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Abstract

Among the enteric viruses implicated in foodborne outbreaks, the human norovirus and hepatitis viruses A and E (HAV and HEV) represent a serious public health concern. International standard ISO 15216 proposes methods for detecting HAV and norovirus (genogroups I and II) RNA from soft fruit, leaf, stem and bulb vegetables, bottled water or food surfaces. These methods had not previously been validated for detecting the targeted viruses in other foodstuffs such as multicomponent foods, nor for detecting other viruses in foodstuffs. The aim of this study was to characterise a method derived from the vegetable method described in ISO 15216 to detect HAV, HEV and norovirus in artificially-contaminated multicomponent foodstuffs according to the recent international standard ISO 16140-4.

Results showed that the mean recovery rates for all settings did not differ according to the operator. The mean extraction yields ranged from 0.35% to 40.44% for HAV, 5.19% to 100% for HEV, 0.10% to 40.61% for norovirus GI and 0.88% to 69.16% for norovirus GII. The LOD_{95} was 10^2 genome copies/g for HAV, HEV and norovirus GII and 10^3 genome copies/g for norovirus GI. The LOQ was $2.90x10^4$, $1.40x10^3$, $1.60x10^4$ and $1.30x10^4$ genome copies/g for HAV, HEV, norovirus GI and norovirus GII respectively. The MNV-1 process control was detected in 120 out of 128 RNA extracts analysed and was recovered with an efficiency of between 3.83% and 50.22%. The mean inhibition rates of quantitative real-time RT-PCR reaction ranged from 3.25% to 28.70% and varied significantly with the type of food matrix. The described method could be used to detect viruses in composite food products for routine diagnosis needs.

Keywords: Multicomponent foodstuff; Human norovirus; Hepatitis virus (A, E); quantitative real-time RT-PCR; Detection; Process control

1. Introduction

Viruses are a leading cause of foodborne disease worldwide. Human norovirus and hepatitis viruses (hepatitis A (HAV) and hepatitis E (HEV)) are recognised to be the main viruses of public health importance. Enteric viruses are primarily transmitted via the faecal-oral and vomit-oral routes, including direct person-to-person contact, consumption of contaminated food or water, contact with contaminated environmental surfaces (Kotwal and Cannon, 2014; Matthews et al., 2012) and for hepatitis E virus direct contact with infected animals (Dalton et al., 2013; Meng, 2010; Pavio et al., 2006, 2010). Various food products, such as bivalve molluscs, fresh fruit and vegetables including different types of lettuce, onions or berries have been involved in foodborne disease outbreaks worldwide (Bernard et al., 2014; Donnan et al., 2012; Ethelberg et al., 2010; Fournet et al., 2012; Gallot et al., 2011; Herman et al., 2015; Le Guyader et al., 2010; Muller et al., 2016; Sarvikivi et al., 2012; Thebault et al., 2013; Wadl et al., 2010). A wide variety of foodstuffs have been implicated and mixed foods and ready to eat meals have frequently been considered as the carriers. In France, in addition to the high-risk food categories, meat, water, dishes with mixed foods or ready to eat meals have also been found to be responsible for 33% of foodborne illnesses and more specifically for 31% of viral foodborne disease outbreaks (*Santé publique France*, 2017). Virus outbreaks have been much more frequently reported in settings using catering services and in restaurants (*Santé publique France*, 2017). Food contamination mainly occurs in restaurants during food preparation by infected workers, and is most often associated with

food service settings (Baert et al., 2008; Barrabeig et al., 2010; Franck et al., 2015; Ronnqvist et al., 2014; Stals et al., 2013).

Most foodborne viruses are currently difficult or impossible to cultivate (Hamza et al., 2011), and sensitive molecular methods are therefore used to detect them in food. International standard ISO 15216 proposes methods for detecting HAV and norovirus (genogroup I and II) RNA from soft fruit, leaf, stem and bulb vegetables, bottled water or food surfaces. However, these methods have neither been validated to detect other targeted viruses in other foodstuffs, such as multicomponent foods.

The aim of this study was to validate a method adapted from ISO 15216-1 for the detection of HAV, HEV and norovirus in multicomponent foodstuffs based on the recent international standard ISO 16140-4 (Microbiology of the food chain — Method validation —Part 4: Protocol for method validation in a single laboratory) (Anonymous, 2017) to ensure the safety of these products.

2. Materials and methods

2.1. Viruses and cells

The FRhK-4 (foetal rhesus monkey kidney) cell line was purchased from the American Type Culture Collection (ATCC) (ATCC® CRL-1688™) (LGC standards SARL, Illkirch, France). These epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco™) supplemented with non-essential amino acids (NEAA, Gibco™) and 10% of heat-inactivated foetal bovine serum (FBS, Gibco™) (Thermo Fisher Scientific, Waltham, MA, 209 USA). Cells were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

HAV strain HM175/18f, clone B (VR-1402), was obtained from ATCC. This clone replicates rapidly and has cytopathic effects in cell cultures (Lemon et al., 1991). HAV stock was produced by propagation in foetal rhesus monkey kidney (FRhK-4) cells (ATCC, CRL-1688) (Cromeans et al., 1987). The virus production titre was determined in HAV RNA genomic copies using a quantitative real-time RT-PCR standard curve obtained from the ten-fold diluted *in vitro* RNA transcripts as previously described (Fraisse et al., 2017). The HAV stock had a titre of 2.90×10^9 genome copies/mL.

A clarified HEV genotype 3e suspension was obtained from faecal samples of infected swine provided by the ANSES Maisons-Alfort Laboratory for Animal Health. The partial sequence of ORF2 had previously been deposited with GenBank (accession number JF718793). The faecal sample was suspended in 10 mM Phosphate Buffered Saline (PBS), pH 7.4, to obtain a final 10% suspension (w/v), vortexed and centrifuged at 4000 g for 20 min at 4°C. Aliquots of supernatants containing viral particles were then stored at −80°C. The number of HEV RNA copies in the faecal suspension was quantified by ausing the standard curve obtained with the quantified *in vitro* RNA transcripts as previously described (Martin-Latil et al., 2012). The clarified suspension stock of HEV had a titre of 1.40×10^7 genome copies/mL.

Stool samples of norovirus GI (E8050) and norovirus GII (E7022) from infected humans were provided by the French National Reference Center for Gastroenteritis Viruses in Dijon, France. The faecal samples were suspended in 10 mM phosphate-buffered saline (PBS), pH 7.4 to obtain a final 10% suspension (w/v), and then vortexed and centrifuged at 4,000 x g for 20 min at 4°C. Aliquots of 100 µL were frozen and kept at -80°C for later use. The genomic titres of the clarified faecal suspensions were determined by quantitative real-time

RT-PCR using a quantitative real-time RT-PCR standard curve obtained with the 10-fold diluted *in vitro* RNA transcripts as previously described (Hennechart-Collette et al., 2014). The clarified suspension stocks of norovirus GI and norovirus GII had titres of approximately 1.60×10^7 and 1.30×10^7 genome copies/mL respectively.

The murine norovirus MNV-1 (CW1 strain) was provided to the ANSES Fougères Laboratory (Fougères, France) by Dr. H. Virgin from Washington University (Saint Louis, MO, USA), and was propagated in a mouse leukaemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). RAW 264.7 was grown at 37°C in an atmosphere containing 5% CO_2 in DMEM supplemented with GlutaMAX™, 1% non-essential amino acids and 10% foetal bovine serum (Life Technologies, Saint Aubin, France). The production stock of MNV-1 had titres of approximately 2.15×10^7 $TCID_{50}$/mL.

2.2. Experimental design and artificial contamination of multicomponent foodstuffs

Twelve multicomponent foodstuff samples (samples 1 to 12 including vegetables, mixed vegetables, meals including meat or fish, soup or sauce) were purchased from a local supermarket. These samples were selected by taking account of food processing factors such as freezing, vacuum packaging or canning. Table 1 describes the selected samples according to matrix type and processing factor. The selected food samples were classified according to the EFSA FoodEx2 classification (https://data.food.gov.uk/codes/foodtype/hierarchy/main/_report).

The experimental design described in ISO 16140-4:2017 was used. Multicomponent foods were artificially contaminated at four contamination levels for all four viruses (HAV, HEV,

137 norovirus GI and GII). The concentration levels for HAV, HEV and norovirus were obtained by
138 using different inoculum dilution levels. The four inoculation levels ranged from 2.90×10^3 to
139 2.90×10^6 genome copies for HAV, 1.40×10^2 to 1.40×10^5 genome copies for HEV, 1.60×10^3 to
140 1.60×10^6 genome copies for norovirus GI and 1.30×10^3 to 1.30×10^6 genome copies for
141 norovirus GII. The food samples were randomly allocated to four different settings (R1, R2,
142 R3 and R4). For each setting, four different matrices per contamination level were analysed
143 by two operators (operators A and B). Table 2 describes the allocation of the food matrices
144 to the different settings along with virus level. Thus, for each virus, four settings (R1 to R4)
145 with four inoculation levels and two replicates (operators A and B) were tested
146 corresponding to 32 analyses in total. Tests were conducted on three different days to
147 evaluate the reproducibility of the method. R1 and R2 settings were analysed on the same
148 day, R3 and R4 on two different days.
149 Each sample was co-inoculated with 2.15×10^4 $TCID_{50}$ of MNV-1 (process control virus) just
150 before adding elution buffer. For each matrix, one food sample inoculated only with sterile
151 water was used as a negative control during the entire sample processing and viral detection
152 procedure.
153

154 ***2.3. Sample processing***

155 The method used to recover the viruses was adapted from the ISO 15216-1 procedure
156 described for vegetables by adding a final purification step with chloroform-butanol. Briefly,
157 each inoculated sample (25 g) placed in a 400 mL polypropylene bag containing a filter
158 compartment was soaked in 40 mL of elution buffer (Tris-HCl 100 mM, glycine 50 mM, 1%
159 beef extract (Sigma-Aldrich, Saint-Quentin Fallavier, France)) at pH of 9.5. The rinsing fluid
160 was removed via the bag's filter compartment and centrifuged at 10,000 g for 30 min at 4°C

to pellet the food particles. The pH of the decanted supernatant was adjusted to 7.2 ± 0.2 by the addition of 5 N HCl while the fluid was swirled constantly. The neutralised supernatant was supplemented with 10% (w/v) polyethylene glycol (PEG) 8,000 and 0.3 M NaCl (Sigma-Aldrich), and was then incubated with constant shaking at 4°C for one hour. The viruses were concentrated by centrifuging the solution at 10,000 g for 30 min at 4°C. The supernatant was discarded then what remained was centrifuged again at 10,000 g for 5 min at 4°C to compact the pellet. The pellet was then suspended in 500 µL of PBS and vortexed with 500 µL of chloroform: butanol, 1:1 (v/v). The suspension was then incubated for 5 min at room temperature, and centrifuged at 8,000 g for 15 min at 4°C. The upper aqueous phase containing viruses was directly processed by the nucleic acid extraction procedure.

2.4. Viral RNA extraction

NucliSENS® easyMAG™ lysis buffer (BioMérieux, Marcy l'Etoile, France) was added to the virus suspension (up to 3 mL) and total nucleic acid was extracted using the NucliSENS® easyMAG™ platform with the "off-board Specific A" protocol according to the manufacturer's instructions. Nucleic acids were eluted in 100 µL of elution buffer and stored at -80°C.

2.5. Primers and probes

The primers and probes used to quantify HAV, norovirus GI and norovirus GII have already been described in the literature (Costafreda et al., 2006; da Silva et al., 2007; Kageyama et al., 2003; Loisy et al., 2005; Pinto et al., 2009; Svraka et al., 2007) and are recommended in

183 ISO 15216 standards. The primers and probe used to quantify HEV were adapted from the
184 model described by Jothikumar et al. (2006) and have been previously described (Martin-
185 Latil et al., 2012b, 2014). The primers and the TaqMan® probe targeting the ORF1
186 polyprotein of the murine norovirus (MNV-1), which were designed using Beacon Designer
187 software (Bio-Rad, Marnes-la-Coquette, France), have also been described in the literature
188 (Martin-Latil et al., 2012b). All the primers and probes were purchased from Eurofins MWG
189 Operon (Les Ulis, France).
190

191 ***2.6. Quantitative real-time RT-PCR conditions***

192 One-step quantitative real-time RT-PCR amplifications were performed in duplicate on the
193 CFX96™ real-time PCR detection system (Bio-Rad). Reactions were performed in a 25 µL
194 reaction mixture containing 1X of RNA UltraSense™ master mix and 1.25 µL of RNA
195 UltraSense™ enzyme mix, which are components of the RNA UltraSense™ One-Step
196 Quantitative RT-PCR System (Life Technologies), 2 U RNase inhibitor (Life Technologies),
197 1.25µg of bovine serum albumin (Life Technologies), 500 nM of forward primer, 900 nM of
198 reverse primer, 250 nM of probe and 5 µL of RNA extract.Positive controls containing RNA
199 extracted from virus suspensions and a negative control containing all the reagents except
200 the RNA template were included with each set of reaction mixtures. The one-step
201 quantitative real-time RT-PCR programme involved 60 min of reverse transcription of RNA at
202 55°C, followed by a 5 min denaturation step at 95°C, and finally 45 cycles of 15 s at 95°C, 1
203 min at 60°C and 1 min at 65°C. Fluorescence was recorded by the apparatus at the end of
204 the elongation steps (1 min at 65°C) for each amplification cycle. All the samples were
205 characterised by a corresponding cycle threshold (Ct) values. Negative samples gave no Ct

206 value. A standard curve for each viral target was generated with RNA extracts resulting from
207 the serial dilution of viral stock suspension in distilled water. The slopes (S) of the regression
208 lines were used to calculate the amplification efficiency (E) of the quantitative real-time RT-
209 PCR reactions according to the formula $E=10^{|1/s|}-1$ to determine the performance of
210 quantitative real-time RT-PCR assays. HAV, HEV, norovirus GI, norovirus GII, and MNV-1
211 recovery rate percentages from spiked samples were calculated by using the standard curves
212 obtained with viral inoculum dilution and the following formula: quantity of virus recovered
213 after spiking experiments/quantity of viral inoculum X100. The HAV, HEV, norovirus GI or
214 norovirus GII RNA transcript was used as an external amplification control (EAC) to monitor
215 RT-PCR inhibition in samples. This approach has been described in ISO 15216-1, where an
216 external control RNA (i.e. an RNA species carrying the target sequence of interest) is added
217 to an aliquot of RNA sample. The degree of RT-PCR inhibition in each tested sample is
218 obtained by comparing these results with the results of EAC RNA in the absence of sample
219 RNA (i.e. in water).
220

221 ***2.7. Statistical analysis***

222 All statistical analyses were performed using the Statgraphics Centurion XVII software
223 (Statgraphics Centurion Version 17.1.04). The influence of the operator factor on virus
224 recovery rates (norovirus, HAV, HEV and MNV) was first assessed by using one-way analysis
225 of variance (ANOVA). The result of the ANOVA is a p value associated with the hypothesis
226 that the mean recovery rates of all groups were the same. The influence of additional factors
227 on extraction yields of pathogenic viruses (norovirus, HAV, HEV and MNV) was studied by
228 using a one-way ANOVA. The effect of virus (norovirus, HAV, HEV and MNV-1) on virus

recovery rates was first evaluated. Because the extraction yields were statistically different according to the virus used (ANOVA, $p < 0.05$), a multiple-comparison procedure (Fisher's least-significant-differences (LSD)) was applied to determine which viruses, provided the highest extraction yields. Graphs plotting the mean and its standard error for each group illustrate the multiple comparison procedure. When confidence intervals for means do not overlap, the difference between two groups of a factor is significant. Next, four factors were tested on extraction yields: (1) dilution of RNA extracts (pure vs. 10-fold diluted), (2) the inoculated level of viruses, (3) setting experiments (R1 to R4) and (4) EAC recovery rates according to the type of food matrix.

The limit of detection values (LOD) which correspond respectively to 50% (LOD_{50}) and 95% (LOD_{95}) of the probability of detection were calculated with the method for estimating POD (probability of detection) function and the LOD of a qualitative microbiological measurement method as described by Wilrich et al. (2009). The POD-LOD calculation software was used (version 9, dated 2017-09-23) (Wilrich et al., 2009). This program can be freely downloaded from www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html. The limit of quantification (LOQ) was estimated for each virus using the total error approach based on the accuracy profile (Hubert et al, 2007).

The accuracy profile is calculated by a two-sided β expectation tolerance interval (β-ETI) for each level from the results of the validation experiments expressed, such as recovery (trueness) and precision (repeatability and intermediate precision). Data are log_{10} transformed and we defined the acceptability limits (λ) at ± 95 % of bias because the expected recovery is low. To calculate the accuracy profile a specified proportion has been defined. This proportion is fixed to 80% (β) and corresponds to future measurements would

253 fall within the acceptability limits. The complete theory and calculation of accuracy profiles
254 are described in the references (Hubert et al, 2007a, Hubert et al, 2007b, Hubert et al, 2008)
255 and a fully developed application is presented for microbiological methods by Feinberg et al
256 (2009) and Boubetra et al (2011). The procedure reported here is simplified and tailored to
257 molecular methods, such as published by Saint-Cyr et al (2014).
258 The results are presented through a graphical representation as follows: the horizontal (x)
259 axis shows the reference level in $\log_{10}$ concentration level (genome copies/g) and the vertical
260 (y) axis shows at each level the bias, the acceptability limits (λ), and the β-ETI limits.
261

262 **3. Results**

263 ***3.1 Mean virus recoveries of norovirus, HAV and HEV according to operator***

264 To determine whether the operator factor influenced virus recovery rates, the mean
265 recovery rates obtained for all settings were compared for each operator (A and B) (Figure
266 1). These results showed that the mean recovery rates for all settings were 8.04% and
267 13.81% for HAV, 41.25% and 49.59% for HEV, 9.95% and 6.55% for norovirus GI, 15.78% and
268 14.88% for norovirus GII, 18.18% and 13.38% for MNV-1 for operator A and operator B
269 respectively. The statistical analysis revealed that the operator factor did not influence virus
270 recovery from multicomponent food (one-way ANOVA; p-value=0.3215).
271

272 ***3.2 Virus recoveries and limit of detection***

273 Table 3 gives the mean extraction yields obtained for norovirus GI, norovirus GII, HAV, HEV
274 and the process control virus according to the inoculum level and repeat experiments (R1 to
275 R4). As expected, no viral RNA was detected in the uninoculated samples. The average of

recoveries with pure RNA extracts ranged from 0.35% to 40.44% for HAV, from 5.19% to 100% for HEV, from 0.1% to 40.61% for norovirus GI and 0.88% to 69.16% for norovirus GII. The mean recovery rates obtained for HAV, HEV, norovirus GI and norovirus GII showed that the recovery rates from composite matrices vary according to the virus inoculated (one-way ANOVA; p-value<0.001) (Figure 2). More specifically, the multiple comparison tests showed that the recovery rates of HEV were higher and significantly different from other viruses.

By testing the 10-fold diluted RNA extracts, the extraction yields obtained for HAV, HEV and norovirus in composite matrices were improved by a factor that ranged from 0.0 to 25.7 (Table 3).

The process control (MNV-1) was detected in 120 out of 128 RNA extracts analysed and was recovered with an efficiency of between 3.83% and 50.22% for all four food settings.

The limits of detection (LOD) for the method were calculated by the Wilrich approach for each virus in each setting with the twelve samples. The LOD_{50} and LOD_{95} values for HAV, HEV, norovirus GI and norovirus GII are shown in Table 4.

The LOD_{95} values were 10^2 genome copies/g for HAV, HEV and norovirus GII and 10^3 genome copies/g for norovirus GI. The LOD_{50} values were between 80 and 92 genome copies/g for HAV, HEV and norovirus GII and 360 genome copies/g for norovirus GI.

The limit of quantification (LOQ) was assessed for different viruses using the accuracy profile. Figure 3 shows a typical accuracy profile for HAV. Table 5 summarizes the results for the LOQ and LOD and some performance characteristics (repeatability and intermediate precision). The LOQ was 2.90×10^4, 1.40×10^3, 1.60×10^4, 1.30×10^4 genome copies/g for HAV, HEV, norovirus GI and norovirus GII respectively. The LOD for HAV, HEV, norovirus GI and norovirus GII was established at 0.97×10^4, 0.47×10^4, 0.53×10^4 and 0.43×10^4 genome copies/g respectively.

3.3 Influence of experimental factors on virus extraction yield

To assess the influence of the different experimental factors on HAV, HEV and norovirus extraction from multicomponent matrices, the mean recovery rates from virus-spiked samples were compared. Figure 4 shows the recovery rates for HAV, HEV and norovirus according to their inoculation levels. The statistical analysis indicated that the recovery rates were not significantly different for norovirus GII regardless of the inoculation level (one-way ANOVA; p-value=0.2942 for norovirus GII), but were significantly different for norovirus GI, HAV and HEV when different inoculation levels were tested (one-way ANOVA; p-value<0.001 for HAV and norovirus GI, and p-value<0.0243 for HEV).

More specifically, the multiple-comparison tests showed that inoculations with $2.90x10^3$ HAV genome copies, $1.40x10^2$ HEV genome copies and $1.6x10^3$ norovirus GI genome copies differed significantly from other inoculation levels of HAV, HEV and norovirus GI.

Figure 5 shows the mean recovery rates of HAV, HEV, norovirus in the four repeat experiments. The differences between R1 to R4 were not significant for the extraction yield of norovirus GI (one-way ANOVA; p-value=0.0736) but were significant for the extraction yield of norovirus GII, HAV and HEV (one-way ANOVA; p-value=0.0028 for HAV, one-way ANOVA; p-value<0.001 for norovirus GII and for HEV). More specifically, the multiple-comparison tests showed that R3 was significantly different from R1, R2 and R4 for HAV. Both R1 and R3 were significantly different from R4 for HEV, and R4 was significantly different from R1, R2 and R3 for norovirus GII.

The statistical analysis also revealed that the allocated food setting (factor R1 to R4) influences mean MNV-1 recoveries (one-way ANOVA; p-value=0.0046). The multiple-comparison tests showed that R1 and R2 were significantly different from R3 and R4. Mean

MNV-1 recoveries from allocated food settings R1 and R2 were higher than allocated food settings R3 and R4.

3.4 Recovery rates for external amplification control (EAC)

An EAC corresponding to the viral target was used to examine quantitative real-time RT-PCR inhibition. Table 6 shows the mean percentages of quantitative real-time RT-PCR inhibition for each of the twelve samples. The mean percentages of quantitative real-time RT-PCR inhibition varied from 3.25% to 28.70%. They vary significantly with the type of food matrix (one-way ANOVA; p-value=0.0209) but not with repeated experiments (R1 to R4) (one-way ANOVA; p-value=0.0554).

4. Discussion

Enteric viruses are the leading cause of foodborne outbreaks. Their detection is challenging because many of them are either difficult or impossible to replicate in cell culture, and the number of viral particles present in the food may be very low. Today, quantitative real-time RT-PCR is widely used for virus detection because it is sensitive, specific, rapid and can deliver quantitative data. ISO standards 15216-1 and 15216-2 were published in 2017 and 2019 respectively for detecting and quantifying norovirus and HAV in high-risk food categories such as shellfish, bottled water and vegetables. The method used in this study to recover viruses from composite foods is adapted from the ISO 15216 procedure described for vegetables. The LOD_{95} varied from 10^2 to 10^3 genome copies per g regardless of the virus analysed. Because of the complexity of the food matrices used in our study, the LOD_{95} values obtained for norovirus and HAV in composite foods and multicomponent foodstuffs were higher than the LOD_{95} recently reported for lettuce or raspberries (Lowther et al., 2019).

Derived from the LOQ estimated by accuracy profile, the LOD in our study ranged between 4.33x$10^3$and 9.67x10^3 genome copies per g. These values are slightly different than the estimated LOD_{95} but the difference, which is around one log, can be explained by the definition of these parameters. LOD_{95} is the level at which the probability of detection is equal to 95 % (ISO standard 16140-1), whereas the LOD is estimated using a quantitative approach based on a chemical method. Classically, the LOD is the lowest level that differs from the background noise with an accepted confidence level. Armbruster et al. (2008) have previously explained that the LOD is a value as mean + k SD with k, the coverage factor, ranged between 2 and 10. The common value for k is 3 for LOD and if k = 10 it is the LOQ. However, the LOQ is the lowest level quantifiable with a defined trueness and precision (ISO standard 16140-1). In our case, we defined the LOQ using an accuracy profile with a defined trueness and precision, then derived the LOD from this LOQ. These two approaches are totally different because one is for a qualitative approach and the other for a quantitative approach. The results are nonetheless of the same magnitude and consequently the LOD confirms the LOD_{95} values.

Various methods have been described for extracting viruses from vegetables, composite foods or dairy products (Baert et al., 2008; Blaise-Boisseau et al., 2010; Cheong et al., 2009; Coudray et al., 2013; Dubois et al., 2002; El Sanousy et al., 2013; Hennechart et al., 2017; Hida et al., 2013; Hyeon et al., 2011; Kim et al., 2008; Morillo et al., 2012; Pan et al., 2012; Sanchez et al., 2012; Scherer et al., 2010 ; Schwab et al., 2000; Stals et al., 2011a, 2011b). The PEG concentration method was used in our study to concentrate viruses from multicomponent foods. Our results are in agreement with data reported in other studies on food. Virus recovery rates ranged from 0.35% to 40.44% for HAV, 5.19% to 100% for HEV and

371 0.10% to 69.16% for norovirus. The PEG concentration method was previously developed
372 and applied to recover viruses from suspected food such as shellfish, smooth-surfaced, semi-
373 dried tomatoes or food samples such as spaghetti, chicken, ham and sausages (Martin-Latil
374 et al., 2014, 2012a; Saito et al., 2015; Summa et al., 2012; Rutjes et al., 2006). The norovirus
375 recovery rates in the literature varied between 0.02% and 47%, while the HEV recovery rate
376 from pig liver sausage ranged from 3.94 to 18.38% and the HAV recovery rate from semi-
377 dried tomatoes ranged from 2.91% to 50.92% (Hennechart et al., 2017; Martin-Latil et al.,
378 2014, 2012a; Saito et al., 2015; Summa et al., 2012; Rutjes et al., 2006). Taking into account
379 the mean recovery rates obtained with other matrices, our results lie within the same range.
380
381 ISO standard 15216-1 was validated for the detection and quantification of HAV and
382 norovirus in seven food matrices: bottled water, food surfaces, Pacific oysters (*Crassosttrea*
383 *gigas*), common mussels (*Mytilus edulis*), raspberries, lettuce and green onions (Lowther et
384 al., 2017). Other methods for the detection of viruses in semi-dried tomatoes, raw pig livers
385 and dairy products have also been characterised (Hennechart et al., 2017; Martin-Latil et al.,
386 2014, 2012a). However, to our knowledge, no publications until now have reported the
387 validation of a method for detecting norovirus, HAV or HEV from multicomponent
388 foodstuffs.
389
390 The experimental design from ISO standard 16140-4:2017 was applied to characterise a
391 method used to detect norovirus, HAV and HEV in multicomponent foodstuffs. In addition to
392 the factors studied in the ISO 16140 standard, part 4 describes the calculation of
393 repeatability and reproducibility. The experimental design allowed us to study a method
394 without a reference method. Twelve different foodstuffs (vegetables, mixed vegetables,

meals including meat or fish, soup or sauce) were selected and analysed in this study. This selection allowed us to take into account a wide selection of multicomponent foods representative of samples analysed in the laboratory when investigating viral foodborne outbreaks. The items selected are consumed in a variety of forms and are major components of meals.

To calculate repeatability, foods were randomly allocated to four different settings (R1, R2, R3 and R4) and results show that the differences between repeated experiments (R1 to R4) were significant for the extraction rate of norovirus GII, HAV and HEV**.** Different studies have shown that the composition of food products can affect virus extraction (Blaise-Boisseau et al., 2010; Butot et al., 2007; Summa et al., 2012; Yavarmanesh et al., 2013, 2010). Due to the presence of substances that can inhibit PCR amplification, the implementation of different controls such as the virus process control and EAC are necessary to validate results. According to the recommendations in ISO 15216, the inhibition rates for RNA extracted from food samples have to be lower than 75% and virus process control extraction yields higher than 1%. In this study, the mean percentages of quantitative real-time RT-PCR inhibition were always lower than 75% and the recovery rate of MNV-1 was more than 1% in 93% of the RNA extracts analysed. In our study, MNV-1 was successfully tested as a process control virus for detecting HAV, HEV and norovirus in multicomponent foodstuffs. Its use had previously been described in the literature for different types of water, shellfish, milk products, meat such as sausages, soft red fruits, lettuces and ready-to-eat foods (Coudray et al., 2013; Hennechart et al., 2015; Martin-Latil et al., 2014; Sanchez et al., 2012; Stals et al., 2011a, 2011b).

According to the ISO 16140 procedure, analyses were conducted by two operators. An evaluation of operator influence showed no significant difference between the two

operators. The calibration and maintenance of equipment should also limit the difference between operators working at the same laboratory. To evaluate the quality of virus detection from multicomponent foodstuffs for other laboratories, it should be necessary to carry out an inter-laboratory assay.

To conclude, the method described herein could be used to detect viruses in composite food products for routine diagnosis needs. This research underscores the importance of further research to develop, standardise and validate methods for detecting viruses from other food matrices or other viruses than those described in standard ISO 15216.

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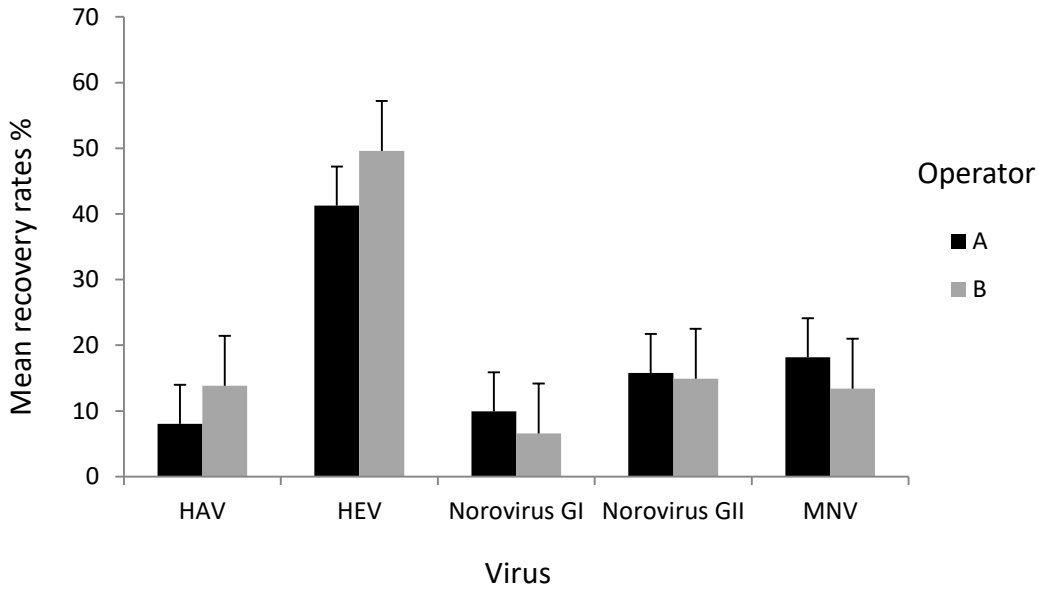


Figure 1: Comparison of mean recovery rates for HAV, HEV, norovirus GI, norovirus GII and MNV according to the operator factor (operator A and operator B). For each virus, 16 analyses were performed by each operator (four foodstuffs for all settings with all levels of inoculation) and RNA extracts were analysed in duplicate (pure and ten-fold diluted) with a quantitative real-time RT-PCR assay

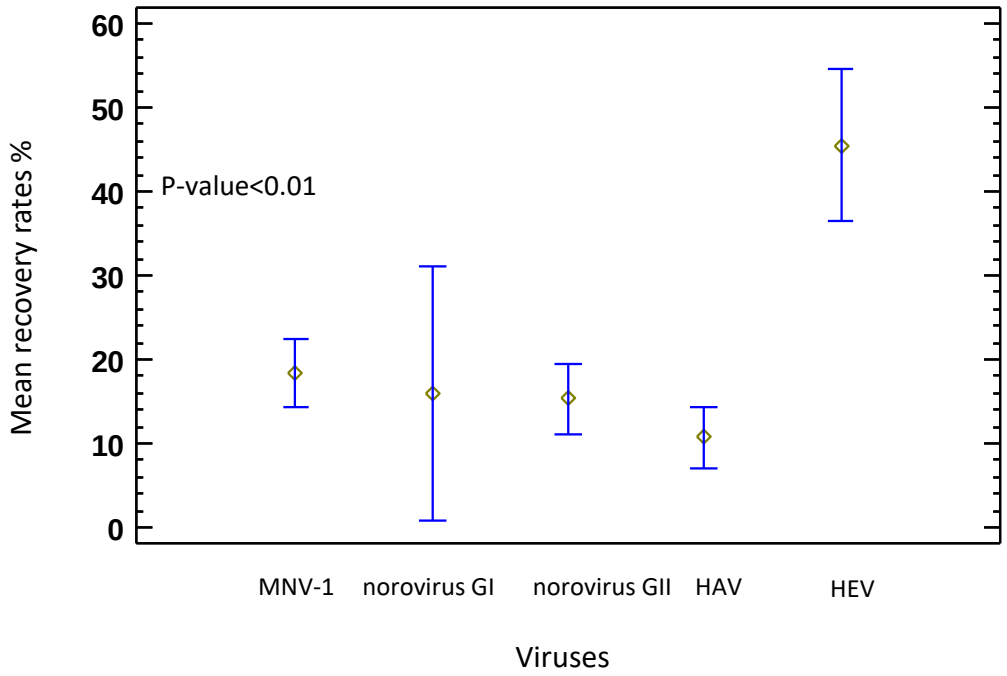


Figure 2: Comparison of mean virus recovery rates from spiked samples for all settings

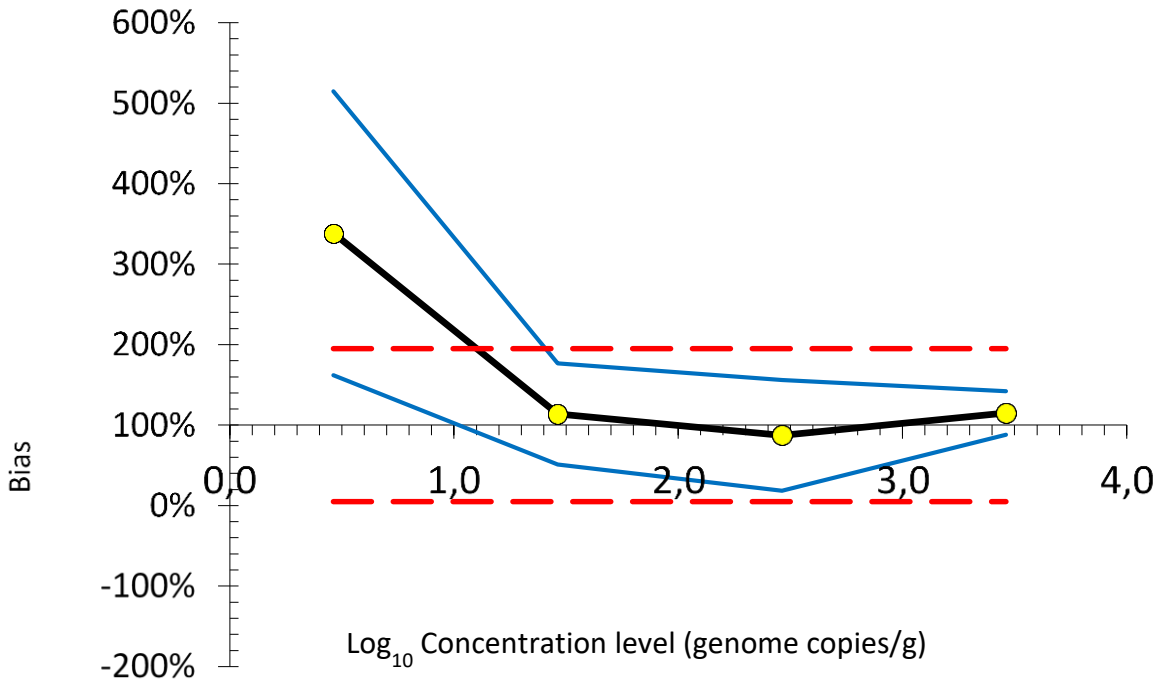


Figure 3: Example of accuracy profile for HAV. The black line is the bias and blue lines are the tolerance limits that define the 80% tolerance interval around the bias. Yellow points are the concentration levels tested. Dotted lines are the acceptance limit.

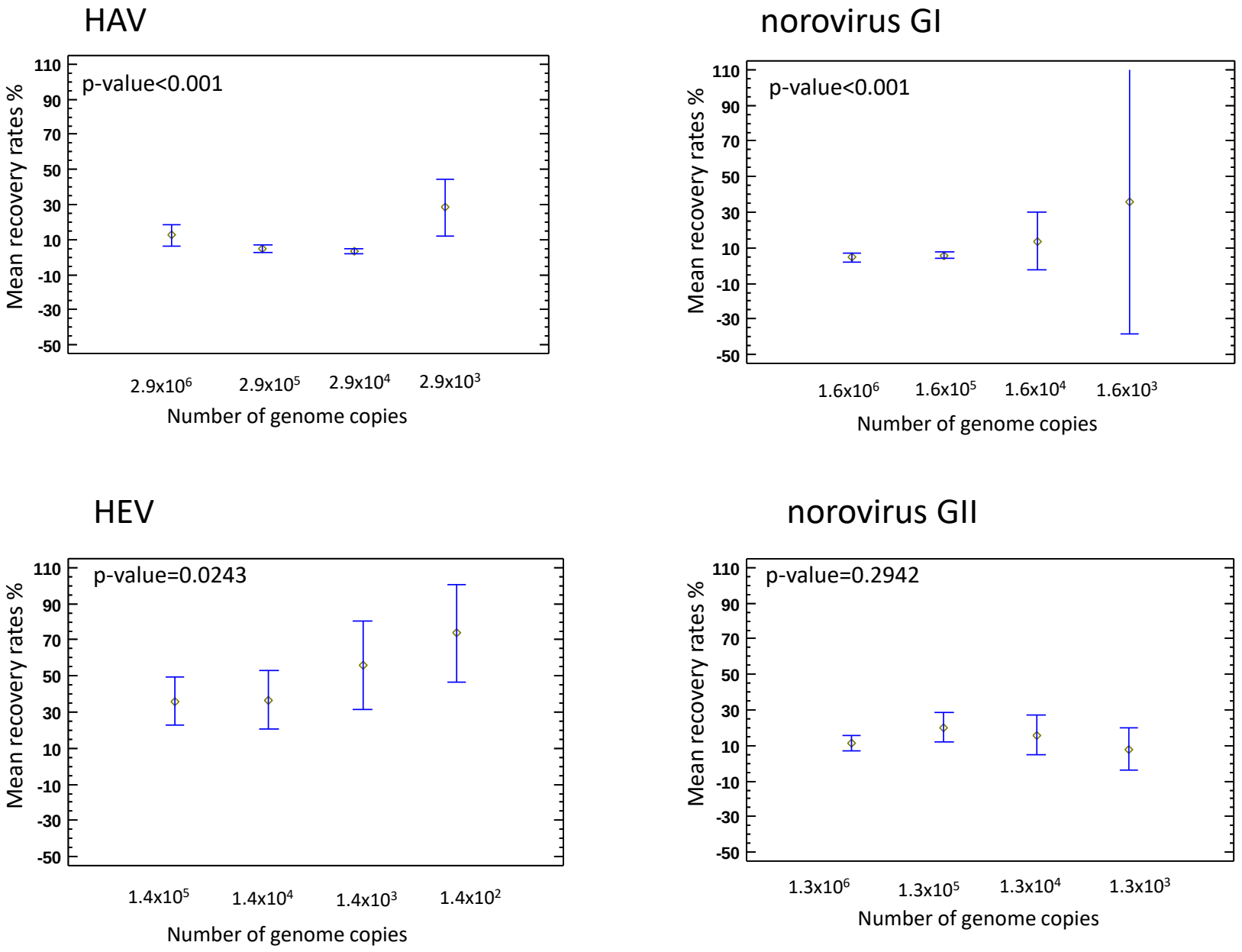
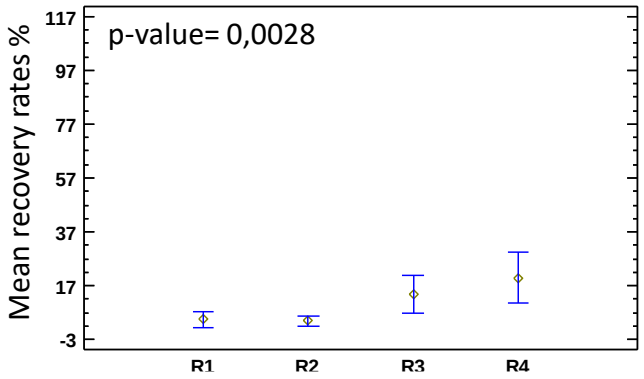
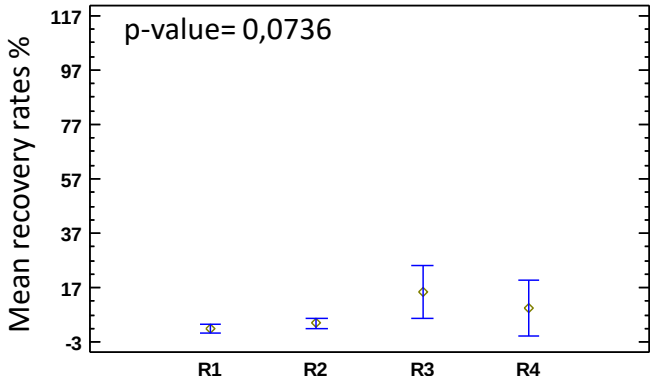


Figure 4: Recovery rates for HAV, HEV and norovirus with respect to artificial inoculation levels

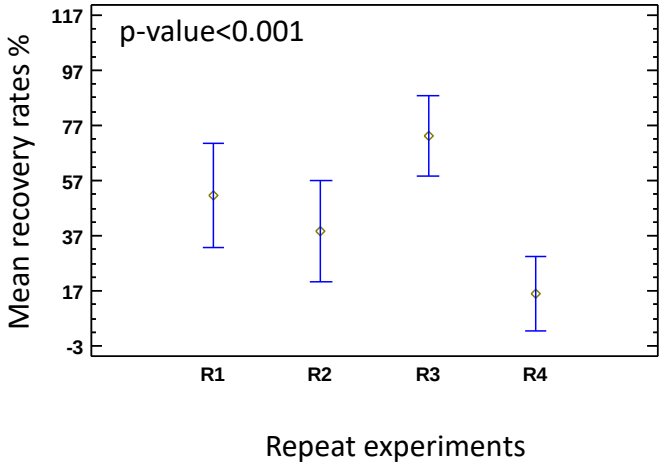
HAV



norovirus GI



HEV



norovirus GII

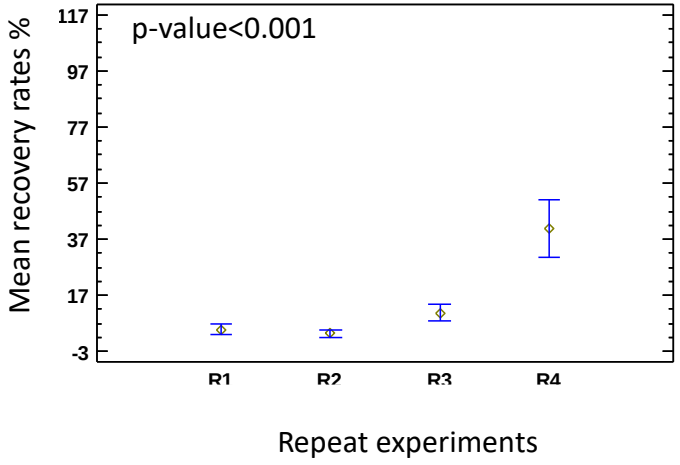


Figure 5: Recovery rates for HAV, HEV and norovirus in repeat experiments (R1 to R4)

Table 1: Selected food samples according to the matrix type and processing factor

Processing factor	Matrix type	Matrix (EFSA FoodEx2 classification)	Sample number
Tin can	Composite dishes with meat	Meat stew (A03VY)	1
		Vol-au-vent (A040G)	2
	Vegetables	Spinach (A00MH)	3
		Corn salad (A05EA)	4
		French beans (A00PG)	5
	Mixed vegetables	Ratatouille (A03YH)	6
	Soup	Vegetable soup (A041S)	7
	Sauce	Ketchup (A044P)	8
Chopped	Composite dishes with meat or fish	Spaghetti bolognese (A040V)	9
		Paella (A041D)	10
		Mixed salmon and spinach (A0C75 and A00MH)	11
Frozen	Mixed vegetables	Mushrooms (A03YV)	12

Table 2: Experimental design for detection of each enteric virus (HAV, HEV and norovirus) in multicomponent foodstuffs adapted from the ISO standard 16140-4:2017 procedure performed by two operators

		Repeat experiments			
		R1	R2	R3	R4
Virus contamination levels	Very low	Sample 8	Sample 4	Sample 5	Sample 6
	Low	Sample 1	Sample 11	Sample 6	Sample 3
	Medium	Sample 2	Sample 10	Sample 7	Sample 5
	High	Sample 12	Sample 9	Sample 8	Sample 4

Table 3: Mean percentage recovery calculated for four inoculum levels of HAV, HEV, norovirus GI or GII in the presence of MNV-1

Virus	Number of genome copies	RNA extracts	Repeated experiment R1 (%±SD)	(F)	Repeated experiment R2 (%±SD)	(F)	Repeated experiment R3 (%±SD)	(F)	Repeated experiment R4 (%±SD)	(F)
HAV	$2.90x10^6$	pure	1.56±1.31 (4/4)	1.6	2.80±1.05 (4/4)	1.2	32.53±3.99 (4/4)	1.5	4.39±0.72 (4/4)	0.8
		10-fold diluted	2.58±2.54 (4/4)		3.28±1.80 (4/4)		48.81±3.96 (4/4)		3.50±0.80 (4/4)	
	$2.90x10^5$	Pure	3.72±4.01 (4/4)	1.5	3.62±2.34 (4/4)	1.1	7.26±8.27 (4/4)	1.7	0.41±0.67 (4/4)	
		10-fold diluted	5.57±4.52 (4/4)		4.05±2.91 (4/4)		12.16±2.18 (3/4)		nd	
	$2.90x10^4$	Pure	3.11±3.49 (4/4)	1.5	3.45±3.36 (4/4)	0.9	4.75±4.06 (4/4)	0.9	4.02±2.54 (4/4)	1.0
		10-fold diluted	4.56±5.29(3/4)		3.21±2.67 (4/4)		4.33±6.70 (2/4)		0.40±0.63 (4/4)	
	$2.90x10^3$	Pure	24.99±21.17 (4/4)	2.3	19.06±16.29 (3/4)	1.2	40.44±14.48 (2/4)	0.1	0.35 (1/4)	
		10-fold diluted	57.90±49.40 (4/4)		22.55 (1/4)		3.13±0.43 (2/4)		nd	
MNV-1	MNV		8.49±9.84		8.12±6.22		12.28±17.52		3.83±4.85	
	Total samples with recovery rates >1%				27/32					

Virus	Number of genome copies	RNA extracts	Repeated experiment R1 (%±SD)	(F)	Repeated experiment R2 (%±SD)	(F)	Repeated experiment R3 (%±SD)	(F)	Repeated experiment R4 (%±SD)	(F)
HEV	**$1.40x10^5$**	pure	29.14±13.87 (4/4)	2.1	6.24±4.92 (4/4)	2.7	59.04±9.99 (4/4)	1.7	16.65±18.31 (4/4)	0.3
		10-fold diluted	60.22±40.64 (4/4)		16.92±10.99 (4/4)		100.00±0.00 (4/4)		4.62±2.21 (3/4)	
	$1.40x10^4$	pure	33.64±45.25 (4/4)	2.8	57.34±38.49 (3/4)	1.0	27.49±16.87 (4/4)	2.2	5.19±6.09 (2/4)	0.9
		10-fold diluted	94.45 (1/4)		57.63±51.29 (2/4)		59.87±14.20 (2/4)		4.87±5.96 (3/4)	
	$1.40x10^3$	pure	16.09±11.75 (2/4)	6.2	17.90 (1/4)	11.1	100.00±0.00 (3/4)		nd	
		10-fold diluted	100.00±0.00 (3/4)		99.67±99.50 (2/4)		nd		14.62±10.48 (3/4)	
	$1.40x10^2$	pure	55.79±62.52 (2/4)		11.64 (1/4)	8.6	100.00±0.00 (3/4)		50.64±53.76 (2/4)	1.7
		10-fold diluted	nd		100.00±0.00 (2/4)		nd		86.35	
MNV-1	MNV		18.43±11.74		15.40±16.00		12.78±8.44		12.33±5.39	
	Total samples with recovery rates >1%				30/32					

Virus	Number of genome copies	RNA extracts	Repeated experiment R1 (%±SD)	(F)	Repeated experiment R2 (%±SD)	(F)	Repeated experiment R3 (%±SD)	(F)	Repeated experiment R4 (%±SD)	(F)
norovirus GI	$1.60x10^6$	pure	0.10±0.08 (4/4)	0.6	1.77±0.12 (4/4)	0.5	10.95±6.63 (4/4)	1.5	1.42±0.41 (4/4)	1.3
		10-fold diluted	0.06±0.05 (2/4)		0.93±0.36 (4/4)		16.29±11.10 (4/4)		1.91±0.63 (4/4)	
	$1.60x10^5$	pure	3.36±0.64 (4/4)	1.4	8.24±2.95 (4/4)	0.4	5.59±2.79 (4/4)	2.2	5.44±0.96 (4/4)	0.9
		10-fold diluted	0.48 (1/4)		2.96±3.33 (3/4)		12.01±10.46 (3/4)		5.18±4.23 (3/4)	
	$1.60x10^4$	pure	2.55±3.34 (2/4)	4.6	nd		3.89±3.14 (4/4)	25.7	11.68±9.93 (3/4)	0.1
		10-fold diluted	11.96 (1/4)		9.76 (1/4)		100.00 (1/4)		1.68 (1/4)	
	$1.60x10^3$	pure	1.14±0.54 (2/4)		nd		40.61 (1/4)		nd	
		10-fold diluted	nd		nd		nd		100.00 (1/4)	
MNV-1	MNV		11.99±12.50		14.75±12.65		9.38±8.34		15.49±8.83	
	Total samples with recovery rates >1%				31/32					

Virus	Number of genome copies	RNA extracts	Repeated experiment R1 (%±SD)	(F)	Repeated experiment R2 (%±SD)	(F)	Repeated experiment R3 (%±SD)	(F)	Repeated experiment R4 (%±SD)	(F)
norovirus GII	$1.30x10^6$	pure	1.85±0.67 (4/4)	1.0	2.08±1.59 (4/4)	1.0	10.16±6.97 (3/4)	1.8	21.63±1.76 (4/4)	1.5
		10-fold diluted	1.78±0.79 (4/4)		2.09±2.09 (4/4)		18.60±4.04 (4/4)		32.06±2.60 (4/4)	
	$1.30x10^5$	pure	8.10±1.89 (4/4)	0.4	7.98±1.47 (4/4)	0.4	14.24±0.79 (4/4)	0.9	49.94±12.26 (4/4)	1.2
		10-fold diluted	3.39±2.35 (4/4)		3.46±4.11 (4/4)		12.95±3.93 (3/4)		58.92±17.38 (4/4)	
	$1.30x10^4$	pure	10.32±1.70 (4/4)	0.0	0.88±0.63 (4/4)		8.04±6.01 (4/4)	0.1	69.16±6.90 (4/4)	0.04
		10-fold diluted	0.34 (1/4)		nd		0.37 (1/4)		2.56±2.06 (3/4)	
	$1.30x10^3$	pure	4.35 (1/4)		nd		8.89±8.67 (3/4)		nd	
		10-fold diluted	nd		nd		nd		nd	
MNV-1	MNV		50.22±41.50		35.41±35.44		12.08±4.45		11.84±5.04	
	Total samples with recovery rates >1%				32/32					

The mean of operator A and B replicates was used for each inoculation level sample. Results are expressed as means +/- SD. The number of positive Ct determinations is mentioned for HAV, HEV, norovirus GI and GII. RNA extracts were tested twice for each operator, resulting in four cycle threshold (Ct) values for each sample. The ratio between the mean values for extraction yields obtained with undiluted RNA extracts and those obtained with 10-fold diluted RNA extracts were calculated to determine whether the dilution of RNA extracts enhanced mean extraction yields (F).

nd: not detected.

Table 4: LOD_{50} and LOD_{95} calculated by Wilrich approach for HAV, HEV and norovirus

Virus target	Genome copies/g	R1	R2	R3	R4	All settings
HAV	LOD_{50}	<116*	$6.00x10^1$	$1.16 x10^2$	$2.08x10^2$	$8.00 x10^1$
	LOD_{95}	<116*	$2.52x10^2$	$4.80 x10^2$	$9.20x10^2$	$3.52x10^2$
HEV	LOD_{50}	$3.00x10^1$	$1.84x10^2$	$1.37x10^1$	$1.60x10^2$	$9.20x10^1$
	LOD_{95}	$1.28 x10^2$	$8.00x10^2$	$6.00x10^1$	$6.80x10^2$	$3.96x10^2$
norovirus GI	LOD_{50}	$3.44x10^2$	$1.24x10^3$	$1.16x10^2$	$2.72x10^2$	$3.60x10^2$
	LOD_{95}	$1.48x10^3$	$5.20x10^3$	$4.80x10^2$	$1.16x10^3$	$1.56x10^3$
norovirus GII	LOD_{50}	$1.16x10^2$	$1.52x10^2$	$2.60x10^1$	$1.52x10^2$	$9.20x10^1$
	LOD_{95}	$4.00x10^2$	$6.40x10^2$	$1.16x10^2$	$6.40x10^2$	$4.00x10^2$

(*LOD_{50} and LOD_{95} values were estimated. It was not possible to determine values because the LOD was not reached)

Table 5: performance characteristics for all viruses

	Virus			
	HAV	HEV	norovirus GI	norovirus GII
LOQ (genome copies/g)	2.90×10^4	1.40×10^3	1.60×10^4	1.30×10^4
LOD (genome copies/g)	0.97×10^4	0.47×10^4	0.53×10^4	0.43×10^4
Validated range level (genome copies/g	2.90×10^4 to 2.90×10^6	1.40×10^3 to 1.40×10^5	1.60×10^4 to 1.60×10^6	1.30×10^4 to 1.30×10^6
Repeatability (genome copies/g)	0.08-0.66	0.48-0.58	0.06-0.59	0.03-0.29
Intermediate precision (genome copies/g)	0.61-1.17	0.69-0.75	0.20-0.86	0.45-0.89

Table 6: Mean percentages of quantitative real-time RT-PCR inhibition for each sample

Sample number	Mean inhibition recovery rates (%±SD)
1	15.71±8.56 (N= 8)
2	6.44±9.15 (N= 8)
3	3.25±5.77 (N= 8)
4	8.75±9.29 (N= 16)
5	12.99±23.26 (N= 16)
6	13.67±25.95 (N= 16)
7	6.27±11.60 (N= 8)
8	28.70±27.00 (N= 16)
9	22.00±13.36 (N= 8)
10	16.51±10.33 (N= 8)
11	15.44±13.08 (N= 8)
12	26.84±13.39 (N= 8)