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Extended direct lysis method for virus detection on berries including droplet digital RT-PCR or real time RT-PCR with reduced influence from inhibitors

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ABSTRACT

Detection of viruses on berries is a challenging task, often hampered by the presence of RT-qPCR inhibiting substances from berry juice. A direct extraction method for virus detection (murine norovirus and GA phage) on frozen raspberries was previously published. We expanded (different types of berries and viruses) and improved the method using MobiSpin S400 columns that filter nucleic acids based on size-exclusion chromatography. While no inhibition was detected in filtered RNA, unfiltered RNA needed from 1:2 to more than 1:8 dilution in order to remove inhibition. The modified method gave recoveries of bovine norovirus around 40.8 ± 4.5% (40.0 ± 7.0%), 48.0 ± 26.0% (50.5 ± 7.8%), 28.3 ± 2.6% (45.8 ± 6.6%) from frozen (fresh) raspberries, strawberries and blueberries, respectively. For the same samples, recoveries of hepatitis A virus were 34.0 ± 5.9% (34.0 ± 6.0%), 40.0 ± 13.3% (34.2 ± 10.5%) and 23.0 ± 6.8% (31.5 ± 7.9%). For adenovirus40 (DNA virus), recoveries were 21.2 ± 8.6%, 16.0 ± 3.2% and 5.7 ± 0.2% from fresh raspberries, strawberries and blueberries respectively and column filtration did not add any improved effect. The modified method is effective and timesaving for detection of viral RNA from both fresh and frozen berries. As an emerging detection and direct quantification method, droplet digital RT-PCR was compared to RT-qPCR and was much less influenced by inhibitors when detecting mengovirus in unfiltered RNA from berries. However, for low levels of pure RNA, RT-qPCR showed slightly higher sensitivity and more stable results.

1. Introduction

During production of berries (pre harvest, harvest and post harvest), human viral pathogens such as norovirus (NoV) and hepatitis A virus (HAV) may contaminate products through irrigation water and workers' hands. As NoV and HAV are stable from days to weeks at room temperature, environmental contamination and poor hygiene routines may eventually result in outbreaks of disease. A total of 1589 cases of hepatitis A were linked to the consumption of frozen berries in European countries from January 2013 to August 2014 (Severi et al., 2015). Regarding NoV, contaminated frozen strawberries caused gastroenteritis in more than 10 000 people in Germany in 2012 and frozen raspberries caused multiple outbreaks in Finland in 2009 (Sarvikivi et al., 2012; Mäde et al., 2013).

Detection of viruses in food matrixes is important in the study of food borne outbreaks and in surveillance of virus contamination in foods. However, virus detection on berries has been a difficult task, mainly due to the fragile texture of strawberries and raspberries and various substances in berry juice that inhibit detection by (RT)-qPCR. Phenols and polyphenols (e.g. anthocyanin, flavonol, ellagitannin, proanthocyanidin, phenolic acids, tannic acid) could cross-link nucleic acids (NA), impede resuspension of pellets that contain NA and chelate metal ions necessary for enzyme catalyzed (RT)-qPCR (Peist et al., 2001). Further, the primary cell wall of plants contains a structural heteropolysaccharide, pectin, that inhibits (RT)-qPCR reactions (Wei et al., 2008). Inhibitors, together with low efficiency of eluting virus from berries, often reduce the sensitivity of detection and may cause false negative results (Mäde et al., 2013).

In order to concentrate virus and remove inhibitors, several methods have been developed, including polyethylene glycol (PEG) precipitation (Butot et al., 2007; Scherer et al., 2010; Mäde et al., 2013), capture of NoV using magnetic beads coated with porcine gastric mucin (Tian et al., 2005), receptor-binding capture and magnetic sequestration (RBCMS) (Pan et al., 2012), and direct RNA extraction using Trizol (Baert et al., 2008). The International Organization for Standardization (ISO) has adopted the PEG protocol for detection of HAV and NoV in fresh fruits and vegetables (ISO, 2017). This method is time consuming and extracted RNA may still contain RT-PCR inhibitors

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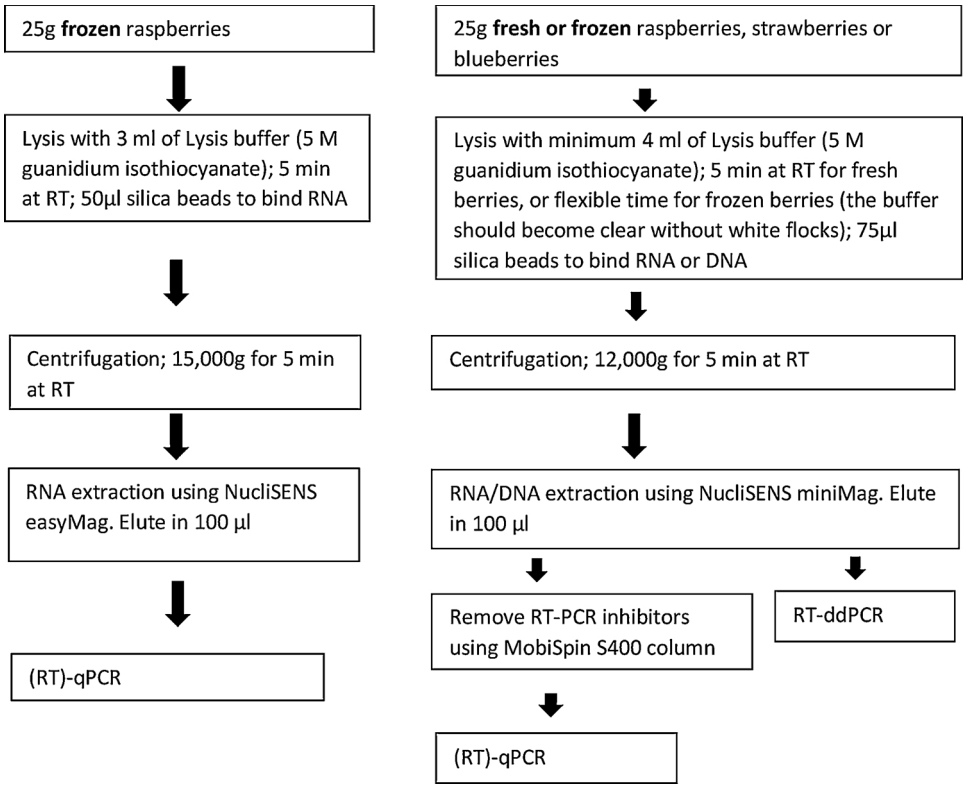

Fig. 1. Flowchart of the original (left) and modified method (right) for virus detection in berries. As chemicals in the lysis buffer precipitate on contact with the frozen berries, the incubating time should be flexible until the chemicals solubilize and the lysis buffer becomes clear. More juice will be released into the buffer during prolonged incubation and with ruptured berries. The volume ratio of lysis buffer and juice should be kept at 4: 1 and extra buffer may be added before the centrifugation step. A sediment of jelly-like substances will appear after centrifugation, while viral RNA/DNA remains in the supernatant.

(Summa et al., 2012; Bartsch et al., 2016). A more simple and rapid direct lysis method for detecting viral genomes (murine NoV and bacteriophage GA) on frozen raspberries has been published (Perrin et al., 2015). This method was later compared to the ISO method regarding detection of human NoV in strawberries (Bartsch et al., 2016), showing comparable low recoveries (0.5 ± 0.54 versus 1.7 ± 2.3%). However, using the ISO method and inhibitor removal from RNA with MobiSpin S400 columns, a recovery of 5.6 ± 1.6 to 15.3 ± 9.7% was found (Bartsch et al., 2016). In order to increase the usefulness of the rapid method of Perrin et al, the present work developed it further, combined it with MobiSpin columns and tested it on several types of berries (strawberries, blueberries and raspberries), using different viruses. Furthermore, the original direct lysis method requires a freezing step before RNA extraction to reduce leakage of raspberry juice inhibitors into the washing buffer. As the freezing step reduces the convenience for onsite field investigation, the method was also optimized for virus detection in fresh berries. In addition, droplet digital RT-PCR (RT-ddPCR) was compared with RT-qPCR regarding tolerance to inhibitors during detection of the viral RNA.

2. Materials and methods

2.1. Viruses

Hepatitis A virus (HAV pHM175 43c) was kindly provided by professor Albert Bosch (University of Barcelona), and propagated in fetal rhesus monkey kidney cell line (FRhK-4/R) (ATCC CRL-1688), as previously described (Flehmig, 1980). The human adenovirus 40 (AdV40) (ATCC VR-931) was cultured in PLC/PRF/5 cells (ATCC CRL-8024) according to a previous report (Grabow et al., 1992). Mengovirus (strain MC_0, kindly provided by professor Albert Bosch) was prepared according to ISO method (2017). A bovine NoV (BNoV, GIII.2) originated from a fecal sample which was diluted with PBS (1:10), vortexed for 30 s, centrifuged (1200 *g* for 20 min) and aliquoted. Aliquots of cultured viruses and fecal samples were stored at −80 °C before use.

2.2. Artificial contamination of berries

Fresh berries originating from Morocco, Spain and Norway were purchased from local food stores. Samples of fresh berries (each of 25 g), consisting of 1–2 strawberries, 8 raspberries or 16–20 blueberries, were spiked with 40 µl mixed virus suspension in PBS which contained 3.8×10^5 copies of HAV, 3×10^4 copies of BNoV and 9.6×10^5 copies of AdV40 by evenly spreading drops on the surface. Berries spiked with 40 µl PBS, and no virus, were included as negative controls. The berries were air dried in a fume hood at room temperature (RT) for 1 h. “Fresh berries” were immediately used for further processing, while “frozen berries” were kept at −20 °C overnight before use.

2.3. Processing of berries for virus detection using direct lysis

Each sample of fresh or frozen raspberries and blueberries was divided between two 50 ml Falcon tubes, while strawberries were put in a plastic bag with zipper. A total of 4 ml lysis buffer (RT) (NucliSENS, Biomerieux, France) was added directly to each berry sample. Mengovirus was spiked into the samples in order to study its applicability as a process control. For fresh berries, the tubes were gently rotated for around 5 min, while for frozen berries the incubating time could be flexible (5–10 min, depending on berry size). As chemicals (mainly guanidium isothiocyanate) in the lysis buffer precipitate after contacting with frozen berries, it is important to wait for complete solubilizing and the buffer to regain the clear look. The lysis buffer was removed with a pipette, centrifuged at 12 000 *g* for 5 min at RT, and used for RNA and DNA isolation.

2.4. Extraction and purification of RNA and DNA

Nucleic acids were isolated from the total buffer volume using the NucliSENS MiniMag and 75 µl magnetic silica particles. The particles were washed twice with 400 µl buffer 1, twice with 500 µl buffer 2 and the NA from each sample were eluted in 100 µl buffer. In order to

evaluate MobiSpin S400 columns (Mobitec, Germany) for the capacity of reducing inhibitors, NA from two parallel samples (2 × 25 *g* of berries) were pooled and mixed carefully. Half of the volume was purified according to the MobiSpin manual (filtered sample) and the other half served as an unfiltered control. Briefly, the column resin was re-suspended by vortexing and the column placed in a 2 ml micro centrifuge tube (1.5 ml tubes should be avoided in this step) and pre-spun for 1 min at 800 *g*. The tube was discarded, and the column placed in a new 2 ml tube. One hundred µl NA was applied to the matrix surface, and the column spun for 2 min at 800 *g*. Filtered and unfiltered NA were aliquoted and kept at −80 °C until (RT)-qPCR analysis. The steps in the original method published by Perrin and the present modified method are presented in Fig. 1.

2.5. (RT)-qPCR

Primers and probe for HAV, mengo, AdV40 and BNoV were as previously described (Pintó et al., 2009; ISO, 2017; Jothikumar et al., 2005; Costafreda et al., 2006; Christensen et al., 2017). The (RT)-qPCR was carried out using the AriaMx Realtime PCR System (Agilent Genomics, USA). RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, USA) was used to detect viral RNA (HAV, mengo and BNoV), while TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, USA) was used for AdV40 DNA. Two µl of NA was used in a total volume of 20 µl, and two parallels were analyzed for each sample. The RT-qPCRs were run at 50 °C for 30 min, 95 °C for 3 min, and finally 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The AdV40 qPCR was run at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 60 °C for 45 s.

2.6. Droplet digital RT-PCR (RT-ddPCR) for mengovirus

The RT-ddPCR was carried out using the QX200TM Droplet digital™PCR system (Bio-Rad, CA, USA) and the One-step RT-ddPCR advanced kit (Bio-Rad). Final concentrations were 900 nM of primers and 250 nM probe as recommended by the kit manufacturer. For RT-ddPCR, two µl RNA was used in the initial 20 µl reaction mixture which, together with the droplet generating oil, resulted in a final volume of 40 µl. Thermal cycling was performed on a Bio-Rad C1000 using the following protocol: 50 °C for 1 h, 95 °C for10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and a final step of 98 °C for 10 min. The ramp rate was set to 2 °C/second and results were analyzed by using the Quantasoft 1.7.4.0917 software (Bio-Rad).

2.7. Testing of (RT)-qPCR inhibition and the effect of MobiSpin columns on NA from berries processed by direct lysis

Each berry sample was spiked with 3.8×10^5 copies of HAV, 3×10^4 copies of BNoV and 9.6×10^5 copies of AdV40 and processed as described. In order to find any inhibitor-reducing effects of the Mobispin column, two µl filtered and non-filtered NA from frozen and fresh berries were tested in RT-qPCR, using dilutions 1:1, 1:2, 1:4 and 1:8 (NA: DEPC water). Inhibition was evaluated by comparing the Ct values from the dilution series. A difference of 1.0 between the Ct values for a given sample and its 1:2 dilution indicates absence of inhibition when the amplification efficiency is 100%. The experiment was performed three times and two parallels of each sample (type of berry and fresh/frozen) were included in each setup. In order to increase the sensitivity of detection, increased volumes of filtered NA from fresh and frozen berries were also tested for HAV (four and eight µl) and AdV40 (four µl) as models for RNA and DNA viruses, respectively.

2.8. Estimation of virus recovery rate (RR)

Samples from the inhibition study were further used to estimate virus recovery. Based on the results in that study, filtered NA was used for detection of viral RNA and filtered/unfiltered for detection of AdV DNA. The RR was calculated by comparing the Ct value of the control (virus mixture used for spiking) to the Ct value of samples (spiked berries). The following formula was used in which E denotes the efficiency of amplification (Livak and Schmittgen, 2001):

$$RR\% = (1 + E)^{(Ct\ control - Ct\ sample)} \times 100\%$$

E was calculated from standard curves based on serial dilutions of homologous viral NA using software version 1.1 of the AriaMx Realtime PCR System (Agilent Genomics, USA). The efficiency was 0.91 for HAV, 0.95 for mengovirus, 0.99 for AdV40, and 1.03 for BNoV.

2.9. Limit of detection (LOD) of viruses on strawberries

In order to estimate the LOD for HAV, BNoV and AdV40, samples of strawberries (25 g) were spiked as described in 2.2. Two-fold serial dilutions of mixed virus were used for spiking and the amount of each virus was in the range 25 to 400 copies per sample. Then berry samples were frozen before processing by direct lysis and Mobispin filtration of RNA/DNA. The study was performed three times. Four µl of NA was used in each (RT)-qPCR. Absolute quantification of viral NA was performed using standard curves generated from 10-fold serial dilutions of linearized plasmids containing target sequences of HAV, BoNV and AdV40, respectively (GenScript, NJ, USA).

2.10. Comparison of RT-ddPCR and RT-qPCR inhibitor tolerance and sensitivity

Mengovirus was used as a model virus in order to study RT-ddPCR and RT-qPCR for detection of viral RNA in samples with inhibitors (nonfiltered RNA from spiked strawberries, three replicates) and without inhibitors (pure RNA from cultivated virus, two replicates). Thereafter, RNA was serially diluted 2-fold and two µl used in RT-ddPCR and RT-qPCR. To find any difference in sensitivity, endpoint dilution was used for pure RNA.

2.11. Statistical analysis

Any difference in virus recoveries was analyzed by Student's *t*-test. Differences were considered statistical significant with $p < 0.05$.

3. Results

3.1. (RT)-qPCR inhibition and the effect of MobiSpin columns

The original direct lysis method for simple detection of viruses in raspberries includes a freezing step and 5 min incubation in lysis buffer in order to reduce the release of raspberry juice (Fig. 1). Our initial experiments with this method, gave inconsistent results on strawberries, but more stable results on blueberries and raspberries. RNA extracted from these berries needed at least a 1:4 dilution in order to remove RT-qPCR inhibitors (Table 1).

As a possible alternative to dilution of RNA, MobiSpin S400 column filtration was included in the direct extraction protocol for frozen and fresh berries (Fig.1). Table 1 shows the difference in detection of HAV and BNoV RNA and AdV40 DNA with or without column filtration. In general, processing of fresh berries without filtering the RNA resulted in higher Ct-values compared to frozen berries. The degree of inhibition from the different types of berries was strawberries > raspberries > blueberries. However, the MobiSpin S400-Column proved highly efficient in removing inhibitors from all three types of berries. For AdV40, there was no significant qPCR inhibition in DNA isolated from any of the fresh berries (Table 1).

In order to increase the sensitivity of virus detection, larger volumes of filtered NA were tested in (RT)-qPCR for HAV and AdV40. Four µl could be used without any obvious negative effect and generally gave lower Ct values than two µl (Table 2).

Table 1
MobiSpin column filtration of RNA from berries reduces (RT)-qPCR inhibition. RNA/DNA was isolated from virus-spiked strawberries, raspberries and blueberries. The berries were processed fresh or after a freezing step and RNA/DNA was isolated with or without column filtration after extraction by the direct lysis method. The RNA/DNA was twofold serial diluted, and two µl was analyzed in a 20 µl (RT)-qPCR reaction. Ct values are presented as mean ± standard deviation from three individual replicates, two parallel samples and two parallel (RT)-qPCR reactions. Samples showing inhibition are in bold. BNoV: bovine norovirus; HAV: hepatitis A virus; AdV40: adenovirus 40; F: filtered; N-F: non-filtered. An increase of the Ct value of 1 corresponds to ca 50% reduction in virus copy number.

			Ct values in dilutions of RNA/DNA			
Strawberries			1:1	1:2	1:4	1:8
BNoV	Frozen	N-F	**negative**	**32.6±1.1**	**31.1±1.0**	30.1±1.0
		F	26.7±0.8	28.0±0.7	29.2±0.8	31.0±0.9
	Fresh	N-F	**negative**	**negative**	**33.8±1.8**	**32.8±0.8**
		F	26.6±0.3	27.6±0.1	29.0±0.2	30.1±0.3
HAV	Frozen	N-F	**negative**	**33.6, 38.4, negative**	**29.9±0.5**	29.4±0.7
		F	25.8±0.6	27.1±0.4	28.1±0.4	29.9±0.6
	Fresh	N-F	**negative**	**negative**	**negative**	**31.9±0.2**
		F	26.4±0.3	27.0±0.1	28.0±0.1	28.8±0.2
AdV40	Fresh	N-F	25.1±0.5	25.9±0.3	26.8±0.3	27.6±0.4
		F	25.4±0.3	26.4±0.4	27.5±0.4	28.6±0.3
Raspberries						
BNoV	Frozen	N-F	**27.5±1.0**	27.1±0.3	28.3±1.1	28.9±0.2
		F	26.3±0.1	27.6±0.1	28.7±0.3	30.0±0.2
	Fresh	N-F	**29.2±0.8**	**28.2±0.7**	28.2±0.1	29.8±0.4
		F	26.4±0.3	27.6±0.3	28.7±0.5	30.1±0.3
HAV	Frozen	N-F	**28.8±0.8**	**27.9±0.9**	**27.8±0.6**	28.1±0.2
		F	25.7±0.1	26.6±0.3	27.6±0.1	28.8±0.4
	Fresh	N-F	**30.5±1.7**	**29.0±0.5**	**28.2±0.4**	28.7±0.1
		F	25.8±0.2	26.7±0.2	27.8±0.4	28.2±0.3
AdV40	Fresh	N-F	25.1±0.7	25.7±0.6	26.5±0.7	27.3±0.6
		F	25.1±0.6	26.3±0.5	27.5±0.5	28.6±0.6
Blueberries						
BNoV	Frozen	N-F	**27.5±0.3**	28.2±0.3	29.1±0.2	29.9±0.2
		F	27.4±0.2	28.4±0.1	29.5±0.1	30.5±0.4
	Fresh	N-F	**27.5±0.2**	**27.8±0.3**	28.2±0.2	29.0±0.1
		F	26.3±0.3	27.6±0.2	28.9±0.1	30.0±0.1
HAV	Frozen	N-F	**27.1±0.1**	27.8±0.5	28.7±0.3	29.4±0.3
		F	27.0±0.1	27.6±0.1	28.7±0.1	29.4±0.1
	Fresh	N-F	**28.7±0.1**	**27.5±0.3**	28.1±0.1	28.9±0.1
		F	26.2±0.5	27.4±0.2	28.4±0.4	29.2±0.1
AdV40	Fresh	N-F	26.7±0.1	27.4±0.1	28.3±0.2	29.0±0.2
		F	26.8±0.1	28.0±0.2	29.1±0.2	30.5±0.3

3.2. Recovery rates of viruses from berries

Recovery rates for BNoV, HAV, mengovirus and AdV40 using MobiSpin column filtered NA from frozen and fresh strawberries, raspberries and blueberries are shown in Fig. 2. For AdV40, RR was also estimated from non-filtered DNA.

Comparing recoveries of viruses from fresh and frozen berries, there was no statistical significant difference, except that higher BNoV recovery could be achieved in fresh blueberries ($p < 0.05$).

As recoveries for RNA viruses were relatively even for fresh and frozen berries, analysis for AdV40 DNA was only performed on fresh berries. In general, the recovery of AdV40 DNA was lower compared to viral RNA, and especially from blueberries ($p < 0.05$). For raspberries and strawberries, a lower recovery was found for AdV40 compared to BNoV and mengovirus ($p < 0.05$), but there was no significant difference between AdV40 and HAV (p 0.10-0.17). Recovery of AdV40 from blueberries was also lower compared to raspberries and strawberries ($p < 0.05$).

Table 2
The volume of filtered RNA could be increased to 20% of an (RT)-qPCR reaction volume. Two, four and eight µl MobiSpin column-filtered RNA/DNA were tested in a 20 µl total volume. Samples that show inhibition are in bold. ND; not done.

			Ct values from different volumes		
			2 µl	4 µl	8 µl
HAV	Strawberries	Frozen	26.0±0.7	24.8±0.7	24.0±0.3
		Fresh	25.3±0.5	24.7±0.2	**25.0±0.2**
	Raspberries	Frozen	24.9±0.1	24.2±0.2	**24.1±0.4**
		Fresh	25.3±0.3	24.3±0.4	23.7±0.4
	Blueberries	Frozen	26.7±0.3	25.7±0.2	**25.4±0.5**
		Fresh	25.9±0.5	24.9±0.5	**24.8±1.0**
AdV40	Strawberries	Fresh	25.4±0.3	24.7±0.3	ND
	Raspberries	Fresh	25.1±0.6	24.3±0.5	ND
	Blueberries	Fresh	26.8±0.1	26.2±0.1	ND

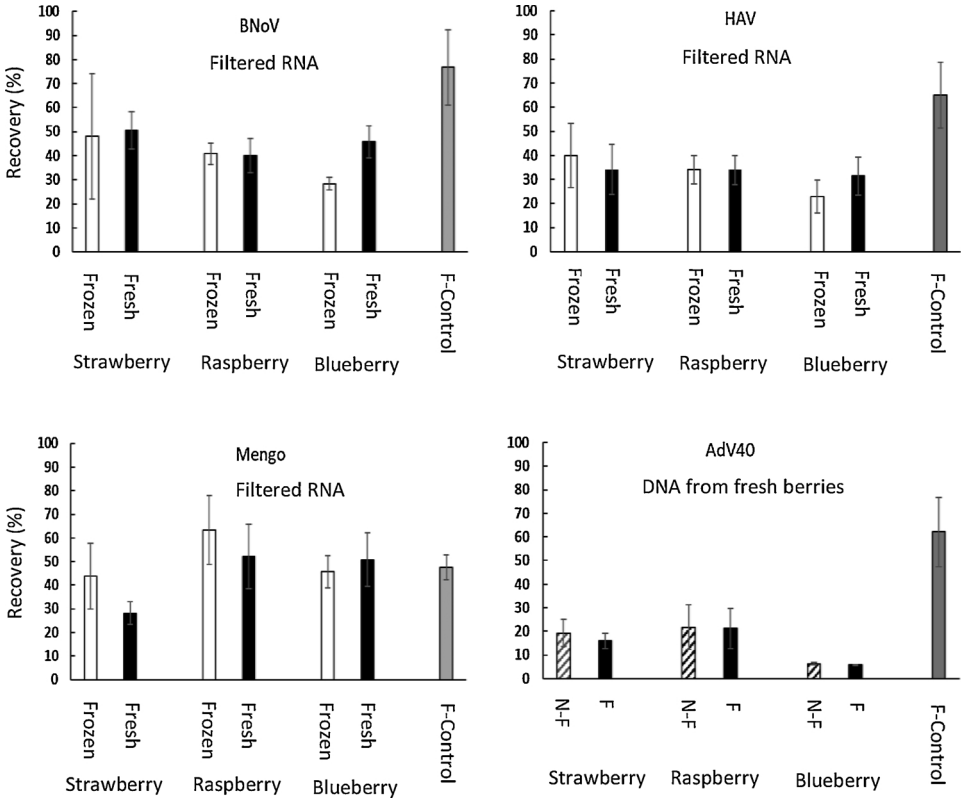

Fig. 2. Recovery rates of viruses in spiked frozen and fresh strawberries, raspberries and blueberries. RNA/DNA was extracted with the direct lysis method combined with or without filtration using MobiSpin columns. Non-filtered controls (viruses in PBS) were defined as 100%. Recovery of viruses in filtered controls and berries was presented as percentage of the non-filtered control. Filtered controls (F-control) were included in order to demonstrate the loss of RNA through filtering. Two µl RNA was analyzed in a 20 µl (RT)-qPCR volume. BNoV: bovine norovirus; HAV: hepatitis A virus; AdV40: adenovirus 40; F: filtered; N-F: non-filtered.

3.3. (RT)-qPCR detection limits of viruses on strawberries

In order to find the LOD for the different viruses, strawberries were spiked with serial dilutions of the mixed virus suspension, frozen and RNA extracted with the optimized direct lysis method and column filtration. Detection limits for HAV, BNoV and AdV40 on artificially contaminated strawberries are listed in Table 3. The LOD could repeatedly reach 100 copies/25 g for HAV and BNoV, and 200 copies/25 g for AdV40.

3.4. Inhibitor tolerance and sensitivity of RT-ddPCR and RT-qPCR

RT-ddPCR showed a tolerance to substances in unfiltered RNA from strawberries that inhibited RT-qPCR (Table 4). For RT-qPCR, at least a 1:8 dilution of unfiltered RNA was needed to get positive results in all samples. However, when comparing sensitivity of detection using pure RNA, more consistent results on low copy numbers were achieved by RT-qPCR (Table 4).

Table 3
Detection limits for hepatitis A virus (HAV), bovine norovirus (BNoV) and adenovirus 40 (AdV40) on artificially contaminated strawberries (25 g per sample) that were frozen before direct extraction and filtration with Mobispin S400 columns. Four µl RNA/DNA was analyzed in a 20 µl (RT)-qPCR.

Spiking level, virus copies /25g	No. positives / No. analyzed		
	HAV	BNoV	AdV40
400	3/3	3/3	3/3
200	3/3	3/3	3/3
100	3/3	3/3	1/3
50	1/3	1/3	0/3
25	0/3	0/3	0/3

4. Discussion

Berries are prone to contamination by viruses through sewage-contaminated irrigation water or by infected farm workers and food handlers. The current method for molecular detection of virus in berries adopted by ISO includes steps of elution and PEG precipitation, which originate from historical protocols used to recover infectious viruses from solid matrices (Lowther et al., 2019). The procedure includes a step of pectinase digestion, as pectin is regarded as a potential RT-qPCR inhibitor. However, there are still other types of inhibitors in strawberries (Bartsch et al., 2016) and raspberries (Summa et al., 2012). In order to improve virus detection, Perrin et al. developed a direct extraction method for raspberries, which achieved a significantly higher viral extraction efficiency (46.2%) of murine NoV and GA phage than the standard ISO method (20.3%) (Perrin et al., 2015). However, this method could not be successfully applied on viruses in strawberries as the RR was 0.52% (Bartsch et al., 2016). Bartsch et al. also found that RNA from different batches of strawberries contained various levels of inhibitors, giving no obvious effect or false negative results if samples were not properly diluted (Bartsch et al., 2016). In a study by Summa et al., 28% of NoV was recovered from raspberries by the standard ISO method, but the RNA had to be diluted 10 times for use in RT-qPCR due to inhibitors (Summa et al., 2012). As it is difficult to estimate the level of inhibitors simply by the appearance of berries, which may pose a risk to consumers even with low levels of virus, a convenient and effective method to remove inhibitors, other than dilution, is vital to make the analysis applicable.

The present paper describes an improved method for virus detection in strawberries, raspberries and blueberries using direct extraction and removal of inhibitors. HAV, AdV40 and BNoV were included, as HAV and human NoV are considered the most important foodborne viral pathogens and AdV40 and 41 have been suggested as indicators of fecal contamination in water (Jothikumar et al., 2005). Bovine NoV was used as a surrogate for human NoV due to the overall similarity of the viruses (Vashist et al., 2009) and because it is non-infectious to humans and

Table 4
Comparison of RT-qPCR and RT-ddPCR regarding inhibitor tolerance (left) and sensitivity (right), using mengovirus RNA. Unfiltered RNA isolated from spiked strawberries was used in the tolerance test, while pure RNA from cell culture supernatant was used in the sensitivity study. The RNA was two-fold serially diluted and two µl used in (RT)-qPCR and RT-ddPCR. Results are presented individually. Ct: Ct-value; copy: RNA copy number; N: negative.

Dilution of RNA	Inhibitor tolerance test on unfiltered RNA from berries						Sensitivity test on pure RNA from cell culture supernatant				
	RT-qPCR (Ct)			RT-ddPCR (copy)			RT-qPCR (Ct)		RT-ddPCR (copy)		
Sample No.	1	2	3	1	2	3	1	2	1	2	2
1:1	***N***	***N***	***N***	2560	3340	3180	33.8	34.5	23	22	16.2
	N	***N***	***N***				34.1	35.0	20	20	
1:2	***N***	***N***	34.2	1280	1980	1880	35.0	36.2	16.6	13.2	4.8
	N	***N***	35.0				35.4	36.3	10.2	4.8	
1:4	32.6	***N***	33.6	688	888	1008	35.5	37.8	6.1	4.8	0
	33.1	***N***	32.8				35.9	38.4	2.8	0	
1:8	31.4	34.2	32.4	312	444	552	36.6	38.1	3.4	3.0	0
	31.7	33.5	32.1				38.3	38.4	2.8	2	
1:16	33.0	32.5	32.5	164	206	238	38.1	N	4.2	3.0	0
	33.3	32.7	33.0				38.3	N	0	0	

therefore safe to handle in the lab. The use of a model virus could influence the results, however, a BNoV with a fecally origin is probably as fit as cultivated murine NoV as a model for human NoV, in berry studies. Initially, strong inhibition could be observed when ruptured, overripe, dark-red strawberries were processed by the original direct lysis method. Also for blueberries and raspberries, different levels of inhibition were due to various ripe conditions, berry intactness and plant type. When including the MobiSpin column to remove inhibitors, detection of all the RNA viruses in all berry types, fresh and frozen, was improved (Table 1) although some RNA was lost (Table 1 and Fig. 2). Purification using the MobiSpin S400 column is based on size-exclusion chromatograph. The column is pre-packed with a Sephacryl HR matrix and designed for a wide variety of separation tasks. It requires the target to be at least 20 times larger than the impurities and has been applied to remove dNTPs, oligos and salt from NA, and dye terminators or unincorporated labelled nucleotides from DNA labelling reactions (Schneider et al., 2004; Sachsenröder et al., 2012). Other chromatography-based columns have been used in removing salts, small proteins and polysaccharides from various sample matrices, such as seminal fluid or stool (Schrader et al., 2012). Bartsch et al. had used Mobispin S400 columns to purify RNA isolated from strawberries by the standard ISO method (Bartsch et al., 2016). For NoV GII in frozen berries, they found an increase in recovery from 2.83 ± 2.92% to 15.28 ± 9.73% (batch 1), and from 0.59 ± 0.49% to 5.60 ± 1.58% (batch 2). In the present study, the recovery of BNoV from strawberries was 48.0 ± 26.0% (frozen) and 50.5 ± 7.8% (fresh), while the recovery of HAV from strawberries was 40.0 ± 13.3% (frozen) and 34.2 ± 10.5% (fresh) (Fig. 2). The recovery of viral RNA in raspberries (34–63%) was comparable to the results from the original research (46.2%) (Perrin et al., 2015), but the present method reduced inhibition by RNA purification instead of dilution (1:10), which is favorable as berries may contain low amounts of virus.

It is difficult to compare recoveries from different studies as berries may contain different levels of RT-qPCR inhibitors or protocols be executed with minor differences. The frozen berries used in the present study may be different to commercial berries, which are sometimes washed before freezing. The intactness of commercial frozen berries is not as good as for fresh berries, and release of juice is often observed after defrosting. In the present study, release of juice was more often observed in fresh berries during shaking with lysis buffer for 5 min. Although 4 ml lysis buffer is suggested for 25 g of berries (Fig. 1), more buffer should be added after shaking to keep the 4:1 ratio of buffer to released juice. Guanidium isothiocyanate is the component in lysis buffer that denatures proteins, lyses viral particles and releases viral NA, and a certain concentration is needed to obtain efficient release of NA. A low viral RR (0.52%) using the direct lysis method may partly be due to only 2 ml lysis buffer being used for 25 g of strawberries (Bartsch et al., 2016).

The LOD for viral RNA from strawberries achieved by the presented method (100 copies /25 g) was lower than the LOD achieved when combining the standard ISO method and column filtration (216 copies of NoV/25 g) (Bartsch et al., 2016). It was also similar to the LOD achieved by another modified direct extraction method on raspberries in which the RNA was diluted to avoid inhibitors (Summa and Maunula, 2018). Summa and Maunula combined direct NA extraction with minor supplement of PEG and got four positive results out of nine with 100 copies of NoV per 25 g raspberries. In the present study viral LOD was estimated for strawberries only, as initial testing showed similar recoveries of viral RNA for the three berry types, and strawberries contained the highest amounts of inhibitors. The relevance of comparing LODs between publications is, however, limited since no reference material has been used in the studies.

The sensitivity of virus detection depends on the RR, purity of viral RNA and the sample volume used in the RT-qPCR. As RNA filtered by the MobiSpin S400 column showed no inhibition (Table 1), the RNA volume could be doubled to four µl (20% of the total volume) in the RT-qPCR (Table 2). Although column filtering reduced the final amount of RNA with an average of 37% (Fig. 2), doubling the volume used for analysis is beneficial when analyzing berries that might contain low level of virus (giving pure RNA in an amount of 126% compared to using two µl of unfiltered RNA). In comparison, unfiltered RNA from fresh strawberries needed at least 1:8 dilutions to remove the influence of inhibitors, reducing the RNA amount to 12.5%.

The column filtration step was not necessary when viral DNA was the target, which indicates that the inhibition mainly lies in the reverse transcription (RT) of RNA (Table 1 and 2). The RR of AdV DNA was low compared with viral RNA, especially from blueberries (Fig. 2), despite a relatively low interference from inhibitors. The low recovery of AdV DNA from blueberries may be due to substances that bind to viral DNA or silica particles and thereby interfere with isolation of NA, or due to viral DNA being more easily adsorbed on blueberry matrix after lysis of the virus particles. Although the RR of AdV DNA was low compared to the RNA viruses, screening of adenoviruses could be used as a parameter for fecal contamination of strawberries and raspberries. However, further studies should be performed in order to optimize elution of viral DNA from berries.

Virus detection in food matrices should include a process control in order to monitor the quality of the total operation. As the standard ISO method includes mengovirus, the applicability of this virus as a process control for the optimized direct lysis method, was tested. Recoveries of mengovirus were higher than for HAV in blueberries and raspberries ($p < 0.05$), but not in strawberries (Fig. 2). Recoveries of mengovirus

were not significantly different to BNoV in raspberries (p 0.06-0.24). The results indicate that the recovery of mengovirus is relatively stable and that this virus could be used as a process control for the optimized protocol. The recovery of mengovirus was higher from raspberries and blueberries than from the control sample after filtration (Fig. 2). This can partly be explained by the variation of the results, but also by the fact that mengovirus was added directly to the berries soaked in lysis buffer and not onto the berries before drying, as for the other viruses.

Recently ddPCR has been confirmed to be a sensitive method enabling accurate quantification of a target NA sequence (Pinheiro et al., 2012). As ddPCR is based on the "most probable number" approach, it was interesting to compare RT-ddPCR with RT-qPCR regarding inhibitor tolerance and sensitivity of virus detection. Detection of unfiltered mengovirus RNA from strawberries was used to test the "proof of principle" as these extracts were highly inhibitory (Fig. 2). The results show that RT-ddPCR had a tolerance to substances that inhibited RT-qPCR (Table 4). For RT-qPCR, at least 1:8 dilutions of the RNA were necessary to remove the influence of inhibitors. However, when comparing the methods for the analysis of low levels of pure RNA, RT-qPCR seemed to give results that were more consistent compared to the RT-ddPCR (Table 4). The phenomenon of qPCR showing higher sensitivity and less variability than ddPCR with low viral load has also been noticed for the DNA viruses cytomegalovirus (Hayden et al., 2013, 2016) and hepatitis B virus (Boizeau et al., 2014). Digital droplet PCR includes automatic partitioning of samples (20 µl into 20,000 droplets) and three steps of sample transfer, which may result in lower repeatability for detection of low copy numbers (< 10 copies) of pure NA. However, for detection of RNA in environmental or food samples that contain inhibitors, as demonstrated in the present study, RT-ddPCR has the advantages of direct absolute quantification and higher tolerance to RT-inhibitors compared to RT-qPCR. As RT-qPCR inhibition was found for all three RNA viruses used in the present study, RT-ddPCR diagnostic is most likely advantageous for a range of RNA viruses in berries.

The conclusion based on the present study is that the optimized direct lysis method, which included column purification of RNA, was time-saving, efficient, and could be used in (RT)-qPCR analysis of different viruses from several types of berries under different conditions (fresh/frozen, intact/ruptured). As column purification also reduces the amount of RNA, RT-ddPCR, which showed resistance to inhibitors, is a very suitable alternative for detection and direct quantification of viral RNA in the berries.

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