CPS 2024 RFP – FINAL PROJECT REPORT

Project Title:
Hydrogel affinity particles to enable high throughput screening of soft fruits for intact HAV virions

Project Period:
January 1, 2025 – December 31, 2025 (extended to January 31, 2026)

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Objectives:

1. Adapt existing RT-qPCR HAV assays to multiplex RT-dPCR format and assess performance for quantification and detection of HAV RNA.
2. Assess the HAV RNA extraction efficiencies of two RNA extraction kits compatible with an automated workflow.
3. Compare Nanotrap concentration to azo-dye pretreatment for the detection and quantification of HAV RNA within intact virus capsids.
4. Assess the efficiency and performance characteristics of the complete workflow for the recovery and quantification of HAV RNA within intact virus capsids.

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FINAL REPORT

Summary of Findings and Recommendations

In this one-year proof-of-concept project a novel workflow using Nanotrap hydrogel affinity particles and reverse transcription digital PCR was developed and assessed for recovering hepatitis A virus (HAV) RNA from soft fruits. The complete workflow demonstrated an average recovery efficiency of 28.9% for blueberries inoculated with an HAV control material consisting of intact capsids. Crucially, the Nanotrap recovery efficiency for RNA molecules outside of a virus capsid (free RNA) was 13 times lower than for RNA within a capsid, indicating the preferential recovery of intact virus capsids, which are associated with a higher likelihood of infectivity, over free RNA, which is not infectious. When the workflow was modified to accommodate implementation on a liquid handling robot, the recovery efficiencies of an archival culture fluid from a wild-type strain of HAV for both blueberries and strawberries was significantly lower than the protocol optimized using the intact capsid control material. Nonetheless, the proof-of-concept results strongly suggest the Nanotrap particle concentration could increase the whole process efficiency and the ability to discriminate between intact capsids and free RNA. Future work should validate under what conditions and formats the performance can be extended to wild-type HAV strains.

Abstract

Currently, screening of soft fruits for hepatitis A virus (HAV) is performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). While RT-qPCR can detect extremely low amounts of RNA, it cannot discriminate between residual HAV genetic material (free RNA), which is harmless, and intact HAV virions, which are potentially infectious, due to the long persistence of RNA compared to infectious virions. In this one-year proof-of-concept project a novel method using Nanotrap particles and RT-digital PCR (RT-dPCR) was assessed for recovering intact HAV virions from soft fruit. When adapted to duplexed RT-dPCR format, standardized HAV assays (FDA and ISO) showed strong linearity for quantifying HAV cDNA and RNA with the 95% limit of detection (3.5 gene copies (GC)/reaction) for the FDA assay approaching the theoretical limit (3 GC/reaction). For blueberries inoculated with a custom control material consisting of intact capsids (Armored RNA Quant HAV), a workflow comprised of elution with glycine beef extract, concentration by Nanotrap Microbiome A particles, extraction and purification by AllPrep PowerViral DNA/RNA, and quantification by RT-dPCR (FDA assay) demonstrated an average whole process recovery efficiency of 28.9% ± 15.5%. Importantly, the Nanotrap particles concentrated the intact capsids 13 times more efficiently than free RNA molecules, suggesting the particles show some discriminatory power between intact virions and free RNA. When the fully optimized workflow was modified (extraction kit change) to accommodate automation on a liquid handling platform and applied to strawberries and blueberries inoculated with an archived culture fluid from wild-type HAV, the whole process recovery efficiencies were significantly lower, although the form of HAV RNA in the fluid (within capsid versus free) was unknown. Future work should further explore the application of the method to wild-type HAV strains, including intact virions derived from fresh cell culture.

Background

In the United States, foodborne outbreaks of hepatitis A virus (HAV) are exceptionally rare compared to person-to-person outbreaks.[1] Nonetheless, there have been occasional HAV outbreaks associated with frozen pomegranate seeds (2013), frozen scallops (2016), fresh blackberries (2019), and frozen or fresh

strawberries (2016, 2022, 2023). The sporadic nature of HAV foodborne outbreaks is substantiated by surveillance of HAV genetic material (*i.e.*, RNA) on produce. Since 2019, the US Food & Drug Administration (FDA) has detected HAV RNA on only 3 of 524 domestic berry samples and 6 of 989 imported frozen berry samples.[2] Similar trends have been observed in Canada from with no detection of HAV RNA among 926 fresh and 3,292 frozen berry samples from 2016 to 2019.[3]

The standard method of testing food products for HAV is reverse transcription quantitative PCR (RT-qPCR), which detects the virus's RNA genetic material.[4,5] While RT-qPCR is analytically sensitive, detection of HAV RNA alone is insufficient to establish the presence of an infectious virus, which requires an intact capsid and functional proteins in addition to its RNA. Recently, RNA genetic material has been observed to persist much longer than infectious virus particles (*i.e.*, virions) for both SARS-CoV-2[6] and norovirus.[7] The long persistence of RNA increases the likelihood of RT-qPCR false positives for infectious HAV on food products. This was exemplified during Canadian Food Inspection Agency (CFIA) surveillance from 2014 to 2016, which found no reported illnesses associated with 7 berry samples testing positive for virus RNA.[8] Nonetheless, as currently formulated, regulatory guidance does not require confirmatory testing to better assess the likelihood of infectious viruses in response to RT-qPCR positivity. In the absence of an improved method of discerning infectious HAV on soft fruits, there is an ongoing risk of large economic losses associated with recalls triggered by RNA positivity during RT-qPCR screening of produce.

The aim of this proof-of-concept project was to use hydrogel affinity trap particles (*i.e.*, Nanotraps) and reverse transcription digital polymerase chain reaction (RT-dPCR) to concentrate and quantify HAV RNA molecules contained within intact capsids from soft fruits. Nanotraps are hydrogel nanoparticles functionalized with histology stains and magnetic material for efficient concentration of viruses from dilute solutions via automated workflows.[9] Preliminary studies indicate Nanotraps, such as those widely used for SARS-CoV-2 testing in wastewater, preferentially bind intact bacteria and virions.[10] While, existing HAV RT-qPCR methods could potentially be modified with azo-dye pretreatments to measure only RNA within intact capsids, such dye treatments could further exacerbate the time and labor intensity of standard HAV screening methods for fruits, thus further limiting throughput and increasing expense.[11] Nanotraps, on the other hand, could enable efficient concentration of intact virus capsids using automated workflows for high throughput screening of soft fruits. Pilot testing indicates hydrogel particles are compatible with fruit rinsate for HAV testing via RT-qPCR.[12] Importantly, magnetic bead separations have also demonstrated compatibility with azo-dye pretreatment if further enrichment of intact capsids is required. DNA and RNA can be extracted from Nanotrap-concentrated viruses and purified using a variety of commercial kits available in automated formats. The purified genetic material is compatible with a variety of molecular testing endpoints including reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid screening[13] and RT-qPCR (standard method), RT-dPCR, and sequencing (genotyping for outbreak investigation[14]).

Research Methods and Results

We developed and assessed a four-step method using Nanotrap particles and RT-dPCR to recover, enrich, and measure RNA from intact capsids containing RNA from HAV. In the first step of the method, virions were eluted from soft fruits (frozen) using a tris/glycine/beef extract (TGBE) buffer adopted from existing standard methods.[4,5] Second, intact virions were captured and concentrated from the TGBE buffer using Nanotrap Microbiome A particles functionalized for virus capture and magnetic separation and implemented on a KingFisher Apex platform.[12] Third, the Nanotrap-captured virions were lysed and extracted with commercial RNA extraction kits. Fourth and finally, RNA targets will be measured using RT-dPCR with adapted RT-qPCR assays. [4,5] The method was developed, optimized, and assessed using HAV

control materials inoculated into the workflow in a step-by-step fashion proceeding upstream from the analytical endpoint, RT-dPCR interrogation, to the soft fruit elution. Here the methods and results from each step are described in the same order from downstream to upstream, which is the order in which the experiments were performed beginning first with the control materials used for the experiments.

HAV Control Materials

The preliminary method development experiments in this project were performed using an Armored RNA Quant molecular quality control (Bio-Techne, Minneapolis, Minnesota, USA). Armored RNA consists of lab-synthesized intact male-specific coliphage-like capsid containing a DNA or RNA template.[15] Phage-like capsid control materials have been used as whole process controls for the detection of RNA viruses in various matrices, including vegetable eluates.[16] The Armored RNA Quant HAV contained a 500 base RNA transcript (Strain HM-175, wild type, M14707: 51 – 550) from the HAV 5' untranslated region that contains the target sequences for RT-qPCR assays from both the International Organization for Standardization (ISO)[5] and the FDA.[4] The RNA transcript was in vitro transcribed to maintain fidelity with naturally occurring RNA features and the Armored RNA Quant HAV was delivered at a concentration of 5×10^7 gene copies (GC)/mL. Working aliquots (20 µL) of Armored RNA Quant HAV were prepared by diluting 10,000-fold with TSMIII buffer (10 mM Tris, 100 nM NaCl, 1 mM $MgCl_2$, 0.1% gelatin, 0.3% Microcide III, pH 7.0) and were stored at -20 °C per the manufacturer's instructions. For each experiment, the required number of Armored RNA Quant HAV aliquots were thawed on ice immediately prior to use to avoid excessive freeze thawing. Following the preliminary experiments, the whole process recovery was also assessed using aliquots of culture fluid from HAV strain HM175/18f (VR-1402, American Type Culture Collection, Manassas, Virginia, USA), which were stored at -80 °C prior to single use. To preserve other control materials for workflow development, the initial RT-dPCR optimization experiments were performed using Ultramer DNA oligonucleotides (Integrated DNA Technologies, Coralville, Iowa, USA) of the ISO assay sequence (174 bases) and the FDA assay sequence (90 bases).

HAV Assays by RT-dPCR

To quantify HAV RNA, the existing FDA[4] and ISO[5] RT-qPCR assays were adapted and optimized for RT-dPCR performed on a QIAcuity One 5plex dPCR System (Qiagen, Hilden, Germany) using the QIAcuity OneStep Advanced Probe Kit (Qiagen). The HAV assays were duplexed with Dual-labeled Probes (Biosearch Technologies, Petaluma, California, USA) with the FDA probe in the green channel (FAM) and the ISO probe in the yellow channel (TAMRA) and forward and reverse primers (Integrated DNA Technologies). Reactions were prepared per the manufacturer's recommendations to a final volume of 40 µL with RNA or DNA template volumes added ranging from 2 µL to up to 10 µL depending on the experiment. Thermal cycling was performed as per the manufacturer's recommendations with a 40 minute at 50 °C reverse transcription step prior to PCR amplification. Each RT-dPCR experiment included no-template and/or negative controls as per the experimental design. After amplification and imaging, each reaction was manually partitioned such that all negative controls were negative for the target (*i.e.*, no positive partitions) and then most probable template copy numbers in the experimental reactions were estimated via Poisson statistics.[17] The RNA copy number in the sample matrix was then calculated per the material flows of the workflow.

As shown in **Figure 1A**, during the initial RT-dPCR experiments with DNA templates in a 4-step dilution series, both the FDA and ISO RT-dPCR assays showed strong linearity with r^2 = 0.994 and 0.992, respectively. For the FDA assay the coefficients of variation (CoVs) ranged from 3.43% (12,500 GC/reaction) to 34.1% (33 GC/reaction), while for the ISO assay the CoVs ranged from 5.6% (6300 GC/reaction) to 78% (6.5 GC/reaction). Following heat extraction per the manufacturer's recommendations (75 °C, 3 minutes), the initial working stock Armored RNA Quant HAV titer by RT-dPCR was 3.91 ± 0.05 $\log_{10}$ GC/µL by the ISO assay and 3.67 ± 0.05 $\log_{10}$ GC/µL by the FDA assay. Subsequent

RT-dPCR experiments (**Figure 2**) indicated the ISO HAV assay template portion of the Armored RNA was decreasing with time in -20 °C storage from 400 GC/µL to 64 GC/µL from June to September while the FDA assay template copy number remained stable (235 GC/µL versus 246 GC/µL) over the same interval. For this reason, Armored RNA Quant HAV results from subsequent experiments were only considered valid and reported for the FDA assay. Over an RNA dilution series ranging from 2.41 $\log_{10}$ GC/µL to 0.45 $\log_{10}$ GC/µL, the RT-dPCR FDA assay demonstrated reasonable linearity (r^2 = 0.964), even at low template copy numbers (**Figure 1B**). The 95% limit of detection (LOD) for the RT-dPCR FDA assay was estimated to be 3.5 GC/reaction suggesting analytical sensitivity approaching the theoretical limit (3 GC/reaction). When quantifying RNA from extracted HAV culture fluid, the estimated titer by the RT-dPCR FDA assay was 5.32 ± 0.01 $\log_{10}$ GC/µL while the ISO assay was 5.19 ± 0.06 $\log_{10}$ GC/µL indicating the RT-dPCR assays demonstrated reasonable agreement for quantifying wild type HAV virions.

The results from the RT-dPCR validation experiments indicate the existing RT-qPCR assays for HAV RNA measurement are readily transferrable to RT-dPCR. RT-dPCR provides reliable quantification and detection of HAV RNA with performance comparable to RT-qPCR.

HAV RNA Extractions

During the initial extraction experiments, RNA was extracted from the Armored RNA Quant HAV using two bead-based extraction kits implemented on a KingFisher Apex (ThermoFisher Scientific, Waltham, Massachusetts, USA). The first kit trialed was the MagMax Microbiome Ultra Nucleic Acid Isolation Kit (ThermoFisher Scientific) and the second was the NucleoMag DNA/RNA Water Extraction Kit (Macherey-Nagel, Düren, Germany). RNA extractions with each kit were performed per the manufacturer's KingFisher protocols with heat lysis of the Armored RNA performed at both 95 °C and 75 °C and in the presence or absence of Ambion carrier RNA (ThermoFisher Scientific). In addition to the bead-based extractions performed on KingFisher, manual Armored RNA Quant HAV extractions were also performed using the AllPrep PowerViral DNA/RNA Kit (Qiagen) per the manufacturer's instructions with the addition of carrier RNA. After extraction, the purified RNA produced by each kit was measured using the FDA assay on RT-dPCR as previously described. During each experiment, an aliquot of Armored RNA Quant HAV was heat extracted in parallel and also quantified by RT-dPCR. The percentage recovery for each kit and condition was then computed as the ratio of the RNA quantity from the kit to the RNA quantity from heat lysis multiplied by 100.

As shown in **Table 1**, the RNA recoveries for each bead-based kit were well below 10%. The maximum recovery for both kits was achieved with 75 °C lysis and carrier RNA where MagMax Microbiome Ultra averaged a 1.7% recovery and NucleoMag DNA/RNA averaged 5.6%. Under each condition the NucleoMag DNA/RNA recovery was higher than the MagMax Microbiome Ultra; however, the results indicate the extraction of the Armored RNA Quant HAV was extremely inefficient for both bead-based kits. We hypothesized this could be due to poor performance of the bead-based kits with the low molecular weight RNA molecule (500 bases) in the Armored RNA. To assess the performance of a silica column kit, we also performed Armored RNA extractions using the AllPrep Power Viral, which achieved notably improved performance with a mean recovery of 43.3%. It is notable that recovery was well below 100% for all kits and conditions trialed suggesting that RNA extraction and purification could be a meaningful, even if overlooked, source of analyte loss in HAV workflows.

HAV Concentration from Fruit Wash with Nanotrap Particles

To assess the performance of Nanotrap Microbiome A Particles (Ceres Nanosciences, Manassas, Virginia, USA) for concentrating HAV virions from fruit wash, 10 mL aliquots of glycine beef extract buffer (GBE) were prepared to the specification described in the FDA protocol for Concentration and Extraction of Enteric Viruses from soft fruit: fresh and frozen. Each aliquot was seeded with Armored RNA Quant HAV,

amended with pectinase, and subjected to a manual Nanotrap capture procedure followed by RNA extraction with the AllPrep PowerViral as detailed in Ceres Nanosciences APP-088 Revision 2. The resulting purified RNA was quantified by RT-dPCR using the FDA assay as previously described. To examine the effects of fruit debris on RNA recovery, in follow on experiments, approximately 5 g of freshly thawed blueberries were added to each 10 mL aliquot of GBE and shaken at 150 rpm for 15 minutes at room temperature. The resulting supernatant was recovered and then seeded with Armored RNA Quant HAV and pectinase and processed with a manual Nanotrap capture procedure and RNA exaction with AllPrep PowerViral as previously described. A 10-fold dilution series of fruit debris (neat, 10-fold, 100-fold) was also prepared by using GBE as a diluent and then seeding each step of the dilution series with Armored RNA and pectinase. The resulting samples were processed as previously described and the resulting RNA was quantified by RT-dPCR. The fruit debris dilution series experiments were also repeated with seeding of HAV RNA that had been heat lysed (*i.e.*, extra-capsidular RNA) to assess the recovery efficiency or RNA enclosed within a capsid compared to RNA alone. In each experiment, aliquots of Armored RNA Quant HAV were heat extracted and the resulting RNA quantified by RT-dPCR in parallel. The percentage recovery was then computed as the ratio of the RNA quantity from the experimental sample to the RNA quantity from heat lysis multiplied by 100.

As shown in **Figure 3A**, during the first batch of experiments performed with GBE in the absence of fruit debris HAV RNA was not recovered during Nanotrap concentration and extraction. In the case of the blueberry debris with no dilution (neat, n = 10) average recovery was 30.8% ± 19.6%. When the fruit debris was diluted 10-fold the average recovery decreased to 23.5% ± 6.25% while at 100-fold dilution the average recovery increased to 52.0% ± 33.2%, although the differences in recovery were not significant ($p > 0.9999$). The variation in the recovery efficiency at each fruit debris level was substantial with coefficients of variation ranging from 26.6% (10-fold) to 64% (neat, 100-fold). Nonetheless, our results clearly demonstrate the recovery of HAV RNA by the Nanotrap particles is dependent on the presence of fruit debris within the matrix. We hypothesize the molecules released from the epicuticular wax and the pericarp during elution (polysaccharides, lipids, etc.) serve as an intermediary between the Nanotrap particles and the virions with virions potential adhering to the blueberry-derived particulates and then the blueberry particulates adhering to the Nanotraps. The expected workflow efficiency is best estimated by the 30.8% average recovery from the experiments with neat fruit eluate. This efficiency includes both the concentration and RNA extraction step in series (*i.e.,* multiplying the efficiency of the concentration by the efficiency of the RNA extraction). Given the average efficiency for RNA extraction in the previous experiments (43.3%), the efficiency of the concentration step is estimated at nearly 71% (*i.e.,* 30.8/43.3). Another, and perhaps more likely, possibility is that the presence of the fruit debris enhanced the efficiency of both the Nanotrap concentration and the downstream RNA extraction simultaneously.

The recovery of the extra-capsidular RNA along the 10-fold fruit debris dilution series was 2.37%, 2.60%, and 4.15% for neat, 10-fold, and 100-fold, respectively (**Figure 3B**). Comparing the average recovery of the RNA within a capsid (30.8%) with the average of the RNA alone (2.37%) indicates that the Nanotrap particles recover HAV RNA within a virus capsid 13 times more efficiently than RNA molecules alone. While this is not perfect discrimination between RNA in capsids versus RNA alone, the result suggests that, all things being equal, HAV RNA detections resulting from the Nanotrap concentration method are more likely to have come from intact virions than not.

Whole Process HAV Recovery Efficiency

With the concentration and extraction efficiencies established, the next set of experiments was intended to determine the whole process efficiency for the entire workflow (elution, concentration, extraction). Samples of frozen blueberries (~50 g per sample) were thawed at room temperature and then inoculated with 20 µL of Armored RNA Quant HAV by pipetting 10 spots of 2 µL onto the surface of randomly selected

spots on the blueberries. The inoculated blueberries were then carefully transferred into a Whirl-Pak filter bag and eluted with 30 mL of GBE as previously described. The resulting supernatant was aspirated into a clean 50 mL centrifuge tube, divided into three roughly 8 mL aliquots. Each aliquot was subjected to a manual Nanotrap capture procedure followed by RNA extraction with the AllPrep PowerViral as previously described. The resulting purified HAV RNA was quantified using RT-dPCR with the FDA assay. In each experiment, aliquots of Armored RNA Quant HAV were heat extracted and the resulting RNA quantified by RT-dPCR in parallel. The percentage recovery was then computed as the ratio of the RNA quantity from the experimental sample to the RNA quantity from heat lysis multiplied by 100.

To assess the recovery efficiency of HAV RNA in a format with both the Nanotrap concentration and RNA extraction performed onboard the KingFisher Apex with wild-type HAV virions, we also performed whole process experiments where samples of blueberries and strawberries (~50 g per sample) were inoculated with HAV strain HM175/18f culture fluid in a manner similar to what was previously described for Armored RNA Quant HAV. Nanotrap concentration and RNA extraction were performed onboard the KingFisher following the protocol outlined in Ceres Nanosciences APP-114, Revision 0. Since the AllPrep PowerViral DNA/RNA extraction must be performed manually, HAV RNA was instead purified using the NucleoMag DNA/RNA Water Extraction Kit (Macherey-Nagel, Düren, Germany) also onboard the KingFisher. The resulting purified HAV RNA was quantified using RT-dPCR with both the FDA and ISO assays. In each experiment, aliquots of HAV strain HM175/18f culture fluid were directly extracted using the NucleoMag kit on the KingFisher and the purified RNA was also quantified by the FDA and ISO assays using RT-dPCR in parallel with the other samples. The percentage recovery was then computed as the ratio of the RNA quantity resulting from each inoculated sample to the RNA quantity from direct extraction multiplied by 100. Importantly, in this instance since the control was also extracted by the same method as the samples, the recovery estimated is for the elution and concentration steps in series and the extraction efficiency cannot be estimated due to the experimental design.

During the Armored RNA blueberry inoculation experiments, the average whole process recovery efficiency among batches ranged from 38.5% to 18.8% (**Figure 4A**), although the efficiency was highly variable within batches with CoVs from 38.1% to 68.9%. The average recovery efficiency among all samples was 28.9% ± 15.5%. This suggests that even under ideal and tightly controlled experiments the recovery efficiency is highly variable. The overall process efficiency is the product of the individual efficiencies of each step in the workflow (elution, concentration, extraction). Based on the estimated efficiency of concentration and extraction (30.8%), the efficiency of the elution step is estimated to be 93.8% (28.9/30.8). For Armored RNA Quant HAV inoculated onto blueberries, eluted per the FDA protocol, with manual Nanotrap concentration and extraction with AllPrep PowerViral, the estimated efficiencies for each step are 43.3% for the extraction, 71.1% for the concentration, and 93.8% for the elution. These results indicate the GBE elution followed by Nanotrap concentration are highly efficient (66.7%) for the Armored RNA Quant HAV control material.

Given that the objective for the project was a fully automated workflow performed using a KingFisher Apex, the final experiments included two key changes from the fully vetted workflow developed with Armored RNA. First, the extraction method was change to the NucleoMag DNA/RNA kit, so as to be compatible with the KingFisher Apex. Second, given the depletion of the Armored RNA stocks, a wild-type HAV strain (HM175/18f) in culture fluid was used to inoculate the fruit. With these two changes, the workflow performance deviated drastically. As shown in **Figure 4B**, the average elution and concentration efficiency for the blueberry experiments was 1.07% when measured by the FDA assay and 0.91% when measured by the ISO assay. In the case of the strawberries, the mean efficiencies were less than 1%. One possible explanation for the deviance is the form of the HAV RNA in the culture fluid. Notably, the culture fluid used in these experiments was an archival sample purchased from ATCC and was not freshly

cultivated. If most of the RNA in the culture fluid is not contained within intact capsids, then the Nanotrap concentration efficiency would be expected to be roughly 13 times less efficient based on the RNA only concentration experiments. If the elution efficiency were simultaneously reduced 5-fold for RNA versus RNA within a capsid, then the drastically reduced performance is possible while still being consistent with the previous Armored RNA experimental results. Unfortunately, the experiments to assess the proportion of free RNA versus RNA contained within capsids in the culture fluid are not yet complete as of the time of this writing.

Outcomes and Accomplishments

This one-year proof-of-concept delivered severable notable outcomes and accomplishments. First, two standardized RT-qPCR assays for HAV, the ISO and FDA assays, were formatted successfully to duplex RT-dPCR. The assays demonstrated strong linearity for quantifying both HAV cDNA and HAV RNA with analytical sensitivity (3.5 GC/reaction) approaching the theoretical limit (3 GC/reaction). The experimental results indicate RT-dPCR provides reliable quantification and detection of HAV RNA. A comparison of three different extraction methods indicated the silica column-based kit provided superior efficiency (43.3%) for the extraction of an HAV Armored RNA control, although the extraction could not be performed on the KingFisher Apex platform and had to be implemented manually. During concentration with Nanotrap particles, it was found that the presence of debris from the fruit itself released during elution is crucial to enhancing recovery with a 30.8% increase in recovery efficiency versus when fruit debris was absent. Crucially, the recovery efficiency of HAV Armored RNA (*i.e.,* RNA within a capsid) was 13-fold higher than the recovery of RNA molecules alone, indicating some discrimination between intact HAV capsids and free RNA. When adapted to a fully automated workflow consisting of elution per the FDA method followed by concentration and extraction performed on a KingFisher Apex platform, the recovery efficiency of an archival wild-type HAV culture fluid was extremely low, although experimental results to confirm whether the culture fluid consisted of intact virions are not yet available. The Nanotrap concentration method showed strong performance for concentrating an HAV control material known to consist of intact capsids. Further work should closely examine under what conditions this performance extends to wild-type HAV.

APPENDICES

Publications and Presentations

There are no publications or presentations to report outside of the project update provided at the Center for Produce Safety Research Symposium in Summer 2025. The full experimental results are currently being prepared for publication.

Budget Summary

This project was awarded $57,341 in research funds.

Invoicing and Billing:

- Salary: $19,009.02
- Fringe: $1,764.40
- Supplies and Materials: $21,786.33
- Other Costs (Tuition Remission): $5,473.37
- Indirect: $1,661.89
- Travel: $3,043.08 (2025 Symposium)

Total Invoicing and Billing: $52,738.09
Balance: $4,602.91 (Travel to 2026 Symposium)

Table and Figures

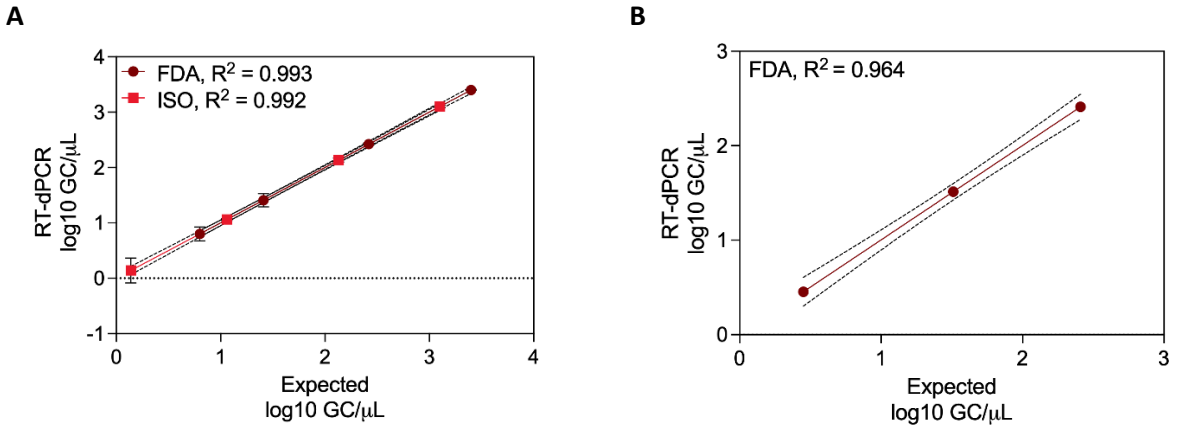

Figure 1. Linearity of the FDA and ISO RT-dPCR assays for quantifying DNA oligomer control material (A) and linearity of the FDA RT-dPCR assay for quantifying Armored RNA Quant HAV RNA (B).

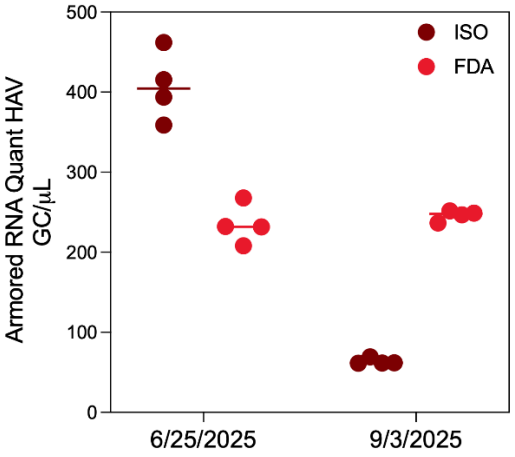

Figure 2. Stability of the ISO and FDA templates of the Armored RNA Quant HAV control material stored at -20 °C from June to September 2025.

Table 1. HAV RNA recovery efficiencies from Armored RNA Quant HAV extracted using various combinations of extraction kit, lysis temperature, and carrier RNA

Method	dPCR Assay	Recovery (%) Mean ± Standard Deviation
MagMax	FDA	0.63 ± 0.29
NucleoMag	FDA	1.47 ± 0.54
MagMax (95C lysis)	FDA	0.53 ± 0.44
NucleoMag (95C lysis)	FDA	2.01 ± 0.43
MagMax (75C lysis) + Carrier RNA	FDA	1.71 ± 0.72
NucleoMag (75C lysis) + Carrier RNA	FDA	5.59 ± 0.19
AllPrep PowerViral + Carrier RNA	FDA	43.32 ± 11.41

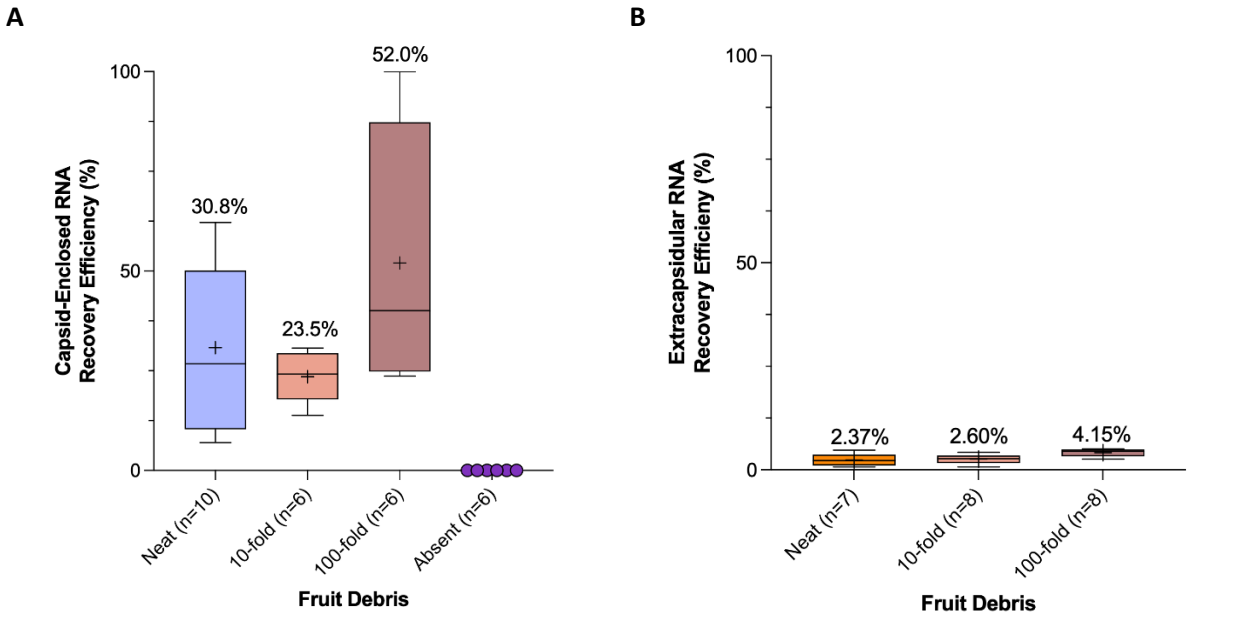

Figure 3. (A) Armored RNA Quant HAV recovery from GBE buffer containing debris from blueberry elution along a 10-fold dilution from neat to 100-fold and from GBE buffer without debris from blueberry elution (absent). (B) RNA-only recovery from GBE buffer containing debris from blueberry elution along a 10-fold dilution from neat to 100-fold.

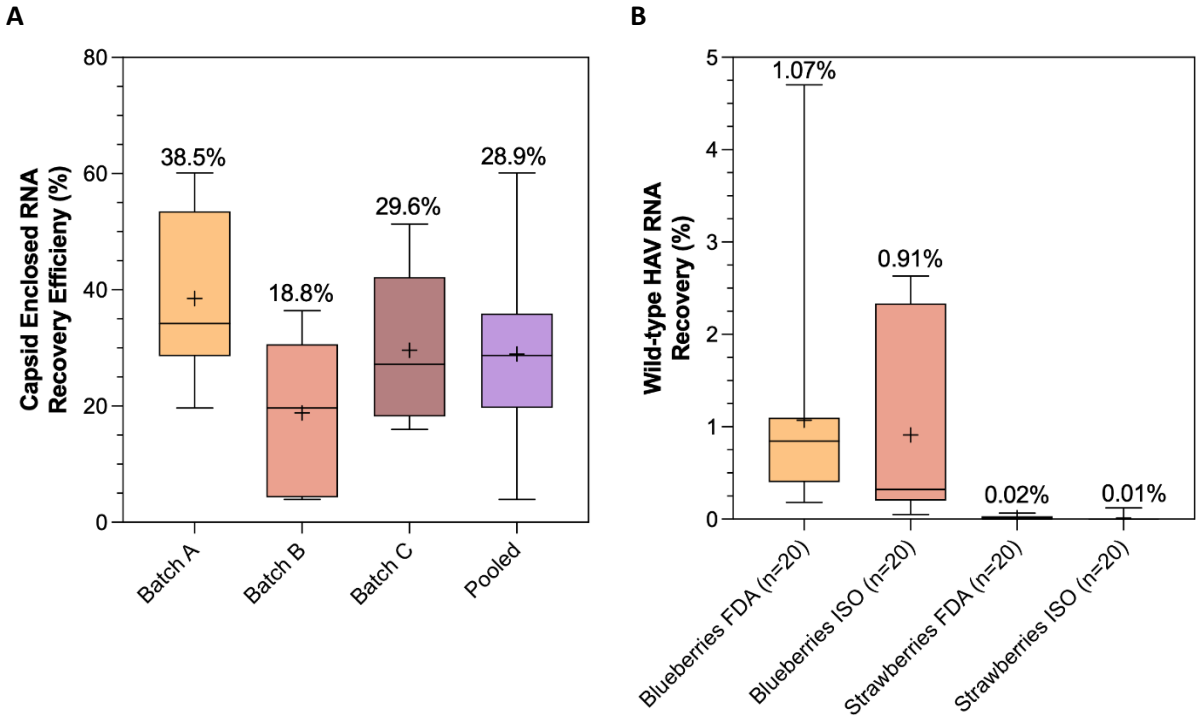

Figure 4. (A) Armored RNA Quant HAV recovery from three batches of inoculated blueberries with the average recovery efficiency for each batch denoted above the box and whiskers plot. (B) Recovery efficiency of HAV RNA from blueberries and strawberries inoculated with HAV strain HM175/18f culture fluid.

References

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