Procedure for Rapid Concentration and Detection of Enteric Viruses from Berries and Vegetables

S. Butot, T. Putallaz, and G. Sánchez*

Quality & Safety Assurance Department, Nestlé Research Center, Lausanne, Switzerland

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Several hepatitis A virus (HAV) and norovirus (NV) outbreaks due to consumption of berries and vegetables have been reported during recent years. To facilitate the detection of enteric viruses that may be present on different fresh and frozen products, we developed a rapid and sensitive detection method for HAV, NV, and rotavirus (RV). Initial experiments focused on optimizing the composition of the elution buffer, improving the viral concentration method, and evaluating the performance of various extraction kits. Viruses were extracted from the food surface by a direct elution method in a glycine-Tris (pH 9.5) buffer containing 1% beef extract and concentrated by ultrafiltration. Occasionally, PCR inhibitors were present in the processed berry samples, which gave relatively poor detection limits. However, this problem was overcome by adding a pectinase treatment in the protocol, which markedly improved the sensitivity of the method. After optimization, this concentration method was applied in combination with real-time reverse transcription-PCR (RT-PCR) using specific primers in various types of berries and vegetables. The average detection limits were 1 50% tissue culture infective dose (TCID₅₀), 54 RT-PCR units, and 0.02 TCID₅₀ per 15 g of food for HAV, NV, and RV, respectively. Based on our results, it is concluded that this procedure is suitable to detect and quantify enteric viruses within 6 h and can be applied for surveillance of enteric viruses in fresh and frozen products.

Recent epidemiological evidence indicates that enteric viruses, in particular noroviruses (NV), which cause acute gastroenteritis, but also hepatitis A virus (HAV) and rotavirus (RV), are the leading cause of food-borne illness in developed countries (16, 29, 54).

While consumption of raw or improperly cooked shellfish remains the major risk factor for food-borne outbreaks, many types of berries increasingly are being recognized as vehicles of viral gastroenteritis (9, 18, 22, 24, 30, 33, 39, 48) or hepatitis A (5, 25, 37, 40, 41) outbreaks. The increased awareness that berries can transmit viruses is due to epidemiological surveys that have played an important role in identifying sources of infection, to the availability of improved methods for diagnosis of viral infections, and perhaps also to an increased occurrence of contaminated berries, which in turn may be related to imports from developing countries. About 15 berry-related outbreaks were reported in Finland between 1998 and 2001, resulting in a ban on the use of unheated berries in all catering and other large-scale kitchens (39). Recently, six norovirus outbreaks that occurred in Europe were associated with the consumption of frozen berries imported from Poland and involved up to 1,100 people (9, 15, 30).

Vegetables, including different types of salads and green onions, have also been associated with viral hepatitis and gastroenteritis (3, 12, 20, 35, 42). Recently, a hepatitis A outbreak caused by the ingestion of contaminated green onions resulted in three deaths among a total of 601 cases (53).

Reported RV food-borne outbreaks due to the consumption of berries or vegetables are scarce, but the involvement of fecally contaminated foods is often suspected (6, 10, 17). In addition RV, as well as HAV, was detected in lettuce in Costa Rican markets (23).

Although viral food-borne disease is a significant problem, foods are rarely tested for viral contamination, and such testing is usually limited to shellfish. Frequently, the cause of an outbreak is suspected to be of viral origin, but due to the lack of sensitive and reliable methods, this assumption can be confirmed by experimental results only rarely (43). More-sensitive techniques are required to detect viruses in food samples, in which viral loads are typically much lower than those found in clinical samples, e.g., 0.2 to 224 infectious particles per 100 g shellfish meat (55) or 10⁵ to 10⁶ genomes per gram of clam (8). Therefore, the two greatest challenges for the detection of enteric RNA viruses in food are to overcome the effects of inhibitory materials coextracted with viral RNA and to concentrate the low levels of viruses into a volume that is small enough for reverse transcription-PCR (RT-PCR) analysis. Moreover, the detection of viruses in berries, especially raspberries and blackberries, can be hampered by the presence of inhibitors and a low pH. For these reasons, the presence of viruses has been demonstrated for only a few naturally contaminated vegetables or berry samples (5, 18, 23, 27, 33).

Many different methods have been described for enteric virus detection (4, 11, 13, 33, 34, 43–46, 49, 52), but none has been validated for detecting the three viruses studied herein in a wide range of berries and vegetables (Table 1). Only few laboratories are using such methods, since they are currently too expensive and time-consuming for the routine screening of food (36). Therefore, the purpose of this work was to develop a rapid, specific, sensitive, and reliable analytical procedure to detect enteric viruses in different types of berries, vegetables, and herbs.

* Corresponding author. Mailing address: Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland. Phone: 41 21 785 8092. Fax: 41 21 785 8553. E-mail: gloria.sanchez-moragas@rdls.nestle.com.

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MATERIALS AND METHODS

Cells, viruses, and infections. A clinical stool sample positive for NV (geno-
group I, Valetta strain; kindly provided by the RIVM, Bilthoven, The Nether-
lands) was used as NV reference material. The cytopathogenic HM-175 strain of HAV (courtesy of A. Bosch, enteric
virus group, University of Barcelona, Spain) and the human rotavirus strain Wa (ATCC, VR-2018) were propagated and assayed in FRhK-4 and MA-104 cell
monolayers, respectively. Semipurified stocks were thereafter produced in the
(Applied Biosystems) consisting of 1
/H11003
real-time PCR was performed using a TaqMan Universal PCR master mix
mega, Madison, Wis.), and 10
/H9262
primer, 1
/H9262
Germany) consisting of 1
formed at 41°C for 60 min using a Sensiscript RT kit (QIAGEN GmbH, Hilden,
(PE Applied Biosystems, Foster City, Calif.). The NV RT reaction was per-
formed at 43°C for 60 min using a Sensiscript RT kit (QIAGEN, Hilden,
Germany) consisting of 1
/H9262
PCR inhibitors. RT was performed at 55°C for 10 min, and amplification was
which markedly improved the sensitivity of the method due to the blocking of
supplemented with 5% of dimethyl sulfoxide (Merck, Darmstadt, Germany),
extracted RNA was transferred into a capillary containing 7.5
/H9262
/H9262
lo f
/H9262
curve was generated by performing real-time RT-PCR on 10-fold dilutions of
extracted RNA. The crossing points obtained from the assay of each dilution
were used to plot a standard curve by assigning the corresponding TCID50
viral genome quantification. HAV RNA was quantified with a LightCycler
hepatitis A virus quantification kit (Roche Diagnostics, Mannheim, Germany) on
a LightCycler instrument as described previously (47). Briefly, a total of 2.5 µl of extracted RNA was transferred into a capillary containing 7.5 µl of master mix
supplemented with 5% of dimethyl sulfoxide (Merck, Darmstadt, Germany),
which markedly improved the sensitivity of the method due to the blocking of
PCR inhibitors. RT was performed at 55°C for 10 min, and amplification was
performed for 1 cycle of 95°C for 30 s and 45 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 12 s. The LightCycler hepatitis A virus quantification kit contains
intrinsic HAV RNA standards that allow the number of RNA copies per sample
to be estimated.

Norovirus GI RNA was quantified using a specific assay for the Valetta strain
as described elsewhere (32) on an ABI Prism 7700 sequence detection system
(PE Applied Biosystems, Foster City, Calif.). The NV RT reaction was per-
formed at 43°C for 60 min using a Sensiscript RT kit (QIAGEN GmbH, Hilden,
Germany) consisting of 1× RT buffer, 500
µM of NVP3-F and NVP3-R primers, 0.2
µM of the TaqMan probe, 0.1 µM of the TaqMan probe, and 0.05 µM of the TaqMan
probe, 0.2 µM of NVP3-F and NVP3-R primers, 0.2 µM of the TaqMan probe, and
8 µl of RNase inhibitor. Five microliters of RNA was denatured by heating for
minutes and transferred into a capillary containing 15 µl of master mix. RT
was performed at 60°C for 30 min; amplification was performed for 1 cycle of
95°C for 15 min and 45 cycles of 95°C for 0 s and 60°C for 1 min. An RV standard
curve was generated by performing real-time RT-PCR on 10-fold dilutions of
Wa-extracted RNA. The crossing points obtained from the assay of each dilution
were used to plot a standard curve by assigning the corresponding TCID50

 Experimental protocol. Locally purchased berries (strawberries, raspberries,
blueberries, blackberries, and black currants), vegetables/herbs (lettuce, green
onions, mint, parsley, and basil), and a sugared berry mix (consisting of raspber-
ries, blueberries, and 10% sugar) were used in this study. Dilutions of viruses in
buffered saline (PBS) were tested by counting 30 µl over 10 spots
on the surfaces of fresh or frozen berries and vegetables (15 g). Inoculated
samples were air dried in a laminar flow hood for 60 min, then stored at
4°C overnight (4, 13). Viruses were released from the food surface by gentle shaking
with 60 ml of elution buffer (50 mM glycine, 100 mM Tris, 1% [wt/vol] beef
electrophoretic (PE Applied Biosystems, Foster City, Calif.), and centrifuged in a laminar flow hood for 60 min, then stored at
4°C overnight (4, 13). Viruses were released from the food surface by gentle shaking
with 60 ml of elution buffer (50 mM glycine, 100 mM Tris, 1% [wt/vol] beef
extract [pH 9.5]) for 15 min at room temperature (Fig. 1). The elution buffer was
then transferred into a Falcon tube containing a nylon cell strainer of 40-µm pore
size (BD Falcon, Basel, Switzerland) to remove particulate debris. Berries or
vegetables and the cell strainer were rinsed with 6 ml and 2 ml of elution buffer,
respectively. The recovered elution buffer was then adjusted to pH 7.0 ± 0.2 with
9.5 M HCl and centrifuged at 3,500 × g for 15 min. Further improvement of
the detection of viruses in berries (except blueberries) was achieved by adding 300 µl of
pectinase (Pectinex; Sigma, Buchs, Switzerland) in the elution buffer prior to
the centrifugation and gently shaking for 30 min at room temperature. The
extract was centrifuged at 3,500 × g for 15 min, and the supernatant was then
transferred to a Centrifloc Plus-70 centrifugal filter device (100 k NMWL; Mil-
limed, Molsheim, France) and centrifuged at 3,500 × g to concentrate the virus
particles in a volume of ca. 400 µl. The centrifugal filter device was subsequently
rinsed with 200 µl of elution buffer, pH 7.0 ± 0.2, as preliminary tests had shown
improvement in the virus recovery (Fig. 1).

The RNA extraction from concentrated samples (ca. 600 µl) was performed with a QIAamp viral RNA mini kit (QIAGEN) according to the manufacturer's
instructions. Concentrated samples of raspberries were pretreated with the Plant
RNA Isolation Aid product (Ambion, Cambridge, United Kingdom) to
remove the residual particles that could inhibit the real-time RT-PCR (see
Results). For this purpose, 600 µl of the eluate was mixed with 150 µl of the Plant
RNA Isolation Aid and 2,400 µl AVL buffer containing carrier RNA (QIAamp
viral RNA mini kit) and subjected to pulse-vortexing for 1 min. Afterwards, the
homogenate was centrifuged for 5 min at 13,000 × g to remove the debris. The
supernatant was subsequently processed using the QIAamp viral RNA mini kit.

The RNA extraction from concentrated samples (ca. 600 µl) was performed
using a NucliSens magnetic kit (bioMérieux, Genève, Switzerland) according to the
manufacturer's instructions, and a NucliSens miniMAG instrument (bio-
Mérieux) was used to collect and wash the magnetic silica particles.

RNA extracts were either immediately analyzed by real-time RT-PCR or
stored at −80°C until use. Suspensions of nucleic acids were analyzed twice by
the specific real-time RT-PCR method, which also allowed us to estimate the
number of recovered viruses or RNA copies. Positive controls, with the same
viral concentration as the suspension that was used to inoculate berries and
vegetables, were analyzed in parallel by real-time RT-PCR to determine the
recovery of the elution and concentration procedure.

Eluting conditions for berry analyses. Fifteen-gram portions of fresh straw-
berries and frozen raspberries were inoculated with ca. 120 TCID50 of HAV, 540

| Table 1. Methods for enteric virus detection in vegetables and berries |
|--------------------------|--------------------------|--------------------------|
| Food sample(s) (reference) | Sample size (g) | Method (as indicated on flow chart) | Detection limit(s) (per quantity of analyzed product) |
| Green onions (21) | 25 | Alkaline elution and PEG precipitation | 1 TCID50 HAV; 1 PCRU NV |
| Green onion and strawberry rinses (49) | 25 (only 0.1 g analyzed) | PBS elution and immunomagnetic separation | 0.5 PFU HAV |
| Lettuce (19) | 10 | Guanidinium thiocyanate elution | 10 PFU HAV; 50 PCRU NV |
| Lettuce (46) | 6 | Alkaline elution and PEG precipitation | 10 PFU HAV |
| Lettuce and strawberries (4) | Not specified | PBS elution, immunomagnetic separation, and filtration with positively charged filters | 10% recovery* |
| Raspberries (44) | 60 | Alkaline elution and ultracentrifugation | 1 × 103 to 1 × 106 PCRU HAV or 5.6 × 102 to 1 × 103 TCID50 HAV |
| Raspberries and strawberries (45) | 60–90 | Alkaline elution and ultracentrifugation | 50 PFU HAV |
| Vegetables (14) | 25 | PBS elution and filtration | 10% recovery* |
| Vegetables (13) | 100 | Alkaline elution and PEG precipitation | 50 TCID50 HAV; 1.2 × 103 PCRU NV |

* Undiluted RNAs were not tested and RT-PCR units were not quoted. ** Detection limit in infectious viruses was calculated taking into account the infectious titer of the HAV stock given by ATCC.

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PCRU of NV, and 26 TCID<sub>50</sub> of RV. Each experimental condition was analyzed in duplicate and processed the same day as described above. The following elution buffers were tested: (i) 50 mM glycine, 1% (wt/vol) beef extract (pH 9.5); (ii) 50 mM glycine, 100 mM Tris, 1% (wt/vol) beef extract (pH 9.5); and (iii) 50 mM glycine, 100 mM Tris (pH 9.5).

Comparison of virus concentration methods. Sixty-gram portions of fresh strawberries and raspberries were inoculated with ca. 480 TCID<sub>50</sub> of HAV, 2160 PCRU of NV, and 104 TCID<sub>50</sub> of RV and analyzed as a single sample in order to avoid variability. The elution buffer volumes were adjusted according the ratios mentioned above. After the pectinase treatment, the supernatant was split in four equivalent samples to compare in duplicate the efficiencies of virus concentration by polyethylene glycol (PEG) precipitation (PEG 6000; Fluka, Buchs, Switzerland) and by ultrafiltration (Centricon Plus-70).

For PEG precipitation, 68 ml of the elution buffer rinses was supplemented with 10% PEG and 0.3 M NaCl and shaken for 2 h at 4°C. Viruses were further concentrated by centrifugation at 10,000 × g for 30 min at 4°C. The pellet was immediately resuspended with 2.4 ml of AVL buffer containing carrier RNA (QIAamp viral RNA mini kit) and processed further according to the manufacturer’s instructions.

Virus detection limit and efficiency of the procedure. The detection limit of our procedure was experimentally determined by inoculating 15-g portions of berries and vegetables/herbs with serial dilutions of the three viral strains. Each experimental condition was analyzed in triplicate. The inoculated samples were processed as described above. At the end of the procedure, two values, corresponding to the level of virus present in the processed food sample on the one hand and the level of virus present in the positive controls on the other, were obtained for HAV, NV, and RV. The efficiencies of the whole procedure from different food matrices were estimated by comparing the number of viruses, or RNA copies, recovered from the food sample and the number of viruses from each positive control.

RESULTS

Comparison of the performances of different elution buffers. During the initial elution trials with berries, a pH drop of the eluate, which was probably due to the release of large quantities of acidic fruit juice, was often observed. Since the low pH may impair the release of the viruses from the food surface (13), the elution buffer was supplemented with 100 mM Tris to increase the buffering capacity. The addition of beef extract into the elution buffer improved the efficiency of the procedure 7, 3.5, and 5.7 times for HAV, NV and RV, respectively, with frozen raspberries, whereas with fresh strawberries the improvement was less marked (Table 2).

Comparison of the performances of different concentration methods. The concentration method developed in this study is a modification of an elution-concentration methodology previously reported for virus concentration from water (32). The sequential steps of elution and ultrafiltration were evaluated as a primary virus concentration step for the berry samples, and results were compared with those ob-

![Flow chart of the method](FIG. 1)

**TABLE 2. Comparison of elution efficiencies with different buffers for fresh strawberries and frozen raspberries**

<table>
<thead>
<tr>
<th>Food sample</th>
<th>HAV (120 TCID&lt;sub&gt;50&lt;/sub&gt;/15 g)</th>
<th>NV (540 PCRU/15 g)</th>
<th>RV (26 TCID&lt;sub&gt;50&lt;/sub&gt;/15 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris buffer</td>
<td>Tris buffer + 1%</td>
<td>Tris buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>beef extract</td>
<td></td>
</tr>
<tr>
<td>Frozen raspberries</td>
<td>2.3 ± 0.61</td>
<td>15.8 ± 0.31</td>
<td>3.07 ± 0.70</td>
</tr>
<tr>
<td>Fresh strawberries</td>
<td>1.99 ± 0.49</td>
<td>2.29 ± 0.30</td>
<td>0.42 ± 0.07</td>
</tr>
</tbody>
</table>

*The different efficiencies are indicated beneath the viruses, and the units by which the efficiencies were measured are indicated in parentheses. Each experimental condition was analyzed in duplicate. Results are means ± standard errors.*
tained by the PEG precipitation method. The results obtained with raspberry and strawberry samples showed that ultrafiltration gave better virus recoveries than PEG precipitation (Fig. 2).

RNA extraction kit selection. A variety of methods have been developed to extract viral RNA from complex matrices while simultaneously reducing the level of PCR inhibitors. In the case of foods, most of these methods have been tested on shellfish, and only a few have been reported for other food commodities (26, 43, 46, 50). We evaluated two commercially available RNA extraction kits, the QIAamp viral RNA mini kit (QIAGEN) and the Nuclisens magnetic kit (bioMérieux). Both kits were found to be suitable for HAV and NV detection in food samples, but the bioMérieux kit failed to detect rotaviruses in berry samples (Table 3). For this reason, the QIAGEN kit was selected for the final procedure.

Removal of RT-PCR inhibitor substances. Initial tests with berry samples often yielded the appearance of a pectinaceous gel-like substance that led to invalid results. We therefore included a pectinase treatment as described by Rzezutka et al. (44, 45) and found that this treatment indeed eliminated the problem of false-negative results except for raspberry samples. Attempts were made to improve the recovery from raspberries by pretreating the concentrated sample before the RNA extraction by using the Plant RNA Isolation Aid (Ambion), which proved to be more effective than Concert plant RNA reagent (Invitrogen) (data not shown). Combining the pectinase treatment with the use of Plant RNA Isolation Aid, it was indeed possible to detect and quantify the three viruses from inoculated raspberry samples (Table 4).

Detection limits and recovery. To establish the detection limit and virus recovery, 15-g portions of different types of berries and vegetables were surface inoculated in triplicate with different serially diluted suspensions of virus strains and subsequently analyzed with the optimized concentration and detection protocol (Fig. 1). The average recoveries for the different types of berries were ca. 14% (from 37.5 to 21.1%), 6.8% (from 19.6 to 0.5%), and 3.3 (from 14 to 0.3%) for HAV, NV, and RV, respectively, with fresh raspberries and blueberries giving the best results. The presence of sugar in the berry mix did not show cross-reactivity in the virus recoveries, since the recoveries were similar to those obtained with frozen raspberries (Table 4). Frozen berries gave rather poor detection limits, which was probably due to the release of large amounts of fruit juice.

The average recoveries from the fresh vegetables were ca. 42% (from 83.1 to 9.8%), 25% (from 46.3 to 9.5%), and 6.7% (from 21.2 to 1.6%) for HAV, NV, and RV, respectively (Table 5). Of the vegetables tested, lettuce and mint were best suited for virus recovery.

Of the three viruses studied, RV showed the lowest recoveries in all the tested food matrices except for fresh strawberries (Tables 4 and 5). This would lead to an underestimation of the RV recovery due to the heterogeneity of the RV stock, given an estimation that 1 TCID<sub>50</sub> corresponds to 6,000 RT-PCR units (data not shown). Free RNA molecules and defective particles were detected in the positive control submitted only to the RNA extraction, whereas in food samples those free RNAs and defective particles were most likely lost during the concentration steps.

Detection limits were 1.2 TCID<sub>50</sub>, 54 PCRU, and 0.02 TCID<sub>50</sub> for HAV, NV, and RV, respectively, per 15 g of berries or vegetables (Tables 4 and 5). By use of the standard curve generated with the LightCycler hepatitis A virus quantification kit, the assayed HAV stock containing 1.2 × 10<sup>6</sup> TCID<sub>50</sub> was estimated to have 1.9 × 10<sup>8</sup> RNA copies, which corresponds to an average detection limit of 190 RNA copies in 15 g of berry and vegetable samples. As few as 19

![FIG. 2. Comparison of PEG precipitation and ultrafiltration for virus concentration. Enteric virus recovery from fresh strawberries and raspberries is shown. Each experimental condition was analyzed in duplicate. Recoveries of HAV, NV, and RV from berries are depicted in white, gray, and black, respectively.](http://aem.asm.org/)

### TABLE 3. Comparison of methods of extracting viral RNA from inoculated frozen raspberries

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of samples positive for indicated virus/no. tested&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAV (TCID&lt;sub&gt;50&lt;/sub&gt;/15 g)</td>
</tr>
<tr>
<td>QIAGEN kit</td>
<td>2/2</td>
</tr>
<tr>
<td>bioMérieux kit</td>
<td>2/2</td>
</tr>
</tbody>
</table>

<sup>a</sup> The different detection limits are indicated beneath the viruses, and the units by which the detection limits were measured are indicated in parentheses.
RNA copies were detectable in lettuce and basil samples (Table 5).

**DISCUSSION**

The importance of viral food-borne diseases is increasingly recognized, and the World Health Organization has signaled an upward trend in their incidence. This recognition is reflected by the attention that national and international organizations give to considering the control of food-borne viral infection in the report of the Advisory Committee on the Microbiological Safety of Food (2). This is particularly pertinent to fruits and vegetables that most likely will not be cooked before consumption. Berries and vegetables are prone to be contaminated through sewage-contaminated surface water or by infected food handlers during harvesting, packaging, or food preparation, where the viruses are likely to be on the food surface. In order to monitor the presence of enteric viruses, there is a need to develop better methods for virus recovery from a range of fresh produce items. This work presents an optimized methodology for sensitive and accurate detection of HAV, NV, and RV from berry and vegetable surfaces that consists of viral elution from the food surface, concentration by ultrafiltration, and real-time RT-PCR.

All methods proposed so far, including the one described in this study, consist of an elution step to release the viruses from the food surface, because it is assumed that naturally contaminated samples carry virus particles only on the surface. Artificially contaminated samples, mimicking the most likely natural mode of contamination, were used to validate this method; however, a new mechanism of HAV contamination of green onions has recently been proposed. Chancellor et al. (7) found HAV particles trapped inside growing green onions taken up intracellularly through the roots, even though survival of the virus was not evaluated. This mechanism warrants further examination, and if confirmed for other viruses and for different fresh products it will definitely change future approaches for the detection of viruses in vegetables.

Several strategies for virus concentration, e.g., immunocentrification, PEG precipitation, ultrafiltration, and ultracentrifugation, have been described. Immunological methods have been applied to virus detection in food samples (4, 27, 49, 50); however, NV immunocentrification is unlikely to be adapted broadly for norovirus detection due to the difficulties in obtaining antibodies and its variability at the capsid level. As this study aimed at developing a method for virus detection for a broader range of berries and vegetables, the elution buffer and the method of concentration were optimized using the most difficult matrix (13), i.e., berry samples. The adaptation of this

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**TABLE 4. Detection of HAV, NV, and RV in spiked berry samples by real-time RT-PCR**

<table>
<thead>
<tr>
<th>Berry sample</th>
<th>No. of samples positive for indicated virus/no. tested&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAV (TCID&lt;sub&gt;50&lt;/sub&gt;/15 g)</td>
</tr>
<tr>
<td></td>
<td>120 12 1.2 0.1</td>
</tr>
<tr>
<td>Fresh blackberries</td>
<td>3/3 (5.6)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh raspberries</td>
<td>3/3 (37.5)</td>
</tr>
<tr>
<td>Fresh strawberries</td>
<td>3/3 (2.1)</td>
</tr>
<tr>
<td>Frozen black currants</td>
<td>3/3 (3.6)</td>
</tr>
<tr>
<td>Frozen blueberries</td>
<td>3/3 (35.5)</td>
</tr>
<tr>
<td>Frozen raspberries</td>
<td>3/3 (4.4)</td>
</tr>
<tr>
<td>Sugared berry mix</td>
<td>3/3 (9.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The different detection limits are indicated beneath the viruses, and the units by which the detection limits were measured are indicated in parentheses next to the viruses. The numbers in parentheses next to the fractional data indicate the percent extraction efficiencies.<br><sup>b</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by HAV RNA copies with the external standards provided in the LightCycler HAV kit.<br><sup>c</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by NV PCRU.<br><sup>d</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by means of infectious rotavirus.

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**TABLE 5. Detection of HAV, NV, and RV seeded in fresh vegetables by real-time RT-PCR**

<table>
<thead>
<tr>
<th>Vegetable</th>
<th>No. of samples positive for indicated virus/no. tested&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAV (TCID&lt;sub&gt;50&lt;/sub&gt;/15 g)</td>
</tr>
<tr>
<td></td>
<td>120 12 1.2 0.1</td>
</tr>
<tr>
<td>Basil</td>
<td>3/3 (52.3)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green onions</td>
<td>3/3 (9.8)</td>
</tr>
<tr>
<td>Lettuce</td>
<td>3/3 (83.1)</td>
</tr>
<tr>
<td>Mint</td>
<td>3/3 (51.6)</td>
</tr>
<tr>
<td>Parsley</td>
<td>3/3 (12.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The different detection limits are indicated beneath the viruses, and the units by which the detection limits were measured are indicated in parentheses next to the viruses. The numbers in parentheses next to the fractional data indicate the percent extraction efficiencies.<br><sup>b</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by HAV RNA copies with the external standards provided in the LightCycler HAV kit.<br><sup>c</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by means of NV PCRU.<br><sup>d</sup> Each spiked sample was extracted and the RNA extract titrated by real-time RT-PCR using a standard curve made by means of infectious rotavirus.
procedure to the vegetable samples was straightforward; only the pectinase treatment was not included. The concentration step is particularly critical when the viral load in samples is expected to be low and samples are further analyzed by RT-PCR, which requires a suitably concentrated small volume. In this context, two current viral concentration approaches were evaluated. Compared with PEG precipitation, the use of the ultrafiltration columns is less labor-intensive and time-consuming and, on average, doubles the virus recovery. However, other authors found better results with PEG precipitation than with ultrafiltration; this difference depends mainly on the RNA kit used thereafter or the type of food matrix (43).

Processing of berries and vegetables by use of the described method successfully removed PCR inhibitors in the RNA extract. This is a substantial improvement over results achieved with other published methods, which depend on dilution to circumvent the effect of PCR inhibitors to the detriment of the detection limit (13, 33, 34, 44).

Real-time RT-PCR methods were chosen for this study for a more accurate estimation of the viral recovery, but some caution is necessary when interpreting results. Quantitative RT-PCR methods presume that the target and the environmental sample are amplified with similar efficiencies. However, differences in the viral target sequences from the unknown samples may significantly reduce the efficiency of the RT-PCR and affect the final quantification. It is worth mentioning that the NV real-time method used in this study was designed specifically for the detection of the Valetta strain; therefore, for NV screening, the molecular detection method must be adapted.

This study reports detection limits of HAV and RV in the range of 1 or 0.02 infectious particles per 15 g of berries or vegetables, respectively, whereas the detection limit of noroviruses was ca. 50 PCR units. Virus recovery with berry samples was consistently low compared to that with vegetables, which confirms the findings of Dubois et al. (13). This has been attributed to virus inactivation on the berry surface by a pH drop (28). HAV, NV, and RV detection efficiencies from blueberry surfaces were higher than those from other berries, probably because it is easier to release viruses from smooth surfaces and also because blueberries have a relatively thick and waxy skin, which limits the exudation of antiviral substances (28). Additionally, the influence of individual food matrices and viral strains on the overall efficiency is also likely to be significant. The differences in the viral strains may be correlated with different stabilities under drying conditions and different levels of attachment to the food surface and the viral resistance at low pH (1, 31, 51). Recently, Vega et al. (51) demonstrated that viruses exhibited different attachment patterns depending on their isoelectric point, suggesting that the use of a particular pH buffer to release viruses from food surfaces could selectively recover a particular type or group of viruses.

The detection limits obtained with our procedure are similar to or even better than the lowest HAV and NV detection limits reported previously (Table 1) with the exception of the work of Guevremont et al. (21), who reported as little as 1 PCRU NV in green onions, whereas we found 50 PCRU NV. Nevertheless, this method was not validated for other food matrices, and comparable performances were obtained for HAV. Recently, similar detection limits were reported by Shan et al. (49), who detected 0.5 PFU HAV in green onions and strawberries. However, in this work HAV was inoculated in the rinse instead of on the food surface; hence, the efficacy of the PBS buffer used to release the viruses from the food surface was not proven. The detection limits obtained using our procedure would suit the needs of epidemiological investigations, since HAV, NV and RV infectious doses in food samples range from 10 to 100 viral particles. HAV has recently been quantified for naturally contaminated shellfish samples showing titers ranging from $1 \times 10^5$ to $1 \times 10^6$ HAV genomes per gram of clam (8).

Considering that our HAV detection limit was 12 RNA copies per gram of berry or vegetable sample, viral contamination levels even 10 to 50 times lower could be detected by use of our methodology with berries and vegetables.

To our knowledge, this is the first reported methodology to detect norovirus, rotavirus, and hepatitis A virus in a broad range of berries and vegetables. This method has proven to be a reliable tool for concentrating and detecting enteric viruses in berries and vegetables within 6 h, a period of time that includes the real-time RT-PCR amplification step. The concentration and extraction method can eventually be applied for the detection of other food-borne viruses by adapting the molecular detection method. It would also be very useful during the epidemiological investigation of food-borne outbreaks and for a routine surveillance of enteric viruses from fresh and frozen berries and vegetables.

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