OPTIMIZING THE FDA/BAM STANDARDIZED METHOD FOR THE RECOVERY OF INFECTIOUS VIRUSES FROM STRAWBERRIES

by

AKHILA PATI

(Under the Direction of MALAK A. ESSEILI)

ABSTRACT

Human norovirus (HuNoV) and hepatitis A virus (HAV) are responsible for several foodborne outbreaks associated with berries. The FDA/BAM/26 method for virus detection from berries relies on RT-qPCR for the detection of viral RNA, which does not indicate infectious viruses. The objectives of this study were to detemine the method's limit of detection (LOD), the relationship between Ct and virus infectivity and to examine the BAM steps for recovery of infectious virus from strawberries. Tulane virus (TV), a HuNoV surrogate and HAV were used. Results showed that the LODs for TV and HAV were not significantly different. At Ct value >36, there is a low probability of directly recovering infectious viruses, while Ct values>40-44 predict the presence of one $TCID_{50}$ infectious viral unit on strawberries. The elution buffer's pH significantly affected the recovery of infectious TV. Overall, this study provides better insights into the detection of infectious viruses on strawberries.

INDEX WORDS: Human Norovirus, Hepatitis A Virus, Tulane Virus, Berries, FDA/BAM26, ISO 15216, infectious virus detection, RT-qPCR Ct values, limit of detection

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DEDICATION

This work is dedicated to my parents for their constant support. Their belief in me and the sacrifices they made have helped shape my academic journey. They have been by my side through every challenge and success, always guiding and encouraging me.

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CHAPTER 1

INTRODUCTION

Foodborne illnesses pose a significant threat to public health with a variety of pathogens contributing to outbreaks worldwide. Among these, human norovirus (HuNoV) and Hepatitis A virus (HAV) have gained attention due to their association with the highest number of foodborne outbreaks in berries. Transmission of HuNoV and HAV can occur through contact with infected individuals, as well as via contaminated food, water, or surfaces. Moreover, strawberries are often associated with HuNoV and HAV outbreaks (Bozkurt et al., 2021). Identifying viral pathogens responsible for outbreaks linked to berry consumption poses a challenge in virus detection. The United States Food and Drug Administration (FDA) developed a method published in Bacteriological Analytical Manual (BAM/Chapter 26) for the recovery of enteric viruses from fresh and frozen soft fruits. However, the approach utilized by this BAM method, which relies on eluting viruses using a specific elution buffer and then concentrating the viruses using ultracentrifugation, was designed for the detection of viral RNA rather than the detection of infectious viruses. It is important to note that the presence of viral RNA does not necessarily indicate the presence of infectious viruses. Therefore, this study is to understand how virus RNA detected from strawberries relates to virus infectivity and which step of the method is the most critical to allow enhanced recovery of infectious viruses.

CHAPTER 2

LITERATURE REVIEW

Human norovirus:

Globally, the World Health Organization (WHO) estimates that there are approximately 600 million cases of foodborne illnesses annually (WHO, 2015). These illnesses are attributed to at least 31 etiological agents, primarily microbiological and chemical in nature. Among these, microbiological agents are the leading contributors, with foodborne viruses being particularly significant (WHO, 2015). Out of these, HuNoV stands out due to its persistence and the severity of the illness it causes in the elderly, the children and the immunocompromised individuals (FAO, 2023). HuNoV is responsible for 23% of all foodborne illnesses, amounting to an estimated 125 million cases each year (WHO, 2015). In Canada, HuNoV infections resulting from food consumption exceed 1 million cases annually, representing approximately 65% of all foodborne illnesses in the country (Trudel-Ferland, Collard, et al., 2024). In the United States, HuNoV is the leading cause of acute gastroenteritis across all age groups (CDC, 2024a). Each year, HuNoV is responsible for an estimated 19 to 21 million illnesses, 465,000 emergency department visits—primarily among young children—and 2.27 million outpatient clinic visits (CDC, 2024a). The virus leads to approximately 109,000 hospitalizations and 900 deaths annually, with most fatalities occurring in adults aged 65 years and older. Human norovirus is also the leading cause of foodborne illness in the United States, accounting for 58% of foodborne illnesses acquired each year (CDC, 2024a). The economic impact of foodborne HuNoV infections is significant, costing the U.S. around $2 billion annually in healthcare expenses and lost productivity. Among children under five years, HuNoV contributes to nearly 1 million pediatric medical care visits annually. By the age of five, 1 in 7 children will have visited an outpatient clinic, 1 in 40 will have sought care in the emergency department, 1 in 160 will have been hospitalized, and 1 in 110,000 will have died due to HuNoV (CDC, 2024a). This underscores the significant public health burden posed by HuNoV infections, particularly among young children and older adults. Human norovirus outbreaks are more frequent during cooler months, typically occurring from November to April in countries above the equator and from May to September in countries below the equator (Steele et al., 2022). Near the equator, the seasonality of outbreaks is less pronounced. Transmission predominantly occurs through direct contact with infected individuals or through shared items such as food and utensils. Globally, HuNoVs remain the leading cause of acute gastroenteritis outbreaks (Steele et al., 2022).

Human noroviruses are highly infectious viruses belonging to the *Norovirus* genus within the *Caliciviridae* family. These non-enveloped viruses have a non-segmented, positive-strand RNA genome of approximately 7.5 k (De Graaf et al., 2016). HuNoVs are classified into ten genogroups (GI–GX), with genogroups I (GI) and II (GII) being the predominant foodborne pathogens responsible for the highest incidence of illnesses and hospitalizations globally (Chhabra et al., 2019; Steele et al., 2022). Infection with this virus occurs through multiple routes, including direct person-to-person contact, consumption of contaminated food or water, exposure to infectious aerosols generated by vomiting, or via the fecal-oral route (CDC, 2011). Symptoms of infection, including vomiting and diarrhea, typically appear within 1–2 days of exposure and last for 2–3 days. Notably, up to 30% of infected individuals may remain asymptomatic but can still shed the virus, contributing to its spread (CDC, 2011). Human noroviruses are highly infectious, requiring only a minimal dose of 18 viral particles to cause illness (Teunis et al., 2008). Additionally, infected individuals can shed substantial amounts of the virus, with median peak levels reaching 9×10^9 genomic copies (GC) per gram of feces (Bozkurt et al., 2021; Teunis et al., 2008).

Since 2002, genogroup II genotype 4 (GII.4) viruses have accounted for the majority of outbreaks worldwide (CDC, 2024b). Historically, new GII.4 variants emerged every 2–4 years; however, the GII.4 Sydney strain has been the dominant genotype since 2012. Occasionally, non-GII.4 genotypes, such as GII.17 and GII.2, have temporarily replaced GII.4 strains in certain regions, particularly in parts of Asia (CDC, 2024b). Emergence of new strains often correlates with a 50% increase in norovirus illnesses, emphasizing the virus's genetic adaptability and its significant impact on public health (CDC, 2024b).

HuNoV Surrogates:

Studying HuNoV has been challenging because of the lack of a reliable cell culture model to grow large amounts of viruses. Recently, the virus has been shown to replicate in B cells and in enteroids, but the virus cannot be propagated to generate more viruses for research purposes (Ahmed et al., 2020). Until better a cell culture or animal models are developed, surrogate viruses remain important tools for studying HuNoV(Ahmed et al., 2020).

Common HuNoV surrogates include murine norovirus (MNV), feline calicivirus (FCV), Tulane virus (TV), and bacteriophage MS2. Researchers also use virus-like particles (VLPs) that mimic HuNoV's outer structure but do not contain its genetic material (Ahmed et al., 2020).

Tulane virus was isolated from rhesus macaques' stools and is part of the Recovirus genus of the *Caliciviridae* family (Yu et al., 2013). Tulane virus is a small non-enveloped icosahedral structure with a positive sense, single-stranded RNA genome of approximately 6.7 kb (Yu et al., 2013). Tulane virus shares key characteristics with HuNoV, such as the same genetic organization and a similar capsid structure (Yu et al., 2013). Importantly, both HuNoVs and TV are enteric viruses causing gastroenteritis, and they recognize histo-blood group antigens (HBGAs) as attachment factors for infection (Yu et al., 2013). Consequently, TV is regarded as an excellent surrogate for investigating HuNoV-host interactions, especially in understanding the mechanisms of viral attachment and entry into host cells through interactions with cellular receptors and/or co-receptors (Tan et al., 2015).

Hepatitis A virus:

HAV is a small, nonenveloped virus with a diameter of 27–32 nm and a positive-strand RNA genome approximately 7.5 kb in length. HAV belongs to the Hepatovirus genus within the *Picornaviridae* family (Gholizadeh et al., 2023). HAV consists of seven distinct genotypes, four of which can infect humans (I, II, III, and VII). Among these, genotypes I and III are the most prevalent in humans (Lemon et al., 1992).

According to the CDC's assessment of foodborne disease, HAV is the second most contagious human pathogenic virus, after HuNoV (CDC). This virus is highly contagious, causing an illness lasting from a few weeks to several months. Most individuals recover fully without long-term liver damage, but in rare cases, particularly among older adults and those with chronic liver disease, HAV can lead to liver failure and even death. Outbreaks of HAV have been reported across multiple states since 2016, predominantly resulting from person-to-person transmission. These outbreaks have primarily affected people who use drugs, those experiencing homelessness, and men who have sex with men (CDC, 2025). In 2022, nearly half (49%) of all reported HAV cases occurred in individuals aged 30–49 years (CDC, 2025). While there was a significant 60% decline in newly reported cases compared to 2021, the number of cases in 2022 remained almost double that of 2015 (CDC, 2025). Symptoms of hepatitis A, which are more common in adults than children, usually appear 2–7 weeks after exposure and last less than two months in most cases, though some individuals may feel sick for up to six months. These symptoms include dark urine, clay-colored stools, diarrhea, fatigue, fever, joint pain, loss of appetite, nausea, stomach pain, vomiting, and jaundice (yellowing of the skin or eyes). However, not everyone infected with the virus develops symptoms (CDC, 2025). Hepatitis A virus is primarily spread through person-to-person contact or by ingesting contaminated food or drink, even in small amounts. Anyone who has not been vaccinated or previously infected is at risk, particularly individuals with certain life circumstances and behaviors that increase exposure. Vaccination is the most effective way to prevent HAV (CDC, 2025).

Berries and foodborne viruses:

Contaminated fruits and vegetables can cause foodborne illnesses. In the United States, from 2004 to 2013, 36% of foodborne illness cases were linked to contaminated produce (Oteiza et al., 2022). Additionally, data from the CDC's Foodborne Disease Outbreak Surveillance System between 1998 and 2013 showed an increase in outbreaks attributed to raw produce, rising from 8% in 1998–2001 to 16% in 2010–2013. Among these raw produce outbreaks, the most common sources were vegetable row crops (38%), fruits (35%), and seeded vegetables (11%) (Oteiza et al., 2022). In recent years, the consumption of berries, including raspberries, blueberries, blackberries, and currants, increased significantly (Bozkurt et al., 2021). Berries are produced by various plant types, such as low-growing ground bushes, small shrubs, or taller plants, and are cultivated using different agricultural methods like organic farming for blueberries and open field cultivation for strawberries (Ochmian et al., 2020). Due to their high juice content and delicate nature, berries like strawberries, raspberries and blackberries which are intended for the fresh market are harvested by hand to avoid the damage, while blueberries and berries intended for frozen market are harvested either manually or mechanically. Berries primarily consumed fresh are not processed further to remove harmful microorganisms such as viruses (Oteiza et al., 2022). Their short growing season and limited shelf life prompted the development of various artisanal methods to extend their usability. Berries can be dehydrated for use in products like breakfast cereals, teas, snacks, and powders, or transformed into juices, purees, and pulps (Aguilera, 2024).

The Food and Agriculture Organization (FAO) and the WHO have ranked berries as the second most concerning food in terms of produce safety, following leafy greens(Bozkurt et al., 2021). A review by (Bozkurt et al., 2021) highlighted that from 1983 to 2018, fresh and frozen berries were associated with 68 outbreaks, leading to 18,851 reported cases of illness globally. Among these outbreaks, HuNoV was the predominant virus, linked to 46 outbreaks and over 15,000 cases (Bozkurt et al., 2021). The most frequently implicated berries in these outbreaks were strawberries, raspberries, and berry mixes. Notably, frozen raspberries were responsible for over 80% of HuNoV outbreaks, while frozen berry mixes were linked to the majority of HAV outbreaks (44%). These outbreaks were reported by 18 different countries and occurred throughout the year. An example of the global spread of viral contamination through berries occurred in 2012, when a HuNoV outbreak in eastern Germany impacted 390 facilities, mostly schools, across five federal states. The outbreak resulted in 10,950 cases of gastroenteritis, with 38 hospitalizations. Investigations traced the source of the outbreak to a shipment of frozen strawberries imported from China (Mäde et al., 2013). Contamination was often traced back to producers in countries such as Poland, China, and the Republic of Serbia. The most common food preparation settings for these outbreaks were restaurants (23%) for HuNoV and private homes (35%) for HAV, with food handlers frequently identified as the source of contamination.

Sources of contamination of berries:

Berries can become contaminated with human pathogenic microbes at any point in the supply chain—preharvest, harvest, or postharvest—especially through human contact. Viral transmission occurs via direct or indirect contact with feces, vomit, or aerosolized droplets. Contamination can happen preharvest through exposure to contaminated water or soil, and postharvest due to contact with infected food handlers, contaminated surfaces, or water. Food handlers include field workers, production staff, chefs, and even individuals preparing food at home (Bozkurt et al., 2021).

Irrigation water

Irrigation water, sourced from surface or subsurface systems, plays a critical role in agriculture but can also serve as a vehicle for microbial contamination of fresh produce like berries. Sources such as municipal water, deep wells, and rainwater are less vulnerable to viral contamination, while delivery methods include furrow, drip, and sprinkler systems. Though irrigation water quality is vital for food safety, current microbiological guidelines focus on bacterial indicators like *E. coli*, which may not reliably predict viral contamination due to viruses' longer survival rates (Bozkurt et al., 2021).

Soil

Viral contamination of agricultural soils can occur through leaking septic tanks or farming practices such as irrigation with fecally contaminated water or the application of manure and biosolids. Biosolids, originating from wastewater processing, may contain high levels of pathogens. While biosolids recycle organic matter and nutrients, regulations on their use vary across countries (Bozkurt et al., 2021). Although contaminated soil has not been directly linked to viral outbreaks in berries, a previous review study indicated that viruses like MNV can attach to produce through contact with treated sludge and manure (Bozkurt et al., 2021). Factors such as soil type, pH, organic matter, water saturation, and conductivity influence virus transport (Bozkurt et al., 2021). Furthermore, sandy soils are less absorbent of viruses compared to fine-textured soils with 30–50% clay content, which are better at retaining viruses (Bozkurt et al., 2021).

Different berries have varied soil requirements. While most tolerate a range of soils, blueberries and cranberries require sandy loam soils with specific pH (4.2–4.8) and organic matter (>4%). Sandy loam soil, however, allows virus movement to other matrices like groundwater or produce. Wet soil conditions reduce virus attachment, promoting their transfer to berries, as berries cannot tolerate standing water during the growing season. The low infectious dose of enteric viruses heightens the risk, even with minimal soil contamination (Bozkurt et al., 2021).

During Harvesting

Contamination of berries during harvesting can occur through direct contact with infected food handlers, contaminated surfaces, or water. Poor personal hygiene, particularly among berry pickers, is a significant source of contamination, with fecally contaminated fingers transferring viruses to berries, door handles, and work surfaces (Bozkurt et al., 2021).

A previous review study indicated higher viral transfer rates from berries to gloves (15–16%) compared to gloves to berries (0.15–0.2%) (Bozkurt et al., 2021). In addition, virus transfer rates from fingertips to berries or stainless steel range from 20–70% under wet conditions and 4–12% under dry conditions, with drying time significantly influencing transfer rates (Bozkurt et al., 2021).

Postharvest handling

Cross-contamination of berries can occur from machinery used in sorting, processing, packing, storage, and transport, as well as from waste from previously contaminated batches. Common treatments for frozen berries, such as washing, freezing, and storage, are ineffective at removing or inactivating HuNoV or HAV. Additionally, processing steps involving human contact pose a high risk for viral transmission (Bozkurt et al., 2021).

Virus attachment to berries:

For berries to pose a viral risk, the initial attachment of enteric viruses is a crucial step in the contamination process. Viruses need to attach to the berry surface and remain there until consumption. The attachment process is influenced by electrostatic, hydrophobic, and van der Waals forces (Bozkurt et al., 2021). Electrostatic forces are related to viral properties like the isoelectric point (pI) of the virus, the presence of food-specific ligands, and surface acid-base groups. Attachment also depends on factors like surface structure, pH, ionic strength, and temperature. HuNoV capsids have a pI of 5.5-6.0, while HAV has a lower pI of 2.8 (Bozkurt et al., 2021), so when HuNoV encounters a low pH food matrix like berries, the virus may be attracted to the berry surface. It is thought that electrostatic forces play a major role in HuNoV adsorption to berries, while hydrophobic interactions are more significant for HAV attachment, especially due to polyphenols in berries (Bozkurt et al., 2021).Additionally, histo-blood group antigen-like (HBGA-like) molecules in fresh produce may help the attachment of HuNoV capsid proteins to surfaces like lettuce (Bozkurt et al., 2021). These molecules could also be involved in viral attachment and transmission in fruits and vegetables. Berries, with their soft flesh and irregular surface morphology, are more susceptible to contamination compared to fruits like blueberries, which have a smooth surface and waxy skin that can limit virus attachment (Bozkurt et al., 2021). The surface features of berries, including the presence of stigma on aggregate fruits like raspberries and blackberries, provide more sites for viral attachment.

Standard detection methods for viruses in berries:

Methods employed for the detection of viruses need to exhibit high sensitivity because contaminated foods may have very low viral loads (Hida et al., 2018). Low HuNoV levels can cause infection in humans because previous studies estimated a low 50% infectious dose for HuNoV (~18 viral particles) (Teunis et al., 2008). Therefore, low levels of virus present in the foods must be concentrated for downstream detection assays (Hida et al., 2018).

The United States FDA developed a Bacteriological Analytical Manual (BAM) method for virus elution and concentration from fresh and frozen soft fruits (FDA/BAM, 26). This method relies on first eluting viruses from berries by using 6% Tris-glycine beef extract (TGBE) (pH 9.5) as an elution buffer. Then the food matrix is clarified by centrifugation at 12,000 x g for 15 minutes. To concentrate viruses, ultracentrifugation at a speed of 170,000 x g for 45 minutes is used

(FDA/BAM, 26). The International Organization for Standardization (ISO) published in 2017, the ISO 15216 method, which differs from the FDA method in both the elution step by using the beef extract at 1% (1% TGBE, pH 9.5) and in the concentration step by using polyethylene glycol (PEG) precipitation (Stals et al., 2012). Polyethylene glycol is a water-soluble polymer that is commercially available in various molecular weights (such as PEG 6000, PEG 8000) that is often used in virus precipitation (Vajda, 1978). The ISO 15216 method uses PEG8000 mixed with 1.5 M NaCl to precipitate viruses eluted from berries. The final concentrated samples in both the ISO and BAM methods are then subjected to RNA extraction. Therefore, these methods were both developed for the detection of viral RNA by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and not cell-based infectivity assay.

Optimization of virus recovery methods for viruses on berries:

Before and after the publication of ISO and BAM standardized methods, many studies attempted to improve the recovery of viruses from berries by testing different elution buffers and concentration methods.

Optimizing the elution step of virus detection methods from berries:

The first step in all virus recovery methods from berries utilizes an elution buffer. Previous studies tested a number of elution buffers, or varied the duration of elution, or added enzymes to the elution buffer (Table 1). For example, (Dubois et al., 2002) assessed the pH stability of various elution buffers during the elution step from frozen raspberries. The buffers tested included phosphate buffered saline (PBS), 50 mM glycine, 50 mM glycine with 3% beef extract, 100 mM Tris with 50 mM glycine and 3% beef extract, and 500 mM Tris with 50 mM glycine and 3% beef extract.

Thawed raspberries were found to release acids that reduced the pH of water and low-buffered solutions, such as PBS, 50 mM glycine (pH 9.5), and 50 mM glycine with 3% beef extract (pH 9.5). Depending on their quality, the acidity of berries often caused the pH of these buffers to drop below 7. In contrast, buffered solutions such as those containing 100- or 500-mM Tris combined with 50 mM glycine and 3% beef extract (pH 9.5) maintained a stable pH near 9. In another study by Butot et al. fresh or frozen produce, including strawberries, raspberries, blueberries, blackberries, lettuce, green onions, and herbs were contaminated with HAV and then the samples were shaken with various elution buffers for 15 minutes to release the viruses. Elution buffers used included Tris buffer alone and a combination of Tris buffer with 1% beef extract (50 mM glycine, 100 mM Tris, 1% beef extract, pH 9.5) for extracting viruses from frozen raspberries and fresh strawberries. The combination of Tris buffer and beef extract provided higher virus infectivity recovery rates, with 15.8% recovery from frozen raspberries and 2.99% recovery from fresh strawberries for HAV(Butot et al., 2007). Similarly, in a related study by Kim et al. the performance of different elution buffers was tested on strawberries and raspberries. Among the buffers assessed, 3% beef extract demonstrated the highest elution efficiency, achieving an 85% recovery rate for HuNoV GII.4 RNA from strawberries (Kim et al., 2008). In a study by Hida et al. various buffers were tested, including 1% TGBE (0.1 M Tris-HCl, 0.05 M glycine, 1% beef extract, pH 9.2) and several variations of PBS-based buffers to recover HAV from sliced tomatoes. Among all buffers tested, 1% TGBE provided the best recovery, achieving 36.7% recovery rate for HAV RNA(Hida et al., 2013)

The majority of these previous studies emphasize the effectiveness of using TGBE-based buffers in virus recovery from produce. In contrast, Cheng et al. found that another buffer outperformed 1% TGBE. Specifically, the authors optimized a method for detecting HuNoV in artificially contaminated salad products, including fruit salads and vegetable salads. The samples were inoculated with HuNoV GI, HuNoV GII, and MS2 viruses and then eluted with 135 mL of various buffers, including 1% TGBE, PBS, MG (0.5% skimmed milk in glycine buffer), and KNT (0.05M KH_2PO_4, 1.0 M NaCl, 0.1% Triton X-100, pH 9.2). The study compared different concentration methods, including ultrafiltration, PEG, and filtration. Buffer KNT yielded the highest recovery, with 43.8% recovery of HuNoV GI RNA from fruit salad and 55.2% recovery from vegetable salad. However, 1% TGBE yielded 41.4 % HuNoV GI RNA with a pectinase treatment. The addition of pectinase to KNT elution buffer enhanced HuNoV GII virus RNA recovery from fruit salads from 59% to 63 %(Cheng et al., 2018). Other studies also explored the addition of enzymes to the elution buffers. For example, enzyme treatments like cellulase and pectinase were tested for recovery of HAV from tomatoes using PBS elution buffer. The combination of cellulase and pectinase yielded the highest virus recovery (17 %) in comparison to single enzymes (6%) (Hida et al., 2013). Specifically, the study found that the addition of 28 units of pectinase for fruit salad and 42 units for vegetable salad had the best results, although the difference was not statistically significant (Hida et al., 2013).

Optimizing the concentration step of virus detection methods:

Several studies have explored different methods for concentrating HuNoV and other enteric viruses from fresh produce and soft fruits, with a particular focus on improving recovery rates and detection accuracy. Table 2 provides a summary of various concentration methods employed and their corresponding virus recovery rates. One of the widely employed methods for viral concentration is polyethylene glycol (PEG) precipitation, which has been evaluated in several studies. Polyethylene glycols are typical condensation polymers. X-ray diffraction and infrared spectroscopic studies suggest that they have a spiral structure (Vajda, 1978). PEGs are water-soluble, non-ionic polymers available in various molecular weights. PEGs with molecular weights ranging from 2000 to 6000 (e.g., PEG2000, PEG4000, and PEG6000) are commonly used for virus precipitation. However, PEGs with higher molecular weights (e.g., PEG20000, PEG40000) are less practical due to the highly viscous solutions they form, which complicate centrifugation and other procedures. One advantage of PEG is that being non-ionic, i.e. interacts less strongly with biological materials. This is beneficial, as stronger interactions, may damage the materials being separated such as viruses (Vajda, 1978).

A previous study by Oteiza et al. used PEG 6000 precipitation following the ISO 15216 guidelines to concentrate viral particles from soft fruit samples, including strawberries, blueberries, and raspberries. The study showed that the process control virus, FCV, was recovered by PCR from berries at 23 ± 12% (Oteiza et al., 2022). Another study by Hennechart-Collette et al. also used the ISO method to detect various enteric viruses, including HuNoV, in artificially contaminated mixed vegetables. This study reported varying recoveries for different viruses, with HuNoV RNA recovery ranging from 0.1% to 40.61% (Hennechart-Collette et al., 2021). While the PEG precipitation method is widely used, the variation in recovery rates suggests that further optimization may be necessary for more consistent results across different food matrices. For example, (Kim et al., 2008) explored the influence of PEG molecular weight on the efficiency of PEG precipitation in different buffers. Their study found that PEG 8000 and PEG 10,000 achieved higher recovery rates when compared to PEG 6000 and PEG 20,000, with an average recovery of 79% for HuNoV GII4 viral RNA from strawberries and 6% for HuNoV GII4 viral RNA from raspberries. These findings underscore the importance of selecting the appropriate PEG molecular weight and optimizing precipitation conditions to improve viral recovery rates in different types of produce. In another study, Summa et al. (Summa M, 2012 Aug; Summa et al., 2012) compared PEG 8000 precipitation with other methods for virus recovery in lettuce, ham, and raspberries. They found that PEG precipitation was particularly effective for raspberries, with recovery efficiency of 28% of the HuNoV GII.4 viral RNA, which was higher than the recovery rates obtained using other methods like ultracentrifugation (4%) and immunomagnetic separation (4%) (Summa et al., 2012). Similarly, Bartsch et al. tested the ISO 15216 method, which includes PEG 8000 precipitation, for detecting HuNoV RNA in frozen strawberries. Although the recovery percentage using this method was low (1.71 ± 2.31%), the study recommended optimizing the method by adding an RNA purification step to improve recovery, particularly in frozen samples that are prone to PCR inhibition (Bartsch et al., 2016). This indicates that while PEG precipitation is a reliable method, its efficiency may be affected by factors such as sample type and the presence of inhibitors, suggesting a need for further method refinement. A study by Stals et al. utilized the ISO method for detecting HuNoV in food samples, including raspberries and strawberries. In this study, the sample was washed with an elution buffer and treated with Pectinex for 20 minutes, followed by centrifugation and the addition of PEG 6000 and NaCl. After overnight shaking, the sample was centrifuged again, and RNA extraction was done using an RNeasy Mini kit. The study used MNV as a process control, with qualitative analysis revealing that recovery success varied depending on the fruit type. In strawberries, the recovery success rate was 19 out of 20, while in raspberries, it was 8 out of 10. The mean recovery percentage from successfully recovered MNV viral RNA is consistent across fruit types, with a mean recovery of 12.8 ± 5.6% from strawberries (Stals et al., 2011). This study illustrates that the ISO method can be effective, but recovery rates are variable depending on the fruit matrix.

Other concentration methods, such as ultracentrifugation, ultrafiltration, and filtration, have also been evaluated for their ability to improve virus recovery from food samples. For example, Hida et al. compared ultracentrifugation and PEG precipitation for detecting HuNoV in produce items such as cucumber, lettuce, and grapes. The study found that ultracentrifugation consistently outperformed PEG precipitation in terms of virus recovery, with HuNoV RNA recovery reaching up to 13.67 ± 0.28% for cucumber, compared to 2.54 ± 2.48% for PEG precipitation. The addition of polyvinylpyrrolidone to the elution buffer in lettuce further increased MNV RNA recovery (from 5.67 ± 1.06 % to 10.65 ± 1.34%), suggesting that ultracentrifugation could be more effective if optimized further (Hida et al., 2018). Similarly, a study by Cheng et al. tested multiple concentration methods, including ultrafiltration, PEG, and filtration, for detecting HuNoV in fruit and vegetable salads. They found that filtration was more effective for fruit salads, achieving recovery rates of about 40% for HuNoV GI RNA and 60% for HuNoV GII RNA, while ultrafiltration and PEG were more effective for vegetable salads, providing higher recovery rates (41.13 %) compared to fruit salads (38.57%)(Cheng et al., 2018). These results suggest that ultrafiltration and filtration methods may be preferred for certain types of salads, highlighting the importance of selecting the right concentration method based on the food matrix. Butot et al. also compared PEG precipitation and ultrafiltration for virus recovery in berries and vegetables. Their study found that ultrafiltration was more effective in berries, with a recovery rate of 13% for HAV RNA compared to 5% when using PEG precipitation. This supports the findings of other studies that suggested ultrafiltration can be a more efficient method for virus recovery, particularly when dealing with certain types of produce such as berries and vegetables. PEG precipitation remains a widely used method due to its simplicity and applicability across a range of matrices. Despite its lower recovery rates in certain cases, PEG precipitation is still a reliable method for detecting enteric viruses in fresh produce, but for more efficient virus recovery, ultracentrifugation and ultrafiltration may be preferred, depending on the specific food matrix and virus being targeted(Butot et al., 2007)

The study by Trudel-Ferland et al. aimed to optimize the concentration step by using ultrafiltration for detecting HAV and HuNoV in various fresh and frozen produce, including strawberries, raspberries, blackberries, lettuce, and green onions. The researchers compared the ultrafiltration method to the ISO 15216 method, with elution times ranging from 5 to 20 minutes. No significant effect of elution time on HAV recovery was observed in fresh strawberries. The study found that for strawberries, the concentration method and the state of the berries (fresh or frozen) had no impact on the recovery of Mengo virus (MGV), HAV, or HuNoV GI.7. However, for HuNoV GII.4, there was an interaction between these factors, with the ultrafiltration method being more effective for fresh strawberries, while the ISO method performed better for frozen strawberries. Detection efficiency was affected by virus type, food matrix, and sample condition, with HAV being more likely to be detected than HuNoV GII.4. For frozen strawberries, neither the concentration method nor virus type significantly influenced detection, but the viral load did. Higher genome copy numbers resulted in better detection outcomes. The ultrafiltration method reduced processing time from 190 minutes to 130 minutes for six samples, making it faster than the ISO method, though it was less effective for frozen raspberries, likely due to high concentrations of PCR inhibitors such as polysaccharides and phenolic compounds. The study used RT-qPCR for viral detection, but this method does not assess virus infectivity, leaving the actual risk posed by the detected viruses unclear(Trudel-Ferland, Levasseur, et al., 2024)

Another study by Trudel-Ferland et al. found that the use of OneStep PCR Inhibitor Removal Kit (Zymo Research) on frozen berry samples after nucleic acid extraction, reduced PCR inhibition and led to less sample-associated inhibition of the PCR. Also, the authors found that the use of the automated method reduced this PCR inhibition to 56.5% in comparison to 95% using manual extractions. This demonstrated that the automated system could improve detection efficiency by reducing PCR inhibition, thus offering a potential improvement over the traditional semi-automated method(Trudel-Ferland, Collard, et al., 2024).

Finally, a novel concentration method based on magnetic silica beads (MSB) was tested by Raymond et al. to extract HuNoV from frozen raspberries. The extraction process involved eluting the virus from the sample with an elution buffer, followed by clarification via centrifugation. The MSB method was faster than the ISO 15216 method, taking only 7 hours compared to the ISO method's 9 hours, and the recovery rates for HuNoV GII.4 were 2.6% for the MSB method and 1.8% for the ISO method. For HuNoV GI.5, the recovery was 3.6% with MSB, while MNV RNA recovery was 2.8%. The MSB method also involved the use of Pluronic F-127 and other reagents to improve virus extraction efficiency, resulting in a more rapid but comparable recovery rate to the ISO method (Raymond et al., 2021).

Prevalence of foodborne viruses on berries:

The prevalence of enteric viruses in frozen berries has been a growing concern due to their potential association with foodborne illness outbreaks. In a large-scale microbiological sampling assignment conducted by the FDA from 2019 to 2023, the prevalence HAV and HuNoV was assessed in 1,558 frozen berry samples, including 585 strawberries, 528 raspberries, and 445 blackberries. The study aimed to estimate the prevalence of these viruses in both domestically produced and imported frozen berries, determine potential risk factors, and evaluate the effectiveness of current food safety measures. Samples were collected from processors, distribution centers, storage facilities, ports of entry, and retail establishments across 22 countries, with 538 samples sourced domestically and 1,020 samples from imports, primarily from Chile, Mexico, and Serbia. The results indicated that HAV was detected in 8 samples (0.5% prevalence). Specifically, one positive in strawberries, five in raspberries, and two in blackberries, Furthermore, HuNoV was found in 10 samples (0.6% prevalence), comprising 3 positives in strawberries, 3 in raspberries, and 4 in blackberries. All positive detections were in finished packaged products, with no virus found in bulk containers. While the prevalence of HAV and HuNoV in frozen berries was relatively low (FDA, 2025), knowing the prevalence of viruses on berries is important for assessing food safety risks and implementing effective control measures. Around the world several studies attempted to determine the prevalence of viruses in berries. These studies were summarized in Table 3.

In Spain, Stals et al. conducted a study screening for HuNoV contamination in various fruit products, including raspberries (imported from Serbia and Poland), cherry tomatoes and strawberries (Spain), and fruit salads (prepared in Belgium). A total of 75 fruit samples were tested for the presence of HuNoV using MNV as a process control. Virus extraction was done following the ISO method, which involved the use of PEG 6000 and NaCl. Out of the 75 fruit samples, 18 tested positives for HuNoV GI and/or GII (24%). Notably, HuNoV was detected in 6 out of 20 strawberry samples, with genomic copies ranging from 2.29 (Ct 41.97) to 4.10 (Ct 39.27) per 10 g of the sample. The detection was not consistently confirmed across all tests, with positive results only obtained in one out of four RT-qPCR tests(Stals et al., 2011).

In a study performed across Belgium, Canada and France, Baert et al. investigated the prevalence of HuNoV on fresh produce, including 867 samples of leafy greens, 180 samples of fresh soft red fruits, and 57 samples of other types of fresh produce (such as tomatoes, cucumbers, and fruit salads). The study utilized real-time RT-PCR to detect HuNoV viral RNA, and the results showed that 28.2% of the leafy green samples tested in Canada, 33.3% of fresh soft red fruits tested in Belgium, and 50% of leafy greens tested in France were HuNoV positive. For soft red fruits, 34.5% of the samples tested positive in Belgium, while 6.7% of the samples tested positive in France. In Belgium, 55.5% of the other fresh produce samples tested positive for HuNoV using real-time RT-PCR (Baert et al., 2011).

In a European multinational study, Maunula et al. monitored the entire food production chain across four countries—Czech Republic, Finland, Poland, and Serbia—to investigate possible routes of viral contamination in berry fruits. A total of 785 samples were collected during two growing seasons from various sources, including irrigation water, animal feces, food handlers' hand swabs, toilet swabs from farms, conveyor belts at processing plants, and raspberries or strawberries at points-of-sale. These samples were analyzed for human and animal enteric viruses using ISO 15216 method and real-time PCR. The study found that human adenovirus (hAdV) was detected in irrigation water (9.5%), food handlers' hands (5.8%), and toilets (9.1%) at berry production sites, suggesting potential contamination routes. At processing plants, hAdV was found in 2.0% of hand swabs, while at point-of-sale, it was detected in 0.7% of fresh raspberries, 3.2% of frozen raspberries, and 2.0% of fresh strawberries. Among human enteric viruses, HuNoV GII was found in 3.6% of irrigation water samples, while hepatitis E virus (HEV) was detected in 2.6% of frozen raspberry samples. No HAV was detected in any samples. Additionally, the presence of porcine adenovirus (pAdV) and bovine polyomavirus (bPyV) indicated fecal contamination from animal sources. At point-of-sale, 5.7% of fresh berries and 1.3% of frozen berries tested positive for pAdV. The findings highlight irrigation water and food handlers' hands as potential vehicles for virus transmission, emphasizing the need for strict adherence to good agricultural and hygienic practices to minimize contamination risks in the berry supply chain(Maunula et al., 2013).

In Italy, a study by Macori et al. investigated the presence of HAV and HuNoV GI and GII in fresh berries from a major production area. A total of 50 berry producers were sampled to assess viral contamination at the primary production stage. The viruses were detected using the ISO 15216 method with RT-qPCR. The results showed that HAV and HuNoV were not detected in any of the fresh fruit samples, indicating a low risk of viral contamination in the region's berry production (Macori et al., 2018).

In China, a survey was conducted by Gao et al. to investigate the presence of HuNoV in commercial fresh and frozen berries collected from a major berry-producing region, during 2016-2017. A total of 2,477 fresh and frozen berry samples, including strawberry, blueberry, raspberry, cranberry, blackberry, and blackcurrant, were analyzed using the ISO 15216 method and real-time PCR to detect HuNoV viral RNA. The results revealed that 9% (81/900) of frozen berry samples and 12.11% (109/900) of fresh domestic retail berry samples tested positive for HuNoV viral RNA. Among the positive samples, 35.80% (29/81) of frozen berries and 29.36% (32/109) of fresh berries were contaminated with genotype GI viral RNA, while 54.32% (44/81) of frozen berries and 60.55% (66/109) of fresh berries had genotype GII viral RNA. Additionally, 9.88% (8/81) of frozen berries and 10.09% (11/109) of fresh berries contained both GI and GII viral RNA. No HuNoV viral RNA contamination was found in the 677 frozen berry samples intended for export(Gao et al., 2019).

In Argentina, a study by Oteiza et al. explored the viral quality of berries by analyzing a total of 184 soft fruit samples, including strawberries, blueberries, raspberries, blackberries, currants, pomegranate arils, cassis, and elderberries, collected from production plants and retail markets between 2016 and 2020. The viruses tested included HuNoV GI and GII, HAV, rotavirus, and enterovirus. The results revealed that viral contamination was detected in only 1 out of 184 berry samples (0.5%), with HuNoV GII identified in a raspberry sample(Oteiza et al., 2022).

In Ireland, a study conducted by Bennett et al. investigated the presence of HAV, HEV, HuNoV, HAdV, and Sapovirus (SaV) in ready-to-eat (RTE) berries collected from retail stores between May and October 2018. Out of the 239 berry samples tested, 16 samples (6.7%) showed the presence of viral genetic material using the ISO method. The detected viruses included HAV in 5 samples, HAdV in 5 samples, HEV in 3 samples, and HuNoV GII in 3 samples. Sapovirus RNA was not detected in any of the tested samples. However, the levels of viral RNA detected were low, with the average Ct value ranging from 33 to 44 (Bennett et al., 2023).

In all these prevalence studies, although the levels of viruses detected were low, these findings suggest that contamination could have occurred at some point in the supply chain. However, low levels should not be immediately interpreted as a significant public health risk, though the potential for foodborne transmission cannot be ruled out entirely. A major limitation of these studies is the inability to confirm whether the detected nucleic acid of viruses represented viable viruses capable of causing infection. Thus, viral detection in berries highlights the challenges of assessing the potential health risks associated with low levels of viral contamination in food products.

Persistence of viruses on berries:

In order to provide evidence that inactivated viruses can be present on berries, the persistence of heat-inactivated HAV across various surfaces, water, and on blueberries was evaluated in a previous study (Trudel-Ferland et al., 2021). On blueberries, inactivated HAV remained detectable by RT-qPCR at −20 °C for up to 90 days at high viral loads (2.5×10^6 GC/μl), demonstrating that non-infectious virus can persist in food matrices under freezing conditions. However, at 4 °C, there was a notable decrease in detection within 24 hours (22%), with only 5% of the virus detectable on day 16. At a lower load of 25,000 GC/berry, detection decreased to just 2% on day 16, with minimal temperature effects. These findings indicate that the stability of non-infectious HAV in food products like blueberries can vary based on both the temperature and the initial viral load. This study showed that standard methods for the detection of viruses that rely on RT-qPCR can detect non-infectious viruses which are shown to persist in food matrices.

The persistence of HuNoV and HAV in various environmental conditions was recently reviewed (Kotwal & Cannon, 2014). This review study highlights how these viruses can remain viable on surfaces, foods, and in water, contributing to prolonged outbreaks. On stainless steel and plastic surfaces, HuNoV RNA remained detectable for up to 56 days at 7 °C under high humidity conditions, whereas at room temperature (20 °C) with low humidity, persistence was significantly reduced to 7 days. Similarly, HuNoV RNA and infectious HAV persisted on lettuce, turkey, and berries for up to 7 days at refrigeration temperatures (10 °C). However, higher viral reductions were observed in berries stored at 21°C, indicating that temperature plays a critical role in viral stability. In water, enteric viruses demonstrated extended persistence, with HuNoV RNA detected for up to 1266 days (3.5 years) in groundwater, whereas infectious HuNoV was confirmed to persist for at least 61 days using human volunteers.

The persistence of HAV, HuNoV and FCV in frozen berries was investigated in a study by Butot et al. to assess the survival of these viruses during long-term frozen storage (Butot et al., 2008) . The study aimed to evaluate whether freezing at -20°C for 90 days could effectively reduce viral contamination in strawberries, raspberries, and blueberries. Hepatitis A virus showed no significant reduction in viral RNA or infectivity across all tested berry types. Similarly, HuNoV GI and GII remained largely stable, with only a 0.9 log reduction observed in blueberries over 90 days, while in raspberries, HuNoV GII exhibited no measurable decline. In strawberries, HuNoV GI and GII demonstrated minimal reductions ranging from 0.1 to 0.4 log. In contrast, FCV, a common surrogate for HuNoV in experimental studies, was the most sensitive to freezing. A 2.7 log reduction in FCV infectivity was observed in strawberries, while raspberries showed a 1.1 log decline over the same period. These results demonstrated that freezing had limited effects on the stability of HuNoV and HAV, emphasizing the resilience of foodborne viruses in frozen produce.

The persistence of HuNoV GII and GI and MNV on soft berries under different storage conditions was also examined (Verhaelen et al., 2012). The authors aimed to assess the survival of these viruses on raspberries and strawberries stored at 4, 10, and 21 °C**,** as well as in PBS for comparison. Specifically, at 4 and 10 °C**,** all tested viruses exhibited high stability in PBS and on raspberries, with minimal reductions in viral titers over time (≤ 0.5 log reduction). This suggests that storage at refrigeration temperatures does not significantly inactivate these viruses, allowing them to persist on berries for extended periods. At 21°C, on raspberries, MNV was inactivated by 1 log in 3 days. In contrast, on strawberries, infectious MNV decayed rapidly showing 1 log reduction in 1 day. The latter indicated that strawberries do not support viral persistence as well as raspberries under room temperature conditions. Additionally, HuNoV GI RNA exhibited a 1 log reduction in 2 days on strawberries, while HuNoV GII RNA exhibited a 0.5 log reduction in 3 days. Therefore, HuNoV GII exhibited greater stability on strawberries, exceeding the shelf life of the fruit at 21°C.

Physical and chemical Treatments for reducing viral contamination on berries:

A variety of sanitation methods have been studied for their effectiveness in removing HuNoV and HAV from strawberries and other berries. Washing with potable water alone generally results in less than 1 log reduction, similar to the effectiveness of 100 mg/L chlorine (Bozkurt et al., 2021). However, berries intended for fresh consumption are typically not washed due to their delicate nature, whereas frozen berries undergo washing during postharvest handling. Studies indicate that washing berries with tap water at 18 °C achieves less than 1.5 log reduction in viral titers, with minor improvements when using warm water (43 °C) or hand rubbing (Butot et al., 2008). Additionally, household washing methods, such as using salt (2% NaCl) or commercial produce washes, provided no significant additional viral reduction (Bozkurt et al., 2021).

Among chemical sanitizers, chlorine (200 ppm) is the most widely used and has been shown to reduce HuNoV and HAV on berries by up to 3.4 log in blueberries, 3.0 log in strawberries, and 2.4 log in raspberries after 10 minutes of treatment (Butot et al., 2008). However, chlorine efficacy is reduced by the presence of organic matter, and its use is restricted in some European countries due to concerns about harmful disinfection by-products (Bozkurt et al., 2021). Chlorine dioxide (ClO_2), considered a safer alternative, has shown promising results in virus suspensions, achieving 4 log reductions of HAV at concentrations as low as 0.6–0.8 ppm. However, its effectiveness on berries is lower, with less than 2 log reductions in HAV on raspberries and parsley when used at higher concentrations (10 ppm) (Butot et al., 2008). Gaseous ClO_2 has shown better penetration, achieving reductions of 2.9 to 4.1 log in various berries, but its effectiveness against HuNoV requires further validation (Bozkurt et al., 2021).

Other potential sanitizers studied for viral inactivation in berries include hydrogen peroxide, trisodium phosphate, sodium dodecyl sulfate (SDS), cetylpyridinium chloride (CPC), quaternary ammonium compounds (QAC), and sodium hypochlorite, though their effectiveness remains limited. Combining SDS (50 ppm) with chlorine (200 ppm) for 2 minutes enhanced virus inactivation, achieving up to 3 log reductions in strawberries and raspberries (Bozkurt et al., 2021). However, the combination of peroxyacetic acid and hydrogen peroxide was only effective at four times the manufacturer's recommended concentration, raising concerns about potential chemical residues on berries (Bozkurt et al., 2021). Overall, chlorinated water (200 ppm) was found to be more effective than chlorine dioxide (10 ppm) in reducing HuNoV and HAV contamination on berries, but complete virus removal remains challenging (Butot et al., 2008).

Tables

Table 1: Summary of elution buffers and recovery percentages for enteric viruses' detection in fresh and frozen produce gathered from previous studies.

Virus	Fruit/vegetable	Elution buffer	Recovery	Reference
HAV Infectivity	Fresh or frozen strawberries, raspberries, blueberries, blackberries, lettuce, green onions	50 mM glycine, 100 mM Tris, 1% beef extract, pH 9.5, Tris buffer alone, Tris buffer + 1% beef extract	Tris buffer + 1% beef extract -15.8% from frozen raspberries, 2.99% from fresh strawberries	(Butot et al., 2007)
HuNoV GII-4 RNA	Strawberries and raspberries	3% beef extract	85%	(Kim et al., 2008)
HAV RNA	Sliced tomatoes	1% TGBE (0.1 M Tris-HCl, 0.05 M glycine, 1% beef extract, pH 9.2)	36.70%	(Hida et al., 2013)
HuNoV GI, HuNoV GII RNA	Fruit salads and vegetable salads	1% TGBE, PBS, MG (0.5% skimmed milk in glycine buffer), and KNT (0.05M KH2PO4, 1.0 M NaCl, 0.1% Triton X-100, pH 9.2)	KNT-43.89± 8.21% recovery of HuNoV GI RNA from fruit salad .55.21% from vegetable salad,1% TGBE -41.42± 9.23% HuNoV	(Cheng et al., 2018)

Table 2: A list of concentration methods and recoveries (%) for enteric viruses in fresh and frozen produce from summarized from previous studies

Virus type	Fruit/Vegetable tested	Concentration method	Recovery	Reference
HAV Infectivity	Berries and vegetables	PEG, ultrafiltration	Berries-PEG-5%, Ultrafiltration- 13%	(Butot et al., 2007)
HuNoV GII.4 RNA	Strawberries, raspberries	PEG 6000, 8000,10000,20000	PEG8000, 10000 strawberries- 79%, 6% from raspberries	(Kim et al., 2008)
HuNoV GII.4 RNA	lettuce, ham, raspberries	PEG 8000, ultracentrifugation, immunomagnetic separation	PEG-28% ultracentrifugation- 4%, immunomagnetic separation-4%	(Summa et al., 2012)
HuNoV RNA	Frozen strawberries	PEG 8000	1.71 ± 2.31%	(Bartsch et al., 2016)
HuNoV RNA	cucumber, lettuce, and grapes	PEG, Ultracentrifugation	Cucumber- ultracentrifugation- 13.67 ± 0.28% , PEG-2.54 ± 2.48%	(Hida et al., 2018)
HuNoV RNA	Mixed vegetables	PEG 6000	0.1% to 40.61%.	(Hennechart-Collette et al., 2021)
FCV RNA	Strawberries, blueberries, raspberries	PEG 6000	23 ± 12%	(Oteiza et al., 2022)

Table 3: Summary of enteric viruses' prevalence in fresh and frozen produce gathered from previous studies.

Virus	Fruit/Vegetable	Sample Size	Method	Ct or Infectivity	Reference
HuNoV	Raspberries (from Serbia and Poland), cherry tomatoes and strawberries(Spain), and fruit salads (prepared in Belgium)	75 fruit samples	ISO/ PCR	Ct values: 39.27 to 41.97 18 samples shown positive	(Stals et al., 2011)
HuNoV	Fresh soft red fruits and leafy greens. Belgium, Canada and France	180 samples of fresh soft red fruits (strawberries and raspberries) and 57 samples of other types of fresh produce (tomatoes, cucumber and fruit salads), 867 samples of leafy greens	ISO/ PCR	Strawberries and raspberries-34.5% of samples in Belgium, 6.7% of samples in France (Ct values from 30-40)	(Baert et al., 2011)
HAdV	Raspberries, strawberries at the point of sale (Czech Republic, Finland, Poland and Serbia)	785 samples	ISO/ PCR	Prevalence-Fresh raspberries- 0.7%, frozen raspberries- 3.2%, fresh strawberries- 2%	(Maunula et al., 2013)

HuNoV GII	Food production chain (irrigation water, animal feces, food handlers' hand swabs, swabs from toilets on farms, from conveyor belts at processing plants)	785 samples	ISO/PCR	detected in two (3.6%) water samples at berry production	(Maunula et al., 2013)
HAV, HuNoV GI, GII,	Blackberry, blueberry and raspberry	50 producers	ISO/PCR	Not detected	(Macori et al., 2018)
HuNoV GI and GII	Fresh and frozen Berries (Strawberry, raspberry, blueberry, blackberry, blackcurrant, cranberry), China	900 frozen and 900 batches fresh domestic, 677 batches of frozen export berry samples	ISO/PCR	frozen berries-9% , fresh berries-12.11%, Frozen berries GI- 35.8%, GII-54.32%, both GI and GII-9.88% Fresh berries-GI-29.36%, GII-60.55%, Both GI and GII- 10.09%	(Gao et al., 2019)
HuNoV GI, GII, HAV, rotavirus, enterovirus	Strawberries, blueberries, raspberries, blackberries,currants,pomegranate arils, cassis, and elderberries between 2016 and 2020	184 samples	ISO/PCR	Only 1 tested positive with HuNoV GII	(Oteiza et al., 2022)
HAV, HEV, NoV, HAdV and SaV	Ready to eat berries collected from retail stores in Ireland	239 berry samples	ISO/PCR	Ct values: 33 to 44	(Bennett et al., 2023)

CHAPTER 3

MATERIALS AND METHODS

Virus stock preparation:

The Tulane virus stock was prepared by propagating TV on LLC-MK2 cells, a Rhesus Monkey Kidney Epithelial cell line, using M199 media (Fisher Scientific, MA, USA) supplemented with 5% horse serum (Fisher Scientific, MA, USA) and 1% antibiotic/antimycotic solution (Fisher Scientific, Waltham, MA, USA). The cells were cultured until they reached 95% confluency before being infected with TV. Infection media was prepared with M199, 2% fetal bovine serum (FBS) (VWR, USA), and 1% antibiotic-antimycotic (AA) solution. TV of known titer (a generous gift from Dr. Tibor Farkas at Taxes A&M University) was added to the infection media. After removing the old media, the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) (Fisher Scientific, VA, USA) before adding the virus. The flasks were incubated at 37°C for 48-72 hours until at least 80% cytopathic effect was observed. Following incubation, the flasks underwent three cycles of freeze-thaw. The cells were then scraped from the surfaces of the flasks and centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatants were ultra-filtered using Amicon 100KDa Ultra-15 centrifugal filter tubes (Millipore, MA, USA) at 3000 rpm for 30 minutes to eliminate any remaining cell lysates and concentrate the virus by a factor of 10x. Finally, the ultra-filtered viruses were aliquoted and stored at -80 °C.

Hepatitis A virus (HM175/8f strain) stock (ATCC, VA, USA) was prepared by propagating the virus on FRhK-4 (Fetal Rhesus Monkey Kidney) cells. The FRhK-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (VWR, USA) supplemented with 10% FBS and 1% AA. Once the cells reached 95% confluency, they were infected with HAV stock purchased from ATCC. Infection media was prepared by adding 2% FBS and 1% AA to DMEM. The old media was removed, and the cells were rinsed with DPBS. The HAV inoculum was added in infection media, and the flasks were incubated at 37 °C for 90 minutes, swirling every 15 minutes for optimal virus attachment. After incubation, the inoculum was removed, and fresh infection media was added. The infected cells were incubated for 6-7 days at 37 °C. Upon observing ≥ 80% cytopathic effect (CPE), the virus was harvested using three freeze-thaw cycles. The virus was centrifuged at 3000 rpm for 30 minutes at 4°C, and the supernatant was ultra-filtered at 3000 rpm for 30-35 minutes to remove cell lysates and concentrate the virus by a factor of 10x. The ultrafiltered virus was aliquoted and stored at –80°C for future use.

Virus infectivity quantification by TCID50 assay:

The tissue culture 50% infectious dose (TCID50) assay is a cell-based infectivity assay used to determine the presence of infectious virus in a sample. The TCID50 assay is the most common method for assessing the infectivity of TV and HAV. This technique involved the observation and measurement of visible CPE in cell lines that indicate the replication of viruses. For TV, LLC-MK2 cells were plated into 96-well plates at a density of 1.6×10^4 cells per well and incubated at 37 °C with 5% CO_2 for one day until they reached 95% confluency. Similarly, for HAV, FRhK-4 cells were seeded into 96-well plates at a density of 5.2×10^5 cells per well and incubated under the same conditions for three days to reach confluency. Once the cells were ready, the media was removed, and fresh infection media was added—M199 supplemented with 2% FBS and 1% AA for TV, and DMEM supplemented with 2% FBS and 1% AA for HAV. Meanwhile, the virus samples to be tested were vortexed and 10-fold serially diluted. Each dilution was then tested on quadruplicate wells of the respective cell plates. The cell culture plates were then placed in an incubator for 6-7 days for TV and 7-10 days for HAV to observe the dilution at which 50% of the infected cells exhibited CPE.

Strawberry Inoculation with viruses:

Fresh strawberries were purchased from local grocery stores in Griffin, GA. The calyx portion was removed from the strawberries using a sterile scalpel, and they were weighed to make approximately 50 g per replicate. A 100 µl (10 µl droplets) of viruses was inoculated onto the surface of the strawberries and subsequently left to dry inside a biological safety cabinet (~ 45 minutes).

The BAM 26 method for recovering viruses from strawberries:

The virus-inoculated strawberries (50 g per replicate) were placed into Whirl-Pak filter bags containing 30 ml of the elution buffer 6% TGBE (pH 9.5). A 50 µl of pectinase, prepared by dissolving 1.25 g of 10,000 U pectinase from *Aspergillus niger* (MP Biochemicals, USA) in 5 ml of RNase-free water, was added to the buffer. The bags were shaken at 150 rpm for 15 minutes at room temperature (RT). The pH was monitored and adjusted to 8.0 ± 0.5 using 2.5 M NaOH, if necessary. Following the shaking period, the liquid was then transferred to 50 ml tubes, and the tubes were centrifuged using JA-10 rotor (Beckman Coulter, CA, USA) at 12,000 x *g* for 15 minutes at 4 °C to remove debris from the solution. The supernatants were then transferred to Thin wall ultra-clear™ centrifuge tubes (Beckman Coulter) and centrifuged using SW 32 Ti rotor

(Beckman Coulter, Optima XE-90 Ultracentrifuge) at 170,000 x g for 45 minutes at 4 °C to concentrate the viruses. The resulting pellets were resuspended in 1 ml PBS and stored at -80 °C. These samples were later used for quantifying virus infectivity through the TCID50 assay and viral RNA using RNA extraction followed by RT-qPCR.

The ISO 15216 method for recovering viruses from strawberries:

The virus-inoculated strawberries were placed into Whirl-Pak filter bags containing elution buffer 1% TGBE (pH 9.5). A 50 µL of pectinase was added to the buffer as described above for the BAM method. The bags were then shaken at room temperature for 15 minutes at 150 rpm. Subsequently, the liquid was collected into tubes, and centrifuged using JA-10 rotor (Beckman Coulter) at 10,000 rpm for 15 minutes at 4 °C to remove the strawberry debris. The supernatants were transferred to 50 ml tubes, and the pH was adjusted to 7–7.5 using 5 M HCl. The latter step was first performed on control negative strawberry samples (i.e. without any viruses) to determine pH of the samples and the required volume of 5M HCl to adjust the pH to 7. Then, a 0.25 volume of 5x PEG 8000 (Sigma Aldrich, USA) mixed with 1.5M NaCl (Fisher Scientific, USA) solution was added to the samples. The 5x PEG/NaCl solution was added as 7.5 ml per 30 ml volume. The samples were vortexed for 1 minute and then shaken at 60 rpm for 1 hour inside a cold room (4 °C). The samples were then centrifuged using JA-10 rotor at 10,000 rpm for 30 minutes at 4 °C. The supernatants were discarded, and the samples were centrifuged again at 10,000 rpm for 5 minutes at 4 °C to compact the pellets. The resulting pellets were resuspended in 1 ml PBS and stored at -80 °C. These samples were used for quantifying virus infectivity by TCID50 assay and viral RNA using RNA extraction followed by RT-qPCR.

Determining the limits of detection (LOD) for the BAM and ISO methods

The LODs were determined by preparing 10-fold serial dilutions (from 10^{-1} to 10^{-6}) of TV and HAV and spiking strawberries with each dilution in addition to undiluted viruses. Viruses were recovered using the BAM and ISO methods as described above. Both the serially diluted virus inocula and the recovered viruses from the contaminated strawberry samples were tested by infectivity assay and RT-qPCR.

Virus RNA extraction:

Viral RNA extraction was performed using the QIAamp Viral RNA Mini Kit (QIAGEN, MA, USA) according to the manufacturer's instructions. Briefly, 560 µL of AVL buffer containing carrier RNA was added to each sample. The mixture was vortexed for 20 seconds and incubated at room temperature for 10 minutes. Next, 100 µL of 2 M potassium acetate solution was added to the samples, and the samples were incubated on ice for 15 minutes. Following this, the samples were centrifuged at 4 °C for 10 minutes at 14,000 rpm, and the supernatants were transferred to new 2 mL centrifuge tubes.

A 700 µL volume of the supernatants was transferred to QIAshredder columns (QIAGEN) and centrifuged at 14,000 rpm for 2 minutes. The supernatants of the flow-through fractions were transferred to new 2 mL tubes to which 700 µL of 95–100% ethanol was added and mixed. A 630 µL volume of the resulting solutions was applied to QIAamp Mini columns and centrifuged at 8,000 rpm for 1 minute. The flow-through was discarded, and the spin columns were placed in new collection tubes. The application of the sample solutions was repeated until all had been processed. A 500 µL volume of buffer AW1 was added to the columns and centrifuged at 8,000 rpm for 1 minute, with the flow-through discarded. The spin columns were then transferred to new collection tubes, and 500 µL of buffer AW2 was added and centrifuged at 14,000 rpm for 3 minutes. The flow-through was discarded, and the spin columns were placed in new 1.5 mL tubes. A 50 µL volume of buffer AVE was added to the columns and centrifuged at 8,000 rpm for 1 minute. The eluted 50 µL was pipetted back onto the column, and an additional 50 µL of buffer AVE was added and centrifuged for 1 minute at 8,000 rpm. The columns were discarded, and the tubes containing the RNA were placed on ice.

To remove PCR inhibitors, the Zymo columns (One Step PCR Inhibitor Removal Kit) (Zymo Research, CA, USA) were inserted into collection tubes, and 600 µL of the prep solution provided in the kit was added. The mixture was centrifuged at 8,000 rpm for 3 minutes. The Zymo columns were then transferred to new 1.5 mL tubes, and 100 µL of viral RNA was added to the columns and centrifuged again at 8,000 rpm for 3 minutes.

Quantification of TV and HAV RNA using RT-qPCR

The 4X TaqPath™ One-Step RT-qPCR Master Mix (MM) (Thermo Fisher Scientific) was used. The MM also contained 50 µM virus forward primer, 50 µM virus reverse primer, and 5 µM virus-specific probe. A 15 µL volume of the master mix was aliquoted into MicroAmp Optical 8-tube Strips (Applied Biosystems). The primer and probe sequences for TV (Tian et al., 2013) and HAV were used as reported previously (FDA/BAM, 26). Appropriate controls, such as no-template controls (MM C-) and positive controls, were included. A 5 µL volume from each viral RNA sample was added to the PCR tubes. Each sample was tested in duplicate.

The RT-qPCR assay was performed using a thermal cycler (QuantStudio 5 system) with the following cycling conditions for TV: reverse transcription at 50 °C for 30 minutes, polymerase activation at 95 °C for 15 minutes, followed by 45 cycles of 95 °C for 15 seconds, 53 °C for 20 seconds, and 60 °C for 50 seconds (Tian et al., 2013). The RT-qPCR amplification of HAV was carried out under the following conditions: reverse transcription at 50 °C for 50 minutes, polymerase activation at 95 °C for 15 minutes, followed by 50 cycles of 95 °C for 10 seconds, 53 °C for 25 seconds, and 64 °C for 70 seconds, as reported in the BAM 26 method (FDA/BAM, 26).

Optimization of BAM elution step for recovering infectious TV from Strawberries:

The BAM 26 method elution step is based on 6% TGBE (pH 9.5). This buffer was compared to two other elution buffers for the recovery of infectious TV from strawberries: (1) infection media, DMEM, supplemented with 2% FBS and 1% AA (pH ~7) “DMEM/2% FBS”, and (2) commercially available carbonated water (pH: 4.1) (Brand: SARATOGA) (Summa et al. 2017). Following elution, the rest of the BAM steps were followed as described above. Furthermore, another modification tested was the use of homogenated strawberries in DMEM/2% FBS instead of whole strawberries. Briefly**,** fresh strawberries without the calyx were weighed, cut into pieces, and transferred to a beaker, in which they were homogenized in infection media using a juice blender. The liquid was strained using a sterile strainer and collected in falcon tubes. TV was then spiked into these strawberry homogenates and processed according to the steps of the BAM method. The recovery of infectious TV was quantified. In addition, elution and recovery of viruses as done in the ISO method using 1% TGBE (pH 9.5) was included for comparisons.

Finally, the elution step alone was examined by incubating TV in 1 ml of the BAM (6% TGBE, pH 9.5) and DMEM/2% FBS (pH 7) for 15 minutes at room temperature. In addition, TV incubations in 1% TGBE, pH 9.5 as done in the ISO method were included for comparisons. Following this step, the infectivity of the viruses was directly quantified using TCID50 assay.

Optimizing the clarification step for recovering infectious TV from strawberries:

The clarification step in the BAM method is done by centrifugation at 12,000 x *g* for 15 minutes at 4 °C. To test the effect of speed on recovery of infectious viruses, TV was added to 1 ml aliquots of 6% TGBE (pH 9.5) and DMEM/2% FBS (pH 7), and then directly centrifuged at various speeds (2000, 4000, 6000, 8000 and 10,000 rpm) for 15 minutes at 4 °C. Following centrifugation, both the pellet and supernatant fractions were collected and tested for recovery of infectious TV. In addition, clarification as done in the ISO method using 1% TGBE (pH 9.5) was included for comparisons.

Optimizing the concentration step for recovering infectious TV from strawberries:

In the BAM method, ultracentrifugation at 170,000 x *g* for 45 minutes is used. To test whether speed affect virus recovery in the pellets, the standard speed was compared to slower but longer period of centrifugation (125,000 x *g* for 120 minutes) using TV spiked in 30 ml of DMEM (pH 7). Following ultracentrifugation, both the pellet and supernatant fractions were collected and tested for recovery of infectious TV. In addition, the virus concentration by PEG ± NaCl as used in the ISO method was included for comparisons of virus recovery in the pellet fractions only.

Statistical analysis:

Each experiment was repeated independently a total of three times, using three technical replicates for each matrix, virus or method tested. Percent virus recovery for BAM And ISO methods was determined based on input and recovered virus titters. Percent virus recovery for viruses in pellets and supernatants for the clarification and concentration steps were calculated based on control samples not subjected to centrifugations. The entire data set was $\log_{10}$-transformed. Statistical analyses were performed using GraphPad Prism version 10 (Graph Pad Software, USA). Analysis of variance (ANOVA) with Bonferroni post-test was utilized for comparing multiple means. Linear regression analyses were performed between initial virus infectivity titers spiked on strawberries and Ct values obtained from recovered viruses from strawberries. The slope and Y-intercept of the equation as well as the goodness-of-fit of the linear regression (R^2) was determined. Multiple logistic regression analysis was performed using Ct-values and their corresponding presence/absence of infectious virus (Yes/No outcome). Significance was determined when the *P* value was less than 0.05, denoted in the tables and figures by different alphabets or by asterisks.

CHAPTER 4

RESULTS

Objective 1: To determine the FDA/BAM chapter 26 and ISO limit of detection and the relationship between RT-qPCR Ct values and virus infectivity

1) Determining the BAM and ISO method limit of detection for virus infectivity from strawberries

Ten-fold serially diluted TV inocula were prepared and used to contaminate strawberries. Strawberries were then processed following the standardized BAM methods to recover the viruses. The ISO method was used for comparisons. Using the BAM method, the lowest recovered infectious TV was 1.8 ± 0 log $TCID_{50}$/50 g strawberries, corresponding to Ct 33.2 ± 2.2 (Table 4). Using the ISO method, the lowest recovered infectious virus was 1.7 ± 0.1 log $TCID_{50}$/50 g of strawberries, corresponding to Ct 36.2 ± 0.5 (Table 4). These Ct values were not significantly different between the BAM and ISO methods (Table 4). Similar experiments were repeated using HAV spiked on strawberries. Using the BAM method, the lowest infectious HAV recovered from strawberries was 1.6 $TCID_{50}$/50 g, corresponding to Ct 34.1 ± 0.4 (Table 4). The ISO method gave similar results, whereby, the lowest recovered infectious HAV was 1.8 $TCID_{50}$/50g, equivalent to Ct 35.6 ± 4.5 (Table 4). These Ct values were not significantly different between the BAM and ISO methods (Table 4).

2) Determining the BAM and ISO method's limit of detection for virus RT-qPCR Ct values from strawberries

The RT-qPCR LODs for TV on strawberries for BAM and ISO were at Ct values of 36.5 ± 5 and 38.5 ± 2, respectively (Table 4). At these LOD Ct values no infectious viruses can be directly detected from strawberries by the TCID50 infectivity assay (Table 4). The RT-qPCR LODs for the BAM and ISO for HAV on strawberries were at Ct values of 35.2 ± 0.1 and 37.8 ±1.2, respectively, which also did not yield any infectious viruses by TCID50 assay (Table 4). Comparing all recovered HAV and TV from strawberries by both BAM and ISO methods revealed no statistically significant difference between the processing methods for RT-qPCR Ct values (Table 4).

3) Determining the relationship between Ct values and infectivity for viruses recovered from strawberries

Because there were no significant differences between the BAM and ISO methods for Ct values between TV and HAV recovered from strawberries, logistic regression model was fitted to the overall TV and HAV Ct values and their corresponding infectivity status (i.e. Yes/No outcome). This model showed that there is a low probability (< 0.5) of directly detecting infectious viruses from strawberries when $Ct > 36$ (Figure 1). This model was statistically significant with a p-value $=0.04$ and $R^2= 0.64$ (Figure 1). Also, the model had a positive and negative predictive power of 93.7 and 75 %, respectively.

4) Predicting the initial virus infectivity titer on strawberries using Ct values of recovered virus from strawberries

Linear regression analyses were performed between the initial infectivity titers of the viruses that were spiked on strawberries and the corresponding Ct values of the viruses recovered from strawberries. For TV, there was a significant linear relationship between these two variables using either the BAM or ISO methods, with R^2= 0.98 and 0.97, respectively (Figure 2). The slopes of the two best fit lines obtained from the linear regression analyses for the BAM and ISO methods were not significantly different from each other's (p=0.90). The latter indicates that both methods performed similarly over a wide range of TV infectivity titers. Similarly, for HAV, a significant linear relationship between these two variables was found, whether using the BAM or ISO methods (R^2 =0.95 and 0.93) (Figure 3). Again, the slopes of the best fit lines obtained from BAM and ISO methods for HAV were not significantly different (p=0.53). The latter indicates that both methods performed similarly over a wide range of HAV titers.

Using the linear regression equations for both TV and HAV for both the BAM and ISO methods (Figure 2 and 3), at Ct between 40-44 it can be predicted that the strawberries initially had one-unit infectious virus (1 $TCID_{50}$) per 50g.

Objective 2: To examine the BAM steps for recovery of infectious TV from strawberries.

1) Optimizing the elution step of the BAM method for recovery of infectious TV from strawberries

Infectious TV (~$5x10^4$ to $9x10^4$ $TCID_{50}$/50 g of strawberries) was dried on the surface of strawberries or suspended in elution buffer alone, before being processed using the BAM method. The results showed that the baseline average recovery of infectious TV from strawberries using BAM was at ~10 % (Figure 4A). The recovery of TV suspended in elution buffer processed following the BAM method was not significantly different from strawberries, at ~16 % (Figure 4A).

To optimize the BAM method, DMEM/2% FBS (pH 7), and carbonated water (pH 4) were tested as elution buffers. Results showed that DMEM/2% FBS (pH 7), recovered infectious TV at a similar % from strawberries and buffer alone, ~11 and 7%, respectively (Figure 4B). In contrast, carbonated water (pH 4) recovered infectious TV at a significantly higher recovery from strawberries as compared to carbonated water alone, ~5 and 1%, respectively (Figure 4C). Another way for eluting viruses was tested by homogenization of the strawberries in DMEM/2% FBS (pH 7) followed by the step of the BAM method. Results showed that homogenization recovered from infectious TV at ~23%, while the recovery of infectious TV in buffer alone was ~ 18% (Figure 4D). For reference the ISO method recovered infectious TV from strawberries and 1% TGBE (pH 9.5) alone at 14 and 1%, respectively (Figure 4E). Comparing all the recovery (%) from various tested buffers revealed that there were no significant differences for elution in DMEM/2% FBS, carbonated water, homogenization or ISO method as compared to the BAM method (Table 5). Two-way ANOVA performed on the overall data of recovery (%) of infectious TV, whether from strawberry or buffer alone (matrix factor) and elution buffer type (method factor), showed that the matrix did not significantly affect the variation in the overall data, however the elution type did.

Next, the first step of the BAM method (shaking at 150 rpm for 15 min at room temperature) was examined separately from the other steps. TV was incubated in 6% TGBE (pH 9.5), 1% TGBE (pH 9.5) and DMEM/2% FBS (pH 7) (used as a control) for 15 min at room temperature and then the virus was quantified by TCID50 assay. It was observed that infectious TV inactivated in both the 1% TGBE (pH 9.5) and 6% TGBE (pH 9.5) by ~ 0.98 to 1 log $TCID_{50}$/ml (~90%), respectively in comparison to TV in DMEM/2% FBS (Figure 5). The latter indicated that the pH of the buffer significantly affected the recovery of infectious TV, while the percent beef extract did not. To confirm this observation, the BAM method was re-tested by using the TGBE elution buffer at two different percentages of beef extract while lowering the pH to 8. Specifically, the 6% TGBE and 1% TGBE at pH 8, gave statistically similar recovery of infectious TV at ~62 and 76%, respectively (Figure 6). The recovery of infectious TV in the 6% TGBE and 1% TGBE at pH 8 buffers alone was at 50 and 57%, respectively (Figure 6). The recovery of infectious TV by both two tested buffers alone and on strawberries was significantly higher from the recovery of TV by the BAM method (Table 5). Therefore, lowering the pH of the BAM elution buffer significantly improved the recovery of infectious TV as compared to the standardized elution buffer of the BAM method.

2) Optimizing the clarification step of the BAM method for recovery of infectious TV from elution buffers

The second step in the BAM method is intended to separate the eluted viruses from the strawberry debris recovered in the elution buffer from the first step. The latter is achieved by centrifuging the recovered viruses in buffers at 12,000 x g for 15 min at 4 °C. By the end of this centrifugation step, the viruses are expected to stay in the supernatants while the pellet is usually discarded. To test the effect of centrifugation speed on the recovery of infectious viruses, TV was suspended directly in the standard elution buffers (6% or 1 % TGBE, pH 9.5) in 1 ml aliquots and then subjected to various centrifugation speeds (2,000 to 10,000 rpm for 15 min at 4 °C). In addition, TV was also suspended in DMEM/2% FBS (used as a control) and subjected to the same centrifugation speeds. After centrifugation, the supernatants and the pellets were collected, and infectious viruses were tested in both fractions.

Results showed that for TV suspended DMEM/2% FBS, there were no significant differences in the average recovery of infectious TV across all speeds tested (Figure 7A). Specifically, infectious TV was recovered at 79 to 82% at the speeds of 2000 to 10,000 x *g*, respectively (Figure 7A). While in the pellets, infectious TV was recovered at 0.5 to 1.6% at the speeds of 2000 to 10,000 rpm, respectively (Figure 7A). In contrast, by using the 6% TGBE (pH 9.5) of the BAM method, the recovery of infectious TV in the supernatant increased non-significantly from ~45% to 85% as the centrifugation speed increased from 2000 to 10,000 rpm, respectively (Figure 7B). However, the increase in speed significantly increased the percent recovery of infectious TV in the pellet from 5 to 12% (Figure 7B). As for the 1% TGBE (pH 9.5) of the ISO method, the recovery of infectious viruses was not significantly different across all the speeds, averaging at 120 to 117% as the speed increased from 2000 to 10,000 rpm, respectively (Figure 7C). However, the recovery of infectious TV in the pellet increased significantly from 1 to 13%, as the speed increased from 2000 to 10,000 rpm, respectively (Figure 7C).

Comparing all the recovery (%) from various tested speeds for TV in the supernatants revealed that there were no significant differences between buffers for any speed (Table 6). However, for TV in pellets, the 6% TGBE pH (9.5) showed the highest infectious TV recovered at the speeds of 6,000 and 8,000 x rpm (8-9%, respectively) as compared to TV in pellets of DMEM/2% FBS (1-1.3%, respectively) (Table 6). Two-way ANOVA was performed on the overall recovery (%) data of infectious TV in the supernatants, while accounting for the elution buffers (elution buffer factor) and the various centrifugation speeds (speed factor). This analysis revealed that the speed did not significantly affect the overall variation in the recovery of infectious TV in the supernatants, while the type of elution buffer did. When performing two-way ANOVA for the overall recovery data of infectious TV in the pellets, it was revealed that both the speed and elution type significantly affected the variation in the data.

3) Optimizing the concentration step of the BAM method for recovery of infectious TV from elution buffer

Because the BAM method elutes viruses from 50 g samples of strawberries in 30 ml of elution buffer, the third step is to concentrate this volume into smaller volumes of ~ 1 ml, which are more suitable for molecular analyses by RT-qPCR. For this reason, ultracentrifugation at very high speeds (170,000 x *g* for 45 min at 4 °C) is used in the BAM method. Following ultracentrifugation, it is expected that the viruses will be concentrated in the pellet, while the supernatant is usually discarded. To determine the effect of speed and time on the concentration of the viruses in the pellets, another speed at a longer period (125,000 x *g* for 120 min) was tested and compared to the BAM's combinations of speed and time. Following ultracentrifugation, both the supernatant and the pellet fractions were collected and tested for recovery of infectious viruses.

The results showed that using BAM speed and time combinations, infectious TV was recovered in the pellet at 1.8-fold increase, while the supernatant still contained infectious viruses at 0.3-fold of what was initially present before ultracentrifugation (Figure 8). The lower speed for longer period of ultracentrifugation (125,000 x *g* for 120 min) improved the viruses in pellets to ~2.3-fold increase; however, this was not significantly different than the speed/time used by BAM (Figure 8). Furthermore, at 125,000 x *g* for 120 min, infectious viruses were still detectable in the supernatant at a similar level (0.26-fold) to the BAM speed and time (Figure 8). Overall, both tested speeds and time combinations resulted in about 0.26 to 0.30 (i.e. 26-30%) infectious TV being lost in the supernatants.

The ISO method adopted a simpler way of pelleting viruses through shaking the virus in the clarified 1% TGBE (pH adjusted to 7) in PEG mixed with NaCl for 1 h at 4 °C, then performing regular centrifugation at 10,000 x *g* for 30 min at 4 °C. Therefore, this virus precipitation method was evaluated for recovery of infectious TV in the pellet. The results showed that infectious TV was concentrated at 2.6-fold of what was initially present prior to addition of PEG+NaCl (Figure 9). Retesting this precipitation method without the addition of NaCl showed a similar fold increase of infectious TV in the pellet (~2.1 fold) (Figure 9). Comparing the ISO and BAM ways of concentrating viruses in the pellets revealed no significant difference in fold-increase of infectious viruses in the pellets (Table 4).

Figures

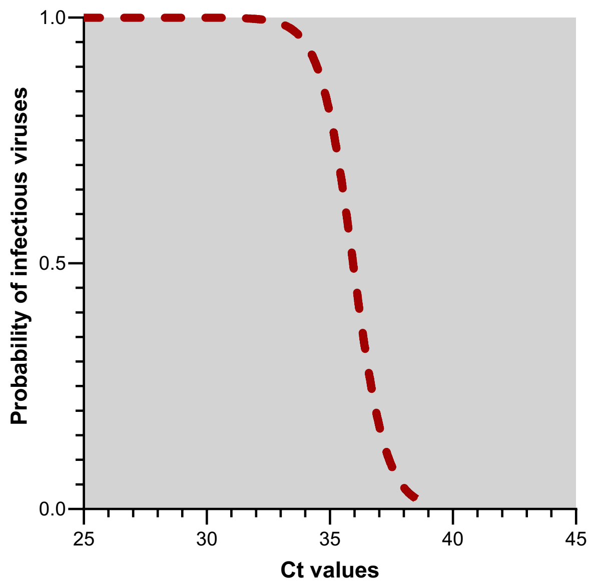

Figure 1: Logistic regression model to predict the probability of recovering infectious viruses using the overall BAM and ISO data for TV and HAV Ct values from strawberries and their infectivity status (Yes/No outcome).

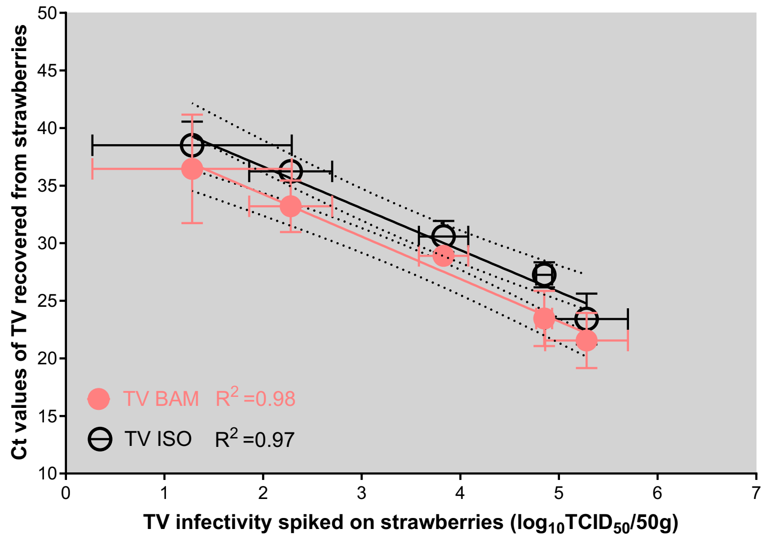

Figure 2: Regression analyses between original TV infectivity titers spiked on strawberries and the Ct values for TV recovered from strawberries using both the BAM and ISO methods. Solid lines represent the best fit lines for each method and the dashed lines are the 95% confidence bands of the best fit lines.

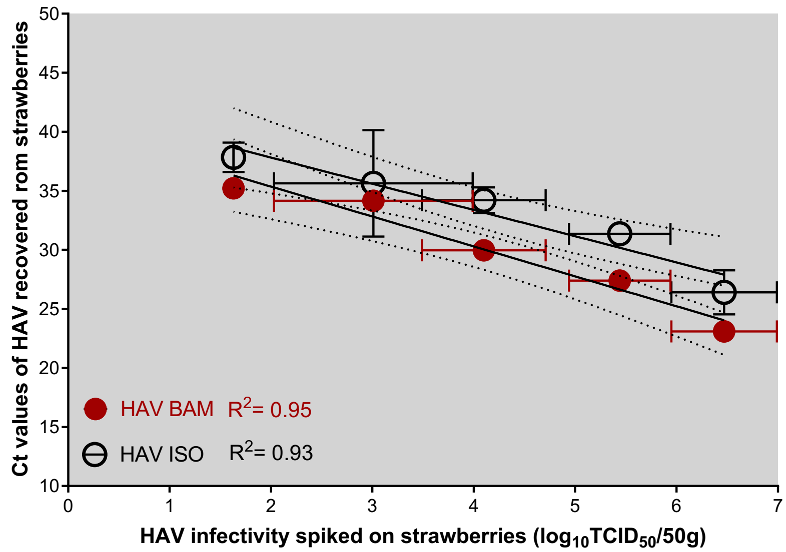

Figure 3: Regression analyses between original HAV infectivity titers spiked on strawberries and the Ct values for HAV recovered from strawberries using both the BAM and ISO methods. Solid lines represent the best fit lines for each method and the dashed lines are the 95% confidence bands of the best fit lines.

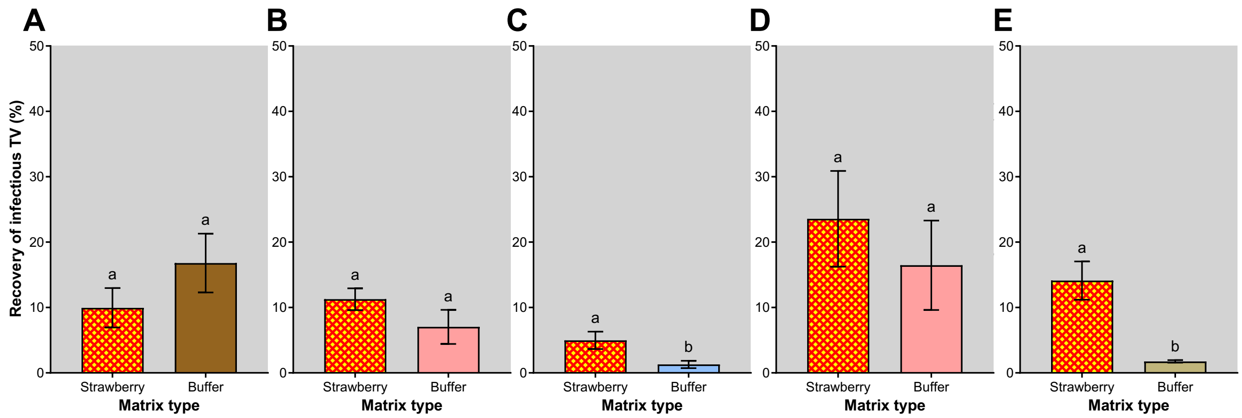

Figure 4: Examining the BAM method for recovery (%) of infectious TV. The virus was eluted from strawberries or buffers using the BAM method with variation in the elution step: (A) standardized 6% TGBE (pH 9.5), (B) DMEM/2% FBS (pH 7), (C) Carbonated water (pH 7) and homogenization in DMEM/2% FBS (pH 7) followed by the BAM steps. (E) Recovering TV from strawberries and in buffer by the ISO method was included for comparisons. Means with different letters indicate significant differences ($p < 0.05$).

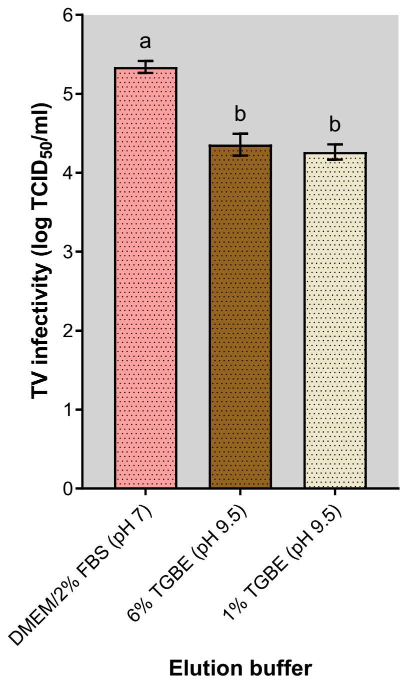

Figure 5: Examining the BAM elution step: Infectious Tulane virus was incubated in 1 ml aliquots of the 1 and 6% TGBE (pH 9.5) and DMEM/2% FBS (pH 7) for 15 min at RT and then directly tested by TCID50 assay. Means with different letters indicate significant differences ($p < 0.05$).

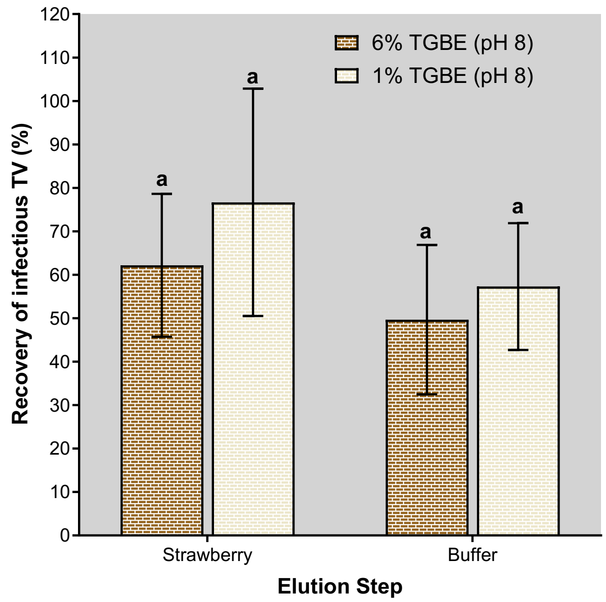

Figure 6: Re-examining the BAM method for recovery of infectious Tulane virus. The virus was eluted from strawberries using the BAM TGBE elution buffer at different beef extract percent and pH. Means with different letters indicate significant differences ($p < 0.05$).

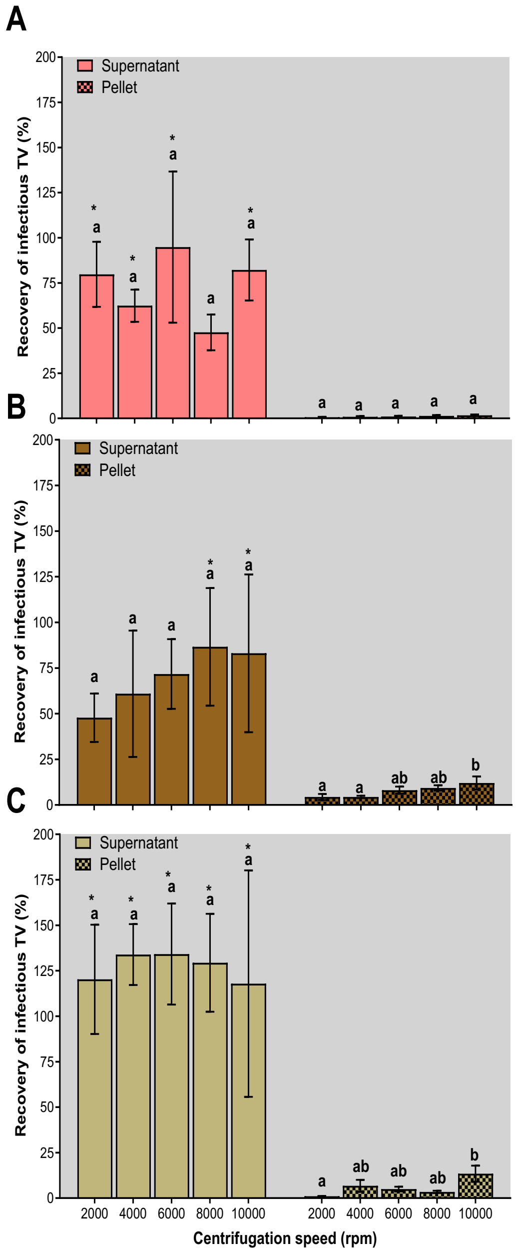

Figure 7: Examining the BAM clarification step. The virus was suspended in (A) DMEM/2% FBS pH 7, (B) 6% TGBE, pH 9.5 and (C) 1% TGBE pH 9.5 and then centrifuged at different centrifugation speeds for 15 min at 4 °C. The virus was tested by TCID50 assay in both the supernatant and pellet fractions. Means with different letters indicate significant differences ($p < 0.05$). Asterisks indicate significant differences between corresponding fractions.

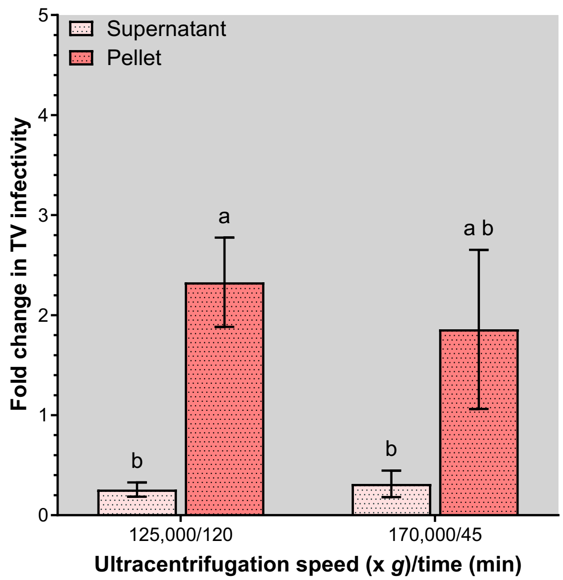

Figure 8: Examining the BAM concentration step. TV was suspended in 30 ml DMEM (pH 7) and then tested at a lower speed for longer duration in comparison to the standard BAM speed & time. Fold change was calculated from pre-ultracentrifugation virus titers. Means with different letters indicate significant differences ($p < 0.05$).

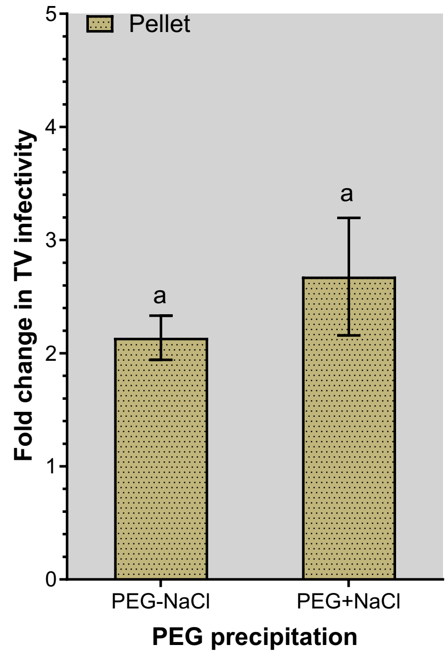

Figure 9: Examining the ISO virus concentration step: TV was suspended in clarified 1% TGBE, pH adjusted to 7 after which PEG was added with and without NaCl and the samples were incubated shaking for 1h at 4 °C. Fold change was calculated from pre-PEG virus titers. Means with different letters indicate significant differences ($p < 0.05$).

Tables:

Table 4: Summary of the BAM And ISO methods' infectivity and RT-qPCR limit of detection (LOD) for TV and HAV recovered from artificially contaminated strawberries. The recovery of viruses was done using the standardized BAM and ISO methods. Values represent mean ± standard deviations. Similar alphabets indicate no statistical significance ($P<0.05$) in the ANOVA tests comparing HAV and TV across methods. UD refers to undetected.

	Infectivity LOD & corresponding Ct		**Ct LOD & corresponding infectivity**	
Viruses on strawberries	Infectivity log $TCID_{50}$/50g	Ct	Infectivity log $TCID_{50}$/50g	Ct
TV /BAM	1.8 ± 0	33.2 ± 2.2a	UD	36.5 ± 5a
TV/ ISO	1.7 ± 0.1	36.2 ± 0.5a	UD	38.5 ± 2a
HAV/BAM	1.6 ± 0	34.1 ± 0.4a	UD	35.2 ± 0.1a
HAV/ISO	1.8 ± 0	35.6 ± 4.5a	UD	37.8 ± 1.2a

Table 5: Recovery (%) of infectious TV using the BAM and ISO methods, with variations in the elution step. Values represent mean ± standard error. Similar alphabets indicate no statistical significance (P<0.05) in the ANOVA tests across all elution types across methods (Column comparisons). Asterisks indicate significant difference between recovery from strawberries and buffer only (Row comparisons)

	% recovery of infectious TV	
	From Strawberries	From buffer only
Elution type (BAM)		
6% TGBE, pH 9.5	9.9 ± 3 bc	16.8 ± 4.5 bc
DMEM/2% FBS, pH 7	11.2 ± 1.6 bc	7 ±2.6 c
Carbonated Water, pH 4	4.9 ±1.3 c	1.3 ± 0.5 c
Homogenization in DMEM/2% FBS	23.5 ± 7.3 ac	18.5 ± 6.9 ac
6% TGBE, pH 8	62.1 ± 16.4 ab	49.6 ±17.2 ab
1% TGBE, pH 8	76.6 ± 26.1 a	57.3 ± 14.5 a
Elution type (ISO)		
1% TGBE, pH 9.5 (ISO)	14.1 ± 2.5* bc	1.75 ± 0.18 c

Table 6: Summary of TV recovery (%) for the clarification step: Infectious TV was centrifuged for 15 min at 4 C in the elution buffers of BAM and ISO methods as compared to control DMEM/2% FBS (pH 7). Values represent mean ± standard error. Similar alphabets indicate no statistical significance (P<0.05) in the ANOVA tests across all supernatants or pellets for a specific speed (Column comparisons). Asterisks indicate significant difference within the speeds of specific elution buffer as compared to 2000 x *g* (row comparisons).

% recovery of infectious TV					
Supernatants					
Centrifugation speed (rpm)	**2000**	**4000**	**6000**	**8000**	**10, 000**
DMEM/2%FBS (pH 7)	79 ± 18 a	62 ± 9 a	94 ± 41 a	47 ± 10 a	82 ± 16 a
6% TGBE (pH 9.5)	47 ± 13 a	60 ± 34 a	71 ±19 a	86 ± 32 a	83 ± 43 a
1% TGBE (pH 9.5)	120 ± 30 a	133 ± 17 a	134 ± 27 a	129 ±27 a	117 ± 62 a
Pellets					
DMEM/2%FBS (pH 7)	0.6 ± 0.1 a	0.8 ± 0.4 a	1 ± 0.4 b	1.3 ± 0.5 b	1.6 ± 0.5 a
6% TGBE (pH 9.5)	4 ± 1.6 a	4 ± 0.6 a	8 ±1.9 a	9 ± 1 a	12 ± 3 a
1% TGBE (pH 9.5)	1 ± 0.1 a	6 ± 3 a	5 ±1 ab	3 ± 0.6 ab	13 ± 4 a

Table 7: Summary of TV recovery (%) for the concentration step: Infectious TV was ultracentrifuged at 170,000 xg for 45 min at 4 C in DMEM and compared to 125,000 xg for 120 min. The ISO method of virus pelleting in PEG+NaCl was included for comparisons. Values represent mean ± standard error. Similar alphabets indicate no statistical significance (P <0.05) in the ANOVA test between pellets (Row comparisons). Presence of asterisks indicate significant difference between fractions (Column comparisons). ND indicates not done.

TV infectivity fold change				
	BAM 26 (DMEM, pH 7)		**ISO 15216 (PEG -NaCl)**	**ISO 15216 (PEG -NaCl)**
Ultracentrifugation speed and time	**170,000 x *g* (45 min)**	**125,000 x *g* (120 min)**	**10,000 x *g* (30 min)**	**10,000 x *g* (30 min)**
Supernatants	0.31 ± 0.1* a	0.26 ± 0.0* a	ND	ND
Pellets	1.86 ± 0.8 a	2.33 ± 0.4 a	2.67 ± 0.5 a	2.14 ± 0.1 a

CHAPTER 5

DISCUSSION

The relationship between viral RNA detection via RT-qPCR and the infectivity of viruses such as TV and HAV is an important aspect of food virology. Because HuNoV and HAV are difficult to assess in cell culture, surrogate virus TV, and cell-culture adapted strain of HAV were used to explore how the infectivity of these viruses on strawberries correlates with RT-qPCR Ct values. Our results demonstrated a clear difference between the detection of viral RNA, which is measured by RT-qPCR, and the virus's ability to infect cells. While RT-qPCR is sensitive and can detect viral RNA at low levels, it does not accurately reflect the virus's ability to cause infection.

Our findings showed that the lowest infectious titers of TV and HAV detected by the BAM and ISO methods on strawberries were similar at ~ 1.6 to 1.8 log $TCID_{50}$/ 50 g (i.e. 39 to 63 viral particles) with corresponding Ct values that were not significantly different. Most of the previous studies using the BAM or ISO methods did not report on the LOD of the method used (Bartsch et al., 2016; Raymond et al., 2021; Trudel-Ferland, Collard, et al., 2024) or reported an LOD for viral RNA without giving the corresponding Ct values (Hennechart-Collette et al., 2021; Hida et al., 2018). For example, a previous study following the ISO method reported LODs of 10^2 GC/g for HAV and HuNoV GII and 10^3 GC/g for HuNoV GI from multicomponent foodstuffs without mentioning Ct values (Hennechart-Collette et al., 2021). On the other hand, only one previous study determined the infectivity LOD for HAV from strawberry and lettuce which was ~5-10 PFU/ml (Bidawid et al., 2000). However, in that study, the authors eluted the viruses from the exact spots they inoculated the viruses on the produce, using very small volumes of PBS (1ml), and thus there was no need for processing the produce, which led to a high virus recovery of ~85% and lower LOD as compared to our study. The authors acknowledged that LOD may be influenced by several factors, including virus capture efficiency, suggesting that only a fraction of the virus particles in the sample are detected. Therefore, our study fills a knowledge gap in our understanding of the BAM and ISO detection limits for infectious viruses using the TCID50 assay.

Our results suggest that the BAM and ISO methods are similar in recovering infectious viruses, which was confirmed in our second objective (~10-14%, respectively). The similar BAM and ISO infectivity LODs may reflect a limitation by the TCID50 assay which is based on visual observation of CPE under microscopy. Using this TCID50 assay, the lowest observable CPE was in the first row of infected cells which corresponds to about 1.8 log $TCID_{50}$/ml. Including control negative strawberries (i.e. without viruses added) samples within each experiment allowed the differentiation between CPE and cytotoxicity due that may occasionally occur due to concentrated berry or remaining beef extract molecules in the pellets. Therefore, using the BAM and ISO methods to detect infectious viruses is limited by the detection limit of the TCID50 assay itself. Future advancement in virus infectivity detection may allow recovery of lower infectious titers for viruses recovered using the BAM And ISO methods.

Additionally, our study revealed that at virus Ct values >36, the probability of detecting infectious viruses directly from strawberries decreases. In order to avoid biases in Ct values obtained, all virus samples recovered from strawberries were subjected to the PCR inhibitor cleanup using the Zymo kit, as recommended by the BAM method. In a previous study, the rate of virus infectivity reduction was shown to be faster than virus RNA reduction for MNV, FCV, and poliovirus in surface and groundwater microcosms incubated at 25 °C (Bae & Schwab, 2008). The latter indicates that the presence of viral RNA does not necessarily indicate the presence of infectious viruses. Additionally, the authors found that Ct values of 36-38.8 (obtained without an RNase pre-treatment) corresponded to 1 PFU for Poliovirus, FCV, and MNV in environmental waters and therefore at higher Ct values, viruses are less likely to be infectious (Bae & Schwab, 2008). While another study used human intestinal enteroids (HIE) to evaluate the persistence of infectious HuNoV in raw surface freshwater (Esseili et al., 2025), found that the persistence of infectious HuNoV in freshwater microcosms ranged from ≤1 day to ≥7 days. However, the decay rates for RNA from intact HuNoV capsids ranged from 0.04 to 0.54/day, predicting a much longer RNA persistence: 4.2 to 57.5 days for a 1 log reduction in viral RNA. The study found that HuNoV RNA Ct values <32 predicted a higher probability of detecting infectious HuNoV in contaminated raw freshwater using HIE, suggesting that HuNoV in raw freshwater is less likely to be infectious at Ct values >32. In the latter study, the samples were treated with RNase prior to RNA extraction to reduce detection of free RNA from damaged viruses by RT-qPCR. Treating samples with RNase will reduce the total RNA detected by RT-qPCR and thus will result in higher Ct values. Both the BAM and ISO methods do not mention treating recovered virus samples with RNase prior to RNA extraction. Therefore, it is difficult to compare our study to these previous studies due to different matrices and sample pre-treatments. Overall, these previous studies highlight that Ct values cutoff for when viruses are deemed less likely to be infectious may depend on the virus and the matrix as well as whether the sample was subjected to prior RNase treatment or not. In addition, the % positive and negative predictive powers of the logistic model used can affect the results.

Other studies recognized the limitations of RT-qPCR in determining viral infectivity (Trudel-Ferland, Levasseur, et al., 2024). This is because RT-qPCR can detect even small amounts of viral

RNA, which may not come from infectious virus (Steele et al., 2022). The persistence of viral RNA in food, even after the virus has lost its ability to infect, has also been documented in other studies. For example, Tan et al. observed that viruses on contaminated produce can be detected by RT-qPCR, but the viruses lost their infectivity (Tan et al., 2015). Thus, RT-qPCR assay cannot differentiate between live and dead viruses, further emphasizing the need to use infectivity assays alongside RT-qPCR for a more accurate assessment of infection risk (Trudel-Ferland, Levasseur, et al., 2024). This limitation is reflected in our study, where at the RT-qPCR LOD Ct values of 35.2 to 38.5, no infectious viruses were detected using the TCID50 assay. Furthermore, the detection and quantification of HuNoV and HAV RNA in frozen raspberries using RT-qPCR remain challenging due to the low concentration of viral particles and the presence of RT-qPCR inhibitors (Larocque et al., 2022). The lack of an easy cell culture model for wildtype HAV and HuNoV is a key limitation. While RT-qPCR is useful for detecting viral RNA, it may not always reflect the actual risk of infection. Without infectivity assays, the presence of HAV and HuNoV GII RNA do not confirm the viability of the virus, emphasizing the need for complementary methods, such as receptor-binding assays, antibody coated magnetic beads or ideally the use of HIE assays, to accurately assess infection risks (Oteiza et al., 2022).

Knowing that infectious viruses are lost during the processing steps of the BAM or ISO method (10-14% recovery), and not all viruses are easily culturable, using statistical models to predict initial virus infectivity from Ct values obtained from viruses on strawberries become important. In our study linear regression analysis indicated that at Ct values between 41 and 44 for TV and 40 to 42 for HAV, the initial viral infectivity titers on strawberries are predicted to be as low as 1 $TCID_{50}$/50 g. The Ct values is consistent with quantification limits of RT-qPCR cycle number of 45, beyond which quantification is deemed not reliable (Bae & Schwab, 2008). Previous studies on virus prevalence on berries that detected HuNoV or HAV reported very high Ct values of 39-41 (Stals et al., 2011). At these Ct values, our logistic regression model would indicate a low probability of being able to directly detect infectious viruses from these berry samples. Other studies reported a wide range of Ct values for viruses recovered from berries, such as Ct values between 30 and 40 (Baert et al., 2011) and between 33 and 44 (Bennett et al., 2023), which would suggest a combination of infectious and non-infectious viruses being present on various berries, depending on the Ct value. Overall, our linear regression model provides better insight into the interpretation of Ct values obtained for viruses in berries, but ultimately a more sensitive infectivity assay is needed to confirm infectivity.

Prior to the publication of the ISO and BAM methods for viruses on berries, there were a wide range of virus recovery methods from berries with different virus extraction and concentration methods which can impact the virus recovery (%) and accuracy of results. Studies by (Mäde et al., 2013) and (Hida et al., 2018) revealed that varying protocols could lead to differences in the virus recovery rate from food matrices, further underscoring the importance of standardized protocols to ensure reliable results. The recovery rates of infectious TV from strawberries using the BAM and ISO methods were approximately 10% and 14%, respectively. For comparison, previous studies achieved the following recovery rates from different food matrices. Kim et al. (2008) achieved a recovery of 79% for HuNoV GII.4 RNA in strawberries using PEG 8000, though recovery from raspberries was significantly lower at 6%. Oteiza et al. (2022) found a recovery rate of 23% for FCV in strawberries and blueberries using PEG 6000, which is higher than the recovery rate for TV in our study. Summa et al. (2012) reported variable recoveries for HuNoV GII.4 RNA across different methods. Specifically, from fruit salads, the recovery for PEG, ultracentrifugation and immunomagnetic separation was 28, 4 and 4%, respectively. These results highlight that recovery rates vary depending on the food matrix and the method used. Also, the significant difference in recovery may be attributed to differences in virus infectivity versus total RNA which is a reflection of the loss of virus infectivity during processing while RNA can still be detected from damaged and non-damaged viral particles.

In an effort to optimize the BAM method, alternative elution buffers such as DMEM/2% FBS (pH 7) and carbonated water (pH 4) were tested. However, these buffers showed no significant improvement over the standard BAM method. Similarly, the ISO method, using 1% TGBE (pH 9.5), recovered infectious TV at 14% from strawberries, which is comparable to the BAM method. This aligns with prior studies, such as Hida et al. (2013), who reported a 36.7% recovery of HAV infectivity from sliced tomatoes using 1% TGBE (pH 9.2), indicating that pH variations and differences in matrix composition can affect recovery rates. A slight improvement in TV recovery was observed when homogenization of strawberries in DMEM/2% FBS was incorporated, leading to a 23% recovery. This suggests that mechanical processing may enhance viral release from the strawberry matrix. However, the recovery was still lower than that reported for RNA-based studies. Kim et al. (2008) reported recovery of HuNoV GI RNA at 43.89% from fruit salad and 55.21% from vegetable salad using KNT buffer (pH 9.2), while HuNoV RNA recovery using 1% TGBE was 41.42%. These results indicate that while RNA extraction methods yield higher recovery percentages, infectious virus recovery remains a challenge, potentially due to virus inactivation during processing.

Further investigation into the BAM method revealed that the pH of the elution buffer significantly impacted infectious TV recovery. Infectious TV was inactivated by approximately 90% in 6% TGBE and 1% TGBE at pH 9.5, emphasizing the detrimental effect of high pH on viral infectivity.

In contrast, adjusting the elution buffer pH to 8 resulted in a significant increase in recovery (~62–76%). These findings align with Butot et al. (2006), who reported lower HAV recovery (2.99–15.8%) from fresh and frozen berries using a high-pH elution buffer (50 mM glycine, 100 mM Tris, 1% beef extract, pH 9.5), confirming that high-pH elution buffers can compromise the infectivity of enteric viruses. A previous study showed that TV infectivity inactivated on average by 0.2 and 2 log when incubated at 37 °C for 30 min in 100 mM carbonate buffer, pH 9 and pH 10, respectively (Cromeans et al., 2014). However, when TV was incubated at room temperature for 90 minutes in M199 culture media adjust to pH 9 and 10, ~0.5 and 2 log reductions occurred, respectively (Arthur & Gibson, 2015). Therefore, TV infectivity is highly susceptible to pH variation between 9 and 10, where the pH of elution buffers used in BAM and ISO falls.

When comparing the BAM and ISO virus concentration methods, our study showed that ultracentrifugation yielded a 1.8-fold increase in infectious TV in the pellet which was similar to 2.6-fold increase achieved by the ISO method. To my knowledge no previous studies performed side-by-side comparison of just the virus concentration steps of the ISO and BAM methods. However, Oteiza et al. (2022) used PEG 6000 to recovery 23% of FCV from strawberries and blueberries, and suggested that PEG is more effective than ultracentrifugation for certain viruses. Summa et al. (2012) found that the combination of PEG and ultracentrifugation provided variable results for HuNoV. Specifically, PEG yielded a recovery rate of 28% from fruit salad while ultracentrifugation provided a recovery of 4% from fruit salad. It is likely that the upstream steps impacted those recoveries and thus this is not a side-by-side comparison of the virus concentration step only to draw conclusions on PEG versus ultracentrifugation alone.

One of the limitations of our study is the use of TV as a surrogate for HuNoV. While TV shares several structural, binding and genomic similarities with HuNoV, it may not fully mimic HuNoV attachment, persistence and elution from strawberries. For example, no previous studies were performed to compare the pH effect on TV versus HuNoV infectivity. As pH was found to significantly affect the elution of TV from strawberries, knowledge about how pH affects the infectivity of HuNoV is important as this may introduce discrepancies in virus recovery efficiency and detection accuracy. Other limitations of our study include the short duration allowed for viruses to attach to strawberries surfaces. Viral spots were left to dry for ~ 45 minutes and then subjected to elution by ISO or BAM. Viruses on strawberries in real life scenarios may have been sitting on the surface of the fruit for longer time which may affect its binding and the methods' ability to elute them. Additionally, this study was conducted using good looking strawberries without any visible damage, which may not fully represent field or retail strawberries that may have more surface blemishes allowing viruses to be protected from being eluted. Furthermore, the logistic regression had an $R^2 \sim 0.6$ and did not have 100% positive and negative predictive powers, therefore, inclusion of more data derived from other infectious enteric viruses recovered from artificially spiked strawberries can help improve this model.

Future research should focus on the use of the actual HuNoV pathogen and the HIE to assess the virus infectivity recovered from strawberries, allowing for a more realistic assessment of virus detection in food matrices. Integrating HIE with advanced detection assays could enhance our understanding of virus persistence and transmission, ultimately bridging the gap between surrogate-based studies and real-world foodborne contamination scenarios. Also, future research could explore how temperature fluctuations, relative humidity, UV exposure, and packaging materials influence the presence and detection of viruses on fresh produce.

CHAPTER 6

CONCLUSION

The FDA/BAM method consists of three main steps that basically allow the elution, separation and concentration of viruses from berries. The final detection of viruses is achieved by RT-qPCR which quantifies viral RNA through Ct values. These Ct-values are inversely proportional to the amount of viral RNA; however, their relationship to virus infectivity is not well delineated. Results from this study showed that the RT-qPCR LOD of the FDA/BAM method was found to be similar to the ISO 15216 method for both HAV and TV. Whether using the BAM or ISO methods, infectious HAV and TV are less likely to be directly recovered from strawberries when the RT-qPCR Ct values are >36. Furthermore, linear regression analyses showed that Ct values between 40 and 44, obtained by BAM or ISO methods, predict the presence of one TCID50 infectious unit (HAV or TV) per 50 g of strawberries.

Results from the BAM method optimizations indicated that the first step is the most critical step for improving recovery of infectious viruses from strawberries. The pH of the elution buffer significantly affected the recovery of infectious TV. Using 6 or 1 % TGBE pH 8, improved the recovery of infectious viruses from baseline 10% to 62-76%. The second step did not affect much the recovery of infectious viruses. However, for the third step, there is still a need for further improvement in the concentration step to capture the infectious viruses lost in the supernatants.

Overall, while RT-qPCR is an effective tool for detecting viral RNA, it cannot provide an accurate representation of viral infectivity. Our study showed that RT-qPCR should be used alongside infectivity assays, when possible, to more reliably assess the risk of foodborne viral infections. In the absence of an easy, cost-effective and scalable cell culture method for HuNoV and HAV from berries, results from this study provide better insights into the interpretation of virus RT-qPCR Ct values obtained from strawberries.

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