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Stability and Sensory Properties of Rice Bran Peptide Fraction Incorporated Orange Juice

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STABILITY AND SENSORY PROPERTIES OF RICE BRAN PEPTIDE FRACTION INCORPORATED ORANGE JUICE
STABILITY AND SENSORY PROPERTIES OF RICE BRAN PEPTIDE FRACTION INCORPORATED ORANGE JUICE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science

By

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ABSTRACT

Functional foods offer consumers added benefits beyond the sole nutritional content of the food. Food companies are aggressively seeking new and cheaper sources of functional ingredients. Kannan et al. (2008) characterized anticancer peptides and peptide fraction from rice bran. However, information about their application and stability in food is lacking. In this study the stability of the peptide fraction in water at pH 7.2, at pH 3.5, and in orange juice was studied. The effect of the addition of the peptide fraction on the quality parameters of orange juice including sensory was also assessed. The results showed that the peptide fraction is stable at 4°C in water at pH 3.5 and in orange juice for 42 days; however, it was not stable in water at pH 7.2. A drop in pH to 5.5 from 7.2 in water and a decrease in the amount of peptide fraction were observed after 21 days. The orange juice’s pH, color, and vitamin C content were not affected by the addition of the peptide; however, the total soluble solid content was significantly lower in the control compared to the orange juice with the peptide, a possible sign for interaction of peptide fraction with the orange juice components. A triangle test and a 9-point hedonic scale test were conducted with 36 panelists on freshly prepared control orange juice and freshly prepared orange juice with peptide fraction as well as control orange juice and orange juice with peptide fraction that has been stored at 4°C for 14 days. The sensory panelists did not report any differences between the control and the peptide fraction incorporated orange juice (p=0.05838) that were freshly prepared; however, the difference in flavor was reported when stored for 14 days at 4°C. These results show that there is a potential for using orange juice as a carrier for the bioactive peptide fraction.
This thesis is approved for recommendation to the Graduate Council.

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Chapter 1—Introduction

Consumers have changed their perception about food in the last couple of decades. Food has moved from being a means to satisfy hunger to a tool that can help maintain a healthier lifestyle. Consequently, the trend in the food industry has shifted towards developing food that meets the demands of consumers. This trend is that of foods that can provide health benefits to the body functions, thus the trend of “functional foods” emerged (Day and others 2009).

To be categorized as a “functional food”, a food should convey a positive effect on the body beyond the basic nutritional values that every food usually imparts (Lennie 2001). Although there is no universal definition for “functional foods”, several descriptions have been proposed (Gray and others 2003). According to the Japanese regulatory authorities, a functional food should satisfy three main qualities: 1. be a naturally occurring substance that is not in the form of a capsule, 2. be consumable on a daily basis, and 3. impact health in a positive way by preventing diseases or enhancing the health of a body (Hardy 2000). Functional foods were defined by Hamada (2000) as materials from plant or microbial origins that have antimicrobial, anticarcinogenic, or other health promoting properties such as fiber, vitamins, polyphenols, phytic acid, tannins, and others. Fruits and vegetables are thought to be the simplest type of functional foods since they are rich in vitamins and bioactive phytochemicals such as polyphenols, anthocyanins, and carotenoids with antioxidant properties that can help reduce the risk of certain types of cancers (Day and others 2009). Although some nutraceutical components
are naturally available, some are available in by-products and need to be extracted and concentrated to be more effective.

Rice bran is the product of rice milling. The defatted residue of rice bran contains 15.4% protein (Hamada 2000). Rice bran protein is hypoallergenic, widely available, of a high nutritional value, and inexpensive; however, rice bran is only used as a cheap animal feed (Saunders 1990; Hamada 2000; Lima and others 2002).

The proteins from defatted rice bran can be used as nutraceuticals to develop new functional food products (Tang and others 2003). Rice bran extract was prepared for use as a functional ingredient (Parrado and others 2006) and rice bran peptide fraction were found effective against human colon and liver cancer cell lines (Kannan and others 2008). This latter study focused on the benefits of rice bran peptide fraction consisting of a pool of peptides that were less than 5 kDa in size, which resulted in significant inhibition of human cancer cell lines by up to 80% of growth compared to controls that were not treated by rice bran peptides. The peptide fraction were prepared to gastrointestinal resistance. Therefore, the peptides fraction can potentially be added to food products, be consumed, and remain intact after passing the human gastrointestinal tract. However, the application of the bioactive peptide fraction in a food product and the stability of the peptide fraction in the food need further investigation.

Since the rice bran peptide fraction is soluble in water, they can be added to beverages. Orange juice is the preferred fruit juice among American consumers and hence, can serve as a vehicle to deliver bioactive peptides (Pollack and others 2003).
Shelf life is a very important factor for every product in the market; it is a part of the product development process and can give very important data on the stability of a product on shelf. Several quality parameters are conducted for a shelf life study. For orange juice, color, soluble solids, and sensory properties are the major parameters among others that may affect the life of the product in the market (Polydera and others 2003).

The consumers have the option to choose among several products in the market. Competition is increasing and only 10 to 20% of new products will survive more than one year in the market (Moskowitz and others 2006). Since food flavor and overall acceptability are the most important factors when a consumer purchases a product (Luckow and Delahunty 2004), it is important to conduct a sensory analysis on products during development for it to be successful in the market.

The objectives of this study were therefore to:

1. Test the stability of the peptide fraction in water at pH 7.2 and pH 3.5

2. Prepare orange juice from concentrate and test the peptide fraction’s stability in orange juice

3. Study the sensory attributes of orange juice compared to peptide fraction incorporated orange juice.
References


Moskowitz HR, Beckley JH, Resurreccion AVA. 2006. Sensory and consumer research in food product design and development. Wiley Online Library.


Chapter 2—Literature review

1. Consumers and functional food purchase

When a consumer is in a store picking up a product from the shelf, the decision to purchase is made in a matter of seconds. Those few seconds will decide the product’s success or failure in the market. Wennström (2009) presents in his book, “Four Factors of Success”, the four decision making questions a consumer asks himself before buying a product:

1-“Do I need this product?”. Is a question asked for all products purchased by consumers. Just like any other products purchased, functional foods should be a product that offers the consumer an added value or benefit.

2-“Do I accept the ingredients in this product?”. The consumers have sometimes some knowledge about the ingredients that certain products contain. The knowledge that consumers have could have come from various sources and can affect the decision of purchase of a product. If the consumers are not familiar with the ingredients in a product, they might base their decision on whether they understand the benefits of the ingredients and if this information is not available, then they might not buy the product.

3-“Do I understand the benefits this product offers me?”. If the consumers understand how the product affects their health they are more likely to buy the product.

4-“Can I trust this brand?”. Brands that consumers are already familiar with will be trusted faster than new brands that are introduced, especially when it comes to trusting
product containing new ingredients. It is essential to deliver enough information about the product for the consumers to gain their trust.

2. Functional foods market

The rising cost of healthcare, the increase in baby boomers average age, and the increase in life expectancy create a higher demand for functional food to improve health and avoid disease (Gray and others 2003).

According to a report published in 2009 by Marigny Research Group, Inc. (MRG), consumers are taking a more proactive approach when dealing with their health. They do not wait until they get sick to treat themselves; they want to avoid that by acquiring a healthy lifestyle, by exercising, and by consuming healthier food. The consumers are demanding products that could offer benefits beyond basic nutrition (Gray and others 2003).

Sloan (2010), investigated the top 10 functional foods in the United States. On top of the list were traditional foods including whole grains. Second were foods that are naturally rich in phytochemicals and functional ingredients, such as fresh fruits and vegetables. Since consumers are looking for ways to introduce more flavonoids and polyphenols in their diets, they are hence consuming more natural fruit drinks and blends. Healthy snacks are ranked third. Consumers enjoy snacking and appreciate the option of a healthy snack rich in grains, low in calories, and free of added sugar.

Nutraceuticals and functional foods are growing in the US at a level of 5% per year (Hardy 2000). The United States has become the biggest market for functional foods.
followed by Europe and Japan (Siró and others 2008). In 2008, 30% of all functional foods launched originated from the United States (Heller 2009). The United States of America is an appealing market for functional food with more than 50% of the worldwide functional food market (Siro and others 2008). The worldwide functional food market is expected to reach $21.8 billion in a study made by Freedonia Group. Mintel’s Global New Products Database recorded 785 new functional foods and beverages in 2008 and 770 in 2009. These products stretch to all food categories and have positive effects on a wide variety of health related issues.

Consumers are well aware of the ingredients that were used several years ago to enhance food products. These ingredients included vitamins and minerals. The acceptance of more novel functional ingredients that have health benefits, such as peptides, is increasing since people are getting more educated and familiar with these new types of ingredients (Luckow and Delahunty 2004). Therefore, it is important to focus on what consumers look for in food products which Gray (2003), summarizes in three main points: health, convenience, and pleasure. These three pillars form the base for developing a new functional food product that could have a good potential market (Gray and others 2003).

In the Western world, functional foods are seen as an extension of products with additional functionality. The market for functional food is on the increase. There is a new European Union project consensus document that provides a definition for functional foods stating that a product can be labeled functional if it was shown to effectively benefit one or more target functions of the body (Urala and Lähteenmäki 2007).
Although in the United States functional foods do not have a specific label to differentiate them from other foods in the market, the sales of functional foods and beverages in the U.S. in 2009 reached a total of $37.4bn (Sloan 2010). Table 1 shows the functional beverage market which is expected to reach around $19.7bn in the US alone by 2013.

Consumers are more attracted by food products having physiological health claims rather than psychological health claims. They tend to accept more functional foods if the food carrier itself has a healthy image and if it is widely consumed. A product that is consumed and loved by the consumers makes a good carrier (Siegrist and others 2008).

Consumers consider flavor and taste of the food product as primary attributes to base their purchases. Soft drinks and coffee are popular drinks; however, the consumption of soft drinks and coffee goes together with the health concerns related to a high consumption. Natural fruit juices are also widely consumed. On an average, 21% of the Americans consume orange juice per day (Pollack and others 2003), and 60% of the adult Americans bought a functional food or beverage in 2009 (Sloan 2010).

3. The economical crisis and its influence on the functional food market

The economical crisis in late 2008 had a big influence on the food market. It reinforced the food trends that already existed and slowed down newer trends. Functional foods and nutraceuticals have been in the food market for the past two decades and health and nutrition are two emerging trends in food and marketing (Mellentin 2009). During the economical crisis in 2008-2009 the functional food market stagnated; however, as of
2010, the functional foods and especially functional beverages market has experienced a new cycle of growth.

Since Americans were worried about the recession that hit the United States and reduced their eating-out habits, some shifted towards eating their meals at home. However, the trend of healthy foods remains very important and it is positively changing the grocery shopping habits of Americans.

4. **Beverage as a vehicle for functional ingredients**

Using the staple food of the country as a food vehicle to develop functional foods is a way to reach consumers (Fogliano and Vitaglione 2005). People consume staple foods regularly; therefore, food companies will expect to have a higher acceptance by the consumers if they use already accepted carriers. The staple food differs from one country to another. While in Italy the staple food might be pasta or tomatoes, in northern European countries they are dairy spreads and products and in Asian countries such as China, Japan, Malaysia, India, and Sri Lanka, rice is the staple food. In northern America, the melting pot of the different ethnicities offers a wide range of staple foods that cater to the different nationalities. However, research has shown that many adults in America obtain up to 20% of their daily caloric intake from beverages (Duffey and Popkin 2006).

5. **Challenges in using beverages as a vehicle**

The main challenge that is facing the functional food industry is the ongoing use of sugar and artificial ingredients that governs the beverage industry. The consumption of soft drinks has been linked to obesity in several studies (Harnack and others 1999;
Health and nutrition specialists are partly blaming high fructose corn syrup for the high obesity rates in children and teenagers (Bray and others 2004). Therefore food developers are looking at developing products that not only have functional ingredients, but are also free of any unhealthy ingredients. Milk and fruits products can therefore be good vehicles. On a single day, more than one fifth of the American population consumes orange juice which makes it an ideal product to develop as functional beverage since the consumers are already familiar with it and they accept it (Pollack and others 2003).

6. Functional ingredients in food

Several ingredients have been used as functional ingredients. Conjugated linoleic acid (CLA) is used for health and weight control (Kovacs and Mela 2006) and since it gained the ‘Generally Recognized As Safe’ (GRAS) status in 2008, it has been added to a range of food products such as milk, yogurt, nutritional bars, fruit juices, and others.

Flaxseed contains gums that play a role in reducing diabetes and coronary heart disease by acting as dietary fibers. The gums also act as viscous fibers that have the ability to reduce blood glucose response and decrease blood glucose profile. Flaxseed contains proteins that could also have a positive effect on blood glucose since they simulate insulin secretion which in turn can reduce the glycemic response. Flaxseed contains lignin phytochemicals that have been shown to have anticancer properties. Flaxseed also contains higher amounts of Omega-3 fatty acid and alpha linoleic acid (ALA) and essential fatty acids that are necessary for our body health (Oomah 2001). Flaxseed is found in granola cereals, spaghetti, brownies mix, and others. Green tea and
oolong tea showed positive results for short term fat oxidation and energy expenditure (Kovacs and Mela 2006).

If we screen the products on the market, we notice that there are several other ingredients that are used as functional ingredients; minerals such as calcium, magnesium, or iron, phytosterols to lower cholesterol levels, lactoferrin as antibacterial and antiviral agent, peptides, and probiotics. These ingredients are most commonly found in food products such as yoghurts, drinks, and cereals.

Wansink (2007) discusses in his book, “Marketing Nutrition”, whether the ingredients health claims on food labels are understood by the consumers and whether consumers are encouraged to consume healthier food by reading the health claims. The FDA health claims are generally phrased to help consumers understand the diet-health relationship of the food they are about to consume; however, the challenge remains in making consumers believe these health claims and in changing a person’s eating habits to consume “healthier” foods. To change consumers’ diet, it is important for them to understand the relationship between their health and their diet (Wansink 2007).

7. Amino acids and peptides in food products

Most foods contain proteins, peptides, and/or amino acids. Amino acids have an amino group, a carboxylic group, and a side chain group that varies from one amino acid to the other (Figure 1). Peptides are made of several amino acids linked by peptide bonds (Figure 2). Peptides can act as antioxidants, antimicrobial agents, as well as interfacial agents, flavoring agents, sweet or bitter agents (Gonzales de llano D. and others 2004).
8. Proteins, peptides and amino acids as functional ingredients

Dietary proteins are a source of many biologically active peptides. These peptides are usually inactive within the protein sequence. During gastrointestinal ingestion or food processing they are released and can influence different physiological functions. These bioactive peptides are usually 3 to 20 amino acids per molecule and are proven to have antithrombotic, anti-hypertension, and immunomodulation activity (Korhonen and Pihlanto 2003). Recently many of the bioactive compounds found in food are proteins and peptides since they are active in small doses and are not very expensive (Tewes and others 2006).

The use of proteins and peptides as functional ingredients is becoming widely popular. The proteins and peptide sources include soy, wheat, fermented milk, eggs, fish, bovine lactoferrin, and rice (Mine 2010)

Soy proteins are shown to reduce the LDL cholesterol concentration by up to 10% and also slightly increase the HDL having a positive effect on lipoprotein and plasma lipid concentrations (Clarkson 2002). Patterson (2008) studied the bioactivity of several proteins and peptides and reviewed these for the Agriculture and Agri-Food in Canada.

Some of the proteins studied are: 1. lunasin peptides of soy, it has been shown to have anticancer effects, soy proteins are also used as a nutraceutical for antiobesity, gluten and gliadin from wheat help in the regulation of the nervous system, 2. thionin peptides found in wheat, barley, rye, and oats have antimicrobial properties, 3. casein and whey peptides from bovine milk as well as ovalbumin from the egg white are ACE-
inhibitory peptides with antihypertensive properties, 4. lactoferricin, and beta-lactoglobulin proteins from milk have antimicrobial properties, 5. the bovine serum albumins from milk have anticarcinogenic properties, 6. immunoglobulin from milk and eggs have immunomodulatory properties, and 7. milk proteins have also positive effect as mineral sequestering and nervous system regulators.

Muscle peptides of certain types of fish such as salmon, sardine, and tuna have antihypertensive activities (Patterson C.A. 2009). Other fish such as mudfish, catfish, and sole contains peptides that have antimicrobial properties. Rice proteins oryzatensin showed an immunomodulatory property. Other food such as peas, chickpeas, potatoes, soybean, and lupin also contain proteins and peptides that have been shown to have positive nutraceutical effect on the human body in studies (Patterson C.A. 2009). Table 2 summarized the information by listing the benefits of each protein.

9. Rice grain and rice bran peptides

Rice (Figure 3) is a grain from the family of Gramineae, genus Oryze (Ensminger 1995). It is considered as the staple food for more than half of the world’s population (Hui 2000). Every year, more than 700 million tons of rice are produced in hundreds of countries (IRRI 2011).

The amino acids in rice protein were compared to those of casein and soy protein isolate in fulfilling the amino acid requirements for children between the ages of 2 and 5 years old. The amino acids in rice protein were found to be better than those in casein and soy protein (Wang and others 1999).
Rice bran is the outer layer of the grain (Herbst and Herbst 2007). It constitutes the pericarp and germ of Oryza sativa seeds and represents 8% of the rice grain. Rice bran is the result of the removal of the outer brown layer from brown rice by the use of an abrasive milling which will result in the white rice grain. The brown layer that is separated is known as the rice bran (Saunders 1990).

In the United States alone more than 500,000 tons of rice are produced annually (Ali and others 2010). The bran is made up of fibers (such as hemicelluloses and glucofructans), ash, enzymes, vitamins, and storage proteins (Ali and others 2010). Scientists have been researching the benefits of rice bran and found that is has many potential benefits such as high nutritional value and high protein content (Saunders 1990).

Rice bran is rich in vitamin B, minerals, soluble fibers, and oils which all impart health benefits (Hamada 2000). Recent studies showed that rice bran can help reduce cholesterol levels (Herbst and Herbst 2007). It was also found to be a good source of protein, fat, and antioxidants. The bran contains between 12 to 20% protein that are considered the best quality proteins of all cereals (Saunders 1990; Ockerman 1991). Rice bran is also rich in hypoallergenic proteins. The major proteins in rice bran are albumin and globulin (Prakash 1996). Rice bran proteins contain high amounts of lysine, an essential amino acid, an amount higher than those found in any other cereal grain (Juliano 1985).

Rice bran contains lipase; however, lipase can cause fat oxidation and results in rancidity in foods. To avoid this, lipase is usually denatured. The defatted rice bran is also
stabilized to decrease the free fatty acid levels. It can be stabilized by various methods such as dry extrusion or heat. The result of heat stabilization is the heat-stabilized defatted rice bran product also known as HDRB.

There is an increase in the demand for cheaper sources of protein that can be incorporated in food, and research on going to help produce more functional food at a lower cost (Chandi and Sogi 2007). Since rice bran is a cheap by-product it can be economical source of nutrients including high quality proteins that can be used to obtain functional peptides.

Rice bran proteins have been used in a wide variety of food products aiding in product development. Some examples include rice bran protein as flavor enhancers. Glutamines and asparagines are deaminated and serve as perfect flavor enhancers for food products. Rice bran proteins are also widely used in bakery products to help in dough consistency (Ali and others 2010).

10. Preparation of anticancer peptide fraction from heat stabilized defatted rice bran (HDRB)

Kannan et al. (2008) prepared anticancer peptide fraction from HDRB. The HDRB was hydrolyzed using alcalase enzyme. The solution was then centrifuge and the supernatant was collected and passed through gastric and intestinal juice simulated treatments to obtain protein hydrolysates that were then fractionated. The fractionation was carried out using a Romicon ultrafiltration system. The hydrolysate solution was run sequentially through columns of 50000 Da, 10000 Da, and then 5000 Da, respectively.
The resulting solution that passed through the 5000 Da was collected, freeze-dried, and stored at 4°C.

Kannan et al. (2010) went further in the study by purifying a peptide that has showed a high anti-cancer activity. The amino acid analysis determined its sequence made of 5 amino acids.

However, the purification procedure is labor intensive and therefore the entire peptide fraction below 5 kDa that have shown gastrointestinal resistance and anticancer properties were used in this study.

11. Peptide fraction in orange juice

Orange juice is the number one juice that is consumed in the United States. Americans consume two and a half times more orange juice than apple juice which is ranked in number two (Pollack and others 2003). Therefore orange juice is an already popular product that consumers enjoy. Orange juice is almost 88% water which makes it a good vehicle for nutrients supplementation especially for those ingredients that are water soluble. Different brands of orange juice have already been fortified with Omega 3 (Tropicana, healthy heart), plant sterols (Devaraj and others 2004), vitamins and minerals. Minute Maid® is fortified with up to 16 essential vitamins and minerals including vitamin A, D, E, B, Zinc, Selenium, Calcium, Phosphorus, Magnesium, Chromium (Tangpricha and others 2003), and fructooligosaccharide (Renuka and others 2009).
Peptide fraction is readily soluble in water and therefore can be incorporated in many water based beverages. Water can be a first choice to test the peptide fraction stability. Since orange juice is a popular beverage choice, orange juice can also be tested as a carrier and test if the peptide fraction addition affects the deterioration process of the product.

12. **Orange juice shelf life and deterioration**

Food naturally deteriorates; however, food scientists try to slow down the process of deterioration to maximize the shelf life. Physical deterioration can frequently occur during handling, and results in products getting bruised, especially fruits and vegetables. Chemical or microbiological spoilage can also be triggered by a physical damage. Dented cans and opened containers can lead to enhanced chemical or microbiological deterioration.

Chemical deterioration such as oxidation occurs and could result in off-flavors such as fat oxidation, loss in color for example color pigment oxidation, or vitamin oxidation such as vitamin C loss in citrus juice. Oxidation in the orange juice can lead to the Maillard reaction which will result in change in color of the juice.

Microbiological deterioration results from spoilage bacteria such as lactic acid bacteria that were found to be spoilage bacteria in some fruit juices. *Lactobacillus* spp. and *Leuconostoc* spp. can cause undesirable flavors and odors in the juice though they are unlikely to flourish in orange juice because of the high sugar concentration and low storage temperature (Polydera and others 2003). Acetic acid bacteria as well as yeast and
molds can spoil the juice if it is not held at appropriate low temperature. Yeasts can spoil the juice even when chilled; therefore, it is important to pasteurize the juice (Polydera and others 2003). However pasteurization could also lead to loss in color and ascorbic acid, and could affect the organoleptic properties of the orange juice (Polydera and others 2003). That is why monitoring the pasteurization is crucial.

13. Evaluation of functional products in general and of functional peptide fraction incorporated in orange juice

Sensory science is defined as: “A discipline dealing with human sensory perceptions of and affective responses to food, beverages and their components.”(Tuorila and Monteleone 2009). It uses the five senses of sight, smell, taste, touch, and hearing to answer questions related to discrimination, description, and preference of a food product (Carpenter and others 2000).

There are several tests under the sensory analysis such as duo-trio test, triangle test, ranking test, magnitude test, hedonic rating, ratio scales, and others. Each of these tests has a specific purpose to help scientists in developing a product or controlling the quality of a product (Carpenter and others 2000).

There are many biases and error that could occur during a sensory test, these include: association effects, presentation order, expectation, habituation, distractions, and others. However sensory scientists usually design their experiments in a way to minimize the effect of these biases and analyzed their data correctly and draw relevant conclusions (Carpenter and others 2000).
In sensory science descriptive methods are used to differentiate between products whereas quantitative descriptive uses independent panelists who give scores and statistical analysis follow to get conclusions (Tuorila and Cardello 2002). Each method serves a different purpose. In product development acceptance and preference tests are conducted (Moskowitz and others 2006).

In the acceptance test, panelists are given a scale, verbal or numerical, and are asked to give a grade to the product they are given. The most common used scale is the 9-point hedonic scale. The sample is given for the panelists and they have to rate the sample by choosing a point on a scale ranging from “dislike extremely” to “like extremely” for different attributes of the product.

Whereas in preference tests, the panelists are given more than one sample at the same time and are asked to give their opinion on which one they prefer. In case more than two samples are offered, the panelists are asked to rank them or find the odd one out (Drake 2007). The triangle test is an example of a preference test. There are usually two samples A and B and to each panelist we give two As and one B or one A and two Bs and the panelist is asked to choose the sample he thinks is different from the other two.

Verbeke (2006) found that consumers are generally not willing to sacrifice intrinsic product attributes even if the product is labeled as functional and healthy. Consumers are still looking for taste, aroma, and flavor before healthiness when purchasing a product (Gray and others 2003). Therefore, to be able to keep the products successful in the market, it is very important to respond to consumers’ demand for high sensory attributes of products (Verbeke 2006). The sensory attributes of the product such
as appearance, flavor, and smell should be the center of the product development objectives and ranked before the nutritional aspect of the products (Day and others 2009).

Consumers tend to accept and like the product more whenever they are informed of its actual health benefits and when they can feel a tangible and concrete health benefit when they are having a blind test and comparing two products one containing phytonutrients and the other not (Frewer and others 2003). Urala et al. (2007) said that the reward that is perceived from the functional food consumption is the strongest reason for consuming them. However, studies have shown that consumers are first drawn by the taste of the product and then by its health benefits (Tuorila and Cardello 2002; Urala and Lähteenmäki 2007).
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Table 1. United States Functional Drinks Market Value -USD Billions (2007-2013E)

<table>
<thead>
<tr>
<th>Year</th>
<th>U.S. functional beverage market value (USD Billions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>6.0</td>
</tr>
<tr>
<td>2008</td>
<td>7.6</td>
</tr>
<tr>
<td>2009 E</td>
<td>9.7</td>
</tr>
<tr>
<td>2010 E</td>
<td>12.2</td>
</tr>
<tr>
<td>2011 E</td>
<td>14.8</td>
</tr>
<tr>
<td>2012 E</td>
<td>17.0</td>
</tr>
<tr>
<td>2013 E</td>
<td>19.7</td>
</tr>
</tbody>
</table>

Reference: Adapted from DATAMONITOR: functional drinks in the U.S. (December 2008)
Figure 1. Alpha amino acid generic structure

Figure 2. Peptide structure: formed of five amino acids.
Table 2. Peptides and proteins from various plant and animal sources and their health benefits. (Clarkson 2002; Patterson C.A. 2009)

<table>
<thead>
<tr>
<th>Peptide/protein</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy proteins</td>
<td>Reduce LDL cholesterol and increase HDL, and antiobesity</td>
</tr>
<tr>
<td>Lunasin peptides of soy</td>
<td>Anticancer effects</td>
</tr>
<tr>
<td>Gluten and gliadin from wheat</td>
<td>Regulation of the nervous system</td>
</tr>
<tr>
<td>Thionin from wheat, barley, rye, and oats</td>
<td>Antimicrobial properties</td>
</tr>
<tr>
<td>Casein and whey peptides from bovine milk and ovalbumin from egg white</td>
<td>ACE-inhibitors with antihypertensive properties</td>
</tr>
<tr>
<td>Lactoferrin and beta-lactoglobulin from milk</td>
<td>Antimicrobial properties</td>
</tr>
<tr>
<td>Bovine serum albumins from milk</td>
<td>Anticarcinogenic properties</td>
</tr>
<tr>
<td>Immunoglobulin from milk and eggs</td>
<td>Immunomodulatory properties</td>
</tr>
<tr>
<td>Milk proteins</td>
<td>Positive effect as mineral sequestering and nervous system regulators</td>
</tr>
<tr>
<td>Fish muscle peptides (salmon, sardine, tuna)</td>
<td>Antihypertensive activity</td>
</tr>
<tr>
<td>Mudfish, catfish, and sole peptides</td>
<td>Antimicrobial properties</td>
</tr>
<tr>
<td>Rice proteins oryzatensin</td>
<td>Immunomodulatory properties</td>
</tr>
<tr>
<td>Peas, chickpeas, potatoes, soybean, lupin proteins and peptides</td>
<td>Positive nutraceutical effects of the body</td>
</tr>
</tbody>
</table>
Figure 3. A longitudinal section of a rice grain and its components: endosperm (70%), bran (8%), germ (1%). (Encyclopædia Britannica 1996).

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CHAPTER 3 - The Stability and Shelf Life of Rice Bran Anticancer Peptide Fraction in Water at pH 3.5, at pH 7.2, and in Orange Juice, and the Quality Attributes of Orange Juice with Peptide Fraction

1. Abstract

The functional beverages sales rose to $22 billion in 2010 and are expected to be higher in 2011. Recently, peptide fraction and pure peptide from rice bran were characterized with anti-cancer properties on human cancer cell lines. The overall goal was to use the bioactive rice bran peptide fraction (<5kDa) in a beverage as a functional ingredient. The specific objective of the study was to evaluate the stability of the peptides in water at its pH of ~7.2, at pH 3.5, and in orange juice; then, to evaluate the quality attributes of orange juice with and without peptide fraction during storage. Three replicates of each treatment were prepared with a concentration of 3000 µg/mL of peptide fraction. The samples were stored at 4°C and evaluated at 0, 1, 3, 7, 10, 14, 21, 28, 35, and 42 days. The changes in pH and peptide fraction concentrations were tested for the water sample; while the peptide fraction incorporated orange juice were evaluated for pH, color, total soluble solids, vitamin C concentration, and peptide fraction concentration. The results showed that the concentration of peptides was reduced to nearly half the concentration and the pH dropped from 7.2 to 5.5 after 28 days, possibly due to hydrolysis which lead to instability. The peptide fraction was more stable at a pH of 3.5 after 42 days and the pH of the solution remained constant throughout the study. The orange juice with the peptide fraction showed similar quality attributes (pH, color, and vitamin C content) when compared with the control orange juice. Furthermore, the concentration of the peptide fraction in orange juice remained comparable to the
concentration of the peptide fraction in water at pH 3.5. This study demonstrated that the <5kDa peptide fraction was stable at acidic pHs of orange juice for a period of at least 42 days.

2. Introduction

a. Functional foods market

Food has moved from being a sole source of energy to also being a source of biologically active compounds with health benefits. Consumers are interested in getting more information about the nutritional benefits of foods. They are becoming more concerned about the food they eat and want to avoid diseases by maintaining a healthy diet and lifestyle. Therefore, the demand for food that is healthy and offers additional benefits such as added antioxidants or vitamins is increasing.

Functional foods are foods that offer consumers added benefits beyond the nutritional content of the product (Gray and others 2003; Granato and others 2010). The functional food market witnessed an increase of 2.7% between 2008 and 2009 (Sloan 2010). The functional beverage market is also growing; according to Mintel group, it is worth $8.6 billion and is expected to grow by 19% by 2014 (Koski 2010). Moreover, The U.S. sales of functional beverages in 2010 alone were over $22 billion, with half of adult buyers consuming functional drinks (Sloan 2011). Different types of new food ingredients are now considered nutraceutical ingredients since they prevent diseases or enhance health.
Functional foods are most popular in Europe, Japan, and the United States. In most countries functional foods are under strict regulations. In Japan the label FOSHU (Food for Specified Health Uses) is found on food products that are mainly marketed for their health benefits (Siró and others 2008). In the United States, functional foods are not differentiated from other food products and have no specific label. The ADA (American Dietetic Association) claims that all food can be functional foods to some extent when a person consumes varied types of food in moderation. The health claims on food labels are monitored by the FDA (Food and Drug Administration) and can play the role of informing the consumer about specific health benefits of various types of food. As an example, foods that contain antioxidants or vitamins will have labels that provide information on the added ingredients and their health benefits. The FDA health claims include a relationship between food and health such as the consumption of calcium and decreased chances of osteoporosis, consumption of oats and decrease risks of heart disease, and many others. One of the sources of bioactive ingredients is rice bran.

b. Rice bran as a source of functional ingredients

The rice grain has an outer layer called the rice bran which is a by-product of rice milling. Rice bran is a by-product of rice milling; it is cheap and used as animal feed (Piyaratne and others 2009)(Piyaratne and others 2009; Piyaratne and others 2009; Piyaratne and others 2009). Rice bran is rich in vitamin B, minerals, soluble fibers, and oils which all impart health benefits (Hamada 2000). Recent studies showed that rice bran can help reduce cholesterol levels (Herbst and Herbst 2007). It was also found to be a good source of protein, fat, and antioxidants. The bran contains between 12 to 20% protein that are considered the best quality proteins of all cereals (Saunders 1990;
Ockerman 1991). The major proteins in rice bran are albumin and globulin (Prakash 1996). They contain high amounts of lysine, an essential amino acid, higher than those found in any other cereal grain (Juliano 1985). Kannan et al. (2008) studied rice bran protein hydrolysate, the <5 kDa peptide fractions showing anticancer activities on human colon and liver cancer cells. To deliver these bioactive ingredients to consumers, a food vehicle is needed.

c. **Choice of food as vehicle for functional ingredients**

Beverages are simple to manipulate and control when it comes to functional food. Since beverages usually contain fewer ingredients than other solid foods, ingredients interaction can be minimized (Day and others 2009).

Fruit juices are thought to be a good medium for serving functional beverages (Tuorila and Cardello 2002). Fruit juices are positioned as a healthy product in the market and the fruit juice market is very big (Luckow and Delahunty 2004). Orange juice is the number one juice that is consumed in the United States; it is consumed 2.5 times more than apple juice which is ranked in number two. On average a person consumes the juice of 64.1 pounds of oranges per year (Pollack and others 2003).

d. **New product development: Monitoring ingredient interactions**

A major challenge in the food development is ingredients interaction. In functional foods, added ingredients are also part of the food system and are prone to interact with other components of the food. This could either lead to a significant change in the food attributes and potentially the consumers could reject the product. On the other hand an equally important attribute is the stability of the nutraceutical ingredient when
stored in the food product itself (Day and others 2009). If it loses its functional properties when added to the system, then the product loses its functionality. Therefore testing the stability of products in food is a first step towards developing functional food products.

Proteins and peptide have many side groups that could interact with other components in food. Measuring the quality attributes of the orange juice such as its color and its soluble solids throughout the shelf life can provide some information on the interaction of peptide fraction with components in the orange juice which could affect the quality properties of the juice. Moreover, all food products are subject to spoilage after a certain period of time. A long shelf life is always preferred, and artificial preservatives are getting a general opposition from the consumers. Therefore, the aim of food scientists is to provide products that are healthy, can last long, have high quality, are natural, and contain no additives.

The loss of one or more product constituents such as nutrients or flavors, or the formations of a certain compound such as an off-flavor are the limits that set the shelf life for certain food products. In Europe, to maintain a high quality orange juice, the Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Union require that orange juice contains at least 200mg/L of ascorbic acid at its expiration date (Polydera and others 2003).

Having a soluble ingredient is a prerequisite however the stability of the ingredient in the food product for a long period of time is crucial. Testing the peptide stability in water and monitoring the degradation pathway is important to understand how the peptide fraction can be eventually used in commercial products.
The overall objective was to develop a functional product using the anticancer rice bran peptide fraction developed by protein laboratory (Hettiarachchy) at the University of Arkansas. The first objective of this study was to determine the stability of peptide fraction (<5kDa) in water at two different pHs, the pH of water for potential use of water as a carrier and in an acidic pH of 3.5 for potential use of acidic juice drinks such as orange juice as carriers. The second objective was to determine the stability of the peptide fraction in orange juice and analyze the effect of adding the peptide fraction on the quality parameters of orange juice when compared to a control orange juice.

3. Materials and Methods

a. Materials

Rice bran peptide fraction (<5KDa) were obtained from protein laboratory, Food Science department, University of Arkansas. Orange juice concentrates were obtained from Southern Gardens Citrus Processing (Florida). 1000mL, 250-mL, and 8-mL PET bottle were purchased from VWR, International (Bridgeport, NJ). Double-distilled water used was filtered through a nylon membrane of 0.45 µm. High-Performance Liquid Chromatography with biopore C-18 preparative HPLC column, pH meter (orion), Refractometer (Bausch&Lomb), HPLC analytical column, Chroma meter (Minolta). All chemicals for HPLC were HPLC grade and purchased from Sigma (St. Louis, MO).
b. Methods

1. Preparation of a standard curve to determine peptide fraction concentrations

A preparative scale peptide-specific column (Biopore Prep ID 22 xL 250 mm parts # 34955) was used to separate peptide fraction originally derived from rice bran. The samples were run in HPLC following the method used by Kannan et al. (2010). The injection volume was 1000 µL. The gradient was changed from solvent A (0.12% TFA in deionized water) to solvent B (0.1% TFA in acetonitrile:water 50:50) following the program: 0min-5min: 100% A, 5min-60min: 90% A, 60min-75min: 30% A 75min-80min: 0% A with 2 mL/min flow rate and was monitored at 215 nm. Peak areas were used to prepare a standard curve using three difference concentrations of the peptide fraction in water: 400µg/mL, 500µg/mL, and 600µg/mL.

Figure 1 shows the standard curve with the following equation

\[
\text{Concentration (µg/mL)} = \frac{(\text{Area} + 163006)}{902.6} \quad \text{(Equation 1)}
\]

To calculate the concentration, the area of the peak at minute ~58 was recorded and the concentration was calculated using equation 1.

2. Preparing standard curves for ascorbic acid.

Vitamin C is one of the most important constituents of orange juice and the % Vitamin C is available on the label and should therefore be constant throughout the shelf life. To monitor the vitamin C concentration High Performance Liquid Chromatography (Hewlett Packard system) with C-18 column was used. The procedure used follows the
method described by TOSOH Bioscience. Water: Acetonitrile (9:1)+0.1% TFA solvent was used, 10 µl was injected at a flow rate of 1ml/min and the peaks were detected at 280 nm (TOSOH).

A calibration curve was prepared using ascorbic acid standard obtained from Sigma, USA. Three difference concentrations were prepared: 0.1 mg/ml, 0.2 mg/ml, and 0.3 mg/ml standards. Using the area of the peak in the output and the calibration curve equation, the concentration of ascorbic acid in the sample was calculated.

To determine the vitamin C concentration a standard curve (Figure 2) was plotted and had the following equation.

\[
\text{Vitamin C concentration} = \frac{\text{Peak Area} - 21.518}{0.4453} \quad \text{(Equation 2)}
\]

The area of the peaks was recorded and equation 2 was used to calculate the concentration of the vitamin C in the orange juice samples.

3. **Experimental design**

The experiment followed the statistical model of a repeated measure over time that was treated as a whole plot. The between subjects in this experiment are the treatments (orange juice with and without peptide fraction) and the split plot is time (days at which the measurements were done) (Appendix A). The test was spread over 42 days. Measurements of pH, peptide concentration, color, total soluble solids, and vitamin C concentration were made on Days: 0, 1, 3, 7, 10, 14, 17, 21, 28, 35, and 42. One sample from each triplicate of each treatment was withdrawn and the samples were rapidly
sealed and placed back in the refrigerator at 4°C. The triplicates were run through the HPLC in a random order to minimize any experimental errors.

4. Testing the stability of the peptide fraction in water

1. Preparation of the samples for shelf life peptide fraction stability

Two treatments were prepared for the study for measuring the stability of peptide fraction at pH 7.2 and pH 3.5. For the pH 7.2 study, 3 PET bottles were filled with 200 mL of DI water and 600mg (concentration of 3000µg/mL) of the peptide fraction were added to each bottle. For the pH 3.5 study, the pH of the water was modified to 3.5 using 1N HCl and 0.1N HCl, accordingly. Three PET bottles were filled with water at pH 3.5, and 600 mg of the peptide fraction were added to each bottle. All bottles were capped and tightened, mixed to homogeneity, labeled, and then stored at 4°C.

2. Peptide fraction concentration and pH measurements

The pH of each sample was recorded prior to the HPLC run to determine stability. The area of the peaks corresponding to retention times obtained from Kannan et al. (2009, 2010) was run for a period of 42 days for all samples.

5. Testing the stability of the peptide fraction in orange juice

1. Preparation of protein fraction (<5kDa) incorporated orange juice

Orange juice was prepared by mixing one part orange juice concentrates (Southern Gardens Citrus Processing, Florida) with 6.5 part water to achieve 11.8°brix orange juice, the recommended soluble solids for orange juice consistency (CFR
The soluble solids content (brix) was determined using a refractometer. The orange juice was filled into 2, 1000-mL PET bottles. To one of the bottles containing orange juice, 3g of the peptide fraction was added (3mg/mL), the other one did not contain peptide fraction and was treated as the control. The bottles were then pasteurized at 90˚C for 10 seconds. 6-mL of each treatment was distributed into 12 sterile 8-mL PET bottles to be used for the shelf life test. The bottles were sealed, labeled, stored at 4˚C.

2. Testing for quality control of the juice

Three replicates of each treatment; control orange juice and orange juice with peptide fraction were tested for the following quality attributes.

a) pH

The pH meter was calibrated with standard pH buffer solutions (pH= 4.01, and pH=7.00). Then, the pHs of the samples were measured.

b) Total soluble solids

The soluble solids were determined using a refractometer.

c) Color

Color was measured using a chroma-meter. The chroma-meter was standardized using a white color. The orange juice samples were measured in triplicates. The L*, a*, b* color values were recorded.

To determine the grade of quantitative difference of Hue parameter with reference to grey color the chroma (Cr.) was calculated as follow.

\[ Cr = (a^2 + b^2)^{1/2} \]
The hue angle $\vartheta_H$ is the qualitative attribute of color and it defines the difference of a color compared to grey and was calculated as follow

$$\vartheta_H = \arctan(b/a)$$

d) Sample preparation for HPLC

HPLC was used to measure the vitamin C concentration and the peptide fraction concentration in orange juice. To prepare the samples for HPLC, they were centrifuged at 7000 x g for 5 minutes. The supernatant was then filtered through 25 µm syringe filter into the sample holders. The concentration of peptide fractions remained the same after centrifugation and filtration; this was tested by comparing the concentration added and HPLC peak areas.

e) Vitamin C content

Vitamin C content of the orange juice samples was quantified using HPLC (Hewlett Packard system) with C-18 column using the same method described for the standard curve preparation.

f) Peptide profile

The HPLC method used by Kannan and others (2010) was used to study the stability of the peptides. The same method used to prepare the standard curve for peptide fraction concentration was followed.

g) Microbiology

To test the effect of pasteurization in controlling microorganisms in orange juice, a microbiology test for total plate count was done. Three replications were taken
randomly from different bottles. The samples were diluted using 0.1% (w/v) peptone water and were plated onto total plate count agar (PCA) to determine Total Plate Count. The PCA plates were incubated at 30˚C for 48 hours and then the colonies were counted (Evrendilek and others 2000).

6. Statistical analysis

The data were analyzed using JMP® 9.0. Each treatment was run as separate experiments however the conditions were close enough to be able to compare. The model is a split plot where the between subject is the treatment and the within subjects is time. However time cannot be randomized between each treatment; therefore, the randomization comes from the replications within the treatments.

The model repeated measures through time was treated as a whole plot where the response is Y. The replications were nested in treatments and randomized to give the error variation within bottles. This model is used to analyze all the data unless otherwise specified.

\[ Y_{ijk} = \mu + \alpha_i + \rho_{ij} + \gamma_k + (\alpha\gamma)_{ik} + e_{ijk} \]

where

- \( i \) = “pH 7.2”, “pH 3.5”, control orange juice, orange juice with peptide fraction
- \( \rho_{ij} \) is the error coming from the between subjects randomization of replication in treatments
- \( k = 0, 1, 3, 7, 14, 17, 21, 28, 35, 42 \)
$e_{ijk}$ is the residual error

Therefore each response will be analyzed to see if it was statistically different between the treatments, if it was statistically different with time, and if it was statistically different with time by treatment interaction meaning if one treatment was acting significantly different from the other treatment with time.

4. Results and Discussion

c. Stability of the peptide fraction in water

1. pH stability

The pH of the tap water “as is” was ~ 7.2 and this is designated as “pH 7.2”. The water at pH 3.5, designated “pH 3.5” was chosen because the orange juice beverage and many fruit juices are acidic and their pH ranges ~ 3.5. More specifically the pH of orange juice, a potential vehicle for the rice bran peptide fraction, varies usually between 3.0 and 4.0.

Figure 4 shows the pH stability of the two treatments within the 0 - 42 days’ time period. The Figure shows that the “pH 3.5” remained almost constant throughout the 42 days of testing; the small variations observed can be due to technical errors. In the “pH 7.2” peptide fraction the pH was stable for 10 days and then it gradually dropped until 28 days and then remained almost unchanged until Day 42.

The pH stability test was highly significant between Treatments, Time and Time*Treatment. The pH is significantly different between treatments because the two
treatments had varying pHs “3.5” and “7.2” at the beginning of the experiment and their pHs remained different until Day 42 “3.5” and “5.5”.

To understand the variations in each of the treatment, we looked at the test detail that compared “Day 0” of “pH 3.5” with each consecutive day. The comparison of “Day 0” to the end of the test “Day 42” was not significant; therefore, the pH remained constant after 42 days.

As for “pH 7.2”, the comparison between pH of “Day 0” and “Day 42” was significant (Appendix B for detailed statistical analysis results). At the end of the study, the pH of the solution reached ~5.5. These changes in pH could lead to structural changes and amino acid release which might lead to a loss of the bioactivity of the peptide (Korhonen and others 1998).

The pH was more stable in “pH 3.5” however; the concentration of the peptide fraction still remains the most important factor for determining the shelf life of the product since the concentration factor refer to the added functional ingredient in a product.

2. Peptide fraction stability

Figure 5 shows the concentration of the peptide fraction from “Day 0” to “Day 42”. The concentration of the peptide in treatment “pH 7.2” remained stable until “Day 14”; however, in “pH 3.5” it remained constant until the end of the study. In the treatment “7.2” as of day 17 the concentration started to drop. The standard deviation also increased and this was mainly due to the concentration of one of the triplicates, replication1, which
dropped faster than the other two, dropping to zero by Day 28. The container of this replication cracked during handling and the content of the bottle was transferred to another bottle; therefore, the increased subjection to ambient air might have caused a contamination and increased the rate of degradation of the peptide fraction. If this replication was considered as an outlier, the concentration would have still dropped, however at a slower rate and the standard deviation would have decreased as it is shown in Figure 6.

For the analysis, the replication 1 of “pH 7.2” will be considered an outlier and the data will be excluded. To be able to analyze the data one replication of the treatment “pH 3.5” was randomly selected and the data was excluded from the analysis.

The ANOVA showed that the change in concentration was significant (p<0.0001). The concentration of the peptide fraction seems to be significantly different through Time (p<0.0001) and throughout Time*Treatment (p<0.0001).

The “Day 0” was compared to each of the following measurement days in the treatment “pH 7.2”. The results showed that as of “Day 21” and until “Day 42” the concentration significantly decreased. The peptide fraction was stable in the “pH 7.2” for 21 days after which the degradation resulted in a decrease in concentration significantly (More detailed statistical analyses in Appendix C).

The pH and the peptide concentration seemed to have a relationship. The drop in pH was accompanied by a drop in peptide fraction concentration in the “pH 7.2”
treatment. The treatment “pH 3.5” seems to have had a similar parallelism between pH and peptide fraction concentrations where both seemed to be stable throughout the study.

In this study the concentration and the stability of the peptide fraction were based on the concentration and stability of the < 5kDa fraction characterized by Kannan et al. (2008). At pH 7.2, glutamic acid and aspartic are negatively charged and if available in the peptide fraction, they could have moved the pH of the solution to a more acidic region. Since the drop in the pH was accompanied by a drop in peptide concentration it is likely that the release of glutamic acid and aspartic acid from the peptide chain might have lead to a drop in the peptide concentration. An amino acid analysis needs to be done to confirm this.

The pH seemed to play an important role in the stability of peptide fraction in solutions. The peptide fraction was more stable at an acidic pH of 3.5 rather than at neutral water pH of 7.2. Since the “pH 7.2” dropped to a pH of 5.5 and stabilized, it would be interesting to investigate the stability of the peptide fraction at lower pHs between 3.5 and 5.5 to find the optimum pH at which the peptide fraction are most stable for an extended period of time. The activation energy and therefore the degradation of different peptides can vary with varying pHs and varying temperature (Bell 1997). Aspartame is a commonly used dipeptide in beverages, especially carbonated beverages that have low pH that is around 2.6 (Jaruratanasirikul and Kleepkaew 1997; Lin and others 2008); however when tested beverages with higher pHs such as dairy beverages with pH 6-7, aspartame has lower stability. At various pHs the stability of the peptide can
be altered by various mechanisms (Oliyai and Borchardt 1993). Therefore finding the pH at which the peptide fraction is most stable at is crucial for product application.

Based on the findings, beverages that have pHs close to 3.5 should be considered as potential carriers for the peptide fraction since the peptide fraction showed better stability at this pH rather than at a pH of 7.2. Orange juice pH ranges between 3.0 and 4.0, it is a popular drink that is widely available and could be an ideal functional product for anti-cancer rice bran peptide fraction. Therefore to evaluate the stability of the peptide fraction in orange juice a shelf life study was conducted.

d. Quality attributes of orange juice with and without peptide fraction

3. Orange juice safety

No microbiological growth was observed in any of the samples showing that the pasteurization was effective in inhibiting the growth of spoilage pathogens. All samples showed a total count of less than 20 cfu/mL which is within acceptable limits (Sharma and others 1998).

To evaluate the stability of peptide fraction incorporated orange juice, the analysis studied and contrasted the pH, Hue, Chroma, total soluble solids and vitamin C of control orange juice and orange juice with peptide fraction.

4. pH stability

The test effects show that the pH was only significant through Time. That means that the treatments are not acting differently with time; however, the pH of both
treatments, on average, is changing throughout the time. Figure 7 shows that both samples are showing similar data on each measurement day.

To monitor the changes in time within each treatment, a contrast with “Day 0” was done to compare the pH at “Day 0” with each of the consecutive days. For control orange juice and for orange juice with peptide fractions, the pH at “Day 0” was not significantly different from the pH at the final day of the test “Day 42”. Therefore the pH of the orange juice with and without peptide fractions remained constant throughout the study. Few studies have been done on the addition of peptides to orange juice; however, similar observations were reported in a study of whey protein incorporated orange juice (Kazmerski and others 2003).

5. **Color stability**

Color can be an important quality factor that can give the consumer an idea about the freshness and eventually the taste of a food product. To study the color of orange juice the analysis was based on the Lightness (L), the Chroma, and the Hue.

Figures 8, 9, and 10 show the color by three different parameters: L, Chroma, and Hue, respectively. From “Day 0” to “Day 1” there is a drop in L and chroma; and there is an increase in the Hue. The model showed that these changes are significant (p=0.0302 for Lightness, p=0.0441 for Chroma and p<0.0001 for Hue) between treatments and time. Although these changes are significant through time but they are not significant between treatments; therefore, the orange juice with the peptide acted similarly to the control orange juice and the differences seen in the color through time were seen for both samples. To verify if the color has significantly changed for consumers, a sensory test
assessing the color and appearance of the orange juice was performed as described later on in the study (More detailed statistics in Appendix E).

6. Total soluble solids stability

Total soluble solid (TSS) is an important factor in orange juice that defines the consistency of a product. In orange juice, total soluble solid is usually 11.8°brix (CFR 146.145). Figure 11 seems to show that orange juice with peptide has a higher TSS at days 7, 17, 21, 35, and 42. The analysis of the data showed that the test was significant (p<0.0001) (Figure 12); and therefore we looked at each effect’s significance.

The test effects, (Figure 13), show that the TSS is significantly different between treatment with Time and with Time by Treatment. Therefore there is interaction and the two treatments are acting differently with time.

Slicing Time by Treatment effect shows at what point exactly the difference between treatments is significant. The slicing shows that the TSS is significantly different between the control orange juice and the orange juice with the peptide fraction at days 7, 17, 21, 35, and 42. The TSS in orange juice with peptide fraction is higher than the TSS for control orange juice at Day 42. However, the consistency of the orange juice was not visibly different between the two treatments. These additions of the peptide fraction are increasing the TSS that might be due to the formation of soluble solids that are the result of the interaction of the peptide fraction with the component of the orange juice. Corrective action such as encapsulating the peptides for decreased ingredients interaction can be a solution in case the problem is persistent as is affecting the organoleptic acceptability of the orange juice.
7. *Vitamin C stability*

Vitamin C is an important constituent of orange juice. Since the label of orange juice contains the %RDA of vitamin C that is available in the bottles of orange juice; therefore, it is important to give the consumer the exact amount of vitamin C the label shows.

Figure 14 shows that both the control orange juice and the orange juice with the peptide vitamin C content drops from ~200mg/L to ~20mg/L after 3 days. The model is significant (p<0.0001); however, the change in vitamin C is only significant through Time and not through Time by Treatment. Therefore, both treatments are acting similarly and the drop in the vitamin C was not caused by the addition of the peptide fraction.

Since vitamin C is very heat sensitive, the loss of vitamin C observed in the results might be linked to pasteurization. It is reported that more than 15% of the vitamin C in orange juice can be lost in a heat treatment (Sharma and others 1998). Pressure or pulsed electric field treatments can be alternative ways for treating orange juice (Basak and Ramaswamy 1996; Torregrosa and others 2006). These methods have already showed better results in maintaining the vitamin C levels for longer periods of time. Fortification of the orange juice with Vitamin C can also be done to make sure that the orange juice will have at least the amount of vitamin C written on the label even by the end of its shelf life (Choi and others 2002). Calculations of the exact amount of vitamin C needed at manufacturing should be done to have a final product that meets the label requirements and that has ~200mg/L of Vitamin C (Polydera and others 2003).
8. Stability of peptide fraction in orange juice

Figure 15 shows the concentration of the peptide fraction in water at pH 3.5 and in orange juice. The concentrations seemed to be close until “Day 42”. At this point the peptide in orange juice dropped and the standard deviation increased. The data showed that one of the replication in orange juice reached a zero concentration of peptide fraction by “Day 42”.

The difference in peptide concentration between the two treatments was not significant. However since the peptide fraction concentration dropped to zero in one of the replication further studies while storing and monitoring the peptide fraction for a longer period of time would give a better understanding on how long the peptide fraction can remain stable in orange juice.

The peptide fraction could affect the orange juice environment by affecting the stability and other quality parameters of the orange juice. Microencapsulation can be a novel and efficient way to extend the shelf life of the peptide fraction encapsulated orange juice while protecting the organoleptic properties of the orange juice.
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Figure 1. Standard curve for peptide fraction concentration

Three different concentrations were chosen to plot the standard curves $R^2=0.9946$

Figure 2. Standard curve for vitamin C concentration

Three different concentrations were chosen to plot the standard curves $R^2=0.9983$
Figure 3. Preparation of orange juice from concentrate

*TSS: Total Soluble Solids
Figure 4. The stability of the peptide fraction (<5kDa) in water at pH 3.5 and pH 7.2

*Samples were run in triplicates ± Standard Error of the Means (SEM). JMP® 9.0 was used to analyze the data.
Figure 5. Stability of peptide fraction in water at pH 3.5 and pH 7.2

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.

Figure 6. Stability of peptide fraction in water at pH 3.5 and pH 7.2 (excluding rep 1 from treatment “pH 7.2” and excluding rep 3 from “pH 3.5”)

*Samples were run in duplicates ±SEM. JMP® 9.0 was used to analyze the data.
Figure 7. pH stability of control orange juice and orange juice with peptide

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.
Figure 8. Change in Lightness of the color of orange juice

Figure 9. Change in Chroma of color of orange juice

Figure 10. Change in Hue color of color of orange juice

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.
Figure 11. Change in Total soluble solids (TSS) in orange juice

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.
**Figure 12. ANOVA table of TSS of control orange juice and orange juice with peptide fraction**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>25</td>
<td>2.2559091</td>
<td>0.090236</td>
<td>7.8466</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Error</td>
<td>40</td>
<td>0.4600000</td>
<td>0.011500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>65</td>
<td>2.7159091</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 13. Test statistics of peptide fraction concentration of water treatments “pH 3.5” and “pH 7.2”**

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>MS Num</th>
<th>DF Num</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.12742</td>
<td>0.12742</td>
<td>1</td>
<td>3.0582</td>
<td>0.1552</td>
</tr>
<tr>
<td>Time</td>
<td>1.53091</td>
<td>0.15309</td>
<td>10</td>
<td>13.3123</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>0.43091</td>
<td>0.04309</td>
<td>10</td>
<td>3.7470</td>
<td>0.0013*</td>
</tr>
</tbody>
</table>
Figure 14. Stability of Vitamin C concentration of orange juice with and without peptide fraction

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.
Figure 15. Stability of peptide fraction in water at pH 3.5 and in orange juice

*Samples were run in triplicates ±SEM. JMP® 9.0 was used to analyze the data.
Appendix A – The experimental design model

The model: Between subjects are the treatments and the within subjects is time. The time represents the days at which the tests were done.

<table>
<thead>
<tr>
<th>Treatment pH 3.5 (Whole plot 1)</th>
<th>Treatment pH 7.2 (Whole plot 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 0</td>
</tr>
<tr>
<td>Day 1</td>
<td>Day 1</td>
</tr>
<tr>
<td>Day 3</td>
<td>Day 3</td>
</tr>
<tr>
<td>Day 10...Day 28</td>
<td>Day 10...Day 28</td>
</tr>
<tr>
<td>Day 35</td>
<td>Day 35</td>
</tr>
<tr>
<td>Day 42</td>
<td>Day 42</td>
</tr>
</tbody>
</table>
Appendix B - pH in water statistical analysis

Table 1 shows the ANOVA table for the pH test. The test is highly significant with a p-value of <0.0001. The $R^2$ is 0.998 showing that more than 99.8% of the variation is accounted for by the model used (table 1.)

To understand the change in pH between treatments and time, the individual F ratios for the test are analyzed. Table 2 shows that the pH is significant between Treatments, through Time and through Time*Treatment.

The sum of square slicing was done to understand if the pH is changing in one treatment more than in the other throughout the time (Table 3.). Both treatments seem to be significant with a p-value of 0.0032 for the “pH 3.5” and a p-value of <0.001 for the “pH 7.2” treatment. The sum of squares for the treatment 3.5 is $SS_1=0.333$ and the sum of square of the treatment of pH 7.2 $SS_2=16.33$ therefore although both are significantly different, “pH 7.2” has a much higher significance than “pH 3.5” which was also shown in the Figure 5.

To understand the variations in each of the treatment, we looked at the test detail that compares Day 0 with each consecutive day and to compare the significance (Table 4 and Table 5). Looking at the contrasts and the significance of the test for the pH 3.5 in Table 4., only two contrast are significant at Day 21 and Day 35; however, at the end of the test which is Day 42, the test was not significant, therefore the fluctuation that are due to experimental error, such as poor calibration, might be the cause of these differences.
seen on Day 21 and Day 35. We can therefore say that the pH remained constant until day 42.

As for “pH 7.2” in Table 5, the contrasts show that the difference was significant for all days except “Day 3” and “Day 10”; however, the sum of squares of the “Day 1” to “Day 10” account for a very small part of the whole sum of square and therefore we can consider that as of day 14 the difference in the pH was clearly significantly different from “Day 0”.

Table 1. ANOVA analysis of pH of water treatments “pH 3.5” and “pH 7.2”

Actual by Predicted Plot

Summary of Fit

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RSquare</td>
<td>0.998011</td>
</tr>
<tr>
<td>RSquare Adj</td>
<td>0.996767</td>
</tr>
<tr>
<td>Root Mean Square Error</td>
<td>0.100157</td>
</tr>
<tr>
<td>Mean of Response</td>
<td>4.900303</td>
</tr>
<tr>
<td>Observations (or Sum Wgts)</td>
<td>66</td>
</tr>
</tbody>
</table>
### Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>25</td>
<td>201.29773</td>
<td>8.05191</td>
<td>802.6613</td>
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<td>Error</td>
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<td>0.40126</td>
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<tr>
<td>C. Total</td>
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<td>201.69899</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Test statistics of pH of water treatments “pH 3.5” and “pH 7.2”

Tests wrt Random Effects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>MS Num</th>
<th>DF Num</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>184.001</td>
<td>184.001</td>
<td>1</td>
<td>1167.135</td>
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</tr>
<tr>
<td>Time</td>
<td>7.86739</td>
<td>0.78674</td>
<td>10</td>
<td>78.4268</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>8.79913</td>
<td>0.87991</td>
<td>10</td>
<td>87.7148</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

### Table 3. Test slicing of pH of water treatment “pH 3.5” and “pH 7.2” by treatment

<table>
<thead>
<tr>
<th>Slice Treatment=3.5</th>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.333</td>
<td>10</td>
<td>40</td>
<td>3.3234</td>
<td>0.0032*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slice Treatment=7.2</th>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16.33</td>
<td>10</td>
<td>40</td>
<td>162.8182</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>
Table 4. Significance of the change in pH between Day 0 and subsequent days in treatment “pH=3.5”

<table>
<thead>
<tr>
<th></th>
<th>Day 0 to Day 1</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Error</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
</tr>
<tr>
<td>Prob&gt;</td>
<td>t</td>
<td></td>
<td>0.518</td>
<td>0.8395</td>
<td>0.1501</td>
<td>0.2442</td>
<td>0.1198</td>
<td>0.1025</td>
<td>0.0336</td>
<td>0.2956</td>
<td>0.0368</td>
</tr>
<tr>
<td>SS</td>
<td>0.0043</td>
<td>0.0004</td>
<td>0.0216</td>
<td>0.014</td>
<td>0.0254</td>
<td>0.028</td>
<td>0.0486</td>
<td>0.0113</td>
<td>0.0468</td>
<td>0.0014</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Significance of the change in pH between Day 0 and subsequent days in treatment “pH=7.2”

<table>
<thead>
<tr>
<th></th>
<th>Day 0 to Day 1</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Error</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
<td>0.0818</td>
</tr>
<tr>
<td>Prob&gt;</td>
<td>t</td>
<td></td>
<td>0.0278</td>
<td>0.0947</td>
<td>0.0441</td>
<td>0.4198</td>
<td>3.5e-6</td>
<td>5e-11</td>
<td>1e-14</td>
<td>1e-22</td>
<td>3e-22</td>
</tr>
<tr>
<td>SS</td>
<td>0.0523</td>
<td>0.0294</td>
<td>0.0434</td>
<td>0.0067</td>
<td>0.2904</td>
<td>0.7921</td>
<td>1.4113</td>
<td>4.15</td>
<td>3.9204</td>
<td>2.7068</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C - Peptide fraction concentration in water statistical analysis

The ANOVA showed that the change in concentration was significant (Table 6). The concentration of the peptide fraction seems to be significantly different through Time and throughout Time*Treatment (Table 7). Slicing gave a better understanding of the change of concentration of the peptide fraction within Time*Treatment as shown in Table 8.

The test slice (Table 9) shows that the concentration was not significantly different through time for the treatment “pH 3.5” however it was significant for the “pH 7.2” and; therefore, at the “pH 3.5” the peptide fraction was stable and so was the pH of the solution. To better understand how the “pH 7.2” changes with time, the contrast in table 9 compared “Day 0” to each of the following measurement days. The contrast showed that the concentration was significantly different from “Day 0” as of “Day 21”; therefore, the peptide fraction was stable in the “pH 7.2” for 21 days after which the degradation brings down the concentration significantly.
Table 6. Change in concentration by omission of rep 1 of treatment “pH 7.2” and rep 3 of treatment “pH 3.5”

Actual by Predicted Plot

Summary of Fit

RSquare 0.907887
RSquare Adj 0.801957
Root Mean Square Error 156.4662
Mean of Response 2576.05
Observations (or Sum Wgts) 44

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
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<tbody>
<tr>
<td>Model</td>
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<td>4825923.2</td>
<td>209823</td>
<td>8.5706</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
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<td>24482</td>
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<tr>
<td>C. Total</td>
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<td>5315556.3</td>
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<td></td>
</tr>
</tbody>
</table>

Table 7. Test statistics of peptide fraction concentration of water treatments “pH 3.5” and “pH 7.2”

Tests wrt Random Effects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>MS Num</th>
<th>DF Num</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>42.2371</td>
<td>42.2371</td>
<td>1</td>
<td>0.0013</td>
<td>0.9744</td>
</tr>
<tr>
<td>Time</td>
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<td>233205</td>
<td>10</td>
<td>9.5257</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>2429288</td>
<td>242929</td>
<td>10</td>
<td>9.9229</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>
Table 8. Test slicing of peptide fraction concentration of water treatments “pH 3.5” and “pH 7.2”

<table>
<thead>
<tr>
<th>Slice Treatment=3.5</th>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3e+5</td>
<td>10</td>
<td>20</td>
<td>1.0487</td>
<td>0.4416</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slice Treatment=7.2</th>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5e+6</td>
<td>10</td>
<td>20</td>
<td>18.3999</td>
<td>&lt;.0001*</td>
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</table>

Table 9. Significance of the change in peptide fraction concentration between Day 0 and subsequent days in treatment “pH=7.2”

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimate</td>
<td>55.919</td>
<td>60.965</td>
<td>139.54</td>
<td>245.27</td>
<td>351.29</td>
<td>133.28</td>
<td>-443.6</td>
<td>-968.9</td>
<td>-761.7</td>
</tr>
<tr>
<td>Std Error</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
<td>156.47</td>
</tr>
<tr>
<td>Prob&gt;</td>
<td>t</td>
<td></td>
<td>0.7245</td>
<td>0.7009</td>
<td>0.3831</td>
<td>0.1327</td>
<td>0.0362</td>
<td>0.4044</td>
<td>0.0102</td>
</tr>
<tr>
<td>SS</td>
<td>3126.9</td>
<td>3716.7</td>
<td>19472</td>
<td>60158</td>
<td>123402</td>
<td>17764</td>
<td>196794</td>
<td>938851</td>
<td>580170</td>
</tr>
</tbody>
</table>
Appendix D - pH change in orange juice statistical analysis

Table 10 shows the significance of each test by itself through time to be significant. The pH changed significantly for both treatments through time (Table 11). To monitor the changes in time within each treatment a contrast with “Day 0” was done (Table 12 and 13). For control orange juice, only “Day 28” pH is significantly different from the pH at “Day 0”; however, the pH of the final day of the test at “Day 42” is not significant. As for the orange juice with the peptides, the pH at “Day 14” and “Day 17” are significantly different from “Day 0”; however the last day of the study “Day 42” is not significantly different from “Day 0”. Therefore the pH of the orange juice with and without peptide fraction remained constant throughout the study.

Table 10. ANOVA graph and table of pH of control orange juice and orange juice with peptide fraction

Actual by Predicted Plot

<table>
<thead>
<tr>
<th>pH Actual</th>
<th>pH Predicted</th>
<th>R$^2$=0.70</th>
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<tr>
<td>3.4</td>
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<td></td>
</tr>
<tr>
<td>3.6</td>
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<td>3.8</td>
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<td>4.2</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

Summary of Fit

- RSquare: 0.697624
- RSquare Adj: 0.508639
- Root Mean Square Error: 0.105457
- Mean of Response: 3.731364
- Observations (or Sum Wgts): 66
### Analysis of Variance

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<th>Source</th>
<th>DF</th>
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<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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</thead>
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<td>0.041053</td>
<td>3.6914</td>
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<tr>
<td>Error</td>
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<td>C. Total</td>
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<td>1.4711773</td>
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Tests wrt Random Effects

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<th>DF Num</th>
<th>F Ratio</th>
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**Table 11. Test slicing of pH of control orange juice and orange juice with peptide**

**Slice Treatment=OJ**

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**Slice Treatment=OJ+peptide**

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<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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</thead>
<tbody>
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<td>0.541</td>
<td>10</td>
<td>40</td>
<td>4.8646</td>
<td>0.0001*</td>
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</tbody>
</table>

**Table 12. Significance of the change in pH between “Day 0” and subsequent days in treatment OJ**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>-0.023</td>
<td>0.17</td>
<td>0.0133</td>
<td>0.0433</td>
<td>0.1267</td>
<td>0.0667</td>
<td>-0.22</td>
<td>-0.087</td>
<td>-0.06</td>
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<td>0.0861</td>
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<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
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<tr>
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<td>0.0145</td>
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<td>0.0002</td>
<td>0.0008</td>
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<td>0.0003</td>
<td>0.0028</td>
<td>0.0241</td>
<td>0.0067</td>
<td>0.0726</td>
<td>0.0113</td>
<td>0.0054</td>
</tr>
</tbody>
</table>

**Table 13. Significance of the change in pH between “Day 0” and subsequent days in treatment OJ+peptide**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0533</td>
<td>0.0333</td>
<td>-0.043</td>
<td>0.1033</td>
<td>0.2533</td>
<td>0.3333</td>
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<td>-0.043</td>
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<tr>
<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
<td>0.0861</td>
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<tr>
<td>0.5392</td>
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<tr>
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<td>0.0017</td>
<td>0.0028</td>
<td>0.016</td>
<td>0.0963</td>
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<td>0.0104</td>
<td>0.0028</td>
<td>0.0096</td>
</tr>
</tbody>
</table>
Appendix E - Color change in orange juice statistical analysis

For the response L, the model was significant with time and time*treatment significance. However when the test is sliced, looking at each time point the difference was significant between the control and the orange juice with the peptide at “Day 0” and at “Day 14”. However since the graph seems to be pretty stable after “Day 14”, especially at the end of the trial at “Day 42”; therefore, we can say that the lightness of the color between control and treatment is similar.

The chroma and the hue showed significance; however, the test was only significant through Time and not significant through Time*Treatment. Since there was not significant change between treatments and between treatment and time therefore we can consider that the orange juice with the peptide acted similarly to the control.
CHAPTER 4 - The Sensory Properties of Rice Bran Peptide Fraction Incorporated Orange Juice

1. Abstract

To assess the sensory properties of peptide fraction incorporated orange juice a triangle test and a 9-points hedonic scale test were run. 36 panelists assessed the control orange juice “as is” and an orange juice with peptide fraction that were prepared on the same day of the sensory trial, and 36 different panelists assessed the same samples that were stored for 14 days. The panelists were not able to differentiate between the control and the peptide fraction incorporated orange juice (p=0.05838) that were freshly prepared; however, the difference was detected when they were stored for 14 days at 4°C. The control scored significantly higher, 7.1 on Day 0 and 7.3 on Day 14 and a score of 6.6 for orange juice with peptide fraction on both days for overall liking. This observation was based on the flavor profile of the peptide fraction incorporated orange juice.

2. Introduction

It is reported that American adult consumers gain more than 20% of their caloric needs for the day from beverages (Duffey and Popkin 2006). Beverages are easy to consume since no preparation is usually needed. However, beverages are also the easiest form of food since they usually contain the least amounts of ingredients and have a simple formulation (Day and others 2009). Therefore the addition of bioactive ingredients can create value-added products.
The demands for healthy products have been increasing recently. The baby-boomers are getting older and more prone to disease; it is expected that between 2010 and 2030 the population of the 65 year olds and older will constitute 20% of the total population (Dziegielewski and others 2010). Also, people are getting more educated about the health benefits of specific types of food and are becoming more proactive when dealing with their health. The majority of the population is looking for alternative choices of food that will help them maintain a healthier lifestyle (Gray and others 2003). This demand for healthier food is increasing the demand for functional food products and more specifically functional beverages.

On a single day, more than one fifth of the American population consumes orange juice (Pollack and others 2003). Orange juice is consumed two and a half times more frequently than apple juice which is ranked as number two (Pollack and others 2003). Orange juice is almost 88% water which makes it a good vehicle for nutrients supplementation especially for those ingredients that are water soluble. The insoluble bioactive ingredients can be dispersed using emulsifiers in beverages. Different brands of orange juice have already been fortified with Omega 3 (Tropicana, healthy heart), plant sterols (Devaraj and others 2004), vitamins and minerals. Minute Maid® is fortified with up to 16 essential vitamins and minerals including vitamin A, D, E, B, Zinc, Selenium, Calcium, Phosphorus, Magnesium, Chromium (Tangpricha and others 2003), and fructooligosaccharide (Renuka and others 2009). Orange juice is an ideal vehicle to develop functional beverages since it is widely accepted and consumed (Pollack and others 2003).
Verbeke (2006) found that consumers are generally not willing to sacrifice intrinsic product attributes even if the product is labeled as functional and healthy. Consumers prefer pleasant taste, aroma, and flavor before healthiness when purchasing a product (Gray and others 2003). Therefore, to be able to keep the products successful in the market, it is very important to respond to consumers’ demand for high sensory attributes of products (Verbeke 2006). The sensory attributes of the product such as appearance, flavor, and aroma should be taken into consideration in new product developments and must be placed before the nutritional aspect of the products (Day and others 2009).

Proteins and peptides can have various taste effect on food products. Some peptides have been characterized as having savory flavors such as those extracted from fish proteins (Park and others 2002). Other peptides can have an umami, sweet, or salty taste. However in other cases, peptides can have a bitter taste and could affect the taste of the whole product (Li-Chan and Cheung 2010). Therefore after studying the stability of a new food product a sensory analysis should be conducted to asses any changes in sensory attributes that might have been caused by the addition of the peptide into the food or beverages of choice.

The overall objective was to add value to a functional beverage using peptide fraction that demonstrated anti-cancer proliferation in human cancer cell line (Kannan and others 2008; Kannan and others 2009). The rice bran peptide fraction was incorporated in orange juice. To get a better understanding of the potential acceptance of the peptide fraction incorporated orange juice, a triangle test and hedonic scale test were
conducted for control orange juice and orange juice with added peptide fraction and the effect of storage of the peptide fraction incorporated orange juice on the organoleptic properties of the juice.

3. Material and methods


Rice bran peptide fraction (<5KDa) (protein laboratory, Food Science department, University of Arkansas) was used as a bioactive ingredient. Orange juice concentrates were obtained from Southern Gardens Citrus Processing, Florida. 1-mL PET bottles were purchased from VWR, International (Bridgeport, NJ) for storage. Water was used for diluting the orange juice concentrate; 2 oz. plastic cups were used for sensory testing.

b. Methods

1. Sample preparation and experimental design.

Orange juice was prepared by mixing one part orange juice concentrates (Southern Gardens Citrus Processing, Florida) with 6.5 part water to achieve 11.8°brix orange juice, the recommended soluble solids for orange juice consistency (CFR 146.145). The soluble solids content (brix) was determined using a refractometer. The orange juice was filled into sixteen 1 liter PET bottles. To 8 of the bottles, 3g of the peptide fraction was added (3mg/mL) to each bottle; the other 8 bottles were left without peptide fraction as a control. The juice bottles were then pasteurized at 90°C for 10 seconds. The bottles were then sealed and transferred into an ice bath to cool.
Four bottles (PET 1L bottles) of each treatment, with and without peptide fraction, were then tested on the same day “Day 0” for the sensory attributes. The 36 panelists on Day 0 were presented orange juice with and without peptide that was prepared on the same day. The rest of the bottles were stored in the refrigerator at 4°C for 14 days. At Day 14, 36 different panelists were presented orange juice with and without peptides that have been prepared and stored for 14 days. Two different sets of panelists participated in the test since the test was done with volunteers that were available at the sensory testing premises at the time of the test.

2. *Triangle test*

IRB approval (#11-04-598) was obtained for conducting a sensory analysis with panelists. 36 panelists participated in the trial. To balance the test, the six triads of sample A: original orange juice, and sample B: orange juice with peptide fraction, were presented equally among the panelists AAB, ABA, BAA, BBA, BAB, and ABB. Each combination appeared 6 times throughout the test.

The samples were place in 2 oz. plastic cups; each plastic cup had a random three digit number assigned. The panelists were informed to start tasting from the right to the left and to choose which sample they think is different than the other two.

3. *Preference test*

The 36 panelists were given the sample A or B in a 2 oz. plastic cup and were asked to take a sip and rate the overall liking for the sample, then to rate the color, the texture, and finally the flavor of the orange juice on a 9-point hedonic scale. After they
were done with the first sample they were given water and crackers to cleanse their palate and were given the other sample to rate following the same questionnaire.

The same tests were done after the 14th day with 36 panelists. 16 of the panelists that had already participated in the test of the day 0 also participated in the test at Day “14”. In the test at Day 14, the panelists were also asked to add any comments about their likes or dislikes of the product.

The panelists were asked at the beginning of each test their overall general liking for orange juice. They were also asked demographic questions such as age, income, and sex at the end of the test.

4. Data analysis

The data were analyzed using Excel for triangle test and JMP 9.0 using a paired t-test ANOVA test to compare the score of the sample A to sample B hedonic scale.

Using Excel the p-value for the triangle test was calculated using the formula, since we had 16 correct answers form a total of 36 questions, with a probability of 1/3 of getting the correct answer by chance:

\[
p\text{-value: } = \text{BINOMDIST}(16,36,1/3,\text{FALSE})
\]

Using JMP to analyze the hedonic scale test data, a paired t-test was done for overall acceptability, color, flavor, and texture.
4. Results and Discussion

The test was run on two days Day 0, with fresh orange juice with and without peptides, and on Day 14 with orange juice with and without peptides that have been stored for 14 days at 4˚C. The test was done at the sensory laboratory at the University of Arkansas. The demographics of the test done on Day 0 showed that the majority of the panelists (61%) ranged between 18 and 35 years old (Figure 1). The set of panelists was composed of 44% males and 56% females. As for their frequency in the consumption of orange juice 83% of the panelists were reported to have consumed orange juice at least one time in the past month (Figure 3). All the participants liked orange juice, their likings ranged from “slightly like” to “extremely like”, but none of the panelists disliked orange juice (Figure 5).

The first test done was the triangle test. The hedonic test offers the panelists two samples one after the other, the panelists rank several attributes of the orange juice. To avoid “expectation errors” that is the error from knowing that panelists are presented two different samples (Carpenter and others 2000), the triangle test is done first and the panelists are given three samples and told to chose the odd one out without any guidance on whether the color, the flavor, or the texture could be different.

The total number of tests done was 36 therefore n=36, with alternating 6 combinations (AAB, ABA, BAA, BBA, BAB, ABB) of sample A and sample B. The null hypothesis would be that A and B are the same. The alternative hypothesis would be that A is different from B.
The null hypothesis was that A and B are the same with no detectable differences. The panelists had a probability of \( \frac{1}{3} \) to get the correct answer by chance. With an \( \alpha \)-level of 0.05, the p-value for the test was 0.05838 > \( \alpha \), therefore we failed to reject the null hypothesis which means that at Day 0 the samples A and B did not seem to taste differently for the panelists.

To understand if A and B could be different and to assess which of the two is preferred, the 9-point hedonic scale was conducted with the same 36 panelists. The panelists were asked to rank both samples A and B without any knowledge of which sample they were ranking first.

Table 1 summarizes the results from the hedonic scale test. The control orange juice scored significantly higher on the overall liking (7.1) and flavor (7.1) compared to the overall liking of the peptide fractions orange juice (6.6) and its flavor (6.4). The color and mouthfeel scored the same for both products samples with 7.3 and 6.9, respectively.

The same tests were done with orange juice that has been stored for Day 14. At Day 14, 80% of the panelists’ age ranged from 18 to 35 (Figure 2), with 36 % males and 64% females. 95% of the panelists consumed orange juice at least once in the past month (Figure 4) and all panelists liked orange juice slightly, moderately, a lot, or extremely (Figure 6).

The p-value for the triangle test was 0.00058 < \( \alpha \) therefore, we rejected the null hypothesis. The panelists were able to detect a difference between samples A and B. Since A and B are different for the panelists, one of the samples can be preferred over the
other. To assess the preference between samples, a hedonic scale test for A and for B followed the triangle test.

The overall acceptability was significantly different between the control orange juice (7.3) and the orange juice with the peptide fraction (6.6). Flavor played a major role in their opinion; the original orange juice flavor scored significantly higher (7.2) compared to the orange juice with the peptide (6.3). However color and mouthfeel scored the same, 7.6 and 7.1, respectively.

Based on the triangle test results, panelists failed to see any difference between A and B at day zero; however, they were able to detect a difference at Day 14. Since the panelists on Day 0 were not the same panelists than those on Day 14, it is likely that individual variations may have contributed to these observations. Considering the demographics, panelists on Day “14” were 85% between 18 and 35 years old (Figure 2), whereas at Day “0”, 61% were between 18 and 35 years old (Figure 1). The relationship between age and food perception may have contributed to these differences (Mojet and others 2004; Kremer and others 2007).

Furthermore, the storage of the orange juice for 14 days might have caused interactions of the peptide fraction with other components in the orange juice which might have contributed to the formation of flavors that lead the panelists to better distinguish the orange juice with peptides from the control orange juice.

To better assess whether the differences in results were due to sample differences or panelists food perception, the same panelists should asses orange juice with peptide
fraction that has been freshly prepared and orange juice with peptide fraction that has been stored for 14 days. Instead of using control orange juice and orange juice with peptide fraction for the triangle test, the two samples used would be orange juice with peptide that has been freshly prepared and another that has been stored for 14 days. If the test is significantly different then the findings in this study would be confirmed and the differences would be due storage and the interaction of the peptide fraction with orange juice components.

As for the hedonic scale, the first questions asked to the panelists was to rate the overall acceptability before rating specific characteristics of the orange juice to minimize the errors that could occur from the influence of the rating of one characteristic to the rating of the overall liking. Since in both studies, the overall liking and flavor were significantly higher for the control orange juice therefore panelists were obviously able to detect difference between samples which could lead to the assumption that the addition of the bioactive ingredient was the reason the ranking of the orange juice dropped.

Several studies have investigated the effect of the addition of functional ingredients on the taste and liking of the product by consumers. The off-flavors produced by nutraceutical ingredients generally decreased the liking. Several peptides and protein hydrolysates with bioactive activity were found to have a bitter taste (Möller and others 2008). In some cases the knowledge that the product is a functional food product increases the rating; however, an off-flavor in a product does not play the role of an indicator that the food is functional (Tuorila and Cardello 2002). A new technology called QSAR (quantitative structure-analysis relationship) can help in the prediction of
whether a peptide will have a bitter taste or not (Li-Chan and Cheung 2010). This study could give product developers previous knowledge on the probable effects of the peptide on the overall flavor of the products so they can develop the product accordingly.

Turila et al. (2002) asked panelists to rate a control orange juice to which the rating ranged from 6-7; however, when the functional ingredients were added the rating dropped between 6 and 2. Therefore the scores that the orange juice with the peptide received are comparable to scores that orange juice with no added functional ingredients scores. Although the demographics of the panelists such as age and ethnicity can affect the data; however, a big number of panelists can give enough data, allowing us to compare our scores to other published scores.

On Day 0 we noticed that the control orange juice scored higher than the orange juice with the peptide; therefore, on “Day 14” the panelists were asked to leave comments about their likes and dislikes. While 40% liked the taste of the orange juice with peptide fraction, 10% were neutral and 50% disliked it, off-flavor and after taste comments were provided by seven panelists. Other comments included “not too sweet”, “earthy flavor”, “bitter”, “bland”, and “tastes different than normal orange juice”. The control orange juice was generally liked and the dislikes comments included “bitter”, “not flavorful”, “prickly after taste”, and “very acidic, has lemon flavor” among others. Therefore the brand of the orange juice used might affect the overall liking of the orange juice despite the use of the peptide fractions. Several different brands should be tested with the addition of the peptide fraction to asses any possible likes and dislikes caused by the use of a specific orange juice brand.
The data showed that the peptide fraction might affect the orange juice flavor by the creation of subtle off-flavors that were detected by some panelists. However, the concentration used in this experiment was 3000µg/mL, lower concentrations should also be tested, if at lower concentrations the peptide would still be effective in inhibiting the cancer cells growth, then the off-flavors due to the peptide might be less perceived. Also storing the orange juice with the peptide fraction seem to have affected the taste, thus storing the orange juice with the peptide fraction for a longer period of time should also be tested to evaluate whether the peptide fraction will result in more off-flavors when stored for a longer period of time; therefore, maximizing the shelf life of the product.

Flavor and taste are the main attributes that consumers look for in a food product. They are generally unwilling to sacrifice taste for health (Verbeke 2006). If the peptide fraction creates off-flavors that are disliked by some of the panelists and by consumers in general, flavor masking agents that maintain the original orange juice flavor can be investigated to be used to cover any bitterness or off-flavor. Also encapsulation can be used to avoid the interaction of orange juice constituents with the peptide fraction and can help by avoiding the formation of off-flavors and potentially increasing the shelf life of the peptide fraction in the orange juice.
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Figure 1. Age groups distribution for panelists on day Day 0

![Pie chart showing age distribution on Day 0.]

- 18-25 years old: 30%
- 26-35 years old: 31%
- 36-45 years old: 14%
- 46-54 years old: 11%
- 55-64 years old: 11%
- Over 65 years old: 3%

Figure 2. Age distribution for panelists on “Day 14”

![Pie chart showing age distribution on Day 14.]

- 18-25 years old: 47%
- 26-35 years old: 33%
- 36-45 years old: 6%
- 46-54 years old: 3%
- 55-64 years old: 11%
- Over 65 years old: 3%
Figure 3. Consumption of orange juice in the past month for panelists on Day 0

- Never: 17%
- 1-2 times: 19%
- 3-4 times: 11%
- 5 or more times: 53%

Figure 4. Consumption of orange juice in the past month for panelists at “Day 14”

- Never: 5%
- 1-2 times: 25%
- 3-4 times: 17%
- 5 or more times: 53%
Figure 5. How much do you like orange juice in general for panelists on Day 0

Figure 6. How much do you like orange juice in general for panelists on “Day 14”
Table 1. Hedonic scale means results of control orange juice orange juice with peptide fraction and significant difference

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control OJ</td>
<td>OJ + peptide</td>
</tr>
<tr>
<td><strong>Overall liking</strong></td>
<td>7.1 a*</td>
<td>6.6 b</td>
</tr>
<tr>
<td><strong>Color</strong></td>
<td>7.3 a</td>
<td>7.3 a</td>
</tr>
<tr>
<td><strong>Flavor</strong></td>
<td>7.1 a</td>
<td>6.4 b</td>
</tr>
<tr>
<td><strong>Texture</strong></td>
<td>6.9 a</td>
<td>6.9 a</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different at 0.05 α-level for each test day.
Chapter 5 - Conclusion

The stability of rice bran peptide fraction was evaluated in water at pH~7.2, at pH~3.5, and in orange juice. The results were used to draw conclusions on whether orange juice is a suitable beverage to be a carrier for the peptide fraction. pH seemed to play an important role since the peptide fraction were more stable in a pH of 3.5 (in water and orange juice) rather than a pH of 7.2 for a period of 42 days. The quality attributes of orange juice with peptide fraction were compared to the quality attributes of a control orange juice and showed similar conditions in pH, color, and vitamin C content. The total soluble solids (TSS) showed a significant difference between the two treatments where TSS were significantly higher in the orange juice with the peptide than in the control orange juice. To assess whether the organoleptic properties of orange juice were affected by the addition of the peptide fraction a triangle test showed that after 14 days of storage the panelists were able to detect that there is a difference and that the control orange juice is preferred over the orange juice with the peptide. Some panelists were able to detect off-flavors in the functional orange juice and therefore further sensory studies could give a better understanding on the acceptability and rating of the functional orange juice to optimize all conditions for longer shelf life and better organoleptic properties.