapter II. Samples were homogenized using an Omni mixer homogenizer (Omni International Waterbury, CT) at speed control 3 for 1 min at room temperature (20°C). Maceration was allowed for 3 h at room temperature for M and MH solutions and 1 h for the EH solutions for a complete extraction. Centrifugation was done at 5°C at 11872 x g for 15 min. The supernatant fluids were kept at -76°C in airtight bottles and filtered through a Whatman N°4 filter paper before analysis (Wada and Ou 2002; Li and others 2007). Samples were analyzed in triplicate.

Measurement of total anthocyanins

An aliquot of 0.8 mL of RRJ (11°Brix) extract was diluted with potassium chloride buffer pH 1 to a volume of 10 mL, to obtain an absorbance of the sample at the wavelength $\lambda_{vis\text{-}max}$ (520 nm) within the linear range of the spectrophotometer (< 1.2). No initial dilution with distilled water was necessary (Giusti and Wrolstad 2005). The final volume of the sample was divided by the sample volume to obtain the dilution factor (DF). The same procedure was followed for CB, RB, CM and RM, but considering a 20% concentration and no extra dilution was necessary. Previously, a check was run to see if the assay deviates from Lambert-Beers' Law as recommended by Wrolstad (1976). A series of dilutions of RRJ were prepared with pH 1.0 buffer and the absorbance measured at 520 nm. A plot of absorbance vs. concentration is shown in **Figure 2** where a straight line passes through the origin.

Two dilutions of the sample were prepared, one with potassium chloride buffer, pH 1.0 and the other with sodium acetate buffer, pH 4.5, following the previous DFs determined. The dilutions were equilibrated for 15 min. The absorbance of the solution was measured at a $\lambda_{vis\text{-}max}$ of 520 nm (De Ancos and others 2000) and at 700 nm (to correct for haze), against a blank cell filled with distilled water, using an Ultrospec 4000 UV/Visible Spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England). To calculate the absorbance (A) of the sample the next formula (**1**) was used:

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5} \quad \ldots\ldots (1)$$

Cyanidin-3-glucoside is the most common anthocyanin in nature (Lee and others 2005), so the average total anthocyanins was calculated for each sample and expressed according to equation (**2**).

$$\text{Concentration (mg/g)} = (A/\varepsilon \times L)\,(MW \times DF \times 1000) \times (L_{solvent}/g_{sample}) \quad \ldots\ldots (2)$$

where:
A = Absorbance of the sample
ε = molar absorptivity of cyanidin-3-glucoside
L = pathlength in cm = 1
MW =molecular weight of cyanidin-3-glucoside
DF = dilution factor
$L_{solvent}$: Volume of solvent used for extraction of the sample
g_{sample}: Amount of sample used for extraction

The pH differential method is a measure of the monomeric anthocyanin pigments and the results may not correlate with the color intensity of the extract samples as they are judged visually (Wrolstad 1976).

The % recovery of TACY from RRJ incorporated muffin was calculated based on:

% Recovery =

mg/g DM of TACY detected in RM – mg/g DM of TACY detected in CM x 100……(1)
mg of TACY added from RRJ estimated for g of raspberry muffin on DM basis

The estimated concentration in muffins on a dry matter basis was calculated as:

Estimated amount =

concentration of TACY of RRJ x 2.4% RRJ in raspberry muffin on DM basis …… (2)

HPLC analysis of anthocyanins

Preparation of the extract

Five g of freeze-dried red raspberry juice, batter and muffin samples (3 replicates) were blended in a homogenizer (Ultra Turrax T25 basic) with 20 mL of acidified solution (80% ethanol, 1% formic acid). Each of the replications was decanted into 150 mL round bottom flask using a small funnel and Whatman #4 filter paper. The extraction procedure was repeated with 10 mL of the acidified solution. Fifteen mL of acidified solution (5 % ethanol, 1% formic acid) was added as a 3rd extraction, continuing with the homogenization and decanting. The ethanol extracts were dried to 10 mL in a rotary

evaporator (Brinkmann Model: B-169 Vacuum aspirator) at 60°C and transferred into 50 mL Pyrex test tubes.

a) Removal of carotenoids and chlorophylls:

The samples were mixed with 5 mL of ethyl ether (100%) twice and inverted in a test tube with a Teflon lid. The solution was allowed to sit for 5 min for separation of two phases. The ether phase (top fraction) was discarded and the aqueous fraction retained and placed in 25 mL beakers in the hood to evaporate the remaining ether.

b) Anthocyanin compound purification:

The aqueous fractions (extracts) were combined with 2.5 mL of a solution of 20% ammonium sulfate, 20% ethanol, 2% metaphosphoric acid; and 5 mL of HPLC grade ethyl acetate for anthocyanins partition into the aqueous phase. The addition of ethyl acetate was repeated twice and the solution was inverted and allowed to separate.

The ethyl acetate fraction (top phase) was removed using a glass pipette and discarded. The aqueous fractions were syringe filtered through 0.45 μm Durapore® membrane filters (Type HV) (Millipore Corporation, Burlington, MA) into glass vial with Teflon lids (VWR International, LLC., Brisbane, CA) and held at -76°C for anthocyanin analysis (Macheix and others 1990). The same procedure was followed for the cranberry and blackberry samples.

HPLC analysis

HPLC analysis was performed on an Agilent 1100 series that consisted of an auto-sampler, a quaternary pump system, a photodiode array detector (DAD) and a Chemstation data system (Agilent Technologies, Inc., Palo Alto, CA). Extracts were injected onto the HPLC system equipped with a 5μM C-18 Zorbax-SB column 150 x 4.6 mm and a guard column 12.5 x 4.6 mm (Agilent Technologies Inc., New Castle, DE).

The absorption spectra were recorded from 200 to 700 nm for all peaks. The mobile phase included phosphoric acid (0.5%) (solvent A) and methanol (100%) (solvent B) in the following gradient system: 90:10 of A:B from 0 to 10 minutes, a linear gradient to 30:70 over 42 minutes, maintained to 0:100 for 5 minutes, cleaned with 100% methanol for 13 minutes, and then re-equilibrated to 90:10 for 5 minutes. The total running time was 65 minutes. The volume of sample injection was 10 μL, flow rate was set at 1.0 mL/min and the primary selection at λ 520 nm. Each of the solvent reservoirs was degassed adequately to eliminate air within the mobile phases. Identification and peak assignments of anthocyanins in all samples was based on comparison of their retention times (RT) and spectral data (200 – 700 nm) with those of blackberry and cranberry fruits, anthocyanin profiles for red raspberry found in the literature and an authentic standard, cyanidin-3-galactoside chloride (Ideain chloride). Additional information of anthocyanins present in the samples was carried out by spiking the raspberry products with the anthocyanin standard.

Statistical analysis

Data were reported as mean ± standard deviation (SD). A Randomized Complete Block Split Plot design was analyzed using a mixed model (SAS Institute Inc. v. 9.1, Cary, NC, USA). An analysis of variance procedure was used to determine significant differences ($p<0.05$) among treatments. In addition, Tukey's Honestly Significant Difference (HSD) test was used for pairwise comparisons of treatments. Regression analysis was conducted using Microsoft Office Excel 2003 (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

Batter and Muffin properties

For the measurement of anthocyanins, RRJ was around 10°Brix (11) so no dilution was necessary before the evaluation. This was also the case for other samples that reported lower °Brix values than the RRJ using the same dilution factor of 1:10 (sample:water) **(Table 1).**

When CB and RB were compared for pH, a decrease from 7.5 to 7.1 was observed due to the incorporation of raspberry juice. In addition, color changes occurred during the mixing process (**Figure 3**) due to the pH variation from 3.5 (RRJ) to 7.1 (muffin ingredients + RRJ). According to Mazza and Miniati (1993) color change occurs mostly at pH 4 to 6 and the color goes from bright red to purple (colorless) as was corroborated during the muffin mix preparation. The red color was observed at the beginning of the mixing operation, but after 10 min, a light purple color remained. Mazza (1997) noted that in aqueous media, most of the natural anthocyanins behave as pH

indicators, being red at low pH, bluish at intermediate pH, and colorless at high pH. This phenomenon was observed at the time of making muffins as was mentioned before.

It has been demonstrated that in acidic or neutral media, four anthocyanin structures exist in equilibrium: the flavylium cation AH^+, the base A, the carbinol pseudo-base B, and the chalcone C (**Figure 4**). At pH less than 2, red color of anthocyanins is present and the anthocyanin exists primarily in the form of the red (R_3 = *O*-sugar) or yellow (R_3=H) flavylium cation (AH^+). As the pH increases, rapid proton loss occurs to yield the red or blue forms (A). On standing, a hydration of the flavylium cation occurs to give the colorless carbinol or pseudo-base. This reaction mostly occurs at pH range from 3 to 6. Between pH 4 and 6 very little color remains in the anthocyanin since the amount of the colored forms AH^+ and A are very small (Mazza and Miniati (1993). Cabrita and others (2000) found during a study of six common anthocyanidins that 3-glucosides of pelargonidin, peonidin and malvidin, showed bathochromic shifts up to pH 6. Between pH 6 and 7.6, shifts increased and the bluish color did not change above pH 8. The same trend was observed for the 3-glucosides of cyanidin, delphinidin and petunidin. These anthocyanins were more than 70% stable after 60 days at pH 1-3 where an intense reddish color was observed. At higher pH values near the neutrality, anthocyanins stability and color intensity decreased.

After baking, pH of the RB decreased from 7.1 to 6.3 (**Table 1**), possibly due to the rupture of some compounds of RRJ such as ellagitannins (EA), which form ellagic acids that could decrease the pH level of the medium. Clifford and Scalbert (2000) cited that EA content in raspberries can be found in the range of 1.2-1.5 mg/g DM being the highest content in the seeds (88%), which in our study were not used. The same reduction

was not found in the control sample where the pH was 7.5 and 7.6 for the CB and CM, respectively.

Total anthocyanin content (TACY) in red raspberry juice, batter and muffin

Dried raspberry juice was analyzed using a pH-differential method, which is a principal spectrophotometric method to examine total anthocyanin content (TACY) (Wu and others 2006).

In **Table 2**, pH and color of the different extracts obtained are shown. The color of extract solutions of RRJ was ochre in M, and red in MH and EH due to different pH levels. This comparative study of extraction methods using 100% methanol (M), methanol:HCl (MH) and ethanol:HCl 1N (EH) showed that no significant differences were found between MH and EH solvents; although the highest TACY values for RRJ and RB were found using MH (**Table 3**).

In **Table 3**, TACY content of the different samples using the three different solvents is observed. TACYs in RRJ extracted with MH was greater than the average over the RRJ extracted with M and EH. For raspberry batter (RB), there was not enough evidence to indicate that significant differences ($p<0.05$) were found between the average TACYs determined with the three different system solvents: M, MH and EH. For control batter (CB) and raspberry muffin (RM), only M resulted in detectable TACY. In the case of control muffin, TACYs were not detectable with the solvents proposed in this study. Considering that 10% of RRJ was used in the raspberry batter and muffin samples, a normalization of the ACYs of RRJ was done by multiplying that value by 0.024 due to the presence of 24 mg of RRJ solids in 1 g of dry solids sample to compare the values

obtained. In addition, **Table 3** showed that using MH solvent a small reduction of TACYs was determined at the time of preparing the RB. TACY of RRJ (normalized value) decreased from 0.080 to 0.046 mg cyanidin-3-glucoside/g DM equal to 42.5% loss. In the cases of M and EH loss of TACY was ca. 53% and 56%, respectively. Interaction of anthocyanins present in RRJ with other compounds from other ingredients during batter preparation could have influenced stability and reduction of TACYs. Li and others (2007) concluded that muffin ingredients likely interacted with antioxidant components in the evaluation of antioxidant activity of purple wheat bran muffins.

Looking at **Figure 5**, it could be assumed that the best extraction was obtained with 100% methanol because a pale residual color was obtained after the extraction process. This was not the case considering that a bright final color was obtained after extraction on all the residues containing raspberry using MH and EH**,** which gave us the highest values on TACY for RRJ in comparison to M solvent. Then, anthocyanins color is due to the effect of pH of the solvent system used for extraction and does not reflect the large or small presence of anthocyanin content in the residue of the sample. Markakis (1982) pointed out that anthocyanin color is really stable only in acidic media. At low pH, anthocyanins are expected to show a stable color since the equilibrium between the colored flavylium and the colorless pseudo-base is shifted toward the flavylium, which is much more stable than the pseudo-base (Markakis 1974). Regarding the final color of the solutions prepared for the measurement of ACY, using the three different solvents and after diluting with sodium acetate buffer (pH 4.5), a light color was observed **(Table 4)**. As mentioned by Giusti and Wrolstad (2005), the presence of artificial dyes could be suspected if a bright red color is found at pH 4.5.

Regarding the ACY values obtained with MH extracts, a significant difference was found in the TACYs between RRJ and RB according to the Tukey method (adjusted p-value) at the 0.05 simultaneous level of significance (**Table 5**). Anthocyanins were found in a highest concentration in RRJ (3.3 mg cyanidin 3-glucoside/g DM) whereas the concentration of anthocyanins in RB, which contained 10% RRJ, was significantly lower (0.046 cyanidin 3-glucoside/g DM) (**Table 5**). Data presented by Beekwilder and others (2005a) confirmed that the amount of ACY present in red raspberries was in the range of 2.8 – 3.3 mg cyanidin 3-glucoside/g DM. Pantelidis and others (2007) found that cv. Meeker had 0.43 mg cyanidin 3-glucoside/g FW. Wada and Ou (2002) reported a value of 4.64 cyanidin-3-glucoside mg/g DM for red raspberries that were grown near Salem, OR.

TACYs were not detected in control batter. Yu (2008) reported that small amounts of anthocyanins have been reported in flour-based products due to the presence of flavonoids. Among the major flavonoids found in wheat, flavonols and anthocyanins are the most typical. Wu and others (2006) reported that in processed foods where foods containing ACY were added, such as the case of bread, cereals, and baby foods, ACY could not be detected. This was the case of RM acidified methanolic extract where detectable ACYs were not found through the spectrophotometric method. These extremely low concentrations may result from poor stability and destruction during processing. In this regard, Rupasinghe and others (2008) pointed out that anthocyanins are not stable and tend to lose color during processing. Also, they added that cyanidin glycosides, which have been found in raspberries as mentioned by others (Boyles and Wrolstad 1993; Mullen and others 2002; Beekwilder and others 2005b), are hydrolyzed

to unstable cyanidin aglycone during processing resulting in the formation of various products.

The portion of anthocyanins in total phenolic content (TPC determined in the previous chapter), was evaluated by calculating TACY/TP ratio, although units were different for each one of the measured parameters. The concentrations of TACY and TACY/TP ratio are shown in **Table 5**. Anthocyanins represented a small portion of the TPC of RRJ (14%) indicating a higher proportion of other polyphenols in raspberries. That value was similar to 13% reported by Jakobek and others (2007b) where the same pH differential method was used; although extraction system and method was different from that used in our study.

Considering that RB and RM were the samples where RRJ was added, the recovery of the ACYs provided by the juice before and after baking process was determined. The highest recovery of ACY was found in RB after its extraction with MH solution (59%). Comparing the recovery of ACY of RB and RM using M extract, a decrease was observed from 48% to 30% (**Table 6**). There was obvious degradation of ACYs under the conditions selected possibly due to the baking treatment and pH effect Regarding anthocyanins in raspberries, Francis (1989) referred that a marked loss of that component was found in raspberries canned in syrups with basic pH. Li and others (2007) showed the complete destruction of anthocyanins during production of untreated or heat-treated purple wheat bran muffins baked at 177°C for 7-12 min. A similar result was obtained in this study when no TACYs were detected in RM using the MH solvent. Rupasinghe and others (2008) reported that an interaction of apple skin powder (ASP) with wheat protein via hydrogen bonding during muffin preparation could have caused

poor recovery of phenolics where anthocyanins were included. Cyanidin-3-galactoside showed a recovery of 24.8%.

As was cited in Chapter II, the highest TPC in RM was found using MH. Meanwhile no ACY in RM was detected using the same extraction solvent. For RB, no differences were found in ACY content due to extraction solvents; although MH gave the highest mean value (0.046 mg of cyanidin 3-glucoside/g DM).

The content of ACYs is different from plant to plant. In raspberries cv. Meeker, the TACY increased fourfold in the ripe fruit. The relative proportion of the four major pigments, which were all found in unripe fruit, did not vary appreciably with maturity (Mazza and Miniati 1993).

The correlation plot of TACY and AA (evaluated in Chapter II) showed that a highest correlation exists between the two parameters mentioned, RRJ (normalized values) and RB (r=0.95) (**Figure 6**). Liu and others (2002) confirmed the same fact when after evaluation of four different cultivars of raspberry (Heritage, Kiwigold, Goldie and Anne) determined that higher anthocyanin content in raspberries contributed to their higher AA. Comparing with the correlation coefficient obtained for TPC vs. AA (r=0.99), a similar coefficient was obtained for TACY vs. AA. This differs from the results obtained by Jakobek and others (2007b) who found a larger correlation for TPC vs. AA.

Identification of anthocyanins in red raspberry juice, batter and muffin

Identification of anthocyanins was complicated by the fact that there are a large number of anthocyanins found in nature, and standards are not readily available for most

of them. Even though a large amount of published data is available, methods used are different that make comparison of results difficult.

Considering those difficulties, chromatograms and spectra from blackberry and cranberry were obtained from the literature and also samples (frozen blackberries from a local supermarket and cranberries Washington grown) were analyzed and the compounds identified (**Figures 7 and 8).**

Peak identification and assignment

Identification and peak assignment of anthocyanins of RRJ and batter and muffin products were based on comparison of their retention times (RT) and area with that of other fruits, published data, and Ideain (cyanidin-3-galactoside chloride) (**Figure 9**). Blackberry and cranberry fruits, which have been studied extensively, served as references for identification purposes. Only one representative chromatogram from every sample (RRJ, RB and RM) is presented due to a highly reproducible chromatogram obtained for the three replications (**Figures 10, 11 and 12**).

Based on the blackberry sample, peaks were identified using a well known chromatogram from the literature (Hong and Wrolstad 1990), the presence of cyd-3-glu in RRJ was confirmed. This peak came out at the same RT in RRJ and blackberry sample (ca. 18.3 min) (**Figure 7A**). The same elution program was used for the analysis of the blackberry sample; therefore, results were able to be compared. The same procedure was followed for the cranberry sample that also was used as a reference (Durst and Wrolstad 2005) and the same RT (ca. 18.4 min) was detected for cyd-3-glu (**Figure 8A**).

With the Ideain standard, the chromatogram showed the highest peak at the RT of 17.2 min (**Figure 9**). RRJ did not show the presence of cyanidin-3-galactoside chloride (Ideain) considering that the fruit had four mean peaks with an average of RT for each as follows and shown in **Figure 10**: peak 1, 16.5 min; peak 2, 18 min; peak 3, 18.2 min; and peak 4, 19.7 min. This finding was verified with the results obtained by Boyles and Wrolstad (1993) who did not find cyanidin-3-galactoside in twelve cultivars of raspberry where cv. Meeker was included.

According to Meyer (1999), the retention time of a component is constant under identical chromatographic conditions. In raspberry, although RTs were not similar to the values reported by Jakobek and others (2007a) due to different experimental conditions and the origin of the raspberries (Slavonia region, Croatia), the comparison was based on the similarity of the chromatograms obtained by them to ours. In addition, data found by Spanos and Wrolstad (1987), Boyles and Wrolstad (1993), Mullen and others (2002) and Kim and Padilla-Zakour (2004), complemented the results obtained. The four peaks were identified as peak 1, cyanidin-3-sophoroside (cyd-3-sop); peak 2, cyanidin-3-glucosylrutinoside (cyd-3-glurut); peak 3, cyanidin-3-glucoside (cyd-3-glu); peak 4, pelargonidin-3-sophoroside (pg-3-sop) in RRJ (**Figure 10)** where cyd-3-sop is a major and characteristic anthocyanin in raspberries (Torre and Barritt 1977; Rommel and others 1990; De Ancos and others 1999; Mullen and others 2002). Wu and Prior (2005) corroborated the presence of a trisaccharide glycoside in raspberry (cyanidin 3-(2^{G}-glucosylrutinoside) of peak 2. As cited by Rommel and others (1990) red raspberry anthocyanins are di- and triglycosides except for the monoglycoside cyanidin-3-glucoside. Barritt and Torre (1975) reported that cyd-3-glurut is present in Meeker but

not in Willamete cv. Anthocyanins found in the RRJ are indicated in **Table 7**. In a reversed-phase system, the order of elution of anthocyanidins is as follows: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Mazza and others 2004). As the degree of glycosylation increases, the anthocyanins are less well retained, have a shorter retention time and the structural stability and water stability of the parent anthocyanidin increases. The elution order of the cyanidin pigments was as follows: cyd-3-sop, cyd-3-glurut, and cyd-3-glu. This order reverses the general rule that the elution is tri-, di-, mono-sacharide of the same aglycone. This suggests that the hydrophobic methyl group (CH_3) of rhamnose of the rutinose contained in cyd-3-glurut caused a higher RT (Spanos and Wrolstad 1987; Mullen and others 2002; Mazza and others 2004). For that reason, cyd-3-sop eluted before cyd-3-glurut. Structure of the anthocyanins identified in RRJ are shown in **Figure 13.**

When RB and RM were evaluated, only three peaks were detected (peak 1, cyd-3-sop; peak 2, cyd-3-glurut; peak 3, cyd-3-glu) (**Figure 11 and 12**). A different absorbance scale is used in the figures for RB and RM in comparison to RRJ, where greater absorbance values were detected. Markakis (1974) noted that pg-3-glu heated in aqueous solution (50-100°C), loses color and a short time high temperature heating processing was recommended for better pigment retention. As pg-3-glu was degraded by the thermal treatment, pg-3-sop also seemed to have been affected by the baking process, which resulted in the complete absence of the compound. In addition, anthocyanins are also altered by oxygen that contributes to their degradation. This may be the case for pg-3-sop, which was not found after the mixing process that last 10 min under air. For the same anthocyanins, as the pH of the medium increased with the addition of other

ingredients, an destabilizing effect on the cited anthocyanins is also produced. Moreover, Rommel and others (1990) found that pg-3-sop decreased by fermentation combined with depectinization in wine after six months of storage. Regarding the presence of pg-3-sop in RRJ, the smallest peak was observed as is shown in **Figure 10**. Then, it can be assumed that a negative effect was also produced on the other anthocyanins compounds, although no disappearance was observed considering that these anthocyanins, cyd-3-sop, cyd-3-glurut, cyd-3-glu, showed larger absorbance values in comparison to pg-3-sop.

The peak areas, as mean percentage of total peak area, are shown in **Figure 14** for RRJ, RB and RM. When RRJ was analyzed, cyd-3-sop was 73.06% total peak area, while cyd-3-glu and pg-3-sop made up 4.23% and 18.15% total peak area, respectively. Jakobek and others (2007a) found that cyd-3-sop represented 79.8% of the TACY in RRJ; meanwhile, cyd-3-glu and pg-3-sop were found in relatively smaller amounts. This percentage, 73.06% of cyd-3-sop, is similar to the value reported by Jakobek and others (2007a) (79.8%). Boyles and Wrolstad (1993) determined cyd-3-sop (71.6%), cyd-3-glurut (9.9%); cyd-3-glu (8.1%) and pg-3-sop (5.5%). Differences in the methods used, solvent systems, maturity and origin of samples, and other factors influenced the differences among the averaged areas for the anthocyanins determined. Comparing the percentages of the samples evaluated, an increase of the area% of cyd-3-sop was observed when the RB was prepared (82.55%). In the case of cyd-3-glu, a decrease from 18.15% (RRJ) to 10.11% (RB) was observed. RM in comparison to RB, showed a decrease for cyd-3-sop from 82.55% (RB) to 78.92% (RM) because of the heat treatment effect. The same effect was seen for cyd-3-glurut, which presented a value of 3.94% that was less than the 4.40% and 4.23% found in RB and RRJ, respectively. For the third

compound, cyd-3-glu, an increase was produced from 10.11% (RB) to 12.32% (RM). The statistical analysis showed that significant differences between the cyd-3-sop content in RRJ and RB were found and between RRJ and RM as well ($p<0.05$). No significant differences ($p<0.05$) were found between RB and RM for cyd-3-sop. Regarding the cyd-3-glurut, no significant differences were determined as can be clearly observed in **Figure 14**. Finally, cyd-3-glu content was greater in RRJ than the content in RB and RM. No significant differences in the cyd-3-glu content were found between RB and RM ($p<0.05$).

Regarding the effect of heating on cyd-3-glu, Kim and Padilla-Zakour (2004) found complete destruction occurred during the production of jam (104-105°C final boiling point). Havlíková and Míková (1985) showed that pH effect depends on the type of anthocyanins glycosidation. For example, 3,5-diglycosides have the highest stability at pH 3-4. In contrast, highest stability of anthocyanidin -3 monoglucosides was observed in the pH range 1.8-2.0.

Total peak areas (mAU*s) found for RRJ and RB were directly proportional and highly correlated to the spectrophotometric TACY values determined using the pH differential method ($r=0.95$) (**Figure 15**). This analysis was done considering only the TACY values found for RRJ (normalized values) and RB through the pH differential method due to the lack of sensitivity of this procedure. In the case of the reversed-phase HPLC method, anthocyanins compounds were detectable in all the samples evaluated.

Spectral analysis of RRJ and juice-containing batter and muffin

Spectral characteristics of peak 1, cyd-3-sop; peak 2, cyd-3-glurut; peak 3, cyd-3-glu; peak 4, pg-3-sop are shown in **Figure 16**. All the peaks found in the three different products (RRJ, RB and RM) shared the same spectral pattern. Maxima and shapes of the absorption curves were not different regardless of RRJ, RB or RM as that was clearly seen for cyd-3-sop (**Figures 16A, 16B, 16C**). Sági and others (1974) reached the same conclusion after studying the effects of ripening of raspberries on absorption spectrum. In our study, cyd-3-sop and cyd-3-glurut exhibited a visible λ_{max} at about 520 nm, meanwhile cyd-3-glu and pg-3-sop have a visible λ_{max} at 516 - 520 nm. The visible wavelength maxima for the anthocyanins detected in RRJ and subproducts were similar. As can be observed in **Figure 16**, absorbance detected for RB and RM differs from the absorbance determined for RRJ in all their anthocyanins compounds. Looking at **Figure 16A**, the absorbance at all wavelength maxima detected (216, 280 and 520 nm) of cyd-3-glu (peak 3) was lower than cyd-3-glurut (peak 2). In the case of RB and RM (**Figures 16B and 16C**), a decrease of absorbance was produced for cyd-3-sop at all wavelengths determined (208-212, 280, 520 nm). From the records of spectral curves, the absorbance reported for cyd-3-sop in RRJ at λ_{max} = 520 nm (**Figure 17**) decreased dramatically after mixing (RB), which seemed to have a larger effect than baking process (RM) as was concluded from spectrophotometrical ACY content data. A decrease of absorbance of cyd-3-glurut and cyd-3-glu at λ_{max} = 516 - 520 nm was also observed in RB and RM (**Figures 18 and 19**). One reason for decrease of absorbance is the oxidation of anthocyanins considering that air exposure was not controlled during the muffin process (Havlíková and Míková 1985). If we compare the absorbance values reported for RRJ

(**Figure 16A**) with the reported for RB (**Figure 16B**), a dramatic decrease was observed for all the compounds including the disappearance of pg-3-sop in RB. According to Havlíková and Míková (1985), who considered the theory of equilibrium shift between the cation and pseudo-base, decrease of absorbance is due to increase in pH value (from 3.42 for RRJ to 7.1 for RB). This pH effect was highest than the effect produced afterwards by baking.

CONCLUSIONS

Red raspberries are a potential source of anthocyanins in baked products, although a great reduction is observed after processing through mixing with other ingredients and further baking. RRJ was used in muffin formulation and degradation of the anthocyanins in RRJ resulted. TACY content in RRJ, RB and RM was determined through the spectrophotometric method. No ACYs were detected in CM and RM through the spectrophotometric method, but they were found through an HPLC reverse-phase method. HPLC allowed us to find four main anthocyanins in RRJ (cyd-3-sop, cyd-3-glurut, cyd-3-glu and pg-3-sop). After mixing and baking pg-3-sop disappeared likely due to the effect that produces an increase of pH and temperature on this anthocyanin.

During a HPLC analysis, retention time data is the important information provided together with absorbance detection. Determination of retention time of reference standards is essential in order to confirm the identity of the compounds (peaks) with greater certainty. This becomes more important when complex mixtures are analyzed as in was in our study.

The degradation of anthocyanins observed during the muffin process is a challenge for the food manufacturing industry as the beneficial effects of these compounds are diminished.

FUTURE RESEARCH

Due to the unavailability of standards for the identification of anthocyanins present in fruit juices and mixes based on RRJ or others, use of HPLC coupled with mass spectrometry could provide more useful structural information regarding molecular weight and fragmentation. Reduction of reliance on retention time and UV-visible spectra for identification would take place if a more powerful method for routine analysis of anthocyanins such as mass spectrometry were used.

Considering that the structure and stability of anthocyanins depend on the pH of the medium, implementation of new techniques has to take place in order to keep a high quality and benefits that anthocyanins-containing products have as is the case of the raspberry muffins.

Other varieties with greater acidity values could be used in muffin formulations considering that is expected to enhance the color strength of anthocyanins according to De Ancos and others (1999). Finding a suitable cv. for processing could allow us to obtain a raspberry baked product with a higher stability of the anthocyanins red pigments and a higher nutritional value after processing. In addition, different formulations of muffins might be tried in order to avoid drastic modifications of the pH of the medium. Also, lowering the pH within acceptable limits may contribute to the stabilization of anthocyanins in red raspberries.

As was mentioned in Chapter II, encapsulation of the whole raspberry using several coatings could be included in future studies to protect the fruit from external changes such as pH and temperature.

Table 1- pH and °Brix of red raspberry juice and juice-containing batter and muffins

Samples	pH*	°Brix
Red raspberry juice (100%)	3.4 ± 0.021	11
Red raspberry juice**	3.5 0.087	1
Control Batter**	7.5 ± 0.021	2.8
Raspberry Batter**	7.1 ± 0.10	2.9
Control Muffin**	7.6 ± 0.11	3.9
Raspberry Muffin**	6.3 ± 0.16	3.5

* Results reported are mean values of three replications ± standard deviation.

** Diluted samples (1:10, sample:water)

Table 2- Color and pH of anthocyanins extracts using different solvent systems

Product	**Solvent System***		
	M	**MH**	**EH**
Red raspberry juice	Ochre (5.00)	Dark red (0.68)	Dark red (1.53)
Control Batter	Clear (8.76)	Clear (0.69)	Light pink (1.30)
Raspberry Batter	Light ochre (8.30)	Red (0.67)	Pink (1.27)
Control Muffin	Clear (7.70)	Clear (0.68)	Clear (1.25)
Raspberry Muffin	Clear (6.92)	Red (0.67)	Pink (0.59)

* M, extracts in 100% methanol, MH, extracts in methanol:HCl, EH, extracts in ethanol 95%:HCl 1N.

** Results reported in parenthesis are pH of the media solution

Table 3- Total anthocyanin content* of red raspberry juice and juice-containing batter and muffins** using different solvent systems (N=3)

Product	Solvent System**		
	M	**MH**	**EH**
Red raspberry juice***	0.068 ± 0.0051[a]	0.080 ± 0.0051[b]	0.073 ± 0.010[ab]
Control Batter	0.015 ± 0.019	nd	nd
Raspberry Batter	0.032 ± 0.011[a]	0.046 ± 0.0083[a]	0.032 ± 0.010[a]
Control Muffin	nd	nd	nd
Raspberry Muffin	0.017 ± 0.089	nd	nd

* Results reported are mean values of three determinations ± standard deviation expressed as mg of cyanidin-3-glucoside/g DM). Sample means containing different letters in the same row are significantly different from one another ($p<0.05$).

** M, extracts in 100% methanol, MH, extracts in methanol:HCl, EH, extracts in ethanol 95%:HCl 1N.

*** A normalization was done by multiplying TACY values of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

nd = Not detected

Table 4- Observed color of sample extracts at pH 1 and 4.5

Samples	Extraction solvent					
	100% Methanol		**Methanol:HCl**		**Ethanol:HCl 1N**	
	pH 1.0	**pH 4.5**	**pH 1.0**	**pH 4.5**	**pH 1.0**	**pH 4.5**
RRJ	Bright red	Light red	Bright red	Light red	Bright red	Light red
CB	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
RB	Pink	Cloudy light pink	Pink	Cloudy pink	Pink	Cloudy pink
CM	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
RM	Light pink	Cloudy light pink	Light pink	Cloudy light pink	Light pink	Cloudy light pink

Table 5- Total anthocyanin content* and TACY/TPC ratio of red raspberry juice and juice-containing batter and muffins using methanol:HCl extraction solvent (N=3)

	TACY (mg of cyaniding 3-glucoside/g DM)	TACY/TPC
Red raspberry juice**	0.080 ± 0.0051^{b}	0.14
Control Batter	nd	
Raspberry Batter	0.046 ± 0.0083^{a}	0.066
Control Muffin	nd	
Raspberry Muffin	nd	

* Results reported are mean values of three determinations ± standard deviation. Sample means containing different letters in the same column are significantly different from one another ($p<0.05$).

** A normalization was done by multiplying TACY values of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

nd = Not detected

Table 6- The mean percent recovery of anthocyanin compounds of red raspberry juice before and after baking in a model system muffin (N=3)

		Total Anthocyanin Content * **(mg cyanidin 3-glucoside/g DM)**		**Recovery of anthocyanins (%)****
		Estimated amount**	Detected amount***	
Raspberry batter	100% MeOH	0.068 ± 0.0051	0.033 ± 0.012	48
	MeOH:HCl	0.080 ± 0.0051	0.047 ± 0.006	59
	EtOH:HCl	0.073 ± 0.010	0.030 ± 0.010	41
Raspberry muffin	100% MeOH	0.068 ± 0.0051	0.017 ± 0.010	30
	MeOH:HCl	0.080 ± 0.0051	nd	nd
	EtOH:HCl	0.073 ± 0.010	nd	nd

* Results reported are mean values of three determinations ± standard deviation.

** Estimated amount is based on the amount of RRJ added to the muffin mixture. A normalization was done by multiplying TACY values of RRJ by 0.024 due to 24 mg of RRJ solids were contained in 1 g of batter and muffin dry matter samples.

*** Detected amount for Raspberry Batter and Raspberry Muffin was based on the amount of TACY detected in each product because no TACYs were detected in Control samples

**** %Recovery of anthocyanins measured is based on all the anthocyanins originated from RRJ

Table 7 – Identification of anthocyanins in red raspberry juice

Peak N°	RT (min)	Anthocyanins
1	16.5	cyanidin 3-sophoroside
2	18.0	cyanidin-3-glucosylrutinoside
3	18.2	cyanidin 3-glucoside
4	19.7	pelargonidin 3-sophoroside

Anthocyanidin	R_1	R_2
pelargonidin	H	H
cyanidin	OH	H
peonidin	OCH_3	H
delphinidin	OH	OH
malvidin	OCH_3	OCH_3
petunidin	OCH_3	OH

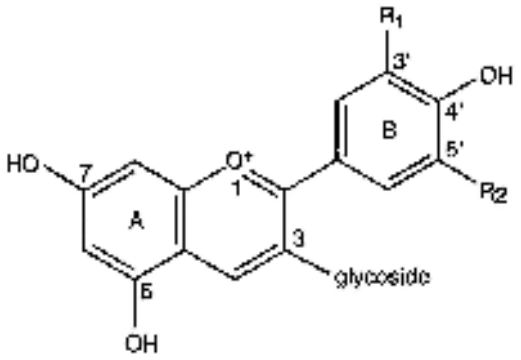


Figure 1 – Anthocyanin structure

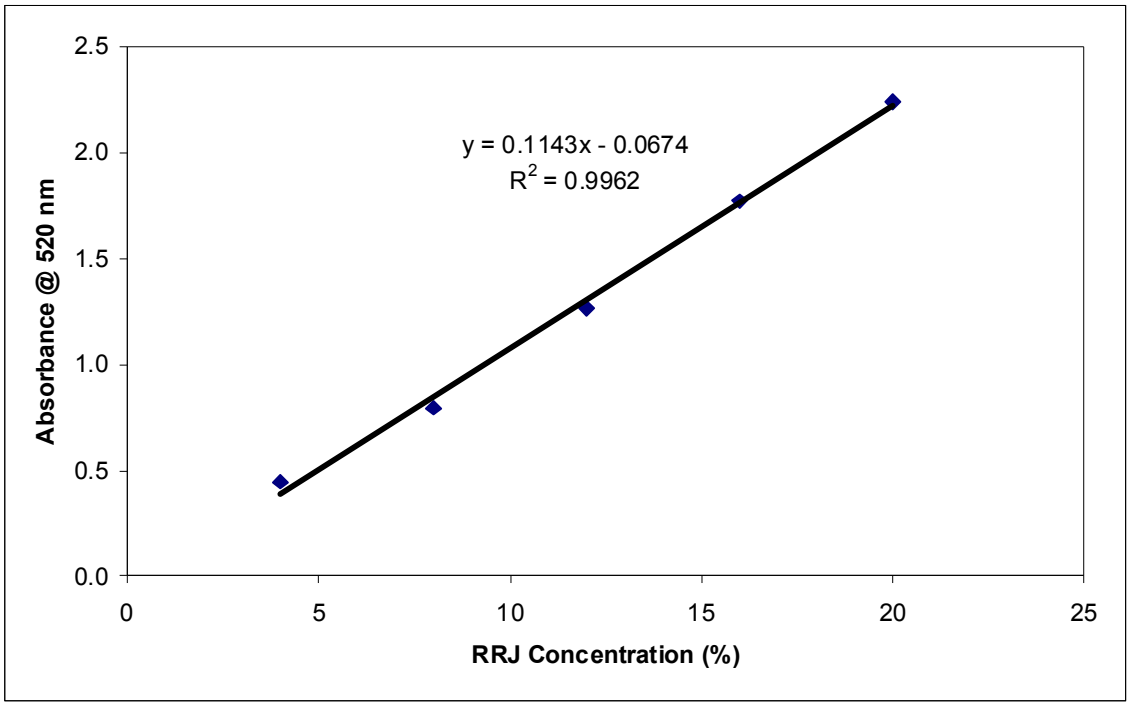


Figure 2-Relationship between absorbance and concentration of acidified methanolic red raspberry juice extract diluted with pH 1 buffer

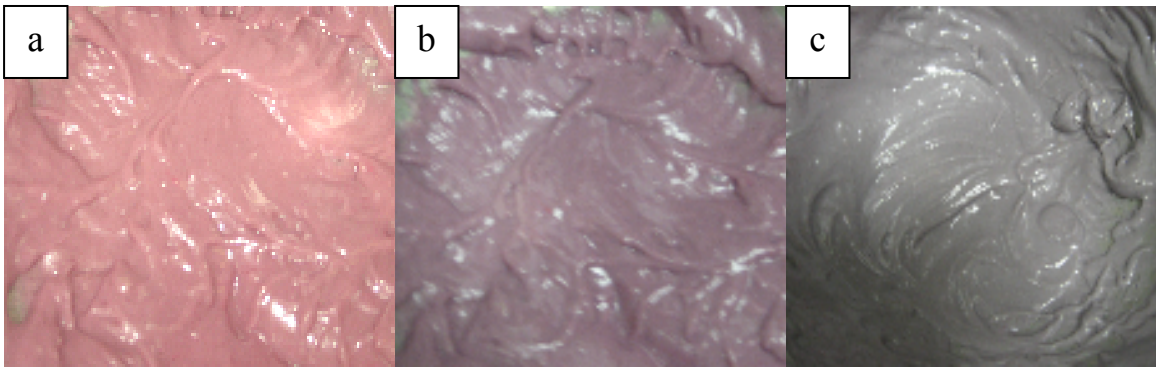


Figure 3- Color changes of raspberry batter after mixing:

a) 3 min; b) 5 min; c) 10 min

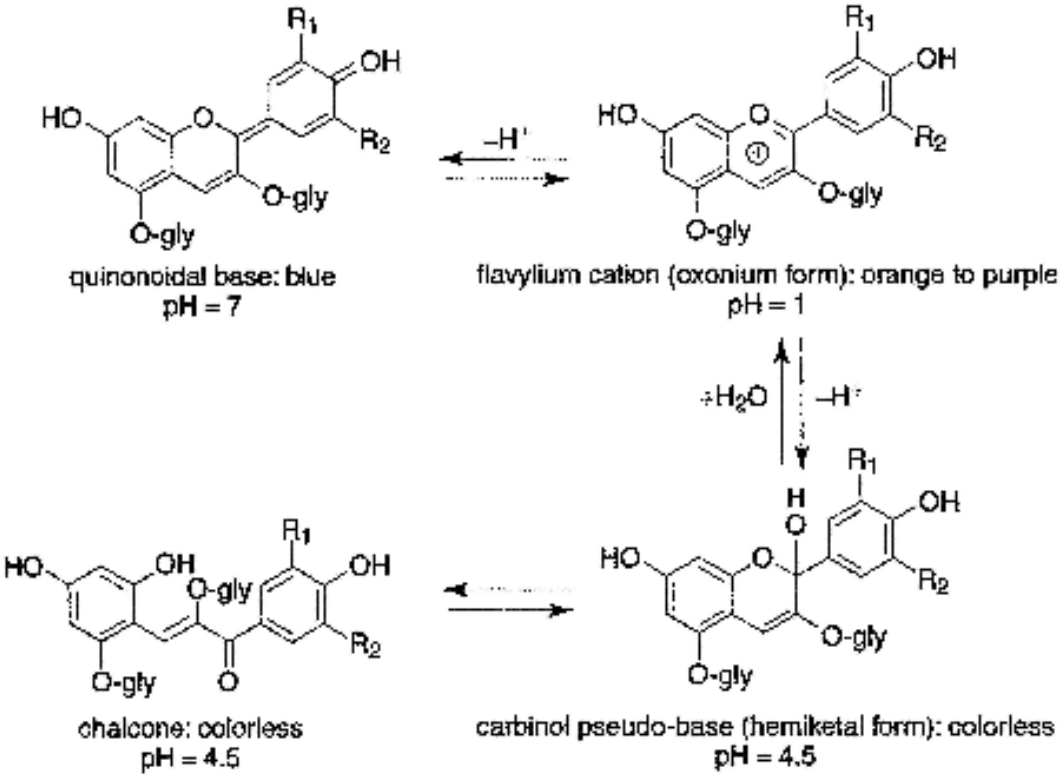


Figure 4- Structural transformations of anthocyanins at different pH levels

Samples	Extraction solvents		
	100% Methanol	Methanol:HCl	Ethanol:HCl 1N
RRJ			
CB			
RB			
CM			
RM			

Figure 5- Residue of samples after extraction process

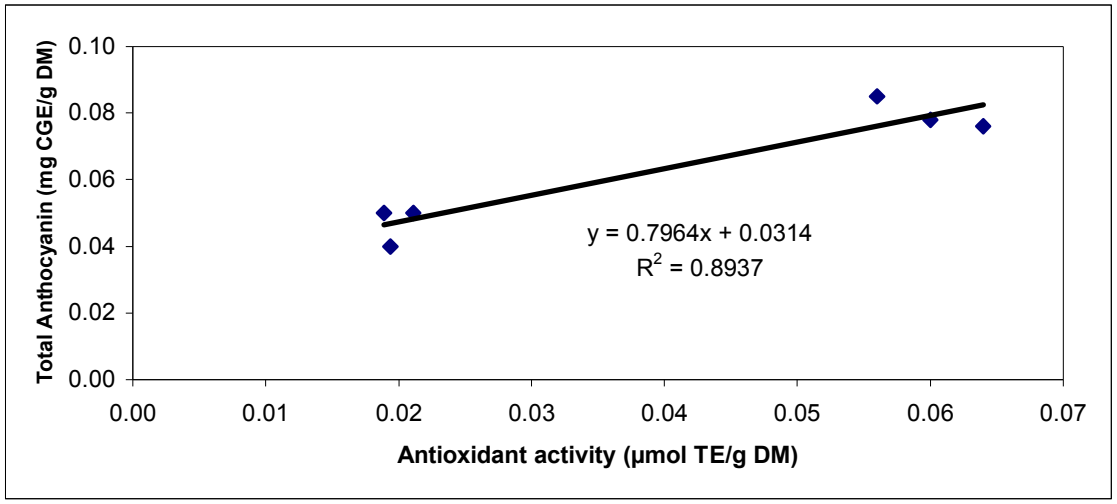


Figure 6-Correlation plot of TACY versus AA of red raspberry juice and batter

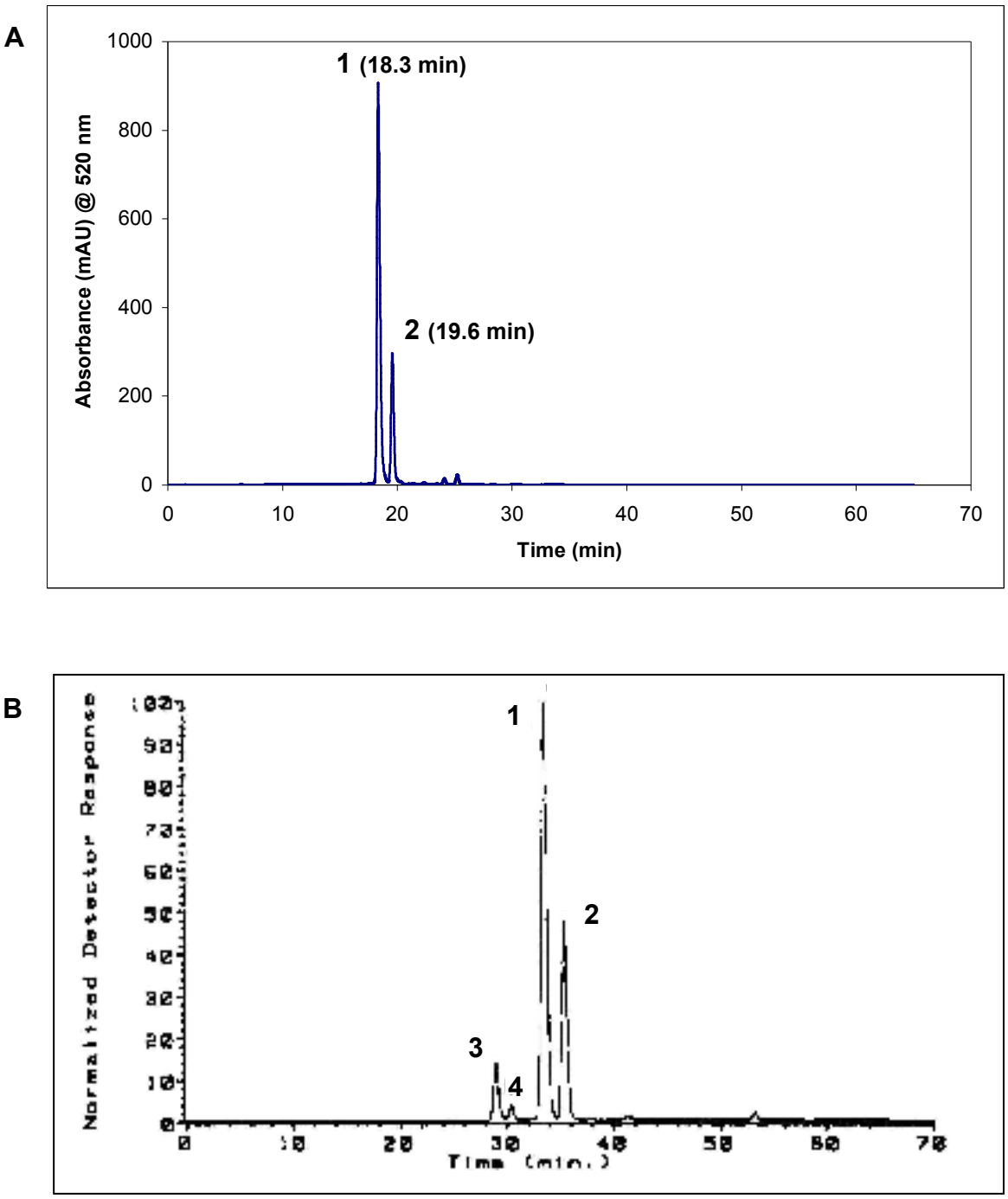


Figure 7- HPLC chromatogram of the anthocyanins compounds of blackberry @ 520 nm:

(A) Detection on-site (B) From literature (Hong and Wrolstad 1990)

Peaks identification: 1, cyanidin-3-glucoside ; 2, cyanidin-3-rutinoside; 3, cyanidin-3-sophoroside; 4, cyanidin-3-glucosylrutinoside

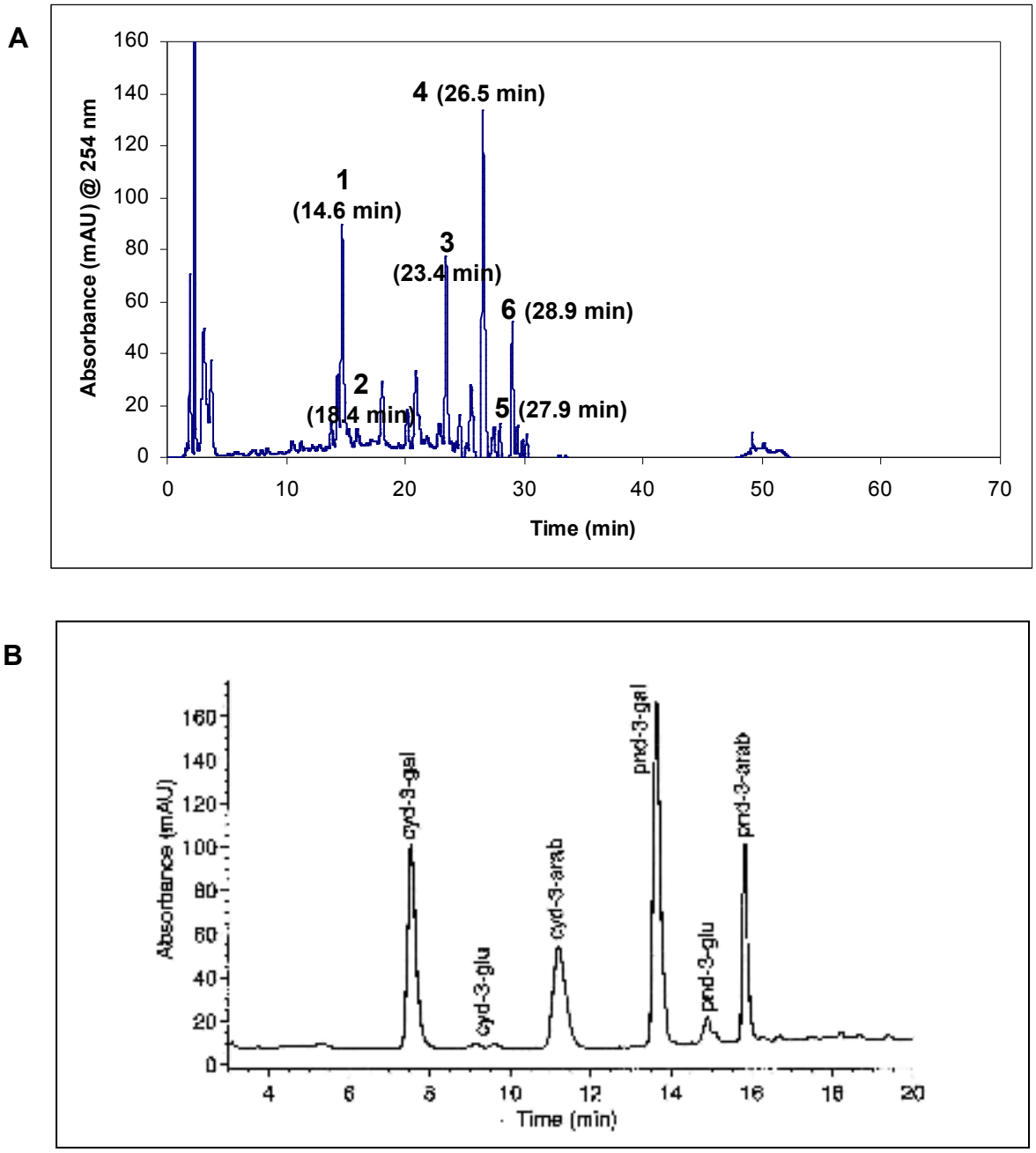


Figure 8- HPLC chromatogram of the anthocyanins compounds of cranberry:

(A) Detection on-site @ 254 nm (B) From literature @ 520 nm (Durst and Wrolstad 2005)

Peaks identification: 1, cyanidin-3-galactoside; 2, cyanidin-3-glucoside; 3, cyanidin-3-arabinoside; 4, peonidin-3-galactoside; 5, peonidin-3-glucoside; 6, peonidin-3-arabinoside

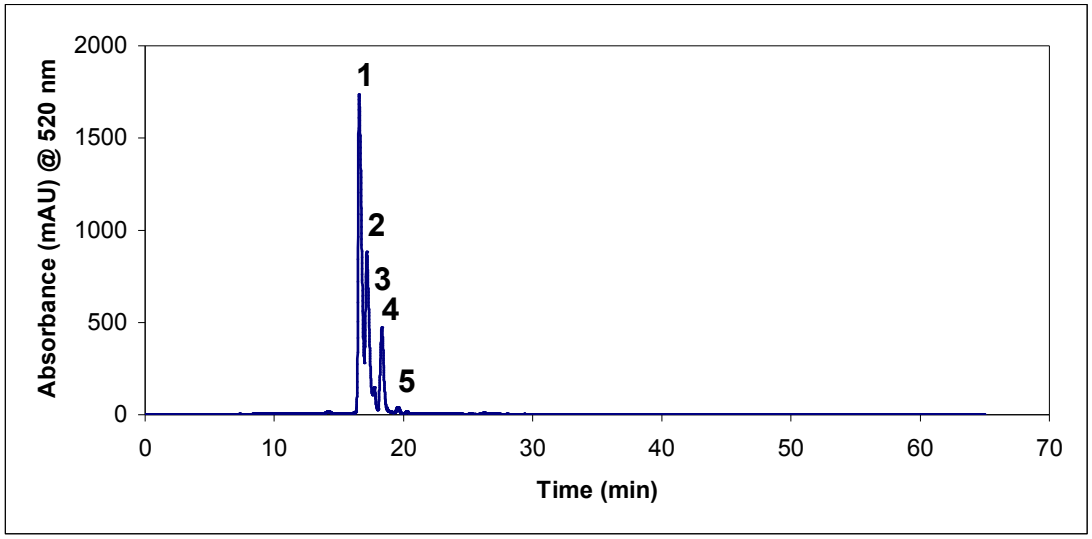


Figure 9- HPLC chromatogram of spike (RRJ + Ideain (cyanidin-3-galactoside))

Peaks identification: 1, cyanidin-3-sophoroside (16.6 min); 2, cyanidin-3-galactoside (17.2 min); 3, cyanidin-3-glucosylrutinoside (17.7 min); 4, cyanidin-3-glucoside (18.3 min); 5, pelargonidin-3-sophoroside (19.6 min)

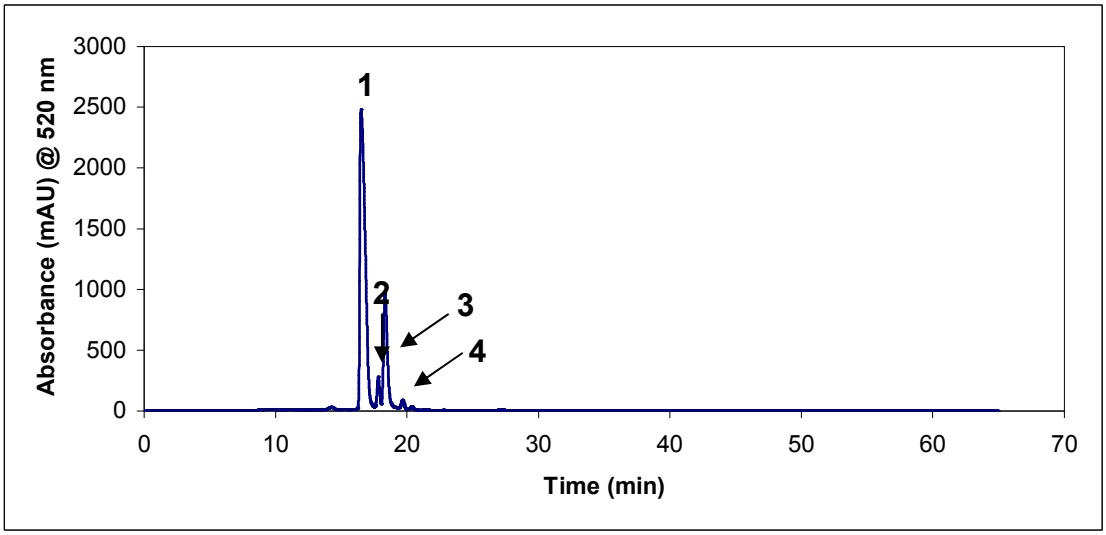


Figure 10- HPLC chromatogram of the anthocyanins compounds of red raspberry juice:

Peaks identification: 1, cyanidin-3-sophoroside (16.5 min); 2, cyanidin-3-glucosylrutinoside (18 min); 3, cyanidin-3-glucoside (18.2 min); 4, pelargonidin-3-sophoroside (19.7 min)

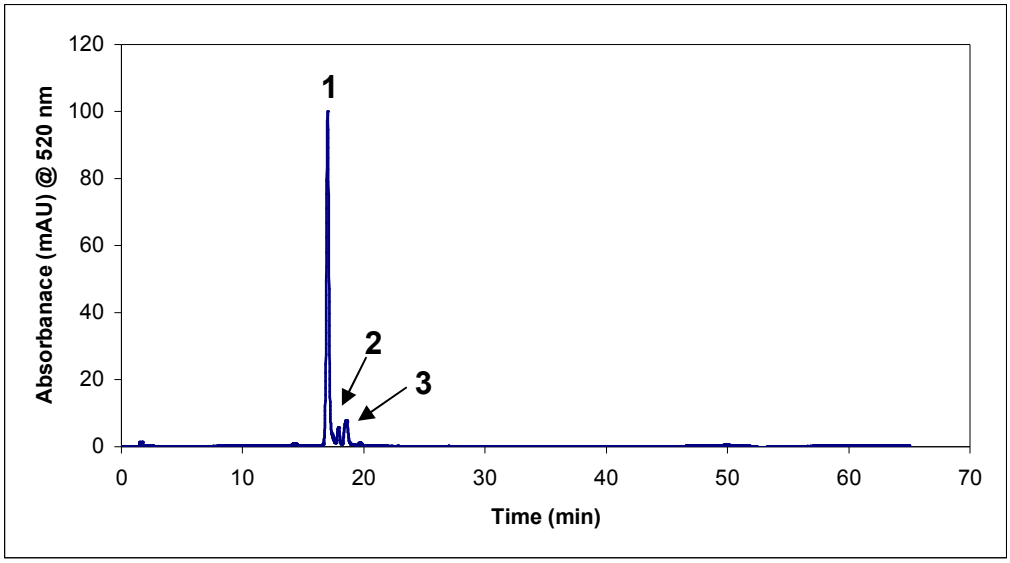


Figure 11- HPLC chromatogram of the anthocyanins compounds of raspberry batter

Peaks identification: 1, cyanidin-3-sophoroside (17 min); 2, cyanidin-3-glucosylrutinoside (17.9 min); 3, cyanidin-3-glucoside (18.5 min)

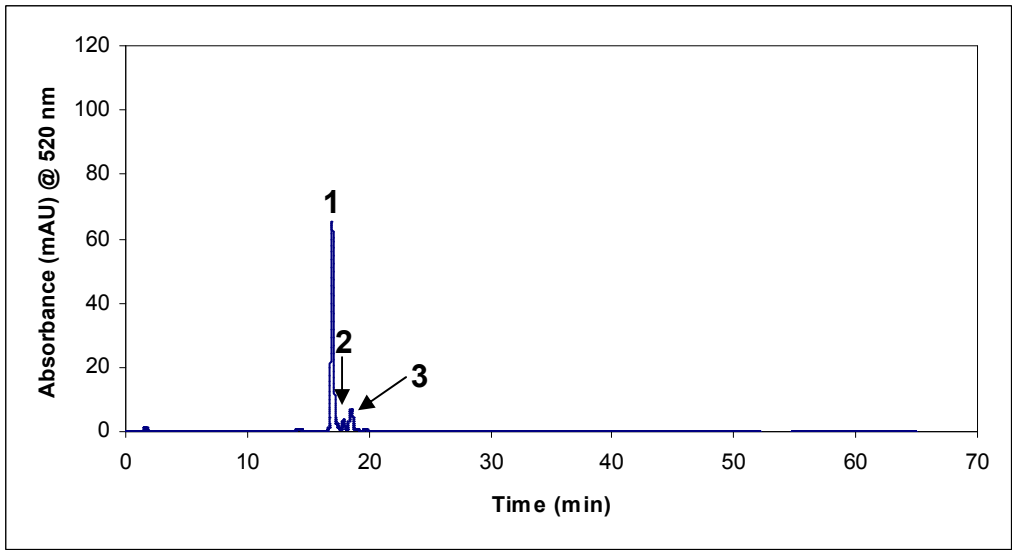
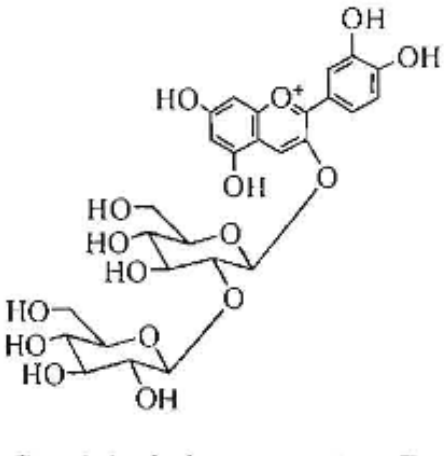
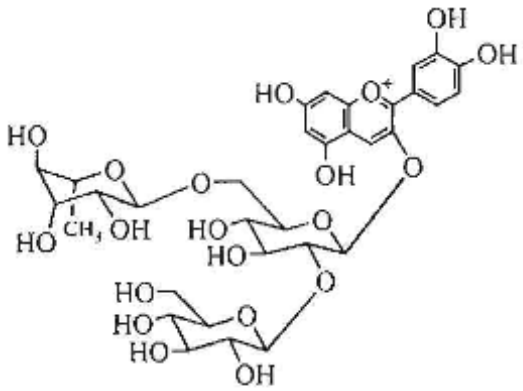


Figure 12- HPLC chromatogram of the anthocyanins compounds of raspberry muffin

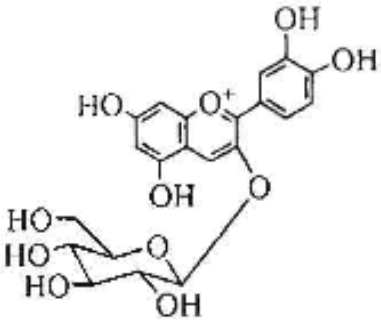
Peaks identification: 1, cyanidin-3-sophoroside (17 min); 2, cyanidin-3-glucosylrutinoside (17.9 min); 3, cyanidin-3-glucoside (18.6 min)



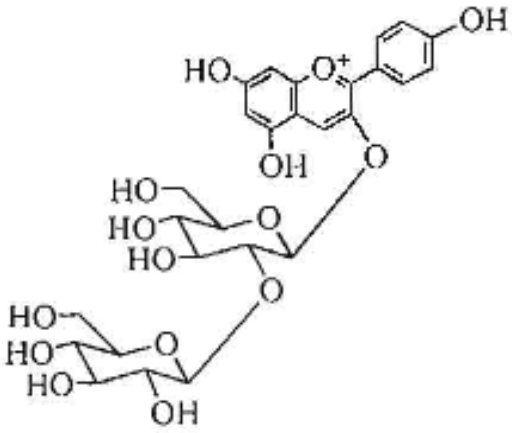
Cyanidin-3-sophoroside



Cyanidin-3-glucosylrutinoside



Cyanidin-3-glucoside



Pelargonidin-3-sophoroside

Figure 13- Structure of anthocyanins identified in red raspberry juice

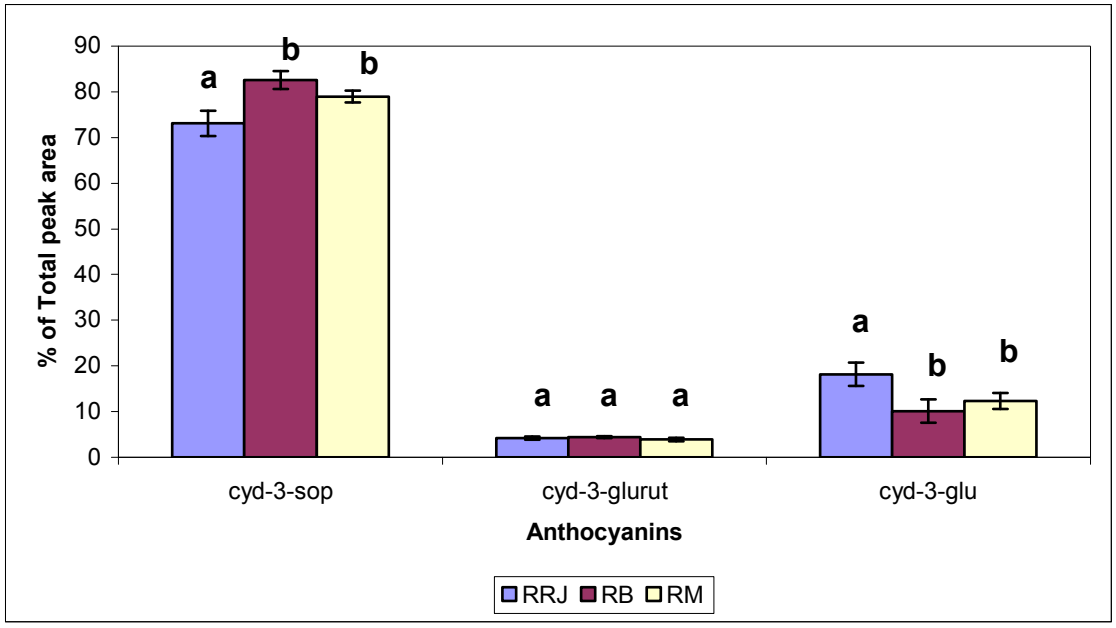


Figure 14- Changes in anthocyanin content in red raspberry juice, raspberry batter and raspberry muffin. Same letters mean that values are not significantly different ($p < 0.05$)

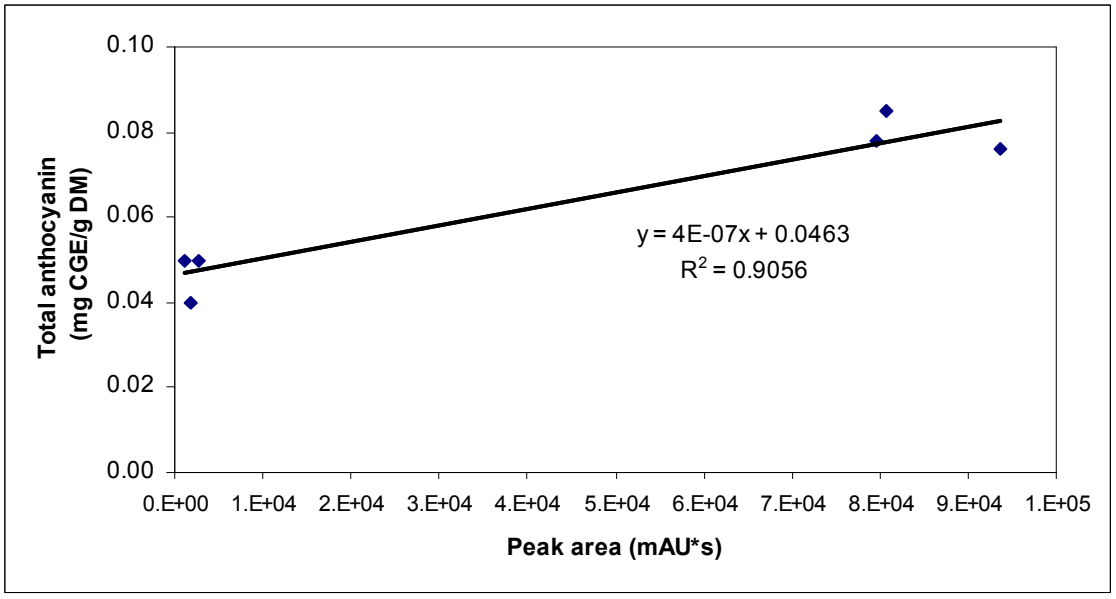


Figure 15- Relationship between the spectrophotometric measurement of anthocyanins and the peak area of anthocyanins (HPLC method)

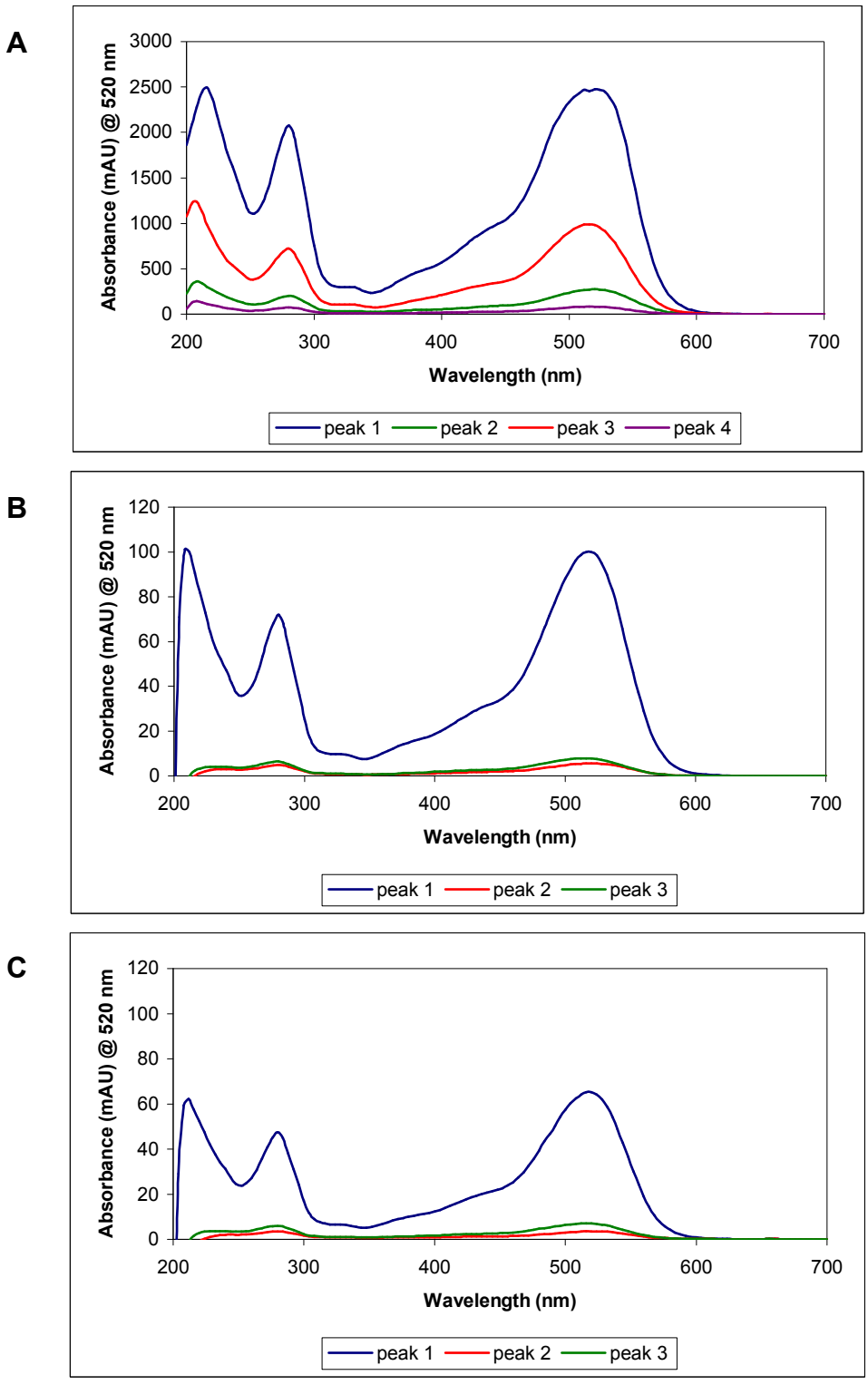


Figure 16- Spectral changes of anthocyanins: Peak 1, cyanidin-3-sophoroside; peak 2, cyanidin-3-glucosylrutinoside; peak 3, cyanidin-3-glucoside; peak 4, pelargonidin-3-sophoroside in A) Red raspberry juice; B) Raspberry Batter; C) Raspberry Muffin

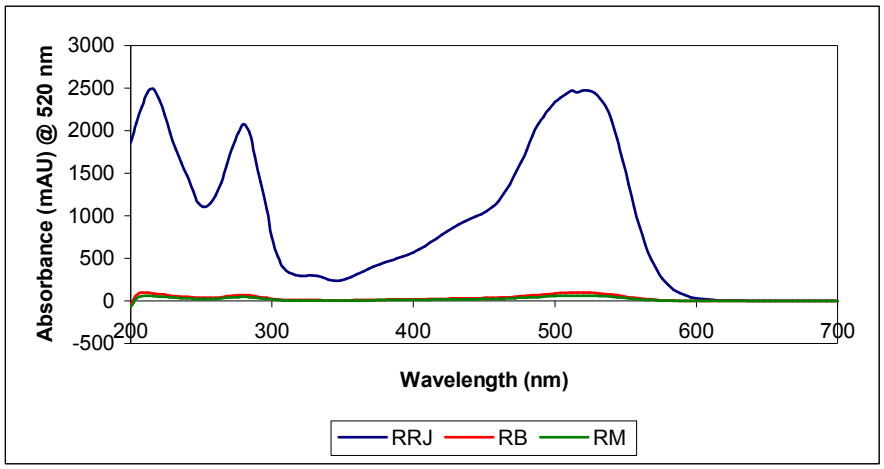


Figure 17- Spectral changes of cyanidin-3-sophoroside in RRJ, RB and RM

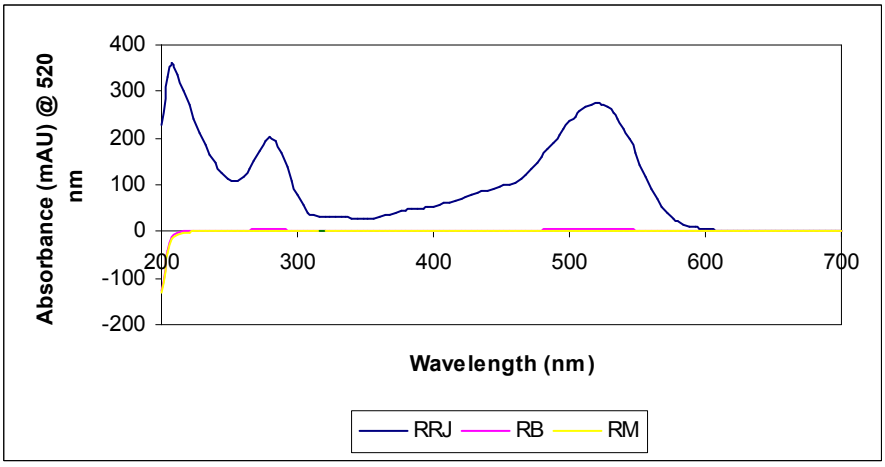


Figure 18- Spectral changes of cyanidin-3-glucosylrutinoside in RRJ, RB and RM

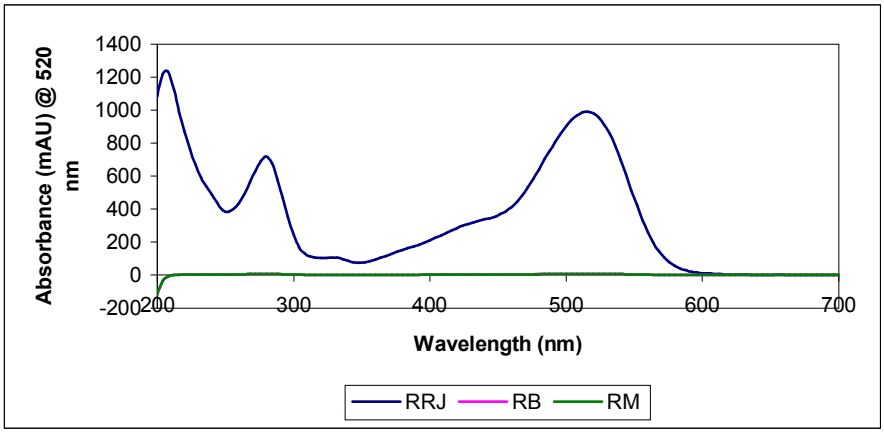


Figure 19- Spectral changes of cyanidin-3-glucoside in RRJ, RB and RM

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APPENDIX

PRELIMINARY STUDY MUFFIN PREPARATION

Considering the selected formulation for the muffin preparation, two modifications were applied in order to observe the effects on the final product. First, 10% flour was replaced by water, and second, 10% water was replaced by RRJ. Results of the preliminary experiments are shown next:

A

B

Figure 1- Control muffin prepared with 10% water replacing flour

A

B

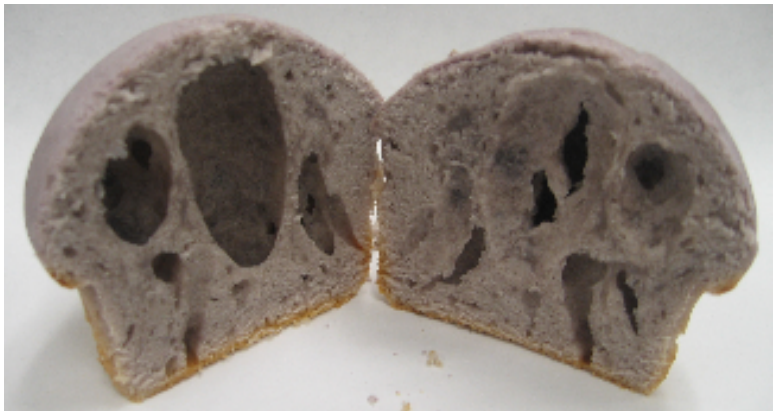

Figure 2- Raspberry muffin prepared with 10% RRJ replacing water

Replacement of flour with water did affect the volume of the final product. As is observed in **Figure 1**, the volume shown of the final product is lower than the CM prepared in this study. Although, the same baking time was used, it seems the product was not well-cooked looking at the transversal cut of the muffin. In the case of the replacement of water with RRJ, an increase of volume was observed in comparison to the low volume obtained using the formulation selected in this study. A high volume of the product could be a determinant factor to make a product more appealing to the

consumers. On the contrary, looking at the internal structure of the samples, the size of the tunnels present in the RM (**Figure 2**) increased in comparison to the sample prepared using the regular formulation.

EFFECT OF ACIDIFIED SOLUTION ON ANTIOXIDANT ACTIVITY

As was determined in the study of antioxidant activity, acidified extracts did not provide an optimum pH for the extraction of the antioxidants present in the samples evaluated. In consequence, the use of 100% methanol was determined as the preferred solvent for the DPPH antioxidant activity test because no interference was found considering that the same solvent was used for dilution of the DPPH reagent. In addition, Trolox standard was dissolved in the same solvent.

A previous study was done using a different solvent for the dilution of Trolox standard and DPPH reagent. A solution of methanol:HCl (36.5-38%) (99:1, v/v) was used for the construction of the Trolox calibration curve at different concentrations (0, 25, 50, 75 and 100 µM). The absorbance results are shown in **Figure 3**:

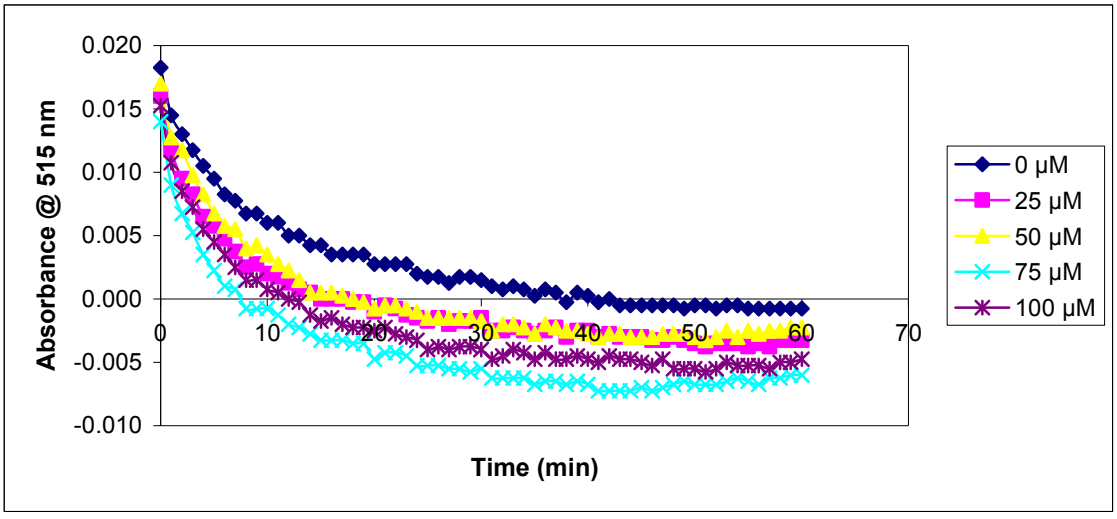

Figure 3- DPPH behavior in the presence of Trolox antioxidant standard solution prepared with an acidified methanolic solution

Figure 3 shows the reaction progress absorbance of the mixture, DPPH + Trolox solution at different concentrations that was monitored at 515 nm for 60 min. As is observed, a rapid decrease of the absorbance values were determined before 10 min of reaction to reach values below zero at the end of the evaluation time. Therefore, DPPH reagent was not stable in acidic conditions (pH < 1). In addition, DDPH dissolved in the acidified solution showed an orange color different from the violet color obtained when the reagent was dissolved in 100% methanol. No variation of the orange color was observed after reaction of the antioxidant (Trolox) with the DPPH reagent after 60 min.