INACTIVATION OF MURINE NOROVIRUS BY THERMAL TREATMENT AND GRAPE SEED EXTRACT

by

KRISTYN CARA NOCK

(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Human norovirus is the leading cause of foodborne gastroenteritis outbreaks worldwide. This study evaluates the thermal inactivation behavior of MNV-1 with and without the addition of grape seed extract (GSE) in a model juice system. The antioxidant activity of GSE was measured using the DPPH assay. The antioxidant and virucidal activities of GSE were reduced when suspended in an acidic buffer (citrate buffer, pH 2.3). Thermal treatment at 56, 60, and 65°C resulted in $t_D$ values of 8.24 ± 0.07, 2.39 ± 1.10, and 0.59 ± 0.38 min, respectively. When 500 ppm GSE suspended in citrate buffer (pH 4.0) was included with thermal treatment, $t_D$ values of 6.19 ± 3.73, 6.41 ± 5.46, and 0.45 ± 0.28 min were found for the same temperatures. Although results suggest there may be no synergism of treatments for improved viral inactivation, the efficacy of thermal treatment alone and GSE alone was established.

INDEX WORDS: Norovirus, Murine norovirus, Inactivation, Thermal treatment, Grape seed extract, DPPH antioxidant assay, Synergism

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# CHAPTER 1

# INTRODUCTION

An estimated 9.4 million cases of foodborne illness occur yearly in the United States which are caused by 31 major pathogens (*101*). Viral pathogens are the cause of 59% of total cases, and human norovirus (NoV) is responsible for a majority of all foodborne illnesses (5.5 million cases) (*101*). NoV is the leading cause of non-bacterial acute gastroenteritis in all age groups worldwide. Human NoV belongs to the *Norovirus* genus of the *Caliciviridae* family. The *Norovirus* genus is divided into six genetic groups (GI-GVI), which are classified based on capsid sequence (*45*). The most prevalent human-infecting genogroups, GI and GII, can be divided into at least 9 and 22 distinct genotypes, respectively (*65*). The most prevalent genotype, GII.4, is constantly undergoing genetic change. This leads to the emergence of new variants, giving rise to gastroenteritis epidemics worldwide (*31*). NoV is transmitted through the fecal-oral route by ingestion of contaminated food or water, and only a small number of viral particles are needed for host infection (*64*).

Human NoV is responsible for an estimated 21,800 deaths in children less than 5 years of age and 1.1 million hospitalizations per year worldwide (*80*). In terms of outcome severity, the greatest impact of NoV infection is to the population aged greater than 65 years, as 90% of deaths from infection fall in this age group (*48*). Infection with NoV commonly induces diarrhea and vomiting. In institutions such as hospitals, nursing homes, and day cares, NoV can be easily transmitted person-to-person through aerosolized vomitus or indirectly via contaminated fomites or environmental surfaces.

Although the majority of NoV outbreaks are transmitted through person-to-person contact, many cases are linked to virus-contaminated food and water (*98*). Foods may become contaminated with NoV anywhere along the food chain from harvesting and processing to final product handling. The most commonly implicated foods include leafy greens, fruits/nuts, and shellfish (*48*). In 2006, four isolated NoV gastroenteritis outbreaks in Sweden were linked to raspberries (*52*). Outbreaks traced back to frozen raspberries have also been reported in Denmark and France in 2005 (*27*, *35*). In 2012 in Germany, frozen strawberries imported from China were identified as the most likely vehicle of contamination with human NoV in an outbreak of 11,000 gastroenteritis cases (*9*). The current study uses citrate buffer as the sample medium in order to represent a juice or mildly acidic beverage product. Although NoV may not have been associated with large outbreaks from contaminated juice, orange juice has been implicated in outbreaks of hepatitis A virus (HAV). In 2004, an outbreak involving 351 cases of HAV identified orange juice as the most likely vehicle of contamination (*39*). Foodborne viruses such as NoV are inherently resistant to environmental conditions and are able to withstand many bacterial-targeted minimal processing technologies implemented during food processing, preservation, and storage (*50*). For this reason, there is a need for alternative preventative and control measures that will be effective in reducing potential viral contamination of food products.

The uncultivability of human NoV has historically led to the use of culturable surrogates to mimic the behavior of NoV in survival and inactivation studies. A novel method utilizing human B cells for propagation of human NoV has recently been developed (*54*), but surrogates will continue to be utilized for human NoV research until this model is validated for routine use in laboratories. Commonly used surrogates, such as murine norovirus (MNV-1), feline calicivirus (FCV) and Tulane Virus (TV) are similar to NoV in terms of structure, size, genome

organization, and environmental stability and persistence (*50*, *73*, *122*, *126*). In a study comparing NoV surrogates, MNV-1 was found to be more resistant to pH extremes (pH 2 to 10) than FCV. In the same study, FCV demonstrated greater thermal stability to inactivation than MNV-1 at 56°C, but differences were not significant at 63 and 72°C (*21*). For its tolerance to environmental conditions and genetic and transmission similarities to NoV, MNV-1 is a suitable surrogate for studies of inactivation of human NoV by acidic and thermal treatments.

Thermal treatment is a commonly studied method for foodborne viral inactivation. D-values, the time needed at a given temperature to reduce a microbial population by 90%, are reported for viruses in order to describe their resistance to thermal applications. Once D-values have been determined, the z-value, the required temperature to reduce the D-value by 90% can be calculated. Establishment of these thermal inactivation parameters gives the ability to design effective processes for microbial inactivation in the food industry. D-values have been reported in studies for viruses in various mediums and food matrices to give a broad range of thermal inactivation data for these pathogens. At 56°C and 72°C using a capillary tube method, D-values for MNV-1 were reported to be 3.47 min and 0.17 min, respectively (*21*). A study of thermal inactivation of human NoV surrogates in spinach reported the D-value for MNV-1 at 56°C to be 4.09 min. An increase in temperature to 72°C resulted in a D-value of 0.22 min in this same study (*17*). A much higher D-value was found at 56°C when blue mussel samples were used as the medium (11.44 min). However, this study revealed a quicker inactivation at 72°C for blue mussel samples than was found for spinach, with a reported D-value of 0.15 min (*18*). A study of mild pasteurization of raspberry puree found 30 seconds at 65°C to reduce MNV-1 by 1.86 $\log_{10}$ PFU/10 g raspberry puree. A pasteurization process at 75°C for 15 seconds resulted in a 2.81 $\log_{10}$ PFU/10 g reduction of MNV-1 in the same study (*6*). As reported D-values in the literature

considerably vary for MNV-1, it is important to establish microbial thermal death times for a specific type of food or beverage application. The current study determines thermal death times for MNV-1 that can be used to develop thermal processing parameters for the inactivation of NoV in a juice or acidic beverage product.

The majority of published studies use first order models to create survivor curves for determination of D-values. The first order model is commonly used in the food industry and describes an exponential decrease in the number of survivors across a treatment time at a constant temperature (*90*). However, non-linear patterns such as shoulders (lag times) and tails or upward and downward concavity of survival curves have been observed (*117*). Since non-linear behavior is common for microorganisms during thermal treatment, more recent studies have applied mathematical modeling to describe the inactivation kinetics of human NoV surrogates. The Weibull model is a non-linear model that has been shown to better represent inactivation data than traditional first order models (*117*).

The application of natural antimicrobial substances is another method that has been investigated as a means of viral inactivation in foods. Introduced as alternatives to synthetic antimicrobials used in foods, natural antimicrobials such as plant-derived extracts, essential oils, and juices have been shown to exhibit antimicrobial properties. Naturally occurring antimicrobials frequently used in food products are thymol, eugenol, and cinnamaldehyde (*118*). Carvacrol, the primary active component of oregano oil, has exhibited antiviral activity against FCV, MNV-1, and hepatitis A virus (*33*, *100*). Grape seed extract (GSE), typically taken as a dietary supplement for its reported antioxidant, anticarcinogenic, and anti-inflammatory properties, has recently been shown to also have antibacterial and antiviral effects. Room temperature (20-25°C) incubation with GSE at concentrations of 0.25 mg/mL and 1.0 mg/mL for

2 h reduced MNV-1 by 1.37 and 1.67 $\log_{10}$ PFU/ml, respectively (*110*). Another study found GSE at a concentration of 1 mg/mL in apple juice to reduce MNV-1 to undetectable levels after 1 h, while 1 mg/mL GSE in milk reduced MNV-1 by 1 $\log_{10}$ PFU/ml after 24 h (*55*).

The ability of GSE to be used as an antioxidant has been frequently studied. One of the most commonly used methods to measure the antioxidant activity of GSE is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. DPPH is a stable free radical that is characterized by a deep violet color that can be detected spectrophotometrically at a wavelength of 517 nm. The addition of a substance with antioxidant properties will reduce the DPPH radical, resulting in loss of violet color (*59*). Radical scavenging activity is then calculated from the loss in absorbance between the negative control and the sample. One study reported the half maximal inhibitory concentration ($IC_{50}$) of GSE from a red grape variety (*Vitis labrusca*) as 56.7 ± 3.7 μg/mL (*87*). Another study found the $IC_{50}$ value of GSE from white grape varieties (*Vitis vinifera*) to be 104.5 ± 17 μg/mL (*77*). The discrepancy between reported $IC_{50}$ values may have to do with the difference in grape varieties and/or extraction methods. The current study uses commercial GSE powder extracted from *Vitis vinifera*.

The current study aims to evaluate the efficacy of thermal treatment alone and in combination with GSE alone for reduction of MNV-1 in an acidic buffer system, representative of a moderately acidic juice or beverage. With the intent of applying results to an acidic beverage product, this study determines the antioxidant and virucidal behavior of GSE when suspended in an acidic environment. Furthermore, the effectiveness of GSE concentration for inactivation of MNV-1 in a model juice product is established. There are limited studies describing synergism between physical and chemical treatments for reduction of enteric viruses, and there are no studies in the current literature that describe the combination of GSE and thermal treatment for

viral inactivation. Thus, to further characterize processing methods for reduction of viral contamination in acidic beverages, the combination of the two treatments was assessed for potential synergistic effects for inactivation of MNV-1. The antioxidant activity of GSE was verified before its subsequent use for inactivation of MNV-1, helping to verify the functionality of GSE and describe its benefits as a food additive. In summary, experiments were performed to determine the antioxidant activity of GSE, to evaluate citrate buffer as the medium to represent a juice or acidic beverage product, to assess GSE as a treatment for MNV-1 inactivation at room temperature, and to determine the effect of pH on the antioxidant and virucidal activity of GSE. The primary objective of this study was to compare the thermal inactivation behavior of MNV-1 with and without the addition of GSE in order to determine whether synergistic effects exist for a greater degree of inactivation.

# CHAPTER 2

# LITERATURE REVIEW

## Foodborne Viruses

Viral gastroenteritis is the primary cause of foodborne illness in the United States, as at least 58% (5.5 million) of all illnesses have been associated with viruses, compared to 33% for bacterial pathogens (*101*). Foodborne viruses include human norovirus (NoV), hepatitis A virus, hepatitis E virus, rotavirus, astrovirus, sapovirus, and aichivirus (*64*). Of these viruses, human NoV and hepatitis A virus (HAV) are known to be the most significant with regard to the number of outbreaks they cause and the number of people they affect (*94*).

Viruses are obligate parasites, meaning they require living cells to replicate. Although they cannot replicate in the environment, a contaminated food still poses a high risk to humans as foodborne viruses are typically quite stable outside of the host and in some cases, only a small number of viral particles are needed for host infection (*64*).

The stability of foodborne viruses is a result of their unique structure. All viruses possess some form of a protein capsid encompassing their genetic material. Many species of viruses also have an outer lipid envelope, making them more susceptible to damage from harsh environmental conditions. However, most human foodborne enteric viruses, such as human NoV and hepatitis A virus, are non-enveloped. The structure of the protein capsids of these viruses make them exceptionally hardy and resistant to harsh environmental conditions and some food processing treatments (*1*). Many bacteria-targeted minimal processing technologies applied during food processing, preservation, and storage are relatively ineffective against viruses (*50*).

Therefore, there is a need for preventative measures to reduce potential viral contamination on these products.

## Human NoV

### *Epidemiology and Significance*

NoVs are the most common cause of epidemic acute gastroenteritis in the United States and other high-income nations. Foodborne infections may be traced back to food that was contaminated by an infected food handler during preparation or service (*4*). In January of 2015, a NoV outbreak was reported among customers of a restaurant in Finland. Upon investigation, four kitchen staff tested positive for a strain of GI NoV, and it was noted that the kitchen personnel's hygiene level was inadequate (*119*). A NoV outbreak of 450 employees occurred after a lunch banquet in Taiwan in February 2015. Pork liver and lamb chops thought to have been contaminated by asymptomatic infected food handlers were determined to be the source of the outbreak (*24*).

NoV gastroenteritis outbreaks have also been sourced to production and distribution events. The most commonly implicated foods in NoV outbreaks are leafy greens, fruits and nuts, and shellfish (*48*). Frozen raspberries have been a frequent source of contamination in outbreaks in European countries. In 2005, Denmark and France experienced outbreaks traced back to frozen raspberries (*27*, *35*). A year later, four isolated NoV gastroenteritis outbreaks in Sweden were also linked to frozen raspberries (*52*). Again, frozen raspberries were the most likely vehicle in an outbreak following two meetings held at a conference center in Norway (*34*). In 2012, frozen strawberries imported from China were consistently identified as the most likely source of contamination in over 11,0000 gastroenteritis cases in Germany (*9*). In 2003, simultaneous clusters of NoV gastroenteritis cases occurred in France and Italy and were thought

to have been associated with the consumption of oysters harvested from lagoons in the south of France (*46*). In December 2013, a NoV outbreak was linked to insufficient heat treatment of shellfish soup served at a Christmas gathering in Norway. NoV strains from two genogroups were subsequently detected in the remaining raw shellfish (*75*).

In the U.S. population, there is an estimated national incidence of 399,000 hospital emergency department visits and 1.7 million outpatient visits in all age groups (*40*). NoVs cause an estimated 64,000 hospitalizations and 900,000 medical visits among children in developed countries (*89*). Although children have the highest rates of NoV-associated medical visits, outbreaks can occur in people of all age groups (*40*). The greatest impact in terms of disease outcome severity is in the population aged ≥65 years. In the United States, 90% of deaths resulting from a NoV infection fall in this age group (*47*). This high fatality rate for the elderly age group may be partially attributed to large number of outbreaks that occur in institutions such as nursing homes and hospitals (*64*).

NoV is transmitted through the fecal-oral route, which includes person-to-person and fomite contact, as well as ingestion of contaminated food or water. NoV particles may be shed in feces at numbers as high as $9.5 \times 10^{10}$ genomic copies/gram of feces 2-5 days postinfection. Shedding may continue to occur 10 days after initial infection (*4*). The NoV infectious dose is thought to be between 17 and 2,800 virus particles (*5*, *115*). In a 2013 human challenge study, of the 21 susceptible people infected with human NoV, 67% developed gastroenteritis. The 50% human infectious dose was then calculated to be around 2,800 NoV genomic equivalents, which is higher than previously reported and similar to other human RNA viruses (*5*). The disease has an incubation period of 1 to 2 days with symptoms typically lasting from 24 to 60 h.

Asymptomatic infections are seen in up to 30% of cases and asymptomatic persons can shed virus at low titers (*4*, *44*, *92*).

***Classification and Characteristics***

Human NoVs, previously referred to as Norwalk-like viruses, were originally identified in an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio in 1968 (*97*). They belong to the family *Caliciviridae,* which is comprised of five genera, *Norovirus, Sapovirus, Lagovirus, Vesivirus,* and *Nebovirus.* Three additional genera have been proposed in this family, *Recovirus, Valovirus,* and *Chicken calicivirus* (*36*, *69*, *123*). The *Norovirus* genus can be divided into six genogroups (GI-GVI), determined by phylogenic analysis of the major capsid protein sequence. Genogroups I and II are the most prevalent genogroups among humans, although outbreaks with strains from genogroup IV have also been documented among humans (*30*). Porcine noroviruses belong to genogroup II, genogroup III contains bovine noroviruses, genogroups IV includes canine and feline noroviruses, murine noroviruses belong to genogroup V, and genogroup VI contains canine noroviruses (*79*, *81*, *93*, *109*, *127*).

GI and GII NoVs exhibit the most genetic diversity as they can be divided into at least 9 and 22 distinct genotypes, respectively (*65*). NoV genotype classification is most commonly based on the open reading frame 2 (ORF2) nucleotide sequence, which encodes the major structural capsid protein (*127*). One single genotype, GII.4, is by far the most prevalent and undergoes constant genetic change. This leads to the frequent emergence of new GII.4 variants, coinciding with new acute gastroenteritis epidemics worldwide (*31*). At least 8 GII.4 variants have been reported in the past two decades which are estimated to have been responsible for 70-80% of all NoV outbreaks (*66*, *105*).

Human NoVs are icosahedral, nonenveloped viruses that are approximately 27 to 38 nm in diameter. The NoV viral capsid contains a single-stranded positive-sense RNA genome ranging from 7.4 to 8.3 kb in length which is composed of three open reading frames (ORFs). ORF1 encodes non-structural proteins including those important for replication. ORF2 encodes the major structural protein, VP1, and ORF3 encodes the minor structural protein, VP2. VP1 consists of the amino-terminal shell domain (S), which forms the interior of the icosahedral shell and the carboxyl-terminal protruding domain (P), which forms the cup-like protrusions on the capsid surface. The P domain is further divided into subdomains, P1 and P2. P1 is more interiorly located and has a fairly conserved sequence, while P2 is located at the exterior surface and contains a highly variable sequence. Because P2 is located at the capsid surface and contains the most highly variable sequence, it has been assumed that the P2 subdomain is important for host cell interaction (*114*). Human NoVs recognize human histo-blood group antigens (HBGAs) as receptors, which are complex carbohydrates present on the surface of red blood cells, gastrointestinal and respiratory epithelial cells, and as free antigens in biological fluids such as milk, saliva, and intestinal contents (*78*).

**Human NoV Surrogates**

Past studies attempting to culture and propagate human NoV *in vitro* have been unsuccessful; therefore laboratory research of human NoV has utilized viral surrogates as comparable indicators. In the past year, a method utilizing human B-cells for the propagation of human NoV was developed (*54*). However, human NoV surrogates will continue to be utilized until the model is validated for routine use in laboratories. Ideal surrogates should be similar to human NoV in terms of structure and size, environmental survival and persistence, and transmission via the fecal-oral route. Furthermore, surrogates should be chosen based on specific

inactivation parameters, where the surrogate should be more resistant to the treatment being investigated than is human NoV (*19*). This laboratory approach will generate data that can be applied for a fail-safe treatment design in which inactivation parameters are set based on the more resistant surrogate and should therefore be effective for inactivation of human NoV. Three viral surrogates that are commonly employed for human NoV research are murine norovirus (MNV-1), Tulane virus (TV) and feline calicivirus (FCV).

MNV was discovered in 2003 and possesses many similar traits to human NoV. However, unlike human NoV, MNV belongs to genogroup V and infects mice. Transmission of MNV-1 is analogous to NoV, as the virus is shed in mice feces and transmitted via the fecal-oral route (*57*). The viral structure and genome organization is very similar to human NoVs, as MNV-1 has also a 28-35 nm icosahedral, non-enveloped capsid containing a single-stranded RNA genome (*122*). However, MNV is dissimilar to human NoV in terms of its receptor usage. While human NoV targets histo-blood group antigen receptors for infection of intestinal epithelial cells, MNV-1 uses sialic acid as a receptor to infect macrophages and dendritic cells (*62*). Like human NoVs, MNV-1 is stable and persistent in the environment and has a high resistance to heat and acidity (*21*, *71*). A study comparing NoV surrogates found that MNV-1 was more resistant to both high and low pH values (pH 2 to 10) than was FCV (*21*). While resistant to conditions such as heat and acidity, MNV-1 is effectively inactivated by high-pressure processing (*74*). MNV-1 has also exhibited high sensitivity to alcohols (*28*).

TV is a member of the proposed genus *Recovirus* of the *Caliciviridae* family. Although the genus classification is dissimilar, TV possesses qualities of close relation to GII NoVs. With a diameter of ~40 nm, the capsid organization and protein structure closely resembles human NoV (*126*). Tulane virus uses HBGAs as its receptor, further suggesting it may have a more

similar capsid structure to human NoVs than does MNV-1 (*51*). In terms of thermal inactivation sensitivity, TV responds similarly to heat treatments as MNV-1 but is more sensitive to pH variations. For example, a study found that TV was significantly inactivated at pH 2.0, 9.0, and 10.0, whereas MNV-1 was only slightly inactivated at pH 10.0 (*51*). Pressure applications have shown that both MNV-1 and TV are fairly resistant in acidic environments and become more sensitive as they approach neutral pH. However, in cell culture media, blueberries, and oysters, TV showed greater sensitivity to pressure than MNV-1 (*73*).

FCV is also a member of the *Caliciviridae* family, but belongs to the genus *Vesivirus* and causes respiratory illness in cats (*96*). Discovered much earlier than most other commonly used surrogates, it has been the preferred human NoV surrogate since the 1970s (*28*). The virus is ~35 nm in diameter and, like MNV-1, targets sialic acid receptors (*96*). The more recent discovery of MNV has decreased the use of FCV as the preferred human NoV surrogate as it has shown to be more sensitive to many environmental conditions. These conditions include acidic and alkaline pH environments, chloroform treatment, UV irradiation, and pressure treatment. Although FCV has been shown to be more stable than MNV-1 at 56°C, differences were not significant at 63 and 72°C (*21*).

**Natural Antimicrobials**

Antimicrobial agents are used in and on foods to inactivate or inhibit the growth of spoilage or pathogenic microorganisms. When dealing with an antimicrobial that will come in contact with food, it is important to consider how the consumer will perceive the safety and wholesomeness of the food product. For this reason, natural antimicrobials have begun to be investigated as alternatives to the common antimicrobials that have been utilized in foods for years. Many of the natural antimicrobials that have been tested for their use in foods are plant-

derived extracts, essential oils, and juices. Plant extracts have been shown to contain an abundance of secondary metabolites that have antimicrobial properties (*33*). Essential oils can be produced from plant materials such as leaves, fruits, flowers, and bark and are composed of a diverse mixture of volatile compounds. There are over 3,000 known essential oils, and while most are used for flavors or fragrance, many have been important for bacterial inactivation. Three significant essential oils with antimicrobial properties that are used in the food industry are thymol, eugenol, and cinnamaldehyde (*118*). *Alpinia galangal* (L.) Willd., a member of the ginger family, has antibacterial properties against *Listeria monocytogenes* and *Staphylococcus aureus* (*20*). Another member of the ginger family, *Alpinia katsumadai*, has exhibited anti-*Campylobacter* and antiviral properties. Also, components of tea such as epigallocatechin gallate have been shown to have antimicrobial and antiviral properties (*61*).

While many natural antimicrobials are used primarily to target bacteria, some have shown to be effective against viruses. Carvacrol is the primary active component of oregano oil and has antiviral activity against FCV, MNV-1, and HAV. A concentration of 0.5% carvacrol inactivated the two NoV surrogates completely, while a 1% concentration was necessary to achieve a 1 log reduction of HAV (*33*, *100*). Cranberry juice and its derived proanthocyanidins have exhibited antiviral properties against enteric virus surrogates. At a starting titer of 5 $\log_{10}$ PFU/ml, cranberry juice and cranberry juice proanthocyanidins at concentrations of 0.30, 0.60, and 1.20 mg/ml reduced FCV to undetectable levels. The same tested substances reduced MNV-1 by 2.06-2.95 $\log_{10}$ PFU/ml, MS2 bacteriophage by 0.55-1.14 $\log_{10}$ PFU/ml, and PhiX174 bacteriophage by 1.79-4.98 $\log_{10}$ PFU/ml (*111*). Polyphenols in pomegranate juice have also demonstrated antiviral effects against MNV-1. At pomegranate polyphenol (PP) concentrations of 4, 8, and 16 mg/ml, MNV-1 at a low starting titer was reduced by 1.30, 2.11, and 3.61 $\log_{10}$

PFU/ml, respectively. At a high initial titer and the same PP concentrations, MNV-1 was reduced by 1.56, 1.48, and 1.54 $\log_{10}$ PFU/ml (*112*).

## Grape Seed Extract

### *Characteristics and Antimicrobial Properties*

With an annual production of approximately 58 million metric tons, grapes (*Vitis vinifera)* are the world's largest fruit crop (*128*). Extract from grape seeds, a major byproduct from grape and wine production, has been recognized for its many health benefits. GSE is used as a dietary supplement for its reported antioxidant, anticarcinogenic, and anti-inflammatory properties. It also has demonstrated cardioprotective, hepatoprotective, neuroprotective, and antidiabetic effects as well as inhibition of ulcer formation (*99*, *110*). Recently, it has been reported to have antimicrobial properties against a range of different microorganisms (*110*). The pharmacological, biological, and medicinal properties of GSE is due to its rich source of phenolic compounds. Grape seeds are composed of a mixture of monomers, oligomers, and polymers of (+)-catechins, (-)-epicatechin, (-)-epicatechin-3-O-gallate, and (-)-epigallocatechin (*99*). These polyhydroxyflavan-3-ol units are linked together with C4-C8 or C4-C6 bonds and are known as proanthocyanidins (*76*).

GSE has shown success as an antibacterial and antiviral agent. GSE was tested against *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa,* and was found to inhibit Gram-positive bacteria completely at 850-1,000 ppm and Gram-negative bacteria at 1,250-1,500 ppm (*53*). At a concentration of 0.25 mg/mL (250 ppm), GSE reduced *Listeria monocytogenes* by $10^6$ to $10^7$ CFU/mL or to undetectable levels within 10 min (*95*). When investigated as a produce wash, 0.125% (1250 ppm) GSE reduced *L. monocytogenes* by ~2 $\log_{10}$ from a ~5 $\log_{10}$ starting titer on tomato

surfaces, indicating a potential inexpensive solution to reduce *L. monocytogenes* on fresh produce (*10*). At 1 mg/mL (1000 ppm) concentration, a study found GSE to inhibit *S. aureus* growth by 99% (*56*). Gallic acid is the active component for inhibition of *E. coli* and *Salmonella* Enteritidis by GSE. The three hydroxyl groups located on the benzene ring were revealed to be important for this antibacterial activity, while all of benzene's constituents were found to be effective against *S. aureus* (*104*).

Past studies have revealed GSE to possess antiviral properties against NoV surrogates and HAV. Incubation of high viral titers (~7 $\log_{10}$ PFU/ml) at room temperature with 0.25 mg/mL (250 ppm) and 1.0 mg/mL (1000 ppm) GSE for 2 h reduced MNV-1 by 0.44 and 1.06 $\log_{10}$ PFU/mL, respectively. At corresponding conditions, HAV was reduced by 0.86 and 1.90 $\log_{10}$ PFU/mL. When lower viral titers were used (~5 $\log_{10}$ PFU/mL), log reductions increased. At 0.25 mg/mL and 1.0 mg/mL, MNV-1 was reduced by 1.37 and 1.67 $\log_{10}$ PFU/mL, and HAV was reduced by 2.40 and 3.01 $\log_{10}$ PFU/mL, respectively. With an increase in incubation temperature to 37°C, 0.25 mg/mL and 1.0 mg/mL GSE reduced high-titer MNV-1 by 0.92 and 1.73 $\log_{10}$PFU/mL and low-titer MNV-1 by 1.49 and 1.97 $\log_{10}$PFU/mL, respectively. At the same conditions, high-titer HAV was reduced by 1.81 and 3.20 $\log_{10}$ PFU/mL and low-titer HAV was reduced by 1.86 and 2.89 $\log_{10}$ PFU/mL, respectively (*110*). An investigation of the mechanism of action revealed FCV, MNV-1, and HAV reduction by GSE was primarily a consequence of interference of viral adsorption with minor effects on replication (*110*).

***Antioxidant Activity***

Numerous studies have measured the antioxidant activity of GSE using assays such as the Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Potential assay (FRAP), Trolox equivalent antioxidant capacity (TEAC), and 2,2'-azinobis (3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. The DPPH assay is one of the most frequently used to measure the antioxidant capacity of GSE. This assay uses the stable free radical, 2,2,-diphenyl-1-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, MW=394.33), which contains a nitrogen atom with a single unpaired electron. The delocalization of the unpaired electron over the molecule as a whole gives DPPH its characteristic deep violet that can be detected spectrophotometrically at a wavelength of 517 nm. Upon mixing DPPH solution with an antioxidant substance, the substance will reduce the free radical by donating a hydrogen atom, resulting in loss of violet color (*59*). The loss in absorbance between the negative control and sample can then be used to calculate the radical scavenging activity of the substance.

A study reported 45.6% and 87% radical scavenging activity for GSE at concentrations of 25 ppm and 50 ppm, respectively (*53*). The half maximal inhibitory concentration ($IC_{50}$) values, the concentration of GSE required to scavenge 50% of the DPPH radical, have been reported as 56.7± 3.7 μg/mL and 104.5 ± 17 μg/mL (*76*, *87*). Other studies using Trolox as a standard reported GSE antioxidant data as μmol Trolox/g dry weight (DW). One study reported antioxidant activity of grape pomace seed extracts from various Mediterranean grape varieties to range from 410.79 ± 43.30 to 1050.59 ± 30.11 μmol Trolox/g DW (*67*). Another study reported the antioxidant activity of 800 μg/mL GSE to be 79.73 ± 19.90 μmol Trolox/capsule of GSE (*106*). The antioxidant activity of GSE is due to the reducing power of proanthocyanidins. These phenolic compounds inhibit free radicals by donating a hydrogen atom or by reacting with them directly to stabilize them and terminate the free radical reaction chain (*53*).

Polyphenols from grape seeds are highly sensitive to acid, alkakine, oxygen, and light, but are less sensitive to heat (*102*). In applications where GSE is subjected to a heat treatment and still expected to maintain it original functional properties, it is important to verify its thermal

stability. One study found GSE in raw and cooked pork patties to have similar antioxidant activities (*22*). An alternative study found that although bread with the addition of GSE had greater antioxidant activity than bread without GSE, thermal processing caused the antioxidant activity to decrease by 30-40% (*91*). It is important to consider, however, that this bread was baked for 43 min at high temperatures (which could range from ~176-218°C). Another study investigated the thermal stability of procyanidins in grape and blueberry pomace by heating with a forced air oven at 40, 60, 105, and 125°C for 72, 48, 16, and 8 h, respectively. With the exception of 40°C for 72 h, procyanidin concentrations in both pomaces decreased significantly ($p < 0.05$). The study found that procyanidin reductions began at 60°C, but did not increase from 105 to 125°C. Overall, procyanidins may retain their thermal stability at lower temperatures for up to 3 days, but higher temperatures for more than 8 h results in considerable thermal degradation (*60*). Therefore, the degree of GSE functionality loss by thermal degradation is greatly dependent on the time and temperature of the applied process.

***Sensory Effects in Foods***

With the intent of using this antimicrobial as a food additive, a consideration of the sensory properties of GSE is necessary. Several studies have conducted sensory tests of food products formulated with and without GSE. The detection or sensory influence of GSE varied greatly with the product it was included in. In a study of the sensory and analytical effects of adding GSE to red wine at concentrations of 0.0, 0.5, 1.0, 2.5 and 5.0 g/L (0-5,000 ppm), GSE was positively associated with astringency ($R^2 = 0.841$) and woody/earthy aroma ($R^2 = 0.933$). It was also observed that GSE suppressed the fruity aroma of the red wine (*26*). Perceptions of astringency were also noted in a sensory study of cookies enriched with GSE. Quantitative Descriptive Analysis (QDA®) trained panelists found GSE enriched cookies to be similar in

aroma and flavor to cookies baked with standard whole grain flour but with an added astringency. The reason for this astringency is the interaction of the polyphenolic compounds within GSE, primarily procyanidins, with glycoproteins in saliva (*29*). In some products, however, this astringency may not be detected. For example, a study found the sensory aspects of cooked pork patties to be unaffected by GSE additions at 400 and 1,000 ppm concentrations (*22*). GSE is also beginning to be included as an ingredient in commercial food products, such as energy bars (*125*).

**Thermal Inactivation of MNV-1**

Thermal applications have historically been utilized as successful measures of viral inactivation. Many studies have observed the thermal response of MNV-1 by measuring the decimal reduction times (D-values). D-values have been reported for MNV-1 at a range of temperatures to give a foundation for understanding its thermal sensitivity.

D-values for MNV-1 at temperatures ranging from 50 to 80°C have been experimentally determined in previous studies. One study reports D-values for MNV-1 at 50 to 80°C to range from 36.28 to 0.15 min (*13*). Using a volume of 50 µL with the capillary tube method, D-values for MNV-1 at 56, 63, and 72°C were found to be 3.47, 0.44, and 0.17 min, respectively (*21*). When repeated using the same capillary tube method, another study found D-values at 50, 56, 60, 65, and 72°C to be similar to those reported by Cannon et al (*15*). When sample volumes were increased to 2 mL, MNV-1 D-values at 60, 65, and 72°C were higher than those found by the capillary tube method, but there were no statistically significant differences at 50 and 56°C ($p>0.05$) (*14*). Another study found that MNV-1 was quickly inactivated at 60 and 63°C, while 20 min were required for a 5 log inactivation at 56°C (*28*). When spinach was used as the medium for viral thermal inactivation, D-values were reported for MNV-1 at 56, 60, 65, and

72°C to be 4.09, 1.11, 0.47, and 0.22 min, respectively (*17*). Thermal treatment of blue mussels at the same temperatures resulted in D-values of 11.44, 3.01, 0.42, and 0.15 min. When comparing D-values of MNV-1 in suspension and in dried mussels, a study reported D-values at 60°C to be 7.79 min in suspension and 9.01 min in dried mussels (*88*). In turkey deli meat, MNV-1 D-values at 56, 60, 65, and 72°C were 6.7, 2.8, 0.9, and 0.3 min, respectively (*16*).

The mechanism in which viruses become inactivated in the presence of heat is associated with alterations to the viral capsid. When mild temperatures (<56°C) are applied, inactivation is due to destruction of specific capsid structures that are necessary for host cell binding and recognition (*121*). At higher temperatures (>60°C), the capsid protein tertiary structure is altered, facilitating access of thermal energy to the viral genetic material (*58*).

**Synergism of Chemical and Physical Inactivation Treatments**

The proceeding research project will measure the synergism of a chemical procedure (the application of GSE) and a physical procedure (thermal treatment) in inactivation of MNV-1. A previous study observed synergy between radio frequency and various antimicrobials in inactivation of *Escherichia coli* inoculated in ground beef homogenate. Inoculated beef homogenate without the addition of antimicrobials was heated in the radio frequency oven to a temperature of 55°C and resulted in an *E. coli* reduction of 0.94 logs. With the separate additions of 2.5% potassium bicarbonate, 0.5% citric acid, and blends of citric acid and potassium bicarbonate (0.5% citric acid with 0.5% citric acid and 1.5% potassium bicarbonate), inoculated beef homogenate heated in the RF oven to 55°C resulted in more than 5 log reductions of *E. coli* (*84*). Another study aimed to determine potential combined effects of chemical disinfectants and UV treatments in reduction of coliphage MS2. At a dose of 7.0 mg/l, peracetic acid (PAA) reduced MS2 by 0.86 log units. When UV was applied at a dose of 38 mWs/cm$^2$, a 1.40 log MS2

reduction was observed. The combination of PAA and UV resulted in a 2.37 log reduction, demonstrating a moderate amount of synergy between the two treatments (*63*).

The primary goal of the proceeding research project is to reveal novel processes for reduction of foodborne viruses in commonly contaminated foods. This project will measure the reduction ability of MNV-1 by GSE alone, by thermal treatment alone, and by the combination of GSE and thermal treatment. Each treatment will be performed in an acidic buffer system found to be analogous to a fruit juice or beverage, so results will be most directly applied to this product. The overall objective of this study is to compare the thermal inactivation behavior of MNV-1 with and without the addition of GSE.

# CHAPTER 3

# MATERIALS AND METHODS

## Viruses and Cell Lines

Murine norovirus (MNV-1), strain P3, was provided by Dr. Skip Virgin (Washington University, St Louis, MO) and RAW 264.7 cells (ATCC TIB-71) were used for MNV-1 infectious plaque assays. Hepatitis A Virus (HAV) and its host FRhK-4 cells were obtained from ATCC (Manassas, VA).

## Infectious Plaque Assays

Cell culture plates (60 mm diameter, Corning Inc., Tewksbury, MA) were seeded with RAW 264.7 cells at a density of 2 x $10^6$ cells per plate before 5 ml of Minimum Essential Medium (MEM) (Corning Inc., Tewksbury, MA) was added to the plates and the cells were grown to 80 to 90% confluence at 37°C in a 5% $CO_2$ incubator. Cell monolayers were infected with 10-fold serial dilutions of samples containing MNV-1 for 1 h at 37°C. The inoculum was then removed and 5 ml of overlay medium containing 1X MEM and 0.5% agarose (GoldBio, Inc., St. Louis, MO) was added to the cells. After incubation for 44-48 h, liquid samples were visually observed for obvious plaques. Approximately 2-3 mL of a 10% formaldehyde (Acros Organics, NJ) solution was then added directly to the agar layer. Plates were left at room temperature for 6-48 hrs, the formaldehyde solution was removed, and plates were subsequently stained with Crystal Violet (Alfa Aesar, Haverhill, MA) solution (2.5 g crystal violet, 50 mL methanol, 197.5 Milli-Q® water). Resulting plaques were counted 1-8 h later and plates with 5 to

50 plaques were selected for calculation of MNV-1 log plaque forming unit (PFU) recovery from samples.

**Determination of Viral Inactivation by Citrate Buffers**

Experiments were performed to determine the virucidal properties of citrate buffer (pH 2.3, pH 3.0, pH 4.0) before it was used as the diluent for the GSE antimicrobial in subsequent experiments. Citrate buffers were prepared with varying ratios of 0.1M-citric acid monohydrate (Macron Fine Chemicals, Center Valley, PA) and 0.2M-sodium phosphate, dibasic (Fisher Chemical, Fair Lawn, NJ). Phosphate-buffered saline (PBS) was prepared with sodium chloride, potassium chloride, potassium phosphate, and sodium phosphate dibasic all purchased from Fisher Chemical (Fair Lawn, NJ). Neutralization buffer was made with 10% fetal bovine serum (FBS) (HyClone Characterized, GE Healthcare) in PBS and 1M-sodium bicarbonate (J.T. Baker, Avantor Performance Materials, Inc., Phillipsburg, NJ).

The stability of MNV-1 was determined by mixing 10 µL of virus stock (~8 $\log_{10}$ PFU/ml) with 90 µL of each buffer (citrate buffer pH 2.3, 3.0, 4.0) and incubation at room temperature. Initial experiments were performed using citrate buffer (pH 3.0 and 4.0) with a 5 min contact time. A subsequent experiment was performed using citrate buffer (pH 2.3) for 80 min of contact time, representing the longest treatment time used for thermal inactivation experiments. Once the contact time was achieved, the pH was adjusted to 7.0 by addition of neutralization buffer. Neutralization controls were prepared by adding neutralization buffer to citrate buffers prior to addition of MNV-1. These samples were used as a control and were the basis for calculating $\log_{10}$ PFU/ml reduction after citrate buffer treatments. Negative controls and cytotoxicity controls, prepared with PBS instead of virus were also included. The negative control was included for verification that sample reagents were not contaminated and the

cytotoxicity control was included to verify that sample reagents did not produce cytotoxic effects for RAW 264.7 cells. Samples were stored in a -80°C freezer until analysis by plaque assay. Citrate buffer (pH 3.0 and 4.0) samples were prepared in triplicate and citrate buffer (pH 2.3) samples were prepared in duplicate.

**Comparison of Viral Inactivation by Various GSE Solution Preparations**

To assess the effect of the citrate buffer pH on the ability of GSE to inactivate MNV-1, GSE solutions were prepared by dissolving GSE powder (>95% Proanthocyanidins, BulkSupplements.com, Henderson, NV) at concentrations of 200 ppm, 500 ppm, and 800 ppm in citrate buffer (pH 2.3), citrate buffer (pH 4.0), and sterile Milli-Q® water (pH 6.25). Samples were prepared by mixing a virus cocktail of MNV-1 (~9 $\log_{10}$ PFU/ml) and HAV (~9 $\log_{10}$ PFU/ml) with GSE solutions and incubating for 1 min at room temperature. GSE solutions were subsequently adjusted to pH 7.0 with a neutralization buffer prepared with FBS and 10-20 µL additions of 1M-sodium bicarbonate or 6M-hydrochloric acid. Neutralization controls were prepared by adding neutralization buffer to GSE solutions prior to addition of the virus cocktail, which were included for calculating $\log_{10}$ PFU/ml reduction after citrate buffer treatments. Negative controls and cytotoxicity controls prepared with PBS instead of virus cocktail were included. Samples were stored in a -80°C freezer until analysis by plaque assay. Two experimental replicates were performed with duplicate samples.

**Heating Rate of Citrate Buffer Compared to Various Juices**

Citrate buffer (pH 4.0) was chosen to represent various juices of similar acidities. Apple juice (pH 3.79) (100% Juice From Concentrate, The Kroger Co., Cincinnati, OH), orange juice (pH 4.00) (Original No Pulp, Tropicana Manufacturing Company, Inc., Bradenton, FL), and grape juice (pH 3.65) (Organic Concord Grape Juice, Knudsen & Sons, Inc., Chico, CA) were

purchased from a local grocery store. Heating rates of citrate buffer and juices were determined by heating samples in a circulating water bath (Thermo Scientific, NESLAB RTE 10, Newington, NH). Glass vials (12x32 mm Glass Screw Thread, Scientific Specialties Service, Inc., Hanover, MD) with T-Thermocouples (Omega Engineering Inc., Stamford, CT) inserted through a hole and rubber pore in the vial lid were connected to a data logger (HotMux, DCC Corporation, Pennsauken, NJ) that was used to record the time taken to reach the target temperature (come-up time) for 300 μL volumes of citrate buffer, apple juice, orange juice, or grape juice submerged in water baths set to temperatures of 56, 60, 65, and 72°C. Samples were performed in triplicate at each temperature and experimental replicates were performed in triplicate.

**GSE Radical Scavenging Activity Determination by the DPPH Method**

The free-radical scavenging activity of varying concentrations of GSE was determined using a modified version of the method by Xu and Chang (*124*). Test solutions were prepared by dissolving GSE Powder (>95% Proanthocyanidins, BulkSupplements.com, Henderson, NV) in methanol (Macron Fine Chemicals, Center Valley, PA). Ascorbic acid positive controls were prepared by dissolving L-ascorbic acid (Macron Fine Chemicals, Center Valley, PA) in methanol. A 60 μM DPPH solution was prepared by dissolving 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Alfa Aesar, Haverhill, MA) in methanol. As the absorbance of a DPPH solution has been shown to decrease over a 1 h time period (*103*), the solution was allowed to stabilize in the dark for 1-2 h before use.

GSE solutions of concentrations ranging from 25-200 ppm were added in 0.1 mL doses to 3.9 mL of the DPPH solution. After vortexing for 1 min, the samples were allowed to react for 30 min in the dark before measuring the absorbance at 517 nm using a UV-Vis

spectrophotometer (Life Science UV/VIS Spectrophotometer, Multicell Module DU 530, Beckman, Brea, CA). Pure methanol was used as the blank. Sample absorbance ($A_s$) was compared against the absorbance of a negative control ($A_0$) prepared with methanol in place of extract to determine radical scavenging activity (% radical scavenging activity (SA) = [($A_0 - A_s$) / $A_0$] x 100). Results are expressed as free radical scavenging activity half maximal inhibitory concentration ($IC_{50}$) value, the concentration of GSE required to scavenge 50% of the DPPH radical.

The free-radical scavenging activity of 2 alternative brands of GSE were determined for comparison against the GSE powder used in later thermal inactivation experiments. GSE powder (95% Proanthocyanidins, NuSci, Walnut, CA) was purchased from HerbstoreUSA.com and Gravinol-S™ (95% Proanthocyanidins, OptiPure, Commerce, CA) was kindly provided by Dr. Doris D'Souza from The University of Tennessee in Knoxville, Tennessee. The DPPH assay was performed as previously described and the radical scavenging activity was calculated for 50, 100, 150, and 200 ppm GSE solutions and L-ascorbic acid positive controls. Results are expressed as free radical scavenging activity $IC_{50}$ values.

**Comparison of Radical Scavenging Activity Across Varying GSE Concentrations and Solvents**

To observe the effect of citrate buffer pH on GSE radical scavenging activity, GSE solutions were prepared at 200 ppm, 500 ppm, and 800 ppm in citrate buffer, pH 2.3, and citrate buffer, pH 4.0. GSE solutions made with sterile water were prepared at the same concentrations for comparison to those made with citrate buffer. Ascorbic acid positive controls were prepared at the same concentrations (200, 500, and 800 ppm) and in the same solvents (citrate buffer (pH

2.3 and 4.0) or sterile water). The DPPH assay was performed as previously described and the radical scavenging activity was calculated for each solution.

**Thermal Effects on GSE Antioxidant Capacity**

To determine potential thermal effects on the antioxidant activity of GSE, 500 ppm GSE solutions in glass vials were heated for 6 min at 72°C using a circulating water bath. This represented the most extreme conditions viral samples endured in subsequent thermal inactivation experiments. Triplicate 12x32 mm glass vials containing 300 µL of 500 ppm GSE in citrate buffer (pH 4.0) and L-ascorbic acid positive controls were prepared. A circulating water bath (Thermo Scientific, NESLAB RTE 10, Newington, NH) was set to 72°C and allowed 15 min of temperature stabilization before use. Three vials containing GSE solution were fixed with T-Thermocouples in the same manner as stated above. The sample vials and thermocouple vials were inserted into the water bath and the come-up time was recorded. Once the temperature of the solution reached 72°C, the samples remained in the water bath for 6 min and were then promptly removed and placed on ice for 3 min. To determine the thermal effects on GSE antioxidant activity, the heated solutions were compared against GSE solutions left at room temperature. The DPPH assay was performed using a 60 µM DPPH solution and the radical scavenging activity was calculated to compare the effect of heating on GSE.

**Viral Inactivation by GSE and Thermal Treatments Used Alone and in Combination**

Citrate buffer (pH 4.0) was selected as the diluent for GSE powder (>95% Proanthocyanidins, BulkSupplements.com, Henderson, NV), which was used at the 500 ppm concentration. After buffer preparation, it was sterilized using a syringe filter (0.2 µm pore size, VWR, Radnor, PA). A neutralization buffer was prepared with fetal bovine serum (HyClone Characterized, GE Healthcare) and 1M-sodium bicarbonate (Fisher Chemical, Fair Lawn, NJ). A

virus cocktail consisting of MNV-1 (~9 $\log_{10}$ PFU/ml) and HAV (~9 $\log_{10}$ PFU/ml) was used for sample inoculation.

A DPPH antioxidant assay was performed in order to measure the radical scavenging activity of the 500 ppm GSE solution that was subsequently used for viral inactivation. Sample sets receiving four different treatments were prepared in order to determine viral inactivation by GSE and thermal treatments used alone and in combination. Full treatment samples were treated with 500 ppm GSE and received thermal treatment (56, 60, 65, and 72°C). Temperature control samples were treated with 500 ppm GSE and were kept at room temperature (20-25°C). GSE control samples were prepared with citrate buffer, pH 4.0, and received thermal treatment. Positive control samples were prepared with citrate buffer, pH 4.0, and were kept at room temperature.

For each treatment temperature (56, 60, 65, and 72°C), specific time points were established for sample treatment (e.g. at a 56°C treatment temperature, time points ranged from 0-80 min). The circulating water bath (Thermo Scientific, NESLAB RTE 10, Newington, NH) was set to the target temperature and allowed to stabilize with minimal temperature fluctuation (± 0.1°C) for 15 min. 270 µL of 500 ppm GSE solution or citrate buffer, pH 4.0, was added to glass vials (12x32 mm Glass Screw Thread, Scientific Specialties Service, Inc., Hanover, MD) corresponding to predetermined time points. Each vial was quickly inoculated with 30 µL virus cocktail, vortexed, and positioned into a floating tube rack. Non-inoculated vials with GSE solution were fixed with T-thermocouples (Omega Engineering Inc., Stamford, CT) and placed into separate locations in the rack. Thermocouples were connected to a temperature data logger (HotMux, DCC Corporation, Pennsauken, NJ). The floating tube rack was placed into the circulating water bath and the time taken for the samples to reach the target temperature (come-

up time) was recorded. The time from inoculation until submerging vials in the circulating water bath was also recorded. When the samples reached target temperature, the 0 min tubes were removed by hand, placed on ice, and a timer was started. At each time point, samples were removed and placed on ice for 1.25 min before adjusting to pH 7.0 with 900 µL neutralization buffer kept room temperature (20-25°C).

For each treatment set, a control sample (Before = "B") was inoculated and neutralized before water bath treatment to establish thermal effects during the come-up time when compared against the 0 min time point. To verify the efficacy of the neutralization buffer, controls were prepared by neutralizing the non-inoculated 500 ppm GSE solution and were included with the other samples in the water bath treatment. At the longest time point, the neutralization controls were removed and placed on ice for 3 min before inoculation with virus cocktail.

The sample sets that did not receive thermal treatment were performed at room temperature (20-25°C) for the same time points. GSE solution or citrate buffer was added to microcentrifuge tubes, inoculated with virus cocktail, and vortexed. Samples were allowed to sit for the same amount of time between inoculation and achievement of target temperature that was recorded for the thermal treatment samples. When this time was reached, another timer was started and the 0 min tubes were neutralized after 1.25 min. At each subsequent time point, corresponding samples were neutralized after 1.25 min.

A negative control and cytotoxicity control prepared with PBS instead of virus cocktail were included. Samples were stored in a -80°C freezer until analysis by plaque assay.

**Weibull Model**

The Weibull model was used for calculation of $t_D$ values, the time required to reduce the virus population by a factor of 10 (analogous to the D-value) at a specific temperature. In

contrast to the linear first-order kinetics model, the Weibull model assumes that the survival curve is a cumulative distribution of lethal effects. The model was applied as previously described by Bozkurt et al. (*14*). The $t_D$ value can be calculated by the equation,

$$t_D = \alpha(-\ln(10^{-D}))^{1/\beta}$$

where α ($min^{-1}$) is the scale parameter, β (-) is the shape parameter, and D represents decades (log) reduction of a microbial population. Once $t_D$ values were established, the z-value was calculated to determine the temperature required to decrease the $t_D$ value by a factor of 10.

**Statistical Analysis**

Linear regression analysis for calculation of DPPH radical scavenging activity $IC_{50}$ values and generation of graphs were performed using JMP Pro 11 (SAS Institute, Cary, NC). For thermal inactivation, non-linear regression analysis was performed using SPSS Statistics 23.0 (IBM, Armonk, NY). JMP Pro 11 was used for nonparametric one-way analysis of variance (Kruskal-Wallis). Significant differences between group means ($\alpha = 0.05$) were compared using the Tukey HSD test.

# CHAPTER 4

# RESULTS

## Antioxidant Activity Assays for Selection of Grape Seed Extracts

Due to its rich phenolic composition, GSE is recognized as having benefits as an antioxidant. To quantify its antioxidant activity, the radical scavenging activity (SA) was determined for commercial GSE powders using a range of GSE concentrations suspended in methanol as compared to L-ascorbic acid, which was used as a positive control. Figure 1 shows the relationship between concentration (mg/mL) and radical scavenging activity for three brands of commercial GSE powders and the L-ascorbic acid control. Linear regression was used for calculation of half maximal inhibitory concentration ($IC_{50}$) values, the concentration of GSE required to scavenge 50% of the DPPH radical (Table 1). The $IC_{50}$ values of the commercial GSE suspensions ranged from 0.094 to 0.105 mg/mL, none of which were significantly different from that of L-ascorbic acid (0.092 ± 0.009 mg/mL) ($p > 0.05$). The similarities in GSE and L-ascorbic acid $IC_{50}$ values further support the use of GSE as an antioxidant.

## Evaluation of Citrate Buffer for Suspension of Grape Seed Extract

Citrate buffer was selected for use as the solvent for GSE solution preparation as it could be induced to have a similar pH as several fruit juices; apple juice (pH 3.79), orange juice (pH 4.0), and grape juice (pH 3.65). However, it was important to determine if citrate buffer produced a similar temperature profile and come-up time as the juices when heated to temperatures of 56, 60, 65, and 72°C. Figure 2 is provided as an example of the typical heating profiles recorded for juices and citrate buffer. The come-up times for each juice at each

temperature are presented in Table 2 and compared with a representative citrate buffer preparation (pH 4.0). Although there was some variability in the come-up times recorded, a nonparametric one way analysis of variance test stratifying results by temperature revealed no significant difference between the juice and buffer come-up times. At each temperature, citrate buffer and apple juice had the shortest and most comparable come-up times. Orange juice had come-up times ranging from 7.4 to 19.3 seconds higher than citrate buffer, and grape juice exhibited the highest come-up times at each temperature.

In order to be used as a suspension medium for GSE, it was also important to test the potential for viral inactivation (measured by log PFU/ml reduction of MNV-1) by citrate buffers alone at varying acidities. Inactivation by each citrate buffer (pH 2.3, 3.0, and 4.0) was determined by comparison to its respective neutralization control, in which the citrate buffer was adjusted to pH 7.0 prior to addition of MNV-1. Log PFU/ml reductions of MNV-1 by citrate buffers are shown in Table 3. MNV-1 was reduced by less than 0.5 $\log_{10}$ PFU/ml by each buffer. No significant differences in mean log reductions of infectious MNV-1 were observed when citrate buffers were compared ($p > 0.05$).

**Effect of pH and Concentration on Antioxidant and Virucidal Efficacy of Grape Seed Extract Solutions**

Experiments preceding the current project demonstrated the virucidal efficacy of 200 ppm GSE suspended in sterile water (pH 6.25) after a 1-min contact time (data not shown). However, it was important to compare this performance with citrate buffer preparations with acidities similar to those of fruit juices.

Experiments were performed to compare the efficacy of varying concentrations (200, 500, and 800 ppm) of GSE solutions prepared with citrate buffers (pH 2.3 and pH 4.0) or sterile

water (pH 6.25) for inactivation of MNV-1 after a 1-min contact time. The virucidal efficacy of 200, 500, and 800 ppm GSE solutions prepared with citrate buffer (pH 2.3), citrate buffer (pH 4.0), and water (pH 6.25) are shown Table 4. All solutions of 200 ppm GSE gave significantly smaller $\log_{10}$ PFU/ml reductions of MNV-1 than 500 and 800 ppm solutions. At a concentration of 200 ppm, no inactivation of MNV-1 was observed when GSE was dissolved in citrate buffer (pH 2.3) and citrate buffer (pH 4.0). At 500 and 800 ppm concentrations, solutions prepared with citrate butter (pH 2.3) resulted in significantly smaller $\log_{10}$ PFU/ml reductions of MNV-1 than solutions prepared with citrate buffer (pH 4.0) and water ($p < 0.05$). GSE solutions prepared with water gave the largest $\log_{10}$ PFU/ml reductions of MNV-1 at every concentration. While $\log_{10}$ PFU/ml reductions of MNV-1 by solutions prepared with water were significantly larger than solutions prepared with citrate buffers at 200 and 800 ppm, results given by 500 ppm GSE in citrate buffer (pH 4.0) and 500 ppm GSE in water were not significantly different ($p > 0.05$). Overall, $\log_{10}$ PFU/ml reductions of MNV-1 increased with increasing concentration and solvent pH.

To support the results indicating an alteration of GSE virucidal efficacy by solvents of varying acidities, DPPH assays were performed to determine whether similar changes to antioxidant activity are shown by these GSE solutions. Results of % radical scavenging activity are presented in Table 5. At each concentration, the radical scavenging activities of the GSE solutions suspended in citrate buffer (pH 2.3) were significantly different from those suspended in citrate buffer (pH 4.0) and water ($p <0.05$). When using methanol as the solvent for GSE in preliminary DPPH antioxidant assays, a 200 ppm solution exhibited a 92.21 ± 1.88% radical scavenging activity (Figure 1). In preliminary DPPH experiments performed, the maximum observable radical scavenging activity was around 93-94%, exhibited by concentrations slightly

higher than 200 ppm (225-250 ppm). Therefore, 200 ppm GSE approached the limit of quantification and exhibited maximal radical scavenging activity when methanol was used as the solvent.

Results presented in Table 5 for solutions of 200 ppm GSE demonstrate the effect that solvent pH has on the radical scavenging activity of GSE. Each 200 ppm solution showed decreased radical scavenging activities when compared to methanolic GSE solutions, but to different degrees. When water was used as the solvent, the radical scavenging activity decreased by 8%. 200 ppm GSE solutions prepared with citrate buffer (pH 4.0) and citrate buffer (pH 2.3) were 24.75% and 47.48% lower than 200 ppm methanolic GSE solutions, respectively. Increasing the solution concentrations to 500 and 800 ppm revealed a decrease in radical scavenging activity when solutions were prepared with citrate buffer (pH 2.3). At a 500 ppm concentration, radical scavenging activities of GSE solutions prepared with citrate buffer (pH 2.3) were around 18-19% lower than solutions prepared with citrate buffer (pH 4.0) and water. Radical scavenging activity of 800 ppm GSE solutions suspended in citrate buffer (pH 2.3) were 6-7% lower than solutions prepared with citrate buffer (pH 4.0) and water at the same concentration. Radical scavenging activities of solutions prepared with citrate buffer (pH 4.0) and water may have been reduced to a degree as they were with 200 ppm solutions, however the concentrations were high enough that they exhibited maximal radical scavenging activity for the assay used. These results further support the assumption that solvent pH decreases the functionality of GSE as an antiviral and antioxidant agent. Figure 3 shows the relationship between percent radical scavenging activity and resulting $\log_{10}$ PFU/ml reduction of MNV-1 using all GSE solutions. In a test of multicollinearity, a pearson correlation coefficient of 0.792 was found for the two response variables, indicating a high degree of positive correlation

between radical scavenging activity and $\log_{10}$ PFU/ml reduction of MNV-1 ($p < 0.01$). These results indicate that the antioxidant activity of GSE may be a good indicator of its antiviral activity.

Prior to thermal inactivation experiments, experiments were performed to identify any potential thermal effects the heating process may have on the antioxidant activity of GSE. Samples of 500 ppm GSE in citrate buffer (pH 4.0) were heated at 72°C for 6 min and the radical scavenging activity was compared to that of unheated 500 ppm GSE samples. The radical scavenging activity of the unheated and heated samples was 94.22% and 94.23%, respectively, showing no significant difference ($p > 0.05$). This suggests that this specific heating process does not affect the antioxidant activity of GSE.

**Viral Inactivation by GSE and Thermal Treatments Used Alone and in Combination**

Prior to beginning thermal inactivation experiments, a DPPH antioxidant assay was used to determine the antioxidant activity of the prepared 500 ppm GSE solution. The radical scavenging activity for the 500 ppm GSE solutions used in all experiments was 94.82 ± 0.27%. Table 6 displays the log recoveries of infectious MNV-1 at time points in the thermal inactivation process with and without the addition of GSE. At 56°C, recovered MNV-1 titers following thermal treatment with and without the addition of GSE were significantly different at every time point ($p < 0.05$) except for the 5 min treatment time. At 60°C, the addition of GSE gave significantly different results than thermal treatment alone at 0 and 2 min time points. At increased temperatures of 65 and 72°C, there was no significant difference in recovered MNV-1 titers between treatments for all time points. Significant differences for a majority of the control samples (Control B) demonstrated the efficiency of GSE for inactivation of MNV-1 at room temperature. Control samples (Control B) that were not subjected to thermal treatment were

prepared in order to quantify viral inactivation during temperature come-up times when compared to the 0 min samples. During the experiments at 56°C, MNV-1 was reduced by 0.55 ± 0.05 $\log_{10}$ PFU/ml during the come-up time (93.5 ± 34.6 s). The addition of GSE resulted in a significant increase to 1.63 ± 0.30 $\log_{10}$ PFU/ml reduction during the come-up time to 56°C ($p < 0.05$). Thermal effects during come-up times to 60°C (79.0 ± 31.1 s) and 65°C (76.5 ± 27.6 s) resulted in MNV-1 reductions of 1.25 ± 0.30 and 2.70 ± 0.37 $\log_{10}$ PFU/ml, respectively. With the addition of GSE, viral inactivation during the come-up time to 60°C increased with a reduction of 1.84 ± 0.68 $\log_{10}$ PFU/ml. A 2.28 ± 0.11 $\log_{10}$ PFU/ml MNV-1 reduction was observed during the come-up time to 65°C when GSE was included in the treatment. Reductions in recovered MNV-1 titers during come-up times to 60 and 65°C following addition of GSE were not significantly different than reductions following thermal treatment alone ($\alpha = 0.05$). At 72°C, thermal effects during the come-up time (86.5 ± 2.1 s) resulted in a >5 $\log_{10}$ PFU/mL reduction of MNV-1 after thermal treatment alone and a complete inactivation of MNV-1 with the addition of GSE, showing no significant difference between treatments ($\alpha = 0.05$).

To best compare the thermal inactivation kinetics for MNV-1 with and without the addition of GSE, the data was fitted into a Weibull model. The treatment time of zero, the point in which the samples reach target temperature was used as the starting titer for reduction calculations. For each temperature, a $t_D$ value (analogous to a D-value) was calculated using the shape ($\beta$) and scale ($\alpha$) parameters, which describes the time needed to destroy 1 log (D=1) of MNV-1. Results for thermal inactivation experiments without and with the addition of GSE are presented in Tables 7 and 8, respectively. At 56°C, the $t_D$ value for thermal treatment alone was calculated to be 8.24 ± 0.07 min. When GSE was added to the process, the $t_D$ value decreased to 6.19 ± 3.73 min. A $t_D$ value of 2.39 ± 1.10 min was found for MNV-1 at 60°C. However, the

addition of GSE to a 60°C thermal treatment increased the $t_D$ value to 6.41 ± 5.46 min. At 65°C, thermal treatment alone produced a $t_D$ value of 0.59 ± 0.38 min and the combination of the treatments resulted in a decreased $t_D$ value of 0.45 ± 0.28 min. At each temperature, there was no significant difference between $t_D$ values with and without the addition of GSE ($p > 0.05$). As indicated by reported $R^2$ values, the addition of GSE to each thermal process resulted in atypical inactivation patterns. Instead of observing a gradual decrease along time points, the addition of GSE produced sporadic decreases and increases in $\log_{10}$ PFU/ml recoveries of MNV-1 along sequential time points. At 72°C, results from both replicates of thermal treatment alone showed a 1.00 ± 1.41 $\log_{10}$ PFU/mL recovery of MNV-1 at the point in which the samples reached 72°C (0 min) (Table 6). Later time points resulted in slightly higher log recoveries in some cases, preventing the calculation of an accurate $t_D$ value using the Weibull model. When GSE was added during the 72°C thermal treatment, no log recoveries of MNV-1 were observed at the 0 min time point, once again preventing the calculation of $t_D$ values.

A set of samples where MNV-1 was treated with 500 ppm GSE at room temperature for contact time intervals identical to those used for thermal treatments was performed. Table 9 lists the log recoveries of MNV-1 from room temperature (20-25°C) samples treated with GSE for intervals up to 80 min, the longest treatment time used in thermal inactivation experiments at 56°C. The samples are compared to the positive control, which was prepared with citrate buffer without the addition of 500 ppm GSE solution. The 'control (B)' samples had a contact time identical to the 'control (B)' samples from the thermal treatment sample set of the same replicate; the time between virus inoculation and insertion of samples into the water bath. This time averaged 3.65 ± 0.32 min from replicates of all temperatures. A previous experiment showed a 1.64 ± 0.22 $\log_{10}$ PFU/ml reduction of MNV-1 using 500 ppm GSE in citrate buffer

(pH 4.0) after 1 min of contact time (Table 4). When compared to the 1.72 ± 0.19 $\log_{10}$ PFU/ml reduction seen during this 3.65 ± 0.32 min contact time employed for the control (B) samples, it seems that the majority of the immediate inactivation happens within the first min of contact time. As the contact time increases, gradual inactivation continues to occur. At 80 min of contact time, MNV-1 was reduced by 3.01 ± 0.19 $\log_{10}$ PFU/ml. Results from Weibull model analysis of MNV-1 inactivation by GSE at room temperature is presented in Table 8. After the initial 1-2 log reduction observed within the first few min of contact time, 15.59 ± 2.38 min were required to achieve another 1 log reduction of MNV-1.

Z-values for MNV-1 from thermal treatment with and without the addition of GSE were calculated from $t_D$ values. The z-values for thermal treatment alone and thermal treatment with GSE are 7.77 ± 1.91°C and 8.80 ± 4.51°C, respectively. There is no significant difference between the two treatments ($p > 0.05$).

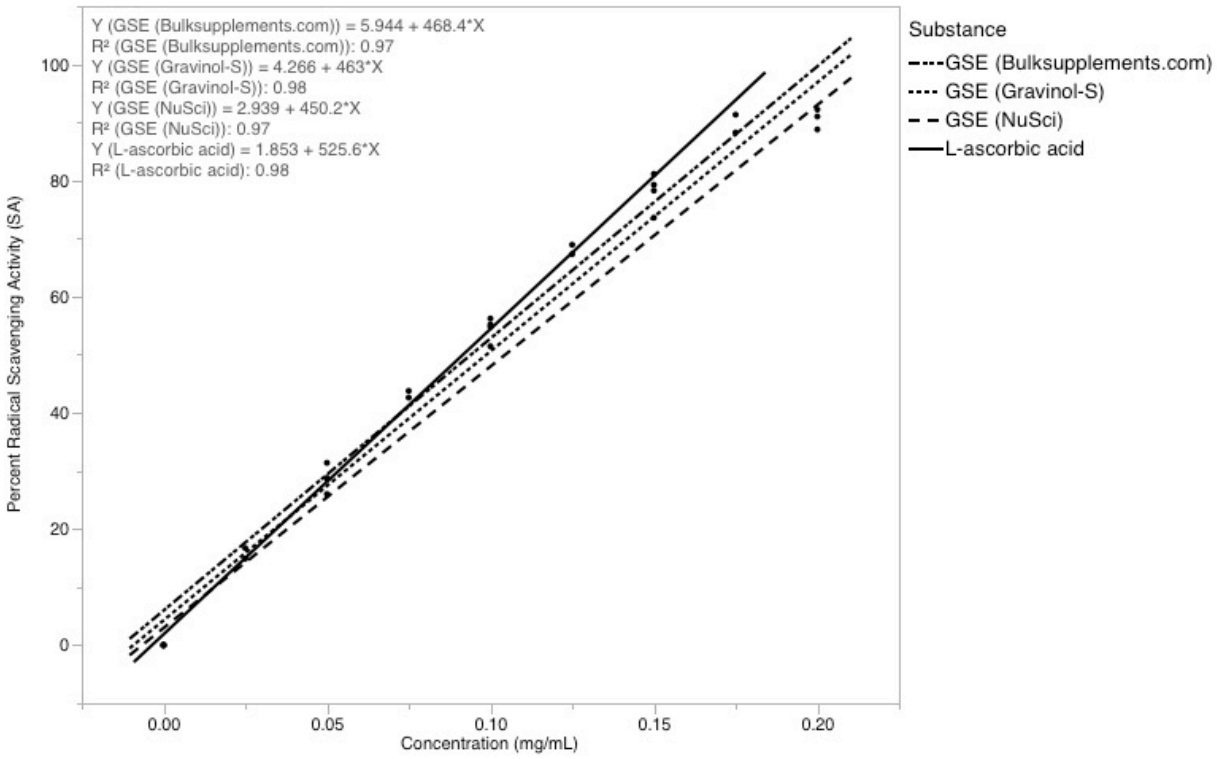


**Figure 1.** Percent radical scavenging activity of different grape seed extract brands and L-ascorbic acid at concentrations from 0.0 – 0.20 mg/mL.

**Table 1.** Half maximal inhibitory concentration ($IC_{50}$) values of L-ascorbic acid and commercial grape seed extract powders of different brands.

| **Grape Seed Extract Brand** | **$IC_{50}$ ± SD (mg/ml)** |
|---|---|
| Bulksupplements.com | $0.094 \pm 0.007^{a}$ |
| Gravinol-S | $0.099 \pm 0.006^{a}$ |
| NuSci | $0.105 \pm 0.010^{a}$ |
| L-Ascorbic Acid | $0.092 \pm 0.009^{a}$ |

[a]Superscript means with different letters are significantly different ($\alpha = 0.05$).

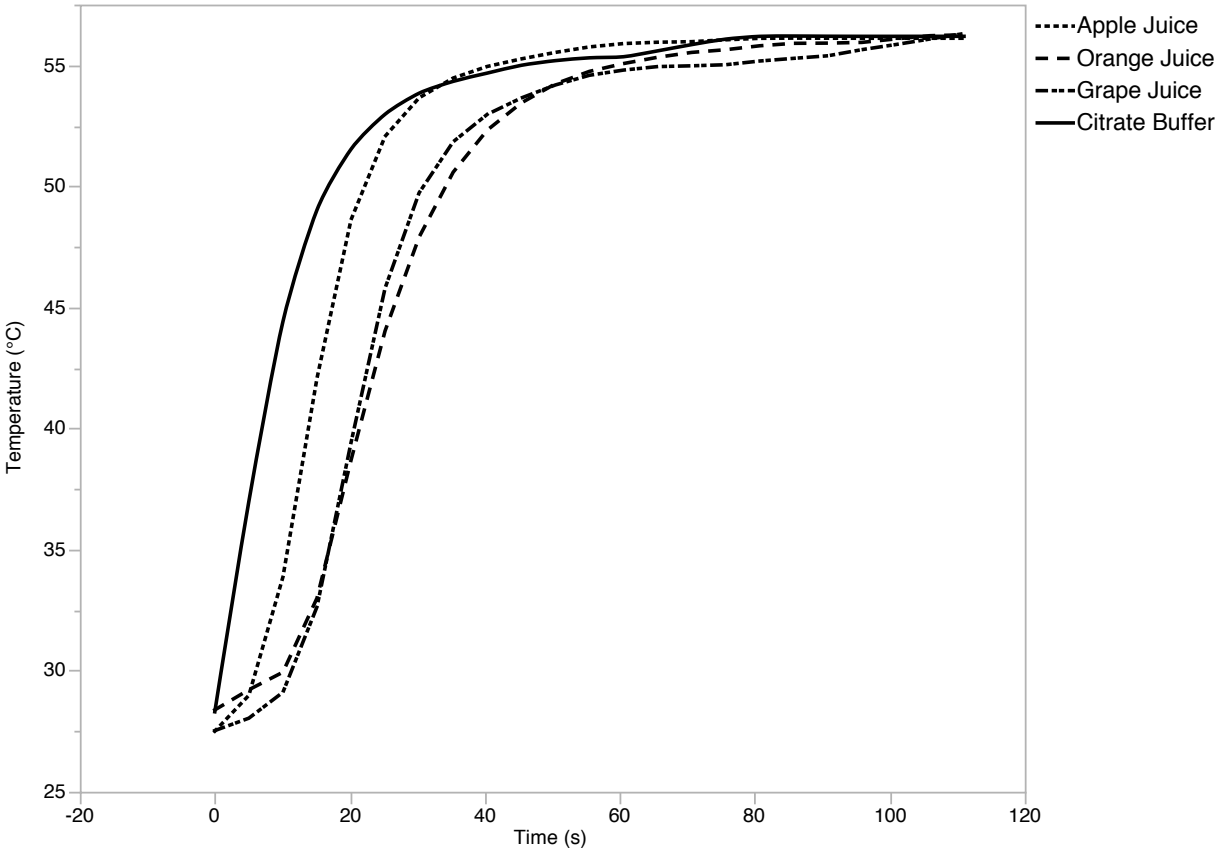


**Figure 2.** Heating profiles of juices and citrate buffer at 56°C.

**Table 2.** Come-up times of citrate buffer and various juices at 56, 60, 65, and 72°C.

| Temperature (°C) | Substance | Come-up Time ± SD (sec) |
|---|---|---|
| 56 | Apple Juice, pH 3.79 | 85.8 ± 38.6[a] |
| | Orange Juice, pH 4.0 | 96.1 ± 41.5[a] |
| | Grape Juice, pH 3.65 | 111 ± 27.3[a] |
| | Citrate buffer, pH 4.0 | 80.3 ± 36.1[a] |
| 60 | Apple Juice, pH 3.79 | 67.9 ± 25.8[b] |
| | Orange Juice, pH 4.0 | 86.0 ± 24.7[b] |
| | Grape Juice, pH 3.65 | 103 ± 33.0[b] |
| | Citrate buffer, pH 4.0 | 66.7 ± 36.2[b] |
| 65 | Apple Juice, pH 3.79 | 68.4 ± 28.7[c] |
| | Orange Juice, pH 4.0 | 79.6 ± 29.0[c] |
| | Grape Juice, pH 3.65 | 80.0 ± 19.7[c] |
| | Citrate buffer, pH 4.0 | 68.3 ± 19.0[c] |
| 72 | Apple Juice, pH 3.79 | 92.7 ± 32.5[d] |
| | Orange Juice, pH 4.0 | 97.2 ± 32.9[d] |
| | Grape Juice, pH 3.65 | 101 ± 31.9[d] |
| | Citrate buffer, pH 4.0 | 89.8 ± 26.4[d] |

[a,b,c,d]Superscript means with different letters in the same column at the same temperature are significantly different ($\alpha = 0.05$).

**Table 3.** $Log_{10}$ PFU/mL reduction of MNV-1 with citrate buffers.

| Solvent | $Log_{10}$ Reduction ± SD (PFU/mL) | Contact Time (min) |
|---|---|---|
| CB[β], pH 2.3 | 0.12 ± 0.15[a] | 80 |
| CB, pH 3.0 | 0.05 ± 0.10[a] | 5 |
| CB, pH 4.0 | 0.29 ± 0.22[a] | 5 |

[β]CB = citrate buffer

[a]Superscript means with different letters are significantly different ($\alpha = 0.05$).

**Table 4.** $Log_{10}$ PFU/mL reductions of MNV-1 by GSE solutions prepared with citrate buffer (pH 2.3 and 4) and water at different concentrations.

| Concentration (ppm) | Solvent | $Log_{10}$ Reduction ± SD (PFU/mL) | P-value |
|---|---|---|---|
| 200 | CB[β], pH 2.3 | -0.21 ± 0.12[a] | 0.0218 |
| | CB, pH 4.0 | -0.27 ± 0.14[a] | |
| | Water[γ] | 0.93 ± 0.49[b] | |
| 500 | CB, pH 2.3 | 0.05 ± 0.43[a] | 0.0231 |
| | CB, pH 4.0 | 1.64 ± 0.22[b] | |
| | Water | 1.98 ± 0.52[b] | |
| 800 | CB, pH 2.3 | 0.70 ± 0.22[a] | 0.0073 |
| | CB, pH 4.0 | 1.79 ± 0.12[b] | |
| | Water | 2.38 ± 0.37[c] | |

[β]CB = citrate buffer
[γ]Water = pH 6.25
[a,b,c]Superscript means with different letters in the same column at the same concentration are significantly different (α = 0.05).

**Table 5.** Percent radical scavenging activity of GSE solutions prepared with citrate buffer (pH 2.3 and 4) and water at different concentrations.

| Concentration (ppm) | Solvent | %SA ± SD | P-value |
|---|---|---|---|
| 200 | CB[β], pH 2.3 | 44.73 ± 0.31[a] | 0.0273 |
| | CB, pH 4.0 | 67.46 ± 0.82[b] | |
| | Water[γ] | 84.20 ± 12.56[b] | |
| 500 | CB, pH 2.3 | 75.19 ± 0.71[a] | 0.0273 |
| | CB, pH 4.0 | 94.54 ± 0.14[b] | |
| | Water | 93.59 ± 0.39[b] | |
| 800 | CB, pH 2.3 | 87.80 ± 0.21[a] | 0.0321 |
| | CB, pH 4.0 | 95.01 ± 0.26[b] | |
| | Water | 93.82 ± 0.78[b] | |

[β]CB = citrate buffer
[γ]Water = pH 6.25
[a,b]Superscript means with different letters in the same column at the same concentration are significantly different (α = 0.05).

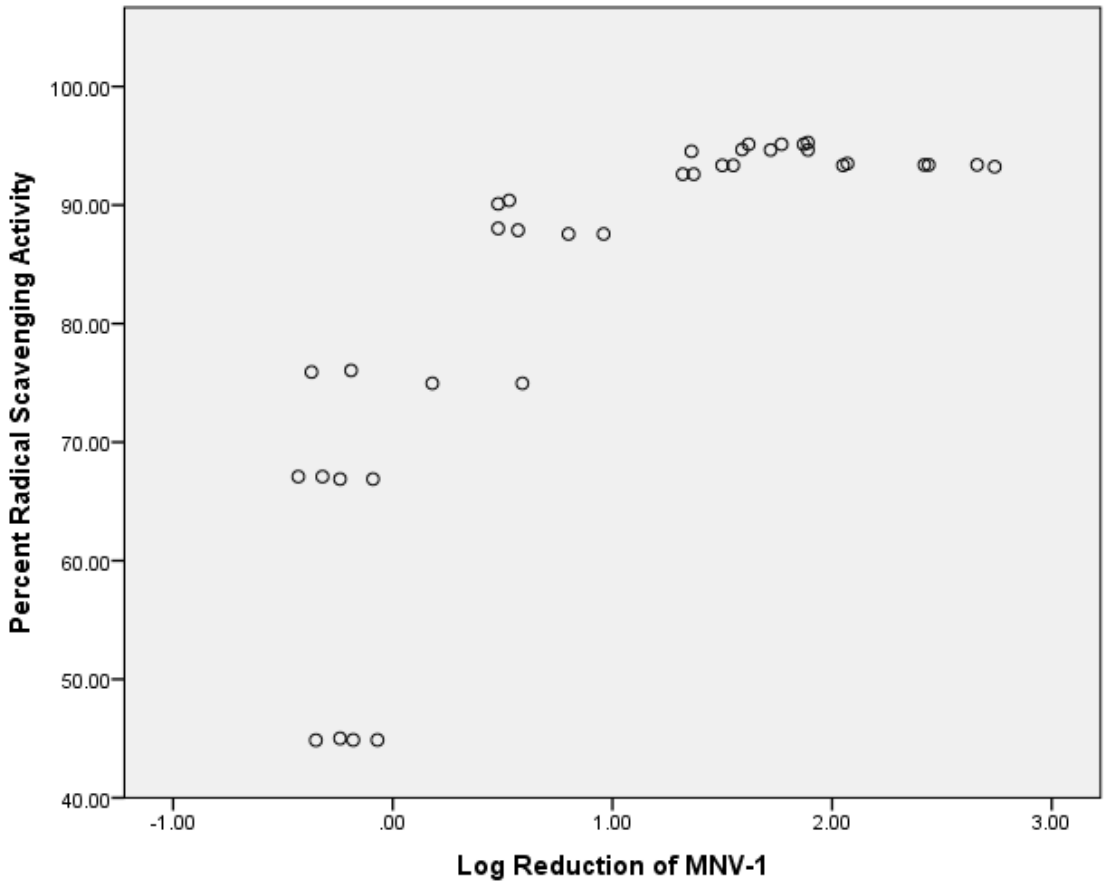


**Figure 3.** Relationship between percent radical scavenging activity and $\log_{10}$ PFU/ml reduction of MNV-1 for all GSE solutions. Pearson correlation = 0.792 ($p < 0.01$)

**Table 6.** $Log_{10}$ PFU/mL recoveries of MNV-1 following thermal treatment with and without the addition of GSE.

| Temp (°C) | Come-up Time ± SD (sec) | Treatment | Recovered titer log (PFU/ml) (- GSE) | Recovered titer log (PFU/ml) (+ GSE) |
|---|---|---|---|---|
| 56 | 93.5 ± 34.6 | Control (Before= B) | 6.25 ± 0.01[a] | 4.72 ± 0.04[b] |
| | | 0 min | 5.71 ± 0.04[a] | 3.09 ± 0.34[b] |
| | | 1.5 min | 5.52 ± 0.11[a] | 2.88 ± 0.04[b] |
| | | 2 min | 5.34 ± 0.02[a] | 3.11 ± 0.42[b] |
| | | 2.5 min | 5.34 ± 0.20[a] | 2.17 ± 0.01[b] |
| | | 5 min | 5.08 ± 0.14[a] | 3.70 ± 0.98[a] |
| | | 7.5 min | 4.93 ± 0.04[a] | 0.77 ± 1.09[b] |
| | | 10 min | 4.59 ± 0.24[a] | 1.69 ± 0.55[b] |
| 60 | 79.0 ± 31.1 | Control (B) | 6.26 ± 0.02[a] | 4.70 ± 0.06[b] |
| | | 0 min | 5.01 ± 0.32[a] | 2.86 ± 0.62[b] |
| | | 0.5 min | 4.75 ± 0.25[a] | 2.97 ± 1.09[a] |
| | | 1 min | 4.46 ± 0.01[a] | 2.41 ± 0.95[a] |
| | | 2 min | 4.00 ± 0.21[a] | 2.26 ± 0.33[b] |
| | | 3 min | 3.64 ± 0.09[a] | 1.65 ± 0.66[a] |
| | | 7 min | 3.07 ± 0.23[a] | 2.40 ± 0.93[a] |
| 65 | 76.5 ± 27.6 | Control (B) | 6.60 ± 0.85[a] | 4.57 ± 0.38[a] |
| | | 0 min | 3.91 ± 0.47[a] | 2.29 ± 0.27[a] |
| | | 0.25 min | 3.50 ± 0.03[a] | 3.25 ± 0.96[a] |
| | | 0.5 min | 3.19 ± 0.74[a] | 2.00 ± 0.56[a] |
| | | 0.75 min | 2.99 ± 0.42[a] | 2.86 ± 1.35[a] |
| | | 1 min | 2.04 ± 0.22[a] | 1.74 ± 2.46[a] |
| 72 | 86.5 ± 2.1 | Control (B) | 6.36 ± 0.45[a] | 4.14 ± 0.06[b] |
| | | 0 min | 1.00 ± 1.41[a] | 0.00 ± 0.00[a] |
| | | 3 sec | 1.37 ± 1.94[a] | 1.38 ± 0.96[a] |
| | | 5 sec | 0.35 ± 0.49[a] | 0.80 ± 1.13[a] |
| | | 10 sec | 2.60 ± 0.43[a] | 1.19 ± 1.68[a] |
| | | 15 sec | 1.21 ± 1.70[a] | 0.59 ± 0.83[a] |
| | | 20 sec | 1.37 ± 1.94[a] | 0.70 ± 0.99[a] |
| | | 25 sec | 0.35 ± 0.49[a] | 0.00 ± 0.00[a] |

[a]Superscript means with different letters in the same row at the same temperature are significantly different ($\alpha = 0.05$).

**Table 7.** Weibull model coefficients for the survival curves of MNV-1 during thermal inactivation alone.

| Temp (°C) | β | α (min) | $t_{D=1}$ | $R^2$ |
|---|---|---|---|---|
| 56 | 0.70 ± 0.02[γ] | 2.49 ± 0.06[γ] | 8.24 ± 0.07[γ] | 0.98 |
| 60 | 0.67 ± 0.06 | 0.71 ± 0.38 | 2.39 ± 1.10 | 0.99 |
| 65 | 1.10 ± 0.63 | 0.33 ± 0.34 | 0.59 ± 0.38 | 0.94 |

[γ]Shape (β), scale (α), and $t_D$ values are the mean ± standard deviation of 2 experimental replicates.

**Table 8.** Weibull model coefficients for the survival curves of MNV-1 during thermal inactivation with the addition of 500 ppm GSE.

| Temp (°C) | β | α (min) | $t_{D=1}$ | $R^2$ |
|---|---|---|---|---|
| 56 | 1.26 ± 0.78[γ] | 4.01 ± 4.14[γ] | 6.19 ± 3.73[γ] | 0.69 |
| 60 | 0.90 ± 0.52 | 3.12 ± 3.59 | 6.41 ± 5.46 | 0.65 |
| 65 | 9.08 ± 11.60 | 1.07 ± 1.16 | 0.45 ± 0.28 | ---[ε] |
| Room Temp[δ] | 0.34 ± 0.12 | 2.35 ± 1.16 | 15.59 ± 2.38 | 0.98 |

[γ]Shape (β), scale (α), and $t_D$ values are the mean ± standard deviation of 2 experimental replicates.
[ε]$R^2$ could not be generated for one replicate.
[δ]Room temp = 20-25°C

**Table 9.** $Log_{10}$ PFU/mL recoveries of MNV-1 following GSE treatment at room temperature.

| Treatment | Recovered titer log ± SD (PFU/ml) |
|---|---|
| Positive Control | 6.19 ± 0.24 |
| Control (B)[γ] | 4.48 ± 0.20 |
| 0 min | 4.53 ± 0.26 |
| 10 min | 4.00 ± 0.14 |
| 20 min | 3.80 ± 0.25 |
| 40 min | 3.70 ± 0.18 |
| 60 min | 3.45 ± 0.13 |
| 80 min | 3.23 ± 0.26 |

[γ]Time from inoculation until B: 3.65 ± 0.32 min

# CHAPTER 5

# DISCUSSION

Grape seed extract is largely composed of phenolic compounds such as gallic acid, monomeric anthocyanins and catechins, and dimeric, trimeric, and polymeric proanthocyanidins. All phenolic compounds contain an aromatic ring with one or more hydroxyl groups. Because of the ability to donate hydrogen atoms from the multiple hydroxyl groups as well as to quench singlet oxygen, phenolic compounds possess high antioxidant potentials (*102*). GSE is therefore widely recognized as possessing benefits as an antioxidant.

The current study reported half maximal inhibitory concentrations ($IC_{50}$) as 0.094 to 0.105 mg/mL for commercial GSE powders. Studies have reported $IC_{50}$ values for grape seed extracts produced by various extraction methods. The $IC_{50}$ values for *Vitis vinifera* seed extracts have been reported as 0.15 ± 0.10 mg/mL and 0.10 ± 0.02 mg/mL (*72*, *77*). These values were found when extractions from grape seeds were performed using ethanol. When ethyl acetate was used for extraction, a much lower $IC_{50}$ value was found (0.06 ± 0.01 mg/mL). This lower value correlated with higher contents of polyphenols and flavan-3-ols in the ethyl acetate extractions when compared to the ethanolic extractions (*77*). Another study reported the $IC_{50}$ value of an ethanolic extract of *Vitis vinifera* seeds as 0.07 mg/mL (*113*). When a methanolic extraction procedure was performed on *Vitis labrusca* seeds, an $IC_{50}$ value of 0.06 ± 0.00 mg/mL was reported (*87*). Results from the current study show antioxidant activity similarities between commercial GSE powders and ethanolic extracts of *Vitis vinifera* seeds. No alternative studies have reported antioxidant activity $IC_{50}$ values of commercial GSE powders.

Multiple considerations must be taken when selecting a buffer for suspension of GSE to be used for viral inactivation experiments. One important such consideration is the buffer's pH. The control of pH has historically been used for inactivation and growth prevention of microorganisms in foods and beverages. Therefore, it was important to consider how a buffer's pH may influence viral inactivation. With inactivation of MNV-1 at levels below 0.5 $\log_{10}$ PFU/ml after 1 min of contact time with citrate buffers at pH 2.3 and 4.0, buffer acidity showed minimal effect on viral inactivation. These results are comparable to a previous study in which MNV was reduced by 0.68 ± 0.46 and 0.27 ± 0.35 $\log_{10}$ PFU/mL after 30 min incubation at room temperature (20-25°C) in citrate buffers of pH 2.0 and 4.0, respectively (*51*). Similarly, incubation of MNV-1 at 37°C for 30 min with citrate buffers of the same acidities resulted in 0.6 and 0.5 $\log_{10}$ PFU/mL reductions, respectively (*21*).

Results from experiments using GSE solutions prepared with citrate buffer (pH 2.3 and 4.0) and water demonstrated the loss of antioxidant and virucidal activity of GSE in a low pH environment. The antioxidant activity and stability of polyphenolic compounds is affected by the pH of the surrounding environment (*43*). Previous studies have noted that alterations in pH result in changes in dissociation rates of oxygen-containing chemical groups in anthocyanins and hydroxyflavones (*12*, *68*). It is likely that the radical scavenging activity of GSE polyphenols may be influenced by pH in a similar manner. A study reported that the antioxidant activity of palm wine and palm vinegar increases as pH increased (*42*). Another study observed that the radical scavenging capacity of catechins significantly increases with increasing pH. As epicatechin, gallocatechin, epigallocatechin, and epicatechin 3-*O*-gallate are a few of the major polyphenols contained within grape seeds, these results are comparable to what was observed in the current study (*83*). The effect of pH on the antibacterial/virucidal activity of polyphenol-

containing antimicrobials is not clearly defined in the literature. However, the correlation between radical scavenging activity and virucidal activity of GSE when suspended in different pH citrate buffers provides further support for the ability to use antioxidant activity as an indicator of virucidal activity of GSE.

Experiments in the current study found no thermal degradation of GSE based on unchanged radical scavenging activity of unheated GSE samples and GSE samples heated to 72°C for 6 min. Similarly, a previous study found that 100°C furnace treatment for 15, 30, and 60 min did not affect antioxidant activity, polyphenol content, tannin content, and procyanidin components of GSE (*23*). Another study reported antioxidant activity of GSE in cooked pork patties (180°C for >8 min), further demonstrating the thermal stability of GSE (*22*).

Thermal inactivation experiments with and without the addition of GSE allowed for calculation of $t_D$ values for MNV-1 at 56, 60, and 65°C using parameters generated by the Weibull model. The Weibull distribution corresponds to a concave downward survival curve if $\beta>1$, a concave upward curve if $\beta<1$, and follows an exponential distribution if $\beta=1$. In other words, a shape factor of >1 means the remaining microbial population becomes increasingly damaged, and a shape factor <1 means the remaining population is adapting to the applied stress (*117*). The scale parameter, $\alpha$, describes the mean of the distribution of death times of each microorganism in the microbial population, indicating the effect of the heating environment on inactivation. Results from this study fall within the ranges of decimal reduction times presented for the same temperatures by multiple previous studies.

Past thermal inactivation studies of NoV surrogates have reported varying D-values for MNV-1. These deviations may be the result of experimental factors such as sample size, sample container, sample matrix, and heating method. At 56°C in cell culture media, studies found the

time taken to achieve a 1 log reduction of MNV-1 to range from 3.47 to 12.39 min when using a circulating water bath as the method of thermal inactivation. The study presenting 3.47 min as the D-value for MNV-1 used 50 µL of sample contained within capillary tubes, while the study presenting the higher D-value treated 100 µL of sample in an undefined reaction vessel (*21*, *120*). In two sequential studies, MNV-1 was treated in cell culture media at volumes of 50 µL in capillary tubes and 2 mL in glass screw-capped vials. Thermal treatment at 56°C gave a 3.65 min D-value when the capillary tube method was used as compared to a 3.74 min D-value for 2 mL samples (*14*, *15*).

Another study investigated the effect of change in sample matrix on inactivation kinetics of MNV-1. A comparison of thermal inactivation at 56°C between 500 µL samples of cell culture media with and without the addition of stool gave D-values of 4.21 and 1.06 min, respectively (*116*). A more complex sample matrix does not always result in quicker inactivation times, as D-values at 56°C for spinach, mussel, and turkey deli meat were reported as 3.29, 6.12, and 7.30 min, respectively (*16*, *17*, *18*). When using a PCR machine as the method of thermal treatment, a D-value of 1.18 min was found for MNV-1 at 55°C (*51*). A D-value of 0.57 min was reported when 60°C heat treatment using a water bath was applied to 50 µL cell culture media samples in capillary tubes (*15*). When sample size was increased to 2 mL, D-values of 1.09 and 7.79 min were found when 2 ml glass vials and 15 mL conical tubes were used, respectively (*14*, *88*). Whether this discrepancy stems from differences in container material (i.e. glass or polymer) or container size, this difference suggests that the treatment vessel is a source of variation in thermal inactivation studies.

A D-value of 0.64 min was reported for a 60°C thermal treatment using a PCR machine (*51*). 60°C treatment of spinach, turkey deli meat, mussel, and dried mussel samples resulted in

D-values of 0.98, 2.74, 2.64, and 9.01 min, respectively. At 65°C, similar D-values of 0.4, 0.41, and 0.44 min were reported for spinach, mussel, and raspberry puree samples, respectively (*6*, *16*, *17*, *18*, *88*). When cell culture media samples were used, D-values ranged from 0.3 min to 0.77 min for 50 µL and 2 mL samples, respectively (*14*, *15*). At 72°C, D-values from treatment with the capillary tube method range from 0.15 min for 50 µL samples to 0.72 min for 80 µL samples (*3*, *14*). At the same temperature, D-values from treatment of food samples are reported as 0.16, 0.22, and 0.5 min for spinach, turkey deli meat, and milk, respectively (*16*, *17*, *49*).

Decimal reduction times found in the current study for 56, 60, and 65°C treatment alone falls within the range of previously reported D-values at the same temperatures. With $t_D$ values of 8.24 ± 0.07 and 2.39 ± 1.10 min for 56 and 60°C, the times taken to achieve a 1 log reduction of MNV-1 fall on the higher end of reported D-values. These higher times are likely due to the relatively large container used for treatment of 300 µL samples. As the thermal conductivity of air is less than that of liquids, the heat transfer may be less efficient when there is a more headspace within the tube. At 20°C, the thermal conductivity of air is 0.026 W $m^{-1}$ $K^{-1}$, which is lower than that of citric acid solutions which can range from 0.209 to 0.598 W $m^{-1}$ $K^{-1}$, depending on concentration (*2*, *25*). This difference in thermal conductivity affects heat conduction of the sample and a larger headspace can results in more heat loss to the lid.

A study investigated the influence of heat transfer within mashed potato samples within tubes of varying sizes on thermal inactivation of *Escherichia coli*. The come-up time to 60°C using capillary tubes was 0.27 min, and the resulting survival curves of *E. coli* K-12 followed first-order kinetics. For the larger 13 and 20 mm tubes, the come-up times to 60°C were 3 and 7 min, respectively. The survival curves showed a broad shoulder of minimal inactivation during the initial treatment times after the target temperature was reached. After a period of around 2

min for the 13 mm tubes and 4 min for the 20 mm tubes, the survival curve began to follow a log linear pattern. The D-values for samples in the capillary tubes were 1.6 to 4.5 times smaller at every temperature than were reported for the larger tubes (*25*). Although this study uses solid media and come-up times are longer than have been found for GSE in citrate buffer samples (Table 6), similar trends between different tube sizes have been observed for bacteria in liquid samples such as milk and saline (*32*, *107*).

Container and/or sample size may affect the heat transfer rate of the sample, causing differences in the time to reach the target temperature (come-up time) (*25*). For this reason, it is important to consider the come-up time in thermal inactivation studies. The come-up times reported from Bozkurt et al. for 56, 60, 65, and 72°C were 66, 116, 142, and 158 s, respectively. The discrepancies in come-up times between the present study and those reported by Bozkurt et al. may be attributed to sample size or differences in thermocouples used. With such a small sample size for the vial used (300 µL sample in a 2 mL vial), a slight change in thermocouple position may have been a cause of variations between come-up times of replicates in the present study. In some cases, the sample would slightly evaporate and condense within the vial, which may cause come-up time differences. The contribution of thermal inactivation during the come-up time is an important consideration for design of a thermal process to achieve a desired reduction in a target microbial population. Therefore, the contribution of the come-up time ($t_c$) can be calculated from the equation, $0.4*t_c$ (in min) and added to the calculated D-value for a particular temperature (*7*).

Z-values reported in this study for MNV-1 after thermal treatment alone and thermal treatment with the addition of GSE were 7.77 ± 1.91°C and 8.80 ± 4.51°C, respectively. Previous studies have reported z-values for MNV-1 in cell culture media to be 9.31°C and 10.37°C (*14*,

*15*). Reported z-values for MNV-1 in mussel, spinach, and turkey deli meat samples were 11.62, 10.98, and 12.83 (*16*, *17*, *18*).

The calculation of $t_D$ values for MNV-1 at 72°C was not possible due to atypical fluctuations in inactivation levels across sequential time points. These results may have been due to experimental error. Fluctuations may have been caused by some samples being more submerged in the water bath than others, causing differences in the inactivation rate. Also, samples could have been placed on ice at varying depths, causing some samples to cool down faster than others. It is also possible that the fluctuations were the result of normal variations that are seen from plaque assay results when samples contain a low concentration of virus. With higher concentrations of virus, normal variations in plaque assay results appear small and are less apparent. With low virus concentrations, results may approach the limit of quantification for the plaque assay. With the small amount of plaques appearing on plates, variations in number of plaques result in log recoveries that appear to have much greater deviations. Therefore, these fluctuations may be typical for what is seen for low virus concentrations and may not have been due to experimental error. Using the $t_D$ value for 56°C and the calculated z-value, it can be predicted that the $t_D$ value for 72°C would be around 5 seconds.

Thermal treatment alone resulted in fairly steady decreases in log recoveries of MNV-1 over time at 56 ($R^2$=0.98), 60 ($R^2$=0.99), and 65°C ($R^2$=0.94). However, the addition of GSE to the thermal processes caused atypical fluctuations for all temperatures studied. Both treatments were performed at the same time and vials for identical time points from each treatment were situated adjacent to one another in the water bath and removed and placed on ice at the same time. For this reason, it is likely that fluctuations in log reduction resulted directly from the addition of GSE to the samples. It is possible that GSE does not fully inactivate MNV-1 virus

particles, but may cause them to aggregate, preventing attachment to and infection of RAW 264.7 cells. If this is the case, the aggregation of virus particles may serve as protection during thermal treatment, causing inconsistent inactivation across the process time span. A similar phenomenon was seen in a study that treated poliovirus with formaldehyde followed by subsequent heat treatment at 50°C, in which an evident reduction in the rate of inactivation was observed as the process time increased (*11*, *108*). One possible rationalization offered was that virus particles might have been protected from heat treatment by aggregation. Certain compounds such as chemicals in environmental water samples have been shown to cause the aggregation or deaggregation of virus particles, leading to a respective decrease or increase in infective virus units (*38*, *41*). Furthermore, it has been established that virus aggregation may enhance or decrease efficiency of chemical and physical treatments such as disinfection and UV treatment (*8*, *37*).

When 500 ppm GSE was allowed to react with MNV-1 at room temperature, a rapid inactivation of MNV-1 by 1-2 $\log_{10}$ PFU/ml was observed within the first few min of contact time with an additional 1 log of inactivation occurring after 15.59 ± 2.38 min. A previous study found 0.5 mg/ml (500 ppm) GSE to reduce high titer MNV-1 (~7 $\log_{10}$ PFU/mL) by 0.72 $\log_{10}$ PFU/mL after incubation for 2 h at room temperature, while low titer MNV-1 (~ 5 $\log_{10}$ PFU/mL) was reduced by 1.48 $\log_{10}$ PFU/mL (*110*). Another study found 0.5 mg/mL GSE to reduce MNV-1 titers by 1.05 and 3.08 $\log_{10}$ PFU/mL after incubation for 24 h at room temperature and 37°C, respectively. GSE at concentrations of 1, 2 and 4 mg/mL in apple juice (pH 3.6) reduced MNV-1 to undetectable levels after 30 min. When 1, 2, and 4 mg/mL GSE were prepared in neutralized apple juice, MNV-1 reductions after 30 min were 0.89, 0.89, and 0.91 $\log_{10}$ PFU/mL, respectively. However, an increase to 3 h of incubation time resulted in

undetectable levels of MNV-1 for all concentrations. GSE prepared in 2% milk caused no significant reduction in MNV-1 titers after 24 h (*55*). An alternate study found similar results after adding dried milk to a 0.2 mg/mL GSE solution. A 0.2 mg/mL GSE solution prepared in PBS reduced MNV-1 by >3 $\log_{10}$ PFU/mL after incubation at 37°C for 1 h. As no significant reduction was observed by 0.2 mg/mL GSE solution prepared in dried milk, the researchers concluded milk proteins were most likely interfering with the anti-NoV effects of GSE (*70*).

To understand the mechanism of GSE on MNV-1, a previous study treated RAW 264.7 cells with GSE pre- and post-infection to define the effect on viral adsorption and replication, respectively. With resulting MNV-1 reductions of 0.23 and 0.50 $\log_{10}$ PFU/mL, it was concluded that GSE has only minor effects on both mechanisms, but the greater effect was on viral adsorption (*110*). It was suggested that GSE might block either host cell receptors or viral binding sites. With relatively small log reductions compared to direct contact of GSE with the virus for 2 h (4.10 $\log_{10}$ PFU/mL reduction with 500 ppm GSE), it is likely that the primary mechanism is through direct alteration of virus particles with GSE. This observation supports the hypothesis that GSE may be blocking viral binding sites through coating of the virus particles or through triggering viral particle aggregation.

Grape seed phenolic compounds have a high affinity for some proteins and certain protein building blocks such as proteins rich in proline (*102*). Hence, it is expected that GSE would bind viral capsid proteins. The effect of GSE on the morphology of human NoV GII.4 VLPs was investigated by examination of VLPs with transmission electron microscopy before and after treatment with GSE (*70*). Treatment with 0.2 mg/mL (200 ppm) GSE caused VLPs to clump together and larger particles appeared to be deformed and inflated. At a concentration of 2 mg/mL (2000 ppm), the spherical structure of VLPs was destructed and a large amount of

protein debris was observed. These results serve as evidence that GSE directly targets the NoV capsid protein, causing aggregation at lower concentrations and further damage as dose increases. The size, shape, and viral capsid proteins of MNV-1 are analogous to human NoVs, therefore the mechanism of inactivation by GSE should be the same for MNV-1.

In order to compare the applicability of MNV-1 as a surrogate for human NoV in studies of efficiency of GSE as an antiviral, the researchers ran an assay to measure the specific binding ability of human NoV after GSE treatment. Binding levels of GII.4 NoVs to Caco-2 cells, which express H types 1 and 2 of HBGAs, were reduced by 1 and 2 log genomic compies/mL after treatment with 0.2 and 2 mg/mL GSE, respectively, when measured by RT-PCR. This difference in reduction, when compared to a >3 $\log_{10}$ PFU/mL in MNV-1 after treatment with 0.2 mg/mL GSE, indicates that MNV-1 may be more susceptible to GSE than human NoV. However, the cell-binding RT-PCR only measures NoV binding ability, whereas plaque assays measures infectivity of MNV-1. Treatment with GSE may have caused a larger proportion of GII.4 NoV to lose their infectivity and still retained their ability to bind (*70*).

This study is successful in showing the efficacy of two separate treatments for the inactivation of MNV-1. Although no significant increase in inactivation was observed when thermal treatment and GSE were combined, insight is given into the antioxidant and virucidal mechanism of GSE and its behavior in different conditions. The functionality loss of GSE was observed in low pH environments, and a protective effect for MNV-1 by GSE during thermal treatment is suggested. Overall, this study contributes data for the development of thermal and antimicrobial treatments for the inactivation of NoV in juices.

# CHAPTER 6

# CONCLUSIONS

This study was conducted to expand on the knowledge of thermal inactivation kinetics of a human NoV surrogate and to further characterize whether the addition of GSE, a successful antiviral, to the thermal process produces synergistic effects for improved viral inactivation. Citrate buffer was chosen for suspension of GSE to represent a model juice system. Experiments using citrate buffer (pH 2.3 and 4.0) and water for suspension of GSE revealed a significant decrease in virucidal and antioxidant functionality of GSE at a low pH (pH 2.3). 500 ppm GSE suspended in citrate buffer (pH 4.0) was shown to be an effective treatment method for reduction of MNV-1 and exhibits high antioxidant activity, further establishing its use as a food additive.

Thermal treatment alone was effective for inactivation of MNV-1 at temperatures of 56, 60, 65, and 72°C. The juice hazard analysis and critical control point (HACCP) regulation requires a process to achieve a 5-log reduction in the most pertinent pathogen (*86*). High temperature short time pasteurization (HTST) applies a 72°C heat treatment for 15 seconds. Regular batch pasteurization applies 63°C heat treatment for 30 minutes. These pasteurization processes are generally effective for reduction of NoV, although few studies have shown pasteurization to be insufficient for NoV inactivation (*82*). Although the current study does not apply 63°C heat treatment, $t_D$ values indicate a 5-log reduction of MNV-1 would be achieved from 11.95 min and 2.95 min treatment at 60 and 65°C, respectively. This data suggests that a 30 min treatment at 63°C would be sufficient for a 5-log reduction of MNV-1. Sterilization processes (i.e., aseptic and retort sterilization) are performed at temperatures above 100°C and

are designed to inactivate all microorganisms including bacterial spores. The survival of human NoV would therefore not be a concern for sterilized beverages. Using a more mild 56°C heat treatment, a 8.24 $t_D$ value indicates that a 41.2 min thermal treatment would be necessary for a 5-log reduction of MNV-1. In buffets, hot foods should be held at an internal temperature of 60°C (*85*). Data presented in this study suggested that if a food served in a buffet was contaminated with NoV, it may be safe for consumption after 11.95 min if held at the correct temperature.

Although synergistic effects were not observed from the combination of GSE and thermal treatments, similar studies should be conducted with human NoV in order to investigate the suggestion that GSE offers protection through viral aggregation. Using citrate buffer as a model juice system, this study gives insight into the development of human NoV prevention or inactivation strategies for a juice or other mildly acidic beverage.

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