STABILIZATION OF RESVERATROL THROUGH MICROENCAPSULATION AND INCORPORATION INTO FOOD PRODUCTS

BY

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DISSERTATION

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ABSTRACT

There is an increasing prevalence of chronic disease and obesity in the United States. Bioactive compounds which have been shown to prevent and alleviate disease states can aid in decreasing this prevalence. The addition of these compounds to food products would help to deliver these health benefits to consumers.

Challenges such as instability in environmental conditions and the digestive system, as well as negative sensory properties of the bioactive compounds limits the addition of these compounds to food products. Encapsulation is a processing technique that can help to overcome these challenges and increase the range of food products into which the compounds can be incorporated within. The long term goal of this research is to enhance stability and ease of consumption of bioactive compounds by way of utilizing microencapsulation, which will increase the consumption of these healthful ingredients.

This research utilized resveratrol, a polyphenol found in red grapes and wine, as a model compound which encapsulation was applied in order to overcome the light instability and bitterness of the compound. Resveratrol was encapsulated within a protein matrix through spray drying. Encapsulated resveratrol within a sodium caseinate matrix had a higher UVA light and digestive stability (0.63 *trans:cis* resveratrol ratio and 84%) than unencapsulated resveratrol (0.49 *trans:cis* resveratrol ratio and 47%). In addition, the encapsulation of resveratrol decreased the detection of the compound in comparison to unencapsulated resveratrol. The resveratrol microcapsules were added to snack bars and gummies in order to show application of the stabilized resveratrol. A group of consumers was identified who's overall liking for the food products were maintained with the addition of the encapsulated resveratrol.

The encapsulation-system approach developed in this research can be extended to other bioactive compounds in order to increase stability and minimize negative sensory properties of the target compounds, while delivering the health benefits of the compounds. This

research serves as a basis upon which additional research can tailor the encapsulation-system approach for the specific properties of the compound.

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CHAPTER 1: INTRODUCTION

1.1 Motivation

In the United States, more than 1/3 of adults are obese which poses concern as many chronic diseases, such as heart disease, stroke, diabetes, cancer, liver problems, and respiratory problems, are related with obesity [1, 2]. The overall medical cost in the United States related to obesity in 2008 was $147 billion dollars [2].

Healthy eating habits and regular exercise can help to combat obesity and rising prevalence of chronic disease in the United States. With this being said, it is often difficult for people to change their lifestyles. Therefore, the addition of healthful compounds to food products can provide the health benefits in easy-to-consume foods and provide a convenient means for the compounds to be delivered to consumers.

Resveratrol was a compound of interest due to its association with beneficial effects on heart disease, cancer, diabetes, and neurological function [3-6]. Some of the challenges with the incorporation of resveratrol into food products are instability in light, bitterness, and low bioaccessibility through the human digestive system [7-9]. The ***long term goal*** of this research is to enhance stability and ease of consumption of bioactive compounds by way of utilizing microencapsulation, which will increase the consumption of these healthful ingredients. The ***central hypothesis*** is microencapsulation of resveratrol within a protein matrix will affect stability and sensory properties of the compound.

1.2 Project Objectives

Objective 1

The first objective of this research was to stabilize *trans*-resveratrol in the presence of light and through the human digestive system. A common processing technique and cost-effective encapsulation material would be desirable in order to make scale-up and application in the food industry feasible. The working hypothesis for this objective was the use of

microencapsulation will increase the stability of resveratrol as evaluated through UV light and *in-vitro* digestion testing.

Objective 2

The second objective of this research was to evaluate the sensory properties of the stabilized resveratrol. Taste is one of the most important considerations when consumers are purchasing a product [10]. Even if a product contains a healthful compound, it needs to have a high acceptability in order for consumers to repeatedly purchase the product. The working hypothesis for Objective 2 was the incorporation of resveratrol into a microcapsule will increase the taste detection threshold of the compound, and further, not compromise consumer acceptance of food products with encapsulated resveratrol.

1.3 Outline of Thesis

Chapter 2 is a literature review that provides an overview of resveratrol, associated health benefits, regulations, challenges with incorporation into food products, binding between resveratrol and protein, resveratrol recovery, bioaccessibility, processing methods to stabilize resveratrol, and sensory properties of the compound.

Chapter 3 evaluates the stability of resveratrol in the four resveratrol microcapsule formulations under UVA light and *in-vitro* digestion testing. Two proteins (whey protein concentrate and sodium caseinate) and the effect of fat within the encapsulation matrix were compared as wall materials in the microcapsules. In addition, the quantification of binding between resveratrol and protein was explored. Resveratrol recovery from the microcapsules was limited, thereby, prompting interest in the investigation of the interaction between resveratrol and protein which may limit the ability for resveratrol to be extracted from the microcapsules. It was thought that binding between resveratrol and protein plays a significant role in stability of the compound.

Chapter 4 investigates the effect of plasticizers in the resveratrol microcapsule formulation in terms of morphology of the microcapsules and UV stability of resveratrol. The effect of plasticizer concentration and the use of protein denaturation on resveratrol stability within the microcapsule was also studied in terms of UV stability.

Chapter 5 compares the taste detection threshold of resveratrol and resveratrol encapsulated within a protein matrix. The R-index measure by the signal detection rating method in comparison to the noise and signal sample was utilized in this study. Resveratrol was tested in 6-concentration levels that differed in 3-fold increments, ranging from 2.2-540 mg/L for unencapsulated resveratrol and between 4.4-1080 mg/L for encapsulated resveratrol.

Chapter 6 assesses consumer acceptance of bars and gummies with added resveratrol microcapsules and unencapsulated resveratrol. Resveratrol was added to the food products at concentrations of 10 and 40 mg/serving. These levels of resveratrol have been shown to provide health benefits in humans [4, 11]. Panelists rated the overall liking of each product along with indicating which attributes they liked and disliked about the products. This testing gives indication of consumer perception of products with added resveratrol.

Chapter 7 concludes this dissertation with a summary of research findings, implications to the food industry, and explanation of future research.

1.4 References

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Resveratrol (3,4',5-trihydroxystilbene) found in red grapes and peanuts has been shown to have numerous health benefits associated with heart disease, cancer, diabetes, neurological function and other biological functions [1-6]. It has also been shown to have anti-microbial and anti-oxidant properties [7, 8]. The *trans*-isomer of resveratrol is the form which has been associated with health benefits, but instability in light, insolubility in aqueous solutions, low bioaccessibility and bitterness limit the range of products into which the compound can be incorporated [9-12].

Various processing methods are being used to overcome the challenges of resveratrol incorporation into foods. Processing methods can aid in the innovation of enhancing stability and decreasing negative sensory properties of resveratrol. Innovative processing methods can aid in the incorporation of resveratrol into foods with the long term goal of providing the health benefits of the compound to consumers. In terms of stability, prior research was mainly focused on the encapsulation of resveratrol in order to limit isomerization of the compound. The encapsulation techniques which have been explored in prior research are cyclodextrin complexes, porous microspheres, cross-linked chitosan, pectinate beads, lipid nanoparticles and carriers, double emulsions, nanoencapsulation, and niosomes [13-19]. In addition, processing methods to increase the solubility of resveratrol through addition of stevioside and application to food systems such as hazelnut paste and edible films have been researched [20-22]. There are limited published studies available on the sensory properties of resveratrol.

2.2 Health Benefits

Extensive research supports resveratrol is associated with health benefits related to heart disease, cancer, diabetes, neurological function, and other biological activities [1-6].

Table 2.1 summarizes the extensive body of literature on various health benefits according to the type of animal model used in the research.

2.2.1 Cardiovascular Disease

Research findings support that resveratrol lowered platelet aggregation in platelet-rich plasma and isolated human platelets [23, 24]. It was also able to lower total cholesterol and low-density lipoprotein (LDL) levels in hypercholesterolaemic rats [1]. In addition, resveratrol was able to improve vascular function by decreasing total plasma cholesterol and cholesterol in lipoprotein fractions in mice [25]. An increased recovery at reperfusion and significant vasodilation which indicates cardioprotection was also found in rats [26]. The effects of resveratrol on heart disease were also investigated in guinea pigs and found to increase cardiac DT-diaphorase and catalase activity. It also resulted in relaxation of arteries which supported the cardioprotective effects of the compound [27, 28]. In rabbits, resveratrol intake helped to suppress atherosclerosis and ADP-induced platelet aggregation, indicating prevention of coronary heart disease [24, 29]. Furthermore, a dose dependent inhibition of platelet aggregation and increased cerebral blood flow were reported when resveratrol was administered to humans, which indicated a decreased risk of cardiovascular disease [3, 24, 30].

2.2.2 Cancer

A second health benefit of resveratrol is its ability to prevent cancer and slow tumor growth. It is thought that resveratrol can inhibit carcinogenesis at multiple stages. Resveratrol has been tested against ovarian carcinoma cell lines, and it was found to inhibit the proliferation and survival of these cells; thereby causing autophagocytosis that helps to maintain homeostasis in cells [31]. In other cell lines, resveratrol helped to decrease basal production of PGE2, inhibit PMA-mediated activation of protein kinase C and COX-2, and decrease ornithine decarboxylase activity [32-34]. These changes in biomarkers were indications of inhibiting cancer proliferation. The administration of resveratrol to rat and mice models helped to prevent initiation and progression of tumor growth, even decreasing tumor size and increasing survival

of the animal [1, 35-46]. Healthy adults were given 0.5, 1, 2.5 or 5 g of resveratrol/day and a decrease in IGF-1 and IGFBP-3 was observed, which indicated chemoprotection [47].

2.2.3 Diabetes Mellitus

Thirdly, resveratrol has been associated with the prevention and treatment of diabetes mellitus. Resveratrol has been found to have an anti-hyperglycemic effect and also reduced glycosylated hemoglobin that further confirmed a reduction in blood sugar [48-51]. Resveratrol facilitated intracellular glucose transport even in the absence of insulin [52]. Furthermore, insulin sensitivity was increased in healthy rats and also in insulin-resistant mice on a high-fat diet after the consumption of resveratrol [51, 53]. In diabetic males, resveratrol has been shown to decrease blood glucose and insulin resistance, showing positive effects on diabetes [4, 5].

2.2.4 Neurological Function

The fourth health benefit of resveratrol is an increase in neurological function. It has been found that resveratrol helped to reduce neuronal cell death and neurotoxicity in an Alzheimer's mouse model [2]. Another study found resveratrol was able to reduce amyloid plaques in mice which is thought to be associated with decreased glutathione and increased cysteine [54]. Furthermore, other biomarkers such as NF-kappaB p65 expression, and decreased malondialdehyde levels are thought to be related to improved neurological function [55-57]. Research have shown that resveratrol intake stimulated Sir1, that inhibited nuclear factor κB signaling which helped to prevent β-amyloid toxicity and showed a therapeutic effect against Alzheimer disease [58].

2.2.5 Lifespan

Not only has resveratrol been associated with the prevention of disease states but also an increase in lifespan. Sirtuins are a group of genes associated with the aging pathway; therefore, the up-regulation of these genes is correlated with increased lifespan. In yeast, resveratrol has been shown to activate Sir2 and increase lifespan by 70% [59]. Another study has paralleled studies in yeast finding that resveratrol was able to increase lifespan in flies, fish,

worms, and mice [60-63]. Lagouge and others (2006) found that resveratrol intake prevented diet-induced obesity, increased aerobic capacity and inhibited muscle fatigue in mice [64].

2.2.6 Obesity

Resveratrol has also been shown to have positive effects on obesity. A resveratrol supplementation of 0.4% to mice on a high-fat diet was shown to decrease body weight gain, visceral fat, and plasma biomarkers related to adipogenesis and inflammation [65]. In non-human primates, it was found that 200 mg resveratrol/kg body weight/day reduced energy intake by 13% and increased resting metabolic rate by 29% [66]. It is hypothesized that resveratrol can increase satiety thereby decreasing energy intake and increasing energy expenditure.

2.3 Anti-microbial and Anti-oxidant Functions

Additional functional properties of resveratrol include its ability to serve as anti-microbial and anti-oxidant agents. At concentrations of 11 and 22 mg/L, resveratrol was shown to inhibit the growth of *Saccharomyces cerevisiae, Penicillium expansum, and Aspergillus niger* [7]. The effectiveness of resveratrol against HIV has also been shown [67, 68]. The ability of resveratrol to increase oxidative stress tolerance was studied in yeast. The yeasts were exposed to oxidative agents such as CCl_4 and H_2O_2. In the presence of resveratrol, there was a significant increase in survival rate and reduction in lipid peroxidation [8].

2.4 Resveratrol Sources

Resveratrol is naturally found in low concentrations in some fruits, wine, and nuts. The concentrations of resveratrol in red grapes, red wine, white wine, peanuts, and blueberries are 0.050 mg/100 g, 0.002-0.653 mg/L, trace-0.100 mg/L, 0.002-.0179 mg/100 g, and trace-0.003 mg/100 g, respectively [69-72]. The higher resveratrol content in peanuts than red grapes may be due to the high water content in grapes in comparison to peanuts. Therefore, it appears there is a lower concentration of resveratrol in red grapes.

2.4.1 Resveratrol as a Defense Mechanism in Plants

In plants, resveratrol may be produced as a defense mechanism to fungal invasion and other physical damage [71]. This defense mechanism has been specifically observed in peanuts that may provide an explanation for the wide range of resveratrol concentration found in this food source. When peanut seedling leaves were exposed to UVC irradiation, it was found that endogenous resveratrol increased significantly within 24 hours of exposure [73]. The effects of UVC irradiation was also explored in grape leaves [74]. Endogenous resveratrol was shown to increase 180-196 fold in comparison to the control (not exposed to UVC light) at 16 hr and 24 hr. Stilbene synthase, which is an important enzyme in the resveratrol biosynthesis, was also increased in the grape leaves after UVC exposure. Aluminum chloride has also been used to enhance accumulation of resveratrol in grape vine leaves [75]. All concentrations of aluminum chloride which were tested, 7-90 mM of aluminum chloride, were shown to induce resveratrol production. In addition, resveratrol has been shown to be produced as a defense response to *Botrytis* in grapevines. The level of resveratrol production was correlated to the level of pathogen infection [76]. Resveratrol has also been shown to be produced when grapvines were infected with mildew disease [77].

2.4.2 Transgenic Fruit with High Resveratrol Content

The concentration of resveratrol shown to have biological effects in humans is higher than that naturally found in plants. Therefore, transgenic produce have been developed in order to increase resveratrol concentration in natural sources of resveratrol. A stilbene synthase gene, associated with resveratrol production in fruits and vegetables, has been overexpressed in order to increase resveratrol content in lettuce [78]. Resveratrol content in the lettuce reached 56.40 ± 5.52 ug/g. Stilbene synthase gene has also been used to increase resveratrol glucoside, a resveratrol derivative in apples [79].

2.5 Regulations

Currently, resveratrol is not regulated as a drug in the United States and instead is considered a dietary supplement [80]. The Dietary Supplement Health and Education Act of 1994 made it the responsibility of manufacturers to determine the safety of compounds such as resveratrol. Under this law, the manufacturers can provide basic information about the mechanism by which the supplement affects "the structure or function of humans" but claims about the treatment or prevention of diseases cannot be made [81, 82]. Little is known about the amount of resveratrol intake that is considered safe.

The safety of resveratrol consumption is a viable concern when considering the incorporation of the compound into food products. No adverse effects were seen in rats when fed 50-700 mg resveratrol/kg bw/day for 28-90 days [83]. Other research found a decrease in white blood cells when rats were fed 450 mg resveratrol/kg bw/day for 56 days [53]. No serious effects were seen when healthy adults were given a 0.5-5 g single dose, but minor effects such as increased bilirubin and alanine aminotransferase were seen at a 1 g dosage [84]. Headache and dizziness were also seen in healthy adults when given a total of 150-900 mg resveratrol/day in small dosages every 4 hours [85]. The effects of resveratrol in rats and humans have been shown to not be serious, and the dosages that have been used in safety testing are well above those which have been shown to have biological effects.

2.6 Challenges

The incorporation of resveratrol into food products would help to deliver the health benefits of the compound to consumers, but resveratrol is unstable in certain environmental conditions such as light, pH and heat. The activation energy to isomerize *trans*-resveratrol to *cis*-resveratrol has been found to be ~3.7 kcal/M [72]. This activation energy is relatively low as the activation energy to go from $H_2O_2 \rightarrow H_2O + O_2$ was ~10 kcal/M [86]. The biggest challenge behind the addition of resveratrol to food products is instability of resveratrol in the presence of

light [9, 87]. After one hour of exposure to light, 80-90% of *trans*-resveratrol (bioactive) was converted to *cis*-resveratrol (bio-inactive) [87]. Another study found that about 90% of *trans*-resveratrol was converted to *cis*-resveratrol after UV exposure for 120 min at 366 nm [9]. *Trans*-resveratrol was most stable between pH 5-8 as the isomerization was slowest in this pH range [88]. Heat is another factor that can lead to instability of *trans*-resveratrol. Exposure to 190°C for 18 min has been shown to degrade 17-46% of resveratrol in blueberries and biliberries [70].

Another challenge with resveratrol incorporation into food products is its limited solubility in both aqueous and lipid solutions. The reported amount of soluble resveratrol was 0.03 mg/mL in water [10] and 0.18 mg/mL in coconut oil [89]. It is soluble in ethanol at 50 mg/mL and also in dimethyl sulfoxide at 16 mg/mL [10]. Additionally, relative humidity has not been found to affect isomerization of *trans*-resveratrol significantly at 25°C when relative humidity was increased from 75% to 90% [90].

2.7 Bioavailability and Bioaccessibility

An important consideration when processing resveratrol for incorporation into food products is the bioavailability and digestibility of the resveratrol. When resveratrol was administered orally through a drink, serum resveratrol levels peaked at about 30 minutes post-consumption and less than 2% of resveratrol was found in the *trans*-isomer [91]. Other research showed that resveratrol glucuronides and sulfates were mainly found in the plasma and were detected for a prolonged amount of time. Walle and others (2004) found that in addition to glucuronic acid and sulfate metabolites, the hydrogenation of aliphatic double bonds also occured. The majority of the metabolites were found in the urine [92]. These findings suggested that the resveratrol was partly metabolized in the small intestine and then distributed to various parts of the body in conjugated form [11]. This theory was supported by another research study as 50% of *trans*-resveratrol and derived metabolites administered to pigs was

found in the jejunum and ileum of the small intestine [93]. Resveratrol metabolites have been found to accumulate in the liver probably due to partial metabolism of the compound in the liver and also the transport of metabolites from the intestine. In the kidneys, resveratrol levels gradually decreased with time that suggested that renal excretion is one of the predominant means of elimination [11]. Therefore, these findings support that resveratrol readily breaks down in the body resulting in low bioaccessibility and bioavailability.

2.8 Increasing Stabilization and Bioaccessibility

Research has explored various ways to overcome the challenges of resveratrol related to instability and low bioaccessibility. The main method of stabilizing resveratrol is encapsulation that helps to prevent isomerization and maintain the bioactivity.

2.8.1 Cyclodextrin Complexes

Cyclodextrin complexes are the most common form of resveratrol stabilization. The number of glucose residues connected by α(1→4) glycosidic bonds can range from six to eight. Cyclodextrins can form inclusion complexes with a wide range of compounds as they have a hydrophobic inner cavity and hydrophilic capsule walls. In this way, resveratrol can form an inclusion complex with the hydrophobic cavity thereby protecting resveratrol [13]. In all the studies involving encapsulation of resveratrol by cyclodextrin complexes, antioxidant testing was the primary means to evaluate the microcapsules. β- and maltosyl-β-cyclodextrins have been used to form complexes with resveratrol and testing supported that biological activities were maintained. Oxidation of resveratrol by lipoxygenase was tested for both cyclodextrins [13]. Mantegna and others (2012) formed β-cyclodextrin and resveratrol complexes through ultrasound-assisted extraction from *Polygonum cuspidatum.* The complexation helped to increase antioxidant capacity that was tested through DPPH radical scavenging and $ORAC_{FL}$ test [94]. The cyclodextrins also helped to increase dispersibility and reduced time needed to extract resveratrol from the root. Unmodified β-cyclodextrin was thought to be unsafe due to

nephrotoxicity. Therefore, the use of modified hydroxypropyl-β-cyclodextrin as a complexation agent of resveratrol has also been explored [95]. This modified cyclodextrin has been found to form a similar 1:1 inclusion complex, but with a larger inclusion constant that indicates a greater inclusive potential. The solubility and antioxidant efficacy of β-cyclodextrins and hydroxypropyl-β-cyclodextrins were investigated. The resveratrol-cyclodextrin complexes had a higher solubility and similar antioxidant activity to free resveratrol [95]. The use of hydropropyl-β-cyclodextrin for resveratrol complexation decreased oxidation of the compound by free radicals and increased antioxidant activity [96]. The cytotoxicity of resveratrol complexed with β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin was compared [97]. The complex of resveratrol and 2-hydroxypropyl-β-cyclodextrin showed a higher cytotoxicity against two cancer cells in comparison to β-cyclodextrin complexation with resveratrol. Resveratrol was incorporated into the hydrophobic cavity of the cyclodextrin, therefore, hydrophobic interactions occurred between the compound and cyclodextrin [13]. In comparison, other encapsulation methods of resveratrol, such as pectinate beads, emulsions, and nanoparticles, utilize physical entrapment of resveratrol within a wall structure.

2.8.2 Porous Microspheres

Prior research utilized porous polymeric microspheres for the stabilization of resveratrol, looking specifically at the wetting time and cyano-functional groups [14]. The polymer particles are an effective means to stabilize resveratrol because they scatter light that limits light exposure to the compound. Additionally, resveratrol was maintained within the microsphere through hydrogen bonding of the compound and cyano-functional groups of the particles. The particles were produced by first forming monodisperse porous polymer particles containing cyano groups by dispersion polymerization. Then, the porous particles underwent a wetting period of 24 hours before resveratrol was immobilized within the porous particles by dropping an ethanol and resveratrol mixture into the porous particle solution. The results showed that the encapsulation helped to preserve 93% of the antioxidant activity and maintained bioactivity for 5

weeks. Confocal laser scanning microscopy analysis confirmed that the resveratrol was evenly distributed within the porous particle, and it was hypothesized that hydrogen bonding helps provide stability to the compound.

2.8.3 Cross-linked Chitosan

Cross-linked chitosan microspheres are another method that has been utilized to increase resveratrol stability. Chitosan is commonly used in the food industry and is a desirable material for stabilization because it is non-toxic, bio-compatible and able to form films. Cross-linkers were used for chitosan microspheres to increase stability and control the release of the bioactive compounds [15]. Vanillin is an ideal cross-linker because it is generally regarded as safe and has bioactive properties [98, 99]. The microspheres were formed by an emulsion and chemical cross-linking method. Encapsulation efficiency measurements showed 93.68% efficiency for the chitosan microspheres with vanillin cross-linking. The microspheres were exposed to UV light for 1 hr and temperatures of 60°C and 70°C for 15 days. The results support that the chitosan microspheres were able to preserve the resveratrol retention [15].

2.8.4 Pectinate Beads

Calcium-pectinate beads have been used to stabilize resveratrol in order to delay the release of the compound from the capsule [16, 100]. This method was selected in order to achieve colon-specific delivery of resveratrol for the treatment of lower gastro-intestinal disorders. The capsules were formed through ionotropic gelation where a resveratrol and pectin solution was crosslinking into a calcium, chloride, and polyethyleneimine solution. The beads were then washed with water and dried. Various parameters were altered to form capsules with a delayed drug release profile and high encapsulation efficiency. It was found that 0.1% polyethyleneimine solution with 2 hour crosslinking time and 0.2% polyethyleneimine with 0.5 hour crosslinking time showed an optimum drug release profile and high encapsulation efficiency.

Zinc-pectinate beads have also been used to encapsulate resveratrol [101]. It was found that zinc-pectinate beads had a better delayed release than calcium-pectinate beads. The release of resveratrol from the beads followed zero-order kinetics, and the beads were stable at room temperature and 4°C.

2.8.5 Lipid Nanoparticles and Carriers

Solid lipid nanoparticles and nanostructured lipid carriers have also been utilized to encapsulate and protect resveratrol [102]. High shear homogenization was used to produce the nanoparticles. Both particles were able to decrease reactive oxygen species, but nanostructure lipid carriers were able to permeate deeper into the skin. Solid lipid nanoparticles have also been produced through solvent diffusion-solvent evaporation [103]. Encapsulation efficiency of these particles was 88.9 ± 3.1%, and the *in-vitro* drug release was up to 120 hours.

2.8.6 Double Emulsion

A water-in-oil-in-water double emulsion is where an aqueous solution is first dispersed in oil to form the primary emulsion which is then re-emulsified in water. These types of double emulsions have been utilized to stabilize resveratrol. A benefit of multiple emulsions is that it provides protection against oxidation of the target compound. The concentration of resveratrol used was 200 mg/L in order to ensure dissolution of the compound. It was found that less than 10% of the initial resveratrol content migrated to the external aqueous phase after 2 weeks of storage [17]. Response surface methodology was used to find the optimum ratio of primary and secondary emulsifiers which were as follows: 1:2 ratio of core material to coating material, 1.25% w/v primary emulsifier concentration, 1:1.23 ratio of W/O emulsion to secondary coating material, and 1.21% w/v secondary emulsifier concentration [104]. Whey protein was found to be the most stable secondary coating material. Further research can investigate the use of higher concentrations of resveratrol in emulsions and different emulsion materials in order to minimize the passive diffusion of resveratrol into surrounding aqueous phase.

2.8.7 Nanoencapsulation

Nanoencapsulation is when a bioactive compound is entrapped within a nano-sized capsule or emulsion. Resveratrol nanoemulsions have also been utilized to increase stability and bioavailability of the compound [18]. Nanoencapsulation is beneficial because the small size is thought to increase cellular uptake. The emulsions were composed of peanut oil and 0.01% by weight resveratrol processed with high-pressure homogenization. The chemical stability of the emulsions was tested under different storage temperatures of 4ºC, 30ºC, and 55ºC for 30 days. There was no *cis*-resveratrol detected in the encapsulated samples. Storage at 4ºC degraded about 50% of resveratrol but at 30ºC and 55ºC, resveratrol was stable. With an exposure to UV-C light for 2 hours, the nanoemulsions showed a decreased amount of *cis*-resveratrol formation and a lower rate of degradation. In addition, *in-vitro* testing of the stomach and small intestine processes showed that resveratrol nanoemulsions stabilized resveratrol and preserved the *trans*-isomer throughout these processes. Nanoparticle system of resveratrol have also been utilized to increase the solubility while increasing the hepatoprotective effect [105]. Positive effects such as reduction in oxidative stress and inflammatory cytokines were observed from the nanoparticle system.

2.8.8 Niosomes

Niosomes are non-ionic surfactant vesicles and another means by which resveratrol has been stabilized with a controlled release of the compound. Niosomes were chosen for research because they can encapsulate hydrophilic compounds in the aqueous layer and lipophilic compounds on the vesicular membrane. In addition, niosomes have a relatively low cost and high chemical stability in comparison to liposomes [19]. Span 60 and Span 80-cholesterol were the encapsulation material in the niosomes. Span 80 niosomes had low entrapment efficiency while Span 60 niosomes had a higher entrapment efficiency and slower rate of release. Stability testing of the niosomes by light backscattering analysis showed that the Span 80 niosomes are more stable than the Span 60 niosomes [19].

2.8.9 Encapsulation Combination

Resveratrol has been encapsulated in combination with other bioactive ingredients. An oil-in-water emulsion was utilized to enable the co-delivery of compounds that differ in solubility: resveratrol, tributyrin and fish oil [106]. The emulsion was stabilized by heated milk protein, glucose and modified resistant starch. This study forms the basis for the incorporation of various bioactive ingredients into a single product which can deliver similar or synergistic health benefits. The stability of multiple compounds within an emulsion was not compared to a single compound, but future research can investigate this comparison.

2.9 Increasing Solubility

Resveratrol has a limited solubility in aqueous solutions [10]. Stevioside has been added to resveratrol in order to increase the solubility of resveratrol in aqueous solutions [20]. It is hypothesized that there is an interaction between the hydrophobic core of the stevioside and the resveratrol thereby enhancing the solubility of the resveratrol. The resveratrol and stevioside complex was 45 times more soluble in aqueous solution compared to the pure compound, and the addition of the complexes increased oxidative stability of soy protein emulsions.

2.10 Protein Binding

Resveratrol has been shown to bind with protein, and this interaction can further enhance stability of the compound. It has been found that resveratrol interacts with β-lactoglobulin in a 1:1 ratio. This binding was not able to completely prevent the conversion of *trans* to *cis*-resveratrol, but it was capable of delaying the conversion. In addition, the interaction helped to increase the solubility of resveratrol [107]. The binding between resveratrol and β-lactoglobulin has been found to be on the outer surface of the ligand near Trp19-Arg124. The complex was stable at acidic pH with a binding constant of ~10^4 M^{-1} [108].

Binding between resveratrol and proteins (sodium caseinate and whey protein) have been investigated [109, 110]. Fluorescence has been utilized to explore binding of resveratrol. For sodium caseinate, the binding constant was found to be 3.7-5.1 x 10^5 M^{-1}. Hydrogen bonding and hydrophobic interaction are thought to be responsible for the static and dynamic binding between resveratrol and sodium caseinate. The fraction of binding sites available for complexation was 1.20 at 25°C. For whey protein, the binding constant was between 1.7 x 10^4 – 1.2 x 10^5 M^{-1}. Whey protein and resveratrol formed a 1:1 complex, which did not affect the secondary structure of resveratrol. Dipole-dipole and Van der Waal interactions were thought to be responsible for the binding between resveratrol and whey protein.

2.11 Resveratrol Recovery

Various means to increase stability of resveratrol have been developed therefore it is necessary to find methods to characterize the complexes or microcapsules that are formed. Recovery and efficiency are currently used for characterization, but different methods and calculations are used across research studies, making it difficult to cross compare the results.

Nanoparticle system using polyvinyl alcohol and a cationic copolymer has been used to encapsulate resveratrol [105]. Resveratrol recovery was 96.3% and defined as the actual amount of resveratrol in the nanoparticle system compared to the theoretical maximum of resveratrol in the nanoparticle system. Ethanol was used to extract resveratrol extensively from vanillin cross-linked chitosan microspheres, and the resveratrol recovery was 93.68% [15]. Resveratrol recovery was also measured in resveratrol liposomes in which free resveratrol was first removed by dialysis and then 0.025% non-ionic surfactant, Triton X-100, was used to extract the resveratrol [103]. The resulting recovery was about 76%. The decreased amount of recovery may be because dialysis was first used prior to the recovery analysis. In calcium-pectinate beads with resveratrol, the compound was extracted by pectinase enzyme and methanol was added to increase the solubility of the resveratrol [16]. Resveratrol recovery in

the calcium-pectinate beads was between 60-98%, depending on the various factors tested (cross-linking time, pectin:resveratrol, and polyethyleneimine concentration).

Encapsulation efficiency is often used to compare the effectiveness of encapsulation methods. This method is an indirect measurement of resveratrol content in the microcapsule as it takes into account the amount of resveratrol that is easily washed from the surface of the microcapsules rather than the amount of resveratrol extracted from the microcapsule. When resveratrol was incorporated in liposomes, the technique used to produce the liposomes had a significant effect on encapsulation efficiency, which is explained by the following equation:

$$\text{Encapsulation efficiency} = \left(\frac{resveratrol\ load - resveratrol\ in\ supernatant}{resveratrol\ load}\right) \qquad \textbf{(eq 1.1)}$$

Liposome prepared through sonication had an encapsulation efficiency of 44-56% and those prepared by extrusion had an encapsulation efficiency of 92-96% [111]. Also, the encapsulation efficiency of resveratrol nanoparticles in a methoxy poly(ethylene glycol)-poly(caprolactone) matrix was found to be about 91% [112]. When resveratrol was encapsulated in zinc-pectinate or calcium-pectinate, the encapsulation efficiency was relatively high, between 97-99% [100, 101]. In comparison, resveratrol in solid lipid nanoparticles had an encapsulation efficiency between 64-89%, and nanostructured lipid carriers had an encapsulation efficiency between 65-77% [113]. Differences in encapsulation efficiency may be due to the use of different processing techniques, wall materials, and method of resveratrol extraction.

2.12 Food and Non-Food Applications

The ultimate goal is to incorporate resveratrol into food products, but there is very limited research which shows application of resveratrol. One example of this incorporation for functionality within a food matrix is the nanoencapsulated resveratrol was incorporated into hazelnut paste in order to prevent oxidation of the paste [114]. The results showed that the oxidation of hazelnut paste was reduced through the addition of encapsulated resveratrol.

Another study investigated resveratrol incorporation into edible film formation. Chitosan and methylcellulose films were both used to incorporate the resveratrol. The addition of resveratrol altered the film properties such as decreased ability to stretch and glossiness, and increased resistance to fracture, opaqueness, and antioxidant activity [22].

Resveratrol has also been utilized for non-food application. Poly(L-lactic acid) films have been formulated with various levels of both α-Tocopherol and resveratrol. The goal was to develop a functional membrane which could be used for controlled release of antioxidants in packaging. The equilibrium time for the diffusion of the antioxidants into ethanol decreased with increased temperature. The addition of the antioxidants to the film resulted in a decreased glass transition and melting temperature and increased thermal stability [115, 116]. These findings are significant because they provide a model for incorporation of antioxidants into packaging in order to reduce negative effects such as oxidation of the contents within the packaging through chelation of the free radicals.

2.13 Sensory Analysis

There is limited research in the sensory attributes of resveratrol. One study investigated the sensory and chemical characteristics of Riesling and Cabernet Sauvignon wines fortified with two concentrations of *trans*-resveratrol: 20 and 200 mg/L. Analysis was completed at various time points up to 58 weeks after bottling. The Riesling wine fortified with 20 mg/L *trans*-resveratrol had a significantly higher bitterness than the unfortified control, but the highest bitterness rating was the Riesling wine fortified with 200 mg/mL *trans*-resveratrol [12]. Another research study identified the *trans*-resveratrol concentrations and sensory scores of various wines. The results show there was no direct correlation between the *trans*-resveratrol concentrations and the sensory scores of the wines, which were general ratings [117]. If specific attributes were evaluated individually, then significant differences might have been correlated with varying *trans*-resveratrol content.

2.14 Conclusion

The health benefits of resveratrol provide a valid rationale to add the bioactive form of resveratrol into food products in order to disseminate the health benefits to the consumers. Current research is investigating innovative approaches to overcome the challenges of resveratrol incorporation into food products. Most of these approaches are not suitable for mass production and have not been applied to commercial products. Future research can investigate techniques such as spray drying which is widely available in the food industry and easy to scale up. It is important to ensure that resveratrol is incorporated into food grade matrices as the intent is to incorporate the stabilized resveratrol into food products. Protein may be a desirable encapsulation material for resveratrol due to the binding of resveratrol and protein which can further enhance stability. The relatively low cost of protein also makes sodium caseinate a desirable encapsulation material. The sensory properties of resveratrol are not fully understood. Therefore, it would also be valuable if future research utilized sensory testing such as consumer testing to evaluate overall acceptance of products with added resveratrol. Descriptive analysis could also be utilized to evaluate the effect of resveratrol on attribute intensity of food products.

Overall, future research should focus on innovative processing approaches to increase resveratrol stability and minimize negative sensory properties of the compound. Thereby, providing bioactive resveratrol to the consumer and increasing consumer acceptance of products with added resveratrol.

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2.15 Figures and Tables

Table 2.1: Research supporting the health benefits of resveratrol (bw=body weight)

In-vitro			
Author	**Oral Dosage**	**Cell Model**	**Findings**
Bertelli et al [23]	3.56 mg/L	Platelet rich plasma	Lowered platelet aggregation
Wang et al [24]	10-1000 mM	Isolated human platelets	Inhibited platelet aggregation in concentration dependent manner
Li et al [118]	30 μM	Endothelial cells	Stimulated K_{Ca} channels in endothelial cells
Subbaramaiah et al [32]	30 μM	Human mammary epithelial cells	70% decrease in basal production of PGE_2, inhibited PMA-mediated activation of protein kinase C and COX-2
Schneider et al [33]	25 μM	CaCo-2 cells	70% growth inhibition, decreased ornithine decarboxylase activity
Ferry-Dumazet et al [34]	100 mM	Normal and leukemic hematopoietic cells	Inhibited proliferation and induced apoptosis of cells
Opipari et al [31]	50-200 μM	Human ovarian carcinoma cell lines	Induced cell death in cancer cells separate from apoptosis
Cao and Li [119]	50-100 μM	H9C2 cells	Resveratrol pretreatment increased protection against cytotoxicity
Mice/Rats			
Author	**Oral Dosage**	**Animal Model**	**Findings**
Sharma et al [48]	5, 15, and 50 mg/kg bw/day for 4 weeks	Obese mice	Antihyperglycemic activity and improvement in insulin levels
Palsamy et al [49]	5 mg/kg bw/day for 30 days	Diabetic rats	Decreased blood glucose, improvements in plasma insulin and hemoglobin
Andersen et al [53]	300 mg/kg bw/day for 8 weeks	Healthy male rats	Improved insulin sensitivity
Su et al [52]	0.1-0.75 mg/kg bw	Streptozotocin-induced diabetic mice	Decreased insulin secretion, delayed onset of insulin resistance, increased glucose uptake
Palsamy and Subramanian [50]	5 mg/kg bw/day	Diabetic rats	Decreased hyperglycemia and increased insulin secretion

Table 2.1 (cont.)

Barger et al [120]	4.9 mg/kg bw/day for 16 months	Mice on high calorie diet	Prevented decline of cardiac function with age and prevented 93% of age-induced gene expression-similar to results seen with calorie restriction
Pearson et al [25]	5-200 mg/kg bw/day for 6 months	Mice	Protected against vascular or kidney dysfunction, reduced total plasma cholesterol and amount of cholesterol carried in lipoprotein fractions
Bradamante et al [26]	25 mg/L for 15 days	Rats	Increased recovery at reperfusion and significant vasodilation
Hudersen et al [36]	45µg/kg bw/day for 60 days	Mice	Inhibited colon tumorigenesis
Bishayee et al [37]	50, 100, and 200 mg/kg bw/ day	Rats with liver cancer	Reduced size of cancer nodules by inhibiting cell proliferation
Jang et al [38]	1-25µM, twice a week for 18 weeks	Mouse skin cancer model	Inhibited development of preneoplastic lesions and inhibit tumorigenesis
Miura et al [1]	50 ppm for 20 days	Hepatoma-bearing rats	Solid tumor growth and metastasis was suppressed, serum triglyceride and VLDL + LDL-cholesterol levels suppressed
Kimura et al [39]	2.5 and 10 mg/kg/bw	Lung carcinoma-bearing mice	Reduced tumor volume and weight and lung metastasis
Chen et al [40]	40 mg/kg for 28 days	Mice with neuroblastoma tumors	Suppressed growth rate of subcutaneous neuroblastomas
Brakenhielm et al [41]	1.2 µg/day	Mice	Inhibited murine fibrosarcoma growth
Revel et al [42]	50 mg/kg/week	Balb-C mice	Prevented BaP-induced CYP1A1 expression, related to prevention of lung cancer
Schneider et al [43]	0.3-0.4 mg/mouse	C57BL/6J-*ApcMin* male mice	Prevented colon tumor formation and reduced formation of small intestine tumors by 70%
Garvin et al [44]	100 µM	MDA-MB-231 tumors in nude mice	Lowered tumor growth, decreased angiogenesis, increased apoptotic index
Zhou et al [45]	500, 1000, 1500 mg/kg	Gastric cancer cells in nude mice	Induced apoptosis of transplanted tumor cells
Tseng et al [46]	40 mg/kg/day	Rats	Slower tumor growth, increased survival of animal

Table 2.1 (cont.)

Bishayee and Dhir [121]	50, 100, 300 mg/kg bw/day	Sprague-Dawley rats	Decreased incidence, number and multiplicity of visible hepatocyte nodules
Kim et al [2]	50-500 nM	Mouse model of Alzheimer's disease	Decreased neurodegeneration and prevent learning impairment
Wang et al [55]	10^{-8}, 10^{-7}, 10^{-6} g/kg, IV	Cerebral artery occlusion model in Wistar rats	Neuroprotective effect and NF-kappaB p65 expression
Karuppagounder et al [54]	300 mg/kg bw/day for 45 days	Mice	Diminished plague formation, brain glutathione decreased and cysteine increased
Gupta et al [57]	20 and 40 mg/kg ip	Male Wistar rats	Malondialdehyde levels reduced showing protective effect against seizures
Gupta et al [56]	20 and 40 mg/kg ip	Male Wistar rats	Reduced incidence of convulsions, increased malondialdehyde
Baur et al [51]	22.4 mg/kg bw/day	Middle aged mice on high calorie diet	Prevent harmful effects of high calorie intake, increased survival of rats on high calorie diet, increased insulin sensitivity, AMPK, mitochondrial number and improved motor function
Martin et al [122]	5-10 mg/kg/day	Rats	Reduced degree of colonic injury, the index of neutrophil infiltration and levels of the cytokine, decreased PG_2 and COX-2 expression
Lagouge et al [64]	200 and 400 mg/kg bw/day for 15 weeks	Mice	Increased aerobic capacity, consumption of oxygen in muscle fibers, protected against diet-induced obesity and insulin resistance
Mizutani et al [123]	1 mg/kg bw/day, gastric intubation	Stroke prone spontaneously hypertensive rats	Suppressed oxidative DNA damage and glycoxidative stress
Wu et al [124]	25, 50, 100 mg/kg, intraperitoneal	Wistar rats after liver transplant	Immuno-suppressive property and protective effect on hepatocytes
Feng et al [125]	0.75-6 µM	Mice	Promoted lymphocyte proliferation and IL-2 production

Table 2.1 (cont.)

Liu et al [126]	2 mg/kg bw	Weanling mice	Shorten vaginal opening latency periods, enhanced keratinization of vaginal epithelium
Giovannini et al [127]	0.23 µg/kg	Male Wistar rats	Reduced mortality of ischemic rats and reduced renal damage, inhibited renal peroxidation
Gentilli et al [128]	2 mg/kg	Rats	Reversed hyperalgesia for 48 hours
Docherty et al [129]	6.25 and 12.5% cream	Mice	Delayed or abolished development of herpes
Guinea Pigs			
Author	**Oral Dosage**	**Animal Model**	**Findings**
Floreani et al [27]	14 mg/kg for 16 days	Guinea Pigs	Increased cardiac DT-diaphorase and catalase activity
Naderali et al [28]	5-70 µM/L	Female guinea pigs	Concentration dependent relaxation of preconstricted mesenteric and uterine arteries
Rabbits			
Author	**Oral Dosage**	**Animal Model**	**Findings**
Wang et al [24]	4 mg/kg bw/day	Hypercholesterolemic rabbits	Inhibited ADP-induced platelet aggregation
Wang et al [29]	3 mg/kg bw/day	Hypercholesterolemic rabbits	Cardioprotective properties, suppressed atherosclerosis
Elmali et al [130]	10 µM/kg for 2 weeks, IV	Rabbits	Decreased cartilage tissue destruction
Humans			
Author	**Oral Dosage**	**Animal Model**	**Findings**
Wang et al [24]	10-1000 µM	Normotensive males	Inhibited platelet aggregation in concentration dependent manner
Kennedy et al [30]	250 and 500 mg dose	Healthy adults	Dose dependent increase in cerebral blood flow and deoxyhemoglobin
Wong et al [3]	30, 90, 270 mg	Overweight/obese men or post-menopausal women	Improved flow-mediated dilation
Brasnyo et al [4]	10 mg/day for 4 weeks	Type 2 diabetic patients	Reduced insulin resistance and urinary ortho-tyrosine excretion
Brown et al [47]	0.5, 1, 2.5, 5 g/ day for 29 days	Healthy adults	Decrease IGF-1 and IGFBP-3 indicating chemoprotection

Table 2.1 (cont.)

Timmers et al [5]	150 mg/day for 30 days	Healthy, obese men	Reduced sleeping and resting metabolic rate, increased SIRT1 and PDC-1 protein levels, decreased intrahepatic lipid content, circulating glucose, triglycerides and systolic blood pressure
Ghanim et al [6]	40 mg/day for 6 weeks	Healthy, normal weight	Reduced reactive, oxygen species and inflammation

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CHAPTER 3: STABILITY AND BINDING OF *TRANS*-RESVERATROL ENCAPSULATED IN A PROTEIN MATRIX PRODUCED USING SPRAY DRYING

3.1 Abstract

Resveratrol has demonstrated the potential to provide therapeutic and preventive activities against diseases such as heart disease and cancer. The incorporation of resveratrol into food products would allow for wide access of these health benefits to a larger population, but this strategy is limited by instability of resveratrol under environmental conditions and within the digestive system due to the isomerization of *trans*-resveratrol (bioactive form) to *cis*-resveratrol (bio-inactive form). The overall goal of this research was to stabilize the bioactive form of resveratrol through an innovative processing approach, microencapsulation. *Trans*-resveratrol was encapsulated using whey protein concentrate or sodium caseinate, with or without anhydrous milk fat (AMF). The binding of protein and resveratrol is of interest because it may help to stabilize resveratrol. Resveratrol-protein binding was calculated utilizing the Stern-Volmer equation and the developed resveratrol binding sites equation. The stability of encapsulated resveratrol was evaluated after exposure to Ultraviolet A (UVA) light and *in-vitro* digestion. The samples were exposed to UVA light, equivalent to about 4 min of sunlight, in aqueous solution. Digestive stability was assessed using a 3-phase *in-vitro* digestion, which included oral, gastric and small intestine phases. Resveratrol isomers were quantified by reverse phase HPLC with coulometric detection. After UVA light exposure, sodium-caseinate-based microcapsules retained a significantly higher *trans:cis* resveratrol ratio (0.63) than whey-protein-concentrate-based microcapsules (0.43) and unencapsulated resveratrol (0.49). In addition, all encapsulated resveratrol had an increased digestive stability and bioaccessibility, respectively, in comparison to unencapsulated resveratrol (47% and 23%), with sodium caseinate providing a higher digestive stability (84% and 60%) compared to whey-protein-concentrate-based microcapsules (70% and 53%). The addition of AMF within formulations did not affect UVA light and *in-vitro* digestion stability. In conclusion, microencapsulation with

sodium caseinate increased the stability of resveratrol after UVA light exposure and *in-vitro* digestion conditions. This encapsulation-system-approach can be extended to other labile, bioactive polyphenols.

Keywords: resveratrol, light stability, bioaccessibility, binding, microencapsulation

3.2 Introduction

Resveratrol is a polyphenol found in grapes and peanuts that has gained interest due to the numerous health benefits associated with this compound. Resveratrol naturally occurs in low amount in red grapes (0.050 mg/100 g), red wine (0.002-0.653 mg/L) and peanuts (0.002-.0179 mg/100 g) [1-3]. The incorporation of resveratrol into food products would allow the intake of biologically active dosages of resveratrol and disseminate its health benefits to a larger population. The health benefits of resveratrol have been extensively investigated over the years in a wide range of *in-vitro*, pre-clinical and clinical settings. *In-vitro* studies showed that resveratrol can lower platelet aggregation thereby decreasing cardiovascular disease and promoting cell death in cancer cells [4, 5]. In mice and rat models, resveratrol has been shown to decrease blood glucose related to diabetes and reduce plasma cholesterol linked to a reduction in risk of cardiovascular disease [6, 7]. Resveratrol has also been shown to decrease tumor growth and neurodegeneration in mice and rat models [8, 9]. In humans, several randomized control trials have shown the effects of resveratrol on improvement of cerebral blood flow and flow mediated dilation which are related to decreased risk of stroke, reduced insulin resistance, modulation of biochemical pathways associated with chemoprotection and inflammation [10-14].

The preferred dietary form of resveratrol which has been associated with benefits is *trans*-resveratrol while the form which has questionable health benefits is *cis*-resveratrol. The activation energy to cause the isomerization from the *trans* to *cis*-isomer was about 3.7 kcal/M which is a relatively small amount of energy in comparison to the activation energy to convert

hydrogen peroxide to oxygen, which was about 10 kcal/M [15, 16]. The conversion from the bioactive to bio-inactive form of resveratrol is induced by environmental conditions such as light, heat and pH [17-20]. Under UV light, 90.6% of *trans*-resveratrol in solution was converted to *cis*-resveratrol after 120 min exposure at 366 nm [17]. Also, one hour of exposure to sunlight resulted in 80-90% conversion of *trans*-resveratrol to *cis*-resveratrol [18]. Resveratrol is more stable to heat than light. In blueberries and bilberries, 17-46% of resveratrol was degraded after heating for 18 min at 190°C [19]. *Trans*-resveratrol was stable in a range of pH conditions, from pH 3 to 7 for one month, but unstable at pH 12 with a half-life ranging from 10-20 hr [20]. Another study found that *trans*-resveratrol was stable between pH 1-7 for 28 days and the half-life of *trans*-resveratrol in pH 10 was 1.6 hr [17]. Therefore, prior research showed that resveratrol is unstable in certain environmental conditions.

Digestive stability and bioaccessibility of resveratrol is another important consideration in order to ensure bioactivity of the compound when consumed. An oral dose of resveratrol has been shown to have 70% absorption, but only trace amounts of *trans*-resveratrol were found in the blood after consumption of the compound. Resveratrol conjugated with sulfates and glucuronic acid were the most common metabolites found in the urine and plasma [21]. After oral ingestion of resveratrol, trace amounts of the ingested resveratrol was detected in plasma and glucuronides were the most common form of resveratrol found in the urine [22]. Peak plasma concentration of *trans*-resveratrol was less than 2% of the oral dose and occurred about 30 min after consumption [23]. Thus, this low digestive stability and bioavailability of resveratrol provides further rationale to stabilize resveratrol in order to ensure the health benefits are delivered to the consumer.

Encapsulation is a viable processing method that can help to stabilize the bioactive form of resveratrol to environmental and digestive stress. This technique is used to incorporate labile or desirable compounds into microcapsules utilizing a stable wall material. The core ingredient can be protected from environmental conditions such as light, heat, pH and moisture. In the

food industry, encapsulation is common for flavors, enzymes, colors, preservatives, antioxidants, nutrients and artificial sweeteners [24]. Encapsulation can be accomplished using several methods which include spray drying, liposomes, coacervation, and emulsions [25]. In the food industry spray drying is favored because it is economical, flexible, a continuous operation, and produces particles of high quality [25]. One limitation of spray drying is the wall material needs to be water soluble to form a solution [26]. Therefore, the selection of wall materials is limited by their solubility in water. Some wall materials that have been used in spray drying are maltodextrins, chitosan, gum acacia and protein-lipid mixtures [26, 27]. In a prior study, porous polymeric microspheres were used to encapsulate resveratrol and the wetting time and presence of cyano-functional groups were measured. In addition, antioxidant potential was measured and it was found that encapsulation helped to preserve 93% of the antioxidant activity and maintain the bioactivity for 5 weeks [28]. These results suggested that the encapsulation of resveratrol will help to stabilize the compound which in turn may enhance bioaccessibility. In another study, the incorporation of resveratrol into cyclodextrin complexes was shown to maintain its antioxidant potential and increase solubility of the compound [29]. Cross-linked chitosan and pectinate beads have also been utilized to encapsulate resveratrol [30, 31]. Results from these encapsulation studies indicate that protection of resveratrol is feasible, but requires optimization. The current methods, although useful, are limited for their wide use in industry, since they are more appropriate for small scale production. Protein such as sodium caseinate has been used in the past for encapsulation of curcumin, thymol, and a combination of bioactive compounds [32-34].

In this study, spray drying with protein-based material was used as the encapsulation method. Sodium caseinate and whey protein concentrate were chosen as wall materials to encapsulate resveratrol because they are common dairy protein utilized in the food industry and can be readily leveraged in many product formats, making commercialization more feasible. In addition, resveratrol has been previously shown to bind with protein thereby stability of the

compound would be enhanced [35-37]. The binding of resveratrol to β-lactoglobulin decreased the isomerization of resveratrol in comparison to resveratrol not bound to protein [38]. Future research can utilize plant based proteins such as soy protein and pea protein, in order to make the encapsulation system suitable for vegans or those with sensitivities to dairy.

The objective of this study was to evaluate the stability of the bioactive form of resveratrol, *trans*-resveratrol, against light and digestive conditions, after microencapsulation. The microcapsules were characterized and evaluated on the basis of stability under UVA light and *in-vitro* digestion. The hypothesis was that microencapsulation of resveratrol within a protein matrix will change the stability of resveratrol in comparison to unencapsulated resveratrol. The second objective of this study was to quantify the amount of binding between resveratrol and protein utilizing two equations and methods of calculation. The hypothesis was that protein binding plays a significant role in the stability of resveratrol which can explain the partial recovery of resveratrol from the microcapsules.

3.3 Materials and Methods

3.3.1 Materials

Trans-resveratrol was supplied by Dutch State Mines (DSM, Parsippany, NJ); its purity was $\geq$99% according to the manufacturer. Oxy-resveratrol was used as an internal standard for high performance liquid chromatography (HPLC), and it was purchased from Cayman chemicals (Ann Arbor, MI); its purity was $\geq$98%. Whey protein concentrate and sodium caseinate were supplied by Agropur (La Crosse, WI). Anhydrous milk fat was purchased from Danish maid (Chicago, IL). Methanol was HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO). Materials used for the *in-vitro* digestion study were all purchased from Sigma-Aldrich (St. Louis, MO). The filters used were 0.2 µM PTFE filter (VWR, Radnor, PA).

3.3.2 Methods

3.3.2.1 Microcapsule Production

Resveratrol microcapsules were spray dried in duplicates according to the formulas displayed in Table 3.1 and a schematic diagram of the microcapsule production process is shown in Figure 3.1. Whey protein concentrate or sodium caseinate (Agropur Ingredients, La Crosse, WI) were mixed thoroughly with deionized water and heat treated at 80ºC in a water bath shaker (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ) at 100 rpm for 25 min (whey protein concentrate) or 2 hours (sodium caseinate). The extended period of heat treatment of sodium caseinate was used to enable even dispersion of the protein within the solution. Anhydrous milk fat (AMF) and *trans*-resveratrol were mixed together for 2 min at 15,200 rpm using a hand mixer (IKA Works, Wilmington, NC). Then, the AMF and resveratrol mixture was added into the protein solution. High-pressure homogenization (APV Gaulin Inc., Wilmington, MA) at 55 MPa was, then, used to disperse the mixture evenly and create an emulsion. The solution was passed through the homogenizer twice. The homogenized sample was incubated at 45ºC in a water bath for an hour to ensure it reached temperature equilibrium throughout the solution. The temperature of incubation was chosen because it is above the melting point of anhydrous milk fat [39].

A spray dryer (B-290 Buchi Corporation, New Castle, DE) was used to produce the encapsulated resveratrol particles. The spray drying conditions were: inlet temperature = 160ºC, outlet temperature = 90ºC, flow rate = 5-7 g/min, air pressure = 5 kPa and nozzle diameter = 0.7 mm. Flow rate was adjusted to maintain outlet temperature. Throughout the process, aluminum foil was used to protect the resveratrol from direct exposure to light.

3.3.2.2 High-performance Liquid Chromatography

The reverse phase high-performance liquid chromatography (HPLC) with coulometric detection consisted of Waters 717plus autosampler (Milford, MA) and ESA CoulArray detector (ThermoScientific, Sunnyvale, CA), with a Phenomenex Gemini 5u, C18, 110A, 150x4.6 mm

column (Phenomenex, Torrance, CA). A binary mobile phase was used, 100% methanol and 25 mM sodium acetate at pH 4.5 (Sigma, St. Louis, MO). A gradient separation method was used starting with 30% methanol and held for 2 min. Then, methanol was increased to 60% during 14 min and held for 3 min. Then, the gradient was brought back to initial conditions over 2 min and held constant for 2 min. Oxy-resveratrol was used as an internal standard in HPLC analysis. External standard curves were constructed with pure compounds of *trans*-resveratrol, *cis*-resveratrol and oxy-resveratrol.

3.3.2.3 Moisture Content and Water Activity Measurements

Moisture content was analyzed by HR83 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH), and water activity was assessed by Aqua Lab 4TE (Aqua Lab Technologies, Riverside, CA). Samples from each replication of spray drying were measured in triplicates.

3.3.2.4 Morphology and Particle Size

Microcapsule morphology was observed through scanning electron microscope (XL30 ESEM-FEG, FEI Company, Hillsboro, OR) at Beckmann Institute for Advanced Science and Technology (Urbana, IL). Samples were coated with gold-palladium using a sputter coater (Desk-1 TSC, Denton Vacuum, Moorestown, NJ). Hivac mode was used to observe the morphology of the resveratrol microcapsules at a voltage of 5 kV. Particle size was estimated from the scanning electron microscope images.

3.3.2.5 Microencapsulation Efficiency Measurements

Microencapsulation efficiency (ME) was defined as the fraction of resveratrol that is not washed off by a solvent. Fifty mg of resveratrol microcapsules and 5 mL methanol were combined in a flask and placed on a shaker at 100 rpm for 15 min at room temperature. The solution was centrifuged at 1775 g with a Sorvall Legend Micro 17 centrifuge (ThermoScientific, Sunnyvale, CA) for 5 min at 23°C with and resveratrol content of the supernatant was measured by HPLC. Samples were filtered with 0.2 µM PTFE filters before HPLC injection. Microencapsulation efficiency was calculated using the following equation [40]:

$$\text{ME (\%)} = \frac{(\boldsymbol{total\ resveratrol - surface\ resveratrol})}{\boldsymbol{resveratrol\ load}} * \mathbf{100} \qquad \textbf{(eq 3.1)}$$

3.3.2.6 Resveratrol Recovery Measurements

Resveratrol was extracted from the microcapsules using methanol coupled with sonication and volume standardization. A Qsonica probe sonicator (Cole Parmer, Vernon Hills, IL) was used to sonicate 10 mg resveratrol microcapsules mixed with 4 mL methanol in a 15 mL round bottom tube. The sonication probe was 3.22 mm and 50% amplitude was used. Sample tubes were placed in ice water, during sonication for three cycles of 30 sec continuous sonication followed by 30 sec no sonication, in order to reduce heat buildup of samples. The solutions were transferred to volumetric flasks and brought up to 25 mL in order to account for methanol evaporation during the sonication process. Samples were filtered with 0.2 µM filters before HPLC analysis. Three measurements of each spray dried replication were taken for resveratrol recovery. Resveratrol recovery was calculated using the following equation:

$$\text{Resveratrol Recovery (\%)} = \frac{\boldsymbol{resveratrol\ extracted}}{\boldsymbol{resveratrol\ load}} * \mathbf{100} \qquad \textbf{(eq 3.2)}$$

3.3.2.7 Fluorescence

Fluorescence was used to indirectly measure the binding between resveratrol and sodium caseinate. The method was adapted from prior research measuring resveratrol and sodium caseinate binding [35]. SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA) was used to measure fluorescence with an excitation of 280 nm and emission of 350 nm. Five replications of each sample were taken. An aliquot of 200 µl was loaded into each well of a black, 96-well polystyrene plate (Costar, Corning Incorporated, Corning, NY).

3.3.2.8 Fluorescence of Resveratrol and Ethanol

Protein is able to fluoresce due to natural fluorophores in the protein, which are aromatic amino acids [41]. When protein is bound to resveratrol, the protein no longer fluoresces. The change in fluoresce has been used to calculate the amount of protein bound to resveratrol [35, 36]. Background fluorescence for all components was measured in order to control from non-

specific signals from components in the sample other than protein. Fluorescence measurements ranged from 175 to 250 for various ethanol concentrations and from 110 to 250 for various resveratrol concentrations solubilized in 2.5% EtOH (Figure 3.2). The average fluorescence of a blank well was 243 (±7). Therefore, it was assumed that the fluorescence of ethanol and resveratrol alone do not significantly contribute to the overall fluorescence of the samples which fluorescence measurements ranged between 1200 and 2000. Therefore, the fluorescence of resveratrol was not taken into account for the actual samples and it was assumed that the decrease in fluorescence as resveratrol concentration increase was due to binding between the protein and resveratrol.

3.3.2.9 Fluorescence of Solution with Spiked Resveratrol with Various Resveratrol Concentrations

Sodium caseinate solutions were prepared in the same manner as the resveratrol microcapsules using partial denaturation of the protein. The resveratrol stock solution was 500 µg resveratrol/100 µl 50% EtOH (w/w). Resveratrol was spiked into 1 mg/mL sodium caseinate solution at the following levels: 12.5, 25, 50, 100, and 200 µg/mL sodium caseinate solution (0.055, 0.110, 0.219, 0.438, and 0.876 µM). The standard curve encompassed 8 concentrations of sodium caseinate: 0.007, 0.016, 0.031, 0.063, 0.125, 0.250, 0.500, and 1 mg/mL. The ratio of resveratrol:sodium caseinate was the same as the 4.8% (dry basis, w/w) resveratrol microcapsule.

3.3.2.10 Fluorescence of Microcapsules with Varying Resveratrol Concentrations

When testing the fluorescence of microcapsules with varying resveratrol concentrations, standard curves were built from sodium caseinate microcapsules without any resveratrol content at 7 dilutions. The concentrations of sodium caseinate in the standard curve were 31.25, 62.5, 125, 250, 500, 1000, and 2000 µg/mL of sodium caseinate. Resveratrol microcapsules were produced with various concentrations of resveratrol: 1.2%, 2.4%, 4.8%, 9.1%, 16.7% (% in final product, w/w). All sodium caseinate microcapsules in solution and

resveratrol microcapsule in solution were stored at 5°C overnight to allow protein to fully hydrate before fluorescence measurements.

3.3.2.11 Stern-Volmer Equation

The Stern-Volmer equation was developed by Otto Stern and Max Volmer and it indicates fluorescence quenching, which is a decrease in quantum yield of fluorophore fluorescence due to interaction with a quencher molecule [42, 43]. This equation was modeled after a simple mechanism utilizing one fluorophore, one quencher, one excited state and an irreversible quenching mechanism [44]. A linear plot of the equation indicates a single class of fluorophores while a curvature indicates complex formation of both static and dynamic binding [43]. This equation has been used to measure binding affinity between flavonoids and bovine serum albumin and resveratrol and proteins [35, 36, 43]. The Stern-Volmer equation is as follows:

Stern-Volmer equation = $$\frac{F_0}{(F_0 - F)} = \frac{1}{f} + \frac{1}{f}\frac{1}{ksv}\frac{1}{[Q]}$$ **(eq 3.3)**

F_0 represents the fluorescence of protein without resveratrol and F represents fluorescence of protein with resveratrol. *Ksv* is the quenching constant and *f* is the fraction of binding sites. [Q] is the concentration of resveratrol. The absolute concentration of protein and resveratrol was calculated for each sample. The linear equation from the standard curve of protein was used to determine the F_0 of each sample by plugging the absolute concentration of protein (x) and finding the fluorescence measurement (y). The *f*, fraction of binding sites, was obtained from the best linear fit of 1/[Q] (M^{-1}) on the x-axis and $F_0/(F_0-F)$ on the y-axis (Figure 3.3). The concentration of sodium caseinate was multiplied by *f* to calculate the concentration of resveratrol bound to protein.

3.3.2.12 Equation Based on Number of Resveratrol Binding Sites

The three amino acids that are able to fluoresce are tryptophan, tyrosine and phenylalanine [35]. It is thought that tryptophan is mainly responsible for the fluorescence of

protein because tyrosine is easy quenched and phenylalanine has low quantum yield thereby causing emission from these amino acids to be very low [35, 41]. Therefore, tyrosine and phenylalanine have negligible effects on the fluorescence of proteins. It can be assumed that the binding between resveratrol and tryptophan is responsible for the decrease in fluorescence when resveratrol is present with protein.

The absolute amount of resveratrol and sodium caseinate was calculated for each sample. The average molecular weight used for sodium caseinate was 22,814 g/M which was derived from the molecular weight of each fraction and the percentage of each fraction in sodium caseinate. The percentage by weight of each fraction of α(S1), α(S2), β-casein and κ-casein in sodium caseinate used for the average molecular weight were respectively: 40%, 10%, 35%, and 15%. Amino acid sequences were found on the Uniprot database and sequences from *Bos taurus* (bovine) were used [45]. The reference codes in the database for α(S1), α(S2), β-casein and κ-casein were P02662, P02663, P02666 and P02668, respectively. The molecular weight and number of tryptophan in each fraction were as follows: 23,000 g/M and 2 tryptophan for α(S1)-casein, 25,230 g/M and 2 tryptophan for α(S2)-casein, 24,000 g/M and 1 tryptophan for β-casein, and 19,000 g/M and 1 tryptophan for κ-casein. The molecular weight, percentage of each fraction in sodium caseinate and the number of tryptophan in each protein fraction were taken from the literature and it is important to note that these are assumptions.

The number of tryptophan in each sodium caseinate fraction along with the percentage of each fraction in the total protein were the factors that were taken into account when deriving the resveratrol binding site equation (shown in Table 3.2). Therefore, the assumption of this equation is that tryptophan is the only binding site of resveratrol. The molecular weight of each protein fraction was also taken into account when deriving the equation. For each protein fraction, the percentage of each protein fraction in the total protein was divided by the molecular weight of the protein fraction and the resulting value was multiplied by the number of tryptophan

in the protein fraction. The sum of this value was calculated for each protein fraction and the sum is the constant term in the resveratrol binding sites equation for sodium caseinate, shown below.

$$\text{Fraction of bound resveratrol} = \frac{\mathbf{6.5188E^{-5}} * [\boldsymbol{P}]}{[\boldsymbol{R}]} \qquad \textbf{(eq 3.4)}$$

[P] = concentration of sodium caseinate (g/L)

[R] = molar concentration of resveratrol (M)

$$\text{Constant} = \frac{\#\ of\ tryptophan\ per\ protein\ molecule}{molecular\ weight} = \frac{1.49}{22{,}814\ g/M} = 6.5188E^{-5}\ (M/g)$$

The resveratrol binding sites equation was also developed for whey protein. The fractions of whey protein used in the equation were β-lactoglobulin, α-lactalbumin, and serum albumin which were respectively: 66%, 25% and 9% of the total protein. Molecular weights and number of tryptophan units in each fraction were 18,300 g/M and 2 tryptophan for β-lactoglobulin, 14,200 g/M and 4 tryptophan for α-lactalbumin, and 66,400 g/M and 2 tryptophan for serum albumin [46-48]. The molecular weight, percentage of each fraction in whey protein and the number of tryptophan in each protein fraction were taken from the literature and it is important to note that these are assumptions. The number of resveratrol binding sites for whey protein was calculated using the following equation:

$$\text{Fraction of bound resveratrol} = \frac{\mathbf{1.4527E^{-4}} * [\boldsymbol{P}]}{[\boldsymbol{R}]} \qquad \textbf{(eq 3.5)}$$

[P] = concentration of whey protein (g/L)

[R] = molar concentration of resveratrol (M)

$$\text{Constant} = \frac{\#\ of\ tryptophan\ per\ protein\ molecule}{molecular\ weight} = \frac{2.64}{18{,}173\ g/M} = 1.4527E^{-4}\ (M/g)$$

3.3.2.13 UVA Stability Measurements

Solutions of 10 mg resveratrol microcapsules and 4 mL Nanopure™ water filtered through a Barnstead water purification system (Waltham, MA) were prepared in 15 mL tubes.

The solutions were exposed to UVA light with a wavelength of 365 nm using a Benchtop 2UV Transilluminator (LM-20E, Ultra-Violet Products Ltd, Upland, CA). Samples were placed directly on top of the UVA light source. This amount of UVA light translates to about 3.7 min of sunlight. Resveratrol was extracted from the samples, in the same way, as the resveratrol recovery measurements. Resveratrol isomers were quantified on HPLC and the ratios of *trans*:*cis* resveratrol were calculated.

3.3.2.14 In-Vitro Digestion Test

Digestive stability and bioaccessibility of *trans*-resveratrol was evaluated using a 3-phase digestion model simulating oral, gastric and intestinal phases; utilizing a modified method from Green and others (2007) [49]. Twenty-five mg of microcapsules were used for each measurement and 3 measurements were taken for each spray dried replication. The oral phase consisted of the addition of 8 mg of α-amylase (Sigma A3176) to each sample and tubes were placed in a 37ºC water bath at 90 rpm for 10 min. Base solution for the oral phase consisted of potassium chloride (1.792 g/L), sodium phosphate (1.776 g/L), sodium sulfate (1.140 g/L), sodium chloride (0.596 g/L) and sodium bicarbonate (3.388 g/L). For the gastric phase, 20 mg of pepsin (Sigma P7125, final concentration 0.5 g/L in sample) in 2 mL of 0.1 M HCl was added and samples were acidified to pH 2.5 using 1 M HCl (~200 µl). Sample volumes were brought up to 40 mL with saline and tubes were placed in a shaking water bath for 1 hr at 37ºC. For the intestinal phase, 2 mL 0.1 M $NaHCO_3$ solution containing 40 mg pancreatin (Sigma P1750, final concentration 0.8 g/L) and 20 mg lipase (Sigma L3126, final concentration 0.4 g/L) and 3 mL 0.1 M Na HCO_3 solution containing 120 mg bile (Sigma B8631, final concentration 1.8 g/L) were added to each sample. The pH of samples was adjusted to 6.5 using 1 M $NaHCO_3$ (<50 µl, if needed) and sample volumes brought up to 50 mL with saline (0.9% NaCl). Samples were placed in the water bath for 2 hr at 37ºC. An aliquot of the resulting sample was taken and this was referred to as the digesta. In order to isolate the aqueous fraction, the remaining sample was centrifuged at 10,000 g for 1 hr (Allegra X-22R, Beckman Coulter, Brea, CA) and filtered

with 0.2 µM filters. Samples were placed immediately on ice between phases and blanketed with nitrogen before incubations and storage to minimize oxygen in the sample tubes. Digestive stability is the amount of resveratrol left in the sample after the completion of the small intestinal phase (digesta) in comparison to the amount of *trans*-resveratrol loaded into the microcapsules (resveratrol load).

$$\text{Digestive stability (\%)} = \frac{\textit{trans-resveratrol in digesta}}{\textit{resveratrol load}} * 100\% \qquad \textbf{(eq 3.6)}$$

Bioaccessibility is the experimentally determined estimate of what is available for intestinal absorption by virtue of being solubilized in the continuous aqueous fraction of the digesta. This was the amount of resveratrol partitioned into the micellar fraction, after high speed centrifugation at 10,000 x g for 60 min. It was the comparison of the amount of *trans*-resveratrol in the aqueous fraction in comparison to the resveratrol load.

$$\text{Bioaccessibility (\%)} = \frac{\textit{trans-resveratrol in aqueous fraction}}{\textit{resveratrol load}} * 100\% \qquad \textbf{(eq 3.7)}$$

HPLC was utilized to measure the amount of *trans*-resveratrol in each digestive fraction. Samples were filtered with 0.2 µM filters (VWR, Radnor, PA) before HPLC analysis.

3.3.2.15 Statistical Analysis

Formulations presented in Table 3.1 were spray dried in duplicates. Microsoft Excel (Microsoft, Redmond, WA) was used to analyze data related to resveratrol-protein binding which includes the equation of the best fit line of fluorescence data, calculating percentage of bound resveratrol, and fraction of binding sites. Microencapsulation efficiency, resveratrol recovery, UVA stability, digestion analysis, moisture content and water activity were completed in triplicates. The software, Statistical Analysis System (Cary, NC), was used to conduct analysis of variance (ANOVA, $p<0.05$) and least significant difference (LSD) testing on the data.

3.4. Results and Discussion

3.4.1 Moisture Content and Water Activity Measurements

Moisture content and water activity of the microcapsule formulations are represented in Table 3.3. Moisture content was not significantly different between spray dried replications. When whey protein was used to encapsulate avocado oil through spray drying, the moisture content ranged from 2.24-2.89% with a standard deviation up to about 1% [50]. These results were comparable to the moisture content found in our study when using whey protein concentrate with the inclusion of fat (2.36-2.54%). Water activity of all resveratrol microcapsule formulations varied between 0.06-0.15. This range of water activity was lower than that found for *bifidobacteria* encapsulated in whey protein-based microcapsules in which the water activity was between 0.14-0.18 [51]. The variation in water activity may be attributed to the different encapsulation technique (spray drying vs. freeze drying) and the type of core material, as the optimum condition for *bifidobacteria* is a water activity between 0.11-0.22 [52].

3.4.2 Morphology and Particle Size

Scanning electron microscope (SEM) images of resveratrol microcapsules showed that the microcapsules had a particle size between 1-20 µm and they were non-spherical (Figure 3.4). They appear to have a wrinkled or dehydrated appearance which may be attributed to the nature of the spray drying process when using protein as an encapsulation matrix. Void space was created at the core of the spray dried particle due to expansion and shrinkage during the spray drying process. Other research supports that spray drying with protein as a wall material will produce this type of morphology [53, 54]. Morphology images of dried egg and skimmed milk showed similar morphology as the resveratrol microcapsules [53]. Skimmed milk particles were found to be hollow with an outer shell thickness between 20-60 µm. In addition, morphology of pea protein microcapsules produced through spray drying had a similar morphology to the resveratrol microcapsules [54].

3.4.3 Microencapsulation Efficiency

Microencapsulation efficiency measurements of the samples are shown in Table 3.4. Whey-protein-concentrate-based microcapsules had a higher microencapsulation efficiency than sodium-caseinate-based microcapsules ($p<0.05$). Furthermore, the inclusion of anhydrous milk fat led to a decrease in microencapsulation efficiency (6%) in whey-protein-based microcapsules only ($p<0.05$).

The microencapsulation efficiency of resveratrol was studied within liposomes and a significant difference was found between the encapsulation methods tested [55]. Liposomes prepared through sonication had 44-56% microencapsulation efficiency and those prepared through extrusion had 92-96% microencapsulation efficiency. The low microencapsulation efficiencies of liposomes produced through sonication may have been due to the high shear force used in this processing method which probably resulted in resveratrol movement out of the microcapsule. Microencapsulation efficiency of resveratrol within a solid lipid nanoparticle was 64-89% and within a nanostructured lipid carrier was 65-77% [56]. Our results were similar to those found in the nanoparticles and nanostructured lipid carriers. Differences in microencapsulation efficiencies between studies may be due to the level of solubility of resveratrol in different mediums and the partitioning of resveratrol between these mediums.

3.4.4 Resveratrol Recovery

Recovery of resveratrol using sonication and methanol was not complete. Table 3.4 shows the recovery for most formulations was less than 66% after probe sonication. Therefore, this suggests that the decreased recovery may be attributed to nonspecific binding between the resveratrol and protein. Prior research supported that proteins can bind with resveratrol. The binding constants of whey protein and sodium caseinate with resveratrol are 1.7×10^4 - 1.2×10^5 M^{-1} and 3.7×10^5 M^{-1} - 5.1×10^5 M^{-1}, respectively [35, 36]. The binding between resveratrol and whey protein was thought to be due to dipole-dipole and Van der Waal forces [36]. For sodium caseinate, hydrogen bonding and hydrophobic interactions were thought to be

responsible for resveratrol-protein-binding [35]. The binding between resveratrol and sodium caseinate was investigated, utilizing two equations: Stern-Volmer equation and resveratrol binding sites equation. It was thought that the low resveratrol recovery from the microcapsules was attributed to resveratrol-protein binding.

3.4.4.1 Fluorescence

Fluorescence of the proteins decreased as the resveratrol concentration in the sample increased (Figure 3.5). The change in fluorescence indicated that the resveratrol was binding with the protein and binding increased as resveratrol concentration increased.

3.4.4.2 Estimated Resveratrol Binding Using Stern-Volmer Equation

The increased stability of encapsulated resveratrol may decrease the recovery of resveratrol from the microcapsules. It was thought that the binding between resveratrol and protein inhibits the compound from being fully recovered from the microcapsules using only probe sonication. Resveratrol may be fully extracted from the microcapsules using enzymes and other physical treatments. Thereby, the quantification of binding between resveratrol and protein is necessary in order to provide an explanation for the limited recovery of resveratrol using only probe sonication.

The Stern-Volmer equation (eq 3.3) was used to calculate resveratrol-protein binding. An *f* value of 0.35 and K_{sv} of 22,727 was obtained when fluorescence was measured of the resveratrol microcapsules (Table 3.6). When sodium caseinate solutions were spiked with resveratrol and Stern-Volmer equation used, it was calculated that the *f* and K_{sv} values were 1.04 and 9,640, respectively. An *f* value of 1.20 and K_{sv} of 29,600 were reported in the literature for sodium caseinate solutions with spiked resveratrol [35]. The *f* value reported in the literature was close to that of our sodium caseinate solutions with spiked resveratrol but different from that of our resveratrol microcapsules with varying resveratrol concentrations.

The *f* value is a component in the calculation for bound resveratrol, therefore, the amount of bound resveratrol in microcapsules was significantly less than in protein solutions.

An *f* value slightly greater than 1 may be due to a combination of dynamic and static quenching [35]. Therefore, it was assumed that the lower *f* value in the resveratrol microcapsules indicated that there was limited dynamic binding while the higher *f* value in the sodium caseinate solutions indicated a higher dynamic binding. In the protein solutions, there was more fluidity of components within the matrix therefore providing more opportunity for binding. In comparison, there was less fluidity within the microcapsules thereby resulting in less binding between the protein and resveratrol. The resveratrol microcapsules also had a lower R^2 than the sodium caseinate solutions which suggests that the additional processing in these samples may result in a higher variation in the fluorescence measurements of the samples. These results suggest that the Stern-Volmer equation was more suitable to compare samples with both dynamic and static binding. This is reasonable as the equation was originally modeled for dynamic binding [57, 58]. Also, it would not be appropriate to compare samples of different nature (microcapsule vs. protein solution). Although the encapsulated resveratrol was mixed with water, it was assumed that the resveratrol and protein generally remain within the microcapsule as resveratrol has limited solubility in aqueous solution.

3.4.4.3 Effect of pH on Stern-Volmer Calculations

Fluorescence measurements of the resveratrol microcapsules were measured with and without pH adjustment. The pH of samples without buffer or pH adjustment ranged from 6.4 to 7.3. Phosphate buffered saline, 1x pH 7.4 (Quality Biological Inc, Gaithersburg, MD) was used to buffer protein standards and resveratrol microcapsule samples. The pH was adjusted to 7.4 ± 0.05 with 1 M HCl and 1 M $NaHCO_3$.

The fraction of binding sites and percent bound resveratrol were 0.35 and 6% with pH adjustment and 0.44 and 8% without pH adjustment (Table 3.7). Fluorescence procedures utilized by other research groups to measure protein and resveratrol binding adjusted pH of samples to be pH 7 [36, 37], while other research groups did not control the pH [35]. This

suggests that pH may not be a significant factor in samples within the narrow pH range of 6.4 - 7.3.

3.4.4.4 Estimated Resveratrol Binding Using Resveratrol Binding Sites Equation

Results from using the derived resveratrol binding sites equation to calculate the amount of maximum binding potentials of resveratrol are shown in Figure 3.6. According to the resveratrol binding sites equation, all the resveratrol in the 1.2% resveratrol microcapsule for both whey protein concentrate and sodium caseinate had the potential to bind to the protein. The equation estimated that there was more bound resveratrol in whey-protein-concentrate-based microcapsules because there are more tryptophan binding sites per mole in comparison to sodium caseinate.

The structures of whey protein and sodium caseinate may also play a role in resveratrol-protein binding as it affects the ability of binding sites to be accessed by resveratrol. Sodium caseinate has a more open configuration without any tertiary structure while whey protein is a globular protein [59-61]. Although sodium caseinate may have few tryptophan per protein molecule, the protein structure may allow a higher percentage of these binding sites to be accessible. In comparison, whey protein has more tryptophan per protein molecule but less of these binding sites are accessible to bind with resveratrol.

3.4.4.5 Compare the Resveratrol Binding Using Stern-Volmer Equation and Resveratrol Binding Sites Equation

$$\text{Amount of bound resveratrol (\%)} = \frac{[P]* f}{[R]} * 100\% \qquad \textbf{(eq 3.8)}$$

[P] = molar concentration of protein (M)

[R] = molar concentration of resveratrol (M)

f = fraction of binding sites available

The amount of bound resveratrol was compared using the microcapsules containing 4.8% resveratrol (dry basis, w/w). The calculation of maximum binding potential of resveratrol

by tryptophan in the microcapsule differs significantly when using the Stern-Volmer equation (eq 3.3) (6%) and the resveratrol binding sites equation (eq 3.4) (26%) (Table 3.8). The difference in the results may be due to the fact that one result was experimentally determined while the other solely accounts for the number of tryptophan in the protein. In addition, the resveratrol binding sites equation is the maximum binding potential of resveratrol to tryptophan while the Stern-Volmer equation is not an indication of the maximum binding potential. In addition, the molecular weight of the protein fractions are assumptions as crude systems were used to measure resveratrol-protein binding. The Stern-Volmer equation compares change in fluorescence of the protein in the presence and absence of the resveratrol. The change in fluorescence was assumed to be due to binding of the protein that prevents the protein from fluorescing. The resveratrol binding sites equation solely accounts for the number of tryptophan as it is the main amino acid responsible for binding with resveratrol [35].

The assumptions of the resveratrol binding site equation were that 1) one resveratrol molecule binds with each binding site, 2) only tryptophan is the binding site for resveratrol and 3) all tryptophan in the protein are available for binding.

3.4.4.6 Total Resveratrol Accounted For

Table 3.9 shows the total resveratrol accounted for according to resveratrol binding sites equation for both sodium caseinate and whey-protein-concentrate-based microcapsules. The compilation of both experimentally determined resveratrol recovery data and the estimation of bound resveratrol from the resveratrol binding sites equation indicate that sodium caseinate microcapsules have approximately 90% resveratrol accounted for and whey protein concentrate microcapsules have about 97.5% resveratrol accounted for. The resveratrol that was unaccounted for may be lost in processing of the microcapsules broken down to metabolites.

3.4.4.7 Binding of Resveratrol and Protein

The stability of resveratrol was enhanced by the binding of resveratrol and protein. It was found that specifically β-lactoglobulin, can form a 1:1 complex with resveratrol, binding to

the surface of the β-lactoglobulin with a binding constant of 10^4-10^6 M^{-1}. The binding delayed the conversion of *trans* to *cis*-resveratrol, thereby increasing the photo stability of resveratrol [37]. Molecular docking research showed that the resveratrol was bound to the surface of β-lactoglobulin by two hydrogen bond interactions [62]. One study compared different fractions of whey protein and found that whey protein binds with resveratrol in a 1:1 complex, similar to the results found of β-lactoglobulin [36]. The ratio of bound whey protein:resveratrol was determined from fluorescence measurements. The binding constant of resveratrol and whey protein was between 1.7 x 10^4 to 1.2 x 10^5 M^{-1} [36]. Binding between whey protein and resveratrol was thought to be dipole-dipole and Van der Waal interactions. In sodium caseinate, resveratrol was found to have a binding constant of 3.7 to 5.1 x 10^5 M^{-1}. Binding between sodium caseinate and resveratrol was thought to be hydrogen bonding and hydrophobic interaction [35]. The binding constant of resveratrol with α-casein was 1.9 x 10^4 M^{-1} and with β-casein it was 2.3 x 10^4 M^{-1} [63].

Our results differ from the literature as sodium caseinate was found in the literature to have a higher binding constant than whey protein. In our results, we found that there was more potential for resveratrol to bind with whey protein than sodium caseinate. The difference in bound resveratrol was most likely because the resveratrol binding sites equation solely takes into account the number of tryptophan within a protein. In addition, different testing conditions such as temperature and ratio of resveratrol:protein were used among different researchers. Our sample matrix was resveratrol microcapsules and due to the lack of dynamic binding in this matrix, it may not be accurate to cross-compare our results to other research studies.

In addition, nonpolar interactions may be attributed to the binding between resveratrol and protein. These interactions have been found between resveratrol and human islet amyloid polypeptide oligomers and fibrils [64].

Overall, this research study showed a significant difference between the binding calculations of the Stern-Volmer equation and the resveratrol binding sites equation. The Stern-

Volmer equation was experimentally determined through fluorescence while the resveratrol binding sites equation takes into account the number of tryptophan in the protein. Whey protein was shown to have a higher binding potential than sodium caseinate according to the resveratrol binding sites equation, in order to increase resveratrol stability.

3.4.5 UVA Stability

The results from UVA stability tests are in the form of *trans:cis* resveratrol ratio as shown in Table 3.4. Absolute amounts of *trans*- and *cis*-resveratrol in the samples were estimated according to the ratios found after UVA light exposure (Table 3.5). Ratios of *trans:cis* resveratrol were used to evaluate the stability because not all the resveratrol was able to be recovered after the extraction. Therefore, the comparison of *trans*-resveratrol concentration between formulations would not be a true representative of UVA stability. For this comparison, it was assumed that the unrecovered resveratrol existed in the same *trans:cis* resveratrol ratio as what was extracted. The results showed that sodium-caseinate-based microcapsules was able to preserve the *trans*:*cis* resveratrol ratio in a higher proportion than the whey-protein-concentrate-based microcapsules and unencapsulated resveratrol. This showed that sodium caseinate is a better encapsulation material to maintain resveratrol bioactivity. UV stability of resveratrol encapsulated with chitosan cross-linked with vanillin has been investigated [31]. The recovery of *trans*-resveratrol after UV exposure of these microcapsules was about 78%. The amount of recovered *trans*-resveratrol was maintained in the bioactive form and extracted from the microcapsule. The UV light used in this study was 16 W at about 20 cm distance but the condition used in our study was 8 W at 0 cm distance from the light source. The difference in power and distance of the sample from the UV source may be attributed to differences seen in the retention of *trans*-resveratrol between the studies but it is difficult to cross-compare the studies because different UV light sources were used.

3.4.6 In-Vitro Digestions

Digestive stability of *trans*-resveratrol within the microcapsules (68-86%) was higher than the unencapsulated resveratrol (47%) ($p<0.05$, Figure 3.7). The digestive stability was generally higher in the sodium-caseinate-based microcapsules in comparison to the whey-protein-concentrate-based microcapsules. In terms of bioaccessibility, all microcapsule formulations increased bioaccessibility of resveratrol (48-60%) in comparison to unencapsulated resveratrol (23%) (Figure 3.8). This suggests that the encapsulation of resveratrol decreased the isomerization or degradation of resveratrol throughout digestion as evaluated by the *in-vitro* digestion. Encapsulation may also have increased the solubility of resveratrol thereby playing a factor in increased *in-vitro* stability. Sodium caseinate proved to be a better encapsulation matrix than whey protein concentrate for resveratrol and the inclusion of anhydrous milk fat in the formulation did not significantly affect stability. Therefore, it is not necessary to include anhydrous milk fat in the formulation.

Encapsulation can aid in the protection of resveratrol throughout the digestion system and enable targeted release of the compound in the human body. Our results suggested that the bioavailability would be increased and degradation of resveratrol during digestion would be decreased. This assumption is supported by the higher bioaccessibility and digestive stability of the encapsulated resveratrol compared to unencapsulated resveratrol within the 3-phase *in-vitro* digestion model.

When humans were fed 25 mg resveratrol orally, 70% of the compound was absorbed but only trace amounts of *trans*-resveratrol were detected in the plasma. Most of the *trans*-resveratrol was metabolized to sulfates and glucuronic acid found in the urine [21]. Research by Azorin-Ortuno and others (2011) supported this theory as 50% of *trans*-resveratrol and derived metabolites administered to pigs was found in the jejunum and ileum of the small intestine [65]. Therefore, stabilization of resveratrol in the digestive tract is an important

consideration in order to deliver the compound in a bioactive form to the targeted part of the digestive tract.

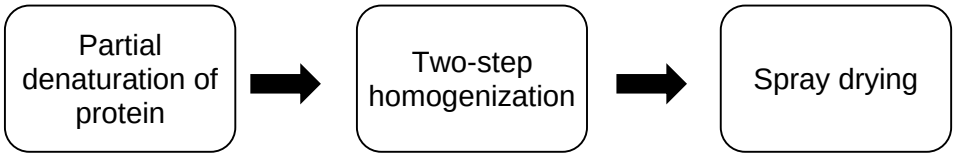
3.5 Conclusions

The resveratrol binding sites equation provides simple quantification of bound resveratrol without experimentation. This method can be extended to other polyphenols or bioactive compounds which bind to sodium caseinate and whey protein concentrate in a similar manner as resveratrol. The equation can also be altered to fit the parameters of other types of protein where the amino acid composition is known. The use of fluorescence and the resveratrol binding sites equations have limitations as they only take into account binding of resveratrol with aromatic amino acids. Further research can investigate if resveratrol can bind with additional amino acids, aside from tryptophan or other components of the protein. This understanding will provide a more accurate estimate of bound resveratrol and allow comparison of different proteins as an encapsulation material.

The combination of spray drying as an encapsulation method and sodium caseinate as an encapsulation matrix proved to be an effective approach to produce stabilized resveratrol. This processing technique and encapsulation material are desirable as they are widely available at relatively low cost. Thus, sodium-caseinate-based microcapsules can be further investigated to increase stability in UV light and *in-vitro* digestion. One limitation of the findings was that the microscopic images of the resveratrol microcapsules showed the morphology was non-spherical. Future research can investigate the incorporation of specific components, such as plasticizers, in resveratrol microcapsules and their effect on morphology and stability of resveratrol within the microcapsule. The encapsulation-system-approach utilized in this research can be extended to other labile, bioactive compounds such as quercetin or lutein to increase their stability.

3.6 Figures and Tables

Figure 3.1: Schematic diagram of resveratrol microcapsule production



Inlet temp. = 160ºC
Outlet temp. = 90ºC
Flow rate = 5-7 g/min
Air pressure = 5 kPa
Nozzle diameter = 0.7 mm

Figure 3.2: Fluorescence measurements from spectrofluorometer of: A) Ethanol concentrations without resveratrol, B) Various resveratrol concentrations solubilized in 2.5% ethanol

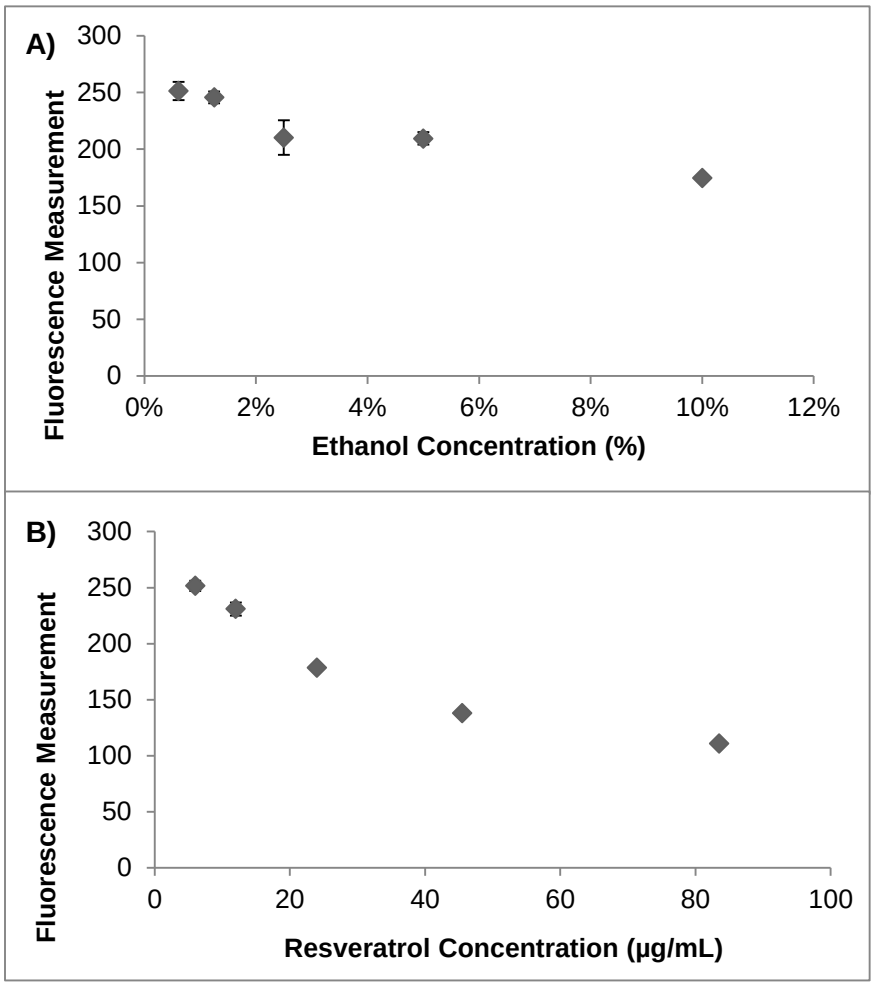
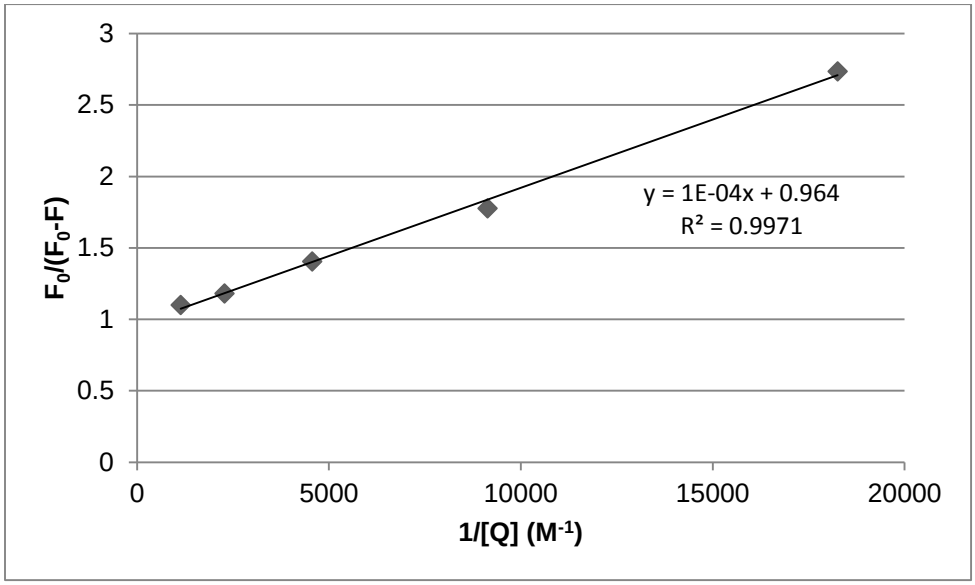
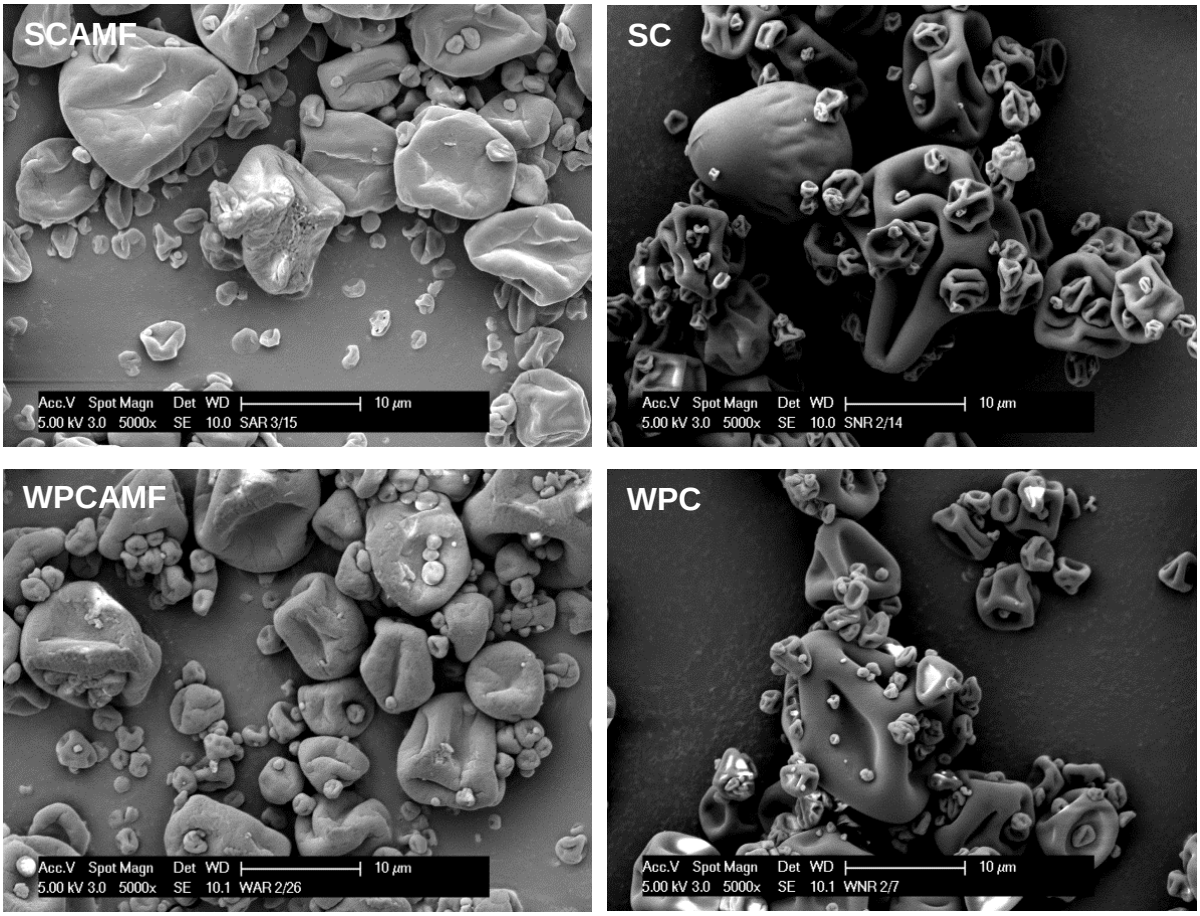


Figure 3.3: Best fit linear line of 1/[Q] (M^{-1}) and $F_0/(F_0-F)$, components of the Stern-Volmer equation. Fraction of binding sites calculated by 1/intercept of best fit line.



1/[Q] (M^{-1}) and $F_0/(F_0-F)$ from Stern-Volmer equation (eq 3.3)
[Q]: Resveratrol concentration
F_0: Fluorescence measurement without resveratrol
F: Fluorescence measurement in the presence of resveratrol

Figure 3.4: Scanning electron microscope images of resveratrol microcapsules



AMF: Anhydrous milk fat
SCAMF: Sodium caseinate with AMF, SC: Sodium caseinate without AMF
WPCAMF: Whey protein concentrate with AMF, WPC: Whey protein concentrate without AMF

Figure 3.5: Fluorescence measurements of sodium caseinate solutions with various resveratrol concentrations showing that as resveratrol increases, fluorescence decreases

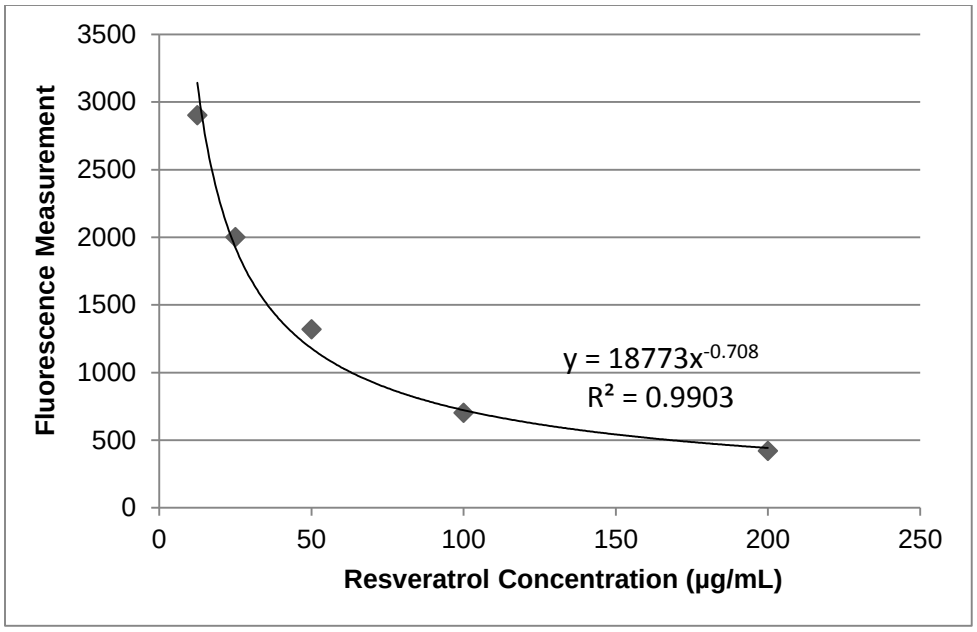
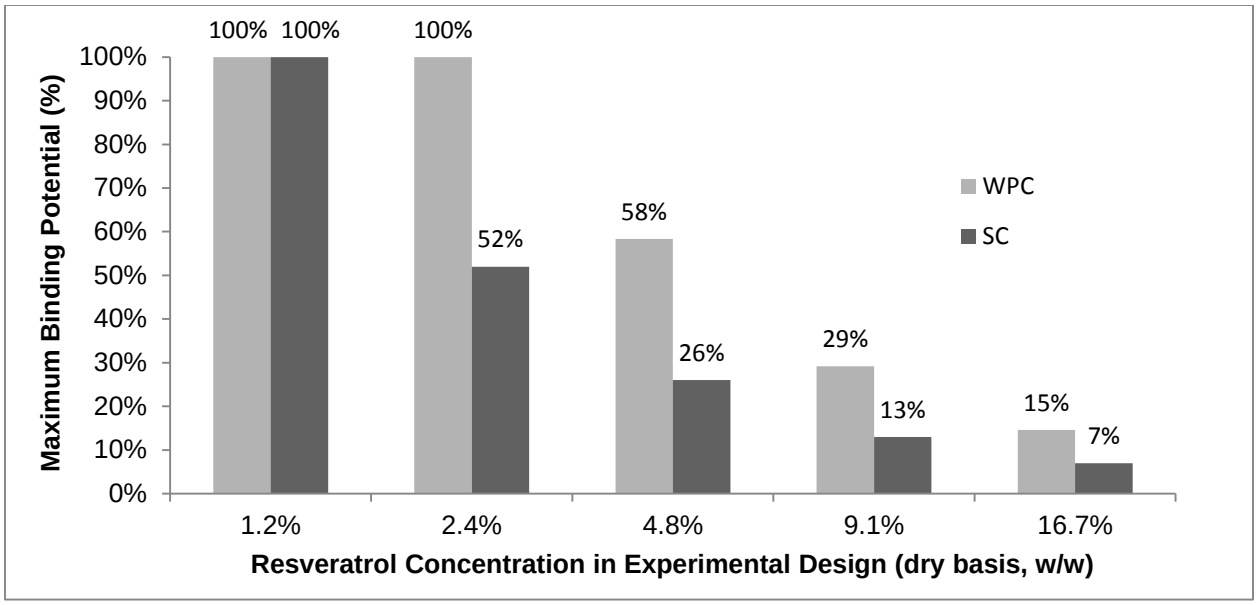
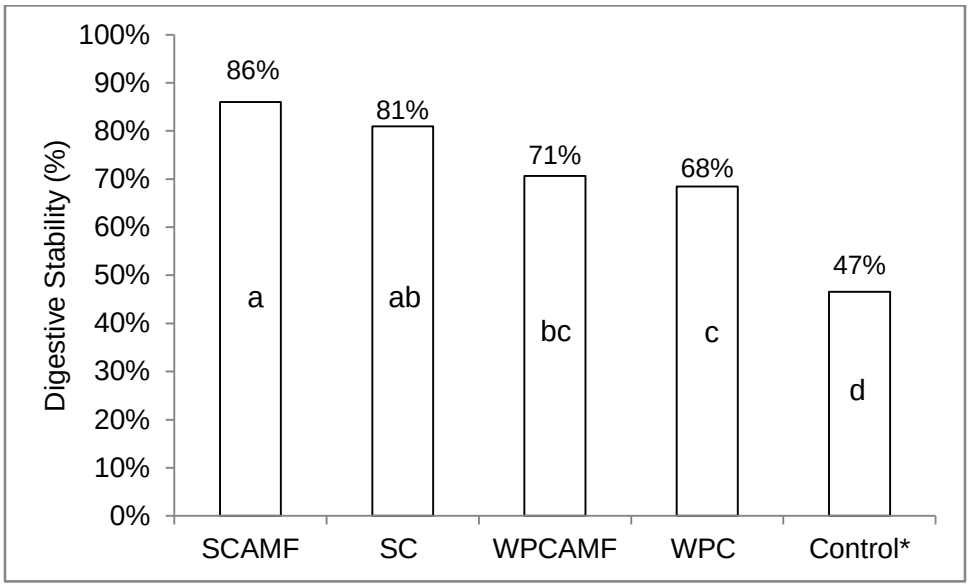


Figure 3.6: Maximum binding potential of resveratrol in sodium-caseinate and whey-protein-concentrate-based microcapsules with various resveratrol concentrations according to resveratrol binding sites equation



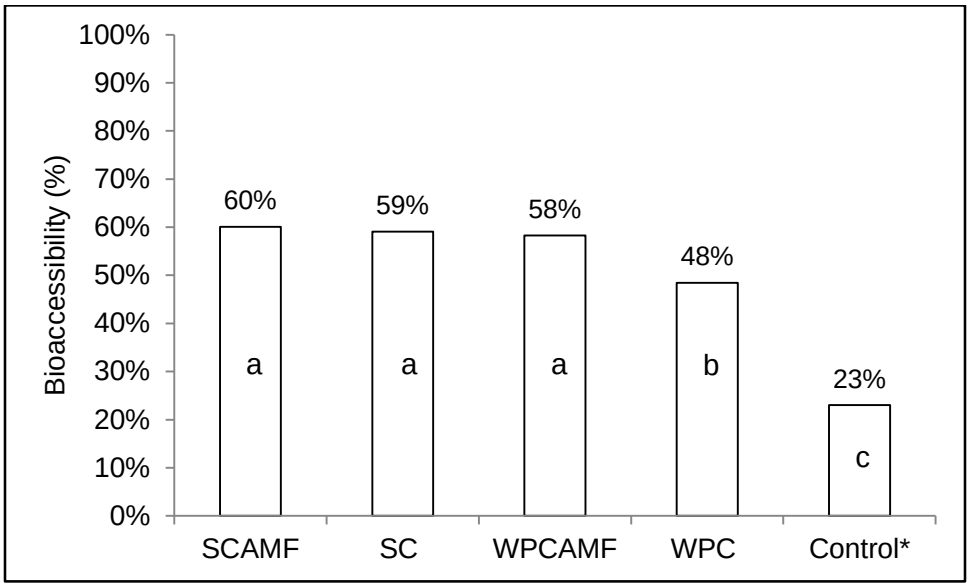
WPC: Whey-protein-concentrate-based microcapsules
SC: Sodium-caseinate-based microcapsules

Figure 3.7: Digestive stability (%) of resveratrol in microcapsules after 3-phase *in-vitro* digestion



Values represent average of two spray dried replicates
Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD)
Anhydrous milk fat (AMF); sodium caseinate with AMF (SCAMF); sodium caseinate without AMF (SC); whey protein concentrate with AMF (WPCAMF); whey protein concentrate without AMF (WPC)
* Unencapsulated resveratrol

Figure 3.8: Bioaccessibility (%) of resveratrol in microcapsules after 3-phase *in-vitro* digestion



Values represent average of two spray dried replicates
Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD)
Anhydrous milk fat (AMF); sodium caseinate with AMF (SCAMF); sodium caseinate without AMF (SC); whey protein concentrate with AMF (WPCAMF); whey protein concentrate without AMF (WPC)
* Unencapsulated resveratrol

Table 3.1: Formulas for resveratrol microcapsules using sodium caseinate and whey protein concentrate, with and without anhydrous milk fat

Component	***SCAMF***	***SC***	***WPCAMF***	***WPC***
Sodium Caseinate	80 g	80 g	0 g	0 g
Whey Protein Concentrate	0 g	0 g	80 g	80 g
Anhydrous milk fat	100 g	0 g	100 g	0 g
Deionized Water	920 g	920 g	920 g	920 g
Trans-Resveratrol (% by total solids)	9 g (4.8%)	4 g (4.8%)	9 g (4.8%)	4 g (4.8%)

Table 3.2: Percent of total protein, molecular weight, and number of tryptophan for each protein fraction in A) sodium caseinate and B) whey protein concentrate

A)

Protein Fraction	% of total protein by weight	Molecular Wt	# of tryptophan
αS1-casein	40	23000	2
αS2-casein	10	25230	2
β-casein	35	24000	1
κ-casein	15	19000	1
		Weighted Avg:	1.49

B)

Protein Fraction	% of total protein by weight	Molecular Wt	# of tryptophan
B-lactoglobulin	66	18300	2
alpha-lactalbumin	25	14200	4
serum albumin	9	66400	2
		Weighted Avg:	2.64

Table 3.3: Percent moisture content and water activity, mean (±SD), of resveratrol microcapsules

Sample	Moisture Content	Water Activity
SCAMF R1	2.42[a]	0.12[b]
SCAMF R2	2.17[ab]	0.10[d]
SC R1	2.12[ab]	0.07[g]
SC R2	2.35[a]	0.11[c]
WPCAMF R1	2.36[a]	0.15[a]
WPCAMF R2	2.54[a]	0.06[h]
WPC R1	1.73[bc]	0.09[e]
WPC R2	1.51[c]	0.07[f]

(±standard deviation), R = replication
AMF: Anhydrous milk fat
SCAMF: Sodium caseinate with AMF, SC: Sodium caseinate without AMF
WPCAMF: Whey protein concentrate with AMF, WPC: Whey protein concentrate without AMF
Same letters within each column represent not significantly different values after ANOVA and LSD ($p<0.05$).

Table 3.4: Efficiency of microencapsulation, recovery and UVA stability ratio (*Trans:Cis* Ratio) of resveratrol in microcapsules

Sample	Efficiency	Recovery	UVA Stability
SCAMF	68%[c]	66%[b]	0.62[a]
SC	68%[c]	62%[b]	0.64[a]
WPCAMF	77%[b]	42%[c]	0.46[b]
WPC	83%[a]	37%[c]	0.39[b]
Control	---	93%[a]	0.49[b]

Values represent average of two spray dried replicates
Same letters within each column represent not significantly different values after ANOVA and LSD ($p<0.05$).
Anhydrous milk fat (AMF); sodium caseinate with AMF (SCAMF); sodium caseinate without AMF (SC); whey protein concentrate with AMF (WPCAMF); whey protein concentrate without AMF (WPC); Unencapsulated resveratrol (Control)

Table 3.5: Estimated absolute values of *trans*- and *cis*-resveratrol in the resveratrol microcapsules after UVA light testing

Sample	*Trans:Cis* resveratrol Ratio	*Trans*-resveratrol (µg)	*Cis*-Resveratrol (µg)
SCAMF	0.62	184	296
SC	0.64	187	293
WPCAMF	0.46	151	329
WPC	0.39	135	345
Control	0.49	158	322

Estimated absolute values are calculated according to the resveratrol load and ratio of *trans:cis* resveratrol after UVA light testing at 365 nm for 1 hour. Resveratrol load in 10 mg of microcapsules is 480 µg resveratrol.
AMF: Anhydrous milk fat, Control: Unencapsulated resveratrol
SCAMF: Sodium caseinate with AMF, SC: Sodium caseinate without AMF
WPCAMF: Whey protein concentrate with AMF, WPC: Whey protein concentrate without AMF

Table 3.6: Comparison of the fraction of binding sites (*f*), quenching constant of sodium caseinate by resveratrol (K_{sv}) and correlation coefficient (R^2) of resveratrol microcapsules, sodium caseinate solutions with spiked resveratrol, and values reported in the literature (similar procedure to sodium caseinate solutions with spiked resveratrol)

Sample	*f*	K_{sv}	R^2
Resveratrol microcapsules	0.35	22,727	0.897
Sodium caseinate solution with spiked resveratrol	1.04	9,640	0.997
Literature [32] at 25ºC	1.20	29,600	0.978

Values are components of the Stern-Volmer equation

Table 3.7: Fraction of binding sites (f) and bound resveratrol in resveratrol microcapsules with and without pH adjustment to pH 7.4

Sample	*f*	Bound Resveratrol (%)
With pH adjustment (pH = 7.4)	0.35	6%
Without buffer or pH adjustment (~pH 6.4-7.3)	0.44	8%

f value is component of the Stern-Volmer equation

Table 3.8: Estimated maximum binding potential of resveratrol by tryptophan in microcapsule with various resveratrol concentrations using number of resveratrol binding sites in the protein

Sample	Maximum Binding Potential of Resveratrol by Tryptophan (w/w %)
Stern-Volmer Equation – Resveratrol Microcapsules	6%
Stern-Volmer Equation – Sodium Caseinate Solution with Spiked Resveratrol*	18%
Resveratrol Binding Site Equation	26%

* Ratio of resveratrol:sodium caseinate was the same as the 4.8% (dry basis, w/w) resveratrol microcapsule

Table 3.9: Total resveratrol accounted for according to resveratrol binding sites equation for 4 resveratrol microcapsule formulations

Sample	***Recovery*** [1]	***Maximum Binding Potential by Tryptophan*** [2]	***Total Resveratrol*** [3]
SCAMF	66%	26%	92%
SC	62%	26%	88%
WPCAMF	42%	58%	100%
WPC	37%	58%	95%

[1]Experimental resveratrol recovery using probe sonication
[2]Maximum binding potential of resveratrol by tryptophan according to resveratrol binding sites equation
[3]Total resveratrol (%) = Resveratrol recovery (%) + Maximum binding potential of resveratrol by tryptophan (%)
SCAMF: sodium caseinate w/anhydrous milk fat microcapsule
SC: sodium caseinate w/o anhydrous milk fat microcapsule
WPCAMF: whey protein concentrate w/anhydrous milk fat microcapsule
WPC: whey protein concentrate w/o anhydrous milk fat microcapsule

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CHAPTER 4: EFFECT OF PLASTICIZERS ON STABILITY OF ENCAPSULATED RESVERATROL

4.1 Abstract

Microencapsulation in a sodium-caseinate-based microcapsule has been shown to provide higher UV stability of resveratrol compared to unencapsulated resveratrol, in our prior research (Chapter 3). Scanning electron microscope images showed that the morphology of the microcapsules was non-spherical, and this provided rationale to enhance the spherical nature of the microcapsules through the addition of plasticizers. The increased spherical nature would reduce surface area of the microcapsule thereby increasing stability of resveratrol. Therefore, the objective of this research was to evaluate the effect of plasticizers (propylene glycol, sorbitol, sucrose) on the UV stability of encapsulated resveratrol and morphology of the microcapsules. It was found that the addition of plasticizers in the concentration range of 31.7-47.6% (dry basis, w/w) to the resveratrol microcapsules decreased the *trans:cis* resveratrol ratio after UV light exposure, thereby indicating a decreased light stability in comparison to the formulation without added plasticizers. The UV light stability of encapsulated resveratrol within a protein matrix with the inclusion of a plasticizer was not significantly different across the three plasticizer types. Morphology images showed that the resveratrol microcapsules with sorbitol used as a plasticizer exhibited a more spherical shape. The use of plasticizers also resulted in the microcapsule wall being thin and fragile which may be related to the decreased light stability of resveratrol in these microcapsules. Since the addition of plasticizers did not increase stability of resveratrol nor enhance the spherical nature of the microcapsule, the secondary objective was to evaluate the effect of protein denaturation on UV stability of resveratrol within a microcapsule. The exclusion of protein denaturation in the preparation of the resveratrol microcapsules resulted in a lower *trans:cis* resveratrol ratio after UV stability testing in comparison to microcapsules in which the preparation included protein denaturation. The stability of resveratrol within microcapsules without protein denaturation was not significantly

different than that of microcapsules with added plasticizers. Overall, the use of plasticizers and absence of protein denaturation in the processing of resveratrol microcapsules did not enhance the UV stability of the microcapsules.

Keywords: plasticizers, microencapsulation, resveratrol, stability, morphology

4.2 Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a polyphenol found in red grapes, red wine, peanuts and blueberries [1-4]. This compound has been associated with health benefits related to the prevention and alleviation of disease states related to cancer, diabetes, and heart disease [5-8]. Some of the challenges with resveratrol incorporation into food systems are bitterness and light instability [9-11]. Wines fortified with 20 and 200 mg resveratrol/L were found to have an increased bitterness in comparison to wines without fortification [9]. In terms of light instability, exposure of resveratrol to UV light for 1-2 hours isomerized 80-90% of resveratrol from the bioactive form to the bio-inactive form [10, 11]. The incorporation of resveratrol into a microcapsule with a stable wall material can help to overcome these challenges through the innovative processing of resveratrol utilizing spray drying. Microencapsulation is readily used in the food industry to protect micronutrients and also enable the delayed release of flavor compounds [12, 13].

Our prior investigation was focused on the stability of resveratrol encapsulated in a sodium caseinate matrix (Chapter 3). Encapsulation was able to increase both UV light and *in-vitro* digestion stability of resveratrol. Scanning electron microscope images showed that the resveratrol microcapsules were non-spherical and contained many folds and crevices. It was hypothesized that the inclusion of plasticizers may further increase stability of the resveratrol by enhancing the spherical shape of microcapsules thereby decreasing the surface area of the compound that is exposed to environmental conditions.

The effect of plasticizers has been previously investigated in hydroxypropyl methylcellulose (HPMC) based microcapsules produced through spray drying [14]. Plasticizers that were used in the HPMC microcapsules were triethylcitrate, propylene glycol, polyethylene glycol, glycerin and citric acid. Cohesiveness of the microcapsules was increased with the use of plasticizers. Triethylcitrate microcapsule had a rapid release of the core material due to the porous nature of the microcapsules. Propylene glycol, glycerin, and citric acid were shown to have positive effects on microcapsule wall formation and drug release kinetics. Citric acid has also been used as a plasticizer in theophylline particles with a cellulose polymer [15]. The plasticizer addition increased the spherical nature of the particle and also the size of the drug crystals.

There is a wide range of research that looked into the effect of plasticizers on film formation that potentially can be translated to microcapsule formation. Sorbitol has been added to sodium caseinate films in ratios of plasticizer to sodium caseinate ranging from 1:65 to 2:1 [16]. Partial specific volume of the sodium caseinate solution was reduced with the inclusion of sorbitol, which indicated a more ordered structure of the protein. In addition, sorbitol led to a decrease in glass transition temperature. The effect of plasticizers (sorbitol, glycerol, polyethylene glycol) was investigated in fish protein films [17]. The use of sorbitol increased mechanical resistance and decreased film flexibility. In comparison, glycerol and polyethylene glycol exhibited a lower mechanical resistance and higher film flexibility. Sucrose had a more significant effect as a plasticizer on elongation at break than invert sugar in cassava starch films [18]. In β-lactoglobulin films, the effect of glycerol, sorbitol, polyethylene glycol, and sucrose were compared according to the elastic modulus, tensile strength, and elongation percentage [19]. The plasticizer efficiency in the films was ranked from most to least efficient as glycerol, polyethylene glycol, sorbitol, and sucrose.

The objective of this study was to evaluate the effect of plasticizers on the stability of resveratrol in sodium-caseinate-based microcapsules and the morphology of the microcapsules.

The three plasticizers that were compared in terms of UV stability and morphology were propylene glycol, sorbitol, and sucrose. The hypothesis was that plasticizers will help to enhance the spherical shape of the resveratrol microcapsules that would in turn increase the UV stability of resveratrol within the microcapsule. The secondary objective was to evaluate the effect of protein denaturation in the microcapsule processing on the UV stability of resveratrol in microcapsules. The hypothesis was that protein denaturation would enhance UV stability of resveratrol in the microcapsule by resulting in a more open protein structure thereby providing more access to resveratrol binding sites.

4.3 Material and Methods

4.3.1 Materials

Propylene glycol (USP) had a purity of 99.8%, and sorbitol was a 70% solution (USP/FCC); both were donated by Archer Daniels Midland (Chicago, IL, U.S.A.). Sucrose used as a plasticizer was from C&H (Crockett, CA, U.S.A.). Sodium caseinate was donated by Agropur (La Crosse, WI, U.S.A.), and resveratrol was donated by Dutch State Mines (DSM, Parsippany, NJ, U.S.A.). All solvents used were HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The internal standard used was oxy-resveratrol with $\geq$98% purity from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Filters, used before HPLC analysis, were 0.2 uM PTFE (VWR, Radnor, PA, U.S.A.).

4.3.2 Microcapsule Production

The formulations of resveratrol microcapsules are shown in Table 4.1. All formulations were spray dried in duplicates. Sodium caseinate solution was partially denatured for 2 hr in a shaking water bath (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ, U.S.A.) at 100 rpm and 80°C. Plasticizers were added to the microcapsules in a 1:1 ratio of protein to plasticizer. This level of plasticizer was chosen because it was the average of prior studies that used sorbitol and glycerol as plasticizers [16, 20]. Resveratrol and plasticizer were mixed into

the protein solution at 15,200 rpm with a hand mixer (IKA Works, Wilmington, NC, U.S.A.). The resulting solution underwent 2 cycles of high-pressure homogenization (APV Gaulin Inc., Wilmington, MA, U.S.A.) at 55 MPa in order to create a homogeneous dispersion. A lab-bench spray drier (B-290 Buchi Corporation, New Castle, DE, U.S.A.) was then used to produce resveratrol microcapsules with an inlet temperature of 160°C and outlet temperature of 90°C. Additional parameters of spray drying were flow rate between 5-7 g/min, air pressure of 5 kPa and nozzle diameter of 0.7 mm. Aluminum foil was used to protect sample solutions from light exposure during the microcapsule production process.

SC-SOR microcapsules with a 2:1 ratio of sodium caseinate to sorbitol was spray dried with the same methodology as previously explained in this section. The purpose was to test if the ratio of protein to plasticizer had a significant effect on resveratrol stability within the sodium-caseinate-based microcapsule. In addition, SC microcapsules without protein denaturation were also spray dried in order to observe the effect of protein denaturation on UV stability of resveratrol within the microcapsule.

4.3.3 Morphology and Particle Size

Scanning electron microscope (XL30 ESEM-FEG, FEI Company, Hillsboro, OR, U.S.A.) at Beckmann Institute for Advanced Science and Technology (Urbana, IL, U.S.A.) was used to observe the morphology of resveratrol microcapsules. In order to prevent surface charging, gold-palladium through sputter coating (Desk-1 TSC, Denton Vacuum, Moorestown, NJ, U.S.A.) was used to coat samples before viewing with a scanning electron microscope. Hivac mode was used to observe the morphology of the resveratrol microcapsules at a voltage of 5 kV. Particle size was estimated from the scanning electron microscope images.

4.3.4 Moisture Content and Water Activity

Moisture content and water activity were measured in three replications with HR 83 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH, U.S.A.) and Aqua Lab 4TE (Aqua Lab Technologies, Riverside, CA, U.S.A.), respectively.

4.3.5 HPLC

Resveratrol isomers were quantified on high-performance liquid chromatography using coulometric detection (ESA CoulArray detector, ThermoScientific, Sunnyvalle, CA, U.S.A.) and a C18 column (Phenomenex Gemini 5u, 110A, 150x4.6 mm, Torrance, CA, U.S.A.). Standards of both resveratrol isomers and oxy-resveratrol (Cayman Chemicals, Ann Arbor, MI) were used to construct external standard curves. The HPLC method utilized a gradient of two mobile phases, 100% methanol (Sigma-Aldrich, St. Louis, MO) and 25 mM sodium acetate (Sigma-Aldrich, St. Louis, MO), pH 4.5. The starting condition was 30% methanol which was held for 2 min and gradually increased to 60% methanol at 14 min. These conditions were held for 3 min, and the amount of methanol was decreased back to 30% within 2 min and held for an additional 2 min.

4.3.6 UV Stability

Ten mg of resveratrol microcapsules was added to 4 mL ultra-filtered water, to form a solution, in 15 mL centrifuge tubes (Thermo-Scientific, Rochester, NY, U.S.A.). Samples were exposed to 365 nm of UVA light for 1 hr in Benchtop 2UV Transilluminator (LM-20E, Ultra-Violet Products Ltd., Upland, CA, U.S.A.). Resveratrol was extracted from the microcapsules with probe sonication (Qsonica probe sonicator, Cole Palmer, Vernon Hills, IL, U.S.A.), using 3 cycles of 30 sec continuous sonication then 30 sec no sonication. The amplitude used in the sonication was 50%, and the diameter of the probe was 3.22 mm. Sample tubes were placed in ice water during probe sonication in order to minimize heat buildup within the samples. Samples were filtered and then injected to HPLC to quantify resveratrol isomers. The ratio *of trans*:*cis* resveratrol was compared among microcapsule formulations to evaluate UV stability of the encapsulated resveratrol. UV stability testing was conducted in triplicates for each spray dried replicate.

4.3.7 Statistics

Statistical analysis system (SAS, Cary, NC, U.S.A.) was used to conduct analysis of variance (ANOVA) and least significant difference testing (LSD) on the data. Microsoft Excel (Microsoft, Redmond, WA) was used to calculate the *trans:cis* resveratrol ratio of samples after UV light exposure.

4.4 Results and Discussion

4.4.1 Morphology and Particle Size

Scanning electron microscope images of resveratrol microcapsules are shown in Figure 4.1. Particle size for resveratrol microcapsules with sorbitol or sucrose used as a plasticizer ranged between 1-30 µm. Resveratrol microcapsules with propylene glycol as a plasticizer ranged between 1-20 µm. Sorbitol, as a plasticizer, helped to improve the spherical shape of the microcapsules in comparison to the formulation without the addition of plasticizer. Sucrose and propylene glycol had a minimal effect on the microcapsule morphology. Microcapsule walls appeared to be thin and fragile, according to the microscopic images at 10,000-20,000 magnification (Figure 4.2). In films, the addition of plasticizers has been shown to increase tensile strength that can decrease flexibility of the film thereby resulting in a brittle film [21]. Propylene glycol as a plasticizer in β-lactoglobulin films resulted in a brittle film and the concentration of plasticizer was independent of mechanical strength [19]. Therefore, it is hypothesized that the addition of plasticizers in the resveratrol microcapsules caused the capsule wall to be weak and easily broken. The wall flexibility was probably decreased and tensile strength increased, resulting in a capsule that was more rigid and broken with moderate force. The walls may have been easily broken in the spray drying process as the particle expands and contracts onto itself due to the heat used in the drying process.

4.4.2 Moisture Content and Water Activity

Resveratrol microcapsules with propylene glycol as a plasticizer had a significantly higher moisture content than the sodium-caseinate-based resveratrol microcapsules without any added plasticizers (Table 4.2). The resveratrol microcapsules with added propylene glycol also had the highest water activity out of all microcapsule samples. Propylene glycol has a lower molecular weight than the other two plasticizers used in resveratrol microcapsules (sorbitol and sucrose). Therefore, there were a larger number of propylene glycol molecules in the microcapsules than the other plasticizers, thereby providing more opportunity for the plasticizer to bind with the protein. This decreased water binding with the protein may have resulted in a higher moisture content and water activity of the microcapsule.

In addition, the use of sorbitol in the resveratrol microcapsules in a 2:1 ratio of sodium caseinate to plasticizer resulted in significantly lower moisture content than the sodium-caseinate-based resveratrol microcapsules. The addition of glycerol in chitosan films has been shown to have an increased moisture content with increasing concentration of the plasticizer, which disagrees with the findings of our study [22]. This occurrence indicated that the use of plasticizers can alter the moisture content and water activity of the matrix. Water sorption in films has been shown to be affected by a range of factors including size of the plasticizer, interaction between water molecules, plasticizers and polymers [23]. Some plasticizers may increase the water activity or moisture content while other plasticizers may decrease these measurements.

4.4.3 UV Stability

The *trans:cis* resveratrol ratios of microcapsules with added plasticizers after UV light exposure was significantly lower than microcapsules without plasticizers and unencapsulated resveratrol (Figure 4.3). Therefore, the addition of plasticizers to resveratrol microcapsules decreased the *trans:cis* resveratrol ratio after UV exposure. The effect of plasticizers in tara gum films have been studied, and it was found that the plasticizers increased mobility of the

polymers within the film by decreasing intermolecular forces [24]. These results suggest that the addition of plasticizers in the resveratrol microcapsules caused an increased mobility of molecules that in turn may result in a higher concentration of resveratrol migrating to the surface of the microcapsule.

It has been found that the size, shape and composition of a plasticizer can affect the ability of a protein film from forming hydrogen bonding [19]. Therefore, incorporation of plasticizers into the resveratrol microcapsules may have affected the stability of the compound as the plasticizer binds with the protein, thereby reducing the number of binding sites available to resveratrol.

There was no significant difference between the type of plasticizer used in the microcapsule in terms of UV stability of resveratrol. Sorbitol and sucrose have been previously compared as plasticizers in protein films, and it was found that sorbitol was a more effective plasticizer in terms of elasticity, tensile strength and elongation [19]. In our research, the plasticizers were compared only on the basis of UV stability that is aligned with evaluating the light stability of resveratrol. The parameters measured in the protein films with added plasticizers may not be directly correlated to UV stability of resveratrol in protein-based microcapsules.

When the amount of sorbitol in the microcapsule was decreased to a 2:1 ratio of protein to plasticizer, the *trans:cis* resveratrol ratio did not change significantly from microcapsules with a 1:1 ratio of protein to plasticizer (Figure 4.3). The ratio of sodium caseinate to sorbitol did not significantly affect the UV stability of resveratrol within the microcapsules. In addition, the lack of denaturation of sodium caseinate significantly decreased the *trans:cis* resveratrol ratio after UV exposure in comparison to the sodium-caseinate-based microcapsule with protein denaturation. Therefore, protein denaturation plays a significant role in enhancing UV stability of resveratrol within a protein microcapsule. Denaturation of protein has been defined as “a major change from the original native structure, without alteration of the amino acid sequence”

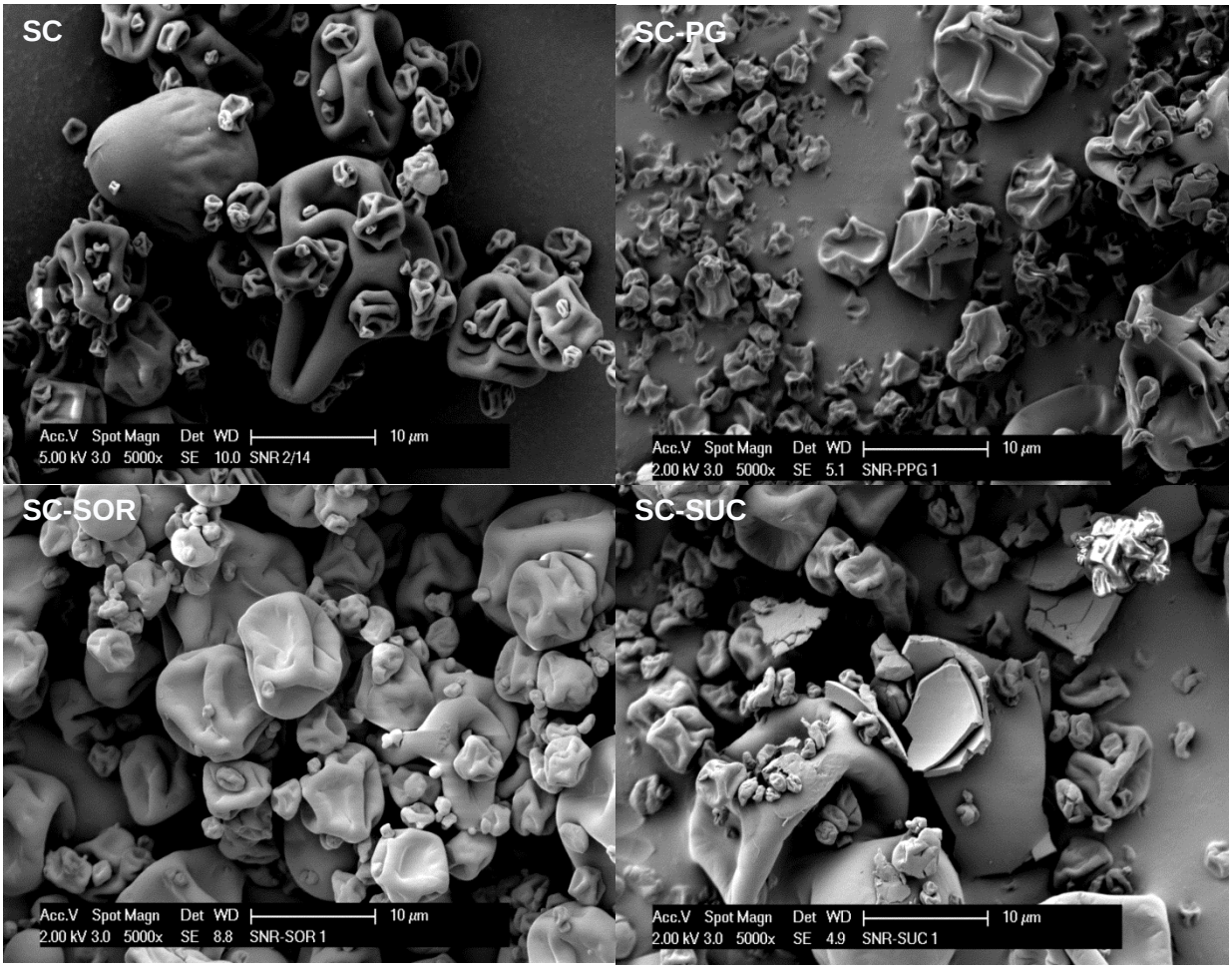
[25]. Thus, the change in the native structure of sodium caseinate resulted in an increased UV stability, perhaps by increasing the number of binding sites available on the protein for resveratrol to bind.

4.5 Conclusions

Plasticizers have been shown to increase tensile strength of protein gels but when they were applied to microcapsules with the objective of protecting resveratrol, they were not able to increase stability of the compound. Tensile strength and other parameters commonly measured in films may not be directly related to enhanced stability of bioactive compounds in microcapsule matrixes. The use of stability testing is the most direct means to evaluate the effect of additional components in microcapsules. Moisture content and water activity are thought to be factors that affect the stability of these bioactive compounds, due to their effect on mobility of the compound within the matrix. A limitation of this study was the effect of plasticizers was only investigated in sodium-caseinate-based microcapsules. In order to protect resveratrol from environmental factors, the incorporation of plasticizers into other types of protein-based microcapsules can be further investigated in the future. Another limitation of this research was the morphology of the microcapsules were only qualitatively evaluated by scanning electron microscope imaging and future research can evaluate morphology through a quantitative measure. In addition, it is important to note that the morphology of the particles may be different with scale up using a larger spray dryer.

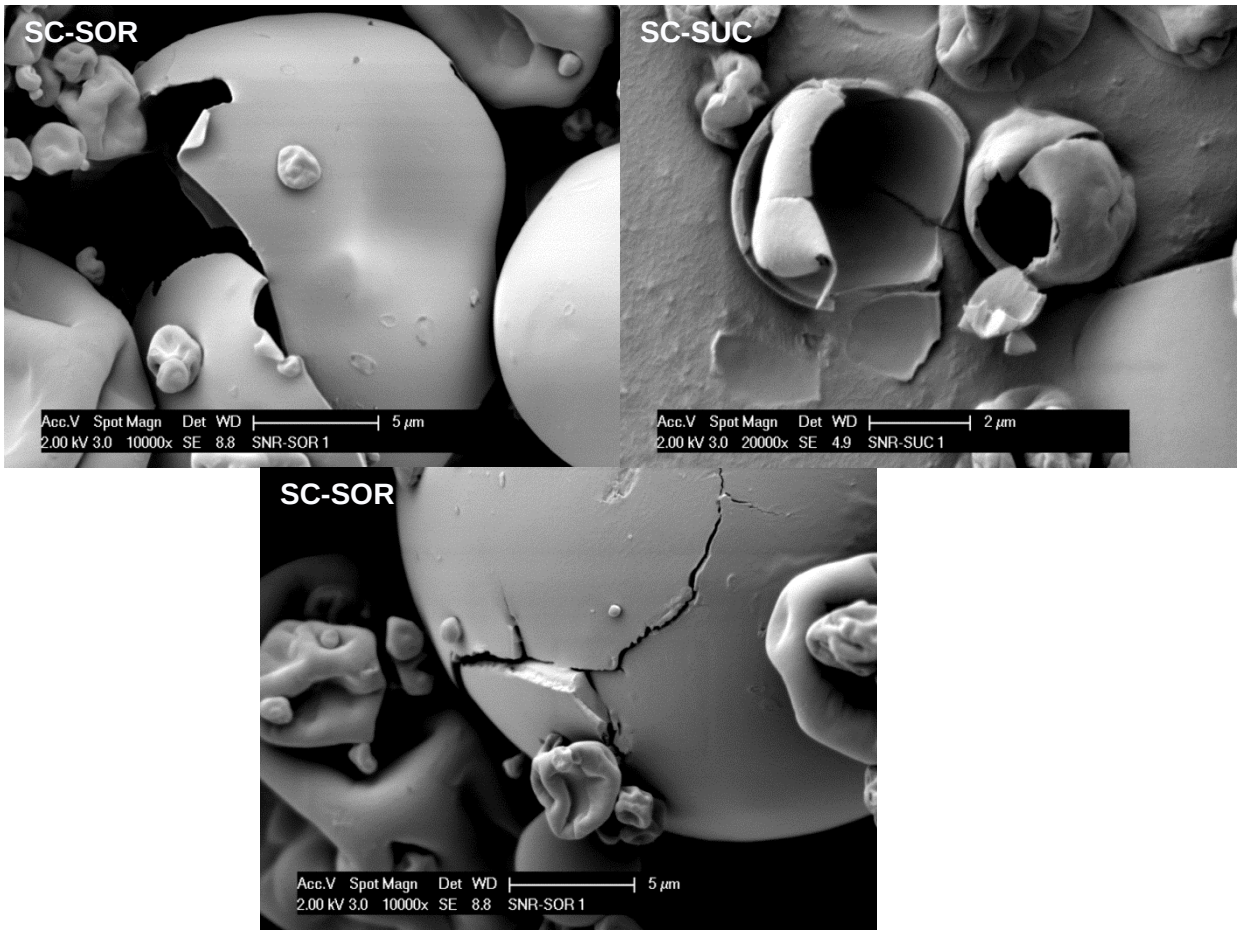
4.6 Figures and Tables

Figure 4.1: Scanning electron microscope images of sodium-caseinate-based resveratrol microcapsules with and without plasticizers



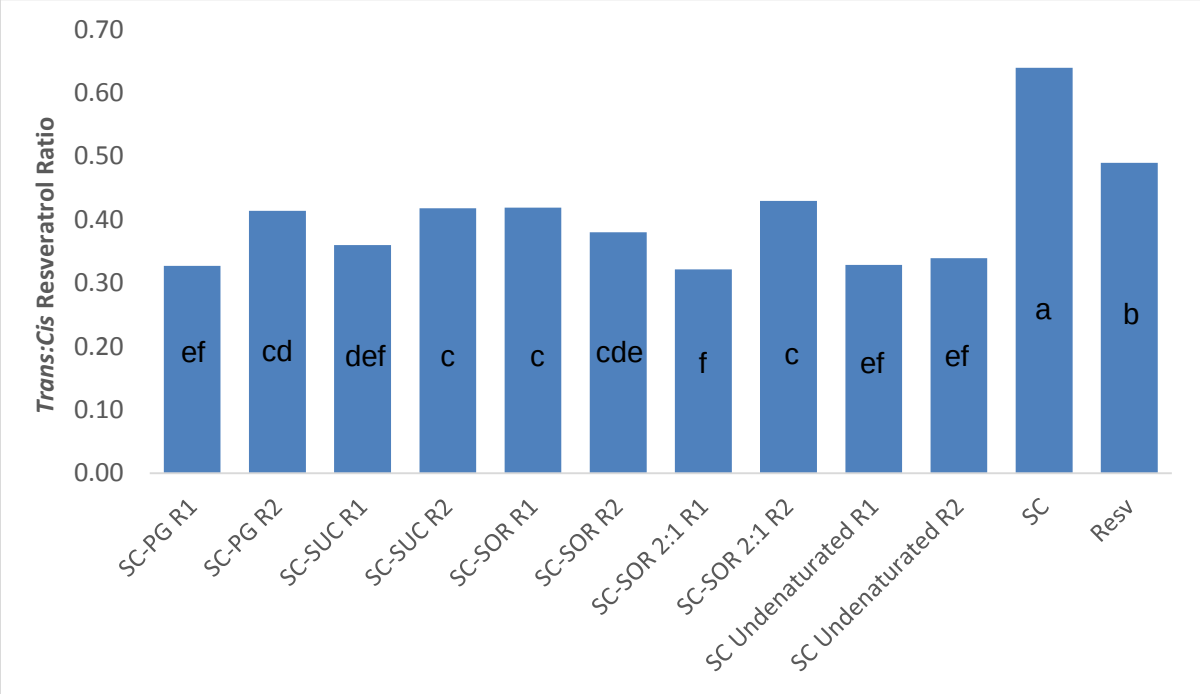
SC: sodium caseinate without plasticizer
SC-PG: sodium caseinate with propylene glycol
SC-Sor: sodium caseinate with sorbitol
SC-Suc: sodium caseinate with sucrose

Figure 4.2: Scanning electron microscope at 10,000x and 20,000x magnification of resveratrol microcapsules with added plasticizers



SC-Sor: sodium caseinate with sorbitol
SC-Suc: sodium caseinate with sucrose

Figure 4.3: *Trans:Cis* resveratrol ratio of resveratrol microcapsules with and without plasticizers in comparison to unencapsulated resveratrol (Resv)



SC-PG: sodium caseinate with propylene glycol (1 protein:1 plasticizer)
SC-Suc: sodium caseinate with sucrose (1 protein:1 plasticizer)
SC-Sor: sodium caseinate with sorbitol (1 protein:1 plasticizer)
SC-Sor 2:1: sodium caseinate with sorbitol (2 protein:1 plasticizer)
SC Undenaturated: sodium caseinate without plasticizer, protein undenatured
SC: sodium caseinate without plasticizer
R: replication
Resv: unencapsulated resveratrol

Table 4.1: Resveratrol microcapsule formulations with the addition of plasticizers and the sodium-caseinate-based microcapsule without plasticizer (SC)

Component	SC	SC-PG	SC-Sor	SC-Suc
Sodium Caseinate	80 g	80 g	80 g	80 g
Plasticizer Purity	---	99.8%	70%	~100%
Plasticizer Amount	---	80 g	114 g	80 g
Deionized Water	920 g	920 g	886 g	920 g
***Trans*-Resveratrol (% by total solids)**	4 g (4.8%)	8 g (4.8%)	8 g (4.8%)	8 g (4.8%)

SC: sodium caseinate without plasticizer
SC-PG: sodium caseinate with propylene glycol
SC-Sor: sodium caseinate with sorbitol
SC-Suc: sodium caseinate with sucrose
Sorbitol was a 70% solution with the remaining 30% being water. The purity of this plasticizer was accounted for in the microcapsule formulations.

Table 4.2: Resveratrol microcapsule analyses of mean moisture content (%) and mean water activity of resveratrol microcapsules with the addition of plasticizers, sodium-caseinate-based microcapsule without protein denaturation (SC-Undenat) and sodium-caseinate-based microcapsule without plasticizer (SC)

	Moisture Content	***Water Activity***
SC-PG R1	3.14^{a}	0.33^{a}
SC-PG R2	2.87^{ab}	0.27^{b}
SC-Sor R1	1.67^{abc}	0.08^{g}
SC-Sor R2	2.81^{efg}	0.18^{c}
SC-Suc R1	1.88^{ef}	0.10^{e}
SC-Suc R2	1.70^{efg}	0.06^{i}
SC-Sor2:1 R1	1.61^{fg}	0.08^{f}
SC-Sor2:1 R2	1.34^{g}	0.07^{g}
SC-Undenat R1	2.53^{bcd}	0.07^{h}
SC-Undenat R2	2.41^{bcd}	0.05^{j}
SC R1	2.12^{de}	0.07^{h}
SC R2	2.35^{cd}	0.11^{d}

R = replication
SC-PG: sodium caseinate with propylene glycol
SC-Sor: sodium caseinate with sorbitol
SC-Suc: sodium caseinate with sucrose
SC-Sor 2:1: sodium caseinate with sorbitol (2 protein:1 plasticizer)
SC-Undenat: sodium caseinate with plasticizer, protein not denatured
SC: sodium caseinate without plasticizer

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CHAPTER 5: TASTE DETECTION THRESHOLDS OF RESVERATROL

5.1 Abstract

Resveratrol is a polyphenol that is associated with numerous health benefits related to heart disease, cancer, diabetes and neurological function. The addition of this compound to food products would help to deliver these health benefits to the consumer. However, bitterness associated with resveratrol may impart negative sensory qualities on the food products, which may decrease consumer acceptability. This concern may be resolved by encapsulating resveratrol through spray drying, an innovative processing technique. The objectives of this research were to: 1) compare taste detection thresholds of unencapsulated resveratrol (unencapsulated) and encapsulated resveratrol and 2) determine if the inclusion of anhydrous milk fat in the formulation affects the taste detection threshold of resveratrol within the microcapsules. Resveratrol microcapsules were produced by encapsulating resveratrol in a protein matrix through spray drying. R-index measure by the rating method was used to find the average taste detection threshold and the pooled group taste detection threshold. The average and pooled group taste detection thresholds for resveratrol, sodium-caseinate-based resveratrol microcapsule without fat (SC), and sodium-caseinate-based resveratrol microcapsule with fat (SCAMF) were: 90 and 47 mg resveratrol/L, 313 and 103 mg resveratrol/L, 334 and 108 mg resveratrol/L of resveratrol, respectively. The findings demonstrate that the encapsulation of resveratrol decreased the detection of the compound and provided a means to incorporate the resveratrol into food products without imparting negative sensory properties.

Keywords: Resveratrol, threshold, encapsulation, R-index, bitterness

5.2 Introduction

Resveratrol is one of the polyphenols found in the skin of red grapes and red wine, which contributes to the health benefits of red wine [1, 2]. It has been shown to have positive impacts on heart disease, cancer, diabetes and neurological function [3-6]. The addition of resveratrol to food products would help to offer biologically active concentrations of the compound in easy-to-consume foods in order to provide the health benefits to the consumer.

One of the challenges with the incorporation of resveratrol into products is the astringent and bitter perception of the compound being a polyphenol [7]. However, there has been limited research related to the sensory perception of resveratrol. Descriptive analysis testing was utilized to evaluate bitterness of Riesling wine fortified with resveratrol [7]. A significant difference in bitterness existed between wines fortified with resveratrol at 20 mg/L and the control without any resveratrol. Significant differences in bitterness also existed between wines fortified with 20 and 200 mg resveratrol/L, with the higher concentration being rated as more bitter.

Chemical analysis on 23 Italian wines showed the concentration of resveratrol in wine ranged from 0.18-5.44 mg resveratrol/L with the exception of one wine that had no resveratrol content [8]. For the sensory evaluation of the Italian wines, a trained panel rated samples according to a criteria commonly used for wine judging competitions according to the *Associazione Enotecnici Italiani-Organizzazione Nazionale Assagiatori Vini* model which provided one sensory evaluation score that took into account all categories such as appearance, taste and bouquet. There was no direct correlation between the overall sensory ratings and the resveratrol content. Overall sensory ratings were the only form of sensory evaluation conducted and specific attributes were not evaluated individually.

There is very limited prior research on the sensory properties of resveratrol and none that investigated the taste detection threshold of this compound. In addition, no research has explored encapsulation of resveratrol as a way to mask the unattractive sensory perception of

resveratrol, which, in turn, could maintain consumer acceptance of products with added resveratrol. In this research study, signal detection rating method was used to measure taste threshold values of unencapsulated resveratrol and encapsulated resveratrol in solution. The first objective of this research was to compare the taste detection threshold of resveratrol microcapsules with a protein matrix to that of unencapsulated resveratrol. The hypothesis was that the resveratrol within the microcapsules would have a higher taste detection threshold than unencapsulated resveratrol. The second objective was to determine the effect of anhydrous milk fat in the microcapsule formulation on taste detection threshold of resveratrol within the microcapsule. Due to the higher fat solubility of resveratrol, the hypothesis was that the resveratrol would be dispersed within the fat and the protein would form a protective layer around the fat. Therefore, the taste detection threshold of resveratrol when encapsulated with fat and protein combined was hypothesized to be higher than that of resveratrol microcapsules that only utilized protein as a matrix. This research can provide an indication of the ideal level at which encapsulated resveratrol microcapsules can be added to food products without a negative impact on sensory properties of the product.

5.3 Materials and Methods

5.3.1 Encapsulated Resveratrol Production

Taste detection threshold was evaluated for two formulations of encapsulated resveratrol. The first formulation contained 95.2% sodium caseinate (Agropur Ingredients, La Crosse, WI, U.S.A.) and 4.8% resveratrol (DSM, Parsippany, NJ, U.S.A.) by solid weight basis. The second formulation contained 52.9% anhydrous milk fat (Danish Maid, Chicago, IL, U.S.A.), 42.3% sodium caseinate and 4.8% resveratrol, by solid weight basis. The amount of protein in the sample solution (8%) before spray drying was determined from prior research in our lab to be a level at which the protein could be solubilized in solution [9]. The amount of anhydrous milk was slightly higher than the amount of protein in the formulations. The increased amount of

fat was used to help solubilize the resveratrol within the solution [10]. Resveratrol concentration was kept consistent among formulations at 4.8% resveratrol (dry basis, w/w). This resveratrol concentration was chosen to provide more wall material than resveratrol.

Two formulations of resveratrol microcapsules were tested in order to compare the effect of anhydrous milk fat in the microcapsule formulation on the taste detection threshold of resveratrol. Sodium caseinate was partially denatured for 2 hours at 80ºC and 100 rpm in a shaking water bath (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ, U.S.A.). Anhydrous milk fat and resveratrol were mixed into the protein solution using a hand mixer (IKA Works, Wilmington, NC, U.S.A.) at 15,200 rpm for 3 min. An evenly dispersed solution was obtained through a two-stage high-pressure homogenization (APV Gaulin Inc., Wilmington, MA, U.S.A.) at 55 MPa. Resveratrol microcapsules were produced through spray drying with Buchi B-290 (New Castle, DE, U.S.A.) spray dryer. The following spray drying conditions were used: 160ºC inlet temperature, 90ºC outlet temperature, 0.7 mm nozzle diameter, 4.5-7.5 g/min flow rate and 7 kPa.

5.3.2 Sample Preparation

Unencapsulated resveratrol and the two types of encapsulated resveratrol were tested at five concentrations, in three-fold increments. The concentrations of unencapsulated resveratrol tested were 2.2, 6.7, 20, 60, and 180 mg resveratrol/L. The water solubility of resveratrol is 0.03 mg/mL; therefore, it is assumed that 30 mg/L of resveratrol is soluble and the remaining resveratrol concentration is dispersed in solution [11]. The concentrations of resveratrol in the microcapsules tested were 4.4, 13.3, 40, 120, and 360 mg resveratrol/L. These levels were established by 2 rounds of preliminary testing with individuals who were familiar with threshold testing. The first round of preliminary testing used 12 panelists (4 males and 8 females) and 1 replication of each concentration/panelist was tested. The concentrations tested for unencapsulated resveratrol were 0.625, 1.25, 2.5 and 40 mg resveratrol/L. The concentrations

tested for encapsulated resveratrol were 0.625, 1.25, 2.5 and 80 mg resveratrol/L. The rinse protocol consisted of 1) warm water and 2) room temperature water.

The second round of preliminary testing used 13 panelists (5 males and 8 females) and two replications of each concentration/panelist were tested. The concentration tested for unencapsulated resveratrol was 20 mg resveratrol/L and for encapsulated resveratrol was 40 mg resveratrol/L. The rinse protocol consisted of 1) room temperature water (Absopure, Urbana, IL, U.S.A), 2) ice cream (Meijers, Grand Rapids, MI, U.S.A.), 3) carbonated water (Meijers, Grand Rapids, MI, U.S.A.) and 4) room temperature water (Absopure, Urbana, IL, U.S.A). The inclusion of the ice cream and carbonated water in the rinse protocol helped to further cleanse the palette in comparison to the first testing.

In both preliminary tests, testing was conducted under incandescent lighting in a conference room and responses were collected on paper ballots. The noise sample was 0.4% ethanol solution for unencapsulated resveratrol and spring water for the resveratrol microcapsules. The signal sample was the highest concentration of resveratrol being tested. Panelists were first asked to familiarize themselves with both the signal and noise samples. Once familiar with these samples, they were presented with the test samples and rated each sample according to four options: 1) Signal Sure, 2) Signal Unsure, 3) Noise Unsure, and 4) Noise Sure.

For the actual testing, six replications of the noise and each concentration were tested. The noise sample was the background solution which unencapsulated resveratrol or the microcapsules were dispersed within. In order to solubilize resveratrol, it was dissolved in 1.2% ethanol (EtOH), 190 Proof, USP (New Brunswick, NJ, U.S.A.). It was confirmed by HPLC that 1.2% EtOH (v/v) was sufficient to dissolve 180 mg resveratrol/L (Appendix A). The encapsulation of resveratrol helped to increase its solubility; therefore, it was not necessary to use an ethanol solution for these samples. Resveratrol microcapsules without anhydrous milk in the formulation were added to a base protein solution. The sodium caseinate added to the

base solution differed for each microcapsule concentration in order for the sum of protein in the microcapsules and in the base solution to be equal to that in the highest concentration of microcapsules. A similar procedure was applied to microcapsules with the inclusion of anhydrous milk fat, the difference being that the base solution included both anhydrous milk fat and protein, in order to keep the amount of protein and fat consistent across all microcapsule concentrations. All base solutions underwent two-stage high-pressure homogenization (APV Gaulin Inc., Wilmington, MA, U.S.A.) at 55 MPa in order to keep the solutions homogeneous.

All samples were prepared the day before testing, in a room without windows and under amber light, in order to minimize light degradation of resveratrol. The wavelength associated with yellow or amber light is the least degradative to light-sensitive resveratrol [12]. Twenty mL of sample was placed in 60 mL clear, plastics containers with lids. Samples were put on trays, and the entire tray was covered with foil and stored between 3-5°C, overnight. Samples were labeled with a 3-digit code and served at room temperature.

5.3.3 Rating Method and R-index Measurement

R-index by rating method and the American Society of Testing and Materials (ASTM) method of ascending limits are the most commonly used threshold methods [13-18]. R-index measurement by rating has been used in relation to the noise and signal samples [19]. R-index by rating method and ASTM method have been found to be similar when the thresholds of caffeine solutions determined by the two methods were compared [13]. R-index by rating method was proposed to be a more efficient method than ASTM, because fewer samples were needed [13, 20].

5.3.4 Taste Detection Threshold Testing

This study utilized 33 panelists between 18-45 years of age, 11 males and 22 females. Panelists were asked to not eat, drink, or smoke at least 30 minutes prior to the testing. They were also instructed to not wear strong cosmetics that may interfere with their perception of the

samples. In the first session, panelists received a brief presentation on the testing method in order to familiarize them with the rating method used to determine the R-index measure.

The testing was completed in a booth setting with red lighting, positive air flow, and room temperature of 25ºC. Sample cups were placed on top of red colored paper on trays to help mask color differences across the samples. Compusense® *five* Plus (version 5.6, Guelph ON, Canada) was used to determine a randomized complete block design for six replications of five concentration levels and the noise. Each panelist rated six replications of each concentration of unencapsulated resveratrol and encapsulated resveratrol.

The taste detection threshold testing began with a warm-up phase, in which panelists were presented with two samples: Noise and Signal. The noise sample was 1.2% ethanol for unencapsulated resveratrol samples. For resveratrol microcapsules, the noise sample was the base solution to which the microcapsules were added, as described in the sample preparation section. The signal sample was the highest concentration of the sample being tested in the test design. Panelists were instructed to swirl the samples on the tray for 2-3 seconds, then, place the sample in their mouth and swish around for 2-3 seconds. After waiting 10 seconds, the panelists were asked to focus on the perception of the sample, in order to fully assess astringency which can be a delayed perception. Panelists rinsed their mouth with the same rinse protocol before and between all samples. The rinse protocol was as follows: 1) heavy whipping cream (Land O'Lakes, Arden Hills, MN, U.S.A.), 2) carbonated water (Meijers, Grand Rapids, MI, U.S.A.), 3) room temperature water (Absopure, Urbana, IL, U.S.A). All samples and rinses were expectorated.

Once the panelists were familiar with the Noise and Signal samples, they were presented with the first sample tray which contained six samples (5 concentration levels of resveratrol and one noise sample). The panelists rated all samples in comparison to the noise and signal samples, choosing one of the four options: Signal Sure, Signal Unsure, Noise Unsure, Noise Sure. The testing protocol of the samples was similar to the warm-up phase,

where panelists were instructed to swirl the cup on the tray before tasting and a 10-second timer was embedded to the program to force panelists to wait 10-seconds before rating each sample. Panelists were allowed to re-taste the noise and signal samples at any time.

After the completion of the first sample set, there was a 2-minute break, during which time the panelists held ¼ of a slice of Sara Lee Soft and Smooth bread with no crust (Chicago, IL, U.S.A.) in their mouth compressed between the top of the mouth and tongue to soak up any residue sample or rinses. A built-in 2-minute timer in the Compusense® *five* Plus system (version 5.6, Guelph ON, Canada) reinforced the break. After compressing the bread in the mouth, panelists repeated the rinse protocol to cleanse the palate. Then, the panelists rated another six samples that consisted of five concentration levels and the noise.

5.3.5 Testing of Additional Concentration Level

The empirical threshold is defined as 50% above the chance probability of selecting the correct answer (R-index of 50%) [21], an R-index of 75% is the empirical threshold [13, 20, 22]. A number of panelists (11 panelists for unencapsulated resveratrol, 16 panelists for SC, 20 panelists for SCAMF) did not reach their individual threshold in the five concentration levels of unencapsulated resveratrol and encapsulated resveratrol testing. The pooled R-index reached above 75% only for the resveratrol testing range. Therefore, an additional testing was completed, which included the highest concentration from the original testing, a concentration that was 3-fold higher than the highest level from the original testing, and the noise to more accurately determine the pooled and individual threshold values. Resveratrol was dissolved in 3.6% ethanol. Ethanol content was increased 3-fold from the original testing because the concentration of resveratrol was tripled.

The additional testing was conducted in a similar manner to the original testing, but panelists evaluated a total of 15 samples per session. Sample order was randomized using Compusense® *five* Plus (version 5.6, Guelph ON, Canada) program, between and across all sample sets. Five replications of each sample were tested with three sample trays of five

samples per tray. The same warm-up and rinse protocol were used in the additional testing. Five samples were served on each tray with a 2-minute break between each sample tray, in which panelists held ¼ of a slice of bread compressed between the tongue and top of the mouth. After the 2-min break, panelists rinsed their mouth with the established protocol.

5.3.6 Post-Questionnaire

At the completion of the threshold testing, all panelists filled out a questionnaire that was aimed at gathering 1) demographic information, 2) information on a suitable product for a health claim related to resveratrol, and 3) knowledge of resveratrol. Some of the questions included: "What product would be best aligned with the health claim, may decrease cancer risk, increase heart health and neurological function?" and "Which of the following health benefits do you associate with resveratrol?" Responses were collected on paper ballots, shown in Appendix B.

5.3.7 Data Statistical Analysis

Data were collected using Compusense® *five* Plus (version 5.6, Guelph ON, Canada). This software assigned randomized sample orders to each panelist and presented the testing questions along with the embedded timer to panelists throughout the testing. Data were exported into Microsoft Excel (Microsoft, Redmond, WA, U.S.A.). Statistical Analysis System (SAS, Cary, NC, U.S.A) was used to conduct analysis of variance (ANOVA) and least significant difference (LSD) test on the data. Nonparametric LSD was used to find where the difference existed between check-all-that-apply questions in the post questionnaire related to health benefits of resveratrol.

5.3.8 Taste Detection Threshold Calculations

Taste detection thresholds were calculated for all pooled data and each panelist using the R-index response matrix method [22]. For each panelist, resveratrol concentration (x-axis) was plotted against the R-index percentage (y-axis). A linear line was constructed between the two points above and below an R-index of 75%. Then, this linear equation was used to calculate the resveratrol concentration (x-axis) at which the R-index (y-axis) was 75%. The R-

index of panelists who participated in the additional testing was calculated by the additional testing data of the 180 mg resveratrol/L and 540 mg resveratrol/L in the microcapsules. Panelists who did not have a taste detection threshold within the concentration range tested in both the original and additional testing were not included in the average or pooled threshold calculations. The sample size (N) in Table 5.1 indicates the number of panelists who had a taste detection threshold within the tested concentration range. Pooled taste detection thresholds (mg/L) were calculated by compiling the individual data from all panelists who reached threshold in the first testing and data from the second testing of those who reached threshold during the additional testing. The sample size for the pooled threshold was 198 (6 replications of each sample per panelist with a total of 33 panelists).

5.4 Results and Discussion

5.4.1 Taste Detection Threshold

The average individual panelist thresholds for encapsulated resveratrol were significantly higher than unencapsulated resveratrol (Table 5.1). This indicated that the unencapsulated resveratrol was detected at a lower concentration than resveratrol in the microcapsules. The findings demonstrated that encapsulation of resveratrol within a protein or protein and fat matrix successfully masked the detection of the compound in aqueous solutions. The presence of fat within the formulation did not significantly decrease the detection of resveratrol as hypothesized, which was evidenced by no difference between the thresholds of SC and SCAMF formulations ($P>0.05$). These results were contrary to our expectations. The anhydrous milk fat is solid at room temperature, which is the temperature that samples were served. Therefore, the resveratrol would be physically encapsulated within the fat of the microcapsules. Opposed to the microcapsules without anhydrous milk fat, in which resveratrol could be more easily extracted from the microcapsules when in solution. One possible explanation is that the resveratrol microcapsules were hollow as shown by scanning electron microscope images

(Chapter 3). Therefore, the water can diffuse into the microcapsules when mixed into a solution. In this way, the resveratrol may diffuse into the aqueous phase and reach equilibrium between the inside and outside of the microcapsule.

The pooled threshold of each sample was 2- to 3-fold lower than the average of the individual panelists' threshold (Table 5.1). In a past study on mouthfeel detection threshold for sucrose and high fructose corn syrup (HFCS), the average of individual panelist thresholds and pooled taste detection thresholds were similar to each other [14]. In comparison, the pooled threshold of sulfur compounds in wines was 2- to 3-fold higher than the average individual panelists' threshold [23]. The discrepancy seen in our study between individual panelists' average taste threshold and pooled threshold may have been due to the variation between the propylthiouracil (PROP) status of panelists [24, 25]. PROP tasters may have been able to detect the bitterness of resveratrol at a lower concentration than panelists who are not PROP tasters. Therefore, the taste threshold of individuals who are PROP tasters would have a lower individual taste detection threshold than panelists who are non-PROP tasters. The number of replications to calculate the pooled taste threshold was 198, while the number of replications for each sample for individual taste threshold was 6. The difference in threshold levels may be attributed to the difference in the number of replications. The larger sample size used for the pooled threshold may have minimized the variability across individual panelists [26]. Although the pooled and average of the individual thresholds were different, the encapsulated resveratrol had a higher threshold than the unencapsulated resveratrol in both the pooled and average threshold results. Thereby, this supported that the effectiveness of the encapsulation of resveratrol to mask the detection of the compound.

The number of panelists who had a taste detection threshold in the original testing for unencapsulated resveratrol, SC, and SCAMF was 22, 17, and 13, respectively. These findings suggested that as the complexity of the sample increased, the ability for the panelists to detect the compound decreased. SCAMF had the most complex sample matrix with the inclusion of

resveratrol within sodium caseinate and anhydrous milk fat. It was assumed that the sodium caseinate and milk fat contributed to the flavor notes of the samples that may have helped to mask the bitterness or astringency of resveratrol. These results were in line with previous research that compared the thresholds of pure limonin and naringin in different matrices which included distilled water, sucrose solutions, citric acid solutions and citrus juice model systems [27]. Limonin and naringin in a juice model system increased the threshold several folds in comparison to the compounds in distilled water. Another study investigated the recognition threshold of limonin and naringin in orange juice and found that the addition of sucrose to the matrix decreased the bitterness of the compounds [28]. Therefore, an increased complexity of the sample matrix can increase taste detection threshold of a compound within that matrix.

The increased threshold of resveratrol in the microcapsules also aligned with prior findings which showed that the addition of sodium caseinate can lower the bitterness of olive oil phenolics in an oil-free and 65% oil-in-water emulsion [29]. Our findings demonstrated that encapsulation with sodium caseinate can mask bitterness of polyphenols in aqueous solutions.

Taste detection threshold testing using the R-index measure by rating method has been completed for isoflavonoids such as genistein and daidzein [30]. The respective taste detection thresholds for these compounds were 1080 mg/L and 740 mg/L. In addition, caffeine taste detection thresholds have been found to be between 148-260 mg/L [13]. The taste detection thresholds of resveratrol was lower than genistein, daidzein, and caffeine indicating the importance of the use of innovative processing methods to decrease the detection of resveratrol.

5.4.2 Post Questionnaire

Rank analysis in regards to the type of food product that would be best aligned with a health statement related to resveratrol found that there was a significant difference across the product choices. Yogurt had a significantly higher preference rank than all other products,

followed by drink and snack bars, which were not significantly different from one another. Cookies had the lowest preference rank among all the food products.

When panelists were asked what health benefits they associated with resveratrol, “anti-aging” was the most recognized (14 out of 33), followed by “prevent/treat cancer” (12 out of 33). The option of “lower blood sugar levels” was selected the fewest number of times (5 out of 33). “Anti-aging” was chosen significantly more than “lower blood sugar levels”, while the other options were not significantly different from one another. The health benefits selected most often were different than those normally associated with wine, which may indicate that the panelists were not making a direct association between the health benefits of wine and resveratrol. The findings suggested that panelists associated resveratrol as an anti-aging compound. Thus, the ideal marketing of a product that contains resveratrol may need to include the health benefits of the compound in order to show consumers the range of health benefits associated with resveratrol.

5.5 Conclusions

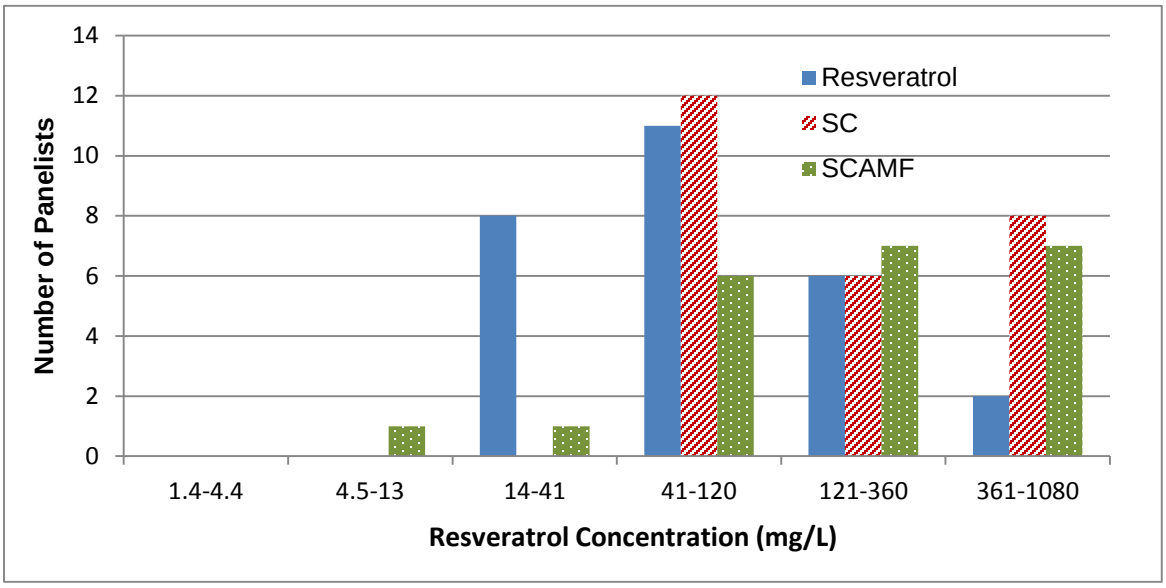
This research sheds light on the taste detection threshold of resveratrol and encapsulated resveratrol. The encapsulation of resveratrol significantly increased the taste detection threshold of the compound in comparison to unencapsulated resveratrol, suggesting that microencapsulation successfully masked taste or other sensory characteristics of the compound. Therefore, the encapsulation of resveratrol provides the food industry a means to add resveratrol into a wide range of foods products without negatively affecting sensory attributes of the product. Use of spray drying as the processing method and sodium caseinate as an encapsulation matrix are good options to produce resveratrol microcapsules due to the wide availability and low cost of this method and ingredient.

One limitation of this study was that all samples were expectorated which is not a typical way consumers would consume food products. The bitterness of resveratrol is often a delayed

perception which may be decreased when samples are not swallowed. We decided to have panelists expectorate all samples in order to minimize fatigue and instead enforced a 10-second timer before samples were rated. Yet, the results still demonstrated a significant difference between taste detection of unencapsulated resveratrol and encapsulated resveratrol. Another limitation was that this research only focused on encapsulation of resveratrol with sodium caseinate as a wall material, and the findings from this study cannot be extrapolated to the taste detection threshold of resveratrol encapsulated within other wall materials. Future research could investigate the taste detection threshold of resveratrol encapsulated within different types of encapsulation material such as cyclodextrins and gums. In addition, descriptive analysis testing could be conducted in order to determine the specific sensory properties that may differ between resveratrol and encapsulated resveratrol in solution and food products. This would help to gain a better understanding of the effects of resveratrol on sensory properties when added to food products.

5.6 Figures and Tables

Figure 5.1: Distribution of individual panelists' taste detection thresholds of unencapsulated resveratrol, resveratrol encapsulated in SC (sodium caseinate) matrix, and resveratrol encapsulated in SCAMF (sodium caseinate with anhydrous milk fat) matrix



Total number of panelists who reached threshold was 27 for resveratrol, 26 for SC, and 22 for SCAMF.
Resveratrol: unencapsulated resveratrol
SC: sodium-caseinate-based microcapsule without anhydrous milk fat
SCAMF: sodium-caseinate-based microcapsule with anhydrous milk fat

Figure 5.2: Check-all-that-apply results from health benefits associated with resveratrol

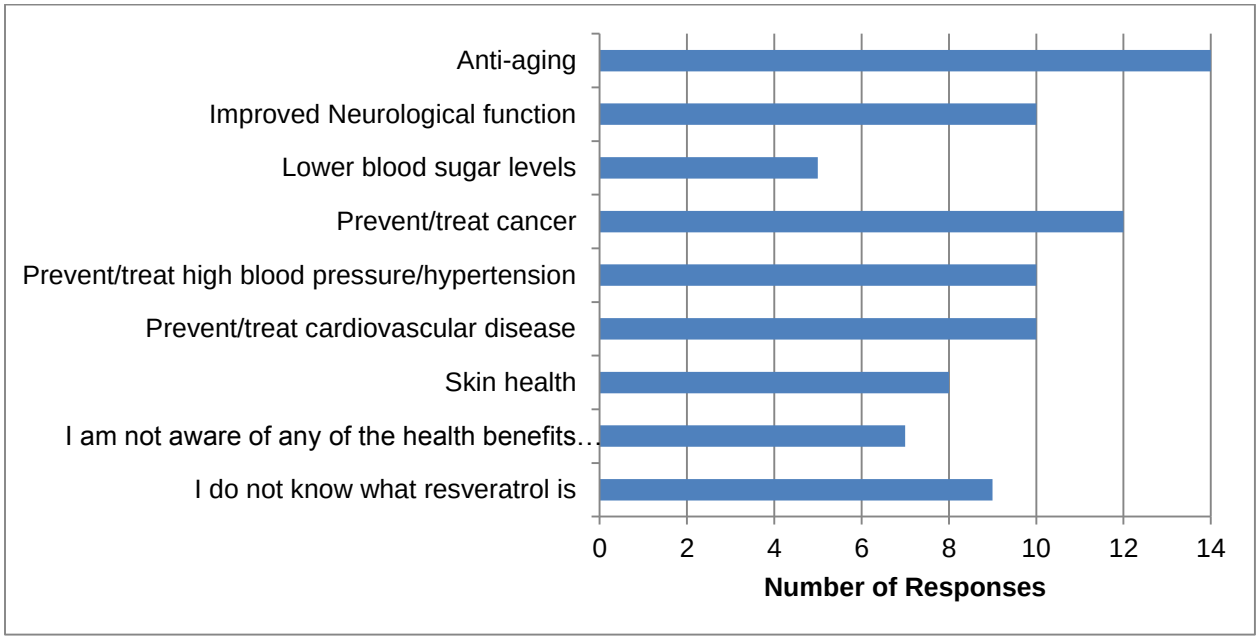


Table 5.1: Pooled threshold, average of individual panelist threshold, range of individual panelist thresholds and number of panelists who reached threshold in the concentration range tested (N), out of 33 total panelists in the taste detection threshold testing of resveratrol solutions

	Resveratrol	SC	SCAMF
Pooled threshold	47	103	108
Average Individual Panelist Threshold	90^{A} (±96)	313^{B} (±286)	334^{B} (±314)
Range of Individual Panelist Threshold	17-390	47-990	9.38-998
N	27	26	22

Threshold and range expressed in mg/L.
Same letter superscripts for the average threshold represent not significantly different values ($p<0.05$).
Resveratrol: unencapsulated resveratrol
SC: sodium-caseinate-based microcapsule without anhydrous milk fat
SCAMF: sodium-caseinate-based microcapsule with anhydrous milk fat

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CHAPTER 6: CONSUMER ACCEPTANCE OF BARS AND GUMMIES WITH RESVERATROL AND ENCAPSULATED RESVERATROL

6.1 Abstract

The addition of resveratrol, a polyphenol found in red wine and peanuts, to food products would help to provide the health benefits associated with the compound to the consumer in a wide array of food matrices. The bitterness of resveratrol and instability in light are two major challenges with the incorporation of the compound into food products. In this research, microencapsulation in a sodium caseinate matrix was utilized as a strategy to overcome these challenges. The objective of this research was to show the application of the resveratrol microcapsules in easy-to-consume foods. Consumer acceptance was evaluated for gummies and bars with encapsulated resveratrol in comparison to the controls. Two concentrations of resveratrol that have been shown to have therapeutic effects in humans were tested (10 and 40 mg/serving). The overall liking of bars with 10 mg of encapsulated resveratrol did not differ significantly from the sample without any added resveratrol or protein or from bar samples with equivalent protein and/or resveratrol concentrations. For gummies, the samples with the resveratrol microcapsules had a significantly lower overall liking than samples with the same protein and/or resveratrol content. The differing results across bars and gummies suggest that a more complex matrix may help to mask the negative sensory properties of resveratrol. A cluster of panelists was identified who preferred the bar samples with 10 mg encapsulated resveratrol (SC 10) more than bar samples with the same amount of protein and unencapsulated resveratrol (P+R 10) and the bar sample with the same protein concentration only (PRO 10). The findings suggest that complexity of the food matrix may affect the masking of negative sensory properties of resveratrol. Future research can identify which component of the matrix (ex. fat, sugar) has the most significant effect on masking these properties.

Keywords: consumer testing, overall liking, CATA, resveratrol, microencapsulation

6.2 Introduction

Resveratrol is a polyphenol found in the skin of red grapes, red wine and peanuts [1-3]. This compound may be of interest to the food industry because it has been extensively shown through both *in-vitro and in-vivo* studies to provide health benefits [4-10]. It has been associated with reduction in blood glucose and improvement in plasma glucose in diabetic rats, that indicates positive improvements related to diabetes [6]. Resveratrol has also been demonstrated to prevent and slow down tumor growth, related to positive effects on cancer [11-13]. In humans, it has been shown to inhibit platelet aggregation and increase cerebral blood flow which decreases the risk of cardiovascular disease [8, 9, 14].

Due to the numerous health benefits associated with resveratrol, it would be valuable for the food industry to incorporate the compound into easy-to-consume foods in order to deliver the health benefits to consumers. Resveratrol could be incorporated into food products at levels which have been shown to be biologically active. These levels are much higher than the levels naturally found in foods with resveratrol [1-3]. One drawback is the instability of resveratrol in the presence of light, in which about 90% of the bioactive form of resveratrol was converted to the bio-inactive form of resveratrol after 100 min of light exposure at 366 nm [15]. Another limitation with the incorporation of resveratrol into food products is the bitterness associated with the compound. Riesling wine fortified with resveratrol at 20 and 200 mg/L were found to be significantly more bitter than the control wine without added resveratrol [16]. Research on the sensory properties of resveratrol is very limited and further research should be conducted to gain a better understanding, especially related to consumer acceptance of food products with added resveratrol.

Consumer testing can measure the acceptance of products with added resveratrol. This type of testing uses untrained panelists who are familiar with the type of product being tested. The product acceptance can be affected by attributes such as appearance, aroma, taste, and texture, but it has been found that taste has the greatest influence on acceptance [17]. The 9-

point hedonic scale is most commonly used in consumer testing to measure degree of liking. This type of scale is easy for panelists to use and easy for researchers to implement [18]. In addition to overall liking questions using the 9-point hedonic scale, check-all-that-apply (CATA) questions can be used in consumer testing to evaluate product attributes. There is some disagreement on whether the use of CATA questions may bias the hedonic scales when used in the same testing procedure. It has been found that CATA questions have less of an effect on hedonic scale ratings in comparison to just-about-right and intensity scale ratings [19]. On the other hand, it was found that the completion of attribute analysis along with hedonic ratings biased overall ratings [20]. Research supported that rotating presentation order of CATA terms or using a forced choice CATA format did not have a significant influence on hedonic scale ratings [21]. Consumer testing using the 9-point hedonic scale along with CATA questions with a rotating presentation order can be used to evaluate food products with added resveratrol. Ratings from this testing can evaluate acceptance of products with encapsulated resveratrol in comparison to the unencapsulated resveratrol.

The results of our prior research supported that resveratrol encapsulated within a sodium caseinate matrix had a significantly higher stability (Chapter 3) and higher taste detection threshold in comparison to unencapsulated resveratrol (Chapter 5). This indicated that the encapsulation within a sodium caseinate matrix provided stability and masked negative sensory attributes of resveratrol. Therefore, the objective of this research was to show application of the resveratrol microcapsules to easy-to-consume food products and evaluate consumer acceptance of these products. The hypothesis was that products with encapsulated resveratrol will have a consumer acceptance not significantly different than the sample without any added resveratrol or protein. The second hypothesis was that there will be a higher consumer acceptance for products with encapsulated resveratrol in comparison to the product with unencapsulated resveratrol.

6.3 Materials and Methods

6.3.1 Materials

Resveratrol and the sodium caseinate used were from Dutch State Mines (DSM, Parsippany, NJ) and Agropur (La Crosse, WI), respectively. The oatmeal, almond milk and distilled water were all Meijer® brand (Grand Rapids, MI). Nestlé® chocolate chips (Vevey, Canton of Vaud, Switzerland) and Skippy® peanut butter (Austin, MN) were used for the bar samples. Knox® gelatin (Kraft Foods, Chicago, IL), grape flavored Kool-Aid® (Kraft Foods, Chicago, IL), and Jello-O® (Kraft Foods, Chicago, IL) were used for the gummy samples.

6.3.2 Encapsulated Resveratrol Production

Our previous research used 4.8% resveratrol (dry basis, w/w) in the microcapsules but the concentration of resveratrol was increased for the consumer testing to minimize the amount of microcapsules which needed to be added to the food products. The microcapsules used in the consumer testing contained 9.1% resveratrol and 90.9% sodium caseinate (dry basis, w/w). An 8% sodium caseinate solution was mixed, then partially denatured at 80°C for 2 hours in a shaking water bath (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ). The resveratrol was mixed into the sodium caseinate solution before high-pressure homogenization (APV Gaulin Inc., Wilmington, MA) at 55 psi, for two passes. The resulting solution was spray dried in a lab bench spray drier (Buchi B-290, New Castle, DE). The inlet and outlet temperatures for spray drying were 160°C and 90°C, respectively. The nozzle diameter was 0.7 mm and flow rate was 4.5-7.5 g/min, in order to maintain a constant outlet temperature.

6.3.3 Preparing Samples

Bar and gummy samples were prepared according to the formulations in Table 6.1. The serving size for bars and gummies were 31 g and 25 g, respectively. These serving sizes were averages of bars and gummies currently on the market.

6.3.4 Bars

For the bars, all ingredients except oatmeal were microwaved for 2 min on high, then mixed by hand for 15 sec. The heating and mixing was repeated a second time before an additional 1 min of heating. The resveratrol microcapsules, protein, and resveratrol were mixed into the heated mixture until thoroughly dispersed. Then, the oatmeal was added. Fifteen grams of the sample was weighed out and hand-shaped into circular bars. The samples were put into the refrigerator for 30 min in order to set. Then, the bars were transferred to plastic containers and placed in the refrigerator until the day before testing when samples were transferred to 60 mL plastic cups with lids.

6.3.5 Gummies

All ingredients were combined and mixed together with a spatula. Then, the mixture was microwaved for 2 min and 45 sec. The mixture was vortexed to evenly disperse the resveratrol microcapsules, protein, and resveratrol in the mixture. The resulting solution was placed in a 45°C water bath for 30 minutes under vacuum in order to minimize air incorporated in the solution. Gummies were molded in 1 cm x 1 cm x 1 cm cubes and placed in the freezer to harden for 30 min. Samples were stored in the refrigerator for about 5-7 days in plastic containers until the day before testing when samples were transferred to 30 mL plastic cups with lids.

6.3.6 Resveratrol Dosage

For both bars and gummies, 10 and 40 mg resveratrol/serving were tested as these are effective levels that align with the therapeutic dosages. Prior research has found 10 mg and 40 mg of resveratrol to be biologically effective in humans [10, 22, 23]. Resveratrol has been shown to be effective in reducing insulin resistance and urinary ortho-tyrosine excretion related to positive effects on diabetes at 10 mg/day and having an anti-oxidant and anti-inflammatory effect related to anti-aging at 40 mg/day.

6.3.7 Panelists

There were a total of 100 panelists ranging from 18 to over 65 years of age. There were 35 males and 63 females reported. Two panelists did not answer the question regarding gender. Panelists' demographics are shown in Figure 6.1.

6.3.8 Consumer Testing

The testing took place in the Bevier Hall Spice Box, a large room where meals are commonly served in the food science building. There were two days of testing in which gummies were tested on the first day and bars were tested on the second day. Four different controls were used for both gummies and bars: 1) without any resveratrol or protein (Plain), 2) unencapsulated resveratrol (Resv), 3) sodium caseinate and unencapsulated resveratrol just mixed without encapsulation (P+R), and 4) sodium caseinate only (PRO). The unencapsulated resveratrol and sodium caseinate were added in the same concentration as the resveratrol microcapsules which had a resveratrol concentration of 10 and 40 mg. Therefore, there were a total of 9 samples for each of the testing days. Amounts of protein, resveratrol and microcapsules added to each sample are shown in Table 6.2.

All samples were served to the panelists at once, in a multiple presentation format. Samples were served at room temperature and covered with foil until being served to panelists. Sample order was randomized among panelists using 25 different sample orders determined by the RAND function in Microsoft Excel (Microsoft, Redmond, WA). All responses were collected on paper ballots and a copy of the ballot can be found in Appendix C. Panelists were asked to rate overall liking of each of the samples along with check-all-that-apply (CATA) questions regarding what attributes they LIKE and DISLIKE about the samples. The attributes included in the CATA questions were developed through preliminary testing with 7 panelists who were experienced in generating descriptive attributes, specifically for these products. The order that the CATA options were presented were randomized using 25 different orders.

6.3.9 Post-Questionnaire

Panelists were asked two questions related to health benefits and label claims related to resveratrol: “Which of the following health benefits do you associate with resveratrol? (Check all that apply)” and “If a product was labeled with “May decrease cancer risk, increase heart health and neurological function”, would this increase the chance that you purchase this product over another product without a health claim?” These questions were intended to gauge the panelists’ knowledge of resveratrol and purchase intent. Appendix D shows a copy of the post-questionnaire.

6.3.10 Data Analyses

All responses were entered from the paper ballots into Microsoft Excel (Microsoft, Redmond, WA). Statistical Analysis Software (SAS, version 9.3, SAS Inst. Inc., Cary, NC) was used to analyze overall liking and CATA data. Analysis of variance (ANOVA) found if there was a significant difference across average overall liking of samples and Fisher’s least significant difference (LSD) test identified where the difference existed across average overall liking scores of samples. The data generated from the overall liking scores was assumed to be continuous data thereby allowing the use of ANOVA and LSD to analyze the data.

Cochran’s Q Equation, shown below, was used to calculate the significance level of each attribute for the CATA data, separately for LIKE and DISLIKE, of each product.

Cochran’s Equation: Q= $\frac{nk\,(nk-1)\sum_{k=1}^{nK}(Tk-T)\text{^}2}{nk\sum_{j=1}^{nj}Rj-\sum_{j=1}^{nj}Rj\text{^}2}$ **(eq 6.1)**

nk: number of products
Tk: number of times attribute was chosen for specific product
T: total number of times attribute was chosen across all products
nj: number of panelists
Rj: number of times attribute was chosen across all products by a specific panelist

Cochran’s Q-value was converted into the p-value using the Chi-squared distribution function in Microsoft Excel. The nonparametric LSD was utilized to find where the difference

existed across samples in terms of the number of times an attribute was chosen for LIKE and DISLIKE, separately. XLSAT 2014.4.09 (Addinsoft, New York, NY) was used for agglomerative cluster analysis and correspondence analysis. Agglomerative cluster analysis grouped panelists according to their overall liking scores of products with added resveratrol microcapsules for gummies and bars, separately. Correspondence analysis provided a visual depiction of the relationship of attributes to each other according to determined groups of factors.

6.4 Results and Discussion

6.4.1 Overall Liking

The average overall liking scores for all samples are shown in Figure 6.2. There was no significant difference in overall liking for bars when comparing samples with equivalent protein and/or resveratrol content to SC 10. In addition, there was no significant difference in overall liking between SC 10 bar and the Plain bar. These results indicated that the addition of 10 mg of encapsulated resveratrol to bar samples did not significantly change the overall liking of the product. For the gummies, samples with both concentrations of microcapsules had a significantly lower overall liking in comparison to other samples with the equivalent concentration of protein and/or resveratrol. This suggested that the complexity of the food matrix affected overall liking scores across samples with encapsulated resveratrol and unencapsulated resveratrol. Bars are complex food matrices and there was no significant difference across SC and other samples, while there was a significant difference across SC and other samples in gummies.

Panelists were clustered according to their ratings of samples with resveratrol microcapsules (Figure 6.3 and Table 6.3). Therefore, there was similarity in overall liking ratings of panelists within each cluster. The overall liking scores of the cluster of panelists who rated SC samples highest in comparison to the other clusters of panelists (N=23 for bars) show

that, for bars, SC 10 had a significantly higher overall liking score than P+R 10 and PRO 10 (Figure 6.4 and Table 6.4). The panelists in this cluster liked SC 10 significantly more than samples with the same protein content. For gummies, the overall liking scores of the cluster of panelists who rated SC samples highest in comparison to the other clusters of panelists (N=28 for gummies) were not significantly different than other samples with the same resveratrol and/or protein concentrations, for both concentrations of resveratrol microcapsule. Therefore, the resveratrol microcapsules in gummies had no significant effect on overall liking in comparison to other samples with the same protein and/or resveratrol concentration. Also, the overall liking scores from this cluster of panelists for SC 10 and SC 40 bar and gummy samples were not significantly different from the Plain samples. Thereby, this finding indicated that the addition of encapsulated resveratrol maintained the overall liking of the sample.

6.4.2 Check-all-that-apply

The attributes that showed a significant difference (α=0.05) across the number of times the attribute was chosen across products are shown in bold in Table 6.5. For bars, bitter and sweet were significant attributes for both LIKE and DISLIKE. Sweetness has been shown to be most resistant to being masked by other tastes [24]. Therefore, the significant differences seen in both LIKE and DISLIKE are in line with the literature showing that sweetness is not masked by changes in bitterness or sourness. Chalky and mouth coating were significant attributes for both LIKE and DISLIKE in the gummies. This demonstrates that attributes related to taste differentiated bar samples and attributes related to texture differentiated gummy samples. Therefore, an increased complexity of the sample matrix may help to mask textural impacts of added resveratrol microcapsules.

The results of the nonparametric LSD performed on the CATA data are shown in Table 6.6. The number of times bitter was chosen as a DISLIKE attribute for bars with added resveratrol microcapsules was significantly higher than all other products with equivalent protein and/or resveratrol concentrations. When comparing the non-parametric LSD for gummies, Resv

40 was selected significantly more times for chewy, mouth coating and smooth as a LIKE attribute than SC 40. There was no significant difference across the number of times SC 40 was chosen as a LIKE attribute in comparison to PRO 40 and P+R 40 for chewy, mouth coating and smooth. Therefore, the protein content in the sample was most likely attributed to the difference in chewy, mouth coating and smooth for LIKE attributes of gummies. Sweet was selected as a DISLIKE attribute the most for Plain, which was significantly higher than all products with unencapsulated resveratrol and encapsulated resveratrol. These results are not in agreement with prior research as it has been found that bitterness and sweetness were negatively correlated so increased levels of bitterness resulted in decreased ratings of sweetness [25]. Therefore, it would be expected that samples with resveratrol would have sweet chosen as a DISLIKE option significantly more than samples without resveratrol. This may be an indication that the Plain sample was the least sweet and consumers did not like it because the sweetness was not on par with the other samples. The CATA questions indicate differences between the number of times a specific attribute was chosen for LIKE and DISLIKE. These questions do not indicate intensity of the attributes but descriptive analysis could be used to accomplish this.

Complexity of the samples may be attributed to differences seen across the CATA results of bars and gummies since the matrix of bars was more complex than that of gummies. Discrimination testing between caffeinated and uncaffeinated solutions showed that ability to discriminate between samples was affected by complexity of the beverage such as flavor, sweetness and carbonation [26]. It has also been found that increasing the complexity of odor-taste mixtures decreased that ability of trained panelists to identify specific components [27, 28].

According to correspondence analysis for the LIKE attributes of bars, the attributes that were closely related to each other but separated from the other attributes were 1) bitter and sour, 2) chalky and hard, and 3) astringent (Figure 6.5). For the LIKE attributes of gummies, the attributes that were separated from the others but also not associated with each other were 1)

hard, astringent, foamy, bitter and 2) chalky. The attributes that were separated from the internal cluster of attributes are the drivers of liking and disliking between the products. Future research could utilize descriptive analysis to evaluate the intensity of specific attributes of the bar and gummy samples, in order to confirm the findings from the correspondence analysis. The overall findings of this study suggested that the complexity of the food matrix can affect whether a difference exists across the overall liking of products with added resveratrol microcapsules and the controls. Also, there may be a segment of consumers who prefer products with encapsulated resveratrol in comparison to products with the same concentration of protein and/or unencapsulated resveratrol. For this segment of consumers, it was also found that overall liking of both bars and gummies with encapsulated resveratrol was maintained in comparison to the Plain sample. The CATA data indicated that taste differentiated the bar samples while texture distinguished gummy samples.

6.4.3 Post-Questionnaire

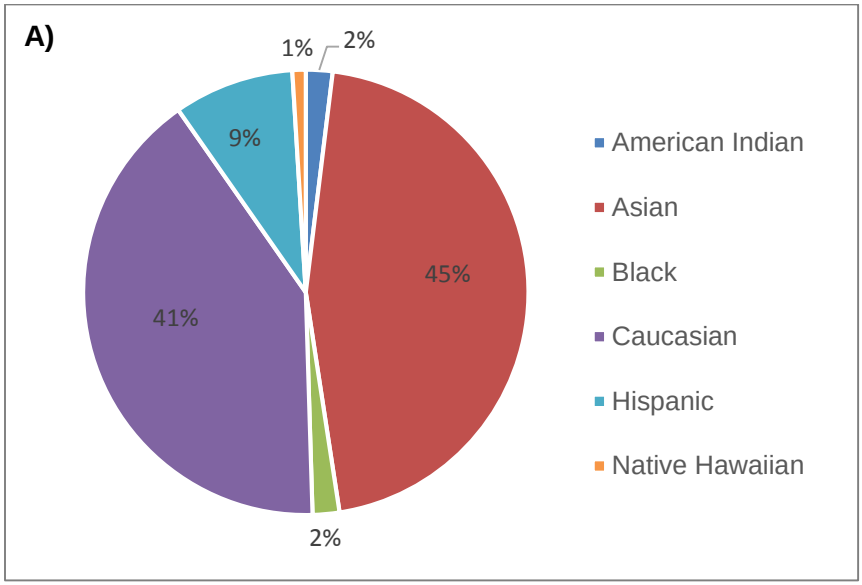
The post-questionnaire asked the panelists about prior knowledge of the health benefits related to resveratrol and the effect of a health claim related to resveratrol on purchase intent. For the questions related to prior knowledge of resveratrol, the option “I don’t know what resveratrol is” was chosen that most, followed by “Prevent/treat cardiovascular disease” and “anti-aging” (Figure 6.6). Also, most panelists indicated that the health claim related to resveratrol would increase their purchase intent of the product. The results of the health claim related to resveratrol showed that the claim would increase the chance that consumers would purchase the product (Figure 6.7). Therefore, in order to positively influence the purchase intent of consumers, it would be better to include health claims related to resveratrol rather than just stating the resveratrol content since most panelists did not know of resveratrol.

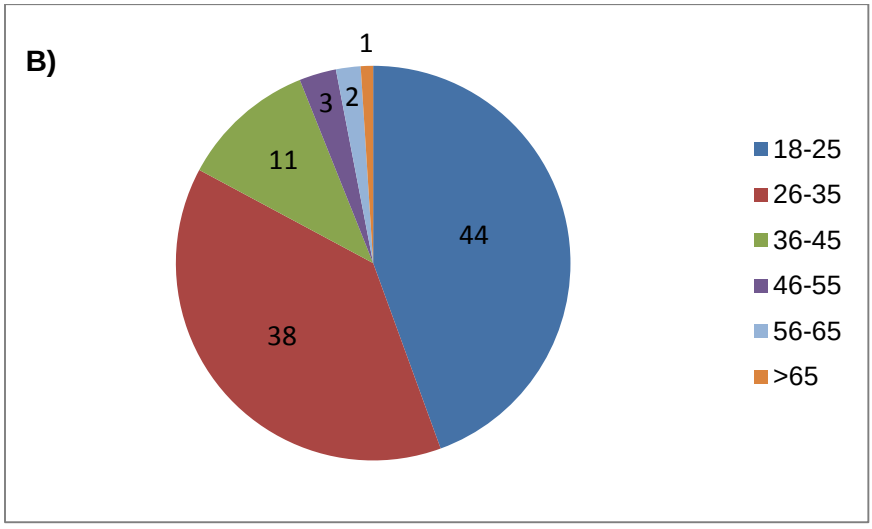
6.5 Conclusion

This research provides a basis upon which the food industry can add stabilized resveratrol into food products in order to deliver the health benefits to the consumer. The benefit of using resveratrol microcapsules is that the encapsulation matrix helps to protect the compound and maintain its bioactivity. The overall liking of SC 10 in bars was not significantly different than that of the Plain sample. In addition, a group of panelist was identified who had a higher overall liking for the SC 10 bar sample in comparison to the bar sample with both unencapsulated resveratrol and protein (P+R 10) and the bar sample with equivalent amount of protein (PRO 10). Therefore, these types of products with added resveratrol should be targeted towards a segment of the market who prefer these products. Some limitations of this study were that only two levels of resveratrol concentration and only two types of food products were tested, as consumer acceptance of additional food matrixes may differ from those tested in this research. Future research could evaluate consumer acceptance of other complex food matrices utilizing additional resveratrol concentrations that have been shown to have therapeutic effects. Another limitation of the study is that for the CATA questions, it is not possible to determine if the panelists checked an attribute because it was present or not present. Therefore, descriptive analysis would be helpful to determine attribute intensities of products with added resveratrol.

6.6 Figures and Tables

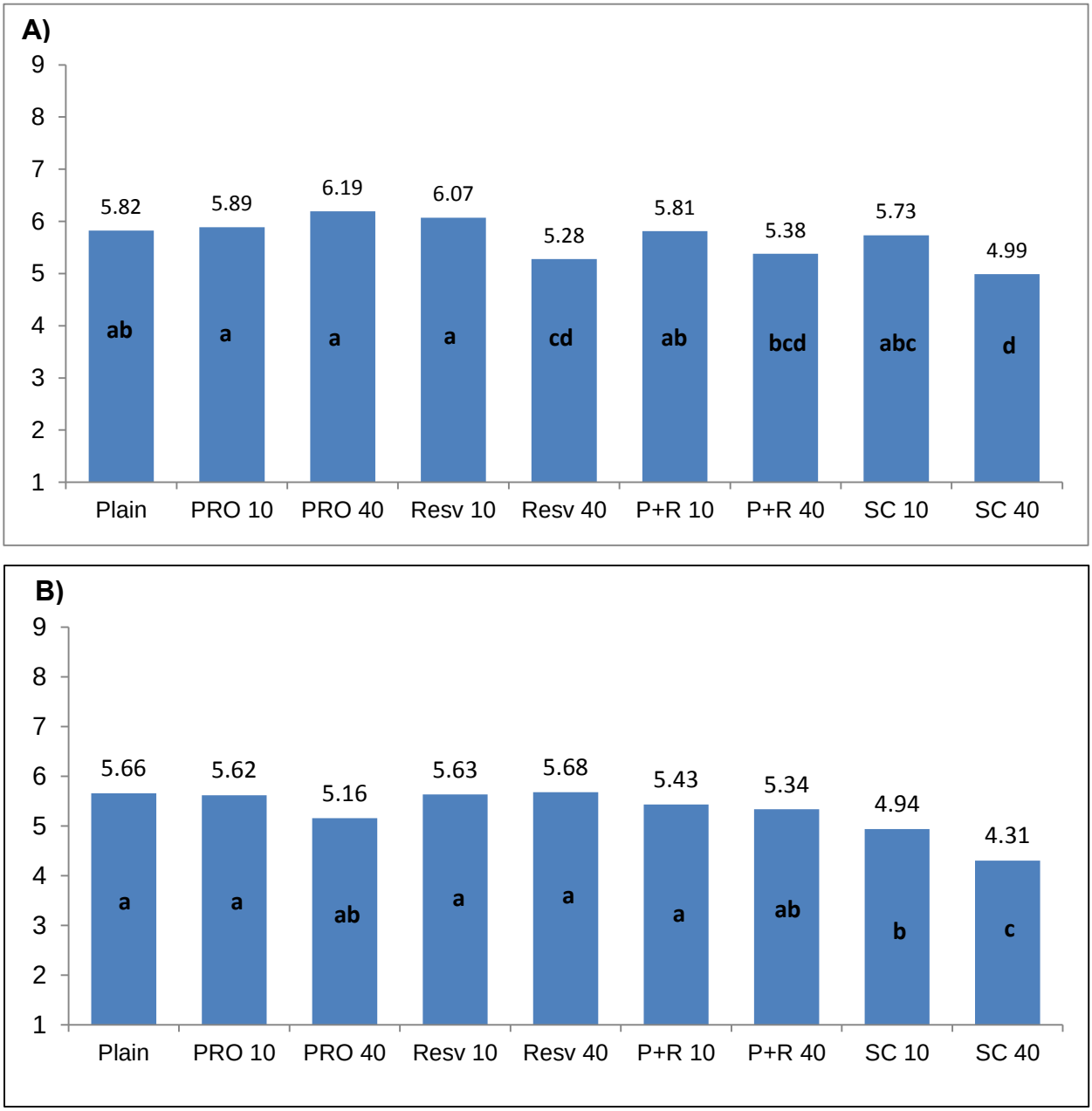
Figure 6.1: Demographics of panelists who participated in consumer testing of food products with added resveratrol: A) Ethnicity, B) Age (years of age)





There were a total of 100 panelists who participated in the consumer testing.

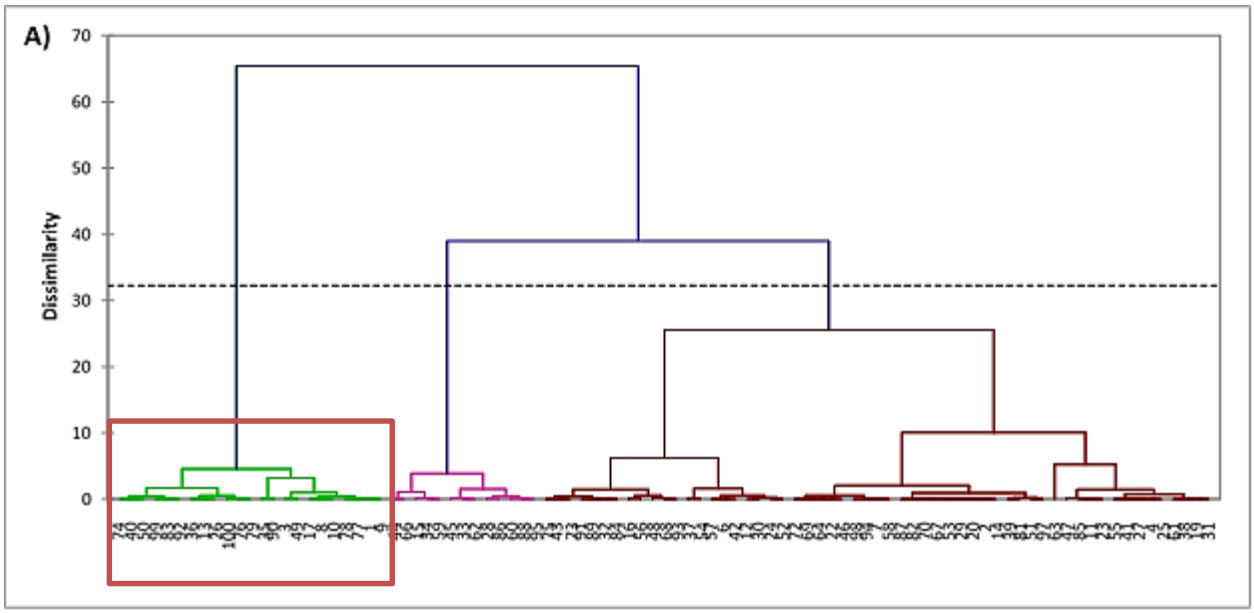
Figure 6.2: Average overall liking scores of panelists from the consumer testing of A) Bars and B) Gummies, with added unencapsulated resveratrol and encapsulated resveratrol.

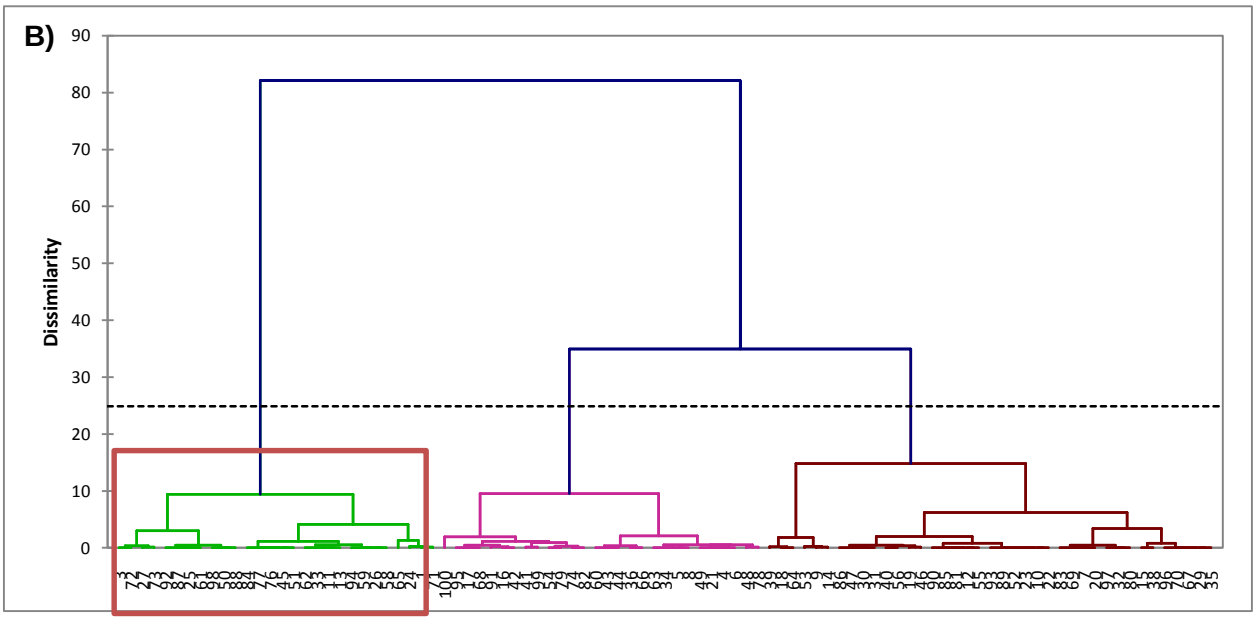


Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD)
Average overall liking scores indicated above each sample bar
Plain: no added resveratrol or protein, PRO 10: same sodium caseinate concentration as SC 10, PRO 40: same sodium caseinate concentration as SC 40, Resv 10: 10 mg of unencapsulated resveratrol, Resv 40: 40 mg of unencapsulated resveratrol, P+R 10: same sodium caseinate and resveratrol concentration as SC 10, P+R 40: same sodium caseinate and resveratrol concentration as SC 40, SC 10: 110 mg resveratrol microcapsule, SC 40: 439.6 mg resveratrol microcapsules.

There were a total of 100 panelists who participated in the consumer testing.

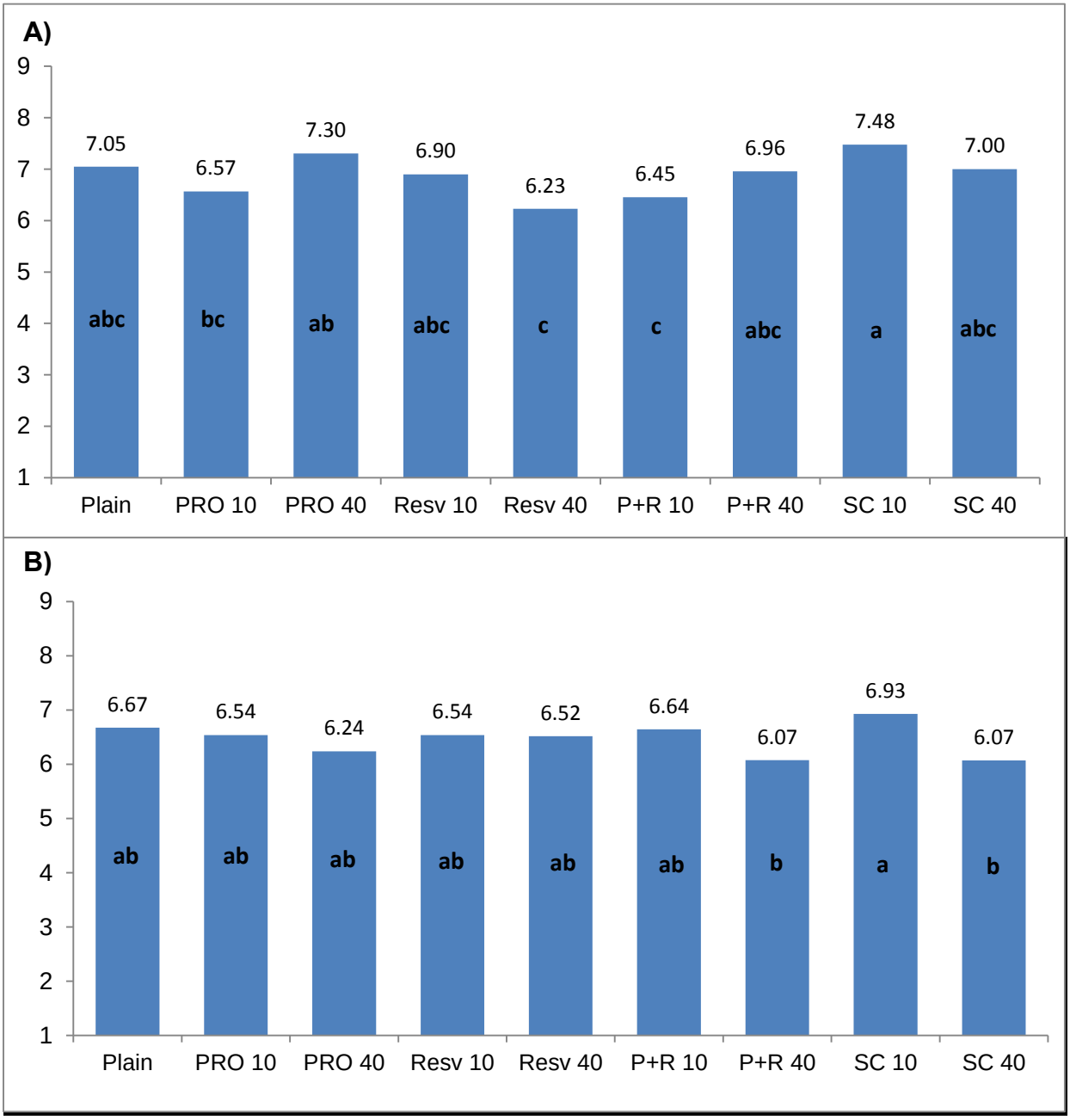
Figure 6.3: Cluster analysis of panelists based on overall liking ratings of samples with encapsulated resveratrol for: A) Bars and B) Gummies.





The concentration of encapsulated resveratrol within the food products was 10 and 40 mg/serving. The cluster with a box around it represents panelists who rated the samples with encapsulated resveratrol higher than the other two clusters of panelists.
There were a total of 100 panelists who participated in the consumer testing.
Panelists were clustered using agglomerative cluster analysis.

Figure 6.4: Average overall liking scores for cluster of panelists who rated samples with resveratrol microcapsules highest for: A) Bars (N=23) and B) Gummies (N=28)



Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD)
Average overall liking scores indicated above each sample bar
Plain: no added resveratrol or protein, PRO 10: same sodium caseinate concentration as SC 10, PRO 40: same sodium caseinate concentration as SC 40, Resv 10: 10 mg of unencapsulated resveratrol, Resv 40: 40 mg of unencapsulated resveratrol, P+R 10: same sodium caseinate and resveratrol concentration as SC 10, P+R 40: same sodium caseinate and resveratrol concentration as SC 40, SC 10: 110 mg resveratrol microcapsule, SC 40: 439.6 mg resveratrol microcapsules

Figure 6.5: Correspondence analysis from check-all-that-apply questions on attributes that are liked and disliked for A) Bars LIKE, B) Bars LIKE (zoomed in on central cluster) C) Bars LIKE for only significant attributes according to Cochran's Q equation, D) Bars DISLIKE, E) Bars DISLIKE for only significant attributes according to Cochran's Q equation F) Gummies LIKE, G) Gummies LIKE for only significant attributes according to Cochran's Q equation, H) Gummies DISLIKE, I) Gummies DISLIKE for only significant attributes according to Cochran's Q equation

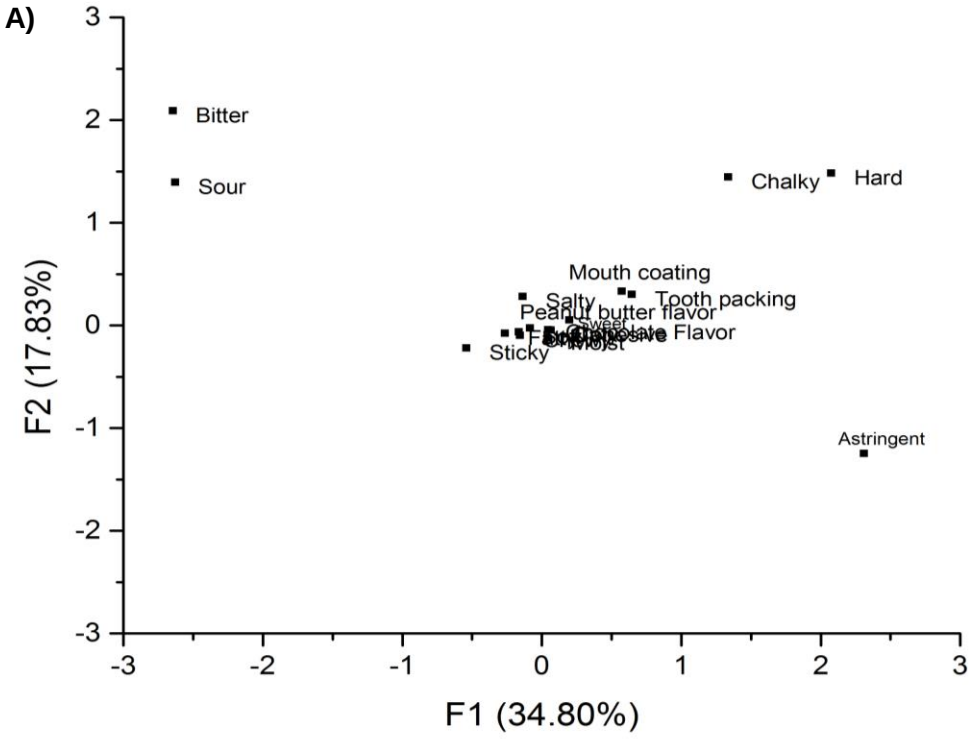
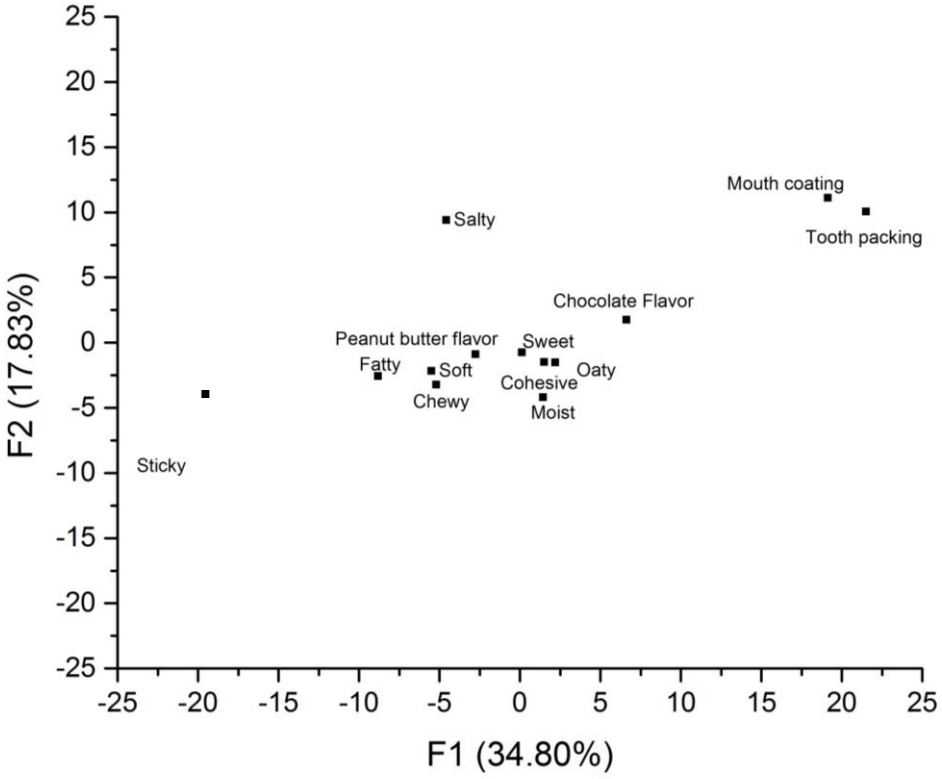
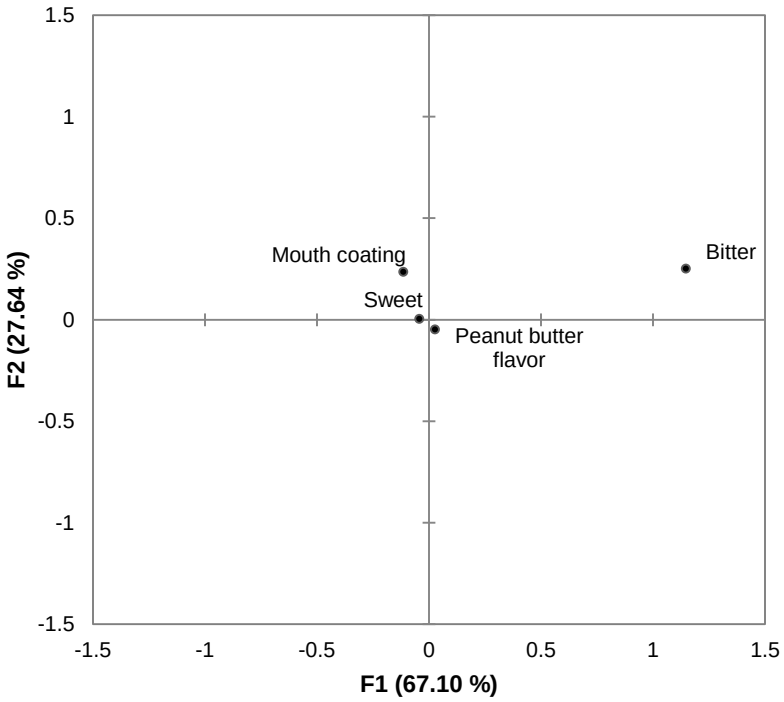


Figure 6.5 (cont.)

B)



C)



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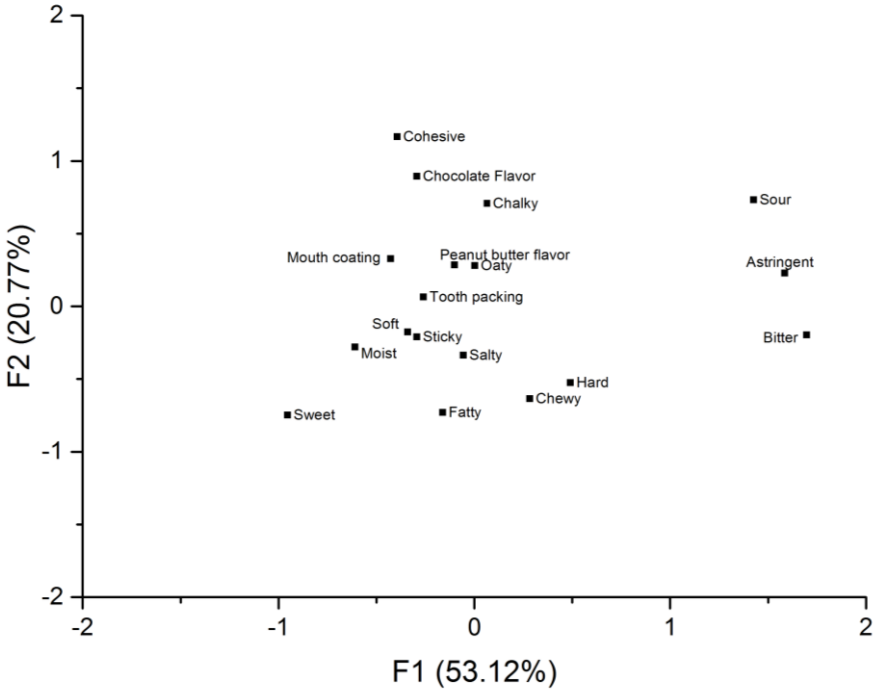
D)



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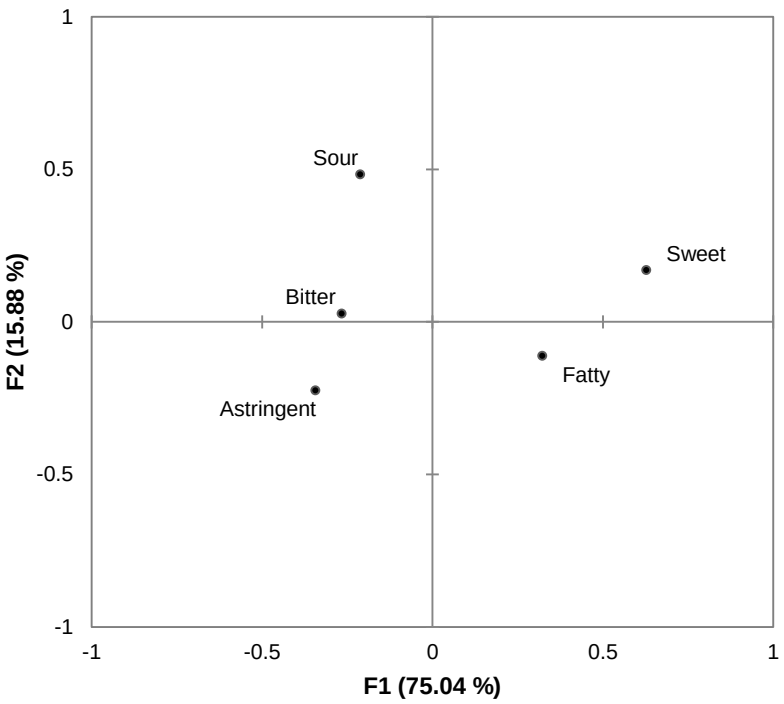
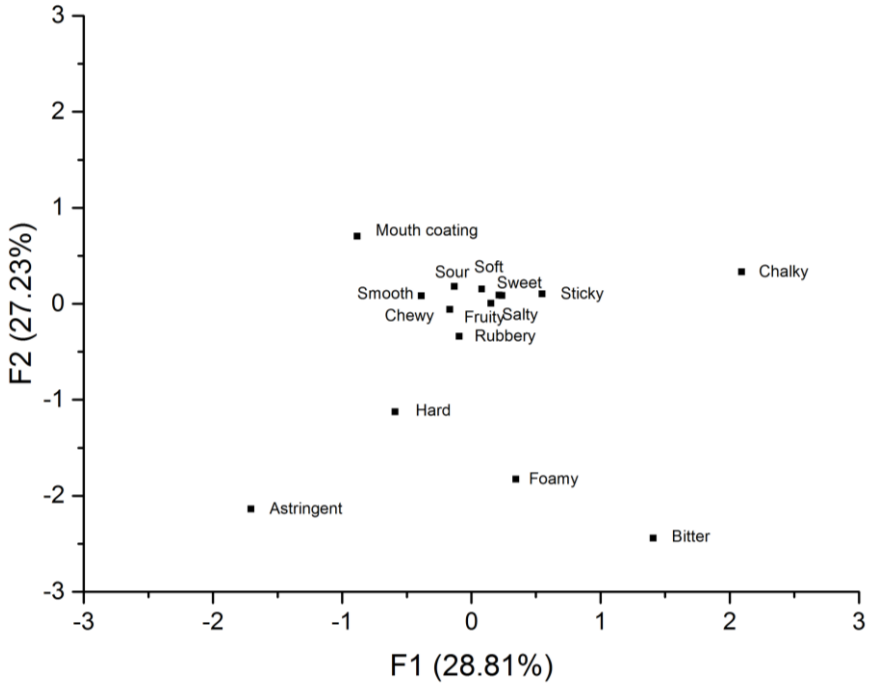


Figure 6.5 (cont.)

F)



G)

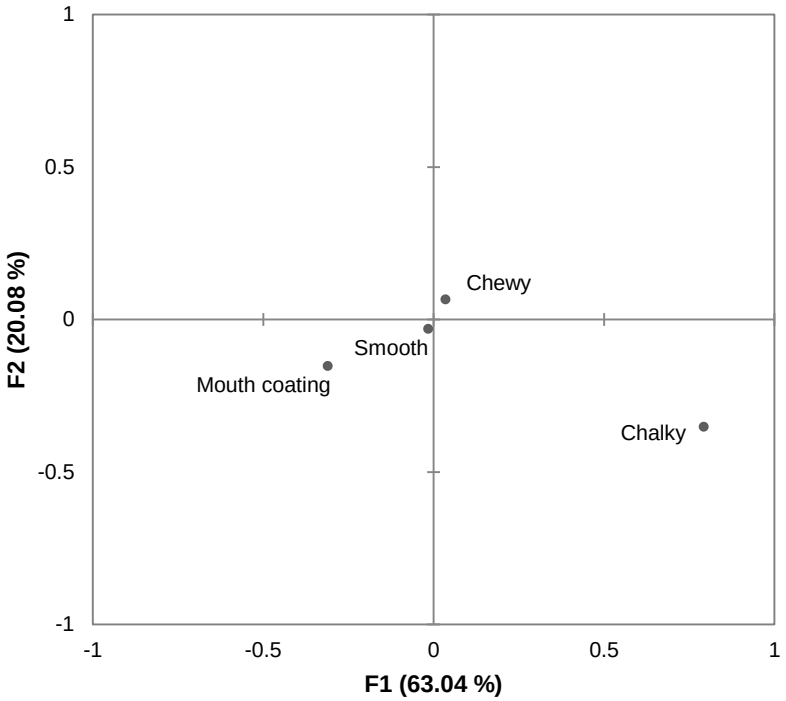
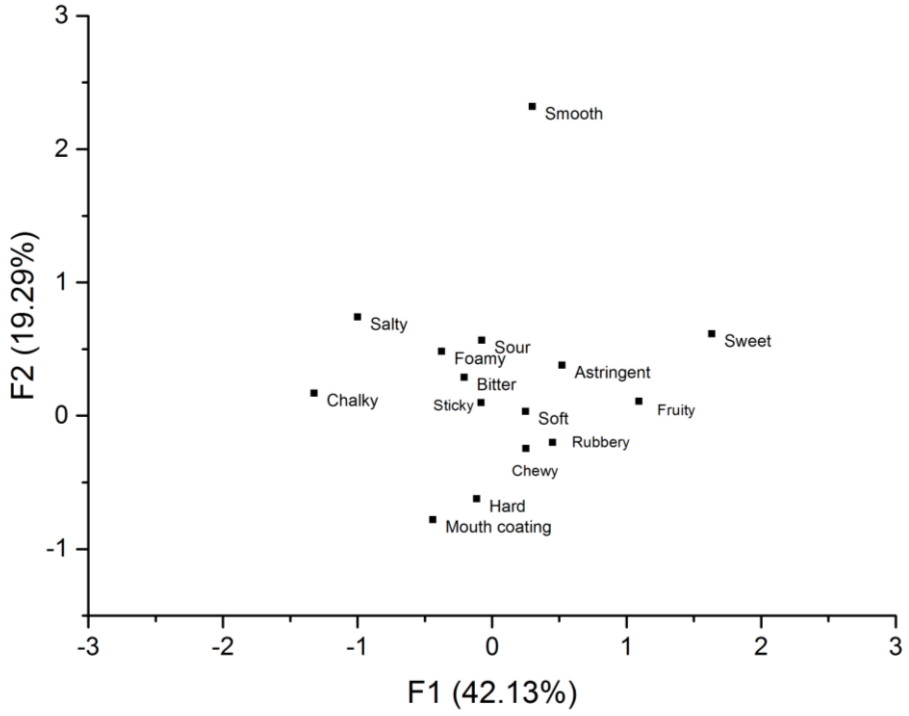


Figure 6.5 (cont.)

H)



I)

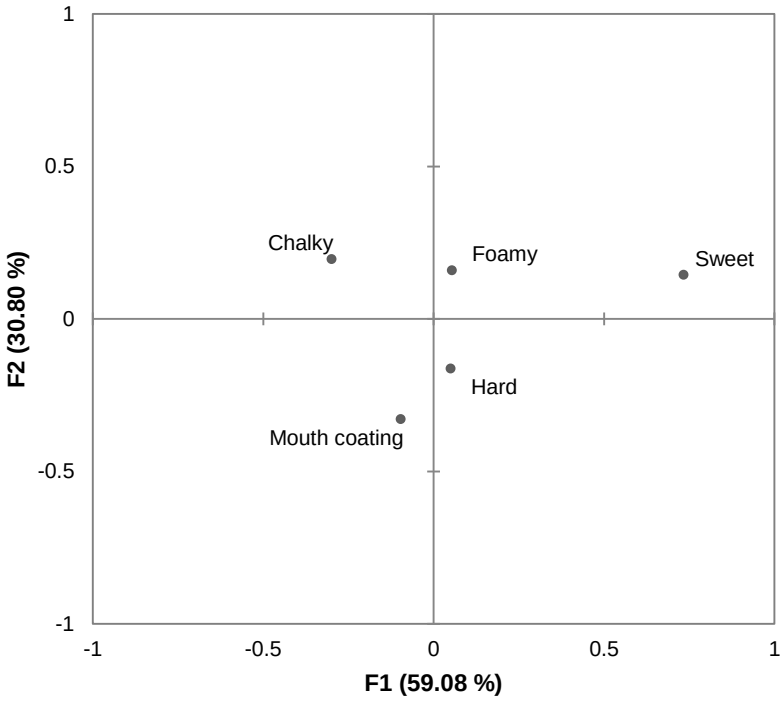
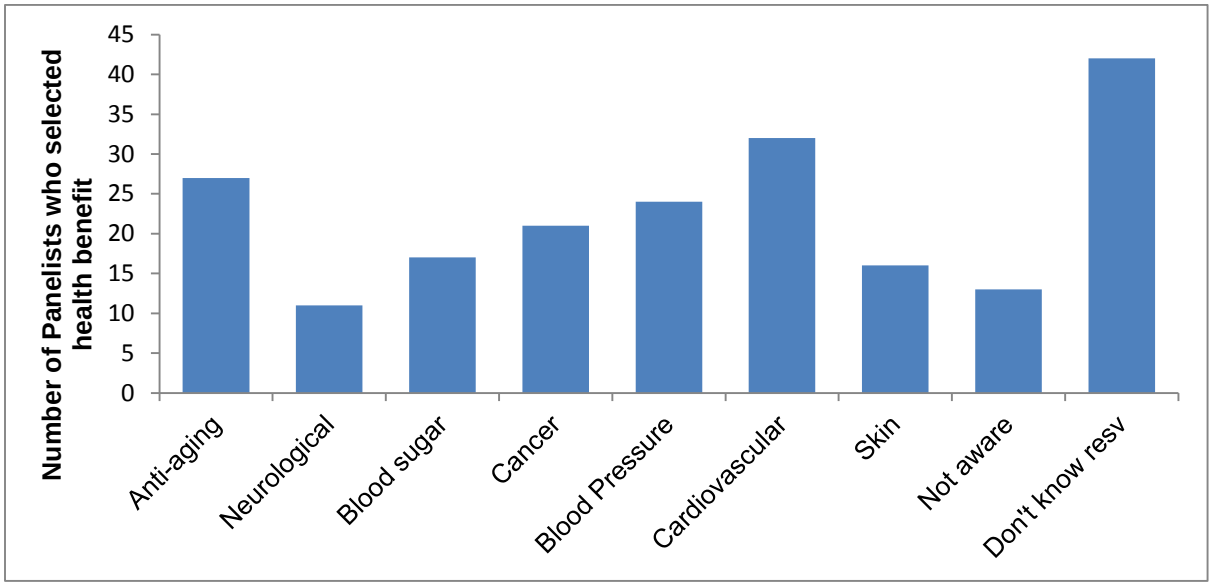
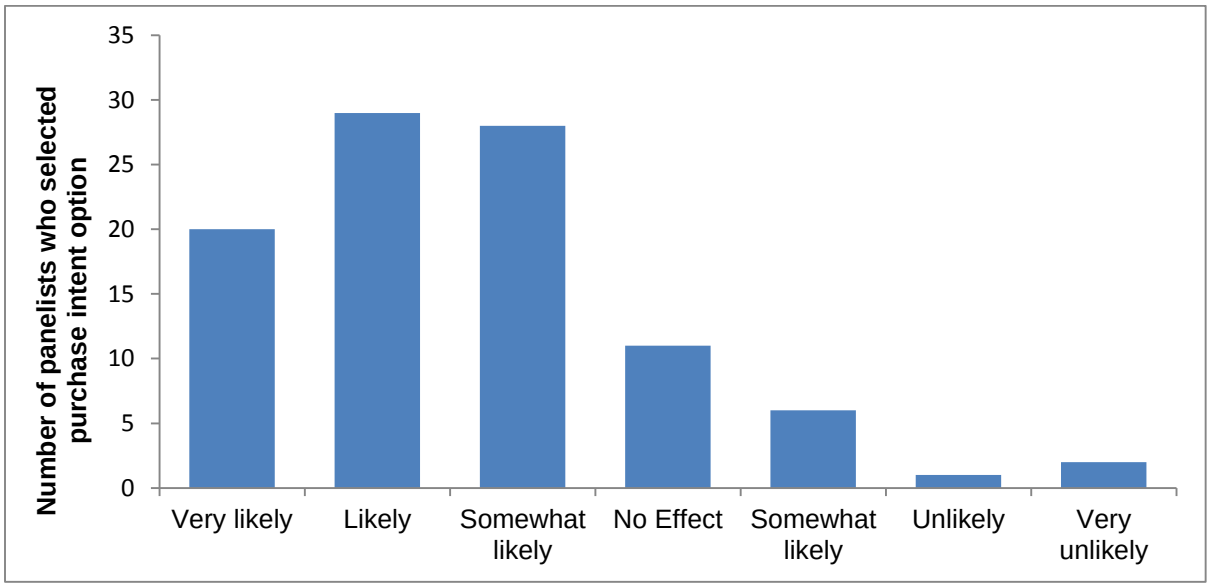


Figure 6.6: Prior knowledge of resveratrol health benefits of panelists who participated in consumer testing of products with added resveratrol



There were a total of 100 panelists who participated in the consumer testing.

Figure 6.7: Effect of health claim related to resveratrol on purchase intent of product for panelists who participated in consumer testing of products with added resveratrol



There were a total of 100 panelists who participated in the consumer testing.

Table 6.1: Formulation of bar and gummy samples used in consumer testing of products with added resveratrol

Bar (per serving)
8 g oatmeal (Meijer®, Grand Rapids, MI)
6 g milk chocolate chips (Nestlé®, Vevey, Canton of Vaud, Switzerland)
11 g peanut butter (Skippy®, Austin, MN)
6 g almond milk (Meijer®, Grand Rapids, MI)

Gummy (per serving)
2.95 g gelatin (Knox®, Kraft Foods, Chicago, IL)
2.25 g kool-aid (Kool-Aid®, Kraft Foods, Chicago, IL)
3.9 g jello (Jell-O®, Kraft Foods, Chicago, IL)
15.9 g distilled water (Meijer®, Grand Rapids, MI)

Serving size of bars are 31 g and serving size of gummies are 25 g.

Table 6.2: Sample codes and amount of unencapsulated resveratrol, sodium caseinate, and encapsulated resveratrol added to each serving in consumer testing of food products with added resveratrol

Sample Code	Unencapsulated resveratrol	Sodium Caseinate	Encapsulated Resveratrol
Plain	--	--	--
PRO 10	--	100 mg	--
PRO 40	--	399.6 mg	--
Resv 10	10 mg	--	--
Resv 40	40 mg	--	--
P+R 10	10 mg	100 mg	--
P+R 40	40 mg	399.6 mg	--
SC 10	--	--	110 mg
SC 40	--	--	439.6 mg

Plain: no added resveratrol or protein, PRO 10: same sodium caseinate concentration as SC 10, PRO 40: same sodium caseinate concentration as SC 40, Resv 10: 10 mg of unencapsulated resveratrol, Resv 40: 40 mg of unencapsulated resveratrol, P+R 10: same sodium caseinate and resveratrol concentration as SC 10, P+R 40: same sodium caseinate and resveratrol concentration as SC 40, SC 10: 110 mg resveratrol microcapsule, SC 40: 439.6 mg resveratrol microcapsules.

Table 6.3: Number of panelists in each cluster (N), average overall liking scores of SC 10 and SC 40 according to cluster analysis of overall liking scores of products with resveratrol microcapsules

	Bars	Gummies
Cluster 1	N = 23 SC 10 average = 7.0 SC 40 average = 7.5	N = 28 SC 10 average = 6.9 SC 40 average = 6.1
Cluster 2	N = 57 SC 10 average = 4.8 SC 40 average = 5.6	N = 28 SC 10 average = 4.7 SC 40 average = 2.1
Cluster 3	N = 13 SC 10 average = 2.3 SC 40 average = 3.2	N = 39 SC 10 average = 3.6 SC 40 average = 4.6

Panelists were clustered according to their overall liking scores of SC 10 and SC 40, separately for bars and gummies.
SC 10: 110 mg resveratrol microcapsule, SC 40: 439.6 mg resveratrol microcapsules.
There were a total of 100 panelists who participated in the consumer testing.

Table 6.4: Overall liking of samples for each cluster of panelists for A) Bars and B) Gummies

A)

Sample Code	Cluster 1	Cluster 2	Cluster 3
Plain	7.05^{abc}	5.64^{a}	4.85^{a}
PRO 10	6.57^{bc}	5.98^{a}	4.69^{ab}
PRO 40	7.30^{ab}	5.88^{a}	5.54^{a}
Resv 10	6.90^{abc}	5.93^{a}	4.82^{ab}
Resv 40	6.23^{c}	5.43^{ab}	2.92^{cd}
P+R 10	6.45^{c}	5.71^{a}	5.00^{a}
P+R 40	6.96^{abc}	5.05^{bc}	3.58^{bc}
SC 10	7.48^{a}	5.61^{a}	3.15^{cd}
SC 40	7.00^{abc}	4.77^{c}	2.31^{d}

B)

Sample Code	Cluster 1	Cluster 2	Cluster 3
Plain	6.67^{ab}	5.11^{ab}	5.37^{ab}
PRO 10	6.54^{ab}	5.19^{ab}	5.41^{a}
PRO 40	6.24^{ab}	4.96^{ab}	4.68^{bc}
Resv 10	6.54^{ab}	5.60^{a}	5.05^{abc}
Resv 40	6.52^{ab}	5.68^{a}	5.03^{abc}
P+R 10	6.64^{ab}	4.93^{ab}	5.00^{abc}
P+R 40	6.07^{b}	5.44^{ab}	4.78^{abc}
SC 10	6.93^{a}	4.71^{b}	3.64^{d}
SC 40	6.07^{b}	2.07^{c}	4.64^{c}

Panelists were clustered according to their ratings of SC 10 and SC 40 using agglomerative cluster analysis.
Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD).
Plain: no added resveratrol or protein, PRO 10: same sodium caseinate concentration as SC 10, PRO 40: same sodium caseinate concentration as SC 40, Resv 10: 10 mg of unencapsulated resveratrol, Resv 40: 40 mg of unencapsulated resveratrol, P+R 10: same sodium caseinate and resveratrol concentration as SC 10, P+R 40: same sodium caseinate and resveratrol concentration as SC 40, SC 10: 110 mg resveratrol microcapsule, SC 40: 439.6 mg resveratrol microcapsules.

Table 6.5: P values for each attribute from Cochran's Q equation which determines if a significant difference exists across the number of times an attribute was chosen in the check-all-that-apply questions on ballot of LIKE and DISLIKE across products as evaluated in consumer testing of A) bars and B) gummies with added resveratrol

A)

BAR	LIKE	DISLIKE
Astringent	0.433	***0.003**
Bitter	***0.042**	***<0.001**
Chalky	0.055	0.154
Chewy	0.395	0.336
Chocolate Flavor	0.285	0.232
Cohesive	0.362	0.510
Fatty	0.903	***0.006**
Hard	0.226	0.633
Moist	0.120	0.066
Mouth Coating	***0.027**	0.412
Oaty	0.255	0.065
Peanut butter Flavor	***0.005**	0.742
Salty	0.553	0.633
Soft	0.389	0.452
Sour	0.473	***0.029**
Sticky	0.201	0.076
Sweet	***0.004**	***0.034**
Tooth Packing	0.881	0.825

B)

GUMMY	LIKE	DISLIKE
Astringent	0.310	0.703
Bitter	0.473	0.125
Chalky	***0.018**	***<0.001**
Chewy	***0.047**	0.269
Foamy	0.126	***0.003**
Fruity	0.104	0.699
Hard	0.074	***0.019**
Mouth Coating	***0.004**	***0.004**
Rubbery	0.394	0.099
Salty	0.721	0.084
Smooth	***0.001**	0.389
Soft	0.604	0.555
Sour	0.381	0.286
Sticky	0.528	0.064
Sweet	0.190	***0.002**

*Significant values (α=0.05) are shown in bold

Table 6.6: Nonparametric LSD results for check-all-that-apply questions showing differences across the number of times attributes were chosen across samples for A) Bars and B) Gummies, as evaluated by consumer testing on food products with added resveratrol

A)

	LIKE				DISLIKE				
Sample	**Bitter**	**Mouth Coating**	**Peanut Butter Flavor**	**Sweet**	**Astringent**	**Bitter**	**Fatty**	**Sour**	**Sweet**
Plain	1^{B}	19^{A}	64^{ABC}	54^{AB}	4^{BCD}	13^{CD}	15^{ABC}	0^{B}	8^{A}
PRO 10	2^{AB}	6^{B}	66^{ABC}	47^{AB}	0^{D}	8^{D}	14^{ABC}	2^{AB}	8^{A}
PRO 40	0^{B}	13^{AB}	59^{C}	58^{A}	5^{BCD}	8^{D}	6^{C}	1^{B}	1^{B}
Resv 10	0^{B}	12^{AB}	75^{A}	57^{A}	3^{BCD}	10^{D}	13^{ABC}	2^{AB}	4^{AB}
Resv 40	5^{A}	10^{B}	62^{ABC}	43^{B}	8^{AB}	31^{B}	21^{A}	2^{AB}	5^{AB}
P+R 10	1^{B}	10^{B}	67^{ABC}	53^{AB}	2^{CD}	11^{D}	14^{ABC}	3^{AB}	8^{A}
P+R 40	1^{B}	9^{B}	56^{C}	42^{B}	6^{ABC}	31^{B}	11^{BC}	6^{A}	5^{AB}
SC 10	0^{B}	11^{AB}	73^{AB}	51^{AB}	4^{BCD}	22^{BC}	19^{AB}	1^{B}	4^{AB}
SC 40	2^{AB}	8^{B}	60^{BC}	41^{B}	11^{A}	49^{A}	11^{BC}	6^{A}	2^{AB}

B)

	LIKE				DISLIKE				
Sample	**Chalky**	**Chewy**	**Mouth Coating**	**Smooth**	**Chalky**	**Foamy**	**Hard**	**Mouth Coating**	**Sweet**
Plain	1^{B}	41^{AB}	6^{ABC}	31^{ABC}	6^{B}	11^{AB}	11^{BC}	3^{C}	13^{A}
PRO 10	1^{B}	39^{AB}	11^{AB}	30^{ABC}	4^{B}	8^{B}	14^{ABC}	7^{BC}	9^{AB}
PRO 40	4^{A}	30^{B}	4^{BC}	24^{BCD}	26^{A}	12^{AB}	15^{ABC}	12^{AB}	7^{ABC}
Resv 10	1^{B}	37^{AB}	8^{ABC}	33^{AB}	11^{B}	8^{B}	16^{ABC}	10^{ABC}	3^{BC}
Resv 40	1^{B}	45^{A}	13^{A}	37^{A}	7^{B}	8^{B}	7^{C}	7^{BC}	6^{BC}
P+R 10	1^{B}	37^{AB}	6^{ABC}	30^{ABC}	9^{B}	8^{B}	16^{ABC}	18^{A}	3^{BC}
P+R 40	1^{B}	38^{AB}	9^{ABC}	19^{CD}	26^{A}	18^{A}	11^{BC}	9^{BC}	5^{BC}
SC 10	1^{B}	44^{A}	4^{BC}	26^{ABCD}	23^{A}	9^{B}	19^{AB}	13^{AB}	6^{BC}
SC 40	3^{A}	30^{B}	2^{C}	17^{D}	32^{A}	19^{A}	23^{A}	13^{AB}	2^{C}

Same letters represent not significantly different values ($p<0.05$, ANOVA and LSD)

6.7 References

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CHAPTER 7: SUMMARY

Bioactive compounds, such as resveratrol, can help to prevent and alleviate certain disease states. The incorporation of these compounds in food products can provide convenient means to deliver the health benefits to consumers. Microencapsulation is an innovative processing method that can help to stabilize the compounds during processing, storage, and digestion, and minimize negative sensory properties of the compounds.

Whey protein and sodium caseinate were compared as encapsulation materials evaluated by UVA light testing and a 3-stage *in-vitro* digestion model. After exposure to UVA light, the *trans:cis* resveratrol ratios of samples were compared and a higher *trans:cis* resveratrol ratio indicated higher UVA light stability. Sodium caseinate as a wall material for encapsulation enhanced UVA light stability of resveratrol (0.63 *trans:cis* resveratrol ratio) in comparison to whey protein concentrate (0.43 *trans:cis* resveratrol ratio) and unencapsulated resveratrol (0.49 *trans:cis* resveratrol ratio). In addition, stability through the human digestive system evaluated by digestive stability and bioaccessibility of sodium-caseinate-based microcapsules, whey-protein-concentrate-based microcapsules and unencapsulated resveratrol were 84% and 60%, 70% and 53%, and 47% and 23%, respectively. The addition of anhydrous milk fat in the formulation did not have a significant effect on the stability of resveratrol within the microcapsule. The addition of plasticizers to the microcapsule formulations caused a decrease in UV stability of resveratrol (0.39 *trans:cis* resveratrol ratio) compared to the original microcapsule formulation (0.64 *trans:cis* resveratrol ratio). The encapsulation of resveratrol also helped to decrease the detection of resveratrol. The taste threshold of encapsulated resveratrol (313-334 mg resveratrol/L) was significantly higher than that of the unencapsulated resveratrol (90 mg resveratrol/L). In terms of consumer acceptance of products with added resveratrol, a segment of consumers was identified whose overall liking scores of both bars and gummies with encapsulated resveratrol were not significantly different from the plain samples (without any

resveratrol). Therefore, overall liking of the products was maintained when encapsulated resveratrol was added to the products.

Stabilization of resveratrol was achieved through microencapsulation within a protein matrix using spray drying. In the food industry, spray drying is a common technique and the equipment is readily available to make the scale up and production of resveratrol microcapsules feasible. In addition, the relatively low cost of protein helps to minimize the cost of the encapsulation, thereby minimizing the additional cost of providing a stabilized form of resveratrol to the consumer.

The developed encapsulation system is valuable as it can serve as a model into which other components can be incorporated, such as bioactive polyphenols such as quercetin, micronutrients such as iron, probiotics and flavor compounds for protection and controlled release. Future research can also compare the ability of other types of proteins, such as soy protein and pea protein, to stabilize resveratrol utilizing encapsulation. These proteins are more suitable for individuals who are vegan or have dietary sensitivities to dairy foods. Future research could also evaluate taste detection thresholds of resveratrol within food products as these levels may be different than those in aqueous solutions. It would also be interesting to add the resveratrol microcapsules to other complex food products, chocolate and protein shakes, and evaluate consumer acceptance of these products. Consumer testing could also be completed on food products with added resveratrol, with and without the information regarding health benefits of resveratrol. In this way, the effect of the information about resveratrol on consumer acceptance of the product could be determined. In addition, further research can utilize descriptive analysis to evaluate product attribute intensities in order to confirm CATA question results regarding attributes related to LIKE and DISLIKE attributes.

CHAPTER 8: APPENDICES

Appendix A: Apparent solubility of 180 mg Resveratrol/L in 1.2% Ethanol Solution

	Resveratrol Concentration (µg/mL)
Amount Detected in Sample	181
Standard Deviation	9.3

Appendix B: Post-Questionnaire for Threshold Testing of Resveratrol

DEMOGRAPHICS QUESTIONNAIRE

We want to ask you a few questions about yourself. This information will help us compare opinions of people with different backgrounds. All information is confidential and will not be identified with your name. You may choose not to answer questions, if you wish, as your participation is voluntary.

1. **How old are you?**

- ☐ 18-25 years old
- ☐ 26-35 years old
- ☐ 36-45 years old
- ☐ 46-55 years old
- ☐ 56-65 years old
- ☐ Over 65 years old

2. **What is your gender?**

- ☐ Male
- ☐ Female

3. **How do you describe yourself? (check all that apply)**

- ☐ American Indian or Alaska Native
- ☐ Asian
- ☐ Black or African American
- ☐ Caucasian
- ☐ Hispanic or Latino
- ☐ Native Hawaiian or Other Pacific Islander
- ☐ Other: _____

Appendix B (cont.)

4. **When purchasing a food product which of the following criteria are most important to you? (Rank from 1 = most important to 5 = least important, no ties)**

   ____ Convenience

   ____ Health benefits

   ____ Nutrient content

   ____ Price

   ____ Taste

5. **What product would be *best* aligned with the health claim "May decrease cancer risk, increase heart health and neurological function"? (Rank from 1 = most important to 4 = least important, no ties)**

   ____ Snack Bar

   ____ Yogurt

   ____ Cookie

   ____ Drink

6. **Which of the following health benefits do you associate with resveratrol? (Check all that apply)**

   ☐ Anti-aging

   ☐ Improved Neurological Function

   ☐ Lower blood sugar levels

   ☐ Prevent/treat cancer

   ☐ Prevent/treat high blood pressure/hypertension

   ☐ Prevent/treat cardiovascular diseases

   ☐ Skin health

   ☐ I am not aware of any of the health benefits associated with resveratrol

   ☐ I do not know what resveratrol is

   ☐ Other: _____

Appendix C: Ballot for Consumer Testing on Food Products with Resveratrol

OVERALL ACCEPTANCE OF NUTRACEUTICAL BARS

Instructions:

1. ***Before each sample please rinse*** *in the following manner:*
    a. *Rinse your mouth with* ***warm water****.*
    b. *Rinse your mouth with* ***carbonated water.***
    c. *Rinse your mouth with* ***room temperature water****.*
2. *Check to* ***ensure that the 3-digit code on the sample cup matches*** *the one written in above the question.*
3. ***Repeat the rinse procedure between each sample*** *and evaluate the samples in the order they are presented on this page.*

Sample Number: 506

How much do you like this sample overall?

☐	☐	☐	☐	☐	☐	☐	☐	☐
1	2	3	4	5	6	7	8	9
Dislike extremely				*Neither like nor dislike*				*Like extremely*

What attributes of the product do you LIKE? (check all that apply)

- ☐ Peanut butter flavor
- ☐ Bitter
- ☐ Sour
- ☐ Astringent
- ☐ Soft
- ☐ Salty
- ☐ Hard
- ☐ Tooth packing
- ☐ Cohesive
- ☐ Sticky
- ☐ Moist
- ☐ Mouth coating
- ☐ Chewy
- ☐ Fatty
- ☐ Oaty
- ☐ Chocolate flavor
- ☐ Chalky
- ☐ Sweet

Appendix C (cont.)

What attributes of the product do you DISLIKE? (check all that apply)

- ☐ Peanut butter flavor
- ☐ Bitter
- ☐ Sour
- ☐ Astringent
- ☐ Soft
- ☐ Salty
- ☐ Hard
- ☐ Tooth packing
- ☐ Cohesive
- ☐ Sticky
- ☐ Moist
- ☐ Mouth coating
- ☐ Chewy
- ☐ Fatty
- ☐ Oaty
- ☐ Chocolate flavor
- ☐ Chalky
- ☐ Sweet

Other Comments:

PLEASE RINSE NOW: First with warm water, then with carbonated water, and then with room temperature water.

Appendix D: Post-Questionnaire for Consumer Testing on Food Products with Resveratrol

DEMOGRAPHICS QUESTIONNAIRE

We want to ask you a few questions about yourself. This information will help us compare opinions of people with different backgrounds. All information is confidential and will not be identified with your name. You may choose not to answer questions, if you wish, as your participation is voluntary.

1. **How old are you?**

- ☐ 18-25 years old
- ☐ 26-35 years old
- ☐ 36-45 years old
- ☐ 46-55 years old
- ☐ 56-65 years old
- ☐ Over 65 years old

2. **What is your gender?**

- ☐ Male
- ☐ Female

3. **How do you describe yourself? (check all that apply)**

- ☐ American Indian or Alaska Native
- ☐ Asian
- ☐ Black or African American
- ☐ Caucasian
- ☐ Hispanic or Latino
- ☐ Native Hawaiian or Other Pacific Islander
- ☐ Other: _____

Appendix D (cont.)

4. **Which of the following health benefits do you associate with resveratrol? (Check all that apply)**

- ☐ Anti-aging
- ☐ Improved Neurological Function
- ☐ Lower blood sugar levels
- ☐ Prevent/treat cancer
- ☐ Prevent/treat high blood pressure/hypertension
- ☐ Prevent/treat cardiovascular diseases
- ☐ Skin health
- ☐ I am not aware of any of the health benefits associated with resveratrol
- ☐ I do not know what resveratrol is
- ☐ Other: _____

5. **If a product was labeled with "May decrease cancer risk, increase heart health and neurological function", would this increase the chance that you purchase this product over another product without a health claim?**

- ☐ Very Likely
- ☐ Likely
- ☐ Somewhat Likely
- ☐ No Effect
- ☐ Somewhat Unlikely
- ☐ Unlikely
- ☐ Very unlikely

You have now completed the test. Thank you for your participation.
Please return your ballot.